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Molecular and phenotypic characterization of *Vibrio aestuarianus* subsp. *francensis* subsp. nov., a pathogen of the oyster *Crassostrea gigas*

Matthieu Garnier[a], Yannick Labreuche[a] and Jean-Louis Nicolas[a, *]

[a] Ifremer, Laboratoire de Physiologie des Invertebrés, PFOM, UMR PE2M, Centre de Brest BP 70, 29280 Plouzané, France

*: Corresponding author : Jean-Louis Nicolas : Jean.Louis.Nicolas@ifremer.fr

Abstract:

Eleven *Vibrio* isolates invading the hemolymph of live and moribund oysters (*Crassostrea gigas*) collected in the field and from a hatchery in France, were characterized by a polyphasic approach. Phylogenetic analysis of 16S rRNA, *gyrB* and *toxR* genes indicated high homogeneity between these strains and the *Vibrio aestuarianus* type strain (ATCC35048^T), and confirmed previous 16S rRNA analysis. In contrast, DNA:DNA hybridization was from 61% to 100%, while phenotypic characters and virulence tests showed a large diversity between the strains. Nevertheless, several common characters allowed the isolates to be distinguished from the reference strain. On the basis of several distinct phenotypic characteristics, it is proposed to establish two subspecies within the *V. aestuarianus* spp. group, *V. aestuarianus* subsp. *aestuarianus* [D. Tison, R. Seidler, *Vibrio aestuarianus*: a new species from estuarine waters and shellfish, Int. J. Syst. Bacteriol. (1983) 699–702] and *V. aestuarianus* subsp. *francensis* for these French isolates. The characters that differentiate the new strains from *V. aestuarianus* subsp. *aestuarianus*^{T} are virulence (positive for 63% of the isolates) and 12:0 fatty acid content. The colonies were smaller and uncoloured, whereas no growth occurred at 35 °C or on TCBS, and the strains did not utilize several substrates, including l-serine, *α*-cyclodextrin, d-mannitol, *α*-glycyl-l-aspartic acid, l-threonine and glucose-1-phosphate.

Keywords: *Vibrio aestuarianus*; French isolates; Pathogen; *Crassostrea gigas*; Summer mortalities; Polyphasic approach

1. Introduction

Summer mortality of the oyster *Crassostrea gigas* is a complex syndrome involving physiological and environmental factors as well as pathogens, whose role remains controversial. A recently published epidemiological investigation [7] demonstrated the occurrence of septicaemic *Vibrio* spp. in dying adult oysters collected during summer mortality events in a hatchery and nursery. Moribund oysters collected in the field were found to be infected either by a mixture of several bacteria (40% of cases) or by a dominant *Vibrio* strain (40%), which could sometimes be isolated in pure culture from hemolymph. In the hatchery and nursery, spontaneous mortalities of adult and spat oysters were almost systematically correlated with the presence of only one *Vibrio* species in their hemolymph.
Preliminary characterization by 16S rRNA sequencing showed that 53% of the dominant bacteria could be affiliated to the *Vibrio aestuarianus* group, about 30% to the *Vibrio splendidus* polyphyletic group and 5% to *Pseudoalteromonas* spp.
The first isolation and description of *V. aestuarianus* was reported by Tison and Seidler [26] who isolated it along with other *Vibrio* spp. from seawater, oysters, clams and crabs from the Oregon and Washington coasts (USA). More recently, it has been found in association with *Ostrea edulis* culture [11] on the Galician coast (Spain). The species had never been reported as a pathogen until the studies made by Labreuche et al. [13,14], who demonstrated its pathogenicity following injection of live bacterial cells or their extracellular products into oysters. Despite numerous recent publications indicating its ubiquitous presence on the coasts of the Baltic Sea [3], Spain [11,20] and Hong Kong [27], and, apart from some identification based on phenotypic characters or 16S rRNA sequencing, the diversity of strains from different geographic areas had not been investigated.
The aim of this study was, therefore, to characterize several strains of *V. aestuarianus* isolated over a three year period (2001 to 2003) from oysters sampled during summer mortality events in France. The strains were identified using a polyphasic approach that included morphological, physiological and biochemical characteristics, phylogenetic analysis and virulence tests.

