Nocardioides daeguensis sp. nov., a nitrate-reducing bacterium isolated from activated sludge of an industrial wastewater treatment plant

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A Gram-reaction-positive, rod-shaped, non-spore-forming bacterium (strain 2C1-5^T) was isolated from activated sludge of an industrial wastewater treatment plant in Daegu, South Korea. Its taxonomic position was investigated by using a polyphasic approach. On the basis of 16S rRNA gene sequence similarity, the closest phylogenetic relatives were the type strains of *Nocardioides nitrophenolicus* (98.6 % similarity), *N. kongjuensis* (98.5 %), *N. caeni* (98.4 %), *N. simplex* (98.3 %), *N. aromaticivorans* (98.1 %) and *N. ginsengisoli* (97.5 %); the phylogenetic distance from other species with validly published names within the genus *Nocardioides* was greater than 3 %. Strain 2C1-5^T was characterized chemotaxonomically as having LL-2, 6-diaminopimelic acid in the cell-wall peptidoglycan, MK-8(H_4) as the predominant menaquinone and iso-$C_{16:0}$, $C_{16:0}$ and $C_{17:1}\omega6c$ as the major fatty acids. The G+C content of the genomic DNA was 74.9 mol%. These chemotaxonomic properties and phenotypic characteristics supported the affiliation of strain 2C1-5^T to the genus *Nocardioides*. The results of physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain 2C1-5^T from existing species with validly published names. Therefore, strain 2C1-5^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides daeguensis* sp. nov. is proposed, with the type strain 2C1-5^T (=JCM 17460^T=KCTC 19799^T).

The genus *Nocardioides* was proposed by Prauser (1976) with *Nocardioides albus* as the type species. Since then, species affiliated with the genus *Nocardioides* have been actively isolated and studied taxonomically. At the time of writing, the genus comprises 57 species with validly published names, including the recently described species

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2C1-5^T is HQ246164.

Two supplementary tables are available with the online version of this paper.

Nocardioides caricicola (Song *et al.*, 2011), *N. hungaricus* (Tóth *et al.*, 2011), *N. ginsengisegetis* (Im *et al.*, 2011), *N. maradonensis* (Lee *et al.*, 2011), *N. iriomotensis* (Yamamura *et al.*, 2011), *N. ultimimeridianus* (Lee *et al.*, 2011), *N. alpinus* (Zhang *et al.*, 2012), *N. daejeonensis* (Woo *et al.*, 2012) and *N. ginsengagri* (Lee *et al.*, 2012).

Polychlorinated phenols have been released into the environment as a result of their wide usage as biocides, wood preservatives and organic precursors in the synthesis of chlorophenoxyacetate herbicides. They are frequently transformed by anaerobic micro-organisms to various lower-chlorinated phenols, which can be further dechlorinated to chlorophenol or phenol (Boyd & Shelton, 1984; Cole *et al.*, 1994; Mikesell & Boyd, 1986). Since chlorophenol cannot be degraded easily under anaerobic conditions, chlorophenol and phenol may constitute two major byproducts of anaerobic polychlorophenol degradation (Madsen & Aamand, 1992; Woods *et al.*, 1989). Therefore, a micro-organism that can degrade both compounds simultaneously would be very useful.

In this study, we describe the physiological, biochemical and phylogenetic analysis of a *Nocardioides*-like strain, 2C1-5^T. We also report briefly on its utilization of phenolic compounds (phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, *N*-nitrophenol and dibenzofuran) on agar plates and in liquid medium.

