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# Ecology, evolution and virulence of environmental vibrios

Adèle James

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# Sorbonne Université

Ecole doctorale Complexité du vivant

*Laboratoire de biologie intégrative des modèles marins / UMR 8227*

## **Ecology, Evolution and virulence of environmental vibrios**

Par Adèle James

Thèse de doctorat de Microbiologie moléculaire

Dirigée par Frédérique Le Roux

Présentée et soutenue publiquement le 24 septembre 2018

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# Ecology, evolution and virulence of environmental vibrios

## Abstract

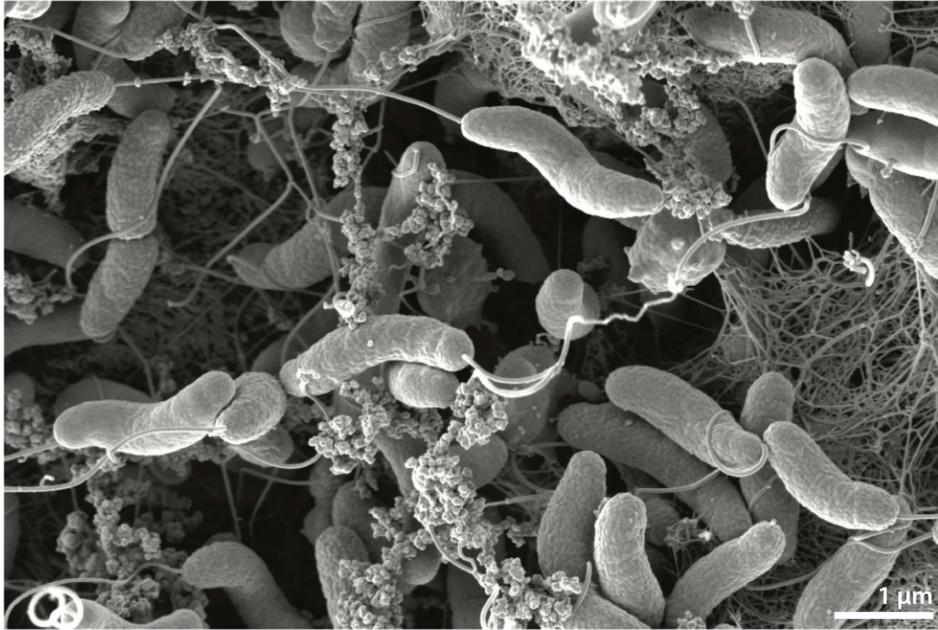
Global change, including anthropogenic activities such as aquaculture, have been associated with an increase in the incidence of *Vibrio*-associated illnesses (Le Roux et al., 2015; Vezzulli et al., 2016; Baker-Austin et al., 2017). Contrary to human-pathogenic *Vibrio*, fairly little is known about vibrios affecting marine animals. Considering the ecological and economic consequences of these infections, there is an urgent need to develop basic knowledge about vibrios in the environment. Investigating pathogen eco-evolutionary history and exploring their virulence mechanisms is an essential step to understand and further manage environmental vibrio-associated diseases. In this PhD project, we took advantage of the oyster *Crassostrea gigas* as a model of infection to answer two main questions: i) How widespread is pathogenic potential of vibrios in the wild? ii) Does oyster farming select for emergent pathogen(s)? To address these questions, we combined experimental ecology, a high throughput infection assay, genome sequencing and inverse genetics. We found that a number of environmental populations are virulent toward oysters thanks to virulence mechanisms ancestrally acquired and involved in ecological differentiation. We showed that the Herpes virus OsHV-1, often recognized as the aetiological agent of oyster mortalities is neither sufficient nor essential to cause oyster death. We further observed that diseased juvenile oysters are always colonized by a diversity of vibrios. *V. crassostreae* was found to be abundant in diseased oyster. Its virulence is dependent on a conjugative plasmid that notably encodes a type VI secretion system whose mechanism remains to be determined. This plasmid is only detected in high-density oyster farming areas, suggesting that intensive aquaculture settings have selected higher virulent genotypes. We further showed that plasmid transfer and/or selection is enhanced in oyster compared to *in vitro* conditions, suggesting that oysters represent a niche for HGT and emergence of pathogens.

# Introduction

## I The *Vibrionaceae* represent a major part of bacterial biomass in the ocean

Marine bacteria have been described as “**the unseen majority**” (about  $10^6$  bacteria per milliliter) because they represent a large proportion of genetic diversity and overall biomass in the ocean while being microscopic (Whitman et al., 1998). Bacteria are ubiquitous in the marine environment, where they play major roles in several biogeochemical processes. They notably regulate atmospheric carbon dioxide by recycling it into organic compounds and they participate in oceanic primary production (Falkowski et al., 1998). In spite of being highly abundant and playing critical functions in their environment, there is still a lot of progress required to explore diversity, structural organization and functional potential of bacteria. Advances in community DNA shotgun sequencing, has considerably improved our knowledge of the taxonomic diversity and genetic content of ocean microbial communities. However, considering the tremendous genetic diversity of the bacterial kingdom and difficulties in sampling some extreme environments, the vast majority of the global microbiome is still unknown. The large-scale sampling campaign Tara Oceans, covering 64 sites worldwide and several seawater depths, highlighted that microbial communities were highly heterogeneous, and revealed strong disparity in abundance, diversity and turn-over of the microbial composition (Sunagawa et al., 2015). To date, *Alphaproteobacteria*, *Gammaproteobacteria*, *Cyanobacteria* and *Deferribacteres* are considered to be the most abundant classes of bacteria in the ocean.

Within the *Gammaproteobacteria* class, the *Vibrionaceae* (figure 1), thereafter named vibrios usually only represent a small percentage of total bacteria (Gilbert et al., 2012). However, they have a relatively big size compared to other marine bacteria with a single-cell biomass ten times bigger than *Prochlorococcus* (*Cyanobacteria*) and one hundred times bigger than *Pelagibacter* (*Alphaproteobacteria*) (Cermak et al., 2017). This distinctive feature highlights the importance of the *Vibrionaceae* in biogeochemical cycles and more broadly in ecological interactions in the ocean. In addition, the family *Vibrionaceae* is of major interest due to the health and ecological impacts it has on its environment (Dubert et al., 2017; Rosenberg et al., 2007).

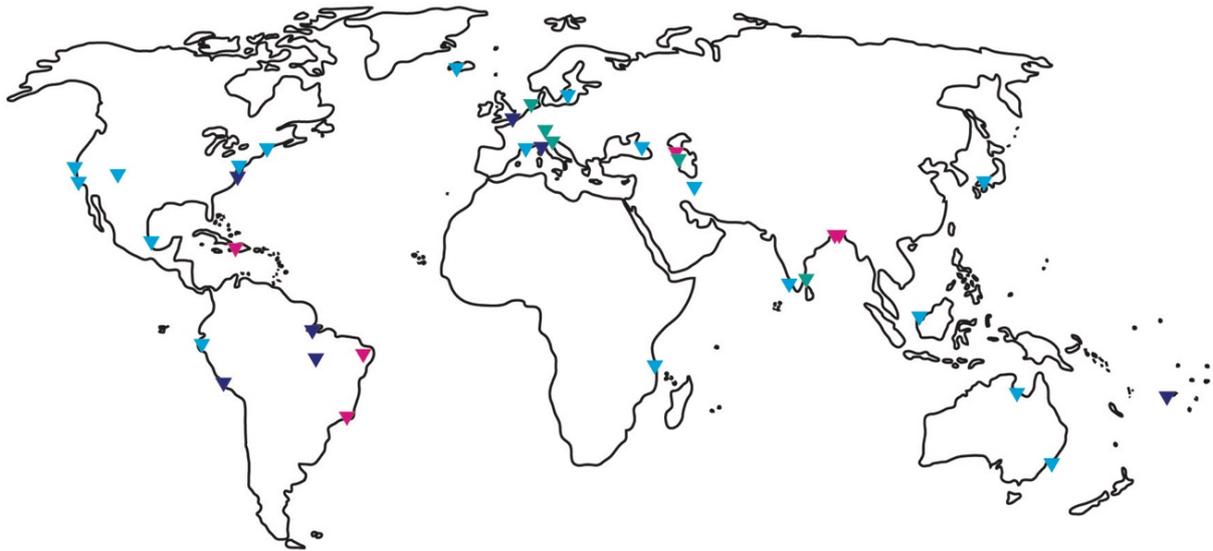


**Figure 1 | Imaging of *Vibrio cholerae* forming a biofilm on chitinous surface obtained by electron microscopy.** *V. cholerae* is a curved rod-shaped bacterium. Vibrios measure between 1.5 to 3μm long and 0.5μm across. From Le Roux and Blokesch, 2018.

## II The *Vibrionaceae* are opportunitroph

### II.A The *Vibrionaceae* abundance is highly dynamic

Vibrios are ubiquitous aquatic bacteria. Salinity and temperature are the main abiotic factors shaping their distribution (Singleton et al., 1982; Takemura et al., 2014). According to their halophilic status, they can thus be found in fresh waters, estuaries, brackish waters and oceans (Barbieri et al., 1999; Eyisi et al., 2013; West and Lee). For example, *Vibrio cholerae* has better survival in a low salinity environment and is found in estuarine and coastal regions of the globe (figure 2) (Lutz et al., 2013). In the ocean, according to their tolerance to abiotic factors, vibrios can occupy the whole seawater column, from the surface to high depths and can be found at very distant latitudes. They have been isolated from the boreal (Eiler et al., 2006) to the tropical waters (Shibata et al., 2013) and can survive in extreme environments such as the hypersaline lake Assal (Brisou et al., 1974) and in deep-sea hydrothermal vents (Raguénès et al., 1997).



**Figure 2 | Global distribution of *V.cholerae* as an example for vibrio distribution.** Triangles indicate where *V.cholerae* has been detected. Red triangles indicate O1/O139 detection, light blue triangles non-O1/non-O139 detection and dark blue triangles not specified. This map has been built on more than 30 studies. From Lutz et al., 2013.

Relative abundance of vibrios is highly dynamic. Indeed, in favorable conditions, vibrios can go from barely detectable to being the predominant bacteria in term of abundance in a very short-time (<day) (Gilbert et al., 2012; Martin-Platero et al., 2018). Blooms of vibrios are linked to their fast replication abilities and depend on biotic and abiotic factors. For instance, a high abundance of vibrios is related to plankton blooms as they result in an increase in nutrients that vibrios can exploit to feed on. Decline in abundance derives from lower available nutrients, less favorable conditions and also from predation. In fact, the relatively big size of vibrios makes them subject to a higher predation rate by protozoa and phages, which significantly contributes to population control (Faruque, 2014; Matz et al., 2005). In addition, when they are facing unfavorable environmental changes, vibrios can adopt a survival mode *e.g* a viable but non-culturable state (Colwell, 2000).

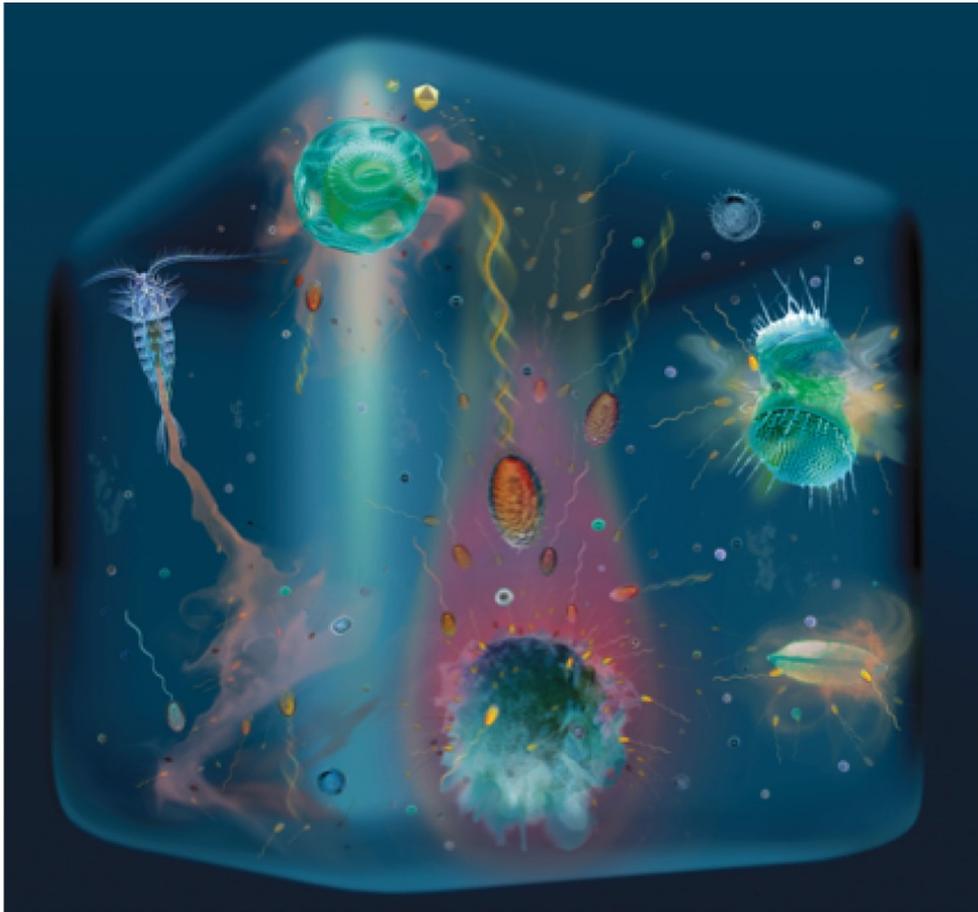
## II.B Vibrios can adopt several feeding strategies to survive

Vibrios are heterotrophic bacteria, which means that they use organic matter as a carbon source. Aquatic environments represent a heterogeneous habitat in which there are areas of very

low abundance in nutrients contrary to rich zones of dissolved organic compounds. To survive in this patchy environment, vibrios have adopted several ecological strategies: they can exploit the generally lower, ephemeral, but more evenly distributed dissolved nutrients, they can attach to particles or to phyto- and zooplankton (Stocker et al., 2008), or they can initiate a mutualistic or pathogenic interaction with higher organisms.

### II.B.1 Adopting a free-living lifestyle assures more abundant food

The first strategy is adopted by the free-living bacteria, also called planktonic bacteria. They feed on dissolved nutrients derived from zooplankton excretions, plumes of exudates in the wake of sinking or swimming phyto- and zooplankton, and from macroscopic organic aggregates described as marine snow (figure 3) (Alldredge and Silver, 1988; Stocker and Seymour, 2012). These nutrient patches can also come from the lysis of organisms following viral infection. Once the patch does not provide enough nutrients, vibrios move rapidly to reach another richer patch (Yawata et al., 2014). The free-living lifestyle is thought to be adopted by a majority of vibrios. Indeed, it confers the ability to reach a new source of food when facing micro-environments deprived of nutrients and to flee deleterious substances.



**Figure 3 | View of the ocean at the microscale, emphasizing the heterogeneity of resources and related microbial interactions and distributions.** Organic substrates diffuse from a range of sources, including zooplankton excretions (left), phytoplankton exudation (the “phycosphere”) (top; bottom right), phytoplankton lysis (top right), and settling marine snow particles (center bottom). The last, in particular, can produce intense, comet-like plumes of dissolved matter. Approximate scale for the image: 1 cm. From Stocker and Seymour, 2012.

### II.B.2 Attachment to particles or plankton confers a stable environment

Vibrios can also live attached to large organic particles or living organisms, the latter constituting a commensal interaction. Attached-feeding mode represents a much more stable micro-environment in which bacteria can metabolize plant and algal polysaccharides (Hehemann et al., 2016), digest dead host tissue or metabolize the chitinous exoskeleton and many other compounds when attached to zooplankton (Heidelberg et al., 2002). In addition to food, attachment also provides protection against environmental stress, predation by phages and grazers and thus constitutes a niche for persistence in the aquatic environment (Alam et al., 2007; Beyhan and Yildiz, 2007; Faruque et al., 2006; Matz et al., 2005). Particles or organisms

to which vibrios attach can also serve as a disease vector, as has been previously shown for *V. cholerae* attached to zooplankton (Lipp et al., 2002). As particles or organisms are often colonized by complex microbial communities (Simon et al., 2002), adopting this feeding strategy requires the ability to cooperate with and/or compete against other colonizers in order to survive.

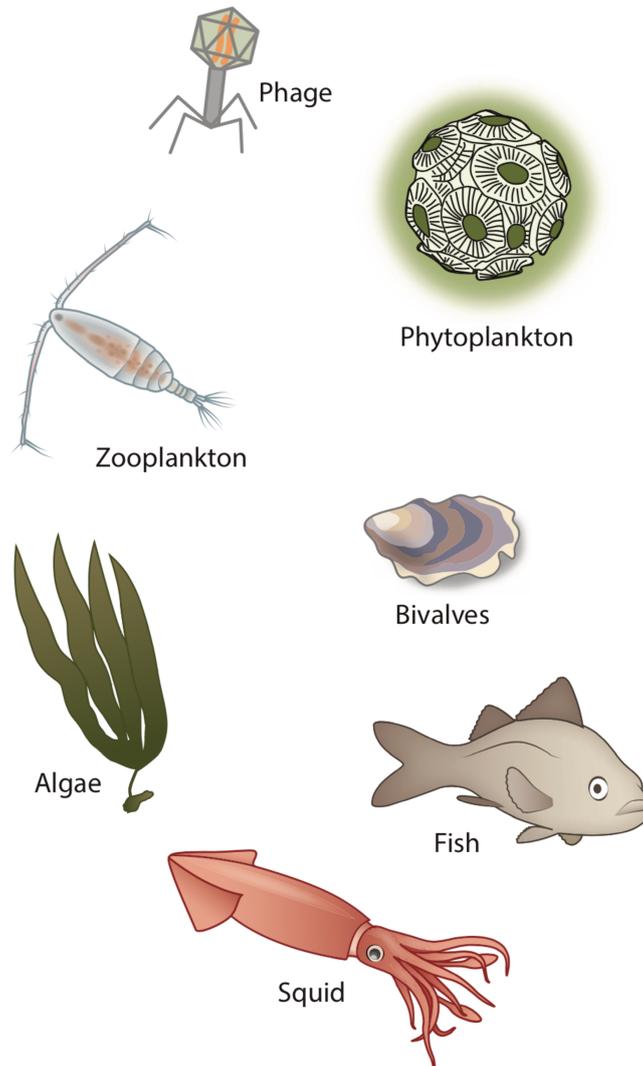
### II.B.3 Mutualistic and pathogenic interactions provide a specific source of nutrients

Another way to feed is to engage in a mutualistic or pathogenic interaction with another living organism. Specific associations have been suggested many times between vibrios and living organisms (Reen et al., 2006; Thompson et al., 2004). However, specific interactions are rarely demonstrated formally. Indeed, co-occurrence does not mean that organisms interact with each other, in particular when the sampling is not based on time series, or metapopulation analysis (i.e. comparing host/seawater). In addition, isolation of certain vibrios can only result from the feeding mode of their apparent host. Vibrios presence can also be fortuitous and dependent on other biotic/abiotic parameters that are not related to the apparent host. However, some types of interactions have been explored in detail.

A well described example of **mutualism** (positive interaction for both partners) is the relation between *Vibrio fischeri* and the squid *Euprymna scolopes* (Mandel and Dunn, 2016; Ruby and Lee, 1998). *V. fischeri* colonizes the light organ of the squid which constitutes a very specific and safe niche for this species. Colonization of the host is initiated by the production of the regulator of symbiotic colonization sensor RscS that regulates the “symbiosis polysaccharide” locus *syp* (Yip et al., 2006). Once the symbiont has colonized the squid, the ciliated appendages of the host light organ undergo apoptosis to avoid further colonization. This regression is induced by the *V. fischeri* envelope shedding components binding to host receptors (Koropatnick et al., 2004). When they reach a certain density, the *V. fischeri* strains provide a costly service to their host by producing bioluminescence thanks to *lux* operon encoding the luciferase (*luxAB*), proteins synthesizing the aldehyde substrate (*luxCDE*) and quorum sensing transcriptional regulators *luxR* and *luxI* (Fuqua et al., 1996; Visick et al., 2000). This light removes the animal shadow on the ground and thus allows the squid to avoid predation by upper organisms (Miyashiro and Ruby, 2012).

Numerous interactions involving vibrios as pathogens have been reported. Vibrios induce damage to their hosts and feed on degraded tissues. Hosts are various and encompass

unicellular host such as amoeba (Van der Henst et al., 2016), animals including humans (Islam et al., 2017), and algae (Hehemann et al., 2016). Pathogenic interactions will be detailed in section V. Overall, vibrios interact with diverse organisms (figure 4) and adopt diverse strategies to survive in the environment.

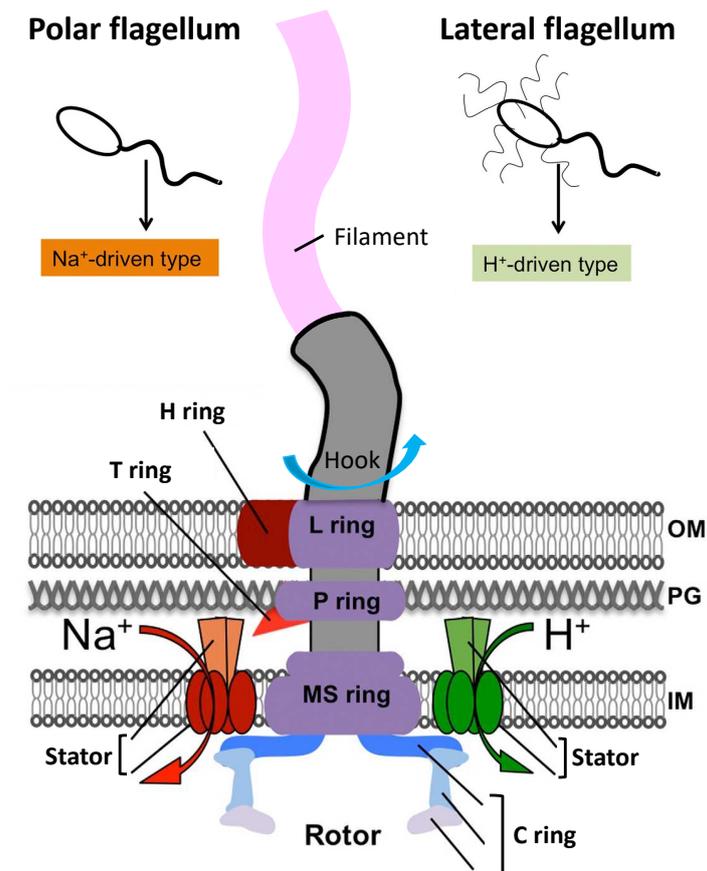


**Figure 4 | The *Vibrio* universe.** In the aquatic environment, vibrios engage in organismal interactions, which can include phages, phytoplankton and zooplankton, bivalves, algae, squid, and fish. From Le Roux and Blokesch, 2018.

## II.C Mechanisms involved in high colonization potential

### II.C.1 Motility

The feeding strategies described above require the ability to find and reach nutrient-rich areas, particles or hosts. *Vibrio* species are motile thanks to one or several flagella. The polar flagellum confers an ability to swim in liquid environment while the lateral flagella are involved in moving over surfaces or swimming in viscous liquids (McCarter, 2004). The flagellum consists of a molecular motor at the inner membrane of the cell, a hook-basal body complex which forms an export channel, a hook and the rotating filament (Aizawa et al., 2000). Rotation of the flagellum is dependent on an energy source that will activate the molecular motor: within the *Vibrio*, polar flagella are driven by a sodium-motive force while lateral flagella are driven by a proton-motive force (Asai et al., 1999)(figure 5). The rotary filament produces a propulsive force on water in a movement similar to an endless screw. Flagella are big organelles; their production, assembly and functioning are very costly for the cell and require the involvement of about 50 genes (Macnab, 2003). Assembly is tightly regulated and starts from the inner to the outer membrane of the cell e.g from the basal body to the filament (Zhu et al., 2013). Remarkably, the sodium-driven flagellum of vibrios can spin much faster than the proton-driven flagellum of *Salmonella* (1700 vs 300 Hz), indicating that vibrios are relatively fast swimmers (Magariyama et al., 1994; Minamino and Imada, 2015).



**Figure 5 | Flagellar structure of *Vibrio*.** Schematic diagram of the structure of polar flagellum and lateral flagella in some *Vibrio* species. H,T,L,P,MS rings, stator and rotor constitute the basal body complex. The characteristics of the polar flagellum are the H ring and the T ring, shown in red. Adapted from Zhu et al., 2013.

## II.C.2 Chemotaxis

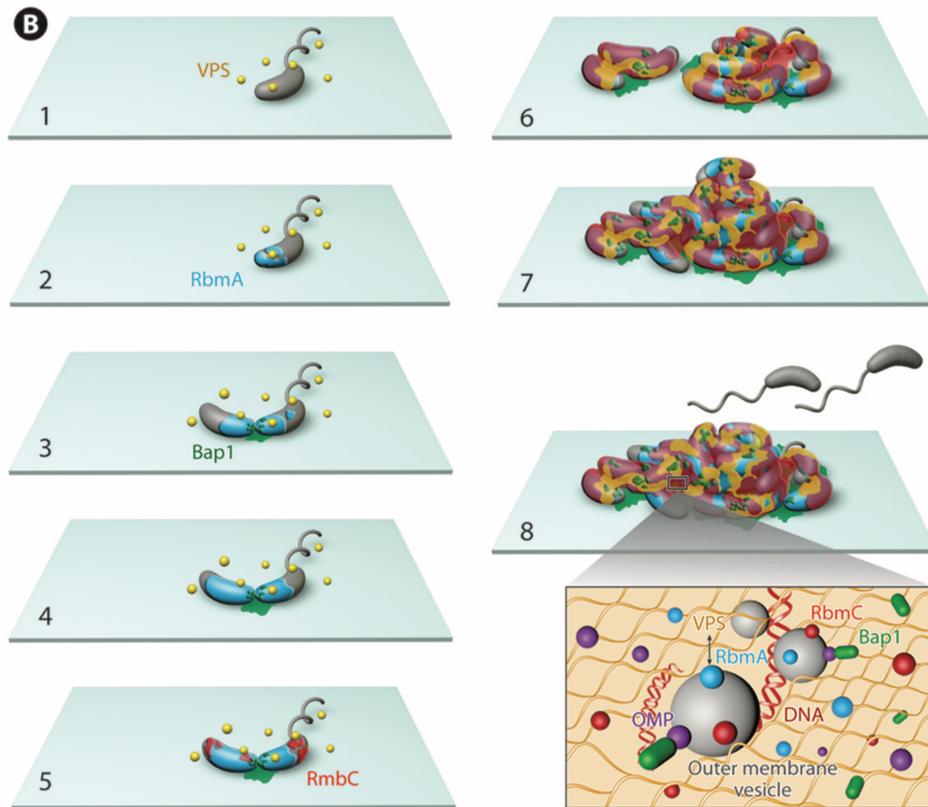
Bacterial motility is not always random and is tightly linked to chemotaxis (Szurmant and Ordal, 2004). Chemotaxis corresponds to the process by which an organism orientates itself according to a chemical gradient, inducing a movement toward an attractive substance or away from a repulsive one. Chemotaxis starts with the binding of a specific ligand to methyl-accepting chemotaxis proteins (MCPs). Those chemoreceptors transduce the signal to the chemosensor system and is further relayed to the flagellum motor (Lux and Shi, 2004). Sense of rotation of the flagellum depends on whether the detected chemical is attractant or repulsive (Armitage, 1999). In the case of the free-living feeding strategy, bacteria detect nutrient gradients and move toward highly concentrated food patches. In the attached strategy, the

attractant chemicals are molecules secreted by the future host or bacteria already attached to the host/particle (Zhu et al., 2013).

### II.C.3 Attachment and biofilm formation

Biofilms are complex communities of microorganisms attached to surfaces or associated with interfaces. Those cell aggregates are surrounded by a matrix of heterogeneous biochemical composition. This assemblage induces an enhanced fitness for members of the biofilm communities and resistance to environmental stressors such as predation and antibiotics. Biofilm formation is thus a strategy to persist and survive in the aquatic environment but requires bacteria to coexist and compete for limited space and nutrients (Rendueles and Ghigo, 2015). Biofilm genesis has been extensively studied for *V.cholerae* (Teschler et al., 2015). Following attraction toward their host or particle, cells scan the surface in an orbiting or roaming trajectory. Orbiting enables *V. cholerae* to loiter over the surface and to interact more strongly with it thanks to its MSHA pili, whereas roaming produces a weaker interaction (Utada et al., 2014). Both of these trajectories require the mannose-sensitive haemagglutinin (MSHA) type IV pili and the polar flagella (Utada et al., 2014). Initial attachment derives from a mechano-chemical interaction between the type IV pili and the surface. Two other pili; the toxin co-regulated pili (TCP) and the chitin-regulated pili (ChiRP) also contribute to biofilm formation. TCP is involved in microcolony formation and ChiRP in competitive attachment to chitin surface (Yildiz and Visick, 2009). The flagellar motor may act as a mechanosensor enabling vibrios to recognize when it encounters a surface (Lauriano et al., 2004; Watnick et al., 2001) and hence stops the scanning motion. Initial attachment is followed by polysaccharide (*Vibrio* polysaccharide VPS) excretion, that will last throughout the whole biofilm formation process to form a mature three-dimensional matrix. Then, the biofilm matrix protein RbmA accumulates on the cell surface and is involved in cell-cell and cell-surface adhesion (Fong and Yildiz, 2007). Two other biofilm matrix proteins, Bap1 and RbmC are further excreted (Berk et al., 2012). VPS, RbmA, Bap1 and RbmC form a flexible envelope allowing cell division and mature biofilm formation. Outer membrane vesicles, extracellular DNA and other matrix components are also found in mature biofilms. Biofilm formation is a costly process and results in major biological consequences; it allows a better fitness in some conditions but might also be deleterious in others, notably when nutrient sources are being over-exploited. Biofilm

formation is thus tightly regulated, which enables biofilm exit and strain dispersal according to the environmental conditions experienced (Teschler et al., 2015, figure 6).



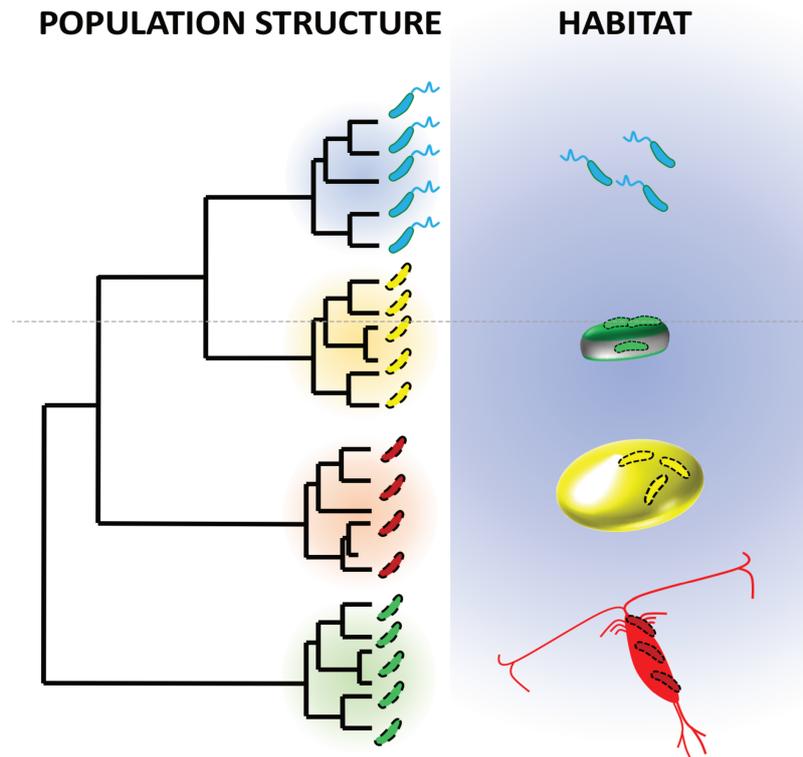
**Figure 6 | Building a *V. cholerae* biofilm.** Soon after initial attachment, *Vibrio* polysaccharide (VPS) is excreted from cell surfaces (B1), and VPS extrusion is observed throughout biofilm formation. Next, the biofilm matrix protein RbmA accumulates on the cell surface (B2). During cell division, the daughter cell remains attached to the founder cell (also known as parental cell), confirming the role of RbmA in cell-cell adhesion, and the biofilm matrix protein Bap1 is excreted between the two cells and on the substrate nearby (B3). Bap1 gradually radially accumulates on nearby surfaces, although the concentration of Bap1 remains the highest near the founder cell. Subsequently, the biofilm matrix protein RbmC is excreted and found on discrete sites on the cell surface (B4). As biofilms develop, VPS, RbmC and Bap1 form envelopes that can grow as cells divide (B5). The mature biofilm is a composite of organized clusters composed of cells, VPS, RbmA, Bap1 and RbmC, in addition to other matrix components, such as outer membrane vesicles (OMVs) and extracellular DNA (eDNA) (inset). Outer membrane proteins (OMPs) associate with Bap1 in OMVs and bind to antimicrobial peptides thereby increasing *V. cholerae* resistance. The last stages in biofilm development are dispersal, whereby exiting *V. cholerae* cells seek out and colonize new resources (B6). From Teschler et al., 2016.

In this section, we showed that *Vibrio* are highly motile bacteria, they can move among or attach to nutrient sources and they can sense and interact with their environment, resulting in a high colonization potential. They can adapt to various environmental conditions, exploit many alternative niches and use a variety of substrates as energy and carbon sources, which indicates a high metabolic diversity (Polz et al., 2006). Due to these abilities, vibrios have been classified as “opportunitrophs” (Singer et al., 2011).

### III Population structure of *Vibrio*

#### III.A Vibrios are organized into ecological populations

Vibrios are ubiquitous marine bacteria that are ecologically and metabolically diverse members of planktonic- and animal-associated microbial communities (Polz et al., 2006). Polz and collaborators proposed to explore to what extent ecological differentiation is reflected by genetic diversification. To determine whether phylogenetic clusters are groups of strains sharing a common habitat, they applied an evolutionary model (AdaptML) to a collection of co-occurring *Vibrionaceae* isolated from coastal seawater at different time of the year (season) and in different seawater fraction of isolation (associated with large (L), intermediate, small (S) particles/zoo-phytoplankton or free-living) that represent their “habitat”. This study demonstrated that phylogenetic clusters were coherent with habitat (Hunt et al., 2008, figure 7) and led to the definition that population structure of vibrios is organized into “ecological populations” defined as groups of **phylogenetically closely related strains that form an ecological unit** (Preheim et al., 2011a; Shapiro and Polz, 2014).



**Figure 7 | Population structure coincides with habitat.** Vibrios are organized into ecological populations that correspond to phylogenetic clusters containing strains sharing the same habitat (season (not represented) and free-living or particle/organism-attached lifestyle). Adapted from Hunt et al., 2008.

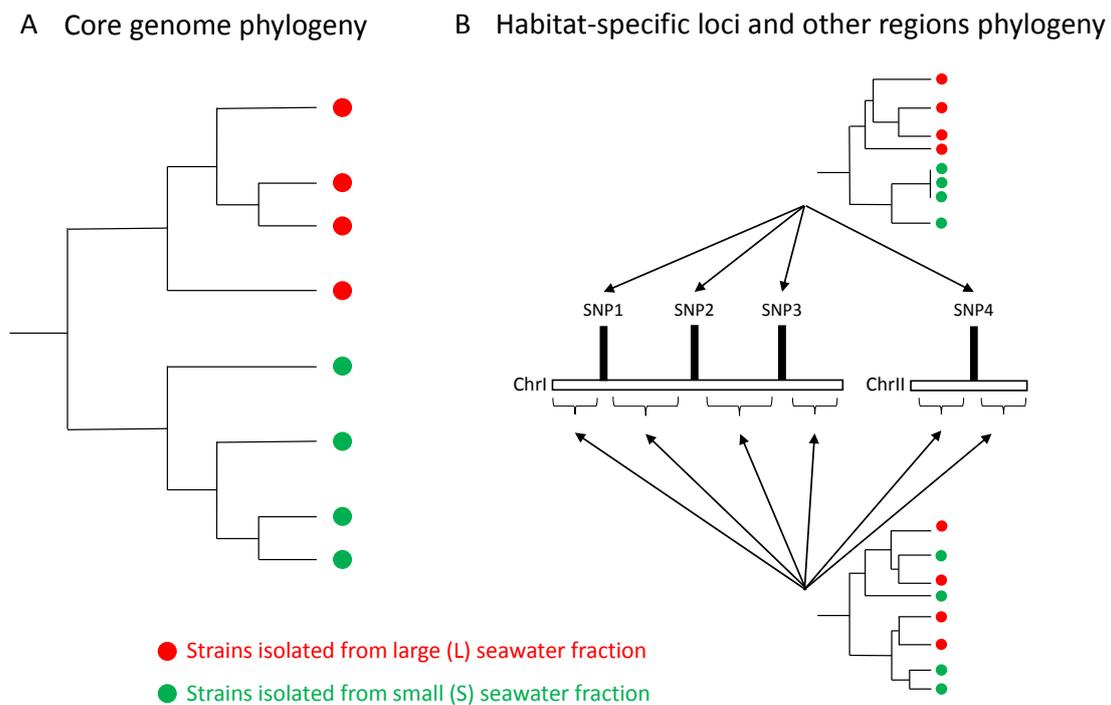
### III.B Ecological populations are congruent with taxonomic assignation

Overall, ecologically cohesive populations are predominantly congruent with taxonomic *Vibrio* species previously characterized as genotypic clusters by multilocus sequence analysis (MLST) (Preheim et al., 2011b; Sawabe et al., 2013). There are to date 110 described *Vibrio* species (Lovell, 2017), that have been classified into 17 clades by Sawabe et al. in 2013. The Splendidus clade contains 12 species, including *Vibrio crassostreae* and *Vibrio splendidus*, that are of special interest for the up-coming chapters (figure 8).



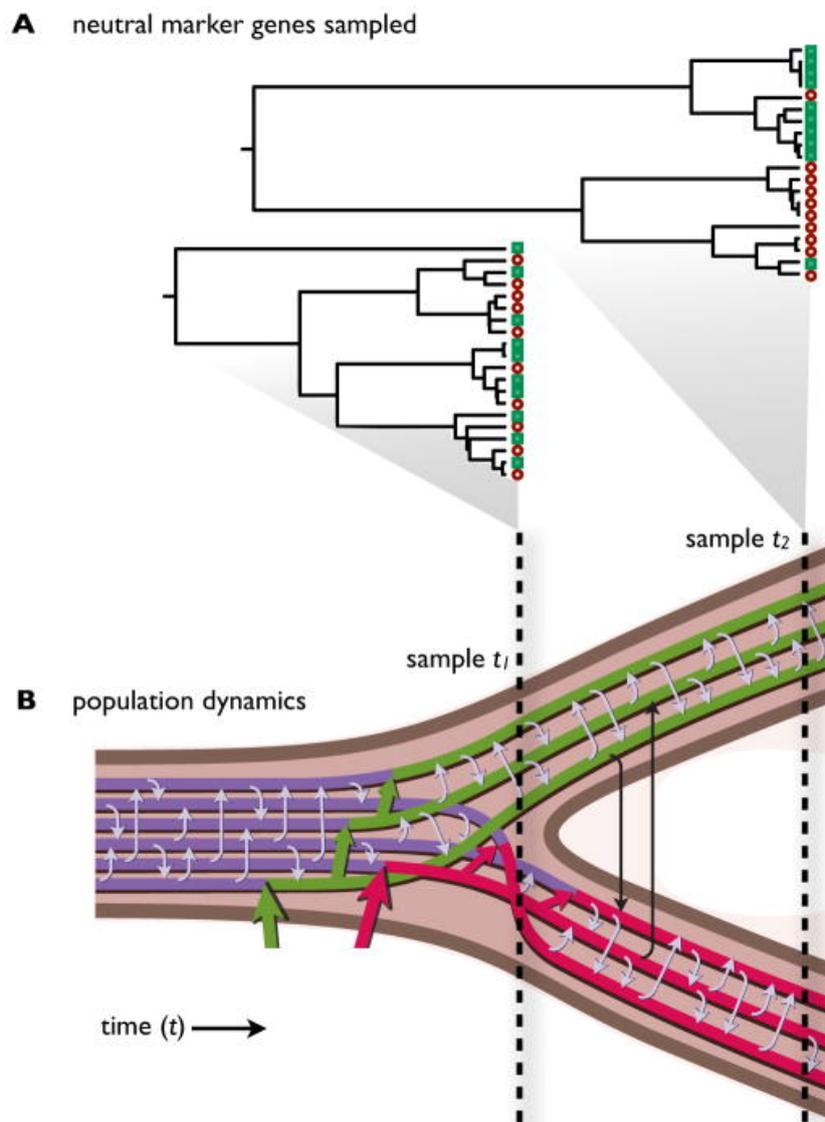
### III.D Ecological populations represent gene flow units

Ecological differentiation is a dynamic process that can lead to phylogenetic differentiation. As the population structure of vibrios highlighted some extremely closely-related clusters that have different ecology; it offers an opportunity to study evolutionary mechanisms behind this differentiation. A recent differentiation event has been highlighted in two closely related populations from *V. cyclitrophicus* (Shapiro et al., 2012). These populations are respectively associated with two different size fractions: the large (L) and the small (S) of seawater. Although those size fractions only represent proxies for habitat, comparative genomics revealed that few loci, called habitat-specific loci (HSL), were congruent with L or S ecology (figure 9). This result validates that fractionation is biologically meaningful as being associated with one habitat indeed requires specific function.



**Figure 9 | Phylogeny follows ecology at just a few habitat-specific loci.** **A** Core genome phylogeny is congruent with ecology: strains isolated from L (red dots) or S (green dots) seawater fractions are clustered in distinctive groups. **B** Representation and schematic tree topology of habitat-specific loci and other genomic regions of chromosome I and II of *V. cyclitrophicus*. Phylogeny of HSLs is congruent with ecological differentiation between L and S strains while phylogeny of the other regions is not always coherent with ecology. HSL3 shows allelic differentiation in the *syd* cluster, involved in polysaccharide synthesis that is essential in biofilm formation. Other HSL functions seem less related to S or L ecology. Adapted from Shapiro et al., 2012.

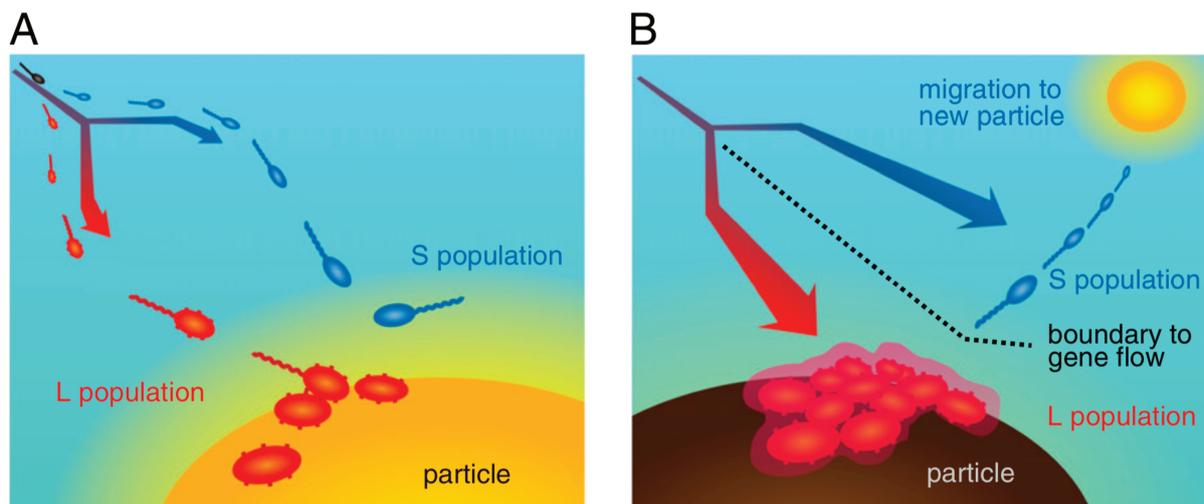
Further analyses showed that genetic diversity within 3/4 habitat-specific loci was reduced compared to the one observed in the rest of the genome. This suggests that these loci were recently acquired by homologous recombination and then fixed within S or L populations, respectively. Barriers to gene flow then arise between ecological populations, isolating them from the others in a process leading to ecological differentiation (figure 10).



**Figure 10 | Ecological differentiation in recombining microbial populations (A)** Example genealogy of neutral marker genes sampled from the S and L population(s) at different times. **(B)** Underlying model of ecological differentiation. Thin grey or black arrows represent recombination within or between

ecologically-associated populations. Thick colored arrows represent acquisition of adaptive alleles for red (L) or green (S) habitats. From Shapiro et al., 2012.

A further study showed that these two populations pursue different behavioral strategies of feeding in the heterogeneous aquatic environment. The two populations show the same ability in motility and orientation thanks to chemotaxis; however, L population is specialized in colonizing particles and forming biofilm while the S population, due to its free-living lifestyle, is able to disperse among particles by rapidly detecting and swimming toward new particles (Yawata et al., 2014) (figure 11). Notably, at the genomic level, Shapiro et al. showed that allelic differentiation in the habitat-specific *syp* operon, involved in polysaccharide synthesis, was congruent with L and S ecology. Yawata's study further revealed that this operon was not complete within the S strain genomes, thus conferring a reduced ability to form biofilm.

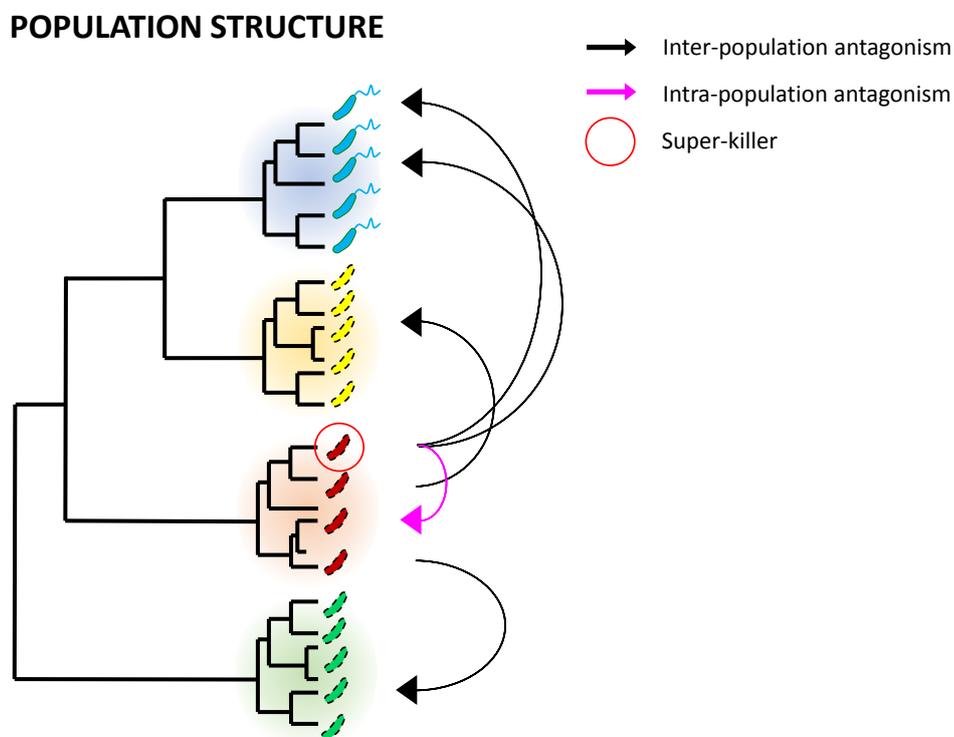


**Figure 11 | Model for the tradeoff between the feeding adaptations of the two *V. cyclitrophicus* populations.** (A) Both populations swim and respond to chemical gradients, but only the L population attaches and forms biofilms on particles, whereas the S population hovers near the surface. (B) When a more nutrient-rich particle appears nearby, only the S population is ready to rapidly respond by migrating to the new source. From Yawata et al., 2014.

### III.E Ecological populations form socially cohesive units

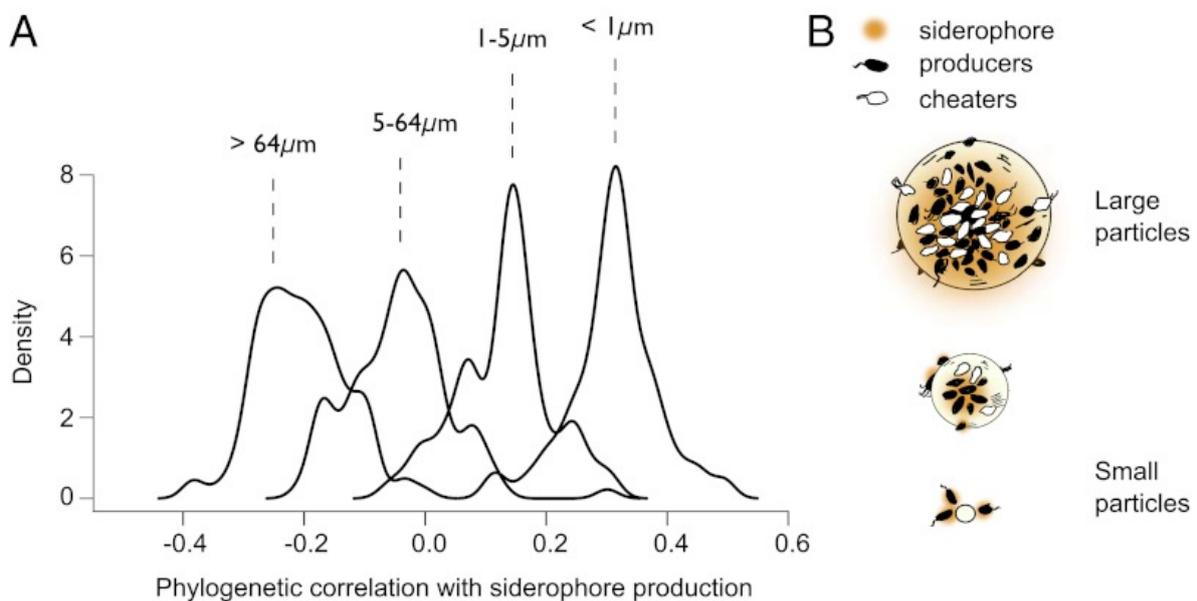
In addition to genotypic cohesion and differential environmental distributions, Polz and coll. further explored whether other factors, such as social behavior, could enforce the vibrio population structure described above. A first study focused on interference competition among individuals from different populations and tested growth inhibition in an all-versus-all

framework of 185 strains. The network of antagonistic interactions (schematic representation in figure 12) revealed that the potential for interference competition was much lower within than between populations. Within a chosen population, they showed that the antagonistic activity was only mediated by a single specific antibiotic biosynthesis mediated by an antimicrobial peptide and that few genotypes produced antibiotics to which strains from their own population was resistant. This study suggests that antibiotics can be considered as public goods within populations, benefiting nonproducing but resistant conspecifics. Some genotypes might favor their conspecifics and cooperation may occur within populations while competition is observed between populations (Cordero et al., 2012a).



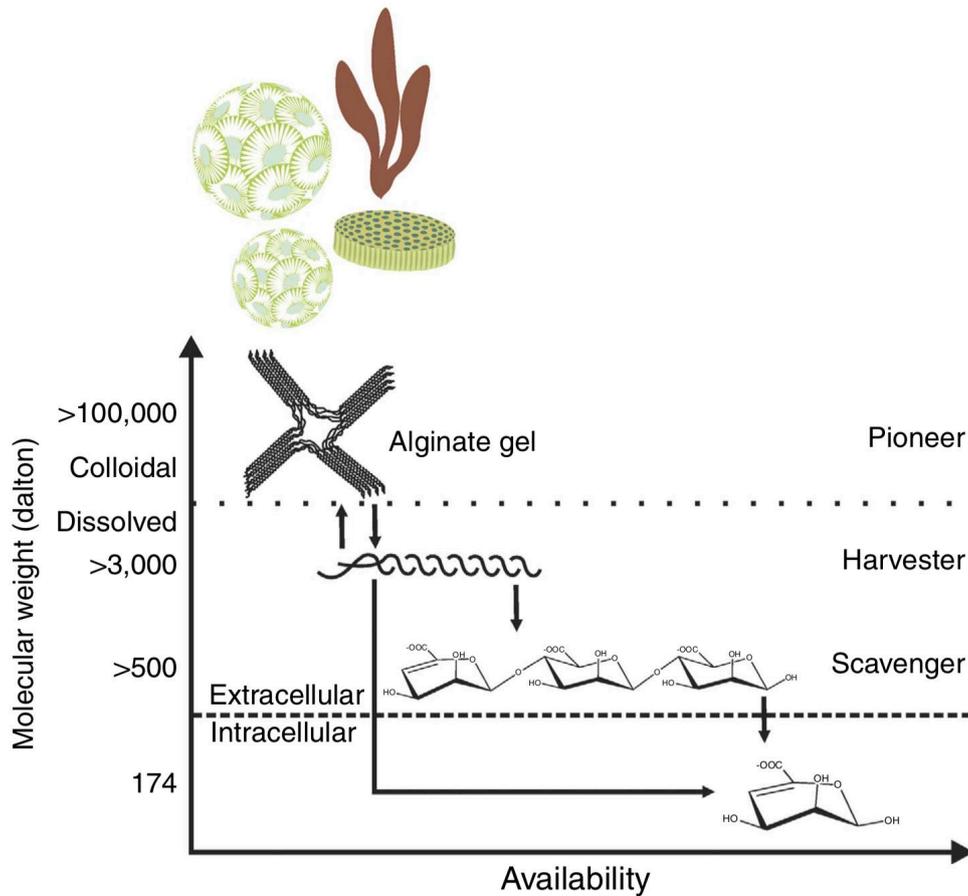
**Figure 12 | Schematic representation of competition by interference in relation to *Vibrio* phylogeny and genetic distance.** Competition by interference was assessed by growth inhibition assay in an all-versus-all framework. Inter-population antagonism (black arrows) is more frequent than intra-population antagonism (pink arrow). Some strains can kill several strains or can be killed by several ones. Some strains are classified as super-killers if they can kill numerous other strains. This scheme only represents antagonism mediated by one population, but it can be generalized to the others. Adapted from Cordero et al., 2012a.

Among bacteria, siderophore production represents the paradigm of public goods. Siderophores are organic compounds that bind and chelate iron, making it available for microorganisms living in iron-limited environments (Ahmed and Holmström, 2014). These siderophores are released into the environment and can be exploited by cheaters e.g. benefit recipients that are non-producers. Cordero et al. wanted to determine whether public good games could support the existence of stable social structure in bacteria and thus explore the evolutionary and ecological dynamics of siderophore production and cheating in natural ecological populations of vibrios. They found that within ecological populations, siderophores are secreted only by a subset of the population members, the producers, while the whole population still harbor the specific transporters necessary to internalize chelated iron. Non-producers have lost the siderophore biosynthesis genes and therefore act as cheaters by using the ones secreted by the producers of their own population. As non-producers cannot survive in iron-limited environments without producers, a stable cheater/producer equilibrium is maintained according to the environment. It has been shown that cheaters are relatively more frequent on large particles than on smaller ones (figure 13). Larger particles constitute the micro-environment harboring more stable and high-density communities. As the presence of producers is more likely to be assured on larger particles, in those conditions, evolving as a cheater constitutes a fitness advantage. Altogether, this study suggests that there are differences in the structure of social interactions between particle-attached vs. free-living populations with cooperation being more likely to occur in particle-attached populations than in the free-living ones (Cordero et al., 2012b).



**Figure 13 | Cheaters within the *V. splendidus*-like clade are more successful in larger particles than in smaller ones.** (A) Association with large particle sizes (>64  $\mu\text{m}$ ) in the ocean is negatively correlated with siderophore production, whereas the free-living stage (<1  $\mu\text{m}$ ) is positively correlated with siderophore production. (B) Cartoon representation of the impact of particle size on frequency of siderophore producers. These results suggest that social interactions are more relevant in large particles, possibly owing to the higher cell densities and long periods of attachment facilitating the accumulation and exploitation of public goods. From Cordero et al., 2012b.

Finally, a study focused on behavioral unit by studying ecophysiological strategies and the ability of vibrio populations to degrade diverse algal glycans (Hehemann et al., 2016). Genomic analyses between several populations of vibrios showed that the alginate degradation pathway has assembled primarily by horizontal gene transfer and further undergone extensive evolutionary changes across the majority of populations. Its architecture differentiates within closely related populations; resulting in populations adapted to degrade different forms of alginate according to their alginate lyase contents. The alginate degradation pathways are constituted by one to four polysaccharide lyase families with different molecular functions. Ability to degrade more or less complex sugars allows the discrimination of different feeding behavior. Pioneers colonize and degrade intact polymers into more soluble forms of polymers or oligomers that can then be consumed by scavengers. Scavengers can only degrade oligomers and thus need the prior action of pioneers in order to feed on partially degraded sugars. Harvesters represent an intermediate between the two other categories. They can degrade polymers but also take advantage of oligomers produced by the pioneers (figure 14). Different degradation pathway composition is assumed to mitigate competitive exclusion and to reinforce interdependency/cooperation between populations.



**Figure 14 | Alginate degradation cascade of substrates varying in solubility and chain length.** *Vibrios* diversified into different populations characterized by their ability to consume insoluble alginate polysaccharide and soluble alginate oligosaccharides of different chain lengths. Pioneers are specialized in consumption of native, insoluble alginate due to their endowment with broadcast alginate lyases. These enzymes can diffuse freely into the alginate gel and depolymerize the alginate into soluble oligosaccharides. Harvester populations with secreted but tethered alginate lyases can exploit the range of soluble alginate substrates including medium and small oligosaccharides liberated by pioneers. Scavenger populations devoid of any alginate lyases can only use the smallest alginate oligosaccharides. Adapted from Hehemann et al., 2016.

Overall, ecological populations are defined as genetic units representing cohesive ecology, gene flow and social attributes. As such, these populations fit species concepts that are typically used for plants and animals. However, we note that they are at a finer evolutionary divergence than traditionally used to separate species in bacterial taxonomy. Furthermore, the ecological population paradigm allows us to go beyond the classical methods to study microbial evolution and ecology i.e. based on the snapshot of the species at a specific location.

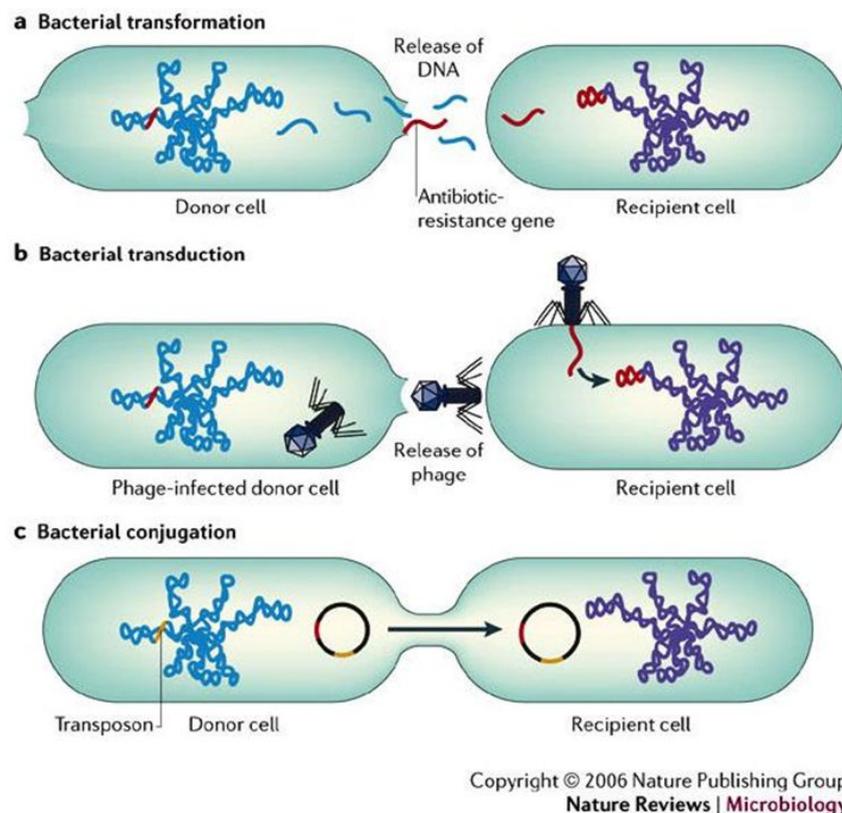
## IV Genome plasticity as a major driver of *Vibrio* evolution

As described above, vibrios are ecologically very diverse whether it concerns abiotic factor tolerance, feeding strategies, niche colonization or interactions with their hosts. This phenotypic diversity results from a **high adaptive potential** that derives from a **high genetic diversity** in part due to horizontal gene transfer (HGT).

### IV.A Mechanisms of HGT

HGT is a major driver in genome evolution of vibrios (Hazen et al., 2010). It is a process by which foreign genetic material is acquired in the genome of a strain. It is opposed to the vertical transmission where genetic information is transferred from the parent to the offspring. HGT contribute to important variation in gene content and genome size (Thompson et al., 2005). Within the vibrios, genome size ranges from 3.9Mb (*V. ordalii*) to 6.4 Mb (*V.nigripulchritudo*) (Okada et al., 2005), and genome size had been positively correlated with functional and ecological complexity (Konstantinidis and Tiedje, 2004; Cordero and Hogeweg, 2009).

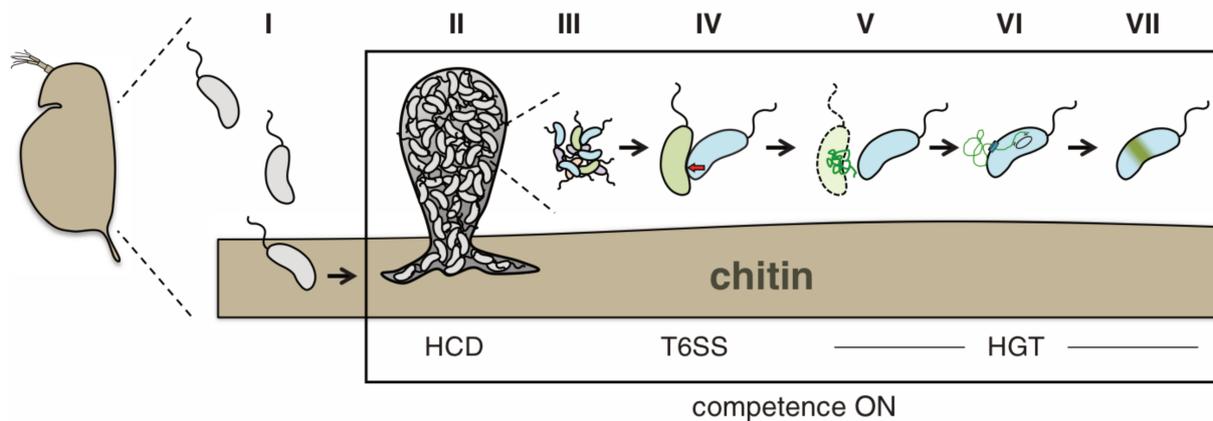
HGT outcome can be positive for the recipient cell by conferring a selective advantage, allowing fast adaptation to new environments or to stressful conditions (Groisman and Ochman, 1996). For example, HGTs are major drivers of antibiotic resistance spreading (Barlow, 2009) and there are numerous studies of HGT involved in virulence (Hazen et al., 2010) and adaptation to a new niche (Wiedenbeck and Cohan, 2011). There are three well described mechanisms of HGT and that occur in vibrios (figure 15).



**Figure 15 | Schematic of the three processes of horizontal gene transfer.** From Furuya and Lowy, 2006.

**Conjugation** corresponds to the transfer of genetic material from a donor to a recipient bacterium and requires a cell-to-cell contact. Conjugation is mediated by a type IV secretion system, a relaxase and a type IV coupling protein (T4CP), which constitute a large macromolecular complex involved in DNA transport (Cabezón et al., 2015) (figure Y). **Transduction** is the mechanism by which exogenous DNA is introduced into a recipient cell by a bacteriophage and further integrated to bacterial genome. Finally, **natural competence for transformation** is the ability of a recipient cell to uptake free DNA from its environment followed by incorporation into the recipient cell genome. The competent state is transient and Meibom et al. showed in 2005 that *V. cholerae* competence is induced by chitin (Meibom et al., 2005). After binding to chitinous surface, *V. cholerae* produces the transformation regulator TfoX, whose mechanism still remains unknown. TfoX is assumed to regulate genes involved in biosynthesis and structural components of the DNA-uptake machinery (Metzger and Blokesch, 2016). This DNA-uptake machinery is composed of a type IV pilus structure (Seitz and Blokesch, 2013; Borgeaud et al., 2015) (figure 16). Another protein apparatus, a type VI secretion system (T6SS), favors DNA release and confers an opportunity to acquire foreign

DNA, thus promoting bacterial evolution and adaptation. The T6SS is a syringe-like protein apparatus that inflicts damages to eukaryotic or prokaryotic cells by delivering effector molecules directly in target cell cytoplasm (Ho et al., 2014). On chitin, expression of the T6SS of *V. cholerae* is induced and provoke neighboring cell killing. Released DNA from prey cells constitutes the genetic material that is internalized thanks to the pilus structure (Borgeaud et al., 2015).



**Figure 16 | Chitin fosters the DNA uptake machinery and the T6SS expression.** In its natural environment, *V. cholerae* often colonizes the exoskeleton molts of zooplankton. Upon initial attachment (I), the bacteria form a three-dimensional biofilm (II) on the chitinous surface, thereby reaching high cell density (HCD). Within these biofilms, different *V. cholerae* strains carrying diverse and noncompatible effector-immunity modules (shown by the different colors of bacteria) most likely form mixed communities (III). Under such chitin-dependent and HCD conditions, *V. cholerae* induces its natural competence program. The T6SS (indicated by the red arrow) is part of the competence regulon and is induced upon the growth of *V. cholerae* on chitin substrates. The T6SS is active against nonimmune neighboring cells (IV), leading to their lysis (V). The DNA released by the lysed cells can be taken up by a competent predator cell (VI), leading to its natural transformation and evolution in the case that new DNA material is incorporated (VII). From Borgeaud et al., 2015.

#### IV.B Mobile genetic elements (MGE)

Mobile genetic elements are segments of DNA that encode proteins that mediate the movement of DNA within and between bacterial chromosomes or genomes (depending on their integrated or episomic status). They have been called “agents of open source evolution” (Frost et al., 2005) and partially exploit their host machinery to persist or to move from one cell to another one.

**Phage-mediated DNA** transfer occurs after a lysogenic infection. DNA brought by the phage will be injected in the bacterial cytoplasm, it can remain in an episomic form or be incorporated by recombination in specific regions called attachment sites (*attP*) in the recipient cell chromosome. Moreover, if bacterial DNA is transferred by the phage, integration by homologous recombination can occur. When integrated, phage DNA is replicated together with the host chromosome. Occasionally, often induced by stressful conditions, the prophage excise from the host chromosome, eventually bringing bacterial genes, and enter the lytic cycle (and further infect new hosts). Phage DNA and protein synthesis are assembled in the host before its lysis, releasing phages in the environment. The best described phage-mediated DNA among the vibrios is the CTX phage in *V. cholerae*. The CTX phage encodes the cholera toxin, one of the main virulence factors of the etiological agent of the cholera (Kim et al., 2014). The CTX phage requires the toxin co-regulated pilus, itself encoded by a MGE, for attachment to *V. cholerae*. It further exploits host tyrosine recombinases XerC and XerD to integrate its single-stranded DNA at the specific site *dif1/dif2* into its host chromosome (Waldor and Mekalanos, 1996; Das, 2014).

**Transposon** or transposable element is a DNA sequence (400bp to 40kb) that can jump from one position to another one in the genome of a strain by a “cut and paste” or a “copy and paste” mechanism. It is composed of an inverted repeat sequence at each end, a transposase that catalyses the transposon excision and cuts the target insertion site and eventually other genes (Hochhut et al., 2001). Transposable element can also jump on plasmids and then be transferred, with potential neighboring genes, by HGT. It can also be transferred via transformation. **Integrative and conjugative elements (ICEs)** were previously called conjugative transposons. As for transposons, ICEs are integrated in the host chromosome and are passively propagated during cell division in “normal” environmental condition and contain genes needed for their integration and excision. Contrary to transposons, ICEs encode a type IV secretion system that mediates their own transfer to the recipient cells (Burrus et al., 2002). When facing a stressful situation, genes involved in excision and conjugation are expressed, the ICE excises from the host chromosome and is transferred via conjugation to the recipient cell. Their size ranges from 20 to 500kb (Johnson and Grossman, 2015). The STX<sup>ET</sup> element from *V. cholerae*, previously named “constin” for its properties of conjugation, self-transmission and integration is an ICE that encodes resistance genes to the following antibiotics: sulfamethoxazole, trimethoprim, chloramphenicol and streptomycin. (Hochhut et al., 2001). Due to their self-transmissibility and

self-excision from host chromosome, ICE are major drivers of generalized antibiotic resistance in bacteria (Burrus et al., 2006).

**Plasmids** are extrachromosomal genetic elements that replicate autonomously from the host chromosome as they harbor their own origin of replication. They are classified as conjugative plasmid if they harbor their own functional conjugation machinery (T4SS). Plasmids that do not carry a T4SS exploit the ones from other conjugative elements and are named mobilizable plasmids but they still possess their own relaxases and T4CP. Plasmids that are neither conjugative, nor mobilizable (~ half of the plasmids) are thought to be transferred by transduction or natural transformation (Smillie et al., 2010). Plasmid conjugation requires a relaxase, a key protein encoded by both conjugative and mobilizable plasmids. The relaxase binds the origin of transfer of the plasmid and catalyzes the initial cleavage of the double-stranded structure to produce the DNA strand that will be transferred to the recipient cell. T4CP connects the relaxosome (DNA bound to the relaxase) to the transport channel formed by the T4SS (Llosa et al., 2003). The relaxosome is transferred through the recipient cell and the relaxase is involved in ligation of the single strand in the recipient cell at the termination of conjugation (de la Cruz et al., 2010) (figure X). Double-stranded structure is restored thanks to a rolling-circle replication in both recipient and donor cells (Ruiz-Masó et al., 2015). Plasmid size ranges from 2kb to 1Mb, but the biggest plasmid described to date within the *Vibrionaceae* has been identified in the strain FORC\_036 from *Vibrio vulnificus* with a size of 925kb (NCBI database).

#### IV.C *Vibrios* possess a multipartite genome shaped by HGT

The *Vibrio* genome is divided in two chromosomes (diCenzo and Finan, 2017). The bigger chromosome (ChrI) has a size ranging from 2.8 to 4.1 Mb (NCBI database) and contains most of the essential genes (Reen et al., 2006; Le Roux et al., 2009). The secondary chromosome (ChrII), the smaller one, is assumed to derive from domestication of a large plasmid (Heidelberg et al., 2000) and its size ranges from 0.9 to 2.4 Mb (NCBI database). It seems that there is a correlation between ChrI and ChrII sizes: strains having a relatively big ChrI tend to have a bigger ChrII as well (Okada et al., 2005). ChrII contains some housekeeping genes that assure its maintenance but mainly contains genes related to adaptation to specific niches or stressful conditions (Cooper et al., 2010). Replication termination of the two

chromosomes is synchronous and constitutes a strong constraint at the molecular level (Val et al., 2016). It has been shown that genes evolve faster on the smaller chromosome (Cooper et al., 2010). However, it implies a trade-off between adaptation/gain of new functions by HGT and survival enhanced by a short generation-time.

Several transcriptomic analyses revealed that ChrII genes are relatively more expressed in stressful/hostile growth conditions compared to *in vitro*/optimal growth conditions (Xu et al., 2003; Toffano-Nioche et al., 2012; Vanhove et al., 2016) For example, in *V. cholerae*, the comparison of gene expression in *in vitro* and *in vivo* (rabbit intestine) growth showed that many more genes from the ChrII were expressed in *in vivo* conditions. Indeed, intestine constitute a harsh environment to grow in, it is stressful for the cell that need to express genes to adapt and to survive in this hostile environment. Those “adaptation” genes are encoded by the secondary chromosome (Xu et al., 2003). *Vibrio tasmaniensis* is an oyster pathogen that is able to survive into hemocytes (Duperthuy et al., 2011). This particular lifestyle involves the ability to resist to the hostile environment that constitutes the hemocyte. Notably, copper efflux is a function highly induced intracellularly and is a major vibrio adaptive trait for oyster tissue colonization and virulence. Most of the genes involved in copper efflux and that are highly overexpressed in hemocytes are encoded in ChrII of *V. tasmaniensis* (Vanhove et al., 2016).

#### IV.D The superintegron plays a major role in evolution of the *Vibrio*.

**Chromosomal superintegrons (SI)** play an important role in bacterial evolution. They are massive gene-capturing platforms that incorporate exogenous genetic material by site-specific recombination and ensures exogenous DNA correct expression. SI possess a gene encoding an integrase, a primary recombination site and a promoter that directs transcription of the captured genes (Mazel, 2006). SI are at the margins of MGE as they do not encode proteins involved in their own excision and cannot be transferred from one site to another one in the chromosome neither they can move from one cell to another one. Genes integrated into SI are acquired thanks to transposable elements and conjugative plasmids present in the host cell. Within the *Vibrionaceae*, SI contain a large number of gene cassettes, from 72 in *V. parahaemolyticus* to more than 200 in *V. vulnificus*. *V. cholerae* SI represent ~3% of its genome size (Rowe-Magnus et al., 2003) and 10% of its ChrII. SI are powerful agents of evolution as they possess genes coding a vast variety of functions. They confer a rapid adaptation potential

in changing environments (Escudero et al., 2015) and notably encode virulence factors within *V. cholerae* and *V. vulnificus*.

## V Emergence of pathogens from environmental vibrios

Numerous bacterial pathogens have emerged from environmental populations following acquisition of genes through horizontal transfer that are involved in host colonization, intra-host survival or host-tissue damaging, all of these factors being critical for a successful infection (van Baarlen et al., 2007). Some vibrios are pathogenic and infect human and a multitude of marine wild and farmed animals. The following section details the evolutionary history leading to pathogen emergence in some *Vibrio* species.

