Pleomorphomonas oryzae gen. nov., sp. nov., a nitrogen-fixing bacterium isolated from paddy soil of *Oryza sativa*

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Five strains of free-living diazotrophs isolated from rice were characterized by using a polyphasic approach. The strains were found to be very closely related, with 99–100% 16S rRNA gene sequence similarity and DNA–DNA hybridization values greater than 70%, suggesting that they represent a single species. When compared with other recognized species, they showed not more than 93 and 89% similarity for the 16S rRNA and *nifH* gene sequences, respectively. Phylogenetic distances showed that these isolates were distinct from other taxa within the α -*Proteobacteria*. Chemotaxonomic characteristics of these isolates included the DNA G+C content (62.1–63.1 mol%), the major quinone system (Q-10), predominant fatty acids (18:1 ω 7c, cyclo 19:0 ω 8c and 16:0) and major hydroxy fatty acids (14:0 3-OH, 18:0 3-OH and 16:0 3-OH). Based on phylogenetic and phenotypic analyses, these isolates are considered to represent a novel genus and species, for which the name *Pleomorphomonas oryzae* gen. nov., sp. nov. is proposed. The type strain is F-7^T (=IAM 15079^T = ATCC BAA-940^T = DSM 16300^T).

Rice serves as a principal food in the Asia–Pacific region. The association of free-living nitrogen-fixing bacteria with rice plants has been investigated since 1982 (Fujie *et al.*, 1987; Sato, 1994; Barraquio *et al.*, 1997; Elbeltagy *et al.*, 2001) and many novel micro-organisms have been isolated from rice plants, such as species of *Azospirillum*, *Burkholderia*, *Herbaspirillum* and *Klebsiella*, *Sphingomonas paucimobilis*, *Azorhizobium caulinodans* and *Azospira oryzae* (Reinhold-Hurek & Hurek, 2000; Engelhard *et al.*, 2000; Eckert *et al.*, 2001; Kirchhof *et al.*, 2001). Oyaizu-Masuchi & Komagata (1988) isolated and identified 74 bacterial strains that showed acetylene reduction activity from the rice rhizosphere and classified them into ten groups based on phenotypic and chemotaxonomic characteristics. Here we report on the classification of five of these strains, F-7^T,

B-18, B-4, B-24 and B-32, which belong to group 6 in their original scheme.

Strains F-7^T, B-4 and B-18 were isolated from *Oryza sativa* C5444, whereas strains B-24 and B-32 were isolated from *Oryza sativa* T65 in Japan. The strains were maintained in ampoules after isolation from the roots of rice plants in 1982. Bacterial strains were grown at 25 °C in nitrogen-free medium (10.0 g glucose, 0.1 g CaCl₂·2H₂O, 0.1 g MgSO₄·7H₂O, 0.9 g K₂HPO₄, 0.1 g KH₂PO₄, 5 g CaCO₃, 10 mg FeSO₄·7H₂O, 5.0 mg Na₂MoO₄·2H₂O, 1.0 litre distilled water, pH 7.3, or supplemented with 0.5 g yeast extract l⁻¹). Accumulation of poly- β -hydroxybutyrate (PHB) granules, DNA base composition, major quinones and acetylene reduction were determined according to the methods of Oyaizu-Masuchi & Komagata (1988). Cellular fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (MIS) as described by Xie & Yokota (2003). The fatty acid composition could not be clearly identified by using the MIDI system (Microbial ID, Inc.). Therefore, summed features 2 and 3 were further analysed as follows: the fatty acid samples, together with non-polar fatty acid and hydroxy fatty acid standards used for comparison, were developed on TLC plates (silica-gel F254; Merck) with hexane/ethyl ether (1:1), sprayed with a 0.02% dichlorofluorescein solution in ethanol, dried and detected under UV light. The separated spots of the non-polar fatty acids and hydroxy fatty acids were scraped from the plates, transferred to tubes

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Abbreviation: PHB, poly- β -hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains F-7^T, B-24, B-4, B-18 and B-32 are AB159680 and AB159682–AB159685, respectively, and for the *nifH* gene sequences they are AB161454, AB182912, AB159683, AB182911 and AB182913, respectively.

