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

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Abstract :

Oyster diseases caused by pathogenic vibrios pose a major challenge to the sustainability of oyster farming. In France, since 2012 a disease affecting specifically adult oysters has been associated with the presence of *Vibrio aestuarianus.* Here, by combining genome comparison, phylogenetic analyses and high-throughput infections of strains isolated before or during the recent outbreaks, we show that virulent strains cluster into two *V. aestuarianus* lineages independently of the sampling dates. The bacterial lethal dose was not different between strains isolated before or after 2012. Hence, the emergence of a new highly virulent clonal strain is unlikely. Each lineage comprises nearly identical strains, the majority of them being virulent, suggesting that within these phylogenetically coherent virulent lineages a few strains have lost their pathogenicity. Comparative genomics allowed the identification of a single frameshift in a non-virulent strain. This mutation affects the *varS* gene that codes for a signal transduction histidine-protein kinase. Genetic analyses confirmed that *varS* is necessary for infection of oysters and for a secreted metalloprotease expression. For the first time in a *Vibrio* species, we show here that VarS is a key factor of pathogenicity.

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50

INTRODUCTION

52

53 The development of aquaculture has been the source of anthropogenic changes on a massive
54 scale, characterized by displacements of aquatic animals from their natural habitats, farming
55 under high stocking density and exposition to environmental stresses. At the same time, over-
56 exploitation of some species and anthropogenic stress on aquatic ecosystems have placed
57 pressure on wild populations, providing opportunities for the emergence of an expanding
58 array of new diseases (Harvell et al., 1999). This can be illustrated by the outbreaks of
59 *Crassostrea gigas* oyster's diseases over the past decade threatening the long-term survival of
60 commercial and natural stocks (Renault, 2011).

61

62 A disease affecting oyster spat since 2008 has been linked to the presence of an oyster herpes
63 virus (OsHV-1 μvar) (Segarra et al., 2010) and to vibrio populations related to the *Splendidus*
64 clade (Lemire et al, *in press*). In addition, over the last four years, the number of reported
65 cases of adult mortalities associated with the presence of *Vibrio aestuarianus* has increased
66 considerably (Garnier et al., 2007; Vezzulli et al., 2014). Interestingly, during the 2008-2012
67 period, this bacterial species had been rarely isolated from moribund oysters, suggesting the
68 possible (re)-emergence of *V. aestuarianus* as an oyster pathogen.

69

70 Emergent infectious diseases can arise from genomic modifications of an infectious agent
71 (Keim and Wagner, 2009). Such genomic alterations frequently result in the optimization of
72 virulence genes, the acquisition of resistance cassettes, changes in prevalence, and/or
73 adaptation to a new host. Thus, understanding an emerging disease requires investigating the
74 pathogen and its evolution at the gene and genome level. If the recent emergence of *V.*

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75 *aestuarianus*-caused diseases is due to a new virulent clonal strain, one can expect differences
76 in the lethal doses between strains isolated during the recent outbreaks (heightened virulence)
77 and the ones sampled a decade ago. Furthermore, one can hope that sequencing closely
78 related isolates with contrasting virulence status and performing whole genome comparative
79 analyses would lead to the identification of genomic modification(s) correlating with
80 increased virulence.
81
82 Indeed, whole genome comparative analyses combined with mutagenesis of candidate genes
83 have been successfully used to identify virulence determinants of vibrio pathogenic to oysters
84 such as *V. crassostreae* (Lemire et al., *in press*) or shrimp such as *V. nigripulchritudo*
85 (Goudenege et al., 2013). However such a strategy requires the sequencing of several closely
86 related strains with contrasting virulence and is still limited to genetically tractable strains. To
87 date attempts to perform reverse genetics in *V. aestuarianus* (strain 01_032) have proven
88 unsuccessful (Labreuche et al., 2010) limiting the investigation of virulence mechanisms in
89 this species.
90
91 In the present study, we explored the virulence potential and genome diversity of *V.*
92 *aestuarianus* isolates. We asked whether the recent adult mortality outbreaks are due to the
93 emergence of a specific genotype. To address this question, we performed a high throughput
94 sequencing (HTS)-based comparative genome analysis of 14 *V. aestuarianus* strains isolated
95 before or during the recent outbreaks along with bacterial lethal dose determination by
96 experimental challenges. We then took advantage of the near identity of some strains with
97 contrasting virulence properties to identify key factor(s) of *V. aestuarianus* pathogenicity by
98 comparative and functional genomics.
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101

102 **RESULTS AND DISCUSSION**

103

104 ***V. aestuarianus* comprises virulent and non-virulent strains**

105 To explore the virulence potential and genome diversity of *V. aestuarianus*, strains isolated
106 from diseased oysters before (four strains, named 01_xxx to 07_xxx, the first two numbers
107 corresponding to the year of isolation, *i.e.* 01 for 2001) and during the recent mortality
108 outbreaks (six strains, named 12_xxx) were selected (Table 1). In addition, four strains
109 isolated from healthy oysters, cockles or zooplankton and not linked to mortality events were
110 added to our analysis (Table 1).

111

112 The strains were first injected into specific-pathogen-free (SPF) standardized oysters (Petton
113 et al., 2013) at 10^7 CFU/animal, a bacterial concentration previously used in experimental
114 infections (Garnier et al., 2007). At 6 days post injection, 10/14 strains caused mortality rates
115 >80% (Fig. 1, black bars). We subsequently injected lower bacterial concentrations to oysters
116 (ranging from 10^6 to 10^2 CFU/animal). Surprisingly, when injected at 10^2 CFU /animal, the
117 strain 02_041 isolated in 2002 and six strains isolated in 2012 were still able to cause >80%
118 mortality (Fig.1, grey bars).

