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HAL Authorization

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

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6 **Running Head:** *V. crassostreae* cytotoxicity relies on a T6SS

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25 **ORIGINALITY-SIGNIFICANCE STATEMENT** [L
SEP]

26 A recent study highlighted the role of a herpes virus as primary etiological agent of Pacific oyster
27 mortality syndrome (POMS), which affects juveniles of the oyster *Crassostrea gigas*. We show
28 here that the selection of virulent bacteria in oyster farms is also an important piece of the POMS
29 puzzle. This bacteria taxonomically assigned to *Vibrio crassostreae* species, carries a plasmid
30 that encodes a Type 6 Secretion System (T6SS), which increases its ability to kill the major
31 cellular players of oyster immunity, the hemocytes. This T6SS was identified in two additional
32 species that infect mollusks, suggesting a parallel evolution of these pathogens. Finally, our
33 results indicate that broad range of pathogens kill their hosts via hemocyte cytotoxicity.

34

35 **ABSTRACT**

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

49 **INTRODUCTION**

50 The Pacific oyster mortality syndrome (POMS) affects juveniles of *Crassostrea gigas*, the main
51 oyster species exploited worldwide. This syndrome occurs when the seawater temperature
52 reaches 16°C and is caused by multiple infections with an initial and necessary step relying on
53 infection of the hemocytes, the oyster immune cells, by the endemic Ostreid herpesvirus OsHV-1
54 μ Var (de Lorgeril et al., 2018). Viral replication leads to the host entering an immune-
55 compromised state, evolving towards subsequent bacteremia involving opportunistic bacteria
56 such as *Vibrio sp.* Exploring POMS in an oyster farming area from the French North Atlantic coast
57 (Brest), we showed previously that the onset of disease is associated with progressive
58 replacement of diverse benign colonizers by members of a phylogenetically coherent virulent
59 population, *V. crassostreae* (Lemire et al., 2015). The virulent population is genetically diverse
60 but most members of the population can cause disease. We further demonstrated that *V.*
61 *crassostreae* virulence depends on the presence of a large mobilizable plasmid, pGV1512
62 (hereafter named pGV for simplicity) although the mechanisms underpinning virulence remain to
63 be elucidated (Bruto et al., 2017). Having observed that juvenile infection by *V. crassostreae* is
64 recurrent in the POMS occurring in Brest (Bruto et al., 2017, de Lorgeril et al., 2018, Lemire et
65 al., 2015), the questions arose whether oyster farms create conditions that lead to the selection of
66 this virulence plasmid and whether oysters (farmed or wild) represent a reservoir of virulent *V.*
67 *crassostreae*. Indeed, it has been suggested that, during cold months, oysters act as a reservoir for
68 *V. aestuarianus* (Goudenege et al., 2015, Parizadeh et al., 2018), a pathogen that primarily targets
69 adult animals and hence is not thought to be involved in POMS (Azema et al., 2017).

70

71 Pathogenic lifestyles are typically associated with horizontal acquisition of virulence genes (Le
72 Roux and Blokesch, 2018), but pre-existing genomic features might be necessary for the
73 acquisition and/or the functionality of these virulence genes (Shapiro et al., 2016). Indeed, we
74 showed that a core gene, *r5.7*, which encodes an exported protein of unknown function, is
75 necessary for full virulence in *V. crassostreae* (Lemire et al., 2015). This gene is widely
76 distributed across the Splendidus clade, a large group of closely-related species (e.g., *V.*
77 *splendidus*, *V. crassostreae*, *V. cyclitrophicus*). The *r5.7* gene was acquired by the common
78 ancestor of this group and co-diversified in some populations while being lost from non-virulent
79 populations (Bruto et al., 2018). The widespread occurrence of *r5.7* across environmental *Vibrio*
80 populations suggests that it has an important biological role but its frequency also indicates that
81 this role is population-specific. Indeed, it was recently showed that *r5.7* is involved in population-
82 specific mechanisms of hemocyte cytotoxicity (Rubio et al., in press). In *V. crassostreae*
83 hemocyte cytotoxicity is contact-dependent and requires *r5.7*. The R5.7 protein is not lethal when
84 injected into oysters, but this protein is able to restore virulence when co-injected with a mutant
85 lacking the *r5.7* gene (Bruto et al., 2018). This suggests that R5.7 interacts with the external
86 surface of *Vibrio* and / or with a cellular target. Whether *r5.7* and the virulence gene(s) encoded
87 by the pGV plasmid act in concert or independently to promote *V. crassostreae* virulence and
88 cytotoxicity was a goal of this study.

89
90 Here, we explored the distribution and functional interaction of two *V. crassostreae* virulence
91 determinants, R5-7 and the plasmid pGV. *V. crassostreae* strains were collected from Brest
92 (France), an area of intense oyster farming that is experiencing recurrent mortality events, and in
93 Sylt (Germany) where a massive oyster invasion formed natural beds that have not yet suffered
94 from *Vibrio*-related disease outbreaks (Reise et al., 2017). While the *r5.7* gene was detected at

95 high frequency in *V. crassostreae*, the pGV plasmid was detected only in isolates from Brest and
96 its presence correlated with virulence as assessed by experimental oyster infections. We further
97 showed that, at a temperature of <16°C, oysters act as a reservoir of *V. crassostreae* strains.
98 Exploring genetically the virulence determinants carried by the plasmid we showed that a
99 transcriptional regulator is necessary for pGV-mediated virulence. This regulator induces the
100 expression of a molecular killing device called the type 6 secretion system (T6SS) which is also
101 necessary for full virulence. RNA sequencing (RNAseq) followed by transcriptional fusion
102 analysis led us to identify a bidirectional promoter within the T6SS genes cluster that is up-
103 regulated by the transcriptional activator. Gene deletions and complementation experiments
104 further confirmed the role of the *r5.7* and the T6SS in hemocyte cytotoxicity and indicated that
105 they act in an additive manner. Finally, the identification of a similar type of T6SS in *V.*
106 *aestuarianus* and *V. tapetis* led us to hypothesis a parallel evolution of mollusk pathogens.

107

108

109 **RESULTS**

110

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

112 We previously hypothesized that the introgression of the virulence plasmid pGV into
113 *V. crassostreae* might have been favored by elevated host density in farming areas (Bruto et al.,
114 2017). However, wild oyster beds can also reach high densities, as exemplified by the recent
115 invasion of *C. gigas* oysters into the Wadden sea (North Sea) (Reise et al., 2017). To date, no
116 *Vibrio*-associated mass mortalities have been observed in this area, in contrast to observations in
117 heavily farmed areas. We thus investigated the presence and frequency of the pGV plasmid in
118 *V. crassostreae* strains sampled from Sylt. For this, 910 *Vibrio* strains were isolated from

119 seawater fractions and oysters from Sylt, genotyped by partial *hsp60* gene sequencing and
120 assigned to *Vibrio* populations as described previously (Figure S1). Multi Locus Sequencing
121 Typing (MLST) further confirmed the taxonomic assignment of 47 *V. crassostreae* strains
122 isolated from Sylt (Figure 1, beige squares) as well as 42 isolates from Brest (Figure 1, brown
123 squares) (Table S1). The phylogenetic structure partitioned these strains into two clades
124 representing the two locations. The first clade contained the majority of strains from Sylt (68%,
125 32 out of 47), while the second clade principally contained strains from Brest (80%, 34 out of
126 42). The pGV *repB* gene was never detected in isolates from Sylt and was mainly detected in
127 strains from Brest that belonged to clade 2 (Figure 1, plain blue circles). Only one clade 1 strain
128 (8T5_11), originating from Brest, was found to be positive for *repB*. The presence of the plasmid
129 was confirmed by sequencing the genome of the 8T5_11 strain (Table S2). We next explored the
130 virulence of these isolates by experimental infection. When the 47 and 42 *V. crassostreae* strains
131 isolated from Sylt and Brest, respectively, were injected individually into oysters, we observed
132 that virulence was strongly correlated with the presence of the plasmid (50 to 100% oyster
133 mortalities, 24 hours post injection), supporting previous findings (Bruto et al., 2017). Only three
134 strains carrying the plasmid (8T5_11, 7T7_10 and 8T7_10) induce a weak mortality (<20%)
135 (Figure 1). Gene loss could explain this non-virulent phenotype. Indeed, comparative genomic
136 analyses identified 44 genes that were absent from the 8T5_11 genome but were present in all of
137 the sequenced virulent strains of *V. crassostreae* (Figure 1; Table S3). These 44 genes included
138 *r5.7*, which is necessary for virulence and is located in a region that was previously identified as
139 being specific to *V. crassostreae* (Lemire et al., 2015). However the expression of *r5.7* from a
140 plasmid had no effect on 8T5_11 virulence (Figure S2). Furthermore the *r5.7* gene was detected
141 by PCR in the non-virulent strains 7T7_10 and 8T7_10 that carry the pGV plasmid (Figure 1,

142 black squares). Together these results indicate a role for pGV in virulence but additional genomic
143 components appear to be necessary.

144

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

146 *V. crassostreae* infection has been recurrently associated with POMS events that affect juvenile
147 oysters at a temperature threshold of 16°C (Bruto et al., 2017, de Lorgeril et al., 2018, Lemire et
148 al., 2015). In oyster farming areas such as Brest, roughly 700 tons of farmed oysters are
149 introduced into a site where 10'000 tons of wild oysters reside (Pouvreau, personal
150 communication). We thus asked whether oysters may asymptotically host *V. crassostreae* and
151 hence play a role as a reservoir of this pathogen. Wild adult animals were collected from Brest at
152 12°C and returned to the laboratory where they were transferred into a tank at 21°C, a procedure
153 previously shown to allow the development and transmission of oyster diseases (Petton et al.,
154 2015a, Petton et al., 2015b, Petton et al., 2013). Mortality started at day 8, reached 90% after day
155 14, and were accompanied by the presence of *V. crassostreae* in the water tank and in the
156 hemolymph of moribund animals (Figure S3). The pGV plasmid was detected in 39 of 41 (95%)
157 *V. crassostreae* strains isolated during this experiment. We noted that *V. aestuarianus* was not
158 isolated on *Vibrio* selective media (TCBS, see material and method), although it was detected by
159 PCR in animal tissues, co-occurring or not with *V. crassostreae*. On the other hand, OsHV-1 was
160 never detected in DNA extracted from the oysters. Contaminated seawater (CSW) was collected
161 at day 11 from the tank containing the moribund wild oysters and three-month-old specific
162 pathogen free oysters (SPF juveniles) were exposed to this CSW at 21°C (Petton et al., 2013).
163 Mortalities of the juveniles started at day 3 and reached 100% after 6 days. No mortality occurred
164 when SPF juveniles were kept in filtered seawater at the same temperature. *V. crassostreae* and
165 *V. aestuarianus*, but not OsHV-1, were detected in moribund animal tissues. These results

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

168

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

170 Having shown that oyster farming correlates with a high prevalence of the virulence plasmid, we
171 next explored the virulence trait(s) encoded by pGV. A previous study identified a region within
172 pGV (Px3, Figure 2A) that is necessary for virulence in *V. crassostreae* (Bruto et al., 2017).
173 Manual annotation of the genes within this region did not reveal any known virulence
174 determinants, but a putative transcriptional regulator (labelled VCR9J2v1_750086 in J2-9 and
175 hereafter named TF for simplicity) was identified. We assessed the importance of TF for
176 virulence using a genetic knockout approach. Deletion of this gene (Δtf) resulted in a significant
177 decrease in mortality after oyster injection (Figure 2B). Constitutive expression of *tf* from a
178 plasmid was sufficient to restore virulence both in the Δtf mutant and in a mutant lacking the
179 complete Px3 region ($\Delta Px3$). On the other hand, expression of *tf* in a pGV-cured strain did not
180 result in increased mortality (Figure 2B). These results showed that the gene encoding the TF
181 regulator is the only gene involved in Px3-mediated virulence but that additional determinant(s),
182 carried by this plasmid, are involved in *V. crassostreae* virulence.

183

184 *V. crassostreae* virulence has been recently demonstrated to be intimately related with its
185 cytotoxic effects on hemocytes (Rubio et al., in press). Here, using flow-cytometry, we observed
186 that *V. crassostreae* effects on hemocyte viability require the presence of pGV. Deletion of the
187 Px3 region or of the *tf* gene also led to an attenuation of cytotoxicity (Figure 2C). Expression of
188 the *tf* gene *in trans* complemented the $\Delta Px3$ deletion with respect to hemocyte toxicity, mirroring

189 the phenotype observed following oyster injection. This result was surprising as pGV was
190 previously described as dispensable for *V. crassostreae* cytotoxicity (Rubio et al., in press). This
191 discrepancy might be explained by the different methodological approaches used to assess cell
192 viability. In the previous study, bacteria were added to hemocyte monolayers at a multiplicity of
193 infection (MOI) of 50 and viability monitored for 15 hours by a Sytox green assay (Rubio et al.,
194 in press). Here, exposition of hemocytes to vibrios was performed in a cell suspension at a MOI
195 of 10 for 6 hours before addition of SYBR Green-I and propidium iodide to determine cell
196 viability by flow cytometry. To verify that the plasmid is essential for toxicity, we thus incubated
197 the hemocytes with a wild type *V. crassostreae* strain (J2-9) or with a plasmid-cured strain
198 (Δ pGV) at MOIs of 10 or 100 for 6 hours. These tests revealed a dose-dependent effect in which
199 low levels of the plasmid-cured strain were less cytotoxic while high levels could overcome the
200 plasmid deficiency (Figure S4). Altogether, our results showed that the TF regulator controls
201 plasmid-carried genes involved in hemocyte cytotoxicity.

