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

N. Faury,[1] D. Saulnier,[1] F. L. Thompson,[2] M. Gay,[1] J. Swings[2] and F. Le Roux[1]

Correspondence
F. Le Roux
fleroux@ifremer.fr

[1]Laboratoire de Génétique et Pathologie, Institut français de recherche pour l'exploitation de la mer (Ifremer), 17390 La Tremblade, France

[2]Laboratory for Microbiology and BCCM™/LMG Bacteria Collection, Laboratory for Microbiology, Ghent University, K.L. Ledeganckstraat 35, Ghent 9000, Belgium

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, NO_2 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 related, with 54 % similarity (Fig. 3). The FAFLP data suggest that the isolates indeed belong to a novel *Vibrio* species.

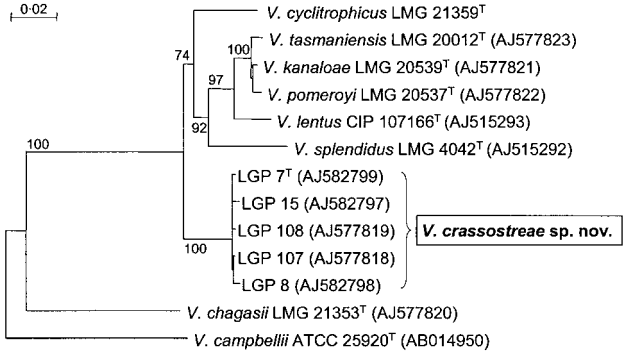

Fig. 1. Phylogenetic tree of partial *gyrB* sequences. The *Vibrio campbellii* homologue was used as the outgroup; 1064 gap-free sites were compared. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap percentages from 1000 replicates appear next to the corresponding branch.

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 : 1ω7*c* and/or 15 : 0 iso 2-OH, 18 : 1ω7*c*, 12 : 0, 14 : 0, 12 : 0 3-OH (Supplementary Table C). The fatty acids 16 : 0 iso and

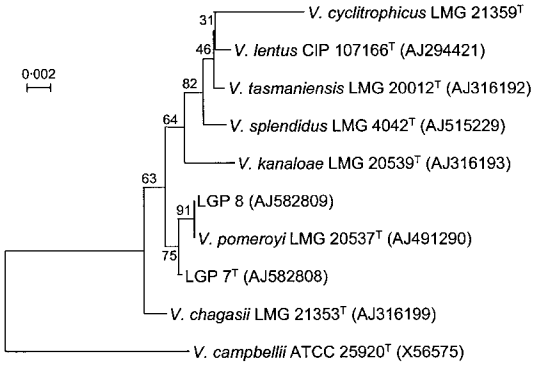

Fig. 2. Phylogenetic tree of partial 16S rRNA gene sequences; 1200 gap-free sites were compared. Other features as in Fig. 1.

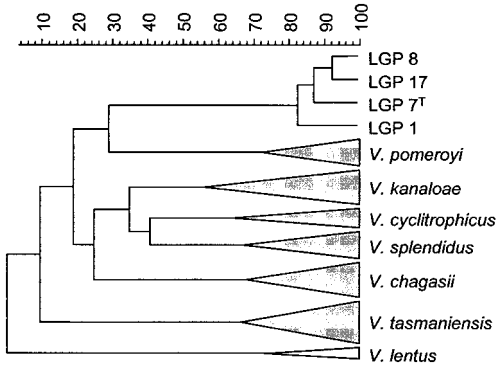

Fig. 3. Dendrogram (Dice, Ward) of the FAFLP band patterns of *V. crassostreae* sp. nov. and all other known *V. splendidus*-related species.

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 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 NO_2. 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 : 1ω7*c* and/or 15 : 0 iso 2-OH), 16 : 0 (17·3±1·3 %), 16 : 0 iso (8·7±0·2 %), 18 : 1ω7*c* (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 : 1ω8*c* (1·3 %) and 14 : 0 iso 3-OH (1·2 %).

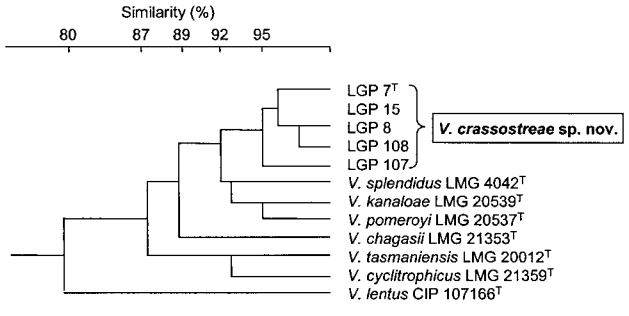

Fig. 4. Cluster analysis of phenetic data for *Vibrio* species using simple matching-similarity coefficient and unweighted pair group method (expressed as a percentage on the scale bar) for 12 strains related to *V. splendidus*.

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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