Variovorax ginsengisoli sp. nov., a denitrifying bacterium isolated from soil of a ginseng field

Wan-Taek Im,¹ Qing-Mei Liu,² Kang-Jin Lee,¹ Se-Young Kim,¹
Sung-Taik Lee² and Tae-Hoo Yi¹

Correspondence
Tae-Hoo Yi
drhoo@khu.ac.kr

¹Department of Oriental Medicinal Material and Processing College of Life Science, Kyung Hee University, Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-701, Republic of Korea

²Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Guseong-dong, Yuseong-gu, Daejeon 305-701, Republic of Korea

A Gram-negative, aerobic or facultatively anaerobic, non-spore-forming, motile, rod-shaped bacterium (strain Gsoil 3165^T) was isolated from soil of a ginseng field in Pocheon, South Korea. Its taxonomic position was determined by using a polyphasic approach. On the basis of 16S rRNA gene sequence analysis, strain Gsoil 3165^T was shown to belong to the family *Comamonadaceae*, class *Betaproteobacteria*, and was related most closely to the type strains of *Variovorax boronicumulans* (98.9% similarity), *Variovorax paradoxus* (98.3%), *Variovorax soli* (98.2%) and *Variovorax dokdonensis* (96.6%). Levels of 16S rRNA gene sequence similarity between strain Gsoil 3165^T and the type strains of other species in the family *Comamonadaceae* were less than 97.0%. The G+C content of the genomic DNA of strain Gsoil 3165^T was 66 mol%. Phenotypic and chemotaxonomic data (Q-8 as the major ubiquinone; C_{16:0} and C_{17:0} cyclo as major fatty acids) supported the affiliation of strain Gsoil 3165^T to the genus *Variovorax*. The results of physiological and biochemical tests allowed the genotypic and phenotypic differentiation of strain Gsoil 3165^T from recognized *Variovorax* species. Gsoil 3165^T is therefore considered to represent a novel species of the genus *Variovorax*, for which the name *Variovorax ginsengisoli* sp. nov. is proposed. The type strain is Gsoil 3165^T (=KCTC 12583^T =LMG 23392^T).

The genus *Variovorax* was erected with the reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus* (Willems *et al.*, 1991). Phylogenetic analyses based on 16S rRNA gene sequences showed that the genus *Variovorax* falls within the family *Comamonadaceae*, class *Betaproteobacteria* (Anzai *et al.*, 2000). At the time of writing, the genus comprises four recognized species, *V. paradoxus*, *Variovorax dokdonensis* (Yoon *et al.*, 2006), *Variovorax soli* (Kim *et al.*, 2006) and *Variovorax boronicumulans* (Miwa *et al.*, 2008).

During the course of a study on the culturable aerobic bacterial community in soil of a ginseng field of Pocheon Province, South Korea, a large number of novel bacterial strains were isolated (Im *et al.*, 2005). In the present study, we conducted a phylogenetic analysis on the basis of 16S rRNA gene sequences and examined phenotypic, genotypic and chemotaxonomic characteristics to determine the precise taxonomic position of one of these strains (Gsoil 3165^T). On the basis of the results obtained, we propose that strain Gsoil 3165^T should be placed in the genus *Variovorax* as the type strain of a novel species.

Strain Gsoil 3165^T was originally isolated from soil of a ginseng field. The soil sample was serially diluted with 50 mM phosphate buffer (pH 7.0) and the suspension was spread on one-fifth-strength modified R2A agar plates (per litre distilled water: 0.25 g tryptone, 0.25 g peptone, 0.25 g yeast extract, 0.125 g malt extract, 0.125 g beef extract, 0.25 g Casamino acids, 0.25 g soytone, 0.5 g glucose, 0.3 g soluble starch, 0.2 g xylan, 0.3 g sodium pyruvate, 0.5 g KNO₃, 0.3 g K₂HPO₄, 0.05 g MgSO₄, 0.05 g CaCl₂ and 15 g agar). The plates were incubated at room temperature (25 °C) for 1 month in an anaerobic chamber (Three-shine) containing N₂/CO₂/H₂ (80:15:5). Single colonies on the plates were purified by transferring them to new plates and incubating them again on modified R2A agar or half-strength modified R2A agar plates. Purified colonies were tentatively identified by partial sequences of the 16S rRNA gene (Im *et al.*, 2005). Strain Gsoil 3165^T was one of the isolates that appeared on the modified R2A agar plates under anaerobic conditions. It was also routinely cultured under aerobic conditions on R2A agar (Difco) at 30 °C and maintained as a glycerol suspension (20%, w/v) at –70 °C.

