Vol. 62: 65–74, 2004 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published November 23

Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations

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ABSTRACT: For several years, strains phenotypically related to *Vibrio splendidus* have been associated with mortality outbreaks of molluscs. A former study on *Crassostrea gigas* demonstrated the genetic diversity of *V. splendidus* strains associated with diseased animals. Another study suggested that different strains may act in an additive/synergistic way leading to higher *C. gigas* mortality rates. Here, a strain pair (31+32) was characterised at taxonomic and virulence levels. Using a polyphasic approach, these strains were confirmed to be *V. splendidus*-related, without a clear discrimination between *V. kanaloae* and *V. pomeroyi* since hybridisation rates with both these strains were above 70%. Following experimental infection of *C. gigas* by injection in the adductor muscle or in the pallial cavity, the host alterations induced were described. After injection of strains 31 and/or 32, bacteria were localised at the periphery of the muscle and induced extensive lesions of the translucent part of the adductor muscle. Muscle alterations were of 3 kinds: (1) presence of isolated rounded muscular fibres containing non-homogenous granular material and surrounded by a translucent halo; (2) presence of non-homogenous granular material in the cytoplasm of entire muscle bands; (3) affection of wide muscle areas with extremely condensed muscle fibres. Infiltration associated with these lesions was notably absent in the vast majority of the individuals.

KEY WORDS: Vibriosis · Agonism · Molecular taxonomy · Histopathology

INTRODUCTION

Vibrio splendidus (Baumann et al. 1980, Baumann & Schubert 1984) is widely distributed in marine ecosystems (Nealson et al. 1993, Farto et al. 1999). It has long been considered an environmental organism with no pathogenic significance (Baticados et al. 1990, Paillard & Maes 1990, Myhr et al. 1991, Castro et al. 1992). However, for several years, different strains phenotypically related to this species have been associated with mortalities of molluscs, including *Pecten maximus* (Nicolas et al. 1996), *Crassostrea gigas* (Sugumar et al. 1998, Lacoste et al. 2001, Waechter et al. 2002) and *Octopus vulgaris* (Farto et al. 2003), and fish, *Scophthalmus maximus* (Gatesoupe et al. 1999). The present controversial status of *V. splendidus* (pathogenic/opportunistic) seems to be due to a lack of pertinent diagnostic tools for its identification and for the evaluation of its potential pathogenic capacity.

Epidemiological studies of *Vibrio splendidus* strains associated with mollusc mortality outbreaks have demonstrated the genetic diversity within this group and suggested its polyphyletic nature (Le Roux et al. 2002, 2004). At least 4 species, *V. lentus*, *V. kanaloae*, *V. pomeroyi* and *V. chagasii*, phenotypically related to *V. splendidus* have been described on a molecular basis (gyrase B subunit, *gyrB*, and 16S rRNA sequences, and Amplified Fragment Length Polymorphisms), although no biochemical method is available to clearly discriminate species within this group (Macian et al. 2001,

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Thompson et al. 2003a,b). Therefore, taxonomic identification of *V. splendidus* related strains should be conducted using a polyphasic approach employing new phenotypic tests and molecular techniques (Thompson et al. 2001, Stackenbrandt et al. 2002, Le Roux et al. 2004).

A wide range of virulence among strains related to *Vibrio splendidus* has been observed by experimental exposure on different animal models (Sugumar et al. 1998, Farto et al. 1999, Gatesoupe et al. 1999, Le Roux et al. 2002, Gay et al. 2004). Studies of ribosomal and *gyrB* gene polymorphisms or RAPD have not led to the identification of markers associated with pathogenicity. Thus, so far, there is no phenotypic or genotypic feature to distinguish pathogenic from non-pathogenic strains, and the only way to determine the virulence of a given strain remains experimental infection. However, results may vary from one trial to another. The development of cellular and/or molecular tests to evaluate the potential virulence of strains is necessary. The development of such bioassays requires preliminary descriptions of host alterations to define the virulence mechanisms implicated in the pathogenesis.

Although *Vibrio splendidus*-related strains have been associated with outbreaks of mortality among several aquatic animals, pathogenesis has not yet been well defined. Martin et al. (2002) described some *V. splendidus* strains inducing tissue necrosis and mass mortality in gorgons (*Paramuricea clavata*, *Eunicella cavolinii* and *E. singularis*). A *V. lentus* strain, isolated from diseased octopus (*Octopus vulgaris*), is able to induce round, hard lesions in the arm or head mantle (Farto et al. 2003). However, so far, there has been no histological description following experimental or natural exposure of bivalves to *V. splendidus* related strains.

In a former study, a collection of *Vibrio* strains isolated from *Crassostrea gigas*, genotyped by *gyrB*-based phylogenetic analysis and screened for their virulence by experimental infection, was established (Gay et al. 2004). Few strains displayed an individual pathogenicity; however, quite an important number of strains displayed an enhanced virulence when concomitantly inoculated, suggesting an agonistic action. In particular, this collaboration has been shown to be statistically significant for the strains 31 and 32. These 2 strains appear closely related but may each possess a specific virulence feature, which, when simultaneously expressed, leads to an increased pathogenicity.

