Aquatic Living Resources

Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution

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Abstract – The main microbial diseases affecting marine cultured bivalves have been revised on the basis of the etiologic agents, pathogenesis and pathogenicity. Several recent bivalve-interaction models have been studied, including *Pecten* larvae-*Vibrio pectinicida*, brown ring disease, juvenile oyster disease, Pacific oyster nocardiosis and summer mortalities of oysters. In addition, the taxonomy and phylogeny of new potential bivalve pathogens and their virulence factors have been established. Facing the difficulty of identifying bacterial strains associated with molluscan diseases (mainly vibriosis), polyphasic approaches have been developed to correlate the phenotype and genotype of potential pathogens. By evaluating likely virulence mechanisms, developing biotests to screen virulent strains and characterising the genes implicated in pathogenesis, a new generation of diagnostic tools, based on potential virulence, will be developed. Acquisition of pertinent diagnostic tools will be of major benefit in disease management, health surveillance and monitoring will contribute to maintaining sustainable aquaculture industries.

Key words: Vibriosis / Bivalve mollusc / Etiology / Pathogenesis / Pathogenicity / Virulence factors / Diagnostic tools

Résumé – Les maladies bactériennes chez les bivalves marins, synthèse des travaux récents : tendances et évolution. Les principales maladies bactériennes affectant les bivalves marins aquacoles ont été synthétisées en se basant sur trois aspects, la démarche étiologique, l'étude de la pathogenèse et le mode d'action du pathogène. Plusieurs modèles d'interactions chez les bivalves ont été récemment étudiés, tels que *Pecten-Vibrio pectinicida*, la maladie de l'anneau brun chez les palourdes japonaises, la maladie des juvéniles d'huîtres américaines, la Nocardiose et les mortalités estivales chez les huîtres du pacifique. Ainsi, la taxonomie et la phylogénie de ces nouveaux pathogènes ont pu être établis et des facteurs de virulence identifiés. Face à la difficulté d'identification des souches bactériennes associées aux maladies des mollusques (principalement des vibrioses), une approche polyphasique a été développée afin de corréler le phénotype et le génotype des pathogènes potentiels. Des bio-tests utilisant toute une nouvelle génération d'outils sont en cours de développement afin de cribler les souches virulentes, d'évaluer les mécanismes potentiels de virulence et de caractériser les gènes impliqués dans la pathogenèse. L'acquisition de ces outils diagnostics permettra de mieux contrôler l'état sanitaire des bivalves aquacoles et contribuera ainsi à maintenir toute une filière industrielle conchylicole performante.

1 Introduction

In Europe, mollusc aquaculture is a traditional well established industry and is a major contribution to the socio-economic development of most of the coastal regions. There are many examples of the severe impact of disease outbreaks and mass mortality which are increasingly recognized as a significant constraint to aquaculture production and trade. The disease affects any stage of production, including larvae, juveniles or adults and whatever farming technique is used. Major disease outbreaks can also occur through translocation of infected stock and is a particular problem associated with mollusc culture. Furthermore, species diversification in aquaculture, as well as increased hatchery production raises the demand for trans-boundary movements and transfers of live molluscs. The development of reliable and useful diagnostic tools is of central importance to the prevention and control of diseases mainly with bivalve production highly vulnerable to the risk of disease.

Until recently, most of bivalve diseases were reported to be provoked by protozoans, such as *Bonamia*, *Haplosporidium*, *Marteilia* and *Perkinsus*, fungi or by virus (see reviews in Lauckner 1983; Sindermann 1990; Bower et al. 1994; McGladdery 1999). Bacterial diseases were commonly described in larval stages and were associated with high mortalities in hatcheries (Lauckner 1983; Sparks 1985; Sinderman 1990). To date, three bacterial diseases of adults have been reported: Brown Ring Disease (BRD) in clams,

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Ruditapes philippinarum and R. decussatus (Paillard et al. 1989; Paillard and Maes 1990), the nocardiosis in the Pacific oyster, *Crassostrea gigas* (Friedman et al. 1991a,b) and Juvenile Oyster Disease (JOD) in the eastern oyster, *C. virginica* (Boettcher et al. 1999, 2000; Bricelj et al. 1992; Ford and Borrero 2001; Paillard et al. 1996). In addition, other diseases and mortalities of bivalves are suspected to be provoked by bacteria, such as the shell disease in the pearl oysters, *Pinctada maxima* and *P. margaritifera* (Pass et al. 1987; Marin and Dauphin 1991), and in the recent phenomenon of "summer mortalities" of the Pacific cup oyster, *C. gigas*, which has beeen associated with some *Vibrio* of the *V. splendidus* polyphyletic group (Lacoste et al. 2001a,b; Waechter et al. 2002; Le Roux et al. 2002). The emergence of *Vibrio* spp. as etiological agents of disease in cultured bivalves will be undoubtedly one of the major problems in the next few years. Ocean warming and biological invasions of exotic species might facilitate the spread of marine bacterial diseases. Therefore, there is an urgent need to evaluate the impact of the disease on mollusc aquaculture and to determine the present and future risk by better understanding the nature of the interaction between *Vibrio* spp. and shellfish. Advances in molecular biology, genetics, analytical chemistry and other sciences has allowed to develop cellular and molecular tools to detect smaller amounts of infectious agents and contaminants and light biological changes as well as to identify mechanisms at the cellular and molecular levels. This progress in the molecular biological approaches has provoked advances in the study, prevention and control of microbial diseases in aquaculture.

New concepts in pathology have emerged and new definitions for pathogen, etiology, pathogenesis and pathogenicity may be established. Several recent studies on bacterial diseases in bivalves have been conducted based on these new approaches and are described here. Bacterial taxonomy and phylogeny will be revisited and innovative new methods for prokaryotic systematics will be proposed. Although many virulence factors of human and fish pathogenic *Vibrio* have been identified, little such knowledge exists for *Vibrio* spp. pathogenic for bivalves. Methods of screening gene-encoding virulence factors for various disease models are suggested and discussed.

2 Emergence of new diseases in bivalves: Terminology and methodology evolution

Before describing precisely the general approach, it will be useful to agree on pathology terminologies. First, what is really meant by a pathogen? A pathogen is generally define as a microorganism capable of causing disease. But recently, certain authors have proposed new definitions encompass classical pathogens and opportunistic pathogens and they define a pathogen as a microorganism able of causing host damage. The host damage can result from either direct microbial action or the host immune response, see Casadevall and Pirofski (2002).

The general approach to demonstrate that a new disease occurs in bivalves is firstly to identify the cause of the disease (i.e. Etiology); secondly to describe the host syndromes associated with the disease (i.e. Pathogenesis); and thirdly, to determine the mode of action of the pathogen (i.e. Pathogenicity).

2.1 Etiology

Etiology used to be defined according to Koch's postulates. A bacterium will be pathogen if four postulates can be demonstrated.

1. Transmissibility of the disease from a diseased bivalve to a healthy one.
2. Isolation of the bacterial pathogen and phenotypic and molecular characterization.
3. Experimental reproduction of the disease by exposure to the bacterium and systematic detection of the bacterium in all the bivalves experimentally challenged.
4. Systematic detection of the pathogen in all the naturally diseased bivalves originating from different locations.

To fulfill Postulate 1, transmission can be conducted either by contact or by inoculation of an extract of the diseased bivalve (see methodology in Paillard et al. 1989). When extracts are used, differential filtration can be used to better define the nature of the etiologic agent. For Postulate 3, in bacterial infections, predominant or systematic bacteria are generally isolated just before the peak of disease or mortalities to obtain a higher probability to identify the pathogenic ones. These experiments performed in postulates 1 and 3, must reproduce the disease following the most closely the normal route of infection, either by bathing, or by inoculation in the pallial cavity (see methodology in Paillard and Maes 1990). To explore different way of the pathogenicity, the bacterial agent could be also injected in vivo directly at the infection site into cibled target tissues or fluids (hemolymph, pallial or extrapallial fluids) see methodology in Allam et al. (2002). It is important to remember that the injection into tissues, hemolymph and extrapallial fluids passes through the immune barriers of the host.

Successful reproduction of disease or mortalities in bivalves can be also dependent on the dose of inoculum. Dose effect must be considered as a function of bacterial strains, and size and species of bivalve. In larval challenge by bathing, a decrease in larval survival is observed in *C. virginica* with 10^3 CFU per ml of *Vibrio* spp. (Elston and Leibovitz 1980) and in *Argopecten purpuratus* with 10^6 CFU/ml of *V. anguillarum* (Riquelme et al. 1995). The adult bivalve may tolerate higher concentration of pathogenic bacteria. In the case of BRD, a concentration of 10^7 CFU/clam of *V. tapetis*, injected in the pallial cavity, is required to induce 70−90% prevalence of conchiolin deposit on the inner shell of *R. philippinarum* (Paillard and Maes 1990; Novoa et al. 1998). In order to fulfill Koch's postulates as stated above, the pathogen must be isolated and cultured. For bacteria that could not be cultured, a molecular adaptation of Koch's postulates was proposed by Falkow (1988). A bacterial component will present a role in pathogenicity if it satisfy four conditions:

1. This component is found only in the pathogen species for a target host.
2. Inactivation by mutation of one or more gene(s) associated with pathogenicity must reduce the virulence.
3. Reintroduction in the mutant of the wild gene, must restore the virulence
4. The gene (s) must be expressed in vivo, during the infectious process.

Neither of these Koch and Falkow's postulates takes into account pathogens that cause disease only in the presence of certain other pathogens (co-infections or synergistic infections) or under environmental conditions favorable for the pathogen and not for the host, or only in immunodepressed host. In these latter cases, the situation is more complicated and thus may require other strategies. To demonstrate their pathogenic role in the disease, in case of cultured bacteria, both pathogens or the pool of pathogens suspected to act in synergy, must be inoculated together to reproduce the disease and then, each pathogen of the pool must be systematically detected in experimentally-and naturally-diseased bivalves. In case of non-culturable bacteria, the molecular demonstration must be performed for both or the pool pathogenic bacteria. This requires a multifactorial molecular strategy especially if several genes are involved.

2.2 Pathogenesis

Description of damages caused to a host by microbial pathogens is crucial to the elucidation of two points: first in the guarantee that the etiologic agent(s) is(are) responsible for the disease and secondly, in researching the potential mode of action of the pathogen. This approach is necessary to detect and quantify pathogenesis in term of damage to the host other than only in term of mortalities which could be associated with multifactorial causes. The study of pathogenesis is a critically important early step in the process of disease characterisation.

