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Christophe Lambert, Jean-Louis Nicolas, Valérie Cilia, Sophie Corre. *Vibrio pectenica* sp. nov., a pathogen of scallop (*Pecten maximus*) larvae.. *International Journal of Systematic Bacteriology*, 1998, 48 (2), pp.481-487. <10.1099/00207713-48-2-481>. <hal-00447706>

**HAL Id: hal-00447706**

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Submitted on 1 Feb 2010

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## ***Vibrio pectenica* sp. nov., a pathogen of scallop (*Pecten maximus*) larvae**

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**Abstract:** Five strains were isolated from moribund scallop (*Pecten maximus*) larvae over 5 years (1990–1995) during outbreaks of disease in a hatchery (Argenton, Brittany, France). Their pathogenic activity on scallop larvae was previously demonstrated by experimental exposure. The phenotypic and genotypic features of the strains were identical. The GMC content of the strains was in the range 39–41 mol%. DNA–DNA hybridization showed a minimum of 73% intragroup relatedness. Phylogenetic analysis of small-subunit rRNA sequences confirmed that these strains should be affiliated within the family *Vibrionaceae* and that they are closely related to *Vibrio tapetis* and *Vibrio splendidus*. Phenotypic and genotypic analyses revealed that the isolates were distinct from these two vibrios and so constitute a new species in the genus *Vibrio*. They utilized only a limited number of organic substrates as sole carbon sources, including betaine and rhamnose, but did not utilize glucose and fructose. In addition, their responses were negative for indole, acetoin, decarboxylase and dihydrolase production. The name *Vibrio pectenica* is proposed for the new species; strain A365 is the type strain (=CIP 105190T).

**Keywords:** *Vibrio pectenica* sp. nov., *Pecten maximus*, phenotypic analysis, genotypic analysis, bivalve larvae.

**Abbreviation:** SSU, small subunit.

The EMBL accession numbers for the sequences reported in this paper are X16895, X74685–X74687, X74689, X74690, X74692, X74693, X746998, X74700, X74701, X74703–X74706, X747010, X74711, X74714–X74720, X74722–X74726, Y08430 and Y13830.

### **INTRODUCTION**

The identity of species of the genus *Vibrio* was recently confirmed by phylogenetic analysis deduced from small-subunit (SSU) rRNA sequences (24), with the exception of *Vibrio marinus*, which was placed in the *Pseudoalteromonas* branch, and *Vibrio damsela*, which was renamed *Photobacterium damsela* (12). These results further indicated affiliation to the genus *Vibrio*, previously determined by phenotypic analysis. However, some isolates that are pathogenic to aquacultured species remain unnamed or have not been correctly placed phylogenetically. To avoid misidentification and to allow the comparison of strains isolated by different laboratories, it seems necessary to study the phenotypic and genetic aspects of new isolates from aquaculture environments. The isolates studied here were systematically found (22) in moribund scallop larvae (*Pecten maximus*) over 5 years of sampling at experimental and commercial hatcheries (Argenton, Brittany, France). They may be ubiquitous in this area, since they appeared as soon as antibiotic was no longer added to the larval cultures. Five strains were examined by DNA–DNA hybridization and extensive phenotypic and phylogenetic analysis. As a result, a new species, named *Vibrio pectenica*, is proposed.

### **METHODS**

**Bacterial strains and growth conditions.** Five strains of *V. pectenica* isolated over 5 years from scallop larvae reared without antibiotics were used in this study (T for type strain) : A060 (isolated in 1990), A365T (1991), A496 (1992), A601 (1993) and A700 (1995). They were maintained on Marine Agar 2216 (Difco) at 4 °C or stored frozen in Marine Broth 2216 (Difco) supplemented with 5% (v/v) DMSO (Sigma) at -80 °C. Incubations were carried out at 22–25 °C.

**Electron microscopy.** Flagella characteristics were determined by transmission electron microscopy. Cells were negatively stained. After rinsing twice with 0.1 M ammonium acetate (pH 7.2) and once with

1% uranyl acetate, cells were placed on a 3 mm Cu grid coated with carbon film (300 mesh) for observation.

