Brevibacterium daeguense sp. nov., a nitrate-reducing bacterium isolated from a 4-chlorophenol enrichment culture

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A Gram-reaction-positive, non-spore-forming, aerobic actinobacterial strain (2C6-41^T) was isolated from the activated sludge from 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, closest phylogenetic relatives to strain 2C6-41^T were *Brevibacterium pityocampae* DSM 21720^T (97.2 %), *Brevibacterium salitolerans* KCTC 19616^T (96.7 %), *Brevibacterium album* KCTC 19173^T (96.2 %) and *Brevibacterium samyangense* KCCM 42316^T (96.2 %). The DNA G+C content of strain 2C6-41^T was 66.4 mol%. Chemotaxonomic data, which included MK-8(H_2) as the major menaquinone; *meso*-diaminopimelic acid, glutamic acid and alanine as cell-wall amino acids; ribose, mannose and glucose as major cell-wall sugars; and anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, $C_{16:0}$ and iso-$C_{15:0}$ as major fatty acids, supported the affiliation of strain 2C6-41^T to the genus *Brevibacterium*. The aromatic ring cleavage enzyme catechol 1,2-dioxygenase was not detected in strain 2C6-41^T, but catechol 2,3-dioxygenase was detected. The results of physiological and biochemical tests, and the low level of DNA–DNA relatedness to the closest phylogenetic relative enabled strain 2C6-41^T to be differentiated genotypically and phenotypically from recognized species of the genus *Brevibacterium*. The isolate is therefore considered to represent a novel species in the genus *Brevibacterium*, for which the name *Brevibacterium daeguense* sp. nov. is proposed. The type strain is 2C6-41^T (=KCTC 19800^T=JCM 17458^T).

The genus *Brevibacterium* was proposed by Breed (1953) with *Brevibacterium linens* as the type species. Since then, many species affiliated to the genus *Brevibacterium* have been isolated and taxonomically studied. At the time of writing, the genus comprised 26 recognized species, including the recently described *Brevibacterium pityocampae* (Kati *et al.*, 2010), *Brevibacterium salitolerans* (Guan *et al.*, 2010) and *Brevibacterium sandarakinum* (Kämpfer *et al.*, 2010).

Polychlorinated phenols have been released into the environment because of their wide usage as biocides, wood

Abbreviations: C12O, catechol 1,2-dioxygenase; C23O, catechol 2,3-dioxygenase.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 2C6-41^T is HQ246162.

One supplementary figure is available with the online version of this paper.

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 easily degraded under anaerobic conditions, chlorophenol and phenol may constitute two major by-products of the anaerobic degradation of polychlorophenol (Madsen & Aamand, 1992; Woods *et al.*, 1989). Several aerobic bacteria have been reported to degrade 4-chlorophenol through the *ortho*- or *meta*-cleavage pathway, which involves catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O), respectively (Bae *et al.*, 1996). Therefore, it is useful for a micro-organism to contain these two genes and to be able to degrade phenol and 4-chlorophenol simultaneously.

In this study, we describe a novel species of the genus *Brevibacterium* on the basis of physiological, biochemical and phylogenetic analysis. We also briefly report the utilization of phenolic compounds (phenol, 2-chlorophenol, 3-chlorophenol and 4-chlorophenol) in agar plates and liquid broth by the type strain of this species.

Strain 2C6-41^T was isolated from activated sludge from an industrial wastewater treatment plant in Daegu, South Korea, during screening for 4-chlorophenol-biodegrading bacteria. A 1 % suspension of activated sludge in water was initially stimulated with 50 p.p.m. of 4-chlorophenol for 2 weeks at 30 °C and was then diluted serially in 0.85 % saline solution. Aliquots of each dilution were spread on half-strength R2A agar (Difco) and incubated at 30 °C for 2 weeks. Single colonies appearing on the half-strength R2A plates were purified by transfer onto fresh half-strength R2A plates and incubation under the same conditions. A 16S rRNA gene sequence was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments of the sequences 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 constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods in the MEGA4 program (Tamura *et al.*, 2007), with bootstrap values based on 1000 replications (Felsenstein, 1985).

