environmental microbiology

Environmental Microbiology (2019) **00**(00), 00–00 doi:10.1111/1462-2920.14776

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

Damien Piel,[1,2†] Maxime Bruto,[2†] Adèle James,[1,2†] Yannick Labreuche,[1,2] Christophe Lambert,[3] Adrian Janicot,[2] Sabine Chenivesse,[2] Bruno Petton,[1,3] K. Mathias Wegner,[4] Candice Stoudmann,[5] Melanie Blokesch[5] and Frédérique Le Roux [1,2*]

[1]*Unité Physiologie Fonctionnelle des Organismes Marins ZI de la Pointe du Diable, CS 10070, Ifremer, F-29280, Plouzané, France.*

[2]*Sorbonne Universités, UPMC Paris 06, CNRS, UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff cedex, France.*

[3]*Laboratoire des Sciences de l'Environnement Marin, UMR 6539 CNRS UBO IRD IFREMER, Institut Universitaire Européen de la Mer, Technopôle Brest-Iroise – Rue Dumont d'Urville, F-29280, Plouzané, France.*

[4]*AWI - Alfred Wegener Institut, Helmholtz-Zentrum für Polar und Meeresforschung, Coastal Ecology, Wadden Sea Station Sylt, 25992, Hafenstrasse 43,List, Germany.*

[5]*Laboratory of Molecular Microbiology, Global Health Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, CH-1015, Lausanne, Switzerland.*

Summary

Pacific oyster mortality syndrome affects juveniles of *Crassostrea gigas* oysters and threatens the sustainability of commercial and natural stocks of this species. *Vibrio crassostreae* (*V. 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 haemocyte (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 haemocyte cytotoxicity is a lethality trait shared by a broad range of mollusc pathogens, and we speculate that T6SS was involved in parallel evolution of pathogen for molluscs.

Received 24 June, 2019; revised 1 August, 2019; accepted 5 August, 2019. *For correspondence. E-mail frederique.le-roux@sb-roscoff.fr; Tel. (+33) 2 98 29 56 47; Fax (+33) 2 98 29 23 24. †These authors contributed equally to this work.

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 the infection of the haemocytes, the oyster immune cells, by the endemic ostreid herpesvirus 1 (OsHV-1) µVar (de Lorgeril *et al*., 2018). Viral replication leads to the host entering an immune-compromised state, evolving towards subsequent bacteraemia involving opportunistic bacteria such as *Vibrio* sp. Exploring POMS in an oyster-farming area from the French North Atlantic cost (Brest), we showed previously that the onset of the disease is associated with progressive replacement of diverse benign colonizers by the members of a phylogenetically coherent virulent population, *Vibrio crassostreae* (*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 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* and *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 haemocyte cytotoxicity (Rubio *et al*., 2019). In *V. crassostreae*, haemocyte 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 from 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 < 16 °C, 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 haemocyte 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 mollusc pathogens.

Results

The virulence plasmid is widespread in Vibrio crassostreae *population occurring in oyster farms*

We previously hypothesized that the introgression of the virulence plasmid pGV into *V. crassostreae* might have been favoured 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 (Supporting Information Fig. S1). Multilocus sequence typing further confirmed the taxonomic assignment of 47 *V. crassostreae* strains isolated from Sylt (Fig. 1, beige squares) as well as 42 isolates from Brest (Fig. 1, brown squares) (Supporting Information 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 (Fig. 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 (Supporting Information 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%–100% oyster mortalities, 24 h postinjection), supporting previous findings (Bruto

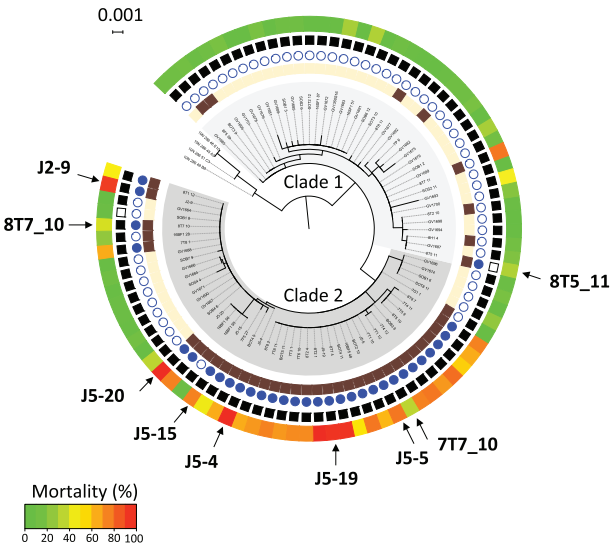

Fig. 1. The presence of the pGV plasmid is correlated with the geographic origin and virulence of *V. crassostreae* strains. Phylogenetic tree of 89 *V. crassostreae* isolates based on the *gyrB/rctB/rpoD* gene fragments. Dark/light shades of grey indicate the two clades within the species. Rings, from 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%–100%) induced by individual strains 24 h 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).

