Pseudoclavibacter caeni sp. nov., isolated from sludge of a sewage disposal plant

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A Gram-positive, strictly aerobic, rod-shaped, non-motile bacterial strain, designated MJ28^T, was isolated from a sludge sample from the Daejeon sewage disposal plant in South Korea. A polyphasic approach was applied to study the taxonomic position of strain MJ28^T. Strain MJ28^T showed highest 16S rRNA gene sequence similarity to *Pseudoclavibacter soli* KP02^T (95.2%). Levels of 16S rRNA gene sequence similarity to the type strains of other *Pseudoclavibacter* species were less than 94.0%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain MJ28^T belonged to the clade formed by members of the genus *Pseudoclavibacter* in the family *Microbacteriaceae*. The G+C content of the genomic DNA of strain MJ28^T was 65.8 mol%. The chemotaxonomic characteristics of strain MJ28^T showed features typical of the genus *Pseudoclavibacter*, with MK-9 as the predominant respiratory quinone, 2,4-diaminobutyric acid as the diamino acid in the peptidoglycan, and anteiso-C_{17:0} (44.6%), anteiso-C_{15:0} (35.7%) and C_{16:0} (9.5%) as the major fatty acids. On the basis of phylogenetic inference, fatty acid profile and other phenotypic properties, strain MJ28^T is considered to represent a novel species of the genus *Pseudoclavibacter*, for which the name *Pseudoclavibacter caeni* sp. nov. is proposed. The type strain is MJ28^T (=KCTC 19773^T=JCM 16921^T).

The genus *Pseudoclavibacter* was created by Manaia *et al.* (2004) with the reclassification of *Brevibacterium helvolum*. At the time of writing, the genus comprised three recognized species: *Pseudoclavibacter soli* KP02^T, isolated from a ginseng field (Kim & Jung, 2009); *Pseudoclavibacter helvolus* DSM 20419^T, isolated from butter (Manaia *et al.*, 2004); and *Pseudoclavibacter chungangensis* CAU 59^T, isolated from activated sludge (Cho *et al.*, 2010). The genus comprises Gram-positive rods with a B2 γ -type peptidoglycan and MK-9 as the predominant respiratory quinone (Manaia *et al.*, 2004).

In the course of collecting micro-organisms from sludge at the Daejeon sewage disposal plant, South Korea, a Gram-positive, non-motile, aerobic, non-spore-forming bacterial strain, designated MJ28^T, was isolated. On the basis of 16S rRNA gene sequence analysis, strain MJ28^T was considered to belong to the genus *Pseudoclavibacter*. Strain MJ28^T was subjected to a

polyphasic taxonomic investigation and the results indicated it represents a novel *Pseudoclavibacter* species.

Strain MJ28^T was originally isolated from sewage sludge. One gram of the sludge sample was mixed with 50 ml saline [0.85% (w/v) NaCl], vortexed, serially diluted and 100 μ l of each dilution was spread onto 0.1 $\times$ R2A agar (Difco) and incubated at 30 °C for 1 week. Single colonies of strain MJ28^T were transferred to new plates and the purified colonies were tentatively identified by analysis of partial 16S rRNA gene sequences. The isolate was routinely cultured on nutrient agar (NA; Difco) at 30 °C and preserved as a suspension in Luria–Bertani broth (LB; Difco) with 20% glycerol (w/v) at –70 °C.

The genomic DNA of strain MJ28^T 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 nearly complete sequence of the 16S rRNA gene (1464 nt) was compiled with SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MJ28^T is HQ266601.

A supplementary table is available with the online version of this paper.

edited by using the program BioEdit (Hall, 1999). Multiple alignments were performed with the program CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distances were calculated according to the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods (Fig. 1) in MEGA3 (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

On the basis of levels of 16S rRNA gene sequence similarity, the closest relatives of strain MJ28^T were *P. soli* KP02^T (95.2 %), *Zimmermannella alba* IFO 15616^T (94.1 %) and *P. chungangensis* CAU 59^T (93.9 %). 16S rRNA gene sequence similarity to *P. helvolus* DSM 20419^T was ≤93 %. Consequently, the type strains of recognized *Pseudoclavibacter* species were obtained and grown under the same conditions as strain MJ28^T and used as reference strains for phenotypic and chemotaxonomic experiments.

