The ISME Journal
April 2017, Volume 11, Issue 4, Pages 1043-1052
http://dx.doi.org/10.1038/ismej.2016.162
http://archimer.ifremer.fr/doc/00360/47137/
© 2016 International Society for Microbial Ecology All rights reserved

Achimer
http://archimer.ifremer.fr

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

Bruto Maxime [1, 2], James Adèle [1, 2], Petton Bruno [1, 3], Labreuche Yannick [1, 2], Chenivesse Sabine [2], Alunno-Bruscia Marianne [1, 3], Polz Martin F [4, *], Le Roux Frédérique [1, 2, *]

[1] Ifremer, Unité Physiologie Fonctionnelle des Organismes Marins, ZI de la Pointe du Diable, Plouzané, France
[2] Sorbonne Universités, UPMC Paris 06, CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France
[3] Ifremer, LEMAR UMR 6539, 11 presqu'île du Vivier, Argenton-en-Landunvez, France
[4] Parsons Laboratory for Environmental Science and Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA

* Corresponding authors : Frédérique Le Roux, email address : frederique.le-roux@sb-roscoff.fr ; Martin F. Polz, email address : mpolz@mit.edu

Abstract :

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.

Please note that this is an author-produced PDF of an article accepted for publication following peer review. The definitive publisher-authenticated version is available on the publisher Web site.

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 *V. 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 7.3 L h^{-1} gr^{-1} dry tissue weight) (Cognie et al., 2003), which act like a sieve capturing food particles in the optimum range of 5-10 µM. 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 (AMPs) that constitute a chemical barrier in the colonization process (Destoumieux-Garzon et al., 2016; Lokmer et al., 2016; Pales Espinosa et al., 2016). Finally, since 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.

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., 2015a; Petton et al., 2015b; Petton et al., 2013) 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 <1 cfu 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, *i.e.*, 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.

MATERIAL 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. Since previous work has demonstrated that the disease occurs beyond a temperature threshold of ~16°C (Petton et al., 2015a), we tested infection at two time points in the summer (>16°C)

and, for comparison, two time points in spring (<16°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°C (previously shown to allow the disease to proceed more rapidly (Lemire et al., 2014)) 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°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 µm 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 µm plankton net and sequentially filtered through 5, 1 and 0.22 µm pore size filters. All fractions were collected in four replicates.

Bacterial isolation and gene sequencing

The 5, 1 and 0.22 µm 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 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 g/l agar, 4 g/l bactopeptone and 1 g/l yeast extract in artificial sea water, 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 1,635 *hsp60* sequences were obtained from the 4 experiments (Table S1).

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; Fig. S1) while the others were unknown species. For one representative strain of each identified taxon, the assignment was confirmed by multilocus sequence typing using 3 additional protein-coding genes (*rpoD*, *gyrB* and *rctB*) (Table S2).

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: >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 since 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, 2015; URL http://www.R-project.org/). 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-val* ≤ 0.05. Statistical analysis of population distribution in oyster tissues was tested using the parametric test of ANOVA since 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 plasmid used and construct in the present study are described in Table S3 and S4. *Vibrio* isolates were grown in Zobell broth or agar (4 g/l bactopeptone and 1 g/l yeast extract in artificial sea water, pH 7.6), 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 (5 or 25µg/ml for *Vibrio* and *E. coli,* respectively), spectinomycin (100µg/ml), thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction of the P_{BAD} 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 (Le Roux et al., 2011; Rui et al., 2010) was used to introduce green or red fluorescent protein (GFP or RFP) constitutively expressed from the P_{LAC} promoter into the *Vibrio* strains. Alternatively, the P_{LAC}-*gfp* and Cm^R 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^R marked version of the pGV1512 (pGV1512::Cm^R). To this end, a 500-bp fragment of pGV1512 (transposase fragment, position 90957 to 91768 in J5-20) was PCR amplified using Primer pGV1512-1 and 2 (Table S5) and cloned in pSW23T (Demarre et al., 2005). After conjugative transfer, selection of the plasmid-borne drug marker (Cm^R) 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 $Spec^R$ cassette in a chromosomal locus (ΔR1::$Spec^R$) by allelic exchange as described previously (Lemire et al., 2014).

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_{BAD} (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 500bp 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^R) and recipient (*e.g.,* ΔR1::Spec^R) at a ratio of 1/1 on LBA + NaCl 0.5M and incubating at 20°C for 24 hours.

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 (Clontech) (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) 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 (10^6 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 2.5 L aquarium) containing 1 liter of aerated 5 µm-filtered seawater at 20°C, kept under static conditions. Experiments were performed in duplicate and mortality was assessed after 24 hours. 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 (10^5 cfu/animal, *i.e.* sub-lethal dose) or added to the oyster tank (10^7cfu/mL). Hemolymph was collected after 24 h, serial diluted and spread on LB-agar (LBA) + NaCl 0.5M supplemented with Cm.

RESULTS

Population structure and dynamics

To compare the *Vibrio* population structure in seawater and oyster tissues, a total of 1,635 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 (Fig. 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 (Fig. 1 and 2). While 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 (*e.g.*, 5 May: *Vibrio sp*. #9; 29 July: *V. chagasii* #3) Fig.2) suggesting short-lived population expansions. Since 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

While the comparison of population frequency in the water and oyster samples suggested little discrimination for many populations, some were unequally distributed (Fig.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 (Table S6, Fig.S2). The analysis revealed a significant positive correlation between oysters and 10 of 24 populations (*V. harveyii* #5, *V. crassostreae* #11, *V. splendidus* #17 and 19, *V. tasmaniensis* # 23 and 24, *Vibrio* spp. #6, 7, 8, 12) (Table S6, in bold; Fig.S2E). However, of these, only *V. crassostreae* showed a negative association with all seawater fractions (Table S6). This suggests that *V. crassostreae* is the only population specifically associated with oysters since all others also occur in seawater fractions at considerable 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 (Table S7). Among the populations positively associated with oysters (Table S7, 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 (Fig. 2). No mortalities were observed in spring (<16°C), whereas 50% cumulative mortalities were detected in summer (>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 (Fig. 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., 2014) 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., 2014), 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., 2014). 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 >50% mortality) while the remainder induced <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 (Fig. S3A). 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 (Fig. 3A). This plasmid was then identified in all virulent strains with a sequence identity of 99.4% in the common regions (Fig. 3A). Finally, the existence of the replicon detected by gel staining was confirmed by Southern blot (Fig. S3B). This plasmid was successfully detected in 1/10 (10%) and 152/196 (77%) of *V. crassostreae* strains isolated from seawater and oysters, respectively (Fig. S4). 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 (Fig.3B).

The manual annotation of the pGV1512 plasmid revealed 206 open reading frames (ORFs) (Fig.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 IV secretion system, T4SS, the coupling protein and the relaxase, Fig.S5), (iii) a heavy metal resistance cluster (Cus/Cop), and (iv) a type VI secretion system (T6SS, Fig.S6). 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 J5-20, J2-9 and the J2-9-Spec^R derivative ΔR1 (Fig. 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 (Fig 4B). A restoration of the ΔR1 phenotype was observed after pGV1512 mobilization into J2-9 ΔR1-ΔpGV1512 (J2-9 ΔR1-ΔpGV1512+pGV1512Cm^R), 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 (Fig.3A) did not result in virulence attenuation (Fig.S7). However, the deletion of the regions between *cus/cop* and T6SS (Px2), T6SS and T4SS (Px3) (Fig.3A), dramatically affected the virulence of *V. crassostreae* (Fig.S7). No putative virulence factor could be identified in the Px2 and 3 loci based on sequence homology searches (Table S8), suggesting that virulence determinants encoded by pGV1512 are novel.

The pGV1512 plasmid is not necessary for oyster colonization

Since 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 (10^7cfu/ml) and their ratios determined (Fig.5A). The colonization by *V. crassostreae* was found to remain high (10^4-10^5 cfu/ml hemolymph) for up to four days, whereas *V. cyclitrophicus* was initially slow to colonize, then briefly rose to higher density but decreased dramatically on days 3 and 4 (10^3 cfu/ml). This result was independent of *V. crassostreae* carrying the plasmid (Fig. 5A) and was further confirmed by using strains expressing the GFP from a chromosomal locus (Fig. S8). Here again for three days we observed a two logs difference in colonization between a *V. crassostreae* cured from the plasmid (10^5 cfu/ml, and *V. cyclitrophicus* (10^3 cfu/ml), although from day 5 both strain loads was found to be 10^3-10^2 cfu/ml.

