

1 **Comprehension of Antimicrobial Peptides Modulation of the Type VI Secretion System**

2 **in *Vibrio cholerae***

3 **Annabelle Mathieu-Denoncourt and Marylise Duperthuy\*.**

4

5 Département de Microbiologie, infectiologie et immunologie, Faculté de médecine,

6 Université de Montréal, Montréal, H3T 1J4, Quebec, Canada.

7

8 \*Corresponding author: Marylise Duperthuy, Université de Montréal, C.P. 6128, succursale

9 Centre-ville, Montréal, QC, H3C 3J7, tel : 514 343-6111, fax: 514 343-5701,

10 [marylise.duperthuy@umontreal.ca](mailto:marylise.duperthuy@umontreal.ca)

11

12 **Abstract**

13

14 The Type VI secretion System (T6SS) is a versatile weapon used by bacteria for virulence,  
15 resistance to grazing and competition with other bacteria. We previously demonstrated that  
16 the role of the T6SS in interbacterial competition and in resistance to grazing is enhanced in  
17 *Vibrio cholerae* in the presence of subinhibitory concentrations of polymyxin B (PmB). In  
18 this study, we performed a global quantitative proteomic analysis by liquid chromatography  
19 coupled to mass spectrometry and a transcriptomic analysis by quantitative PCR of the T6SS  
20 known regulators in *V. cholerae* grown with and without PmB. The proteome of *V. cholerae*  
21 is greatly modified in the presence of PmB at subinhibitory concentrations with more than 39  
22 % of the identified cellular proteins displaying a difference in their abundance, including  
23 T6SS-related proteins (Hcp, VasC, TsaB and ClpV). We identified a regulator whose  
24 abundance and expression are increased in the presence of PmB, *vxrB*, the response regulator  
25 of the two-component system VxrAB. In a *vxrAB* deficient mutant, the expression of *hcp*  
26 measured by quantitative PCR, although globally reduced, was not modified in the presence  
27 of PmB, confirming its role in *hcp* upregulation with PmB. The upregulation of the T6SS in  
28 the presence of PmB appears to be, at least in part, due to the two-component system VxrAB.

29

30 **Keywords**

31

32 *Vibrio cholerae*, Type VI Secretion System, Regulation, Polymyxin B, Proteome,  
33 antimicrobial peptides, two-component regulatory systems

34

35 **Importance**

36

37 The type VI secretion system is important for bacterial competition, virulence and resistance  
38 to grazing by predators. In this study, we investigated the regulation leading to the type VI  
39 secretion system activation in the presence of polymyxin B (PmB), an antimicrobial used in  
40 veterinary and human health to treat infection caused by multi-resistant Gram-negative  
41 bacteria, in *V. cholerae*. In addition to making an overall portrait of the modifications to the  
42 proteome, we identified the VxrAB two-component system as the main regulator responsible  
43 for this activation. Our results provide evidence that subinhibitory concentrations of  
44 antimicrobials are responsible for important modifications of the proteome of pathogenic  
45 bacteria, inducing the production of proteins involved in virulence, host colonisation,  
46 resistance and environmental survival.

47

## 48 **Introduction**

49

50 *Vibrio cholerae* is a ubiquitous Gram-negative bacterium found in brackish rivers, coastal  
51 water and estuaries, or associated with fishes, shellfishes and zooplankton (1). There are more  
52 than 200 serotypes of *V. cholerae*, the O1 and O139 causing the cholera disease. The O1 El  
53 Tor biotype is responsible for the 7<sup>th</sup> ongoing pandemic (2). Cholera is acquired by the  
54 consumption of contaminated water or food and is characterized by an acute diarrhea, leading  
55 to dehydration and eventually to death if not treated (1). *V. cholerae* is endemic in regions  
56 where water sanitation facilities are inadequate and access to drinking water is precarious (2,  
57 3). Many virulence and competition effectors allow *V. cholerae* to cause infections, the most  
58 important being the cholera toxin and the toxin co-regulated pilus (4, 5).

59

60 The type VI secretion system (T6SS), found in more than 25% of Gram-negative bacteria, has  
61 been discovered a decade ago in *V. cholerae* (6). The T6SS is a molecular syringe analogous  
62 to T4 bacteriophage. It allows the translocation of various toxic effectors into target  
63 neighbouring cells. A contraction event of the VipA/B sheath propels a hemolysin-  
64 coregulated protein (Hcp) nanotube that punctures the target cell's envelope (7-9). The  
65 effectors have many intracellular targets and modes of action in both eukaryotic and  
66 prokaryotic cells, such as disruption of the cytoskeleton by actin cross-linking (10, 11),  
67 disruption of the cell membrane (12, 13) and peptidoglycan degradation (14, 15). It makes the  
68 T6SS an important weapon against competitor bacteria and predators (12, 13, 16, 17). The  
69 T6SS genes are divided in multiple gene clusters, *i.e.* a large cluster (VCA0105-VCA0124)  
70 that encodes for the major structural proteins, a protease and the internal regulator *vasH*  
71 (VCA0117), and at least 2 auxiliary clusters (VCA0017-VCA0022 and VC1415-VC1421),  
72 harbouring *hcp*, *vgrG*, adaptor and effector/immunity proteins (6, 12, 18). The expression of

73 the T6SS genes is complex and involves different regulators (reviewed in (7)), including  
74 *vasH*. VasH orchestrates, along with the alternative sigma factor *rpoN*, the expression of the  
75 large and auxiliary clusters (7, 19). Most studies on the T6SS in *V. cholerae* were done with  
76 non-O1/non-O139 environmental strains that constitutively secrete through their T6SS.  
77 However, the regulation appears to vary between strains and seems to differ in clinical  
78 isolates as the secretion needs to be triggered. In clinical isolates, high osmolarity and high  
79 cell density, involving the regulators *oscR* and *hapR* respectively, are both required for  
80 secretion, even though the T6SS is constitutively expressed and produced (20, 21). Globally,  
81 the T6SS regulation also depends on other environmental signals, such as bile (22),  
82 nucleosides levels (23) and chitin (24-27) through the chitin competence pathway. The global  
83 regulators LonA and TsrA also play a role in T6SS regulation. Cold shocks lead to the  
84 production of the regulator *cspV*, which modulates the expression of many genes involved in  
85 cold shock response, biofilm formation and to the up-regulation of the T6SS (28, 29). The  
86 T6SS is also up-regulated by cell wall damages through the two-component system (TCS)  
87 VxrAB (30). Bacteria use TCS, a signal transduction device, to regulate the expression of  
88 diverse stress or virulence factors in response to variations in their environment and  
89 throughout infection (31). TCS are generally composed of a membrane-bound sensor histidine  
90 kinase (HK) and a response regulator (RR), which are activated through subsequent  
91 phosphorylation. In most TCS, the detection of stimuli by HK leads to the  
92 autophosphorylation of its histidine residue, then to the transphosphorylation of the aspartate  
93 residue of the RR (32). The RR acts afterwards as a transcriptional regulator. TCS are  
94 implicated in various processes such as virulence, stress response, environmental adaptation,  
95 and quorum sensing, among others (33). In *V. cholerae*, *vxA* and *vxB* are encoded on the  
96 conserved *vxrABCDE* (VCA0565 - VCA0569) (34). VxrC appears to have an inhibitory role  
97 in biofilm formation (30). As for VxrDE, their role is still unknown although it has been

98 suggested that VxrDE have a minor role in *vpsL* expression without affecting the biofilm  
99 formation (30). While VxrAB are essential for the colonization of infant mouse model, the  
100 contribution of VxrCDE is minor (34).

101

102 Antimicrobial peptides (AMPs) are ubiquitous, small, and mostly cationic peptides produced  
103 by bacteria and host cells for competition, prevention of infections, or to control the  
104 microbiota (35). AMPs are produced in the gut by the microbiota and host cells, and are  
105 effective against a broad range of microorganisms such as viruses, fungi and bacteria (36).  
106 AMPs have many cellular targets, but the most common mechanism of action is an  
107 electrostatic interaction with the negative bacterial membrane that leads to pore formation, the  
108 loss of cytoplasmic content and eventually, cell death (37). Polymyxin B (PmB), a cationic  
109 AMP produced by *Paenibacillus polymyxa*, binds to the lipid A portion of the LPS at the  
110 outer membrane (38). Polymyxins are commonly used in prevention of infections in  
111 veterinary medicine and in the treatment of multidrug resistant infections in human health  
112 (39). Since the absorption of polymyxins is low, a high percentage is excreted through urine  
113 and can accumulate in the environment, including soil and water (40). Polymyxins can thus be  
114 found in subinhibitory concentrations in water (41, 42). Subinhibitory concentrations of  
115 AMPs are known to have a modulatory effect on virulence, persistence and resistance factors  
116 in Gram-negative bacteria (43, 44), including on *V. cholerae* (45-48). El Tor strains are  
117 resistant to PmB conversely to the classical strains they replaced (38).

118

119 We previously demonstrated that subinhibitory concentrations of PmB increase the expression  
120 of Hcp, the structural component of the T6SS syringe in a pandemic strain of *V. cholerae*  
121 (46). PmB alone was not sufficient to induce the secretion through the T6SS in an O1 El Tor  
122 strain in low osmolarity conditions, but increased its production and secretion in a dose

123 dependant manner. Although it did not increase the resistance of *V. cholerae* to antimicrobial  
124 peptides or antibiotics, the increased secretion through the T6SS due to the PmB led to a more  
125 efficient elimination of the competitor bacteria *E. coli* and an increased cytotoxicity towards  
126 amoebas in a T6SS-dependant manner. Furthermore, the increased expression of both *hcp*  
127 genes lets us believe that PmB could act as an activating signal for T6SS production and  
128 secretion (44, 46). In this study, we aim to identify the regulatory pathways involved in the  
129 over expression and over secretion of Hcp in the presence of subinhibitory concentrations of  
130 PmB. A proteomic approach of the cell fraction coupled with a transcriptomic approach using  
131 quantitative PCR, allowed to determine that the presence of subinhibitory concentrations of  
132 PmB has a complex impact on the proteome of *V. cholerae* and greatly modulates the  
133 production of proteins implicated in cellular and metabolic processes, regulation and binding  
134 and catalytic activities. Among the proteins that were more abundant in the presence of PmB,  
135 we identified VxrB, part of the VxrAB TCS and involved in the T6SS regulation. We further  
136 constructed a deficient mutant of *vxrAB* and its Hcp production and expression (*hcp*) were  
137 assessed by western blot and quantitative PCR. Our results suggest that *vxrAB* is, at least  
138 partly, responsible for the increased expression and secretion of Hcp in the presence of PmB  
139 in *V. cholerae*.

## 140 **Material & methods**

141

### 142 **Strains used in this study**

143 *Vibrio cholerae* O1 El Tor strain A1552, an Inaba clinical strain isolated in 1992 from a  
144 Peruvian tourist, as well as its T6SS defective isogenic mutant A1552 $\Delta$ *hcp1-2* were used for  
145 this study (20, 49). Bacterial strains obtained by mutagenesis by natural competence as  
146 previously described (see below) (50) and plasmids used in this study are listed in Table I. *V.*  
147 *cholerae* strains were grown on LB agar plates at 37°C. Isolated colonies were inoculated in 5

148 mL LB broth and incubated 16 h at 37°C with agitation. To obtain the final cultures, 100 µL  
149 of the bacterial suspension were transferred into 5 mL of LB broth with 2% NaCl (LB-  
150 2%NaCl), with or without 3 µg/mL of PmB (Sigma-Aldrich) and incubated at 37°C with  
151 agitation until they reached an optical density at 600nm (OD<sub>600nm</sub>) of 2. When needed, L-  
152 arabinose (0.05 % w/v) or antibiotics (chloramphenicol (2 µg/mL), carbenicillin (50 µg/mL))  
153 were added to the media.

154

### 155 **Proteomic analysis**

156 Proteomic analysis of the bacterial cells grown with or without PmB (3 µg/mL) was  
157 performed by liquid chromatography coupled with tandem mass spectrometry, as described  
158 before (46). The cellular fraction of *V. cholerae* A1552 cultures at OD<sub>600nm</sub> of 2 in  
159 LB2%NaCl in the presence or in absence of PmB were analyzed. The experiment was  
160 conducted in two biological replicates. The proteins were identified using the genome of *V.*  
161 *cholerae* N16961, a strain very closely related to A1552. The data were analyzed using the  
162 Scaffold V.5 software (protein threshold: 99%, with at least two peptides identified and a  
163 false discovery rate of 1%) and we removed the proteins identified in only one of the  
164 replicates. The proteins with differential abundance were then subjected to a Gene Ontology  
165 (GO) term enrichment (51).

