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

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**Word count for the abstract :** 232

**Word count for the text :** 4677

**ABSTRACT**

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

## IMPORTANCE

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

## INTRODUCTION

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

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

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

94 mortalities were linked to the emergence of a new genotype of OsHV-1 (µvar) (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 µvar (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

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

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

## RESULTS

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

To explore the virulence potential and genome diversity of *V. aestuarianus* strains, 4 and 6 strains isolated from diseased oysters during "summer mortality" events and the recent 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 ≤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 $10^2$
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+ 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_*ΔvarS*). 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_*ΔvarS* 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).

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

The two-component regulatory system VarA/S (VarS being the sensor histidine-kinase and VarA the response regulator) has been involved in the regulation of the secreted hemagglutinin/metalloprotease expression HapA in *V. cholerae* (26). Here, the protease activity measured in the ECPs of the 12_016a_*ΔvarS* mutants (Fig. 5B and C, lanes 2 and 3) was found to be 3 times lower than that of the wild type virulent strain 12_016a (Fig. 5B and C, lane 1) and in the range of the intermediate wild-type strain 07_115 (Fig. 5B and C, lane 4). The SDS-PAGE protein profiles of the ECPs prepared from 07_115 and two clones of 12_016a_*ΔvarS* were found to be very similar and significantly different from this of 12_016a (Fig.5A). A band found more intense in 12_016a (Fig.5A, lane 1) was excised from the gel, analyzed by µLC-ESI MS/MS and demonstrated to correspond to the Vam metalloprotease, previously reported to be involved in *V. aestuarianus* toxicity (27). Unfortunately, several attempts to generate a *Δvam* mutant were unsuccessful (100% wild type reversion after the second recombination), preventing a definitive conclusion about the direct role of Vam in virulence. In conclusion, we have demonstrated that VarS is a key regulator of *V. aestuarianus* pathogenicity, secreted proteolytic activity and extracellular Vam production.

## DISCUSSION

The rise of aquaculture has been the source of anthropogenic changes on a massive scale, characterized by displacements of aquatic animals from their natural habitats, farming under 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

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

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

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

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

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

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

## MATERIALS AND METHODS

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

**Genome sequencing, assembly and annotation.** The complete genome sequence of 02_041 strain was obtained using two sequencing technologies: 1) A Sanger library was constructed after mechanical shearing of DNA and cloning of 10 kpb fragments into pCNS (pSU18 derived). Plasmids were purified and end-sequenced using dye-terminator chemistry on ABI3730 sequencers leading to a 4-fold coverage. 2) A 454 single read library was constructed and sequenced to a 16-fold coverage. The reads obtained using the two technologies were assembled using Newbler (www.roche.com). Then, primer walks, PCRs and transposon bombs were performed to finish the sequence of the *V. aesturianus* reference 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 (*ΔvarS*-1+2 and *ΔvarS*-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 $P_{LAC}$ 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 Π3813 and β3914 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 $OD_{600nm}$ 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 Δ*dapA* donor was
438 done by plating on a medium devoid of DAP, supplemented with chloramphenicol and 1%
439 glucose. $Cm^R$ 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 *ΔvarS*-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 tissus).
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

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

**ACKNOWLEDGEMENTS**

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

**Author contributions**

DG, MAT and AL contribute equally to this work.

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

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## TITLES AND LEGENDS TO FIGURES