2. Materials and Methods

2.1. Bacterial strains

Eleven strains of *Vibrio aestuarianus* (Table 1) were chosen to be representative of isolates collected during summer mortality events along the shore and in hatchery and nursery facilities. All were septicaemic and their concentrations exceeded 10^8 cfu ml^{-1} in the hemolymph, except for strain 02/103 that was isolated from an apparently healthy oyster at 10^4 cfu ml^{-1}.
The strains were compared with the type strain *Vibrio aestuarianus* ATCC35048^T. For DNA:DNA hybridization, *Vibrio orientalis* (CIP 102891^R) and *Vibrio pomeroyi* (LMG 21352) [25] were obtained from microbial collections (Collection of Institut Pasteur, and Belgian Co-ordinated Collections of Micro-organisms, respectively). All bacteria were grown routinely in Marine Broth (Difco) at 20 °C and stored at –80 °C in the same medium containing 5% DMSO.

2.2. Cell morphology

Motility was observed by phase contrast microscopy of an exponentially growing culture. The shape and flagella were examined by transmission electron microscopy (TEM). After rinsing twice with 0.1 M ammonium acetate (pH 7.2) and staining with 1% uranyl acetate, cells were placed on a 3 mm copper grid coated with carbon film (300 mesh, Oxford Instruments). Grids were examined by TEM [15] after air drying the grid.

2.3. Biochemical characterization

Biochemical and nutritional profiles were determined using API 20E and API 50CH commercial kits (Bio-Merieux, France) and Biolog GN microplates (Biolog Inc.). Fresh colonies were suspended to obtain about 2 x 10^9 cells ml^{-1} in 2% NaCl for API 20E or in the medium from the API 50CH kit supplemented with 2% NaCl. After inoculation, the strips were incubated for 48 h at 20 °C and the results were determined according to the manufacturer's instructions.
Fermentative and/or oxidative metabolism was determined in O/F medium (Merck) with 1% added sugar (glucose, fructose and galactose in equal concentrations of 0.33% each), and oxidase activity was determined using oxidase discs (Sanofi-Pasteur). Growth at different salinities was observed after 48 h in peptone broth (15 g l^{-1}) with 0, 2, 4, 6, 8, or 10% NaCl at 20 °C. Growth at different temperatures was recorded after 72 h culture in peptone broth (15 g l^{-1}) with 2% NaCl at 4, 20, 30, 35 and 40 °C. Numerical

analysis of phenotypic characters was carried out using simple matching coefficients and an unweighted pair group method [19].
Sensitivity to antibiotics was examined according to the disc diffusion method on Marine Agar (Oxoid).

2.4. Phylogenetic analysis

Template genomic DNA was prepared according to Sambrook's method [22]. Partial *gyr*B and *tox*R genes were amplified, cloned and sequenced as described by Le Roux et al. [17]. Phylogenetic analyses were performed using the GCG Wisconsin Package (Accelrys) and Phylowin software [6]. Sequences were aligned and the phylogenetic trees were constructed using the neighbour-joining method. Distance matrices were calculated using Kimura's 2-parameter distances [1]. Robustness of topologies was assessed by the bootstrap method with 500 replicates.

2.5. Genotypic analysis

Quantitative DNA:DNA hybridizations were performed on membranes, as described by Jensen et al [12], and validated by comparison with the classical method of nuclease S1 [17]. Chromosomal DNA of all the *V. aestuarianus* isolates (the French isolates and ATCC35048^T), *V. orientalis* CIP102391^T, and *V. pomeroyi* LMG21352 were purified on caesium chloride gradients [23]. Four 300 ng replicates of each were then blotted onto Hybond N membranes (Amersham). Chromosomal DNA of *Vibrio aestuarianus* ATCC35048^T and 01/032 were then labelled with DIG (Dioxygenin) and used successively as probes. After overnight hybridization at 55 °C and two washings, probes were revealed with CDP-star RPB3682 (Amersham) and quantified with a FLUOR-S^{TM} Multi-Imager (BIO-RAD) equipped with a Nikon 50 mm lens. The average chemiluminescence level (cpm mm^{-2}) reached by the four replicates of homologous DNAs was considered as the 100% re-association rate, and the level reached by herring sperm DNA as 0%.