The 16S rRNA gene sequence of strain 2C6-41^T was a continuous stretch of 1365 bp. Sequence similarity calculations after an alignment analysis indicated that the closest relatives were *B. pityocampae* DSM 21720^T (Kati *et al.*, 2010), *B. salitolerans* KCTC 19616^T (Guan *et al.*, 2010), *Brevibacterium album* KCTC 19173^T (Tang *et al.*, 2008) and *Brevibacterium samyangense* KCCM 42316^T (Lee, 2006). The relationship between strain 2C6-41^T and other members of the genus *Brevibacterium* is shown in the neighbour-joining phylogenetic tree (Fig. 1). Strain 2C6-41^T formed a monophyletic clade with the four species of the genus *Brevibacterium* mentioned above with a moderate bootstrap value of 71 % and was well separated from other species of the genus *Brevibacterium*. On the basis of phylogenetic inference, the above four type strains were selected as the closest neighbours of strain 2C6-41^T. *B. pityocampae* DSM 21720^T, *B. album* KCTC 19173^T, *B. salitolerans* KCTC 19616^T and *B. samyangense* KCCM 42316^T were 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 was observed with an Olympus light microscope (1000× magnification) using 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 and additional physiological tests were performed using API 20NE and API ID 32 GN strips (bioMérieux) according to the instructions of the manufacturer. Growth at various temperatures (4, 10, 15, 20, 25, 30, 37, 42 and 45 °C) was tested on R2A agar after incubation for 5 days. Salt tolerance was tested on R2A agar supplemented with 1, 2, 3, 4, 5, 6, 7, 8 and 10 % (w/v) NaCl, and observed after incubation for 5 days at 30 °C. Growth on Luria–Bertani (LB) agar, nutrient agar, trypticase soy agar and MacConkey agar (all Difco) was also evaluated after incubation at 30 °C for 7 days. Anaerobic growth was tested in serum bottles by adding thioglycolate (1 g l^{-1}) to R2A broth and substituting the upper air layer with nitrogen gas and resazurin oxygen indicator.

For measurement of the G+C content of the chromosomal DNA, genomic DNA of the strain was extracted and purified as described by Moore & Dowhan (1995), and the DNA G+C content was determined as described by Mesbah *et al.* (1989) using reversed-phase HPLC. Cellular fatty acid profiles were determined for strains grown in trypticase soy broth (TSB; Difco) at 30 °C for 5 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids analysed by GC (6890; Hewlett Packard) were identified and compared using the Microbial Identification System software package, version 2.95 (Sasser, 1990) and the TSBA 3.9 database. For chemotaxonomic analysis (isoprenoid quinones and cell-wall peptidoglycan type), strain 2C6-41^T was grown on R2A agar 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, 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 previously described (Hiraishi *et al.*, 1996). The isomer of the diamino acid of