A neighbour-joining tree based on *nifH* gene sequences showing the position of the novel strains is available as supplementary material in IJSEM Online.

and extracted with ethyl ether. The extracts were then concentrated under a stream of nitrogen gas and dissolved in hexane/methyl tert-butyl ether (1:1). The separated and purified non-polar fatty acids and hydroxy fatty acids were then detected by using the MIDI system again.

DNA–DNA hybridization was performed by the photo-biotin-labelling method of Ezaki *et al.* (1989) using a Multi-well Plate Reader (CytoFluoR; Perseptive Biosystems). The hybridization temperature was 52 °C and reciprocal experiments were performed as follows: the DNA of strain F-7^T was used as a probe to hybridize the DNA of itself and strains B-32, B-24, B-4 and B-18. PCR-mediated amplification of the 16S rRNA gene and sequencing of the PCR products were carried out as described by Xie & Yokota (2003). A 411 bp fragment of the *nifH* gene (encoding the iron protein of nitrogenase) was amplified from the extracted DNA using the forward primer IGK (5'-TACGGYAARGGBGGYATCGG-3') and the reverse primer AQE (5'-GACGATGATYTCCTG-3') (Xie & Yokota, 2004). The DNA sequences were compared with those obtained from NCBI GenBank and aligned using the CLUSTAL W software package (Thompson *et al.*, 1994) and evolutionary distances and K_{nuc} values (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were excluded in the calculations. A phylogenetic tree based on comparison of 1236 bases was constructed using the neighbour-joining method (Saitou & Nei, 1987). The topology of this tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates, and similarity values were calculated using PAUP 4.0b1 (Swofford, 1997). Using the same method, 328 bases of the *nifH* gene sequence were also aligned and a phylogenetic tree was constructed.

Morphological and physiological characteristics of the five strains showed no significant differences and are given in the species description below. The strains showed very high acetylene reduction activity and were found to possess the *nifH* gene, suggesting that the bacterial species they represent is able to fix nitrogen. The quinone system identified supports the proposed affiliation of the isolates to the α -Proteobacteria, the majority of species of which typically have ubiquinone Q-10 as the major quinone (Lechner *et al.*, 1995; Yokota *et al.*, 1992; Kämpfer *et al.*, 2003). The cellular fatty acids of the five strains were examined by using the MIDI system and unidentified elements (summed features 2 and 3) were further characterized (14:0 3-OH and 16:1 ω 7c, respectively). The results are in excellent agreement with the previous studies by Oyaizu-Masuchi & Komagata (1988) using GLC. Fatty acid data from two different methods could therefore be used for comparison of the isolates. Cellular fatty acid profiles included fatty acids 18:1 ω 7c (11.0–36.7%), cyclo 19:0 ω 8c (27.9–52.7%), 16:0 (12.0–15.3%), 18:0 (4.6–7.9%), 11-methyl 18:1 ω 7c (1.8–3.1%), 20:2 ω 6,9c (1.2–1.9%), cyclo 17:0 (1.1–2.2%), 14:0 (0.4–1.1%) and summed feature 7 (comprising one or more of 18:1 ω 7c, 18:1 ω 9t

and 18:1 ω 12t) (0–2.1%). The hydroxy fatty acids identified were 14:0 3-OH (2.9–3.0%), 18:0 3-OH (2.2–2.9%) and 16:0 3-OH (0.3–0.6%), which is a unique profile, different from those of other nitrogen-fixing taxa such as *Methylosinus*, *Methylocystis*, rhizobia, *Rhodopseudomonas*, *Azospirillum* and *Xanthobacter* (Tighe *et al.*, 2000; Bowman *et al.*, 1993; Hiraishi & Ueda, 1995; Oyaizu-Masuchi & Komagata, 1988). It should be noted that the isolates appear to be similar to the nitrogen-fixing genera *Azospirillum* and *Xanthobacter* based on their fatty acid composition and pleomorphic morphology, but levels of DNA–DNA relatedness were quite low (14.2–17.8%; Oyaizu-Masuchi & Komagata, 1988). The G + C contents of the DNA of strains F-7^T (63.1 mol%), B-4 (62.2 mol%), B-18 (62.9 mol%), B-24 (62.6 mol%) and B-32 (63.1 mol%) were 5–10 mol% lower than those of *Azospirillum* and *Xanthobacter*. Moreover, the novel bacterial isolates can be differentiated from the genera *Azospirillum* and *Xanthobacter* based on colony colour and motility (Table 1).

