Pusillimonas ginsengisoli sp. nov., isolated from soil of a ginseng field

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Two novel strains of Gram-negative, non-sporulating, short rod-shaped, motile bacteria, designated DCY25^T and DCY28, were isolated from soil of a ginseng field in South Korea and characterized in order to determine their taxonomic position. 16S rRNA gene sequence analysis showed that strains DCY25^T and DCY28 belonged to the *Betaproteobacteria*, the highest sequence similarities being found with *Pusillimonas noertemannii* BN9^T (96.9%), *Bordetella trematum* DSM 11334^T (95.9%), *Achromobacter denitrificans* DSM 30026^T (95.9%), *Achromobacter insolitus* LMG 6003^T (95.8%) and *Pigmentiphaga kullae* K24^T (95.5%). Chemotaxonomic data revealed that both strains DCY25^T and DCY28 possessed ubiquinone Q-8. Fatty acid analysis of strain DCY25^T demonstrated the presence of 19:0 cyclo ω 8c (22.8%) and 16:0 (16.6%). The polar lipid profiles of strains DCY25^T and DCY28 included phosphatidylethanolamine, phosphatidylglycerol, two unknown aminolipids and diphosphatidylglycerol. The G+C contents of strains DCY25^T and DCY28 were 57.3 and 57.2 mol%, respectively. DNA–DNA relatedness values, biochemical and physiological characteristics strongly supported the genotypic and phenotypic differentiation of strains DCY25^T and DCY28 from *Pusillimonas noertemannii*. Therefore, strains DCY25^T and DCY28 should be classified in a novel species, for which the name *Pusillimonas ginsengisoli* sp. nov. is proposed. The type strain is DCY25^T (=KCTC 22046^T =JCM 14767^T); strain DCY28 (=KCTC 22047=JCM 14768) is a reference strain.

The family *Alcaligenaceae* (De Ley *et al.*, 1986) belongs to the order *Burkholderiales* within the *Betaproteobacteria*. The genus *Pusillimonas* was proposed by Stolz *et al.* (2005) and currently contains only one species with a validly published name, *Pusillimonas noertemannii*.

During a series of studies to investigate the community structure of micro-organisms in ginseng fields with a culture-dependent method, strains DCY25^T and DCY28 were isolated from surface soil from a ginseng field near Daejeon city in South Korea. A soil sample (1 g) was mixed with 50 ml saline solution, vortexed and serially diluted and an aliquot (0.1 ml) was spread on 1/10 R2A agar (Difco). The plates were incubated at 30 °C for 3 days and

single colonies were purified by repeated subcultivation. Cells of pure cultures were tentatively identified after partial 16S rRNA gene sequencing.

Morphology and motility of cells from 3-day-old colonies grown on R2A agar at 30 °C were observed with a Nikon light microscope (magnification $\times$ 1000). Gram reactions were conducted according to the non-staining method as described by Buck (1982). Oxidase activity was tested by oxidation of 1% *p*-aminodimethylaniline oxalate. Catalase activity was tested by bubble production from 3% (v/v) hydrogen peroxide solution (Cappuccino & Sherman, 2002). Growth at 4, 15, 18, 25, 30, 37 and 42 °C and pH 5.0–10.0, at intervals of 0.5 pH units, was assessed in R2A broth. Anaerobic growth was tested in serum bottles containing 1 g thioglycolate I^{−1} in R2A broth and nitrogen gas as the gaseous layer. The API 20NE, API 50CH, API ZYM and API ID 32GN systems (bioMérieux) were used according to the manufacturer's instructions.

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified with TLC and analysed by

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains DCY25^T and DCY28 are EF672088 and EF672089, respectively.

A chromatogram of polar lipids of strain DCY25^T and a table of DNA–DNA relatedness values between strains DCY25^T and DCY28 and the type strains of type species of closely related genera are available with the online version of this paper.

HPLC as described by Collins & Jones (1981) and Shin *et al.* (1996). To determine the cellular fatty acids, each strain was grown on TSA (Difco) for 48 h at 30 °C and two loopfuls of cell material were then harvested and fatty acid methyl esters were prepared, separated and identified with the Sherlock Microbial Identification System (Sasser, 1990). Polar lipids were extracted and examined by two-dimensional TLC (Minnikin *et al.*, 1977).

