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## Selection of *Vibrio crassostreae* relies on a plasmid expressing a type 6 secretion system cytotoxic for host immune cells

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### Abstract :

Pacific oyster mortality syndrome affects juveniles of *Crassostrea gigas* oysters and threatens the sustainability of commercial and natural stocks of this species. *Vibrio crassostreae* has been repeatedly isolated from diseased animals and the majority of the strains have been demonstrated to be virulent for oysters. In this study we showed that oyster farms exhibited a high prevalence of a virulence plasmid carried by *V. crassostreae* while oysters, at an adult stage, were reservoirs of this virulent population. The pathogenicity of *V. crassostreae* depends on a novel transcriptional regulator, which activates the bidirectional promoter of a Type 6 Secretion System (T6SS) genes cluster. Both the T6SS and a second chromosomal virulence factor, r5.7, are necessary for virulence but act independently to cause to hemocyte (oyster immune cell) cytotoxicity. A phylogenetically closely related T6SS was identified in *V. aestuarianus* and *V. tapetis*, which infect adult oysters and clams, respectively. We propose that hemocyte cytotoxicity, is a lethality trait shared by a broad range of mollusk pathogens and we speculate that T6SS was involved in parallel evolution of pathogen for mollusks.

## INTRODUCTION

The Pacific oyster mortality syndrome (POMS) affects juveniles of *Crassostrea gigas*, the main oyster species exploited worldwide. This syndrome occurs when the seawater temperature reaches 16°C and is caused by multiple infections with an initial and necessary step relying on infection of the hemocytes, the oyster immune cells, by the endemic Ostreid herpesvirus OsHV-1  $\mu$ Var (de Lorgeril et al., 2018). Viral replication leads to the host entering an immune-compromised state, evolving towards subsequent bacteremia involving opportunistic bacteria such as *Vibrio sp.* Exploring POMS in an oyster farming area from the French North Atlantic coast (Brest), we showed previously that the onset of disease is associated with progressive replacement of diverse benign colonizers by members of a phylogenetically coherent virulent population, *V. crassostreae* (Lemire et al., 2015). The virulent population is genetically diverse but most members of the population can cause disease. We further demonstrated that *V. crassostreae* virulence depends on the presence of a large mobilizable plasmid, pGV1512 (hereafter named pGV for simplicity) although the mechanisms underpinning virulence remain to be elucidated (Bruto et al., 2017). Having observed that juvenile infection by *V. crassostreae* is

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recurrent in the POMS occurring in Brest (Bruto et al., 2017, de Lorgeril et al., 2018, Lemire et al., 2015), the questions arose whether oyster farms create conditions that lead to the selection of this virulence plasmid and whether oysters (farmed or wild) represent a reservoir of virulent *V. crassostreae*. Indeed, it has been suggested that, during cold months, oysters act as a reservoir for *V. aestuarianus* (Goudenege et al., 2015, Parizadeh et al., 2018), a pathogen that primarily targets adult animals and hence is not thought to be involved in POMS (Azema et al., 2017).

Pathogenic lifestyles are typically associated with horizontal acquisition of virulence genes (Le Roux and Blokesch, 2018), but pre-existing genomic features might be necessary for the acquisition and/or the functionality of these virulence genes (Shapiro et al., 2016). Indeed, we showed that a core gene, *r5.7*, which encodes an exported protein of unknown function, is necessary for full virulence in *V. crassostreae* (Lemire et al., 2015). This gene is widely distributed across the Splendidus clade, a large group of closely-related species (*e.g.*, *V. splendidus*, *V. crassostreae*, *V. cyclitrophicus*). The *r5.7* gene was acquired by the common ancestor of this group and co-diversified in some populations while being lost from non-virulent populations (Bruto et al., 2018). The widespread occurrence of *r5.7* across environmental *Vibrio* populations suggests that it has an important biological role but its frequency also indicates that this role is population-specific. Indeed, it was recently showed that *r5.7* is involved in population-specific mechanisms of hemocyte cytotoxicity (Rubio et al., in press). In *V. crassostreae* hemocyte cytotoxicity is contact-dependent and requires *r5.7*. The R5.7 protein is not lethal when

injected into oysters, but this protein is able to restore virulence when co-injected with a mutant lacking the *r5.7* gene (Bruto et al., 2018). This suggests that R5.7 interacts with the external surface of *Vibrio* and / or with a cellular target. Whether *r5.7* and the virulence gene(s) encoded by the pGV plasmid act in concert or independently to promote *V. crassostreae* virulence and cytotoxicity was a goal of this study.

Here, we explored the distribution and functional interaction of two *V. crassostreae* virulence determinants, R5-7 and the plasmid pGV. *V. crassostreae* strains were collected from Brest (France), an area of intense oyster farming that is experiencing recurrent mortality events, and in Sylt (Germany) where a massive oyster invasion formed natural beds that have not yet suffered from *Vibrio*-related disease outbreaks (Reise et al., 2017). While the *r5.7* gene was detected at high frequency in *V. crassostreae*, the pGV plasmid was detected only in isolates from Brest and its presence correlated with virulence as assessed by experimental oyster infections. We further showed that, at a temperature of <math>16^{\circ}\text{C}</math>, oysters act as a reservoir of *V. crassostreae* strains. Exploring genetically the virulence determinants carried by the plasmid we showed that a transcriptional regulator is necessary for pGV-mediated virulence. This regulator induces the expression of a molecular killing device called the type 6 secretion system (T6SS) which is also necessary for full virulence. RNA sequencing (RNAseq) followed by transcriptional fusion analysis led us to identify a bidirectional promoter within the T6SS genes cluster that is up-regulated by the transcriptional activator. Gene deletions and complementation experiments further confirmed the role of the *r5.7* and the T6SS in hemocyte cytotoxicity and indicated that

they act in an additive manner. Finally, the identification of a similar type of T6SS in *V. aestuarianus* and *V. tapetis* led us to hypothesize a parallel evolution of mollusk pathogens.

