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1 **Sleeping ribosomes: bacterial signaling triggers RaiA mediated persistence to aminoglycosides.**

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14

15 **Abstract**

16 Indole is a molecule proposed to be involved in bacterial signaling. We find that indole secretion is
17 induced by sublethal tobramycin concentrations and increases persistence to aminoglycosides in *V.*
18 *cholerae*. Indole transcriptomics showed increased expression of *raiA*, a ribosome associated factor.
19 Deletion of *raiA* abolishes the appearance of indole dependent persisters to aminoglycosides, while its
20 overexpression leads to 100-fold increase of persisters, and a reduction in lag phase, evocative of
21 increased active 70S ribosome content, confirmed by sucrose gradient analysis. We propose that,
22 under stress conditions, RaiA-bound inactive 70S ribosomes are stored as so-called “sleeping
23 ribosomes”, and are rapidly reactivated upon stress relief. Our results point to an active process of
24 persister formation, through ribosome protection during translational stress (e.g. aminoglycoside
25 treatment), and reactivation upon antibiotic removal. Translation is a universal process, and these
26 results could help elucidate a mechanism of persistence formation in a controlled, thus inducible way.

27 **Introduction**

28 Antibiotic resistance is a major public health concern leading to increased health care costs and
29 mortality (Opatowski et al., 2019; Touat et al., 2019). Although the majority of studies address the
30 response of bacteria to lethal doses of antibiotics, the effect of low doses of antibiotics on bacteria has
31 also recently started to draw attention. Antibiotic concentrations lower than the minimal inhibitory
32 concentration (sub-MICs) have historically been proposed to serve as signaling molecules (Davies et
33 al., 2006), provoking considerable changes in transcription and triggering a wide variety of cellular
34 responses in different bacterial species (Andersson and Hughes, 2014) and mutagenesis (Gutierrez et
35 al., 2013). In *V. cholerae*, sub-MIC aminoglycosides (AGs) are known to activate various stress response
36 pathways, such as the SOS (Baharoglu et al., 2014; Baharoglu and Mazel, 2011) and RpoS stress
37 responses (Baharoglu et al., 2013), allowing cells to cope with increased reactive oxygen species (ROS)
38 levels and DNA breaks (Negro et al., 2019). AGs are bactericidal antibiotics that are known to enter the
39 bacterial cell through the proton motive force (Frimow et al., 1991; Herisse et al., 2017; Taber et al.,
40 1987). AGs target the ribosome, leading to mistranslation and eventually cell death (Davis, 1987).

41 Interestingly, sub-MIC antibiotics, among which AGs, have been shown to stimulate the production of
42 a small molecule, indole (Han et al., 2011). Indole is a byproduct of tryptophan degradation by
43 tryptophanase TnaA (Evans et al., 1941) in both Gram+ and Gram- bacterial species (Lee and Lee,
44 2010), together with pyruvate and ammonia. While pyruvate and ammonia are respectively sources of
45 carbon and nitrogen, the role of indole is not well understood. Indole is also found in plants and
46 animals, and was linked with signaling and human diseases (for a review, (Lee et al., 2015)). Common
47 indole concentrations in the human gut are in the order of 250-1100 μ M, and up to 200 μ M in blood
48 and other tissues. Regulation of indole production has been described, namely through carbon source
49 utilization (Botsford and DeMoss, 1971) and catabolic repression (Yanofsky et al., 1991), amino acid

50 availability (Newton and Snell, 1965), cold temperature (Lee et al., 2008), heat shock (Li et al., 2003)
51 and growth phase (Kobayashi et al., 2006). Particularly, indole is produced during transition from
52 exponential to stationary phase (Lelong et al., 2007).

53 In *E. coli*, indole is nontoxic at physiologic concentrations (below 1 mM) (Lee et al., 2007), and does
54 not change the growth rate (Lee et al., 2008). At high concentrations however (above 1-3 mM), indole
55 inhibits cell division (Chant and Summers, 2007; Chimere et al., 2012). In *V. cholerae*, indole secretion
56 reaches its maximum at 600 μ M during transition from midlog to stationary phase (Howard et al., 2019;
57 Mueller et al., 2009) and was not observed to have any effect on the polarity of the *V. cholerae* cell
58 membrane at this concentration (Mueller et al., 2009).

59 Indole can pass across the cell membrane without the need for a transporter (Pinero-Fernandez et al.,
60 2011), and was proposed to act as an interkingdom signaling molecule (Martino et al., 2003; Wang et
61 al., 2001). An effect of indole in persistence to antibiotics has also been observed. At toxic
62 concentrations (1-2 mM), where indole behaves as a membrane ionophore, it was observed to reduce
63 persistence of stationary phase cultures to tested antibiotics (ciprofloxacin, ampicillin) (Hu et al., 2015).
64 However, studies in *E. coli* show that lower concentrations of indole increase survival/persistence to
65 lethal concentrations of ofloxacin, ampicillin and kanamycin (Vega et al., 2012; Vega et al., 2013),
66 suggesting that the protective effect of indole is not specific to one family of antibiotic. Studies also
67 pointed to the involvement of indole secretion in the cooperation between antibiotic resistant and
68 sensitive populations during antibiotic stress (Lee et al., 2010). Notably, a recent study identified indole
69 production as a potential target for the increased activity of quinolones against persisters in *E. coli*
70 (Zarkan et al., 2020). Since indole appears to be beneficial for bacteria in the presence of antibiotics,
71 we addressed whether indole production is increased upon sub-MIC AG treatment in *V. cholerae* and
72 whether this can lead to improved response to lethal antibiotic concentrations.

73 Importantly, we find that indole strongly increases persistence to AGs through the action of RaiA. We
74 find that transcription from the *raiA* gene promoter is highly upregulated in the presence of indole in
75 exponential phase *V. cholerae* cells. RaiA was shown to be a ribosome associated protein, in the same
76 conditions as Rmf (ribosome modulation factor) and Hpf (hibernation promoting factor) (Maki et al.,
77 2000). The two latter factors cause dimerization of vacant 70S into inactive 100S ribosome dimers, in
78 a process called ribosome hibernation during stationary phase (Gohara and Yap, 2018), whereas RaiA
79 mostly binds to free 70S monosomes (Maki et al., 2000; Sabharwal et al., 2015). *E. coli* mutants lacking
80 these ribosome-associated factors do not show any growth defect during exponential growth, which
81 is consistent with the fact that their expression is specific to stationary phase and stress (Prossliner et
82 al., 2018). RaiA was observed to protect the 70S ribosome from degradation (Agafonov et al., 1999; Di
83 Pietro et al., 2013), and was also observed to block the binding of tRNA to the ribosomal A site in a cell
84 free translation system (Agafonov et al., 2001), and during cold shock (Vila-Sanjurjo et al., 2004). In the
85 present study, characterization of the *raiA* deletion mutant shows that RaiA is instrumental in the
86 appearance of persister cells to AGs. We propose here a new mechanism of induced persistence to
87 AGs by which RaiA positively affects the intact ribosome content of the cell, and facilitates regrowth
88 after removal of the antibiotic.

89 **Results**

90 **Indole is produced in response to sub-MIC tobramycin and increases persistence to AGs**

91 Since the antibiotics ampicillin and kanamycin increase indole levels in *E. coli* (Han et al., 2011), we
92 measured indole secretion (Saint-Ruf et al., 2014) in *V. cholerae* to determine whether the
93 aminoglycoside tobramycin also impacts indole levels in this case. We found increased extracellular
94 indole concentrations in the presence of sub-MIC tobramycin (TOB 0.15 μ g/ml, 20% of the MIC, **Figure**
95 **1**). We next addressed whether indole has an impact on the growth of *V. cholerae*, using an indole
96 concentration of 350 μ M. This concentration was previously shown to be physiologically relevant in *V.*
97 *cholerae* and was observed to have no inhibitory effect on growth, and to complement the biofilm
98 formation defect of a Δ *tnaA* mutant deficient for indole production (Mueller et al., 2009). We found

99 that 350 μ M indole does not affect growth in the absence of antibiotics, but improves growth in sub-
100 MIC antibiotics tobramycin (**Figure S1A**).

101 We next addressed the effect of indole in the response to lethal concentrations of antibiotics, by
102 measuring persister cells formation in *V. cholerae*. In order to do so, we adapted to *V. cholerae*, a
103 protocol developed for *E. coli* (Ivan Matic and Wei-Lin Su, personal communication). Early exponential
104 phase cultures were treated with lethal doses of antibiotics (5 to 10 times the MIC) for 20 hours. We
105 first confirmed that cells surviving after 20 hours of antibiotic treatment and which grow upon
106 antibiotic removal, were indeed persister cells, by performing survival curves (**Figure S2A**). The biphasic
107 profiles of the killing curves we obtained was consistent with the formation of persister cells after 5
108 hours (Brauner et al., 2016). Furthermore, these cells were not resistant to the antibiotic, as no growth
109 was observed when streaked on antibiotic containing plates (not shown). We thus carried on with the
110 quantification of persisters at 20 hours of antibiotic treatment. We found that *V. cholerae* cultures
111 grown in the presence of indole yielded higher numbers of persister cells to the AGs TOB and
112 gentamicin (GEN) (**Figure 2AB**), as previously observed for *E. coli* treated with kanamycin and ofloxacin
113 (Vega et al., 2012), but we observed no effect for persistence to carbenicillin (CRB), or trimethoprim
114 (TMP) (**Figure 2CD**). Furthermore, a strain deleted for *tnaA* yielded less persisters to tobramycin than
115 the WT strain (**Figure S2B**), and the effect was reversed by indole complementation, consistent with a
116 link between indole and persistence.

117 **Indole, at concentrations allowing growth, does not affect AG entry**

118 We next addressed whether the beneficial effect of indole treatment in the presence of AGs is due to
119 modifications in membrane potential, which could lead to decreased AG entry into the cell. AG uptake
120 by the bacterial cell is known to be linked to the proton motive force (PMF). At high concentrations (5
121 mM) indole is a proton ionophore which blocks cell division by dissipating the PMF (Kralj et al., 2011),
122 and was observed to interact with the cell membrane and change its physical structure (Mitchell,
123 2009). In order to measure AG entry in the bacterial cell, we used the aminoglycoside neomycin
124 coupled to the fluorophore Cy-5, which was previously synthesized for aminoglycoside uptake studies
125 in bacteria and demonstrated to bear the properties of aminoglycosides for uptake, mode of action
126 and activity against Gram negative bacteria (Sabeti Azad et al., 2020), (Pierlé et al, unpublished). We
127 found that indole at the physiological concentration of 350 μ M does not affect AG entry into the
128 bacterial cell (**Figure S3A**), ruling out the possibility of decreased AG entry due to modifications of PMF
129 in the presence of indole. Moreover, the presence of 350 μ M indole did not change the MIC of two
130 aminoglycosides: tobramycin and gentamicin (**Figure S3B**). The beneficial effect of indole in the
131 presence of AGs is thus not through reduced antibiotic entry.

132 **Indole induces RaiA (VC0706) expression.**

133 In order to shed light into mechanisms allowing for more efficient response to antibiotic stress upon
134 indole treatment, we decided to study the transcriptomic changes of *V. cholerae* in response to 350
135 μ M indole. mRNA sequencing of exponential phase cultures shows differential regulation of 260 genes
136 shown in **Table S1** and represented in **Figure 3A** (>2-fold change, adjusted *p* value <0.01 as in (Krin et
137 al., 2018)). The most affected categories were respiration (31 genes upregulated), electron transfer
138 and iron uptake (55 genes downregulated). Indole mediated protection against antibiotic killing was
139 previously proposed to be through upregulation of efflux pumps (Blair et al., 2013; Hirakawa et al.,
140 2005; Kobayashi et al., 2006; Lee and Lee, 2010; Nikaido et al., 2012), or through an increase in OxyR
141 associated oxidative stress response (Vega et al., 2013). However, our RNA-seq data show no induction
142 of oxidative stress response related genes in *V. cholerae* (*oxyR*, *soxRS*, *katG*) by indole, and rather
143 suggest decreased expression of proteins linked to iron uptake, suggesting decreased iron and ROS
144 levels upon indole treatment (Baharoglu et al., 2013; Mehi et al., 2014). The second most affected
145 category belongs to translation related genes (31 genes, approximately 10% of total differentially
146 regulated genes, with $p < 3.00E-4$). Notably, one translation related gene was markedly upregulated:

147 *raiA* (VC0706, 20-fold up), together with *rmf* (VC1484, 3-fold up), which are both described as factors
148 associated with inactive ribosomes in stationary phase (Agafonov et al., 1999; Di Pietro et al., 2013).
149 *raiA* expression is known to be triggered by transition to stationary phase (Maki et al., 2000), and RaiA
150 is usually weakly expressed during exponential phase. RT-qPCR on *raiA* (Figure 3B) and fluorescence
151 associated flow cytometry on cells carrying GFP fused to the *raiA* promoter (Figure 4A and S4AB)
152 confirmed upregulation of *raiA* by indole during exponential phase, and increased expression of *raiA*
153 in stationary phase. Since transcriptomic data pointed RaiA as one of the most differentially regulated
154 genes by indole, we next decided to address the contribution of RaiA in the indole associated
155 phenotypes in *V. cholerae*.

156 **Absence of *raiA* reduces persistence to AGs and abolishes induction by indole.**

157 In order to address the involvement of RaiA in indole induced persistence, we constructed a *V. cholerae*
158 Δ *raiA* mutant, and measured the frequency of persistence to tobramycin (TOB), gentamicin (GEN),
159 carbenicillin (CRB) and trimethoprim (TMP) in exponential phase cultures, grown in the absence and
160 presence of indole (Figure 2ABCD). We found that *V. cholerae* Δ *raiA* generally formed slightly less
161 persister cells to TOB, although the difference was not statistically significant when we compare
162 persistence frequency, probably because at exponential phase *raiA* expression is not strong enough in
163 the untreated WT strain, and due to variability between experiments. However, the ratio of persisters
164 calculated separately for each experiment shows significant 10-fold decrease in Δ *raiA* compared to WT
165 (Figure S5A). Strikingly, the deletion of *raiA* completely abolished induction of persistence by indole to
166 both tested AGs (TOB, GEN, Figure 2AB), pointing to a role of RaiA in persister cell formation. No effect
167 of neither indole nor *raiA* was observed in the formation of persisters to CRB or TMP (Figure 2CD),
168 suggesting that the effect of RaiA in persister formation is specific to AGs. The involvement of RaiA in
169 persistence to AGs also appears to be conserved in *E. coli* as the *raiA* deficient mutant yielded less
170 persisters to TOB (Figure S5B). Finally, as performed above in the WT *V. cholerae* strain, we confirmed
171 that the presence of indole does not affect the AGs MIC of the Δ *raiA* strain, and that deletion of *raiA*
172 does not affect the MIC and AG uptake (Figure S3AB), meaning that the phenotypes we observe are
173 not due to reduced entry of AGs or increased resistance. On the other hand, RaiA is dispensable for
174 growth improvement by indole (Figure S1B), because indole still improves growth in TOB when *raiA* is
175 deleted, showing that the mechanism of AG persister induction by indole (antibiotic concentration
176 >MIC) is different than the mechanism of growth improvement in sub-MIC AGs. It is worth mentioning
177 here that the growth of Δ *raiA* strain appeared to be slightly slower than the WT strain, suggesting that
178 RaiA may also have a role during exponential growth, despite its low level of expression.

179 **RaiA overexpression increases persistence to AGs and promotes earlier exit from stationary phase.**

180 In order to mimic conditions of RaiA induction, we cloned it under a controlled *Para* promoter, which
181 is repressed by glucose and induced by arabinose. Since the presence of different carbon sources may
182 differentially affect growth and the response to aminoglycosides (Pierlé et al., unpublished), and also
183 because arabinose was shown to have an impact on growth in *V. cholerae*, we compared the
184 persistence levels of cells carrying the empty vector (p0 in Figure 5ABCDEF) or the pBAD-RaiA plasmid,
185 in the presence of arabinose (RaiA overexpression conditions). Overexpression of RaiA strongly
186 increased persisters formation in three aminoglycosides: TOB, GEN and NEO (Figure 5ABC), but not in
187 three non-aminoglycoside antibiotics from different families: CRB, TMP and ciprofloxacin (CIP) (Figure
188 5DEF), indicating that RaiA is directly and specifically involved in the persistence mechanism to
189 aminoglycosides. Additionally, the MIC showed no difference between the *raiA* overexpression strain
190 compared to the strain with empty plasmid p0 and pRaiA+ (MIC = 0.75 μ g/ml for both on MH plates
191 containing arabinose), ruling out a potential effect on resistance.

192 We next asked whether such increased persistence could be due to slower growth when RaiA is
193 overexpressed. We monitored growth in conditions where 1) RaiA is not overexpressed previous to
194 inoculation and only overexpressed during the growth curve, and 2) RaiA is previously overexpressed

195 in cells used for inoculation (**Figure S6A**). First, our results show no difference in growth rate (slope) in
196 presence or absence of RaiA overexpression (**Figure 6AD and S6B**), indicating that RaiA overexpression
197 does not cause a slow growth phenotype, which discards the hypothesis linking RaiA-mediated
198 persistence to slow growth/dormancy. Interestingly, we observe in cells where RaiA overexpression
199 was pre-induced, that these cells start growing faster due to a reduction in lag phase (**Figure 6BC and**
200 **Figure S6B**). Such a reduction in lag phase is reminiscent of increased active ribosome content which
201 allows faster resumption of growth at the exit of stationary phase (Condon et al., 1995). As a corollary,
202 when *raiA* was deleted, the lag phase was increased (**Figure 7A and Figure S6C**), suggesting that RaiA
203 levels affect inactive but “ready to use” ribosome content, which we called *sleeping ribosomes*, in
204 stationary phase.

205 **The effect of RaiA on lag phase and persistence is independent of ribosome hibernation factors** 206 **Rmf/Hpf**

207 RaiA was previously found to be associated with the inactive ribosomes at stationary phase, in the
208 same conditions as Rmf and Hpf factors. Rmf and Hpf are known to dimerize ribosomes into so called
209 hibernating 100S ribosomes, whereas RaiA associates with monomeric 70S ribosomes (Gohara and
210 Yap, 2018; Maki et al., 2000; Prossliner et al., 2018). In order to address whether Hpf/Rmf dependent
211 ribosome hibernation also favors rapid exit from stationary phase, we performed deletion and
212 overexpression experiments similar to what we did for RaiA. Deletion of *rmf* or *hpf* has no effect on lag
213 phase nor growth (**Figure 7A and S6C**). Our results showed that in contrast to overexpression of RaiA
214 which decreases the lag phase, overexpression of Rmf or Hpf rather increase lag phase (**Figure 7B and**
215 **S6C**). These findings are consistent with a model where increased ribosome dimerization by Hpf/Rmf
216 would require action of dissociation factors in order to resume growth whereas spontaneous
217 dissociation of RaiA from inactive 70S ribosomes (Agafonov et al., 2001; Maki et al., 2000), is sufficient
218 for growth restart. Furthermore, when we overexpressed RaiA in Δhpf or Δrmf mutants, we observed
219 a reduction of lag phase similar to what is observed upon overexpression of RaiA in the WT strain
220 (**Figure 7DC**), meaning that RaiA action is not dependent of Hpf/Rmf mediated ribosome hibernation.

221 In addition, we addressed the effect of hibernation factors on persistence in exponential phase. Unlike
222 for RaiA overexpression, no increase in persistence to TOB was detected upon overexpression of Hpf
223 or Rmf, excluding an effect of 100S ribosome dimer formation on persistence to AGs in exponential
224 phase (**Figure 5A**). Unexpectedly, persistence levels even decreased upon overexpression of Hpf,
225 suggesting that 70S-RaiA (sleeping ribosome) and 100S-Rmf/Hpf (hibernating ribosome) complexes
226 may have opposite effects on persistence to AGs in exponentially growing bacteria. We also tested the
227 persistence levels of *rmf* and *hpf* deletion mutants. Surprisingly again, and consistent with
228 overexpression results, we found that deletion of *rmf* or *hpf* hibernation factors increases persistence
229 to AGs (**Figure S7**). The increase of persistence due to deletion of *rmf* is dependent on the presence of
230 *raiA*, as the double mutant *raiA rmf* shows reduced persistence compared to WT, and similar to the
231 *raiA* mutant. This can be due to the observed increased expression of *raiA* in the absence of *rmf*
232 (Sabharwal et al., 2015) or amplified ribosome-RaiA complex formation in the absence of *rmf*, due to
233 decreased 100S formation (Ueta et al., 2005). Finally, it is important to note that the *hpf raiA* double
234 mutant could not be constructed despite the use of two different strategies, implying synthetic
235 lethality. Overall these results suggest an equilibrium between Rmf/Hpf and RaiA actions, consistent
236 with previous literature that showed a combined role for these proteins in ribosome hibernation and
237 antagonizing regulation of *rmf/hpf* and RaiA in *V. cholerae* (Sabharwal et al., 2015).

238 **RaiA overexpression increases 70S ribosome proportion over 50S and 30S subunits in stationary** 239 **phase.**

240 In order to address whether intact 70S ribosomes are protected/stored upon RaiA overexpression, we
241 measured the ribosome contents of WT and RaiA overexpressing cells, as well as the strain deleted for

242 *raiA*, by performing 10-50% sucrose gradients on cellular extracts from 24h stationary phase cultures.
243 The profiles obtained were consistent with well described peaks for 30S and 50S subunits followed by
244 a third peak corresponding to the 70S ribosome (Ueta et al., 2013). No clear 100S peak was observed
245 in any of the experiments. Deletion of *raiA* leads to a slight increase in the proportion of dissociated
246 subunits compared to 70S ribosome. We found that the proportion of 70S ribosomes is increased
247 compared to 30S+50S dissociated subunits upon RaiA overexpression (**Figure 8AB**). This agrees with
248 the hypothesis that RaiA would stabilize the intact 70S ribosome and increase functional ribosome
249 pools, reducing the lag phase of the population upon growth restart.