Strain 2C1-5^T was isolated from activated sludge of an industrial wastewater treatment plant in Daegu, South Korea, during screening of 4-chlorophenol-degrading bacteria. A 1 % suspension of this activated sludge was initially stimulated with 50 p.p.m. 4-chlorophenol for 2 weeks and was then diluted serially in 0.85 % saline solution. Aliquots of each serial dilution were spread on 1/2 R2A agar (Difco) and incubated at 30 °C for 2 weeks. Single colonies that appeared on the 1/2 R2A plates were then purified by transferring onto fresh 1/2 R2A plates and incubating under the same conditions. The almost-complete sequence of the 16S rRNA gene of strain 2C1-5^T was compiled using the SeqMan software (DNASTAR). 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by using the CLUSTAL X program (Thompson *et al.*, 1997) and gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms in the MEGA4 program (Tamura *et al.*, 2007) and by using the maximum-likelihood algorithm (Felsenstein, 1981) in PHYLIP, version 3.69 (Felsenstein, 2009), based on bootstrap values from 1000 replications (Felsenstein, 1985).

The obtained 16S rRNA gene sequence of strain 2C1-5^T was a continuous stretch of 1393 bp. The isolate was identified using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) on the basis of 16S rRNA gene sequence data. Sequence similarity calculations after alignment indicated that the closest relatives were the type strains of *Nocardioides nitrophenolicus* (98.6 % similarity) (Yoon *et al.*, 1999), *N. kongjuensis* (98.5 %) (Yoon *et al.*, 2006), *N. caeni* (98.4 %) (Yoon *et al.*, 2009), *N. simplex* (98.3 %) (Yoon *et al.*, 1997), *N. aromaticivorans* (98.1 %) (Kubota *et al.*, 2005) and *N. ginsengisoli* (97.5 %) (Cui *et al.*, 2009). Relationships between strain 2C1-5^T and other members of the genus *Nocardioides* are shown in the phylogenetic tree in Fig. 1, which was reconstructed by the neighbour-joining and maximum-parsimony methods. Strain 2C1-5^T formed a monophyletic group with these six species of the genus *Nocardioides* with a high bootstrap value (99 %). The isolate was clearly differentiated from other species in the genus *Nocardioides*. On the basis of phylogenetic inference, these six species were the closest neighbours of strain 2C1-5^T; *N. aromaticivorans* DSM 15131^T, *N. caeni* KCTC 19600^T, *N. ginsengisoli* Gsoil 1124^T, *N. kongjuensis* KCTC 19054^T, *N. nitrophenolicus* KCTC 0457BP^T and *N. simplex* DSM 20130^T were therefore obtained from culture collections, grown under the same conditions and used as reference strains in most phenotypic tests.

Gram-reaction testing was performed by the non-staining method as described by Buck (1982). Cell morphology and motility were observed with an Olympus light microscope ($\times$1000 magnification) using the hanging drop technique with cells grown for 2 days at 30 °C on R2A agar and nutrient agar. Catalase activity was determined by using bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined using 1 % (w/v) tetramethyl *p*-phenylenediamine. Carbon source utilization tests, acid production tests and additional physiological tests were performed using the API 20NE and API ID 32 GN kits (bioMérieux) according to the instructions of the manufacturer. Growth at 4, 10, 15, 20, 25, 30, 37, 42 and 45 °C was assessed on R2A agar after 5 days of incubation. Salt tolerance was determined on R2A agar supplemented with 1, 2, 3, 4, 5, 6, 7, 8 and 10 % (w/v) NaCl, and observed after 5 days of incubation at 30 °C. Growth on LB agar, nutrient agar, trypticase soy agar and MacConkey agar (all from BD) was also evaluated after 7 days of incubation at 30 °C. Growth at pH 5.0–11.0 (at intervals of 0.5 pH units) was tested by incubating the strain in R2A broth adjusted to the appropriate pH for 5 days at 30 °C. Anaerobic growth was tested in serum bottles after adding thioglycolate (1 g l^{-1}) and resazurin oxygen indicator to R2A broth and replacing the upper air layer with nitrogen gas.

