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## Interplay between Sublethal Aminoglycosides and Quorum Sensing: Consequences on Survival in *V. cholerae*

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1 **Interplay between sublethal aminoglycosides and quorum sensing: consequences on survival in**  
2 ***V. cholerae***

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10

11 **ABSTRACT**

12

13 Antibiotics are well known drugs which when present above certain concentrations are able to  
14 inhibit the growth of certain bacteria. However, a growing body of evidence has shown that even  
15 when present at lower doses, unable to inhibit or affect microbial growth, antibiotics work as  
16 signaling molecules, affect gene expression and trigger important bacterial stress responses.  
17 However, how subMIC antibiotic signaling interplays with other well-known signaling networks in  
18 bacteria (and the consequences of such interplay) is not well understood. In this work, through  
19 transcriptomic and genetic approaches, we have explored how quorum-sensing (QS) proficiency of  
20 *V. cholerae* affects this pathogen's response to subMIC doses of the aminoglycoside tobramycin.  
21 We show that the transcriptomic signature of *V. cholerae* in response to subMIC TOB highly depends  
22 on the presence of QS master regulator HapR. In parallel, we show that subMIC doses of tobramycin  
23 are able to negatively interfere with the AI-2/LuxS QS network of *V. cholerae*, which seems critical  
24 for survival to aminoglycoside treatment and tobramycin-mediated induction of SOS response in  
25 this species. This interplay between QS and aminoglycosides suggests that targeting QS signaling  
26 may be a strategy to enhance aminoglycoside efficacy in *V. cholerae*.

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## 33 INTRODUCTION

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35 Many bacterial species secrete small diffusible signaling molecules to synchronize  
36 multicellular behaviors which allow them to adapt and survive in natural environments [1,2]. The  
37 most studied intercellular communication mechanism is quorum-sensing (QS), which monitors local  
38 population density [3,4]. QS is achieved *via* the production and detection of extracellular small  
39 molecules called autoinducers. At low cell density autoinducers diffuse away but at high cell density  
40 their concentration increases and triggers synchronization of gene expression in bacterial  
41 populations. Gram-negative bacteria are able to produce and detect several classes of autoinducers.  
42 Autoinducer 1 (AI-1) is a species-specific signaling molecule while autoinducer 2 (AI-2), which is  
43 produced by Gram-negative and Gram-positive bacteria, is able to mediate both intra and  
44 interspecies QS communication [5,6].

45 *Vibrio cholerae*, the causative agent of cholera disease, produces both autoinducers. AI-1,  
46 called cholera AI-1 (CAI-1), is produced by the CqsA protein and sensed by CqsS, while AI-2 is  
47 produced by LuxS and sensed by LuxQ, via LuxP periplasmic protein. The QS regulatory network of  
48 *V. cholerae* relies on a well described phosphorylation cascade (Fig. 1). At low cell density, CqsS and  
49 LuxPQ work as kinases and phosphorylate LuxU which will then transfer the phosphate to the  
50 regulator LuxO. Phosphorylated LuxO will then trigger the expression of four small RNAs (qrr1-4)  
51 which in turn allow for translation of AphA (master regulator of low cell density), while inhibiting  
52 that of HapR, the LuxR family master regulator of high cell density in *V. cholerae*. By contrast, at high  
53 cell numbers, both CAI-1 and AI-2 accumulate and bind the cognate receptors CqsS and LuxPQ which  
54 will now act as phosphatases and inhibit the phosphorylation cascade described above. This leads  
55 to an absence of qrr1-4 sRNAs and, consequently, the absence of AphA. Concomitantly, HapR is  
56 produced, inducing the expression of several genes involved in group behavior [7,8].

57 *Escherichia coli* can also detect autoinducers produced by other bacteria and react to them  
58 via SdiA, a LuxR protein homologue [9,10]. *E. coli* AI-2 is produced by the LuxS protein and sensed  
59 by the proteins encoded by the *lsr* operon [11,12].

60 Interestingly, bacterial communication through small molecule signaling can induce  
61 antibiotic tolerant phenotypes [13–15]. In parallel, it is also known that antibiotics at low doses can  
62 work as signaling molecules [16]. While studying the bacterial response to antibiotics, we showed  
63 that antibiotics from different families induce stress responses in Gram-negative bacteria, at  
64 concentrations below the minimal inhibitory concentration (subMIC), namely the SOS response

65 [17,18]. SOS induction reflects the presence of a genotoxic stress to which the bacterial cell responds  
66 by triggering mutagenic DNA repair and recombination pathways, as well as rearrangements in the  
67 Superintegron carried by the *V. cholerae*'s second chromosome, which carries antibiotic resistance  
68 and adaptation genes [19,20]. We have pursued with the study of the response to aminoglycosides  
69 (AGs), which is a class of antibiotics that target the ribosome and induce mistranslation [21]. The  
70 AG-mediated SOS induction that we observed in *V. cholerae* is conserved among distantly related  
71 Gram-negative pathogens, such as *Photobacterium luminescens* and *Klebsiella pneumoniae* [18]. This  
72 observation was puzzling because AGs do not directly target DNA synthesis or DNA molecules.  
73 Strikingly, we have observed that the induction of SOS by low doses of AGs appeared to be  
74 dependent on HapR [17], because SOS induction by the aminoglycoside tobramycin was prevented  
75 in the *V. cholerae* strain lacking *hapR*. This observation suggested that QS could play a significant  
76 role in the evolution of antibiotic resistance.

77 We thus decided to study the impact of quorum sensing on the effect of sub-inhibitory  
78 concentrations of AGs in *V. cholerae*. We constructed mutants deriving from the N16961 HapR+  
79 strain (referred to as wild-type), deleted for the genes *cqsA* (deficient for CAI intra-species signaling),  
80 *luxS* (deficient for AI-2 inter-species signaling), *luxPQ* (deficient for AI-2 sensing), *luxO* ("locked" in  
81 high cell density state) and *aphA* (the master regulator of low cell density). We asked which QS  
82 pathway(s) are involved in the response to sub-inhibitory concentrations of aminoglycosides, and  
83 how QS is involved in modulation of gene expression patterns by treatment with sub-inhibitory  
84 concentrations of AGs.

