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## ***Vibrio* - bivalve interactions in health and disease**

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### **Abstract :**

In the marine environment, bivalve mollusks constitute habitats for bacteria of the Vibrionaceae family. Vibrios belong to the microbiota of healthy oysters and mussels, which have the ability to concentrate bacteria in their tissues and body fluids, including the hemolymph. Remarkably, these important aquaculture species respond differently to infectious diseases. While oysters are the subject of recurrent mass mortalities at different life stages, mussels appear rather resistant to infections. Thus, *Vibrio* species are associated to the main diseases affecting the worldwide oyster production. Here we review the current knowledge on *Vibrio*-bivalve interaction in oysters (*Crassostrea* sp.) and mussels (*Mytilus* sp.). We discuss the transient versus stable associations of vibrios with these bivalves as well as technical issues limiting the precise monitoring of vibrios in health and disease. Based on the current knowledge of oyster/mussel immunity and their interactions with *Vibrio* species pathogenic for oyster, we discuss how differences in immune effectors could contribute to the higher resistance of mussels to infections. Finally, we review the multiple strategies evolved by pathogenic vibrios to circumvent the potent immune defenses of bivalves and how key virulence mechanisms could have been positively or negatively selected in the marine environment through interactions with predators.

## Introduction

Animal-bacteria association has a long evolutionary history that could go as far back as to the origins of multicellularity (McFall-Ngai *et al.*, 2013). The presence of complex host-associated microbial communities is a characteristic shared by most animal species (Fraune and Bosch, 2010) and the type of interactions that they establish with their host can range from mutualistic to pathogenic. The capacity for microbes to colonize a host depends on both host and microbial determinants. On the one hand, healthy metazoans control their microbial environment with physical and chemical barriers, which from early stages of development shape the assemblage of their associated microbiota (for review see (Bevins and Salzman, 2011; McFall-Ngai *et al.*, 2013)). On the other hand, microbes have evolved strategies to overcome a series of innate immune mechanisms conserved across metazoans, which control both infections and host-microbiome homeostasis (Brennan and Gilmore, 2018).

In marine environments, metazoans constitute habitats for bacteria of the *Vibrionaceae* family (vibrios). These  $\gamma$ -proteobacteria are ubiquitous in marine and brackish environments representing one of the most abundant culturable fraction of the marine microbial community (Ceccarelli *et al.*, 2019). They possess a high colonization potential partially due to their metabolic versatility and genetic variability (Le Roux *et al.*, 2016). Their associations with plants, algae, zooplankton and other animals have been widely documented (Takemura *et al.*, 2014; Le Roux and Blokesch, 2018). In particular, some species of mollusks have established very intimate associations with *Vibrio* species, as illustrated by the mutualistic symbiosis of the squid *Euprymna scolopes* (Cephalopod) and *Vibrio fischeri* (McFall-Ngai, 2014). Bivalves, such as the oyster *Crassostrea gigas*, were shown to host a diversity of *Vibrio* populations, defined as members of a same species sharing a common gene pool and habitat (Le Roux *et al.*, 2016), in both health and disease (Bruto *et al.*, 2017). *Vibrio* associations with bivalves can be also neutral. Indeed, as filter feeders straining food particles from the seawater, bivalves accumulate exogenous bacteria, often transiently (Froelich and Oliver, 2013). Moreover, *Vibrio* species attach to the

chitinaceous surfaces of invertebrates, which provide a nutrient-rich habitat (Dana E. Hunt *et al.*, 2008), but do not always colonize their tissues. However, intimate interactions have also been described. Recently, evidence of co-evolution between vibrios and bivalves has been acquired in natural oyster beds (Wegner *et al.*, 2019).

Parasitic interactions are among the more documented *Vibrio*-bivalve associations, as they cause major damages to the host (Goudenège *et al.*, 2015; Lemire *et al.*, 2015; Bruto *et al.*, 2018; Dias *et al.*, 2018), with important consequences on the sustainability of the shellfish farming industry (Travers *et al.*, 2015). A key question for predicting and managing disease is whether *Vibrio* associations with bivalves result from specific (genome-encoded) abilities or disability to colonize these animal species and how much they depend on environmental constraints. A series of genetic determinants favoring bivalve colonization and disease expression have been identified in vibrios so far (Duperthuy *et al.*, 2011; Lemire *et al.*, 2015; Bruto *et al.*, 2017; Rubio *et al.*, 2019). They will be reviewed here in details. Among environmental factors, temperature is known to significantly affect the *Vibrio* communities associated with bivalves (Alfaro *et al.*, 2019). In fact, vibrios are strongly thermo-dependent and many species thrive in warm water exceeding 17 °C (Ceccarelli *et al.*, 2019). The threat posed by vibrios to the shellfish industry can be thus exacerbated by the impact of climate change, in particular sea surface warming, on their global spread (Vezzulli *et al.*, 2016; Baker-Austin *et al.*, 2017). Additional effects include the temperature-dependent expression of virulence factors and the modifications of host physiology (reproductive effort, immune status, etc.) (Travers *et al.*, 2009), which in bivalves can indirectly favor colonization by opportunistic vibrios (Le Roux *et al.*, 2016).

In this review, we will focus on the oyster *Crassostrea gigas* and the mussels *Mytilus edulis* and *Mytilus galloprovincialis*, which are bivalves of economic interest and ecological relevance with widely documented interactions with a diversity of vibrios. We will discuss successively *Vibrio* associations with bivalves, the key role of bivalve cellular defenses in controlling vibrios, the multiple adaptive strategies of vibrios to

their bivalve hosts, and the role of the biotic environment in the selection (both positive and negative) of adapted genotypes.

Accepted Article

## 1. **Vibrios as key components of bivalve microbiota**

Vibrios account for a significant proportion of bacteria found in healthy and diseased bivalves, where they can reach concentrations about 100-fold higher than those in ambient water (DePaola *et al.*, 1990; Šolić *et al.*, 1999; Shen *et al.*, 2009). Approaches to study *Vibrio* communities include both culture-dependent protocols and global DNA sequencing approaches. Whole body homogenates or single tissues (e.g., hemolymph, gut, hepatopancreas) are often analyzed. A large fraction of these bacteria might go undetected using culture-dependent methods due to metabolic requirements and culture conditions (e.g. food source, temperature, generation time). Sequencing-based approaches allow to overcome most of the problems related to cultivation (Lokmer and Wegner, 2015; Lokmer, Goedknecht, *et al.*, 2016; Lokmer, Kuenzel, *et al.*, 2016) and to access to the hidden non culturable vibrios. However, species-level resolution among genetically-related *Vibrio* species is often poor using the most commonly employed 16S rRNA marker, thereby limiting the fine-scale evaluation of taxonomic heterogeneity within the genus (Poretsky *et al.*, 2014; King *et al.*, 2019). Additional phylogenetic markers such as the heat shock protein hsp60 (Jesser and Noble, 2018; King *et al.*, 2019) were proposed as alternatives to 16S-barcoding to discriminate members of the *Vibrio* genus more precisely. Still, one single marker often provides insufficient resolution, due to the high genomic plasticity of vibrios (Le Roux and Blokesch, 2018), and culture-dependent methods coupled to Multiple Locus Sequence Analyses (MLSA) or core gene phylogenies are generally required for finest resolution analysis (Sawabe *et al.*, 2013).