For the determination of the DNA G + C content, genomic DNA was extracted and purified with the Qiagen Genomic-tip system 100/G (Qiagen) and degraded enzymically into nucleosides. The nucleosides were analysed using HPLC as described by Tamaoka & Komagata (1984) and Mesbah *et al.* (1989). DNA–DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and micro-dilution wells. For each sample, five replicate hybridizations were conducted, the highest and lowest values were excluded and the DNA–DNA relatedness value was calculated as the mean of the remaining three values.

The 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primer set fD1 and rP1 (Weisburg *et al.*, 1991). Purified PCR products were sequenced by Genotec (Daejeon, Korea; Kim *et al.*, 2005) and the sequences were compiled with SeqMan software (DNASTAR Inc). 16S rRNA gene sequences of related taxa were obtained from GenBank and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). A phylogenetic tree was constructed with the neighbour-joining method (Saitou & Nei, 1987) in MEGA3 (Kumar *et al.*, 2004). Bootstrap analysis with 1000 replicates was conducted in order to obtain confidence levels for the branch nodes (Felsenstein, 1985).

After growth on R2A agar at 30 °C for 5 days, colonies of strains DCY25^T and DCY28 were pale yellow and circular. Cells were aerobic, Gram-negative, motile coccobacilli and grew optimally at 30 °C. The physiological characteristics of strains DCY25^T and DCY28 are given in the species description and in Table 1.

The cellular fatty acids in strain DCY25^T were 19:0 cyclo ω 8c (22.8%), 16:0 (16.6%), summed feature 2 (iso-16:1 I and/or 14:0 3-OH; 15.3%), 17:0 cyclo (8.7%), summed feature 3 (16:1 ω 6c and/or 16:1 ω 7c; 8.6%), summed feature 8 (18:1 ω 7c and/or 18:1 ω 6c; 7.0%), 14:0 (6.4%) and 18:0 (6.2%). Table 2 shows the fatty acid profiles of strains DCY25^T and DCY28 and the type strains of the type species of closely related genera. The major fatty acids of strains DCY25^T and DCY28 were similar to those of *Pusillimonas noertemannii* DSM 10065^T but differed in quantities. The predominant quinone of strains DCY25^T and DCY28 was Q-8, which supported our assignment of the strains to the order *Burkholderiales*, in which the majority of species, including *Pusillimonas noertemannii*,

Table 1. Differential characteristics between strains DCY25^T and DCY28 and the type strains of the type species of closely related genera

Strains: 1, *Pusillimonas ginsengisoli* sp. nov. DCY25^T; 2, *Pusillimonas ginsengisoli* sp. nov. DCY28; 3, *Pusillimonas noertemannii* DSM 10065^T; 4, *Achromobacter xylosoxidans* DSM 10346^T; 5, *Bordetella pertussis* DSM 5571^T; 6, *Pigmentiphaga kullae* DSM 13608^T. Data were obtained in this study unless indicated otherwise. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5	6
Colony colour (LB agar)*	PY	PY	B	PW	WC	WC
Cell dimensions						
Length (µm)	0.5–0.8	0.5–0.8	1–1.5 ^{a†}	2.5–3.0 ^b	1.0–2.8 ^c	1.3–4 ^d
Width (µm)	0.3–0.6	0.4–0.7	0.5–0.5 ^a	0.8–1.2 ^b	0.4–0.7 ^c	0.7–1.2 ^d
Production of:						
Nitrite from nitrate	+	+	–	+	–	–
Acid from glucose	–	–	–	–	w	+
Enzyme activity (API ZYM)						
N-Acetyl-β-glucosaminidase	–	–	+	–	–	–
Acid phosphatase	+	+	–	+	–	+
α-Chymotrypsin	+	+	–	–	–	+
Cystine arylamidase	+	+	–	–	–	–
α-Galactosidase	–	–	+	–	–	–
α-Glucosidase (starch hydrolysis)	–	–	+	–	–	–
Leucine arylamidase	+	+	–	w	w	+
Naphthol-AS-BI-phosphohydrolase	+	+	–	+	+	+
Trypsin	+	+	–	–	–	+
Urease	–	–	+	–	+	+
Assimilation (API ID 32GN, API 20NE) of:						
3-Hydroxybenzoate	–	–	+	–	+	+
4-Hydroxybenzoate	–	–	+	–	+	+
Adipate	+	+	–	+	–	+
Caprate	–	–	+	+	w	–
Citrate	+	+	–	+	–	+
Malonate	+	–	–	–	–	–
Phenyl acetate	+	+	–	+	–	+
Propionate	+	+	–	w	w	+
Suberate	–	–	+	+	+	w
n-Valerate	+	+	w	w	w	+
D-Glucose	–	–	+	+	+	–
D-Ribose	–	+	–	–	–	–
myo-Inositol	–	–	+	w	w	–
L-Alanine	+	+	–	–	–	–
DNA G + C content (mol%)	57.3	57.2	61.8 ^a	65–70 ^b	63.8 ^c	68.5 ^d

