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Termination factor Rho mediates transcriptional reprogramming of *Bacillus subtilis* stationary phase

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28

29 **Abstract**

30 Reprogramming of gene expression during transition from exponential growth to stationary
31 phase is crucial for bacterial survival. In the model Gram-positive bacterium *Bacillus subtilis*,
32 this process is mainly governed by the activity of the global transcription regulators AbrB,
33 CodY and Spo0A. We recently showed that the transcription termination factor Rho, known for
34 its ubiquitous role in the inhibition of antisense transcription, is involved in Spo0A-mediated
35 regulation of differentiation programs specific to the stationary phase in *B. subtilis*. To identify
36 other aspects of the regulatory role of Rho during adaptation to starvation, we have constructed
37 a *B. subtilis* strain that expresses *rho* at a relatively stable high level in order to circumvent its
38 decrease occurring in the wild-type cells entering the stationary phase. We show that *B. subtilis*
39 cells stably expressing Rho fail to sporulate and to develop genetic competence, which is
40 largely, but not exclusively, due to abnormally low expression of the master regulator Spo0A.
41 Moreover, in addition to a global decrease of antisense transcription, these cells exhibit
42 genome-wide alterations of sense transcription. A significant part of these alterations affects
43 genes from global regulatory networks of cellular adaptation to the stationary phase and reflects
44 the attenuated de-repression of the AbrB and CodY regulons and the weakened stringent
45 response. Accordingly, stabilization of Rho level reprograms stationary phase-specific
46 physiology of *B. subtilis* cells, negatively affects cellular adaptation to nutrient limitations and
47 alters cell-fate decision-making to such an extent that it blocks development of genetic
48 competence and sporulation. Taken together, these results indicate that the activity of
49 termination factor Rho constitutes a previously unknown layer of control over the stationary
50 phase and post-exponential adaptive strategies in *B. subtilis*, from the adjustment of cellular
51 metabolism to the activation of survival programs.

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59 Introduction

60 Transcription termination is a critical step in regulation of gene expression in all living
61 organisms. In bacteria, termination is achieved by two mechanisms: factor-independent, which
62 is associated with specific sequence forming an RNA terminator hairpin, and factor-dependent,
63 which relies mostly on the activity of an RNA helicase–translocase, transcription termination
64 factor Rho [1-3].

65 Since its initial characterization in 1993 [4], termination factor Rho was repeatedly shown to
66 be dispensable for the model Gram-positive bacterium *Bacillus subtilis* in laboratory growth
67 conditions [5-8]. On the contrary, the viability of numerous Gram-negative bacteria depends
68 strictly on active Rho (reviewed in [9]). While, the essentiality of the *rho* gene varies among
69 different bacterial species, Rho is recognized now as the major factor controlling pervasive
70 antisense transcription in both Gram-positive and Gram-negative bacteria [6, 10-12]. Moreover,
71 Rho inactivation alters significantly the expression of protein-coding genes by a combination
72 of direct *cis* and indirect *trans* effects in bacterial species in which the *rho* gene is non-essential,
73 as reported for *B. subtilis*, *Staphylococcus aureus*, and *Bacillus thuringiensis* [8, 11, 12].

74 In *B. subtilis*, we have shown, using $\Delta\rho$ mutant, that a significant part of the Rho-controlled
75 transcription is connected to the regulation of three mutually exclusive differentiation
76 programs: cell motility, biofilm formation, and sporulation. To a large extent, the choice
77 between these and other cell fates (e.g., genetic competence, cannibalism toxins production)
78 upon entry into the stationary phase depends on cellular levels of the phosphorylated active
79 form of the master regulator Spo0A (Spo0A~P). Only cells expressing high levels of Spo0A~P
80 can commit to sporulation, an ultimate survival option of *B. subtilis* cells at stationary phase
81 [14, 15]. We have established that deletion of the *rho* gene prevents Rho-dependent intragenic
82 termination of the *kinB* transcript encoding the sensory kinase KinB, thereby activating the
83 Spo0A phosphorelay and increasing cellular levels of Spo0A~P to a threshold triggering
84 sporulation. Thus, Rho inactivation increases the efficiency of sporulation and inhibits the
85 alternative cell fates [8].

86 In the human pathogens *S. aureus*, *Mycobacterium tuberculosis* and *Clostridioides difficile*,
87 Rho inactivation induces the expression of virulence factors essential for the successful host
88 colonization and infection [12, 16, 17]. Likewise, Rho affects expression of genes involved in
89 cellular differentiation, colonization and pathogenesis in *B. thuringiensis* [13].

90 Overall, these data indicate that in *B. subtilis* and other Gram-positive bacteria, Rho plays an
91 important role in the regulation of different phenomena associated with the stationary phase.

92 This specific physiological state of growth caused by nutrients depletion is characterized by
93 slowdown of macromolecular synthesis, reorientation of the cellular metabolism towards
94 alternative metabolic pathways, activation of the stringent response and alternative sigma
95 factors [18].

96 Along with Spo0A, two other key transcriptional regulators, AbrB and CodY, sensing
97 environmental and intracellular metabolic status drive the reprogramming of metabolism and
98 the initiation of stationary phase-specific developmental programs in *B. subtilis* [19]. During
99 exponential growth, AbrB suppresses transcription of over two hundred genes that are switched
100 ON upon AbrB depletion during the transition to the stationary phase [20-22]. AbrB plays an
101 important role in the interconnected regulatory networks governing the initiation of sporulation
102 and development of genetic competence by controlling the expression of transition-phase sigma
103 factor SigH and competence transcription factor ComK [23, 24]. Thus, AbrB depletion is
104 important for cells entering the stationary phase. Expression of the *abrB* gene is repressed by
105 Spo0A~P, which also indirectly controls AbrB DNA binding activity [21, 25, 26]. In addition,
106 AbrB is an unstable protein and its concentration decreases rapidly due to degradation of the
107 *abrB* mRNA triggered by small regulatory RNA, RnaC [27].

108 The pleiotropic regulator CodY directly and indirectly represses transcription of the numerous
109 genes required for adaptation to nutrient limitation [28, 29]. This repression is released to
110 activate the alternative nutrient acquisition pathways when cells enter into the stationary phase
111 [30, 31]. CodY is also implicated in the control of genetic competence and sporulation [32, 33].
112 CodY modulates its own DNA-binding affinity by sensing two metabolites: branched-chain
113 amino acids (BCAA) isoleucine, leucine, and valine and the nucleoside triphosphate GTP. In
114 the absence of any of these ligands, the ability of CodY to bind DNA is impaired [30, 34].

115 In that way, activity of CodY is linked to stringent response, a widespread stress resistance
116 mechanism essential for stationary phase survival [35-37]. It is characterized by the synthesis
117 of the alarmone guanosine-(penta)tetra-phosphate ((p)ppGpp), mainly provided by a
118 bifunctional synthetase/hydrolase Rel sensing starved ribosomes [38-40]. In *B. subtilis*,
119 (p)ppGpp modifies genome-wide transcription indirectly, by causing a decrease in GTP levels
120 due to the inhibition of activity of GTP-synthesizing enzymes and consumption of GTP during
121 synthesis of (p)ppGpp [41-43]. A decrease in the GTP levels causes de-repression of genes
122 from the CodY regulon, negatively affects transcription from promoters of stable RNA
123 synthesis genes (e. g., genes involved in the ribosome biogenesis) and re-directs RNA
124 polymerase from these GTP-initiating promoters to promoters of biosynthetic genes [44-46]. In
125 addition, (p)ppGpp directly represses the activity of the DNA primase, thereby regulating DNA

126 replication [47] and inhibits protein synthesis [48-50]. Furthermore, it is known that burst of
127 (p)ppGpp upon entering the stationary phase contributes to the induction of genetic competence
128 and sporulation [33, 51, 52].

129 It is important to note that in the wild type cells *rho* mRNA and Rho protein levels decrease
130 during transitional and stationary growth phases [6, 8, 11]. By analogy with AbrB, this phase-
131 dependent depletion of Rho suggests that, in addition to controlling long-term survival
132 strategies, Rho may participate in the regulation of the earlier stages of physiological adaptation
133 to stationary phase and, in a broader sense, in the control of transition and stationary phase-
134 specific transcriptomes. Such hypothetical Rho activity could not be detected using the Δrho
135 mutant. Thus, we assumed that the stabilization of Rho levels over an extended period of growth
136 would provide an experimental model to study this aspect of Rho regulatory activity and thereby
137 to expand our knowledge about Rho-mediated regulation of gene expression and its effect on
138 the cellular physiology of *B. subtilis*.

139 To evaluate this hypothesis, we used a combination of experimental approaches, including
140 genome-wide transcriptional analysis, monitoring of time-course activity of selected promoters,
141 morphological and functional studies of the *B. subtilis* strain (hereinafter, Rho⁺), in which Rho
142 was maintained at a relatively stable high level throughout exponential and stationary growth
143 phases. We show that stable expression of the *rho* gene causes system-level alterations of
144 genome transcription. Rho reprograms cellular physiology and starvation-specific
145 developmental programs driven by the activity of key transcriptional regulators AbrB, CodY,
146 ComK, and Spo0A. Moreover, Rho⁺ strain exhibits partially relaxed phenotype characterized
147 by a weakened stringent response.

148 Altogether, these findings provide new functional insights into the role of the transcription
149 termination factor Rho in the physiology of *B. subtilis* and indicate that strict regulation of Rho
150 expression is crucial for the functionality of the complex gene networks governing the
151 stationary-phase adaptation in this model Gram-positive bacterium.

152

153

154 **Results**

155

156 **Heterogenic expression system assures Rho expression at a steady high level**

157 To evaluate the impact of the stabilization of Rho levels on stationary phase-specific
158 phenomena, we first conceived a system that would maintain relatively stable Rho amounts

159 over exponential and stationary growth. The plasmid that we previously used for Rho over-
160 expression seemed unsuitable for this purpose due to the copy number heterogeneity [8, 53],
161 and the presence of the 5'UTR region of *rho* gene previously implied in auto-regulation of Rho
162 [5]. To overcome these limitations, we have disconnected the expression of *rho* from any
163 regulatory circuits acting at its natural locus by placing a copy of the *rho* gene under the control
164 of heterogenic expression signals at an ectopic location. Briefly, we substituted the native *rho*
165 promoter with a well-characterized constitutive *B. subtilis* promoter P_{veg} [54, 55], replaced the
166 5'UTR of the *rho* gene with the ribosome binding site of the *tagD* gene [56] and placed this *rho*
167 expression unit at the *amyE* locus. Expression of the *rho* gene driven by these regulatory
168 elements had no effect on growth rate of the resulting *B. subtilis* strain (hereinafter, Rho⁺) in
169 rich LB medium over an extended period (~10 h), ranging from exponential into the stationary
170 phase (Fig 1A).

171 To evaluate expression levels of Rho protein under these growth conditions, we used the
172 relative to WT and Rho⁺ strains expressing the SPA-tagged Rho protein to reveal Rho content
173 by immunoblotting [8]. In the WT cells, Rho-SPA protein levels steadily decreased during
174 transition into the stationary phase in LB medium (Fig 1B). In contrast, Rho⁺ cells showed
175 relatively stable levels of Rho-SPA during the exponential growth and after entering the
176 stationary phase (Fig 1B). The decrease of Rho-SPA observed in the Rho⁺ cells during the late
177 stationary phase could be due to a decline of the P_{veg} promoter's activity at this stage as reported
178 previously [55].

179 We concluded that using the heterogenic expression system assures a steady high level of Rho
180 expression in the stationary-phase *B. subtilis* cells.

181

182 **Rho⁺ strain exhibits sporulation-deficient phenotype.**

183 We initiated the analysis of physiological effects of a steady high Rho content by assessment
184 of the sporulation capacity of Rho⁺ cells.

185 The WT, Δrho mutant (RM) and Rho⁺ cells were cultured in the sporulation-inducing DS
186 medium and compared for the ability to form heat-resistant spores (Materials and Methods).
187 Depending on the experiment, 20% to 40% of the WT cells developed spores under the used
188 conditions, while *rho* deletion increased the sporulation rate to its maximum as previously
189 reported (Fig 2A; [8]). In contrast, the efficiency of spore formation by the Rho⁺ strain was
190 reduced up to 10⁻⁵. The rare spores isolated from Rho⁺ cultures appeared to be suppressor
191 mutants able to form thermo-resistant spores with a near-WT efficiency (S1 Table).

192 Analysis of eight independent sporulation-proficient Rho⁺ suppressors revealed six different
193 point mutations within the *rho* coding sequence of the ectopic *rho* expression unit; two
194 mutations were isolated twice. This reaffirms the determining role of Rho in the sporulation-
195 deficient phenotype of Rho⁺ cells (S1 Table). Two mutant Rho proteins were truncated by a
196 stop codon at the position 146 (Q146Stop) and six others had single amino acid changes:
197 A177T, N274H (isolated twice), G286R, G287R and P335R (S1 Fig). The primary sequence
198 of *B. subtilis* Rho subunit displays some characteristic motifs identified previously by studies
199 of different Rho proteins [57-60]; (S1 Fig). In accordance with these data, three of the identified
200 point mutations might have drastic effect on Rho activity. Replacement of glycine by arginine
201 at the positions 286 and 287 (G286R and G287R, respectively) could destroy the highly
202 conserved Q-loop forming a secondary RNA binding site, while the substitution of alanine177
203 localized within one of the Walker motifs by threonine (A177T) could affect ATP binding.
204 Indeed, in a complementation assay using the $\Delta\rho$ mutant we showed that suppressor
205 mutations G287R, G286R and A177T completely inactivate Rho protein, while N274H and
206 P335R mutant proteins remain partially active (S1 Table).

207 Considering that Rho affects sporulation by controlling activity of the Spo0A phosphorelay
208 (Fig 2B); [8], strong inhibition of sporulation in Rho⁺ cells suggested that stabilization of the
209 Rho level during stationary phase effectively suppresses the accumulation of active Spo0A~P.
210 To assess the activity of Spo0A~P in Rho⁺ cells, we first analyzed the real-time expression of
211 the *spoIIAA-AB-sigF* operon using the transcriptional fusion of its promoter to the firefly
212 luciferase gene *luc* ($P_{spoIIAA-luc}$) [8]. Expression of the *spoIIAA-AB-sigF* operon depends on
213 the alternative sigma factor SigH and is activated at a high threshold level of Spo0A~P [26,
214 61-63]. As shown in Fig 2C, whereas in WT cells grown in DS medium the $P_{spoIIAA}$ promoter
215 was switched ON roughly three hours after the entry into stationary phase, no expression could
216 be detected in Rho⁺ cells, suggesting that cells failed to accumulate sufficient amount of
217 Spo0A~P.

218 To further characterize activation of Spo0A in Rho⁺ cells, we analyzed the expression of the
219 *spo0A* gene itself. Transcription of *spo0A* is driven by two promoters, the vegetative SigA-
220 dependent P_v and the SigH/Spo0A-controlled P_s, which is activated at the onset of sporulation
221 at a low level of Spo0A~P [13, 62, 64, 65]. The reporter $P_{spo0A-luc}$ fusion, which we used for
222 analysis, is established at the natural *spo0A* locus and monitors activity of both P_v and P_s
223 promoters [8, 52].

224 During exponential growth, the $P_{spo0A-luc}$ expression was rather similar in WT and Rho⁺ cells
225 suggesting that P_v promoter was not affected by Rho (Fig 2D). In contrast, two hours after the

226 entry into stationary phase, $P_{spo0A-luc}$ activity greatly increased in WT cells, but remained low
227 in Rho^+ cells. We noticed that expression kinetics of the $P_{spo0A-luc}$ in Rho^+ cells was very similar
228 to that observed in the *sigH* mutant, in which the activity of Ps promoter is abolished (Fig 2D);
229 [64, 65]. This suggests that promoter Ps of the *spo0A* gene was inactive in Rho^+ cells either due
230 to the Spo0A~P level lower than required for promoter's activation or, not mutually exclusive,
231 due to the low activity of SigH.

232 Taken together, these results demonstrated that stably expressing *rho* drastically reduces
233 accumulation of the active Spo0A~P under sporulation-stimulating conditions.