### **Vibrios associated with bivalves**

Over the past 30 years, many studies carried out in different geographical areas have highlighted the complex structure of *Vibrio* populations commonly associated with bivalves (Romalde *et al.*, 2014; Chen *et al.*, 2016; Bruto *et al.*, 2017). Species belonging to the clades Splendidus and Harveyi are among the most frequently

found in association with healthy oysters and mussels (Romalde *et al.*, 2014; Le Roux *et al.*, 2016). Their concentration, diversity and dynamics are strongly dependent upon environmental parameters, such as temperature and salinity (Romalde *et al.*, 2014).

Most of our knowledge on vibrios associated with bivalves derives from studies focusing on pathological contexts. Indeed, a number of bacterial species, which include many *Vibrio* species, have been associated with mortality outbreaks of cultivated bivalves (oysters, clams, mussels) (Saulnier *et al.*, 2010; Travers *et al.*, 2015; Le Roux *et al.*, 2016; Dubert *et al.*, 2017). These *Vibrio* species can be primary pathogens, capable of causing pathological changes associated with disease in a healthy bivalve, or opportunistic pathogens, which cause disease when bivalves are compromised by a break in protective barriers or immunosuppression,

Expression of *Vibrio* diseases depends on host factors, essentially host genetics (species, population), life stage (larval, juvenile or adult form) and life history traits, e.g. co-infection by other pathogens (viruses as for example), the weakening of immune capacity due to environmental stressors (Zannella *et al.*, 2017), or immune priming following previous encounter with vibrios (Zhang *et al.*, 2014; Rey-Campos *et al.*, 2019).

Thanks to highly developed oyster production systems that go from hatcheries to nurseries and grow-out farming, we have a good knowledge of *Vibrio* species infecting different developmental stages of *Crassostrea* sp.. Vibrios associated with diseases in oyster spat and/or larvae include bacteria belonging to the species *V. anguillarum*, *V. alginolyticus*, *V. tubiashii*/*V. europaeus*, *V. ostreicida*, *V. coralliilyticus*, *V. neptunius*, *V. bivalvicida*, and to the Splendidus clade (for review see Romalde and Barja, 2010; Travers *et al.*, 2015; Dubert *et al.*, 2017). Closely-related species of the Splendidus clade (e.g., *V. splendidus*, *V. tasmaniensis*, *V. cyclitrophicus*, and *V. crassostreae*) have been repeatedly isolated from *C. gigas* juveniles (less than 12 months old) affected by the Pacific Oyster Mortality Syndrome (POMS) triggered by the Ostreid herpesvirus OsHV-1  $\mu$ Var. (Lemire *et al.*, 2015; Bruto *et al.*, 2017; de Lorgeril

et al., 2018). *V. aestuarianus* (Anguillarum clade) was found to cause mass mortality of adult *C. gigas* in France since 2001 and since 2011 in Ireland, Scotland and Spain (Garnier et al., 2008; Garcia et al., 2014; Travers et al., 2017)

High taxonomic resolution analysis of the bivalve pathobiota applied on healthy and diseased *C. gigas* samples using a new target enrichment next-generation sequencing approach revealed dominance of primary pathogens such as *V. aestuarianus* or OsHV-1 in tissues of diseased *C. gigas* collected during mass mortality events affecting juveniles and/or adults at different European farming sites (Lasa et al., 2019). Albeit at lower relative abundance, other bacterial species were exclusively identified in infected oysters, particularly *Vibrio* sp. (*V. alginolyticus*, *V. coralliilyticus*, *V. crassostreae*, *V. splendidus*, *V. tasmaniensis*) and *Arcobacter* sp.

Thorough studies on vibrios pathogenic for oysters have shown that the functional unit of pathogenesis can be a clone (e.g. *V. aestuarianus*), a population (e.g. *V. crassostreae*) or a consortium, and this is further complicated by *Vibrio* interactions with the host microbiota (Le Roux et al., 2016). Recently, de Lorgeril et al. (2018) demonstrated that the POMS is caused by multiple infections with an initial and necessary step involving OsHV-1  $\mu$ Var. The virus leads the host to enter an immunocompromised state preceding the invasion by opportunistic pathogenic bacteria (including pathogenic vibrios) that kill the oyster. It can be speculated that POMS-associated mortalities are caused by multiple species/populations of pathogens (e.g. *Vibrio*, *Arcobacter* sp.) that collaborate to kill oysters (Russell and Cavanaugh, 2017; de Lorgeril et al., 2018), but the role of the non-virulent microbiota should not be neglected as non-virulent *Vibrio* strains have the potential to dramatically increase the host damages caused by virulent strains (Lemire et al., 2015). The role of these bacterial consortia should be deeply analyzed in future functional studies to shed light on the mechanisms underlying polymicrobial infections in bivalves (Lasa et al., 2019).

In contrast to oyster diseases, less information is available on pathogenic *Vibrio* diseases affecting mussels (*Mytilus* spp), which also are other important farmed

bivalves. Actually, although mussels can concentrate vibrios in their tissues (Stabili *et al.*, 2005), they are considered particularly resistant to microbial infections, in particular vibrioses (Lupo and Le Bouquin, 2019). They rarely experience mass mortalities (Domeneghetti *et al.*, 2014; Romero *et al.*, 2014) although recent outbreaks of unknown etiology have been reported (Charles, Bernard, *et al.*, 2020). Among the rare reported cases of *Vibrio* infection, *V. splendidus*-related strains were isolated from diseased *Mytilus edulis* adults and the isolated strains induced mortalities when injected to mussels (Ben Cheikh *et al.*, 2016, 2017). Strains identified as *V. splendidus* and *V. coralliilyticus/neptunius*-like caused high mortality rates in larvae of the greenshell mussel *Perna canalicula* (*Mytilidae*) (Kesarcodi-Watson *et al.*, 2009). Moreover, strains of *V. coralliilyticus* isolated from bleached corals had strong and concentration-dependent immunotoxicity and embryotoxicity in mussel *in vitro* (Balbi *et al.*, 2019). Interestingly, the susceptibility of mussels to vibrios of the Splendidus clade (oyster pathogens) was shown to be dependent on host physiology (Charles, Trancart, *et al.*, 2020). Whether or not vibrios are causal agents in mussel mortalities observed in the field remains unclear (Charles, Bernard, *et al.*, 2020).

A recent study investigated possible links between the different susceptibility to *Vibrio* infection of oysters and mussels and their microbiota (Vezzulli *et al.*, 2018). Using 16S rRNA gene-based analysis, the authors provided the first comparison of microbiota profiles associated with hemolymph and digestive gland of *C. gigas* and *M. galloprovincialis* co-cultured at the same aquaculture site. Vibrios accounted for a larger fraction of the microbiota in *C. gigas* compared to *M. galloprovincialis*, suggesting that oysters might provide better conditions than mussels for survival of these bacteria. More recently, Pierce and Ward (2019), by comparing the gut microbiome of the oyster *C. virginica* and the mussel *M. edulis*, found that while the two bivalves harbor microbial communities with similar composition, on a functional level, the microbial community varies according to host species and season. The authors suggested that host species intrinsic factors affect the composition of the bivalve microbiota independently of environmental conditions and diet.