166

### 167 **Mutant construction and complementation**

168 A1552Δ*vxrAB*::CmR and A1552Δ*cspV*::CmR defective mutants were obtained using the  
169 optimized natural competence protocol by Marvig & Blokesch (50). All the PCR amplicons  
170 were obtained with Thermo Fisher™ oligonucleotide primers listed in Table II, using Taq  
171 DNA Polymerase with Standard buffer from New England Biolabs®, according to the  
172 manufacturer's instructions. The chloramphenicol resistance cassette (CmR), with its



173 promoter and terminator from pCas9 CR4 (52) (Table I), was amplified by PCR. Up and  
174 downstream regions of target genes of about 1000 bp were amplified from A1552 genomic  
175 DNA by PCR using primers adding 15 bp homology to CmR 5' and 3' extremities (Table II).  
176 The upstream region, the CmR cassette and the downstream region were bound together by  
177 overlap PCR and purified using Monarch DNA Gel extraction kit (New England Biolabs®).

178

179 For the natural transformation, briefly, *V. cholerae* wild type strain A1552 was grown over  
180 night in LB at 30°C with agitation. The culture was diluted 1:50 in fresh LB and incubated at  
181 30°C until it reached an OD<sub>600nm</sub> of 0.5. Bacteria were washed by centrifugation at 5500 x g  
182 for 5 min and suspended in M9 minimal media (Sigma) supplemented with 32 mL of MgSO<sub>4</sub>  
183 1 M and 5.1 mL of CaCl<sub>2</sub> 1 M per liter (50). The bacteria were diluted 1:2 in fresh M9 media.  
184 Chitin powder (Sigma-Aldrich) was added, and the culture was grown overnight at 30°C with  
185 agitation. Two hundred ng of purified PCR amplicon were added to the culture, and the  
186 bacteria were further incubated 24 h at 30°C. To detach the bacteria from chitin, fresh M9  
187 medium was added to the culture and the bacteria were vortexed vigorously for 2 min. The  
188 chitin was pelleted by quick centrifugation and the supernatant containing free bacteria was  
189 centrifuged at 5000 x g for 5 min. The bacteria were suspended in 500 µL of PBS and plated  
190 on LB agar supplemented with 2 µg/mL chloramphenicol (Fisher). Plates were incubated at  
191 30°C for 3 days. Colonies grown on agar supplemented with chloramphenicol were further  
192 isolated on agar plates with chloramphenicol to confirm the phenotype. Deficient mutants  
193 were confirmed with PCR using either *cspV* – verif or *vxrAB* – verif primers (Table II) and  
194 sequencing of the insertion region.

195

196 For complementation, the complete open reading frame of *cspV* or *vxrAB* was amplified by  
197 PCR from purified A1552 genomic DNA using the Thermo Fisher™ oligonucleotide primers

198 adding restriction sites listed in Table II. The amplicons and pBAD24 vector (Table I) were  
199 digested using HindIII and EcoRI (*cspV*) or PstI and KpnI (*vxrAB*) from New England  
200 Biolabs® according to the manufacturer's instructions. Plasmids and amplicons were purified  
201 from agarose gel using Monarch Gel purification kit (New England Biolabs®) and ligated  
202 using T4 ligase from New England Biolabs®. The final construction was introduced by heat  
203 shock into thermocompetent *E. coli* DH5 $\alpha$  at 42°C for 30 s (53). The bacteria were then  
204 suspended in fresh LB and incubated 1.5 h at 37°C. Bacteria were selected on LB agar  
205 supplemented with carbenicillin (50  $\mu$ g/mL) (Fisher). Plasmids were extracted from *E. coli*  
206 using PureYield™ Plasmid Miniprep System (Promega) and suspended in milliQ water  
207 (Thermo Fisher).

208

209 *V. cholerae* was grown in LB for 16 h at 37°C and were made electrocompetent by successive  
210 washes in sterile water supplemented with 10% glycerol. The constructions were  
211 electroporated in the corresponding strains at 1.275 kV, 25  $\Omega$  in 1 mm electroporation  
212 cuvettes (Thermo Fisher). The bacteria were incubated 1.5 h at 37°C in LB, then plated on  
213 LB agar containing 50  $\mu$ g/mL carbenicillin. Colonies were screened by PCR using pBAD24 –  
214 *verif* primers (Table II) to verify the construction.

215

## 216 **Growth curves**

217 *V. cholerae* was grown 16 h at 37 °C with agitation in LB, with carbenicillin when needed. A  
218 1:50 dilution in fresh LB was done, and the bacteria were grown at 37°C to an OD<sub>600nm</sub> of 0.2.  
219 Then, 0.05 % L-arabinose was added, and the bacteria were grown until they reached OD<sub>600nm</sub>  
220 = 0.3. They were further diluted 1:50 in LB2%NaCl - 0.05 % L-arabinose, with or without 3  
221  $\mu$ g/mL of PmB, in 50 ml Falcon tubes, or 1:3000 in 96 wells plates. The bacterial growth was

222 followed by reading the OD<sub>600nm</sub> every 15 min, at 37°C with agitation. Data were obtained  
223 from at least three independent experiments, in technical triplicates.

224

### 225 **RNA extraction, cDNA synthesis and quantitative PCR (RT-qPCR)**

226 *V. cholerae* was grown to an OD<sub>600nm</sub> of 0.5 at 37°C in 10 mL of LB2%NaCl, with or without  
227 3 µg/mL of PmB. The bacteria were suspended in 1 mL TRIzol solution (Invitrogen) and the  
228 total RNA was extracted according to the manufacturer's instructions. Five hundred  
229 nanograms of RNA were retrotranscribed to cDNA using QuantiNova Reverse Transcription  
230 Kit (Qiagen). RNA and cDNA purity and quality were assessed by nanodrop and migration  
231 on 2% agarose gel, respectively. Quantitative PCR analysis was done as described before (46)  
232 with primers listed in Table II. The relative *hcp* and regulators' expression was calculated in  
233 PmB treated bacteria in comparison to non-treated cells using QuantStudio™ Desing and  
234 Analysis Software (Thermo Fisher) v1.5.1 and normalized using *recA*. The results were  
235 obtained from at least 3 independent experiments, in technical triplicates.

236

### 237 **Western blot**

238 Bacteria were grown to an OD<sub>600nm</sub> of 2 in LB2%NaCl, a condition that activates the T6SS  
239 secretion in *V. cholerae* A1552, supplemented with – 0.05 % L-arabinose and 3 µg/mL of  
240 PmB. Samples were treated and western blot conducted as described before (46). Results are  
241 representative of at least three independent experiments.

242

### 243 **Statistical Analysis**

244 All data are expressed as mean ± SD and were analyzed for significance using the SigmaPlot  
245 (version). Student's *t*-tests were used to compare conditions between 2 groups. Single way

246 ANOVA was used for multiple groups comparison. A result was considered as significant  
247 when  $p$  value  $< 0.05$  (\*).

248

## 249 **Results**

250

### 251 **Proteomic analysis of *V. cholerae*'s cells grown with subinhibitory concentrations of**

#### 252 **PmB**

253 To identify the cellular proteins whose abundance is modified in the presence of PmB, a  
254 quantitative proteomic analysis by liquid chromatography coupled to mass spectrometry was  
255 performed on *V. cholerae* A1552 bacterial cells grown in LB2%NaCl with or without 3  $\mu\text{g/ml}$   
256 of PmB, a concentration that does not affect bacterial growth but significantly increases the  
257 expression and secretion of Hcp (46). Our proteomic analysis identified a total of 22,819  
258 peptides corresponding to 473 proteins of 7 to 184 kDa. After data curation, 454 proteins  
259 were obtained. We calculated the relative abundance of each protein in the presence of PmB  
260 in comparison with the control without PmB and determined that 177 proteins (39%) showed  
261 a modified abundance in the presence of PmB (Tables IV to VII). Among them, 130 were  
262 more abundant in the presence of PmB (Table IV), 17 were less abundant in its presence  
263 (Table V), 26 were only found in its presence (Table VI), and 4 were found only in its  
264 absence (Table VII). The proteins showing no modulation are presented in Table SI. The  
265 correlation of the biological replicates was analyzed using scatter plots and demonstrated a  
266 good correlation with R-squared of more than 0.9 (Figure 1AB).

267

268 An analysis of the proteins that are modulated by PmB using Go Annotation showed that  
269 most modulated proteins have catalytic and/or binding function, with 99 and 75 proteins with  
270 these functions, respectively (Figure 1C). Regulatory proteins, transporters, transducers and

271 sensor proteins were also modulated by PmB. The analysis showed that the proteins that are  
272 more abundant in the presence of PmB were mainly implicated in biological (59) and cellular  
273 (75) processes (Figure 1C). Regulation, response to stimulus and locomotion were the  
274 biological processes that were greatly modified by PmB.

275

276 Among the proteins with an increased abundance in the presence of PmB, many have a role in  
277 AMPs or PmB resistance (54). For example, VexB, which is part of the efflux system VexAB  
278 (55), was 2.7 times more abundant in the presence of PmB, and AlmG and AlmE, which are  
279 directly responsible for PmB resistance by LPS modification (56), were 15 and 3.8 times  
280 more abundant with PmB, respectively (Table IV). VprB, only present in the presence of  
281 PmB (Table VI), is part of the TCS VprAB that activates the expression of AlmGE (57). The  
282 protease DegS was also 2.3 times more abundant in the presence of PmB (Table IV). It is  
283 responsible for the activation of the alternate sigma factor RpoE, which activates the repair of  
284 AMPs induced damages in the bacterial envelope (58). VarF is part of an antibiotic efflux  
285 pump (59) and is only found in the presence of PmB (Table VI). OmpR, the RR to the EnvR-  
286 OmpR TCS, is a repressor of virulence factors in response to stress envelope, was also more  
287 abundant in the presence of PmB (Table IV) (60).

288

289 As for the T6SS related proteins, many showed an increased abundance in the presence of  
290 PmB, although a lot of the structural components were not identified in our analysis. As  
291 expected, the main component of the T6SS syringe, Hcp (VC\_1415, VC\_A0017), was also  
292 more abundant (2.3 times) in the presence of PmB (46). VasC (VC\_A0112), a cytoplasmic  
293 protein with an FHA domain essential for secretion (61), and ClpV (VC\_A0116), the ATPase  
294 responsible for recycling of the contractile sheath, presented abundances that were 2.7 and 3.0  
295 times higher in the presence of PmB, respectively. The abundance of VasK (VC\_A0120), a

296 part of the inner-membrane complex (62) was increased by 5.5 times by PmB (Table IV).  
297 TsaB (VC\_1989), the immunity protein to the VgrG-3 that targets the peptidoglycan, had an  
298 increased abundance (2.0 times) in the presence of PmB (Table IV). The abundance of VipA  
299 (VC\_A0107) and VipB (VC\_A0108), the small and large subunits of the contractile sheath,  
300 was not modified by PmB (Table S1).

301

302 We paid a particular attention to the effect of PmB on the T6SS known regulators. As shown  
303 in the table III, our proteomic analysis identified many of them. The abundance of most of  
304 these regulators was not significantly affected by the presence of PmB, except for VxrB that  
305 was 2.7 times more abundant in the presence of PmB (Table III).

306

### 307 **Transcriptomic analysis of known T6SS regulators in the presence of PmB**

308 To confirm the results obtained in the proteomic analysis and to include regulators that were  
309 not identified in this analysis, we performed a transcriptomic analysis of all the known T6SS  
310 regulators of *V. cholerae* A1552 grown in LB2%NaCl supplemented or not with 3 µg/ml of  
311 PmB. To do so, a quantitative PCR approach was used and the relative expression of known  
312 T6SS regulators was calculated by comparing the number of transcripts from bacterial cells  
313 grown with and without PmB and normalized with *recA* (Figure 2A). Although statistically  
314 significant, the expression of cyclic AMP receptor protein (CRP) (1.23x), *tfoY* (1.15x), *tsrA*  
315 (1.17x) and *osrC* (1.26x) was only slightly higher in the presence of PmB, and was not  
316 considered for further analysis. Interestingly, the expression of *vxrB* was 2.1 times higher in  
317 the presence of PmB than in its absence, while the expression of *cspV* was decreased by 2.43  
318 times in its presence.

319

320 We also assessed the expression of 2 genes encoding structural proteins of the T6SS, the main  
321 component of the contractile sheath, VipB, and ClpV, the ATPase protein responsible for the  
322 recycling of the sheath after a contraction event (Figure 2B) (63, 64). Our results showed that  
323 the expression of *vipB* is not modified by PmB (Figure 2B). This result is in line with our  
324 previous work and the proteomic analysis of this study that showed no difference in protein  
325 abundance in the bacterial cell (46). The expression of *clpV* is 1.54 times higher in the  
326 presence of PmB (Figure 2B), which is also in line with our proteomic analysis.

327

328 Based on the results of both proteomic and transcriptomic analysis showing an increased  
329 abundance and expression of VxrB and *vxrB*, respectively, in the presence of PmB, and a  
330 decreased expression of *cspV* in the presence of PmB, we constructed defective mutants of the  
331 regulators *vxrAB* and *cspV* using natural competence (50) and complemented strains using  
332 pBAD24 vector (65). The deletion of *vxrAB* (Figure S1A) or *cspV* (Figure S2A) had no major  
333 consequence on bacterial growth. The growth was slightly impaired by complementation and  
334 PmB for A1552Δ*vxrAB*::CmR (Figure S1B), but not for A1552Δ*cspV*::CmR (Figure S2B).  
335 Like A1552, A1552Δ*cspV*::CmR and A1552Δ*vxrAB*::CmR grew in the presence of PmB at a  
336 concentration up to 100 ug/ml, although the growth of A1552Δ*vxrAB*::CmR was impaired in  
337 all PmB conditions (result not shown), an observation in line with previous studies (66).

