Vibrio ostreicida sp. nov., a new pathogen of bivalve larvae

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The taxonomic position of the bivalve pathogen PP-203[T] was studied together with those of two similar isolates (PP-200 and PP-204). The bacterial strains were isolated from samples of young oyster spat in a bivalve hatchery in Galicia (NW Spain), which was continually affected by outbreaks of disease and severe mortalities. On the basis of 16S rRNA gene sequencing, the three strains formed a cluster within the genus *Vibrio* and were most closely related to *Vibrio pectenicida* DSM 19585[T] (97.9 % similarity). Additional multilocus sequence analysis, including sequences of the housekeeping genes *rpoA*, *recA*, *pyrH*, *gyrB* and *ftsZ*, and DNA–DNA hybridization experiments indicated that the strains were distinct from currently known species of the genus *Vibrio* and confirmed the clustering of the three isolates. Several phenotypic features, such as growth in TCBS medium and nitrate reduction, proved useful for distinguishing the proposed novel species from its closest relatives. The findings support the description of a novel species to include the three isolates, for which the name *Vibrio ostreicida* sp. nov. (type strain PP-203[T]=CECT 7398[T]=DSM 21433[T]) is proposed.

Mollusc hatcheries frequently suffer disease outbreaks that cause heavy mortalities in the productive stocks. In most cases, members of different species of the genus *Vibrio* have been identified as the responsible agent (Tubiash *et al.*, 1965; Brown & Losee 1978; DiSalvo *et al.*, 1978; Elston & Leibovitz 1980; Brown 1981; Jeffries 1982; Sutton & Garrick 1993; Riquelme *et al.*, 1995; Sáinz *et al.*, 1998; Gómez-León *et al.*, 2005; Elston *et al.*, 2008; Kesarcodi-Watson *et al.*, 2009), including novel species described in the last decade (Nicolas *et al.*, 1996; Prado *et al.*, 2005).

Several episodes of mortalities affecting the flat oyster *Ostrea edulis* were investigated in hatcheries in Galicia (NW Spain), to establish the relationship between microbiota and mortalities (Prado *et al.*, 2005). Strain PP-203[T] was isolated from the inner surface of the bins in a nursery

suffering from repeated disease outbreaks. This was the only isolate, among the different types of bacteria found, that was pathogenic to larval stages, as demonstrated in laboratory experiments. Another two strains, PP-200 and PP-204, were obtained from different bins during the same outbreak.

The results of a polyphasic approach, including multilocus sequence analysis (MLSA) analysis, DNA–DNA hybridization (DDH), chemotaxonomic techniques and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, support the classification of these isolates within a novel species of the genus *Vibrio*, for which we propose the name *Vibrio ostreicida* sp. nov.

Isolation of strain PP-203[T] (=CECT 7398[T]=DSM 21433[T]) from the surfaces of nursery culture containers was carried out as previously described (Prado *et al.*, 2005). The pathogenicity of the strain was demonstrated in challenge experiments with flat oyster larvae. Isolates PP-200 (=CECT 7399=DSM 21434) and PP-204, obtained from different containers during the same episode, were included in this study because of their similar phenotypic traits. Other strains were obtained from reference collections (*Vibrio pectenicida* DSM 19585[T], *Vibrio aestuarianus* ATCC 35048[T], *Vibrio corallilyticus* LMG 20984[T] and *Vibrio neptunius* DSM 17183[T]). Bacteria were cultured in marine agar (MA) or marine broth (MB) (Pronadisa, Lab Conda) for at least 24 h at 25 °C.

Phenotypic characterization was carried out as previously described (Prado *et al.*, 2005; Prado 2006). Additional analyses were performed with miniaturized systems. The API 20E, API 20NE and API ZYM systems (bioMérieux) and Biolog GN2 Microplates (Biolog) were used following the manufacturers' instructions, except that inocula were prepared in saline solution (0.85 %, w/v, NaCl). For the API 50CH system (bioMérieux), used to evaluate acid production under anaerobiosis, the inocula were prepared in a modified ZOF-medium (Prado, 2006). Type strains of the closest relatives were included in the study with this miniaturized system.

The three strains (PP-203[T], PP-200 and PP-204) were Gram-stain-negative, oxidase- and catalase-positive motile rods. They were fermentative in OF and ZOF (Lemos *et al.*, 1985) media with glucose, susceptible to vibriostatic agents and grew in thiosulfate-citrate-bile-sucrose TCBS medium (Oxoid), forming green colonies.