In addition to vibrios pathogenic for bivalves, both oysters and mussels can concentrate within their tissues other vibrios pathogenic for humans. Since edible bivalves, especially oysters, are often eaten raw or undercooked, they represent an important vehicle for the transmission of these pathogens to humans. Most relevant *Vibrio* species associated with seafood-related diseases are *V. parahaemolyticus*, *V. vulnificus* and, to a lesser extent, non-toxigenic *V. cholerae* (Jones *et al.*, 2014; Froelich and Noble, 2016; Williams *et al.*, 2017; Tack *et al.*, 2019). These species, as far as we know, do not affect bivalve health but represent a serious and growing threat to public health (Tack *et al.*, 2019).

### **Stable versus transient associations**

It has been debated whether the presence of vibrios in bivalves results from stable associations or from the non-specific uptake of vibrios from food or seawater. In a pioneer study, Polz and collaborators (Dana E Hunt *et al.*, 2008) analyzed *Vibrio* population structures in mussels and in the water column. Diversity and frequency of populations identified by MLSA was similar in mussels and water samples, suggesting that mussels do not represent a strongly selective habitat for vibrios. However, population structure does not imply absence of selection in mussel, but suggests that selection is balanced by migration and/or adaptation to different environments (Preheim *et al.*, 2011). More recently, Le Roux and collaborators have deeply analyzed *Vibrio* associations with oysters and relationships between vibrios in the water column and in oyster tissues (Le Roux *et al.*, 2016; Bruto *et al.*, 2017). By comparing the frequency and abundance between *Vibrio* populations in these two compartments, Bruto *et al.*, (2017) showed that several populations were unequally distributed. Among them, *V. crassostreae* and some *V. splendidus* populations were positively and preferentially associated with oyster tissues.

Because of habitat preferences related to their metabolic requirements, resistance/tolerance mechanisms and specific colonization determinants, distinct

*Vibrio* species can colonize diverse tissues in bivalves. Total bacterial 16S has proven that hemolymph, which carries the bivalve immune cells, harbors a microbiota highly distinct from the rest of the tissues (Lokmer, Kuenzel, et al., 2016). Strikingly, vibrios can survive in this immune tissue, which is likely a colonization route toward deeper tissues. Outside pathological contexts, hemolymph colonization is rapidly controlled by hemocytes, the bivalve immune cells (Rubio et al., 2019). In contrast, pathogenic vibrios escape from hemocyte control and colonize the connective tissue, which appears as a site of proliferation leading to fatal bacteremia (Parizadeh et al., 2018; Rubio et al., 2019) (Figure 1).

New technologies such as metagenomics-based tools combined with manipulative ecological and physiological approaches are expected to significantly increase our current knowledge on *Vibrio* association with bivalves and their colonization mechanisms.

## **2. Vibrios facing bivalve cellular defenses**

Upon host colonization, microbes are recognized by pattern-recognition receptors that initiate cellular-based signal transduction cascades connecting microbe recognition signals to expression and secretion of immunomodulatory cytokines and chemokines (Reddick and Alto, 2014) and contribute to control infections by triggering the expression of immune effectors that modify the microbe habitat. Effectors of metazoan innate immunity include antimicrobial peptides (AMPs), reactive oxygen species (ROS), nitric oxide (NO) and heavy metals (copper, zinc), present at epithelial/mucosal surfaces, in phagocytes and body fluids, which create a hostile environment for microbes (Hood and Skaar, 2012; Franzenburg et al., 2013). These conserved innate immune mechanisms are key components of the defense of bivalves against infections.

## The key role of hemocytes

Bivalves are endowed with a powerful innate immune system, mainly based on the activity of circulating immune cells, the hemocytes, soluble plasma factors, as well as on tissue-mediated immune responses (Bachère *et al.*, 2015; Canesi and Pruzzo, 2016; Gerdol *et al.*, 2018), creating a hostile environment for vibrios (Schmitt, Rosa, *et al.*, 2012; Destoumieux-Garzón *et al.*, 2014). Due to the presence of an open circulatory system, bivalve hemocytes are not only circulating in the hemolymph but they also infiltrate tissues.

Hemocytes of both oysters and mussels can kill vibrios through phagocytosis, production of highly reactive ROS and NO, as well as a number of antimicrobial peptides (AMPs) and hydrolytic enzymes (Canesi *et al.*, 2002; Bachère *et al.*, 2015; Canesi and Pruzzo, 2016) (Figure 1). Hemocytes populations (granular, semi-granular or agranular cells) (Bachère *et al.*, 1988; Chagot *et al.*, 1992) vary according to bivalve species, physiological status, and environmental factors (Smith *et al.*, 2016). In *C. gigas*, specialized populations of semi-granular and granular cells harbor strong phagocytic capacity and produce highly reactive molecules (ROS and NO) in response to *V. splendidus*; they also express lysozymes and AMPs in agreement with their immunological roles (Bachère *et al.*, 2015; Wang *et al.*, 2017). Similarly, in *M. galloprovincialis*, granular and semi-granular cells harbor phagocytic activity, they produce ROS and NO; small hyaline cells produce NO only (García-García *et al.*, 2008). Enzymes involved in oxidative stress (dual oxidases, NADPH oxidases) and hydrolases including lysozymes, which participate in the killing of phagocytized bacteria by degrading bacterial peptidoglycan (Costa *et al.*, 2009; Itoh *et al.*, 2010; Xue *et al.*, 2010) are expressed by hemocytes of mussels and oysters (Rosa *et al.*, 2012; Campos *et al.*, 2015; Wang *et al.*, 2017; de Lorgeril *et al.*, 2018; Jiang *et al.*, 2018). The respiratory burst generated by hemocytes is a major microbicidal reaction in bivalves, which also triggers the formation of DNA Extracellular traps (ETosis). In oysters and mussels ETosis contributes to control *Vibrio* infections (Altincicek *et al.*, 2008; Poirier *et al.*, 2014; Romero *et al.*, 2020).

More recently, mechanisms involved in heavy metal homeostasis have been shown to play a key role in the antibacterial response of oysters (Vanhove *et al.*, 2015; Shi *et al.*, 2019)(Rubio *et al.*, 2019)(Shi *et al.*, 2019). Copper/zinc redistribution upon *Vibrio* infections is accompanied in hemocytes by changes in the expression levels of metallothioneins as well as copper and zinc transporter genes (Ctr1, ATP7A, ZIP1, and Znt2), which across animal species mediate the uptake of heavy metals and their accumulation in intracellular compartments for intracellular killing of phagocytized bacteria (Neyrolles *et al.*, 2015). It is worth noting that bivalves have widely differing concentrations of heavy metals in their tissues, with oysters (*Crassostrea* sp.) showing the highest body burden of copper and zinc (Pan and Wang, 2009; Shi *et al.*, 2019). Although in mussels the role of heavy metals in the immune response to vibrios has been not investigated in such detail, sequences of ATP7A, Ctr1, ZIP1, Znt1 and Znt2 belonging to evolutionarily conserved metal transport systems are present in the genome of *M. galloprovincialis* (Gerdol M., pers. comm), which suggests they could play a similar role in mussel defense. So far in *Mytilus* sp., available data indicate that metal exposure can result in increases or decreases in hemocyte immune parameters, depending on the exposure conditions (Parry and Pipe, 2004; Höher *et al.*, 2013). In this light, the role of copper/zinc homeostasis in mussel immune response deserves further investigation.