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 (Fig.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., 2014). Again we did not observe any clear effect of the plasmid loss on hemolymph colonization (Fig. S9), confirming that the plasmid is not necessary for colonization and/or survival in the host.

DISCUSSION

Although vibrios are frequently associated with mortality of farmed species, their role as causative 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 (*e.g.,* 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. tasmaniensis* (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., 2014). Interestingly some of these genes encode for colonization factors (*tad* gene cluster, (Tomich et al., 2007)) and for resistance to AMPs (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 microenvironments 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.

ACKNOWLEDGEMENTS

We thank the staff of the station Ifremer Argenton and Bouin, the ABIMS and CRBM (Roscoff) and LABGeM (Evry) plateforms for technical support. The present study has been supported by the ANR (13-ADAP-0007-01« OPOPOP», MB fundings), Ifremer (MB and AJ fundings) and Region Bretagne (AJ funding).

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. There is no conflict of interest. The material represents an original result and has not been submitted for publication elsewhere.

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, and 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 (IncPalpha) 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* **371**:.

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. *Cellular microbiology* **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. *Isme J* **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 *Crassostreae gigas*. *Aquaculture 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*. *Aquacult 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.

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. *Nature Reviews Microbiology* **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 genetics* **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**: D636-647.

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.

TITLES AND LEGENDS TO FIGURES

Figure 1: Population structure prediction of *Vibrionaceae* bacteria recovered from different seawater fractions or oyster tissues in two seasons. (**A**) Phylogenetic tree of 1,635 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 grey 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 >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 (*i.e*., 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 fractions/oysters replicates 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).

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 shows the open reading frames encoding replication and partitioning; a type4 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 (grey) or 50 strains without (white) plasmid (detected by a PCR against a pGV1512 fragment). A total of 10^6 cfu of the strains was intramuscularly injected into oysters (n= 20, in duplicate). Mortality (%) was assessed after 24 hours.

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. 2014, for details). **B.** Virulence assessment of the different genetic constructs (x-axis) after injection of strains (10^6 cfu/animal) in 20 oysters and counting the percentage of mortalities (y-axis).

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^R cassette into the *Vibrio* strains. **A-** GFP labeled strains (*V. cyclitrophicus,* strain 7T5-8, grey triangle; *V. crassostreae*, strain J2-9, black circle; J2-9ΔpGV1512 white circle) were individually added to the oyster tank (10^7cfu/ml). 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 cfu/ml of hemolymph retrieved on plates. The horizontal bar in each experiment indicates the mean of log cfu/ml. **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.

FIGURE 1.

