Vibrio areninigrae sp. nov., a marine bacterium isolated from black sand

Ho-Won Chang,[1] Seong Woon Roh,[1,2] Kyoung-Ho Kim,[1] Young-Do Nam,[1,2] Che Ok Jeon,[3] Hee-Mock Oh[1] and Jin-Woo Bae[1,2,3]

Correspondence
Jin-Woo Bae
baejw@kribb.re.kr

[1]Biological Resources Center, KRIBB, Daejeon 305-806, Republic of Korea

[2]Korea University of Science & Technology, 52 Eoeun-dong, Daejeon 305-333, Republic of Korea

[3]Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea

A Gram-negative, facultatively anaerobic, motile, slightly curved rod-shaped bacterial strain was isolated from black sand collected from Soesoggak, Jeju island, Korea. The strain, designated J74^T, was able to grow in the presence of 1–7.5 % NaCl, at temperatures of 4–45 °C and at pH 5–10. Strain J74^T was oxidase- and catalase-positive, arginine dihydrolase-negative and sensitive to the vibriostatic agent O/129. Strain J74^T was characterized based on physiology, morphology, biochemical features and 16S rRNA gene sequence analysis. The isolate required sodium ions for growth and utilized a wide range of compounds as sole sources of carbon and energy. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain J74^T belongs to the class *Gammaproteobacteria*. It was found to be associated with the genus *Vibrio* and was phylogenetically related most closely to the type strain of *Vibrio hispanicus* (98.7 % 16S rRNA gene sequence similarity). However, DNA–DNA hybridization experiments between strain J74^T and *V. hispanicus* KCTC 12827^T revealed a level of relatedness of 37.7 %. Thus, phenotypic and phylogenetic data suggested that J74^T should be placed in the genus *Vibrio* as representing a novel species, for which the name *Vibrio areninigrae* sp. nov. is proposed. The type strain is J74^T (=KCTC 22122^T =JCM 14949^T).

At the time of writing, the genus *Vibrio* Pacini 1854 contains 64 recognized species (http://www.bacterio.cict.fr/uw/vibrio.html). Members of the genus *Vibrio* are found in a variety of marine environments, and are frequently isolated from estuarine and coastal waters (Thompson *et al.*, 2004). Several species of *Vibrio* are well-known pathogens of human and marine animals, such as crustaceans, molluscs, corals, fish, shrimp and zooplankton (Austin, 1988; Hjeltnes & Roberts, 1993; Kushmaro *et al.*, 2001; Lightner, 1993; Thompson *et al.*, 2004). Several species of *Vibrio* exist in symbiotic relationships with marine fish and squid (Thompson *et al.*, 2004). A *Vibrio*-like, Gram-negative, rod-shaped bacterial strain, designated J74^T, was recently isolated from black sand collected from Soesoggak, Jeju island, Korea. In the present study, phenotypic, chemotaxonomic and phylogenetic analyses were used to establish the taxonomic position of this strain.

Strain J74^T was isolated by growth at 30 °C for 3 days on marine agar 2216 (MA; Difco) with repeated restreaking to obtain a pure culture. NaCl growth tolerance and requirements were investigated by using marine broth (MB; Difco) medium supplemented with various concentrations of NaCl. Growth at various temperatures and pH values was measured in MB. Reference strain *Vibrio hispanicus* KCTC 12827^T was obtained from the Korean Collection for Type Cultures (Daejeon, Korea) and was grown under the same conditions. All phenotypic growth tests were carried out on the novel isolate and *V. hispanicus* KCTC 12827^T. Bacterial cultures of strain J74^T and *V. hispanicus* KCTC 12827^T were stored at −80 °C in MB containing 20 % glycerol. For morphological and physiological characterization, cells were cultured in MB at 30 °C with shaking. Growth under anaerobic conditions was determined by incubation for 7 days in Gaspak jars (BBL) containing an atmosphere of 80 % N_2, 10 % CO_2 and 10 % H_2. The cell morphology of strain J74^T was examined by light microscopy (E600; Nikon) and transmission electron microscopy. The cellular motility of the novel isolate was observed in fresh wet mounts of young bacterial cultures in MB by the hanging drop method. For observation of flagella, cells from exponentially growing cultures were investigated by transmission electron microscopy. The Gram reaction was determined by using a Gram stain kit (Difco) according to the manufacturer's instructions. Catalase and oxidase activities were determined by

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain J74^T is EU143360.

