Castellaniella caeni sp. nov., a denitrifying bacterium isolated from sludge of a leachate treatment plant

Qing-Mei Liu,[1] Leonid N. Ten,[2] Wan-Taek Im[1] and Sung-Taik Lee[1]

[1]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1 Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

[2]Department of Biology & Medicinal Sciences, Pai Chai University, #14 Yonja-1st Street, Seo-Gu, Daejeon 302-735, Republic of Korea

Correspondence
Wan-Taek Im
wandra@kaist.ac.kr

Sung-Taik Lee
e_stlee@kaist.ac.kr

A Gram-negative, non-motile, facultatively anaerobic, denitrifying bacterial strain, designated Ho-11^T, was isolated from sludge of a leachate treatment plant and was characterized taxonomically by using a polyphasic approach. The G+C content of the genomic DNA was 63.5 mol%. Strain Ho-11^T contained ubiquinone Q-8 as the major respiratory lipoquinone and putrescine as the predominant polyamine. The major fatty acids were summed feature 4 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; 29.3 %), $C_{16:0}$ (28.0 %) and summed feature 7 ($C_{18:1}\omega7c$, $C_{18:1}\omega9t$ and/or $C_{18:1}\omega12t$; 19.8 %). Comparative 16S rRNA gene sequence analysis showed that strain Ho-11^T belonged to the family *Alcaligenaceae*, class *Betaproteobacteria*, and joined the evolutionary radiation enclosed by the genus *Castellaniella*. 16S rRNA gene sequence similarities between strain Ho-11^T and the type strains of the two recognized species of the genus, *Castellaniella denitrificans* DSM 11046^T and *Castellaniella defragrans* DSM 12141^T, were 97.8 and 97.4 %, respectively. Levels of similarity between strain Ho-11^T and all other recognized species of the family *Alcaligenaceae* were below 95.6 %. Strain Ho-11^T exhibited relatively low levels of DNA–DNA relatedness with respect to *C. denitrificans* DSM 11046^T (33 %) and *C. defragrans* DSM 12141^T (28 %). On the basis of its phenotypic and genotypic properties together with phylogenetic distinctiveness, strain Ho-11^T (=KCTC 12197^T=LMG 23411^T) should be classified in the genus *Castellaniella* as the type strain of a novel species, for which the name *Castellaniella caeni* sp. nov. is proposed.

The genus *Castellaniella* was described recently 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, putrescine as the predominant polyamine, and $C_{16:0}$, $C_{16:1}\omega7c$, $C_{17:0}$ cyclo, $C_{18:1}\omega7c$ and/or $C_{14:0}$ 3-OH as major fatty acids. At the time of writing, the genus comprised two recognized species, *Castellaniella defragrans* and *Castellaniella denitrificans* (Kämpfer *et al.*, 2006). The former was reclassified from *Alcaligenes defragrans* DSM 12141^T (Foss *et al.*, 1998) and was established as the type species of the new genus, and the latter species was proposed for two strains, NKNTAU (Denger *et al.*, 1997) and TJ4 (Baek *et al.*, 2003), previously identified as strains of *Alcaligenes defragrans*.

During the course of screening micro-organisms from sludge of a leachate treatment plant located in Daejeon (South Korea), a Gram-negative, denitrifying bacterial strain, designated Ho-11^T, was isolated. On the basis of preliminary 16S rRNA gene sequence analysis, strain Ho-11^T was considered to belong to the genus *Castellaniella*. To determine its exact taxonomic position, strain Ho-11^T was subjected to a detailed polyphasic taxonomic investigation, including genotypic, chemotaxonomic and classical phenotypic analyses. These results indicate that strain Ho-11^T represents a novel species of the genus *Castellaniella*.

Strain Ho-11^T was isolated from sludge of the aerobic treatment tanks of a municipal leachate treatment plant. The sludge was suspended and spread on R2A agar plates (Difco) 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 new plates and incubating them again under the same conditions. The isolate was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at −70 °C. *C. denitrificans* DSM

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ho-11^T is AB166879.

11046^T and *C. defragrans* DSM 12141^T, obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ, Braunschweig, Germany), were used as reference strains for DNA–DNA hybridization and other experiments.

