Vibrio pacinii sp. nov., from cultured aquatic organisms

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Three strains were isolated from cultured aquatic organisms. They were Gram-negative, oxidase-positive, motile, fermentative, arginine dihydrolase-positive, lysine and ornithine decarboxylase-negative and sensitive to vibriostatic agent O/129. These strains differ from other related *Vibrio* species by several phenotypic features, which include acetoin and indole production and utilization of amygdalin and D-mannitol. Comparison of 16S rDNA sequences showed a close relationship to the recently described species *Vibrio kanaloae* (96.6 %) and *Vibrio pomeroyi* (96.4 %) and to *Vibrio furnissii* (96.6 %), but DNA–DNA hybridization experiments showed that the three isolates form a tight novel species with ≤ 30 % DNA–DNA similarity to its closest phylogenetic neighbours. *Vibrio pacinii* sp. nov. is proposed, with LMG 19999^T (= CAIM 530^T = STD3-1057^T; DNA G + C content, 44.9 mol%) as the type strain.

Vibrio is an important genus in the culture of marine and estuarine animals, in that many species have been responsible for causing disease in fish (Hjeltnes & Roberts, 1993), crustaceans (Lightner, 1993) and molluscs (Austin, 1988). Some *Vibrio* species may also have a beneficial effect in host organisms, either as symbionts [e.g. of squids (McFall-Ngai, 2002)] or probionts (Gomez-Gil *et al.*, 2000). Several studies have investigated phenotypic diversity of vibrios in aquaculture environments (Vandenberghe *et al.*, 1998, 2003). Recently, genotypic techniques have also been employed to better characterize the diversity of vibrios: Thompson *et al.* (2001) characterized 506 strains by using fluorescent amplified fragment length polymorphism (FAFLP) analysis and found many strains that did not cluster with any known type strains and might thus be considered as potential novel species of the genus *Vibrio*. This analysis disclosed a group (A47) of three tightly related strains (LMG 13245, LMG 19999^T and LMG 21514). These strains were grouped apart from known type strains of *Vibrio* species, with >70 % FAFLP pattern similarity between them but only 52 % similarity to that of their closest FAFLP neighbour, strain LMG 10953 (FAFLP group A1). Phenotypic analysis of 1476 *Vibrio* isolates with the Biolog GN system clustered strains LMG 19999^T and LMG 13245 in a separate group (STD3-1057) that was related to *Vibrio logei*, and strain LMG 21514 clustered in the *Vibrio ordalii*

group (Vandenberghe *et al.*, 2003). Austin *et al.* (1995) reported that strain LMG 13245 had unique ribotype and lipopolysaccharide profiles and no plasmid content, belonged to an unknown serogroup and was pathogenic for Atlantic salmon (*Salmo salar*). Phenotypic and genotypic methodologies clearly show that bacterioflora, particularly vibrios, found in aquaculture environments represent a highly diverse group with many potential novel species that are yet to be detected and described.

Strain LMG 19999^T (= CAIM 530^T = STD3-1057^T) was isolated from healthy shrimp larvae (*Penaeus chinensis*) in the Dahua hatchery in Laizhou (Shandong Province, China) during the spring of 1996 (Vandenberghe *et al.*, 1998). Strain LMG 13245 (= CAIM 526 = VIB 218) was isolated from sea bass (*Dicentrarchus labrax*) in Spain 1991, while strain LMG 21514 (= CAIM 466 = VIB 847) was isolated from Atlantic salmon (*Salmo salar*) in Tasmania during the 1990s. All strains have been deposited at the BCCM/LMG bacteria collection and at the CAIM (Collection of Aquacultural Important Microorganisms, CIAD, A. C. Mazatlán, México).

Strains were grown aerobically on tryptone soy agar (TSA; Oxoid) supplemented with 2 % (w/v) NaCl for 24 h at 28 °C unless otherwise stated. Phenotypic characterization was done with the API 20E system (bioMérieux) to determine the biochemical and nutritional properties of the strains tested, the API ZYM system (bioMérieux) for evaluation of enzymes produced by the strains and the Biolog GN2 system to test the strains' ability to utilize

Abbreviations: FAFLP, fluorescent amplified fragment length polymorphism; FAME, fatty acid methyl ester.

