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ORIGINAL ARTICLE

Vibrio inhibens sp. nov., a novel bacterium with inhibitory activity against *Vibrio* species

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Strain BFLP-10^T, isolated from faeces of wild long-snouted seahorses (*Hippocampus guttulatus*), is a Gram-negative, motile and facultatively anaerobic rod. This bacterium produces inhibitory activity against *Vibrio* species. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BFLP-10^T was a member of the genus *Vibrio* and was most closely related to *Vibrio owensii* (99%), *Vibrio communis* (98.9%), *Vibrio sagamiensis* (98.9%) and *Vibrio rotiferianus* (98.4%). However, multilocus sequence analysis using *gyrB*, *pyrH*, *recA* and *topA* genes revealed low levels of sequence similarity (<91.2%) with these closely related species. In addition, strain BFLP-10^T could be readily differentiated from other closely related species by several phenotypic properties and fatty acid profiles. The G+C content of the DNA was 45.6 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain BFLP-10^T represents a novel species within the genus *Vibrio*, for which the name *Vibrio inhibens* sp. nov. is proposed. The type strain is BFLP-10^T (=CECT 7692^T=DSM 23440^T).
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INTRODUCTION

The family *Vibrionaceae* currently comprises six validly published genera: *Vibrio*,[1] *Photobacterium*,[2] *Salinivibrio*,[3] *Enterovibrio*,[4] *Grimontia*[5] and *Aliivibrio*.[6] *Vibrio* species are common inhabitants of aquatic environments, and they can be isolated under a wide range of salinity and temperature conditions from oysters, clams, mussels, and fish, as well as from sediment and plankton.[7,8]

Some species have been reported to cause infections in humans and aquatic animals,[9,10] whereas a small number of other species have been used as probiotics in aquaculture.[11,12] In the present study, a Gram-negative bacterium (BFLP-10^T), with inhibitory activity against *Vibrio* species, was isolated from faeces of wild long-snouted seahorses (*H. guttulatus*) captured in northwest Spain. The phylogenetic analysis based on the 16S rRNA gene of BFLP-10^T indicated that it is closely related to members of the Harveyi clade. Thus, we characterize strain BFLP-10^T and describe the identification of this novel species.

MATERIALS AND METHODS

Culture conditions

Strain BFLP-10^T was isolated from faeces of wild long-snouted seahorses by using the standard dilution plating method on marine agar at 20 °C for 72 h. The strain was subcultured on the same medium at 22 °C for 24 h. Stock cultures were stored at −80 °C in marine broth with 30% (v/v) glycerol.

Physiological and biochemical characterization

Gram reaction was determined using the non-staining (KOH) method.[13] Cell morphology and motility were studied using phase-contrast microscopy and electron microscopy as previously described by Herrera *et al.*[14] NaCl growth tolerance and requirements were investigated by using nutrient broth (0.5% peptone from casein, 0.3% meat extract, 0.3% yeast extract, and adjusted to pH 7.2) supplemented with various concentrations of NaCl (0–15% at intervals of 1%). The pH range for growth was determined in marine broth that was adjusted to various pH values with acetic acid-sodium acetate (pH 4.0–4.5, 100 mM), MES (pH 5.0–6.0, 50 mM), MOPS (pH 6.5, 50 mM), Tris (pH 7.0–9.0, 50 mM) or CHES (pH 9.5–10.0, 50 mM). Growth temperature between 5 and 40 °C was tested in nutrient broth supplemented with 2% w/v NaCl for 48 h with shaking. Anaerobic growth was assessed at 22 °C in anaerobic chambers with an H_2/CO_2 atmosphere (BioMérieux, Marcy l'Etoile, France).

Catalase activity was determined by assessing bubble production in 3% (v/v) H_2O_2; oxidase activity was determined using 1% (w/v) tetramethyl-*p*-phenylenediamine as described by Lim *et al.*[15] Some physiological characteristics were performed using API 20E, API 20NE and API ZYM (BioMérieux). Cells for inoculation of the strips were grown for 24 h at 22 °C on marine agar and the results were visually interpreted according to the manufacturer's instructions.

