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1 **Deficiency in cytosine DNA methylation leads to high chaperonin expression and**
2 **tolerance to aminoglycosides in *Vibrio cholerae***

3

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24 **ABSTRACT**

25

26 Antibiotic resistance has become a major global issue. Understanding the molecular
27 mechanisms underlying microbial adaptation to antibiotics is of keen importance to fight
28 Antimicrobial Resistance (AMR). Aminoglycosides are a class of antibiotics that target the
29 small subunit of the bacterial ribosome, disrupting translational fidelity and increasing the
30 levels of misfolded proteins in the cell. In this work, we investigated the role of VchM, a
31 DNA methyltransferase, in the response of the human pathogen *Vibrio cholerae* to
32 aminoglycosides. VchM is a *V. cholerae* specific orphan m5C DNA methyltransferase that
33 generates cytosine methylation at 5'-RCCGGY-3' motifs. We show that deletion of *vchM*,
34 although causing a growth defect in absence of stress, allows *V. cholerae* cells to cope with
35 aminoglycoside stress at both sub-lethal and lethal concentrations of these antibiotics.
36 Through transcriptomic and genetic approaches, we show that *groESL-2* (a specific set of
37 chaperonin-encoding genes located on the second chromosome of *V. cholerae*), are
38 upregulated in cells lacking *vchM* and are needed for the tolerance of *vchM* mutant to lethal
39 aminoglycoside treatment, likely by fighting aminoglycoside-induced misfolded proteins.
40 Interestingly, preventing VchM methylation of the four RCGGY sites located in *groESL-2*
41 region, leads to a higher expression of these genes in WT cells, showing that the expression
42 of these chaperonins is modulated in *V. cholerae* by DNA methylation.

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45 **AUTHOR SUMMARY**

46

47 Bacteria are organisms with a remarkable ability to adapt to several stress conditions,
48 including to the presence of antibiotics. The molecular mechanisms underlying such
49 adaptation lead, very often, to phenomena like antimicrobial tolerance and resistance,
50 responsible for the frequent failure of antibiotic treatment. The study of these molecular
51 mechanisms is thus an important tool to understand development of antimicrobial
52 resistance in bacteria. In this work, we show that abrogating cytosine DNA methylation in
53 *Vibrio cholerae* increases its tolerance to aminoglycosides, a class of antibiotics that cause
54 protein misfolding. DNA methylation is known to affect gene expression and regulate
55 several cellular processes in bacteria. Here we provide evidence that DNA methylation also
56 has a more direct role in controlling antibiotic susceptibility in bacteria. Consequently, the
57 study of bacterial DNA methyltransferases and DNA methylation should not be overlooked
58 when addressing the problem of antimicrobial tolerance/resistance.

59

60 **INTRODUCTION**

61 In the past decades, the over/misuse and large-scale production of antibiotics has
62 created a serious ecological problem with important consequences for the emergence of
63 antimicrobial resistance (AMR). In fact, a large proportion of the antibiotics ingested are
64 released intact in the environment (1, 2) and found at trace levels or as gradients in various
65 environments (3, 4). Hence, in these environments, one can find the presence of very low

66 doses of drugs commonly referred as subMIC, i.e. under the MIC (Minimal Inhibitory
67 Concentration). Although not enough to kill or prevent the growth of bacterial populations,
68 subMIC doses of antibiotics are proposed to work as signaling molecules (5) and trigger
69 important stress mechanisms that often result in development of antibiotic resistance (4,
70 6–8). We have previously shown that subMIC of antibiotics, such as aminoglycosides, trigger
71 common and specific stress responses in Gram-negative bacteria (9, 10).

72 Aminoglycosides (AGs) are positively charged molecules that bind 16S rRNA at the
73 30S ribosomal subunit and negatively affect translation. Specifically, AGs (e.g. tobramycin,
74 streptomycin, kanamycin, gentamicin and neomycin) are known to disrupt translational
75 fidelity and increase the levels of mistranslation, i.e. the misincorporation of certain amino
76 acids in proteins (11, 12). In turn, high levels of mistranslation result in the production and
77 accumulation of aberrant proteins in the cell, which contribute to the collapse of important
78 cell processes and ultimately lead to cell death (13, 14).

79 *V. cholerae* is a water-borne gram-negative bacterium, human pathogen and the
80 causative agent of cholera disease. As part of its life cycle, *V. cholerae* often transits
81 between the human gut and the external environment where it can find low doses of
82 antibiotics. During our studies to better understand adaption of *V. cholerae* to
83 aminoglycosides (15), we observed that a mutant of a *V. cholerae*'s specific DNA
84 methyltransferase (*vca0198* - VchM) was less susceptible to aminoglycosides than its
85 isogenic WT strain, suggesting that DNA methylation could play a role in *V. cholerae*
86 adaptation to AGs. *vchM* codes for an Orphan m5C DNA methyltransferase that causes DNA
87 methylation at 5'-RCCGGY-3' motifs (16). DNA methylation is catalyzed by enzymes called

88 DNA methyltransferases (DNA MTases) that transfer a methyl group from S-adenosyl-L
89 methionine (SAM) to adenine and cytosine in specific DNA motifs (17, 18). As a result, one
90 can find the existence of small amounts of N6-methyl-adenine (6mA), C5-methyl-cytosine
91 (5mC) and N4-methyl-cytosine (4mC) in the DNA of both eukaryotes and prokaryotes. In
92 bacteria, the existence of such modified DNA bases have been shown to play a critical role
93 in processes such as protection against invasive DNA, DNA replication and repair, cell cycle
94 regulation and control of gene expression (19–23).

95 While it was previously proposed that VchM plays a role in the cell envelope stress
96 response of *V. cholerae* (23), no link between this DNA MTase and antibiotic stress has yet
97 been established. Here, we show that deletion of *vchM* (although causing a growth defect
98 in absence of stress) allows *V. cholerae* cells to better deal with the effect of
99 aminoglycosides. In fact, not only the *vchM* mutant is a better competitor during growth in
100 presence of subMIC doses of aminoglycosides, it is also more tolerant to killing by lethal
101 doses of these antibiotics. Transcriptome analysis of a $\Delta vchM$ strain revealed the
102 upregulation of *groESL-2* genes, a specific set of chaperonin-encoding genes located on the
103 second chromosome of *V. cholerae*. High expression of *groESL-2* genes (but not of
104 chromosome one *groESL-1* homologues) determines the higher tolerance of $\Delta vchM$ to
105 lethal AG treatment, suggesting a new and specific role of *groESL-2* in managing AG-
106 mediated proteotoxic stress. Interestingly, we observed the presence of four VchM motifs
107 in *groESL-2* region. Preventing methylation of all these sites in the WT strain by disrupting
108 such motifs results in increased expression of these genes. Intriguingly, the high expression
109 of *groESL-2* does not seem to contribute to the competitive advantage of the $\Delta vchM$ strain

110 grown under subMIC AG which suggests the involvement of additional players in the global
111 response of $\Delta vchM$ to aminoglycosides.

112

113 RESULTS

114

115 ***V. cholerae* cells lacking *vchM* cope better with subMIC doses of AGs**

116

117 In order to explore a possible role of *vchM* in the response of *V. cholerae* O1 El Tor
118 N16961 to aminoglycosides, we constructed an in-frame deletion mutant of *vchM* by allelic
119 replacement with an antibiotic resistance cassette, and compared its growth to the isogenic
120 wild-type (WT) strain, in rich media, with or without increasing concentrations of subMIC
121 tobramycin (Fig 1). As previously described (23), this mutant exhibits a reduced doubling
122 rate when grown in monoculture in antibiotic free rich media. However, the difference in
123 growth between WT and $\Delta vchM$ strains observed in absence of antibiotics becomes
124 gradually more negligible with increasing concentrations of subMIC TOB. At higher
125 concentrations (90% of the MIC), $\Delta vchM$ even displays a clear advantage over the WT (Fig
126 1A). Importantly, a $\Delta vchM$ strain harboring a low-copy number plasmid with *vchM* gene
127 under the control of its own promoter behaves as the WT strain in absence of tobramycin
128 and even slightly worse in presence of higher doses of this drug (Fig 1A), showing that the
129 observed growth phenotypes are due to the absence of *vchM*.

130 Next, we asked whether the growth phenotype observed in monocultures was translatable
131 to a higher relative fitness in co-cultures in the presence of subMIC doses of tobramycin and
132 other AGs. For that, we competed both WT and $\Delta vchM$ strains (both $lacZ^+$) with an isogenic
133 $\Delta lacZ$ mutant (initial ratio of 1:1), in MH or MH supplemented with subMIC concentrations
134 (50% MIC) of the aminoglycosides tobramycin (TOB), gentamicin (GEN) and neomycin
135 (NEO). We assessed relative fitness by plating cultures after 20 hours of growth and
136 counting the final proportion of $lacZ^+/lacZ^-$ colonies. Competition of WT against the $lacZ^-$
137 mutant served as a control to account for any effect of $lacZ$ deletion on growth. Supporting
138 the previous results in monocultures, $\Delta vchM$ is outcompeted by the $lacZ^-$ mutant in MH
139 (≈ 10 -fold difference) (Fig 1B). More importantly, in presence of low concentrations of
140 aminoglycosides, $\Delta vchM$ is either equally competitive or even displays a clear growth
141 advantage over the reference strain (Fig 1B). Additionally, in order to test whether these
142 results hold for drugs other than aminoglycosides we performed competitions in the
143 presence of chloramphenicol (CAM) and the beta-lactam carbenicillin (CARB). Unlike AGs,
144 the presence of low concentrations of these drugs did not increased the relative fitness of
145 the $\Delta vchM$ mutant.

146 Altogether, these results confirm that lack of $vchM$ in *V. cholerae* negatively impacts growth
147 in antibiotic-free media (23) but confers a selective advantage to *V. cholerae* in presence of
148 subMIC doses of AGs (Fig 1). In order to test if deleting $vchM$ affects the MIC of these drugs,
149 we measured the MIC of both WT and $\Delta vchM$ mutant and found no difference (Table 1).

150

151

152

Table 1. MICs ($\mu\text{g/ml}$) of the different antibiotics tested for *V. cholerae* N16961 WT and $\Delta vchM$ strains

153

Strain	TOB	GEN	NEO	CAM	CARB
WT	1-2	1	4	1	15
$\Delta vchM$	1-2	1	4	1	7.5

154

155

TOB, tobramycin; GEN, gentamicin; NEO, neomycin; CAM, chloramphenicol; CARB, carbenicillin

156

157

158 **VchM deficiency promotes higher tolerance to lethal doses of aminoglycosides**

159

160 It has been previously shown that certain mutations affect functions conferring
161 bacterial populations a tolerant phenotype towards a specific drug (24). Such bacterial
162 populations can transiently withstand lethal doses of that drug without necessarily any
163 impact on the MIC of the population (24).

