Pseudomonas pachastrellae sp. nov., isolated from a marine sponge

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Two Gram-negative, non-fermentative, non-denitrifying, non-pigmented, rod-shaped bacteria that were motile by means of polar flagella, designated strains KMM 330^T and KMM 331, were isolated from a deep-sea sponge specimen and subjected to a polyphasic taxonomic study. The new isolates exhibited 16S rRNA gene sequence similarity of 99.9%, and their mean level of DNA–DNA relatedness was 82%. Phylogenetic analysis based on their 16S rRNA gene sequences placed the strains within the genus *Pseudomonas* as an independent deep clade. Strain KMM 330^T shared highest sequence similarity (96.3%) with each of *Pseudomonas fulva* NRIC 0180^T, *Pseudomonas parafulva* AJ 2129^T and *Pseudomonas luteola* IAM 13000^T; sequence similarity to other recognized species of the genus *Pseudomonas* was below 95.7%. The marine sponge isolates KMM 330^T and KMM 331 could be distinguished from the other recognized *Pseudomonas* species based on a unique combination of their phenotypic characteristics, including growth in 8 or 10% NaCl, the absence of pigments, the inability to denitrify and lack of carbohydrate utilization. On the basis of phylogenetic analysis, physiological and biochemical characterization, strains KMM 330^T and KMM 331 should be classified as a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas pachastrellae* sp. nov. is proposed. The type strain is KMM 330^T (=JCM 12285^T =NRIC 0583^T =CCUG 46540^T).

Pseudomonads are well-known and widespread micro-organisms, which have been isolated from a variety of natural sources, soil, plants, mineral waters and clinical specimens, and they are characterized by a high level of metabolic diversity (Rosselló *et al.*, 1991; Moore *et al.*, 1996). The vast majority of species formerly assigned to *Pseudomonas* and recovered from marine sources are now classified within other genera (Kerstens *et al.*, 1996; Brown *et al.*,

2001; Yoon *et al.*, 2003). Relatively few species originating from marine environments are included within *Pseudomonas* (*sensu stricto*), including *Pseudomonas stutzeri* (later synonym *Pseudomonas perfectomarina*) (Baumann *et al.*, 1983; Rosselló-Mora *et al.*, 1993), *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes* (Palleroni, 1984) and *Pseudomonas alcaliphila* (Yumoto *et al.*, 2001) from sea waters, and some strains of *Pseudomonas balearica* (Bennasar *et al.*, 1996) have been recovered from marine sediments.

In the present study two *Pseudomonas*-like bacteria isolated from a deep-sea sponge specimen were investigated by using a polyphasic taxonomic approach. The new strains KMM 330^T and KMM 331 appeared to have homogeneous phenotypic characteristics and to be closely related based on phylogenetic and DNA–DNA hybridization experiments, but clearly distinct from all recognized *Pseudomonas* species. Based on the phenotypic and molecular data obtained, a novel species, *Pseudomonas pachastrellae* sp. nov., is described.

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Abbreviations: DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of KMM 330^T and KMM 331 are AB125366 and AB125367, respectively.

Images of cell morphology, a thin-layer chromatogram of polar lipids and a maximum-likelihood tree are available as supplementary figures in IJSEM Online.

