



**HAL**  
open science

## A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters

David Goudenège, Marie Agnès Travers, Astrid Lemire, Philippe Haffner, Yannick Labreuche, Delphine Tourbiez, Bruno Petton, Sophie Mangenot, Alexandra Calteau, Didier Mazel, et al.

### ► To cite this version:

David Goudenège, Marie Agnès Travers, Astrid Lemire, Philippe Haffner, Yannick Labreuche, et al.. A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. *Environmental Microbiology*, 2015, 17 (11), pp.4189-4199. 10.1111/1462-2920.12699 . hal-01444897

**HAL Id: hal-01444897**

**<https://hal.science/hal-01444897v1>**

Submitted on 14 Oct 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## **A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters**

David Goudenège, Marie Agnès Travers, Astrid Lemire, Bruno Petton, Philippe Haffner, Yannick Labreuche, Delphine Tourbiez, Sophie Mangenot, Alexandra Calteau, Didier Mazel, et al.

► **To cite this version:**

David Goudenège, Marie Agnès Travers, Astrid Lemire, Bruno Petton, Philippe Haffner, et al.. A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in oysters. *Environmental Microbiology*, Wiley-Blackwell, 2015, 17 (11), pp.4189-4199. 10.1111/1462-2920.12699 . hal-01444897

**HAL Id: hal-01444897**

**<https://hal.archives-ouvertes.fr/hal-01444897>**

Submitted on 14 Oct 2019

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **A single regulatory gene is sufficient to alter *Vibrio aestuarianus* pathogenicity in**  
2 **oysters**

3  
4 **Running Title :** Comparative genomics of *Vibrio aestuarianus*

5  
6 D. Goudenège<sup>1,2,3</sup>, MA. Travers<sup>4</sup>, A. Lemire<sup>1,2,3</sup>, P. Haffner<sup>4</sup>, Y. Labreuche<sup>1,2,3</sup>, B. Petton<sup>1</sup>, D. Tourbiez ,S.  
7 Mangenot<sup>5</sup>, A. Calteau<sup>5,6</sup>, D. Mazel<sup>7,8</sup>, J.L. Nicolas<sup>1</sup>, A. Jacq<sup>9</sup> & F. Le Roux<sup>1,2,3\*</sup>

8  
9 <sup>1</sup>Ifremer, Unité Physiologie Fonctionnelle des Organismes Marins, ZI de la Pointe du Diable,  
10 CS 10070, F-29280 Plouzané, France.

11 <sup>2</sup>Sorbonne Universités, UPMC Univ Paris 06, UMR 8227, Integrative Biology of Marine  
12 Models, Station Biologique de Roscoff, CS 90074, F-29688, Roscoff Cedex, France.

13 <sup>3</sup>CNRS UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff,  
14 CS 90074, F-29688, Roscoff Cedex, France.

15 <sup>4</sup>Ifremer, SG2M-LGPMM, Laboratoire de Génétique et Pathologie des Mollusques Marins  
16 Avenue de Mus de Loup, 17390 La Tremblade, France

17 <sup>5</sup>Commissariat à l’Energie Atomique et aux Energies Alternatives (CEA), Direction des  
18 Sciences du Vivant (DSV), Institut de Génomique (IG), Genoscope

19 <sup>6</sup>CNRS UMR 8030, Laboratoire d’Analyse Bioinformatiques en Génomique et Métabolisme  
20 (LABGeM), 2 rue Gaston Crémieux, CP 5706, 91057 Evry cedex, France

21 <sup>7</sup>Institut Pasteur, Unité Plasticité du Génome Bactérien, Département Génomes et Génétique,  
22 75015 Paris, France

23 <sup>8</sup>CNRS UMR3525, F-75015 Paris, France

24 <sup>9</sup>Institut de génétique et Microbiologie, UMR8621, CNRS-Université Paris-Sud, 91405 Orsay  
25 Cedex, France

26

27 \***Corresponding author:** Frédérique Le Roux

28 Equipe « Génomique des Vibrio », Station Biologique de Roscoff, Place Georges Teissier,

29 29688 Roscoff cedex, France.

30 Tel 33 2 98 29 56 47, Fax 33 2 98 29 83 84, frederique.le-roux@sb-roscoff.fr

31

32 **Word count for the abstract :** 232

33 **Word count for the text :** 4677

34 **ABSTRACT**

35

36 Oyster diseases caused by pathogenic vibrios pose a major challenge to the sustainability of  
37 both commercial and natural oyster stocks. Problematically, vibrios form a very diverse group  
38 of microorganisms with virulence not necessarily assigned to any specific lineages within it.  
39 Furthermore, the required factors for virulence in vibrios pathogenic to oysters is still poorly  
40 understood. Since 2012, a disease affecting specifically adult oysters has been associated with  
41 the presence of *Vibrio aestuarianus* suggesting the possible (re) emergence of this organism  
42 as a pathogen. Here, by combining genome comparison, phylogenetic analysis and high  
43 throughput infection, we show that virulent strains are grouped into two *V. aestuarianus*  
44 lineages independent of the sampling date. The bacterial lethal dose was not different between  
45 strains isolated before or during the recent outbreaks. Hence the emergence of a new virulent  
46 clonal strain is unlikely. Each lineage comprises nearly identical strains and the majority of  
47 the strains are virulent. This suggests that within these phylogenetically coherent virulent  
48 lineages a few strains lost their pathogenicity. Comparative genomics allowed for the  
49 identification of a single frameshift in a non-virulent strain. The mutation affects the *varS*  
50 gene that codes for a signal transduction histidine-protein kinase. Genetic analyses confirmed  
51 that *varS* is necessary for infection of oysters and for a secreted metalloprotease expression.  
52 Hence our results for the first time in a *Vibrio* species show that VarS is a key factor of  
53 pathogenicity.

54

55 **IMPORTANCE**

56

57 Vibrios have been associated with successive mortality outbreaks of oyster beds (*Crassostrea*  
58 *gigas*) in France that have resulted in losses up to 100% of production. Given the actual quasi  
59 monoculture of *C. gigas*, there is an urgent need to understand the epidemiology of these  
60 outbreaks. Here we show that *V. aestuarianus* strains, isolated from diseased oysters, can be  
61 grouped into two phylogenetic lineages containing a majority of virulent strains. Genome  
62 sequence comparison between virulent versus non-virulent strains led us to identify a key  
63 regulator of *V. aestuarianus* pathogenicity. In the future, identification of the genes that are  
64 controlled by this regulator should help in understanding the virulence mechanisms of *V.*  
65 *aestuarianus*. Finally our results suggest that the recent adult mortality outbreaks of *C. gigas*  
66 are not due to the emergence of a new genotype of *V. aestuarianus*.

67

68

69 **INTRODUCTION**

70

71 The Pacific oyster *Crassostrea gigas* is by far the major mollusk species cultivated in France.  
72 However, this situation of near monoculture significantly weakens this economic sector,  
73 especially in the case of emergent diseases. This is illustrated by a recent decline of oyster-  
74 producing companies due to successive mortality outbreaks presenting distinct  
75 epidemiological characteristics (1).

76

77 For the last 20 years, French oyster production (mainly spat, i.e. oyster <12 months) has  
78 experienced periodical mass mortalities on the west coast of France, known as “summer  
79 mortalities” (2). This syndrome is the result of multiple factors including infectious agents (an  
80 ostreid herpes virus designated OsHV-1 and bacteria of the genus *Vibrio*), elevated  
81 temperature (>19°C), physiological stress associated with maturation, host genetic traits and  
82 aquaculture practices, none of these individual factors being consistently responsible for the  
83 syndrome. Although OsHV-1 was detected in half the mortality events, its pathogenicity was  
84 experimentally demonstrated in oyster larvae only (3, 4). Regarding bacteria, vibrios related  
85 to the species *V. aestuarianus* and to the clade *Splendidus* (containing several species) were  
86 the most common taxons isolated from moribund animals (5). The virulence of some strains  
87 belonging to either taxons was demonstrated by experimental infection on spat and adults (5-  
88 7).

89

90 Over the past six years, the frequency of oyster spat mortality outbreaks has increased  
91 considerably. Compared with the “summer mortalities”, these events occurred at a lower  
92 threshold temperature (16°C) and are characterized by a geographical extension to all French  
93 coasts (Atlantic, Channel and Mediterranean). Epidemiological analyses suggested that these

94 mortalities were linked to the emergence of a new genotype of OsHV-1 ( $\mu$ var) (8) probably  
95 associated with environmental changes and/or an increased fragility of the oysters. Although  
96 oyster spat experimental infections confirmed pathogenicity of the OsHV-1  $\mu$ var (9), the  
97 relationship between genome diversity and the infectious virus titer is currently not  
98 established. In addition, oysters spat were shown to harbor multiple vibrio genotypes. We  
99 recently demonstrated that the mortality onset is linked to a progressive replacement within  
100 the oyster microflora of diverse benign colonizers by members of a phylogenetically coherent  
101 virulent population (Lemire et al., ISME J. under revision). This phylogenetic cluster includes  
102 the *V. crassostreae* type strain and belongs to the *Splendidus* clade (10).

103

104 While the outbreaks described above concerned mainly oyster spats, the number of reported  
105 cases of adult mortalities associated with the presence of *V. aestuarianus* has increased  
106 considerably over the last 3 years (Francois et al., 2014). During the same period, other  
107 infectious agents such as the herpes virus or *Splendidus* related strains were rarely detected  
108 suggesting the possible emergence of *V. aestuarianus* as a pathogen for adult oysters.  
109 Emergent infectious diseases can be caused by genomic modifications of an infectious agent  
110 that can improve its virulence (11). In such a scheme, experimental infections should  
111 highlight differences in the lethal doses between strains isolated during the recent outbreaks  
112 and a decade ago. Then sequencing closely related isolates with contrasted virulence and  
113 performing whole genome comparative analyses, should lead to correlate genomic  
114 modification(s) and virulence improvement.

