1 **The *Vibrio vulnificus* stressosome is dispensable in nutrient-replete** 2 **conditions.**

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9 **ABSTRACT**

10 The stressosome is a protein complex that has been demonstrated to sense environmental
11 stresses and mediate the stress response in several Gram-positive bacteria, through the
12 activation of the alternative sigma factor SigB. The *in vivo* characterisation of this complex
13 has never been performed in *Vibrio vulnificus* or any other bacteria that do not possess SigB.
14 The elucidation of the role of the stressosome in *V. vulnificus* would provide elements to
15 elaborate a functional model of the complex in a Gram-negative bacterium and identify the
16 regulatory output in the absence of SigB. The stressosome locus is only found in 44% of *Vibrio*
17 *vulnificus* isolates raising the question as to whether the role of stressosome is essential or
18 modulatory in this bacterial species.

19 In this work, the expression of the stressosome genes was proven in nutrient-replete
20 conditions and the co-transcription as one operonic unit of the stressosome locus and its
21 putative downstream regulatory locus was demonstrated.

22 Moreover, the construction of a stressosome mutant lacking the four genes constituting the
23 stressosome complex allowed us to examine the role of this complex *in vivo*. The initial
24 established mutagenesis strategy relied on rifampicin-resistant *V. vulnificus* to select
25 recombinant bacteria. Our data clearly showed that the influence of the Rif^R allele on stress
26 and virulence characteristics overshadowed any effects of the stressosome. Therefore, we
27 established an alternative mutagenesis strategy with a non-modified *V. vulnificus* parental
28 strain and a DAP auxotrophic *E. coli* donor strain. Extensive phenotypic characterisation of
29 the successfully-generated mutant in nutrient-replete conditions showed that the
30 stressosome does not significantly contribute to the growth, of *V. vulnificus*. The stressosome
31 did not modulate the response of *V. vulnificus* to the range of stresses tested – Ethanol,

32 osmolarity, temperature, and salinity. Furthermore, the stressosome is dispensable for
33 motility and exoenzyme production of *V. vulnificus*.

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38 INTRODUCTION

39 *Vibrio vulnificus* is a human foodborne pathogen that causes severe human infections, with
 40 a fatality rate higher than 50% in the United States (1). It populates coastal waters and is
 41 bioaccumulated by many bivalves, amongst which are oysters and other shellfish used for
 42 human consumption (2). Two different infection pathways have been described for this
 43 pathogen, gastrointestinal and wound infections (2-4). The first one occurs when the bacteria
 44 is ingested through the consumption of raw molluscs (1), the latter is caused by the contact
 45 of contaminated water with pre-existing wounds and it represents the most common
 46 infection route in the US (5-7). The fatality rate of the latter pathway is high but the severity
 47 of the infection generally depends on underlying health conditions in the patient (2). *V.*
 48 *vulnificus* is considered an opportunistic pathogen and severe infections have been observed
 49 in immunocompromised patients and in the presence of pathological conditions that
 50 increase the blood level of iron, such as liver disease (8, 9). Moreover, the distribution of this
 51 pathogen is dependent on environmental factors, such as water temperature and salinity
 52 this results in regionality of the cases of *V. vulnificus* infection (10, 11). A key step to
 53 understanding and predicting the distribution of the microorganism and the occurrence of
 54 the infection is the elucidation of the mechanisms that constitute the bacterial stress
 55 response.

56 The bacterial stress response is the set of physiological changes and molecular mechanisms
 57 that a bacterium puts in place to survive changes in the environment that would otherwise
 58 be lethal (12). The stress response is a fundamental step to persist in the environment and
 59 to guarantee a successful infection in the case of pathogenic bacteria (13, 14). It does require
 60 substantial changes in the physiology of the bacteria that are often in contrast with normal
 61 growth and propagation (15). For this reason, it is essential to regulate the stress response
 62 and limit such changes to the right time and environmental conditions. To do so, bacteria
 63 have developed complex regulation mechanisms, often characterised by a fast activation and
 64 equally important and efficient inactivation systems. To optimally coordinate the stress
 65 response with the outside world, bacteria have evolved signalling complexes that sense the
 66 stress and integrate the signal to modulate the cell response. Amongst these is the bacterial
 67 stressosome, a 1.8 MDa complex that has been discovered and extensively characterised in
 68 the Gram-positive *Bacillus subtilis* (16-18). In this organism and other Gram-positive bacteria,
 69 the stressosome has been found to activate the alternative sigma factor σ^B (SigB), following
 70 the sensing of environmental stresses (19). SigB in its active form binds to the RNA

71 polymerase (RNAP) and promotes the expression of hundreds of genes involved in stress
72 response (20).