All strains were positive for acid production from D-glucose and for amylase, gelatinase and lipase (Tween 80) activities. They were negative for gas production from glucose, arginine-dihydrolase (Thornley, Baumann & Baumann 1981, and Moeller's media, Decarboxylase medium base, Difco), lysine and ornithine decarboxylases, hydrolysis of aesculin, H_2S production, urease, indole production and nitrate reduction. They grew in media containing between 1.5 and 6.0 % (w/v) NaCl, at between 15 and 25 °C and at pH 5.6–9.3, but not with 0.5 or 8.0 % (w/v) NaCl, at 8 °C or at 35 °C.

The strains were susceptible to amikacin (30 µg), erythromycin (5 µg), tetracyclin (30 µg), oxytetracycline (30 µg), chloramphenicol (30 µg), enrofloxacin (5 µg), norfloxacin (10 µg) and sulphamethoxazole-trimethoprim (25 µg), and they were resistant to penicillin G (10 units), amoxicillin (25 µg), ampicillin (10 µg), ticarcillin (75 µg), cephalexin (30 µg), cephalotin (30 µg) and clindamycin (2 µg).

The phenotypic characters that differentiated *V. ostreicida* sp. nov. from closely related species of the genus *Vibrio* are listed in Table 1. The features found to vary among the isolates of *V. ostreicida* are included in Table S1 (available in the online Supplementary Material).

Fatty acid methyl esters were prepared, separated and identified by the Sherlock Microbial Identification System (MIDI), as described by Sasser (1990), using 24 h cultures from TSA (trypticase soy agar; Pronadisa) supplemented with 0.5 % (w/v) NaCl incubated at 24 ± 1 °C. The fatty acid profiles of the three novel isolates were very similar. The major fatty acids in *V. ostreicida* sp. nov. were, in descending order (mean percentage of the three strains analysed; maximum, minimum of the total fatty acid content), as follows: summed feature 3, $C_{16:1}\omega7c/C_{16:1}\omega6c$ (33.6; 34.9, 31.7), $C_{16:0}$ (21.4; 22.2, 20.6), $C_{18:1}\omega7c$ (15.1; 15.8, 14.2), $C_{14:0}$ (6.1; 6.2, 6.0), $C_{12:0}$ 3-OH (2.8; 3.0, 2.7), $C_{12:0}$ (2.6; 3.0, 2.5), $C_{17:0}$ (2.5; 3.0, 1.9), summed feature 2, $C_{14:0}$ 3-OH / $C_{16:1}$ iso I (2.3; 2.3, 2.3) and $C_{17:0}$ iso (1.7;

Table 1. Features useful in differentiating *Vibrio ostreicida* sp. nov. from closely related species of the genus *Vibrio*

Taxa 1, *Vibrio ostreicida* sp. nov. (*n*=3); 2, *V. pectenicida* DSM 19585[T]; 3, *V. aestuarianus* ATCC 35048[T]; 4, *V. coralliilyticus* LMG 20984[T]; 5, *V. neptunius* DMS 17183[T]. + Positive; −, negative; TR: trace (<1.0 %); ND: not detected. All data were obtained in this study except where indicated otherwise. All strains are Gram-stain-negative motile rods. They are positive for oxidase, catalase, gelatinase, amylase, lipase, susceptibility to vibriostatic agent, fermentation of glucose and growth with 3 % (w/v) NaCl or at 15–25 °C. The following tests are negative: lysine and ornithine decarboxylases, luminescence, growth with 0 % or 10 % (w/v) NaCl. All the strains produce acid from D-glucose, *N*-acetyl glucosamine, maltose, trehalose, starch and glycogen. None of them produce acid from erythritol, D- and L-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, inulin, melezitose, raffinose, xylitol, D-lyxose, D-tagatose, D- and L-fucose, L-arabitol, potassium 2-ketogluconate or potassium 5-ketogluconate.