338

339 **Quantitative PCR analysis of *hcp* shows that *vxrAB*, but not *cspV*, is responsible for its**  
340 **upregulation in the presence of PmB**

341 To determine the effect of the *vxrAB* and *cspV* deletion on the regulation of *hcp* in the  
342 presence of PmB, we performed a quantitative PCR analysis. *V. cholerae* A1552,  
343 A1552Δ*cspV*::CmR and A1552Δ*vxrAB*::CmR were grown in LB2%NaCl with or without  
344 PmB. We quantified the expression of *hcp1* or *hcp2* by quantitative PCR in the presence of

345 PmB, in comparison to the non-treated cells, and normalized using *recA* (Figure 3 and S3).  
346 The expression of *hcp1* and *hcp2* in A1552 treated with PmB was 3.03 and 2.16 times higher,  
347 respectively, similarly to what we observed before (46) (Figure 3). For A1552 $\Delta$ *cspV*::CmR,  
348 the expression of *hcp1* and *hcp2* is similar to the wildtype strain, and an upregulation of both  
349 *hcp1* and *hcp2* is still observed in the presence of PmB (Figure S3)., indicating that, upon  
350 *cspV* mutation, the T6SS is activated by PmB. However, upon *vxrAB* mutation, the expression  
351 of *hcp1* and -2 was significantly reduced, as expected (34), but was not increased in the  
352 presence of PmB (Figure 3), indicating that the upregulation due to the PmB is no longer  
353 effective.

354

355 The secretion of Hcp in A1552 pBAD24, A1552 $\Delta$ *vxrAB*::CmR pBAD24 and the  
356 complemented strain A1552 $\Delta$ *vxrAB*::CmR pBAD24-*vxrAB* in the presence of PmB was  
357 assessed by western blot (Figure 3C). We previously demonstrated that Hcp secretion by *V.*  
358 *cholerae* A1552 is active in the presence of PmB (46). As expected, the secretion of Hcp is  
359 greatly reduced upon *vxrAB* mutation (Figure 3C). However, a *vxrAB* complementation  
360 completely restored, or even slightly increased, the secretion of Hcp to the level of the WT in  
361 the presence of PmB (Figure 3C).

362

## 363 **Discussion**

364

365 In this study, we aim to determine the impact of subinhibitory concentrations of PmB on the  
366 proteome of *V. cholerae* and to identify the regulatory pathways involved in the over  
367 expression and over secretion of Hcp in the presence of subinhibitory concentrations of PmB.  
368 To do so, we performed a proteomic analysis of the cellular fraction of *V. cholerae* O1 El Tor  
369 strain A1552 in the presence and in absence of PmB. We identified a total of 22,819 peptides



370 corresponding to 454 proteins of 7 to 184 kDa, of which 177 had a modified abundance in the  
371 presence of PmB.

372

373 Several proteins were identified in both our last and current proteomic analysis. It is the case  
374 of Hcp that is more abundant in supernatant and bacterial cells in the presence of PmB, and of  
375 the proteases VesC and DegP, which was expected since they are secreted components. VesC  
376 is secreted through the Type II Secretion System (67), while DegP is secreted by membrane  
377 vesicles (68). A putative hydrolase (VC\_1485) and an immunogenic protein (VC\_0430) were  
378 also more abundant in both analysis in the presence of PmB. While the abundance of the  
379 lipoprotein Lpp and SucD ( $\alpha$  subunit of succinyl-CoA synthetase) was decreased by PmB in  
380 the secretome, they were more abundant in the cell fraction. Two proteins were only present  
381 in the supernatant in the presence of PmB, and more abundant in the cellular fraction, the  
382 amino-acid transporter VC\_0010 and the uncharacterized protein VC\_0483. One protein,  
383 VxrD, was less abundant in both supernatant and bacterial cells in the presence of PmB. Our  
384 results show that *V. cholerae* modulates a large proportion of its components in response to  
385 PmB, as 39% of the identified proteins had their abundance modified in its presence. Most  
386 modulated proteins with increased abundance are implicated in cellular and metabolic  
387 processes, with binding, catalytic or transporter activities, that suggests an adaptation to  
388 survive the presence of the toxic AMP. This is further supported by the upregulation of efflux  
389 systems (VexAB, VprAB, VarF), LPS modifying enzymes (AlmGE), proteases (DegS) and  
390 RpoE activating proteins, all known mechanisms of antimicrobials resistance. Numerous  
391 proteins displaying a modified abundance in the presence of PmB are yet to be described.

392

393 Our previous study has shown that Hcp, the T6SS syringe major component, was more  
394 abundant in the secretome of *V. cholerae* under PmB activation (46). The Hcp syringe is

395 wrapped by the contractile sheath composed of multiple helical polymers of VipAB,  
396 assembled in an extended form (69-71). This extended form provides enough energy, upon a  
397 contraction signal, for a contraction and rotation of VipAB helical polymers that propel the  
398 Hcp syringe, along with the effectors, through the bacterial envelope. Based on the fact that,  
399 conversely to the Hcp secreted protein, the abundance of VipB, a structural recycled  
400 component of the T6SS, was not modified, we hypothesized that PmB might increase the  
401 secretion through the T6SS rather than the number of assembled systems at the bacterial  
402 surface. Our current proteomic analysis of bacterial cells confirms that the contractile sheath  
403 components VipA and VipB are not more abundant in the presence of PmB. ClpV is a AAA+  
404 (ATPases associated with various cellular activities) that hydrolyzes ATP to reshape, or  
405 recycle, various substrates (71). In *V. cholerae*, ClpV recognizes the contracted state of the  
406 T6SS sheath and forces its disassembly by unfolding VipB (70). To our knowledge, VipA/B  
407 are the only recycled proteins through ClpV activity. The recycling of the contractile sheath is  
408 essential and facilitates the effectors' secretion because it makes VipA/B available for further  
409 secretion (70, 72). The presence of more ClpV could help the contractile sheath recycling in a  
410 cell with a T6SS activated by PmB.

411 Our proteomic analysis also revealed that the immunity protein TsaB (14, 73), the inner-  
412 membrane complex subunit VasK (74), and the FHA protein VasC (TagH), required for  
413 secretion (61), are more abundant in the presence of PmB. VasK (TssM) is part of the inner-  
414 membrane complex (74) along with VasF (TssL) and VasD (TssJ), a complex randomly  
415 distributed in the membrane. Once the inner-membrane complex is formed, it recruits the base  
416 plate components, then the Hcp syringe along with the VipA/B sheath (75). It has been  
417 suggested that the inner-membrane complexes are pre-assembled in the membrane, more  
418 abundant than the syringe portion and that they are reused for further secretion events using  
419 new syringe complexes (74). Altogether, our results suggest that more T6SS anchoring

420 complexes might be formed and ready for secretion, while the recycled components  
421 abundance remains constant, and the secreted effectors are more produced to increase the  
422 number of contraction events and the global T6SS activity in the presence of PmB.

423

424 Our proteomic analysis identified many known regulators of the T6SS in *V. cholerae*, *i.e.*  
425 CytR, CRP, TfoY, RpoN, TsrA, OscR and VxrB, but only VxrB had a modified abundance in  
426 the presence of PmB. The expression of *crp*, *tfoY*, *tsrA* and *oscR*, although significantly  
427 higher, was only slightly modified by PmB, while the expression of *cytR* and *rpoN* was  
428 similar to the control. OscR is a transcriptional regulator that represses T6SS genes expression  
429 at low osmolarity (21). CytR is activated upon nucleosides starvation, that further activates  
430 the expression of T6SS (23). RpoN, along with the internal regulator VasH, coordinates the  
431 expression of the different T6SS genes clusters (6). TsrA is a global regulator (76), while  
432 TfoY and CRP are produced in response to c-di-GMP and cyclic-AMP, respectively. While  
433 CspV was not identified in our proteomic analysis, our transcriptomic results showed that  
434 PmB decreases the expression of *cspV*. However, since its expression is reduced in the  
435 presence of PmB and known to be produced upon cold shock (29), it is not surprising that it  
436 was not identified in the proteomic analysis. We constructed a mutant in which *cspV* is  
437 deleted and compared the expression of *hcp1* and *hcp2* in absence and in the presence of PmB  
438 for the wild-type strain and the *cspV* mutant. The expression pattern of both *hcp* genes was  
439 similar for the two strains, thus suggesting that *cspV* is not responsible for the increased *hcp*  
440 expression in the presence of PmB.

441

442 Our proteomic and transcriptomic analysis showed that the presence of PmB increased the  
443 production and expression of VxrB, the response regulator of the two-component system  
444 VxrAB (34). VxrB is a known regulator of *V. cholerae* T6SS, as its deletion decreases the

445 expression of many T6SS related genes, including Hcp (34). In this study, we constructed a  
446 A1552 $\Delta$ *vxrAB*::CmR mutant, the only essential components of the *vxrABCDE* locus, and a  
447 complemented strain. As previously reported, the deletion of *vxrAB* strongly diminished *hcp*  
448 expression (34). The complementation of *vxrAB* using pBAD24 restored the secretion of Hcp  
449 in the presence of PmB. Conversely to the wild type strain, the presence of PmB did not  
450 increase *hcp* expression in A1552 $\Delta$ *vxrAB*::CmR, demonstrating that it is implicated in the  
451 upregulation of *hcp* in the presence of PmB. VxrAB (also known as WigKR) was first  
452 identified for its implication in colonization in the infant mouse model and T6SS regulation  
453 (34). VxrAB has already been described to regulate other systems implicated in antimicrobial  
454 resistance, such as biofilm formation, motility and cell shape maintenance and homeostasis in  
455 the presence of cell wall targeting antimicrobials (30, 66, 77). In the later, they demonstrated  
456 that the expression of *vxrAB* is increased by antibiotic-induced cell wall damage, and that  
457 VxrAB regulates the entire cell wall synthesis pathway, leading to *V. cholerae*'s tolerance to  
458 antibiotics (66). Even though we detected no major damages in the cell wall of *V. cholerae*  
459 A1552 at a concentration of PmB as high as 25  $\mu$ g/mL (47), we observed, in this study, a  
460 significant upregulation of *vxrB* in the presence of 3  $\mu$ g/mL of PmB. Taken together, our  
461 results suggest that in *V. cholerae* O1 El Tor the T6SS is up regulated by PmB, and that the  
462 upregulation involves the TCS VxrAB. The over production of ClpV and VasK also suggests  
463 that the T6SS are more efficiently recycled to facilitate the secretion of effectors.

464

#### 465 **Acknowledgment**

466

467 The authors would like to thank Dre Wai from Laboratory for Molecular Infection Medicine  
468 Sweden (MIMS) at Umeå University for bacterial strains and the RAQ (Ressources  
469 Aquatiques Québec), an inter-institutional group supported financially by the Fonds de

470 recherche du Québec – Nature et technologies (FRQNT) for their support. The proteomic  
471 analysis was performed by the Center for Advanced Proteomics Analyses, a Node of the  
472 Canadian Genomic Innovation Network supported by the Canadian Government through  
473 Genome Canada. This work was supported by the Natural Sciences and Engineering Research  
474 Council of Canada (NSERC; [http://www.nserc-crsng.gc.ca/index\\_eng.asp](http://www.nserc-crsng.gc.ca/index_eng.asp)) Discovery grant  
475 number RGPIN-2017-05322. AM-D received financial support from the NSERC scholarship  
476 program (BESC D3 – 558624 – 2021).  
477