## RESULTS

### **The virulence plasmid is widespread in *V. crassostreae* population occurring in oyster farms**

We previously hypothesized that the introgression of the virulence plasmid pGV into *V. crassostreae* might have been favored by elevated host density in farming areas (Bruto et al., 2017). However, wild oyster beds can also reach high densities, as exemplified by the recent invasion of *C. gigas* oysters into the Wadden sea (North Sea) (Reise et al., 2017). To date, no *Vibrio*-associated mass mortalities have been observed in this area, in contrast to observations in heavily farmed areas. We thus investigated the presence and frequency of the pGV plasmid in *V. crassostreae* strains sampled from Sylt. For this, 910 *Vibrio* strains were isolated from seawater fractions and oysters from Sylt, genotyped by partial *hsp60* gene sequencing and assigned to *Vibrio* populations as described previously (Figure S1). Multi Locus Sequencing Typing (MLST) further confirmed the taxonomic assignment of 47 *V. crassostreae* strains isolated from Sylt (Figure 1, beige squares) as well as 42 isolates from Brest (Figure 1, brown squares) (Table S1). The phylogenetic structure partitioned these strains into two clades representing the two locations. The first clade contained the majority of strains from Sylt (68%, 32 out of 47), while the second clade principally contained strains from Brest (80%, 34 out of

42). The pGV *repB* gene was never detected in isolates from Sylt and was mainly detected in strains from Brest that belonged to clade 2 (Figure 1, plain blue circles). Only one clade 1 strain (8T5\_11), originating from Brest, was found to be positive for *repB*. The presence of the plasmid was confirmed by sequencing the genome of the 8T5\_11 strain (Table S2). We next explored the virulence of these isolates by experimental infection. When the 47 and 42 *V. crassostreae* strains isolated from Sylt and Brest, respectively, were injected individually into oysters, we observed that virulence was strongly correlated with the presence of the plasmid (50 to 100% oyster mortalities, 24 hours post injection), supporting previous findings (Bruto et al., 2017). Only three strains carrying the plasmid (8T5\_11, 7T7\_10 and 8T7\_10) induce a weak mortality (<20%) (Figure 1). Gene loss could explain this non-virulent phenotype. Indeed, comparative genomic analyses identified 44 genes that were absent from the 8T5\_11 genome but were present in all of the sequenced virulent strains of *V. crassostreae* (Figure 1; Table S3). These 44 genes included *r5.7*, which is necessary for virulence and is located in a region that was previously identified as being specific to *V. crassostreae* (Lemire et al., 2015). However the expression of *r5.7* from a plasmid had no effect on 8T5\_11 virulence (Figure S2). Furthermore the *r5.7* gene was detected by PCR in the non-virulent strains 7T7\_10 and 8T7\_10 that carry the pGV plasmid (Figure 1, black squares). Together these results indicate a role for pGV in virulence but additional genomic components appear to be necessary.

### **Oysters act as reservoir of the *V. crassostreae* pathogen**

*V. crassostreae* infection has been recurrently associated with POMS events that affect juvenile oysters at a temperature threshold of 16°C (Bruto et al., 2017, de Lorgeril et al., 2018, Lemire et al., 2015). In oyster farming areas such as Brest, roughly 700 tons of farmed oysters are introduced into a site where 10'000 tons of wild oysters reside (Pouvreau, personal communication). We thus asked whether oysters may asymptotically host *V. crassostreae* and hence play a role as a reservoir of this pathogen. Wild adult animals were collected from Brest at 12°C and returned to the laboratory where they were transferred into a tank at 21°C, a procedure previously shown to allow the development and transmission of oyster diseases (Petton et al., 2015a, Petton et al., 2015b, Petton et al., 2013). Mortality started at day 8, reached 90% after day 14, and were accompanied by the presence of *V. crassostreae* in the water tank and in the hemolymph of moribund animals (Figure S3). The pGV plasmid was detected in 39 of 41 (95%) *V. crassostreae* strains isolated during this experiment. We noted that *V. aestuarianus* was not isolated on *Vibrio* selective media (TCBS, see material and method), although it was detected by PCR in animal tissues, co-occurring or not with *V. crassostreae*. On the other hand, OsHV-1 was never detected in DNA extracted from the oysters. Contaminated seawater (CSW) was collected at day 11 from the tank containing the moribund wild oysters and three-month-old specific pathogen free oysters (SPF juveniles) were exposed to this CSW at 21°C (Petton et al., 2013). Mortalities of the juveniles started at day 3 and reached 100% after 6 days. No mortality occurred when SPF juveniles were kept in filtered seawater at the same temperature. *V. crassostreae* and *V. aestuarianus*, but not OsHV-1, were detected in moribund animal tissues. These results

showed that wild adult oysters are reservoirs of virulent *V. crassostreae* and increasing the temperature can induce disease symptoms.

### **A transcriptional regulator is necessary for pGV-mediated virulence and cytotoxicity.**

Having shown that oyster farming correlates with a high prevalence of the virulence plasmid, we next explored the virulence trait(s) encoded by pGV. A previous study identified a region within pGV (Px3, Figure 2A) that is necessary for virulence in *V. crassostreae* (Bruto et al., 2017). Manual annotation of the genes within this region did not reveal any known virulence determinants, but a putative transcriptional regulator (labelled VCR9J2v1\_750086 in J2-9 and hereafter named TF for simplicity) was identified. We assessed the importance of TF for virulence using a genetic knockout approach. Deletion of this gene ( $\Delta tf$ ) resulted in a significant decrease in mortality after oyster injection (Figure 2B). Constitutive expression of *tf* from a plasmid was sufficient to restore virulence both in the  $\Delta tf$  mutant and in a mutant lacking the complete Px3 region ( $\Delta Px3$ ). On the other hand, expression of *tf* in a pGV-cured strain did not result in increased mortality (Figure 2B). These results showed that the gene encoding the TF regulator is the only gene involved in Px3-mediated virulence but that additional determinant(s), carried by this plasmid, are involved in *V. crassostreae* virulence.