164 To continue exploring the different susceptibility of the $\Delta vchM$ strain to aminoglycosides
165 we assessed the survival rate of *V. cholerae* WT and $\Delta vchM$ strains during treatment with
166 lethal doses of tobramycin and gentamicin at 20x and 10x the MIC, respectively (Fig 2).
167 Given the inherent growth defect of VchM deficiency, we performed time-dependent killing
168 curves on stationary phase cells, where both WT and $\Delta vchM$ strains are no longer actively
169 growing, excluding a possible link between growth rate and aminoglycoside lethality as
170 previously shown (25). Strikingly, survival to both antibiotics was increased 10-1000 fold in
171 the $\Delta vchM$ mutant, suggesting that the absence of VchM allows *V. cholerae* to transiently
172 withstand lethal doses of these aminoglycosides (Fig 2). We additionally tested the

173 tolerance of exponential phase cells of WT and $\Delta vchM$ mutant strains to lethal
174 concentrations of tobramycin and observed similar results (S1 Fig).

175 One crucial aspect that determines the efficacy of aminoglycoside treatment is the uptake
176 of these drugs by the bacterial cell. This process is energy dependent and requires a
177 threshold membrane potential (26). We used a previously reported assay that measures
178 cell fluorescence after incubation with fluorescent-marked neomycin (neo-cy5) (27) as a
179 proxy for aminoglycoside uptake. We did not observe any difference in fluorescence
180 between WT and $\Delta vchM$ mutant strains (S2 Fig). Thus, differential uptake of
181 aminoglycosides is unlikely the reason for the increased tolerance to these drugs in $\Delta vchM$.

182

183

184 **A specific set of chaperonins is upregulated in $\Delta vchM$ cells**

185

186 To understand the high tolerance to aminoglycosides observed in $\Delta vchM$, we
187 performed RNA-seq on stationary phase cells of WT and $\Delta vchM$ strains grown in rich, stress-
188 free media. The analysis of the transcriptome of $\Delta vchM$ *V. cholerae* O1 El Tor N16961 strain
189 reveals the significant upregulation (fold change ≥ 2 , $p < 0.01$) and downregulation (fold
190 change ≤ -2 , $p < 0.01$) of 68 and 53 genes, respectively (S1 Table). Among the differentially
191 expressed, we found four genes directly involved in protein folding to be upregulated in
192 $\Delta vchM$ strain. Those are the molecular chaperones GroEL and co-chaperonins GroES (Table
193 2). In many bacterial species, GroEL and its co-chaperonin GroES form a molecular machine
194 essential for folding of large newly synthesized proteins also helping re-folding of proteins

195 damaged by proteotoxic stress (28). Interestingly, overexpression of GroES and GroEL
196 proteins was found to promote short-term tolerance to aminoglycoside-induced protein
197 misfolding in *E. coli* (29).

Table 2. Protein folding and stabilization genes upregulated in $\Delta vchM$ (fold change > 2, p-value < 0.01)

Locus	Name	Fold change ($\Delta vchM$ /WT)	Annotation
vc2665	groEL-1	2.24	Chaperonin, 60-kDa subunit
vca0820	groEL-2	3.54	Chaperonin, 60-kDa subunit
vc2664	groES-1	2.60	Chaperonin, 10-kDa subunit
vca0819	groES-2	6.78	Chaperonin, 10-kDa subunit

198

199 *V. cholerae* is one of, at least, seven *Vibrio* species harboring two copies of *groES* –
200 *groEL* (*groESL*) bicistronic operons (30). Whereas *groESL-1* is encoded in chromosome 1
201 (*vc2664-vc2665*), *groESL-2* is located in chromosome 2 (*vca0819-0820*) (Fig 3A) (30). Based
202 on our RNA-seq data, the latter manifested a larger fold change (Table 2). In order to
203 confirm differential expression of these genes in $\Delta vchM$, we measured *groES-1* and *groES-*
204 *2* relative gene expression in exponential and stationary phase cells of WT and mutant
205 strains, using digital qRT-PCR with the housekeeping *gyrA* gene as reference (31). The
206 results confirm a higher induction of *groES-2* genes in both exponential and stationary
207 phase $\Delta vchM$ cells with a fold change (over the WT) of ca. 10X and 5X, respectively (Fig 3B).
208 However, *groES-1* fold change was only slightly increased in exponential and unnoticeable
209 in stationary phase.

210 Induction of *groESL* genes is usually associated to perturbations in proteostasis
211 which leads to activation of the heat-shock response (32). Indeed, expression of both

212 *groESL-1* and *groESL-2* is controlled by the heat-shock alternative sigma factor RpoH in *V.*
213 *cholerae* (33). However, the upregulation of *groESL-2* genes in $\Delta vchM$ cells is likely
214 independent of heat-shock activation as i) we do not observe any other genes of the heat-
215 shock regulon being upregulated in the mutant (for example, *dnaKJ/grpE*, *clpB*, *ibpAB*) and
216 ii) expression of *groESL-1* is not as increased as expression of *groESL-2* (Fig 3B).

217 Altogether, these results confirm that in absence of *VchM*, expression of *groESL-2*
218 genes is markedly increased in *V. cholerae* and suggest that regulation of *groESL-2* operon
219 in the $\Delta vchM$ mutant can be independent of heat-shock response.

220

221 **Deletion of *groESL-2* operon abolishes $\Delta vchM$ high tolerance to lethal doses of tobramycin**

222 In bacteria that harbor a single copy of this operon, GroESL are essential proteins for
223 cell viability (34). However, possible redundancy between *groESL-1* and *groESL-2* could
224 allow for the deletion of one or the other operon in *V. cholerae*. Thus, we attempted to
225 delete *groESL-1* and *groESL-2* from *V. cholerae* WT and $\Delta vchM$ strains. While $\Delta groESL-2$ and
226 $\Delta vchM groESL-2$ strains were easily obtained, we could not manage to delete *groESL-1* in
227 any background despite several attempts. Moreover, deletion of *groESL-2* did not affect the
228 growth of *V. cholerae* in rich medium (Fig 4A). Respectively, GroES-1 and GroEL-1 share 80%
229 and 87% amino acid identity with the only and essential GroES and GroEL proteins of *E. coli*,
230 but lower (66% and 76%) amino acid identity with GroES-2 and GroEL-2 (S3 Fig). These
231 observations suggest that i) GroESL-1 (but not GroESL-2) is essential for *V. cholerae* viability
232 and ii) GroESL-1 is probably the main housekeeping chaperonin system while the divergent

233 GroESL-2 could act synergistically in response to high levels of misfolding or having specific
234 substrates upon protein damage caused by specific stresses. Surprisingly, competition of
235 $\Delta groESL-2$ with a lacZ⁻ strain shows that loss of these proteins is not detrimental for growth
236 of *V. cholerae* in presence of subMIC TOB (S4A Fig). Similarly, survival of the $\Delta groESL-2$ strain
237 to lethal doses of TOB does not differ from that of the WT (S4B Fig). However, these genes
238 are intrinsically highly expressed in $\Delta vchM$ strain, where they may confer a selective
239 advantage in presence of AG stress. In this case, deletion of *groESL-2* in $\Delta vchM$ background
240 would affect the mutant's tolerance. Indeed, when we compared the survival to lethal AG
241 treatment of $\Delta vchM$ to that of a $\Delta vchM groESL-2$ double mutant, we found that the absence
242 of *groESL-2* abolishes high tolerance to tobramycin and gentamicin in $\Delta vchM$ (Fig 4B),
243 without affecting growth in absence of stress (Fig 4A). These results show that the higher
244 expression of *groESL-2* is required for the high tolerance of the $\Delta vchM$ mutant to lethal AG
245 treatment. Importantly, we assessed whether *groESL-2* locus is part of *V. cholerae*'s intrinsic
246 stress response to AGs, and we observed induction of this locus by both subMIC and lethal
247 concentrations of tobramycin (S5 Fig)"

248 We then tested whether the high tolerance of this mutant relies on general higher
249 levels of chaperonins or if it specifically linked to GroESL-2 chaperonins. We thus tried to
250 complement the $\Delta vchM groESL-2$ mutant by ectopically expressing *groESL-1* or *groESL-2*
251 and assessed survival to lethal doses of AGs. Strikingly, only overexpression of *groESL-2* is
252 able to promote survival levels similar to those observed in $\Delta vchM$ (Fig 4C), suggesting a
253 specific role for GroESL-2 in managing AG-mediated proteotoxic stress in *V. cholerae* cells
254 lacking VchM. Interestingly, we observed no difference in the relative fitness of $\Delta vchM$ and

255 *ΔvchM groESL-2* mutants in competitions in presence of subMIC doses of tobramycin, which
256 shows that *groESL-2* it is not implicated in *ΔvchM* higher relative fitness during growth in
257 subMIC AGs (Fig 4D).

258 **VchM controls *groESL-2* expression in part through direct DNA methylation**

259 Knowing the role of VchM in regulating gene expression in *V. cholerae* (23), we asked
260 whether VchM controls *groESL* expression directly through DNA methylation. VchM
261 methylates the first cytosine in 5'-RCCGGY-3' motifs (16). This prompted us to search for
262 such motifs in both *groESL* operons. While we couldn't detect any of these sites along the
263 *groESL-1* locus, we found a total of four VchM motifs in *groESL-2* region: motif #1 within the
264 5' UTR of the operon, 47 bp away from the initiation codon; motif #2 is within the coding
265 region of *groES-2* while motifs #3 and #4 are located within the coding region of *groEL-2*
266 (Fig 5A). We hypothesized that the methylation state of these motifs could modulate the
267 transcription of *groESL-2* genes. To test this, we generated a mutant by replacing all RCCGGY
268 motifs in *groESL-2* region by non-consensus motifs but maintaining the amino acid
269 sequence of GroESL-2 proteins intact (Fig 5A, mut#1-4). Additionally, we created a mutant
270 where only the RCCGGY #1 was altered in order to investigate if this site, for being in the
271 regulatory region of this operon, had a stronger contribution in modulating gene expression
272 (Fig 5A, mut#1). We then measured *groES-2* expression in both mutants and observed that
273 disruption of RCCGGY #1 lead to a very weak increase in *groES-2* expression relative to the
274 WT, while disruption of all four sites led to a significantly higher expression of this gene (Fig
275 5B), although not as high as in the *ΔvchM* mutant. We additionally tested *groEL-2*
276 expression and observed similar results (S6 Fig). Supporting our hypothesis that this

277 regulation is methylation-dependent, we did not observe any difference in *groES-2* or
278 *groEL-2* expression when we mutated sites #1-4 in the $\Delta vchM$ background (S6 Fig). It is
279 worth mentioning that, in these experiments, the expression of *groES-2* in the $\Delta vchM$ strain
280 was consistently higher than in the WT mut#1-4 (Fig 5B) suggesting that an additional factor
281 (e.g. an unknown DNA methylation-sensitive transcription factor), in synergy with the
282 methylation of RCCGGY sites, controls expression of *groES-2*. Nonetheless, overall these
283 results show that a specific set of chaperonin encoding genes is under the control of DNA
284 cytosine methylation in *V. cholerae*, linking DNA methylation to modulation of chaperonin
285 expression and tolerance to antibiotics.

