

1 **Macromolecular crowding links ribosomal protein gene dosage to growth rate in *Vibrio***  
2 ***cholerae*.**

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27 **Abstract:** Ribosomal protein (RP) genes locate near the replication origin (*oriC*) in fast-  
28 growing bacteria, which is thought to have been selected as a translation optimization strategy.  
29 Relocation of *S10-spc- $\alpha$*  locus (S10), which codes for most of the RP, to ectopic genomic  
30 positions shows that its relative distance to the *oriC* correlates to a reduction on its dosage, its  
31 expression, and bacterial growth rate. Deep-sequencing revealed that S10 relocation altered  
32 chromosomal replication dynamics and genome-wide transcription. Such changes increased as  
33 a function of *oriC*-S10 distance. Strikingly, in this work we observed that protein production  
34 capacity was independent of S10 position. Since RP constitute a large proportion of cell mass,  
35 lower S10 dosage could lead to changes in macromolecular crowding, impacting cell  
36 physiology. Accordingly, cytoplasm fluidity was higher in mutants where S10 is most distant  
37 from *oriC*. In hyperosmotic conditions, when crowding differences are minimized, the growth  
38 rate and replication dynamics were highly alleviated in these strains. Therefore, on top of its  
39 essential function in translation, RP genomic location contributes to sustain optimal  
40 macromolecular crowding. This is a novel mechanism coordinating DNA replication with  
41 bacterial growth.

## 42 **Introduction:**

43 Replication, gene expression and segregation are tightly coordinated with the cell cycle to  
44 preserve homeostasis (1, 2). Genome structure is a plausible factor contributing to integrate  
45 these many simultaneous processes occurring on the same template. The relative simplicity and  
46 the increasing amount of available data render bacterial genomes ideal models to study this  
47 subject (3-6).

48 Bacterial chromosomes are highly variable in their gene content, but highly conserved in terms  
49 of the order of core genes in the chromosomes. Replication begins at a sole replication origin  
50 (*oriC*), proceeding bidirectionally along two equally sized replichores until the terminal region  
51 (*ter*). This organizes the genome along an *ori-ter* axis that interplays with cell physiology (Fig.  
52 1a) (4, 5, 7). For instance, essential genes are overrepresented in the replicative leading strand  
53 to avoid head-on collisions between the replication and transcription machineries (8). Large  
54 inversions occur preferentially symmetrically with respect to the *ori-ter* axis to avoid the  
55 emergence of replichore size imbalance (9, 10). Recent studies indicate that gene order within  
56 the chromosome may play a relevant role in harmonizing the genome structure with cell  
57 physiology. Remarkably, key genes coding for nucleoid associated proteins, RNA polymerase  
58 modulators, topoisomerases and energy production are arranged along the *ori-ter* axis following  
59 the temporal order of their expression during growth phases (11, 12). In addition, recent studies  
60 have showcased an increasing number of traits whose expression is influenced by the genomic  
61 position of its encoding genes (13-15).

62 Notable cases are genes encoding the flux of the genetic information. In fast-growing bacteria,  
63 the genes coding for transcription and translation machineries locate near the *oriC* (16, 17).  
64 These microorganisms divide faster than the time required for genome duplication.  
65 Consequently, chromosomes trigger replication more than once before cytokinesis, overlapping  
66 successive DNA duplication rounds, a phenomenon called multi-fork replication (Fig. 1a). This

67 leads to replication-associated gene dosage gradients along the *ori-ter* axis during exponential  
68 growth (Fig. 1a) (14). Therefore, it was proposed that the *oriC*-proximal location of ribosomal  
69 and transcription genes allows the recruitment of multi-fork replication for growth optimization  
70 purposes (5, 16, 17). Thus, the dosage and expression of the aforementioned genes peak during  
71 exponential growth phase (Fig. 1a, right) when the transcriptional activity and ribosome  
72 numbers increase by 10 and 15-fold respectively (18).

73 In previous works (19, 20), we tackled this issue in *Vibrio cholerae*, the causative agent of  
74 cholera disease. This microorganism is also a model for multi-chromosomal bacteria, a trait  
75 found in ~10% of these microorganisms (21). *V. cholerae* harbors a main chromosome (Chr1)  
76 of 2.96 Mbp and a 1.07 Mbp secondary replicon (Chr2). Their replication is coordinated along  
77 the cell cycle: the *oriC* of Chr2 (*ori2*) fires only after 2/3 of Chr1 duplication has elapsed,  
78 finishing the process synchronously (22, 23). *V. cholerae* is among the fastest-growing bacteria  
79 and therefore it displays particularly high replication-associated gene dosage effects (16). Its  
80 transcription and translation genes map close to the *oriC* of Chr1 (*ori1*) (19). Among them, *S10*-  
81 *spc-a* (*S10*) is a 13.4 Kbp locus harboring half of the ribosomal protein genes (RP) located 0.19  
82 Mbp away from *ori1* (19). Using recombineering techniques, we built a set of *S10* *movants* (i.e.  
83 isogenic strains where the genomic position of *S10* locus is modified) to uncover interplays  
84 between the chromosomal position of the locus and cell physiology. We found that its  
85 maximum growth rate ( $\mu$ ) decreased as a function of the distance between *S10* and *ori1* (Fig.  
86 1b and 1c). Also, *S10* genomic location impacted on *V. cholerae* fitness and infectivity (19, 20).  
87 In line with prior bioinformatics studies (16, 17), we showed that *oriC*-proximity of *S10*  
88 provides optimal dosage and expression to attain the maximal growth capacity (19). We also  
89 found that *S10* position impacts bacterial fitness in absence of multi-fork replication (20). This  
90 suggests that the RP gene location affects cell physiology even in slow-growing bacteria (20).

91 In sum, our previous work and the cited examples (14) support the notion that gene order  
92 conditions cell physiology, shaping genome structure along the evolution.

93 However, although we proved that the current S10 genomic location maximizes *V. cholerae*  
94 fitness (19, 20), we still lack a mechanism explaining this phenomenon. Here, we addressed  
95 this issue through the most straightforward hypothesis that is S10 relocation far away from *oriI*  
96 diminishes ribosome component availability. This in turn, should reduce ribosomal activity,  
97 impacting cell physiology globally through the general impairment of protein synthesis. In this  
98 work, we quantified the global protein production in the parental strain and in the most affected  
99 derivatives (Fig. 1b and 1c). RNA and DNA deep-sequencing revealed genome-wide alterations  
100 in gene transcription and replication dynamics. Surprisingly, we found no differences in global  
101 protein production at the population level. This suggests the existence of global mechanisms  
102 linking S10 dosage to cell physiology not linked to protein biosynthesis capacity.

103 The intracellular milieu has a very high concentration of macromolecules that reaches 400  
104 mg/mL in *Escherichia coli*. Consequently, the cytoplasm does not behave as an ideal solution  
105 since this large quantity of macromolecules occupies 20-30% of its volume, which is physically  
106 unavailable to other molecules. Such steric exclusion creates considerable energetic  
107 consequences, deeply impacting intracellular biochemical reactions. This phenomenon,  
108 referred to as macromolecular crowding (24, 25), has received little attention in *in vivo* systems  
109 (26, 27). Protein accounts for ~55% of the bacterial cell mass (18, 24) , with RP representing  
110 one third of them (28). We hypothesized that S10 expression reduction, would lead to lower  
111 macromolecular crowding within the bacterial cytoplasm, globally affecting cell physiology  
112 (24, 26, 27). Here, we gathered evidence supporting the idea that S10 relocation mainly impacts  
113 cellular physiology of *V. cholerae* by altering cytoplasm homeocrowding (i. e. macromolecular  
114 crowding homeostasis) (24).

115 **Results:**

116 **S10 relocation does not cause ribosomal activity reduction at the population level.** We  
117 recently settled that S10 relocation impacts cell physiology in a dosage-dependent manner (19,  
118 20). However, how S10 dosage reduction affects cell physiology was still unknown. The most  
119 plausible explanation is that a reduction of RP levels upon S10 locus relocation affects ribosome  
120 biogenesis leading to a reduction in protein synthesis. To inquire if S10 relocation impairs  
121 protein production, we created strains expressing GFP by inserting *gfpmut3\** (29) under a  
122 strong constitutive promoter into an innocuous intergenic space (Table S1). The direct  
123 quantification of fluorescence, allows for estimation of protein production capacity in each  
124 strain (30). First, we followed in time the optical density (OD) and the fluorescence signal of  
125 these derivatives. We estimated translation capacity by plotting fluorescence as a function of  
126 OD (Fig. 2a). Fluorescence increased exponentially as the OD incremented ( $R^2 > 0.99$ , Table  
127 S2). Although the curves differed slightly between strains, there was no significant correlation  
128 between S10 genomic position and GFP production (Pearson's Test,  $r=0.1$ ,  $p=0.86$ ). We next  
129 subjected cultures of these strains to flow cytometry during the early exponential phase, when  
130 S10 dosage differences among the movants are maximal. This method allows to simultaneously  
131 observe the average GFP production per cell with higher sensitivity and the distribution of  
132 fluorescence among the cells in the populations (Fig. 2b). All tested strains showed similar  
133 signal levels and the same distribution pattern. In sum, we found no link between GFP  
134 production and S10 genomic location.

135 To confirm that these results were not due to lack of sensitivity, we used the *Renilla* Luciferase  
136 (RL) as a reporter of protein synthesis capacity. RL detection shows higher sensitivity than GFP  
137 due to lower background, higher signal amplification and a larger dynamic range, making it  
138 suitable to reveal more subtle differences otherwise impossible to differentiate (31). We built  
139 S10 movant strains constitutively expressing RL at high levels (Table S1). Again, no

140 differences in luciferase activity arose between the parental strain, S10Tnp-35, S10Tnp-1120  
141 and S10TnpC2+479 (Fig. 2c), suggesting similar translation capacity at the population level.