119

120 These results allowed us to classify seven strains (12_063, 12_128a, 12_130, 12_142,
121 12_055, 12_016a and 02_041) as highly virulent (Vir+) (*i.e.* inducing >50% mortalities at 10^2
122 CFU/animal), and four strains (12_122, 11_U17, 11_KB19 and 01_151) as non-virulent (Vir-
123) (*i.e.* inducing <50% mortalities at 10^7 CFU/animal). Three strains (07_115, 01_308, 01_032)
124 were defined as intermediate (*i.e.* pathogenic only at 10^7 CFU/animal).

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125

126 General features of the *V. aestuarianus* genomes

127 The genome of strain 02_041 was assembled to near completion (8 contigs in total) and
128 manually annotated. It consists of two circular chromosomes of 2.98 (chromosome 1; 4
129 contigs) and 1.21 Mb (chromosome 2, 4 contigs) with an average GC content of 43.11 and
130 42.16% respectively (Table 1; Fig.S1). Chromosomes 1 and 2 contain 7 and 0 rRNA operons,
131 74 and 10 tRNA genes, respectively. However because the genome is not fully assembled,
132 some rRNA and tRNA genes may have been missed.

133

134 The genome sequences of the 13 other strains were partially assembled, with contig numbers
135 per strain ranging from 38 to 732 and approximate genome sizes ranging from 4.2 to 4.99 Mb
136 compared to 4.19 Mb for strain 02_041 (Table 1). The difficulty to achieve a better genome
137 assembly may be attributed to i) a high number of transposition elements (184 transposase
138 genes in the strain 02_041); ii) the large size of the chromosomal integron (Mazel et al., 1998)
139 (94 cassettes in the chromosome 2 of strain 02_041) (Fig. S1).

140

141 Genes differentiating *V. aestuarianus* from other *Vibrionaceae*

142 A phylogenetic analysis based on concatenated nucleic acid sequences derived from 50 shared
143 genes from 223 *Vibrionaceae* genome sequences including 14 *V. aestuarianus* strains and
144 using *Shewanella baltica* as an outgroup demonstrated the cohesive genotypic structure of *V.*
145 *aestuarianus* with relatively little diversity among genomes (Fig. S2). The clade *V.*
146 *aestuarianus* is sister to a clade that contains two species previously associated with farmed
147 fish diseases, *V. ordalii* and *V. anguillarum* (Austin, 2011). Our analyses confirmed that *V.*
148 *aestuarianus*, *V. ordalii* and *V. anguillarum* are grouped in the *Anguillarum* clade (Sawabe et
149 al., 2013).

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150

151 Intraspecific genomic comparisons identified 2866 genes that are shared by all sequenced *V.*
152 *aestuarianus* strains (Fig. S1) of which only 40 genes were found in ≤5 other *Vibrionaceae*
153 genomes (Table S1). Among these *V. aestuarianus*-specific genes, we identified a cluster of
154 genes homologous to the Toxin co-regulated (Tcp) pilus biosynthesis cluster encoded by a
155 pathogenicity island in *V. cholerae* that is necessary for intestine colonization (Davis and
156 Waldor, 2003). However in the strain 02_041, the *tcp* gene cluster is interrupted by a
157 transposon, and genes encoding the accessory colonization factors (*acf*) are absent (Fig.S3)
158 suggesting that this *tcp* like cluster may play a distinct role, if any, in *V. aestuarianus*.
159

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

161 The phylogenetic relationships based on the core genome of the *V. aestuarianus* strains
162 included in this study were investigated (Fig.2). The main outcome of this analysis was the
163 grouping of 6/7 Vir+ isolates into a clade A, which also contains one Vir-, and two
164 intermediate strains. Clade A is a sister of Clade B containing one Vir+ and one intermediate
165 strain. Both clades A and B show very little intra-clade diversity (>99 % average nucleotide
166 identity – ANI– value) (Konstantinidis and Tiedje, 2005). Inter-clade diversity was also low
167 as determined by the ANI value calculation (>98.4 %) and by the number of clade-specific
168 genes (≃180 genes, essentially in a clade B-specific phage). Vir- strains isolated from oysters
169 in Spain, zooplankton in Italy or cockles in Brittany were found to be more diverse.
170

171 As a consequence of the low inter-clade diversity, the genes commonly used for multilocus
172 sequence analysis (*hsp60, pyrH, atpA, gyrB, recA, topA*) did not allow the separation of
173 clades A and B with a high bootstrap value. Thus, we compared the phylogenetic
174 relationships of each core gene (2866 trees) and identified 55 genes allowing the placement of

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175 isolates in clade A or B with a high bootstrap value. Among them, a gene encoding a putative
176 D-lactate dehydrogenase (VIBAEv3_A30718) was selected to explore the genetic structure of
177 *V. aestuarianus* using a larger collection of strains (n=116) isolated from diseased animals
178 (Table S2). Phylogenetic analyses reveal that 87/116 (75%) and 29/116 (25%) of these strains
179 belong to clade A and B, respectively (Fig.3). When injected intramuscularly to oysters at 10^2
180 CFU /animal, 81/87 (93%) and 23/29 (79%) strains from respectively clade A and B were
181 classified as Vir+ (Fig.3). The remaining strains (indicated with an asterisk in Fig.3) were
182 defined as intermediate (i.e. inducing >50% mortalities when injected at 10^7 CFU/animal,
183 M.A. Travers, pers.com.). The dominance of clade A and Vir+ strains (belonging to either
184 clade A or B) was observed during the summer mortality events and the more recent
185 outbreaks, whatever the age of the diseased oysters (> or <12months). Altogether these data
186 demonstrate that strains belonging to *V. aestuarianus* and isolated from diseased oysters can
187 be grouped into two lineages containing a majority of Vir+ strains. However, since we did not
188 observe any correlation between *V. aestuarianus* lethal dose, genotype and isolation date, the
189 hypothesis of the emergence of a new virulent clonal strain is unlikely.
190
191 An alternative hypothesis is that physiological alteration(s) of the oysters leading to an
192 increased sensitivity to *V. aestuarianus* may explain the increased number of reported cases of
193 mortalities. Such physiological disorders may result from environmental factors (acquired
194 sensitivity), genetic trade-offs (innate sensitivity) or a combination of both. Experimental
195 infections using wild stock of “naive” oysters that have never experienced the spat disease or
196 selected lineages resistant to one/several infectious agents may help in testing this hypothesis.
197 Finally the identification of habitat(s) and a spatio-temporal survey of *V. aestuarianus* should
198 help in understanding the ecological parameters that modulate virulence, persistence and/or
199 prevalence of this pathogen.