et al., 2017). Only three strains carrying the plasmid (8T5_11, 7T7_10 and 8T7_10) induce a weak mortality (< 20%) (Fig. 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* (Fig. 1 and Supporting Information 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 (Supporting Information Fig. S2). Furthermore, the *r5.7* gene was detected by polymerase chain reaction (PCR) in the non-virulent strains 7T7_10 and 8T7_10 that carry the pGV plasmid (Fig. 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 asymptomatically 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 was accompanied by the presence of *V. crassostreae* in the water tank and in the haemolymph of moribund animals (Supporting Information Fig. 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 *Materials and methods*), 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, Fig. 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 (Δtf) resulted in a significant decrease in mortality after oyster injection (Fig. 2B). Constitutive expression of *tf* from a plasmid was sufficient to restore virulence both in the Δtf mutant and in a mutant lacking the complete Px3 region (ΔPx3). On the other hand, expression of *tf* in a pGV-cured strain did not result in

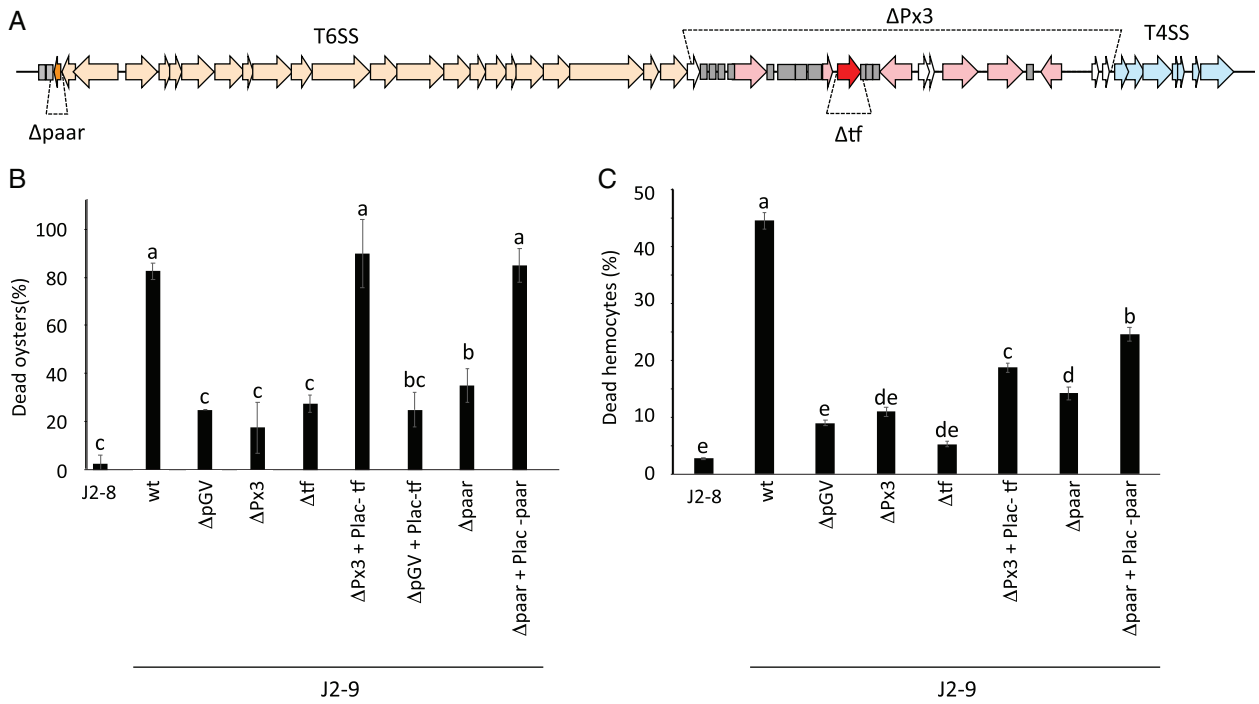

Fig. 2. Experimental assessment of pGV loci as virulence determinants. A. The indicated region or genes were deleted by allelic exchange, and the virulence of *V. crassostreae* J2-9 WT, mutants of specific loci (Δ) and complemented strains (+Plac_tf or *paar*) was compared by (B). B. Injection of strains (10^6 or 10^7 cfu depending on the cohort susceptibility, see *Materials and methods*) in 20 oysters and counting the percentage of mortalities after 24 h. C. Haemocyte cell viability evaluated by flow cytometry using a double-staining procedure (SYBR Green and 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 ($p < 0.05$).

increased mortality (Fig. 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 haemocytes (Rubio *et al*., 2019). Here, using flow cytometry, we observed that *V. crassostreae* effects on haemocyte viability require the presence of pGV. Deletion of the Px3 region or of the *tf* gene also led to an attenuation of cytotoxicity (Fig. 2C). Expression of the *tf* gene *in trans* complemented the ΔPx3 deletion with respect to haemocyte 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*., 2019). This discrepancy might be explained by the different methodological approaches used to assess cell viability. In the previous study, bacteria were added to haemocyte monolayers at a multiplicity of infection (MOI) of 50 and viability monitored for 15 h by a SYTOX green assay (Rubio *et al*., 2019). Here, the exposition of haemocytes to Vibrios was performed in a cell suspension at an MOI of 10 for 6 h before addition of SYBR Green I and propidium iodide (PI) to determine cell viability by flow cytometry. To verify that the plasmid is essential for toxicity, we thus incubated the haemocytes with a wild-type (WT) *V. crassostreae* strain (J2-9) or with a plasmid-cured strain (ΔpGV) at MOIs of 10 or 100 for 6 h. 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 (Supporting Information Fig. S4). Altogether, our results showed that the TF regulator controls plasmid-carried genes involved in haemocyte cytotoxicity.