Antimicrobial activity was determined as described by Balcázar *et al.*[16] Briefly, strain BFLP-10^T was grown in 100 ml of marine broth without agitation at 22 °C for 48 h. After incubation, bacteria were removed by centrifugation (2000 *g*), and cell-free culture supernatant was recovered by passage through 0.22 μm pore size filters. The cell-free culture supernatant was adjusted to pH 6.5 with 5M NaOH to eliminate the inhibitory effects produced by organic acids. Moreover, sensitivity of cell-free culture supernatant to

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trypsin and proteinase K (Sigma Chemical Co., St Louis, MO, USA) at a final concentration of 1.0 mg ml^{-1} was also tested in buffers recommended by the supplier. Samples with and without enzymes were incubated at 37 °C for 2 h and residual activity was determined. To exclude potential inhibition by hydrogen peroxide, we added catalase (Sigma Chemical Co.) at a final concentration of 0.5 mg ml^{-1} and incubated at 37 °C for 30 min. All assays were independently repeated at least two times for reproducibility.

Five strains, *Vibrio alginolyticus* N26-1, *Vibrio harveyi* HT351, *Vibrio ichthyoenteri* HT21, *Vibrio parahaemolyticus* HT352 and *Vibrio splendidus* HT29, which have been isolated and identified in previous studies, were used as indicator bacteria.[10,16] Briefly, bacterial strains were grown in 5 ml of marine broth at 22 °C for 24 h. The cells were harvested by centrifugation (2000 *g*), washed twice with sterile saline solution and resuspended in 5 ml of the same solution. The bacterial suspensions were spread on marine agar plates. Four wells were made in each agar plate with a sterile Pasteur pipette, and cell-free culture supernatants (10 µl) from strain BFLP-10^T were placed into each well. The plates were incubated aerobically at 22 °C for 24 h and then examined for zones of inhibition.

Genotypic characterization

Genomic DNA extraction, PCR amplification and sequencing of the 16S rRNA, DNA gyrase B subunit (*gyrB*), urydilate kinase (*pyrH*), recombination repair protein (*recA*) and topoisomerase I (*topA*), were carried out as described previously by Sawabe *et al*.[17] and Balcázar *et al*.[18] The sequences obtained were compared against the sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLASTN.[19] Phylogenetic analysis was performed using the software MEGA version 4.0 (Center for Evolutionary Medicine and Informatics, Tempe, AZ, USA) after multiple alignments of data by CLUSTAL X.[20,21] Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-parsimony methods were determined using bootstrap values based on 1000 replications.

For base composition analysis, DNA was prepared according to Chun & Goodfellow.[22] The G + C content of the DNA was determined using the thermal denaturation method.[23] DNA from *Escherichia coli* ATCC 11775^T and *Vibrio azureus* LMG 25266^T were used as a reference for determination of the thermal-melting profile (T_m).

Chemotaxonomic analysis

Whole-cell fatty acids from the isolate were extracted from biomass grown on tryptone soy agar (TSA) supplemented with 1.5% NaCl (w/v) and analysed according to the standard protocol of the Sherlock Microbial Identification System, version 4.5 (MIDI Inc., Newark, DE, USA).

RESULTS AND DISCUSSION

Phenotypic characteristics

Phenotypically, strain BFLP-10^T can be clearly assigned to the genus *Vibrio*, and belongs to the arginine-dihydrolase-negative, lysine-and ornithine-decarboxylase-positive species.[24] Cells of strain BFLP-10^T were slightly curved rods, Gram-negative, oxidase- and catalase-positive, motile and facultatively anaerobic. The novel strain also showed prolific growth on thiosulphate-citrate-bile salts-sucrose agar, forming green colonies. Strain BFLP-10^T can be differentiated from related species on the basis of some biochemical properties such as the lack of mannose assimilation and sucrose fermentation. Other physiological characteristics of strain BFLP-10^T are shown in Table 1 and also in the species description.