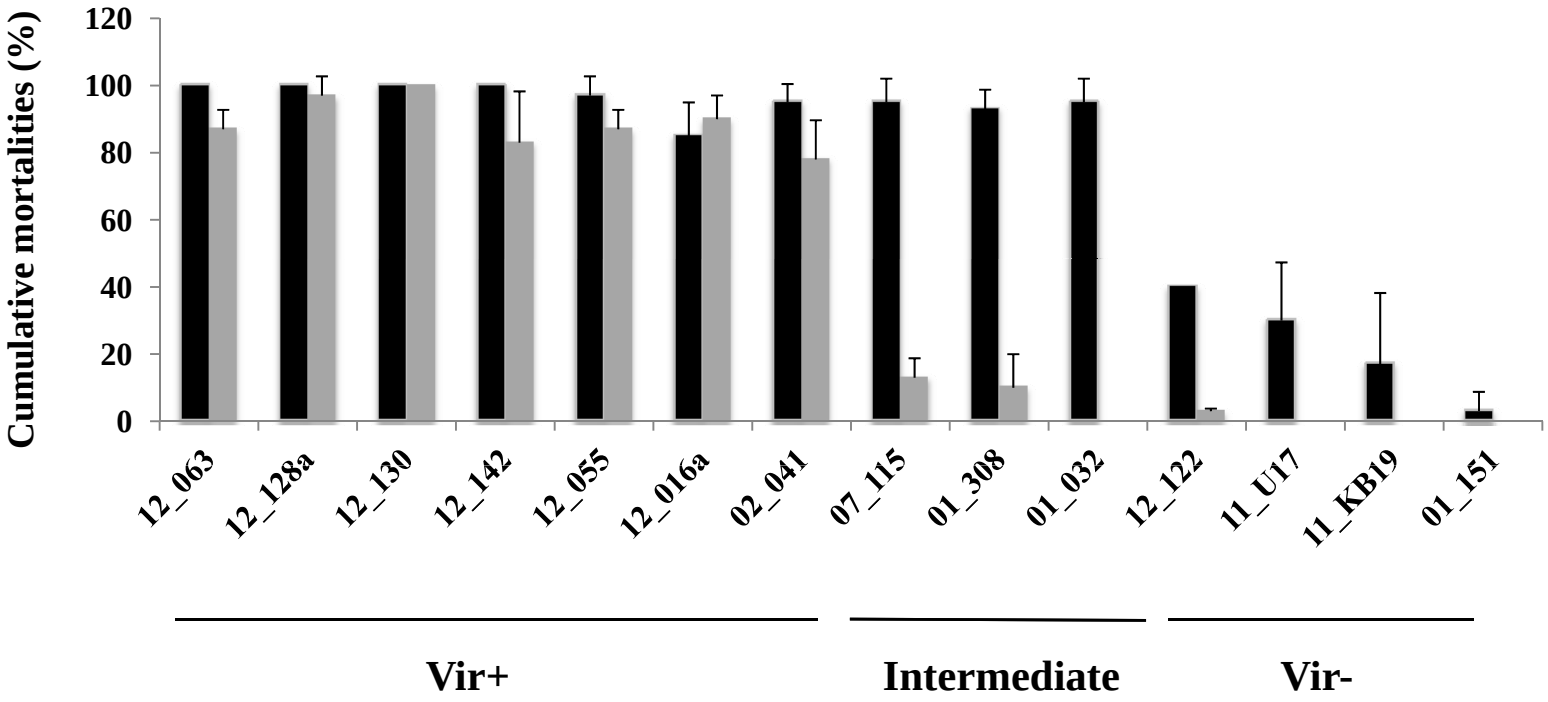
**Figure 1:** Oyster mortality in response to experimental infection with *V. aestuarianus* strains selected for genome sequencing. $10^7$ CFU (black bar) or $10^2$ CFU (grey bar) of the tested strain were intramuscularly injected into oysters (n= 10, in duplicate). Cumulative mortality (%) was assessed 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).