Cell morphology and motility were observed with an Olympus light microscope (1000× magnification) with cells grown for 24 h in nutrient broth at 30 °C. For electron microscopy, cells were gently resuspended in one drop of deionized H₂O, and samples were placed on carbon- and Formvar-coated nickel grids for 30 s. Grids were floated on

one drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then viewed with a Carl Zeiss LEO912AB transmission electron microscope at 100 kV under standard operating conditions. The Gram reaction was performed by using the non-staining method, as described by Buck (1982). Catalase activity was assessed by bubble production in 3 % (v/v) H₂O₂ and oxidase activity was assessed using 1 % (w/v) tetramethyl *p*-phenylenediamine (Cappuccino & Sherman, 2002). Anaerobic growth was examined in serum bottles with sodium thioglycolate (1 g l⁻¹) added to nutrient broth and the upper air layer was replaced with nitrogen. Physiological characteristics were determined with API 20NE, API ID 32 GN and API ZYM galleries (bioMérieux) according to the instructions of the manufacturer. Growth at 4, 10, 20, 25, 28, 30, 37, 40, 42 and 45 °C and at pH 3.0–12.0 (at 0.5 pH unit intervals) was assessed in LB broth for 5 days at 30 °C. The pH of the medium was adjusted by using diluted NaOH and HCl and then sterilized by autoclaving. Salt tolerance was tested in R2A broth supplemented with 0–10 % (w/v) NaCl incubated for 5 days at 30 °C. Growth on trypticase soy agar (TSA; Difco), R2A agar and LB agar was also evaluated at 30 °C. The phenotypic characteristics of strain MJ28^T are given in the species description below and those that differentiate it from closely related type strains are listed in Table 1.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strain MJ28^T, recognized *Pseudoclavibacter* species and representatives of other members of the family *Microbacteriaceae*. Bootstrap values >70 % (percentages of 1000 replications) 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, 0.01 substitutions per nucleotide position. *Brevibacterium linens* DSM 20425^T was used as an outgroup.

Table 1. Phenotypic characteristics that differentiate strain MJ28^T from related species

Strains: 1, MJ28^T; 2, *P. soli* KCTC 19255^T; 3, *P. chungangensis* KCTC 22691^T; 4, *P. helvolus* KCTC 19531^T; 5, *Zimmermannella alba* KCTC 19528^T; 6, *Zimmermannella bifida* KCTC 19529^T; 7, *Zimmermannella faecalis* KCTC 19530^T. All data are from this study under the same experimental conditions. All strains are Gram-positive, non-motile and rod-shaped. w, Weakly positive reaction.

Characteristic	1	2	3	4	5	6	7
Cell size (µm)							
Length	0.3–0.7	0.3–0.5	1.3–1.7	0.4–0.5	0.5–1.1	1.5–3.0	0.5–1.1
Width	0.2–0.3	0.2–0.3	0.1–0.2	0.9–1.1	0.3–0.4	0.3–0.4	0.3–0.4
Growth at/with (LB agar):							
20 °C	+	–	+	+	w	–	–
pH 4	w	–	+	–	–	–	–
5 % (w/v) NaCl	+	w	–	+	w	w	w
6 % (w/v) NaCl	+	–	–	+	–	–	–
Enzyme activity (API ZYM):							
Acid phosphatase	+	+	–	+	w	–	–
Alkaline phosphatase	–	+	+	+	+	–	+
Cystine arylamidase	w	+	–	+	–	+	–
Esterase (C4)	+	+	+	+	–	w	–
Esterase (C8)	w	+	+	+	+	–	–
β-Glucosidase (aesculin hydrolysis)	–	–	–	–	+	+	+
Lipase (C14)	w	+	–	+	+	–	w
Naphthol-AS-BI-phosphohydrolase	w	+	–	+	w	–	–
Protease (gelatin hydrolysis)	–	–	–	–	+	–	w
Acid production from (API 50CH):							
5-Ketogluconate	+	–	–	–	w	+	w
D-Fructose	+	w	–	–	w	–	+
Lactose	+	–	+	–	–	–	–
Maltose	–	–	w	–	–	–	+
Raffinose	–	–	+	–	–	–	–
D-Xylose	+	–	–	–	–	–	+
Glycerol	+	w	+	+	w	+	w
Inositol	+	–	–	+	–	–	+
Mannitol	–	–	+	–	–	–	+
Assimilation as sole carbon sources (API ID32GN):							
Acetate	w	+	–	–	+	+	+
Caprate	w	+	w	–	–	–	–
DL-Lactate	–	–	–	+	+	–	–
L-Malate	+	–	–	–	w	+	+
Malonate	+	+	+	w	w	w	–
n-Valerate	w	+	–	–	w	–	+
L-Arabinose	+	+	w	w	–	–	–
D-Glucose	–	–	+	+	–	–	+
Sucrose	–	–	+	+	–	–	–
D-Mannitol	–	–	+	+	–	–	+
L-Alanine	w	w	+	+	–	–	–
N-Acetyl-D-glucosamine	–	–	+	+	–	–	–
DNA G + C content (mol%)	65.8	64.4	66.2	67.0	68	62	67

Isoprenoid quinones were extracted from bacterial cells of strain MJ28^T with chloroform/methanol (2:1, v/v), evaporated under vacuum and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane quinone solution was then purified by using silica Sep-Pak Vac cartridges (Waters) and was subsequently analysed by HPLC, as described by Hiraishi *et al.* (1996). Strain MJ28^T contained menaquinone MK-9 as the major respiratory quinone and

minor amounts of MK-8. The peptidoglycan was analysed as described by Schleifer & Kandler (1972) by using TLC on cellulose sheets instead of paper chromatography. Strain MJ28^T possessed 2,4-diaminobutyric acid, alanine, glutamate and glycine. Whole-cell sugars contained rhamnose and ribose, as is common for *Pseudoclavibacter* species. Polar lipids were extracted and examined by two-dimensional TLC (Minnikin *et al.*, 1977). The polar lipid profile of

strain MJ28^T included predominantly diphosphatidylglycerol and phosphatidylglycerol and minor amounts of two aminolipids and one glycolipid.

