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Note

Vibrio rotiferianus sp. nov., isolated from cultures of the rotifer *Brachionus plicatilis*

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Five Gram-negative bacterial strains, oxidase-positive, motile by means of more than one polar flagella, facultative anaerobe, arginine dihydrolase-negative, lysine- and ornithine decarboxylase-positive, sensitive to the vibriostatic agent O/129, were isolated from a flow-through rotifer culture system in Gent, Belgium, and previously characterized by fluorescent amplified fragment length polymorphism. Comparison of the 16S rDNA sequence of strain LMG 21460^T indicated close relationships (~99 % similarity) to *Vibrio campbellii*, *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. However, DNA hybridization experiments revealed similarity values below 70 % with its closest species *V. campbellii* and *V. harveyi*. Additionally, the analysed strains differ from related *Vibrio* species by the utilization of melibiose and production of acid from L-arabinose and amygdalin. Among the strains analysed, differences were observed in some phenotypic characters, particularly susceptibility to ampicillin, polymyxin B and amikacin, and urease activity. The major fatty acids identified were 16 : 0, 18 : 1ω7*c*, 14 : 0, 12 : 0 3-OH and 18 : 0. *Vibrio rotiferianus* sp. nov. is proposed, with type strain LMG 21460^T (=CAIM 577^T); it has a DNA G+C content of 44·5 ± 0·01 mol%.

Rotifers are an important nutritional source for the culture of many aquatic organisms' larvae, especially fish and crustaceans. Bacteria present in rotifer cultures can reach high numbers and are transmitted to the target larvae with the rotifers at feeding (Munro *et al.*, 1994), and thus may cause poor survival and growth of the fish larvae (Gatesoupe, 1989). Other bacteria may enhance the growth of rotifers (Douillet, 2000) and of fish larvae (Skjermo & Vadstein, 1999). The principal genera identified in rotifer cultures have been *Pseudomonas*, *Vibrio*, *Moraxella* and *Flavobacterium* (Verdonck *et al.*, 1994, 1997). *Vibrio* was the dominant genus in rotifer cultures, constituting up to 56 % of the bacterial community, with *Vibrio anguillarum*, *Vibrio alginolyticus*, *Vibrio diazotrophicus*, *Vibrio mediterranei* and *Vibrio tubiashii*-like as representative species (Verdonck *et al.*, 1997). Understanding the bacterial composition of rotifers and rotifer cultures is important for the aquaculture industry.

Several bacteria were isolated from rotifers and from the water of a flow-through rotifer system during August 1999 at the Artemia Reference Centre, University of Ghent, Belgium. The rotifer rearing system and bacterial isolation procedures have been described by Suantika *et al.* (2001). Samples of rotifer culture water and from rotifers + water were homogenized and serially diluted in sterile saline solution (SSS; 1·5 % NaCl, w/v), plated onto marine agar (Difco) and thiosulphate-citrate-bile salts-sucrose agar (TCBS; Difco), and incubated for 24–48 h at 25 °C. Five isolates (LMG 21456, LMG 21457, LMG 21458, LMG 21459 and LMG 21460^T) were analysed by Thompson *et al.* (2001) by fluorescent amplified fragment length polymorphism (FAFLP) and 16S rDNA sequencing. They showed (1) that these strains formed a tight cluster, and (2) that no known *Vibrio* type species grouped into this cluster. Therefore, all five isolates were considered as potentially novel species of *Vibrio*.

The five strains were phenotypically analysed by API 20E and API ZYM (bioMérieux) and Biolog GN2 according to the manufacturers' instructions, except that SSS was used to prepare the inocula. Other phenotypic tests were performed following the methodologies of Lanyi (1987). Presence of flagella was determined with Gray's stain (Murray *et al.*, 1994). Antibiotic sensitivity was estimated by the disk diffusion test (Bauer *et al.*, 1966) in Iso-sensitest agar (Oxoid) + 1·5 % NaCl (w/v). Fatty acid analysis was

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Abbreviation: FAFLP, fluorescent amplified fragment length polymorphism.

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Vibrio rotiferianus* LMG 21460^T is AJ316187.

performed as described by Osterhout *et al.* (1991), except that the cells were grown on Tryptone Soya Agar (TSA; Oxoid) + 1·5 % NaCl (w/v) and incubated at 28 °C for 24 h. The 16S rDNA sequence of strain LMG 21460^T (GenBank/EMBL accession no. AJ316187) was compared with sequences deposited in EMBL (FASTA; Pearson & Lipman, 1988) and in the Ribosomal Database Project (RDP; Maidak *et al.*, 1999) to specify the closest related species. Sequences of relevant taxa and of strain LMG 21460^T were aligned by means of CLUSTAL X version 1.8 (Thompson *et al.*, 1997). Distance estimations (Jukes & Cantor, 1969), tree topology [neighbour-joining, Saitou & Nei (1987), with 0·4 gamma correction and pairwise deletion] and stability of groupings (Bootstrap analysis, 1000 replicates) were performed with MEGA version 2.1 software (Kumar *et al.*, 2001) with *Vibrio cholerae* as outgroup. The DNA G+C content was determined as described by Mesbah *et al.* (1989) using the modifications proposed by Logan *et al.* (2000). DNA–DNA hybridization analysis was carried out at stringent conditions (39 °C) following the methodology described by Willems *et al.* (2001).