85 RNA-seq performed on both QS proficient (HapR+) and QS deficient (HapR-) *V. cholerae*  
86 strains points to major differences on global gene expression in response to subMIC tobramycin  
87 (TOB) treatment. Moreover, transcriptomic data suggests that subMIC AG treatment may interfere  
88 with the quorum sensing pathways and lead to the activation of the AphA low cell density regulon.  
89 We find that supplementation of growth media with AI-2 alleviates SOS induction by subMIC TOB.  
90 We further show that deletion of *luxS* (and to a lesser degree *cqsA*) is strongly detrimental for  
91 growth in presence of sublethal AGs concentrations and also for survival to lethal doses of this  
92 antibiotic family. These observations strongly suggest that QS signaling plays an important role in  
93 the response to antibiotics.

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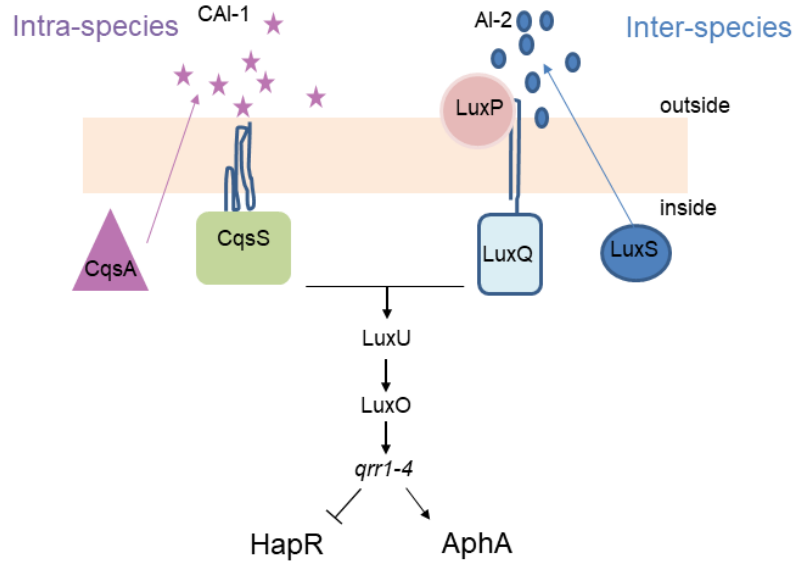
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**Figure 1. Simplified QS network of *V. cholerae*.** Intra and Inter-species autoinducers activate a phosphorylation cascade through LuxU and LuxO, leading to the expression of Qrr1-4 sRNAs which in turn promote expression of the Apha while inhibiting expression of HapR.

## MATERIALS AND METHODS

### Bacterial strains and plasmids.

Strains and plasmids are described in Table 1. Primers used in this work are listed in Table S1. *V. cholerae* N16961 hapR<sup>+</sup> derivatives were constructed by natural transformation as described [22]. Allelic replacements were performed using an assembly PCR fragment carrying 500 bp up and down regions of the gene to be deleted, and replaced by *aadA* spectinomycin resistance gene, using specified primers. Selection was done using spectinomycin 100µg/mL.

122 **Table 1. Strains and plasmids used in this study**

Strains and plasmids	Strain #	construction	
<i>E. coli</i> MG1655			Laboratory collection
<i>P. luminescens</i> TT01 $\Delta$ uvrY	PL2105		[30]
<i>V. cholerae</i> N16961 hapR-	7805		Laboratory collection
<i>V. cholerae</i> N16961 hapR+	8637/F606		Laboratory collection
<i>V. cholerae</i> N16961 hapR+ derivatives			
luxO(VC1021):: spectinomycin	J419	PCR assembly and natural transformation	ZIP413/414 and ZIP415/416 on gDNA. ZB47/48 on pAM34. Assembly ZIP413/416
cqsA(VCA0523):: spectinomycin	J422	PCR assembly and natural transformation	ZIP81/82 and ZIP83/84 on gDNA. ZB47/48 on pAM34. Assembly ZIP81/84
luxS(VC0557)::spectinomycin	J439	PCR assembly and natural transformation	ZIP87/88 and ZIP89/90 on gDNA. ZB47/48 on pAM34. Assembly ZIP87/90
luxPQ(VCA0736- VCA0737)::spectinomycin	F562	PCR assembly and natural transformation	ZIP191/192 and ZIP193/194 on gDNA. ZB47/48 on pAM34. Assembly ZIP191/194
<i>pTOPO-PintIA350-gfp</i> kanamycin	9192	350bp region upstream of <i>intIA</i> was fused to <i>gfp</i> .	[22]

123

124

125 **Tolerance assays**

126 Tolerance assays were performed on early stationary phase cultures. Overnight *V. cholerae* cultures  
127 were diluted 1000x in 10 mL fresh Mueller-Hinton (MH) medium and incubated at 37°C with shaking.  
128 When cultures reached an OD<sub>600</sub> 1.0, aliquots were serial diluted and spotted on MH plates. 3 ml of

129 cultures were then collected into 14 mL Falcon tubes and treated with lethal doses of desired  
130 antibiotics (10 to 20 times the MIC: tobramycin 20 µg/mL and gentamicin 10 µg/mL), for 4 hours at  
131 37°C with shaking in order to guarantee oxygenation. Serial dilutions were then spotted on MH agar  
132 without antibiotics. Experiments were performed 3 times.

133

#### 134 **MIC determination using *Etests*.**

135 Stationary phase cultures were diluted 20 times in PBS, and 300 µL were plated on MH plates and  
136 dried for 10 minutes. *Etests* (Biomérieux) for Tobramycin and Gentamicin were placed on the plates  
137 which were then incubated overnight at 37°C.

#### 138 **RNA-seq.**

139 Overnight cultures of the O1 biovar El Tor N16961 *hapR+* or *hapR-* *V. cholerae* strain were diluted  
140 100x and grown in triplicate in MH medium until an OD<sub>600</sub> of 0.4 with or without 0.02 µg/mL  
141 tobramycin. Sample collection, total RNA extraction, library preparation, sequencing and analysis  
142 were performed as previously described [23].

143

#### 144 **Growth curves.**

145 Overnight cultures were diluted 100x in fresh medium, on 96 well plates. Each well contained 200  
146 µl. Plates were incubated with shaking on TECAN device at 37°C, OD<sub>600</sub> was measured every 15  
147 minutes.

#### 148 **SOS induction**

149 SOS induction measurements by flow cytometry was performed as previously described [17,18,24].  
150 Briefly, overnight cultures were diluted 100 fold in MH or MH supplemented with subMIC  
151 tobramycin (0.02 µg/mL), subMIC ciprofloxacin (0.005 µg/mL) and/or AI-2 (10 µM) and were  
152 incubated overnight at 37°C. Fluorescence was then measured in 100 000 cells on the Miltenyi  
153 MACSquant device. The fluorescence values in each condition were normalized to the fluorescence  
154 values obtained in MH.