234

235 **Synthetic over-production of sensor histidine kinases KinA or KinC does not rescue the** 236 **sporulation-deficient phenotype of Rho^+ strain.**

237 To investigate whether the sporulation-negative phenotype of Rho^+ cells was solely due to a
238 low level of active Spo0A~P, we attempted to boost Spo0A phosphorylation (Fig 2B). To this
239 end, we first used a system over-expressing the major kinase of the phosphorelay, KinA, from
240 an IPTG-inducible promoter ($P_{hyspnc-kinA}$); [13]. We transferred this system in WT and Rho^+
241 cells and assessed their sporulation in DS medium at different concentrations of IPTG inducer.
242 As shown in Fig 2E, addition of IPTG at 10 μ M and 50 μ M (concentrations shown to induce
243 the *kinA* gene to a saturation level [14]), triggered sporulation in ~100 percent of WT cells. The
244 over-expression of KinA in Rho^+ cells also increased the sporulation frequency ~10³- fold,
245 which remained, however, much below the sporulation level of the non-induced WT cells (Fig
246 2E). Thus, artificially triggering the phosphorelay appeared insufficient to restore sporulation
247 in Rho^+ cells and suggested that other roadblocks could exist either within or outside the
248 phosphorelay.

249 To relieve the accumulation of Spo0A~P from the control of phosphorelay, we further used an
250 IPTG-regulated system over-producing the sensor histidine kinase KinC, known to transfer
251 phosphate directly to Spo0A [13, 66, 67]. As shown in Fig 2F, induction of KinC expression at
252 IPTG concentrations of 5 to 10 μ M, previously shown to be optimal for proper activation of
253 Spo0A [68], stimulated sporulation in WT cells to maximal levels, but resulted only in a partial
254 restoration of sporulation efficiency in Rho^+ strain, as in the case of KinA over-production.

255 Overall, we concluded that over-production of sensor kinases ensuring consequent increase of
256 Spo0A phosphorylation either directly (KinC) or via the phosphorelay (KinA) cannot fully
257 suppress the sporulation-negative phenotype of Rho^+ cells. This indicates that Rho negatively
258 affects sporulation not only by repressing Spo0A activation, but also at other stages.

259

260

261 **Rho⁺ strain exhibits competence-deficient phenotype.**

262 The intermediate Spo0A~P level, which is raised transitionally during the late exponential
263 growth without the activation of the sporulation-specific *spo0A* promoter Ps, was shown to be
264 crucial for development of genetic competence (Fig 3A); [69, 70]. We noticed that, contrarily
265 to WT, Rho⁺ strain was not transformable using a common two-step transformation procedure
266 (Materials and Methods). We set to characterize this phenotype of Rho⁺ cells in more details.

267 Since competence is a transient state, we constantly monitored its development, testing cells for
268 genetic transformation during three hours after they were transferred from a rich defined growth
269 medium to a competence-inducing medium (Materials and Methods).

270 As shown in Fig 3B, the efficiency of transformation of WT cells by homologous genomic
271 DNA gradually increased during ~2.5 hours of growth in competence medium and declined
272 later. In the same conditions, Rho⁺ cells remained transformation-deficient all over the
273 experiment. Similarly, it appeared impossible to transform Rho⁺ cells with plasmid DNA (S2
274 Fig). The primary role of Rho in the competence-negative phenotype of Rho⁺ cells was further
275 confirmed by the WT-like transformation efficiency of the Rho⁺_{Q146Stop} suppressor mutant
276 selected as restoring sporulation (see above; Fig 3B).

277 To determine whether the competence-negative phenotype of Rho⁺ cells is caused by low
278 expression of the master regulator of competence ComK [71], we followed the activity of the
279 *comK* promoter (P_{comK}) during growth in competence medium using a P_{comK}-*luc* transcriptional
280 fusion [70]. In accordance with previously published data [70], we observed an increasing
281 expression of the P_{comK} promoter in WT cells up to the entry into the stationary phase (Fig 3C).
282 In the same time, P_{comK} activity was reduced about three-fold in Rho⁺ cells compared to WT
283 (Fig 3C), although remained higher than the basal expression level observed in the *spo0A*
284 mutant (S3 Fig). Thus, *comK* expression in Rho⁺ cells appears insufficient to assure a threshold
285 level of ComK required for competence induction [72].

286 In exponentially growing *B. subtilis* cells, transcription of *comK* is repressed by AbrB, Rok,
287 and CodY [32, 73, 74]; (Fig 4A) and is activated by the raise of Spo0A~P, which also relieves
288 the AbrB- and Rok-mediated repression thus opening a temporary “competence window” [70,
289 75]. Considering the low levels of Spo0A expression in Rho⁺ background in DS medium (see
290 above), it was plausible that Spo0A-mediated de-repression of *comK* was inefficient in Rho⁺
291 cells. Thus, we attempted to increase expression of *comK* by inactivating its known repressors.
292 Introduction of single *abrB* and *rok* mutations in WT cells increased activity of the P_{comK}
293 promoter about two- and three-fold, respectively (Fig 3D and E), and simultaneous inactivation

294 of both repressors synergistically stimulated *comK* expression (Fig 3F). Concordantly, we
295 observed the increased transformation efficiencies of the mutants in our test for the competence
296 state (Fig 3G). Inactivation of *abrB* or *rok* genes in Rho⁺ cells also led to the de-repression of
297 P_{*comK*}, close to or above WT levels, respectively (Fig 3D and E), and the combination of both
298 mutations led again to a synergetic five-fold increase of *comK* expression compared to WT cells
299 (Fig 3F). However, despite the strong stimulation of *comK* expression Rho⁺ cells mutated for
300 *rok* and *abrB* remained non-transformable (Fig 3G).

301 Repression activity of CodY does not depend on Spo0A~P as it relies on the nutrient and energy
302 cellular status [30, 32]. To evaluate the significance of CodY-mediated regulation of *comK*
303 expression in the context of high Rho amount, we compared the effect of the *codY* mutation on
304 P_{*comK-luc*} activity in WT and Rho⁺ cells. In our experiments, the *codY* mutation reduced the
305 growth rate of both strains in the competence-inducing medium, which explains a delayed
306 induction of P_{*comK*} compared to CodY⁺ cells, and had a variable effect on its activity (S4 Fig).
307 We attribute this discrepancy to some uncontrolled fluctuations in the nutrient content between
308 the experiments. The relatively small effect of the *codY* mutation on de-repression of *comK*
309 was observed previously [72]. More importantly, the expression of the *comK* gene in Rho⁺ cells
310 mutated for *codY* always remained below the WT level (S4 Fig). Not surprisingly, Rho⁺ *codY*
311 mutant strain appeared non-transformable (Fig 3G).

312 Altogether, these results show that a steady high level of Rho caused complex and efficient
313 repression of the competence transcription factor ComK. They also pinpoint the existence of
314 other road-blocks acting downstream of ComK which contribute to the loss of genetic
315 transformation in Rho⁺ cells.

316

317 **Comparative transcriptome analysis of *B. subtilis* WT and Rho⁺ strains.**

318 To gain deeper insight into the origins of competence- and sporulation-deficient phenotypes of
319 Rho⁺ cells and to reveal other modifications of the transcription conceivably caused by stable
320 expression of *rho*, we performed comparative RNAseq transcriptome analyses of *B. subtilis*
321 WT, Rho⁺ and $\Delta\rho$ strains grown in LB medium. Two time points corresponding to the mid-
322 exponential and early stationary phase were selected for this comparison (Materials and
323 Methods).

324 On RNAseq data, we conducted differential expression (DE) analyses of sense and antisense
325 strands of the native transcription regions (TRs) composed of 4,292 Genbank-annotated genes
326 and 1,583 other TRs (so called “S-segments”) comprising sense and antisense RNAs (asRNAs)
327 identified in WT from a large collection of expression profiles [6]. In addition to the DE analysis

328 whose results are detailed in S2 Table, we used Genoscapist [76] to set-up a web-site for online
329 interactive exploration of strain- and condition-dependent transcriptional profiles up to single-
330 nucleotide resolution; these profiles are illustrated in Fig 4 and can be accessed at
331 http://genoscapist.migale.inrae.fr/seb_rho/.

332 The RNAseq data obtained for the Δrho mutant confirmed elevated levels of antisense
333 transcription (Fig 5A, S2 Table) seen in previously published *B. subtilis* Δrho transcriptomes
334 [6, 8]. Reciprocally, we observed a global down-regulation of the antisense transcription in
335 Rho^+ cells compared to WT (Fig 5A; S2 Table), which is consistent with the well-established
336 role of Rho in the suppression of antisense transcription. Namely, we detected that transcription
337 of the antisense strands of 338 GenBank-annotated genes was down-regulated ($\log_2 Rho^+/WT$
338 ≤ -1 ; q-value ≤ 0.05) in the exponentially growing Rho^+ cells, and this trend was even more
339 pronounced in the stationary phase where antisense transcription of 1,550 genes was down-
340 regulated (Fig 4, Fig 5A and S2 Table). In particular, out of the 90 S-segments expressed in WT
341 during the stationary phase (cutoff expression level of $\log_2(fpkm+5) \geq 5$) and previously
342 documented as antisense transcripts [6], 58 S-segments were down-regulated in Rho^+ (\log_2
343 $Rho^+/WT \leq -1$; q-value ≤ 0.05). Nevertheless, only a small fraction of the down-regulated
344 antisense transcripts in Rho^+ cells are expressed in WT at levels that would be considered
345 relevant for classical genes (e.g. 24/338 and 96/1,550 for cutoff expression level of
346 $\log_2(fpkm+5) \geq 5$ in Fig 5A). This observation is consistent with relatively low levels of
347 antisense transcription in WT bacteria [77]. It explains that only a minority of the genes that
348 we report here as with decreased antisense transcription in Rho^+ were previously documented
349 as being subject to antisense transcription in WT [6].

350 Examination of transcriptional profiles along the genome revealed modifications in Rho^+ cells
351 that are typical for enhanced termination of transcription at a number of weak intrinsic and Rho-
352 specific terminators, preventing read-through transcription often in antisense of downstream
353 genes (Fig 4A). Further supporting this observation of enhanced termination in Rho^+ cells, we
354 counted that 57 out of 107 S-segments previously described [6] as resulting from a partial
355 termination or exhibiting a 3' extended mRNA in WT, displayed a decreased expression level
356 in Rho^+ ($\log_2 Rho^+/WT \leq -1$).

357 Over-expression of *rho* also caused considerable modifications of the sense-strand transcription
358 (Fig 5B and S2 Table). However, in contrast to antisense transcription for which inactivation
359 and over-expression of Rho mediated globally opposite effects (up-regulation in Δrho vs.
360 down-regulation in Rho^+), sense transcription of the Δrho and Rho^+ strains differed from WT
361 by specific patterns of up- and down-regulations on regions of low and high expression (Fig

362 5B). This is consistent with changes of the sense-strand transcription caused by a combination
363 of direct effects downstream of Rho-dependent termination sites and indirect effects resulting
364 from their propagation into regulatory cascades, as already described for Δrho [8]. With 739
365 up-regulated Genbank-annotated genes detected in the comparison Rho^+ vs. WT in stationary
366 phase, out of which 553 resulting in $\log_2(\text{fpkm}+5) \geq 5$; the increased level of Rho in the
367 stationary phase apparently caused the greatest number of indirect effects.

368 To be able to retrace the propagation of effects into regulatory cascades, we examined the
369 correlation between DE and known regulons and functional categories as defined in *SubtiWiki*
370 database ([78]; <http://subtiwiki.uni-goettingen.de>). The complete list of statistically significant
371 associations for up- and down-regulated genes in Rho^+ and Δrho (Fisher exact test p-value \leq
372 $1e-4$) is presented in S2 and S3 Tables, and was further used to investigate alterations of gene
373 expression caused by steady high level of Rho in *B. subtilis* cells. The three strongest statistical
374 associations between regulons and DE gene sets for the comparison Rho^+ vs. WT in the
375 stationary phase were for AbrB, CodY, and the stringent response regulons (p-value $\leq 1e-12$,
376 S3 Table) which are all three known as global regulatory pathways governing the transition to
377 the stationary phase.

378 Taking into account the pronounced transcriptional changes induced by Rho over-expression
379 in the stationary-phase cells, and the phenotypes of Rho^+ strain described above, we examine
380 below more closely the expression of genes controlled by ComK, AbrB, and CodY, and the
381 stringent response.

382

383 **Suppression of the ComK regulon in Rho^+ cells.**

384 As presented above, over-expression of Rho led to the inhibition of the *comK* promoter activity.
385 In line with this, the amount of the *comK* transcript was significantly reduced in Rho^+ compared
386 to WT under exponential and stationary conditions (four- and 15-fold, respectively) (S2, S3
387 Tables and Fig 6A, B). Thus, transcriptome analysis confirmed that the P_{comK} promoter was not
388 de-repressed upon entering the stationary phase in Rho^+ cells. Since ComK activates its own
389 transcription, insufficient ComK amount would impede a positive feedback autoregulation of
390 *comK* and activation of the ComK-controlled genes (Smits *et al.*, 2005). Indeed, the 34 out of
391 60 genes belonging to the ComK regulon were down-regulated more than two-fold upon entry
392 into the stationary phase; expression of 20 of them was reduced already during the exponential
393 growth (Fig. 6A, B; S2 and S3 Tables). The strongest transcriptional decrease was detected for
394 the genes involved in binding and uptake of DNA (van Sinderen *et al.*, 1995): the *comC*,

395 *comEA-EB-EC*, *comFA-FB-FC*, and *comGA-GB-GC-GD-GE-GF-GG-spoIIIL* operons with a
396 maximum reduction for the *comGC-GD-GE-GF* genes (more than 300-fold; S2 Table).

397 None of the genes involved in DNA recombination and repair (*recA*, *addAB*, *sbcD*, *ssbA*, *radA*
398 and *yisB*) were repressed in Rho⁺ cells during either exponential growth or stationary phase.
399 While the weak dependence of the *addAB* genes on ComK activity was noticed previously [79-
400 81], the stable expression of other genes might be explained by the presence of additional
401 regulatory elements.

402 In agreement with our previous results [6, 8], no significant differences were found for the
403 expression patterns of the ComK-controlled genes between WT and Δrho strains grown either
404 exponentially or stationary (S6 Fig A, B; and S2, S3 Tables).

405

406 **Rho attenuates de-repression of the AbrB-controlled genes.**

407 The amount of *B. subtilis* transition state regulator AbrB decreases rapidly upon entering the
408 stationary phase, causing subsequent de-repression of the AbrB regulon of genes [21].

409 In Rho⁺ cells, the *abrB* gene was up-regulated two-fold in the exponential- and stationary-phase
410 cells (Fig 6C, D and S2, S3 Tables). This most probably resulted from the inefficient activation
411 of Spo0A (see above), which is responsible for the *abrB* repression [25, 26]. In addition, the
412 observed three-fold decrease in *rnaC* sRNA can contribute to stabilization of the *abrB* mRNA
413 in Rho⁺ [27].

414 Accordingly, 36% and 62% of the negatively controlled genes from the AbrB regulon were
415 significantly down-regulated in Rho⁺ cells compared to WT during exponential growth and in
416 the stationary phase, respectively (Fig 6C, D; and S2, S3 Tables). For example, while the
417 operons encoding antimicrobial compounds (*pksCDE-ACP-pksFGHIJLMNR*, *ppsABCDE*,
418 *sunA*, *sboA-albG* and *bacAF*) (81 Strauch *et al.*, 2007) were effectively activated in the
419 stationary WT cells, their expression remained at a lower level in Rho⁺ (Fig 6C, S2 Table). The
420 strong decrease in transcription was also found for the *sdpABC* and the *skfABCEFGH* operons
421 encoding SdpC sporulation delay toxin and the SkfA killing factor, respectively [83]; (Fig 6C,
422 S2 Table). Transcription of the *sigH* gene, which is negatively controlled by AbrB [84],
423 increased upon entry into the stationary phase in WT cells, but was four-fold lower in Rho⁺ (S2
424 Table). This effect propagated into the SigH regulon, since almost half of the corresponding
425 genes were down-regulated in Rho⁺ cells compared to WT (S2 Table). In accordance with the
426 results showing inefficient de-repression of negatively controlled genes, we noted that several
427 operons activated by AbrB (the *rbsRKDACB*, *glpD*, *glpFK*, and *gmuBACDREFG*) [84], were
428 upregulated in the exponential Rho⁺ cells compared to WT (S2 Table). Upon entering the

429 stationary phase, the expression of all of them (with the exception of the *rbsRKDACB* operon)
430 exceeded significantly the WT level (S2 Table).

431 This analysis demonstrated that, being at a high level, Rho attenuates de-repression of the AbrB
432 regulon and thereby reduces the expression of numerous transition state genes essential for the
433 adaptation to unfavorable growth conditions.

