Variovorax dokdonensis sp. nov., isolated from soil

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A Gram-negative, motile and oval- or rod-shaped bacterial strain, DS-43^T, was isolated from soil from Dokdo, Korea, and its taxonomic position was investigated by using a polyphasic approach. The strain grew optimally at pH 7.0–8.0 and at 30 °C in the presence of 0.5 % (w/v) NaCl. Strain DS-43^T had Q-8 as the predominant ubiquinone and C_{16:0}, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{18:1}ω7c and C_{17:0} cyclo as the major fatty acids, which are consistent with the corresponding data for the type strain (KCTC 12459^T) of *Variovorax paradoxus*. The DNA G + C content was 66.0 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain DS-43^T was most closely related to *Variovorax paradoxus*, the only recognized species of the genus. The level of 16S rRNA gene sequence similarity between strain DS-43^T and *V. paradoxus* IAM 12373^T was 97.3 %. DNA–DNA relatedness data and differential phenotypic properties demonstrated that strain DS-43^T is distinguishable from *V. paradoxus*. On the basis of phenotypic, phylogenetic and genetic data, it is proposed that strain DS-43^T (= KCTC 12544^T = CIP 108838^T) be classified in the genus *Variovorax* as a member of a novel species, *Variovorax dokdonensis* sp. nov.

The genus *Variovorax* was proposed by reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus*, the single recognized species of the genus (Willems *et al.*, 1991). Phylogenetic analyses based on 16S rRNA gene sequences showed that the genus *Variovorax* falls within the family *Comamonadaceae* of the *Betaproteobacteria* (Anzai *et al.*, 2000). In this study, we describe a *Variovorax*-like bacterial strain, DS-43^T, which was isolated from a soil from Dokdo, Korea, and provide an assessment of its taxonomic position.

Strain DS-43^T was isolated by the standard dilution plating technique at 25 °C on 10 × diluted nutrient agar (NA; Difco). *V. paradoxus* KCTC 12459^T, which was used as a reference strain for isoprenoid quinone and fatty acid analyses and DNA–DNA hybridization, was obtained from the Korean Collection for Type Cultures, Taejeon, Korea. The morphological, physiological and biochemical characteristics of strain DS-43^T were investigated by using routine cultivation on trypticase soy agar (TSA; Difco) at 30 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. The presence of flagella was determined by transmission electron microscopy using cells from exponentially growing cultures. For transmission electron microscopic observation, the cells

were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after air-drying with a Philips CM-20 transmission electron microscope. The Gram reaction was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4–45 °C) was measured on TSA. Growth in the absence of NaCl and at various NaCl concentrations [0.5 % (w/v) and 1.0–5.0 % (w/v) at intervals of 1.0 %] was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. The pH range for growth was determined in nutrient broth (Difco) adjusted to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units). The pH was adjusted to various levels prior to sterilization by the addition of HCl or Na₂CO₃. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on TSA and on TSA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were studied as described by Lanyi (1987). Sensitivity to antibiotics was tested on TSA plates using antibiotic discs containing: polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) or carbenicillin (100 µg). Other physiological and

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biochemical properties were tested by using the API 20E, API 20NE and API ZYM systems (bioMérieux).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained from cultures grown in trypticase soy broth (Difco) at 30 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For fatty acid methyl ester analysis, cell mass of strain DS-43^T and *V. paradoxus* KCTC 12459^T was harvested from TSA plates after incubation for 3 days at 30 °C. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by using the method of Ezaki *et al.* (1989) using 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 were quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain DS-43^T are given in the species description (see later) or are shown in Table 1. The almost complete 16S rRNA gene sequence of strain DS-43^T determined in this study comprised 1486 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analyses showed that strain DS-43^T was phylogenetically most closely related to the genus *Variovorax* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain DS-43^T joined the phylogenetic lineage of *V. paradoxus* with a bootstrap confidence level of 75.3 % (Fig. 1). The relationship between strain DS-43^T and *V. paradoxus* was also recovered in the trees based on the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain DS-43^T had a 16S rRNA gene sequence similarity value of 97.3 % to *V. paradoxus* IAM 12373^T and of <96.1 % to all other species included in the phylogenetic analysis.

The predominant respiratory lipoquinone detected in strain DS-43^T was ubiquinone-8 (Q-8) at a peak area ratio of approximately 97 %. In this study, *V. paradoxus* KCTC 12459^T was also found to contain Q-8 as the predominant respiratory lipoquinone. The fatty acid profiles of strain DS-43^T and *V. paradoxus* KCTC 12459^T were similar to each other (Table 2). The two strains contained large amounts of

Table 1. Differential phenotypic characteristics of *Variovorax dokdonensis* sp. nov. and *Variovorax paradoxus*

Species: 1, *V. dokdonensis*; 2, *V. paradoxus*. +, Positive reaction; –, negative reaction; D, 11–79 % of strains are positive; [+], 80 % or more of strains are positive; data in parentheses are for the type strain. The two species are positive for catalase*, oxidase*, urease*, hydrolysis of Tween 80*, utilization of glucose and gluconate, and susceptibility to polymyxin B, streptomycin, chloramphenicol, gentamicin, tetracycline, kanamycin and neomycin. Both are negative for the Gram stain*, anaerobic growth with nitrate*, hydrolysis of starch*, utilization of maltose and caprate, and susceptibility to penicillin G, ampicillin, novobiocin, lincomycin and oleandomycin.

