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TAXONOMIC DESCRIPTION

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Vibrio aestuarianus subsp. *cardii* subsp. nov., pathogenic to the edible cockles *Cerastoderma edule* in France, and establishment of *Vibrio aestuarianus* subsp. *aestuarianus* subsp. nov. and *Vibrio aestuarianus* subsp. *francensis* subsp. nov.

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Abstract

Cockle mortality events have been reported in northern France since 2012. In the present study, we describe and investigate the implication of a potential bacterial causative agent in cockle mortality. Bacteria isolated from five different cockle mortality events were characterized and studied. Using phenotypic analysis combined with DNA–DNA hybridization (DDH) and whole genome sequencing, the isolates were shown to belong to *Vibrio aestuarianus*, a species regularly detected in France during oyster mortality events. Comparison of the strains from cockles with strains from French oysters and the type strain showed that the strains from cockles were genetically different to those from oysters and also different to the *V. aestuarianus* type strain. Moreover, the cockle and oyster strains were classified into two different, but close, groups both separated from the type strain by: (1) analyses of the *ldh* gene sequences; (2) DDH assays between 12/122 3T3^T (LMG 31436^T=DSM 109723^T), a representative cockle strain, 02/041^T (CIP 109791^T=LMG 24517^T) representative oyster strain and *V. aestuarianus* type strain LMG 7909^T; (3) average nucleotide identity values calculated on the genomes; and (4) phenotypic traits. Finally, results of MALDI-TOF analyses also revealed specific peaks discriminating the three representative strains. The toxicity of representative strains of these cockle isolates was demonstrated by experimental infection of hatchery-produced cockles. The data therefore allow us to propose two novel subspecies of *Vibrio aestuarianus*: *Vibrio aestuarianus* subsp. *cardii* subsp. nov. for the cockle strains and *Vibrio aestuarianus* subsp. *francensis* subsp. nov. for the Pacific oyster strains, in addition to an emended description of the species *Vibrio aestuarianus*.

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Keywords: bacterial pathogen; Bivalvia; cockle; mortality; shellfish; *Vibrio aestuarianus*.
Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; FAME, fatty acid methyl ester; SASW, sterilized artificial seawater; TCBS, thiosulfate citrate bile salts sucrose agar.
The GenBank/EMBL/DDBJ accession numbers for the whole genome sequences of *Vibrio aestuarianus* subsp. *cardii* subsp. nov. 12/122 3T3^T (=LMG 31436^T=DSM 109723^T), 15/061 4T2 (=LMG 31438=DSM 109725), 15/064 3T2 (=LMG31439=DSM 109719) and 15/075 3T2 (=LMG 31440=DSM 109720) are JAAKZK000000000, JAAKZL000000000, JAAKZM000000000 and JAAKZN000000000, respectively, under BioProject accession number PRJNA607444. Accession numbers for the 16S, *gyrB* and *ldh* sequences of the same strains are MK307684, MK307691, MK307694, MK307697; MK315009, MK315016, MK315019, MK315022; and MK315026, MK315033, MK315036, MK315039, respectively. Lastly, for these three genes in strains LMG 31437 (=DSM 109724), 12/122 1T3, 12/122 2T3, 12/122 4T3, 12/122 5T3, 15/061 1T1, 15/061 2T2, 15/061 3T1, 15/061 3T2, 15/061 4T4, 15/061 5T2, 15/064 4T2, 15/075 1T2 of *Vibrio aestuarianus* subsp. *cardii* subsp. nov., the accession numbers are MK307681–307683, MK307685–307690, MK307692, MK307693, MK307695, MK307696; MK315006–315008, MK315010–315015, MK315017, MK315018, MK315020, MK315021; and MK315023–315025, MK315027–315032, MK315034, MK315035, MK315037, MK315038, respectively.
For *Vibrio aestuarianus* subsp. *francensis* subsp. nov., accession numbers for strains 01/308, 01/151, 01/032, 02/041^T and 07/115 are JABANK000000000, JABANJ000000000, JABANI000000000, JAAZTV000000000 and JABANH000000000, respectively, under BioProject accession number PRJNA623953.
Finally, for *Vibrio aestuarianus* subsp. *aestuarianus* subsp. nov. LMG 7909T, the whole genome accession number is JAAZTU000000000.
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Five supplementary tables and three supplementary figures are available with the online version of this article.
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INTRODUCTION

The edible cockle *Cerastoderma edule* is one of the most common bivalve species and is widely distributed along the European coasts. It is commercially important in the UK, Netherlands, Spain and France. For many years, mortalities of cockles and dramatic declines in their populations have been reported [1]. Predation, diseases, climatic events, pollution, poor recruitment and over-fishing have all been suggested as driving factors [2]. Cockles are host to a wide variety of viruses, bacteria, fungi and protistans. Many of these micro-organisms are normally innocuous but can become problematic if prevailing environmental conditions are poor. Some parasites have been isolated during mass mortality events (review [1]): Protistan *Marteilia cochillia* in Spain [3], gregarines *Nematopsis scheideri* and *Nematopsis incognito* in Portugal [4, 5], *Amoeba* [6], *Haplosporidia* [7], and a wide range of *Digenea* (trematodes) [8, 9]. Cockles also host emerging parasites, such as *Perkinsus chesapeaki*, newly described in Europe [10]. They can also suffer from a transmissible disease known as disseminated neoplasia [11, 12]. Some studies have suggested that different factors, such as genetic alterations, viruses, retrotransposons and contaminants [12], can act in concert [13]. However, bacteria have rarely been isolated associated with mortalities, apart from mycoplasma-like organisms in Spain [14].

Vibrio are the most abundant cultivable bacteria found in the marine environment. Free, associated with plankton or with marine animals, bacteria belonging to this genus are classified in clades [15–18] clustering more than 100 different species [19]. Some of these species have virulent strains implicated in fish and shellfish diseases either alone, in bacterial consortia [20–22] or in concert with other pathogens [23]. For bivalves, the main pathotypes belong to the *Splendidus* clade (*Vibrio tasmaniensis*, *Vibrio crassostreae*, *Vibrio splendidus*) or the species *Vibrio tapetis*, *Vibrio tubiashii*, *Vibrio bivalvicida*, *Vibrio europaeus* or *Vibrio aestuarianus* [19, 24].