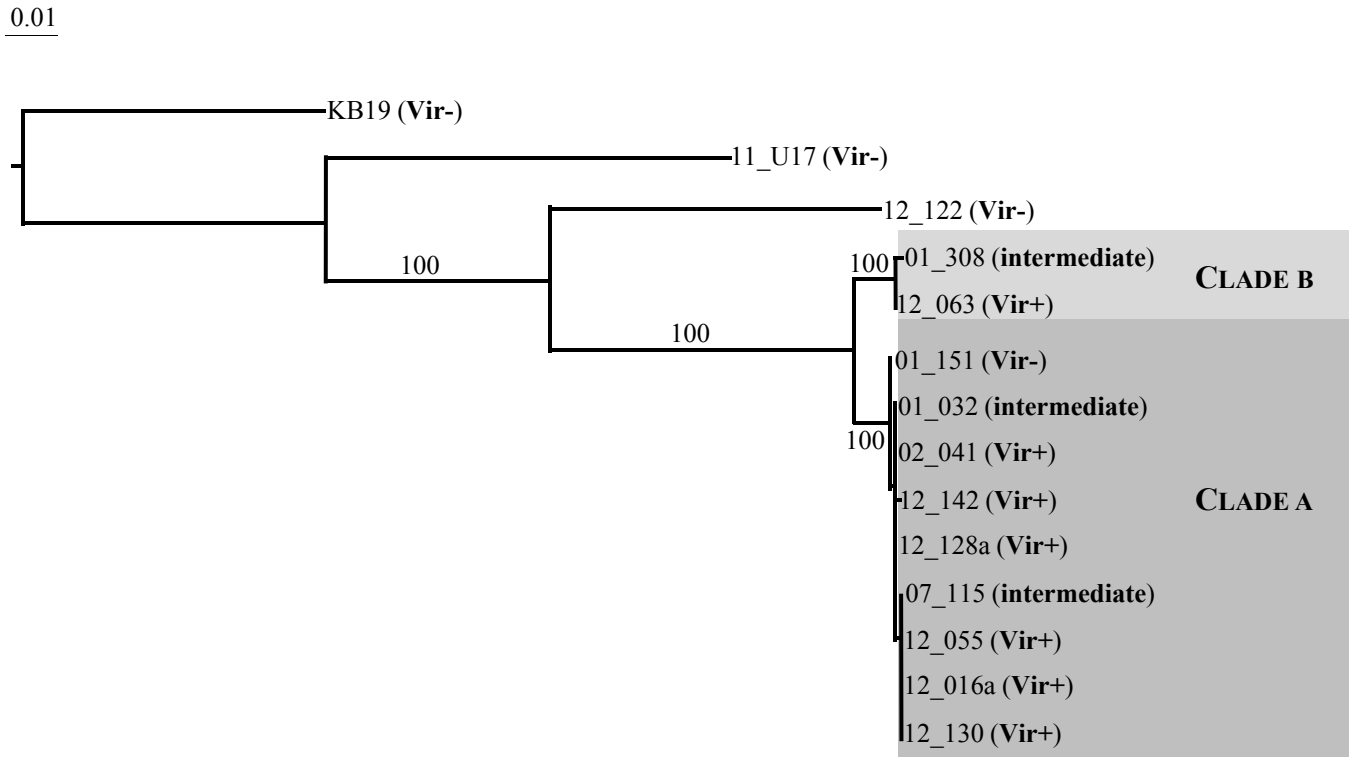
**Figure 2:** Phylogenetic analysis based on concatenated alignments of nucleic acid sequences of 2866 core genes from 14 *V. aestuarianus* strains, KB19 as an outgroup. Tree was built by the Maximum-Likelihood method based on sequences aligned using Muscle. Branch lengths are drawn to scale and are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

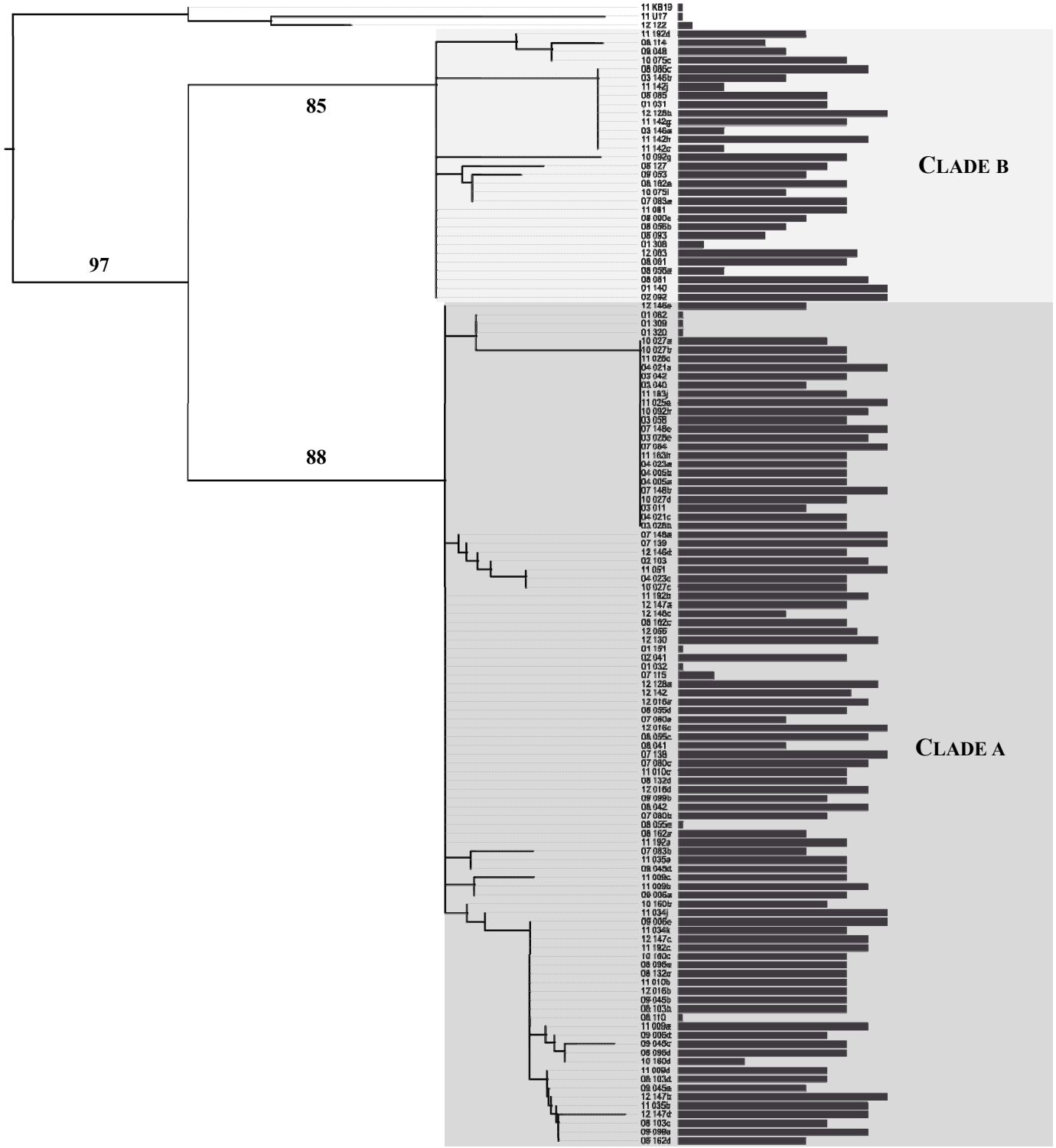
**Figure 3:** Virulence onto *V. aestuarianus* isolates phylogeny inferred by maximum likelihood analysis of partial D-lactate dehydrogenase gene sequences. The tree was built by the Maximum-Likelihood method based on sequences aligned using Muscle. Branch lengths are drawn to scale and are proportional 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).

