Pusillimonas soli sp. nov., isolated from farm soil

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A Gram-negative, motile, non-spore-forming bacterial strain, designated MJ07^T, was isolated from a farm soil and was characterized to determine its taxonomic position by using a polyphasic approach. Comparative 16S rRNA gene sequence analysis showed that strain MJ07^T belongs to the family *Alcaligenaceae*, class *Betaproteobacteria*, and is related most closely to *Pusillimonas ginsengisoli* KCTC 22046^T (98.6 % sequence similarity) and *Pusillimonas noertemannii* BN9^T (96.9 %). The levels of 16S rRNA gene sequence similarity between strain MJ07^T and members of all other recognized species of the family *Alcaligenaceae* were below 95.2 %. The G+C content of the genomic DNA of strain MJ07^T was 59.4 mol%. The detection of a quinone system with ubiquinone Q-8 as the major respiratory lipoquinone, putrescine as the predominant polyamine, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and two unknown aminolipids as major polar lipids and a fatty acid profile with $C_{16:0}$ (32.0 %), $C_{17:0}$ cyclo (24.7 %) and $C_{19:0}$ cyclo ω8*c* (11.5 %) as the major components supported the affiliation of strain MJ07^T to the genus *Pusillimonas*. Strain MJ07^T exhibited relatively low levels of DNA–DNA relatedness with respect to *P. ginsengisoli* KCTC 22046^T (50 ± 8 %) and *P. noertemannii* KACC 13183^T (18 ± 7 %). On the basis of its phenotypic and genotypic properties together with its phylogenetic distinctiveness, strain MJ07^T (=KCTC 22455^T =JCM 16386^T) should be classified in the genus *Pusillimonas* as the type strain of a novel species, for which the name *Pusillimonas soli* sp. nov. is proposed.

The genus *Pusillimonas*, belonging to the family *Alcaligenaceae*, was proposed by Stolz *et al.* (2005) and was defined as comprising Gram-negative, oxidase-positive rods with ubiquinone Q-8 as the predominant respiratory quinone and putrescine, spermidine and 2-hydroxyputrescine as the major polyamines. The description of the genus also stated that cells contained phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unknown aminolipids as the major polar lipids and $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8*c*, $C_{16:0}$ and summed feature 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH) as the major fatty acids. At the time of writing, the genus contains only one recognized species, *Pusillimonas noertemannii* (Stolz *et al.*, 2005), but the description of a second member of the genus, *Pusillimonas ginsengisoli*, is available ahead of print (Srinivasan *et al.*, 2010). In the present paper, we describe a bacterial strain, designated MJ07^T, which was isolated from a soil sample. On the basis of 16S rRNA gene sequence data, the strain was found to be a member of the genus *Pusillimonas*. Further study of this strain was based on a polyphasic approach that included chemotaxonomic and physiological analyses and DNA–DNA hybridization, and confirmed its position as a representative of a novel species within the genus *Pusillimonas*.

Strain MJ07^T was isolated from a farm-soil sample collected near Daejeon, South Korea. The sample was suspended and spread on Luria–Bertani (LB) agar (Difco) plates after being serially diluted with 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30 °C for 2 weeks. Single colonies on the plates were purified by transferring them onto fresh plates and incubating them again under the same conditions. Strain MJ07^T was routinely cultured on LB agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at −70 °C. *P. noertemannii* KACC 13183^T and *P. ginsengisoli* KCTC 22046^T were used as reference strains for DNA–DNA hybridization and other experiments.

For the phylogenetic analysis of strain MJ07^T, genomic DNA was extracted by using a commercial genomic DNA-extraction kit (Solgent) and 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 GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MJ07^T is GQ241322.

A 2D TLC plate showing polar lipids of strain MJ07^T is available as supplementary material with the online version of this paper.

The full sequence of the 16S rRNA gene was compiled by using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the program CLUSTAL_X (Thompson *et al.*, 1997) and gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated by using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA4 program (Tamura *et al.*, 2007), with bootstrap values based on 1000 replications (Felsenstein, 1985).