Vibrio aestuarianus was first isolated in the 1980s in the USA from seawater and several shellfish including oysters, clams and crabs presenting no signs of disease [25]. This species was isolated for the first time in France in 2001, during mortality events of Pacific oyster, *Crassostrea gigas*. Garnier *et al.* [26] compared the French strains with the American one and found that the French strains had some different biochemical and pathogenic characters. These authors concluded that the French strains could constitute a new subspecies within *V. aestuarianus* and proposed to name the American strain *V. aestuarianus* subsp. *aestuarianus* and the French strains *V. aestuarianus subsp. francensis*, although these two subspecies were never officially validated.

The present study describes the isolation and classification of bacteria from cockle mortality events that occurred in France in 2012 and 2015. Strains were described and sequenced that allowed us to reproduce and describe this new cockle disease. These strains were compared with the French 'oyster' strains [26] and with the type strain of *V. aestuarianus* (LMG 7909^T) [25], and led us to propose two novel subspecies of *V. aestuarianus*.

ISOLATION AND ECOLOGY

In 2012, mass mortality events of *Cerastoderma edule* were reported in two wild beds on the English Channel coast: Baie des Veys and Baie de Somme, the latter of which is the largest cockle bed in France. In 2015, another mortality event occurred in both these locations as well as in Binic, also on the English Channel coast (Fig. S1, available with the online verion of this article). Fishers reported these mortality events to the competent authority and national network for surveillance and monitoring of mollusc health, REPAMO (REseau de PAthologie des MOllusques), in charge of recording mortality cases and analysing representative samples associated with them. In 2012 and 2015, 15–30 adult cockles were collected from the different wild beds. Each collected individual was examined for macroscopic characteristics and processed using either histological or bacteriological analysis in addition to molecular analyses (Table S1). The mortality rate in 2012 was estimated at 80–89% according to site. For each site, the mortality rate was lower in 2015 than in 2012 ranging from 33 to 49% (Table S1). In all cases, cockle mortality was uniform, massive and sudden, concerning mainly adults. No other species were affected by these mortality events except in Baie des Veys where a number of stocks of *Crassostrea gigas* spat and juveniles also suffered mortalities. The cockle mortality occurred during summer and lasted from 1 to 2 months, according to fishermen. No specific environmental events were reported during the summers of 2012 or 2015, except a high level of macroalgae at Binic. The sampled individuals were mainly moribund, lying on the bottom and characterized by very slow valve closing valves and limited intrapalleal fluid. No specific macroscopic signs such as pustules, conchyolin deposits or necrosis and degradation of the hinge could be observed.

For the detection and quantification of bacterial pathogens, pieces of gill, muscle and mantle of five moribund cockles per sample were individually crushed in 150 µl sterile seawater, diluted 100-fold and 10000-fold and spread onto marine agar (Conda) incubated for 48–72 h at 20 °C. Colonies were enumerated and those bacterial colonies found in abundance were isolated and tested by duplex real time PCR for the detection of *V. aestuarianus* and bacteria of the *Splendidus* clade [27]. Pure cultures of these bacterial strains were conserved frozen at −80 °C in Zobell broth with 15% glycerol. These bacteriological analyses revealed that all analysed samples yielded numerous bacterial colonies. The species *V. aestuarianus* was detected in all samples after 48 h at 20 °C (Table S2). Bacteria of the *Splendidus* clade were also isolated in some samples but these bacteria were not the dominant strains in the Petri dishes. Moreover, *V. aestuarianus* DNA was detected by molecular analyses [28] in all samples, at a generally high detection frequency. DNA of OsHV-1 (Ostreid Herpevirus type 1) known to contribute to mortality events in *Crassostrea gigas* [23, 29], was not detected in any of the samples (Table S2).

Fifteen to twenty individuals per sample were also processed for histological analyses. After 48 h in Davidson's fixative,