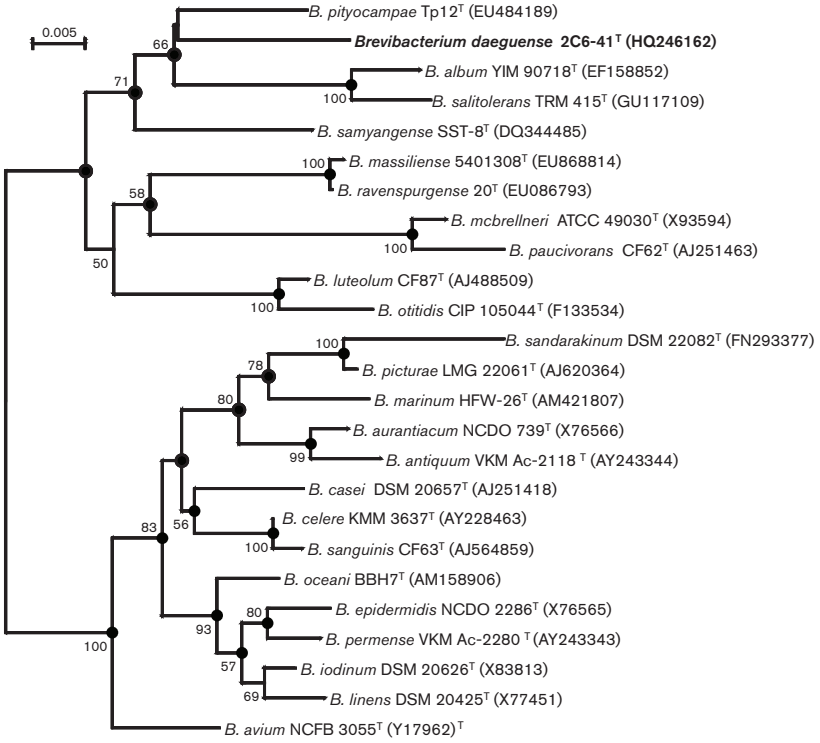

Fig. 1. Neighbour-joining phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strain 2C6-41^T (1365 bp) with related species. Filled circles at nodes indicate corresponding branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1000 replications) of $\geqslant$50 % are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

the cell-wall peptidoglycan was determined by using TLC after cell-wall hydrolysis with 6 M HCl at 100 °C for 18 h, as described by Komagata & Suzuki (1987). Cell-wall sugars were analysed as described by Staneck & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1977).

DNA–DNA hybridization experiments were performed between strain 2C6-41^T and *B. pityocampae* DSM 21720^T according to the method described by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and micro-dilution wells. Hybridization was performed 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.

The presence of a wide variety of C12O and C23O genes was detected as described previously (Sei *et al.*, 1999). The utilization of aromatic compounds as sole carbon sources was determined by using a minimal salt medium (Bae *et al.*, 1996) containing 2 % (w/v) Noble agar (Difco). After sterilization, aromatic compounds [phenol (400, 500, 600, 700, 800 and 1000 p.p.m.), 2-chlorophenol (50, 100, 200, 300, 400 and 500 p.p.m.), 3-chlorophenol (50, 100, 200, 250, 300, 350 and 400 p.p.m.) and 4-chlorophenol (50, 100, 150, 200, 250, 300 and 400 p.p.m.)] were added separately to the basal medium. The aromatic compound utilization ability of strain 2C6-41^T in liquid broth with the same components but without Noble agar was also assessed. To minimize the evaporation of phenolic compounds, these tests were performed in 160 ml serum bottles with butyl rubber stoppers. After sterilization, aromatic compounds [phenol (10, 25 and 50 p.p.m.), 2-chlorophenol (10, 25 and 50 p.p.m.), 3-chlorophenol (10, 25 and 50 p.p.m.) and 4-chlorophenol (10, 25 and 50 p.p.m.)] were added separately. Changes in the aromatic compounds in liquid broth were monitored as described previously (Bae *et al.*, 1996).

Cells of strain 2C6-41^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 were yellow to pale yellow, circular, opaque, convex and shiny with a diameter of 1–1.2 mm after incubation for 3 days at 30 °C. The phenotypic characteristics of strain 2C6-41^T are summarized in the species description and compared with those of the closest phylogenetic relatives in Table 1. The utilization of phenolic compounds as sole carbon sources on agar plates is given in the species description. The aromatic compounds in liquid broth showed no changes in the presence of strain 2C6-41^T during incubation periods of 1 month. This might be because the washed Noble agar still contained traces of nutrients that allowed bacterial growth on the agar plates. Catechol dioxygenase C23O was detected in strain 2C6-41^T by PCR amplification, but C12O was not detected.