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201 **Non-virulent strains have undergone genetic modification(s)**

202 Phylogenetic analysis of whole genomes revealed that virulent strains are grouped into two *V.*
203 *aestuarianus* lineages, containing nearly identical strains. As each lineage contains a majority
204 of highly virulent strains, we hypothesized that their common ancestor was virulent, and that a
205 few modern strains might have undergone genetic modification(s) leading to loss of
206 pathogenicity. We therefore performed comparative genomic analyses to identify these
207 genetic modification(s).

208

209 In clade B, 49 genes localized in seven genomic regions were present in the Vir+ strain
210 12_063 but not in the intermediate strain 01_308 (Table S3). These regions encode common
211 phage-related proteins (*e.g.,* integrase, helicase, relaxase and restriction endonuclease system)
212 as well as other proteins of unknown function. However, none of these genes were found in
213 the Vir+ strains from clade A. Finally, a frameshift was observed in 13 genes of strain
214 01_308, the majority of them coding for proteins of unknown function. However it should be
215 noted that comparative genomic analyses within this clade B were hampered by the small
216 number of sequenced strains (one Vir+ and one intermediate) and by genome fragmentation.

217

218 In clade A, we could not identify any genes specific of the Vir+ strains. However, in the Vir-
219 strain 01_151, we detected a frameshift in three genes encoding respectively an exported
220 protein of unknown function (VIBAEv3_A31414 in strain 02_041), a putative
221 acetyltransferase (VIBAEv3_A10934) and a membrane protein of unknown function
222 (VIBAEv3_A20116). Interestingly, a single frameshift was identified in the intermediate
223 strain 07_115 in a gene that codes for a signal transduction histidine-protein kinase (VarS)
224 (Lenz et al., 2005). The *varS* gene was found intact in the 13 others strains. The *varS* gene

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225 (VIBAEv3_A30043 in strain 02_041) codes for a protein of 925 amino acids (aa) and
226 contains six domains (Fig.4A): an uncharacterized signal transduction histidine kinase domain
227 (DUF2222), a cytoplasmic helical linker and methyl-accepting protein domain (HAMP), a
228 phosphoacceptor domain (HisKA), an ATPase domain (HATPase_c), a response regulator
229 receiver domain (response reg) and a histidine-containing phosphotransfer domain (HPt). In
230 the strain 07_115 the deletion of one nucleotide results in a stop codon, generating a 677 aa
231 protein that lacks the response reg and HPt domains (Fig. 4A).
232
233 **Disruption of *varS* is sufficient to alter *V. aestuarianus* pathogenicity**
234 The sensory system VarS/VarA (VarS being the sensor histidine-kinase and VarA the
235 response regulator) has been implicated in the pathogenicity of a variety of Gram-negative
236 bacteria, including among others, *Escherichia coli* (BarA/UvrY), *Salmonella typhimurium*
237 (BarA/SirA), and *Pseudomonas aeruginosa* (GacS/GacA) (Chavez et al., 2010; Gooderham
238 and Hancock, 2009; Jones, 2005; Timmermans and Van Melderen, 2010). Hence, we assessed
239 the importance of *varS* for *V. aestuarianus* virulence using a previously described genetic
240 approach relying on a suicide vector, which can be transferred by conjugation to potentially
241 any *Vibrio* strain (Le Roux et al., 2007). However, a dramatic difference in DNA delivery (10^{-}
242 ^{4} to 10^{-6} transconjuguant per recipient cells) and allelic exchange efficiency (0 to 10^{-8}
243 integration per recipients) was observed between nearly clonal strains. These data highlight
244 the limitations of genetic methods when working with environmental non-model strains.
245 Limitations can occur at several levels from the DNA delivery inside the cells to the allelic
246 exchange efficiency and the availability of selective genes.
247
248 We obtained a successful integration of the suicide plasmid by a single crossover in only one
249 out of seven virulent strains (12_016a), showing intra-specific variation. After the second

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250 recombination event leading to plasmid excision, 30% of the colonies carried the *varS*
251 deletion (strains 12_016a_Δ*varS*). For two isolates selected randomly, this deletion did not
252 impair bacterial growth in culture media, but resulted in a dramatic decrease in mortality rates
253 induced after bacteria injection in oysters (Fig. 4B, lanes 3 and 4 compared to lane 1). Upon
254 constitutive expression of *varS in trans* from a replicative plasmid, the virulence of the mutant
255 12_016a_Δ*varS* was partially restored (Fig. 4B, lane 5 compared to 3) and this of the
256 intermediate strain 07_115 was increased (Fig. 4B, lane 6 compared to 2). These
257 complementation experiments confirmed that *varS* is necessary to 12_016a pathogenicity and
258 that the frameshift in *varS* is also involved in the 07_115 virulence attenuation.
259
260 **The metalloprotease Vam production and/or secretion is regulated by VarS**
261 The two-component regulatory system VarA/S has been involved in the regulation of the
262 secreted hemagglutinin/metalloprotease gene *hapA* in *V. cholerae* (Jang et al., 2010). Here,
263 the protease activity measured in the extracellular products (ECPs) of the 12_016a_Δ*varS*
264 mutants (Fig. 5B, lanes 2 and 3) was found to be 3 times lower than that of the wild type
265 virulent strain 12_016a (Fig. 5B, lane 1) and in the range of the intermediate wild-type strain
266 07_115 (Fig. 5B, lane 4). The SDS-PAGE protein profiles of the ECPs prepared from 07_115
267 and two independent clones of 12_016a_Δ*varS* were found to be very similar and
268 significantly different from this of 12_016a (Fig.5A). A band (25-35 kDa) found more intense
269 in 12_016a (Fig.5A, lane 1) was excised from the gel, analyzed by µLC-ESI MS/MS and
270 demonstrated to correspond to a peptide derived from the Vam metalloprotease
271 (VIBAEv3_B10595 in strain 02_041) an homologue of HA/P, the *hapA* gene product.
272
273 The Vam metalloprotease of the *V. aestuarianus* strain 01_032 has been previously
274 demonstrated to be lethal to *C. gigas* oysters (Labreuche et al., 2010). The expression of this