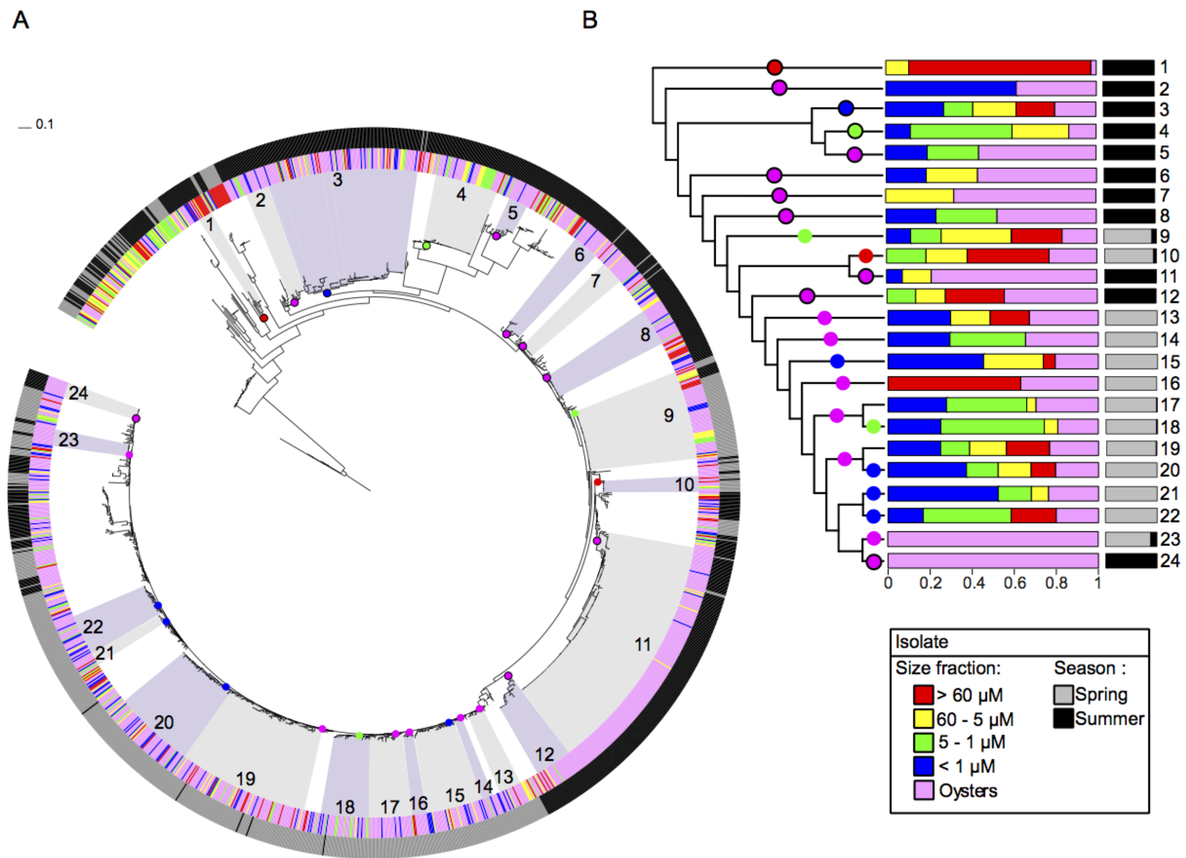


FIGURE 2

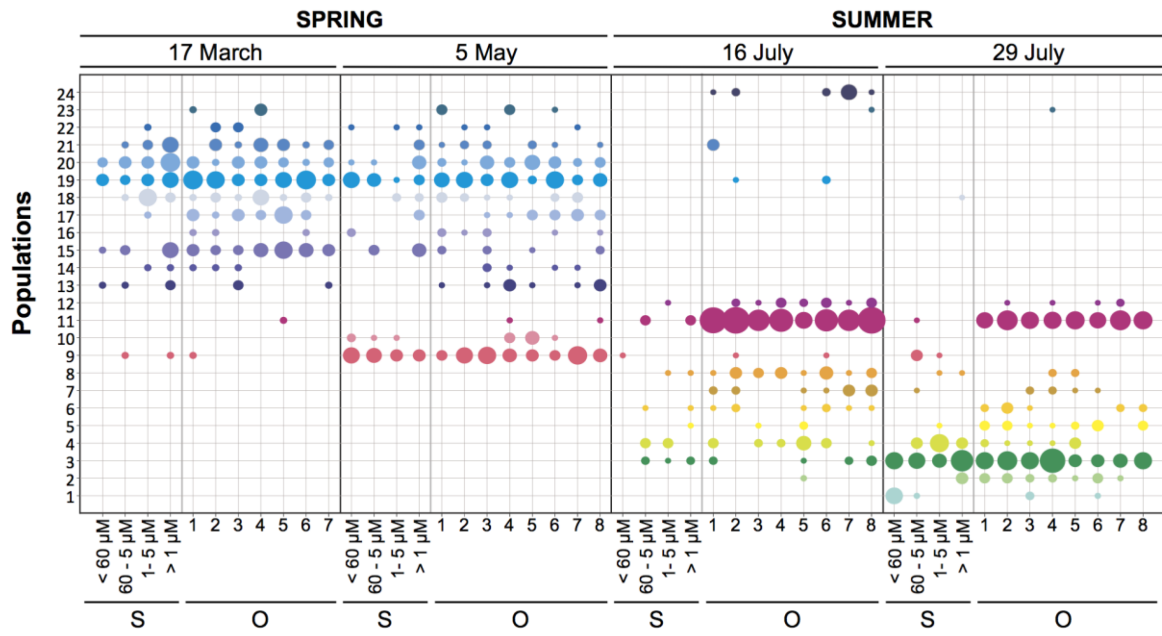


FIGURE 3

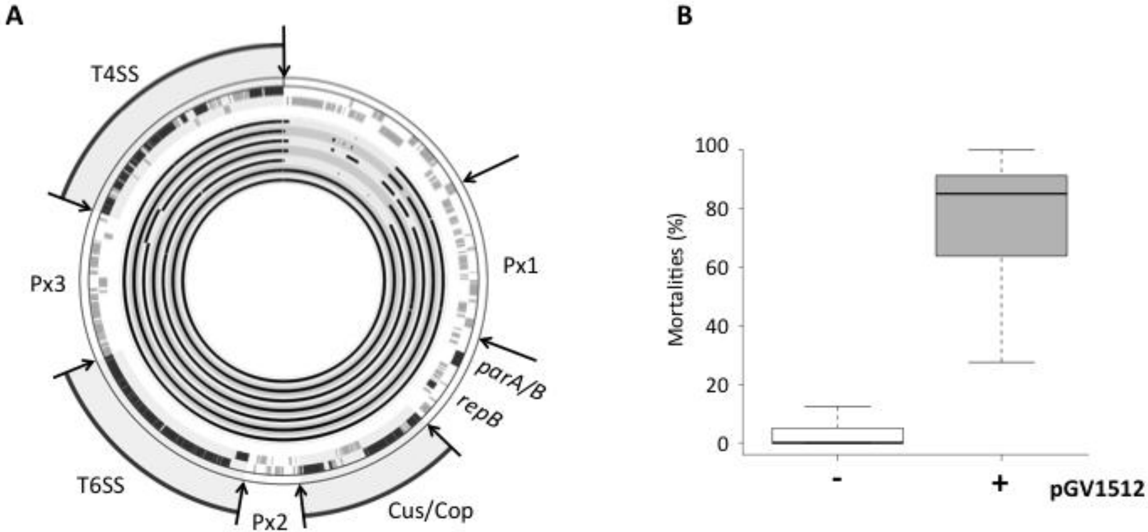
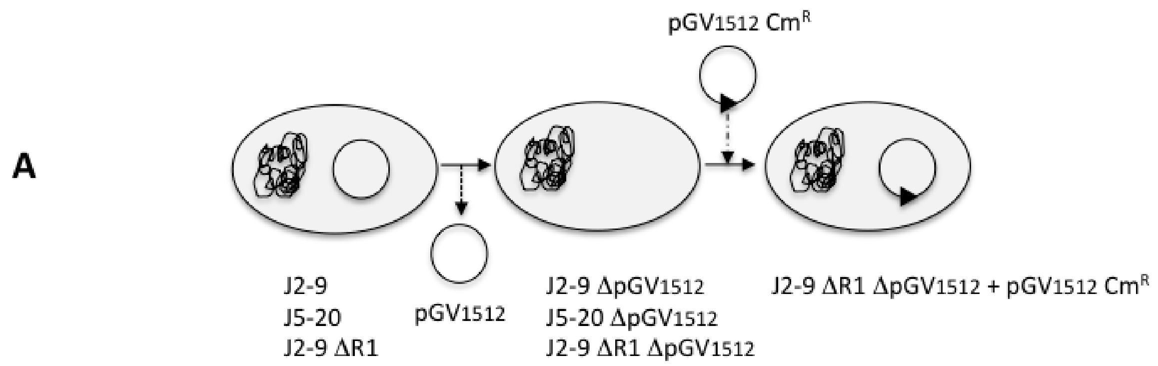


FIGURE 4



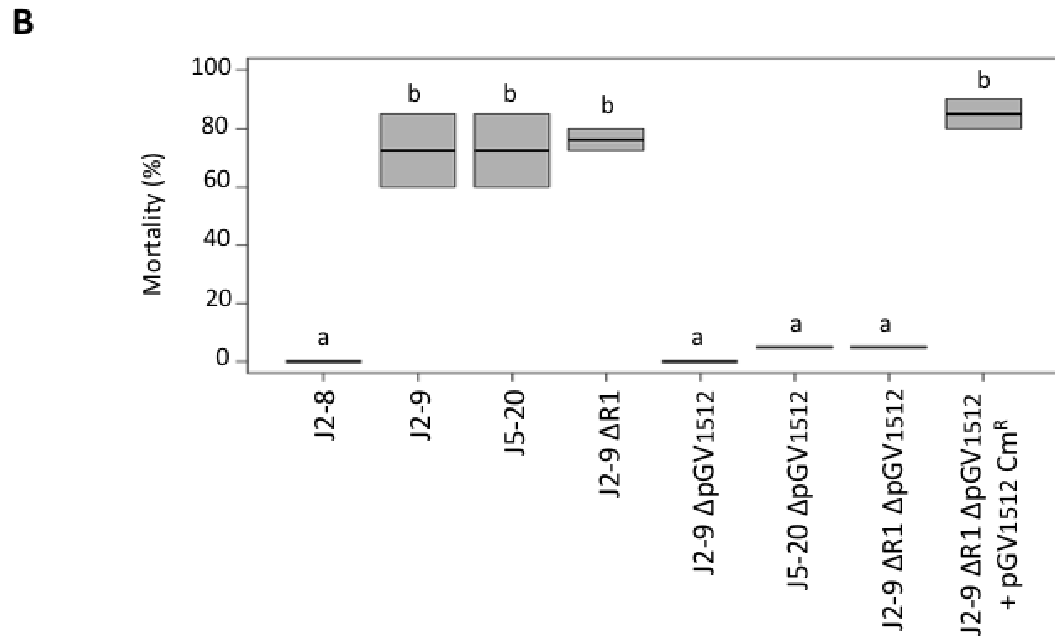
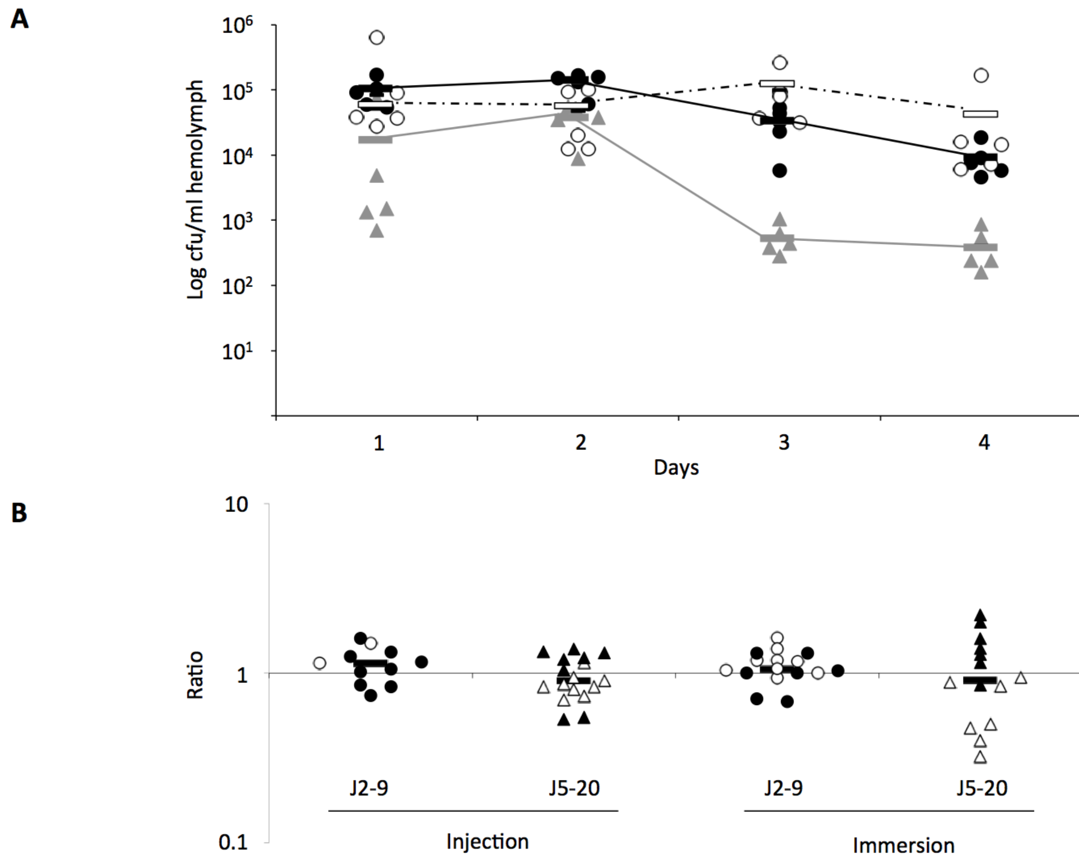


FIGURE 5



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 spermatozoids 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 begininng 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 µm3 µl−1 (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 tissus) 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 $MgCl_2$ 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 bactopeptone 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 $Spec^R$ recipient (J2-9ΔR1), three independent PCR amplifications were performed using primers ΔT6SS-1 to 6 (Table S5) and ΔR1-1 to 6 respectivelly. 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_{600nm} 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^R 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 (IncPalpha) 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.