434

435 **Rho restrains de-repression of the CodY regulon upon entry into the stationary phase.**

436 The global transcription regulator CodY controls a number of genes essential for the successful
437 transition from the exponential to stationary phase in accordance to the nutrient and energy
438 cellular status [30, 86]. During the exponential growth, all genes repressed by CodY are
439 transcribed at a low level until the cells reach the stationary phase when the activity of CodY
440 starts to decline. In line with this, our analysis showed that over 90% of the CodY-controlled
441 genes were reliably repressed in WT cells during the exponential growth in the rich LB medium
442 compared to the stationary phase (S2 Table). In Rho⁺ cells, this repression was not wholly taken
443 off during the stationary phase for more than 66% of these genes (Fig 6E and 6F; S2 and S3
444 Tables). Most of them encode proteins involved in amino acid metabolism and are controlled
445 by one or more additional regulators (e.g., AbrB, TnrA or AhrC) responding to other
446 intracellular and/or environmental signals. Others genes, like the *dppA-E* operon encoding a
447 dipeptide permease or the *nupN-Q* operon encoding the guanosine transporter, are under the
448 sole control of CodY. Expression of these genes was strongly decreased in Rho⁺ cells compared
449 to WT (from 25- to 190-fold for *dppA* and *dppE*, respectively, and 36-fold for *nupN-O* genes;
450 S2 Table).

451 Under nutrient-rich conditions, CodY prevents the development of competence and sporulation
452 [30, 32]. Besides the direct negative effect on the genetic competence through repression of the
453 *comK* promoter, CodY controls negatively the *srfAA-AB-comS-AC-AD* operon, which encodes
454 an essential component of the competence activation pathway, ComS [87]. Transcription of the
455 *comS* gene was significantly down-regulated (more than three-fold, S2 Table) in the stationary
456 Rho⁺ cells compared to WT. CodY controls directly several genes essential for the initiation of
457 sporulation, including the *kinB* gene. In the stationary-phase Rho⁺ cells, expression level of
458 the *kinB* gene was decreased 14-fold (S2 Table). However, we attributed this effect mainly to
459 the improved Rho-dependent intragenic transcription termination of *kinB* [8]. Transcription of
460 the *phrA* and *phrE* genes encoding the regulatory peptides of the Spo0F-specific phosphatases
461 RapA and RapE was repressed ~27- and nearly three-fold, respectively. All these changes fit
462 with the observed competence- and sporulation-deficient phenotypes of Rho⁺ strain.

463 We noted that genes strongly down-regulated in Rho⁺ cells fall into the clusters of genes
464 associated with the strongest CodY-binding sites, which were shown to be repressed at an
465 intermediate concentration of active CodY [88, 89]. Thus, we suggested that in the stationary-
466 phase Rho⁺ cells, growth-dependent inactivation of CodY is delayed, and CodY retains the
467 ability to control gene expression. The intermediate levels of active CodY are known to increase
468 the activity of promoters jointly repressed by CodY and another transcriptional factor, ScoC,
469 via feed-forward regulatory loop [90, 91]. Indeed, the *braB* gene and the *oppA-B-C-D-F* operon
470 known to be under the interactive CodY-ScoC regulation were up-regulated in Rho⁺ cells (five-
471 and three-fold, respectively; S2 Table). CodY-controlled gene expression pattern was not
472 significantly modified in the Δrho mutant strain (S2 and S3 Tables, S6 Fig).

473 Thus, transcription analysis indicates an ineffective inactivation of CodY in Rho⁺ cells entering
474 the stationary phase compared to WT cells. Considering that the modification of CodY activity
475 is caused by changes in intracellular pools of GTP and/or BCAA [30, 34, 92], we suggested
476 that Rho modifies the content of activated CodY through noticeable alterations in the
477 availability of CodY effectors.

478

479 **Expression patterns of the stringently controlled genes in WT and Rho⁺ cells are** 480 **significantly different.**

481 Since in *B. subtilis*, CodY activity and the stringent response are tightly linked [36], inefficient
482 de-repression of the CodY regulon in stationary Rho⁺ cells should be associated with altered
483 expression of stringently controlled genes. This prompted us to compare transcription patterns
484 of the stringently regulated genes in WT, Rho⁺ and Δrho strains.

485 We found no significant difference in the expression of the stringent regulon genes in the
486 exponentially growing WT and Rho⁺ cells (Fig 6E, S2 and S3 Tables). As expected,
487 transcription of the negatively regulated stringent response genes was decreased in WT cells
488 entering the stationary phase. Of these genes, 87% (124 out of 142) showed at least a two-fold
489 decrease in the expression levels compared to the exponential phase (S2 and S3 Tables). In
490 accordance with the former transcriptome analysis of the stringent response [93], genes
491 encoding the components of translational apparatus, including ribosomal proteins (r-proteins)
492 and translation factors were considerably repressed. Of these genes, 91% showed at least a four-
493 fold decrease in their expression levels, while 62% of them were down-regulated more than 10-
494 fold (S2 Table). Conversely, the *kinA*, *kinB*, *ftsW* and *pycA* genes previously shown to be under
495 positive stringent control [94, 95] were up-regulated from four- to eight-fold. These values fit

496 well the changes in the expression of the stringent regulon genes detected earlier in WT cells
497 [6].

498 The transcription pattern of the stringent regulon was remarkably different in the stationary
499 Rho⁺ cells (Fig 6F, S2 and S3 Tables). In total, 60% of genes negatively regulated by stringent
500 response (86 out of 142) were transcribed from two- to 9-fold more efficiently in Rho⁺ cells
501 than in WT. Almost all genes encoding r-proteins were significantly up-regulated (from two-
502 to five-fold) in Rho⁺ cells compared to WT. The relative increase in transcription of genes
503 encoding the translation factors (*tufA*, *tsf*, *fusA*, *efp*, *frr*, *fusA*, *infA*, *infB*, *infC*, *rbfA*) varied from
504 two- to four-fold. In addition, a similar increase was observed for genes involved in RNA
505 synthesis and degradation (e.g., *nusA*, *nusB*, *rnc*, and *rpoA*). On the contrary, the *kinA*, *kinB* and
506 *ftsW* genes positively regulated by the stringent response [94, 95] were less efficiently
507 transcribed in Rho⁺ cells (Fig 6F and S2 Table). The expression level of the *hpf* gene, which
508 encodes a ribosome hibernation-promoting factor and is considered as a reporter for the
509 activation of stringent response [96], was reduced two-fold in Rho⁺ compared to WT (S2
510 Table).

511 We noticed also that some genes whose transcription in response to starvation was shown to be
512 changed in a Rel-independent manner [93] had the opposite behavior in WT and Rho⁺ cells.
513 Indeed, while most of the genes encoding aminoacyl-tRNA synthetases were repressed in the
514 stationary WT cells, their transcription slightly increased in Rho⁺. In accordance with the
515 previous analyses [93, 97], we observed a decrease in transcription of genes involved in purine
516 biosynthesis in the stationary WT cells. In contrast, the *purE-K-B-C-S-Q-L-F-M-N-H-D* operon
517 genes were expressed from three- to six-fold and the *xpt-pbuX* genes up to 20-fold higher in
518 Rho⁺ cells compared to WT (S2 Table).

519 Comparison of WT and Δrho mutant strains did not reveal any global changes in the
520 transcription of the stringent regulon genes in these growth conditions (S6 Fig and S2, S3
521 Tables).

522 Overall, the present analysis revealed significant differences between the expression patterns
523 of the stringent regulon in Rho⁺ and WT cells, which suggests that when steadily expressed,
524 Rho restrains activation of the stringent response upon entry into the stationary phase.
525 Following this hypothesis, we assessed the physiological consequences of *rho* over-expression
526 on characteristic phenotypes achieved through induction of the stringent response in *B. subtilis*.

527

528 **Rho⁺ strain exhibits a modified cell morphology and decreased stationary-phase survival.**

529 In *B. subtilis* as well as in other bacteria, induction of the (p)ppGpp synthesis under nutrient
530 limitation and activation of the stringent response at stationary phase causes cell size reduction
531 [98-102]. In accordance, the (p)ppGpp-deficient cells are longer than WT cells, which
532 correlates with an altered expression of genes involved in cell shape determination and the
533 biosynthesis of fatty acids and cell wall components [93, 99, 101].

534 Microscopy analysis of the cellular morphology detected no difference between WT and Rho⁺
535 strains during exponential growth. However, while WT cells were effectively reduced in size
536 and appeared as short rods with the average length of $1.9 \pm 0.6 \mu\text{m}$ upon entering the stationary
537 phase, Rho⁺ cells remained significantly longer (average cell length $3.2 \pm 1.0 \mu\text{m}$; Fig 7A, B).
538 Consistently with the observed cell size reduction, 53% of genes involved in cell wall synthesis
539 (the gene functional categories as defined in *SubtiWiki*; [78]) were repressed from two- to ten-
540 fold in WT cells after transition to stationary phase (S2 Table). In contrast, most of these genes
541 displayed a higher expression level in Rho⁺ cells than in WT cells, during stationary phase. For
542 instance, the *murE-mraY-murD-spoVE-murG-B* gene cluster involved in the biosynthesis of
543 peptidoglycan precursors [103]; the *cwlO*, *lytE* and *ftsX* genes encoding lytic enzymes critical
544 for the cell elongation [104]; and the *mreB-C-D* genes responsible for the cell shape
545 determination [105] were expressed up to four-fold higher in the stationary Rho⁺ cells than in
546 WT (S2 Table).

547 To assess whether an abnormal size of the stationary-phase Rho⁺ cells correlates with a reduced
548 level of (p)ppGpp alarmon, we compared the ppGpp pools in WT and Rho⁺ cells using high
549 performance liquid chromatography (HPLC). Indeed, we found that Rho⁺ cells grown
550 stationary accumulated about two-fold less ppGpp compared to WT cells (Fig 7C).

551 Considering the crucial role of the stringent response in the adaptation and survival under
552 starvation conditions [100, 106-109], we next examined whether a steady high Rho amount
553 affects a long-term survival of *B. subtilis*. The growth rate and viability of Rho⁺ cells during the
554 exponential growth in LB medium were identical to those of WT (Fig 1, Fig 7D). However,
555 while almost 50% of WT cells remained viable for at least 48 hours, the viability of the Rho⁺
556 strain decreased significantly during this time, as estimated by colony formation (Fig 7D). It is
557 noteworthy that a decreased long-term survival of Rho⁺ strain does not depend on its failure to
558 sporulate, since LB medium does not support efficient sporulation, and at 48 hours, lesser than
559 0.5% of WT cells formed spores.

560 These results are consistent with RNAseq data, and show that stationary Rho⁺ cells exhibit
561 characteristic features associated with a decrease in intracellular (p)ppGpp levels.

562

563 **Rho⁺ strain exhibits phenotypic amino acid auxotrophy.**

564 *B. subtilis* mutants deficient in (p)ppGpp production ((p)ppGpp⁰) are characterized by
565 phenotypic auxotrophy for amino acids, in particular, BCAA, threonine, histidine, arginine,
566 tryptophan and methionine, provoked by a deregulation of GTP homeostasis [42, 46, 110].
567 Therefore, we tested the ability of Rho⁺ cells to form colonies on a minimal MS medium either
568 in the presence or absence of amino acids. Both WT and Rho⁺ strains grew equally well on MS
569 medium supplemented either with casamino acids (CAA) or with eight amino acids listed above
570 (Fig 8A; data shown for MS medium supplemented or not with CAA). However, omission of
571 CAA had a strong inhibitory effect on the colony-forming ability of the Rho⁺ strain, contrary
572 to WT or the Rho⁺ Q146Stop suppressor mutant of sporulation-deficiency (Fig 8A). Therefore, the
573 increased amount of active Rho protein causes phenotypic amino acid auxotrophy of *B. subtilis*.
574 It was previously shown that lowering intracellular level of GTP restores the viability of *B.*
575 *subtilis* (p)ppGpp⁰ cells in minimal medium without CAA [42, 46, 110]. To analyze whether
576 this was also true for cells over-expressing Rho, we decided to weaken the level of GTP in Rho⁺
577 strain by mutating *guaB*, the essential gene of the GTP biosynthesis pathway. This was
578 performed by introducing, in the WT and Rho⁺ strains, of the partial loss-of-function point
579 mutations *guaB* T139I and *guaB* S121F, which were previously isolated as spontaneous
580 suppressors of the poly-auxotrophy of *B. subtilis* (p)ppGpp⁰ mutant [42]. Both T139I and
581 S121F *guaB* mutations rescued the auxotrophic phenotype of Rho⁺ strain (Fig 8B), reinforcing
582 potential link between a high Rho content and the shortage of (p)ppGpp.
583 However, Rho⁺ cells did not exhibit all known phenotypes characteristic to the absence of
584 (p)ppGpp. For example, it has been shown that (p)ppGpp⁰ cells adapt poorly to a sudden
585 nutrient downshift, which results in a failure to survive the transition from amino acid-replete
586 medium to amino acid-limited medium [46]. Contrary to (p)ppGpp⁰ cells, Rho⁺ cells
587 propagated in liquid MS medium containing CAA formed colonies at a solid MS medium
588 supplemented with only eight amino acids similarly to WT strain (Fig 8C).
589 Then we tested whether Rho⁺ cells could survive treatment with a nonfunctional amino acid
590 analog arginine hydroxamate (RHX), an inhibitor of arginyl-tRNA synthesis and a powerful
591 activator of the stringent response. Rapid death upon RHX treatment is a characteristic feature
592 of (p)ppGpp⁰ cells [42, 110]. However, using the same protocol of pulse treatment of cells with
593 RHX [42], we observed a rather minor effect of RHX on the viability of Rho⁺ cells. While
594 (p)ppGpp⁰ cells survive very poorly to sudden starvation provoked by the RHX treatment, the
595 survival rate of the Rho⁺ strain was about 50% compared to 98% of WT (Fig 8D). In addition,
596 HPLC analysis revealed very similar increase of ppGpp pool in the exponentially growing WT

597 and Rho⁺ cells in response to RHX treatment (Fig 8E). Nevertheless, Rho⁺ cells appeared
598 highly sensitive to constant exposure to RHX during growth in rich LB medium or in MS
599 medium supplemented with CAA (Fig 8F).

600 Taken together, these findings converge to the conclusion that Rho⁺ strain differs markedly
601 from WT by its reduced capacity to synthesize (p)ppGpp and to induce the stringent response
602 under some stressful conditions.

603

604 **Rho weakens survival of *B. subtilis* cells under fatty acid starvation and heat stress.**

605 It has been shown that (p)ppGpp deficiency caused specifically by inhibition of the synthetic
606 activity of the bifunctional synthetase-hydrolase Rel, results in a high sensitivity to fatty acid
607 starvation and heat stress [101, 111]. Therefore, we analyzed the Rho⁺ strain for these
608 phenotypes.

609 We assessed the ability of Rho⁺ cells to adapt to fatty acid starvation using cerulenin, an
610 inhibitor of the fatty acid synthesis enzyme FabF [112]. As reported previously [101], treatment
611 with cerulenin did not affect viability of WT cells but appeared highly toxic for the (p)ppGpp⁰
612 strain. Addition of the drug had a less pronounced but significant inhibitory effect on Rho⁺ cells
613 resulting in an efficient growth arrest and concomitant loss of viability as assessed by colony
614 formation (Fig 9A and B).

615 Next, we examined the growth capacity of WT and Rho⁺ cells at 55°C, the temperature shown
616 to be non-permissive for both the (p)ppGpp⁰ and synthetase-deficient *relA* mutants [111]. While
617 growth of WT cells plated at solid LB medium was not affected at 55°C, Rho⁺ strain did not
618 form colonies at this temperature (Fig 9C). Furthermore, thermo-sensitive phenotype of Rho⁺
619 strain allowed us to isolate the mutants able to grow at 55°C. The subsequent analysis of several
620 thermo-resistant Rho⁺ clones revealed mutations of the ectopic *rho* expression unit, similarly
621 to the suppressors of the Rho⁺ sporulation deficiency (S1 Table). These findings confirm that a
622 high level of Rho underlies the heat sensitivity of Rho⁺ cells. Notably, neither *guaB* T139I nor
623 *guaB* S121F mutation did not improve the resistance of Rho⁺ strain to high temperature (S7
624 Fig), indicating that lowering the cellular GTP level is not sufficient to confer thermo-resistance
625 to *B. subtilis* (p)ppGpp-deficient cells as was shown previously [111].

626 Taken together, these results demonstrate the involvement of Rho in the control of specific
627 stress survival and suggest that synthetic activity of the main (p)ppGpp synthetase-hydrolase
628 Rel is altered in Rho⁺ cells.