In the present study, these strains were taxonomically characterised using a polyphasic approach (16S and *gyrB* gene sequence analysis, DNA/DNA hybridisation, and numerical taxonomy). Light and transmission electron microscopy examination was carried out on oysters following different inoculation methods (intrapallial or intramuscular injection) of the strains 31 and/or 32 to describe common and specific tissular alterations.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study were either isolated from haemolymph of oysters suffering from summer mortality outbreaks (31, 32, 47) (Gay et al. 2004) or purchased from national collections (CIP: Collection de l'Institut Pasteur, Paris, France; LMG: Laboratorium voor Mikrobiologie, Universiteit Gent, Belgium). Strains are maintained at the European Community Reference Laboratory for Molluscs Diseases as part of the collection (Centre de Ressources Biologiques, Laboratoire de Génétique et Pathologie, IFREMER, La Tremblade, France).

Phenotypic characterisation. Phenotypic profiles of strains and numerical taxonomy were determined from 80 classical biochemical tests as described in Le Roux et al. (2004). The following reference strains were included in the numerical analysis: *Vibrio splendidus* (LMG 4042^T), *V. lentus* (CIP 107166^T), *V. chagasii* (TNEMF6), *V. aestuarianus* (01/32), *V. mediterranei* (CIP 103203^T), *V. campbellii* (CIP 7067), *V. alginolyticus* (LMG 4409), *V. harveyi* (CIP 105197), *V. pelagius* LMG 3897^T), *V. fluvialis* (CIP 103355^T), *V. natriegens* (LMG 10935^T).

Phylogenetic analyses. DNA extraction, 16S rDNA and DNA gyrase subunit (*gyrB*) amplification and sequencing were performed using primers and methods previously described (Le Roux et al. 2004). Sequences were aligned and phylogenetic analyses were performed using Seaview and Phylo-win programs (Galtier et al. 1996). Phylogenetic trees were built using the BIONJ method (Gascuel 1997) applied to Kimura's 2-parameter distances. Reliability of topologies was assessed by the bootstrap method with 1000 replicates.

Quantitative DNA-DNA hybridisation. Labelling of DNA probes with tritium-labelled nucleotides was performed by the random primed method (Megaprime labelling kit, Amersham) and hybridisation was carried out at 60°C by the S1-nuclease method (Crosa et al. 1973, Grimont et al. 1980) with adsorption of S1-resistant onto DE81 filters (Whatman).

Experimental infection. Bacteria were grown at 20°C for 36 h in marine broth (Diagnostic Pasteur) and harvested in autoclaved (121°C for 15 min) seawater at a concentration of 8×10^8 CFU ml^{-1} evaluated by spectrophotometer (Eppendorf) at 600 nm. This bacterial concentration was accurately determined by enumeration on marine agar plates using a conventional dilution plating technique with incubation at 20°C for 2 d (not shown).

Intrapallial injection: Oyster spat originated from natural beds, measured 4 to 6 cm and were 12 to 18 mo old. The shell was filed away on the dorsal side to form a gap between the valves. The conditions tested were

strains 31, 32 or 31+32 in triplicate. A volume of 50 µl of the bacterial suspension (i.e. 4×10^7 CFU) was injected into the pallial cavity. Negative controls consisted of groups of 30 oysters injected with sterile seawater (SSW). After injection, oysters were held out of water overnight at 20°C. They were then transferred to aquaria (30 oysters per 2.5 l aquarium) containing aerated 5 µm-filtered seawater at 20°C, kept under static conditions and fed daily with a mixture of *Isochrisis galbana* and *Chaetoceros calcitrans*. Mortality was recorded daily. The water was changed once a week.

Bath exposure: The oyster spat used originated from the IFREMER nursery (La Tremblade, France), measured 1 to 2 cm and were 4 mo old. The conditions tested SSW, strain 47 (strain of lower virulence and phylogenetically related to *Vibrio splendidus*), 31, 32 or 31+32 in triplicate. One hundred juvenile oysters were kept overnight in 400 ml of bacterial suspension (10^7 CFU ml^{-1}) in aerated 5 µm-filtered seawater supplemented with a mixture of *Isochrisis galbana* and *Chaetoceros calcitrans*. Two litres of 5 µm-filtered seawater were then added per aquarium. Oysters were kept as described above. The water was changed once a week.

Intramuscular injection: Oysters were intramuscularly injected with bacterial strains as described by Gay et al. (2004). Conditions were SSW, 47, 31, 32 and 31+32.

Histology and electron microscopic examination. As soon as mortality was observed in the individuals experimentally challenged by injection in the adductor muscle, moribund and live oysters in all conditions were fixed in Davidson's or Carson's fixative (Renault & Cochennec 1995). For oysters injected in the pallial cavity, individuals were fixed in Davidson's fixative as soon as they displayed a weakness of the adductor muscle. Individuals in Davidson's fixative were prepared according to Renault & Cochennec (1995). Slides were stained with haematoxylin and eosin. Slides were examined on an Olympus BX50 optical microscope.