250 **RaiA expression is under environmental control and linked to the Fur iron sensing regulon in *V.*** 251 ***cholerae***

252 RaiA is known to be expressed in stationary phase and upon temperature stress (Agafonov et al., 1999;
253 Agafonov et al., 2001; Maki et al., 2000; Sabharwal et al., 2015; Slamti et al., 2007). Regulation of *raiA*
254 was also described to occur through carbon catabolite control (CRP-cAMP) (Manneh-Roussel et al.,
255 2018; Shimada et al., 2013). Our transcriptomic data suggests that indole affects genes from the Fur
256 regulon (**Table S1**). Fur responds to iron levels and is generally known to be a repressor of iron/heme
257 transport genes (such as *hutA*, *hutXW*, *tonB*, *fbpA*, *viuB*, among others) and also activates a small
258 number of genes (namely the *napABC* operon and *menB* in *V. cholerae*) (Mey et al., 2005). The *V.*
259 *cholerae raiA* gene promoter, appears to carry sequences similar to Fur boxes described in *V. cholerae*
260 (Davies et al., 2011). In order to shed light in the means by which indole induces expression from the
261 *raiA* promoter in exponential phase, we constructed deletion mutants for *fur*, *crp* and for the stationary
262 phase sigma factor *rpoS*. We used our *PraiA-gfp* transcriptional fusion to measure expression in the
263 presence and absence of indole in the mutants compared to wild type strain.

264 As expected, in WT *V. cholerae*, fluorescence was triggered by indole at 3 hours of culture which
265 corresponds to early exponential phase (OD 0.2 to 0.3), and accumulated in stationary phase (**Figure**
266 **4A and S4AB**). Deletion of *crp* strongly decreases *raiA* expression (25x in exponential phase, **Figure 4B**
267 **and S4AB**), confirming that CRP is a prevailing activator of *V. cholerae raiA*. On the other hand, deletion
268 of *rpoS* had no major effect on *V. cholerae raiA* expression (1.25x decrease). In the Δfur strain, *raiA*
269 expression was increased 2-fold (**Figure 3B and S4AB**), suggesting Fur dependent repression of the *raiA*
270 promoter. No major effect on *raiA* promoter was observed upon treatment with iron, possibly because
271 iron is already in excess levels for the cells during growth. Strikingly, treatment with dipyriddy (DP), an
272 iron chelator which mimics conditions of iron starvation strongly induced fluorescence (**Figure 4A**). To
273 confirm that these effects were specific to the *raiA* promoter, we introduced constitutively expressed
274 *gfp* using the same plasmid vector in the strains WT, Δcrp and Δfur and observed no effect on
275 fluorescence (**Figure S8**). This was also the case upon indole treatment of the WT strain. DP appears to
276 decrease fluorescence for constitutive promoter, while increasing it 4x from *raiA* promoter (**Figure 3B**).
277 Overall, these data confirm that decreased fluorescence in Δcrp and increased fluorescence in Δfur or
278 upon indole and DP treatments are specific to an effect on transcription from the *raiA* promoter.

279 These results show a link between extracellular iron levels and RaiA expression. Together with the CRP-
280 cAMP control, RaiA expression appears to be under environmental control, highlighting a link between
281 bacterial persistence and environmental stress.

282 **The effect of RaiA overexpression is conserved in the Gram-negative pathogen *Pseudomonas*** 283 ***aeruginosa*.**

284 We next asked whether RaiA could have a similar function in other Gram-negative pathogens such as
285 *Pseudomonas aeruginosa*, an organism associated with antibiotic resistance and persistence (Koeva et
286 al., 2017; Spoering and Lewis, 2001) and of high concern regarding resistant infections. *P. aeruginosa*
287 RaiA exhibits 37% protein identity with RaiA from *V. cholerae*. We overexpressed *V. cholerae* RaiA in
288 *P. aeruginosa* and assessed lag phase and persistence, as we performed for *V. cholerae*. We found that

289 upon RaiA overexpression, lag phase is also decreased in *P. aeruginosa* (Figure 9A and S6D), and
290 strikingly, persistence to tobramycin is increased (Figure 9B). These results show that RaiA-protected
291 sleeping ribosomes can be involved in persistent infections by various pathogenic bacteria.

292 Discussion

293 We show here that indole is produced upon sub-MIC aminoglycoside treatment in *V. cholerae* and at
294 physiological concentrations, and increases appearance of persister cells in lethal concentrations of
295 AGs. We find that such increase in persistence occurs through an inducible mechanism involving RaiA
296 (previously called pY or *yfiA* in *E. coli*, and *vrp* in *V. cholerae*). While indole improved growth in the
297 presence of sub-MIC antibiotics seems to be nonspecific to an antibiotic class and independent of RaiA,
298 increased persistence involving RaiA is specific to AGs in exponentially growing bacteria. Since RaiA is
299 regulated by several environmental cues and signaling molecules, our findings highlight a new,
300 inducible mechanism of persistence, based on increased protection of ribosomes during stress, rather
301 than slowdown of the metabolism.

302 *In vitro* characterization of RaiA in *E. coli*, has previously shown association with ribosomes during cold
303 shock and stationary phase, but not during growth at 37°C, suggesting that binding of RaiA is prompted
304 by stress (Agafonov et al., 2001). Based on crystal structures, RaiA was suggested to arrest translation
305 (Vila-Sanjurjo et al., 2004). However, our results in *V. cholerae* do not support such a role, since there
306 is no impact of RaiA overexpression on growth, and rather point to a protective effect of RaiA under
307 ribosomal stress caused by AG treatment.

308 Alternatively, since RaiA is able to stabilize the 70S ribosome monomers against dissociation *in vitro*
309 (Agafonov et al., 1999; Di Pietro et al., 2013), it was proposed to constitute a pool of inactive 70S
310 ribosomes preserved from degradation in bacteria (Giuliodori, 2016; Giuliodori et al., 2007; Gualerzi
311 et al., 2011).

312 *In vivo* effects of RaiA on bacterial phenotypes are less well described. A protective effect of RaiA during
313 stress, such as starvation, was previously observed in the Gram-positive species *Mycobacterium*
314 *tuberculosis* (Li et al., 2018) and *Lactococcus lactis* (Puri et al., 2014). Yet, the mechanisms remained
315 enigmatic. Our results show a role of RaiA on survival to antibiotic stress in Gram-negative pathogens.
316 One known mechanism of protection of non-translating ribosomes is ribosome hibernation. The
317 ribosome hibernation factors, Rmf (ribosome modulation factor) and Hpf (hibernation promoting
318 factor) (Maki et al., 2000), dimerize 70S ribosomes (monosomes) into 100S hibernating ribosome
319 dimers. The importance of ribosome hibernation in stress survival is well established in various bacteria
320 (McKay and Portnoy, 2015; Tkachenko et al., 2017), as 100S dimers are less susceptible to degradation
321 by RNases (Feaga et al., 2020; Prossliner et al., 2018; Prossliner et al., 2021; Wada et al., 2000;
322 Yamagishi et al., 1993). Ribosome hibernation factors were even proposed as potential new targets for
323 antibiotics (Matzov et al., 2019). However, in some cases, 70S particles appear to be more robust
324 during heating than 100S dimers which dissociate into 30S and 50S subunits more rapidly (Niven,
325 2004).

326 RaiA was previously identified as bound to the ribosome together with Rmf and Hpf, but its role in
327 relation with ribosome hibernation is unclear. In *V. cholerae*, RaiA shows a synergistic effect with Hpf
328 for survival to starvation (Sabharwal et al., 2015), and we observe a synthetic lethal phenotype for the
329 deletion of *raiA* and *hpf*. Despite such apparent synergy with hibernation factors, RaiA was shown to
330 inactivate 70S ribosomes without forming 100S dimers (Polikanov et al., 2012; Ueta et al., 2005). RaiA
331 thus appears to act in a process different than ribosome hibernation, maybe by blocking breaking down
332 of ribosomes into 30S and 50S subunits by ribosome recycling factors (Rrf/EF-G) (Agafonov et al., 1999;
333 Janosi et al., 1996).

334 In *E. coli*, RaiA can even prevent 100S dimer formation by Hpf and Rmf (Maki et al., 2000; Ueta et al.,
335 2005). According to cryo-EM data, RaiA can compete with Rmf for ribosome binding, hence shifting
336 the ribosome content from a Rmf-mediated dimeric inactive form to a RaiA-bound monomeric inactive
337 form (Franken et al., 2017). Recent work has identified RaiA as a player in persister formation together
338 with hibernation factors, through a dormancy related mechanism linked to stringent response/ppGpp

339 (Song and Wood, 2020; Wood and Song, 2020). In these studies, persister formation was assessed on
340 near stationary cell cultures (OD 0.8), where these factors are highly expressed, and persistence is
341 largely due to dimerization of inactive ribosomes, leading to a general dormancy state which also
342 favors persister formation to other antibiotics like ampicillin and ciprofloxacin. In contrast, the present
343 study shows in exponentially growing cells and without nutrient limitation, a specific mechanism
344 involving the induction of RaiA, triggering persistence specifically to aminoglycosides (and not to other
345 antibiotics), without affecting growth (**Figure 10A**). The mechanism we describe is also different than
346 the ppGpp-independent persistence caused in slow growing bacteria (Pontes and Groisman, 2019),
347 since increased RaiA does not affect growth rate in our study. In this regard, the RaiA-dependent
348 persistence mechanism observed in the present study is not a dormancy-like or slow growth
349 mechanism, unlike the increased persistence triggered by ppGpp to antibiotics from various families,
350 in stationary phase. Moreover, no effect of hibernation factors Rmf/Hpf is observed, suggesting
351 different persistence mechanisms than ribosome dormancy formation in growing cells. Recent studies
352 also show the existence of mechanisms triggering persistence independently of ppGpp or hibernation
353 factors in bacteria (Hossain et al., 2021; Wood et al., 2021). We show here that RaiA is one player
354 enhancing persister formation in the Gram-negative bacterium *V. cholerae*.

355 While RaiA, Hpf and Rmf are rapidly released from ribosomes when normal growth conditions are
356 restored (Agafonov et al., 2001; Maki et al., 2000), ribosome reactivation necessitates dissociation of
357 hibernating 100S ribosome dimers into monomers by HflX and other factors (Basu and Yap, 2017),
358 whereas no dissociation factor is needed for the reactivation of the RaiA inactivated 70S ribosome.
359 There may thus be an interplay and equilibrium between hibernating 100S-Hpf/Rmf and “sleeping”
360 70S-RaiA forms.

361 Such synergy or antagonism between inactive 70S and 100S ribosome pools can however depend on
362 the nature of the stress and the bacterial species. We show here that persistence to aminoglycosides
363 is better achieved in the presence of RaiA (70S), rather than hibernation factors (100S) in *V. cholerae*.
364 Here, we propose that increased production of RaiA (e.g. upon stress) leads to preservation of non-
365 translating ribosomes in a pool of inactive and intact 70S “sleeping” ribosomes. In that scenario,
366 bacteria can keep growing in the absence of the antibiotic, since RaiA will not bind to actively
367 translating ribosomes. Upon stress affecting translation (e.g. aminoglycosides), ribosomes may stall or
368 stop, which would lead to the dissociation from the mRNA. If RaiA is induced, we propose that such
369 non-translating 70S ribosomes will be bound and protected by RaiA, and avoid death upon antibiotic
370 treatment, not by entering a dormant state, but by inducing protection of ribosomes. We propose that,
371 in contrast to hibernating ribosomes which need recycling factors to dissociate into 70S monosomes,
372 such sleeping ribosomes can be rapidly reactivated upon stress relief through spontaneous dissociation
373 of RaiA from the intact 70S monosome (Agafonov et al., 2001; Maki et al., 2000), conferring an
374 advantage for stress survival (**Figure 10AB**).

375 In line with this, a recent study showed that the greater the ribosome content of the cell, the faster
376 persister cells resuscitate (Kim et al., 2018). In parallel, protein synthesis was shown to be necessary
377 for persister cell formation in *V. cholerae* (Paranjape and Shashidhar, 2019), consistent with the fact
378 that we see no effect of RaiA overexpression on growth rates, thus on protein synthesis during growth.
379 Our results do not exclude however another role for RaiA on translating ribosomes.

380 Increased RaiA levels thus allow higher persistence, but what is controlling RaiA expression? RaiA is
381 expressed in stationary phase and during cold shock (Agafonov et al., 1999; Agafonov et al., 2001; Maki
382 et al., 2000; Sabharwal et al., 2015). Other known factors are the heat shock in *V. cholerae* (Slamti et
383 al., 2007), stringent response (Prossliner et al., 2018), envelope stress and the carbon catabolite
384 response (Manneh-Roussel et al., 2018; Shimada et al., 2013). We additionally show that *raiA*
385 expression is linked to iron levels and responds to Fur regulation. Interestingly, iron associates with
386 ribosomes (Bray et al., 2018) and a link between iron and modulation of ribosome function during
387 stress has been described (Zinskie et al., 2018). Iron control of RaiA may thus allow protection of

388 ribosomes from iron related damage. Altogether, RaiA appears to be part of the bacterial response to
389 environmental stress.
390 Results of the present study constitute a link between bacterial signaling, ribosome protection and
391 persistence. Moreover, since RaiA action appears to be conserved in various pathogens (Gram-
392 negatives and positives, such as *S. aureus* and *M. tuberculosis*), it may be one factor involved in the
393 failure of treatment in persistent infections. It would be interesting to ask now whether RaiA can be
394 used as an early indicator of persistence, which would allow isolation of persisters within a
395 heterogeneous population and further studies using single cell approaches.

396

397 **Limitation of Study**

398 This study shows that indole is secreted under sub-MIC aminoglycosides treatment and increases *raiA*
399 expression. It also shows that indole is implicated in *raiA*-dependent persister formation under lethal
400 aminoglycosides treatment. However, we do not address whether indole is also produced under lethal
401 treatment because it is challenging to quantify indole production or *tnaA* expression on dying cells.
402 Development of this method in our lab could be the purpose of a future work.

403 In addition, *raiA*-dependent persister formation is shown here to be specific to aminoglycosides
404 (bactericidal), which target translation. Bacteriostatic antibiotics such as chloramphenicol or
405 tetracycline also target the ribosome, but persistence assays are usually not performed using these
406 antibiotics. Our study does not exclude a role for *raiA* protection on sleeping in the response to such
407 translation targeting antibiotics.

408

409 **Author Contributions**

410 Experiments were designed by Z.B. Experiments were conducted by M.L., E. K., C.K. and Z. B., and
411 results were interpreted by Z.B. and M.L. RNA-seq library preparation, sequencing and statistical
412 analysis was performed by O. S., H.V. and J-Y. C. The manuscript was written and reviewed by Z.B., M.L.
413 and D.M. All authors read and approved the final manuscript.

414 **Declaration of Interests**

415 The authors declare no competing interests.

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424

425 **Main Figure Titles and Legends**

426 **Figure 1. Indole is produced during growth in sub-MIC tobramycin and induces *raiA*.** Measure of
427 extracellular indole concentrations of bacterial cultures grown overnight in rich medium MOPS
428 (Teknova EZ rich defined medium) with and without tobramycin at 0.10 µg/ml (TOB 0.10) or 0.15 µg/ml
429 (TOB 0.15) using the Kovacs reagent (Saint-Ruf et al., 2014). $\Delta tnaA$ strain was used as negative control
430 without tobramycin. Experiments were performed in triplicates, and statistical analysis was performed
431 (***: $p < 0.001$). Error bars represent standard deviation.

432 **Figure 2. Modulation of persistence of exponential phase WT and $\Delta raiA$ *V. cholerae* by indole. A to**
433 **D:** Early exponential phase of wild-type (WT) and $\Delta raiA$ *V. cholerae* cultures were treated with lethal
434 doses of the specified antibiotics for 20 hours. The y axis represents survival, as the number of CFU

435 growing after antibiotic treatment and removal divided by the total number of CFU at time zero (before
436 antibiotic treatment). Bars represent geometric means and error bars represent geometric standard
437 deviation. Tobramycin (TOB): 10 µg/ml, gentamicin (GEN): 5 µg/ml, carbenicillin (CRB): 100 µg/ml,
438 indole (IND): 350 µM. Experiments were performed 3 to 6 times, and statistical analysis was performed
439 (*: $p < 0.05$; **: $p < 0.01$).

440 **Figure 3. Indole production during growth in sub-MIC tobramycin induces *raiA*.** A. Volcano plot
441 showing differentially expressed genes upon indole treatment. X axis represents log 2-fold change, Y
442 axis represents the negative log 10 of the *p* value. *raiA*, *rmf* and *hpf* are indicated. The dashed line
443 represents a *p* value of 0.01, all of the dots above thus show a *p* value < 0.01. Red dots indicate genes
444 linked with respiration, green with sugar metabolism, dark blue with iron, purple with amino-acid
445 metabolism, and light blue with translation. RNA-seq was performed in triplicates for each condition.
446 See also Table S1. B. *raiA* mRNA levels measured by RT-qPCR on exponential phase *V. cholerae* cultures
447 in presence or absence of indole. Statistical analysis was performed (**: $p < 0.01$). Error bars represent
448 standard deviation.

449 **Figure 4. Environmental stress induce *raiA* expression in exponential phase *V. cholerae*.** Fluorescence
450 quantification of GFP expression from the *raiA* promoter by flow cytometry in MH media in exponential
451 phase (except for "Stat"). A. in WT *V. cholerae*. NT: Non-treated, IND: indole (350 µM), Fe: iron (18
452 µM), DP: 2,2'-Dipyridyl (500 µM), Glc: Glucose (1%), Mal: Maltose (1%), Stat: stationary phase. The y
453 axis represents the fluorescence ratio of the treated over non-treated (NT) strain. B. in indicated *V.*
454 ***cholerae* deletion mutants.** The y axis represents the fluorescence ratio of the mutant over wild type
455 (WT) strain. Mean fold change values are indicated within histogram bars. Experiments were
456 performed at least 3 times, and statistical analysis was performed (**: $p < 0.01$; ****: $p < 0.0001$). Error
457 bars represent standard deviation. See also Figure S4.

458 **Figure 5. Modulation of persistence of exponential phase *V. cholerae* by RaiA.** Early exponential
459 phase of wild-type (WT) *V. cholerae* carrying either the empty pBAD vector (p0) or with specified gene
460 (pGene) cultures were treated with lethal doses of the specified antibiotics for 20 hours. The y axis
461 represents survival, as the number of CFU growing after antibiotic treatment and removal divided by
462 the total number of CFU at time zero (before antibiotic treatment). Tobramycin (TOB): 10 µg/ml,
463 gentamicin (GEN): 5 µg/ml, carbenicillin (CRB): 100 µg/ml, ciprofloxacin (CIP): 0.025 µg/ml,
464 trimethoprim (TMP): 50 µg/ml, neomycin (NEO): 30 µg/ml, indole (IND): 350 µM. Experiments were
465 performed 3 to 6 times, and statistical analysis was performed (*: $p < 0.05$; **: $p < 0.01$).

466 **Figure 6. RaiA influences lag phase upon growth restart after the stationary phase.** Growth is
467 measured on a TECAN plate reader. The curves of wild type *V. cholerae* with the empty plasmid are
468 compared with plasmid carrying *raiA* under inducible promoter in MH media containing either glucose
469 or arabinose. The promoter is repressed using glucose (GLC) and induced using arabinose (ARA).
470 Growth was performed as specified, with RaiA expression repressed or induced in the overnight culture
471 used for inoculum (overnight culture supplemented or not with ARA; indicated as "during pre-
472 cultures"), or/and with RaiA expression induced or not during growth in the microplate reader (growth
473 media supplemented or not with ARA in the microplate; indicated as "during sub-cultures") : A.
474 GLC/ARA, B. ARA/GLC, C. ARA/ARA, D. GLC/GLC. A scheme of the experimental setup is found on Figure
475 S5A. Experiments were performed in triplicates and geometric means are represented. Error bars
476 represent the geometric standard deviation. See also Figure S6B.

477
478 **Figure 7. The effect of RaiA on lag phase is independent of ribosome hibernation factors Rmf/Hpf.**
479 A. Growth in MH media of WT and mutant strains. B. Growth of *V. cholerae* overexpressing hibernation

480 factors or empty plasmid in MH media containing arabinose (ARA/ARA). **CD**: Growth of hibernation
481 factor mutants Δrmf and Δhpf overexpressing RaiA in MH media containing arabinose (ARA/ARA).
482 Experiments were performed in triplicates and geometric means are represented. Error bars represent
483 the geometric standard deviation. See also **Figure S6C**.
484

485 **Figure 8. RaiA levels influence stationary phase 70S ribosome content relative to 50S+30S subunits**
486 **in *V. cholerae*.** **A.** Cellular extracts of 24 hours cultures ($\Delta raiA$ and WT *V. cholerae* carrying the
487 p0/pBAD-RaiA vector, in MH media containing spectinomycin and arabinose) were separated on 10-
488 50% sucrose density gradient. Ribosomal RNA content was measured at OD 260 nm using a
489 spectrometer coupled to a pump and time on X axis represents samples from less dense (upper
490 fragments, smaller complexes) to denser (bottom of the tube, heavier complexes). Lysis was
491 performed in the presence of 10 mM $MgCl_2$. Cell debris eluting before 550 seconds are not shown.
492 Graphs are normalized to total OD 260 nm =1 for each sample. Mean values are indicated within
493 histogram bars. Error bars represent standard deviation. **B.** Percentage of 70S ribosomes over total
494 ribosome subunits (70S)/(70S+50S+30S). Error bars represent standard deviation (**: $p < 0.01$).