In case of cultured pathogens, the infection process must be followed after inoculation of the pathogenic bacteria, at the level of the site of infection, in relationships with the defense system (hemolymph, extrapallial fluids, etc.) and also in other compartments which are affected indirectly by the disease. In case of both non-culturable and cultured pathogens, research on damage to the host should be performed on bivalves sampled in field during critical period (before, during and after the maximum prevalence). This strategy allows definition of different stages of the disease in order to establish a classification scale. The description of different stages of the disease should be performed at tissue, cellular and molecular level using techniques the most appropriate to the specific disease such as macroscopic and microscopic observations and quantification by image analysis (TEM, SEM, Confocal microscopy, video in four-dimensions, flow cytometry, Fluorescence In Situ Hybridation (FISH), In Vivo Expression Technology (IVET) (Falkow 1997; Moxon and Tang 2000).

2.3 Pathogenicity

Different terms are used to describe pathogenicity and virulence. The definition differs according to the authors and scientific interest. In medical glossaries, pathogenicity defined as the capacity to induce a disease. This term is used (qualitatively) to describe or to compare bacterial species. The term virulence indicates the degree or intensity of pathogenicity. The determinants of the virulence of a pathogen are the genetic, biochemical, and structural features that enable it to inflict damage on a host. Virulence can be measured experimentally with the determination of the lethal dose (LD_{50}) or infectious dose (ID_{50}). This term is often used to describe or compare different strains of the same bacterial species.

The definitions of pathogenicity and virulence are primarily pathogen centered and do not include any host response. Their definitions can be very modified if we attempt to find precise and basic definitions of virulence and pathogenicity that incorporated the contributions of both the host and pathogen; see review by Casadevall and Pirofsky (1999) who defined pathogenicity as the ability to cause host damage. The definition can encompass classical pathogens and opportunists; host damage can be due to either direct microbial action or the host immune response. These authors revisited the concept and suggested a classification system for microbial pathogens based on their ability to cause damages as a function of host's immune response.

Infection may imply colonization, multiplication, invasion or persistence of the bacteria on or within the host. In general, pathogens share similar mechanisms and traits: invasiveness which encompasses mechanisms for colonization, the ability to overcome host defense mechanisms and the production of toxins. These traits and mechanisms of pathogenicity are in general similar between human and shellfish pathogens. More specifically, however, the fact that bivalve anatomy present some particularities compared to vertebrates and other invertebrates, such as the presence of internal and pseudo-internal shell cavity compartments related to the shell structure (Paillard et al. 1996; Allam and Paillard 1998) may allow the potential for different strategies for bacteria pathogenic to bivalve.

Considering that bacterial pathogenicity cannot be evaluated without considering host damage, in vitro tests of interactions between host cells and bacteria are generally used. In human and other vertebrates, pathogenicity tests are generally performed using cell cultures (Biosca and Amaro 1996; Basu et al. 1999; Bejar et al. 1997; Ormonde et al. 2000). In bivalves, for which cell cultures are not available, in vitro bioassays have been developed using tissue or larvae-bacteria interaction (McHenery and Birkbeck 1986; Nottage and Birkbeck 1987a) or more recently, hemocyte-bacteria interactions (Nottage and Birkbeck 1989, 1990; Noël et al. 1993; Lambert and Nicolas 1998; Lane and Birkbeck 1999; Lambert et al. 2001; Choquet et al. 2003). Bacterial suspensions (from petri dishes or broth culture), extracellular products (ECP) or purified toxins are used in these bioassays, depending on the mechanism of pathogenicity to be studied.

3 Status of the art

The difficulties in identifying pathogenic bacteria in bivalves is essentially due to the fact that bivalves normally accumulate and harbor a very rich bacterial commensal microbiota, composed of various species, mainly belonging to the genus *Vibrio*, *Pseudomonas*, *Alcaligenes*, *Aeromonas*, *Flavobacterium*, but also Gram positive of the genus *Bacillus* and *Micrococcus*, to name a few (Colwell and Liston 1960, 1962; Colwell and Sparks 1967; Murchelano and Bishop 1969; Brisou et al. 1962; Prieur 1976, 1982, etc.). Some of them, mostly opportunistic and pathogenic bacteria, could colonize and invade the host, depending on the environmental factors modulating the bivalve-bacteria interactions.

Bacterial diseases affect bivalves differently according to their life stage. The role of bacteria in diseases and larval mortalities were documented in the 1960s (See reviews of Lauckner 1983; Sindermann 1990) (Table 1). Bacteria that provoke severe mortalities in larval cultures belong generally to the genera *Vibrio* (*V. alginolyticus*, *V. anguillarum*, *V. tubiashi* and other *Vibrio* spp.), *Pseudomonas* and *Aeromonas* (Tubiash et al. 1965, 1970; Colwell and Sparks 1967; Brown 1973, 1981; Le Pennec and Prieur 1977; DiSalvo et al. 1978; Elston and Leibovitz 1980; Elston 1984; Hada et al. 1984; Grizel 1987; Olafsen et al. 1993; Kaspar and Tamplin 1993). Disease symptoms in moribund larvae were described first in *C. gigas* and *Mercenaria mercenaria* by Tubiash (1965) and Elston and Leibovitz (1980) and named Bacillary Necrosis. The necrosis is characterized by deciliation and loss of velar epithelial cells, growth of bacteria along the internal shell and mantle, and abnormal swimming behaviour. After experimental challenge, a rapid mortality was observed within 48 hours (Elston 1993). Tissues of abnormal larval bivalve with bacillary necrosis have been well described with histological, immunofluorescent and ultrastructure techniques (Leibovitz 1978; Elston 1979; Elston et al. 1981; Elston and Leibovitz 1980). These latter authors described three patterns of disease that they designated them types I, II, III pathogenesis. The types I and III become sedentary and can be detected at the bottom with the substrate; the type I pathogenesis occurs when bacteria colonize the inner shell along the mantle before invading the visceral cavity; type II exhibits progressive and extensive visceral atrophy before the invasion of bacteria by focal lesions into the organs of the digestive tract. Abnormal larvae of type II, can first be detected in the water column. Abnormal larvae remain active despite showing a variety of velar damage, deformation without bacterial invasion, abnormal swimming, and subsequent invasion, which may occur with an intact velum. Early diagnosis using this type pathogenesis can aid in prevention and management of the disease in hatcheries (Elston and Leibovitz 1980). More recently, three new species of vibrios were isolated and associated with high mortalities of bivalve larvae in hatcheries. In 1986, severe mortalities occurred in clam, *R. philippinarum* larvae and were related to a *Vibrio* named VTP *(Vibrio Tapes philippinarum)* (Nicolas et al. 1992). Variants of vibrios designated as "*V. anguillarum-like*" (VAR) were isolated from an epizootic occurring in a commercial hatchery in Chile, producing the scallop, *Argopecten purpuratus* (Riquelme et al. 1995). A second pathogen of scallop larvae, *V. pectenicida* was also identified as being responsible for mortality outbreaks in hatcheries in France (Lambert et al. 1998) (Plate 1). The experimental reproduction, pathogenesis, pathogen identification and pathogenicity are only documented in *V. pectenicida* model (Lambert 1998; Lambert and Nicolas 1998; Lambert et al. 2001) (Table 1). This pathogen does not induce mortalities in adult scallops. In general, adult bivalves do not suffer high mortality when experimentally challenged with larval pathogens. Tubiash (1973) found that *V. anguillarum* (strain ATCC 1909, concentrations between 10^6 and 10^7 cells ml^{-1}) is highly pathogenic to experimentally infected larval causing 90% mortalities after 48 hours, however, it produces no significant mortality in adult oyster under similar experimental conditions, even after five month maintained in flowing water througout the winter.

Hatchery epizootics associated with chlamydia infections have been reported in larval and post-metamorphic bay scallops, *Argopecten irradians* (Leibovitz 1989). No specific clinical signs of disease or mortalities were associated with the presence of intracellular inclusions in adult or juvenile bay scallops (Leibovitz 1989).

Numerous authors have suspected bacteria to induce mortalities in adult and juveniles of bivalves (Tubiash et al. 1973; Colwell and Sparks 1967; Dybdahl and Pass 1985; Pass et al. 1987). At this date, only four bacterial diseases have been reported to affect particularly bivalve juveniles, such as Juvenile Oyster Disease (JOD) in *C. virginica* (Bricelj et al. 1992; Boechter 1999, 2000), hinge ligament erosion disease in *C. gigas* (Dungan and Elston 1988; Dungan et al. 1989), the chronic abcess syndrome in *C. gigas* (Elston et al. 1999) and recently the summer oyster mortalities in *C. gigas* in France (Lacoste et al. 2001; Waechter et al. 2002). Only the chronic abcess syndrome affects specifically the juveniles (Elston et al. 1999). The three other ones can also affect adult bivalve but with little or no mortality.

Exept for summer oyster mortalities in *C. gigas*, the clinical signs of disease all include lesions of the mantle and the structure it produces (e.g. the shell organic matrix, ligament). The ligament disease is characterized by a destruction of the hinge ligament caused by *Cytophaga* sp.-like gliding bacteria (Table 2, Plate 2). It was commonly detected in cultured juvenile bivalves, mostly in oysters of different species, *C. virginica*, *Ostrea edulis*, and *C. gigas* but also in clams, *M. mercenaria* (Elston 1982; Dungan and Elston 1988; Dungan et al. 1989). This disease can be also observed in adult oysters and some hinge ligament degrading bacteria have been isolated from altered hinge ligaments of adult *C. gigas* grown in open waters near Sequim, Washington (Dungan, personal communication). Juvenile oyster disease (JOD) in cultured *C. virginica* is characterized by an anomalous conchiolin deposit on the inner valves (Bricelj et al. 1992; Ford and Borrero 2001) (Plate 3). Similar symptoms can be also observed in adults maintained in stressful conditions (Ford, personal communication). Chronic mortalities were recently described in juvenile *C. gigas* and were associated with an abcess in the extrapallial space resulting from an invasion by bacterial rods along the inner shells (Elston et al. 1999) (Plate 4). This disease provokes alterations of the underlying mantle and abnormal shell deposition. This disease has been reported only in very small juvenile pacific oysters with a maximum of 1 cm shell height but usually only 2 to 6 mm (Elston, personal communication). Microscopical examination of mantle and shell in *C. gigas* affected by chronic abcess (Plate 4, No 15) has demonstrated that it is different from hinge ligament infections and Brown Ring Disease (Plate 5, Elston et al. 1999). In the case of summer mortalities in *C. gigas* juveniles, to date no consistent clinical signs have been observed in oysters during an epizootic in the field. Description of this recent phenomenon in juvenile *C. gigas* is developed further in this review (see paragraph, Recent studies of some major bivalve-bacteria interaction models).