Small pieces of tissue (3 to 4 mm) from individuals fixed in Carson's fixative were processed for transmission electron microscopy according to Renault & Cochennec (1995). Ultrathin sections were then examined in a JEOL JEM 1200EX electron microscope at 80 kV.

RESULTS

Taxonomic characterisation

Following phenotypic analysis and numerical taxonomy, based on 80 biochemical tests, strains 31 and 32 displayed more than 80% similarity with type strains belonging to *Vibrio splendidus* polyphyletic group i.e. *V. kanaloae*, *V. pomeroyi*, *V. tasmaniensis*, *V. lentus*, *V. chagasii* and *V. splendidus*. Table 1 sums up the discriminant tests between strains 31 and 32 and the *V. splendidus*-related type strains.

Table 1. Biochemical characteristics of 31, 32 and type strains belonging to *Vibrio splendidus* polyphyletic group. Only the 20 discriminant tests are given

	31	32	*V. kanaloae* LMG 20539^T	*V. tasmaniensis* LMG20012^T	*V. pomeroyi* LMG20537^T	*V. chagasii* LMG 21353^T	*V. splendidus* LMG 4042^T	*V. lentus* CIP 107166^T
ADH	+	+	+	−	+	+	−	−
β-galactosidase	−	−	+	−	+	−	+	+
NO_2 production	−	+	−	+	−	−	−	−
Gelatinase	−	+	+	−	+	+	+	+
Catalase	+	+	+	+	+	−	+	+
TDA	−	−	+	−	+	+	−	−
Acid from								
Mannitol	+	−	+	+	+	+	+	+
Saccharose	+	−	+	−	−	+	−	−
Melibiose	−	−	−	−	−	−	+	−
Amygdaline	+	+	+	−	+	−	+	−
Glycerol	+	+	+	−	−	−	+	−
Galactose	−	−	+	−	+	−	+	−
Esculine	−	+	+	+	+	+	+	−
Mannose	+	+	+	+	+	−	+	+
Glycogene	+	+	+	−	+	+	+	+
Amidon	+	+	+	−	+	+	+	−
Growth								
4°C	+	+	+	−	+	−	+	−
35°C	−	−	+	−	−	−	+	−
40°C	−	−	+	−	−	−	−	−
6% NaCl	−	−	+	+	+	+	−	−

Phylogenetic analysis of *Vibrio splendidus* related strains was carried out by comparison of the nucleotide sequences (1200 bp) of the small subunit ribosomal RNA genes (16S rDNA, Fig. 1) and gyrase B subunit (*gyrB*) genes. In the 16S rDNA tree, clustering of strain 31, 32 and *V. lentus*, *V. splendidus*, *V. tasmaniensis* type strains was supported by a bootstrap value of 95%. In the *gyrB* tree, the same clustering as formerly described (Gay et al. 2004) was obtained despite a longer sequence analysed (1200 bp instead of 588). Strain 32 was separated from a cluster containing strain 31, *V. kanaloae*, *V. tasmaniensis* and *V. pomeroyi* type strains with a bootstrap value of 100%.

DNA-DNA hybridisation was performed with DNA of strains 31, 32, LMG 20539^T (*V. kanaloae*), LMG 20537^T (*V. pomeroyi*), CIP 107166^T (*V. lentus*), LMG 4042^T (*V. splendidus*), LMG 21359^T (*V. cyclitrophicus*) as targets and 31 or 32 DNA as probes (Table 2). Rates of hybridisation ranged between 40% (31-*V. cyclitrophicus*) and 92% (31-*V. kanaloae*). Both strain 31 and strain 32 probes hybridised at more than 70% with *V. kanaloae* (92 and 87%, respectively) and with *V. pomeroyi* (72 and 76%, respectively).

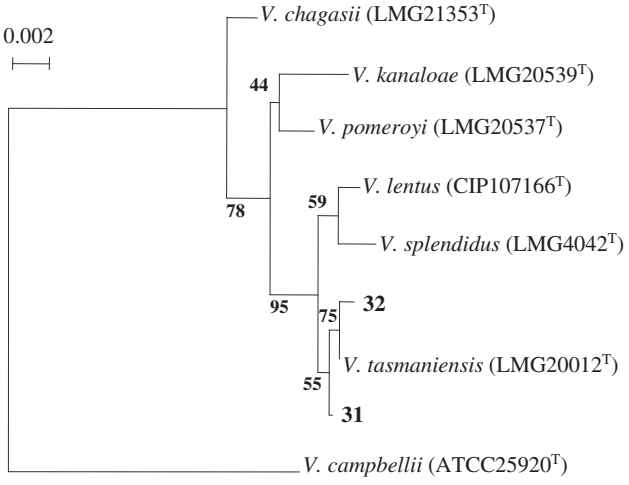

Fig. 1. Phylogenetic tree of partial 16S rDNA. The *Vibrio campbellii* homologue was used as outgroup. 1200 gap-free sites were compared. Horizontal branch lengths are proportional to evolutionary divergence. Bootstrap values (percent of 1000 replicates) appear next to the corresponding branch

Experimental infection

Intrapallial injection. When strains were injected in the pallial cavity, mortality began 7 d post injection. The mean highest mortality rate at 21 d post injection was induced by 31+32 (36%), followed by 31 (29.5%), then 32 (19.2%) and SSW (18.6%).