202

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

204 The *tf* gene encodes a putative transcriptional regulator of the AraC family that contains two
205 domains: a N-terminal domain with putative Class I glutamine amidotransferase function and a
206 C-terminal helix-turn-helix DNA binding domain (Figure S5). To identify its target gene(s) we
207 conducted a RNAseq analysis to compare the transcriptomes of a *V. crassostreae* derivative Δ Px3
208 constitutively expressing either the *tf* or the gene encoding the green fluorescent protein (*gfp*), as
209 a control. Expression of *tf* resulted in significant changed mRNA levels for only 27 predicted
210 protein-coding genes (Log2Fold change >2, Table S4) of which 6 and 21 genes were down- and
211 up-regulated, respectively, in a TF-dependent manner. All 21 up-regulated genes were located on

212 the virulence plasmid and encode a putative T6SS (here after named T6SS_{pGV}) (Figure 3). The
213 induction of two of the T6SS_{pGV} genes (*vipA* and *vgrG*, the first gene of each operon) by TF was
214 further validated by RT-PCR in two biologically independent experiments (Figure S6).

215
216 The T6SS_{pGV} locus is organized into at least two operons with *vgrG*, a gene encoding unknown
217 function and *paar* being expressed in the opposite direction compared to the rest of the T6SS_{pGV}
218 genes. Between these two operons, we predicted a bidirectional promoter (-10/-35 boxes on each
219 operon site) as well as a putative TF target site that comprised a palindromic sequence of 6
220 nucleotides spaced by 5 nucleotides (Figure 4). This motif was not identified at other loci within
221 the *V. crassostreae* genome. To test whether the transcription factor and this putative promoter
222 region were sufficient to drive expression of adjacent genes in a heterologous host, we cloned the
223 promoter between GFP- and DsRed-encoding genes in a replicative plasmid. Next, we
224 transformed this reporter plasmid into an unrelated *Vibrio* species (in this case *V. cholerae*),
225 which had been engineered to chromosomally encode *tf* under the control of an arabinose-
226 inducible promoter (P_{BAD}) (see Materials and methods for details). Induction of *tf* expression by
227 arabinose resulted in the production of both GFP and DsRed demonstrating that the promoter was
228 indeed bidirectional and activated by TF (Figure 4). Deletion of the palindromic sequence altered
229 the induction capacity of TF, while inversion or mutation of one of the 6 nucleotide sites did not
230 abrogate gene activation (Figure 4). We therefore concluded that the TF transcription factor
231 drives T6SS expression in *V. crassostreae*.

232
233 **The T6SS_{pGV} is involved in virulence and hemocyte cytotoxicity**
234 T6SSs are contact-dependent contractile nanomachines used by many Gram-negative bacteria as
235 weapons against a variety of prokaryotic and eukaryotic organisms (Cianfanelli et al., 2016).

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

246
247 A genetic approach was therefore used to test the importance of the T6SS_{pGV} for *V. crassostreae*
248 virulence. We had previously generated a knockout mutant that lacked this locus and observed no
249 effect on virulence (Bruto et al., 2017). However, re-investigating this mutant we identified an
250 unexpected duplication of this region resulting in one deleted and one whole T6SS cluster.
251 Several attempts to delete the *vgrG* or *vipA* genes were unsuccessful, repeatedly resulting in
252 complete loss of the plasmid, suggesting that these mutations come at a cost for the bacteria.
253 However, deletion of the T6SS *paar* gene was successful ($\Delta paar$) and led to decreased virulence
254 (Figure 2A). Complementation by constitutively expressing *paar in trans* restored the virulence
255 potential to similar levels as observed for the WT.

256
257 Having demonstrated a role for the T6SS in virulence, we next explored its cellular target. In
258 many bacterial models, T6SSs are used to kill competing bacteria (Cianfanelli et al., 2016). We

259 thus asked whether *V. crassostreae* that constitutively expressed *tf* would be able to kill bacteria
260 in an *in vitro* killing assay (Borgeaud et al., 2015). When the *tf*-expressing strain was used as a
261 predator and *E. coli*, *V. cholerae*, or a collection of 40 diverse *Vibrio* strains isolated from oysters
262 were used as prey, we did not observe any killing under the tested conditions. The T6SS has also
263 been demonstrated to mediate toxicity for eukaryotic cells. For example, non-pandemic *V.*
264 *cholerae* exhibits T6SS-mediated cytotoxicity towards macrophages and the soil amoeba
265 *Dictyostelium discoideum* (Pukatzki et al., 2007), while the aquatic amoebae *Acanthamoeba*
266 *castellanii* is not affected (Van der Henst et al., 2018). Here, we observed that the *V. crassostreae*
267 Δ *paar* mutant has decreased cytotoxicity towards hemocytes compared to the WT and that
268 expression of the *paar* gene *in trans* partially restored cytotoxicity (Figure 2C). Our results
269 therefore suggest a critical role for the virulence plasmid, TF, and T6SS_{pGV} in *V. crassostreae*-
270 mediated killing of oyster immune cells and therefore pathogenicity towards this animal host.

271 Looking at the distribution of the T6SS_{pGV} in publicly available *Vibrio* genomes, we found that
272 closely related loci are present in *V. aestuarianus* (11/11 genomes) and *V. tapetis* (1/1 genome),
273 which are pathogens of adult oysters and clams, respectively (Travers et al., 2015). Overall the
274 synteny and amino acid identities between core components of the T6SSs were high with the
275 exception of genes localized after the *vasK* gene that could be candidate effectors (Figure 5). In
276 *V. aestuarianus*, a specific gene (VIBAEv3_A30819 in the strain 02-041) encodes a protein with
277 weak sequence identity (25%) with a T3SS effector from *Bordetella bronchiseptica* named BteA.
278 This secreted protein has been reported to inhibit phagocytosis by macrophage and induce
279 necrosis through an actin cytoskeleton-signalling pathway (Kuwaie et al., 2016). In the T6SS_{pGV} a
280 specific gene (VCRJ2v1_750073 in strain J2-9) encodes a protein with 38% similarity and 13%
281 identity to the C-terminal and N-terminal domains of an insecticidal delta-endotoxin found in

282 *Bacillus thuringiensis*. Unfortunately, deletion of this gene in *V. crassostreae* also resulted in loss
283 of pGV preventing further functional analysis. An ortholog of VCRJ2v1_750073 in *V. tapetis* has
284 been pseudogenized, potentially leading to its functional inactivation. On the other hand, a
285 second, species-specific gene in the *V. tapetis* T6SS encodes a protein with only 60% similarity
286 and 29% identity within 45 amino acids of the central domain of nigratoxin, a toxin for
287 crustaceans and insects (Labreuche et al., 2017). Hence while annotation and localization of these
288 genes suggests a role as T6SS effectors for the three pathogens, the formal demonstration of their
289 function remains to be done.

290 **The T6SS_{pGV} and R5.7 protein act independently to mediate *V. crassostreae* cytotoxicity**

291 We showed in a previous study (Bruto et al., 2018) that *V. crassostreae* evolution as pathogen
292 involved sequential acquisition of virulence genes, including i) acquisition of the *r5.7* gene,
293 which encodes an exported protein that may be involved in the contact-dependant cytotoxicity
294 (Rubio et al in press) and ii) more recent acquisition of T6SS_{pGV} that, in our experimental design,
295 appeared necessary for the killing of host immune cells. It is therefore tempting to hypothesize
296 that these two virulence traits work in concert to mediate cytotoxicity, R5.7 potentially favouring
297 attachment of the vibrio to the hemocyte and facilitating anchorage of the T6SS_{pGV}, which then
298 injects a toxic effector into the cell. Under such an hypothesis, deletion of the *r5.7* gene ($\Delta r5.7$) or
299 curing of the plasmid (ΔpGV) should decrease the cytotoxicity of *V. crassostreae* to a similar
300 level to that observed with the double mutant $\Delta pGV1512\Delta r5.7$. However, as we observed that the
301 cytotoxicity of the double mutant was significantly more attenuated than that of the single
302 mutants (Figure 6), we suggest that these virulence factors act additively rather than being
303 functionally connected.

304

305 **DISCUSSION**

306

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

321 Oyster-associated vibrios have been previously analyzed in the context of a metapopulation
322 framework, *i.e.*, by considering potential overlap or differences in populations collected from
323 spatially and temporally distinct habitats, which are connected by dispersal (Bruto et al., 2017).
324 This study showed that *V. crassostreae* was abundant in diseased animals while nearly absent in
325 the surrounding seawater, suggesting that its primary habitat is not the water column. Potential
326 alternative reservoirs for *V. crassostreae* at temperature $<16^{\circ}\text{C}$ were still undetermined. Here, we
327 showed that oysters that reside in farming areas year-round asymptotically host

328 *V. crassostreae* and hence potentially serve as a pathogen reservoir. An increase of temperature
329 triggered active multiplication of *V. crassostreae* leading to a sufficiently high bacterial load
330 and/or virulence state allowing the pathogen to colonize and infect juvenile oysters. As *V.*
331 *aestuarianus* was detected in both adult and the juvenile oysters, it is impossible to discriminate
332 the respective roles of *V. crassostreae* and *V. aestuarianus* in the induction of oyster mortality in
333 the present experiment. It should be notice, however, that *V. aestuarianus* virulence seems to be
334 restricted to the adult stage of oyster (Azema et al., 2017). Importantly, OsHV-1 μ Var was never
335 detected in our experiments, confirming previous observations that infection of juveniles can
336 occur in the absence of OsHV-1 μ Var (Petton et al., 2015b). Hence our present results suggest that
337 oyster mortality syndrome might have different etiologies. It remains to be determined how
338 temperature acts on *V. crassostreae* infective status. In the context of global warming, how
339 temperature influences the virulence of these pathogens as well as oyster resistance or resilience
340 is a major concern to predict sustainability of commercial and natural stocks of this species.

341
342 Another argument strengthening a role for *V. crassostreae* in oyster juvenile mortality syndrome
343 is the high frequency of the pGV plasmid in farming areas that are affected by the syndrome.
344 Although we were able to isolate *V. crassostreae* from oysters in Sylt, none of these isolates were
345 virulent in an infection assay. This observation is consistent with the absence of the pGV plasmid
346 in these isolates and strengthens our hypothesis that the introgression of pGV into the *V.*
347 *crassostreae* population has played a major role in its emergence as a pathogen (Bruto et al.,
348 2017). By identifying virulence traits of *V. crassostreae* encoded by this plasmid, *i.e.* the
349 T6SS_{pGV} and its transcriptional activator TF, we deciphered a mechanism that increases
350 hemocyte cytotoxicity of *V. crassostreae* worsens oyster disease. In the future, identification of
351 the effector protein(s) of the T6SS_{pGV} should help decipher its effect on hemocytes. In addition,

352 exploring the role of the T6SSs and its effector(s) in the virulence of *V. aestuarianus* and *V.*
353 *tapetis* may support a parallel evolution from harmless to pathogenic states of these mollusk
354 pathogens.

355
356 We also demonstrated that the T6SS and R5.7 are not co-dependent for their function, ruling out
357 the hypothesis that R5.7 acts as a facilitator of T6SS-mediated injection of a toxic effector into
358 hemocytes. Within the Splendidus clade, a few populations have lost the *r5.7* gene and are not
359 able to kill oysters (Bruto et al., 2018). When infecting the host, these non-virulent strains are
360 highly controlled by cellular (phagocytosis) and humoral (antimicrobial peptides, reactive oxygen
361 species, and heavy metals) immunity mediated by the hemocytes (Rubio et al., in press).
362 However, several *V. tasmaniensis* strains isolated from diseased oysters (Le Roux et al., 2009,
363 Lemire et al., 2015) that do not carry the *r5.7* gene, were able to induce mortalities when injected
364 to oysters. Compared to *V. crassostreae*, the hemocyte cytotoxicity of these strains was
365 demonstrated to be dependent on phagocytosis and required a distinct T6SS localised on the
366 chromosome 1 of the strain LGP32 (T6SS_{Chr1-LGP32}, Rubio et al in press) (Figure 5C).
367 Consideration of this data led to the hypothesis that R5.7 may act as an inhibitor of phagocytosis
368 and *V. tasmaniensis* secondary evolved as pathogen by the acquisition of T6SS_{Chr1-LGP32} that is
369 active at the intracellular stage as described for the *V. cholerae* T6SS (Ma et al., 2009).
370 Alternatively, the acquisition of a T6SS_{Chr1-LGP32} that is functions exclusively during the
371 intracellular stage may have further selected for *r5.7* loss. Hence in addition to Rubio et al.
372 article, the present study suggests multiple evolutionary scenarios leading to the emergence of
373 pathogenic populations with common and specific virulence traits converging on a common
374 objective: killing of the major actors of the oyster immune response. Finally our results confirm
375 the functional diversity of the T6SS nanomachine and its effectors, acting against bacterial

376 competitors (Unterweger et al., 2014) against amoeba or phagocytic cells at an intracellular stage
377 (Ma et al., 2009) or directly by contact with the target eukaryotic cell.