**Figure 4:** Role of *varS* in *Vibrio aestuarianus* pathogenicity**. A-** Schematic representation of the VarS functional domains identified in the Vir+ strain 02_041 and the truncated protein resulting from a frameshift in the intermediate strain 07_115. DUF2222 corresponds to an uncharacterized signal transduction histidine kinase domain; HAMP, a cytoplasmic helical linker domain and methyl-accepting proteins; HisKA, a phosphoacceptor domain; HATPase_c, an ATPase domain; Response reg, a response regulator receiver domain; HPt, an histidine-containing phosphotransfer domain. **B-** Experimental infection of wild type *V. aestuarianus, ΔvarS* mutants and complemented *ΔvarS* mutants. $10^2$ CFU of the tested strains (lane 1: strain 12_016a wild type; lane 2: strain 07_115 wild type; lanes 3 and 4: GV1124 and 1125, two distinct clones of 12_016_ *ΔvarS*; lane 5: GV1124 i.e. 12_016_ *ΔvarS* carrying an expression vector for VarS, pMRB-$P_{LAC}$*varS*; lane 6: 07_115 carrying pMRB-$P_{LAC}$*varS)* was intramuscularly injected into oysters (n= 20, in duplicate). Mortality (%) was assessed after 6 days.

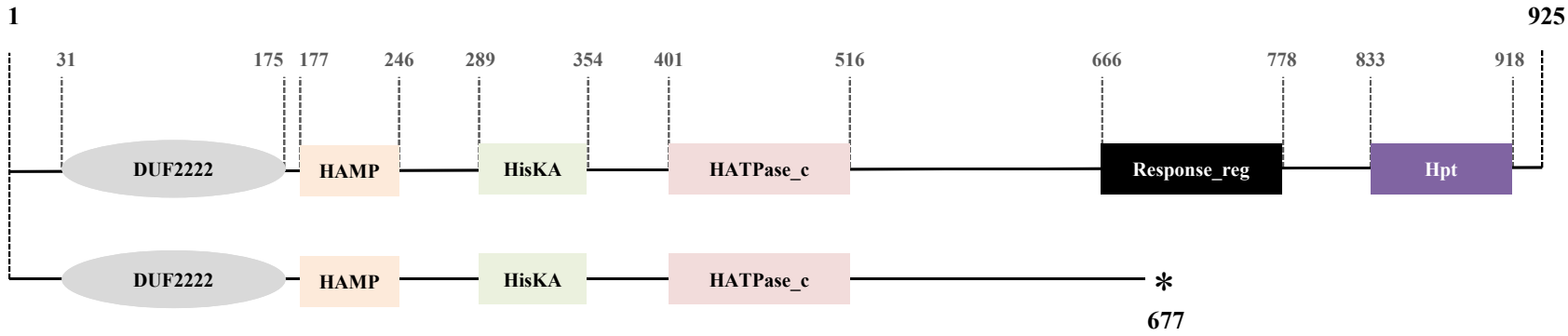
**Figure 5:** Role of *varS* in *Vibrio aestuarianus* metalloprotease expression**. A-** Extracellular product analysis by Coomassie blue stained 10% SDS-PAGE gel (lane 1: strain 12_06 wild type; lane 2 and 3: GV1124 and 1125, two distinct clones of 12_016_ *ΔvarS*; lane 4: 07_115 wild type). Arrow indicates the Vam metalloprotease identified by MS/MS. **B-** Azocasein-SDS-polyacrylamide gel (protease which degraded the gelatin are detected by zones of clearing). **C-** Proteolytic activities of ECPs determined by an azocasein assay (absorbance at 440 nm).