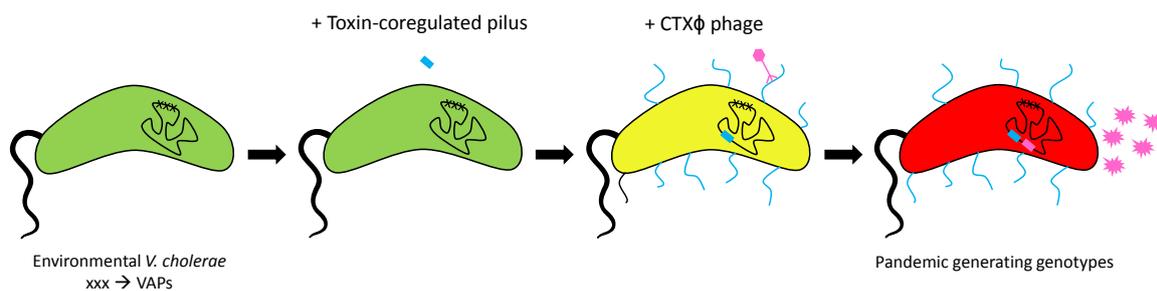
### V.A *Vibrio cholerae*

One of the best described examples of pathogen emergence from environmental strains is *Vibrio cholerae*, the etiological agent of the cholera. In the environment, *V. cholerae* is a diverse species, containing numerous genotypes inhabiting aquatic environment. Among them, only a subset is able to cause the severe diarrheal disease cholera and constitutes a clonal group named “pandemic generating” (PG) genotypes. Those PG genotypes evolved from environmental genotypes to become highly pathogenic (Islam et al., 2017).

All *V. cholerae* genotypes are predisposed to survive in the human body as they can live in low-salinity environment, grow well at 37°C and have the abilities to form biofilm and resist acidic passage in the stomach (Islam et al., 2017). Then, a study exploring the evolutionary origins of the PG group showed that these PG genotypes possess a particular genomic background containing alleles, called virulence adaptive polymorphisms (VAPs) that serve as “preadaptations” to give rise to pandemic disease. However, some environmental strains also possess some PG-like alleles, but only strains possessing an optimal combination of alleles can take advantage of the major virulence factors further acquired by HGT and emerge as PG genotypes (Shapiro et al., 2016).

Environmental strains possessing the “preadaptation” alleles successively acquired three genomic islands, leading to clonal expansion of the pandemic lineage (figure 17). First, the *Vibrio* Pathogenicity Island 1 (VPI1) encodes a toxin-coregulated pilus (TCP) and constitutes an essential colonization factor. In a second step, the CTXΦ phage had been acquired (Islam et al., 2017) and carries the second major virulence factor, the cholera toxin

(CT). The TCP is the receptor for the CTX $\Phi$  phage (figure 17). Finally, the *Vibrio* Pathogenicity Island 2 (VPI2) encodes several genes, including genes involved in transport and catabolism of sialic acid. The ability to utilize sialic acid confers a competitive advantage of PG lineage against other bacteria in the mouse gut (Almagro-Moreno and Taylor, 2013, Almagro-Moreno and Boyd, 2009). PG genotypes associated with the current 7<sup>th</sup> pandemic further acquired by HGT two *Vibrio* Seventh Pandemic Islands, VSP1 and VSP2. VSP1 plays a role in intestine colonization while VSP2 is still poorly described (Dziejman et al., 2002).



**Figure 17 | Schematic representation of emergence and evolution of pandemic *V. cholerae*.** Environmental *V. cholerae* strains possessing the right combination of VAPs acquired the toxin coregulated pilus and the cholera toxin via HGT. These HGT events lead to the emergence of the clonal pandemic group. Adapted from Islam et al., 2017.

## V.B *Vibrio parahaemolyticus*

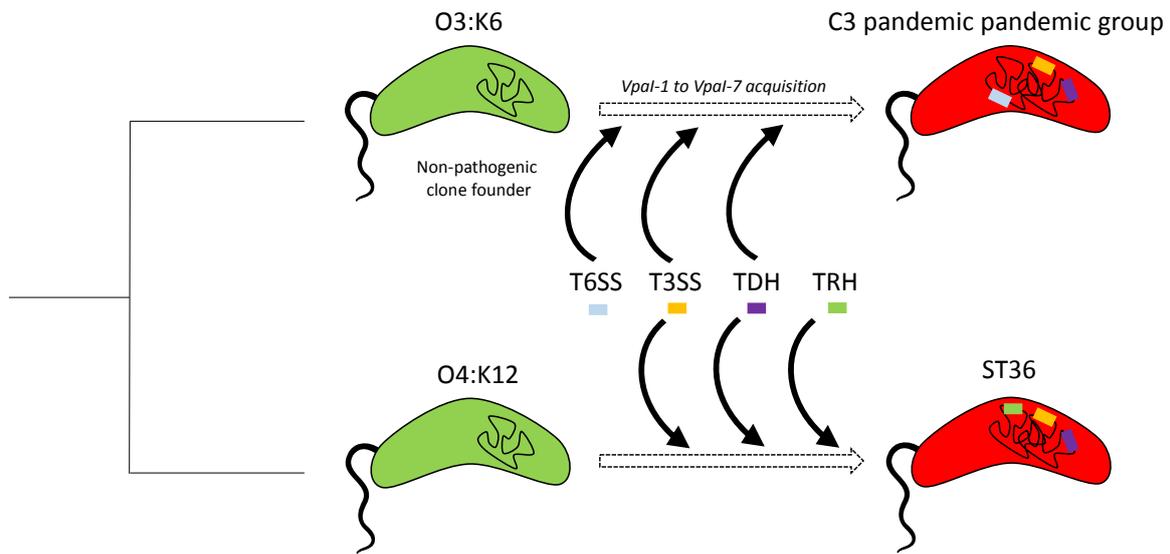
*Vibrio parahaemolyticus* is responsible for a foodborne gastroenteritis in human following raw or undercooked contaminated fish or seafood consumption. As for *V. cholerae*, *V. parahaemolyticus* is genetically and serotypically diverse and only a few genotypes can cause severe diarrhea in human. In the past, outbreaks were geographically isolated and associated with diverse serotypes (Chowdhury et al., 2004). However, over the last 2 decades, epidemiological changes induced transition from locally restricted strain dominance to emergence and transcontinental expansion of two strains that are genetically and biochemically distinct. The first *V. parahaemolyticus*-associated pandemic occurred in India and was attributed to **the clonal group C3** that further spread worldwide (Nair et al., 2007; Okuda et al., 1997, Daniels et al., 2000; González-Escalona et al., 2005; Martínez-Urtaza et al., 2005). The second expansion was first restricted to the Pacific Northwest (PNW) area and attributed to the **sequence type 36 (ST36)** (Turner et al., 2013). In 2012, this group was associated with

illnesses reported in the Northeast coast of the USA (Martinez-Urtaza et al., 2013) and in the Northwest of Spain (Martinez-Urtaza et al., 2016).

Comparative genomics of pre-pandemic and pandemic isolates showed that C3 derived from a non-pathogenic-clone-founder of serotype **O3:K6**. This founder strain acquired a T6SS and at least seven genomic islands on its secondary chromosome (VPaI-1 to 7) by horizontal transfer (Espejo et al., 2017; Makino et al., 2003). One of the two sets of type III secretion system gene clusters presents in *V. parahaemolyticus* was encoded by VPaI-7 (T3SS2) and was demonstrated to play a role in virulence (Piñeyro et al., 2010, Zhou et al., 2013). VPaI-7 also possesses two copies of the *tdh* gene, that is specific to pathogenic strains, and encodes a thermostable direct hemolysin that plays a role in invasiveness of the bacterium in humans (Letchumanan et al., 2014). C3 strains evolve rapidly and the clonal pandemic group contains to date 27 pandemic serovariants.

The PNW complex derives from an ancestral strain of serotype **O4:K12**. Clinical isolates belonging to the PNW complex possess the *tdh* gene and, in addition, a gene encoding the TDH-related hemolysin (*trh*), that is not detected in the C3 pandemic clone. Strains from the PNW complex also possess 2 T3SSs (Paranjpye et al., 2012).

To sum up, *V. parahaemolyticus* outbreaks are caused by worldwide clonal expansion of strains that acquired specific sets of genes (figure 18). These genes give them a pathogenic potential and differentiate them from environmental strains.



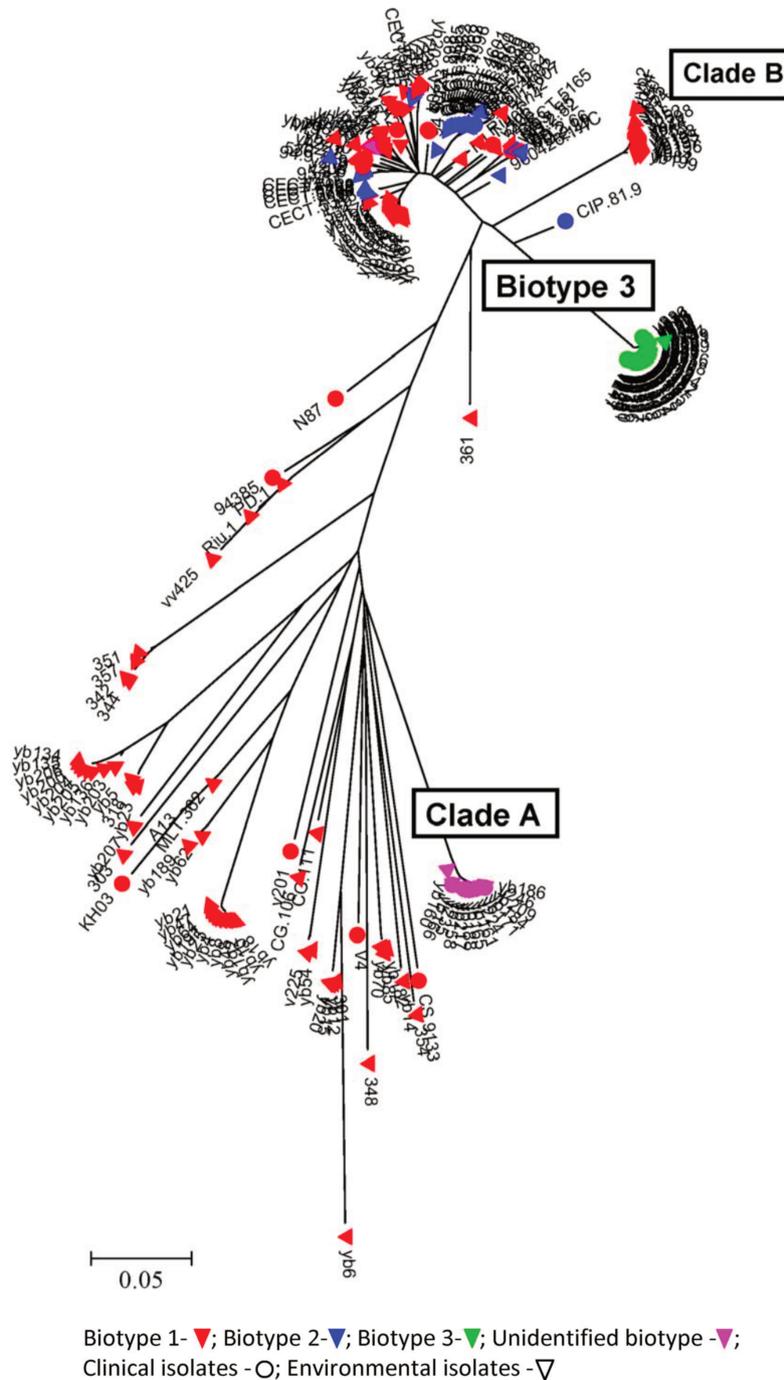
**Figure 18 | Schematic representation of the plausible origin of the two pathogenic groups in *V. parahaemolyticus*.** Based on articles cited in section V.B and focused on main virulence factors acquisition.

### V.C *Vibrio vulnificus*

*V. vulnificus* causes invasive septicemia, skin necrosis or gastroenteritis in human. It is the most virulent foodborne opportunistic pathogen in human (Oliver, 2005, Baker-Austin and Oliver, 2018) and it also infects fish. *V. vulnificus* is commonly found in temperate to warm estuarine and coastal environments. Contrary to the pathogens *V.cholerae* and *V. parahaemolyticus*, pathogenic strains from *V.vulnificus* show high phenotypic and genotypic heterogeneity. Many attempts in identifying virulence markers to distinguish pathogenic from non-pathogenic *V. vulnificus* strains had been made, but candidate genes for virulence were not specific to virulent genotypes (Phillips and Satchell, 2017).

*V. vulnificus* has thus been classified based on biotypes of the strains and it has been divided into three groups: **biotype 1** contains diverse genotypes and is involved in human infection worldwide, **biotype 2** contains diverse genotypes as well but it mostly infects teleost fish (e.g eels). **Biotype 3** is the only clonal biotype and it is responsible for wound infection in human associated with tilapia aquaculture, and has only been reported in Israel (Koton et al.,

2015, Oliver, 2015). Genotypic classification divided *V. vulnificus* biotype 1 into two clusters, one dominated by environmental strains constituting the **E genotypes**, and the second one dominated by human clinical isolates regrouping the **C genotypes**. Genetic dimorphisms between E and C genotypes occur in genes coding ecological preferences (Gonzalez-Escalona et al., 2007; Rosche et al., 2005; Morrison et al., 2012). However, further analyses revealed that the genotypic profiles were not sufficient predictors of virulence (Kim and Cho, 2015). To better understand the evolution of this pathogen, genome-wide SNP genotyping on 254 isolates was performed. *V. vulnificus* was divided into three phylogenetic lineages (LI to LIII). Biotype I strains were split into LI and LIII, biotype 2 was present in LI and biotype 3 strains form a separate, clonal cluster (LII) (Raz et al., 2014, figure 19). Biotype 1 is the most ancient and diverse biogroup. Biotype 3 recently emerged from two distinct biotype 1 populations. It acquired several genes by HGT in its specific environment that induced a biotype change (Raz et al., 2014). Altogether, the difficulty to highlight genomic features to distinguish pathogenic strains is suggested to be due to high rate HGT of chromosomal segments between *V. vulnificus* strains that lead to continuous evolution of this pathogen (Raz et al., 2014).



**Figure 19 | Phylogenetic relationships among 254 *V. vulnificus* isolates based on variation data at 570 SNP loci.** The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances were computed using the Kimura two-parameter method with bootstrap analysis of 1000 replicates. From Raz et al., 2014.

Despite difficulties in identifying genomic features specific to virulence in *V. vulnificus*, the ability for biotype 2 strains to infect eels relies on the virulence plasmid pVvBt2 (Lee et al., 2008). It contains a MARTX toxin involved in resistance to phagocytosis (Lee et al., 2015) and

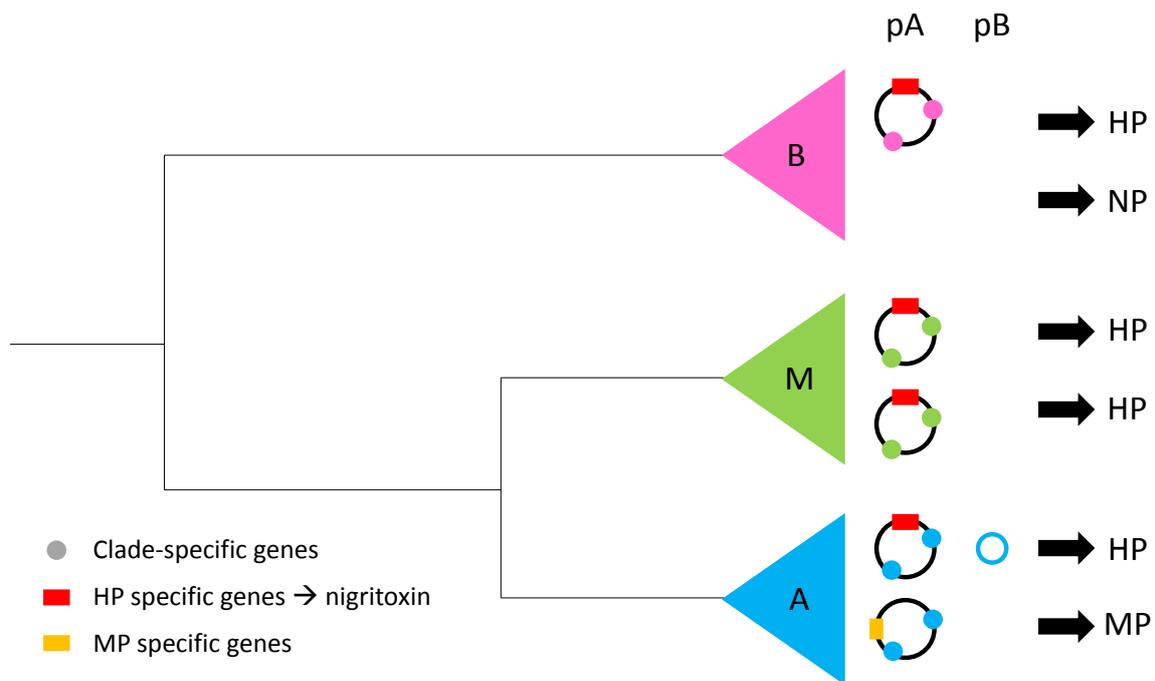
an iron-repressed outer membrane protein Vep20 involved in iron acquisition via eel transferrin binding (Pajuelo et al., 2015) that allows pathogen survival in fish blood and tissue. Biotype 2 is mostly pathogenic for eel due to the specificity of Vep20 that binds more efficiently eel transferrin than other fish transferrin.

#### V.D Plasmid acquisition can lead to pathogen emergence

As for *V. vulnificus* biotype 2, several pathogens emerged following plasmid acquisition. This is notably the case of the fish pathogen, *Photobacterium damsela* *subsp. damsela* (*Vibrionaceae*), whose virulence relies on the plasmid pPHDD1. It encodes two hemolysins named damselysin Dly and phobalysin P PhlyP. In addition, all hemolytic strains possess a copy of the gene encoding a phobalysin C PhlyC on their chromosome. Phylogenetic analyses revealed that PhlyC and PhlyP originated from gene duplication of Phly that was acquired by HGT. All three hemolysins are secreted by a type II secretion system and constitute the major virulence factors of *P. damsela subsp. damsela*. There is an additive effect between PhlyC and PhlyP in virulence and a synergistic effect between Dly and either PhlyP or PhlyC. Those hemolysins induce severe hemorrhages to fish but the synergy between Dly and PhlyP or C is important for full virulence. Remarkably, whether they are carried by MGE or on the chromosome, those hemolysins are regulated by a unique regulator RstB encoded on the secondary chromosome of the bacterium (Rivas et al., 2011; 2013; 2014; 2015; Terceti et al., 2017).

*V. nigripulchritudo* is a shrimp pathogen that has been associated with various diseases. In New Caledonia, it has been isolated from shrimp suffering a winter disease, named “Syndrome 93” (S93) in “cold” tropical water (<25°C) and was also isolated from moribund shrimp suffering a “Summer Syndrome” (SS). Mortalities induced by *V. nigripulchritudo* were also reported in Japan and Madagascar (Sakai et al., 2007 and E.Chung pers. com). Strains isolated from SS and S93 grouped into two distinct clusters, clade A and B respectively and a third lineage comprises strains isolated from moribund shrimp in Madagascar (clade M). Those three clades show very low intra-clade diversity and contain diverse pathotypes: highly pathogenic strains (HP), moderately virulent (MP) and non-virulent (NP) (figure 20). A plasmid (pA) (~250kb) was detected in virulent and non-virulent strains from all clades suggesting it is extensively circulating among *V. nigripulchritudo* and confers a selective advantage to its host.

pA shows a modular structure, and one module, specific to HP strains (Goudenège et al., 2013), encodes a new toxin, named nigrifoxin, that is able to target Pancrustacea-specific pathways (Labreuche et al., 2017). Another plasmid, pB (11.2kb), was found to be specific to HP strains from clade A and both pA and pB were necessary for full virulence (Reynaud et al., 2008; Le Roux et al., 2011).



**Figure 20 | Schematic representation of plasmid acquisition and composition in *V. nigripulchritudo*.** pA possesses pathotype-specific genes and geographic-specific genes. pB is only found in HP strains from clade A. Adapted from Goudenège et al., 2013.

#### V.E A consortium of vibrios can contribute to pathogenesis

While septicemia in shrimp are associated with a clonal expansion of the pathogen *V. nigripulchritudo*, in other marine animals, diseased animals are associated with a diversity of strains or species, which could indicate that a consortium of vibrios is necessary to induce some diseases. This hypothesis has been proposed for some coral vibrioses. In another way than coral bleaching that is caused by the disruption of the symbiosis between the cnidarian host and their photosynthetic microalgal endosymbionts and has been correlated with increasing sea temperature (Brown, 1997; Hoegh-Guldberg, 1999), vibrioses affecting corals, sometimes inducing a bleaching phenomenon as well, result in irreversible necroses of the cnidarians. A

first study suggested that a consortium of 4 *Vibrio* species is responsible for the Yellow Band Disease in the Indo-Pacific and Caribbean reefs. *Vibrio rotiferianus*, *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio proteolyticus* induce symbiotic algae dysfunction that lead to coral death (Cervino et al., 2008). Another study showed that *V. coralliilyticus* and *V. mediterranei* were associated with diseased corals *Oculina patagonica* sampled in the Mediterranean Sea. Inoculation of these pathogens were able to induce tissue damage in experimental conditions from 24°C. Surprisingly, coral disease signs were observed at 20°C following the co-inoculation of the two pathogens, a temperature at which necrosis is not normally observed (Rubio-Portillo et al., 2014) and suggests a synergistic effect of these infectious agents in coral disease.

Several lines of evidence also suggest that juvenile oyster disease is enhanced by a consortium of bacteria. First, diseased oysters are systematically colonized by several ecological populations of *Vibrio* (Petton et al., 2015; Wendling et al., 2014; Gay et al., 2004a; Lemire et al., 2015). Experimental infections have demonstrated that some moderately virulent strains have a heightened virulence when co-injected with other strains (Gay et al., 2004b). In addition, reduction of the injected dose of a virulent strain by dilution in the culture media significantly reduced oyster mortality. In contrast, when the same virulent strain was injected at low dose but diluted in a non-virulent strain culture, mortality rates were markedly increased (Lemire et al., 2015). Thus, the presence of non-virulent bacteria may significantly increase the virulence of virulent genotypes at the onset of infection, suggesting an effect of bacterial density upon virulence. Hence, although non-virulent strains are not sufficient for pathogenesis, they may contribute to virulence by generating sufficiently high bacterial loads to either overcome host defenses or to induce expression of virulence factors via quorum sensing (Le Roux et al., 2016).

Overall, HGT largely shaped emergence of human and marine organism pathogens. Diseases caused by vibrios can either derive from infection by a single clone, by diverse genotypes from the same species or even by a consortium of vibrios, in which the different populations play a different/additive role in infection, that ultimately lead to host damage/death.

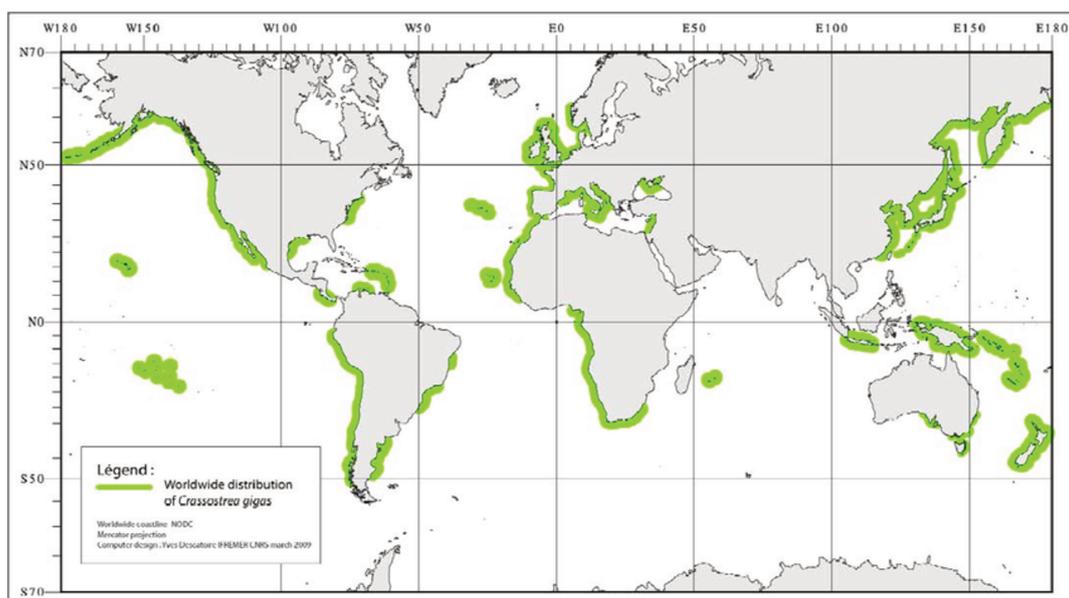
## V.E Vibrios and global warming

Change in environment such as global warming and anthropogenic activities have caused a worldwide increase in *Vibrio*-associated infectious diseases (Harvell et al., 2002). Environmental changes have been shown to affect marine organism physiological functioning, behavior and demographic traits (Doney et al., 2012). Those disruptions can be worsening according to the reproductive state and abiotic factors tolerance limits of organisms that subsequently lead to an increased sensitivity to opportunistic pathogens. In parallel of weakened host, vibrio abundance is positively correlated with sea surface temperature rising (Vezzulli et al., 2012). Rationally, if sea surface temperature goes in the direction of optimal growth temperature for pathogens or their reservoirs (such as copepods whose abundance has been correlated to cholera outbreaks in Bangladesh (Constantin de Magny et al., 2011)), occurrence of those vibrios is expected to increase. In a context of global warming and considering the “opportunitroph” status of vibrios due to their high colonization and adaptation potential, an increase in severity of vibriosis is likely to happen (Baker-Austin et al., 2010; 2013; 2016). However, in comparison to human pathogens, fairly little is known about the requirements for virulence in pathogenic vibrios of marine organisms.

## VI Vibrioses affecting oysters can be used as a model of infection

### VI.A Oyster-farming has been facing recurrent diseases in France

Aquaculture is the fastest-growing food industry worldwide and oyster-farming represents a major part of fish and shellfish farming. Indeed, its production is only outdistanced, in tons, by the production of herbivore carps (5.4 vs 6 million tons in 2016, FAO). France is the first European oyster producer, contributing at up to 90% to its production (FAO). Oyster farming industry had been shaped by successive infectious diseases in France. The endemic oyster *Ostrea edulis* endured severe mortality outbreaks in 1970 and 1980 respectively, caused by two parasites *Marteilia refringens* et *Bonamia ostrea* (His et al., 1976; Comps, 1980) that led to production cessation. The Portuguese oyster *Crassostrea angulata* had been introduced at the end of the XIXth century but an iridovirus, causing gills damage, decimated this species between 1969 and 1973. (Comps et al., 1976; Comps, 1988). Due to its rapid growth and its ability to adapt to various environmental conditions, the Pacific cupped oyster *Crassostrea gigas* had thus been introduced in France in the 1970s from British Columbia (Canada). Today, oyster-farming worldwide is mainly based on this species, which represents 98% of French and international production (figure 1) (FAO).



**Figure 21 | Worldwide distribution of the Pacific oyster *Crassostrea gigas*.** From Miossec et al., 2009

However, in France, *C. gigas* has been facing a series of diseases during the last twenty years, becoming a major threat for oyster-farming industry. Between 1998 and 2008, mortality events have been associated with a multifactorial disease, termed “summer mortality syndrome” (Samain, 2008). It was dependent on host genetic background and physiological state (stress and maturation), happened when seawater temperature reached a threshold of 19°C (Samain, 2008) and was associated with the presence of several pathogenic microorganisms: the ostreid Herpes virus OsHV-1 and vibrios mainly belonging to the Splendidus clade and *Vibrio aestuarianus* (Renault et al., 1995; Lacoste et al., 2001; Gay et al., 2004).

Since 2008, distribution and severity of this phenomenon have been increasing. It mainly occurs on juvenile oysters and arises when seawater temperature reaches 16°C and can result in losses of up to 100% of production in some farms (Samain, 2008) and all French coasts are affected by this disease. *C. gigas* mortality events have also been reported worldwide: in Northern America (Caceres-Martinez et al., 2018; Burge et al., 2006) and in South America (Mello et al., 2017), in Oceania (Go et al., 2017), in Southern (Domeneghetti et al., 2014) and in Northern Europe (Reise et al., 2017). These mortalities are associated with the detection of the Herpes virus OsHV1  $\mu$ var and bacteria from the genus *Vibrio* isolated from diseased oysters (Segarra et al., 2010; Schikorski et al., 2011; Martenot et al., 2013; Gay et al., 2004; Bruto et al., 2017). Since 2012, reported cases of adult oyster mortalities have strongly increased and are associated with clonal strains of the pathogen *V. aestuarianus* (Goudenège et al., 2015).

## VI.B Virulence mechanisms of juvenile oyster pathogens

Despite strong economic and ecological consequences, to date, knowledge on oyster infection by vibrios is limited and is mainly based on very few model strains and experimental infection studies in the laboratory with unknown relevance for disease in the wild.

Vibrios from the Splendidus clade have been isolated for years from diseased juvenile oysters (Waechter et al., 2002; Gay et al., 2004). Among them, the virulent strain LGP32 from the species *Vibrio tasmaniensis* became a model to study the oyster/*Vibrio* interaction. Mechanisms of virulence described so far are the followings. LGP32 is an intracellular pathogen of oyster hemocytes, which are cells involved in numerous physiological processes including host defense reactions (Duperthuy et al., 2011). Hemocytes invasion is mediated by the outer membrane protein OmpU that allows LGP32 to adhere and to invade hemocytes,

making LGP32 able to survive into phagosomes (Duperthuy et al., 2011). An ATPase CopA, involved in copper resistance, is overexpressed by LGP32 when it is in the hemocyte and plays a role in intracellular survival (Vanhove et al., 2016). OmpU plays a role in resistance to antimicrobial peptides involved in *C. gigas* immunity (Duperthuy et al., 2010), as well as outer-membrane vesicles (OMVs) produced by LGP32 (Vanhove et al., 2015). OMVs contain proteases, the major one being a vesicular serine protease (Vsp). Vsp deletion results in a reduced virulence in experimental infection of oysters. Finally, the Vsm metalloprotease has been demonstrated to be the major toxicity factor of LGP32 extracellular products (Le Roux et al., 2007, Binesse et al., 2008).

In another population isolated from haemolymph of diseased oyster (Faury et al., 2004), *V. crassostreae*, the virulence seems to rely on distinct mechanisms since its cytotoxicity is not dependent on hemocytes invasion (T. Rubio pers. com). Contrary to LGP32, at the beginning of my PhD project, few virulence factors had been evidenced in this population. Only a gene encoding a putative outer membrane protein of unknown function (R5.7) has been demonstrated to play a role in oyster infection (Lemire et al., 2015).

In a situation of quasi-monoculture of *C. gigas* and with the increase of mortality events reported worldwide, the sustainability of oyster-farming relies on management strategies of the disease that could arise from understanding the fine mechanisms of this infectious disease.

### VI.C *Crassostreae gigas* as a model to study infection in the wild

In addition to being a species of prime interest for aquaculture, the relation *C. gigas*/vibrios has been proposed as a model to explore disease dynamics in the wild and can be exploited to understand the complex ecological and evolutionary dynamics of infectious agents. First, most of the vibrios are easily cultured and are able to grow on the selective medium Thiosulfate-citrate-bile salt-sucrose agar (TCBS), which facilitates their isolation. Then, delineation of vibrios in ecological populations (Hunt et al., 2008) and the possibility to genetically manipulate several *Vibrio* strains (Le Roux et al., 2016) allow to investigate microbe evolution at both gene and genome level and to determine the consequence of this evolution on microbe ecology and host damages.

The recent development of Specific-Pathogen-Free (SPF) oyster (Petton et al., 2013) constitutes an important step forward towards investigating natural infection mechanisms. SPF oysters are descendants of a pool of genitors that are produced in hatcheries under highly controlled conditions to minimize the influence of genetic and environmental parameters that could affect the host sensitivity to the disease (Petton et al., 2013;2015a;2015b). SPF oysters are not axenic, but they contain a low dose of *Vibrio* and the herpes virus is not detected, both infectious agents being associated with diseased oysters. Microbiome composition in SPF oyster remains unknown but it is not sufficient to induce a disease. SPF oysters can be placed in the environment where they can be naturally infected. Onset of mortality and cumulative mortality rates can be monitored under natural conditions. Once infected, alive animals can be reintroduced in laboratory to explore the diversity and dynamics of microbes during disease progression. Moreover, these SPF oysters can be used for high-throughput experimental infections with hundreds of bacterial isolates (Goudenège 2015, Lemire 2014; Petton 2015). Those elements offer a unique opportunity to study the evolutionary adaptation of these bacteria to oyster environment, and pathogen emergence.

#### VI.D Objectives

During this PhD project, we took advantage of the infection model *C. gigas* to fill the lack of basic knowledge about vibrios in the environment. Investigating pathogen evolutionary history and exploring their virulence mechanisms is an essential step to understand and further manage environmental vibrio-associated diseases. In this PhD project, we addressed two main questions: i) How widespread is pathogenic potential of vibrios in the wild? ii) Does oyster farming select for emergent pathogen(s)? To address these questions, we combined experimental ecology, a high throughput infection assay, genome sequencing and inverse genetics. We found that a number of environmental populations are virulent toward oysters thanks to virulence mechanisms likely acquired during ecological differentiation. We observed that diseased juvenile oysters are always colonized by diverse vibrios and proposed a scenario on the role of vibrios in mortality events. *V. crassostreae* was found to be abundant in diseased oyster. Its virulence is dependent on a type VI secretion system carried by a plasmid. This plasmid is only detected in high-density oyster farming areas, suggesting that intensive aquaculture settings have selected higher virulent genotypes. We further showed that plasmid

transfer and/or selection is enhanced in oyster compared to *in vitro* conditions. This result suggests that oysters represent a niche for HGT and emergence of pathogens.

## Chapter I Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations

*Vibrios* have been associated with mortality events of numerous wild and farmed marine organisms. The number of cases of these diseases are rising worldwide and are occurring coincidentally with global changes. To better understand and manage these diseases, the ecology and the evolution of these infectious agents need to be explored. The first question we wanted to address is how widespread potential pathogens are in the wild. **Are they already present in the environment or do they emerge following environmental changes, such as those induced by farming at high density and/or animal transfers?**

Previous studies conducted on these pathogenic agents have relied on samplings that are often performed on diseased animals. This presents a major bias resulting from pathogens expansion in diseased animals. To address this issue, we used a collection of strains isolated from Plum Island (MA, USA), an area with no high-density oyster farming. This collection contains representatives of fifteen ecological populations that had been previously well described in *Vibrio* population structure analyses. To assess the virulence potential of these strains, we used specific pathogen free oysters as a model for experimental infection that allows rapid, high throughput and replicable screening of virulent strains. Mapping virulence potential on the population structure allows us to explore the functional unit of pathogenesis, i.e. a clone that recently emerged after the acquisition of virulence via HGT or a population within which strains ancestrally acquired and maintain virulence factors that are selected in the niche.

Injection of individual strains into oysters showed that some populations only contain non-virulent strains (9/15), while the remaining ones contain virulent strains (6/15) (figure 1). As most of the populations containing virulent strains belong to the Splendidus clade (5/6), we conducted further tests to distinguish whether the ability to kill oysters is an ancestral trait for the entire clade.

Within the Splendidus clade, two closely related populations showed contrasting virulence potential: the first one only contains virulent strains (thereafter named virulent population) while the other only contains non-virulent strains (non-virulent population). Comparative genomics allowed us to identify 24 genomic regions that are specific to the virulent population. Each of these 24 regions were genetically deleted in order to explore their role in virulence; we found that only one locus, Rg, is necessary for full virulence.

This locus encodes two exported proteins of unknown function named R5-7 and R5-8. Deletion of the R5-7 gene results in reduced virulence when injected in to oyster. Full virulence restoration was obtained after co-injection of the mutant with the purified R5-7 protein, suggesting that this protein interacts with the external surface of the bacterium or the host target cells. However, this protein was not sufficient to induce mortality when injected alone or co-injected with a non-virulent strain, suggesting that R5-7 alone does not have a lethal effect. R5-8 gene deletion did not impair virulence. Overall, our results suggest that only R5-7 is important for Rg virulence.

The R5-7/8 cluster is found exclusively in the genome of strains that belong to the Splendidus clade. An ancestral character state reconstruction suggests that this cluster has been acquired by the most recent common ancestor (MRCAs) of the Splendidus clade and has then been lost by some populations. The presence/absence pattern of R5-7/8 correlates with population virulence potential. This cluster is, in most cases, transferred vertically to the offspring. Its high frequency in virulent populations suggests that it is maintained by positive selection. Finally, ancestral acquisition, high frequency or loss at the level of population and vertical transmission strongly suggest that this cluster plays a role in adaptation to the niche and that it has therefore not been selected as a virulence factor.

Within *V. splendidus* and *Vibrio cyclitrophicus* populations, few non-virulent strains harbor this gene cluster, suggesting that it is necessary but not sufficient for virulence and therefore other genes must play a role in virulence. By comparative genomics within *V. splendidus* strains, we identified a cluster encoding a multifunctional-autoprocessing-repeats-in-toxin (MARTX) toxin and its transporter whose presence was strongly correlated with high virulence potential. Deletion of this cluster impaired virulence towards oysters. This is the first demonstration of the involvement of a MARTX toxin in virulence towards marine invertebrates. MARTX toxins are composed of a core structure containing conserved N- and C- terminal regions and a cysteine protease domain, and heterogeneous composition of effector domains. Only one effector domain, the Ras/Rap1-specific endopeptidase (RRSP), is conserved among all *V. splendidus* MARTX toxins from our strain collection; suggesting an essential role in the MARTX toxin activity. Ras and Rap1 proteins are involved in intracellular signaling and membrane trafficking, including the Toll-like receptor 4 trafficking implicated in animal innate immunity. Hence, RRSP domain may be important in the MARTX activity by affecting host immunity. The variability in effector domain composition suggests that the different variants play distinct roles in the niche, which has previously been proposed for *V. vulnificus*.

In the Splendidus clade, MARTX toxin is only found in *V. splendidus*, and in 24/35 strains. However, it is present in other pathogenic species at variable frequencies; suggesting different selective pressure is ongoing within this cluster. The MARTX toxin is a large secreted protein, its production is thus very costly for the cell. Within *V. splendidus*, the intermediate frequency, the large size and the diversity of the effector domain composition suggest that the MARTX toxin is under frequency-dependent selection, therefore genotypes carrying this cluster are only favored when they represent a small proportion of the population.

In this study, we show that virulence towards juvenile oysters is an ancestral trait in the Splendidus clade. Virulence potential is a phenotype shared by the whole population and is thus not related to clonal emergence. R5-7 is necessary but not sufficient for virulence, additional genes may be involved in virulence. In addition to R5-7, we identified a MARTX toxin that plays a role in the virulence of *V. splendidus*.

More broadly, we showed that several environmental *Vibrio* populations were virulent, independent of intensive aquaculture settings. As virulence is a core genome function for those virulent populations, specific markers, like the R5-7/8 gene cluster, can be used for risk assessment. To manage oyster disease, an important step will be to explore the specific ecological conditions that occur as virulent populations increase in the environment. As virulent populations possess population-specific virulence genes, co-occurrence of such populations could result in cooperation or even synergistic interactions. We already have substantial evidence to suggest that intermediate virulence of a strain can be increased by mixing it with another strain. The hypothesis of a polymicrobial disease inducing juvenile oyster mortalities needs to be further explored.

This work is detailed in the article entitled “**Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations**” (accepted in the ISME Journal in July 2018). [In this study, I performed the high throughput experimental infection assays, mutagenesis and statistical analyses.](#)



# Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations

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## Abstract

Diseases of marine animals caused by bacteria of the genus *Vibrio* are on the rise worldwide. Understanding the eco-evolutionary dynamics of these infectious agents is important for predicting and managing these diseases. Yet, compared to *Vibrio* infecting humans, knowledge of their role as animal pathogens is scarce. Here we ask how widespread is virulence among ecologically differentiated *Vibrio* populations, and what is the nature and frequency of virulence genes within these populations? We use a combination of population genomics and molecular genetics to assay hundreds of *Vibrio* strains for their virulence in the oyster *Crassostrea gigas*, a unique animal model that allows high-throughput infection assays. We show that within the diverse Splendidus clade, virulence represents an ancestral trait but has been lost from several populations. Two loci are necessary for virulence, the first being widely distributed across the Splendidus clade and consisting of an exported conserved protein (R5.7). The second is a MARTX toxin cluster, which only occurs within *V. splendidus* and is for the first time associated with virulence in marine invertebrates. Varying frequencies of both loci among populations indicate different selective pressures and alternative ecological roles, based on which we suggest strategies for epidemiological surveys.

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These authors contributed equally: Maxime Bruto, Yannick Labreuche

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## Introduction

Global change due to rising temperature and ocean acidification but also more directly due to anthropogenic influences, such as widespread use of aquaculture, is predicted to lead to a worldwide increase in *Vibrio*-associated illness of marine organisms [1–3]. This development is illustrated by recent outbreaks of disease in *Crassostrea gigas* (oyster) farms in France [4, 5], in which strains related to the Splendidus clade have been implicated [6–10]. The Splendidus clade is a large group of closely related species (e.g., *V. splendidus*, *V. crassostreae*, *V. tasmaniensis*, and *V. cyclitrophicus*) containing several facultatively pathogenic members that affect diverse marine organisms [11, 12]. Elevated host density due to farming is a likely factor involved in *Vibrio*-associated disease. However, it is less well understood whether the bacteria are drawn from environmental reservoirs of potential pathogens or have arisen by recent acquisition of virulence traits in response to increased host availability due to aquaculture. The latter appears to be the case in *V. crassostreae*, which we recently demonstrated to have emerged as an oyster pathogen at least partially as a result of the population-specific spread of a

virulence plasmid [6]. However, collections of environmental pathogens are typically biased toward isolates from diseased animals [13, 14]. It is thus difficult to assess how broadly distributed virulence potential is among *Vibrio* spp., i.e., whether virulence is limited to few strains within populations or is a trait of the entire species. We therefore reasoned that assessment of virulence properties within populations of *Vibrio* spp. in natural environments where oysters are not farmed, could be used to determine what level of taxonomic resolution has the greatest predictive power for disease risk assessment in oyster-farming areas.

Here we determine the virulence potential of a large collection of *Vibrio* strains using specific pathogen-free (SPF) oysters as a model. The bacterial strain collection derives from a single location without intense oyster farming, on the Atlantic coast of the United States (Massachusetts, Plum Island). Extensive genetic and microhabitat characterization of these isolates has been previously reported and identified genetically defined groups that correspond to ecologically distinct habitats. This is indicative of different lifestyles (free-living or attached to different types of particulate organic matter and zoo- or phytoplankton) between these groups [15–18]. Subsequent work has found multiple lines of evidence these *Vibrio* groups represent biologically distinct populations with respect to gene flow [19, 20] and interaction with other organisms (social/behavioral networks) [21–23]. As such, these populations fit species concepts as typically used in plant and animal ecology; however, we note that they are at a finer evolutionary divergence than those traditionally used to separate species in bacterial taxonomy. To assess the virulence potential of these populations, we injected a large number of strains into juvenile SPF oysters and monitored their mortality. These animals are descendants of a pool of genitors that are produced in hatchery under highly controlled conditions to minimize the influence of genetic and environmental parameters that could affect the host sensitivity to the disease [10, 24, 25]. Moreover, these SPF oysters can be used for high-throughput experimental infections with hundreds of individual bacterial isolates [9, 10, 26]. Assaying the virulence of strains by injection has drawbacks, as it fails to capture the importance of initial infection events such as mobility and chemotaxis. However, *Vibrio* spp. related to *Splendidus* (both virulent and non-virulent) naturally colonize the oyster hemolymph [6] and injection in the adductor muscle allows the direct transfer of bacteria in the hemolymph. Here screening *Vibrio* strains by injection allows the identification of traits that cause death in the animals. It is thus possible to ask whether the functional units of pathogenesis for oysters are either clones that have emerged after recent acquisition of virulence factors by horizontal gene transfer (HGT) or populations of diverse genotypes with virulence as a core function [27].

We show that the ability to trigger oyster mortality is an ancestral trait of populations within the *Splendidus* clade and depends on at least two loci. While in some populations all members are potentially virulent, the trait occurs intermittently in other populations and some populations have lost it altogether. The ancestrally acquired gene R5.7 is necessary for full virulence and while it has co-diversified with populations, it was lost in the non-virulent populations. Across populations, additional genes may, however, play a role in virulence. This is illustrated by the occurrence of several different types of MARTX gene clusters in *V. splendidus* for which frequency-dependent selection is suggested. The widespread occurrence of virulence genes across environmental *Vibrio* populations suggests an important biological role but their different frequency also indicates that the role is population specific.

## Materials and methods

### Genome sequencing, assembly, and annotation

In the present study, a total of 37 strains (Table S1) were sequenced (Plateforme génomique, Institut Pasteur, Paris) using the Illumina HiSeq2000 technology with ~50-fold coverage as described previously [9]. Contigs were assembled de novo using Velvet [28]. Computational prediction of coding sequences together with functional assignments was performed using the automated annotation pipeline implemented in the MicroScope platform [29]. Some gene annotations were manually curated using InterPro, FigFam, PRIAM, COGs, PsortB, TMHMM, and synteny group computation.

### Protein family identification

The proteome of each strain was first compared by performing a Blastp all-vs-all. The SiLiX software [30] was used to reconstruct protein families based on reciprocal alignment length (at least 80% of sequence length) and different thresholds of identity depending on the analysis. A threshold of 60% identity was used when comparing two populations, while a threshold of 80% was used to reconstruct families within a population.

### Phylogenetic tree construction

Species trees were reconstructed based on core genes present in a single copy. Protein sequences of each family were first aligned with Muscle, filtered using Gblocks with relaxed parameters [31] and concatenated using a custom script (available at <https://github.com/mbruto/OPOPOP>). Phylogenetic reconstruction was done using RAxML [32]

on this concatemer using a LG model of evolution with a gamma model and default parameters. For single-marker gene trees, we aligned the sequences with Muscle and performed the phylogenetic reconstruction with RAxML using a GTR model of evolution with a gamma model and default parameters.

### Phylogenetic logistic regression analysis

The correlation between gene family presence/absence and virulence (percentage of induced mortality) was computed using the phylogenetic logistic regression implemented in the R package “*phylolm*” [33]. Virulence was first standardized to have a mean equal to 0 and standard deviation equal to 1, as suggested in previous work [34]. The following command was used to infer a correlation coefficient: *phyloglm(Fam ~ Mort, phy = Tree, data = Corr\_data, method = “logistic\_MPLE”)*. The variable *Corr\_data* is a table containing the presence/absence of a single family in the first column (named *Fam*), while the second column contains virulence values that were standardized to have a mean of 0 and a standard deviation of 1 (named *Mort*). The *Tree* variable is the species tree of the strains considered. We used the *logistic\_MPLE* model as it appears to be the most general one and can be applied on a wider range of data sets [33].

### Ancestral character reconstruction

Ancestral character reconstructions were performed with the version 3.31 of Mesquite (<http://mesquiteproject.org>) using the maximum parsimony method. The presence or absence of the Splendidus specific locus R5.7/8 was coded as 1 or 0 in a character matrix. Together with the species tree, this matrix was used as an input to reconstruct the presence or absence of the cluster on each node using the unordered parsimony model, in which acquisitions and losses have an equal cost.

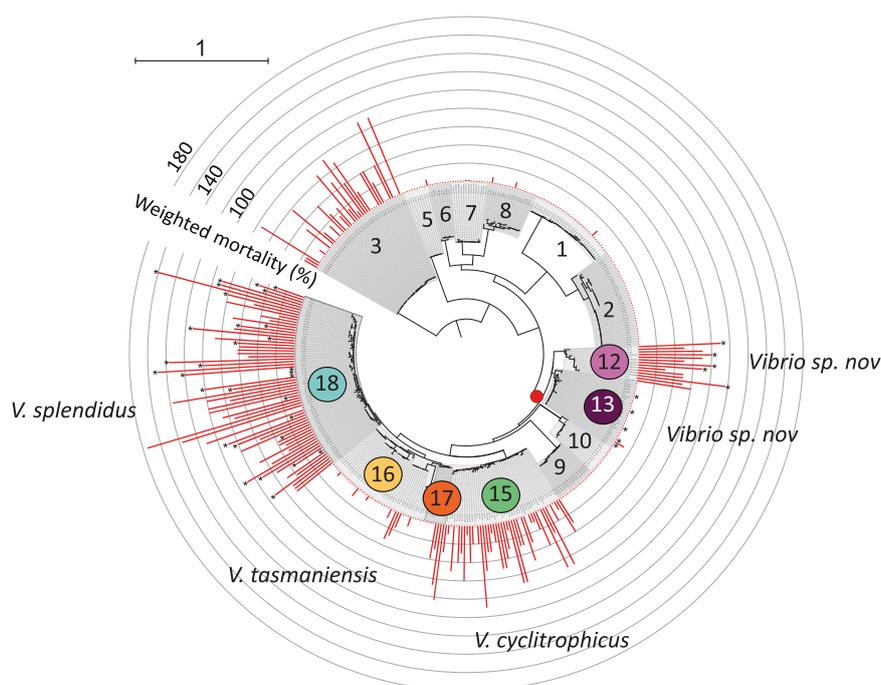
### Molecular microbiology

The *Vibrio* collection used for the high-throughput infection assays (population F1–18), i.e., Plum Island collection, has been described previously [15]. The *Vibrio* strains isolated from a French oyster farming area have been described by Bruto et al. [6]. Strains and plasmids used or constructed in the present study are described in Table S2 and S3. *Vibrio* isolates were grown at 20 °C in Zobell broth or agar (4 g l<sup>-1</sup> bactopeptone and 1 g l<sup>-1</sup> yeast extract in artificial seawater, pH 7.6), Luria-Bertani (LB), or LB-agar (LBA) + 0.5 M NaCl. *Escherichia coli* strains were grown at 37 °C in LB or on LBA for conjugation experiments and in ZYP 5052 medium [35] for induction of protein expression in *E. coli*,

strain BL21 (DE3). Chloramphenicol (Cm, 5 or 25 µg ml<sup>-1</sup> for *Vibrio* and *E. coli*, respectively), carbenicillin (100 µg ml<sup>-1</sup>), thymidine (0.3 mM), and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction of the P<sub>bad</sub> promoter was achieved by the addition of 0.2% L-arabinose to the growth media, and conversely, was repressed by the addition of 1% D-glucose. Deletion of selected regions or genes was performed by allelic exchange using the pSW7848T suicide plasmid [36, 37]. To this end, two 500-bp fragments flanking the target region or gene were amplified (see primer details in Table S4), cloned into pSW7848T as previously described [9], and transferred by conjugation from *E. coli* as a donor to *Vibrio* as a recipient. Subsequently, the first and second recombinations leading to pSW7848T integration and elimination were selected on Cm/glucose and arabinose-containing media, respectively. Gene inactivation was performed by cloning 500 bp of the target gene in pSW23T [38] and selecting on Cm the suicide plasmid integration obtained by a single recombination [8]. For complementation experiments, the R5.7 gene was cloned in Apa1/Xho1 sites of the pMRB plasmid known to be stable in *Vibrio* spp. [39], resulting in a constitutive expression from a P<sub>lac</sub> promoter [9]. Conjugation between *E. coli* and *Vibrio* was performed at 30 °C as described previously [36]. The BL21(DE3) strains expressing the R5.7\_GFP fusion proteins were constructed using the Gibson assembly method (New England Biolabs, NEB) as previously described [40]. Briefly, the fragment encoding the green fluorescent protein (GFP) was amplified from plasmid pMRB-GFP [6] (see primer details in Table S4). R5.7<sub>F12</sub> and R5.7<sub>crass</sub> genes, including a flexible linker were PCR-amplified from genomic DNA of strains B8 and J2-9, respectively. The pFO4 vector, that contains a His6-tag at the N terminus for affinity purification, was amplified by PCR inside-out. All constructs were confirmed by sequencing prior to electrocompetent BL21(DE3) transformation.

### Purification of recombinant proteins

Recombinant R5.7<sub>F12</sub> and R5.7<sub>crass</sub> proteins were purified as previously described [40]. Briefly, after expression of the proteins in ZYP 5052 (20 °C, 72 h) and chemical cell lysis, the lysate was clarified at 13,865 × g for 45 min at 4 °C. The supernatant was loaded on a 5-ml GE HisTrap HP column, washed twice with 20 mM Na<sub>3</sub>PO<sub>4</sub>, pH 8.0, 290 mM NaCl, and 5 mM imidazole, and eluted using a gradient of 1–100% 20 mM Na<sub>3</sub>PO<sub>4</sub>, pH 8.0, 290 mM NaCl, and 1 M imidazole. Fractions containing the protein of interest were pooled, concentrated (molecular weight cutoff of 10 kDa), and loaded onto a Superdex S200 column in HEPES that contains 20 mM NaCl at 150 mM, pH 7.4. Fractions were again pooled and concentrated. The concentration was calculated from A<sub>280</sub> and the extinction coefficient was



**Fig. 1** Mortality induced in oysters by *Vibrio* populations. The virulence potential mapped onto the isolate phylogeny (*Vibrio* collection from Plum Island, MA, USA). The tree is based on the genetic marker *hsp60* and comprises different genotypic clusters previously found to have a cohesive ecology and hypothesized to represent samples from natural ecological populations [15]. The closest named species to populations are as follows: F1: *Enterovibrio calviensis*; F2: *Enterovibrio norvegicus*; F3: *V. ordalii*; F4: *V. rumoiensis*; F5: *V. alginolyticus*; F7 *Aliivibrio fischeri/logei*; F8: *A. fischeri*; F9: *V. superstes*; F15: *V. cyclitrophicus*; F16–17: *V. tasmaniensis*; F18: *V. splendidus*. Populations F6, 10, 12, and 13 were not assigned to a species and were thus named here as *Vibrio sp. nov.* The node leading to the Splendidus

clade in this tree is indicated by a red dot and the six populations belonging to the Splendidus clade are each indicated by distinct colors (also used in subsequent figures). Note that the presence of populations F9 and F10 within this cluster on the *hsp60* tree is an artifact of a recombination event in that gene and they are not members of the Splendidus clade, as determined by a full genome analysis. Red bars indicate weighted mortality (using *V. crassostreae* J2-9 mortality rate as a reference to evaluate variability between experiments) induced by individual strains 24 h after injection in oysters ( $n = 20$ ). The range of variability for J2-9 between experiments (50–90%, data not shown) was lower than the variability between populations. Stars indicate the available genome sequences

calculated using the ProtParam tool from ExPASy ( $\epsilon_{0.1\%}$  of 1.04).

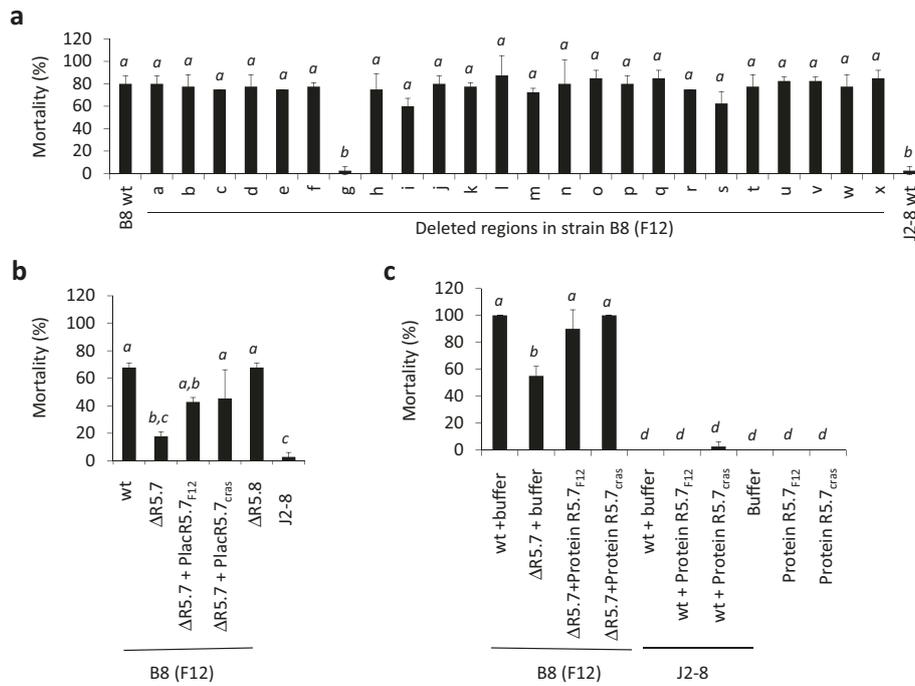
### Experimental determination of mortality

To determine the virulence of isolates, bacteria were grown under constant agitation at 20 °C for 24 h in Zobell media. One hundred microliters of the culture ( $10^8$  colony-forming units, cfu) were injected intramuscularly into oysters. Co-injections of the  $\Delta R5.7$  mutant with the recombinant purified R5.7<sub>F12</sub> or R5.7<sub>crass</sub> proteins were performed following the same procedure, except that the tested protein (30  $\mu$ g/animal) or the corresponding volume of protein solubilization buffer (as a control) were added to the bacterial suspension 1 h before injection. The bacterial concentration was confirmed by conventional dilution plating on Zobell agar. After injection, the oysters were transferred to aquaria (20 oysters per 2.5 l of aquarium) containing 1 l of aerated 5- $\mu$ m-filtered seawater at 20 °C, and kept under static conditions. Experiments were performed in duplicate at least twice, and mortality was assessed after 24 h.

## Results

### Virulence is an ancestral trait of several ecological populations

We first investigated the frequency of virulence among 405 strains representing 15 ecological populations sampled randomly from Plum Island (MA) [15, 17] by injecting them individually into SPF oysters. This showed that nine populations contained exclusively non-virulent strains, while six of them were potentially virulent with the majority (5/6) of these being in the Splendidus clade (Fig. 1, populations belonging to the Splendidus clade are each indicated by distinct colours). All the strains of population F12, which represents a new species closely related to *V. crassostreae* [41], induced high levels of oyster mortality, while populations F15 (taxonomically assigned to the species *V. cyclitrophicus*), F17 (*V. tasmaniensis*), and F18 (*V. splendidus*) are predominantly virulent, and population F16 (*V. tasmaniensis*) mostly contains non-virulent strains. The only highly virulent population outside the Splendidus clade



**Fig. 2** Oyster mortality in response to experimental infection with *Vibrio* wild-type strains and derivatives. **a** Twenty four population-specific regions were deleted in *Vibrio sp. nov* F12 strain B8. Virulence of the wild-type B8 strain (wt), derivatives (deletion of regions a to x), and *Vibrio sp. nov* F13-like strain J2-8 as a negative control was compared after injection of strains ( $10^8$  cfu/animal) into 20 oysters and counting the percent mortality at 24 h (y axis). **b** Comparison of mortality induced by B8 wild-type strain (wt), the mutants obtained by R5.7 or R5.8 single-gene deletion ( $\Delta R5.7$  or  $\Delta R5.8$ ), and the complementation assay in  $\Delta R5.7$  after the transfer of a plasmid that

expressed R5.7<sub>F12</sub> or R5.7<sub>cras</sub> constitutively from a Plac promoter (P<sub>lac</sub>R5.7<sub>F12</sub> and P<sub>lac</sub>R5.7<sub>cras</sub>). **c** Complementation assays using recombinant purified protein R5.7, the wild-type strains (B8, J2-8) injected with buffer (wt + buffer), the mutant obtained by R5.7 single-gene deletion with buffer ( $\Delta R5.7 + buffer$ ), the mutant  $\Delta R5.7$  or J2-8 co-injected with the recombinant purified proteins R5.7<sub>F12</sub> or R5.7<sub>cras</sub>, the recombinant proteins, or buffer as control. All experiments were performed in duplicate and at least twice. Means with the same letter in italic are not significantly different from each other (ANOVA,  $p < 0.05$  and Tukey HSD test)

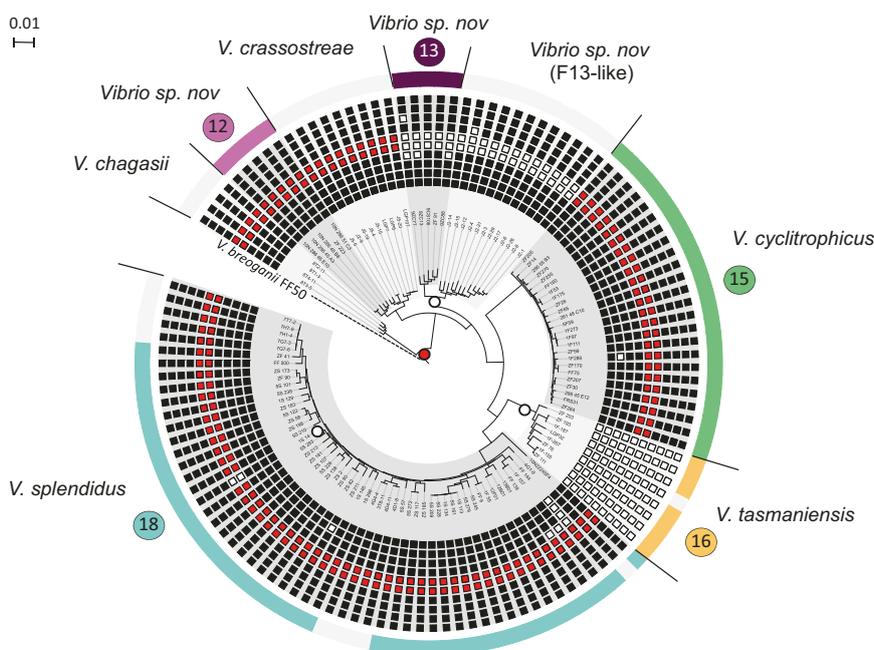
is F3, which is taxonomically assigned to *V. ordalii*, a species that has been associated with fish disease [42]. The observed high prevalence of virulence among populations in the Splendidus clade led us to test the hypothesis that the ability to kill oysters might be an ancestral trait for the entire clade by (i) identifying putative virulence genes by comparative genomics and genetic knockout and (ii) reconstructing the acquisition and inheritance of the virulence genes among populations.

**The same genes are necessary for full virulence across the Splendidus clade**

To test the hypothesis of ancestral virulence in the Splendidus clade, we first sought to identify genes potentially responsible for virulence using comparative genomics of populations F12 (*Vibrio sp. nov*) and F13 (*Vibrio sp. nov*), since these are the most closely related populations that contain exclusively virulent and non-virulent strains, respectively (Fig. 1). Using genomes of five isolates from each population, we identified 120 genes to be specific to the virulent population F12, of which 87 genes were localized in

24 genomic regions, designated regions a through x (i.e., Ra through Rx) (Table S5). Annotation implicates many of these genes in regulation, detoxification, xenobiotic degradation, siderophore production, or acquisition, i.e., the ability to respond to transient selective pressure in the environment.

Using a genetic knockout approach, we next investigated the importance of these loci for the virulence of population F12 strain 10N.286.48.B8 (hereafter named B8). None of the 24 deletions impaired bacterial growth in culture media (data not shown) and only the deletion of region Rg resulted in a decrease in oyster mortalities after experimental infection (Fig. 2a). This region contains two genes VB12B8\_v1\_40197 and VB12B8\_v1\_40198, each encoding an exported protein of unknown function. Notably, these genes showed 91 and 90% identity to the R5.7 and R5.8 proteins of *V. crassostreae*, respectively [9]. For simplicity, these genes are subsequently referred to as R5.7/8<sub>cras</sub> or R5.7/8<sub>F12</sub> for *V. crassostreae* and population F12, respectively. A mutant lacking the R5.7<sub>F12</sub> gene ( $\Delta R5.7_{F12}$ ) revealed that this gene contributes to B8 virulence (Fig. 2b), as previously shown for R5.7<sub>cras</sub>. When constitutively expressed in trans from a plasmid, R5.7<sub>F12</sub> or R5.7<sub>cras</sub> were



**Fig. 3** Distribution of the R5.7 and R5.8 cluster and the surrounding genes in the Splendidus clade. The phylogenetic tree was reconstructed on an alignment of 707 concatenated core proteins and rooted with *V. breoganii* FF50. The length of the outgroup branch has been arbitrarily defined (dashed line) to increase the resolution of the tree. The eight species considered are highlighted in alternating grays. Strains isolated from Plum Island are indicated by the same color code as in Fig. 1 (outer-circle). Species assignment and correspondence to population assignment in Fig. 1 are also indicated. Strains isolated from oyster

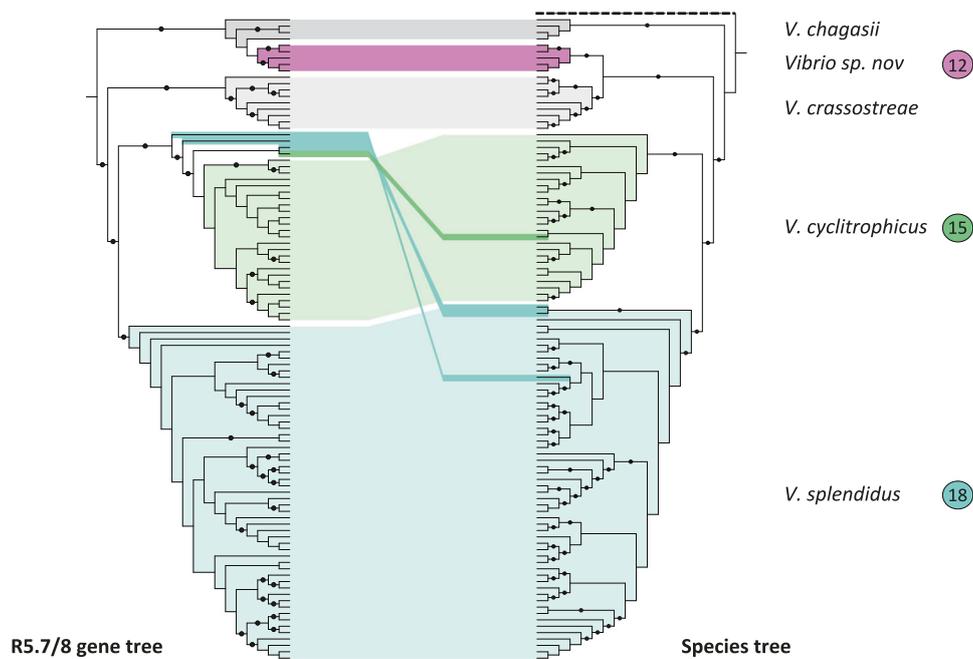
farming areas in France, i.e., *V. chagasii*, *V. crassostreae*, and *Vibrio sp. nov* F13-like are indicated in gray (outer-circle). The absence and presence of the R5.7/8 cluster are represented by white and red squares, respectively. The presence of the surrounding genes is indicated by a black square if they belong to the same syntenic group of the R5.7/8 gene cluster (see also Figure S1). Note that in *V. splendidus*, only the strain 1S\_14 lacks the R5-7/8, while strains FF\_139, FF-144, and 1S-157 lack at least one flanking gene. Red and white circles on the tree indicate acquisition and loss of R5.7/8, respectively

sufficient to partially restore the virulence of the mutant  $\Delta R5.7_{F12}$ . Furthermore, a complete restoration of virulence was observed by co-injecting the  $\Delta R5.7_{F12}$  with the recombinant purified R5.7<sub>F12</sub> or R5.7<sub>cras</sub> proteins (Fig. 2c). On the other hand, these proteins did not induce mortality when injected alone or co-injected with the non-virulent strain J2-8, showing that R5.7 is not sufficient to induce mortality. Finally, the deletion of the R5.8 gene did not alter the virulence of the B8 strain, showing that only R5.7 is necessary for the contribution of Rg to virulence.

R5.7/8 appear highly specific to the Splendidus clade, as evidenced from the distribution in 872 available genomes from 30 different species of *Vibrio*. First, R5.7 is always colocalized with R5.8 (Fig. S1). Second, this gene cluster is restricted to the Splendidus clade encompassing the populations F12 (*Vibrio sp. nov*), F15 (*V. cyclitrophicus*), and F18 (*V. splendidus*) from the Plum Island collection, as well as populations of strains previously isolated from a French oyster farming area and assigned to *V. chagasii*, *V. splendidus*, and *V. crassostreae* [6, 9] (Fig. 3). We note that population F17 (*V. tasmaniensis*) shown in Fig. 1 from the Plum Island collection was not evaluated since there are currently no genomes available. We also confirmed the importance of

the R5.7 homolog for virulence in *V. chagasii* and *V. splendidus* by inactivating this gene in strains representative of these species (Fig. S2). Finally, the genomic comparison showed that the R5.7/8 gene cluster is absent in three out of eight populations within the Splendidus clade (Fig. 3), populations F13 (*Vibrio sp. nov*) and F16 (*V. tasmaniensis*) from the Plum Island collection, and also in *Vibrio sp. nov* F13-like isolated in France. Moreover, a single non-virulent *V. splendidus* strain, 1S\_14, was lacking the gene (confirmed by PCR, data not shown). Altogether, these results suggest that the R5.7 gene is necessary for full virulence across the entire Splendidus clade.

We next tested whether the R5.7/8 gene cluster distribution in the Splendidus clade is best explained by (i) ancestral acquisition followed by multiple losses or (ii) multiple independent acquisitions. Reconstruction of the most parsimonious ancestral character state of the R5.7/8 genes suggests an acquisition in the most recent common ancestor (MRCA) of the Splendidus clade followed by three distinct losses in the MRCAs of populations F13, F16, and F13-like (Fig. 3). The R5.7/8 gene tree is highly congruent with the species tree (Fig. 4), suggesting vertical inheritance and persistence of the gene cluster through speciation events that likely involved differential ecological specialization of



**Fig. 4** Comparison of the R5.7 and R5.8 concatenated gene tree with the species tree for populations of the Splendidus clade. The species tree (right) was reconstructed based on an alignment of 707 concatenated core proteins and rooted with *V. breoganii* FF50. Both trees are represented without branch lengths and the outgroup is represented with a dashed line on the tree. Black points on branches indicate a support value above 80% based on the rapid bootstrap analysis

the populations. The main exception to this vertical inheritance pattern is population F12, which is most closely related to *V. crassostreae* in the species tree but is sister to *V. chagasii* in the R5.7/8 tree indicating horizontal acquisition. Although the R5.7/8 gene tree clusters the vast majority of strains within their previously assigned populations, four strains are an exception: one *V. cyclitrophicus* (out of 27) and three *V. splendidus* (out of 56) appear to have acquired the R5.7/8 locus horizontally (Fig. 4). This more recent acquisition most likely involved homologous recombination since annotation of genes present within 20 kb upstream and downstream of the R5.7/8 locus did not identify any potential insertion sites for mobile genetic elements. However, such gene conversions appear to be the exception and our analysis predicts that R5.7/8 was ancestrally acquired and subsequently predominantly vertically inherited in the Splendidus clade.

### Acquisition and loss of MARTX toxin modulates virulence

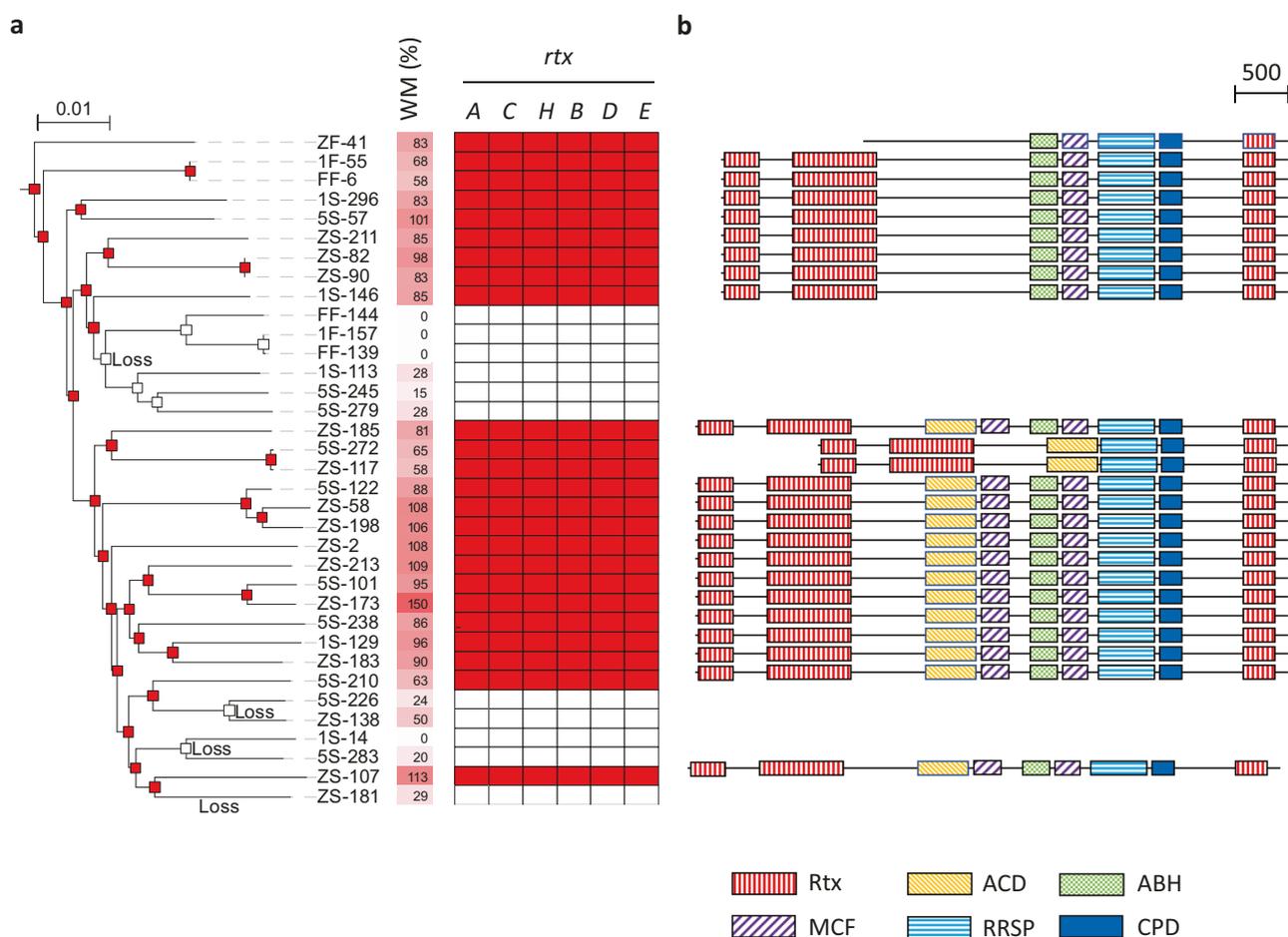
The above genomic analysis also showed that R5.7 was present in non-virulent strains belonging to populations F15 and F18 (Figs. 1 and 3), suggesting that the gene is necessary but not sufficient for virulence and that other

implemented in RAxML. Species are linked with different colors in both trees, and population correspondence with Fig. 1 is also indicated. Supported incongruences between R5.7/8 gene cluster (left) and species trees were identified for *Vibrio sp. nov.* F12 (pink), one *V. cyclitrophicus* (darker green), and three *V. splendidus* strains (darker turquoise)

genes may play a role that these strains might have lost or never acquired. To test this hypothesis of additional virulence genes, we first computed the correlation between gene family presence/absence and induced mortalities using phylogenetic logistic regression in population F18 (*V. splendidus*) (see Materials and methods).