478 **Bibliography**

479

- 480 1. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. Cholera. *Lancet*.  
481 2012;379(9835):2466-76.
- 482 2. Deen J, Mengel MA, Clemens JD. Epidemiology of cholera. *Vaccine*. 2020;38 Suppl 1:A31-A40.
- 483 3. Jutla A, Whitcombe E, Hasan N, Haley B, Akanda A, Huq A, et al. Environmental factors  
484 influencing epidemic cholera. *Am J Trop Med Hyg*. 2013;89(3):597-607.
- 485 4. Vanden Broeck D, Horvath C, De Wolf MJ. *Vibrio cholerae*: cholera toxin. *Int J Biochem Cell*  
486 *Biol*. 2007;39(10):1771-5.
- 487 5. Mathieu-Denoncourt A, Giacomucci S, Duperthuy M. The Secretome of *Vibrio cholerae*. In:  
488 Huang L, Li J, editors. *Vibrios2021*.
- 489 6. Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, et al. Identification of a  
490 conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model  
491 system. *Proc Natl Acad Sci U S A*. 2006;103(5):1528-33.
- 492 7. Joshi A, Kostiuk B, Rogers A, Teschler J, Pukatzki S, Yildiz FH. Rules of Engagement: The Type  
493 VI Secretion System in *Vibrio cholerae*. *Trends Microbiol*. 2017;25(4):267-79.
- 494 8. Crisan CV, Hammer BK. The *Vibrio cholerae* type VI secretion system: toxins, regulators and  
495 consequences. *Environ Microbiol*. 2020;22(10):4112-22.
- 496 9. Unterweger D, Kostiuk B, Ojtjengerdes R, Wilton A, Diaz-Satizabal L, Pukatzki S. Chimeric  
497 adaptor proteins translocate diverse type VI secretion system effectors in *Vibrio cholerae*. *EMBO J*.  
498 2015;34(16):2198-210.
- 499 10. Pukatzki S, Ma AT, Revel AT, Sturtevant D, Mekalanos JJ. Type VI secretion system  
500 translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc Natl Acad*  
501 *Sci U S A*. 2007;104(39):15508-13.
- 502 11. Durand E, Derrez E, Audoly G, Spinelli S, Ortiz-Lombardia M, Raoult D, et al. Crystal structure  
503 of the VgrG1 actin cross-linking domain of the *Vibrio cholerae* type VI secretion system. *J Biol Chem*.  
504 2012;287(45):38190-9.
- 505 12. Zheng J, Ho B, Mekalanos JJ. Genetic analysis of anti-amoebae and anti-bacterial activities of  
506 the type VI secretion system in *Vibrio cholerae*. *PLoS One*. 2011;6(8):e23876.
- 507 13. Miyata ST, Kitaoka M, Brooks TM, McAuley SB, Pukatzki S. *Vibrio cholerae* requires the type  
508 VI secretion system virulence factor VasX to kill *Dictyostelium discoideum*. *Infect Immun*.  
509 2011;79(7):2941-9.
- 510 14. Brooks TM, Unterweger D, Bachmann V, Kostiuk B, Pukatzki S. Lytic activity of the *Vibrio*  
511 *cholerae* type VI secretion toxin VgrG-3 is inhibited by the antitoxin TsaB. *J Biol Chem*.  
512 2013;288(11):7618-25.
- 513 15. Altindis E, Dong T, Catalano C, Mekalanos J. Secretome analysis of *Vibrio cholerae* type VI  
514 secretion system reveals a new effector-immunity pair. *mBio*. 2015;6(2):e00075.
- 515 16. MacIntyre DL, Miyata ST, Kitaoka M, Pukatzki S. The *Vibrio cholerae* type VI secretion system  
516 displays antimicrobial properties. *Proc Natl Acad Sci U S A*. 2010;107(45):19520-4.
- 517 17. Drebes Dorr NC, Blokesch M. Interbacterial competition and anti-predatory behaviour of  
518 environmental *Vibrio cholerae* strains. *Environ Microbiol*. 2020;22(10):4485-504.
- 519 18. Crisan CV, Chande AT, Williams K, Raghuram V, Rishishwar L, Steinbach G, et al. Analysis of  
520 *Vibrio cholerae* genomes identifies new type VI secretion system gene clusters. *Genome Biol*.  
521 2019;20(1):163.
- 522 19. Kitaoka M, Miyata ST, Brooks TM, Unterweger D, Pukatzki S. VasH is a transcriptional  
523 regulator of the type VI secretion system functional in endemic and pandemic *Vibrio cholerae*. *J*  
524 *Bacteriol*. 2011;193(23):6471-82.
- 525 20. Ishikawa T, Rompikuntal PK, Lindmark B, Milton DL, Wai SN. Quorum sensing regulation of  
526 the two hcp alleles in *Vibrio cholerae* O1 strains. *PLoS One*. 2009;4(8):e6734.

- 527 21. Ishikawa T, Sabharwal D, Bröms J, Milton DL, Sjöstedt A, Uhlin BE, et al. Pathoadaptive  
528 conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. *Infect Immun*.  
529 2012;80(2):575-84.
- 530 22. Bachmann V, Kostiuk B, Unterweger D, Diaz-Satizabal L, Ogg S, Pukatzki S. Bile Salts Modulate  
531 the Mucin-Activated Type VI Secretion System of Pandemic *Vibrio cholerae*. *PLoS Negl Trop Dis*.  
532 2015;9(8):e0004031.
- 533 23. Watve SS, Thomas J, Hammer BK. CytR Is a Global Positive Regulator of Competence, Type VI  
534 Secretion, and Chitinases in *Vibrio cholerae*. *PLoS One*. 2015;10(9):e0138834.
- 535 24. Borgeaud S, Metzger LC, Scignari T, Blokesch M. The type VI secretion system of *Vibrio*  
536 *cholerae* fosters horizontal gene transfer. *Science*. 2015;347(6217):63-7.
- 537 25. Lo Scudato M, Blokesch M. A transcriptional regulator linking quorum sensing and chitin  
538 induction to render *Vibrio cholerae* naturally transformable. *Nucleic Acids Res*. 2013;41(6):3644-58.
- 539 26. Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK. Chitin induces natural  
540 competence in *Vibrio cholerae*. *Science*. 2005;310(5755):1824-7.
- 541 27. Metzger LC, Stutzmann S, Scignari T, Van der Henst C, Matthey N, Blokesch M. Independent  
542 Regulation of Type VI Secretion in *Vibrio cholerae* by TfoX and TfoY. *Cell Rep*. 2016;15(5):951-8.
- 543 28. Townsley L, Sison Mangus MP, Mehic S, Yildiz FH. Response of *Vibrio cholerae* to Low-  
544 Temperature Shifts: CspV Regulation of Type VI Secretion, Biofilm Formation, and Association with  
545 Zooplankton. *Appl Environ Microbiol*. 2016;82(14):4441-52.
- 546 29. Datta PP, Bhadra RK. Cold shock response and major cold shock proteins of *Vibrio cholerae*.  
547 *Appl Environ Microbiol*. 2003;69(11):6361-9.
- 548 30. Teschler JK, Cheng AT, Yildiz FH. The Two-Component Signal Transduction System VxrAB  
549 Positively Regulates *Vibrio cholerae* Biofilm Formation. *J Bacteriol*. 2017;199(18).
- 550 31. . !!! INVALID CITATION !!! (73, 74).
- 551 32. Kamal F, Liang X, Manera K, Pei TT, Kim H, Lam LG, et al. Differential Cellular Response to  
552 Translocated Toxic Effectors and Physical Penetration by the Type VI Secretion System. *Cell Rep*.  
553 2020;31(11):107766.
- 554 33. Krell T, Lacal J, Busch A, Silva-Jimenez H, Guazzaroni ME, Ramos JL. Bacterial sensor kinases:  
555 diversity in the recognition of environmental signals. *Annu Rev Microbiol*. 2010;64:539-59.
- 556 34. Cheng AT, Ottemann KM, Yildiz FH. *Vibrio cholerae* Response Regulator VxB Controls  
557 Colonization and Regulates the Type VI Secretion System. *PLoS Pathog*. 2015;11(5):e1004933.
- 558 35. Boparai JK, Sharma PK. Mini Review on Antimicrobial Peptides, Sources, Mechanism and  
559 Recent Applications. *Protein Pept Lett*. 2020;27(1):4-16.
- 560 36. Bevins CL, Salzman NH. Paneth cells, antimicrobial peptides and maintenance of intestinal  
561 homeostasis. *Nat Rev Microbiol*. 2011;9(5):356-68.
- 562 37. Le CF, Fang CM, Sekaran SD. Intracellular Targeting Mechanisms by Antimicrobial Peptides.  
563 *Antimicrob Agents Chemother*. 2017;61(4).
- 564 38. Matson JS, Yoo HJ, Hakansson K, Dirita VJ. Polymyxin B resistance in El Tor *Vibrio cholerae*  
565 requires lipid acylation catalyzed by MsbB. *J Bacteriol*. 2010;192(8):2044-52.
- 566 39. Trimble MJ, Mlynarcik P, Kolar M, Hancock RE. Polymyxin: Alternative Mechanisms of Action  
567 and Resistance. *Cold Spring Harb Perspect Med*. 2016;6(10).
- 568 40. Couet W, Gregoire N, Gobin P, Saulnier PJ, Frasca D, Marchand S, et al. Pharmacokinetics of  
569 colistin and colistimethate sodium after a single 80-mg intravenous dose of CMS in young healthy  
570 volunteers. *Clin Pharmacol Ther*. 2011;89(6):875-9.
- 571 41. Serwecinska L. Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment  
572 and to Public Health. *Water*. 2020;12(12):3313.
- 573 42. Davis CA, Janssen EM. Environmental fate processes of antimicrobial peptides daptomycin,  
574 bacitracins, and polymyxins. *Environ Int*. 2020;134:105271.
- 575 43. Duperthuy M. Antimicrobial Peptides: Virulence and Resistance Modulation in Gram-  
576 Negative Bacteria. *Microorganisms*. 2020;8(2).
- 577 44. Linares JF, Gustafsson I, Baquero F, Martinez JL. Antibiotics as intermicrobial signaling agents  
578 instead of weapons. *Proc Natl Acad Sci U S A*. 2006;103(51):19484-9.

- 579 45. Duperthuy M, Sjöström AE, Sabharwal D, Damghani F, Uhlin BE, Wai SN. Role of the *Vibrio*  
580 *cholerae* matrix protein Bap1 in cross-resistance to antimicrobial peptides. *PLoS Pathog.*  
581 2013;9(10):e1003620.
- 582 46. Mathieu-Denoncourt A, Duperthuy M. Secretome analysis reveals a role of subinhibitory  
583 concentrations of polymyxin B in the survival of *Vibrio cholerae* mediated by the type VI secretion  
584 system. *Environ Microbiol.* 2022;24(3):1133-49.
- 585 47. Giacomucci S, Cros CD, Perron X, Mathieu-Denoncourt A, Duperthuy M. Flagella-dependent  
586 inhibition of biofilm formation by sub-inhibitory concentration of polymyxin B in *Vibrio cholerae*.  
587 *PLoS One.* 2019;14(8):e0221431.
- 588 48. Giacomucci S, Mathieu-Denoncourt A, Vincent AT, Jannadi H, Duperthuy M. Experimental  
589 evolution of *Vibrio cholerae* identifies hypervesiculation as a way to increase motility in the presence  
590 of polymyxin B. *Front Microbiol.* 2022.
- 591 49. Allue-Guardia A, Echazarreta M, Koenig SSK, Klose KE, Eppinger M. Closed Genome Sequence  
592 of *Vibrio cholerae* O1 El Tor Inaba Strain A1552. *Genome Announc.* 2018;6(9).
- 593 50. Marvig RL, Blokesch M. Natural transformation of *Vibrio cholerae* as a tool--optimizing the  
594 procedure. *BMC Microbiol.* 2010;10:155.
- 595 51. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: a library  
596 of protein families and subfamilies indexed by function. *Genome Res.* 2003;13(9):2129-41.
- 597 52. Reisch CR, Prather KL. The no-SCAR (Scarless Cas9 Assisted Recombineering) system for  
598 genome editing in *Escherichia coli*. *Sci Rep.* 2015;5:15096.
- 599 53. Chang AY, Chau VW, Landas JA, Pang Y. Preparation of calcium competent *Escherichia coli*  
600 and  
601 heat-shock transformation *UJEMI.* 2017;1:22-5.
- 602 54. Conner JG, Teschler JK, Jones CJ, Yildiz FH. Staying Alive: *Vibrio cholerae*'s Cycle of  
603 Environmental Survival, Transmission, and Dissemination. *Microbiol Spectr.* 2016;4(2).
- 604 55. Bina JE, Provenzano D, Wang C, Bina XR, Mekalanos JJ. Characterization of the *Vibrio*  
605 *cholerae* vexAB and vexCD efflux systems. *Arch Microbiol.* 2006;186(3):171-81.
- 606 56. Henderson JC, Herrera CM, Trent MS. AlmG, responsible for polymyxin resistance in  
607 pandemic *Vibrio cholerae*, is a glycytransferase distantly related to lipid A late acyltransferases. *J Biol*  
608 *Chem.* 2017;292(51):21205-15.
- 609 57. Herrera CM, Crofts AA, Henderson JC, Pingali SC, Davies BW, Trent MS. The *Vibrio cholerae*  
610 VprA-VprB two-component system controls virulence through endotoxin modification. *mBio.*  
611 2014;5(6).
- 612 58. Mathur J, Waldor MK. The *Vibrio cholerae* ToxR-regulated porin OmpU confers resistance to  
613 antimicrobial peptides. *Infect Immun.* 2004;72(6):3577-83.
- 614 59. Lin HV, Massam-Wu T, Lin CP, Wang YA, Shen YC, Lu WJ, et al. The *Vibrio cholerae* var  
615 regulon encodes a metallo-beta-lactamase and an antibiotic efflux pump, which are regulated by  
616 VarR, a LysR-type transcription factor. *PLoS One.* 2017;12(9):e0184255.
- 617 60. Kunkle DE, Bina TF, Bina XR, Bina JE. *Vibrio cholerae* OmpR Represses the ToxR Regulon in  
618 Response to Membrane Intercalating Agents That Are Prevalent in the Human Gastrointestinal Tract.  
619 *Infect Immun.* 2020;88(3).
- 620 61. Wang G, Fan C, Wang H, Jia C, Li X, Yang J, et al. Type VI secretion system-associated FHA  
621 domain protein TagH regulates the hemolytic activity and virulence of *Vibrio cholerae*. *Gut Microbes.*  
622 2022;14(1):2055440.
- 623 62. Nazarov S, Schneider JP, Brackmann M, Goldie KN, Stahlberg H, Basler M. Cryo-EM  
624 reconstruction of Type VI secretion system baseplate and sheath distal end. *EMBO J.* 2018;37(4).
- 625 63. Kapitein N, Bonemann G, Pietrosiuk A, Seyffer F, Hausser I, Locker JK, et al. ClpV recycles  
626 VipA/VipB tubules and prevents non-productive tubule formation to ensure efficient type VI protein  
627 secretion. *Mol Microbiol.* 2013;87(5):1013-28.