2.6. Challenge test

The strains were cultured in Marine Broth at 20 °C for 24 h with agitation (180 rpm). Bacterial cells were subsequently collected by centrifugation (6000 g, 10 min), rinsed with filtered sterile seawater (FSSW) and resuspended in FSSW. Bacterial cell concentration was estimated by optical density (OD; 540 nm) and adjusted to 10^9 cell ml^{-1} according to a relationship previously established between plate counts and OD. Oysters of the treatment batch (n=30) were anaesthetised for 3 h in a $MgCl_2$ bath at a final concentration of 50 g l^{-1} (2/3 v/v seawater/freshwater), and then injected with 0.1 ml of the bacterial suspension (10^8 per individual) into the adductor muscle. Each oyster in the control batch (n=30) was injected with 0.1 ml of sterile seawater. Immediately after injection, animals were transferred into 30 l tanks containing aerated filtered seawater (20 l) at 20 ± 0.5 °C. Oysters were fed with algae (*Isochrysis affinis galbana* clone T-iso and *Pavlova lutheri* at about 10^5 cell m1^{-1} final concentration) and their seawater was changed twice a week. Mortalities were observed every day for the 10 days following injection. Animals were considered to be dead when the valves did not close and the mantle did not react after stimulation by a needle-prick. An aliquot of hemolymph from any dying oyster was streaked onto Marine Agar (MA) to verify if the septicaemic bacteria corresponded to *V. aestuarianus*.

3. Results

3.1. Phenotypic characteristics

After 48 h incubation on MA at 20 °C, colonies of the French isolates measured 1-2 mm diameter, were circular, slightly white and translucent. Colonies of the reference strain were more opaque and larger (2-3 mm) than the French isolates. Cells resembled curved rods, 0.4-0.5 µm wide and 1-1.5 µm in length, motile with a polar flagellum visible by TEM. Neither luminescence nor swarming was observed. The strains were sensitive to chloramphenicol, erythromycin, furans, kanamycin, norfloxacin, and piperacillin.
Fermentation and utilization of substrates were generally variable and two of the strains did not respond to any test: 01/031 on API 50 CH and 01/151 on the GN Biolog plate.
Numerical analysis was performed on the 42 Biolog characters positive for at least one strain. This clustered the strains with 45 to 90% similarity (see dendrogram figure in supplementary material). The reference strain, which gave the most positive responses, appeared outside the cluster of the French isolates.
The French isolates did not grow on TCBS, in 6% NaCl (except 02/041) or at 35 °C and nor did they utilize L-serine, α-cyclodextrin, D-mannitol, α-glycyl-L-aspartic acid, L-threonine, and glucose-1-phosphate, which were utilized by the reference strain (Table 2). All these characters clearly separated the two types of *V. aestuarianus.*

Characters that differentiated the *V. aestuarianus* strains from *V. anguillarum*, *V. pomeroyi*, *V. chagasii* and *V. lentus* are indicated in Table 2. There were higher levels of the 16:1 (n-7) and 18:1 (n-7) fatty acids than in the closely-related *Vibrio* spp. and an absence of 12:03-OH.

3.2. Phylogenetic and genotypic analyses

Phylogenetic trees based on partial *gyrB* and *toxR* sequences showed a strong relationship between the *V aestuarianus* strains, including the reference strain, with similarities between 99 and 100% (Fig. 1). Previously, the 16S rRNA sequences displayed 99% similarity at least between strains. On the other hand, quantitative DNA:DNA hybridization performed with the labelled 01/032 DNA probe showed 17 and 9% hybridization rates with *V. orientalis* and *V. pomeroyi*, respectively, but only 61% with the *V. aestuarianus* reference strain (ATCC35048^T) and between 81 and 100% with all the other French strains (Table 1). The hybridization rates obtained using *V. aestuarianus*^{T} as a probe did not reach 70% with seven of the strains: 01/031 01/032, 01/064, 01/140, 01/151, 02/041, 02/093, but exceeded this threshold with strains 01/308, 02/103, 02/114 and 03/015.

3.3. Virulence

The two other experimental infections confirmed a similar ranking of virulence between the strains, but with higher mortalities. In every moribund oyster analyzed, the hemolymph contained septicaemic bacteria whose colonies on MA resembled those of *Vibrio aestuarianus*.

4. Discussion

The strains of *V. aestuarianus* isolated from French oysters form clearly recognizable colonies on Marine Agar (MA), which are less than 1 mm in diameter after 24 h at 20 °C, circular, white and translucent. These French strains grew slower on MA than most other bacteria found in oysters, such as other *Vibrio* spp. and *Pseudoalteromonas* spp. [7], and they did not grow on TCBS. The detection of *V. aestuarianus* colonies in mixed growth therefore requires special attention. This could explain why the species has not been isolated more frequently and has never been described as an opportunist.

The phenotypic characters of *V. aestuarianus* appear very diverse. However, some common characters allow them to be distinguished from other vibrios associated with oysters, such as *V. anguillarum*, *V. pomeroyi*, *V. lentus* and *V. chagasii*.

