

Vibrio crassostreae sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*)

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Polyphasic analysis of five new *Vibrio* isolates originating from the haemolymph of diseased cultured oysters is described. The new isolates were closely related to *Vibrio splendidus*, having 98% 16S rRNA gene sequence similarity. *gyrB* phylogenetic analysis, fluorescent amplified-fragment length polymorphism (FAFLP) fingerprinting and DNA–DNA hybridization experiments clearly showed that the new isolates form a tight genomic group that is different from the currently known *Vibrio* species. It is proposed to accommodate these isolates in a novel species, *Vibrio crassostreae* sp. nov. (type strain LGP 7^T = LMG 22240^T = CIP 108327^T). Phenotypic and chemotaxonomic features that differentiate *V. crassostreae* from other known *Vibrio* species include arginine dihydrolase, utilization and fermentation of various carbon sources, β -galactosidase activity, NO₂ production and the presence of the fatty acids 14:0 iso and 16:0 iso.

Vibrio splendidus-related species have been associated with mortality of molluscs and fish (Nicolas *et al.*, 1996; Sugumar *et al.*, 1998; Gatesoupe *et al.*, 1999; Lacoste *et al.*, 2001; Waechter *et al.*, 2002; Farto *et al.*, 2003). Epidemiological studies of *V. splendidus* strains associated with oyster mortality outbreaks have demonstrated a high genetic diversity within this group and suggested its polyphyletic nature (Le Roux *et al.*, 2002, 2004). Six species, *Vibrio lentus*, *Vibrio kanaloae*, *Vibrio pomeroyi*, *Vibrio tasmaniensis*, *Vibrio chagasii* and *V. splendidus*, have been described within this group so far, but there exist only a limited number of diagnostic biochemical features that allow clear species discrimination within this group (Macián *et al.*, 2001; Thompson *et al.*, 2003a, b). In a previous study we investigated a collection of *V. splendidus*-related isolates originated from the haemolymph of oysters that are potentially pathogenic for the oyster *Crassostrea gigas* (Gay *et al.*, 2004). These strains were characterized by DNA gyrase subunit B (*gyrB*) gene sequencing (Le Roux

et al., 2004). Several strains clustered together but could not be assigned to any known *Vibrio* species. In the present study we present a detailed polyphasic analysis of a group of five *Vibrio* isolates, including 16S rRNA and *gyrB* gene sequencing, fluorescent amplified-fragment length polymorphism (FAFLP) fingerprinting, DNA–DNA hybridizations and biochemical tests. Collectively, the results suggest that the five isolates belong to a novel species, for which we propose the name *Vibrio crassostreae* sp. nov.

The strains used in this study were purchased from national collections (*V. splendidus* LMG 4042^T, *V. tasmaniensis* LMG 20012^T, *V. kanaloae* LMG 20539^T, *V. pomeroyi* LMG 20537^T, *Vibrio cyclitrophicus* LMG 21359^T, *V. chagasii* LMG 21353^T, *V. lentus* CIP 107166^T) or isolated from the haemolymph of cultured *C. gigas* in La Tremblade (France) [LMG 22240^T (=LGP 7^T = CIP 108327^T), LMG 22241 (=LGP 8 = CIP 108328), LMG 22248 (=LGP 15 = CIP 108329), LMG 22249 (=LGP 107 = CIP 108330), LMG 22242 (=LGP 17), LMG 22243 (=LGP 1) and LGP 108] and deposited in the BCCM/LMG Bacteria Collection (Gent, Belgium) and in the Institut Pasteur Bacteria Collection (CIP; Paris, France). All strains were cultured on tryptone soy agar (TSA; Oxoid) supplemented with 2% (w/v) NaCl for 48 h at 20 °C.

PCR amplification, cloning and sequencing of the 16S rRNA and *gyrB* gene fragments were done as described previously (Yamamoto & Harayama, 1995; Lambert *et al.*, 1998; Le Roux *et al.*, 2004). Sequences were aligned and phylogenetic analyses were performed with SEAVIEW and

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Abbreviation: FAFLP, fluorescent amplified-fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of LGP 7^T are AJ582808 and AJ582799, respectively.

Tables of supplementary DNA–DNA hybridization, phenotypic and fatty acid data are available in IJSEM Online.

PHYLO_WIN software (Galtier *et al.*, 1996). Phylogenetic trees were constructed using neighbour-joining, maximum-likelihood and maximum-parsimony. For neighbour-joining analysis, distance matrices were calculated by using Kimura's 2-parameter distances (Gascuel, 1997). Reliability of topologies was assessed by the bootstrap method with 1000 replicates. FAFLP analysis was carried out as described previously (Thompson *et al.*, 2001).

For DNA–DNA hybridization experiments, *in vitro* labelling of the DNA with tritium-labelled nucleotides was performed by the random primer method (Megaprime labelling kit; Amersham) and hybridization was carried out at 60 °C by the S1-nuclease method (Crosa *et al.*, 1973; Grimont *et al.*, 1980) with adsorption of S1-resistant DNA onto Whatman DE81 filters.

