Extremophiles (2001) 5:343–349
Digital Object Identifier (DOI) 10.1007/s007920100199

ORIGINAL PAPER

**Isao Yumoto · Teruo Kusano · Tomohiro Shingyo
Yoshinobu Nodasaka · Hidetoshi Matsuyama
Hidetoshi Okuyama**

Assignment of *Pseudomonas* sp. strain E-3 to *Pseudomonas psychrophila* sp. nov., a new facultatively psychrophilic bacterium

Received: January 30, 2001 / Accepted: April 12, 2001 / Published online: August 11, 2001

Abstract A facultatively psychrophilic bacterium, previously described as *Pseudomonas* sp. strain E-3, has been reassigned by phenotypic characterization, chemotaxonomic analysis, DNA–DNA hybridization, and 16S rRNA gene phylogenetic analysis. The organism was a gram-negative, aerobic, straight rod with polar flagella. It was catalase positive and oxidase positive, able to grow at –1°C but not at 40°C, and produced acid from D-glucose under aerobic conditions. The major isoprenoid quinone was ubiquinone-9, and the DNA G + C content was 57.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequencing indicated that the bacterium is a member of the genus *Pseudomonas* and was closest to *Pseudomonas fragi*. Determination of the DNA–DNA relatedness between strain E-3 and *P. fragi* revealed too low a level of homology (47.9%–51.3%) to identify them as the same species. On the basis of phenotypic characteristics, phylogenetic analysis, and DNA–DNA relatedness data, it is concluded that strain E-3 represents an individual species. Accordingly, the name *Pseudomonas psychrophila* is proposed. The type strain is E-3^T (= JCM 10889).

Key words *Pseudomonas psychrophila* · Facultatively psychrophilic · 16S rRNA · DNA–DNA hybridization

Communicated by K. Horikoshi

I. Yumoto (✉) · T. Shingyo · H. Okuyama
Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology (AIST), Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan
Tel. +81-11-8578925; Fax +81-11-8578900
e-mail: i.yumoto@aist.go.jp

T. Kusano · H. Okuyama
Laboratory of Environmental Molecular Biology, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo, Japan

T. Shingyo · H. Matsuyama
Department of Bioscience and Technology, School of Engineering, Hokkaido Tokai University, Sapporo, Japan

Y. Nodasaka
Laboratory of Electron Microscopy, School of Dentistry, Hokkaido University, Sapporo, Japan

Introduction

Temperature is one of the most important factors determining the distribution and biological functions of organisms. More than 80% of the Earth is at temperatures below 10°C, such as in deep oceans, high mountains, and the polar regions. Even in such extremely cold environments, it is known that cold-adapted microorganisms, psychrophiles, can be found. Psychrophiles are divided into obligate psychrophiles and facultative psychrophiles. Morita (1975) defined these microorganisms as follows: the former is defined by a maximum growth temperature of 20°C or lower, an optimum growth temperature of 15°C or lower, and a minimum growth temperature of 0°C or lower, and the latter is distinguished from the former or mesophiles as those microorganisms that grow at 0–5°C and have optimum and maximum growth temperatures above 20°C.

It has been considered that obligate psychrophiles are distributed in permanently cold environments in nature whereas facultative psychrophiles are distributed in quite common habitats such as soil, water, and foods. Until now, to understand the psychrophily of microorganisms, facultative psychrophiles have been studied. These studies concern such aspects as cellular membrane fluidity, cold-shock or cold-acclimation proteins, and cold-adapted enzymes (Gerday et al. 1999; Gounot and Russell 1999). Industrial applications of facultative psychrophiles and their cold-adapted enzymes have also been considered (Ohgiya et al. 1999; Okuyama et al. 1999).

A facultative psychrophilic bacterium, *Pseudomonas* sp. strain E-3, has been isolated to investigate its psychrophily (Saruyama et al. 1978). The cellular phospholipid and fatty acid composition as related to growth temperature (Wada et al. 1987), the biosynthesis of unsaturated fatty acids (Wada et al. 1989, 1991), and activities of the *cis–trans* isomerization of the double bond of monounsaturated fatty acids (Okuyama et al. 1996, 1998) of the strain have been studied extensively.

Up to the present, many *Pseudomonas* strains able to grow at 0–5°C have been isolated. It has been considered that most of these strains are identified as *Pseudomonas*

fragi, *Pseudomonas lundensis*, or *Pseudomonas fluorescens* (Molin and Ternström 1986). In this study, phenotypic and chemotaxonomic characteristics, the phylogenetic position and DNA-DNA relatedness in relation to other strains of strain E-3 were examined; the results show that the strain should be classified as a new species.