142 As an alternative approach to look for differences on ribosomal activity, we measured the  
143 minimum inhibitory concentration (MIC) of ribosome-targeting antibiotics such as  
144 chloramphenicol (Cm), gentamicin (Gm) and erythromycin (Er). A reduction in the number of  
145 ribosomes increases sensitivity to these antibiotics (32). We measured MIC for Cm, Gm and Er  
146 using E-tests (Fig. 1d). All generated mutants derive from a *V. cholerae* strain sensitive to Er  
147 and harboring Gm resistance gene (Table S1). Strains that only differed in the genomic location  
148 of S10, had their growth inhibited at the same Er and Gm concentrations (Fig. 2d) suggesting  
149 no differences in ribosomal numbers. In parallel, the parental, S10Tnp-1120 and the S10Md(-  
150 1120;C2+479) strains harbor the Cm resistance gene (*cat*) linked to the S10 locus, therefore the  
151 location of the resistance gene differed among them (Fig. 2d). Cm resistance was higher in the  
152 Parental strain when *cat* is closer to the *oriI* and lower in S10Tnp-1120 and S10Md(-  
153 1120;C2+479) when the resistance marker is nearby the *terI* region. Hence, as in other genetic  
154 systems (33), Cm sensitivity varied according to *cat* genomic location independently of S10  
155 copy number (compare S10Tnp-1120 to S10Md(-1120;C2+479)). Therefore, even though this  
156 assay is sensitive enough to capture the effects caused by differences in *cat* location, it showed  
157 no antibiotic susceptibility differences related to S10 dosage. The lack of effects of S10  
158 relocation on MIC when using any of the three different ribosome-targeting antibiotics,  
159 possessing different tolerance levels, suggests that the number of ribosomes is not affected by  
160 the genomic location of S10.

### 161 **S10 genomic location causes changes in GFP synthesis capacity at the single cell level:**

162 Since we did not detect differences in ribosomal activity at the population level, we measured  
163 GFP production at the single cell level using Fluorescence Recovery After Photobleaching  
164 (FRAP). In this assay individual cells expressing *gfpmut3\** were photo-bleached and followed

165 over time for at least 5 minutes. Then, we quantified the percentage of fluorescence recovery.  
166 In the parental strain, ~95% of the cells displayed a recovery of at least 20% (mean=53.8%,  
167 n=108) of the initial signal after 3 minutes, to reach a plateau until the end of the observation  
168 (Fig. S1a). The addition of Cm up to the MIC inhibited the fluorescence increase (mean=15.8%,  
169 n=21), suggesting that signal recovery corresponds to GFP re-synthesis. Meanwhile, we  
170 observed lower average recovery in the most physiologically affected movants S10Tnp-1120  
171 (20.1%, n=42) and S10TnpC2+479 (25.8%, n=82), Fig. S1b) suggesting that they produced less  
172 GFP. Therefore, at the single cell level, the parental strain displayed a higher protein synthesis  
173 capacity than the most affected S10 movants.

174 **S10 relocation alters the ribosomal sedimentation profile.** Reduction in RP expression can  
175 lead to problems in ribosome assembly due to modifications in the stoichiometry of its  
176 components. To detect alterations in ribosome assembly, reflected in changes in ribosomal  
177 subunits composition, we performed ribosome preparations followed by analytical  
178 ultracentrifugation (AUC) in the parental and the physiologically impaired S10TnpC2+479  
179 strain. We also analyzed a merodiploid strain where most of the growth deficiency is rescued  
180 but still display a reduced  $\mu$  (S10Md(-1120;C2+479)) (19). We expected that growth  
181 impairment would correlate with a reduction in the proportion of assembled ribosomes (i. e. the  
182 70s peak), when compared to free ribosomal subunits (30s and 50s peaks). Figure 2e shows that  
183 parental strain displayed a 53,97% of the signal in the peak corresponding to the 70s while 50s  
184 and 30s peaks represented 19.4 and 20.8% respectively. In the S10TnpC2+479 movant, we  
185 observed an increase in the 70s proportion to the 75.85% of the signal while the free ribosomal  
186 subunits lowered to 5.5% and 14.8% of the signal for 50 and 30s subunits respectively. In the  
187 S10Md(-1120;C2+479) strain, showing an intermediate growth phenotype, 70s, 50s and 30s  
188 represented 71%, 8.3% and 15.8% of the signal respectively. Our data shows that a reduction  
189 in S10 expression led to an increase of the proportion of assembled ribosomes and a reduction



190 of free ribosomal subunits. Therefore, movant strains might compensate lower S10 expression  
191 engaging more free subunits into translation. This could explain the relatively low impact of  
192 S10 relocation on translation capacity.

193 **Dosage reduction of S10 non-ribosomal genes does not impact cell physiology:** Since  
194 reduction of protein biosynthesis upon S10 relocation was mild, we reasoned that it cannot  
195 explain the drastic changes observed in fitness and growth rate ( $\mu$ ). Meanwhile, S10 harbors  
196 genes not related to ribosome biogenesis: *rpoA*, the gene encoding for the  $\alpha$ -subunit of RNA  
197 polymerase and *secY*, which encodes a sub unit of the Sec translocon (34), essential for protein  
198 export. We wondered whether dosage reduction of *rpoA* and/or *secY* could contribute to the  
199 phenotype caused by S10 relocation by provoking a reduction of the transcription rate and/or  
200 by hampering the normal protein export process. To test this, we cloned *rpoA* and *secY* on a  
201 low copy-number plasmid with inducible expression. The parental strain (Table S1, Parental)  
202 and the two most affected movants, S10Tnp-1120 and S10TnpC2+479 were transformed with  
203 either of these plasmids or the empty vector. Next, the  $\mu$  of the transformed strains was  
204 determined through automated growth curves. If lower RNAP and/or translocon activity were  
205 involved in the observed phenotypes, growth rate differences between the parental and movant  
206 strains should lessen or disappear upon *rpoA* and *secY* overexpression. Results on Figure S2  
207 show that the growth rate was significantly lower in the movants compared to the parental strain  
208 independently of the genes expressed on the plasmid vector. Since the plasmids expressing  
209 *rpoA* or *secY* did not rescue the growth defect, the impact of S10 relocation on cell physiology  
210 results from dosage reduction of RP genes within the locus.

211 **Transcriptome analysis of the movant strain set:** Since the physiological effects of S10  
212 relocation are due to dosage reduction of RP genes and the effects on translation were only  
213 observed at single cell level, we reasoned that alternative mechanisms must explain the effects  
214 observed at the population level. To detect genes whose transcription was affected by S10

215 relocation and search for metabolic pathways responding to RP dosage alterations we  
216 characterized the full transcriptome of: S10Tnp-35, the movant in which S10 was slightly  
217 moved presenting no phenotype; and the physiologically impaired strains S10Tnp-510,  
218 S10Tnp-1120 and S10TnpC2+479 (Fig. 1b). We collected the samples in fast growing  
219 conditions during exponential phase ensuring maximal S10 dosage differences, and then we  
220 compared each movant's transcriptome to the one of the parental strain.

221 We first looked at the read coverage along the chromosomes, a parameter accounting for the  
222 genome-wide transcriptional activity. In fast growing conditions, we observed that the  
223 transcription of the *ori1*-region decreased as a function of the distance between S10 and *ori1*  
224 (Fig. 3a). To quantify this effect, we calculated the read coverage of the 400 Kbp flanking *ori1*  
225 (35). While S10Tnp-35 displays no significant transcriptional alteration within this genomic  
226 region, a significant reduction was observed in S10Tnp-510 (-1.042 fold change,  $p < 10^{-13}$ ),  
227 S10Tnp-1120 (-1.056,  $p < 10^{-25}$ ) and S10TnpC2+479 (-1.044,  $p < 10^{-8}$ ) (Figs. 3a and S3). This  
228 was not the case for Chr2 where the *ori2* region displayed no transcriptional activity differences  
229 across the strains. The sole exception was a small increase in the transcriptional activity of the  
230 superintegron (36) in S10Tnp-1120 movant (Fig. S4). Therefore, a global, yet relatively small,  
231 reduction of transcriptional activity of the *ori1* region is observed upon relocation of S10 far  
232 from *ori1*.

233 **Replication dynamics are altered in the most affected movants.** Given that a specific  
234 mechanism regulating the expression of such a wide genomic region seems unlikely, we  
235 wondered if the change in the expression of *ori1* region was linked to changes in global  
236 replication pattern. To assess this, we studied the replication dynamics of the genome of the  
237 whole strain set using Marker Frequency Analysis (MFA). For this, we aligned genomic DNA  
238 reads from exponentially growing cells of each strain to the *V. cholerae* genome. For each  
239 replicon, there is a linear relationship between the  $\text{Log}_2$  number of reads covering the locus and

240 its genomic position between the *oriC* and the *ter* (37) (Figure 3b). This allows for robust  
241 quantification of replication dynamics across the bacterial genome with unprecedented  
242 resolution of replication fork speed and the *ori* and *ter* region locations (23, 37-39). To better  
243 quantify these differences, we calculated the average slope ( $\text{Log}_2(\text{frequency})/\text{Kbp}$ ) along both  
244 replichores, which estimates the replication speed for each strain (Fig. 3c). MFA analysis  
245 revealed significant differences in replication dynamics across the strain set. The parental strain,  
246 the S10Tnp+166 and the S10Tnp-35 displayed a similar slope (Table S3). Conversely, the most  
247 affected movants, S10Tnp-1120 and S10TnpC2+479, where S10 was relocated at the termini  
248 of Chr1 and Chr2, showed a significantly lower slope ( $p < 0.01$ , Fig. 3b, 3c and Table S3).  
249 S10Tnp-510 and S10TnpC2+37 displayed an intermediate value not significantly different  
250 from either group. Coincidentally, the calculated slope closely correlated to the S10 locus  
251 genomic position ( $r = -0.78$ ,  $p < 0.05$ ), its dosage ( $r = 0.8$ ,  $p < 0.05$ ), the *ori1/ter1* ratio ( $r = 0.91$ ,  
252  $p < 0.005$ ) and  $\mu$  ( $r = 0.9$ ,  $p < 0.01$ ) (Fig. S5). This suggests that the genomic location of S10  
253 impacts DNA replication activity, slowing down replication when S10 is far from *ori1*. These  
254 data (Fig. 3b, 3c and Table S3) indicates that DNA coverage decreases at the *ori1* region with  
255 increasing *ori1*-S10 distance. This trend matched the changes in transcriptional coverage  
256 observed in RNA-seq data.