495 **Figure 9. RaiA overexpression in *Pseudomonas aeruginosa* increases tolerance to tobramycin in**
496 **exponential phase and promotes earlier exit from stationary phase.** **A.** Growth is measured on a
497 TECAN plate reader. The curves of WT *P. aeruginosa* with the empty plasmid (p0) are compared with
498 the plasmid carrying *raiA* of *V. cholerae* under inducible promoter in MH media containing arabinose.
499 See also **Figure S6D B**. Early exponential phase of WT *P. aeruginosa* cultures were treated with lethal
500 doses of tobramycin (TOB: 10 $\mu g/ml$) for 20 hours. The y axis represents survival, as the number of CFU
501 growing after antibiotic treatment and removal divided by the total number of CFU at time zero (before
502 antibiotic treatment) (*: $p < 0.05$). Experiments were performed in triplicates.

503 **Figure 10. Summary model: RaiA mediated ribosome protections increases persistence to**
504 **aminoglycosides and decreases lag phase during growth restart.** Diagrams depict RaiA induced
505 conditions. **A. Persistence.** During exponential growth, treatment with lethal doses of aminoglycosides
506 leads to disruption of translation and stalling of translating 70S ribosomes, and eventually to their
507 dissociation into 50S+30S subunits and degradation of these ribosomes, resulting in cell death. Upon
508 induction of RaiA (e.g. by indole signaling), we propose that RaiA does not affect translating ribosomes,
509 but binds non-translating 70S ribosomes to form sleeping ribosomes, and protects them from
510 dissociation into subunits and degradation. This results in increased persistence. **B. Length of lag**
511 **phase.** In a stationary phase culture (e.g. overnight culture), translation is highly decreased, leading to
512 dissociation from the mRNA ribosome degradation. Upon growth restart, a lag phase thus occurs
513 where ribosomes are resynthesized. We propose that when RaiA production is increased before
514 entering the stationary phase, an increased proportion of non-translating ribosomes are bound and
515 are preserved as 70S by RaiA, rather than being degraded. The presence of such an increased pool of
516 intact 70S ready-to-use sleeping ribosomes gives an advantage on translation reactivation, while
517 degraded ribosome have to be recycled or de novo synthesized, decreasing the lag phase necessary
518 for the synthesis of a sufficient number of ribosomes.

519

520 **STAR METHODS**

521 **RESOURCE AVAILABILITY**

522 **Lead contacts**

523 Further informations and requests for resources and reagents should be directed to and will be fulfilled
524 by the Lead Contacts, Didier Mazel (didier.mazel@pasteur.fr) or Zeynep Baharoglu
525 (baharogl@pasteur.fr).

526 **Materials availability**

527 This study did not generate new unique reagents.

528 **Data and code availability**

529 RNA-seq data are deposited at GEO: GSE182561

530 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

531 **Bacterial Strains and Plasmids**

532 All *V. cholerae* strains used in this study are derivative of *V. cholerae* N16961 *hapR+*, and were
533 constructed by allelic exchange. All *E. coli* strains used in this work are derivatives of *E. coli* MG1655,
534 and were constructed by transduction using *E. coli* Keio knockouts strains. Strains and plasmids are
535 listed in Key Resources Table, and Table S2 for more details.

536 **Media and Growth Conditions**

537 *Vibrio cholerae*: Colonies on plates grew at 37°C, in MH media. Plates are conserved at room
538 temperature and should not be placed at 4°C. Liquid cultures grew at 37°C in appropriate media (see
539 Methods), in aerobic conditions, with 180 rotations per minute. *E. coli* and *P. aeruginosa*: Colonies on
540 plates grew at 37°C, in MH media, and are conserved at 4°C. Liquid cultures grew 37°C in aerobic
541 condition in appropriate media (see Methods), with 180 rotations per minute.

542 **METHOD DETAILS**

543 **Persistence tests.** Persistence tests were performed on early exponential phase cultures. In order to
544 clear the culture from previously non-growing cells that could potentially be present from the
545 stationary phase inoculum, we performed a two-step dilution protocol, before antibiotic treatment.
546 For overexpression experiments, glucose 1% was added to the overnight cultures to repress the pBAD
547 promoter. Overnight *V. cholerae* cultures were first diluted 1000x in 4 ml fresh Mueller-Hinton (MH)
548 medium, without indole (with the antibiotic allowing to maintain the plasmid and incubated at 37°C
549 with shaking. For overexpression experiments, arabinose 0.2% was added at this first dilution step to
550 the fresh MH media to induce the pBAD promoter. When the OD 620 nm reached ~0.2, cultures were
551 diluted 1000x a second time, in order to clear them from non-growing cells, in Erlenmeyers containing
552 25 ml fresh MH medium, without or with indole at 350 µM (with the antibiotic allowing to maintain
553 the plasmid, when the strain carried a plasmid), and were allowed to grow at 37°C. For overexpression
554 experiments, arabinose 0.2% was added again at this second dilution step to the fresh MH media to
555 induce the pBAD promoter. When cultures reached an OD 620 nm between 0.25 and 0.3 (early
556 exponential phase), appropriate dilutions were plated on MH plates to determine the total number of
557 CFUs in time zero untreated cultures. Note that for *V. cholerae*, it was important to treat cultures at
558 the precise OD 620 nm 0.25-0.3, as persistence levels seem to be particularly sensitive to growth phase
559 in this species, where they decline in stationary phase, and because we wanted to avoid any stationary
560 phase protein expression such as *raiA* or *rpoS* at later growth. 5 ml of cultures were collected into 50
561 ml Falcon tubes and treated with lethal doses of desired antibiotics (5-10 times the MIC: tobramycin
562 10 µg/ml, gentamicin 5 µg/ml, neomycin 30 µg/ml, carbenicillin 100 µg/ml, ciprofloxacin 0.025 µg/ml,
563 trimethoprim 50 µg/ml) for 20 hours at 37°C with shaking in order to guarantee oxygenation. The same

564 protocol was used for persistence assays on *P. aeruginosa*. Appropriate dilutions were then plated on
565 MH agar without antibiotics and proportion of growing CFUs were calculated by doing a ratio with total
566 CFUs at time zero. Experiments were performed 3 to 6 times.

567 **Quantification of extracellular indole concentrations.** Extracellular indole concentration was
568 measured on bacterial cultures grown overnight with and without antibiotics using the Kovacs reagent
569 (Saint-Ruf et al., 2014). First, we established an indole concentration standard curve using 1 ml culture
570 medium (without bacteria) supplemented with indole 0 to 1000 μM (100 μM steps). After adding 500
571 μl KOVACS reagent, 100 μl of the top layer of the reaction was mixed with 800 μl isoamyl-HCl and OD
572 was read at 570 nm. For indole measurement on bacterial samples, 1 ml of culture was subjected at
573 the same protocol. Measured indole concentration was normalized to the bacterial dry mass based on
574 the assumption that for an OD 600 nm = 1 bacterial dry mass is 0.3 mg/ml (Soini et al., 2008). We
575 detected no indole production in MH medium in any condition. In order to quantify secreted indole in
576 the presence and absence of sub-MIC antibiotics, we thus used the defined rich MOPS transparent
577 medium (Teknova EZ rich defined medium), where MIC TOB is 0.75 $\mu\text{g/ml}$. All the following
578 experiments (persistence, growth) were conducted in MH, as it allows to study the impact of defined
579 indole concentrations added to the media.

580 **Quantification of fluorescent neomycin uptake** was performed as described (Pierlé et al, manuscript
581 in revision, (Okuda, 2015; Sabeti Azad et al., 2020)). Neo-cy5 is an aminoglycoside coupled to the
582 fluorophore Cy5, and has been shown to be active against Gram- bacteria (Okuda, 2015; Sabeti Azad
583 et al., 2020). Briefly, overnight cultures were diluted 100-fold in rich MOPS (Teknova EZ rich defined
584 medium). When the bacterial strains reached an OD 620 nm of 0.25, they were incubated with 0.4 μM
585 of Cy5 labeled Neomycin for 15 minutes at 37°C. 10 μl of the incubated culture were then used for flow
586 cytometry, diluting them in 250 μl of PBS before reading fluorescence. WT *V. cholerae*, was incubated
587 simultaneously without Neo-Cy5 as a negative control. Flow cytometry experiments were performed
588 as described (Baharoglu et al., 2010) and repeated at least 3 times. For each experiment, 100,000
589 events were counted on the Miltenyi MACSquant device.

590 **MIC determination using etests.** Stationary phase cultures were diluted 20 times in PBS, and 300 μl
591 were plated on MH plates and dried for 10 minutes. etests (Biomérieux) were placed on the plates and
592 incubated overnight at 37°C.

593 **RNA-seq.** Overnight cultures of the O1 biovar El Tor N16961 *hapR+* *V. cholerae* strain were diluted
594 100x and grown in triplicate in MH medium until an OD 620 nm = ~ 0.4 with or without 350 μM indole.
595 Sample collection, total RNA extraction, library preparation, sequencing and analysis were performed
596 as previously described (Krin et al., 2018).

597 ***raiA* qRT-PCR.** Total RNA was extracted and purified from exponential phase cultures in MH in
598 presence or absence of indole, as previously described (Krin et al., 2018). Reverse transcription (RT)
599 was performed on 100 ng total RNA using SuperScript® III First-Strand Synthesis System for RT-PCR
600 (Invitrogen). Quantitative PCR was performed on 2 μl RT sample diluted 10-fold using SYBR Green PCR
601 Master Mix (APPLIED) and QuantStudio 6. Quantification was performed using standard range.
602 Expression values were normalized against *gyrA* as previously described in *V. cholerae* (Liu et al., 2010;
603 Lo Scudato and Blokesch, 2012).

604 **Quantification of *raiA* expression by fluorescent flow cytometry using a *gfp* fusion.** *gfp* was amplified
605 by PCR using primers carrying the *raiA* promoter region and cloned into pTOPO-TA cloning vector. The
606 *PraiA-gfp* fragment was then extracted using EcoRI and cloned into the low copy plasmid pSC101 (1 to
607 5 copies per cell). The plasmid was introduced into desired strains, and fluorescence was measured on

608 indicated conditions, by counting 100,000 cells on the Miltenyi MACSquant device. Likewise, the
609 control plasmid Pc-gfp (constitutive) was constructed using primers ZIP513/ZIP200 and similarly cloned
610 in pSC101.

611 **Growth curves.** Overnight cultures were diluted 100x in fresh medium, on 96 well plates. Each well
612 contained 200 μ l. For overexpression lag experiments, glucose 1% was added to the media to repress
613 the pBAD promoter while arabinose 0.2% was added to the media to induce the pBAD promoter, and
614 same growth conditions (glucose or arabinose) were compared in order to avoid noise due to the
615 effects of arabinose on growth and cells shape in *V. cholerae* (Espinosa et al., 2020). Spectinomycin
616 was also added during each experiment to maintain the plasmid. Plates were incubated with shaking
617 on TECAN plate reader device at 37°C, OD 620 nm was measured every 15 minutes.

618 **Preparation of cell lysate for the analysis of ribosome content.** The protocol was adapted from (Qin
619 and Fredrick, 2013). Since we used stationary phase cultures instead of exponential phase, presence
620 of polysomes is not expected. 10 ml of 20 hours cultures were centrifuged in ice cold 50 ml Falcon
621 tubes for 15 minutes at 5000 rpm at 4°C. Pellets were resuspended in 500 μ l lysis buffer (10 mM Tris-
622 HCl, pH 8, 10 mM MgCl₂, Lysozyme 1 mg/ml, protease inhibitor), transferred in ice cold 1.5 ml tubes
623 and incubated with 12 μ l Ribolock RNase inhibitor (Thermo scientific) and DNaseI (5 U/ml) at 4°C for
624 15 minutes. Cell lysis was performed through 3 cycles of flash-freezing in dry ice and thawing in a water
625 bath at 4°C. 15 μ l of 10% sodium deoxycholate were added and cell lysate was obtained after
626 centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet containing cell debris was discarded.
627 Lysate was kept at -80°C until sucrose gradient ultracentrifugation.

628 **Sucrose gradient.** 10-50% sucrose gradient tubes (Beckman ULTRA CLEAR) were prepared. 2U of OD
629 260 nm of each cell extracts were deposited on sucrose gradient tubes. Ultracentrifugation was
630 performed at 39,000 rpm at 4°C for 2 hours 45 minutes. Fractions were collected using a pump coupled
631 to a spectrometer at OD 260 nm, and plotted as a function of time (seconds).

632 **Quantification and statistical analysis.** First an F-test was performed in order to determine whether
633 variances are equal or different between comparisons. For comparisons with equal variance, Student's
634 t-test was used. For comparisons with significantly different variances, we used Welch's t-test. For
635 multiple comparisons, we used one-way ANOVA. We used GraphPad Prism to determine the statistical
636 differences between groups. **** means $p < 0.0001$, *** means $p < 0.001$, ** means $p < 0.01$, * means
637 $p < 0.05$. Number of replicates for each experiment was $3 < n < 6$. Means and geometric means for
638 logarithmic values were also calculated using GraphPad Prism. For persistence tests, data were first
639 log transformed in order to achieve normal distribution, and statistical tests were performed on these
640 log-transformed data.

641 **Supplemental Data**

642 **Table S1. Differentially expressed genes upon indole treatment. Related to Figure 3.**

643 **Table S2. Strains and plasmids. Related to STAR Methods.**

644 **Supplementary figures legends.**

645 **Figure S1. Indole improves growth of *V. cholerae* in the presence of tobramycin. A. WT. B. Δ raiA.**
646 Growth is measured on a TECAN plate reader. IND: indole 350 μ M. TOB: tobramycin sub-MIC (0.6
647 μ g/ml). Experiments were performed in triplicates and geometric means are represented. Error bars
648 represent the geometric standard deviation. Related to **Figure 1.**
649

650 **Figure S2. Persistence and the effect of indole. A.** Kinetics of survival of WT *V. cholerae* to tobramycin
651 10 µg/ml in MH media. Time zero corresponds to the total number of CFU before addition of
652 antibiotics, to an early exponential phase culture (OD 620 nm 0.25-0.3), as described in the methods
653 section. The proportion of surviving cells is calculated after plating and counting growing colonies, and
654 is represented for each time point. Curves represent geometric means of at least 3 replicates for each
655 time point and error bars represent geometric standard deviation. **B.** Persistence of *V. cholerae* WT
656 and $\Delta tnaA$ mutants in exponential phase (in LB instead of MH, to allow indole production, see
657 methods) after 20 hours treatment with specified antibiotics. Tobramycin (TOB): 10 µg/ml,
658 carbenicillin (CRB): 100 µg/ml. Related to **Figure 2**.

659 **Figure S3. Indole does not apparently influence aminoglycoside entry and resistance in *V. cholerae***
660 **WT and $\Delta raiA$.** **A.** Intracellular level of neomycin coupled to the fluorophore Cy5 measured by
661 fluorescence associated flow cytometry. Error bars represent standard deviation. **B.** Minimal inhibitory
662 concentrations of tobramycin (TOB) and gentamicin (GEN) measured using *etests* in *V. cholerae*, in the
663 absence (MH) and presence of indole (IND), and indicated in µg/ml by a numeral on each image.
664 Related to **Figure 2**.

665 **Figure S4. Fluorescence detection from the *raiA* promoter-*gfp* fusion by flow cytometry.**
666 Representative acquisitions are shown. Each plot represents one experiment. Each experiment was
667 performed at least 3 times and data and statistical significance are shown in the histograms in the main
668 manuscript. **A:** histogram curves: GFP fluorescence is represented in the x-axis (FITC channel), the y-
669 axis represents the number of events corresponding to the number of cells, normalized to height (same
670 number of total cells for both conditions). **B:** dot plots: Bacterial cells' size and shape (rugosity). FSC:
671 forward scatter. SSC: size scatter. The SSC (size scatter) vs FSC (forward scatter) graphs show the
672 distribution of cells by size and shape and no major difference in cell size/shape is observed between
673 the WT and mutants with or without indole. Related to **Figure 4**.

674 **Figure S5. RaiA is involved in persistence in *V. cholerae* and *E. coli*.** **A.** Increase of surviving colonies
675 (persisters) after 20 hours of treatment with tobramycin (TOB) 10 µg/ml (ratio over WT surviving
676 colonies) in *V. cholerae*. Error bars represent geometric standard deviation **B.** Persistence of *E. coli* WT
677 and *raiA* mutant, at late exponential phase (OD 620nm 0.5), after 20 hours treatment with 10 µg/ml
678 tobramycin (TOB) in MH media. Serial dilutions are spotted on plates for estimation of survival. Spot
679 assays were performed at least 3 times. Related to **Figure 2**.

680 **Figure S6. Influence of RaiA on the length of lag phase upon growth restart after the stationary**
681 **phase.** **A.** Experimental set-up. Repression was achieved with 1% glucose (GLC), induction was
682 achieved with 0.2% arabinose (ARA). **B.** Lag time (minutes) defined as time to reach OD 620 nm 0.15
683 in WT *V. cholerae* carrying empty vector (p0) or pBAD-RaiA vector (see ABCD in Figure 6). **C.** Lag time
684 (in minutes) defined as time to reach OD 620 nm 0.15 in *V. cholerae* $\Delta raiA$, Δrmf and Δhpf and WT *V.*
685 *cholerae* carrying empty vector (p0) or hibernation factors (pRaiA, pRmf, pHpf) (Figure 7AB) **D.** Lag time
686 (in minutes) defined as time to reach OD 620 nm 0.15 in WT *P. aeruginosa* (Figure 9A) carrying empty
687 vector (p0) or pBAD-RaiA vector. Experiments were performed in triplicates and statistical analysis was
688 performed (**: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant). Related to **Figure 6, 7AB** and
689 **9A**.

691 **Figure S7. Influence of RaiA, Rmf and Hpf on persistence.** Persistence of *V. cholerae* WT and mutants
692 in early exponential phase, after 20 hours treatment with 10 µg/ml tobramycin (TOB) in MH media.

693 Serial dilutions are spotted on plates for estimation of survival. Spot assays were performed at least 3
694 times. Related to **Figure 7**

695 **Figure S8. Expression of *gfp* from constitutive promoter (Pc) in conditions where expression from**
696 **the *raiA* promoter is up or down-regulated.** Fluorescence quantification of GFP expression from
697 constitutive promoter by flow cytometry in MH media in exponential phase, in WT and indicated *V.*
698 *cholerae* deletion mutants. IND: indole (350 μ M), DP: 2,2'-Dipyridyl (500 μ M). The Y axis represents
699 fluorescence ratio of the mutant over wild type (WT) strain. Error bars represent standard deviation.
700 Related to **Figure 4.**

