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 σ^{70} 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, NO_2 production, growth at 35 °C, and utilization of sucrose, melibiose, amygdalin, glycerol, galactose, starch and glycogen. The type strain is LGP 13^T (=LMG 22741^T=CIP 108656^T).

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 σ^{70} 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 19031^T, *Vibrio tasmaniensis* LMG 20012^T, *Vibrio kanaloae* LMG 20539^T, *Vibrio pomeroyi* LMG 20537^T, *Vibrio cyclitrophicus* LMG 21359^T, *Vibrio chagasii* LMG 21353^T, *Vibrio lentus* CIP 107166^T) or isolated from the haemolymph of cultured *Crassostrea gigas* in La Tremblade (France) [*V. crassostreae* LGP 7^T and LGP 8; strains LGP 13^T (=LMG 22741^T=CIP 108656^T), LGP 16 (=LMG 22742=CIP 108655), LGP 37 and LGP 45] and deposited in the BCCM/LMG Bacteria Collection (Ghent, Belgium) and in the Institut Pasteur Bacteria Collection

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Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *gyrB*, *rpoD*, *rctB* and *toxR* gene sequences of strains LGP 13^T, LGP 16, LGP 37 AND LGP 45 determined in this study are given in Figs 1–4 and Supplementary Fig. S1.

A phylogenetic tree based on 16S rRNA gene sequences, a dendrogram based on FAFLP band patterns, and data on primer sequences used in PCR, intra- and interspecific DNA–DNA relatedness, phenotypic characteristics and fatty acid composition of *Vibrio gigantis* sp. nov. and other *Vibrio splendidus*-related species, are available as supplementary material in IJSEM Online.

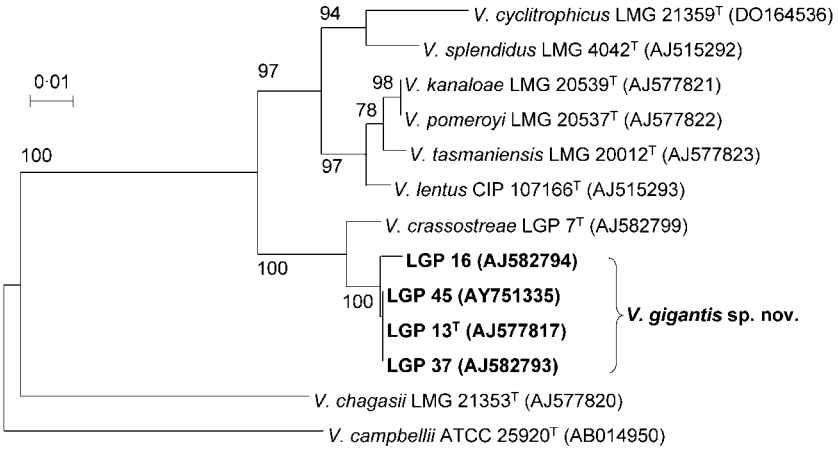

Fig. 1. Phylogenetic tree based on partial *gyrB* sequences. The *Vibrio campbellii* homologue was used as the outgroup; 1064 gap-free sites were compared. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap percentages from 1000 replicates appear next to the corresponding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

(CIP; Paris, France). Strains LGP 13^T, 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).

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 and 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).

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 13^T 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 13^T, 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. kanaloae*, *V. tasmaniensis*, *V. cyclitrophicus* and *V. chagasii* (Fig. 1). Similarities between *gyrB* sequences ranged between 98 % (LGP 13^T and *V. crassostreae* LGP 7^T) and 85 % (LGP 13^T and *V. chagasii* LMG 21353^T). For several species related to the *V. splendidus* group, i.e. *V. pomeroyi*, *V. kanaloae* 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 13^T, 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 13^T and *V. crassostreae* LGP 7^T) and 92 % (LGP 13^T and *V. lentus* CIP 107166^T). Similarities between *rctB* sequences ranged between 89 % (LGP 13^T and *V. crassostreae* LGP 7^T) and 85 % (LGP 13^T and *V. cyclitrophicus* LMG 21359^T). Finally, similarities between *toxR* sequences ranged between 91 % (LGP 13^T and *V. crassostreae* LGP 8) and 72 % (LGP 13^T and *V. lentus* CIP 107166^T).