378

379

380 **MATERIAL AND METHODS**

381

382 **Isolation of bacteria and gene sequencing**

383 In July 2015 and 2016, 24 live oysters, together with surrounding seawater (temperature 18°C),
384 were collected in Sylt. To collect zooplankton, large phytoplankton and organic particles, a 50 L
385 sample was filtered through a 60 µm plankton net and the collected material was subsequently
386 washed with sterile seawater. Small organic particles and free-living bacterial cells were collected
387 from 2L water samples pre-filtered through the 60 µm plankton net and sequentially filtered
388 through 5 µm, 1 µm and 0.22 µm pore size filters. These filtrates were directly placed onto *Vibrio*
389 selective media (Thiosulfate-citrate-bile salts-sucrose agar, TCBS). The zooplankton and oyster
390 tissues were ground in sterile seawater (10 mL/g of wet tissue) and streaked onto TCBS. About
391 150 colonies per seawater fraction and 300 colonies per oyster tissue sample were randomly
392 picked and re-streaked on TCBS first and subsequently on Zobell agar (15 g/l agar, 4 g/l
393 bactopectone and 1 g/l yeast extract in artificial sea water, pH 7.6). All isolates were genotyped
394 by partial *hsp60* gene sequencing and stored in 10% DMSO at -80°C. A total of 910 *hsp60*
395 sequences were obtained from the two samplings performed in Sylt. This set of data was
396 complemented with 719 *hsp60* sequences obtained from previous samplings at Brest in 2014
397 (Bruto et al., 2017) and 2016 (seawater temperature above 18°C).

398

399 **Strains, plasmids and culture conditions.** The strains used in this study are described in Table
400 S5. *Vibrio* isolates were grown at 20°C in Zobell broth or agar, Luria-Bertani (LB) or LB-agar
401 (LBA) + 0.5M NaCl. *Vibrio cholerae*, strain A1552, was grown in LB at 30°C. *Escherichia coli*
402 strains were grown at 37°C in LB or on LBA. Chloramphenicol (5 or 25µg/ml for *Vibrio* and *E.*
403 *coli*, respectively), spectinomycin (100µg/ml), kanamycin (75ug/ml for *V. cholerae*), thymidine
404 (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. Induction
405 of the P_{BAD} promoter was achieved by the addition of 0.2% L-arabinose to the growth media, and
406 conversely, was repressed by the addition of 1% D-glucose where indicated.

407
408 **Vector construction and mutagenesis.** All plasmids used or constructed in the present study are
409 described in Table S6. Deletion of selected regions or genes was performed by allelic exchange
410 using the pSW7848T suicide plasmid (Le Roux et al., 2007, Val et al., 2012). To this end, two
411 500 bp fragments flanking the target region or gene were amplified, (see primer details in Table
412 S7), assembled by PCR and cloned into pSW7848T as previously described (Lemire et al., 2015).
413 The suicide plasmid was then transferred by conjugation from *E. coli* as donor to *Vibrio* as
414 recipient. Subsequently, the first and second recombinations leading to pSW7848T integration
415 and elimination were selected on Cm/glucose and arabinose containing media, respectively. For
416 the complementation experiments, genes were cloned into the Apa1/Xho1 (*paar*) or EcoR1/Xho1
417 (TF) sites of the pMRB plasmid, which is stable in *Vibrio spp.* (Le Roux et al., 2011), resulting in
418 constitutive expression from a P_{lac} promoter. Conjugations between *E. coli* and *Vibrio* were
419 performed at 30°C as described previously (Le Roux et al., 2007). The T6SS intergenic region
420 (i.e. putative promoter region) was PCR amplified, digested, and cloned into *Sma*I and *Stu*I sites
421 in pBR-GFP_dsRed (Lo Scudato and Blokesch, 2012) before being transferred to the *V.*
422 *cholerae* strain A1552 carrying the arabinose-inducible *tf* on a mTn7 transposon. Mutagenesis of

423 the palindromic region was performed by PCR assembly as described earlier (Matsumoto-
424 Mashimo et al., 2004).

425

426 **Fluorescence microscopy**

427 *V. cholerae* cells were back-diluted (1:100) from an overnight culture and grown for 2h at 30C in
428 LB medium containing kanamycin. At this point, 0.2% arabinose was added to the culture where
429 indicated and the growth was continued for 2h before the bacteria were mounted onto agarose
430 pads (in 1% PBS) and imaged with a Plan-Apochromat 100×/1.4 Ph3 oil objective using a Zeiss
431 Axio Imager M2 epifluorescence microscope. Image acquisition occurred with the Zeiss
432 AxioVision software. Depicted images are representative of three independent biological
433 replicates.

434

435 **SDS-PAGE and Western blotting**

436 *V. cholerae* cells were grown for 5h at 30C in LB medium with or without 0.2% arabinose
437 supplementation (after 3h of growth) to induce *tf* in the respective strains. Cells were lysed by
438 resuspension in 2x Laemmli buffer (100 µl of buffer per OD₆₀₀ unit of 1) and boiling at 95°C for
439 15 min. Proteins were separated by SDS-PAGE (10% resolving gels) and blotted onto PVDF
440 membranes. Detection of proteins was carried out as described (Lo Scudato and Blokesch, 2012)
441 using primary antibodies against GFP (Roche, #11814460001; diluted 1:5'000) and mCherry
442 (BioVision, #5993-100; diluted 1:5'000). Anti-mouse-HRP (Sigma, #A5278; diluted 1:20'000)
443 and anti-rabbit-HRP (Sigma, #A9169; diluted 1:20'000) were used as secondary antibodies. An
444 anti-RNA Sigma70-HRP conjugate (BioLegend; # 663205; diluted 1:10'000) was used to
445 validate equal loading. Lumi-Light^{PLUS} (Roche) served as an HRP substrate and the signals were

446 detected using a ChemiDoc XRS+ station (BioRad). Western blots were performed three
447 independent times with comparable results.

448

449 **Experimental infections.**

450 Animals.

451 Three-month-old Specific Pathogen Free (SPF) oysters were descendants of a pool of 100
452 genitors that were produced in a hatchery under highly controlled conditions to minimize the
453 influence of genetic and environmental parameters that could affect host sensitivity to the disease
454 (Petton et al., 2015a, Petton et al., 2015b, Petton et al., 2013). These animals were used for
455 experimental infections by immersion (see below) or by intramuscular injections of bacteria into
456 the adductor muscle. Triploid adult oysters (24 to 30-months-old) were provided by a local oyster
457 farm (Coïc, Pointe du Château, Logonna-Daoulas, France) and were used to collect hemolymph
458 for cytotoxicity assays. Wild adult *C. gigas* oysters (n=50) were collected from Bay of Brest
459 (Pointe du Château, 48° 20' 06.19" N, 4° 19' 06.37" W) in April 2019 (seawater temperature
460 12°C).

461

462 Disease monitoring in wild adult oysters.

463 After sampling in the Bay of Brest, wild adult oysters were immediately returned to the
464 laboratory (Station Biologique de Roscoff, Roscoff, France). Upon arrival, the animals were first
465 cleaned using a bristle brush and briefly rinsed to remove sand, sediments and other shell debris
466 before being placed in a 300-L tank under static conditions (no change of seawater) with aerated
467 5-µm-filtered seawater at 21°C. Mortality was recorded daily for 14 days. Vibrios were isolated
468 daily from the tank seawater (100 µl) or from the hemolymph of moribund animals (10 µl) by
469 plating onto selective media (thiosulfate-citrate-bile salts-sucrose agar (TCBS), Difco, BD,

470 France). Randomly selected colonies were mixed into 20 µl of molecular biology grade water and
471 heated using a thermal cycler (2720 thermal cycler, Applied Biosystems) at 98°C for 10 min and
472 stored at -20°C for PCR testing.

473

474 Infection by immersion in contaminated seawater.

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

481

482 **Nucleic acid extraction and PCR**

483 Hemolymph of moribund wild adult oysters was withdrawn from the adductor muscle using a
484 1 mL plastic syringe fitted with a 25-gauge needle, centrifuged for 5 min at 5000 rpm and the cell
485 pellet kept at -20°C until further use. In the case of 3-month-old juvenile oysters, the whole wet
486 body of dead animals was crushed in marine broth (1 mg/ml) using a Tissue Lyser II (Qiagen).
487 Genomic DNA was purified from homogenized oyster tissues or hemocyte cell pellets by
488 resuspension in lysis buffer (NaCl 0.1M, pH8 EDTA 0.025M, SDS 1%, proteinase K 100 µg/ml)
489 for 16 h (56°C) followed by Phenol:Chloroform:Isoamyl Alcohol (Sigma-Aldrich, #77617)
490 extraction.

491 The primer pairs and PCR conditions used for the detection of *V. crassostreae* (de Lorgeril et al.,
492 2018), *V. aestuarianus* (Saulnier et al., 2010) and the herpes virus OsHV-1 (Martenot et al.,
493 2010) have been described elsewhere. PCRs were performed on 300 ng oyster DNA for oyster

494 pathogen detection or on 1 µl cell lysate obtained from *Vibrio* randomly picked on TCBS for
495 *V. crassostreae* identification.

496

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

498 Several cohorts of SPF-oysters were used to perform experimental infections by intramuscular
499 injections of bacteria into the adductor muscle. Because the susceptibility to bacterial infection of
500 these cohorts may have varied over the course of this study depending on biotic (size) and abiotic
501 (temperature) parameters, each cohort was systematically submitted to an experimental infection
502 by injection with 3 different concentrations (1X, 0.1X and 0.01X) of the pathogenic *V.*
503 *crassostreae* wt strain J2-9 used here as a reference. The bacterial concentration determined to
504 induce between 50-90% mortality was subsequently used on the considered cohort to evaluate
505 bacterial virulence. Bacteria were grown under constant agitation at 20°C for 24 h in Zobell
506 media. One hundred microliters of the culture (10^6 or 10^7 colony forming unit, cfu, depending on
507 the susceptibility of the considered cohort) were injected intramuscularly into oysters. The
508 bacterial concentration was confirmed by conventional dilution plating on Zobell agar. After
509 injection, the oysters were transferred to aquaria (20 oysters per 2.5 L aquarium) containing 1 L
510 of aerated 5 µm-filtered seawater at 20°C, and kept under static conditions. Experiments were
511 performed in duplicate and repeated at least once. Mortality was assessed after 24 hours.

512

513 ***In vitro* cytotoxicity assays**

514 Hemolymph was withdrawn from the adductor muscle through a notch previously ground in the
515 oyster shell using a 1 mL plastic syringe fitted with a 25-gauge needle. After bleeding, syringes
516 were maintained on ice and individually controlled by microscope observation to retain only

517 hemolymph that was free of contaminating particles (sperm, ovocytes, small debris...). Selected
518 samples were filtered through a 80 µm mesh to eliminate aggregates or large pieces of debris (to
519 avoid clogging of the flow-cytometer flow-cell) and pooled.

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

528 After hemocyte counting, the hemolymph pool was divided into 200 µL sub-samples maintained
529 on ice. Each sub-sample received 200 µL of the different bacterial suspensions (wild-type or
530 derivatives) at a multiplicity of infection (MOI) of 10:1 or 200 µL of FSSW as a control. Each
531 condition was tested in 3 replicates and the experiment was performed twice. Tubes were
532 maintained at 18°C for 5.5 h. Then SYBR®Green I and Propidium Iodide (PI, Sigma–Aldrich)
533 were added to each tube at final concentrations of 1× and 10 µg mL⁻¹, respectively and
534 incubation was continued for another 30 min (6h total incubation time): PI only permeates
535 hemocytes that lose membrane integrity and are considered to be dead cells, whereas
536 SYBR®Green I permeates both dead and living cells. SYBR Green and PI fluorescence were
537 measured on the FITC detector (527/32 nm) and on the PerCP-Cy5-5 detector (700/54 nm)
538 respectively. Results are expressed as percent dead hemocytes.

