Variovorax defluvii sp. nov., isolated from sewageLong Jin,¹ Kwang Kyu Kim,² Chi-Yong Ahn¹ and Hee-Mock Oh¹

Correspondence

Chi-Yong Ahn

cyahn@kribb.re.kr

Hee-Mock Oh

heemock@kribb.re.kr

¹Environmental Biotechnology Research Centre, Korea Research Institute of Bioscience & Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea²Korean Collection for Type Cultures, Biological Resource Centre, Korea Research Institute of Bioscience & Biotechnology, 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea

A polyphasic taxonomic study was carried out on 2C1-b^T and 2C-21, two strains isolated from sewage flowing into River Geumho in Korea. Cells of the two strains were Gram-negative, non-spore-forming, motile and oval or rod-shaped. Comparative 16S rRNA gene sequence studies showed a clear affiliation of these two isolates with members of the *Betaproteobacteria*; they were most closely related to *Variovorax boronicumulans* KCTC 22010^T, *Variovorax dokdonensis* KCTC 12544^T, *Variovorax ginsengisoli* KCTC 12583^T, *Variovorax paradoxus* ATCC 17713^T and *Variovorax soli* KACC 11579^T showing 16S rRNA gene sequence similarities of 97.4–98.8 % with these strains and shared 100 % similarity with each other. The genomic DNA G + C contents of strains 2C1-b^T and 2C-21 were 65.5 and 65.2 mol%, respectively. Phenotypic and chemotaxonomic data [Q-8 as the major ubiquinone; C_{16:0}, summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{17:0} cyclo and summed feature 7 (C_{18:1}ω7c and/or ω9t and/or ω12t) as major fatty acids] supported the affiliation of strains 2C1-b^T and 2C-21 to the genus *Variovorax*. Based on evidence derived from this polyphasic analysis, it is proposed that strains 2C1-b^T and 2C-21 represent a novel species for which the name *Variovorax defluvii* sp. nov. is proposed; the type strain is 2C1-b^T (=KCTC 12768^T=JCM 17804^T).

Chlorophenols belong to a group of toxic xenobiotics that are resistant to microbial attack and are widely used in industry as wood preservatives, resulting in accumulation of these compounds in soils. Among the proteobacteria, members of the genera *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Burkholderia*, *Pseudomonas*, *Ralstonia* and *Rhodococcus* have been reported to degrade chlorophenols (Apajalahti & Salkinoja-Salonen, 1987; Häggblom, 1990, 1992; Li *et al.*, 1991; Martin-Le Garrec *et al.*, 2001). The genus *Variovorax* within the family *Comamonadaceae* was proposed by reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus*, the type species of the genus (Willems *et al.*, 1991). At the time of writing, the genus comprised five recognized species, *Variovorax paradoxus* (Willems *et al.*, 1991), *V. dokdonensis* (Yoon *et al.*, 2006), *V. soli* (Kim *et al.*, 2006), *V. boronicumulans* (Miwa *et al.*, 2008) and *V. ginsengisoli* (Im *et al.*, 2010).

Two strains, 2C1-b^T and 2C-21, were isolated from sewage flowing into River Geumho in South Korea, during screening for 2-chlorophenol-degrading bacteria. Sewage samples were initially stimulated with 100 p.p.m. 2-chlorophenol; the stimulated cultures were serially diluted in

0.85 % saline solution. Aliquots of each serial dilution were spread on R2A agar (Difco) and incubated at 25 °C for 7 days. Two yellow colonies, designated 2C1-b^T and 2C-21, were isolated. Both of the isolates were subcultivated on R2A agar at 30 °C for 48 h. Based on phylogenetic, phenotypic and chemotaxonomic analysis, the two strains should be placed in the genus *Variovorax*. The strains were further investigated using a polyphasic approach.

