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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 implicated (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 $7.3 \text{ l h}^{-1} \text{ g}^{-1}$ dry tissue weight; Cognie *et al.*, 2003), which act like a sieve capturing food particles in the optimum range of 5–10 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 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 <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 ~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.*, 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 °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^{-1} 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^{-1} agar, 4 g l^{-1} bactopectone and 1 g l^{-1} 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: $>60 \mu\text{m}$; $60\text{--}5 \mu\text{m}$; $5\text{--}1 \mu\text{m}$; $1\text{--}0.2 \mu\text{m}$ 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 $P\text{-value} \leq 0.05$. 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 $P \leq 2.2\text{e-}16$) 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 g l^{-1} bactopectone and 1 g l^{-1} 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 $25 \mu\text{g ml}^{-1}$ for *Vibrio* and *E. coli*, respectively), spectinomycin ($100 \mu\text{g ml}^{-1}$), 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 (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_{LAC} promoter into the *Vibrio* strains. Alternatively, the $P_{\text{LAC-gfp}}$ and Cm^R cassettes were integrated in non-essential chromosomal genes (transposases *orfA* and *B*) using pSW3654, a R6K $\gamma\text{-ori}$ -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 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^R) resulted from integration of pSW23T in the pGV1512 plasmid by a single crossover. This strategy was also used to stabilize the pGV1512 in $\Delta\text{cus}/\text{cop}$ 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 ($\Delta\text{R1::Spec}^R$) 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_{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 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^R) and recipient (for example, Δ R1::Spec^R) 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 90 957 to 91 768 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 (10⁶ 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 2.5 l aquarium) containing 1 l of aerated 5 μ 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 (10⁵ c.f.u. per animal, that is, sub-lethal dose) or added to the oyster tank (10⁷ c.f.u. ml⁻¹). 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

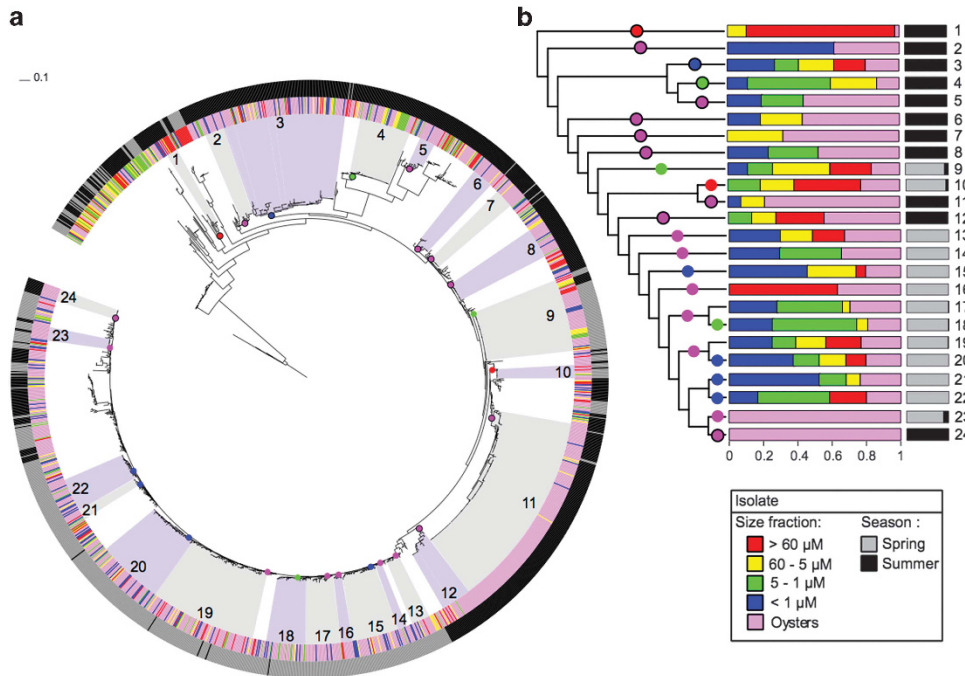


Figure 1 Population structure prediction of *Vibrionaceae* bacteria recovered from different seawater fractions or oyster tissues in two seasons. (a) Phylogenetic tree of 1635 isolates based on partial *hsp60* marker genes. The inner ring indicates whether a strain was isolated from a specific seawater 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 > 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).