257 **Differentially expressed genes upon S10 relocation:** We next analyzed the transcriptomic  
258 data to find which genes and pathways were differentially transcribed with respect to the  
259 parental strain in S10Tnp-35 and in the affected movants S10Tnp-510, S10Tnp-1120 and  
260 S10TnpC2+479 (Fig. 1b and 1c).

261 First, using volcano plots, we analyzed the statistical significance of the changes in transcription  
262 of each gene ( $-\text{Log}_{10}(p\text{-value})$ ) as a function of its transcriptional  $\text{Log}_2$  of fold change ( $\text{Log}_2$   
263 (FC)) compared to the parental strain. We observed more transcriptionally altered genes with  
264 higher distances between the S10 locus and *ori1* (Fig. 4a). S10Tnp-35, a strain presenting no

265 phenotype used as a control of the neutrality of the relocation process, displayed only 8 genes  
266 with significant ( $p < 0.05$ ) transcriptional change (Table 1, Data Set 1). S10Tnp-510, displaying  
267 a slight  $\mu$  reduction (Fig. 1c), showed 111 genes with significantly altered transcription (Table  
268 1, Fig. 4a, Data Set 1). Finally, the most affected movants, S10Tnp-1120 and S10TnpC2+479,  
269 displayed a significant transcriptional change in 664 and 742 genes, representing 17.95% and  
270 20.06% of their gene repertoire, respectively. Most of altered genes in the movants were up  
271 regulated (Fig. 4b and Table1). These transcriptional perturbations were relatively small in  
272 magnitude since only a 26%, a 10.8% and a 14.15% of altered genes presented alterations  
273 greater than 2-fold in S10Tnp-510, S10Tnp-1120 and S10TnpC2+479 respectively.  
274 Meanwhile, up-regulated genes showed 2.8-fold, 1.6-fold and 1.7-fold average increases  
275 respectively (Table 1, Fig. 4b, Data Set 1). In the three movants, the down-regulated genes  
276 displayed a smaller perturbation of  $\sim 1.4$ -fold (Table 1).

277 Most of the transcriptional alterations were found in the same genes across the S10 movants  
278 (Figure 4c). A large fraction of transcriptionally altered genes in a movant were also regulated  
279 in either of the other two movants (Table S4). Shared genes showed similar levels of  
280 transcriptional change across the movants (Fig 4d and Table S4). For example, the degree of  
281 change in altered genes of S10Tnp-510 and S10Tnp-1120 were highly correlated ( $r = 0.927$ ,  
282  $p < 10^{-24}$ ). The differentially expressed genes were not confined to specific chromosome regions  
283 nor associated to a specific replicon: S10 relocation produced homogeneously distributed  
284 changes in *V. cholerae* gene transcription (Fig. S6).

285 To identify the functions or metabolic pathways altered by S10 relocation, we classified *V.*  
286 *cholerae* genes in 25 functional categories using the [EMBL](#) eggNOG database v.4.0 (40)(Supp.  
287 Text). We then identified the categories with over or under-representation of genes with altered  
288 transcription levels in S10Tnp-510, S10Tnp-1120 and S10TnpC2+479 with respect the full  
289 repertoire of *V. cholerae* genome (Data Set 1, Table S5, Fig. S7)

290 Genes from the category ‘Translation, ribosomal structure and biogenesis’ (J) were not  
291 significantly altered, which is consistent with the results above showing that S10 relocation did  
292 not alter the translation capacity (Fig. 2). The category ‘Amino acid transport and metabolism’  
293 (E) was statistically altered in all three movants. The category “Posttranslational modification,  
294 protein turnover, chaperones” (O) was the most affected category in S10Tnp-1120 and  
295 S10TnpC2+479, since about 65% of its genes showed higher transcription in the movants  
296 (Table S5, Data Set 1). The list of up-regulated genes was dominated by chaperones and heat-  
297 shock proteins. Strikingly, the highest transcriptional changes occurred in the main pathway for  
298 cytosolic protein folding (41): *grpE* (VC0854), *dnaKJ*(VC0855-6) and both copies of the  
299 *groEL-groES* system (VC2664-5 and VCA0819-20). Many transcriptionally altered genes were  
300 involved in protein export and ion transport, belonging to several significantly perturbed  
301 categories such as: “V, Defense mechanisms” (e.g. VC0590 coding for an ABC-2 type  
302 transporter), “U, Intracellular trafficking, secretion, and vesicular transport” (*secA*, VC2462),  
303 and “N, cell motility” (some *fli* and *fla* genes) (Table 1 and Data Set 1). Some particularly  
304 induced genes of “P, Inorganic ion transport and metabolism” group were iron (*hutX*, *hmuV*,  
305 *hmuU*, *exbD1*, *tonB*; ~2.8 FC) and sulfur (*sbp*, *cysHI*, *cysDNC*; ~8 FC ) transporters. Based on  
306 the analysis of functional categories, we observed that *V. cholerae* responds to S10 relocation  
307 by altering amino acid synthesis pathways, increasing the transcription of chaperones and  
308 proteases probably to degrade misfolded proteins and by activating the expression of  
309 transporters and permeases.

310 **Cytoplasm is more fluid in the most affected movants.** During exponential growth,  
311 ribosomes account for up to 30% of bacterial dry weight (42). S10 encodes half of the ribosomal  
312 proteins, which are very highly expressed constituting more than a third of total cell proteins in  
313 *E. coli* (28). Therefore, it is likely that a reduction in S10 expression results in macromolecular  
314 crowding alterations as observed in other systems (43, 44). Macromolecular crowding is

315 crucially important in biochemical reactions, however how it impacts cellular physiology  
316 remains mostly unexplored (24-26). It is well documented that it influences protein folding,  
317 aggregation and perturbs protein-nucleic acids interactions (45). On the other hand, DNA  
318 replication has an absolute dependence on macromolecular crowding (44, 46). Therefore, the  
319 reduction in replication fork dynamics (Figs. 3b and c), the alteration of genes linked to protein  
320 folding, protein degradation, permeases and transport systems (Data Set 1, Table 1) observed  
321 upon S10 relocation can be interpreted in light of changes in macromolecular crowding caused  
322 by a lower RP concentration.

323 To test this hypothesis, we measured the viscosity of the cytoplasm in the parental strain and in  
324 the most affected movants, S10Tnp-1120 and S10TnpC2+479. We expected a more viscous  
325 cytoplasm in the parental strain since it expresses S10 genes at higher levels generating a greater  
326 concentration of RPs than the movant strains. Differences in cytoplasm viscosity can be  
327 uncovered by FRAP experiments on GFP expressing strains. For this, the fluorescence recovery  
328 time is measured after bleaching a part of the bacterial cytoplasm (47, 48). Since the small size  
329 and the comma-shape of *V. cholerae* complicates the procedure, we generated elongated cells  
330 by deleting the Chr2 replication-triggering site (*crtS*) (23) in cells expressing GFP. These  
331 mutants present a defective replication of the secondary chromosome. Therefore,  
332 S10TnpC2+479 should have even less copies of S10 per cell and, concomitantly, display higher  
333 cytoplasmic fluidity than S10Tnp-1120.

334 In the *gfpmut3\**  $\Delta$ *crtS* context (Table S1), the parental strain displayed a significantly longer  
335 half-time recovery of fluorescence ( $\tau$ ) than the movants (Fig. 5a, Supp. Text). The collected  
336 data showed a high dispersion due to biological variability, however,  $\tau$  distribution was  
337 different in the movants when compared to the parental strain (Fig. 5b) which displayed a  $\tau$  of  
338 139.7 ms (95% confidence interval (CI) (120.4-158.9)ms; median=110 ms; n=104 ). As  
339 expected, S10Tnp-1120 showed a  $\tau$  of 97.3 ms (95% CI (88.31-106.3)ms; median=90 ms;

340 n=128) significantly shorter than the parental strain ( $p < 0.0001$ ). S10TnpC2+479 displayed a  $\tau$   
341 of 107.5 ms (95% CI (97.39-117.52) ms; median= 100 ms; n= 92), statistically lower than the  
342 parental strain ( $p < 0.05$ ) but not significantly different from S10Tnp-1120. The more fluid  
343 cytoplasm in movants could be a consequence of fewer S10-encoded RP suggesting that S10  
344 relocation far from *oriI* reduces cytoplasm macromolecular crowding.