155

156

157 **Luminescence measurements in *P. luminescens***

158 Overnight cultures in Schneider media supplemented with 10  $\mu$ M Na-borate, were diluted at OD<sub>600</sub>  
159 0.15 at grown at 30°C. When cultures reached an OD<sub>600</sub> of 0.9, 0.5  $\mu$ g/mL tobramycin were added.  
160 After four hours, luminescence was measured as previously described [25]. Relative value was  
161 calculated: luminescence/ OD<sub>600</sub>. Experiment was performed at least three times.

162 **Statistical analysis.**

163 Student's t-test (unpaired) was performed using GraphPad Prism to determine the statistical  
164 differences between two groups. \* indicates p<0.05. Number of replicates for each experiment was  
165 3<n<6. Means were also calculated using GraphPad Prism.

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168 **RESULTS**

169 **Quorum sensing proficiency influences the response of *V. cholerae* to subMIC tobramycin**

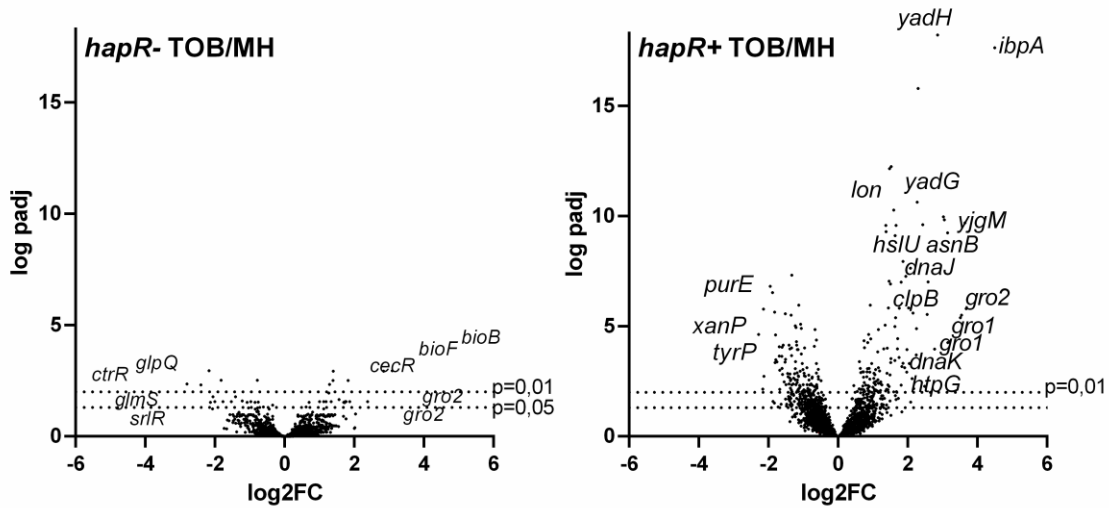
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171 In order to have a global view on the gene expression patterns and their alterations by  
172 treatment with sub-inhibitory concentrations of tobramycin, we undertook a global study using  
173 RNA-seq.

174 Since TOB at 2% of the MIC was previously shown to differently impact stress responses of  
175 *V. cholerae* in a QS proficient *hapR*<sup>+</sup> strain [17] and in the N16961 strain carrying a frameshift  
176 inactivating the *hapR* gene, we performed RNA-seq in exponential phase cultures of *V. cholerae* of  
177 both strains treated or not with TOB at 2% of the MIC.

178 Strikingly, we observed that the number of significantly differentially regulated genes by  
179 TOB in the HapR proficient strain was nearly twice the number of those differentially regulated by  
180 TOB in the *hapR*<sup>-</sup> strain: 366 and 566 genes were at least 1.5-fold up- and down- regulated in *hapR*<sup>+</sup>  
181 against 259 and 238 genes in *hapR*<sup>-</sup>, respectively (Fig. 2). Similarly, 70 genes with  $\geq$  3-fold change  
182 were found to be affected in *hapR*<sup>+</sup> strain, against 45 in *hapR*<sup>-</sup> (Fig. 2).





184

185 **Figure 2. Transcriptomic profiles of *V. cholerae* cells treated with subMIC TOB depend on HapR**  
 186 **proficiency.** Volcano plots depicting gene expression changes caused by subMIC TOB (2% MIC) in  
 187 HapR deficient (left panel) or HapR proficient (right panel) *V. cholerae* cells. The names of the genes  
 188 with the strongest fold changes are represented. The x-axis represents the  $\log_2$  of the fold change  
 189 plotted against the  $\log_{10}$  of the adjusted p-value.

190

191 First, looking at general effects of subMIC TOB on both strains, our analysis reveals the upregulation  
 192 of chaperones and protein degradation factors, usually involved in the response to heat shock and  
 193 protein stress (e.g. GroEL-ES, IbpA, Lon), showing that, even at doses that do not affect growth (here  
 194 50-fold lower than the MIC), TOB still yields protein stress. However, such stress seems to be more  
 195 important in the *hapR+* strain, as the induction of the two *groEL-ES* operons is 11- and 13-fold in  
 196 *hapR+* versus 3- and 4-fold for *hapR-*. Similarly, *ibpA* is induced 22-fold in *hapR+* against 3-fold in  
 197 *hapR-* (Fig. S1A).

198 Other categories of modulated gene expression are sugar metabolism and transport, as well as iron  
 199 related genes. Markedly, sugar transport and metabolism genes are strongly downregulated by TOB  
 200 in the *hapR-* strain (Fig. S1B). This effect is also found in the *hapR+* strain but to a lesser degree,  
 201 which may be explained by the fact that the level of these RNAs are already lower in the *hapR+*  
 202 strain compared to *hapR-*.