A high number of highly diversified AMPs have also been identified in oysters and mussels, in which they are expressed by hemocytes and/or epithelia. Some of them, but not all, have been purified and characterized for their various immune functions. Remarkably, mussels and oysters show contrasting antimicrobial responses. Indeed, AMP expression is potent in mussel (Pallavicini *et al.*, 2008) as opposed to mild in oysters (Schmitt, Rosa, *et al.*, 2012). Both species express highly diversified Csq $\beta$ -defensins (Hubert *et al.*, 1996; Mitta *et al.*, 1999; Gueguen *et al.*, 2006; Schmitt *et al.*, 2010) and big defensins (Rosa *et al.*, 2012; Gerdol *et al.*, 2019,2020; Loth *et al.*, 2019) as well as bactericidal/permeability-increasing proteins (Gonzalez *et al.*, 2007). These antimicrobial peptides/proteins are expressed in hemocytes and/or epithelia either

constitutively or in response to bacterial challenge (Schmitt, Rosa, *et al.*, 2012). Although the multiple mechanisms of action of bivalve AMPs are far from being uncovered, activities against vibrios have been evidenced, ranging from potent (e.g. big-defensins) to weak (e.g. defensins) (Duperthuy *et al.*, 2010; Schmitt, Lorget, *et al.*, 2012; Loth *et al.*, 2019). Remarkably, beyond these shared peptide families, the AMP repertoire of oysters and mussels contains species-specific AMP families. In *M. galloprovincialis*, these families include myticins, mytilins and mytimicins, which are also the most expressed AMPs (Pallavicini *et al.*, 2008). Similarly, in *C. gigas*, they include proline-rich peptides, molluscidins and a Macrophage expressed gene 1-like (for review see Bachère *et al.*, 2015). However, some AMP families like mussel myticins are likely not involved in the response to vibrios but other functions such as antiviral responses (Balseiro *et al.*, 2011) or wound healing (Rey-Campos *et al.*, 2020).

Environmental factors are known to significantly affect immune responses. Thus, immune parameters of mussels and oysters (total hemocyte counts, proportion of hemocyte populations, immune gene expression) vary with temperature and exposure to pollutants, including heavy metals and nanoparticles (Parry and Pipe, 2004; Höher *et al.*, 2013; Wendling and Wegner, 2015; Canesi and Corsi, 2016; Auguste *et al.*, 2020), which may have important consequences on bivalve susceptibility to pathogens. In addition, recent studies have revealed that previous encounter with pathogens is a life history trait that defines the resistance/tolerance of bivalves to infections (*i.e.* immune priming). In the oyster *C. gigas*, priming of the antiviral response conferred long-term protection against the OsHV-1 virus, which was controlled by a sustained antiviral response (Lafont *et al.*, 2017, 2020); moreover a primary stimulation with *V. splendidus* enhanced immune signaling and conferred higher phagocytosis capacities to oyster hemocytes (Zhang *et al.*, 2014; Wang *et al.*, 2020). In the mussel *M. galloprovincialis* priming with vibrios modified immune responses allowing mussels to tolerate the infection (Rey-Campos *et al.*, 2019). Interestingly, Wendling and Wegner (2015) showed that in selective environments, *i.e.* at elevated temperatures reflecting patterns of disease outbreaks in natural populations, oysters can rapidly adapt to widespread communities of pathogenic

vibrios. Oysters showing higher resistance to sympatric vibrios showed higher hemocyte counts and phagocytosis rate.

### Responses to vibrios in oysters and mussels

Long term records on mussels (*Mytilus* spp) and oysters (*Crassostrea* spp.) have revealed they experience rare or frequent pathologies, respectively, in particular in relation to *Vibrio* diseases. Although no data on species-specific comparison are available, the main immune mechanisms and effectors seem to be shared by the hemocytes of different mollusks, as reported in Escoubas *et al.* (2016). Experimental infections with different *Vibrio* species and strains have revealed how the complex machinery of immune pathways in hemocytes, together with soluble plasma factors, is activated when facing a *Vibrio* challenge. Here some examples of responses of mussels and oysters to challenge with pathogenic vibrios are summarized. Oyster pathogens have been often used to challenge both species of bivalves. Thus, most data have been collected on the responses to the oyster pathogens *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 (both from the Splendidus clade), and *V. aestuarianus* 01/032 (Anguillarum clade) (Labreuche, Lambert, *et al.*, 2006; Balbi *et al.*, 2013; Green *et al.*, 2016; Vanhove *et al.*, 2016; Rubio *et al.*, 2019).

One important question is whether the host can discriminate commensals from pathogenic strains. A recent study in which vibrios of the Splendidus clade were injected to oysters revealed that all *Vibrio* strains were recognized by oyster hemocytes independently of their virulence status (Rubio *et al.*, 2019). They induced three major immune signaling pathways, namely the interleukin 17 (IL-17), tumor necrosis factor (TNF), and Toll-like receptor (TLR) pathways. They also induced the production of AMPs (big defensins), ROS (dual oxidase expression), and heavy metals (metallothioneins and metal transporters). However, pathogenic strains were also shown to manipulate bivalve immune responses for successful colonization. These species-specific mechanisms are detailed below.

- **Responses to *V. tasmaniensis*.** *V. tasmaniensis* LGP32 was isolated from moribund *C. gigas* oysters by Le Roux and collaborators (Gay *et al.*, 2004). LGP32 interactions with its natural host have been widely documented. LGP32 is a facultative intracellular pathogen of oyster immune cells (Duperthuy *et al.*, 2011). In *C. gigas*, LGP32 uses the major outer membrane protein OmpU to attach to and enter the hemocytes; opsonization is mediated by the major plasma protein Cg-EcSOD recognized by  $\beta$ -integrins at the hemocyte surface (Figure 2). Consistent with a manipulation of hemocyte phagocytosis, LGP32 infection leads to an altered expression of oyster genes involved in cytoskeleton dynamics (Rubio *et al.*, 2019). As LGP32 is recognized by the oyster immune system, it induces antimicrobial defenses based on AMPs (big defensins), ROS (dual oxidase) and heavy metals (Rubio *et al.*, 2019). However, inside the phagosome, it escapes from host cellular defenses by avoiding acidic vacuole formation and by limiting the production of ROS (Duperthuy *et al.*, 2011). This is accompanied by a repression of the NADPH-oxidase expression (Rubio *et al.*, 2019) and the activation of mechanisms of resistance/tolerance to antimicrobials (see section 3). Inside the hemocytes, LGP32 induces lysosomal disruption (Balbi *et al.*, 2013) and causes hemocyte lysis through the intracellular delivery of type 6 secretion system (T6SS) effectors (Vanhove *et al.*, 2016; Rubio *et al.*, 2019) (Figure 2).