629

630

631 Discussion

632

633 Soil-dwelling bacterium *B. subtilis* adapts to adverse environmental conditions by various
634 survival strategies from the adjustment of metabolic processes via the stringent response to
635 sporulation as an ultimate survival option. Here we provide evidence that the transcription
636 termination factor Rho is involved in the control of the gene regulatory networks that govern
637 adaptation of *B. subtilis* to the stationary phase and thereby in survival under suboptimal
638 environmental conditions.

639 Previously, using a Δrho mutant we have shown that Rho negatively affects the activity of
640 Spo0A, the master regulator of *B. subtilis* differentiation, by reducing the expression of the
641 major sensor kinase, KinB. Consistently, in the Δrho cells, Spo0A~P rapidly reaches a high,
642 sporulation-triggering, threshold due to an increased activity of the phosphorelay, which causes
643 accelerated sporulation above the wild type level [8]. We have proposed that Rho-mediated
644 control of the phosphorelay is an essential element of the gene regulatory network centered on
645 Spo0A. In order to maintain the proper rate of sporulation, this control should be released by a
646 programmed decrease of Rho abundance on the onset of sporulation [8].

647 To assess further the potential regulatory role of Rho, here we used the opposite experimental
648 approach by artificially maintaining *rho* transcription at a relatively high stable level to
649 compensate for the drop of *rho* expression in WT strain upon entering the stationary phase (Fig
650 1). We show that maintaining stable *rho* expression causes transcriptional reprogramming of *B.*
651 *subtilis* stationary phase and leads to considerable physiological changes, thereby affecting
652 adaptation of cells to nutrient limitations and cell-fate decision-making to such an extent that it
653 blocks competence development and sporulation.

654 While inhibition of sporulation due to the repression of *spo0A* transcription at a high Rho level
655 was expected, to some extent, as oppositely mirroring its acceleration in the *rho* mutant cells,
656 loss of genetic competence revealed a novel aspect of Rho-mediated regulation. The master
657 regulator Spo0A~P plays an essential role in the development of genetic competence by
658 relieving the *comK* gene transcription from the repression by AbrB and Rok [24, 70]. Thus,
659 strong Rho-mediated repression of the *spo0A* gene is consistent with low expression of *comK*
660 and, consequently the ComK regulon.

661 However, in spite of de-repression of the *comK* promoter in the Rho⁺ *rok*, *abrB* cells, which
662 should provide a threshold ComK level sufficient for activation of the competence genes, this
663 strain retained a competence-negative phenotype manifested by the absence of genetic

664 transformation (Fig 3G). While emphasizing a low Spo0A~P as a main cause of inefficient
665 expression of the *comK* gene in Rho⁺ cells, these results strongly suggest that a stable high level
666 of Rho also impedes the expression of other gene(s) directly or indirectly involved in the
667 development of genetic competence. There is nothing unusual about this, since many proteins
668 that have been shown to be essential for genetic transformation act independently and
669 downstream of ComK [80, 114-116]. Interestingly, many of these proteins play essential roles
670 in the RNA metabolism [114-116]. As in the case of Rho, their respective roles in the
671 development of genetic competence remain elusive [114, 116]. Further research is needed to
672 identify the potential Rho targets that may be crucial for genetic transformation.

673 In *B. subtilis*, the role of Spo0A~P goes beyond the regulation of genetic competence and
674 sporulation. Through the control of the transition state regulator AbrB, Spo0A~P mediates the
675 de-repression of genes important for adaptation to the stationary phase [21, 85]. Consequently,
676 extended repression of the AbrB regulon observed in Rho⁺ cells (Fig 6D, S2 and S3 Tables) is
677 consistent with a low Spo0A~P, which appears insufficient for negative regulation of AbrB.

678 Notwithstanding the important role of Spo0A in the cellular adaptive response to nutrient
679 limitations, our results demonstrate that a steady low level of Spo0A~P is not a sole cause of
680 the complex and coordinated reprogramming of the stationary-phase gene expression in Rho⁺
681 cells.

682 Expression of CodY, the second major regulator of the transition to the stationary phase, is
683 independent from Spo0A~P. The activity of CodY is controlled by the GTP levels, which
684 decrease upon transition to stationary phase due to an increasing synthesis of (p)ppGpp [41-43,
685 51]. Therefore, the GTP/ (p)ppGpp switch manifests itself in the synchronized activation of the
686 CodY regulon and repression of genes from the stringent regulon, which are required for growth
687 and division [36]. The RNAseq analysis unveils that, in deep contrast with WT strain, in which
688 the amount of Rho decreases upon entering the stationary phase, maintaining Rho at a roughly
689 constant level in Rho⁺ strain impedes de-repression of the CodY regulon and detains the
690 stringent response-related transcriptional changes (Fig 6F, S2 and S3 Tables).

691 There are no significant differences in the expression levels of genes from the GTP biosynthesis
692 pathways between Rho⁺ and WT strains (S2 Table). This indicates a less efficient accumulation
693 of (p)ppGpp in Rho⁺ cells, rather than an increase in GTP biosynthesis. Concordantly, we
694 detected ppGpp at about twice-lower level in stationary-phase Rho⁺ cells compared to WT and
695 demonstrated that Rho⁺ strain exhibits phenotypic poly-auxotrophy, a hallmark of *B. subtilis*
696 (p)ppGpp-deficiency ([46]; Fig 7C and 8A, B). This growth defect of Rho⁺ strain was rescued
697 by mutations that reduce the synthesis of GTP (Fig 8B), which reflects the restoration of the

698 GTP/ (p)ppGpp balance necessary for the activation of amino acids biosynthetic pathways [46,
699 110].

700 In *B. subtilis* cells, accumulation of (p)ppGpp is determined by joint activities of the alarmone
701 synthetases and hydrolases [96, 117-119]. Whereas the expression of both small (p)ppGpp
702 synthetases RelP and RelQ is mainly controlled at the transcriptional level and depends on the
703 growth phase, the bifunctional synthetase-hydrolase enzyme Rel is regulated at the allosteric
704 level [39, 40]. In addition, the activation of RelQ, which is mainly present in a “passive” state,
705 requires (p)ppGpp provided by the bi-functional Rel enzyme [120, 122]. According to the
706 RNAseq analysis, the transcription levels of the *relA*, *relP* and *relQ* genes, and (p)ppGpp
707 hydrolase gene *nahA* (*yvcI*) were similar in Rho⁺ and WT cells (S2 Table). Thus, a decreased
708 level of (p)ppGpp in stationary Rho⁺ cells cannot be caused by changes in the expression of
709 enzymes synthesizing or hydrolyzing (p)ppGpp. In this context, it is important to note that Rho⁺
710 cells exhibit a thermo-sensitive phenotype, a decreased viability during fatty acid starvation and
711 a reduced long-term survival (Fig 7D and Fig 9). Considering that the bi-functional synthetase-
712 hydrolase Rel is the main source of (p)ppGpp necessary for the survival of *B. subtilis* under
713 these stressful conditions [109, 101, 111], we assume that partially relaxed phenotype of Rho⁺
714 strain is determined by insufficient accumulation of (p)ppGpp mediated by Rel.

715 The bifunctional Rel protein can be present in a cell in two alternative states: synthetase-
716 ON/hydrolase-OFF and synthetase-OFF/hydrolase-ON for alarmone synthesis and hydrolysis,
717 respectively [119]. Accordingly, the balance between the synthetase and hydrolase activities of
718 Rel determines the intracellular levels of (p)ppGpp. The Rel-specific synthesis of (p)ppGpp is
719 triggered by ribosomal complexes harboring uncharged tRNA in the ribosomal A-site upon
720 amino-acid starvation [39, 40].

721 It is known that the enzymatic activity of divers (p)ppGpp synthetic and/or hydrolytic enzymes
722 is modulated by direct interaction with other proteins. In *E. coli*, synthetic activity of
723 bifunctional synthetase-hydrolase SpoT is triggered by the YtfK protein [123] and the acyl
724 carrier protein [124], while its hydrolase activity is stimulated by the anti-sigma factor Rsd
725 [125]. The activity of *E. coli* monofunctional (p)ppGpp synthetase RelA is inhibited by specific
726 interaction with NirD, a small subunit of the nitrite reductase [126]. In *B. subtilis* and Gram-
727 positive pathogen *Listeria monocytogenes*, cyclic-di-AMP-binding proteins DarB and CbpB,
728 respectively, stimulate the synthesis of (p)ppGpp by Rel through direct protein-protein
729 interaction under specific conditions of low intracellular cyclic-di-AMP (c-di-AMP) level [127,
730 128]. Considering that no difference was found between Rho⁺ and WT cells in the expression
731 of the *ktrA* gene, which is controlled by a c-di-AMP dependent riboswitch ([129]; S2 Table),

732 we concluded that the level of c-di-AMP remains unchanged in both strains under the
733 experimental conditions used. Thus, any particular involvement of DarB into Rel activity in
734 Rho⁺ cells seems unlikely. The late competence protein ComGA interacts with Rel, inhibiting
735 its hydrolase activity, which leads to a temporary increase of the (p)ppGpp pool in competent
736 cells [113]. One can assume that the absence of ComGA due to the inhibition of *comK*
737 expression may contribute to the stabilization of the synthetase-OFF/hydrolase-ON state of Rel
738 enzyme in Rho⁺ cells. However, the previous transcriptional analysis of the *comK* mutant [80]
739 did not reveal changes in gene expression indicative of an altered Rel activity upon entering the
740 stationary phase. In addition, unlike Rho⁺ cells, the *comK* and *comGA* mutants are heat-resistant
741 (S8 Fig), which indicates a sufficient level of (p)ppGpp synthesis mediated by Rel in these
742 strains. Altogether, these data imply that repression of *comGA* cannot underlay a decreased
743 accumulation of (p)ppGpp in Rho⁺ cells.

744 As a central element of adaptation to various stressful conditions, the stringent response
745 alarmone (p)ppGpp has a strong influence on the cell fate decision-making, regulating the
746 corresponding gene networks at different levels [41, 51, 109, 113, 120, 121, 130].

747 The (p)ppGpp is involved in the control of genetic competence through the modulation of
748 CodY activity by lowering GTP level [32, 33] and the inhibition of cell growth caused by the
749 ComGA-mediated increase of (p)ppGpp pool [113]. Thus, insufficient synthesis of (p)ppGpp
750 in Rho⁺ cells probably contributes to repression of the *comK* gene by CodY, although a low
751 Spo0A~P level appears more important for this phenomenon.

752 A sharp drop in the GTP level is one of the well-known sporulation triggers [41, 51, 130, 131].
753 It has been shown that *relA* and (p)ppGpp⁰ mutants delay *spo0A* transcription due to a weak
754 activity of the SigH-dependent promoter Ps [52, 132]. In addition, the balance between
755 (p)ppGpp and GTP has a strong effect on transcription of the *kinA* and *kinB* genes, which are
756 under positive stringent control depending on adenine as the transcription initiation nucleotide
757 [94, 95]. Thus, attenuation of (p)ppGpp synthesis provides additional negative regulation of the
758 phosphorelay, contributing to a low level of Spo0A~P in Rho⁺ cells. That is probably why the
759 artificially increased levels of KinA or KinC kinases, able to trigger sporulation even in
760 nutrient-rich conditions [14, 133], did not completely rescue the sporulation-negative
761 phenotype of Rho⁺ cells (Fig 2E, F).

762 From this point, it is important to note that the activity of Rel appears crucial throughout the
763 entire pathway of sporulation. The direct evidence for this was provided by the study of Relacin,
764 a potent inhibitor of the Rel-mediated (p)ppGpp production [109]. Authors demonstrated that
765 Relacin strongly inhibited formation of spores regardless the time at which it was added to cells

766 committed to sporulation. In addition, given an important and ever growing number of genes
767 involved in the process of spore formation downstream of the Spo0A phosphorelay [115, 134,
768 135], we cannot exclude that Rho might negatively regulate some of them.

769 Taken together, these results show that the transcription termination factor Rho imposes a new
770 layer of control over the stationary phase and post-exponential adaptive strategies. This
771 pinpoints that a *programmed* decrease of Rho levels during the transition to stationary phase is
772 crucial for the adaptation of *B. subtilis* to nutrient starvation: from the adjustment of cellular
773 metabolism and to the activation of survival programs.

774 Previous studies have shown that in *B. subtilis* and other bacteria, *rho* is negatively auto-
775 regulated through transcription attenuation mechanism at its leader mRNA [5, 136]. In
776 *Salmonella*, the small noncoding RNA SraL was shown to base pair with *rho* mRNA
777 upregulating its expression in several growth conditions [137]. This highlights the importance
778 for cells to regulate the levels of Rho, although the exact mechanism of this control in *B. subtilis*
779 remains to be established.

780 We propose that in *B. subtilis*, in addition to controlling the Spo0A phosphorelay, Rho
781 participates in the regulation of stationary phase-associated phenomena by tuning the enzymatic
782 ON/OFF balance between the synthetase and hydrolase activities of the bi-functional Rel
783 protein, thereby limiting the (p)ppGpp accumulation upon different stresses.

784 As a key player of the physiological regulation, (p)ppGpp has been shown to be important for
785 bacterial virulence, survival during host invasion, antibiotic resistance and persistence in both
786 Gram-negative and Gram-positive bacteria [138-141]. Consequently, (p)ppGpp metabolism is
787 currently recognized as a potential target for improving antimicrobial therapy [109, 139, 141].
788 Albeit the precise molecular mechanism by which Rho delays the accumulation of (p)ppGpp
789 and weakens the stringent response awaits further investigation, the unexpected involvement of
790 Rho in the metabolism of (p)ppGpp should be of particular interest, given the importance of
791 this second messenger for bacterial physiology.

792 There is increasing evidence for the important role of Rho in controlling various processes
793 connected with (p)ppGpp metabolism (cell fate decisions, virulence and antibiotic
794 susceptibility; [8, 12, 13, 16, 17, 142-144, 147]). Remarkably, mutations of the *rho* gene
795 altering Rho activity were shown to increase adaptation of *E. coli* and *B. subtilis* cells to various
796 stresses and survival under restrictive conditions [145-149].

797 Taken together, these and the present study highlight the importance of Rho-mediated
798 regulation of genes expression for adaptation to nutrient deprivation and/or other stresses, as

799 well as for the activation of the alternative survival strategies. They unveil Rho as a novel
800 stationary phase regulator and encourage future research.

801

802

803 **Materials and methods**

804 **Bacterial strains and growth conditions.**

805 *B. subtilis* strains used in the work are listed in S4 Table. Cells were routinely grown in Luria-
806 Bertani liquid or solidified (1.5% agar; Difco) medium at 37°C. Where indicated, S7 defined
807 synthetic medium [150] containing 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) and
808 supplemented with 0.1% (wt/vol) glutamate, 0.5% (wt/vol) glucose, 0.5% (wt/vol) Casamino
809 Acids (Bacto Casamino Acids), and 0.01% (wt/vol) tryptophan was used. To perform
810 fluorescence microscopy, *B. subtilis* WT and Rho⁺ cells were grown in LB medium to
811 exponential and early stationary phase (optical density OD₆₀₀ 0.2-0.3 and 1.6, respectively,
812 measured with NovaspecII Visible Spectrophotometer, Pharmacia Biotech). For amino acids
813 auxotrophy tests cells were plated on 1.5% (wt/vol) agar with Spizizen minimal salts (SM; [150]
814 supplemented with 0.5% (wt/vol) glucose, 0.1% (wt/vol) glutamate and 0.5% or 0.004%
815 (wt/vol) Casamino Acids. Standard protocols were used for transformation of *E. coli* and *B.*
816 *subtilis* competent cells [150].

817 Sporulation was analyzed in supplemented Difco Sporulation medium (Difco) [151]. To
818 determine the level of ppGpp, cells were grown in the defined SM medium. When required for
819 selection, antibiotics were added at following concentrations: 100 μg per ml of ampicillin,
820 100 μg per ml of spectinomycin, 0.5 μg per ml of erythromycin, 3 μg per ml of phleomycin, 5
821 μg per ml of kanamycin, and 5 μg per ml of chloramphenicol. IPTG (isopropyl- β-D-1-
822 thiogalactopyranoside) inducer was added to cells at concentrations indicated in the main text.

823

824 **Strains and plasmid construction.**

825 *E. coli* TG1 strain was used for plasmids construction. All *B. subtilis* constructions were
826 performed at the basis of BSB1 strain. The used oligonucleotides are listed in S6 Table.

