Castellaniella daejeonensis sp. nov., isolated from soil

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A Gram-negative, non-spore-forming, motile, facultatively anaerobic bacterium, designated strain MJ06^T, was isolated from oil-contaminated soil and was characterized taxonomically by using a polyphasic approach. Strain MJ06^T contained ubiquinone Q-8 as the major respiratory lipoquinone, putrescine as the predominant polyamine and phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol as major polar lipids. The G + C content of the genomic DNA of strain MJ06^T was 66.2 mol%. The major fatty acids were summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 32.5 %), C_{16:0} (22.8 %) and summed feature 7 (one or more of C_{18:1}ω7c, C_{18:1}ω9t and C_{18:1}ω12t; 14.9 %). Comparative 16S rRNA gene sequence analysis showed that strain MJ06^T belonged to the family *Alcaligenaceae*, class *Betaproteobacteria*, and joined the evolutionary radiation enclosed by the genus *Castellaniella*. Levels of 16S rRNA gene sequence similarity between strain MJ06^T and its phylogenetically closest relatives, *Castellaniella denitrificans* NKNTAU^T, *Castellaniella defragrans* 54Pin^T, *Castellaniella ginsengisoli* DCY36^T and *Castellaniella caeni* Ho-11^T, were 98.6, 98.3, 97.8 and 97.3 %, respectively. Levels of similarity between strain MJ06^T and the type strains of all other recognized species in the family *Alcaligenaceae* were below 95.0 %. Strain MJ06^T exhibited relatively low levels of DNA–DNA relatedness with respect to *C. defragrans* DSM 12141^T (52 %), *C. denitrificans* DSM 11046^T (31 %), *C. ginsengisoli* KCTC 22398^T (18 %) and *C. caeni* KCTC 12197^T (15 %). On the basis of its phenotypic and genotypic properties together with phylogenetic distinctiveness, strain MJ06^T is considered to represent a novel species of the genus *Castellaniella*, for which the name *Castellaniella daejeonensis* sp. nov. is proposed. The type strain is MJ06^T (=KCTC 22454^T =JCM 16240^T).

The genus *Castellaniella* was described by Kämpfer *et al.* (2006) to accommodate species characterized by Gram-negative, short rods that are motile, facultatively anaerobic and denitrifying, and contain ubiquinone Q-8 as the major quinone, phosphatidylethanolamine as the major polar lipid, putrescine as the predominant polyamine and C_{16:0}, C_{16:1}ω7c, C_{17:0} cyclo and C_{18:1}ω7c and/or C_{14:0} 3-OH as major fatty acids. The type species of the genus, *Castellaniella defragrans* (Kämpfer *et al.*, 2006), was reclassified from

Alcaligenes defragrans (Foss *et al.*, 1998) and *Castellaniella denitrificans* was proposed to accommodate two strains, NKNTAU (Denger *et al.*, 1997) and TJ4 (Baek *et al.*, 2003), previously identified as strains of *Alcaligenes defragrans*. Two other members of the genus, *Castellaniella caeni* (Liu *et al.*, 2008) and *Castellaniella ginsengisoli* (Kim *et al.*, 2009), were subsequently described. The present paper reports on the characterization of a bacterial strain, designated MJ06^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 *Castellaniella*. Further study of this strain, based on a polyphasic approach that included chemotaxonomic, physiological and DNA–DNA hybridization analyses, confirmed the position of this strain as a representative of a novel species of the genus *Castellaniella*.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain MJ06^T is GQ241321.

A two-dimensional thin-layer chromatograph of polar lipids of strain MJ06^T and fatty acid profiles of strain MJ06^T and its phylogenetically closest relatives are available as supplementary material with the online version of this paper.

Strain MJ06^T was isolated from oil-contaminated soil collected near an oil reservoir in Daejeon, South Korea. The sample was suspended and spread on R2A 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 on to fresh plates and incubating them again under the same conditions. The isolate was routinely cultured on R2A agar at 30 °C and was maintained as a glycerol suspension (20 %, w/v) at –70 °C. *C. denitrificans* DSM 11046^T, *C. defragrans* DSM 12141^T, *C. caeni* KCTC 12197^T and *C. ginsengisoli* KCTC 22398^T were used as reference strains for DNA–DNA hybridization and other experiments.