In addition, cell-free culture supernatant from strain BFLP-10^T exhibited antibacterial activity against all indicator strains. Inhibitory activity of the cell-free culture supernatant was inactivated by enzyme treatment, which indicates that the inhibitory compound is proteinaceous. This action against closely related species provides evidence that the inhibitor involved could be a bacteriocin.[25]

Table 1 Characteristics of strain BFLP-10^T and some related species

Characteristic	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Lysine decarboxylase	+	+	+	−	+	−	+
Ornithine decarboxylase	+	+	+	−	+	−	+
Urease	+	−	−	−	+	−	−
Acetoin production	−	−	+		−	−	−
Assimilation of:							
L-Arabinose	−	−	−	−	+	w	−
D-Mannitol	+	+	+	−	−	+	+
D-Mannose	−	+	+	−	−	+	+
Citrate	−	−	−	−	−	−	w
Enzyme activities of:							
β-galactosidase	+	−	+	−	−	−	+
Lipase (C14)	+	−	+	+	−	+	+
N-acetyl-*β*-glucosaminidase	+	−	+	−	−	+	−
Fermentation of:							
Amygdalin	+	+	+	−	+	+	−
Melibiose	−	−	−	−	+	−	+
Sucrose	−	+	+	−	+	−	+

Abbreviations: +, positive; −, negative; w, weak reaction. Strains: (1) *V. inhibens* sp. nov. BFLP-10^T; (2) *V. owensii* LMG 25443^T (Cano-Gómez *et al*.[32]); (3), *V. communis* R-40496^T (Chimetto *et al*.[33]); (4) *V. sagamiensis* LC2-047^T (Yoshizawa *et al*.[34]); (5) *V. rotiferianus* LMG 21460^T; (6) *Vibrio campbellIii* LMG 11216^T; (7) *V. harveyi* LMG 4044^T.

Phylogenetic analysis

The 16S rRNA gene sequence of strain BFLP-10^T was a continuous stretch of 1484 bp. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (Figure 1), strain BFLP-10^T was closely related to *V. owensii* DY05^T (99.0%), *V. communis* R-40496^T (98.9%), *V. sagamiensis*^{T} (98.9%) and *V. rotiferianus* LMG 21460^T (98.4%). Similar results were obtained with the maximum-parsimony algorithm (Supplementary Information, Figure S1). Multilocus sequencing analysis of housekeeping genes has been used previously for inferring evolutionary relationships among members of the genus *Vibrio*.[17,26] In the present study, we used four housekeeping genes, *gyrB*, *pyrH*, *recA* and *topA*, in order to establish the taxonomic position of strain BFLP-10^T. The phylogenetic trees based on *gyrB*, *pyrH*, *recA* and *topA* gene sequences revealed low levels of similarity (<91.2%) between strain BFLP-10^T and the most closely related species (Figure 2). Previous studies have demonstrated that multilocus sequencing analysis has a higher discriminatory power than DNA–DNA hybridization.[27,28] In fact, Thompson *et al*.[29] have reported that when two *Vibrio* strains share more than 95% similarity by multilocus sequencing analysis, they are the same species. These results demonstrate that strain BFLP-10^T is distinct from any recognized species of the genus *Vibrio*.

Chemotaxonomic characteristics and DNA base composition

The major fatty acids in strain BFLP-10^T were $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$ (42.6%), $C_{18:1}\omega7c$ (19.6%), $C_{16:0}$ (16.2%) and $C_{12:0}$ (3.8%), which comprise approximately 82.2% of the total fatty acid methyl esters detected. Fatty acids $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$, $C_{16:0}$, $C_{18:1}\omega7c$, $C_{14:0}$, $C_{12:0}$ and $C_{16:0}$ iso are typically the major fatty acids found in *Vibrio* species.[30] However, strain BFLP-10^T and most closely related-type strains, *V. owensii*, *V. communis*, *V. sagamiensis* and *V. rotiferianus*, could be clearly distinguished from each other based on the relative fatty acid concentration (Table 2). The DNA G + C content was calculated to be 45.6 mol%. This value is within the range for the genus *Vibrio*.[31]