827 To construct the system for stable Rho expression, *rho* open reading frame was fused by PCR
828 to the ribosome-binding site and spacer sequence of *B. subtilis tagD* gene using BSB1
829 chromosome as a template and oligonucleotides eb424 and eb458. The amplified fragment was
830 cloned downstream P_{veg} promoter at pDG1730 plasmid using the blunted NheI and EagI sites.

831 The resulting plasmid was transformed into *B. subtilis* BSB1 cells with selection for

832 spectinomycin-resistance leading to the integration of the *Pveg-rho* expression unit at
833 chromosomal *amyE* locus by double crossover.

834 To construct a similar system expressing the tagged Rho, *rho-SPA* DNA fragment was
835 amplified from the chromosome of BRL415 strain [8] using oligonucleotides eb458 and op148-
836 R, digested by EagI endonuclease and cloned at pDG1730 between EagI and the filled-in NheI
837 sites. The *Pveg-rho-SPA* expression unit was inserted into the BSB1 chromosome as above.

838 To overexpress Rho in the strain BRL1250 containing *P_{hy-spac}-kinC* fusion (Spec^R), *P_{veg}-rho*
839 expression unit was amplified from the chromosome of BRL802 strain using the
840 oligonucleotides opv1730-B and veb596, digested by BamHI endonuclease and cloned between
841 BamHI and blunted EcoRI sites of pSWEET vector. The resulting plasmid was used to reinsert
842 the *P_{veg}-rho* expression unit at the *amyE* locus of BSB1 strain as above with selection for
843 chloramphenicol-resistance. The resulting strain BRL1248 was controlled for sporulation- and
844 competence-negative phenotypes associated with Rho overexpression. Finally, *Pveg-rho* fusion
845 was transformed into BRL1250 cells with selection for chloramphenicol-resistance.

846 The *B. subtilis* partial loss-of-function mutants *guaB S121F* and *guaB T139I* were constructed
847 as follows. The corresponding single-nucleotide mutations *c362t* and *c416t* (coordinates
848 starting from the *guaB* +1 nucleotide) were introduced into the *guaB* gene by the two-step site-
849 directed mutagenesis. First, the DNA fragments were PCR-amplified using the complementary
850 mutagenic oligonucleotides veb852, veb853 (for *c362t*), and veb855, veb856 (for *c416t*) in
851 pairs with correspondent primers veb857, veb858 (for *c362t*), and veb857, veb859 (for *c416t*).
852 Next, the respective fragments were joined by PCR using the primers veb857 and veb858 (for
853 *c362t*) and veb857 and veb859 (for *c416t*) and cloned at the thermo-sensitive shuttle plasmid
854 pMAD [152] between Sall and EcoRI sites. The resulting plasmids were transformed in BSB1
855 cells with the selection for erythromycin-resistance at non-permissive 37°C. In this way, the
856 plasmids integrated at the chromosomal *guaB* locus by single crossover leading to *guaB*
857 duplication. The selected clones were propagated without selection at permissive 30°C to
858 induce plasmid replication and its segregation from the chromosome due to the recombination
859 between the flanking *guaB* copies. The erythromycin-sensitive clones which lost the plasmid
860 were tested for the presence of *guaB* mutations by PCR using common primer veb857 and the
861 oligonucleotides veb851 and veb854, specific for *c362t* and *c416t* mutations, respectively. The
862 selected *guaB* mutants were controlled by sequencing.

863

864 **Luciferase assay.**

865 Analysis of promoters' activity using luciferase fusions was performed as described previously
866 with minor modifications [52]. Cells were grown in LB medium to mid-exponential phase
867 (optical density OD₆₀₀ 0,4-0,5 with NovaspecII Visible Spectrophotometer, Pharmacia
868 Biotech), after which cultures were centrifuged and resuspended to OD 1.0 in fresh DS media,
869 to follow expression of *spo0A-luc* and *spoIIA-luc* fusions during sporulation, or in competence-
870 inducing MM media, to analyze *comK-luc* activity during competence development. Upon OD
871 verification, these pre-cultures were next diluted in respective media to an OD₆₀₀ 0.025. The
872 starter cultures were distributed by 200µl in a 96-well black plate (Corning, USA) and
873 Xenolight D-luciferin K-salt (Perkin, USA) was added to each well to final concentration 1.5
874 mg/mL. The cultures were incubated at 37°C with agitation and analyzed in Synergy 2 Multi-
875 mode microplate reader (BioTek Instruments). Relative Luminescence Units (RLU) and OD₆₀₀
876 were measured at 5 min intervals. Each fusion-containing strain was analyzed at least three
877 times. Each experiment included four independent cultures of each strain.

878

879 **Epifluorescence microscopy, image processing and cell measurements.**

880 Cultures of *B. subtilis* were performed as described above. Cultures were sampled during
881 exponential growth (OD₆₀₀ 0.2) and stationary phase (OD₆₀₀ 1.3), and cells were mixed with
882 Nile Red (10 µg/ml final concentration) before mounting on a 2% agarose pad and topped with
883 a coverslip. Bacteria were imaged with an inverted microscope (Nikon Ti-E), controlled by the
884 MetaMorph software package (v 7.8; Molecular Devices, LLC), equipped with a 100× oil
885 immersion phase objective. Epifluorescence images were recorded on phase-contrast and
886 fluorescence channels (ex. 562 ± 40/em. 641 ± 75 nm filters) with an ORCA-R2 camera
887 (Hamamatsu), and 100 ms exposure time. The post-acquisition treatment of the images was
888 done with the Fiji software [153, 154]. The mean cell lengths were determined with the
889 ChainTracer plugin of the Fiji software [155] on two independent experiments with N >140
890 ($N_{\text{avg}} = 290$).

891

892 **Sporulation assay.**

893 For sporulation assay, cells were diluted in LB in a way to obtain the exponentially growing
894 cultures after over-night incubation at 28°C. The pre-cultures were diluted in pre-warmed liquid
895 DS medium at OD₆₀₀ 0.025 and incubated at 37°C for 20 or 24 hours. To determine quantity
896 of the spores, half of a culture was heated at 75°C for 15 min and cells from heated and non-
897 heated samples were plated in sequential ten-fold dilutions at LB-agar plates. Colonies were

898 counted after 36 h of incubation at 37°C, and the percentage of spores was calculated as the
899 ratio of colonies forming units in heated and unheated samples. In the sporulation experiments
900 employing the IPTG-inducible systems for *kinA* or *kinC* expression, cells were let to sporulate
901 in the presence of IPTG at concentrations indicated in the text.

902 Each experiment included three independent isogenic cultures. Four independent experiments
903 were performed to establish sporulation efficiency of each strain.

904

905 **Genetic competence assay.**

906 To establish kinetics of competence development using a two-step transformation procedure
907 [150]. *B. subtilis* cells were grown in a rich defined medium SpC to stationary phase (OD₆₀₀
908 1.5) and diluted 7-fold in a competence-inducing medium SpII; at 30-min intervals, culture
909 samples (0.25 ml) were mixed with *B. subtilis* BSF4217 genomic DNA (100 ng) or pIL253
910 plasmid DNA (500 ng), incubated for 30 min at 37°C and plated at LB plates containing
911 erythromycin. Plates were incubated at 37°C for 18 h before colonies counting.

912

913 **Western blotting.**

914 The crude cell extracts were prepared using Vibracell 72408 sonicator (Bioblock scientific).
915 Bradford assay was used to determine total protein concentration in each extract. Equal amounts
916 of total proteins were separated by SDS-PAGE (10% polyacrylamide). After the run, proteins
917 were transferred to Hybond PVDF membrane (GE Healthcare Amersham, Germany), and the
918 transfer quality was evaluated by staining the membrane with Ponceau S (Sigma-Aldrich). The
919 SPA-tagged Rho protein was visualized by hybridization with the primary mouse ANTI-FLAG
920 M2 monoclonal antibodies (Sigma-Aldrich; dilution 1:5,000) and the secondary goat
921 peroxidase-coupled anti-mouse IgG antibodies A2304 (Sigma-Aldrich; dilution 1:20,000). The
922 control Mbl protein was visualized using primary rabbit anti-Mbl antibodies (dilution 1:10,000)
923 and the secondary goat peroxidase-coupled anti-rabbit IgG antibodies A0545 (Sigma-Aldrich;
924 dilution 1:10,000). Three independent experiments were performed, and a representative result
925 is shown in Fig 1B

926

927 **ppGpp determination.**

928 To determine intracellular ppGpp level, *B. subtilis* cells were grown in the defined MS medium
929 supplemented with 0.5% (wt/vol) glucose, 0.1% (wt/vol) glutamate and 0.5% (wt/vol)
930 Casamino Acids to optical densities OD₆₀₀ 0.5 (for arginine hydroxamate treatment analysis)
931 or OD₆₀₀ 1.5 (for the stationary phase analysis).

932 Bacterial cultures in triplicates (20 ml each) were rapidly centrifuged at 4°C and cellular pellets
933 were frozen in liquid nitrogen. All extraction steps were performed on ice. Cellular pellets were
934 deproteinized by addition of an equal volume of 6% perchloric acid (PCA) and incubation on
935 ice for 10 min with two rounds of vortex-mixing for 20 s. Acid cell extracts were centrifuged
936 at 13,000 rpm for 10 min at 4°C. The resulting supernatants were supplemented with an equal
937 volume of bi-distilled water, vortex-mixed for 60 s, and neutralized by addition of 2 M Na₂CO₃.
938 After filtration (3kDa cut off), extracts were injected onto a C18 Supelco 5 μm (250 × 4.6 mm)
939 column (Sigma) at 45°C. The mobile phase was delivered using the stepwise gradient of buffer
940 A (10 mM tetrabutylammonium hydroxide, 10 mM KH₂PO₄ and 0.25% MeOH; adjusted with
941 1M HCl to pH 6.9) and buffer B (5.6 mM tetrabutylammonium hydroxide, 50 mM KH₂PO₄
942 and 30% MeOH; adjusted with 1 M NaOH to pH 7.0) at a flow-rate of 1 ml/min and elution
943 program: from 60%A + 40%B at 0 min to 40%A+60%B at 30 min and 40%A+60%B at 60
944 min.

945 Detection was done with a diode array detector (PDA). The LC Solution workstation
946 chromatography manager was used to pilot the HPLC instrument and to process the data.
947 Products were monitored spectrophotometrically at 254 nm, and quantified by integration of
948 the peak absorbance area, employing a calibration curve established with various nucleoside
949 standards. ppGpp standard was purchased from Jena Bioscience GmbH (Germany). Finally, a
950 correction coefficient was applied to correct raw data for minor differences in the densities of
951 bacterial cultures.

952

953 **Transcriptome profiling by RNA sequencing.**

954 RNA was extracted from independent cultures of *B. subtilis* BsB1 WT, $\Delta\rho$ and Rho⁺ strains
955 grown in LB medium at 37°C under vigorous agitation up to mid exponential or early stationary
956 phase of growth (OD₆₀₀ ~0.5 and ~2.0, respectively). Experiments were performed in
957 duplicates for WT and mid-exponential samples and triplicates for early stationary phase of
958 $\Delta\rho$ and Rho⁺.

959 RNA preparation and DNase treatment were done as described [6]. Quality and quantity of
960 RNA samples were analyzed on Bioanalyzer (Agilent, CA). The Next Generation Sequencing
961 (NGS) Core Facility (Institute of Integrative Biology of the Cell, Gif-sur-Yvette, France;
962 <https://www.i2bc.paris-saclay.fr/sequencing/ng-sequencing/addon-ng-sequencing>) prepared
963 the RNAseq libraries with ScriptSeq protocol using RiboZero for rRNA-depletion (Illumina,
964 San Diego, California) and generated strand-specific paired-end reads of 40 bp on an Illumina
965 NextSeq platform (NextSeq 500/550 High Output Kit v2).

966 Reads were trimmed to remove adapters and low-quality ends using Cutadapt (v1.15,
967 DOI:10.14806/ej.17.1.200) and Sickle (v1.33, options: -t sanger -x -n -q 20 -l 20) and mapped
968 onto AL009126.3 reference genome assembly using Bowtie2 (v2.3.5.1; options "-N 1 -L 16 R
969 4", [156]). Counts of the number of read pairs (fragments) overlapping the sense and antisense
970 strand of each transcribed region (AL009126.3-annotated genes and S-segments from [6]) were
971 obtained with Htseq-count (v0.11.0; options "--m union --nonunique=all"; 156 Anders *et al.*,
972 2015).

973 Since Δrho , WT and Rho^+ exhibited different levels of pervasive transcription leading to global
974 changes in low expression values and antisense signal; we selected a subset of well-expressed
975 genes whose sense signal is in principle less impacted and thus most relevant for sequencing
976 depth normalization. To this end, we selected the 728 AL009126.3-annotated genes satisfying,
977 for all 4 WT samples, $\log_2(\text{fpkm_raw}+5) > 7$, where fpkm_raw refers to the fpkm (fragments
978 per kilobase of transcript per million mapped fragments) value obtained when library size is
979 simply estimated of as the sum of counts. Differential gene expression analysis between
980 conditions and strains, including sequencing depth normalization, was then conducted with R
981 library "DESeq2" (v1.32.0; [158]). DESeq2 p-values for each pairwise comparison and each
982 strand were converted into q-values using R library "fdrtool" (v1.2.17; [159]). Genes were called
983 differentially expressed between strains or conditions when the estimated q-value ≤ 0.05 and
984 $|\log_2\text{FC}|$ exceeded the cut-off specified in the text (0.5 or 1) for the considered strand (sense or
985 antisense). Data was deposited in GEO (accession number GSE195579).

986 Graphical representations of the expression level of a gene in a given strain and condition used
987 the geometrical mean of $\log_2(\text{fpkm}+5)$ values, where FPKM was computed with the DESeq2-
988 estimated library size factors multiplied by the median of sample count sums. To allow
989 interactive exploration of the sense and antisense signal along the genome with bp-resolution,
990 we also implemented in Genoscapist [76] the representation of a new data type corresponding
991 to RNAseq coverage. For this purpose, count values are extracted with "bedtools genomecov"
992 (version 2.27.0, 156.; [160]) and represented as a step function with breakpoints corresponding
993 to extremities of mapped read pairs along the genome sequence. To make these counts
994 comparable between different samples and with gene-level expression values, coverage counts
995 are converted to fpkm, using the formula $\text{fpkm_cov}(t) = \text{cov}(t) * (10^3/F) * (10^6/L)$, where $\text{cov}(t)$
996 is the coverage count for genome position t , L is the library size used to compute gene-level
997 FPKM, and F is the average fragment length (from 178 bp to 200 bp across samples) obtained
998 from the distance between extremities of inward oriented read pairs returned by "samtools stats"

999 (version 1.10, 157.; [161]). The bp-level signal, displayed as $\log_2(\text{fpkm_cov}(t)+5)$, can be
1000 accessed via the website http://genoscapist.migale.inrae.fr/seb_rho/.

1001

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1007

1008

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1012

1013

1014 **References**

1015

- 1016 1. Roberts JW. Termination factor for RNA synthesis. *Nature*. 1969; 224: 1168-1174.
- 1017 2. Richardson JP. Rho-dependent termination and ATPases in transcript termination. *BBA-*
1018 *Gene Structure and Expression*. 2002; 1577: 251-260.
- 1019 3. Boudvillain M, Figueroa-Bossi N, Bossi L. Terminator still moving forward: expanding
1020 roles for Rho factor. *Curr Opin Microbiol* 2013; 1: 118-124.
- 1021 4. Quirk PG, Dunkley Jr, E. A., Lee, P., & Krulwich TA. Identification of a putative *Bacillus*
1022 *subtilis* rho gene. *J Bacteriol*. 1993; 175: 647-654.
- 1023 5. Ingham CJ, Dennis J, Furneaux PA. Autogenous regulation of transcription termination
1024 factor Rho and the requirement for Nus factors in *Bacillus subtilis*. *Mol Microbiol*. 1999;
1025 31: 651-663.
- 1026 6. Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, *et al*. Condition-
1027 dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*.
1028 *Science*. 2012; 335: 1103-1106.
- 1029 7. Liu B, Kearns DB, Bechhofer DH. Expression of multiple *Bacillus subtilis* genes is
1030 controlled by decay of slrA mRNA from Rho-dependent 3' ends. *Nucleic Acids Res*. 2016;
1031 44: 3364-3372.