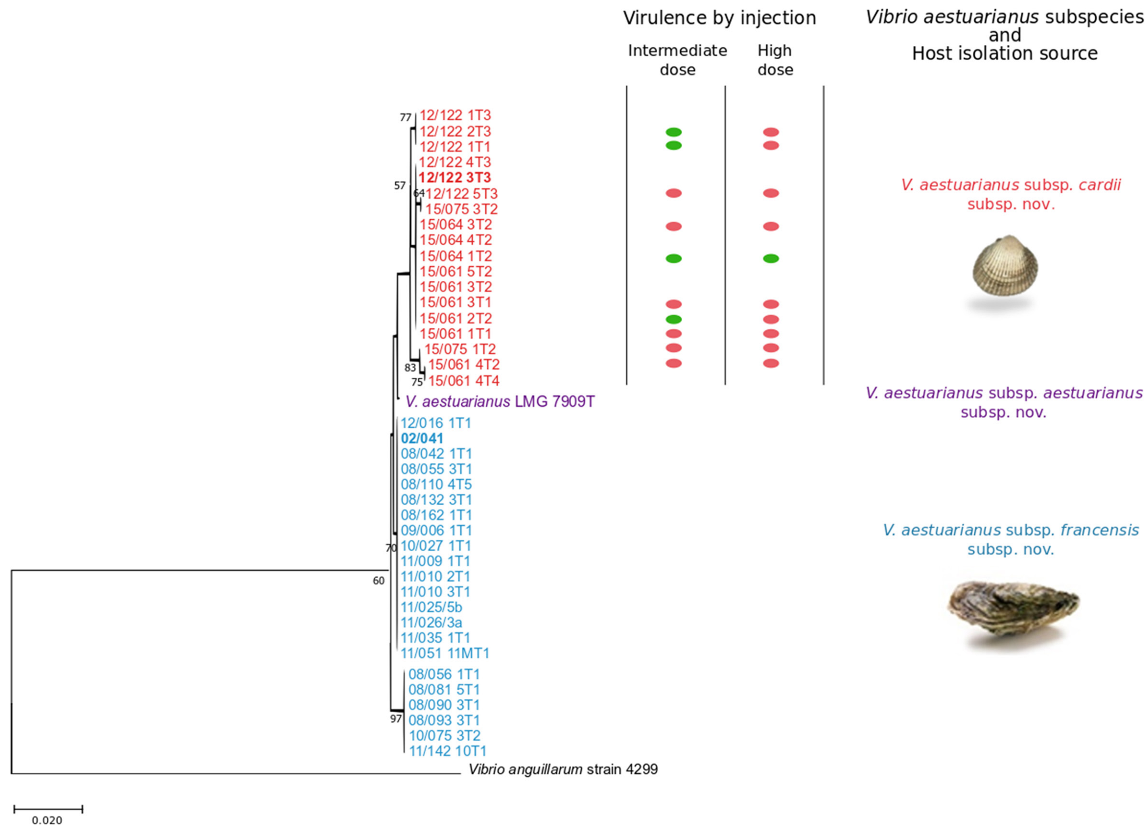


Fig. 1. Phylogenetic position of *Vibrio aestuarianus* subsp. *cardii* subsp. nov. and *V. aestuarianus* subsp. *francensis* subsp. nov. strains and virulence of *V. aestuarianus* subsp. *cardii* subsp. nov. Molecular phylogenetic analysis by a maximum-likelihood method based on the Kimura two-parameter model conducted in MEGA X. The analysis involved 42 nucleotide sequences with a total of 570 positions. Proposed type strains are indicated in bold. Virulence was tested by intramuscular injection of hatchery-produced cockles, using two bacterial doses (high, OD 600 nm=0.4; intermediate, OD 600 nm=0.04). Strains causing more than 70% mortality are indicated by a red oval.

tissues were maintained in 70% ethanol until histological preparation by dehydration and embedding in paraffin following standard procedures. Sections of 2–3 µm thickness were stained with haematoxylin and eosin. Histological analyses revealed the presence of other organisms at low infection intensity (a few parasites per individual), despite a high prevalence in some cases such as gregarine spores or rickettsia-like organisms (Table S2).

16S rRNA GENE PHYLOGENY

A total of 59 bacterial strains were isolated during periods when the REPAMO surveillance network reported high levels of cockle mortalities. After isolation, the predominant bacteria appeared small (1–2 mm), translucid, regular and white to cream-coloured on marine agar after 48 h at 20 °C (Fig. S2). For genotyping, DNA was extracted by boiling in distilled water [30] and the *ldh* (lactate dehydrogenase) gene was amplified and sequenced according to methods previously described [20], as were the 16S rRNA and *gyrB* genes [17] (Table S4). Sequences were aligned and phylogenetic trees built using MEGA X [31]. The trees were drawn using the neighbour-joining method with the Kimura two-parameter model [32] and the maximum-parsimony method [33]. A total of 17 isolates (isolated in 2012 and 2015 from cockle mortality events) were identified as belonging to *V. aestuarianus* by qPCR (Table S3) and gene sequencing (16S rRNA and *gyrB*; Table S4). They showed a high degree of similarity to the 16S rRNA gene reference sequences of *V. aestuarianus* (99% to the type strain LMG 7909^T). Phylogenetic analyses based on the *ldh* gene sequence of these strains led to their affiliation to the species *V. aestuarianus*, in a group close to but apart from the oyster strains and type strain. Moreover, oyster strains formed an individual branch clearly separated from the type strain of *V. aestuarianus* (Fig. 1) [26].

This classification was confirmed by DNA–DNA hybridization (DDH) performed by the BCCM/LMG laboratory at Ghent University (Belgium). After genomic DNA extraction according to a modified Wilson protocol [34], hybridizations were performed in microplates at 40 °C [35] and DNA relatedness values (%) based on means of a minimum of six hybridizations. DDH of two strains isolated from diseased cockles in 2012 and 2015 and one strain isolated

Table 1. DDH values (%) measured by comparing *Vibrio aestuarianus* strains from cockles and oysters with *V. aestuarianus* LMG 7909^T and other close species

Strains: 1, *V. aestuarianus* subsp. *cardii* subsp. nov. 12/122 3T3^T; 2, *V. aestuarianus* subsp. *cardii* subsp. nov. 15/061 4T2; 3, *V. aestuarianus* subsp. *francensis* subsp. nov. 02/041^T; 4, *V. aestuarianus* subsp. *aestuarianus* subsp. nov. LMG 7909^T; 5, *Vibrio furnisii* LMG 7910^T; 6, *Vibrio ordalii* LMG 13544^T; 7, *Vibrio salilacus* JCM 19265^T.

Strain	1	2	3	4	5	6	7
V. aestuarianus subsp. *cardii* subsp. nov. 12/122 3T3^T	100						
V. aestuarianus subsp. *cardii* subsp. nov. 15/061 4T2	84 (7)	100					
V. aestuarianus subsp.*francensis* subsp. nov. 02/041^T	80 (18)	80 (11)	100				
V. aestuarianus subsp.*aestuarianus* subsp. nov. LMG 7909^T	70 (2)	71 (5)	79 (22)	100			
V. furnisii LMG 7910^T	14 (3)	18 (4)	16 (5)	17 (20)	100		
V. ordalii LMG 13544^T	14 (12)	16 (5)	16 (5)	9 (16)	8 (19)	100	
V. salilacus JCM 19265^T	15 (4)	16 (4)	12 (2)	ND	ND	ND	100