Table 1. Bacterial pathogens to bivalve larvae. For each pathogen, are given, depending author reference, the host species, its ability to reproduce host mortality, its impact on host, its characteristics (taxonomy, phylogeny, pathogenicity). nd: non done. Bn: Bacillary necrosis.

| Bacterial pathogens to bivalve larvae	Host	Experimental Reproduction of mortalities	Pathogenesis	Pathogen taxonomy/ pathogenicity	References
Chlamydia	*Argopecten irradians*	nd	Digestive diverticula (histology and ultrastructure)	nd/nd	(Leibovitz 1989)
V. tubiashii	*C. virginica*	+	Bn	nd/nd	(Tubiash et al. 1965)
Vibrio tubiashi	*O. edulis*	+	Bn	nd/ Extracellular thermostable toxin	(Lodeiros et al. 1987)
	C. gigas	+	Bn	nd	(Takahashi et al. 2000)
V. alginolyticus	*O. edulis*	+	Bn	nd	(Tubiash et al. 1970)
	O. edulis	+	Bn	nd/nd	(Lodeiros et al. 1987)
	M. mercenaria/ C. virginica	nd	Bn	Taxonomy (ATCC 19108)/nd nd/nd	(Tubiash et al. 1970)
	A. ventricosus/ N. subnodosus/ A. maura/C. gigas	+ (dose effect)	Bn		(Luna-Gonzales et al. 2002)
V. anguillarum	*C. virginica M. mercenaria*	+	Bn (histology, ultrastructure)	nd/nd	(Tubiash 1975)
	O. edulis	+	nd	nd/Heat stable exotoxin	(DiSalvo et al. 1978)
	O. edulis	+	Bn	nd/nd	(Lodeiros et al. 1987)
	M. mercenaria/ C. virginica	nd	Bn	Taxonomy (ATCC 19105,6,9)/nd	(Tubiash et al. 1970)
V. anguillarum related	*A. purpuratus*	+	nd	+	(Riquelme et al. 1995)
V. anguillarum related	*C. virginica*	nd	Abnormal shell shape	nd/nd	(Brown 1981)
V. splendidus	*C. gigas*	+	nd	nd	(Sugumar et al. 1998)
V. pectinicida	*P. maximus*	+	Bn	Phylogeny	(Nicolas et al. 1996)
	P. maximus	nd	nd	Taxonomy (CIP 105190[T]) Toxin	(Lambert et al. 1998) (Lambert et al. 2001)
V. tapesi	*T. philippinarum*	+	Bn	nd/nd	(Nicolas et al. 1992)
Vibrio spp.	*C. virginica*	+	Bn		(Brown 1973)
	C. virginica	-	Bn	nd/nd	(Leibovitz 1978)
	C. virginica				(Brown and Losee 1978)
	C. virginica	+(dose effect)	early diagnosis type I, II, III (histology, ultrastructure)	nd/nd	(Elston et al. 1980)
	C. virginica	nd	histology and immunofluorescent detection of *V*. spp		(Elston et al. 1981)
	C. virginica	+	Abnormal shell shape	Phenotype	(Brown and Tettelbach 1988)
	M. mercenaria	+	Abnormal shell shape	phenotype	
	M. mercenaria				(Guillard 1959)
	M. mercenaria	+	Bn	Phenotype	(Tubiash et al. 1965)
	C. virginica	+	Bn	Phenotype	
	A. irradians	+	Bn		
	Teredo navalis	+	Bn		
	C. gigas	+(dose effect)	Bn	nd/nd	(Garland et al. 1983)
	C. gigas	+(dose effect)	Bn	nd	(Jeffries 1982)
	O. edulis	+	Bn		
Aeromonas sp.	*C. virginica,*	+	Bn	Phenotype	(Tubiash et al. 1965)
	M. mercenaria	+	Bn	Phenotype	
Alteromonas spp.	*C. gigas*	+	Bn	nd	(Garland et al. 1983)
Pseudomonas spp.	*C. virginica*	?			(Brown 1973)
	M. mercenaria				(Guillard 1959)
	O. edulis	+	Bn	nd/nd	(Lodeiros et al. 1987)
	Euvola ziczac	+	Bn	Phenotype, endotoxin	(Lodeiros et al. 1992)
Not cited	*O. edulis*				Walne (1956, 1958)
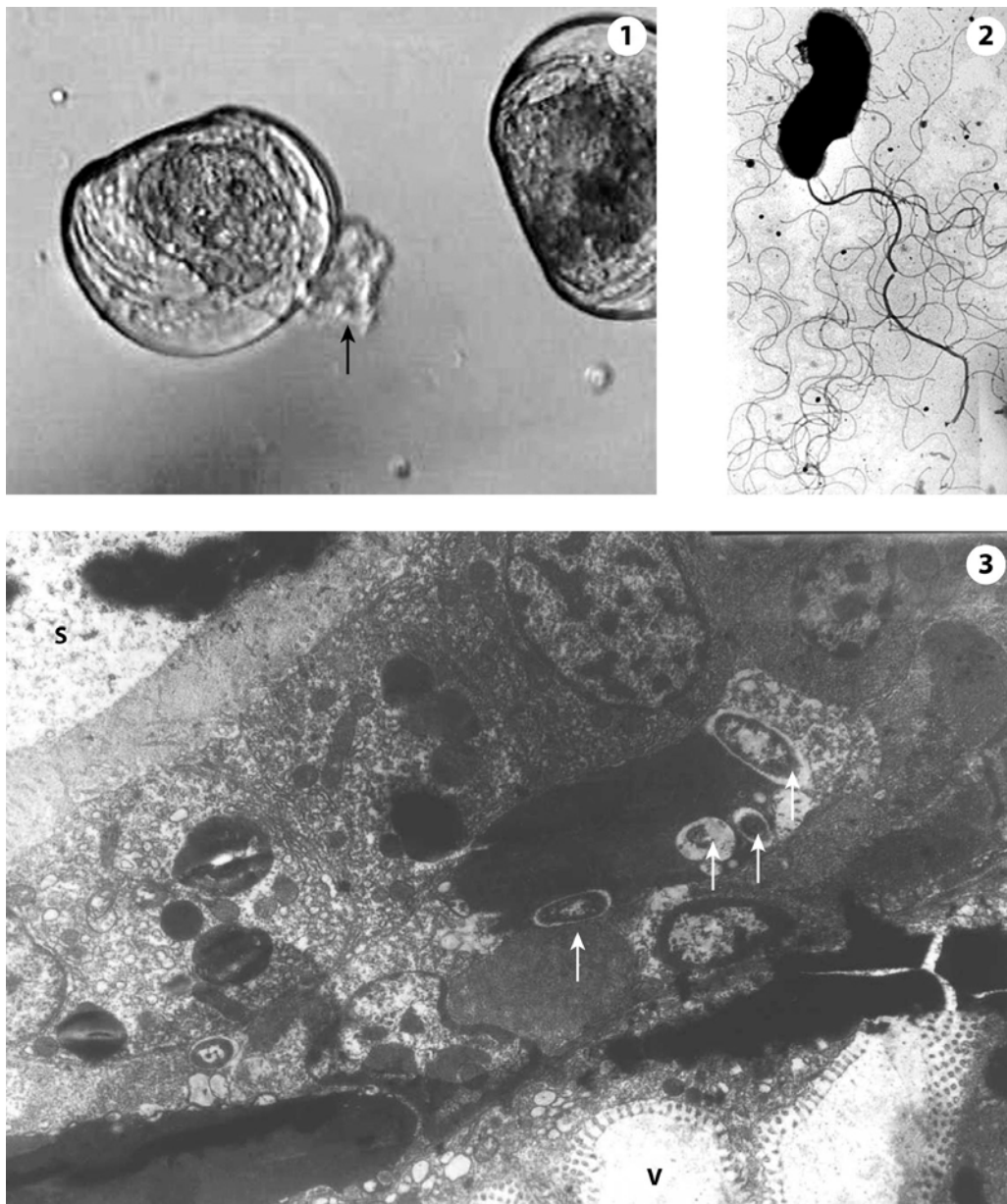
Plate 1. *Pecten maximus* larvae- *Vibrio pectenicida* (photographs by courtesy of C. Lambert, reprinted with permission from Lambert et al. 1998). 1: Diseased *Pecten* larvae, showing velar damage, 48 hours after *V. pectenicida* challenge (×450).
2: Electron micrograph of a negatively stained cell of *Vibrio pectenicida*, strain A365^T (Marine Agar growth) showing polar flagellum and lateral flagella (×8000).
3: Electron micrograph of a section of *Pecten maximus* larvae after *Vibrio pectenicida* challenge. Several bacteria at different stages of degradation, included into phagosome or lysozome, are localised between the stomach and velum fold (×4000).

All the other bacterial diseases cited in Table 2 affect both juveniles and mainly adults. In 1983, Lauckner writes that the pathogenic role of bacteria had never been well demonstrated in adult bivalves. To date, the nocardiosis affecting *C. gigas* (Friedmann et al. 1988, 1991a,b; Friedmann and Hedrick 1991) and the Brown Ring Disease in clams due to *V. tapetis* (Paillard et al. 1994), are the only bacterial disease of adult bivalves for which the etiology has been clearly defined (Table 2). Generally, in other bacterial diseases affecting bivalves, the experimental reproduction of symptoms is not clearly proved, and Koch's postulates are not completely demonstrated.