701

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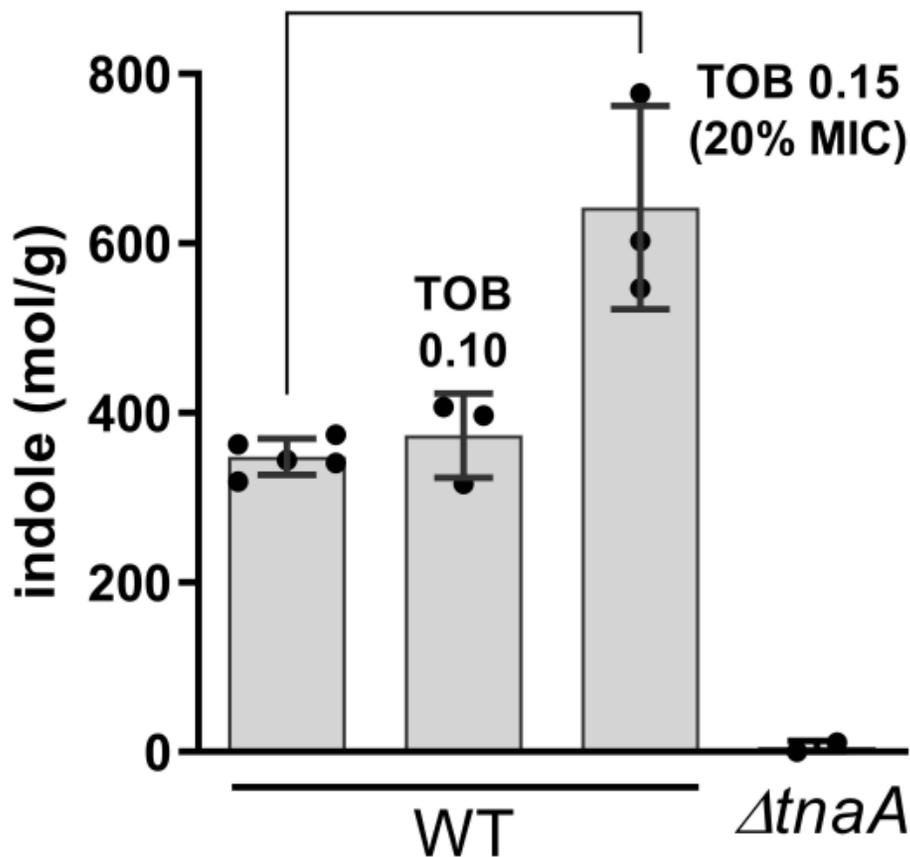
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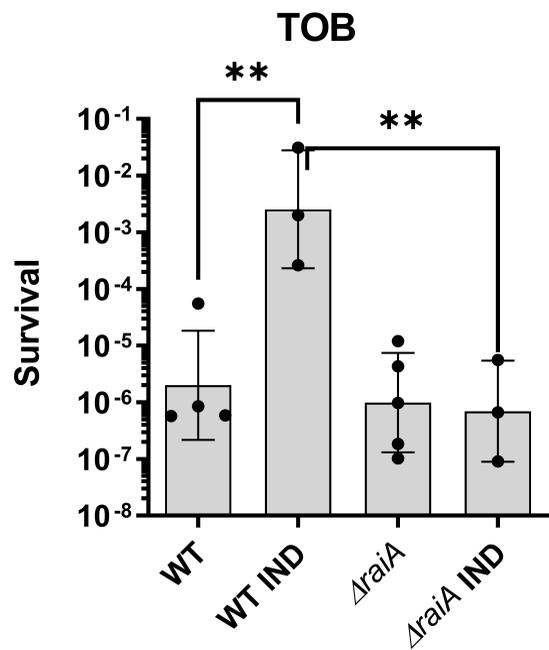
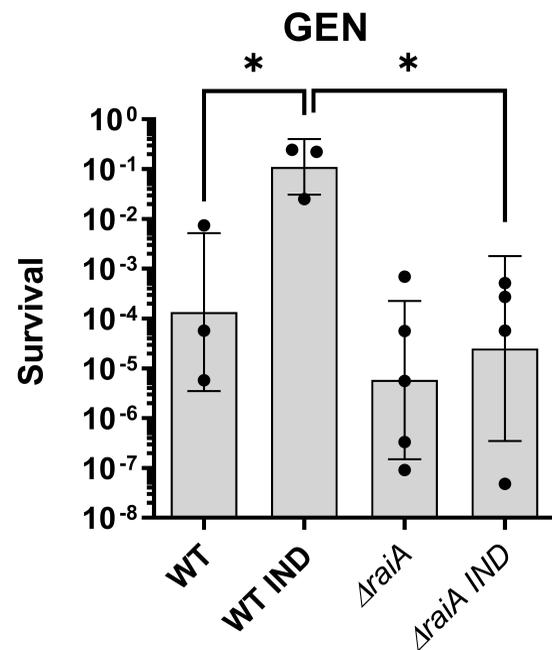
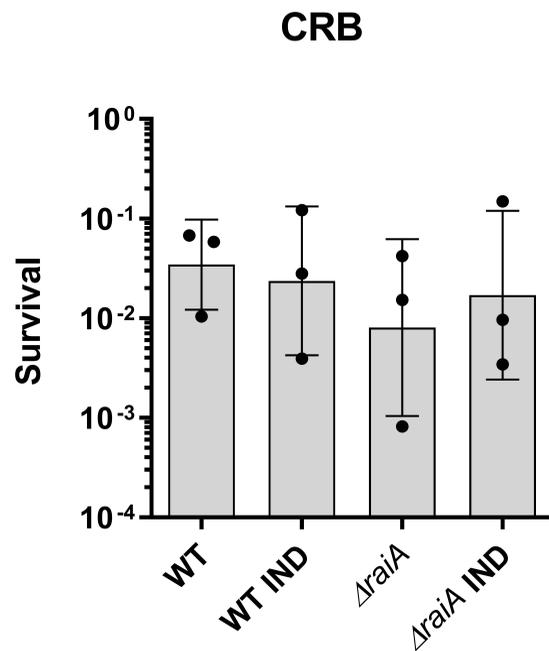
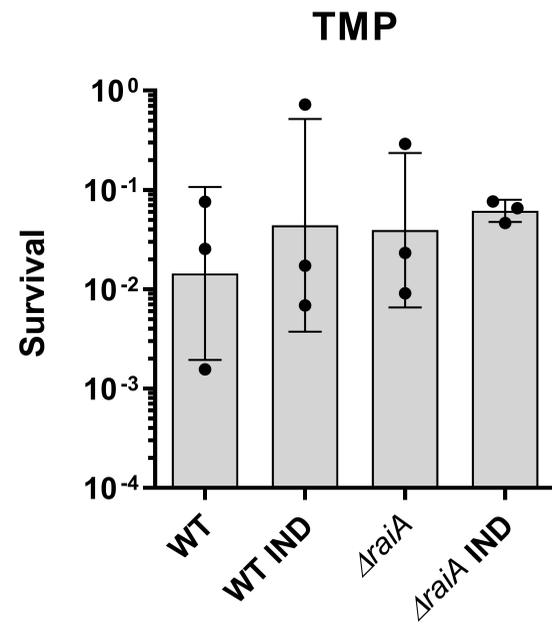
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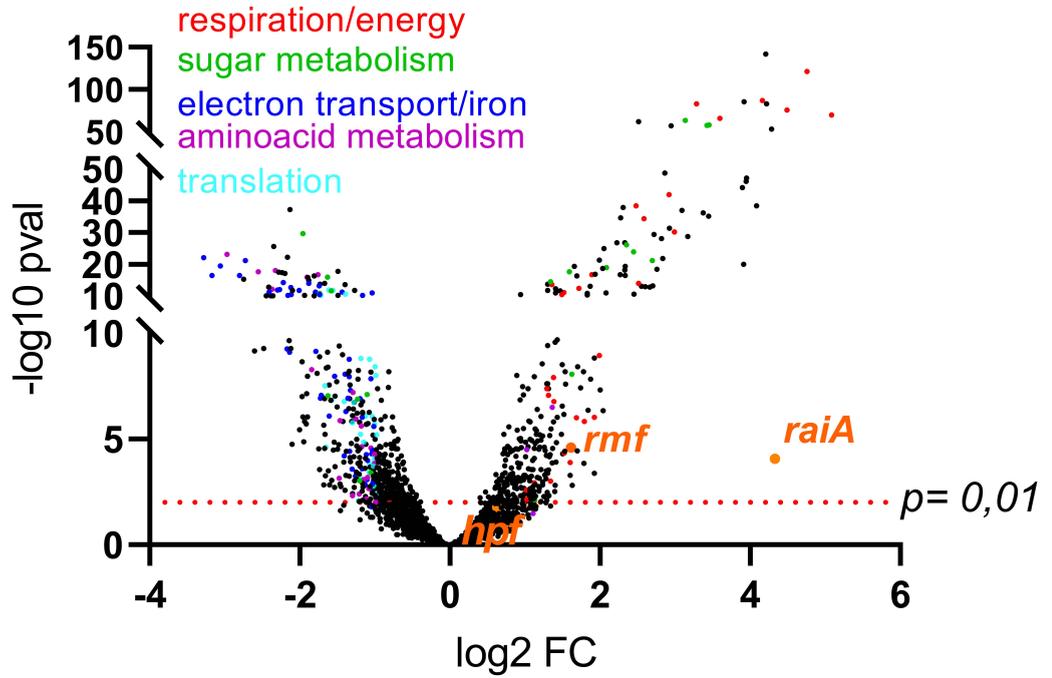
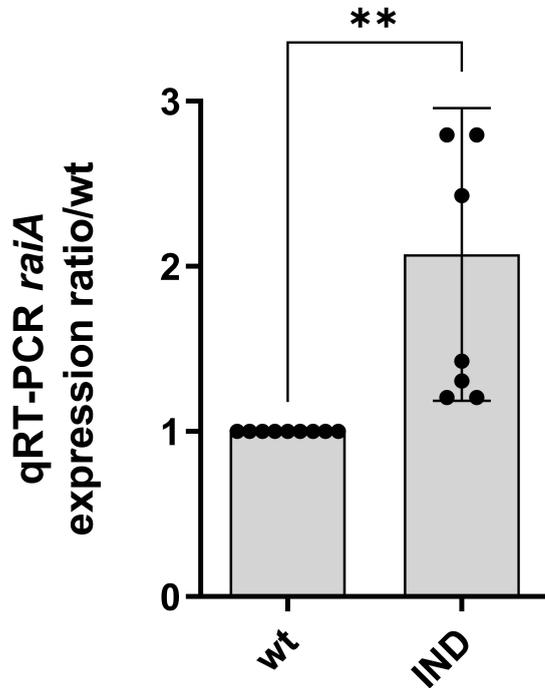
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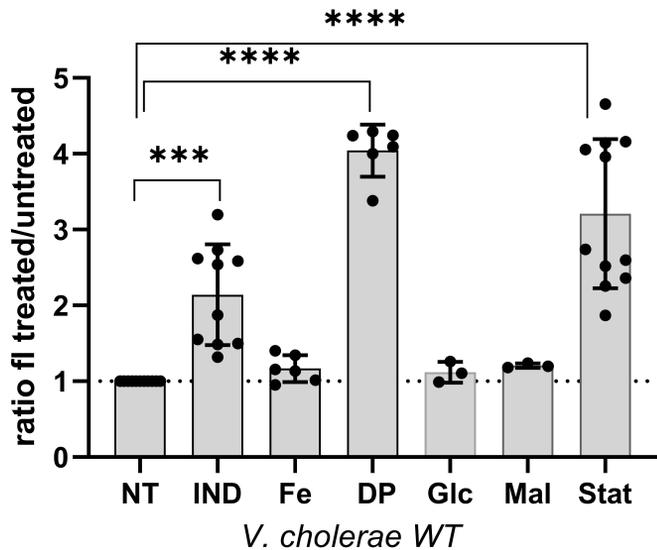
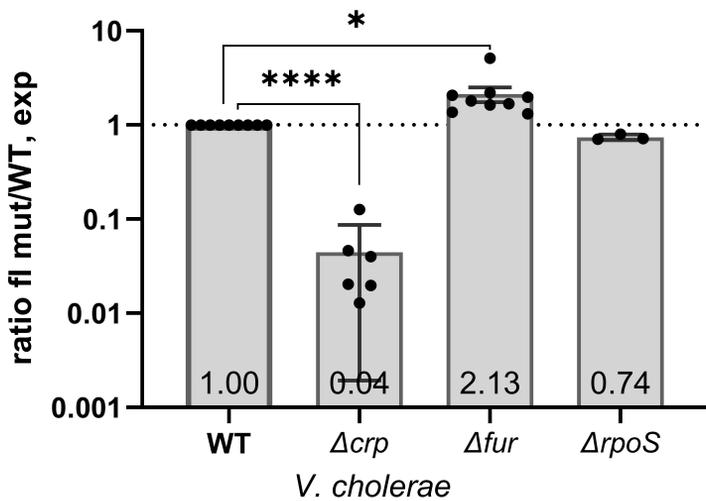
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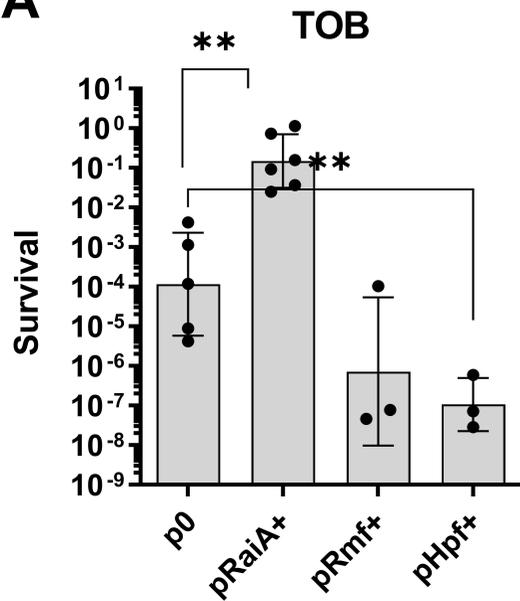
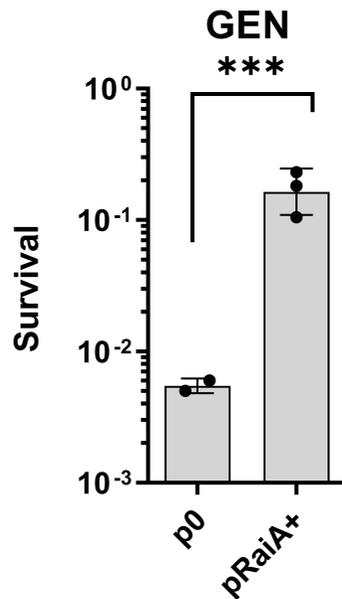
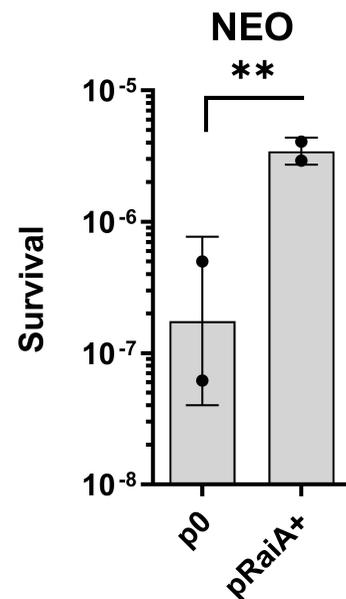
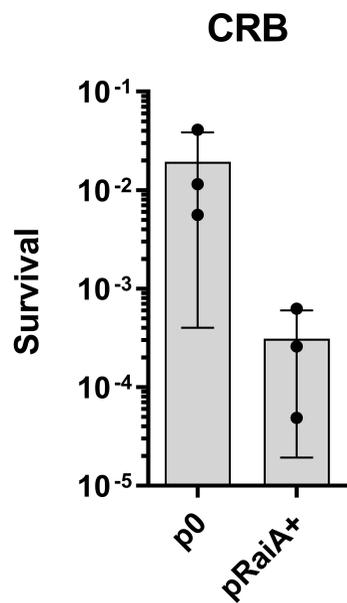
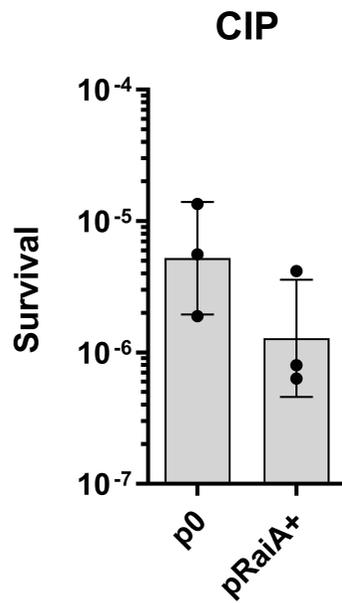
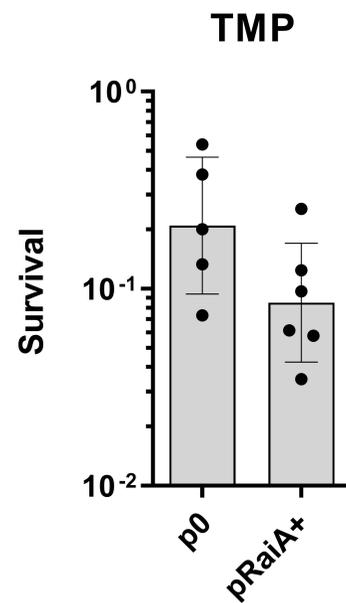
937



A**B****C****D**

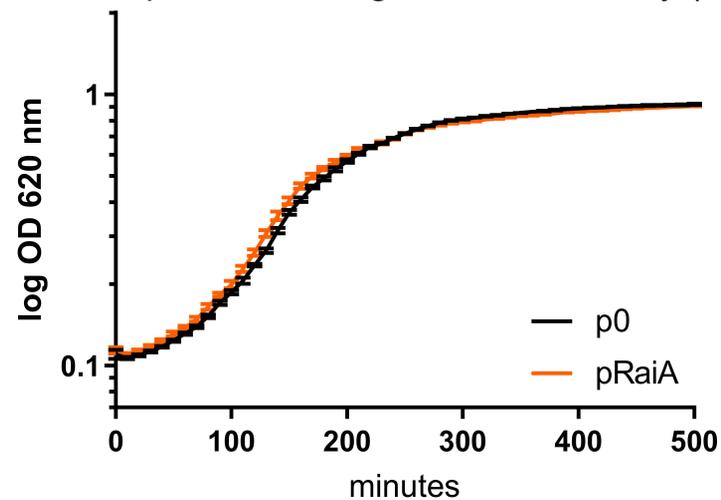
A**B**

A**B**

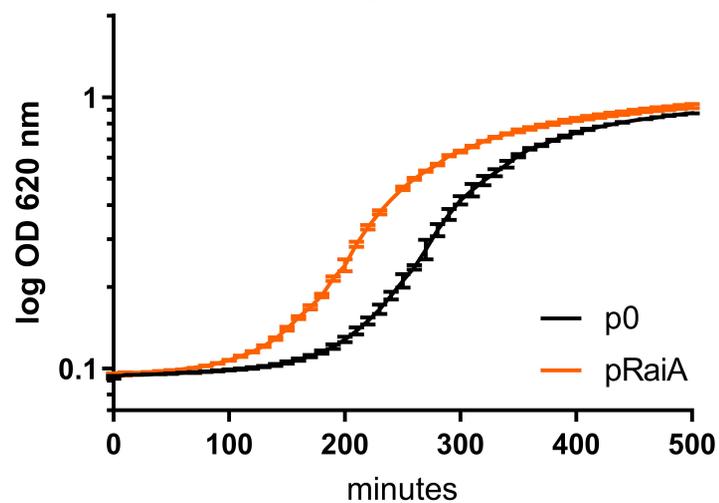
A**B****C****D****E****F**

A

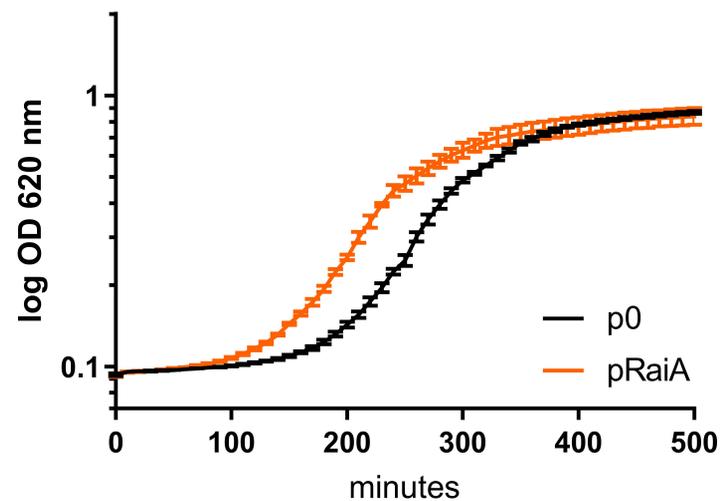
RaiA overexpressed during sub-cultures only (GLC/ARA)

**B**

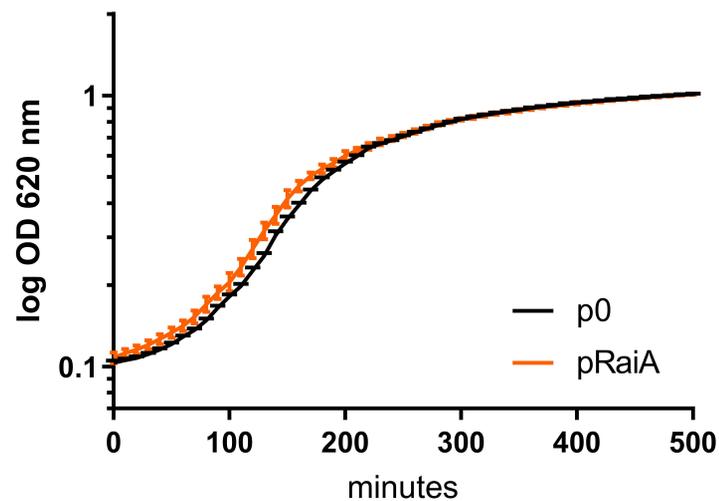
RaiA overexpressed during pre-cultures only (ARA/GLC)