A nearly complete 16S rRNA gene sequence of strain MJ07^T (1454 bp) was obtained. Preliminary sequence comparison against 16S rRNA gene sequences deposited in the GenBank database indicated that strain MJ07^T belonged to the family *Alcaligenaceae*, class *Betaproteobacteria*. On the basis of 16S rRNA gene sequence similarity, the closest relatives of strain MJ07^T were *P. ginsengisoli* DCY25^T (98.6 %) and *P. noertemannii* BN9^T (96.9 %). The levels of similarity between strain MJ07^T and members of all other recognized species of the family *Alcaligenaceae* were below 95.2 %. This relationship between strain MJ07^T and other members of the family *Alcaligenaceae* was also evident in the neighbour-joining phylogenetic tree (Fig. 1). Strain MJ07^T, *P. noertemannii* BN9^T and *P. ginsengisoli* DCY25^T formed a coherent cluster with relatively high bootstrap values in both the neighbour-joining and maximum-parsimony trees. These data indicate that strain MJ07^T can be clearly separated from other members of the family *Alcaligenaceae* with the exception of the two species of *Pusillimonas* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). To differentiate strain MJ07^T from its phylogenetically closest relatives, DNA–DNA hybridization experiments were performed.

The Gram reaction was performed by using the non-staining method, as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at $\times 1000$ magnification with cells grown for 3 days at 30 °C on LB agar. Catalase and oxidase tests were performed as outlined by Cappuccino & Sherman (2002). Assimilation of single carbon sources, enzyme activities and other physiological characteristics were determined with the API ID 32 GN, API ZYM, API 20NE and API 50CH galleries according to the manufacturer's instructions (bioMérieux). Tests for degradation of DNA [DNase agar (Scharlau), with DNase activity detected by flooding plates with 1 M HCl], casein, chitin, starch (Atlas, 1993), xylan and hydroxyethyl (HE)-cellulose (Ten *et al.*, 2004) were performed and evaluated after 7 days. Growth at 10, 15, 20, 25, 30, 37, 42 and 45 °C was assessed on R2A agar, nutrient agar, LB agar, trypticase soy agar (TSA; Difco) and MacConkey agar after 5 days of