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

	Spring		Summer	
Category	**17 March**	**5 May**	**16 July**	**29 July**
> 60 µM	30	32	22	43
60-5 µM	20	34	24	46
5-1 µM	36	35	21	41
1-0.2□□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*).

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

Table S3: Strains used in this study.

Strain	Description	Reference
Π3813	*lacIQ, thi1, supE44, endA1, recA1, hsdR17, gyrA462, zei298::Tn10*, *ΔthyA::(erm-pir116)* [Tc^R Erm^R]	(Le Roux et al., 2007)
β3914	(F^-) RP4-2-Tc::Mu ΔdapA ::*(erm-pir116), gyrA462, zei298::Tn10* [Km^R Em^R Tc^R]	(Le Roux et al., 2007)
GV1460	J2-8, *Vibrio sp.* (accession number PRJEB5890)	(Lemire et al., 2014)
GV1508	J2-9, *V. crassostreae* (accession number PRJEB5876)	(Lemire et al., 2014)
GV1512	J5-20, *V. crassostreae* (accession number PRJEB5882)	(Lemire et al., 2014)
GV218	J2-5, *V. cyclitrophicus*	(Lemire et al., 2014)
GV973	J2-9 *ΔR1::(aadA7)* [$Spec^R$]	(Lemire et al., 2014)
GV1633	7T5-8, *V. cyclitrophicus*	This study
GV1634	7T2-5, *V. crassostreae*	This study
GV1635	7T8-11, *V. crassostreae*	This study
GV1636	7T7-1, *V. crassostreae*	This study
GV1637	8D7-1, *V. crassostreae*	This study
GV1638	8H2-1, *V. crassostreae*	This study
GV1639	8H8-3, *V. crassostreae*	This study
GV1476	J2-9 + pMRB-P_{LAC}*gfp*	This study
GV1478	J2-9 + pMRB-P_{LAC}*rfp*	This study
GV1488	J5-20 + pMRB-P_{LAC}*gfp*	This study
GV1490	J5-20 + pMRB-P_{LAC}*rfp*	This study
GV1470	J2-8 + pMRB-P_{LAC}*gfp*	This study
GV1471	J2-8 + pMRB-P_{LAC}*rfp*	This study
GV1398	7T5-8 + pMRB-P_{LAC}*gfp*	This study
GV1397	7T5-8 + pMRB-P_{LAC}*rfp*	This study
GV1474	J2-9ΔpGV1512+ pMRB-P_{LAC}*gfp*	This study
GV1475	J2-9ΔpGV1512+ pMRB-P_{LAC}*rfp*	This study
GV1472	J5-20ΔpGV1512+ pMRB-P_{LAC}*gfp*	This study

GV1473	J5-20ΔpGV1512+ pMRB-P_{LAC}*rfp*	This study
GV1440	J5-20 *ΔpGV1512*	This study
GV1438	J2-9 *ΔpGV1512*	This study
GV1436	J2-9 *ΔR1::(aadA7)*, *ΔpGV1512* [Spec ^{R}]	This study
GV1424	J5-20 *pGV1512-Tr::pSW23T* [Cm^{R}]	This study
GV1458	GV1436 + *pGV1512::pSW23T* [Spec ^{R} Cm^{R}]	This study
GV1539	J5-20 *pGV1512-ΔT6SS::(aadA7)* [Spec ^{R}]	This study
GV1589	J5-20 *pGV1512-Δcus/cop-Tr::pSW23T* [Cm^{R}]	This study
GV1593	J2-9 *pGV1512-ΔT4SS-Tr::pSW23T* [Cm^{R}]	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) $_{LGP32}$, P_{LAC}-*gfp* □Cm^{R}□ *ΔpGV1512*	This study
GV1596	J5-20 :: pSW23T :: (II900891-901985) $_{LGP32}$, P_{LAC}-*gfp* □Cm^{R}□ *ΔpGV1512*	This study

Table S4: Plasmids used in this study

Plasmid	Description	Reference
pSW23T	*oriV*$_{R6K}$; *oriT*$_{RP4;}$ Cm^{R}	(Demarre et al., 2005)
pSW23T::Fvir	pSW23T *::* (2700-3400) $_{pGV1512}$ Cm^{R}	This study
pSW7848T	*oriV*$_{R6K}$; *oriT*$_{RP4}$; *araC*-P$_{BAD}$*ccdB;* Cm^{R}	(Val et al., 2012)
pSW7848T::Fvir	pSW7848T *::* (2700-3400) $_{pGV1512}$ Cm^{R}	This study
pSW3654T	pSW23T :: (II900891-901985) $_{LGP32}$, P$_{LAC}$-*gfp* Cm^{R}	(Duperthuy et al., 2011)
pSWδcus/copT	pSW7848T:: *Δcus/cop*	This study
pSWδT4SST	pSW7848T:: *ΔT4SS*	This study
pSWδT6SST	pSW7848T :: *ΔT6SS*	This study
pSWδPx1T	pSW7848T :: *ΔPx1*	This study
pSWδPx2	pSW7848T :: *ΔPx2*	This study
pSWδPx3	pSW7848T :: *ΔPx3*	This study
pMRB-P$_{LAC}$*gfp*	*oriV*$_{R6K}$; *oriT*$_{RP4}$; *oriV*$_{pB1067\,;}$ P$_{LAC}$-*gfp* Cm^{R}	(Le Roux et al., 2011)
pMRB-P$_{LAC}$*rfp*	*oriV*$_{R6K}$; *oriT*$_{RP4}$; *oriV*$_{pB1067\,;}$ P$_{LAC}$- *rfp* Cm^{R}	(Le Roux et al., 2011)

Table S5: Primers used in this study

Primer	Sequence 5'-3'
Hsp60F	GAATTCGAIIIIGCIGGIGAYGGIACIACIAC
Hsp60R	CGCGGGATCCYKIYKITCICCRAAICCIGGIGCYTT
pGV1512-1	GCCCGGATCCATGAATAAGAAAACATTACC
pGV1512-2	GCCCGGATCCGTTGCGTTGCTTGGACTGAG
SW-F	GAATTCCTGCAGCCCGGGGG
SW-R	GAATTCGATATCAAGCTTATCGATAC
ΔT6SS-1	GTATCGATAAGCTTGATATCGAATTCGTTACCAGCAGCAAGAGCAA
ΔT6SS-2	CGTCACAGGTATTTATTCGGCGGCCAATTAAGACGGTTGGCA
ΔT6SS-3	TGCCAACCGTCTTAATTGGCCGCCGAATAAATACCTGTGACG
ΔT6SS-4	ACGAGCTATGCTATTGGCACGATATCGTCGCAGACCAAAACG
ΔT6SS-5	CGTTTTGGTCTGCGACGATATCGTGCCAATAGCATAGCTCGT
ΔT6SS-6	CCCCCGGGCTGCAGGAATTCGTGTCGGTAGCATAGCAAGA
ΔCus/Cop-1	GTATCGATAAGCTTGATATCGAATTCCCTGTCCAGTGTTGAATAGG
ΔCus/Cop-2	CGTCGGTCAAAGTAGACGGCCGTTCACTCTAAGAAACCGC
ΔCus/Cop-3	GCGGTTTCTTAGAGTGAACGGCCGTCTACTTTGACCGACG
ΔCus/Cop-4	CCCCCGGGCTGCAGGAATTCGAAGGAAACCCAAACCGGCC
ΔT4SS-1	GTATCGATAAGCTTGATATCGAATTCGCTGCTGAGGAAAATGTGCG
ΔT4SS-2	GCGATACGCATGGATGAAGGCGACTTTGCTTCGTACATGG
ΔT4SS-3	CCATGTACGAAGCAAAGTCGCCTTCATCCATGCGTATCGC
ΔT4SS-4	CCCCCGGGCTGCAGGAATTCGGATCTTCTGTTGATGCTGC
ΔPx1-1	GTATCGATAAGCTTGATATCGAATTCGCTCATACTTATCGCCTCAC
ΔPx1-2	CCTTTGCGTGTTCAAGTCACGTCAAAATACCAAGGGTGGC
ΔPx1-3	GCCACCCTTGGTATTTTGACGTGACTTGAACACGCAAAGG
ΔPx1-4	CCCCCGGGCTGCAGGAATTCCATTGGGTGCCCTTAATGCC
ΔPx2-1	GTATCGATAAGCTTGATATCGAATTCCCTGTCCAGTGTTGAATAGG
ΔPx2-2	CGTCGGTCAAAGTAGACGGCCGTTCACTCTAAGAAACCGC
ΔPx2-3	GCGGTTTCTTAGAGTGAACGGCCGTCTACTTTGACCGACG