Some characters (arginine dihydrolase, gelatinase, melibiose and glycerol) were negative for the reference strain in our study, in contrast to the first description [26]. However, the methods used to determine these characters were different in the present study, which could explain these negative responses.

The French strains did not grow in 6% NaCl (except 02/041) or at 35 °C, in contrast to the reference strain, which can support more saline and warmer conditions.

Using the reference strain as a probe, the DNA:DNA hybridization rates with the French isolates ranged from 54 to 87%. Strains 01/308 02/103, 02/114, 03/015 could be formally affiliated to *V. aestuarianus*, while 01/031, 01/032, 01/140, 02/041, 01/064, 01/151, and 01/093 could constitute another species [10]. However, the French strains themselves are closely related since their DNA:DNA hybridizations using the 01/032 strain as a probe gave values higher than 81%. Consequently, the delineation of a new species could not be made. Moreover, the phylogenetic analyses unambiguously affiliated all strains to *V. aestuarianus*. The *rpoA* and *recA* sequences of some isolates, including 01/064 (AM884020, AM884017), 01/308 (AM884021, AM884018), and 02/041 (AM884022, AM884019) also confirmed their proximity to the reference strain (AJ842554, AJ842369) with 99% homology [unpublished data]. The high conservation and specificity of the *gyrB*, *toxR*, *rpoA* and *recA* sequences may enable future applications, such as specific diagnostic molecular probes, to be designed [9,23].

The experimental infections gave variable mortality rates from one experiment to another, but the ranking of virulence between the strains remained similar. This variability could be linked to the genetic background and physiological status of individual oysters, that may modify their susceptibility to infection, as reported for oyster summer mortalities [4,21].

In this study, no phenotypic or genotypic character could differentiate the virulent strains (>50% mortality in one challenge at least) from the avirulent ones (<15% mortality). To date, the only possibility for discriminating between these types of isolate has been based on experimental challenges. The identification of molecular markers specific to pathogenic isolates appears therefore essential, as these markers could be used as rapid, reliable and sensitive tools for discrimination between isolates. Recently, Labreuche et al. [unpublished results], demonstrated the implication of a zinc-dependent metalloprotease in the virulence of strain 01/032. However, although the corresponding gene was found with 100% identity in another virulent strain (02/041), it was also characterized in three avirulent ones (01/064, 02/114, ATCC35048^T) [unpublished

data]. Research is still ongoing to identify other virulence factors and their corresponding genes, which may be used for rapid detection of pathogenic strains.
The strains isolated from dying oysters were not systematically pathogenic. This could be the result of a loss of immunological defences due to a deficient physiological status of the oyster in conjunction with environmental stresses, favouring the invasion by bacteria able to colonise the tissues quickly.
However, the presence of septicaemic bacteria is generally due to their virulence. Therefore, the presence of avirulent bacteria at a high concentration in dying oysters may be explained by the simultaneous presence of different strains of *V. aestuarianus* with various virulence levels inside one host. This hypothesis is supported by the study of Gay et al. [8] who observed the simultaneous presence of several *V. splendidus* strains in the same oyster and demonstrated a cooperative effect between two isolates that killed the oysters. The diversity of *V. aestuarianus* strains found during the same mortality event was not really studied, except for strains 01/031, 01/032 and 01/064 isolated from the same batch of moribund oysters but they did not display the same characteristics, including phenotypic characters and virulence. For a further study, it would be interesting to know the extent of this diversity by identifying ten or more strains of *V. aestuarianus* isolated from the same moribund individuals.

4.1. Emended description of *Vibrio aestuarianus*

The characteristics modified or added to those reported in the original description recorded by Tison and Seidler, 1983 are as follows:
The colonial morphology is variable according to the subspecies. All strains reduce nitrate to nitrite. Amylase is positive. Gelatinase is variable. Most of strains (more than 82%) utilize succinic acid, L-alanine, L-aspartic acid, N-acetyl-D-glucosamine, D-galactose, a-D-glucose, D-mannose, D-fructose, sucrose, and D-trehalose. Most strains (more than 82%) produce acid from acetylglucosamine, galactose, and fructose. The following substrates are fermented by the majority of strains (58% or more): ribose, maltose, starch, glycogen, and potassium glucose Na Te. The major fatty acids are: iso14:0 (1.19±0.65%), iso16:0 (4.31±1.63%), 14:0 (6.92±2.29%), 15:0 (1.04±0.27%), 16:0 (19.11±2.76%), 16:1(n-7) (45.58±2.76%), 17:1(n-8) (1.08±0.42%), 18:1(n-7) (12.77±4.60%), and iso17:0 (1.23±0.77%). Other characters are shown in supplementary material.