Few Gram-positive bacteria are involved in adult bivalve disease. One of the most well-known disease is the nocardiosis, which is provoke by a Gram-positive bacterium, *Nocardia crassostreae* (Plate 6) (Friedman and Hedrick 1991; Friedman et al. 1991) (see paragraph, Recent studies of some major bivalve-bacteria interaction models). Similar pathological changes and a Gram-positive bacteria was previously reported during mass mortalities of oysters in Japan in 1964
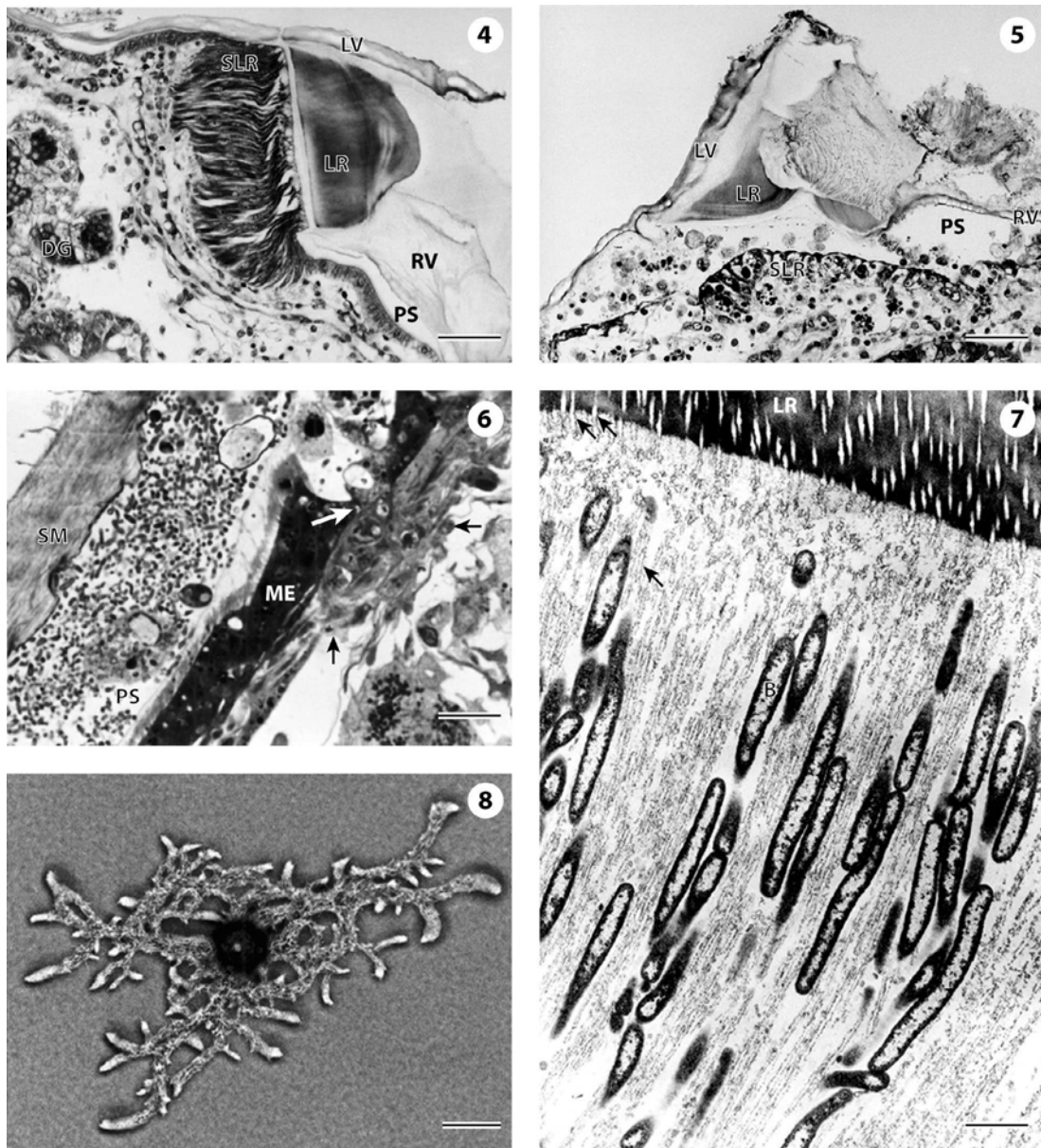
Plate 2. Hinge Ligament Erosion in *Crassostrea gigas* juveniles (photographs by courtesy of C. Dungan, reprinted with permission from Dungan et al. 1989).

4: Normal morphology of the hinge ligament and associated tissues in juvenile *C. gigas*. Transverse histological sections through the hinge ligament resilium (LR), secretory subligament ridge (SLR), pallial space (PS), left valve (LV), right valve (RV), and digestive gland (DG). The SLR is intimately apposed to the internal surface of the resilium. Neither the resilium is colonized by bacteria. Bar = 100 μm.

5: Affected juvenile oyster whose hinge ligament has been nearly perforated by bacterial erosion. Transverse histological section through the hinge ligament resilium (LR) which separates the left (LV) and right (RV) valves. Ligament-eroding bacteria appear as fibers oriented at right angles to the eroding resilium margin. The SLR appears necrotic, and is separated from the internal resilium surface by a wide pallial space (PS) containing bacteria and free oyster cells. Bar = 100 μm.

6: Histological section showing juvenile oyster response to bacterial proliferation within the pallial space. A dense bacterial population is seen in the pallial space (PS) which occurs between the valve (SM) and the mantle epithelium (ME). Oyster hemocyte aggregate at the ME basal lamina (small arrows), and migrate through the epithelium to phagocytise bacteria within the pallial space (large arrows). Bar = 10 μm.

7: Electron micrograph showing homogenous population of bacteria (B) associated with the eroding surface of the hinge ligament resilium (LR). By reference to the eroding resilium margin, material surrounding bacteria is identified as degraded resilium by the presence of remnants of mineral fibers (arrows), which are seen as light figures embedded within the dense protein matrix of intact resilium. Bar = 1 μm.

8: Characteristic colony morphology of oyster hinge ligament *Cytophaga*-like bacteria (CLB) cultured on *Cytophaga* agar, formulated with 50% of sea water (SWCA) was observed by means of a differential interference contrast microscopy. In this young (24 h) colony, rhizoid colony margins typical of many CLB can be seen to result from migration of cells along common routes and away from the colony center. Bar = 50 μm.

Table 2. Bacterial pathogens to juvenile and (or) adult bivalve. For each pathogen, are given, depending author reference, the host species and its size, its ability to reproduce host mortality and symptoms, its impact on host, its characterisation and its pathogenicity. nd: non done.

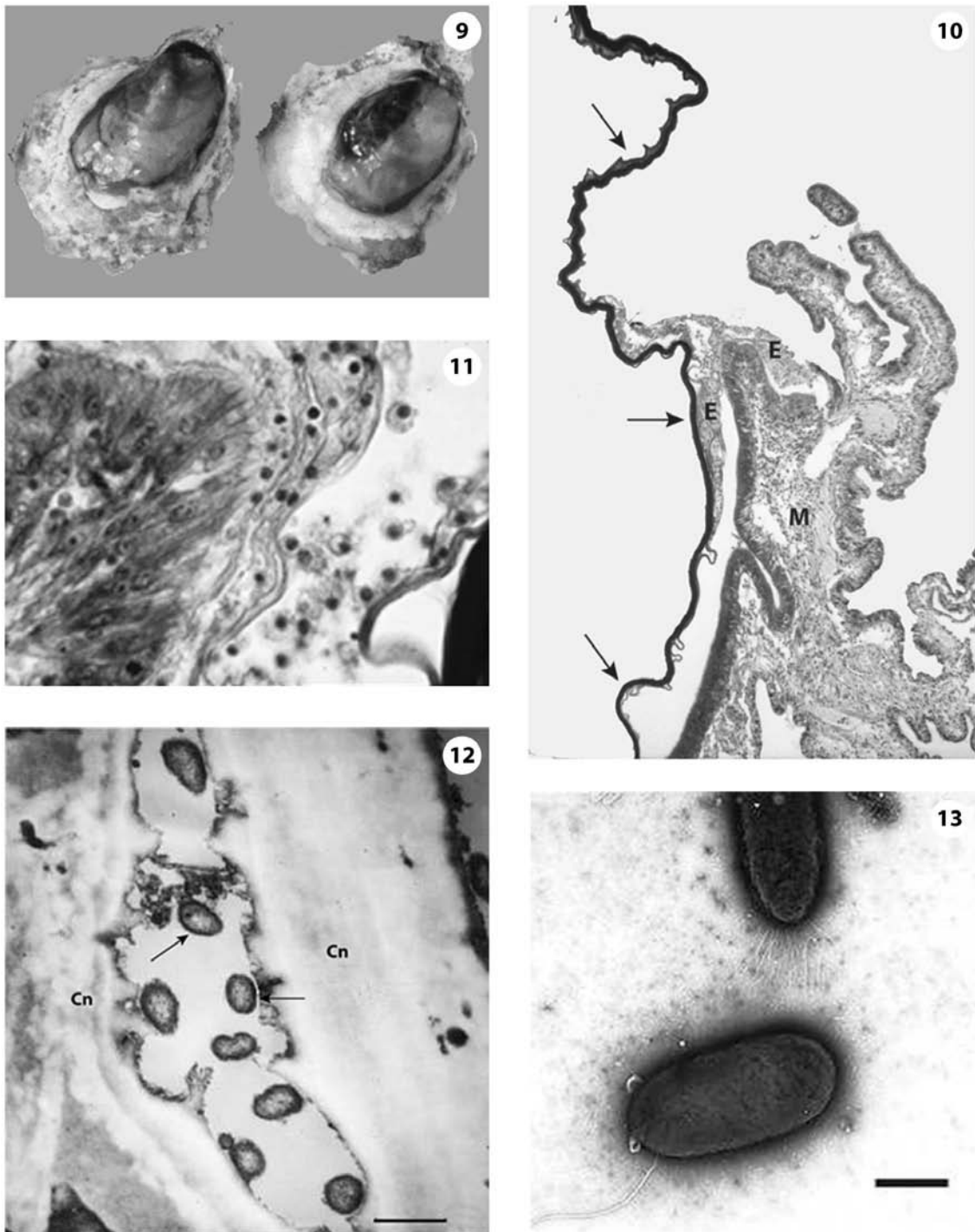| Bacterial pathogens to juvenile and (or adult) bivalve	Disease name	Host	size	Experimental Reproduction of symptoms	Pathogenesis (site of infection, symptom)	Pathogen characterisation	Pathogenicity	Référence
Rod shaped bacteria nd		*Crassostrea gigas*	Juvenile	nd	+ mantle, abcess in the extrapallial space	nd	nd	Elston et al. 1999
Cytophaga sp.	Hinge ligament erosion	*C. gigas*	**Juvenile** adult	+	+ ligament erosion			Dungan & Elston 1988 Dungan et al. 1989
Gram positive, nd	Bacterial abscess disease (BAD)	*Placopecten magellanicus*	adult	nd	+ adductor muscle Haemocyte infiltration s	nd	nd	Sherburne & Bean 1986 Getchell 1991 Mc Gladerry et al. 1993
Nocardia	Nocardiosis	*C. gigas*	adult	+	+ adductor muscle, pustules	+	nd	Friedman et al. 1991
Mycoplasme		*Patinopecten yessoensis* *Cerastoderma edule*	adult adult	+ (mortalities) -	+ (adductor muscle) + (gills)	+ +	nd nd	Bower et al. 1991, 1992; Bower & Meyer 1994 Azevedo 1993
Ricketssia		*C. gigas* *H. hippopus* *Tridacna gigas* *Meretrix lusoria* *P. yessoensis,* *Tapes japonica* *magellanicus* *Pecten maximus*	adult adult adult adult adult adult adult adult	nd nd nd nd nd + nd	+ (gill) + (siphon mantle + (mantle) + (gill) + (gill, digestive) + (gill, digestive) + myodegeneration + (gill)	+ nd nd nd nd nd (cultured) nd	nd nd nd nd nd nd nd nd	Azeverdo & Viallalba 1991 Norton et al. 1993 Norton et al. 1993 Wen et al. 1993 Elston 1986a Elston 1986a Gulka et al. 1983 Gulka & Chang 1984 Le Gall et al. 1988
Chlamydia		*C. gigas* *Argopecten irradians* *P. maximus*	adult adult adult	nd	+ (gill, mantle) +	+ +	nd nd	Renault & Cochennec 1994, 1995 Getchell 1991 Le Gall et al. 1988, 1992
Procaryote, Nuclear inclusion X		*Siliqua patula*	adult	nd	+ (gills)	nd	nd	Elston 1986b
Gram negative, nd		*Pinctada maxima* *Pinctada. margaritifera*	adult	nd	+ (pericardial enlargment)	nd	nd	Pass et al. 1987 Marin & Dauphin 1991
Vibrio sp.	Conchiolin infection	*Crassostrea virginica* *Ostrea edulis* *Mercenaria mercenaria*	juvenile		ligament periostracum	phenotype		Elston et al. 1982
V. tapetis	Brown Ring Disease (BRD)	*Ruditapes philippinarum* *Ruditapes decussatus*	juvenile **adult**	+	+ mantle, extrapallial fluids CD	phenotype phylogeny	nd nd + + +	Paillard et al. 1989 Paillard & Maes 1990 Paillard & Maes 1995a,b Paillard et al. 1994 Borrego et al. 1996
Proteobacteria	Juvenile oyster disease (JOD)	*C. virginica*	**juvenile** adult	negative with bacteria nd nd transmission + (CD)	+, mantle extrapallial fluids CD	+	nd	Boechter 1999, 2000 Bricelj et al. 1992 Ford & Borrero 2001 Paillard et al. 1996
V. splendidus		*P. maximus*	adult	nd	+ mantle extrapallial fluids CD	phylogeny	nd	Lambert et al. 1999
Gram negative, nd		*Pinctada maxima* *P. margaritifera*	adult		+ (pericardial enlargment)	nd	nd	Pass et al. 1987 Marin & Dauphin 1991
V. anguillarum		*Crassostrea gigas*	adult	+ (mortalities)	nd	+ phenotype	nd	Grischowski & liston 1974
V. anguillarum		*C. gigas*	adult	+ Cardiac edema	+	+	nd	Tubiash 1973