115

116 In comparison to human pathogens, little is known about the requirements for virulence in  
117 vibrios pathogenic to oysters. The genome sequencing and development of new genetic tools  
118 in the strain LGP32 isolated from a “summer mortality” outbreak and pathogenic to oysters



119 (*V. tasmaniensis*, clade *Splendidus*), has become a model to investigate *Vibrio*-oyster  
120 interaction (12-17). However, it has not been possible to apply the knowledge gained in this  
121 model system to other *Vibrio* pathogens due to inter-species variations such as cellular  
122 interactions with the oyster immune-competent cells, the hemocytes, For instance, *V.*  
123 *aestuarianus* strain 01\_032 inhibits phagocytosis (18) whereas *V. tasmaniensis* strain LGP32  
124 invades hemocytes but resists intracellular elimination (14). Thus, extending genomic and  
125 genetic analyses to other *Vibrio* species such as *V. aestuarianus* is an essential step towards  
126 understanding the role of bacteria in oyster mortality outbreaks.

127

128 In the present study, we explored the virulence potential and genome diversity of *V.*  
129 *aestuarianus* isolates. We asked whether the recent adult mortality outbreaks are due to the  
130 emergence of a specific genotype. To address this question, we performed high throughput  
131 sequencing (HTS)-based comparative genome analysis of 14 *V. aestuarianus* strains isolated  
132 before or during the recent outbreaks in parallel to bacterial lethal dose determination by  
133 experimental challenge. We then took advantage of the near identity of some strains with  
134 contrasted virulence to identify key factor(s) of *V. aestuarianus* pathogenicity by comparative  
135 and functional genomics.

136

137

## 138 **RESULTS**

139

### 140 ***V. aestuarianus* comprises virulent and non-virulent strains**

141 To explore the virulence potential and genome diversity of *V. aestuarianus* strains, 4 and 6  
142 strains isolated from diseased oysters during “summer mortality” events and the recent  
143 mortality outbreaks respectively were selected (Table 1). In addition, 4 strains isolated from

144 healthy oysters, cockles or zooplankton and not linked to mortality events were added to our  
145 analysis.

146

147 The strains were first injected into specific-pathogen-free (SPF) standardized oysters (19) at  
148  $10^7$  CFU/animal, a bacterial concentration previously used in experimental infections (6, 18).

149 At 6 days post injection, 10/14 strains induced mortality rates >80% (Fig. 1, black bars). We

150 subsequently injected lower bacterial concentrations to oysters (ranging from  $10^6$  to  $10^2$

151 CFU/animal). Surprisingly, when injected at  $10^2$  CFU /animal, the strain 02\_041 isolated in

152 2002 and 6 strains isolated in 2012 were still able to induce >80% mortality (Fig.1, grey bars).

153 When injected at  $<10^6$  CFU /animal, *V. tasmaniensis* and *V. crassostreae* strains, both species

154 related to *Splendidus* clade, did not cause mortality (Lemire et al., ISME J. under revision)

155 suggesting that the lethal dose of  $10^2$  CFU /animal is specific to some *V. aestuarianus* strains

156 isolated in 2002 or 2012.

157

158 These results allowed us to classify 7 strains (12\_063, 12\_128a, 12\_130, 12\_142, 12\_055,

159 12\_016a and 02\_041) as highly virulent (Vir+) (i.e. inducing >50% mortalities at  $10^2$

160 CFU/animal), and 4 strains (12\_122, 11\_U17, 11\_KB19 and 01\_151) as non-virulent (Vir-)

161 (i.e. inducing <50% mortalities at  $10^7$  CFU/animal). Three strains (07\_115, 01\_308, 01\_032)

162 were defined as intermediate (i.e. pathogenic only at  $10^7$  CFU/animal).

163

#### 164 **General features of the *V. aestuarianus* genomes**

165 The genome of strain 02\_041 was the most completely assembled and manually annotated. It

166 consists of two circular chromosomes of 2.98 (chromosome 1; 4 contigs) and 1.21Mb

167 (chromosome 2, 4 contigs) with an average GC content of 43.11 and 42.16% respectively

168 (Table 1; Fig.S1). Chromosomes 1 and 2 contain 7 and 0 rRNA operons, 74 and 10 tRNA

169 genes, respectively. However because the genome is not fully assembled, some rRNA and  
170 tRNA genes may have been missed.

171

172 The genome sequences of the 13 other strains were partially assembled, with contig numbers  
173 per strain ranging from 38 to 732 and approximate genome sizes ranging from 4.2 to 4.99 Mb  
174 compared to 4.19 Mb for strain 02\_041 (Table 1). The difficulty to achieve a better genome  
175 assembly may be attributed to i) a high number of transposition elements (184 transposase  
176 genes in the strain 02\_041); ii) the large size of the chromosomal integron (20) (94 cassettes  
177 in the chromosome 2 of strain 02\_041) (Fig. S1).

178

#### 179 **Genes differentiating *Vibrio aestuarianus* from other *Vibrionaceae***

180 A phylogenetic analysis based on concatenated nucleic acid sequences derived from 50 shared  
181 genes from 223 *Vibrionaceae* genome sequences including 14 *V. aestuarianus* strains and  
182 *Shewanella baltica* as an outgroup demonstrated the cohesive genotypic structure of *V.*  
183 *aestuarianus* with relatively little diversity among genomes (Fig. S2). The clade *V.*  
184 *aestuarianus* is sister to a clade that contains two species previously associated with farmed  
185 fish diseases, *V. ordalii* and *V. anguillarum* (21). Our analyses confirmed that *V.*  
186 *aestuarianus*, *V. ordalii* and *V. anguillarum* are grouped in the *Anguillarum* clade (10).

187

188 Intraspecific genomic comparisons identified 2866 CDSs that are shared by all sequenced *V.*  
189 *aestuarianus* strains (Fig. S1) of which only 40 proteins were found in  $\leq 5$  other *Vibrionaceae*  
190 genomes (Table S1). Among these *V. aestuarianus*-specific genes, we identified a cluster of  
191 genes homologous to the Toxin co-regulated (Tcp) pilus biosynthesis cluster encoded by a  
192 pathogenicity island in *V. cholerae* that is necessary for colonization to the intestine (22).  
193 However in the strain 02\_041, the *tcp* gene cluster is interrupted by a transposon, and genes

194 encoding the accessory colonization factors (*acf*) are absent (Fig.S3) suggesting that this *tcp*  
195 like cluster may play a distinct, if any, role in *V. aestuarianus*.

196

197 **Within *V. aestuarianus*, two lineages A and B contain a majority of Vir+ strains**

198 The phylogenetic relationships based on the core genome of the *V. aestuarianus* strains  
199 included in this study were investigated (Fig.2). The main outcome of this analysis was the  
200 grouping of 6/7 Vir+ isolates into a clade A, which also contains 1 Vir-, and 2 intermediate  
201 strains. Clade A is a sister of Clade B containing 1 Vir+ and one intermediate strain. Both  
202 clades A and B show very little intra-clade diversity (>99 % average nucleotide identity –  
203 ANI– value) (23). Inter-clade diversity was also low as determined by the ANI value  
204 calculation (>98.4 %) and by the number of clade-specific genes (180 CDSs, essentially in a  
205 clade B-specific phage). Vir- strains isolated from oysters in Spain, zooplankton in Italy or  
206 cockles in Brittany were found to be more diverse.

207

208 As a consequence of the low inter-clade diversity, the genes commonly used for multilocus  
209 sequence analysis (*hsp60*, *pyrH*, *atpA*, *gyrB*, *recA*, *topA*) do not allow the separation of clades  
210 A and B with a high bootstrap value. Thus, we compared the phylogenetic relationships of  
211 each core gene (2866 trees) and identified 55 genes allowing the placement of isolates in  
212 clade A or B with a bootstrap value of 100%. Among them, a gene encoding a putative D-  
213 lactate dehydrogenase (VIBAEv3\_A30718) was selected to explore the genetic structure of *V.*  
214 *aestuarianus* using a larger collection of strains (n=116) isolated from diseased animals  
215 (Table S2). Phylogenetic analyses reveal that 87/116 (75%) and 29/116 (25%) of these strains  
216 belong to clade A and B, respectively (Fig.3). When injected intramuscularly to oysters at 10<sup>2</sup>  
217 CFU /animal, 81/87 (93%) and 23/29 (79%) strains from respectively clade A and B were  
218 classified as Vir+ (Fig.3). The remaining strains were defined as intermediate (i.e. pathogenic

219 at  $10^7$  CFU/animal). The dominance of clade A and Vir+ strains (belonging to either clade A  
220 or B) was observed during summer mortalities and the recent outbreaks, whatever the age of  
221 the diseased oysters (> or <12months). Altogether these data demonstrate that strains  
222 belonging to *V. aestuarianus* and isolated from diseased oysters can be grouped into two  
223 lineages containing a majority of Vir+ strains. However, since we did not find a correlation  
224 between virulence, genotype and date of isolation, the hypothesis of a specific genotype  
225 emergence is unlikely.