For measurement of the G+C content of the chromosomal DNA, genomic DNA was extracted and purified as described by Moore & Dowhan (1995), and the G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Cellular fatty acids were analysed from organisms grown on R2A agar for 48 h at 30 °C. The cellular fatty acids were liberated, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) version 3.0 (Sasser, 1990). The fatty acid methyl esters were then analysed by GC (Agilent 6890; Hewlett Packard) using the Aerobe database (TSBA6, version 6.0) of the Sherlock Microbial Identification software package. For chemotaxonomic analysis (isoprenoid

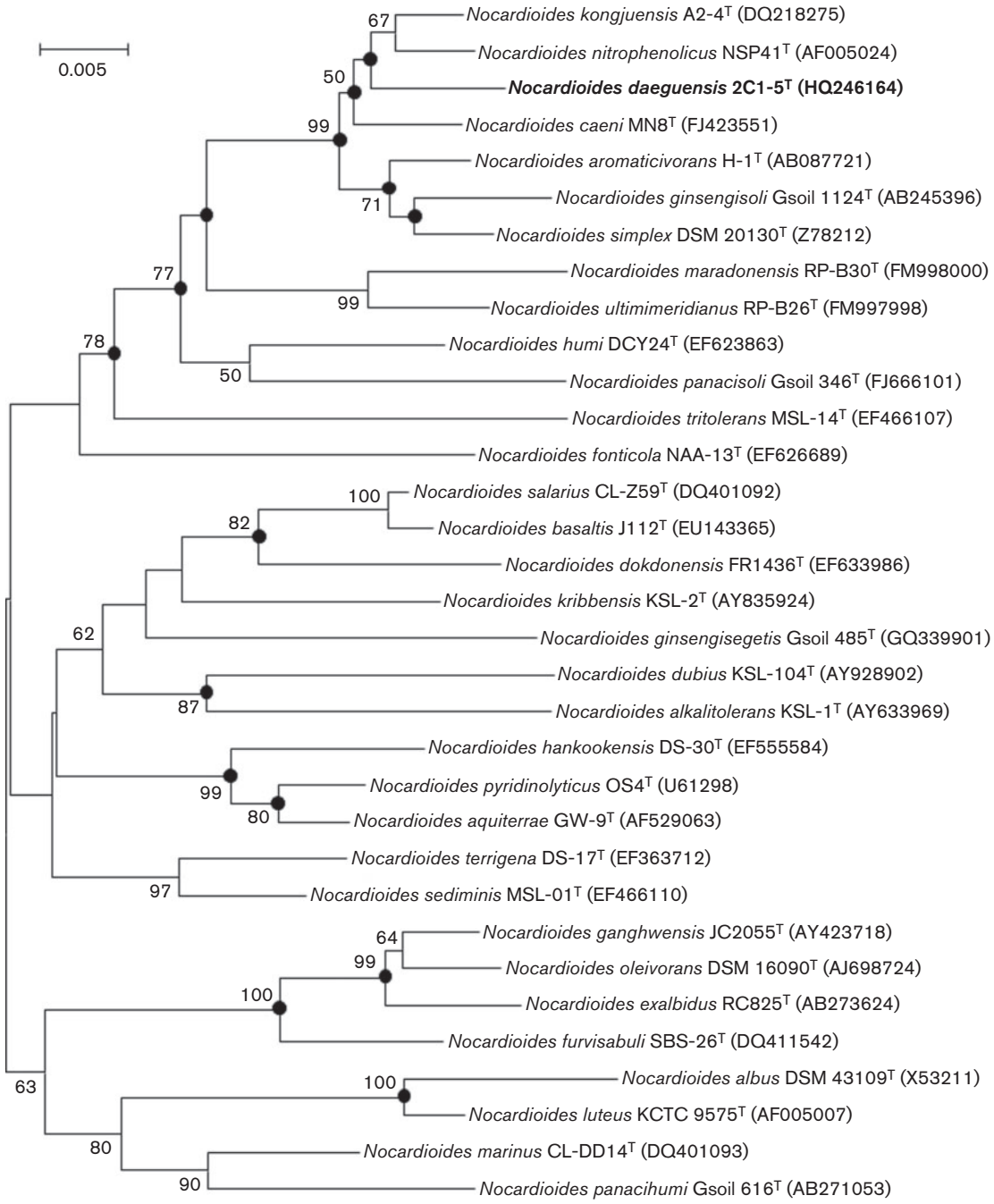

Fig. 1. Neighbour-joining phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships between *Nocardioides daeguensis* sp. nov. 2C1-5^T and type strains of related species of the genus *Nocardioides*. Filled circles at nodes indicate generic branches that were also recovered by using the maximum-parsimony and maximum-likelihood algorithms. Bootstrap values (expressed as percentages of 1000 replications) $\geqslant$50 % are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

quinones and cell-wall peptidoglycan type), strain 2C1-5^T was grown on R2A for 5 days at 30 °C. Collected cells were lyophilized for 24 h. 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 n-hexane–quinone solution was purified using a Sep-Pak Vac silica cartridge (Waters) and subsequently analysed by HPLC as described previously (Hiraishi *et al.*, 1996). The isomer type of the diamino acid of the cell-wall peptidoglycan was determined by using TLC after

hydrolysis with 6 M HCl at 100 °C for 18 h, as described by Komagata & Suzuki (1987).

DNA–DNA hybridization experiments were performed between strain 2C1-5^T and the reference strains with the method described by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed reciprocally 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 were converted to percentage DNA–DNA relatedness values.