Among 12,158 families, only six genes, which were all colocalized in a single locus, showed a high correlation factor ( $c = 4.01$ ;  $P = 0.005$ ) (Fig. 5a). This locus encodes a putative toxin (MARTX encoded by the gene *rtxA*), a putative acyltransferase (*rtxC*), an uncharacterized protein (*rtxH*), and a putative type-I secretion system (*rtxBDE*). Because this comparison strongly suggested *rtxACHBDE* as additional virulence genes, we genetically assessed the importance of this locus for *V. splendidus* virulence.

The deletion of the complete *rtxACHBDE* locus in three strains (ZS\_173, ZS\_185, and ZS\_213) did not impair growth in culture media, but resulted in a considerable decrease in mortalities after injection of bacteria (Fig. 6), demonstrating a role of this locus in *V. splendidus* virulence. MARTX toxins have been defined as “multifactorial effector cargo translocation, processing and delivery machines” (reviewed by refs. [43, 44]). As in other bacteria, the MARTXs found in *V. splendidus* (MARTX<sub>vs</sub>) are extremely large (3646–5097 aa) and



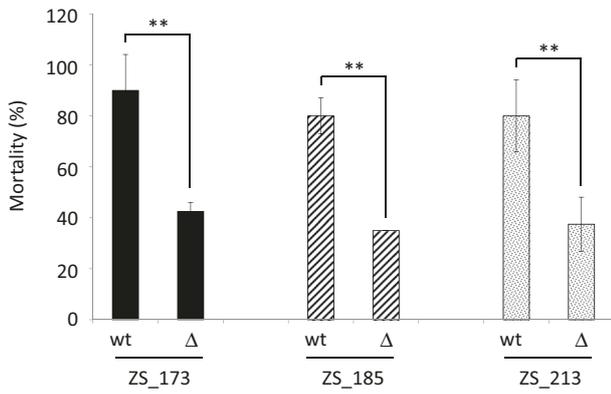
**Fig. 5** Distribution, evolutionary history, and domain variation of the *rtxACHBDE* cluster in *V. splendidus* related to induced mortality in oysters. **a** The phylogenetic tree is based on an alignment of 2290 concatenated core genes. Weighted mortalities (WM, using *V. crassostreae* J2-9 mortality rate as a reference to evaluate variability between experiments) after injection in oysters ( $n = 20$ ) are indicated by a color gradient from white (0%) to red (150%). The range of variability for J2-9 between experiments (50–90%, data not shown) was lower than the variability between populations. Gene presence and

absence is represented by red and white squares, respectively. Losses are indicated when the *rtxACHBDE* cluster is predicted to be absent on a node (or leaf) while being present in the parent node. **b** Diversity of MARTX identified in *V. splendidus*. The domain abbreviations are Rtx repeats in toxin, ACD actin cross-linking domain, ABH alpha/beta hydrolase, MCF makes caterpillars floppy, RRSP Ras/Rap1-specific endopeptidase, CPD cysteine protease domain [44]. A scale bar indicating protein length in amino acids is present on the top right of the figure

contain several domains (Fig. 5b). MARTX<sub>Vs</sub> is predicted to contain the three domains found in all MARTXs, i.e., the core structure composed of two conserved regions at the amino- and carboxyl-termini and a cysteine protease domain (CPD) [45]. Only one effector domain, a Ras/Rap1-specific endopeptidase (RRSP) [46, 47] is present in all MARTX<sub>Vs</sub>. A homolog of the actin cross-linking domain (ACD) [48] allows distinguishing two main variants of MARTX<sub>Vs</sub> but the presence of ACD is not associated with heightened virulence among strains. The  $\alpha/\beta$  hydrolase (ABH) and the cysteine protease (MCF) domains [49] are absent from only two *V. splendidus* strains (5S-272 and ZS-117) out of 24, that are still virulent. Finally, the Rho inactivation domain (RID) [50] found in *V. cholerae* and *V. vulnificus* and associated with

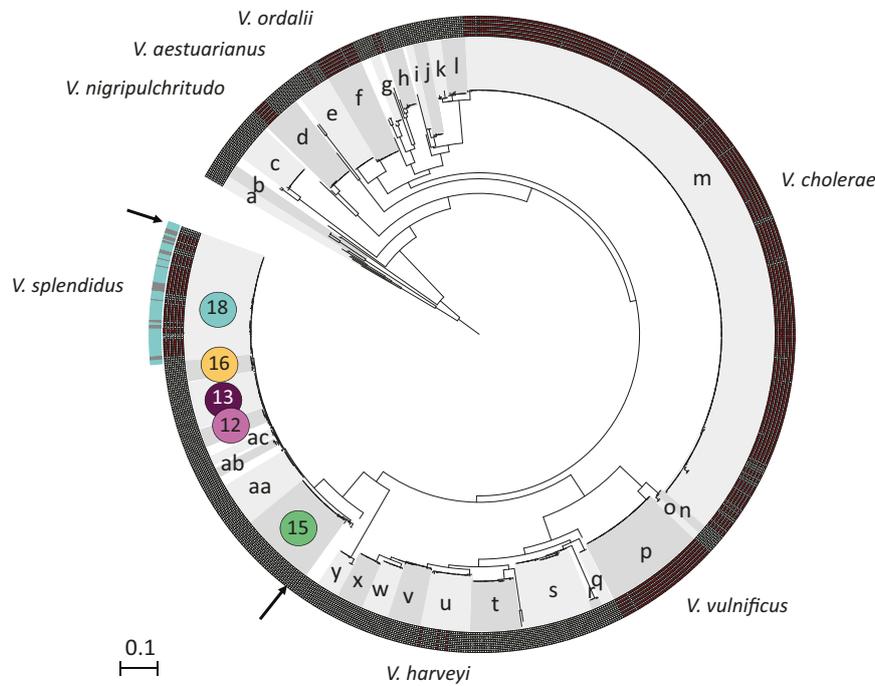
a “cell rounding” effect is absent from *V. splendidus*. Hence, none of the domains ACD and RID previously demonstrated to affect polymerized actin in target eukaryotic cells, seem to be key domains of *V. splendidus* MARTX activity, while the RRSP may be hypothesized as an essential domain for its activity.

In the Splendidus clade, the *rtxACHBDE* cluster is found only in *V. splendidus* but is also present in several more distantly related *Vibrio* species (Fig. 7). Within *V. splendidus*, the locus is present in 24/35 members (Fig. 5a), is chromosomally encoded, and does not show any signs of recent acquisition by HGT involving non-homologous mechanisms. Across all 872 genomes of *Vibrionaceae* (Fig. 7), this cluster is identified in seven additional species that have all been associated with disease in humans or



**Fig. 6** Oyster mortality in response to experimental infection with *V. splendidus* wild-type strains and  $\Delta$ *rtxACHBDE* derivatives. The *rtxACHBDE* locus was deleted in three *V. splendidus* strains (ZS\_173, ZS\_185, and ZS\_213). Virulence of the wild type (wt) and derivative ( $\Delta$ ) was compared after injection of strains ( $10^8$  cfu/animal) in 20 oysters and counting the percentage of mortalities at 24H (y axis). Experiments were performed in duplicate and repeated twice. Two stars indicate highly significant differences between treatments (Wilcoxon test,  $p > 0.005$ )

animals [43, 44]. The frequency of the locus (strains carrying the *rtxACHBDE* cluster/total) appears different between species. In *V. cholerae*, *V. vulnificus*, and *V. ordalii*, the cluster is present in the vast majority of the strains, suggesting strong selection for its maintenance, irrespective of their source of isolation. In *V. cholerae* and *V. vulnificus*, 153 of 167 and 15 of 16 clinical isolates, and 37 of 43 and 16 of 16 environmental isolates contained the *rtxACHBDE* cluster, respectively. Conversely, in the shrimp pathogen *V. nigripulchritudo*, the cluster is restricted to six strains that belong to the same pathogen-containing lineage and is carried by a plasmid [51, 52]. Similarly, in *V. harveyi* and *V. aestuarianus*, the locus is detected in only 3 of 23 and 1 of 12 strains, respectively, and annotation of flanking regions indicates a recent acquisition by HGT (plasmid genes, transposase, and syntenic rupture). Hence *rtxACHBDE*, although widespread among *Vibrio* spp., appears to be present at variable frequencies within species, indicating potentially different selective pressures on the gene cluster.



**Fig. 7** Distribution of the *rtxACHBDE* cluster in the genomes of 30 *Vibrio* species. The phylogenetic tree was reconstructed using the RctB protein sequence. The inner circle indicates the presence (red square) or absence (white square) of the six genes in the *rtxACHBDE* cluster. The outer circle indicates the isolation site of the *V. splendidus* strains with a turquoise for Plum Island and gray for oyster-farming areas in France. The 30 different *Vibrio* species and two *Vibrionaceae* genus outgroups are represented using alternating grays with the following letters: (a) *Photobacterium* spp.; (b) *Enterovibrio* spp.; (c) *V. breoganii*; (d) *V. nigripulchritudo*; (e) *V. aestuarianus*; (f) *V. ordalii*; (g) *V. scophthalmi*; (h) *V. coralliilyticus*; (i) *V. furnissii*; (j) *V. fluvialis*;

(k) *V. metoecus*; (l) *V. mimicus*; (m) *V. cholerae*; (n) *V. navarrensis*; (o) *V. cidicii*; (p) *V. vulnificus*; (q) *V. natrigens*; (r) *V. parahaemolyticus*; (s) *V. alginolyticus*; (t) *V. rotiferianus*; (u) *V. harveyi*; (v) *V. campbellii*; (w) *V. owensii*; (x) *V. jasicida*; (y) *V. mediterranei*; (F15) *V. cyclitrophicus*; (aa) *V. crassostreae*; (ab) *V. chagasii*; (ac) *Vibrio sp. nov* F13-like; (F12) *Vibrio sp. nov*; (F13) *Vibrio sp. nov*; (F16) *V. tasmaniensis*; (F18) *V. splendidus*. The tree was rooted with *Photobacterium* spp. and *Enterovibrio* spp. Populations of the *Splendidus* clade are grouped between the two arrows and population correspondence with Fig. 1 is indicated by the same color code

## Discussion

Ecological and evolutionary dynamics of virulence genes have been poorly documented for *Vibrionaceae* in the wild, and most knowledge stems from a handful of human pathogens isolated from clinical cases where the focus has been on pathogen emergence by gene acquisition and expansion of virulent clones [53–56]. Here we show that virulence toward juvenile oysters appears to be an ancestral trait in the Splendidus clade triggered by the acquisition and subsequent vertical inheritance of the R5.7/8 gene cluster. Populations F12 (closely related to *V. crassostreae*), F15 (*V. cyclitrophicus*), and F18 (*V. splendidus*) have all previously been implicated in mortality of a wide range of animals [8, 57], and our results show that the ability to be virulent is characteristic of the entire populations, rather than recently emerged clones. Consequently, non-virulent populations and strains have evolved by gene loss and expansion. However, we also show that increased virulence likely involves additional gene acquisitions, as demonstrated for the *rtxACHBDE* gene cluster, which in our collection was specifically acquired by *V. splendidus*. Because our results indicate that the R5.7 gene is necessary but not sufficient for virulence, we hypothesize that the other virulent populations within the Splendidus clade (e.g., F15, *V. cyclitrophicus*) may contain additional yet-to-be-determined virulence genes.

The only virulent population outside the Splendidus clade in our collection is F3, *V. ordalii*, which has been associated with fish disease [42] and belongs to the Anguillarum clade that contains the fish pathogen *V. anguillarum* and the oyster pathogen *V. aestuarianus* [26]. This widespread distribution of pathogenicity among closely related species suggests that similar to the Splendidus clade, virulence traits might have been acquired by the MRCA of the Anguillarum clade. One candidate gene is a zinc metalloprotease, which has been demonstrated in *V. aestuarianus* to be sufficient to cause exotoxicity [58] and to be necessary for virulence in *V. anguillarum* [59]. Moreover, we also found this gene in all *V. ordalii* genomes (not shown). Hence, as is the case in the Splendidus clade, this zinc metalloprotease may be an ancestral virulence determinant gene that has been preserved through speciation events and that plays an important role in the biology of this clade.

Within the Splendidus clade, all virulent populations share the R5.7/8 gene cluster for which we show this to be ancestrally acquired. Only R5.7 seems necessary for full virulence and complementation assays using the purified R5.7<sub>F12</sub> or R5.7<sub>cras</sub> proteins strongly suggest that these conserved proteins interact with the external surface of *Vibrio* and/or its cellular target in oysters. The injection of the R5.7 protein alone or in the presence of non-virulent *Vibrio* strains did not affect oyster viability, showing that

the protein alone does not have a lethal effect. Finally, a strong genetic linkage between R5.7/8 suggests functional interaction between these exported proteins of unknown function, in a mechanism that remains to be determined.

The R5.7/8 cluster can be present in non-virulent strains demonstrating that this cluster is necessary but not sufficient for virulence. Indeed, we show that an additional locus, the *rtxACHBDE* cluster, is necessary for full virulence towards oysters in *V. splendidus*. In other *Vibrio* species, the toxin MARTX has been demonstrated to work in concert with a cytotoxin (in *V. vulnificus*) and a hemolysin (in *V. cholerae*), and it has been associated with the severity of infection by contributing to the evasion of innate immune defense [44]. An accessory role of MARTX in virulence might be expected in the shrimp pathogen *V. nigripulchritudo* [52] since it has been recently demonstrated that a lethal toxin, nigrifactor, is the major virulence factor of this pathogen [40, 51]. Among *V. splendidus* strains, MARTX contains diverse effector domains and only RRSP is shared by all strains. This domain encoding an endopeptidase has been implicated in other species in the cleavage of Ras and Rap1 proteins [46, 47]. Importantly these small GTPases play a role as regulatory nodes of intracellular signaling and membrane trafficking, including trafficking of Toll-like receptor 4 (TLR4) that is involved in innate immune response in both vertebrates and invertebrates [60]. Hence the presence and conservation of a RRSP domain suggest that in *V. splendidus*, MARTX might be involved in virulence by impairing the host innate immune responses.

The R5.7/8 and *rtxACHBDE* loci showed differential evolutionary dynamics that indicate distinct selective regimes. The high frequency within virulent populations and mostly vertical transmission of R5.7/8 suggest that it is maintained by positive selection. That three non-virulent populations within the Splendidus clade have lost the gene cluster further suggests that it is involved in niche differentiation at the species level. On the other hand, the *rtxACHBDE* cluster has been independently acquired by *V. splendidus* and distantly related other species, and this gene cluster varies in frequency across these species. Moreover, within *V. splendidus*, and similarly in *V. vulnificus* [61, 62], the composition of the effector domains appears heterogeneous, ranging from 4 to 6 effectors per toxin with only one, RRSP, found in all MARTX<sub>vs</sub>. In *V. vulnificus*, such compositional variation has been shown to result from homologous recombination, shuffling effector domains such that different strains within the same species can express variants of the toxins that are likely to have distinct roles in the niche [44, 61–63]. Among the diverse MARTX<sub>vs</sub>, 15 and 22 of 24 share an ACD and ABH domain, respectively. Moreover, 22 of 24 MARTX<sub>vs</sub> share at least one MCF domain with the MARTX type III of *V. vulnificus*. Although the functional consequences of this domain variation are not

fully understood, the MARTX type III of *V. vulnificus* appears to play a dual role in eel infection as well as in protection from predation by amoebae by causing the lysis of this grazer [64]. In both *V. splendidus* and *V. vulnificus*, the intermittent presence and domain diversity of the MARTX cluster are potential indicators of frequency-dependent selection [65]. Because MARTX is a very large secreted protein and hence costly to express, we hypothesize that public good dynamics may arise where producers are favored only when low in frequency within the population [66]. Additionally, variation in toxin types may arise as a result of the evolutionary arms race between bacteria and predators or host immune system and, hence, is similar to frequency-dependent selection imposed on O-antigen structure or outer membrane proteins [67–70].

Our analysis shows that a number of environmental *Vibrio* populations present a risk of virulence independent of intensive aquaculture settings. Because virulence represents a function encoded in the core genome of these populations, specific markers can be used for risk assessment rather than overall *Vibrio* abundance, as is frequently the practice. At a minimum, the R5.7/8 gene cluster may be used to indicate overall risk. However, population specific markers will facilitate a more detailed evaluation of the epidemiology of oysters. An important component of such surveys is the determination of population dynamics outside the oyster host to specifically recognize ecological conditions that may trigger population increases and hence risk of infection. Importantly, recent analysis of microbial dynamics in the coastal ocean has shown rapid, near weekly, turnover of communities that are characterized by short-lived blooms of different bacteria and eukaryotes [71]. Exploration to what extent such transient communities allow for specific blooms of different *Vibrio* populations may elucidate whether there are periods of increased risk from multiple co-occurring populations or whether risk is similar across extended periods due to sequential blooms of populations. In support of the former, we previously showed that several virulent populations of *Vibrio* can co-occur in diseased oysters, e.g., *V. crassostreae* with *V. splendidus* or *V. chagasii* [6]. We identified a plasmid that is present specifically in virulent strains of *V. crassostreae* and necessary for full virulence [6]. Here we show that the MARTX gene cluster is involved in *V. splendidus* virulence and absent from the other virulent populations within the Splendidus clade. An interesting hypothesis to explore in the future is that other virulent populations such as *V. chagasii* or *V. cyclitrophicus* carry specific virulent traits. In a context of oyster infection by multiple co-occurring populations, these diverse virulence traits could act synergistically, increasing the vulnerability of the host. Indeed, experimental infections have demonstrated that some strains, belonging to the same [72] or different species [73],

are moderately virulent when injected into oyster individually, and display heightened virulence in mixed experimental infections. Hence oyster disease may result from microbial interaction within and between populations and should be evaluated as potentially polymicrobial.

## Disclaimer

The material represents an original result and has not been submitted for publication elsewhere.

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**Author contributions** YL, AJ, DP, SC, BP, and FLR performed experiments. MB performed the *in silico* analyses. MB, FLR, and MFP designed the experiments, interpreted results, and wrote the paper.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest. The material represents an original result and has not been submitted for publication elsewhere.

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## References

1. Baker-Austin C, Trinanés JA, Taylor NGH, Hartnell R, Siitonen A, Martínez-Urtaza J. Emerging *Vibrio* risk at high latitudes in response to ocean warming. *Nat Clim Change*. 2013;3:73–7.
2. Le Roux F, Wegner KM, Baker-Austin C, Vezzulli L, Osorio CR, Amaro C, et al. The emergence of *Vibrio* pathogens in Europe: ecology, evolution, and pathogenesis (Paris, 11–12th March 2015). *Front Microbiol*. 2015;6:830.
3. Vezzulli L, Grande C, Reid PC, Helaouet P, Edwards M, Hofle MG, et al. Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. *Proc Natl Acad Sci USA*. 2016;113:E5062–71.

4. Barbosa Solomieu V, Renault T, Travers MA. Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. *J Invertebr Pathol*. 2015;131:2–10.
5. Travers MA, Boettcher Miller K, Roque A, Friedman CS. Bacterial diseases in marine bivalves. *J Invertebr Pathol*. 2015;131:11–31.
6. Bruto M, James A, Petton B, Labreuche Y, Chenivresse S, Alunno-Bruscia M, et al. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J*. 2014;11:1043–52.
7. Gay M, Berthe FC, Le Roux F. Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Dis Aquat Organ*. 2004;59:49–56.
8. Le Roux F, Zouine M, Chakroun N, Binesse J, Saulnier D, Bouchier C, et al. Genome sequence of *Vibrio splendidus*: an abundant planktonic marine species with a large genotypic diversity. *Environ Microbiol*. 2009;11:1959–70.
9. Lemire A, Goudenege D, Versigny T, Petton B, Calteau A, Labreuche Y, et al. Populations, not clones, are the unit of *Vibrio* pathogenesis in naturally infected oysters. *ISME J*. 2014;9:1523–31.
10. Petton B, Bruto M, James A, Labreuche Y, Alunno-Bruscia M, Le Roux F. *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Front Microbiol*. 2015;6:686.
11. Perez-Cataluna A, Lucena T, Tarazona E, Arahal DR, Macian MC, Pujalte MJ. An MLSA approach for the taxonomic update of the *Splendidus* clade, a lineage containing several fish and shellfish pathogenic *Vibrio* spp. *Syst Appl Microbiol*. 2016;39:361–9.
12. Sawabe T, Koizumi S, Fukui Y, Nakagawa S, Ivanova EP, Kita-Tsukamoto K, et al. Mutation is the main driving force in the diversification of the *Vibrio splendidus* clade. *Microbes Environ*. 2009;24:281–5.
13. Spratt BG, Maiden MC. Bacterial population genetics, evolution and epidemiology. *Philos Trans R Soc Lond B Biol Sci*. 1999;354:701–10.
14. Sundberg LR, Ketola T, Laanto E, Kinnula H, Bamford JK, Penttinen R, et al. Intensive aquaculture selects for increased virulence and interference competition in bacteria. *Proc Biol Sci*. 2016;283:20153069.
15. Hunt DE, David LA, Gevers D, Preheim SP, Alm EJ, Polz MF. Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science*. 2008;320:1081–5.
16. Preheim SP, Boucher Y, Wildschutte H, David LA, Veneziano D, Alm EJ, et al. Metapopulation structure of *Vibrionaceae* among coastal marine invertebrates. *Environ Microbiol*. 2011;13:265–75.
17. Preheim SP, Timberlake S, Polz MF. Merging taxonomy with ecological population prediction in a case study of *Vibrionaceae*. *Appl Environ Microbiol*. 2011;77:7195–206.
18. Szabo G, Preheim SP, Kauffman KM, David LA, Shapiro J, Alm EJ, et al. Reproducibility of *Vibrionaceae* population structure in coastal bacterioplankton. *ISME J*. 2012;7:509–19.
19. Hehemann JH, Arevalo P, Datta MS, Yu X, Corzett CH, Henschel A, et al. Adaptive radiation by waves of gene transfer leads to fine-scale resource partitioning in marine microbes. *Nat Commun*. 2016;7:12860.
20. Shapiro BJ, Friedman J, Cordero OX, Preheim SP, Timberlake SC, Szabo G, et al. Population genomics of early events in the ecological differentiation of bacteria. *Science*. 2012;336:48–51.
21. Cordero OX, Ventouras LA, DeLong EF, Polz MF. Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *Proc Natl Acad Sci USA*. 2012;109:20059–64.
22. Cordero OX, Wildschutte H, Kirkup B, Proehl S, Ngo L, Hussain F, et al. Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. *Science*. 2012;337:1228–31.
23. Yawata Y, Cordero OX, Menolascina F, Hehemann JH, Polz MF, Stocker R. Competition-dispersal tradeoff ecologically differentiates recently speciated marine bacterioplankton populations. *Proc Natl Acad Sci USA*. 2014;111:5622–7.
24. Petton B, Boudry P, Alunno-Bruscia M, Pernet F. Factors influencing disease-induced mortality of Pacific oysters *Crassostrea gigas*. *Aquac Environ Interact*. 2015;6:205–22.
25. Petton B, Pernet F, Robert R, Boudry P. Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. *Aquac Environ Interact*. 2013;3:257–73.
26. Goudenege D, Travers MA, Lemire A, Petton B, Haffner P, Labreuche Y, et al. A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. *Environ Microbiol*. 2015;17:4189–99.
27. Le Roux F, Wegner KM, Polz MF. Oysters and Vibrios as a model for disease dynamics in wild animals. *Trends Microbiol*. 2016;24:568–580.
28. Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res*. 2008;18:821–9.
29. Vallenet D, Belda E, Calteau A, Cruveiller S, Engelen S, Lajus A, et al. MicroScope--an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res*. 2013;41:D636–47. (Database issue)
30. Miele V, Penel S, Duret L. Ultra-fast sequence clustering from similarity networks with SiLiX. *BMC Bioinform*. 2011;12:116.
31. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol*. 2000;17:540–52.
32. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*. 2006;22:2688–90.
33. Ho L, Ane C. A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. *Syst Biol*. 2014;63:397–408.
34. Ives AR, Garland T Jr. Phylogenetic logistic regression for binary dependent variables. *Syst Biol*. 2010;59:9–26.
35. Studier FW. Protein production by auto-induction in high density shaking cultures. *Protein Expr Purif*. 2005;41:207–34.
36. Le Roux F, Binesse J, Saulnier D, Mazel D. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl Environ Microbiol*. 2007;73:777–84.
37. Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, Mazel D. Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS Genet*. 2012;8:e1002472.
38. Demarre G, Guerout AM, Matsumoto-Mashimo C, Rowe-Magnus DA, Marliere P, Mazel D. A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate *Escherichia coli* host strains. *Res Microbiol*. 2005;156:245–55.
39. Le Roux F, Davis BM, Waldor MK. Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res*. 2011;39:1004–13.
40. Labreuche Y, Chenivresse S, Jeudy A, Le Panse S, Boulo V, Ansquer D, et al. Nigritoxin is a bacterial toxin for crustaceans and insects. *Nat Commun*. 2017;8:1248.
41. Faury N, Saulnier D, Thompson FL, Gay M, Swings J, Le Roux F. *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). *Int J Syst Evol Microbiol*. 2004;54:2137–40. (Pt 6)
42. Austin B. Taxonomy of bacterial fish pathogens. *Vet Res*. 2011;42:20.

43. Gavin HE, Satchell KJ. MARTX toxins as effector delivery platforms. *Pathog Dis.* 2015;73:ftv092.
44. Satchell KJ. Multifunctional-autoprocessing repeats-in-toxin (MARTX) toxins of Vibrios. *Microbiol Spectr.* 2015 Jun;3. <https://doi.org/10.1128/microbiolspec.VE-0002-2014>.
45. Satchell KJ. MARTX, multifunctional autoprocessing repeats-in-toxin toxins. *Infect Immun.* 2007;75:5079–84.
46. Antic I, Biancucci M, Zhu Y, Gius DR, Satchell KJ. Site-specific processing of Ras and Rap1 Switch I by a MARTX toxin effector domain. *Nat Commun.* 2015;6:7396.
47. Biancucci M, Rabideau AE, Lu Z, Loftis AR, Pentelute BL, Satchell KJF. Substrate recognition of MARTX Ras/Rap1-specific endopeptidase. *Biochemistry .* 2017;56:2747–57.
48. Cordero CL, Kudryashov DS, Reisler E, Satchell KJ. The actin cross-linking domain of the *Vibrio cholerae* RTX toxin directly catalyzes the covalent cross-linking of actin. *J Biol Chem.* 2006;281:32366–74.
49. Agarwal S, Zhu Y, Gius DR, Satchell KJ. The Makes Caterpillars Floppy (MCF)-like domain of *Vibrio vulnificus* induces mitochondrion-mediated apoptosis. *Infect Immun.* 2015;83:4392–403.
50. Ahrens S, Geissler B, Satchell KJ. Identification of a His-Asp-Cys catalytic triad essential for function of the Rho inactivation domain (RID) of *Vibrio cholerae* MARTX toxin. *J Biol Chem.* 2013;288:1397–408.
51. Goudenege D, Labreuche Y, Krin E, Ansquer D, Mangenot S, Calteau A, et al. Comparative genomics of pathogenic lineages of *Vibrio nigripulchritudo* identifies virulence-associated traits. *ISME J.* 2013;7:1985–96.
52. Le Roux F, Labreuche Y, Davis BM, Iqbal N, Mangenot S, Goarant C, et al. Virulence of an emerging pathogenic lineage of *Vibrio nigripulchritudo* is dependent on two plasmids. *Environ Microbiol.* 2011;13:296–306.
53. Boucher Y. Sustained local diversity of *Vibrio cholerae* O1 biotypes in a previously cholera-free country. *MBio.* 2016 May 3;7. pii: e00570-16. <https://doi.org/10.1128/mBio.00570-16>.
54. Martinez-Urtaza J, van Aerle R, Abanto M, Haendiges J, Myers RA, Trinanes J, et al. Genomic variation and evolution of *Vibrio parahaemolyticus* ST36 over the course of a transcontinental epidemic expansion. *MBio.* 2017 Nov 14;8. pii: e01425-17. <https://doi.org/10.1128/mBio.01425-17>.
55. Raz N, Danin-Poleg Y, Hayman RB, Bar-On Y, Linetsky A, Shmoish M, et al. Genome-wide SNP-genotyping array to study the evolution of the human pathogen *Vibrio vulnificus* biotype 3. *PLoS ONE* 2014;9:e114576.
56. Shapiro BJ, Levade I, Kovacicova G, Taylor RK, Almagro-Moreno S. Origins of pandemic *Vibrio cholerae* from environmental gene pools. *Nat Microbiol.* 2016;2:16240.
57. Roux F, Austin B . The Vibrios. . Washington, DC: ASM Press; 2006. 285–296 Chapter 21
58. Labreuche Y, Le Roux F, Henry J, Zatylny C, Huvet A, Lambert C, et al. *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. *Fish Shellfish Immunol.* 2010; 29:753–8.
59. Milton DL, Norqvist A, Wolf-Watz H. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. *J Bacteriol.* 1992;174:7235–44.
60. Luo L, Wall AA, Yeo JC, Condon ND, Norwood SJ, Schoenwaelder S, et al. Rab8a interacts directly with PI3Kgamma to modulate TLR4-driven PI3K and mTOR signalling. *Nat Commun.* 2014;5:4407.
61. Kwak JS, Jeong HG, Satchell KJ. *Vibrio vulnificus* rtxA1 gene recombination generates toxin variants with altered potency during intestinal infection. *Proc Natl Acad Sci USA.* 2011;108: 1645–50.
62. Roig FJ, Gonzalez-Candelas F, Amaro C. Domain organization and evolution of multifunctional autoprocessing repeats-in-toxin (MARTX) toxin in *Vibrio vulnificus*. *Appl Environ Microbiol.* 2011;77:657–68.
63. Dolores J, Satchell KJ. Analysis of *Vibrio cholerae* genome sequences reveals unique rtxA variants in environmental strains and an rtxA-null mutation in recent altered El Tor isolates. *MBio.* 2013;4:e00624.
64. Lee CT, Amaro C, Wu KM, Valiente E, Chang YF, Tsai SF, et al. A common virulence plasmid in biotype 2 *Vibrio vulnificus* and its dissemination aided by a conjugal plasmid. *J Bacteriol.* 2008;190:1638–48.
65. Cordero OX, Polz MF. Explaining microbial genomic diversity in light of evolutionary ecology. *Nat Rev Microbiol.* 2014;12: 263–73.
66. Jousset A, Rochat L, Pechy-Tarr M, Keel C, Scheu S, Bonkowski M. Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. *ISME J.* 2009;3:666–74.
67. Rodriguez-Valera F, Martin-Cuadrado AB, Rodriguez-Brito B, Pasic L, Thingstad TF, Rohwer F, et al. Explaining microbial population genomics through phage predation. *Nat Rev Microbiol.* 2009;7:828–36.
68. Seed KD, Yen M, Shapiro BJ, Hilaire IJ, Charles RC, Teng JE, et al. Evolutionary consequences of intra-patient phage predation on microbial populations. *Elife .* 2014;3:e03497.
69. Wildschutte H, Preheim SP, Hernandez Y, Polz MF. O-antigen diversity and lateral transfer of the wbe region among *Vibrio splendidus* isolates. *Environ Microbiol.* 2010;12:2977–87.
70. Seed KD, Faruque SM, Mekalanos JJ, Calderwood SB, Qadri F, Camilli A. Phase variable O antigen biosynthetic genes control expression of the major protective antigen and bacteriophage receptor in *Vibrio cholerae* O1. *PLoS Pathog.* 2012;8:e1002917.
71. Martin-Platero AM, Cleary B, Kauffman K, Preheim SP, McGillicuddy DJ, Alm EJ, et al. High resolution time series reveals cohesive but short-lived communities in coastal plankton. *Nat Commun.* 2018;9:266.
72. Gay M, Renault T, Pons AM, Le Roux F. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Dis Aquat Org.* 2004;62:65–74.
73. Saulnier D, De Decker S, Haffner P, Cobret L, Robert M, Garcia C. A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. *Microb Ecol.* 2010;59:787–98.

## Supplementary information

### Tables

**Table S1:** Features of genomes sequenced in the present study

Population	Strain name	Number of contigs	Genome size (Mb)	Number of CDSs	Accession number
F12	10N.286.45.A3	126	5.48	5124	PIGF00000000
	10N.286.46.E10	134	5.39	5009	PIGE00000000
	10N.286.48.B8	60	5.23	4822	PIGD00000000
	10N.286.51.C3	50	5.19	4758	PIGC00000000
	ZF 223	32	5.15	4727	PIGB00000000
F18	1F_55	117	5.74	5349	PIGA00000000
	1S_113	62	5.33	4842	PIFZ00000000
	1S_129	84	5.36	4875	PIFY00000000
	1S_14	41	5.33	4833	PIFX00000000
	1S_146	73	5.40	4934	PIFW00000000
	1S_296	48	5.40	4905	PIFV00000000
	5S_122	94	5.51	4998	PIFU00000000
	5S_210	41	5.28	4982	PIFT00000000
	5S_226	67	5.34	5070	PIFS00000000
	5S_238	68	5.41	4861	PIFR00000000
	5S_245	60	5.31	4781	PIFQ00000000
	5S_272	105	5.35	4853	PIFP00000000
	5S_279	69	5.40	4914	PIFO00000000
	5S_283	57	5.33	5050	PIFN00000000
	5S_57	53	5.41	5106	PIFM00000000
	FF_139	196	5.84	5641	PIFL00000000
	FF_144	195	5.90	5616	PIFK00000000
	ZS_107	56	5.46	4920	PIFJ00000000
	ZS_117	101	5.35	4847	PIFI00000000
	ZS_138	90	5.34	4865	PIFH00000000
	ZS_173	58	5.49	4906	PIFG00000000
	ZS_181	69	5.28	4968	PIFF00000000
	ZS_183	64	5.35	4841	PIFE00000000
	ZS_185	102	5.45	4960	PIFD00000000
	ZS_198	82	5.39	4873	PIFC00000000
	ZS_2	80	5.47	4966	PIFB00000000
	ZS_211	270	5.56	5282	PIFA00000000
	ZS_213	96	5.47	4953	PIEZ00000000
	ZS_58	78	5.37	4869	PIEY00000000
	ZS_82	53	5.38	5089	PIEX00000000
ZS_90	46	5.38	5085	PIEW00000000	
ZF_41	87	5.74	5310	PIEV00000000	

**Table S2:** Strains used in the present study

Name	Description	Reference
PIR2	(F-) $\Delta$ lac169 rpoS(am) robA1 creC510 hsdR514 endA recA1 uidA( $\Delta$ Mlu I)::pir	Invitrogen, USA
$\beta$ 3914	$\beta$ 2163 <i>gyrA462</i> , <i>zei298::Tn10</i> [Km <sup>R</sup> Em <sup>R</sup> Tc <sup>R</sup> ]	Le Roux <i>et al.</i> , 2007
BL21 (DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3)	Novagen, USA
GV1300	BL21 (DE3) + pFO4_GFP_R5-7 <sub>crass</sub>	This study
GV1549	BL21 (DE3) + pFO4_GFP_R5-7 <sub>F12</sub>	This study
TOP10	F- <i>mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ <i>lacX74</i> <i>recA1 araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galU galK rpsL(Str<sup>R</sup>) endA1 nupG</i>	Invitrogen, USA
GV1460	J2-8 ( <i>Vibrio</i> sp. nov F13-like)	Lemire <i>et al.</i> , 2015
GV1422	J2-9 ( <i>V. crassostreae</i> )	Lemire <i>et al.</i> 2015
GV1495	J2-9 $\Delta$ R5.7	Lemire <i>et al.</i> , 2015
GV1040	10N.286.48.B8 (hereafter named B8)	Hunt <i>et al.</i> , 2008
GV1826	B8 $\Delta$ R-a	This study
GV1872	B8 $\Delta$ R-b	This study
GV1906	B8 $\Delta$ R-c	This study
GV1874	B8 $\Delta$ R-d	This study
GV1895	B8 $\Delta$ R-e	This study
GV1897	B8 $\Delta$ R-f	This study
GV1899	B8 $\Delta$ R-g	This study
GV1830	B8 $\Delta$ R-h	This study
GV1601	B8 $\Delta$ R-i	This study
GV1870	B8 $\Delta$ R-j	This study
GV1876	B8 $\Delta$ R-k	This study
GV1908	B8 $\Delta$ R-l	This study
GV1832	B8 $\Delta$ R-m	This study
GV1828	B8 $\Delta$ R-n	This study
GV1903	B8 $\Delta$ R-o	This study
GV1891	B8 $\Delta$ R-p	This study
GV1887	B8 $\Delta$ R-q	This study
GV1889	B8 $\Delta$ R-r	This study
GV1884	B8 $\Delta$ R-s	This study
GV1885	B8 $\Delta$ R-t	This study
GV1858	B8 $\Delta$ R-u	This study
GV1850	B8 $\Delta$ R-v	This study
GV1901	B8 $\Delta$ R-w	This study
GV1905	B8 $\Delta$ R-x	This study
GV1832	B8 $\Delta$ R-m	This study
GV1828	B8 $\Delta$ R-n	This study
GV1903	B8 $\Delta$ R-o	This study

GV1568	B8 $\Delta R5.7$	This study
GV2825	B8 $\Delta R5.8$	This study
GV2620	B8 $\Delta R-g + pMRB-P_{LACR-5.7_{F12}}$	This study
GV2621	B8 $\Delta R5-7 + pMRB-P_{LACR-5.7_{F12}}$	This study
GV2288	8T2_11 ( <i>V. chagasii</i> , #3)	Bruto et al., 2016
GV2455	8T2_11 $\Delta R5.7$	This study
GV2423	7T7_2 ( <i>V. splendidus</i> , #24)	Bruto et al., 2016
GV2493	7T7_2 $\Delta R5.7$	This study
GV1054	ZS_173 ( <i>V. splendidus</i> , F18)	Hunt <i>et al.</i> , 2008
GV2266	ZS_173 $\Delta MARTX$	This study
GV1065	ZS_185 ( <i>V. splendidus</i> , F18)	This study
GV1882	ZS_185 $\Delta MARTX$	This study
GV2466	ZS_185 $\Delta R5-7$	This study
GV1067	ZS_213 ( <i>V. splendidus</i> , F18)	Hunt <i>et al.</i> , 2008
GV2267	ZS_213 $\Delta MARTX$	This study

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**Table S3:** Plasmids used and constructed in this study

<b>Name</b>	<b>Description</b>	<b>Reference</b>
pSW23T	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; $\square$ Cm <sup>R</sup> $\square$	Demarre <i>et al.</i> , 2005
pSW $\delta$ R5.7	pSW23T; $\Delta$ R5.7 <sub>8T2_11</sub> & ZS_185	This study
pSW7848T	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; <i>araC-P<sub>BADccdB</sub></i> ; $\square$ Cm <sup>R</sup> $\square$	Le Roux <i>et al.</i> , 2007
pSW $\delta$ R-a	pSW7848T; $\Delta$ R-a	This study
pSW $\delta$ R-b	pSW7848T; $\Delta$ R-b	This study
pSW $\delta$ R-c	pSW7848T; $\Delta$ R-c	This study
pSW $\delta$ R-d	pSW7848T; $\Delta$ R-d	This study
pSW $\delta$ R-e	pSW7848T; $\Delta$ R-e	This study
pSW $\delta$ R-f	pSW7848T; $\Delta$ R-f	This study
pSW $\delta$ R-g	pSW7848T; $\Delta$ R-g	This study
pSW $\delta$ R-h	pSW7848T; $\Delta$ R-h	This study
pSW $\delta$ R-i	pSW7848T; $\Delta$ R-i	This study
pSW $\delta$ R-j	pSW7848T; $\Delta$ R-j	This study
pSW $\delta$ R-k	pSW7848T; $\Delta$ R-k	This study
pSW $\delta$ R-l	pSW7848T; $\Delta$ R-l	This study
pSW $\delta$ R-m	pSW7848T; $\Delta$ R-m	This study
pSW $\delta$ R-n	pSW7848T; $\Delta$ R-n	This study
pSW $\delta$ R-o	pSW7848T; $\Delta$ R-o	This study
pSW $\delta$ R-p	pSW7848T; $\Delta$ R-p	This study
pSW $\delta$ R-q	pSW7848T; $\Delta$ R-q	This study
pSW $\delta$ R-r	pSW7848T; $\Delta$ R-r	This study
pSW $\delta$ R-s	pSW7848T; $\Delta$ R-s	This study
pSW $\delta$ R-t	pSW7848T; $\Delta$ R-t	This study
pSW $\delta$ R-u	pSW7848T; $\Delta$ R-u	This study
pSW $\delta$ R-v	pSW7848T; $\Delta$ R-v	This study
pSW $\delta$ R-w	pSW7848T; $\Delta$ R-w	This study
pSW $\delta$ R-x	pSW7848T; $\Delta$ R-x	This study
pSW $\delta$ R5.7 <sub>F12</sub>	pSW7848T; $\Delta$ R5.7 <sub>F12</sub>	This study
pSW $\delta$ $\square$ 5.8 <sub>F12</sub>	pSW7848T; $\Delta$ R5.8 <sub>F12</sub>	This study
pSW $\delta$ MARTX	pSW7848T; $\Delta$ MARTX	This study
pMRB-P <sub>LAC</sub> R-5.7 <sub>F12</sub>	<i>oriV<sub>R6K<math>\gamma</math></sub></i> ; <i>oriT<sub>RP4</sub></i> ; <i>oriV<sub>pB1067</sub></i> ; P <sub>lac</sub> R-5.7 <sub>F12</sub> [Cm <sup>R</sup> ]	This study
pMRB-P <sub>LAC</sub> GFP	<i>oriV<sub>R6K<math>\gamma</math></sub></i> ; <i>oriT<sub>RP4</sub></i> ; <i>oriV<sub>pB1067</sub></i> ; P <sub>lac</sub> GFP [Cm <sup>R</sup> ]	Bruto <i>et al.</i> , 2016
pFO4	Amp <sup>R</sup> , T7lac promoter, His.Tag	Groisillier <i>et al.</i> , 2010
pFO4-R5.7 <sub>F12</sub>	pFO4-HisTag-GFP-R5.7 <sub>F12</sub>	This study
pFO4-R5.7 <sub>crass</sub>	pFO4-HisTag-GFP-R5.7 <sub>crass</sub>	This study

**Table S4:** Primers used in this study

Use	Name	Sequence 5'-3' <sup>a</sup>
Deletion of R-a	R-a-1	GCCCGAATTCGACATATCCCGGTGGTTCAC
	R-a-2	CCAATAAAGAGAGGAGCCTGGGAAGTTAGTGG GCTTTGTG
	R-a-3	CACAAAGCCCACTAACTTCCCAGGCTCCTCTCT TTATTGG
	R-a-4	GCCCGAATTCCAACACACCACCAACCTACG
Deletion of R-b	R-b-1	GCCCGAATTCATCGCCCCAGATTTACTGG
	R-b-2	CACTCGTTATGCTGGTGAGTGGAGTCGTTTGTG TAAAGGG
	R-b-3	CCCTTTACACAAACGACTCCACTCACCAGCATA ACGAGTG
	R-b-4	GCCCGAATTCGTGTGGTTACGCCTTCATCG
Deletion of R-c	R-c-1	GCCCGAATTCCTTGCCGCTCTCGTAGATTG
	R-c-2	CGATACTTCTGGCTGCCATAGTGAGGCATTGAG CTTTGTG
	R-c-3	CACAAAGCTCAATGCCTCACTATGGCAGCCAG AAGTATCG
	R-c-4	GCCCGAATTCGTCGCGATAAAAGTAACGC
Deletion of R-d	R-d-1	GCCCGAATTCGCGATTTCATCAGAGTTCCAG
	R-d-2	CAGGGTTACTGGAATTGTCCGCAAAGGTGAGG GTTTCAGG
	R-d-3	CCTGAAACCCTCACCTTTGCGGACAATTCCAGT AACCTG
	R-d-4	GCCCGAATTCGGTAAACGTAAACGTGATGCG
Deletion of R-e	R-e-1	GCCCGAATTCGGGTAAGTGTGCTACTGTCTC
	R-e-2	CAACTAGTGCAATGAGAGCCAAGCCAGTCAAT AGACTGGC
	R-e-3	GCCAGTCTATTGACTGGCTTGGCTCTCATTGCA CTAGTTG
	R-e-4	GCCCGAATTCCTTGGCATCGCTTCAACACC
Deletion of R-f	R-f-1	GCCCGAATTCCTGCATTGCTGAGTCGAAG
	R-f-2	GAGCCTGAAGCGTATCAGATCCATGCTATGTCG GCCTTTG
	R-f-3	CAAAGGCCGACATAGCATGGATCTGATACGCT TCAGGCTC
	R-f-4	GCCCGAATTCCAAGTTACTCTCCCACGGAG
Deletion of R-g	R-g-1	GCCCGAATTCCTACGCAGCCAACATGCAAC
	R-g-2	GGCAAAGGCATTTTACCGTGCCATAAAAAGG CCGACATC
	R-g-3	GATGTCGGCCTTTTTATGGCACGGTGAAAATGC CTTTGCC
	R-g-4	GCCCGAATTCCTCGATCTCGATTGGACTGC

Deletion of R-h	R-h-1	GCCCGAATTCGTGATCCAGCCTTTGTTTCAG
	R-h-2	GATATTCGCGAAGCAGCACCCCTGTTGAGTTGACAGGAGG
	R-h-3	CCTCCTGTCAACTCAACAGGGGTGCTGCTTCGC GAATATC
	R-h-4	GCCCGAATTCCTTTGGAGCTGCGTTCAAAC
Deletion of R-i	R-i-1	GCCCGAATTCGGTAGTGGCTGATGAAGTTC
	R-i-2	GCTGCCGTGGTGATATGTATAAAATGTCCCGCA TTAGGCG
	R-i-3	CGCCTAATGCGGGACATTTTATACATATCACCA CGGCAGC
	R-i-4	GCCCGAATTCCTGGCGCTGTATGACCAAAC
Deletion of R-j	R-j-1	GCCCGAATTCGCAGAAGTATGGTAAGTCCC
	R-j-2	GAAGTACCCAGGGTCGTTTGAATGCCAATACC AAGCGCAG
	R-j-3	CTGCGCTTGGTATTGGCATTCAAACGACCCTGG GTACTTC
	R-j-4	GCCCGAATTCATGCTTTAGGTGCACACGC
Deletion of R-k	R-k-1	GCCCGAATTCAGACACAGACATCGGCTATG
	R-k-2	GCATTGAAGTAGGGGACATCGTGAGCAAATAG CGTACCTG
	R-k-3	CAGGTACGCTATTTGCTCACGATGTCCCCTACT TCAATGC
	R-k-4	GCCCGAATTCGGTATTGGTGATGTAGCCTC
Deletion of R-l	R-l-1	GCCCGAATTCAGATAACGTAGCAGCAGG
	R-l-2	GTGCCTACAGTCCGAGTAATAAACAGCCAGCA TAAGGCTG
	R-l-3	CAGCCTTATGCTGGCTGTTTATTACTCGGACTG TAGGCAC
	R-l-4	GCCCGAATTCCTGCGCTGTCGTTAGCAAAG
Deletion of R-m	R-m-1	GCCCGAATTCACCAACCAACGAAAGTGGTG
	R-m-2	CAAGATGTTACCTCTGCGCTGTGTTGCAGCTTT CATTGGC
	R-m-3	GCCAATGAAAGCTGCAACACAGCGCAGAGGTA ACATCTTG
	R-m-4	GCCCGAATTCCTTCCATAACCTCTGTGC
Deletion of R-n	R-n-1	GCCCGAATTCACAAGACACAATCCATCACC
	R-n-2	CAGTGGTGAAGTTCAGAAGCTATTAGGTGACC TCCAACG
	R-n-3	CGTTGGGAGGTCACCTAATAGCTTCTGAACTTC ACCACTG
	R-n-4	GCCCGAATTCGACATCGAGAACTTGGTC
Deletion of R-o	R-o-1	GCCCGAATTCGTTGCGTTTGCGGTATCAAG
	R-o-2	CCCGAAGGCTACACTTATTCGACTCTGTGAGCA TGTTTGG

	R-o-3	CCAAACATGCTCACAGAGTCGAATAAGTGTAG CCTTCGGG
	R-o-4	GCCCGAATTCATAGTCTATCGTGCTGCAGG
Deletion of R-p	R-p-1	GCCCGAATTCCTAATTCAGGATCGCCATC
	R-p-2	CATACGCTCAGCAGTATTCGCAAGCACGATGA CAGTTGAC
	R-p-3	GTCAACTGTCATCGTGCTTGCGAATACTGCTGA GCGTATG
	R-p-4	GCCCGAATTCGGTCAGATCAAAGACCGATG
Deletion of R-q	R-q-1	GCCCGAATTCGAGACTTCGTGTGTCTCTGC
	R-q-2	GTGGGCTTTTTAACGTCTGCCTGGTACATTAGC CAGCTTC
	R-q-3	GAAGCTGGCTAATGTACCAGGCAGACGTTAAA AAGCCAC
	R-q-4	GCCCGAATTCGCAATGGAAGAGCAAGACAG
Deletion of R-r	R-r-1	GCCCGAATTCCTCCTGAACAGTAAGCGGAC
	R-r-2	GAACATCGATTCTCAGCCTCAATTGGGGAGAG CATTAGGC
	R-r-3	GCCTAATGCTCTCCCAATTGAGGCTGAGAATC GATGTTT
	R-r-4	GCCCGAATTCGGTAGGATGGATGAGGAAAC
Deletion of R-s	R-s-1	GCCCGAATTCGCATCGAACAATGGGATTGG
	R-s-2	CGGTATTACCCAACGGAAACGACATAGAGTAA GACCTCGC
	R-s-3	GCGAGGTCTTACTCTATGTCGTTTCCGTTGGGT AATACCG
	R-s-4	GCCCGAATTCCTACTTCTCGGCCATCAAAC
Deletion of R-t	R-t-1	GCCCGAATTCCTAACAGTACTCAGCGCTC
	R-t-2	CTCGTATCTAGCTACCCTTCGTAAGCAAGCCAA GCATCAG
	R-t-3	CTGATGCTTGGCTTGCTTACGAAGGGTAGCTAG ATACGAG
	R-t-4	GCCCGAATTCGTTCTACGTGAACTACCAG
Deletion of R-u	R-u-1	GCCCGAATTC AAGGCTATCGTGCCGCATTC
	R-u-2	CCAATACTGCCACAAAGTGCATTTGTGCGGAC AACCTACG
	R-u-3	CGTAGGTTGTCCGCACAAATGCACTTTGTGGCA GTATTGG
	R-u-4	GCCCGAATTCCTGAGGTTGACCACGTTTCG
Deletion of R-v	R-v-1	GCCCGAATTCCTGAACAAGCGTGTGATCACC
	R-v-2	GGTAAGGGGTCGACTTTACCGCTCACGCTAAA GTAACCAC
	R-v-3	GTGGTTACTTTAGCGTGAGCGGTAAAGTCGACC CCTTACC
	R-v-4	GCCCGAATTCGCACACTATCGACATGACAG

Deletion of R-w	R-w-1	GCCCGAATTCCACAATGGACAAGTCCCATC
	R-w-2	GACTTGTGTCAGCCAAACACCTTCCATGCCCAA ACTGGAG
	R-w-3	CTCCAGTTTGGGCATGGAAGGTGTTGGCTGAC AAGTGTC
	R-w-4	GCCCGAATTCGCACCGCTAAGTCATCAACC
Deletion of R-x	R-x-1	GCCCGAATTC AAGTAATGCGGGACTGGAC
	R-x-2	CAACCCAGTTCTCAACAGGGATGTTGCAACG TTACCGAC
	R-x-3	GTCGGTAACGTTGCAACATCCCTGTTGAGAACT GGGGTTG
	R-x-4	GCCCGAATTCATGTTCAAGTGCCTCACCTC
Deletion of R5.7	R5.7-1	GTATCGATAAGCTTGATATCGAATTCCTCTTAC CATCGCTCTCAG
	R5.7-2	CCAAAAGGCCGACAAGATGGGGTAGCAGAA ACCAACACC
	R5.7-3	GGTGTGGTTTCTGCTACCCCATCTTGTCGGCC TTTTTGG
	R5.7-4	CCCCGGGCTGCAGGAATTCCTACGCAGCCAA CATGCAAC
Deletion of R5.8	R5.8-1	GTATCGATAAGCTTGATATCGAATTCCTCTCCTG CAATCACAGTCAC
	R5.8-2	CTAATCACAAATCTAACAGGGATAAAACTCCA TACCCATCGACAAATAG
	R5.8-3	CTATTTGTCGATGGGTATGGAGTTTTATCCCTG TTAGATTTGTGATTAG
	R5.8-4	CCCCGGGCTGCAGGAATTCACCTCTTAGTTAT TTGGCACAG
Inactivation of R5.7	0504-1	CGTATGCCTGAACATAGTTAG
	0504-2	GGGATCTGATGATCACCGAG
Deletion of <i>rtxACHBDE</i>	MX1	GCCCGAATTCAGATACATCGGACCATGCTG
	MX2	GGATACTCAGGTACAGCCTAAGGAAGTGACGT GATGGTAC
	MX3	GTACCATCACGTCACCTCCTTAGGCTGTACCTG AGTATCC
	MX4	GCCCGAATTCGCTCGAATTGATAGGTTGCG
R5.7 <sub>F12</sub> in MRB	1102-1	GTGAGCGGATAACAAAGGAAGGGCCCATGAG AATTCTACCGATTATTATTTCTC
	1102-2	CGACGCGTCTGCAGCTCGAGTCAGTTAGAACA ACTGTTGCCATTC
	0207-1	CTCGAGCTGCAGACGCGTCG
	0207-2	GGGCCCTTCCTTTGTTATCCGCTCAC
R5.7 <sub>F12</sub> in pFO4	1102-3	GGTGGTGGTTCTGGTGGTGGTTCTAGTACTGG CTAGAGCTTAATAAC

	1102-4	CATGCATGCTAGCCTCGAGTTAGTTAGAACAAC TGTTGCCATTC
	0904-1	CATCACCATCACCATGGATCCAGTAAAGGAGA AGAACTTTTC
	0904-2	AGAACCACCACCAGAACCACCACCTTTGTATA GTTTCATCCATGC
	2003-1	GGA TCC ATG GTG ATG GTG ATG
	2003-2	CTC GAG GCT AGC ATG CAT G
<hr/>		
R5.7 <sub>cras</sub> in pFO4	0904-5	GGTGGTGGTTCTGGTGGTGGTTCTAGCGACTGG CTTGA ACTG
	0904-6	CATGCATGCTAGCCTCGAGTTAATTAGAGCAGC TATTGCC
		<hr/>

<sup>a</sup> restriction sites are indicated in bold

**Table S5:** Annotation of *Vibrio* sp. F12 specific regions.

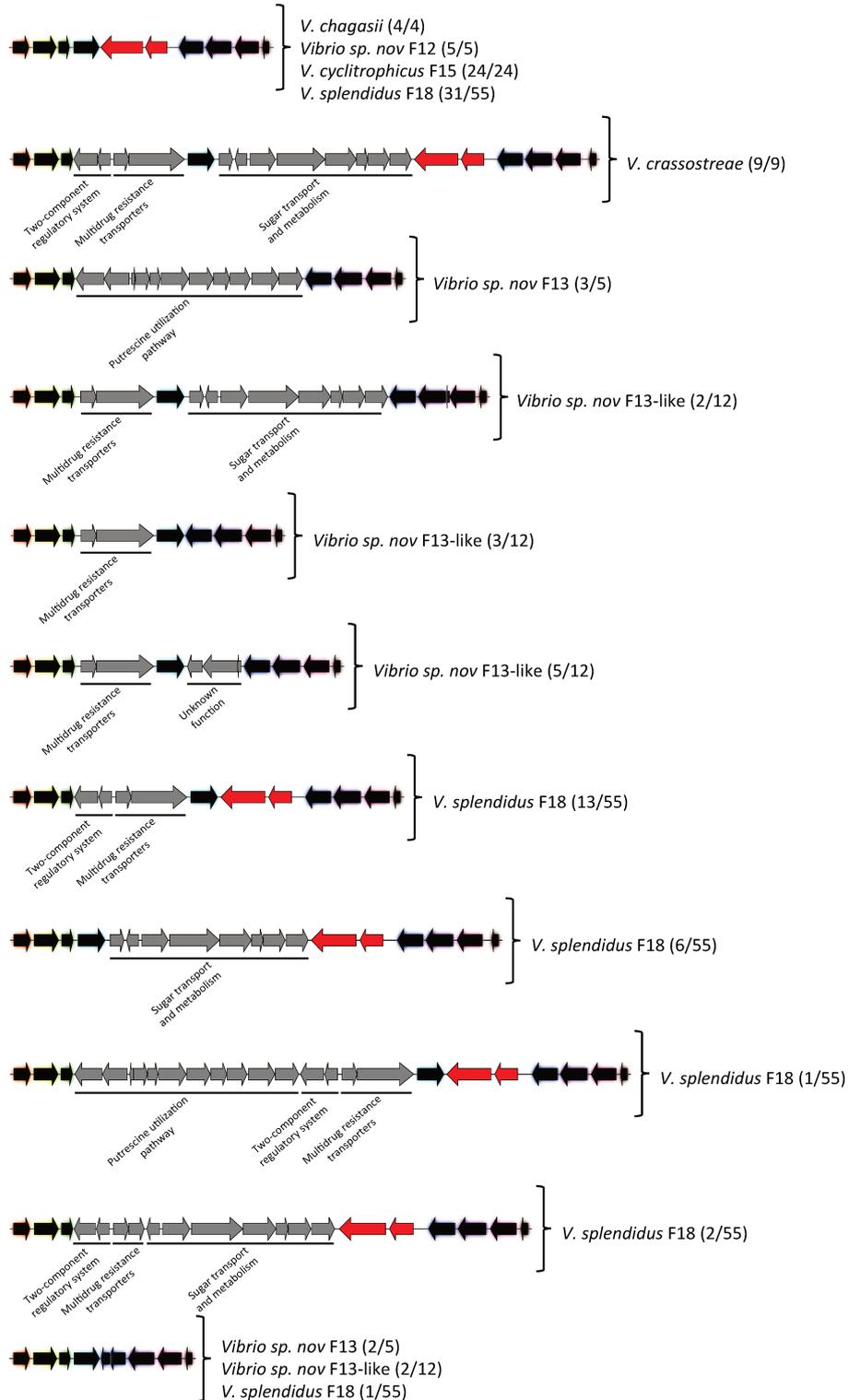
<b>Region</b>	<b>Locus tag<sup>a</sup></b>	<b>Product</b>
R-a	160071	AraC-type DNA-binding domain-containing protein
	160070	Putative MOSC domain protein
R-b	180051	Iron-hydroxamate transporter permease subunit
	180052	Ferrichrome-binding protein
	180053	Ferrichrome outer membrane transporter
	180055	Transcriptional activator
	180056	Conserved protein of unknown function with ferric iron reductase domains
	180057	Iron-hydroxamate transporter subunit ; ATP-binding component of ABC superfamily
R-c	10026	Putative Transcription regulator, AsnC-type protein
	10027	Conserved membrane protein of unknown function
R-d	10384	Putative Transcription regulator, TetR-like
	10385	Putative dihydrofolate reductase
	10386	Proton/sodium-glutamate symport protein
R-e	40063	NADH:flavorubredoxin oxidoreductase
	40065	Flavorubredoxin oxidoreductase
R-f	40179	Transcriptional regulator, LysR family protein
	40180	Conserved exported protein of unknown function
R-g	40197	Conserved exported protein of unknown function (R5.7)
	40198	Conserved exported protein of unknown function (R5.8)
R-h	40249	Conserved membrane protein of unknown function
	40250	Uncharacterized HTH-type transcriptional regulator
	40251	Putative glutathione S-transferase
	40252	Putative transcriptional regulator, LysR family protein
	40253	Putative methyl-accepting chemotaxis protein
	40254	Cryptochrome-like protein cry2
	40255	Sigma-54 dependent transcriptional regulator
	40256	Aldehyde dehydrogenase B
	40257	Putative NUDIX hydrolase
	40258	Conserved protein of unknown function
	40259	Putative multidrug efflux transporter
	40260	S-formylglutathione hydrolase
	40261	Alcohol dehydrogenase class 3 (Alcohol dehydrogenase class III)
	40262	Quaternary ammonium compound-resistance protein QacE
	200002	Murein peptide amidase A
	200003	Periplasmic oligopeptide-binding protein
	200004	Putative Ethylbenzene dehydrogenase
200006	Putative cytochrome c-type protein NrfB	
200007	Putative tetraheme cytochrome c3	
R-i	200027	Putative Organic hydroperoxide resistance protein
	200028	Putative Organic hydroperoxide resistance transcriptional regulator
R-j	200054	Putative two component histidine kinase

	200055	Two-component response regulator of C4-dicarboxylate transport
	200056	Putative glycerophosphoryl diester phosphodiesterase
	200057	Putative Phosphonate-binding periplasmic transporter
	200058	Putative Phosphonates import ATP-binding protein PhnC
	200059	Putative PhnE phosphonate ABC transporter, permease
	200060	Putative Phosphonate ABC transporter permease protein phnE1
R-k	310012	Conserved protein of unknown function
	310011	Conserved exported protein of unknown function
R-l	130064	Putative 5'-nucleotidase
	130062	Multiple antibiotic resistance protein marC
R-m	100119	Putative membrane bound peptidase; NefD homolog
	100118	Putative Stomatin like transmembrane protein
R-n	100095	Conserved exported protein of unknown function
	100094	Conserved protein of unknown function
R-o	30035	Putative Signal transduction protein containing an EAL and a GGDEF domains
	30034	Putative ABC-type sugar transport system, periplasmic component
	30033	Putative Ribose ABC transporter, periplasmic ribose-binding protein
R-p	170092	Conserved exported protein of unknown function
	170091	Conserved exported protein of unknown function
	170090	Conserved protein of unknown function
	170089	Conserved exported protein of unknown function
	170088	Conserved exported protein of unknown function
	170087	Putative Transcriptional regulator, LuxR family
	170081	Endonuclease/Exonuclease/phosphatase family protein
R-q	80118	GGDEF family protein
	80117	Putative Outer membrane protein and related peptidoglycan-associated (lipo)proteins
	80116	Conserved protein of unknown function
	80115	Conserved protein of unknown function
	80114	TadE-like protein
	80113	Conserved exported protein of unknown function
	80112	Conserved membrane protein of unknown function
	80111	Putative Flp pilus assembly protein TadB
	80109	Putative Flp pilus assembly ATPase TadZ/CpaE
	80108	Conserved exported protein of unknown function
	80106	Putative Flp pilus assembly protein RcpC/CpaB
	80105	Conserved membrane protein of unknown function
	80104	Putative pilin Flp
	80103	Bacterial regulatory helix-turn-helix , lysR family protein
R-r	80026	Conserved exported protein of unknown function
	80025	Conserved exported protein of unknown function
	80024	Conserved protein of unknown function
	80023	Conserved exported protein of unknown function
R-s	80012	Fe <sup>2+</sup> /Zn <sup>2+</sup> uptake regulation protein

	80011	Adenine deaminase family protein
	80010	Putative carbonate dehydratase
	80009	Putative Cobalamin (vitamin B12) biosynthesis CobW-like
R-t	250021	Transcriptional regulator
	250022	Conserved membrane protein of unknown function
R-u	60205	Conserved exported protein of unknown function
	60204	Conserved protein of unknown function
R-v	60093	Putative Cytochrome c556
	60092	Hydrogenase cytochrome B-type subunit-like protein
	60091	Transcriptional regulator, MarR family protein
R-w	60041	Transcriptional regulator
	60040	Conserved exported protein of unknown function
R-x	350020	Predicted Na <sup>+</sup> /dicarboxylate symporter
	440001	Coenzyme A disulfide reductase
	440002	Conserved exported protein of unknown function

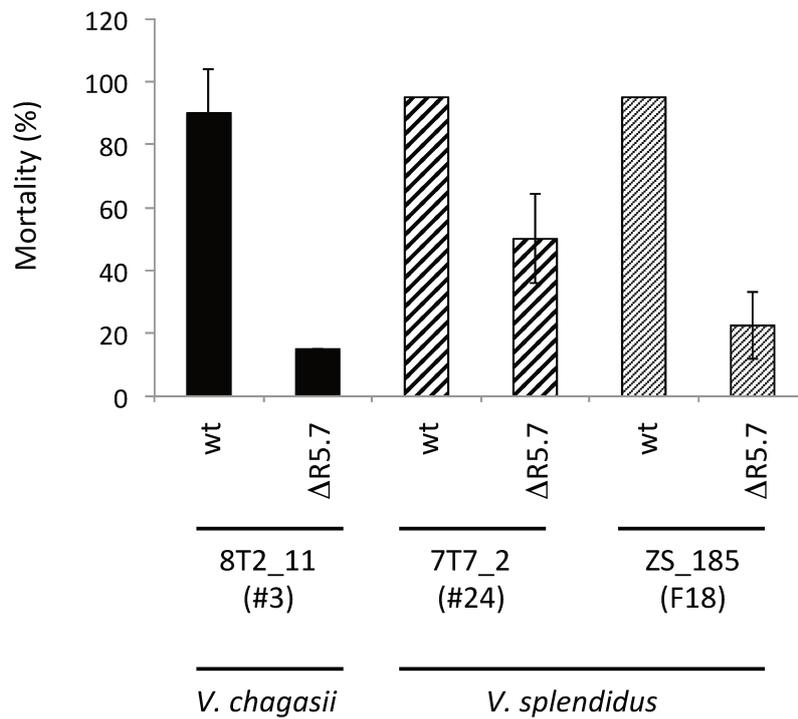
<sup>a</sup>Locus tag in B8 (VB12B8\_v1\_XXXXXX)

## Figures



**Figure S1: Comparison of the genetic organization of the R5-7/8 locus in the Splendidus clade.**

The first genetic organization (top of the figure) is mostly found in the populations of the Splendidus clade and was used as a reference for Figure 3. The R5.7 and R5.8 genes are represented with red arrows. The surrounding genes of R5-7/8 are represented by black arrows shrouded by colors to keep track in the different genetic organization shown in this figure. Genes in grey are intermittently present in the R5-7 locus and encode for different metabolic activities that are indicated when known. The distribution among different species the Splendidus clade of the different genetic organization of the R5-7/8 locus is shown on the right.



**Figure S2: Virulence of R5.7 inactivated strains belonging to the Splendidus clade.** Comparison of mortalities induced by wild type strain (wt) and R5.7 inactivated mutant of a strain representative of *V. chagasii* (8T2\_11) and *V. splendidus* (7T7\_2) isolated in France (population #3 and 24 in Bruto et al., 2016) and *V. splendidus* isolated in Plum Island (population F18 in Hunt et al., 2008). All infections were performed in duplicate and at least twice by intramuscular injection of strains ( $10^7$  cfu/animal) in 20 oysters and counting the percentage of mortalities at 24H (y-axis).

## Additional information

Table A1 | Accession number of R5-7 and R5-8 genes

<b>Gene</b>	<b>Mage locus tag</b>	<b>NCBI locus tag</b>
R5-7	VB12B8_v1_40197	VCR9J2_730269
R5-8	VB12B8_v1_40198	VCR9J2_730268

## Chapter II Emergence of pathogenic vibrios in oyster farms

### II.A *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease

In France, the oyster-farming industry is facing mortality outbreaks that have been rising over the past decades. The death of juvenile oysters has been associated with the presence of a new genotype of the herpes virus, OsHV-1  $\mu$ var and several populations of vibrios. Although experimental infection confirmed the virulence of both infectious agents in laboratory condition, knowledge on the complexity of infections occurring in the wild remains limited. **Here, we wanted to explore the role of each of these putative infectious agents in oyster disease.**

To study the infectious agents' dynamics leading to oyster mortality, specific-pathogen free (SPF) oysters were naturally infected in Brest oyster-farming area during a mortality event (when seawater temperature  $>16^{\circ}\text{C}$ ). Cumulative mortalities were recorded on field and viral, total culturable bacteria and *Vibrio* loads were quantified from ten oysters sacrificed at different intervals throughout the disease development. We found that a rapid *Vibrio* colonization precedes viral replication and oyster mortality induction. No correlation was found between vibrios and viral load in co-infected animals. However, OsHV-1 load is a proxy to predict if animals will die.

To assess the importance of bacteria in oyster mortality induction, a cohabitation experiment was performed between a batch of SPF oysters infected in the field as “donor” and a SPF “recipient” batch. Cohabitation was made in aquaria in the presence or absence of an antibiotic. We demonstrated that in the absence of bacteria, a high virus load was not sufficient to induce high mortality rate. This result suggests that bacteria play an important role in the induction, transmission and development of oyster disease.

Finally, cohabitation experiments were designed to determine whether oysters carry putative pathogens outside mortality events (seawater temperature  $< 16^{\circ}\text{C}$ ). First, a cohabitation experiment was conducted between spats coming from natural settlement zones from 6 different geographic origins as “donor” batch and SPF hatched spats as “recipient” batches. 4/6 donor batches were able to induce mortalities in the recipient batches. Detection of OsHV-1 was always correlated with mortalities in donor and recipient batches. A second experiment was

performed between adult farmed or wild oysters from different geographical origins as “donors” and juvenile SFP oysters of different ages as “recipients”. Only 1/10 set of wild oyster donors expressed mortalities and was able to transmit the disease to the recipients whilst 13/18 donor batches of farmed oysters were capable, suggesting that oysters can host putative pathogens (e.g OsHV1 herpesvirus) without apparent sign of disease. In this experiment on adults, the virus was only detected once in a recipient batch. This last result shows that OsHV-1 does not play a significant role in adult disease and confirms that the disease affecting adult oysters is different from the one killing juvenile oysters.

Overall, this study reveals the importance of vibrios in oyster disease induction and development. Due to its systematic detection in diseased juvenile oysters, the virus OsHV-1 is a good indicator of the disease but it is not sufficient to induce high mortalities. Oysters may act as reservoirs of pathogenic vibrios and disease expression depends on geographical origin, age and farming practices of oysters.

This work is detailed in the article entitled “***Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease**” published in *Frontiers in Microbiology* in July 2015. [In this study, I performed the DNA extraction and \*hsp60\* amplification for sequencing and statistical analyses.](#)

# *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease

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Successive disease outbreaks in oyster (*Crassostrea gigas*) beds in France have resulted in dramatic losses in production, and subsequent decline in the oyster-farming industry. Deaths of juvenile oysters have been associated with the presence of a herpes virus (OsHV-1  $\mu$ var) and bacterial populations of the genus *Vibrio*. Although the pathogenicity of OsHV-1  $\mu$ var, as well as several strains of *Vibrio* has been demonstrated by experimental infections, our understanding of the complexity of infections occurring in the natural environment remains limited. In the present study, we use specific-pathogen-free (SPF) oysters infected in an estuarine environment to study the diversity and dynamics of cultured microbial populations during disease expression. We observe that rapid *Vibrio* colonization followed by viral replication precedes oyster death. No correlation was found between the vibrio concentration and viral load in co-infected animals. We show that the quantity of viral DNA is a predictor of mortality, however, in the absence of bacteria, a high load of herpes virus is not sufficient to induce the full expression of the disease. In addition, we demonstrate that juvenile mortalities can occur in the absence of herpes virus, indicating that the herpes virus appears neither essential nor sufficient to cause juvenile deaths; whereas bacteria are necessary for the disease. Finally, we demonstrate that oysters are a reservoir of putative pathogens, and that the geographic origin, age, and cultivation method of oysters influence disease expression.

**Keywords:** pacific oysters, summer mortality, herpes virus, vibrio pathogenicity, experimental infection

## Introduction

The oyster aquaculture industry in France has been shaped by various infectious diseases caused by bacteria, viruses, or parasites (Grizel and Héral, 1991; Cochenne et al., 2000; Le Roux et al., 2001). Today, cultivation of the imported species, *Crassostrea gigas* (Pacific oyster), constitutes the vast majority of oyster farming (Rohfritsch et al., 2013), and diseases affecting this species have been steadily rising over the past decades, threatening the long-term survival of farmed and natural stocks. For example, a syndrome known as “summer mortality” has been affecting oyster production on the west coast of France (Samain, 2008). This syndrome has been associated with an ostreid herpes virus, designated OsHV-1, and bacteria of the genus *Vibrio* as infectious agents

(Samain, 2008). “Summer mortality” is also thought to be influenced by elevated temperature (> 19°C), physiological stress associated with maturation, genetic traits of the host, and aquaculture practices. However, none of these individual factors have been shown to consistently be responsible for the syndrome.

