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Vibrio hannami sp. nov., Isolated from Seawater

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Abstract A Gram-reaction negative, aerobic, motile, non-pigmented and rod-shaped bacterium, designated as 168GH5-2-16^T, was isolated from seawater Jeju island. Phylogenetic analysis based on 16S rRNA gene sequence comparison revealed that the strain formed a distinct lineage within the genus *Vibrio* and was most closely related to *Vibrio variabilis* R-40492^T (96.0%). The DNA G+C content was 49.3 mol%. The major polar lipids were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The predominant quinone was ubiquinone-8 (Q-8). The major fatty acids were $C_{16:0}$, summed feature 3 (comprising $C_{16:1}$ $\omega7c$/$C_{16:1}$ $\omega6c$) and summed feature 8 ($C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega6c$) supported the affiliation of 168GH5-2-16^T to the genus *Vibrio*. Moreover, the physiological, biochemical, and taxonomic analysis allowed the phenotypic and genotypic differentiation of strain 168GH5-2-16^T from the recognized species of the genus *Vibrio*. Therefore, strain 168GH5-2-16^T represents a novel species of the genus *Vibrio*, for which the name *Vibrio hannami* sp. nov. is proposed, with the type strain 168GH5-2-16^T (=KACC 19277^T = DSM105032^T).

Keywords Vibrio hannami · 16S rRNA gene sequence · Polyphasic taxomony

Introduction

The genus *Vibrio* is on of the most diverse group of facultative anaerobes of the phylum Proteobacteria. Species of the genus *Vibrio* are commonly isolated from aquatic environments, usually from marine environments, both as free-living bacteria and as symbionts or parasites of fish, crustaceans, and molluscs [22]. Vibrios are Gram-negative, halophilic, usually motile rods, mesophilic and chemo-organotrophic, which have the facultatively fermentative metabolism [2]. Affiliates of the genus *Vibrio* obtained from shellfish and cultured fish around the world have provided more than 30% of the species currently recognized in the genus. Recently, based on multi-locus sequence analysis (MLSA) the species of genus *Vibrio* have been grouped in 23 clades [1]. At the time of writing, the genus *Vibrio* comprises more than 120 recognized species with validly published names (http://www.bacterio.net/vibrio.html).

In this study, we describe the taxonomic characterization of novel strain 168GH5-2-16^T which belongs to the genus *Vibrio*.

Ga-Eun Lee and Wan-Taek Im have contributed equally to do this work.

The digital protologue database (DPD) number of Current Microbiology for the strain 168GH5-2-16^T is TA00219.

The GenBank accession number for the 16S rRNA gene sequence of strain 168GH5-2-16^T is KY451770.

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Materials and Methods

Isolation of the Bacterial Strain

The water samples were collected from seawater (Goheung, South Korea), and after collection the samples were thoroughly suspended with 0.85% sterilized saline, following serial dilution, and then was spread onto marine agar medium (BD). The plates were incubated at 30 °C for 2 weeks. Single colonies were purified by subculture. Strain 168GH5-2-16^T was seek out, and then it was routinely cultured on marine agar at 30 °C and maintained as a glycerol suspension (25%, v/v) at −80 °C.

Physiological, Morphological, and Biochemical Characteristics

The Gram-reaction was determined using the non-staining method, as described previously [3]. Cell motility was determined using the hanging drop method, while cell morphology was examined with the transmission electron microscope (SU-3500, Hitachi), using cells grown for 2 day at 30 °C on MA (Marine agar) medium. Oxidase activity was determined using 1% (w/v) *N,N,N,N*-tetramethyle-1,4-phenylenediamine reagent (bioMe′rieux). Catalase activity was determined by the production of bubbles from 3% (v/v) H_2O_2 solution. Hydrolysis activity was tested using the following substrates: starch, casein, DNase (DNase agar medium, Sharlau), Tween 80, and carboxyl methyl cellulose (CMC) [20]. All tests were performed and evaluated after 2 days of incubation at 30 °C. Biochemical tests in the commercial API kits [API ZYM, API 20NE, and API 32GN (bioMerieux)] were generally performed according to the manufacturer's instructions. The API ZYM test strips were read after 4 h of incubation at 37 °C, and the other API streps were examined after 2 days of incubation at 30 °C. Growth at different temperatures (4, 10, 15, 25, 30, 37, and 40 °C) and various pH values (pH 4–10 at intervals of 0.5 and 1 pH units) was assessed after 3 days of incubation at 30 °C. The following buffers (final concentration, 50 mM) were used to adjust the pH of nutrient broth. Acetate buffer was used for pH 4.0–5.5; phosphate buffer was used for pH 6.0–8.0; and Tris buffer was used for pH 8.5–10.0. Salt tolerance test was evaluated on marine agar medium supplemented with 2–8% (w/v at intervals of 0.5 and 1% unit) NaCl and growth was assessed after 7 days of incubation at 30 °C. Growth on different media was also tested by using nutrient agar (NA, Difco), trypticase soy agar (TSA, Difco), lysogeny broth (LB) agar (Difco), and MacConkey agar (Difco) at 30 °C for 1 week.

