Pseudomonas duriflava sp. nov., isolated from a desert soil

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A Gram-negative, rod-shaped, non-motile, non-spore-forming bacterium, designated strain HR2^T, was isolated from a soil sample from the Taklimaken Desert in Xinjiang Province, China. Strain HR2^T grew optimally at pH 7.0–8.0 and 30–37 °C in the presence of 0–1 % (w/v) NaCl. An analysis of 16S rRNA gene sequences revealed that strain HR2^T fell within the radiation of the genus *Pseudomonas*, the highest level of similarity being found with respect to *Pseudomonas luteola* IAM 13000^T (97.5 %); the levels of sequence similarity with respect to other recognized *Pseudomonas* species were <96.4 %. DNA–DNA hybridization showed that the genetic relatedness between strain HR2^T and *P. luteola* IAM 13000^T was 53.2 %. The G + C content of the genomic DNA of strain HR2^T was 55.2 mol%. The major fatty acids were 18 : 1, summed feature 3 and 16 : 0. The hydroxylated fatty acids 10 : 0 3-OH, 12 : 0 3-OH and 12 : 0 2-OH were also present. The data obtained in this polyphasic study indicated that this isolate represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas duriflava* sp. nov. is proposed. The type strain is HR2^T (=KCTC 22129^T=CGMCC 1.6858^T).

The genus *Pseudomonas sensu stricto* originally comprised only species belonging to *Pseudomonas* RNA homology group I (Palleroni, 1984) in the *Gammaproteobacteria* (Kerstens *et al.*, 1996). However, the genus was subdivided into two main intragenetic clusters (as reviewed by Anzai *et al.*, 2000): the first cluster comprised six groups and the second had only one group. *Pseudomonads* are abundant in various environments, such as water, air and soil. In our search for organisms capable of growing in desert ecosystems, a bacterial strain, designated HR2^T, was isolated and analysed using polyphasic taxonomy. An analysis of 16S rRNA gene sequence similarities indicated that the isolate was closely related to the *Pseudomonas aeruginosa*/*Pseudomonas stutzeri* group within the first cluster and formed a distinct line. On the basis of phenotypic and genotypic evidence, it is proposed that strain HR2^T represents a novel species of the genus *Pseudomonas*.

Strain HR2^T was isolated from a desert soil sample by using the usual dilution plating technique on Luria–Bertani (LB) agar (1⁻¹: 5 g yeast extract, 10 g tryptone, 10 g NaCl, pH 7.0) incubated for 2 days at 30 °C under aerobic conditions. The isolate was cultivated routinely on LB agar or in LB broth at the same temperature. The strain was maintained in a glycerol suspension (20 %, v/v) at –70 °C.

Amplification and sequencing of the 16S rRNA gene were performed according to a method described by Cui *et al.* (2001), but which was modified slightly by us: the primers used for the PCR amplification were 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-AAGGAGGTGATCCAAGCCGCA-3' (reverse), corresponding to positions 8–27 and 1521–1540, respectively, in the 16S rRNA sequence of *Escherichia coli* (Brosius *et al.*, 1978). The resultant sequence (1404 bp) of strain HR2^T was compared with sequences available from the GenBank database, using a BLAST search to determine an approximate phylogenetic affiliation. Phylogenetic trees were inferred using PHYLIP (Felsenstein, 1993) and MEGA (version 3.1; Kumar *et al.*, 2001) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (using distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HR2^T is EU046271.

Transmission electron micrographs of strain HR2^T are available as supplementary material with the online version of this paper.

the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. The bootstrap resampling method of Felsenstein (1985) (1000 replicates) was used to assess the reliability of the phylogenetic trees. Comparisons of 16S rRNA gene sequences showed that strain HR2^T should be positioned within the genus *Pseudomonas*, being most closely related to *Pseudomonas luteola* IAM 13000^T (97.5 % similarity); the sequence similarities with respect to type strains of other established *Pseudomonas* species were less than 96.4 %. A phylogenetic tree based on the neighbour-joining method indicated that strain HR2^T formed a monophyletic clade adjacent to *P. luteola* IAM 13000^T with high bootstrap support (94 %) (Fig. 1).

Chromosomal DNA from strain HR2^T was extracted and purified according to the procedure of Yoon *et al.* (1996). The DNA G+C content was measured according to the method of Cashion *et al.* (1997) and was found to be 55.2 mol%. This value is slightly below the range (58–70 mol%) mentioned by Palleroni (1984) for the genus *Pseudomonas* and is close to that of *P. luteola* IAM 13000^T

(54.6 mol%; data from this study). DNA–DNA hybridization between strain HR2^T and *P. luteola* IAM 13000^T was measured as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program described by Jahnke (1992). At the DNA–DNA level, there was 53.2 % genetic relatedness between strain HR2^T and *P. luteola* IAM 13000^T; as the recommended threshold value for the delineation of genomic species is considered to be 70 % DNA–DNA relatedness (Stackebrandt *et al.*, 2002), these two strains could be confirmed as belonging to different species.