Plate 3. Juvenile Oyster Disease (JOD) in *Crassostrea virginica.*

9: Conchiolin deposits on surface of inner valves, with raised edge. Note fouling on exposed shell external to deposit.

10: Histological sections showing justaposition of mantle (M) and conchiolin deposit (arrows). Exudate of hemocytes, debris, and organic material. Bar = 100 μm.

11: Mantle epithelium (ME) with exudate of hemocytes and debris being deposited, between organic sheets (arrows), against primary conchiolin deposit (*). Bar = 10 μm (photographs No 9, 10 and 11 by courtesy of S. Ford, reprinted with permission from Ford and Borrero 2001).

12: Chamber between layers of anomalous conchiolin (Cn) in which gram negative bacteria (arrows) are present. Bar = 0.5 μm (photograph by courtesy of F. Perkins, reprinted with permission from Bricelj et al. 1992).

13: Electron micrograph of a negatively stained cell of *Roseobacter* sp. showing one bacteria with a flagellum, and the other (partial) with a tuft of polar fimbriae (After). Bar = 0.2 μm (original micrograph provided by K. Boettcher).
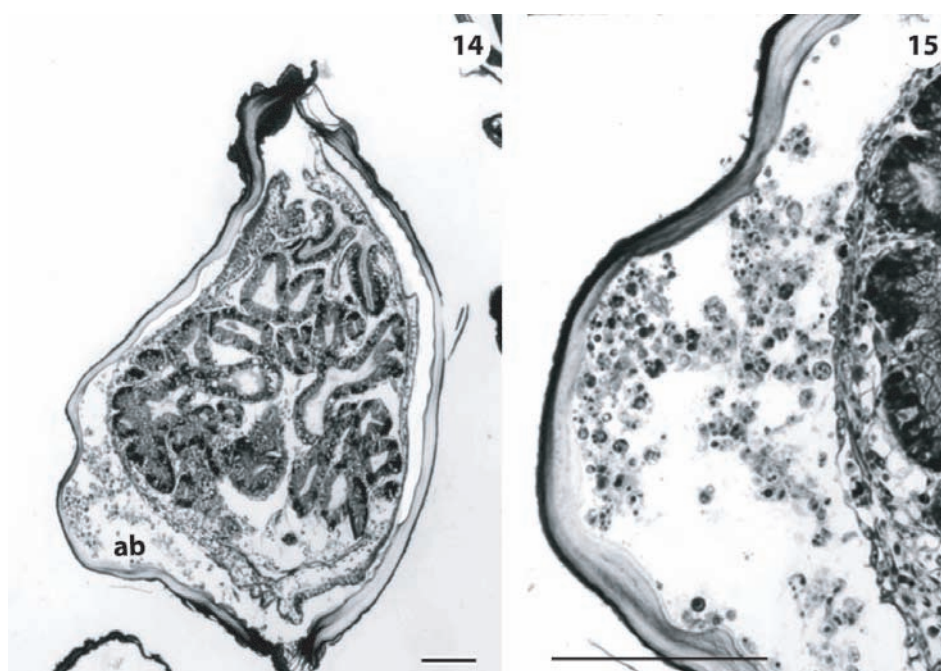
Plate 4. Bacterial Abcess Disease (BAD) in *Crassostrea gigas* juveniles (photographs by courtesy of R. Elston, reprinted with permission from Elston et al. 1999).
14: Transverse section through juvenile oyster, C. gigas, showing an abscess (ab) in the extrapallial space. The extrapallial space on the left hand aspect of the oyster is normal. Giesma stain. Bar = 100 μm.
15: Enlarged area of the abcess containing cell debris and rounded detached host cells. The mantle epithelium is flattened and contains rounded degenerating cells (arrows). Giemsa stain, Bar = 100 μm.

(Numachi et al. 1965; Imai et al. 1965). In *Pecten magellanicus*, some gram-positive bacteria were also suspected to produce brown spots along with necrosis and abscesses in the adductor muscle (Sherburne and Bean 1986; Getchell 1991; McGladdery et al. 1990).

Bacteria belonging to *Rickettsia*, *Chlamydia* and *Mycoplasma* genera infect a wide range of adult bivalve molluscs, and are reported generally as potential pathogens (Comps et al. 1977; Joly and Comps 1980; Meyers 1981; Elston 1986; Le Gall et al. 1988; Azevedo and Villalba 1991; Norton et al. 1993; Renault and Cochennec 1995). Ricketssial and chlamydial agents are closely related to Gram-negative and are generally intra-cytoplasmic, detected mainly in the epithelium of gills, siphons and mantle. *Mycoplasma* presumably evolved by degenerative evolution from Gram-positive bacteria and are phylogenetically most closely related to clostridia. Some of *Rickettsia*, *Chlamydia*, and *Mycoplasma* have been associated with host damages, but only occasional mortalities have been associated with their infections; this is the case for all the references cited in Table 2.

Most of the bacterial diseases in adult bivalves are provoked by Gram-negative bacteria and mostly of the genus *Vibrio* (Table 2). When pathogenesis process are described, in the majority of disease cases affecting various bivalves (conchiolin infection in *C. virginica*, *O. edulis*, *M. mercenaria* and *P. maximus*, BRD in clams, *R. philippinarum* and *R. decussatus*, JOD in *C. virginica*) the pathogen induces typical clinical signs including abnormal Conchiolin Deposit (CD) (Elston et al. 1982; Bricelj et al. 1992; Paillard and Maes 1995a,b;

Lambert et al. 1999) (Table 2, Plates 3 and 5). Different Gram-negative bacteria species can produce CD and susceptibility is generally host specific. For example, *V. tapetis* induces high CD prevalence only in *R. philippinarum* (Paillard et al. 1997; Novoa et al. 1998); *V. splendidus* provokes CD in *P. maximus* (Lambert et al. 1999). One other group of bacteria, the alpha-proteobacteria corresponding to the *Roseobacter* clade, has been also associated with this symptom in *C. virginica*, but to date experimental reproduction of CD has been unsuccessful (Boettcher et al. 2000). Another symptom, the cardiac oedema, has been described in oysters associated with *Vibrio anguillarum*, however, this infection in adult bivalves is infrequent (Tubiash et al. 1973). In the case of other Gram-negative bacterial diseases, clinical signs are poorly described, or not yet known in the case of emergent diseases. In many instances, only mortality episodes are followed, often associated with Gram negative bacteria of the genus *Vibrio* (Pass et al. 1987; Lacoste et al. 2001; Waechter et al. 2002). Two research teams have recently independently implicated a same bacterium species, however, they identified different biovar, *V. splendidus* biovar I (Lacoste et al. 2001) and *V. splendidus* biovar II (Waechter et al. 2002). Summer mortality of oysters and BRD of clams are described further in this review (see paragraph, Recent studies of some major bivalve-bacteria interaction models).