73 Interestingly, the genetic locus encoding for the stressosome proteins has been found in
74 several phyla and in bacteria that do not possess SigB, like *V. vulnificus* (21). In this organism,
75 the stressosome locus contains an upstream and a downstream module. The first formed by
76 the genes encoding for the three stressosome proteins VvrsbR, VvrsbS, VvrsbT (RsbR, RsbS
77 and RsbT in *B. subtilis*) and the phosphatase VvrsbX (RsbX) and the latter encoding for a
78 putative regulatory output of the stressosome, a two-component system (TCS) most likely
79 involved in c-di-GMP hydrolysis (22). This locus has been identified in approximately 50% of
80 the sequenced strains and its expression has been proved both in nature and laboratory
81 conditions (23-25). Moreover, the heme-binding globin domain at the N-terminal of VvuR
82 and the biochemical characterisation of these proteins from *V. brasiliensis* has shown a
83 potential role in surviving or tolerating anaerobic conditions (26). This would be relevant
84 both for the persistence of *V. vulnificus* in the environment and for survival during the
85 infection process. For this reason, this work has been focused on characterising, for the first
86 time *in vivo*, the role of the stressosome in this marine pathogen through the construction
87 and phenotypical characterisation of a stressosome mutant, lacking the whole upstream
88 module. This preliminary characterisation confirmed that the stressosome genes are
89 expressed in LB + 2.5% NaCl (LBN), a rich media commonly used to culture this bacterium
90 and focused on the effects of the stressosome on the growth and stress response of *V.*
91 *vulnificus*, with particular attention to anaerobiosis and oxidative stress. Moreover, due to
92 the potential role in regulating the levels of c-di-GMP, motility and other virulence traits were
93 tested to elucidate the role of the stressosome in the virulence of *V. vulnificus*.

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95 MATERIAL AND METHODS

96 Strains, plasmids and growth conditions.

97 The clinical strain *Vibrio vulnificus* CMCP6 (27) was used for the construction of the
98 stressosome mutant in this work (see below). The auxotrophic *E. coli* β 2163 was used to
99 perform bacterial conjugation (28). *Vibrio vulnificus* was cultured in lysogeny broth medium
100 with an additional 0.4M NaCl (LBN) at 30°C or 37°C, as specified. LB medium supplemented
101 with 0.3 mM 2,6-Diaminopimelic acid (DAP) was used to culture *E. coli* β 2163 at 37°C.
102 Overnight broth cultures were grown in 2 ml in 15 ml bacterial culture tubes, at 37 °C with
103 agitation at 150 rpm. Specific growth conditions, different from the one indicated above, are
104 described in the appropriate sections. All chemicals and reagents were supplied by Sigma,
105 unless indicated otherwise.

106 RNA extraction and one-step RT-PCR analysis.

107 For RNA extraction, *V. vulnificus* wild-type was grown overnight in LBN at 30°C and 37°C. One
108 volume of bacterial culture containing approximately 0.2 OD₆₀₀ of cells was mixed with 2
109 volumes of RNeasy Protect (Qiagen) and processed according to the manufacturer's
110 instructions. The pellet was used for RNA extraction, within 24 h of treatment with
111 RNeasy Protect. The pellet was resuspended in 200 μ l of TE buffer (30 mM Tris-HCl, 1mM EDTA,
112 pH 8.0) + 15 mg/ml lysozyme + 10 μ l ready-to-use QIAGEN Proteinase K (20 mg/ml) and
113 incubated 10 min at room temperature to achieve cell lysis. RNA extraction on the lysate was
114 performed with RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The total
115 RNA was eluted in 30 μ l RNase-free water and traces of DNA were removed using the TURBO
116 DNA-free kit (Life Technologies), according to the manufacturer instruction. The
117 concentration and quality of the extracted RNA were assessed with a NanoDrop
118 spectrophotometer and only samples with a 260/280 ratio $\geq$ 1.9 were used for the RT-PCR
119 protocol, and RNA integrity was verified on a 1.5% agarose gel. To assess the presence of the
120 target mRNA a one-step RT-PCR protocol was performed, using the QIAGEN OneStep RT-PCR
121 Kit, according to the manufacturer instruction. Briefly, the cycle consisted of two subsequent
122 steps, one of reverse transcription (RT) at 50°C for 30 min and one traditional PCR cycle. The
123 primers for the PCR reactions (Table 1) were designed using Primer-BLAST (29). An RT
124 negative control, that skipped the step at 50°C, was included to confirm the absence of DNA
125 contamination. A PCR negative control in which the template was substituted with PCR-grade
126 water was included for each pair of primers. The gene *tuf* (elongation factor Tu) was used as
127 endogenous control. PCR products were visualised on a 1.2% agarose gel.

128 Construction of the *V. vulnificus* Δ RSTX knock-out mutant in a rifampicin-resistant *V.*
129 *vulnificus* background.

130 The construction of a knock-out mutant lacking the upstream module of the stressosome
 131 locus consisting of the *VvrsbR*, *VvrsbS*, *VvrsbT* and *VvrsbX* genes (*V. vulnificus* Δ RSTX), was
 132 first achieved using a classical conjugation protocol with a spontaneous rifampicin-resistant
 133 strain of *V. vulnificus* (Rif^R) and *E.coli* SM10 λ pir. The use of a rifampicin-resistant *V. vulnificus*
 134 has been previously used (30, 31) and it is necessary to counter-select for the donor strain
 135 after conjugation. The knockout cassette (Eurofins) contained a deletion from nucleotide 4
 136 of *VvrsbR* to nucleotide 577 of *VvrsbX*. The cassette was digested with *Xba*I and *Zra*I and
 137 cloned into the pDS132 suicide vector (32), between the *sacB* promoter and the *lac* operator,
 138 resulting in the knockout plasmids pDS_ Δ RSTX. This was transformed by electroporation into
 139 *E. coli* SM10 λ pir and transformant colonies selected on LB + 25 μ g/mL Chloramphenicol. Bi-
 140 parental conjugation was performed using the *E. coli* strain containing the pDS_ Δ RSTX
 141 plasmid as a donor strain and the Rif^R *V. vulnificus* as parental strain. *V. vulnificus* CMCP6 and
 142 *E. coli* SM10 λ pir pDS_ Δ RSTX were grown overnight at 37°C in LBN and LB + 25 μ g/mL
 143 Chloramphenicol, respectively. Cultures were washed in LB broth, the two strains were
 144 mixed at a 1:1 ratio (v/v), spotted onto LB agar plates and incubated for 5h at 37°C. *V.*
 145 *vulnificus* transconjugants carrying the knock-out plasmid integrated into the chromosome
 146 by homologous recombination were selected on LBN + 5 μ g/mL Chloramphenicol + 50 μ g/mL
 147 Rifampicin. To promote the excision of the plasmid from the chromosome of *V. vulnificus*,
 148 the second event of homologous recombination was favoured by culturing the first
 149 recombinant cells in LBN broth without selective antibiotic. Second recombinant cells were
 150 selected on LBN + 5% sucrose (w/v) because the *sacB* gene present in the suicide plasmid
 151 does not allow growth in the presence of sucrose and growth on this media is a sign of a
 152 successful excision of the plasmid from the bacterial genome. This event can equally
 153 generate wild-type cells or deletion mutants. For this reason, mutants were selected by PCR
 154 screening of colonies grown on LBN + 5% sucrose using Taq polymerase (Bioline) and the
 155 primers RSTX_For (5'-GTCACGGGTTGATTGATTCGCAT-3') and RSTX_Rev (5'-
 156 CTCACCGAGACGTAACATATGAATGT-3'). PCR products of different sizes for the mutant and
 157 the wild-type were visualised through agarose gel electrophoresis.