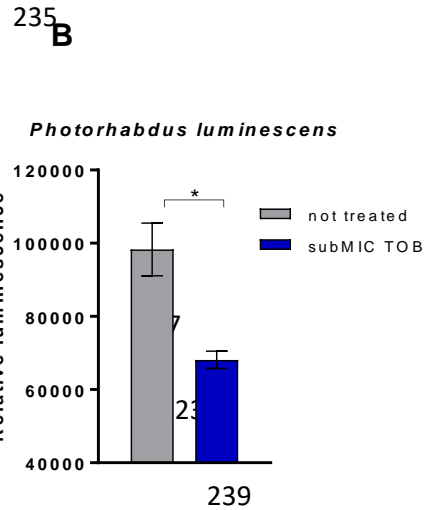
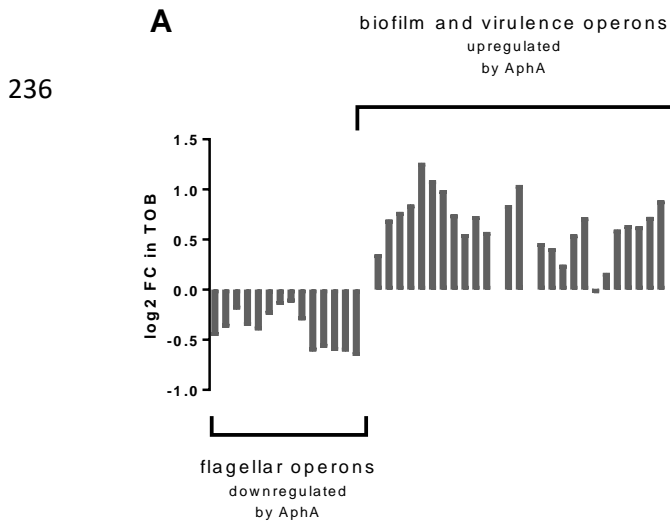
203 Our second observation is that there are also dissimilarities between the two strains in pathways  
204 that are mobilized in response to TOB. Major differences are on translation (mostly downregulated  
205 by TOB in *hapR+*) (Fig. S1C) and iron related genes (mostly up in *hapR-*) (Fig. S1D). Strikingly,  
206 ribosomal protein expression is oppositely modulated in the two strains (Fig. S1C). Since AGs target  
207 translation, reduction of translation in response to low levels of TOB can be an adaptive response,  
208 especially in *hapR+* strain. Importantly, the basal expression levels of ribosomal genes appear to be  
209 already higher in the absence of TOB in the *hapR+* strain and TOB reduces their expression to the  
210 *hapR-* levels. Iron and energy related genes are markedly increased by TOB in *hapR-* with while no  
211 major change was observed in *hapR+* (Fig. S1D).

212

213 **TOB influences QS response of *V. cholerae* and interferes with AI-2 signaling of *Photobacterium***  
214 ***luminescens***

215         Regarding QS, we observed that genes known to be activated or inhibited by AphA [26,27]  
216 in *V. cholerae* are, respectively, up and downregulated by TOB in *hapR+* strain (Fig. 3A), suggesting  
217 activation of the AphA low cell density regulon upon TOB treatment. In *V. cholerae*, the level of  
218 AphA protein is known to be negatively controlled by the concerted action of AI-2, CAI-1 and DPO  
219 (an autoinducer that is part of a third QS pathway in *V. cholerae* [28]), with AphA being barely  
220 detectable upon the simultaneous presence of these autoinducers [29]. However, eliminating AI-2  
221 signaling is sufficient for AphA to be detected, even though at low levels [29]. Thus, in order to  
222 determine whether TOB has a positive or negative influence on AI-2 regulated phenotypes, we  
223 tested the effect of sublethal TOB treatment on bioluminescence in *Photobacterium luminescens* [30],  
224 where natural luminescence is induced by elevated AI-2 levels [25]. We used an *uvrY* deficient strain  
225 as reference strain, as a previously shown decreased amount of AI-2 in this strain allows more  
226 sensitive measurements of AI-2 dependent luminescence, in comparison to the wild-type [25]. We  
227 found that subMIC TOB treatment significantly decreases luminescence in *P. luminescens* (Fig. 3B).  
228 In order to prove that the negative effect of tobramycin on bioluminescence production was indeed  
229 by targeting of the QS network of *P. luminescens* we performed the same experiment using a *luxS*  
230 deficient strain (Fig. S2). The results show that in absence of *luxS*, tobramycin no longer impacts  
231 bioluminescence production in *P. luminescens* (Fig. S2). Together with the activation of AphA  
232 regulon, these observations suggest that subMIC TOB interferes with AI-2 signaling and mimic a low  
233 cell density state.

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243 **Figure 3. subMIC TOB interferes with QS signalling.** **A.** Expression changes induced by subMIC TOB  
 244 (2% MIC) of genes belonging to operons known to be regulated by AphA. **B.** Effect of subMIC TOB  
 245 (50% MIC) on QS dependent luminescence of *P. luminescens*. Error bars indicate standard  
 246 deviations. \* p-value < 0.05 by Student's *t*-test.

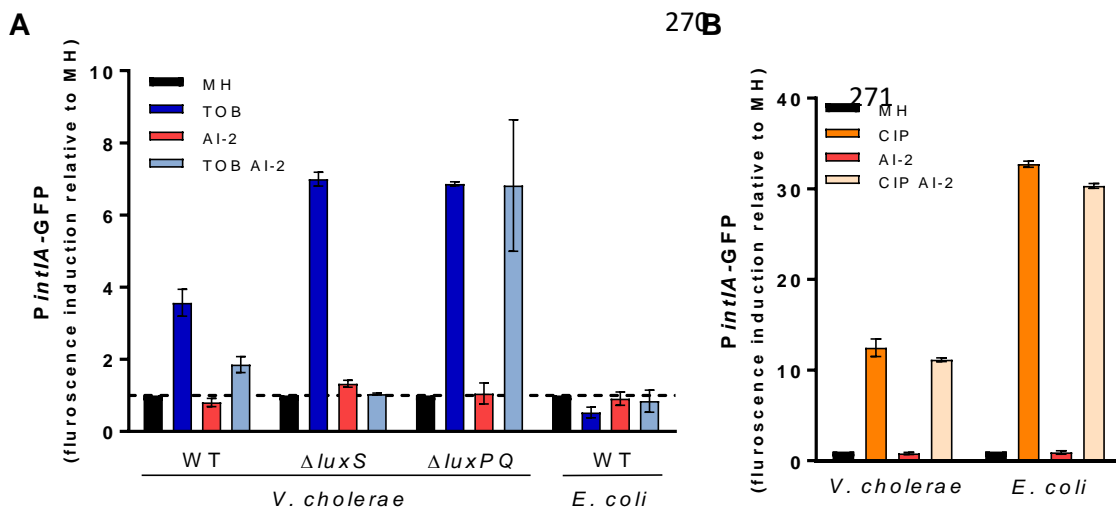
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248 **AI-2 signaling alleviates SOS response induction by subMIC TOB**