Little is known on the pathogenicity of bacteria involved during bivalve infections compared to the studies on the pathogeny processes for vertebrates and plants pathogens (see reviews for bacterial pathogens of plants and animal,
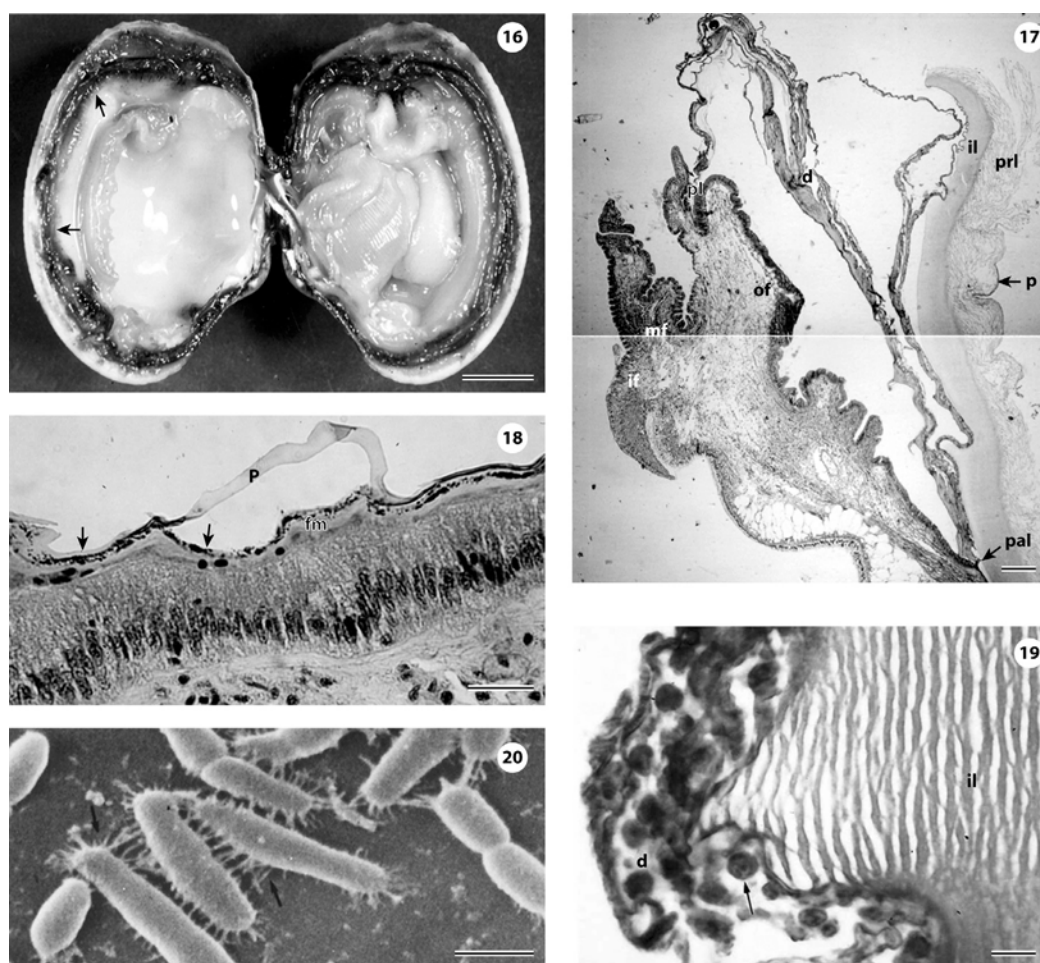
Plate 5. Brown Ring Disease (BRD) in *Ruditapes philippinarum* juveniles and adults.

16: Adult clam, *R. philippinarum*, naturally affected by the Brown Ring Disease (BRD) and exhibited the characteristic brown conchiolin deposit. Bar = 1 cm.

17: Transversal sections of the mantle and shell edge after shell decalcification of a clam, *R. philippinarum* presenting brown ring deposit. The conchiolin deposit is clearly visible between the pallial line and the shell edge. The periostracal lamina is very thick and covered by many debris. Light micrograph and Masson-Trichrome coloration. Bar = 200 μm (micrograph reprinted with permission from Paillard and Maes 1995b).

18: Light micrograph of the outer fold (of) of the mantle of *R. philippinarum*. Numerous bacteria are present within the periostracal lamina, which is composed on two layers, the periostracum (p) and the fibrous matrix (fm). Hematoxylin-eosine coloration. Bar = 10 μm (micrograph reprinted with permission from Paillard et al. 1994).

19: Light micrograph of conchiolin deposit (d) adhering to the inner shell layer (il) of *R. philippinarum*. Accumulation of dark granules and cells (arrows) colored by Masson-Trichrome. Bar = 10 μm (micrograph reprinted with permission from Paillard and Maes 1995b).

20: Scanning electron micrograph of the shell edge of *R. philippinarum* 12 hours after *V. tapetis* challenged. Numerous *V. tapetis* adhere to the surface of the periostracum lamina which is still attached to the shell edge. Note the pili (arrow) which mediate adherence of *V. tapetis*. Bar = 1 μm (micrograph reprinted with permission from Paillard et al. 1994).

Hueck 1998; Soto and Hultgren 1999; Schmiel and Miller 1999; Brodgen et al. 2000; Cossart et al. 2000; Donnenberg 2000). It is generally known that bacterial pathogenicity is associated both with structural components of the microbial cells (e.g. capsules, fimbriae, lipopolysaccharides, LPS, endotoxins, other cell wall components) or active secretions of substances that either damage host tissues or protect the bacteria against host defences (invasin, enzyme, haemolysin, coagulase and various toxins such as, i.e. diphtheria, botulism, tetanus or cholera). Recently, pathologists working on human bacterial pathogens have begun to describe genes involved in regulating

virulence factors (Smith 2000). Some virulence determinants such adherence factors, LPS and toxins have been described in marine bacterial pathogens such as the vibrios *V. anguillarum*, *V. vulnificus*, *V. harveyi* and *V. penaeicida* isolated from fish and crustaceans (Goarant et al. 2000; Milton et al. 1992, 1996; Amaro et al. 1997; Babelona et al. 1995, 1998a,b; Paranjpye et al. 1998; Montero et al. 1999; Gorant et al. 2000). Exotoxins (hemolysin, protease, ciliostatic toxin) have been identified in vibrios pathogenic of bivalve (DiSalvo et al. 1978; Brown and Roland 1984; Elston 1984; Lodeiros et al. 1987; Nottage and Birkbeck 1986, 1987a,b; Nottage et al. 1989;
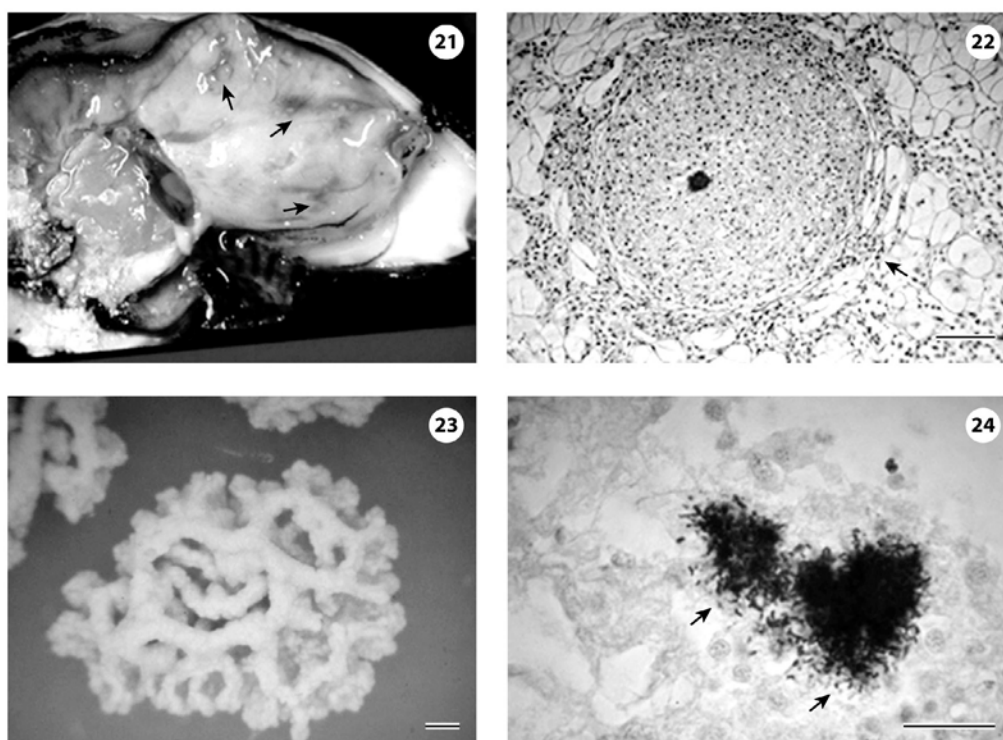
Plate 6. Nocardiosis in *Crassostrea gigas* adults (photographs by courtesy of C.S. Friedman, reprinted with permission from Friedman et al. 1991).

21: Lesions observed on adult pacific oysters with *Nocardia crassostreae* infections. Heavy infection in which large nodules can be seen in most tissues (arrows).

22: Brown-Brenn Gram-stained tissue section of a naturally infected Pacific oyster showing *Nocardia crassostreae* infection and associated pathological changes. Arrows point to bacterial colonies and indicate the filamentous nature of the bacterium within oyster connective tissues. Bar = 120 μm.

23: A *Nocardia crassostreae* colony cultured on Brain Heart Infusion agar plates with supplemental NaCl 2%, observed by means of a differential interference contrast microscopy. After 1–2 week at 22 °C, the colony is dry, waxy, irregular and wrinkled. It produces a well developed mycelium which fragmented into irregular rod-shaped forms. Bar = 9 μm.

24: Gram stained lesion smear of mantle. *Nocardia crassostreae* colonies are Gram + to Gram variable pleomorphic, beaded, branched bacterium. Bar = 1 μm.

Borrego et al. 1996a; Lambert et al. 2001) (Table 1). By use of in vitro bioassays of hemocyte-bacteria interactions, Lane and Birkbeck (1999) have demonstrated that *V. anguillarum* and *V. alginolyticus* possess a cytotoxic effect on mussel hemocytes causing hemocyte rounding. A degree of specificity in the bacteria-hemocyte interactions was demonstrated for particular model such as *V. pectinicida-P. maximus*, *V. tapetis-Ruditapes* spp. and *Vibrio* V110-*Ostrea edulis*; all these bivalve pathogens tested caused a high degree of cell rounding in hemocytes of the species which they were isolated (Lane and Birkbeck 2000; Choquet et al. 2003). Some of these exotoxins have been characterised such as a protease of 38–41 kDa in *V. alginolyticus* (Nottage and Birkbeck 1987a), ciliostatic toxins of low molecular weight (<1 kDa) in *V. alginolyticus* or *Vibrio* spp. (Nottage et al. 1989; Birkbeck et al. 1987) and a *Vibrio* Hemocyte-Killer Toxin (VHKT) of low molecular weight (<3 kDa) in *V. pectenicida* (Lambert et al. 2001). Other virulence factors such as pili, which facilitate attachment to shell secretions in the host have been implicated in pathogenicity of *V. tapetis* (Paillard and Maes 1995a,b).

4 Recent studies of some major bivalve-bacteria interaction models

4.1 Pecten larvae - V. pectenicida

Scallop *P. maximus* larvae are generally cultivated in hatcheries using antibiotic treatments. Without antibiotics, high mortalities occur between day 12 and day 19 after hatching. Two clusters of vibrios present at high densities were identified only in larval cultures in which antibiotics were not used (Nicolas et al. 1996). Experimental challenge with some strains belonging to these clusters at a final concentration of 10^4 cell ml^{-1} caused mortalities in scallop larvae within 48 h. Over 5 years of sampling at the Argenton hatchery (Brittany, France), these pathogenic isolates were systematically detected in moribund scallop larvae (Lambert et al. 1998). Phylogenetic analysis confirmed that these strains are closely related to *V. tapetis* and *V. splendidus*, but DNA-DNA hybridisation-confirmed them as a new species, which was named *V. pectenicida* (Plate 2, No 2) (Lambert et al. 1998).