158 Construction of the *V. vulnificus* Δ RSTX knock-out mutant in a wild-type *V. vulnificus*
159 background.

To avoid the use of a rifampicin-resistant strain of *V. vulnificus* and pleiotropic effects previously reported associated with such mutations (33) and observed also in this work to interfere with the phenotypic characterisation of the mutant, the construction of the stressosome knock-out mutant was performed in a wild-type background. To do so, we used for the first time a conjugation protocol with *V. vulnificus* and a DAP (diaminopimelic acid)-auxotrophic strain of *E. coli*. The same knock-out cassette (Eurofins) and plasmid described in the previous section were used and transformed by electroporation into *E. coli* β 2163 and transformant colonies selected on LB + 25 μ g/mL Chloramphenicol + 0.3 mM 2,6-DAP agar plates. Bi-parental conjugation was performed as previously described, with the only difference that 0.3 mM 2,6-DAP were supplemented to all the growth media to allow growth of the *E. coli* strain. Conjugation time was extended from 5h to 7h and *V. vulnificus* transconjugants were selected on LBN + 5 μ g/mL Chloramphenicol. In this last medium, the absence of additional DAP allowed the counter-selection of the donor strain. Second recombination and selection of the mutant were performed as described in the previous section.

Whole Genome Sequencing (WGS) analysis.

One putative mutant was selected for each mutagenesis protocol and Whole Genome Sequencing (WGS) analysis was performed. In addition, the *V. vulnificus* CMCP6 and the Rif^R strain were also sequenced. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. Genomic DNA was sequenced by MicrobesNG (Birmingham, UK) using Illumina technology. Average read lengths were between 168 and 645 nucleotides for each sample and average fold coverage was between 52 and 1162. BreSeq was used to call base substitution mutations Read Alignment evidence using Consensus mode, with a mutation E-value cut-off of 10 and frequency cut-off of 0.8 (80 %).

Growth characterisation in LBN.

Growth was assessed at 30°C in LBN, in the presence and reduced oxygen condition and at 42°C. Overnight cultures were grown in 2 mL LBN at 37°C and diluted in LBN at an initial OD of 0.01. 200 μ L of cell suspension were incubated in a microtiter plate and incubated at the appropriate temperature. To achieve oxygen-depleted growth conditions a higher volume of cell suspension (approximately 300 μ L) was used to completely fill the microtiter well and the plate was sealed with a sterile adhesive plastic film before incubation at the required temperature. To test growth at 42°C, the plate was set up as previously described and

193 incubated first at 37°C for one hour and then at 42°C. At least three biological replicates were
194 used for each strain and each of them was assessed in two technical replicates. The plates
195 were statically incubated in a Sunrise microtiter plate reader at the specified temperature
196 and the OD₅₉₅ was measured every 30 min for 24h.

197 **Stress survival assays.**

198 In order to test stress survival in LBN, the survival of both *V. vulnificus* wild-type and the
199 ΔRSTX mutant was assessed in LBN in the presence of different stresses. Overnights cultures
200 were grown at 37°C in 2 mL LBN for no longer than 18h. The cultures were then diluted to
201 OD 0.1 in 2 mL of the appropriate stress media and incubated at 30°C (except the samples
202 tested for temperature stress). Stresses (and media compositions) were: ethanol stress (LBN
203 + 10% ethanol (v/v)), oxidative stress (LBN + 2mM H₂O₂), acid stress (LBN pre-acidified to pH
204 4 with HCl) and temperature stress (45°C in a temperature-controlled block). 100 μL samples
205 were withdrawn at different time points and used for serial dilution and plate counting. At
206 least two biological replicates were tested for each strain and stress condition, each one
207 assessed in two technical replicates for the plate counting.

