Pleomorphomonas koreensis sp. nov., a nitrogen-fixing species in the order *Rhizobiales*

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A Gram-negative, non-motile, non-spore-forming, rod-shaped bacterium (strain Y9^T) was isolated from a contaminated culture of the phototrophic bacterium *Rhodospseudomonas palustris*, and was investigated using a polyphasic taxonomic approach. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain Y9^T belonged to the order *Rhizobiales* in the *Alphaproteobacteria*. Comparison of phylogenetic data indicated that it was most closely related to *Pleomorphomonas oryzae* (98.5% similarity of 16S rRNA gene sequence), and the phylogenetic distance from any other species of the order *Rhizobiales* with a validly published name was greater than 7.5% (i.e. less than 92.5% similarity). The predominant ubiquinone was Q-10 and the major fatty acids were C_{18:1}, C_{16:0}, C_{19:0} cyclo ω 8c and C_{18:0}. The G + C content of genomic DNA of strain Y9^T was 65.1 mol%. The results of DNA–DNA hybridization in combination with chemotaxonomic and physiological data demonstrated that strain Y9^T represents a novel species within the genus *Pleomorphomonas*, for which the name *Pleomorphomonas koreensis* sp. nov. is proposed. The type strain is Y9^T (=KCTC 12246^T=NBRC 100803^T).

In the most recent edition of *Bergey's Manual of Systematic Bacteriology* (Garrity & Holt, 2001), the order *Rhizobiales* is the biggest group within the α -2 subgroup of the *Proteobacteria* and constitutes a heterogeneous group from both a physiological and an ecological perspective. According to *Bergey's Manual*, the order *Rhizobiales* is divided into ten families on the basis of 16S rRNA gene phylogenetic analyses: *Bartonellaceae*, *Beijerinckiaceae*, *Bradyrhizobiaceae*, *Brucellaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, *Methylocystaceae*, *Phyllobacteriaceae*, *Rhizobiaceae* and *Rhodobiaceae*. The order *Rhizobiales* is characterized chemotaxonomically by its major respiratory ubiquinone (Q-10) and by the presence of octadecenoic acid (C_{18:1}) as a major cellular fatty acid (like other members of the *Alphaproteobacteria*), and it contains plant and animal pathogens associated with eukaryotic cells (e.g. the families *Bartonellaceae* and *Brucellaceae*), plant endosymbionts (*Rhizobiaceae*), methane-oxidizing bacteria (*Methylobacteriaceae* and *Methylocystaceae*) and, in particular, a large number of nitrogen-fixing species (distributed throughout almost all of its families).

In this study, a nitrogen-fixing, rod-shaped strain (Y9^T) was isolated from a contaminated culture of the phototrophic bacterium *Rhodospseudomonas palustris*. On the basis of 16S rRNA gene sequence data, strain Y9^T was found to be closely related to *Pleomorphomonas oryzae* (order *Rhizobiales* of the *Alphaproteobacteria*) and was subjected to a taxonomic investigation. The aim of this study was to determine the taxonomic position of strain Y9^T by using chemotaxonomic, physiological and DNA–DNA hybridization analyses. The results provided evidence that Y9^T is a representative of a novel bacterial species.

Strain Y9^T was isolated from a contaminated culture of the phototrophic bacterium *R. palustris* by direct plating on R2A agar (Difco). For analysis of quinones and DNA extraction, cell biomass was obtained from a culture grown in R2A broth at 30 °C. Strain Y9^T was cultivated on a horizontal shaker at 150 r.p.m., and the purity was checked using a light microscope prior to harvesting by centrifugation. Unless indicated otherwise, the strain was grown on R2A agar or broth incubated at 30 °C and maintained as a glycerol suspension (20%, w/v) at –70 °C.

Cell morphology and motility were observed under a Nikon light microscope ($\times 1000$ magnification) using cells grown for 2 days at 30 °C on R2A agar. The Gram reaction was performed using the non-staining method described by

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Y9^T is AB127972.

Buck (1982). Tests for catalase and oxidase were performed by using the procedures outlined by Cappuccino & Sherman (2002). Growth at various temperatures (4, 15, 25, 30, 37 and 42 °C) was investigated on R2A agar. Growth at 30 °C on nutrient agar, trypticase soy agar and MacConkey agar was also examined. Utilization of various substrates as sole carbon sources was investigated, together with some physiological characteristics, using the API 32GN and API 20NE galleries according to the instructions of the manufacturer (bioMérieux).