539

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

548

549 ***In silico* analyses.**

550 Species trees were reconstructed based on a MLST (Multi Locus Sequence Typing) containing 3
551 markers for *V. crassostreae* isolates phylogeny (*gyrB*, *rctB* and *rpoD*). Nucleotide sequences
552 were aligned with Muscle and concatenated using Seaview (Gouy et al., 2010). Phylogenetic
553 reconstruction was done using RAxML (Stamatakis, 2006) on this concatemer with the GTR
554 model. Tree visualization was performed with iTOL (Letunic and Bork, 2011).

555

556 **RNA-seq experimentation**

557 The *Vibrio* strains J2-9 Δ Px3 constitutively expressing *tf* or *gfp* from a plasmid (pMRB) were
558 grown in LB-NaCl. Bacteria were sampled at OD 0.3, 0.6 and 1.0 and RNA extraction was
559 performed using TRIzol reagent and following manufacturer's instructions (Invitrogen). Total
560 nucleic acids were quantified based on absorption at 260 nm and RNA integrity was verified by
561 gel electrophoresis. DNA was removed by DNase I digestion using the Turbo DNA-free kit
562 (Ambion). RNAs from the 3 OD conditions were pooled. The experiment was performed three

563 times. Directional cDNA libraries were constructed with the ScriptSeq RNA-Seq Library
564 Preparation Kit (Illumina). Sequencing was done with the NextSeq 500/550 Mid Output Kit v2
565 (Illumina) on a NextSeq 500Mid (Illumina) by the “Plateforme de Séquençage haut-débit” at
566 I2BC-UMR9198. Data treatment and mapping onto *V. crassostreae* J2-9 reference genome was
567 performed with the TAMARA pipeline hosted by the MAGE platform
568 ([http://www.genoscope.cns.fr/agc/microscope/transcriptomic/NGSProjectRNAseq.php?projType](http://www.genoscope.cns.fr/agc/microscope/transcriptomic/NGSProjectRNAseq.php?projType=RNAseq)
569 [=RNAseq](http://www.genoscope.cns.fr/agc/microscope/transcriptomic/NGSProjectRNAseq.php?projType=RNAseq)).

570

571 **Statistical analyses.**

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

581

582

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584

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594

595

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707

708 **TITLES AND LEGENDS TO FIGURES**

709

710 **Figure 1. The presence of the pGV plasmid is correlated with the geographic origin and**
711 **virulence of *V. crassostreae* strains.** Phylogenetic tree of 89 *V. crassostreae* isolates based on
712 the *gyrB/rctB/rpoD* gene fragments. Dark/light shades of gray indicate the two clades within the
713 species. Rings, from inside to outside, indicate i) the geographic origin of the isolates (Brest,
714 brown square; Sylt, beige square); ii) the presence (blue circles) or absence (white circles) of
715 pGV-like plasmids; iii) the presence (black squares) or absence (white squares) of the *r5-7* gene
716 and iv) the mortality rate (colour gradient from green to red corresponding to 0 to 100%) induced
717 by individual strains 24h after injection in oysters (n=20). Experiments were performed in
718 duplicate with two distinct oyster batches. The arrows highlight the virulent strains previously
719 sequenced (Lemire et al., 2015) the non-virulent strain from clade 1(8T5_11) and the two
720 plasmid-carrying but non-virulent strains from clade 2 (7T7_10 and 8T7_10).

721

722 **Figure 2. Experimental assessment of pGV loci as virulence determinants. A-**The indicated
723 region or genes were deleted by allelic exchange and the virulence of *V. crassostreae* J2-9 wild-
724 type (wt), mutants of specific loci (Δ) and complemented strains (+Plac_tf or paar) was compared
725 by **B-** injection of strains (10^6 or 10^7 cfu depending on the cohort susceptibility, see Material and
726 Methods) in 20 oysters and counting the percentage of mortalities after 24 hours; **C-** hemocyte
727 cell viability evaluated by flow cytometry using a double staining procedure (SYBR® Green and
728 propidium iodide, PI, Sigma). Injection and cell viability assays were performed in duplicate and
729 triplicate, respectively, and reproduced at least twice. A single experiment is represented here for
730 each method. Letters indicate significant differences of mortality assessed by simultaneous tests

731 for general linear hypotheses with Tukey contrasts ($P < 0.05$).

732

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

737

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

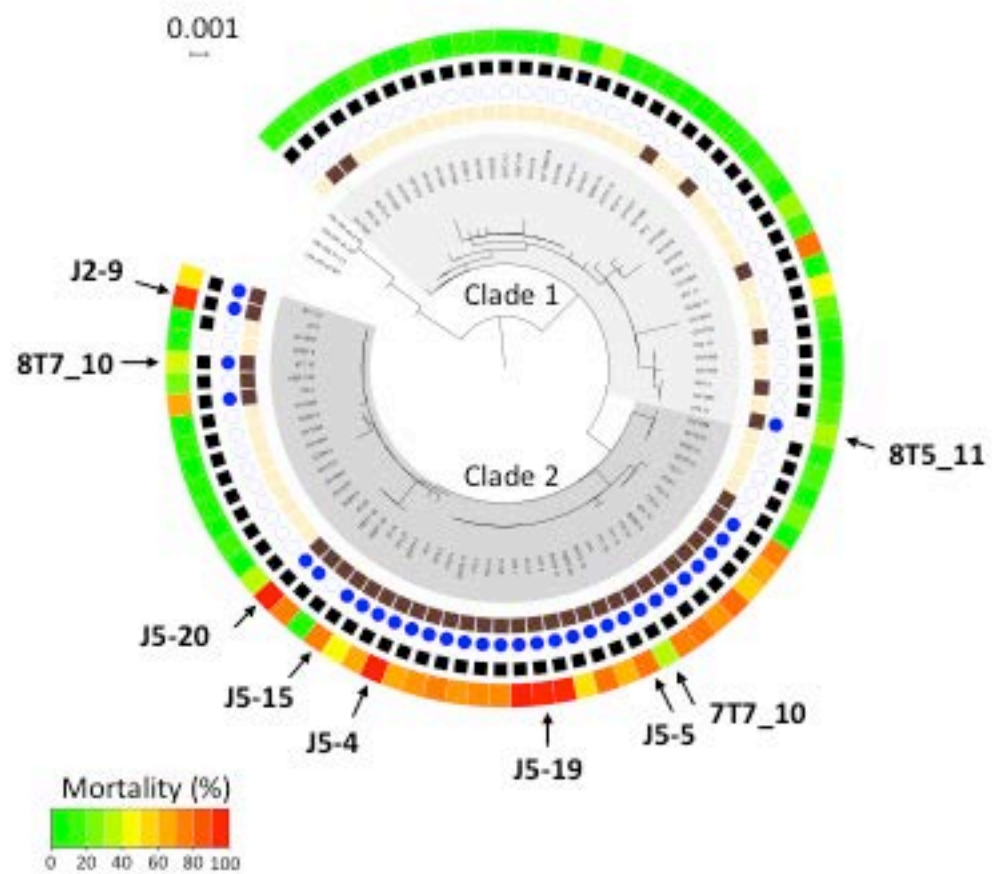
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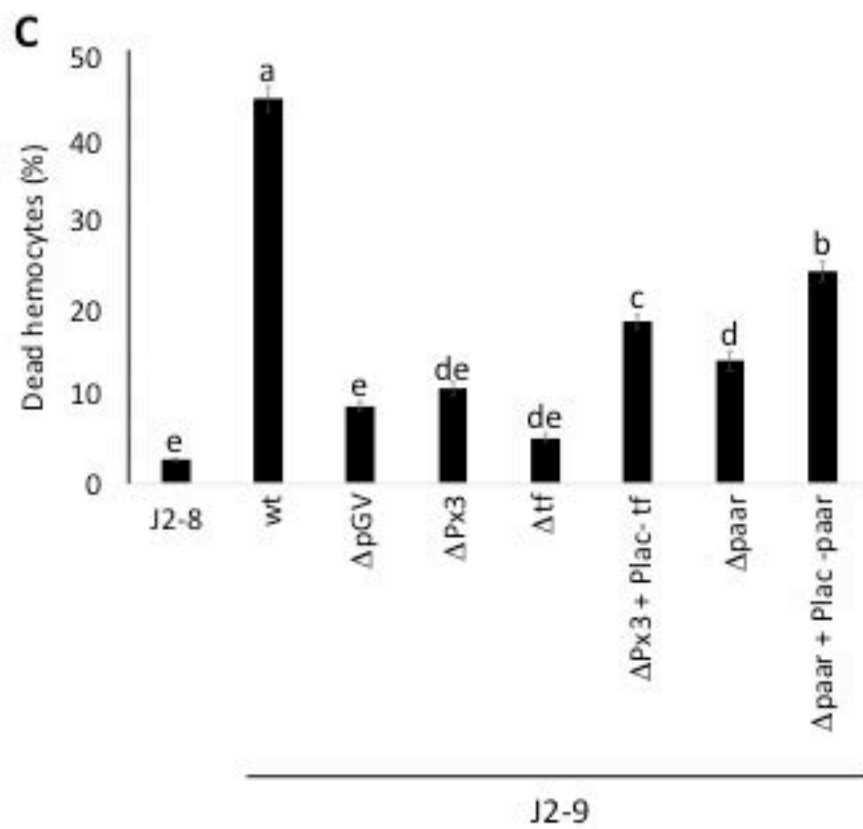
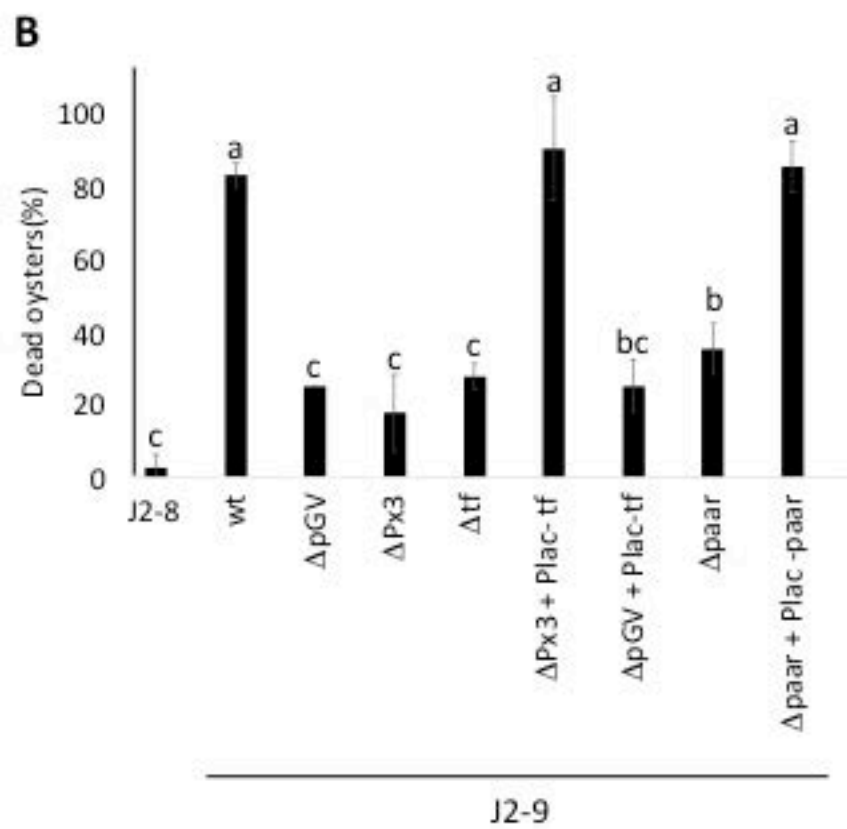
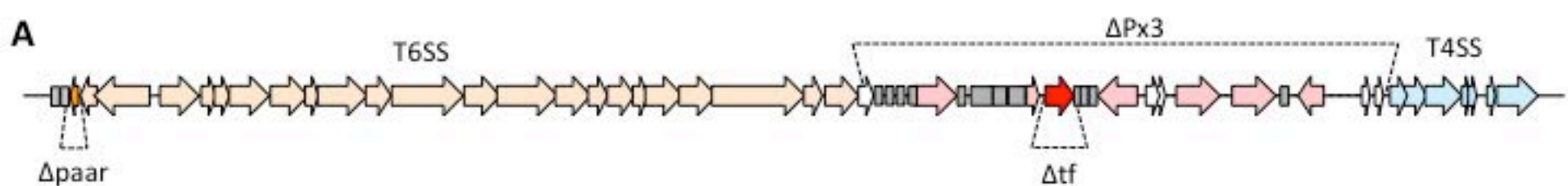
745 **Figure 5 Comparative genomic of *V. crassostreae*, *V. aestuarianus* and *V. tapetis* T6SS and**
746 **putative effectors.** A. Synteny of the T6SS in the three strains compared. Genes with the same
747 colour code are homologous (>40% amino acid identity). Specific genes in each T6SS are shaded
748 in yellow and described in B. Schematic representation of the sequence identity or structural
749 similarity of the putative effector of *V. crassostreae* T6SS_{pGV} in strain J2-9, *V. aestuarianus* 02-
750 041 and *V. tapetis* CECT4600. Structural similarities were identified with Phyre2. C. Phylogeny
751 based on a concatemer of T6SS homologs found in *V. crassostreae* (J5-4; LGP7; J2-9; J5-20), *V.*
752 *tapetis* (CECT4600), *V. aestuarianus* (07-115; 02-041; 12-128a; 01-032) and *V. tasmaniensis*