208 **Stress tolerance assays.**

209 To test the ability of *V. vulnificus* wild-type and the ΔRSTX mutant to tolerate stress and to
210 grow in non-lethal stress conditions, we tested the growth of the two strains on LBN agar
211 supplemented with several stressors. Overnights cultures were grown at 37°C in 2 mL LBN
212 for no longer than 18h and then diluted to OD 1 in LBN broth. Cells suspensions were then
213 tenfold serial diluted up to 10⁻⁷ and 3 μL of each dilution spotted on the appropriate LBN agar
214 plate and incubated at 30°C. Stresses (and media compositions) were: oxidative stress (LBN
215 agar + 0.5 mM H₂O₂), osmotic stress (LB agar + 0.8 M NaCl). To test growth ability in complete
216 anaerobic conditions, the plates were incubated in a 2.5L anaerobic jar in the presence of an
217 Oxoid AnaeroGen 2.5L Sachet that generates an atmosphere with less than 1% oxygen,
218 according to the manufacturer. Pictures of the plates were taken after 48h growth.

219 **Motility assay.**

220 In order to evaluate the effects of the stressosome mutation on the motility of *V. vulnificus*,
221 the two strains were tested for swimming on rich motility agar plates (10 g tryptone, 20 g
222 NaCl and 3.35 g agar per litre). Overnights cultures were grown overnight in 2 mL LBN broth
223 at 37°C. A sterile metal wire was then immersed in the cell suspension and used to pierce

224 the motility plate. Plates were incubated at 30°C or 37°C and motility zone measured after
225 16h. At least three biological strains were tested for each strain.

226 **Exoenzyme production.**

227 The wild-type and the Δ RSTX mutant strains were tested for the production of two
228 exoenzymes, haemolysin and protease. The strains were grown overnight in 2 mL LBN broth
229 at 37°C. The cell suspension was then inoculated on LBN agar plates containing 5 % (v/v)
230 defibrinated sheep blood (ThermoFisher Scientific) for haemolysin assay and 1 % (w/v) skim
231 milk for protease assay. The plates were incubated at 37°C and pictures were taken after 48
232 h and 24 h, for haemolysin and protease test respectively.

233 **Cross-protection assay.**

234 Cross-protection experiments were performed as previously described (34) to test the
235 effects of nutrient downshift on temperature survival in *V. vulnificus* wild-type and Δ RSTX.
236 Briefly, the strains were grown overnight in 2 mL LBN at 37°C. Cultures were then diluted in
237 2 mL of fresh LBN to OD₆₀₀ 0.05 and grown to mid-log phase (OD₆₀₀ 0.4-0.6). Cell cultures
238 were then diluted 1:100 in CDM (9.94 mM Na₂HPO₄, 10.03 mM KH₂PO₄, 0.81 mM
239 MgSO₄·7H₂O, 9.35 mM NH₄Cl, 856 mM NaCl, 0.75 µM FeCl₃) in the absence of carbon source.
240 Cell suspensions were incubated at 37°C and 1 mL withdrawn at different time points (0h,
241 0.5h, 1h, 2h, 4h, 24h) to test survival to high temperature. Plate counting was performed, at
242 each time point, before and after 1h of incubation at 45°C. Mid-log phase cells diluted in LBN
243 rather than CDM were used as control, to confirm temperature sensitivity before nutritional
244 downshift.

245

246 RESULTS

247 The stressosome locus is expressed in rich media with co-transcription of all modular 248 genes.

249 Previous reports have shown that *VvrsbR*, *VvrsbS*, *VvrsbT* and the downstream TCS (two-
250 component system) are expressed in artificial seawater (ASW) (24) but little information is
251 available on the expression and possible role of the stressosome in *V. vulnificus* during
252 growth in rich media (35). Moreover, although the locus is predicted to be an operon, due to
253 close proximity, sometimes overlapping, of the genes to one another, no proof of co-
254 transcription has been provided to date. To address these two questions, the presence of
255 single transcripts and co-transcripts was investigated in cells growing in LBN to stationary
256 phase, through RNA extraction and non-quantitative Reverse Transcriptase PCR (RT-PCR).
257 First, the presence of the transcripts of the *VvrsbR* and *VvrsbD1* genes was confirmed in cells
258 growing in LBN, demonstrating that the locus is expressed in nutrient-replete conditions
259 (Figure S1). Moreover, the detection of RT-PCR products with the use of primers in the
260 proximity of intergenic regions at the 3' and 5' ends of adjoining genes (Figure 1A) indicated
261 the presence of co-transcripts between each pair of genes within each module and between
262 *VvrsbX* and *VvD1* (Figure 1B), showing that the upstream and downstream module genes are
263 co-transcribed. The expression of the stressosome and its downstream module genes in LBN
264 suggested a role of this complex in rich media, while their proximity on the chromosome and
265 the suggested presence of a common mRNA support the idea of the two modules being
266 functionally related to each other.

267 Phenotypic characteristics of the stressosome mutant were overshadowed by the 268 pleiotropic effects of the Rif^R allele.

269 Phenotypic characterisation of the stressosome mutant constructed in the *V. vulnificus*
270 rifampicin-resistant background was performed in order to identify the *in vivo* role of the
271 stressosome in this human pathogen. The mutant was tested for growth, stress survival and
272 tolerance, and for the main virulence characteristics and compared to the *V. vulnificus*
273 CMCP6 wild-type strain. The use of the rifampicin-resistant parental strain as control,
274 allowed us to identify possible effects of the rifampicin resistance and validate the use of the
275 classical mutagenesis protocol when downstream applications include stress response and
276 virulence characterisation of the mutants. In some cases, the mutant showed differences
277 compared to the wild-type strain but all ascribable to pleiotropic effects in the rifampicin-
278 resistant parental strain. In particular, reduced motility, ethanol survival and hyperosmotic

279 stress tolerance were observed (Figure S2). The same effects were observed, to the same
280 extent, in the rifampicin-resistant parental strain in line with previously published work (33).
281 This is an indication that the construction of the stressosome mutant in a Rif^R strain might
282 not allow the observation of differences caused by the *VvrsbRSTX* deletion, where these
283 overlap with pleiotropic effects associated with rifampicin resistance. Based on this, a
284 different mutagenesis protocol was successfully optimised during this work with the
285 successful construction of the stressosome mutant in a wild-type background. This allowed
286 a direct comparison with the wild-type and extensive characterisation of the role of the
287 stressosome in LBN medium.