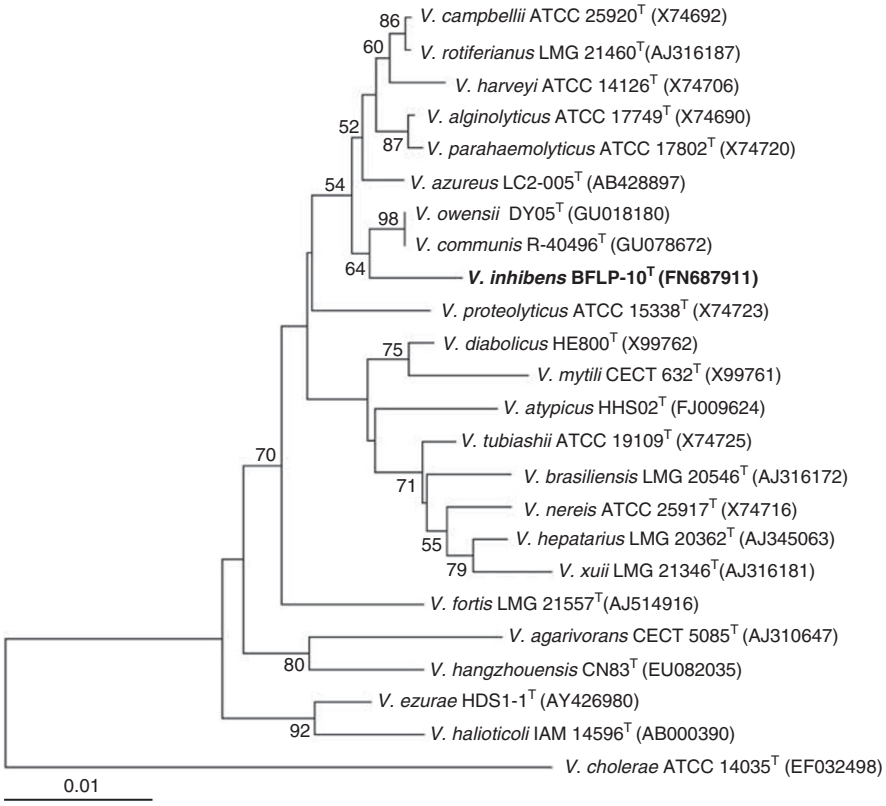

Figure 1 Phylogenetic dendrogram of strain BFLP-10^T with the most closely related *Vibrio* species, based on 16S rRNA gene sequences and constructed by the neighbour-joining method. Bootstrap percentages (>50%) based on 1000 replications are shown at branch nodes. *Vibrio cholerae* ATCC 14035^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

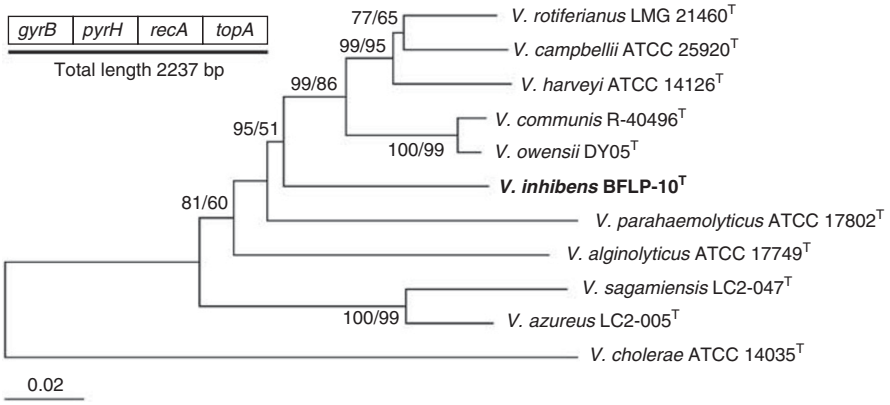

Figure 2 Phylogenetic tree based on the concatenated sequences of the four housekeeping genes (2237 bp). This tree combines the results of both the neighbour-joining (NJ) and maximum-parsimony (MP) methods. The topology shown was obtained by using the NJ method. Bootstrap percentages based on 1000 replications are shown at branch nodes (NJ/MP). *V. cholerae* ATCC 14035^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Therefore, the phenotypic and genotypic properties of strain BFLP-10^T support its description as a novel species within the genus *Vibrio*, for which the name *V. inhibens* sp. nov. is proposed.

Description of *V. inhibens* sp. nov.