Phylogenetic Tree Construction and Determination of DNA G+C Content (mol%)

For phylogenetic analysis, the genomic DNA was extracted with a commercial genomic DNA extraction kit (Solgent). The bacterial universal primer sets 27F, 800R, 518F, and 1492R were used to amplify the 16S rRNA gene sequence [13]. The purified PCR product was sequenced by Genotech according to Kim et al. [10]. Nearly full-length sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/genbank) or http://www.ezbiocloud.net/eztaxon, [11]. Multiple sequence alignments were performed via the Clustal_X program [23]. Gaps were edited in the BioEdit program [8] and 1370 nucleotides were used for phylogenetic tree construction. For maximum-likelihood tree analysis, evolutionary distances were calculated using Kimura two-parameter model [12] and the gapes were treated by complete deletion. The neighbor-joining tree was constructed by using the same model with complete deletion of gaps [18]. Similarly, maximum-parsimony tree was made with Subtree–Pruning–Regrafting (SPR) heuristic method and the gaps were edited with complete deletion [7] using MEGA6 program [21] with bootstrap values based on 1000 replications [6].

To analyze the DNA G+C content, the genomic DNA was extracted and purified as previously described [17], degraded enzymatically into nucleosides, and the DNA G+C content was determined as described by Mesbah et al. [15] using a reverse-phase HPLC.

Chemotaxonomic Analysis

The novel isolate was examined for their polar lipid contents as described by Minnikin et al. [16] and the polar lipids were developed in the first direction by using the chloroform/methanol/water (65:25:4, by v/v), while in the second direction, it was developed by chloroform/acetic acid/methanol/water (80:15:12:4, by v/v). Isoprenoid quinone of the isolate was extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum condition, and re-extracted in *n*-hexane/water (1:1, v/v). The crude *n*-hexane-quinone solution was purified using Sep-Pak Vac cartridges silica (Waters) and subsequently analyzed by HPLC as previously described [9]. Cellular fatty acid profiles were determined after 48 h of growth at 30 °C on R2A agar medium. The cellular fatty acids were saponified, methylated, and extracted according to the described method of Sherlock Microbial Identification System (MIDI). The fatty acids analyzed by a gas chromatograph (Hewlett Packard 6890) were identified by the Microbial Identification software package based on Sherlock Aerobic Bacterial Database (TSBA60) [19].

Results and Discussion

Morphological and Phenotypic Characteristics

Cells of strain 168GH5-2-16^T were Gram-reaction-negative, aerobic, motile, and rod-shaped Fig. S1. Colonies of strain 168GH5-2-16^T grown on marine agar were circular, convex, opaque, and non-pigmented after 48 h of incubation at 30 °C. The isolate did not grow on LB (BD), NA (BD), and MacConkey agar (Difco), whereas weakly grew on R2A (BD) and TSA (Difco) at 30 °C. Strain 168GH5-2-16^T negative for the hydrolysis of starch, casein, cellulose, and DNase. Furthermore, physiological characteristics of strain 168GH5-2-16^T is summarized in the species description and a comparison of selective characteristics of the isolated strain and related type strain is given in Table 1.