288 **Knockout mutation of the stressosome does not influence growth of *V. vulnificus* in LBN,**
289 **at various oxygen concentrations and temperatures.**

290 In order to test the physiological role of the stressosome in *V. vulnificus*, a stressosome
291 mutant lacking the upstream module (*V. vulnificus* Δ RSTX) was successfully constructed and
292 used for phenotypic characterisation in rich media in comparison to the wild-type strain.
293 Firstly, growth characterisation of the two strains was performed in LBN at 30°C and 37°C in
294 aerobic conditions, to eliminate any possible influence of differential growth rate on the
295 phenotypic characteristics analysed. The 2 strains grew with similar kinetics in these
296 conditions (Fig S3)

297 Next, growth characterisation of the two strains was performed in LBN at 30°C and 37°C, in
298 aerobic and O₂-depleted conditions. *V. vulnificus* experiences variation in O₂ levels both in
299 the environment and in the human host (25). Moreover, the *V. brasiliensis* stressosome has
300 been shown to bind O₂ *in vitro* (26). For these reasons, growth curves in aerobic and reduced
301 O₂ conditions at 30°C (Figure 2A) were compared. Interestingly, the oxygen depletion does
302 not cause any growth rate variation in the wild-type strain, but only a reduction in the overall
303 biomass accumulation, indicating a good adaptation of *V. vulnificus* to hypoxia. Moreover,
304 the growth profile of the mutant was comparable to the one of the wild-type strain, thus
305 indicating no significant role of the stressosome in this adaptation to low oxygen levels.

306 During the infection of the human host, *V. vulnificus* often faces not only reduced oxygen
307 levels but also increasing temperatures due to the occurrence of fever in the patient (36).
308 For this reason, the ability of the two strains to grow at 42°C was tested after a brief time of
309 growth at 37°C (Fig 2B). In this experimental setup, both the wild-type and the mutant strain
310 showed slightly reduced growth when compared to the optimal growth temperature but no
311 significant differences were observed between the two strains. These growth experiments

312 demonstrate that the presence of the stressosome does not influence growth rates in LBN,
313 nor is it required for the processes of adaptation to low oxygen levels or high temperatures
314 in nutrient-replete conditions.

315 **The stressosome mutation does not alter the ability of *V. vulnificus* to survive lethal**
316 **environmental stress in LBN.**

317 In Gram-positive bacteria, such as *Listeria monocytogenes* and *Bacillus subtilis*, the
318 stressosome is part of the signalling hub that ultimately leads to the activation of the
319 alternative sigma factor σ^B (22). This controls the transcription of hundreds of genes involved
320 in the stress response and contributes to the survival of the bacteria in harsh environmental
321 conditions (37-39). To study a possible role of the stressosome in the survival of *V. vulnificus*
322 to lethal stresses, survival assays in the presence of a range of different stressors were
323 performed using the wild-type *V. vulnificus* CMCP6 strain and the Δ RSTX strain (Figure 3). We
324 tested several stresses, including 10% ethanol (Figure 3A), 2 mM H₂O₂ (Figure 3B), pH 4
325 (Figure 3C) and high temperature (45°C) (Figure 3D). Ethanol was used to test whether the
326 difference observed for the Rif^R stressosome mutant was due to the rifampicin resistance
327 and not to the *VvrsbRSTX* mutation. Oxidative, acid and temperature stresses were chosen
328 because they are relevant to the life cycle of *V. vulnificus* and in particular to survive in the
329 human host. Moreover, response mechanisms to these stresses have been only partially
330 elucidated in *V. vulnificus* (40-42). The experiments performed showed that the two strains
331 equally survived the tested stresses. In particular, no difference at all was observed in the
332 presence of 10% ethanol and at pH 4, confirming that the use of the Rif^R parental strain was
333 the cause of the previously observed difference. A faster death was occasionally observed in
334 the presence of H₂O₂ (Figure 3B) and after a shift to 45°C (Figure 3.D). This might have a
335 biological meaning and indicate a potential modulatory involvement of the stressosome or
336 simply be due to overall higher variability in these stress conditions.

337 Higher temperatures (48°C and 50°C) and hyperosmotic stress (LBN + 1.2 M NaCl) were also
338 tested but these were respectively too harsh or too mild conditions to appreciate differences
339 between the strains (data not shown). In fact, the temperatures tested caused the complete
340 death of both strains in less than 20 min thus making it challenging to perform a time-series
341 experiment. The presence of an additional 1.2 M NaCl, on the contrary, did not cause
342 bacterial death. These results indicate that, in our experimental conditions, the stressosome
343 does not contribute to the survival of *V. vulnificus* to environmental stresses and its deletion

344 does not affect the ability of this human pathogen to survive several stresses that can be
345 encountered both in the environment and in the human host.