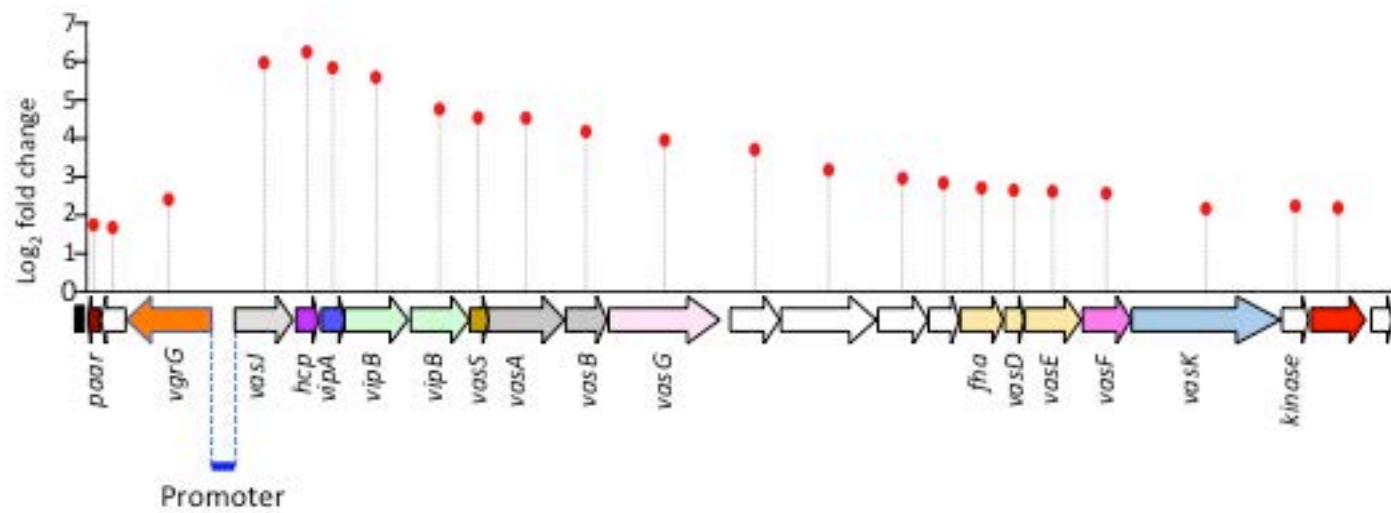
753 (LGP32; J0-13). The matrix shows the conservation of the different T6SS homologs with
754 T6SS_{pGV} as a reference. A scale bar indicating amino acid sequence identity is located to the right
755 of the matrix.

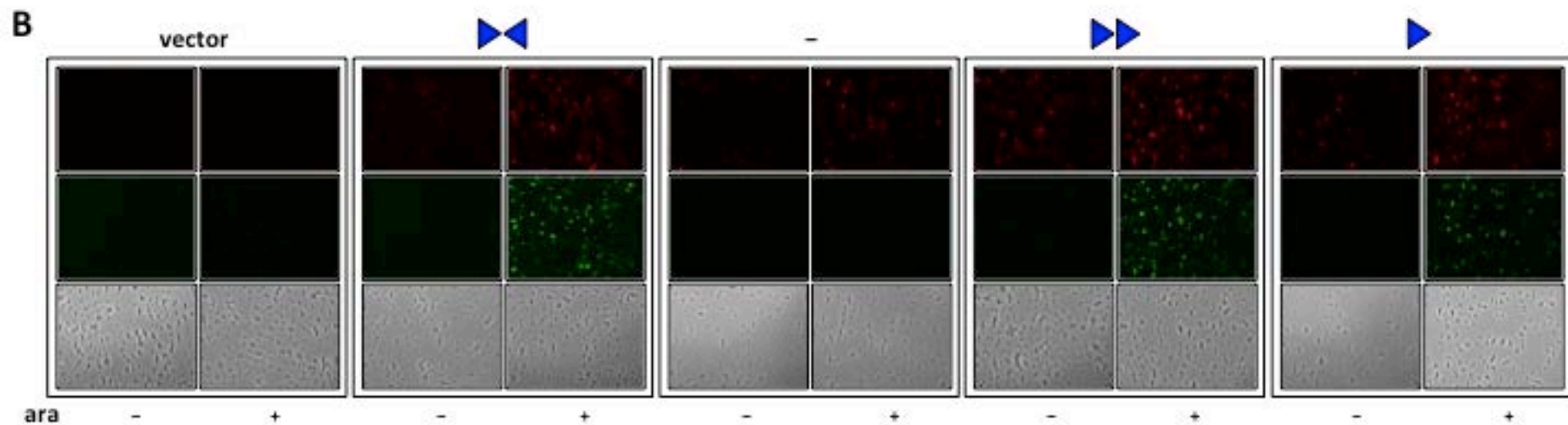
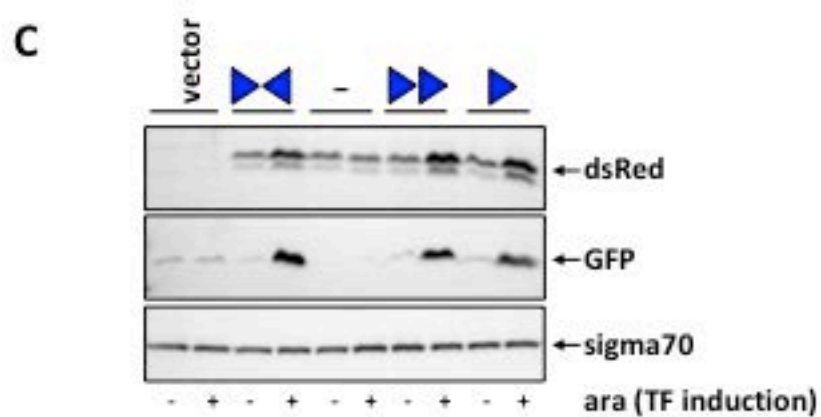
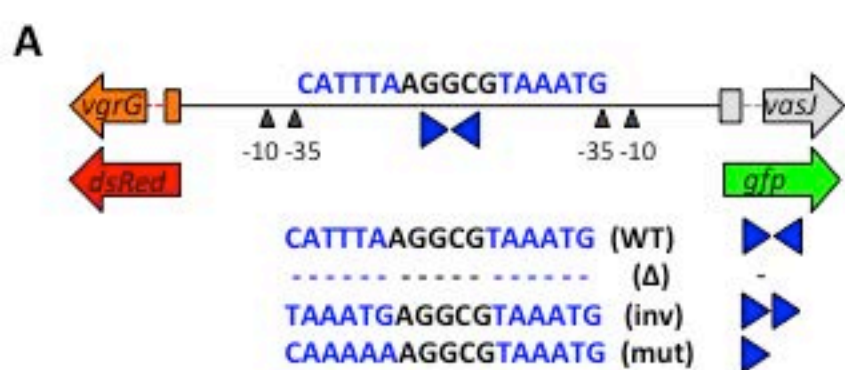
756

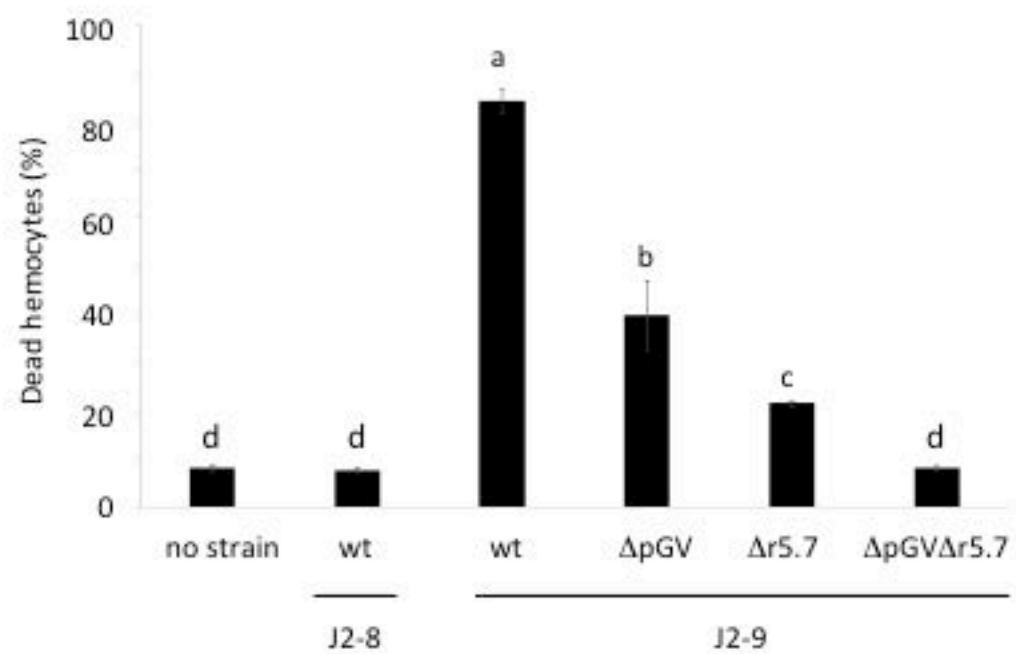
757 **Figure 6. Cytotoxic activities of T6SS and R5.7.** The cytotoxicity of *V. crassostreae* wt or
758 mutant strains (Δ) was assessed by flow cytometry using a double staining procedure. Control
759 hemocytes were either incubated in the absence of any bacteria or with a non-virulent strain (J2-
760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











Strains	Isolation site	Isolation date	Fraction	Plasmid	Oyster batch 1	
					Mortality (%)	Deviation
16BF1_28	Brest	2016	1-5 µM	0	20.0	14.1
16BF1_56	Brest	2016	1-5 µM	1	70.0	14.1
16BF1_95	Brest	2016	1-5 µM	0	5.0	0.0
16BF5_48	Brest	2016	5-1 µM	1	72.5	17.7
7F5_27	Brest	2014	5-1 µM	1	40.0	21.2
7G1_1	Brest	2014	Oyster	0	0.0	0.0
7P_6	Brest	2014	1-0.2 µM	0	7.5	0.0
7T1_10	Brest	2014	Oyster	1	30.0	0.0
7T1_12	Brest	2014	Oyster	1	70.0	7.1
7T3_1	Brest	2014	Oyster	1	65.0	7.1
7T4_11	Brest	2014	Oyster	1	60.0	14.1
7T4_12	Brest	2014	Oyster	1	72.5	3.5
7T5_9	Brest	2014	Oyster	1	52.5	3.5
7T6_10	Brest	2014	Oyster	1	67.5	3.5
7T8_1	Brest	2014	Oyster	1	60.0	7.1
7T8_11	Brest	2014	Oyster	1	65.0	7.1
8F5_39	Brest	2014	5-1 µM	0	7.5	10.6
8H1_4	Brest	2014	Oyster	0	10.0	7.1
8T1_12	Brest	2014	Oyster	1	45.0	7.1
8T2_1	Brest	2014	Oyster	1	92.5	3.5
8T2_10	Brest	2014	Oyster	0	5.0	7.1
8T2_4	Brest	2014	Oyster	1	67.5	3.5
8T5_10	Brest	2014	Oyster	1	75.0	14.1
8T5_11	Brest	2014	Oyster	1	27.5	10.6
8T7_10	Brest	2014	Oyster	1	35.0	7.1
8T7_11	Brest	2014	Oyster	0	22.5	3.5
8T7_4	Brest	2014	Oyster	1	90.0	7.1
8T8_11	Brest	2014	Oyster	0	2.5	3.5
8T8_2	Brest	2014	Oyster	1	60.0	0.0
8T8_7	Brest	2014	Oyster	1	70.0	0.0
BOB3_6	Brest	2016	Oyster	1	65.0	7.1
BOT2_10	Brest	2016	Oyster	1	57.5	3.5
BOT3_9	Brest	2016	Oyster	0	10.0	7.1
BOT4_11	Brest	2016	Oyster	1	47.5	10.6
BOT4_5	Brest	2016	Oyster	1	60.0	14.1
BOT5_11	Brest	2016	Oyster	1	70.0	0.0
J2-9	Brest	2011	Oyster	1	87.1	9.1
J5-15	Brest	2011	Oyster	1	70.0	14.1
J5-19	Brest	2011	Oyster	1	90.0	0.0
J5-20	Brest	2011	Oyster	1	92.5	3.5
J5-4	Brest	2011	Oyster	1	92.5	3.5