*V. crassostreae* virulence has been recently demonstrated to be intimately related with its cytotoxic effects on hemocytes (Rubio et al., in press). Here, using flow-cytometry, we observed



that *V. crassostreae* effects on hemocyte viability require the presence of pGV. Deletion of the Px3 region or of the *tf* gene also led to an attenuation of cytotoxicity (Figure 2C). Expression of the *tf* gene *in trans* complemented the  $\Delta$ Px3 deletion with respect to hemocyte toxicity, mirroring the phenotype observed following oyster injection. This result was surprising as pGV was previously described as dispensable for *V. crassostreae* cytotoxicity (Rubio et al., in press). This discrepancy might be explained by the different methodological approaches used to assess cell viability. In the previous study, bacteria were added to hemocyte monolayers at a multiplicity of infection (MOI) of 50 and viability monitored for 15 hours by a Sytox green assay (Rubio et al., in press). Here, exposition of hemocytes to vibrios was performed in a cell suspension at a MOI of 10 for 6 hours before addition of SYBR Green-I and propidium iodide to determine cell viability by flow cytometry. To verify that the plasmid is essential for toxicity, we thus incubated the hemocytes with a wild type *V. crassostreae* strain (J2-9) or with a plasmid-cured strain ( $\Delta$ pGV) at MOIs of 10 or 100 for 6 hours. These tests revealed a dose-dependent effect in which low levels of the plasmid-cured strain were less cytotoxic while high levels could overcome the plasmid deficiency (Figure S4). Altogether, our results showed that the TF regulator controls plasmid-carried genes involved in hemocyte cytotoxicity.

### **The TF transcriptional regulator activates a Type 6 Secretion System (T6SS).**

The *tf* gene encodes a putative transcriptional regulator of the AraC family that contains two domains: a N-terminal domain with putative Class I glutamine amidotransferase function and a

C-terminal helix-turn-helix DNA binding domain (Figure S5). To identify its target gene(s) we conducted a RNAseq analysis to compare the transcriptomes of a *V. crassostreae* derivative  $\Delta$ Px3 constitutively expressing either the *tf* or the gene encoding the green fluorescent protein (*gfp*), as a control. Expression of *tf* resulted in significant changed mRNA levels for only 27 predicted protein-coding genes (Log2Fold change >2, Table S4) of which 6 and 21 genes were down- and up-regulated, respectively, in a TF-dependent manner. All 21 up-regulated genes were located on the virulence plasmid and encode a putative T6SS (here after named T6SS<sub>pGV</sub>) (Figure 3). The induction of two of the T6SS<sub>pGV</sub> genes (*vipA* and *vgrG*, the first gene of each operon) by TF was further validated by RT-PCR in two biologically independent experiments (Figure S6).

The T6SS<sub>pGV</sub> locus is organized into at least two operons with *vgrG*, a gene encoding unknown function and *paar* being expressed in the opposite direction compared to the rest of the T6SS<sub>pGV</sub> genes. Between these two operons, we predicted a bidirectional promoter (-10/-35 boxes on each operon site) as well as a putative TF target site that comprised a palindromic sequence of 6 nucleotides spaced by 5 nucleotides (Figure 4). This motif was not identified at other loci within the *V. crassostreae* genome. To test whether the transcription factor and this putative promoter region were sufficient to drive expression of adjacent genes in a heterologous host, we cloned the promoter between GFP- and DsRed-encoding genes in a replicative plasmid. Next, we transformed this reporter plasmid into an unrelated *Vibrio* species (in this case *V. cholerae*), which had been engineered to chromosomally encode *tf* under the control of an arabinose-

inducible promoter ( $P_{BAD}$ ) (see Materials and methods for details). Induction of *tf* expression by arabinose resulted in the production of both GFP and DsRed demonstrating that the promoter was indeed bidirectional and activated by TF (Figure 4). Deletion of the palindromic sequence altered the induction capacity of TF, while inversion or mutation of one of the 6 nucleotide sites did not abrogate gene activation (Figure 4). We therefore concluded that the TF transcription factor drives T6SS expression in *V. crassostreae*.

### **The T6SS<sub>PGV</sub> is involved in virulence and hemocyte cytotoxicity**

T6SSs are contact-dependent contractile nanomachines used by many Gram-negative bacteria as weapons against a variety of prokaryotic and eukaryotic organisms (Cianfanelli et al., 2016). Indeed, T6SSs allow bacteria to translocate a wide variety of toxic effectors into target cells. Formed by a minimum of 13 conserved ‘core’ components, T6SSs are made up of three large sub-structures: a trans-membrane complex, a baseplate and a tail composed of an inner tube formed by hexamers of hemolysin-coregulated protein (Hcp) encased within an outer VipA/VipB sheets complex and topped with a VgrG spike, which can be extended by a final tip formed by a PAAR-motif protein. T6SS effectors are frequently fused to C-termini of T6SS structural proteins, such as VgrG or PAAR (Shneider et al., 2013). However, *in silico* analysis did not predict any C-terminal extension of the VgrG or PAAR proteins of *V. crassostreae*. We also failed to identify any putative effector protein using a public database (<http://db-mml.sjtu.edu.cn/SecReT6/>).

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A genetic approach was therefore used to test the importance of the T6SS<sub>pGV</sub> for *V. crassostreae* virulence. We had previously generated a knockout mutant that lacked this locus and observed no effect on virulence (Bruto et al., 2017). However, re-investigating this mutant we identified an unexpected duplication of this region resulting in one deleted and one whole T6SS cluster. Several attempts to delete the *vgrG* or *vipA* genes were unsuccessful, repeatedly resulting in complete loss of the plasmid, suggesting that these mutations come at a cost for the bacteria. However, deletion of the T6SS *paar* gene was successful ( $\Delta paar$ ) and led to decreased virulence (Figure 2A). Complementation by constitutively expressing *paar in trans* restored the virulence potential to similar levels as observed for the WT.