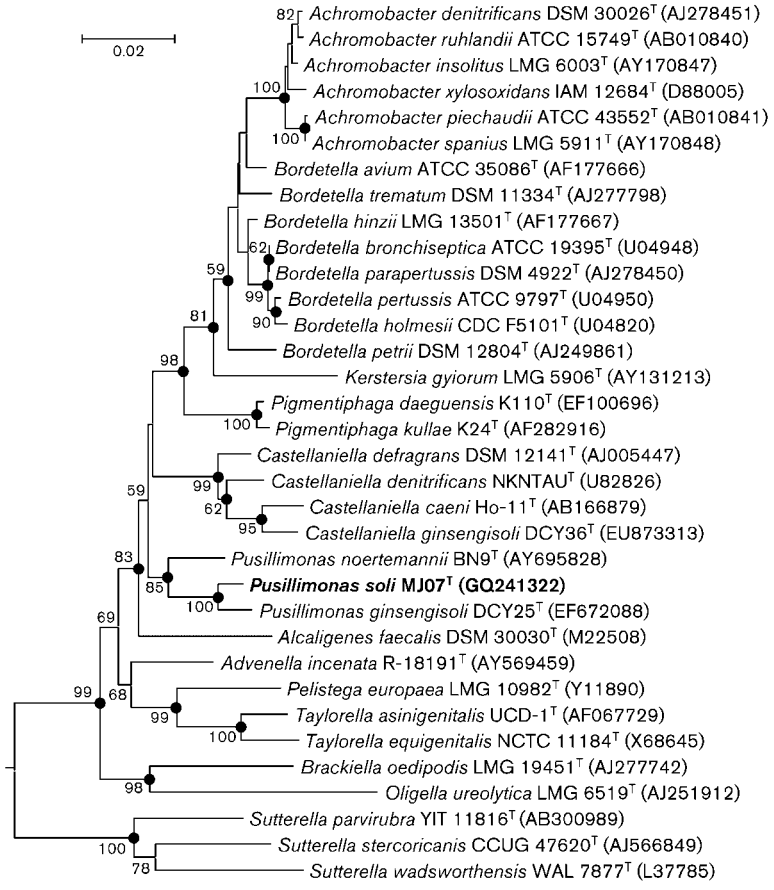

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strain MJ07^T among neighbouring species selected from the *Betaproteobacteria*. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. The sequence of *Escherichia coli* ATCC 11775^T (GenBank accession no. X80725) was used as an outgroup (not shown). Bar, 0.02 substitutions per nucleotide position.

incubation. Growth at pH 5.0–11.0 (at intervals of 0.5 pH units) was evaluated in LB broth at 30 °C.

Cells of strain MJ07^T were Gram-negative, motile, non-spore-forming rods, 0.3–0.5 μm wide and 0.7–1.0 μm long. On LB agar, colonies reached a diameter of about 1–3 mm within 5 days at 25 or 30 °C and they were yellow, irregularly circular and low-convex. Colonies grown on R2A, TSA, MacConkey and NB agar plates at the same temperatures were only 0.3–0.5 mm in diameter. With the exception of MacConkey agar, weak growth occurred on the above-mentioned complex media at 42 °C. The biochemical characteristics of strain MJ07^T were similar to those reported for *P. noertemannii* BN9^T (Stolz *et al.*, 2005) and *P. ginsengisoli* DCY25^T (Srinivasan *et al.*, 2010), i.e. the strain was negative for acid production from various sugars and sugar alcohols (e.g. lactose, sucrose, D-arabitol and D-mannitol), assimilation of carbohydrates (e.g. L-arabinose, D-mannose and maltose) and production of hydrolysing enzymes (e.g. β-galactosidase and α-glucosidase). Phenotypic and chemotaxonomic characteristics that differentiate strain MJ07^T from *P. noertemannii* KACC 13183^T and *P. ginsengisoli* KCTC 22046^T are listed in Table 1. In particular, our strain could be differentiated from its closest relatives based on its ability to utilize carbohydrates such as L-rhamnose, D-ribose and D-sorbitol and to produce acid from L-arabinose, arbutin and D-glucose.

For the measurement of chromosomal DNA G+C content, genomic DNA of the strain was extracted and purified as described by Moore & Dowhan (1995) and degraded enzymically into nucleosides; the DNA G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v), evaporated under vacuum conditions and re-extracted in n-hexane/water (1 : 1, v/v). The crude quinone in n-hexane was purified using Sep-Pak Vac silica cartridges (Waters) and then analysed by HPLC, as described previously (Hiraishi *et al.*, 1996). Cellular fatty acid profiles were determined for strains grown on TSA for 2 days at 30 °C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acid methyl esters were then analysed by GC (model 6890; Hewlett Packard) by using the Microbial Identification software package (Sasser, 1990). Polyamines were extracted and analysed according to the methods of Busse & Auling (1988) and Schenkel *et al.* (1995). Polar lipids were extracted using the procedure described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents as described previously (Lee *et al.*, 2008).

Cellular fatty acid profiles of strain MJ07^T, *P. ginsengisoli* KCTC 22046^T and *P. noertemannii* KACC 13183^T are shown in Table 2. All strains contained $C_{16:0}$, $C_{17:0}$ cyclo, $C_{19:0}$ cyclo ω8*c* and summed feature 3 (iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH; referred to as summed feature 2 by Stolz *et al.*,

Table 1. Differential phenotypic characteristics between strain MJ07^{T}and its phylogenetically closest relatives

Strains: 1, MJ07^T (*Pusillimonas soli* sp. nov.); 2, *P. ginsengisoli* KCTC 22046^T; 3, *P. noertemannii* KACC 13183^T. All data are from the present study except where indicated. In API 20 NE tests, all strains were positive for oxidase and negative for aesculin and gelatin hydrolysis, arginine dihydrolase and indole production. In API ID 32 GN, API ZYM and API 50CH tests, all strains showed identical biochemical characteristics except those indicated here (see species description).