In mussels, LGP32 shows a lower pathogenic potential and induces lower hemocyte damage than in oysters (Balbi *et al.*, 2013). LGP32 is rapidly phagocytized by *M. galloprovincialis* hemocytes, although phagocytosis does not require opsonization by plasma components, and it elicits release of hemocyte antibacterial effectors (Balbi *et al.*, 2013) (Figure 2). When internalized, LGP32 remains viable within intracellular vacuoles, escaping lysosomal degradation. Accordingly, *V. splendidus* is able to proliferate in mussel hemolymph *in vivo*. The observed resistance of LGP32 to the bactericidal activity of mussel hemocytes was related to alterations of PI-3 Kinase signaling, leading to impairment of the endo-lysosomal system, which suggests that

LGP32 could interfere with host cell signaling pathways, thereby escaping its bactericidal activity. In agreement, in *M. galloprovincialis* infected with LGP32, hemocytes show an altered expression of genes involved in innate immune response, inflammatory response, cell migration and defense responses to bacteria (Rey-Campos *et al.*, 2019). Although less documented, cellular interactions of LGP32 with *M. edulis* hemocytes revealed an effect on hemocyte adhesion, phagocytosis and oxidative burst (Tanguy *et al.*, 2013). *In vitro*, hemocytes of *M. edulis* exposed to LGP32 showed an altered expression of genes involved in recognition (lectins), immune signaling (TLRs, MyD88), antimicrobial defenses (AMPs, lysosomal hydrolases oxidative stress) and apoptosis (Tanguy *et al.*, 2018).

Overall, studies on LGP32 interactions with bivalves (both mussels and oysters) identify this strain as a facultative intracellular pathogen, which uses host immune cell manipulation as a key feature of its biology. The mechanisms underpinning LGP32/oyster hemocyte interactions are conserved within the species *V. tasmaniensis* and the damages caused to oyster hemocytes might be a consequence of *V. tasmaniensis* intracellular stages (Rubio *et al.*, 2019). From our current knowledge, the mechanistic interactions of *V. tasmaniensis* LGP32 with mussel and oyster hemocytes differ and the damages caused by LGP32 to hemocytes during its intracellular stages appear higher in oyster (Figure 2).

- **Responses to *V. aestuarianus*.** *V. aestuarianus* subsp. *francensis* 01/032 was isolated from adult moribund oysters by Nicolas and collaborators during a French disease outbreak in 2001 (Garnier *et al.*, 2007, 2008). This strain produces extracellular products (ECPs) containing the main virulence factor of *V. aestuarianus*: the metalloprotease Vam (Labreuche *et al.*, 2010). *In vitro*, these ECPs were shown to alter phagocytosis by *C. gigas* hemocytes, adhesion and ROS production (Labreuche, Soudant, *et al.*, 2006). High doses of bacterial cells also affect hemocyte activities *in vivo* after a few days (Labreuche, Lambert, *et al.*, 2006). It was therefore proposed that ECPs allow 01/032 to avoid phagocytosis by oyster hemocytes (Figure

2), which likely favors oyster colonization. In contrast, hemocytes of *M. galloprovincialis* are able to efficiently kill *V. aestuarianus* 01/032, both *in vitro* and *in vivo* (Balbi *et al.*, 2013). The different susceptibility of 01/032 to the antibacterial activity of oyster and mussel hemolymph was dependent on the presence of the extrapallial-like protein (MgEP), the main protein in *Mytilus* hemolymph, which increases adhesion and killing of 01/032 by mussel hemocytes (Pezzati *et al.*, 2015) (Figure 2). Interestingly, upon addition of purified MgEP, oyster hemocytes acquired the capacity to kill *V. aestuarianus* 01/032. MgEP thus can play a key role in promoting mannose-sensitive interactions of this strain (adhesion and killing) with both mussel and oyster hemocytes, by serving as an opsonin (Pezzati *et al.*, 2015; Canesi and Pruzzo, 2016). These data further highlight the importance of soluble plasma factors in mediating hemocyte-*Vibrio* interactions in different bivalve species, which can play a key role in the pathogenic outcome (Pruzzo *et al.*, 2005).

Overall data on *V. aestuarianus* 01/032 interactions with hemocytes reveal an opposite outcome in oysters and mussels. In *C. gigas*, where it expresses pathogenic potential, 01/032 is able to avoid phagocytosis, one of the main component of bivalve defenses, whereas in mussels, where it does not cause disease, 01/032 is opsonized by a major plasma component, MgEP, and killed by the hemocytes (Figure 2).

However, *V. aestuarianus* 01/032 was recently demonstrated to be moderately virulent, i.e. it induces mortalities upon injection of high doses ( $5 \times 10^7$  bacteria/animal) (Goudenège *et al.*, 2015) compared to highly virulent strains (e.g. strain 12\_016a isolated during an oyster mortality episode) that kill at low doses ( $10^2$  bacteria/animal) as well as through immersion procedures (Parizadeh *et al.*, 2018). Therefore strain 01/032 might be well adapted to oyster as a host but does not possess the complete virulence repertoire that triggers disease. Only a few studies have focused on oyster responses to highly virulent strains of *V. aestuarianus*. In 2011, de Lorgeril *et al* provided a first catalog of genes related to cellular / immune functions differentially expressed in individuals surviving an infection with the strain

02/041. Genes differentially expressed in susceptible and resistant oyster lineages included genes involved in recognition (L-rhamnose-binding lectin), cell adhesion ( $\beta$ -Integrin) and antioxidants (Cg-EcSOD) (Azéma *et al.*, 2015), in agreement with a manipulation of phagocytosis. Still, a clear picture of the mechanisms allowing *V. aestuarianus* to proliferate within oyster hemolymph is still missing, and we still ignore if like strain 01/032, highly virulent strains of *V. aestuarianus* manipulate oyster hemocytes to achieve successful infections.

- **Responses to other vibrios.** Although less data is available and no specific comparison has been documented between oyster and mussel responses, there are at least two additional examples that evidence close interactions between pathogenic vibrios and hemocytes. First, *V. crassostreae* J2-9, which is associated with oyster juvenile mass mortalities (POMS) (Lemire *et al.*, 2015), tends to dampen several immune defenses in oyster. Like LGP32, it represses the NADPH-mediated production of ROS and C-type lectin-mediated antibacterial immunity (Rubio *et al.*, 2019). It also represses the expression of C1q domain-containing (C1qDC) proteins, a family of complement-related proteins, which, in oysters, can serve as opsonins that enhance *Vibrio* phagocytosis (Lv *et al.*, 2018). J2-9 remarkably alters cellular defenses by inducing massive hemocyte lysis through a contact-dependent mechanism (Rubio *et al.*, 2019). Whether or not the observed repression of immune genes is related to the vibrio-triggered lysis of specific hemocyte populations remains to be clarified. Similar to other pathogenic vibrios, *V. splendidus* 10/068, which was isolated from moribund mussels in France in 2010 (Ben Cheikh *et al.*, 2017), was shown to alter different functions of hemocytes including motility, adhesion, ROS production, phagosome maturation and viability. Moreover, its extracellular products inhibited cellular responses such as ROS production (Ben Cheikh *et al.*, 2016) through an unknown mechanism.