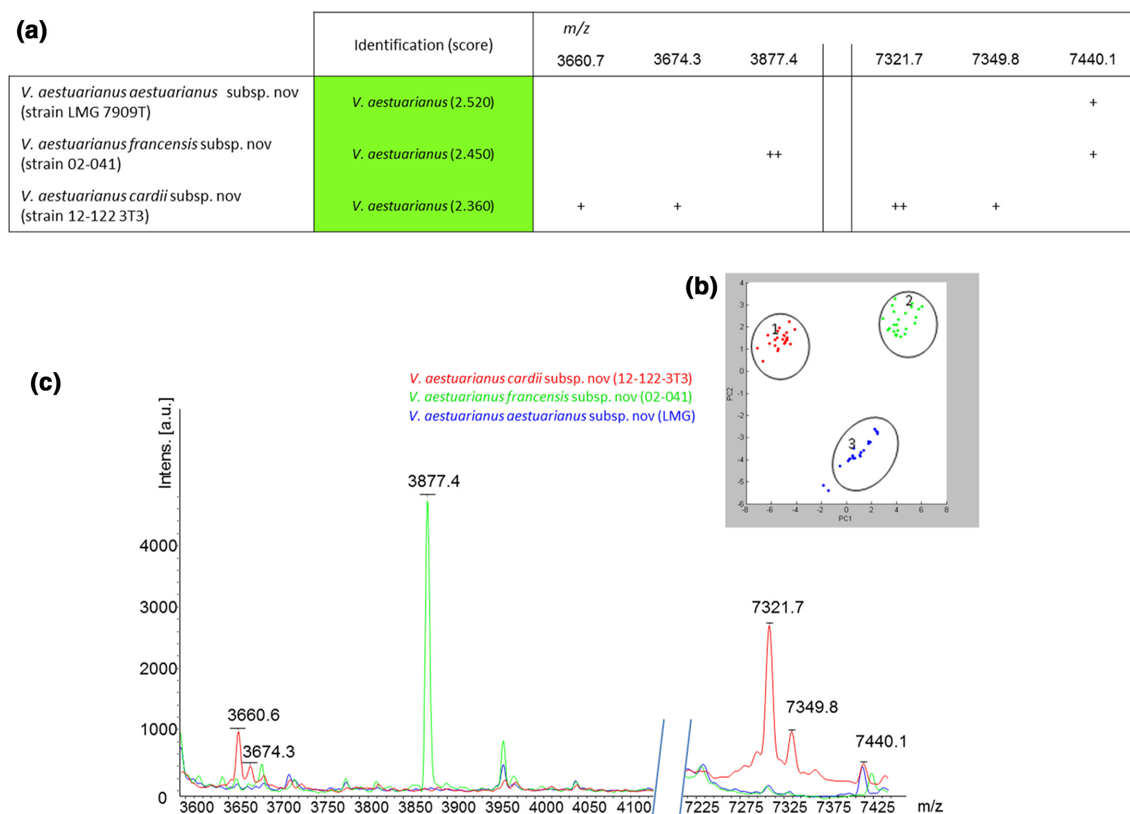
from diseased oysters were performed with *V. aestuarianus*, *V. ordalii* and *V. salilacus* reference strains. With more than 70% hybridization to the *V. aestuarianus* type strain, we confirmed the affiliation of the cockle and oyster strains to the species *V. aestuarianus*. Indeed, hybridizations were 70, 71 and 79% with the *V. aestuarianus* type strain for strains 12/122 3T3^T, 15/061 4T2 and 02/041^T, respectively, and were 80 and 80% with the *V. aestuarianus* oyster strain for cockle strains 12/122 3T3^T and 15/061 4T2, respectively (Table 1). Garnier *et al.* generally found similar results for hybridizations between the *V. aestuarianus* type strain and different oyster strains: their hybridizations were between 54 and 87% [26].

The three representative strains for the three subspecies were further studied by whole-cell MALDI-TOF MS profiling. MALDI-TOF mass spectrum measurements and processing were carried out as described by Mougin *et al.* [36]. The analyses were performed using Flex Analysis (version 3.4) and ClinProTool (version 3.0) software (Bruker Daltonics). Fig. 2 shows the comparison of the MALDI-TOF spectra. First, all three strains were identified with Bruker database as *V. aestuarianus* with correct scores. Moreover, significant differences observed between spectra (12 per strain) with prominent mass ions at 3877.4 and 7321.7, characteristic for the *V. aestuarianus* subsp. *francensis* subsp. nov. type strain and the *V. aestuarianus* subsp. *cardii* subsp. nov. type strain, respectively. The dendrogram based on whole-cell MALDI-TOF mass spectra as well as principal component analysis demonstrated a clear separation of the three strains. Comparison in other isolates is now needed to confirm the peak combination allowing discrimination [37].

Genome features

The existence of three distinct and coherent groups was also supported by results from the comparison of the complete genomes of four cockle isolates, the genome of the *V. aestuarianus* type strain and the five genomes already available for different *V. aestuarianus* oyster strains. Briefly, whole genomes of cockle isolates were sequenced by Biofidal (Vaulx-en-Velin, France) after DNA isolation using NucleoSpin microbial DNA (Macherey-Nagel). DNA libraries were made from 1 ng genomic DNA using Nextera XT DNA kit (Illumina). Paired-end isolate sequencing (2×300) was performed on a MiSeq system (Illumina). Genomes were assembled *de novo* using SPAdes (version 3.11.0 [38]), with read error corrections and 20× coverage cut-off. Assembly quality was checked using QUAST [39]. Assemblies were added in MAGE [40], excluding contigs with lengths below 1000 bp. Oyster and cockle *V. aestuarianus* strain draft genomes presented typical features for the species. They ranged between 4.02 and 4.51 Mb, with a relatively close number of CDSs, genes coding for transfer RNA (between 91 and 102 for cockle isolates and between 84 and 85 for oyster isolates) and G+C contents (42.68–42.89 mol% for the cockle isolates and 42.52–42.83 mol% for the oyster isolates) (Table 2). 16S sequences and whole genome comparisons were performed by calculating average nucleotide identity (ANI) between strains (JSpeciesWS [41]). A distance matrix was calculated by inversing ANI results based on whole genomes. Similarity percentages calculated between 16S sequences extracted from whole genomes ranged from 99.09–100% (data not shown). All values were above the 98.7% cut-off generally accepted for differentiating two species [42]. Moreover, ANI values calculated with whole genomes indicated an intra-clade similarity of 99.21–99.99% (mean value of 99.69%) for the *V. aestuarianus* oyster strains and an intra-clade similarity ranging from 97.88 to 99.99% (mean value of 98.47%) for the cockle isolates. The inter-clade similarity ranged from 96.83 to 97.8% depending on the isolates (Table S5) and confirmed that all strains belong to the same species (ANI >95%) but can be classified into three different subspecies.