345 **Growth rate and replication dynamics alterations in movants are alleviated in**  
346 **hyperosmotic conditions.** In line with lower macromolecular crowding, we observed a  
347 reduction in cytoplasm viscosity in the movants. To test the possible impact of such molecular  
348 crowding alterations on the physiology of the movants, we employed an osmotic stress  
349 approach (49-51). This consists of subjecting strains to a hyperosmotic environment. In these  
350 culture conditions, water exits the cell reducing the macromolecular crowding differences  
351 between the strains. Therefore,  $\mu$  differences between the parental strain and the movants should  
352 be reduced with increasing solute concentration. To test this, we performed automated growth  
353 curves in rich media with increasing NaCl concentrations, comparing the  $\mu$  of the parental strain  
354 to S10Tnp-1120 and S10TnpC2+479 movants. As depicted in Figure 6a, growth rate  
355 differences between the parental strain and the movants were reduced as NaCl concentration  
356 increased. Since this phenomenon could be explained by the nature of the solute of choice (e.g.  
357 putative differential sensitivity to NaCl), we repeated these assays using sucrose as an  
358 alternative compound. As shown in Figure 6b, results were very similar, suggesting that this  
359 phenomenon depends on osmotic changes and cannot be attributed to the nature of the solute.

360 Notably, the  $\mu$  of the parental strain was not significantly reduced in the range of 5 to 20 gr/L  
361 NaCl (Fig. S8). Meanwhile, the growth of movant strains varied significantly along this  
362 concentration range, displaying a reduced growth at 5 gr/L and 10 gr/L and reaching its  
363 maximum at 20gr/L (Fig. S8). Consequently, growth differences observed are not due to  
364 impairment of the parental strain in hyperosmotic conditions. We conclude that  $\mu$  differences

365 caused by S10 relocation far from *oriI* can be counterbalanced by artificially increasing  
366 cytoplasmic crowding.

367 Upon S10 relocation far from *oriI*, we observed a lower replication speed in the movants  
368 suggesting that DNA replication activity diminished, suggesting a lower replication speed in  
369 the movants (Fig. 3c). Since, molecular crowding is crucial for chromosome replication (44,  
370 46) we used the osmotic stress approach to test if the observed replication dynamics defects in  
371 movants could be compensated. For this, we performed MFA analyses of the parental strain  
372 and the S10Tnp-1120 and S10TnpC2+479 movants in the presence of 5 or 20 gr/L of NaCl. In  
373 these culture conditions, the parental  $\mu$  is unaffected. In contrast, movant strains grew 10-15%  
374 slower than the parental strain but they were able to rescue the growth defect at higher NaCl  
375 concentrations (Figs 6a and S8). As in earlier experiments, MFA analyses revealed that the  
376 movants have a significantly lower slope than the parental strain. Increasing NaCl concentration  
377 to 20 gr/L made their slopes converge diminishing replication dynamics differences (Fig. 6c  
378 and 6d). The integration of these and the previous observations, suggests that lower expression  
379 of RP caused by S10 relocation (Fig. 1b) leads to lower molecular crowding (Fig. 5), which  
380 negatively impacts replication (Fig. 3b). This fits the observation that addition of external NaCl,  
381 causing water loss and thus narrowing differences in macromolecular crowding, produces more  
382 similar replication dynamics between the parental and the movant strains (Fig. 6d).

### 383 **Discussion:**

384 Comparative genomics suggests that gene order coordinates cell cycle to the expression of key  
385 functions necessary for cellular homeostasis (4, 11, 16, 17) but few papers provided  
386 experimental support (13, 14, 52). A notable case is that of ribosomal genes which are located  
387 near the *oriC* in fast growing bacteria (16, 17). By systematically relocating S10, the main  
388 cluster of RP genes (Fig. 1c), we proved that its genomic location determines its dosage and  
389 expression in *V. cholerae* (Fig. 1b). S10 repositioning far from *oriI* leads to larger generation



390 times, lower fitness and less infectivity (19, 20). These effects are dependent on S10 dosage.  
391 However, the mechanism explaining how RP dosage affects cell physiology was still missing.  
392 The most straightforward explanation was that high RP dosage due to multi-fork replication  
393 increases their expression maximizing protein biosynthesis capacity (16, 17). Our initial  
394 hypothesis was that movants in which S10 was far from *oriI* would have a lower translation  
395 capacity, easily explaining lower growth and fitness of these movants. Surprisingly, we found  
396 that in the most affected movants, translation capacity reduction could not explain the observed  
397 physiological changes (Fig. 2). We do not rule out that translation impairment may have an  
398 effect in the cellular physiology, however, it must have a secondary role in the phenotypes  
399 displayed in the affected movants. Slight differences in protein production between the parental  
400 strain and the most affected movants could only be detected at single cell level (Fig. S1). The  
401 movants displayed a larger proportion of assembled ribosomal subunits. This might compensate  
402 putative deficiencies in the translation apparatus (Fig. 2e). Interestingly, the S10TnpC2+479  
403 displayed a small peak of ~21s that might correspond to precursors of 30s subunit typically  
404 associated to cells displaying ribosome assembly deficiencies (53). Meanwhile,  
405 complementation of movants with *secY* and *rpoA*, two S10 genes not related to ribosome  
406 biogenesis, failed to rescue the growth defect demonstrating the relevance of RP in the observed  
407 phenotype. In sum, although dosage reduction of S10-encoded RP genes caused the observed  
408 phenotypes, it is unlikely that this is a consequence of translation defects.  
409 Deep sequencing techniques revealed less transcriptional activity in the region flanking *oriI*  
410 (Fig. 3a) and lower replication velocity in the most affected movants (Figs. 3b, 3c and 6c). Since  
411 highly expressed genes that account for a large majority of transcriptional activity in the cell  
412 (i.e. *rrn*, ribosomal protein genes, etc.) cluster at this chromosomal region, slight changes in its  
413 dosage may globally impact cell physiology (4, 11) and may be responsible for the slight  
414 reduction in translational activity observed at single cell level (Fig. S1). Meanwhile, differential

415 expression analysis revealed that the transcriptional response is not limited to the *oriI* region  
416 (Fig. S6), and encompasses a large number of genes that show slightly but consistently altered  
417 transcription in the most affected movants (Fig 4). Furthermore, the number of these genes  
418 increases with distance between S10 and *oriI* (Table 1, Fig. 4a, 4b and S6). The latter  
419 observation corresponds to biologically meaningful transcriptional changes since furthest  
420 relocations caused larger perturbations (Figs. 4a and 4b), the majority of altered genes were  
421 common to the different movants (Fig 4c), where they showed similar transcriptional changes  
422 (Fig. 4d). This strongly suggests the presence of a common mechanism that slightly affects gene  
423 expression at a large scale. Amino acid metabolism and transport genes were less transcribed  
424 while there was an up-regulation of genes helping protein folding and cellular transporters  
425 (Table S5, Data set 1). Importantly, and in line with previous data (Fig. 2), the transcription of  
426 translation genes seems to be unaffected in the movants reinforcing the notion that lower protein  
427 biosynthesis capacity was not enough to explain the physiological alterations that we observed.  
428 Molecular crowding has a well-known key role in biochemical reactions. Even if its impact on  
429 physiological processes has been poorly studied (26), two processes - DNA replication and  
430 protein folding - are strongly influenced by macromolecular crowding (27). Since the discovery  
431 of DNA replication, the presence of crowding agents such as polyethylene glycol was shown  
432 to be absolutely necessary to reproduce DNA polymerase activity *in vitro* (44, 46). In parallel,  
433 macromolecular crowding greatly impacts protein aggregation and folding (27), although the  
434 *in vivo* consequences of how the latter occurs are still a matter of debate (45, 54). It was recently  
435 shown that ribosomes are important contributors of macromolecular crowding in the cytoplasm  
436 both in prokaryotic and eukaryotic systems (43, 44). All this information leads us to suggest  
437 that upon S10 relocation, the consequent fewer RP may lead to homeocrowding (24)  
438 perturbations. Interestingly, to the best of our knowledge this is first study exploring the  
439 consequences of lower macromolecular crowding conditions since most works linking this

440 physicochemical factor to physiology focus on situations of increased crowding (44, 55, 56).  
441 Concomitantly, we observed reduced replication activity (Fig. 3c), as well as induction of  
442 proteases and chaperones to cope with protein aggregation and misfolding (Table 1 and Fig.  
443 S6). Notably, in the most affected movants, the genes coding for the three main chaperone  
444 systems –*grpE*, *dnaKJ* and *groEL-groES* (41)-were among the most strongly induced. The  
445 lower transcription of protein and ion transporters could be used for intracellular environment  
446 restoration (Table S4, Fig. S6) and could be a natural consequence of the change in cytoplasm  
447 osmotic pressure. We next tested experimentally if S10 relocation could alter homeocrowding.  
448 First, using FRAP, we observed slight but statistically significant alterations in the fluidity of  
449 the cytoplasm of the most affected movants compared to the parental strain (Figs. 5a and 5b,  
450 Supplementary Text). This supports the notion that lower expression of RP associated with  
451 movants lowers cytoplasm macromolecular crowding. In the  $\Delta crtS$  context, we did not detect  
452 differences in cytoplasmic fluidity between the S10Tnp-1120 and S10TnpC2+479 movants,  
453 expected from lower S10 copy number in the latter by Chr2 loss. We believe that the detrimental  
454 effects of *crtS* deletion (23) can explain this. In the S10TnpC2+479 movant, S10 dosage  
455 reduction enhances fitness loss, as reflected by slower growth and the presence of small non-  
456 viable cells in the microscope not further analyzed (data not shown). When Chr2 replication is  
457 inhibited, the fusion of both chromosomes -mainly between their terminal regions- occurs at  
458 relatively high frequency (57). Therefore, the S10TnpC2+479  $\Delta crtS$  population might in part  
459 consist of cells with fused chromosomes. In this scenario S10 dosage would not decrease below  
460 1 copy per cell.

