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Pseudomonas extremaustralis sp. nov., a Poly(3-hydroxybutyrate) Producer Isolated from an Antarctic Environment

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Abstract A Gram-negative, mobile, rod-shaped, non-spore-forming bacterium (strain 14-3^T) was isolated from a temporary pond in Antarctica. On the basis of 16S rRNA gene sequence similarity, strain 14-3^T was shown to belong to the genus *Pseudomonas sensu stricto.* Physiological and biochemical tests supported the phylogenetic affiliation. Strain 14-3^T is closely related to *Pseudomonas veronii* DSM 11331^T, sharing 99.7% sequence similarity. DNA–DNA hybridization experiments between the two strains showed only moderate reassociation similarity (35.1%). Tests for arginine dihydrolase and nitrate reduction were positive, while those for denitrification, indol production, glucose acidification, urease, ß-galactosidase, esculin, caseine and gelatin hydrolysis were negative. Growth of this bacterium occurred in a range from 4 to 37°C but not at 42°C. It accumulated poly(3-hydroxybutyrate) when grown on sodium octanoate medium. Strain 14-3^T therefore represents the type strain of a new species, for which the name *Pseudomonas extremaustralis* sp. nov. is proposed. The type strain 14-3^T has been deposited as DSM 17835^T and as CIP 109839^T.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 14-3^T is AJ583501.

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Introduction

The genus *Pseudomonas,* first described by Migula in 1894 [26], currently comprises a large number of species. Over the years, many studies have been conducted on this genus to differentiate it from other genera and to define intrageneric clusters [2, 25]. Due to their metabolic versatility and ubiquity in nature, new species of the genus inhabiting different kinds of environments are constantly described, including species from extreme areas such as Antarctica [8, 10, 22, 24, 30, 32].

Pseudomonas sp. 14-3 is a strain isolated from a temporary pond in Antarctica. This strain shows a high heat and oxidative stress resistance [3] and increased cold tolerance [6] in association with its high capacity of accumulate large amounts of polyhydroxybutyrate (PHB), the best known polyhydroxyalkanoate (PHA). We have previously performed several genetic studies regarding PHA metabolism of this strain [4, 5]. In this work, we characterized strain 14-3. Polyphasic taxonomy revealed that it represents a novel species for which the name *Pseudomonas extremaustralis* sp. nov. is proposed.

Methods

Sampling Area and Isolation Procedure

Samples from several Antarctic habitats at Cierva Point, located on the Danco Coast on the west side of the Antarctic Peninsula, at 64°09′S, 60°57′W, were screened for

PHA producing bacteria. Samples from this area were taken during cruise CAV95 from Instituto Antártico Argentino (IAA) in 1995. During a study devised for the detection of spore-forming PHA producers, water samples from a temporary water body were heated at 80°C during 10 min and then plated on nutrient agar (NA) supplemented with 30 mM sodium octanoate and incubated at 30°C as described [3] . PHA accumulation was detected by microscopic Nile Blue stain [28] and verified by gas chromatography [9]. Strain 14-3^{T} was chosen because of its high PHB production (80% polymer/dry weight).

Morphological and Biochemical Characterization

Bacterial cultures in different growth phases were used to check bacterial shape and motility by microscopy. Cells were stained according to the classical Gram procedure. Several biochemical determinations including catalase, amylase, glycerol and L-phenylalanine utilization, casein hydrolysis and denitrification were performed as individual tests. Oxidase testing was assayed using commercial disks (Britania, Argentina). Other biochemical tests were carried out using API 20 NE (bioMérieux, Argentina).

Phylogenetic Analysis

DNA extraction and PCR amplification were performed as described by Rainey and Stackebrandt [29]. The 16S rRNA gene was amplified using a universal primer set corresponding to positions 8-27 (forward primer) and 1505-1525 (reverse primer) based on *Escherichia coli* sequence [29]. The sequence of the 16S ribosomal RNA gene was determined using a SeqiTherm Long-Read cycle sequencing kit (Epicentre Technologies, Madison, WI, USA) in a model 4000L semiautomatic DNA sequencer (LI-COR). The nucleotide sequence of the 16S rRNA gene of strain 14-3^{T} is available under EMBL Nucleotide Sequence Database accession number AJ583501. The 16S rRNA gene sequence was initially analyzed by using BLAST program (http://www.ncbi.nlm.nih.gov/blast) [1]. Multiple alignment of sequences was performed using CLUSTAL X [34]. The phylogenetic tree was constructed using the neighbor-joining method of Saitou and Nei [31], and checked by UPGMA and maximum parsimony methods all included in MEGA 3 package [23]. The tree topology was tested by bootstrap analysis of 1000 resamplings. *Pseudomonas aeruginosa* was used as an outgroup.