Vibrio aestuarianus subsp*. aestuarianus* subsp. nov. Tison and Seidler 1983 (a.es.tu.ri.a'nus. N.L. masc. adj. aesturianus, pertaining to an estuary) (Type strain = OY-0-002 = ATCC 35048 = CAIM 592 = CCUG 28583 = CIP 102971 = NBRC 15629 = LMG 7909). Displays typical characteristics for the species *V. aestuarianus*. Colonies are opaque, 2-3 mm in diameter on Marine Agar after 24 h at 20 °C. It grows on TCBS, with 6% salt and at 35 °C. It utilizes L-serine, α-cyclodextrin, D-mannitol, α-glycyl-L-aspartic acid, L-threonine, glucose-1-phosphate, and melibiose. The fatty acid 12:0 content is very low at 0.38%.

Vibrio aestuarianus subsp. *francensis*, subsp. nov. (fran.cen'sis. N.L. masc. adj. francensis: of, or belonging to, France) (Type strain = 02/041 = LMG 24517 = CIP 109791). Displays typical characteristics for the species *V. aestuarianus*. Colonies are clearly recognizable on Marine Agar and are less than 1 mm in diameter after 24 h at 20 °C. No strain can grow in the presence of 6% NaCl, except for strain 02/041, and on TCBS. The majority of strains are pathogenic to the Pacific oyster, *Crassostrea gigas.* Growth occurs from 4 °C to 30 °C but not at 35 °C. The fatty acid 12:0 content is 2.20±0.26%. It can be differentiated from *V. aestuarianus* subsp *aestuarianus* by the inability to utilize several substrates (Table 2 and supplementary material). Isolates exhibit a broad phenotypic diversity, as the Biolog tests demonstrate (see supplementary material). They are frequently found in healthy and diseased oysters, and in sediment.
The type strain was isolated from a diseased oyster maintained in the Argenton experimental hatchery (Ifremer, France).

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Fig. 1. Neighbour-joining phylogenetic trees based on partial *gyrB* and *toxR* gene sequences. Bootstrap values are expressed as a percentage of 500 replicates. Maximum likelihood and maximum parsimony methods gave similar results.