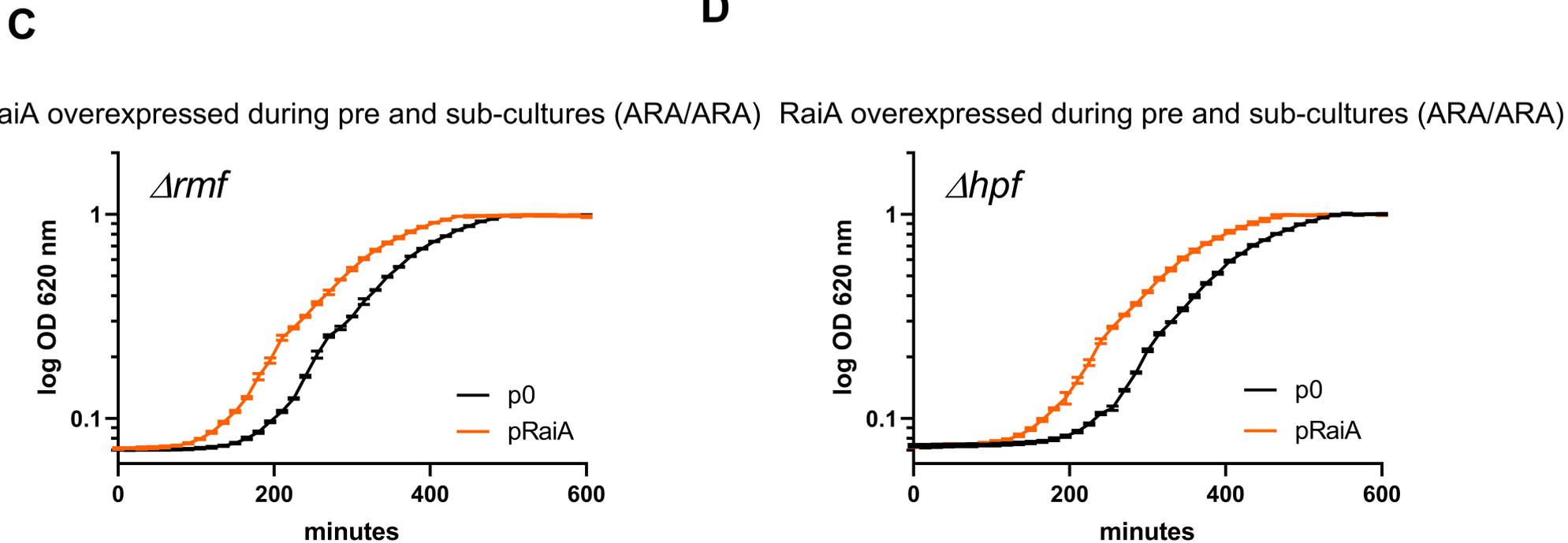
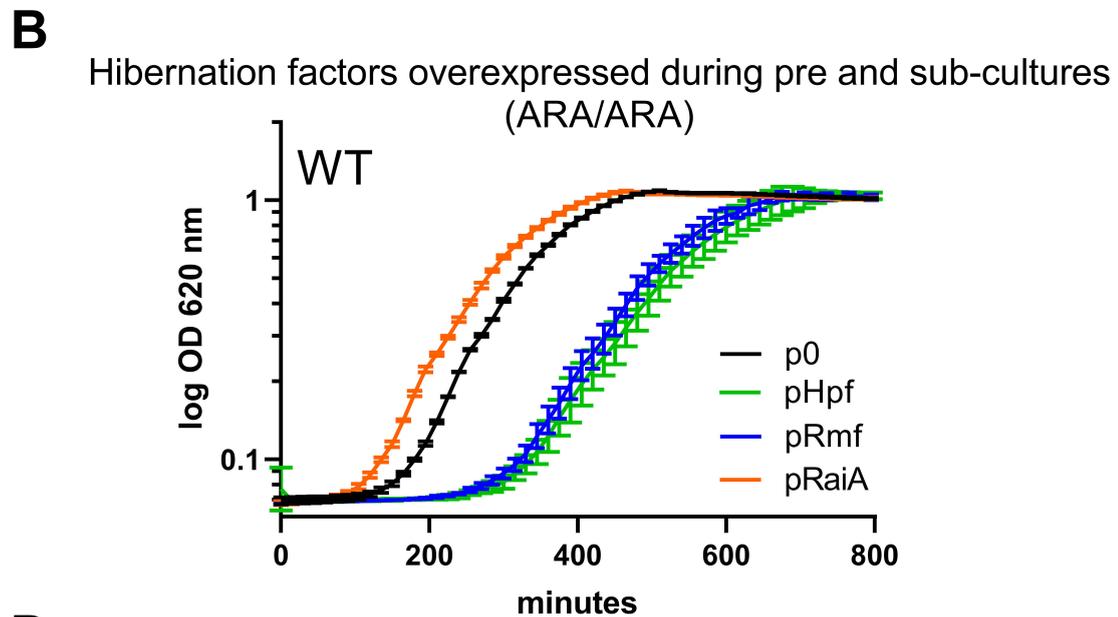
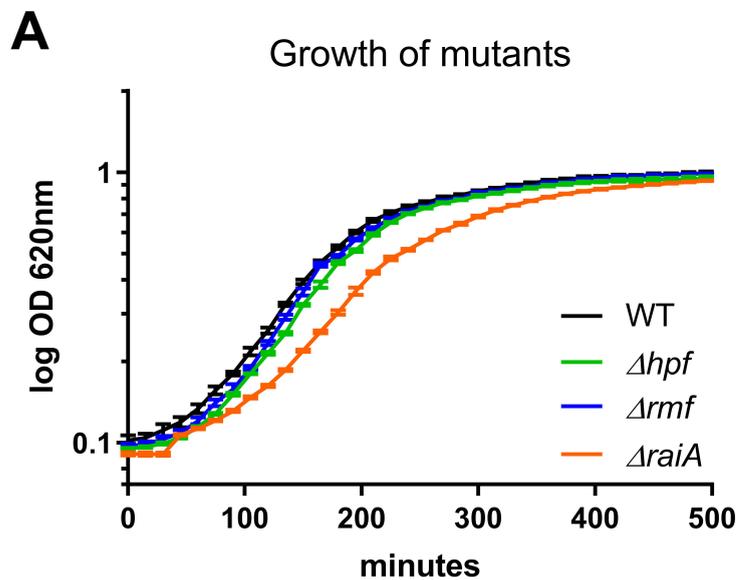
**C**

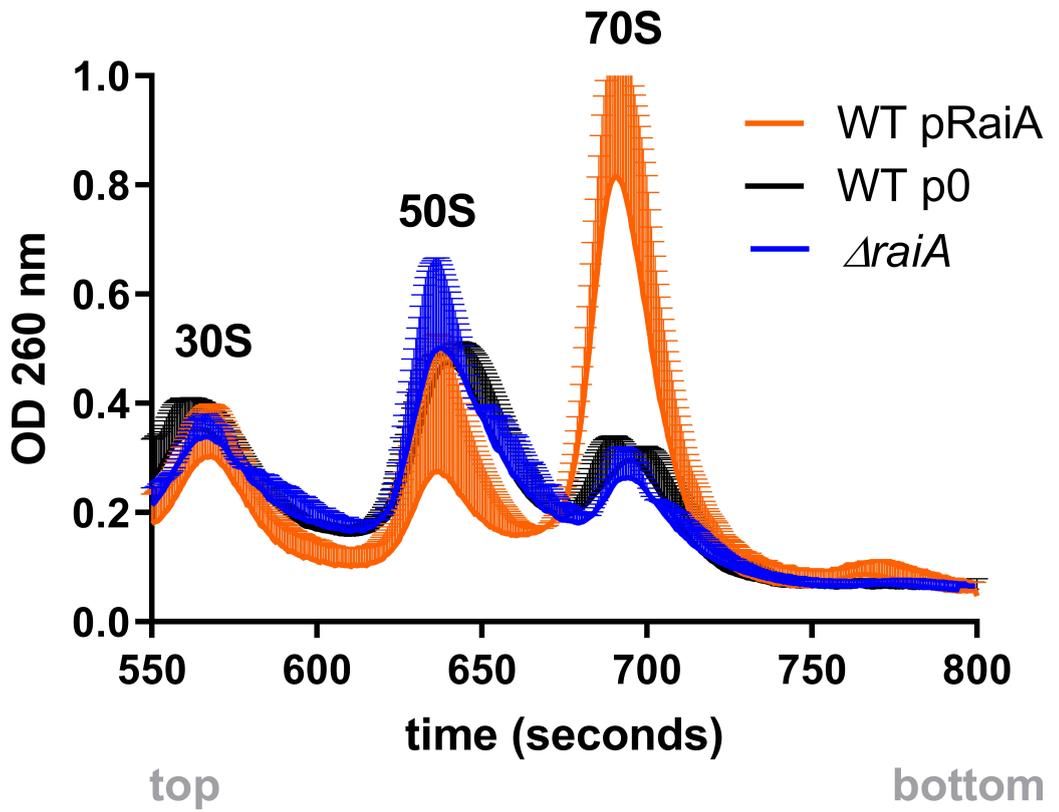
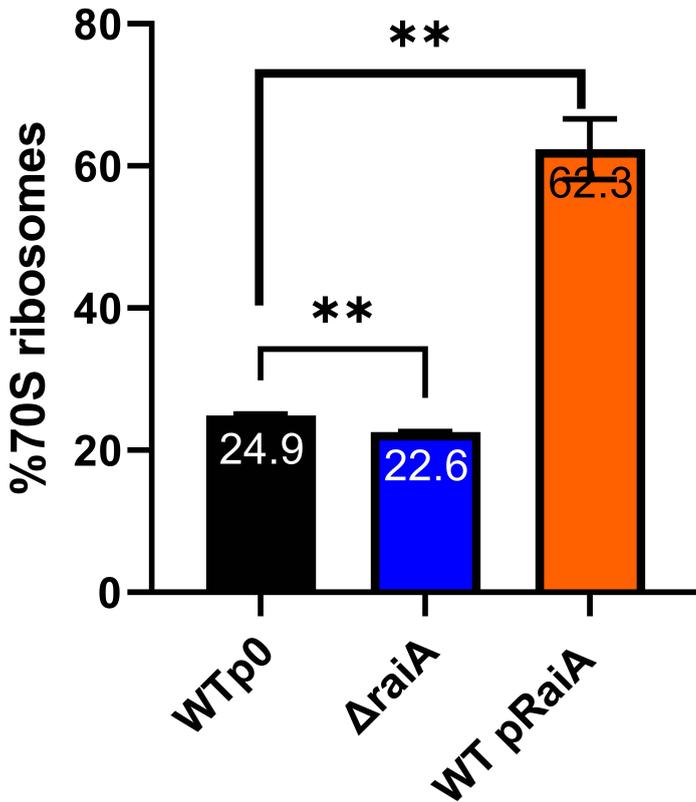
RaiA overexpressed during pre and sub-cultures (ARA/ARA)

**D**

RaiA never overexpressed (GLC/GLC)

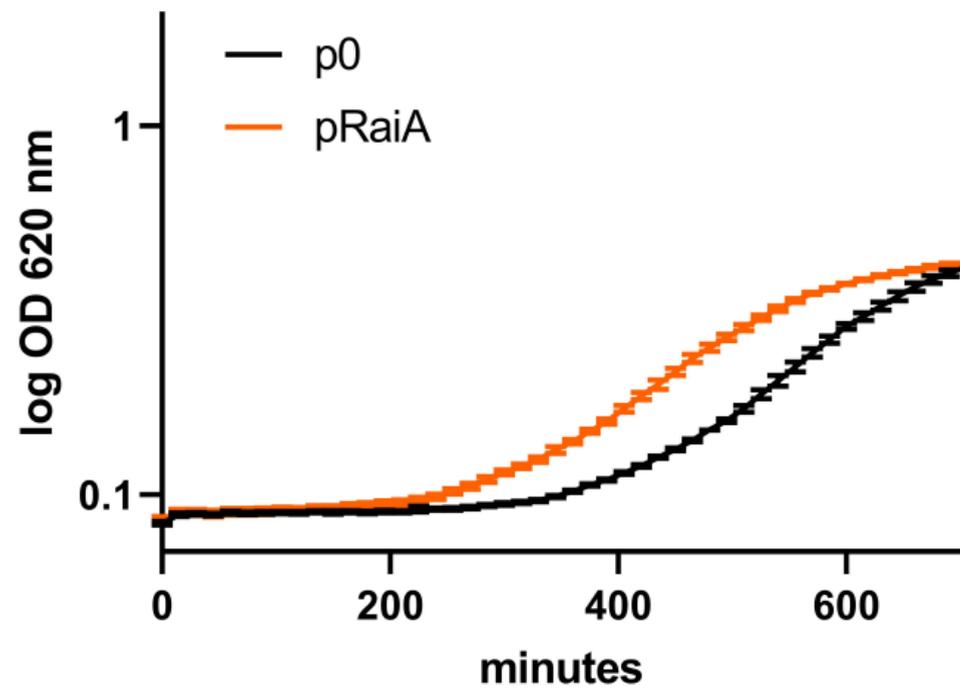
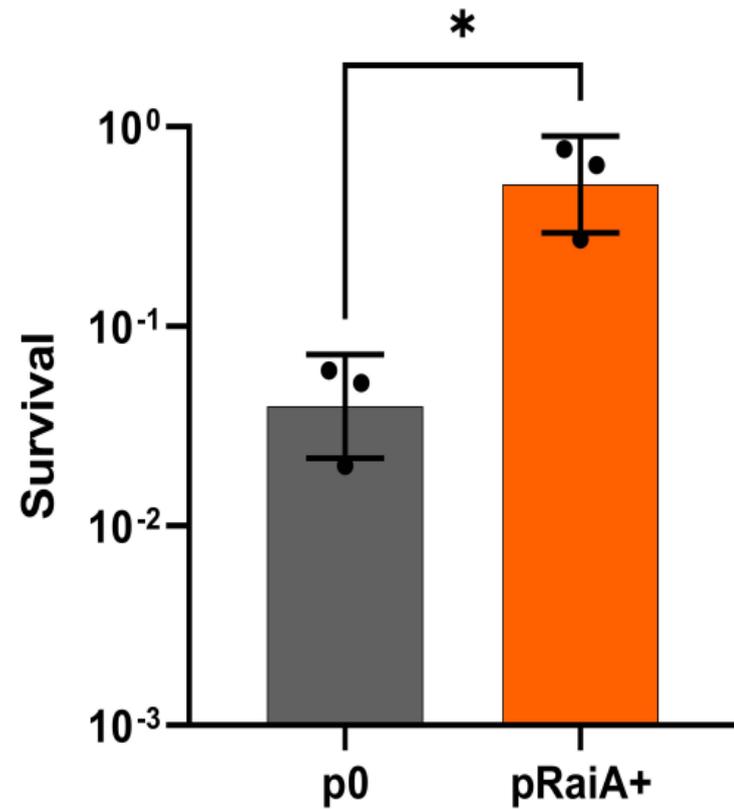




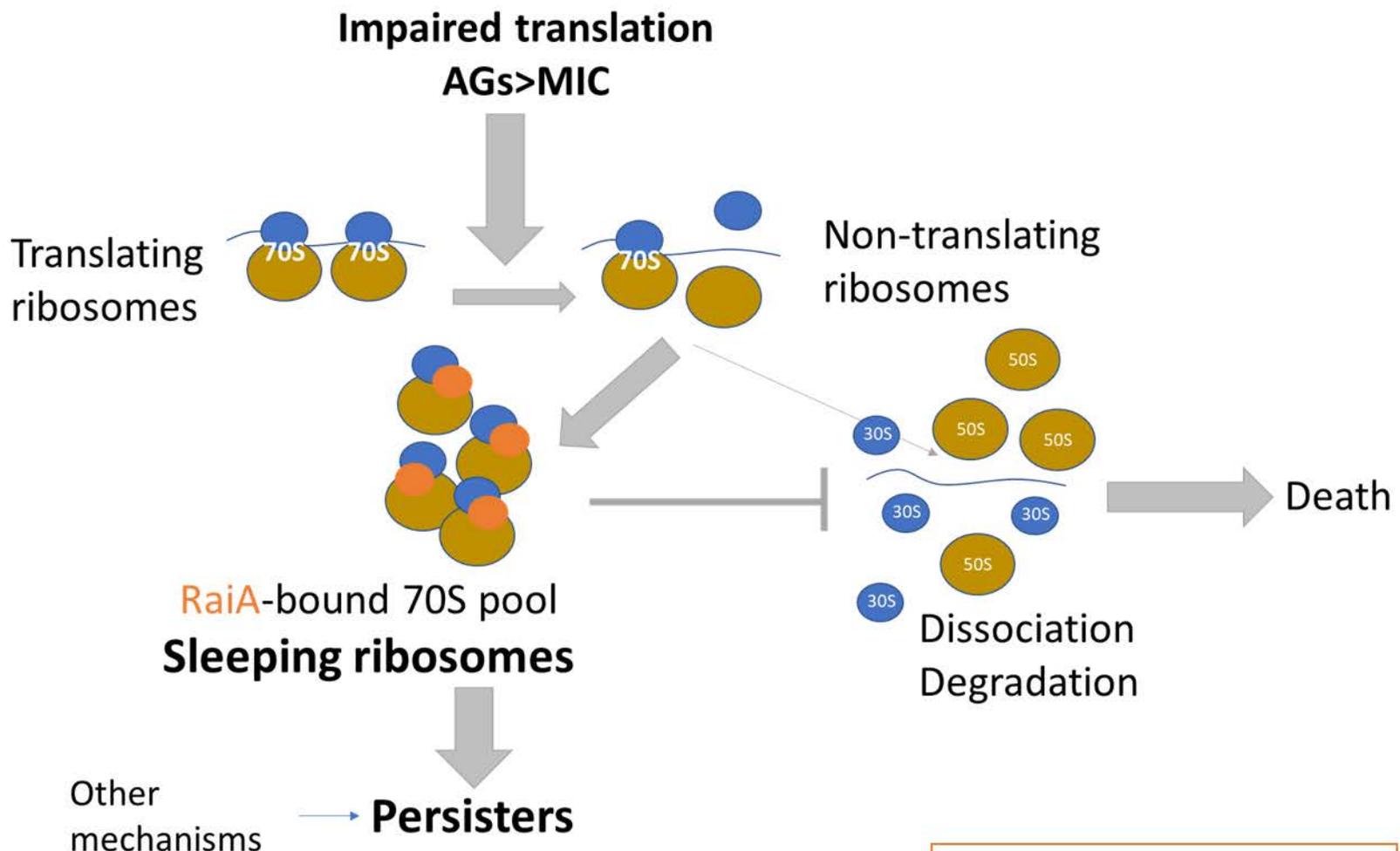
A**B**

A

RaiA overexpressed before and during growth curves
P. aeruginosa

**B**

A Persistence



B Lag period

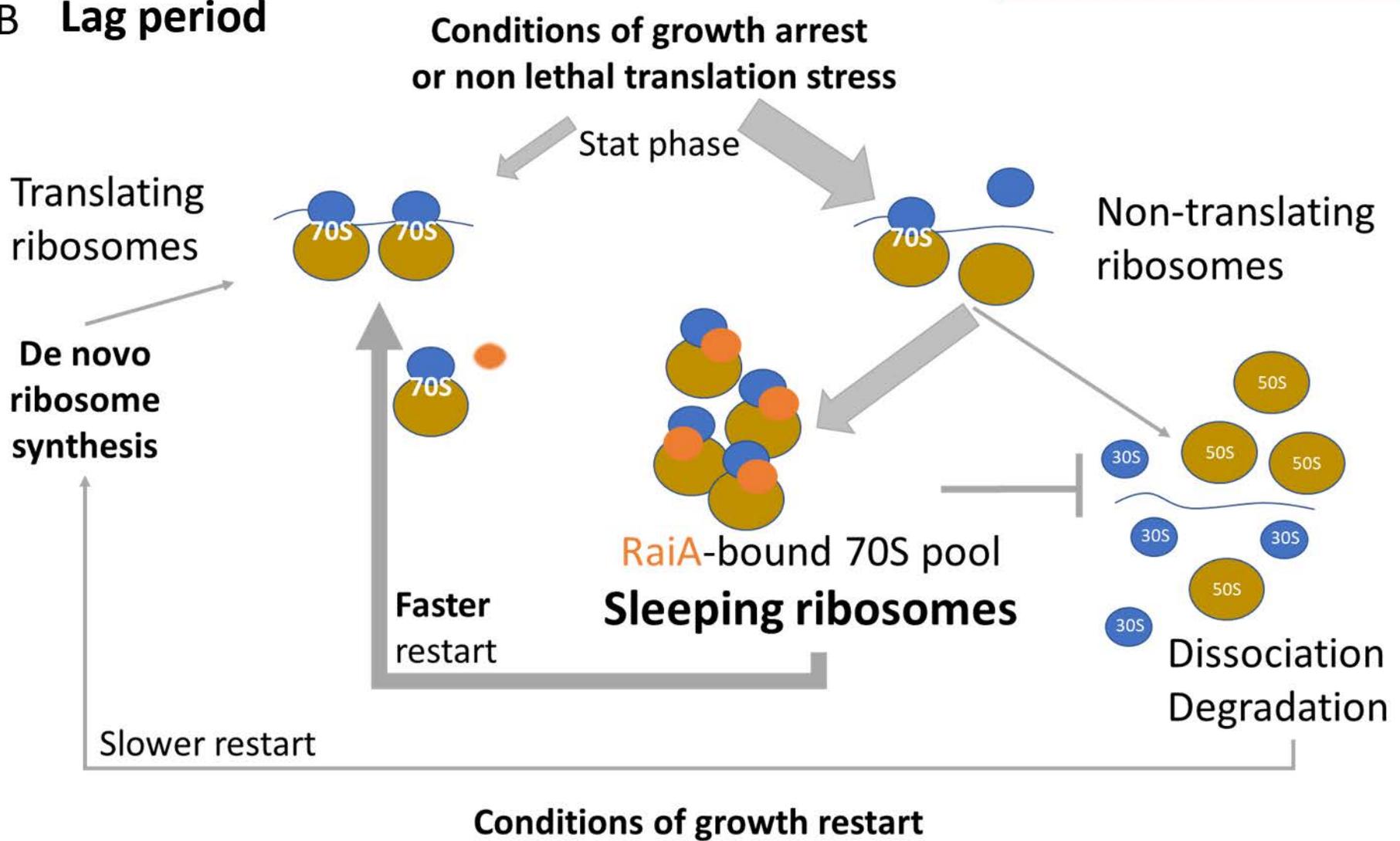


Figure S1. Indole improves growth of *V. cholerae* in the presence of tobramycin. A. WT. B. Δ *raiA*. Growth is measured on a TECAN plate reader. IND: indole 350 μ M. TOB: tobramycin sub-MIC (0.6 μ g/ml). Experiments were performed in triplicates and geometric means are represented. Error bars represent the geometric standard deviation. Related to **Figure 1**.