Two strains, KMM 330^T and KMM 331, were isolated from a sponge specimen of *Pachastrella* sp., collected in 1991 from the Philippine Sea at a water depth of 750 m. A small amount of internal tissue of the sponge was homogenized and aliquots of the diluted homogenates were spread on agar plates of sea water medium (SWM), containing: 5.0 g peptone l⁻¹, 2.5 g yeast extract l⁻¹, 1.0 g glucose l⁻¹, 0.2 g K₂HPO₄ l⁻¹, 0.05 g MgSO₄ l⁻¹ and 15.0 g agar l⁻¹ in 750 ml sea water/250 ml distilled water. The inoculated plates were incubated for 14 days at 28 °C. Each colony was picked, re-streaked on the agar nutrient media and tested. The bacteria were grown aerobically on marine 2216 agar (MA) or marine broth (MB; Difco) and trypticase soy agar (TSA) at 25–28 °C. They were also stored at –80 °C in the liquid medium supplemented with 30 % (v/v) glycerol. Strains KMM 330^T and KMM 331 have been deposited in the Collection of Marine Micro-organisms (KMM) of the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia. The following *Pseudomonas* type strains, *P. stutzeri* CIP 103022^T, *P. alcaligenes* CIP 101034^T, *P. pseudoalcaligenes* CIP 66.14^T and *Pseudomonas mendocina* CIP 75.21^T, were kindly provided by the Collection de l'Institut Pasteur, Paris, France. Motility was observed by the hanging drop method. Cell morphology was examined by transmission electron microscopy from exponential phase cells grown in MB. Cells were negatively stained with potassium phosphotungstate (1 %, w/v; pH 7.0). Production of pyocyanin and formation of fluorescent pigments were tested on King A and King B medium, respectively (King *et al.*, 1954). The Gram-reaction, oxidase and catalase reactions and production of amylase, caseinase, DNase, gelatinase and lipase (Tween 80) were determined according to the methods described by Smibert & Krieg (1994). Acid production from carbohydrates was examined by using the medium of Leifson (1963) for marine bacteria. The API 20NE, API 32GN and API ZYM systems (bioMérieux) were additionally used to test biochemical properties and these tests were performed according to the manufacturer's instructions. The temperature range for growth was determined on TSA and MA at 4, 7, 12, 28, 37, 41, 42 and 43 °C. The pH range for growth (5.0–10.0) was tested using trypticase soy broth (TSB) and MB. Growth requirement for sodium ions and salt tolerance were examined on SWM prepared on a distilled water base supplemented with an appropriate amount of NaCl ranging from 0 to 15 % (w/v). DNA was isolated by the procedure of Marmur (1961). DNA base composition was determined as described by Marmur & Doty (1962) and Owen *et al.* (1969). The level of DNA–DNA relatedness between strains KMM 330^T and KMM 331 was measured spectrophotometrically by using the initial renaturation rate method of De Ley *et al.* (1970). The photobiotin-labelled DNA probe microplate method of Ezaki *et al.* (1989) was used to determine genetic relatedness of strain KMM 330^T and selected type strains of *Pseudomonas* species, including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas fulva*, *Pseudomonas luteola*, *Pseudomonas parafulva*, *Pseudomonas putida*, *Pseudomonas straminea* and *P. stutzeri*. Bacteria were grown on TSA

at 28 °C for 48 h and whole-cell fatty acids were determined in accordance with the procedure described by Svetashev *et al.* (1995). Cellular quinones were determined by HPLC (LC-6A; Shimadzu) using a Cosmosil 5C18 column (4.6 × 150 mm), with acetonitrile/2-propanol (50:50, v/v) as the eluant (0.5 ml min⁻¹), at a temperature 40 °C and with an SPD-2AM (270 nm) UV detector. Polar lipids were examined as described by Vaskovsky & Terekhova (1979). 16S rRNA gene sequences of the strains tested were determined and compared as described by Shida *et al.* (1997). The sequences obtained were compared with 16S rRNA gene sequences retrieved from the EMBL/GenBank/DDBJ databases by using the FASTA program (Pearson & Lipman, 1988). Distances were calculated according to the method of Jukes & Cantor (1969). Phylogenetic trees were constructed by the neighbour-joining method of Saitou & Nei (1987) with the CLUSTAL X program (version 1.8; Thompson *et al.*, 1997) and by the maximum-likelihood method using the BIOEDIT program (Hall, 1999).

The marine isolates KMM 330^T and KMM 331 were Gram-negative, non-pigmented, rod-shaped encapsulated bacteria that were motile by means of a single polar flagellum (see Supplementary Fig. A in IJSEM Online). Physiological, biochemical and chemotaxonomic characteristics of strains KMM 330^T and KMM 331 are given in Table 1 and under the species description below.