DNA–DNA Hybridization

DNA was isolated using a French pressure cell (Thermo Spectronic, Rochester, NY, USA) and was purified by hydroxyapatite chromatography as described by Cashion et al. [11]. DNA–DNA hybridization was carried out as described by De Ley et al. [12], with the modifications of Huss et al. [17] and Escara and Hutton [16], using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostated 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian, Victoria, Australia).

Fatty Acids Profile

Fatty acids were extracted and analyzed [27] according to the standard protocol of the Microbial Identification System (MIDI; Microbial ID). Extracts were analyzed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame ionization detector as described by Kämpfer and Kroppenstedt [19].

Results and Discussion

The phenotypic characterization showed this isolate to be a Gram-negative, non-sporulating bacterium with straight and mobile rods. It grew on E minimal medium [35], presented a non-fermentative metabolism on a wide range of sugars, was oxidase-positive and synthesized a fluorescent pigment when grown on King B medium [21]. Growth of this bacterium was observed from 4 to 37°C but not at 42°C. No reaction was observed for indol production, glucose acidification, urease, ß-galactosidase, esculin, caseine, lecithin, starch and gelatin hydrolysis, whilst arginine dihydrolase and nitrate reduction were positive. The strain was capable of utilizing D-glucose, L-arabinose, D-mannitol, potassium gluconate, glycerol, capric acid, adipic acid, malic acid and trisodium citrate. D-Mannose, *N*-acetyl-glucosamine, L-phenylalanine and D-maltose were not utilized. The phenotypic studies showed that the isolate displayed characteristics consistent with those described for the genus *Pseudomonas*.

Comparison of the 16S rRNA gene sequence of strain 14-3^{T} with those of other type strains of *Pseudomonas* showed the highest sequence similarity with *Pseudomonas veronii* DSM 11331^{T} (99.7% identity), followed by *Pseudomonas trivialis* DSM 14937^{T} (99.6% identity) and *Pseudomonas poae* DSM 14936^{T} (99.5% identity). Comparison of phenotypic characteristics that differentiate strain 14-3^{T} from *P. veronii, P. poae* and *P. trivialis* are shown in Table 1. Strain 14-3^{T} exhibited minor sequence similarity with the Antarctic isolates, *Pseudomonas antarctica* DSM 15318^{T} (98.7%), *Pseudomonas meridiana* CMS 38^{T} (98.6 %), *Pseudomonas proteolytica* CMS 64^{T} (98.2%) and *Pseudomonas guineae* LMG 24016^{T} (95. 3%), and differed from these species in several phenotypic characters (Table 1). Strain 14-3^{T} and *P. veronii* presented

Table 1 Phenotypic characteristics that differentiate strain 14-3^T from other closely phylogenetically related and Antarctic species

Characteristic	1	2	3	4	5	6	7	8
Gelatin hydrolysis	−	D	D	D	−	−	+	−
Arginine dihydrolase	+	+	−	−	−	−	−	−
Esculin hydrolysis	−	−	+	+	−	−	−	−
Urease	−	D	NR	NR	+	W	−	−
PHB production	+	−	NR	NR	−	−	−	NR
Production of fluorescent pigment on King's B medium	+	+	+	+	−	+	NR	−
Oxidase	+	+	W	W	+	+	+	+
Nitrate reduction	+	+	−	−	+	+	+	−
Denitrification	−	+	−	−	NR	NR	NR	−
Utilization of								
D-Mannose	−	+	+	+	+	+	+	−
N-Acetyl-glucosamine	−	D	D	D	NR	NR	NR	−
D-Phenylalanine	−	+	−	−	NR	NR	+	NR

1 = strain 14-3^T; 2 = *P. veronii* DSM 11331^T; 3 = *P. poae* DSM 14936^T; 4 = *P. trivialis* DSM 14937^T; 5 = *P. antarctica* DSM 15318^T; 6 = *P. meridiana* CMS 38^T; 7 = *P. proteolytica* CMS 64^T; 8 = *P. guineae* LMG 24016^T). Data were obtained in this and other studies [7, 8, 15, 18, 30]