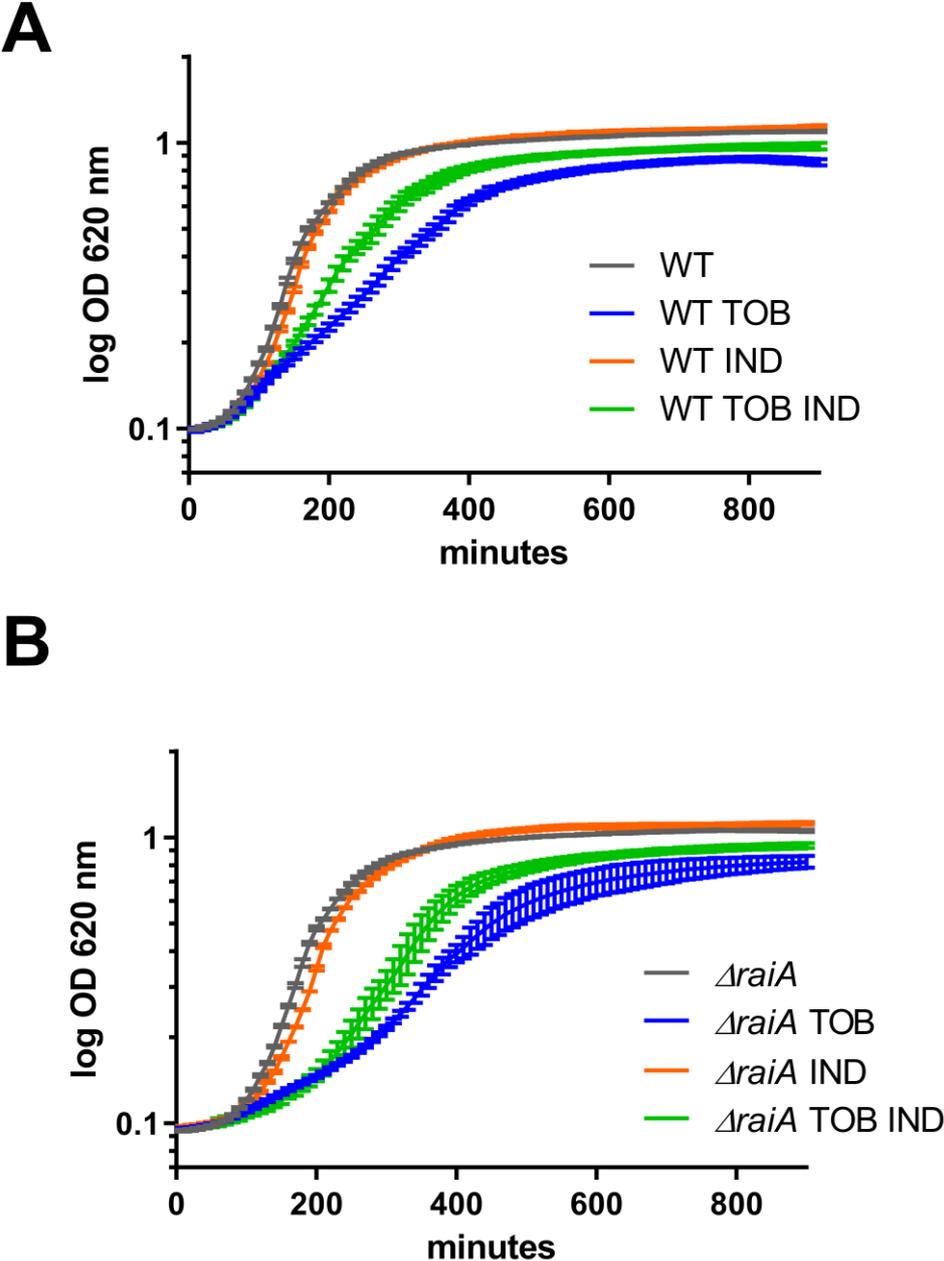
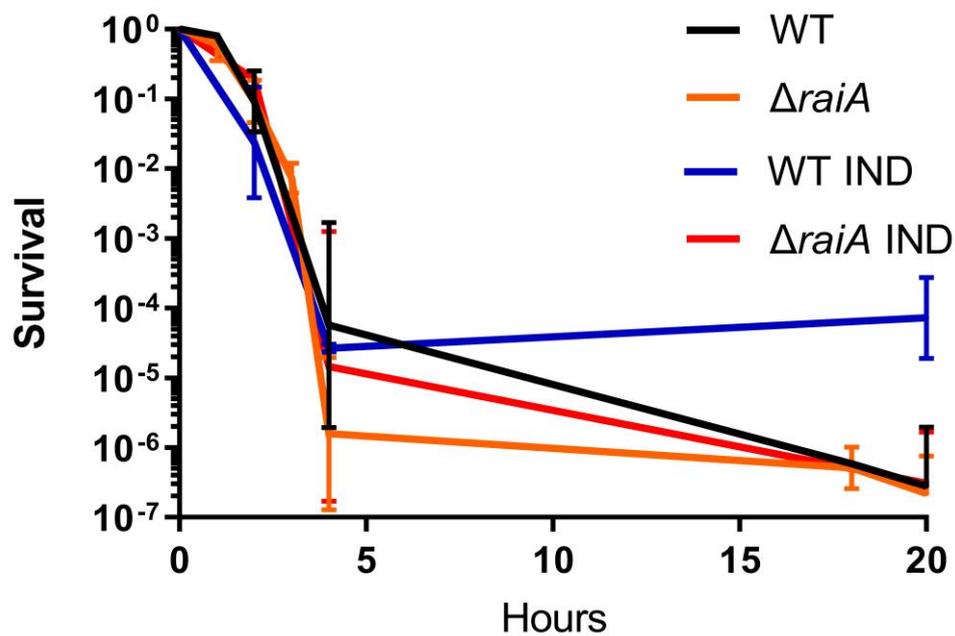


Figure S2. Persistence and the effect of indole. **A.** Kinetics of survival of WT *V. cholerae* to tobramycin 10 µg/ml in MH media. Time zero corresponds to the total number of CFU before addition of antibiotics, to an early exponential phase culture (OD 620 nm 0.25-0.3), as described in the methods section. The proportion of surviving cells is calculated after plating and counting growing colonies, and is represented for each time point. Curves represent geometric means of at least 3 replicates for each time point and error bars represent geometric standard deviation. **B.** Persistence of *V. cholerae* WT and $\Delta tnaA$ mutants in exponential phase (in LB instead of MH, to allow indole production, see methods) after 20 hours treatment with specified antibiotics. Tobramycin (TOB): 10 µg/ml, carbenicillin (CRB): 100 µg/ml. Related to **Figure 2**.

A



B

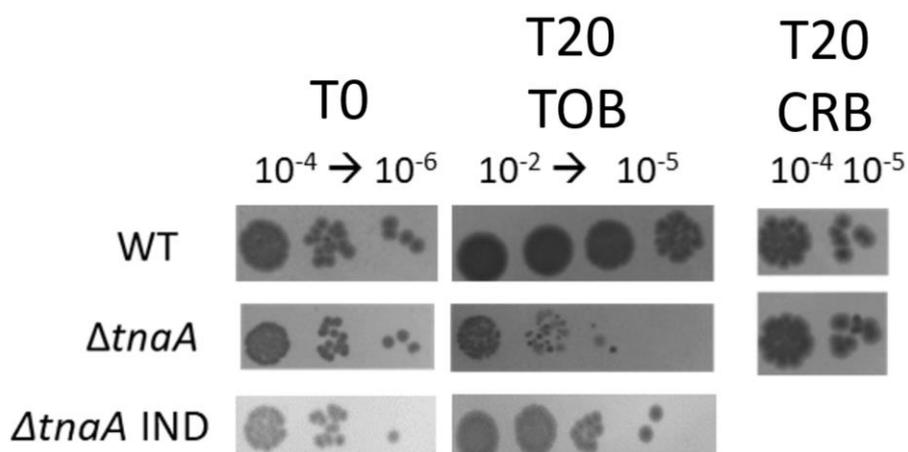
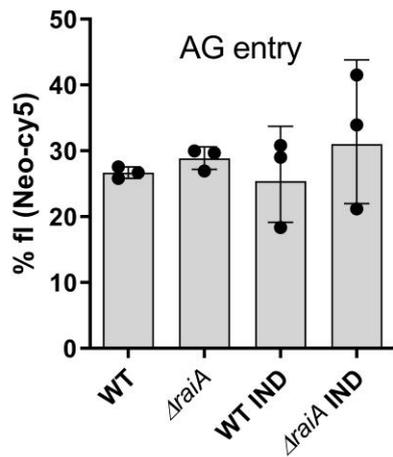


Figure S3. Indole does not apparently influence aminoglycoside entry and resistance in *V. cholerae* WT and Δ *raiA*. **A.** Intracellular level of neomycin coupled to the fluorophore Cy5 measured by fluorescence associated flow cytometry. Error bars represent standard deviation. **B.** Minimal inhibitory concentrations of tobramycin (TOB) and gentamicin (GEN) measured using *etest*s in *V. cholerae*, in the absence (MH) and presence of indole (IND), and indicated in μ g/ml by a numeral on each image. Related to **Figure 2**.

A



B

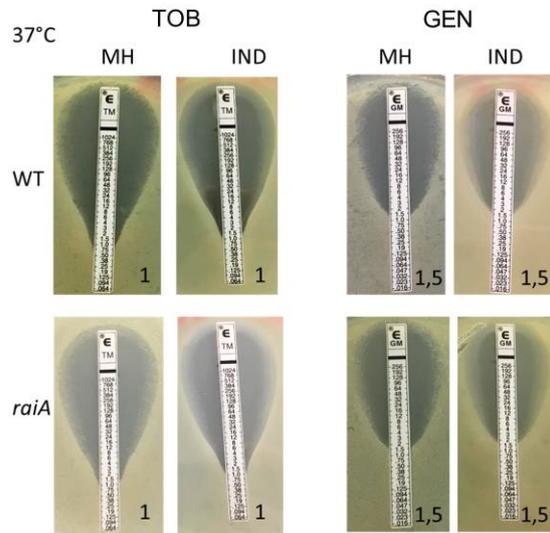


Figure S4. Fluorescence detection from the *raiA* promoter-*gfp* fusion by flow cytometry. Representative acquisitions are shown. Each plot represents one experiment. Each experiment was performed at least 3 times and data and statistical significance are shown in the histograms in the main manuscript. **A:** histogram curves: GFP fluorescence is represented in the x-axis (FITC channel), the y-axis represents the number of events corresponding to the number of cells, normalized to height (same number of total cells for both conditions). **B:** dot plots: Bacterial cells' size and shape (rugosity). FSC: forward scatter. SSC: size scatter. The SSC (size scatter) vs FSC (forward scatter) graphs show the distribution of cells by size and shape and no major difference in cell size/shape is observed between the WT and mutants with or without indole. Related to **Figure 4**.

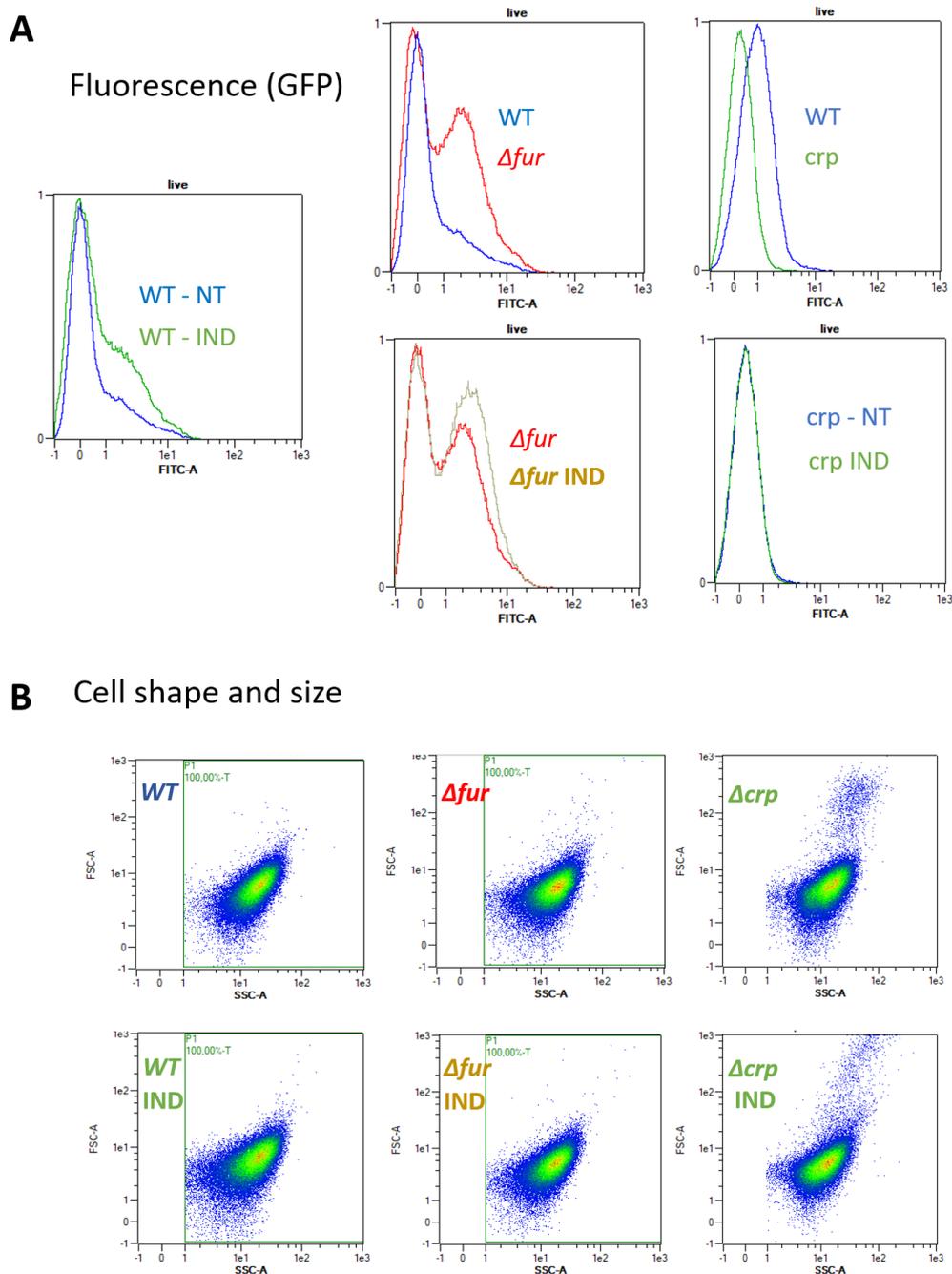
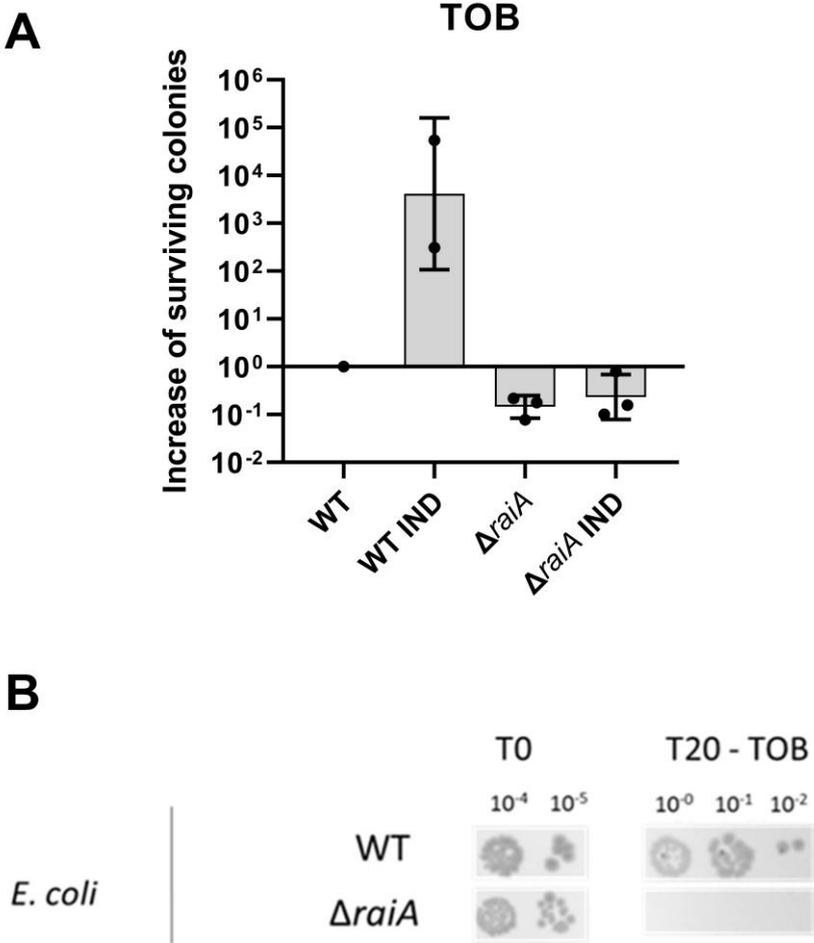


Figure S5. RaiA is involved in persistence in *V. cholerae* and *E. coli*. **A.** Increase of surviving colonies (persisters) after 20 hours of treatment with tobramycin (TOB) 10 µg/ml (ratio over WT surviving colonies) in *V. cholerae*. Error bars represent geometric standard deviation **B.** Persistence of *E. coli* WT and *raiA* mutant, at late exponential phase (OD 620nm 0.5), after 20 hours treatment with 10 µg/ml tobramycin (TOB) in MH media. Serial dilutions are spotted on plates for estimation of survival. Spot assays were performed at least 3 times. Related to **Figure 2**.



*

Figure S6. Influence of RaiA on the length of lag phase upon growth restart after the stationary phase. **A.** Experimental set-up. Repression was achieved with 1% glucose (GLC), induction was achieved with 0.2% arabinose (ARA). **B.** Lag time (minutes) defined as time to reach OD 620 nm 0.15 in WT *V. cholerae* carrying empty vector (p0) or pBAD-RaiA vector (see ABCD in Figure 6). **C.** Lag time (in minutes) defined as time to reach OD 620 nm 0.15 in *V. cholerae* Δ raiA, Δ rmf and Δ hpf and WT *V. cholerae* carrying empty vector (p0) or hibernation factors (pRaiA, pRmf, pHpf) (Figure 7AB) **D.** Lag time (in minutes) defined as time to reach OD 620 nm 0.15 in WT *P. aeruginosa* (Figure 9A) carrying empty vector (p0) or pBAD-RaiA vector. Experiments were performed in triplicates and statistical analysis was performed (**: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant). Related to **Figure 6, 7AB** and **9A**.

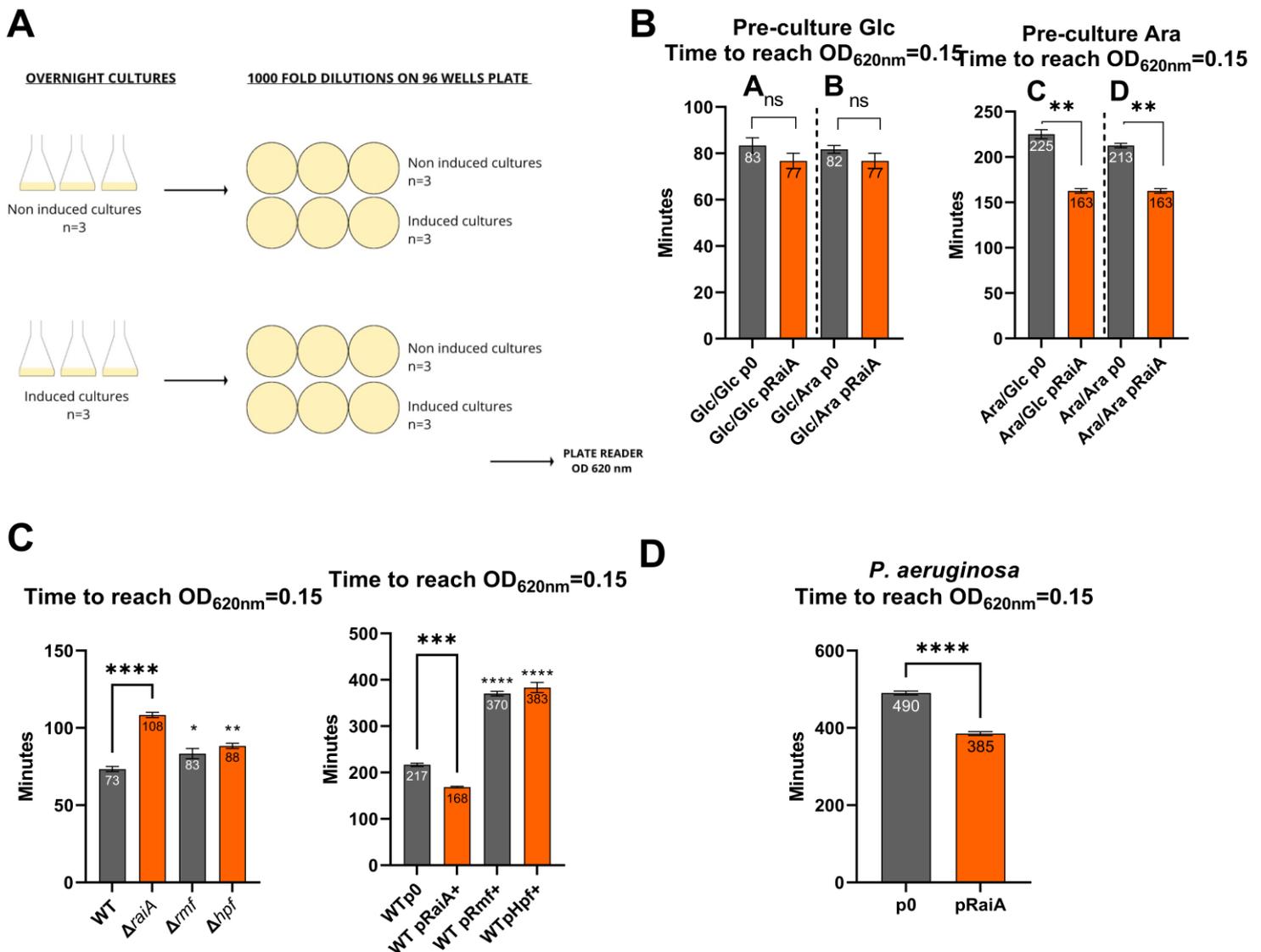


Figure S7. Influence of RaiA, Rmf and Hpf on persistence. Persistence of *V. cholerae* WT and mutants in early exponential phase, after 20 hours treatment with 10 µg/ml tobramycin (TOB) in MH media. Serial dilutions are spotted on plates for estimation of survival. Spot assays were performed at least 3 times. Related to **Figure 7**.

