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 7T = LMG 22240T = CIP 108327T). 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 kanaloeae, 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 (Macíán 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 4042T, V. tasmaniensis LMG 20012T, V. kanaloeae LMG 20539T, V. pomeroyi LMG 20537T, Vibrio cyclitrophicus LMG 21359T, V. chagasii LMG 21353T, V. lentus CIP 107166T) or isolated from the haemolymph of cultured C. gigas in La Tremblade (France) [LMG 22240T (= LMG 7T = CIP 108327T), LMG 22241 (= LMG 8 = CIP 108328), LMG 22248 (= LMG 15 = CIP 108329), LMG 22249 (= LMG 107 = CIP 108330), LMG 22242 (= LMG 17), LMG 22243 (= LMG 1) and LMG 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
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 (MegaPrimer 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 7T, 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 7T 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 (Macian 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.

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

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 ω7c and/or 15 : 0 iso 2-ΟΗ, 18 : 1 ω7c, 12 : 0, 14 : 0, 12 : 0 3-ΟΗ (Supplementary Table C). The fatty acids 16 : 0 iso and

*V. crassostreae sp. nov.*
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 NO2. The following compounds are utilized as sole carbon sources: glucose, sucrose, melibiose, amygdalin, glycerol, ribose, galactose, D-mannose, mannitol, N-acetylgalactosamine, aesculin, cellobiose, starch, glycogen and L-fucose. None of the strains utilize inositol, rhamnose, arabinose, erythritol, D- or L-arabinose, D- or L-xylitol, adonitol, methyl β-D-xylidine, L-sorbitol, 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% ±, comprising 16:1ω7c and/or 15:0 iso 2-0H), 16:0 (17±3% ±±, 16:0 iso (8±7% ±±-2_), 18:1ω7c (7±3% ±±), 12:0 (5±5% ±±-3_), 14:0 (5±4% ±±), 12:0 3-0H (3±3% ±± -2_), summed feature 2 (2±6% ±±-0), comprising 14:0 3-0H and/or 16:1 iso I and/or unidentified fatty acid with equivalent chain-length value of 10±9 and/or 12±0 ALDE), 14:0 iso (1±6_), 17:0 (1±4_), 17:1ω8c (1±3_) and 14:0 iso 3-0H (1±2_).

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

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**References**