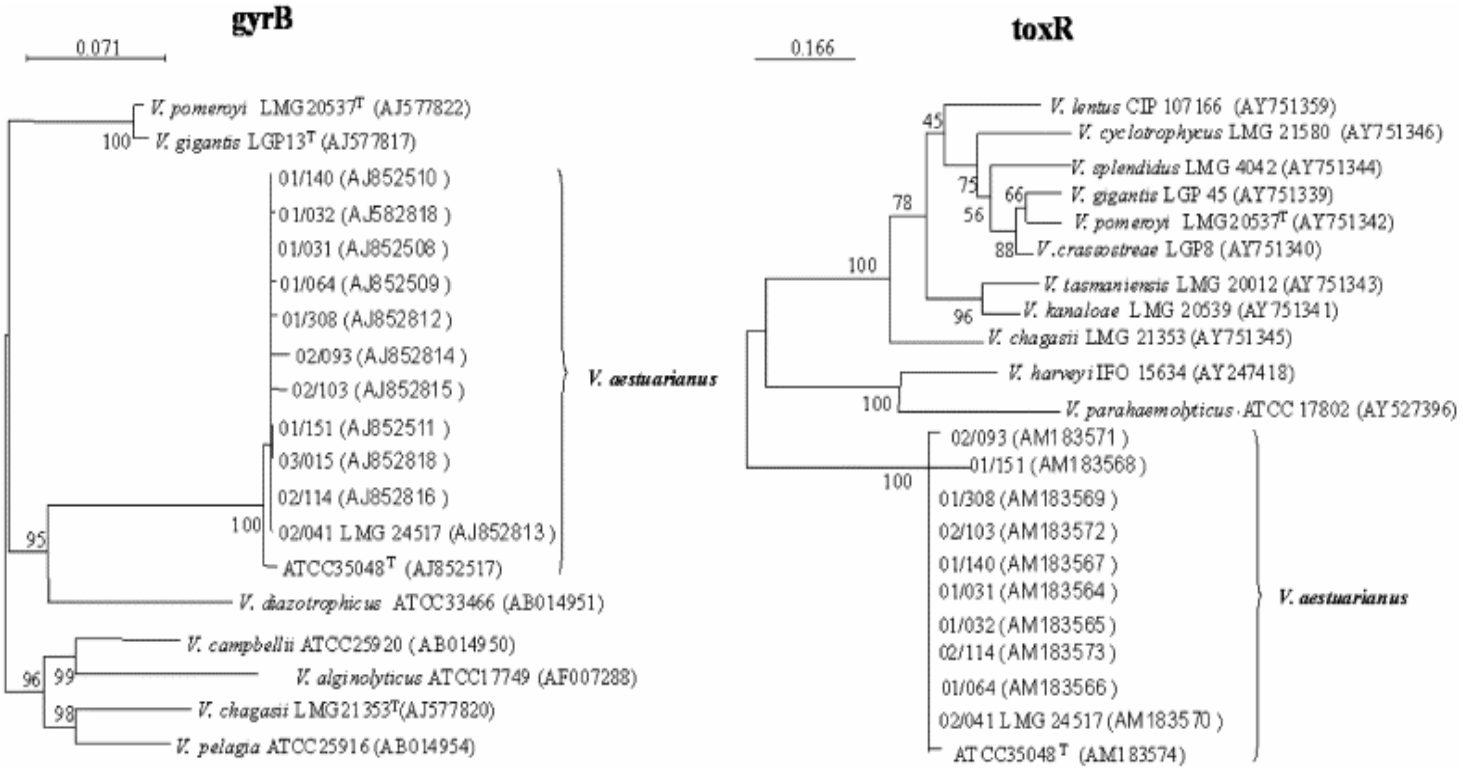


Table 1. Origin of the strains and *p*ercentage of DNA:DNA hybridization (4 replicates). Genomic DNA of 01/032 and ATCC35048^T were labelled with digoxygenin and used as probes to detect the DNA of the different strains isolated. Values superior to 70% are shown in bold.

	Isolation	DNA:DNA hybridization	
Strain	Location/date	01/032 % hybridization	ATCC35048^T % hybridization
01/031	Argenton (hatchery) 01/06/2001	**96**	65
01/032	Argenton (hatchery) 01/06/2001	**100**	54
01/064	Argenton (hatchery) 15/06/2001	**100**	55
01/140	Arcachon 02/07/2001	**81**	56
01/151	La Trinité 11/07/2001	**95**	57
01/308	Normandy 29/08/2001	**100**	**81**
02/041 (LMG24517)	Argenton (hatchery) 05/06/2002	**86**	62
02/093	Bouin (nursery) 23/07/2002	**81**	63
02/103	Baie des Veys 05/09/2002	**100**	**87**
02/114	Argenton (hatchery) 24/09/2002	**94**	**75**
03/015	La Tremblade 24/09/2002	**90**	**80**
V. aestuarianus ATCC35048^T	Oregon, USA 1983	61	**100**
V. orientalis CIP102891^T		17	17
V. pomeroyi LMG21352		9	10