Characteristic	1	2
Flagellation	Peritrichous	1–2
Nitrate reduction	–	D* (+)†
Hydrolysis of:		
Gelatin	–	D* (–)†
Utilization of:‡		
Arabinose	–	(+)
Mannose	–	(+)
Mannitol	–	(+)
Adipate	+	[+]* (+)
Citrate	–	+* (–)
Phenylacetate	–	D* (–)
Susceptibility to antibiotics:‡		
Cephalothin (30 µg)	+	(–)
Carbenicillin (100 µg)	+	(–)
DNA G + C content (mol%)	66.0	66.8–69.4 (67.0)

*Data for *V. paradoxus* from Kersters & De Ley (1984).

†Data for the type strain of *V. paradoxus* from this study.

‡Susceptibility to antibiotics for the type strain of *V. paradoxus* from this study.

straight, unsaturated, cyclo and hydroxy fatty acids; the major components (>10 % of the total fatty acids) were C_{16:0}, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{18:1}ω7c and C_{17:0} cyclo (Table 2). The DNA G + C content of strain DS-43^T was 66.0 mol%.

The mean DNA–DNA relatedness value between strain DS-43^T and the type strain (KCTC 12459^T) of *V. paradoxus* was 13 %, 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). Strain DS-43^T also differed from *V. paradoxus* in several phenotypic characteristics, as shown in Table 1. The phylogenetic and genetic distinctiveness and differential phenotypic properties were sufficient to categorize strain DS-43^T as a member of a species distinct from *V. paradoxus* (Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, DS-43^T is considered to be the type strain of a novel species of the genus *Variovorax*, for which the name *Variovorax dokdonensis* sp. nov. is proposed.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Variovorax dokdonensis* DS-43^T and some related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points. *Escherichia coli* ATCC 11775^T was used as an outgroup (not shown). Dots indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

Description of *Variovorax dokdonensis* sp. nov.

Variovorax dokdonensis (dok.do.nen'sis. N.L. masc. adj. *dokdonensis* of Dokdo, a Korean island, from where the organism was isolated).

Table 2. Percentage cellular fatty acid content of *Variovorax* species

Strain: 1, *V. dokdonensis* DS-43^T; 2, *V. paradoxus* KCTC 12459^T (data from this study). Fatty acids that represented <0.5% in the two strains are omitted. —, Not detected.

Fatty acid	1	2
Straight-chain fatty acid		
C _{10:0}	0.7	—
C _{12:0}	3.1	3.1
C _{14:0}	1.1	0.9
C _{15:0}	1.8	6.7
C _{16:0}	27.9	26.8
C _{17:0}	2.1	1.8
Unsaturated fatty acid		
C _{15:1} ω6c	1.1	0.9
C _{16:1} ω5c	0.3	2.2
C _{18:1} ω7c	16.1	12.6
Hydroxy fatty acid		
C _{10:0} 3-OH	2.0	2.1
C _{12:0} 3-OH	2.4	—
C _{14:0} 2-OH	—	2.2
C _{16:1} 2-OH	—	0.6
Iso-C _{17:0} 3-OH	—	1.4
Cyclo fatty acid		
C _{17:0} cyclo	15.1	26.3
C _{19:0} cyclo ω8c	—	0.7
Summed feature 3*	25.8	10.1

*Summed feature represents groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Cells are Gram-negative and oval- or rod-shaped (0.3–0.5 µm × 0.7–2.8 µm). Colonies on TSA are circular, slightly convex, smooth, glistening, pale yellow in colour and 1.0–2.0 mm in diameter after 3 days incubation at 30 °C. Optimal temperature for growth is 30 °C. Growth occurs at 10 and 40 °C, but not at 4 or 41 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 5.0 and 9.5, but not at pH 4.5 or 10.0. Growth occurs in the presence of 0–5% (w/v) NaCl; optimal growth occurs in the presence of 0.5% (w/v) NaCl. Anaerobic growth does not occur on TSA or on TSA supplemented with nitrate. Hypoxanthine and Tweens 20, 40 and 60 are hydrolysed, but aesculin, casein, L-tyrosine and xanthine are not. H₂S and indole are not produced. N-Acetylglucosamine and malate are not utilized. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present and α-glucosidase is weakly present, but lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. The predominant ubiquinone is Q-8. The major fatty acids are C_{16:0}, C_{16:1}ω7c and/or iso-C_{15:0} 2-OH, C_{18:1}ω7c and C_{17:0} cyclo. The DNA G+C content is 66.0 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, DS-43^T (= KCTC 12544^T = CIP 108838^T), was isolated from soil from Dokdo, Korea.

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