346 Concerning stress survival, several cases of cross-protection mechanisms have been
347 described in *Vibrio* spp. (43) and in *V. vulnificus* specifically (34, 44), where pre-exposure to
348 sub-lethal stresses (such as nutrient downshift) causes a general stress adaptation response
349 that results in a significant increase in survival to subsequent exposure to lethal conditions.
350 In this work, the role of the stressosome in the general stress adaptation response was
351 assessed by determining survival to high temperature following nutrient downshift, well-
352 described in *V. vulnificus* (34). We confirmed that the shift from a rich to a chemically defined
353 media, lacking a carbon source, rapidly caused a significant transient resistance to lethal heat
354 stress (1h at 45°C) but no differences were observed between the wild-type and the
355 stressosome mutant (Figure 4). The cross-protective adaptive response occurred within 5
356 minutes following nutrient downshift and persisted for 2 h, after which time the adaptive
357 response was resolved and normal responses returned. These experiments suggest that the
358 stressosome does not regulate stress survival, neither directly nor indirectly through cross-
359 protection adaptive mechanisms.

360 **The stressosome does not modulate stress tolerance in *V. vulnificus* in nutrient replete**
361 **media.**

362 Bacterial stress response refers not only to survival to extreme lethal stresses but also to
363 stress tolerance mechanisms that allow the microorganisms to grow in the presence of
364 milder stresses. This is essential to allow a bacterium or a bacterial community to reproduce
365 and grow in the environment, which must be considered as a dynamic system in which small
366 changes happen continuously due to natural fluctuations, human intervention and the
367 presence of other organisms and microorganisms (45). To test the ability of our strains to
368 tolerate, adapt and grow in the presence of mild stresses, we analysed their growth on LBN
369 agar in the presence of different stressors. Amongst these, we tested 0.5 mM H₂O₂,
370 anaerobiosis and hyperosmotic stress (LB + 0.8 M NaCl) (Figure 5). Various degrees of growth
371 effects were observed in the presence of these stresses for the wild-type, with the 0.5 mM
372 H₂O₂ being the one causing the strongest inhibitory effect, with a reduction of five log in CFU
373 numbers when compared to the LBN plate. Also the presence of extra salt caused a
374 significant reduction in the growth ability of *V. vulnificus*, while the anaerobiosis condition
375 only caused an effect on the amount of biomass present (i.e. smaller colonies indicative of a
376 reduced replication rate) but no changes in the ability to initiate growth (i.e. equal number

377 of colonies). The stressosome mutant strain suffered growth reductions comparable to those
378 of the wild-type in all tested conditions. This confirms that the stressosome is not involved
379 in the stress response of *V. vulnificus* in nutrient-replete conditions.

380 **The stressosome does not affect motility and exoenzyme production.**

381 The previous experiments indicate the lack of a significant role of the stressosome in
382 regulating growth and stress response in *V. vulnificus* in nutrient-replete media, in terms of
383 stress survival, stress adaptation and stress tolerance. This leaves an open question on the
384 physiological role of this complex in *V. vulnificus* that can explain the presence of the genetic
385 locus in a large proportion of strains of this species and active transcription of the locus in
386 LBN. To address this question, we investigated another fundamental aspect of the life cycle
387 of a human pathogen: virulence characteristics. Virulence includes a wide range of
388 physiological processes from motility to biofilm formation, through exoenzyme and toxin
389 production, and it is intrinsically connected to both growth and stress response (46, 47).

390 In this work, we assessed the ability of both strains to swim on tryptone motility agar plates
391 and their ability to produce haemolysin and protease exoenzymes (Figure 6). The motility
392 was tested at both 30°C and 37°C (Figure 6A) and the results show a clear effect of the
393 temperature but no effect of the stressosome mutation on the swimming of *V. vulnificus*.
394 Haemolysin (Figure 6B) and protease (Figure 6C) production were tested at 37°C on LBN
395 supplemented with sheep blood and skim milk respectively. Both *V. vulnificus* wild-type and
396 Δ RSTX showed degradation of the substrates, indicating that they are actively producing and
397 secreting both haemolysin and protease. Although precise quantification is not possible
398 using this method, no significant difference was observed between the two.

399 This set of experiments showed that the stressosome is not involved in motility and
400 exoenzyme production in nutrient-replete conditions.

401

402 DISCUSSION

403 The elucidation of the role of the stressosome in *V. vulnificus* could constitute an important
404 piece of information towards the understanding of distribution and infection of a deadly and
405 enigmatic human pathogen. Moreover, it would represent the first characterisation of the
406 stressosome in a Gram-negative bacterium. This would lay the basis to elaborate a functional
407 model of the stressosome different from the one observed in *B. subtilis* and other Gram-
408 positive bacteria and to understand the evolution of this complex across different bacterial
409 species (22).

410 In this work, we focused on the phenotypical characterisation of a stressosome mutant
411 lacking the whole upstream module of the stressosome genetic locus. This includes the genes
412 encoding the three proteins forming the stressosome (VvrsbR, VvrsbS and VvrsbT) and the
413 phosphatase VvrsbX that has been shown to switch off the stressosome-mediated response
414 and avoid the activation of SigB in the absence of stress in other microorganisms (48, 49).
415 We decided to perform this extensive characterisation in the rich medium LBN. This is one
416 of the most used media to study the physiology of *V. vulnificus* and it prevents the occurrence
417 of nutritional stress, thus allowing us to focus exclusively on the environmental stress
418 response. This is the route of SigB activation that is known to be mediated by the
419 stressosome in *B. subtilis* (19).