Characteristic	1	2	3
Growth at 25 °C	+	+	−
Enzyme activities (API ZYM)			
Acid phosphatase	+	+	−
Alkaline phosphatase	+	+	−
α-Chymotrypsin	−	+	−
Cystine arylamidase	−	+	−
Leucine arylamidase	+	+	−
Naphthol-AS-BI-phosphohydrolase	+	+	−
Trypsin	−	+	−
Valine arylamidase	−	+	−
Assimilation of (API ID 32GN and API 20 NE):			
Adipate	+	+	−
L-Alanine	+	+	−
Caprate	−	−	+
Citrate	+	+	−
L-Histidine	+	−	−
3-Hydroxybenzoate, 4-hydroxybenzoate	+	−	+
myo-Inositol	+	−	−
Itaconate	+	+	−
L-Malate	+	+	−
Malonate	+	+	−
Phenylacetate	+	+	−
L-Proline	+	+	−
Propionate	+	+	−
L-Rhamnose	+	−	−
D-Ribose	+	−	−
L-Serine	+	+	−
D-Sorbitol	+	−	−
Suberate	+	−	+
Valerate	+	+	−
Acid production from (API 50 CHB):			
L-Arabinose	+	−	−
Arbutin	+	−	−
Gluconate	+	−	−
D-Glucose	+	−	−
Glycerol	+	−	+
D-Fucose, L-fucose	−	−	+
Maltose	−	+	−
D-Ribose	+	−	−
Trehalose	−	+	−
β-D-Xylopyranoside	+	−	−
DNA G+C content (mol%)	59.4	57.3*	61.8†

*Data from Srinivasan *et al.* (2010).
†Data from Stolz *et al.* (2005).

Table 2. Cellular fatty acid profiles of strain MJ07^T and its phylogenetically closest relatives

Strains: 1, MJ07^T (*P. soli* sp. nov.); 2, *P. ginsengisoli* KCTC 22046^T (data from Srinivasan *et al.*, 2010); 3, *P. noertemannii* KACC 13183^T. Unless indicated, data are from the present study; strains were grown on TSA at 30 °C for 48 h prior to fatty acid analysis. Values are percentages of total fatty acids. −, Not detected/not reported; tr, trace amount (<1 %).

Fatty acid	1	2	3
$C_{12:0}$	8.4	tr	10.0
$C_{14:0}$	−	6.4	−
$C_{16:0}$	32.0	16.6	21.9
$C_{17:0}$	−	2.6	−
$C_{17:0}$ cyclo	24.7	8.7	25.7
$C_{18:0}$	2.2	6.2	5.1
$C_{12:0}$ 2-OH	tr	−	5.3
$C_{16:0}$ 3-OH	−	−	2.0
$C_{19:0}$ cyclo $\omega 8c$	11.5	22.8	12.7
Summed feature 3*	5.9	15.3	16.8
Summed feature 4*	9.3	8.6	tr
Summed feature 7*	5.9	7.0	tr

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH; summed feature 4 contained $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH; summed feature 7 contained one or more of $C_{18:1}\omega 7c$, $C_{18:1}\omega 9t$ and $C_{18:1}\omega 12t$.