For most experiments, all strains were cultivated on R2A agar or broth (Difco) at 30 °C for 48 h. *V. boronicumulans* KCTC 22010^T, *V. dokdonensis* KCTC 12544^T, *V. ginsengisoli* KCTC 12583^T, *V. paradoxus* ATCC 17713^T and *V. soli* KACC 11579^T were used as reference strains under the same conditions.

The Gram reaction was performed with 2 % (w/v) crystal violet, iodine fixation and decolorization. Decolorization was accomplished using 95 % ethanol followed by a distilled water wash and counterstaining with safranin (Gerhardt *et al.*, 1994). Cell morphology and motility were observed under a phase-contrast microscope (Nikon Optiphot; 1000 × magnification) with cells grown on R2A agar for 1–3 days. Oxidase activity was tested using 1 % tetramethyl-*p*-phenylenediamine (Tarrand & Gröschel, 1982) and catalase activity was tested using 3 % H₂O₂. Growth was investigated on R2A agar at different temperatures (4, 8, 10, 15, 20, 25, 30, 32, 37, 42 and 45 °C), NaCl concentrations (1, 2, 3 and 5 %) and

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 2C1-b^T and 2C-21 are HQ385753 and HQ385754, respectively.

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

pH 5–10 (at intervals of 1 pH unit). Different pH values were achieved using appropriate biological buffers: $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer and $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer were used for pH values of 5–7 and pH values of 8–11, respectively (Bates & Bower, 1956; Gomori, 1955). Degradation of DNA [using DNA agar (Difco) supplemented with 0.01 % toluidine blue (Merck)], degradation of casein, chitin and starch (Atlas, 1993), and degradation of cellulose and xylan (Ten *et al.*, 2004) were investigated. Antibiotic susceptibility tests were done in duplicate using filter-paper disks containing the following: ampicillin (20 µg), chloramphenicol (100 µg),

erythromycin (25 µg), gentamicin (30 µg), kanamycin (30 µg), lincomycin (15 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin G (20 IU), streptomycin (50 µg) and tetracycline (30 µg) (Sigma). Carbon source utilization tests, acid production tests and additional physiological tests were performed using API 20NE, API ID 32GN, API 50CH and API ZYM galleries according to the instructions of the manufacturer (bioMérieux).

Fatty acid methyl esters were prepared from bacteria grown in R2A medium for 2 days at 30 °C and saponified,

Table 1. Phenotypic characteristics that distinguish strains 2C1-b^T and 2C1-21 from other *Variovorax* species

Strains: 1, 2C1-b^T; 2, 2C1-21; 3, *V. boronicumulans* KCTC 22010^T; 4, *V. dokdonensis* KCTC 12544^T; 5, *V. ginsengisoli* KCTC 12583^T; 6, *V. paradoxus* ATCC 17713^T; 7, *V. soli* KACC 11579^T. All data are from this study unless indicated. All strains were positive for catalase, oxidase, esterase lipase (C8) and leucine arylamidase but negative for nitrite reduction, indole production, glucose fermentation, arginine dihydrolase, gelatin hydrolysis, *N*-acetyl- β -glucosaminidase, α -chymotrypsin, cystine arylamidase, α -fucosidase, α -galactosidase, β -galactosidase, β -glucosidase, β -glucuronidase, lipase (C14), α -mannosidase and trypsin. All strains assimilated L-arabinose, histidine, 4-hydroxybenzoate, 3-hydroxybutyrate, 2-ketogluconate and suberate. None of the strains assimilated *N*-acetylglucosamine, caprate, maltose, rhamnose, inositol, sucrose, glycogen, L-serine, melibiose or salicin. All strains were grown on R2A for 2 days. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Nitrate reduction	+	+	–	–	+	+	+
Urease	–	–	–	+	–	–	–
Aesculin hydrolysis	+	+	–	–	–	–	–
Assimilation of:							
Acetate	+	+	w	+	+	–	+
Adipate	+	+	–	+	–	+	+
L-Alanine	w	w	w	+	+	–	+
L-Fucose	–	–	–	–	–	+	–
Glucose	–	–	+	+	–	+	–
Gluconate	+	+	–	+	–	+	+
3-Hydroxybenzoate	+	+	+	+	–	+	+
Itaconate	+	+	–	–	–	–	–
5-Ketogluconate	–	–	+	–	–	+	–
DL-Lactate	+	+	w	+	+	–	+
Malate	+	+	+	+	–	+	+
Malonate	+	+	–	–	+	–	–
D-Mannitol	–	–	+	–	+	+	+
D-Mannose	–	–	–	–	+	+	–
Phenylacetate	–	–	–	–	+	+	+
L-Proline	–	–	+	+	+	+	+
Propionate	+	+	+	+	+	–	+
D-Ribose	–	–	+	–	–	+	–
D-Sorbitol	–	–	+	–	–	+	–
Valerate	+	+	+	+	+	–	+
Enzyme activities:							
Acid phosphatase	–	–	w	+	+	–	+
Alkaline phosphatase	–	–	+	+	w	+	+
Esterase (C4)	+	+	–	w	+	+	+
α -Glucosidase	–	–	–	+	+	+	–
Naphthol-AS-BI-phosphohydrolase	–	–	–	–	–	–	+
Valine arylamidase	–	–	–	–	w	+	–
DNA G + C content (mol%)	65.5	65.2	71.2 ^{a*}	66.0 ^b	66.0 ^c	67.0 ^d	67.1 ^e