Characteristic	1	2	3	4	5
TCBS*	+ (g)	−	+ (y)	+ (y)	+ (y)
Arginine dihydrolase (Moeller)	−	−	+	+	+
Nitrate reduction	−	+	+	+	+
Indole production	−	−	+	−	−
Citrate (Simmon's)	−	−	+	−	−
ONPG (β-galactosidase)	−	−	+	+	−
Swarming	+	+	−	−	−
Growth with/at:					
0.5 % (w/v) NaCl	−	−	+	−	−
4 °C	−	+	+	−	−
37 °C	−	−	+	+	+
Acid from:					
D-Ribose	+	−	+	+	+
D-Fructose	+	−	+	+	+
D-Mannose	+	−	+	+	+
D-Mannitol	+	−	+	+	−
Melibiose	−	−	+	−	−
DNA G+C content (mol%)	53.0	41.0[a]†	43.2[b]	45.6[c]	46.0[d]
Fatty acid content (%)					
Summed $C_{16:1}\omega7c/$ $C_{16:1}\omega6c$	33.6	43.5	43.1	37.3	38.8
$C_{16:0}$	21.1	28.3	23.5	16.7	15.5
$C_{18:1}\omega7c$	15.1	6.4	15.7	20.5	16.3
$C_{17:1}\omega8c$	2.9	1.0	TR	TR	2.8
$C_{17:0}$ iso	1.7	ND.	ND	1.9	3.5

*(y), Yellow colonies; (g) green colonies.
†Data from: *a*, Lambert *et al.* (1998); *b*, Tison & Seidler (1983); *c*, Ben-Haim *et al.* (2003); *d*, Thompson *et al.* (2003a).

2.1, 1.1) (the complete profile is shown in Table S2). In contrast to *V. pectenicida* DSM 19585[T], *V. ostreicida* contained the fatty acid $C_{17:0}$ iso. Furthermore, the percentages of the fatty acids $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{16:0}$

and $C_{14:0}$ were lower than those of *V. pectenicida* DSM 19585[T], while the quantity of fatty acid $C_{18:1}\omega7c$ was higher.

The MALDI-TOF profiles were obtained at CECT following the method proposed by Maier *et al.* (2006). The species limit value considered was 2300. MALDI-TOF analysis of strains PP-203[T] and PP-200 confirmed their distinctiveness as the same species (score 2853) and their distance from the closest species in the database, *V. pectenicida* DSM 19585[T] (score 1256).

DNA was extracted and purified, with an Instagene kit (Bio-Rad), from pure isolates, which were cultured for 24 h at 24 ± 1 °C on MA. A fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene (27F and 1510R) (Lane, 1991). The genes encoding the RNA polymerase alpha subunit gene (*rpoA*), RNA recombinase alpha subunit gene (*recA*), uridine monophosphate kinase (*pyrH*), gyrase beta subunit (*gyrB*) and a cell-division protein (*ftsZ*) were used for MLSA (Sawabe *et al.*, 2007). PCR conditions were as previously described (Yamamoto & Harayama 1995; Thompson *et al.*, 2005, 2007a), although the annealing temperature was adjusted in a few cases to provide specific amplification. These genes were amplified using Ready To Go PCR-beads (Amersham Pharmacia Biotech). The sequences obtained were analysed with the DNASTAR Lasergene Seqman program. Comparative analysis was carried out using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) and BLAST (Altschul *et al.*, 1997). Sequence data of the most closely related species of the genus *Vibrio* were obtained from GenBank and from the online electronic taxonomic scheme for vibrios (http://www.taxvibrio.lncc.br) (Table S3). The MEGA package, version 5.05 (Tamura *et al.*, 2011), was used to align and analyse the sequences. The neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods were used. The statistical reliability of the tree topologies was established by bootstrapping analysis based on 1000 tree replicates.

Analysis of the 16S rRNA gene sequences classified the three strains in the genus *Vibrio* and identified the strains as being very closely related to each other ($\geqslant99.9$ % similarity). The type strain *V. pectenicida* A365[T] was the closest relative (97.9 %). The topologies of the phylogenetic trees reconstructed by the NJ, MP and ML methods (Fig. 1) supported, with high bootstrap values, the notion that strains formed a stable clade, with *V. pectenicida* as the closest phylogenetic neighbour.

Partial sequences of the housekeeping genes *gyrB* (1143-1163 bp), *rpoA* (923 bp), *recA* (820–825 bp), *pyrH* (558–597 bp) and *ftsZ* (573–578 bp) were determined, and the results confirmed the group formed by the three isolates, with similarities higher than 98.3 % and $\geqslant99$ % bootstrap support in all the NJ, MP and ML phylogenetic trees reconstructed individually for each of the genes evaluated.