All five isolates grew well on TCBS agar as bright non-luminescent yellow colonies and unpigmented translucent colonies in marine agar. Phenotypically, the five strains can be clearly assigned to the genus *Vibrio* (Alsina & Blanch, 1994), and present many characters that clearly distinguish them from similar species (Table 1 and description). Of particular interest is the capacity to utilize melibiose, a feature only observed in *Vibrio nigripulchritudo*, *Vibrio agarivorans* and some strains of *Vibrio natriegens*, but in none of the arginine-dihydrolase-negative, lysine- and ornithine-decarboxylase-positive species. Differences were observed in the phenotypic characters among the five strains analysed (see Table 2).

Fatty acid analysis showed a distinct pattern from its closest phylogenetic neighbours, *Vibrio harveyi* and *Vibrio campbellii*. The mean percentage of the fatty acid 14 : 0 was 9·52 % (max. 10·31 %, min. 8·89 %), while in *V. harveyi* and *V. campbellii* it was 4·88 and 4·28 %, respectively; 16 : 0 was 25·40 % (max. 28·47 %, min. 21·18 %) compared to 13·94 and 17·04, respectively; and 18 : 1ω7*c* was 10·79 % (max. 12·34 %, min. 9·13 %) against 21·05 and 22·55 %, respectively. For other fatty acids, see species description, but no clear differences with the other type strains were observed. In general, the identified fatty acids of strain LMG 21460^T were in agreement with the fatty acid signature of the genus *Vibrio*; only 14 : 0 was slightly above the maximum reported for the genus (8·63 %) (Bertone *et al.*, 1996).

The 16S rDNA sequence clearly classified strain LMG 21460^T in the genus *Vibrio*. The closest phylogenetic neighbours were *V. campbellii* (99·86 % FASTA and 99·2 % RDP) and *V. harveyi* (99·11 and 96·7 %) (Fig. 1). Phylogenetic analysis with maximum-likelihood and maximum-parsimony treeing methods produced congruent results with the

Table 1. Phenotypic characters that differentiate *Vibrio rotiferianus* sp. nov. from related arginine-dihydrolase-negative, lysine- and ornithine-decarboxylase-positive (A−, L+, O+) *Vibrio* species

Strains: 1, *V. rotiferianus* (*n*=5); 2, *V. alginolyticus*; 3, *V. campbellii*; 4, *V. cholerae*; 5, *V. fischeri*; 6, *V. harveyi*; 7, *V. logei*; 8, *V. mimicus*; 9, *V. parahaemolyticus*; 10, *V. splendidus* I; 11, *V. splendidus* II; 12, *V. vulnificus*. Data for related A−, L+ and O+ *Vibrio* species were taken from Alsina & Blanch (1994) and Baumann & Schubert (1984). Percentages indicate positive results; +, positive for >90 %; (+), positive for 75–89 %; −, negative for <10 %; (−), negative for 25–11 %; V, variable for 26–74 %; ND, no data; d, discrepancies between authors.

Test	1	2	3	4	5	6	7	8	9	10	11	12
Citrate utilization*	−	+	V	V	−	v	−	+	+	d	d	+
Voges–Proskauer*	−	(+)	−	+	−	−	−	(−)	−	−	−	−
Growth in the presence of:												
0 % NaCl (w/v)*	−	−	−	+	−	−	−	+	−	−	−	−
8 % NaCl (w/v)*	−	+	V	−	−	(+)	−	−	+	v	V	−
Utilization of:												
L-Arabinose*	+	−	−	−	−	V	−	−	−	−	−	−
D-Mannitol	−	+	(+)	+	v	+	+	ND	+	+	+	d
D-Mannose	+	V	+	V	−	+	v	+	−	−	−	−
Melibiose	+	−	−	−	−	−	−	−	−	−	−	−
Acid from:												
L-Arabinose	+	−	−	−	−	−	−	−	−	−	−	−
Amygdalin	+	−	−	−	−	−	+	−	−	−	−	−
Activity of:												
α-Chymotrypsin	+	−	+	−	−	+	−	−	−	−	ND	−

*Test useful to differentiate A−, L+, O+ *Vibrio* species according to the scheme of Alsina & Blanch (1994).