Overall, current insights into *Vibrio*-bivalve interactions show a key role of hemocytes in controlling infections through phagocytosis and its associated arsenal of antimicrobial compounds (ROS, heavy metals and AMPs). Hemocyte reactions also include ETosis which is triggered by the oxidative burst and could participate in containing vibrios inside the hemolymph compartment. While the cellular defense arsenal is generally comparable in oyster and mussels, it appears to mainly differ in the AMPs expressed by immune cells and tissues and in the major soluble components present in the plasma, which serve as opsonins. Both AMPs and soluble plasma factors might contribute to the distinct susceptibility of oysters and mussels to infections.

### 3. Multifaceted adaptive responses of vibrios to their molluscan hosts

According to natural selection, microbes adapted for growth in metazoan hosts have selected strategies to evade, inhibit or manipulate immune responses (Stubbendieck *et al.*, 2016). Commonly, counter-measures to host innate immunity include active dampening of immune defenses, *i.e.* immune suppression, escape from host recognition (usually by disguising their immunogenic surface components), or resistance/tolerance to antimicrobials (Hornef *et al.*, 2002; Reddick and Alto, 2014). This evasion/tolerance scenario applies to species belonging to the resident microbiota of metazoans as well as to new invaders. In *Vibrio* species infecting or simply colonizing bivalves several of these strategies have been recently identified. These recent data highlight the multifaceted nature of immune evasion in *Vibrio* populations positively associated with bivalves.

Resistance/tolerance to host antimicrobials has been evidenced as a key determinant of *Vibrio* infectivity in oysters. Most data were obtained in the facultative intracellular pathogen *V. tasmaniensis* (Duperthuy *et al.*, 2011). In this species, resistance to ROS and to heavy metals was identified as essential. Indeed, both antioxidants (SodA, AhpCF, catalase) and copper efflux machineries (CopA,

CusABC) were highly induced upon contact with hemocytes and they determined *Vibrio* resistance to intracellular killing (Vanhove *et al.*, 2016). Mechanisms that confer increased resistance to oyster AMPs were also evidenced (Destoumieux-Garzón *et al.*, 2014). Inside the phagosomes of oyster hemocytes, *V. tasmaniensis* LGP32 was shown to release outer membrane vesicles that entrap AMPs and increase antimicrobial resistance (Vanhove *et al.*, 2015). An OmpU-mediated protection against AMPs was also evidenced in *V. tasmaniensis* LGP32 (Duperthuy *et al.*, 2010), which likely results from the OmpU-mediated induction of the envelope stress response, as demonstrated in *V. cholerae* (Mathur and Waldor, 2004; Mathur *et al.*, 2007). However, up to now potent mechanisms that modify the electronegativity of the lipopolysaccharides (LPS) and increase resistance to AMPs (Hankins *et al.*, 2011, 2012; Cullen *et al.*, 2015) have not been identified in *Vibrio* strains associated with bivalves. RND efflux machineries that detoxify *Vibrio* cells from the host antimicrobials (Bina *et al.*, 2008) are present in the genomes of *Vibrio* strains colonizing bivalves, but their role has not been studied. Moreover, *Vibrio* strains positively and negatively associated with oysters could not be discriminated on the basis of their resistance to AMPs (Oyanedel *et al.*, unpublished). Therefore, it is unclear how much resistance to AMPs contributes to colonization success in oysters. This may differ for *Vibrio* species associated with mussels, which express AMPs in large amounts (Mitta *et al.*, 2000; Pallavicini *et al.*, 2008), or with oysters, which express AMPs in small amounts (Schmitt, Lorget, *et al.*, 2012).

Mechanisms of immune cell lysis needed to escape from hemocyte control were identified in *V. tasmaniensis* and *V. crassostreae* (Vanhove *et al.*, 2016; Piel *et al.*, 2019; Rubio *et al.*, 2019). Such an active dampening of oyster cellular defenses was shown to depend on distinct molecular effectors: a chromosome-encoded T6SS active against eukaryotic cells in *V. tasmaniensis*, and the ancestral R5-7 virulence factor together with a T6SS carried by a plasmid (T6SSpGV) in *V. crassostreae* (Piel *et al.*, 2019; Rubio *et al.*, 2019). Both mechanisms enable virulent *Vibrio* to escape hemocyte control and cause systemic infections (Figure 1). Recently, strains of *V. splendidus* able to colonize oyster tissues were also found to be cytotoxic toward

hemocytes (Oyanedel *et al.*, 2020). Similar evolution toward cytotoxic phenotypes illustrates the importance for successful colonizers to overcome the major cellular defenses of oysters: they create a less hostile habitat that enables colonization of oyster tissues. Cytotoxicity is therefore an important determinant of strain infectivity, which enables virulence expression when *Vibrio* strains harbor virulence factors.

Some bacterial species have acquired the capacity to escape from host recognition by becoming less immunogenic or by reducing the host immune responses elicited by the release of microbe-associated molecular patterns such as LPS and peptidoglycan (Charroux *et al.*, 2018). Such mechanisms of immune evasion remain poorly described in *Vibrio* species. However, in a recent study of a *V. splendidus* population associated with oysters, distinct structures of LPS O-antigen were identified. Manipulations of the *wbe* hypervariable genomic region, which determines the O-antigen structure in *V. splendidus* (Wildschutte *et al.*, 2010) lead to less immunogenic structures that favored oyster colonization (Oyanedel *et al.*, 2020). This identifies immune evasion as a novel adaptive mechanism underpinning colonization in *Vibrio* associated with bivalves.

Within metazoan hosts, microbes are also engaged in interbacterial competition for survival among the diverse resident microbial communities and against new colonizers (Fraune *et al.*, 2015). Bacteria have adapted to growth with neighbors (Stubbendieck and Straight, 2016). Tools for competition between bacteria can point to the fight for vital resources, growth inhibition or direct killing of rivals by the delivery of toxins and AMPs such as bacteriocins (Stubbendieck and Straight, 2016). The fight for iron, present in limiting concentrations within metazoan hosts, was evidenced in the transcriptomic activity of vibrios colonizing bivalves with a major induction of siderophore biosynthesis and iron-uptake machineries (Vanhove *et al.*, 2016; Rubio *et al.*, 2019). This likely has major impact on colonization success, as earlier shown in *V. vulnificus* infecting mice (Arezes *et al.*, 2015). While several studies have evidenced bacteria harboring antimicrobial activity in the hemolymph of bivalves (Defer *et al.*, 2013; Desriac *et al.*, 2014), the production of bacteriocins and resistance to such non-

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host-derived immune effectors remains a poorly explored aspect of *Vibrio* infectivity. However, it was suggested that cooperation exists within *Vibrio* populations based on bacteriocin production and uptake (Cordero *et al.*, 2012). Similarly, recent studies have revealed the broad distribution of interbacterial T6SS in *Vibrio* species, a killing mechanism that uses contact-dependent toxin delivery and toxin-resistance mechanism to inhibit competitors (Ho *et al.*, 2015). In *Vibrio* species pathogenic for oysters such T6SS systems are induced upon host colonization although a formal role in interbacterial competition has not been demonstrated (Rubio *et al.*, 2019). Such a demonstration was achieved in *V. vulnificus*, in which two T6SS are involved in inter-/intra-species competition within oyster hosts (Hubert, 2019), and in *V. fischeri*, in which a T6SS controls competitions during squid colonization (Speare *et al.*, 2018).