The TF transcriptional regulator activates a T6SS

The *tf* gene encodes a putative transcriptional regulator of the AraC family that contains two domains: an N-terminal domain with putative Class I glutamine amidotransferase function and a C-terminal helix–turn–helix DNA binding domain (Supporting Information Fig. S5). To identify its target gene(s), we conducted an RNAseq analysis to compare the transcriptomes of a *V. crassostreae* derivative ΔPx3 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; Supporting Information 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 (hereafter named $T6SS_{pGV}$) (Fig. 3). The induction of two of the $T6SS_{pGV}$ genes (*vipA* and *vgrG*, the first gene of each operon) by TF was further validated by RT-PCR in two biologically independent experiments (Supporting Information Fig. S6).

The $T6SS_{pGV}$ locus is organized into at least two operons with *vgrG*, a gene encoding unknown function and *paar* being expressed in the opposite direction compared with the rest of the $T6SS_{pGV}$ 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 six nucleotides spaced by five nucleotides (Fig. 4). This motif was not identified at other loci within the *V. crassostreae* genome. To test whether the transcription factor (TF) and this putative promoter region were sufficient to drive the 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 the TF (Fig. 4). Deletion of the palindromic sequence altered the induction capacity of TF, while inversion or mutation of one of the six nucleotide sites did not abrogate gene activation (Fig. 4). We therefore concluded that the TF drives T6SS expression in *V. crassostreae*.

The $T6SS_{pGV}$ is involved in virulence and haemocyte 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 substructures: a trans-membrane complex, a baseplate and a tail composed of an inner tube formed by hexamers of haemolysin-coregulated protein 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/).

A genetic approach was therefore used to test the importance of the $T6SS_{pGV}$ 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 (Δ*paar*) and led to decreased virulence (Fig. 2A). Complementation by

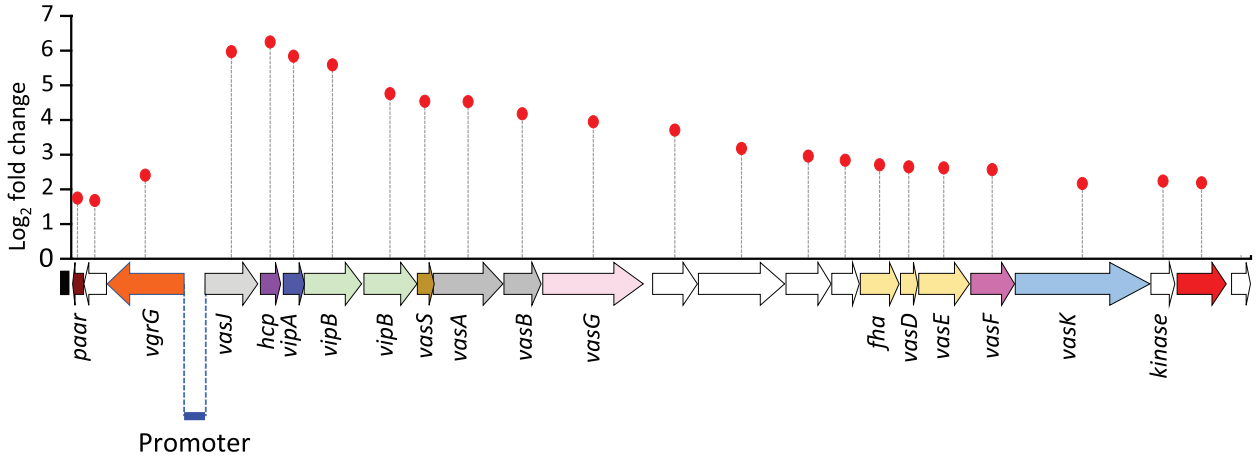

Fig. 3. The identified TF activates both $T6SS_{pGV}$ operons. RNAseq analyses revealed that the expression of *tf* resulted in changed mRNA levels (Log2Fold change on the y-axis) of 21 genes belonging to the $T6SS_{pGV}$ cluster (x-axis). The $T6SS_{pGV}$ locus is organized into two putative operons expressed in opposite directions.