ΔPx2-4	CCCCCGGGCTGCAGGAATTCGAAGGAAACCCAAACCGGCC
ΔPx3-1	GTATCGATAAGCTTGATATCGAATTCCTGAAACGCATGTCAGCTCG
ΔPx3-2	CCGTGTCTTTCATGGCTTGCGTGTGCGAAAGCACTTGACC
ΔPx3-3	GGTCAAGTGCTTTCGCACACGCAAGCCATGAAAGACACGG
ΔPx3-4	CCCCCGGGCTGCAGGAATTCCCCACCAATAAAGTGACAGG

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

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

	Gills	**Digestive gland**	**Hemolymph**
V. fortis (#2)	o	o	-
V. chagasii (#3)	-	+	o
Vibrio sp.(#4)	o	+	o
***V. harveyi* (#5)**	o	o	o
***Vibrio* sp. (#6)**	+	-	o
***Vibrio* sp. (#7)**	o	o	o
***Vibrio* sp. (#8)**	+	-	o
Vibrio sp. (#9)	o	o	o
***V. crassostreae* (#11)**	+	-	+
***Vibrio* sp. (#12)**	o	o	o
V. splendidus (#13)	o	o	o
V. splendidus (#14)	o	o	o
***V. splendidus* (#17)**	o	o	o
V. splendidus (#18)	o	o	o
***V. splendidus* (#19)**	o	+	-
V. splendidus (#20)	o	o	o
V. splendidus (#21)	o	o	o

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

Region	Gene label	Annotation	Size (bp)
Px2	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
Px3	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

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.

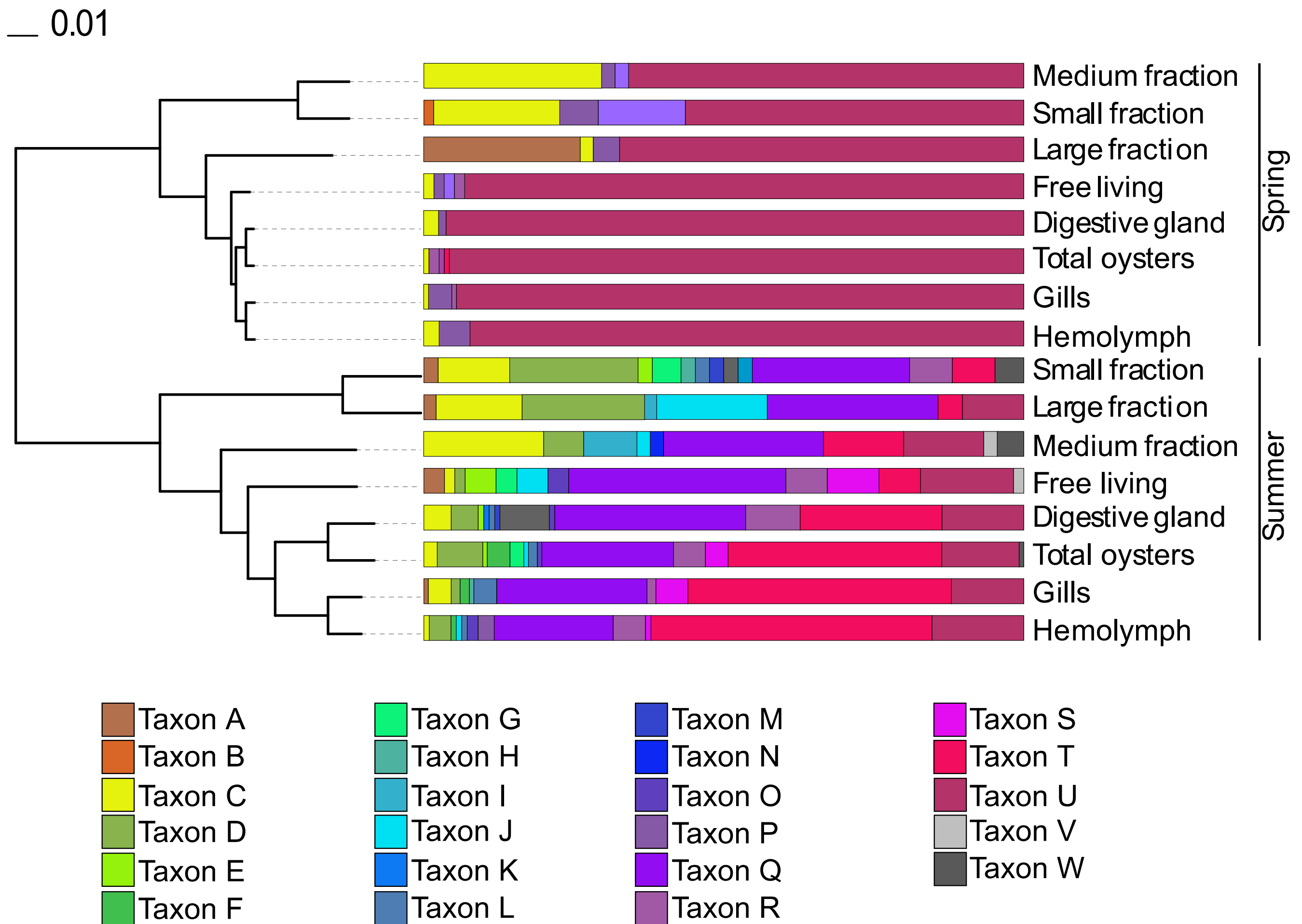
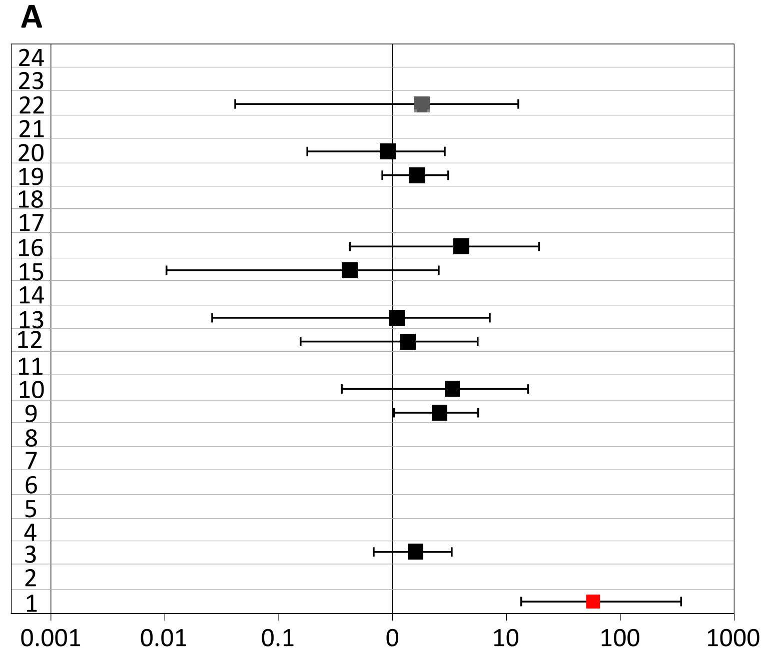
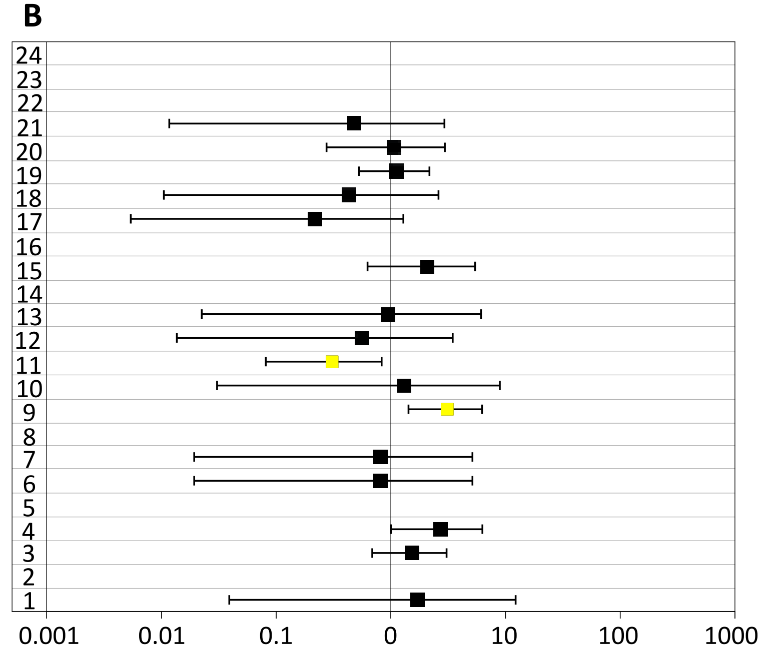
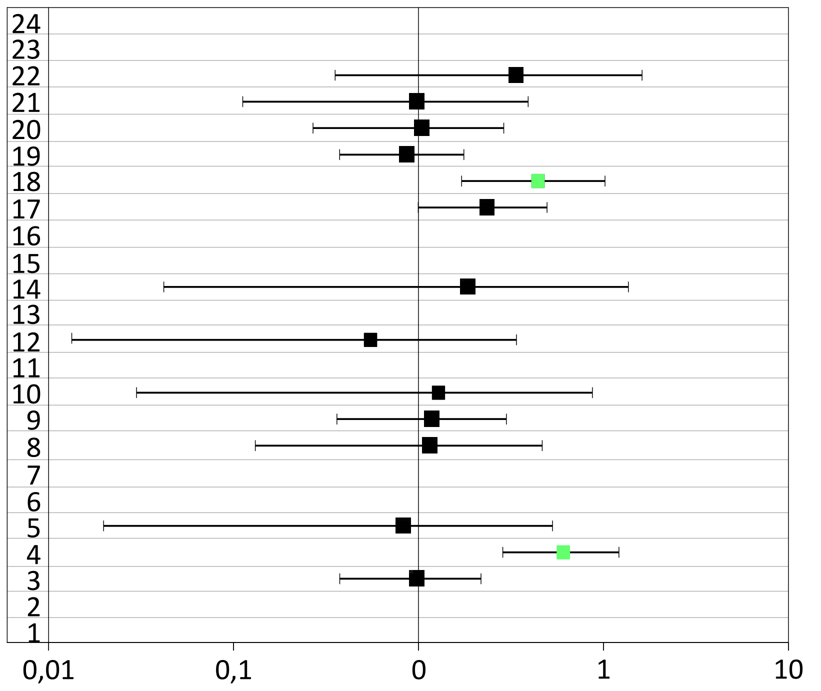