A wide variety of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) genes were detected as described previously (Sei *et al.*, 1999). Utilization of aromatic compounds as sole carbon sources was determined by using a minimum salt medium (Bae *et al.*, 1996) with 2 % Noble agar (Difco). After sterilization, aromatic compounds (400, 500, 600, 700, 800 and 1000 p.p.m. phenol, 50, 100, 200, 300, 400 and 500 p.p.m. 2-chlorophenol, 50, 100, 200, 250, 300, 350 and 400 p.p.m. 3-chlorophenol, 50, 100, 150, 200, 250, 300 and 400 p.p.m. 4-chlorophenol, 50, 100, 150 and 200 p.p.m. *N*-nitrophenol and 50, 100, 150 and 200 p.p.m. dibenzofuran) were added separately. We also detected the ability of 2C1-5^T to use aromatic compounds in liquid medium with the same components without Noble agar. To minimize evaporation of phenolic compounds, these tests were performed in 160 ml serum bottles with butyl robber stoppers. After sterilization, aromatic compounds (phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, *N*-nitrophenol and dibenzofuran, each at 10, 25 and 50 p.p.m.) were added separately. The change of aromatic compounds in liquid medium was detected as described previously (Bae *et al.*, 1996).

Cells of strain 2C1-5^T were Gram-reaction-positive, aerobic, non-spore-forming, short rods. Colonies of the isolate cultured on R2A agar, nutrient agar, LB agar and trypticase soy agar plates were pale yellow, circular, opaque and convex, with a diameter of 1–2 mm after 2 days at 30 °C. The phenotypic characteristics of strain 2C1-5^T are summarized in the species description and compared with those of the closest reference strains in Table 1. Utilization of phenolic compounds as sole carbon sources on agar plates is described in the species description. Aromatic compounds in liquid medium were not used by strain 2C1-5^T after 1 month. This might be because the washed Noble agar still contained some nutrients that allowed bacterial growth on agar plates but not in liquid medium. Catechol dioxygenase C23O was detected in strain 2C1-5^T by PCR amplification, but C12O was not detected.