- 1032 8. Bidnenko V, Nicolas P, Grylak-Mielnicka A, Delumeau O, Auger S., Aucouturier, A., ... &
1033 Bidnenko, E. Termination factor Rho: from the control of pervasive transcription to cell fate
1034 determination in *Bacillus subtilis*. *PLoS Genet.* 2017; 13(7), e1006909.
- 1035 9. Grylak-Mielnicka A, Bidnenko V, Bardowski J, Bidnenko E. Transcription termination
1036 factor Rho: a hub linking diverse physiological processes in bacteria. *Microbiology.* 2016;
1037 162: 433-447.
- 1038 10. Peters JM, Mooney R A, Grass JA, Jessen ED, Tran F, Landick R. Rho and NusG suppress
1039 pervasive antisense transcription in *Escherichia coli*. *Genes Dev.* 2012; 26: 2621-2633.
- 1040 11. Mäder U, Nicolas P, Depke M, Pané-Farré J, Debarbouille M, van der Kooi-Pol M. M, *et*
1041 *al.* *Staphylococcus aureus* Transcriptome Architecture: From Laboratory to Infection-
1042 Mimicking Conditions. *PLoS Genet.* 2016; 1: e1005962.
- 1043 12. Lin Y, Alstrup M, Pang JKY, Maróti G, Er-Rafik M, Tourasse N, ... & Kovács ÁT.
1044 Adaptation of *Bacillus thuringiensis* to Plant Colonization Affects Differentiation and
1045 Toxicity. *Msystems.* 2021; 6: e00864-21.
- 1046 13. Fujita M, & Losick R. Evidence that entry into sporulation in *Bacillus subtilis* is governed
1047 by a gradual increase in the level and activity of the master regulator Spo0A. *Genes Dev.*
1048 2005; 19: 2236-2244.
- 1049 14. Eswaramoorthy P, Duan D, Dinh J, Dravis A, Devi SN, Fujita M. The threshold level of the
1050 sensor histidine kinase KinA governs entry into sporulation in *Bacillus subtilis*. *J Bacteriol.*
1051 2010; 192: 3870-388.
- 1052 15. Nagel A, Michalik S, Debarbouille M, Hertlein T, Gesell Salazar M,... & Mäder U.
1053 Inhibition of rho activity increases expression of SaeRS-dependent virulence factor genes
1054 in *Staphylococcus aureus*, showing a link between transcription termination, antibiotic
1055 action, and virulence. *MBio.* 2018; 9: e01332-18.
- 1056 16. Botella L, Vaubourgeix J, Livny J, & Schnappinger D. Depleting *Mycobacterium*
1057 tuberculosis of the transcription termination factor Rho causes pervasive transcription and
1058 rapid death. *Nature Commun.* 2017; 8:1-10.
- 1059 17. Trzilova, D., Anjuwon-Foster, B. R., Torres Rivera, D., & Tamayo, R. Rho factor mediates
1060 flagellum and toxin phase variation and impacts virulence in *Clostridioides difficile*. *PLoS*
1061 *pathogens.* 2020; 16: e1008708.
- 1062 18. Jaishankar J & Srivastava P. Molecular basis of stationary phase survival and applications.
1063 *Frontiers Microbiol.* 2017; 8: 2000.
- 1064 19. Phillips ZEV & Strauch MA. *Bacillus subtilis* sporulation and stationary phase gene
1065 expression. *Cell Mol Life Sciences.* 2002; 59: 392-402.

- 1066 20. Strauch MA, Spiegelman GB, Perego M, Johnson WC, Burbulys D, & Hoch JA. The
1067 transition state transcription regulator AbrB of *Bacillus subtilis* is a DNA binding protein.
1068 *The EMBO J*, 1989; 8: 1615-1621.
- 1069 21. Banse AV, Chastanet A, Rahn-Lee L, Hobbs EC, & Losick, R. Parallel pathways of
1070 repression and antirepression governing the transition to stationary phase in *Bacillus*
1071 *subtilis*. *Proc Natl Acad Sci U S A*. 2008; 105: 15547-15552.
- 1072 22. Chumsakul O, Nakamura K, Kurata T, Sakamoto T, Hobman JL, Ogasawara N, Oshima T,
1073 Ishikawa S. High-resolution mapping of in vivo genomic transcription factor binding sites
1074 using in situ DNase I footprinting and ChIP-seq. *DNA Res*. 2013; 20:325-38. doi:
1075 10.1093/dnares/dst013.
- 1076 23. Weir J, Predich M, Dubnau E, Nair G, & Smith I. Regulation of spo0H, a gene coding for
1077 the *Bacillus subtilis* sigma H factor. *J Bacteriol*. 1991; 173: 521-529.
- 1078 24. Hahn J, Roggiani M, & Dubnau D. The major role of Spo0A in genetic competence is to
1079 downregulate abrB, an essential competence gene. *J Bacteriol*. 1995; 177: 3601-3605
- 1080 25. Perego M, Spiegelman GB, Hoch JA. Structure of the gene for the transition state regulator,
1081 abrB: regulator synthesis is controlled by the spo0A sporulation gene in *Bacillus subtilis*.
1082 *Mol Microbiol*. 1988; 2:689-99. doi: 10.1111/j.1365-2958.1988.tb00079.x.
- 1083 26. Strauch M, Webb V, Spiegelman G, Hoch JA. The SpoOA protein of *Bacillus subtilis* is a
1084 repressor of the abrB gene. *Proc Natl Acad Sci USA*. 1990; 87: 1801-1805.
- 1085 27. Mars RA, Nicolas P, Ciccolini M, Reilman E, Reder A,... & Denham EL. Small regulatory
1086 RNA-induced growth rate heterogeneity of *Bacillus subtilis*. *PLoS Genet*. 2015; 11:
1087 e1005046.
- 1088 28. Sonenshein AL. CodY, a global regulator of stationary phase and virulence in Gram-
1089 positive bacteria. *Curr Opin Microbiol*. 2005; 8:203-207. doi:10.1016/j.mib.2005.01.001
- 1090 29. Sonenshein AL. Control of key metabolic intersections in *Bacillus subtilis*. *Nat Rev*
1091 *Microbiol*. 2007; 5(12):917-927. doi:10.1038/nrmicro1772
- 1092 30. Ratnayake-Lecamwasam M, Serron P, Wong KW, and Sonenshein AL. *Bacillus subtilis*
1093 CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev*. 2001; 15:
1094 1093–1103. doi: 10.1101/gad.874201
- 1095 31. Molle V, Nakaura Y, Shivers RP, Yamaguchi H, Losick R, Fujita Y, & Sonenshein AL.
1096 Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin
1097 immunoprecipitation and genome-wide transcript analysis. *J Bacteriol*. 2003; 185: 1911-
1098 1922.

- 1099 32. Serror P, & Sonenshein AL. CodY is required for nutritional repression of *Bacillus subtilis*
1100 genetic competence. *J Bacteriol.* 1996; 178: 5910-5915.
- 1101 33. Inaoka T, & Ochi K. RelA protein is involved in induction of genetic competence in certain
1102 *Bacillus subtilis* strains by moderating the level of intracellular GTP. *J Bacteriol.* 2002; 184:
1103 3923-3930.
- 1104 34. Shivers RP, & Sonenshein AL. Activation of the *Bacillus subtilis* global regulator CodY by
1105 direct interaction with branched-chain amino acids. *Mol Microbiol.* 2004; 53:599-611.
1106 doi:10.1111/j.1365-2958.2004.04135.x
- 1107 35. Potrykus K, & Cashel M (p) ppGpp: still magical? *Annu Rev Microbiol.* 2008; 62: 35-51.
- 1108 36. Geiger T, & Wolz C. Intersection of the stringent response and the CodY regulon in low
1109 GC Gram-positive bacteria. *Int J Med Microbiol.* 2014; 304:150-155.
1110 doi:10.1016/j.ijmm.2013.11.013
- 1111 37. Steinchen W. & Bange G. The magic dance of the alarmones (p) ppGpp. *Mol Microbiol.*
1112 2016; 101: 531-544.
- 1113 38. Arenz S, Abdelshahid M, Sohmen D, Payoe R, Starosta AL,... & Wilson DN. The stringent
1114 factor RelA adopts an open conformation on the ribosome to stimulate ppGpp synthesis.
1115 *Nucleic Acids Res.* 2016; 44: 6471-6481.
- 1116 39. Takada H, Roghanian M, Caballero-Montes J, Van Nerom K, Jimmy S, Kudrin P ... &
1117 Haurlyliuk V. Ribosome association primes the stringent factor Rel for tRNA-dependent
1118 locking in the A-site and activation of (p) ppGpp synthesis. *Nucleic Acids Res.* 2021; 49:
1119 444-457.
- 1120 40. Pausch P, Abdelshahid M, Steinchen W, Schäfer H, Gratani FL, Freibert SA, ... & Bange
1121 G. Structural basis for regulation of the opposing (p) ppGpp synthetase and hydrolase within
1122 the stringent response orchestrator Rel. *Cell Reports.* 2020; 32: 108157.
- 1123 41. Lopez JM, Dromerick A, & Freese E. Response of guanosine 5'-triphosphate concentration
1124 to nutritional changes and its significance for *Bacillus subtilis* sporulation. *J Bacteriol.* 1981;
1125 146: 605-613.
- 1126 42. Kriel A, Bittner AN, Kim SH, Liu K, Tehranchi AK, Zou WY, ... & Wang JD. Direct
1127 regulation of GTP homeostasis by (p) ppGpp: a critical component of viability and stress
1128 resistance. *Mol Cell.* 2012; 48: 231-241.
- 1129 43. Anderson BW, Liu K, Wolak C, Dubiel K, She F, Satyshur KA, ... & Wang JD. Evolution
1130 of (p) ppGpp-HPRT regulation through diversification of an allosteric oligomeric
1131 interaction. *Elife.* 2019; 8: e47534.

- 1132 44. Krásný L, & Gourse RL. An alternative strategy for bacterial ribosome synthesis: *Bacillus*
1133 *subtilis* rRNA transcription regulation. *The EMBO J.* 2004; 23: 4473-4483.
- 1134 45. Krásný L, Tišerová H, Jonák J, Rejman D, & Šanderová H. The identity of the transcription
1135 +1 position is crucial for changes in gene expression in response to amino acid starvation
1136 in *Bacillus subtilis*. *Mol Microbiol.* 2008; 69: 42-54.
- 1137 46. Kriel A, Brinsmade S R, Tse JL, Tehranchi AK, Bittner AN., Sonenshein AL, & Wang JD.
1138 GTP dysregulation in *Bacillus subtilis* cells lacking (p) ppGpp results in phenotypic amino
1139 acid auxotrophy and failure to adapt to nutrient downshift and regulate biosynthesis genes.
1140 *J Bacteriol.* 2014; 196: 189-201.
- 1141 47. Wang JD, Sanders GM, & Grossman AD. Nutritional control of elongation of DNA
1142 replication by (p) ppGpp. *Cell.* 2007; 128: 865-875.
- 1143 48. Corrigan RM, Bellows LE, Wood A, & Gründling A. ppGpp negatively impacts ribosome
1144 assembly affecting growth and antimicrobial tolerance in Gram-positive bacteria. *Proc Natl*
1145 *Acad Sci USA.* 2016; 113: E1710-E1719.
- 1146 49. Wood A, Irving SE, Bennison DJ, & Corrigan RM. The (p) ppGpp-binding GTPase Era
1147 promotes rRNA processing and cold adaptation in *Staphylococcus aureus*. *PLoS Genet.*
1148 2019; 15: e1008346.
- 1149 50. Diez S, Ryu J, Caban K, Gonzalez R L, & Dworkin J. The alarmones (p) ppGpp directly
1150 regulate translation initiation during entry into quiescence. *Proc Natl Acad Sci USA.* 2020;
1151 117:15565-15572.
- 1152 51. Lopez JM, Marks CL, Freese E. The decrease of guanine nucleotides initiates sporulation
1153 of *Bacillus subtilis*. *Biochim Biophys Acta.* 1979; 587:238-252. doi:10.1016/0304-
1154 4165(79)90357-x
- 1155 52. Mirouze N, Prepiak P, & Dubnau D. Fluctuations in spo0A transcription control rare
1156 developmental transitions in *Bacillus subtilis*. *PLoS Genet.* 2011. 7: e1002048.
- 1157 53. Bron S, & Luxen E. Segregational instability of pUB110-derived recombinant plasmids in
1158 *Bacillus subtilis*. *Plasmid.* 1985. 14: 235-244.
- 1159 54. Lam KHE, Chow KC, & Wong WKR. Construction of an efficient *Bacillus subtilis* system
1160 for extracellular production of heterologous proteins. *J Biotechnol.* 1998; 63: 167-177.
- 1161 55. Radeck J, Kraft K, Bartel J, Cikovic T, Dürr F, Emenegger J, ... & Mascher T. The *Bacillus*
1162 BioBrick Box: generation and evaluation of essential genetic building blocks for
1163 standardized work with *Bacillus subtilis*. *J Biol Engineering.* 2013; 7: 29.

- 1164 56. Bhavsar AP, Zhao X, & Brown ED. Development and Characterization of a Xylose-
1165 Dependent System for Expression of Cloned Genes in *Bacillus subtilis*: Conditional
1166 Complementation of a Teichoic Acid Mutant. *Appl Environ Microbiol.* 2001; 67: 403-410.
- 1167 57. Skordalakes E, & Berger JM. Structure of the Rho transcription terminator: mechanism of
1168 mRNA recognition and helicase loading. *Cell.* 2003; 114: 135-146.
- 1169 58. Thomsen ND, & Berger JM. Running in reverse: the structural basis for translocation
1170 polarity in hexameric helicases. *Cell.* 2009; 139: 523-534.
- 1171 59. Balasubramanian K, & Stitt BL. Evidence for amino acid roles in the chemistry of ATP
1172 hydrolysis in *Escherichia coli* Rho. *J Mol Biol.* 2010; 404: 587-599.
- 1173 60. D'Heygèr F, Schwartz A, Coste F, Castaing B, & Boudvillain M. ATP-dependent motor
1174 activity of the transcription termination factor Rho from *Mycobacterium tuberculosis*.
1175 *Nucleic Acids Res.* 2015; 43: 6099-6111.
- 1176 61. Wu JJ, Howard MG, Piggot PJ. Regulation of transcription of the *Bacillus subtilis* spoIIA
1177 locus. *J Bacteriol.* 1989; 171: 692-698.
- 1178 62. Chibazakura, T, Kawamura F, & Takahashi H. Differential regulation of spo0A
1179 transcription in *Bacillus subtilis*: glucose represses promoter switching at the initiation of
1180 sporulation. *J Bacteriol.* 1991; 173: 2625-2632.
- 1181 63. Fujita M, González-Pastor JE, Losick R. High-and low-threshold genes in the Spo0A
1182 regulon of *Bacillus subtilis*. *J Bacteriol.* 2005; 187: 1357-1368.
- 1183 64. Chibazakura T, Kawamura F, Asai K, & Takahashi H. Effects of spo0 mutations on spo0A
1184 promoter switching at the initiation of sporulation in *Bacillus subtilis*. *J Bacteriol.* 1995;
1185 177: 4520-4523.
- 1186 65. Chastanet A, & Losick R. Just-in-time control of Spo0A synthesis in *Bacillus subtilis* by
1187 multiple regulatory mechanisms. *J Bacteriol.* 2011; 193: 6366-6374.
- 1188 66. LeDeaux JR, Grossman AD. Isolation and characterization of kinC, a gene that encodes a
1189 sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus*
1190 *subtilis*. *J Bacteriol.* 1995; 177:166-175. doi:10.1128/jb.177.1.166-175.
- 1191 67. Kobayashi K, Shoji K, Shimizu T, Nakano K, Sato T, Kobayashi Y. Analysis of a
1192 suppressor mutation ssb (kinC) of sur0B20 (spo0A) mutation in *Bacillus subtilis* reveals
1193 that kinC encodes a histidine protein kinase. *J Bacteriol.* 1995; 177:176-182.
1194 doi:10.1128/jb.177.1.176-182.1995.
- 1195 68. Vishnoi M, Narula J, Devi SN, Dao HA, Igoshin OA, & Fujita M. Triggering sporulation
1196 in *Bacillus subtilis* with artificial two-component systems reveals the importance of proper
1197 Spo0A activation dynamics. *Mol Microbiol.* 2013; 90: 181-194.