Bath exposure. At 30 d post exposure to SSW, suspensions of 47, 31, 32 or 31+32, no mortality had occurred.

Histology and electron microscopy examination

Oysters were histologically analysed after injection of SSW, strain 47, 31, 32, or 31+32 in the adductor muscle (Gay et al. 2004) or after injection of SSW, 31, 32, or 31+32 in the pallial cavity. After the injection in the adductor muscle, some live oysters were sampled as soon as mortality began i.e. 24 h. Beginning at 1 wk after the injection in the pallial cavity, oysters showing signs of weakness of the adductor muscle were sampled daily. For both types of inoculation, bacterial localisations and induced alterations were similar.

Bacterial localisation. Oysters injected with 47 showed scarce bacteria in the adductor muscle; these were located between the muscular fibres. In oysters injected with 31, 32, or 31+32, bacteria were localised in foci either between muscular fibres at the periphery of the adductor muscle (Fig. 2a) or in the connective tissue surrounding this organ (Fig. 2b). Some foci were also observed in the kidney close to the injected muscle, whereas no foci were observed in mantle, gills, or digestive gland. Transmission electron microscopy of pieces of the adductor muscle led to the observation of bacterial foci in the connective tissue between muscular fibres (Fig. 3).

Tissular and cellular alterations. After injection of SSW (Fig. 4a) or 47, all the organs appeared unaltered, except in the case of a few individuals injected with 47, which showed slight and localised alterations of the adductor muscle.

Following the injection of 31 and/or 32, the most altered organ was the translucent part of the adductor muscle, whereas the crescent shaped white part did not generally display any lesion. The other organs were undamaged, except for some individuals displaying localised infiltration in the gills and connective tissues.

Table 2. Intraspecific DNA-DNA homology among strains 31 and 32 and type strain belonging to *Vibrio splendidus* polyphyletic group. Numbers in bold represent hybridisation rates over 70%

	31	32
31	100	81
32	79	100
V. kanaloae (LMG 20539^T)	**92**	**87**
V. pomeroyi (LMG 20537^T)	**72**	**76**
V. lentus (CIP 107166^T)	64	63
V. splendidus (LMG 4042^T)	56	62
V. cyclitrophicus (LMG 21359^T)	40	53

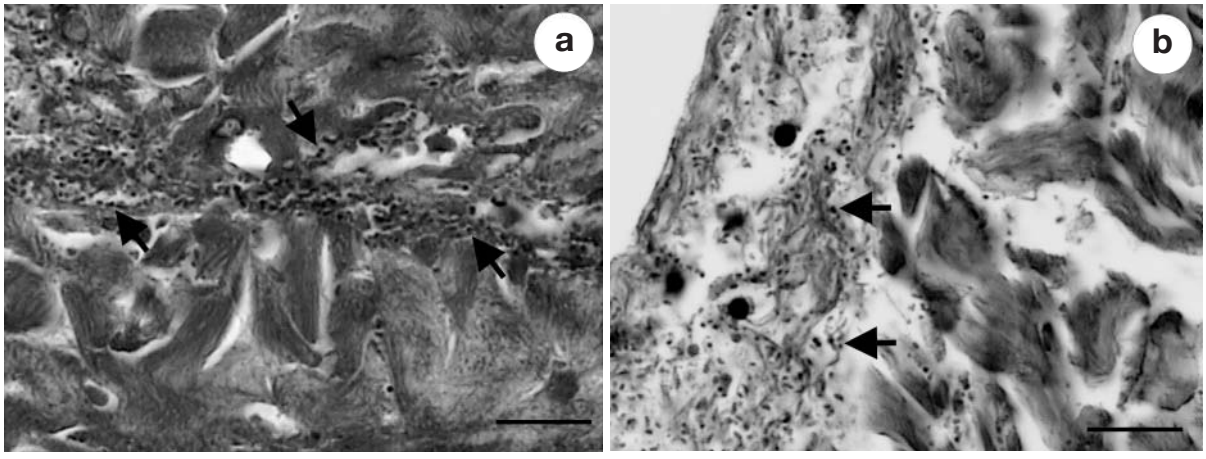

Fig. 2. *Crassostrea gigas.* Localisation of the bacteria (arrows) after experimental infection by injection in the adductor muscle. (a) Between muscular fibres at the periphery of the adductor muscle, (b) in the connective tissue surrounding the adductor muscle. HE staining, scale bars = 10 µm