Comparison of predicted CDSs revealed a core genome, shared by all three strains, with 2858 CDSs and specific genomes containing 611–797 specific CDSs (Fig. S3). As oyster and cockle isolates are mollusc pathogens, the predicted virulome was compared in the three representative strains. Globally, the three strains contain homologous



(a)

	Identification (score)	*m/z* 3660.7	3674.3	3877.4	7321.7	7349.8	7440.1
V. aestuarianus aestuarianus subsp. nov (strain LMG 7909T)	*V. aestuarianus* (2.520)						+
V. aestuarianus francensis subsp. nov (strain 02-041)	*V. aestuarianus* (2.450)			++			+
V. aestuarianus cardii subsp. nov (strain 12-122 3T3)	*V. aestuarianus* (2.360)	+	+		++	+	

Fig. 2. MALDI TOF analyses. Spectra of three representative strains were assigned and compared by principal component analysis: LMG7909^T for *V. aestuarianus* subsp. *aestuarianus* subsp. nov 02/041^T for *V. aestuarianus* subsp. *francensis* subsp. nov. and 12/122 3T3^T for *V. aestuarianus* subsp. *cardii* subsp. nov. (a) Bacterial strain identification by MALDI-TOF analysis using the Bruker database. Presence/absence of discriminatory peaks revealed by PCA is also indicated. (b) Twelve spectra were compared by PCA clustering with the MBT compass. (c) MALDI-TOF spectra overview. Major discriminatory peaks are indicated.

CDSs encoding putative virulence effectors, virulence regulators, secretion systems (SST1, SST2, SST4 and SST6) and pili/flagella/chemotaxis-related proteins. But, some predicted virulence factors are missing in the different strains as haemolysin co-regulated protein in LMG7909^T, zonular occludens toxin zot and T1SS-2 in 12/122 3T3^T and a complete T6SS locus in 02/041^T. However, their implication in pathogenesis must definitively be demonstrated.

PHYSIOLOGY AND CHEMOTAXONOMY

The phenotypes of selected isolates were characterized in different ways. Bacterial growth was observed on marine agar, thiosulfate citrate bile salts sucrose agar (TCBS) medium (AES Chemunex) and CHROMagar *Vibrio* medium (CHROMagar) (Fig.S2). The capacity of bacteria to grow in Zobell broth with 0–10% NaCl at 20 °C or with 2% NaCl at 4, 10, 37 and 40 °C was determined (Table 3).

Antibiograms were carried out using disc-diffusion assays on Mueller–Hinton agar (Oxoid) with commercial impregnated discs (Oxoid): flumequine 30 µg (UB30), kanamycin 30 µg (K30), tetracycline 30 µg (TE30), sulfamethoxazole/trimethoprim 19:1 25 µg (SXT25), streptomycin 25 µg (S25), sulfonamides 300 µg (S3), erythromycin 30 µg (E30), penicillin 10 UI (6 µg) (P10), Trimethoprim 5 µg (W5), Chloramphenicol 30 µg (C30), ampicillin 10 µg (AMP10), gentamicin 30 µg (CN30) or the vibriostatic agent, pteridine 150 and 10 µg (O129/150 and O129/10). Inhibition was observed after 24–48 h at 22 °C.

API 20E, API 20NE and API 50CH kits (bioMérieux) were used in accordance with the manufacturer's recommendations. Fresh colonies, obtained from culture bacteria on tryptone soya agar (Difco) adjusted to 2% NaCl, were suspended in SASW to obtain about OD_{600}=0.1. After inoculation, the strips were incubated for 48 h at 20 °C and results were determined according to the manufacturer's instructions. Finally, FAME (fatty acid methyl ester) determination was performed by the BCCM/LMG laboratory at Ghent University using a standard commercial identification system (MIDI) and gas chromatography.

The 17 cockle strains and two oyster strains were able to grow in Zobell liquid medium at different temperatures from 4 to 37 °C, with a limited growth rate at the extremes. However, no growth was observed at 40 °C, with or without 6% salt. Only the *V. aestuarianus* type strain was able to

Table 2. Genome characteristics for *Vibrio aestuarianus* subsp. *cardii* subsp. nov., *V. aestuarianus subsp. francensis* subsp. nov and *V. aestuarianus subsp. aestuarianus* subsp. nov. type strain (LMG 7909[T])

Strain	Source	Genome size (bp)	No. of CDS	No. of tRNA	G+C content (%)	N_{50} (bp)	L_{50}	CheckM Completness	CheckM contamin.	Accession no.	No. of reads
12/122 3T3[T]	*Cerastoderma edule*	4350835	4449	97	42.73	50782	25	100%	0.91%	JAAKZN000000000	694942
15/061 1T1	*Cerastoderma edule*	4099086	4205	102	42.89	43962	29	100%	0.91%	JAAKZK000000000	843350
15/064 3T2	*Cerastoderma edule*	4024371	4101	91	42.79	44282	29	99.93%	0.83%	JAAKZM000000000	703751
15/075 3T2	*Cerastoderma edule*	4394098	4614	98	42.68	55001	26	99.90%	1.10%	JAAKZL000000000	1091426
LMG 7909[T]	Type strain	4229739	4020	103	42.67	96002	16	100%	0.09%	JAAZTU000000000	897933
01/032	*Crassostrea gigas*	4204160	4188	84	42.83	–	–	99.72	0.12%	JABANI000000000	–
01/151	*Crassostrea gigas*	4368412	4350	84	42.73	–	–	100%	0.12%	JABANJ000000000	–
01/308	*Crassostrea gigas*	4510343	4534	85	42.52	–	–	100%	0.16%	JABANK000000000	–
02/041[T]	*Crassostrea gigas*	4196441	4063	84	42.83%			99.72%	0.12%	JAAZTV000000000	–
07/115	*Crassostrea gigas*	4245988	4243	84	42.79	–	–	99.72%	0.12%	JABANH000000000	–