V. inhibens (in.hi′bens. L. part. adj. *inhibens*, inhibiting). Cells are Gram-negative, motile, facultatively anaerobic and slightly curved rod-shaped (0.6 × 2.0–2.5 μm). Colonies on TSA supplemented with 1.5% (w/v) NaCl are cream coloured, circular and 1.5–2.0 mm in diameter. Growth occurs at NaCl concentrations between 1.0 and 8.0% (w/v), but not without NaCl or in the presence of >9.0% NaCl (w/v); grows at 10–35 °C (optimum 22 °C), but not at 5 and 40 °C; grows at pH 5.0–10.0 (optimum pH 7.0), but not at pH 4.5 or pH 10.5. Positive for catalase, oxidase, lysine-and ornithine-decarboxylase,

Table 2 Cellular fatty acid composition of *V. inhibens* sp. nov. and related *Vibrio* species

Fatty acid	*1*	*2*	*3*	*4*	*5*	*6*	*7*
C12:0	3.8	2.3	2.6	3.0	3.0	4.5	3.5
C12:0 3-OH	2.1	1.0	2.8	–	1.3	2	2.2
C14:0	3.4	6.3	5.8	8.2	7	10	4.7
C15:0	–	–	–	4.4	–	–	–
C16:1 ω7c alcohol	–	–	–	–	–	–	–
C16:0 iso	0.7	3.5	2.5	–	–	–	3.8
C16:1 ω9c	0.6	–	–	–	–	–	–
C16:0	16.2	16.7	14.3	15.1	28.6	30	15.6
C17:1 ω8c	0.4	1.7	1.5	3.6	–	–	–
C17:1 ω6c	–	–	–	1.2	–	–	–
C17:0	0.4	1.8	2.3	1.8	–	–	–
C18:1 ω7c	19.6	14.6	16.6	13.7	12.7	8	24
C18:0	0.4	1.0	–	1.5	1.2	1	1.6
Summed feature 2	3.6	2.2	4.5	2.0	3.2	6.9	4.4
Summed eature 3	42.6	37.5	37.3	38.6	39.9	37.9	34.7

Strains: (1) *V. inhibens* sp. nov. BFLP-10^T; (2) *V. owensii* LMG 25443^T (Cano-Gómez *et al.*[32]); (3) *V. communis* R-40496^T (Chimetto *et al.*[33]); (4) *V. sagamiensis* LC2-047^T (Yoshizawa *et al.*[34]); (5) *V. rotiferianus* LMG 21460^T; (6) *V. campbellii* LMG 11216^T; (7) *V. harveyi* LMG 4044^T. Summed feature 2 comprises $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I. Summed feature 3 comprises $C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$. Data are expressed as percentages of total fatty acids.

indole production, nitrate reduction to nitrite, urease, aesculin, tryptophane deaminase, gelatine hydrolysis, *N*-acetyl-β-glucosamine, assimilation of D-glucose, D-mannitol and malate. Negative for acetoin production, arginine dihydrolase, production of H_2S, assimilation of L-arabinose, D-mannose, D-maltose, potassium gluconate, caprate, citrate, adipate and phenyl-acetate. Acid is produced from amygdalin, D-glucose, D-fructose, D-mannitol, D-cellobiose, D-trehalose, amidon and glycogen but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-mannose, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, arbutin, salicin, D-maltose, D-lactose, D-melibiose, D-saccharose, inulin, D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol and L-arabitol. API ZYM tests show activities for alkaline phosphatase, β-galactosidase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but not for α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase. The major fatty acids are $C_{16:0}$, $C_{12:0}$ and $C_{18:1}\omega7c$; smaller amounts of $C_{14:0}$ and $C_{12:0}$ 3-OH are present. Undefined fatty acids are also observed, summed feature 2 ($C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I) and summed feature 3 ($C_{15:0}$ iso 2-OH and/or $C_{16:1}\omega7c$). The G + C content of the type strain is 45.6 mol% (T_m).

The type strain is BFLP-10^T (= CECT 7692^T = DSM 23440^T), isolated from the faeces of wild seahorses captured in northwest Spain (Toralla, Galicia).

Accession numbers

The GenBank/EMBL/DDBJ accession numbers for the sequences of *V. inhibens* sp. nov. are FN687911 (16S rRNA gene), FR669655 (*recA* gene), HE588134 (*gyrB* gene), HE588135 (*pyrH* gene) and HE588136 (*topA* gene).

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