Table 2. Phenotypic differences among the five strains of *Vibrio rotiferianus* sp. nov.

Strains: 1, LMG 21460^T; 2, LMG 21459; 3, LMG 21457; 4, LMG 21456; 5, LMG 21458. W, Weak reaction; R, resistant; I, intermediate; S, sensitive.

Test	1	2	3	4	5
α-Lactose	−	+	−	−	W
DL-Lactic acid	W	W	+	W	W
Bromosuccinic acid	−	+	W	W	−
L-Glutamic acid	−	+	W	W	−
L-Threonine	−	+	−	W	W
Urease	+	+	+	+	−
Susceptibility to:					
Ampicillin (30 μg)	R	I	S	R	I
Polymyxin B (300 U)	R	R	I	R	R
Amikacin (30 μg)	I	R	R	R	R

neighbour-joining method regarding the positioning of the type strain LMG 21460^T. Strain LMG 21460^T clustered within the group of *Vibrio* species called the *V. harveyi* group (Reichelt *et al.*, 1976), and later called the core group of the *Vibrio* genus (Dorsch *et al.*, 1992). This group has had little taxonomic change over time; the last species described was *Vibrio vulnificus* (Farmer, 1980).

The DNA G+C content determined was 44·5 ± 0·01 mol% (n=3); this value is within the range of values reported for *Vibrio* (Baumann & Schubert, 1983). Strain LMG 21460^T was hybridized with its two closest neighbours (by 16S rDNA) *V. campbellii* (LMG 11216^T) and *V. harveyi* (LMG 4044^T) showing 65 and 66 % reassociation, respectively. The DNA reassociation between *V. campbellii* and *V. harveyi* was 69 %, a similar result to the 65 % obtained by Reichelt *et al.* (1976).

These results clearly showed that strain LMG 21460^T is closely related to *V. campbellii* and *V. harveyi*, but it can be differentiated from these taxa by means of FAFLP (Thompson *et al.*, 2001), DNA–DNA similarity, as well as by several phenotypic traits, i.e. utilization of melibiose and acid formation of L-arabinose and amygdalin (Table 1).

Description of *Vibrio rotiferianus* sp. nov.

Vibrio rotiferianus (ro.ti.fer.i.a′nus. English n. *rotifer*; L. masc. suff. *-ianus* pertaining to; N.L. masc. adj. *rotiferianus* pertaining to rotifers).

Gram-negative curved rods (0·8–1·2 × 2·0–3·5 μm), facultative anaerobic, motile by means of more than one polar flagella. Non-pigmented, translucent, non-luminescent colonies on marine agar with no swarming. Bright, round, 2–3 mm yellow colonies, with umbilicated growth in TCBS agar. No growth occurs without NaCl ions in the culture medium; growth occurs in the presence of 1·5, 3·0 and 6·0 % NaCl (w/v), but not at 8 or 10 %; grows at 28–40 °C, but not at 4 °C. Susceptible to chloramphenicol (30 μg), tetracycline (30 μg), oxolinic acid (2 μg), oxytetracycline (30 μg), and to the vibriostatic agent O/129 at 10 and 150 μg; resistant to kanamycin (30 μg), streptomycin (25 μg) and gentamicin (10 μg). Arginine-dihydrolase-negative, lysine- and ornithine-decarboxylase-positive, ferments glucose without producing gas; positive for indole, oxidase, urease, tryptophan deaminase and gelatinase. Voges–Proskauer-, H_2S- and citrate-negative. Phenotypic differences are observed between the strains (Table 2). Utilizes the following substrates as sole carbon source: alaninamide, α-cyclodextrin, α-D-glucose, methyl β-D-glucoside, cellobiose, dextrin, D-fructose, D-galactose, D-gluconic acid, D-glucuronic acid, D-mannose, D-melibiose, D-raffinose, D-serine, D-trehalose, gentiobiose, glucose 6-phosphate, glycogen, glycyl-L-aspartic acid, inosine, L-alanine (LMG 21460^T and LMG 21458 weakly positive), L-alanine-glycine (LMG 21460^T and LMG 21458 weakly positive), L-arabinose, L-asparagine, L-aspartic acid, L-serine, maltose, *N*-acetyl-D-glucosamine, psicose, sucrose, thymidine and uridine. None of the strains utilizes the following carbon sources: 2,3-butanediol, 2-aminoethanol, acetic acid, adonitol, α-D-lactose lactulose, α-hydroxybutyric acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, β-hydroxybutyric acid, *cis*-aconitic acid, citric acid, DL-α-glycerol phosphate, DL-carnitine, D-alanine, D-arabitol, D-galactonic acidolactone, D-galacturonic acid, D-glucosaminic acid, D-mannitol, D-saccharic acid, D-sorbitol, formic