Phylogenetic and DNA G+C Content Analysis

The almost complete 16S rRNA gene sequence of strain 168GH5-2-16T (1,427 nt) was determined and subjected to comparative analysis. Phylogenetic analysis using the maximum-likelihood method based on 16S rRNA gene sequences indicated that strain clustered within the genus *Vibrio* (Fig. 1) and form a monophyletic clad with *Vibrio aestivus* KCTC 23860^T. Moreover, this relationship was also evident in phylogenetic trees based on the neighbor-joining and maximum-parsimony methods. *Vibrio variabilis* LMG 25438^T showed the highest sequence similarity (96%) to the new isolate.

Based on 16S rRNA gene sequence and phylogenetic trees analysis, *Vibrio variabilis* LMG25438^T, *Vibrio aestivus* KCTC 23860^T, and *Vibrio maritimus* LMG 25439^T were selected as the closest recognized neighbor of strain 168GH5-2-16^T and used as reference strains in most of the subsequent phenotypic analysis.

DNA G+C contents of the strain 168GH5-2-16^T were 49.3 mol%, which was similar to those of the described species of the genus *Vibrio* (Table 1).

Chemotaxonomic Characteristics

The main polar lipids of strain 168GH5-2-16^T were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG); the minor polar lipids were two unknown polar lipids (L1, L2), one unknown phospholipid (PL) three unknown aminolipids (AL1–AL3) and one unknown amino phospholipid (APL) (Fig. S2). Based on the polar lipid analysis, strain 168GH5-2-16^T share similar major polar lipid PE and PG with the recently described species of the genus *Vibrio* [5]. The major respiratory quinone was Q-8. The major fatty acids of strain 168GH5-2-16^T were $C_{16:0}$ (11.7%), summed feature 3 [(comprising $C_{16:1}$ $\omega 7c$ and/

Table 1 Differential characteristics between strain 168GH5-2-16T^T and of related species of the genus *Vibrio*

Characteristic	1	2	3	4
Enzyme activity				
Acid phosphatase	+	+	+	−
Arginine dihydrolase	+	−	−	−
N-acetyl-β-glucosaminidase	−	w	+	−
Cystine arylamidase	−	−	+	−
α-Galactosidase	−	−	+	+
β-Galactosidase	−	−	+	+
α-Glucosidase	+	−	+	+
β-Glucosidase	+	w	−	−
Esterase	w	−	+	+
Esterase lipase	w	−	+	+
Indole production	+	−	+	+
Lipase	−	+	−	−
Nitrate reduction	−	−	+	+
Trypsin	−	−	+	−
Valine arylamidase	−	w	+	−
Utilization of				
Acetate	+	+	−	+
Adipate	+	−	−	+
L-Arabinose	+	+	−	−
Caprate	−	−	−	+
Citrate	+	−	−	−
L-Fucose	+	+	−	−
Gluconate	+	+	+	−
Glycogen	−	−	+	+
3-Hydroxy-butyrate	−	w	+	−
Inositol	+	+	−	+
Itaconate	+	−	−	−
5-Ketogluconate	+	−	+	+
Malonate	+	−	+	+
D-Mannose	−	w	+	+
D-Mannitol	−	+	+	+
D-Melibiose	+	−	−	+
Phenyl-acetate	−	−	+	−
Propionate	+	w	−	−
L-Proline	+	w	−	−
L-Rhamnose	+	+	−	−
Salicin	+	−	+	+
D-Sorbitol	+	−	+	+
Suberate	−	−	+	−
Valerate	−	−	−	−
G+C content (mol %)	49.3	44.8[a]	46.8[b]	46.3[b]

The data presented here are taken from the current study. In API kit system (ZYM, 20NE, and 32GN), all strains were positive for D-glucose, *N*-acetyl-glucosamine, D-maltose, malate, L-histidine, 2-ketogluconate, 4-hydroxy-benzoate, D-ribose, D-sucrose, D-maltose, L-alanine, and L-serine. Negative for urea, gelatin, α-chymotrypsin, β-glucuronidase, α-mannosidase, α-fucosidase, and 3-hydroxy-benzoate. + Positive, *w* weak positive, − negative

Table 1 (continued)

Strain: *1* 168GH5-2-16^T, *2 Vibrio aestivus* KCTC 23860^T, *3 Vibrio variabilis* LMG 25438^T, *4 Vibrio maritimus* LMG 25439^T