286

287

288 **DISCUSSION**

289 Antimicrobial resistance (AMR) is currently one of the biggest threats to global
290 health (35). It is thus urgent to not only find new and alternative ways to fight bacterial
291 infections but also to understand how bacteria adapt to the presence of antibiotics and
292 study the molecular mechanisms they use to circumvent antibiotic action.

293 In this study, we establish a previously unknown link between VchM-mediated DNA
294 methylation and aminoglycoside susceptibility in the human pathogen *V. cholerae*. VchM is
295 a relatively understudied orphan DNA methyltransferase only found in *V. cholerae* species,
296 known to methylate the first cytosine at 5'-RCCGGY-3' DNA motifs (16, 23). VchM is
297 necessary for the optimal growth of *V. cholerae*, both *in vitro* and *in vivo*, and it was shown

298 to repress the expression of a gene important for cell envelope stability through direct DNA
299 methylation (23).

300 Here we show that despite the growth defect in stress-free medium, cells lacking
301 VchM are also less susceptible to aminoglycoside toxicity. Specifically, we show that these
302 cells have a higher relative fitness in presence of low AG concentrations. The reason for this
303 can be inferred from the growth curves in presence of subMIC TOB (Fig 1A) where it is clear
304 that small increments in TOB concentration lead to a higher toxicity in the WT strain when
305 compared to the $\Delta vchM$. Moreover, even though the MIC values for the tested AGs are the
306 same in both strains, $\Delta vchM$ displays a higher tolerance to lethal aminoglycoside treatment
307 (Fig 2).

308 Aminoglycosides are a well-known class of antimicrobial drugs that cause disruption
309 of the translation process and consequently protein misfolding (14, 36). The exact
310 mechanism underlying the bactericidal activity of aminoglycosides has been subject of
311 debate in the literature (37) but it is generally accepted that killing by AGs involves i) the
312 uptake of the AG into the cytoplasm (11, 38, 39) and ii) membrane disruption mediated by
313 insertion of misfolded proteins in the membrane as consequence of AG binding to the
314 ribosomes and disruption of translational fidelity (11–13, 40). Indeed, mechanisms
315 modulating aminoglycoside tolerance/resistance in different bacterial species (in
316 exponential or stationary phase) have been shown to be associated either to AG uptake
317 (41–44) or to translational fidelity and proteostasis (14, 29, 40, 45). Our results revealed a
318 higher relative abundance of *groESL-2* transcripts in bacterial cells lacking VchM, which led
319 us to hypothesize that such increased expression of these chaperonins could underlie the

320 high tolerance to AGs observed in this mutant, as it had been previously observed in *E. coli*
321 (29). In fact, we show that stationary phase cells lacking both *vchM* and *groESL-2* genes have
322 similar or even lower tolerance to lethal AG treatment compared to the WT strain. However,
323 we could not observe a significant increase in tolerance upon overexpression of *groESL-2* in
324 the WT strain (S7 Fig), suggesting that high *groESL-2* levels alone do not explain the high
325 tolerance to lethal AG treatment. Instead, it is possible that high levels of GroESL-2
326 chaperone system counteract AG-mediated misfolding of specific substrates present only
327 in cells devoid of VchM.

328 *V. cholerae* harbors two copies of *groESL* operon in its genome, thus belonging to
329 the group of 30% of bacterial species that contains multiple copies of these chaperonins
330 (46, 47). An interesting question to ask is whether these extra copies of chaperonins are
331 functionally redundant or have a more specialized role in the cell, as it had been observed
332 for *Myxococcus xanthus* (46–48). Supporting the latter hypothesis, we show here that the
333 high tolerance observed in $\Delta vchM$ is dependent on the high expression of *groESL-2* but not
334 on the high expression of *groESL-1* (Fig 4B). Amino acid identity comparison between these
335 proteins suggests that *Vc* GroESL-1 is likely the orthologue of the housekeeping GroESL of
336 *E. coli* whereas *Vc* GroESL-2, thought to have appeared by duplication in *V. cholerae* (30),
337 differ equally from both (S3 Fig). Thus, we speculate that *V. cholerae* GroESL-2 constitutes
338 an alternative chaperone system capable of helping the folding of specific substrates
339 important for survival to specific stresses. The existence of an alternative *groESL-2* locus in
340 *V. cholerae* that is needed for aminoglycoside tolerance only in certain contexts is a new
341 finding and it paves the way for the study of potential alternative roles of otherwise thought

342 redundant chaperonin systems in bacteria. It would be interesting, in future studies, to
343 assess in depth how GroESL-2 interacts with specific substrates and which stress conditions
344 demand one of the chaperonins in preference to the other.

345 Interestingly, even though essential for the higher tolerance to lethal
346 aminoglycoside treatment, *groESL-2* is not involved in the increased relative fitness of the
347 $\Delta vchM$ mutant in presence of subMIC doses of aminoglycosides (Fig 4C). This suggests that
348 the mechanisms operating in $\Delta vchM$ cells that increase their relative fitness during growth
349 in subMIC AGs are not the same that increase their tolerance to lethal doses of these drugs.
350 In fact, it has been recently shown that the type of translation errors occurring at lower
351 streptomycin (another AG) concentrations differ from those found in high concentrations
352 of this aminoglycoside, with the latter being associated to a higher misfolding propensity
353 (12). Thus, it seems plausible that, in $\Delta vchM$ cells, the higher expression of *groESL-2* is likely
354 to be more important at high concentrations of AGs, when the abundance of misfolded
355 proteins tend to increase. The mechanisms driving $\Delta vchM$ higher relative fitness at lower
356 doses of aminoglycosides remain to be elucidated in future work.

357 DNA methylation controls gene expression through modulation of protein-DNA
358 interactions (49). In most of the cases, the methylated base interferes with the binding of
359 transcription factors and/or the RNA polymerase at the regulatory region of a gene,
360 affecting transcription (50–52). However, there is also evidence that the presence of
361 methylated DNA bases that occur along the coding region of genes could also directly affect
362 their expression in bacteria, even though the precise mechanism is still unknown (20, 22,
363 23). In eukaryotes, cytosine methylation tends to repress gene expression. A recent study

364 shedding light on how cytosine methylation affect DNA mechanical properties shows that
365 cytosine methylation stabilizes the DNA helix and slows transcription in eukaryotic cells
366 (53). Thus, a similar m5C-mediated transcriptional hindrance is likely to happen also in
367 prokaryotes. Here we support this view by showing that abrogation of VchM-dependent
368 methylation of cytosines at the four RCGGY motifs in *groESL-2* region increased its
369 expression in WT cells (Fig 5B). However, this is unlikely the sole mechanism responsible
370 for the high expression of *groESL-2* genes in $\Delta vchM$ cells, as this mutant has even higher
371 expression levels of *groESL-2*. This suggests that the pleiotropic effects resulting from VchM
372 deficiency also affect, indirectly, the expression of these genes maybe through regulation
373 of a methylation sensitive transcription factor. Thus, VchM controls *groESL-2* expression in
374 part in a direct and in an indirect way.

375 Our work shows that a *V. cholerae* deletion mutant of the orphan DNA methyltransferase
376 VchM have a general higher tolerance towards aminoglycosides. It remains to be explored
377 whether *V. cholerae* WT cells can modulate VchM expression and, consequently, alter the
378 levels of cytosine methylation. Bisulfite sequencing analysis of *V. cholerae* genome shows
379 that all cytosines within RCGGY motifs were methylated in *V. cholerae*, during exponential
380 and stationary phases, with the exception of three of these sites which had been previously
381 shown to be constantly undermethylated in this species (54). However, these studies were
382 conducted in cells cultured in LB stress-free media or collected from frozen rabbit cecal
383 fluid, and thus may not reflect the m5C profile of *V. cholerae* during other stress conditions.
384 Moreover, bisulfite sequencing allows for cytosine methylation analysis of the total
385 population at a specific time and thus it is not suitable to detect potential transient changes

386 in small subpopulations of cells. Such changes could be mediated, for example, by altering
387 the levels of VchM through gene expression. Little is known about *vchM* regulation but it
388 was recently shown that the *V. cholerae* quorum sensing low density transcriptional
389 regulator AphA is able to bind the *vchM* region (55) leaving the possibility that *vchM* may
390 be regulated by quorum sensing. Moreover, *vchM* was previously found to be differentially
391 expressed between different stages of human infection (56), suggesting the possibility that
392 modulation of cytosine methylation levels can be adaptative during *V. cholerae*'s life cycle.
393 In line with our work, lowering VchM levels could lead to a trade-off, where low m5C levels
394 would be detrimental for fitness in stress-free contexts, but highly advantageous in
395 presence of specific stress conditions, such as antibiotic exposure.

396 With this study, we show for the first time that in *V. cholerae* (and potentially other
397 clinically relevant species) the high expression of specialized chaperonins are crucial in
398 certain conditions where specific proteins may be affected by AGs. Moreover, we show here
399 that there is a link between chaperonin expression and DNA cytosine methylation, which
400 has not previously been observed, thus linking DNA methylation levels with response to
401 proteotoxic stress.

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410 **MATERIALS AND METHODS**

411

412 **Strains, media and culture conditions**

413 *V. cholerae* was routinely cultured at 37°C in Mueller-Hinton (MH) medium. Plasmids were
414 introduced in *V. cholerae* by electrotransformation. Strains containing the pSC101 plasmid
415 were grown in presence of 100 µg/mL carbenicillin for plasmid maintenance. All *V. cholerae*
416 mutant strains are derived from *Vibrio cholerae* serotype O1 biotype El Tor strain N16961
417 hapR+. Mutants were constructed by homologous recombination after natural
418 transformation or with a conjugative suicide plasmid as previously described (15, 57–59).
419 Primers, strains and plasmids used in this study, and their constructions, are listed in Table
420 S2. For routine cloning we used chemically competent *E. coli* One Shot TOP10 (Invitrogen).
421 All strains and plasmids were confirmed by sanger sequencing.

422 **Mutation of RCCGGY sites #1-4 in *groESL-2* region**

423 In order to mutate all four RCCGGY sites present in *groESL-2* (*vca0819-0820*) region we
424 generated a DNA fragment (S2 Table) with these sites containing the following nucleotide
425 changes: #1- ACCGGC changed to ATCGGC; #2- ACCGGC changed to ACGGGC; #3- GCCGGC
426 changed to GCGGGC and #4- ACCGGC changed to ACGGGC. This fragment was then
427 introduced in *V. cholerae* at the endogenous locus by allelic replacement as described in
428 Table S2.