Phenotypic characterization of the strains was done using the following commercially available kits: the Gram kit (bioMérieux), oxidase (Bactident oxidase; Merck), respiratory activity (meat liver medium; Diagnostic Pasteur), glucose metabolism (MEVAG; Diagnostic Pasteur), API 20E and API 50CH (bioMérieux) with the modification suggested by MacDonell *et al.* (1982), namely 2% NaCl was added to the bacterial suspension. Motility, NaCl requirement and tolerance (0, 2, 4, 6, 8 and 10%, w/v) and temperature tolerance (4, 20, 35 and 40 °C) were tested in 1.5% (w/v) peptone broth (Diagnostic Pasteur). Numerical analysis of phenotypic features was performed using simple matching coefficients (Sneath, 1972) and the unweighted pair group method (Sneath & Sokal, 1973). Sensitivity to O/129 (150 µg per disc) was determined with Oxoid discs. Fatty acid methyl ester analysis was carried out as described by Huys *et al.* (1994).

The phylogenetic tree based on the *gyrB* nucleotide sequences (1064 gap-free sites long) confirmed the clustering of *V. crassostreae* sp. nov. strains LGP 7^T, LGP 8, LGP 15, LGP 107 and LGP 108 with a bootstrap value of 100% and their distinction from their closest phylogenetic neighbours *V. cyclitrophicus*, *V. lentus*, *V. pomeroyi*, *V. kanaloae*, *V. tasmaniensis* and *V. splendidus* (Fig. 1). The phylogenetic tree based on almost-complete sequences of the 16S rRNA gene does not allow clear differentiation of the two representative isolates (LGP 7^T and LGP 8) from other species phenotypically related to *V. splendidus* (Fig. 2). Results are in accordance with previous studies, showing that the 16S rRNA gene sequences of *V. splendidus*-related strains are very similar (Macián *et al.*, 2001; Le Roux *et al.*, 2002, 2004; Thompson *et al.*, 2003a, b). Similar results were obtained by maximum-parsimony and maximum-likelihood analyses (data not shown).

The FAFLP patterns of four representative *V. crassostreae* strains consisted of 125 bands (± 9 SD). The mutual FAFLP pattern similarity among these strains was at least 86%. Strains of *V. crassostreae* sp. nov. were clearly differentiated from all the other currently known species of the *Vibrionaceae*, *V. splendidus* and *V. kanaloae* being the most closely

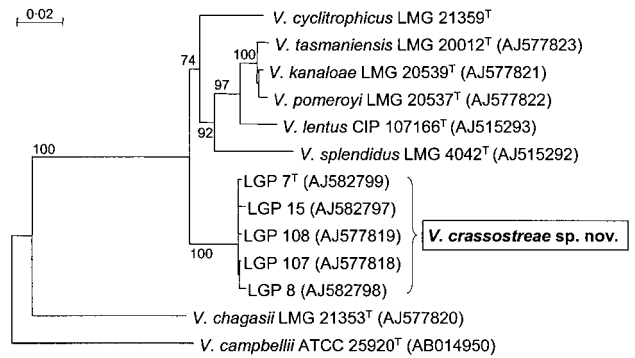


Fig. 1. Phylogenetic tree of partial *gyrB* sequences. The *Vibrio campbellii* homologue was used as the outgroup; 1064 gap-free sites were compared. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap percentages from 1000 replicates appear next to the corresponding branch.

related, with 54% similarity (Fig. 3). The FAFLP data suggest that the isolates indeed belong to a novel *Vibrio* species.

DNA–DNA hybridization experiments confirmed the grouping found with FAFLP and *gyrB*. *V. crassostreae* strains had at least 78% DNA–DNA relatedness, but at maximum 61% towards five other *V. splendidus*-related species (Supplementary Table A in IJSEM Online).

Strains of *V. crassostreae* sp. nov. could be differentiated from their closest phylogenetic neighbours by 17 phenotypic characters analysed in this study (Supplementary Table B). Those tests were coded as 1 (positive result) or 0 (negative result) and numerical analysis was performed using simple matching coefficients (Fig. 4). *V. crassostreae* sp. nov. has the main fatty acid traits of vibrios, i.e. 16:1 ω 7c and/or 15:0 iso 2-OH, 18:1 ω 7c, 12:0, 14:0, 12:0 3-OH (Supplementary Table C). The fatty acids 16:0 iso and

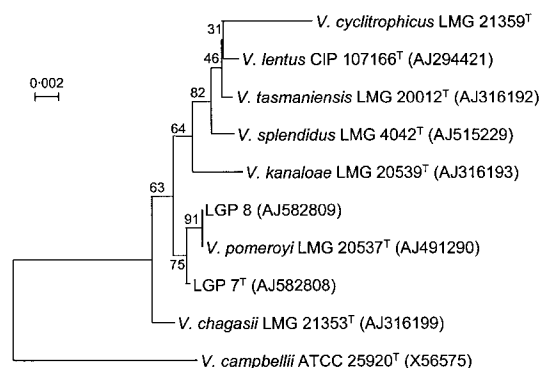


Fig. 2. Phylogenetic tree of partial 16S rRNA gene sequences; 1200 gap-free sites were compared. Other features as in Fig. 1.