Materials and methods

Bacterial strains and cultivation

The strain examined was *Pseudomonas* sp. E-3^T (T = type strain) (Saruyama et al. 1978). It was isolated from a cold room for food storage. The organism was cultivated aerobically at 27°C in nutrient medium with 0.5% NaCl (pH 7.0) containing 5 g peptone (Kyokuto, Tokyo, Japan), 3 g yeast extract (Kyokuto), 5 g NaCl, and 15 g agar (if needed) in 1 l distilled water. *Pseudomonas fragi* IFO 3458^T, *Pseudomonas aureofaciens* NCIMB 9030^T, *Pseudomonas lundensis* NCIMB 12337^T, and *Pseudomonas fluorescens* JCM 5963^T were used as reference strains for DNA–DNA relatedness. The microorganisms were cultivated using nutrient broth with 0.5% NaCl (pH 7.0) with shaking (130 rpm) until the late exponential phase of growth at 27°C.

Physiological and biochemical characteristics

For phenotypic characterization, nutrient broth with 0.5% NaCl was used as the basal medium and the culture was incubated at 27°C for 2 weeks; the experiment was performed more than twice unless otherwise stated. Determination of substrate utilization as the sole carbon and energy source was performed in utilization of substrates for neutrophiles (USN) medium containing 0.2% carbohydrate or 0.1% amino acid as a substrate, 2 g NH_4Cl, 2 g Na_2HPO_4, 1 g KH_2PO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2{\cdot}2H_2O$, and 1 ml metal mixture in 1 l distilled water (pH 7.0). The metal mixture contained the following (per 100 ml distilled water): 1.8 g ethylenediaminetetraacetic acid (EDTA), 5.0 g $ZnSO_4{\cdot}7H_2O$, 5.0 g $FeSO_4{\cdot}7H_2O$, 1.5 g $MnSO_4{\cdot}5H_2O$, 0.4 g $CuSO_4{\cdot}5H_2O$, 0.25 g $Co(NO_3)_2{\cdot}6H_2O$, and 0.1 g H_3BO_3. The requirement and tolerance for NaCl were determined using a medium containing 2 g glucose, 1 g yeast extract (Kyokuto), 1 g peptone (Nihon Pharmaceutical, Tokyo, Japan), 0–200 g NaCl, 1 g sodium acetate, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2{\cdot}2H_2O$, 0.001 g $MnSO_4{\cdot}nH_2O$, and 0.005 g $ZnSO_4{\cdot}7H_2O$ in 1 l distilled water (pH 7.0). Other physiological and biochemical characteristics were examined according to the methods described in Cowan and Steel's manual (Barrow and Feltham 1993).

Electron microscopy

For observation of negatively stained cells under a transmission electron microscope (TEM), the following processes were carried out. Cells grown on nutrient agar with 0.5% NaCl were suspended in physiological saline solution. A small drop of suspension was placed on a carbon-coated grid and the cells were negatively stained with 1% phosphotungstic acid for observation under the TEM (model H-800; Hitachi, Tokyo, Japan).

Analysis of isoprenoid quinones

Isoprenoid quinones were extracted by treating 0.5 g freeze-dried cells, which were cultivated on nutrient agar with 0.5% NaCl at 27°C, with 150 ml chloroform/methanol (2:1, v/v) for 2 h in a reciprocal shaker (120 strokes min^{-1}) at room temperature. The extracted solution was concentrated by evaporation to dryness and the resulting precipitate was dissolved in acetone. The resulting solution was then concentrated and separated by thin-layer chromatography (TLC) using benzene as the solvent, and the isoprenoid quinones were analyzed using a high performance liquid chromatograph (HPLC) equipped with a 3.9×150 mm Novapak C_{18} column (Waters); methanol/2-propanol (1:1, v/v) was used as the solvent at room temperature.