249 We have previously found that subMIC TOB induces SOS response in *V. cholerae*, but not in  
 250 *E. coli* [17]. However, in *V. cholerae*, subMIC TOB only induces the SOS response in the *hapR+* but  
 251 not in the *hapR-* strain, suggesting that QS is important for the effect of subMIC TOB ([17] and Fig.  
 252 S2). Since bioluminescence data suggests modulation of *luxS* pathway by TOB, we asked whether  
 253 the levels of AI-2 could have an effect on SOS response induction by TOB. We used GFP reporters of  
 254 the SOS response regulated *intI*A promoter as previously described [17] and confirmed that TOB  
 255 induces SOS in *V. cholerae hapR+* but not in *E. coli* (Fig. 4A). Interestingly, addition of exogenous AI-  
 256 2 alleviates the SOS induction by TOB, suggesting that AI-2 counteracts the effect of TOB. In order  
 257 to confirm that this effect was specifically due to sensing of AI-2 by the bacterial cells, we

258 constructed a strain deleted for the AI-2 sensor *luxPQ* operon. We confirmed that SOS response is  
 259 also induced in the strain lacking *luxPQ* but the negative effect of AI-2 on TOB mediated SOS was  
 260 lost. This is consistent with the hypothesis that sensing of AI-2 by *V. cholerae* minimizes the toxic  
 261 effect of subMIC TOB. We also tested the effect of AI-2 on ciprofloxacin (CIP) induced SOS response  
 262 in both *V. cholerae* and *E. coli* (Fig. 4B). While both species induce SOS in response to CIP (as  
 263 expected), we found no effect of AI-2. CIP induces SOS through direct DNA damage and our results  
 264 show that AI-2 specifically interferes with subMIC TOB induced SOS, and does not prevent this  
 265 induction by DNA damage. Furthermore, we constructed a *V. cholerae* strain deleted for *luxS*, and  
 266 observed that SOS response induction by subMIC TOB was increased in this strain, and addition of  
 267 AI-2 was sufficient to counteract this effect (Fig. 4A). Altogether, these data show that interspecies  
 268 QS signaling interferes with the effect of subMIC TOB in *V. cholerae*.

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274 **Figure 4. Aminoglycoside-mediated SOS induction in *V. cholerae* depends on LuxS/AI-2 signalling.**

275 SOS induction by (A) subMIC tobramycin (TOB, 0.02  $\mu\text{g}/\text{mL}$ ) or (B) subMIC ciprofloxacin (CIP, 0.005  
 276  $\mu\text{g}/\text{mL}$ ) in *V. cholerae* and *E. coli* cells.

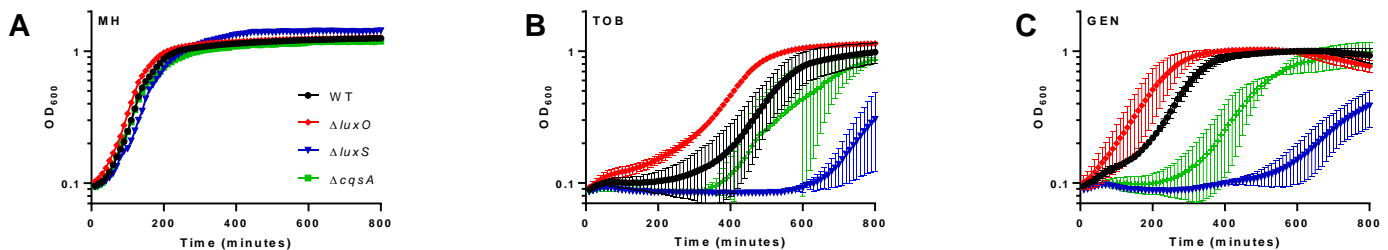
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280 **AI-2 signaling improves growth in subMIC TOB and tolerance to lethal TOB concentrations**

281 We next tested the impact of QS on the susceptibility to antibiotics. We first tested the  
282 ability of QS mutants to grow in the presence of subMIC of the aminoglycosides TOB and gentamicin  
283 (GEN) (Fig. 5). We used a higher subMIC (50% of the MIC) to assess growth of WT and different QS  
284 mutants: *cqsA* (deficient in CAI-1), *luxS* (deficient in AI-2 signaling) and *luxO* deletion mutant, which  
285 is “locked” in a high cell density state. In fact, the *luxO* deletion mutant mimics a state where CAI-1  
286 and AI-2 levels are high. We measured growth in microtiter plates by following the OD<sub>600</sub> for 16  
287 hours. We found that deletion of *luxS* highly impacts growth in subMIC TOB and GEN (Fig. 5B and  
288 C). As a corollary, deletion of *luxO* slightly improves growth in presence of both antibiotics. On the  
289 other hand, deletion of *cqsA* negatively impacts growth in presence of gentamicin but to a lesser  
290 extent when compared to the *luxS* mutant (Fig. 5C). This suggests a protective role of AI-2 signaling  
291 against aminoglycoside action in *V. cholerae* cells.



292

293 **Figure 5. Growth curves of *V. cholerae* QS mutants in (A) absence or (B) presence of sublethal**  
294 **concentrations of tobramycin (TOB, 0.5 μg/mL) and (C) gentamicin (GEN, 0.5 μg/mL). Error bars**  
295 **indicate standard deviation.**

296 We further asked whether deletion of *luxS* increases susceptibility to lethal doses of  
297 antibiotics (Fig. 6). We observed no difference in the MIC of neither tobramycin nor gentamicin  
298 when we compared different mutants to wild type (Table 2). We thus treated *V. cholerae* cultures  
299 grown in rich media at OD<sub>600</sub> 1.0 with these antibiotics at 10 to 20x the MIC for 4 hours, and counted  
300 the proportion of surviving CFUs (Fig. 6). Strikingly, when compared to the WT, the number of  
301 surviving bacteria is highly decreased in the *ΔluxS* strain ( $\approx$  2 and 4 logs difference in gentamicin and  
302 tobramycin, respectively) with *ΔcqsA* and *ΔluxO* mutants exhibiting equivalent survival to WT strain.