Nitrogen-fixing ability was determined by growing strain Y9^T in 50 ml nitrogen-free medium (DSMZ medium no. 3; Deutsche Sammlung von Mikroorganismen und Zellkulturen) contained in a 125 ml serum bottle. The medium contained the following (in 1 l distilled water): 5.0 g glucose, 5.0 g mannitol, 0.1 g CaCl₂·2H₂O, 0.1 g MgSO₄·7H₂O, 5.0 mg Na₂MoO₄·2H₂O, 0.9 g K₂HPO₄, 0.1 g KH₂PO₄, 0.01 g FeSO₄·7H₂O, 5.0 g CaCO₃ and 1 ml trace element mixture. The trace element mixture (SL-6, in DSMZ medium no. 27) contained the following (1⁻¹ distilled water): 0.1 g ZnSO₄·7H₂O, 0.03 g MnCl₂·4H₂O, 0.3 g H₃BO₃, 0.2 g CoCl₂·6H₂O, 0.01 g CuCl₂·2H₂O and 0.02 g NiCl₂·6H₂O. Acetylene reduction was performed for all liquid cultures by injecting purified acetylene into appropriate containers closed with rubber stoppers to yield 15 % acetylene (v/v); this was followed by incubation for up to 24 h. Ethylene was measured using a Hewlett Packard 5890A gas chromatograph equipped with a flame-ionization detector and a prepacked column (HayeSep N; Supelco). Additionally, the presence of the *nifH* gene was determined by a PCR with primers and reaction conditions as described by Poly *et al.* (2001).

The G + C content of the chromosomal DNA of strain Y9^T was determined as described by Mesbah *et al.* (1989), using reversed-phase HPLC. Quinones were extracted from cells grown on nutrient broth (Difco), and analysed as described by Shin *et al.* (1996), using reversed-phase HPLC. Cellular fatty acids were analysed in organisms grown on trypticase soy agar (Difco) for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids, analysed by GC (6890; Hewlett Packard), were identified using the Microbial Identification software package (Sasser, 1990). DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989), with photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values are quoted as DNA relatedness values.

PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim *et al.* (2005). The complete 16S rRNA gene sequence was compiled using SeqMan software (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from

the GenBank database. Multiple alignments were performed using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). The phylogenetic tree was constructed by using a neighbour-joining method (Saitou & Nei, 1987) and maximum parsimony (Fitch, 1972) in the MEGA 3 program (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985).

Strain Y9^T consisted of Gram-negative, non-spore-forming, non-motile rods. The cells were 0.3–0.5 µm in diameter and 1.5–3.0 µm in length. After 2 days incubation on R2A agar, colonies were smooth, low-convex, circular, translucent, non-shiny and white in colour. Strain Y9^T grew at 15–42 °C on R2A agar but not at 4 °C or above 45 °C. Strain Y9^T was able to reduce acetylene to ethylene at a mean rate of 57.5 µmol min⁻¹ (mg wet weight)⁻¹ at an incubation temperature of 30 °C. An internal region of *nifH* (about 360 bp) was clearly amplified, and strain Y9^T grew well in a nitrogen-free liquid medium. Other physiological characteristics of strain Y9^T are summarized in the species description, and some characteristics that differentiate it from *P. oryzae* F-7^T are presented in Table 1.

The 16S rRNA gene sequence of strain Y9^T determined in this study was a continuous stretch of 1417 bp. A sequence similarity calculation performed after a neighbour-joining analysis indicated that the closest relative of strain Y9^T was *P.*

Table 1. Characteristics that differentiate strain Y9^T from *P. oryzae* F-7^T

Both strains are Gram-negative, non-motile, white-pigmented, rod-shaped bacteria. Both strains exhibit catalase, oxidase, urease and β-galactosidase activities, fix nitrogen and possess the *nifH* gene and reduce nitrate to nitrite. Both strains are positive for the assimilation of L-rhamnose, D-glucose, D-mannose, L-arabinose, D-ribose, sucrose, D-fructose, inositol, D-mannitol and D-sorbitol and negative for the assimilation of L-alanine, L-histidine, 3-hydroxybutyrate, malonate and itaconate.

Characteristic	<i>P. koreensis</i> Y9 ^T	<i>P. oryzae</i> F-7 ^T
Indole production	–	+
β-Glucosidase (aesculin hydrolysis)	+	–
Protease (gelatin hydrolysis)	+	–
Acid from glucose	–	+
Utilization as carbon source		
L-Proline	–	+
L-Serine	–	+
Acetate	–	+
Malate	–	+
D-Maltose	+	–
Gluconate	–	+
DNA G + C content (mol%)	65.1	63.1

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of strain Y9^T and related taxa. Filled circles at nodes indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1000 replications) greater than 65 % are shown at the branch points. Bar, 0.02 substitutions per nucleotide position.

oryzae F-7^T (98.5 %). Lower levels of similarity (<92.5 %) were found with respect to all available sequences of species of the *Rhizobiales* with validly published names. This relationship between strain Y9^T and other members of the order *Rhizobiales* was also evident in the phylogenetic tree (Fig. 1). Strain Y9^T and *P. oryzae* formed a monophyletic clade with a high bootstrap value (100 %) that was supported by both types of tree-making method employed in this study.

