Vibrio litoralis sp. nov., isolated from a Yellow Sea tidal flat in Korea

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Two Gram-negative, facultatively anaerobic bacterial strains, MANO22D^T and MANO22P, were isolated from a tidal flat area of Dae-Chun, Chung-Nam, Korea. The isolates were rod-shaped and were motile by means of one or more polar flagella. They grew at 1–12 % NaCl, 4–45 °C and pH 4.1–8.8 and were oxidase-positive, arginine dihydrolase-negative and sensitive to the vibriostatic agent O/129. The isolates required Na^+ for growth, produced acid, but no gas, from D-glucose under anaerobic conditions and utilized a wide range of compounds as sole carbon and energy sources. A phylogenetic analysis based on 16S rRNA gene sequences revealed that the strains belong to the *Gammaproteobacteria* and are specifically related to *Vibrio* species. They were most closely related to *Vibrio rumoiensis* FERM P-14531^T, with which they were found to share 98.65 % 16S rRNA gene sequence similarity. In the phylogenetic tree, the two novel strains comprised a relatively long subline of descent, sharing a branching point with the outlying species *V. rumoiensis*, and were found to occupy a phylogenetically distant position on the main *Vibrio* branch. The levels of DNA–DNA hybridization with respect to *V. rumoiensis* FERM P-14531^T, which is their most closely related phylogenetically related *Vibrio* species, were 7.4 % (MANO22D^T) and 3.9 % (MANO22P). Thus, the two novel isolates appear to represent a novel species within the genus *Vibrio*, for which the name *Vibrio litoralis* sp. nov. is proposed. The type strain is MANO22D^T (=KCTC 12520^T=DSM 17657^T).

The genus *Vibrio* belongs to the family *Vibrionaceae* (Baumann & Schubert, 1984), which includes the genera *Photobacterium* (Baumann & Baumann, 1984), *Salinivibrio* (Mellado *et al.*, 1996) and *Grimontia* (Thompson *et al.*, 2003) amongst others. Micro-organisms belonging to the genus *Vibrio* occur frequently in aquatic environments, particularly in marine and estuarine waters, where they are often found associated with various organisms ranging from plankton to fish (Thompson *et al.*, 2004). While some *Vibrio* species act beneficially in host organisms as probionts (Gomez-Gil *et al.*, 1998, 2000, 2002), others are known to cause disease in fish (Hjeltnes & Roberts, 1993), crustaceans (Lightner, 1993) and molluscs (Austin, 1988). The number of species assigned to the genus *Vibrio* increased from 20 in 1981 to 63 in 2004 (Thompson *et al.*, 2004); at the time of writing, this genus includes 76 species with validly published names.

To clarify our understanding of the genus *Vibrio*, we searched for novel strains of this taxon in getbol, the Korean term for tidal flats. The western and south-western coastlines of the Korean peninsula consist primarily of such tidal flats (Kim *et al.*, 2004). They are unique among other marine sediments as they alternately undergo flooding with seawater and exposure to air (Kim *et al.*, 2005). Two novel strains were isolated from a tidal flat area near Dae-Chun, Chung-Nam, Korea (36° 17′ 45.2″ N 126° 31′ 9.5″ E)

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MANO22D^T and MANO22P are DQ097523 and DQ097524, respectively.

by employing the dilution plating technique and culturing the micro-organisms at 25 °C for 3 days on marine agar 2216 (MA; Difco). These strains were designated as MANO22D^T and MANO22P. Below, we report the phenotypic, genetic and chemotaxonomic analyses that we performed to elucidate the taxonomic position occupied by these strains. These analyses revealed that both strains are very similar; they belong to the family *Vibrionaceae* and are most closely related to *Vibrio rumoiensis*.