The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of strain LMG 19999^T is AJ316194.

different carbon sources. All systems were used according to the manufacturers' instructions, but sterile saline solution [1.5 % (w/v) NaCl] was used to prepare the inocula in every case. Additional phenotypic tests and confirmation of doubtful test results obtained by these systems were performed by following the methodologies of Lanyi (1987) and Austin & Lee (1992). Antibiotic sensitivity was estimated by the disc-diffusion test (Bauer *et al.*, 1966) on Iso-Sensitest agar (Oxoid) with 1.5 % (w/v) NaCl. GLC analysis of methylated fatty acids was performed as described by Osterhout *et al.* (1991), but the cells were grown on TSA (Difco) with 1.5 % (w/v) NaCl and incubated at 28 °C. Determination of G+C content of the DNA of strain LMG 19999^T was determined according to Mesbah *et al.* (1989) and modified as described by Logan *et al.* (2000). Strain LMG 19999^T (GenBank/EMBL accession no. AJ316194) was analysed further by sequencing the 16S rDNA as described by Thompson *et al.* (2001). Sequence similarities were obtained with the Jukes-Cantor model [gamma 0.4, pairwise deletion; standard error (SE) estimated by bootstrap method, 1000 replications and random number seed=67137] with the MEGA program (version 2.1; Kumar *et al.*, 2001). The LMG 19999^T sequence was also compared to the sequences in GenBank (BLASTN; Altschul *et al.*, 1990) and the Ribosomal Database Project (RDP; Maidak *et al.*, 1999). Sequences of the closest species and of isolate LMG 19999^T were aligned with CLUSTAL X (version 1.8; Thompson *et al.*, 1997). Tree topology (neighbour-joining; Saitou & Nei, 1987) and stability of groupings (bootstrap analysis, 1000 replicates) were performed with PHYLO_WIN software (Galtier *et al.*, 1996) with *Vibrio cholerae* as the outlier. DNA-DNA hybridization was done following the methodology described by Willems *et al.* (2001) at 39 °C.

The three isolates grew as round, yellow, bright colonies on TCBS (thiosulfate/citrate/bile/sucrose) agar; no swarming or luminescence was observed. The isolates were arginine dihydrolase-positive and lysine and ornithine decarboxylase-negative (A+, L-, O-). A+, L-, O- vibrios represent a diverse group that includes many species (Alsina & Blanch, 1994a, b). According to the identification scheme provided by Alsina & Blanch (1994b), strains LMG 19999^T, LMG 21514 and LMG 13245 would be identified as *Vibrio splendidus* II or *Vibrio furnissii*. All three isolates presented many characters that differentiated them from other A+, L-, O- vibrios, e.g. a positive Voges-Proskauer reaction (except for LMG 13245; positive only in *Vibrio anguillarum*, *Vibrio metschnikovii* and *Vibrio tapetis*); negative for indole production (also negative for *Vibrio mytili* and *V. tapetis*, variable for *V. splendidus* II); and utilization of amygdalin (also by *Vibrio diabolicus*) but not of D-mannitol (also negative in *Vibrio nereis* and *V. tapetis*) (Table 1). Fatty acid content of the strains varied considerably: 28 were detected in strain LMG 13245, 23 in LMG 19999^T and only 10 in LMG 21514 (see species description and Table 2). The fatty acid methyl ester (FAME) profiles are in good agreement with those of representatives of the

genus *Vibrio* (Bertone *et al.*, 1996), although two fatty acids were present in higher concentrations. Fatty acids useful for differentiation are shown in Table 1.

16S rDNA sequence similarities from distance matrix calculation and comparison of LMG 19999^T to sequences deposited in GenBank (BLASTN) and the Ribosomal Database Project (RDP) II indicated a close relationship to several *Vibrio* species (Fig. 1), including *V. metschnikovii* CIP 69.14^T ($d=0.028$, SE=0.005; 97.4 % BLASTN; 87.0 % RDP, 1360 uniquely occurring oligomers), *Vibrio kanaloae* LMG 20539^T ($d=0.029$, SE=0.005; 96.6 % BLASTN), *Vibrio pomeroyi* LMG 20537^T ($d=0.029$, SE=0.005; 96.4 % BLASTN), *Vibrio aestuarianus* ATCC 35048^T ($d=0.031$, SE=0.005; 97.7 % BLASTN; 87.7 % RDP, 1345 uniquely occurring oligomers), *V. tapetis* CECT 4600^T ($d=0.033$, SE=0.005; 97.3 % BLASTN; 87.2 % RDP, 1387 uniquely occurring oligomers) and *V. furnissii* ATCC 35016^T ($d=0.035$, SE=0.005; 96.6 % BLASTN; 87.2 % RDP, 1453 uniquely occurring oligomers). 16S rDNA sequence distance within the *Vibrio* genus for the isolates was 0.028–0.091.