461 The osmotic shock approach provided strong evidence supporting the notion that S10 dosage  
462 deficit perturbs cellular homeocrowding. In rich medium, movant strains grow slower than the  
463 parental strain. With increasing solute concentrations this growth deficit is reduced (Figs. 6a  
464 and 6b). In the case of NaCl, the parental strain grew normally in the range from 5 to 20 gr/L.

465 Outside of this range, growth rate was reduced. Growth was particularly impaired at  
466 concentrations below 5 gr/L where culture development was very variable due to hyposmotic  
467 stress (Fig. 5b and Data not shown). Interestingly, movants looked more sensitive than the  
468 parental strain to lower solute concentrations. We think that movants express less ribosomal  
469 proteins which account for a large fraction of the bacterial proteome, which in turn constitutes  
470 a large proportion of the cytoplasmic macromolecules (58). It is known that about 0.5 gr of  
471 water is bound per gram of cytoplasmic macromolecules (49, 59). Therefore movants may lose  
472 their capacity to retain water, suffering from a situation similar to being exposed to hyposmotic  
473 conditions. Meanwhile, the  $\mu$  of the parental and the movants was similar when exposed to 30  
474 gr/L of NaCl. This indicates that detrimental hyperosmotic conditions altered the strains  
475 similarly.

476 Recent work shows that specific ribosomal protein genes link cell growth to replication in  
477 *Bacillus subtilis* (60). We observed similar effects since S10 dosage correlated growth rate and  
478 *oriC*-firing frequency (Fig. 3b, 3c, S6 and Table S3). In the cited study, the authors attribute  
479 this effect to ribosomal function. Although in our system the effects were milder, we do not  
480 rule out the possibility that S10 relocation alters cellular physiology through a reduction in  
481 protein synthesis. But this effect is unlikely to account for the full magnitude of the observed  
482 phenotypes (Fig. 2) especially as it is relieved in hyperosmotic conditions. We believe that this  
483 could be due to a number of factors including: i) the many regulatory mechanisms that control  
484 ribosomal protein expression at the translation level, which could partially compensate  
485 transcription reduction; ii) the fact that ribosomal subunits are found in excess with respect to  
486 assembled ribosomes; iii) the possibility that an eventual reduction in functional ribosomes can  
487 be compensated by faster translation rates (61-63); iv) finally, it has been described, particularly  
488 in *Vibrio* sp. CCUG 15956 (64), that ribosomes are available in excess of numbers needed for  
489 exponential growth. Such large ribosome quantities would have been selected as an ecological

490 survival strategy that allows for fast growth restoration after its arrest in rapidly changing  
491 environmental conditions (65). Hence, lower S10 expression could be buffered at many levels  
492 and protein production might be only mildly impacted. Molecular crowding reduction might  
493 however not be as easily compensated. Therefore, movant strains possess a less crowded  
494 cytoplasm where DNA polymerase activity is reduced and more chaperones are needed. This  
495 would embody a novel mechanism which could explain how ribosomal protein gene position  
496 influences growth rate.

497 Bacterial growth closely correlates to ribosomal protein content. This has been attributed to the  
498 role ribosomes have in protein synthesis (66, 67). We propose that, on top of that, ribosome  
499 concentration may change the macromolecular crowding conditions to optimize biochemical  
500 reactions, in particular in protein folding and DNA replication (26, 27). We provide evidence  
501 indicating that this is the case for replication dynamics in *V. cholerae*. Our experiments suggest  
502 that the genomic position of S10 contributes to generate the RP levels necessary to attain  
503 optimal cytoplasmic macromolecular crowding. Besides connecting ribosomal gene position to  
504 growth in *V. cholerae*, this mechanism could link ribosome biogenesis to cell cycle in bacteria.  
505 During exponential phase, when RP production is maximal and ribosomes represent 30 % of  
506 cell weight, crowding peaks. This leads to the highest *oriC*-firing frequency. Upon nutrient  
507 exhaustion, ribosome production is reduced, the cytoplasm macromolecular crowding  
508 diminishes, slowing down replisome dynamics. This scenario, which is beyond the scope of our  
509 study, deserves to be tested in other model microorganisms.

#### 510 **Materials and methods:**

511 **General procedures.** Genomic DNA was extracted using the GeneJET Genomic DNA  
512 Purification Kit while plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit  
513 (Thermo Scientific). PCR assays were performed using Phusion High-Fidelity PCR Master Mix

514 (Thermo Scientific). Strains and plasmids used in this study are listed in Table S1. Details of  
515 culture conditions and selection can be found in Supp. Text.

516 **Automated growth curve measurements:** ON cultures were diluted 1/1000 in LB. Bacterial  
517 preparations were distributed at least by triplicate in p96 microplates. Growth-curve  
518 experiments were performed using a TECAN Infinite Sunrise microplate reader, following the  
519 OD<sub>600nm</sub> every 5 minutes at 37°C on maximum agitation. Growth rate was obtained using a  
520 custom Python script coupled to the Growthrates program (68).

521 **Protein production capacity:** For estimating GFP production we performed *V. cholerae*  
522 *gfpmut3\** automated growth curves in a TECAN Infinite 200 microplate reader, following  
523 OD<sub>600nm</sub> and GFP fluorescence over time. Data was analyzed using GraphPad Prism 6. For flow  
524 cytometry strains were grown in fast growing conditions until early exponential phase  
525 (OD<sub>450</sub>~0.2). Then 50 µL were diluted in 800 µL of PBS. The fluorescence of 20.000 events  
526 was recorded in a MACSQuant 10 analyzer (Miltenyi Biotec). Cells were detected using Side  
527 Scatter Chanel (SSC) in log<sub>10</sub> scale. Data analysis was done using Flowing Software 2.5.1  
528 ([www.flowingsoftware.com](http://www.flowingsoftware.com)). For luciferase activity measurement, *Vibrio cholerae::RL* strains  
529 were cultured until OD<sub>450nm</sub>~0.2. For each experiment, three samples of 20 µL were harvested  
530 and directly measured using the Renilla Luciferase Assay System (Promega).

531 **Ribosome profiling:** Ribosomal 70s, 50s and 30s species from the indicated *V. cholerae* strains  
532 were isolated as previously described (69, 70). Early exponential phase cultures (OD<sub>450nm</sub>~0.2)  
533 were harvested by centrifugation. Subsequent steps were performed at 4°C. The pellet was  
534 resuspended in ice-cold Buffer A (20 mM HEPES pH 7.5, 50 mM NH<sub>4</sub>Cl, 10 mM MgCl<sub>2</sub>, 5  
535 mM β-mercaptoethanol, 0.1 mM PMSF) in the presence of Ribolock (Thermo Fisher  
536 Scientific). DNase I was added up to 2 µg/mL and kept for 20 min at 4°C. Cells were lysed by  
537 two passes at 11,000-15.000 psi using Emulsiflex. Cell debris were removed by two

538 centrifugation steps at 30,000g for 30 min. Then 0.8 mL of Cold 60% sucrose buffer A was  
539 added to RNase-free 5 mL Ultraclean tubes for ultracentrifugation in a SW55Ti (Beckman).  
540 The ribosome-containing supernatant was used to fill these tubes and an ultracentrifugation step  
541 was performed for 16 hs at 150.000g. Ribosomes were recovered from the bottom 0.8 mL of  
542 60% sucrose Buffer A and dialyzed using a Float-a-lyzer G2 in Buffer A. Sedimentation  
543 velocity was determined in a Beckman XL-I Analytical Ultracentrifuge. Double sector quartz  
544 cells were loaded with 400 µl of Buffer A as reference and 380 µl of sample (3 µm), and data  
545 were collected at 120,000 rpm from 5.8 to 7.3 cm using a step size of 0.003 cm without  
546 averaging. Sedimentation velocity data were analyzed using the continuous size-distribution  
547 model employing the program SEDFIT.

548 **FRAP:** For measurement of GFP synthesis, stationary phase cultures of *V. cholerae* strains  
549 were diluted 1/300 in fresh LB. Then 6 µL were distributed on an LB agar pad within a Gene  
550 Frame (Thermo-Fisher) and covered with a cover slip. When indicated, the agar pad was  
551 supplemented with Cm at MIC. Cells were then visualized and recorded in a Spinning-Disk  
552 UltraView VOX (Perkin-Elmer) equipped with two Hamamatsu EM-CCD (ImageEM X2)  
553 cameras. Photobleaching was done using 5-20 % of laser power. Image analysis is detailed in  
554 Supp Text.

555 **Transcriptomic analysis:** Preparation of RNA and libraries is detailed in Supp. Text. four  
556 independent biological replicates for each sample were done for statistical analysis which is  
557 also detailed in the Supp. Text. Trimmed reads were aligned to the *V. cholerae* reference  
558 genome using Bowtie (71) with default parameters. Aligned reads were counted using HTSeq  
559 Count (72). Further quality control and differential expression analysis was performed using  
560 methods described in supplementary methods (73-75). Graphics were done using Graph Pad  
561 software, specific online service for Venn diagram  
562 (<http://bioinformatics.psb.ugent.be/webtools/Venn/>) and Circos Plot (76). The sequence data

563 was submitted to the GenBank Sequence Read Archive. Accession numbers for these samples  
564 are: SRR8316520, SRR8316521, SRR8316528, SRR8316529, SRR8316526, SRR8316527,  
565 SRR8316524, SRR8316525, SRR8316522, SRR8316523, SRR8316530, SRR8316531,  
566 SRR8316518, SRR8316519, SRR8316516, SRR8316517, SRR8316514, SRR8316515,  
567 SRR8316512 and SRR8316513.