Having demonstrated a role for the T6SS in virulence, we next explored its cellular target. In many bacterial models, T6SSs are used to kill competing bacteria (Cianfanelli et al., 2016). We thus asked whether *V. crassostreae* that constitutively expressed *tf* would be able to kill bacteria in an *in vitro* killing assay (Borgeaud et al., 2015). When the *tf*-expressing strain was used as a predator and *E. coli*, *V. cholerae*, or a collection of 40 diverse *Vibrio* strains isolated from oysters were used as prey, we did not observe any killing under the tested conditions. The T6SS has also been demonstrated to mediate toxicity for eukaryotic cells. For example, non-pandemic *V. cholerae* exhibits T6SS-mediated cytotoxicity towards macrophages and the soil amoeba *Dictyostelium discoideum* (Pukatzki et al., 2007), while the aquatic amoebae *Acanthamoeba castellanii* is not affected (Van der Henst et al., 2018). Here, we observed that the *V. crassostreae*

*Δpaar* mutant has decreased cytotoxicity towards hemocytes compared to the WT and that expression of the *paar* gene *in trans* partially restored cytotoxicity (Figure 2C). Our results therefore suggest a critical role for the virulence plasmid, TF, and T6SS<sub>pGV</sub> in *V. crassostreae*-mediated killing of oyster immune cells and therefore pathogenicity towards this animal host.

Looking at the distribution of the T6SS<sub>pGV</sub> in publicly available *Vibrio* genomes, we found that closely related loci are present in *V. aestuarianus* (11/11 genomes) and *V. tapetis* (1/1 genome), which are pathogens of adult oysters and clams, respectively (Travers et al., 2015). Overall the synteny and amino acid identities between core components of the T6SSs were high with the exception of genes localized after the *vasK* gene that could be candidate effectors (Figure 5). In *V. aestuarianus*, a specific gene (VIBAEv3\_A30819 in the strain 02-041) encodes a protein with weak sequence identity (25%) with a T3SS effector from *Bordetella bronchiseptica* named BteA. This secreted protein has been reported to inhibit phagocytosis by macrophage and induce necrosis through an actin cytoskeleton-signalling pathway (Kuwae et al., 2016). In the T6SS<sub>pGV</sub> a specific gene (VCRJ2v1\_750073 in strain J2-9) encodes a protein with 38% similarity and 13% identity to the C-terminal and N-terminal domains of an insecticidal delta-endotoxin found in *Bacillus thuringiensis*. Unfortunately, deletion of this gene in *V. crassostreae* also resulted in loss of pGV preventing further functional analysis. An ortholog of VCRJ2v1\_750073 in *V. tapetis* has been pseudogenized, potentially leading to its functional inactivation. On the other hand, a second, species-specific gene in the *V. tapetis* T6SS encodes a protein with only 60% similarity and 29% identity within 45 amino acids of the central domain of nigratoxin, a toxin for

crustaceans and insects (Labreuche et al., 2017). Hence while annotation and localization of these genes suggests a role as T6SS effectors for the three pathogens, the formal demonstration of their function remains to be done.

### **The T6SS<sub>pGV</sub> and R5.7 protein act independently to mediate *V. crassostreae* cytotoxicity**

We showed in a previous study (Bruto et al., 2018) that *V. crassostreae* evolution as pathogen involved sequential acquisition of virulence genes, including i) acquisition of the *r5.7* gene, which encodes an exported protein that may be involved in the contact-dependant cytotoxicity (Rubio et al in press) and ii) more recent acquisition of T6SS<sub>pGV</sub> that, in our experimental design, appeared necessary for the killing of host immune cells. It is therefore tempting to hypothesize that these two virulence traits work in concert to mediate cytotoxicity, R5.7 potentially favouring attachment of the vibrio to the hemocyte and facilitating anchorage of the T6SS<sub>pGV</sub>, which then injects a toxic effector into the cell. Under such an hypothesis, deletion of the *r5.7* gene ( $\Delta r5.7$ ) or curing of the plasmid ( $\Delta pGV$ ) should decrease the cytotoxicity of *V. crassostreae* to a similar level to that observed with the double mutant  $\Delta pGV1512\Delta r5.7$ . However, as we observed that the cytotoxicity of the double mutant was significantly more attenuated than that of the single mutants (Figure 6), we suggest that these virulence factors act additively rather than being functionally connected.

## DISCUSSION

In recent years, a syndrome affecting juveniles of *Crassostrea gigas* (POMS) has become panzootic, being observed in all coastal regions of France and numerous other countries worldwide, threatening the long-term survival of commercial and natural stocks of oysters (Le Roux et al., 2015). A study recently demonstrated that this syndrome results from an intense replication of the oyster herpes virus OsHV-1  $\mu$ Var, creating an immune-compromised state that permits secondary infections by opportunistic bacteria (de Lorgeril et al., 2018). An unresolved question, however, is whether diverse bacterial species can be considered to be opportunistic or whether specific bacterial species cooperate to induce this syndrome. Here, we provide evidence that *V. crassostreae* is a major player of this syndrome. First, we propose that the recurrent detection of *V. crassostreae* in an area affected by POMS might indicate that it originates from a reservoir in oysters. Second, a high prevalence of a virulence plasmid is observed in oysters affected by POMS, suggesting that strains carrying this plasmid have a selective advantage. Third, cellular characterization of virulence traits sequentially acquired by *V. crassostreae*, revealed a lethal activity on hemocytes by distinct pathways.

Oyster-associated vibrios have been previously 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 (Bruto et al., 2017). This study showed that *V. crassostreae* was abundant in diseased animals while nearly absent in

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the surrounding seawater, suggesting that its primary habitat is not the water column. Potential alternative reservoirs for *V. crassostreae* at temperature  $<16^{\circ}\text{C}$  were still undetermined. Here, we showed that oysters that reside in farming areas year-round asymptotically host *V. crassostreae* and hence potentially serve as a pathogen reservoir. An increase of temperature triggered active multiplication of *V. crassostreae* leading to a sufficiently high bacterial load and/or virulence state allowing the pathogen to colonize and infect juvenile oysters. As *V. aestuarianus* was detected in both adult and the juvenile oysters, it is impossible to discriminate the respective roles of *V. crassostreae* and *V. aestuarianus* in the induction of oyster mortality in the present experiment. It should be notice, however, that *V. aestuarianus* virulence seems to be restricted to the adult stage of oyster (Azema et al., 2017). Importantly, OsHV-1  $\mu\text{Var}$  was never detected in our experiments, confirming previous observations that infection of juveniles can occur in the absence of OsHV-1 $\mu\text{Var}$  (Petton et al., 2015b). Hence our present results suggest that oyster mortality syndrome might have different etiologies. It remains to be determined how temperature acts on *V. crassostreae* infective status. In the context of global warming, how temperature influences the virulence of these pathogens as well as oyster resistance or resilience is a major concern to predict sustainability of commercial and natural stocks of this species.