Fatty acid methyl esters were prepared and identified according to the instructions of the Microbial Identification system (MIDI), as described by Sasser (1990), from cultures of strain HR2^T grown on tryptic soy agar (Merck) for 2 days at 30 °C. Type strains of the closely related species *P. luteola* (IAM 13000^T) and *Pseudomonas oryzihabitans* (IAM 1568^T) were also cultured under the same nutrient and temperature conditions and characterized concurrently with strain HR2^T. The results of

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain HR2^T and some related *Pseudomonas* species. Bootstrap percentages (based on 1000 resamplings) greater than 50 % are shown at nodes. The maximum-likelihood tree showed essentially the same topology (data not shown). Bar, 0.005 substitutions per nucleotide position.

Table 1. Fatty acid content (%) of strain HR2^T and the type strains of phylogenetically related species of the genus *Pseudomonas*

Strains: 1, HR2^T; 2, *P. luteola* IAM 13000^T; 3, *P. oryzihabitans* IAM 1568^T; 4, *Pseudomonas psychrotolerans* LMG 2197^T; 5, *Pseudomonas oleovorans* IAM 1508^T; 6, *P. stutzeri* CCUG 11256^T. For 1–3, data are from this study; for 4 and 5, data are from Hauser *et al.* (2004); for 6, data are from Manaia & Moore (2002). tr, Trace (<1.0%); –, not detected.

Fatty acid	1	2	3	4	5	6
10:0 3-OH	2.1	2.0	2.8	2.6	3.3	3.2
12:0 3-OH	3.5	4.3	3.9	3.0	3.3	3.2
12:0 2-OH	0.7	1.5	2.1	1.8	–	–
10:0	0.2	0.1	–	–	0.2	tr
11:0	–	–	–	–	0.1	tr
12:0	6.6	6.9	5.8	4.5	7.7	7.5
14:0	0.8	1.7	–	–	0.2	1.3
15:0	–	–	–	–	0.2	tr
16:0	11.2	23.0	21.5	22.6	12.0	19.7
cyclo-17:0	–	–	0.5	–	0.6	tr
18:1	46.3	35.6	41.8	49.1	46.5	36.5
18:0	0.7	0.6	0.3	–	0.5	tr
cyclo-19:0 ω 8c	4.6	6.8	2.7	–	–	tr
Summed feature 3*	22.8	15.7	17.1	16.4	22.9	27.1

*16:1 ω 7c and/or iso-15:0 2-OH.

the chemotaxonomic analyses are given in Table 1; all of the strains were shown to have rather similar fatty acid profiles. The presence of high levels of 18:1, summed feature 3 and 16:0, along with 10:0 3-OH, 12:0 3-OH and 12:0 2-OH, supported the view that strain HR2^T belonged to the genus *Pseudomonas* (Oyaizu & Komagata, 1983).

Cell morphology and motility were examined using light microscopy (model BH 2; Olympus) and transmission electron microscopy (model H-7650; Hitachi) after 48 h growth on LB agar at 30 °C. For the transmission electron microscopy observations, cells were negatively stained with 1% (w/v) phosphotungstic acid and air-dried (see Supplementary Fig. S1 available in IJSEM Online). Gram-staining and endospore formation were investigated according to the methods described by Smibert & Krieg (1994). Growth at different temperatures and pH values was investigated as described by Xu *et al.* (2005), using LB as the basal medium. NaCl tolerance was tested using LB broth supplemented with 0, 1, 2, 3, 4 or 5% (w/v) NaCl. Growth was tested on nutrient agar, Simmons' citrate agar and MacConkey agar. The production of fluorescent pigment was tested on King's medium B (King *et al.*, 1954). Oxidase, catalase and phenylalanine deaminase activities and the hydrolysis of casein, gelatin, Tweens 80 and 20, aesculin, DNA, starch and carboxymethylcellulose were investigated as described by Smibert & Krieg (1994). Acid production from carbohydrates was determined using the medium and method described by Yamaguchi & Yokoe (2000). Studies designed to determine the utilization of substrates as single carbon sources were carried out as described by Zhou *et al.* (2007). Sensitivity to antibiotics was tested using the diffusion method, as described by Park *et al.* (2007), on LB agar at 30 °C with filter-paper discs

(8 mm diameter; Sanofi Pasteur) containing the following antibiotics: streptomycin (10 µg), penicillin G (10 IU), ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), tetracycline (30 µg), ofloxacin (5 µg), amoxicillin (10 µg), cefamezin (30 µg), rifampicin (5 µg), gentamicin (10 µg), ceftazidime (30 µg), vancomycin (30 µg), kanamycin (30 µg), carbenicillin (100 µg) and polymyxin B (30 µg). All tests were performed at 30 °C and properties were recorded after 2 days. Additional biochemical features were tested using API 20E kits according to the instructions of the manufacturer (bioMérieux). *P. luteola* IAM 13000^T and *P. oryzihabitans* IAM 1568^T were included in these studies and analysed under the same conditions. Phenotypic characteristics of strain HR2^T are given in the species description and in Table 2. As shown in Table 2, our isolate can be readily differentiated from its closest phylogenetic relatives in the genus *Pseudomonas sensu stricto* on the basis of several phenotypic properties.

