Pseudomonas taiwanensis sp. nov., isolated from soil

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A novel Gram-negative, rod-shaped, motile, non-spore-forming bacterial strain, CMS^T, isolated from soil was characterized using phenotypic and molecular taxonomic methods. 16S rRNA gene sequence analysis revealed that the organism belongs phylogenetically to the genus *Pseudomonas*. *Pseudomonas monteilii*, *P. plecoglossicida* and *P. mosselii* were the most closely related species, with 16S rRNA gene sequence similarities to the respective type strains of 99.79, 99.73 and 99.59%. Relatively low *gyrB* gene sequence similarities (<90%) and DNA–DNA reassociation values (<51%) were obtained between the strain and its phylogenetically closest neighbours. The G + C content of strain CMS^T was 62.7 mol%. The major cellular fatty acids were C_{18:1}ω7c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{16:0} and C_{10:0} 3-OH. Based on the phenotypic and genetic evidence, the strain is suggested to represent a novel species, for which the name *Pseudomonas taiwanensis* sp. nov. is proposed. The type strain is CMS^T (=BCRC 17751^T =DSM 21245^T).

The genus *Pseudomonas* represents a group of Gram-negative bacteria that are non-spore-forming, motile and rod-shaped (Palleroni, 1984). Pseudomonads are widespread in various natural environments, such as soil, plants, animals, air and water. The genus *Pseudomonas sensu stricto* is now restricted to *Pseudomonas* RNA homology group I (Palleroni, 1984) in the *Gammaproteobacteria* (Kerstens *et al.*, 1996). The taxonomy of the genus *Pseudomonas* has been revisited several times on the basis of phenotypic features (Sneath *et al.*, 1981; Palleroni, 1984), 16S rRNA gene sequence similarity (Anzai *et al.*, 2000), *gyrB* and *rpoD* gene sequences (Yamamoto *et al.*, 2000) and chemotaxonomic data (Oyaizu & Komagata, 1983; Vancanneyt *et al.*, 1996).

Strain CMS^T was isolated from soil taken from Tamkang University (Tamsui, Taipei, Taiwan) in 2005. For isolation of strain CMS^T, 1 g soil sample was suspended in sterile distilled water, plated onto agar medium (0.5% shrimp shell powder, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, pH 7, 1.5% agar) and incubated at 30 °C for 3 days under aerobic conditions. Chitin-degrading strains were isolated.

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA and *gyrB* gene sequences of strain CMS^T are EU103629 and FJ418634, respectively.

Maximum-parsimony and maximum-likelihood phylogenetic trees based on 16S rRNA and *gyrB* gene sequences and comparisons of phenotypic properties and cellular fatty acid profiles are available as supplementary material with the online version of this paper.

The strain was cultivated on nutrient agar (NA; Difco) at 30 °C for 24–48 h. It was obtained in pure culture after three successive transfers to fresh agar medium. The pure strain was preserved at –80 °C in nutrient broth (NB; Difco) containing 20% (v/v) glycerol or by lyophilization. In addition to strain CMS^T, the reference strains *Pseudomonas monteilii* BCRC 17520^T, *P. plecoglossicida* BCRC 17517^T, *P. mosselii* BCRC 17518^T, *P. fulva* BCRC 17506^T, *P. parafulva* BCRC 17511^T, *P. putida* BCRC 10459^T, *P. oryzihabitans* BCRC 16007^T, *P. fuscovaginae* BCRC 17126^T, *P. asplenii* BCRC 17131^T, *P. cremoricolorata* BCRC 17510^T, *P. mohnii* BCRC 80051^T, *P. moorei* BCRC 80052^T and *P. japonica* BCRC 80101^T were used for comparative studies. A polyphasic taxonomic study of the above strains and strain CMS^T was performed using phenotypic characterization as well as phylogenetic and genetic methods. The results of this study consistently indicated that strain CMS^T represents a novel *Pseudomonas* species.