grow in the presence of 6% salt. All the strains, like the type strain, were shown to be sensitive to the tested antibiotics (Table 3). Cockle and oyster strains appeared under blue light on CHROMagar medium and, unlike the type strain, had a limited growth capacity on TCBS medium, where a few yellow colonies appeared (Fig. S2). The observations of the oyster strains and the type strain were similar to those made by Garnier *et al*. [26]. Additional biochemical characterization analyses were performed to test this proposition, using API20E and 50CH strips (Table 4) and FAME analyses (Table 5). The strains isolated from cockles and oysters, like the *V. aestuarianus* type strain, could form acids from D-glucose, sucrose, trehalose, *N*-acetyl-D-glucosamine and had β-galactosidase activity and indole production. Cockle strains could be distinguished from oyster strains by their utilization of D-mannitol and arginine and fermentation of starch, D-ribose and glycogen. They could be distinguished from the type strain by the non-fermentation of potassium gluconate. The oyster strains could not use D-mannitol and arginine. However, as fermentation and utilization of substrates appeared variable among the cockle and oyster strains, no character clearly separated the three groups of strains.

Table 3. Key phenotypic characteristics (antibiotic sensitivity and growth capacity) of *Vibrio aestuarianus* strains isolated from cockles and oysters compared with the *V. aestuarianus* type strain

Strains: 1, 17 strains from cockles; 2, *V. aestuarianus* LMG 7909[T]; 3, oyster strain 02/041[T]; 4, oyster strain 02/093; 5, Results of Garnier *et al*. for 11 oyster strains [26]. Flumequine 30 µg (UB30), kanamycin 30 µg (K30), tetracycline 30 µg (TE30), sulfamethoxazole/trimethoprim 19:1 25 µg (SXT25), streptomycin 25 µg (S25), sulfonamides 300 µg (S3), erythromycin 30 µg (E30), penicillin 10 UI (6 µg) (P10), trimethoprim 5 µg (W5), chloramphenicol 30 µg (C30), ampicillin 10 µg (AMP10), gentamicin 30 µg (CN30) or the vibriostatic agent, pteridine 150 µg and 10 µg (O129/150 and O129/10). +, Positive; –, negative; ND, no data.

Characteristic	1	2	3	4	5
AMP10, C30, E30, K30, TE30	+	+	+	+	+
P10, UB30, SXT25, S25, S3, W5, CN30, O129/150, O129/10	+	+	+	+	ND
Growth at 4 and 20 °C	+	+	+	+	+
Growth at 35 and 37 °C	+	+	+	+	–
Growth at 40 °C or in absence of salt	–	–	–	–	–
Growth in 6% NaCl	–	+	–	–	–
Growth on TCBS	–	+	–	–	–

Table 4. Key differential phenotypic characteristics (API) of *Vibrio aestuarianus* strains isolated from cockles and oysters from the *Vibrio aestuarianus* type strain

Strains: 1, 17 strains from cockles; 2, *V. aestuarianus* LMG 7909[T]; 3, oyster strain 02/041[T]; 4, oyster strain 02/093; 5, results of Garnier *et al.* for 11 oyster strains [26]. +, Positive for >90% of the strains; (+), positive for 75–89%; –, negative for <10%; (–), negative for 25–11%; v, variable for 26–74%.

Characteristic	1	2	3	4	5
Fermentation of:					
D-Glucose	+	+	–	–	(+)
N-Acetyl-D-glucosamine	(+)	+	+	+	(+)
Sucrose and trehalose	+	+	+	+	+
Potassium gluconate	–	+	+	–	v
D-Galactose	+	+	–	–	+
Maltose	(+)	+	–	+	v
Cellobiose	(+)	+	–	–	v
D-Fructose and D-mannose	+	+	+	–	+
Starch, D-ribose and glycogen	(+)	+	–	–	v
Arginine dihydrolase	+	+	+	+	(–)
β-Galactosidase	+	+	+	+	+
Tryptophan deaminase	–	–	–	–	v
Gelatinase	v	+	–	+	v
Indole production	+	+	–	+	+
Utilization of D-mannitol	v	–	–	–	–
Utilization of citrate	(–)	–	–	–	v
LDC, ODC, VP, H2S, URE, INO, SOR, RHA, MEL, ARA, GLY, ERY, DXYL, LXYL, ADO, MDX, SBE, DUL, MDM, MDG, AMY, ARB, ESC, SAL, LAC, INU, MLZ, RAF, XLT, GEN, TUR, LYX, TAG, DFUC, LFUC, DARL, LARL, 2KG, 5KG	–	–	–	–	–

Other characters that can also differentiate the strains include their fatty acids (Table 5). *V. aestuarianus* strains can be distinguished from *V. ordalii* and *V. furnisii* by their lower level of 3 hydroxy-dodecanoic acid and higher level of iso-$C_{16:0}$. Between the *V. aestuarianus* strains, differences were observed for $C_{12:0}$ 3-OH, iso-$C_{14:0}$ and $C_{14:0}$, for which the cockle strains appeared intermediate and the oyster strains had a high level compared with the type strain. However, the most remarkable difference concerned $C_{18:1}$ ω7*c*, which was present at a higher level in the type strain compared with the oyster (low level) or cockle strains (intermediate level). In contrast, the oyster and cockle strains showed a high level of the $C_{16:0}$ compared to the type strain.