For phylogenetic analysis of strain MJ06^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). Full sequences of the 16S rRNA gene were compiled by using SeqMan software (DNASTAR). 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). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated by using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed with 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 MJ06^T (1443 bp) was obtained. Preliminary sequence comparisons against 16S rRNA gene sequences deposited in the GenBank database indicated that strain MJ06^T belonged to the family *Alcaligenaceae*, class *Betaproteobacteria*. On the basis of levels of 16S rRNA gene sequence similarity, the closest recognized relatives of strain MJ06^T were *C. denitrificans* NKNTAU^T (98.6 %), *C. defragrans*

54Pin^T (98.3 %), *C. ginsengisoli* DCY36^T (97.8 %) and *C. caeni* Ho-11^T (97.3 %). Levels of 16S rRNA gene sequence similarity between strain MJ06^T and the type strains of other recognized species within the family *Alcaligenaceae* were less than 95 %. This relationship between strain MJ06^T and other members of the family *Alcaligenaceae* was also evident in the neighbour-joining phylogenetic tree (Fig. 1). Strain MJ06^T, *C. denitrificans* NKNTAU^T, *C. defragrans* 54Pin^T, *C. caeni* Ho-11^T and *C. ginsengisoli* DCY36^T formed a coherent cluster with high bootstrap values in both the neighbour-joining and maximum-parsimony trees. These data indicate that strain MJ06^T can be clearly separated from other members of the family *Alcaligenaceae* with the exception of the four strains given above (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). To differentiate strain MJ06^T from its phylogenetically closest relatives, DNA–DNA hybridization experiments were performed.

The Gram reaction for strain MJ06^T was performed by using the non-staining method, as described by Buck (1982). Cell morphology was observed under a Nikon light microscope at ×1000 magnification with cells grown for 2 days at 30 °C on R2A agar. Catalase and oxidase tests were performed as outlined by Cappuccino & Sherman (2002). Anaerobic growth was determined at 30 °C as described by Liu *et al.* (2008). Assimilation of single carbon sources, enzyme activities and other physiological characteristics were determined with the API ID 32GN, 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 cellulose (Ten *et al.*, 2004) were performed and then evaluated after 7 days. Growth at different temperatures (10, 15, 20, 25, 30, 37, 42 and 45 °C) was assessed on R2A agar, nutrient agar, Luria–Bertani (LB) agar (Difco), trypticase soy agar (TSA; Difco) and MacConkey agar after 5 days of incubation. Growth at pH 5.0–11.0 (at intervals of 0.5 pH units) was evaluated in R2A broth at 30 °C.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strain MJ06^T among neighbouring species selected from the *Betaproteobacteria*. Bootstrap values (expressed as percentages of 1000 replications) of >70 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 1 substitution per 100 nucleotide positions.

Colonies of strain MJ06^T grown on R2A agar plates for 2 days at 30 °C were 0.5–2.0 mm in diameter, circular, smooth, convex, shiny, transparent and yellowish. On R2A agar, LB agar, TSA and nutrient agar the isolate was able to grow at 25–42 °C, but not at 20 or 45 °C. On MacConkey agar, growth occurred at 30–42 °C, but not at 25 °C. The biochemical characteristics of strain MJ06^T were similar to those reported for members of the genus *Castellaniella* (Kämpfer *et al.*, 2006), i.e. positive for assimilation of a number of organic acids (e.g. acetate, caprate and valerate) and amino acids (e.g. L-alanine and L-proline) but negative for β -galactosidase, utilization of sugars (e.g. sucrose, melibiose and glycogen) and acid production from glucose, sucrose and other carbohydrates. Phenotypic and chemotaxonomic characteristics that differentiate strain MJ06^T from its phylogenetically closest relatives are listed in Table 1.