Analysis of whole-cell fatty acids

Whole-cell fatty acids were extracted from 100 mg of freeze-dried cells that were cultivated on nutrient agar with 0.5% NaCl at 27°C, esterified by acid methanolysis, and analyzed using a gas-liquid chromatograph (GLC) equipped with a flame-ionization detector (model GC 353; GL Sciences, Tokyo, Japan) and a 0.25-mm (inner diameter) × 100-mm, 0.2-μm film SP-2560 column (Supelco, Bellefonte, PA, USA) at an oven temperature of 140°C (initial, 15 min) which was increased to 240°C in increments of 4 °C/min. Fatty acids were identified by comparing them with fatty acid methyl ester standards purchased from Supelco and GL Science, and using GC/MS (model INCOS 50; Finnigan mat) connected to a GLC (model 3400; Varian, Walnut Creek, CA, USA).

DNA base composition and DNA–DNA hybridization

DNA was prepared from bacterial cells according to the method of Marmur (1961). The G+C content of the DNA was determined according to the method of Tamaoka and Komagata (1984). The prepared DNA was digested with nuclease P1 (Yamasa Shoyu, Choshi, Japan). The resulting nucleotides were analyzed by HPLC with a 4.6×250 mm Inertsil C_4 column (GL Sciences) with 0.2 M $NH_4H_2PO_4$ (pH 4.5) as the solvent at room temperature. The HPLC system was as already described. The UV spectrophotometric detector was set at 265 nm. An equimolar mixture of four deoxyribonucleotides (Yamasa Shoyu) was used as the standard. The levels of DNA relatedness were determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labeled DNA probes and black microplates.

Amplification of 16S rRNA gene and sequencing

The almost complete 16S rRNA gene of strain E-3^T was amplified by polymerase chain reaction (PCR). The sequences of primers used for amplification were 5′-AGAGTTTGATCCT-GGCTC-3′ and 5′-AAGGAGGTG ATCCAGCCGCA-3′, corresponding to nucleotides 8 to 25 and 1,521 to 1,540, respectively, in the 16S rRNA sequence of *Escherichia coli* (Brosius et al. 1978). The amplified DNA fragment was cloned into a plasmid vector, and the nucleotide sequence was determined by the Thermo Sequence fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) with a DNA sequencer (model 4000L, LI-COR). Multiple alignments of the sequence were performed, nucleotide substitution rates (K_{nuc} value) were calculated, and a neighbor-joining phylogenetic tree (Kimura 1980; Saitou and Nei 1987) was constructed using the CLUSTAL W program (Thompson et al. 1994). The similarity value of the sequence was calculated using the GENETXY computer program (Software Development, Tokyo, Japan). The 16S rRNA gene sequences were placed with GenBank/EMBL/DDBJ under accession number AB041885.

Results

Morphology

Colonies of strain E-3 on a nutrient medium with 0.5% NaCl were circular and colorless. The cells are gram-negative, polarly flagellated straight rods, 0.5–0.9 by 1.2–2.8 μm in size (Fig. 1). Spore formation was absent.

Phenotypic characteristics

Catalase and oxidase reactions were positive. The strain was able to grow well in media supplemented with 0–4% NaCl but not in media with salinity higher than 8% NaCl. Growth occurred at –1° to 35°C, but no growth was observed at 40°C or higher. The optimum growth temperature of the strain was 25°C. Methyl red, urease, and arginine dihydrolase tests were positive, but the Voges–Proskaur test and tests for indole, H_2S production, lysine, and ornithine decarboxylase were negative. The nitrate reduction test was weakly positive. Acid was produced from D-glucose, L-arabinose, D-fructose, D-mannose, melibiose, sucrose, D-xylose, raffinose, mannitol, galactose, and trehalose. No acid was produced from maltose, inositol, sorbitol, or rhamnose. The strain hydrolyzed Tween-20, -40, and -60, but not casein, gelatin, starch, DNA, alginic acid, esculin, or Tween-80. It utilized L-arabinose, D-fructose, D-glucose, glycerol, D-mannose, melibiose, raffinose, sucrose, D-xylose, trehalose, mannitol, D-galactose, L-proline, L-arabinose, succinate, glutamate, and citrate but not maltose, sorbitol, rhamnose, cellobiose, inositol, or *N*-acetyl-D-glucosamine.