Altogether, recent advances in *Vibrio*-bivalve interactions highlight the adaptive potential of bivalve colonizers, which have not only developed strategies to circumvent the potent cellular defenses of their natural hosts but also tools for competition with the abundant microbial communities hosted by bivalves.

#### 4. The selection of adapted genotypes in bivalves and the aquatic environment

As free-living and non-obligatory parasites, vibrios engage in diverse biotic interactions in the environment outside of their bivalve hosts. Among these biotic interactions they encounter two major groups of bacterial predators, *i.e.* bacteriophages and bacterivorous protists (grazers), *i.e.* ciliates, flagellates and amoebozoae (Pernthaler, 2005). As predator-prey interactions are some of the strongest forces driving rapid co-evolution of both partners, often referred to as an arm-race (Dawkins *et al.*, 1979), predator-prey interactions in bacterial communities significantly influence and shape bacterial adaptive strategies and favor the selection of predation resistance traits.

Grazers can be found in most ecological niches inhabited by bacterial communities, and their predation activity shares similar cellular and molecular

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processes with their metazoan immune cells counterparts, the phagocytes, *i.e.* the hemocytes in the case of bivalves. Phagocytosis and intracellular antimicrobial processes are indeed highly conserved from protozoans to mammals (Boulais *et al.*, 2010). Hence, bacterial predation by grazers has often been suggested to favor coincidental selection of bacterial virulence factors, including in *Vibrionaceae* (Erken *et al.*, 2013). A diversity of survival adaptations against predation by grazers can be found in bacteria and classified in two main groups: either **pre-ingestional** or **post-ingestional** adaptations (Matz and Kjelleberg, 2005). Pre-ingestional adaptations including high motility, filamentation, surface masking or toxin release are commonly found in extracellular pathogens; whereas post-ingestional adaptations including digestion resistance through vacuolar trafficking inhibition or vacuolar escape, and intracellular toxin release are commonly found for intracellular pathogens and tend to favor bacterial growth (Matz and Kjelleberg, 2005).

**Escape from phagocytosis.** Among pre-ingestional adaptations some are widely shared by *Vibrionaceae* as they are flagellated and highly motile. In addition some of them can form biofilms, as e.g. *V. cholerae* that produces *Vibrio* Polysaccharides (VPS) favoring resistance to grazing (Henst *et al.*; Sun *et al.*, 2013). Interestingly the oyster *V. tasmaniensis* LGP32 has been suggested to form biofilms (Vezzulli *et al.*, 2015), although a potential role of this process in resistance to grazers remains to be investigated.

**Cytotoxic activities.** Toxin/protease release and cytotoxic activities can both play a role as pre-ingestional or post-ingestional adaptations to grazers (Matz and Kjelleberg, 2005). **Protease secretion** is widespread among vibrios and it has been described for most strains pathogenic toward bivalves (Binesse *et al.*, 2008; Labreuche *et al.*, 2010). Some of these proteases favor resistance to grazing by amoeba and ciliates as shown for the metalloprotease Vsm secreted by *V. tasmaniensis* LGP32 (Robino *et al.*, 2019) and the protease PrtV secreted by *V. cholerae* (Vaitkevicius *et al.*, 2006). To date in vibrios, they represent some of the best examples of factors under environmental selective pressure that contribute to

virulence/toxicity in a metazoan host. *V. tasmaniensis* and *V. crassostreae* also harbor cytotoxic capacities that are necessary for their pathogenicity (Rubio *et al.*, 2019), among them MARTX toxins and T6SS components (Bruto *et al.*, 2017; Piel *et al.*, 2019; Rubio *et al.*, 2019). However, up to now, none of these systems were shown to protect bivalve pathogens against grazing. The role of **MARTX toxins** in resistance to grazing was shown for *V. vulnificus*, in which a MARTX involved in eel infection confers protection against amoeba grazing (Lee *et al.*, 2013). However, such a role has not yet been investigated in bivalve pathogens. Similarly, the **T6SS** of *V. cholerae* has shown cytotoxic activity on the slime mold *Dictyostelium discoideum* during its actively grazing unicellular life stage (Pukatzki *et al.*, 2006). However, in the oyster pathogen *V. tasmaniensis* LGP32, where a T6SS determines cytotoxicity towards oyster hemocytes (Rubio *et al.*, 2019), no effect was found in the interaction with the amoebae *Vannella* sp. 1411 (Robino *et al.*, 2019). Hence the role of T6SS in biotic interactions outside bivalves remains to be determined.

**Resistance to heavy metals.** Evidence of post-ingestional adaptations in vibrios are limited to a number of pathogenic strains that adopt intracellular stages and survive intracellular digestion (Rosenberg and Falkovitz, 2004; Ma *et al.*, 2009; Vidal-Dupiol *et al.*, 2011; Ritchie *et al.*, 2012; de Souza Santos and Orth, 2014; Van der Henst *et al.*, 2016), as shown for *V. tasmaniensis* LGP32 in hemocytes of bivalves (Duperthuy *et al.*, 2011; Balbi *et al.*, 2013; Vanhove *et al.*, 2016; Rubio *et al.*, 2019). Such properties are rather unusual for pathogenic vibrios and potentially reminiscent of post-ingestional adaptation to grazers predation. Indeed, LGP32 was found resistant to grazing by the free-living amoeba *Vannella* and some virulence factors, such as the p-ATPase copper efflux pump CopA, were found to play a role in this resistance (Robino *et al.*, 2019). Interestingly the CopA-mediated resistance to copper is also necessary for *V. fischeri* colonization in the bobtail squid (Brooks *et al.*, 2014). Therefore, copper resistance favoring resistance to amoeba predation is also a key determinant of *Vibrio* associations with molluscan hosts, both in parasitic and mutualistic interactions.

**Adsorption inhibition.** In addition to grazers, bacteriophages are an important class of predators which are abundant and highly diverse in marine environments; they contribute to the evolution of bacteria by mediating horizontal gene transfer and genomic rearrangements, as well as by bactericidal selection (Dy *et al.*, 2014). A critical step in their interaction with bacteria is the attachment to cell wall components (membrane receptors, porins, LPS, flagella, pili) thus exerting an important selective pressure on cell wall composition and cell wall plasticity. Hence bacteria adaptation to phage predation can share similarities with pre-ingestional masking strategies to avoid recognition by grazers. Among adaptations to phages that have been described for vibrios, LPS modifications (Seed *et al.*, 2012) play a key role and OMVs release can be used as decoy strategy (Manning and Kuehn, 2011) in a similar manner than OMVs play a role as decoy against AMPs during bivalve immune response (Vanhove *et al.*, 2015). Interestingly OMVs are involved in *V. cholerae* resistance to phages (Reyes-Robles *et al.*, 2018) as well as in resistance against the amoeba *Acanthamoebae castellanii* (Valeru *et al.*, 2014)