The DNA G+C content of strain 2C6-41^T was 66.4 mol%, which is lower than the values for *B. pityocampae* DSM 21720^T (69.8 mol%; Kati *et al.*, 2010), *B. salitolerans* KCTC 19616^T (69 mol%; Guan *et al.*, 2010), *B. album* KCTC

Table 1. Physiological characteristics of strain 2C6-41^T and related type strains of species of the genus *Brevibacterium*

Strains: 1, 2C6-41^T; 2, *B. album* KCTC 19173^T; 3, *B. pityocampae* DSM 21720^T; 4, *B. salitolerans* KCTC 19616^T; 5, *B. samyangense* DSM 19451^T. All strains were negative for assimilation of capric acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, inositol, itaconic acid, mannitol, melibiose, phenylacetate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-rhamnose and D-ribose. All strains were positive for assimilation of suberic acid. All strains were negative for indole production, acid production from glucose, arginine dihydrolase, urease and β-galactosidase. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5
Assimilation of:					
N-Acetylglucosamine	W	W	+	−	+
Adipic acid	+	+	+	−	−
L-Alanine	−	+	+	+	−
L-Arabinose	−	W	+	−	−
L-Fucose	−	−	−	+	−
Gluconic acid	W	+	+	+	+
Glucose	−	W	+	−	+
Glycogen	W	−	−	+	−
L-Histidine	−	+	+	−	−
3-Hydroxybutyric acid	+	+	+	+	−
DL-Lactic acid	W	W	+	+	+
DL-Malic acid	W	W	+	+	+
Maltose	−	−	−	−	+
Mannose	−	W	+	−	+
D-Sorbitol	−	−	−	+	−
L-Proline	−	+	+	−	+
Propionic acid	−	+	−	+	+
Sucrose	−	−	W	−	+
L-Serine	−	+	−	+	−
Sodium acetate	+	W	+	+	+
Sodium malonate	−	W	+	−	+
Trisodium citrate	−	+	−	−	+
Valeric acid	+	−	W	−	+
Enzymes					
Nitrate reductase	+	−	−	−	−
β-Glucosidase	−	−	−	+	−
Gelatin protease	−	+	−	+	−

19173^T (70 mol%; Tang *et al.*, 2008) and *B. samyangense* KCCM 42316^T (70.7 mol%; Lee, 2006). The major menaquinone was MK-8 (H_2), which is consistent with the majority of species in the genus *Brevibacterium*. Strain 2C6-41^T and its four closest phylogenetic relatives contained *meso*-diaminopimelic acid in the cell-wall peptidoglycan. The fatty acid profiles of strain 2C6-41^T and the most closely related species of the genus *Brevibacterium* are summarized in Table 2. According to the results of fatty acid analysis, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$ comprised about 50–67 % of the total fatty acids in all compared strains, which supported the affiliation of strain 2C6-41^T to the genus *Brevibacterium*. DNA–DNA relatedness between strain 2C6-41^T and *B. pityocampae*

Table 2. Cellular fatty acid compositions of strain 2C6-41^T and type strains of related species of the genus *Brevibacterium*

Strains: 1, 2C6-41^T; 2, *B. album* KCTC 19173^T; 3, *B. pityocampae* DSM 21720^T; 4, *B. salitolerans* KCTC 19616^T; 5, *B. samyangense* DSM 19451^T. All strains were cultured in TSB for 5 days at 30 °C. Fatty acids that account for <1.0 % of the total fatty acids are not shown. Major fatty acids (>10 %) are shown in bold type. −, Not detected or <1.0 %.