The species in the *Coralliilyticus* clade (Sawabe *et al.*, 2007), *V. neptunius* LMG 20536[T] and *V. coralliilyticus* LMG

20984[T], were the closest relatives, on the basis of *rpoA*, *recA* and *pyrH* partial sequences, although with similarities $\leqslant93.8$, 84.2 and 86.1 %, respectively. In these three cases, the highest similarities were lower than the species limits established for the different genes: 98 % for *rpoA*, 94 % for *recA* and *pyrH* (Thompson *et al.*, 2005). The position of *V. ostreicida* sp. nov. in the phylogenetic trees reconstructed by all three methods was similar, supporting the grouping of the three strains. The NJ trees are shown in Figs S1 (*rpoA*), S2 (*recA*) and S3 (*pyrH*).

Vibrio orientalis was the nearest species according to *gyrB* gene sequence similarity (80.7 %), as observed in the NJ (Fig. S4), MP and ML trees. Only the sequence of the *ftsZ* gene showed highest similarity with that of *V. pectenicida* LMG 19642[T] (86.2 %) (Fig. S5).

Phylogenetic trees based on concatenated sequences (4747 bp) of the 16S rRNA (1332 bp), *gyrB* (764 bp), *rpoA* (895 bp), *recA* (729 bp), *pyrH* (533 bp) and *ftsZ* (494 bp) genes were reconstructed using the NJ, MP and ML methods (Fig. 2). In all cases, strains PP-203[T], PP-200 and PP-204 formed a stable clade, supporting the view that they represented a novel species in the genus *Vibrio*. The novel isolates clustered with the species *V. pectenicida*, close to the *Coralliilyticus* clade, with high bootstrap values. Interestingly, members of all these species have been reported to be larval pathogens in hatcheries globally and responsible for the mortality of different bivalves, including oysters, scallops and mussels (Jeffries, 1982; Lambert *et al.*, 1998; Prado *et al.*, 2005; Kesarcodi-Watson *et al.*, 2009). Thompson *et al.* (2009) suggested that species of the genus *Vibrio* share more than 95 % similarity among MLSA datasets. Distance matrices for the MLSA combined sequence dataset (data not shown) provided additional evidence that supported the clustering of PP-203[T], PP-200 and PP-204 (similarity $\geqslant99.9$ %) and their substantial genetic divergence from other closely related species of the genus *Vibrio* (highest similarity 88.5–88.0 %, with the species *V. neptunius*, *V. coralliilyticus*, *V. orientalis*, *Vibrio ichthyoenteri* and *V. pectenicida*).