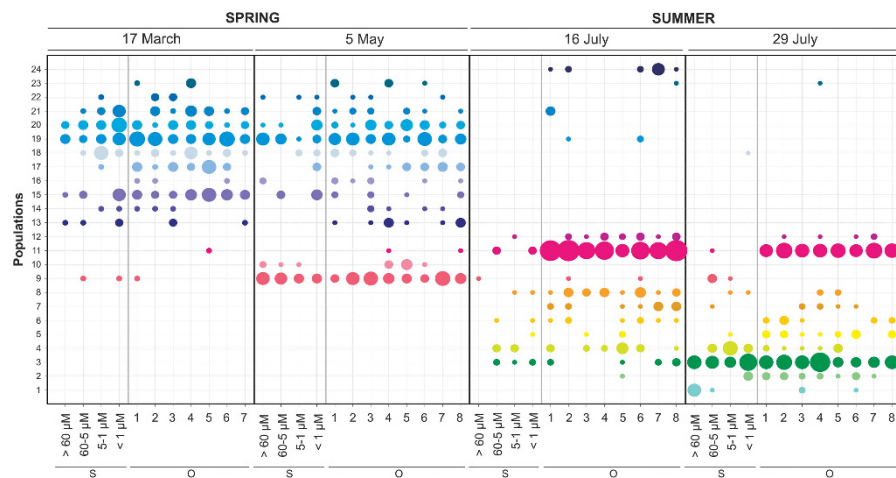


Figure 2 Relative abundance of *Vibrio* populations predicted by AdaptML by sampling categories and date. The size of each circle is proportional to the relative abundance of a population in a sample category (normalized by the number of isolates in each fraction/oyster replicate to account for uneven 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 (<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 (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

>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 (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

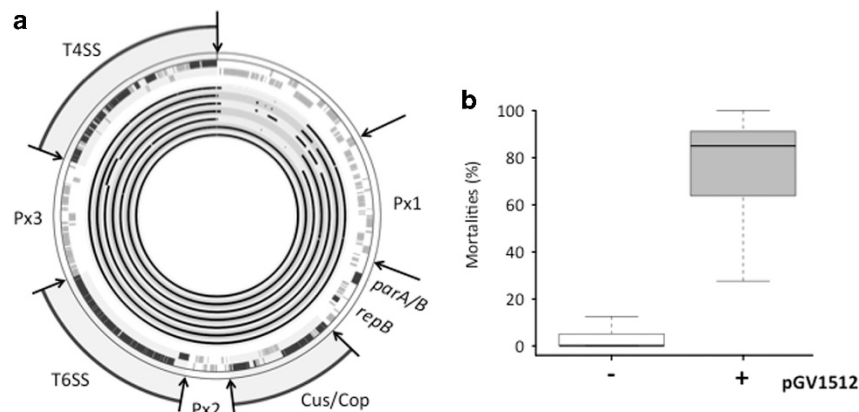


Figure 3 Correlation between pGV1512 presence and *V. crassostreae* virulence. (a) Circular representation of the *V. crassostreae* (strain J5-20) pGV1512 plasmid. From the outside inwards: the 1st and 2nd circles show the open reading frames encoding replication and partitioning; a type 4 secretion 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 10^6 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 J5-20, J2-9 and the J2-9-Spec^R derivative Δ R1 (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 Δ pGV1512, J5-20 Δ pGV1512, J2-9 Δ R1- Δ pGV1512) injection (Figure 4b). A restoration of the Δ R1 phenotype was observed after pGV1512 mobilization into J2-9 Δ R1- Δ pGV1512 (J2-9 Δ R1- Δ pGV1512 + 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

(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, J2-9 Δ pGV1512 and, as a control, in *V. cyclitrophicus* strain 7T5-8. Both fluorescent strains were then added to oyster tanks (10^7 c.f.u. ml⁻¹) and their ratios determined (Figure 5a). The colonization by *V. crassostreae* was found to remain high (10^4 – 10^5 c.f.u. ml⁻¹ 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 (10^3 c.f.u. ml⁻¹). 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 (10^5 c.f.u. ml⁻¹), and *V. cyclitrophicus* (10^3 c.f.u. ml⁻¹), although from day 5 both strain loads were found to be 10^3 – 10^2 c.f.u. ml⁻¹.