J5-5	Brest	2011	Oyster	1	72.5	24.7
16SF1_51	Sylt	2016	1-5 µM	0	27.5	24.7
16SF1_87	Sylt	2016	1-5 µM	0	0.0	0.0
S16	Sylt	2016	Oyster	0	25.0	14.1
GV1664	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1666	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1667	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1671	Sylt	2016	1-0.2 µM	0	2.5	3.5
GV1672	Sylt	2016	1-0.2 µM	0	2.5	3.5
GV1674	Sylt	2016	1-0.2 µM	0	12.5	3.5
GV1675	Sylt	2016	1-0.2 µM	0	12.5	3.5
GV1676	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1677	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1678	Sylt	2016	1-0.2 µM	0	12.5	3.5
GV1679	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1680	Sylt	2016	1-0.2 µM	0	7.5	3.5
GV1681	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1682	Sylt	2016	1-0.2 µM	0	7.5	3.5
GV1683	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1684	Sylt	2016	1-0.2 µM	0	5.0	0.0
GV1685	Sylt	2016	1-0.2 µM	0	7.5	3.5
GV1687	Sylt	2016	1-0.2 µM	0	15.0	7.1
GV1688	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1689	Sylt	2016	1-0.2 µM	0	12.5	3.5
GV1690	Sylt	2016	1-0.2 µM	0	10.0	0.0
GV1691	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1692	Sylt	2016	1-0.2 µM	0	5.0	0.0
GV1693	Sylt	2016	1-0.2 µM	0	42.5	3.5
GV1694	Sylt	2016	1-0.2 µM	0	2.5	3.5
GV1695	Sylt	2016	1-0.2 µM	0	5.0	0.0
GV1696	Sylt	2016	1-0.2 µM	0	5.0	7.1
GV1698	Sylt	2016	1-0.2 µM	0	0.0	0.0
GV1699	Sylt	2016	1-0.2 µM	0	10.0	7.1
GV1700	Sylt	2016	1-0.2 µM	0	17.5	10.6
GV1701	Sylt	2016	1-0.2 µM	0	7.5	10.6
SOB1_2	Sylt	2016	Oyster	0	25.0	14.1
SOB1_3	Sylt	2016	Oyster	0	0.0	0.0
SOB1_6	Sylt	2016	Oyster	0	2.5	3.5
SOB1_8	Sylt	2016	Oyster	0	5.0	7.1
SOB4_6	Sylt	2016	Oyster	0	30.0	7.1
SOB6_12	Sylt	2016	Oyster	0	7.5	3.5
SOB7_9	Sylt	2016	Oyster	0	7.5	10.6
SOS2_11	Sylt	2016	Oyster	0	0.0	0.0
SOS2_6	Sylt	2016	Oyster	0	7.5	3.5

SOS4_4	Sylt	2016	Oyster	0	5.0	0.0
SOT2_12	Sylt	2016	Oyster	0	0.0	0.0
SOT3_10	Sylt	2016	Oyster	0	0.0	0.0
SOT8_11	Sylt	2016	Oyster	0	20.0	7.1

Oyster batch 2

Mortality (%)	Deviation
15.0	7.1
72.5	3.5
2.5	3.5
67.5	17.7
45.0	7.1
5.0	7.1
3.5	0.0
67.5	3.5
80.0	14.1
80.0	14.1
67.5	3.5
35.0	7.1
57.5	3.5
70.0	7.1
82.5	3.5
65.0	7.1
5.0	7.1
10.0	7.1
50.0	21.2
97.5	3.5
12.5	3.5
57.5	3.5
57.5	3.5
15.0	0.0
62.5	10.6
2.5	3.5
97.5	3.5
7.5	10.6
82.5	10.6
72.5	3.5
70.0	21.2
67.5	10.6
45.0	7.1
67.5	3.5
67.5	17.7
57.5	10.6
89.6	6.6
77.5	3.5
100.0	0.0
75.0	14.1
100.0	0.0

82.5	10.6
5.0	7.1
0.0	0.0
35.0	21.2
2.5	3.5
0.0	0.0
0.0	0.0
0.0	0.0
2.5	3.5
15.0	7.1
27.5	10.6
0.0	0.0
10.0	7.1
0.0	0.0
5.0	7.1
7.5	3.5
2.5	3.5
10.0	7.1
7.5	10.6
15.0	0.0
47.5	10.6
17.5	3.5
40.0	7.1
20.0	7.1
2.5	3.5
2.5	3.5
12.5	3.5
50.0	7.1
10.0	0.0
5.0	0.0
2.5	3.5
2.5	3.5
5.0	7.1
27.5	3.5
0.0	0.0
42.5	17.7
5.0	0.0
32.5	3.5
0.0	0.0
27.5	10.6
5.0	7.1
0.0	0.0
10.0	7.1
10.0	7.1

10.0	7.1
7.5	3.5
17.5	24.7
20.0	7.1

Strain name	8T5_11
Number of contigs	29
Genome size	5383205
Number of CDSs	4742
pGV1512-like size	142833
pGV1512-like CDS number	170
Number of genes shared with pGV1512	160
Genome Accession number	NZ_RJJZ01000000.1
pGV1512-like accession number	RJJZ01000012.1

Label in J2-9 (VCR9J2v1_ in MAGE; VCR9J2 in NCBI)

10047
40072
40090
50182
60118
60146
100001
130061
150072
150073
150074
180046
180047
180060
190033
720145
730019
730020
730260
730261
730262
730263
730264
730265
730266
730267
730268
730269
760172
920002
920039
920043
960144
1330033
1350183
1420001
1440001
1570004
1570005
1570006
1600060
1620002
1620025

1630029

Product

Protein of unknown function
Conserved membrane protein of unknown function
Protein of unknown function
Maltoporin
Protein of unknown function
Hypothetical protein
Putative transposase, IS116/IS110/IS902
Conserved protein of unknown function
Putative LysR family transcriptional regulator
Putative exported metal-dependent hydrolase
Exported protein of unknown function
Putative Integral membrane protein, two-component signal transducer
Protein of unknown function
Protein of unknown function
Hypothetical protein
Conserved protein of unknown function
Conserved exported protein of unknown function
Conserved exported protein of unknown function
Putative outer membrane protein
Putative Transcriptional regulator
Putative Phosphotransferase system, fructose-specific IIC component (FruA)
Putative Alpha-mannosidase
Putative sucrose phosphorylase
Putative PTS fructose-specific enzyme IIA component-like protein
Glycerate kinase
Mannose-6-phosphate isomerase
Conserved exported protein of unknown function (R5.7)
Conserved exported protein of unknown function
Conserved protein of unknown function
Hypothetical protein
Conserved protein of unknown function
Conserved hypothetical protein
Conserved hypothetical protein
Conserved membrane protein of unknown function
Protein of unknown function
Transposase
Transposase (fragment)
Putative membrane-fusion protein
Putative N-acetylglucosaminyltransferase
Conserved hypothetical protein
Conserved protein of unknown function
Protein of unknown function
Protein of unknown function

Protein of unknown function

Label (VCR9J2v1_)	Name
30006	argB
30005	argC
720146	hisM
720148	artI
720147	-
720149	artP
750071	<i>tssM (vasK)</i>
750073	-
750072	-
750053	<i>tssI (vgrG)</i>
750070	<i>tssL (vasF)</i>
750069	<i>tssK (vasE)</i>
750068	<i>tssJ (vasD)</i>
750067	<i>fha</i>
750066	-
750065	-
750064	-
750063	-
750062	<i>tssH (vasG)</i>
750061	<i>tssG (vasB)</i>
750060	<i>tssF (vasA)</i>
750059	<i>tssE (vasS)</i>
750058	<i>tssC-2 (vipB-2)</i>
750057	<i>tssC-1 (vipB-1)</i>
750056	<i>tssB (vipA)</i>
750054	<i>tssA (vasJ)</i>
750055	<i>tssD (hcp)</i>
750086	-

Product	Begin
Acetylglutamate kinase	454077
N-acetyl-gamma-glutamyl-phosphate reductase	453060
Histidine transport system permease protein hisM	2950116
Arginine ABC transporter: substrate binding protein	2951471
ABC transporter: transmembrane protein; Arginine uptake	2950781
Arginine transporter: ATP-binding protein	2952323
Putative type VI secretion protein IcmF/VasK/VtsI	3615265
Conserved protein of unknown function	3619396
Conserved protein with serine threonine kinase domain	3618736
Putative type VI secretion protein VgrG	3592082
Putative type VI secretion protein VasF/VtsH/DotU	3614121
Putative type VI secretion protein VasE/VtsG/ImpJ	3612797
Putative type VI secretion protein VasD-1 (VtsF)	3612330
Putative type VI secretion protein VasC/VtsE with forkhead domain (FHA)	3611302
Putative type VI secretion protein VtsD	3610581
Putative type VI secretion protein VtsC	3609412
Putative type VI secretion protein VtsB	3607172
Putative type VI secretion protein VtsA D-alanine-D-alanine ligase	3606015
Type VI secretion protein VasG (ClpV1)	3603206
Putative type VI secretion protein VasB/ImpH	3602217
Putative type VI secretion protein VasA/ImpG/TssF	3600415
Putative type VI secretion protein VasS	3600000
Putative type VI secretion protein, tail sheath-like, (VasRB)	3598642
Putative type VI secretion protein, tail sheath-like (VasRA/ImpC)	3597115
Putative type VI secretion protein Hcp2/ VasQ/ImpB/VipA	3596606
Putative type VI secretion protein VasJ	3594584
Putative type VI secretion protein, hemolysin-coregulated protein (Hcp1)	3595998
Putative transcriptional regulator (FT1512)	3627769

End	Length	Frame	Normalized average read count
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454064	1005	3	1055
2950784	669	-3	176
2952202	732	-1	515
2951467	687	-1	395
2953066	744	-1	608
3618720	3456	1	9708
3620673	1278	1	1.49e+4
3619368	633	1	4688
3594070	1989	-1	5560
3615263	1143	3	3046
3614113	1317	2	3996
3612794	465	3	1451
3612333	1032	1	2832
3611300	720	3	2200
3610584	1173	1	3497
3609415	2244	2	6116
3607169	1155	3	5249
3605818	2613	2	1.50e+4
3603179	963	3	7245
3602217	1803	1	8209
3600422	423	3	2482
3600003	1362	1	1.04e+4
3598593	1479	1	2.07e+4
3597115	510	2	1.79e+4
3595966	1383	2	3.45e+4
3596531	534	3	2.74e+4
3628749	981	1	1.78e+4

Log2 fold change	Adjusted pvalue (FDR)
-2.22	3.42e-44
-2.85	5.73e-73
-2.94	2.92e-55
-2.97	1.19e-66
-3.21	4.93e-72
-3.74	4.51e-98
2.17	2.11e-51
2.19	2.11e-51
2.24	7.53e-55
2.41	3.37e-62
2.57	5.69e-68
2.62	1.26e-71
2.65	5.23e-65
2.71	1.89e-74
2.84	3.97e-78
2.96	3.09e-88
3.18	2.24e-101
3.71	2.61e-131
3.95	4.18e-142
4.18	2.19e-159
4.53	6.50e-181
4.54	5.92e-168
4.76	2.40e-193
5.59	1.89e-234
5.84	4.07e-253
5.97	5.62e-240
6.25	9.85e-265
9.19	0

Strain

П3813

β3914

GV1975

GV1460

GV2798

GV1438

GV1542

GV2445

GV2702

GV2800

GV2470

GV3196

GV2829

GV2833

A1552

A1552-TnTF1512

GV1495

GV2723

GV1141

GV1484

GV3226

GV3225

Description

lacIQ, thi1, supE44, endA1, recA1, hsdR17, gyrA462, zei298::Tn10, DthyA::(erm-pir116) [Tc^R Erm (F⁻) RP4-2-Tc::Mu ΔdapA ::(erm-pir116), gyrA462, zei298::Tn10 [Km^R Em^R Tc^R]
8T5_11; *V. crassostreae*
J2-8, *Vibrio sp.* (accession number PRJEB5890)
J2-9, *V. crassostreae* (accession number PRJEB5876)
J2-9 ΔpGV1512
J2-9 ΔPx3 (deletion of the Px3 region within the pGV1512 plasmid)
J2-9 Δtf (deletion of the transcriptional activator *tf* within the pGV1512 plasmid)
J2-9 ΔPx3 + Plac-tf (constitutive expression of *tf*, from a pMRB plasmid)
J2-9 ΔpGV + Plac-tf
J2-9 Δ*paar*
J2-9 Δ*paar* + Plac-*paar* (constitutive expression of *paar*, from a pMRB plasmid)
J2-9 ΔPX3 + Plac-tf
J2-9 ΔPX3 + Plac-gfp (constitutive expression of *gfp*, from a pMRB plasmid)
V. cholerae O1 El Tor Inaba; WT (#1)
A1552 carrying arabinose-inducible TF on mTn7 transposon (#6624)
J2-9 Δr5-7
J2-9 Δr5-7 ΔpGV1512
J2-9 Δr5-7 + Plac-r5-7 (constitutive expression of *r5.7*, from a pMRB plasmid)
J2-9 Δp5-7 + Plac-gfp
8T5_11 + Plac-r5-7
8T5_11 + Plac-gfp