Figure S2: Fisher exact-test for the 24 populations in seawater fractions (**A** : >60µm ; **B** : 60-5µm ; **C** : 5-1µm ; **D** : >1µm) and oysters (**E**). The x-axis represents the odds-ratio retrieved from the test. The error bars indicate the range of the 95% confidence interval. The y-axis represents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

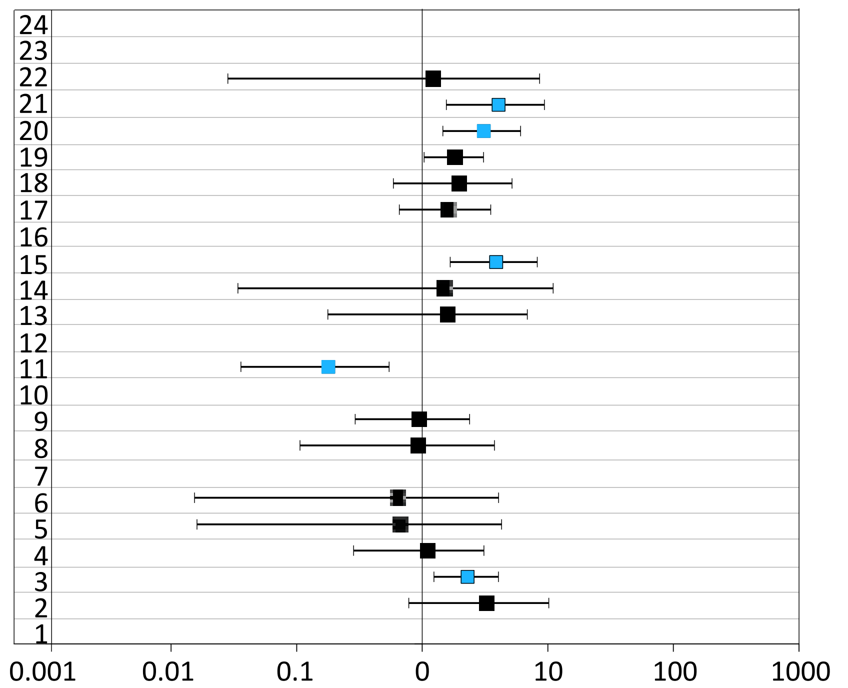




C



D



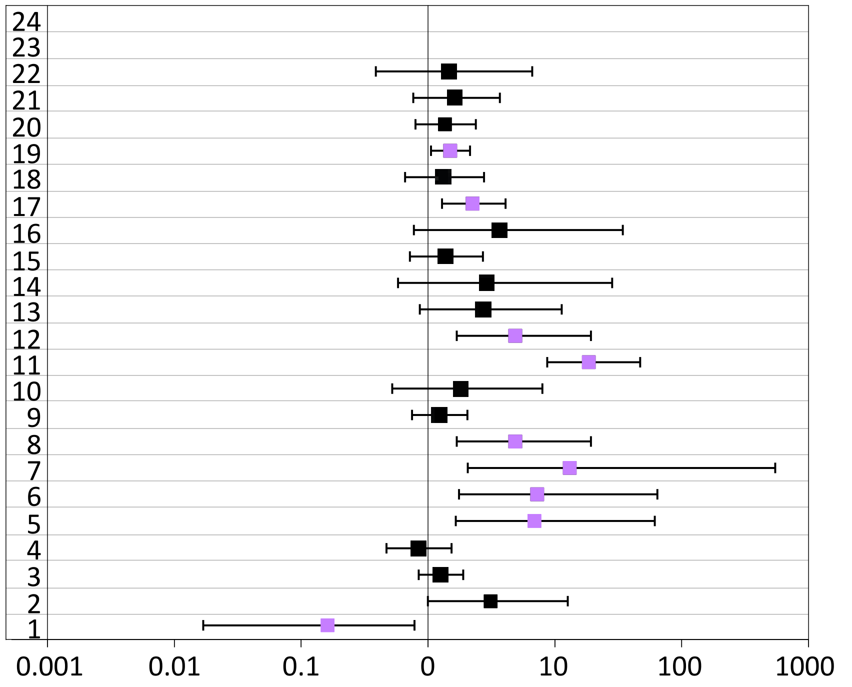
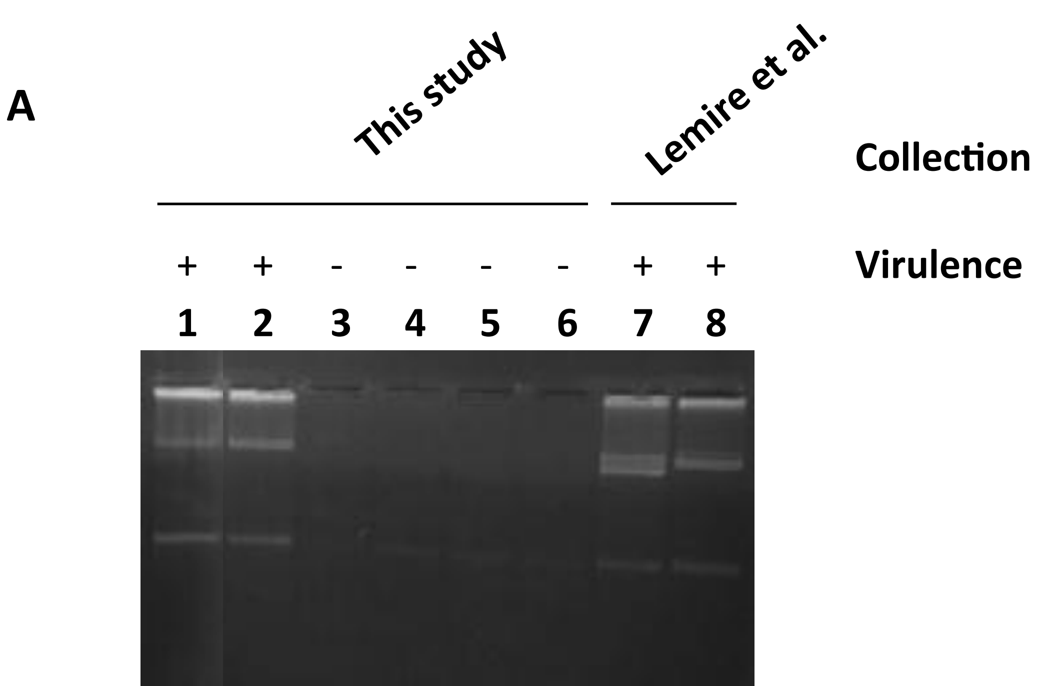

E
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0.001
0.01
0.1
0
10
100
1000

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.