429

430

431 **Growth curves**

432 Overnight cultures from single colonies were diluted 1:100 in Mueller-Hinton (MH) rich
433 media or MH + subMIC antibiotics at different concentrations, in 96-well microplates. OD₆₀₀
434 was measured in a Tecan Infinite plate reader at 37°C, with shaking for 20 hours.
435 Measurements were taken every 10 minutes.

436

437 **MIC determination**

438 MICs were determined by microtiter broth dilution method (60) with an initial inoculum
439 size of 10⁵ CFUs/mL. The MIC was interpreted as the lowest antibiotic concentration
440 preventing visible growth.

441

442 **Neo-Cy5 uptake**

443 Quantification of fluorescent neomycin (Neo-cy5) uptake was performed as described (61).
444 Neo-cy5 is an aminoglycoside coupled to the fluorophore Cy5, and has been shown to be
445 active against Gram- bacteria (27). Briefly, overnight cultures were diluted 100-fold in rich
446 MOPS (Teknova EZ rich defined medium). When the bacterial cultures reached an OD₆₀₀ of
447 0.25, they were incubated with 0.4 μM of Cy5 labeled Neomycin for 15 minutes at 37°C. 10
448 μL of the incubated culture were then used for flow cytometry, diluting them in 250 μL of

449 PBS before reading fluorescence. Flow cytometry experiments were performed as
450 described (62). For each experiment, 100000 events were counted on the Miltenyi
451 MACSquant device.

452

453 **Competitions experiments**

454 Overnight cultures from single colonies of lacZ⁻ and lacZ⁺ strains were washed in PBS
455 (Phosphate Buffer Saline) and mixed 1:1 (500µl + 500µl). At this point 100µl of the mix were
456 serial diluted and plated in MH agar supplemented with X-gal (5-bromo-4-chloro-3-indolyl-
457 β-D-galactopyranoside) at 40 µg/mL to assess T0 initial 1:1 ratio. At the same time, 10 µl
458 from the mix were added to 2 mL of MH or MH supplemented with subMIC tobramycin at
459 0.6µg/mL and incubated with agitation at 37°C for 20 hours. Cultures were then diluted and
460 plated in MH agar plates supplemented with X-gal. Plates were incubated overnight at 37°C
461 and the number of blue and white CFUs was assessed. Competitive index was calculated by
462 dividing the number of blue CFUs (lacZ⁺ strain) by the number of white CFUs (lacZ⁻ strain)
463 and normalizing this ratio to the T0 initial ratio.

464

465 **Survival assays**

466 Bacterial cultures from single colonies were cultured at 37°C for 16 h with agitation in 10
467 mL of MH medium. Aliquots from these cultures were removed, serial diluted and plated in
468 MH agar plates to assess CFUs formation prior antibiotic treatment (T0). In addition, 5 mL
469 of these aliquots were subjected to antibiotic treatment and incubated with agitation at

470 37°C. At the indicated time points, 500uL of these cultures were collected, washed in PBS,
471 serial diluted and plated in MH agar plates. The plates were then incubated overnight at
472 37°C. Survival at each time point was determined by dividing the number of CFUs/mL at
473 that time point by the number of CFUs/mL prior treatment. Antibiotics were used at the
474 following final concentrations: 20 µg/mL Tobramycin (TOB) and 10 µg/mL Gentamicin
475 (GEN). Experiments were repeated at least two to three times.

476

477 **Digital qRT-PCR**

478 For RNA extraction, overnight cultures of three biological replicates of strains of interest
479 were diluted 1:1000 in MH media and grown with agitation at 37°C until an OD₆₀₀ of 0.3
480 (exponential phase) or an OD₆₀₀ of 1.0 or 2.0 (stationary phase). 0.5 mL of these cultures
481 were centrifuged and supernatant removed. Pellets were homogenized by resuspension
482 with 1.5 mL of cold TRIzol Reagent. Next, 300 µL chloroform were added to the samples
483 following mix by vortexing. Samples were then centrifuged at 4°C for 10 minutes. Upper
484 (aqueous) phase was transferred to a new 2mL tube and mixed with 1 volume of 70%
485 ethanol. From this point, the homogenate was loaded into a RNeasy Mini kit (Quiagen)
486 column and RNA purification proceeded according to the manufacturer's instructions.
487 Samples were then subjected to DNase treatment using TURBO DNA-free Kit (Ambion)
488 according to the manufacturer's instructions. RNA concentration of the samples was
489 measured with NanoDrop spectrophotometer and diluted to a final concentration of 1-10
490 ng/µL.

491 qRT-PCR reactions were prepared with 1 μ L of diluted RNA samples using the qScript XLT 1-
492 Step RT-qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA) within Sapphire
493 chips. Digital PCR was conducted on a Naica Geode (programmed to perform the sample
494 partitioning step into droplets, followed by the thermal cycling program suggested in the
495 user's manual. Primer and probe sequences used in digital qRT-PCR reaction are listed in
496 Table S3. Image acquisition was performed using the Naica Prism3 reader. Images were
497 then analyzed using Crystal Reader software (total droplet enumeration and droplet quality
498 control) and the Crystal Miner software (extracted fluorescence values for each droplet).
499 Values were normalized against expression of the housekeeping gene *gyrA* as previously
500 described (31).

501

502

503 **RNA-seq**

504 For RNA extraction, overnight cultures of three biological replicates of WT and $\Delta vchM$
505 strains were diluted 1:100 in MH medium and grown with agitation at 37°C until cultures
506 reach an OD₆₀₀ of 2.0. Total RNA extraction, library preparation, sequencing and analysis
507 were performed as previously described (63). The data for this RNA-seq study has been
508 submitted in the GenBank Sequence Read Archive (SRA) under project number
509 PRJNA509113.

510

511

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520

521

522 **FIGURE CAPTIONS**

523 **Fig 1. *V. cholerae* N16961 $\Delta vchM$ is less susceptible to subMIC aminoglycosides** **A.** Growth
524 curves in absence (MH) or presence of subMIC doses of tobramycin. Bars represent SD (n=3)
525 **B.** *In vitro* competitions of WT and mutant strains against isogenic $\Delta lacZ$ reference strain in
526 absence or presence of different antibiotics at subMIC concentrations (TOB, 0.6 $\mu\text{g/ml}$; GEN,
527 0.5 $\mu\text{g/ml}$; NEO, 2.0 $\mu\text{g/ml}$; CAM, 0.4 $\mu\text{g/ml}$; CARB, 2.5 $\mu\text{g/ml}$). Box plots indicate the median
528 and the 25th and 75th percentiles; whiskers indicate the min and max values (n=6).

529 **Fig 2. $\Delta vchM$ strain is more tolerant to lethal aminoglycoside treatment.** Survival of
530 stationary-phase WT and $\Delta vchM$ cells exposed to lethal doses of tobramycin (TOB) **(A)**, and
531 gentamicin (GEN) **(B)**. Survival represents the number of bacteria (CFU/mL) after treatment

532 divided by the initial number of bacteria prior treatment. Time in the X-axis represents the
533 duration of the antibiotic treatment in hours. Means and SD are represented, n=3.

534 **Fig 3. *groESL-2* operon is upregulated in $\Delta vchM$ strain.** **A.** Schematic representation of both
535 *groESL* operons in *V. cholerae*. The four RCCGGY sites present along the *groESL-2* region are
536 represented by the inverted orange triangles. **B.** Fold change ($\Delta vchM$ /WT) of the relative
537 expression levels of *groES-1* and *groES-2* in cultures at exponential phase (Exp, OD₆₀₀ \approx 0.3)
538 or stationary phase (Stat, OD₆₀₀ \approx 1.8-2.0). Means and SD are represented, n=3.

539 **Fig 4. *groESL-2* is needed for the increased tolerance of $\Delta vchM$ to lethal AG treatment.** **A.**
540 Growth curves in MH medium. Means and SD are represented, n=3. **B.** Survival of
541 stationary-phase WT and $\Delta groESL-2$ cells exposed to lethal aminoglycoside treatment for 7
542 hours. Box plots indicate the median and the 25th and 75th percentiles; whiskers indicate the
543 min and max values (n=6 from two independent experiments). **C.** Survival (after 7 hours AG
544 treatment) of $\Delta vchM groESL-2$ double mutant harboring an empty plasmid or a plasmid
545 expressing either *groESL-1* or *groESL-2*, relative to survival of the $\Delta vchM$ with the control
546 plasmid. Box plots indicate the median and the 25th and 75th percentiles; whiskers indicate
547 the min and max values (n=6 from two independent experiments). In **B** and **C** statistically
548 significant differences were determined using Friedman's test with Dunn's post-hoc test for
549 multiple comparisons. * P<0.05, ** P<0.01, ns= not significant. **D.** *In vitro* competitions of
550 $\Delta vchM$ and $\Delta vchM groESL-2$ double mutant strains against isogenic $\Delta lacZ$ reference strain
551 in absence or presence of subMIC TOB, 0.6 μ g/ml; Error bars indicate SD (n=6).

552 **Fig 5. Disrupted VchM sites in *groESL-2* region leads to increased gene expression in the**
553 **WT. A.** Schematic representation of mutants with abrogated VchM sites. **B.** Relative
554 expression of *groES-2* in the different strains at OD₆₀₀ of 1.0. Box plots indicate the median
555 and the 25th and 75th percentiles; whiskers indicate the min and max values (n= 5). Statistical
556 significance was determined by Kruskal-Wallis test with Dunn's post-hoc test for multiple
557 comparisons. * P<0.05, ** P<0.01, ns = not significant

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737 **SUPPORTING INFORMATION**

738 **S1 Table. Differentially regulated genes in $\Delta vchM$ strain**

739 **S2 Table. Strains, plasmids and primers used in this study**

740 **S3 Table. Primer and probe sequences used in digital qRT-PCR**

741 **S1 Fig. $\Delta vchM$ exponential phase cells are more tolerant to lethal aminoglycoside**
742 **treatment.** Survival of exponential phase cells (OD_{600} 0.3-0.4) of WT and $\Delta vchM$ strains
743 exposed to lethal doses of tobramycin (TOB) for a period of 3.5 hours. The Y-axis represents
744 the \log_{10} CFUs/mL after 3.5 hours growth with 0, 3, 5 or 10 $\mu\text{g}/\text{mL}$ of Tobramycin (3x, 5x or
745 10x higher than the MIC, respectively). Means and SD are represented, n=3.

746 **S2 Fig. Neo-cy5 uptake is not decreased in $\Delta vchM$.** Percentage of neo-cy5 positive cells
747 analyzed by flow cytometry after incubation with fluorescent marked neomycin. Means and
748 SD are represented, n=3.

749

750 **S3 Fig. Comparison of GroESL proteins from *E. coli* and *V. cholerae*.** Amino acid identity
751 between GroES and GroEL proteins of *E. coli* MG1655 (*Eco*) and *V. cholerae* O1 El Tor
752 N16961 (*Vch*) computed by BLASTP. Values represent percentage identity between
753 proteins.