226

### 227 **Non-virulent strains have undergone genetic modifications(s)**

228 Although the Vir+ strains belong to near clonal lineages, each lineage contains a low  
229 proportion of intermediate strains (7 and 21% for A and B respectively, [table S2](#)). We  
230 therefore performed comparative genomic analyses to identify Vir+ specific genes or alleles.  
231 In the clade B, 49 genes localized in 7 genomic regions were found in the Vir+ strain 12\_063  
232 but not in the intermediate strain 01\_308 ([Table S3](#)). These regions encode common phage-  
233 related proteins (e.g., integrase, helicase, relaxase and restriction endonuclease system) as  
234 well as other proteins of unknown function. None of these genes were found in the Vir+  
235 strains from clade A. Finally, a frameshift was observed in 13 genes of strain 01\_308, the  
236 majority of them coding for proteins of unknown function. However it should be noted that  
237 comparative genomic analyses within this clade are hampered by the reduced number of  
238 strains (one Vir+ and one intermediate) and by genome fragmentation.

239

240 In clade A, we could not identify genes present in all Vir+ and absent from the intermediate or  
241 Vir- strains. However, in the Vir- strain 01\_151, we detected a frameshift in three genes  
242 encoding respectively an exported protein of unknown function (VIBAEv3\_A31414 in strain  
243 02\_041), a putative acetyltransferase (VIBAEv3\_A10934) and a membrane protein of

244 unknown function (VIBAEv3\_A20116). Interestingly, a single frameshift was identified in  
245 the intermediate strain 07\_115 in a gene that codes for a signal transduction histidine-protein  
246 kinase (VarS) (24). In the Vir<sup>+</sup> strain 02\_041 the *varS* gene (VIBAEv3\_A30043) codes for a  
247 protein of 925 amino acids (aa) and contains 6 domains (Fig.4A): an uncharacterized signal  
248 transduction histidine kinase domain (DUF2222), a cytoplasmic helical linker domain and  
249 methyl-accepting proteins (HAMP), a phosphoacceptor domain (HisKA), an ATPase domain  
250 (HATPase\_c), a response regulator receiver domain (response reg) and a histidine-containing  
251 phosphotransfer domain (HPt). In the strain 07\_115 a deletion of a nucleotide result in a stop  
252 codon, generating a 677 aa protein that lacks the response reg and HPt domains (Fig. 4A). In  
253 *V. cholerae*, the VarS/VarA-CsrA/B/C/D system has been demonstrated to control the  
254 expression of virulence genes (25). Hence, based on genome comparison, we have identified a  
255 gene affected by a frameshift in an attenuated strain that could encode for a virulence  
256 regulator.

257

### 258 **Disruption of *varS* is sufficient to alter *Vibrio aestuarianus* pathogenicity**

259 We assessed the importance of *varS* for *V.aestuarianus* virulence using a genetic knockout  
260 approach described previously (15). We obtained a successful integration of the suicide  
261 plasmid by a single crossover in only one out of seven virulent strains (12\_016a). After the  
262 second recombination event leading to plasmid excision, 30% of the colonies carried the  
263 deletion of *varS* (strains 12\_016a\_Δ*varS*). For two isolates selected randomly, this deletion  
264 did not impair bacterial growth in culture media, but resulted in a dramatic decrease in  
265 mortalities induced after bacteria injection in oysters (Fig. 4B, lanes 3 and 4 compared to lane  
266 1). When constitutively expressed *in trans* from a replicative plasmid, the virulence of the  
267 mutant 12\_016a\_Δ*varS* was partially restored (Fig. 4B, lane 5 compared to 3) and sufficient  
268 to increase the virulence of the intermediate strain 07\_115 (Fig. 4B, lane 6 compared to 2).

269 These complementation experiments confirm that *varS* is necessary to 12\_016a pathogenicity  
270 and that the frameshift in *varS* is responsible of the 07\_115 virulence attenuation.

271

272 The two-component regulatory system VarA/S (VarS being the sensor histidine-kinase and  
273 VarA the response regulator) has been involved in the regulation of the secreted  
274 hemagglutinin/metalloprotease expression HapA in *V. cholerae* (26). Here, the protease  
275 activity measured in the ECPs of the 12\_016a\_Δ*varS* mutants (Fig. 5B and C, lanes 2 and 3)  
276 was found to be 3 times lower than that of the wild type virulent strain 12\_016a (Fig. 5B and  
277 C, lane 1) and in the range of the intermediate wild-type strain 07\_115 (Fig. 5B and C, lane  
278 4). The SDS-PAGE protein profiles of the ECPs prepared from 07\_115 and two clones of  
279 12\_016a\_Δ*varS* were found to be very similar and significantly different from this of 12\_016a  
280 (Fig.5A). A band found more intense in 12\_016a (Fig.5A, lane 1) was excised from the gel,  
281 analyzed by μLC-ESI MS/MS and demonstrated to correspond to the Vam metalloprotease,  
282 previously reported to be involved in *V. aestuarianus* toxicity (27). Unfortunately, several  
283 attempts to generate a Δ*vam* mutant were unsuccessful (100% wild type reversion after the  
284 second recombination), preventing a definitive conclusion about the direct role of Vam in  
285 virulence. In conclusion, we have demonstrated that VarS is a key regulator of *V.*  
286 *aestuarianus* pathogenicity, secreted proteolytic activity and extracellular Vam production.

287

288

## 289 **DISCUSSION**

290

291 The rise of aquaculture has been the source of anthropogenic changes on a massive scale,  
292 characterized by displacements of aquatic animals from their natural habitats, farming under  
293 high stocking density and exposition to environmental stresses. At the same time, over-

294 exploitation of some species and anthropogenic stress on aquatic ecosystems have placed  
295 pressure on wild populations, providing opportunities for the emergence of an expanding  
296 array of new diseases (28). In France, since 2012, a disease affecting specifically adult oysters  
297 has been associated with the presence of *Vibrio aestuarianus* suggesting the possible (re)  
298 emergence of this organism as a pathogen. However in the present study, we did not observe  
299 any correlation between *V. aestuarianus* lethal dose, genotype and isolation date suggesting  
300 that the hypothesis of the emergence of a new virulent clonal strain is unlikely.

301

302 Environmental changes have been shown to affect marine organism physiological  
303 functioning, behavior and demographic traits subsequently leading to an increased sensitivity  
304 to opportunistic pathogens, and/or amplification of resident infectious agents (29). Hence  
305 physiological alteration (s) of the oyster leading to an increased sensitivity to *V. aestuarianus*  
306 may also explain the recent outbreaks. Such physiological disorders may result from  
307 environmental factors or the presence of other infectious agents. It is also possible that trade-  
308 offs may have occurred between high levels of spat disease resistance and low levels of adult  
309 disease resistance (30). Experimental infections using wild stock of “naive” oysters that have  
310 never experienced the spat disease or selected lineages resistant to one/several infectious  
311 agents may help testing of this hypothesis. Finally the identification of habitat(s) and a spatio-  
312 temporal survey of *V. aestuarianus* will help in understanding the ecological parameters that  
313 modulate the virulence, persistence and/or prevalence of this pathogen.

314

315 Phylogenetic analysis of whole genomes revealed that virulent strains are grouped into two *V.*  
316 *aestuarianus* lineages, containing nearly identical strains. As each lineage contains a majority  
317 of highly virulent strains, we hypothesized that their common ancestor was virulent, and that a  
318 few modern strains might have undergone genetic modification (s) leading to loss of



319 pathogenicity. This was illustrated by the identification of a unique frameshift in a strain  
320 showing intermediate virulence. A single nucleotide deletion generates a truncation of one  
321 third of the C-term part of the VarS protein, containing two functional domains. The deletion  
322 of the *varS* gene in a Vir+ strain confirmed the role of this regulator in the virulence of *V.*  
323 *aestuarianus*.

324

325 VarS is a component of the sensory system VarS/VarA implicated in pathogenesis of a variety  
326 of Gram-negative bacteria, including among others, *V. cholerae* (VarS/VarA), *Escherichia*  
327 *coli* (BarA/UvrY), *Salmonella typhimurium* (BarA/SirA), and *Pseudomonas aeruginosa*  
328 (GacS/GacA) (31-34). In *V. cholerae* the VarS/VarA system is involved in the expression of  
329 the metalloprotease HapA and TcpA protein and in biofilm production (25, 26, 35). Here the  
330 comparison of the proteins excreted by the Vir+ wild type compared to  $\Delta varS$  strain  
331 demonstrates that the secretion and activity of a metalloprotease (Vam) is VarS dependant.  
332 The Vam metalloprotease of the *V. aestuarianus* strain 01\_032 has been previously  
333 demonstrated to display lethality in *C. gigas* oysters (27). The expression of this gene by a  
334 non-toxicogenic vibrio strain (*V. tasmaniensis* LMG20012<sup>T</sup>) induces the same  
335 immunosuppressant effects on hemocytes as those observed for *V. aestuarianus* ECPs  
336 showing that this protein is sufficient to induce immunosuppression in oysters (18). However  
337 the formal demonstration of the predicted, or supposed, role of a candidate gene requires a  
338 gene knock out strategy (15).

339

340 In the present study, a dramatic difference in DNA delivery ( $10^{-4}$  to  $10^{-6}$  transconjugant per  
341 recipient cells) and allelic exchange efficiency (0 to  $10^{-8}$  integration per recipients) was  
342 observed between nearly clonal strains. We were able to delete the *varS* gene in 1/7 virulent  
343 strain (12\_016a); but could not generate a *vam* mutant, preventing a definitive conclusion

344 regarding the role of Vam in virulence. This suggests that the presence of this gene is  
345 essential in this strain in this culture condition. It is important to note that the lack of a second  
346 usable resistance marker prevented the demonstration that a *vam* mutant could be constructed  
347 when the gene was provided *in trans*. We are currently exploring a larger panel of antibiotic  
348 resistance genes to allow the development of such strategy in the future. Our study highlights  
349 the limitations of genetic methods when working with environmental non-model strains.  
350 Limitations can occur at several levels from the DNA delivery inside the cells, to the allelic  
351 exchange efficiency and the availability of selective genes. Consequently we strongly  
352 recommend testing the feasibility of genetic approaches from a collection of strains rather  
353 than a single isolate before starting genomic projects. Parameters such as antibiotic's  
354 resistance, conjugation and integration frequency should be tested to select the model strain to  
355 be used.