*Data from: a, Miwa *et al.* (2008); b, Yoon *et al.* (2006); c, Im *et al.* (2010); d, Willems *et al.* (1991); e, Kim *et al.* (2006).

methyated, extracted and analysed (Sasser, 1990) according to the protocol of the Sherlock Microbial Identification System (MIDI). Extracts were analysed by GC (Hewlett Packard 6890) and identified by comparing the fatty acid profiles to the TSBA 3.9 database provided with the Sherlock software 2.95. Isoprenoid quinones were analysed by HPLC as described by Groth *et al.* (1996).

Extraction of genomic DNA, PCR-mediated amplification of the 16S rRNA genes and sequencing of purified PCR products were carried out according to Rainey *et al.* (1996). 16S rRNA gene sequences were aligned with published sequences retrieved from GenBank/EMBL using CLUSTAL_X (Thompson *et al.*, 1997) and edited using BIOEDIT (Hall, 1999). The phylogenetic tree was reconstructed on the basis of the neighbour-joining method (Saitou & Nei, 1987); distances were estimated using the Kimura two-parameter model (Kimura, 1983) with MEGA version 3.1 (Kumar *et al.*, 2004). The resultant tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resampled dataset. DNA G+C content was determined by HPLC after hydrolysis as described by Tamaoka & Komagata (1984) and non-methylated λ DNA (Sigma) was used as a standard. DNA–DNA hybridization to determine genomic relatedness was performed fluorometrically by the method of Ezaki *et al.* (1989) using DNA probes labelled with photobiotin (Sigma) and microdilution wells (Greiner Bio-one; 96-well microplate).

Strains 2C1-b^T and 2C1-21 formed visible colonies on R2A agar incubated at 30 °C within 48 h. Growth occurred at temperatures ranging from 8 to 37 °C, but no growth was observed at 4 °C or at 42 °C. Growth occurred at pH 6–9, but no growth was observed at pH 5 or 10. Cells were Gram-negative, motile and oval- or rod-shaped. Colonies on R2A were circular, slightly convex, smooth, glistening

and yellow in colour. Detailed physiological and biochemical characteristics are summarized in Table 1 and in the species description.