Cumulative mortalities (%)
120
100
80
60
40
20
0
12_063
12_128a
12_130
12_142
12_055
12_016a
02_041
07_115
01_308
01_032
12_122
11_U17
11_KB19
01_151
Vir+
Intermediate
Vir-

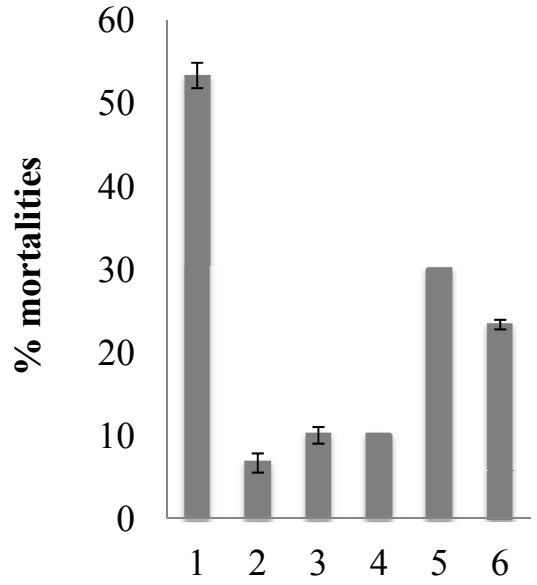

0.01
KB19 (Vir-)
11_U17 (Vir-)
12_122 (Vir-)
100
100
100
100
01_308 (intermediate)
12_063 (Vir+)
CLADE B
01_151 (Vir-)
01_032 (intermediate)
02_041 (Vir+)
12_142 (Vir+)
12_128a (Vir+)
07_115 (intermediate)
12_055 (Vir+)
12_016a (Vir+)
12_130 (Vir+)
CLADE A


0.001
85
97
88
CLADE B
CLADE A

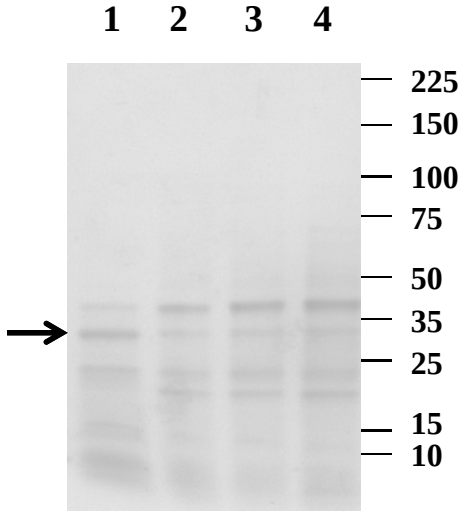
**A**


1
925
31
175
177
246
289
354
401
516
666
778
833
918
DUF2222
HAMP
HisKA
HATPase_c
Response_reg
Hpt
DUF2222
HAMP
HisKA
HATPase_c
*
677