Muscle of individuals injected with SSW displayed a homogeneous structure where muscle fibres formed dense bands surrounded by connective tissue (Fig. 4a). In cross sections, bands appeared as separate packed units (Fig. 4a). Haemocytes were usually present between the fibres and in the connective tissue. Muscle fibres were long cylindrical cells including an oval shaped nucleus near the cell surface, and a homogenous cytoplasm. In individuals injected with strains 31 and/or 32, the translucent part of the adductor muscle displayed several kinds of lesions (Fig. 4b–d). The first type of alteration was characterised by the presence of peculiar muscle cells, arbitrarily distributed throughout the organ. These cells were oval or round and contained non-homogenous granular material surrounded by a translucent halo (Fig. 4b). These structures were interpreted as degenerating cells. The second type of alteration affected entire muscle bands. Granular and non-homogenous material was observed in the muscle cells as in the first type of alteration. Most of the damaged cells were of an unmodified shape, except for a few round cells, as in the first kind of alteration (Fig. 4c). Whole damaged muscle bands displayed a general swelling of the cells (rounded or not) as observed when Fig. 4b,c were compared. This type of lesion was more extensive than before but still quite localised. The third type of alteration usually affected wide muscle areas. Muscle fibres appeared extremely condensed resulting in large clear gaps (Fig. 4d).

Even though some cells appeared as degenerating, these 3 types of lesions were not associated with any noticeable infiltration of haemocytes (Fig. 4b–d). Nevertheless, rare individuals displayed massive infiltrations where large areas of the adductor muscle were infiltrated by haemocytes with some condensed muscular fibres in between (Fig. 5).

Transmission electron microscopy (Fig. 6) confirmed the lesions observed in light microscopy. Healthy and damaged muscular fibres were seemingly arbitrarily distributed throughout the adductor muscle. Whereas the cytoplasmic content of these damaged cells was severely degenerating, an apparently intact cytoplas-

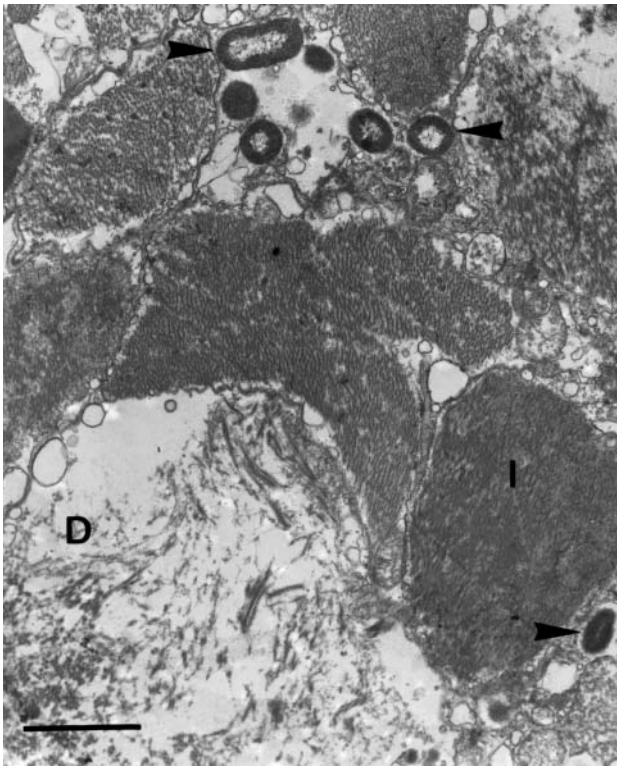

Fig. 3. *Crassostrea gigas.* Localisation of the bacteria (arrowheads) after experimental infection by injection in the adductor muscle. I: intact muscle fibres, D: degenerating muscle fibres. TEM, scale bar = 2 µm