The DNA G+C content of strain 2C1-5^T was 74.9 mol%, a little higher than the DNA G+C contents reported for *N. aromaticivorans* (72.0–72.4 mol%; Kubota *et al.*, 2005), *N. caeni* (71.5 mol%; Yoon *et al.*, 2009), *N. ginsengisoli* (70.2 mol%; Cui *et al.*, 2009), *N. kongjuensis* (72.1 mol%; Yoon *et al.*, 2006), *N. nitrophenolicus* (71.4 mol%; Yoon *et al.*, 1999) and *N. simplex* (72.0–73.5 mol%; Yoon *et al.*, 1997). The respiratory quinone system supported the affiliation of strain 2C1-5^T to the genus *Nocardioides*, as the majority of species in the genus *Nocardioides* have MK-8(H_4) as the predominant quinone. Strain 2C1-5^T and the four closest strains contained LL-2,6-diaminopimelic acid in the cell-wall peptidoglycan. The fatty acid profiles of

Table 1. Physiological characteristics of *Nocardioides daeguensis* sp. nov. 2C1-5^T and type strains of related species of the genus *Nocardioides*

Strains: 1, 2C1-5^T; 2, *N. aromaticivorans* DSM 15131^T; 3, *N. caeni* KCTC 19600^T; 4, *N. ginsengisoli* Gsoil 1124^T; 5, *N. kongjuensis* KCTC 19054^T; 6, *N. nitrophenolicus* KCTC 0457BP^T; 7, *N. simplex* DSM 20130^T. All experimental results are from the present study. API 20NE and API ID 32 GN (bioMérieux) were used according to the instructions of the manufacturer. All strains were negative for assimilation of L-fucose, inositol, itaconic acid, lactic acid, potassium 2-ketogluconate, potassium 5-ketogluconate, sodium malonate, suberic acid and D-sorbitol. All strains were negative for arginine dihydrolase activity, indole production, reduction of nitrate to nitrogen and urease activity. +, Positive; –, negative; W, weakly positive.

Characteristic	1	2	3	4	5	6	7
Assimilation of:							
Adipic acid	–	W	+	W	–	W	–
Capric acid	–	–	–	–	–	+	–
Gluconic acid	W	+	+	+	+	+	+
3-Hydroxybenzoic acid	–	–	–	+	–	+	–
4-Hydroxybenzoic acid	–	+	–	+	+	+	–
3-Hydroxybutyric acid	+	+	–	+	W	+	–
Malic acid	+	W	+	+	+	+	W
Phenylacetic acid	W	–	–	–	–	–	–
Propionic acid	+	–	–	+	W	+	–
Sodium acetate	+	–	W	+	+	+	–
Trisodium citrate	–	–	–	W	–	–	W
Valeric acid	+	–	–	+	+	+	–
L-Arabinose	–	+	–	+	–	–	+
D-Glucose	W	+	–	+	+	+	+
Maltose	–	+	–	+	W	W	–
Mannose	–	–	–	+	W	–	+
Melibiose	–	+	–	–	–	–	+
L-Rhamnose	–	+	+	+	–	+	–
D-Ribose	–	+	–	–	–	+	+
Sucrose	+	+	+	W	+	+	+
N-Acetylglucosamine	W	–	–	+	–	–	+
L-Alanine	–	–	–	+	+	W	+
L-Histidine	–	–	–	+	–	W	–
L-Proline	W	W	–	+	–	+	W
Salicin	–	–	–	–	–	–	+
L-Serine	–	W	–	–	W	–	–
Glycogen	W	W	–	–	–	–	–
D-Mannitol	–	+	–	+	–	–	+
Reduction of nitrate to nitrite	+	–	–	–	–	–	–
Acidification of glucose	–	–	–	+	–	–	–
β-Galactosidase	+	+	–	+	+	+	+
β-Glucosidase	+	+	–	–	+	+	+
Protease	–	–	+	–	–	+	–

2C1-5^T and the type strains of the most closely related species of the genus *Nocardioides* are summarized in Table S1, available in IJSEM Online. According to the results of fatty acid detection, iso-$C_{16:0}$, $C_{16:0}$ and $C_{17:1}\omega6c$ made up about 50–70 % of the total fatty acids in all compared strains, again supporting the conclusion that 2C1-5^T belonged to the genus *Nocardioides* (Yoon *et al.*, 1999; Cui *et al.*, 2009). Strain 2C1-5^T exhibited relatively low levels of DNA–DNA relatedness with respect to the type strains of *N. nitrophenolicus* (35.2 %), *N. simplex* (30.8 %), *N. kongjuensis* (28.9 %), *N. aromaticivorans* (19.6 %), *N. caeni* (11.2 %) and *N. ginsengisoli* (8.5 %); these values are low enough to assign strain 2C1-5^T to a novel species of the genus *Nocardioides* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Detailed DNA relatedness results are given in Table S2.