2005) as the major fatty acids, but some quantitative differences were found between the three strains in the amounts of these components. Apart from these differences, strain MJ07^T differed from its closest neighbour, *P. ginsengisoli* KCTC 22046^T, by the presence of larger amounts of $C_{12:0}$ and by the absence of $C_{14:0}$ and $C_{17:0}$. Strain MJ07^T contained predominantly putrescine, with a moderate amount of 2-hydroxyputrescine, a smaller amount of spermidine and a minor amount of cadaverine. Ubiquinone Q-8 was identified as the major respiratory lipoquinone. The DNA G+C content of strain MJ07^T was 59.4 mol%. All these data are in good agreement with the description of the genus *Pusillimonas* (Stolz *et al.*, 2005) and support the affiliation of the isolate to the genus. Strain MJ07^T contained a large amount of phosphatidylethanolamine, rather large amounts of phosphatidylglycerol, diphosphatidylglycerol and unknown aminolipid AL2, a smaller amount of AL1 and minor amounts of unidentified aminophospholipid APL1 and polar lipid L1 (Supplementary Fig. S1, available in IJSEM Online). These lipids were also detected in *P. noertemannii* BN9^T (Stolz *et al.*, 2005) and *P. ginsengisoli* DCY25^T (Srinivasan *et al.*, 2010). However, the presence of minor amounts of unknown polar lipids APL6, L9–L12 and PL7 could be useful for the differentiation of strain MJ07^T from its closest relatives.

DNA–DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes (Sigma) and microdilution wells (Greiner), 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–DNA hybridization values. Standard deviations were also calculated based on these three values. Strain MJ07^T exhibited relatively low levels of DNA–DNA relatedness with respect to *P. ginsengisoli* KCTC 22046^T (50±8 %) and *P. noertemannii* KACC 13183^T (18±7 %), indicating that it is not related to them at the species level (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). These results are also in good agreement with the recent recommendation that proposed an increase from 97 % to 98.7–99 % in the 16S rRNA gene sequence similarity threshold used to determine the uniqueness of a new strain (Stackebrandt & Ebers, 2006).

The phenotypic and phylogenetic data presented here indicate that strain MJ07^T belongs to the genus *Pusillimonas*. The phylogenetic distinctiveness, together with the DNA–DNA hybridization data, confirmed that this isolate represents a species that is distinct from *P. ginsengisoli* and *P. noertemannii*. There are some phenotypic differences between strain MJ07^T and its phylogenetically closest relatives (Table 1). Therefore, on the basis of the data presented, strain MJ07^T should be classified within the genus *Pusillimonas* as the type strain of a novel species, for which the name *Pusillimonas soli* sp. nov. is proposed.

Description of *Pusillimonas soli* sp. nov.

Pusillimonas soli (so′li. L. neut. gen. n. *soli* of soil, the source of the type strain).

Cells are Gram-negative, non-spore-forming, motile rods, 0.3–0.5 μm wide and 0.7–1.0 μm long. The optimum temperature for growth is 30 °C. Grows at pH 5.0–9.0, with optimum growth between pH 6.5 and 7.0. Positive for oxidase and catalase. Reduces nitrate to nitrogen gas. Casein, chitin, DNA, HE-cellulose, starch and xylan are not hydrolysed. Carbon source utilization (API ID 32 GN and API 20NE; bioMérieux) and enzyme activities (API ZYM; bioMérieux) are reported in Table 1. In addition, acetate, DL-3-hydroxybutyrate and DL-lactate are utilized and *N*-acetyl-D-glucosamine, L-arabinose, gluconate, D-glucose, glycogen, L-fucose, 2-ketogluconate, 5-ketogluconate, maltose, D-mannitol, D-mannose, melibiose, salicin and sucrose are not utilized. Esterase (C4) and esterase lipase (C8) are positive and *N*-acetyl-β-glucosaminidase, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14) and α-mannosidase are negative. In API 50 CHB tests, acid is not produced from any substrate except those indicated in Table 1. The major fatty acids are $C_{16:0}$, $C_{17:0}$ cyclo and $C_{19:0}$ cyclo $\omega 8c$. The DNA G+C content of the type strain is 59.4 mol%. The major polar lipids detected are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and two unknown aminolipids.

The type strain, MJ07^T (=KCTC 22455^T =JCM 16386^T), was isolated from a farm soil near Daejeon, South Korea.

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