356

357 VarS is a key regulator of *V. aestuarianus* virulence and Vam seems to be a target of this  
358 regulator. Previous studies have examined the contribution to the virulence of various vibrio  
359 metalloproteases in animal experimental models (15, 36-39) but no conclusive evidence about  
360 the role of the protease in virulence was found, since mutants deficient in protease showed  
361 comparable virulence levels than their parental strains. There are only a few examples of  
362 toxins (such as diphtheria or tetanus), which act as single determinants to produce disease.  
363 Microbial pathogenesis is often multifactorial, and pathogens use several biochemical  
364 mechanisms operating in concert to produce infection and disease (40). For instance, the  
365 HA/P metalloprotease from *V. cholerae* was reported to activate proteolytically both the El  
366 Tor cytolysin/haemolysin (41) and the cholera toxin CT, an ADP-ribosylating enterotoxin  
367 inducing a highly secretory diarrhea (42). Research is now ongoing to identify other genes  
368 that are regulated by VarS and the protein targets that are processed by Vam in the ECP

369 fraction. Finally, due to the near clonality of strains within clades A and B, *V. aestuarianus*  
370 appears as a great model for comparative genome analysis, leading to the identification of a  
371 restricted number of virulence candidate genes. In the future, each of these genes will be  
372 knocked out to investigate their role in virulence.

373

374

## 375 **MATERIALS AND METHODS**

376

377 **Strains and culture conditions.** The strains used for genomic analyses are described in [Table](#)  
378 [1](#). Other bacterial strains are described in [Table 2](#). *Vibrio* isolates were grown in Zobell or  
379 Zobell agar, Luria-Bertani (LB) or LB-agar (LBA) + NaCl 0.5M, at 20°C. *Escherichia coli*  
380 strains were grown in LB or on LBA at 37°C. Chloramphenicol (5 to 25µg/ml), thymidine  
381 (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary.  
382 Induction of the P<sub>BAD</sub> promoter was achieved by the addition of 0.2% L-arabinose to the  
383 growth medium, and conversely, repression was obtained by the addition of 1% D-glucose.

384

385 **Genome sequencing, assembly and annotation.** The complete genome sequence of 02\_041  
386 strain was obtained using two sequencing technologies: 1) A Sanger library was constructed  
387 after mechanical shearing of DNA and cloning of 10 kpb fragments into pCNS (pSU18  
388 derived). Plasmids were purified and end-sequenced using dye-terminator chemistry on  
389 ABI3730 sequencers leading to a 4-fold coverage. 2) A 454 single read library was  
390 constructed and sequenced to a 16-fold coverage. The reads obtained using the two  
391 technologies were assembled using Newbler ([www.roche.com](http://www.roche.com)). Then, primer walks, PCRs  
392 and transposon bombs were performed to finish the sequence of the *V. aestuarianus* reference  
393 genome. The 13 other *V. aestuarianus* strains were sequenced using the Illumina HiSeq2000

394 technology with ~50-fold coverage. Contigs were assembled *de novo* using Velvet (43) and  
395 genome assembly was improved by contig mapping against the 02\_041 reference genome.  
396 Computational prediction of coding sequences and other genome features (RNA encoding  
397 genes, ribosome binding sites, signal sequences, etc...), together with functional assignments  
398 were performed using the automated annotation pipeline implemented in the MicroScope  
399 platform (44). An extensive manual curation of the genes, which includes correction of the  
400 start codon positions and of the functional assignments, was performed. This expert procedure  
401 was supported by functional analysis results [e.g., InterPro, FigFam, PRIAM, COGs (Clusters  
402 of Orthologous Groups), PsortB] which can be queried using an exploration interface, and by  
403 synteny groups computation visualized in cartographic maps to facilitate genome comparison.

404

405 ***In silico* analyses.** To investigate the core and flexible genomes, an all-versus-all BlastP  
406 search was performed using genomic sequences of 209 *Vibrionaceae* and *Shewanella baltica*  
407 (strain OS155) available in Genbank and 14 *V. aestuarianus* sequenced in the present study  
408 (Table 1). A dedicated precomputing repository (marshalling) was created to perform  
409 comparative genomic and phylogenomic analyses. Orthologous proteins were defined as  
410 reciprocal best hit proteins with 80% MaxLrap and a minimum of 30% and 60% identity  
411 cutoff was used for intra- and inter-species analysis, respectively (45). The nucleic acid  
412 sequences were aligned using Muscle (46) and filtered by BMGE (47). Phylogenetic trees  
413 were built using the parallel version of PhyML applied to Maximum-likelihood algorithm and  
414 GTR model as parameters (48). Reliability was assessed by the bootstrap method with 100  
415 replicates.

416

417 **Vector construction.** Cloning was performed using the Gibson assembly method according  
418 to the manufacturer's instructions (New England Biolabs, NEB). For the *varS* deletion, two

419 independent PCR amplifications of the regions (500 bp) encompassing the *varS* gene were  
420 performed using two primer pairs ( $\Delta varS$ -1+2 and  $\Delta varS$ -3+4) (Table 2). An inside out PCR  
421 was performed using pSW7848T suicide vector DNA (49) and primer pair (*pSW-F* and *pSW-*  
422 *R*) (Table 2). For the cloning of *varS* gene under a P<sub>LAC</sub> promoter in a pMRB plasmid (50),  
423 two independent PCR amplifications of the gene and plasmid were performed using the  
424 primers *varS-F+R* and *pMRB-F+R* reciprocally. After purification and quantification, 100 ng  
425 of the PCR products were mixed with Gibson assembly Master Mix and incubated for 60  
426 minutes at 50°C. Samples were diluted at 1/3 before *E. coli* transformation. Clones were  
427 controlled by digestion with restriction enzyme and sequencing using the primers described in  
428 Table 2. Strains II3813 and  $\beta$ 3914 were used as a plasmid host for cloning and conjugation,  
429 respectively (15). Plasmids and strains used and established in the present study are presented  
430 in Table 2.

431

### 432 **Conjugation**

433 Overnight cultures of donor and recipient were diluted at 1:100 in culture media without  
434 antibiotic and grown at 30°C to an OD<sub>600nm</sub> of 0.3. The different conjugation experiments  
435 were done by a filter mating procedure described previously (15) with a donor/recipient ratio  
436 of 1ml/10ml. Conjugations were performed overnight on filters incubated on LBA + NaCl  
437 0.5N + diaminopimelic acid (DAP) plates at 30°C. Counter-selection of  $\Delta dapA$  donor was  
438 done by plating on a medium devoid of DAP, supplemented with chloramphenicol and 1%  
439 glucose. Cm<sup>R</sup> resistant colonies were grown in LB + NaCl 0.5N up to late logarithmic phase  
440 and spread on plates containing 0.2% arabinose. Mutants were screened by PCR using  
441 primers  $\Delta varS$ -1+4 (Table 2).

442

443 **Extracellular products analyses.** Bacterial extracellular products (ECPs) were produced by  
444 the cellophane overlay method as described previously (15). The protein concentration of the  
445 ECPs was measured by the method of Bradford with bovine serum albumin as the standard  
446 and normalized (BioRad). Protease activity was measured by the azocasein procedure as  
447 described previously (51). In addition, protease activity of separated proteins in an SDS-  
448 polyacrylamide gel was detected by copolymerizing 0.2% azocasein in the polyacrylamide  
449 matrix (Zymography) as described previously (15).

450 After concentration by ultrafiltration (Centricon® 10 Kda), twenty micrograms of crude ECPs  
451 were analyzed on a 4-15% Mini-PROTEAN® TGX Precast Gels. The differentially expressed  
452 protein band was manually excised from the gel, in-gel digested using trypsin and subjected  
453 to MS and MS/MS analyses for protein identification, following previously described  
454 protocols (52).

455

#### 456 **Production of “pathogen free” oysters**

457 Oysters (18 to 36 months; n=40) collected in Fouras Bay (Marennes- Oléron, France) were  
458 transferred to the Ifremer facility located at Argenton (Brittany, France) for maturation  
459 conditioning as described previously (19). After gamete stripping and fertilization, obtained  
460 larvae, then spat, were reared under controlled conditions up to 12-13 months. PCR detection  
461 of Herpes was performed to confirm the negative status of oyster (19). Vibrios isolation on  
462 selective culture medium (Thiosulfate-citrate-bile salts-sucrose agar, TCBS) confirmed a low  
463 vibrio presence (~10 cfu/gr tissue).

464

465 **Virulence studies using oysters.** Bacteria were grown under constant agitation at 20°C for  
466 24 h in Zobell. One hundred microliters of the diluted culture ( $10^7$  to  $10^2$  CFU) were injected  
467 intramuscularly to anaesthetized SPF oysters (12-13 months old, 1.5 g, s.d. 0.2). The bacterial

468 concentration was confirmed by conventional dilution plating on Zobell agar. After injection,  
469 the oysters were transferred to aquaria (10 oysters per aquarium) containing 2.5 liter of  
470 aerated 5 µm-filtered and UV-treated seawater at 20°C and kept under static conditions for 6  
471 days. Each bacterial treatment was performed in duplicates and mortality was recorded daily.