303

Table 2. MIC measured by *E-test* ( $\mu\text{g/mL}$ )

Strain	Tobramycin	Gentamicin
WT	1	1
$\Delta\text{luxS}$	1	1
$\Delta\text{cqsA}$	1	1
$\Delta\text{luxO}$	1	1

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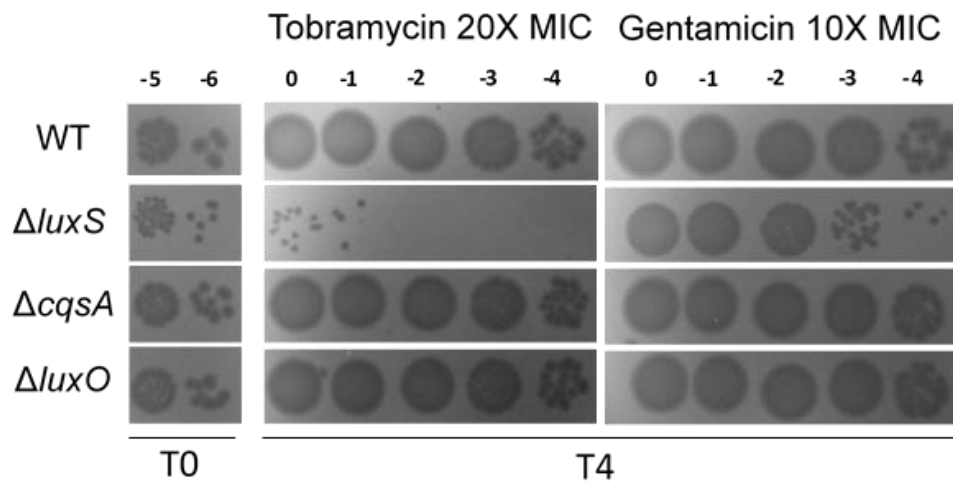
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**Figure 6. Tolerance of *V. cholerae* QS mutants to lethal concentrations of aminoglycosides.**

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Bacterial populations were treated with lethal concentrations of tobramycin (20X MIC) and

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gentamicin (10X MIC) for 4 hours. Represented are 10  $\mu\text{L}$  drops of the indicated dilutions prior

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treatment (T0) and post treatment (T4). This is a representative experiment of three independent

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experiments with similar results.

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## 325 DISCUSSION

326 We based this study on our previous findings that SOS is induced by subMICs of  
327 aminoglycosides in QS proficient (hapR+) but not in QS deficient (hapR-) *V. cholerae* ([17] and Figure  
328 S2). The observation that *V. cholerae* strain lacking HapR fails to trigger aminoglycoside-mediated  
329 SOS induction prompted us to investigate how the responses to subMIC aminoglycosides vary in  
330 different QS contexts in this species. The transcriptomic analysis of both HapR- or HapR+ cells  
331 treated with 2% MIC of tobramycin revealed substantially different gene expression profiles  
332 between the two strains, specifically regarding the expression of genes involved in translation, cell  
333 energy and sugar transport processes (Fig. S1), which are known to modulate the physiological  
334 activity of aminoglycosides in bacteria [14,31–34]. Aminoglycosides are antibiotics known to target  
335 the ribosome, generating mistranslation and protein stress [35]. In agreement with this, we  
336 observed the induction of several members of the heat-shock regulon by subMIC TOB in both  
337 strains, showing that even very low concentrations of these drugs are able to generate protein stress  
338 in *V. cholerae*. However, the extent of this induction seems to be dependent on the QS state of the  
339 cells as we noticed a greater induction of the heat-shock regulon in *hapR+* cells, thus suggesting a  
340 link between QS and response to aminoglycoside treatment in *V. cholerae*. Moreover, when we  
341 treated the *hapR+* strain of *V. cholerae* with subMIC TOB we observed the upregulation of several  
342 genes whose expression is positively controlled by AphA, and the downregulation of genes known  
343 to be repressed by AphA [26,27] (Fig 3A). This suggests that subMIC tobramycin treatment mimics  
344 a state of low cell density, which is characterized by the absence (or low concentration) of  
345 autoinducers and the lack of activation of the respective QS systems. How subMIC TOB leads to the  
346 activation of the low cell density regulon is not clear but it is possible that subMIC tobramycin  
347 interferes with one or several of these QS systems. In fact, we show that subMIC TOB seems to  
348 interfere with AI-2 QS signaling, as we observed that AI-2–dependent bioluminescence production  
349 in *Photobacterium luminescens* is halted by subMIC TOB. Thus, by interfering with LuxS/AI-2 system,  
350 subMIC TOB could partially inhibit the LuxS – LuxO phosphorylation cascade and lead to an increase  
351 of AphA protein levels in the cell with the activation of AphA regulon. However, it has been  
352 suggested that the QS network of *V. cholerae* is quite robust and resilient to signal perturbations by  
353 relying on four functionally redundant QS circuits [8] and that full QS network activation requires  
354 the concerted action of AI-2, CAI-1 and DPO molecules, which act together to fully repress AphA  
355 [29]. Thus, it is possible that some other additional factors, together with low AI-2 signaling, can be  
356 involved in the AphA regulon activation by subMIC TOB.

357           Given the lack of AG-mediated SOS induction in *V. cholerae hapR*- (Fig. S3), and the  
358 observation that AI-2 signaling seems to be affected by tobramycin (Fig. 3B), we also sought to  
359 determine whether AG-mediated SOS induction in *V. cholerae* relies on this interspecies QS system.  
360 We found that subMIC TOB generates higher levels of genotoxic stress in absence of AI-2 signaling,  
361 as we observed a greater induction of SOS response in the *luxS* mutant (Fig. 4). Moreover, deficiency  
362 of LuxS is highly detrimental for *V. cholerae* growth in subMIC aminoglycosides (Fig. 5) and survival  
363 to lethal doses of these antibiotics (Fig. 6).

364           The results described here suggest an interplay between aminoglycoside activity and QS in  
365 *V. cholerae*: on one hand, we show that subMIC TOB affects AI-2 signaling. On the other, we  
366 demonstrate the QS state of the cells (specially mediated by the *luxS*/AI-2 system) seems to dictate  
367 the response of *V. cholerae* to aminoglycosides.

368 Several studies have demonstrated that QS signaling in bacteria often controls a multitude of  
369 processes that promote tolerance and resistance to several antibiotics. For example, expression of  
370 the MexAB-OprM efflux pump is positively controlled by QS and promotes resistance to beta-  
371 lactams in *Pseudomonas aeruginosa* [36]. Also, biofilm formation, which is critical to aminoglycoside  
372 susceptibility [37], is known to be controlled by QS [38,39] and it has been shown that the  
373 susceptibility of *P. aeruginosa* biofilms to aminoglycosides increases in presence of QS inhibitors of  
374 the LasI/LasR and RhlI/RhIR systems [40].