DDH values were determined for type strain PP-203[T] and the other *V. ostreicida* sp. nov. isolates, PP-200 and PP-204, and the reference type strains *V. pectenicida* DSM 19585[T] and *V. aestuarianus* ATCC 35048[T]. DDH experiments were performed by the hydroxyapatite method, in microtitre plates (Ziemke *et al.*, 1998) at a hybridization temperature (T_m) of 60 °C. DNA–DNA relatedness values of PP-203[T] were 86.3 and 85.0 % with PP-200 and PP-204, respectively, but 58.6 % with *V. pectenicida* DSM 19585[T] and 66.2 % with *V. aestuarianus* ATCC 35048[T]. High values of DNA–DNA relatedness ($\geqslant65$ %) between different species of the genus *Vibrio* have been reported: 66 % for *V. neptunius*–*V. coralliilyticus*, *Vibrio fortis*–*Vibrio pelagius*, *Vibrio hepatarius*–*V. orientalis* and *Vibrio rotiferianus*–*Vibrio harveyi* (Thompson *et al.*, 2003a, b; Gómez-Gil *et al.*, 2003), 67 % for *Vibrio mimicus*–*Vibrio cholerae* (Davis *et al.*, 1981), 68 % for *Vibrio plantisponsor*–*Vibrio diazotrophicus*
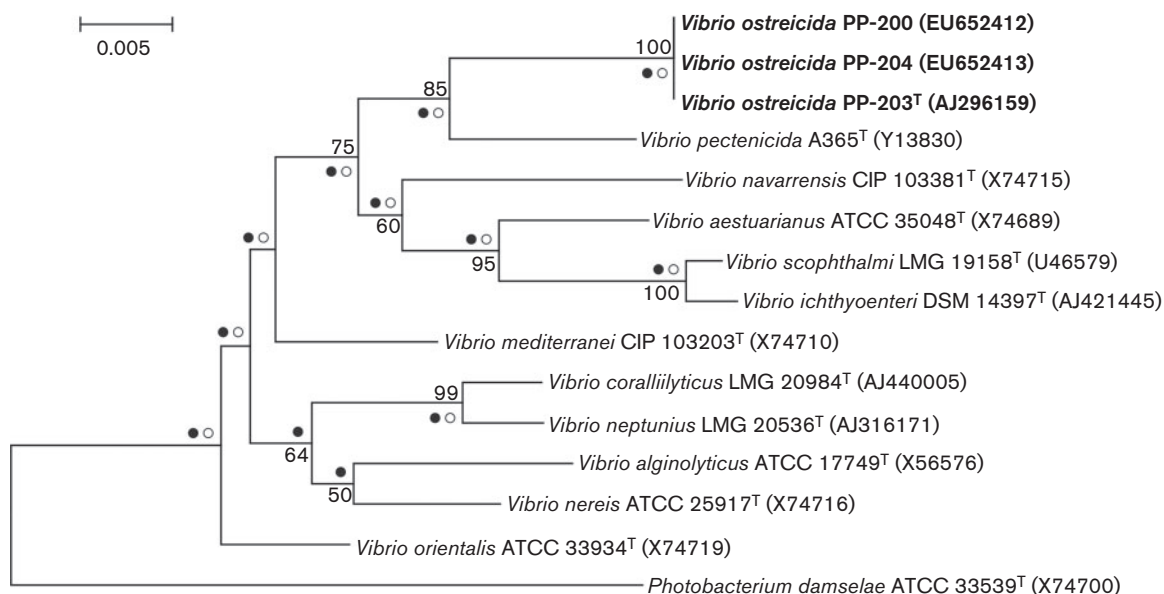
Fig. 1. Phylogenetic tree based on almost complete 16S rRNA gene sequences reconstructed by the neighbour-joining method. *Photobacterium damselae* was used as the outgroup. GenBank sequence accession numbers are given in parentheses. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap values ($\geqslant 50\%$) from 1000 replicates appear next to the corresponding branch. Bar, 0.005 substitutions per nucleotide position. Circles indicate that corresponding nodes were recovered in trees generated with the ML method (filled circles) and MP method (open circles).
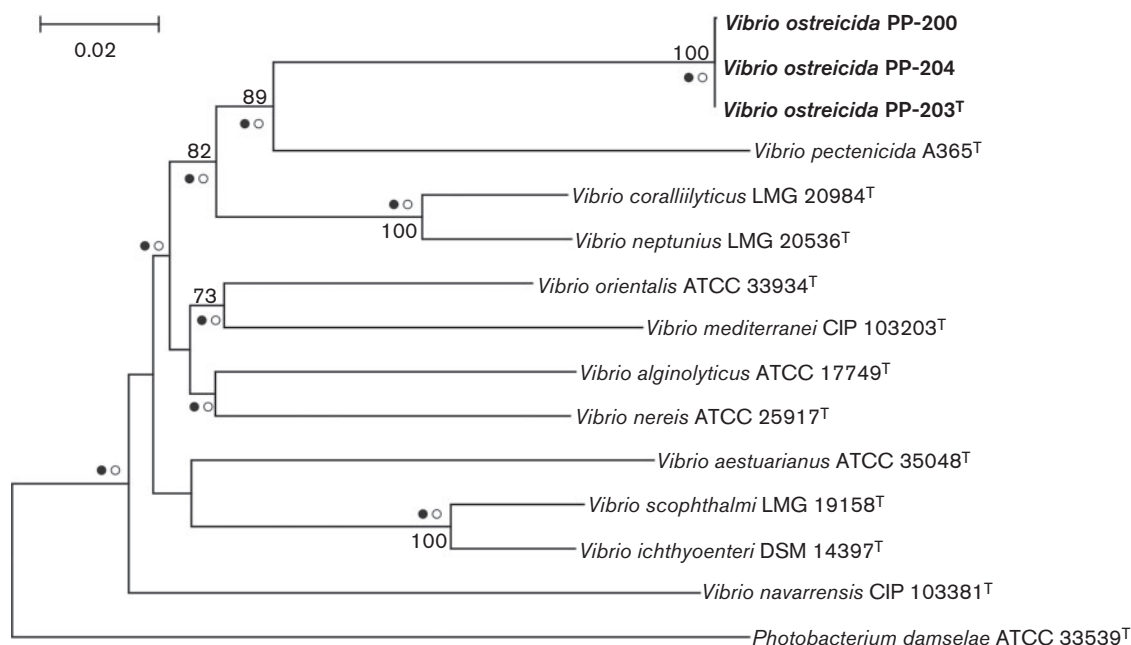