472

473

#### 474 **ACKNOWLEDGEMENTS**

475 We warmly thank Dr Maurizio Labbate (University of Technology, Sydney, Australia) for  
476 critically reading the manuscript. We also thank Joël Henry and Céline Zatylny-Gaudin  
477 (Université de Caen Basse-Normandie, Institut de Biologie Fondamentale et Appliquée) for  
478 kindly performing mass spectrometry analyses. We acknowledge the staff of the station  
479 Ifremer Argenton, La Tremblade, Bouin, particularly Max Naury, the ABIMS (SBR Roscoff)  
480 and LABGeM (Evry) platforms for technical support. The present study has been supported  
481 by the DPMA (Convention DPMA 2013- IFREMER 12/1210320/NYF), the ANR blanc (11-  
482 BSV7-023-01 « VIBRIOGEN », DG funding), the EMBRC France (AL funding) and the  
483 ANR bioadapt (13-ADAP-0007-01« OPOPOP»).

484

#### 485 **Author contributions**

486 DG, MAT and AL contribute equally to this work.

487 MAT, AL, PH, YL, BP, DT, SM, JLN performed experiments. DG, AC and AJ performed the  
488 *in silico* analyses. FLR, designed experiments, interpreted results, and wrote the paper with  
489 the help of YL, MAT, AJ and DM.

490 **REFERENCES**

491

- 492 1. **Renault T.** 2011. A review of mortality outbreaks in the Pacific oyster, *Crassostrea*  
493 *gigas*, reported since 2008 in various European Union Member States and the related  
494 implementation of Council Directive 2008/88/EC. Bulletin OIE **4**:51-52.
- 495 2. **Samain et al.** 2008. Summer mortality of Pacific oyster *Crassostrea gigas*. The  
496 Morest Project, Editions Quae.
- 497 3. **Renault T, Le Deuff RM, Chollet B, Cochenec N, and Gerard A.** 2000.  
498 Concomitant herpes-like virus infections in hatchery-reared larvae and nursery-  
499 cultured spat *Crassostrea gigas* and *Ostrea edulis*. Dis. Aquat. Organ. **42**:173-183.
- 500 4. **Davison AJ et al.** 2005. A novel class of herpesvirus with bivalve hosts. J. Gen. Virol.  
501 **86**:41-53
- 502 5. **Garnier M, Labreuche Y, Garcia C, Robert M, and Nicolas JL.** 2007. Evidence  
503 for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster  
504 *Crassostrea gigas*. Microbial ecology **53**:187-196.
- 505 6. **Gay M, Berthe FC, and Le Roux F.** 2004. Screening of *Vibrio* isolates to develop an  
506 experimental infection model in the Pacific oyster *Crassostrea gigas*. Dis. Aquat.  
507 Organ. **59**:49-56.
- 508 7. **Saulnier D et al.** 2010. A large-scale epidemiological study to identify bacteria  
509 pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and  
510 metalloprotease-like activity. Microbial ecology **59**:787-798.
- 511 8. **Segarra A et al.** 2010. Detection and description of a particular *Ostreid herpesvirus 1*  
512 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea*  
513 *gigas*, in France in 2008. Virus Res. **153**:92-99.



- 514 9. **Schikorski D et al.** 2011. Experimental *ostreid herpesvirus 1* infection of the Pacific  
515 oyster *Crassostrea gigas*: kinetics of virus DNA detection by q-PCR in seawater and  
516 in oyster samples. *Virus Res.* **155**:28-34.
- 517 10. **Sawabe T et al.** 2013. Updating the *Vibrio* clades defined by multilocus sequence  
518 phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp.  
519 nov. *Front. Microbiol.* **4**:414.
- 520 11. **Keim PS and Wagner DM.** 2009. Humans and evolutionary and ecological forces  
521 shaped the phylogeography of recently emerged diseases. *Nat. Rev. Microbiol.* **7**:813-  
522 821.
- 523 12. **Binesse J et al.** 2008. Metalloprotease *vsm* is the major determinant of toxicity for  
524 extracellular products of *Vibrio splendidus*. *Applied and environmental microbiology*  
525 **74**:7108-7117.
- 526 13. **Duperthuy M et al.** 2010. The major outer membrane protein OmpU of *Vibrio*  
527 *splendidus* contributes to host antimicrobial peptide resistance and is required for  
528 virulence in the oyster *Crassostrea gigas*. *Environmental microbiology* **12**:951-963.
- 529 14. **Duperthuy M et al.** 2011. Use of OmpU porins for attachment and invasion of  
530 *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proc. Natl.*  
531 *Acad. Sci. U S A* **108**:2993-2998.
- 532 15. **Le Roux F, Binesse J, Saulnier D, and Mazel D.** 2007. Construction of a *Vibrio*  
533 *splendidus* mutant lacking the metalloprotease gene *vsm* by use of a novel  
534 counterselectable suicide vector. *Applied and environmental microbiology* **73**:777-  
535 784.
- 536 16. **Le Roux F et al.** 2009. Genome sequence of *Vibrio splendidus*: an abundant  
537 planctonic marine species with a large genotypic diversity. *Environmental*  
538 *microbiology* **11**:1959-1970.

- 539 17. **Vanhove AS et al.** 2014. Outer membrane vesicles are vehicles for the delivery of  
540 *Vibrio tasmaniensis* virulence factors to oyster immune cells. Environmental  
541 microbiology. Jun 11. doi: 10.1111/1462-2920.12535.
- 542 18. **Labreuche Y, Soudant P, Goncalves M, Lambert C and Nicolas JL.** 2006. Effects  
543 of extracellular products from the pathogenic *Vibrio aestuarianus* strain 01/32 on  
544 lethality and cellular immune responses of the oyster *Crassostrea gigas*. Dev. Comp.  
545 Immunol. **30**:367-379.
- 546 19. **Petton B, Pernet F, Robert R, and Boudry P.** 2013. Temperature influence on  
547 pathogen transmission and subsequent mortalities in juvenile Pacific oysters  
548 *Crassostrea gigas*. Aquacult. Environ. Interact. **3**:257-273.
- 549 20. **Mazel D, Dychinco B, Webb VA and Davies J.** 1998. A distinctive class of integron  
550 in the *Vibrio cholerae* genome. Science **280**:605-608.
- 551 21. **Austin B.** 2011. Taxonomy of bacterial fish pathogens. Vet. Res. **42**:20.
- 552 22. **Davis BM and Waldor MK.** 2003. Filamentous phages linked to virulence of *Vibrio*  
553 *cholerae*. Current opinion in microbiology **6**:35-42.
- 554 23. **Konstantinidis KT and Tiedje JM.** 2005. Genomic insights that advance the species  
555 definition for prokaryotes. Proc. Natl. Acad. Sci. U S A **102**:2567-2572.
- 556 24. **Lenz DH, Miller MB, Zhu J, Kulkarni RV and Bassler BL.** 2005. CsrA and three  
557 redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. Molecular  
558 microbiology **58**:1186-1202.
- 559 25. **Jang J, Jung KT, Park J, Yoo CK and Rhie GE.** 2011. The *Vibrio cholerae*  
560 VarS/VarA two-component system controls the expression of virulence proteins  
561 through ToxT regulation. Microbiology **157**:1466-1473.

- 562 26. **Jang J, Jung KT, Yoo CK and Rhie GE.** 2010. Regulation of  
563 hemagglutinin/protease expression by the VarS/VarA-CsrA/B/C/D system in *Vibrio*  
564 *cholerae*. Microb. Pathog. **48**:245-250.
- 565 27. **Labreuche Y et al.** 2010. *Vibrio aestuarianus* zinc metalloprotease causes lethality in  
566 the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses.  
567 Fish Shellfish Immunol **29**:753-758.
- 568 28. **Harvell CD, et al.** 1999 Emerging marine diseases--climate links and anthropogenic  
569 factors. Science **285**:1505-1510.
- 570 29. **Doney SC et al.** 2012. Climate change impacts on marine ecosystems. Ann. Rev. Mar.  
571 Sci. **4**:11-37.
- 572 30. **Cotter SC, Kruuk LE and Wilson K.** 2004. Costs of resistance: genetic correlations  
573 and potential trade-offs in an insect immune system. J. Evol. Biol. **17**:421-429.
- 574 31. **Timmermans J and Van Melderren L.** 2010. Post-transcriptional global regulation  
575 by CsrA in bacteria. Cellular and molecular life sciences **67**:2897-2908.
- 576 32. **Chavez RG, Alvarez AF, Romeo T and Georgellis D.** 2010. The physiological  
577 stimulus for the BarA sensor kinase. Journal of bacteriology **192**:2009-2012.
- 578 33. **Jones BD.** 2005. *Salmonella* invasion gene regulation: a story of environmental  
579 awareness. Journal of microbiology **43**:110-117.
- 580 34. **Gooderham WJ and Hancock RE.** 2009. Regulation of virulence and antibiotic  
581 resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. FEMS  
582 Microbiol. Rev. **33**:279-294.
- 583 35. **Tsou AM, Liu Z, Cai T, and Zhu J.** 2011. The VarS/VarA two-component system  
584 modulates the activity of the *Vibrio cholerae* quorum-sensing transcriptional regulator  
585 HapR. Microbiology **157**:1620-1628.