In addition to “summer mortality,” since 2008, juvenile oyster (<12 months) deaths have increased considerably, and current mortality levels range between 60 and 90% (Martenot et al., 2011). These events are more pervasive because they occur at a lower threshold temperature (16°C) and geographically extend to all coastal regions in France (Atlantic, Channel, and Mediterranean; Pernet et al., 2012; Petton et al., 2013). This dramatic mortality level coincides with an increase in the detection frequency of herpes virus and with the identification of OsHV-1 variants, the OsHV-1  $\mu$ var being the most predominant genotype (Segarra et al., 2010; Martenot et al., 2011, 2013). It has been postulated that the variant OsHV-1  $\mu$ var is more virulent than the reference strain (OsHV-1) and could be the etiological agent of juvenile mortalities (Segarra et al., 2010; Martenot et al., 2011, 2013; Schikorski et al., 2011a,b). However, these assumptions are speculative (to the authors’ knowledge, no published data has formally demonstrated that the pathogenicity of OsHV-1  $\mu$ var is higher than that of OsHV-1) and await further confirmations. In particular, recent technological improvements in the procedures used to detect the viral DNA load in animal tissues now offer an opportunity revisiting these assumptions. The real-time PCR-based assays recently developed and now widely used as detection tools have been shown to be at least two orders of magnitude more sensitive than earlier quantitative PCR assays (Arzul et al., 2002; Pepin et al., 2008; Martenot et al., 2010). Hence, the determination of the viral DNA quantity in archived samples (collected before 2008) using the older and newer protocols should be performed to verify that the increase of mortality correlates with an increase in virus prevalence. Therefore, it is yet to be determined if the increase in juvenile oyster mortalities since 2008 corresponds to a new disease or to a worsening summer mortality syndrome, emphasizing the need to continue investigating the diversity and functioning of all oyster disease agents.

This is particularly important for oyster farming because the only regulation implemented to help the oyster industry to date is based on the hypothesis that juvenile mortalities are due to the emergence of a new disease connected to OsHV-1  $\mu$ var. The European Union Regulation (175/2010), implemented in March of 2010, suggests that when the presence of OsHV-1  $\mu$ var is detected, disease control measures should be implemented, including the establishment of a containment area to restrict the movement of *C. gigas* oysters. Thus, demonstrating that the herpes virus is a marker of the disease and/or the unique etiological agent requires further investigations.

Previous research efforts have mainly focused on the viral hypothesis and knowledge on the role of bacteria remains limited. We recently investigated the oyster disease ecology of microdiverse *Vibrio* genotypes using a new field-based approach (Lemire et al., 2014). We took advantage of recently developed specific-pathogen-free (SPF) juveniles of *C. gigas*, which become naturally infected when placed in an estuarine

environment (Petton et al., 2013). We showed that the onset of disease in oysters is associated with progressive replacement of diverse and benign colonizers by members of a non-clonal but phylogenetically coherent virulent population of *Vibrio crassostreae*. While the detection of putative pathogens (OsHV-1- $\mu$ var and *V. crassostreae*) coincides with oyster deaths, the respective role of each infectious agent in the disease remains unresolved. When oysters are co-infected by the herpes virus and vibrios, one can ask if a synergy between these microbes occurs and, if so, is required to the disease. Finally as the European Union regulation implies the establishment of containment areas of infected oysters their role as reservoirs of pathogens needs to be demonstrated.

In the present study, we investigated the respective roles of herpes virus and vibrios in causing oyster mortality. The first issue we address is the potential for co-infection by the herpes virus and *Vibrio* spp. in oysters infected in the field. To this end, we use standardized SPF oysters, descendants of a pool of genitors produced in hatchery under highly controlled conditions, in order to decrease the influence of genetic and environmental parameters that could affect the host sensitivity to the disease. SPF oysters represent a suitable model to investigate the role of each pathogenic agent in the disease process, because the vibrio fraction is low in SPF oyster tissue and herpes virus undetected. Second, we investigate whether the quantity of virus and/or *Vibrio* spp. in infected oysters can be used as a predictor of mortality. Third, we aim at differentiating the roles of *Vibrio* spp. and the herpes virus as causative agents of the disease. Finally, we investigate the role of oysters as a reservoir of putative pathogens.

## Materials and Methods

### Specific-Pathogen-Free Juveniles

Wild oyster spats were collected in Fouras (Marennes-Oléron, France) in 2011 and were moved to grow-up areas located at Paimpol and at Aber Benoit (northern Brittany, France) between 2012 until 2014. These animals were exposed to diseases during the spring 2012 and suffered mortality > 50% (Petton et al., 2013). In January and in April 2014, 60 individuals were successively transferred to the experimental Ifremer facilities located at Argenton (Brittany, France) and treated by chloramphenicol (8 mg/L) for 5 days prior to maturation conditioning as described previously (Petton et al., 2013). After gamete stripping and fertilization, animals were reared under controlled conditions up to 5 months, or until reaching a mean individual wet mass between 0.5 and 3.0 g. Quantification of the herpes virus was performed by qPCR at the different steps of the SPF production, i.e., from the broodstock at the beginning of their conditioning to the larval and post-larval stages and spats.

### Natural Infection in the Field and Cohabitation Experiments in the Laboratory

SPF oysters were maintained in Bay of Brest (Pointe du Château, 48° 20' 06.19" N, 4° 19' 06.37" W) during a disease outbreak (seawater temperature > 16°C). Total mortalities resulting in

the field were recorded daily for 57 days (from 7 August 2014 to 3 October 2014). For cohabitation experiments, field-exposed oysters (i.e., “donors”) were brought back to the laboratory and cohabited in a tank with SPF oysters (i.e., “recipients”) at 21°C under controlled conditions of water renewal (Petton et al., 2015). When indicated, chloramphenicol (8 mg/L) was added in tanks every 2 days to remove the cultivable microbiota in the oyster tissues. Alive and dead donors and recipients were daily counted, and dead animals were removed from the tanks.

### Bacterial Isolation and Identification

Oysters were sacrificed, dissected to remove the digestive gland, and ground in sterile seawater (10 mL/g of wet tissue). The total cultivable bacteria and *Vibrio* microbiota was quantified (cfu/mg of tissue) using serial dilutions on Marine agar (Difco) and TCBS selective media, respectively. In addition, in two distinct experiments, the hemolymph of 40 field-exposed oysters (8 to 11 July; 25 to 29 July 2014) was non-destructively collected from marked individuals that were held for 10 days in 1-L tanks to identify animals that died or survived. Vibrios isolated from the hemolymph were quantified on TCBS. Randomly selected colonies (~80/from 4 animals that died after 24 h; ~80/from 4 animals that survive after 9 days) were re-streaked two times on TCBS, cultivated in Zobell media (4 g/L bactopectone and 1 g/L yeast extract in artificial sea water, pH 7.6) and stored at -80°C. For DNA sequencing, purified isolates were grown in Zobell overnight and their DNA was extracted using a DNA extraction kit (Wizard, Promega). The partial *hsp60* gene was amplified for all isolates as described previously (Hunt et al., 2008). The PCR conditions were: 3 min at 95°C followed by 30 cycles of 30 sec each at 95°C, 37°C and 1 min at 72°C with a final step of 5 min at 72°C. Genes were sequenced using the reverse primer and sequencing was performed by GATC-biotech (<https://www.gatc-biotech.com>). The partial *hsp60* gene sequences were aligned using Muscle (Edgar, 2004). Phylogenetic trees were built using PhyML applied to maximum-likelihood algorithm and GTR model as parameters (SPR,  $\gamma_4$ , invariant site; Guindon et al., 2010). Reliability was assessed by the bootstrap method with 100 replicates. Circular tree figures were drawn using the online iTOL software package (Letunic and Bork, 2011).

### OsHV-1 DNA Quantification

The OsHV-1 DNA was quantified from ground tissues by real time PCR using the SYBR®Green chemistry (Labocea Quimper, France) as described previously (Pepin et al., 2008). The TaqMan® chemistry, previously demonstrated to be more sensitive (Martenot et al., 2010) was used to quantify the herpes virus from hemolymph samples (Labéo, Laboratoire Frank Duncombe, Caen, France).

### Virulence Studies using Oysters

Bacteria were grown under constant agitation at 20°C for 24 h in Zobell media. One hundred mL of the pure culture ( $10^7$  cfu) was injected intramuscularly into anesthetized SPF oysters. The bacterial concentration was confirmed by conventional dilution plating on Zobell agar. After injection, the oysters were transferred to aquaria (20 oysters per aquarium of 2.5 L)

containing 1 liter of aerated 5  $\mu$ m-filtered seawater at 20°C, kept under static conditions for 24 h.

### Statistical Analysis

Statistical analysis was performed using the computing environment R Development Core Team. For all statistical comparisons, we first tested normality of distribution. For normal distributions we compared variables using Student's *t*-test. For non-normal distributions, we compared variables using Wilcoxon rank test and tested correlations using the Spearman correlation coefficient.

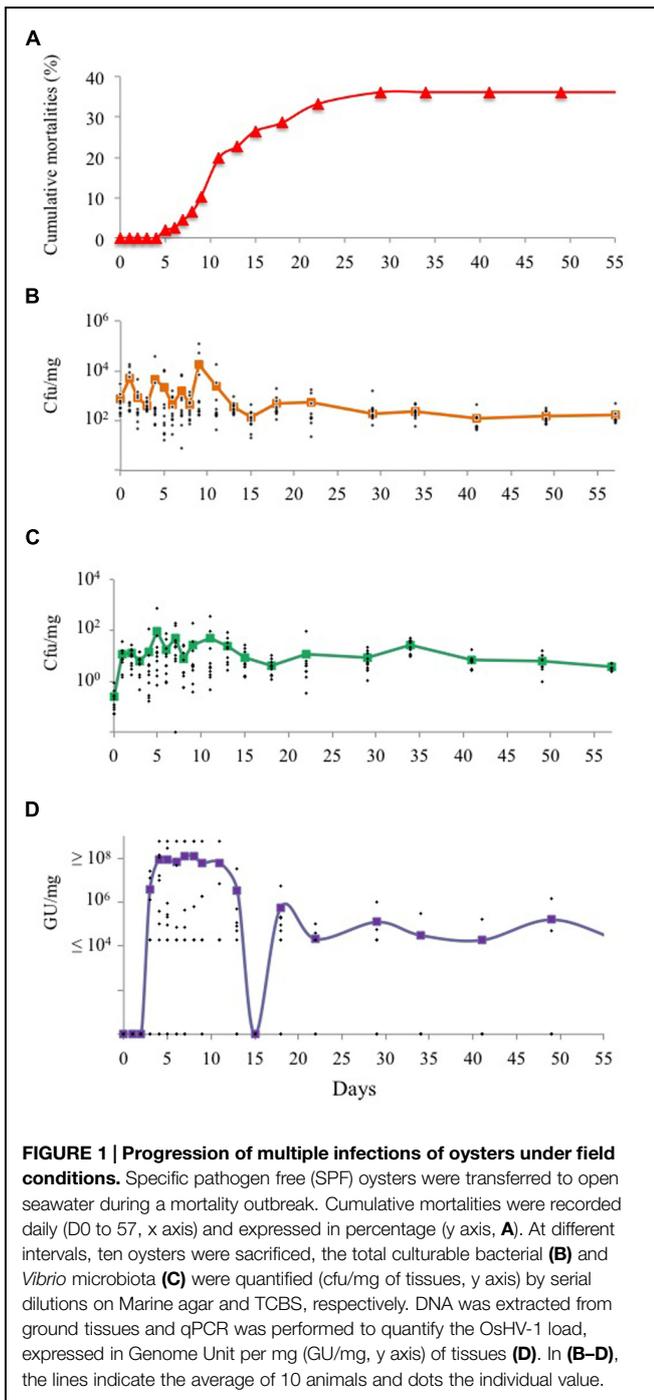
## Results

### Rapid *Vibrio* Colonization followed by Viral Replication Precedes Oyster Death

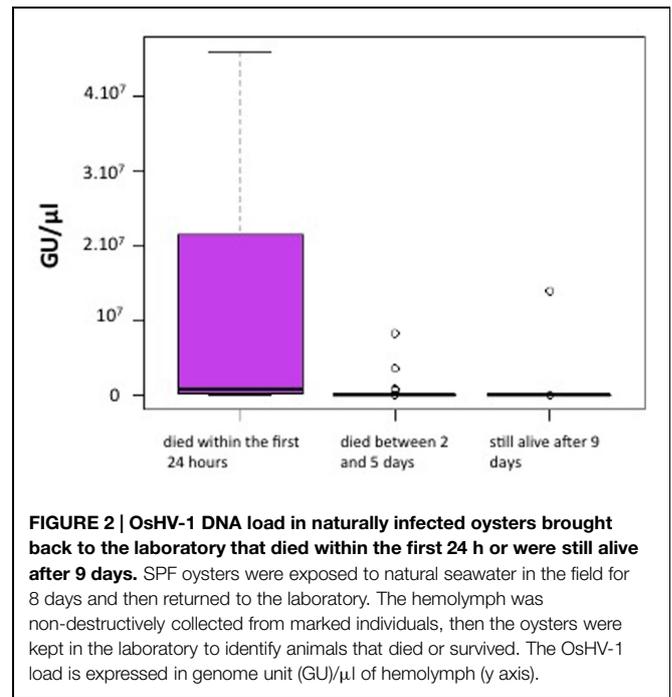
Specific-pathogen-free oysters were maintained in the field during a disease outbreak. Mortalities began at day 5, increased dramatically until day 15, and then stabilized, reaching a final percentage of 36% cumulative mortalities after day 30 (Figure 1A). The starting concentration of cultivable bacterial microbiota was about  $10^3$  cfu/mg of oyster-wet tissue prior to deployment in the field (Figure 1B) and did not change significantly after field exposure. However, the vibrio concentration did increase significantly after the first day in the field ( $p = 0.0001$  using Wilcoxon test) and reached a maximum of  $10^2$  cfu/mg (Figure 1C). After day 15, the vibrio concentration was stabilized to 10 cfu/mg. The ratio of vibrio to total cultivable microbiota was  $10^{-4}$  in SPF oysters and  $10^{-2}$  in animals maintained under field conditions. The OsHV-1 DNA was detected after day 3 and the amount of virus DNA reached a maximum of  $10^8$  genome units (GUs)/mg between days 5–13 (Figure 1D). Surprisingly, at day 15, no viral DNA was detected from the 10 sampled oysters. Finally, from day 20 to the end of the experiment, the viral load was maintained around  $10^5$  GU/mg. The inter-individual variability in microbial counts was higher during the first 15 days of exposure (Figures 1B–D). No correlation was found between the vibrio concentration and viral load. This result was confirmed by a Spearman's test ( $p$ -value  $\geq 0.05$ ;  $R^2 = 0.009$ ). Altogether our results show that while diseased oysters are co-infected by vibrios and virus, the presence of the herpes virus does not seem to influence the infection by vibrios and vice versa.

### Oyster Mortalities Correlate with a Higher Quantity of Herpes DNA

We asked whether the quantity of OsHV-1 and vibrios could predict oyster mortalities. In two separate experiments in July 2014, SPF oysters were exposed to natural seawater for 8 days in the field and then returned to the laboratory before the onset of death. The hemolymph was non-destructively collected from marked individuals, and the oysters were kept in individual tanks in the laboratory to observe the onset of the disease. Mortalities started at day 1, reached 30% (first experiment) or 50% (second experiment) at day 5 and then leveled off (Supplementary Figure S1). The hemolymph sampling did not cause any effect on



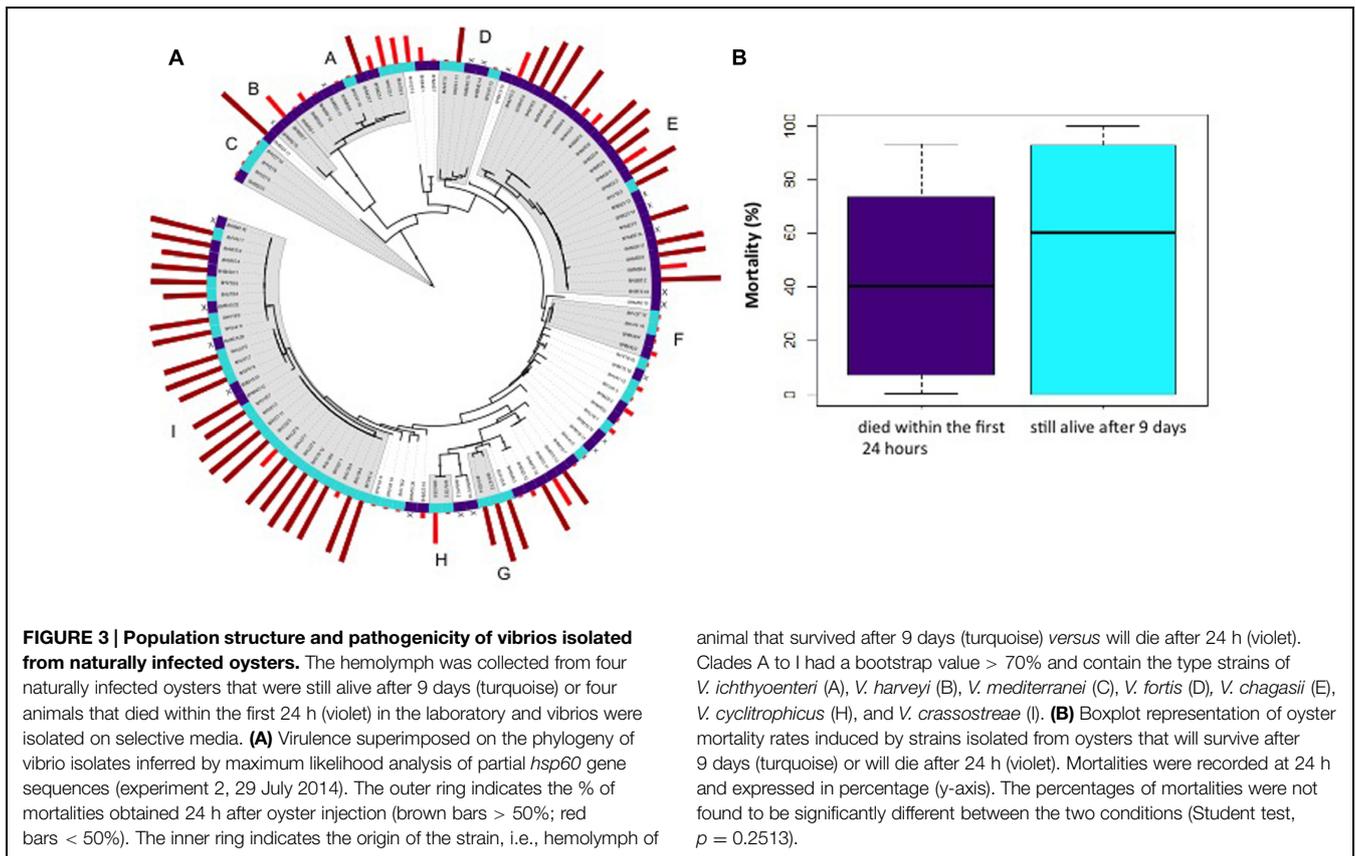
the mortality rate and disease kinetics as the sampled infected animals showed the same rate of mortality as the unsampled infected animals (Supplementary Figure S1). The quantity of OsHV-1 DNA detected in the hemolymph of animals that died within the first 24 h (Figure 2) was found to be significantly higher (up to 4 log units) than in the hemolymph of animals that were still alive after 9 days (pairwise comparison using Wilcoxon rank sum test;  $p = 0.0008$ ). In contrast, the concentration of vibrios isolated from the hemolymph varied between individuals,



but was not correlated with the mortality occurrence (data not shown).

Previous results have suggested that only a subset of vibrio strains are virulent by experimental infection (Lemire et al., 2014). Here, we investigated the diversity and pathogenicity of vibrios isolated from oysters that died and compared it to the diversity of those that survived. A total of 247 vibrio isolates sampled from the hemolymph of animals that died within the first 24 h and from animals that were still alive after 9 days were characterized by partial sequencing of a protein-coding gene (*hsp60*). Phylogenetic analysis allowed the grouping of 148/247 isolates into 11 clades (designated from A to K) with a bootstrap value  $>70\%$  (Supplementary Figure S2). Some of these clades could be matched with named species using type strains: *V. ichthyenteri* (A), *V. harveyi* (B), *V. mediterranei* (C), *V. fortis* (D), *V. chagasii* (E), *V. cyclitrophicus* (H), *V. tasmaniensis* (J), *V. breoganii* (K), and *V. crassostreae* (I). We did not observe a genotype signature in oysters that died with the first 24 h.

To address the pathogenic potential of individual strains, we used an injection model of infection, which allows for rapid infection in the laboratory (Figure 3). A total of 91 *Vibrio* strains, isolated in the second experiment (29 July), were injected individually into oysters at a concentration of  $10^7$  cfu/animal (Figure 3A). The percentages of mortalities induced by strains isolated from the hemolymph of animals that died within the first 24 h and strains isolated from hemolymph of oysters that were still alive after 9 days were not found to be significantly different (Student test,  $p = 0.2513$ ; Figure 3B). Half of the strains with  $>50\%$  mortality rates belonged to the *hsp60* clade I (Figure 3A) and matched to the phylogenetically coherent virulent population described recently as *V. crassostreae* (Lemire et al., 2014). Hence, our results show that viral load is sufficient to predict mortalities.



### In the Absence of Bacteria, a High Load of Herpes Virus is not Sufficient for a Full Expression of the Disease

Antibiotic treatments are typically used to investigate the role of bacteria in a specific disease. SPF oysters were exposed to natural seawater in the field and then returned to the laboratory after 10 days (see Material and Methods). These animals (referred to as “donors”) were kept together with SPF oysters (referred to as “recipients”) in the presence or absence of chloramphenicol for 14 days. In the absence of the antibiotic, donor mortalities started at day 2 and reached a cumulative value of 50%. In comparison, recipients began to die at day 8 and reached a cumulative mortality of 36% (Figure 4A). The antibiotic treatment resulted in a 2- and 4-fold decrease in mortality for donors and recipients, respectively, demonstrating a role of bacteria in the disease transmission and development. While chloramphenicol exposure completely removed the cultivable vibrio microbiota in the oyster tissues (data not shown), no significant effect was observed on the OsHV-1 DNA quantity detected in the donors or the recipients until day 8 (Figure 4B).

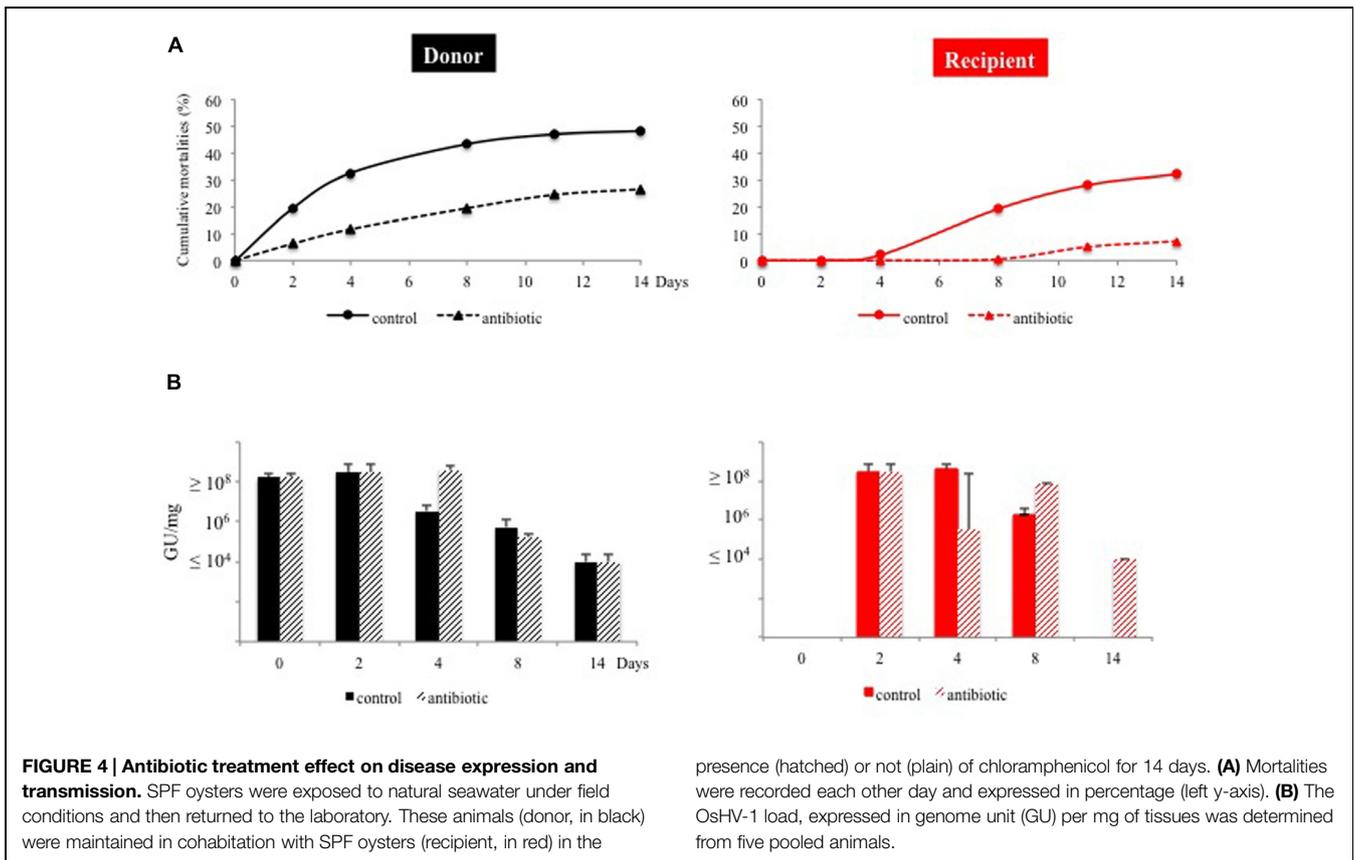
### Oysters are Reservoirs of Pathogens

The results above demonstrate that oysters can asymptotically host putative pathogens, raising concern about the role of oysters as pathogen reservoirs. Using a cohabitation experiment, we asked whether oysters carry putative pathogens at temperatures of <math>16^{\circ}\text{C}</math> (i.e., excluding mortality context). French oysters are

cultivated to the size of “spats,” the point at which they attach themselves to a substrate. These spats originated from natural settlement zones (wild seeds) or were produced in hatchery (hatched seeds). Wild adult oysters (i.e., not farmed) can also be collected from the environment. Hence, we also determined whether the geographic origin, age, and farming of oysters influence the disease(s) transmission.

The first experiment was performed with juvenile oysters originated from wild seed collected in 6 different geographic areas as donors, and 3-months-old SPF hatch oysters as recipients (Table 1). Over the course of the experiment (30 days), the hatched animals did not die on their own (not shown). On the contrary, death occurred in oysters originated from wild seeds collected in four regions (Arcachon, Pertuis Charentais, Bay of Vilaine, and Bay of Brest). In this case, 11/15 batches of juveniles originating from these regions were able to transmit the disease to the recipients. Finally, six batches originating from two regions (Thau and Bay of Bourgneuf) did not show significant mortality rates and did not cause mortality in the recipients. Mortalities always coincided with the detection of OsHV-1 in both donors and recipients. Interestingly, in 7/21 batches of donors, the virus was not detected at the beginning of the experiment but was found at the end of the trial, confirming that a thermal stress in the mesocosm (see Materials and Methods) can be used to highlight viral infection (Petton et al., 2015).

The second experiment was performed with farmed or wild adults (> 18 months) sampled from four different geographic



areas as donors, and 3-, 6-, and 15-months-old SPF oysters as recipients (Table 2). Only 1/10 sets of wild oysters expressed mortalities and transmitted the disease to the recipients. On the contrary, 13/18 (72%) batches of farmed oysters originating from the four regions showed mortalities and caused the disease in the recipients. A Wilcoxon test confirmed that the farmed animals had a significantly higher death rate and transmitted more of the disease than the wild oysters ( $p = 0.0007$ ). In all of these cases, the mortality rate was negatively correlated with the age of recipient animals. Interestingly, OsHV-1 was detected only once in the recipients (not shown), suggesting no significant role for the virus in the disease transmitted by adults (Table 2). Altogether, our results demonstrate that oysters are a reservoir of putative pathogens. Only juvenile oysters were shown to carry and transmit a detectable amount of OsHV-1, while adult animals carried infectious agents that are able to induce mortality of juveniles in the absence of the virus.

## Discussion

In this study, we investigated the respective roles of herpes virus and vibrios in the juvenile oyster disease by exposing SPF oysters in the field during a disease outbreak. Prior to field exposure, the cultivable fraction of the SPF oyster microbiota was determined to be  $10^3$  cfu/mg tissue, and a low vibrio presence ( $\leq 1$  cfu/mg) was detected in these animals (Figure 1). Hence,

these animals represent a suitable model to investigate the role of each pathogenic agent in the disease process, both independently and together.

The limited sensitivity in detecting viral DNA ( $10^4$  GU/mg tissue), prevented the monitoring of initial infection kinetics for the animals. Hence, the detection of viral DNA after 3 days of exposition under field conditions likely resulted from the viral replication culminating at  $10^8$  GU/mg (Figure 1). While the mortalities stabilized after 15 days, the viral load was maintained at  $10^4$  GU/mg, and can thus be considered as an asymptomatic carriage. Whether this load corresponds to a latent phase of the virus or to a limited shedding of particles remains to be determined, but our results highlight the interest of our model to explore the herpes virus biological cycle (i.e., latent and lytic cycle; cellular tropism). Whereas previous data correlating the virus load with mortalities have been based on moribund animal sampling (Sauvage et al., 2009; Oden et al., 2011), we performed a non-destructive collection of hemolymph and showed that viral DNA quantification ( $> 10^7$  GU/mg) can predict the mortalities of juvenile oysters (Figure 2). However, when using an antibiotic treatment, we also demonstrated that in the absence of bacteria, a load of herpes virus  $\geq 10^8$  GU/mg is not sufficient to induce oyster mortalities in a range classically ascribed to this disease (Figure 4). These results suggest that viral replication may be a consequence rather than the cause of the disease. Recently, Green and Montagnani (Green et al., 2014) observed that a poly I:C treatment decreases the OsHV-1

**TABLE 1 | Cohabitation experiments performed for 30 days with juvenile oysters collected in six different geographic areas as donors and 3 months-old-SPF oysters as recipients (nd, not detected; d, detected).**

Geographic origin	Wild seed collecting sector	Donors			Recipients		
		Mortality (%) at day 30	OsHV-1 $\mu$ var at day 0	OsHV-1 $\mu$ var at day 30	Mortality (%) at day 30	OsHV-1 $\mu$ var at day 0	OsHV-1 $\mu$ var at day 30
Thau	Marseillan	3 $\pm$ 1	nd	d	0	nd	nd
	Loupian	6	nd	nd	0	nd	nd
	Loupian	2 $\pm$ 1	nd	nd	0	nd	nd
Arcachon	Lucarnan	25 $\pm$ 1	nd	d	46 $\pm$ 1	nd	d
	Villa Algèrienne	22 $\pm$ 25	nd	d	13 $\pm$ 17	nd	d
	Graouères	5 $\pm$ 5	nd	nd	0	nd	nd
	Comprian	19 $\pm$ 0	nd	d	0	nd	d
	Bélisaire	16 $\pm$ 2	d	d	35 $\pm$ 9	nd	d
Pertuis Charentais	Les Longès	1 $\pm$ 0	nd	nd	0	nd	nd
	Seudre	67 $\pm$ 4	d	d	42 $\pm$ 5	nd	d
	Mouclières	0	nd	nd	0	nd	nd
Bay of Bourgneuf	Les Moutiers	0	d	d	0	nd	nd
	Bernerie Nord	0	d	d	0	nd	d
	Bernene Sud	0	nd	d	0	nd	nd
Bay of Vilaine	Tréguier (gros)	54 $\pm$ 1	nd	d	63 $\pm$ 5	nd	d
	Tréguier (petit)	27 $\pm$ 17	nd	d	16 $\pm$ 22	nd	d
Bay of Brest	Le Pryoldi	56 $\pm$ 1	d	d	36 $\pm$ 10	nd	d
	Mengleuz	57 $\pm$ 4	d	d	39 $\pm$ 6	nd	d
	Lomergat	68 $\pm$ 1	d	d	39 $\pm$ 9	nd	d
	Pte du Château	79 $\pm$ 6	d	d	49 $\pm$ 3	nd	d
	Pte du Château	80 $\pm$ 2	d	d	51 $\pm$ 3	nd	d

replication in *C. gigas* infected by injection. Hence developing poly I:C and/or RNA interference approaches (Labreuche and Warr, 2013; Huvet et al., 2015) in oysters would be of prime interest to decipher the direct role of this virus in the disease process.

Having shown that oysters are naturally co-infected by the herpes virus and vibrios, we next ask if a synergy between these microbes is fundamental to the disease. In a first scenario, oysters infected with herpes may be more susceptible to subsequent *Vibrio* infection. However, this hypothesis is contradicted by the fact that we did not observe a correlation between the herpes load and vibrio concentration at the individual level. In a second scenario, oysters infected with *Vibrio* may be more susceptible to herpes replication. However, as in antibiotic treated oysters the viral load is similar to that of untreated animals (Figure 4), this scenario seems also to be unlikely. In any case, diseased oysters that are co-infected by several putative pathogens (virus and bacteria) should sustain much greater losses.

Since we aimed at differentiating the roles of *Vibrio* spp. and the herpes virus as causative agents of oyster deaths, we used an antibiotic treatment and highlighted an essential role of bacteria in the disease process (Figure 4). As chloramphenicol treatment is not restricted to vibrio, the next step will be to demonstrate that vibrio(s) is (are) the causative agent(s) of the death. We observed that the quantity, genotype and virulence of vibrios (determined by the injection of single strain) cannot predict the mortalities (Figure 3). Our results seem to contradict a

recent study that describes the microbial communities in oysters challenged by a virulent vibrio strain (Lokmer and Wegner, 2015). Lokmer and Wegner found that dead and moribund oysters displayed signs of community structure disruption characterized by a very low diversity and proliferation of few taxons. However, their analysis used barcoded 16S rRNA 454 amplicon sequencing, the genetic resolution of which is not fine scale enough to analyze the vibrio diversity. Furthermore, the bacterial challenge was based on single strain injection, which does not capture the complexity of infection within the natural environment as described in the present study. We previously hypothesized that *Vibrio* diversity may have significance in the disease onset. In a first study we showed that heightened virulence is observed when combination of virulent strains are used in experimental infections, suggesting that virulence of vibrio results from a combination of factors, each associated with different strains within the population (Gay et al., 2004). More recently, we demonstrated that naturally infected oysters initially contain a large proportion of non-virulent strains, and that these are progressively replaced by a virulent population that comprise ~50% of the bacterial isolates at the peak of mortality (Lemire et al., 2014). We suspected that this result reflects a contribution of the non-virulent strains to the development of disease. We further demonstrated that the presence of non-virulent bacteria dramatically increases the virulence of a virulent strain at low doses. Thus, non-virulent strains must have some features that contribute either directly or indirectly to the pathogenicity of virulent isolates. In the future

**TABLE 2 | Cohabitation experiments performed for 30 days with farmed or wild adults sampled in 4 different geographic areas as donors and 3-, 6-, and 15-months-old SPF oysters as recipients.**

Geographic origin	Collecting sector	Donors		Recipients		
		Farmed/wild	Mortality (%)	Mortality (%) 3 months	Mortality (%) 6 months	Mortality (%) 15 months
Bay of Veys	Géfosse	Farmed	38	64	43	29
Aber Benoit	Beg ar Vill	Farmed	20	64	26	20
	Beg ar Vill	Farmed	31	57	30	24
	Beg ar Vill	Farmed	59	55	33	28
	Beg ar Vill	Farmed	46	48	26	21
	Beg ar Vill	Farmed	20	63	37	28
Bay of Bourgneuf	La Coupelasse	Farmed	5		5	8
	Cob	Farmed	64		51	17
	Roche aux Bretons	Farmed	69		25	25
	Grill Nod	Farmed	54		28	29
	Graisseloup	Farmed	57		33	25
	La Coupelasse	Wild	2		0	0
	Cob	Wild	0		1	0
	Graisseloup	Wild	1		0	0
	Roche aux Bretons	Wild	2		0	0
Bay of Brest	Pte du Château	Farmed	0	0	0	0
	Pte du Château	Farmed	8	28	7	17
	Pte du Château	Farmed	0	0	0	0
	Pte du Château	Farmed	5	0	0	0
	Pte du Château	Farmed	0	0	0	1
	Pte du Château	Farmed	17	66	23	20
	Pte du Château	Farmed	91	78	59	54
	Pte du Château	Farmed	3	70	14	22
	Le Prioldy	Wild	3	0	0	0
	Le Loc'h	Wild	0	0	0	0
	Pte du Château	Wild	31	45	19	15
	Pte du Château	Wild	0	0	0	0
	Pte du Château	Wild	0	0	0	0
	Pte du Château	Wild	8	57	7	0
	Pte du Château	Wild	1	1	0	0

we will investigate the significance of *Vibrio* population assembly in diseased oysters with emphasis on cooperative behavior and weapons sharing that may be an important feature of the disease.

From the data collected in this study, the oyster physiology seems to be a key feature of this disease. We observed that when oysters are naturally co-infected by the herpes virus and pathogenic vibrios, only 30% to 50% of animals died. This suggests that viral replication and/or the vibrio's pathogenic effect depends on oyster susceptibility. This susceptibility may result from environmental factors (acquired susceptibility) and/or genetic traits (innate susceptibility). It may implicate the physiology of the animal itself and its associated microbial communities (Lokmer and Wegner, 2015). It has been demonstrated that resistance toward oyster juvenile disease has a genetic basis (Huvet et al., 2004; Segarra et al., 2014). More recently, Wendling and Wegner suggested that a simple genetic resistance mechanism of the oyster is matched to a common virulence mechanism shared by sympatric *Vibrio* strains (Wendling and Wegner, 2015). In the future, we will

investigate the genetic bases of oyster susceptibility to the vibrio isolated in the present study.

Both adult and juvenile oysters were shown to act as reservoirs of pathogens which were able to induce mortalities of young animals. Only the juveniles were demonstrated to transmit the herpes virus. This suggests that the diseases transmitted by the juveniles or by the adults are distinct. However, it also demonstrates that the virus infection is not a prerequisite for juvenile mortality outbreaks. Farmed animals seem to present more risk of disease transmission than wild animals. In addition, the geographic origin of oysters influences the carriage of pathogen. Though our data suggest that some farming zones are less infected than others they also indicate that movements of animals between ecosystems are very noxious in terms of disease-spreading. Beyond the environmental factors that are known to potentially influence mechanisms of disease transmission in marine systems, i.e., hydrodynamics, biomass of infected animals (Petton et al., 2015), identifying other potential reservoirs of pathogens and the source(s) of the disease is a future avenue to qualify infected vs. non-infected areas.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.00686>

## References

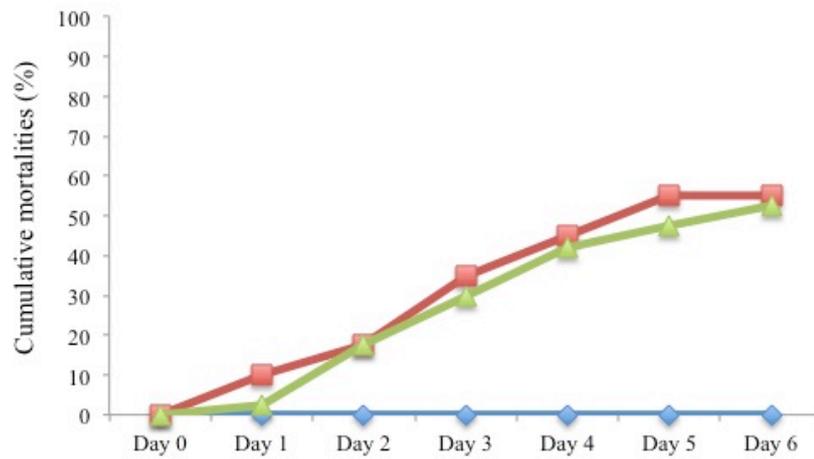
- Arzul, I., Renault, T., Thebault, A., and Gerard, A. (2002). Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Res.* 84, 151–160. doi: 10.1016/S0168-1702(02)00007-2
- Cochennec, N., Le Roux, F., Berthe, F., and Gerard, A. (2000). Detection of *Bonamia ostreae* based on small subunit ribosomal probe. *J. Invertebr. Pathol.* 76, 26–32. doi: 10.1006/jipa.2000.4939
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340
- Gay, M., Renault, T., Pons, A. M., and Le Roux, F. (2004). Two vibrio splendidus related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Dis. Aquat. Organ* 62, 65–74. doi: 10.3354/dao062065
- Green, T. J., Montagnani, C., Benkendorff, K., Robinson, N., and Speck, P. (2014). Ontogeny and water temperature influences the antiviral response of the Pacific oyster, *Crassostrea gigas*. *Fish Shellfish Immunol.* 36, 151–157. doi: 10.1016/j.fsi.2013.10.026
- Grizel, H., and Héral, M. (1991). Introduction into France of the Japanese oyster (*Crassostrea gigas*). *J. Cons. Int. Explore Mer.* 47, 399–403. doi: 10.1093/icesjms/47.3.399
- Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010
- Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J., and Polz, M. F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science* 320, 1081–1085. doi: 10.1126/science.1157890
- Huvet, A., Béguel, J.-P., Cavaleiro, N. P., Thomas, Y., Quillien, V., Boudry, P., et al. (2015). Disruption of amylase genes by RNA interference affects reproduction in the Pacific oyster *Crassostrea gigas*. *J. Exp. Biol.* 218, 1740–1747. doi: 10.1242/jeb.116699
- Huvet, A., Herpin, A., Degremont, L., Labreuche, Y., Samain, J. F., and Cunningham, C. (2004). The identification of genes from the oyster *Crassostrea gigas* that are differentially expressed in progeny exhibiting opposed susceptibility to summer mortality. *Gene* 343, 211–220. doi: 10.1016/j.gene.2004.09.008
- Labreuche, Y., and Warr, G. W. (2013). Insights into the antiviral functions of the RNAi machinery in penaeid shrimp. *Fish Shellfish Immunol.* 34, 1002–1010. doi: 10.1016/j.fsi.2012.06.008
- Lemire, A., Goudenege, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., et al. (2014). Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *ISME J.* 9, 1523–1531. doi: 10.1038/ismej.2014.233
- Le Roux, F., Lorenzo, G., Peyret, P., Audemard, C., Figueras, A., Vivares, C., et al. (2001). Molecular evidence for the existence of two species of *Marteilia* in Europe. *J. Eukaryot. Microbiol.* 48, 449–454. doi: 10.1111/j.1550-7408.2001.tb00178.x
- Letunic, I., and Bork, P. (2011). Interactive tree of life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res.* 39, W475–W478. doi: 10.1093/nar/gkr201
- Lokmer, A., and Wegner, K. M. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J.* 9, 670–682. doi: 10.1038/ismej.2014.160
- Martenot, C., Oden, E., Travaille, E., Malas, J. P., and Houssin, M. (2010). Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. *J. Virol. Methods* 170, 86–89. doi: 10.1016/j.jviromet.2010.09.003
- Martenot, C., Oden, E., Travaille, E., Malas, J. P., and Houssin, M. (2011). Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. *Virus Res.* 160, 25–31. doi: 10.1016/j.virusres.2011.04.012
- Martenot, C., Travaille, E., Lethuillier, O., Lelong, C., and Houssin, M. (2013). Genome exploration of six variants of the Ostreid Herpesvirus 1 and characterization of large deletion in OsHV-1muVar specimens. *Virus Res.* 178, 462–470. doi: 10.1016/j.virusres.2013.08.006
- Oden, E., Martenot, C., Berthaux, M., Travaille, E., Malas, J. P., and Houssin, M. (2011). Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: determination of a viral load threshold to prevent summer mortalities. *Aquaculture* 317, 27–31. doi: 10.1016/j.aquaculture.2011.04.001
- Pepin, J. F., Riou, A., and Renault, T. (2008). Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. *J. Virol. Methods* 149, 269–276. doi: 10.1016/j.jviromet.2008.01.022
- Pernet, F., Barret, J., Gall, P. L., Corporeau, C., Dégremont, L., Lagarde, F., et al. (2012). Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practises in the Thau lagoon. *Aquac. Environ. Interact.* 2, 215–237. doi: 10.3354/aei00041
- Petton, B., Boudry, P., Alunno-Bruscia, M., and Pernet, F. (2015). Factors influencing disease-induced mortality of Pacific oysters *Crassostrea gigas*. *Aquac. Environ. Interact.* 6, 205–222. doi: 10.3354/aei00125
- Petton, B., Pernet, F., Robert, R., and Boudry, P. (2013). Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. *Aquac. Environ. Interact.* 3, 257–273. doi: 10.3354/aei00070
- Rohfritsch, A., Bierne, N., Boudry, P., Heurtebise, S., Cornette, F., and Lapegue, S. (2013). Population genomics shed light on the demographic and adaptive histories of European invasion in the Pacific oyster, *Crassostrea gigas*. *Evol. Appl.* 6, 1064–1078. doi: 10.1111/eva.12086
- Samain. (2008). *Summer Mortality of Pacific Oyster Crassostrea gigas*. Versailles: Editions Quae, The Morest Project, 379.
- Sauvage, C., Pepin, J. F., Lapegue, S., Boudry, P., and Renault, T. (2009). Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: differences in viral DNA detection and quantification using real-time PCR. *Virus Res.* 142, 181–187. doi: 10.1016/j.virusres.2009.02.013
- Schikorski, D., Faury, N., Pepin, J. F., Saulnier, D., Tourbiez, D., and Renault, T. (2011a). Experimental ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. *Virus Res.* 155, 28–34. doi: 10.1016/j.virusres.2010.07.031
- Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., and Pepin, J. F. (2011b). Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. *Vet. Res.* 42:27. doi: 10.1186/1297-9716-42-27

- Segarra, A., Mauduit, F., Faury, N., Trancart, S., Degremont, L., Tourbiez, D., et al. (2014). Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. *BMC Genomics* 15:580. doi: 10.1186/1471-2164-15-580
- Segarra, A., Pepin, J. F., Arzul, I., Morga, B., Faury, N., and Renault, T. (2010). Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* 153, 92–99. doi: 10.1016/j.virusres.2010.07.011
- Wendling, C. C., and Wegner, K. M. (2015). Adaptation to enemy shifts: rapid resistance evolution to local *Vibrio* spp. in invasive Pacific oysters. *Proc. Biol. Sci.* 282:20142244. doi: 10.1098/rspb.2014.2244

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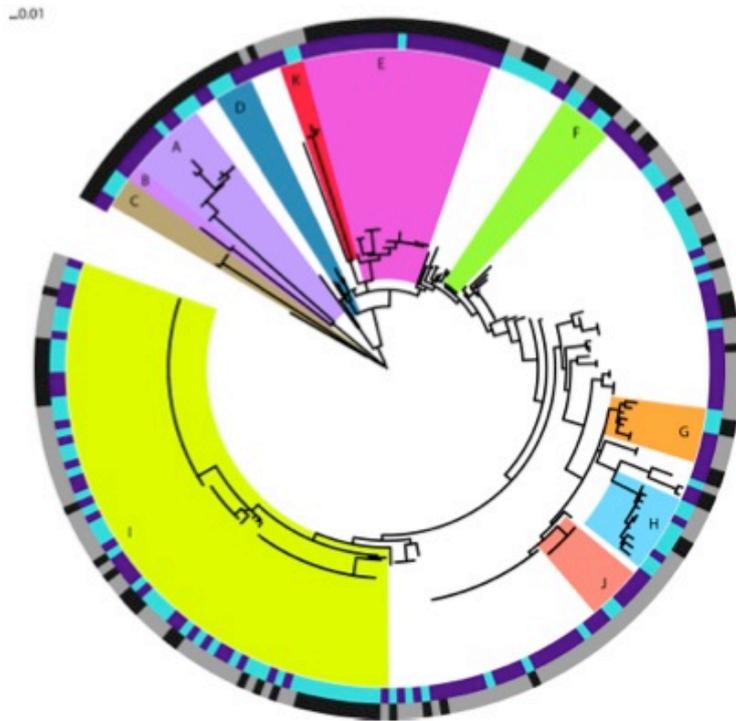
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FIGURE S1



**Figure S1:** Specific pathogen free oysters were transferred to open seawater and then transferred to the laboratory before the onset of death. The hemolymph was non-destructively collected from marked individuals (red line) or not (green line). The oysters were kept in individual tanks in the laboratory to observe the onset of the disease. SPF oysters (in blue) were used as control. Mortalities were recorded daily (Day 0 to 6) and expressed in percentage (y-axis).

FIGURE S2



**Figure S2: *Vibrio* population structure in infected oysters that died within the first 24 hours or were still alive after 9 days.** In two separate experiments in July 2014, the hemolymph was collected from 4 naturally infected oysters that survived after nine days or 4 animals that died after 24 hours in the laboratory and vibrios were isolated on selective media. The phylogeny of bacterial isolates inferred by maximum likelihood analysis of partial *hsp60* gene sequences, with outer and inner rings indicating the date of sampling (11 July 2014: grey; 29 July 2014: black) and hemolymph of animal that survived after nine days (turquoise) versus will die after 24 hours (violet). Clades A to K had a bootstrap value >70% and contain the type strains of *V. ichthyenteri* (A), *V. harveyi* (B), *V. mediterranei* (C), *V. fortis* (D), *V. chagasii* (E), *V. cyclitrophicus* (H), *V. tasmaniensis* (J), *V. breoganii* (K) and *V. crassostreae* (I).

## II.B *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition

The previous studies show that some populations of vibrios are virulent and suggest a role of vibrios in oyster juvenile mortality events. Due to filter-feeding mode of nutrition, it has been suggested that *Vibrio* assembly in oyster is neutral, meaning that the population structure within oysters reflects that of the surrounding seawater (Wendling et al., 2014). However, in this study, phylogenetic and ecological resolution was not sufficient to discriminate ecological populations and the sampled site was exempt of mortality events. Here, we ask: **i) to what extent are oysters passively colonized by *Vibrio* populations present in the surrounding water, (ii) how populations turn over during pathogenicity events?**

We compared population structures of vibrios between the seawater column and oysters to explore the hypothesis of neutral association and/or population shift during a disease event. We performed two samplings in spring (no oyster mortality) and two in summer (during mortality events) and isolated 1635 strains. Phylogeny of these strains on a marker gene coupled with the use of AdaptML, an evolutionary model that groups strains sharing the same ecological preference, allowed the identification of 24 ecological populations. Spring samples were dominated by *V. splendidus*-related populations, but no mortality was recorded in spring, suggesting that other factors, biotic and/or abiotic, are necessary for oyster disease induction. Summer samples showed a much higher diversity, with various populations associated with diseased oysters. Overall, most populations are found both in seawater and in oyster, suggesting a neutral association. Nevertheless, 10 out of 24 of them were unequally distributed, showing a specific association with oyster. Among them, we found notably *V. crassostreae*, *V. splendidus* (2 populations) and *V. tasmaniensis* (2 populations).

Interestingly, among them, only *V. crassostreae* was detected in each oyster sampled during mortality events at a high abundance and was nearly completely absent from healthy oysters sampled in spring and in seawater. Additionally, this population was the only one positively associated with oyster hemolymph; suggesting an ability to escape host immunity and to survive in such a hostile niche.

Dynamics of *V. crassostreae* suggests that it plays a major role in oyster mortality. While all members possess the R5-7/8 gene cluster (see chapter I), we found few *V. crassostreae* strains with low virulence, confirming previous data that showed that R5-7/8 is necessary but not sufficient for virulence. Furthermore, virulence of some strains could be reduced after lab-handling; suggesting an involvement of a plasmid in virulence. We thus

explored this hypothesis by comparing extrachromosomal DNA content of virulent or non-virulent strains and found that a large plasmid was only detected in the first group. This plasmid was named pGV1512. Thereafter using a larger collection of strains, we showed that pGV1512 presence was strongly correlated with strain virulence potential. Plasmid curing decreased mortality induced by *V. crassostreae*. whilst transferring it back to the cured strain restored its virulence, thus demonstrating its role in virulence. On the other hand, competition assays showed that a strain carrying the plasmid did not outcompete the cured one. Colonization assay further confirmed that *V. crassostreae*, with or without pGV1512, survived more successfully in oyster than a population showing a neutral association with oyster and seawater (*V. cyclitrophicus*). Hence, the ability of *V. crassostreae* to colonize and survive within oyster hemolymph is likely encoded by core genome genes

In our infection model, it appears that *V. crassostreae* is a benign colonizer and that plasmid invasion within this population dramatically increases the virulence. As the plasmid is detected in the majority of strains isolated from farmed oyster and seems absent from its closest phylogenetic neighbor isolated in the US (*V. sp. nov.* 12 in chapter I), we could question if oyster farming led to the selection of pGV1512 and therefore a high prevalence of the plasmid in this environment. Future research will therefore focus on whether the virulence plasmid is specifically enriched in populations associated with diseased oysters and the underlying mechanisms of virulence of this plasmid will be further explored.

This work is detailed in the article entitled “***Vibrio crassostreae*, a benign colonizer turned into a pathogen after plasmid acquisition**” published in the ISME journal in April 2017. [In this study, I made the infection assays, the mutagenesis of the different regions of the plasmid pGV1512, the strain curing and labelling and the colonization and competition assays. I also performed the statistical analyses.](#)

## ORIGINAL ARTICLE

# Vibrio crassostreae, a benign oyster colonizer turned into a pathogen after plasmid acquisition

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Vibrios are frequently associated with oyster mortality; however whether they are the primary causative agent or secondary opportunistic colonizers is not well understood. Here we combine analysis of natural infection dynamics, population genomics and molecular genetics to ask (i) to what extent oysters are passively colonized by *Vibrio* population present in the surrounding water, (ii) how populations turn over during pathogenicity events and (iii) what genetic factors are responsible for pathogenicity. We identified several populations of *Vibrio* preferentially associated with oyster tissues. Among these, *Vibrio crassostreae* is particularly abundant in diseased animals while nearly absent in the surrounding water, and its pathogenicity is correlated with the presence of a large mobilizable plasmid. We further demonstrate that the plasmid is essential for killing but not necessary for survival in tissues of oysters. Our results suggest that *V. crassostreae* first differentiated into a benign oyster colonizer that was secondarily turned into a pathogen by introgression of a virulence plasmid into the population, possibly facilitated by elevated host density in farming areas.

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## Introduction

Disease in aquaculture systems has been steadily rising over the past decade, in many cases threatening the long-term survival of commercial and natural stocks (Le Roux et al., 2015). A well-documented example is successive mass mortalities of juvenile oysters (*Crassostrea gigas*) in France in which bacteria of the genus *Vibrio* have been implemented (Petton et al., 2015b). A previous study demonstrated that the onset of disease in oysters coincides with progressive replacement of diverse benign colonizers by members of a phylogenetically coherent virulent population (Lemire et al., 2015) that was assigned to the previously characterized species *Vibrio crassostreae* (Faury et al., 2004). However, how pathogen populations assemble in oysters from the environment

remains poorly understood yet is important both for understanding of disease outbreaks and devising appropriate monitoring tools (Le Roux et al., 2016).

Because oysters are filter feeders they are intricately connected to the microbial community in the water column; however, colonization may be a multifactorial process. Oysters pump water through their gills (up to  $7.31 \text{ h}^{-1} \text{ g}^{-1}$  dry tissue weight; Cognie et al., 2003), which act like a sieve capturing food particles in the optimum range of 5–10  $\mu\text{m}$ . Vibrios attached to particulate organic matter or larger organisms may therefore have a higher probability of contact and infection (Froelich et al., 2012). In addition, the gill epithelium and its associated microbiota may constitute a first line of defense against vibrios by producing antimicrobial peptides that constitute a chemical barrier in the colonization process (Destoumieux-Garzon et al., 2016; Lokmer et al., 2016; Pales Espinosa et al., 2016). Finally, as oysters have a semi-open circulatory system, the hemocytes (or oyster immuno-competent cells) are not confined to the blood vessels but can invade or reside in multiple other tissues (Bachere et al., 2015). Hence although oyster may take up vibrios from seawater at high rates, mechanical, chemical and cellular selectivity may influence the assembly of *Vibrio* populations in oyster tissues.

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A key question is to what extent oysters mirror *Vibrio* population structure in the water, and if pathogenic populations first bloom in the water or reproduce more specifically in oysters (Le Roux et al., 2016). These questions can be addressed by combining a population genetic framework with ecological sampling. Previous work has shown that vibrios are differentiated into closely related but differentially distributed populations (among organic particles, larger organisms or free-living) in the same water samples (Hunt et al., 2008; Szabo et al., 2012). This approach has enabled the study of population assembly in various marine animals including mussels for which it was shown that population frequencies in the water resembled those in mussels, likely due to passive transfer by filter-feeding (Preheim et al., 2011). Similarly, neutral association was also suggested for vibrios and *C. gigas* (Wendling et al., 2014) although in this study phylogenetic and ecological resolution may not have been sufficient to delineate ecological and/or virulent population. In addition, the sampling site was not affected by mass mortality events (Watermann et al., 2008). Hence, it remains poorly understood to what extent (i) oysters mirror *Vibrio* population structure in the water and (ii) populations turn over during pathogenicity events.

Here we address the above problem by using specific pathogen free (SPF; Petton et al., 2013, 2015a, b) oysters in a field-based approach to investigate the disease ecology of *Vibrio* populations in an oyster farming area. SPF oysters are descendants of a pool of genitors raised under controlled conditions and shown to have  $\leq 1$  c.f.u. of vibrios per mg tissue. These oysters were deployed in the environment to monitor *Vibrio* population assembly before and during disease events. Oyster-associated vibrios are analyzed in the context of a metapopulation framework, that is, by considering potential overlap or differences in populations collected from spatially and temporally distinct habitats, which are connected by dispersal. Our analysis reveals that several populations of *Vibrio* were preferentially associated with specific oyster tissues. Among these, *V. crassostreae* is particularly abundant in diseased animals and its pathogenicity is correlated with the presence of a large mobilizable plasmid. We further demonstrate that although the plasmid is essential for killing it is not necessary for survival in the host. Our results suggest that *V. crassostreae* first differentiated into a benign oyster commensal that secondarily turned into a pathogen by invasion of a virulence plasmid.

## Materials and methods

### Oyster natural infection

We developed a natural infection scheme to mimic disease progression in the environment by deploying SPF oysters (Supplementary Methods) and monitoring

disease outbreak. As previous work has demonstrated that the disease occurs beyond a temperature threshold of  $\sim 16^\circ\text{C}$  (Petton et al., 2015a), we tested infection at two time points in the summer ( $4$   $16^\circ\text{C}$ ) and, for comparison, two time points in spring ( $0$   $16^\circ\text{C}$ ). To differentiate oysters infected with the primary disease-causing agent from moribund animals that might suffer from secondary opportunistic infections, we divided the deployed SPF oysters into 'sentinel' and 'experimental' specimens. Each experiment was initiated by transferring a set of sentinel SPF oysters ( $n=200$ ) to the farming area. After 2 days, a set of 'experimental' SPF oysters ( $n=200$ ) was placed in the farm next to the sentinel oysters. The viability of 'sentinel' animals was recorded daily and at the first observation of oyster mortality (16 July and 29 July 2014), the 'experimental' batch was returned to the laboratory to (i) allow development of the disease in tanks at  $21^\circ\text{C}$  (previously shown to allow the disease to proceed more rapidly (Lemire et al., 2015)) and (ii) to isolate vibrios from oysters ( $n=8$ ). In the laboratory tanks, the cumulative mortality reached 50% after 5 days, similar to the observed mortality for 'sentinel' oysters left in the field for one month. These results confirmed that the batches of oysters transferred to the lab were affected by the disease. In spring, the sampling dates (17 March and 5 May 2014) correspond to 15 days of incubation of 'experimental' oysters in the field. No mortality was observed for the 'sentinel' animals after one month in the field and for the 'experimental' animals maintained in laboratory tanks at  $21^\circ\text{C}$  for 15 days.

### Oyster and seawater sampling

On each sampling date, hemolymph was collected from eight 'experimental' living oysters (Supplementary Methods). The animals were then dissected to collect the gills, digestive gland and remaining tissues and each tissue was ground separately in sterile seawater. On each sampling date, seawater was also collected at high tide and size fractionated. To collect zooplankton, large phytoplankton and organic particles, a 50 l sample was filtered through a  $60\ \mu\text{m}$  plankton net and the collected material subsequently washed with sterile seawater. Smaller organic particles and free-living bacterial cells were collected from 0.1 to 0.5 ml water samples pre-filtered through the  $60\ \mu\text{m}$  plankton net and sequentially filtered through 5, 1 and  $0.22\ \mu\text{m}$  pore size filters. All fractions were collected in four replicates.

### Bacterial isolation and gene sequencing

The 5, 1 and  $0.22\ \mu\text{m}$  filters were directly placed on *Vibrio* selective media (thiosulfate-citrate-bile salts-sucrose agar, TCBS) (Supplementary Methods). The hemolymph was directly streaked onto TCBS plates. The zooplankton/large particle fractions and oyster

tissues (gills, digestive gland and remaining tissues) were ground in sterile seawater (10 ml g<sup>-1</sup> of wet tissue) and streaked on TCBS. About 50 colonies per seawater fractions and 100 colonies per type of oyster tissue were randomly picked, re-streaked first on TCBS and then Zobell agar (15 gl<sup>-1</sup> agar, 4 gl<sup>-1</sup> bacto-peptone and 1 gl<sup>-1</sup> yeast extract in artificial seawater, pH 7.6). All isolates were genotyped by hsp60 partial gene sequence and stored in 10% DMSO at -80 °C (Supplementary Methods). A total of 1635 hsp60 sequences were obtained from the four experiments (Supplementary Table 1).

#### Population structure analysis

We first placed the isolates' hsp60 sequences in a taxonomic framework using the software pplacer (Matsen et al., 2010). A reference phylogeny was constructed based on 60 Vibrionaceae type strains and each hsp60 retrieved in this study was placed onto this reference tree. According to their position in the reference tree, hsp60 sequences received a taxonomic affiliation ranging from the genus to the species level. This resulted in the assignment of 1148/1635 isolates to 23 different species (taxon A to W; Supplementary Figure 1) whereas the others were unknown species. For one representative strain of each identified taxon, the assignment was confirmed by multilocus sequence typing using three additional protein-coding genes (rpoD, gyrB and rctB) (Supplementary Table 2).

Secondly, we partitioned the isolates according to their genetic and ecological similarities using the mathematical model implemented using the software AdaptML, which applies a Hidden Markov Model to delineate ecologically distinct groups based on genetic relatedness and similarity in environmental association (Hunt et al., 2008). The following environmental categories were considered in the analysis: (i) the sample categories: 4 60 µm; 60–5 µm; 5–1 µm; 1–0.2 µm seawater fractions and oyster tissue, and (ii) the season: spring and summer. We decided to merge the different oyster compartments as too many ecological categories might yield spurious results. Using a collapse threshold of 0.5 and convergence threshold of 0.001, we consistently and reproducibly obtained the highest number of non-redundant projected habitats. Finally, only ecological populations showing an empirical significance threshold of 99.999% and composed of at least 10 strains were further considered (Preheim et al., 2011).

Statistical analyses were performed using the computing environment R (R Core Team, 2016). To validate the ecological preferences of populations and study the distribution of populations among oyster tissues and hemolymph, Fisher-exact tests were performed with a 2x2 contingency table for each population in each isolation fraction. Significance was assessed using P-value ≤ 0.05. Statistical analysis of population distribution in oyster tissues

was tested using the parametric test of ANOVA (analysis of variance) as the data followed a normal distribution (Shapiro–Wilks test  $P \leq 2.2e-16$ ) and homoscedasticity (Bartlett test  $P = 0.66$ ).

#### Molecular microbiology

The strains and the strains and plasmids used and constructed in the present study are described in Supplementary Tables 3 and 4. *Vibrio* isolates were grown in Zobell broth or agar (4 gl<sup>-1</sup> bacto-peptone and 1 gl<sup>-1</sup> yeast extract in artificial seawater, pH 7.6), Luria-Bertani (LB) or LB-agar (LBA)+NaCl 0.5 M, at 20 °C. *Escherichia coli* strains were grown in LB or on LBA at 37 °C. Chloramphenicol (5 or 25 µg ml<sup>-1</sup> for *Vibrio* and *E. coli*, respectively), spectinomycin (100 µg ml<sup>-1</sup>), thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction of the P<sub>BAD</sub> promoter was achieved by the addition of 0.2% L-arabinose to the growth media, and conversely, was repressed by the addition of 1% D-glucose.

For the colonization and pairwise competition assays, a high-copy-number plasmid (pMRB), which is stably maintained in vivo without selection (Rui et al., 2010; Le Roux et al., 2011) was used to introduce green or red fluorescent protein (GFP or RFP) constitutively expressed from the P<sub>LAC</sub> promoter into the *Vibrio* strains. Alternatively, the P<sub>LAC</sub>-gfp and Cm<sup>R</sup> cassettes were integrated in non-essential chromosomal genes (transposases orfA and B) using pSW3654, a R6K γ-ori-based suicide vector, as described previously (Duperthuy et al., 2011).

For the pGV1512 mobilization experiment, we generated a donor containing a Cm<sup>R</sup> marked version of the pGV1512 (pGV1512::Cm<sup>R</sup>). To this end, a 500-bp fragment of pGV1512 (transposase fragment, position 90 957 to 91 768 in J5-20) was PCR amplified using Primer pGV1512-1 and 2 (Supplementary Table 5) and cloned in pSW23T (Demarre et al., 2005). After conjugative transfer, selection of the plasmid-borne drug marker (Cm<sup>R</sup>) resulted from integration of pSW23T in the pGV1512 plasmid by a single crossover. This strategy was also used to stabilize the pGV1512 in Δcus/cop and ΔT4SS mutants as we observed that the deletion of these operons induces a higher frequency of pGV1512 loss. To generate the recipient, we introduced a Spec<sup>R</sup> cassette in a chromosomal locus (ΔR1::Spec<sup>R</sup>) by allelic exchange as described previously (Lemire et al., 2015).

For plasmid curing, the 500-bp fragment of the pGV1512 described above was cloned in pSW7848T plasmid (Val et al., 2012). This pSW23T derivative vector encodes the ccdB toxin gene under the control of an arabinose-inducible and glucose-repressible promoter, P<sub>BAD</sub> (Le Roux et al., 2007). Selection of the plasmid-borne drug marker on Cm and glucose resulted from integration of pSW7848T in the pGV1512 plasmid by a single crossover. Elimination of the recombinant plasmid was selected by

arabinose induction of the *ccdB* toxin gene. After several re-isolations, the curing of the plasmid was confirmed by PCR using primers pGV1512-1 and 2.

Deletion of selected regions was performed by allelic exchange using the same pSW7848T suicide plasmid (Le Roux et al., 2007). To this end, two 500 bp fragments flanking the region to delete were amplified and cloned into pSW7848T (Supplementary Methods). Subsequently, the first and second recombination leading to pSW7848T integration and elimination was selected on Cm +glucose and arabinose media respectively.

Matings between *E. coli* and *Vibrio* were performed at 30 °C as described previously (Le Roux et al., 2007). Matings between vibrios were performed by mixing the donor (pGV1512::Cm<sup>R</sup>) and recipient (for example,  $\Delta$ R1::Spec<sup>R</sup>) at a ratio of 1/1 on LBA +NaCl 0.5 M and incubating at 20 °C for 24 h.

#### Plasmid extraction, detection and annotation

Strains were screened for plasmid presence by using gel electrophoresis of DNA that had been extracted using a modified NucleoBond plasmid purification kit (Takara-Clontech Bio Inc., Mountain View, CA, USA) (Xue et al., 2015). The pGV1512 identification was then confirmed by Southern blot using a Digoxigenin-labeled probe (transposase fragment, position 90957 to 91768 in pGV1512 of J5-20) (Roche Diagnostics, Meylan Cedex, France) according to the manufacturer's instructions. The detection of the pGV1512 from a larger collection of strains was performed by PCR using the primer pGV1512-1 and 2. The pGV1512 plasmid found in *Vibrio crassostreae* J5-20 was annotated using the MaGe software (Magnifying Genome, (Vallenet et al., 2013) (<http://www.genoscope.cns.fr/agc/mage>) and deposited at Genbank under the accession number KX765275.

#### Experimental challenge

To determine virulence of isolates, bacteria were grown under constant agitation at 20 °C for 24 h in Zobell media. One hundred microliters of the culture (10<sup>6</sup> c.f.u.) were injected intramuscularly into oysters. The bacterial concentration was confirmed by conventional dilution plating on Zobell agar. After injection, the oysters were transferred to aquaria (20 oysters per 2.5l aquarium) containing 1l of aerated 5  $\mu$ m-filtered seawater at 20 °C, kept under static conditions. Experiments were performed in duplicate and mortality was assessed after 24 h. For colonization and competition experiments, a pure culture or a mix (1:1) of GFP-labeled wild type to RFP-labeled mutant or reciprocally RFP-labeled wild type to GFP-labeled mutant were injected into oysters (10<sup>5</sup> c.f.u. per animal, that is, sub-lethal dose) or added to the oyster tank (10<sup>7</sup>c.f.u. ml<sup>-1</sup>). Hemolymph was collected after 24 h, serial diluted and

spread on LB-agar (LBA)+NaCl 0.5 M supplemented with Cm.

## Results

### Population structure and dynamics

To compare the *Vibrio* population structure in seawater and oyster tissues, a total of 1635 strains were isolated in spring and summer and genotypes identified by protein-coding gene sequencing. Mathematical modeling taking into account phylogenetic and ecological structure partitioned these strains into 24 putative populations (Figures 1a and b). Most are well resolved although we note that predictions within the large *V. splendidus* clade (#13 to 22) might be unstable due to high-sequence and habitat similarity (Preheim et al., 2011). Overall, the population structure of *Vibrio* was strongly influenced by season of isolation, with the 24 populations showing strong association with either spring or summer (Figures 1 and 2). Although the spring samples are dominated by *V. splendidus* related populations (#13 to 22), diversity of populations was much higher in summer samples. Many of the populations, including the dominance of *V. splendidus* in spring, were also found at a site on the Northeastern U.S. coast experiencing similar temperature regimes (Hunt et al., 2008; Preheim et al., 2011; Szabo et al., 2012). Moreover, as in this previous work (Szabo et al., 2012), several populations were abundant only on a single sampling date ((for example, 5 May: *Vibrio* sp. #9; 29 July: *V. chagasii* #3) Figure 2) suggesting short-lived population expansions. As these populations were found in both seawater and oysters this observation furthermore suggests a generally high connectivity between oysters and their environment.

### Populations positively associated with oysters and pathogenicity

Although the comparison of population frequency in the water and oyster samples suggested little discrimination for many populations, some were unequally distributed (Figures 1b and 2) suggesting the potential for specific enrichment within oysters. To address this hypothesis we compared the relative abundance of the 24 populations in animals and seawater fractions using Fisher-exact and odds-ratio tests (Supplementary Table 6; Supplementary Figure 2). The analysis revealed a significant positive correlation between oysters and 10 of 24 populations (*V. harveyi* #5, *V. crassostreae* #11, *V. splendidus* #17 and 19, *V. tasmaniensis* #23 and 24, *Vibrio* spp. #6, 7, 8, 12) (Supplementary Table 6, in bold; Supplementary Figure 2E). However, of these, only *V. crassostreae* showed a negative association with all seawater fractions (Supplementary Table 6). This suggests that *V. crassostreae* is the only population specifically associated with oysters since all others also occur in seawater fractions at considerable

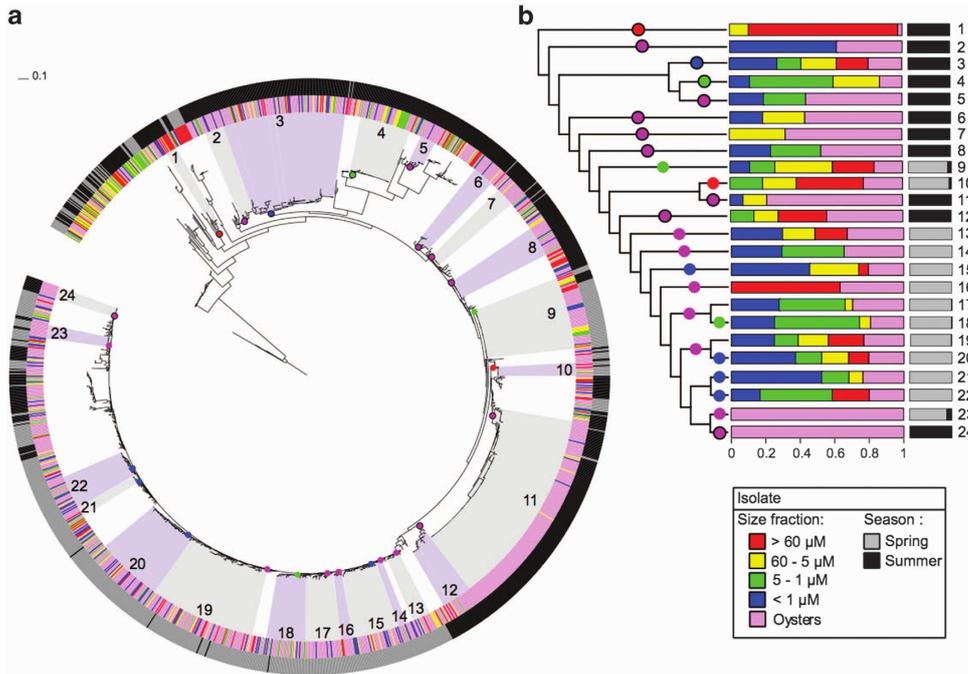


Figure 1 Population structure prediction of Vibriaceae bacteria recovered from different seawater fractions or oyster tissues in two seasons. (a) Phylogenetic tree of 1635 isolates based on partial hsp60 marker genes. The inner ring indicates whether a strain was isolated from a specific seawater fraction or from oysters while the outer ring represents the season of isolation. Ecological populations predicted by the AdaptML algorithm (Hunt et al., 2008) are identified by alternating gray and purple shading if they passed an empirical confidence threshold of 99.9%. (b) Cladogram summarizing the normalized distribution of each population across seasons, seawater fractions and oysters with only populations 4-10 members being represented. A total of 17/24 populations were assigned to a named taxon: *V. orientalis* (#1); *V. fortis* (#2); *V. chagasii* (#3); *V. harveyi* (#5); *V. crassostreae* (#11); *V. splendidus* (#13 to 22), *V. tasmaniensis* (#23 and 24). Other populations were designated *Vibrio* sp. due to lack of closely related reference species (that is, populations 4, 6 to 10 and 12).