- 1198 69. Siranosian KJ, & Grossman AD. Activation of spo0A transcription by sigma H is necessary
1199 for sporulation but not for competence in *Bacillus subtilis*. *J Bacteriol.* 1994; 176: 3812-
1200 3815.
- 1201 70. Mirouze N, Desai Y, Raj A, Dubnau D. Spo0A~P imposes a temporal gate for the bimodal
1202 expression of competence in *Bacillus subtilis*. *PLoS Genet.* 2012; 8(3):e1002586.
1203 doi:10.1371/journal.pgen.1002586.
- 1204 71. van Sinderen D, Luttinger A, Kong L, Dubnau D, Venem, G, & Hamoen L. comK encodes
1205 the competence transcription factor, the key regulatory protein for competence development
1206 in *Bacillus subtilis*. *Mol Microbiol.* 1995; 15: 455-462.
- 1207 72. Smits WK, Eschevins CC, Susanna KA, Bron S, Kuiper OP, & Hamoen LW. Stripping
1208 *Bacillus*: ComK auto-stimulation is responsible for the bistable response in competence
1209 development. *Mol Microbiol.* 2005; 56: 604-614.
- 1210 73. Hoa TT, Tortosa P, Albano M, & Dubnau D. Rok (YkuW) regulates genetic competence in
1211 *Bacillus subtilis* by directly repressing comK. *Mol Microbiol.* 2002; 43: 15-26.
- 1212 74. Hamoen LW, Kausche D, Marahiel MA, van Sinderen D, Venema G, & Serror P. The
1213 *Bacillus subtilis* transition state regulator AbrB binds to the -35 promoter region of comK.
1214 *FEMS Microbiol Lett.* 2003; 218: 299-304.
- 1215 75. Schultz D, Wolynes PG, Ben Jacob E, Onuchic JN. Deciding fate in adverse times:
1216 sporulation and competence in *Bacillus subtilis*. *Proc Natl Acad Sci U S A.* 2009; 106:
1217 21027-21034. doi:10.1073/pnas.0912185106
- 1218 76. Dérozier S, Nicolas P, Mäder U, & Guérin C. Genoscapist: online exploration of
1219 quantitative profiles along genomes via interactively customized graphical representations.
1220 *Bioinformatics.* 2021; 37: 2747-2749. doi: 10.1093/bioinformatics/btab079.
- 1221 77. Lloréns-Rico V, Cano J, Kamminga T, Gil R, Latorre A, Chen WH, ... & Lluch-Senar M.
1222 Bacterial antisense RNAs are mainly the product of transcriptional noise. *Science*
1223 *Advances.* 2016; 2: e1501363.
- 1224 78. Zhu B, & Stülke J. SubtiWiki in 2018: from genes and proteins to functional network
1225 annotation of the model organism *Bacillus subtilis*. *Nucleic Acids Res.* 2018; 46: D743-
1226 D748.
- 1227 79. Berka, RM, Hahn J, Albano M, Draskovic I, ... & Dubnau D. Microarray analysis of the
1228 *Bacillus subtilis* K-state: genome-wide expression changes dependent on ComK. *Mol*
1229 *Microbiol.* 2002; 43: 1331-1345.

- 1230 80. Ogura M, Yamaguchi H, Kobayashi K, Ogasawara N, Fujita Y, & Tanaka T. Whole-
1231 genome analysis of genes regulated by the *Bacillus subtilis* competence transcription factor
1232 ComK. *J Bacteriol* 2002; 184: 2344-2351.
- 1233 81. Boonstra M, Schaffer M, Sousa J, Morawska L, Holsappel S, ... & Kuipers, O. P. Analyses
1234 of competent and non-competent subpopulations of *Bacillus subtilis* reveal yhfW, yhxC and
1235 ncRNAs as novel players in competence. *Environ Microbiol.* 2020; 22: 2312-2328.
- 1236 82. Strauch MA, Bobay BG, Cavanagh J, Yao F, Wilson A, & Le Breton Y. Abh and AbrB
1237 control of *Bacillus subtilis* antimicrobial gene expression. *J Bacteriol* 2007; 189: 7720-
1238 7732.
- 1239 83. González-Pastor, J. E. Cannibalism: a social behavior in sporulating *Bacillus subtilis*.
1240 *FEMS Microbiol Rev.* 2011; 35: 415-424.
- 1241 84. Britton RA, Eichenberger P, Gonzalez-Pastor JE, Fawcett P, Monson R, Losick R, &
1242 Grossman AD. Genome-wide analysis of the stationary-phase sigma factor (sigma-H)
1243 regulon of *Bacillus subtilis*. *J Bacteriol.* 2002; 184: 4881-4890.
- 1244 85. Chumsakul O, Takahashi H, Oshima T, Hishimoto T, Kanaya S, Ogasawara N, Ishikawa S.
1245 Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and
1246 its homolog Abh reveals their interactive role in transcriptional regulation. *Nucleic Acids*
1247 *Res.* 2011; 39:414-28. doi: 10.1093/nar/gkq780.
- 1248 86. Slack FJ, Serror P, Joyce E, & Sonenshein AL. A gene required for nutritional repression
1249 of the *Bacillus subtilis* dipeptide permease operon. *Mol Microbiol*, 1995; 15: 689-702.
- 1250 87. Hamoen LW, Eshuis H, Jongbloed J, Venema G, & van Sinderen D. A small gene,
1251 designated comS, located within the coding region of the fourth amino acid-activation
1252 domain of srfA, is required for competence development in *Bacillus subtilis*. *Mol*
1253 *Microbiol.* 1995; 15: 55-63.
- 1254 88. Belitsky BR, Sonenshein AL. Genome-wide identification of *Bacillus subtilis* CodY-
1255 binding sites at single-nucleotide resolution. *Proc Natl Acad Sci U S A.* 2013; 23:7026-31.
1256 doi: 10.1073/pnas.1300428110.
- 1257 89. Brinsmade SR, Alexander EL, Livny J, ... & Sonenshein AL. Hierarchical expression of
1258 genes controlled by the *Bacillus subtilis* global regulatory protein CodY. *Proc Natl Acad*
1259 *Sci U S A.* 2014; 111: 8227–8232. doi:10.1073/pnas.1321308111
- 1260 90. Belitsky BR, Barbieri G, Albertini AM, Ferrari E, Strauch MA, & Sonenshein AL.
1261 Interactive regulation by the *Bacillus subtilis* global regulators CodY and ScoC. *Mol*
1262 *Microbiol.* 2015; 97: 698-716.

- 1263 91. Belitsky BR, Brinsmade SR, & Sonenshein AL. Intermediate levels of *Bacillus subtilis*
1264 CodY activity are required for derepression of the branched-chain amino acid permease,
1265 BraB. *PLoS Genet.* 2015; 11: e1005600.
- 1266 92. Brinsmade SR, & Sonenshein AL. Dissecting complex metabolic integration provides
1267 direct genetic evidence for CodY activation by guanine nucleotides. *J Bacteriol.* 2011; 193:
1268 5637-5648.
- 1269 93. Eymann, C., Homuth, G., Scharf, C., & Hecker, M. *Bacillus subtilis* functional genomics:
1270 global characterization of the stringent response by proteome and transcriptome analysis. *J*
1271 *Bacteriol.* 2002; 184: 2500-2520.
- 1272 94. Tojo S, Kumamoto K, Hirooka K, Fujita Y. Heavy involvement of stringent transcription
1273 control depending on the adenine or guanine species of the transcription initiation site in
1274 glucose and pyruvate metabolism in *Bacillus subtilis*. *J Bacteriol.* 2010; 192:1573-1585.
1275 doi:10.1128/JB.01394-09
- 1276 95. Tojo S, Hirooka K, & Fujita Y. Expression of kinA and kinB of *Bacillus subtilis*, necessary
1277 for sporulation initiation, is under positive stringent transcription control. *J Bacteriol.* 2013;
1278 195: 1656-1665.
- 1279 96. Tagami , Nanamiya, H, Kazo Y, Maehashi M, Suzuki S, Namba E, ... & Kawamura F.
1280 Expression of a small (p) ppGpp synthetase, YwaC, in the (p) ppGpp⁰ mutant of *Bacillus*
1281 *subtilis* triggers YvyD-dependent dimerization of ribosome. *Microbiol Open*, 2012; 1: 115-
1282 134.
- 1283 97. Anderson BW, Schumacher MA, Yang J, Turdiev A, Turdiev H, He Q, ... & Wang JD. The
1284 nucleotide messenger (p) ppGpp is a co-repressor of the purine synthesis transcription
1285 regulator PurR in Firmicutes. *bioRxiv.* 2020.
- 1286 98. Schreiber G, Ron EZ, and Glaser G. ppGpp-mediated regulation of DNA replication and
1287 cell division in *Escherichia coli*. *Curr Microbiol.* 1995; 30: 27-32.
- 1288 99. Traxler MF, Summers SM, Nguyen HT, Zacharia VM, Hightower GA, Smith JT, &
1289 Conway T. The global, ppGpp-mediated stringent response to amino acid starvation in
1290 *Escherichia coli*. *Mol Microbiol.* 2008; 68: 1128-1148.
- 1291 100. Chatnaparat T, Li Z, Korban SS, & Zhao Y. The bacterial alarmone (p) ppGpp is
1292 required for virulence and controls cell size and survival of *Pseudomonas syringae* on
1293 plants. *Environ Microbiol.* 2015; 17: 4253-4270.
- 1294 101. Pulschen AA, Sastre DE, Machinandiarena F, Crotta Asis A, Albanesi D, de Mendoza
1295 D, & Gueiros-Filho J. The stringent response plays a key role in *Bacillus subtilis* survival
1296 of fatty acid starvation. *Mol Microbiol.* 2017; 103: 698-712.

- 1297 102. Vadia S, Jessica LT, Lucena R, Yang Z, Kellog DR, Wang JD, & Levin PA. Fatty acid
1298 availability sets cell envelope capacity and dictates microbial cell size. *Curr Biol.* 2017; 27:
1299 1757-1767.
- 1300 103. Henriques AO, de Lencastre H, Piggot PJ. A *Bacillus subtilis* morphogene cluster that
1301 includes spoVE is homologous to the mra region of *Escherichia coli*. *Biochimie.* 1992;
1302 74:735-48. doi: 10.1016/0300-9084(92)90146-6.
- 1303 104. Brunet YR., Wang X, & Rudner DZ. SweC and SweD are essential co-factors of the
1304 FtsEX-CwlO cell wall hydrolase complex in *Bacillus subtilis*. *PLoS Genet.* 2019; 15:
1305 e1008296.
- 1306 105. Errington J, & Wu LJ. Cell cycle machinery in *Bacillus subtilis*. *Prokaryotic*
1307 *Cytoskeletons.* 2017; 67-101.
- 1308 106. Primm TP, Andersen S J, Mizrahi V, Avarbock D, Rubin H, & Barry III CE. The
1309 stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J*
1310 *Bacterial.* 2000; 182: 4889-4898.
- 1311 107. Dahl JL, Kraus CN, Boshoff HI, Doan B, Foley K, Avarbock D, ... & Barry CE. The
1312 role of RelMtb-mediated adaptation to stationary phase in long-term persistence of
1313 *Mycobacterium tuberculosis* in mice. *Proc Natl Acad Sci U S A.* 2003; 100: 10026-10031.
- 1314 108. Magnusson LU, Farewell A, & Nyström T. ppGpp: a global regulator in *Escherichia*
1315 *coli*. *Trends Microbiol.* 2005; 13: 236-242.
- 1316 109. Wexselblatt E, Oppenheimer-Shaanan Y, Kaspary I, London N, Schueler-Furma O, Yavin
1317 E, ... & Ben-Yehuda S. Relacin, a novel antibacterial agent targeting the stringent response.
1318 *PLoS Pathog.* 2012; 8:e1002925. doi: 10.1371/journal.ppat.1002925.
- 1319 110. Osaka N, Kanesaki Y, Watanabe M, Watanabe S, Chibazakura T, Takada H, ... & Asai
1320 K. Novel (p) ppGpp0 suppressor mutations reveal an unexpected link between methionine
1321 catabolism and GTP synthesis in *Bacillus subtilis*. *Mol Microbiol.* 2020; 113: 1155-1169.
- 1322 111. Schäfer H, Beckert B, Frese CK, Steinche W., Nuss AM, Beckstette M, ... & Turgay K.
1323 The alarmones (p) ppGpp are part of the heat shock response of *Bacillus subtilis*. *PLoS*
1324 *Genet.* 2020; 16: e1008275.
- 1325 112. Schujman GE, Choi KH, Altabe S, Rock CO, de Mendoza D. Response of *Bacillus*
1326 *subtilis* to cerulenin and acquisition of resistance. *J Bacteriol.* 2001; 183:3032-40. doi:
1327 10.1128/JB.183.10.3032-3040.2001
- 1328 113. Hahn J, Tanner AW, Carabetta VJ, Cristea IM, Dubnau D. ComGA-RelA interaction
1329 and persistence in the *Bacillus subtilis* K-state. *Mol Microbiol.* 2015; 97:454-71. doi:
1330 10.1111/mmi.13040.

- 1331 114. Figaro S, Durand S, Gilet L, Cayet N, Sachse M, & Condon C. *Bacillus subtilis* mutants
1332 with knockouts of the genes encoding ribonucleases RNase Y and RNase J1 are viable, with
1333 major defects in cell morphology, sporulation, and competence. *J Bacteriol.* 2013; 195:
1334 2340-2348.
- 1335 115. Koo BM, Kritikos G, Farelli JD, Todor H, Tong K, Kimsey H, ... & Gross CA.
1336 Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell*
1337 *Systems.* 2017; 4: 291-305.
- 1338 116. Benda M, Schulz LM, Stülke J, & Rismondo J. Influence of the ABC Transporter
1339 YtrBCDEF of *Bacillus subtilis* on competence, biofilm formation and cell wall thickness.
1340 *Frontiers Microbiol.* 2021; 12: 761.
- 1341 117. Nanamiya H, Kasai K, Nozawa A, Yun CS, Narisawa T, Murakami K, ... & Tozawa Y.
1342 Identification and functional analysis of novel (p) ppGpp synthetase genes in *Bacillus*
1343 *subtilis*. *Mol Microbiol.* 2008; 67: 291-304.
- 1344 118. Yang J, Anderson B W, Turdiev A, Turdiev H, Stevenson DM, Amador-Noguez D, ...
1345 & Wang JD. The nucleotide pGpp acts as a third alarmone in *Bacillus*, with functions
1346 distinct from those of (p) ppGpp. *Nature Comm.* 2020; 11: 1-11.
- 1347 119. Irving S, Choudhury NR, & Corrigan RM. The stringent response and physiological
1348 roles of (pp) pGpp in bacteria. *Nature Rev Microbiol.* 2021; 19: 256-271.
- 1349 120. Ababneh QO, & Herman JK. RelA inhibits *Bacillus subtilis* motility and chaining. *J*
1350 *Bacteriol.* 2015; 197: 128-137.
- 1351 121. Ababneh QO, & Herman JK. CodY regulates SigD levels and activity by binding to
1352 three sites in the *fla/che* operon. *J Bacteriol.* 2015; 197: 2999-3006.
- 1353 122. Steinchen W, Vogt MS, Altegoer F, Giammarinaro PI, Horvatek P, Wolz C, Bange G.
1354 Structural and mechanistic divergence of the small (p)ppGpp synthetases RelP and RelQ.
1355 *Sci Rep.* 2018; 1:2195. doi: 10.1038/s41598-018-20634-4.
- 1356 123. Germain E, Guiraud P, Byrne D, Douzi B, Djendli M, & Maisonneuve E. YtfK activates
1357 the stringent response by triggering the alarmone synthetase SpoT in *Escherichia coli*.
1358 *Nature Comm.* 2019; 10: 1-12.
- 1359 124. Battesti A, & Bouveret E. Acyl carrier protein/SpoT interaction, the switch linking
1360 SpoT-dependent stress response to fatty acid metabolism. *Mol Microbiol;* 2006; 62: 1048-
1361 1063.
- 1362 125. Lee JW, Park YH, and Seok YJ. Rsd balances (p)ppGpp level by stimulating the
1363 hydrolase activity of SpoT during carbon source downshift in *Escherichia coli*. *Proc Natl*
1364 *Acad Sci USA.* 2018; 115: E6845–E6854. doi: 10.1073/pnas.1722514115