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275 gene by a non-toxicogenic *Vibrio* strain (*V. tasmaniensis* LMG20012^{T}) induced the same
276 immunosuppressant effects on hemocytes as those observed for *V. aestuarianus* ECPs
277 showing that this protein is sufficient to induce immunosuppression in oysters (Labreuche et
278 al., 2006). However the formal demonstration of the predicted, or supposed, role of a
279 candidate gene requires a gene deletion strategy. Several attempts to generate a Δ*vam* mutant
280 were unsuccessful (100% wild type reversion after the second recombination event),
281 preventing the drawing of a definitive conclusion about the direct role of Vam in virulence.
282 This suggests that the presence of this gene is essential in this strain in our culture conditions.
283 It is important to note that the lack of a second usable resistance marker prevented the
284 demonstration that a *vam* mutant could be constructed when the gene was provided *in trans*.
285 We are currently exploring a larger panel of antibiotic resistance genes to allow the
286 development of such strategy in the future.
287
288 Our results show that VarS is a key regulator of *V. aestuarianus* virulence and Vam secretion
289 and/or activity and/or production. Several studies have examined the contribution to virulence
290 of various *Vibrio* metalloproteases in animal experimental models (Finkelstein et al., 1992;
291 Jeong et al., 2000; Le Roux et al., 2007; Milton et al., 1992; Shao and Hor, 2000) but no
292 conclusive evidence about the role of proteases in virulence was found, since mutants
293 deficient in secreted proteases showed comparable virulence levels to their parental strains.
294 There are only a few examples of toxins (such as diphtheria or tetanus), which act as single
295 determinants to produce disease. Microbial pathogenesis is often multifactorial, and
296 pathogens use several biochemical mechanisms operating in concert to produce infections and
297 diseases (Finlay and Falkow, 1997). For instance, the HA/P metalloprotease from *V. cholerae*
298 was reported to activate proteolytically both the El Tor cytolysin/haemolysin (Nagamune et
299 al., 1996) and the cholera toxin CT, an ADP-ribosylating enterotoxin inducing a highly

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300 secretory diarrhea (Booth et al., 1984). Research is now ongoing to identify other genes that
301 are regulated by VarS (at the transcriptional and post-transcriptional levels) and the protein
302 targets that are processed by Vam in the ECP fraction.
303
304 Finally, due to the near identity of strains within clades A and B and existence of ~10% of
305 Vir- isolates, *V. aestuarianus* appears as a great model to investigate by comparative genomic
306 the genetic modification(s) leading to loss of pathogenicity and identify new virulence
307 candidate genes and regulators. In the future, each of these genes will be deleted to investigate
308 their potential respective role in virulence.
309

310 **MATERIALS AND METHODS**

311
312 **Strains and culture conditions.** The strains used for genomic analyses are described in Table
313 1. Other bacterial strains are described in Table S2 and S4. *Vibrio* isolates were grown in
314 Zobell or Zobell agar, Luria-Bertani (LB) or LB-agar (LBA) + NaCl 0.5M, at 20°C.
315 *Escherichia coli* strains were grown in LB or on LBA at 37°C. Chloramphenicol (5 to
316 25µg/ml), thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements
317 when necessary. Induction of the P_{BAD} promoter was achieved by the addition of 0.2% L-
318 arabinose to the growth medium, and conversely, repression was obtained by the addition of
319 1% D-glucose.
320
321 **Genome sequencing, assembly and annotation.** The complete genome sequence of 02_041
322 strain was obtained using two sequencing technologies: 1) A Sanger library was constructed
323 after mechanical shearing of DNA and cloning of 10 kpb fragments into pCNS (pSU18
324 derived). Plasmids were purified and end-sequenced using a dye-terminator chemistry on

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325 ABI3730 sequencers leading to a 4-fold coverage. 2) A 454 single read library was
326 constructed and sequenced to a 16-fold coverage. The reads obtained using the two
327 technologies were assembled using Newbler (www.roche.com). Then, primer walks, PCRs
328 and transposon bombs were performed to finish the sequence of the *V. aestuarianus* reference
329 genome. The 13 other *V. aestuarianus* strains were sequenced using the Illumina HiSeq2000
330 technology with a ~50-fold coverage. Contigs were assembled *de novo* using Velvet (Zerbino
331 and Birney, 2008) and genome assembly was improved by contig mapping against the 02_041
332 reference genome. Computational prediction of coding sequences and other genome features
333 (RNA encoding genes, ribosome binding sites, signal sequences, etc...), together with
334 functional assignments were performed using the automated annotation pipeline implemented
335 in the MicroScope platform (Vallenet et al., 2013). An extensive manual curation of the
336 genes, which includes correction of the start codon positions and of the functional
337 assignments, was performed. This expert procedure was supported by functional analysis
338 results [e.g., InterPro, FigFam, PRIAM, COGs (Clusters of Orthologous Groups), PsortB]
339 which can be queried using an exploration interface, and by synteny group computation
340 visualized by cartographic maps to facilitate genome comparison.
341
342 ***In silico* analyses.** To investigate the core and flexible genomes, an all-versus-all BlastP
343 search was performed using genomic sequences of 209 *Vibrionaceae* and *Shewanella baltica*
344 (strain OS155) available in Genbank and 14 *V. aestuarianus* sequenced in the present study
345 (Table 1). A dedicated precomputing repository (marshalling) was created to perform
346 comparative genomic and phylogenomic analyses. Orthologous proteins were defined as
347 reciprocal best hit proteins with 80% MaxLrap and a minimum of 30% and 60% identity
348 cutoff was used for intra- and inter-species analysis, respectively (Daubin et al., 2002). The
349 nucleic acid sequences were aligned using Muscle (Edgar, 2004) and filtered by BMGE