Genomic DNA was extracted and purified using the Qiagen Blood & Cell Culture DNA kit (Qiagen). The 16S rRNA gene was amplified by PCR and sequenced using the MicroSeq Full Gene 16S rDNA Bacterial Identification kit (Applied Biosystems). Sequencing was performed with a 3730 DNA sequencer (Applied Biosystems and Hitachi). Sequence assembly was performed using the ABI PRISM DNA Sequencing Analysis software (PE Applied Biosystems).

Approximately 1.5 kb of the 16S rRNA gene sequence obtained in this study and sequences taken from GenBank were aligned using the CLUSTAL_X program, version 1.8 (Thompson *et al.*, 1997). Phylogenetic trees based on 1.5 kb 16S rRNA gene sequences (corresponding to positions 30–1502 of the *Escherichia coli* K-12 sequence with gaps) were reconstructed according to the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods using the PHYLIP program package (Felsenstein, 2005). Sequence distances were calculated by the F84 model (Felsenstein, 1984). Bootstrap analysis was made with 1000 replicates (Fig. 1; see also Supplementary Figs S1 and S2, available in IJSEM Online). Tree figures were drawn with TreeView. The results of the phylogenetic analysis indicated that strain CMS^T belongs to the genus *Pseudomonas* and is placed in the subcluster of the *Pseudomonas putida* group (Anzai *et al.*, 2000). Strain CMS^T was included in a monophyletic cluster that contained three closely related species, *P. monteilii*, *P. plecoglossicida* and *P. mosselii* (Fig. 1). The highest levels of sequence similarities were with the type strains of *P. monteilii* (99.79%), *P. plecoglossicida* (99.73%) and *P. mosselii* (99.59%). 16S rRNA gene sequence similarities of strain CMS^T to members of other *Pseudomonas* species were below 99%.

To differentiate further between strain CMS^T and closely related species, the partial sequence for the gene encoding subunit B of DNA gyrase (*gyrB*) was included in the phylogenetic analysis. The *gyrB* gene was amplified by PCR as described previously (Yamamoto & Harayama, 1995). The PCR was performed using the TaKaRa Ex Taq kit. PCR products were purified with the PCR-M clean up system (Viogene) and sequenced with a BigDye Terminator version 3.1 cycle-sequencing kit using primers UP-1S and UP-2Sr (Yamamoto & Harayama, 1995). Using the method described above, approximately 1.0 kb of the sequences

obtained in this study and sequences taken from GenBank were aligned and phylogenetic trees were reconstructed (Supplementary Figs S3–S5). Phylogenetic analysis by the neighbour-joining, maximum-parsimony and maximum-likelihood methods produced similar trees with the exception of minor differences in the topology of the basal branches. The tree based on 1080 bp *gyrB* gene sequences (corresponding to positions 375–1448 of the *E. coli* K-12 sequence with gaps) showed that strain CMS^T formed a distinct branch (Supplementary Fig. S3). Strain CMS^T shared 89.75, 89.54, 89.15 and 89.15% *gyrB* sequence similarity with the type strains of *P. mosselii*, *P. putida*, *P. monteilii* and *P. plecoglossicida*, respectively, whereas the similarities between strain CMS^T and other members of the related cluster were less than 87%. Classification and identification of pseudomonads based on *gyrB* gene sequences can be very useful, as described previously (Yamamoto & Harayama, 1998; Yamamoto *et al.*, 2000; Mulet *et al.*, 2008). The above result indicates that the isolate was differentiated from *P. mosselii*, *P. monteilii* and *P. plecoglossicida* on the basis of *gyrB* gene sequences.

DNA G+C contents and DNA–DNA relatedness were determined as described previously (Tamaoka & Komagata, 1984; Ezaki *et al.*, 1989; Goris *et al.*, 1998; Wang *et al.*, 2007). The DNA G+C content of strain CMS^T was 62.7 mol%. This falls within the range of values of 60–67 mol% reported for closely related *Pseudomonas* species (Table 1). DNA from strain CMS^T was labelled with a hybridization temperature of 50 °C. Low values of reassociation (<51%) were observed with the closely related *Pseudomonas* type strains examined (Table 1). These values are well below the threshold of 70% that has been suggested for bacterial species delineation (Stackebrandt & Goebel, 1994). The results of *gyrB* sequence analysis correlated very well with DNA reassociation values (Yamamoto *et al.*, 1999; Wang *et al.*, 2007). The *gyrB* gene sequence similarities between

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of *Pseudomonas* strains showing the position of strain CMS^T. Only bootstrap percentages (based on 1000 replications) above 50% are shown. Bar, 1 substitution per 100 nucleotide positions.