- 628 64. Kube S, Kapitein N, Zimniak T, Herzog F, Mogk A, Wendler P. Structure of the VipA/B type VI  
629 secretion complex suggests a contraction-state-specific recycling mechanism. *Cell Rep.* 2014;8(1):20-  
630 30.
- 631 65. Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level  
632 expression by vectors containing the arabinose PBAD promoter. *J Bacteriol.* 1995;177(14):4121-30.
- 633 66. Dorr T, Alvarez L, Delgado F, Davis BM, Cava F, Waldor MK. A cell wall damage response  
634 mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. *Proc Natl Acad Sci U S A.* 2016;113(2):404-9.  
635
- 636 67. Sikora AE, Zielke RA, Lawrence DA, Andrews PC, Sandkvist M. Proteomic analysis of the *Vibrio*  
637 *cholerae* type II secretome reveals new proteins, including three related serine proteases. *J Biol*  
638 *Chem.* 2011;286(19):16555-66.
- 639 68. Altindis E, Fu Y, Mekalanos JJ. Proteomic analysis of *Vibrio cholerae* outer membrane vesicles.  
640 *Proc Natl Acad Sci U S A.* 2014;111(15):E1548-56.
- 641 69. Broms JE, Ishikawa T, Wai SN, Sjostedt A. A functional VipA-VipB interaction is required for  
642 the type VI secretion system activity of *Vibrio cholerae* O1 strain A1552. *BMC Microbiol.* 2013;13:96.
- 643 70. Kudryashev M, Wang RY, Brackmann M, Scherer S, Maier T, Baker D, et al. Structure of the  
644 type VI secretion system contractile sheath. *Cell.* 2015;160(5):952-62.
- 645 71. Bonemann G, Pietrosiuk A, Diemand A, Zentgraf H, Mogk A. Remodelling of VipA/VipB  
646 tubules by ClpV-mediated threading is crucial for type VI protein secretion. *EMBO J.* 2009;28(4):315-  
647 25.
- 648 72. Pietrosiuk A, Lenherr ED, Falk S, Bonemann G, Kopp J, Zentgraf H, et al. Molecular basis for  
649 the unique role of the AAA+ chaperone ClpV in type VI protein secretion. *J Biol Chem.*  
650 2011;286(34):30010-21.
- 651 73. Miyata ST, Unterweger D, Rudko SP, Pukatzki S. Dual expression profile of type VI secretion  
652 system immunity genes protects pandemic *Vibrio cholerae*. *PLoS Pathog.* 2013;9(12):e1003752.
- 653 74. Durand E, Nguyen VS, Zoued A, Logger L, Pehau-Arnaudet G, Aschtgen MS, et al. Biogenesis  
654 and structure of a type VI secretion membrane core complex. *Nature.* 2015;523(7562):555-60.
- 655 75. Stietz MS, Liang X, Li H, Zhang X, Dong TG. TssA-TssM-TagA interaction modulates type VI  
656 secretion system sheath-tube assembly in *Vibrio cholerae*. *Nat Commun.* 2020;11(1):5065.
- 657 76. Zheng J, Shin OS, Cameron DE, Mekalanos JJ. Quorum sensing and a global regulator TsrA  
658 control expression of type VI secretion and virulence in *Vibrio cholerae*. *Proc Natl Acad Sci U S A.*  
659 2010;107(49):21128-33.
- 660 77. Peschek N, Herzog R, Singh PK, Sprenger M, Meyer F, Frohlich KS, et al. RNA-mediated  
661 control of cell shape modulates antibiotic resistance in *Vibrio cholerae*. *Nat Commun.*  
662 2020;11(1):6067.
- 663 78. Liu J, Chang W, Pan L, Liu X, Su L, Zhang W, et al. An Improved Method of Preparing High  
664 Efficiency Transformation *Escherichia coli* with Both Plasmids and Larger DNA Fragments. *Indian J*  
665 *Microbiol.* 2018;58(4):448-56.
- 666 79. Rogers A, Townsley L, Gallego-Hernandez AL, Beyhan S, Kwuan L, Yildiz FH. The LonA  
667 Protease Regulates Biofilm Formation, Motility, Virulence, and the Type VI Secretion System in *Vibrio*  
668 *cholerae*. *J Bacteriol.* 2016;198(6):973-85.
- 669 80. Joshi A, Mahmoud SA, Kim SK, Ogdahl JL, Lee VT, Chien P, et al. c-di-GMP inhibits LonA-  
670 dependent proteolysis of TfoY in *Vibrio cholerae*. *PLoS Genet.* 2020;16(6):e1008897.

671

672

673 **Tables**

674

675 **Table I Bacterial strains and plasmids used in this study**

Strain/plasmid	General characteristics	Reference
<i>V. cholerae</i>		
A1552	Wild type strain, O1 El Tor, pathogenic strain isolated from human cholera infection	(49)
A1552 $\Delta$ <i>hcp1-2</i>	Hemolysin coregulated protein (Hcp) expression-deficient strain derived from A1552. Deletion of <i>hcp</i> genes from auxiliary clusters 1 and 2. Lacks functional T6SS.	(20)
A1552 $\Delta$ <i>vxrAB</i> ::CmR	A1552 derived strain in which <i>vxrAB</i> (VCA0565-66) have been replaced by a chloramphenicol resistance cassette, with promoter and terminator, from pCas9 CR4	This study
A1552 $\Delta$ <i>cspV</i> ::CmR	A1552 derived strain in which <i>cspV</i> (VCA0933) have been replaced by a chloramphenicol resistance cassette, with promoter and terminator, from pCas9 CR4	This study
<i>E. coli</i>		
DH5 $\alpha$	F- $\phi$ 80 <i>lacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> ) U169 <i>recA1 endA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> , m <sub>K</sub> <sup>+</sup> ) <i>phoA supE44 <math>\lambda</math>-thi-1 gyrA96 relA1</i>	(78)
<b>Plasmids</b>		
pBAD24	Expression vector. Arabinose inducible promoter, resistance cassette to carbenicillin ( <a href="https://www.addgene.org/vector-database/1845/">https://www.addgene.org/vector-database/1845/</a> )	(65)
pBAD24- <i>vxrAB</i>	pBAD24 vector with complete <i>vxrAB</i> open reading frame from A1552 under <i>ara</i> promoter	This study
pBAD24- <i>cspV</i>	pBAD24 vector with complete <i>cspV</i> open reading frame from A1552 under <i>ara</i> promoter	This study
pCas9 CR4	Cas9 nuclease under control of pTet promoter with <i>ssrA</i> tag and constitutive <i>tetR</i> ( <a href="https://www.addgene.org/62655/">https://www.addgene.org/62655/</a> )	(52)

676

677

678 **Table II Primers used in this study**

<b>Mutant construction</b>		
<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
CmR	ACGTTGATCGGCACGTAAGAGGTTCCAAC TTCC ACC	TGGATTCTCACCAATAAAAAACGCCCGGCGGC
<i>vxrAB</i> up	GGCGGCATTAGTCGTTTCTGGCTTATTGCTGAT	CGTGCCGATCAACGTTATCCGGTAAAGAGATA TTCGAGTGTATATTGATGTCAATAATGACA
<i>vxrAB</i> down	GCGTTTTTTATTGGTGAGAATCCAGTGATCATG GTAAAGCCAAATCCTCTTTTATGGTT	GGTTTTGCTAATAACTTGAGATTGTCAATAGC TAGCGGTCCTTGC
<i>vxrAB</i> - cloning	AGCTGGTACCATGCGTTATAGTTTTTGCATGT	AGCTCTGCAGATCAGCTTTCATTTTGTAAACC
<i>vxrAB</i> - verif	TGCAATACTGCACAGCTTTG	GCGTGCTTCAACTGCATAAT
<i>cspV</i> up	CCTTGACCGTTCCAATGCTCATCCAGT	CGTGCCGATCAACGTAGAGACCTCTAGAG ATAATTTT
<i>cspV</i> down	ATTGGTGAGAATCCAAGGAATTCATGTCT CGCAGAAC	CATGAAAATGAGTGGACGACAGAAAAC
<i>cspV</i> - cloning	GCCGAGAAATTCATGTCTACTAAAATGACT GGTT	AGCTTTCGAATTACAGTGCGACTACGTTA GAC
<i>cspV</i> - verif	TAATCTCTTGACAGGGCTTCT	GGATGAGGCAGACATCATTCT
pBAD24 - verif	TTGCCGTCACTGCGTCTTT	CCGCTTCTGCGTTCTGATTTA
<b>qPCR</b>		
<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
CRP	GTCAAATGGCTCGTCGTCT	GAGTCTGTGCGATACGGC
<i>cspA</i>	CTCAAGACAACGGCGGTC	TTGCCTTGCTCAACGGTG
<i>cspV</i>	ACTCAAGACAACGGTGCA	GCCTTTCTACCTTGTTCAACGA
<i>cytR</i>	GCCGAGGTCAGGTATCAATGT	CAGACGGTATGTTGCTGCTG
<i>hapR</i>	GGAGTAGAAGATGCCGTGGA	CCAACCGAACTAACCAACTGC
<i>lonA</i>	CTTCCGCTTTGACTGGGTAC	GTGCGACACCTAAAGATGGC
<i>oscR</i>	GCGTATATGGTGCCTGTCG	CGAAGCAGTGGTTGACTTTGAC
<i>rpoN</i>	TCTACGAACCTGAACCGCA	CGCTGTAGACTTCATCCCAAG
<i>tfoX</i>	GAGCAACATCACCTGAGC	TAGACGACGACTGGCTGCT
<i>tsrA</i>	CGTTCCTGCTTTGATTGCCTA	GCTCGCATTCTGGAACAAC
<i>vxrB</i>	AGATAGTGGCAAGATGACAG	CGTCGTCGGGAAATGGTTA
<i>vipB</i>	GCGTGAATCGGAAGATGCTCG	CCACAGGTAGTGCTCGTG
<i>clpV</i>	CCTTGTCATCAGCGTCA	GCTGCTGCCAACTCTGTGTC
<i>hcp1</i>	CAAACCTAGGGTCTTATCACTGC	GGTCAGTCGGTACAGTACA
<i>hcp2</i>	ATGTACTGCTGACTCTATCGGC	GTCAGTCGGTACGGTTACG
<i>recA</i>	ATTGAAGCGAAATGGGCGATAG	TACACATACAGTTGGATTGCTTGAG

679 CmR: chloramphenicol resistance cassette; Up: 1000 bp upstream region; Down: 1000 bp

680 downstream region; CRP, Cyclic AMP receptor protein.

681

682 **Table III Known regulators of the Type VI secretion system of *V. cholerae* identified in**  
683 **our proteomic analysis.**

Gene		Found in Analysis	Modified abundance in the presence of PmB	Reference
VC_1021	LuxO	No	NA	(20)
VC_2677	CytR	Yes	No	(23)
VC_1153	TfoX	No	NA	(24, 25, 27)
VC_2614	CRP	Yes	No	(20)
VC_0583	HapR	No	NA	(20, 25)
VC_0396	QstR	No	NA	(24)
VC_1722	TfoY	Yes	No	(27)
VC_0117	VasH	No	NA	(6)
VC_2529	RpoN	Yes	No	(6)
VC_0070	TsrA	Yes	No	(76)
VC_A0566	VxrB	Yes	Increased	(34)
VC_A0933	CspV	No	NA	(28)
VC_A0029	OscR	Yes	No	(21)
VC_1920	LonA	No	NA	(79, 80)

684

685

686 **Table IV. Cellular proteins that are more abundant in the presence of polymyxin B in *V.***  
687 ***cholerae* A1552.**