After experimental infection with *V. pectenicida*, pathogenesis in larval scallops was followed by light and transmission electron microscopy (Plate 2, No 1 and 3) (Lambert 1998). Bacillary necrosis was observed in the scallop larvae, which was similar to that observed by Elston and Leibovitz (1980) in other bivalves. Observations of larval gut sections of *P. maximus* after infection by *V. pectenicida* have shown the presence of degraded bacteria in host cells (probably hemocytes) located between the gut epithelium and the velum (Plate 2, No 3) (Lambert 1998). Using in vitro test of vibrio-hemocyte interactions, it has been demonstrated that this pathogenic strain induced specific inhibition of chemiluminescent activity in hemocytes of adult *P. maximus* (Lambert and Nicolas 1998). Moreover, it was confirmed that cytoplasmic extracts of *V. pectenicida,* as well as live bacteria, not only inhibited the chemiluminescent activity of *P. maximus* hemocytes but also provoked death of hemocytes after 6 h (Lambert et al. 2001). One of the active factors, named VHKT (Vibrio Hemocyte-Killer Toxin) has been partially purified and shown to have properties that do not correspond to those of proteins property (Lambert et al. 2001). In terms of thermoresistance and low molecular weight (<3 kDa), VHKT is close to the ciliostatic toxin described by Nottage et al. (1989), but no similar ciliostatic effect has been shown with *V. pectenicida* against *Mytilus edulis* gills (Lambert 1998). Further characterisation of VHKT is still in progress.

4.2 Brown Ring Disease (BRD)

The Brown Ring Disease (BRD) affecting the manila clam, *R. philippinarum* is a bacterial disease, caused by a vibrio, *V. tapetis*, which perturbs the calcification process and provoks a characteristic symptom, a brown deposit on the inner surface of the valves (Plate 5) (Paillard and Maes 1990; Paillard et al. 1994; Borrego et al. 1996b, Paillard 2004b). *V. tapetis* adheres to the peristracal lamina, the site of infection (Plate 5, No 18 and 20). This bacterial colonisation provoks modifications of shell secretions which become not a good substrate for biomineralization process, then these matrix subsequently accumulates and forms a deposit on the inner edge of the shell (Plate 5, No 16, 17, 19). A classification system of the syndrome, based on disease and recovery stages, has been established for use in epidemiological and experimental studies (Paillard and Maes 1994). This disease occurs in wild populations and cultured clam of France, England, Ireland, Spain and occasionally Italy, and has been reported recently in Corea (Paillard and Choi, unpublished data) (Paillard et al. 1994; Castro et al. 1997a; Allam et al. 2000). The BRD like symptom has also been detected in other clam species (*R. decussatus, Tapes rhomboïdes, Venerupis aurea, Dosinia exoleta*) and in other bivalves (*P. maximus, Chlamys varia*) (Maes and Paillard 1992; Paillard, unpubl. data) and in gasteropods such as abalone (*Haliotis tuberculata, H. rubra, H. leviagata, H. scalaris*) (Shepherd and Triantafillos 1997; Shepherd and Huchette 1997; Marshall and Day 2001; Huchette et al. 2002). To date, *V. tapetis* was associated with BRD only in two different species of clams (*R. decussatus, Venerupis aurea)* from France, Spain, England and Ireland using isolation techniques on differential culture media and immunological tests

(Maes 1992; Novoa et al. 1998; Castro et al. 1992, 1995, 1996; Allam et al. 2000). Recently, a *V. tapetis* strain has been isolated from a cultured fish, the wrasse, *Symphodus melops* during mortalities in Norway (Jensen et al. 2003). An other suspected *V. tapetis* strain has been also isolated recently from a mortality outbreak of the cultured fish, the Halibut, *Hippoglossus hippoglossus* (Reid et al. 2003). Some virulence factors of *V. tapetis* have been identified: adherence factors such as pili (Borrego et al. 1995a,b) toxins such as haemolysins and cytotoxins (Borrego et al. 1996a). All isolates contain between two and four large plasmids sizing approximatively 60 to 100 Kbp (Le Chevalier et al. 2003), and several groups of strains may be distinguished on the basis of their pulse-field electrophoretic profiles and ribotyping and plasmid patterns (Castro et al. 1997b). On the basis of RFLP plasmid patterns, the strain from Norway deviated considerably from the French and British strains (Le Chevalier et al. 2003).

Pathogenicity tests have been standardized in vivo, the inoculation is performed either in the pallial cavity to reproduce the conchiolin deposit (CD) symptom (5×10^7 CFU/individual; clam length 15−20 mm, T °C: 14 °C) or in the adductor muscle to induce mortalities (5×10^7 CFU/individual; clam length 30−40 mm, T °C: 14 °C) (Paillard and Maes 1990; Allam et al. 2002a). Hemolymph parameters, i.e. changes in total and differential hemocyte counts and leucine aminopeptidase activity were altered by experimental challenge (Oubella et al. 1993; Oubella et al. 1994; Oubella et al. 1996). Interactions of *V. tapetis* with clam's tissues or hemocytes have been developed in vitro to evaluate bacterial pathogenicity (Castro et al. 1999; Lopez-Cortes et al. 1999; Lopez-Cortes et al. 1999; Allam et al. 2002; Choquet et al. 2003). According to these studies, cytotoxic factors which inhibit pseudopod formation and adherence capacity of hemocytes have been yet identified as virulence factors; the identification and characterisation of the genes involved in *V. tapetis* cytotoxicity are in progress (Choquet et al. 2003). Diagnostic tools have been developed to study *V. tapetis* including polyclonal and monoclonal antibodies, detection by ELISA tests and nucleic acid probes (16S rDNA) (Nöel et al. 1996; Paillard et al. 2001; Paillard et al. 2004a).

4.3 Juvenile Oyster Disease (JOD)

Juvenile oyster disease is a syndrome recognized in 1988. It is characterized by an excessive cupping on the left valve and an anomalous conchiolin deposit around the periphery of the mantle on the inner valves (Bricelj et al. 1992; Ford and Borrero 2001) (Plate 2). The deposit may cover the entire mantle surface and the attachment of the adductor muscle to the shell (Ford and Borrerro 2001). The deposit of organic material on the inner shell surface resembles the Brown Ring symptom of manila clam, *R. philippinarum* (Plate 5) (Paillard and Maes 1995a,b). Transmission experiments were performed by adding uninfected juvenile oysters to aquaria containing recently infected JOD oysters (Lewis et al. 1996). Under these laboratory experiments, the disease was readily transmissible 3 to 7 weeks after exposure to JOD-infected oysters. Mortalities and expression of conchiolin were enhanced by warm temperatures, 22−26 °C, and salinities of 18−30 ppt (Lewis et al. 1996). Transmission experiments using extracts from

JOD-affected oysters demonstrated also that the conchiolin deposit could be reproduced in healthy oysters within 4 weeks the conchiolin deposit (Paillard et al. 1996). Antibacterial agents both delayed JOD-onset and reduced final cumulative mortalities showing an evidence for a primary bacterial etiology (Boettcher et al. 1999). Several bacterial species have been proposed as etiological agents including, various *Vibrio*, *Aeromonas* and *Pseudomonas* spp. (Lee et al. 1996; Paillard et al. 1996). However, recently, bacteriological analysis of JOD-affected animals in Maine has revealed the involvement of a novel species of marine α-proteobacterium (Boettcher et al. 1999, 2000) (Plate 3, No 13). There is strong support for inclusion in the *Roseobacter* clade of the alpha-proteobacteria, however, the phenotypic and phylogenetic data do not warrant placement of this bacterium in any known genus. The designation *Roseimarina crassostreae* (gen. nov., sp. nov.) has been proposed (Plate 3, No 13) (after Boettcher, pers. comm.). All examined isolates have identical 16S rDNA sequences, regardless of the year (1997-2002), location, or severity of the epizootic (Boettcher et al. 1999; Boettcher et al. 2000; Boettcher, unpublished data). Two genotypes have been identified based on the internal transcribed spacer region between the 16S and 23S rRNA genes. The first (GT1) was associated with JOD in Maine in 1997 and 1998. A second slightly different genotype (GT2) was recovered from all examined Maine epizootics in 2000, 2001, and 2002 (Maloy et al. 2002; Boettcher, unpublished data). Mortalities have been reproduced by experimental exposure to the bacterium, although typical JOD-signs (e.g. proteinaceous conchiolin deposits on inner valve surfaces) were not observed (Boettcher et al. 2000). The emaciated appearance of JOD-affected animals is believed to result from an impairment in feeding caused by *R. crassostreae* (Boettcher et al. 2000; Boettcher, unpublished observations). Using a rifampicin-resistant strain of *R. crassostreae*, additional attempts at experimental reproduction of the disease are in progress. Bacteriological analyses using rifampicin-containing medium allows the colonization process to be followed. In the current trials, patterns of conchiolin deposition consistent with JOD, have been specifically induced in some challenged (but not control) animals (Boettcher, unpublished data).

Mortality rates of JOD varied depending on the outbreak, but it is not unusual for growers in Maine to lose 50% of annual production due to JOD (Bricelj et al. 1992; Davis and Barber 1994; Boettcher, personal communication). Very low mortalities (<5% cumulative mortalities) were observed during the 2002 JOD-epizootics in Maine. However, 9% of sampled animals (which were larger than those known to experience high JOD-related mortalities) had conchiolin deposits characteristic of JOD. In these oysters, the presence of conchiolin was 90% correlated with extensive colonization by *R. crassostreae*. However, the soft body tissues in those animals with conchiolin were still in good to fair condition. This was in fact, the first instance in which *R. crassostreae* was isolated from animals with JOD signs but in the absence of significant mortalities (Boettcher, personal communication). As such, it provides more evidence that in previous epizootics, *R. crassostreae* was not present as just an opportunistic colonizer of terminally ill oysters (Boettcher, personal communication and unpublished data).