Fatty acid	1	2	3	4	5
Saturated					
$C_{10:0}$	1.4	−	−	−	3.7
$C_{12:0}$	4.6	−	−	−	7.5
$C_{14:0}$	−	−	−	−	−
$C_{16:0}$	**14.8**	8.3	**17.3**	5.6	**12.9**
$C_{18:0}$	7.2	2.4	8.0	2.7	−
Branched-chain					
iso-$C_{15:0}$	**10.3**	**20.4**	7.2	**10.8**	**15.1**
iso-$C_{16:0}$	−	8.4	7.2	9.7	−
iso-$C_{17:0}$	1.5	3.3	−	3.8	−
anteiso-$C_{15:0}$	**34.5**	**35.8**	**38.2**	**28.3**	**35.0**
anteiso-$C_{15:1}$ A	2.6	−	−	−	−
anteiso-$C_{15:1}$ G	2.4	−	−	−	−
anteiso-$C_{17:0}$	**16.3**	**20.7**	**22.1**	**38.2**	**25.9**
anteiso-$C_{17:1}$ A	4.3	−	−	−	−

DSM 21720^T was 12.94 ± 16.37 %, which is low enough to assign strain 2C6-41^T to a novel species in the genus *Brevibacterium* (Wayne *et al.*, 1987).

In summary, the characteristics of strain 2C6-41^T were consistent with the description of the genus *Brevibacterium* with regard to morphological and chemotaxonomic properties. However, the phylogenetic distance between strain 2C6-41^T and recognized species of the genus *Brevibacterium* species, the unique phenotypic characteristics (Table 1) and the low level of DNA–DNA relatedness (Wayne *et al.*, 1987) warrant assignment of strain 2C6-41^T to the genus *Brevibacterium* as representing a novel species, for which the name *Brevibacterium daeguense* sp. nov. is proposed.

Description of *Brevibacterium daeguense* sp. nov.

Brevibacterium daeguense (da.e.gu.en′se. N.L. neut. adj. *daeguense* of or belonging to Daegu, Korea, from where the type strain was isolated).

Cells are Gram-reaction-positive, aerobic, non-spore-forming and non-motile, 0.5–0.8 μm in diameter and 1–1.5 μm in length after cultivation on R2A agar or nutrient agar for 3 days at 30 °C. Grows well at 10–42 °C, but not at 4 or 45 °C. Growth occurs in the absence of NaCl and with up to 5 % (w/v) NaCl, but not with 6 % NaCl. Grows well on LB agar, nutrient agar and trypticase soy agar, but does not grow on MacConkey agar. Catalase-positive and

oxidase-negative. Reduces nitrate to nitrite. Negative result in tests for β-galactosidase, β-glucosidase, urease, gelatin protease, indole production and acid production from glucose. Utilizes the following compounds as sole carbon sources: adipic acid, 3-hydroxybutyric acid, sodium acetate, suberic acid and valeric acid. Weakly utilizes *N*-acetylglucosamine, gluconic acid, glycogen, DL-lactic acid and DL-malic acid as sole carbon sources, but does not utilize L-alanine, L-arabinose, capric acid, L-fucose, glucose, L-histidine, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, inositol, itaconic acid, maltose, mannitol, mannose, melibiose, phenyl-acetate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-proline, propionic acid, L-rhamnose, D-ribose, D-sucrose, salicin, L-serine, sodium malonate, D-sorbitol or trisodium citrate. Utilizes the following aromatic compounds as carbon sources on agar plates: phenol (400–800 p.p.m.), 2-chlorophenol (50–400 p.p.m.), 3-chlorophenol (50–200 p.p.m.) and 4-chlorophenol (50–300 p.p.m.). MK-8(H_2) is the predominant menaquinone, and anteiso-$C_{15:0}$, anteiso-$C_{17:0}$, $C_{16:0}$ and iso-$C_{15:0}$ are the major cellular fatty acids. *meso*-Diaminopimelic acid, glutamic acid and alanine are components of the peptidoglycan; ribose, mannose and glucose are the major cell-wall sugars. The polar lipids detected are diphosphatidylglycerol, phosphatidylglycerol and two different phosphatidylinositol-mannosides (see Fig. S1, available in IJSEM Online).

The type strain, 2C6-41^T (=KCTC 19800^T=JCM 17458^T), was isolated from activated sludge from an industrial wastewater treatment plant in Daegu, South Korea. The G+C content of the genomic DNA of the type strain is 66.4 mol% (as determined by HPLC).

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