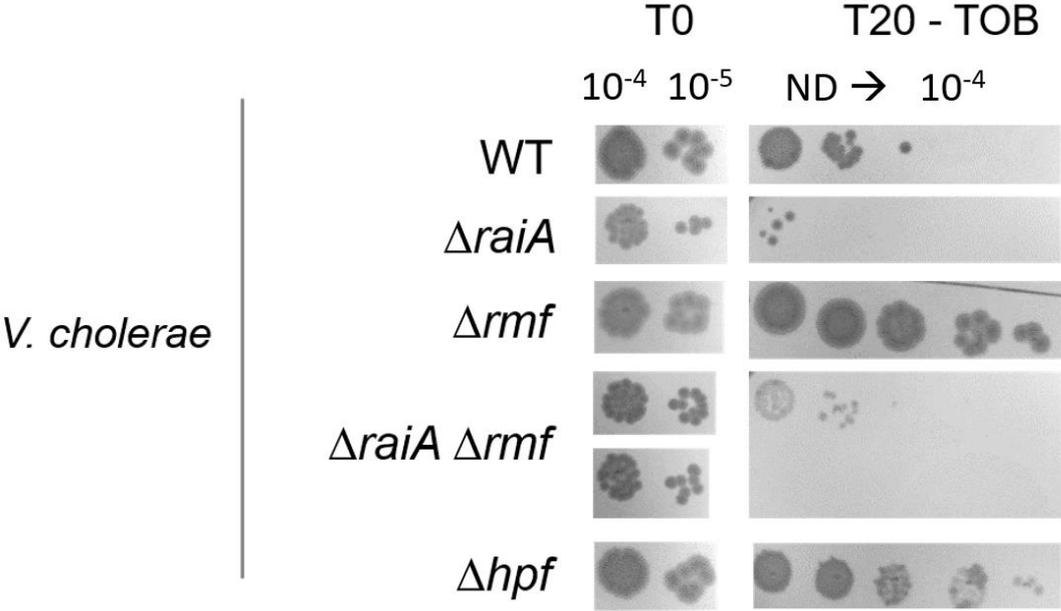


Figure S8. Expression of *gfp* from constitutive promoter (Pc) in conditions where expression from the *raiA* promoter is up or down-regulated. Fluorescence quantification of GFP expression from constitutive promoter by flow cytometry in MH media in exponential phase, in WT and indicated *V. cholerae* deletion mutants. IND: indole (350 μ M), DP: 2,2'-Dipyridyl (500 μ M). The Y axis represents fluorescence ratio of the mutant over wild type (WT) strain. Error bars represent standard deviation. Related to **Figure 4**.

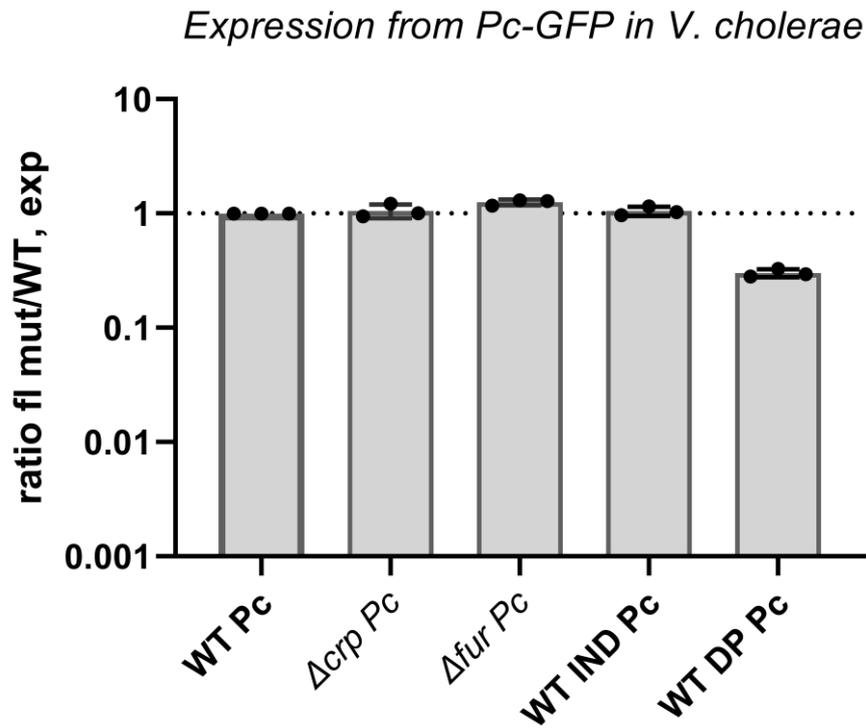


Table S1: Genes differentially regulated by indole. Related to **Figure 3**.

Id	norm MH-A	norm MH-B	norm MH-C	norm IND-A	norm IND-B	norm IND-C	Fold change	p value	adj p value
UP									
VC0706	3903	4103	3710	80171	68404	145086	20,1	8,0E-03	8,9E-03
VC1484	120	111	142	570	232	501	3,1	2,6E-05	1,3E-01
Motility- Chemotaxis									
VC0410	1779	1337	1477	3761	3910	3890	2,5	1,7E-14	8,0E-03
VC1049	413	473	450	2398	2145	2322	4,9	1,1E-38	9,0E-03
VC2370	316	270	299	1469	1694	1286	4,8	2,6E-35	1,0E-02
VC0414	3748	3638	2441	7061	7532	6276	2,1	1,4E-06	1,9E-02
VC0413	658	597	376	1239	1302	1053	2,1	1,2E-05	3,0E-02
VC0411	1939	1418	1067	3411	3912	2746	2,2	1,6E-05	3,9E-02
VCA0536	508	934	598	1748	2438	2062	2,9	6,5E-09	4,6E-02
VCA0954	1750	2718	1721	3886	5756	4105	2,1	2,7E-04	5,9E-02
VC0409	9371	6775	5433	26356	19176	30086	3,3	3,7E-09	6,3E-02
VC0827	1152	1207	1001	3000	7845	2896	3,6	4,4E-07	1,4E-01
VC0826	1967	1966	1336	5009	13957	5414	4,0	4,7E-08	1,5E-01
VC1763	428	1057	267	1554	1847	1495	2,5	3,3E-03	2,0E-01
VC0820	369	623	278	1133	751	2000	2,6	1,5E-03	2,2E-01
Respiration - energy									
VC1115	663	546	508	6006	6126	5482	9,8	6,1E-84	7,9E-03
VC0966	7486	7229	6500	15713	18048	21828	2,6	2,3E-14	1,1E-02
VC2371	163	157	107	1820	1907	1910	12,1	6,9E-67	1,3E-02
VC0477	4301	4841	4095	27291	30017	20290	5,6	4,2E-39	1,5E-02
VCA0511	174	249	197	1325	1518	1124	6,0	4,6E-35	1,8E-02
VCA0665	329	253	320	9542	8962	8507	27,0	5,2E-122	1,8E-02
VCA0665	329	253	320	9542	8962	8507	27,0	5,2E-122	1,8E-02
VC1951	347	334	242	5920	6998	5337	17,9	1,2E-87	2,0E-02
VC1114	430	337	365	1584	1201	1615	3,7	1,7E-17	2,3E-02
VC0356	583	766	592	1725	2375	1675	2,9	7,9E-12	2,4E-02
VC2145	931	952	989	7482	9974	5936	7,6	1,1E-42	2,4E-02
VCA0563	3532	3857	3297	10041	12683	8503	2,8	3,1E-11	2,7E-02
VC2368	8558	11124	8314	25789	18044	26995	2,4	4,1E-08	2,9E-02
VC0278	512	493	641	1311	1930	1207	2,6	1,2E-08	3,1E-02
VC0485	8659	11085	11206	35439	45566	26147	3,3	4,0E-13	3,2E-02
VCA0564	4137	4464	3746	10730	12961	8158	2,5	8,6E-08	3,4E-02
VC2656	941	1355	964	33710	22990	28100	22,5	2,0E-76	4,1E-02
VC2646	2110	1858	1643	4701	6486	4176	2,6	1,7E-07	4,6E-02
VC1687	61	69	112	1751	1301	1331	15,5	6,7E-48	5,0E-02

VC1866	7913	1267 6	7185	81168	10134 9	63711	7,9	6,8E-31	5,3E-02
VC2657	195	285	147	10748	7717	8506	33,9	1,1E-70	7,0E-02
VC1516	769	1005	666	2063	981	2137	2,0	2,6E-03	7,9E-02
VC2699	895	1117	1191	5749	3169	5283	4,0	1,1E-09	9,3E-02
VCA0675	293	225	186	1644	816	2194	5,7	1,1E-14	9,5E-02
VC0965	7652	1711 3	10909	25906	35487	21426	2,2	1,2E-03	9,7E-02
VC1513	979	1330	598	4220	2083	4053	3,2	9,9E-07	1,0E-01
VC2447	2591 1	3603 8	27345	51499	10514 0	37661	2,0	7,0E-03	1,3E-01
VCA0680	583	386	577	2435	870	2782	3,5	1,5E-06	1,3E-01
VCA0679	626	383	350	2412	805	2866	3,8	9,4E-07	1,6E-01
VCA0678	1612	1152	1434	5851	1605	7354	3,0	1,3E-04	1,9E-01
Sugar									
VC2000	3313 4	3852 3	32617	94393	96880	78059	2,5	2,7E-15	9,0E-03
VC0374	3016	2691	2733	9064	9875	7214	3,0	2,5E-18	1,2E-02
VC1973	609	640	690	6655	6173	5216	8,8	4,0E-64	1,3E-02
VC1972	229	204	255	2990	2618	2426	10,7	3,5E-58	2,0E-02
VC1971	280	208	250	3269	2917	2594	11,0	1,4E-58	2,2E-02
VC0487	2056	1941	1773	12473	9768	9035	5,1	6,3E-27	2,8E-02
VCA0011	487	642	554	2564	2618	2403	4,3	9,8E-20	2,9E-02
VC2689	3011	2941	4636	23493	19880	18705	5,4	1,3E-24	3,9E-02
VC2001	1039	618	617	2290	3235	1950	3,1	8,4E-09	5,9E-02
VCA0516	258	256	239	1777	2151	1542	6,5	7,7E-22	6,0E-02
VCA0013	493	1132	476	2028	1287	1755	2,2	7,3E-03	1,7E-01
RRR									
VCA0521	1439	1451	801	3686	3125	3167	2,6	3,1E-07	4,4E-02
VCA0520	694	723	482	1449	1115	1411	2,0	3,1E-05	2,9E-02
VC2386	4774	8304	2645	8736	6458	24643	2,2	3,3E-02	3,6E-01
Metabol									
VC0336	7124	8934	9210	19907	24075	16926	2,4	2,8E-09	1,8E-02
VC0026	268	258	311	1582	1606	1262	5,0	1,6E-27	2,1E-02
VCA0235	3581	3566	3392	8268	5703	8159	2,0	5,8E-05	3,3E-02
VCA0875	2063	2616	1307	5759	4746	4221	2,4	7,0E-06	4,4E-02
VC2698	2245	3759	3380	7765	5269	7196	2,1	6,6E-04	5,7E-02
VC2373	1415	690	1058	2458	3824	2252	2,5	6,8E-05	8,7E-02
VC2361	4108	2170	3027	19721	15393	32664	6,1	1,1E-13	1,3E-01
Nucleotide									
VCA0564	4137	4464	3746	10730	12961	8158	2,5	8,6E-08	3,4E-02
VC1034	470	448	562	1528	1273	1225	2,7	5,9E-13	1,2E-02
regulators									
VC0486	1203	1242	753	11275	8624	10176	8,5	1,0E-37	3,6E-02
VC1719	1362	981	674	5308	5146	6057	5,1	4,1E-20	4,3E-02
VCA0562	824	717	348	1268	1261	1628	2,1	9,6E-04	7,1E-02

VC1919	1270 4	1288 3	7383	28846	15905	39769	2,4	1,1E-03	1,2E-01
VC2366	483	775	287	1133	1549	946	2,2	3,7E-03	1,2E-01
csrD	5813 1	4493 3	26252	10280 2	52169	15791 5	2,2	1,0E-02	1,8E-01
Membrane									
VC1315	54	49	49	1059	987	1071	18,6	6,1E-84	1,0E-08
VC2484	2791	2555	2045	8396	7987	7622	3,2	4,9E-20	1,1E-02
VC2485	2791	2555	2045	8396	7987	7622	3,2	4,9E-21	1,1E-02
VCA0591	531	462	342	1184	1285	1183	2,7	9,1E-12	1,6E-02
VC0976	1443	1713	1027	3220	2738	3167	2,1	1,4E-05	3,1E-02
VC2149	145	110	144	2554	1821	2827	15,4	6,3E-47	5,7E-02
VC0654	606	731	365	2927	5686	3163	5,9	8,2E-14	1,1E-01
VCA0628	680	1842	822	2839	1462	4368	2,2	1,7E-02	2,9E-01
Others									
VC0652	74	71	98	1343	1362	1262	15,1	3,0E-86	1,0E-08
VC0428	523	576	695	4516	4519	4760	7,3	1,6E-49	1,6E-02
VC1892	819	567	543	1182	2075	1046	2,1	5,6E-04	7,2E-02
VC0037	801	555	621	1749	1301	2937	2,8	6,0E-06	8,9E-02
VC1765	1538	4879	1100	7066	10157	6196	2,6	3,1E-03	2,9E-01
Hypothetical									
VCA0718	329	301	343	1989	1854	1881	5,7	6,9E-63	1,0E-08
VC1865	741	620	752	15085	13445	13011	18,5	7,9E-143	7,1E-03
VC1871	630	784	729	5093	6873	5480	7,7	7,7E-58	1,3E-02
VC1077	1496	1106	1197	2628	2923	2918	2,2	1,1E-08	1,4E-02
VCA0236	299	343	418	1039	1012	978	2,8	2,1E-12	1,5E-02
VCA1013	41	54	72	1544	916	1414	19,5	9,5E-54	5,0E-02
VCA0741	8417	7150	6445	35536	27576	52687	4,8	2,1E-17	5,5E-02
VCA0919	1107	806	959	3604	2763	4638	3,6	4,7E-11	5,5E-02
VC0871	209	193	259	1657	1019	2475	6,8	3,3E-19	8,0E-02
VC1853	2696	2439	1227	5889	3740	7203	2,4	3,1E-04	9,6E-02
VC2035	272	247	652	883	1154	1004	2,4	4,9E-04	1,1E-01
VCA0367	870	2025	677	2495	2608	3104	2,1	6,6E-03	1,5E-01
VCA0547	1134	821	2003	3510	1518	3735	2,0	2,3E-02	1,9E-01
VC1770	338	770	245	1049	1704	933	2,4	5,3E-03	2,1E-01
VC2221	97	191	99	3971	1113	3984	15,1	9,8E-21	2,4E-01
VCA0283	721	1944	390	2479	2047	3333	2,2	1,7E-02	2,6E-01
VC1764	769	2351	507	4476	4878	3997	3,0	5,2E-04	2,8E-01
DOWN									
Electron transfer - Ion									
VC2415	6690	8047	8130	1766	1466	1837	0,2	3,9E-40	6,4E-38
VCA0228	703	914	818	60	32	108	0,1	8,9E-25	7,7E-23
VC1265	1746	1306	1376	237	112	243	0,2	8,6E-24	6,8E-22
VCA0227	2696	3888	2205	333	155	375	0,1	4,4E-22	3,3E-20

VCA0230	883	1120	957	128	53	173	0,1	5,5E-19	3,1E-17
VCA0229	545	636	685	68	15	72	0,1	6,8E-19	3,7E-17
VC2559	1531	1298	1238	326	158	308	0,2	1,3E-16	6,1E-15
VC2414	2065 7	2573 6	21516	6744	3908	6762	0,3	2,5E-16	1,2E-14
VC1434	5799	4818	6784	1544	2021	1387	0,3	1,6E-15	6,9E-14
VC1544	860	841	702	211	92	200	0,2	5,8E-15	2,3E-13
VC1266	992	730	899	180	81	218	0,2	2,8E-14	1,1E-12
VC1548	828	977	762	200	71	185	0,2	3,5E-14	1,3E-12
VC1168	2427	1657	3276	600	422	657	0,2	4,4E-14	1,6E-12
VC2560	938	837	656	178	57	151	0,2	1,6E-13	5,6E-12
VC0575	3481	4499	4787	1741	1310	1531	0,4	1,7E-13	5,9E-12
VC0540	1091	965	938	257	204	349	0,3	1,9E-13	6,4E-12
VC1890	4135	4112	4697	2193	1882	2160	0,5	2,8E-13	9,2E-12
VC1267	1006	697	951	208	95	240	0,2	1,2E-12	3,6E-11
VC1625	6073	5424	5715	1808	1036	2011	0,3	1,3E-12	3,8E-11
VC1547	1186	1193	907	270	101	276	0,2	2,0E-12	5,9E-11
VC1623	2356	1972	1754	913	895	853	0,4	2,2E-12	6,6E-11
VC0608	1724	3895	2700	607	375	613	0,2	2,0E-11	5,5E-10
VC1543	1647	1544	1204	468	228	484	0,3	2,7E-11	7,1E-10
VC1264	3449	2931	2531	663	257	830	0,2	3,0E-11	7,8E-10
VC0541	2020	1993	2192	759	645	969	0,4	6,9E-11	1,7E-09
VC0627	1608	1342	1589	479	458	718	0,4	3,9E-10	8,7E-09
VC2413	1118 4	1287 6	15480	4976	2760	5137	0,3	4,8E-10	1,0E-08
VC0574	4963	6302	6116	2629	1626	2374	0,4	5,1E-10	1,1E-08
VCA0779	1014	1043	1299	573	473	544	0,5	6,7E-10	1,4E-08
VC1425	1872 8	1856 8	11818	6031	6511	6308	0,4	2,6E-09	4,9E-08
VC1190	3535	3491	3519	1105	534	1290	0,3	4,7E-09	8,7E-08
VC0539	816	759	978	299	125	291	0,3	5,5E-09	1,0E-07
VC2031	1195	956	1683	516	398	518	0,4	6,3E-09	1,1E-07
VC0538	2463	3016	3026	969	418	946	0,3	6,6E-09	1,2E-07
VC2044	4468	3773	4970	2081	1443	2069	0,4	6,9E-09	1,2E-07
VC1624	2450	2405	2567	1013	552	1133	0,4	3,1E-08	5,0E-07
VC0384	2708	2991	1808	989	444	818	0,3	5,1E-08	8,1E-07
VC1350	1809	1972	2095	700	899	926	0,4	6,0E-08	9,2E-07
VC2099	4134	4299	6134	2367	2308	2392	0,5	1,8E-07	2,5E-06
VC1299	2194	1529	1452	766	807	883	0,5	3,3E-06	3,5E-05
VC2389	1233 5	1044 6	14700	5767	2538	5581	0,4	3,7E-06	3,8E-05
VC0168	4710	4663	3918	2115	1538	2593	0,5	5,0E-06	5,0E-05
VC1439	3658	4680	4161	2062	1186	2295	0,5	5,5E-06	5,5E-05
VC0385	2632	2744	1443	910	465	974	0,4	6,3E-06	6,3E-05
VC0386	1716	1717	944	651	435	748	0,4	2,9E-05	2,5E-04
VCA0554	3640	1975	4155	1213	845	1601	0,4	3,1E-05	2,6E-04