Table 1. DNA base composition, DNA relatedness and gene sequence similarities of strain CMS^T and type strains of closely related *Pseudomonas* species

DNA G + C contents for reference strains were taken from Elomari *et al.* (1997), Nishimori *et al.* (2000), Dabboussi *et al.* (2002), Cámara *et al.* (2007) and Pungrasmi *et al.* (2008); ND, no data available. Reassociation values are means of five determinations.

Strain	DNA G + C content (mol%)	Reassociation (%) with labelled DNA from:		Sequence similarity (%) with respect to strain CMS ^T	
		CMS ^T	<i>P. mosselii</i> BCRC 17518 ^T	16S rRNA gene	<i>gyrB</i>
<i>P. taiwanensis</i> sp. nov. CMS ^T	62.7	(100)	50	(100)	(100)
<i>P. mosselii</i> BCRC 17518 ^T	63	51	(100)	99.59	89.75
<i>P. plecoglossicida</i> BCRC 17517 ^T	62.8	42	53	99.73	89.15
<i>P. monteilii</i> BCRC 17520 ^T	60	45	57	99.79	89.15
<i>P. putida</i> BCRC 10459 ^T	63	48	44	98.90	89.54
<i>P. fulva</i> BCRC 17506 ^T	63	37	37	98.16	85.64
<i>P. parafulva</i> BCRC 17511 ^T	63	40	45	99.11	86.51
<i>P. oryzae</i> BCRC 16007 ^T	67	20	28	98.84	84.46
<i>P. fuscovaginae</i> BCRC 17126 ^T	61	27	13	98.48	85.50
<i>P. asplenii</i> BCRC 17131 ^T	ND	29	15	98.48	84.62
<i>P. cremoricolorata</i> BCRC 17510 ^T	62	45	39	98.63	85.50
<i>P. mohnii</i> BCRC 80051 ^T	ND	23	32	97.86	83.78
<i>P. moorei</i> BCRC 80052 ^T	ND	15	28	97.86	83.99
<i>P. japonica</i> BCRC 80101 ^T	66	20	22	98.71	87.31

strain CMS^T and type strains of closely related species were below 90 % and DNA–DNA relatedness values between the same strains were below 51 %.

The morphological, cultural, physiological and biochemical characteristics of strain CMS^T and reference type strains were determined by using cells grown on tryptic soy agar (TSA; Difco) or tryptic soy broth (TSB; Difco) for 1–2 days at 30 °C under aerobic conditions. Anaerobic growth was not observed after 2 weeks of incubation at 30 °C using a GENbox anaer (bioMérieux). Cellular morphology was observed with a Nikon Eclipse E600 microscope (standard phase-contrast mode). Flagellar morphology was determined by staining flagella by the silver-plating method (West *et al.*, 1977). Gram staining was performed using the 4-Step Gram stain kit (BD) according to the manufacturer's instructions. Catalase activity was detected by production of bubbles after adding 3 % hydrogen peroxide solution to resting cells. The oxidase test was performed with Oxidase reagent droppers (BD). Fluorescent pigment production was observed on *Pseudomonas* agar F (Difco). Growth at 5, 10, 20, 30, 37, 40, 42, 45, 50 and 60 °C and pH 4, 5, 6, 7, 8 and 9 was determined using TSB as the basal medium. Salinity tolerance was analysed using NB supplemented with 0, 2, 3, 4, 5, 6, 7, 8, 9 or 10 % (w/v) NaCl. Additional physiological and biochemical characteristics were tested using the API system (API 20NE; bioMérieux) and Biolog GN2 microplates (MicroLog System) according to the manufacturers' instructions. The results were recorded after 24 or 48 h of incubation at 30 °C. Differential characteristics of strain CMS^T (described in detail in the species description) and closely related *Pseudomonas* species are summarized in Supplementary Table S1.