Accession number	Alternate ID		Molecular weight	PmB 3ug/ml #1	PmB 3ug/ml #2	no PmB #1	No PmB #2	Mean 3µg/ml	Mean No PmB	Ratio 3:0
Q9KQG5_VIBCH	VC_2033	AdhE	96 kDa	79	187	62	72	133.0	67.0	2.0
Q9KTZ9_VIBCH	VC_0731	Prdx	23 kDa	39	130	37	38	84.5	37.5	2.3
Q9KUF5_VIBCH	VC_0566	DegP	48 kDa	50	92	27	21	71.0	24.0	3.0
Q9KUT5_VIBCH	VC_0430	Immunogenic protein	35 kDa	48	78	37	25	63.0	31.0	2.0
Q9KNS5_VIBCH	VC_2656	FrdA	66 kDa	54	83	26	40	68.5	33.0	2.1
Q9KUG7_VIBCH	VC_0554	Putative peptidase	106 kDa	50	79	18	12	64.5	15.0	4.3
Q9KV04_VIBCH	VC_0354	FkpA	28 kDa	35	69	25	24	52.0	24.5	2.1
Q9KTF5_VIBCH	VC_0947	DacA	43 kDa	36	56	24	23	46.0	23.5	2.0
Q9KQB6_VIBCH	VC_2084	<i>sucD</i>	30 kDa	30	61	25	21	45.5	23.0	2.0
Q9KSG3_VIBCH	VC_1293	AspC	45 kDa	30	61	22	23	45.5	22.5	2.0
Q9KRZ8_VIBCH	VC_1485	Putative hydrolase	61 kDa	34	39	19	18	36.5	18.5	2.0
Q9KNA6_VIBCH	VCA_0059	Lpp	13 kDa	30	48	17	17	39.0	17.0	2.3
Q9KPK3_VIBCH	VC_2364	ThrA	89 kDa	20	32	13	13	26.0	13.0	2.0
Q9KQ15_VIBCH	VC_2013	PtsG	52 kDa	13	34	10	11	23.5	10.5	2.2
Q9KV51_VIBCH	VC_0306	TrxA	12 kDa	11	35	12	7	23.0	9.5	2.4
Q9KRJ1_VIBCH	VC_1649	VesC	60 kDa	11	39	9	11	25.0	10.0	2.5
Q9KVM9_VIBCH	VC_0112	Cytochrome C4	24 kDa	12	33	9	8	22.5	8.5	2.6
Q9KR86_VIBCH	VC_1756	VexC	40 kDa	11	21	7	9	16.0	8.0	2.0
Q9KQ20_VIBCH	VC_2185	<i>yehF</i>	42 kDa	11	18	7	7	14.5	7.0	2.1
Q9KQL3_VIBCH	VC_1985	FadD	63 kDa	13	19	7	6	16.0	6.5	2.5
Q9KUV9_VIBCH	VC_0402	MshL	60 kDa	12	16	7	7	14.0	7.0	2.0
Q9KTY7_VIBCH	VC_0743	<i>secD</i>	67 kDa	10	22	6	7	16.0	6.5	2.5
Q9KPC9_VIBCH	VC_2443	Hypothetical protein	23 kDa	10	14	5	7	12.0	6.0	2.0
Q9KUE5_VIBCH	VC_0576	SspA	24 kDa	12	14	6	7	13.0	6.5	2.0
Q9KVI2_VIBCH	VC_0164	VexB	112 kDa	10	20	6	5	15.0	5.5	2.7
Q9KMZ1_VIBCH	VC_A0175	MoxR-related protein	35 kDa	11	17	4	2	14.0	3.0	4.7
Q9KU59_VIBCH	VC_0665	VpsR	50 kDa	10	12	7	3	11.0	5.0	2.2
Q9KP11_VIBCH	VC_2568	FklB	22 kDa	8	15	6	4	11.5	5.0	2.3
Q9KQP8_VIBCH	VC_1950	TorZ	89 kDa	11	12	6	4	11.5	5.0	2.3
H9L4Q3_VIBCH	VC_1415	Hep	19 kDa	10	17	4	8	13.5	6.0	2.3
Q9KUN2_VIBCH	VC_0483	Uncharacterized protein	27 kDa	9	11	4	5	10.0	4.5	2.2
Q9KVV1_VIBCH	VC_0027	<i>ilvA</i>	56 kDa	13	13	2	6	13.0	4.0	3.3
Q9KKS3_VIBCH	VC_A1027	<i>malM</i> , putative	31 kDa	6	15	3	4	10.5	3.5	3.0
Q9KQY8_VIBCH	VC_1859	Methyl-accepting chemotaxis protein	77 kDa	6	15	5	4	10.5	4.5	2.3
Q9KU34_VIBCH	VC_0695	phospho-2-dehydro-3-deoxyheptonate-aldolase	40 kDa	8	11	3	5	9.5	4.0	2.4
Q9KKJ3_VIBCH	VC_A1114	<i>parA2</i>	36 kDa	7	13	1	6	10.0	3.5	2.9
Q9KRS3_VIBCH	VC_1563	VarA	37 kDa	9	9	3	0	9.0	1.5	6.0
Q9KNL8_VIBCH	VC_2714	OmpR	27 kDa	7	13	4	3	10.0	3.5	2.9
Q9KRQ7_VIBCH	VC_1579	AlmE	63 kDa	7	12	1	4	9.5	2.5	3.8

Q9KPD2_VIBCH	VC_2440	Uncharacterized protein	59 kDa	10	10	5	5	10.0	5.0	2.0
Q9KPJ1_VIBCH	VC_2376	GltB	164 kDa	9	13	8	2	11.0	5.0	2.2
Q9KUE3_VIBCH	VC_0578	Putative hemolysin	21 kDa	7	13	1	1	10.0	1.0	10.0
Q9KNB8_VIBCH	VC_A0047	Putative multidrug resistance efflux pump (EmrA)	42 kDa	6	12	2	0	9.0	1.0	9.0
Q9KNP3_VIBCH	VC_2688	GlpX	36 kDa	6	11	4	3	8.5	3.5	2.4
Q9KPM4_VIBCH	VC_2343	<i>rada</i>	49 kDa	7	10	5	3	8.5	4.0	2.1
Q9KQK4_VIBCH	VC_1994	<i>sspA</i> . Protease IV	67 kDa	4	13	2	4	8.5	3.0	2.8
Q9KS77_VIBCH	VC_1382	HrpA	150 kDa	5	9	5	2	7.0	3.5	2.0
Q9KTT2_VIBCH	VC_0806	Uncharacterized protein	59 kDa	7	9	3	1	8.0	2.0	4.0
Q9KU23_VIBCH	VC_0706	Vrp	12 kDa	3	11	3	3	7.0	3.0	2.3
Q9KTY8_VIBCH	VC_0742	yajC	12 kDa	3	13	5	3	8.0	4.0	2.0
Q9KKJ2_VIBCH	VC_A1115	parB2	46 kDa	5	7	2	1	6.0	1.5	4.0
Q9KVN3_VIBCH	VC_0108	polA	104 kDa	6	7	4	2	6.5	3.0	2.2
Q9KRQ9_VIBCH	VC_1577	AlmG	31 kDa	6	9	1	0	7.5	0.5	15.0
Q9KVS7_VIBCH	VC_0062	<i>thiE</i>	49 kDa	4	7	3	2	5.5	2.5	2.2
Q9KSD7_VIBCH	VC_1321	Hypothetical protein	116 kDa	5	9	3	1	7.0	2.0	3.5
Q9KPX3_VIBCH	VC_2239	NprII	13 kDa	3	9	2	4	6.0	3.0	2.0
Q9KTI9_VIBCH	VC_0913	VexG	39 kDa	5	5	3	2	5.0	2.5	2.0
Q9KQD2_VIBCH	VC_2068	FlhF	55 kDa	6	6	2	1	6.0	1.5	4.0
Q9KVX8_VIBCH	VC_0010	Amino acid ABC transporter	27 kDa	3	7	4	1	5.0	2.5	2.0
Q9KM23_VIBCH	VC_A0566	VxrB	28 kDa	4	4	1	2	4.0	1.5	2.7
Q9KUE7_VIBCH	VC_0574	Cytochrome b	48 kDa	6	8	3	4	7.0	3.5	2.0
Q9KS28_VIBCH	VC_1433	Hypothetical protein	35 kDa	5	7	2	3	6.0	2.5	2.4
H9L4Q9_VIBCH	VC_2451	RelA	84 kDa	6	5	1	2	5.5	1.5	3.7
Q9KQ39_VIBCH	VC_2165	ArsC	13 kDa	2	7	1	1	4.5	1.0	4.5
Q9KVF2_VIBCH	VC_0194	gamma-glutamyl transpeptidase	63 kDa	5	6	2	0	5.5	1.0	5.5
Q9KR87_VIBCH	VC_1755	Hypothetical protein	22 kDa	4	4	1	3	4.0	2.0	2.0
Q9KN45_VIBCH	VC_A0120	VasK	135 kDa	5	6	1	1	5.5	1.0	5.5
Q9KNS4_VIBCH	VC_2657	Fumarate reductase	28 kDa	2	6	0	3	4.0	1.5	2.7
Q9KN53_VIBCH	VC_A0112	VasC/Fha	55 kDa	4	4	2	1	4.0	1.5	2.7
Q9KVK8_VIBCH	VC_0134	Hypothetical protein	31 kDa	6	4	2	2	5.0	2.0	2.5
Q9KUU1_VIBCH	VC_0424	rraB	16 kDa	2	5	1	2	3.5	1.5	2.3
Q9KVM5_VIBCH	VC_0116	ClpV	56 kDa	1	8	1	2	4.5	1.5	3.0
Q9KSA9_VIBCH	VC_1350	Peroxiredoxin-2D	17 kDa	2	6	2	2	4.0	2.0	2.0
Q9KT26_VIBCH	VC_1079	Hypothetical protein	36 kDa	4	4	2	2	4.0	2.0	2.0
Q9KKM1_VIBCH	VC_A1082	LapD subunit	43 kDa	4	3	3	0	3.5	1.5	2.3
Q9KP64_VIBCH	VC_2512	Hypothetical protein	14 kDa	4	5	1	2	4.5	1.5	3.0
H9L4Q7_VIBCH	VC_2528	LptB	27 kDa	4	5	1	2	4.5	1.5	3.0
Q9KT57_VIBCH	VC_1048	Hypothetical protein	20 kDa	2	6	2	2	4.0	2.0	2.0
Q9KSQ8_VIBCH	VC_1198	Hypothetical protein	114 kDa	3	5	0	2	4.0	1.0	4.0

Q9KQK9_VIBCH	VC_1989	TsaB	26 kDa	3	5	3	1	4.0	2.0	2.0
Q9KUP6_VIBCH	VC_0469	Ribosomal RNA small subunit methyltransferase E	27 kDa	2	6	1	2	4.0	1.5	2.7
Q9KUD3_VIBCH	VC_0589	Putative ATPase-ABC transporter	34 kDa	4	3	2	1	3.5	1.5	2.3
Q9KUW7_VIBCH	VC_0392	Aminotransferase, class V	41 kDa	3	3	1	2	3.0	1.5	2.0
Q9KUU3_VIBCH	VC_0422	TldD	51 kDa	3	5	0	4	4.0	2.0	2.0
Q9KM14_VIBCH	VC_A0575	LysR	33 kDa	3	4	0	2	3.5	1.0	3.5
Q9KR78_VIBCH	VC_1765	HsdR putative	117 kDa	3	3	2	0	3.0	1.0	3.0
H9L4P8_VIBCH	VC_A0446	Haemagglutinin	7 kDa	3	5	2	2	4.0	2.0	2.0
Q9KRY0_VIBCH	VC_1503	Hypothetical protein	33 kDa	2	4	1	2	3.0	1.5	2.0
Q9KRZ7_VIBCH	VC_1486	<i>uup</i>	72 kDa	3	4	3	0	3.5	1.5	2.3
Q9KU33_VIBCH	VC_0696	<i>tyrA</i>	42 kDa	4	4	1	1	4.0	1.0	4.0
Q9KPA8_VIBCH	VC_2465	RseB	36 kDa	3	4	0	1	3.5	0.5	7.0
Q9KSF6_VIBCH	VC_1300	<i>sdaA-1</i>	49 kDa	4	3	1	2	3.5	1.5	2.3
Q9KMW7_VIBCH	VC_A0199	DUF262 domain-containing protein	74 kDa	1	4	1	1	2.5	1.0	2.5
Q9KQN4_VIBCH	VC_1964	Hypothetical protein	32 kDa	3	6	0	2	4.5	1.0	4.5
Q9KPY9_VIBCH	VC_2223	Pseudo uridine synthase family 1 protein	38 kDa	2	5	2	0	3.5	1.0	3.5
Q9KNW7_VIBCH	VC_2613	<i>prkB</i>	33 kDa	3	2	1	1	2.5	1.0	2.5
Q9KQF4_VIBCH	VC_2044	<i>ydhD</i>	12 kDa	3	4	1	2	3.5	1.5	2.3
Q9KUF6_VIBCH	VC_0565	DegS	38 kDa	2	5	0	3	3.5	1.5	2.3
Q9KPR7_VIBCH	VC_2299	<i>ppiA</i>	21 kDa	2	3	0	2	2.5	1.0	2.5
Q9KQL1_VIBCH	VC_1987	Slp	23 kDa	2	2	1	1	2.0	1.0	2.0
Q9KP37_VIBCH	VC_2542	<i>mpl</i>	49 kDa	2	4	1	1	3.0	1.0	3.0
Q9KQU1_VIBCH	VC_1907	<i>cysB</i>	36 kDa	2	3	0	2	2.5	1.0	2.5
Q9KV68_VIBCH	VC_0289	<i>gnt-I</i> transcriptional repressor	36 kDa	2	4	0	1	3.0	0.5	6.0
Q9KUK0_VIBCH	VC_0519	Hypothetical protein	16 kDa	4	3	1	1	3.5	1.0	3.5
Q9KQT9_VIBCH	VC_1909	Hypothetical protein	30 kDa	3	5	0	2	4.0	1.0	4.0
Q9KNG6_VIBCH	VC_2773	<i>parA</i> homologue	28 kDa	3	3	1	1	3.0	1.0	3.0
Q9KLA6_VIBCH	VC_A0840	Putative deoxycytidylate deaminase	18 kDa	3	2	0	2	2.5	1.0	2.5
Q9KL30_VIBCH	VC_A0919	Hypothetical protein	11 kDa	3	2	0	1	2.5	0.5	5.0
Q9KM74_VIBCH	VC_A0514	Putative Zn-protease	25 kDa	2	2	1	1	2.0	1.0	2.0
Q9KQP7_VIBCH	VC_1951	<i>torY</i>	41 kDa	2	2	1	1	2.0	1.0	2.0
Q9KP72_VIBCH	VC_2504	<i>hprA</i>	35 kDa	1	3	1	1	2.0	1.0	2.0
Q9KT28_VIBCH	VC_1077	Hypothetical protein	18 kDa	2	3	0	1	2.5	0.5	5.0
Q9KVB1_VIBCH	VC_0235	<i>wavJ</i>	39 kDa	2	3	2	0	2.5	1.0	2.5
H9L4R0_VIBCH	VC_0260	<i>wbeU</i>	69 kDa	0	4	1	1	2.0	1.0	2.0
Q9KRI6_VIBCH	VC_1655	<i>mgIE</i>	50 kDa	2	4	1	0	3.0	0.5	6.0
Q9KL70_VIBCH	VC_A0877	Putative hydrolase	39 kDa	2	2	0	2	2.0	1.0	2.0
Q9KM44_VIBCH	VC_A0545	5'-nucleotidase.	64 kDa	2	2	1	0	2.0	0.5	4.0