For phylogenetic analysis of strain Ho-11^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). 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). Gaps were edited in the program BioEdit (Hall, 1999). Evolutionary distances were calculated by using 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 within the program MEGA3 (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

A nearly complete 16S rRNA gene sequence of strain Ho-11^T (1452 bp) was obtained. Preliminary sequence comparison against 16S rRNA gene sequences deposited in the GenBank database indicated that strain Ho-11^T belonged to the family *Alcaligenaceae*, class *Betaproteobacteria*. On the basis of 16S rRNA gene sequence similarity, the closest recognized relatives to strain Ho-11^T were *C. denitrificans* DSM 11046^T (97.8 %) and *C. defragrans* DSM 12141^T (97.4 %). Levels of similarity between strain Ho-11^T and all other recognized species of the family *Alcaligenaceae* were below 95.6 %. This relationship between strain Ho-11^T and other members of the family *Alcaligenaceae* was also evident in the neighbour-joining phylogenetic tree (Fig. 1). Strain Ho-11^T, *C. denitrificans* DSM 11046^T and *C. defragrans* DSM 12141^T formed a coherent cluster with high bootstrap values in both the neighbour-joining and the maximum-parsimony trees. These data indicate that strain Ho-11^T can be clearly separated from other members of the family *Alcaligenaceae* with the exception of the two species given above (Stackebrandt & Goebel, 1994). To differentiate strain Ho-11^T from these closely related species, 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 examined by light microscopy (Nikon

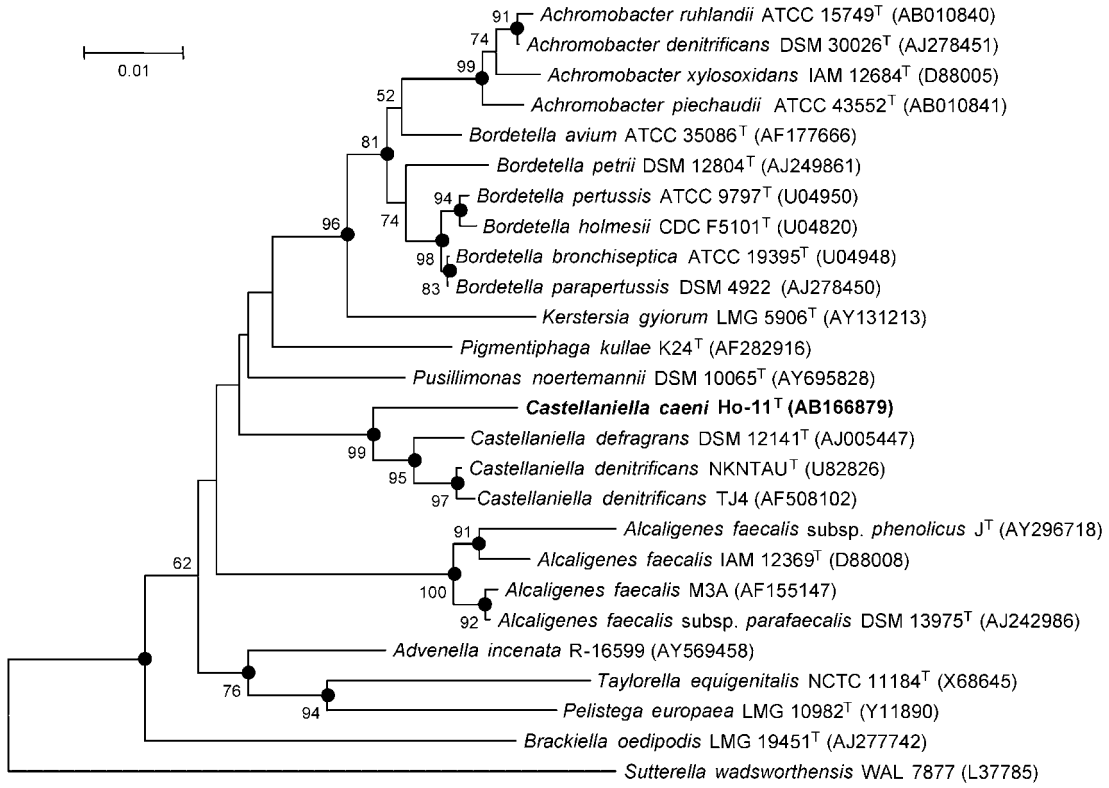

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strain Ho-11^T among neighbouring species selected from the *Betaproteobacteria*. Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 1 substitution per 100 nucleotide positions.