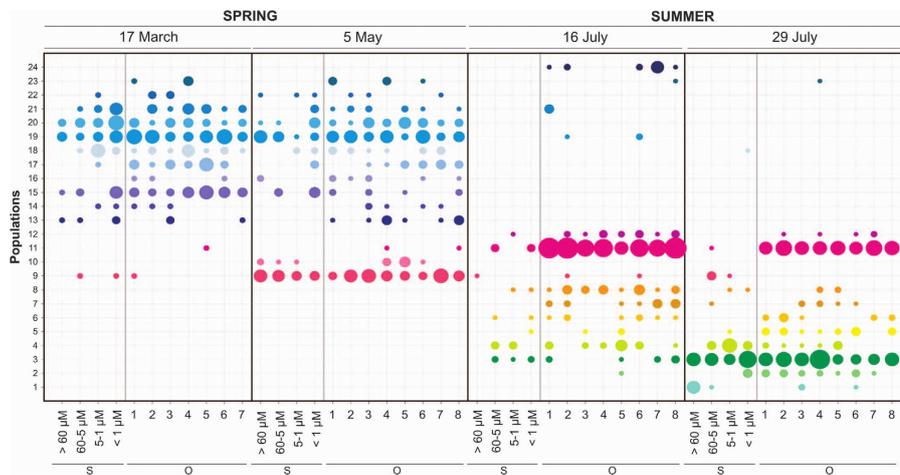


Figure 2 Relative abundance of *Vibrio* populations predicted by AdaptML by sampling categories and date. The size of each circle is proportional to the relative abundance of a population in a sample category (normalized by the number of isolates in each fraction/oyster replicate to account for uneven sampling). Numbering of populations as in Figure 1. S, seawater fraction; O, oyster tissue (digestive gland, gills, hemolymph and remaining tissues).

frequency and their enrichment (#2, 10, 13 to 16 and 22) or depletion in oysters (#1) may simply reflect preferential retention or exclusion.

To further test to what extent oysters represent a specific habitat for some *Vibrio* populations, we

compared their distribution among gills, digestive glands and hemolymph (Supplementary Table 7). Among the populations positively associated with oysters (Supplementary Table 7, in bold), three (*V. crassostreae* #11 and *Vibrio* sp. #6 and 8) show

a significant positive association with gills and only one (*V. crassostreae*) with hemolymph. This result further supports that *V. crassostreae* is specifically adapted to survive within oysters since the circulatory system represents a hostile environment as it is well defended by the immune system, which *V. crassostreae* must therefore be able to overcome.

Because we structured our sampling to span the previously determined temperature threshold for onset of the disease (~16 °C) (Petton et al., 2015a), we were able to observe population changes associated with outbreak of the disease (Figure 2). No mortalities were observed in spring (o 16 °C), whereas 50% cumulative mortalities were detected in summer (4 16 °C) (see materials and methods). Diverse populations infected diseased oysters: however, only *V. crassostreae* was consistently found in all animals at a high relative abundance (Figure 2) and was rarely detected in oysters collected in spring. These environmental dynamics strongly support the previous hypothesis that *V. crassostreae* is the etiological agent of oyster disease (Lemire et al., 2015) which we further test below.

*V. crassostreae* pathogenicity is dependent on a large mobilizable plasmid

Although previous work has suggested that all strains within *V. crassostreae* are virulent (Lemire et al., 2015), our expanded sampling suggests that only a subset of the population is in fact so. Pathogenicity was established for all of 12 *V. crassostreae* isolates from diseased oysters leading to the hypothesis that virulence is a function encoded by the core genome (Lemire et al., 2015). In contrast, here we find by using a larger collection of strains (n=196) isolated from both oysters and seawater that only 75% appeared virulent (inducing

4 50% mortality) while the remainder induced o 20% mortality. In parallel we observed that handling a *V. crassostreae* strain (J2-9) in the laboratory resulted in reduced virulence (data not shown). This led to the changed hypothesis that virulence determinants are carried by a mobile genetic element.

To test this hypothesis, we first investigated the presence of extra-chromosomal DNA in eight *V. crassostreae* strains (four virulent and four non-virulent) by DNA electrophoresis. This showed a large replicon specific to virulent strains (Supplementary Figure 3A). Second, the search for plasmid genes (*parA/B*, *repB* and *tra*) in available genome sequences of *V. crassostreae*, followed by manual scaffolding led to the assembly of a putative gapped sequence for the strain J5-20 (178 kbp) further confirmed by PCR (Figure 3a). This plasmid was then identified in all virulent strains with a sequence identity of 99.4% in the common regions (Figure 3a). Finally, the existence of the replicon detected by gel staining was confirmed by Southern blot (Supplementary Figure 3B). This plasmid was successfully detected in 1/10 (10%) and 152/196 (77%) of *V. crassostreae* strains isolated from seawater and oysters, respectively (Supplementary Figure 4). Among these strains, 50 plasmid carriers and 50 non-carriers were injected into oysters, and we observed that virulence coincides with the presence of the plasmid, strongly suggesting that this replicon, thereafter named pGV1512, encodes virulence factors (Figure 3b).

The manual annotation of the pGV1512 plasmid revealed 206 open reading frames (Figure 3a) with 55% having unknown functions. Like many large plasmids, pGV1512 appears to have a modular architecture: (i) a replication/segregation system (*parA/B* and *repB*), (ii) a conjugative apparatus (Type

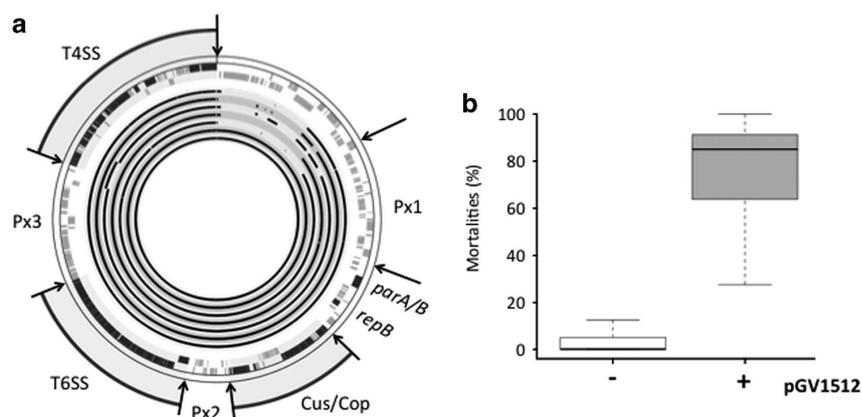


Figure 3 Correlation between pGV1512 presence and *V. crassostreae* virulence. (a) Circular representation of the *V. crassostreae* (strain J5-20) pGV1512 plasmid. From the outside inwards: the 1st and 2nd circles show the open reading frames encoding replication and partitioning; a type 4 secretion system (T4SS); Cus/Cop and regulators; a type 6 secretion system (T6SS); Px1 to 3 regions contain a majority of genes encoding unknown function; the internal circles 3 to 9 show the alignment with contigs for the strain (J5-4; J5-5; J2-9; J5-15; LGP7; LGP15; LGP107). The arrows indicate the site of allelic exchange for deletions. (b) Oyster mortality in response to experimental infection with 50 strains with (gray) or 50 strains without (white) plasmid (detected by a PCR against a pGV1512 fragment). A total of 10<sup>6</sup> c.f.u. of the strains was intramuscularly injected into oysters (n=20, in duplicate). Mortality (%) was assessed after 24 h.

IV secretion system, T4SS, the coupling protein and the relaxase, Supplementary Figure 5), (iii) a heavy metal resistance cluster (*Cus/Cop*) and (iv) a type VI secretion system (T6SS, Supplementary Figure 6). These modules do not permit the classification of the pGV1512 in any previously described plasmid families although both secretion systems have high-sequence similarities with loci found in *V. splendidus*, *V. cyclitrophicus* and *V. tasmaniensis* genome drafts.

We further explored the role of the pGV1512 plasmid in the virulence of *V. crassostreae* using molecular genetics. By introducing a counter selectable marker (*CcdB* toxin) in the plasmid, we successfully cured this replicon in strains J2-9, J2-9 and the J2-9-Spec<sup>R</sup> derivative ΔR1 (Figure 4a). Plasmid loss did not impair bacterial growth in culture media, but resulted in a dramatic decrease in mortality induced after bacterial (J2-9 ΔpGV1512, J5-20 ΔpGV1512, J2-9 ΔR1-ΔpGV1512) injection (Figure 4b). A restoration of the ΔR1 phenotype was observed after pGV1512 mobilization into J2-9 ΔR1-ΔpGV1512 (J2-9 ΔR1-ΔpGV1512 +pGV1512Cm<sup>R</sup>), demonstrating that this plasmid is conjugative and necessary for virulence.

We next assessed the importance of specific loci for virulence using a genetic knockout approach. The deletion of T6SS, T4SS and *Cus/Cop* gene clusters

(Figure 3a) did not result in virulence attenuation (Supplementary Figure 7). However, the deletion of the regions between *cus/cop* and T6SS (Px2), T6SS and T4SS (Px3) (Figure 3a), dramatically affected the virulence of *V. crassostreae* (Supplementary Figure 7). No putative virulence factor could be identified in the Px2 and 3 loci based on sequence homology searches (Supplementary Table 8), suggesting that virulence determinants encoded by pGV1512 are novel.

The pGV1512 plasmid is not necessary for oyster colonization

As we observed that only *V. crassostreae* shows a significant positive association with oyster hemolymph, we addressed whether colonization is related to the presence of pGV1512. First, a replicative plasmid expressing a fluorochrome was introduced in *V. crassostreae* strains J2-9, J2-9ΔpGV1512 and, as a control, in *V. cyclitrophicus* strain 7T5-8. Both fluorescent strains were then added to oyster tanks (10<sup>7</sup>c.f.u. ml<sup>-1</sup>) and their ratios determined (Figure 5a). The colonization by *V. crassostreae* was found to remain high (10<sup>4</sup>–10<sup>5</sup> c.f.u. ml<sup>-1</sup> hemolymph) for up to 4 days, whereas *V. cyclitrophicus* was initially slow to colonize, then briefly rose to higher density but decreased dramatically on days 3 and 4 (10<sup>3</sup> c.f.u. ml<sup>-1</sup>). This result was independent of *V. crassostreae* carrying the plasmid (Figure 5a) and was further confirmed by using strains expressing the GFP from a chromosomal locus (Supplementary Figure 8). Here again for three days we observed a two log difference in colonization between a *V. crassostreae* cured from the plasmid (10<sup>5</sup> c.f.u. ml<sup>-1</sup>), and *V. cyclitrophicus* (10<sup>3</sup> c.f.u. ml<sup>-1</sup>), although from day 5 both strain loads were found to be 10<sup>3</sup>–10<sup>2</sup> c.f.u. ml<sup>-1</sup>.

Secondly, we explored the role of pGV1512 in oyster hemolymph colonization by in vivo competition assays. Oysters were infected by injection or immersion with a 1:1 mix of differentially fluorescently labeled *V. crassostreae* strains that did or did not carry the plasmid (Figure 5b). We did not observe any effect of the plasmid loss on competitiveness, suggesting that the plasmid is not necessary for hemolymph colonization. Further, to control for the potential that the strain without plasmid acts as a cheater with regards to some extracellular product secreted by the wild type strain, we also competed *V. crassostreae* strains with J2-8, a strain that has also been isolated from oysters and belongs to a closely related population that is not implicated in disease (Lemire et al., 2015). Again we did not observe any clear effect of the plasmid loss on hemolymph colonization (Supplementary Figure 9), confirming that the plasmid is not necessary for colonization and/or survival in the host.

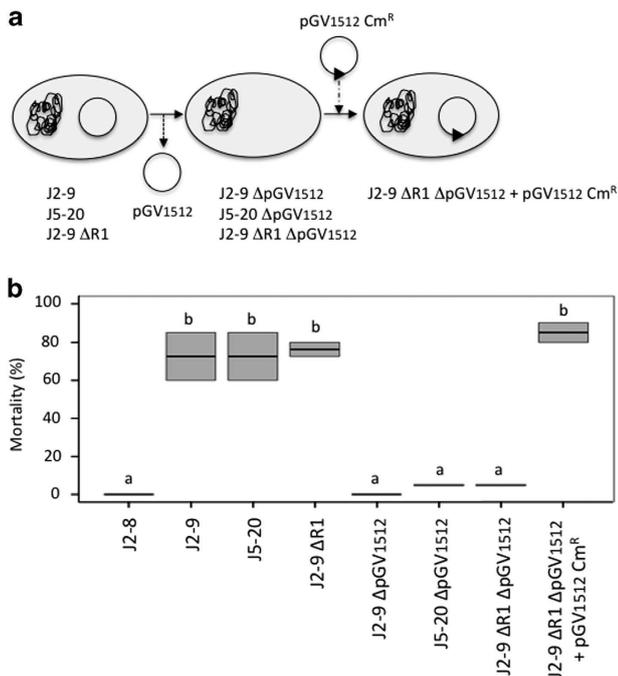


Figure 4 Experimental assessment of the mobilization of the pGV1512 plasmid through conjugative transfer, and its virulence implication in *V. crassostreae*. (a) Different genetic constructs used. First, the plasmid was cured in three *V. crassostreae* strains (J2-9; J2-9 ΔR1 and J5-20). Second, mobilization of the pGV1512 plasmid (modified with the insertion of a chloramphenicol resistance gene) was tested by assessing the possibility of conjugative transfer in J2-9ΔR1 (modified with the insertion of a spectinomycin resistance gene; see Lemire et al., 2015, for details). (b) Virulence assessment of the different genetic constructs (x-axis) after injection of strains (10<sup>6</sup> c.f.u. per animal) in 20 oysters and counting the percentage of mortalities (y-axis).

## Discussion

Although vibrios are frequently associated with mortality of farmed species, their role as causative

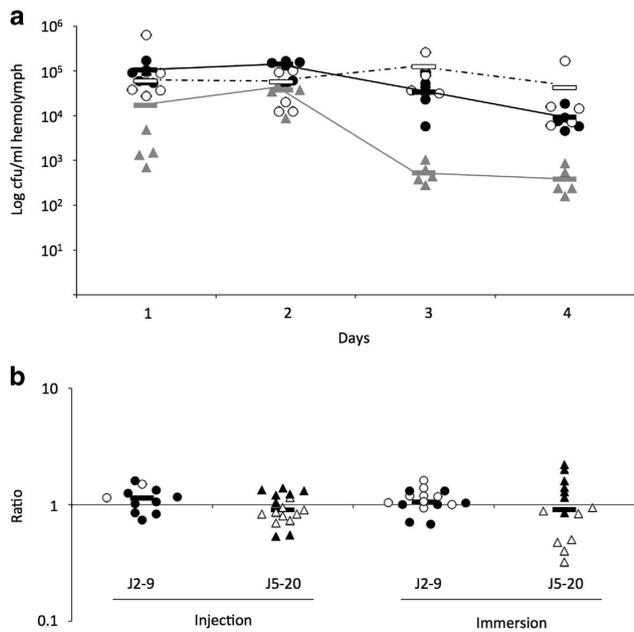


Figure 5 Role of pGV1512 for oyster colonization. A replicative plasmid was used to introduce green or red fluorescent protein (GFP or RFP) and a Cm<sup>R</sup> cassette into the *Vibrio* strains. (a) GFP-labeled strains (*V. cyclitrophicus*, strain 7T5-8, gray triangle; *V. crassostreae*, strain J2-9, black circle; J2-9ΔpGV1512 white circle) were individually added to the oyster tank (10<sup>7</sup>c.f.u. ml<sup>-1</sup>). Hemolymph was collected daily (x-axis) from 5 animals, serially diluted and spread on solid media supplemented with Cm. The y-axis indicates the log c.f.u. ml<sup>-1</sup> of hemolymph retrieved on plates. The horizontal bar in each experiment indicates the mean of log c.f.u. ml<sup>-1</sup>. (b) Colonization capacity assessment by determination of the ratio of WT strains J2-9/J5-20 and derivatives without plasmid retrieved on plates (y-axis) after homogenization of animals that were infected with 1:1 mix by injection or immersion (x-axis). Each dot corresponds to a replicate (up to 10). Black dots: WT tagged with GFP/mutant tagged with RFP; white dots: WT tagged with RFP/mutant tagged with GFP. The horizontal bar in each experiment indicates the mean ratio.

agent or secondary opportunistic colonizers is not well understood. Most analyses are based on moribund animals often infected by multiple agents (viruses, bacteria) and laboratory experiments rely on challenge via injection, a method that does not reflect the natural route of infection and may thus exclude other important factors (for example, chemotaxis, colonization). Here by combining environmental sampling, natural infection and molecular genetics, we attempt to overcome these limitations to explore the role of *Vibrio* in juvenile oyster disease. Our approach identified one dominant population as responsible for disease. This *V. crassostreae* appears to have originated as a benign colonizer of oysters that is capable of extended survival even in the hostile environment of the hemolymph but our data suggest that a subset of the population has been invaded by a pathogenicity plasmid turning it into a pathogen. This large mobilizable plasmid contains two loci essential for virulence; however, none of the genes encoded known virulence factors suggesting that the nature of pGV1512 virulence is novel.

Our results show that although *Vibrio* populations colonizing oysters overall resemble those from the surrounding seawater there is selective enrichment of some populations, suggesting that oysters represent a permissive habitat for these. This contrasts with a previous study that demonstrated neutral assembly of *Vibrio* populations in mussels as indicated by their near identical frequency in seawater and mussel samples (Preheim et al., 2011). This difference may be due to particularities of our sampling area (oyster farm affected by a disease) or properties of oysters themselves. Firstly, high density of oysters in farming areas could result in selection and expansion of vibrios adapted to survival in these animals. Second, vibrios might be more adapted to oysters than mussels. For example, diseases associated with vibrios have not been described in the latter until recently (Ben Cheikh et al., 2016) even when co-cultivated with oysters. Although both are filter feeders and possess innate immunity, mussels and oysters show important physiological differences. Mussels possess a systemic humoral antimicrobial response that seems absent in oysters (Schmitt et al., 2012). Another particularity of *C. gigas* is the high level of copper in the plasma, which is characterized by abundant copper-containing proteins like Zn/Cu SODs (Duperthuy et al., 2011). In fact, copper toxicity appears to be used as a defense mechanism by hosts and could therefore select for resistance in vibrios. This is illustrated by the recent demonstration that the gene *copA*, encoding for a heavy metal efflux protein, is essential for copper resistance, oyster colonization and virulence in vivo in *V. tasmanienensis* (Vanhove et al., 2015). The deletion of the *copA* in *V. crassostreae* strain J2-9 also results in virulence attenuation (not shown) and is likely a key factor for persistence in the hemolymph.

Experimental colonization suggests that members of the *V. crassostreae* population can persist for extended periods in the hemolymph, the most hostile compartment in the animals. Importantly, this colonization does not require the presence of the pGV1512 plasmid, suggesting that this property is likely a core function of this population. Previous genomic comparison identified genes that were shared by all *V. crassostreae* strains and clustered in population-specific genomic regions (Lemire et al., 2015). Interestingly some of these genes encode for colonization factors (*tad* gene cluster, (Tomich et al., 2007)) and for resistance to antimicrobial peptides (PhoPQ, (Otto, 2009)) and to ROS (catalase and superoxide dismutase, (Ibarra and Steele-Mortimer, 2009)). These population-specific genes potentially allow survival vs the host immune response and could explain benign commensal behavior of *V. crassostreae* in oysters.

Although the plasmid is not necessary for colonization its high frequency in the population suggests that it confers a fitness advantage in oysters possibly because pathogenic strains may create damaged

tissue, which they exploit for nutrition. Whether oyster farming itself has selected for the high prevalence of the plasmid in the *V. crassostreae* population analyzed here remains unknown but it is striking that oyster disease appears to be currently absent from areas in Northern Europe where *C. gigas* is abundant but not farmed (Watermann et al., 2008). In this context, an important question will be to what extent *V. crassostreae* is specific to oysters or is currently invading oysters with its ability to survive in the hemolymph being an exaptation. It was the only population negatively associated with seawater suggesting that its primary habitat is not in micro-environments therein. This is confirmed by several years of sampling at a comparable site in the Northwestern Atlantic where no oyster farming occurs and where *V. crassostreae* has not been isolated from seawater (Hunt et al., 2008; Preheim et al., 2011). Future research will therefore focus on determining potential alternative reservoirs for *V. crassostreae* and whether the pathogenicity plasmid is specifically enriched in populations associated with oysters.

## Conflict of Interest

The authors declare no conflict of interest.

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## Author contributions

AJ, BP, YL, SC, MAB and FLR performed experiments. MB performed the in silico analyses, MB and AJ the statistics. MB, FLR and MP designed experiments, interpreted results and wrote the paper. MB and AJ contribute equally to this work. The manuscript has been seen and approved by all of the authors.

## References

Bachere E, Rosa RD, Schmitt P, Poirier AC, Merou N, Charriere GM et al. (2015). The new insights into the oyster antimicrobial defense: cellular, molecular and genetic view. *Fish Shellfish Immunol* 46: 50–64.

Ben Cheikh Y, Travers MA, Morga B, Godfrin Y, Rioult D, Le Foll F. (2016). First evidence for a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities. *Dev Comp Immunol* 57: 107–119.

Cognie B, Barille L, Masse G, Beninger P. (2003). Selection and processing of large suspended algae in the oyster *Crassostrea gigas*. *Mar Ecol Prog Ser* 250: 145–152.

Demarre G, Guerout AM, Matsumoto-Mashimo C, Rowe-Magnus DA, Marliere P, Mazel D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPal-pha) conjugative machineries and their cognate *Escherichia coli* host strains. *Res Microbiol* 156: 245–255.

Destoumieux-Garzon D, Rosa RD, Schmitt P, Barreto C, Vidal-Dupiol J, Mitta G et al. (2016). Antimicrobial peptides in marine invertebrate health and disease. *Philos Trans R Soc Lond B Biol Sci*; e-pub ahead of print 26 May 2016; doi:10.1098/rstb.2015.0300

Duperthuy M, Schmitt P, Garzon E, Caro A, Rosa RD, Le Roux F et al. (2011). Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proc Natl Acad Sci USA* 108: 2993–2998.

Faury N, Saulnier D, Thompson FL, Gay M, Swings J, Le Roux F. (2004). *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). *Int J Syst Evol Microbiol* 54: 2137–2140.

Froelich B, Ayrapetyan M, Oliver JD. (2012). *Vibrio vulnificus* integration into marine aggregates and subsequent uptake by the oyster, *Crassostrea virginica*. *Appl Environ Microbiol* 78: 3885–3889.

Hunt DE, David LA, Gevers D, Preheim SP, Alm EJ, Polz MF. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science* 320: 1081–1085.

Ibarra JA, Steele-Mortimer O. (2009). Salmonella—the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. *Cell Microbiol* 11: 1579–1586.

Le Roux F, Binesse J, Saulnier D, Mazel D. (2007). Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* 73: 777–784.

Le Roux F, Davis BM, Waldor MK. (2011). Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res* 39: 1004–1013.

Le Roux F, Wegner KM, Baker-Austin C, Vezzulli L, Osorio CR, Amaro C et al. (2015). The emergence of *Vibrio* pathogens in Europe: ecology, evolution, and pathogenesis (Paris, 11-12th March 2015). *Front Microbiol* 6: 830.

Le Roux F, Wegner KM, Polz MF. (2016). Oysters and Vibrios as a model for disease dynamics in wild animals. *Trends Microbiol* 24: 568–580.

Lemire A, Goudenege D, Versigny T, Petton B, Calteau A, Labreuche Y et al. (2015). Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *ISMEJ* 9: 1523–1531.

Lokmer A, Kuenzel S, Baines JF, Wegner KM. (2016). The role of tissue-specific microbiota in initial establishment success of Pacific oysters. *Environ Microbiol* 18: 970–987.

Matsen FA, Kodner RB, Armbrust EV. (2010). pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. *BMC Bioinformatics* 11: 538.

- Otto M. (2009). Bacterial sensing of antimicrobial peptides. In: Collin M, Schuch R (eds). Vol. 16. Contributions to Microbiology. KARGER: Basel, pp 136–149.
- Pales Espinosa E, Koller A, Allam B. (2016). Proteomic characterization of mucosal secretions in the eastern oyster, *Crassostrea virginica*. *J Proteomics* 132: 63–76.
- Petton B, Boudry P, Alunno-Bruscia M, Pernet F. (2015a). Factors influencing disease-induced mortality of Pacific oysters *Crassostrea gigas*. *Aquac Environ Interact* 6: 205–222.
- Petton B, Bruto M, James A, Labreuche Y, Alunno-Bruscia M, Le Roux F. (2015b). *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Front Microbiol* 6: 686.
- Petton B, Pernet F, Robert R, Boudry P. (2013). Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. *Aquac Environ Interact* 3: 257–273.
- Preheim SP, Boucher Y, Wildschutte H, David LA, Veneziano D, Alm EJ et al. (2011). Metapopulation structure of Vibrionaceae among coastal marine invertebrates. *Environ Microbiol* 13: 265–275.
- R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing: Vienna, Austria. Available at: <https://www.R-project.org/>.
- Rui H, Ritchie JM, Bronson RT, Mekalanos JJ, Zhang Y, Waldor MK. (2010). Reactogenicity of live-attenuated *Vibrio cholerae* vaccines is dependent on flagellins. *Proc Natl Acad Sci USA* 107: 4359–4364.
- Schmitt P, Rosa RD, Duperthuy M, de Lorgeril J, Bachere E, Destoumieux-Garzon D. (2012). The antimicrobial defense of the pacific oyster, *Crassostrea gigas*. how diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. *Front Microbiol* 3: 160.
- Szabo G, Preheim SP, Kauffman KM, David LA, Shapiro J, Alm EJ et al. (2012). Reproducibility of Vibrionaceae population structure in coastal bacterioplankton. *ISME J* 7: 509–519.
- Tomich M, Planet PJ, Figurski DH. (2007). The tad locus: postcards from the widespread colonization island. *Nat Rev Microbiol* 5: 363–375.
- Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, Mazel D. (2012). Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS Genet* 8: e1002472.
- Vallenet D, Belda E, Calteau A, Cruveiller S, Engelen S, Lajus A et al. (2013). MicroScope—an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res* 41: D636–D647.
- Vanhove AS, Rubio TP, Nguyen AN, Lemire A, Roche D, Nicod J et al. (2015). Copper homeostasis at the host vibrio interface: lessons from intracellular vibrio transcriptomics. *Environ Microbiol* 18: 875–888.
- Watermann BT, Herlyn M, Daehne B, Bergmann S, Meemken M, Kolodzey H. (2008). Pathology and mass mortality of Pacific oysters, *Crassostrea gigas* (Thunberg), in 2005 at the East Frisian coast, Germany. *J Fish Dis* 31: 621–630.
- Wendling CC, Batista FM, Wegner KM. (2014). Persistence, seasonal dynamics and pathogenic potential of *Vibrio* communities from pacific oyster hemolymph. *PLoS One* 9: e94256.
- Xue H, Cordero OX, Camas FM, Trimble W, Meyer F, Guglielmini J et al. (2015). Eco-evolutionary dynamics of episomes among ecologically cohesive bacterial populations. *MBio* 6: e00552–00515.

Supplementary Information accompanies this paper on The ISME Journal website (<http://www.nature.com/ismej>)

## Supplementary Information

### Material and methods

#### **Production of “pathogen free” oysters (Specific Pathogen Free, SPF)**

A pool of 90 genitors (wild seed collected in Fouras Bay, Marennes- Oléron, France) was transferred to the Ifremer facility located at Argenton (Brittany, France; 48° 31' 16'' N, 4° 46' 2''W) and treated for 6 days with chloramphenicol (8mg/L). For maturation induction, animals were held for 6 weeks in 500 L flow-through tanks with seawater enriched with a phytoplankton mixture at a constant temperature of 17°C. Seawater was treated with UV and filtered through 1 µm mesh. The daily mixed diet consisted of *Tisochrysis lutea* (CCAP 927/14) and *Chaetoceros muelleri* (CCAP 1010/3 1:1 in dry weight at a ratio equivalent to 6% of the oyster dry weight. Once the oysters were reproductively mature, gametes from 90 individuals (≈1/3 males, 2/3 females), obtained by stripping, were mixed in a 5 L jar at 50 spermatozooids per oocyte (day 0). The fertilized oocytes completed their embryonic development in 150 L tanks filled with 1 µm filtered and UV-treated seawater at 21°C for 48 h. The D-larvae (day 2) were then collected and reared in flow-through rearing systems at 25°C (Rico-Villa et al., 2008). At the end of the pelagic phase (day 15), all larvae were collected on a 100 µm sieve and allowed to settle on cultch. Postlarvae were maintained in downwelling systems where they were continuously supplied with enriched seawater until the beginning of the experiments. In the larval and post-larval stages, the oysters were fed the same diet as the broodstock at a concentration of 1500 µm<sup>3</sup> µl<sup>-1</sup> (Rico-Villa et al., 2009). Overall, two batches of standardized oyster seed (3 to 6 months old, 0.2 to 1.2 g wet mass) were produced for use in the experiments.

Juvenile disease have been previously associated with the presence of a herpes virus (OsHV-1 µvar) (Segarra et al., 2010) and bacterial populations of the genus *Vibrio* (Lemire et al., 2014). Here the « pathogen free » status of animals was confirmed by i) the absence of OsHV-1 DNA detection by qPCR (Pepin et al., 2008); ii) a low *Vibrio* presence (~10 cfu/gr tissu) determined by isolation on selective culture medium (Thiosulfate-citrate-bile salts-sucrose agar, TCBS) (Petton et al., 2015). Finally all SPF oyster remained free of any abnormal mortality.

#### **Strains isolation and identification**

On each sampling date (17 March, 5 May, 16 July and 29 July), eight “experimental” oysters were anesthetized for 2 h in a MgCl<sub>2</sub> bath (5% w/v in 2/3 v/v seawater/freshwater) prior to hemolymph puncture through the adductor muscle. The animals were then dissected and each tissue were separately grounded in sterile seawater (10 mL/g of wet tissue). Subsamples of these homogenates were spread on TCBS (Difco) medium while filters on which different seawater fractions were collected were directly placed on TCBS plates. TCBS has been shown to be an effective selective medium for *Vibrio* isolation from estuarine and marine waters (Pfeffer and Oliver, 2003). It has been extensively used in the past to recover a large range of *Vibrio* spp. and provides standard approach for comparison with other studies (Turner et al., 2009). Randomly selected colonies (12/animal and 48 by size fractions for the 4 sampling dates) were re-streaked first on TCBS, then on Zobell agar (15g/l agar, 4 g/l bactopectone and 1 g/l yeast extract in artificial sea water, pH7.6), cultivated in Zobell media and stored at -80°C. For DNA sequencing, purified isolates were grown in Zobell overnight and DNA extracted using a DNA extraction kit (Cliniscience). The partial *hsp60* gene was amplified for all isolates as described previously (Hunt et al., 2008) using primers Hsp60 R and F (Table S5). The PCR conditions were: 3 min at 94°C followed by 30 cycles of 30 sec each at 94°C, 55° and 72°C with a final step of 6 min at 72°C. Genes were sequenced using the reverse primer and sequencing was performed at GATC (<https://www.gatc-biotech.com/fr>).

### **Cloning of deleted alleles into a suicide vector**

The cloning was performed using the Gibson assembly method according to the manufacturer’s instructions (New England Biolabs, NEB). For T6SS deletion and to generate a Spec<sup>R</sup> recipient (J2-9ΔR1), three independent PCR amplifications were performed using primers ΔT6SS-1 to 6 (Table S5) and ΔR1-1 to 6 respectively. Primer pairs ΔX-1+ 2 and ΔX-5+ 6 and *V. crassostreae* strain J2-9 DNA as target, were used to amplify the 500 bp fragment located upstream and downstream of the region to be deleted, respectively. Primer pair ΔX-3+ 4 and PSW25T (Demarre et al., 2005) as target allowed the amplification of a spectinomycin resistance cassette to be cloned between the two 500 bp fragments outlined above. For deletion of the other regions (ΔCus/Cop, ΔT4SS, ΔPx1, 2, 3), two independent PCR amplifications of the regions (500 bp) encompassing the gene to be deleted were performed using the primer pairs ΔX-1 and 2, and ΔX-3 and 4 (Table S5). A PCR inside out was performed using PSW7848T suicide vector DNA (Val et al., 2012) and

primer pair (SW-F and SW-R) (Table S5). After purification and quantification, 0.02–0.5 pmols of each PCR products were mixed with Gibson assembly Master Mix and incubated for 60 minutes at 50°C. A dialysis was performed on the samples before electrocompetent *E. coli* transformation. Strains Π3813 and β3914 were used as a plasmid host for cloning and conjugation, respectively (Le Roux et al., 2007). Strains and plasmids used and established in the present study are presented in Table S3 and 4, respectively.

### Conjugation

Overnight cultures of donor (*E. coli* β3914) and recipient (*Vibrio*) were diluted at 1:100 in culture media without antibiotic and grown at 30°C to an OD<sub>600nm</sub> of 0.3. The different conjugation experiments were done by a filter mating procedure described previously (Le Roux et al., 2007) with 1 ml donor and 10ml recipient. Conjugations were performed overnight on filters incubated on LBA + NaCl 0.5N + diaminopimelic acid (DAP) plates at 30°C. Counter-selection of *ΔdapA* donor was done by plating on a medium devoid of DAP, supplemented with chloramphenicol and when required 1% glucose. For mutagenesis, Cm<sup>R</sup> resistant colonies were grown in LB + NaCl 0.5N + glucose 1% up to late logarithmic phase and spread on plates containing 0.2% arabinose. Mutants were screened by PCR using external primers.

### References

Demarre, G., Guerout, A.M., Matsumoto-Mashimo, C., Rowe-Magnus, D.A., Marliere, P., and Mazel, D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalph) conjugative machineries and their cognate *Escherichia coli* host strains. *Res Microbiol* 156, 245-255.

Duperthuy, M., Schmitt, P., Garzon, E., Caro, A., Rosa, R.D., Le Roux, F., Lautredou-Audouy, N., Got, P., Romestand, B., de Lorgeril, J., *et al.* (2011). Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proceedings of the National Academy of Sciences of the United States of America* 108, 2993-2998.

Hunt, D.E., David, L.A., Gevers, D., Preheim, S.P., Alm, E.J., and Polz, M.F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science* 320, 1081-1085.

Le Roux, F., Binesse, J., Saulnier, D., and Mazel, D. (2007). Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl Environ Microbiol* 73, 777-784.

Le Roux, F., Davis, B.M., and Waldor, M.K. (2011). Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res* 39, 1004-1013.

Lemire, A., Goudenege, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., and Le Roux, F. (2014). Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *Isme J* 9, 1523-1531.

Matsen, F.A., Kodner, R.B., and Armbrust, E.V. (2010). pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. *BMC Bioinformatics* 11, 538.

Pepin, J.F., Riou, A., and Renault, T. (2008). Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. *J Virol Methods* 149, 269-276.

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., and Le Roux, F. (2015). *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Front Microbiol* 6, 686.

Pfeffer, C., and Oliver, J.D. (2003). A comparison of thiosulphate-citrate-bile salts-sucrose (TCBS) agar and thiosulphate-chloride-iodide (TCI) agar for the isolation of *Vibrio* species from estuarine environments. *Lett Appl Microbiol* 36, 150-151.

Rico-Villa, B., Pouvreau, S., and Robert, R. (2009). Influence of food density and temperature on ingestion, growth and settlement of Pacific oyster larvae, *Crassostrea gigas*. *Aquaculture* 287, 395-401.

Rico-Villa, B., Woerther, P., Mingant, C., Lepiver, D., Pouvreau, S., Hamon, M., and Robert, R. (2008). A flow-through rearing system for ecophysiological studies of Pacific oyster *Crassostrea gigas* larvae. *Aquaculture* 282, 54-60.

Segarra, A., Pepin, J.F., Arzul, I., Morga, B., Faury, N., and Renault, T. (2010). Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus research* 153, 92-99.

Turner, J.W., Good, B., Cole, D., and Lipp, E.K. (2009). Plankton composition and environmental factors contribute to *Vibrio* seasonality. *Isme J* 3, 1082-1092.

Val, M.E., Skovgaard, O., Ducos-Galand, M., Bland, M.J., and Mazel, D. (2012). Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS genetics* 8, e1002472.

## Tables

**Table S1:** Summary of the number of isolates by sampling date and category.

Category	Spring		Summer	
	17 March	5 May	16 July	29 July
> 60 $\mu$ M	30	32	22	43
60-5 $\mu$ M	20	34	24	46
5-1 $\mu$ M	36	35	21	41
1-0.2 $\mu$ M	43	42	28	44
Digestive gland	61	86	90	70
Gills	32	68	79	84
Hemolymph	32	37	83	62
Total tissues	64	80	90	81

**Table S2:** Comparison of the taxonomic affiliation of isolates determined by *hsp60* sequences using pplacer (Fig. S1) and Multi Locus Sequence Typing (*hsp60*, *rpoD*, *gyrB* and *rctB*).

<b>Taxon</b>	<b>pplacer</b>	<b>MLST</b>
A	<i>Aliivibrio fischeri</i>	<i>Aliivibrio fischeri</i>
B	<i>Aliivibrio salmonicida</i>	nd
C	<i>Vibrio breoganii</i>	<i>Vibrio breoganii</i>
D	<i>Vibrio alginolyticus</i>	<i>Vibrio alginolyticus</i>
E	<i>Vibrio harveyi</i>	Harveyi super clade
F	<i>Vibrio campbellii</i>	Harveyi super clade
G	<i>Vibrio tapetis</i>	<i>Vibrio mediterranei</i>
H	<i>Vibrio ichthyenteri</i>	<i>Vibrio spp</i>
I	<i>Vibrio scophthalmi</i>	<i>Vibrio scophthalmi</i>
J	<i>Vibrio orientalis</i>	<i>Vibrio spp</i>
K	<i>Vibrio corallilyticus</i>	<i>Vibrio corallilyticus</i>
L	<i>Vibrio rotiferianus</i>	Harveyi super clade
M	<i>Vibrio caribbeanicus</i>	<i>Vibrio spp</i>
N	<i>Vibrio maritimus</i>	<i>Vibrio maritimus</i>
O	<i>Vibrio kanaloe</i>	Splendidus super clade
P	<i>Vibrio tasmaniensis</i>	<i>Vibrio tasmaniensis</i>
Q	<i>Vibrio chagasii</i>	<i>Vibrio chagasii</i>
R	<i>Vibrio cyclitrophicus</i>	<i>Vibrio cyclitrophicus</i>
S	<i>Vibrio fortis</i>	<i>Vibrio fortis</i>
T	<i>Vibrio crassostreae</i>	<i>Vibrio crassostreae</i>
U	<i>Vibrio splendidus</i>	<i>Vibrio splendidus</i>
V	<i>Photobacterium damsela</i>	<i>Photobacterium damsela</i>
W	<i>Photobacterium spp.</i>	<i>Photobacterium spp.</i>

**Table S3:** Strains used in this study.

<b>Strain</b>	<b>Description</b>	<b>Reference</b>
Π3813	<i>lacIQ</i> , <i>thi1</i> , <i>supE44</i> , <i>endA1</i> , <i>recA1</i> , <i>hsdR17</i> , <i>gyrA462</i> , <i>zei298::Tn10</i> , <i>ΔthyA::(erm-pir116)</i> [Tc <sup>R</sup> Erm <sup>R</sup> ]	(Le Roux et al., 2007)
β3914	(F <sup>-</sup> ) RP4-2-Tc::Mu <i>ΔdapA</i> ::( <i>erm-pir116</i> ), <i>gyrA462</i> , <i>zei298::Tn10</i> [Km <sup>R</sup> Em <sup>R</sup> Tc <sup>R</sup> ]	(Le Roux et al., 2007)
GV1460	J2-8, <i>Vibrio sp.</i> (accession number PRJEB5890)	(Lemire et al., 2014)
GV1508	J2-9, <i>V. crassostreae</i> (accession number PRJEB5876)	(Lemire et al., 2014)
GV1512	J5-20, <i>V. crassostreae</i> (accession number PRJEB5882)	(Lemire et al., 2014)
GV218	J2-5, <i>V. cyclitrophicus</i>	(Lemire et al., 2014)
GV973	J2-9 <i>ΔRI::(aadA7)</i> [Spec <sup>R</sup> ]	(Lemire et al., 2014)
GV1633	7T5-8, <i>V. cyclitrophicus</i>	This study
GV1634	7T2-5, <i>V. crassostreae</i>	This study
GV1635	7T8-11, <i>V. crassostreae</i>	This study
GV1636	7T7-1, <i>V. crassostreae</i>	This study
GV1637	8D7-1, <i>V. crassostreae</i>	This study
GV1638	8H2-1, <i>V. crassostreae</i>	This study
GV1639	8H8-3, <i>V. crassostreae</i>	This study
GV1476	J2-9 + pMRB-P <sub>LAC</sub> <i>gfp</i>	This study
GV1478	J2-9 + pMRB-P <sub>LAC</sub> <i>rfp</i>	This study
GV1488	J5-20 + pMRB-P <sub>LAC</sub> <i>gfp</i>	This study
GV1490	J5-20 + pMRB-P <sub>LAC</sub> <i>rfp</i>	This study
GV1470	J2-8 + pMRB-P <sub>LAC</sub> <i>gfp</i>	This study
GV1471	J2-8 + pMRB-P <sub>LAC</sub> <i>rfp</i>	This study
GV1398	7T5-8 + pMRB-P <sub>LAC</sub> <i>gfp</i>	This study
GV1397	7T5-8 + pMRB-P <sub>LAC</sub> <i>rfp</i>	This study
GV1474	J2-9 $\Delta$ pGV1512+ pMRB-P <sub>LAC</sub> <i>gfp</i>	This study
GV1475	J2-9 $\Delta$ pGV1512+ pMRB-P <sub>LAC</sub> <i>rfp</i>	This study
GV1472	J5-20 $\Delta$ pGV1512+ pMRB-P <sub>LAC</sub> <i>gfp</i>	This study

GV1473	J5-20ΔpGV1512+ pMRB-P <sub>LAC</sub> rfp	This study
GV1440	J5-20 ΔpGV1512	This study
GV1438	J2-9 ΔpGV1512	This study
GV1436	J2-9 ΔR1::( <i>aadA7</i> ), ΔpGV1512 [Spec <sup>R</sup> ]	This study
GV1424	J5-20 pGV1512-Tr::pSW23T [Cm <sup>R</sup> ]	This study
GV1458	GV1436 + pGV1512::pSW23T [Spec <sup>R</sup> Cm <sup>R</sup> ]	This study
GV1539	J5-20 pGV1512-ΔT6SS::( <i>aadA7</i> ) [Spec <sup>R</sup> ]	This study
GV1589	J5-20 pGV1512-Δ <i>cus/cop</i> -Tr::pSW23T [Cm <sup>R</sup> ]	This study
GV1593	J2-9 pGV1512-ΔT4SS-Tr::pSW23T [Cm <sup>R</sup> ]	This study
GV1506	J2-9 pGV1512-ΔPx1	This study
GV1538	J2-9 pGV1512-ΔPx2	This study
GV1542	J2-9 pGV1512-ΔPx3	This study
GV1537	J5-20 pGV1512-ΔPx2	This study
GV1598	J5-20 :: pSW23T :: (II900891-901985) <sub>LGP32</sub> , P <sub>LAC</sub> - <i>gfp</i> Cm <sup>R</sup> ΔpGV1512	This study
GV1596	J5-20 :: pSW23T :: (II900891-901985) <sub>LGP32</sub> , P <sub>LAC</sub> - <i>gfp</i> Cm <sup>R</sup> ΔpGV1512	This study

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**Table S4:** Plasmids used in this study

<b>Plasmid</b>	<b>Description</b>	<b>Reference</b>
pSW23T	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; Cm <sup>R</sup>	(Demarre et al., 2005)
pSW23T::Fvir	pSW23T :: (2700-3400) <sub>pGV1512</sub> Cm <sup>R</sup>	This study
pSW7848T	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; <i>araC-P<sub>BADccdB</sub></i> ; Cm <sup>R</sup>	(Val et al., 2012)
pSW7848T::Fvir	pSW7848T :: (2700-3400) <sub>pGV1512</sub> Cm <sup>R</sup>	This study
pSW3654T	pSW23T :: (II900891-901985) <sub>LGP32, P<sub>LAC-gfp</sub></sub> Cm <sup>R</sup>	(Duperthuy et al., 2011)
pSW $\delta$ cus/copT	pSW7848T:: <i><math>\Delta</math>cus/cop</i>	This study
pSW $\delta$ T4SST	pSW7848T:: <i><math>\Delta</math>T4SS</i>	This study
pSW $\delta$ T6SST	pSW7848T :: <i><math>\Delta</math>T6SS</i>	This study
pSW $\delta$ Px1T	pSW7848T :: <i><math>\Delta</math>Px1</i>	This study
pSW $\delta$ Px2	pSW7848T :: <i><math>\Delta</math>Px2</i>	This study
pSW $\delta$ Px3	pSW7848T :: <i><math>\Delta</math>Px3</i>	This study
pMRB-P <sub>LACgfp</sub>	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; $\square$ <i>oriV<sub>pB1067</sub></i> ; P <sub>LAC-gfp</sub> Cm <sup>R</sup>	(Le Roux et al., 2011)
pMRB-P <sub>LACrfp</sub>	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; $\square$ <i>oriV<sub>pB1067</sub></i> ; P <sub>LAC-rfp</sub> Cm <sup>R</sup>	(Le Roux et al., 2011)

**Table S5:** Primers used in this study

<b>Primer</b>	<b>Sequence 5'-3'</b>
Hsp60F	GAATTCGAIIIIIGCIGGIGAYGGIACIACIAC
Hsp60R	CGCGGGATCCYKIYKITCICCRAAICCIGGIGCYTT
pGV1512-1	GCCCGGATCCATGAATAAGAAAACATTACC
pGV1512-2	GCCCGGATCCGTTGCGTTGCTTGGACTGAG
SW-F	GAATTCCTGCAGCCCGGGGG
SW-R	GAATTCGATATCAAGCTTATCGATAC
$\Delta$ T6SS-1	GTATCGATAAGCTTGATATCGAATTCGTTACCAGCAGCAAGAGCAA
$\Delta$ T6SS-2	CGTCACAGGTATTTATTCGGCGGCCAATTAAGACGGTTGGCA
$\Delta$ T6SS-3	TGCCAACCGTCTTAATTGGCCGCCGAATAAATACCTGTGACG
$\Delta$ T6SS-4	ACGAGCTATGCTATTGGCACGATATCGTCGCAGACCAAAAACG
$\Delta$ T6SS-5	CGTTTTGGTCTGCGACGATATCGTGCCAATAGCATAGCTCGT
$\Delta$ T6SS-6	CCCCCGGGCTGCAGGAATTCGTGTCTGGTAGCATAGCAAGA
$\Delta$ Cus/Cop-1	GTATCGATAAGCTTGATATCGAATTCCTGTCCAGTGTGAATAGG
$\Delta$ Cus/Cop-2	CGTCGGTCAAAGTAGACGGCCGTTCACTCTAAGAAACCGC
$\Delta$ Cus/Cop-3	GCGGTTTCTTAGAGTGAACGGCCGTCTACTTTGACCGACG
$\Delta$ Cus/Cop-4	CCCCCGGGCTGCAGGAATTCGAAGGAAACCCAAACCGGCC
$\Delta$ T4SS-1	GTATCGATAAGCTTGATATCGAATTCGCTGCTGAGGAAAATGTGCG
$\Delta$ T4SS-2	GCGATACGCATGGATGAAGGCGACTTTGCTTCGTACATGG
$\Delta$ T4SS-3	CCATGTACGAAGCAAAGTCGCCTTCATCCATGCGTATCGC
$\Delta$ T4SS-4	CCCCCGGGCTGCAGGAATTCGGATCTTCTGTTGATGCTGC
$\Delta$ Px1-1	GTATCGATAAGCTTGATATCGAATTCGCTCATACTTATCGCCTCAC
$\Delta$ Px1-2	CCTTTGCGTGTTC AAGTCACGTCAA AATACCAAGGGTGGC
$\Delta$ Px1-3	GCCACCCTTGGTATTTTGACGTGACTTGAACACGCAAAGG
$\Delta$ Px1-4	CCCCCGGGCTGCAGGAATTCATTGGGTGCCCTTAATGCC
$\Delta$ Px2-1	GTATCGATAAGCTTGATATCGAATTCCTGTCCAGTGTGAATAGG
$\Delta$ Px2-2	CGTCGGTCAAAGTAGACGGCCGTTCACTCTAAGAAACCGC
$\Delta$ Px2-3	GCGGTTTCTTAGAGTGAACGGCCGTCTACTTTGACCGACG

$\Delta P_x2-4$  CCCCCGGGCTGCAGGAATTCGAAGGAAACCCAAACCGGCC  
 $\Delta P_x3-1$  GTATCGATAAGCTTGATATCGAATTCCTGAAACGCATGTCAGCTCG  
 $\Delta P_x3-2$  CCGTGTCTTTCATGGCTTGCGTGTGCGAAAGCACTTGACC  
 $\Delta P_x3-3$  GGTCAAGTGCTTTCGCACACGCAAGCCATGAAAGACACGG  
 $\Delta P_x3-4$  CCCCCGGGCTGCAGGAATTCCCCACCAATAAAGTGACAGG

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**Table S6:** Preferential distribution of *Vibrio* populations among seawater fractions and oysters estimated by Fisher's exact test

	Fractions				Oysters
	> 60μM	60-5 μM	5-1 μM	< 1μM	
<i>V. orientalis</i> (#1)	+	o	o	-	-
<i>V. fortis</i> (#2)	o	o	o	o	o
<i>V. chagasii</i> (#3)	o	o	o	+	o
<i>Vibrio</i> sp.(#4)	o	o	+	o	o
<b><i>V. harveyi</i> (#5)</b>	o	o	o	o	+
<b><i>Vibrio</i> sp. (#6)</b>	o	o	o	o	+
<b><i>Vibrio</i> sp. (#7)</b>	o	o	o	o	+
<b><i>Vibrio</i> sp. (#8)</b>	o	o	o	o	+
<i>Vibrio</i> sp. (#9)	o	+	o	o	o
<i>Vibrio</i> sp. (#10)	o	o	o	o	o
<b><i>V. crassostreae</i> (#11)</b>	-	-	-	-	+
<b><i>Vibrio</i> sp. (#12)</b>	o	o	o	o	+
<i>V. splendidus</i> (#13)	o	o	o	o	o
<i>V. splendidus</i> (#14)	o	o	o	o	o
<i>V. splendidus</i> (#15)	o	o	o	o	o
<i>V. splendidus</i> (#16)	o	o	o	o	o
<b><i>V. splendidus</i> (#17)</b>	o	o	o	o	+
<i>V. splendidus</i> (#18)	o	o	+	o	o
<b><i>V. splendidus</i> (#19)</b>	o	o	o	o	+
<i>V. splendidus</i> (#20)	o	o	o	+	o
<i>V. splendidus</i> (#21)	o	o	o	+	o
<i>V. splendidus</i> (#22)	o	o	o	o	o
<b><i>V. tasmaniensis</i> (#23)</b>	o	o	o	o	+
<b><i>V. tasmaniensis</i> (#24)</b>	o	o	o	o	+

**Table S7:** Preferential distribution of populations (n>10) among oysters gills, digestive glands and hemolymph samples.

	Gills	Digestive gland	Hemolymph
<i>V. fortis</i> (#2)	o	o	-
<i>V. chagasii</i> (#3)	-	+	o
<i>Vibrio</i> sp.(#4)	o	+	o
<i>V. harveyi</i> (#5)	o	o	o
<i>Vibrio</i> sp. (#6)	+	-	o
<i>Vibrio</i> sp. (#7)	o	o	o
<i>Vibrio</i> sp. (#8)	+	-	o
<i>Vibrio</i> sp. (#9)	o	o	o
<i>V. crassostreae</i> (#11)	+	-	+
<i>Vibrio</i> sp. (#12)	o	o	o
<i>V. splendidus</i> (#13)	o	o	o
<i>V. splendidus</i> (#14)	o	o	o
<i>V. splendidus</i> (#17)	o	o	o
<i>V. splendidus</i> (#18)	o	o	o
<i>V. splendidus</i> (#19)	o	+	-
<i>V. splendidus</i> (#23)	o	o	o
<i>V. splendidus</i> (#24)	o	o	o

**Table S8:** Gene annotation of the Px2 and Px3 regions in the virulence plasmid pGV1512 of strain J5-20

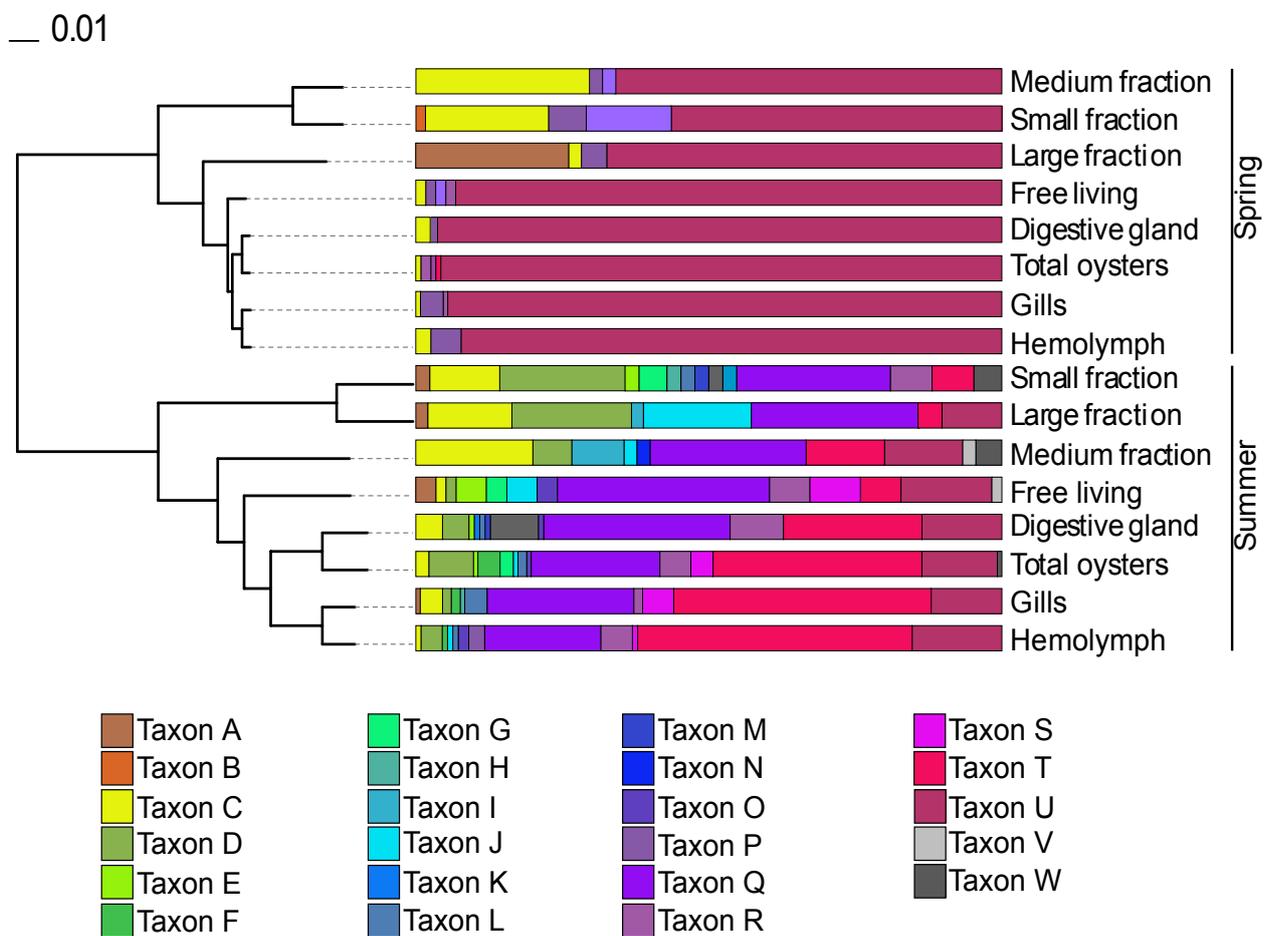
<b>Region</b>	<b>Gene label</b>	<b>Annotation</b>	<b>Size (bp)</b>
<b>Px2</b>	VCR20J5v1_1100011	Exported protein of unknown function	231
	VCR20J5v1_1100010	Gene remnant	126
	VCR20J5v1_1100009	Gene remnant	246
	VCR20J5v1_1100008	Transposase (fragment)	270
	VCR20J5v1_1100007	Gene remnant	123
	VCR20J5v1_1100006	Conserved membrane protein of unknown function	366
	VCR20J5v1_1100005	Transposase (fragment)	765
	VCR20J5v1_1100004	Gene remnant	135
	VCR20J5v1_1100003	Transposase (fragment)	450
	VCR20J5v1_1100002	Transposase (fragment)	171
	VCR20J5v1_1100001	Gene remnant	120
	VCR20J5v1_530001	Transposase (fragment)	816
	VCR20J5v1_770001	Transposase (fragment)	324
	VCR20J5v1_770002	Gene remnant	138
	VCR20J5v1_770003	Transposase (fragment)	141
	VCR20J5v1_770004	Gene remnant	297
<b>Px3</b>	VCR20J5v1_770026	Conserved protein of unknown function	1278
	VCR20J5v1_770027	Conserved protein of unknown function	483
	VCR20J5v1_770028	Transcriptional activator protein CopR (fragment)	123
	VCR20J5v1_770029	Two component sensor protein CopS (fragment)	348
	VCR20J5v1_770030	Two component sensor protein CopS (fragment)	162
	VCR20J5v1_770031	Two component sensor protein CopS (fragment)	216
	VCR20J5v1_770032	Two component sensor protein CusS (fragment)	162
	VCR20J5v1_770033	Putative Methyl-accepting chemotaxis protein	1629
	VCR20J5v1_770034	Transposase (fragment)	282
	VCR20J5v1_770035	Transcription activator ToxR (fragment)	720
	VCR20J5v1_770036	Membrane protein of unknown function	504

VCR20J5v1_770037	Transcription activator ToxR (fragment)	798
VCR20J5v1_770038	Membrane protein of unknown function	480
VCR20J5v1_770039	Putative HTH-type transcriptional regulator	981
VCR20J5v1_770040	Transposase (fragment)	123
VCR20J5v1_770041	Transposase (fragment)	222
VCR20J5v1_770042	Transposase (fragment)	468
VCR20J5v1_770043	Aldehyde dehydrogenase	1506
VCR20J5v1_770044	Protein of unknown function	360
VCR20J5v1_770045	Protein of unknown function	276
VCR20J5v1_770046	Putative Methyl-accepting chemotaxis protein	1614
VCR20J5v1_770047	Putative signalling protein (EAL domain)	1698
VCR20J5v1_770048	Transposase (fragment)	138
VCR20J5v1_1150001	Conserved protein of unknown function	834
VCR20J5v1_1270005	Conserved protein of unknown function	366

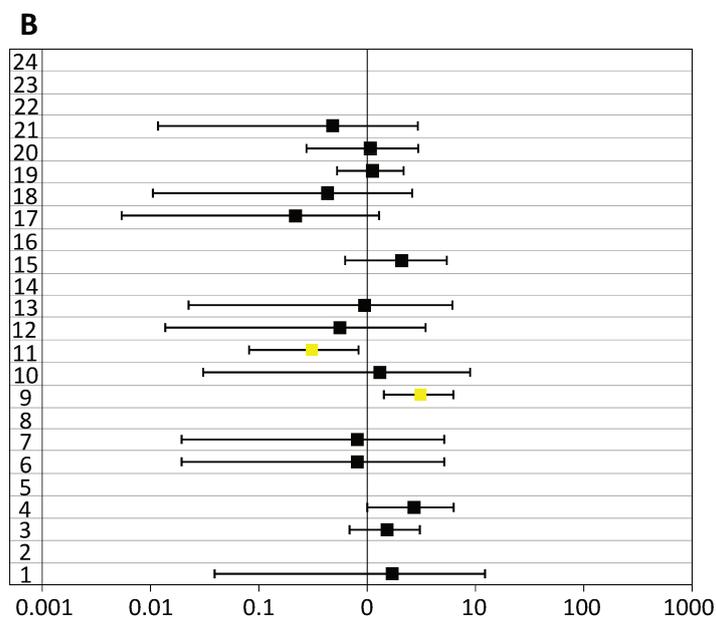
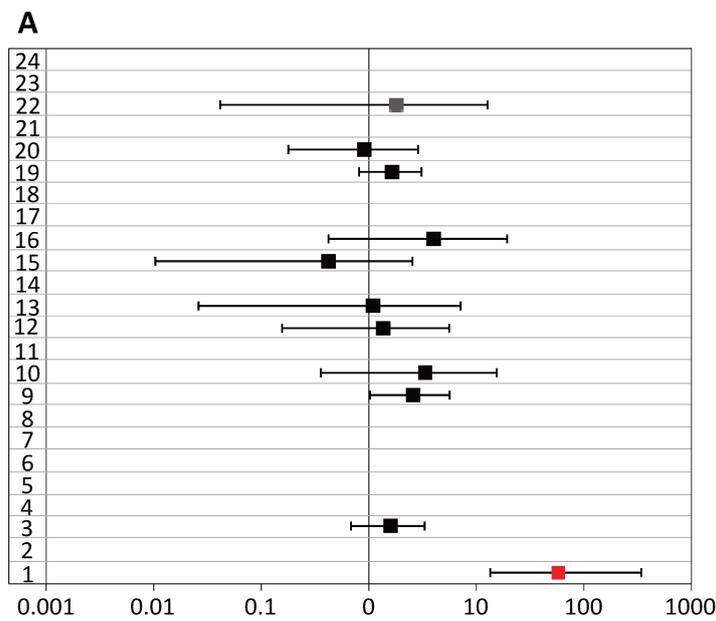
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Figures

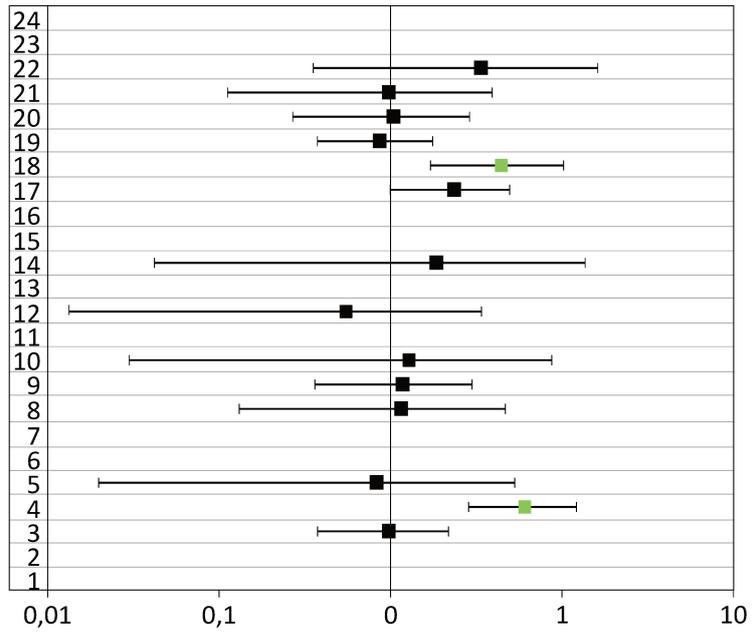
**Figure S1: Clustering of the 16 sample categories isolates were obtained from based on distribution of taxa.** Each *hsp60* sequences was assigned to a taxon by phylogenetic placement using pplacer (Matsen et al., 2010). The different sample categories (fractions, oyster tissues and seasons) are represented by a histogram showing distribution of taxonomic classification. The sample categories are clustered using distribution similarity of taxa as implemented in the Squash method from pplacer.



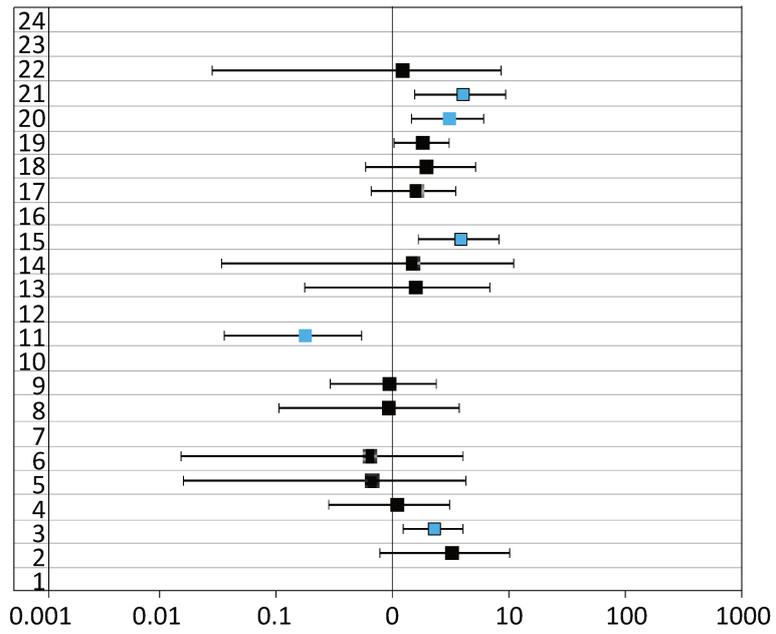
**Figure S2:** Fisher exact-test for the 24 populations in seawater fractions (**A** :  $>60\mu\text{m}$  ; **B** :  $60\text{-}5\mu\text{m}$  ; **C** :  $5\text{-}1\ \mu\text{m}$  ; **D** :  $>1\mu\text{m}$  ) and oysters (**E**). The x-axis represents the odds-ratio retrieved from the test. The error bars indicate the range of the 95% confidence interval. The y-axis represents the population number. A colored square indicates a significant test ( $P_{\text{val}} < 0.05$ ) for association with particles sizes  $>60\mu\text{m}$  (red),  $60\text{-}5\mu\text{m}$  (yellow),  $5\text{-}1\ \mu\text{m}$  (green),  $>1\mu\text{m}$  (blue) or oysters (purple).



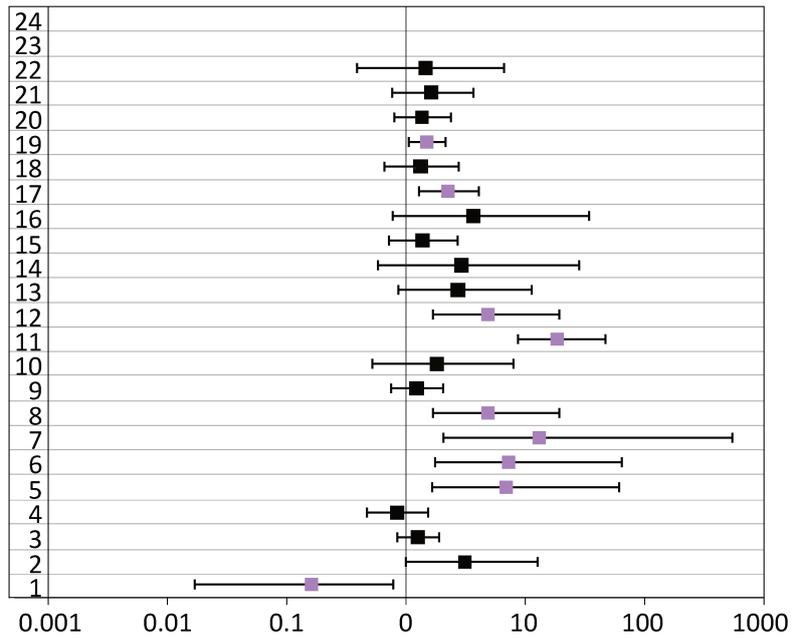
**C**



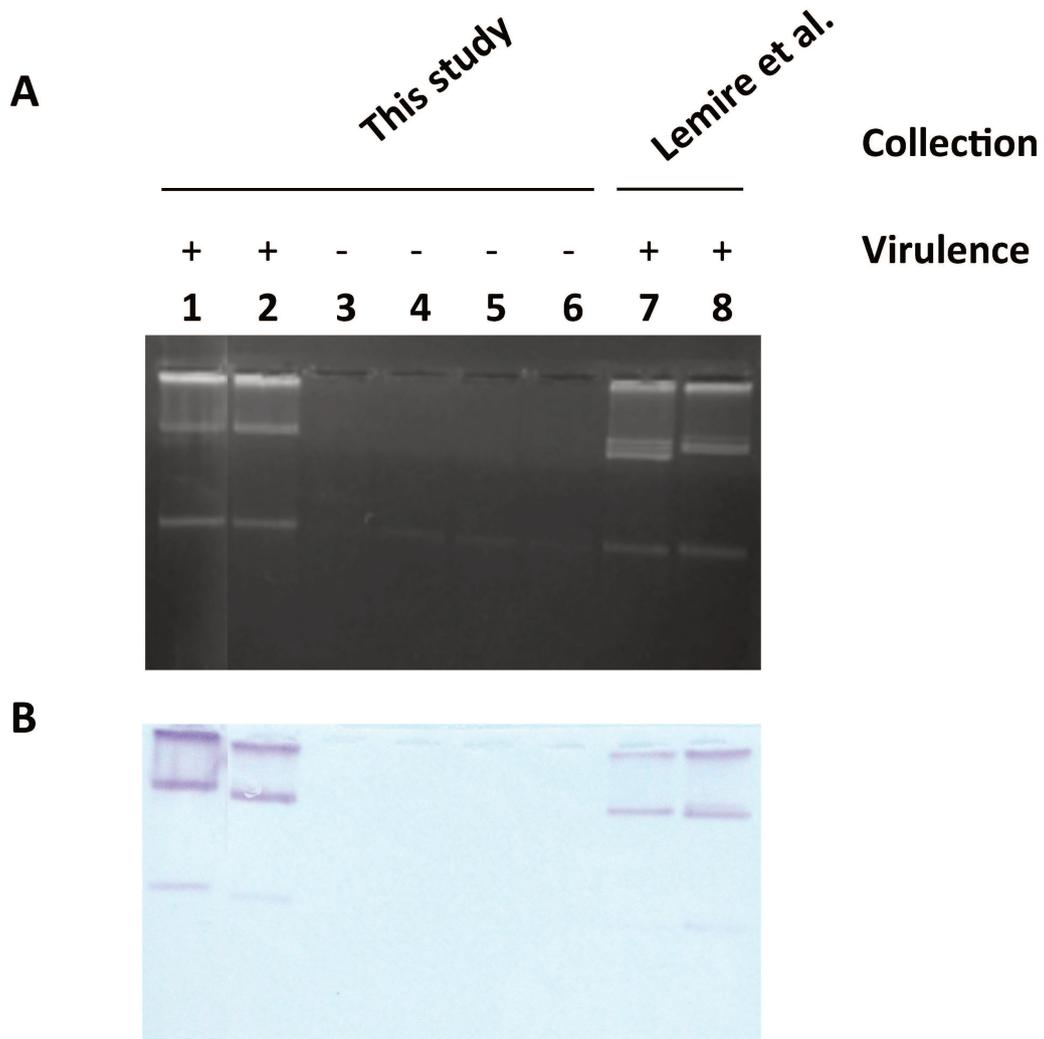
**D**



# E

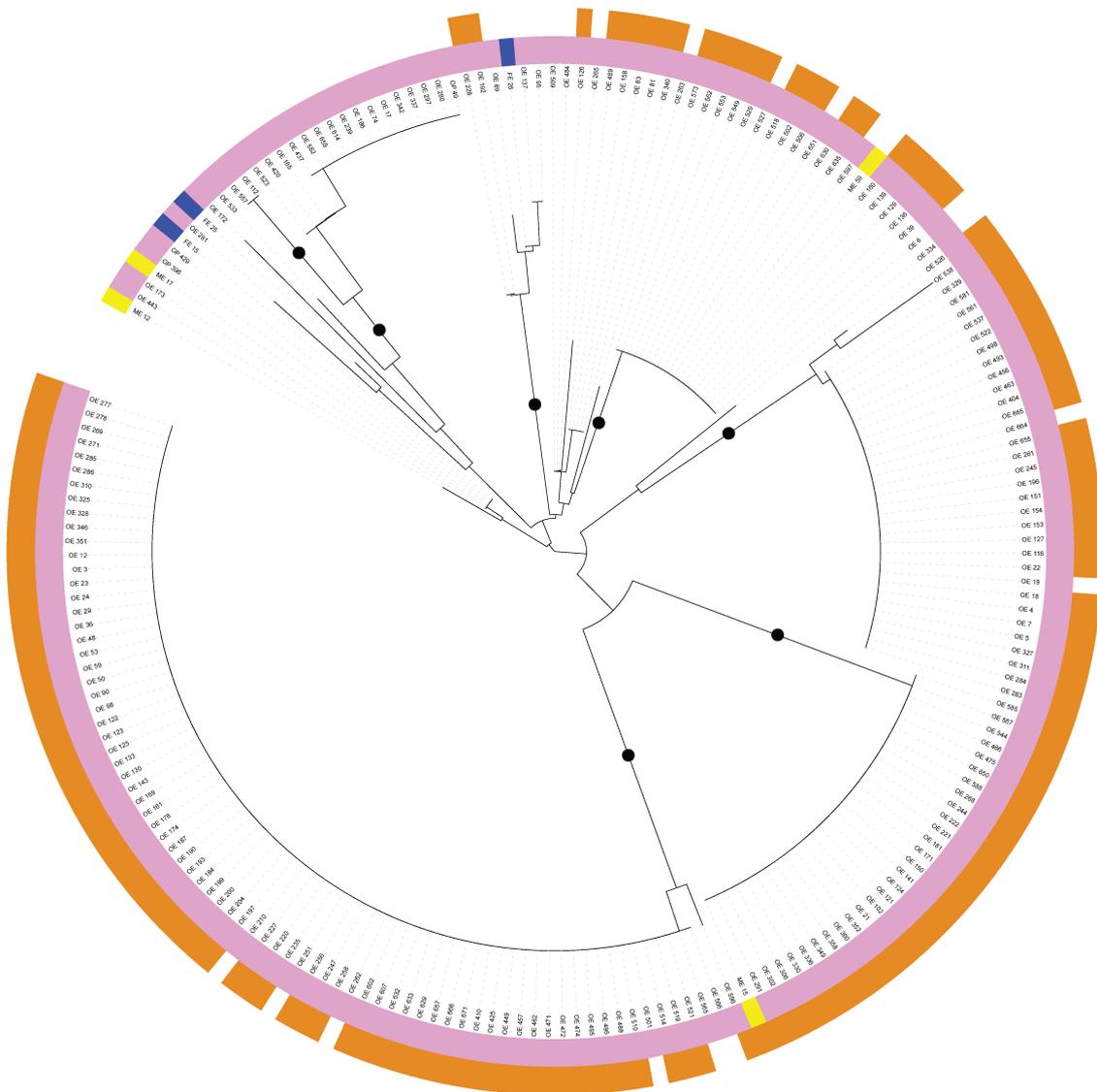


**Figure S3:** Demonstration of the presence of episomes in virulent strains belonging to the *V. crassostreae* population. **A-** Strains isolated in a previous (J2-9 and J5-20, lines 7 and 8) (Lemire et al., 2014) or in the present (7T2-5, 7T8-11, 7T7-1, 8D7-1, 8H2-1, 8H8-3 for lines 1 to 6) study were screened for episome presence by using gel electrophoresis of DNA. **B-** Southern blot analysis using a fragment of pGV1512 as a DIG labeled probe.