Fig. 2. Neighbour-joining tree showing the phylogenetic position of *Vibrio ostreicida* sp. nov., based on concatenated sequences of the 16S rRNA gene and the five housekeeping genes *rpoA*, *recA*, *pyrH*, *gyrB* and *ftsZ* (total length 4747 bp). *Photobacterium damselae* was used as the outgroup. Bootstrap values ($\geqslant 50\%$) based on 1000 replicates are listed as percentages at branching points. Bar, 0.02 substitutions per nucleotide position. Circles indicate that corresponding nodes were recovered in trees generated with the ML method (filled circles) and MP method (open circles).

(Rameshkumar *et al.*, 2011), and up to 74 % for *Vibrio harveyi–Vibrio campbellii* (Thompson *et al.*, 2003c). Moreover, Thompson *et al.* (2005) suggested 80 % DNA–DNA relatedness as the limit for the definition of a species for *Vibrionaceae* taxonomy, rather than the 70 % proposed by Wayne *et al.* (1987).

The $G+C$ content of genomic DNA was determined from the mid-point (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979) as described previously (Ventosa *et al.*, 2004). The DNA $G+C$ content of strain PP-203[T] was 53.0 mol%. This value is within the range for the genus *Vibrio*, but is higher than those of the closest relatives (Table 1).

The characteristics of strains PP-203[T], PP-200 and PP-204 are provided in the species description or are shown in Table 1 and Tables S1 and S2. The genotypic analyses showed that these strains form a stable group closely related to *V. pectenicida*, but which can be differentiated from this and the other closely related species according to their phenotypic and genotypic traits. In summary, on the basis of the data from polyphasic analysis, including MLSA, DDH, chemotaxonomic techniques, MALDI-TOF-MS and phenotypic tests described in this paper, it is concluded that strains PP-203[T], PP-200 and PP-204 represent a novel species that belongs to the genus *Vibrio* for which the name *Vibrio ostreicida* sp. nov. is proposed.

Description of *Vibrio ostreicida* sp. nov.

Vibrio ostreicida [os.tre.i.ci′da. N.L. n. *Ostrea* (from L. n. *ostrea* an oyster) genus name of oysters; L. suff. -*cida* (from L. v. *caedo* to cut or kill) murderer, killer; N.L. n. *ostreicida* oyster-killer].

Gram-stain-negative, oxidase- and catalase-positive motile rods. Unpigmented colonies develop within 24 h on MA, with swarming. They are fermentative in ZOF-glucose, susceptible to vibriostatic agents and grow in TCBS (sucrose-negative). Positive for acid production from glucose and activities of amylase, gelatinase and lipase, and show optimal growth with 3–6 % (w/v) NaCl and at 15–25 °C. Negative for gas production from glucose, arginine dihydrolase (Thornley and Moeller's media), lysine and ornithine decarboxylases, indole production, nitrate reduction, Simmons' citrate, aesculin hydrolysis, H_2S production, urease and growth without NaCl, with 8 % (w/v) NaCl, at 8 °C or at 35 °C. The following enzyme activities are observed: alkaline phosphatase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. Produce acid from D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, trehalose, starch and glycogen. Does not produce acid from erythritol, D- and L-arabinose, D- and L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin/ferric citrate, salicin, cellobiose, melibiose,

sucrose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Utilizes dextrin, glycogen, Tween 40, *N*-acetyl-D-glucosamine, D-fructose, α-D-glucose, maltose, D-mannitol, D-mannose, trehalose, acetic acid, succinic acid, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, D-serine, L-serine, L-threonine, inosine, uridine, glycerol and D-glucose 6-phosphate. The major cellular fatty acids are $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{16:0}$, $C_{18:1}\omega7c$, $C_{14:0}$, $C_{12:0}$, $C_{12:0}$ 3-OH, $C_{17:0}$, $C_{14:0}$ 3-OH/$C_{16:1}$ iso I and $C_{17:0}$ iso.

The type strain, PP-203[T] (=CECT 7398[T]=DSM 21433[T]), was isolated from the inner surface of containers in a mollusc hatchery and were pathogenic to larval cultures of flat oyster. The DNA $G+C$ content of the type strain is 53.0 mol%. The type strain produces acid from glycerol and D-arabitol, and utilizes D-psicose and α-ketobutyric acid.

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