E600). Catalase and oxidase tests were performed as outlined by Cappuccino & Sherman (2002). To study the assimilation of carbon sources, a defined liquid medium containing basal salts was used, containing (per litre distilled water): 1.8 g K_2HPO_4, 1.08 g KH_2PO_4, 0.5 g $NaNO_3$, 0.5 g NH_4Cl, 0.1 g KCl, 0.1 g $MgSO_4$ and 0.05 g $CaCl_2$. A vitamin solution (Widdel & Bak, 1992), a trace elements solution (SL-10; Widdel *et al.*, 1983) and a selenite/tungstate solution (Tschech & Pfennig, 1984) were added to this medium and the pH was adjusted to 6.8. This liquid medium was dispensed into 96-well plates (0.25 ml per well) and filter-sterilized carbon sources were added to each well (at 0.1 %, w/v). Growth in the 96-well plates was examined visually after incubation at 30 °C for 7 days. Negative control wells did not contain any carbon sources. Positive control wells comprised culture growth in R2A broth. Some physiological characteristics were determined with the API 20E, API 20NE and API ID 32 GN galleries according to the manufacturer's instructions (bioMérieux). Anaerobic growth was determined in serum bottles containing R2A broth supplemented with thioglycolate (1 g l^{-1}), and in which the upper air layer had been replaced with nitrogen. Reduction of nitrate and nitrite was determined by using serum bottles containing R2A broth supplemented with KNO_3 (10 mM) and $NaNO_2$ (10 mM), respectively. Reduction of nitrate and nitrite was monitored by ion chromatography on a model 790 personal ion chromatograph (Metrohm) equipped with a conductivity detector and anion exchange column (Metrosep Anion Supp 4; Metrohm). Tests for degradation of DNA [DNase agar (Scharlau), with DNase activity detected by flooding plates with 1 M HCl], casein, chitin, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten *et al.*, 2004) were performed and evaluated after 10 days. Growth at different temperatures (4, 10, 15, 25, 30, 37 and 42 °C) and various pH values (pH 5.0–10.0 at intervals of 0.5 pH units) was assessed after 5 days incubation. Salt tolerance was tested on R2A medium supplemented with 1–10 % (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar (TSA; Difco) and MacConkey agar was also evaluated at 30 °C.

Cells of strain Ho-11^T were facultatively anaerobic, Gram-negative, non-spore-forming, non-motile rods, 0.3–0.5 μm in width and 1.2–2.0 μm in length. The isolate grew well at 37 °C, but growth was not observed at 42 °C. The biochemical characteristics of strain Ho-11^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, lactate and propionate) and amino acids (e.g. L-alanine, L-proline and L-aspartate) but negative for β-galactosidase, utilization of sugars (e.g. L-arabinose, L-rhamnose and D-ribose) and sugar alcohols (e.g. D-mannitol, D-sorbitol and inositol), and acid production from glucose, sucrose and other carbohydrates. Phenotypic and chemotaxonomic characteristics that differentiate strain Ho-11^T from *C. denitrificans* and *C. defragrans* are listed in Table 1.

Table 1. Differential phenotypic characteristics between strain Ho-11^T and recognized *Castellaniella* species

Strains: 1, Ho-11^T (*Castellaniella caeni* sp. nov.); 2, *C. defragrans* DSM 12141^T; 3, *C. denitrificans* DSM 11046^T. Data are from the present study except where indicated. In tests with the API ID 32 GN and API 20NE systems, acetate, lactate, DL-3-hydroxybutyrate, valerate, caprate, propionate, malate, L-alanine and L-proline are used as sole sources of carbon by all of the strains. The following compounds are not assimilated: L-arabinose, D-mannose, D-mannitol, melibiose, D-sorbitol, L-rhamnose, D-ribose, inositol, sucrose, *N*-acetyl-D-glucosamine, malonate, 2-ketogluconate, 5-ketogluconate, salicin and glycogen. All of the strains are positive for β-glucosidase, and negative for arginine dihydrolase, β-galactosidase, urease, gelatin hydrolysis and indole production. +, Positive; −, negative; W, weakly positive; ND, not determined.