Reference

Le Roux et al., 2007

Le Roux et al., 2007

this study

Lemire et al., 2015

Lemire et al., 2015

Bruto et al., 2017

Bruto et al., 2017

This study

This study

This study

This study

This study

This study

This study

Yildiz and Schoolnik, 1998

This study

Lemire et al., 2015

This study

Lemire et al., 2015

Lemire et al., 2015

This study

This study

Plasmid

pSW23T

pSW7848T

pSW7848 Δ Px3

pSW7848 Δ tf

pMRB-P_{LAC}-*gfp*

pMRB-P_{LAC}-tf

pSW7848 Δ paar

pMRB-P_{LAC}-paar

pMRB-P_{LAC}-*R-5.7*

pSW8742D R5-7

pGP704-TnAraC

pGP704-TnTF1512

pBR-GFP_dsRED_Kan

pTFrep - intergenic region containing putative TF1512 binding site (#6650)

pTFrep - pTFrep deleted for palindromic region (#6685)

pTFrep - pTFrep inversion of palindromic region (#6686)

pTFrep - pTFrep side-specific mutagenesis of palindromic region (#6687)

Description

*oriV*_{R6K}; *oriT*_{RP4}; [Cm^R]

*oriV*_{R6K}; *oriT*_{RP4}; *araC*-P_{BAD}*ccdB*; [CmR]

pSW7848T :: ΔP_{X3}

pSW7848T :: ΔP_{X3-2.5}

oriVR6Kg; *oriTRP4*; *oriVpB1067*; P_{LAC}-*gfp* [CmR]

oriVR6Kg; *oriTRP4*; *oriVpB1067* P_{LAC}-P_{X3-2.5} [CmR]

pSW7848T :: Δ*paar*

oriVR6Kg; *oriTRP4*; *oriVpB1067*; P_{LAC}-*paar* [CmR]

*oriV*_{R6Kg}; *oriT*_{RP4}; *oriV*_{pB1067}; P_{LAC}*R-5.7* [Cm^R]

pSW8742T :: *D R5-7*

pGP704 with mini-Tn7 carrying *araC* and PBAD (#5513)

TF cloned behind PBAD promoter of pGP704-TnAraC (#6618)

Promoter-less *gfp* and *dsRed* (*DsRed.T3*[DNT]) reporter genes in *aph*-carrying plasmid (#1650)

pBR-GFP_*dsRED*_Kan with intergenic region containing putative TF1512 binding site (#6650)

pBR-GFP_*dsRED*_Kan with pTFrep deleted for palindromic region (#6685)

pBR-GFP_*dsRED*_Kan with pTFrep inversion of palindromic region (#6686)

pBR-GFP_*dsRED*_Kan with pTFrep side-specific mutagenesis of palindromic region (#6687)

Reference

Demarre et al., 2005

Val et al., 2012

Bruto et al., 2018

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Le Roux et al., 2011

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Lemire et al., 2015

Adams et al., 2019 in press

This study

Lo Scudato and Blokesch, 2012

This study

This study

This study

This study

Primer

hsp60 For

hsp60 Rev

gyrB For

gyrB Rev

rctB For

rctB Rev

rpoD For

rpoD Rev

pGV1512 *repB* For

pGV1512 *repB* Rev

r5.7 detection For

r5.7 detection Rev

V. crassostreae detection For

V. crassostreae detection Rev

OsHV-1 detection For

OsHV-1 detection Rev

V. aestuarianus detection For

V. aestuarianus detection Rev

FT deletion-1

FT deletion-2

FT deletion-3

FT deletion-4

paar deletion-1

paar deletion-2

paar deletion-3

paar deletion-4

FT complementation For

FT complementation Rev

paar complementation For

paar complementation Rev

PCR VipA For

PCR VipA Rev

PCR FT For

PCR FT Rev

PCR repB For

PCR repB Rev

PCR gyrA For

PCR gyrA Rev

PCR vgrG For

PCR vgrG Rev

Mutagenesis in the promoter 1

Mutagenesis in the promoter 2

Mutagenesis in the promoter 3

Mutagenesis inthe promoter 4

Mutagenesis inthe promoter 5

Mutagenesis inthe promoter 6

Clonage DSred/GFP promoter FT in a MRB plasmid 1

Clonage DSred/GFP promoter FT in a MRB plasmid 2

Clonage DSred/GFP promoter FT in a MRB plasmid 3

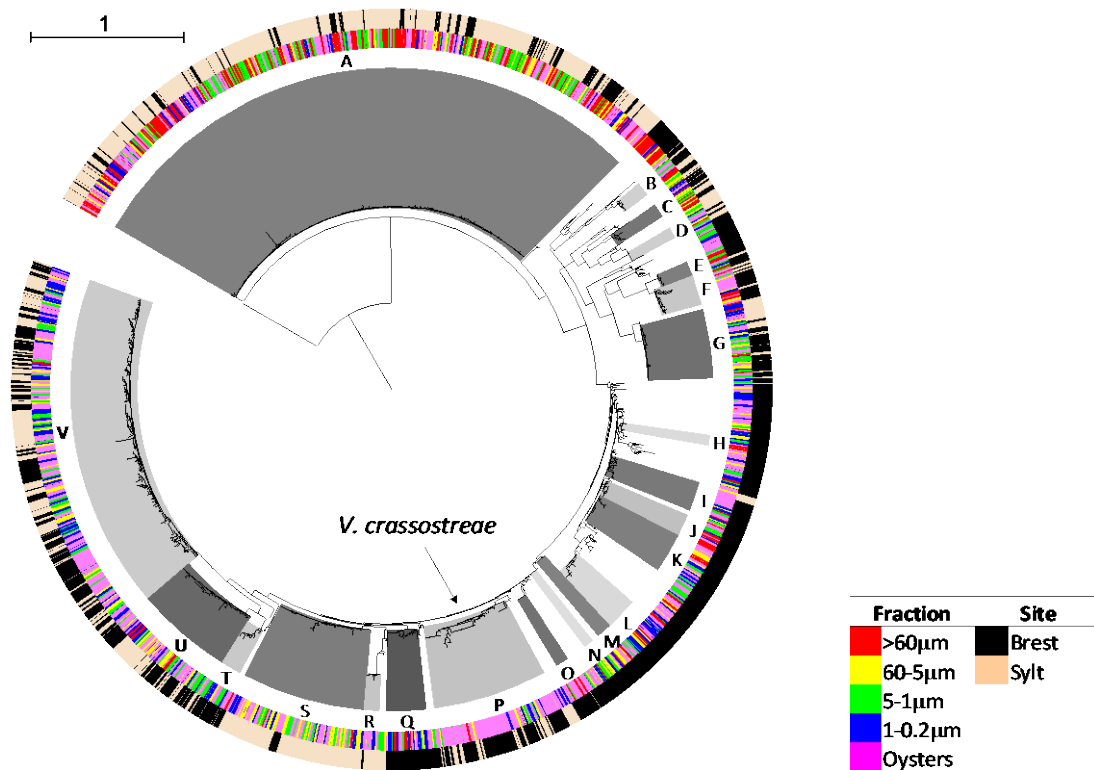
Clonage DSred/GFP promoter FT in a MRB plasmid 4

Sequence 5'-3'

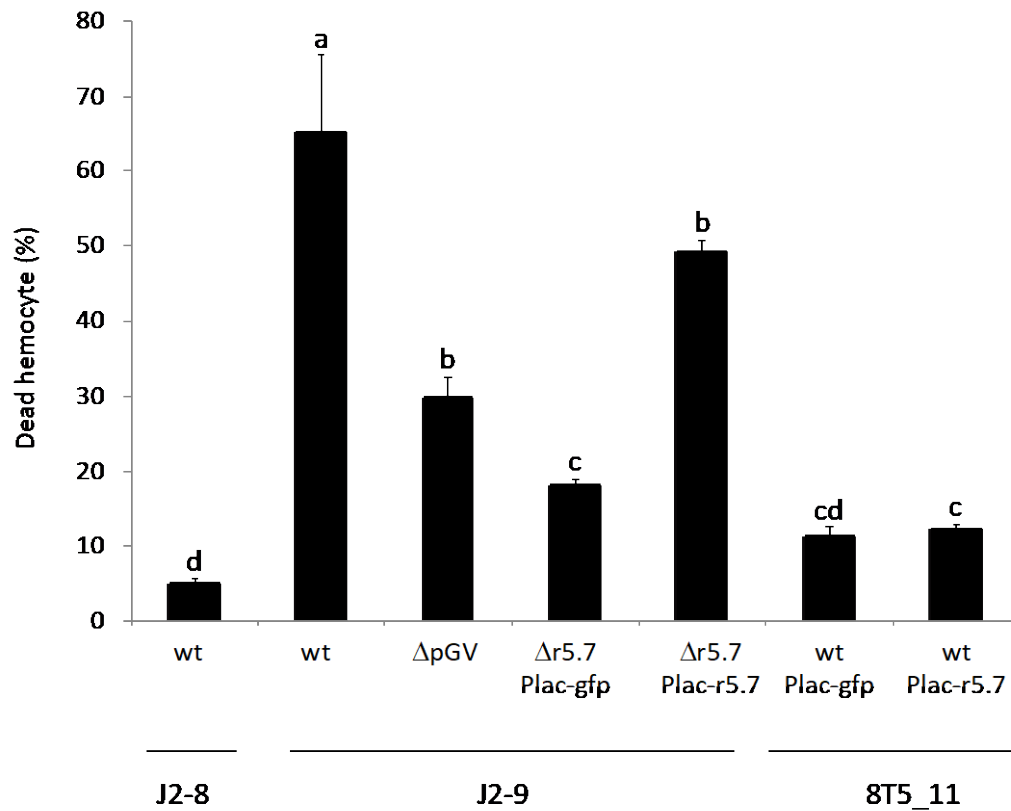
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AGCAGGGTACGGATGTGCGAGCCRTCNCRTCNGCRTCNGYCAT
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GATAAACGACGGCCAYTTNCTYTGHATNGGYTCRAAYTCNCRT
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CCTCTCTCGACTACACGAAG
CATGCAACTTCATTCCAGGC
CGTATGCCTGAACATAGTTAG
GGGATCTGATGATCACCGAG
AGGTCGCCACTTACTTGCTC
TGCCTTCAGTGAGTTGGGTC
ATTGATGATGTGGATAATCTGTG
GGTAAATACCATTGGTCTTGTTCC
GTATGAAATTTTAACTGACCCACAA
CAATTTCTTTCGAACAACCAC
GTATCGATAAGCTTGATATCGAATTCGGAGGAGTTCATGACCTACA
GCAGTCTCTCAATAACACCGCGCTTCTCTTTTGTGTCCCA
TGGGACACAAAAGAGAAGCGCGGTGTTATTGAGAGACTGC
CCCCCGGGCTGCAGGAATTCGCCATTTCCCTATCCAGCGTA
GTATCGATAAGCTTGATATCGAATTCGCCATGCCTTACCTGTCC
GGTACGAATTTTGTAGTAAGTTATTCTAGGCCTCCAACGGTTGTAG
CTACAACCGTTGGAGGCCTAGAATAACTTACTCAAATTCGTACC
CCCCCGGGCTGCAGGAATTCGGTGTCAAGGCCTCGTGGG
GCCCGAATTCATGCTCGACAAAAGAAAACC
GCCCTCGAGCTAAACCTTACAAACCTGTG
GGGCGGGCCATGTTACCAGCAGCAAGAGC
GGGCTCGAGTTAACCGCCAATTAAGACGGTTG
TGTCGGCGTAATTGGCGATT
GAACGGAAGTTCATGCTCAC
GAAATGCTGACAATCGCTGC
CACCACATGCGCCTATTAGT
CACTCTTCTCTCTCGACTA
CATTAAAGCCTACTTTGCGCG
GTTACATACCTAGACTACGCG
ATGCGGGTGGTATTTACCGA
GCCTCAGTTTAGCCTCTATC
CTCAAAGGGTCTTGTGCTTC
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GCCCCTCGAGTTATTTGTATAGTTCATCCATGCC
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SUPPLEMENTARY DATA