Another argument strengthening a role for *V. crassostreae* in oyster juvenile mortality syndrome is the high frequency of the pGV plasmid in farming areas that are affected by the syndrome. Although we were able to isolate *V. crassostreae* from oysters in Sylt, none of these isolates were virulent in an infection assay. This observation is consistent with the absence of the pGV plasmid



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in these isolates and strengthens our hypothesis that the introgression of pGV into the *V. crassostreae* population has played a major role in its emergence as a pathogen (Bruto et al., 2017). By identifying virulence traits of *V. crassostreae* encoded by this plasmid, *i.e.* the T6SS<sub>pGV</sub> and its transcriptional activator TF, we deciphered a mechanism that increases hemocyte cytotoxicity of *V. crassostreae* worsens oyster disease. In the future, identification of the effector protein(s) of the T6SS<sub>pGV</sub> should help decipher its effect on hemocytes. In addition, exploring the role of the T6SSs and its effector(s) in the virulence of *V. aestuarianus* and *V. tapetis* may support a parallel evolution from harmless to pathogenic states of these mollusk pathogens.

We also demonstrated that the T6SS and R5.7 are not co-dependent for their function, ruling out the hypothesis that R5.7 acts as a facilitator of T6SS-mediated injection of a toxic effector into hemocytes. Within the Splendidus clade, a few populations have lost the *r5.7* gene and are not able to kill oysters (Bruto et al., 2018). When infecting the host, these non-virulent strains are highly controlled by cellular (phagocytosis) and humoral (antimicrobial peptides, reactive oxygen species, and heavy metals) immunity mediated by the hemocytes (Rubio et al., in press). However, several *V. tasmaniensis* strains isolated from diseased oysters (Le Roux et al., 2009, Lemire et al., 2015) that do not carry the *r5.7* gene, were able to induce mortalities when injected to oysters. Compared to *V. crassostreae*, the hemocyte cytotoxicity of these strains was demonstrated to be dependent on phagocytosis and required a distinct T6SS localised on the chromosome 1 of the strain LGP32 (T6SS<sub>Chr1-LGP32</sub>, Rubio et al in press) (Figure 5C).

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Consideration of this data led to the hypothesis that R5.7 may act as an inhibitor of phagocytosis and *V. tasmaniensis* secondary evolved as pathogen by the acquisition of T6SS<sub>Chr1-LGP32</sub> that is active at the intracellular stage as described for the *V. cholerae* T6SS (Ma et al., 2009). Alternatively, the acquisition of a T6SS<sub>Chr1-LGP32</sub> that is functions exclusively during the intracellular stage may have further selected for *r5.7* loss. Hence in addition to Rubio et al. article, the present study suggests multiple evolutionary scenarios leading to the emergence of pathogenic populations with common and specific virulence traits converging on a common objective: killing of the major actors of the oyster immune response. Finally our results confirm the functional diversity of the T6SS nanomachine and its effectors, acting against bacterial competitors (Unterweger et al., 2014) against amoeba or phagocytic cells at an intracellular stage (Ma et al., 2009) or directly by contact with the target eukaryotic cell.

## MATERIAL AND METHODS

### Isolation of bacteria and gene sequencing

In July 2015 and 2016, 24 live oysters, together with surrounding seawater (temperature 18°C), were collected in Sylt. To collect zooplankton, large phytoplankton and organic particles, a 50 L sample was filtered through a 60 µm plankton net and the collected material was subsequently washed with sterile seawater. Small organic particles and free-living bacterial cells were collected from 2L water samples pre-filtered through the 60 µm plankton net and sequentially filtered

through 5  $\mu\text{m}$ , 1  $\mu\text{m}$  and 0.22  $\mu\text{m}$  pore size filters. These filtrates were directly placed onto *Vibrio* selective media (Thiosulfate-citrate-bile salts-sucrose agar, TCBS). The zooplankton and oyster tissues were ground in sterile seawater (10 mL/g of wet tissue) and streaked onto TCBS. About 150 colonies per seawater fraction and 300 colonies per oyster tissue sample were randomly picked and re-streaked on TCBS first and subsequently on Zobell agar (15 g/l agar, 4 g/l bactopectone and 1 g/l yeast extract in artificial sea water, pH 7.6). All isolates were genotyped by partial *hsp60* gene sequencing and stored in 10% DMSO at  $-80^{\circ}\text{C}$ . A total of 910 *hsp60* sequences were obtained from the two samplings performed in Sylt. This set of data was complemented with 719 *hsp60* sequences obtained from previous samplings at Brest in 2014 (Bruto et al., 2017) and 2016 (seawater temperature above  $18^{\circ}\text{C}$ ).

**Strains, plasmids and culture conditions.** The strains used in this study are described in Table S5. *Vibrio* isolates were grown at  $20^{\circ}\text{C}$  in Zobell broth or agar, Luria-Bertani (LB) or LB-agar (LBA) + 0.5M NaCl. *Vibrio cholerae*, strain A1552, was grown in LB at  $30^{\circ}\text{C}$ . *Escherichia coli* strains were grown at  $37^{\circ}\text{C}$  in LB or on LBA. Chloramphenicol (5 or  $25\mu\text{g/ml}$  for *Vibrio* and *E. coli*, respectively), spectinomycin ( $100\mu\text{g/ml}$ ), kanamycin ( $75\mu\text{g/ml}$  for *V. cholerae*), thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction of the  $P_{\text{BAD}}$  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 where indicated.