In summary, the characteristics of strain 2C1-5^T are consistent with the description of the genus *Nocardioides* with regard to morphological, biochemical and chemotaxonomic properties. However, the phylogenetic distance between strain 2C1-5^T and recognized species of the genus *Nocardioides*, its unique phenotypic characteristics (Table 1) and the low levels of DNA–DNA relatedness (Wayne *et al.*, 1987) warrant the assignment of strain 2C1-5^T to a novel species of the genus *Nocardioides*, for which the name *Nocardioides daeguensis* sp. nov. is proposed.

Description of *Nocardioides daeguensis* sp. nov.

Nocardioides daeguensis (dae.gu.en′sis. N.L. masc. adj. *daeguensis* of Daegu, Korea, from where the type strain was isolated).

Cells are Gram-reaction-positive, strictly aerobic, non-spore-forming and non-motile, 0.4–0.8 μm in diameter and 0.4–1.5 μm long after culture on R2A or nutrient agar for 2 days. Grows well at 25–42 °C and but not at 20 or 45 °C. Grows at pH 5.5–8.0, with optimum growth at pH 6.5–7.0. Growth occurs in the absence of NaCl and in the presence of up to 5 % (w/v) NaCl, but not at 6 % NaCl or above. Grows well on LB agar, nutrient agar and trypticase soy agar, but not on MacConkey agar. Catalase-positive and oxidase-negative. Reduces nitrate to nitrite, but does not reduce nitrate to dinitrogen gas. Tests for β-galactosidase and β-glucosidase activities are positive; tests for urease activity, indole production, acidification of glucose and protease activity are negative. Utilizes the following compounds as sole carbon sources: 3-hydroxybutyric acid, malic acid, propionic acid, sodium acetate, valeric acid and sucrose. Utilizes the following compounds weakly as sole carbon sources: gluconic acid, phenylacetic acid, D-glucose, *N*-acetylglucosamine, L-proline and glycogen. Does not utilize the following compounds as sole carbon sources: inositol, itaconic acid, suberic acid, sodium malonate, lactic acid, potassium 5-ketogluconate, L-fucose, D-sorbitol, capric acid, potassium 2-ketogluconate, adipic acid, capric acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, trisodium citrate, L-arabinose, maltose, mannose, melibiose, L-rhamnose, D-ribose, L-alanine, L-histidine, salicin, L-serine and D-mannitol. Utilizes the following aromatic compounds as carbon sources on agar plates at the concentrations given: phenol (400–700 p.p.m.), 2-chlorophenol (50–500 p.p.m.) and 4-chlorophenol (50–150 p.p.m.). Does not utilize the following aromatic compounds as carbon sources on agar plates at the concentrations given: phenol (800–1000 p.p.m.), 3-chlorophenol (50–400 p.p.m.), 4-chlorophenol (200–300 p.p.m.), *N*-nitrophenol (50–200 p.p.m.) and dibenzofuran (50–200 p.p.m.). MK-8(H_4) is the predominant menaquinone and iso-$C_{16:0}$, $C_{16:0}$ and $C_{17:1}\omega6c$ are the major cellular fatty acids.

The type strain, 2C1-5^T (=JCM 17460^T=KCTC 19799^T), was isolated from activated sludge of an industrial wastewater treatment plant in Daegu, South Korea. The G+C content of genomic DNA of the type strain is 74.9 mol% (as determined by HPLC).

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