Characteristic	1	2	3
Motility	−	+*	+*
Growth at 42 °C	−	−†	+*
pH range for growth	5.0–8.5	6.3–8.4†	5.5–8.5
Assimilation of:			
Glucose	+	−	−
L-Fucose	+	−	−
3-Hydroxybenzoate	−	+	+
4-Hydroxybenzoate	−	+	+
L-Serine	+	+	−
Gluconate	−	+	+
Itaconate	−	−	+
Phenylacetate	W	W	−
Citrate	−	+	+
Adipate	+	−	−
Suberate	+	−	−
L-Histidine	+	−	−
DNA G+C content (mol%)	63.5	66.9†	ND

*Data from Kämpfer *et al.* (2006).
†Data from Foss *et al.* (1998).

For measurement of the G+C content of the chromosomal DNA, the genomic DNA of strain Ho-11^T was extracted and purified as described by Moore & Dowhan (1995) and enzymically degraded into nucleosides. The G+C content was then 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 and re-extracted in *n*-hexane/water (1 : 1, v/v). The crude quinone in *n*-hexane was purified by using Sep-Pak Vac cartridges (Waters) and subsequently analysed by HPLC, as described by 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). 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 and examined by two-dimensional TLC (Minnikin *et al.*, 1977).

The fatty acid profiles of strain Ho-11^T, *C. defragrans* DSM 12141^T and *C. denitrificans* DSM 11046^T are detailed in Table 2. These three strains contained $C_{16:0}$, summed feature 4 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and summed feature 7 ($C_{18:1}\omega7c$, $C_{18:1}\omega9t$ and/or $C_{18:1}\omega12t$) as the predominant fatty acids. However, some minor qualitative and quantitative differences in fatty acid content could be observed between strain Ho-11^T and its phylogenetically closest relatives. In particular, strain Ho-11^T differed from *C. denitrificans* by higher content of summed feature 7 and from *C. defragrans* by the presence of 3-hydroxyhexadecanoic acid ($C_{16:0}$ 3-OH). Strain Ho-11^T contained ubiquinone Q-8 as the major respiratory lipoquinone and putrescine as the predominant polyamine. The DNA G+C content of strain Ho-11^T was 63.5 mol%. The polar lipids detected were phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol. These data are in good agreement with those for other members of the *Betaproteobacteria* (Busse & Auling, 1988; Auling *et al.*, 1991; Kämpfer *et al.*, 2006).

DNA–DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989), by using photobiotin-labelled DNA probes and microdilution wells (Greiner), with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the mean of the remaining three values is quoted as the DNA–DNA hybridization value. Strain Ho-11^T exhibited relatively low levels of DNA–DNA relatedness to *C. denitrificans* DSM 11046^T (33 %) and *C. defragrans* DSM 12141^T (28 %), indicating that it was not related to them at the species level (Wayne *et al.*, 1987).

Phenotypic and phylogenetic characteristics indicated that strain Ho-11^T was a member of the genus *Castellaniella*. Phylogenetic distinctiveness, together with DNA–DNA hybridization data, confirmed that strain Ho-11^T represents a species that is distinct from recognized *Castellaniella* species. Strain Ho-11^T showed some phenotypic differences to phylogenetically related *Castellaniella* species (Table 1). Therefore, on the basis of the data presented, strain Ho-11^T should be classified within the genus *Castellaniella* as the type strain of a novel species, for which the name *Castellaniella caeni* sp. nov. is proposed.

Table 2. Fatty acid content of strain Ho-11^T and recognized *Castellaniella* species

Strains: 1, Ho-11^T (*Castellaniella caeni* sp. nov.); 2, *C. defragrans* DSM 12141^T; 3, *C. denitrificans* DSM 11046^T. Data are from the present study. Values are percentages of total fatty acids. −, Not detected.