**Vector construction and mutagenesis.** All plasmids used or constructed in the present study are described in Table S6. Deletion of selected regions or genes was performed by allelic exchange using the pSW7848T suicide plasmid (Le Roux et al., 2007, Val et al., 2012). To this end, two 500 bp fragments flanking the target region or gene were amplified, (see primer details in Table S7), assembled by PCR and cloned into pSW7848T as previously described (Lemire et al., 2015). The suicide plasmid was then transferred by conjugation from *E. coli* as donor to *Vibrio* as recipient. Subsequently, the first and second recombinations leading to pSW7848T integration and elimination were selected on Cm/glucose and arabinose containing media, respectively. For the complementation experiments, genes were cloned into the *Apa*I/*Xho*I (*paar*) or *Eco*R1/*Xho*I (TF) sites of the pMRB plasmid, which is stable in *Vibrio spp.* (Le Roux et al., 2011), resulting in constitutive expression from a P<sub>lac</sub> promoter. Conjugations between *E. coli* and *Vibrio* were performed at 30°C as described previously (Le Roux et al., 2007). The T6SS intergenic region (i.e. putative promoter region) was PCR amplified, digested, and cloned into *Sma*I and *Stu*I sites in pBR-GFP\_dsRed (Lo Scudato and Blokesch, 2012) before being transferred to the *V. cholerae* strain A1552 carrying the arabinose-inducible *tf* on a mTn7 transposon. Mutagenesis of the palindromic region was performed by PCR assembly as described earlier (Matsumoto-Mashimo et al., 2004).

### **Fluorescence microscopy**

*V. cholerae* cells were back-diluted (1:100) from an overnight culture and grown for 2h at 30C in LB medium containing kanamycin. At this point, 0.2% arabinose was added to the culture where

indicated and the growth was continued for 2h before the bacteria were mounted onto agarose pads (in 1% PBS) and imaged with a Plan-Apochromat 100×/1.4 Ph3 oil objective using a Zeiss Axio Imager M2 epifluorescence microscope. Image acquisition occurred with the Zeiss AxioVision software. Depicted images are representative of three independent biological replicates.

### **SDS-PAGE and Western blotting**

*V. cholerae* cells were grown for 5h at 30C in LB medium with or without 0.2% arabinose supplementation (after 3h of growth) to induce *tf* in the respective strains. Cells were lysed by resuspension in 2x Laemmli buffer (100 µl of buffer per OD<sub>600</sub> unit of 1) and boiling at 95°C for 15 min. Proteins were separated by SDS-PAGE (10% resolving gels) and blotted onto PVDF membranes. Detection of proteins was carried out as described (Lo Scudato and Blokesch, 2012) using primary antibodies against GFP (Roche, #11814460001; diluted 1:5'000) and mCherry (BioVision, #5993-100; diluted 1:5'000). Anti-mouse-HRP (Sigma, #A5278; diluted 1:20'000) and anti-rabbit-HRP (Sigma, #A9169; diluted 1:20'000) were used as secondary antibodies. An anti-RNA Sigma70-HRP conjugate (BioLegend; # 663205; diluted 1:10'000) was used to validate equal loading. Lumi-Light<sup>PLUS</sup> (Roche) served as an HRP substrate and the signals were detected using a ChemiDoc XRS+ station (BioRad). Western blots were performed three independent times with comparable results.

### **Experimental infections.**

### Animals.

Three-month-old Specific Pathogen Free (SPF) oysters were descendants of a pool of 100 genitors that were produced in a hatchery under highly controlled conditions to minimize the influence of genetic and environmental parameters that could affect host sensitivity to the disease (Petton et al., 2015a, Petton et al., 2015b, Petton et al., 2013). These animals were used for experimental infections by immersion (see below) or by intramuscular injections of bacteria into the adductor muscle. Triploid adult oysters (24 to 30-months-old) were provided by a local oyster farm (Coïc, Pointe du Château, Logonna-Daoulas, France) and were used to collect hemolymph for cytotoxicity assays. Wild adult *C. gigas* oysters (n=50) were collected from Bay of Brest (Pointe du Château, 48° 20' 06.19" N, 4° 19' 06.37" W) in April 2019 (seawater temperature 12°C).

### Disease monitoring in wild adult oysters.

After sampling in the Bay of Brest, wild adult oysters were immediately returned to the laboratory (Station Biologique de Roscoff, Roscoff, France). Upon arrival, the animals were first cleaned using a bristle brush and briefly rinsed to remove sand, sediments and other shell debris before being placed in a 300-L tank under static conditions (no change of seawater) with aerated 5- $\mu$ m-filtered seawater at 21°C. Mortality was recorded daily for 14 days. Vibrios were isolated daily from the tank seawater (100  $\mu$ l) or from the hemolymph of moribund animals (10  $\mu$ l) by plating onto selective media (thiosulfate-citrate-bile salts-sucrose agar (TCBS), Difco, BD, France). Randomly selected colonies were mixed into 20  $\mu$ l of molecular biology grade water and

heated using a thermal cycler (2720 thermal cycler, Applied Biosystems) at 98°C for 10 min and stored at -20°C for PCR testing.

#### Infection by immersion in contaminated seawater.

Contaminated seawater (CSW) containing the oyster-shed bacteria was obtained by sampling the seawater from the 300-L tank in which wild adult oysters had been held for 14 days. SPF oysters were transferred to aerated aquaria (20 oysters per 2.5 L aquarium) filled with either 1L CSW or with fresh 5- $\mu$ m-filtered seawater as a control. Mortality was recorded daily for 6 days and moribund animals were removed and analysed for the presence of *V. crassostreae*, *V. aestuarianus* and OsHV-1.

#### **Nucleic acid extraction and PCR**

Hemolymph of moribund wild adult oysters was withdrawn from the adductor muscle using a 1 mL plastic syringe fitted with a 25-gauge needle, centrifuged for 5 min at 5000 rpm and the cell pellet kept at -20°C until further use. In the case of 3-month-old juvenile oysters, the whole wet body of dead animals was crushed in marine broth (1 mg/ml) using a Tissue Lyser II (Qiagen). Genomic DNA was purified from homogenized oyster tissues or hemocyte cell pellets by resuspension in lysis buffer (NaCl 0.1M, pH8 EDTA 0.025M, SDS 1%, proteinase K 100  $\mu$ g/ml) for 16 h (56°C) followed by Phenol:Chloroform:Isoamyl Alcohol (Sigma-Aldrich, #77617) extraction.