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350 (Criscuolo and Gribaldo, 2010). Phylogenetic trees were built using the parallel version of
351 PhyML applied to Maximum-likelihood algorithm and GTR model as parameters (Guindon et
352 al., 2010). Reliability was assessed by the bootstrap method with 100 replicates.
353
354 **Vector construction.** Cloning was performed using the Gibson assembly method according
355 to the manufacturer's instructions (New England Biolabs, NEB). For the *varS* deletion, two
356 independent PCR amplifications of the regions (500 bp) encompassing the *varS* gene were
357 performed using two primer pairs (Δ*varS*-1+2 and Δ*varS*-3+4) (Table S4). An inside out PCR
358 was performed using pSW7848T suicide vector DNA (Val et al., 2012) and primer pair (*pSW-*
359 *F* and *pSW-R*) (Table S4). For the cloning of *varS* gene under a P_{LAC} promoter in a pMRB
360 plasmid, a replicative plasmid found to be stable in *Vibrionaceae* (Le Roux et al., 2011), two
361 independent PCR amplifications of the gene and plasmid were performed using the primers
362 *varS-F*+R and *pMRB-F*+*R* reciprocally. After purification and quantification, 100 ng of each
363 PCR product were mixed with the Gibson assembly Master Mix and incubated for 60 minutes
364 at 50°C. Samples were diluted at 1/3 before *E. coli* transformation by the reaction product.
365 Clones were controlled by digestion with restriction enzyme and sequencing using the primers
366 described in Table S4. Strains Π3813 and β3914 were used as a plasmid host for cloning and
367 conjugation, respectively (Le Roux et al., 2007). Plasmids and strains used and established in
368 the present study are presented in Table S4.
369
370 **Conjugation**
371 Overnight cultures of donor and recipient were diluted at 1:100 in culture media without
372 antibiotic and grown at 30°C to an OD_{600nm} of 0.3. The different conjugation experiments
373 were done by a filter mating procedure described previously (Le Roux et al., 2007) with a
374 donor/recipient ratio of 1ml/10ml. Conjugations were performed overnight on filters

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375 incubated on LBA + NaCl 0.5N + diaminopimelic acid (DAP) plates at 30°C. Selection of
376 exconjugants and counter-selection of the Δ*dapA* donor was done by plating on a medium
377 devoid of DAP, supplemented with chloramphenicol and 1% glucose. Cm^R resistant colonies
378 were grown in LB + NaCl 0.5N up to late logarithmic phase and spread on plates containing
379 0.2% arabinose. Mutants were screened by PCR using primers Δ*varS*-1+4 (Table S4).
380

381 **Extracellular products analyses.** Bacterial extracellular products (ECPs) were produced by
382 the cellophane overlay method as described previously (Le Roux et al., 2007). The protein
383 concentration of the ECPs was measured by the method of Bradford with bovine serum
384 albumin as the standard and normalized (BioRad). Protease activity was measured by the
385 azocasein procedure as described previously (Miyoshi et al., 1987).
386 After concentration by ultrafiltration (Centricon® 10 Kda), twenty micrograms of crude ECPs
387 were analyzed on a 4-15% Mini-PROTEAN® TGX Precast Gels. The differentially expressed
388 protein band was manually excised from the gel, in-gel digested using trypsin and subjected
389 to MS and MS/MS analyses for protein identification, following previously described
390 protocols (Bernay et al., 2006).
391

392 **Production of "pathogen free" oysters**
393 Oysters (18 to 36 months; n=40) collected in the Fouras Bay (Marennes- Oléron, France)
394 were transferred to the Ifremer facility located at Argenton (Brittany, France) for maturation
395 conditioning as described previously (Petton et al., 2013). After gamete stripping and
396 fertilization, obtained larvae, then spat, were reared under controlled conditions up to 12-13
397 months. PCR detection of oyster herpes virus was performed to confirm the negative status of
398 oysters (Petton et al., 2013). *Vibrio* isolation on selective culture medium (Thiosulfate-citrate-
399 bile salts-sucrose agar) confirmed a low *Vibrio* prevalence (~10 CFU/gr tissues).

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400 **Virulence studies using oysters.** Bacteria were grown under constant agitation at 20°C for
401 24 h in Zobell. One hundred microliters of the diluted culture (10^7 to 10^2 CFU) were injected
402 intramuscularly to anaesthetize SPF oysters (12-13 months old, 1.5 g, s.d. 0.2). The bacterial
403 concentration was confirmed by conventional dilution plating on Zobell agar. After injection,
404 the oysters were transferred to aquaria (10 oysters per aquarium) containing 2.5 liter of
405 aerated 5 µm-filtered and UV-treated seawater at 20°C and kept under static conditions for 6
406 days. Each bacterial treatment was performed in duplicates and mortality was recorded daily.
407
408
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422
423 **Author contributions**
424 DG, MAT and AL contributed equally to this work.
425

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

Figure 2: Phylogenetic analysis based on concatenated alignments of nucleic acid sequences of 2866 core genes from 14 *V. aestuarianus* strains and KB19 as an outgroup. The tree was built by the Maximum-Likelihood method based on a sequence alignment generated by Muscle. Branch lengths are drawn to scale and are proportional to the number of nucleotide changes. Numbers at each node represent the percentage value given by bootstrap analysis of 100 replicates. The pathotype of each *V. aestuarianus* strain (Vir+: virulent; Vir-: non virulent; int: intermediate) is indicated in parentheses.