Finally, the pathogenicity of nine cockle isolates (12/122 1T1, 12/122 2T3, 12/122 3T3[T], 12/122 4T3, 15/061 1T1, 15/061 4T2, 15/064 3T2, 15/075 1T2, 15/075 3T2) on reared cockles was investigated by experimental injections, which also included oyster strain 02/041[T], pathogenic for oysters, and *V. splendidus*-related 15/064 1T2, isolated from cockles (Fig. 1). Cockles were induced to spawn in September 2015 at the Ifremer hatchery in La Tremblade and larvae were grown according to standard protocols used for *C. gigas*, except that seawater temperature was maintained at 20 °C for cockle larvae [43]. Settlement took place from 11 to 16 days post fertilization. All progenies were maintained under controlled environmental conditions in our facilities using unheated UV treated (40 mj cm^{-2}) seawater in a flow-through system. All animals were fed *ad libitum*. Following an initial screening of 30 of these animals (no detection of *Vibrio aestuarianus* on 159 screened colonies, no detection of *V. aestuarianus* or herpesvirus OsHV-1 DNA and no detection of any other pathogens by histology) and in the absence of mortality over the rearing period, we chose to use this biological material for the controlled infections. Experiments were conducted in May and June 2016 when cockles weighed 4.39 g (s.d. 0.80) and measured 23.28 mm (s.d. 1.52).

Bacteria isolated from diseased cockles as well as controls (*V. splendidus*-related from cockles 15/064 1T2 and *V. aestuarianus* oyster strain 02/041[T]) were grown at 20 °C for 20 h in marine broth with constant shaking at 40 r.p.m. (Rotator SB3, Stuart). After an SASW wash, bacterial concentrations were evaluated spectrophotometrically at an optical density (OD)

Table 5. Fatty acid compositions (as percentage of the total fatty acids present) in *Vibrio aestuarianus* strains from cockles and oysters, *V. aestuarianus* type strain and related species

Strains: 1, *V. aestuarianus* subsp. *cardii* subsp. nov. 12/122 3T3^T; 2, *V. aestuarianus* subsp. *francensis* subsp. nov. 02/041^T; 3, *V. aestuarianus* subsp. *aestuarianus* subsp. nov. LMG 7909^T; 4, *V. furnisii* LMG 7910^T; 5, *V. ordalii* LMG 13544^T. TR, Trace; –, not detected. Values are percentages of total fatty acids.

Fatty acid	1	2	3	4	5
$C_{12:0}$	4.4 (0.4)	4	3.2	6.7	–
$C_{12:0}$ 3-OH	2.1 (0.4)	3.4	1.3	6.1	4.5
iso-$C_{14:0}$	1.2 (0.1)	2.1	TR	–	–
$C_{14:0}$	4.8 (1.7)	8.7	4	4.5	5.9
iso-$C_{16:0}$	4.1 (1.4)	5.4	5.6	TR	–
$C_{16:0}$	18.6 (1.5)	16.3	13.4	12	19.2
$C_{18:1}$ ω7*c*	14.5 (2.7)	11.3	22.6	17.1	23.4
Summed feature 2	2.9 (1.6)	2.3	1.1	6.7	1.5
Summed feature 3	41.6 (1.8)	34.6	41.8	37.5	39.8

of 600 nm, adjusted to 0.4 and 0.04 and checked by plating. Cockles were anesthetized for 2 to 4 h in a magnesium chloride solution ($MgCl_2$; Fluka) at a final concentration of 50 g l^{-1} (1/4: v/v seawater/freshwater) under aeration. Subsequently, 50 µl bacterial suspension (10^7 c.f.u. to 10^6 per cockle) was injected into the adductor muscle. A group of cockles were injected with SASW to serve as negative controls. After injection, the cockles were transferred to tanks (triplicates of 10 cockles in 2 l) filled with UV-treated, 1 µm-filtered seawater and maintained under static conditions at 20 °C with aeration. Mortality was monitored twice a day and any newly dead cockles were removed from the tanks over a 7 day period. Animals were considered to be dead when the valves did not close following stimulation.

After 24 h, cumulative mortalities ranged from 0–83% for cockles injected with *V. aestuarianus* cockle strains. Mortalities continued over 7 days, with a peak during the first 2 days. For all moribund cockles, the predominant bacterial isolates were obtained from muscle and haemolymph (205 strains screened from 116 animals, all belonging to *V. aestuarianus* species using the *dnaJ*-qPCR diagnosis assay). We also noticed the high levels of bacteria belonging to the *Splendidus* clade in 31 individuals. No mortality was noted in the control or in cockles injected with *V. splendidus*-related 15/064 1T2 or *V. aestuarianus* oyster strain 02/041^T.

Garnier *et al.* [26] also tested the pathogenicity of 11 *V. aestuarianus* oyster strains and the *V. aestuarianus* type strain by injection of 10^8 bacteria per oyster. They observed mortality ranging between 2 and 55% depending on the oyster strain, whereas a low mortality (10%) was observed with the *V. aestuarianus* type strain. The same observation was made by Goudenège *et al.* [20], who classified oyster strains as either highly virulent for oysters (i.e. inducing $>50\%$ mortalities at 10^2 c.f.u. animal^{-1}) or non-virulent (i.e. inducing $<50\%$ mortalities at 10^7 c.f.u. animal^{-1}).

In summary, *V. aestuarianus* isolates obtained from oysters or cockles present phenotypic and genetic traits distinct from the *V. aestuarianus* type strain and these differences support the separation of these oyster and cockle strains into two novel subspecies of *V. aestuarianus* distinct from *V. aestuarianus* LMG 7909^T and other *V. aestuarianus* strains. Therefore, we propose that *V. aestuarianus* should be divided into three subspecies, for which the names *Vibrio aestuarianus* subsp. *aestuarianus* subsp. nov., *Vibrio aestuarianus* subsp. *francensis* subsp. nov. and *Vibrio aestuarianus* subsp. *cardii* subps. nov. are proposed, with strains LMG 7909^T, 02/041^T and 12/122 3T3^T as the type strains, respectively.