754 **S4 Fig. Deletion of *groESL-2* does not increase susceptibility to tobramycin. A.** *In vitro*
755 competitions of WT and $\Delta groESL-2$ strains against isogenic $\Delta lacZ$ reference strain in absence
756 or presence of tobramycin (TOB) at 0.6 $\mu\text{g}/\text{mL}$; n=3, error bars indicate SD. **B.** Survival of
757 stationary-phase WT and $\Delta groESL-2$ cells exposed to 20X MIC of tobramycin. n=3, error
758 bars indicate SD.

759 **Fig. S5 *groESL-2* is induced in WT cells by tobramycin at both subMIC and lethal**
760 **concentrations.** Relative expression of *groES-2* in WT cells growing in MH, MH with subMIC
761 tobramycin ($\approx 20\%$ MIC) or exponential phase cells following lethal TOB treatment (5X MIC)
762 for a period of 30 minutes. n=3, error bars indicate SD.

763 **S6 Fig. Mutation of VchM sites in *groESL-2* region fails to affect gene expression of the**
764 **operon in absence of VchM.** Relative expression of *groES-2* and *groEL-2* genes in the
765 indicated strains grown at OD_{600} 1.0. n=3, error bars indicate SD.

766 **S7 Fig. Overexpression of *groESL-2* in the WT does not increase tolerance to tobramycin.**
767 Survival (after 7 hours TOB treatment) of WT strain carrying a control plasmid or a plasmid
768 overexpressing *groESL-2* genes. n=6, error bars indicate SD.

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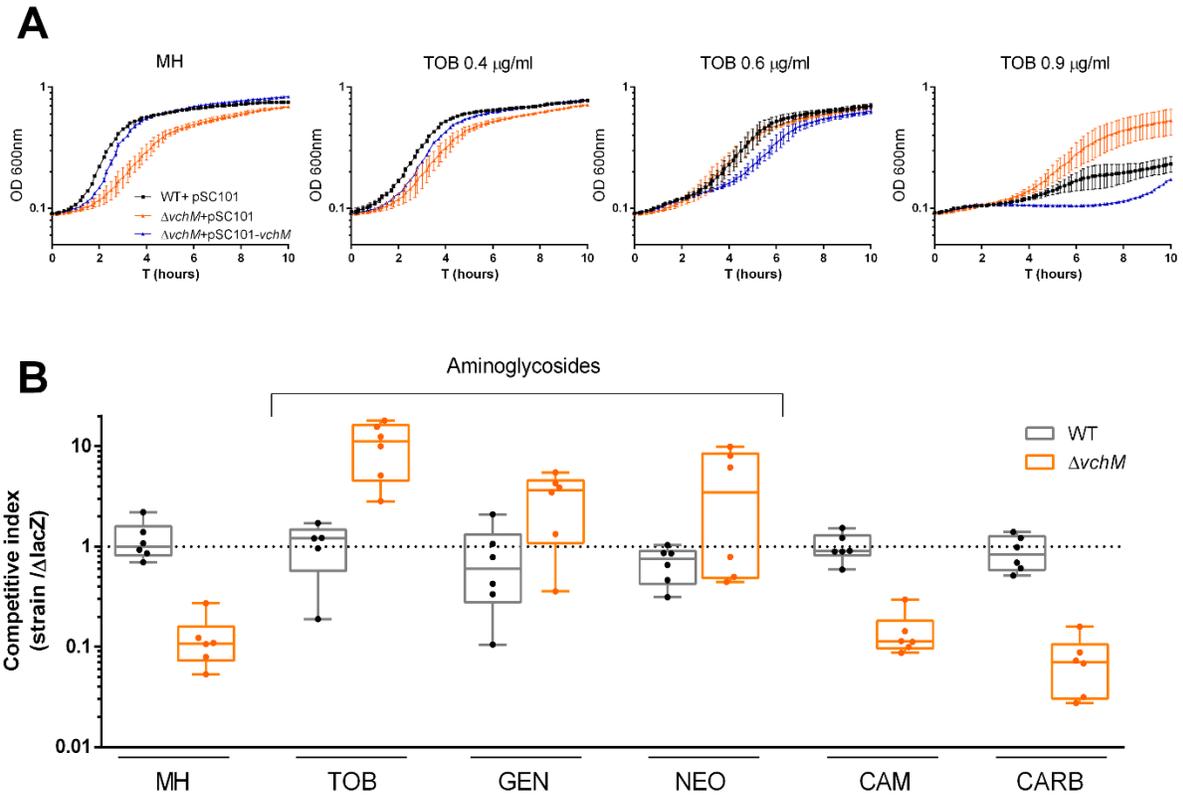
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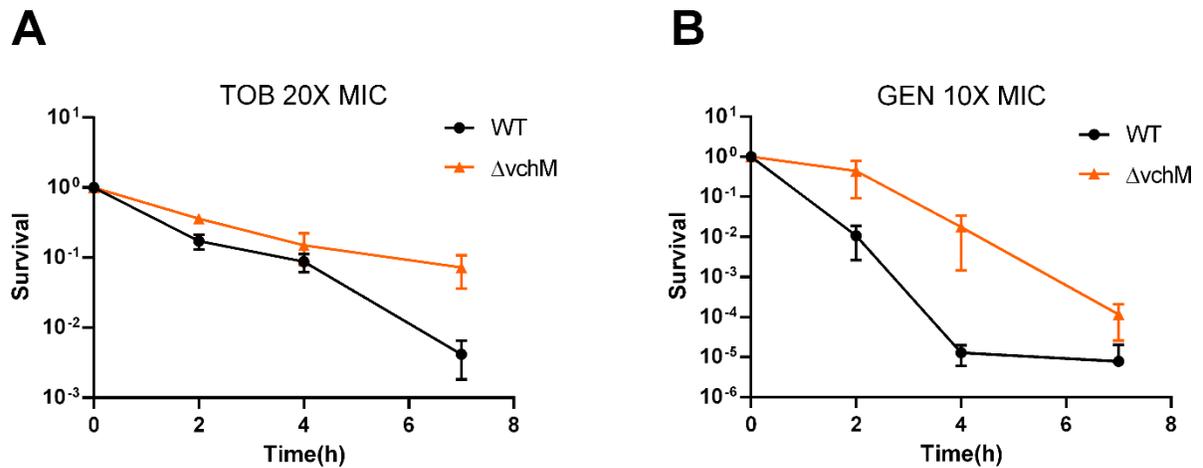
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774 **Figure 1**



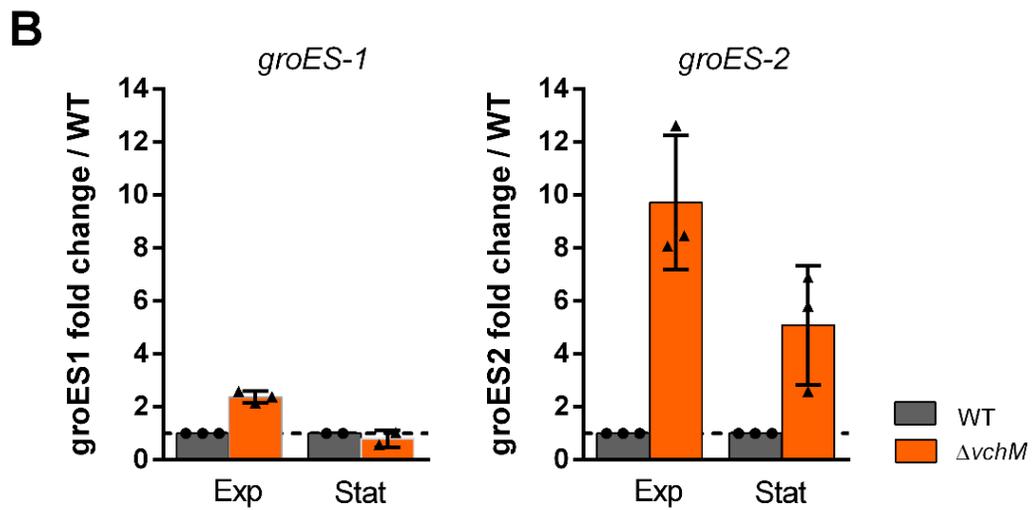
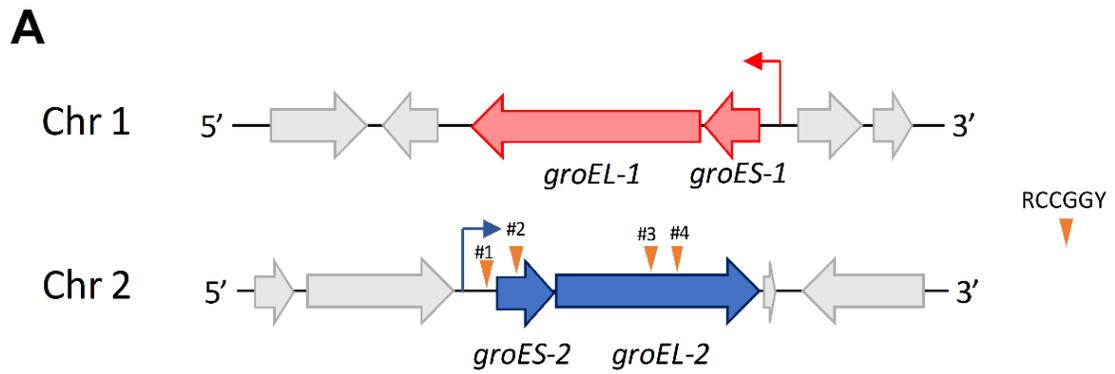
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776 **Figure 2**