**Physiological and conventional characterization.** NaCl (2% w/v) was added to all media, except Marine Agar, Marine Broth and tests at different NaCl concentrations. In experiments using the API 20E identification kit (bioMérieux), 2% NaCl (final concentration) was also added to the distilled water before autoclaving, as recommended by MacDonell *et al.* (20). Fermentation, gas production from glucose and acid production from carbohydrates were tested in Hugh-Leifson medium (OF basal medium; Merck) supplemented with 1% carbohydrate. The cytochrome oxidase test was performed according to the Kovacs method (19). Temperature and NaCl tolerance, NaCl requirements, amylase, deoxyribonuclease, gelatinase and Tween-esterase tests were performed using methods described by West & Colwell (32). Lysine, ornithine decarboxylase and arginine dihydrolase activities were examined by using the API 20E system and Falkow base medium (9) [composition (in g l<sup>-1</sup>): peptone, 5; yeast, 3; d-glucose, 1; bromocresol purple indicator, 0.016] supplemented with 0.5% lysine, ornithine or arginine. Sensitivity to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine) was determined after 48 h on Marine Agar plates with O/129 discs (150 µg; Pasteur Diagnostic). β - Galactosidase activity was tested with discs containing ONPG (Pasteur Diagnostic). Indole production, nitrate reduction and acetoin production were tested with the API 20E system. Catalase activity was determined by the addition of 1 ml H<sub>2</sub>O<sub>2</sub> (3%) to a Marine Broth culture.

**Carbon sources.** The utilization of substrates as sole carbon and energy sources was performed twice, as described by Baumann & Baumann (3), using GN Microplates (Biolog) with media supplemented with 2% NaCl and incubated for 72 h.

**Cluster analysis.** Biolog Microplate tests were coded as 1 (positive result) or 0 (negative result). Cluster analyses were performed using simple matching coefficients (27) and an unweighted pair group method (28).

**Determination of G-C content.** DNA was isolated by the method of Sambrook *et al.* (26). Purity of the DNA was controlled by the method of De Ley (7). The G-C contents (mol%) of the DNA were determined by thermodenaturation (21) with DNAs from *Escherichia coli*, *Clostridium perfringens* and *Micrococcus luteus* as standards (Sigma).

**DNA PCR.** *V. pectenocida* colonies were suspended in 200 µl lysis solution (10 mM Tris/HCl, 1 mM EDTA, 1% Triton X-100, pH 8), heated for 5 min at 100 °C and then placed on ice. After a single chloroform extraction, 5 µl supernatant was used to amplify the SSU rRNA genes. The amplification was effected using two primers, corresponding to positions 8-8 (forward primer) and 1493-509 (reverse primer) of *E. coli* SSU rRNA sequence numbering. The initial denaturation step involved heating the reaction mixture at 95 °C for 3 min, which was followed by an annealing step (52 °C for 1 min) and an extension step (72 °C for 1.5 min).

The thermal profile then consisted of 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 1 min and extension at 72 °C for 1.5 min. A final extension was carried out at 72 °C for 5 min. Total rDNA was precipitated with 20% PEG, 2.5 M NaCl and purified on 1% lowmelting agarose in TAE buffer (40 mM Tris/cetate, pH 7.0; 2 mM EDTA, pH 8.0).

**Sequencing of the 16S rDNA gene** rDNA sequencing was carried out using the method described by Anderson *et al.* (1), modified by Ruimy *et al.* (24). Nine DNA primers were used in the sequencing reactions. These primers corresponded to the following positions in the *E. coli* sequence: S2, 99-119; S4, 342-356; S6, 518-534; S8, 684-702; S10, 906-925; S12, 1099-1114; S15, 1384-1400; and S17, 1493-1509.

**Phylogenetic analysis.** The data described below were obtained by alignment of the different sequences and phylogenetic analysis. All operations were done with computer programs developed in the laboratory of R. Christen (CNRS, Villefranche sur Mer, France). SSU rRNA sequences were aligned by eye. Domains used for deriving phylogenies were restricted to parts of the sequences for which homologies were without doubt and did not include too many undetermined nucleotides.

#### **Phylogenetic methods**

(i) Neighbour-joining algorithm. An algorithm similar to that developed by Saitou & Nei (25) was utilized. The program was rewritten to include inputs and outputs compatible with the ribosomal database and other programs developed by R. Christen.

(ii) Maximum parsimony. The paup program (30) for Macintosh computers was used. All topologies were obtained using the heuristic options. The robustness of the topology was evaluated under maximum-parsimony conditions (heuristic search) through 100 bootstrap replications.

(iii) Maximum likelihood. The fastDNAmI program that was derived from the DNAmI program (11), rewritten by G. J. Olsen (University of Illinois, Urbana, IL, USA) was operated with a SUN station. All analyses were performed using the global options (F, Y and G).

With these phylogenetic methods, all trees obtained were plotted using a Macintosh computer and a program (njplot) developed by M. Gouy (URA 243 CNRS, Université Claude Bernard, Villeurbanne, France), and which allows for transformation of a formal tree representation (Newick's format) into ClarisDraw drawings. Only topologies that were found to be similar by all three methods were retained as 'true trees'. Theoretical works have indeed demonstrated that convergence of the results of all three methods is a very strong indication that the correct phylogeny has been determined and that the tree topology found is robust.