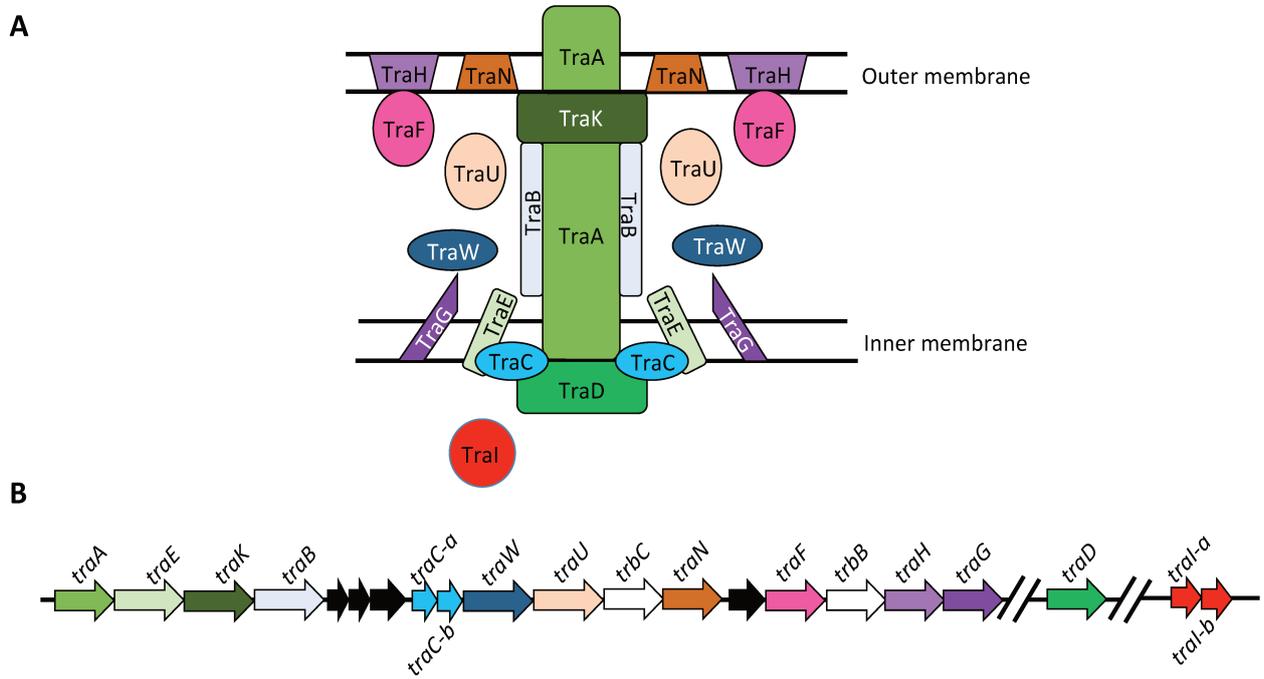


**Figure S4:** Phylogenetic tree of *hsp60* genes built by the Neighbor Joining method (Kimura 2 parameters, 472 sites) of *V. crassostreae* strains isolated in the present study. Bootstrap >90% are mentioned by a black dot. The inner ring indicates whether a strain was isolated from a specific seawater fraction or from oysters (colored as in Fig.1) while the outer ring represents the presence (orange) or absence (white) of pGV1512 plasmid.

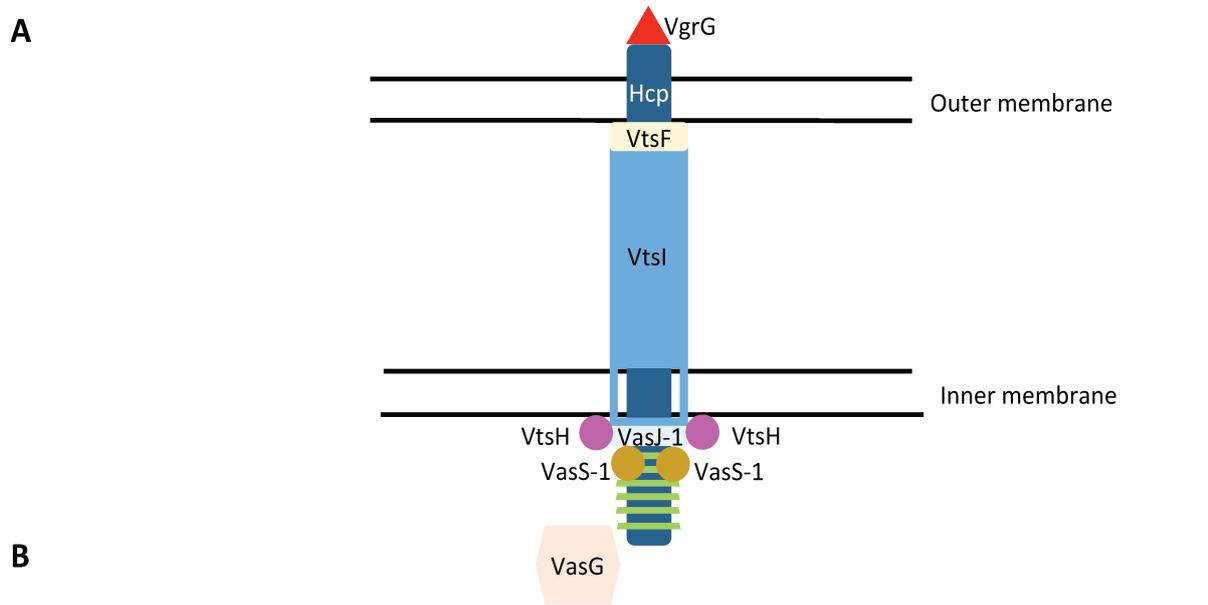
Tree scale: 0.01



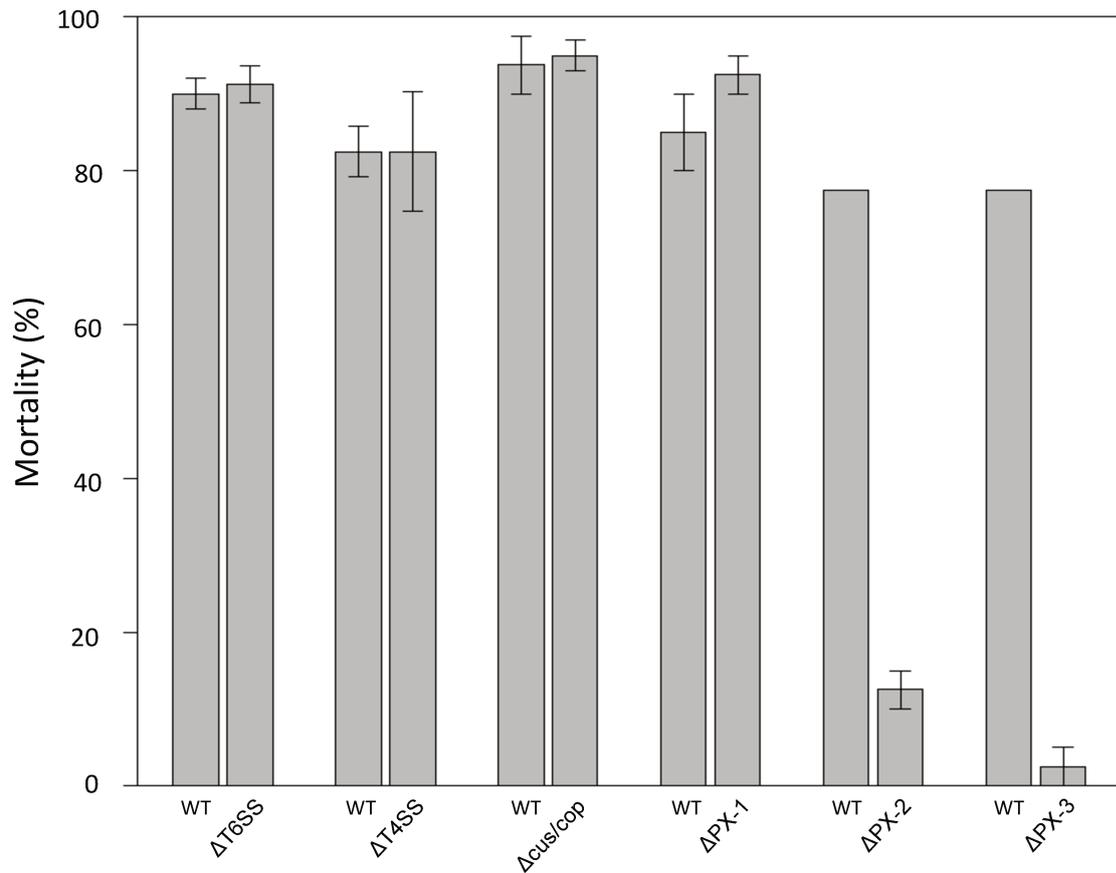
**Figure S5:** The putative conjugative machinery. This cluster includes homologs of the core proteins of a Type IV secretion system, the coupling protein TraD and a relaxase-helicase TraI.



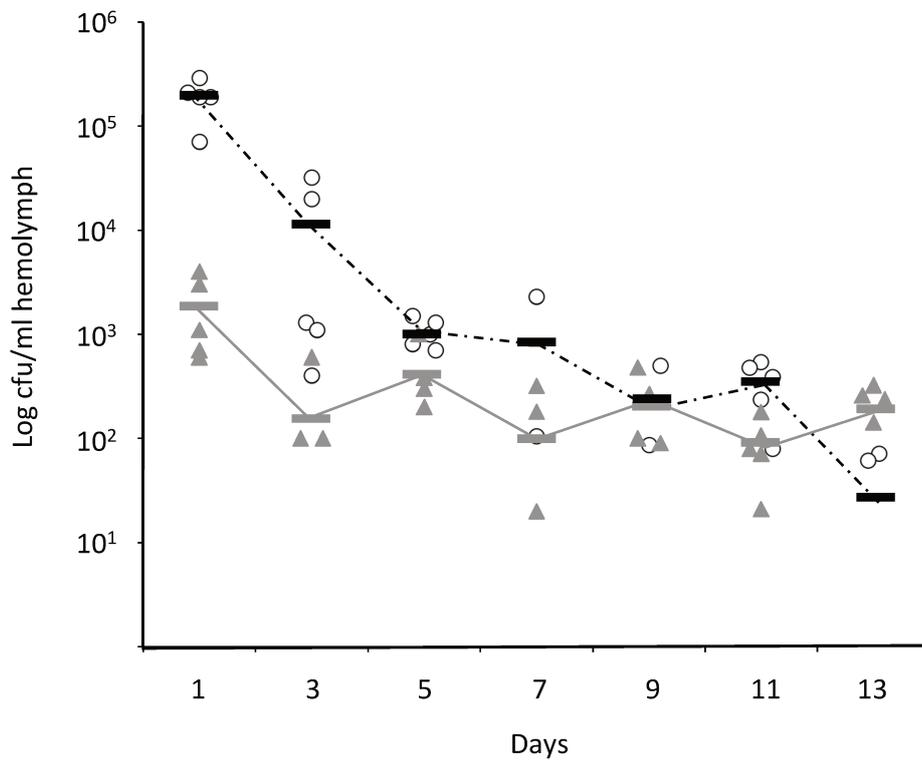
**Figure S6:** The pGV1512 type VI secretion system. **A-** Graphic representation. **B-** Genes cluster



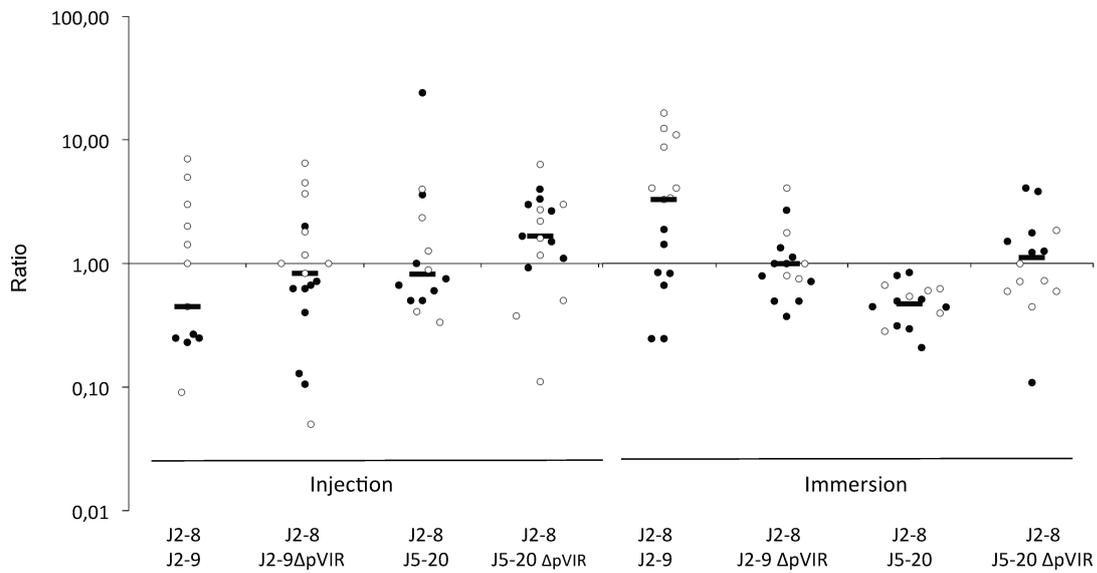
**Figure S7:** Experimental assessment of pGV1512 loci as virulence determinants. The deletion of T6SS, T4SS, and *cus/cop* gene clusters and region Px1 to 3 (Fig.3A) was performed by allelic exchange. The virulence of the wild type and mutants of specific loci (in x-axis) was compared by injection of strains ( $10^6$  cfu) in 20 oysters and counting the percentage of mortalities after 24 hours (y-axis).



**Figure S8:** Comparison of oyster colonization by *V. crassostreae* and *V. cyclitrophicus*. A CmR cassette was integrated in a non-essential chromosomal locus of *V. cyclitrophicus* strain J2-5, black triangle, and *V. crassostreae* strain J5-20  $\Delta$ pGV1512, white circle. Fluorescent strains were individually added to the oyster tank ( $10^7$ cfu/ml). Hemolymph was collected daily (x-axis) from 5 animals, serial diluted and spread on solid media supplemented with Cm. The y-axis indicates the cfu/ml of hemolymph retrieve on plates. The horizontal bar in each experiment indicates the mean cfus/ml.



**Figure S9:** Role of pGV1512 for oyster colonization. Colonization capacity assessment by determination of the ratio of J2-8 strain/ *V. crassostreae* (wild type and derivative without plasmid) retrieved on plates (y-axis) after homogenization of animals that were infected with 1:1 mix by injection or immersion (x-axis). Each dot corresponds to a replicate (up to 10). Black dots: J2-8 tagged with GFP / *V. crassostreae* tagged with RFP; white dots: J2-8 tagged with RFP / *V. crassostreae* tagged with GFP. The horizontal bar in each experiment indicates the mean ratio.



## Chapter III Oysters as a niche for horizontal gene transfer and pathogen emergence

In the previous chapter, we show that the plasmid pGV1512 plays a major role in *V. crassostreae* virulence. In addition, pGV1512 is a conjugative plasmid and was mostly detected in strains isolated from diseased oysters. We also show that there are always several populations colonizing diseased oysters. Those elements led us to explore the distribution of this virulence plasmid. Is pGV1512 restricted to environment experiencing oyster mortality events? Is pGV1512 specific to *V. crassostreae*?

First, the detection of pGV1512 in *V. crassostreae* strains isolated from Brest and Sylt (Germany), that constitute two areas experiencing contrasted oyster mortality events and contrasted oyster-farming densities revealed that the plasmid was restricted to strains isolated in Brest, suggesting that the plasmid could have been selected in high-density oyster farms. We further demonstrated that the plasmid was sufficient for the pathogenic transition of *V. crassostreae* by transferring pGV1512 in non-virulent strains. In a collection of *Vibrio* strains isolated in Brest from seawater and oyster, during and outside mortality events, pGV1512 was detected at high frequency in two populations, both associated from diseased oysters: *V. crassostreae* #11 and *V. splendidus* #24. Although the plasmid can be transferred to several populations and turns them more virulent, this narrow distribution was further experimentally showed to be due to a greater transfer frequency and/or selection of pGV1512 in populations #11 and #24 in oysters. The fact that pGV1512 transfer is enhanced in oysters could indicate that oysters act as a niche for HGT. This hypothesis will be further tested with transfer assays of other plasmids.

We then explored the virulence mechanisms of pGV1512 in *V. crassostreae*. Successive deletions of regions or single genes led to the identification of a transcriptional regulator, named PX3-2.5, whose deletion strongly reduces *V. crassostreae* virulence towards oysters. A RNA-seq analysis showed that genes encoding a type VI secretion system (T6SS) were significantly up-regulated when PX3-2.5 was overexpressed and we further demonstrated that the T6SS was activated by PX3-2.5. Targets of this T6SS, that can be either prokaryotic or eukaryotic cells, remain to be identified in order to deciphering pGV1512 role in oysters.

*NB: This is an updated version of the study sent in July but please note that it is still in progress. Experiments that still need to be performed are mentioned in blue in the text. The data are discussed in the discussion section of this thesis manuscript.*

## Oysters as a niche for horizontal gene transfer and pathogen emergence

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### ABSTRACT

Global change, including anthropogenic activities such as widespread use of aquaculture, is predicted to lead to a worldwide increase in bacterial-associated disease with ecosystem-wide impacts on humans and marine animals. Elevated host density due to farming is a likely factor involved in these diseases, especially as infectious agents often emerge after the acquisition of virulence genes by contact-dependent and contact-independent horizontal transfer events. The question therefore arises whether farmed animals can act as a niche for horizontal gene transfer and pathogen selection. Here we show that oyster farming has selected for the high prevalence of a virulence plasmid in specific *Vibrio* populations. Plasmid transfer and selection of vibrios carrying this plasmid is dramatically enhanced in oysters, although additional genomic background may be involved in the narrow host range of the plasmid observed in nature. This selection, as well as virulence, depends on a molecular killing device called the type VI secretion system together with a gene encoding its transcriptional activator. We propose that

oysters act as a specific niche that actively promotes the emergence of pathogens and we further speculate that antibiotic treatment of these animals may increase spread of resistance genes among the microbial world.

## INTRODUCTION

Global strategies are urgently needed to ensure sustainable and equitable food security in a context of demographic explosion (1). Aquatic products have a critical role in the food system, providing nearly 3 billion people with at least 15% of their animal protein intake and being the main source of omega 3 fatty acids (2). Overexploitation of fisheries had led to the expansion of aquaculture settings. However, exogenous shocks such as climate render marine farms vulnerable to diseases. On the other hand, marine farm can cause deep environmental changes. Indeed, aquaculture is often based on non-endemic and genetically modified or selected animals, which makes hatchery dependent on the use of antibiotics. In coastal marine environments, elevated animal density results in nutrient pollution, animal escapes or invasion and disease spread. Hence, although aquaculture is a promising strategy to face the food crises, sustainable and safe production must be mandatory for its development.

Oyster farming illustrates the risk and vulnerability of aquaculture. It is a major source of contamination of human by pathogens such as *Vibrio parahaemolyticus* the most common bacterial cause of seafood-borne gastroenteritis in the USA, with more than 34,000 annual cases (3). Members of the *Vibrio* genus have also been associated to several mortality outbreaks of *Crassostrea gigas*, the main oyster species produced worldwide (4-6). We previously compared the *Vibrio* population structure in seawater and oysters, sampled in a farming area (Brest, France) (4). By population, we mean a phylogenetic unit representing cohesive ecology, gene flow and social attributes (7-10). We were able to identify populations that were preferentially associated with oyster tissues, suggesting adaptation to survival in these animals. One population, taxonomically assigned to the species *V. crassostreae* (11), was particularly abundant in all animals affected by the juvenile disease and its pathogenicity was related to the presence of a large conjugative plasmid (pGV1512) (4). In addition, *V. crassostreae* was always found to co-occur with other *Vibrio* populations in oysters, questioning about the distribution of this plasmid in other populations as well as its effect on virulence in non-*V. crassostreae* strains. Finally, it remains to be determined whether oyster farming itself has selected for the high prevalence of the plasmid in the *V. crassostreae* population from Brest.

Plasmids are important drivers of rapid adaptation of bacteria to changing environmental conditions (12). Plasmid frequency within a population can thus be used as an indicator of the selective pressure in the niche. Indeed, the pGV1512 plasmid was detected in 1/10 (10%) and

152/196 (77%) of *V. crassostreae* strains isolated from seawater and oysters respectively, suggesting that it confers a fitness advantage in oysters (4). To test this hypothesis, pairwise competition assays using a *V. crassostreae* strain carrying pGV1512 or its plasmid-cured derivative were performed in oyster (4). We did not observe any clear effect of the plasmid loss on hemolymph colonization. However, we noted that neither *V. crassostreae* proliferation nor sustainable maintenance was observed in infected oysters. As fitness is defined as a difference in the number of offspring in the niche, either by increased growth or decreased mortality, the role of the pGV1512 in the fitness of *V. crassostreae* in oyster remains to be determined.

Here, we explored the natural host range of the pGV1512 plasmid in *Vibrio* populations isolated from Brest (France), an area experiencing intense oyster farming and recurrent mortality events and *V. crassostreae* isolated in Sylt (Germany) where a massive oyster invasion formed natural beds that did not suffer from *Vibrio* related disease outbreaks yet (13). The plasmid was detected at high frequency in two out of 24 populations (i.e. *V. crassostreae* and *V. splendidus*) isolated in Brest, while it was absent from strains isolated in Sylt. Conjugative transfer of the plasmid into non-virulent environmental *V. crassostreae* strains isolated in Sylt was however sufficient to increase their pathogenicity. The narrow range of populations carrying this plasmid at high frequency within geographic locations may be explained by enhanced fitness and/or conjugation frequency in oyster. A type 6 secretion system (T6SS) gene cluster, carried by the plasmid, appears to be necessary for full virulence. Its regulation by a plasmid encoded transcriptional factor and bacterial killing activity has been then further explored.

## RESULTS

### **The plasmid is enriched in two populations associated with diseased oysters in a farmed area**

We first explored the distribution of the pGV1512 plasmid in diverse populations of *Vibrio* isolated in Brest. These vibrios have been isolated from seawater fractions or oyster, in spring (no disease) or summer (juvenile disease), and partitioned into 24 populations using mathematical modeling taking into account phylogenetic and ecological structure (4). These populations were further assigned to taxonomic species by Multi Locus Sequencing Tag (MLST), e.g. *V. crassostreae* (#11), *Vibrio sp. nov* (#12) and *V. splendidus* (#13 to 24). Importantly, 13 populations (#1 to 9, 19, 21, 23 and 24) were found to co-occur with *V. crassostreae* in diseased oysters and could thus act as recipient for pGV1512 conjugative transfer.

The detection of the pGV1512 *repB* gene in strains in the original set representing each population (n=120, see Table S1 for details) revealed that pGV1512 is present in several strains of *V. crassostreae* (#11) and *V. splendidus* (#24) (Figure 1A, plain blue circles). Genome sequencing confirmed the presence of the pGV1512 plasmid in these strains with 138-142 (depending on the strain) core genes showing identities in 99% of sites (Table S2). When screening additional strains isolated in Brest, the pGV1512 plasmid was found in 77% (152/196) of *V. crassostreae* (#11) and 100% (10/10) of *V. splendidus* (#24). In addition, one *V. splendidus* strain (3T7-10; #20), isolated from a healthy oyster in spring, was found positive for *repB* (Figure 1A). To obtain a more precise estimate of plasmid occurrence in population #20, we screened 25 additional strains and did not find other *repB* positive strains.

We previously hypothesized that the introgression of the virulence plasmid pGV1512 into *V. crassostreae* is triggered by elevated host density in farming areas (4). Wild oyster beds can also reach very high densities. One example for such high densities can be found after the recent invasion of oysters into the Northern Wadden sea, NE North Sea, close to the island of Sylt (13). Here, no *Vibrio* associated mass mortalities have been observed, providing a natural contrast to the heavily farmed area in Brest. Therefore, we sought to isolate *V. crassostreae* from Sylt in order to explore the presence and frequency of the pGV1512 plasmid in disease-free areas. To this aim, roughly 800 strains were isolated from seawater fractions and oysters collected in Sylt, genotyped by *hsp60* partial gene sequencing and assigned to *Vibrio* population

and species as described previously (Figure S1). MLST further confirmed the taxonomic assignment of 40 *V. crassostreae* strains isolated from Sylt in addition to 49 isolates from Brest (Table S3). The phylogenetic structure partitioned these strains into two clades representing both locations (Figure 2A). The first clade contained the majority of strains (32 out of 40) from Sylt, while the second clade contains the majority of strains from Brest (34 out of 49). The pGV1512 *repB* gene was mainly found in strains belonging to clade 2 and only isolated in Brest (Figure 2A). Only one strain of the clade 1 (8T5\_11), originating from Brest, was found to be positive for *repB*. The presence of the plasmid was confirmed by genome sequencing of the 8T5\_11 strain. The pGV1512 *repB* gene was never detected in isolates from Sylt.

### **The plasmid is sufficient for the pathogenic transition of environmental vibrios**

When 40 and 49 *V. crassostreae* strains isolated respectively in Sylt and Brest were injected individually into oysters, we observed that virulence is strongly correlated with the presence of the plasmid (Student t test,  $p < 0,005$ ), with the exception of the only positive clade 1 strain 8T5-11 that was not able to kill oysters (Figure 2A). This strain lacks the R5-7 gene (not shown), that has been characterized as necessary but not sufficient for virulence in *V. crassostreae* (6,14). We previously showed that pGV1512 is necessary for virulence using plasmid-cured derivative of isolates from Brest (4). Here we tested whether this plasmid is sufficient to confer virulence to non-virulent *V. crassostreae* strains isolated in Sylt. To this end we transferred by *in vitro* mating the pGV1512 plasmid from a donor (*V. crassostreae* from clade 2, strain J2-9) to several recipient strains from clade 1 and 2. To be able to counter-select the conjugant from later one, we first transferred a spectinomycin resistance marked plasmid, pMRB\_Sp<sup>R</sup> (15) in the recipient (Figure 2B). We next transferred a chloramphenicol resistance (Cm<sup>R</sup>) marked version of pGV1512 (pGV\_Cm<sup>R</sup>), from J2-9 to the Sp<sup>R</sup> marked strains. We showed that, in all tested strains, plasmid gain resulted in an increase in oyster mortalities after experimental infection and at a similar level than J2-9 (Figure 2C).

We next asked whether the pGV1512 plasmid is sufficient to increase the virulence of strains belonging to other populations isolated in Brest. The pMRB\_Sp<sup>R</sup> plasmid was transferred successfully to the majority (86/120) of the strains (Figure 1A, plain green circles). When using these Sp<sup>R</sup> marked strain as recipient, we next were able to transfer the pGV1512\_Cm<sup>R</sup> from J2-9 into at least one strain per population except *V. orientalis* (#1), *V. harveyi* (#5), and *Vibrio sp. nov* (#6, 9 and 10) (Figure 1A, plain red circles), indicating that more *Vibrio* population are competent to acquire this plasmid than the natural distribution would suggest. Using oyster

experimental infection, we observed that harboring pGV1512 increased the virulence of strains from diverse populations except *V. fortis* (#2), *V. rotiferianus* (#5), *Vibrio sp. nov* (#12) and *V. splendidus* #14,#15 and #17 (Figure 1B), suggesting that virulence also depends on *Vibrio* genomic background. Similar to *V. crassostreae* strain, the plasmid was also a major virulence factor in *V. splendidus* #24 strains from Brest since plasmid curing resulted in virulence attenuation while virulence could be restored after pGV1512 mobilization in the cured strains (Figure 1B).

### **Virulence depends on a transcriptional activator that regulates a T6SS**

A preliminary exploration of the virulence determinants carried by the pGV1512 plasmid led to the identification of a region that is localized between a conjugative apparatus (T4SS and relaxase) and a T6SS (PX3) and was shown to be necessary for the virulence (4). Deletion of sub-regions of Px3 (Figure 3A), followed by gene knockouts, led to the identification of a single gene (PX3-2.5, label VCR9J2v1\_750086 in J2-9) that was necessary for virulence (Figure 3B). When constitutively expressed in *trans*, this gene was sufficient to restore the virulence of the pGV\_ΔPX3 mutant. The PX3-2.5 gene encodes for a putative transcriptional regulator. This transcriptional regulator contains two domains, a N-terminal domain with putative amidase function and C-terminal Helix-turn-helix DNA binding domain.

We hypothesized that this transcriptional regulator might induce virulence genes. To elucidate the target(s) of the PX3-2.5 regulon, we used RNAseq analysis to compare the transcriptomes of *V. crassostreae* strain J2-9 expressing either the transcriptional factor PX3-2.5 or the green fluorescent protein (GFP) as a control. Expression of PX3-2.5 resulted in changed mRNA levels of 62 predicted protein-coding genes (Log2Fold change >1, Table S4) of which 32 and 30 genes were down and up regulated, respectively in PX3-2.5-dependent manner. Only four genes localized in the J2-9 chromosome were up regulated. These genes encode for putative glycosyltransferase involved in cell wall biogenesis (VCR9J2v1\_960127), a DedA family membrane protein that may be involved in homeostasis (VCR9J2v1\_960126), a small heat shock protein *ibpA* previously associated in *E. coli* with protection against superoxide stress (VCR9J2v1\_130062) and conserved hypothetical proteins. Within the plasmid, all 24 genes of the T6SS-encoding locus (16, 17) were significantly up regulated in the strain expressing PX3-2.5 (Figure 4A). This result was further confirmed by qPCR (Figure S2). The T6SS cluster is organized in at least two operons with *vgrG*, a gene encoding a protein of unknown function and *vasV* (PAAR) being expressed in the opposite direction compared to the residual 21 T6SS

genes. Between these two operons, we predicted a bidirectional promoter (left and right 10/-35 boxes) as well as a putative PX3-2.5 binding site, which constitutes a palindromic sequence of 6 nucleotides spaced by 5 nucleotides (Figure 4B). This motif was not identified in other loci within the J2-9 genome, including upstream regions of the other differentially expressed genes identified by RNAseq. We cloned this promoter between Gfp and DsRed encoding genes in a replicative plasmid and transformed a *V. cholerae* strain (A1552) in which we previously integrated in the chromosome the PX3-2.5 gene under the control of an arabinose inducible promoter (P<sub>BAD</sub>) (see Material and method for details). The induction of PX3-2.5 expression by arabinose resulted in the production of both GFP and DsRed demonstrating that this promoter is activated by PX3-2.5 (Figure 4C).

The T6SS cluster was not only found on the pGV1512-like plasmids carried by strains isolated in Brest but also on the chromosome of 7 out of 102 *V. splendidus* strains that have been isolated in a location without intense oyster farming (Massachusetts, Plum Island and Nahant) (Figure S3). The gene cluster is in synteny in all analyzed genomes and the encoded proteins show high sequence conservation (mean sequence identity 97±3%). The promoter sequence that is localized between the *vgrG* and *vasJ* genes is also conserved in those strains including the palindromic sequence (Figure S4). The gene encoding the transcriptional regulator PX3-2.5 and nearby genes are also present in the vicinity of the T6SS in *V. splendidus* (sequence identity of 78%). Interestingly, we observed multiple insertion sequences of the IS5 family around the chromosomal T6SS and PX3-2.5, suggesting an acquisition by successive HGTs. Similarly, the T6SS localized on the pGV1512 is surrounded by many Tn/IS from different families, including the IS5 family, and most transposases are pseudogenized. This type of organization is similar to composite transposons that are Tn/IS surrounding genes and allowing their mobilization to another position (18).

From the experiments described above, we hypothesized that the regulator PX3-2.5 is necessary for virulence through its induction of the T6SS. This result was surprising as we previously generated a knock out mutant within this locus and observed no effect on virulence (4). Re-investigating this mutant (strain GV878), we identified a duplication of this region, probably resulting from plasmid shuffling, which resulted in duplicated alleles, one deleted and one complete. A new complete deletion of the T6SS gene cluster was therefore constructed (Figure 3A) and the deletion (strain GV2625, hereafter named  $\Delta$ T6SS) was confirmed by PCR using external and internal primers that anneal to the T6SS locus. Comparing the virulence of the WT

strain J2-9 and its  $\Delta$ T6SS derivative revealed that deletion of the T6SS resulted in a slight decrease in oyster mortality after bacterial injection (Figure 3B). Similar decrease in virulence was observed when we deleted the *vasV* gene, encoding for the PAAR-repeat protein. However, we noted that the effect of  $\Delta$ T6SS and  $\Delta$ PAAR never reached the effect of PX3-2.5 deletion with mortalities percentage induced of respectively 70, 60 and 20%. The deletion of a second region (Px2) was also previously demonstrated to result in attenuated virulence. However, this region contains the T6SS *vasV* gene. We therefore constructed a deletion of Px2, excluding the *vasV* gene, and observed no effect on virulence.

The T6SS was reported to contribute directly to virulence of *V. cholerae* by delivering effectors into host eukaryotic cells (19, 20). The T6SS mediated cytotoxicity in *Dictyostelium discoideum* and macrophage has been assigned to a C-terminal extension of the VgrG1 effector protein, which contains an actin cross-linking domain (19). We did not observe any C-terminal extension within the VgrG protein encoded by the T6SS cluster on the plasmid pGV1512, which suggests that a similar cytotoxic effect is unlikely to occur upon oyster injection. Indeed, it is now clear that many, probably the majority, of T6SSs are actually used to target competing bacteria and hence play a critical role in the fitness of bacteria within a specific niche (16, 17). To test this, we asked whether *V. crassostreae* J2-9 expressing PX3-2.5 was able to kill bacteria in an *in vitro* killing assays (21). When such a PX3-2.5 expressing J2-9 strain was used as predators and *E. coli*, *V. cholerae* and 40 diverse *Vibrio* strains isolated in Brest were used as prey, we still did not observe any killing under the tested conditions, indicating that it is not directly killing those bacteria *in vitro*.

***NB: The T6SS might target other bacteria, particularly the ones constituting the oyster microbiote. We are currently performing killing assays with J2-9 as predator and non-Vibrio preys isolated from healthy oyster-microbiote.***

***NB: Mutagenesis of other genes that are up-regulated by Px3-2.5 is currently in progress. The effect of their deletion in virulence will then be tested by injection to oysters.***

### **Oyster acts as a niche for pGV1512 transfer and selection**

The pGV1512 plasmid is present at high frequency in *V. crassostreae* (#11) and *V. splendidus* (#24) and both populations have been demonstrated to be positively associated with oysters (4).

As we were able to transfer this plasmid in diverse strains and populations, the narrow host range unlikely results from transfer, replication or maintenance specificity. Since plasmid host range and/or conjugative transfer efficiency is strongly dependent on the environment (12, 22, 23), we compared the pGV1512 transfer frequency *in vivo* and *in vitro*. First, we performed *in vitro* mating between *V. crassostreae* J2-9 carrying pGV1512\_Cm<sup>R</sup> and representative strains of *V. crassostreae* (#11) and *V. splendidus* (#24) previously cured of pGV1512 or the closely phylogenetically related populations *Vibrio sp. nov* (#12) and *V. splendidus* (#23). Plasmid transfer frequency (conjugant/recipient) ranged from 10<sup>-6</sup> to 10<sup>-8</sup> depending on the strain with a significant lower frequency for *Vibrio sp. nov* (#12) (Figure 5 and S5). Second, we co-injected oysters with sub-lethal (10<sup>7</sup> cfu) and lethal (10<sup>8</sup> cfu) doses of *V. crassostreae* strain J2-9/pGV1512\_Cm<sup>R</sup> together with each of the recipient. Plasmid transfer was only detected when lethal doses of J2-9/pGV1512\_Cm<sup>R</sup> were injected, similarly in dead or alive animals and was highly efficient despite the absence of antibiotic selection (Figure 5). Indeed, *in vivo*, plasmid transfer frequency was increased by up to 10<sup>4</sup> compared to *in vitro* mating. Importantly, the best efficiencies *in vivo* (10<sup>-2</sup>, 10<sup>-3</sup>) were obtained for cured strains belonging to *V. crassostreae* (#11) and *V. splendidus* (#24), closely mimicking plasmid occurrence in natural isolates (Figure 1). This result suggests a high degree of recipient specificity, that together with the increased transfer efficiency in oysters can explain its high abundance within its narrow host range. The dramatic increase of conjugants *in vivo* may result from an enhancement of the transfer and/or selection of the plasmid in oyster.

***NB: To test whether the plasmid is selected and/or more frequently transferred in populations #11 and #24 compared to populations #12 and #23, several experiments are in progress/planned:***

- *Maintenance of pGV1512 in the different strains from populations #11, #23 and #24 will be assessed. A higher maintenance in populations #11 and #24 might indicate a selective advantage conferred by pGV1512.*
- *In vivo and in vitro conjugation assays of pGV1512 wild-type or mutant forms ( $\Delta T6SS$ ,  $\Delta T4SS$ ,  $\Delta Cus/Cop$ ,  $\Delta Px2$ ,  $\Delta Px3$ ) might highlight differential transfer frequency that could indicate a selective advantage mediated by some deleted plasmid region.*
- *Enhancement of the transfer in vivo will be assessed by comparing the tra genes expression in qPCR between in vitro and in vivo mating conditions.*
- *Transfer frequency of other plasmids will also be tested.*

In this study, I performed the *in vitro* and *in vivo* conjugation assays, the mutagenesis of the different regions/genes on pGV1512, the infection assays, the statistics and the samples preparation for the RNAseq.

## MATERIAL AND METHODS

### Molecular microbiology

The strains used in the present study are described in tables S1, S3 and S5. Plasmids and primers used in this study are described in tables S6 and S7. *Vibrio* isolates were grown in Zobell or Zobell agar, Luria-Bertani (LB) or LB-agar (LBA) + NaCl 0.5M, at 20°C. *Escherichia coli* strains were grown in LB or on LBA at 37°C. Chloramphenicol (Cm, 5 or 25µg/ml for *Vibrio* and *E. coli* respectively), spectinomycin (Spec, 100µg/ml), thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction of the P<sub>BAD</sub> promoter was achieved by the addition of 0.2% L-arabinose to the growth media, and conversely, was repressed by the addition of 1% D-glucose.

Clonings were performed using the Gibson method (New England Biolabs, Genopole, Evry, France). For gene knock out by allelic exchange, 500bp of upstream and downstream regions of targeted genes or regions were cloned into a suicide vector. The R6K  $\gamma$ -ori-based suicide vector encodes the *ccdB* toxin gene under the control of an arabinose-inducible and glucose-repressible promoter, P<sub>BAD</sub>. Matings between *E. coli* and *Vibrio* were performed at 30 °C as described above. Selection of the plasmid-borne drug marker (chloramphenicol resistance) resulted in integration of the entire plasmid in the chromosome by a single crossover. Elimination of the plasmid backbone resulting from a second recombination step was selected by arabinose induction of the *ccdB* toxin gene. Mutants were screened by PCR and are described in Table SY. For complementation experiments, the Gibson assembly method was used to clone the PX3-2.5 gene under a constitutive promoter (P<sub>LAC</sub>) in pMRB. This plasmid was then transferred to *Vibrio* by *in vitro* mating.

For *in vitro* mating and *in vivo* conjugation assays between vibrios, a high copy number plasmid, which is stably maintained *in vitro* and *in vivo* without selection (13, 22) was used to introduce green fluorescent protein (GFP) constitutively expressed from the Plac promoter, into the *Vibrio* recipient strains.

For the pGV\_X conjugation experiment, we used donors containing a Cm<sup>R</sup> marked version of the different plasmids (e.g pGV1512\_Cm<sup>R</sup>) as previously described (4) and pGV1512 curing was performed as described previously (4).

Matings between vibrios were performed by mixing the donor (pGV1512\_Cm<sup>R</sup>) and recipient (e.g. J2-8\_Spec<sup>R</sup>) at a ratio of 1/1 on LBA + NaCl 0.5M and incubating at 25°C for 24 hours. To perform in vivo conjugation assay, overnight cultures of donor (pGV1512\_Cm<sup>R</sup>) and recipient (e.g. J2-8\_Spec<sup>R</sup>) were mixed at a ratio 1/1 and one hundred microliters of the mix was injected intramuscularly into oysters. After 24h, dead oyster flesh was ground and spread on a selective medium LBA + NaCl + Cm + Spec.

### **Experimental challenge**

Cohabitation experiments were performed by injecting fifty microliters of a virulent strain culture (10<sup>8</sup> cfu) in the donor oyster batch and fifty microliters of sterile seawater or a non-virulent strain culture (10<sup>7</sup> cfu) in the recipient oyster batch. Mortalities were recorded in both batches and alive recipient oysters were ground and spread on agar plates at 48h and 96h after injection to assess total flora, *Vibrio* and donor strain load.

To determine virulence of isolates, bacteria were grown under constant agitation at 20°C for 24h in Zobell media. One hundred microliters of the culture (10<sup>7</sup> cfu) were injected intramuscularly into oysters. The bacterial concentration was confirmed by conventional dilution plating on Zobell agar. After injection, the oysters were transferred to aquaria (20 oysters per aquarium of 2.5 l) containing 1 liter of aerated 5 µm-filtered seawater at 20°C, kept under static conditions. Mortality was assessed after 24 hours.

### **RNASeq experiments**

5ml, 2ml and 1ml of pGV\_ΔPx3 + pMRB\_TR1512 and pGV\_ΔPx3 + pMRB\_GFP cultures were respectively taken off at OD 0.3, 0.6 and 1. RNA extraction was immediately performed on the different samples using TRIzol Reagent and following manufacturer's protocol (Invitrogen). Samples from the 3 OD per condition were pooled in an equimolar manner and a DNase treatment was performed on the pools using DNA-free DNA Removal kit and following manufacturer's instruction (Invitrogen). Experiments were performed in triplicates. RNASeq library construction and sequencing were performed by the Plateforme de Séquençage Haut Débit I2BC-UMR9198. Directional cDNA libraries were sequenced on Illumina Nextseq 500Mid, in paired-end reads of 2x75bp. Data treatment and mapping to the *V. crassostreae* strain J2-9 reference genome was performed using the TAMARA pipeline hosted by the MAGE platform.

### **Genome sequencing, assembly and annotation.**

Genomes of *V. Splendidus* (#24) were sequenced using Illumina HiSeq 2000 technology with ~40 fold of coverage as described previously (6). Contigs were assembled *de novo* using Velvet (24). Plasmids pGV1512-like were reconstructed by aligning contigs on the reference sequence from *V. crassostreae* J5-20 (Accession Number: KX765275.1). Computational prediction of coding sequences, together with functional assignments, were performed using the automated annotation pipeline implemented in the MicroScope platform (23).

### **Phylogenetic analyses**

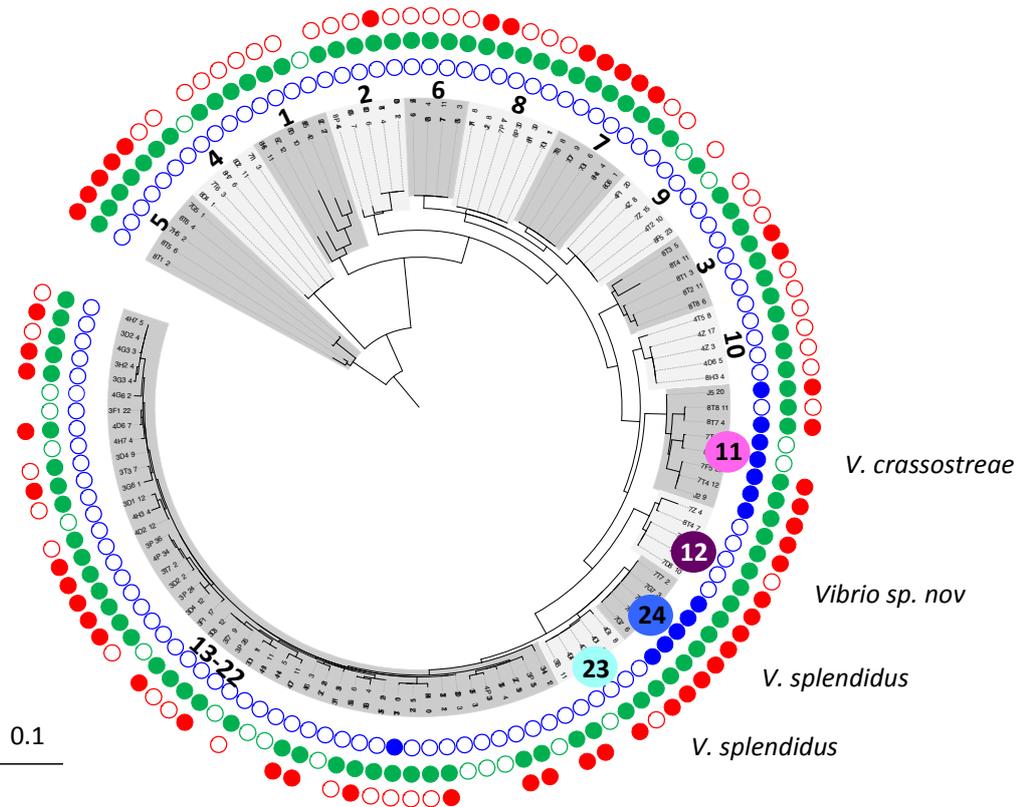
Phylogenetic trees were reconstructed based on the nucleotide alignment of markers by Muscle. Phylogenetic reconstruction was done using RaxML (26) with a GTR model of evolution, a gamma model and default parameters.

### **Statistical analyses**

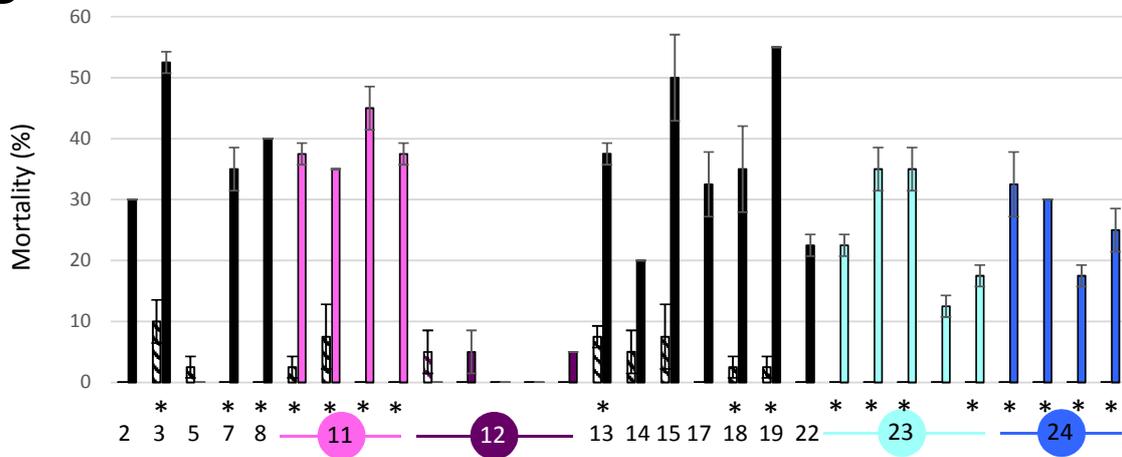
Statistical analyses were performed using the computing environment R (R Core Team, 2016) (27).

FIGURES

A

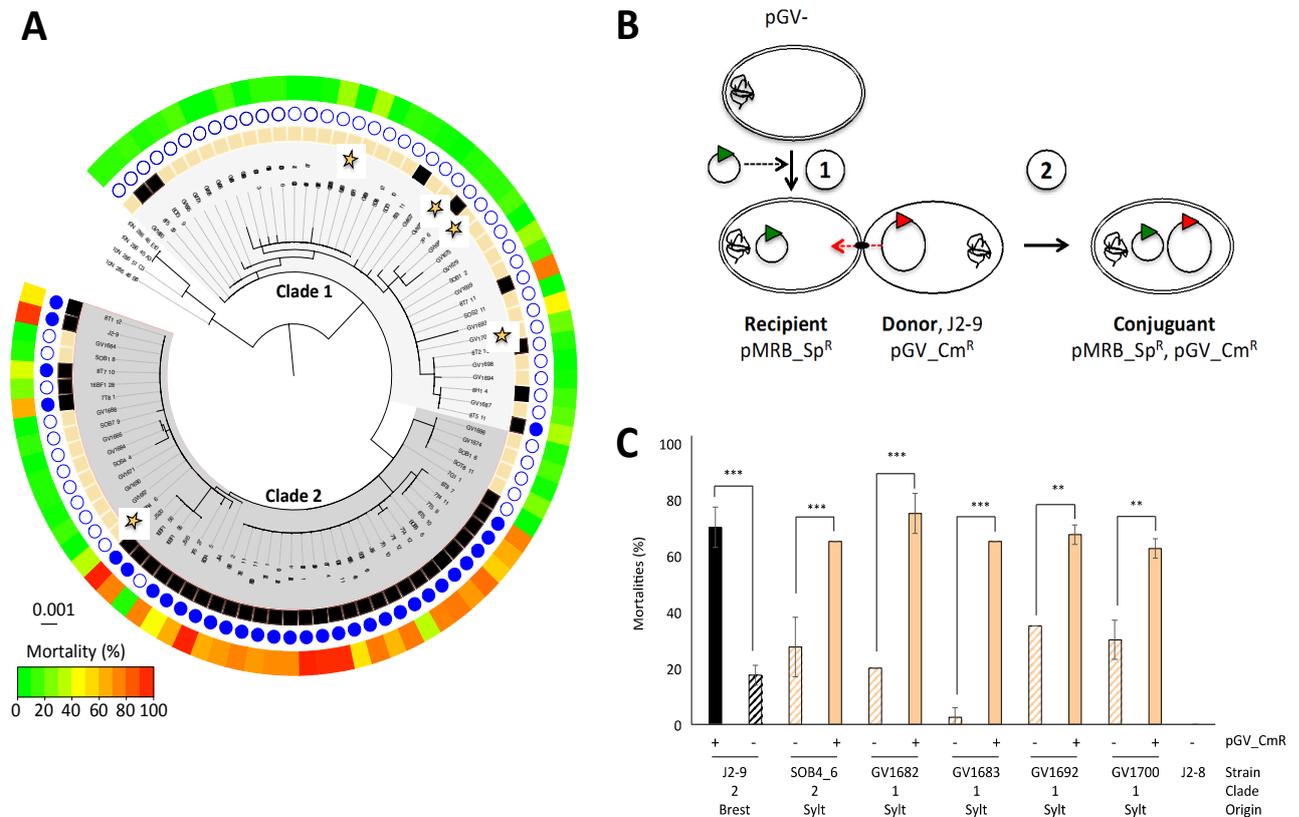


B

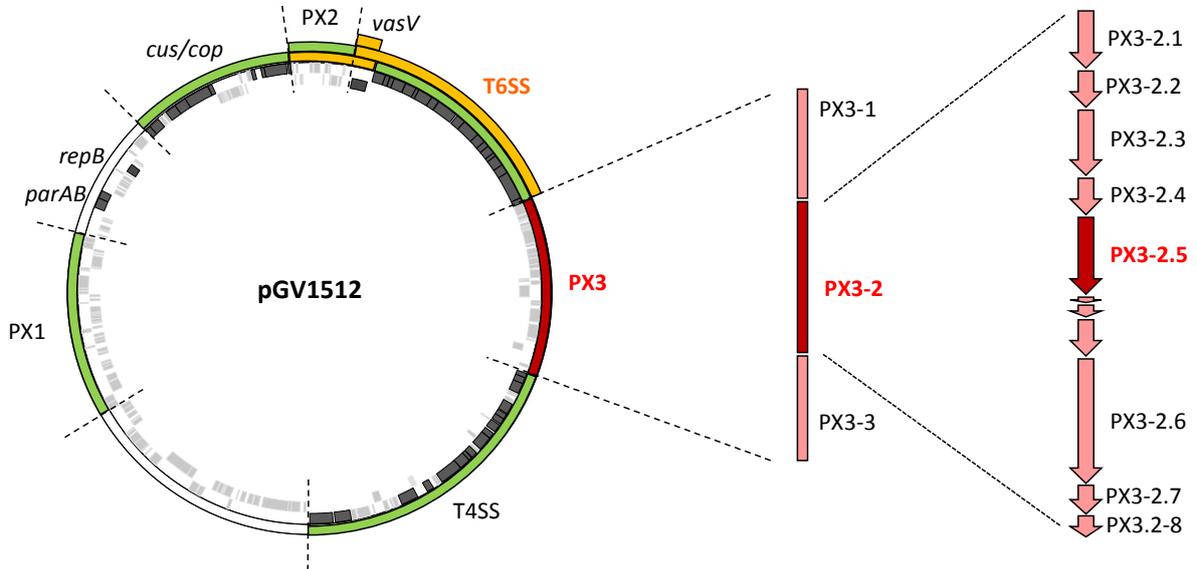
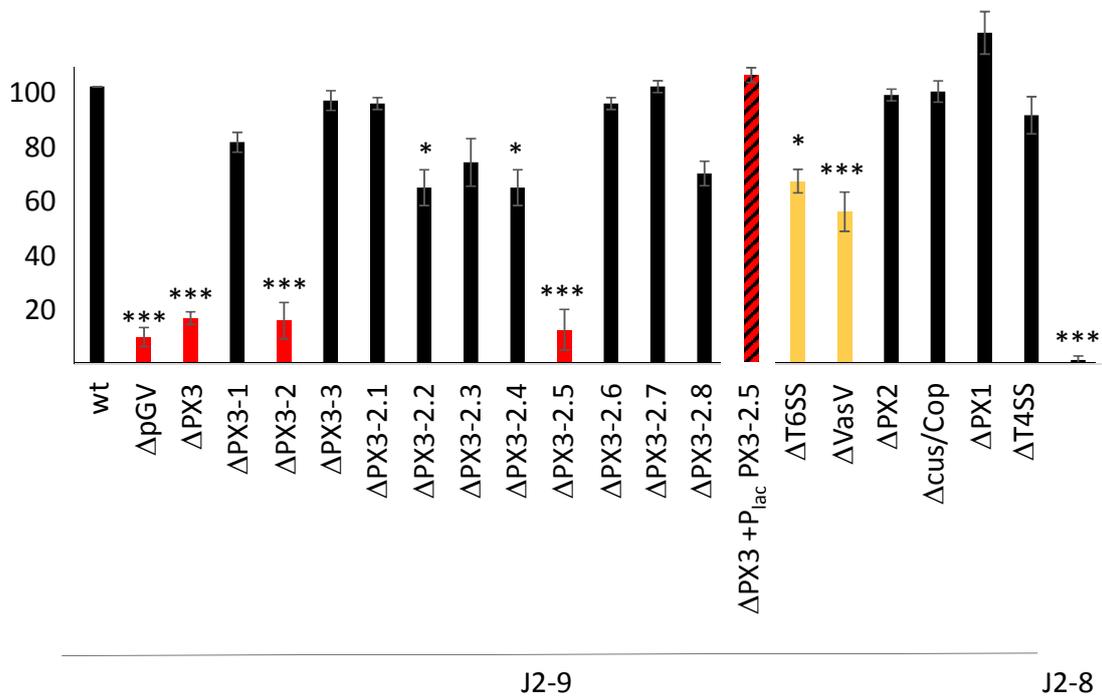


**Figure 1** The narrow host range of the pGV1512 and ability to increase virulence of diverse *Vibrio* populations isolated in Brest. **A.** Phylogenetic tree of 119 isolates (4-5 representative strains per population) based on the *hsp60* marker. Alternating dark/light greys indicate the 24 ecological populations predicted by the AdaptML algorithm (4). A total of 17/24 populations were assigned to a named taxon: *V. orientalis* (#1); *V. fortis* (#2); *V. chagasii* (#3); *V. jasicida* (#4); *V. rotiferianus* (#5); *V. crassostreae* (#11); *V. splendidus* (#13 to 24). Other populations were designated *Vibrio* sp. due to lack of closely related reference species (i.e., populations 6 to 10 and 12). Rings from inside to outside indicate i) the presence (blue circle) or absence (white circle) of pGV1512-like plasmid detected by *repB* PCR in each strains; ii) the successful transfer (green circle) of a spectinomycin resistance marked

pMRB plasmid (pMRB-Sp<sup>R</sup>) by *in vitro* mating from an *E. coli* donor; iii) the successful transfer (red circle) of a chloramphenicol resistance marked pGV1512 plasmid by *in vitro* mating from a *V. crassostreae* donor (strain J2-9, pGV1512-Cm<sup>R</sup>). Populations that naturally possess pGV1512 (#11 and #24) and their closest phylogenetic neighbors (#12 and #23) are highlighted by a colored circle containing their population number. **B.** Histograms representing the mortality rate of strains from all populations with (plain) or without (hatched, numerous strains induced 0% mortality) pGV1512. The x-axis indicates the population number and the y-axis indicates the mortality percentage of oysters 24h after injection. This experiment has been done twice in duplicates. Asterisks indicate population in which pGV1512 induce a significantly higher virulence.

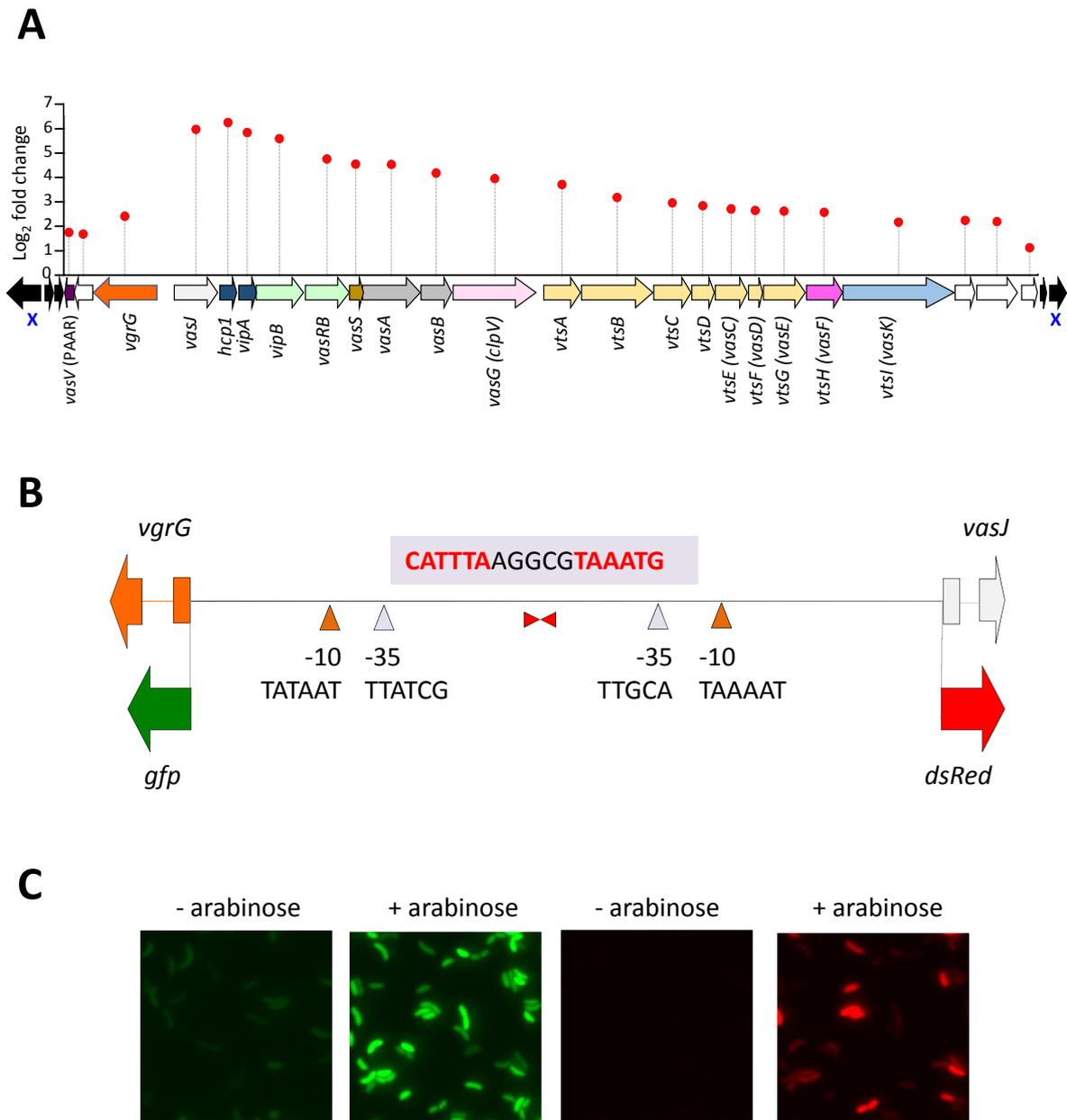


**Figure 2** The presence of the pGV1512 plasmid is correlated with the geographic origin and virulence of *V. crassostreae* strains. **A.** Phylogenetic tree of 89 *V. crassostreae* isolates based on the *gyrB/rctB/rpoD* markers. Dark/light greys indicate the two clades within the species. Rings from inside to outside indicate i) the geographic origin of the isolates (Brest, black square; Sylt, beige square); ii) the presence (blue circle) or absence (white circle) of pGV1512-like plasmid detected by *repB* PCR; iii) the mortality rate (color gradient from green to red corresponding to 0 to 100%) induced by individual strains 24h after injection in oysters (n=20). Experiments were performed in duplicate with two distinct oyster batches. Strains from Sylt that will be used to test the implication of pGV1512 on virulence are indicated by a yellow star. **B.** Schematic representation of the different genetic constructs used to test the implication of pGV1512 on the virulence of Sylt strains. First, a Sp<sup>R</sup> marked pMRB plasmid was transferred from a *E. coli* donor to recipients. Second, the pGV1512 plasmid (modified with the insertion of a chloramphenicol resistance gene, pGV\_Cm<sup>R</sup>) was transferred in pMRB\_spR recipients from *V. crassostreae* J2-9. **C.** Histograms representing the mortality rate of Sylt strains with (plain) or without (hatched) pGV1512. *V. crassostreae* J2-9 (virulent) and *Vibrio* sp. J2-8 (non-virulent) were used as control. The x-axis indicates strains with different metadata (pGV1512 presence/absence, clade in phylogeny and geographical origin) and the y-axis indicates the mortality rate of oysters (n=20) 24h after injection.

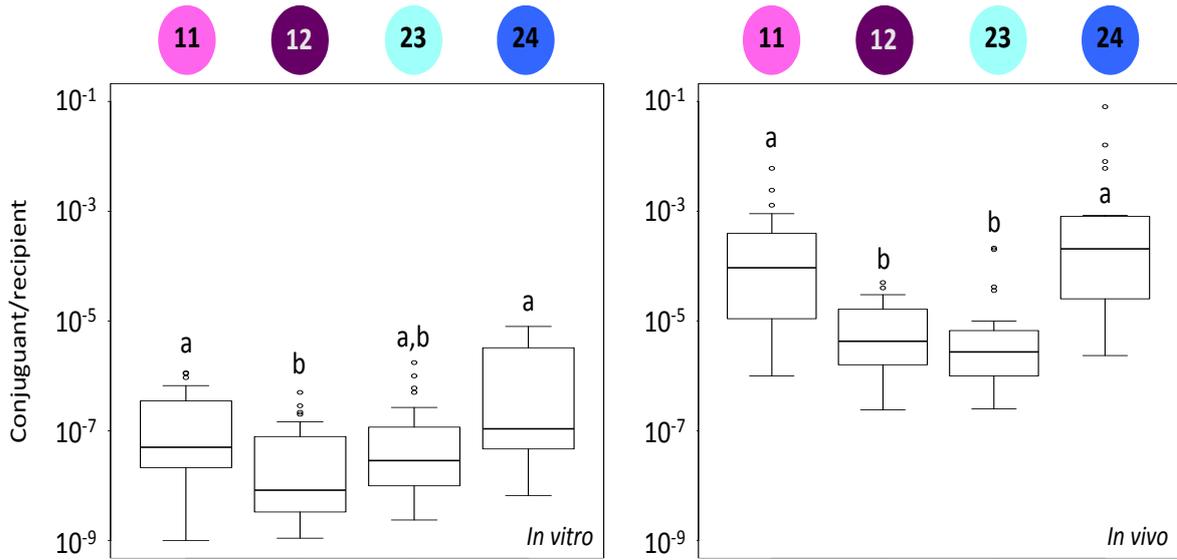
**A****B**

**Figure 3** Experimental assessments of pGV1512 loci as virulence determinants. **A.** Circular representation of the *V. crassostreae* strain J5-20 pGV1512 plasmid and the different loci tested for virulence. The inner circles show the open reading frames encoding replication and partitioning; a type 4 secretion system (T4SS); Cus/Cop and regulators; a type 6 secretion system (T6SS); Px1 to 3 regions

contain a majority of genes encoding unknown function. The deletion of indicated regions or gene was performed by allelic exchange. As some deletions were associated to a plasmid loss in culture, a chloramphenicol resistance suicide plasmid carrying a chloramphenicol resistance marker was introduced by single recombination in a locus encoding a putative transposase (VCR9J2v1\_750048). First outer circle indicates loci deleted in Bruto et al., 2017. Outer circle indicates new deletions performed for the present study. Deletions of PX2 and T6SS loci were misconstrued in Bruto et al., 2017, due to plasmid assemblage uncertainty. Colors correspond to the effect of the different loci in J5-20 virulence (green: no effect; orange: intermediate effect; red: effect on virulence). **B.** The virulence of the wild type and mutants of specific loci was compared by injection of strains ( $10^7$  cfu) in 20 oysters in duplicate and counting the percentage of mortalities after 24 hours. Numbers indicate the ratio of mortality rate obtained for the mutant divided by the rate that resulted from wild type infections. Asterisks indicate a reduced virulence of the mutants compared to the wild type virulence. Deletion of PX3, PX3-2 and PX3-2.5 (red bars) strongly affect virulence of J5-20. Complementation of pGV\_DPX3 with PX3-2.5 (red hatched bar) is sufficient to restore full virulence. Deletion of T6SS and *vasV* gene partially reduced virulence (orange bars).



**Figure 4** Expression and predictions on regulation of the type 6 secretion system. **A.** Genetic organization of the T6SS with differential expression of each gene (condition with overexpression of the regulator versus overexpression of the GFP) indicated above. Black genes indicate the boundaries and the X recombination sites for the suicide plasmid used to delete the complete T6SS. **B.** Prediction of the putative binding site for the PX3-2.5 transcription factor in the promoter region localized between *vgrG* and *vasJ* using RSAT and 10/-35 boxes using BPROM. The ability of the promoter to express genes on both directions will be assessed by cloning this region in a replicative plasmid containing a *gfp* upstream and a *dsRed* downstream. **C.** Fluorescence microscopy of *V. cholerae* strains containing the transcriptional regulator under control of an arabinose inducible promoter and the genetic construct described above. Expression of GFP and DsRed was assessed by the presence of fluorescent cells when arabinose was added.



**Figure 5** Conjugation frequency after *in vitro* mating or injection to oysters. The frequency corresponds to the ratio between conjugants ( $Cm^R$  and  $Sp^R$ ) and recipient ( $Sp^R$ ). Conjugation was performed by *in vitro* mating (left graph) and by injection to oysters (i.e *in vivo* mating, right graph) for different strains from populations #11, #12, #23 and #24, following the same color code than in Figure 1. *In vitro* and *in vivo* conjugation assays have been performed on 4 or 5 strains per populations and in triplicates. Different letters indicate significant differences in conjugation frequency between populations.

## References

1. Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, et al. Food security: the challenge of feeding 9 billion people. *Science*. 2010;327(5967):812-8.
2. Smith MD, Roheim CA, Crowder LB, Halpern BS, Turnipseed M, Anderson JL, et al. Economics. Sustainability and global seafood. *Science*. 2010;327(5967):784-6.
3. Lovell CR. Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Appl Microbiol Biotechnol*. 2017;101(5):1781-94.
4. Bruto M, James A, Petton B, Labreuche Y, Chenivresse S, Alunno-Bruscia M, et al. *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *Isme J*. 2017;11(4):1043-52.
5. Goudenege D, Travers MA, Lemire A, Petton B, Haffner P, Labreuche Y, et al. A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. *Environ Microbiol*. 2015;17(11):4189-99.
6. Lemire A, Goudenege D, Versigny T, Petton B, Calteau A, Labreuche Y, et al. Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *Isme J*. 2014;9(7):1523-31.
7. Cordero OX, Polz MF. Explaining microbial genomic diversity in light of evolutionary ecology. *Nat Rev Microbiol*. 2014;12(4):263-73.
8. Le Roux F, Wegner KM, Polz MF. Oysters and Vibrios as a Model for Disease Dynamics in Wild Animals. *Trends Microbiol*. 2016.
9. Shapiro BJ, Polz MF. Ordering microbial diversity into ecologically and genetically cohesive units. *Trends Microbiol*. 2014;22(5):235-47.
10. Shapiro BJ, Polz MF. Microbial Speciation. *Cold Spring Harb Perspect Biol*. 2015;7(10):a018143.
11. Faury N, Saulnier D, Thompson FL, Gay M, Swings J, Le Roux F. *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). *Int J Syst Evol Microbiol*. 2004;54(Pt 6):2137-40.
12. Smalla K, Jechalke S, Top EM. Plasmid Detection, Characterization, and Ecology. *Microbiol Spectr*. 2015;3(1):PLAS-0038-2014.
13. Reise K, Buschbaum C, Buttger H, Rick J, Wegner KM. Invasion trajectory of Pacific oysters in the northern Wadden Sea. *Mar Biol*. 2017;164(4):68.

15. Le Roux F, Davis BM, Waldor MK. Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res.* 2011;39(3):1004-13.
16. Cianfanelli FR, Monlezun L, Coulthurst SJ. Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon. *Trends Microbiol.* 2016;24(1):51-62.
17. Joshi A, Kostiuk B, Rogers A, Teschler J, Pukatzki S, Yildiz FH. Rules of Engagement: The Type VI Secretion System in *Vibrio cholerae*. *Trends Microbiol.* 2017;25(4):267-79.
18. Vandecraen J, Chandler M, Aertsen A, Van Houdt R. The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit Rev Microbiol.* 2017;43(6):709-30.
19. Ma AT, McAuley S, Pukatzki S, Mekalanos JJ. Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe.* 2009;5(3):234-43.
20. Pukatzki S, Ma AT, Revel AT, Sturtevant D, Mekalanos JJ. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc Natl Acad Sci U S A.* 2007;104(39):15508-13.
21. Borgeaud S, Metzger LC, Scignari T, Blokesch M. The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science.* 2015;347(6217):63-7.
22. Bellanger X, Guilloteau H, Breuil B, Merlin C. Natural microbial communities supporting the transfer of the IncP-1beta plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. *Front Microbiol.* 2014;5:637.
23. Hirt H, Greenwood-Quaintance KE, Karau MJ, Till LM, Kashyap PC, Patel R, et al. *Enterococcus faecalis* Sex Pheromone cCF10 Enhances Conjugative Plasmid Transfer In Vivo. *MBio.* 2018;9(1).
24. Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* 2008;18(5):821-9.
25. Vallenet D, Belda E, Calteau A, Cruveiller S, Engelen S, Lajus A, et al. MicroScope--an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* 2013;41(Database issue):D636-47.
26. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics.* 2006;22(21):2688-90.
27. R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing: Vienna, Austria. Available at: [https:// www.R-project.org/](https://www.R-project.org/).