*B, Brownish; PW, pale white; PY, pale yellow; WC, white cream.

†Data taken from: a, Stolz *et al.* (2005); b, Yabuuchi & Yano (1981); c, von Wintzingerode *et al.* (2001); d, Blümel *et al.* (2001).

Table 2. Cellular fatty acid profiles of strains DCY25^T and DCY28 and the type strains of the type species of closely related genera

Strains: 1, *Pusillimonas ginsengisoli* sp. nov. DCY25^T; 2, *Pusillimonas ginsengisoli* sp. nov. DCY28; 3, *Pusillimonas noertemannii* DSM 10065^T; 4, *Achromobacter xylosoxidans* DSM 10346^T; 5, *Bordetella pertussis* DSM 5571^T; 6, *Pigmentiphaga kullae* DSM 13608^T. Data were taken from this study after culture on TSA agar at 30 °C for 48 h. tr, <1.0 % of the total; –, not detected.

Fatty acid (%)	1	2	3	4	5	6
Saturated						
10:0 3-OH	–	–	–	tr	tr	1.6
12:0	tr	tr	6.7	4.8	3.4	–
12:0 2-OH	–	–	4.6	2.0	3.2	–
14:0	6.4	7.9	1.5	4.7	tr	tr
14:0 2-OH	–	–	–	2.1	1.5	2.4
16:0	16.6	17.3	14.2	27.4	19.8	30.5
16:0 2-OH	–	–	–	tr	tr	2.6
16:0 3-OH	–	–	1.6	tr	1.3	tr
17:0	2.6	3.1	tr	tr	–	–
17:0 cyclo	8.7	5.4	24.9	10.0	16.8	25.2
18:0	6.2	5.6	1.9	tr	4.3	1.1
18:0 10-methyl	–	–	–	–	8.9	–
19:0 cyclo ω8c	22.8	23.2	14.5	0.3	17.5	14.9
Unsaturated						
13:1 at 12–13	tr	tr	2.6	tr	tr	–
15:1 ω8c	tr	tr	tr	–	tr	–
18:1 ω7c	3.7	4.1	2.1	–	–	10.9
18:1 ω9c	–	–	–	tr	3.9	1.3
18:1 2-OH	–	–	1.6	–	2.8	1.8
Summed features*						
2	15.3	12.9	9.5	13.6	5.6	3.9
3	8.6	10.4	9.8	24.8	7.4	1.8
8	7.0	8.3	2.4	6.9	tr	tr

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 2 consisted of iso-16:1 I and/or 14:0 3-OH. Summed feature 3 consisted of 16:1 ω6c and/or 16:1 ω7c. Summed feature 8 consisted of 18:1 ω7c and/or 18:1 ω6c.

exhibit Q-8 as the predominant quinone. The polar lipid compositions of strains DCY25^T and DCY28 were very similar and included phosphatidylethanolamine and phosphatidylglycerol as the predominant lipids, moderate amounts of two unknown aminolipids and diphosphatidylglycerol and minor amounts of an unknown aminophospholipid and two unknown lipids (Supplementary Fig. S1, available in IJSEM Online). The polar lipid profile was similar to that of *Pusillimonas noertemannii* DSM 10065^T, albeit with different quantities.