**Nucleotide sequence accession numbers.** The nucleotide sequences used in this study have the following EMBL database accession numbers: *Vibrio metschnikovii*, X74711; *Vibrio diazotrophicus*, X74701; *Vibrio ordalii*, X74718; *Vibrio anguillarum*, X16895; *Vibrio aestuarianus*, X74689; *Vibrio vulnificus*, X74726; *Vibrio navarrensis*, X74715; *Vibrio tapetis* Y08430; *Vibrio splendidus*, X74724; *Vibrio nereis*, X74716; *Vibrio orientalis*, X74719; *Vibrio tubiashii*, X74725; *Vibrio furnisii*, X74704; *Vibrio fluvialis*, X74703; *Vibrio mediterranei*, X74710; *Vibrio harveyi*, X74706; *Vibrio campbellii*, X74692; *Vibrio parahaemolyticus*, X74720; *Vibrio pelagius*, X74722; *Vibrio natriegens*, X74714; *Vibrio alginolyticus*, X74690; *Vibrio proteolyticus*, X74723; *Vibrio carchariae*, X74693; *Vibrio nigripulchritudo*, X74717; *Vibrio gazogenes*, X74705; *Vibrio cincinnatiensis*, X74698; *Photobacterium leiognathi*, X74686; *P. damsela*, X74700; *Photobacterium phosphoreum*, X74687; *Photobacterium angustum*, X74685; and *V. pectenocida*, Y13830.

**DNA-DNA hybridization.** DNA of strains A365, A060, A496, A601, A700, *V. tapetis* (CECT 4600) and *V. splendidus* (ATCC 33125) was extracted according to the method of Sambrook *et al.* (26). Labelling of the DNA of A365 and A496 was performed by the nick-translation method and hybridization was carried out at 65 °C by the S1-nuclease method (6, 13) with adsorption of S1-resistant DNA onto DE81 filters (Whatman).

## RESULTS AND DISCUSSION

### Identification to the genus level

Five strains exhibited the characteristics that define the genus *Vibrio*: they were Gram-negative bacilli, facultative anaerobes, oxidase-positive, susceptible to vibriostatic agent O/129, required NaCl to grow and were able to produce acid from glucose under anaerobic conditions. The G-C content of the five strains was 39-41 mol%. The bacterial cells examined by electron microscopy exhibited a single polar flagellum (Fig. 1) when grown in liquid medium and unsheathed lateral flagella that were thinner than the polar one when grown on solid media (Fig. 2). These lateral flagella may permit cells to swarm on a solid surface (2).

### Phylogenetic analysis

The SSU rRNA sequence of *V. pectenocida* was aligned with a database containing more than 3000 aligned rRNA sequences of bacteria. The phylogenetic position was investigated with some representatives of the *Vibrio* genus and four representatives of the *Photobacterium* genus, used as outgroups (Fig. 3). Analysis confirmed that *V. pectenocida* belonged to the *Vibrio* genus, supported by a high bootstrap value. Analyses were performed using the three phylogenetic methods.

Results were consistent regardless of which method was used. All three methods placed *V. pectenocida* in a monophyletic group that included *V. splendidus*, *V. tapetis*, *V. nereis*, *V. orientalis*, *V. tubiashii*, *V. furnisii*, *V. fluvialis* and *V. mediterranei*, but this cluster was not supported by a robust bootstrap value. The new bacterium was close to *V. tapetis* and in all analysis clustered with *V. splendidus*. This internal branch was well-supported by a significant positive length at  $P < 0.01$  in the maximum-likelihood analysis and was retrieved from the maximum-parsimony analysis, where a strict consensus of two maximum-parsimony trees having equally high values was constructed (length, 212; consistency index, 0.533; retention index, 0.687). Finally, the sequence similarities with *V. tapetis* and *V. splendidus* were 97.2 and 95.5%, respectively. So, the similarity between 16S rDNA gene

sequences of *V. splendidus* and the proposed new species is on the limit of intraspecies variability (97%) as proposed by Stackebrandt & Goebel (29).

### DNA relatedness

One hybridization was performed with strain A365 DNA as a probe and *V. tapetis* and *V. splendidus* type strain DNA. The result showed a percentage of reassociation of <5% in both cases.

Two hybridizations were performed with strains A365, A496, A060, A601 and A700; labelled A365 and A496 DNAs were used as probes, respectively. Similar rates of reassociation were obtained in both experiments (Table 1); in particular, the lowest rate was observed with strain A060 and a value of 100% was observed with strain A700. Taking into consideration the criteria recommended by Wayne *et al.* (31), the five strains belong to the same species, for which the name *V. pectenica* is proposed.