For fatty acid methyl ester analysis, strain MJ28^T and *P. soli* KCTC 19255^T were grown on TSA for 48 h at 30 °C. Two loopfuls of bacterial mass were collected and subjected to saponification, methylation and extraction by using the methods of Kuykendall *et al.* (1988). The fatty acid methyl ester mixtures were separated by using the Sherlock Microbial Identification System (TSBA version 6.0; MIDI), and were then analysed by GC (Hewlett Packard 6890) and identified by using the Microbial Identification software package (Sasser, 1990). The overall fatty acid compositions of the two strains (see Supplementary Table S1 in IJSEM Online) were very similar, dominated by large amounts of anteiso-branched components. The major components of strain MJ28^T were anteiso-C_{17:0} (44.6 %), anteiso-C_{15:0} (35.7 %) and C_{16:0} (9.5 %), in line with members of the genus *Pseudoclavibacter* (Manaia *et al.*, 2004; Kim & Jung, 2009; Cho *et al.*, 2010). However, there were some qualitative and quantitative differences in the fatty acid contents of the two strains. For instance, strain MJ28^T contained significantly higher amounts of anteiso-C_{17:0}, C_{16:0}, C_{14:0} and C_{18:1 ω 9c} as compared with the reference strains.

For determination of the DNA G+C content, genomic DNA was extracted as mentioned above and enzymically degraded into nucleosides. The nucleosides were analysed by HPLC as described previously (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). The DNA G+C content of strain MJ28^T was 65.8 mol%.

The results of the current polyphasic study clearly indicate that strain MJ28^T represents a novel species of the genus *Pseudoclavibacter*, for which the name *Pseudoclavibacter caeni* sp. nov. is proposed.

Description of *Pseudoclavibacter caeni* sp. nov.

Pseudoclavibacter caeni (cae'ni. L. gen. n. *caeni* of sludge).

Cells are Gram-positive, strictly aerobic, non-motile, non-spore-forming rods, 0.2–0.3 µm in diameter and 0.3–0.7 µm long. After 3 days incubation at 30 °C on R2A agar, colonies are 0.5–0.7 mm in diameter, pale yellow, smooth, convex and circular with regular edges. Growth occurs at 20–42 °C (optimum, 30 °C; weak growth at 15 °C). The pH range for growth is pH 4.0–8.5 (optimum, pH 7.0–7.5). Growth occurs with 0–6 % (w/v) NaCl, but not with 7 %. Growth occurs on TSA, LB agar, NA and R2A agar. Positive for catalase and negative for oxidase. Nitrate is not reduced. In API ZYM and API 20E strips, positive for acid phosphatase, esterase (C4 and C8) and leucine arylamidase activities, weakly positive for cystine arylamidase, esterase lipase, lipase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities, but negative for *N*-acetyl- β -glucosaminidase, alkaline phosphatase, arginine dihydrolase, α -chymotrypsin, α -fucosidase, α -galactosidase, β -galactosidase, gelatinase,

α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase, trypsin, lysine decarboxylase and urease activities. In API ID32GN strips, L-malate, malonate and L-arabinose are assimilated as sole carbon sources, sodium acetate, caprate, citrate, n-valerate and L-alanine are weakly assimilated, but *N*-acetylglucosamine, adipate, L-fucose, gluconate, D-glucose, glycogen, L-histidine, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 3-hydroxybutyric acid, itaconate, DL-lactate, D-mannitol, D-mannose, melibiose, *myo*-inositol, phenyl acetate, potassium 5-ketogluconate, potassium 2-ketogluconate, L-proline, propionate, L-rhamnose, D-ribose, salicin, L-serine, D-sorbitol, suberate and sucrose are not assimilated. The predominant respiratory quinone is MK-9. The diamino acid in the peptidoglycan is 2,4-diaminobutyric acid and the whole-cell hydrolysate shows mainly rhamnose and ribose. The polar lipid profile includes predominantly diphosphatidylglycerol and phosphatidylglycerol, with minor amounts of two aminolipids and one glycolipid. The major fatty acids (>12 %) are anteiso-C_{17:0} and anteiso-C_{15:0}.

The type strain, MJ28^T (=KCTC 19773^T=JCM 16921^T), was isolated from sludge at the Daejeon sewage disposal plant in South Korea. The G+C content of the genomic DNA of the type strain is 65.8 mol%.

Acknowledgements

This subject was supported by the Korea Ministry of Environment via 'The GAIA project (173-101-034)'.

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