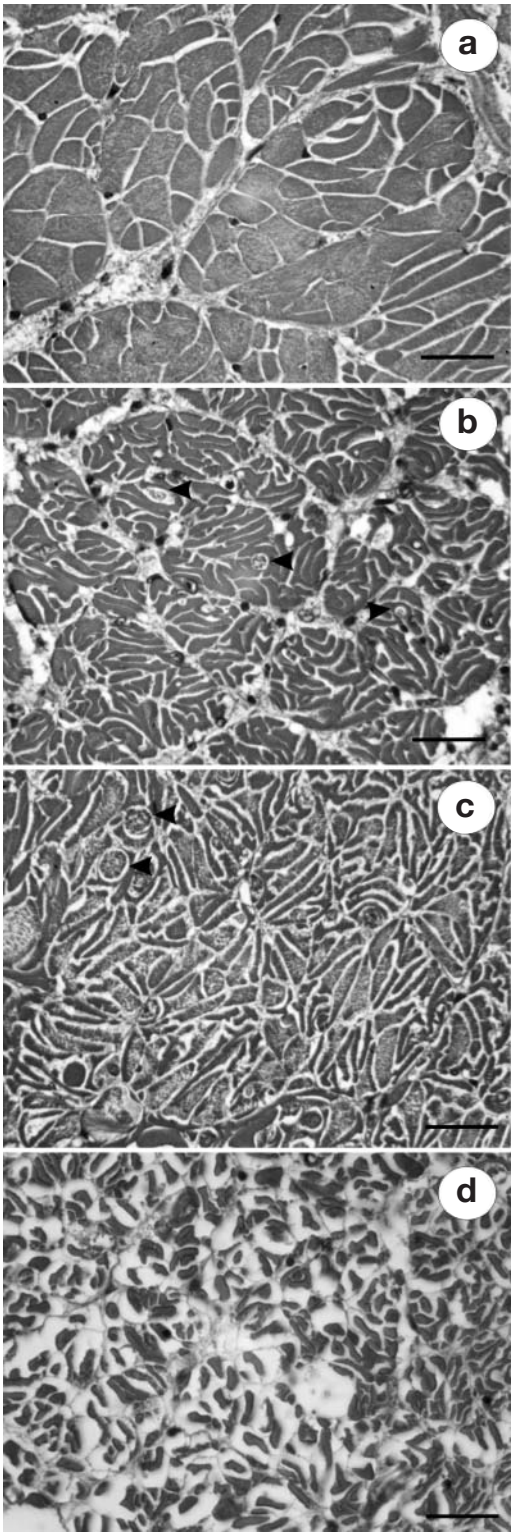

Fig. 4. *Crassostrea gigas.* Adductor muscle following the injection of (a) sterile seawater, (b–d) 31+32 into the adductor muscle. (b) Granular and non-homogenous round structures (arrowheads); (c) entire muscle bands altered with some rounded cells (arrowheads); (d) condensed muscle fibres and clear gaps in between. HE staining, scale bars = 20 µm

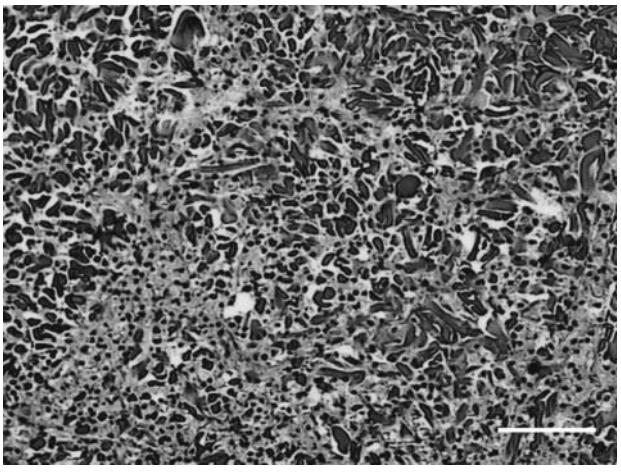

Fig. 5. *Crassostrea gigas.* Intense infiltration following injection in the adductor muscle. HE staining, scale bar = 50 µm

mic membrane was observed surrounding these structures, which could be interpreted as the endomysium (Fig. 6). Some apparently intact mitochondria and clear vacuoles were also observed within these degenerating cells (not shown). The myofibril density decrease induced by the degeneration of myofibrils observed in transmission electron microscopy could be the counterpart of the granular structures of the first and second types of alteration observed in light microscopy.

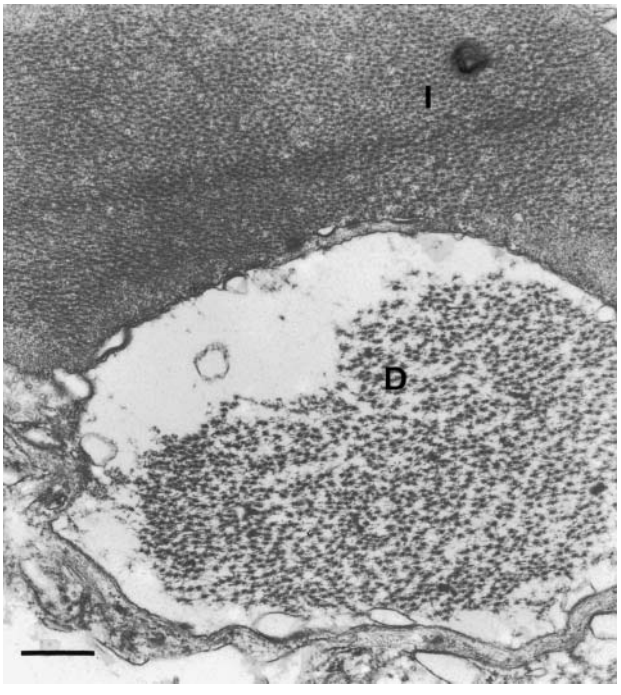

Fig. 6. *Crassostrea gigas.* Myofibril alterations following the injection of 31+32 into the adductor muscle. I: intact muscle fibres, D: degenerating muscle fibres. TEM, scale bar = 500 nm

DISCUSSION

In a former study (Gay et al. 2004), strains 31 and 32 were selected as a model because they were related to *Vibrio splendidus*, a group previously associated with several mollusc and fish mortality events. They illustrate the collaborative effect observed for different strains that will allow us to analyse the shared and/or different mechanisms of virulence implicated in this agonistic action.