Gram-reaction testing was performed by the non-staining method as described by Buck (1982). Cell morphology was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 3165^T is AB245358.

observed at $\times 1000$ magnification with a light microscope (Nikon) by using cells grown for 3 days at 30 °C on R2A agar. Catalase activity was determined based on bubble production in 3% (v/v) H₂O₂ and oxidase activity was determined by using 1% (w/v) tetramethyl *p*-phenylenediamine. Growth and biochemical tests were also carried out by using the API 20 NE, API ID 32GN and API ZYM commercial kits according to the manufacturer's instructions (bioMérieux). Anaerobic growth was tested in serum bottles containing R2A broth supplemented with thioglycolate (1 g l⁻¹) and incubating under nitrogen gas. The anaerobic nitrate reduction test to determine the final electron acceptor was performed in serum bottles by adding 1 g thioglycolate l⁻¹ and 10 mM KNO₃ to the R2A broth, again under nitrogen gas. Aerobic nitrate reduction was later confirmed by inoculation into 12 ml R2A broth supplemented with 10 mM KNO₃ in three 25-ml serum bottles. Nitrate reduction was monitored by using an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and an anion exchange column (Metrosep Anion Supp 4; Metrohm).

Tests for the degradation of DNA [DNase agar (Scharlau), 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 5 days. Growth at 4, 15, 20, 25, 30, 37 and 42 °C and pH 5.0–10.0 (at intervals of 0.5 pH units) was assessed after 5 days incubation. Salt tolerance was tested on R2A agar supplemented with 1–10% (w/v) NaCl after 5 days incubation. Growth on nutrient agar, trypticase soy agar (TSA) and MacConkey agar was also evaluated, at 30 °C. Sensitivity to antibiotics was tested on R2A plates by using antibiotic discs containing the following compounds: streptomycin (10, 25, 50, 100 µg), penicillin (5, 10, 20, 50 U), chloramphenicol (5, 25, 50, 100 µg), ampicillin (5, 10, 20, 50 µg), cephalothin (10, 30, 50, 100 µg), gentamicin (10, 30, 50, 100 µg), novobiocin (1, 2, 5, 10 µg), tetracycline (5, 15, 25, 50 µg), kanamycin (10, 30, 50, 100 µg), lincomycin (3, 5, 15, 30 µg), oleandomycin (1.5, 2.5, 7.5, 15 µg), neomycin (10, 30, 50, 100 µg), carbenicillin (2.5, 7.5, 20, 30 µg) and erythromycin (5, 20, 50, 100 µg).

Extraction of genomic DNA was performed with 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 Ten *et al.* (2008). 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 GenBank. Multiple alignments were performed via the CLUSTAL X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated according to Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) algorithms within the MEGA 3 program

(Kumar *et al.*, 2004) with bootstrap values determined based on 1000 replications (Felsenstein, 1985).

For measurement of the chromosomal DNA G+C content, genomic DNA of strain Gsoil 3165^T was extracted and purified as described by Moore & Dowhan (1995), and was degraded enzymically into nucleosides. The G+C content was 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 conditions and re-extracted in *n*-hexane/water (1:1, v/v). The crude *n*-hexane-quinone solution was purified by using Sep-Pak Vac silica cartridges (Waters) and was subsequently analysed by HPLC as described by Hiraishi *et al.* (1996). Cellular fatty acid profiles were determined for strains grown on R2A agar (Difco) for 2 days. Cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed by GC (Hewlett Packard 6890) and were identified according to the Microbial Identification software package (Sasser, 1990). DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989), with photobiotin-labelled DNA probes and microdilution 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 are quoted as the DNA–DNA relatedness value.

Cells of strain Gsoil 3165^T were Gram-negative, aerobic or facultatively anaerobic, motile rods. The strain was able to grow anaerobically in the presence of nitrate. Colonies grown on R2A agar plates (Difco) for 5 days were undulatory, convex, embedded, yellowish and 2–5 mm in diameter. Strain Gsoil 3165^T grew well on nutrient agar and TSA. On R2A agar medium, it was able to grow at 4–30 °C, but not at 37 °C. Other physiological characteristics of strain Gsoil 3165^T are summarized in the species description below and a comparison of selective characteristics with the type strains of closely related species in the genus *Variovorax* is presented in Table 1.