DNA-DNA hybridization experiments revealed high similarity between pairs LMG 19999^T and LMG 21514 (79 %), LMG 19999^T and LMG 13245 (92 %) and LMG 21514 and LMG 13245 (85 %). Similarity of strain LMG 19999^T to its closest phylogenetic neighbours was, in decreasing order, 30 % (28 % reciprocal value) with *V. aestuarianus* LMG 7909^T, 28 % (24 % reciprocal value) with *V. kanaloae* LMG 20539^T, 28 % (17 % reciprocal value) with *V. pomeroyi* LMG 20537^T, 21 % with *V. furnissii* LMG 7910^T, 18 % (21.8 % reciprocal value) with *V. metschnikovii* LMG 11664^T and 16 % (17 % reciprocal value) with *V. tapetis* LMG 19706^T.

Collectively, phenotypic characterization, 16S rDNA sequences, DNA-DNA similarity and FAFLP fingerprinting (Thompson *et al.*, 2001) provide solid evidence to support the proposal of strains LMG 19999^T, LMG 21514 and LMG 13245 as members of a novel species of the genus *Vibrio*, for which the name *Vibrio pacinii* is proposed.

Description of *Vibrio pacinii* sp. nov.

Vibrio pacinii (pa.ci'ni.i. N.L. gen. n. *pacinii* of Pacini, named after the Italian anatomist Filippo Pacini, who first discovered the causal agent of cholera).

Gram-negative rods, motile, with polar flagella. Non-luminescent, non-pigmented, translucent colonies on marine agar with no swarming. Round, firm, yellow bright colonies on TCBS agar, 1.5–2.8 mm in diameter. Grows in the presence of 1.5, 2.5, 6.0 and 8.0 % NaCl but not at 0, 10.0 or 12.0 %; can grow at 4, 30 and 35 °C but not at 40 °C in TSB. Oxidase- and catalase-positive, ferments glucose and lactose; arginine and L-tyrosine dihydrolase-positive, lysine and ornithine decarboxylase-negative. Positive for Voges-Proskauer test (except for strain LMG 13245), methyl red, tryptophan deaminase and nitrate

Table 1. Phenotypic characteristics that differentiate *Vibrio pacinii* sp. nov. from its closest neighbours and from other arginine dihydrolase-positive, lysine and ornithine decarboxylase-negative *Vibrio* species