For the measurement of chromosomal DNA G + C content, the genomic DNA of strain MJ06^T 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) by 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 by using Sep-Pak Vac silica cartridges (Waters) and was then analysed by HPLC as described by Hiraishi *et al.* (1996). The cellular fatty acid profile of strain MJ06^T was determined from cells 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). Duplicate experiments were performed. Polyamines were extracted and analysed according to the methods of Busse & Auling (1988) and Schenkel *et al.* (1995). Polar lipids were extracted according to the procedure described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with appropriate detection reagents, as described by Lee *et al.* (2008).

The cellular fatty acid profiles of strain MJ06^T and the type strains of the four recognized *Castellaniella* species are given in Supplementary Table S1 available in IJSEM Online. The major components were summed feature 4 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH; 32.5%), C_{16:0} (22.8%), summed feature 7 (one of more of C_{18:1} ω 7c, C_{18:1} ω 9t and C_{18:1} ω 12t; 14.9%), summed feature 3 (iso-C_{16:1} I and/or C_{14:0} 3-OH; 9.8%), C_{12:0} (9.2%) and C_{17:0} cyclo (8.0%), which is a profile typical of members of the genus *Castellaniella* (Kämpfer *et al.*, 2006; Liu *et al.*, 2008; Kim *et al.*, 2009). However, some minor qualitative and quantitative differences in fatty acid content could be observed between strain MJ06^T and its phylogenetically closest relatives. In particular, strain MJ06^T differed in having a lower content of C_{16:0} and a higher content of

C_{12:0}. Strain MJ06^T contained ubiquinone Q-8 as the major respiratory lipoquinone and putrescine as the predominant polyamine. The genomic DNA G + C content of strain MJ06^T was 66.2 mol%, which lies within the range observed for recognized *Castellaniella* species (63.5–66.9 mol%). Two-dimensional TLC of polar lipids extracted from strain MJ06^T (see Supplementary Fig. S1) showed that the predominant component was phosphatidylethanolamine; it also contained moderate amounts of diphosphatidylglycerol and phosphatidylglycerol, smaller amounts of an unidentified aminolipid, an aminophospholipid and a phospholipid and minor amounts of nine other unidentified polar lipids. This polar lipid profile supported the suggestion that strain MJ06^T belongs to the genus *Castellaniella*, as it was almost identical to the polar lipid composition of the type species of the genus (Kämpfer *et al.*, 2006; Stolz *et al.*, 2005).

DNA–DNA hybridization experiments were performed fluorometrically according to the method of Ezaki *et al.* (1989), by 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 levels of DNA–DNA relatedness. Standard deviations were also calculated based on these three values. Strain MJ06^T exhibited relatively low levels of DNA–DNA relatedness with respect to *C. defragrans* DSM 12141^T (52 $\pm$ 8%), *C. denitrificans* DSM 11046^T (31 $\pm$ 6%), *C. ginsengisoli* KCTC 22398^T (18 $\pm$ 7%) and *C. caeni* KCTC 12197^T (15 $\pm$ 6%), indicating that it is not related to them at the species level (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

The phenotypic and phylogenetic characteristics of strain MJ06^T indicated that it was a member of the genus *Castellaniella*. Phylogenetic distinctiveness, together with DNA–DNA hybridization data, confirmed that strain MJ06^T represents a species that is distinct from recognized *Castellaniella* species. There are several phenotypic differences between strain MJ06^T and its phylogenetically closest relatives (Table 1). Therefore, on the basis of the data presented, we suggest that strain MJ06^T represents a novel species of the genus *Castellaniella*, for which the name *Castellaniella daejeonensis* sp. nov. is proposed.

Description of *Castellaniella daejeonensis* sp. nov.

Castellaniella daejeonensis (dae.jeon.en'sis. N.L. fem. adj. *daejeonensis* pertaining to Daejeon, a city in South Korea, from where the type strain was isolated).

Cells are facultatively anaerobic, Gram-negative, non-spore-forming, motile rods, 0.3–0.5 μ m wide and 1.4–1.7 μ m long. Positive for oxidase and catalase. The optimum temperature for growth is 30 °C. The pH optimum for growth is 6.5–7.0. Nitrate is reduced to nitrogen gas. Gelatin and casein (weakly) are hydrolysed, but starch, chitin, xylan, hydroxyethyl cellulose and DNA are not.