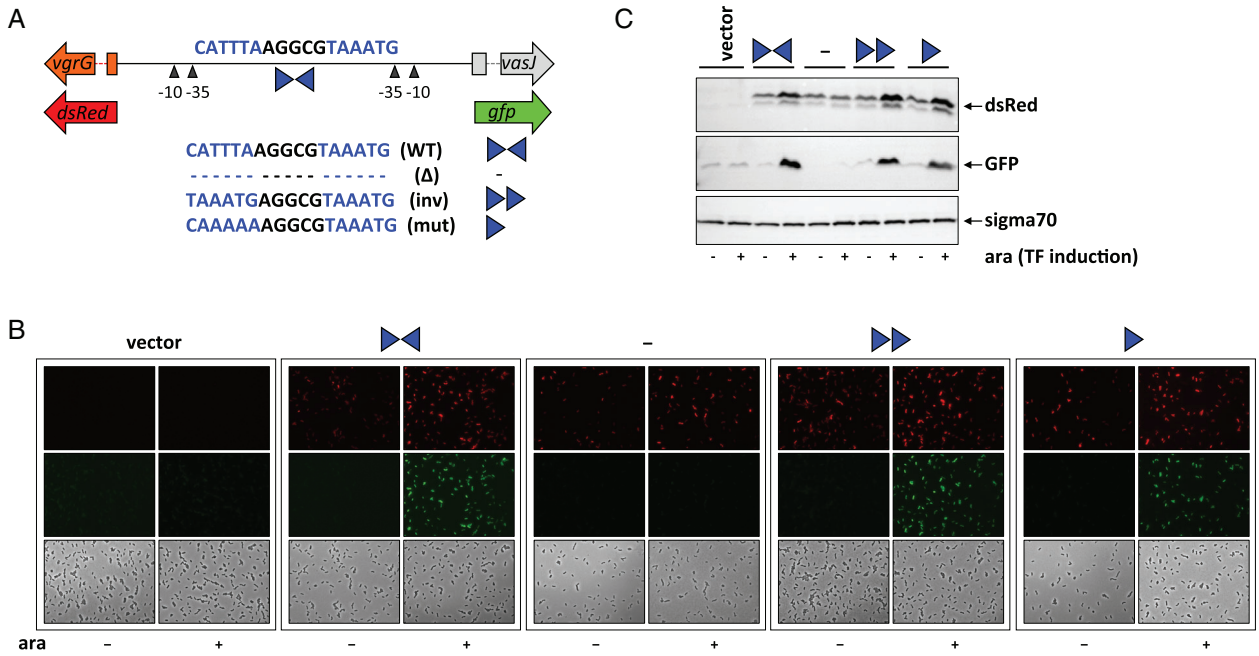

Fig. 4. The identified TF activates a bidirectional promoter. The putative bidirectional promoter containing a palindromic sequence (shown in the middle in A) was cloned between *gfp* and *dsRed* in a replicative plasmid, which was used to transform *V. cholerae* strain A1552-TnTF. This strain carries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

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 amoeba *Acanthamoeba castellanii* is not affected (Van der Henst *et al*., 2018). Here, we observed that the *V. crassostreae* Δ*paar* mutant has decreased cytotoxicity towards haemocytes compared with the WT and that expression of the *paar* gene *in trans* partially restored cytotoxicity (Fig. 2C). Our results therefore suggest a critical role for the virulence plasmid, TF and $T6SS_{pGV}$ in *V. crassostreae*-mediated killing of oyster immune cells and, therefore, pathogenicity towards this animal host.

Looking at the distribution of the $T6SS_{pGV}$ 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 (Fig. 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 $T6SS_{pGV}$, 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 nigritoxin, a toxin for crustaceans and insects (Labreuche *et al*., 2017). Hence, while annotation and localization of these genes suggest a role as T6SS effectors for the three pathogens, the formal demonstration of their function remains to be done.