[a,b]Data from *Vibrio aestivus* KCTC 23860^T [14]; *Vibrio variabilis* LMG 25438^T and *Vibrio maritimus* LMG 25439^T [4]

or $C_{16:1}$ $\omega6c$) 37.4%] and summed feature 8 [(comprising $C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega6c$) 28.6%], which is a typical profile of members of the genus *Vibrio*. However, some qualitative and quantitative differences in the fatty acids distinguished strain 168GH5-2-16^T from the closely related strains *Vibrio aestivus* KCTC 23860^T, *Vibrio variabilis* LMG 25438^T, and *Vibrio maritimus* LMG 25439^T (Table 2).

Taxonomic Conclusions

In summary, the characteristics of strain 168GH5-2-16^T are consistent with descriptions of the genus *Vibrio* with regard to morphological, biochemical, and chemotaxonomic properties. However, on the basis of phylogenetic distance from known *Vibrio* species indicated by 16S rRNA gene sequence similarities and the combination of unique phenotypic characteristics (Table 1), strain 168GH5-2-16^T represents a novel species, for which the name *Vibrio hannami* sp. nov. is proposed.

Description of *Vibrio hannami* sp. nov.

Vibrio hannami (han.nam'i. N.L. masc. gen. n. *hannami* of Hannam University Republic of Korea, where the strain was taxonomically characterized).

The novel isolate is positive for catalase and oxidase activities. Growth occurs at 20–42 °C and pH 6.0–10.0 with 1.0–8.0% NaCl (w/v). Optimum growth occurs at 25 °C and pH 6.0 with 2.0% NaCl (w/v) supplement. In the API kits (API 20NE and 32GN API ZYM) system, positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, indole production, glucose acidification, arginine dihydrolase, β-glucosidase, β-galactosidase, D-glucose, L-arabinose, *N*-acetyl-glucosamine, D-maltose, gluconate, adipate, malate, citrate, D-glucose, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, propionate, citrate, L-histidine, 2-ketogluconate, 4-hydroxy-benzoate, L-proline, L-rhamnose, *N*-acetyl-glucosamine, D-ribose, inositol, D-sucrose, D-maltose, itaconate, malonate, acetate, L-alanine, 5-ketogluconate, and L-serine. List of all negative traits of commercial kits is shown in Table S1. Ubiquinone Q-8 is the predominant respiratory quinone and $C_{16:0}$, summed feature 3 ($C_{16:1}$ $\omega7c$ and/or $C_{16:1}$ $\omega6c$) and summed feature 8 ($C_{18:1}$ $\omega7c$/$C_{18:1}$ $\omega6c$) are the major cellular fatty acids. The major polar lipids were PE and PG. The DNA of the type strain is 49.3 mol%.