Levels of DNA–DNA relatedness of strain F-7^T against the other isolates were 80.5% (B-4), 88.6% (B-18), 92.0% (B-24) and 91.6% (B-32). These high levels of DNA–DNA relatedness (over 70%) strongly suggest that the five strains are representatives of a single species.

The 16S rRNA gene sequences (approximately 1430 bp) of strains F-7^T, B-18, B-24 and B-32 were identical; that of strain B-4 differed from the others by one base. 16S rRNA gene sequence similarity revealed that the closest recognized relatives were members of the genera *Methylosinus* and *Methylocystis*, with 93% similarity (using BLAST in NCBI GenBank). The highest similarities (99%) were to the rice paddy isolates KCB90 (Chin *et al.*, 1999), RR48 (accession no. AB174815) and RR47 (accession no. AB174816), suggesting that such bacteria represent predominant populations in the microbial community of this environment. The phylogenetic tree constructed on the basis of 16S rRNA gene sequence analysis indicated that these strains form a distinct monophyletic clade with 100% bootstrap within the α -Proteobacteria (Fig. 1), and that they represent a sister phyletic group to the nitrogen-fixing genera *Beijerinckia*, *Methylocystis*, *Methylosinus*, *Methylobacterium*, *Bradyrhizobium* and *Rhodopseudomonas* with weak bootstrap support. The new isolates can be easily distinguished from these genera based on their phenotypic characteristics (Table 1). To elucidate further their inferred evolutionary relationships, the *nifH* gene was selected as another phylogenetic marker. The *nifH* gene is currently being examined as a component of the nitrogen-fixation locus (*nifHDK*) that shows strong conservation, and as a molecular marker for phylogenetic analysis of the diazotrophs. A phylogenetic tree based on *nifH* gene sequence analysis was established (available as a supplementary figure in IJSEM Online), and is largely consistent with the 16S rRNA gene phylogeny, except for specific discrepancies with a few taxa (Xie & Yokota, 2004; Moulin *et al.*, 2001; Rosado *et al.*, 1998; Young, 1992). Three of the strains had identical *nifH* gene

Table 1. Differential phenotypic characteristics of *Pleomorphomonas oryzae* gen. nov., sp. nov. and related nitrogen-fixing taxa

Taxa: 1, *Pleomorphomonas oryzae*; 2, *Methylosinus* (data from Bowman *et al.*, 1993); 3, *Methylocystis* (Bowman *et al.*, 1993); 4, *Rhodopseudomonas palustris* ATCC 17001^T (Imhoff & Trüper, 1989); 5, *Bradyrhizobium* (Jordan, 1984); 6, *Azospirillum* (Oyaizu-Masuchi & Komagata, 1988); 7, *Xanthobacter* (Oyaizu-Masuchi & Komagata, 1988). ND, No data.

Characteristic	1	2	3	4	5	6	7
Cell shape	Pleomorphic rods	Vibrioid or pyriform	Coccobacillary or curved rods	Budding rods	Rods	Spiral	Pleomorphic rods
Motility	—	+	—	+	+	+	—
Flagellation	—	> 1, polar	—	Polar	Polar or subpolar	Polar	—
PHB granules	+	+/-	+/-	+	+	+	+
Colour of colonies	—	White or brown	White or brown	Red	—	—	Yellow
Phosphatase	—	-/+	+/-	ND	ND	+	+
Methanotrophy	—	+	+	—	—	—	—
Anaerobic photoheterotrophs	—	—	—	+	—	—	—
Root nodules produced	—	—	—	—	+	—	—
Major fatty acids	cyclo 19:0 ω 8c, 18:1 ω 7c	18:1 ω 8c	18:1 ω 8c	18:1 ω 7c	Summed feature 7*	18:1 ω 7c	18:1 ω 7c
Hydroxy fatty acids	14:0 3-OH, 18:0 3-OH	None	None	14:0 3-OH	None	14:0 3-OH, 16:0 3-OH	18:0 3-OH
G + C content (mol%)	62.1–63.1	63–67	62–67	64.5–65.5	61–65	69–71	65–70
Quinone system	Q-10	Q-8	Q-8	Q-10	Q-10	Q-10	Q-10

*Summed feature 7 contained one or more of 18:1 ω 7c, 18:1 ω 9t and 18:1 ω 12t.