- 586 36. **Finkelstein RA, Boesman-Finkelstein M, Chang Y and Hase CC.** 1992. *Vibrio*  
587 *cholerae* hemagglutinin/protease, colonial variation, virulence, and detachment. *Infect.*  
588 *Immun.* **60**:472-478.
- 589 37. **Jeong KC et al.** 2000. Construction and phenotypic evaluation of a *Vibrio vulnificus*  
590 *vvpE* mutant for elastolytic protease. *Infect. Immun.* **68**:5096-5106.
- 591 38. **Milton DL, Norqvist A and Wolf-Watz H.** 1992. Cloning of a metalloprotease gene  
592 involved in the virulence mechanism of *Vibrio anguillarum*. *Journal of bacteriology*  
593 **174**:7235-7244.
- 594 39. **Shao CP and Hor LI.** 2000. Metalloprotease is not essential for *Vibrio vulnificus*  
595 virulence in mice. *Infect. Immun.* **68**:3569-3573.
- 596 40. **Finlay BB and Falkow S.** 1997. Common themes in microbial pathogenicity  
597 revisited. *Microbiology and molecular biology reviews* **61**:136-169.
- 598 41. **Nagamune K et al.** 1996. *In vitro* proteolytic processing and activation of the  
599 recombinant precursor of El Tor cytolysin/hemolysin (pro-HlyA) of *Vibrio cholerae*  
600 by soluble hemagglutinin/protease of *V. cholerae*, trypsin, and other proteases. *Infect.*  
601 *Immun.* **64**:4655-4658.
- 602 42. **Booth BA, Boesman-Finkelstein M, and Finkelstein RA.** 1984. *Vibrio cholerae*  
603 hemagglutinin/protease nicks cholera enterotoxin. *Infect. Immun.* **45**:558-560.
- 604 43. **Zerbino DR and Birney E.** 2008. Velvet: algorithms for de novo short read assembly  
605 using de Bruijn graphs. *Genome research* **18**:821-829.
- 606 44. **Vallenet D et al.** 2013. MicroScope--an integrated microbial resource for the curation  
607 and comparative analysis of genomic and metabolic data. *Nucleic acids research*  
608 **41**:636-647.

- 609 45. **Daubin V, Gouy M and Perriere G.** 2002. A phylogenomic approach to bacterial  
610 phylogeny: evidence of a core of genes sharing a common history. *Genome research*  
611 **12**:1080-1090.
- 612 46. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and  
613 high throughput. *Nucleic acids research* **32**:1792-1797.
- 614 47. **Criscuolo A and Gribaldo S.** 2010. BMGE (Block Mapping and Gathering with  
615 Entropy): a new software for selection of phylogenetic informative regions from  
616 multiple sequence alignments. *BMC Evol. Biol.* **10**:210.
- 617 48. **Guindon S et al.** 2010. New algorithms and methods to estimate maximum-likelihood  
618 phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* **59**:307-321.
- 619 49. **Val ME, Skovgaard O, Ducos-Galand M, Bland MJ, and Mazel D.** 2012. Genome  
620 engineering in *Vibrio cholerae*: a feasible approach to address biological issues. *PLoS*  
621 *Genet.* **8**:e1002472 .
- 622 50. **Le Roux F, Davis BM and Waldor MK.** 2011. Conserved small RNAs govern  
623 replication and incompatibility of a diverse new plasmid family from marine bacteria.  
624 *Nucleic acids research* **39**:1004-1013.
- 625 51. **Miyoshi N, Shimizu C, Miyoshi S and Shinoda S.** 1987. Purification and  
626 characterization of *Vibrio vulnificus* protease. *Microbiol Immunol* **31**:13-25.
- 627 52. **Bernay B, Baudy-Floc'h M, Gagnon J, and Henry J.** 2006. Ovarian jelly-peptides  
628 (OJPs), a new family of regulatory peptides identified in the cephalopod *Sepia*  
629 *officinalis*. *Peptides* **27**:1259-1268.
- 630
- 631

632 **TITLES AND LEGENDS TO FIGURES**

633

634 **Figure 1:** Oyster mortality in response to experimental infection with *V. aestuarianus* strains  
635 selected for genome sequencing.  $10^7$  CFU (black bar) or  $10^2$  CFU (grey bar) of the tested  
636 strain were intramuscularly injected into oysters (n= 10, in duplicate). Cumulative mortality  
637 (%) was assessed after 6 days. Strains were classified as virulent (Vir+) (i.e. inducing >50%  
638 mortalities at  $10^2$  CFU /animal), non-virulent (Vir-) (i.e. inducing <50% mortalities at  $10^7$   
639 CFU /animal) or intermediate (i.e. pathogenic only at  $10^7$  CFU /animal).

640

641 **Figure 2:** Phylogenetic analysis based on concatenated alignments of nucleic acid sequences  
642 of 2866 core genes from 14 *V. aestuarianus* strains, KB19 as an outgroup. Tree was built by  
643 the Maximum-Likelihood method based on sequences aligned using Muscle. Branch lengths  
644 are drawn to scale and are proportional to the number of nucleotide changes. Number at each  
645 node represents the percentage value given by bootstrap analysis of 100 replicates. The  
646 pathotype of each *V. aestuarianus* strain (Vir+: virulent; Vir-: non virulent; int: intermediate)  
647 is indicated in parentheses.

648

649 **Figure 3:** Virulence onto *V. aestuarianus* isolates phylogeny inferred by maximum likelihood  
650 analysis of partial D-lactate dehydrogenase gene sequences. The tree was built by the  
651 Maximum-Likelihood method based on sequences aligned using Muscle. Branch lengths are  
652 drawn to scale and are proportional to the number of nucleotide changes. Number at each  
653 node represents the percentage value given by bootstrap analysis of 100 replicates. The black  
654 bars indicate the % of mortalities occurring at 6 days post-injection ( $10^2$  CFU/animal).

655

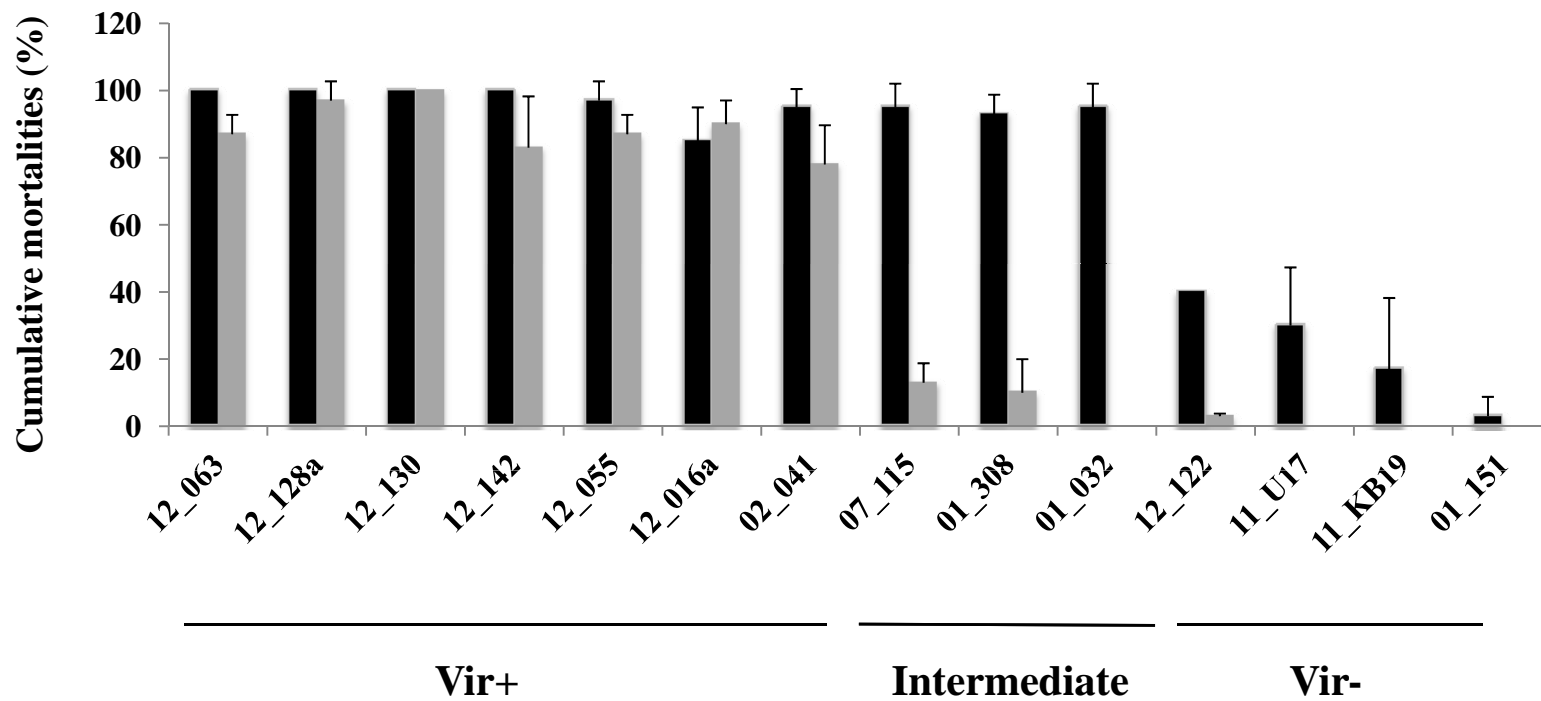
656 **Figure 4:** Role of *varS* in *Vibrio aestuarianus* pathogenicity. **A-** Schematic representation of  
657 the VarS functional domains identified in the Vir<sup>+</sup> strain 02\_041 and the truncated protein  
658 resulting from a frameshift in the intermediate strain 07\_115. DUF2222 corresponds to an  
659 uncharacterized signal transduction histidine kinase domain; HAMP, a cytoplasmic helical  
660 linker domain and methyl-accepting proteins; HisKA, a phosphoacceptor domain;  
661 HATPase\_c, an ATPase domain; Response reg, a response regulator receiver domain; HPt, an  
662 histidine-containing phosphotransfer domain. **B-** Experimental infection of wild type *V.*  
663 *aestuarianus*,  $\Delta varS$  mutants and complemented  $\Delta varS$  mutants.  $10^2$  CFU of the tested strains  
664 (lane 1: strain 12\_016a wild type; lane 2: strain 07\_115 wild type; lanes 3 and 4: GV1124 and  
665 1125, two distinct clones of 12\_016\_  $\Delta varS$ ; lane 5: GV1124 i.e. 12\_016\_  $\Delta varS$  carrying an  
666 expression vector for VarS, pMRB-P<sub>LAC</sub>*varS*; lane 6: 07\_115 carrying pMRB-P<sub>LAC</sub>*varS*) was  
667 intramuscularly injected into oysters (n= 20, in duplicate). Mortality (%) was assessed after 6  
668 days.