Fatty acid	1	2	3
$C_{12:0}$ aldehyde	1.0	0.9	1.0
$C_{12:0}$	6.9	5.7	7.8
$C_{14:0}$	0.6	0.5	1.0
$C_{15:0}$	1.1	0.8	1.3
$C_{16:0}$	28.0	27.9	28.5
$C_{17:0}$	0.5	0.5	0.5
$C_{17:0}$ cyclo	4.4	2.3	7.6
$C_{16:0}$ 3-OH	0.5	−	0.4
$C_{16:1}\omega5c$	−	−	0.4
$C_{17:1}\omega8c$	0.6	0.5	0.5
Summed feature 3*	7.4	6.6	7.5
Summed feature 4*	29.3	36.0	32.7
Summed feature 7*	19.8	18.6	11.0

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contains iso-$C_{16:1}$ I and/or $C_{14:0}$ 3-OH. Summed feature 4 contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH. Summed feature 7 contains $C_{18:1}\omega7c$, $C_{18:1}\omega9t$ and/or $C_{18:1}\omega12t$.

Description of *Castellaniella caeni* sp. nov.

Castellaniella caeni (ca.e′ni. L. gen. neut. n. *caeni* of sludge).

Cells are facultatively anaerobic, Gram-negative, non-motile rods, 0.3–0.5 μm in width and 1.2–2.0 μm in legnth. After 2 days on R2A, colonies are 1–2 mm in diameter, smooth, transparent, circular, convex and brownish. Positive for oxidase, but negative for catalase and lipase. Growth occurs between 10 and 37 °C; optimum temperature for growth is 30 °C. The pH growth range is 5.0–8.5, with an optimum between pH 6.5 and 7.0. Does not require NaCl for growth and can tolerate 5 % (w/v) NaCl. Anaerobically reduces nitrate to nitrite and nitrite to nitrogen gas. Growth occurs on nutrient agar, TSA and MacConkey agar. Aesculin is hydrolysed. Starch, chitin, xylan, CM-cellulose, casein and DNA are not hydrolysed. The following substrates are utilized for growth: D-arabinose, D-glucose, D-galactose, cellobiose, L-fucose, acetate, DL-3-hydroxybutyrate, valerate, fumarate, malate, succinate, formate, propionate, caprate, phenylacetate, glutarate, adipate, lactate, suberate, glycerol, D-adonitol, methanol, L-alanine, L-arginine, L-asparagine, L-aspartate, L-cysteine, L-glutamate, L-glutamine, glycine, L-isoleucine, L-histidine, L-lysine, L-proline, L-serine, L-threonine and L-valine. The following substrates are not utilized for growth: fructose, D-mannose, L-xylose, D-xylose, L-arabinose, D-lyxose, maltose, trehalose, melibiose, raffinose, L-rhamnose, L-sorbose, D-ribose, D-lactose, sucrose, benzoate, citrate, tartrate, gluconate, maleic acid, 3-hydroxybenzoate, 4-hydroxybenzoate, malonate, itaconate, 2-ketogluconate, 5-ketogluconate, oxalate, pyruvate, ethanol, dulcitol, xylitol, D-sorbitol, inositol, D-mannitol, *N*-acetylglucosamine, amygdalin, dextran, inulin, salicin, glycogen, L-leucine, L-methionine, L-phenylalanine, L-tryptophan and L-tyrosine. In API 20E tests, the Voges–Proskauer reaction is positive; gelatin hydrolysis, β-galactosidase, arginine dihy-

drolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, urease, hydrogen sulfide and indole production are all negative. Acid is not produced from L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, glucose or amygdalin. Major fatty acids are summed feature 4 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:0}$ and summed feature 7 ($C_{18:1}\omega7c$, $C_{18:1}\omega9t$ and/or $C_{18:1}\omega12t$). The DNA G+C content of the type strain is 63.5 mol%. Polar lipids detected are phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylglycerol.

The type strain, Ho-11^T (=KCTC 12197^T=LMG 23411^T), was isolated from sludge of the aerobic treatment tanks of a municipal leachate treatment plant located in Daejeon, South Korea.

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