Table 1. Differential phenotypic characteristics between strain MJ06^T and its phylogenetically closest relatives in the genus *Castellaniella*

Strains: 1, MJ06^T; 2, *C. denitrificans* DSM 11046^T; 3, *C. defragrans* DSM 12141^T; 4, *C. ginsengisoli* KCTC 22398^T (data from Kim *et al.*, 2009); 5, *C. caeni* KCTC 12197^T. Data are from the present study except where indicated. In API ID 32GN and API 20 NE tests, acetate, lactate, DL-3-hydroxybutyrate, valerate, caprate, propionate, malate, L-alanine and L-proline are used as sole carbon sources by all of the strains. The following compounds are not assimilated: melibiose, sucrose, 5-ketogluconate, salicin and glycogen. All of the strains are positive for esterase (C4), esterase lipase (C8), leucine arylamidase and aesculin hydrolysis but negative for α -glucosidase, β -glucosidase (6-bromo-2-naphthyl β -D-glucopyranoside), N-acetyl- β -glucosaminidase, arginine dihydrolase, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, lipase (C14), trypsin and urease. +, Positive; –, negative; w, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5
Motility	+	+ ^{a*}	+ ^a	+	– ^b
Growth at 42 °C	–	+ ^a	– ^c	+	– ^b
Growth at 20 °C	–	– ^a	+ ^c	–	+ ^b
pH range for growth	5.0–9.0	5.5–8.5 ^a	6.3–8.4 ^c	5.0–9.0	5.0–8.5 ^b
Gelatin hydrolysis	+	–	–	–	–
Enzyme activities (API ZYM)					
Acid phosphatase	–	+	+	+	+
Alkaline phosphatase	–	+	–	+	–
Cystine arylamidase	–	–	+	–	–
α -Fucosidase	–	–	–	w	w
α -Mannosidase	–	–	–	–	+
Valine arylamidase	–	w	+	w	–
Naphthol-AS-BI-phosphohydrolase	–	+	+	+	+
Assimilation (API ID 32GN and API 20 NE) of:					
L-Arabinose	+	–	–	+	–
D-Glucose	+	–	–	+	+
D-Mannose	+	–	–	+	–
L-Fucose	–	–	–	–	+
D-Mannitol	+	–	–	+	–
D-Sorbitol	+	–	–	–	–
3-Hydroxybenzoate	+	+	+	+	–
Inositol	+	–	–	–	–
Citrate	–	+	+	+	–
N-Acetyl-D-glucosamine	–	–	–	+	–
Malonate	+	–	–	–	–
Itaconate	–	+	–	–	–
2-Ketogluconate	+	–	–	–	–
L-Rhamnose	+	–	–	–	–
D-Ribose	+	–	–	–	–
4-Hydroxybenzoate	+	+	+	+	–
L-Serine	+	–	+	+	+
Gluconate	+	+	+	+	–
Phenylacetate	+	–	+	+	+
Suberate	–	–	–	–	+
Adipate	+	–	–	+	+
L-Histidine	+	–	–	–	+
DNA G + C content (mol%)	66.2	ND	66.9 ^c	63.7	63.5 ^b

*Data obtained from: a, Kämpfer *et al.* (2006); b, Liu *et al.* (2008); c, Foss *et al.* (1998).

Carbon source utilization and enzyme activities are given in Table 1. Chemotaxonomic characteristics, including fatty acid profile and polar lipid pattern, are described in the text and given in Supplementary Table S1 and Supplementary Fig. S1. In API 50 CHB tests, acid is produced without gas from erythritol, mannose, lactose,

D-arabitol and gluconate but not from other substrates. The DNA G + C content of the type strain is 66.2 mol%.

The type strain, MJ06^T (=KCTC 22454^T =JCM 16240^T), was isolated from soil near an oil reservoir in Daejeon, South Korea.

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