568 **Whole chromosome transcriptional activity comparisons:** Reads were mapped as previously  
569 described (35) to a custom assembled linear version of the *V. cholerae* that starts (base 0) at the  
570 *ter* and finishes at the *ter*, with the *oriI* at the center of the sequence. Total reads mapped to this  
571 sequence were counted and normalized as previously described (35). Fold changes were  
572 calculated using normalized values and p-values were calculated as previously described (35).

573 **Functional characterization of the transcriptomic response:** *V. cholerae* N16961 genes  
574 were aligned against the eggNOG database v.4.0 (40). Only hits with at least 50% similarity  
575 and e-value < 0.05 were used. Each protein was assigned to the best functional category,  
576 according to the percentage of similarity and the length of the alignment. We then calculated  
577 the fraction of categories enriched in the fraction of differentially expressed genes, compared  
578 to abundances of the different eggNOG categories in the *V. cholerae* genome. The over-or  
579 under-representation of protein families was assessed statistically using the Pearson Chi square  
580 test with Benjamini–Hochberg correction for multiple test. For further validation, this test was  
581 performed 10,000 times in random sub samples of 30% of the differentially expressed genes.

582 **MIC determination:** The MICs of Gm, Cm and Er were determined using E-test® and the  
583 disk diffusion method following manufacturer's instructions (Biomérieux).

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601

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780

781 **Table 1: Quantitative and qualitative expression changes in the movant strains.** The  
 782 number of differentially expressed genes ( $p < 0.05$ ) compared to parental strain in fast growing  
 783 conditions. The number in parenthesis represents genes whose expression varies more than 2-  
 784 fold. The magnitude of expression change is quantified as the average of the  $\text{Log}_2(\text{FC}) \pm$   
 785 standard deviation.

	<b>-35</b>	<b>-510</b>	<b>-1120</b>	<b>C2+479</b>
<b>Number of upregulated genes</b>	2 (1)	62 (37)	361 (64)	439 (88)
<b>Mean upregulation<sup>a</sup></b>	n/d	$1.5 \pm 0.97$	$0.67 \pm 0.41$	$0.78 \pm 0.56$
<b>Number of downregulated genes</b>	6 (4)	49 (2)	301 (9)	303 (17)
<b>Mean downregulation<sup>a</sup></b>	n/d	$-0.5 \pm 0.24$	$-0.49 \pm 0.26$	$-0.52 \pm 0.29$
<b>Total number of altered genes</b>	8	111 (39)	662 (72)	742 (105)
<b>Altered functions</b>	-	E, P, V	E, O, R, V, N	E, O, R, V, N, F, P, U

786 <sup>a</sup> average of the  $\text{Log}_2(\text{FC}) \pm$  standard deviation. n/d, not determined.

787

788 **Figures legends:**

789 **Figure 1: Genome organization links S10 location to cell physiology. a)** The presence of a  
790 single *oriC* (red dot) organizes the bacterial genome along an *ori-ter* axis (left panel). In slow  
791 growing conditions, genes have between 1 to 2 copies (center). During exponential phase, fast  
792 growing-bacteria overlap replication rounds increasing the dosage of *oriC*-neighboring regions  
793 (right panel). The arrow shows the approximate position of the S10 locus. **b)** The maximum  
794 growth rate ( $\mu$ , black dots) and the relative S10 dosage (gray squares) and expression (white  
795 triangles) with respect to the parental strain were plotted as a function of S10 position along the  
796 *ori-ter* axis within *V. cholerae* genome. **c)** Diagram of the genome of parental, movant, and the  
797 merodiploid strains employed in this study. *ori1* and *ori2* are depicted as dark and light gray  
798 dots, respectively. The orange arrow represents S10 displaying its genomic position and ploidy.  
799 The dashed line represents the S10 location in the parental strain. Chromosomes are drawn  
800 according to their replication timing.

801 **Figure 2: S10 genomic location does not impact ribosome function at the population level.**

802 **a)** The GFP expression and OD<sub>600nm</sub> of the indicated *gfpmut3*<sup>+</sup> strains (Table S1) were measured  
803 along time. The fluorescence mean ( $\pm$ SD) was plotted as a function of the mean ( $\pm$ SD) OD<sub>600nm</sub>.  
804 Figure shows a representative experiment with 4 biological replicates (among three independent  
805 experiments). The parental *gfpmut3*<sup>-</sup> strain is an autofluorescence/light dispersion control. **b)**  
806 The indicated *gfpmut3*<sup>+</sup> strains in early exponential phase (OD<sub>450nm</sub>~0.2) were analyzed by flow  
807 cytometry. Left panel shows the fluorescence signal frequency distribution of the indicated *V.*  
808 *cholerae* strains. Parental *gfpmut3*<sup>-</sup> strain was added negative control. Right panel shows the  
809 Fluorescence intensity with the 95% confidence interval (CI). Points represent individual  
810 biological replicates obtained along at least 2 independent experiments **c)** Parental and movant  
811 strains bearing RLU in the chromosome (Table S1) were grown until early exponential phase.  
812 Then, RL activity, represented as RL units (RLU), was measured in three independent

813 biological replicates for each strain. **d)** Parental and derivative strains present similar resistance  
814 levels to ribosome-targeted antibiotics. On the right panel, chromosomes are represented as in  
815 the previous figure. The encoded antibiotic resistance markers are depicted as boxes: Gm in  
816 violet and Cm in green. Their approximate genomic location is shown in each strain. On the  
817 right the MIC ( $\mu\text{g/mL}$ ) for Cm, Gm and Er for each depicted strain is shown. **e)** Ribosome  
818 profiles for the indicated strains as obtained by analytical ultracentrifugation. Pie charts  
819 quantify polysome, 70s, 50s and 30 s fractions for the indicated strains.

820 **Figure 3: Genome-wide transcription and replication activity along the genome. a)**  
821 **Transcriptional activity across Chr1.** RNA-seq reads were mapped along the Chr1 of *V.*  
822 *cholerae*. The histograms represent mapped read normalized to the genome wide total volume  
823 along both replichores in *ter1-ori1-ter1* order. Normalized Expression Values (NEV) are shown  
824 along the distance from *ori1* in Mbp is shown on top. Each graph represents one strain: Parental  
825 (purple); S10Tnp-510 (green); S10TnpC2+479 (blue). The plots of the whole strain set are in  
826 FigS4. The 400 Kbp flanking *ori1* are highlighted in orange. The arrow indicates the peak  
827 corresponding to the S10 locus. **b)** MFA profiles are obtained by plotting the  $\log_2$  frequency of  
828 reads (normalized against reads from a stationary phase of a parental strain control) at each  
829 position in the genome as a function of the relative position on the *V. cholerae* main  
830 chromosome with respect to *ori1* (to reflect the bidirectional DNA replication) using 1,000-bp  
831 windows. Results for the parental (purple), S10Tnp+166 (black), the S10Tnp-510 (green) and  
832 the S10TnpC2+479 (blue) movants show their differences in read coverage. The arrow  
833 highlights the S10 position in the abscissa, reflecting dosage alterations. **c)** S10 relocation effect  
834 on replication dynamics was quantified by averaging obtained the slope for each replichore for  
835 at least 4 independent MFA experiments. Results are expressed the mean slope with 95% CI.  
836 Statistical significance was analyzed by one-way ANOVA two-tailed test. Then Tukey test was

837 done to compare the mean values obtained for each strain. Statistically different slopes are  
838 indicated as follows: \*\*,  $p < 0.01$  and \*\*\*,  $p < 0.001$ .

839 **Figure 4: S10 relocation impacts gene expression genome-wide in a distance dependent**  
840 **manner. a)** Volcano plot displaying differential expressed genes in S10Tnp-35 (brown),  
841 S10Tnp-510 (green), S10Tnp-1120 (red) and S10TnpC2+479 (blue). Horizontal dotted line  
842 shows  $p = 0.05$ . **b)** The number of coding sequences (CDS) as a function of  $\text{Log}_2(\text{FC})$  of strains  
843 S10Tnp-35 (turquoise), S10Tnp-510 (green), S10Tnp-1120 (red) and S10TnpC2+479 (blue).  
844 **c)** Venn diagram displaying shared genes between S10Tnp-510 (green), S10Tnp-1120 (red) and  
845 S10TnpC2+479 (blue). **d)** Expression correlation between movant strains. Dots correspond to  
846 individual CDS. The  $\text{Log}_2(\text{FC})$  of each gene in S10Tnp-510 (green) or S10Tnp-1120 (red) was  
847 plotted as a function of  $\text{Log}_2(\text{FC})$  in S10TnpC2+479.