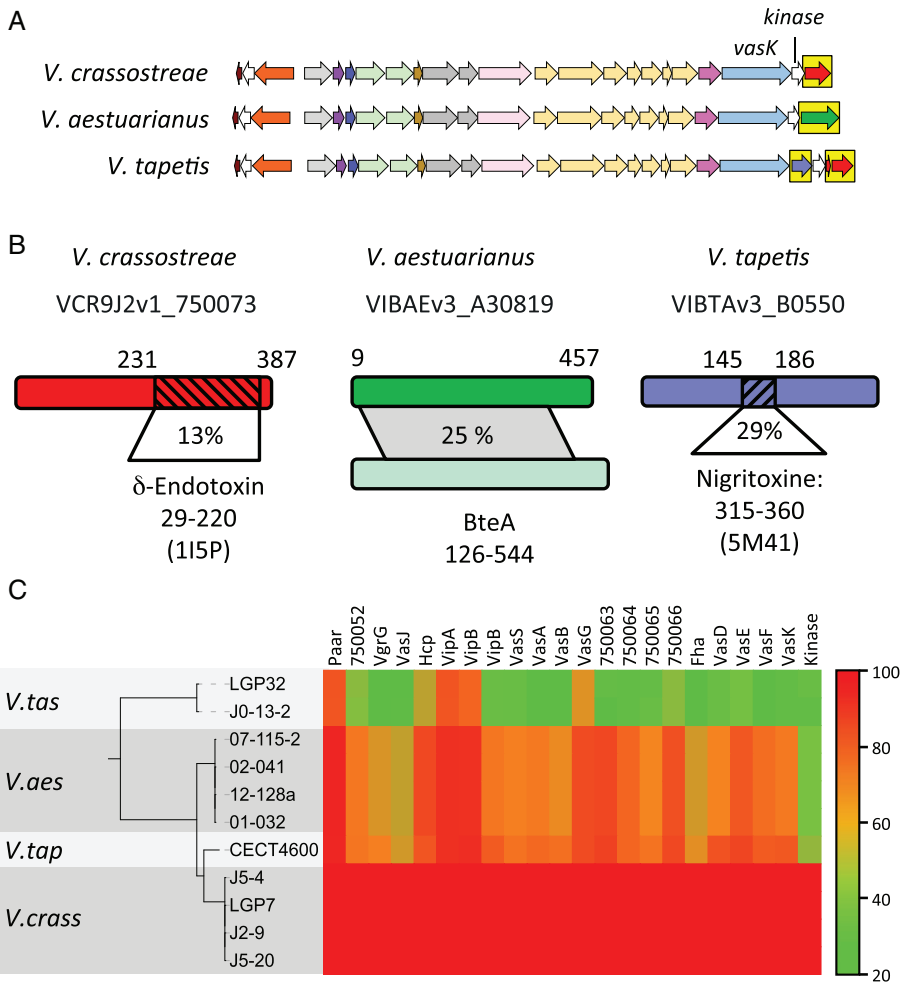

Fig. 5. Comparative genomic of *V. crassostreae*, *V. aestuarianus* and *V. tapetis* T6SS and putative effectors.
A. Synteny of the T6SS in the three strains compared. Genes with the same colour code are homologous (> 40% amino acid identity). Specific genes in each T6SS are shaded in yellow and described in (B).
B. Schematic representation of the sequence identity or structural similarity of the putative effector of *V. crassostreae* $T6SS_{pGV}$ 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 homologues 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 homologues with $T6SS_{pGV}$ as a reference. A scale bar indicating amino acid sequence identity is located to the right of the matrix.

The $T6SS_{pGV}$ 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*., 2019) and (ii) more recent acquisition of $T6SS_{pGV}$ 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 haemocyte and facilitating anchorage of the $T6SS_{pGV}$, which then injects a toxic effector into the cell. Under such a hypothesis, deletion of the *r5.7* gene (Δ*r5.7*) or curing of the plasmid (ΔpGV) should decrease the cytotoxicity of *V. crassostreae* to a similar level to that observed with the double mutant ΔpGV1512Δ*r5.7*. However, as we observed that the cytotoxicity of the double mutant was significantly more attenuated than that of the single mutants (Fig. 6), we suggest that these virulence factors act additively rather than being functionally connected.

Discussion

In recent years, a syndrome affecting juveniles of *C. 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 µVar, 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

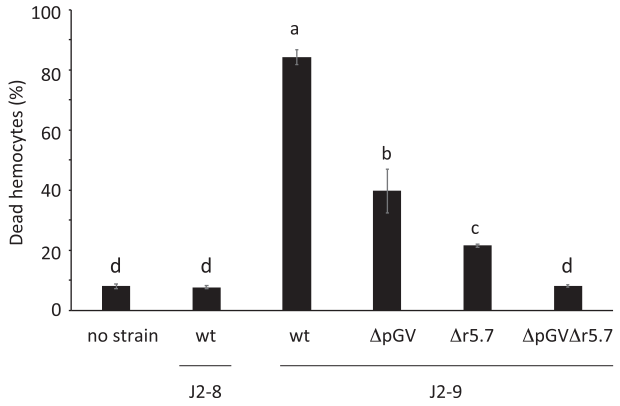

Fig. 6. Cytotoxic activities of T6SS and R5.7. The cytotoxicity of *V. crassostreae* wt. or mutant strains (Δ) was assessed by flow cytometry using a double-staining procedure. Control haemocytes were either incubated in the absence of any bacteria or with a non-virulent strain (J2-8). The experiment 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 ($P < 0.05$).

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 haemocytes by distinct pathways.

Oyster-associated Vibrios have been previously analysed 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 the surrounding seawater, suggesting that its primary habitat is not the water column. Potential alternative reservoirs for *V. crassostreae* at temperature < 16 °C were still undetermined. Here, we showed that oysters that reside in farming areas year-round asymptomatically host *V. crassostreae* and hence potentially serve as a pathogen reservoir. An increase in 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 noticed, however, that *V. aestuarianus* virulence seems to be restricted to the adult stage of oyster (Azema *et al*., 2017). Importantly, OsHV-1 μVar was never detected in our experiments, confirming previous observations that infection of juveniles can occur in the absence of OsHV-1μVar (Petton *et al*., 2015b). Hence, our present results suggest that oyster mortality syndrome might have different aetiologies. 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 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 $T6SS_{pGV}$ and its transcriptional activator TF, we deciphered a mechanism that increases haemocyte cytotoxicity of *V. crassostreae* worsens oyster disease. In the future, identification of the effector protein(s) of the $T6SS_{pGV}$ should help decipher its effect on haemocytes. 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 mollusc 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 haemocytes. 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 haemocytes (Rubio *et al*., 2019). However, several *Vibrio tasmaniensis* (*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 with *V. crassostreae*, the haemocyte cytotoxicity of these strains was demonstrated to be dependent on phagocytosis and required a distinct T6SS localized on the chromosome 1 of the strain LGP32 ($T6SS_{Chr1\text{-}LGP32}$, Rubio *et al*., 2019) (Fig. 5C). Consideration of these 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 $T6SS_{Chr1\text{-}LGP32}$ that is active at the intracellular stage as described for the *V. cholerae* T6SS (Ma *et al*., 2009). Alternatively, the acquisition of a $T6SS_{Chr1\text{-}LGP32}$ that functions exclusively during the

intracellular stage may have further selected for *r5.7* loss. Hence, in addition to Rubio *et al*.'s article (Rubio *et al*., 2019), 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.