The polar lipid compositions of strains KMM 330^T and KMM 331 were identical (see Supplementary Fig. B in IJSEM Online) and consistent in their major components with those previously published for other *Pseudomonas* species. The presence of phosphatidylethanolamine (PE) as the predominant phospholipid, with smaller amounts of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), in the fluorescent pseudomonads and their close relatives has been previously reported by Wilkinson (1988). Polar lipids consisting of PE, PG, DPG, phosphatidylcholine (PC), unknown phospholipids, unknown aminophospholipids and unknown polar lipids were detected in *Pseudomonas oleovorans* and *Pseudomonas psychrotolerans* by Hauser *et al.* (2004). The polar lipids of strains KMM 330^T and KMM 331 did not include PC or aminophospholipid components.

Strains KMM 330^T and KMM 331 contained isoprenoid quinone Q₉ and C_{16:0}, C_{16:1ω9c} and C_{18:1} as the dominant fatty acids, in agreement with data previously reported for *Pseudomonas* species (Oyaizu & Komagata, 1983) and for *P. stutzeri* strains (Rainey *et al.*, 1994), *P. fulva*, *P. parafulva* and related species (Uchino *et al.*, 2001) and *P. alcaliphila* (Yumoto *et al.*, 2001).

The DNA G+C contents of the new isolates KMM 330^T and KMM 331 were 61.1 and 61.5 mol%, respectively, which is within the range reported for most *Pseudomonas* species (Palleroni, 1984).

On the basis of their quinone, polar lipid and fatty acid

Table 1. Phenotypic characteristics of *Pseudomonas pachastrellae* sp. nov. and other *Pseudomonas* species

Strains/species: 1, *P. pachastrellae* sp. nov. strains KMM 330^T and KMM 331; 2, *P. aeruginosa*; 3, *P. fluorescens* biovar 1; 4, *P. putida* biovars; 5, *P. straminea*; 6, *P. luteola*; 7, *P. fulva*; 8, *P. parafulva*; 9, *P. stutzeri*; 10, *P. balearica*; 11, *P. mendocina*; 12, *P. alcaligenes*; 13, *P. pseudoalcaligenes*; 14, *P. alcaliphila*. Data are from the present study and from Palleroni (1984), Uchino *et al.* (2000, 2001), Anzai *et al.* (1997), Bennasar *et al.* (1996) and Yumoto *et al.* (2001). +, Positive; –, negative; d, different reaction between strains; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Production of:														
Yellow–orange water-insoluble pigments	–	–	–	–	+	+	+	+	d	–	+	d	–	–
Fluorescent pigments on King B medium	–	d	+	+	+	–	–	–	–	–	–	–	–	–
Pyocyanin	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Number of flagella	1	1	>1	>1	1	1	1	1	1*	1	1*	1	1	1
Denitrification	–	+	–	–	–	–	–	–	+	+	+	+	d	–
Arginine dihydrolase	–	+	+	+	–	+	+	+	–	–	+	+	d	+
Growth at:														
4 °C	–	–	+	d	+	–	+	d	–	ND	–	–	–	+
41 °C	+	+	–	–	–	+	–	–	+	+	+	+	+	–
Tolerance of 8 % NaCl	+	–	–	–	–	–	–	–	–	+	–	–	–	–
Hydrolysis of:														
Gelatin	–	+	+	–	–	+	–	ND	–	–	–	d	d	+
Starch	–	–	–	–	–	–	–	–	+	+	–	–	–	–
Tween 80	+	d	d	d	–	+	+	–	+	ND	+	d	–	+
Utilization of:														
D-Glucose	–	+	+	+	+	+	+	+	+	ND	+	–	–	+
Maltose	–	–	–	–	–	+	–	–	+	+	–	–	–	–
Xylose	–	–	+	d	+	+	–	–	–	+	–	–	–	–
Fructose	–	+	+	+	+	+	+	+	d	ND	d	–	+	+
D-Mannose	–	–	+	d	+	+	+	+	–	ND	–	–	–	–
Sucrose	–	–	+	d	–	–	–	–	–	ND	–	–	–	–
Trehalose	–	–	+	–	–	+	–	–	–	ND	–	–	–	–
Glycerol	–	+	+	+	–	+	+	+	+	ND	d	–	d	+
D-Mannitol	–	+	+	d	+	+	–	–	d	–	–	–	–	+
Gluconate	–	+	+	+	+	+	+	+	d	ND	+	–	d	ND
Malate	–	+	+	+	–	+	+	+	+	+	–	+	+	+
L-Valine	–	d	+	+	–	–	+	+	+	ND	+	–	–	–
L-Lysine	–	+	+	d	–	+	+	+	–	ND	–	–	–	ND
L-Tyrosine	+	+	+	+	–	+	d	+	d	ND	+	d	d	ND
DNA G + C content (mol%)	61.1–61.5	67.2	60.5	60.7–62.5	62.5–63.7	54.8	60.0–61.5	60.0	60.6–66.3	64.1–64.4	62.8–64.3	64–68	62–64	62.3–63.2

*Lateral short flagella occur under certain conditions.