D reaction differs among strains, *NR* not reported, *W* weakly positive

negative esculin hydrolysis, positive arginine dihydrolase and strong (highly positive) oxidase reaction. Multiple alignment of rRNA gene sequences from strain 14-3^T and other closely related type strains (31 type strains with identity higher than 95%) was used to construct a phylogenetic tree. The resulting tree (Fig. 1) confirmed that strain 14-3^T is a member of the genus *Pseudomonas* closely related to members of the fluorescent group as defined by Anzai et al. [2] . However, strain 14-3^T did not form a robust clade with other *Pseudomonas* which had close percentage of sequence similarity (Fig. 1). A similar result was observed for *P. veronii* which, according to the present tree and previously published data [7, 18, 30], neither formed a robust clade with its closest relatives (Fig. 1).

The comparison of 16S rRNA gene sequences and the phenotypic characterization showed that *P. veronii* DSM 11331^T is the closest relative to strain 14-3^T. Therefore, we determined the genomic relatedness between strain 14-3^T and *P. veronii* DSM 11331^T, by the spectrophotometric DNA–DNA reassociation method. The resulting reassociation value of 35.1% was sufficiently low to separate them into two different genospecies. We also compared the fatty acid profile between strain 14-3^T and *P. veronii* DSM 11331^T. Unsaturated $C_{16:1\varpi}7c$ was the dominant fatty acid in the cell wall of strain 14-3^T (Table 2). Comparison of the fatty acid profile of both strains revealed a similar composition with mainly quantitative differences (Table 2).

Strain 14-3^T accumulates PHB using octanoate as sole carbon source, but not when grown in medium containing glucose. It was shown that PHB accumulation confers high heat tolerance to this strain [3]. This fact could explain its unexpected isolation by a method unsuitable for the members of this genus (heating at 80°C under experiments for the detection of spore-former bacteria). Genetic analysis demonstrated that strain 14-3^T has a complete PHB biosynthesis cluster containing genes *phaR*, *phaA*, *phaB* and *phaC* and that impaired PHB production from glucose is due to a defective *phaA* gene [4]. These genes are located within a genomic island that also contains other genes probably related with its adaptability to Antarctic environments [5]. Inability of PHB-negative mutants to grow and survive under low-temperature conditions constitutes an evidence of the relevance of PHB genes for adaptability to cold environments [6]. According to data available in the literature [15] and analysis performed in our laboratory using glucose and sodium octanoate as carbon sources, *P. veronii*, the closest phylogenetic relative of strain 14-3^T, does not accumulate this polymer (data not shown). Strain 14-3^T accumulated PHB up to 70–80% of its cell dry weight when grown on sodium octanoate [3, 4]. PHB production is a rare characteristic among *Pseudomonas* species, which usually accumulate PHA composed by medium-chain-length monomers when grown on fatty acids or carbohydrates [13, 33], and it has been proposed as an useful negative taxonomic criterion to differentiate bacterial species at the genus level [20]. However, species of the so-called "*P. oleovorans* group" produce PHB during cultivation on octanoate [14]. *Pseudomonas* sp. 14-3 is not phylogenetically related to species of this group.

As strain 14-3^T shows sufficient phenotypic differences and moderate DNA–DNA similarity to its closest relative,