Figure 3: *V. aestuarianus* isolates phylogeny analysis of partial D-lactate dehydrogenase gene sequences and virulence status. The tree was built by the Maximum-Likelihood method based on a sequence alignment generated by Muscle. Branch lengths are drawn to scale and are proportional to the number of nucleotide changes. Number at each node represents the percentage value given by bootstrap analysis of 100 replicates. The black bars indicate the % of mortalities occurring at 6 days post-injection (10^2 CFU/animal). The strains indicated with

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565 an asterisk were defined as intermediate, *i.e.* inducing >50% mortalities when injected at 10^7

566 CFU/animal.

567

568 **Figure 4:** Role of *varS* in *Vibrio aestuarianus* pathogenicity**. A-** Schematic representation of

569 the functional domains of VarS identified in the Vir+ strain 02_041 and the truncated protein

570 resulting from a frameshift in the intermediate strain 07_115. DUF2222 corresponds to an

571 uncharacterized signal transduction histidine kinase domain; HAMP, a cytoplasmic helical

572 linker and methyl-accepting protein domain; HisKA, a phosphoacceptor domain; HATPase_c,

573 an ATPase domain; Response reg, a response regulator receiver domain; HPt, an histidine-

574 containing phosphotransfer domain. **B-** Experimental infection of wild type *V. aestuarianus,*

575 Δ*varS* mutants and complemented Δ*varS* mutants. 10^2 CFU of the tested strains (lane 1: strain

576 12_016a wild type; lane 2: strain 07_115 wild type; lanes 3 and 4: GV1124 and 1125, two

577 independent clones of 12_016a_ Δ*varS*; lane 5: GV1124 i.e. 12_016a_ Δ*varS* carrying an

578 expression vector for *varS*, pMRB-P_{LAC}*varS*; lane 6: 07_115 carrying pMRB-P_{LAC}*varS)* was

579 intramuscularly injected into oysters (n= 20, in duplicate). Mortality (%) was assessed after 6

580 days.

581

582 **Figure 5:** Role of *varS* in *Vibrio aestuarianus* metalloprotease expression**. A-** Extracellular

583 product analysis by Coomassie blue stained 10% SDS-PAGE gel (lane 1: strain 12_016a wild

584 type; lane 2 and 3: GV1124 and 1125, two distinct clones of 12_016a_ Δ*varS*; lane 4: 07_115

585 wild type). Arrow indicates the Vam metalloprotease identified by MS/MS. **B-** Proteolytic

586 activities of ECPs was determined by an azocasein assay as described in Materials and

587 Methods (absorbance at 440 nm).

588

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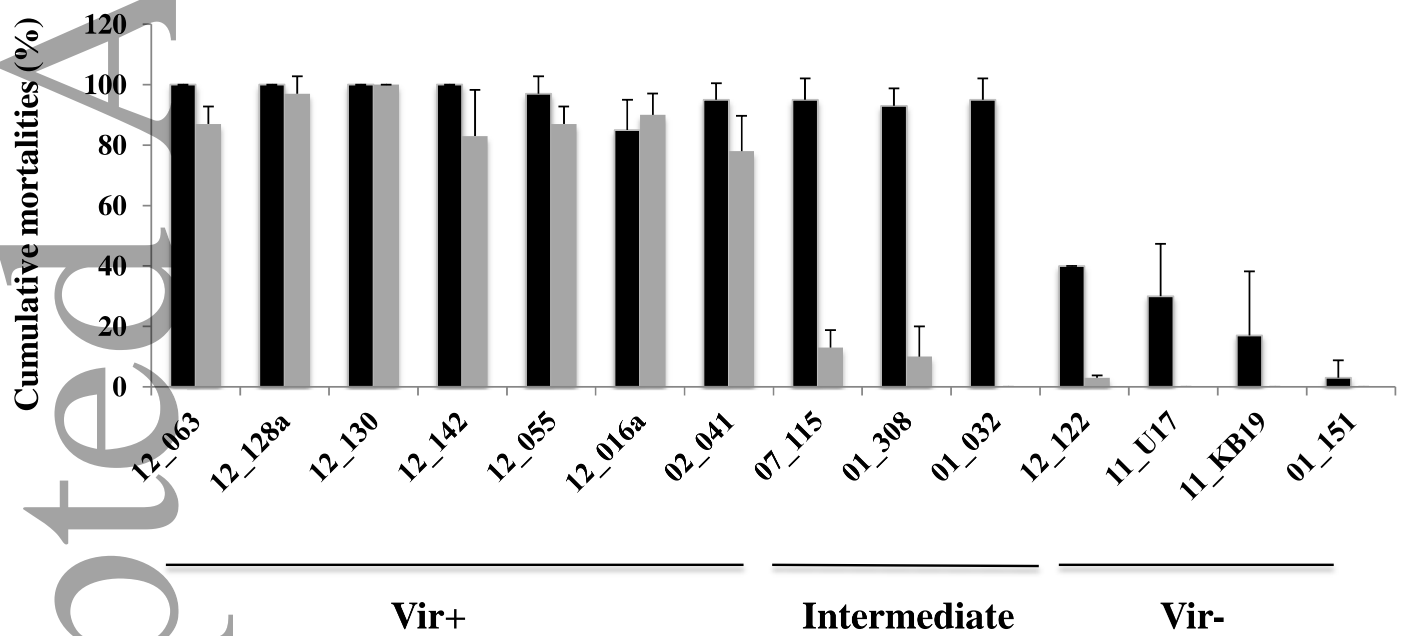