The relationships between strains 31 and 32 and the other *Vibrio splendidus* related new species were evaluated using a polyphasic approach. Neither classical biochemical tests nor phylogenetic study based on 16S rDNA or *gyrB* led to a clusterisation of these strains with a unique species. DNA-DNA hybridisation recognised as the reference method for bacterial systematics (Grimont 1984), led us to define 31 and 32 as belonging to the polyphyletic group *V. splendidus* without clear discrimination between *V. kanaloae* and *V. pomeroyi*. These results could be due either to a lack of discriminatory power of the molecular methods used or to an intermediate position of our strains between the 2 species. Experiments are in progress to develop new phylogenetic analyses using a multilocus scheme.

Putative pathogenic bacteria for *Crassostrea gigas* had been selected by injection of bacterial suspension in the adductor muscle (Lacoste et al. 2001, Le Roux et al. 2002). The results are obtained quickly using this technique since mortality appears as soon as 24 h post injection; however, drawbacks, such as disruption of tissues and variability, were observed. Less invasive methods were tested and the mortality induced was described. The injection in the pallial cavity is an alternative since it allows the transmission of the disease without disrupting the tissues. However, this method does not accurately reflect natural infection due to the artificial crossing of the mechanical barrier (shell and mantle) and the stress induced by a long period of time under static conditions. This method did, however, confirm the collaboration between 31 and 32, and histopathology demonstrated that bacteria localisation and induced lesions were similar following intrapallial or intramuscular inoculation.

Bath exposure did not induce any mortality. However, this is quite often encountered among pathogens of aquatic animals. Li et al. (1998) failed to infect abalone *Haliotis discus hannai* Ino with virulent *Vibrio fluvialis* by bath exposure, whereas abalone infected through foot lesions developed the bacterial disease. Experimental infection of Atlantic salmon *Salmo salar* by *V. salmonicida* and *Aeromonas salmonicida* led to higher reproducible results following intraperitoneal injection compared to cohabitation or bath exposure (Nordmo & Ramstad 1999). Since bath exposure of *Crassostrea gigas* with *V. splendidus* did not lead to any mortality, Lacoste et al. (2001) suggested that *V. splendidus* was transmitted vertically; however, other hypotheses may be proposed for these negative results and for the variability sometimes observed in the different inoculation methods. First, as for the bacteria, culture media or environmental conditions may modulate the expression of virulence factors (Ottaviani et al. 2001), or subcultures may lead to a loss of virulence. Second, as for the oyster, animals may have suffered mortality or stress prior to the experimental infection, or a particular status of the host (genetic and/or physiologic) may be necessary for initiation of pathogenesis and/or susceptibility of the host. For instance, maturation may induce an increased susceptibility to infection. This hypothesis is supported by the knowledge that the gametogenesis is a period of negative energy budget (Soletchnik et al. 1997) where most of the acquired energy is used for the production of gametes to the detriment of the defence mechanisms (Perdue et al. 1981, Myrand et al. 2000). Experiments are in progress to evaluate the role of environmental and host factors in the pathogenesis of disease due to *V. splendidus* related strains.

Host alterations following experimental infection after bacterial inoculation are reported. The only macroscopic sign preceding oyster death was a weakness of the adductor muscle. No alteration or organic deposit was observed on the shell of infected animals, although such macroscopic signs have been previously described following *Vibrio splendidus* infection (Lambert et al. 1999). In oysters injected with 47, few bacteria were observed, suggesting that this strain had been cleared shortly after the injection in contrast to 31 and/or 32 (Kelly & Dinuzzo 1985, Lopez-Cortes et al. 1999). In oysters injected with 31, 32 or 31+32, bacteria were always localised either between muscular fibres at the periphery of the adductor muscle or in the connective tissue surrounding this organ or in the part of the kidney running along the adductor muscle. However, the whole adductor muscle was severely damaged, which is in contrast with the localisation of bacteria only at the periphery of this organ. Based on the literature, several hypotheses may be suggested. Bacteria may produce toxins without the bacteria themselves being present at the altered site. In the withering syndrome of abalone, Rickettsiales-like prokaryotes are localised in gastrointestinal epithelium cells and induce digestive gland degeneration and foot muscle atrophy (Moore et al. 2001, Friedman et al. 2002). Bacterial density may be too low to be detected. In borreliosis, few spirochetes are observed in tissues (Cadavid et al. 2000). The lesions may also be due to the host response which is at first beneficial to help fight the disease but may induce an imbalance resulting in