Taxa: 1, *V. pacinii*; 2, *V. kanaloae*; 3, *V. pomeroyi*; 4, *V. aestuarianus*; 5, *V. anguillarum*; 6, *V. diabolicus*; 7, *Vibrio diazotrophicus*; 8, *Vibrio fluvialis*; 9, *V. furnissii*; 10, *Vibrio mediterranei*; 11, *V. metschnikovii*; 12, *V. mytili*; 13, *V. nereis*; 14, *Vibrio orientalis*; 15, *V. splendidus* I; 16, *V. splendidus* II; 17, *V. tapetis*; 18, *V. tubiashii*. Data were obtained from Pujalte *et al.* (1993), Raguénès *et al.* (1997), Novoa *et al.* (1998), Alsina & Blanch (1994a, b), Farmer (1992), Thompson *et al.* (2003) and Baumann & Schubert (1983). +, Positive; –, negative; v, variable; ND, no data available.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Arginine dihydrolase	+	+	+	+	+	–	v	+	+	v	v	+	+	v	v	v	–	v
Lysine decarboxylase	–	–	–	v	–	+	–	–	–	v	v	–	–	+	–	–	–	–
Ornithine decarboxylase	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	ND	+
Resistance to O/129 (10 µg)	–	–	–	–	+	ND	v	+	+	–	–	ND	v	–	–	v	–	–
Indole	–	+	+	+	v	+	+	v	v	+	+	–	v	+	+	+	–	+
Voges-Proskauer	v	+	v	–	+	+	–	–	–	–	+	–	–	–	–	–	+	–
ONPG	v	–	+	+	+	–	+	v	+	+	+	+	–	+	v	v	+	+
Citrate	+	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+
Gelatinase	v	+	+	+	+	+	–	+	v	–	+	–	v	+	+	+	+	+
Urease	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	v
Swarming	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–
Luminescence	–	–	–	–	–	–	ND	–	–	–	–	–	–	+	–	–	ND	–
Growth in/at:																		
6 % NaCl	+	+	+	+	+	+	+	+	+	+	v	+	+	+	+	v	–	+
8 % NaCl	+	v	+	v	–	ND	+	v	+	v	v	+	+	+	v	v	–	v
10 % NaCl	–	–	–	–	+	ND	v	v	+	–	v	+	v	–	–	–	–	–
4 °C	+	+	+	+	–	+	v	–	–	–	–	–	v	+	v	–	+	–
35 °C	v	–	–	+	+	+	+	+	+	v	+	+	+	+	v	–	–	v
40 °C	–	–	–	–	–	+	v	+	+	–	+	–	v	–	–	–	ND	–
Utilization of:																		
D-Mannitol	–	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+
D-Mannose	+	+	+	+	+	+	–	+	v	+	+	–	–	+	+	–	–	+
myo-Inositol	–	–	–	–	v	–	–	–	–	+	+	–	–	–	–	–	–	–
D-Sorbitol	–	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	–	–	+
Amygdalin	+	–	–	ND	–	+	–	v	–	ND	–	ND	–	ND	–	–	–	–
L-Arabinose	–	+	–	+	+	–	+	+	+	–	–	+	v	–	–	–	–	–
FAME composition:																		
C _{16:0}	18.7	25.6	29.2	21.0	22.0	14.4	24.0	16.0	16.5	17.4	20.0	18.8	12.9	27.6	29.6	ND	1.5	17.3
iso-C _{16:0}	9.0	0.0	0.0	2.7	3.0	1.7	1.9	4.9	1.0	1.9	1.4	1.5	1.1	0.0	0.0	ND	0.0	0.0

reduction; negative for indole production, H₂S and urease. Test for citrate utilization is weakly positive, except for strain LMG 13245, which is strongly positive. Positive activity of acid phosphatase (LMG 13245 is weak), α -glucosidase (LMG 21514 is weak), alkaline phosphatase, esterase (C4), esterase lipase (C8) (LMG 13245 is weak), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase (LMG 13245 and LMG 21514 are weak). Negative activity of α -chymotrypsin (LMG 19999^T is weakly positive), α -fucosidase, α -galactosidase, α -mannosidase, β -glucosidase, β -glucuronidase, lipase (C14), *N*-acetyl- β -glucosaminidase and trypsin. All strains utilize α -D-glucose, cellobiose, dextrin, D-fructose, D-mannitol, D-trehalose, gentiobiose, inosine, L-asparagine,

L-glutamic acid, L-serine, maltose, *N*-acetyl-D-glucosamine, sucrose, thymidine and uridine as sole sources of carbon. None of the strains utilized 2,3-butanediol, 2-aminoethanol, acetic acid, adonitol, alaninamide, α -D-lactose lactulose, α -hydroxybutyric acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, α -lactose, β -hydroxybutyric acid, bromosuccinic acid, *cis*-aconitic acid, citric acid, DL- α -glycerol phosphate, DL-carnitine, D-alanine, D-arabitol, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D-mannose, D-melibiose, D-raffinose, D-saccharic acid, D-serine, formic acid, γ -aminobutyric acid, γ -hydroxybutyric acid, glucose 1-phosphate, glucose 6-phosphate, glucuronamide, glycerol, glycyl L-glutamic acid, hydroxy

Table 2. Phenotypic differences between strains of *V. pacinii* sp. nov.

Strains: 1, LMG 19999^T; 2, LMG 21514; 3, LMG 13245. +, Positive; –, negative; W, weak result; S, sensitive; I, intermediate; R, resistant; ND, not detected.