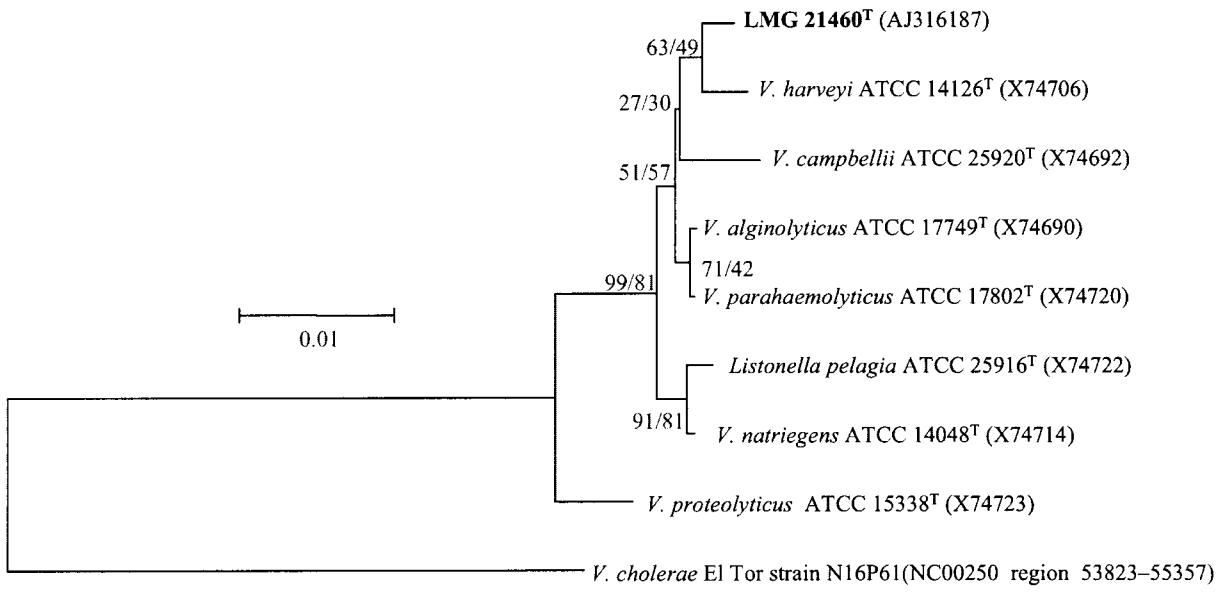

Fig. 1. Phylogenetic dendrogram of strain LMG 21460^T (=CAIM 577^T) and the closest *Vibrio* species derived from the almost complete 16S rDNA sequence data. Neighbour-joining method, 0·4 gamma correction, pairwise deletion, with Jukes–Cantor correction. Numbers at nodes denote the level of bootstrap support based on 1000 replicates, neighbour-joining/maximum-likelihood. Bar, 1 % sequence divergence.

acid, γ-aminobutyric acid, γ-hydroxybutyric acid, glucose 1-phosphate, glucuronamide, glycerol, glycyl-L-glutamic acid, L-hydroxyproline, i-erythritol, itaconic acid, L-fucose, L-histidine, L-leucine, L-ornithine, L-phenyl alanine, L-proline, L-pyro glutamic acid, L-rhamnose, malonic acid, methyl pyruvate, *meso*-inositol, monomethyl succinate, *N*-acetyl-D-galactosamine, phenyl ethylamine, *p*-hydroxyphenylacetic acid, propionic acid, putrescine, quinic acid, sebacic acid, succinamic acid, succinic acid, turanose, Tween 40, Tween 80, urocanic acid or xylitol. All are weakly positive for DL-lactic acid (except LMG 21457, positive). All strains have activities of alkaline phosphatase, esterease (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. None showed activity of lipase (C14), cystine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The following cellular fatty acids are present in descending order (mean percentage of the five strains analysed; maximum, minimum of the total fatty acid content): 16 : 0 (25·40; 28·47, 21·18), 18 : 1ω7*c* (10·79; 12·34, 9·13), 14 : 0 (9·52; 10·31, 8·89), 12 : 0 3-OH (2·91; 3·84, 2·33), 18 : 0 (1·10; 1·35, 0·75). Undefined fatty acids are also observed, summed feature 3 (16 : 1ω7*c* and/or 15 iso 2-OH – 37·14; 39·77, 34·79), summed feature 2 (14 : 0 3-OH and/or 16 : 1 iso I – 7·05; 8·65, 5·98) and one unknown (0·74; 0·98, 5·98). The G+C content of the DNA is 44·5 mol%. The type strain is LMG 21460^T (=CAIM 577^T), reference strains are LMG 21456, LMG 21457, LMG 21458 and LMG 21459; isolated from a rotifer (*Brachionus plicatilis*) flow-through culture system.

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