V. gigantis sp. nov. strains LGP 13^T and LGP 16 had less than 60 % FAFLP band pattern similarity to all other *V. splendidus*-related species (Supplementary Fig. S2 in IJSEM Online), suggesting that these two representative strains

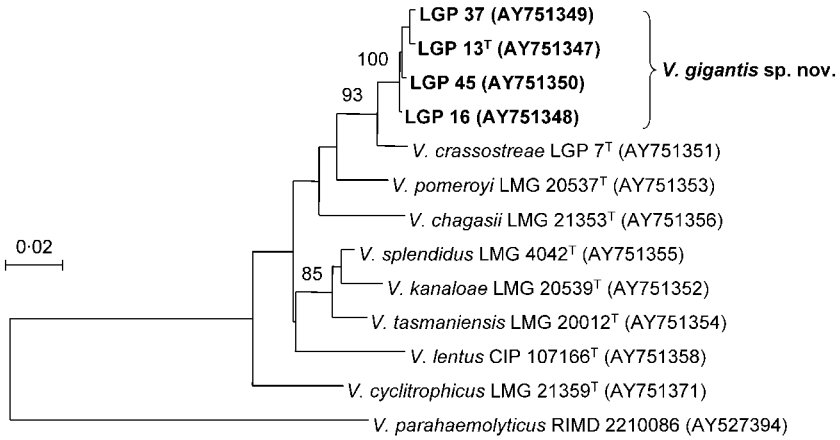

Fig. 2. Phylogenetic tree based on partial *rpoD* sequences. The *Vibrio parahaemolyticus* homologue was used as the outgroup; 500 gap-free sites were compared. Other features as in Fig. 1.

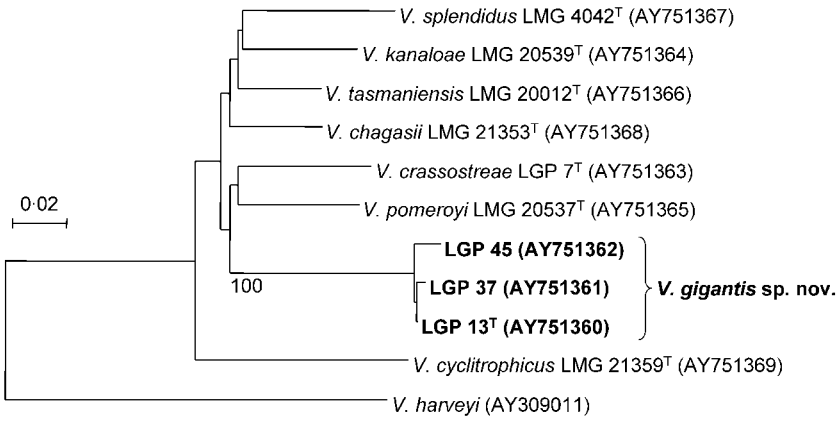

Fig. 3. Phylogenetic tree based on partial *rctB* sequences. The *Vibrio harveyi* homologue was used as the outgroup; 500 gap-free sites were compared. Other features as in Fig. 1.

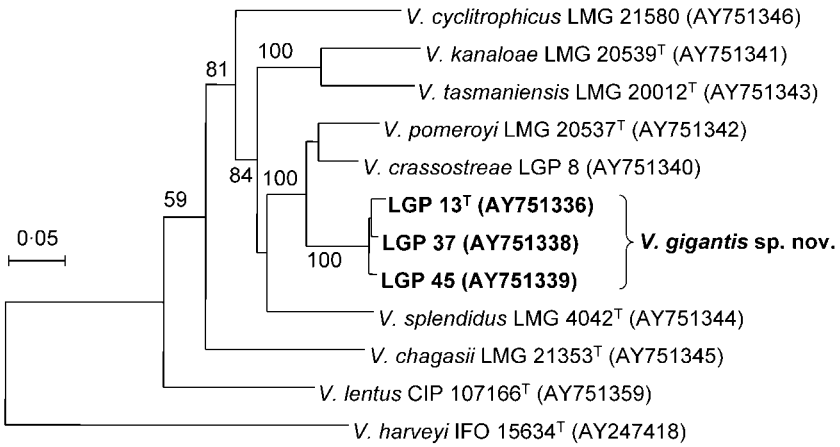

Fig. 4. Phylogenetic tree based on partial *toxR* sequences. The *Vibrio harveyi* homologue was used as the outgroup; 500 gap-free sites were compared. Other features as in Fig. 1.

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. FAFLP 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 and ornithine decarboxylase. Facultatively anaerobic and produces NO_2. The following compounds are utilized as sole carbon sources: glucose, melibiose, amygdalin, glycerol, ribose, galactose, D-mannose, D-fructose, mannitol, *N*-acetylglucosamine, aesculin, cellobiose, trehalose, starch and glycogen. Does not utilize sucrose, inositol, sorbitol, rhamnose, erythritol, D- or L-arabinose, D- or L-xylose, 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 ± 0·4 %; comprising 16 : 1ω7*c* and/or 15 iso 2-OH), 16 : 0 (23·5 ± 1·9 %), 18 : 1ω7*c* (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 I, and/or unidentified fatty acid with equivalent chain-length value of 10·928, and/or 12 : 0 ALDE), 16 : 0 iso (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 : 1ω8*c* (0·9 ± 0·1 %).

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

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