### Phenotypic characterization

Clustering analysis is shown in Fig. 4. The taxonomic study, including the 95 Biolog Microplate tests, clearly indicates that the *V. pectenica* group belongs to the genus *Vibrio* and differentiates it from the other vibrios, especially *V. splendidus*. Only *Vibrio logei* clustered at 90% similarity with *V. pectenica*. Moreover, out of 45 Biolog tests reported by Borrego *et al.* (4) for *V. tapetis*, less than 58% are common with *V. pectenica*. Biochemical and physiological characteristics of the five *V. pectenica* strains are given in Table 2. *V. pectenica* strains were distinct from the already described vibrios by their limited ability to utilize organic substrates (35% utilization) as sole source of carbon according to the method of Baumann & Baumann (3). The other vibrios utilized 50% or more of the tested substrates, except *Vibrio costicola* (38%) and *V. tapetis* (28%, with 21 substrates only) (4). Nevertheless, they grew with betaine and l-rhamnose; both characteristics were shared only by *V. natriegens* and one (growth in betaine) was observed in *Vibrio mytili*. Acid was produced from maltose and d-glucose but not from sucrose. Indole and acetoin production were negative. Finally, although the phylogenetic analysis clustered *V. tapetis* with *V. pectenica*, the G-C content, DNA-DNA homology and a large number of phenotypic tests including growth in 6% NaCl and utilization tests (Biolog GN Microplates) with d- and l-alanine, l-aspartate, d-glucuronate, l-glutamate, glycerol, dl-lactate, l-leucine, maltose, N-acetyl glucosamine, l-proline, methyl pyruvate, l-serine, succinate, d-trehalose, l-threonine and Tween 80 confirm *V. pectenica* as a new species.

Some of the recently described species, including *Vibrio penaeicida* (17), *V. mytili* (23) and *Vibrio ichthyoenteri* (18) can be differentiated from the main group of species with which *V. pectenica* shows the largest phylogenetic affinity (*V. tubiashii*, *V. splendidus*, *V. orientalis*, *V. fluvialis*, *V. nereis*, *V. mediterranei*) by DNA-DNA hybridization. Indeed, the percentage of homology between these species and the *V. pectenica* group is always less than 18% (17, 18, 23). The similarity between 16S rDNA gene sequences of *V. pectenica* and the vibrios not included in the phylogenetic analysis, such as *Vibrio iliopiscarius* (phenotypic characters not available to date), *Vibrio scophthalmi*, *Vibrio salmonicida*, *Vibrio mimicus*, *Vibrio cholerae*, *Vibrio fischeri*, *V. mytili*, *V. logei* and *Vibrio hollisae*, are below 97% (92.5, 95.9, 89.5, 93.4, 93.5, 94.2, 95.9, 93.6 and 91.8%, respectively).

Moreover, a neighbour-joining analysis (not reported here) confirms that these vibrios are not closely related to the *V. pectenica* group. For identification, the main characteristics which differentiate *V. pectenica* from the closest *Vibrio* spp. in the phylogeny (*V. tubiashii*, *V. splendidus*, *V. orientalis*, *V. fluvialis*, *V. nereis*, *V. mediterranei*, *V. tapetis* and *V. furnisii*) and some *Vibrio* spp. With similar phenotypic characteristics (*V. salmonicida*, *V. hollisae*, *V. natriegens*, *V. logei*, *V. penaeicida* and *V. nigripulchritudo*) are indicated in Table 3.