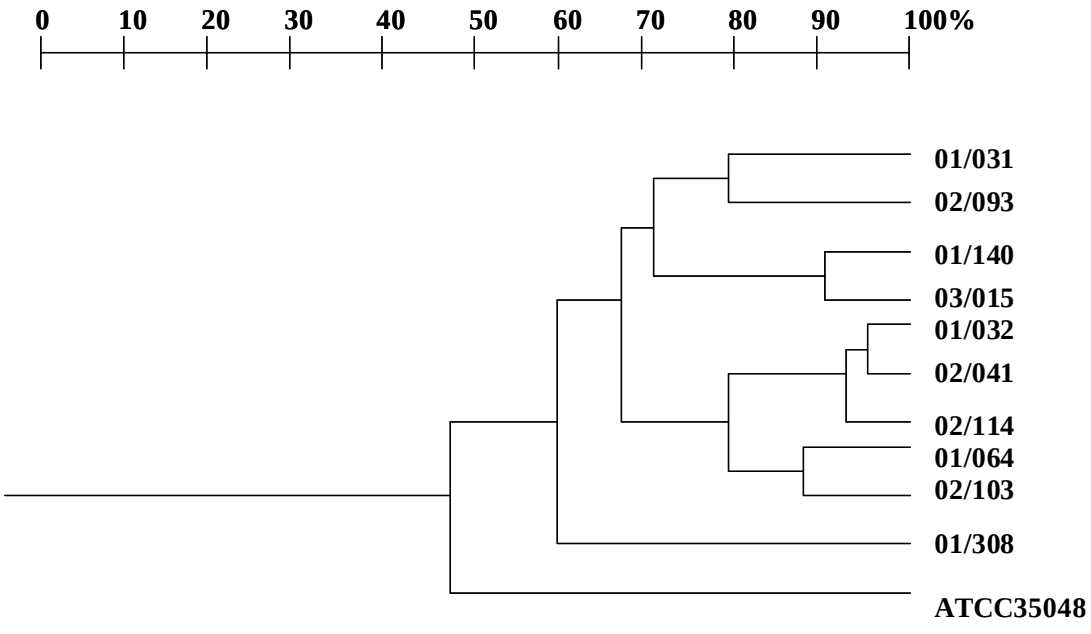
Table 2. Phenotypic characters that differentiate French *V. aestuarianus* isolates (n = 11) (1) from *V. aestuarianus* ATCC35048^T (2), *V. chagasii* (3), *V. anguillarum* (4), *V. lentus* (5), and *V. pomeroyi* (6). +, positive for >90% of strains; (+), positive for 75–89%; -, negative for <10%; (-), negative for 25–11%; (v), variable for 26–74%; nd, no data. Data compiled from The Prokaryotes, [5]; Le Roux et al., [16]; Thompson et al., [25]; Macian et al., [18]; and Austin et al., [2]. The fatty acid composition of *V. anguillarum, V. lentus* is based on one or some of isolates

	1	2	3	4	5	6
Number of isolates	11	1	8	59	12	4
Growth on TCBS	-	+	+	+	+	+
Arginine dihydrolase	(-)	-	v	(+)	+	+
Growth at 35 °C	-	+	+	+	-	+
β-galactosidase	+	+	-	+	nd	+
Indole production	+	+	-	+	v	+
Growth in 6% NaCl	-	+	+	+	+	+
Utilisation of:						
N-acetyl-D-glucosamine	+	+	-	-	+	nd
D-galactose	+	+	-	+	+	+
L-serine	-	+	+	+	+	-
α-cyclodextrin	-	+	-	v	nd	-
D-mannitol	-	+	+	+	+	nd
α-glycyl-L-aspartic acid	-	+	nd	+	nd	nd
L-threonine	-	+	nd	v	+	+
glucose-1-phosphate	-	+	-	+	nd	-
Acid from:						
arabinose	-	-	-	+	-	+
mannose	+	+	-	+	+	v
mannitol	-	+	+	+	+	nd
D-cellobiose	+	+	-	-	+	nd
sucrose	+	+	-	+	-	+
melibiose	-	+	nd	-	+	-
Fatty acids:						
16:1(n-7)"	45.22±2.72	48.42	38.4±3.5	34.7±4.3	41.7	32.9±1.6
16:0"	20.06±2.58	16.01	22.4±3.9	21.9±3.5	24.7	29.2±1.7
18:1(n-7)"	11.90±4.04	19.77	9.7±1.6	7.6	8.7	7.6±1.8
14:0"	7.37±1.98	3.32	7.2±3.5	6	8.8	10.5±0.4
12:0"	2.20±0.26	0.38	3.8±2.0	4.75±1.75	5.3	8.9±1.2

Table 3. Percentage cumulative mortality of *Crassostrea gigas* 10 days after injection of different strains of *Vibrio aestuarianus* with 0.1 ml (10^8 per individual); nd, no data.

Strains	Challenge 1	Challenge 2	Challenge 3
01/308	2	2	nd
01/151	6	nd	nd
02/114	10	nd	nd
ATCC35048^T	10	nd	nd
01/064	15	30	15
01/031	nd	nd	40
02/093	30	45	nd
03/015	35	nd	75
02/103	45	80	nd
01/032	50	70	nd
01/140	55	45	80
02/041 (LMG24517)	55	80	85
Control	0	0	0

Dendrogram of the eleven strains of *V. aestuarianus*, built using 42 differential Biolog tests. The isolate 01/151 had wholly negative responses and was therefore not included.



Biolog characters used to build the dendrogram.