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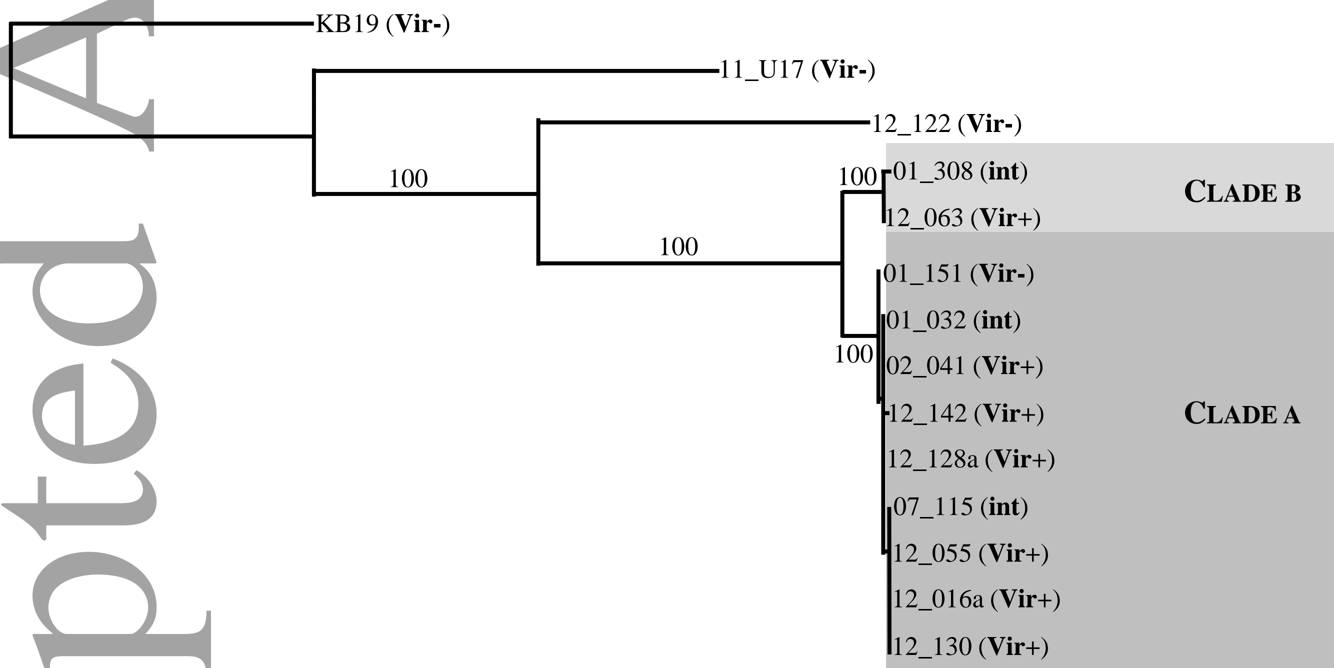

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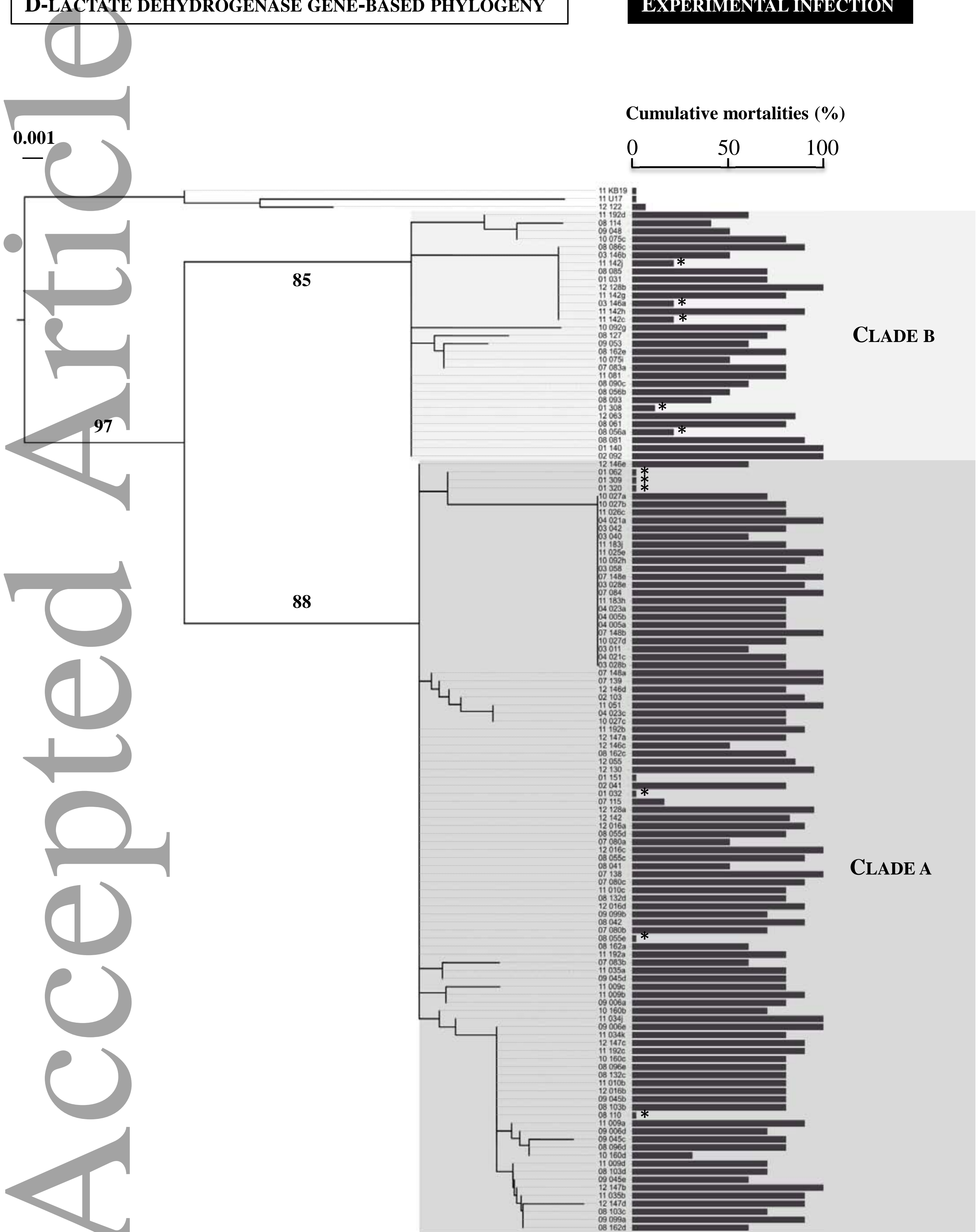