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778 **Figure 3**



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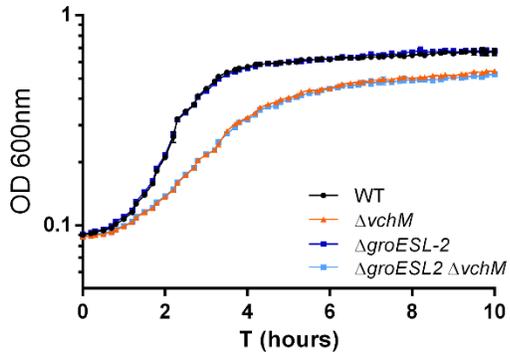
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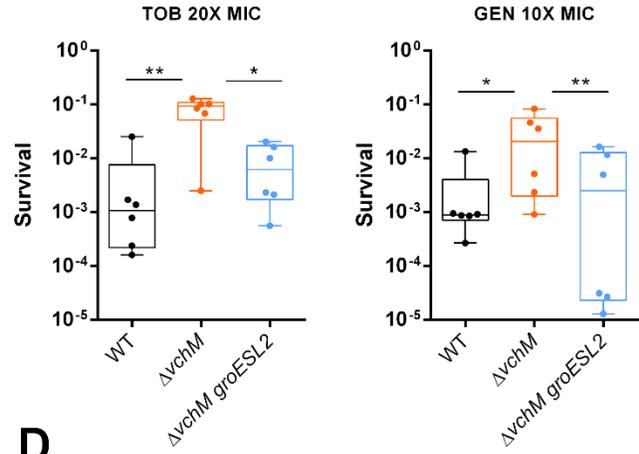
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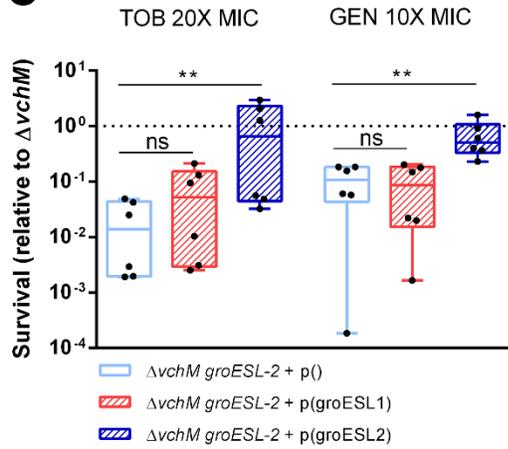
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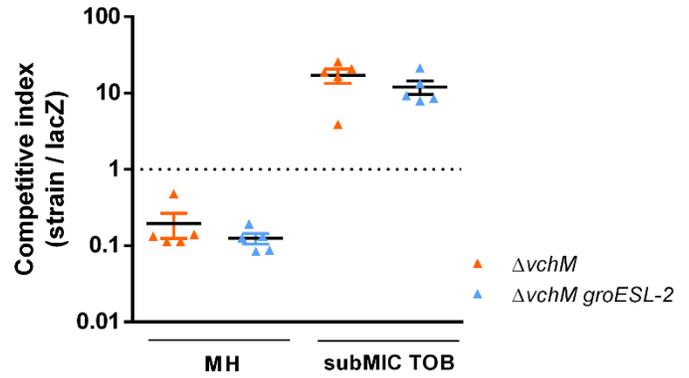
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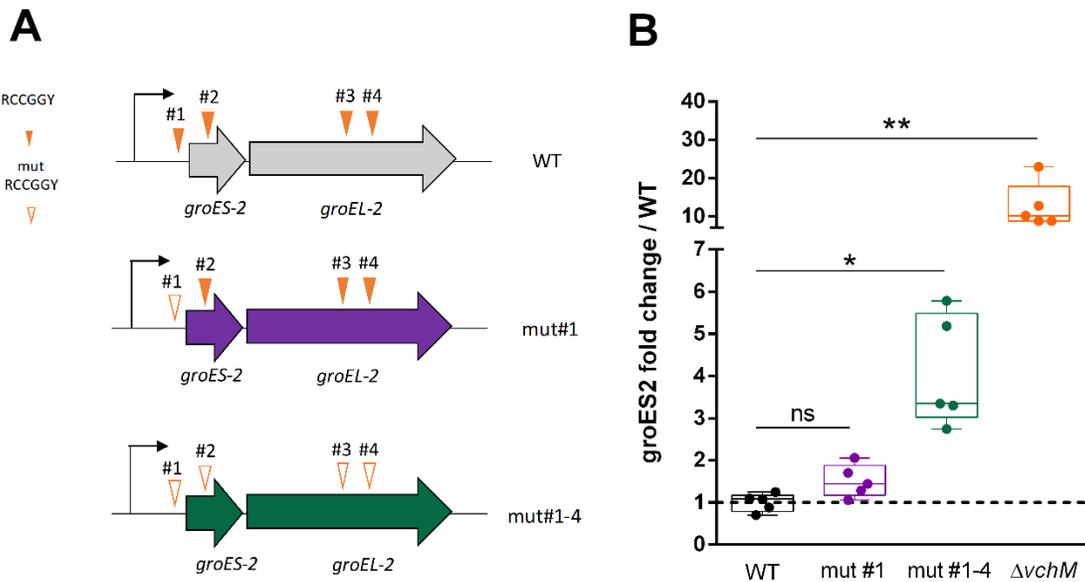


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816 **Figure 5**

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819 **Supplementary data**

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821 **Table S1. Differentially regulated genes in $\Delta vchM$ strain**

Locus	Name	Annotation	Fold change $\Delta vchM/WT$	p-value
UPREGULATED				
<i>vca1028</i>	<i>lamB</i>	maltoporin	52,81	0,0E+00
<i>vca0707</i>	<i>uhpC</i>	MFS transporter	8,76	8,6E-06
<i>vc1368</i>		conserved hypothetical protein	8,18	0,0E+00
<i>vca0819</i>	<i>groES-2</i>	chaperonin	6,78	0,0E+00
<i>vca0200</i>		putative ATPase	6,10	0,0E+00
<i>vca0957</i>	<i>glcB</i>	putative Malate synthase	5,97	0,0E+00
<i>vca0199</i>		conserved hypothetical protein	5,32	5,2E-09
<i>vca1027</i>	<i>malM</i>	putative Maltose operon periplasmic protein MalM	4,14	0,0E+00
<i>vca0201</i>		conserved hypothetical protein	3,97	3,4E-11
<i>vca0946</i>	<i>malK</i>	Maltose/maltodextrin import ATP-binding protein malK	3,91	0,0E+00

vca0469	<i>higA2</i>	plasmid stabilization system HigA protein (antitoxin of TA system HigB-HigA)	3,91	0,0E+00
vca0468	<i>higB2</i>	plasmid stabilization system HigB protein (toxin of TA system HigB-HigA)	3,90	2,3E-10
vca0945	<i>malE</i>	maltose ABC transporter periplasmic binding protein	3,90	0,0E+00
vc1819	<i>aldA</i>	Aldehyde dehydrogenase	3,84	1,7E-12
vc1204	<i>hutG</i>	putative Formimidoylglutamase HutG (histidine utilization)	3,80	2,2E-16
vc1203	<i>hutU</i>	Urocanate hydratase (histidine utilization)	3,60	0,0E+00
<u>vca0820</u>	<u>groEL-2</u>	<u>chaperonin</u>	<u>3,54</u>	<u>2,2E-16</u>
vca0692	<i>secF</i>	fragment of putative SecD/SecF/SecDF export membrane protein (part 2)	3,48	3,2E-08
vc1809	<i>alpA</i>	putative Prophage CP4-57 regulatory protein (AlpA)	3,37	1,8E-09
vc1205	<i>hutI</i>	Imidazolonepropionase (histidine utilization)	3,24	1,1E-11
vc1740	<i>fadE</i>	acyl coenzyme A dehydrogenase	3,22	5,6E-15
vca0944	<i>malF</i>	maltose ABC transporter membrane subunit MalF	3,18	0,0E+00
vc1142	<i>cspD</i>	Cold shock-like protein cspD	2,88	0,0E+00
vc2530	<i>hpf</i>	putative ribosome hibernation promoting factor HPF/sigma 54 modulation protein	2,83	0,0E+00
vca0948	<i>yaeO</i>	putative Rho-specific inhibitor of transcription termination (YaeO)	2,78	0,0E+00
vca0998	<i>xenB</i>	Xenobiotic reductase B	2,73	4,4E-06
vc0486	<i>srlR</i>	putative Transcriptional regulator of sugar metabolism; DeoR family transcriptional regulator	2,65	9,9E-14
vc1874		putative SpoVR family protein	2,64	0,0E+00
vc1202	<i>hutH</i>	Histidine ammonia-lyase	2,61	0,0E+00
vc2265	<i>groES-1</i>	chaperonin	2,60	0,0E+00
vc2615		conserved hypothetical protein	2,55	4,6E-10
vc1678	<i>pspA</i>	Phage shock protein A	2,48	4,6E-10
vc1183		putative 2OG-Fe dioxygenase	2,43	0,0E+00
vca0470		putative Acetyltransferase	2,43	2,1E-08
vca0324	<i>relB1</i>	Plasmid stabilization system protein RelB (Anti Toxin of TA system RelB-RelE)	2,43	1,1E-11
vca0886	<i>kbl</i>	glycine C-acetyltransferase	2,40	2,3E-08
vca0185	<i>arfA</i>	putative Stalled ribosome alternative rescue factor ArfA	2,39	3,0E-13
vca0958		putative transcriptional regulator	2,38	3,9E-10

vca0013	<i>malP</i>	maltodextrin phosphorylase	2,37	3,9E-11
vca0881		conserved hypothetical protein	2,36	1,1E-13
vca0551		conserved hypothetical protein	2,35	0,0E+00
vca0391	<i>higB1</i>	Plasmid stabilization system HigB protein (Toxin of TA system HigB-HigA)	2,34	1,5E-10
vca0923	<i>mlp37</i>	chemoreceptor Mlp37	2,34	2,2E-12
vc2361	<i>grcA</i>	Autonomous glycy radical cofactor	2,30	0,0E+00
vca0392	<i>higA1</i>	Plasmid stabilization system HigA protein (Antitoxin of TA system HigB-HigA)	2,28	0,0E+00
vc0665	<i>vpsR</i>	Fis family transcriptional regulator (sigma-54 dependent transcriptional regulator)	2,28	0,0E+00
vca0720	<i>hnoX</i>	Heme-Nitric Oxide/Oxygen Binding Protein (H-NOX)	2,27	5,1E-10
vc1433	<i>uspE</i>	universal stress protein UspE	2,26	0,0E+00
vc1248	<i>tar</i>	putative Methyl-accepting chemotaxis protein	2,25	0,0E+00
vc2264	<i>groEL-1</i>	chaperonin	2,24	0,0E+00
vc0734	<i>aceB</i>	Malate synthase A	2,24	1,4E-06
vc2507		PhoH family protein	2,23	0,0E+00
vc1344	<i>hppD</i>	4-hydroxyphenylpyruvate dioxygenase	2,22	6,9E-15
vca0219	<i>hlyA</i>	Hemolysin	2,20	5,7E-14
vca0159		putative Universal stress protein family 1	2,17	2,9E-15
vca0987	<i>ppsA</i>	phosphoenolpyruvate synthase	2,15	2,6E-09
vca0359	<i>parE2</i>	Plasmid stabilization system ParE protein (toxin of TA system ParD-ParE)	2,14	9,4E-10
vca0004		conserved hypothetical protein	2,14	0,0E+00
vc1222	<i>ihfA</i>	integration host factor subunit α	2,13	0,0E+00
vc2758	<i>fadB</i>	fatty acid oxidation complex subunit alpha FadB	2,10	6,1E-10
vc2704		conserved hypothetical protein	2,10	8,9E-09
vc1962	<i>nlpE</i>	copper resistance protein NlpE N-terminal domain-containing protein	2,08	3,9E-09
vca0278	<i>glyA</i>	serine hydroxymethyltransferase	2,04	0,0E+00
vca0280	<i>gcvT</i>	glycine cleavage system aminomethyltransferase	2,04	4,4E-16
vc0976	<i>qmca</i>	putative Membrane protease subunits, stomatin/prohibitin homolog qmca	2,03	6,5E-06
vc1539a		hypothetical protein	2,02	6,9E-10
vc0666		conserved hypothetical protein	2,02	1,8E-09
vc2144	<i>flaF</i>	Polar flagellin F	2,01	3,0E-13