wasting, as in some cases of sepsis (Sharma & Anker 2002). Pathogenic bacteria induce a dysfunction of some host proteinases resulting in the lysis of myofibrillar proteins (Hatzizisis et al. 2000, Saouda et al. 2001, Sun et al. 2003). A direct action of the bacteria on other tissue such as the nervous or the vasculatory system may induce muscular lesions as consequences. In the clostridial myonecrosis, bacteria induce a reduction of arteriolar flow impairing oxygen delivery and anoxic necrosis (Dastur & Daver 1980, Stevens 2000). Finally, bacteria may have invaded and damaged the whole muscle and then migrated to other tissues during the time preceding the sampling of individuals for histological examination. Specific molecular tools (*in situ* hybridisation and GFP-expressing bacteria) are being developed to describe the infection route and eventual specific localisation and migrations of both strains.

Muscular lesions were not due to the inoculation method since they were also observed following bacterial injection into the pallial cavity. The observed lesions indicated a degeneration of the adductor muscle, which may explain the weakness of the oysters after experimental infection. The rounded granular structures described in the first type of alterations could have been bacteria globi as described by Lancaster et al. (1983) for *Mycobacterium leprae*. However, following Feulgen staining, these structures were found to be Feulgen negative, thus containing no DNA (not shown). The granular structures of the first and second types of alterations may be due to a large decrease in the myofibril density as observed in transmission electron microscopy. The membrane of the damaged muscular fibres seems to be intact, suggesting a phenomenon other than necrosis, where the first step is membrane disruption leading to influx of water and ions and thus to cell swelling and lysis. Cell alterations observed in the present work have been previously described in the literature; in some cases only one type of alteration was referred to (Abella et al. 2003), in others both were described and called coagulative necrosis, without any further descriptions of the mechanisms induced (Eldar et al. 1999). The 3 types of alterations may be interpreted as a succession of events: first a few muscular fibres were damaged, then the larger part of the adductor muscle displayed granular degenerating muscular fibres and finally, large parts of the organ contained very condensed muscular fibres.

Muscle alteration is one of the signs described in fish and mollusc vibriosis (McCarthy 1976, Bruno et al. 1986, Egidius 1987, Li et al. 1998). Furthermore, other pathogens were described as inducing muscular lesions in marine animals. For instance, the akoya-virus infection in the Japanese pearl oyster *Pinctada fucata martensii* induced necrosis, atrophy, swelling and vacuolisation of the muscle fibres of the adductor, foot, and pallial muscles (Miyazaki et al. 1999, Miyazaki et al. 2000). Co-infection of the giant freshwater prawn *Macrobrachium rosenbergii* by the yeast *Metschnikowia bicuspidata* and the bacteria *Enterococcus faecium* led to important edema and liquefactive necrosis in abdominal, pereipod and pleopod muscles (Chen et al. 2003). In several other bacterial models, muscular lesions seem to be either secondary lesions (Schaible et al. 1989, Ling et al. 2000) or consequences of an action of the pathogenic bacteria on other organs (Hund 2001), whereas in the 31+32 infection model, no other organ appeared damaged.

A few individuals displayed massive infiltrations in the adductor muscle. However, it is noticeable that the vast majority of individuals displayed no infiltration associated with muscular lesions or degenerating cells, whereas inflammation is often observed at the site of infection either as a means of fighting the infectious agent or repairing the damaged tissue (Miyazaki et al. 1999, Neely et al. 2002). Some mollusc pathogens were described as inducing large increases in the circulating haemocytes densities (Oubella et al. 1993) and others were demonstrated to possess chemoattractants for oyster haemocytes (Cheng & Howland 1979, Howland & Cheng 1982). The absence of inflammatory response may be due to different factors. Some bacterial pathogens mimic host structures to avoid this response (Moran et al. 1996), whereas others seem to produce toxins preventing inflammatory response (O'Brien & Melville 2000). The presence of intense inflammation was only observed in some individuals and associated with condensed cells of the third type of alteration. Considering that condensed cells constitute the final stage of the disease, it may be hypothesised that infiltration plays a final role in clearing cell debris and healing the organ rather than being due to the direct action of the pathogenic bacteria (Ford et al. 1993). Experiments will be conducted with various sampling times in order to describe disease development.

The present study made it possible to describe bacterial localisation and muscular alterations following injection in the pallial cavity or in the adductor muscle of strains 31 and/or 32. However, no major difference was observed in light microscopy irrespective of whether the strains were inoculated individually or together. Thus, the strain specific virulence apparently does not induce differential structural changes in altered cells. Other approaches will be tested based on phenotypic features (production of toxic extracellular products) and on genomics (genomic subtraction).

Acknowledgements. The authors acknowledge Mr. Giraudeau for providing oysters and Prof. C. Tinsley for critically reading the manuscript. This study was carried out with financial assistance from the EU Community Reference Laboratory and the Région Poitou-Charentes.

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Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: January 30, 2004; Accepted: June 7, 2004
Proofs received from author(s): October 20, 2004