The primer pairs and PCR conditions used for the detection of *V. crassostreae* (de Lorgeril et al., 2018), *V. aestuarianus* (Saulnier et al., 2010) and the herpes virus OsHV-1 (Martenot et al., 2010) have been described elsewhere. PCRs were performed on 300 ng oyster DNA for oyster pathogen detection or on 1 µl cell lysate obtained from *Vibrio* randomly picked on TCBS for *V. crassostreae* identification.

### **Bacterial virulence determination by intramuscular injection.**

Several cohorts of SPF-oysters were used to perform experimental infections by intramuscular injections of bacteria into the adductor muscle. Because the susceptibility to bacterial infection of these cohorts may have varied over the course of this study depending on biotic (size) and abiotic (temperature) parameters, each cohort was systematically submitted to an experimental infection by injection with 3 different concentrations (1X, 0.1X and 0.01X) of the pathogenic *V. crassostreae* wt strain J2-9 used here as a reference. The bacterial concentration determined to induce between 50-90% mortality was subsequently used on the considered cohort to evaluate bacterial virulence. Bacteria were grown under constant agitation at 20°C for 24 h in Zobell media. One hundred microliters of the culture ( $10^6$  or  $10^7$  colony forming unit, cfu, depending on the susceptibility of the considered cohort) 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 µm-filtered seawater at 20°C, and kept under static conditions. Experiments were performed in duplicate and repeated at least once. Mortality was assessed after 24 hours.



### ***In vitro* cytotoxicity assays**

Hemolymph was withdrawn from the adductor muscle through a notch previously ground in the oyster shell using a 1 mL plastic syringe fitted with a 25-gauge needle. After bleeding, syringes were maintained on ice and individually controlled by microscope observation to retain only hemolymph that was free of contaminating particles (sperm, ovocytes, small debris...). Selected samples were filtered through a 80  $\mu\text{m}$  mesh to eliminate aggregates or large pieces of debris (to avoid clogging of the flow-cytometer flow-cell) and pooled.

In order to adjust the bacteria/ hemocyte ratio, hemocyte and bacterial cell concentrations were measured by incubating 300  $\mu\text{L}$  of the considered suspension (diluted at  $10^{-2}$  in filtered sterile seawater, FSSW, in the case of bacterial suspensions) with SYBR®Green I (DNA marker, Molecular Probes,  $10,000\times$  in DMSO) at  $1\times$  final concentration, in the dark at room temperature for 10 minutes before flow-cytometric analysis (FACSVerse™, Becton Dickinson, CA, USA). Hemocytes or bacterial cells were detected on the FITC detector (527/32 nm) of the flow cytometer and their concentration calculated using the flow rate value given by the Flow-Sensor™ device integrated to the flow cytometer.

After hemocyte counting, the hemolymph pool was divided into 200  $\mu\text{L}$  sub-samples maintained on ice. Each sub-sample received 200  $\mu\text{L}$  of the different bacterial suspensions (wild-type or derivatives) at a multiplicity of infection (MOI) of 10:1 or 200  $\mu\text{L}$  of FSSW as a control. Each

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condition was tested in 3 replicates and the experiment was performed twice. Tubes were maintained at 18°C for 5.5 h. Then SYBR®Green I and Propidium Iodide (PI, Sigma–Aldrich) were added to each tube at final concentrations of 1× and 10 µg mL<sup>-1</sup>, respectively and incubation was continued for another 30 min (6h total incubation time): PI only permeates hemocytes that lose membrane integrity and are considered to be dead cells, whereas SYBR®Green I permeates both dead and living cells. SYBR Green and PI fluorescence were measured on the FITC detector (527/32 nm) and on the PerCP-Cy5-5 detector (700/54 nm) respectively. Results are expressed as percent dead hemocytes.

**Genome sequencing, assembly, and annotation.** Strains were sequenced (Plateforme genomique, Institut Pasteur, Paris; JGI) using Illumina HiSeq2000 technology with ~50-fold coverage as described previously (Lemire et al., 2015). Contigs were assembled *de novo* using Spades (Bankevich et al., 2012). Computational prediction of coding sequences together with functional assignments was performed using the automated annotation pipeline implemented in the MicroScope platform (Vallenet et al., 2013). Some gene annotations were manually curated using InterPro, FigFam, PRIAM, COGs, PsortB, TMHMM and synteny group computation. General features of the genome sequenced in the present study are presented Table S2.

***In silico* analyses.**

Species trees were reconstructed based on a MLST (Multi Locus Sequence Typing) containing 3 markers for *V. crassostreae* isolates phylogeny (*gyrB*, *rctB* and *rpoD*). Nucleotide sequences

were aligned with Muscle and concatenated using Seaview (Gouy et al., 2010). Phylogenetic reconstruction was done using RAxML (Stamatakis, 2006) on this concatemer with the GTR model. Tree visualization was performed with iTOL (Letunic and Bork, 2011).

### **RNA-seq experimentation**

The *Vibrio* strains J2-9  $\Delta$ Px3 constitutively expressing *tf* or *gfp* from a plasmid (pMRB) were grown in LB-NaCl. Bacteria were sampled at OD 0.3, 0.6 and 1.0 and RNA extraction was performed using TRIzol reagent and following manufacturer's instructions (Invitrogen). Total nucleic acids were quantified based on absorption at 260 nm and RNA integrity was verified by gel electrophoresis. DNA was removed by DNase I digestion using the Turbo DNA-free kit (Ambion). RNAs from the 3 OD conditions were pooled. The experiment was performed three times. Directional cDNA libraries were constructed with the ScriptSeq RNA-Seq Library Preparation Kit (Illumina). Sequencing was done with the NextSeq 500/550 Mid Output Kit v2 (Illumina) on a NextSeq 500Mid (Illumina) by the "Plateforme de Séquençage haut-débit" at I2BC-UMR9198. Data treatment and mapping onto *V. crassostreae* J2-9 reference genome was performed with the TAMARA pipeline hosted by the MAGE platform (<http://www.genoscope.cns.fr/agc/microscope/transcriptomic/NGSProjectRNAseq.php?projType=RNAseq>).