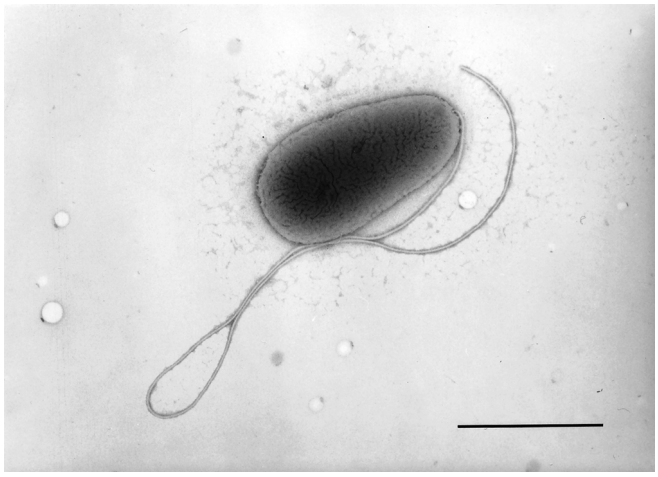

Fig. 1. Transmission electron photomicrograph of a negatively stained cell of *Pseudomonas psychrophila* E-3^T, showing polar flagellation. *Bar* 1 μm

Chemotaxonomic characteristics

The isoprenoid quinone isolated from strain E-3^T using TLC was analyzed by HPLC. Analysis revealed that ubiquinone-9 (Q-9) was the predominant isoprenoid quinone in strain E-3^T. Gas chromatography (GC) analysis of methyl ester derivatives of cellular fatty acids of the strain revealed that the major components were $C_{16:0}$, $C_{16:1(9t)}$, $C_{16:1(9C)}$, $C_{17:0\ cyc}$, $C_{18:1(9c)}$, and $C_{19:0\ cyc}$.

16S rRNA gene sequence analysis

The nucleotide sequence of the 16S rRNA gene amplified by means of PCR from strain E-3^T was determined by direct automated sequencing. The determined sequence of 1,528 bases has 94.7%–98.3% similarity to the 16S rRNA gene sequence of 26 strains of *Pseudomonas* species. A phylogenetic tree constructed by the neighbor-joining method showed that strain E-3^T belongs to the *P. fluorescens* lineage (Moore et al. 1996). The lineage most similar to strain E-3^T in the phylogenetic tree was *Pseudomonas fragi* (Fig. 2). The highest gene sequence similarity to strain E-3^T was 98.3% with *P. fragi*, *Pseudomonas lundensis*, and *Pseudomonas aureofaciens*.

DNA base composition and DNA–DNA hybridization

The DNA G + C content of strain E-3^T was 57.2%. This value falls within the definition range of genus *Pseudomonas*. According to the results of 16S rRNA gene sequence analysis, strain E-3^T had the highest similarity to *P. fragi*, *P. lundensis*, and *P. aureofaciens*. The level of DNA relatedness between strain E-3^T, the three strains, and *P. fluorescens* was estimated (Table 1). The DNA relatedness results indicated that strain E-3^T was obviously different (19.4%–51.3%) from *P. fragi*, *P. lundensis*, *P. taetrolens, P. aureofaciens*, and *P. fluorescens*.

