Vibrio gigantis sp. nov., isolated from the haemolymph of cultured oysters (Crassostrea gigas)

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Polyphasic analysis of four 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. Phylogenetic analysis based on DNA gyrase subunit B (gyrB), RNA polymerase σ70 factor (rpoD), replication origin-binding protein (rctB) and transmembrane regulatory protein (toxR) genes, fluorescent amplified fragment length polymorphism 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 that these new isolates should be accommodated in a novel species, Vibrio gigantis sp. nov.

Phenotypic features that differentiate V. gigantis from other known Vibrio species include arginine dihydrolase, gelatinase and β-galactosidase activities, NO2 production, growth at 35 °C, and utilization of sucrose, melibiose, amygdalin, glycerol, galactose, starch and glycogen. The type strain is LGP 13T (≡LMG 22741T ≡CIP 108656T).

The genus Vibrio (Baumann et al., 1984) comprises bacteria inhabiting aquatic environments, especially marine and estuarine waters, where they are frequently associated with organisms ranging from plankton to fish. Within this genus, the number of species with validly published names increased from 20 in 1981 to 63 in 2004 (Thompson et al., 2004).

The haemolymph of shellfish contains a high abundance of vibrios that may play a role in the health of the host. The diversity of Vibrio splendidus-related strains isolated from the haemolymph of cultured oysters has been characterized (Gay et al., 2004; Le Roux et al., 2004) and the novel species Vibrio crassostreae has been described (Faury et al., 2004).

We present here a polyphasic analysis of four new Vibrio isolates, by means of fluorescent amplified fragment length polymorphism (FAFLP), DNA–DNA hybridization and sequencing of 16S rRNA, DNA gyrase subunit B (gyrB), RNA polymerase σ70 factor (rpoD), replication origin-binding protein (rctB) and transmembrane regulatory protein (toxR) genes (Egan & Waldor, 2003; Le Roux et al., 2004; Lonetto et al., 1992; Osorio & Klose, 2000; Watt & Hickson, 1994; Yamamoto & Harayama, 1998). Overall, the data presented in this study clearly show that the isolates represent a novel species, for which we propose the name Vibrio gigantis sp. nov.

The strains used in this study were purchased from national collections (V. splendidus LMG 19031T, Vibrio tasmaniensis LMG 20012T, Vibrio kandelae LMG 20539T, Vibrio pomeroyi LMG 20537T, Vibrio cyclitrophicus LMG 21359T, Vibrio chagasii LMG 21353T, Vibrio lentus CIP 107166T) or isolated from the haemolymph of cultured Crassostrea gigas in La Tremblade (France) [V. crassostreae LMG 7T and LMG 8; strains LMG 13T (=LMG 22741T =CIP 108656T), LMG 16 (=LMG 22742 =CIP 108655), LMG 37 and LMG 45] and deposited in the BCCM/LMG Bacteria Collection (Ghent, Belgium) and in the Institut Pasteur Bacteria Collection.
Huys et al. (1997) analyzed the phylogenetic relationships of the two representative isolates of *V. gigantis* sp. nov. (LGP 13T and LGP 45) from other species phenotypically related to *V. splendidus* (Supplementary Fig. S1 in IJSEM Online). Similar results were obtained with maximum-parsimony and maximum-likelihood analyses (data not shown). Protein-encoding genes have been reported to evolve much faster than rRNA genes and are therefore expected to provide higher resolution in phylogenetic analysis.

The phylogenetic tree based on almost-complete sequences of the 16S rRNA gene did not permit a clear differentiation of the two representative isolates of *V. gigantis* sp. nov. (LGP 13T and LGP 37) from other species phenotypically related to *V. splendidus* (Supplementary Fig. S1 in IJSEM Online). Similar results were obtained with maximum-parsimony and maximum-likelihood analyses (data not shown). Protein-encoding genes have been reported to evolve much faster than rRNA genes and are therefore expected to provide higher resolution in phylogenetic analysis.

The phylogenetic tree based on gyrB nucleotide sequences (1064 gap-free sites long) confirmed the clustering of *V. gigantis* sp. nov. strains LGP 13T, LGP 16, LGP 37 and LGP 45, with a bootstrap value of 100 %, and their distinction from their closest phylogenetic neighbours *V. crassostreae*, *V. splendidus*, *V. lentus*, *V. pomeroyi*, *V. kanaloeae*, *V. tasmaniensis*, *V. cyclitrophicus* and *V. chagasii* (Fig. 1). Similarities between gyrB sequences ranged between 98 % (LGP 13T and *V. crassostreae* LGP 7T) and 85 % (LGP 13T and *V. chagasii* LGM 21353T). For several species related to the *V. splendidus* group, i.e. *V. pomeroyi*, *V. kanaloeae* and *V. tasmaniensis*, the gyrB gene-based analysis appeared to be less discriminatory than DNA–DNA hybridization or FAFLP fingerprinting (Gay et al., 2004; Thompson et al., 2001).

The clustering of *V. gigantis* sp. nov. strains LGP 13T, LGP 16, LGP 37 and LGP 45 was also observed in phylogenetic trees based on three other genes (Figs 2, 3 and 4). Similarities between *rpoD* sequences ranged between 97 % (LGP 13T and *V. crassostreae* LGP 7T) and 92 % (LGP 13T and *V. lentus* CIP 107166T). Similarities between *rctB* sequences ranged between 89 % (LGP 13T and *V. crassostreae* LGP 7T) and 85 % (LGP 13T and *V. cyclitrophicus* LGM 21359T). Finally, similarities between *toxR* sequences ranged between 91 % (LGP 13T and *V. crassostreae* LGP 8) and 72 % (LGP 13T and *V. lentus* CIP 107166T).