The 16S rRNA gene sequence of strain Gsoil 3165^T determined in this study was a continuous stretch of 1499 bp (base positions 9–1512 with respect to the *Escherichia coli* numbering system). 16S rRNA gene sequence similarity calculations after neighbour-joining analysis indicated that the closest relatives of strain Gsoil 3165^T were the type strains of *V. boronicumulans* (98.9%), *V. paradoxus* (98.3%), *V. soli* (98.2%) and *V. dokdonensis* (96.6%). Lower levels of 16S rRNA gene sequence similarity (<97.0%) were found with the type strains of all other recognized species in the family *Comamonadaceae*. This relationship between strain Gsoil 3165^T and members of the genus *Variovorax* was also evident in the neighbour-joining phylogenetic tree (Fig. 1). Strain Gsoil 3165^T and the type strains of *V. boronicumulans* and *V. paradoxus* formed a clade with a high bootstrap value (98%); this

Table 1. Differential physiological characteristics between strain Gsoil 3165^T and the type strains of recognized *Variovorax* species

Strains: 1, Gsoil 3165^T; 2, *V. dokdonensis* DS-43^T; 3, *V. paradoxus* DSM 66^T (data in columns 1–3 from the present study); 4, *V. soli* GH9-3^T (Kim *et al.*, 2006); 5, *V. boronicumulans* BAM-48^T (Miwa *et al.*, 2008). All strains were Gram-negative, motile, rod-shaped and positive for catalase and oxidase. All produced positive results for alkaline phosphatase, esterase lipase (C8) and leucine arylamidase and negative results for indole production, glucose fermentation, hydrolysis of aesculin and gelatin and activities of arginine dihydrolase, β -galactosidase, lipase (C14), cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. None was able to assimilate *N*-acetyl- β -glucosamine, inositol or L-rhamnose. +, Positive; –, negative; w, weakly positive; NA, no data available.

Characteristic	1	2	3	4	5
Cell shape	Rods	Oval or rods	Rods	Rods	Rods
Cell width (μ m)	0.3–0.5	0.3–0.5	0.5–0.6	0.5–0.7	0.5–0.7
Cell length (μ m)	1.5–3.0	0.7–2.8	1.2–3.0	1.0–1.5	1.0–2.0
Nitrate reduction	+	–	+	+	–
Anaerobic growth with nitrate	+	–	–	–	NA
Urease	–	+	–	–	–
Valine arylamidase	w	–	w	–	–
Acid phosphatase	+	+	–	+	w
Naphthol-AS-BI-phosphohydrolase	–	–	–	+	–
α -Glucosidase	+	w	+	–	–
Utilization of substrates (API results)					
D-Glucose	–	+	+	+	–
L-Arabinose	+	–	+	+	w
D-Mannose	+	–	+	–	–
D-Mannitol	+	–	+	+	w
Gluconate	–	+	+	+	–
Adipate	–	+	+	+	–
Citrate	–	–	–	–	+
Phenylacetate	+	–	+	+	–
D-Sorbitol	–	–	+	+	–
DNA G + C content (mol%)	66.0	66.0	67.0	67.1	71.2

topology was also recovered in the maximum-parsimony phylogenetic tree.

Because levels of 16S rRNA gene sequence similarity between Gsoil 3165^T and the type strains of *V. boronicumulans*, *V. soli* and *V. paradoxus* were above 97 %, DNA–DNA hybridization experiments between these taxa were performed, as described by Ezaki *et al.* (1989). Mean levels

of DNA–DNA relatedness between strain Gsoil 3165^T and *V. boronicumulans* BAM-48^T, *V. soli* GH9-3^T and *V. paradoxus* DSM 66^T were 33, 25, 24 %, respectively, when their DNA was used individually as labelled DNA probes for cross-hybridization. These values indicate that strain Gsoil 3165^T represents a genomic species separate from *V. boronicumulans*, *V. soli* and *V. paradoxus* (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987).

Fig. 1. Neighbour-joining phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships between strain Gsoil 3165^T and related species. Filled circles at nodes indicate branches that were also recovered by using the maximum-parsimony algorithm. Bootstrap values (expressed as a percentage of 1000 replications) of ≥ 70 % are shown at branch points. Bar, 0.005 substitutions per nucleotide position.

The G+C content of the genomic DNA of strain Gsoil 3165^T was 66 mol%. Q-8 was the predominant ubiquinone. The fatty acid profile of strain Gsoil 3165^T (Table 2) mainly comprised C_{16:0} (39.07%) and C_{17:0} cyclo (18.82%). There were no significant differences between the fatty acid profile of strain Gsoil 3165^T and those of recognized *Variovorax* species.