EMENDED DESCRIPTION OF *VIBRIO AESTUARIANUS*

The characteristics modified or added to those reported in original descriptions by Tison and Seidler [25] are as follows:

Vibrio aestuarianus is a Gram-negative, oxidase-positive, motile bacterium. Colony morphology is variable according to the subspecies. Growth occurs on marine agar from 4 to 30 °C with an optimum between 20 and 25 °C and strains do not grow at 40 °C or in the absence of NaCl. Most strains utilize *N*-acetyl-D-glucosamine, β-galactosidase and produce indole. Strains ferment sucrose, D-mannose, D-fructose, D-glucose, D-galactose and trehalose. Strains are susceptible to ampicillin, erythromycin, chloramphenicol, kanamycin and tetracycline. The major fatty acids are $C_{12:0}$ (2.2–4.4%), $C_{12:0}$ 3-OH (1.3–3.4%), $C_{14:0}$ (4–8.7%), iso-$C_{16:0}$ (4.1–5.6%), $C_{16:0}$ (13.4–20.06%) and $C_{18:1}$ ω7*c* (11.3–22.6%).

The genome size is 3.9–4.5 Mb (GenBank genome accession number JAAZTU000000000) and the genomic DNA G+C content is 42.5–42.9 mol%.

The type strain is ATCC 35048^T (=DSM 19606^T=CAIM 592^T=CCUG 28583^T=CECT 625^T=CIP 102971^T=LMG 7909^T=NBRC 15629^T=NCMB 2236^T). The GenBank/EMBL/DDBJ accession number for the genome of the type strain is JAAZTU000000000.

DESCRIPTION OF *VIBRIO AESTUARIANUS SUBSP. AESTUARIANUS* SUBSP. NOV.

Vibrio aestuarianus subsp. *aestuarianus* Tison and Seidler 1983 (a.es.tu.ri.a′nus. N.L. masc. adj. *aestuarianus*, pertaining to an estuary).

The description is essentially the same as that given above for the species *Vibrio aestuarianus*, with the following additions.

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 37 °C. It has gelatinase and arginine dihydrolase activity and utilizes starch, D-ribose, maltose, cellobiose, glycogen and potassium gluconate. Among the three subspecies, it presents the highest

level of $C_{18:1}$ ω7*c* and the lowest level of $C_{12:0}$ 3-OH and iso-$C_{14:0}$ fatty acids. The type strain does not induce mortality of Pacific oyster, *Crassostrea gigas*. The genome size is predicted around 4.2 Mb and genomic DNA G+C content is 42.6mol% (GenBank genome accession number JAAZTU000000000).

The type strain is ATCC 35048^T (=DSM 19606^T=CAIM 592^T=CCUG 28583^T=CECT 625^T=CIP 102971^T=LMG 7909^T=NBRC 15629^T=NCMB 2236^T).

DESCRIPTION OF *VIBRIO AESTUARIANUS SUBSP. FRANCENSIS* SUBSP. NOV.

Vibrio aestuarianus subsp. *francensis* Garnier *et al*. 2008 (fran. cen′sis. N.L. masc. adj. *francensis*: of, or belonging to, France).

It displays typical characteristics for the species *V. aestuarianus*. Colonies are white and slightly opaque on marine agar and are less than 1 mm in diameter after 24 h at 20 °C. No strain can grow in the absence of NaCl or in the presence of 6% NaCl (except for strain 02/041^T) or on TCBS. Growth occurs from 4–30 °C. Most strains have no arginine dihydrolase and cannot use D- mannitol. Some strains use citrate. It presents the highest level of $C_{16:0}$ and $C_{14:0}$ and the lowest level of $C_{18:1}$ ω7*c* compared to the two other subspecies.

The majority of strains, including the type strain 02/041^T, are pathogenic to the Pacific oyster, *Crassostrea gigas*, but the type strain is not pathogenic to edible cockles, *Cerastoderma edule*. The genome size is estimated around 4.18–4.53 Mb with the draft genomes, and genomic DNA G+C content is about 42.52–42.83 mol%.

The type strain is 02/041^T (LMG 24517^T=CIP 109791^T), which was isolated from a diseased oyster maintained in the Argenton experimental hatchery (Ifremer, France). The GenBank genome accession number is JAAZTV000000000.

DESCRIPTION OF *VIBRIO AESTUARIANUS* SUBSP. *CARDII* SUBSP. NOV.

Vibrio aestuarianus subsp. *cardii* (car′di.i. N.L. fam. *cardii* referring to *Cardium* – latin: cockle).

It displays typical characteristics for the species *V. aestuarianus*. Colonies are around 1 mm in diameter after 24 h at 20 °C. No strain can grow in the absence of NaCl and growth was poor on TCBS. Growth occurs from 4 to 30 °C, difficult at 37 °C and not at 40 °C. Strains can use D-mannitol and do not ferment potassium gluconate. Concerning its fatty acid composition, it presents a high level of $C_{16:0}$ and an intermediate level of other fatty acids. Some strains, including the type strain 12/122 3T3^T, are pathogenic to the edible cockle *Cerastoderma edule* but the type strain is not pathogenic to Pacific oyster *Crassostrea gigas*.

The type strain is 12/122 3T3^T (LMG 31436^T=DSM 109723^T), which was isolated from a diseased cockle sampled from a wild bed (Baie de Somme) in France. The GenBank genome accession number is JAAKZK000000000.

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Author contributions
D.T., C.D., A.G.S., B.C., Y.G., D.S., C.G., A.M. and M.A.T. performed the experiments. L.D. produced and conserved the animals. M.A.T. and C.G. supervised the project. D.T., C.D., A.M., C.G. and M.A.T. analysed the data. M.A.T., A.M. and C.G. wrote the manuscript that was revised and approved by all the authors.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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