### **Statistical analyses.**

Survival of oysters after injection with the different genetic construct was analyzed by binomial generalized linear mixed models (GLMM) with logit link function taking the number of survivors vs. the number of dead oysters as response variable and strain identity as predictor. Due to the high number of cells analyzed in flow cytometry assays of hemocyte mortality, we used linear mixed models (LMM) with the proportions of alive and dead cells as response variable. Experimental trial was added as a random to account for differences between independent experiments when repeated trials were performed. To identify pairwise difference between strains we used simultaneous tests for general linear hypotheses implemented in the *multcomp* package (Hothorn et al., 2008) applying Tukey contrasts.

#### **ACKNOWLEDGMENTS**

We are grateful to Mark Cock (Marine station of Roscoff) for his thoughtful comments and english editing, which improved the manuscript. We thank the staff of the station Ifremer Argenton and Bouin, the ABIMS and CRBM (Roscoff) and LABGeM (Evry) platforms for technical assistance. This work was supported by grants from the Agence Nationale de la Recherche (ANR-16-CE32-0008-01 « REVENGE ») to FLR, Ifremer and the Region Bretagne to DP and AJ. MBl. is a Howard Hughes Medical Institute (HHMI) International Research Scholar (grant #55008726) and work in her group was supported by a Swiss National Science Foundation

scientific exchange grant (IZSEZO\_181044) and an ERC Consolidator Grant from the European Research Council (724630-CholeraIndex).

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## TITLES AND LEGENDS TO FIGURES

**Figure 1. The presence of the pGV plasmid is correlated with the geographic origin and virulence of *V. crassostreae* strains.** Phylogenetic tree of 89 *V. crassostreae* isolates based on the *gyrB/rctB/rpoD* gene fragments. Dark/light shades of gray indicate the two clades within the species. Rings, from inside to outside, indicate i) the geographic origin of the isolates (Brest, brown square; Sylt, beige square); ii) the presence (blue circles) or absence (white circles) of pGV-like plasmids; iii) the presence (black squares) or absence (white squares) of the *r5-7* gene and iv) the mortality rate (colour gradient from green to red corresponding to 0 to 100%) induced by individual strains 24h after injection in oysters (n=20). Experiments were performed in duplicate with two distinct oyster batches. The arrows highlight the virulent strains previously sequenced (Lemire et al., 2015) the non-virulent strain from clade 1 (8T5\_11) and the two plasmid-carrying but non-virulent strains from clade 2 (7T7\_10 and 8T7\_10).

**Figure 2. Experimental assessment of pGV loci as virulence determinants.** **A-**The indicated region or genes were deleted by allelic exchange and the virulence of *V. crassostreae* J2-9 wild-type (wt), mutants of specific loci ( $\Delta$ ) and complemented strains (+Plac\_tf or paar) was compared by **B-** injection of strains ( $10^6$  or  $10^7$  cfu depending on the cohort susceptibility, see Material and Methods) in 20 oysters and counting the percentage of mortalities after 24 hours; **C-** hemocyte cell viability evaluated by flow cytometry using a double staining procedure (SYBR® Green and

propidium iodide, PI, Sigma). Injection and cell viability assays were performed in duplicate and triplicate, respectively, and reproduced at least twice. A single experiment is represented here for each method. Letters indicate significant differences of mortality assessed by simultaneous tests for general linear hypotheses with Tukey contrasts ( $P < 0.05$ ).

**Figure 3. The identified transcription factor activates both T6SS<sub>pGV</sub> operons.** RNAseq analyses revealed that the expression of *tf* resulted in changed mRNA levels (Log2Fold change on the y-axis) of 21 genes belonging to the T6SS<sub>pGV</sub> cluster (x-axis). The T6SS<sub>pGV</sub> locus is organized into two putative operons expressed in opposite directions.

**Figure 4. The identified transcription factor activates a bidirectional promoter.** The putative bidirectional promoter containing a palindromic sequence (shown in the middle in A) was cloned between *gfp* and *dsRed* in a replicative plasmid, which was used to transform *V. cholerae* strain A1552-TnTF1512. This strain carries *tf* behind an arabinose inducible promoter within a miniTn7 transposon. Induction of the transcription factor by arabinose resulted in the production of both GFP and DsRed as observed by epifluorescence microscopy (B) or western blotting (C).

**Figure 5 Comparative genomic of *V. crassostreae*, *V. aestuarianus* and *V. tapetis* T6SS and putative effectors.** A. Synteny of the T6SS in the three strains compared. Genes with the same

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colour code are homologous (>40% amino acid identity). Specific genes in each T6SS are shaded in yellow and described in B. Schematic representation of the sequence identity or structural similarity of the putative effector of *V. crassostreae* T6SS<sub>pGV</sub> in strain J2-9, *V. aestuarianus* 02-041 and *V. tapetis* CECT4600. Structural similarities were identified with Phyre2. C. Phylogeny based on a concatemer of T6SS homologs found in *V. crassostreae* (J5-4; LGP7; J2-9; J5-20), *V. tapetis* (CECT4600), *V. aestuarianus* (07-115; 02-041; 12-128a; 01-032) and *V. tasmaniensis* (LGP32; J0-13). The matrix shows the conservation of the different T6SS homologs with T6SS<sub>pGV</sub> as a reference. A scale bar indicating amino acid sequence identity is located to the right of the matrix.

**Figure 6. Cytotoxic activities of T6SS and R5.7.** The cytotoxicity of *V. crassostreae* wt or mutant strains ( $\Delta$ ) was assessed by flow cytometry using a double staining procedure. Control hemocytes were either incubated in the absence of any bacteria or with a non-virulent strain (J2-8). The experiment was performed in triplicate and reproduced twice. A single experiment is represented here. Letters indicate significant differences of mortality assessed by simultaneous tests for general linear hypotheses with Tukey contrasts ( $P < 0.05$ ).