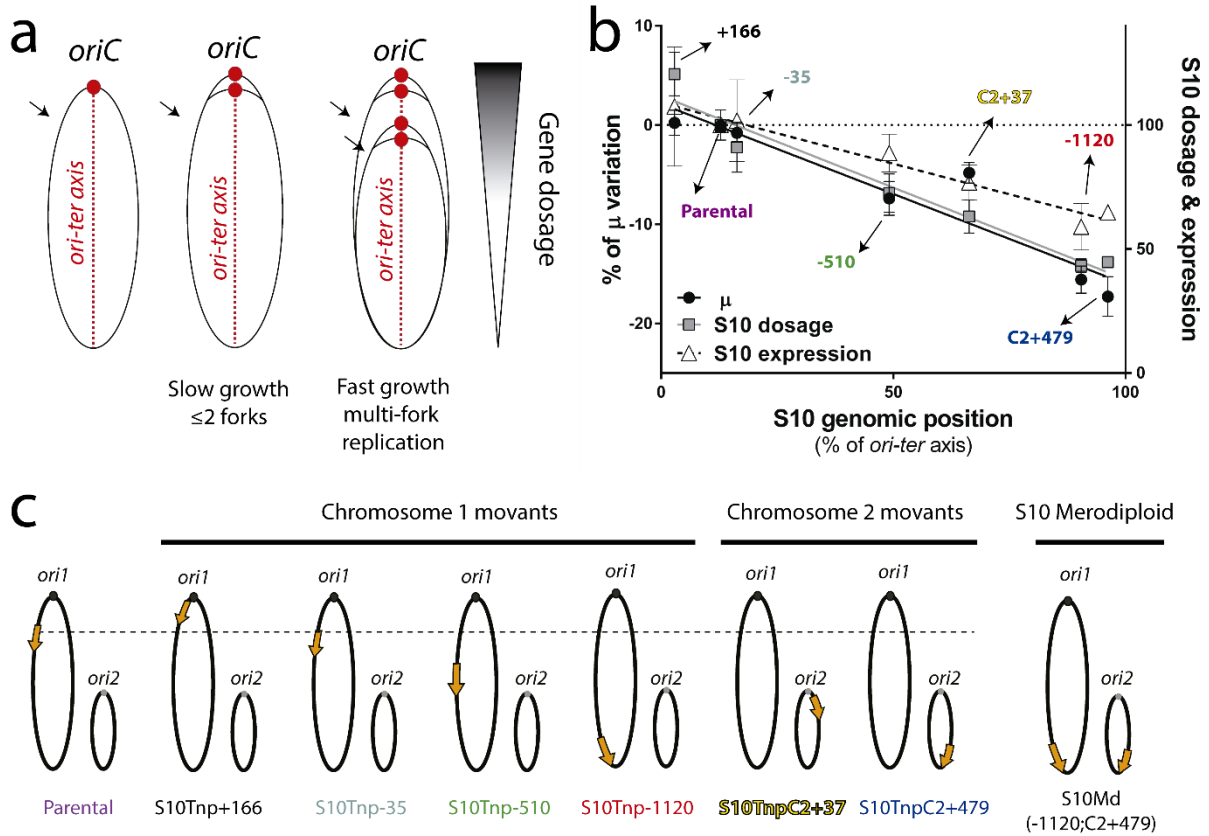
848 **Figure 5: S10 relocation impacts cytoplasm fluidity. a)** Half-time of fluorescence recovery  
849 ( $\tau$ ) in the Parental-1120 (purple,  $n = 104$ ) and the most affected movants S10Tnp-1120 (red,  
850  $n = 128$ ) and S10TnpC2+479 (blue,  $n = 92$ ) in a *gfpmut3\*  $\Delta$ crtS* genetic context. The line indicates  
851 the mean  $\tau$  value and each dot indicates the obtained value for a cell. Statistical significance  
852 was analyzed using Kruskal-Wallis non-parametric tests followed by Dunn's multiple  
853 comparisons using parental as control respectively. \*,  $p < 0.05$ ; \*\*\*\*,  $p < 0.0001$ . **b)** Histogram  
854 showing the relative frequency of  $\tau$  to observe the distribution of the values. The vertical dotted  
855 line shows the mean value as in **a**).

856 **Figure 6: S10 relocation effects are reduced in hyperosmotic conditions. a)** Growth rates of  
857 the parental and the indicated movant strains in LB with increasing NaCl concentrations were  
858 quantified by averaging obtained  $\mu$  in 6 independent experiments with at least 3 biological  
859 replicates. The growth of each movant was normalized to the  $\mu$  of the parental strain and the  
860 percentage of the variation ( $\mu$  %)  $\pm$  SEM with respect to parental strains is shown as a function



861 of NaCl concentration of growth medium. **b)** Changes in growth of the movant strains with  
862 respect to parental strain is shown as a function of sucrose concentration. Data was treated as in  
863 a) but results correspond to 4 independent experiments with at least 3 biological replicates. **c)**  
864 MFA profiles are plotted as in Fig. 3b. Results for the parental (purple), the S10Tnp-1120 (red)  
865 and the S10TnpC2+479 (blue) strains in LB in presence of 5 gr/L (LB, left panel) or 20 gr/L  
866 (LB+NaCl, right panel) are shown. The arrow highlights the S10 position in the abscissa,  
867 reflecting S10 dosage alterations. **d)** Replication dynamics in presence of 5 or 20 gr/L of NaCl  
868 assessed by calculating the slope for each replichore for 2 independent MFA experiments. Dots  
869 indicate mean  $\pm$  SD. Statistical significance was analyzed by one-way ANOVA two-tailed test  
870 and Tukey test for multiple comparisons. Significance is indicated as follows: n.s.: non-  
871 significant; \*,  $p < 0.05$  and \*\*,  $p < 0.01$ .