420 We first confirmed the presence of the stressosome mRNA in wild-type cells grown to
421 stationary phase in LBN, as the expression of these genes has been shown before only in
422 ASW (24). We also investigated the possible co-transcription of the upstream and
423 downstream modules and we confirmed that products of co-transcription were present.
424 This, together with the co-localisation on chromosome two of *V. vulnificus*, supports the
425 hypothesis that the two modules are functionally related and the downstream TCS could be
426 the regulatory output of the stressosome in this bacterium.

427 Following this, construction of the knockout mutant using a classical mutagenesis protocol
428 in a rifampicin-resistant parental background, led to the observation that pleiotropic effects
429 of *rpoB* mutations causing rifampicin resistance can interfere with stress response and
430 virulence characterisation of *V. vulnificus*. For this reason, we successfully optimised a new
431 mutagenesis protocol that allowed construction of the mutant in a wild-type background,
432 thanks to the use of an auxotrophic *E. coli* donor strain. The mutant constructed in the wild-
433 type background was then utilised for an extensive phenotypical characterisation in
434 comparison to the wild-type strain. This started with a growth characterisation in LBN. We

confirmed no differences in the absence of stress, as expected, and focused on the effects of hypoxia and high temperature on growth. Differences between the two strains did not emerge and effects of hypoxia and high temperature were comparable between the two strains. Due to the predicted role of the stressosome in stress sensing and stress response, the next step of this phenotypical characterisation was the assessment of stress survival and tolerance of the stressosome-lacking mutant. Survival was assessed in the presence of several stresses, including acid, ethanol, oxidative and temperature stress, and once again different effects were observed on the wild-type but no differences emerged with the stressosome mutant. Stress tolerance, defined as the ability to grow in the presence of sublethal stresses, was also tested and no effects of the stressosome were observed. According to these results, the presence of the stressosome genes confers no advantage to the human pathogen *V. vulnificus* in growth, stress tolerance and stress survival in LBN. This is surprising considering the previously characterised role of the stressosome in Gram-positive bacteria (18, 22) and the presence of the locus transcript in these experimental conditions.

This lack of detectable function in mediating growth and survival in the presence of sublethal and lethal stresses led to the hypothesis that physiological roles of the stressosome could involve the colonisation and infection process. For this reason, motility and exoenzyme production of *V. vulnificus* wild-type and Δ RSTX mutant were tested. Again, the lack of the stressosome did not affect the ability of *V. vulnificus* to swim and produce enzymes relevant to the infection process, in our experimental condition.

These results could indicate a lack of function of the stressosome in *V. vulnificus* and explain why the stressosome locus is present only in 50% of the sequenced strains. On the other hand, it is possible that the experimental conditions used were not compatible with a stressosome activation, despite the presence of the locus transcripts. A basal level of stressosome expression may occur as a means for the bacterium to monitor and survey for the emergence of stress signal inducers. This could explain the detection of transcription of the stressosome genes in non-stressful conditions. This could be seen as been analogous to the role Toll-Like Receptors in eukaryotic cells acting as surveillance mechanisms for Pathogen-Associated and Damage-Associated Molecular Patterns that upon recognition and activation then initiate signalling cascades leading to inflammation and other cellular responses. Previous works have shown that the stressosome genes are expressed in ASW more than in human serum (24), thus indicating that the stressosome-mediated response could be naturally active in a nutrient-deficient environment rather than in rich media. This

469 first *in vivo* extensive characterisation in a rich medium is the demonstration that the
470 stressosome does not affect *V. vulnificus* physiology in a nutrient-replete environment.
471 Based on this and previous transcriptional analysis, we suggest that a similar characterisation
472 in a minimal, nutrient-depleted media should be performed to identify the role of the
473 stressosome and identify the putative trigger and regulatory output of this complex in a
474 Gram-negative human pathogen.

475

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485 β 2163 strain.

486 **CONFLICTS OF INTEREST**

487 The authors declare that there are no conflicts of interest.

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630 Table 1 Primers for co-transcription experiments of the stressosome locus genes in *V.*
631 *vulnificus* CMCP6 wild-type

Gene	Sequence (5'→3')	632
<i>tuf</i> (VV1_1203)	Forward: AAGTTTACGGCGGTGCTGCT	633
	Reverse: CGTAGTGGCGAGCTGGAGTG	634
<i>VvrsbRS</i>	Forward: GGTTCTGTACCAATGCCACCT	635
	Reverse: TGGTCGATTTACCGCATCT	636
<i>VvrsbST</i>	Forward: GGGGGTTGTGATTGGCCTTA	637
	Reverse: TGCCTACTTCACCACGTTCA	638
<i>VvrsbTX</i>	Forward: CCGGGGATCCACAACATTGA	639
	Reverse: CCAGAGACATATTCGCCGCT	640
<i>VvrsbXD1</i>	Forward: GTGGTCGGAGAATTGTTGCC	641
	Reverse: TAGCCAAAGGCTTGCAGGAT	642
<i>VvD1D2</i>	Forward: CATTAGGCAGACCCAAGCCA	643
	Reverse: TGAGACTGGCACGCAGAAAT	644
<i>VvrsbR</i>	Forward: GGCTCAGAAACACCCCTGAA	645
	Reverse: AGTTGGCAATGGTAAGCCGA	646
<i>VvD1</i>	Forward: GGAAGAGGCGGTTGAAGTGA	647
	Reverse: CATGCTGCTTGGGGGTAAGA	648
		649
		650