669

670 **Figure 5:** Role of *varS* in *Vibrio aestuarianus* metalloprotease expression. **A-** Extracellular  
671 product analysis by Coomassie blue stained 10% SDS-PAGE gel (lane 1: strain 12\_06 wild  
672 type; lane 2 and 3: GV1124 and 1125, two distinct clones of 12\_016\_  $\Delta varS$ ; lane 4: 07\_115  
673 wild type). Arrow indicates the Vam metalloprotease identified by MS/MS. **B-** Azocasein-  
674 SDS-polyacrylamide gel (protease which degraded the gelatin are detected by zones of  
675 clearing). **C-** Proteolytic activities of ECPs determined by an azocasein assay (absorbance at  
676 440 nm).

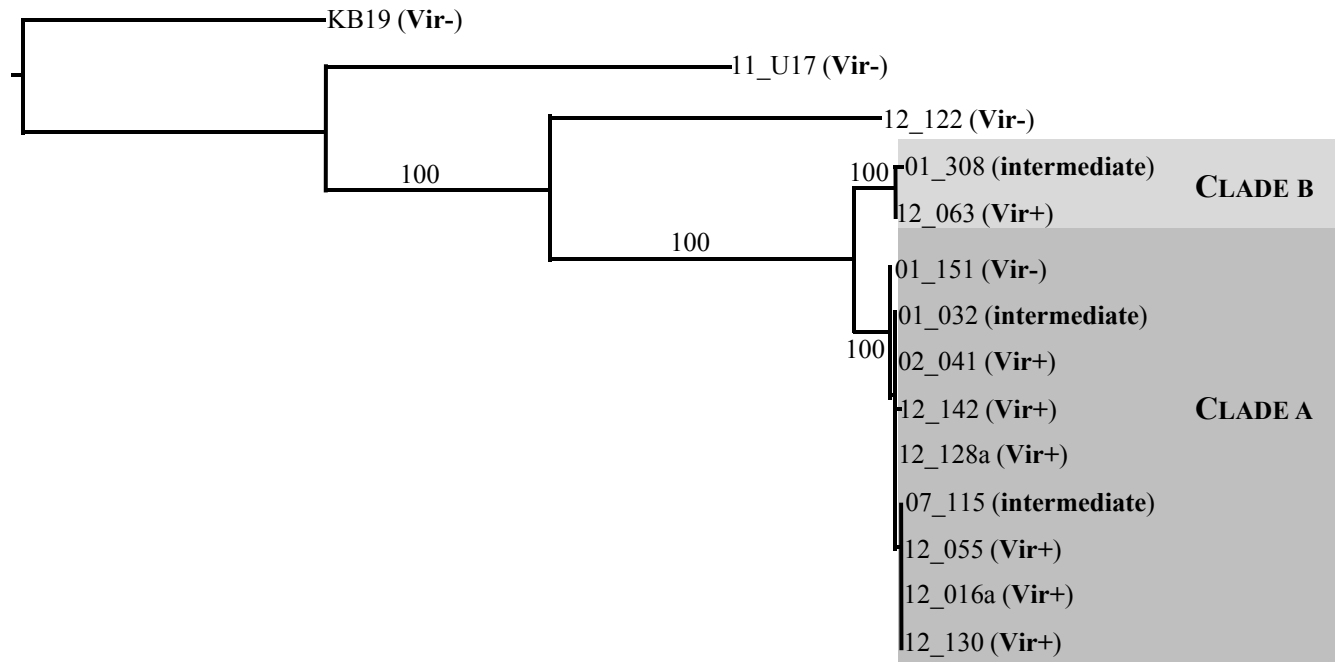
677

678

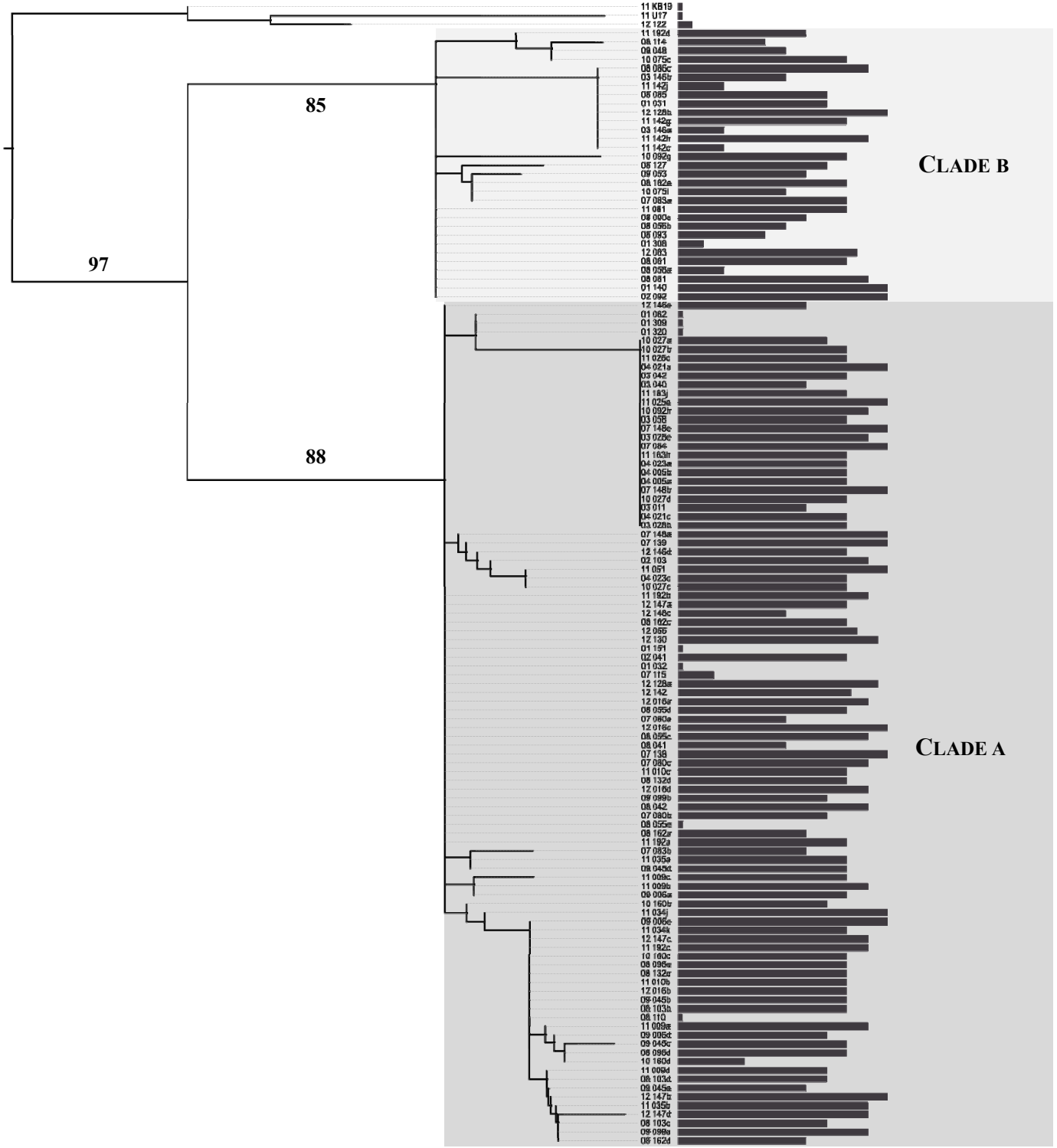


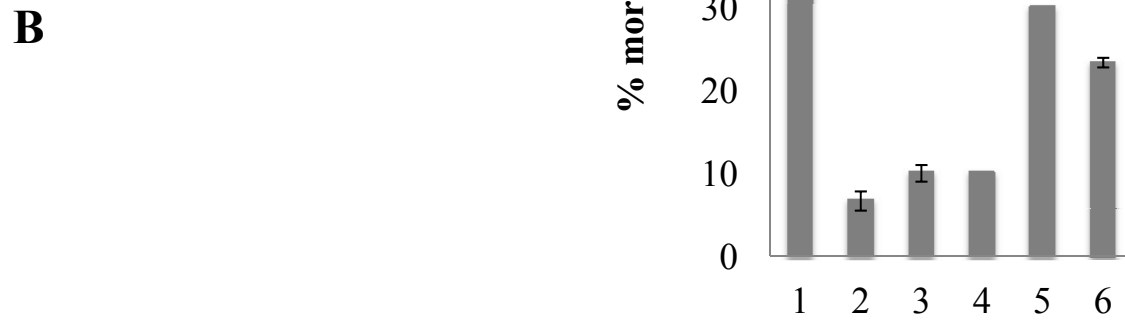
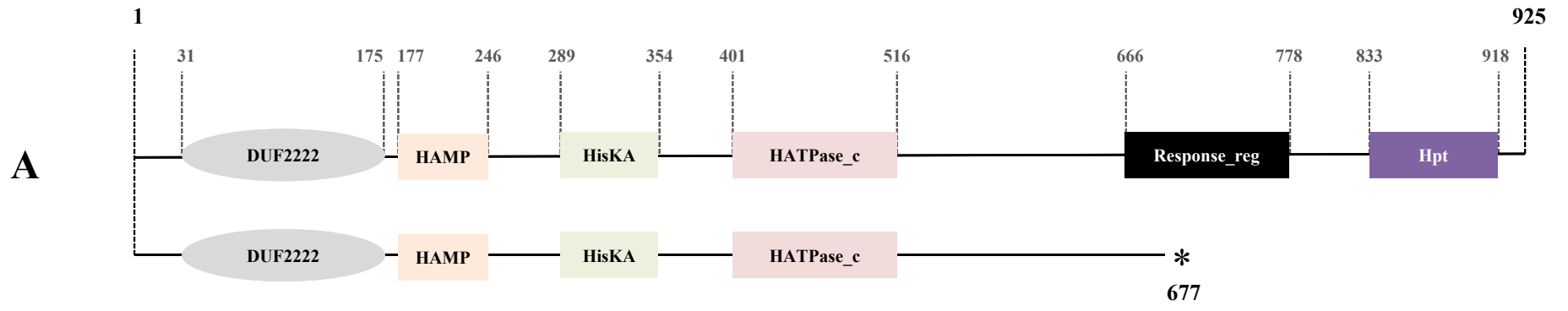