### Description of *Vibrio pectenica* sp. nov.

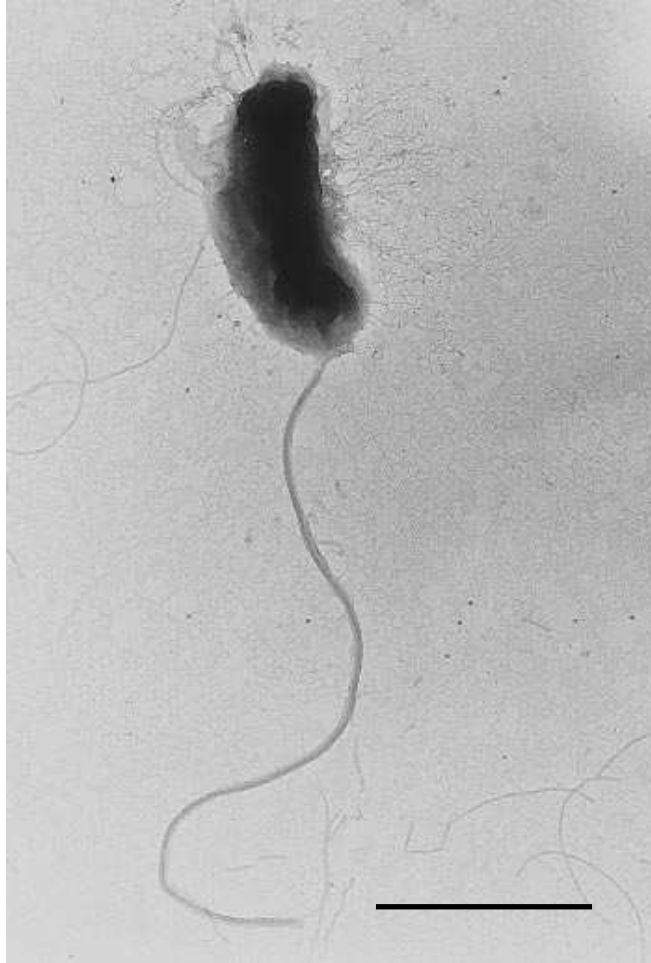
*Vibrio pectenica* (pec.ten.i.ci«da. M.L. n. *Pecten* genus name of scallops; M.L. v. *caedo* to kill; *pectenicida* scallop-killer). Gram-negative rods, motile by means of a single polar flagellum in liquid and unsheathed lateral flagella on solid media. Colonies develop within 24 h at 20 °C, are circular and smooth, unpigmented and began swarming after 48 h. *V. pectenica* strains are not luminescent. No growth occurs on thiosulfate/citrate/bile/sucrose (TCBS) agar. No pigment is produced. Glucose metabolism is fermentative. *V. pectenica* strains are facultatively anaerobic. Acid but no gas is produced from d-glucose and maltose. NaCl is required for growth. Oxidase, catalase, and nitrate reduction to nitrite are positive. *V. pectenica* strains are susceptible to the vibriostatic agent O/129. DNA, gelatin, starch and Tween 80 are hydrolysed extracellularly. Acetoin and indole are not produced. *V. pectenica* uses the following substrates as sole carbon and energy source: maltose, succinate, glycerol, l-rhamnose, isovalerate, pyruvate, l-alanine, l-aspartate, l-lysine, l-histidine, betaine, fumarate, glu glucosamine.

Acid is produced from maltose and glucose, but not from sucrose, l-arabinose, d-cellobiose, d-mannose, d-sorbitol, d-xylose, d-galactose, myoinositol, fructose and l-rhamnose. The G-C content is 39-41 mol% as determined by thermal denaturation. Regularly isolated from moribund scallop (*Pecten maximus*) larvae cultured on the Atlantic French coast (Brittany). Strain A365T is the type strain (CIP 105190T) and had all the properties of the species except for a positive growth response at 30 °C (cf. Table 2). The G-C content of the DNA is 41 mol%.