## Supplementary information

### Tables

**Table S1** Strains representative of the 24 populations of *Vibrio* isolated in an oyster farm in Brest (Figure 1).

Taxonomic species (#Population N°)	Strains details				pGV1512 detected by	
	Name	GV collection	Fraction	Sampling date	PCR repB	Sequences
<i>V. orientalis</i> (#1)	8Z_12	2277	>60 µM	August 2014	-	nd
	8Z_27	2278	>60 µM	August 2014	-	nd
	8F5_42	2279	60-5 µM	August 2014	-	nd
	8T3_10	2280	Oyster	August 2014	-	nd
	8H6_11	2281	Oyster	August 2014	-	nd
<i>V. fortis</i> (#2)	8P_44	2282	1-0.2 µM	August 2014	-	nd
	8T1_4	2283	Oyster	August 2014	-	nd
	8D2_6	2284	Oyster	August 2014	-	nd
	8D3_12	2285	Oyster	August 2014	-	nd
	8G1_7	2286	Oyster	August 2014	-	nd
<i>V. chagasii</i> (#3)	8T1_3	2287	Oyster	August 2014	-	-
	8T2_11	2288	Oyster	August 2014	-	-
	8T3_5	2289	Oyster	August 2014	-	-
	8T4_11	2290	Oyster	August 2014	-	-
	8T8_6	2291	Oyster	August 2014	-	nd
<i>V. jasicida</i> (#4)	7T1_3	2292	Oyster	July 2014	-	-
	7T6_3	2293	Oyster	July 2014	-	-
	8D2_11	2294	Oyster	August 2014	-	-
	8D4_1	2295	Oyster	August 2014	-	nd
	8H7_6	2296	Oyster	August 2014	-	-
<i>V. rotiferianus</i> (#5)	7G5_1	2297	Oyster	July 2014	-	-
	7H5_2	2298	Oyster	July 2014	-	-
	8T1_2	2299	Oyster	August 2014	-	-
	8T5_6	2300	Oyster	August 2014	-	-
	8T6_4	2301	Oyster	August 2014	-	-
<i>Vibrio sp. nov</i> (#6)	7F5_19	2302	60-5 µM	July 2014	-	nd

	7P_16	2303	1-0.2 $\mu$ M	July 2014	-	nd
	7T7_11	2304	Oyster	July 2014	-	nd
	7G1_3	2305	Oyster	July 2014	-	nd
	7G8_4	2306	Oyster	July 2014	-	nd
<i>Vibrio sp. nov</i> (#7)	7T8_8	2307	Oyster	July 2014	-	nd
	7D7_9	2308	Oyster	July 2014	-	nd
	7G1_6	2309	Oyster	July 2014	-	nd
	8G6_1	2310	Oyster	August 2014	-	nd
	8H4_4	2311	Oyster	August 2014	-	nd
<i>Vibrio sp. nov</i> (#8)	7F1_8	2312	5-1 $\mu$ M	July 2014	-	nd
	7P_17	2313	1-0.2 $\mu$ M	July 2014	-	nd
	7G1_11	2314	Oyster	July 2014	-	nd
	8F1_39	2315	5-1 $\mu$ M	August 2014	-	nd
	8P_30	2316	1-0.2 $\mu$ M	August 2014	-	nd
<i>Vibrio sp. nov</i> (#9)	7Z_15	2317	>60 $\mu$ M	July 2014	-	nd
	8F5_23	2318	60-5 $\mu$ M	August 2014	-	nd
	4Z_8	2319	>60 $\mu$ M	April 2014	-	nd
	4F1_20	2320	5-1 $\mu$ M	April 2014	-	nd
	4T2_10	2321	Oyster	April 2014	-	nd
<i>Vibrio sp. nov</i> (#10)	4D6_5	2322	Oyster	April 2014	-	nd
	8H3_4	2323	Oyster	August 2014	-	nd
	4Z_3	2324	>60 $\mu$ M	April 2014	-	nd
	4Z_17	2325	>60 $\mu$ M	April 2014	-	nd
	4T5_8	2326	Oyster	April 2014	-	nd
<i>V. crassostreae</i> (#11)	7T4_12	2327	Oyster	July 2014	+	+
	7T5_9	2328	Oyster	July 2014	+	nd
	8T2_1	2329	Oyster	August 2014	+	+
	8T7_4	2330	Oyster	August 2014	+	+
	8T8_11	2331	Oyster	August 2014	-	-
<i>Vibrio sp. nov</i> (#12)	7Z_4	2332	>60 $\mu$ M	July 2014	-	nd
	7F1_18	2333	5-1 $\mu$ M	July 2014	-	nd
	7T8_6	2334	Oyster	July 2014	-	nd
	7D8_10	2335	Oyster	July 2014	-	nd
	8T4_7	2336	Oyster	August 2014	-	nd
<i>V. splendidus</i> (#13)	4D7_11	2337	Oyster	April 2014	-	nd

	4H4_5	2338	Oyster	April 2014	-	nd
	3P_26	2339	1-0.2 $\mu$ M	March 2014	-	nd
	3T7_9	2340	Oyster	March 2014	-	nd
	3D3_12	2341	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#14)	3F1_38	2342	5-1 $\mu$ M	March 2014	-	nd
	3T2_12	2343	Oyster	March 2014	-	nd
	3D3_3	2344	Oyster	March 2014	-	nd
	4T6_3	2345	Oyster	April 2014	-	nd
<i>V. splendidus</i> (#15)	3Z_39	2346	>60 $\mu$ M	March 2014	-	nd
	3F5_31	2347	60-5 $\mu$ M	March 2014	-	nd
	3P_35	2348	1-0.2 $\mu$ M	March 2014	-	nd
	3T6_4	2349	Oyster	March 2014	-	nd
	4P_33	2350	1-0.2 $\mu$ M	April 2014	-	nd
<i>V. splendidus</i> (#16)	4D2_12	2351	Oyster	April 2014	-	nd
	4D6_8	2352	Oyster	April 2014	-	nd
	4H3_4	2353	Oyster	April 2014	-	nd
	3D1_12	2354	Oyster	April 2014	-	nd
	3G6_1	2355	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#17)	4G6_2	2356	Oyster	April 2014	-	nd
	4H7_4	2357	Oyster	April 2014	-	nd
	3F1_45	2358	5-1 $\mu$ M	March 2014	-	nd
	3T3_7	2359	Oyster	March 2014	-	nd
	3D4_9	2360	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#18)	4D6_7	2361	Oyster	April 2014	-	nd
	3F1_22	2362	5-1 $\mu$ M	March 2014	-	nd
	3P_24	2363	1-0.2 $\mu$ M	March 2014	-	nd
	3D4_12	2364	Oyster	March 2014	-	nd
	3H4_12	2365	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#19)	3Z_28	2366	>60 $\mu$ M	March 2014	-	nd
	3F5_29	2367	60-5 $\mu$ M	March 2014	-	nd
	3F1_5	2368	5-1 $\mu$ M	March 2014	-	nd
	3G1_6	2369	Oyster	March 2014	-	nd
	3G6_4	2370	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#20)	4H3_11	2371	Oyster	April 2014	-	nd
	3Z_15	2372	>60 $\mu$ M	March 2014	-	nd

	3T7_10	2373	Oyster	March 2014	+	nd
	3D1_11	2374	Oyster	March 2014	-	nd
	3H4_10	2375	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#21)	4G3_3	2376	Oyster	April 2014	-	nd
	4H7_5	2377	Oyster	April 2014	-	nd
	3G3_4	2378	Oyster	March 2014	-	nd
	3H2_4	2379	Oyster	March 2014	-	nd
	3D2_4	2380	Oyster	March 2014	-	nd
<i>V. splendidus</i> (#22)	3F1_17	2381	5-1 $\mu$ M	March 2014	-	nd
	3P_36	2382	1-0.2 $\mu$ M	March 2014	-	nd
	3T7_2	2383	Oyster	March 2014	-	nd
	3D2_2	2384	Oyster	March 2014	-	nd
	4P_34	2385	1-0.2 $\mu$ M	April 2014	-	nd
<i>V. splendidus</i> (#23)	4D1_8	2386	Oyster	April 2014	-	-
	4D4_11	2387	Oyster	April 2014	-	-
	4G1_8	2388	Oyster	April 2014	-	-
	4G4_4	2389	Oyster	April 2014	-	-
	3T8_11	2390	Oyster	March 2014	-	-
<i>V. splendidus</i> (#24)	7T7_2	2391	Oyster	July 2014	+	+
	7G7_3	2392	Oyster	July 2014	+	+
	7G7_6	2393	Oyster	July 2014	+	+
	7H1_4	2394	Oyster	July 2014	+	+
	7H7_9	2395	Oyster	July 2014	+	+

**Table S2** General feature of the genome sequence of *V. splendidus* (#24)

<b>Strain name</b>	<b>Number of contigs</b>	<b>Genome size</b>	<b>Number of CDSs</b>	<b>pGV1512 like plasmid size</b>	<b>Number of genes shared with pGV1512</b>
7G7-3	309	5935263	5685	139666	142
7G7-6	286	5980134	5722	138792	141
7H1-4	326	5982032	5769	136138	139
7H7-9	338	6038795	5811	136061	138
7T7-2	314	6008004	5768	137849	139

**Table S3** *Vibrio crassostreae* strain isolated in Brest and Sylt, sequence by MLST (*gyrB*, *rpoD* and *rctB*), screened for pGV1512 *repB* gene presence and used for experimental infection (Figure 2).

Strains	Isolation site	Isolation date	Fraction	Plasmid presence	Oyster batch 1		Oyster batch 2	
					Mortality (%)	Standard deviation	Mortality (%)	Standard deviation
16BF1_28	Brest	July 2016	1-5 µM	0	20.0	14.1	15.0	7.1
16BF1_56	Brest	July 2016	1-5 µM	1	70.0	14.1	72.5	3.5
16BF1_95	Brest	July 2017	1-5 µM	0	5.0	0.0	2.5	3.5
16BF5_48	Brest	July 2018	5-1 µM	1	72.5	17.7	67.5	17.7
7F5_27	Brest	July 2014	5-1 µM	1	40.0	21.2	45.0	7.1
7G1_1	Brest	July 2015	Oyster	0	0.0	0.0	5.0	7.1
7P_6	Brest	July 2016	1-0.2 µM	0	7.5	0.0	3.5	0.0
7T1_10	Brest	July 2017	Oyster	1	30.0	0.0	67.5	3.5
7T1_12	Brest	July 2018	Oyster	1	70.0	7.1	80.0	14.1
7T3_1	Brest	July 2019	Oyster	1	65.0	7.1	80.0	14.1
7T4_11	Brest	July 2020	Oyster	1	60.0	14.1	67.5	3.5
7T4_12	Brest	July 2021	Oyster	1	72.5	3.5	35.0	7.1
7T5_9	Brest	July 2022	Oyster	1	52.5	3.5	57.5	3.5
7T6_10	Brest	July 2023	Oyster	1	67.5	3.5	70.0	7.1
7T8_1	Brest	July 2024	Oyster	1	60.0	7.1	82.5	3.5
7T8_11	Brest	July 2025	Oyster	1	65.0	7.1	65.0	7.1
8F5_39	Brest	August 2014	5-1 µM	0	7.5	10.6	5.0	7.1
8H1_4	Brest	August 2014	Oyster	0	10.0	7.1	10.0	7.1
8T1_12	Brest	August 2014	Oyster	1	45.0	7.1	50.0	21.2
8T2_1	Brest	August 2014	Oyster	1	92.5	3.5	97.5	3.5

8T2_10	Brest	August 2014	Oyster	0	5.0	7.1	12.5	3.5
8T2_4	Brest	August 2014	Oyster	1	67.5	3.5	57.5	3.5
8T5_10	Brest	August 2014	Oyster	1	75.0	14.1	57.5	3.5
8T5_11	Brest	August 2014	Oyster	1	27.5	10.6	15.0	0.0
8T7_10	Brest	August 2014	Oyster	1	35.0	7.1	62.5	10.6
8T7_11	Brest	August 2014	Oyster	0	22.5	3.5	2.5	3.5
8T7_4	Brest	August 2014	Oyster	1	90.0	7.1	97.5	3.5
8T8_11	Brest	August 2014	Oyster	0	2.5	3.5	7.5	10.6
8T8_2	Brest	August 2014	Oyster	1	60.0	0.0	82.5	10.6
8T8_7	Brest	August 2014	Oyster	1	70.0	0.0	72.5	3.5
BOB3_6	Brest	July 2016	Oyster	1	65.0	7.1	70.0	21.2
BOT2_10	Brest	July 2016	Oyster	1	57.5	3.5	67.5	10.6
BOT3_9	Brest	July 2016	Oyster	0	10.0	7.1	45.0	7.1
BOT4_11	Brest	July 2016	Oyster	1	47.5	10.6	67.5	3.5
BOT4_5	Brest	July 2016	Oyster	1	60.0	14.1	67.5	17.7
BOT5_11	Brest	July 2016	Oyster	1	70.0	0.0	57.5	10.6
J2-9	Brest	July 2011	Oyster	1	87.1	9.1	89.6	6.6
J5-15	Brest	July 2011	Oyster	1	70.0	14.1	77.5	3.5
J5-19	Brest	July 2011	Oyster	1	90.0	0.0	100.0	0.0
J5-20	Brest	July 2011	Oyster	1	92.5	3.5	75.0	14.1
J5-4	Brest	July 2011	Oyster	1	92.5	3.5	100.0	0.0
J5-5	Brest	July 2011	Oyster	1	72.5	24.7	82.5	10.6
16SF1_51	Sylt	July 2016	1-5 µM	0	27.5	24.7	5.0	7.1
16SF1_87	Sylt	July 2016	1-5 µM	0	0.0	0.0	0.0	0.0
GV1350/S16	Sylt	July 2016	Oyster	0	25.0	14.1	35.0	21.2

GV1664	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	2.5	3.5
GV1666	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	0.0	0.0
GV1667	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	0.0	0.0
GV1671	Sylt	July 2016	1-0.2 µM	0	2.5	3.5	0.0	0.0
GV1672	Sylt	July 2016	1-0.2 µM	0	2.5	3.5	2.5	3.5
GV1674	Sylt	July 2016	1-0.2 µM	0	12.5	3.5	15.0	7.1
GV1675	Sylt	July 2016	1-0.2 µM	0	12.5	3.5	27.5	10.6
GV1676	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	0.0	0.0
GV1677	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	10.0	7.1
GV1678	Sylt	July 2016	1-0.2 µM	0	12.5	3.5	0.0	0.0
GV1679	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	5.0	7.1
GV1680	Sylt	July 2016	1-0.2 µM	0	7.5	3.5	7.5	3.5
GV1681	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	2.5	3.5
GV1682	Sylt	July 2016	1-0.2 µM	0	7.5	3.5	10.0	7.1
GV1683	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	7.5	10.6
GV1684	Sylt	July 2016	1-0.2 µM	0	5.0	0.0	15.0	0.0
GV1685	Sylt	July 2016	1-0.2 µM	0	7.5	3.5	47.5	10.6
GV1687	Sylt	July 2016	1-0.2 µM	0	15.0	7.1	17.5	3.5
GV1688	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	40.0	7.1
GV1689	Sylt	July 2016	1-0.2 µM	0	12.5	3.5	20.0	7.1
GV1690	Sylt	July 2016	1-0.2 µM	0	10.0	0.0	2.5	3.5
GV1691	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	2.5	3.5
GV1692	Sylt	July 2016	1-0.2 µM	0	5.0	0.0	12.5	3.5
GV1693	Sylt	July 2016	1-0.2 µM	0	42.5	3.5	50.0	7.1
GV1694	Sylt	July 2016	1-0.2 µM	0	2.5	3.5	10.0	0.0

GV1695	Sylt	July 2016	1-0.2 µM	0	5.0	0.0	5.0	0.0
GV1696	Sylt	July 2016	1-0.2 µM	0	5.0	7.1	2.5	3.5
GV1698	Sylt	July 2016	1-0.2 µM	0	0.0	0.0	2.5	3.5
GV1699	Sylt	July 2016	1-0.2 µM	0	10.0	7.1	5.0	7.1
GV1700	Sylt	July 2016	1-0.2 µM	0	17.5	10.6	27.5	3.5
GV1701	Sylt	July 2016	1-0.2 µM	0	7.5	10.6	0.0	0.0
SOB1_2	Sylt	July 2016	Oyster	0	25.0	14.1	42.5	17.7
SOB1_3	Sylt	July 2016	Oyster	0	0.0	0.0	5.0	0.0
SOB1_6	Sylt	July 2016	Oyster	0	2.5	3.5	32.5	3.5
SOB1_8	Sylt	July 2016	Oyster	0	5.0	7.1	0.0	0.0
SOB4_6	Sylt	July 2016	Oyster	0	30.0	7.1	27.5	10.6
SOB6_12	Sylt	July 2016	Oyster	0	7.5	3.5	5.0	7.1
SOB7_9	Sylt	July 2016	Oyster	0	7.5	10.6	0.0	0.0
SOS2_11	Sylt	July 2016	Oyster	0	0.0	0.0	10.0	7.1
SOS2_6	Sylt	July 2016	Oyster	0	7.5	3.5	10.0	7.1
SOS4_4	Sylt	July 2016	Oyster	0	5.0	0.0	10.0	7.1
SOT2_12	Sylt	July 2016	Oyster	0	0.0	0.0	7.5	3.5
SOT3_10	Sylt	July 2016	Oyster	0	0.0	0.0	17.5	24.7
SOT8_11	Sylt	July 2016	Oyster	0	20.0	7.1	20.0	7.1

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**Table S4** RNAseq analysis to compare the transcriptomes of J2-9 expressing constitutively TR1512 or GFP (T6SS genes in bold).

Label VCR9J2v1_	Name	Product	Log2 fold change	Adjusted pvalue (FDR)
10043	-	Conserved protein of unknown function	-1.23	2.40E-12
30005	argC	N-acetyl-gamma-glutamyl-phosphate reductase	-2.85	5.73E-73
30006	argB	Acetylglutamate kinase	-2.22	3.42E-44
30007	argG	Argininosuccinate synthase (Citrulline--aspartate ligase)	-1.55	2.46E-25
50079	-	Conserved hypothetical protein	-1.86	9.15E-27
50083	-	Conserved hypothetical protein	-1.35	1.94E-15
70029	grcA	Autonomous glycyl radical cofactor	-1.14	4.86E-13
70040	-	Putative Radical SAM enzyme	-1.23	5.22E-11
80001	-	Conserved exported protein of unknown function	1.1	9.32E-03
100058	adhB	Alcohol dehydrogenase 2	-1.21	5.45E-12
120021	-	Conserved protein of unknown function	-1.01	1.44E-06
130062	ibpA	Small heat shock protein ibpA	1.38	6.28E-22
150084	-	Putative Cold shock-like protein CspE	-1.62	4.82E-18
150085	-	Conserved protein of unknown function	-1.02	2.29E-08
180032	-	Hypothetical protein	-1.17	2.99E-12
190001	nirB	Nitrite reductase [NAD(P)H] large subunit	-1.03	4.46E-11
190057	-	Conserved hypothetical protein	1.06	6.81E-09
380002	-	Conserved protein of unknown function	-1.37	1.89E-02
700198	-	Putative Peptidase U32	-1.33	5.22E-11
700380	-	Conserved hypothetical protein	-1.74	6.48E-24
720146	hisM	Histidine transport system permease protein hisM	-2.94	2.92E-55
720147	-	ABC transporter: transmembrane protein; Arginine uptake	-3.21	4.93E-72
720148	artI	Arginine ABC transporter: substrate binding protein	-2.97	1.19E-66
720149	artP	Arginine transporter: ATP-binding protein	-3.74	4.51E-98
720273	-	Hypothetical protein	-1.08	8.99E-05
730010	-	Conserved membrane protein of unknown function	-1.59	2.16E-02
730083	-	Conserved hypothetical protein	-1.11	6.56E-11
730173	-	Conserved hypothetical protein	-1.36	5.87E-15
<b>750051</b>	-	<b>Putative type VI secretion protein VasV</b>	<b>1.75</b>	<b>9.41E-21</b>
<b>750052</b>	-	<b>Putative type VI secretion system associated protein of unknown function</b>	<b>1.68</b>	<b>6.27E-23</b>
<b>750053</b>	-	<b>Putative type VI secretion protein VgrG</b>	<b>2.41</b>	<b>3.37E-62</b>
<b>750054</b>	-	<b>Putative type VI secretion protein VasJ</b>	<b>5.97</b>	<b>5.62E-240</b>
<b>750055</b>	-	<b>Putative type VI secretion protein, hemolysin-coregulated protein (Hcp1)</b>	<b>6.25</b>	<b>9.85E-265</b>
<b>750056</b>	-	<b>Putative type VI secretion protein Hcp2/ VasQ/ImpB/VipA</b>	<b>5.84</b>	<b>4.07E-253</b>
<b>750057</b>	-	<b>Putative type VI secretion protein, tail sheath-like (VasRA/ImpC)</b>	<b>5.59</b>	<b>1.89E-234</b>

750058	-	Putative type VI secretion protein, tail sheath-like, (VasRB)	4.76	2.40E-193
750059	-	Putative type VI secretion protein VasS	4.54	5.92E-168
750060	-	Putative type VI secretion protein VasA/ImpG/TssF	4.53	6.50E-181
750061	-	Putative type VI secretion protein VasB/ImpH	4.18	2.19E-159
750062	clpB	Type VI secretion protein VasG (ClpV1)	3.95	4.18E-142
750063	-	Putative type VI secretion protein VtsA D-alanine-D-alanine ligase	3.71	2.61E-131
750064	-	Putative type VI secretion protein VtsB	3.18	2.24E-101
750065	-	Putative type VI secretion protein VtsC	2.96	3.09E-88
750066	-	Putative type VI secretion protein VtsD	2.84	3.97E-78
750067	-	Putative type VI secretion protein VasC/VtsE with forkhead domain (FHA)	2.71	1.89E-74
750068	-	Putative type VI secretion protein VasD-1 (VtsF)	2.65	5.23E-65
750069	-	Putative type VI secretion protein VasE/VtsG/ImpJ	2.62	1.26E-71
750070	-	Putative type VI secretion protein VasF/VtsH/DotU	2.57	5.69E-68
750071	-	Putative type VI secretion protein IcmF/VasK/VtsI	2.17	2.11E-51
750072	-	Putative type VI secretion protein VasX-1	2.24	7.53E-55
750073	-	Conserved protein of unknown function	2.19	2.11E-51
750074	-	Conserved protein of unknown function	1.12	1.56E-12
750086	-	Putative transcriptional regulator (FT1512)	9.19	0
920087	-	Probable anaerobic C4-dicarboxylate transporter dcuC	-1.3	1.04E-12
930011	dmsA	Dimethyl sulfoxide reductase, anaerobic, subunit A	-1.51	2.58E-17
930012	-	Anaerobic dimethyl sulfoxide reductase chain YnfI	-1.06	4.39E-06
930021	-	Nitrite reductase periplasmic cytochrome c552	-1.43	3.13E-17
960126	-	Putative DedA family membrane protein	1.05	3.28E-09
960127	-	Putative Glycosyltransferase involved in cell wall biogenesis	1.07	1.16E-09
1430001	-	Conserved exported protein of unknown function	-1.5	2.14E-05
1500001	-	Serine acetyltransferase (fragment)	1.55	2.03E-02
1560049	argA	Amino-acid acetyltransferase	-1.55	3.27E-21
1590028	raiA	Ribosome-associated inhibitor A	-1.3	5.17E-18

**Table S5** Strains used in the present study

Name	Description	Reference
PIR2	(F-) Δlac169 rpoS(am) robA1 creC510 hsdR514 endA recA1 uidA(ΔMlu I)::pir	Invitrogen, USA
β3914	β 2163 <i>gyrA462</i> , <i>zei298::Tn10</i> [Km <sup>R</sup> Em <sup>R</sup> Tc <sup>R</sup> ]	Le Roux <i>et al.</i> , 2007
GV1460	J2-8	Lemire <i>et al.</i> , 2015
GV1508	J2-9 ( <i>V. crassostreae</i> )	Lemire <i>et al.</i> , 2015
GV2799	J2-9/pGV1512_Cm <sup>R</sup>	Bruto <i>et al.</i> , 2017
GV1438	J2-9 pGV1512-cured	Bruto <i>et al.</i> , 2017
GV1512	J5-20 ( <i>V. crassostreae</i> )	Bruto <i>et al.</i> , 2017
GV1440	J5-20 pGV1512-cured	Bruto <i>et al.</i> , 2017
GV2943	J2-9 pGV_ΔPx2	This study
GV878	J2-9 pGV_ΔT6SS/pGV1512	Bruto <i>et al.</i> , 2017
GV2625	J2-9 pGV_ΔT6SS	This study
GV2490	J2-9 pGV_ΔT4SS	Bruto <i>et al.</i> , 2017
GV1606	J2-9 pGV_ΔPAAR	This study
GV1836	J2-9 pGV_ΔPx3	Bruto <i>et al.</i> , 2017
GV2474	J2-9 pGV_ΔPx3-1	This study
GV2476	J2-9 pGV_ΔPx3-2	This study
GV2478	J2-9 pGV_ΔPx3-3	This study
GV2505	J2-9 pGV_ΔTR1512	This study
GV2829	J2-9 pGV_ΔPx3 + pMRB- <i>P<sub>LAC</sub>TR1512</i>	This study
GV2757	J2-9 pGV1512-cured + pMRB- <i>P<sub>LAC</sub>TR1512</i>	This study
GV2833	J2-9 pGV_ΔPx3 + pMRB- <i>P<sub>LAC</sub>gfp</i>	This study
GV2544	GV2277+ pMRB_Sp <sup>R</sup>	This study
GV2545	GV2279+ pMRB_Sp <sup>R</sup>	This study
GV2546	GV2280+ pMRB_Sp <sup>R</sup>	This study
GV2547	GV2281+ pMRB_Sp <sup>R</sup>	This study
GV2520	GV2282+ pMRB_Sp <sup>R</sup>	This study
GV2521	GV2283+ pMRB_Sp <sup>R</sup>	This study
GV2522	GV2284+ pMRB_Sp <sup>R</sup>	This study
GV2523	GV2285+ pMRB_Sp <sup>R</sup>	This study
GV2524	GV2286+ pMRB_Sp <sup>R</sup>	This study
GV2522	GV2287+ pMRB_Sp <sup>R</sup>	This study
GV2523	GV2288+ pMRB_Sp <sup>R</sup>	This study
GV2524	GV2289+ pMRB_Sp <sup>R</sup>	This study
GV2525	GV2290+ pMRB_Sp <sup>R</sup>	This study
GV2526	GV2291+ pMRB_Sp <sup>R</sup>	This study
GV2527	GV2292+ pMRB_Sp <sup>R</sup>	This study
GV2528	GV2293+ pMRB_Sp <sup>R</sup>	This study
GV2529	GV2294+ pMRB_Sp <sup>R</sup>	This study
GV2530	GV2295+ pMRB_Sp <sup>R</sup>	This study

GV2871	GV2297+ pMRB_Sp <sup>R</sup>	This study
GV2867	GV2298+ pMRB_Sp <sup>R</sup>	This study
GV2529	GV2299+ pMRB_Sp <sup>R</sup>	This study
GV2530	GV2300+ pMRB_Sp <sup>R</sup>	This study
GV2872	GV2301+ pMRB_Sp <sup>R</sup>	This study
GV2531	GV2302+ pMRB_Sp <sup>R</sup>	This study
GV2532	GV2304+ pMRB_Sp <sup>R</sup>	This study
GV2533	GV2305+ pMRB_Sp <sup>R</sup>	This study
GV2534	GV2306+ pMRB_Sp <sup>R</sup>	This study
GV2535	GV2307+ pMRB_Sp <sup>R</sup>	This study
GV2536	GV2308+ pMRB_Sp <sup>R</sup>	This study
GV2537	GV2309+ pMRB_Sp <sup>R</sup>	This study
GV2538	GV2310+ pMRB_Sp <sup>R</sup>	This study
GV2539	GV2311+ pMRB_Sp <sup>R</sup>	This study
GV2553	GV2312+ pMRB_Sp <sup>R</sup>	This study
GV2540	GV2313+ pMRB_Sp <sup>R</sup>	This study
GV2541	GV2314+ pMRB_Sp <sup>R</sup>	This study
GV2542	GV2315+ pMRB_Sp <sup>R</sup>	This study
GV2543	GV2316+ pMRB_Sp <sup>R</sup>	This study
GV2554	GV2317+ pMRB_Sp <sup>R</sup>	This study
GV2555	GV2318+ pMRB_Sp <sup>R</sup>	This study
GV2556	GV2320+ pMRB_Sp <sup>R</sup>	This study
GV2558	GV2322+ pMRB_Sp <sup>R</sup>	This study
GV2559	GV2323+ pMRB_Sp <sup>R</sup>	This study
GV2560	GV2324+ pMRB_Sp <sup>R</sup>	This study
GV2561	GV2325+ pMRB_Sp <sup>R</sup>	This study
GV2562	GV2326+ pMRB_Sp <sup>R</sup>	This study
GV2563	GV2327+ pMRB_Sp <sup>R</sup>	This study
GV2564	GV2330+ pMRB_Sp <sup>R</sup>	This study
GV2565	GV2331+ pMRB_Sp <sup>R</sup>	This study
GV2884	GV2332+ pMRB_Sp <sup>R</sup>	This study
GV2885	GV2333+ pMRB_Sp <sup>R</sup>	This study
GV2568	GV2334+ pMRB_Sp <sup>R</sup>	This study
GV2886	GV2335+ pMRB_Sp <sup>R</sup>	This study
GV2336	GV2336+ pMRB_Sp <sup>R</sup>	This study
GV2569	GV2339+ pMRB_Sp <sup>R</sup>	This study
GV2570	GV2340+ pMRB_Sp <sup>R</sup>	This study
GV2571	GV2341+ pMRB_Sp <sup>R</sup>	This study
GV2572	GV2343+ pMRB_Sp <sup>R</sup>	This study
GV2573	GV2345+ pMRB_Sp <sup>R</sup>	This study
GV2574	GV2346+ pMRB_Sp <sup>R</sup>	This study
GV2575	GV2349+ pMRB_Sp <sup>R</sup>	This study
GV2576	GV2351+ pMRB_Sp <sup>R</sup>	This study

GV2577	GV2353+ pMRB_Sp <sup>R</sup>	This study
GV2578	GV2355+ pMRB_Sp <sup>R</sup>	This study
GV2579	GV2359+ pMRB_Sp <sup>R</sup>	This study
GV2580	GV2360+ pMRB_Sp <sup>R</sup>	This study
GV2581	GV2361+ pMRB_Sp <sup>R</sup>	This study
GV2582	GV2363+ pMRB_Sp <sup>R</sup>	This study
GV2583	GV2365+ pMRB_Sp <sup>R</sup>	This study
GV2584	GV2366+ pMRB_Sp <sup>R</sup>	This study
GV2585	GV2367+ pMRB_Sp <sup>R</sup>	This study
GV2586	GV2369+ pMRB_Sp <sup>R</sup>	This study
GV2587	GV2370+ pMRB_Sp <sup>R</sup>	This study
GV2588	GV2371+ pMRB_Sp <sup>R</sup>	This study
GV2589	GV2372+ pMRB_Sp <sup>R</sup>	This study
GV2590	GV2373+ pMRB_Sp <sup>R</sup>	This study
GV2591	GV2375+ pMRB_Sp <sup>R</sup>	This study
GV2592	GV2376+ pMRB_Sp <sup>R</sup>	This study
GV2593	GV2377+ pMRB_Sp <sup>R</sup>	This study
GV2594	GV2378+ pMRB_Sp <sup>R</sup>	This study
GV2595	GV2379+ pMRB_Sp <sup>R</sup>	This study
GV2596	GV2380+ pMRB_Sp <sup>R</sup>	This study
GV2597	GV2381+ pMRB_Sp <sup>R</sup>	This study
GV2598	GV2382+ pMRB_Sp <sup>R</sup>	This study
GV2599	GV2383+ pMRB_Sp <sup>R</sup>	This study
GV2600	GV2384+ pMRB_Sp <sup>R</sup>	This study
GV2601	GV2385+ pMRB_Sp <sup>R</sup>	This study
GV2602	GV2386+ pMRB_Sp <sup>R</sup>	This study
GV2603	GV2388+ pMRB_Sp <sup>R</sup>	This study
GV2604	GV2389+ pMRB_Sp <sup>R</sup>	This study
GV2605	GV2390+ pMRB_Sp <sup>R</sup>	This study
GV2606	GV2391+ pMRB_Sp <sup>R</sup>	This study
GV2694	GV2392+ pMRB_Sp <sup>R</sup>	This study
GV2695	GV2393+ pMRB_Sp <sup>R</sup>	This study
GV2696	GV2394+ pMRB_Sp <sup>R</sup>	This study
GV2610	GV2395+ pMRB_Sp <sup>R</sup>	This study
GV2644	GV2283+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2645	GV2287+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2646	GV2288+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2647	GV2299+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2878	GV2300+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2648	GV2307+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2649	GV2308+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2650	GV2309+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2651	GV2311+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study

GV2654	GV2312+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2938	GV2327+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2936	GV2330+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2847	GV2332+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2846	GV2334+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2849	GV2335+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2881	GV2336+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2675	GV2339+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2676	GV2343+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2677	GV2345+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2678	GV2346+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2679	GV2351+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2680	GV2359+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2681	GV2361+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2682	GV2365+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2683	GV2366+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2684	GV2370+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2661	GV2378+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2662	GV2379+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2663	GV2380+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2664	GV2381+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2665	GV2382+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2666	GV2383+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2667	GV2384+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2668	GV2388+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2669	GV2389+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2670	GV2390+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2423	GV2391+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2671	GV2392+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2877	GV2393+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2834	GV2394+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2835	GV2395+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV1576	J2-9 pGV1512-cured + pMRB_Sp <sup>R</sup>	This study
GV2685	J2-9 pGV1512-cured 2381+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2693	J5-20 pGV1512-cured + pMRB_Sp <sup>R</sup>	This study
GV2843	J5_20pGV1512-cured 2381+ pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2697	J2-8 + pMRB_Sp <sup>R</sup>	This study
GV2842	J2-8 + pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study
GV2140	GV1664 ( <i>V. crassostreae</i> from Sylt)	This study
GV2704	GV2140 + pMRB_Sp <sup>R</sup>	This study
GV2720	GV2140 + pMRB_Sp <sup>R</sup> , pGV_Cm <sup>R</sup>	This study

**Table S6** Plasmids used and constructed in this study

<b>Plasmid</b>	<b>Description</b>	<b>Reference</b>
pSW23T	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; Cm <sup>R</sup>	(Demarre et al., 2005)
pSW23T::pGV	pSW23T :: (2700-3400) <sub>pGV1512</sub> Cm <sup>R</sup>	(Bruto et al., 2017)
pSW7848T	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; <i>araC-P<sub>BAD</sub>ccdB</i> ; Cm <sup>R</sup>	(Val et al., 2012)
pSW7848T::pGV	pSW7848T :: (2700-3400) <sub>pGV1512</sub> Cm <sup>R</sup>	(Bruto et al., 2017)
pSW $\delta$ T6SS	pSW7848T :: $\Delta$ T6SS	This study
pSW $\delta$ Px2	pSW7848T :: $\Delta$ Px2	This study
pSW $\delta$ PAAR	pSW7848T :: $\Delta$ PAAR	This study
pSW $\delta$ Px3-1	pSW7848T :: $\Delta$ Px3-1	This study
pSW $\delta$ Px3-2	pSW7848T :: $\Delta$ Px3-2	This study
pSW $\delta$ Px3-3	pSW7848T :: $\Delta$ Px3-3	This study
pSW $\delta$ TR1512	pSW7848T :: $\Delta$ TR1512	This study
pMRB-P <sub>LAC</sub> <i>gfp</i>	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; $\square$ <i>oriV<sub>pB1067</sub></i> ; P <sub>LAC</sub> - <i>gfp</i> Cm <sup>R</sup>	(Le Roux et al., 2011)
pMRB-P <sub>LAC</sub> <i>TR1512</i>	<i>oriV<sub>R6K</sub></i> ; <i>oriT<sub>RP4</sub></i> ; $\square$ <i>oriV<sub>pB1067</sub></i> ; P <sub>LAC</sub> - <i>TR1512</i> Cm <sup>R</sup>	This study

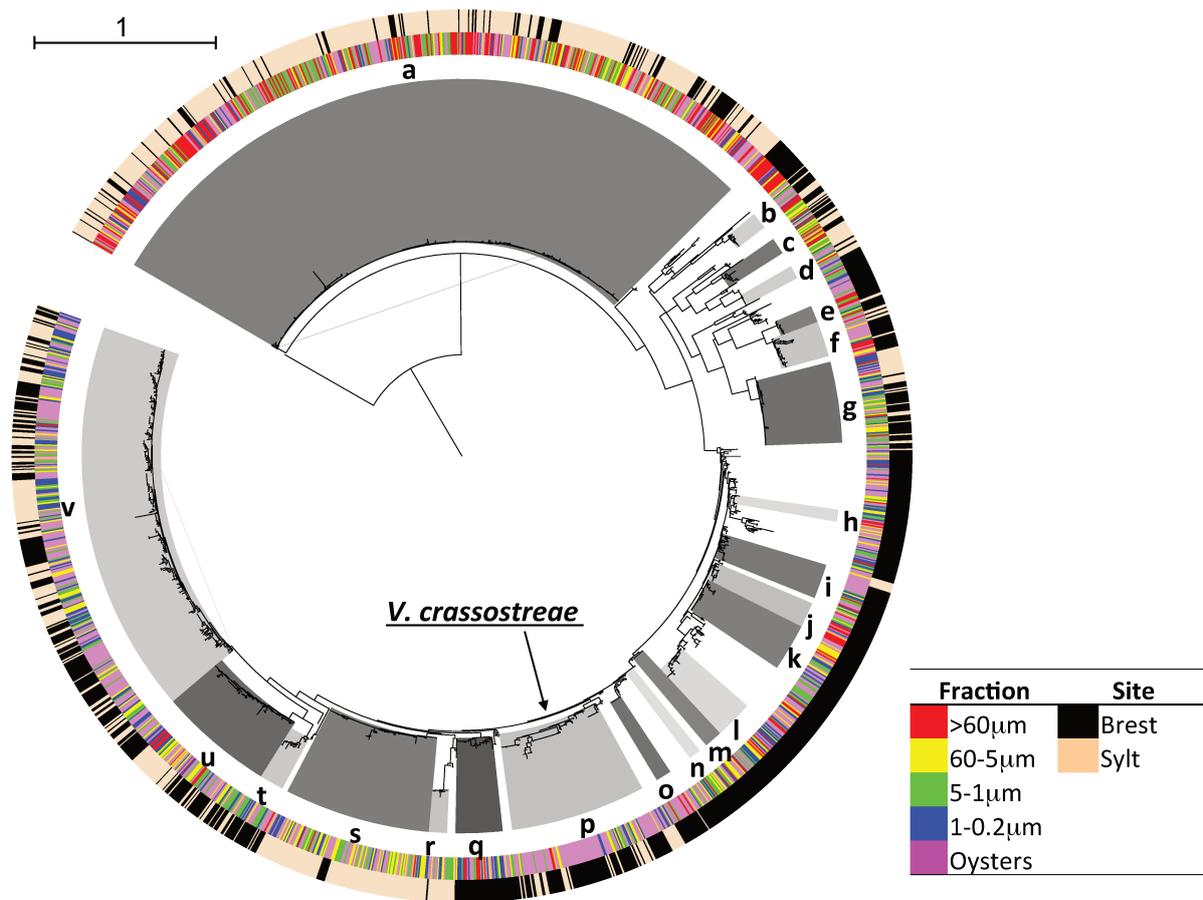
Table S7 Primers used in this study

Primer	Sequence 5'-3'
hsp60F	GAATTCGAIHHGCI GGIGAYGGIACIACIAC
hsp60R	CGCGGGATCCYKIYKITCICCRAAICCGGIGCYTT
gyrBF	GAAGTCATCATGACCGTTCTGCAYGCNNGGNGNAARTTYRA
gyrBR	AGCAGGGTACGGATGTGCGAGCCRTCACRTCNCRCTCNGYCAT
rcfBF	CAGGAAACAGCTATGACCATHGARTTYACNGAYTTYCARYTNCAIY
rcfBR	GATAAACGACGGCCAYTTNCTYTGHATNNGGYTCRAAYTCNCCRT
rpoDF	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT
rpoDR	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT
repF	CCTCTCTCGACTACACGAAG
repR	CATGCAACTTCATTCCAGGC
SW-F	GAATTCCTGCAGCCCCGGGGG
SW-R	GAATTCGATATCAAGCTTATCGATAC
$\Delta$ T6SS-1	GTATCGATAAGCTTGATATCGAATTCGGTGGCAATCTGGAGGTATG
$\Delta$ T6SS-2	CGGATATTCAAGGCACCCTCTGCTCAACCGAGGCATTGTC
$\Delta$ T6SS-3	GACAATGCCTCGGTTGAGCAGAGGGTGCCTTGAATATCCG
$\Delta$ T6SS-4	CCCCCGGGCTGCAGGAATTCGAGATCTGCTGAATGTGCTG
$\Delta$ Px2-1	GTATCGATAAGCTTGATATCGAATTCCTGTCCAGTGTTGAATAGG
$\Delta$ Px2-2	GGCTGATGAAACCTCACATCCGTTCACTCTAAGAAACCGC
$\Delta$ Px2-3	GCGGTTTCTTAGAGTGAACGGATGTGAGGTTTCATCAGCC
$\Delta$ Px2-4	CCCCCGGGCTGCAGGAATTCGGCAGAACTTGAAGACAGAC
$\Delta$ Px3.1-1	GTATCGATAAGCTTGATATCGAATTCCTAATACCTGCGGTGATTCTG
$\Delta$ Px3.1-2	CATTCACACCGACCGTATCTTCACCCGACATCTTCAGATG
$\Delta$ Px3.1-3	CATCTGAAGATGTGCGGGTGAAGATACGGTCGGTGTGAATG
$\Delta$ Px3.1-4	CCCCCGGGCTGCAGGAATTCGAGCTCACCTAAATGACAGC
$\Delta$ Px3.2-1	GTATCGATAAGCTTGATATCGAATTCCTTAAAGTGGCGGGAACAAGC
$\Delta$ Px3.2-2	GGATAACCACCTTTACCACCGCATGTGTAATGCTGGCATC
$\Delta$ Px3.2-3	GATGCCAGCATTACACATGCGGTGGTAAAGGTGGTTATCC
$\Delta$ Px3.2-4	CCCCCGGGCTGCAGGAATTCAGACAATCCTCTAATGCGGC
$\Delta$ Px3.3-1	GTATCGATAAGCTTGATATCGAATTCGGTGGTAAAGGTGGTTATCC
$\Delta$ Px3.3-2	CAAGTACATGGTCAGGATGGAGACAATCCTCTAATGCGGC
$\Delta$ Px3.3-3	GCCGCATTAGAGGATTGTCTCCATCCTGACCATGTAATTG
$\Delta$ Px3.3-4	CCCCCGGGCTGCAGGAATTCAGGCACGAGTTCATCAACAG

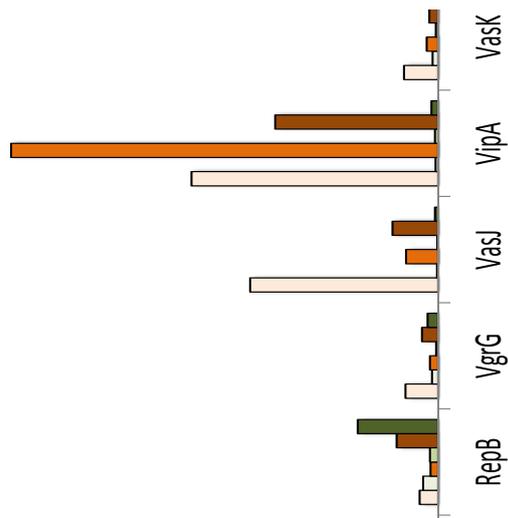
ΔTR1512-1	GTATCGATAAGCTTGATATCGAATTCGGAGGAGTTCATGACCTACA
ΔTR1512-2	GCAGTCTCTCAATAACACCGCGCTTCTCTTTTGTGTCCCA
ΔTR1512-3	TGGGACACAAAAGAGAAGCGCGGTGTTATTGAGAGACTGC
ΔTR1512-4	CCCCCGGGCTGCAGGAATTCGCCATTTCCCTATCCAGCGTA
ΔPAAR-1	GCCCGAATTCCATACCTCCAGATTGCCACC
ΔPAAR -2	CCTGTAGGTCTACAACCGTTCACTTTCTACTAACCCCACC
ΔPAAR -3	GGTGGGGTTAGTAGAAAGTGAACGGTTGTAGACCTACAGG
ΔPAAR-4	GCCCGAATTCTACACATGCCGTTGAGCAAG
pMRB_TR1512F	GCCCGAATTCCTCCATGGTTCCTTTGTTATC
pMRB_TR1512R	GCCCCTCGAGCTAAACTGATGATGAAGCCCC
TR1512F	GCCCGAATTCATGCTCGACAAAAGAAAACC
TR1512R	GCCCCTCGAGCTAAACCTTACAAACCTGTG
extT6SSF	CACTGCCTTTTACCACGATG
extT6SSR	CGCATCAAACCCTTTTCACC
intT6SSF	CCTGGTCAGTAATGTGCAAC
intT6SSR	GCCTAAACGCACCTTTTGTCG

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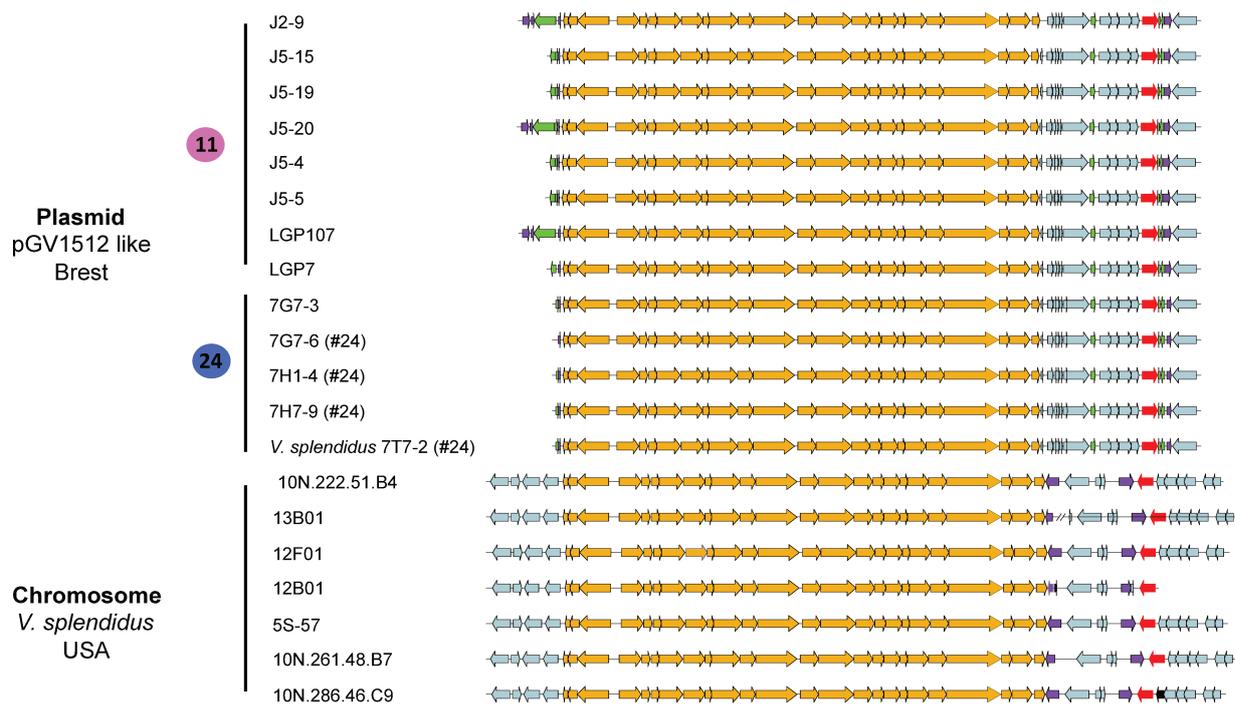
## Figures



**Figure S1** Population structure prediction of *Vibrio* isolates (n=1629) recovered from seawater fractions or oyster tissues from two geographic areas, Brest (France) and Sylt (Germany). Phylogenetic tree (Maximum Likelihood) based on partials *hsp60* sequences. The grey areas correspond to different clades identified by a letter (from a to v) and taxonomically assigned to known *Vibrio* species, i.e. *V. breoganii* (a), *V. pacinii* (b), *V. fischeri* (c), *V. alginolyticus* (e, f), *V. jasicida* (g), *V. chagasii* (l), *V. crassostreae* (p), *V. kanaloae* (t), *V. cyclitrophicus* (u) and *V. splendidus* (v) or *Vibrio sp. nov.* (d, h, I, j, k, m, n, o, q, r, s). The inner and outer ring indicates respectively the origin of the strain and the site of sampling, following the color code given on the right panel.



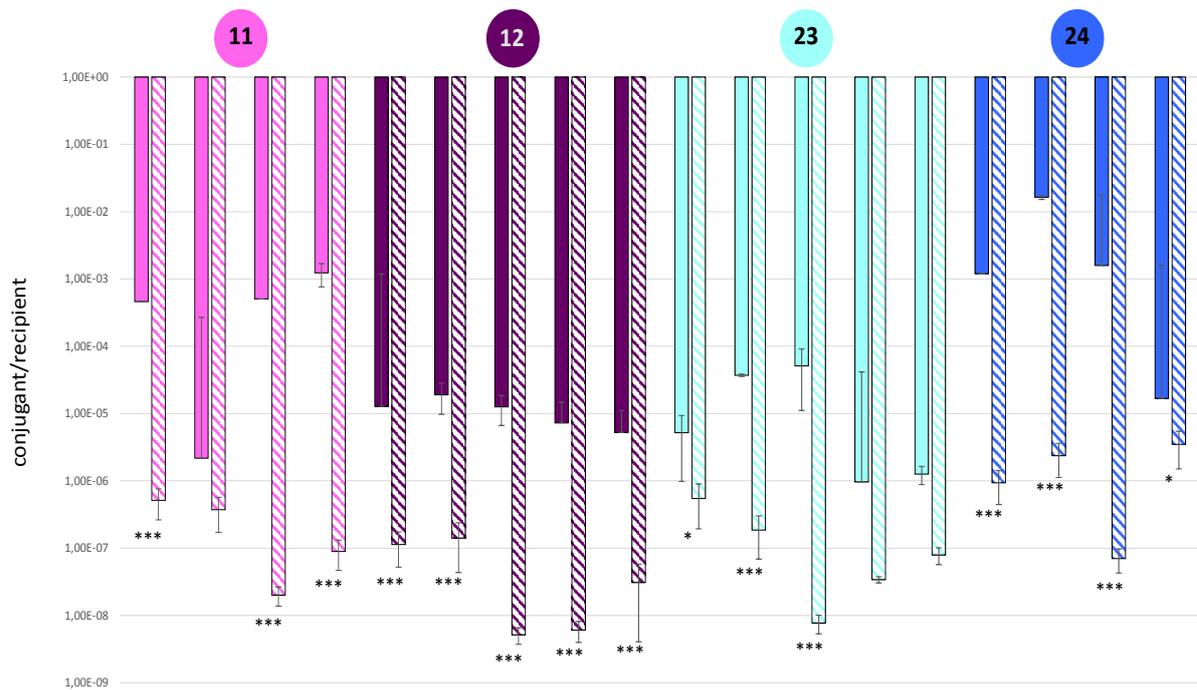
**Figure S2** Differential expression of target genes in cultures of J2-9  $\Delta Px3 + pMRB-P_{LAC}Px3-2.5$  (orange bars) or  $\Delta Px3 + pMRB-P_{LAC}gfp$  (green bars) at DO 0,3/0,6/1 (light to dark bars). Y axis is the ratio of the target genes expression on *gyrA* expression used to normalize the qPCR assays.



**Figure S3** Synteny of the T6SS cluster with surrounding regions. Each arrow represents a CDS that was colored with the following scheme: orange for T6SS; red for TR1512; green for IS/transposase; purple for IS of the IS5 family.



**Figure S4** Conservation of the motif identified in the promoter region of *vgrG* and *vasJ* of *V. crassostreae* and *V. splendidus* strains (Figure S3). The figure was created using WebLogo (<https://weblogo.berkeley.edu/logo.cgi>). The conservation at the position is indicated by the size of the nucleotide (taller is more conserved). The palindromic sequences have been shaded in grey.



**Figure S5** Conjugation frequency after *in vitro* mating or injection to oysters. The frequency corresponds to the ratio between conjugants (Cm and Sp<sup>R</sup>) and recipient (Sp<sup>R</sup>). Conjugation was performed by *in vitro* mating (hatched bars) and by injection to oysters (i.e *in vivo* mating, filled bars) for different strains from populations #11, #12, #23, #24, following the same color code as in Figure 1. *In vitro* and *in vivo* conjugation assays have been performed in triplicates, standard deviation is indicated for each experiment. Asterisks indicate significant difference in pGV1512 transfer frequency between *in vitro* and *in vivo* mating for a same strain.

# Discussion

*Vibrio*-associated disease incidence and prevalence have increased worldwide in the context of anthropogenic perturbations. A major issue is the lack of basic knowledge about population structure and virulence mechanisms of environmental vibrios. My PhD project aimed to explore the ecology, evolution and virulence of environmental *Vibrio* populations, using the oyster *Crassostrea gigas* as a model of infection.

## I Oyster juvenile disease is polymicrobial

In this PhD work, we used *C. gigas* as a model of infection, but it is also naturally affected by recurrent infectious diseases. Diseased juvenile oysters are infected by both the ostreid Herpes virus OsHV-1 and bacteria, including *Vibrio*. We showed that the virus was a good marker of mortalities (Petton et al., 2015). In addition, several studies demonstrated the role of OsHV-1 in oyster mortality induction. Injection of *C. gigas* with viral mimic nucleic acids (such as polyI:C) resulted in an enhanced resistance to further OsHV-1 infection. This was evidenced by a reduced viral replication and an improved survival of infected oysters (Lafont et al., 2017; Green and Speck, 2018). Another study aimed at deciphering the role of the different infectious agents in this polymicrobial disease and showed that OsHV-1 was involved in hemocytes killing, thus damaging host immune response (de Lorgeril et al., under revision).

The monitoring of the dynamic of colonization of juvenile oysters by total cultivable bacteria, vibrio and herpes virus leading to oyster disease showed that vibrio colonization preceded viral replication and oyster mortality induction. An antibiotic treatment revealed that a high viral load was not sufficient to induce high mortality rate in oysters, thus highlighting the importance of bacteria in oyster disease. However, the antibiotic treatment is not only targeting virulent vibrios but also non-virulent vibrios and commensals of the host (Petton et al., 2015). Further studies highlighted that some *Vibrio* genotypes are able to kill oyster in experimental infection and specific virulent genotypes colonization is congruent with disease induction (Lemire et al., 2015; Petton et al., 2013, 2015; Bruto et al., 2017). In addition, we showed that both juvenile and adult oysters are reservoirs of pathogens and were able to transmit the disease to SPF oysters. Finally, adult oysters were able to induce disease to juvenile

oysters without detection of OsHV-1 (Petton et al., 2015). Overall, these elements confirm the importance of vibrios in oyster mortality.

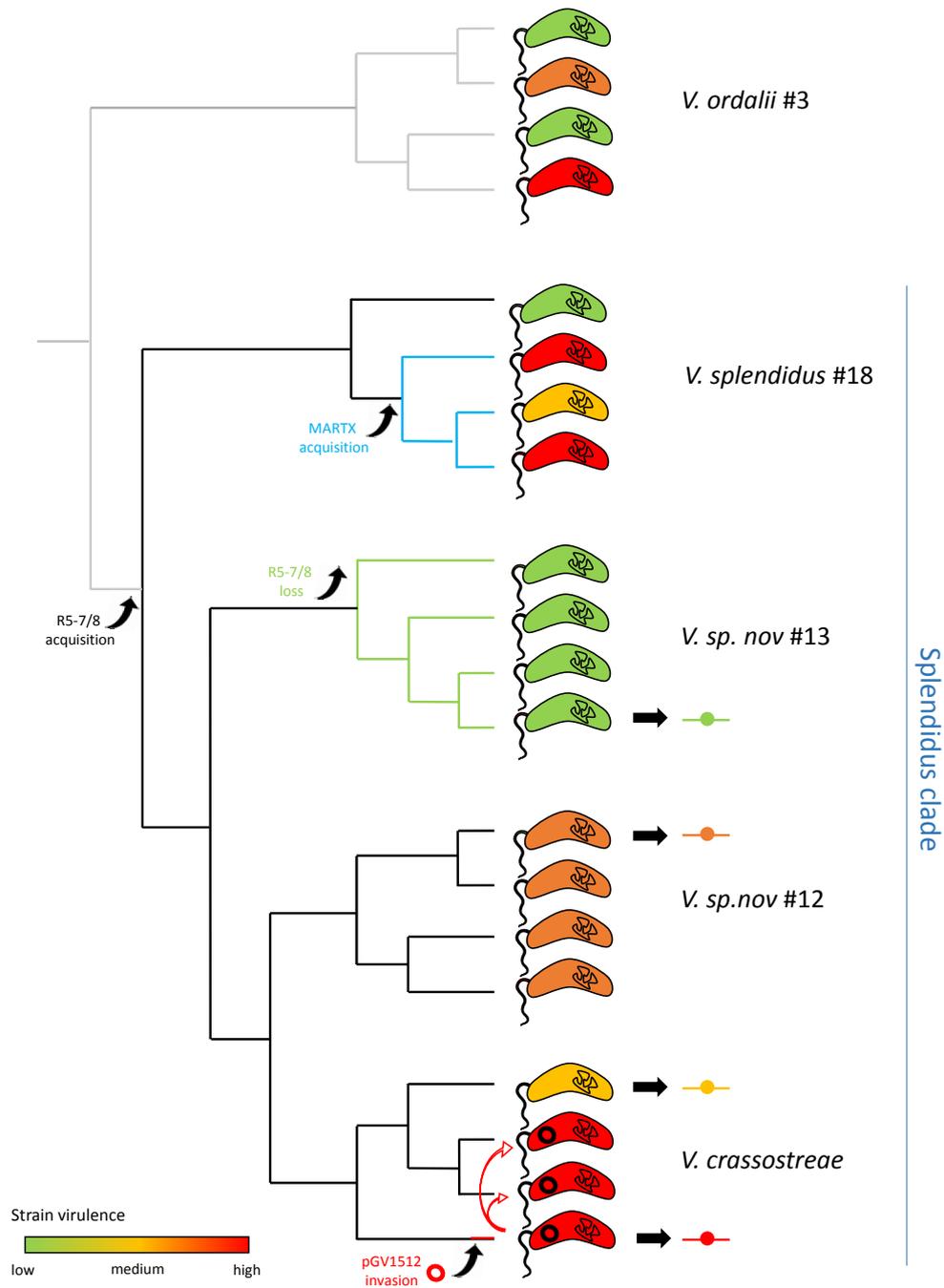
Demonstrating the role of specific *Vibrio* genotypes or populations in oyster disease will require their elimination during infection, such as the one performed on OsHV-1 with polyI:C (Lafont et al., 2017; Green and Speck, 2018). To this aim, the GV laboratory is currently isolating phages infecting *V. crassostreae* and *V. chagasii* in order to deplete specific populations of vibrios in oyster and explore whether it decreases mortalities. Those preliminary experiments could further constitute a basis to build diagnosis/management strategies of oyster disease.

## II Population as a unit of pathogenesis

Contrary to the classic view of clonal expansion of pathogen following recent acquisition of virulence factors by HGT, we identified virulence determinants that are shared by whole *Vibrio* populations, rising the unit of pathogenesis to the population level. This impacts prophylactic and therapeutic approaches and drives important insights into eco-evolutionary mechanisms of virulence acquisition.

One of the consequences of demonstrating that the unit of pathogenesis can be the population is that diagnostic strategies of pathogens in the environment or prior to animal transfer can be based on multi-locus-sequence-typing (MLST) analyses. Then, targeting the R5-7/8 cluster, specific to virulent populations from the Splendidus clade, could be used to indicate the overall risk in a given environment. We also showed that additional genes, that are population-specific, are necessary for virulence: the MARTX toxin and the plasmid pGV1512. A schematic representation of population-specific virulence gene acquisition/loss identified during my PhD project is proposed in figure 1. *V. crassostreae* is repeatedly associated with diseased oysters and its virulence depends on the plasmid pGV1512 (Faury et al., 2004; Lemire et al., 2015; Bruto et al., 2017). Thus, detection of pGV1512 constitutes a reliable marker to assess high risk of oyster disease induction. In the future, detection methods should target population-specific genes to improve their resolution and efficiency. The diagnosis of pathogenic vibrios could thus be achieved by targeting a few markers. Disease management strategies should then develop “ecofriendly” methods (that do not rely on the use of chemicals as antibiotics) such as phage therapy that offers interesting perspectives for prophylaxis in

hatchery. However, efficiency of phage-therapy should rely on large spectrum bacteriophages and phage-cocktail to be efficient against a diversity of virulent genotypes.



**Figure 1 | Schematic representation of gene acquisition and loss in the Splendidus clade and *V. ordalii*.** Strain virulence is indicated by a color gradient, from green (non-virulent strain) to red (highly virulent strain).

In addition, the discovery that population can be the unit of *Vibrio* pathogenesis in oyster opens important perspectives to understand the evolution of these pathogens. The observed population structure yield insight into the evolutionary history of pathogens and types of selection acting on pathogenicity determinant genes. For example, the R5-7/8 gene cluster was ancestrally acquired and plays a role in virulence of different populations belonging to the Splendidus clade. Its high frequency suggests that this cluster plays an essential role in adaptation of those populations in their niche, but its role remains to be determined. On the other hand, the R5-7/8 cluster has been lost by some populations from the Splendidus clade, which indicates that this gene is not important for their survival in the niche.

Within the *V. splendidus* #18 population, the MARTX toxin is detected at medium frequency and shows high diversity in effector composition. Most of those MARTX toxins share at least one ‘Makes Caterpillars Fluffy’ (MCF) domain with the MARTX type III from *V. vulnificus*. The latter is involved in protection against predation by amoebae as it induces lysis of grazers (Lee et al., 2008). To test the role of *V. splendidus* MARTX in resistance to grazing, I co-cultured the grazers *Paraphysomonas sp.* with a *V. splendidus* strain or its  $\Delta$ MARTX derivative. I did not show significant differential growth between the tested strains and therefore I was unable to demonstrate a role of *V. splendidus* MARTX in predation evasion using this model of grazing. On the contrary to phages, to date, grazers are considered to be generalist predators of bacteria, although the fitness of both predator and prey differ between species. An interesting perspective would be to compare different grazers and use a pool of several bacterial culture instead of pure culture of *Vibrio* to explore the role of the MARTX.

*Legionella pneumophila* (Day et al., 2014) and *V. cholerae* (Van der Henst et al., 2015) are able to survive in host phagocytes. This ability is linked to a pre-adaptation that allow them to resist to predation by grazers in their environment. This exemplifies the paradigm of the “coincidental selection” concept. Coincidental selection has been proposed to explain the emergence of many opportunistic pathogens for which virulence factors are selected in a non-parasitic context (Brown et al., 2012). In such a case, virulence factors are selected because they confer a selective advantage for adaptation/survival in a niche that is not the host (Levin and Svanborg Edén, 1990). The virulence factors evidenced in this work, the MARTX, and the R5.7/8 proteins, that are related to the ecological differentiation of *Vibrio* populations, should be explored in the future in regard to the coincidental selection concept, i.e. understand their role in their niche niche in the wild that is not the oyster.

To do so, a time-series sampling followed by sequencing of 18S, 16S and *vibrio* specific gene markers has been performed to explore the dynamic of diverse eukaryotes, total bacteria and vibrios in the environment (ANR Revenge project). This may lead to evidence co-occurrence network associated with pathogenic populations of vibrios and give clues on natural niche of these populations such as unicellular eukaryotes, phytoplankton or zooplankton. In addition, isolation of grazers from the same environment could lead to the development of a new system of interactions in order to test the effect of specific genes on resistance or killing of grazers. Finally, a collaboration with the Melanie Blokesch lab has been initiated to use the lab models *Acanthamoeba* and *Dictyostelium* to depict the molecular mechanisms involved in virulence of environmental vibrios.

### III A consortium as a unit of pathogenesis

Exploration of the diversity of *Vibrio* populations in oysters showed that diseased oysters were systematically colonized by several populations (Bruto et al., 2017). We demonstrated that distinct populations of vibrios have common and specific virulence mechanisms (Bruto et al., 2018), highlighting that several virulence factors are necessary for a strain to kill oysters. These results lead to the hypothesis that several strains or populations cooperate to kill and adopt a “sharing the weapons” strategy. This hypothesis has already been supported in oysters and corals where co-injection of several *Vibrio* strains or species enhances virulence (Gay et al., 2004; Lemire et al., 2015; Rubio-Portillo et al., 2014).

This hypothesis could be explored in the context of oyster disease induction by following the dynamic of infection of vibrios in the wild or in mesocosm. At the individual level, identification of systematic co-colonization or recurrent successive dynamics of the different populations could be highlighted. This experiment requires the use of a metabarcoding approach to discriminate the different *Vibrio* populations. I performed this experiment using the *hsp60* marker used in the Polz laboratory, where I carried out metabarcoding sequencing data analyses. Unfortunately, this technique does not produce conclusive results, mainly due to oyster DNA that is proportionally too important in the samples and involve dilution steps of several log for some samples. The development of a metabarcoding method to discriminate the *Vibrio* populations in oyster samples will be a tool of major interest to study colonization processes in the wild. An alternative method to test the “sharing the weapons” strategy would

be to identify other population-specific virulence genes, notably for populations associated with diseased oysters. Then, in mesocosm, the effect of the reconstitution of various virulent communities on oyster mortality induction could be tested.

#### IV Emergence of virulent genotypes in oyster farms

In our sampling site located in high-density oyster-farming area in Brest, the virulence plasmid pGV1512 was only found in two populations, *V. crassostreae* #11 and *V. splendidus* #24. These two populations are only detected during mortality events, in diseased oysters, and are barely found in surrounding seawater. At a broader spatial scale, pGV1512 was not detected in *V. crassostreae* strains isolated from Northern Europe, where there are no high-density farming and only sporadic mortality events recorded (Waterman et al., 2008). Hence, pGV1512 distribution seems geographically restricted to high-density oyster farms and we suggest that aquaculture settings select higher virulent genotypes. In this sense, oyster may constitute a favorable niche for gene transfer and/or selection. Indeed, we showed that pGV1512 transfer in vibrios was enhanced in oyster compared to *in vitro* condition. However, to qualify oysters as a favorable niche for HGT, transfer of other plasmids need to be demonstrated. The fact that pGV1512 transfer is more frequent in the two populations in which the plasmid is naturally detected can indicate that it is more frequently transferred in those populations or that it is due to a selective advantage mediated by the plasmid in those populations. The selection of pGV1512 might be highlighted by comparing the *in vivo* transfer frequency of pGV1512 WT vs. pGV1512 $\Delta$ X ( $\Delta$ T6SS,  $\Delta$ Px3...). If no difference of frequency is observed between the different plasmids, we could hypothesize that it is not selected. For those conjugation assays, I'm currently using a *V. crassostreae* donor strain. It could be interesting to test other donors to highlight a donor-effect that could promote plasmid transfers.

The facts that the production of oyster spat in hatcheries still requires an antibiotic treatment of genitors (E. Marissal, pers. com) and that MGE transfer might be enhanced in oyster constitute concerning conditions that could result in an increase in the risk of development of multi-resistant bacterial pathogens. First, MGE can simply carry resistance genes that will spread in the niche but these conditions are also favorable to the expression of the SOS response by the bacteria. This mechanism is induced when a high level of single-

stranded DNA deriving from DNA damage or HGT is present in the cell. The SOS response controls genes involved in DNA damage repair and increases mutations and genetic exchanges (Matic et al., 1995; Baharoglu et al., 2010). Mutations acquired during DNA repair can lead to resistance to new substances (Michel, 2005). For example, mutations induced by the SOS response of *Escherichia coli* is responsible for its resistance to ciprofloxacin (Cirz et al., 2005). Hence, multi-resistant strains and further transfer of resistance determinants to human pathogens constitute a threat for human health (Defoirdt et al., 2011).

Virulence of pGV1512 is dependent on a transcriptional regulator that activates a T6SS. T6SSs are molecular machines involved in contact-dependent neighboring cell killing by injection of effector proteins to prokaryotic and eukaryotic preys (Salomon and Orth, 2015). T6SS effectors can be cytotoxic or induce cell membrane disruption in eukaryotes such as amoeba and macrophages (Pukatzki et al., 2006; Pukatzki et al., 2007; Miyata et al., 2011). Cytotoxicity is attributed to the actin cross-linking domain of the VgrG1 effector, but this domain is absent from the T6SS of pGV1512. Another effector, VasX, has been shown to induce lysis of both eukaryotic and prokaryotic cells (Joshi et al., 2017). However, in *V. crassostreae*, pGV1512, and hence its T6SS, does not seem to play a role in hemocyte lysis (T. Rubio, pers. com). Further co-culture experiments with eukaryotic cells, notably grazers and WT or  $\Delta$ T6SS strains could highlight T6SS-mediated selective advantages in some particular conditions.

T6SSs are also involved in inter- and intra-specific antibacterial activity and confer to bacteria a competitive advantage (Unterweger et al., 2012). However, *in vitro* killing assay on more than 40 strains did not reveal any sign of T6SS-mediated competitive advantage. This result could indicate that the preys chosen for this experiment are not the target cells of this machinery. Isolation of commensals from healthy oyster and further killing assays are currently performed in the GV lab. On the other hand, several studies indicate that *in vivo* conditions might be necessary to highlight an effect of the T6SS. A recent study on *V. cholerae* notably showed that the severity of infection by the pathogen was dependent on the effect of the T6SS on commensal bacteria in the gut of *Drosophila melanogaster*. The T6SS of *V. cholerae* needs to be active against commensal bacteria to induce a T6SS-dependant killing of the host. Removal of commensal bacteria or deletion of the T6SS attenuated host-killing by *V. cholerae* (Fast et al., 2018). In parallel, chitin fosters expression of the T6SS in *V. cholerae* (Borgeaud et al., 2015). Melanie Blokesch proposed that attachment to chitin might confer a “ready to

fight” state to vibrios. Thus, following passive colonization of marine animals, through filter-feeding mode in oyster for example, chitinous-particle attached vibrios could directly be highly competitive against cells they encounter thanks to their T6SS (Blokesch, 2015). To explore the role of this secretion system in antibacterial activity *in vivo*, we could compare the bacterial diversity, including microbiota and colonizers, in a recipient oyster batch before and after a cohabitation period with *V. crassostreae* with or without the T6SS. If the T6SS confers a competitive advantage in oysters, preys should be identified.

pGV1512 shows a narrow distribution, indicating that it constitutes a selective advantage only for a few populations in which it is detected at high frequency. Determining the mode of action of the T6SS might give clues that could explain the plasmid specific distribution. Indeed, the T6SS could be involved in killing a population/species whose presence is necessary for survival of other *Vibrio* populations. Another hypothesis is that an element from the core genome of the populations possessing pGV1512 might play a critical role in virulence regulation. Although pGV1512 is able to enhance virulence of numerous strains in injection, a natural process of infection could once again reveal more contrasting effects.

## V Advantages and limitations of the oyster *Crassostrea gigas* as a model of infection

Disease dynamics in the wild are influenced by a number of ecological and evolutionary factors not addressed by traditional laboratory-based characterization of pathogens. The Pacific oyster *Crassostrea gigas* has been proposed as a model to study the interaction between the environment, the pathogens, and the host in disease dynamics (Le Roux et al., 2016). First, whole genome sequence of the Pacific oyster is available (Zhang et al., 2012). Second, oyster antimicrobial effectors are well characterized (Bachère et al., 2004; Schmitt et al., 2010) and these genes can be manipulated as few genes have already been successfully silenced by RNA interference in *C. gigas* (Fabioux et al., 2009). Disease resistance is inheritable (Degremont et al., 2015). Hence, genetic families of oysters (e.g. resistant or sensitive to a disease) can be produced in hatchery by genetic improvement that selects animals possessing the “best genes” to face or to be sensitive to a disease. Third, the recent development of specific-pathogen-free (SPF) oysters has enabled the assessment of the infection process in the field (Petton et al., 2013; 2015a,b). Dynamics of mortalities and colonization by various infectious agents can thus

be recorded. As reductionist approaches are necessary to explore the molecular mechanisms of virulence, SPF oysters can also be used for experimental infections in the laboratory with the possibility to compare hundreds of strains in parallel. Hence, although the exploration of infection by human requires heterologous systems such as mice or infant rabbits (Bishop et al., 2014; Abel and Waldor, 2015), SPF oysters constitute one of the rare models that allows: i) the isolation of infectious agents from the environment; ii) the exploration of the infection by these agents in mesocosms; iii) high throughput experimental infections.

SPF oysters are not axenic and harbor their natural commensal flora, which can be considered as an asset of the model as it offers an opportunity to study the holobiont response to infection. An holobiont represents a complex ecosystem in which the host, the microbiota and the interactions among them are considered (Pita et al., 2018). Indeed, microbiota may be of prime interest in infection process as it may contribute or prevent infection success. In corals, some native commensal bacteria are able to inhibit infection by pathogenic vibrios by competing for space and nutrients. At higher temperature, the microbiota is not able to compete anymore and infection occurs (Frydenborg et al., 2014). It has also been suggested that, by altering the structure of its resident microbiota, corals can adapt to changing environmental conditions more rapidly than with a process depending on evolution/selection. This coral probiotic hypothesis has been proposed to explain how the coral *Oculina patagonica* became resistant to *Vibrio shiloi*, that was responsible for its necrotic bleaching in the past (Rosenberg et al., 2007). In oysters, it has been shown that the microbiota assemblage is vulnerable to environmental disturbance (Wegner et al., 2013) but oysters with stable microbial communities appear more resistant to invasion by pathogens (Lokmer and Wegner, 2015). Overall, a holistic approach taking in consideration the whole community associated with the host (Rosenberg et al., 2007), its stability and the causes of disturbance, is necessary to fully understand a disease in particular when it is polymicrobial. On the other hand, the establishment of genetic lineages of oysters as well as axenic animals would constitute a suitable system to further disentangle the role of diverse pathogens in the progress of the disease.

One limit encountered in this project is that intra-muscular injection of bacterial cultures does not mimic the natural route of infection of vibrios. Infection by bathing, that consists in directly pouring bacterial culture into an aquarium containing oysters, does not lead to neither significant nor replicable mortality rates, although vibrios such as *V. crassostreae* are efficient in colonizing oysters (Bruto et al., 2017). This indicates that the infection process is more

complex. A recent study showed that the adult oyster pathogen *V. aestuarianus* shed from diseased oysters was more virulent (Parizadeh et al., 2018). In this “ecologically realistic” set-up, a donor batch of oysters was injected with *V. aestuarianus*. After 48h, the injected oysters were removed from the aquarium and another SPF oyster batch, the recipient one, was placed in the same aquarium containing contaminated seawater. Those conditions were necessary to induce deaths, suggesting that specific genes are induced *in vivo* and favor further infection, as it has previously been shown for *V. cholerae* (Schild et al., 2007). In our laboratory, a similar set-up with *V. crassostreae* as pathogen still did not induce juvenile oyster deaths. Considering that the presence of non-virulent strains in the recipient batch might promote disease onset (Lemire et al., 2015), we performed a cohabitation experiment between a donor batch injected with *V. crassostreae* and a recipient batch injected with a non-virulent strain and we finally recorded deaths among the recipient oysters. In spite of these promising results, this experiment showed a strong variability in oyster susceptibility to *Vibrio* infection. To reduce variability and to homogenize the disease induction, we could perform this experiment on chosen genetic families of SPF oysters for which sensitivity is high. In addition, preliminary experiments performed in the Destoumieux-Garzon laboratory show that the addition of OsHV-1 to an experimental *Vibrio* infection assay induces oyster mortalities (Destoumieux-Garzon pers. com), hence constituting a new method to induce mortalities without injection needed. Altogether, *Crassostrea gigas* as a model of infection constitutes an advance in the exploration of disease ecology and illustrates how molecular biology (reductionist approach) and systems biology (holistic approach) are actually interdependent and complementary ways to study and make sense of complex phenomena (Fang and Casadevall, 2011).