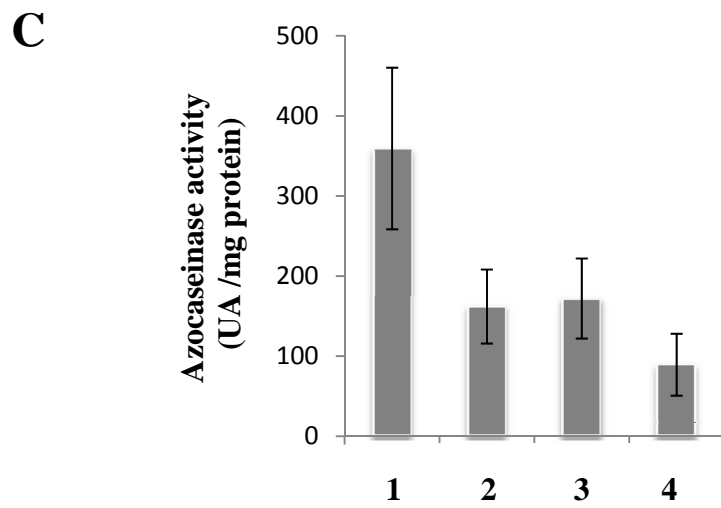
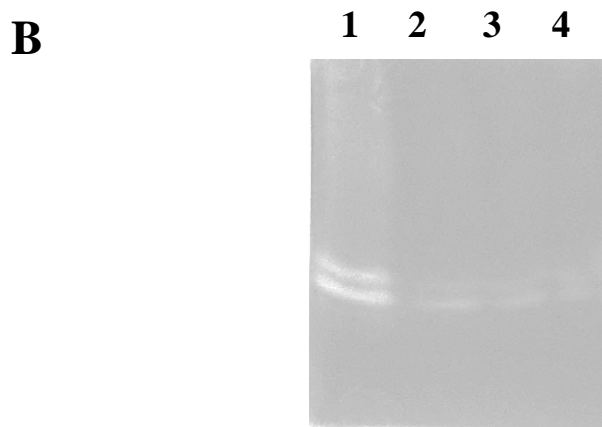
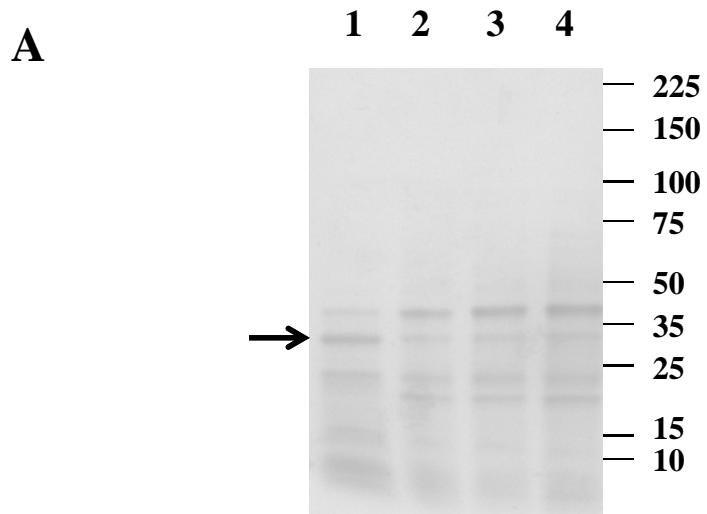
0.01



0.001







Context	Strain	Origin	Mortality on field	Contigs number	Genome size (Mb)	CDSs	Accession number
<b>Summer mortality</b>	01_032	Oyster, September 2001, Argenton, Brittany, France	yes	38	4.20	4180	PRJEB5902
	01_151	Oyster, July 2001, La Trinité, Brittany, France	yes	73	4.36	4339	PRJEB5903
	01_308	Oyster, August 2001, Normandy, France	yes	157	4.49	4533	PRJEB5904
	<b>02_041</b>	<b>Oyster, 2002, Argenton, Brittany, France</b>	<b>yes</b>	<b>8</b>	<b>4.20</b>	<b>4068</b>	<b>PRJEB5915</b>
<b>Recent outbreak</b>	12_016a	Oyster, March 2012, La Tremblade, Charente Maritime, France	yes	52	4.25	4246	PRJEB5906
	12_055	Oyster, June 2012, Agnas, Charente Maritime, France	yes	50	4.25	4237	PRJEB5907
	12_063	Oyster, September 2012, Brest, Brittany, France	yes	141	4.51	4524	PRJEB5908
	12_128a	Oyster, September 2012, Brittany, France	yes	65	4.24	4259	PRJEB5910
	12_130	Oyster, September 2012, Agnas, Charente Maritime, France	yes	80	4.29	4303	PRJEB5911
	12_142	Oyster, Octobre 2012, Normandy, France	yes	115	4.38	4401	PRJEB5912
<b>Other</b>	07_115	Oyster, 2007, Brittany, France	no	44	4.24	4243	PRJEB5905
	11_KB19	Oyster, March 2011, Fangar Bay, Spain	no	707	4.99	5277	PRJEB5913
	11_U17	Zooplankton, May 2011, Goro lagoon, Italy	no	732	4.41	4451	PRJEB5914
	12_122	Cockle, August 2012, Brittany, France	no	399	4.90	4988	PRJEB5909

**Table 1:** Strains used in the study

<b>Strain</b>	<b>Description</b>	<b>Reference</b>
P3813	B462 <i>DthyA::(erm-pir116)</i> [Erm <sup>R</sup> ]	Le Roux et al., 2007
b3914	b2163 <i>gyrA462, zei298::Tn10</i> [Km <sup>R</sup> Em <sup>R</sup> Tc <sup>R</sup> ]	Le Roux et al., 2007
GV1124	02_016 <i>D varS</i> clone 1	This study
GV1125	02_016 <i>D varS</i> clone 2	This study
GV1171	GV1124 + pMRB-P <sub>LAC</sub> <i>varS</i>	This study
GV1174	07_115 + pMRB-P <sub>LAC</sub> <i>varS</i>	This study
<b>Plasmid</b>	<b>Description</b>	<b>Reference</b>
pSW7848T	<i>oriV</i> <sub>R6Kq</sub> ; <i>oriT</i> <sub>RP4</sub> ; <i>araC</i> -P <sub>BAD</sub> <i>ccdB</i> ; [Cm <sup>R</sup> ]	Le Roux et al., 2007
pSWd <i>varS</i>	pSW7848T; <i>DvarS</i>	This study
pMRB-P <sub>LAC</sub> <i>varS</i>	<i>oriV</i> <sub>R6Kq</sub> ; <i>oriT</i> <sub>RP4</sub> ; <i>oriV</i> <sub>pB1067</sub> ; P <sub>LAC</sub> <i>varS</i> [Cm <sup>R</sup> ]	This study
<b>Primer</b>	<b>Sequence 5'-3'</b>	
<i>DvarS</i> -1	GTATCATAAGCTTATATCGAATTCGGGTAACGAGTGGCTATTGT	
<i>DvarS</i> -2	CCATATCCACACCACGATGTAGAATAGGTTGGAGTTGCGC	
<i>DvarS</i> -3	GCGCAACTCCAACCTATTCTACATCGTGGTGTGGATATGG	
<i>DvarS</i> -4	CCCCCGGGCTGCAGGAATTCGGTCAGATGTTGTAGATCGC	
pSW-F	GAATTCCTGCAGCCCCGGGGG	
pSW-R	GAATTCGATATCAAGCTTATCGATAC	
<i>varS</i> -F	GTGAGCGGATAACAAAGGAAGGGCCCATGACCAGATATGGCTTACGC	
<i>varS</i> -R	CGCGTCTGCAGCTCGAGCTAAACCAGATAAGGTTTTGCC	
pMRB-F	CTCGAGCTGCAGACGCGTCCG	
pMRB-R	GGGCCCTTCCTTTGTTATCCGCTCAC	
vector-F seq	GCATGTAGAGTCGGTGCAAG	
vector-R seq	CCAGGCTTTACACTTTATGC	
<i>varS</i> -1 seq	CGTCCATAACCAGCTTTCAAG	
<i>varS</i> -2 seq	GTTCTGACTCTTCAGGCATC	
<i>varS</i> -3 seq	CTGGTGGCTTGCAAATTCAC	
<i>varS</i> -4 seq	GTCGTGCAATAGTCGAAATG	

**Table 2:** Strains, plasmids and primers used in this study