The data presented indicate that strain HR2^T is distinguishable from all recognized species of the genus *Pseudomonas*. Thus HR2^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas duriflava* sp. nov. is proposed.

Description of *Pseudomonas duriflava* sp. nov.

Pseudomonas duriflava (du.ri fla'va. L. adj. *durus* hard; L. adj. *flavus* yellow; N.L. fem. adj. *duriflava* hard yellow).

Cells are Gram-negative, non-motile, non-spore-forming, round-ended, aerobic rods approximately 0.6–0.8 × 0.7–1.1 µm in size. Colonies on LB agar are dry, yellow, circular, convex, translucent, hard, wrinkled and approximately 1.0–2.5 mm in diameter within 2 days at 30 °C.

Table 2. Distinctive phenotypic characteristics of strain HR2^T and related *Pseudomonas* species

Strains: 1, HR2^T; 2, *P. luteola* IAM 13000^T; 3, *P. oryzihabitans* IAM 1568^T; 4, *Pseudomonas psychrotolerans* LMG 21977^T; 5, *Pseudomonas oleovorans* IAM 1508^T; 6, *P. stutzeri* CCUG 11256^T. For 1–3, data are from this study; for 4, data are from Hauser *et al.* (2004). +, Positive; –, negative; d, reaction differs between strains; ND, not determined.

Characteristic	1	2	3	4	5	6
Yellow pigment	+	+	+	+	–*	d‡
Growth at:						
4 °C	–	–	+	+	–*	–‡
42 °C	+	+	–	–	+	ND
Tolerance of 5 % NaCl	–	+	+	+	+	+
Nitrate reduction	–	+	–	ND	ND	+
Hydrolysis of:						
Gelatin	–	+	+	ND	–†	–‡
Starch	+	–	–	ND	–†	–‡
Tween 80	+	+	+	ND	–†	–‡
Aesculin	+	+	–	–	–*	ND
Acid production from:						
D-Xylose	+	+	+	+	–*	ND
D-Mannose	–	+	+	+	–*	ND
L-Rhamnose	+	+	–	d	–*	ND
Maltose	–	–	+	d	–*	ND
Trehalose	–	+	+	+	–*	ND
Salicin	–	+	–	–	–*	ND
Utilization of:						
Gluconate	–	+	+	+	–*	d‡
D-Mannose	–	+	+	+	–*	–‡
D-Xylose	+	+	+	+	–*	–‡
Maltose	–	+	+	+	–*	–‡
Trehalose	–	+	+	+	–*	–‡
D-Sorbitol	–	–	+	+	–*	ND
Mannitol	–	+	+	+	–*	d‡

*Hauser *et al.* (2004).

†Yumoto *et al.* (2001).

‡Romanenko *et al.* (2005).

§Hatayama *et al.* (2005).

Fluorescent pigments are not produced on King's medium B. The temperature, pH and NaCl ranges for growth are 5–42 °C (optimum, 30–37 °C), pH 5.0–9.0 (optimum, pH 7.0–8.0) and 0–2 % (optimum, 0–1 %). Good growth occurs on nutrient agar, MacConkey agar and Simmons' citrate agar. Casein, starch, DNA, Tween 80 and aesculin are hydrolysed, but gelatin, Tween 20 and carboxymethyl-cellulose are not hydrolysed. Positive for catalase, lysine decarboxylase, arginine dihydrolase and β -galactosidase activities. Negative for oxidase, arginine decarboxylase, ornithine decarboxylase, urease, phenylalanine deaminase, indole and hydrogen sulphide production, nitrate reduction and in the Voges–Proskauer reaction. Acid is produced from D-xylose, D-glucose, L-rhamnose and dextrin, but not from D-galactose, D-mannose, maltose, D-fructose, trehalose, D-mannitol, myo-inositol or salicin.

The following substrates are utilized (with no acid production): D-ribose, D-galactose, L-arabinose, sorbose, cellobiose, melezitose, melibiose, aesculin and amygdalin. Lactose, sucrose, maltose, D-fructose, trehalose, raffinose, D-mannose, turanose, glycerol, D-erythritol, D-arabitol, D-mannitol, sorbitol, D-xylitol, adonitol, myo-inositol, inulin, salicin, N-acetylglucosamine, gluconate, acetate and methyl α -glucoside are not utilized. Sensitive to streptomycin, penicillin G, ampicillin, chloramphenicol, erythromycin, tetracycline, ofloxacin, amoxicillin, cefamezin, rifampicin, gentamicin, ceftazidime, kanamycin, carbenicillin and polymyxin B, but resistant to vancomycin. DNA G+C content is 55.2 mol%. The fatty acid pattern is shown in Table 1.

The type strain, HR2^T (=KCTC 22129^T=CGMCC 1.6858^T), was isolated from a desert soil sample collected from the Taklimaken Desert in Xinjiang Province, China.

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