Respiratory quinones were extracted with chloroform/methanol (2:1) and purified by TLC according to the previously described method (Collins & Jones, 1981; Collins, 1985). Quinones were analysed by HPLC (Komagata & Suzuki, 1987) using a previously characterized mixture of various ubiquinones as a reference (Hu *et al.*, 2001). A Nova-Pak C18 column (Waters Corp.) was used. The major quinone was ubiquinone 9 (Q-9), which was in agreement with the results of Uchino *et al.* (2001).

Cellular fatty acids of strain CMS^T and closely related *Pseudomonas* type strains grown on TSA (Difco) for 24 h at 28 °C were prepared and analysed according to the instructions of the Microbial Identification System (MIDI) as described previously (Sasser, 1990; Tai *et al.*, 2006). The full cellular fatty acid profile of strain CMS^T is shown in Supplementary Table S2. The presence of high levels of summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{16:0}, along with C_{10:0} 3-OH, C_{12:0} 3-OH and C_{12:0} 2-OH, supported the classification of strain CMS^T in the genus *Pseudomonas* (Oyaizu & Komagata, 1983). Strain CMS^T had a similar fatty acid profile to *P. plecoglossicida* BCRC 17517^T (Supplementary Table S3). Their major cellular fatty acids were C_{18:1}ω7c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{16:0} and C_{10:0} 3-OH. A difference between the fatty acid profiles of strain CMS^T and its closest relatives was the content of C_{10:0} 3-OH, which was high in strain CMS^T and *P. plecoglossicida* BCRC 17517^T but low in other closely related type strains.

Based on the reported data, the phenotypic characteristics of the isolate were similar to those of its phylogenetically closest relatives. However, *gyrB* gene sequence similarities and DNA–DNA relatedness showed clear separation

between strain CMS^T and its phylogenetically closest relatives. Thus, strain CMS^T represents a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas taiwanensis* sp. nov. is proposed.

Description of *Pseudomonas taiwanensis* sp. nov.

Pseudomonas taiwanensis (tai.wan.en'sis. N.L. fem. adj. *taiwanensis* pertaining to Taiwan, where the type strain was isolated).

Cells are Gram-negative, motile with one or three polar flagella, non-spore-forming, round-ended rods, about 0.7–1.0 × 1.0–2.2 µm. Colonies on TSA are smooth, circular and non-pigmented (creamy yellow). Growth occurs at 5–42 °C (optimum, 30–37 °C), but not at higher temperatures (45–60 °C). Growth occurs at pH 4–9 (optimum, pH 6–8). Grows on media containing 0–6 % (w/v) NaCl; no growth on media containing 7–10 % NaCl. Metabolism is oxidative but not fermentative. Non-haemolytic on blood agar. A fluorescent pigment is produced on *Pseudomonas* agar F, but no pyocyanin is produced on *Pseudomonas* agar P. Catalase, oxidase and arginine dihydrolase are produced. Indole, urease and β-galactosidase are not produced. Does not reduce nitrates to nitrites and denitrification does not occur. Aesculin and gelatin are not hydrolysed. Positive for assimilation of potassium gluconate, capric acid, malic acid, trisodium citrate and phenylacetic acid and negative for the assimilation of adipic acid. Tweens 40 and 80 are hydrolysed. Results obtained with Biolog GN2 microplates indicate that the following substrates are utilized: L-arabinose, D-fructose, α-D-glucose, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, glucuronamide, alaninamide, D- and L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyrogutamic acid, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol and glycerol. The other organic substrates included in the Biolog GN2 microplates are not utilized. The major quinone is ubiquinone 9 (Q-9). The major cellular fatty acids are C_{18:1ω7c}, summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH), C_{16:0} and C_{10:0} 3-OH. The DNA G+C content of the type strain is 62.7 mol%.

The type strain is CMS^T (=BCRC 17751^T =DSM 21245^T), isolated from soil taken from Tamkang University, Tamsui, Taipei, Taiwan, ROC.

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