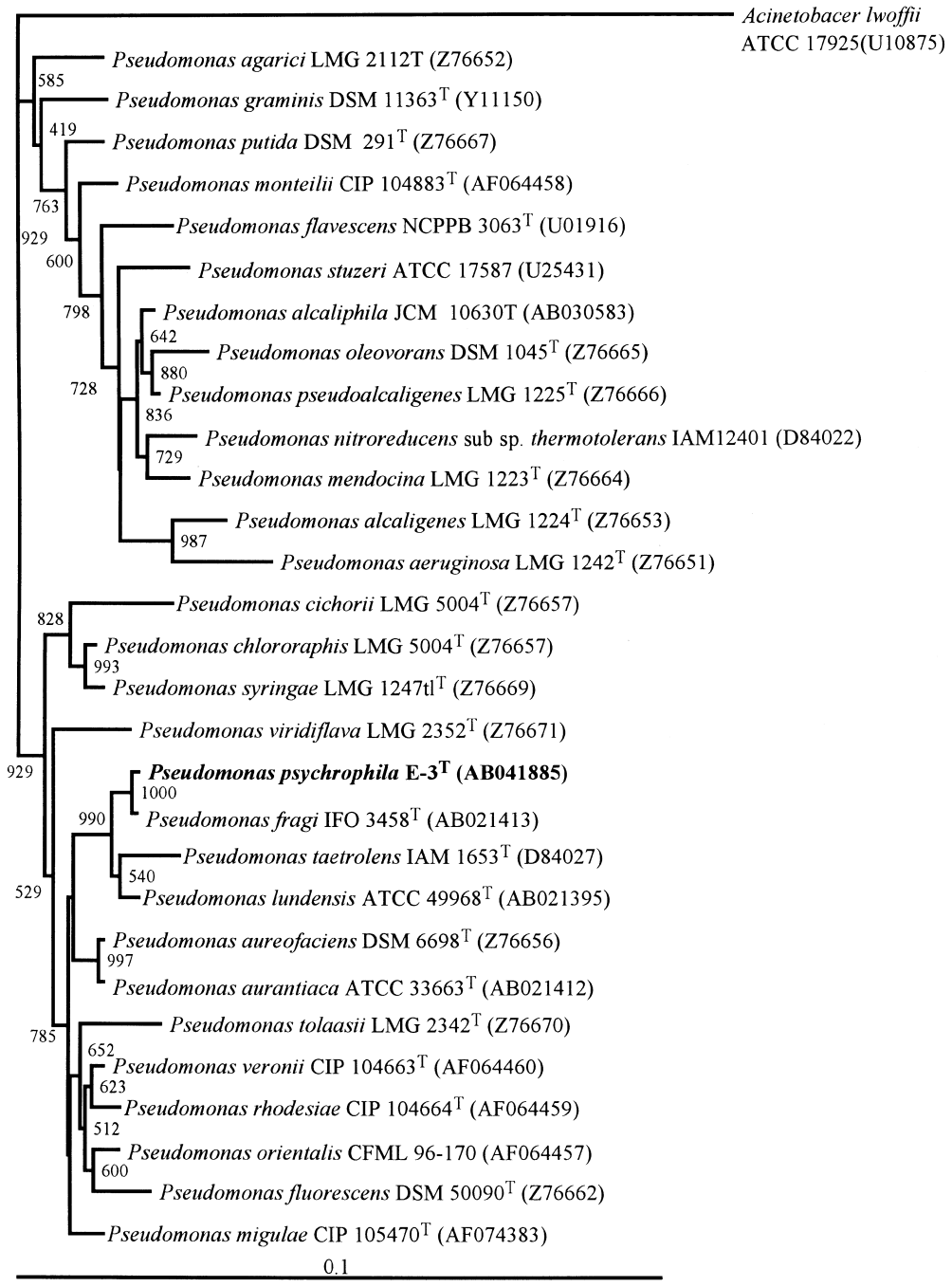

Fig. 2. Phylogenetic tree of *Pseudomonas psychrophila* E-3^T and other *Pseudomonas* strains derived from 1.495 kb of 16S rRNA gene sequence data, using the neighbor-joining method for calculation. *Acinetobacter lwoffii* was used as the outgroup for the phylogenetic tree. *Numbers* indicate bootstrap values greater than 500. *Bar* 0.1 K_{nuc} unit

Discussion

Phenotypic characteristics suggested that strain E-3^T could be classified in genus *Pseudomonas*. The chemotaxonomic data indicated that the isolate belongs in Palleroni's (1984) rRNA group I (Yamada et al. 1982; Oyaizu and Komagata 1983). A phylogenetic analysis based on the 16S rRNA gene sequence showed that strain E-3^T belongs to the *P. fluorescens* lineage with more than 96% sequence similarity to the 16S rRNA gene of strain from the same lineage. Among the lineages, the highest 16S rRNA gene sequence similarity to strain E-3^T was 98.3% with *P. fragi*, *P. lundensis*, and *P. aureofaciens*. The level of DNA relatedness between strain E-3^T and the three strains is less than 52%. These results show that strain E-3^T is different from the three strains tested. Furthermore, strain E-3^T can be differentiated phenotypically from the phylogenetically most closely related species, *P. fragi*, by its inability to produce acid from fructose, mannitol, raffinose, and sucrose and its ability to

Table 1. DNA–DNA relatedness among strains of *Pseudomonas* species examined in this study

Species	Strain	Reassociation (%) with biotinylated DNA from:	
		P. psychrophila E-3^T	*P. fragi* IFO 3458^T
P. psychrophila	E-3^T	100	51.3
P. fragi	IFO 3458^T	47.9	100
P. lundensis	NCIMB 12337^T	34.1	41.1
P. taetrolens	IAM 1653^T	39.2	ND[a]
P. aureofaciens	NCIMB 9030^T	37.8	25.3
P. fluorescens	JCM 5963^T	19.4	30.4

ND, not determined

hydrolyze gelatin (Haynes and Burkholder 1957) (Table 2). The results indicate that strain E-3^T is a novel species.