Fig. 1. Neighbour-joining based on 16S rRNA gene sequence analysis showing relationships between the strains studied and representatives of related families of the α -Proteobacteria. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees; only values greater than 70 % are shown. Bar, 0.02 substitutions per nucleotide position.

sequences, and the sequence from strain F-7^T shared 98.2 % similarity with the other strains. The most closely related organisms based on *nifH* gene sequence analysis were members of *Azospirillum* (89 %). In the *nifH* phylogenetic tree, all five strains form a distinct monophyletic group and are a sister phyletic group to the genus *Azospirillum* with poor bootstrap support. The strains did not cluster close to the phylogenetic neighbours inferred from 16S rRNA gene sequence comparisons, *Beijerinckia*, *Methylocystis* and *Methylosinus*. The novel isolates are phylogenetically distant from other recognized bacteria based on analysis of both 16S rRNA and *nifH* gene sequences. Therefore, they cannot be characterized as members of any recognized genus within the α -Proteobacteria. The name *Pleomorphomonas oryzae* gen. nov., sp. nov. is proposed to accommodate the strains described here.

Description of *Pleomorphomonas* gen. nov.

Pleomorphomonas (Ple.o.mor'pho.mo.nas. Gr. comp. adj. *pleon* more, larger, both of number and of size; Gr. n. *morphe* form, shape; Gr. n. *monas* monad, unit; N.L. fem. n. *Pleomorphomonas* pleomorphic monad).

Cells are Gram-negative, non-motile, pleomorphic and nitrogen-fixing. PHB granules are accumulated. Catalase and oxidase are positive. Predominant fatty acids are 18:1 ω 7c, cyclo 19:0 ω 8c and 16:0; major hydroxy fatty acids are 14:0 3-OH, 18:0 3-OH and 16:0 3-OH. The G+C content of the DNA of the type species is 62.1–63.1 mol%. The respiratory quinone is ubiquinone Q-10. The type species is *Pleomorphomonas oryzae*.

Description of *Pleomorphomonas oryzae* sp. nov.

Pleomorphomonas oryzae (o.ry'zae. L. gen. n. *oryzae* of rice, from which the strains were isolated).

Characteristics are the same as those given for the genus. Colonies are colourless on nitrogen-free agar medium. Old cells became ovoid. Cells are 0.5–1.0 $\times$ 1.0–5.0 μ m. Acids are produced from glucose by fermentative metabolism but the organism cannot grow on tellurite agar medium. Indole production, nitrate reduction, urease and tyrosinase activities are positive. Phosphatase, DNase, tetrazolium chloride reduction and hydrolysis of gelatin, starch and aesculin are negative. Utilizes L-arabinose, D-xylose, D-ribose, L-rhamnose, D-glucose, D-fructose, mannose, D-galactose, trehalose, sorbose, sucrose, lactose, cellobiose, raffinose, salicin, starch, acetate, oxalate, fumarate, DL-malate, pyruvate, gluconate, sorbitol, mannitol, inositol, glycerol, L-serine, L-glutamine, L-aspartate and L-proline as sole carbon sources, but does not utilize maltose, succinate, malonate, glutamate, 2-oxoglutarate, glyoxylate, citrate, DL-lactate, DL-mandelate, DL- β -hydroxybutyrate, itaconate, n-propanol, methanol, ethanol, benzoate, betaine, β -alanine, L-threonine, L-arginine, L-leucine, L-lysine, L-ornithine, L-methionine, L-valine, L-phenylalanine, β -aminobutyrate,

L-histidine or L-cysteine. The G+C content of the type strain is 63.1 mol%.

The type strain, F-7^T (=IAM 15079^T=ATCC BAA-940^T=DSM 16300^T), was isolated from *Oryza sativa* in 1982.

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