observing bubble production in a 3 % (v/v) hydrogen peroxide solution and by using an oxidase reagent (bioMérieux), respectively. Sensitivity to the vibriostatic agent O/129 (150 mg per disc) was determined by using Oxoid discs. API 20NE and API ZYM test strips (bioMérieux) and Biolog GN plates with GN/GP inoculating fluid were used to analyse enzyme activity, substrate utilization from sole carbon sources and acid production from carbohydrates; additional biochemical tests were performed by using the methods and media described by Gordon *et al.* (1973). The method of Gonzalez *et al.* (1997) was used to assess growth on various carbon sources. Bacterial strains grown on MA for 3 days at 30 °C were used for analysis of cellular fatty acid composition. Cellular fatty acids were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Chromosomal DNA was extracted and purified according to the method of Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR by using PCR Master mix solution (iNtRON Biotechnology) and two universal primers, as described by Stackebrandt *et al.* (1993). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (1998). DNA–DNA hybridization was performed by using photobiotin-labelled DNA probes and microwell plates according to Ezaki *et al.* (1989). 16S rRNA gene sequences of strain J74^T and of the type strains of 15 related reference taxa from the NCBI database were aligned by using the multiple sequence alignment program CLUSTAL_X (v1.8) (Fig. 1) (Thompson *et al.*, 1997). Levels of 16S rRNA gene sequence similarity were determined by using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Phylogenetic relationships between representatives of *Vibrio* species were determined by using the MEGA version 3.1 software program. Distance matrices were determined (Kimura, 1980) and these matrices were used to elaborate dendrograms by using the neighbour-joining method (Saitou & Nei, 1987). A bootstrap analysis to investigate the stability of the trees was performed by obtaining a consensus tree that was based on 1000 randomly generated trees.

The cultural, physiological and biochemical characteristics of strain J74^T and of related *Vibrio* species are shown in Table 1. Cells of strain J74^T were Gram-negative, motile and rod-shaped. The novel strain was able to grow at temperatures of 4–45 °C but not above 50 °C. Growth was observed at pH 5–10 but not below pH 4.5 or above pH 11. Growth was observed in the presence of 1–7.5 % NaCl; no growth was detected at concentrations of less than 1 % or more than 10 % NaCl. After 3 days growth on MA, colonies were pale yellow, 1–4 mm in diameter, circular, smooth and low-convex. Details of the fatty acid methyl esters of strain J74^T and of *V. hispanicus* KCTC 12827^T are given in Table 2.

16S rRNA gene sequence analysis indicated that strain J74^T was associated with species belonging to the family

Table 1. Differential characteristics between strain J74^T and the type strains of related species of the genus *Vibrio*

Strains: 1, J74^T; 2, *V. hispanicus* KCTC 12827^T (data from this study); 3, *V. neptunius* LMG 20536^T (data from Thompson *et al.*, 2003); 4, *V. pectenicida* ATCC 700783^T (Lambert *et al.*, 1998); 5, *V. shilonii* ATCC BAA-91^T (Kushmaro *et al.*, 2001). +, Positive; −, negative; ND, no data available; V, variable. All strains grew at 4 °C and reduced nitrate to nitrite.

Characteristic	1	2	3	4	5
Growth at 8 % NaCl	−	+	−	+	−
Production of:					
Arginine dihydrolase	−	+	V	−	−
Esterase	−	+	+	+	ND
Valine arylamidase	+	−	+	ND	ND
Trypsin	+	−	+	ND	ND
Utilization of:					
Succinate	−	−	ND	+	−
Maltose	+	+	+	+	ND
Glycogen	+	−	+	ND	+
Cellobiose	−	+	−	+	+
D-Galactose	−	+	−	+	ND
D-Mannose	−	+	+	+	+
Tween 80	+	−	−	+	+
Citrate	−	+	+	+	ND
Glucose 1-phosphate	+	−	+	ND	+
Glucose 6-phosphate	+	−	+	ND	+
L-Rhamnose	−	+	−	+	+

Table 2. Fatty acid compositions of strain J74^T and *V. hispanicus* KCTC 12827^T

Values are percentages of the total fatty acids. Data were obtained in this study.

Fatty acid	Strain J74^T	*V. hispanicus* KCTC 12827^T
$C_{11:0}$	0.38	0
$C_{12:0}$	4.15	5.36
$C_{14:0}$	6.94	4.66
$C_{15:0}$	0.76	0.64
$C_{16:0}$	26.92	23.96
$C_{17:0}$	0.46	0.59
$C_{18:0}$	0.49	0.81
$C_{16:1}\omega7c$ alcohol	0.44	0.57
$C_{17:1}\omega8c$	0.39	0.44
$C_{18:1}\omega7c$	12.28	14.40
$C_{12:0}$ 3-OH	3.14	4.00
iso-$C_{14:0}$	0.72	0.81
iso-$C_{14:0}$ 3-OH	0	0.61
iso-$C_{16:0}$	2.65	6.48
iso-$C_{18:0}$	0	0.65
$C_{14:0}$ 3-OH/iso-$C_{16:1}$ I	2.23	3.24
$C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH	37.21	30.71
Unknown 12.484	0.84	1.11