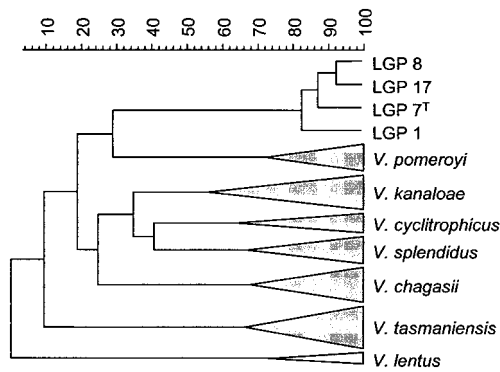


Fig. 3. Dendrogram (Dice, Ward) of the FAFLP band patterns of *V. crassostreae* sp. nov. and all other known *V. splendidus*-related species.

14:0 iso seem to be useful for discriminating among *V. splendidus*-related species.

The present study illustrates the use of the *gyrB*-based phylogenetic structure in an interim period to cluster strains before validation of species affiliation by DNA–DNA hybridization and description of phenotypic features. However, in the case of *V. pomeroyi*, *V. kanaloae* and *V. tasmaniensis*, our *gyrB*-based analysis appears to be less discriminatory than DNA–DNA hybridization or FAFLP fingerprinting (Thompson *et al.*, 2001). Sequence analyses of other loci, including *rpoD* (Yamamoto & Harayama, 1998) and *hsp60* sequences (Kwok *et al.*, 2002), are in progress in order to assess the usefulness of such genes to discriminate *V. splendidus*-related species.

Description of *Vibrio crassostreae* sp. nov.

Vibrio crassostreae (cra.sso.stre'ae. N.L. gen. n. *crassostreae* of *Crassostrea gigas*, the oyster species from which the strains were isolated).

Cells are Gram-negative, curved, 1 µm wide and 2–3 µm

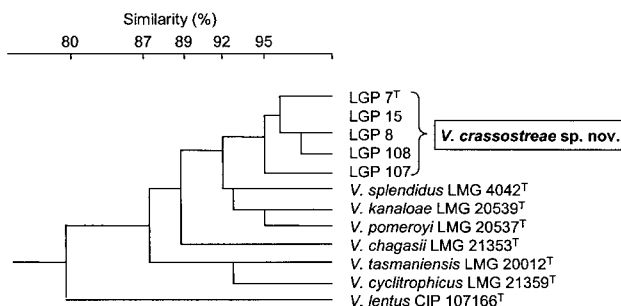


Fig. 4. Cluster analysis of phenetic data for *Vibrio* species using simple matching-similarity coefficient and unweighted pair group method (expressed as a percentage on the scale bar) for 12 strains related to *V. splendidus*.

long. Cells are motile by at least one polar flagellum. Forms translucent, non-swarming, rounded colonies with entire margins on TSA. Strains form yellow, translucent, 5 mm colonies on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Cells grow at 4 °C. None of the strains grows at 0 or 8 % NaCl. All strains are β-galactosidase-negative, arginine dihydrolase- and gelatinase-positive. Oxidase- and catalase-positive and urease-negative. Facultatively anaerobic and produces NO₂. The following compounds are utilized as sole carbon sources: glucose, sucrose, melibiose, amygdalin, glycerol, ribose, galactose, D-mannose, mannitol, N-acetylglucosamine, aesculin, cellobiose, starch, glycogen and L-fucose. None of the strains utilize inositol, rhamnose, arabinose, erythritol, D- or L-arabinose, D- or L-xylose, adonitol, methyl β-D-xyloside, L-sorbose, dulcitol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, amygdalin, arbutin, salicin, lactose, inulin, melezitose, D-raffinose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, D- or L-arabitol, 2-oxoglutarate or 5-oxoglutarate. All strains are sensitive to O/129. Major fatty acids are summed feature 3 (39.4 ± 1 %, comprising 16:1ω7c and/or 15:0 iso 2-OH), 16:0 (17.3 ± 1.3 %), 16:0 iso (8.7 ± 0.2 %), 18:1ω7c (7 ± 1.3 %), 12:0 (5.5 ± 0.3 %), 14:0 (5.4 ± 1 %), 12:0 3-OH (3.3 ± 0.2 %), summed feature 2 (2.6 ± 0.1 %, comprising 14:0 3-OH and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain-length value of 10.928 and/or 12:0 ALDE), 14:0 iso (1.6 %), 17:0 (1.4 %), 17:1ω8c (1.3 %) and 14:0 iso 3-OH (1.2 %).

Type strain LGP 7^T (=LMG 22240^T=CIP 108327^T) was isolated from a diseased oyster (*Crassostrea gigas*) at the laboratoire de genetique et pathologie (Ifremer, France). Reference strains are LMG 22241 (=LGP 8=CIP 108328), LMG 22248 (=LGP 15=CIP 108329), LMG 22249 (=LGP 107=CIP 108330) and LGP 108. The GenBank/EMBL/ DDBJ accession numbers for the 16S and *gyrB* gene sequences of LGP 7^T are AJ582808 and AJ582799, respectively.

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