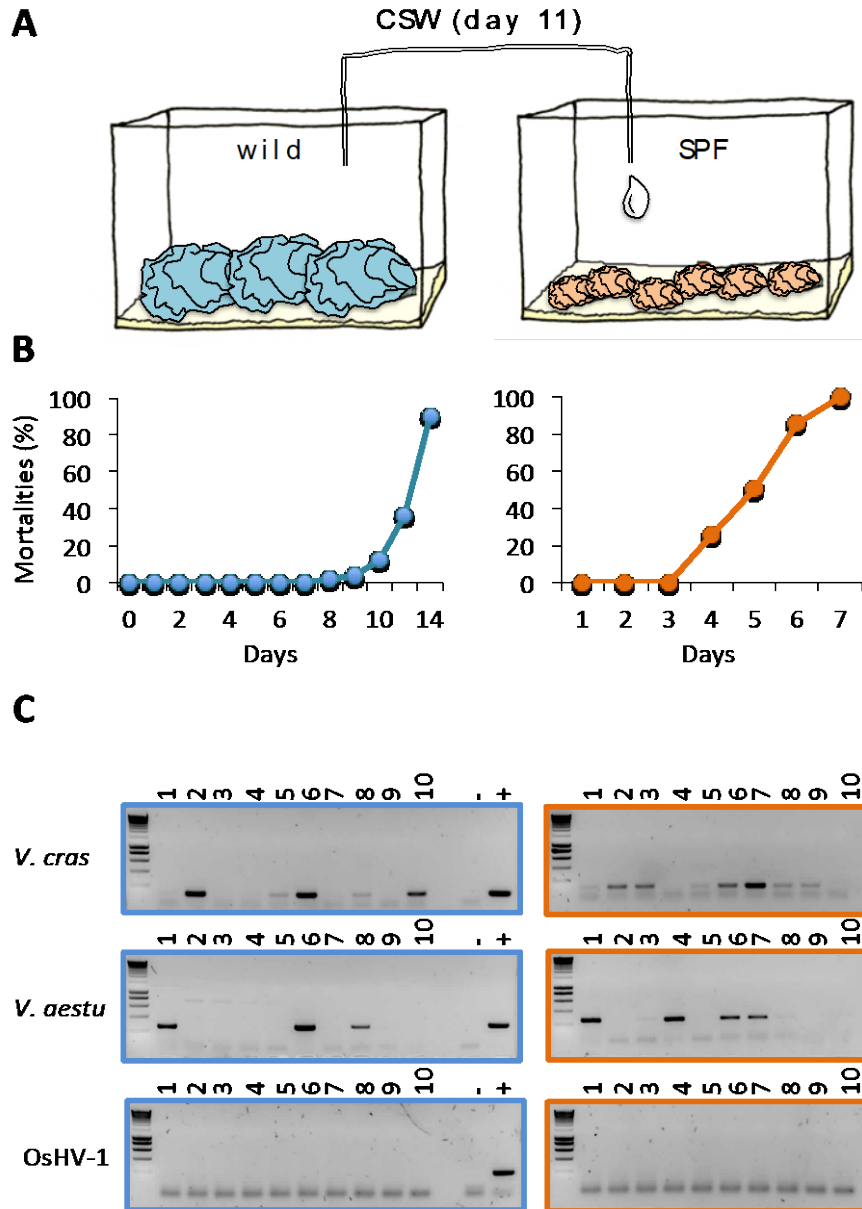


3
4
5 **Figure S1 Population structure of *Vibrio* isolates (n=1629) recovered from seawater**
6 **fractions or oyster tissues from two geographic areas, Brest (France) and Sylt (Germany).**
7 Phylogenetic tree (Maximum Likelihood) based on partial *hsp60* sequences. The grey areas
8 correspond to different clades labelled by letters (from A to V) and taxonomically assigned to
9 known *Vibrio* species, *i.e.* *V. breoganii* (A), *V. pacinii* (B), *V. fischeri* (C), *V. alginolyticus* (E, F),
10 *V. jasicida* (G), *V. chagasii* (L), *V. crassostreae* (P) also indicated with a black arrow, *V.*
11 *kanaloae* (T), *V. cyclitrophicus* (U) and *V. splendidus* (V) or *Vibrio sp. nov.* (D, H, I, J, K, M, N,
12 O, Q, R, S). The inner and outer rings indicate the origin of the strain and the site of sampling,
13 respectively, following the colour code given on the right panel.



15

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



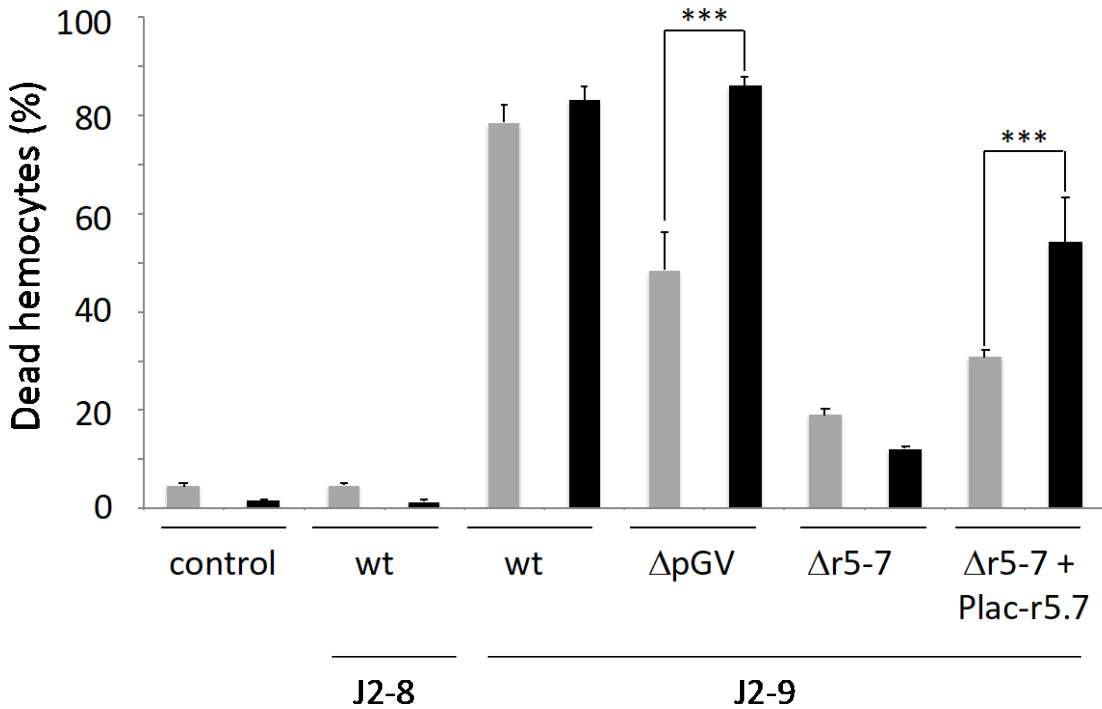
24

25

26 **Figure S3 Experimental infection in mesocosm.** A. Description of the ‘natural’ experimental
 27 infection. Wild adult oysters ($n = 50$) (animals coloured in blue) sampled in Bay of Brest
 28 (seawater temperature of 12°C) were returned to the laboratory and held in a 300-L tank under
 29 static conditions with aerated 5- μ m-filtered seawater at 21°C. At day 11, three-months-old
 30 specific pathogen free oysters ($n = 20$) (animals coloured in orange) were immersed in 1L of

31 contaminated seawater (CSW) collected from the tank containing the moribund wild oysters or in
32 fresh 5- μ m-filtered seawater as a control. **B.** Oyster disease dynamic. Mortality in wild adult
33 oysters (blue line) or in three-months-old juvenile oysters (orange line) was recorded daily for 14
34 days and 6 days, respectively. Cumulative mortality rates are indicated in % (y axis). **C.** PCR
35 detection of different oyster pathogens. *Vibrio crassostreae*, *V. aestuarianus* and the Herpes virus
36 OsHV-1 μ Var were detected in hemolymph of moribund wild adult oysters (blues boxes) sampled
37 at day 10 (lanes 1 to 4) and day 11 (lanes 5 to 10) or from tissues of moribund three-months-old
38 oysters (orange boxes) exposed to contaminated seawater (CSW) and sampled at day 3 (lanes 1 to
39 5), day 4 (lanes 6 to 10), day 5 (lanes 11 to 17) and day 6 (18 to 20) post-immersion. The positive
40 (+) and negative (-) signs indicate the positive and negative controls, respectively.

41



42

43 **Figure S4 Cell viability assay of oyster hemocytes exposed to different ratios of *V.***

44 ***crassostreae* WT or mutants (Δ).** Control hemocytes were incubated without bacteria (control)

45 or with a non-virulent strain (J2-8). Cell viability was evaluated by flow cytometry using a

46 double staining procedure after exposure of the hemocytes to bacteria at a ratio of 10

47 bacteria/hemocyte (grey bars) or 100 bacteria/hemocyte (black bars) for 6 hours. The assay was

48 performed in triplicate. Asterisks indicate significant differences of mortality assessed by

49 simultaneous tests for general linear hypotheses with Tukey contrasts ($P < 0.05$).

50

51



52

53 **Figure S5 Protein domains found in the transcription factor TF.** The protein was annotated

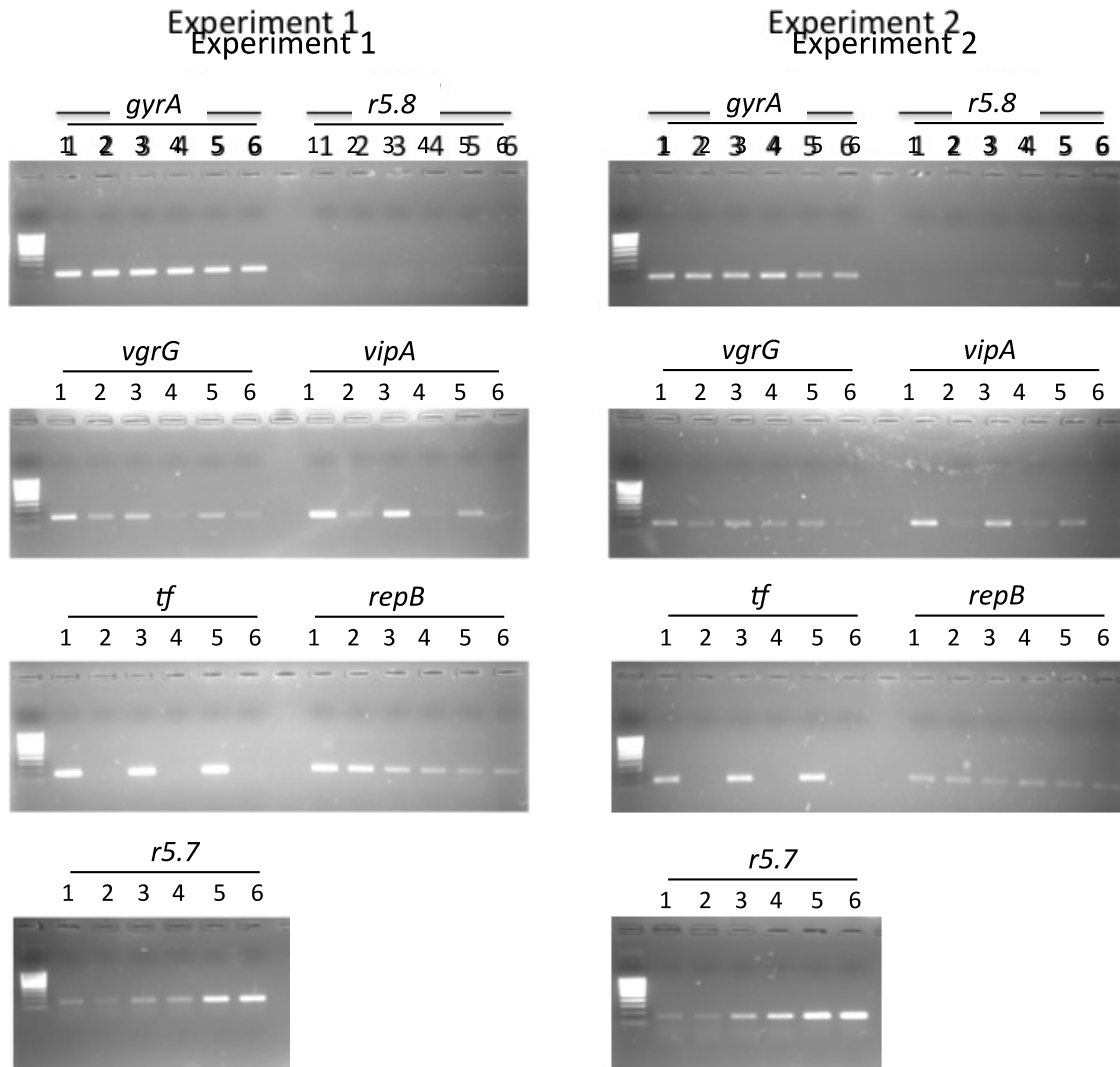
54 with Interproscan. The two domains are represented by colored tubes with numbers indicating the

55 beginning and the end of each domain on the protein. The accession numbers in domain

56 databases (SSF52317 = Superfamily (<http://supfam.org/>); PF12833 = PFAM

57 (<https://pfam.xfam.org/>)) are indicated with their putative function beneath.

58



60

61

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