Vibrionaceae and fell within a cluster comprising *Vibrio* species (Fig. 1). Strain J74^T showed 92.9–98.7 % 16S rRNA gene sequence similarity to the type strains of recognized *Vibrio* species investigated. DNA–DNA reassociation experiments were performed to establish the genomic similarity between strain J74^T and its closest phylogenetic relative, *V. hispanicus* KCTC 12827^T. The two strains showed a level of DNA–DNA relatedness of 37.7 %. Based on phylogenetic, physiological and biochemical characteristics together with DNA–DNA hybridization results, it is thus suggested that strain J74^T represents a novel species of the genus *Vibrio*, for which the name *Vibrio areninigrae* sp. nov. is proposed.

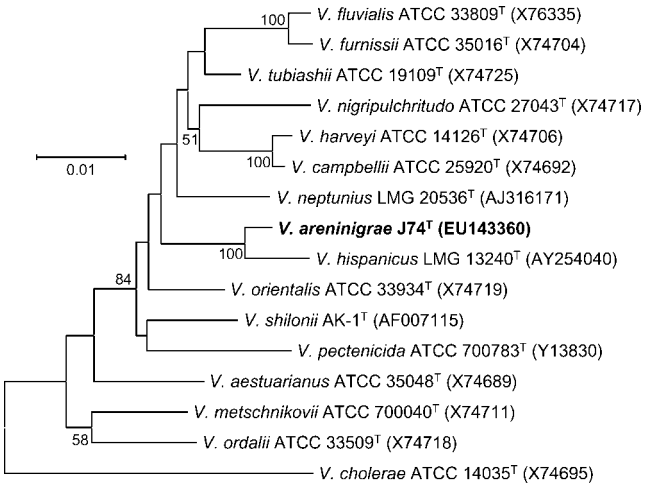

Fig. 1. Consensus phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain J74^T and the type strains of the most closely related *Vibrio* species. The tree was constructed based on the neighbour-joining method and p-distances. Bootstrap analyses were performed with 1000 repetitions; only values >50 % are shown. GenBank accession numbers are given in parentheses. Bar, 0.01 % sequence divergence.

Description of *Vibrio areninigrae* sp. nov.

Vibrio areninigrae (a.re.ni.nig′rae. L. fem. n. *arena* sand; L. adj. *niger -gra -grum* black; N.L. gen. n. *areninigrae* of black sand).

Cells are Gram-negative, facultatively anaerobic, slightly curved and rod-shaped, 0.5–0.8 μm wide by 2.0–3.0 μm long in 3-day cultures growing at 30 °C on MA plates. Cells are motile by means of at least one flagellum per cell. Colonies are circular to slightly irregular, smooth, low-convex, pale yellow and 2–3.5 mm in diameter after incubation for 3 days on MA. Growth occurs in the presence of 1–7.5 % NaCl, but not in the absence of NaCl or in the presence of more than 10 % NaCl. Growth occurs at 4–45 °C and at pH 5–10. No growth occurs below pH 4.5 or above pH 11. Cells are catalase- and oxidase-positive, urease- and arginine dihydrolase-negative and indole-positive. Nitrate is reduced to nitrite. Produces acid but no gas from glucose. Susceptible to the vibriostatic agent O/129. Glycogen, Tween 80, *N*-acetyl-D-glucosamine, D-fructose, gentiobiose, α-D-glucose, maltose, D-mannitol, melibiose, sucrose, trehalose, inosine, glucose 1-phosphate and glucose 6-phosphate can be utilized as sole sources of carbon and energy, but not α-cyclodextrin, dextrin, Tween 40, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, cellobiose, L-erythritol, L-fucose, D-galactose, *myo*-inositol, α-D-lactose, lactulose, D-mannose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, turanose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-, β- or γ-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromo-succinic acid, succinamic acid, glucuronamide, alaninamide, D- or L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D- or L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol or DL-α-glycerol phosphate. Positive for alkaline phosphatase, esterase lipase (C_8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-mannosidase activities (API ZYM). No esterase (C_4), lipase (C_{14}), cystine

arylamidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase or α-fucosidase activity. The predominant fatty acids are $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH, $C_{16:0}$ and $C_{18:1}\omega7c$ (approximately 37.21, 26.92 and 12.28 % of the total, respectively).

The type strain, J74^T (=KCTC 22122^T =JCM 14949^T), was isolated from black sand from Soesoggak, Jeju island, Korea.

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