4.4 Pacific Oyster Nocardiosis (PON)

Summer mortalities among Pacific oysters were first noticed in 1945 in Japan and in 1956 in North America (Perdue et al. 1981; Imai et al. 1965). Physiological stress induced by high metabolism, and gonadal maturation under high nutrient and warm water conditions has been offered as potential causes of these summer mortalities in these oysters (Imai et al. 1965; Mori et al. 1975; Perdue et al. 1981). Pathological changes in the connective tissues surrounding the intestinal duct were suspected to be caused by Gram-positive bacteria (Numachi 1965). In diseased Pacific oysters from Washington State and British Columbia, Friedman and Hedrick (1990) isolated a bacterium belonging to the genus *Nocardia* and renamed the disease as Pacific Oyster Nocardiosis (PON). PON is characterised by few to no external signs of infection in naturally or experimentally infected oysters. Diseased animals showed some mantle lesions with focal areas of discoloration in light infections and with large internal nodules in most tissues in heavy infections (Plate 6) (Friedman et al. 1991). Healthy Pacific oysters inoculated with *Nocardia* isolated from diseased oysters exhibited the same pathological changes as oysters with naturally occurring PON. This pathogen initially identified by morphological stain characteristics has been further phenotypically and genetically characterised and was assigned to a new species, *Nocardia crassostrea* (type strain ATCC 7000418) (Plate 6, No 23) (Friedman et al. 1998).

4.5 Summer mortalities in *C. gigas*

Different bacteria, mostly *Vibrio,* have been involved in *C. gigas* mortalities (Tables 1 and 2); however with the exception of larvae, it remains unclear whether these bacteria act as primary pathogens or as opportunists.

Since 1991 high but sporadic *C. gigas* spat mortality rates (60 to 100%) have been observed during the summer along the coast of Europe, in both wild and cultured oysters. This syndrome called "summer mortality" seems to have a complex etiology with several factors implicated: environmental conditions, physiological and genetic host parameters and infectious agents (Goulletquer et al. 1998). Two bacterial strains have been associated with "summer mortality" outbreaks. These strains, potentially pathogenic for *C. gigas* spat as shown by experimental challenge, have been phenotypically and genotypically identified as *Vibrio splendidus* biovar I (Lacoste et al. 2001a) and biovar II (Waechter et al. 2002).

Recently a comparative analysis of *V. splendidus* related strains isolated during *C. gigas* mortality events was conducted along the French Atlantic coast between 1997-1998 (Le Roux et al. 2002). Strains related to *V. splendidus* were selected and compared to the biovar II pathogenic strain (TNEMF6, Waechter et al. 2002) by classical biochemical tests and genotyping. Only one strain out of 14, was found to be closely related to the pathogenic strain. Neither the phenotypic nor the genotypic markers used in this study were able to distinguish pathogenic from non-pathogenic strains of the widespread *V. splendidus*.

5 Taxonomy and phylogeny of new potential pathogens

The family *Vibrionaceae* (Baumann and Schubert 1984) consists of more than 50 validated species and has been under extensive investigation in the last decades, consequently becoming one of the best documented marine bacterial taxa (Kita-Tsukamoto et al. 1993). The family comprises bacteria inhabiting aquatic environments, especially marine and brackish waters, where they are frequently associated with organisms of a diversity ranging from plankton to finfish. Some species are symbionts, whereas others are known as pathogens of humans as well as marine animals. These bacteria constitute an important percentage of the culturable heterotrophic bacteria associated with marine bivalve, especially oysters and mussels (Kueh and Chan 1985; Prieur et al. 1990; Olafsen et al. 1993; Pujalte et al. 1999; Castro et al. 2002).

Until now, studies on the genetic diversity in the family *Vibrionaceae* have mainly focused on certain pathogenic species, namely *V. anguillarum* (Austin et al. 1995) *Photobacterium (= Vibrio) damselae* (Thyssen et al. 2000), *V. cholerae* (Chun et al. 1999; Jiang et al. 2000a,b) *V. harveyi* (Pedersen et al. 1998), *V. parahaemolyticus* (Maeda et al. 2000), *V. tapetis* (Castro et al. 1997; Romalde et al. 2002; Le Chevalier et al. 2003), *V. vulnificus* (Arias et al. 1997) and *Moritella viscosa* (Benediktsdóttir et al. 2000). However the family includes many taxa not yet described and most *Vibrio* spp. are generally characterised only by phenotypic characters (Urakawa et al. 1999).

In 1994, Alsina and Blanch proposed a set of keys for the biochemical identification of environmental *Vibrio* species. However, the authors noted important discrepancies in the results of certain tests on the same species which increase difficulties for routine purposes in diagnostic laboratories. A diversity of habitat, at the host or environmental levels, could result in a phenotypic diversity as a way of adaptation by physiological flexibility. It could also result in biodiversity in genotype. In the face of these difficulties in characterising marine *Vibrio* spp. molecular approaches to identification should be encouraged.

The report of the Ad Hoc Committee for the re-evaluation of the species definition in Bacteriology has been recently published (Stackebrandt et al. 2002). This report takes into account the introduction of innovative methods, which have provided new opportunities for prokaryotic systematics. These methods are mostly based on gene sequencing (SSU rDNA plus protein-coding genes) and more recently on genome sequencing. New recommendations to investigators provided that maintain a pragmatic delineation of species in the genomic era, while integrating new techniques and new knowledge. Therefore the definition of a new species can be based on genomic methods if there is a sufficient degree of congruence between the technique used and DNA-DNA re-association. Validated polyphasic approaches are encouraged in order to develop new methods to supplement or even supplant DNA-DNA hybridization (Yamamoto et al. 1999; Palys et al. 2000).

SSU rDNA sequence analysis is a standard method to order prokaryotic taxa hierarchically among the ranks of genera and kingdoms (Garrity and Holt 2001; Ludwig and Klenk 2001; Stackebrandt et al. 2002). However, results of SSU rRNA-based analyses often do not correlate with those of DNA-DNA hybridisation which has been used as the key criterion for definition of bacterial species (Fox et al. 1992; Stackebrandt et al. 1994). The use of protein-coding genes may have several advantages over rRNA. Higher levels of sequence variation allow differentiation of closely related strains (Ochmann and Wilson 1987) while the ability to translate DNA to protein sequences permits phylogenetic analysis of distantly related strains and more accurate sequence alignment (Gupta 1998).

DNA gyrase B is a subunit of the enzyme responsible for introducing negative supercoils into bacterial genophores and plays a crucial role in the replication of the bacterial genetic material (Watt and Hickson 1994). The sigma 70 factor is one of the sigma factors that confer promoter-specific trascription initiation on RNA polymerase (Lonetto et al. 1992). Both proteins are ubiquitous in bacteria and they are essential for their growth. Both protein-encoding genes evolved much faster than rDNA and provided higher resolution than the use of 16S rDNA sequences (Yamamoto and Harayama 1998). Since 1995, when universal primers for these genes (*gyrB* and *rpoD*) became available, several studies have suggested that the phylogenetic clustering of strains belonging to a same genus, is almost equivalent to the genomic species delineated by DNA-DNA hybridization (Yamamoto and Harayama 1995; Yamamoto et al. 1999).

6 Diagnostic tools

Different approaches can be proposed in term of diagnostic tools. In the case of specific bacterial pathogens, always associated with disease (i.e. *V. tapetis*, *V. pectenicidae*, *V. harveyi*) isolation, biochemical and/or molecular characterisation should be sufficient to diagnose that a disease is due to the pathogenic micro-organism. However, it is noteworthy that current studies show that strains belonging to the same species demonstrate a clear variability of virulence (i.e. *V. splendidus*). In order to associate a specific strain to a disease, diagnosis can include DNA typing such as those targeting the whole genome (AFLP, RAPD, Rep-PCR, PFGE), a gene cluster (ribotyping of rrn operons), individual genes (ARDRA of 16S rDNA, gyrB, rpoD) and intergenic 16S-23S rDNA spacer regions (ISR) (for review see Vaneechoutte 1996; Rademaker et al. 2000; Gürler and Mayall 2001; Van Belkum et al. 2001). Because genotyping may not lead to the identification of a pathogenicity marker, a complementary approach based on virulence characterisation and gene screening also might be necessary.

7 Screening of gene encoding virulence factor

Depending on the model, different molecular approach can be used to screen gene encoding virulence factors.

7.1 From the phenotype to the genotype

First the virulence of strains can be evaluated by experimental challenges on susceptible hosts and the pathogenesis

studied (host damage and immune response). Bacterial localisation and host damage should give information on virulence mechanisms: In vitro models should be used or developed to select likely virulence mechanisms. Theses models should be validated by comparison to experimental challenge and could provide an alternative approach to evaluate the virulence abilities of a bacterial strain during epidemiological surveys.

From experimental challenges and in vitro tests, restricted mechanims should be selected to study the molecular mechanisms involved. Gene candidates can be screened by PCR or by using genomic library of degenerated oligonucleotides or probe designed in conserved region of gene family.

A post-genomic approach is essential to demonstrate the role of a gene in virulence. The strain can be mutated by the use of transposon for insertional mutagenesis in selected virulence factor genes. The mutant strains is therefore isolated and screened to demonstrate a reduction of their virulence in in vitro and in vivo models.

7.2 From the genotype to the phenotype

Another approach "blind" also can be proposed: random mutagenesis requires a standardised *ex vivo* model to improve the mutant phenotype, substractive genomic requires strains with a high DNA hybridization rate but with opposite virulence, and finally sequence analysis of complete genomes which has provided scientists an immeasurable wealth of information.

In conclusion, the emergence of diseases in marine organisms is worrying (Science 1999). The influence of environmental factors has been demonstrated in major bivalve-bacteria interactions models. One of the key environmental factors triggering bacterial disease is temperature. Major bacterial disease occurs generally with the increasing temperature (it is the case for JOD, PON in United States, Summer mortalities in oysters in France and larval vibriosis in hatcheries from different countries). Rising water temperature were correlated with both increasing vibrio concentrations and in the proportion of ciliostatic-production bacteria in the cases of numerous marine pathogenic vibrios (Birkbeck and Gallacher 1993). A few bacterial diseases are most pronounced at low temperature such as BRD, which has the maximum of prevalence during winter (Paillard et al. 1997; Paillard et al. 2004).

Environmental warming could be associated with many emergent bacterial diseases in bivalves. Moreover, bivalve aquaculture, in rearing larvae and juveniles at artificially high temperatures and densities, and transferring adults for commercialisation, facilitates the acquisition and spread of infectious agents. The increasing number of investigators who now identify the causative agents of what previously were juste "unexplained mortalities" participate also to the phenomena of emergence of bacterial disease in bivalve.

Recently, mathematical models of host-pathogen-environment interactions in bivalves have been developed for two important protozoan parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, affecting the oyster, *C. virginica*. (Hofmann et al. 1995; Powell et al. 1996; Powell et al. 1999; Ford et al. 1999; Paraso et al. 1999). Currently, a numeric model is being developed for a vibriosis disease, BRD in clams (Jean et al. 2001).

Recent researches on bacterial bivalve diseases of bivalve show great promise for the future. New approaches coupling cellular and molecular techniques to study bacterial diseases will permit the development of new concepts on immunity and host resistance, as well as on bacterial virulence and the co-evolution of bacteria and marine invertebrates.

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