DOWNREGULATED				
Locus	Name	Annotation	Fold change $\Delta vchM/WT$	p-value
<i>vca0198</i>	<i>vchM</i>	Cytosine-specific DNA methyltransferase	-12,22	1,4E-24
<i>vca0933</i>	<i>cspE</i>	transcription antiterminator and regulator of RNA stability	-8,41	1,0E-07
<i>vca0017</i>	<i>hcp</i>	Haemolysin co-regulated protein (putative Type VI secretion system effector, Hcp1)	-3,20	1,3E-10
<i>vc2383</i>	<i>ilvY</i>	putative Transcriptional regulator, LysR family	-3,14	5,9E-27
<i>vca0804</i>	<i>deaD</i>	Cold-shock DEAD box protein A	-3,12	6,5E-49
<i>vca0874</i>		conserved hypothetical protein	-3,10	1,1E-05
<i>vc1704</i>	<i>metE</i>	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	-3,02	7,6E-21
<i>vc0768</i>	<i>guaA</i>	GMP synthetase	-2,91	2,7E-72
<i>vca0680</i>	<i>napC</i>	Cytochrome c-type protein napC	-2,87	2,4E-07
<i>vca0679</i>	<i>napB</i>	Diheme cytochrome c napB	-2,82	2,1E-06
<i>vca0935</i>		hypothetical protein	-2,76	1,2E-10
<i>vc1261</i>		putative Sugar efflux transporter B	-2,63	3,9E-07
<i>vc0069</i>	<i>mdtL</i>	putative FLORFENICOL EXPORTER	-2,53	8,1E-14
<i>vca1002</i>		AzIC family ABC transporter permease	-2,49	2,0E-08
<i>vc1415</i>	<i>hcp</i>	type VI secretion system secreted protein Hcp	-2,48	1,0E-06
<i>vc0433</i>	<i>arcD</i>	Arginine/ornithine antiporter	-2,48	1,9E-06
<i>vc1393</i>	<i>sugE</i>	Quaternary ammonium compound-resistance protein sugE	-2,43	3,4E-22
<i>vc1035</i>		conserved hypothetical protein	-2,40	6,3E-06
<i>vc1608</i>		ABC transporter permease	-2,38	2,8E-11
<i>vca0250</i>		Alpha-amylase	-2,37	7,2E-08
<i>vc0767</i>	<i>guaB</i>	inositol-5-monophosphate dehydrogenase	-2,31	3,0E-22
<i>vc1239</i>	<i>cobU</i>	cobinamide-P guanylyltransferase / cobinamide kinase	-2,28	1,2E-09
<i>vc0290</i>	<i>fis</i>	DNA-binding protein fis	-2,27	1,4E-04
<i>vc2227</i>	<i>purN</i>	phosphoribosylglycinamide formyltransferase 1	-2,25	2,6E-10
<i>vc2384</i>		TSUP family transporter	-2,24	8,1E-16
<i>vca1003</i>		AzID domain-containing protein	-2,23	1,5E-11
<i>vca0678</i>	<i>napA</i>	Periplasmic nitrate reductase	-2,22	2,6E-10
<i>vca1031</i>		Methyl-accepting chemotaxis protein	-2,21	7,0E-11
<i>vc1843</i>	<i>cydB</i>	Cytochrome d ubiquinol oxidase subunit 2	-2,21	5,9E-13

vc0191	<i>rhtC</i>	putative Lysine/Homoserine/Threonine exporter protein	-2,20	2,6E-10
vc1609		ABC transporter permease	-2,19	2,2E-10
vc0005	<i>yidD</i>	membrane protein insertion efficiency factor	-2,16	4,9E-10
vc0291	<i>dusB</i>	tRNA-dihydrouridine synthase B	-2,16	3,8E-16
vc1855	<i>dinG</i>	<u>ATP-dependent DNA helicase DinG</u>	-2,15	7,7E-07
vc2382		conserved hypothetical protein	-2,15	3,1E-07
vc1842	<i>cydX</i>	cytochrome bd-I oxidase subunit CydX	-2,14	1,8E-06
vc1629		ABC transporter permease	-2,13	3,0E-06
vc0706	<i>raiA</i>	Ribosome-associated inhibitor A	-2,13	1,1E-08
vc2047		SDR family oxidoreductase	-2,11	3,4E-23
vc1174	<i>trpE</i>	Anthranilate synthase component 1	-2,09	9,9E-18
vc1240		histidine phosphatase family protein	-2,09	1,5E-06
vc0940		conserved hypothetical protein	-2,07	1,3E-07
vc1487		glutaredoxin family protein	-2,06	8,0E-06
vca0214	<i>emrD</i>	multidrug efflux pump EmrD	-2,05	6,0E-12
vca0269		aspartate aminotransferase family protein	-2,04	2,2E-13
vc2647	<i>aphA</i>	Transcriptional regulator PadR family	-2,04	9,6E-56
vc2226	<i>purM</i>	Phosphoribosylformylglycinamide cyclo-ligase	-2,02	9,3E-10
vc0651		putative Peptidase U32	-2,02	2,4E-13
vc1630		putative SalX, ABC-type antimicrobial peptide transport system, ATPase component	-2,02	2,5E-19
vca0684		MFS transporter	-2,02	9,3E-14
vc0717	<i>yegQ</i>	tRNA 5-hydroxyuridine modification protein YegQ	-2,01	4,2E-09
vc1856		TSUP family transporter	-2,00	7,2E-10
vc0987	<i>hemH</i>	ferrochelatase	-2,00	1,9E-17

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824 **Table S2. Strains, plasmids and primers used in this study**

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Strain	Lab Strain number	Source/Construction
<i>Vibrio cholerae</i>		

N16961 hapR+ WT strain	F606	Gift from Melanie Blokesch
<i>ΔvchM</i> (<i>vca0198</i>)	H507	PCR amplification of 500bp up and down genomic regions of VCA0198 using primers ZIP136/137 and ZIP138/139. PCR amplification of <i>aadA7</i> conferring spectinomycin resistance on pAM34 using ZB47/48. PCR assembly of the VCA0198::spec fragment using ZIP136/139 and allelic exchange by natural transformation in F606.
<i>ΔlacZ</i>	K329	Lab collection
<i>ΔgroESL-2</i> (<i>vca0819-0820</i>)	M958	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 (pM340) replacing the gene with <i>frt::kan::frt</i>
<i>ΔvchM</i> <i>ΔgroESL-2</i>	N330	PCR amplification of 500bp up and down genomic regions of VCA0198 using primers ZIP136/137 and ZIP138/139. PCR amplification of <i>aadA7</i> conferring spectinomycin resistance on pAM34 using ZB47/48. PCR assembly of the VCA0198::spec fragment using ZIP136/139 and allelic exchange by natural transformation in M958
WT – VchM site #1 mutated (mut #1)	L900	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 (pL442)
WT – VchM sites #1-4 mutated (mut #1-4)	Q827	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 containing the <i>groESL-2</i> region with sites #1-4 mutated (pQ824) in M958 strain.
<i>ΔvchM</i> mut #1- 4	Q828	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 containing the <i>groESL-2</i> region with sites #1-4 mutated (pQ824) in N330
Plasmids		
pMP7- <i>Δvca0819-0820::kan</i>	M340	gibson assembly using primers MV450/451 for the amplification of pMP7 vector, primers “ <i>vca0819-8205</i> ” and “ <i>vca0819-8206</i> ” for up and down regions of the gene, and primers MV268/269 on pKD4 plasmid for the resistance gene (<i>frt::kan::frt</i>).
pMP7- C->T point mutation	L442	Amplification of 500bp upstream of <i>vca0819</i> with primers 5923/5922; Amplification of 500bp downstream of <i>vca0819</i> with 5924/5921; PCR assembly of the two fragments with

of VchM site #1 in 5' UTR region of vca0819-0820		primers 5923/5924. Note: Primers 5922 and 5921 contain a mismatch to give origin to a C->T point mutation in VchM site #1. The fragment was then cloned in a pTOPO vector and sub cloned into pMP7 using <i>EcoRI</i> restriction sites.
pMP7- sites #1-4 mutated in vca0819-0820 region	Q824	A <i>groESL-2</i> fragment with mutations in VchM sites #2-4 was synthesized and cloned in a pTOPO vector. This fragment was then PCR assembled to the 5' UTR region containing site #1 mutated (from strain L900), which originated a final fragment containing the #1-4 mutated sites. This fragment was then cloned in pTOPO and sub cloned into pMP7 using <i>EcoRI</i> restriction sites.
pSC101- <i>groESL-1</i>	O849	Amplification of <i>vc2664-2665</i> from <i>V. cholerae</i> gDNA with primers AFC046/AFC047. Primer AFC046 contains a P _{trc} promoter. Fragment cloned in pSC101 low copy plasmid (carbenicillin resistant) using <i>EcoRI</i> restriction sites
pSC101- <i>groESL-2</i>	N752	Amplification of <i>vca0819-0820</i> from <i>V. cholerae</i> gDNA with primers AFC029/AFC030. Primer AFC029 contains a P _{trc} promoter. Fragment cloned in pSC101 low copy plasmid (carbenicillin resistant) using <i>EcoRI</i> restriction sites
pSC101- <i>vchM</i>	Q826	Amplification of <i>vca0198 (vchM)</i> with its own promoter from <i>V. cholerae</i> gDNA with primers 5990/5911. Cloning in pTOPO vector and sub cloning in pSC101 low copy plasmid (carbenicillin resistant) using <i>BamHI</i> and <i>PstI</i> restriction sites
Primers		Sequence 5'-3'
ZIP136		GCCGCCGAAGGAAAAACCGTACTATTGC
ZIP137		GCGAGCATCGTTTGTTCGCCAGCTTCTGTATGGAACGGGTAACTGTATCACCATACTACCTCATGG
ZIP138		CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTACATGCTTCACAGCGTAGTCGC
ZIP139		TTAATTTCTCGAGTTTCAGATGC
ZB47		CCCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGC
ZB48		GACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACG
vca0819-8205		CTATTATTTAAACTCTTTCGGTTTTGCCTT
vca0819-8206		TACGTAGAATGTATCAGACTCGCCCAAGGA
5921		AAACAATCCTA <u>T</u> CGGCCTTTTATC
5922		GATAAAAGGCCG <u>A</u> TAGGATTGTTT