Experimental procedures

Isolation of bacteria and gene sequencing

In July 2015 and 2016, 24 live oysters, together with surrounding seawater (temperature 18 °C), were collected from 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 2 l water samples pre-filtered through the 60 μm plankton net and sequentially filtered through 5, 1 and 0.22 μm 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 bactopeptone and 1 g/l yeast extract in artificial seawater, pH 7.6). All isolates were genotyped by partial *hsp60* gene sequencing and stored in 10% dimethylsulphoxide (DMSO) at −80 °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 °C).

Strains, plasmids and culture conditions

The strains used in this study are described in Supporting Information Table S5. *Vibrio* isolates were grown at 20 °C in Zobell broth or agar, Luria-Bertani (LB) or LB-agar (LBA) + 0.5 M NaCl. *Vibrio cholerae*, strain A1552, was grown in LB at 30 °C. *Escherichia coli* strains were grown at 37 °C in LB or on LBA. Chloramphenicol (5 or 25 μg/ml for *Vibrio* and *E. coli* respectively), spectinomycin (100 μg/ml), kanamycin (75 μg/ml for *V. cholerae*), 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 where indicated.

Vector construction and mutagenesis

All plasmids used or constructed in the present study are described in Supporting Information 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 Supporting Information 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 Apa1/Xho1 (*paar*) or EcoR1/Xho1 (tf) sites of the pMRB plasmid, which is stable in *Vibrio* spp. (Le Roux *et al*., 2011), resulting in constitutive expression from a P_{lac} 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 Scrudato 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 2 h at 30 °C in LB medium containing kanamycin. At this point, 0.2% arabinose was added to the culture where indicated and the growth was continued for 2 h 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 5 h at 30 °C in LB medium with or without 0.2% arabinose supplementation (after 3 h of growth) to induce *tf* in the respective strains. Cells were lysed by resuspension in 2× Laemmli buffer (100 μl of buffer per OD_{600} 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 Scrudato 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 Sigma 70-HRP conjugate (BioLegend, #663205; diluted 1:10 000) was used to validate equal loading. Lumi-Light^{PLUS} (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 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 (24–30 months old) were provided by a local oyster farm (Coïc, Pointe du Château, Logonna-Daoulas, France) and were used to collect haemolymph for cytotoxicity assays. Wild adult *C. gigas* oysters (n = 50) were collected from the 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 μm filtered seawater at 21 °C. Mortality was recorded daily for 14 days. Vibrios were isolated daily from the tank seawater (100 μl) or from the haemolymph of moribund animals (10 μl) by plating onto selective media (TCBS; Difco, BD, France). Randomly selected colonies were mixed into 20 μl 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 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 1 l CSW or with fresh 5 μm 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 polymerase chain reaction

Haemolymph 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 three-month-old juvenile oysters, the whole wet body of dead animals was crushed in marine broth (1 mg/ml) using a TissueLyser II (Qiagen). Genomic DNA was purified from homogenized oyster tissues or haemocyte cell pellets by resuspension in lysis buffer (NaCl 0.1 M, pH 8 EDTA 0.025 M, SDS 1%, proteinase K 100 μg/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 three different concentrations (1×, 0.1× and 0.01×) 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; 100 μl of the culture (10^6 or 10^7 colony-forming unit, cfu, depending on the susceptibility of the considered cohort) was 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 h.

In vitro *cytotoxicity assays*

Haemolymph 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 haemolymph that was free of contaminating particles (sperm, ovocytes and small debris). Selected samples were filtered through an 80 μm 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/haemocyte ratio, haemocyte and bacterial cell concentrations were measured by incubating 300 μl 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× in DMSO) at 1× final concentration, in the dark at room temperature for 10 min before flow-cytometric analysis (FACSVerseTM, Becton Dickinson, CA). Haemocytes 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 haemocyte counting, the haemolymph pool was divided into 200 μl subsamples maintained on ice. Each subsample received 200 μl of the different bacterial suspensions (WT or derivatives) at an MOI of 10:1 or 200 μl of FSSW as a control. Each condition was tested in three replicates, and the experiment was performed twice. Tubes were maintained at 18 °C for 5.5 h. Then, SYBR Green I and PI (Sigma-Aldrich) were added to each tube at final concentrations of 1× and 10 $\mu g\ mL^{-1}$, respectively, and incubation was continued for another 30 min (6 h total incubation time): PI only permeates haemocytes 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 percentage of dead haemocytes.

Genome sequencing, assembly and annotation. The 8T5-11 strain was sequenced by the JGI using illumina HiSeq2000 technology and 300bp library. 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 Supporting Information Table S2.

In silico *analyses*

Species trees were reconstructed based on an MLST containing three 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).

RNAseq experimentation

The *Vibrio* strains J2-9 ΔPx3 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 three 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 500 Mid (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 analysed by binomial generalized linear mixed models with logit link function taking the number of survivors versus the number of dead oysters as response

variable and strain identity as predictor. Owing to the high number of cells analysed in flow cytometry assays of haemocyte mortality, we used linear mixed models 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.