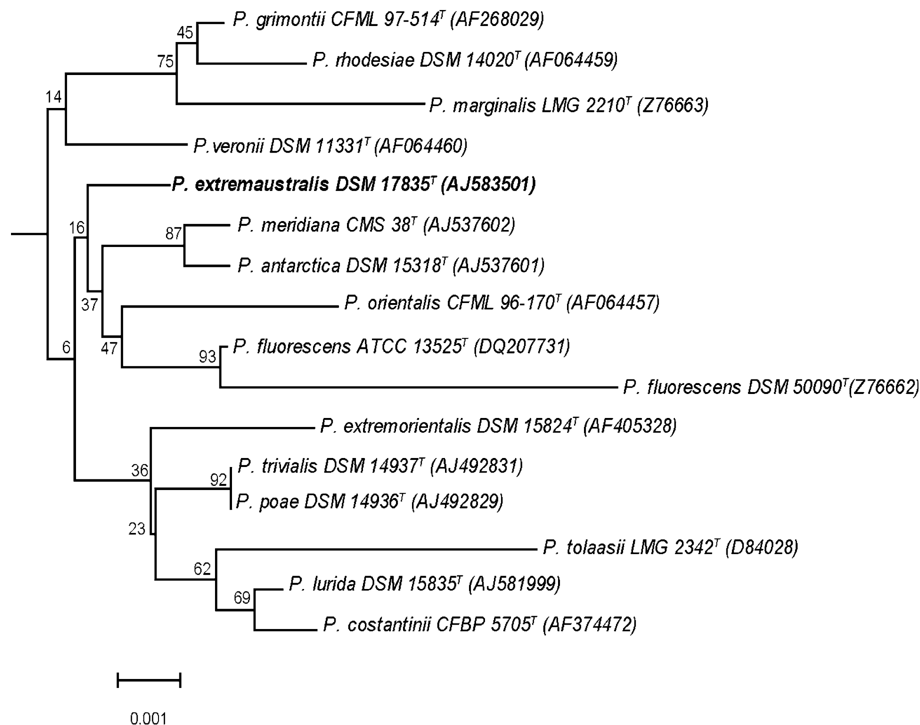

Fig. 1 Phylogenetic tree obtained by neighbor-joining analysis of 16S rRNA gene sequences, showing the position of the Antarctic isolate 14-3^{T} among neighboring species of the genus *Pseudomonas*. Percentages at nodes are levels of bootstrap support based on neighbor-joining analyses of 1000 re-sampled datasets. *P. aeruginosa* DSM 50071^{T} (X06684) was used as outgroup (not shown). Bar 0.001 substitutions per nucleotide position. The accession number of each reference species (DDBJ/EMBL/GenBank) is shown in parenthesis. *LMG* The Belgian Co-ordinated Collections of Microorganisms, *CFBP* Collection Française des Bactéries Phytopathogènes, *CFML* Collection de la Faculté de Médicine de Lille, *ATCC* American Type Culture Collection, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen

Table 2 Cellular fatty acid compositions (%) of strain 14-3^{T} and *P. veronii* DSM 11331^{T}

Fatty acids	Strain 14-3	*P. veronii* DSM 11331^{T}
$C_{10:0}$	0.15	0.18
$C_{10:0}$ 3OH	3.63	3.29
$C_{12:0}$	2.66	2.39
$C_{12:0}$ 2OH	4.60	4.32
$C_{12:0}$ 3OH	3.84	3.48
$C_{14:0}$	0.43	0.69
$C_{16:1\varpi}7c$	37.53	31.45
$C_{16:0}$	29.49	27.96
$C_{17:0}$	0.15	–
$C_{17:0}$ cyclo	1.60	3.58
$C_{18:1\varpi}7c$	15.09	20.80
$C_{18:0}$	0.49	0.83
$C_{11\ \mathrm{methyl}\ 18:1\varpi}7c$	0.11	0.27
$C_{19:0}$ cyclo$_{\varpi}$8c	–	0.56

Empty fields (–) indicate that fatty acids were not detected

P. veronii DSM 11331^{T}, we propose a new species, *Pseudomonas extremaustralis* sp. nov., with the type strain 14-3^{T} (DSM 17835^{T} = CIP 109839^{T}).

Description of *P. extremaustralis* sp. nov.

Pseudomonas extremaustralis (ex.tre.m.aus.tra' lis L. masc.adj. *extrĕmus* furthest end, utmost point; L. fem. adj. australis austral, Southern; N.L. fem. adj. *extremaustralis* from the extreme South, the region of isolation, i.e. Far Southern Antarctica).

Cells are motile, non-spore-forming rods. Gram-negative, oxidase-positive, catalase-positive showing an oxidative metabolism. It produces fluorescent pigment on King B medium. Growth on nutrient agar plates occurs at 4–37°C but not at 42°C. Colonies on this medium are smooth, circular and non-pigmented. PHB is accumulated using sodium octanoate as carbon source. Tests for indol production, glucose acidification, urease, ß-galactosidase, esculin, caseine, lecithin, starch and gelatin hydrolysis are negative, whilst arginine dihydrolase and nitrate reduction are positive. Negative for denitrification. The strain utilizes D-glucose, L-arabinose, D-mannitol, potassium gluconate, glycerol, capric acid, adipic acid, malic acid and trisodium citrate. D-Mannose, *N*-acetyl-glucosamine, L-phenylalanine and D-maltose are not utilized. The fatty acid profile is indicated in Table 2.

Isolated from a temporary water pond in Antarctica (64°09′S, 60°57′W). The type strain is CT14-3^T (= DSM 17835^T = CIP 109839^T).

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