375           In addition, the AI-2/LuxS interspecies QS system has also been shown to modulate  
376 antibiotic resistance mechanisms in several species. Examples include the AI-2/LuxS – dependent  
377 upregulation of MDR efflux pumps which promotes fluoroquinolones resistance in *E. coli* [41] and  
378 *Streptococcus suis* [42] or the AI-2/LuxS dependent upregulation of a two-component system  
379 responsible for increasing vancomycin resistance in *Staphylococcus aureus* [43]. Other examples  
380 linking AI-2/LuxS QS system and drug resistance are reviewed in [44].

381           In *V. cholerae*, the molecular mechanisms behind AI-2-mediated protection against  
382 aminoglycosides remain to be elucidated. Nonetheless, such protection raises the interesting  
383 possibility that even small populations of *V. cholerae*, when in a high cell density multi-species  
384 context, can be less susceptible to aminoglycoside action. This may be of particular importance in  
385 the context of infections in the human gut where AI-2 producing communities may help low loads  
386 of *V. cholerae* to survive aminoglycoside treatment.

387 In parallel, the fact that subMIC TOB interferes with QS signaling may have important consequences  
388 in the context of infection. In fact, AI-2 seems to be the necessary signal to repress biofilm formation



389 and induce dispersal in *V. cholerae* [45]. Thus, by interfering with this QS system, low doses of  
390 aminoglycosides may enhance biofilm formation and virulence of *V. cholerae*. Further work is  
391 necessary to uncover the mechanism by which low doses of tobramycin (and potentially  
392 aminoglycosides in general) disrupt the interspecies QS system. Given that we do not observe any  
393 effect of TOB on the transcription of QS genes in our RNAseq data, one hypothesis may be that  
394 aminoglycosides affect the correct synthesis of specific proteins involved in this system.  
395 Alternatively, aminoglycoside molecules may directly interfere with AI-2 receptors. In fact, subMIC  
396 aminoglycosides attenuate QS-mediated virulence phenotypes in *P. aeruginosa* and they were  
397 found to possess strong binding properties to the QS receptor of *P. aeruginosa*, LasR [46].

398 Overall, the results obtained here contribute to the notion that QS communication and  
399 antibiotic resistance/tolerance mechanisms are linked. A link between bacterial signaling and  
400 antibiotic tolerance was also previously shown for a different signaling system, through indole  
401 secretion [13,15]. Manipulation of cell to cell signaling may thus be a potential way to fight  
402 antimicrobial resistance.

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415 Accession numbers for RNAseq: the data for the RNAseq of the hapR- strain has been submitted in  
416 the GenBank Sequence Read Archive (SRA) under project number: PRJNA506714. For hapR+ strain,  
417 the RNAseq data have been deposited at GEO: GSE182561 and are publicly available as of the date  
418 of publication.

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563

564 **FIGURE LEGENDS**

565 **Figure 1. Simplified QS network of *V. cholerae*.** Intra and Inter-species autoinducers activate a  
566 phosphorylation cascade through LuxU and LuxO, leading to the expression of Qrr1-4 sRNAs which  
567 in turn promote expression of the AphA while inhibiting expression of HapR.

568 **Figure 2. Transcriptomic profiles of *V. cholerae* cells treated with subMIC TOB depend on HapR**  
569 **proficiency.** Volcano plots depicting gene expression changes caused by subMIC TOB (2% MIC) in  
570 HapR deficient (left panel) or HapR proficient (right panel) *V. cholerae* cells. The names of the genes  
571 with the strongest fold changes are represented. The x-axis represents the log<sub>2</sub> of the fold change  
572 plotted against the log<sub>10</sub> of the adjusted p-value.

573 **Figure 3. subMIC TOB interferes with QS signalling.** A. Expression changes induced by subMIC TOB  
574 (2% MIC) of genes belonging to operons known to be regulated by AphA. B. Effect of subMIC TOB  
575 (50% MIC) on QS dependent luminescence of *P. luminescens*. Error bars indicate standard  
576 deviations. \* p-value < 0.05 by Student's t-test.

577 **Figure 4. Aminoglycoside-mediated SOS induction in *V. cholerae* depends on LuxS/AI-2 signalling.**  
578 SOS induction by (A) subMIC tobramycin (TOB, 0.02 µg/mL) or (B) subMIC ciprofloxacin (CIP, 0.005  
579 µg/mL) in *V. cholerae* and *E. coli* cells.

580 **Figure 5. Growth curves of *V. cholerae* QS mutants in (A) absence or (B) presence of sublethal**  
581 **concentrations of tobramycin (TOB, 0.5 µg/mL) and (C) gentamicin (GEN, 0.5 µg/mL).** Error bars  
582 indicate standard deviation.