Acknowledgements

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. The 8T5-11 genome sequence data were produced by the US Department of Energy Joint Genome Institute http://www.jgi.doe.gov/ in collaboration with the user community. MBl is a Howard Hughes Medical Institute (HHMI) International Research Scholar (Grant #55008726), and the work in her group was supported by a Swiss National Science Foundation Scientific Exchange Grant (IZSEZ0_181044) and an ERC Consolidator Grant from the European Research Council (724630-CholeraIndex).

References

Azema, P., Lamy, J.B., Boudry, P., Renault, T., Travers, M. A., and Degremont, L. (2017) Genetic parameters of resistance to *Vibrio aestuarianus*, and OsHV-1 infections in the Pacific oyster, *Crassostrea gigas*, at three different life stages. *Genet Sel Evol* **49**: 23.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., *et al*. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* **19**: 455–477.

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

Bruto, M., James, A., Petton, B., Labreuche, Y., Chenivesse, S., Alunno-Bruscia, M., *et al*. (2017) *Vibrio crassostreae*, a benign oyster colonizer turned into a pathogen after plasmid acquisition. *ISME J* **11**: 1043–1052.

Bruto, M., Labreuche, Y., James, A., Piel, D., Chenivesse, S., Petton, B., *et al*. (2018) Ancestral gene acquisition as the key to virulence potential in environmental *Vibrio* populations. *ISME J* **12**: 2954–2966.

Cianfanelli, F.R., Monlezun, L., and Coulthurst, S.J. (2016) Aim, load, fire: the type VI secretion system, a bacterial nanoweapon. *Trends Microbiol* **24**: 51–62.

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., *et al*. (2018) Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. *Nat Commun* **9**: 4215.

Goudenege, D., Travers, M.A., Lemire, A., Petton, B., Haffner, P., Labreuche, Y., *et al*. (2015) A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. *Environ Microbiol* **17**: 4189–4199.

Gouy, M., Guindon, S., and Gascuel, O. (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* **27**: 221–224.

Hothorn, T., Bretz, F., and Westfall, P. (2008) Simultaneous inference in general parametric models. *Biom J* **50**: 346–363.

Kuwae, A., Momose, F., Nagamatsu, K., Suyama, Y., and Abe, A. (2016) Btea secreted from the *Bordetella bronchiseptica* type III secetion system induces necrosis through an actin cytoskeleton signaling pathway and inhibits phagocytosis by macrophages. *PLoS One* **11**: e0148387.

Labreuche, Y., Chenivesse, S., Jeudy, A., Le Panse, S., Boulo, V., Ansquer, D., *et al*. (2017) Nigritoxin is a bacterial toxin for crustaceans and insects. *Nat Commun* **8**: 1248.

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

Le Roux, F., and Blokesch, M. (2018) Eco-evolutionary dynamics linked to horizontal gene transfer in Vibrios. *Annu Rev Microbiol* **72**: 89–110.

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

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

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

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

Letunic, I., and Bork, P. (2011) Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* **39**: W475–W478.

Lo Scrudato, M., and Blokesch, M. (2012) The regulatory network of natural competence and transformation of *Vibrio cholerae*. *PLoS Genet* **8**: e1002778.

Ma, A.T., McAuley, S., Pukatzki, S., and Mekalanos, J.J. (2009) Translocation of a Vibrio cholerae type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe* **5**: 234–243.

Martenot, C., Oden, E., Travaille, E., Malas, J.P., and Houssin, M. (2010) Comparison of two real-time PCR methods for detection of ostreid herpesvirus 1 in the Pacific oyster *Crassostrea gigas*. *J Virol Methods* **170**: 86–89.

Matsumoto-Mashimo, C., Guerout, A.M., and Mazel, D. (2004) A new family of conditional replicating plasmids and their cognate *Escherichia coli* host strains. *Res Microbiol* **155**: 455–461.

Parizadeh, L., Tourbiez, D., Garcia, C., Haffner, P., Degremont, L., Le Roux, F., and Travers, M.A. (2018) Ecologically realistic model of infection for exploring the host damage caused by *Vibrio aestuarianus*. *Environ Microbiol* **20**: 4343–4355.

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

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

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

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

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

Rubio, T., Oyanedel-Trigo, D., Labreuche, Y., Toulza, E., Luo, X., Bruto, M., *et al*. (2019) Species-specific mechanisms of cytotoxicity toward immune cells determine the successful outcome of *Vibrio* infections. *Proceedings of the National Academy of Sciences* 116:14238–14247. https://doi.org/10.1073/pnas.1905747116

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

Shapiro, B.J., Levade, I., Kovacikova, G., Taylor, R.K., and Almagro-Moreno, S. (2016) Origins of pandemic *Vibrio cholerae* from environmental gene pools. *Nat Microbiol* **2**: 16240.

Shneider, M.M., Buth, S.A., Ho, B.T., Basler, M., Mekalanos, J.J., and Leiman, P.G. (2013) PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* **500**: 350–353.

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

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

Unterweger, D., Miyata, S.T., Bachmann, V., Brooks, T. M., Mullins, T., Kostiuk, B., *et al*. (2014) The *Vibrio cholerae* type VI secretion system employs diverse effector modules for intraspecific competition. *Nat Commun* **5**: 3549.

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

Vallenet, D., Belda, E., Calteau, A., Cruveiller, S., Engelen, S., Lajus, A., *et al*. (2013) MicroScope—an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res* **41**: D636–D647.