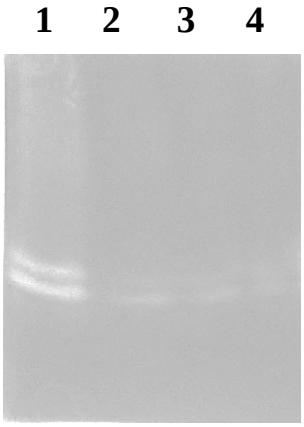
**B**


% mortalities
60
50
40
30
20
10
0
1
2
3
4
5
6

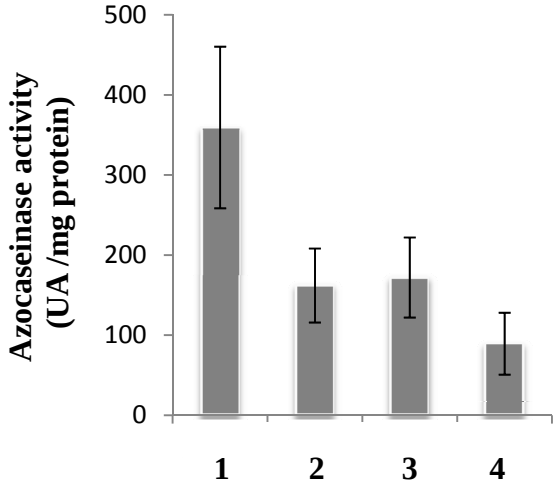
**A**


1
2
3
4
225
150
100
75
50
35
25
15
10

**B**


1
2
3
4

**C**


Azocaseinase activity
(UA /mg protein)
500
400
300
200
100
0
1
2
3
4

| Context | Strain | Origin | Mortality on field | Contigs number | Genome size (Mb) | CDSs | Accession number |
|---|---|---|---|---|---|---|---|
| **Summer mortality** | 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 |
| | ***02_041*** | ***Oyster, 2002, Argenton, Brittany, France*** | ***yes*** | ***8*** | ***4.20*** | ***4068*** | ***PRJEB5915*** |
| **Recent outbreak** | 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 |
| **Other** | 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

| **Strain** | **Description** | **Reference** |
|---|---|---|
| P3813 | B462 *DthyA::(erm-pir116)* [Erm$^R$] | Le Roux et al., 2007 |
| b3914 | b2163 *gyrA462, zei298::Tn10* [Km$^R$ Em$^R$ Tc$^R$] | Le Roux et al., 2007 |
| GV1124 | 02_016 *D varS* clone 1 | This study |
| GV1125 | 02_016 *D varS* clone 2 | This study |
| GV1171 | GV1124 + pMRB-P$_{LAC}$*varS* | This study |
| GV1174 | 07_115 + pMRB-P$_{LAC}$*varS* | This study |
| **Plasmid** | **Description** | **Reference** |
| pSW7848T | *oriV*$_{R6Kg}$; *oriT*$_{RP4}$; *araC*- P$_{BAD}$*ccdB;* [Cm$^R$] | Le Roux et al., 2007 |
| pSW*DvarS* | pSW7848T; *DvarS* | This study |
| pMRB-P$_{LAC}$*varS* | *oriV*$_{R6Kg}$; *oriT*$_{RP4}$; *oriV*$_{pB1067;}$ P$_{LAC}$*varS* [Cm$^R$] | This study |
| **Primer** | **Sequence 5'-3'** | |
| *DvarS*-1 | GTATCATAAGCTTATATCGAATTCGGGTAACGAGTGGCTATTGT | |
| *DvarS*-2 | CCATATCCACACCACGATGTAGAATAGGTTGGAGTTGCGC | |
| *DvarS*-3 | GCGCAACTCCAACCTATTCTACATCGTGGTGTGGATATGG | |
| *DvarS*-4 | CCCCCGGGCTGCAGGAATTCGGTCAGATGTTGTAGATCGC | |
| pSW-F | GAATTCCTGCAGCCCGGGGG | |
| pSW-R | GAATTCGATATCAAGCTTATCGATAC | |
| *varS*-F | GTGAGCGGATAACAAAGGAAGGGCCCATGACCAGATATGGCTTACGC | |
| *varS*-R | CGCGTCTGCAGCTCGAGCTAAACCAGATAAGGTTTTGCC | |
| pMRB-F | CTCGAGCTGCAGACGCGTCG | |
| pMRB-R | GGGCCCTTCCTTTGTTATCCGCTCAC | |
| vector-F seq | GCATGTAGAGTCGGTGCAAG | |
| vector-R seq | CCAGGCTTTACACTTTATGC | |
| *varS*-1 seq | CGTCCATACCAGCTTTCAAG | |
| *varS*-2 seq | GTTCTGACTCTTCAGGCATC | |
| *varS*-3 seq | CTGGTGGCTTGCAAATTCAC | |
| *varS*-4 seq | GTCGTGCAATAGTCGAAATG | |

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