The 16S rRNA gene sequences of strains DCY25^T and DCY28 obtained in this study were continuous stretches of 1479 and 1434 nucleotides, respectively. Comparisons with 16S rRNA gene sequences of related taxa suggested that strains DCY25^T

and DCY28 belong to the *Betaproteobacteria*, order *Burkholderiales*, family *Alcaligenaceae*. High sequence similarities were found between strain DCY25^T and strain DCY28 (99.6 %), *Pusillimonas noertemannii* BN9^T (96.9 %), *Bordetella trematum* DSM 11334^T (95.9 %), *Achromobacter denitrificans* DSM 30026^T (95.9 %), *Achromobacter insolitus* LMG 6003^T (95.8 %) and *Pigmentiphaga kullae* K24^T (95.5 %). The phylogenetic tree (Fig. 1) clearly shows that strains DCY25^T and DCY28 belong to the *Pusillimonas* lineage.

The genomic DNA G + C contents of strains DCY25^T and DCY28 were 57.3 and 57.2 mol%, respectively. Strain DCY25^T exhibited a high level of DNA–DNA relatedness with strain DCY28 (87 %) and a low level with *Pusillimonas noertemannii* DSM 10065^T (20 %). Other DNA–DNA relatedness values are given in Supplementary Table S1.

Thus, the results of our polyphasic analysis support the placement of strains DCY25^T and DCY28 in a separate and previously unrecognized species within the genus *Pusillimonas*, for which the name *Pusillimonas ginsengisoli* sp. nov. is proposed.

Description of *Pusillimonas ginsengisoli* sp. nov.

Pusillimonas ginsengisoli (gin.sen.gi.so'li. N.L. n. *ginsengum* ginseng; L. n. *solum* -i soil; N.L. gen. n. *ginsengisoli* of soil of a ginseng field, the source of the type strain).

Cells are Gram-negative, aerobic, motile, short rods, 0.5–0.8 µm long and 0.3–0.6 µm in diameter. Colonies are pale yellow and circular with an entire margin after growth on nutrient agar at 30 °C for 5 days. Grows at 15–40 °C (optimum 30 °C), but not at 4 or 42 °C, and at pH 5.5–9.0 (optimum pH 7.0). Oxidase- and catalase-positive. Produces acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase. Does not produce *N*-acetyl-β-glucosaminidase, arginine dihydrolase, α-fucosidase, β-galactosidase (PNPG hydrolysis), α-galactosidase, α-glucosidase (starch hydrolysis), β-glucosidase (aesculin hydrolysis), β-glucuronidase, lipase (C14), α-mannosidase, protease (gelatin hydrolysis) or urease. Assimilates acetate, adipate, L-alanine, citrate, 3-hydroxybutyrate, itaconate, DL-lactate, L-malate, phenylacetate, L-proline, propionate, L-serine and n-valerate. Does not assimilate *N*-acetyl-D-glucosamine, L-arabinose, caprate, L-fucose, gluconate, D-glucose, glycogen, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-ketogluconate, 5-ketogluconate, maltose, D-mannitol, D-mannose, melibiose, myo-inositol, L-rhamnose, salicin, D-sorbitol, suberate or sucrose. The predominant quinone is Q-8. The major fatty acids (>5 %) are 19:0 cyclo ω8c, 16:0, summed feature 2 (iso-16:1 I and/or 14:0 3-OH), 17:0 cyclo, summed feature 3 (16:1 ω6c and/or 16:1 ω7c), summed feature 8 (18:1 ω7c and/or 18:1 ω6c), 14:0 and 18:0. The predominant polar lipids are phosphatidylethanolamine and phosphatidylglycerol; moderate amounts of unknown aminolipids and

Fig. 1. Neighbour-joining phylogenetic tree showing the relationships between strains DCY25^T and DCY28 and related strains. Bootstrap values (>50%) based on 1000 replications are shown as percentages at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony algorithm. Bar, 0.02 substitutions per nucleotide position.

diphosphatidylglycerol and minor amounts of unknown aminophospholipids and lipids are also found. The G+C content of the type strain is 57.3 mol% (HPLC).

The type strain, DCY25^T (=KCTC 22046^T =JCM 14767^T), and strain DCY28 (=KCTC 22047 =JCM 14768) were isolated from soil of a ginseng field near Daejeon city, in South Korea.

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