		putative								
Q9KPU2_VIBCH	VC_2270	<i>ribE</i>	24 kDa	2	2	1	1	2.0	1.0	2.0
Q9KQ75_VIBCH	VC_2126	FliM	40 kDa	0	2	0	1	1.0	0.5	2.0
Q9KRN0_VIBCH	VC_1606	Hypothetical protein	52 kDa	1	3	1	1	2.0	1.0	2.0
Q9KVV0_VIBCH	VC_0039	SpoOM-related protein	31 kDa	1	3	1	0	2.0	0.5	4.0
Q9KKM0_VIBCH	VC_A1083	LapD subunit	27 kDa	1	3	1	1	2.0	1.0	2.0
Q9KUU6_VIBCH	VC_0419	<i>cafA</i>	55 kDa	1	2	0	1	1.5	0.5	3.0
Q9KQD9_VIBCH	VC_2061	ParA	29 kDa	1	3	1	0	2.0	0.5	4.0
Q9KT45_VIBCH	VC_1060	SohB	39 kDa	0	3	0	1	1.5	0.5	3.0
Q9KLZ7_VIBCH	VC_A0592	Putative hydrolase	22 kDa	0	3	1	0	1.5	0.5	3.0
Q9KRN4_VIBCH	VC_1602	CheV	37 kDa	0	3	0	1	1.5	0.5	3.0
Q9KMW3_VIBCH	VC_A0205	<i>dcuB</i>	48 kDa	1	3	1	0	2.0	0.5	4.0
Q9KNG2_VIBCH	VC_A0002	<i>rctB</i>	75 kDa	2	0	1	0	1.0	0.5	2.0
Q9KVH2_VIBCH	VC_0174	Hypothetical protein	34 kDa	2	0	0	1	1.0	0.5	2.0
Q9KNF7_VIBCH	VC_A0007	3-hydroxyisobutyrate dehydrogenase, putative	33 kDa	0	2	1	0	1.0	0.5	2.0

688



689 **Table V. Cellular proteins that are less abundant in the presence of polymyxin B in *V.***

690 *cholerae* A1552.

Accession number	Alternate ID		Molecular weight	PmB 3ug/ml #1	PmB 3ug/ml #2	no PmB #1	No PmB #2	Mean 3µg/ml	Mean No PmB	Ratio 3:0
Q9KQZ3_VIBCH	VC_1854	<i>ompT</i>	40 kDa	3	7	25	26	5.0	25.5	0.2
Q9KRP6_VIBCH	VC_1590	AlsS	62 kDa	2	2	4	4	2.0	4.0	0.5
Q9KR21_VIBCH	VC_1826	IIABC mannose permease	66 kDa	1	3	2	8	2.0	5.0	0.4
Q9KM21_VIBCH	VC_A0568	VxrD	42 kDa	0	2	5	5	1.0	5.0	0.2
Q9KS84_VIBCH	VC_1375	Hypothetical protein	27 kDa	0	1	3	1	0.5	2.0	0.3
Q9KL64_VIBCH	VC_A0883	<i>makA</i>	39 kDa	1	0	4	4	0.5	4.0	0.1
Q9KLG5_VIBCH	VC_A0779	Fad-dependant oxidoreductase	53 kDa	0	1	3	2	0.5	2.5	0.2
Q9KT68_VIBCH	VC_1037	<i>mrp</i>	41 kDa	1	1	4	0	1.0	2.0	0.5
Q9KQJ0_VIBCH	VC_2008	Pyruvate kinase II	52 kDa	1	0	2	0	0.5	1.0	0.5
Q9KTX2_VIBCH	VC_0758	Hypothetical protein	43 kDa	1	0	1	4	0.5	2.5	0.2
Q9KTA5_VIBCH	VC_0998	HubP	178 kDa	0	1	2	1	0.5	1.5	0.3
Q9KUQ6_VIBCH	VC_0459	Hypothetical protein	21 kDa	1	0	1	1	0.5	1.0	0.5
Q9KUT7_VIBCH	VC_0428	<i>uspA</i>	16 kDa	1	0	1	3	0.5	2.0	0.3
Q9KPH0_VIBCH	VC_2398	<i>ftsA</i>	46 kDa	1	0	2	2	0.5	2.0	0.3
Q9KT65_VIBCH	VC_1040	Corrinoid adenosyl transferase	22 kDa	0	1	1	1	0.5	1.0	0.5
Q9KMF5_VIBCH	VC_A0405	hypothetical protein	23 kDa	1	0	2	2	0.5	2.0	0.3
E5EUX8_VIBCL	VC_A1025	<i>nagB</i>	23 kDa	1	0	2	0	0.5	1.0	0.5

691

692

693 **Table VI. Cellular proteins that are only found in the presence of polymyxin B in *V.***

694 *cholerae* A1552.

Accession number	Alternate ID		Molecular weight	PmB 3ug/ml #1	PmB 3ug/ml #2	No PmB #1	No PmB #2	Mean 3μg/ml	Mean No PmB
Q9KKN3_VIBCH	VC_A1069	Methyl-accepting chemotaxis protein	70 kDa	6	13	0	0	9.5	0.0
Q9KSD9_VIBCH	VC_1319	VprB	50 kDa	8	11	0	0	9.5	0.0
Q9KXX6_VIBCH	VC_A0974	Methyl-accepting chemotaxis protein	61 kDa	6	8	0	0	7.0	0.0
Q9KV74_VIBCH	VC_0282	Methyl-accepting chemotaxis protein	61 kDa	2	1	0	0	1.5	0.0
Q9KSK4_VIBCH	VC_1252	CinA-like protein	46 kDa	3	7	0	0	5.0	0.0
Q9KQ67_VIBCH	VC_2135	FlrC	52 kDa	2	5	0	0	3.5	0.0
Q9KMZ9_VIBCH	VC_A0167	CalB	29 kDa	2	5	0	0	3.5	0.0
Q9KQ69_VIBCH	VC_2133	FliF	64 kDa	3	7	0	0	5.0	0.0
Q9KP53_VIBCH	VC_2523	Hypothetical protein	35 kDa	2	7	0	0	4.5	0.0
Q9KLR3_VIBCH	VC_A0679	NapB	19 kDa	2	4	0	0	3.0	0.0
Q9KLU7_VIBCH	VC_A0644	NADH oxidase, putative	62 kDa	2	5	0	0	3.5	0.0
Q9KPF1_VIBCH	VC_2417	RecJ	65 kDa	2	4	0	0	3.0	0.0
Q9KQ71_VIBCH	VC_2130	<i>fliL</i>	47 kDa	2	2	0	0	2.0	0.0
Q9KNB7_VIBCH	VC_A0048	Hypothetical protein	15 kDa	1	3	0	0	2.0	0.0
Q9KP12_VIBCH	VC_2567	LysM domain-containing protein	22 kDa	1	1	0	0	1.0	0.0
Q9KV14_VIBCH	VC_0344	AmiB	64 kDa	1	1	0	0	1.0	0.0
Q9KTA8_VIBCH	VC_0995	EIIB/EIIC protein	55 kDa	2	1	0	0	1.5	0.0
Q9KSE9_VIBCH	VC_1308	TyrR	58 kDa	2	2	0	0	2.0	0.0
Q9KQM5_VIBCH	VC_1973	<i>menB</i>	33 kDa	1	4	0	0	2.5	0.0
Q9KTC9_VIBCH	VC_0973	Hypothetical protein	30 kDa	3	0	0	0	1.5	0.0
Q9KKM3_VIBCH	VC_A1080	LapC	54 kDa	1	2	0	0	1.5	0.0
Q9KTD7_VIBCH	VC_0965	PtsI	63 kDa	1	1	0	0	1.0	0.0
Q9KRR8_VIBCH	VC_1568	VarF	25 kDa	1	1	0	0	1.0	0.0
Q9KUR3_VIBCH	VC_0452	MutY	40 kDa	1	2	0	0	1.5	0.0
Q9KT72_VIBCH	VC_1033	Cation transport ATPase	81 kDa	1	2	0	0	1.5	0.0
Q9KMZ2_VIBCH	VC_A0174	DUF58 domain-containing protein	35 kDa	2	1	0	0	1.5	0.0

695

696

697 **Table VII. Cellular proteins that are only found in absence of polymyxin B in *V.***  
 698 ***cholerae* A1552.**

Accession number	Alternate ID		Molecular weight	PmB 3ug/ml #1	PmB 3ug/ml #2	No PmB #1	No PMB #2	Mean 3µg/ml	Mean No PmB
Q9KSG5_VIBCH	VC_1291	Hypothetical protein	73 kDa	0	0	1	1	0.0	1.0
Q9KQK5_VIBCH	VC_1993	<i>fadH</i>	73 kDa	0	0	1	1	0.0	1.0
Q9KVBX0_VIBCH	VC_0018	<i>hspA</i>	17 kDa	0	0	1	1	0.0	1.0
Q9KKW5_VIBCH	VC_A0985	Oxidoreductase/iron-sulfur cluster-binding protein	105 kDa	0	0	2	1	0.0	1.5

699

700

701 **Figures legends**

702

703 **Figure 1. Polymyxin B modifies the proteome of *V. cholerae*.** A quantitative proteomic  
 704 analysis by liquid chromatography coupled to mass spectrometry of *V. cholerae* A1552 grown  
 705 in Type VI Secretion System activating conditions, with or without 3 µg/ml of Polymyxin B  
 706 (PmB) was performed. Scatter plot analysis using the total number of spectra per protein was  
 707 performed to determine the correlation between the biological duplicates of the sample  
 708 without PmB (A) and with 3 µg/ml of PmB (B). A GoAnnotation analysis of the proteins  
 709 identified determined the molecular functions, implication in biological processes and  
 710 localization of the proteins (C).

711

712 **Figure 2. Normalized relative expression of known T6SS regulators (A) and structural**  
 713 **components (B) in *V. cholerae* grown with polymyxin B.** *V. cholerae* A1552 was grown to  
 714 midlog phase in LB2%NaCl supplemented or not with 3 µg/ml of polymyxin B (PmB). Total  
 715 RNA was extracted from cultures and retrotranscribed. The expression of selected regulators  
 716 and structural components was measured by quantitative PCR in the presence of PmB in  
 717 comparison to the non-treated cells, and normalized using *recA*. Data represent mean ± SD of

718 three independent experiments conducted in triplicates. Asterisk represents a significant  
719 difference in expression between treated and non-treated cells ( $P < 0.05$ ). CRP, cyclic AMP  
720 receptor protein.