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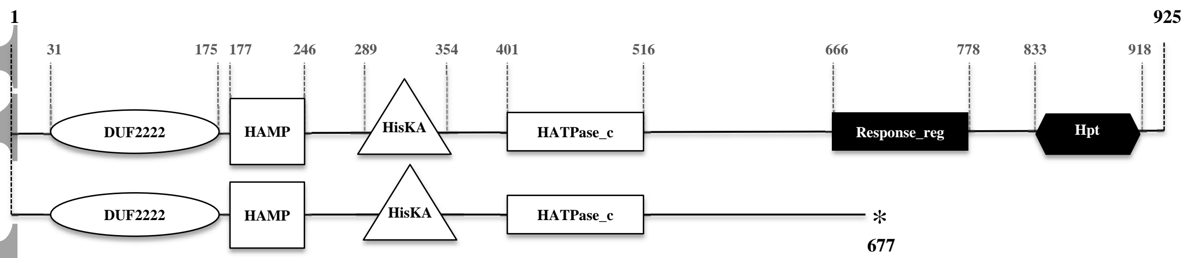

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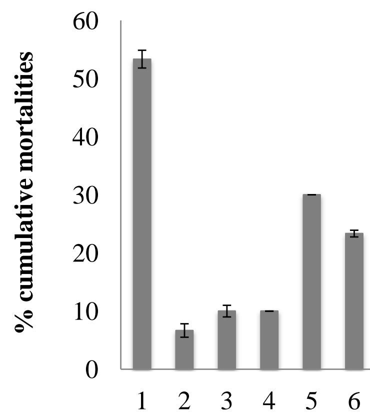

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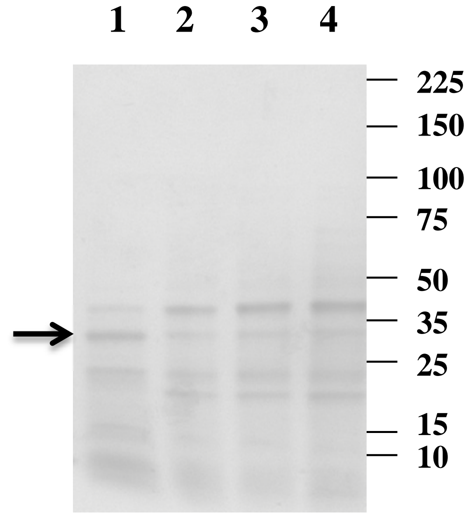

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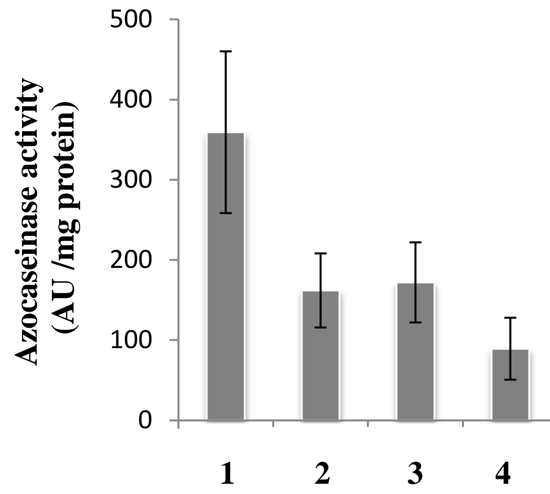

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Context	Strain	Origin	Mortality on field	Contigs number	Genome size (Mb)	Cl
Mortality outbreaks 2001-02	01_032	Oyster, September 2001, Argenton, Brittany, France	yes	38	4.20	41
	01_151	Oyster, July 2001, La Trinité, Brittany, France	yes	73	4.36	43
	01_308	Oyster, August 2001, Normandy, France	yes	157	4.49	45
	02_041	***Oyster, 2002, Argenton, Brittany, France***	***yes***	***8***	***4.20***	***40***
Mortality outbreaks 2012	12_016a	Oyster, March 2012, La Tremblade, Charente Maritime, France	yes	52	4.25	42
	12_055	Oyster, June 2012, Agnas, Charente Maritime, France	yes	50	4.25	42
	12_063	Oyster, September 2012, Brest, Brittany, France	yes	141	4.51	45
	12_128a	Oyster, September 2012, Brittany, France	yes	65	4.24	42
	12_130	Oyster, September 2012, Agnas, Charente Maritime, France	yes	80	4.29	43
	12_142	Oyster, Octobre 2012, Normandy, France	yes	115	4.38	44
Other	07_115	Oyster, 2007, Brittany, France	no	44	4.24	42
	11_KB19	Oyster, March 2011, Fangar Bay, Spain	no	707	4.99	52
	11_U17	Zooplankton, May 2011, Goro lagoon, Italy	no	732	4.41	44
	12_122	Cockle, August 2012, Brittany, France	no	399	4.90	49

Table 1: Strains used in the study

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