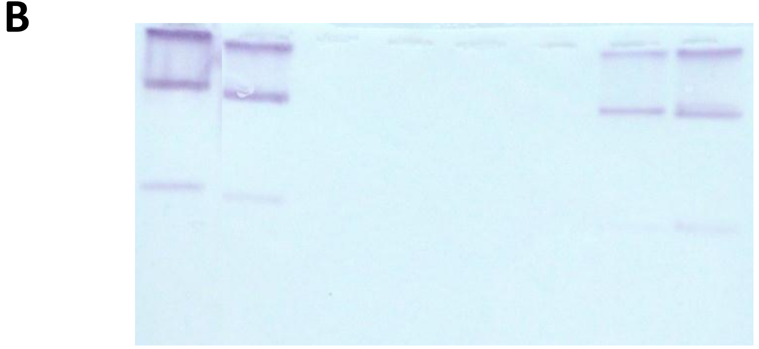


Figure S4: Phylogenetic tree of *hsp60* genes built by the Neighbor Joining method (Kimura 2 parameters, 472 sites) of *V. crassostreae* strains isolated in the present study. Boostratp >90% are mentionned by a black dot. The inner ring indicates whether a strain was isolated from a specific seawater 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

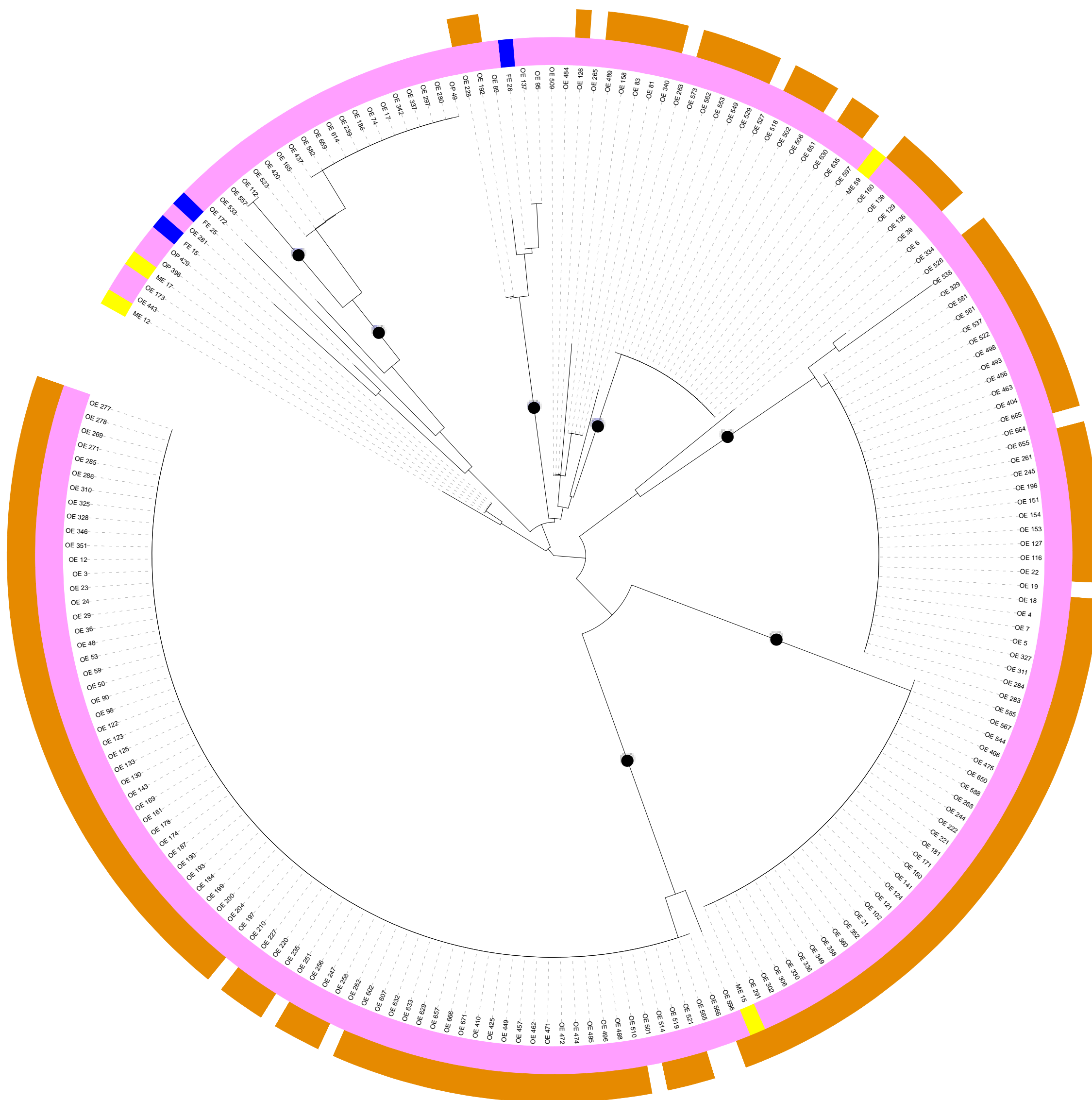
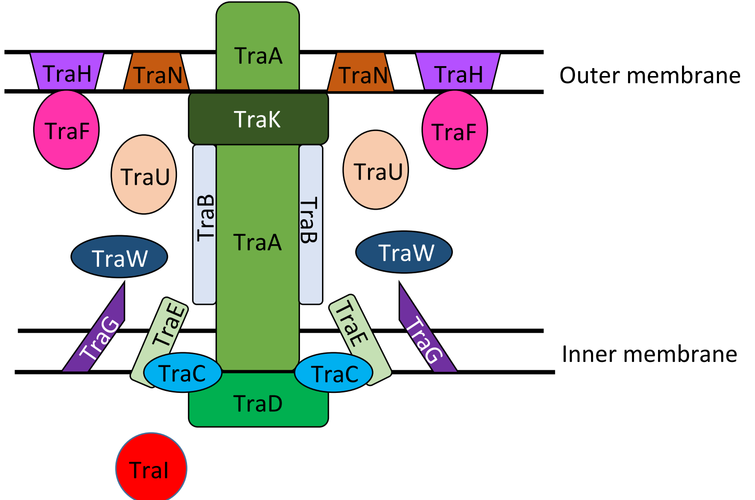


Figure S5: The putative conjugative machinery. This cluster includes homologs of the core proteins of a Type IV secretion system, the coupling protein TraD and a relaxase-helicase TraI.

A



B

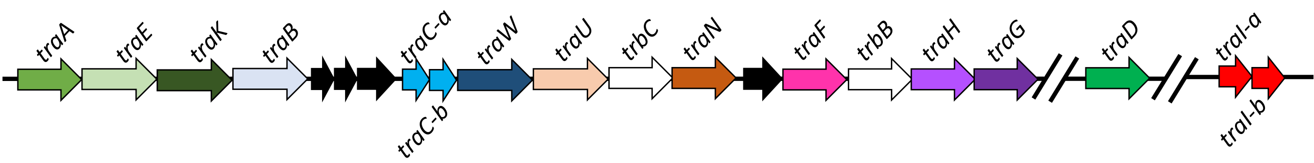
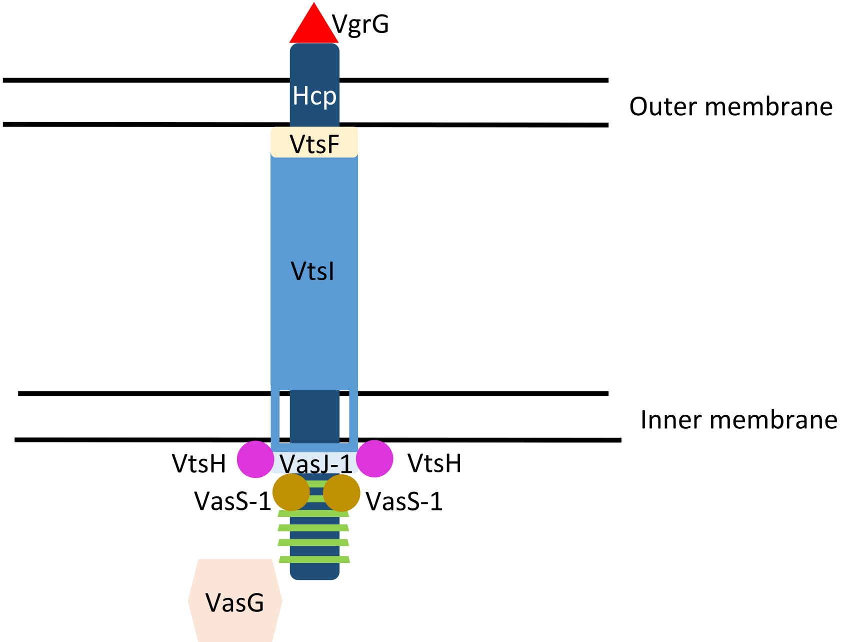