Fig. 1. Phylogenetic relationships of isolates KMM 330^T and KMM 331 and some related *Pseudomonas* species on the basis of 16S rRNA gene sequence analysis. The branching pattern was generated by the neighbour-joining method. Numbers indicate bootstrap values greater than 700. Bar, 0.01 K_{nuc} units. A tree generated by the maximum-likelihood method is available as Supplementary Fig. C in IJSEM Online.

compositions, DNA base composition, cell morphology and motility and physiological characteristics, the isolates can be considered to be members of the genus *Pseudomonas*.

Strains KMM 330^T and KMM 331 had 16S rRNA gene sequence similarity of 99.9%. *P. fulva* NRIC 0180^T, *P. parafulva* AJ 2129^T and *P. luteola* IAM 13000^T were the closest phylogenetic relatives (each 96.3% 16S rRNA gene sequence similarity). Strains KMM 330^T and KMM 331 shared 95.7% 16S rRNA gene sequence similarity with *P. straminea* IAM 1598^T and 95.3% similarity with '*Pseudomonas denitrificans*' IAM 12023. These values fall within the range of 16S rRNA gene sequence similarities (93.7–99.9%) reported for species of the genus *Pseudomonas* (Moore *et al.*, 1996; Anzai *et al.*, 2000). 16S rRNA gene sequence similarity values of below 97% have been stated by Stackebrandt & Goebel (1994) to be an accepted criterion for differentiation of bacteria at the species level. Relatively low 16S rRNA gene sequence similarities (96.3% to *P. luteola* IAM 13000^T, *P. fulva* NRIC 0180^T and *P. parafulva* AJ 2129^T, 95.7% to *P. straminea* IAM 1598^T and values below this to other *Pseudomonas* species) clearly demonstrated that the new isolates could be regarded as members of an independent species. Phylogenetic analysis based on the 16S rRNA gene sequences revealed a clear affiliation of the new isolates to members of the genus *Pseudomonas*. Different algorithms placed strains KMM 330^T and KMM 331 as a separate clade adjacent to *P. luteola* IAM 13000^T and to the *Pseudomonas pertucinogena* IFO 14163^T and '*P. denitrificans*' IAM 12023 cluster (Fig. 1; see also Supplementary Fig. C in IJSEM Online). The species relatedness of the *P. pertucinogena* cluster and

P. luteola among *Pseudomonas* species found in our analyses has been observed in other phylogenetic studies (Anzai *et al.*, 1997, 2000; Peix *et al.*, 2003).

Strains KMM 330^T and KMM 331 possessed a high level of DNA–DNA relatedness (82%), demonstrating their affiliation to the same species in accordance with the cut-off value of 70% recognized by Wayne *et al.* (1987) for discrimination of bacterial species. Strain KMM 330^T exhibited low DNA–DNA relatedness with a range of closely and more distantly related type strains of *P. aeruginosa* (7%), *P. fluorescens* (6%), *P. fulva* (6%), *P. luteola* (3%), *P. parafulva* (7%), *P. putida* (8%), *P. straminea* (9%) and *P. stutzeri* (9%). Based on the 16S rRNA gene sequence similarity values (<97%), their placement in the phylogenetic tree (Fig. 1) and levels of DNA–DNA relatedness, isolates KMM 330^T and KMM 331 should be classified as a novel species within the genus *Pseudomonas*.