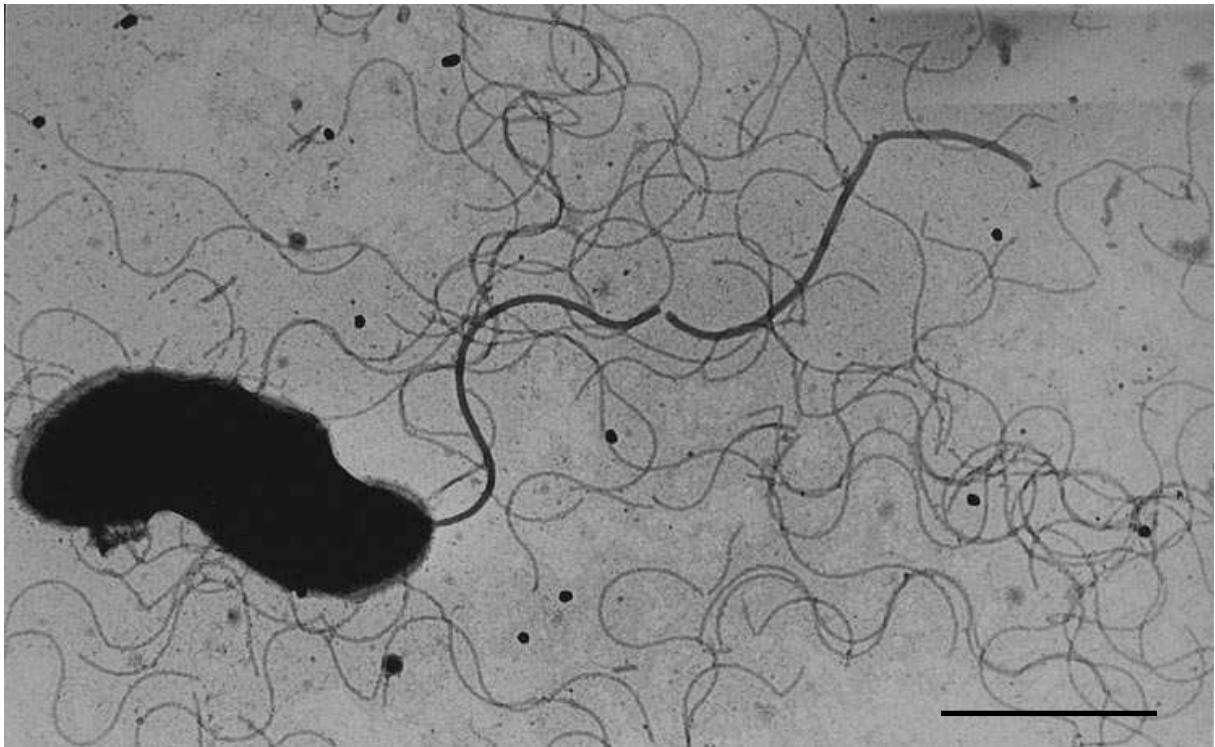
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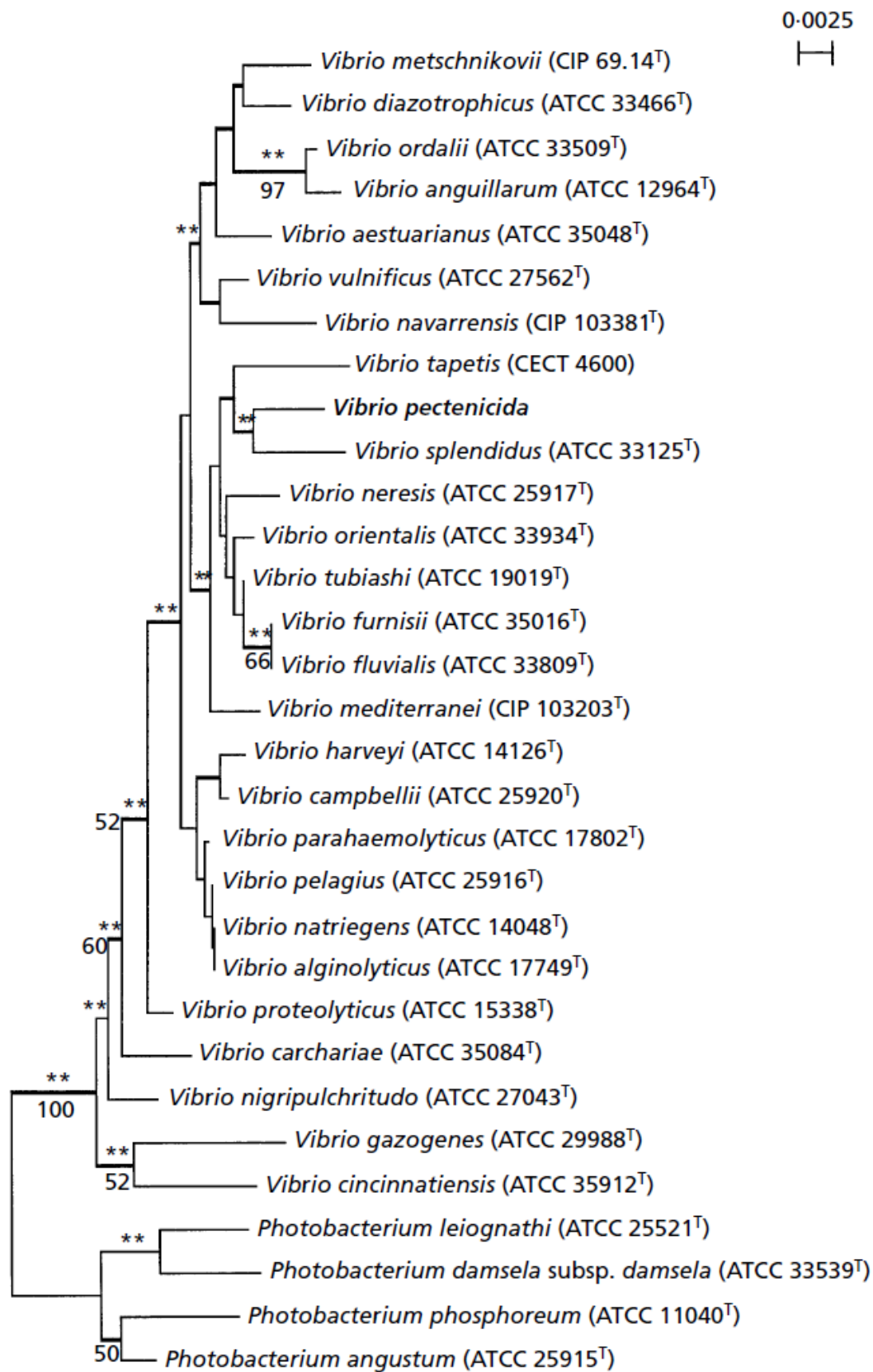
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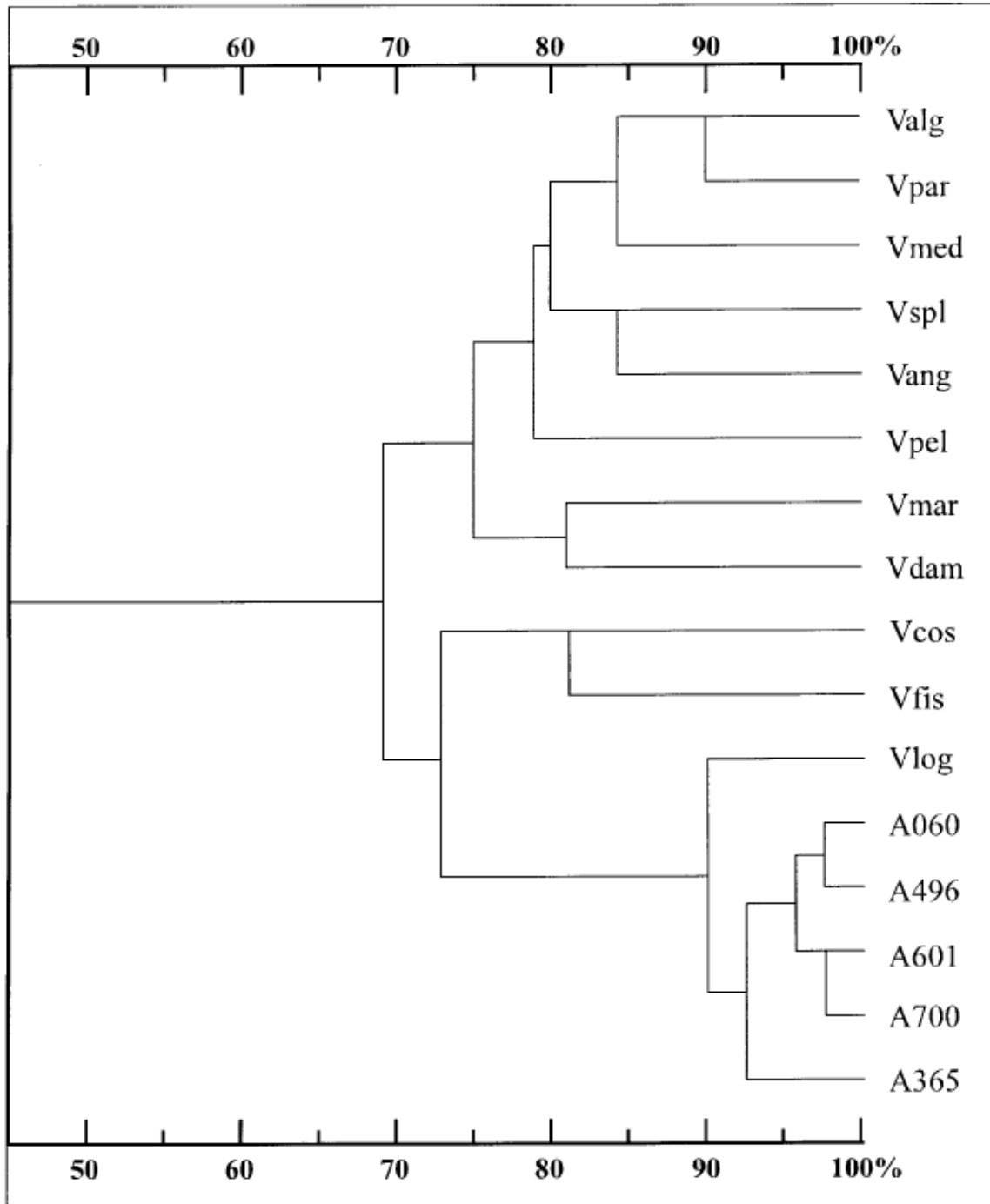
**Fig. 1.** Electron micrograph of a negatively stained cell of *V. pectenica*, strain A365r (Marine Broth growth) showing the single polar flagellum. Bar, 1  $\mu\text{m}$ .