872 Figure 1:

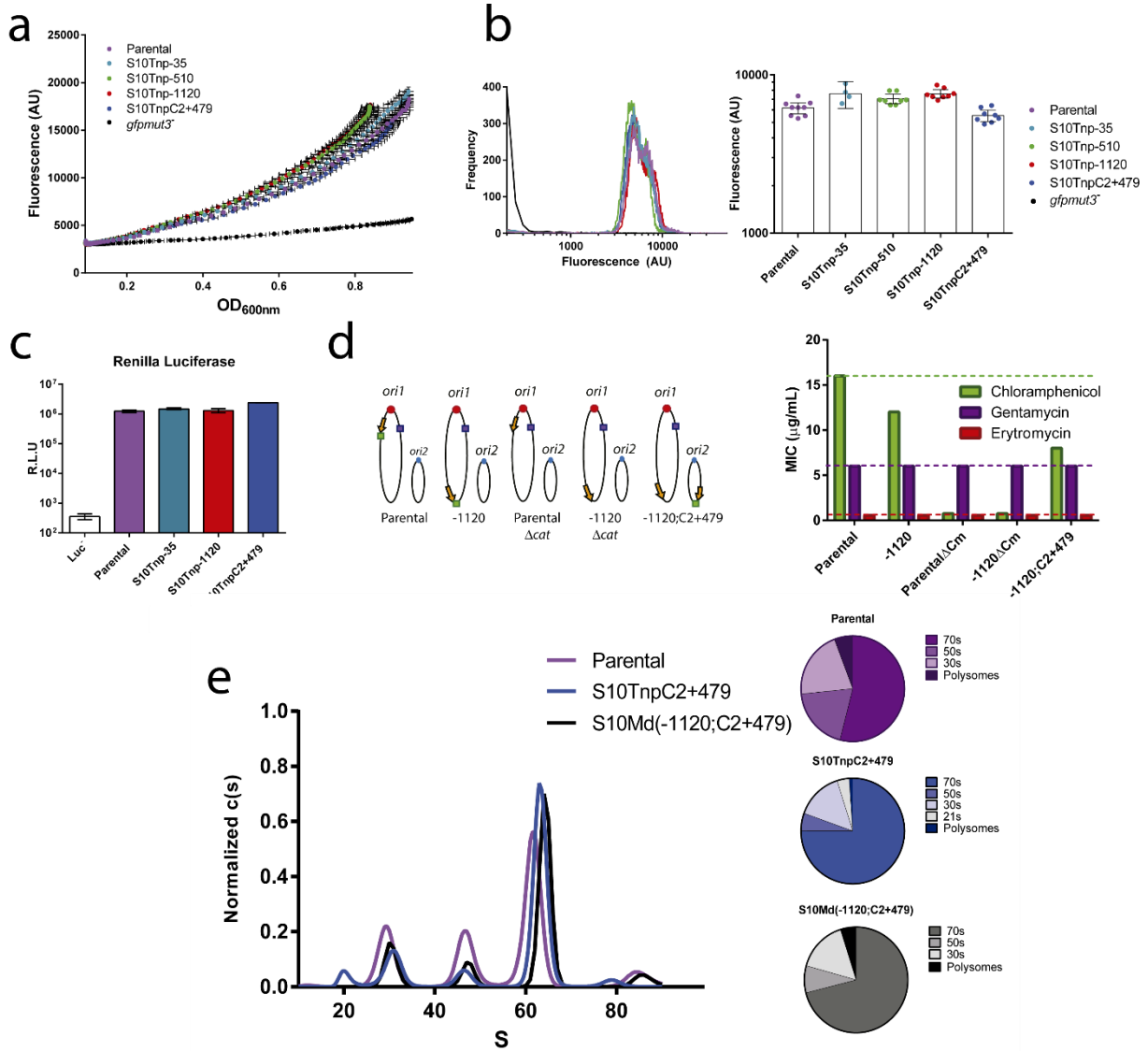


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876 Figure2

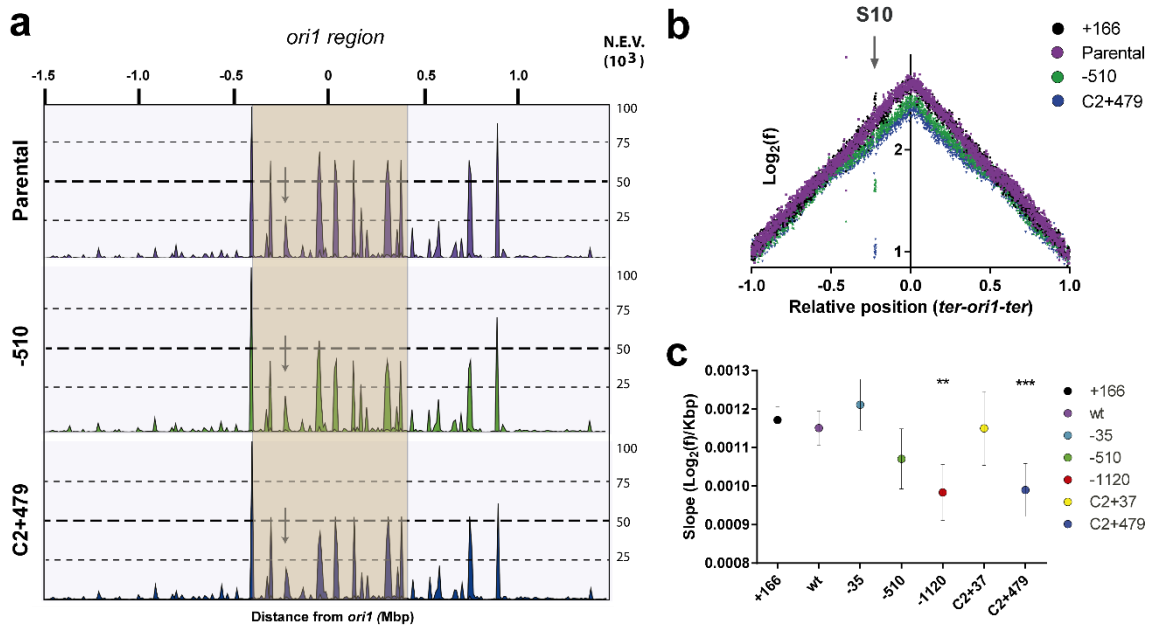


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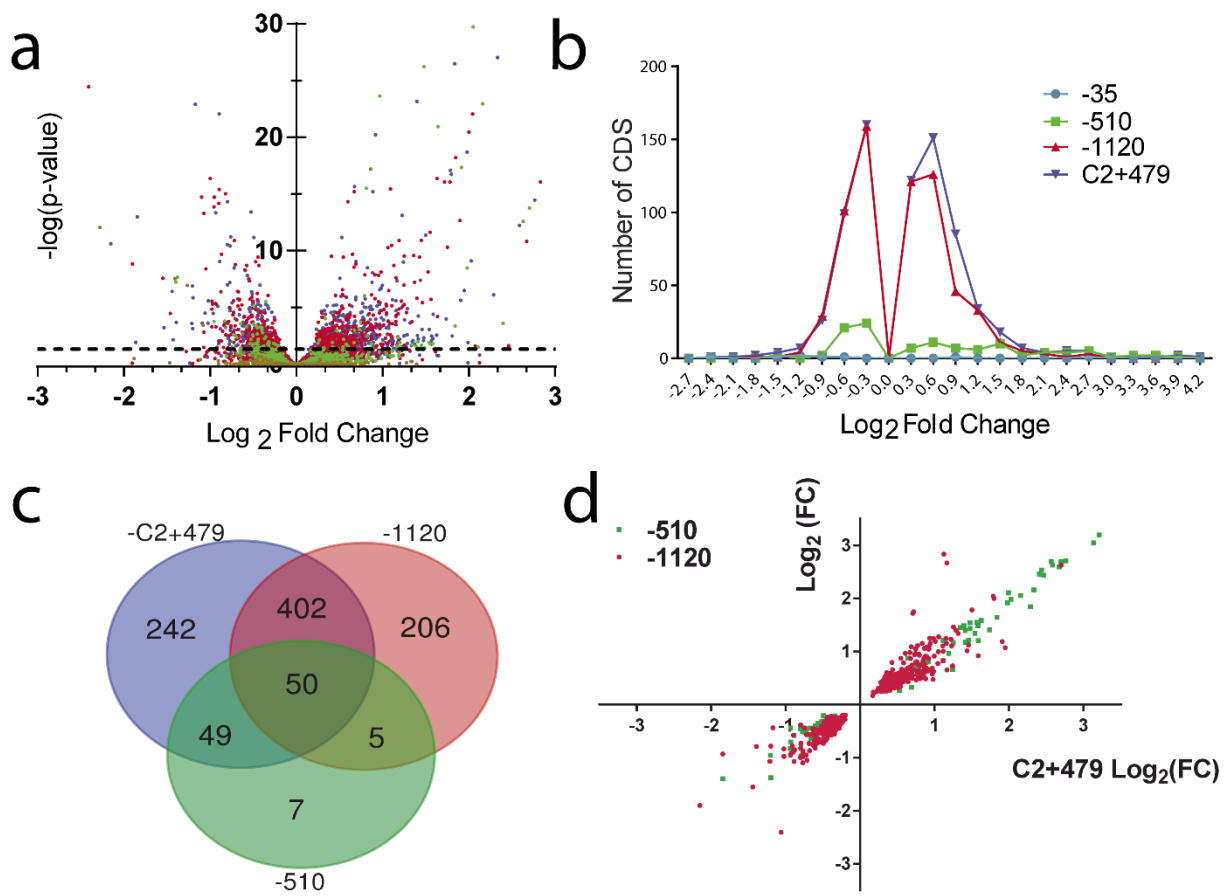
880 Figure 3



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883 Figure 4

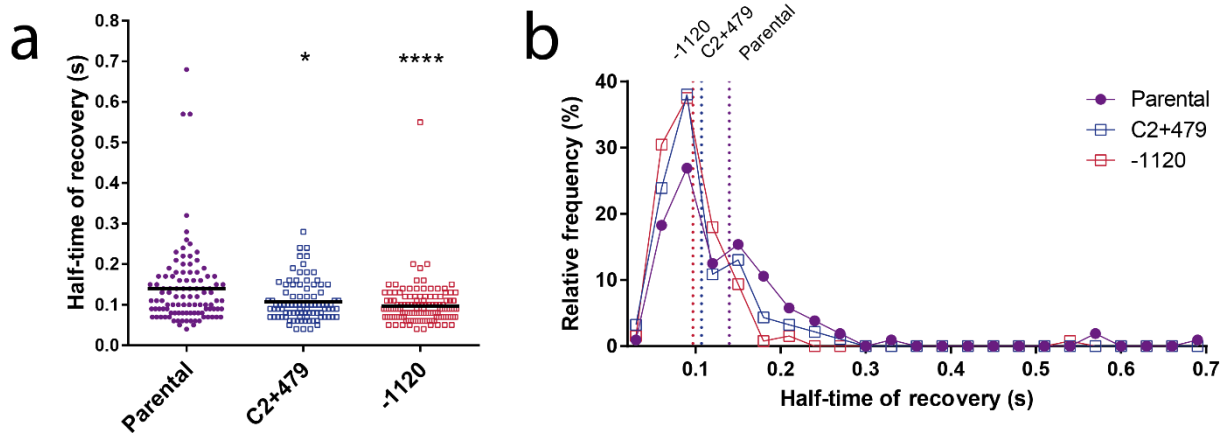


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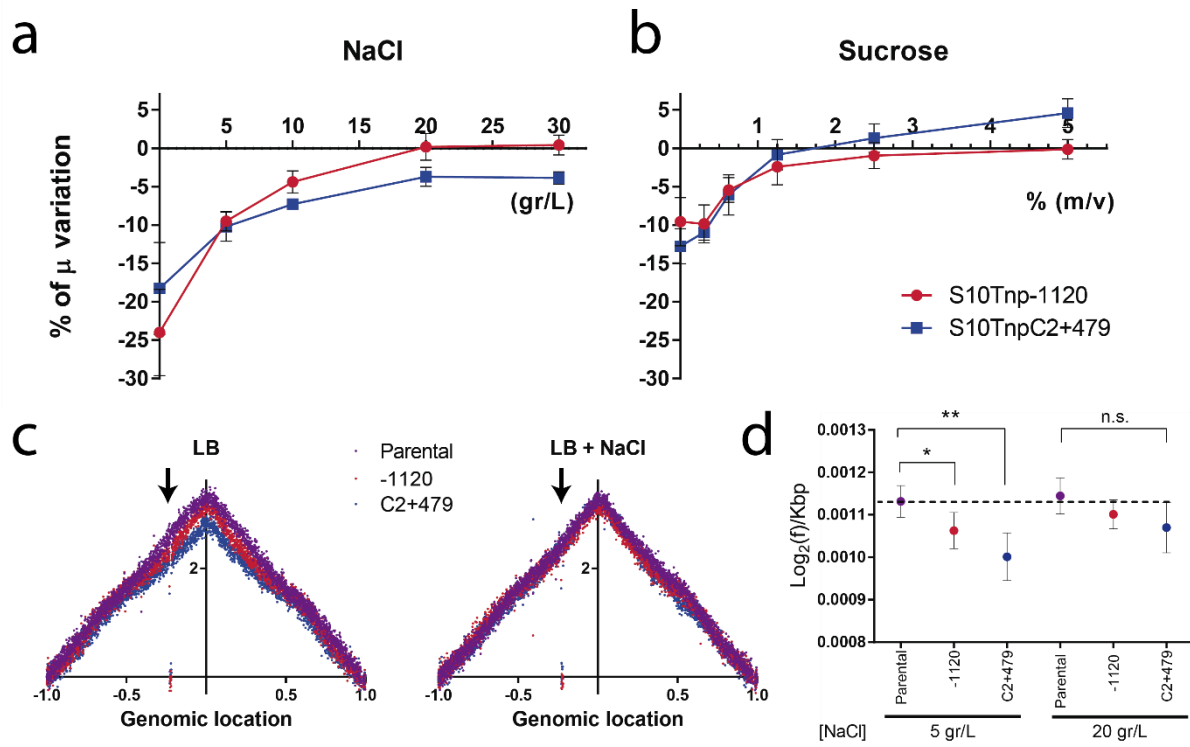
887 Figure 5



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890 Figure 6



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