All of the characteristics determined for strain Gsoil 3165^T were in accordance with those of the genus *Variovorax*. However, on the basis of phylogenetic distance based on 16S rRNA gene sequences (Fig. 1) and the combination of unique phenotypic characteristics (Table 1) and low levels of DNA–DNA relatedness, it is clear that Gsoil 3165^T is not affiliated with any recognized species of the genus *Variovorax*. Therefore, on the basis of the data presented, we consider that strain Gsoil 3165^T represents a novel species of the genus *Variovorax*, for which the name *Variovorax ginsengisoli* sp. nov. is proposed.

Description of *Variovorax ginsengisoli* sp. nov.

Variovorax ginsengisoli (gin.sen.gi.so'li. N.L. n. *ginsengum* ginseng; L. n. *solum* soil; N.L. gen. n. *ginsengisoli* of soil of a ginseng field, the source of the type strain).

Cells are Gram-negative, aerobic or facultatively anaerobic, motile and rod-shaped, 0.3–0.5 µm in diameter and 1.5–3.0 µm long after 3 days of culture on R2A agar. Colonies grown on R2A agar (Difco) for 3 days are undulatory, convex, embedded, yellowish and 2–5 mm in diameter. Grows well at 4–30 °C and at pH 5.0–8.5, but does not

grow at 37 °C. Growth occurs in the absence of NaCl and in the presence of 1.0% (w/v) NaCl, but not in the presence of 2% (w/v) NaCl. Nitrate is reduced aerobically and is reduced to nitrogen under anaerobic conditions. Anaerobic growth does not occur in the absence of nitrate. Unable to degrade DNA, chitin, cellulose, skimmed milk, olive oil or xylan. Utilizes acetate, 3-hydroxybutyrate, valerate, 3-hydroxybenzoate, 4-hydroxybenzoate, propionate, lactate, suberate, alanine, histidine, proline and malonate as sole carbon sources, but not itaconate, malate, D-fucose, D-fructose, L-rhamnose, L-sorbose, D-ribose, N-acetyl-D-glucosamine, maleic acid, salicin, serine or inositol. Other substrate utilization, enzyme production and physiological characteristics are given in Table 1. Susceptible to penicillin G (20 U), erythromycin (25 µg), streptomycin (50 µg), chloramphenicol (100 µg), tetracycline (30 µg), kanamycin (30 µg), neomycin (30 µg), and oleandomycin (15 µg) but resistant to ampicillin (20 µg), novobiocin (5 µg), gentamicin (30 µg) and lincomycin (15 µg). The predominant ubiquinone is Q-8 and C_{16:0} and C_{17:0} cyclo are the major cellular fatty acids. The G+C content of the genomic DNA of the type strain is 66.0 mol% (determined by HPLC).

The type strain, Gsoil 3165^T (=KCTC 12583^T =LMG 23392^T), was isolated from soil of a ginseng field of Pocheon Province, South Korea.

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Table 2. Cellular fatty acid profiles of strain Gsoil 3165^T and the type strains of recognized *Variovorax* species

Strains: 1, Gsoil 3165^T; 2, *V. dokdonensis* DS-43^T; 3, *V. paradoxus* DSM 66^T (data in columns 1–3 from the present study); 4, *V. soli* GH9-3^T (Kim *et al.*, 2006); 5, *V. boronicumulans* BAM-48^T (Miwata *et al.*, 2008). All were grown on R2A for 2 days.

Fatty acid	1	2	3	4	5
Straight-chain					
C _{10:0}	0.53	0.56	—	0.57	—
C _{12:0}	3.65	6.29	3.92	3.29	3.5
C _{14:0}	4.91	1.25	1.37	0.77	1.1
C _{15:0}	0.49	—	7.09	1.17	—
C _{16:0}	39.07	34.95	31.60	38.11	36.1
C _{17:0}	—	—	2.74	1.22	—
C _{10:0} 3-OH	3.54	3.27	2.86	4.77	2.9
C _{17:0} cyclo	18.82	23.12	23.85	22.53	19.0
Summed feature 4*	19.72	23.45	16.98	21.48	21.4
Summed feature 7*	9.28	7.10	11.93	5.09	11.3

*Summed features are groups of two or three fatty acids which cannot be separated by GLC with the MIDI system. Summed feature 4 comprised C_{16:1}ω7*c*/iso-C_{15:0} 2-OH; summed feature 7 comprised C_{18:1}ω7*c*/ω9*t*/ω12*t*.

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