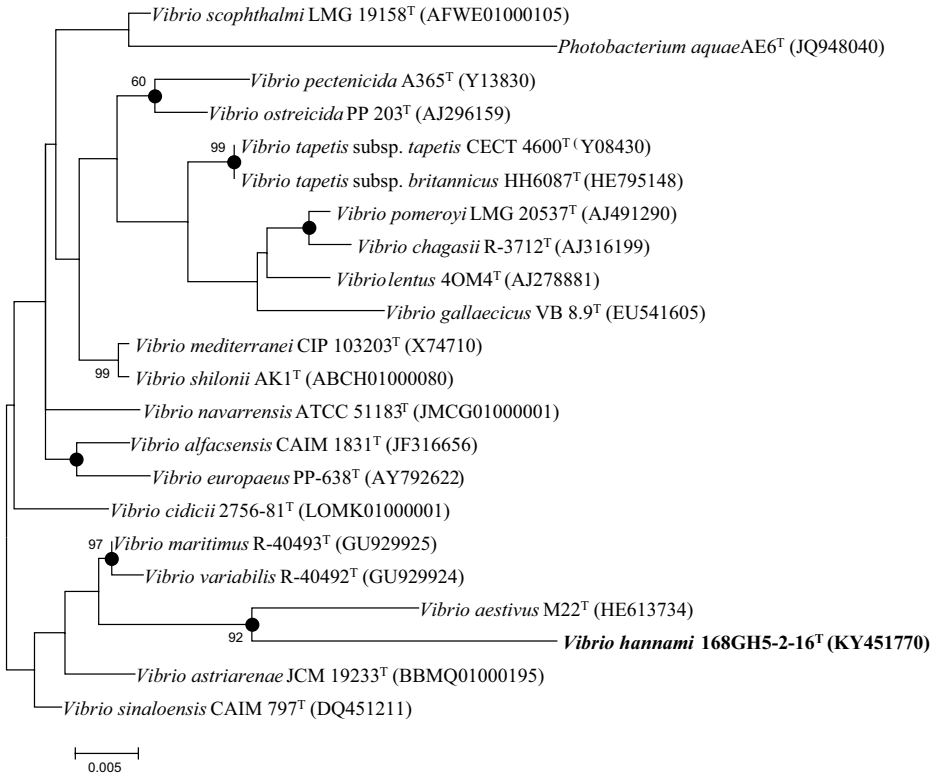

Fig. 1 Phylogenetic relationship between strain 168GH5-2-16^T and other related species of the genus *Vibrio*. The tree was constructed using the maximum-likelihood method based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with maximum-parsimony and neighbor-joining algorithms. *Photobacterium aqua* AE6^T (JQ948040) was used as an outgroup. Scale bar, 0.005 substitutions per nucleotide position

Table 2 Cellular fatty acid profile of strain 168GH5-2-16^T and phylogenetically related species of the genus *Vibrio*

Fatty acids	1	2	3	4
Saturated				
$C_{12:0}$	4.4	4.2	3.0	3.2
$C_{14:0}$	5.2	6.3	6.4	6.3
$C_{15:0}$	0.6	tr	tr	tr
$C_{16:0}$	**11.7**	**18.3**	**17.7**	**20.0**
$C_{17:0}$	0.6	0.6	tr	tr
$C_{18:0}$	1.7	1.1	1.6	1.7
Unsaturated				
$C_{16:1}$ *ω7c* alcohol	tr	tr	tr	0.9
$C_{17:1}$ *ω8c*	0.6	0.8	tr	tr
$C_{20:1}$ *ω7c*	tr	0.6	tr	tr
Branched-chain				
$C_{13:0}$ iso	tr	0.7	0.7	0.9
$C_{14:0}$ iso	tr	0.6	tr	0.5
$C_{15:0}$ iso	tr	1.1	0.7	0.9
$C_{16:0}$ iso	0.7	2.0	0.6	2.0
$C_{17:0}$ iso	tr	0.9	2.0	2.2
$C_{15:0}$ anteiso	tr	2.0	0.6	0.7
$C_{17:0}$ anteiso	tr	1.2	0.7	0.8
Hydroxy				
$C_{12:0}$ 3-OH	3.5	2.8	1.9	1.9
$C_{16:0}$ 3-OH	tr	tr	0.8	0.6
$C_{18:0}$ 3-OH	tr	tr	5.2	tr
Methyl				
$C_{18:1}$ *ω7c* 11-methyl	tr	tr	0.8	tr
Summed feature*				
2; $C_{12:0}$ aldehyde/unknown 10.928	3.0	2.9	3.2	3.0
3; $C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*	**37.4**	**29.9**	**26.0**	**25.9**
7; un 18.846/$C_{19:1}$ *ω6c*/.846/19cy	tr	tr	0.8	tr
8; $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c*	**28.6**	**20.7**	**22.7**	**22.2**

Strain: *1* 168GH5-2-16^T, *2 Vibrio aestivus* KCTC 23860^T, *3 Vibrio variabilis* LMG 25438^T, *4 Vibrio maritimus* LMG 25439^T. All strains were cultured on marine agar for 48 h at 25 °C. Results are presented as percentages of the total fatty acids; the major fatty acids (>10%) are in bold type; fatty acids that represent <0.5% in all strains are omitted. *tr* trace amount

*Summed features represent groups of two or three fatty acids that could not be separated by gas chromatography (GLC) with the MIDI system

The type strain, isolated from seawater, Jeju Island, South Korea, Republic of Korea, is 168GH5-2-16^T (=KACC 19277^T = DSM105032^T).

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