The almost-complete 16S rRNA gene sequences (approx. 1450 nt) of strains 2C1-b^T and 2C1-21 were determined and compared with those of representative species within the genus *Variovorax*. Strains 2C1-b^T and 2C1-21 shared 100 % 16S rRNA gene sequence similarity. The novel strains showed highest levels of 16S rRNA gene sequence similarity to *V. boronicumulans* KCTC 22010^T, *V. dokdonensis* KCTC 12544^T, *V. ginsengisoli* KCTC 12583^T, *V. paradoxus* ATCC 17713^T and *V. soli* KACC 11579^T with similarities of 97.3, 97.6, 97.9, 97.4 and 98.8 %, respectively. In the phylogenetic tree, strains 2C1-b^T and 2C1-21 occupied a distinct position and formed a relatively stable branch with members of the genus *Variovorax* (Fig. 1). DNA–DNA hybridization experiments were performed to confirm the taxonomic status of the novel strains. Strains 2C1-b^T and 2C1-21 shared 90 % DNA–DNA relatedness, and showed levels of relatedness of 19–41 % with *V. boronicumulans* KCTC 22010^T, *V. dokdonensis* KCTC 12544^T, *V. ginsengisoli* KCTC 12583^T, *V. paradoxus* ATCC 17713^T and *V. soli* KACC 11579^T. These values are well below the 70 % cut-off point recommended for the delineation of genomic species (Wayne *et al.*, 1987).

The genomic DNA G+C content of the novel strains was 65.2–65.5 mol%. The major fatty acids were C_{16:0}, summed feature 4 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), C_{17:0} cyclo and summed feature 7 (C_{18:1} ω 7c and/or ω 9t and/or ω 12t). This fatty acid profile was similar to those of the five recognized *Variovorax* species, although there were differences in the proportions of some fatty acids (Table 2). Chemotaxonomic data together with phylogenetic analysis support the affiliation of strains 2C1-b^T and 2C1-21 to the genus *Variovorax*.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences using the neighbour-joining method (Saitou & Nei, 1987) showing the positions of strains 2C1-b^T and 2C1-21 and related taxa. Numbers at branch points refer to bootstrap percentage (1000 resamplings; only values above 50 % shown). Bar, 0.5 substitutions per 100 nt positions.

Table 2. Cellular fatty acid compositions (%) of strains 2C1-b^T and 2C1-21 and the type strains of related *Variovorax* species

Strains: 1, 2C1-b^T; 2, 2C1-21; 3, *V. boronicummulans* KCTC 22010^T; 4, *V. dokdonensis* KCTC 12544^T; 5, *V. ginsengisoli* KCTC 12583^T; 6, *V. paradoxus* ATCC 17713^T; 7, *V. soli* KACC 11579^T. All data are from this study. Cells of all strains were harvested after growth on R2A medium at 30 °C for 2 days. —, Not detected.

Fatty acid	1	2	3	4	5	6	7
C _{10:0}	—	—	0.5	—	0.8	—	0.6
C _{10:0} 3-OH	2.1	1.9	3.2	2.9	2.7	3.5	5.7
C _{12:0}	3.3	3.2	3.1	5.7	3.3	2.7	2.8
C _{12:0} 3-OH	—	—	—	2.4	—	—	—
C _{14:0}	2.0	1.9	1.6	1.4	4.2	1.2	1.2
C _{14:0} 2-OH	2.2	1.9	1.2	—	—	3.3	—
C _{15:0}	1.1	1.4	—	1.2	2.1	5.9	0.7
C _{15:1} ω6c	0.6	0.6	—	0.8	—	1.8	—
C _{16:0}	36.5	35.7	33.7	26.8	37.5	27.2	31.6
C _{16:1} ω5c	—	—	—	—	—	1.9	—
C _{16:1} 2-OH	—	—	—	—	—	0.8	—
C _{17:0}	0.8	0.9	1.2	1.7	—	1.6	1.4
C _{17:0} cyclo	11.8	15.5	21.6	17.3	22.5	16.5	18.3
C _{18:0}	1.1	0.8	—	—	—	—	—
Summed feature 4*	27.8	24.9	20.7	23.3	16.3	21.7	28.8
Summed feature 7†	10.8	11.0	12.8	15.7	8.6	11.6	8.4

*Contains C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

†Contains C_{18:1}ω7c and/or ω9t and/or ω12t.