## General conclusion

In the marine environment, several *Vibrio* populations present a higher risk of virulence for oysters and seem to be involved in oyster diseases. In addition, oysters can act as a niche for horizontal gene transfer and selection of *Vibrio* with higher pathogenicity potential. Indeed, we observed that oyster-farming has selected *V. crassostreae* carrying the pGV1512 plasmid. Increased virulence, colonization potential and probably fitness advantage conferred to *V. crassostreae* by this plasmid relies at least in part on a T6SS. This work provides several interesting perspectives for the future such as: i) exploring the T6SS molecular and cellular mechanisms of action, ii) deciphering the role of oysters as a niche for HGT and consequences for the spread of virulence or antibiotic resistance genes, iii) investigating the coincidental selection and the “sharing the weapons” hypotheses based on the identification of population-specific genes. This project also provides important features for oyster disease management, such as population specific markers that can be used for epidemiological surveys or “eco-friendly” methods to control vibriosis in hatchery. In the context of global changes and demographic explosion, this project and future works will provide scientific knowledge to develop sustainable and safety production of food by aquaculture.

## References

-A-

Abel, S., and Waldor, M.K. (2015). Infant Rabbit Model for Diarrheal Diseases. *Curr. Protoc. Microbiol.* *38*, 6A.6.1-15

Aizawa, S.I., Harwood, C.S., and Kadner, R.J. (2000). Signaling components in bacterial locomotion and sensory reception. *J. Bacteriol.* *182*, 1459–1471.

Agarwal, S., Zhu, Y., Gius, D.R., and Satchell, K.J.F. (2015). The Makes Caterpillars Floppy (MCF)-Like Domain of *Vibrio vulnificus* Induces Mitochondrion-Mediated Apoptosis. *Infect. Immun.* *83*, 4392–4403.

Alam, M., Sultana, M., Nair, G.B., Siddique, A.K., Hasan, N.A., Sack, R.B., Sack, D.A., Ahmed, K.U., Sadique, A., Watanabe, H., et al. (2007). Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc. Natl. Acad. Sci. U. S. A.* *104*, 17801–17806.

Allredge, A.L., and Silver, M.W. (1988). Characteristics, dynamics and significance of marine snow. *Prog. Oceanogr.* *20*, 41–82.

Almagro-Moreno, S., and Boyd, E.F. (2009). Insights into the evolution of sialic acid catabolism among bacteria. *BMC Evol. Biol.* *9*, 118.

Almagro-Moreno, S., and Taylor, R.K. (2013). Cholera: Environmental Reservoirs and Impact on Disease Transmission. *Microbiol. Spectr.* *1*.

Antic, I., Biancucci, M., Zhu, Y., Gius, D.R., and Satchell, K.J.F. (2015). Site-specific processing of Ras and Rap1 Switch I by a MARTX toxin effector domain. *Nat Commun* *6*, 7396.

Armitage, J.P. (1999). Bacterial tactic responses. *Adv. Microb. Physiol.* *41*, 229–289.

Arzul, I., Renault, T., Thébault, A., and Gérard, A. (2002). Detection of oyster herpesvirus DNA and proteins in asymptomatic *Crassostrea gigas* adults. *Virus Res.* *84*, 151–160.

Asai, Y., Kawagishi, I., Sockett, R.E., and Homma, M. (1999). Hybrid motor with H(+)- and Na(+)-driven components can rotate *Vibrio* polar flagella by using sodium ions. *J. Bacteriol.* *181*, 6332–6338.

Austin, B. (2011). Taxonomy of bacterial fish pathogens. *Vet. Res.* *42*, 20.

-B-

van Baarlen, P., van Belkum, A., Summerbell, R.C., Crous, P.W., and Thomma, B.P.H.J. (2007). Molecular mechanisms of pathogenicity: how do pathogenic microorganisms develop cross-kingdom host jumps? *FEMS Microbiol. Rev.* *31*, 239–277.

Bachère, E., Gueguen, Y., Gonzalez, M., de Lorgeril, J., Garnier, J., and Romestand, B. (2004). Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunol. Rev.* *198*, 149–168.

- Bachère, E., Rosa, R.D., Schmitt, P., Poirier, A.C., Merou, N., Charrière, G.M., and Destoumieux-Garzón, D. (2015). The new insights into the oyster antimicrobial defense: Cellular, molecular and genetic view. *Fish Shellfish Immunol.* *46*, 50–64.
- Baharoglu, Z., Bikard, D., and Mazel, D. (2010). Conjugative DNA Transfer Induces the Bacterial SOS Response and Promotes Antibiotic Resistance Development through Integron Activation. *PLoS Genet.* *6*.
- Baker-Austin, C., and Oliver, J.D. (2018). *Vibrio vulnificus*: new insights into a deadly opportunistic pathogen. *Environ. Microbiol.* *20*, 423–430.
- Baker-Austin, C., Stockley, L., Rangdale, R., and Martinez-Urtaza, J. (2010). Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. *Environ. Microbiol. Rep.* *2*, 7–18.
- Baker-Austin, C., Trinanes, J.A., Taylor, N.G.H., Hartnell, R., Siitonen, A., and Martinez-Urtaza, J. (2013). Emerging *Vibrio* risk at high latitudes in response to ocean warming. *Nat. Clim. Change* *3*, 73–77.
- Baker-Austin, C., Trinanes, J.A., Salmenlinna, S., Löfdahl, M., Siitonen, A., Taylor, N.G.H., and Martinez-Urtaza, J. (2016). Heat Wave-Associated Vibriosis, Sweden and Finland, 2014. *Emerg. Infect. Dis.* *22*, 1216–1220.
- Baker-Austin, C., Trinanes, J., Gonzalez-Escalona, N., and Martinez-Urtaza, J. (2017). Non-Cholera Vibrios: The Microbial Barometer of Climate Change. *Trends Microbiol.* *25*, 76–84.
- Barbieri, E., Falzano, L., Fiorentini, C., Pianetti, A., Baffone, W., Fabbri, A., Matarrese, P., Casiere, A., Katouli, M., Kühn, I., et al. (1999). Occurrence, Diversity, and Pathogenicity of Halophilic *Vibrio* spp. and Non-O1 *Vibrio cholerae* from Estuarine Waters along the Italian Adriatic Coast. *Appl. Environ. Microbiol.* *65*, 2748–2753.
- Barbosa Solomieu, V., Renault, T., and Travers, M.-A. (2015). Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. *J. Invertebr. Pathol.* *131*, 2–10.
- Bellanger X, Guilloteau H, Breuil B, Merlin C. Natural microbial communities supporting the transfer of the IncP-1beta plasmid pB10 exhibit a higher initial content of plasmids from the same incompatibility group. *Front Microbiol.* 2014;5:637.
- Ben Cheikh, Y., Travers, M.-A., Morga, B., Godfrin, Y., Rioult, D., and Le Foll, F. (2016). First evidence for a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities. *Dev. Comp. Immunol.* *57*, 107–119.
- Berk, V., Fong, J.C.N., Dempsey, G.T., Develioglu, O.N., Zhuang, X., Liphardt, J., Yildiz, F.H., and Chu, S. (2012). Molecular architecture and assembly principles of *Vibrio cholerae* biofilms. *Science* *337*, 236–239.
- Beyhan, S., and Yildiz, F.H. (2007). Smooth to rugose phase variation in *Vibrio cholerae* can be mediated by a single nucleotide change that targets c-di-GMP signalling pathway. *Mol. Microbiol.* *63*, 995–1007.
- Biancucci, M., Rabideau, A.E., Lu, Z., Loftis, A.R., Pentelute, B.L., and Satchell, K.J.F. (2017). Substrate Recognition of MARTX Ras/Rap1-Specific Endopeptidase. *Biochemistry* *56*, 2747–2757.

Binese, J., Delsert, C., Saulnier, D., Champomier-Vergès, M.-C., Zagorec, M., Munier-Lehmann, H., Mazel, D., and Le Roux, F. (2008). Metalloprotease vsm is the major determinant of toxicity for extracellular products of *Vibrio splendidus*. *Appl. Environ. Microbiol.* *74*, 7108–7117.

Bishop, A.L., Patimalla, B., and Camilli, A. (2014). *Vibrio cholerae*-induced inflammation in the neonatal mouse cholera model. *Infect. Immun.* *82*, 2434–2447.

Blokesch, M. (2015). Competence-induced type VI secretion might foster intestinal colonization by *Vibrio cholerae*: Intestinal interbacterial killing by competence-induced *V. cholerae*. *BioEssays News Rev. Mol. Cell. Dev. Biol.* *37*, 1163–1168.

Borgeaud, S., Metzger, L.C., Scrignari, T., and Blokesch, M. (2015). The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* *347*, 63–67.

Boucher, Y. (2016). Sustained Local Diversity of *Vibrio cholerae* O1 Biotypes in a Previously Cholera-Free Country. *MBio* *7*.

Brisou, J., Courtois, D., and Denis, F. (1974). Microbiological study of a hypersaline lake in French Somaliland. *Appl. Microbiol.* *27*, 819–822.

Brown, B.E. (1997). Coral bleaching: causes and consequences. *Coral Reefs.* *16*, 129–138.

Brown, S.P., Cornforth, D.M., and Mideo, N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol.* *20*, 336–342.

Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivresse, S., Alunno-Bruscia, M., Polz, M.F., and Le Roux, F. (2017). *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J.* *11*, 1043–1052.

Burge, C.A., Griffin, F.J., and Friedman, C.S. (2006). Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA. *Dis. Aquat. Organ.* *72*, 31–43.

Burrus, V., Pavlovic, G., Decaris, B., and Guédon, G. (2002). Conjugative transposons: the tip of the iceberg. *Mol. Microbiol.* *46*, 601–610.

Burrus, V., Marrero, J., and Waldor, M.K. (2006). The current ICE age: biology and evolution of SXT-related integrating conjugative elements. *Plasmid* *55*, 173–183.

-C-

Cabezón, E., Ripoll-Rozada, J., Peña, A., de la Cruz, F., and Arechaga, I. (2015). Towards an integrated model of bacterial conjugation. *FEMS Microbiol. Rev.* *39*, 81–95.

Cáceres-Martínez, J., Vásquez-Yeomans, R., Danigo, P., and Reyes-Roel, C. (2018). Histological Alterations in Pacific Oysters *Crassostrea gigas* that Survived a Summer Mortality Event in Baja California, Mexico. *J. Aquat. Anim. Health* *30*, 31–38.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* *17*, 540–552.

Cermak, N., Becker, J.W., Knudsen, S.M., Chisholm, S.W., Manalis, S.R., and Polz, M.F. (2017). Direct single-cell biomass estimates for marine bacteria via Archimedes' principle. *ISME J.* *11*, 825–828.

Cervino, J.M., Thompson, F.L., Gomez-Gil, B., Lorence, E.A., Goreau, T.J., Hayes, R.L., Winiarski-Cervino, K.B., Smith, G.W., Hughen, K., and Bartels, E. (2008). The *Vibrio* core group induces yellow band disease in Caribbean and Indo-Pacific reef-building corals. *J. Appl. Microbiol.* *105*, 1658–1671.

- Chowdhury, A., Ishibashi, M., Thiem, V.D., Tuyet, D.T.N., Tung, T.V., Chien, B.T., Seidlein L.V., L. von, Canh, D.G., Clemens, J., Trach, D.D., et al. (2004). Emergence and serovar transition of *Vibrio parahaemolyticus* pandemic strains isolated during a diarrhea outbreak in Vietnam between 1997 and 1999. *Microbiol. Immunol.* 48, 319–327.
- Cianfanelli FR, Monlezun L, Coulthurst SJ. (2016). Aim, Load, Fire: The Type VI Secretion System, a Bacterial Nanoweapon. *Trends Microbiol.* 2016;24(1):51-62.
- Cirz, R.T., Chin, J.K., Andes, D.R., de Crécy-Lagard, V., Craig, W.A., and Romesberg, F.E. (2005). Inhibition of Mutation and Combating the Evolution of Antibiotic Resistance. *PLoS Biol.* 3.
- Cochennec, N., Le Roux, F., Berthe, F., and Gerard, A. (2000). Detection of *Bonamia ostreae* based on small subunit ribosomal probe. *J. Invertebr. Pathol.* 76, 26–32.
- Cognie, B., Barillé, L., Massé, G., and Beninger, P.G. (2003). Selection and processing of large suspended algae in the oyster *Crassostrea gigas*. *Marine Ecology Progress Series* 250, 145–152.
- Colwell, R.R. (2000). Viable but nonculturable bacteria: a survival strategy. *J. Infect. Chemother.* 6, 121–125.
- Comps, M. (1988). Epizootic diseases of oysters associated with viral infections. *Am. Fish. Soc. Spec. Publ.* 18, 23–37.
- Comps, M., Bonami, J.-R., and Vago, C. (1976). Une virose de l’huître portugaise (*Crassostrea angulata* LMK). *Comptes Rendus Académie Sci. Paris Sér. D* 282, 139–143.
- Comps, M., Tige, G. and Grizel, H., (1980). Recherches ultrastructurales sur un Protiste parasite de l’Huitre plate *Ostrea edulis* L. *C.R. Acad. Sci. Paris*, 290, Ser. D: 383-384.
- Constantin de Magny, G., Mozumder, P.K., Grim, C.J., Hasan, N.A., Naser, M.N., Alam, M., Sack R.B., Huq A. and Colwell R.R (2011). Role of Zooplankton Diversity in *Vibrio cholerae* Population Dynamics and in the Incidence of Cholera in the Bangladesh Sundarbans . *Appl Environ Microbiol.* 77, 6125–6132.
- Cooper, V.S., Vohr, S.H., Wrocklage, S.C., and Hatcher, P.J. (2010). Why Genes Evolve Faster on Secondary Chromosomes in Bacteria. *PLOS Comput. Biol.* 6, e1000732.
- Cordero, C.L., Kudryashov, D.S., Reisler, E., and Satchell, K.J.F. (2006). The Actin cross-linking domain of the *Vibrio cholerae* RTX toxin directly catalyzes the covalent cross-linking of actin. *J. Biol. Chem.* 281, 32366–32374.
- Cordero, O.X., and Hogeweg, P. (2009). The impact of long-distance horizontal gene transfer on prokaryotic genome size. *Proc. Natl. Acad. Sci. U. S. A.* 106, 21748–21753.
- Cordero, O.X., and Polz, M.F. (2014). Explaining microbial genomic diversity in light of evolutionary ecology. *Nat. Rev. Microbiol.* 12, 263–273.
- Cordero, O.X., Ventouras, L.-A., DeLong, E.F., and Polz, M.F. (2012b). Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20059–20064.
- Cordero, O.X., Wildschutte, H., Kirkup, B., Proehl, S., Ngo, L., Hussain, F., Le Roux, F., Mincer, T., and Polz, M.F. (2012a). Ecological populations of bacteria act as socially cohesive units of antibiotic production and resistance. *Science* 337, 1228–1231.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. (1995). Microbial biofilms. *Annu. Rev. Microbiol.* *49*, 711–745.

de la Cruz, F., Frost, L.S., Meyer, R.J., and Zechner, E.L. (2010). Conjugative DNA metabolism in Gram-negative bacteria. *FEMS Microbiol. Rev.* *34*, 18–40.

-D-

Daniels, N.A., Ray, B., Easton, A., Marano, N., Kahn, E., McShan, A.L., Del Rosario, L., Baldwin, T., Kingsley, M.A., Pühr, N.D., et al. (2000). Emergence of a new *Vibrio parahaemolyticus* serotype in raw oysters: A prevention quandary. *JAMA* *284*, 1541–1545.

Das, B. (2014). Mechanistic insights into filamentous phage integration in *Vibrio cholerae*. *Front. Microbiol.* *5*, 650.

Day, S.R., Sifri, C.D., and Hoffman, P.S. (2013). Infection of nonphagocytic host cells by legionella. *Methods Mol. Biol. Clifton NJ* *954*, 463–478.

Defoirdt, T., Sorgeloos, P., and Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Curr. Opin. Microbiol.* *14*, 251–258.

Dégremont, L., Garcia, C., and Allen, S.K. (2015). Genetic improvement for disease resistance in oysters: A review. *J. Invertebr. Pathol.* *131*, 226–241.

Demarre, G., Guérout, A.-M., Matsumoto-Mashimo, C., Rowe-Magnus, D.A., Marlière, P., and Mazel, D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPalpha) conjugative machineries and their cognate *Escherichia coli* host strains. *Res. Microbiol.* *156*, 245–255.

Destoumieux-Garzón, D., Rosa, R.D., Schmitt, P., Barreto, C., Vidal-Dupiol, J., Mitta, G., Gueguen, Y., and Bachère, E. (2016). Antimicrobial peptides in marine invertebrate health and disease. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* *371*.

diCenzo, G.C., and Finan, T.M. (2017). The Divided Bacterial Genome: Structure, Function, and Evolution. *Microbiol. Mol. Biol. Rev. MMBR* *81*.

Dolores, J., and Satchell, K.J.F. (2013). Analysis of *Vibrio cholerae* genome sequences reveals unique *rtxA* variants in environmental strains and an *rtxA*-null mutation in recent altered El Tor isolates. *MBio* *4*, e00624.

Doney, S.C., Ruckelshaus, M., Duffy, J.E., Barry, J.P., Chan, F., English, C.A., Galindo, H.M., Grebmeier, J.M., Hollowed, A.B., Knowlton, N., et al. (2012). Climate change impacts on marine ecosystems. *Annu. Rev. Mar. Sci.* *4*, 11–37.

Domeneghetti, S., Varotto, L., Civettini, M., Rosani, U., Stauder, M., Pretto, T., Pezzati, E., Arcangeli, G., Turolla, E., Pallavicini, A., et al. (2014). Mortality occurrence and pathogen detection in *Crassostrea gigas* and *Mytilus galloprovincialis* close-growing in shallow waters (Goro lagoon, Italy). *Fish Shellfish Immunol.* *41*, 37–44

Dubert, J., Barja, J.L., and Romalde, J.L. (2017). New Insights into Pathogenic Vibrios Affecting Bivalves in Hatcheries: Present and Future Prospects. *Front. Microbiol.* *8*.

Duperthuy, M., Binesse, J., Le Roux, F., Romestand, B., Caro, A., Got, P., Givaudan, A., Mazel, D., Bachère, E., and Destoumieux-Garzón, D. (2010). The major outer membrane protein OmpU of *Vibrio*

splendidus contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*. *Environ. Microbiol.* *12*, 951–963.

Duperthuy, M., Schmitt, P., Garzón, E., Caro, A., Rosa, R.D., Le Roux, F., Lautrédou-Audouy, N., Got, P., Romestand, B., de Lorgeril, J., et al. (2011). Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proc. Natl. Acad. Sci. U. S. A.* *108*, 2993–2998.

Dziejman, M., Balon, E., Boyd, D., Fraser, C.M., Heidelberg, J.F., and Mekalanos, J.J. (2002). Comparative genomic analysis of *Vibrio cholerae*: Genes that correlate with cholera endemic and pandemic disease. *Proc. Natl. Acad. Sci. U. S. A.* *99*, 1556–1561.

-E-

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* *32*, 1792–1797.

Eiler, A., Johansson, M., and Bertilsson, S. (2006). Environmental Influences on *Vibrio* Populations in Northern Temperate and Boreal Coastal Waters (Baltic and Skagerrak Seas). *Appl. Environ. Microbiol.* *72*, 6004–6011.

Escudero, J.A., Loot, C., Nivina, A., and Mazel, D. (2015). The Integron: Adaptation On Demand. *Microbiol. Spectr.* *3*, MDNA3-0019–2014.

Espejo, R.T., García, K., and Plaza, N. (2017). Insight Into the Origin and Evolution of the *Vibrio parahaemolyticus* Pandemic Strain. *Front. Microbiol.* *8*, 1397.

Eyisi, O.A.L., Nwodo, U.U., and Iroegbu, C.U. (2013). Distribution of *Vibrio* species in Shellfish and Water Samples Collected from the Atlantic Coastline of South-East Nigeria. *J. Health Popul. Nutr.* *31*, 314–320.

-F-

Fabioux, C., Corporeau, C., Quillien, V., Favrel, P., and Huvet, A. (2009). In vivo RNA interference in oyster--vasa silencing inhibits germ cell development. *FEBS J.* *276*, 2566–2573.

Falkowski, P.G., Barber, R.T., and Smetacek, V. (1998). Biogeochemical Controls and Feedbacks on Ocean Primary Production. *Science* *281*, 200–206.

Fang, F.C., and Casadevall, A. (2011). Reductionistic and Holistic Science. *Infect. Immun.* *79*, 1401–1404.

Faruque, S.M. (2014). Role of phages in the epidemiology of cholera. *Curr. Top. Microbiol. Immunol.* *379*, 165–180.

Faruque, S.M., Biswas, K., Udden, S.M.N., Ahmad, Q.S., Sack, D.A., Nair, G.B., and Mekalanos, J.J. (2006). Transmissibility of cholera: in vivo-formed biofilms and their relationship to infectivity and persistence in the environment. *Proc. Natl. Acad. Sci. U. S. A.* *103*, 6350–6355.

Fast, D., Kostiuk, B., Foley, E., and Pukatzki, S. (2018). Commensal pathogen competition impacts host viability. *Proc. Natl. Acad. Sci. U. S. A.* *115*, 7099–7104.

Faury, N., Saulnier, D., Thompson, F.L., Gay, M., Swings, J., and Le Roux, F. (2004). *Vibrio crassostreae* sp. nov., isolated from the haemolymph of oysters (*Crassostrea gigas*). *Int. J. Syst. Evol. Microbiol.* *54*, 2137–2140.

Fong, J.C.N., and Yildiz, F.H. (2007). The *rbmBCDEF* gene cluster modulates development of rugose colony morphology and biofilm formation in *Vibrio cholerae*. *J. Bacteriol.* *189*, 2319–2330.

Frans, I., Michiels, C.W., Bossier, P., Willems, K.A., Lievens, B., and Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention. *J. Fish Dis.* *34*, 643–661.

Froelich, B., Ayrapetyan, M., and Oliver, J.D. (2013). Integration of *Vibrio vulnificus* into marine aggregates and its subsequent uptake by *Crassostrea virginica* oysters. *Appl. Environ. Microbiol.* *79*, 1454–1458.

Frost, L.S., Leplae, R., Summers, A.O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* *3*, 722–732.

Frydenborg, B.R., Krediet, C.J., Teplitski, M., and Ritchie, K.B. (2014). Temperature-dependent inhibition of opportunistic *Vibrio* pathogens by native coral commensal bacteria. *Microb. Ecol.* *67*, 392–401.

Fuqua, C., Winans, S.C., and Greenberg, E.P. (1996). Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators. *Annu. Rev. Microbiol.* *50*, 727–751.

-G-

Gavin, H.E., and Satchell, K.J.F. (2015). MARTX toxins as effector delivery platforms. *Pathog Dis* *73*, ftv092.

Gay, M., Berthe, F.C.J., and Le Roux, F. (2004a). Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Dis. Aquat. Org.* *59*, 49–56.

Gay, M., Renault, T., Pons, A.-M., and Le Roux, F. (2004b). Two *vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. *Dis. Aquat. Organ.* *62*, 65–74.

Gilbert, J.A., Steele, J.A., Caporaso, J.G., Steinbrück, L., Reeder, J., Temperton, B., Huse, S., McHardy, A.C., Knight, R., Joint, I., et al. (2012). Defining seasonal marine microbial community dynamics. *ISME J.* *6*, 298–308.

Go, J., Deutscher, A.T., Spiers, Z.B., Dahle, K., Kirkland, P.D., and Jenkins, C. (2017). Mass mortalities of unknown aetiology in Pacific oysters *Crassostrea gigas* in Port Stephens, New South Wales, Australia. *Dis. Aquat. Organ.* *125*, 227–242.

Godfray HC, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, et al. Food security: the challenge of feeding 9 billion people. *Science.* 2010;327(5967):812-8.

González-Escalona, N., Cachicas, V., Acevedo, C., Rioseco, M.L., Vergara, J.A., Cabello, F., Romero, J., and Espejo, R.T. (2005). *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. *Emerg. Infect. Dis.* *11*, 129–131.

González-Escalona, N., Jaykus, L.-A., and DePaola, A. (2007). Typing of *Vibrio vulnificus* strains by variability in their 16S-23S rRNA intergenic spacer regions. *Foodborne Pathog. Dis.* *4*, 327–337.

Goudenège, D., Labreuche, Y., Krin, E., Ansquer, D., Mangenot, S., Calteau, A., Médigue, C., Mazel, D., Polz, M.F., and Le Roux, F. (2013). Comparative genomics of pathogenic lineages of *Vibrio nigripulchritudo* identifies virulence-associated traits. *ISME J.* *7*, 1985–1996.

Goudenège, D., Travers, M.A., Lemire, A., Petton, B., Haffner, P., Labreuche, Y., Tourbiez, D., Mangenot, S., Calteau, A., Mazel, D., et al. (2015). A single regulatory gene is sufficient to alter *Vibrio*

aestuarianus pathogenicity in oysters. *Environ. Microbiol.* *17*, 4189–4199.

Green, T.J., and Montagnani, C. (2013). Poly I:C induces a protective antiviral immune response in the Pacific oyster (*Crassostrea gigas*) against subsequent challenge with Ostreid herpesvirus (OsHV-1  $\mu$ var). *Fish Shellfish Immunol.* *35*, 382–388.

Green, T.J., Montagnani, C., Benkendorff, K., Robinson, N., and Speck, P. (2014). Ontogeny and water temperature influences the antiviral response of the Pacific oyster, *Crassostrea gigas*. *Fish Shellfish Immunol.* *36*, 151–157.

Green, T.J., and Speck, P. (2018). Antiviral Defense and Innate Immune Memory in the Oyster. *Viruses* *10*.

Grizel, H., and Heral, M. (1991). Introduction into France of the Japanese oyster (*Crassostrea gigas*). *Journal Du Conseil - Conseil International Pour l'Exploration de La Mer* *47*, 399–403.

Groisman, E.A., and Ochman, H. (1996). Pathogenicity islands: bacterial evolution in quantum leaps. *Cell* *87*, 791–794.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* *59*, 307–321.

-H-

Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., and Samuel, M.D. (2002). Climate warming and disease risks for terrestrial and marine biota. *Science* *296*, 2158–2162.

Hazen, T.H., Pan, L., Gu, J.-D., and Sobecky, P.A. (2010). The contribution of mobile genetic elements to the evolution and ecology of *Vibrios*. *FEMS Microbiol. Ecol.* *74*, 485–499.

Hehemann, J.-H., Arevalo, P., Datta, M.S., Yu, X., Corzett, C.H., Henschel, A., Preheim, S.P., Timberlake, S., Alm, E.J., and Polz, M.F. (2016). Adaptive radiation by waves of gene transfer leads to fine-scale resource partitioning in marine microbes. *Nat. Commun.* *7*, 12860.

Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Umayam, L., et al. (2000). DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* *406*, 477–483.

Heidelberg, J.F., Heidelberg, K.B., and Colwell, R.R. (2002). Bacteria of the gamma-subclass Proteobacteria associated with zooplankton in Chesapeake Bay. *Appl. Environ. Microbiol.* *68*, 5498–5507.

Hirt H, Greenwood-Quaintance KE, Karau MJ, Till LM, Kashyap PC, Patel R, et al. (2018). Enterococcus faecalis Sex Pheromone cCF10 Enhances Conjugative Plasmid Transfer In Vivo. *MBio.* 2018;9(1).

His, E., Tige, G., and Rabouin, M.-A. (1976). Observations relatives à la maladie des huîtres plates dans le bassin d'Arcachon, vitesse d'infestation et réactions pathologiques. p.

Ho, L. si T., and Ané, C. (2014). A linear-time algorithm for Gaussian and non-Gaussian trait evolution models. *Syst. Biol.* *63*, 397–408.

Ho, B.T., Dong, T.G., and Mekalanos, J.J. (2014). A view to a kill: the bacterial type VI secretion system. *Cell Host Microbe* 15, 9–21.

Hochhut, B., Lotfi, Y., Mazel, D., Faruque, S.M., Woodgate, R., and Waldor, M.K. (2001). Molecular Analysis of Antibiotic Resistance Gene Clusters in *Vibrio cholerae* O139 and O1 SXT Constins. *Antimicrob. Agents Chemother.* 45, 2991–3000.

Hoegh-Guldberg, O. (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Mar Freshwater Res.* 50,839–866.

Hunt, D.E., David, L.A., Gevers, D., Preheim, S.P., Alm, E.J., and Polz, M.F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science* 320, 1081–1085.

Huvet, A., Herpin, A., Dégremont, L., Labreuche, Y., Samain, J.-F., and Cunningham, C. (2004). The identification of genes from the oyster *Crassostrea gigas* that are differentially expressed in progeny exhibiting opposed susceptibility to summer mortality. *Gene* 343, 211–220.

Huvet, A., Béguel, J.-P., Cavaleiro, N.P., Thomas, Y., Quillien, V., Boudry, P., Alunno-Bruscia, M., and Fabioux, C. (2015). Disruption of amylase genes by RNA interference affects reproduction in the Pacific oyster *Crassostrea gigas*. *J. Exp. Biol.* 218, 1740–1747.

-I-

Ibarra, J.A., and Steele-Mortimer, O. (2009). Salmonella--the ultimate insider. Salmonella virulence factors that modulate intracellular survival. *Cell. Microbiol.* 11, 1579–1586.

Islam, M.T., Alam, M., and Boucher, Y. (2017). Emergence, ecology and dispersal of the pandemic generating *Vibrio cholerae* lineage. *Int. Microbiol. Off. J. Span. Soc. Microbiol.* 20, 106–115.

Ives, A.R., and Garland, T. (2010). Phylogenetic logistic regression for binary dependent variables. *Syst. Biol.* 59, 9–26.

-J-

Johnson, C.M., and Grossman, A.D. (2015). Integrative and Conjugative Elements (ICEs): What They Do and How They Work. *Annu. Rev. Genet.* 49, 577–601.

Joshi, A., Kostiuk, B., Rogers, A., Teschler, J., Pukatzki, S., and Yildiz, F.H. (2017). Rules of Engagement: The Type VI Secretion System in *Vibrio cholerae*. *Trends Microbiol.* 25, 267–279.

Jousset, A., Rochat, L., Péchy-Tarr, M., Keel, C., Scheu, S., and Bonkowski, M. (2009). Predators promote defence of rhizosphere bacterial populations by selective feeding on non-toxic cheaters. *ISME J* 3, 666–674.

-K-

Kim, E.J., Lee, D., Moon, S.H., Lee, C.H., and Kim, D.W. (2014). CTX prophages in *Vibrio cholerae* O1 strains. *J. Microbiol. Biotechnol.* 24, 725–731.

Kim, H.-J., and Cho, J.-C. (2015). Genotypic Diversity and Population Structure of *Vibrio vulnificus* Strains Isolated in Taiwan and Korea as Determined by Multilocus Sequence Typing. *PloS One* 10, e0142657.

Konstantinidis, K.T., and Tiedje, J.M. (2004). Trends between gene content and genome size in prokaryotic species with larger genomes. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3160–3165.

Koropatnick, T.A., Engle, J.T., Apicella, M.A., Stabb, E.V., Goldman, W.E., and McFall-Ngai, M.J. (2004). Microbial factor-mediated development in a host-bacterial mutualism. *Science* 306, 1186–1188.

Koton, Y., Gordon, M., Chalifa-Caspi, V., and Bisharat, N. (2014). Comparative genomic analysis of clinical and environmental *Vibrio vulnificus* isolates revealed biotype 3 evolutionary relationships. *Front. Microbiol.* 5, 803.

Kwak, J.S., Jeong, H.-G., and Satchell, K.J.F. (2011). *Vibrio vulnificus* rtxA1 gene recombination generates toxin variants with altered potency during intestinal infection. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1645–1650.

-L-

Labreuche, Y., Chenivesse, S., Jeudy, A., Le Panse, S., Boulo, V., Ansquer, D., Pagès, S., Givaudan, A., Czjzek, M., and Le Roux, F. (2017). Nigritoxin is a bacterial toxin for crustaceans and insects. *Nat. Commun.* 8, 1248.

Labreuche, Y., Le Roux, F., Henry, J., Zatylny, C., Huvet, A., Lambert, C., Soudant, P., Mazel, D., and Nicolas, J.-L. (2010). *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. *Fish Shellfish Immunol.* 29, 753–758.

Labreuche, Y., and Warr, G.W. (2013). Insights into the antiviral functions of the RNAi machinery in penaeid shrimp. *Fish Shellfish Immunol.* 34, 1002–1010.

Lacoste, A., Jalabert, F., Malham, S., Cuff, A., Gélébart, F., Cordevant, C., Lange, M., and Poulet, S.A. (2001). A *Vibrio splendidus* strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the Bay of Morlaix (North Brittany, France). *Dis. Aquat. Organ.* 46, 139–145.

Lafont, M., Petton, B., Vergnes, A., Pauletto, M., Segarra, A., Gourbal, B., and Montagnani, C. (2017). Long-lasting antiviral innate immune priming in the Lophotrochozoan Pacific oyster, *Crassostrea gigas*. *Sci. Rep.* 7.

Lauriano, C.M., Ghosh, C., Correa, N.E., and Klose, K.E. (2004). The sodium-driven flagellar motor controls exopolysaccharide expression in *Vibrio cholerae*. *J. Bacteriol.* 186, 4864–4874.

Le Roux, F., Binesse, J., Saulnier, D., and Mazel, D. (2007). Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel counterselectable suicide vector. *Appl. Environ. Microbiol.* 73, 777–784.

Le Roux, F., Davis, B.M., and Waldor, M.K. (2011b). Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. *Nucleic Acids Res.* 39, 1004–1013.

Le Roux, F., Labreuche, Y., Davis, B.M., Iqbal, N., Mangenot, S., Goarant, C., Mazel, D., and Waldor, M.K. (2011). Virulence of an emerging pathogenic lineage of *Vibrio nigripulchritudo* is dependent on two plasmids. *Environ. Microbiol.* 13, 296–306.

Le Roux, F., Lorenzo, G., Peyret, P., Audemard, C., Figueras, A., Vivarès, C., Gouy, M., and Berthe, F. (2001). Molecular evidence for the existence of two species of *Marteilia* in Europe. *J. Eukaryot. Microbiol.* 48, 449–454.

Le Roux, F., Wegner, K.M., Baker-Austin, C., Vezzulli, L., Osorio, C.R., Amaro, C., Ritchie, J.M., Defoirdt, T., Destoumieux-Garzón, D., Blokesch, M., et al. (2015). The emergence of *Vibrio* pathogens in Europe: ecology, evolution, and pathogenesis (Paris, 11-12th March 2015). *Front. Microbiol.* 6, 830.

Le Roux, F., Wegner, K.M., and Polz, M.F. (2016). Oysters and *Vibrios* as a Model for Disease

Dynamics in Wild Animals. *Trends Microbiol.* *24*, 568–580.

Le Roux, F., Zouine, M., Chakroun, N., Binesse, J., Saulnier, D., Bouchier, C., Zidane, N., Ma, L., Rusniok, C., Lajus, A., et al. (2009). Genome sequence of *Vibrio splendidus*: an abundant planktonic marine species with a large genotypic diversity. *Environ. Microbiol.* *11*, 1959–1970.

Lee, B.C., Lee, J.H., Kim, M.W., Kim, B.S., Oh, M.H., Kim, K.-S., Kim, T.S., and Choi, S.H. (2008). *Vibrio vulnificus* rtxE is important for virulence, and its expression is induced by exposure to host cells. *Infect. Immun.* *76*, 1509–1517.

Lee, C.-T., Amaro, C., Wu, K.-M., Valiente, E., Chang, Y.-F., Tsai, S.-F., Chang, C.-H., and Hor, L.-I. (2008). A common virulence plasmid in biotype 2 *Vibrio vulnificus* and its dissemination aided by a conjugal plasmid. *J. Bacteriol.* *190*, 1638–1648.

Lee, C.-T., Chen, I.-T., Yang, Y.-T., Ko, T.-P., Huang, Y.-T., Huang, J.-Y., Huang, M.-F., Lin, S.-J., Chen, C.-Y., Lin, S.-S., et al. (2015). The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. *Proc. Natl. Acad. Sci. U. S. A.* *112*, 10798–10803.

Lemire, A., Goudenège, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., and Le Roux, F. (2015). Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *ISME J.* *9*, 1523–1531.

Letchumanan, V., Chan, K.-G., and Lee, L.-H. (2014). *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Front. Microbiol.* *5*, 705.

Letunic, I., and Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res.* *39*, W475-478.

Levin, B.R., and Svanborg Edén, C. (1990). Selection and evolution of virulence in bacteria: an ecumenical excursion and modest suggestion. *Parasitology* *100 Suppl*, S103-115.

Lipp, E.K., Huq, A., and Colwell, R.R. (2002). Effects of global climate on infectious disease: the cholera model. *Clin. Microbiol. Rev.* *15*, 757–770.

Llosa, M., Zunzunegui, S., and de la Cruz, F. (2003). Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc. Natl. Acad. Sci. U. S. A.* *100*, 10465–10470.

Lokmer, A., Kuenzel, S., Baines, J.F., and Wegner, K.M. (2016). The role of tissue-specific microbiota in initial establishment success of Pacific oysters. *Environ. Microbiol.* *18*, 970–987.

Lokmer, A., and Mathias Wegner, K. (2015). Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J.* *9*, 670–682.

Lovell, C.R. (2017). Ecological fitness and virulence features of *Vibrio parahaemolyticus* in estuarine environments. *Appl. Microbiol. Biotechnol.* *101*, 1781–1794.

Luo, L., Wall, A.A., Yeo, J.C., Condon, N.D., Norwood, S.J., Schoenwaelder, S., Chen, K.W., Jackson, S., Jenkins, B.J., Hartland, E.L., et al. (2014). Rab8a interacts directly with PI3K $\gamma$  to modulate TLR4-driven PI3K and mTOR signalling. *Nat Commun* *5*, 4407.

Lutz, C., Erken, M., Noorian, P., Sun, S., and McDougald, D. (2013). Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae*. *Front. Microbiol.* *4*.

Lux, R., and Shi, W. (2004). Chemotaxis-guided movements in bacteria. *Crit. Rev. Oral Biol. Med. Off. Publ. Am. Assoc. Oral Biol.* 15, 207–220.

-M-

Ma AT, McAuley S, Pukatzki S, Mekalanos JJ. (2009). Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe.* 2009;5(3):234-43.

Macnab, R.M. (2003). How bacteria assemble flagella. *Annu. Rev. Microbiol.* 57, 77–100.

Magariyama, Y., Sugiyama, S., Muramoto, K., Maekawa, Y., Kawagishi, I., Imae, Y., and Kudo, S. (1994). Very fast flagellar rotation. *Nature* 371, 752.

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., Yamashita, A., et al. (2003). Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. *The Lancet* 361, 743–749.

Mandel, M.J., and Dunn, A.K. (2016). Impact and Influence of the Natural *Vibrio*-Squid Symbiosis in Understanding Bacterial-Animal Interactions. *Front. Microbiol.* 7, 1982.

Martenot, C., Oden, E., Travaillé, E., Malas, J.-P., and Houssin, M. (2011). Detection of different variants of Ostreid Herpesvirus 1 in the Pacific oyster, *Crassostrea gigas* between 2008 and 2010. *Virus Res.* 160, 25–31.

Martenot, C., Travaillé, E., Lethuillier, O., Lelong, C., and Houssin, M. (2013). Genome exploration of six variants of the Ostreid Herpesvirus 1 and characterization of large deletion in OsHV-1 $\mu$ Var specimens. *Virus Res.* 178, 462–470.

Martinez-Urtaza, J., van Aerle, R., Abanto, M., Haendiges, J., Myers, R.A., Trinanes, J., Baker-Austin, C., and Gonzalez-Escalona, N. (2017). Genomic Variation and Evolution of *Vibrio parahaemolyticus* ST36 over the Course of a Transcontinental Epidemic Expansion. *MBio* 8.

Martinez-Urtaza, J., Simental, L., Velasco, D., DePaola, A., Ishibashi, M., Nakaguchi, Y., Nishibuchi, M., Carrera-Flores, D., Rey-Alvarez, C., and Pousa, A. (2005). Pandemic *Vibrio parahaemolyticus* O3:K6, Europe. *Emerg. Infect. Dis.* 11, 1319–1320.

Martinez-Urtaza, J., Baker-Austin, C., Jones, J.L., Newton, A.E., Gonzalez-Aviles, G.D., and DePaola, A. (2013). Spread of Pacific Northwest *Vibrio parahaemolyticus* strain. *N. Engl. J. Med.* 369, 1573–1574.

Martinez-Urtaza, J., Powell, A., Jansa, J., Rey, J.L.C., Montero, O.P., Campello, M.G., López, M.J.Z., Pousa, A., Valles, M.J.F., Trinanes, J., et al. (2016). Epidemiological investigation of a foodborne outbreak in Spain associated with U.S. West Coast genotypes of *Vibrio parahaemolyticus*. *SpringerPlus* 5, 87.

Martin-Platero, A.M., Cleary, B., Kauffman, K., Preheim, S.P., McGillicuddy, D.J., Alm, E.J., and Polz, M.F. (2018). High resolution time series reveals cohesive but short-lived communities in coastal plankton. *Nat. Commun.* 9, 266.

Matic, I., Rayssiguier, C., and Radman, M. (1995). Interspecies gene exchange in bacteria: the role of SOS and mismatch repair systems in evolution of species. *Cell* 80, 507–515.

Matsen, F.A., Kodner, R.B., and Armbrust, E.V. (2010). pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. *BMC Bioinformatics* *11*, 538.

Matz, C., McDougald, D., Moreno, A.M., Yung, P.Y., Yildiz, F.H., and Kjelleberg, S. (2005). Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U. S. A.* *102*, 16819–16824.

Mazel, D. (2006). Integrons: agents of bacterial evolution. *Nat. Rev. Microbiol.* *4*, 608–620.

McCarter, L.L. (2004). Dual Flagellar Systems Enable Motility under Different Circumstances. *J. Mol. Microbiol. Biotechnol.* *7*, 18–29.

Meibom, K.L., Blokesch, M., Dolganov, N.A., Wu, C.-Y., and Schoolnik, G.K. (2005). Chitin induces natural competence in *Vibrio cholerae*. *Science* *310*, 1824–1827.

Mello, D.F., Danielli, N.M., Curbani, F., Pontinha, V.A., Suhnel, S., Castro, M. a. M., Medeiros, S.C., Wendt, N.C., Trevisan, R., Magalhães, A.R.M., et al. (2018). First evidence of viral and bacterial oyster pathogens in the Brazilian coast. *J. Fish Dis.* *41*, 559–563.

Metzger, L.C., and Blokesch, M. (2016). Regulation of competence-mediated horizontal gene transfer in the natural habitat of *Vibrio cholerae*. *Curr. Opin. Microbiol.* *30*, 1–7.

Michel, B. (2005). After 30 Years of Study, the Bacterial SOS Response Still Surprises Us. *PLoS Biol.* *3*.

Miele, V., Penel, S., and Duret, L. (2011). Ultra-fast sequence clustering from similarity networks with SiLiX. *BMC Bioinformatics* *12*, 116.

Milton, D.L., Norqvist, A., and Wolf-Watz, H. (1992). Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. *J. Bacteriol.* *174*, 7235–7244.

Minamino, T., and Imada, K. (2015). The bacterial flagellar motor and its structural diversity. *Trends Microbiol.* *23*, 267–274.

Miossec, L., Deuff, R.-M.L., and Gouletquer, P. Alien species alert: *Crassostrea gigas* (Pacific Oyster). *46*.

Miyashiro, T., and Ruby, E.G. (2012). Shedding light on bioluminescence regulation in *Vibrio fischeri*. *Mol. Microbiol.* *84*, 795–806.

Miyata, S.T., Kitaoka, M., Brooks, T.M., McAuley, S.B., and Pukatzki, S. (2011). *Vibrio cholerae* requires the type VI secretion system virulence factor VasX to kill *Dictyostelium discoideum*. *Infect. Immun.* *79*, 2941–2949.

Morrison, S.S., Williams, T., Cain, A., Froelich, B., Taylor, C., Baker-Austin, C., Verner-Jeffreys, D., Hartnell, R., Oliver, J.D., and Gibas, C.J. (2012). Pyrosequencing-based comparative genome analysis of *Vibrio vulnificus* environmental isolates. *PloS One* *7*, e37553.

-N-

Nair, G.B., Ramamurthy, T., Bhattacharya, S.K., Dutta, B., Takeda, Y., and Sack, D.A. (2007). Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. *Clin. Microbiol. Rev.* *20*, 39–48.

-O-

Oden, E., Martenot, C., Berthaux, M., Travaillé, E., Malas, J.P., and Houssin, M. (2011). Quantification of ostreid herpesvirus 1 (OsHV-1) in *Crassostrea gigas* by real-time PCR: Determination of a viral load threshold to prevent summer mortalities. *Aquaculture* 317, 27–31.

Okada, K., Iida, T., Kita-Tsukamoto, K., and Honda, T. (2005). Vibrios Commonly Possess Two Chromosomes. *J. Bacteriol.* 187, 752–757.

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A.K., Garg, S., Bhattacharya, S.K., Nair, G.B., and Nishibuchi, M. (1997). Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. *J. Clin. Microbiol.* 35, 3150–3155.

Oliver, J.D. (2005). Wound infections caused by *Vibrio vulnificus* and other marine bacteria. *Epidemiol. Infect.* 133, 383–391.

Oliver, J.D. (2015). The Biology of *Vibrio vulnificus*. *Microbiol. Spectr.* 3.

Otto, M. (2009). Bacterial sensing of antimicrobial peptides. *Contrib Microbiol* 16, 136–149.

-P-

Pajuelo, D., Lee, C.-T., Roig, F.J., Hor, L.-I., and Amaro, C. (2015). Novel host-specific iron acquisition system in the zoonotic pathogen *Vibrio vulnificus*. *Environ. Microbiol.* 17, 2076–2089.

Pales Espinosa, E., Koller, A., and Allam, B. (2016). Proteomic characterization of mucosal secretions in the eastern oyster, *Crassostrea virginica*. *J Proteomics* 132, 63–76.

Paranjpye, R., Hamel, O.S., Stojanovski, A., and Liermann, M. (2012). Genetic Diversity of Clinical and Environmental *Vibrio parahaemolyticus* Strains from the Pacific Northwest. *Appl. Environ. Microbiol.* 78, 8631–8638.

Parizadeh, L., Tourbiez, D., Garcia, C., Haffner, P., Dégremont, L., Le Roux, F., and Travers, M.-A. (2018). Ecologically realistic model of infection for exploring the host damage caused by *Vibrio aestuarianus*. *Environ. Microbiol.*

Pepin, J.F., Riou, A., and Renault, T. (2008). Rapid and sensitive detection of ostreid herpesvirus 1 in oyster samples by real-time PCR. *J. Virol. Methods* 149, 269–276.

Pérez-Cataluña, A., Lucena, T., Tarazona, E., Arahal, D.R., Macián, M.C., and Pujalte, M.J. (2016). An MLSA approach for the taxonomic update of the *Splendidus* clade, a lineage containing several fish and shellfish pathogenic *Vibrio* spp. *Syst. Appl. Microbiol.* 39, 361–369.

Pernet, F., Barret, J., Le Gall, P., Corporeau, C., Dégremont, L., Lagarde, F., Pepin, J.-F., and Keck, N. (2012). Mass mortalities of Pacific oysters *Crassostrea gigas* reflect infectious diseases and vary with farming practices in the Mediterranean Thau lagoon, France. *Aquaculture Environment Interactions* 2, 215–237.

Petton, B., Pernet, F., Robert, R., and Boudry, P. (2013). Temperature influence on pathogen transmission and subsequent mortalities in juvenile Pacific oysters *Crassostrea gigas*. *Aquac. Environ. Interact.* 3, 257–273.

Petton, B., Bruto, M., James, A., Labreuche, Y., Alunno-Bruscia, M., and Le Roux, F. (2015a). *Crassostrea gigas* mortality in France: the usual suspect, a herpes virus, may not be the killer in this polymicrobial opportunistic disease. *Front. Microbiol.* 6, 686.

Petton, B., Boudry, P., Alunno-Bruscia, M., and Pernet, F. (2015b). Factors influencing disease-induced mortality of Pacific oysters *Crassostrea gigas*. *Aquac. Environ. Interact.* *6*, 205–222.

Pfeffer, C., and Oliver, J.D. (2003). A comparison of thiosulphate-citrate-bile salts-sucrose (TCBS) agar and thiosulphate-chloride-iodide (TCI) agar for the isolation of *Vibrio* species from estuarine environments. *Lett. Appl. Microbiol.* *36*, 150–151.

Phillips, K.E., and Satchell, K.J.F. (2017). *Vibrio vulnificus*: From Oyster Colonist to Human Pathogen. *PLoS Pathog.* *13*, e1006053.

Piñeyro, P., Zhou, X., Orfe, L.H., Friel, P.J., Lahmers, K., and Call, D.R. (2010). Development of Two Animal Models To Study the Function of *Vibrio parahaemolyticus* Type III Secretion Systems. *Infect. Immun.* *78*, 4551–4559.

Pita, L., Rix, L., Slaby, B.M., Franke, A., and Hentschel, U. (2018). The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome* *6*, 46.

Polz, M.F., Hunt, D.E., Preheim, S.P., and Weinreich, D.M. (2006). Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* *361*, 2009–2021.

Pukatzki, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., Heidelberg, J.F., and Mekalanos, J.J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. U. S. A.* *103*, 1528–1533.

Pukatzki, S., Ma, A.T., Revel, A.T., Sturtevant, D., and Mekalanos, J.J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc. Natl. Acad. Sci. U. S. A.* *104*, 15508–15513.

Preheim, S.P., Boucher, Y., Wildschutte, H., David, L.A., Veneziano, D., Alm, E.J., and Polz, M.F. (2011a). Metapopulation structure of *Vibrionaceae* among coastal marine invertebrates. *Environ. Microbiol.* *13*, 265–275.

Preheim, S.P., Timberlake, S., and Polz, M.F. (2011b). Merging Taxonomy with Ecological Population Prediction in a Case Study of *Vibrionaceae*. *Appl. Environ. Microbiol.* *77*, 7195–7206.

-R-

R Core Team (2016). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing: Vienna, Austria. Available at: <https://www.R-project.org/>.

Raguénès, G., Christen, R., Guezennec, J., Pignet, P., and Barbier, G. (1997). *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *Int. J. Syst. Bacteriol.* *47*, 989–995.

Raz, N., Danin-Poleg, Y., Hayman, R.B., Bar-On, Y., Linetsky, A., Shmoish, M., Sanjuán, E., Amaro, C., Walt, D.R., and Kashi, Y. (2014). Genome-Wide SNP-Genotyping Array to Study the Evolution of the Human Pathogen *Vibrio vulnificus* Biotype 3. *PLoS ONE* *9*.

Reen, F.J., Almagro-Moreno, S., Ussery, D., and Boyd, E.F. (2006). The genomic code: inferring *Vibrionaceae* niche specialization. *Nat. Rev. Microbiol.* *4*, 697–704.

Reise, K., Buschbaum, C., Büttger, H., Rick, J., and Wegner, K.M. (2017). Invasion trajectory of Pacific oysters in the northern Wadden Sea. *Mar. Biol.* *164*, 68.

- Renault, T., Le Deuff, R.M., Cochenne, N., Chollet, B., and Maffart, P. (1995). Herpes-like viruses associated with high mortality levels in larvae and spat of Pacific oysters, *Crassostrea gigas*: a comparative study, the thermal effects on virus detection in hatchery-reared larvae, reproduction of the disease in axenic larvae. *Vet. Res.* 26, 539–543.
- Rendueles, O., and Ghigo, J.-M. (2015). Mechanisms of Competition in Biofilm Communities. *Microbiol. Spectr.* 3.
- Reynaud, Y., Saulnier, D., Mazel, D., Goarant, C., and Le Roux, F. (2008). Correlation between detection of a plasmid and high-level virulence of *Vibrio nigripulchritudo*, a pathogen of the shrimp *Litopenaeus stylirostris*. *Appl. Environ. Microbiol.* 74, 3038–3047.
- Rico Villa, B., Pouvreau, S., and Robert, R. (2009). Influence of food density and temperature on ingestion, growth and settlement of Pacific oyster larvae, *Crassostrea gigas*. *Aquaculture* 287, 395–401.
- Rico-Villa, B., Woerther, P., Mingant, C., Lepiver, D., Pouvreau, S., Hamon, M., and Robert, R. (2008). A flow-through rearing system for ecophysiological studies of Pacific oyster *Crassostrea gigas* larvae. *Aquaculture* 282, 54–60.
- Rivas, A.J., Balado, M., Lemos, M.L., and Osorio, C.R. (2011). The *Photobacterium damsela* subsp. *damsela* hemolysins *damselysin* and *HlyA* are encoded within a new virulence plasmid. *Infect. Immun.* 79, 4617–4627.
- Rivas, A.J., Balado, M., Lemos, M.L., and Osorio, C.R. (2013). Synergistic and additive effects of chromosomal and plasmid-encoded hemolysins contribute to hemolysis and virulence in *Photobacterium damsela* subsp. *damsela*. *Infect. Immun.* 81, 3287–3299.
- Rivas, A.J., Labella, A.M., Borrego, J.J., Lemos, M.L., and Osorio, C.R. (2014). Evidence for horizontal gene transfer, gene duplication and genetic variation as driving forces of the diversity of haemolytic phenotypes in *Photobacterium damsela* subsp. *damsela*. *FEMS Microbiol. Lett.* 355, 152–162.
- Rivas, A.J., Vences, A., Husmann, M., Lemos, M.L., and Osorio, C.R. (2015). *Photobacterium damsela* subsp. *damsela* major virulence factors *Dly*, plasmid-encoded *HlyA*, and chromosome-encoded *HlyA* are secreted via the type II secretion system. *Infect. Immun.* 83, 1246–1256.
- Rodriguez-Valera, F., Martin-Cuadrado, A.-B., Rodriguez-Brito, B., Pasić, L., Thingstad, T.F., Rohwer, F., and Mira, A. (2009). Explaining microbial population genomics through phage predation. *Nat. Rev. Microbiol.* 7, 828–836.
- Rohfritsch, A., Bierne, N., Boudry, P., Heurtebise, S., Cornette, F., and Lapègue, S. (2013). Population genomics shed light on the demographic and adaptive histories of European invasion in the Pacific oyster, *Crassostrea gigas*. *Evol Appl* 6, 1064–1078.
- Roig, F.J., González-Candelas, F., and Amaro, C. (2011). Domain organization and evolution of multifunctional autoprocessing repeats-in-toxin (MARTX) toxin in *Vibrio vulnificus*. *Appl. Environ. Microbiol.* 77, 657–668.
- Rosche, T.M., Yano, Y., and Oliver, J.D. (2005). A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiol. Immunol.* 49, 381–389.
- Rosenberg, E., Koren, O., Reshef, L., Efrony, R., and Zilber-Rosenberg, I. (2007). The role of microorganisms in coral health, disease and evolution. *Nat. Rev. Microbiol.* 5, 355–362.

Rowe-Magnus, D.A., Guerout, A.-M., Biskri, L., Bouige, P., and Mazel, D. (2003). Comparative analysis of superintegrons: engineering extensive genetic diversity in the Vibrionaceae. *Genome Res.* *13*, 428–442.

Rubio-Portillo, E., Yarza, P., Peñalver, C., Ramos-Esplá, A.A., and Antón, J. (2014). New insights into *Oculina patagonica* coral diseases and their associated *Vibrio* spp. communities. *ISME J.* *8*, 1794–1807.

Ruby, E.G., and Lee, K.H. (1998). The *Vibrio fischeri*-*Euprymna scolopes* Light Organ Association: Current Ecological Paradigms. *Appl. Environ. Microbiol.* *64*, 805–812.

Rui, H., Ritchie, J.M., Bronson, R.T., Mekalanos, J.J., Zhang, Y., and Waldor, M.K. (2010). Reactogenicity of live-attenuated *Vibrio cholerae* vaccines is dependent on flagellins. *Proc. Natl. Acad. Sci. U.S.A.* *107*, 4359–4364.

Ruiz-Masó, J.A., Machón, C., Bordanaba-Ruiseco, L., Espinosa, M., Coll, M., and Del Solar, G. (2015). Plasmid Rolling-Circle Replication. *Microbiol. Spectr.* *3*, PLAS-0035-2014.

-S-

Sakai T, Hirae T, Yuasa K, Kamaishi T, Matsuyama T, Miwa S, et al. Mass mortality of cultured kuruma prawn *Peneaus japonicus* caused by *Vibrio nigripulchritudo*. *Fish Pathol.* 2007;42:141–147.

Salomon, D., and Orth, K. (2015). Type VI secretion system. *Curr. Biol.* *CB 25*, R265-266.

Samain, JF., Summer Mortality of Pacific Oyster *Crassostrea Gigas*: The Morest Project-ebook

Satchell, K.J.F. (2007). MARTX, multifunctional autoprocessing repeats-in-toxin toxins. *Infect. Immun.* *75*, 5079–5084.

Satchell, K.J.F. (2015). Multifunctional-autoprocessing repeats-in-toxin (MARTX) Toxins of *Vibrios*. *Microbiol Spectr* *3*.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., and Garcia, C. (2010). A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. *Microb. Ecol.* *59*, 787–798.

Sauvage, C., Pépin, J.F., Lapègue, S., Boudry, P., and Renault, T. (2009). Ostreid herpes virus 1 infection in families of the Pacific oyster, *Crassostrea gigas*, during a summer mortality outbreak: differences in viral DNA detection and quantification using real-time PCR. *Virus Res.* *142*, 181–187.

Sawabe, T., Koizumi, S., Fukui, Y., Nakagawa, S., Ivanova, E.P., Kita-Tsukamoto, K., Kogure, K., and Thompson, F.L. (2009). Mutation is the Main Driving Force in the Diversification of the *Vibrio splendidus* Clade. *Microbes Environ.* *24*, 281–285.

Sawabe, T., Ogura, Y., Matsumura, Y., Feng, G., Amin, A.R., Mino, S., Nakagawa, S., Sawabe, T., Kumar, R., Fukui, Y., et al. (2013). Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Front. Microbiol.* *4*.

Schikorski, D., Faury, N., Pepin, J.F., Saulnier, D., Tourbiez, D., and Renault, T. (2011b). Experimental ostreid herpesvirus 1 infection of the Pacific oyster *Crassostrea gigas*: kinetics of virus DNA detection by q-PCR in seawater and in oyster samples. *Virus Res.* *155*, 28–34.

Schikorski, D., Renault, T., Saulnier, D., Faury, N., Moreau, P., and Pépin, J.-F. (2011). Experimental infection of Pacific oyster *Crassostrea gigas* spat by ostreid herpesvirus 1: demonstration of oyster spat susceptibility. *Vet. Res.* *42*, 27.

- Schild, S., Tamayo, R., Nelson, E.J., Qadri, F., Calderwood, S.B., and Camilli, A. (2007). Genes induced late in infection increase fitness of *Vibrio cholerae* after release into the environment. *Cell Host Microbe* 2, 264–277.
- Schmitt, P., Rosa, R.D., Duperthuy, M., de Lorgeril, J., Bachère, E., and Destoumieux-Garzón, D. (2012). The Antimicrobial Defense of the Pacific Oyster, *Crassostrea gigas*. How Diversity may Compensate for Scarcity in the Regulation of Resident/Pathogenic Microflora. *Front. Microbiol.* 3, 160.
- Seed, K.D., Faruque, S.M., Mekalanos, J.J., Calderwood, S.B., Qadri, F., and Camilli, A. (2012). Phase variable O antigen biosynthetic genes control expression of the major protective antigen and bacteriophage receptor in *Vibrio cholerae* O1. *PLoS Pathog.* 8, e1002917.
- Seed, K.D., Yen, M., Shapiro, B.J., Hilaire, I.J., Charles, R.C., Teng, J.E., Ivers, L.C., Boncy, J., Harris, J.B., and Camilli, A. (2014). Evolutionary consequences of intra-patient phage predation on microbial populations. *Elife* 3, e03497.
- Segarra, A., Mauduit, F., Faury, N., Trancart, S., Dégremont, L., Tourbiez, D., Haffner, P., Barbosa-Solomieu, V., Pépin, J.-F., Travers, M.-A., et al. (2014). Dual transcriptomics of virus-host interactions: comparing two Pacific oyster families presenting contrasted susceptibility to ostreid herpesvirus 1. *BMC Genomics* 15, 580.
- Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N., and Renault, T. (2010). Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. *Virus Res.* 153, 92–99.
- Seitz, P., and Blokesch, M. (2013). DNA-uptake machinery of naturally competent *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U. S. A.* 110, 17987–17992.
- Shapiro, B.J., and Polz, M.F. (2014). Ordering microbial diversity into ecologically and genetically cohesive units. *Trends Microbiol.* 22, 235–247.
- Shapiro, B.J., Friedman, J., Cordero, O.X., Preheim, S.P., Timberlake, S.C., Szabó, G., Polz, M.F., and Alm, E.J. (2012). Population genomics of early events in the ecological differentiation of bacteria. *Science* 336, 48–51.
- Shapiro, B.J., Levade, I., Kovacikova, G., Taylor, R.K., and Almagro-Moreno, S. (2016). Origins of pandemic *Vibrio cholerae* from environmental gene pools. *Nat. Microbiol.* 2, 16240.
- Shapiro BJ, Polz MF. (2015). Microbial Speciation. *Cold Spring Harb Perspect Biol.* 7(10):a018143.
- Shibata, Y., Nomoto, R., de Vries, G.C., and Osawa, R. (2013). Serendipitous Isolation of Non-*Vibrio* Bacterial Strains Carrying the Cholera Toxin Gene from Environmental Waters in Indonesia. *Int. J. Microbiol.* 2013.
- Simon, M., Grossart, H.-P., Schweitzer, B., and Ploug, H. (2002). Microbial ecology of organic aggregates in aquatic ecosystems. *Aquat. Microb. Ecol.* 28, 175–211.
- Singer, E., Webb, E.A., Nelson, W.C., Heidelberg, J.F., Ivanova, N., Pati, A., and Edwards, K.J. (2011). Genomic Potential of *Marinobacter aquaeolei*, a Biogeochemical “Opportunitroph” ∇. *Appl. Environ. Microbiol.* 77, 2763–2771.
- Singleton, F.L., Attwell, R., Jangi, S., and Colwell, R.R. (1982). Effects of temperature and salinity on *Vibrio cholerae* growth. *Appl. Environ. Microbiol.* 44, 1047–1058.

Smalla K, Jechalke S, Top EM. (2015). Plasmid Detection, Characterization, and Ecology. *Microbiol Spectr.* 2015;3(1):PLAS-0038-2014.

Smillie, C., Garcillán-Barcia, M.P., Francia, M.V., Rocha, E.P.C., and de la Cruz, F. (2010). Mobility of plasmids. *Microbiol. Mol. Biol. Rev. MMBR* 74, 434–452.

Smith MD, Roheim CA, Crowder LB, Halpern BS, Turnipseed M, Anderson JL, et al. Economics. Sustainability and global seafood. *Science.* 2010;327(5967):784-6.

Spratt, B.G., and Maiden, M.C. (1999). Bacterial population genetics, evolution and epidemiology. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 354, 701–710.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22, 2688–2690.

Stocker, R., and Seymour, J.R. (2012). Ecology and physics of bacterial chemotaxis in the ocean. *Microbiol. Mol. Biol. Rev. MMBR* 76, 792–812.

Stocker, R., Seymour, J.R., Samadani, A., Hunt, D.E., and Polz, M.F. (2008). Rapid chemotactic response enables marine bacteria to exploit ephemeral microscale nutrient patches. *Proc. Natl. Acad. Sci. U. S. A.* 105, 4209–4214.

Studier, F.W. (2005). Protein production by auto-induction in high density shaking cultures. *Protein Expr. Purif.* 41, 207–234.

Sunagawa, S., Coelho, L.P., Chaffron, S., Kultima, J.R., Labadie, K., Salazar, G., Djahanschiri, B., Zeller, G., Mende, D.R., Alberti, A., et al. (2015). Structure and function of the global ocean microbiome. *Science* 348, 1261359.

Sundberg, L.-R., Ketola, T., Laanto, E., Kinnula, H., Bamford, J.K.H., Penttinen, R., and Mappes, J. (2016). Intensive aquaculture selects for increased virulence and interference competition in bacteria. *Proc. Biol. Sci.* 283, 20153069.

Szabo, G., Preheim, S.P., Kauffman, K.M., David, L.A., Shapiro, J., Alm, E.J., and Polz, M.F. (2013). Reproducibility of Vibrionaceae population structure in coastal bacterioplankton. *ISME J* 7, 509–519.

Szurmant, H., and Ordal, G.W. (2004). Diversity in chemotaxis mechanisms among the bacteria and archaea. *Microbiol. Mol. Biol. Rev. MMBR* 68, 301–319.

-T-

Takemura, A.F., Chien, D.M., and Polz, M.F. (2014). Associations and dynamics of Vibrionaceae in the environment, from the genus to the population level. *Front. Microbiol.* 5, 38.

Terceti, M.S., Rivas, A.J., Alvarez, L., Noia, M., Cava, F., and Osorio, C.R. (2017). *rstB* Regulates Expression of the *Photobacterium damsela* subsp. *damsela* Major Virulence Factors Damselysin, Phobalysin P and Phobalysin C. *Front. Microbiol.* 8.

Teschler, J.K., Zamorano-Sánchez, D., Utada, A.S., Warner, C.J.A., Wong, G.C.L., Linington, R.G., and Yildiz, F.H. (2015). Living in the matrix: assembly and control of *Vibrio cholerae* biofilms. *Nat. Rev. Microbiol.* 13, 255–268.

Thompson, F.L., Gevers, D., Thompson, C.C., Dawyndt, P., Naser, S., Hoste, B., Munn, C.B., and Swings, J. (2005). Phylogeny and Molecular Identification of *Vibrios* on the Basis of Multilocus Sequence Analysis. *Appl. Environ. Microbiol.* 71, 5107–5115.

Thompson, F.L., Iida, T., and Swings, J. (2004). Biodiversity of vibrios. *Microbiol. Mol. Biol. Rev.* MMBR 68, 403–431, table of contents.

Thompson, J.A., Oliveira, R.A., and Xavier, K.B. (2014). Can chatter between microbes prevent cholera? *Trends Microbiol.* 22, 660–662.

Toffano-Nioche, C., Nguyen, A.N., Kuchly, C., Ott, A., Gautheret, D., Bouloc, P., and Jacq, A. (2012). Transcriptomic profiling of the oyster pathogen *Vibrio splendidus* opens a window on the evolutionary dynamics of the small RNA repertoire in the *Vibrio* genus. *RNA* 18, 2201–2219.

Tomich, M., Planet, P.J., and Figurski, D.H. (2007). The tad locus: postcards from the widespread colonization island. *Nat. Rev. Microbiol.* 5, 363–375.

Travers, M.-A., Boettcher Miller, K., Roque, A., and Friedman, C.S. (2015). Bacterial diseases in marine bivalves. *J. Invertebr. Pathol.* 131, 11–31.

Turner, J.W., Good, B., Cole, D., and Lipp, E.K. (2009). Plankton composition and environmental factors contribute to *Vibrio* seasonality. *ISME J* 3, 1082–1092.

Turner, J.W., Paranjpye, R.N., Landis, E.D., Biryukov, S.V., González-Escalona, N., Nilsson, W.B., and Strom, M.S. (2013). Population structure of clinical and environmental *Vibrio parahaemolyticus* from the Pacific Northwest coast of the United States. *PloS One* 8, e55726.

-U-

Unterweger, D., Kitaoka, M., Miyata, S.T., Bachmann, V., Brooks, T.M., Moloney, J., Sosa, O., Silva, D., Duran-Gonzalez, J., Provenzano, D., et al. (2012). Constitutive type VI secretion system expression gives *Vibrio cholerae* intra- and interspecific competitive advantages. *PloS One* 7, e48320.

Utada, A.S., Bennett, R.R., Fong, J.C.N., Gibiansky, M.L., Yildiz, F.H., Golestanian, R., and Wong, G.C.L. (2014). *Vibrio cholerae* use pili and flagella synergistically to effect motility switching and conditional surface attachment. *Nat. Commun.* 5, 4913.

-V-

Val, M.-E., Skovgaard, O., Ducos-Galand, M., Bland, M.J., and Mazel, D. (2012). Genome engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS Genet.* 8, e1002472.

Val, M.-E., Marbouty, M., de Lemos Martins, F., Kennedy, S.P., Kemble, H., Bland, M.J., Possoz, C., Koszul, R., Skovgaard, O., and Mazel, D. (2016). A checkpoint control orchestrates the replication of the two chromosomes of *Vibrio cholerae*. *Sci. Adv.* 2.

Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A., Le Fèvre, F., Longin, C., Mornico, D., Roche, D., et al. (2013). MicroScope--an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* 41, D636-647.

Vandecraen J, Chandler M, Aertsen A, Van Houdt R. The impact of insertion sequences on bacterial genome plasticity and adaptability. *Crit Rev Microbiol.* 2017;43(6):709-30.

Van der Henst, C., Scignari, T., Maclachlan, C., and Blokesch, M. (2016). An intracellular replication niche for *Vibrio cholerae* in the amoeba *Acanthamoeba castellanii*. *ISME J.* 10, 897–910.

Vanhove, A.S., Duperthuy, M., Charrière, G.M., Le Roux, F., Goudenège, D., Gourbal, B., Kieffer-Jaquinod, S., Couté, Y., Wai, S.N., and Destoumieux-Garzón, D. (2015). Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. *Environ. Microbiol.* 17, 1152–1165.

Vanhove, A.S., Rubio, T.P., Nguyen, A.N., Lemire, A., Roche, D., Nicod, J., Vergnes, A., Poirier, A.C., Disconzi, E., Bachère, E., et al. (2016). Copper homeostasis at the host vibrio interface: lessons from intracellular vibrio transcriptomics. *Environ. Microbiol.* *18*, 875–888.

Vezzulli, L., Brettar, I., Pezzati, E., Reid, P.C., Colwell, R.R., Höfle, M.G., and Pruzzo, C. (2012). Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. *ISME J.* *6*, 21–30.

Vezzulli, L., Grande, C., Reid, P.C., Hélaouët, P., Edwards, M., Höfle, M.G., Brettar, I., Colwell, R.R., and Pruzzo, C. (2016). Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. *Proc. Natl. Acad. Sci. U. S. A.* *113*, E5062–E5071.

Visick, K.L., Foster, J., Doino, J., McFall-Ngai, M., and Ruby, E.G. (2000). *Vibrio fischeri* lux genes play an important role in colonization and development of the host light organ. *J. Bacteriol.* *182*, 4578–4586.

-W-

Waechter, M., Le Roux, F., Nicolas, J.-L., Marissal, E., and Berthe, F. (2002). [Characterization of pathogenic bacteria of the cupped oyster *Crassostrea gigas*]. *C. R. Biol.* *325*, 231–238.

Watnick, P.I., Lauriano, C.M., Klose, K.E., Croal, L., and Kolter, R. (2001). The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. *Mol. Microbiol.* *39*, 223–235.

Waldor, M.K., and Mekalanos, J.J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* *272*, 1910–1914.

Watermann, B.T., Herlyn, M., Daehne, B., Bergmann, S., Meemken, M., and Kolodzey, H. (2008). Pathology and mass mortality of Pacific oysters, *Crassostrea gigas* (Thunberg), in 2005 at the East Frisian coast, Germany. *J. Fish Dis.* *31*, 621–630.

Wegner, K.M., Volkenborn, N., Peter, H., and Eiler, A. (2013). Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiol.* *13*, 252.

Wendling, C.C., Batista, F.M., and Wegner, K.M. (2014). Persistence, seasonal dynamics and pathogenic potential of *Vibrio* communities from Pacific oyster hemolymph. *PLoS One* *9*, e94256.

Wendling, C.C., and Wegner, K.M. (2015). Adaptation to enemy shifts: rapid resistance evolution to local *Vibrio* spp. in invasive Pacific oysters. *Proc. Biol. Sci.* *282*, 20142244.

West, P.A., and Lee, J.V. Ecology of *Vibrio* species, including *Vibrio cholerae*, in natural waters of Kent, England. *J. Appl. Bacteriol.* *52*, 435–448.

Wildschutte, H., Preheim, S.P., Hernandez, Y., and Polz, M.F. (2010). O-antigen diversity and lateral transfer of the wbe region among *Vibrio splendidus* isolates. *Environ. Microbiol.* *12*, 2977–2987.

Wiedenbeck, J., and Cohan, F.M. (2011). Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiol. Rev.* *35*, 957–976.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998). Prokaryotes: The unseen majority. *Proc. Natl. Acad. Sci. U. S. A.* *95*, 6578–6583.

-X-

Xu, Q., Dziejman, M., and Mekalanos, J.J. (2003). Determination of the transcriptome of *Vibrio cholerae* during intrainestinal growth and midexponential phase in vitro. *Proc. Natl. Acad. Sci. U. S. A.* *100*, 1286–1291.

Xue, H., Cordero, O.X., Camas, F.M., Trimble, W., Meyer, F., Guglielmini, J., Rocha, E.P.C., and Polz, M.F. (2015). Eco-Evolutionary Dynamics of Episomes among Ecologically Cohesive Bacterial Populations. *MBio* *6*, e00552-00515.

-Y-

Yawata, Y., Cordero, O.X., Menolascina, F., Hehemann, J.-H., Polz, M.F., and Stocker, R. (2014). Competition-dispersal tradeoff ecologically differentiates recently speciated marine bacterioplankton populations. *Proc. Natl. Acad. Sci. U. S. A.* *111*, 5622–5627.

Yildiz, F.H., and Visick, K.L. (2009). *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol.* *17*, 109–118.

Yip, E.S., Geszvain, K., DeLoney-Marino, C.R., and Visick, K.L. (2006). The symbiosis regulator *rscS* controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. *Mol. Microbiol.* *62*, 1586–1600.

-Z-

Zerbino, D.R., and Birney, E. (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res.* *18*, 821–829.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H., et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* *490*, 49–54.

Zhou, X., Gewurz, B.E., Ritchie, J.M., Takasaki, K., Greenfeld, H., Kieff, E., Davis, B.M., and Waldor, M.K. (2013). A *Vibrio parahaemolyticus* T3SS effector mediates pathogenesis by independently enabling intestinal colonization and inhibiting TAK1 activation. *Cell Rep.* *3*, 1690–1702.

Zhu, S., Kojima, S., and Homma, M. (2013). Structure, gene regulation and environmental response of flagella in *Vibrio*. *Front. Microbiol.* *4*, 410.