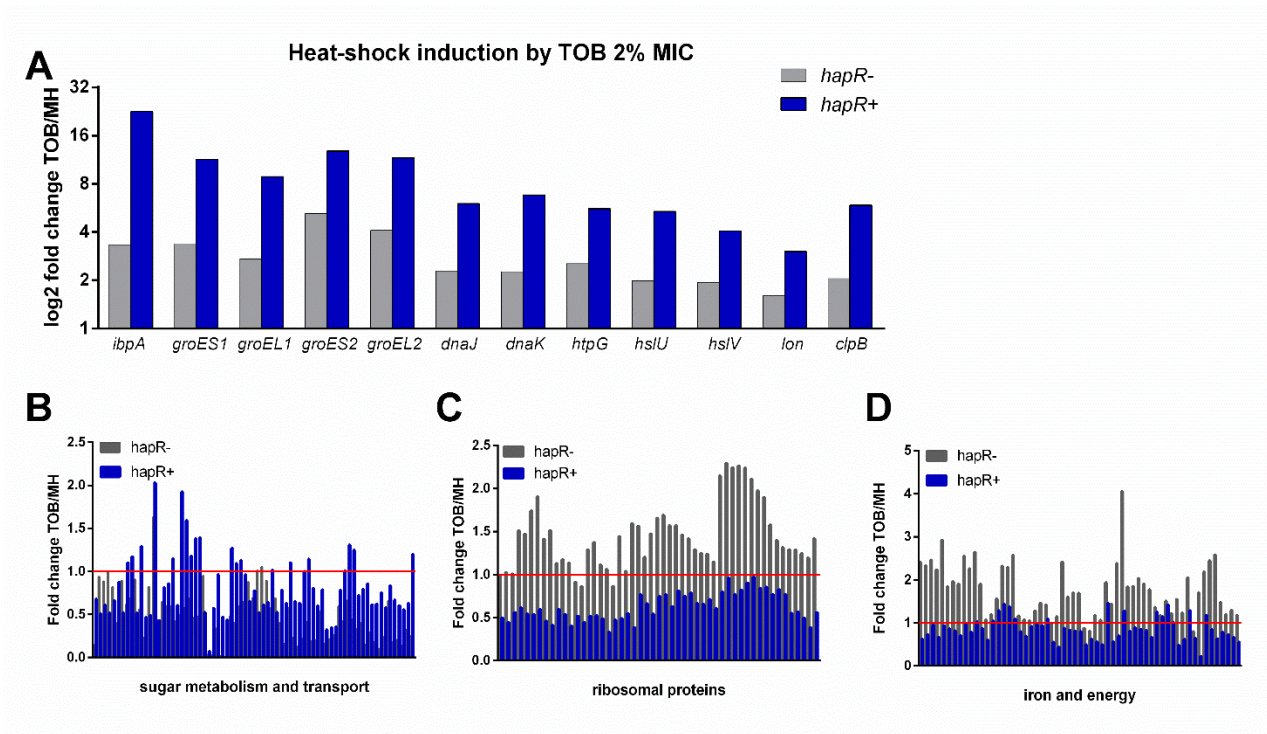
583 **Figure 6. Tolerance of *V. cholerae* QS mutants to lethal concentrations of aminoglycosides.**  
584 Bacterial populations were treated with lethal concentrations of tobramycin (20X MIC) and  
585 gentamicin (10X MIC) for 4 hours. Represented are 10 µl drops of the indicated dilutions prior  
586 treatment (T0) and post treatment (T4). This is a representative experiment of three independent  
587 experiments with similar results.

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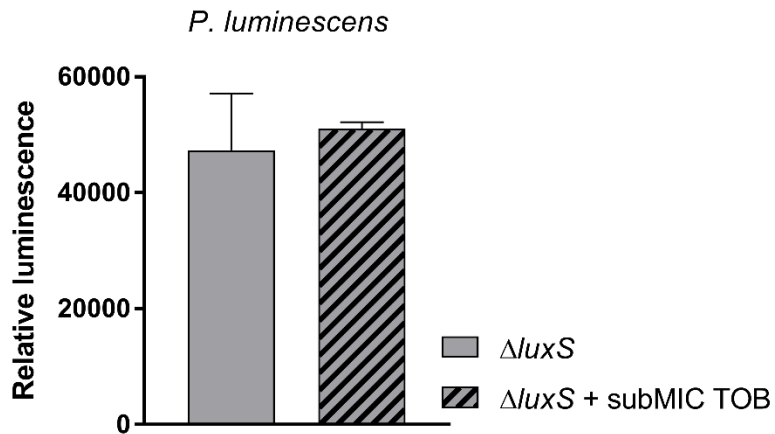
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595 **Figure S1. A.** Fold change values of several members of the heat-shock regulon induced by subMIC  
 596 TOB in the different QS background contexts. **B-D.** subMIC TOB – induced fold change of several  
 597 genes belonging to the specified categories in both hapR- and hapR+ background contexts. All  
 598 represented fold change values are statistically significant with  $p < 0.01$ , as calculated for  
 599 transcriptomics analysis.



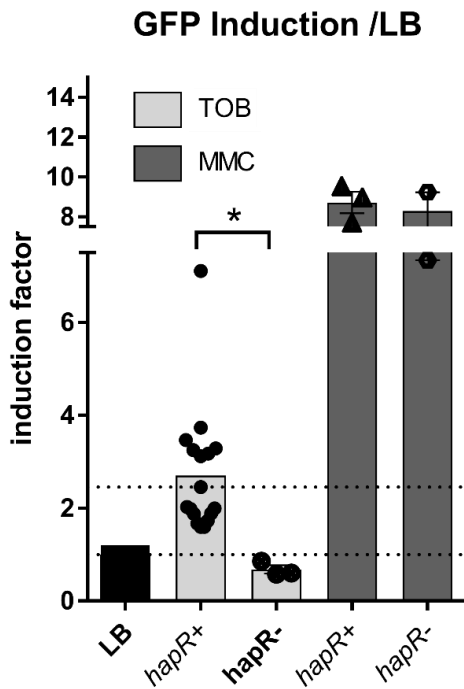


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602 **Figure S2.** subMIC TOB (50% MIC) fails to affect luminescence of a *P. luminescens*  $\Delta luxS$  strain. Error  
 603 bars indicate standard deviations; n=3.

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616 **Figure S3.** SOS induction in both hapR- and hapR+ context of *V. cholerae* by sub inhibitory  
 617 concentrations of tobramycin (TOB) or mitomycin C (MMC). Error bars indicate standard deviations.

618 \*, p-value < 0.05 by Student's t-test.

619 **Table S1. Primers used in this study**

Name	Sequence
ZB47	CCCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGC
ZB48	GACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACG
ZIP413	AAACGTGATGAAATTCATCAGTTGCG
ZIP414	GCGAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGCACCGACGCCGTGTCTTCTACC
ZIP415	CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCGCGATGAGAGAATGGATCAACC
ZIP416	GAATATGGACCTGCAGGACGAATGAGC
ZIP81	GCCAACGATGGTAGTGAAGTGCTCAAGC
ZIP82	GCGAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGATGCATAAATAACAAAACTGCTAAACG
ZIP83	CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCGCAATATATCCTAGTTATAAAAAATTTAACG
ZIP84	TGACAGGGGCTTTAGCGGGCATTGCC
ZIP87	AAGTGGTACTGGAAGGTCGCAAGC
ZIP88	GCGAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGTTCTTTCTCCCTTCTGGCTCGC
ZIP89	CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCGTCGGTTCTGTAAACGTTAAAAATACAAAAGC
ZIP90	GGTACACCGCTTAACGTTCACTGC
ZIP191	CGTGACTGGAGATAAAACGCAGATCTGG
ZIP192	GCGAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGAACAAGATTCAGCAGCACGTATTGC
ZIP193	CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCCTATCAGAAACAGCGGAGATATTAGC
ZIP194	TCACCAAACAGCGCCGATTCAACGG

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