Van der Henst, C., Vanhove, A.S., Drebes Dorr, N.C., Stutzmann, S., Stoudmann, C., Clerc, S., *et al*. (2018) Molecular insights into *Vibrio cholerae's* intra-amoebal host-pathogen interactions. *Nat Commun* **9**: 3460.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Population structure of *Vibrio* isolates (n = 1629) recovered from seawater fractions or oyster tissues from two geographic areas, Brest (France) and Sylt (Germany). Phylogenetic tree (maximum likelihood) based on partial *hsp60* sequences. The grey areas correspond to different clades labelled by letters (from A to V) and taxonomically assigned to known *Vibrio* species, i.e. *V. breogani* (A), *V. pacinii* (B), *V. fischeri* (C), *V. alginolyticus* (E, F), *V. jasicida* (G), *V. chagasii* (L), *V. crassostreae* (P) also indicated with a black arrow, *V. kanaloae* (T), *V. cyclitrophicus* (U) and *V. splendidus* (V) or *Vibrio* sp. *nov* (D, H, I, J, K, M, N, O, Q, R, S). The inner and outer rings indicate the origin of the strain and the site of sampling, respectively, following the colour code given on the right panel.

Fig. S2. Role of R5-7 in the cytotoxic activity of 8 T5-11 strains. The *r5-7* gene or *gfp* as a control were expressed *in trans* from a plasmid in *V. crassostreae* strain 8 T5-11 or in a mutant *V. crassostreae* strains J2-9Δ*r5-7*. Cytotoxic activity was assessed by flow cytometry using a double-staining procedure after exposition of the cells with bacteria at a ratio of 50 bacteria/haemocyte. As control, haemocytes were either incubated with the WT strain J2-9 or with a non-virulent strain (J2-8). The experiment was performed in duplicate. A single experiment is represented here. Letters indicate significant differences of mortality assessed by simultaneous tests for general linear hypotheses with Tukey contrasts ($P < 0.05$).

Fig. S3. Experimental infection in mesocosm.

A. Description of the 'natural' experimental infection. Wild adult oysters (n = 50) (animals coloured in blue) sampled in the Bay of Brest (seawater temperature of 12 °C) were returned to the laboratory and held in a 300 L tank under static conditions with aerated 5 μm filtered seawater at 21 °C. At day 11, three-month-old SPF oysters (n = 20) (animals coloured in orange) were immersed in 1 l of CSW collected from the tank containing the moribund wild oysters or in fresh 5 μm filtered seawater as a control.

B. Oyster disease dynamic. Mortality in wild adult oysters (blue line) or in three-month-old juvenile oysters (orange line) was recorded daily for 14 days and 6 days respectively. Cumulative mortality rates are indicated in % (y axis).

C. PCR detection of different oyster pathogens. *V. crassostreae*, *V. aestuarianus* and the herpes virus OsHV-1 μVar were detected in haemolymph of moribund wild adult oysters (blues boxes) sampled at day 10 (lanes 1 to 4) and day 11 (lanes 5 to 10) or from tissues of moribund three-month-old oysters (orange boxes) exposed to CSW and sampled at day 3 (lanes 1 to 5), day 4 (lanes 6 to 10), day 5 (lanes 11 to 17) and day 6 (18 to 20) post-immersion. The positive (+) and negative (−) signs indicate the positive and negative controls respectively.

Fig. S4. Cell viability assay of oyster haemocytes exposed to different ratios of *V. crassostreae* WT or mutants (Δ). Control haemocytes were incubated without bacteria (control) or with a non-virulent strain (J2-8). Cell viability was evaluated by flow cytometry using a double-staining procedure after exposure of the haemocytes to bacteria at a ratio of 10 bacteria/haemocyte (grey bars) or 100 bacteria/haemocyte (black bars) for 6 h. The assay was performed in triplicate. Asterisks indicate significant differences of mortality assessed by simultaneous tests for general linear hypotheses with Tukey contrasts ($P < 0.05$).

Fig. S5. Protein domains found in the TF. The protein was annotated with Interproscan. The two domains are represented by coloured tubes with numbers indicating the beginning and the end of each domain on the protein. The accession numbers in domain databases (SSF52317 = Superfamily (http://supfam.org/); PF12833 = PFAM (https://pfam.xfam.org/)) are indicated with their putative function beneath.

Fig. S6. Activation of T6SS genes by the transregulator TF. *V. crassostreae* expressing constitutively the transcriptional factor *tf* (lines 1, 3, 5) or, as a control, the *gfp* (lines 2, 4, 6) were cultivated in marine broth to an optical density of 0.3 (lines 1, 2), 0.6 (lines 3, 4) and 1.0 (lines 5, 6), RNA were extracted, reverse transcribed and used for PCR detection of *gyrA* and *repB* (internal controls), *vgrG* and *vipA* (T6SS), *r5.7* and *r5.8* (chromosomal genes) and the *tf* expressed *in trans* from a plasmid. This experiment was performed twice, as indicated.

Table S1. Supplementary Table.

Table S2. Supplementary Table.

Table S3. Supplementary Table.

Table S4. Supplementary Table.

Table S5. Supplementary Table.

Table S6. Supplementary Table.

Table S7. Supplementary Table.