In our analyses, *V. rumoiensis* FERM P-14531^T, obtained from Dr I. Yumoto (Yumoto *et al.*, 1999), served as the reference strain. Bacterial cultures of the isolates and the reference strain were stored at −80 °C on marine broth (MB) containing 20 % glycerol and were cultured in MB at 25 °C, with shaking, for morphological and physiological characterization. API 20NE and API ZYM test strips (bioMérieux) and Biolog GN metabolic fingerprinting plates were used to analyse these strains biochemically and physiologically; other biochemical tests were performed using the methods and media described by Gordon *et al.* (1973). The ability to grow on various carbon sources was tested as described by Gonzalez *et al.* (1997), and catalase activity was determined by assessing bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined by using an oxidase reagent (bioMérieux), while sensitivity to O/129 (150 μg per disc) was determined with Oxoid discs. Growth under anaerobic conditions was determined on MA with anaerobic incubation for 7 days in GasPak jars (BBL) containing an $N_2/CO_2/H_2$ (80 : 10 : 10) atmosphere. Growth in MB at various NaCl concentrations, temperatures and pHs was measured. The Gram reaction was determined by using a Gram-stain kit (Difco) according to the manufacturer's instructions. Cellular morphology and sporulation were determined by using microscopy (E600; Nikon). The cellular motility of young bacterial cultures in MB was observed in fresh wet mounts by using the hanging drop method. For observation using transmission electron microscopy, cells from exponentially grown cultures were negatively stained with 1 % (w/v) phosphotungstic acid. After air drying, the grid was examined using a model H-7600 transmission electron microscope (Hitachi). The strains were grown on MA for 3 days at 25 °C to analyse their fatty acid methyl esters, which 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 as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by using a PCR with two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed according to the methods described by Yoon *et al.* (2003). DNA–DNA hybridization was performed fluorometrically by using the method of Bae *et al.* (2005) with Cy5-labelled DNA probes and genome-spotted microarrays. The 16S rRNA gene sequences of the two novel isolates were aligned with 17 reference sequences obtained from the Ribosomal Database Project (Fig. 1) by using the multiple sequence alignment program CLUSTAL_X (version 1.8) (Thompson *et al.*, 1997). The phylogenetic relationships between representatives of the genus *Vibrio* were determined by using MEGA software (version 2.1). Distance matrices were determined by adopting the assumptions described by Kimura (1980). These matrices were used to elaborate dendrograms by using the neighbour-joining method (Saitou & Nei, 1987). A bootstrap analysis designed to establish the stability of the phylogenetic trees obtained was performed by obtaining a consensus tree based on 1000 randomly generated datasets.

Strains MANO22D^T and MANO22P were largely identical in their morphological, cultural, physiological and biochemical characteristics. The characteristics of the two isolates are shown in Table 1 and are described in the species description below. They were motile and Gram-negative and their cells were rod-shaped (measuring 0.6–0.8 by 2.0–3.0 μm on MA). After 3 days growth on MA, their colonies were pale yellow in colour, 1.5–2.0 mm in diameter, smooth, and circular to slightly irregular in shape. They grew at temperatures ranging from 4 to 45 °C

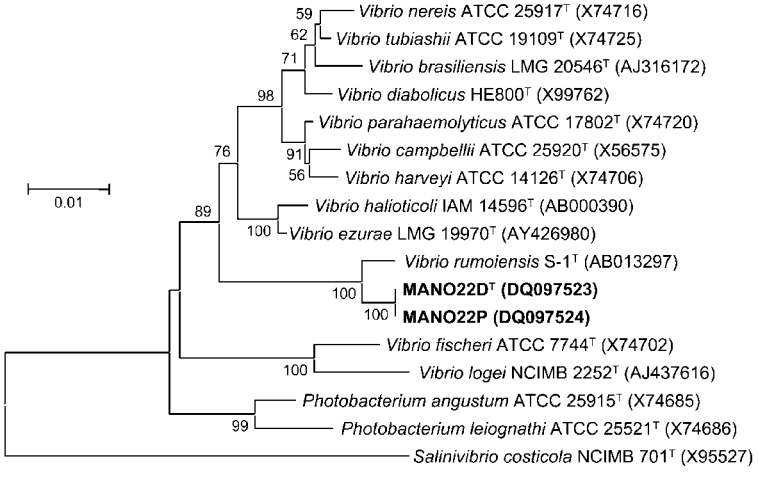

Fig. 1. Consensus neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, that shows how strain MANO22D^T relates to type strains of selected *Vibrio* species and representatives of related genera. Bootstrap analysis was based on 1000 repetitions; only values higher than 50 % are shown. The GenBank accession number for each species is shown in parentheses. Bar, 0.01 substitutions per nucleotide position.

Table 1. Taxonomic characteristics of the novel isolates MANO22D^T, MANO22P and their closest phylogenetic relative, *V. rumoiensis* FERM P-14531^T

Strains: 1, *V. rumoiensis* FERM P-14531^T; 2, MANO22D^T; 3, MANO22P. +, Positive; −, negative; ND, not determined. All strains showed pale-yellow pigmentation, grew at 4 and 35 °C, produced gas from glucose and showed sensitivity to O/129 (150 μg).