**The LPS structure under multiple selective pressures.** Some virulence traits can be under adverse selection in different biotic interactions and generate a tradeoff equilibrium. In a recent study of a *V. splendidus* population associated with oysters, we identified distinct structures of LPS O-antigen encoded by the *wbe* hypervariable genomic region, which determines the O-antigen structure in *V. splendidus* (Wildschutte *et al.*, 2010). Interestingly less immunogenic structures of LPS O-antigen that favored oyster colonization led to attenuated resistance to grazing by the *Vannella* sp. AP1411 free-living marine amoebae, suggesting a tradeoff between grazing resistance and oyster colonization (Oyanedel *et al.*, 2020). Such a tradeoff between virulence and resistance to predators was also reported for *V. cholerae* O-antigen structure, which is under epigenetic control: phase variants of the *wbe* region defective in producing O-antigen are indeed highly resistant to phage infection, but severely attenuated in terms of virulence (Seed *et al.*, 2012). Altogether these studies highlight the critical role of LPS O-antigen in biotic interactions involving vibrios, strains pathogenic for bivalve being no exception. The extreme variability of

this major membrane structure is controlled by genetic or epigenetic determinants favoring environmental persistence but leading to attenuated virulence in bivalves.

Altogether these recent studies show that the multiple biotic interactions of vibrios in the environment can lead to either positive (coincidental selection), neutral (relaxed selection), or negative (conflicting selection) effects on the selection of *Vibrio* traits required for virulence expression in bivalves (Mikonranta *et al.*, 2012) (Figure 1). Predation could favor the selection of some virulence factors that participate to pathogenicity in bivalve host (Adiba *et al.*, 2010). However, as illustrated in the well-known intracellular pathogen *L. pneumophila*, hundreds of virulence factors can interact with a diversity of hosts, without having a similar importance in each interaction (Boamah *et al.*, 2017; Ghosh and O'Connor, 2017; Park *et al.*, 2020). Thus, in *V. cholerae*, some virulence factors involved in the resistance to grazing by *A. castellanii* have a minor role in pathogenesis in human (Van der Henst *et al.*, 2018). They can also have adverse effects as recently illustrated in a *V. splendidus* moderately virulent for oysters (Oyanedel *et al.*, 2020). Vibrios being mostly opportunistic pathogens, they appear to have acquired, on the one hand, "generalist" virulence factors involved in multiple biotic interactions, and on the other hand, "specialist" virulence factors involved in interactions with a particular host, with possible trade-offs on other interactions.

### Concluding Remarks

This review highlights the multifaceted nature of *Vibrio* interactions with mussels and oysters, aquaculture species showing contrasting resistance to infections. While only a limited number of vibrios pathogenic for mussels have been described, the current literature shows that oyster pathogens tend to interact differently with major components of oyster/mussel plasma and with their key immune cells, the hemocytes, which perform phagocytosis and produce highly microbicidal reactive oxygen/nitrogen species and accumulate heavy metals. The potent production of

AMPs in mussels as opposed to oysters could be an important factor controlling disease outcomes. Plasma-soluble recognition proteins specific of the bivalve species also appear to have critical role. However, understanding the higher susceptibility of oysters to infections requires a more systematic comparison of *Vibrio* interactions with mussels and oysters. Thanks to their successful colonization of oysters, different strategies of immune evasion and tolerance/resistance have been evidenced in vibrios pathogenic for oysters. These mechanisms include tolerance to the main cellular defenses of oysters (ROS, AMPs, heavy metals), active dampening of cellular immunity through cytolytic mechanisms (toxins, T6SS) as well as reduced antigenic properties through modifications of LPS, a major and hypervariable component of *Vibrio* outer membrane. Recent knowledge on the interactions of vibrios with environmental predators (phages, grazers) highlight possible mechanisms of coincidental selection as well as tradeoffs acting on these important determinants of *Vibrio* virulence (Figure 1).

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## Figure legend.

**Figure 1. Vibrio-bivalve interactions and the biotic environment.** Vibrios belong to the natural microbiota of oysters and mussels, establishing both transient and stable interactions. Outside pathogenic contexts, vibrio communities are controlled in the hemolymph by key hemocyte responses that include phagocytosis, ETosis and the production/accumulation of microbicidal compounds (ROS, NOS, AMPs, copper, zinc). However vibrios can overcome these potent antimicrobial defenses by inducing the lysis of bivalve hemocytes. Different mechanisms have been evolved by vibrio species leading to this common end. The required effectors can be delivered extracellularly by contact with the hemocyte membrane or intracellularly inside the phagosome. This enables vibrios to colonize deeper tissues and cause systemic infections. Interactions with predators (phages and amoeba) could favor the selection of mechanisms of virulence (cytotoxicity, resistance to bivalve antimicrobial defenses) in the environment as they confer resistance to grazing; in contrast some surface determinants could be counter-selected as they confer an advantage in colonizing oysters but an increased susceptibility to predators.

**Figure 2. A simplified view of hemocyte-vibrio interactions.** The interaction of the oyster pathogens *V. tasmaniensis* LGP32 and *V. aestuarianus* 01/032 with hemocytes of *C. gigas* and *M. galloprovincialis* have been characterized in independent studies. Data highlight different interactions in both species. Left panel: *V. tasmaniensis* LGP32 is readily phagocytized by both oyster and mussel hemocytes,

but entry pathways differ. Opsonisation by the major plasma protein Cg-EcSOD is only required in oyster, in which it mediates uptake through  $\beta$ -integrin recognition. LGP32 survives intracellularly in both species (see section 3 for mechanisms of resistance) and causes hemocyte damages. Hemocyte lysis dampens cellular defenses and is associated to pathogenicity in oyster only. Hemocytes show less damages lower in mussel and respond to LGP32 infection by the release of antibacterial effectors in the plasma. The potent AMPs of mussel hemocytes may play a key role in the control of LGP32 infection. Right panel: *V. aestuarianus* 01/032 secretes extracellular products that inhibit phagocytosis by oyster hemocytes and enable bacterial proliferation. In contrast, in mussels, the plasma protein MgEP recognizes 01/032 and promotes its phagocytosis; 01/032 is then degraded intracellularly. Opsonisation with MgEP is sufficient to restore uptake and killing of 01/032 by oyster hemocytes. MgEP appears as a key factor in the resistance of mussels to 01/032 infections.

Accepted Article

### Vibrio are key component of bivalve microbiota

showing a stable or transient association with different tissues



Hemolymph

Gills



**Pathogenic traits can be selected or counter-selected in the environment**  
by predators : Grazers (amoebae, ciliates and flagelates) or bacteriophages



Grazers



Phages

water column

sediment

### Pathogens or simple colonizers, Vibrio can circumvent bivalve immune responses

They can evade, inhibit or manipulate immune response thanks to various virulence factors



*V. tasmaniensis*  
ompL6SS



*V. crassostreae*  
R5-7 T6SS



Hemocyte lysis

### Bivalve responses are mainly based on hemocytes.

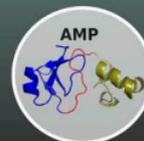
through phagocytosis, ETosis, the production of ROS, NOS, AMPs, hydrolytic enzymes, and the accumulation of heavy metals



Hemocytes



ROS & NOS



AMP

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*V. tasmaniensis* LGP32

*V. aestuarianus* 01/032