	01/031	01/032	01/064	01/140	01/308	02/041	02/093	02/103	02/114	03/015	ATCC 35048
α-cyclodextrin											+
D-mannitol											+
Mono-methyl succinate									+		
Acetic acid					+						
Glycyl-L-aspartic acid											+
L-threonine											+
L-omithine					+						
L-serine											+
Glucose phosphate											+
cis-aconitic acid					+						+
Citric acid					+						+
Uridine						+					+
Tween 40	+						+				+
Bromosuccinic acid	+				+						+
L-asparagine	+				+						+
Glycogen	+				+		+				+
Tween 80	+		+				+				+
D-alanine		+				+			+		+
L-alanyl-glycine	+				+				+		+
L-proline		+			+		+				+
Inosine		+				+			+		+
Cellobiose	+				+		+			+	+
Maltose	+			+			+			+	+
D,L-a-glycerol phosphate	+	+				+			+		+
Glucose-6-phosphate		+				+	+		+		+
Glycerol	+	+				+			+		+
Dextrin	+			+	+		+			+	+
Methyl D-glucoside		+			+	+		+	+		+
D-gluconic acid		+	+			+		+	+		+
D,L-lactic acid		+				+	+	+	+		+
L-glutamic acid	+	+			+	+		+	+		+
Psicose	+	+	+		+	+	+	+	+		
Succinic acid	+	+	+		+	+	+	+	+		+
L-alanine	+	+			+	+	+	+	+	+	+
L-aspartic acid	+	+	+		+	+	+	+	+		+
N-acetyl-D-glucosamine	+	+	+		+	+	+	+	+	+	+
D-galactose	+	+	+		+	+	+	+	+	+	+
a-D-glucose	+	+	+	+		+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+

Characters of API 20E, API 50CH and sensitivity to antibiotics

	01/031	01/032	01/064	01/140	01/151	01/308	02/041	02/093	02/103	02/114	03/015	ATCC 35048
Sensitivity to:												
oxolinic acid	+	+	+	+	+	+	+	+	+	+	-	-
ampicillin	+	+	+	+	+	-	+	+	+	+	+	+
amoxicillin	+	+	+	+	+	-	+	+	+	+	+	+
chloramphenicol	+	+	+	+	+	+	+	+	+	+	+	+
erythromycin	+	+	+	+	+	+	+	+	+	+	+	+
furanes	+	+	+	+	+	+	+	+	+	+	+	+
kanamycin	+	+	+	+	+	+	+	+	+	+	+	+
norfloxacin	+	+	+	+	+	+	+	+	+	+	+	+
piperacillin	+	+	+	+	+	+	+	+	+	+	+	+
tetracycline	+	+	+	+	+	+	-	+	+	+	+	+
API 20E:												
Beta-galactosidase	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	+	+	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-
Use of citrate	-	+	+	+	+	-	+	+	+	-	-	-
Tryptophan deaminase	+	+	-	+	+	+	+	-	+	-	+	+
Indole production	+	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	-	-	+	+	-	-	+	+	-	-	-	-
Glucose	-	-	+	-	-	-	-	-	-	+	-	-
Mannitol	-	+	-	-	+	-	-	-	-	-	-	+
Inositol	-	+	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	+	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	+	-	-	-	-	-	-	-	-	-	-
Sucrose	-	+	+	+	+	-	-	+	-	+	-	+
Melibiose	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	+	-	-	-	+	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	+	-	-	-	-	-	-	-	-	+	-	-
NO_2 production	+	+	+	+	+	+	+	+	+	+	+	+
API 50CH:												
Glycerol	-	-	-	-	-	-	-	-	-	+	-	-
Ribose	-	-	-	-	+	+	+	+	-	+	+	+
Galactose	-	+	+	+	+	+	+	+	+	+	+	+
Glucose	-	+	+	+	+	-	+	+	+	+	+	+
Fructose	-	+	+	+	+	+	+	+	+	+	+	+
Mannose	-	+	+	+	+	+	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-	-	-	-	+	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	+	-	-
Methyl-α-D-glucopyranoside	-	-	-	-	-	-	+	-	-	-	-	-
N-acetylglucosamine	-	+	+	+	+	-	+	+	+	+	+	+
Amygdalin	-	-	-	-	-	-	+	-	-	-	-	-
Cellobiose	-	-	+	+	-	+	-	-	-	+	+	+
Maltose	-	-	+	+	+	+	-	-	-	+	+	+
Sucrose	-	+	+	+	+	-	+	+	+	+	+	+
Trehalose	-	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-
Starch	-	-	-	+	+	+	-	+	-	+	+	+
Glycogen	-	-	-	+	+	+	-	+	-	+	+	+
Potassium gluconate	-	-	+	+	+	-	-	+	+	+	-	+
Other tests	-	-	-	-	-	-	-	-	-	-	-	-