721

722 **Figure 3. The two-component system VxrAB plays a role in *hcp* upregulation in *V.***  
723 ***cholerae* in the presence of subinhibitory concentrations of polymyxin B. *V. cholerae***  
724 **A1552 and A1552 $\Delta$ *vxrAB*::CmR (A1552 $\Delta$ *vxrAB*) were grown to midlog phase in LB2%NaCl**  
725 **with or without 3  $\mu$ g/ml of polymyxin B (PmB). Total RNA was extracted from cultures and**  
726 **retrotranscribed. The expression of *hcp1* (A) or *hcp2* (B) was measured by quantitative PCR**  
727 **in the presence of PmB in comparison to the non-treated cells, and normalized using *recA*.**  
728 **Data represent mean  $\pm$  SD of three independent experiments conducted in triplicates. Asterisk**  
729 **represents a significant difference in expression between treated and non-treated cells**  
730 **( $P < 0.05$ ). ns, non-significative difference. C) The complementation of *vxrAB* restores the**  
731 **level of Hcp in the supernatant of A1552 $\Delta$ *vxrAB*::CmR pBAD24-*vxrAB*. A1552 pBAD24,**  
732 **A1552 $\Delta$ *vxrAB*::CmR pBAD24 and A1552 $\Delta$ *vxrAB*::CmR pBAD24-*vxrAB* were grown to late**  
733 **exponential phase in LB-2%NaCl in the presence of PmB.**

1 – FIGURES –

2 **Comprehension of Antimicrobial Peptides Modulation of the Type VI Secretion System in**

3 *Vibrio cholerae*

4 **Annabelle Mathieu-Denoncourt and Marylise Duperthuy\*.**

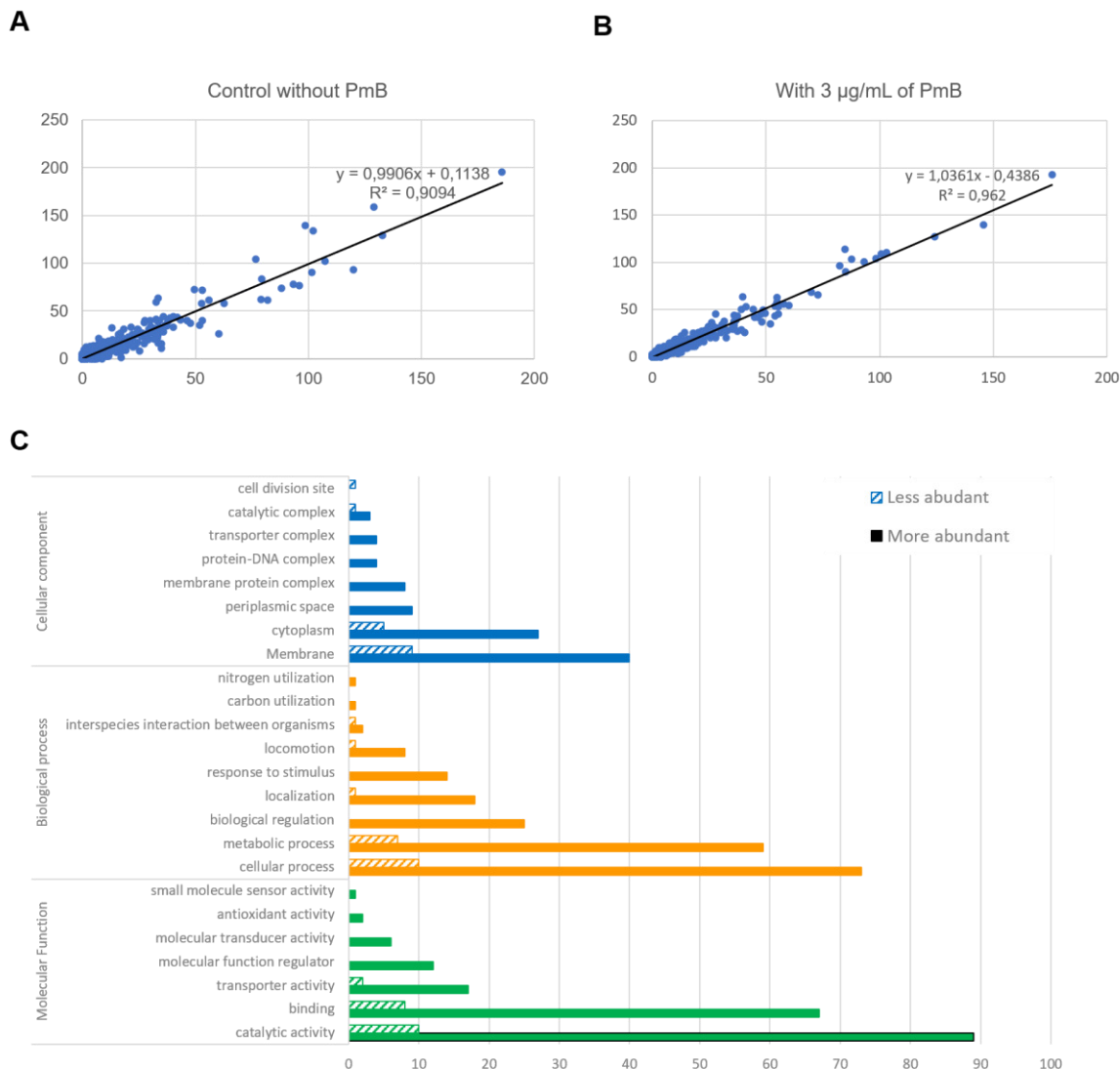
5  
6 Département de Microbiologie, infectiologie et immunologie, Faculté de médecine, Université de  
7 Montréal, Montréal, H3T 1J4, Quebec, Canada.

8  
9 \*Corresponding author: Marylise Duperthuy, Université de Montréal, C.P. 6128, succursale  
10 Centre-ville, Montréal, QC, H3C 3J7, tel : 514 343-6111, fax: 514 343-5701,

11 [marylise.duperthuy@umontreal.ca](mailto:marylise.duperthuy@umontreal.ca)

12

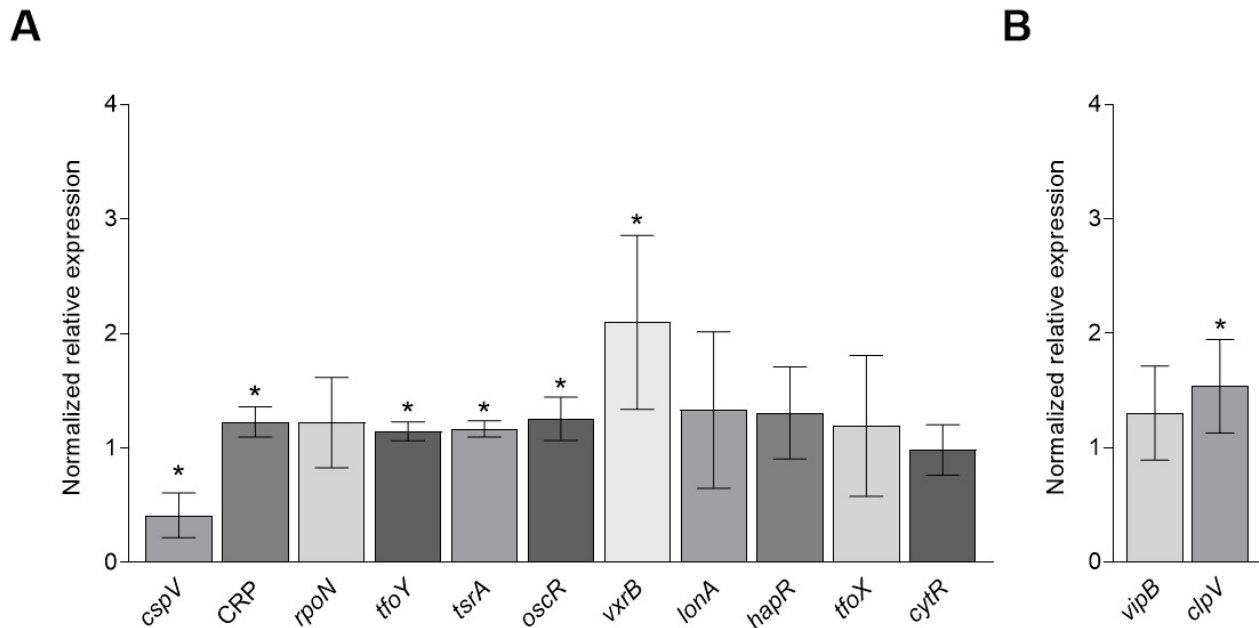
13



14  
15 **Figure 1. Polymyxin B modifies the proteome of *V. cholerae*.** A quantitative proteomic analysis  
16 by liquid chromatography coupled to mass spectrometry of *V. cholerae* A1552 grown in Type VI  
17 Secretion System activating conditions, with or without 3 µg/ml of Polymyxin B (PmB) was  
18 performed. Scatter plot analysis using the total number of spectra per protein was performed to  
19 determine the correlation between the biological duplicates of the sample without PmB (A) and  
20 with 3 µg/ml of PmB (B). A GoAnnotation analysis of the proteins identified determined the  
21 molecular functions, implication in biological processes and localization of the proteins (C).

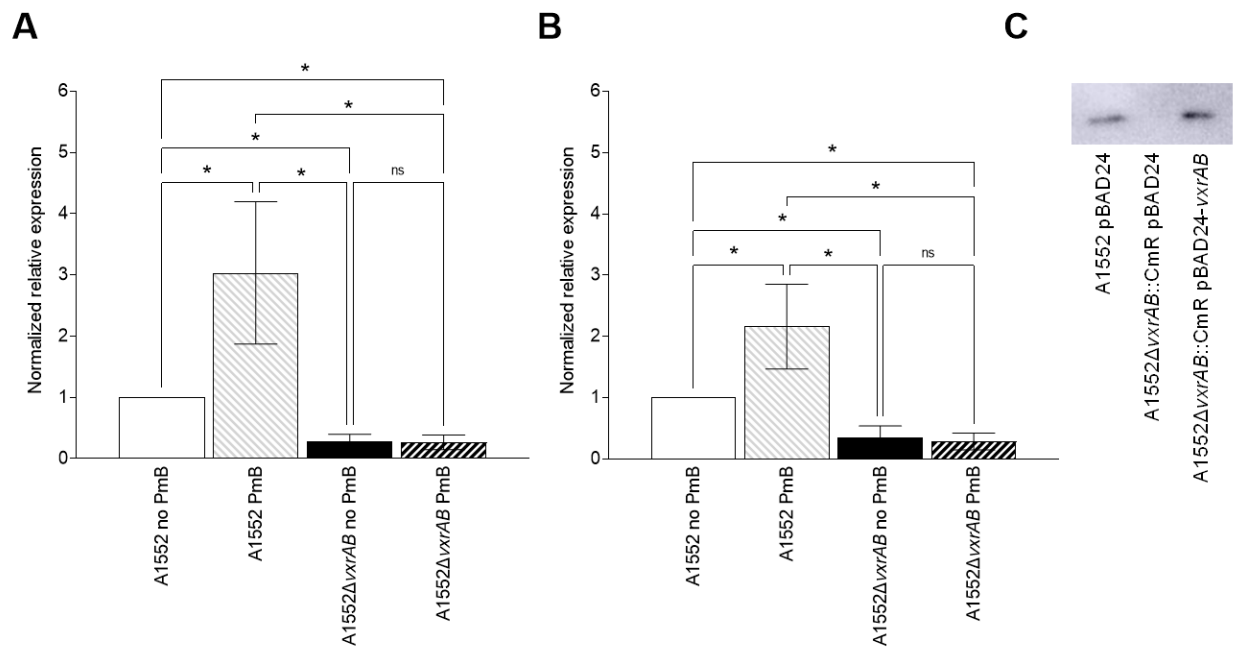
22

23



24 **Figure 2. Normalized relative expression of known T6SS regulators (A) and structural**  
25 **components (B) in *V. cholerae* grown with polymyxin B.** *V. cholerae* A1552 was grown to  
26 midlog phase in LB2%NaCl supplemented or not with 3  $\mu$ g/ml of polymyxin B (PmB). Total RNA  
27 was extracted from cultures and retrotranscribed. The expression of selected regulators and  
28 structural components was measured by quantitative PCR in the presence of PmB in comparison  
29 to the non-treated cells, and normalized using *recA*. Data represent mean  $\pm$  SD of three independent  
30 experiments conducted in triplicates. Asterisk represents a significant difference in expression  
31 between treated and non-treated cells ( $P < 0.05$ ). CRP, cyclic AMP receptor protein.

32



33

34 **Figure 3. The two-component system V<sub>xr</sub>AB plays a role in *hcp* upregulation in *V. cholerae***  
 35 **in the presence of subinhibitory concentrations of polymyxin B.** *V. cholerae* A1552 and  
 36 A1552Δv<sub>xr</sub>AB::CmR (A1552Δv<sub>xr</sub>AB) were grown to midlog phase in LB2%NaCl with or without  
 37 3 μg/ml of polymyxin B (PmB). Total RNA was extracted from cultures and retrotranscribed. The  
 38 expression of *hcp1* (A) or *hcp2* (B) was measured by quantitative PCR in the presence of PmB in  
 39 comparison to the non-treated cells, and normalized using *recA*. Data represent mean ± SD of three  
 40 independent experiments conducted in triplicates. Asterisk represents a significant difference in  
 41 expression between treated and non-treated cells ( $P < 0.05$ ). ns, non-significant difference. C) The  
 42 complementation of v<sub>xr</sub>AB restores the level of Hcp in the supernatant of A1552Δv<sub>xr</sub>AB::CmR  
 43 pBAD24-v<sub>xr</sub>AB. A1552 pBAD24, A1552Δv<sub>xr</sub>AB::CmR pBAD24 and A1552Δv<sub>xr</sub>AB::CmR  
 44 pBAD24-v<sub>xr</sub>AB were grown to late exponential phase in LB-2%NaCl in the presence of PmB.