The mean DNA–DNA relatedness value between strains 2C1-b^T and 2C1-21 and the type strains of members of the genus *Variovorax* was 19–41 % when their DNAs were used individually as labelled DNA probes for cross-hybridization. This value indicated that they represent different genomic species (Wayne *et al.*, 1987). Therefore, on the basis of the data presented, strains 2C1-b^T and 2C1-21 represent a novel species of the genus *Variovorax*, for which the name *Variovorax defluvii* sp. nov. is proposed.

Description of *Variovorax defluvii* sp. nov.

Variovorax defluvii (de.flu'vi.i. L. gen. n. *defluvii* of sewage).

Cells are Gram-negative, non-spore-forming, motile, oval- or rod-shaped, 0.4–0.5 µm in diameter and 0.8–2.0 µm in length. Colonies on R2A agar are circular, slightly convex, smooth, glistening and yellow in colour. Good growth is observed on R2A agar at 28–32 °C. Growth occurs at 8–37 °C, but not at 4 or 42 °C. Optimal pH for growth is 7.0; growth occurs at pH 6.0–9.0, but not at pH 5.0 or 10.0. Growth occurs in the presence of 1 and 2 % NaCl, but not in >3 % NaCl. Oxidase and catalase are positive. Hydrolyses casein but does not hydrolyse chitin, DNA, starch, cellulose or xylan. Positive for nitrate reduction and aesculin hydrolysis but negative for nitrite reduction, indole production, glucose fermentation, arginine dihydrolase, urease and gelatin hydrolysis (API 20NE test strip).

Assimilates acetate, adipate, L-alanine (weakly), L-arabinose, gluconate, histidine, 4-hydroxybenzoate, 3-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 2-ketogluconate, DL-lactate, malate, malonate, propionate, suberate and valerate. Does not assimilate N-acetylglucosamine, caprate, citrate, L-fucose, D-glucose, glycogen, inositol, 5-ketogluconate, maltose, D-mannitol, D-mannose, melibiose, phenylacetate, L-proline, rhamnose, D-ribose, salicin, L-serine, D-sorbitol or sucrose (API 20NE and API ID 32GN test strips). Positive for (API ZYM test strip) esterase (C4), esterase lipase (C8) and leucine arylamidase but negative for N-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase. Acid is produced from aesculin, L-arabinose, fructose (weakly) and D-fucose, but not from N-acetylglucosamine, adonitol, amygdalin, D-arabinose, D-arabitol, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, L-fucose, galactose, gentiobiose, gluconate, glucose, glycerol, glycogen, inositol, inulin, 2-ketogluconate, 5-ketogluconate, lactose, D-lyxose, maltose, mannitol, mannose, melezitose, melibiose, methyl α-D-glucoside, methyl α-D-mannoside, methyl β-D-xylose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, sucrose, starch, D-tagatose, trehalose, turanose, xylitol, D-xylose or L-xylose. Susceptible to ampicillin (20 µg), lincomycin (15 µg), neomycin (30 µg) and penicillin G (20 IU) but resistant to chloramphenicol (100 µg), erythromycin (25 µg), gentamicin (30 µg), kanamycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), streptomycin (50 µg) and tetracycline (30 µg). The predominant ubiquinone is Q-8. The major fatty acids are C_{16:0}, summed feature 4 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{17:0} cyclo and summed feature 7 (C_{18:1}ω7c and/or ω9t and/or ω12t). The G + C content of the genomic DNA is 65.2–65.5 mol%.

The type strain, 2C1-b^T (=KCTC 12768^T=JCM 17804^T), was isolated from sewage flowing into River Geumho in Korea. The genomic DNA G + C content of the type strain is 65.5 mol%.

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