Characteristic	1	2	3
Production of:			
Valine arylamidase	−	+	+
N-Acetyl-β-glucosaminidase	−	+	+
Utilization of:			
Glycogen	+	−	−
Tween 80	−	+	+
N-Acetyl-D-galactosamine	−	+	+
Galactose	−	+	+
D-Mannitol	−	+	+
Sucrose	+	−	−
Monomethyl succinate	+	−	−
D-Gluconic acid	−	+	+
Succinic acid	+	−	−
Bromosuccinic acid	+	−	−
L-Alanine	−	+	−
L-Alanyl glycine	−	+	+
L-Proline	−	+	−
L-Serine	+	−	−
Inosine	−	+	+
Glycerol	−	+	+
Fatty acid content (%)			
16 : 1ω7*c* and/or 15 : 0 iso 2-OH	38.8	38.6	ND

and at pH 4.1–8.8; however, they did not grow at below pH 3.1 or above pH 9.3. They grew in the presence of 1–12 % NaCl, but no growth was observed when NaCl was absent or present at 15 %. They grew under anaerobic conditions, showed catalase, oxidase and urease activities and reduced nitrate to nitrite. They grew on the following carbon sources: glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, malate and citrate. They did not hydrolyse gelatin, urea or aesculin and did not show arginine dihydrolase activity or produce indole. Under anaerobic conditions, they produced acid, but no gas, from D-glucose. They were also susceptible to the vibriostatic agent O/129. The predominant fatty acids of strain MANO22D^T were 16 : 1ω7*c* and/or 15 : 0 iso 2-OH, 18 : 1ω7*c*, 16 : 0 and 14 : 0 3-OH and/or 16 : 1 iso I.

The 16S rRNA gene sequence of MANO22D^T was 93.3–98.65 % similar to those of *Vibrio* and *Photobacterium* strains, its closest phylogenetic neighbour being *V. rumoiensis* S-1^T (=FERM P-14531^T) (98.65 %). A phylogenetic tree constructed by using the neighbour-joining method clearly showed that MANO22D^T and MANO22P both belong to the genus *Vibrio*, albeit constituting a separate branch with respect to the main *Vibrio* group (Fig. 1). DNA–DNA relatedness studies were performed to determine the genomic relationship between MANO22P, MANO22D^T and *V. rumoiensis* FERM P-14531^T. The value for DNA–DNA relatedness between MANO22P and MANO22D^T was 96 %, but the relatedness values between the novel isolates and *V. rumoiensis* FERM P-14531^T were 7.4 % (MANO22D^T) and 3.9 % (MANO22P). Taken together, these observations indicate that strains MANO22D^T and MANO22P represent a novel species of the genus *Vibrio*, for which the name *Vibrio litoralis* sp. nov. is proposed.

Description of *Vibrio litoralis* sp. nov.

Vibrio litoralis (li.to.ra′lis. L. masc. adj. *litoralis* of the shore, a shallow-water dweller).

Cells are Gram-negative, slightly curved rods that are 0.6–0.8 μm wide and 2.0–3.0 μm long. Motile due to the presence of at least one polar flagellum per cell. Colonies on MA are pale yellow, smooth, round or slightly irregular in shape and measure 1.5–2.0 mm in diameter after 3 days culture on MA plates. Bioluminescence is not observed. Growth occurs when 1–12 % NaCl is present, but not when NaCl is absent or at concentrations of 15 %. Growth occurs at 4–45 °C and at pH 4.1–8.8. Optimal conditions are 25–30 °C, pH 6.9 and 3 % NaCl. Susceptible to the vibriostatic agent O/129 and is oxidase- and catalase-positive. Reduces nitrate to nitrite, but not further to N_2. Facultatively anaerobic; acid, but no gas, is produced from glucose. The following substrates can be utilized as sole carbon and energy sources: glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, gluconate, malate and citrate. Gelatin, urea and aesculin are not hydrolysed. API ZYM tests show activities for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. Arginine dihydrolase, lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase activities are not observed. The predominant fatty acids are 16 : 1ω7*c* and/or 15 : 0 iso 2-OH (38.62 %), 18 : 1ω7*c* (19.45 %), 16 : 0 (13.49 %), 14 : 0 3-OH and/or 16 : 1 iso I (7.11 %), 12 : 0 (3.65 %) and 18 : 0 iso (3.44 %).

The type strain, MANO22D^T (=KCTC 12520^T=DSM 17657^T), and a reference strain, MANO22P (=KCTC 12519), were isolated from a tidal flat area of Dae-Chun, Chung-Nam, Korea.

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