The phylogenetic distinctiveness found for strains KMM 330^T and KMM 331 was supported by a combination of phenotypic features that does not allow their assignment to any recognized species of the genus *Pseudomonas*. The new marine isolates displayed a number of phenotypic characteristics that differentiate them from representatives of each of their related phylogenetic groups, including 'fluorescent', non-pigmented denitrifying or intracellular-pigmented *Pseudomonas* species. The phylogenetic relatives *P. fulva*, *P. parafulva* and *P. luteola* could be distinguished from strains KMM 330^T and KMM 331 based on the presence of intracellular pigments and utilization of various compounds. Strains KMM 330^T and KMM 331 were consistent with *P. alcaligenes* in their lack of carbohydrate utilization, but they differed in their inability to denitrify or to produce arginine dihydrolase and in their ability to grow in 8–10% NaCl. In addition, the DNA G+C content of strains KMM 330^T and KMM 331 (61.1–61.5 mol%) clearly distinguished them from *P. alcaligenes* (64–68 mol%) (Palleroni, 1984). Differential physiological and biochemical characteristics of the new marine isolates and a range of *Pseudomonas* species are summarized in Table 1. The most significant differential characteristics for strains KMM 330^T and KMM 331 are the inability to produce pigments, to denitrify, to produce arginine dihydrolase and to grow at 4 °C and the ability to grow in the presence of 8–10% NaCl.

On the basis of combined phenotypic and phylogenetic data, the marine sponge isolates KMM 330^T and KMM 331 are considered to represent a novel species, for which the name *Pseudomonas pachastrellae* sp. nov. is proposed.

Description of *Pseudomonas pachastrellae* sp. nov.

Pseudomonas pachastrellae (pa.cha.strel'lae. N.L. gen. n. *pachastrellae* of *Pachastrella*, the generic name of a sponge).

Gram-negative, aerobic, non-pigmented, encapsulated,

rod-shaped cells, 1.4–1.6 µm long and 0.4–0.5 µm in diameter, that are motile by a single flagellum. Oxidase- and catalase-positive. Forms circular, smooth, non-pigmented, whitish and transparent colonies, 2–3 mm in diameter, on TSA and MA. Pyocyanin on King A medium and fluorescent pigments on King B medium are not produced. A slightly yellowish diffusible pigment is observed during growth of strain KMM 330^T on King A medium. Growth is observed in 0–10 % (w/v) NaCl and at temperatures of 7–41 °C; no growth occurs at 4 or 42 °C. Acid is not produced from D-glucose, D-xylose, rhamnose, galactose, maltose, lactose or mannitol. Variable production of acid from glycerol. Hydrolysis of Tween 40 is positive. In addition to the physiological characteristics given in Table 1, the strains are negative for urease, lysine decarboxylase, ornithine decarboxylase, acetoin production, H₂S production, indole production, aesculin and DNA hydrolysis, haemolysis and utilization of L-arabinose, phenylacetate, DL-norleucine and N-acetylglucosamine and positive for citrate and DL-lactate utilization. In APY ZYM tests, strains are positive for esterase C4, esterase lipase C8, phosphoamidase, leucine arylamidase reaction and negative for alkaline phosphatase, lipase C14, valine arylamidase, cystine arylamidase, α-chymotrypsin, trypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The major isoprenoid quinone is Q₉. The phospholipids are PE, PG, DPG and an unknown phospholipid. Strains KMM 330^T and KMM 331 contain C_{16:0} (20 and 17.6 %, respectively), C_{16:1ω9c} (23.5 and 23.0 %) and C_{18:1} (41.7 and 44.9 %) as major fatty acids, as well as C_{12:0} (6.9 and 4.9 %), C_{12:1}, iso-C_{13:0}, C_{13:0}, C_{13:1}, C_{14:0}, C_{14:1ω7c}, C_{14:0} 3-OH, C_{15:0}, iso-C_{15:0}, iso-C_{16:0}, iso-C_{17:0}, C_{12:0} 3-OH, C_{17:1ω8c}, C_{17:0}, cyc-C_{17:0} and C_{18:0} are present at ≤1 %. The G+C content of the DNA is 61.1–61.5 mol% (determined by the thermal denaturation method).

The type strain, KMM 330^T (=JCM 12285^T=NRIC 0583^T=CCUG 46540^T), was isolated from the sponge *Pachastrella* sp., collected from the Philippine Sea at a water depth of 750 m.

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