Ubiquinone Q-10 was the predominant quinone in strain Y9^T and no menaquinones were detected. Strain Y9^T had a cellular fatty acid profile that contained large amounts of

saturated, unsaturated and cyclo fatty acids. The fatty acid profile of strain Y9^T comprised C_{18:1}ω7*c*/ω9*t*/ω12*t* (63.5 %), C_{16:0} (13.3 %), C_{19:0} cyclo ω8*c* (11.8 %), C_{14:0} 3-OH/C_{16:1} iso (8.1 %) and C_{18:0} (3.3 %) (Table 2). At first sight, the two species appeared to have very different fatty acid profiles. However, the sum of the total amount of C_{19:0} cyclo ω8*c* and C_{18:1}ω7*c*/ω9*t*/ω12*t*, which are interconvertible, was similar. The G + C content of the genomic DNA of strain Y9^T was 65.1 mol%. The phylogenetic, phenotypic and chemotaxonomic results described in this study support classification of strain Y9^T within the genus *Pleomorphomonas* (Xie & Yokota, 2005).

Strain Y9^T showed 98.5 % 16S rRNA gene sequence similarity with respect to the type strain (F-7^T) of *P. oryzae*. Where the level of 16S rRNA gene sequence similarity is greater than 97 %, a DNA–DNA hybridization relatedness of 70 % plays an important role in clarifying the intergenetic relationship between species (Stackebrandt & Goebel, 1994). Strain Y9^T exhibited <29 % DNA–DNA relatedness with the type strain of *P. oryzae*. This level is sufficiently low to permit the classification of strain Y9^T in a distinct species with respect to previously described type strains (Wayne *et al.*, 1987).

On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from the 16S rRNA gene sequence comparisons described above, strain Y9^T should be placed within a novel species, for which we propose the name *Pleomorphomonas koreensis* sp. nov.

Description of *Pleomorphomonas koreensis* sp. nov.

Pleomorphomonas koreensis (ko.re.en'sis. N.L. fem. adj. *koreensis* pertaining to Korea, where the type strain was isolated and researched).

Cells are Gram-negative, oxidase-positive showing an oxidative metabolism, catalase-positive, non-spore-forming, non-motile rods, 0.3–0.5 μm in diameter and 1.5–3.0 μm in length. Growth occurs on R2A agar, nutrient agar and trypticase soy agar at 30 °C and pH 6.0–8.0. Able to

Table 2. Cellular fatty acid profiles of strain Y9^T and *P. oryzae* strains

Data for *P. oryzae* are taken from Xie & Yokota (2005). Fatty acids that account for less than 1 % of the total fatty acids are not shown, so the percentages do not add up to 100 %. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are indicated by the suffixes *c* and *t*, respectively.

Fatty acid	Strain Y9 ^T	<i>P. oryzae</i>
Saturated fatty acids		
C _{16:0}	13.3	12.0–15.3
C _{18:0}	3.3	3.6–7.9
C _{19:0} cyclo ω8 <i>c</i>	11.8	27.9–52.7
Unsaturated fatty acids		
11-Methyl C _{18:1} ω7 <i>c</i>	—	1.8–3.1
C _{20:2} ω6,9 <i>c</i>	—	1.2–1.9
Hydroxy fatty acid		
C _{18:0} 3-OH	—	2.2–2.9
Summed features*		
SF3	8.1	2.9–3.0
SF7	63.5	11.0–36.7

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. SF3, C_{14:0} 3-OH and/or C_{16:1} iso I; SF7, C_{18:1}ω7*c*/ω9*t*/ω12*t*.

assimilate L-fucose, N-acetylglucosamine and D-melibiose as sole carbon sources. Unable to assimilate 4-hydroxybutyrate, caprate, valerate, adipate, suberate, glycogen, 5-ketogluconate or 2-ketogluconate as sole carbon sources. Other substrate-utilization data and details of enzyme production, acid production and physiological characteristics are indicated in Table 1. Predominant quinone is Q-10. Fatty acids consist largely of C_{18:1}, C_{16:0} and C_{19:0} cyclo ω 8c. The G+C content of the genomic DNA of the type strain is 65.1 mol% (determined by HPLC).

The type strain, Y9^T (=KCTC 12246^T=NBRC 100803^T), was isolated from a contaminated culture of *Rhodopseudomonas palustris* in Daejeon, Korea.

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