Test	1	2	3
Voges–Proskauer reaction	+	+	–
Utilization of:			
α-Cyclodextrin	–	–	+
Citrate	W	W	+
Methyl β-D-glucoside	+	–	–
DL-Lactic acid	+	–	+
D-Galactose	+	–	+
D-Sorbitol	–	–	+
Glycogen	–	–	+
Glycyl L-aspartic acid	W	+	–
L-Alanine	+	W	+
L-Alanylglycine	+	W	+
L-Aspartic acid	+	+	–
Methyl pyruvate	+	–	–
Monomethyl succinate	–	+	–
Psicose	+	–	+
Succinic acid	+	+	–
Activity of:			
β-Galactosidase	+	–	+
Cystine arylamidase	+	W	–
Fatty acid content (%):			
iso-C _{16:0}	8·41	ND	9·69
iso-C _{14:0} 3-OH	2·71	ND	1·08
C _{16:0}	14·07	25·55	16·62
Susceptibility to:			
Ampicillin (30 µg)	S	S	R
Amikacin (30 µg)	I	S	R

L-proline, i-erythritol, itaconic acid, L-arabinose, L-fucose, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyrogutamic acid, L-rhamnose, L-threonine, malonic acid, *m*-inositol, *N*-acetyl-D-galactosamine, phenylethylamine, *p*-hydroxyphenylacetic acid, propionic acid, putrescine, quinic acid, sebacic acid, succinamic acid, turanose, Tween 40, Tween 80, urocanic acid or xylitol. The following cellular fatty acids are present in decreasing order (mean percentage of the three strains analysed, minimum and maximum of total fatty acid content): C_{16:0} (18·7 %, 14·1–25·5), C_{18:1ω7c} (11·1 %, 9·9–13·4), iso-C_{16:0} (9·0 %, 8·4–9·7, not detected in LMG 21514), C_{14:0} (6·9 %, 4·7–11·2), C_{12:0} (3·7 %, 1·9–5·2), C_{12:0} 3-OH (3·1 %, 1·5–4·9), iso-C_{14:0} 3-OH (1·9 %, 1·1–2·7, not detected in LMG 21514), iso-C_{17:0} (1·4 %, 0·9–2·0, not detected in LMG 21514), C_{16:1ω7c} and/or iso-C_{15:0} 2-OH (summed feature 3) (38·7 %, 37·8–39·2) and C_{14:0} 3-OH and/or iso-C_{16:1} I (summed feature 2) (2·9 %, 1·5–3·6). Eighteen other fatty acids are present in percentages below 1·0, these are: iso-C_{18:0}, iso-C_{14:0}, C_{16:1ω7c} alcohol, iso-C_{13:0}, iso-C_{15:0}, anteiso-C_{17:0}, C_{18:0}, C_{15:0}, C_{17:0}, 11-methyl C_{18:1ω7c},

Fig. 1. Consensus phylogenetic dendrogram of strain LMG 19999^T (=CAIM 530^T) and the closest *Vibrio* species, derived from almost-complete 16S rDNA sequence data. Tree topology was obtained by neighbour-joining (0·4 γ-correction, pairwise deletion, Jukes–Cantor correction). Numbers at nodes indicate level of bootstrap support (1000 replicates). Bar, 1 % sequence divergence.

C_{17:1ω8c}, iso-C_{13:0} 3-OH, iso-C_{15:0} 3-OH, iso-C_{12:0}, C_{12:0} 2-OH, anteiso-C_{15:0}, C_{15:1ω8c} and C_{16:1ω5c}. Antibiotic susceptibility was observed to chloramphenicol (30 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), polymyxin B (300 U), tetracycline (30 µg); resistant to gentamicin (10 µg) (except for LMG 21514, which is intermediate), kanamycin (30 µg), streptomycin (25 µg) and vibriostatic agent O/129 at 10 and 150 µg. Additional phenotypic features are listed in Table 2. G + C content of the DNA is 44·9 mol%.

The type strain, LMG 19999^T (=CAIM 530^T), was isolated from shrimp larvae (*Penaeus chinensis*) in the Dahua hatchery in Laizhou (Shandong Province, China). Reference strains are LMG 13245 (=CAIM 526) and LMG 21514 (=CAIM 466).

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