Fig. 1. Phylogenetic tree based on partial gyrB sequences. The *Vibrio campbellii* homologue was used as the outgroup; 1064 gap-free sites were compared. 100 % bootstrap percentages from 1000 replicates appear next to the corresponding branch.

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

Phenotypic characterization of the strains was done using commercially available kits: Gram kit (bioMérieux), oxidase (Bactident oxidase; Merck), respiratory activity (meat liver medium; Diagnostic Pasteur), glucose metabolism (MEVAG; Diagnostic Pasteur), and API 20E and API 50 CH (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, 10 %, w/v), and temperature tolerance (4, 20, 35 and 40 °C) were tested in 1-5 % (w/v) peptone broth (Diagnostic Pasteur). Sensitivity to O129 (150 μg per disc) was determined with Oxoid discs. Fatty acid methyl ester analysis was carried out as described by Huys et al. (1994).

(CIP; Paris, France). Strains LGP 13T, LGP 37 and LGP 45 were isolated from distinct animals following separate cohabitation trials (Gay et al., 2004). All strains were cultured on tryptone soy agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 48 h at 20 °C.

PCR, cloning and sequencing of the gene fragments were performed as described previously (Le Roux et al., 2004). Primer sequences and annealing temperatures are given in Supplementary Table S1 in IJSEM Online. Sequences were aligned and phylogenetic analyses were performed using 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 fingerprinting was performed as described previously (Thompson et al., 2001).

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belong to a novel species. Strains of the same species always have more than 60–70 % FAFLP band pattern relatedness (Thompson et al., 2004).

DNA–DNA hybridization experiments confirmed the grouping found with the phylogenetic data. V. gigantis sp. nov. strains had at least 91 % DNA–DNA relatedness, but at maximum 56 % to eight other V. splendidus-related species (Supplementary Table S2 in IJSEM Online).

Although the strains included in this study formed a tight genomic group, the presence of sequence polymorphism and the FAFLP profiles and DNA–DNA hybridization rates led us to exclude a clonal origin for our isolates.
An ad hoc committee for the re-evaluation of the species definition in bacteriology has encouraged investigators to propose novel species based upon genomic methods, e.g. FAP-MLP and multilocus sequence typing (MLST), provided there is a sufficient degree of congruence between the technique used and DNA-DNA reassociation within the taxa studied. In particular, analysis of protein-encoding gene sequences to circumscribe the taxon species and to differentiate it from neighbouring species is recommended (Stackebrandt et al., 2002). The present study illustrates the usefulness of MLST data to elucidate genomic relatedness at inter- and intraspecific levels. Indeed the clustering of three V. gigantis sp. nov. strains was observed for four of 5 MLST-analysed genes and was supported by high bootstrap values. This type of study will certainly improve the taxonomy of the genus Vibrio, making the data more readily available for different purposes.

**Description of Vibrio gigantis sp. nov.**

Vibrio gigantis [gi.gan’tis. L. gen. n. gigantis of Gigas (a giant) and of gigas the specific epithet 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. Forms yellow, translucent, 5 mm colonies on thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Grows at 4°C. Does not grow in 0 or 8% NaCl. β-Galactosidase-negative, and arginine dihydrolase- and gelatinase-positive. Oxidase- and catalase-positive. Negative for urease, lysine negative, and arginine dihydrolase- and gelatinase-positive. Oxidase- and catalase-positive. Negative for urease, lysine and ornithine decarboxylase. Facultatively anaerobic and produces NO2. The following compounds are utilized as sole carbon sources: glucose, melibiose, amygdalin, glycerol, ribose, galactose, D-mannose, D-fructose, mannitol, N-acetylglucosamine, ascinulin, cellobiose, trehalose, starch and glycogen. Does not utilize sucrose, inositol, sorbitol, rhamnose, erythritol, D- or L-arabinose, D- or L-xylene, adonitol, methyl β-D-xyloside, L-sorbose, dulcitol, methyl α-D-mannoside, methyl α-D-glucoside, arbutin, salicin, lactose, inulin, melezitose, D-raffinose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, 2-oxoglutarate or 5-oxoglutarate. Sensitive to O129. Phenotypic characteristics that distinguish V. gigantis from other V. splendidus-related species are available in Supplementary Table S3 in IJSEM Online. Major fatty acids (Supplementary Table S4 in IJSEM Online) are summed feature 3 (40±4±04%); comprising 16:1ω7c and/or 15 iso 2-OH), 16:0 (23±5±1±9%), 18:1ω7c (12±5±0±5%), 12:0 (4±5±0±6%), 14:0 (4±7±0±1%), summed feature 2 (2±3±0±7%); comprising 14:0 3-OH, and/or 16:1 iso 1, and/or unidentified fatty acid with equivalent chain-length value of 10:928, and/or 12:0 ALDE), 16:0 iso 2 (2±4±0±0%), 12:0 3-OH (1±9±0±6%), 17:0 (1±5±0±1%), 18:0 (1±3±0±1%) and 17:1ω8c (0±9±0±1%).

The type strain, LGP 13T (=LMG 22741T =CIP 108656T), was isolated from a diseased oyster (Crassostrea gigas) at the laboratoire de génétique et pathologie (Ifremer, France).

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**References**