Obligate psychrophilic bacteria are able to survive only at cold temperatures (less than 20° or 25°C) and are distributed permanently in cold environments such as polar regions, deep in the sea, and in cold currents in the ocean. On the other hand, facultative psychrophilic bacteria are widespread in natural environments not subject to extremely high temperatures and in most refrigerated foods. Presence of facultative psychrophilic microorganisms can be the cause of food spoilage or poisoning. In fact, it has been an important issue to prevent the growth of the facultative psychrophilic microorganisms in refrigeration for food conservation. The facultative psychrophilic microorganisms in meat, fish, vegetables, and dairy products predominantly consist of gram-negative bacteria, most of which belong to the *Pseudomonas* spp. (Reddy et al. 1969; Edwards et al. 1987; Gram 1993). Several *Pseudomonas* species cause problems in refrigerated foods because of their production of enzymes such as protease and lipase, which are responsible for undesirable odors. It was proposed that in such cases of spoilage *P. fragi* is the dominant microorganism in refrigerated meat from different origins (Rosset 1982; Dainty et al. 1983). Although we do not know if strain E-3^T can cause food spoilage in refrigerated foods, in the present study we found, in a cold room used for food storage, a novel cold-adapted bacterium, which belongs to the genus *Pseudomonas* and is very similar to *P. fragi* phylogenetically. These results are very interesting in terms of the relationship between cold environmental factors and the evolution processes of strains E-3^T and *P. fragi*.

In the present study, we observed the *trans*-unsaturated fatty acid $C_{16:1(9t)}$ in the cellular fatty acid of strain E-3^T as has been previously observed (Okuyama et al. 1996). *trans*-Unsaturated fatty acids have been detected in various bacteria as have the *cis*-unsaturated fatty acid isomers (Keweloh and Heipieper 1996). The mechanism of the conversion of *cis*- into *trans*-unsaturated fatty acids for control of membrane fluidity has been studied in *P. putida* (Heipieper et al. 1992, 1994; Diefenbach and Keweloh 1994; Holtwick et al. 1999). Although the amounts of each of the constituents of fatty acids are different, the fatty acid components of strain E-3^T are very similar to those of *P. putida* (Diefenbach et al. 1992). The ratio of *trans*-unsaturated fatty acids becomes higher when the bacterial cells are exposed to various environmental changes, e.g., in temperature, salinity, and stimulation by toxic substances in *P. putida* (Loffeld and Keweloh 1996). However, it is not known whether environmental stimuli other than temperature can induce the conversion of *cis*- to *trans*-isomerization of the double bond of monounsaturated fatty acid in strain E-3^T.

Description of the new species, *Pseudomonas psychrophila*

Pseudomonas psychrophila (sp. nov.) psy.chro'phi.la M. L. Adj. Psychrophila cold-loving (bacterium).

The cells are gram negative (0.5–0.9 by 1.2–2.8 μm), motile by means of a single polar flagellum. Colonies are circular and colorless. Catalase and oxidase reactions are positive. Growth occurs in media supplemented with 0%–4% NaCl but not in media with salinity higher than 8% NaCl. Growth occurs at –1° to 35°C, but no growth is observed at 40°C or higher. The optimum growth temperature of the strain was 25°C. Methyl red, urease, and arginine dihydrolase tests are positive but the Voges–Proskaur test and tests for indole, H_2S production, lysine, and ornithine decarboxylase are negative. The nitrate reduction test was weakly positive. Acid is produced from D-glucose, L-arabinose, D-fructose, D-mannose, melibiose, sucrose, D-xylose, raffinose, mannitol, galactose, and trehalose. No acid is produced from maltose, inositol, sorbitol, or rhamnose. The strain hydrolyzes Tween-20, -40, and -60, but not casein, gelatin, starch, DNA, alginic acid, esculin, or Tween-80. It utilizes L-arabinose, D-fructose, D-glucose, glycerol, D-mannose, melibiose, raffinose, sucrose, D-xylose, trehalose, mannitol, D-galactose, L-proline, L-arabinose, succinate, glutamate, and citrate but not maltose, sorbitol, rhamnose, cellobiose, inositol, or *N*-acetyl-D-glucosamine. The major isoprenoid quinone is Q-9. The DNA G + C content is 57.2 mol% (determined by HPLC). The major fatty acids are $C_{16:0}$, $C_{16:1(9t)}$, $C_{16:1(9C)}$, $C_{17:0\ cyc}$, $C_{18:1(9c)}$, and $C_{19:0\ cyc}$. The type strain E-3 has been deposited at The Institute of Physical and Chemical Research (RIKEN), Wako, Japan, as a strain of JCM 10889.