**Fig. 2.** Electron micrograph of a negatively stained cell of *V. pectenica*, strain A365r (Marine Agar growth) showing lateral flagella. Bar, 1  $\mu\text{m}$ .



**Fig. 3.** Phylogenetic position of the new bacterium within the genera of *Vibrionaceae*. The topology shown is an unrooted tree obtained with the neighbour-joining algorithm. Branches significantly positive at  $P < 0.01$  with the maximum-likelihood method are labelled with double asterisks. Support from a bootstrap analysis using parsimony is shown above each branch as percentage of replications (only percentages  $> 50\%$  are indicated). Thicker branches are those retrieved within the most parsimonious tree.



**Fig. 4.** Cluster analysis of phenetic data (Biolog tests) using the simple matching similarity coefficient and unweighted pair group method for five strains of *V. pectenocida* and 11 other strains of *Vibrio* species. Valg, *V. alginolyticus* ATCC 17749; Vpar, *V. parahaemolyticus* ATCC 17802; Vmed, *V. mediterranei* ATCC 43341; Vspl, *V. splendidus* ATCC 33125; Vang, *V. anguillarum* ATCC 19264; Vpel, *V. pelagius* ATCC 25916; Vmar, *V. marinus* ATCC 15381; Vdam, *V. damsela* ATCC 33539; Vcos, *V. costicola* ATCC 33508; Vfis, *V. fisheri* ATCC 7744; Vlog, *V. logei* ATCC 15382.

**Table 1.** Intraspecific DNA–DNA homology among strains of *V. pectenicida*

Test strain	Reassociation (%) with labelled strain:	
	A365 <sup>T</sup>	A496
A365 <sup>T</sup>	100	95
A496	99	100
A060	86	73
A601	97	92
A700	100	100

**Table 2.** Biochemical and physiological characteristics of five *V. pectenica* strains: Positive tests for all of the strains were: fermentation of glucose, oxidase, catalase, nitrate reduction, alginase, amylase, gelatinase, deoxyribonuclease, Tween)esterase, growth at 18 and 22 °C, growth in the presence of 1, 3 and 6% NaCl, acid production from maltose, d-glucose and the utilization as a sole carbon source of maltose, succinate, glycerol, l-rhamnose, isovalerate, pyruvate, l-a-alanine, laspartate, l-histidine, betaine and fumarate. Negative tests for all of the strains were: gas produced from glucose, indole production, Voges-Proskauer test, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, ONPG hydrolysis, growth in the presence of 0 and 8% NaCl, acid production from d-mannose, d-xylose, l-arabinose, l-rhamnose, glycerol, melibiose, sucrose, d-galactose, dsorbitol, d-cellobiose, fructose, *myo*-inositol, erythritol and the utilization as a sole carbon source of d-mannose, dgalactose, d-fructose, cellobiose, melibiose, salicin, dgluconate, citrate, erythritol, d-mannitol, d-sorbitol, d-xylose, l-arabinose, d-glucose, trehalose, galacturonate, acetate, propionate, butyrate, dl-hydroxybutyrate, glycine, b-alanine, dl-serine, l-leucine, l-arginine, l-ornithine, l-proline and glucosamine.

Characteristic	Positive strains (%)	Reaction of A365 <sup>T</sup>
Growth at:		
4 °C	80	+
30 °C	40	+
35 °C	20	—
Utilization as sole carbon source:		
Sucrose	20	—
L-Tyrosine	60	+
<i>myo</i> -Inositol	80	+
L-Valine	60	+
L-Glutamate	20	—
L-Lysine	80	+

**Table 3.** Characteristics useful for distinguishing *V. pectenica* from closely related *Vibrio* species; Data from references 4, 5, 8, 10, 14, 15, 16, 17 and 32. (0), *V. pectenica*; (1), *V. tubiashii*; (2)I, *V. splendidus* I; (2)II, *V. splendidus* II; (3), *V. orientalis*; (4), *V. fluvialis*; (5), *V. nereis*; (6), *V. mediterranei*; (7), *V. tapetis*; (8), *V. furnisii*; (9), *V. natriegens*; (10), *V. salmonicida*; (11), *V. logei*; (12), *V. penaeicida*; (13), *V. hollisae*; (14), *V. nigripulchritudo*. -, 90% or more strains are positive; +, 90% or more strains are negative; v, 25-1-74-9% positive; d, 12-89% positive.

Characteristic	(0)	(1)	(2)I	(2)II	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
Swarming	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lateral flagella (solid media)	+	+	-	D	-	D	-	-	-	-	-	-	-	-	-	-
Acetoin production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ONPG hydrolysis	-	-	-	-	-	-	-	-	-	D	-	-	-	+	-	-
Indole	-	+	+	+	+	+	v	+	+	-	-	-	-	v	+	+
Arginine dihydrolase	-	D	+	-	-	+	+	-	-	+	-	-	-	-	-	-
Alginase	+	-	D	-	-	-	-	-	-	-	-	-	-	v	-	-
Growth on:																
D-Glucose	-	-	+	+	-	+	+	-	+	-	+	+	+	-	-	+
L-Rhamnose	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Betaine	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Acid from:																
Arabinose	-	-	-	-	-	+	-	-	-	+	+	-	-	-	+	-
Sucrose	-	+	v	-	+	+	+	+	-	+	+	-	v	-	-	-
Sorbitol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
G+C content (mol%)	39-41	43-45	45-46	45-46	45-46	49-51	46-47	42-43	42-46	-	46-47	42	40-42	46-47	-	46-47