Figure S6: The pGV1512 type VI secretion system. **A-** Graphic representation. **B-** Genes cluster

A



B

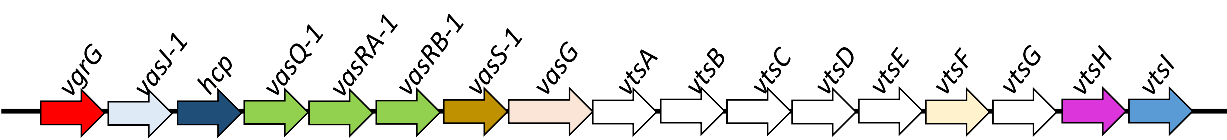


Figure S7: Experimental assessment of pGV1512 loci as virulence determinants. The deletion of T6SS, T4SS, and *cus/cop* gene clusters and region Px1 to 3 (Fig.3A) was performed by allelic exchange. The virulence of the wild type and mutants of specific loci (in x-axis) was compared by injection of strains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

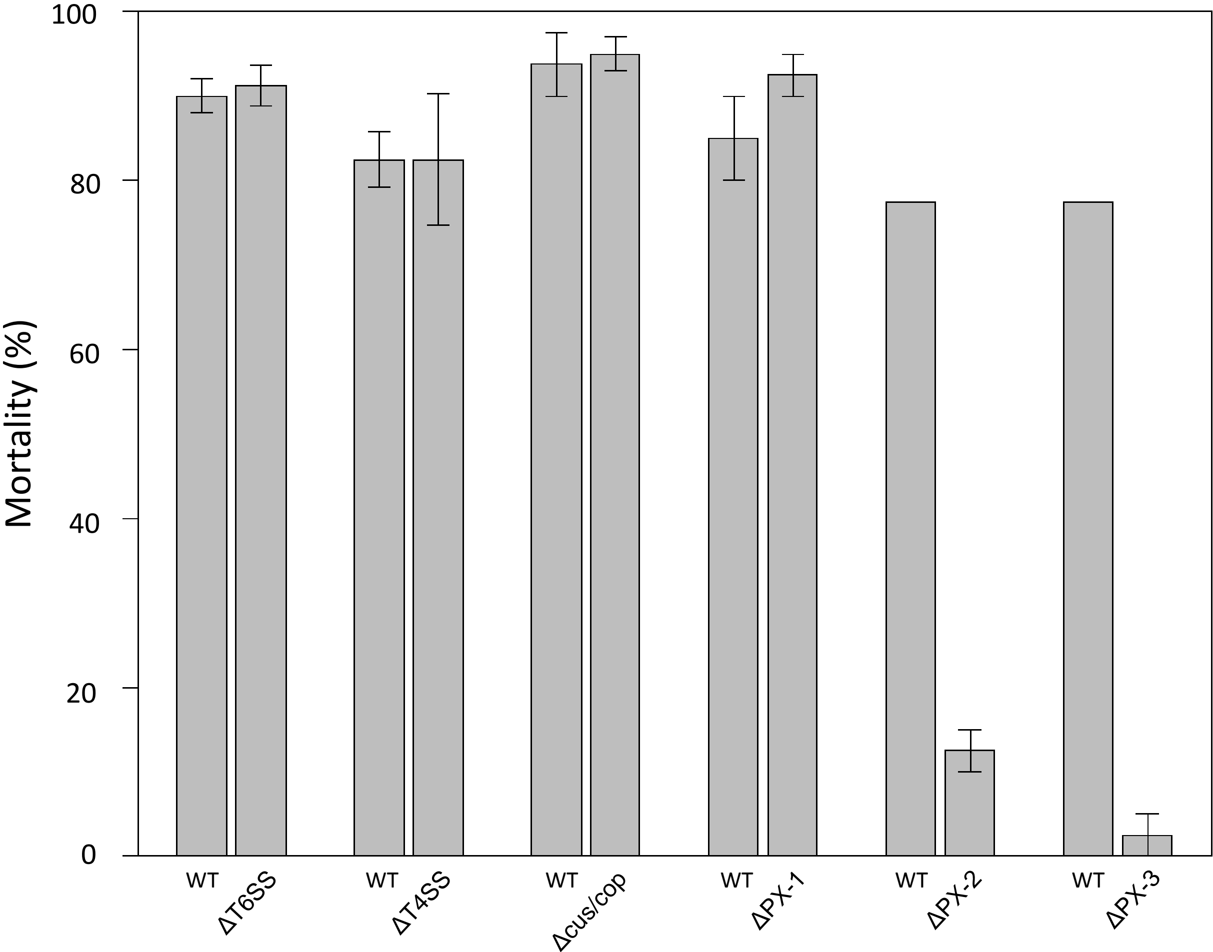


Figure S8: Comparison of oyster colonization by *V. crassostreae* and *V. cyclitrophicus.* A CmR cassette was integrated in a non-essential chromosomal locus of *V. cyclitrophicus* strain J2-5, black triangle, and *V. crassostreae* strain J5-20 ΔpGV1512, white circle. Fluorescent strains were individually added to the oyster tank (10^7cfu/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.

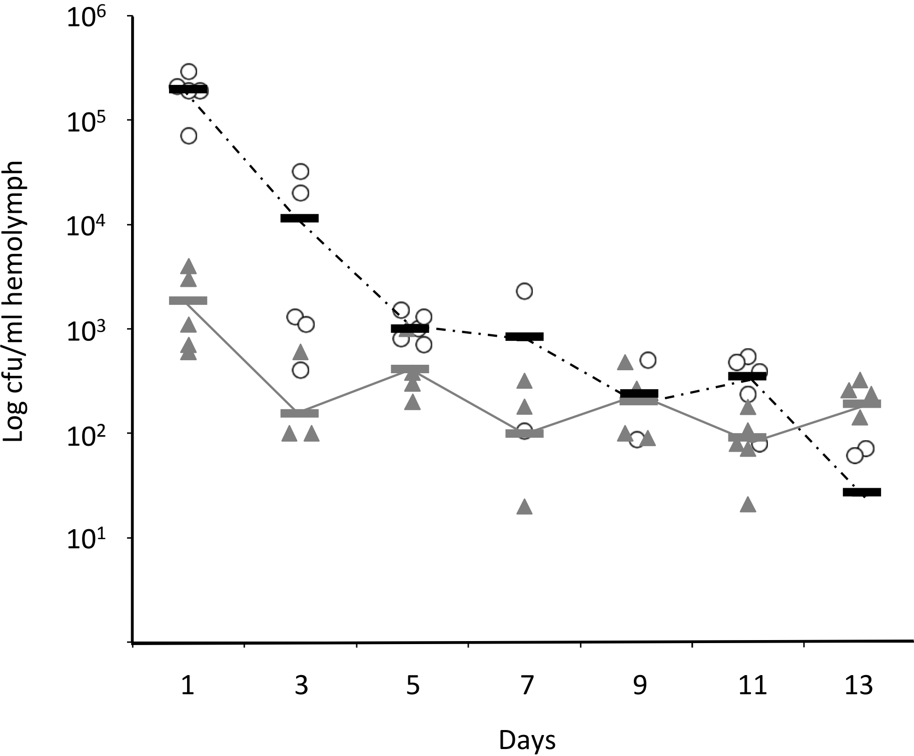


Figure S9: Role of pGV1512 for oyster colonization. Colonization capacity assessment by determination of the ratio of J2-8 strain/ *V. crassostreae* (wild type and derivative without plasmid) retrieved on plates (y-axis) after homogenization of animals that were infected with 1:1 mix by injection or 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.