Table 2. Differential characteristics of *Pseudomonas psychrophila* and facultatively psychrophilic *Pseudomonas* spp. and other related species

Characteristics	*Pseudomonas psychrophila*	*Pseudomonas fragi*	*Pseudomonas lundensis*	*Pseudomonas fluorescens* biovar 1	*Pseudomonas fluorescens* biovar 2	*Pseudomonas fluorescens* biovar 3	*Pseudomonas fluorescens* biovar 4	*Pseudomonas aeruginosa*	*Pseudomonas putida*
Nitrate reduction	W	–	ND	+	+	+	+	+	–
Gelatin hydrolysis	–	D	+	+	+	+	+	+	–
Starch hydrolysis	–	ND	–	–	–	–	–	–	–
Growth at 4°C	+	+	+	+	+	+	+	–	–
Growth at 42°C	–	–	–	–	–	–	–	+	–
Arginine dihydrolase	+	+	+	+	+	D	+	+	+
Acid produced from:									
Glucose	+	+	+	+	+	+	+	+	+
Fructose	+	–	–	–	–	–	–	–	D
Sucrose	+	–	–	D	D	–	D	–	–
Mannitol	+	–	–	D	–	–	–	–	–
Raffinose	+	–	–	–	–	–	–	–	–

+, 90% or more of strains are positive; –, 90% or more of strains are negative; W, weakly positive; D, differ among strains; ND, no data
Characteristics of other *Pseudomonas* species were cited from Molin and Ternström (1986), Barrow and Feltham (1993), Holt et al. (1994), and Kiska and Gilligan (1999)

References

Barrow GL, Feltham RKR (1993) Cowan and Steel's manual for the identification of medical bacteria, 3rd edn. Cambridge University Press, London

Brosius J, Palmer JL, Kennedy JP, Noller HF (1978) Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc Natl Acad Sci USA 75:4801–4805

Dainty RH, Shaw BG, Roberts TA (1983) Microbial and chemical changes in chill-stored red meats. In: Robert TA, Skinner FA (eds) Food microbiology: advances and prospects. Academic Press, London, pp 151–178

Diefenbach R, Keweloh H (1994) Synthesis of *trans* unsaturated fatty acids in *Pseudomonas putida* P8 by direct isomerization of the double bond of lipids. Arch Microbiol 162:120–125

Diefenbach R, Heipieper HJ, Keweloh H (1992) The conversion of *cis*- into *trans*-unsaturated fatty acids in *Pseudomonas putida* P8: evidence for a role in the regulation of membrane fluidity. Appl Microbiol Biotechnol 38:382–387

Edwards RA, Dainty RH, Hibbard CM (1987) Volatile compounds produced by meat pseudomonads and related reference strains during growth on beef stored in air and chill temperature. J Appl Bacteriol 62:403–412

Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution 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

Gerday C, Aittaleb M, Arigny JL, Baise E, Chessa JP, François JM, Garsoux G, Petrescu I, Feller G (1999) Cold enzymes: a hot topic. In: Margesin R, Schinner F (eds) Cold-adapted organisms: ecology, physiology, enzymology and molecular biology. Springer, Berlin Heidelberg New York, pp 257–275

Gounot AM, Russell N J (1999) Physiology of cold-adapted microorganisms. In: Margesin R, Schinner F (eds) Cold-adapted organisms: ecology, physiology, enzymology and molecular biology. Springer, Berlin Heidelberg New York, pp 33–55

Gram L (1993) Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish. Appl Environ Microbiol 59:2197–2203

Haynes W, Burkholder WH (1957) Genus I *Pseudomonas* Migula 1894. In: Breed RS, Murray GD, Smith NR (eds) Bergey's manual of determinative bacteriology, 7th edn. Williams and Wilkins, Baltimore, pp 89–152

Heipieper HJ, Diefenbach R, Keweloh H (1992) Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Appl Environ Microbiol 58:1847–1852

Heipieper HJ, Weber FJ, Sikkema J, Keweloh H, de Bont JAM (1994) Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol 12:409–425

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergy's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore

Holtwick R, Keweloh H, Meinhardt F (1999) *cis*/*trans* Isomerase of unsaturated fatty acids of *Pseudomonas putida* P8: evidence of heme protein of cytochrome *c* type. Appl Environ Microbiol 65:2644–2649

Keweloh H, Heipieper HJ (1996) *trans* Unsaturated fatty acids in bacteria. Lipids 31:129–137

Kimura M (1980) A simple method for estimating evolutionary rates base substitution through comparative studies of nucleotide sequences. J Mol Evol 16: 111–120

Kiska DL, Gilligan PH (1999) *Pseudomonas.* In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of clinical microbiology, 7th edn. American Society for Microbiology, Washington, DC, pp 517–525

Loffeld B, Keweloh H (1996) *cis*/*trans* Isomerization of unsaturated fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8. Lipids 31: 811–815

Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol 3:208–218

Molin G, Ternström (1986) Phenotypically based taxonomy of psychrotrophic *Pseudomonas* isolated from spoiled meat, water, and soil. Int J Syst Bacterial 36:257–274

Moore ERB, Mau M, Arnscheidt A, Bötter EC, Hutson RA, Collins MD, Van De Peer Y, De Wachter R, Timmis KN (1996) The determination and comparison of 16S rRNA gene sequences of species of genus *Pseudomonas* (sensu stricto) and estimation of the natural intergenetic relationship. Syst Appl Microbiol 19:478–492

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Ohgiya S, Hoshino T, Okuyama H, Tanaka S, Ishizaki K (1999) Biotechnology of enzyme from cold-adapted microorganisms. In: Margesin R, Schinner F (eds) Biotechnology application of cold-adapted organisms. Springer, Berlin Heidelberg New York, pp 17–34

Okuyama H, Enari D, Shibahara A, Yamamoto K, Morita N (1996) Identification of activities that catalyze the *cis-trans* isomerization of the double bond of a mono-unsaturated fatty acid in *Pseudomonas* sp. strain E-3. Arch Microbiol 165:415–417

Okuyama H, Ueno A, Enari D, Morita N, Kusano T (1998) Purification and characterization of 9-hexadecenoic acid *cis-trans* isomerase from *Pseudomonas* sp. strain E-3. Arch Microbiol 169:29–35

Okuyama H, Morita N, Yumoto I (1999) Cold-adapted microorganisms for use in food biotechnology. In: Margesin R, Schinner F (eds) Biotechnology application of cold adapted organisms. Springer, Berlin Heidelberg New York, pp 17–34

Oyaizu H, Komagata K (1983) Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference of the existence of 3-hydroxy fatty acids. J Gen Appl Microbiol 29:17–40

Palleroni NJ (1984) Genus I. *Pseudomonas* Migula 1984, 237AL (Nom. Cons. Opin. 5, Jud. Comm. 1952, 237). In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore, pp 141–199

Reddy MC, Bills DD, Lindsay RC (1969) Ester production by *Pseudomonas fragi*. II. Factors influencing ester levels in milk culture. Appl Microbiol 17:779–782

Rosset R (1982) Chilling, freezing and thawing. In: Brown MH (ed) Meat microbiology. Applied Science, New York, pp 265–318

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic tree. Mol Biol Evol 4:406–425

Saruyama H, Ochiai T, Takada Y, Okuyama H, Sasaki S (1978) Isolation and growth temperature of psychrophiles. J Fac Sci Hokkaido Univ Ser 5(11):211–217

Tamaoka J, Komagata K (1984) Determination of base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiol Lett 25:125–128

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Wada M, Fukunaga N, Sasaki S (1987) Effect of growth temperature on phospholipid and fatty acid compositions in a psychrotrophic bacterium, *Pseudomonas* sp. strain E-3. Plant Cell Physiol 28:1209–1217

Wada M, Fukunaga N, Sasaki S (1989) Mechanism of biosynthesis of unsaturated fatty acids in *Pseudomonas* sp. strain E-3, a psychrotrophic bacterium. J Bacteriol 171:4267–4271

Wada M, Fukunaga N, Sasaki S (1991) Aerobic synthesis of unsaturated fatty acids in a psychrotrophic bacterium, *Pseudomonas* sp. strain E-3, having two mechanisms for unsaturated fatty acid synthesis. J Gen Appl Microbiol 37:355–262

Yamada Y, Takinami-Nakamura H, Tahara Y, Oyaizu H, Komagata K (1982) The ubiquinone system in the strains of *Pseudomonas* species. J Gen Appl Microbiol 28:7–22