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.

Discussion

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

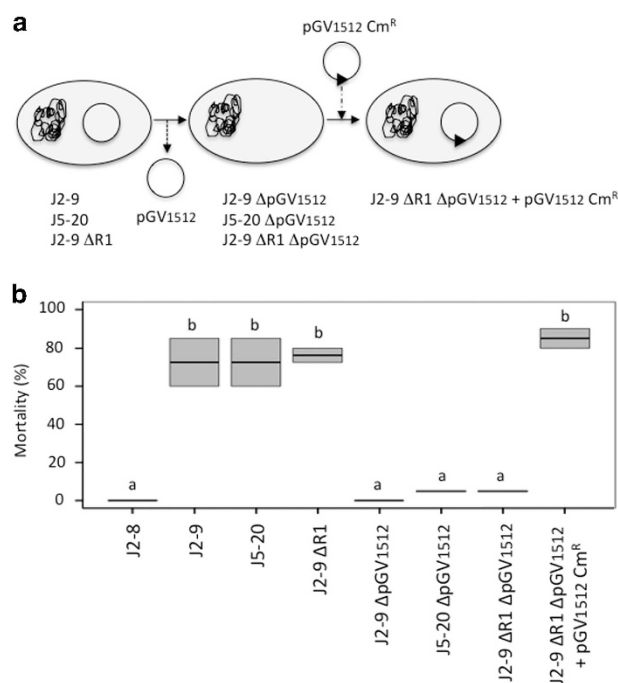


Figure 4 Experimental assessment of the mobilization of the pGV1512 plasmid through conjugative transfer, and its virulence implication in *V. crassostreae*. (a) Different genetic constructs used. First, the plasmid was cured in three *V. crassostreae* strains (J2-9; J2-9 Δ R1 and J5-20). Second, mobilization of the pGV1512 plasmid (modified with the insertion of a chloramphenicol resistance gene) was tested by assessing the possibility of conjugative transfer in J2-9 Δ R1 (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 (10^6 c.f.u. per animal) in 20 oysters and counting the percentage of mortalities (y-axis).

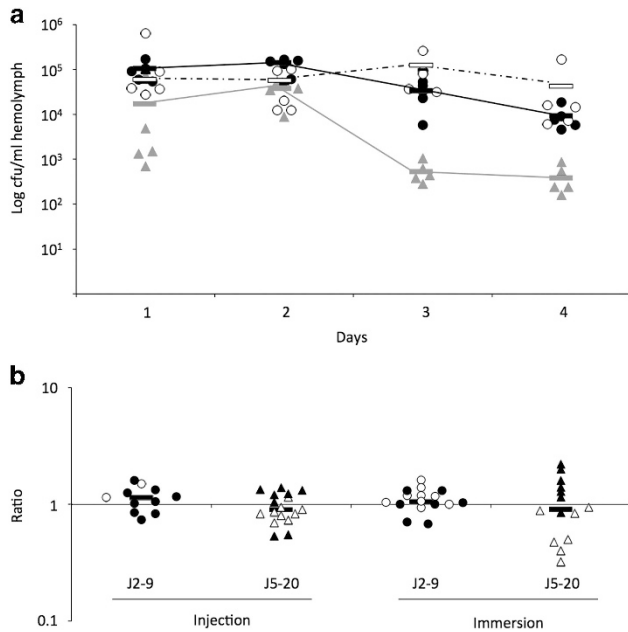


Figure 5 Role of pGV1512 for oyster colonization. A replicative plasmid was used to introduce green or red fluorescent protein (GFP or RFP) and a Cm^R cassette into the *Vibrio* strains. (a) GFP-labeled strains (*V. cyclitrophicus*, strain 7T5-8, gray triangle; *V. crassostreae*, strain J2-9, black circle; J2-9ΔpGV1512 white circle) were individually added to the oyster tank (10⁷c.f.u. ml⁻¹). 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⁻¹ of hemolymph retrieved on plates. The horizontal bar in each experiment indicates the mean of log c.f.u. ml⁻¹. (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. tasmanien-sis* (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.

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