5923		ACTTTGATGGTACGCGCGATG
5924		GATTTATTGAGCACAACATGGCG
AFC029		GTAAGTGAATTCTTGACAATTAATCATCCGGCTCGTATAATGTGTG GAATTGTGAGCGGATAACAATTTACACAGGAAACAGCGCCGCAT GAATATTCGTCCTTTACATG
AFC030		GTAAGTGAATTCATTACGCCGAGACTCTTTGTC
AFC046		GTAAGTGAATTCTTGACAATTAATCATCCGGCTCGTATAATGTGTG GAATTGTGAGCGGATAACAATTTACACAGGAAACAGCGCCGCAT GAATATTCGTCCATTACATGAC
AFC047		GTAAGTGAATTCCTGCTAAGGGGGATGATTACA
5990		GTTTTTGCTGCCGTCTGCTA
5911		GTAGTCGACCCTTTTTACAACTTTCTAGA

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828 **Table S3. Primer and probe sequences used in digital qRT-PCR**

Target	Primers (5'-3')	Probes (5'-3')
<i>gyrA</i>	AATGTGCTGGGCAACGACTG	[Cy5]-CACCTCATGGTGACAGTGCGGTTT-[BHQ2]
	GAGCCAAAGTTACCTTGCC	
<i>groES-1</i>	CGTAGCTTTCTGCGAAGATC	[HEX] -AGCTCAAAGTTGAACTGAACCGTTCTCTA-[BHQ1]
	TGGTGGAATTGTTCTAACTG	
<i>groES-2</i>	GGCGACCAGATCATTTC AAC	[FAM] - TGGACGGTAAAGAGTATCTGATCCTCTCC-[BHQ1]
	TCTACAATCGCTAACACATCAG	
<i>groEL-2</i>	TGCTATCGTTCAGAATCAACC	[HEX] -AAGCTTACCAGCATAGA ACTTGCCAGAGAT-[BHQ1]
	TTTACTTCTCGCTCCCAATCG	

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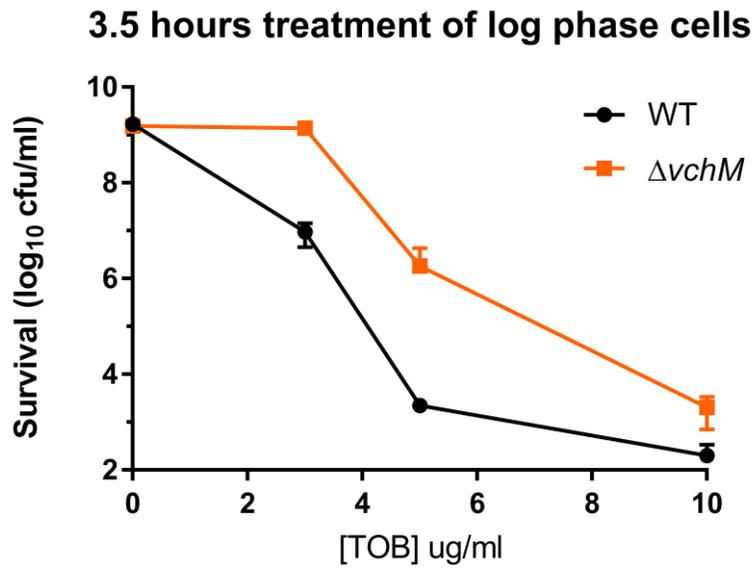
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838 **S1 Figure**

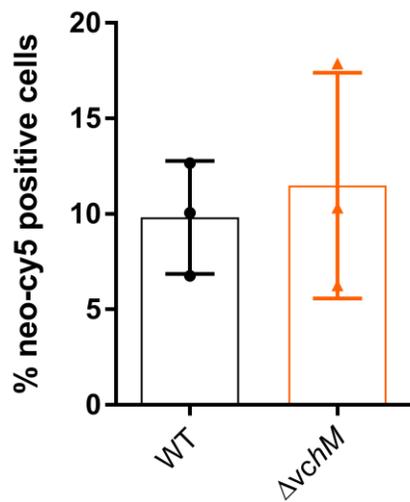


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841 **S2 Figure**

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848 **S3 Figure**

	<i>Eco</i> GroES	<i>Vch</i> GroES-1	<i>Vch</i> GroES-2
<i>Eco</i> GroES (97aa)		80.21	65.62
<i>Vch</i> GroES-1 (96aa)	80.21		65.62
<i>Vch</i> GroES-2 (96aa)	65.62	65.62	

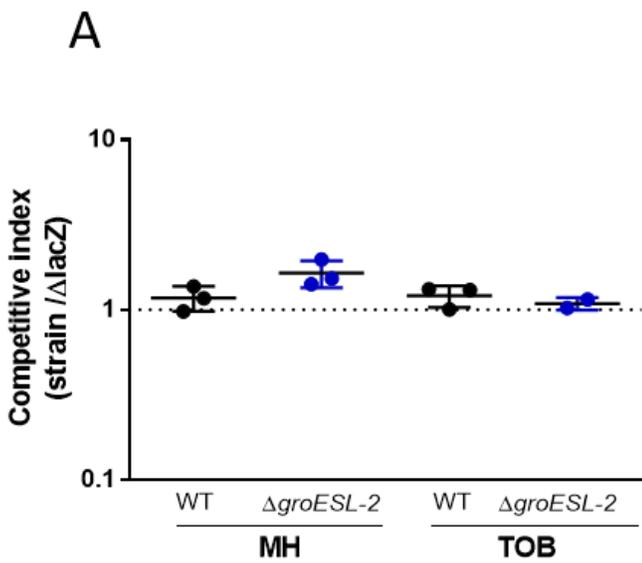
	<i>Eco</i> GroEL	<i>Vch</i> GroEL-1	<i>Vch</i> GroEL-2
<i>Eco</i> GroEL (541aa)		86.77	74.34
<i>Vch</i> GroEL-1 (544aa)	86.77		76.18
<i>Vch</i> GroEL-2 (530aa)	74.34	76.18	

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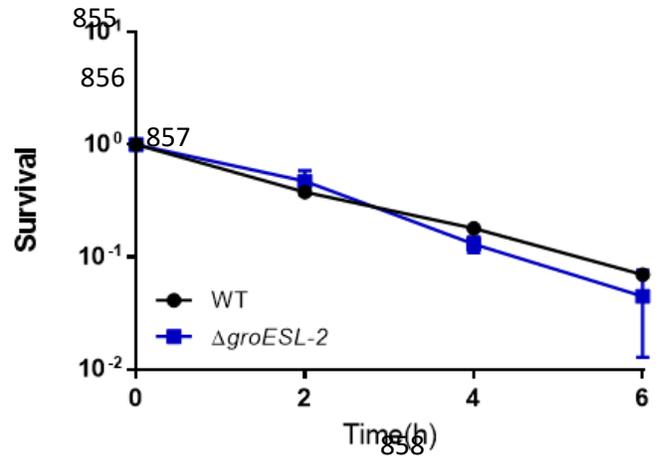
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852 **S4 Figure**



853 **B**
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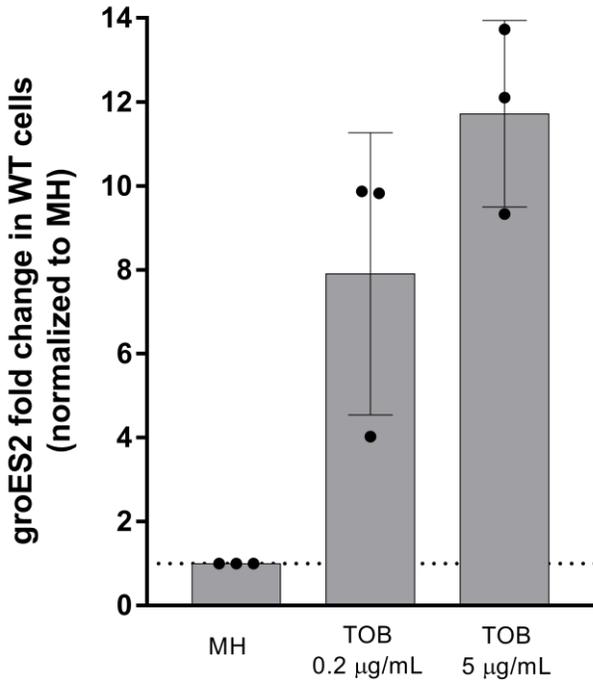
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863 **S5 Figure**



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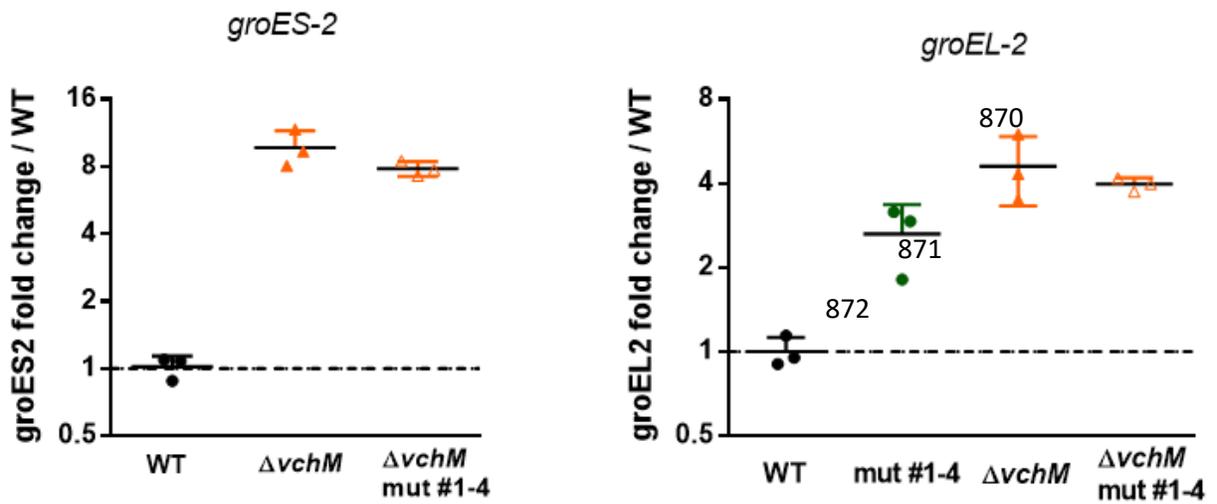
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866 **S6 Figure**

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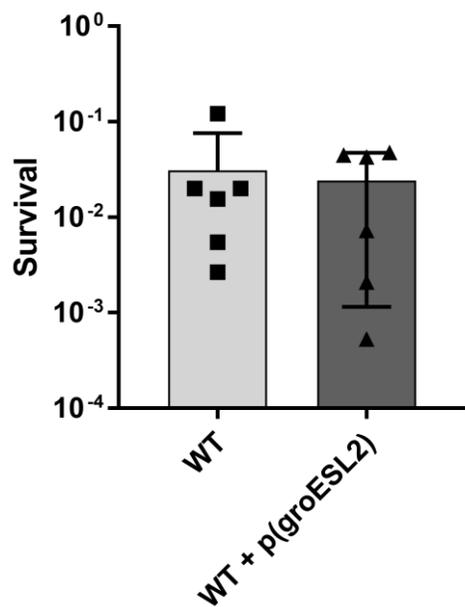
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877 **S7 Figure**

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