VC0731	9328	1866 7	16354	5035	7322	4748	0,4	7,7E-05	5,8E-04
VC0034	1465	2168	3551	936	1054	1174	0,5	1,2E-04	8,2E-04
VC0573	4031	5106	3159	2130	1225	2439	0,5	1,4E-04	9,8E-04
VC1440	803	1073	578	467	268	424	0,5	1,7E-04	1,1E-03
VCA0907	1430	2408	1943	1139	688	868	0,5	2,0E-04	1,3E-03
VC2089	762	1693	896	567	307	554	0,5	2,2E-04	1,4E-03
VC1441	3956	4982	2505	2010	1104	2188	0,5	3,7E-04	2,2E-03
VCA0496	1389	942	2071	426	1108	411	0,5	4,2E-03	1,6E-02
Sugar metabolism									
VCA0896	3304	3007	3497	889	693	872	0,3	1,6E-32	1,8E-30
VC1126	7293	1025 0	7992	2766	2625	2583	0,3	2,4E-18	1,2E-16
VCA0657	685	529	732	221	201	201	0,3	7,2E-14	2,5E-12
VC1004	6034	4770	6826	1934	1231	1837	0,3	4,2E-13	1,4E-11
VC2024	3794	3293	4602	1688	1965	1679	0,5	4,2E-09	8,0E-08
VCA0897	3467	2804	1622	845	579	943	0,3	4,9E-09	8,9E-08
VCA0898	5767	4943	6369	2709	1746	2587	0,4	7,2E-09	1,3E-07
VC0910	3175	3395	3392	1601	1326	1539	0,5	1,1E-05	1,0E-04
VC0911	4479	3736	4242	1881	2225	1776	0,5	2,5E-05	2,2E-04
VC2480	1448	1607	2584	982	741	885	0,5	5,1E-05	4,0E-04
VCA1060	2171	771	1674	633	563	664	0,4	1,4E-04	9,5E-04
VC2183	5155	4222	6751	2904	1660	3070	0,5	2,8E-04	1,7E-03
Aminoacid metabolism									
VC1658	2116	1124	2478	185	194	252	0,1	8,2E-26	7,4E-24
VC0008	724	505	822	117	118	140	0,2	1,2E-20	7,9E-19
VC0009	679	385	742	88	83	99	0,2	4,2E-20	2,6E-18
VC0472	6447	7377	9080	2458	1906	2154	0,3	3,0E-19	1,7E-17
VC1312	2358	2539	3364	624	800	669	0,3	1,9E-18	9,8E-17
VC0010	3040	2440	3330	550	251	670	0,2	2,0E-14	7,7E-13
VC0880	758	657	1076	241	122	270	0,3	2,2E-10	5,2E-09
VCA1073	509	701	623	250	182	291	0,4	3,3E-09	6,3E-08
VC0743	6022	4953	6483	2669	1673	2698	0,4	7,6E-08	1,1E-06
VC1995	1199	1731	2309	639	415	724	0,4	8,9E-08	1,3E-06
VCA0815	3885	3098	3980	1836	1216	1630	0,4	1,7E-07	2,4E-06
VC0907	596	504	737	298	182	300	0,4	2,0E-06	2,3E-05
VC0941	1007 6	7756	8318	4801	2914	4531	0,5	2,3E-06	2,6E-05
VC2682	2219	1588	1853	772	900	1076	0,5	5,2E-06	5,2E-05
VC0947	1067 1	1309 6	9872	4646	6635	4962	0,5	1,5E-05	1,4E-04
VCA0772	607	666	968	364	202	431	0,5	8,6E-05	6,4E-04
VC0275	3965	3080	4200	1403	423	1669	0,4	1,0E-04	7,2E-04
VC2485	1370	3351	2132	1019	971	914	0,5	1,1E-04	8,0E-04
VCA0088	2895	3412	2191	1471	647	1654	0,5	3,7E-04	2,2E-03
VC0662	1746	1104	2625	973	890	798	0,5	3,8E-04	2,2E-03

VC0576	3097	3766	9997	1913	2839	1831	0,4	7,3E-04	3,9E-03
VCA0076	970	1503	1188	541	146	612	0,4	9,4E-04	4,8E-03
VC1492	2786	6743	2369	1909	1011	2088	0,5	1,5E-03	6,9E-03
VC2746	1921 0	2904 5	38036	10763	19348	10475	0,5	2,3E-03	9,8E-03
Nucleotide metabolism									
VC0276	5517	4824	5808	1963	752	2223	0,3	3,1E-06	3,3E-05
VC0767	8972	9299	12691	4208	4262	4502	0,4	2,4E-10	5,7E-09
VC0768	1763 6	1423 7	15989	5714	3303	5613	0,3	1,3E-09	2,7E-08
VC0986	1698 0	1660 9	14315	7288	6361	7172	0,4	9,9E-09	1,7E-07
VC1228	2458	2040	2902	784	365	922	0,3	1,6E-07	2,2E-06
VC1721	2424	2857	3565	1020	1480	1120	0,4	3,7E-08	6,0E-07
VC2171	1886	1374	2592	1049	478	1191	0,5	3,2E-03	1,3E-02
VC2227	7683	7434	9542	1542	600	1786	0,2	3,4E-12	9,6E-11
VC2258	5189	4353	6703	2373	3004	1852	0,5	1,3E-06	1,6E-05
VC2277	6821	6135	7227	1701	1908	1914	0,3	1,0E-18	5,3E-17
VC2352	8009	5520	8212	3958	2318	4075	0,5	3,7E-04	2,2E-03
VC2712	6475	3650	5923	2192	772	2789	0,4	7,2E-04	3,8E-03
VCA0607	1153	603	1425	331	773	334	0,5	3,7E-03	1,5E-02
VC1365	972	1192	1204	492	581	539	0,5	5,0E-07	6,5E-06
Translation									
VC0218	5714 1	3623 0	62003	25376	23118	26376	0,5	6,6E-06	6,5E-05
VC0291	1045 6	1172 6	12645	5607	4153	5227	0,4	6,1E-11	1,5E-09
VC0359	6218 6	4594 2	57114	27307	22476	29864	0,5	1,1E-05	1,0E-04
VC0360	6543 5	4661 3	78079	26811	25570	27739	0,4	4,5E-07	6,0E-06
VC0361	2930 4	3845 5	33303	17321	16307	16634	0,5	4,4E-10	9,8E-09
VC0369	2466 5	2120 0	59251	15852	6855	16988	0,4	1,8E-03	8,1E-03
VC0379	793	970	1720	567	485	531	0,5	4,7E-05	3,8E-04
VC0520	1594 2	1975 4	24705	6763	8291	6571	0,4	9,5E-09	1,6E-07
VC0570	5502 6	7240 5	87623	29769	23007	32471	0,4	1,1E-08	1,9E-07
VC0571	2053 8	2730 7	71337	12432	9502	11204	0,3	2,1E-06	2,4E-05
VC0644	1515	1015	1370	552	434	509	0,4	1,3E-09	2,7E-08
VC0645	2212	1491	2617	645	503	659	0,3	2,2E-12	6,5E-11
VC0646	7442 5	4550 8	52075	24930	23318	30660	0,5	1,7E-05	1,5E-04
VC0659	4553	4864	6873	1701	1920	1491	0,3	3,0E-14	1,1E-12
VC1209	8390	9867	9418	4451	4396	4138	0,5	6,6E-11	1,7E-09
VC1219	1824	1726	2168	991	864	970	0,5	1,6E-10	3,8E-09
VC1220	4783	4380	6074	2824	2175	2437	0,5	1,4E-07	1,9E-06

VC1297	5980	5708	9049	2891	2950	3392	0,5	5,1E-08	8,1E-07
VC1640	4267 2	2758 6	45907	10871	10746	14654	0,3	8,3E-09	1,5E-07
VC1923	1463 5	3008 8	14378	9167	11082	8555	0,5	7,1E-04	3,8E-03
VC2025	1022 3	7808	14105	3020	3736	2634	0,3	1,5E-09	3,0E-08
VC2026	6945 8	5267 6	55871	21480	25695	22013	0,4	1,2E-07	1,7E-06
VC2214	6055	7838	8737	3254	3998	2586	0,5	1,1E-06	1,4E-05
VC2259	3475 4	4409 0	37821	18907	21812	17310	0,5	4,8E-07	6,2E-06
VC2342	6617 0	8699 4	10061 6	45347	39129	36577	0,5	5,5E-08	8,5E-07
VC2461	2014	1761	2572	814	837	695	0,4	9,0E-13	2,9E-11
VC2579	8782	2325 7	18636	7681	5267	7624	0,4	7,6E-05	5,7E-04
VC2583	8239	2080 4	14710	7616	6339	6607	0,5	2,7E-04	1,7E-03
VC2620	1574	1292	1490	785	527	762	0,5	2,2E-05	1,9E-04
VC2664	4009	5269	4052	1503	1695	1142	0,3	4,5E-14	1,6E-12
Others									
csrC2	3606	1920	2667	508	802	551	0,3	9,0E-11	2,2E-09
VIBCH10294	454	271	1091	284	121	291	0,4	5,8E-03	2,1E-02
VC0033	661	587	1321	282	403	265	0,4	1,3E-05	1,2E-04
VC1003	4970	3770	3744	1838	1149	2060	0,4	8,1E-07	1,0E-05
VC1490	685	850	1294	330	344	399	0,4	1,4E-06	1,6E-05
VC1208	3478	4196	5582	2153	1811	1986	0,5	1,4E-07	2,0E-06
VC2460	3572	3075	3149	1084	1300	1026	0,4	2,8E-20	1,8E-18
VC0290	7761	7686	8352	2994	2610	3228	0,4	5,3E-16	2,4E-14
VC2459	960	791	658	428	353	392	0,5	8,0E-08	1,2E-06
VC2647	6843	4552	3914	1936	3109	2207	0,5	1,8E-04	1,2E-03
VC0749	1248	1409	1596	739	601	674	0,5	3,0E-10	6,7E-09
VC0766	1307	1242	3902	984	737	1256	0,5	6,3E-03	2,2E-02
Membrane - transporters									
VC1577	6497	2204	6096	403	1657	363	0,2	1,4E-06	1,7E-05
VC1195	2511	3007	2599	1076	1138	1086	0,4	4,1E-14	1,5E-12
VC1962	1002	693	894	237	323	230	0,3	3,0E-11	7,8E-10
VC1043	2014 9	3465 8	22588	7960	8029	9141	0,3	5,1E-09	9,3E-08
VCA0862	718	539	837	239	239	338	0,4	4,9E-08	7,8E-07
VC1655	1156	809	1960	372	425	333	0,3	6,9E-09	1,2E-07
VC1409	885	311	759	79	245	73	0,3	1,1E-06	1,3E-05
VC1329	2576	1202	2399	450	273	641	0,3	5,4E-09	9,9E-08
VC2305	5931	3437	9844	1711	2267	2010	0,3	1,1E-06	1,4E-05
VCA0554	3640	1975	4155	1213	845	1601	0,4	3,1E-05	2,6E-04
VC2278	7821	6452	9648	2026	1781	2313	0,3	3,4E-18	1,7E-16
VC1319	3625	988	2722	474	1989	485	0,5	1,6E-02	4,9E-02
VCA1071	777	918	739	333	197	313	0,4	1,5E-11	4,0E-10

VC1669	764	292	1161	137	223	135	0,3	1,4E-07	1,9E-06
VC0156	1303 6	1233 6	11471	2758	7063	2800	0,4	6,7E-05	5,1E-04
VC0475	1465	834	1238	579	468	527	0,5	1,5E-06	1,8E-05
Hypothetical									
VC0268	1534	1816	2463	739	1253	701	0,5	5,3E-04	3,0E-03
VC0519	6739 2	7459 1	56109	23575	31849	20968	0,4	1,0E-07	1,5E-06
VC0714	3388	1594	2591	663	2020	811	0,5	8,5E-03	2,9E-02
VC1052	1431	1646	2032	291	395	315	0,2	7,1E-20	4,3E-18
VC1058	762	936	1507	368	638	343	0,5	1,0E-03	5,2E-03
VC1074	639	609	1692	286	317	303	0,3	2,5E-06	2,8E-05
VC1317	4580	788	3907	213	1306	301	0,4	6,8E-03	2,4E-02
VC1574	1767	647	1134	366	505	432	0,4	7,6E-06	7,3E-05
VC1575	786	269	1065	134	207	119	0,3	1,0E-07	1,5E-06
VC1576	2727	1008	1653	330	786	345	0,3	2,5E-06	2,8E-05
VC1578	3800	1491	1767	278	1236	270	0,3	2,3E-04	1,5E-03
VC1832	1010	861	1883	390	605	473	0,4	4,6E-05	3,7E-04
VC1941	3896	3191	4799	1591	1861	1619	0,4	1,5E-08	2,5E-07
VC2093	364	468	980	286	316	262	0,5	1,2E-03	6,0E-03
VC2443	1378	1147	2022	611	768	809	0,5	4,3E-05	3,5E-04
VC2472	1677	1930	2799	671	813	648	0,4	9,3E-10	1,9E-08
VC2706	1479 1	1237 1	19055	2652	2846	3038	0,2	2,8E-28	2,8E-26
VCA0026	2034	2585	2667	1005	1465	984	0,5	7,5E-05	5,6E-04
VCA0921	4725	4659	8190	1557	2134	1754	0,3	8,6E-09	1,5E-07
Metabol									
VC0522	4113	2988	3708	1695	2094	1487	0,5	8,2E-07	1,0E-05
VC1942	1302	1679	3645	440	438	455	0,2	2,0E-11	5,3E-10
VC0869	1473 4	1274 6	18061	3007	1208	3242	0,2	2,7E-12	7,7E-11
VC1040	977	1202	1356	697	434	627	0,5	8,6E-06	8,3E-05
VCA0150	2635	1976	2642	1116	702	1170	0,4	1,4E-05	1,3E-04
VCA0614	2154	1520	2118	759	457	842	0,4	1,2E-07	1,7E-06
VCA0558	2076	607	4525	607	1460	718	0,5	1,0E-02	3,4E-02
VCA0496	1389	942	2071	426	1108	411	0,5	4,2E-03	1,6E-02
VC0992	1408	1336	1672	627	435	786	0,4	1,1E-07	1,6E-06
VCA0843	1854	2880	1583	839	200	997	0,4	4,7E-04	2,7E-03
VC2545	1017 5	1401 6	15506	3639	4482	3767	0,3	1,8E-10	4,4E-09
VC0745	1524 4	1570 8	17276	6155	7574	7144	0,4	3,3E-08	5,3E-07
VCA0863	2047	1494	2025	616	561	814	0,4	3,9E-11	1,0E-09
VCA0661	794	886	1062	483	357	513	0,5	2,9E-06	3,1E-05
VC0051	4018	2862	4106	1016	374	1138	0,3	5,6E-08	8,7E-07
VC0052	2282	2082	3109	621	263	597	0,2	8,0E-12	2,2E-10
VC2226	1185 8	1260 2	16080	2538	1208	2848	0,2	3,7E-15	1,5E-13

VC1625	6073	5424	5715	1808	1036	2011	0,3	1,3E-12	3,8E-11
VC1738	8039	9711	16080	6283	5005	4652	0,5	4,8E-05	3,8E-04
VC2256	2868	2130	4467	1588	1450	1314	0,5	2,5E-05	2,1E-04

Table S2: Strains and plasmids. Related to STAR Methods.

Strain	Strain number	Construction
<i>Vibrio cholerae</i>		
N16961 wt strain		
N16961 hapR+ wt strain	F606	Gift from Melanie Blokesch
Δ <i>raiA</i> (VC0706)	J251	PCR amplification of 500 bp up and down regions of VC0706 using primers ZIP381/384 and ZIP382/383. PCR amplification of <i>aadA7</i> conferring spectinomycin resistance on pAM34 using ZB47/48. PCR assembly of the VCA0706:: <i>aad7</i> fragment using ZIP383/384 and allelic exchange by natural transformation, as described previously (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
Δ <i>rmf</i> (VC1484)	L557	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 L045, replacing the gene with <i>frt::aph::frt</i> as described previously (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
Δ <i>hpf</i> (VC2530)	M566	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 M472, replacing the gene with <i>frt::aph::frt</i> as described previously (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
Δ <i>raiA</i> Δ <i>rmf</i>	L790	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 L045 in J251, replacing the gene with <i>frt::aph::frt</i> as described previously (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
Δ <i>crp</i>	9950	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 8348, replacing the gene with <i>aad7</i> (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
Δ <i>fur</i>	N541	allelic exchange by integration and excision of conjugative suicide plasmid pMP7 N450, replacing the gene with <i>frt::aph::frt</i> as described previously (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
Δ <i>rpoS</i>	A321	Baharoglu <i>et al.</i> , 2013
Δ <i>tnaA</i> (VC0161)	J253	PCR amplification of 500 bp up and down regions of VC0161 using primers ZIP93/94 and ZIP95/96. PCR amplification of <i>aadA7</i> conferring spectinomycin resistance on pAM34 using ZIP97/98. PCR assembly of the VCA0161::spec fragment using ZIP93/96 and allelic exchange by natural transformation, as described previously (Val <i>et al.</i> , 2012, Negro <i>et al.</i> , 2019)
<i>Escherichia coli</i>		
MG1655 wt strain		laboratory collection
Δ <i>raiA</i>	J237	P1 transduction from KEIO strain JW2578-1
<i>Pseudomonas aeruginosa</i> PAO1		laboratory collection
Plasmids		
pBAD43		spectinomycin resistant. Carries Para promoter repressed by glucose 1% and induced by arabinose 0.2%. (Guzman, 1995)
pBAD43-RaiA+	L907	<i>raiA</i> (VC0706) amplified using primers <i>raiA</i> eco/ <i>raiA</i> xba and cloned under Pbad using 5' EcoRI and 3' XbaI restriction sites
pBAD43-Hpf+	M834	<i>hpf</i> (VC2530) amplified using primers <i>hpf</i> eco/ <i>hpf</i> xba and cloned under Pbad promoter using 5' EcoRI and 3' XbaI restriction sites
pBAD43-Rmf+	M831	<i>rmf</i> (VC1484) amplified using primers <i>rmf</i> eco/ <i>rmf</i> xba and cloned under Pbad using 5' EcoRI and 3' XbaI restriction sites

pSC101-PraiA-gfp	L707	raiA promoter region (500 bp) was amplified using primers ZIP383/446 and gfp was amplified using primers zip443/444. 2 fragments were assembled by PCR assembly using primers ZIP383/ZIP443, so that <i>gfp</i> is fused to the <i>raiA</i> promoter at the ATG start codon of <i>raiA</i> . The assembled fragment was cloned into pTOPO and extracted using EcoRI and cloned into pSC101 low copy plasmid (carbenicillin resistant).
pSC101-Pc-gfp	N110	<i>gfp</i> was amplified using primers carrying the promoter zip513/200. The fragment was cloned into pTOPO and extracted using EcoRI and cloned into pSC101 low copy plasmid (carbenicillin resistant).
pMP7- Δ hpf::kan	M472	gibson assembly using primers MV450/451 for the amplification of pMP7 vector, primers VC2530hpf5bis/7 and VC2530hpf6bis/8bis for up and down regions of the gene, and primers MV268/269 on pKD4 plasmid for the resistance gene (frt::kan::frt).
pMP7- Δ rmf::kan	L045	gibson assembly using primers MV450/451 for the amplification of pMP7 vector, primers VC1484rmf5/7 and VC1484rmf6/8 for up and down regions of the gene, and primers MV268/269 on pKD4 plasmid for the resistance gene (frt::kan::frt).
pMP7- Δ crp::aad7	8348	Baharoglu <i>et al.</i> , 2012
pMP7- Δ fur::kan	N450	gibson assembly using primers MV450/451 for the amplification of pMP7 vector, primers VC2106fur5/7 and VC2106fur9/8 for up and down regions of the gene, and primers MV268/269 on pKD4 plasmid for the resistance gene (frt::kan::frt).
Primers		
raiAecoRI		ggaattcaccATGAAAATCAACATCACTGGTAA
raiAxba		gctctagaTTATTCCACTTCTTCGCTCAG
hpfeco		ggaattcaccATGCAAATCAACATTCAAGGCC
hpfxba		gctctagaTTAATGACTACTTAGCTTTTCTTT
rmfeco		ggaattcaccATGAAGAGACAAAAGCGTGAT
rmfxba		gctctagaTTATTTGCAGAGACCAGAAAGTTT
ZB47		CCCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGC
ZB48		GACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACG
ZIP381		GCGAGCATCGTTTGTTCGCCAGCTTCTGTATGGAACGGGTAAATA GAATGATGGGAGATAGCGC
ZIP382		CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCAATGC TTTTCTCTGTGTCATCCCTTATGG
ZIP383		AACCTATCCAGATACCGAAGCGGC
ZIP384		GTTTTGCAGATAGGATTGCTGAAGC
ZIP93		GTCGAATGCCAAACGAAAGCGGAAAATACC
ZIP94		GCGAGCATCGTTTGTTCGCCAGCTTCTGTATGGAACGGGTACGGT ATTGAAAAAGTGCAGCCTGC
ZIP95		CGTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCGCTGG GTATTCCTAAAAATATAAAGTCATGC
ZIP96		CACAACAACCTAGTATCTGGTTTACCCTCG
ZIP97		GGCAGCCTATGCAGGCTGCACTTTTTCAATACCGTCCCGTTCCATAC AGAAGCTGGGCGAACAAACGATGCTCGC
ZIP98		ATGACTTTATATTTAATTTTAGGAATACCCAGCGACATTATTTGCC GACTACCTTGGTGATCTCGCCTTTCACG
ZIP443		TTATTTGTATAGTTCATCCATGCCATGTGTAATCCCAGC
ZIP444		ATGCGTAAAGGAGAAGAAGCTTTTCACTGGAGTTGTCC
ZIP446		GGACAACCTCCAGTGAAAAGTTCTTCTCCTTTACGCATAATGCTTTTTTC CTCTGTGTCATCCCTTATGG
VC2530hpf5bis		CTATTATTTAAACTCTTTCGCCgatggctcaacgatg

VC2530hpf7	CTACACAATCGCTCAAGACGTGagactttccttctctagtttagg
VC2530hpf6bis	TACGTAGAATGTATCAGACTcttcacactgcaatagcacg
VC2530hpf8bis	CTAATTCCCATGTCAGCCGTGCGCCAGATCGCCGAAGCTA
VC1484rmf5	CTATTATTTAAACTCTTTCCggcttggttagatgatc
VC1484rmf7	CTACACAATCGCTCAAGACGTGagttctgtcctcatcacggta
VC1484rmf6	TACGTAGAATGTATCAGACTgttgatgatggatcgcattacc
VC1484rmf8	CTAATTCCCATGTCAGCCGTtcccatacaacaagcttga
VC2106fur5	CTATTATTTAAACTCTTTCCAAGCGGATGCGAACTTCGC
VC2106fur7	CTACACAATCGCTCAAGACGTGATACTTTCTGTTGATGTTCTGC
VC2106fur8	CTAATTCCCATGTCAGCCGTGCTCACAAGCCGAAGAAATAA
VC2106fur9	TACGTAGAATGTATCAGACTccacaaatcgatcagtttatgg
ZIP200	TATCAAGCTTATTTGTATAGTTCATCCATGCC
ZIP513	GAG CTG TTG ACA ATT AAT CAT CCG GCT CGT ATA ATG TGT GGA ATT GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC ACA TAT GCG TAA AGG AGA AGA AC