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653 **Figure 1. Co-transcription of stressosome locus genes in *Vibrio vulnificus*.** A) The genetic
654 stressosome locus in *V. vulnificus* CMCP6 wild-type, formed by the upstream module
655 (*VvrsbR*, *VvrsbS*, *VvrsbT* and *VvrsbX*) in purple and the downstream module (*VvD1* and *VvD2*),
656 in orange. The primers used for the co-transcription analysis are indicated at the top (forward
657 primers) and at the bottom (reverse primers) of each gene. B) Co-transcription analysis of
658 the stressosome locus genes. Electrophoresis analysis of the products of the RT-PCR
659 performed on stationary phase cells, growing in LBN at 37°C. 1: HyperLadder 50bp (Bioline);
660 2: *tuf* RT negative control; 3: *tuf* gene; 4: *tuf* PCR negative control; 5: *VvrsbR*-*VvrsbS* co-
661 transcript; 6: *VvrsbR*-*VvrsbS* PCR negative control; 7: *VvrsbS*-*VvrsbT* co-transcript; 8: *VvrsbS*-
662 *VvrsbT* PCR negative control; 9: *VvrsbT*-*VvrsbX* co-transcript; 10: *VvrsbT*-*VvrsbX* PCR negative
663 control; 11: *VvrsbX*-*VvD1* co-transcript; 12: *VvrsbX*-*VvD1* PCR negative control; 13: *VvD1*-
664 *VvD2* co-transcript; 14: *VvD1*-*VvD2* PCR negative control; 15: HyperLadder 50bp (Bioline).

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666

667 **Figure 2. Growth characterisation of *V. vulnificus* CMP6 wild-type (green) and the Δ RSTX**
668 **mutant (orange) in LBN. A) Growth curve in LBN at 30°C, in the presence of oxygen**
669 **(continuous line) and in reduced oxygen conditions (dashed line). OD₅₉₅ was measured every**
670 **30 min for 24 h. Each curve is the mean of three biological replicates. B) Growth curve in LBN**
671 **at 42°C, after 1h growth at 37°C. OD₅₉₅ was measured every 30 min for 24 h. Each curve is**
672 **the mean of three biological replicates. Y-axis starts at the OD value of the inoculum (0.01).**

673

674

675 **Figure 3. Stress survival of *V. vulnificus* CMP6 wild-type (green) and the Δ RSTX mutant**
676 **(orange) in LBN** A) Survival assay in LBN + 10% Ethanol (v/v). The strains were incubated at
677 30°C and survival was assessed, through plate counting, at five different time points (0 min,
678 20 min, 40 min, 60 min and 120 min). B) Survival assay in LBN + 2 mM H₂O₂. The strains were
679 incubated at 30°C and survival was assessed, through plate counting, at five different time
680 points (0 min, 20 min, 40 min, 60 min and 120 min). C) Survival assay in LBN at pH 4. The
681 strains were incubated at 30°C and survival was assessed, through plate counting, at five
682 different time points (0 min, 20 min, 40 min, 60 min and 120 min). D) Survival assay in LBN
683 at 45°C. The strains were incubated at 45°C and survival was assessed, through plate
684 counting, at five different time points (0h, 1h, 2h, 3h and 4h). For each time point, survival
685 was plotted as Log₁₀(CFU/ml). Three biological replicates for each strain were tested and the
686 reported values are the mean of the three replicates. Student's t-test was performed
687 comparing mutant strains to WT and P values are shown (ns > 0.05). Y-axis starts at Log
688 (CFU/mL) = 2, in proximity of the detection limit value (2.4).

689

690

691 **Figure 4. The cross-protection mechanism in *V. vulnificus* CMCP6 wild-type (green) and the**
692 **Δ RSTX mutant (orange).** A) Cell viability in LBN and CDM + 0.75 μ M FeCl₃ in the absence of
693 glucose. The strains were incubated at 37°C and survival was assessed, through plate
694 counting, before the shift from LBN to CDM and at six different time points (0 h, 0.5 h, 1h,
695 2h, 4h and 24h). A) Survival in LBN at 45°C for 1h. The strains were used for survival assay
696 before the shift from LBN to CDM and at six different time points (0 h, 0.5 h, 1h, 2h, 4h and
697 24h). Survival to high temperature was assessed, through plate counting, after 1h at 45°C.

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699

700 **Figure 5. Growth assessment in stress conditions of *V. vulnificus* CMCP6 wild-type and the**
701 **Δ RSTX mutant in LBN.** The strains were first diluted to OD₆₀₀ = 1 and then 10-fold serial
702 dilutions were performed up to 10⁻⁷ and each dilution was spotted on agar plates and
703 incubated at 30°C. A) LBN agar (LB agar + 0.4M NaCl) after 24 h incubation in atmospheric
704 condition. B) LBN agar after 24 h incubation in oxygen-depleted condition. C) LBN agar + 0.5
705 mM H₂O₂ after 24 h incubation in atmospheric condition. D) LB agar + 0.8 M NaCl after 24 h
706 incubation in atmospheric condition. The pictures shown are representative of at least two
707 biological replicates.

708

709

710 **Figure 6. Motility and exoenzymes production characterisation of *V. vulnificus* CMCP6 wild-**
711 **type (green) and the Δ RSTX mutant (orange) in rich media.** A) Motility assay on tryptone
712 motility plates. The strains were stabbed on motility agar plates and incubated at 30°C and
713 37°C. The motility zone was measured after 16h incubation. Three biological triplicates for
714 each strain were tested and the reported values are the mean of the three replicates. B)
715 Haemolysin production was tested on LBN agar plates + 5% sheep blood (v/v). Pictures were
716 taken after 48h incubation at 37°C. C) Protease production was tested on LBN agar plates +
717 1% skimmed milk. Pictures were taken after 24h incubation at 37°C.

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