- 1365 126. Léger L, Byrne D, Guiraud P, Germain E, & Maisonneuve E. NirD curtails the stringent
1366 response by inhibiting RelA activity in *Escherichia coli*. *Elife*. 2021; 10: e64092.
- 1367 127. Krüger L., Herzberg C, Wicke D, Bähre H, Heidemann JL, Dickmanns A, ... & Stülke
1368 J. A meet-up of two second messengers: the c-di-AMP receptor DarB controls (p) ppGpp
1369 synthesis in *Bacillus subtilis*. *Nature Comm*. 2021; 12: 1-12.
- 1370 128. Peterson BN, Young MK, Luo S, Wang J, Whiteley AT, Woodward JJ, ... & Portnoy
1371 DA. (p) ppGpp and c-di-AMP Homeostasis Is Controlled by CbpB in *Listeria*
1372 *monocytogenes*. *Mbio*. 2020; 11: e01625-20.
- 1373 129. Gundlach J, Herzberg C, Hertel D, Thürmer A, Daniel R, Link H, Stülke J. Adaptation
1374 of *Bacillus subtilis* to Life at Extreme Potassium Limitation. *mBio*. 2017. 8:e00861-17. doi:
1375 10.1128/mBio.00861-17.
- 1376 130. Ochi K, Kandala J, & Freese E. Evidence that *Bacillus subtilis* sporulation induced by
1377 the stringent response is caused by the decrease in GTP or GDP. *J Bacteriol*. 1982; 151:
1378 1062-1065.
- 1379 131. Ochi K, Kandala JC, & Freese E. Initiation of *Bacillus subtilis* sporulation by the
1380 stringent response to partial amino acid deprivation. *J Biol Chem*. 1981; 256: 6866-6875.
- 1381 132. Eymann C, Mittenhuber G, & Hecker M. The stringent response, σ H-dependent gene
1382 expression and sporulation in *Bacillus subtilis*. *Mol Gen Genetics*. 2001; 264: 913-923.
- 1383 133. Chastanet A, Vitkup D, Yuan GC, Norman TM, Liu JS, Losick RM. Broadly
1384 heterogeneous activation of the master regulator for sporulation in *Bacillus subtilis*. *Proc*
1385 *Natl Acad Sci USA*. 2010; 107: 8486-8491.
- 1386 134. Meeske AJ, Rodrigues CD, Brady J, Lim HC, Bernhardt TG, & Rudner DZ. High-
1387 throughput genetic screens identify a large and diverse collection of new sporulation genes
1388 in *Bacillus subtilis*. *PLoS Biology*. 2016; 14: e1002341.
- 1389 135. Shi L, Derouiche A, Pandit S, Rahimi S, Kalantari A, Futo M, ... & Mijakovic I.
1390 Evolutionary analysis of the *Bacillus subtilis* genome reveals new genes involved in
1391 sporulation. *Mol Biol and Evolution*. 2020; 37: 1667-1678.
- 1392 136. Chen J, Morita T, & Gottesman S. Regulation of transcription termination of small
1393 RNAs and by small RNAs: molecular mechanisms and biological functions. *Frontiers Cell*
1394 *Infection Microbiol*. 2019; 9: 201.
- 1395 137. Silva IJ, Barahona S, Eyraud A, Lalaouna D, Figueroa-Bossi N, Massé E, & Arraiano
1396 CM. SraL sRNA interaction regulates the terminator by preventing premature transcription
1397 termination of rho mRNA. *Proc Natl Acad Sci U S A*. 2019; 116: 3042-3051.

- 1398 138. Schofield WB, Zimmermann-Kogadeeva M, Zimmerman M, Barry NA, & Goodman
1399 AL. The stringent response determines the ability of a commensal bacterium to survive
1400 starvation and to persist in the gut. *Cell Host & Microbe*. 2018; 24: 120-132.
- 1401 139. Kushwaha GS, Oyeyemi BF, & Bhavesh NS. Stringent response protein as a potential
1402 target to intervene persistent bacterial infection. *Biochimie*. 2019; 165: 67-75
- 1403 140. Fernández-Coll L, & Cashel M. Possible roles for basal levels of (p) ppGpp: growth
1404 efficiency vs. surviving stress. *Frontiers Microbiol*. 2020; 11
- 1405 141. Pacios O, Blasco L, Bleriot I, Fernandez-Garcia L, Ambroa A, López M, ... & Tomás
1406 M. (p) ppGpp and its role in bacterial persistence: New challenges. *Antimicrobial agents
1407 and chemotherapy*. 2020; 64: e01283-20.
- 1408 142. Lee YH, & Helmann JD. Mutations in the primary sigma factor σ and termination
1409 factor rho that reduce susceptibility to cell wall antibiotics. *J Bacteriol*. 2014; 196: 3700-
1410 3711.
- 1411 143. Liu B, Kearns DB, Bechhofer DH. Expression of multiple *Bacillus subtilis* genes is
1412 controlled by decay of slrA mRNA from Rho dependent 3' ends. *Nucleic Acids Res*. 2016;
1413 44:3364–3372.
- 1414 144. Hafeezunnisa M, & Sen R. The Rho-dependent transcription termination is involved in
1415 broad-spectrum antibiotic susceptibility in *Escherichia coli*. *Front Microbiol*. 2020; 11:
1416 3059.
- 1417 145. Freddolino PL, Goodarzi H, Tavazoie S. Fitness landscape transformation through a
1418 single amino acid change in the Rho terminator. *PLoS Genet*. 2012; 8:e1002744.
- 1419 146. Haft RJ, Keating DH, Schwaegler T, Schwalbach MS, Vinokur J,... & Landick R.
1420 Correcting direct effects of ethanol on translation and transcription machinery confers
1421 ethanol tolerance in bacteria. *Proc Natl Acad Sci U S A*. 2014; 111: E2576-E2585.
1422 doi:10.1073/pnas.1401853111
- 1423 147. Bidnenko E, & Bidnenko V. Transcription termination factor Rho and microbial
1424 phenotypic heterogeneity. *Current Genetics*. 2018; 64: 541-546.
- 1425 148. Hashuel R, & Ben-Yehuda S. Aging of a bacterial colony enforces the evolution of
1426 nondifferentiating mutants. *MBio*. 2019; 10.5: e01414-19
- 1427 149. Yu Y, Dempwolff F, Oshiro RT, Gueiros-Filho FJ, Jacobson SC, & Kearns DB. The
1428 division defect of a *Bacillus subtilis* minD noc double mutant can be suppressed by Spx-
1429 dependent and Spx-independent mechanisms. *J Bacteriol*. 2021; 203: e00249-21.
- 1430 150. Harwood CR and Cutting SM. *Molecular Biological Methods for Bacillus*. Wiley, 1990.

- 1431 151. Schaeffer P, Millet J, & Aubert J-P. Catabolic repression of bacterial sporulation. Proc
1432 Natl Acad Sci USA. 1965; 54: 704–711. pmid:4956288
- 1433 152. Arnaud M, Chastanet A, & Débarbouillé M. New vector for efficient allelic replacement
1434 in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ
1435 Microbiol. 2004; 70: 6887-6891.
- 1436 153. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M,... & Cardona A. Fiji:
1437 an open-source platform for biological-image analysis. Nat Methods 2012; 9: 676-682.
- 1438 154. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, Eliceiri KW.
1439 ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinformatics.
1440 2017; 18: 529.
- 1441 155. Syvertsson S, Vischer NO, Gao Y, and Hamoen LW. When Phase Contrast Fails:
1442 ChainTracer and NucTracer, Two ImageJ Methods for Semi-Automated Single Cell
1443 Analysis Using Membrane or DNA Staining. PLoS One. 2016; 11: e0151267.
- 1444 156. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods.
1445 2012; 4;9(4):357-9. doi: 10.1038/nmeth.1923. PMID: 22388286; PMCID: PMC3322381.
- 1446 157. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput
1447 sequencing data. Bioinformatics. 2015 Jan 15;31(2):166-9. doi:
1448 10.1093/bioinformatics/btu638. Epub 2014 Sep 25. PMID: 25260700; PMCID:
1449 PMC4287950.
- 1450 158. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for
1451 RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550. doi: 10.1186/s13059-014-
1452 0550-8. PMID: 25516281; PMCID: PMC4302049.
- 1453 159. Strimmer K. fdrtool: a versatile R package for estimating local and tail area-based false
1454 discovery rates. Bioinformatics. 2008 Jun 15;24(12):1461-2. doi:
1455 10.1093/bioinformatics/btn209. Epub 2008 Apr 25. PMID: 18441000.
- 1456 160. Quinlan AR. BEDTools: The Swiss-Army Tool for Genome Feature Analysis. Curr
1457 Protoc Bioinformatics. 2014 Sep 8;47:11.12.1-34. doi: 10.1002/0471250953.bi1112s47.
1458 PMID: 25199790; PMCID: PMC4213956.
- 1459 161. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, Whitwham A,
1460 Keane T, McCarthy SA, Davies RM, Li H. Twelve years of SAMtools and BCFtools.
1461 Gigascience. 2021 Feb 16;10(2):giab008. doi: 10.1093/gigascience/giab008. PMID:
1462 33590861; PMCID: PMC7931819.

1463

1464

1465 **Figure legends**

1466

1467 **Fig 1. Heterogenic expression of the transcription termination factor Rho.** *B. subtilis* cells
1468 expressing SPA-tagged Rho protein from natural (WT) and Pveg (Rho⁺) promoters were grown
1469 in LB medium (A) and analyzed for Rho protein content at the indicated time points by
1470 immunoblotting with ANTI-FLAG M2 monoclonal antibodies (Rho-SPA; B). Equal amounts
1471 of total protein extracts were loaded onto the gel according to Bradford assay; samples'
1472 equilibrium between the strains at each time-point and the quality of transfer were controlled
1473 by visualization of Mbl protein using specific anti-Mbl antibodies (Mbl; B). Note that the Mbl
1474 levels decrease in the stationary phase.

1475

1476 **Fig 2. *B. subtilis* Rho⁺ strain exhibits sporulation-negative phenotype.** (A) Sporulation
1477 efficiency of *B. subtilis* WT, Δrho and Rho⁺ cells. Cells inoculated at OD₆₀₀ 0.025 were grown
1478 in DS medium at 37° for 20 hours and analyzed for heat-resistant spores as described in
1479 Materials and Methods. Sporulation efficiency was estimated as proportion of viable cells in
1480 the heated and unheated cultures. Plotted are the mean values from four independent
1481 experiments with three biological replicas for each strain, and the SDs \leq 10%. (B) Schematics
1482 of the multicomponent Spo0A phosphorelay. Only the key elements relevant to this study are
1483 shown. Phosphoryl groups are transferred from sensor protein kinases (KinA-E) to Spo0F,
1484 Spo0B, and ultimately to Spo0A. Sporulation is triggered when the level of Spo0A~P reaches
1485 a high threshold level. The bar-headed line indicates negative Rho-mediated regulation of KinB
1486 expression. (C) Kinetics of luciferase Luc expression from the promoter of early sporulation
1487 gene *spoIIAA* in *B. subtilis* WT (blue line) and Rho⁺ (red line) cells induced for sporulation. (D)
1488 Kinetics of luciferase Luc expression from the promoters of *spo0A* gene in WT (blue line), Rho⁺
1489 (red line) and *sigH* mutant (green line) cells induced for sporulation. In C and D, cells bearing
1490 transcriptional fusions *spoIIAA-luc* and *spo0A-luc*, respectively, were grown in DS medium and
1491 analyzed for luciferase activity at five-minutes intervals in a multimode microplate reader as
1492 described in Materials and Methods. For each strain, plotted are the mean values of
1493 luminescence readings corrected for OD₆₀₀ from four independent cultures analyzed
1494 simultaneously (solid lines with symbols) and characteristic growth curves (double-lined)
1495 measured by OD₆₀₀. The experiments were reproduced at least three times. The results from
1496 the representative experiments are presented. (E and F) Synthetic over-production of sensor

1497 histidine kinases KinA or KinC does not rescue sporulation-negative phenotype of Rho⁺ cells.
1498 Sporulation efficiency of *B. subtilis* WT and Rho⁺ strains and their respective derivatives
1499 expressing *kinA* (E) and *kinC* (F) genes under control of the IPTG-inducible promoter. Cells
1500 were inoculated at OD₆₀₀ 0.025 in DS medium containing IPTG at the indicated concentrations
1501 and grown at 37° during 20 hours; sporulation efficiency was analyzed as described above (Fig
1502 2A) and in Materials and Methods. Plotted are the mean values from three independent
1503 experiments with three biological replicas of each strain (rectangulars) with standard deviation
1504 SD (bar-headed lines).

1505

1506 **Fig 3. *B. subtilis* Rho⁺ exhibits competence–negative phenotype.** (A) Schematics of P_{comK}
1507 regulation in *B. subtilis* cells. Arrows and bar-headed lines represent positive and negative
1508 effects, respectively. (B) Kinetics of competence development in *B. subtilis* WT (blue line),
1509 Rho⁺ (red line) and Rho⁺_{Q146Stop} (red double-line) strains. Cells grown in a defined rich medium
1510 to stationary phase were transferred to the competence-inducing medium (T0) and tested for
1511 transformation by homologous genomic DNA over three hours as described in Materials and
1512 Methods. The experiment incorporated three biological replicas of each strain and was
1513 reproduced three times. Plotted are the mean values and SD from a representative experiment.
1514 (C) Kinetics of luciferase expression in *B. subtilis* WT (blue line) and Rho⁺ (red line) cells
1515 bearing the P_{comK-luc} transcription fusion and grown in competence-inducing medium. Plotted
1516 are the mean values of luminescence readings corrected for OD from four independent cultures
1517 of each strain analyzed simultaneously. The double-lined curves depict characteristic growth
1518 kinetics of cells measured by OD₆₀₀. (D, E and F) Kinetics of luciferase expression in *B.*
1519 *subtilis* mutant strains: (D) *abrB* P_{comK-luc} (green line) and Rho⁺ *abrB* P_{comK-luc} (green double-
1520 line); (E) *rok* P_{comK-luc} (light blue line) and Rho⁺ *rok* P_{comK-luc} (light blue double-lines); (F) *abrB*,
1521 *rok* P_{comK-luc} (purple line) and Rho⁺ *abrB*, *rok* P_{comK-luc} (purple double-line). The indicated
1522 mutant pairs were analyzed in parallel with the control parental strains WT P_{comK-luc} (blue line)
1523 and Rho⁺ P_{comK-luc} (red line). For each strain, data acquisition and processing were performed
1524 as in (C). Each strain was analyzed at least three times. The results of representative
1525 experiments are shown.

1526 (G) Inactivation of the known repressors of *comK* does not rescue competence–negative
1527 phenotype of Rho⁺ cells. Effect of Rho over-production on transformation efficiency of *B.*
1528 *subtilis* WT and Rho⁺ strains and their respective derivatives carrying single mutations in the
1529 *abrB*, *rok*, *codY* genes or double mutations in *abrB*, *rok* genes. Cells were transformed by donor

1530 genomic DNA after two hours of growth in competence-inducing medium as described in
1531 Materials and Methods. Shown are the mean values with SD (in brackets) from two independent
1532 experiments each incorporating three biological replicas of each strain.

1533

1534 **Fig 4. Rho-mediated control of pervasive transcription.** Examples of expression profiles of
1535 *B. subtilis* WT, Rho⁺ and $\Delta\rho$ strains at two loci (**A** and **B**) as measured by RNAseq in
1536 exponential (middle panel) and stationary (bottom panel) growth phases for both strands of the
1537 genome (+ and -). The whole genome can be browsed at
1538 http://genoscapist.migale.inrae.fr/seb_rho. The top panel presents the structural organization of
1539 the region including the GenBank annotation, S-segments and transcription units (TUs) as
1540 determined from a compendium of WT expression profiles by Nicolas *et al.* (2012). Triangle
1541 and square flags positioned on the TU lane (red) represent identified abrupt transcriptional up-
1542 and down-shifts often associated with promoter/terminator activity. The colors of expression
1543 profiles (middle and bottom panels) distinguish strains and growth phases: WT (light and dark
1544 green lines for exponential and stationary phase, respectively), Rho⁺ (orange and red) and $\Delta\rho$
1545 (light and dark blue). Inactivation of Rho in $\Delta\rho$ mutant leads to the mRNA extension of the
1546 3'UTR of *penP* gene (S706, antisense of *yobA-yozU*, sense of *yobB*) (**A**) and increases the
1547 expression of the asRNA (S125, antisense of *tlpC-hxlB-hxlA*, sense of *hxlR*) from its own
1548 promoter (**B**). Opposite effects are observed in Rho⁺. While $\Delta\rho$ and Rho⁺ profiles are clearly
1549 distinguished in both conditions, the WT is intermediate with a position closer to Rho⁺ in
1550 exponential phase and to $\Delta\rho$ in stationary phase (*i.e.* consistent with the decrease of Rho
1551 abundance upon transition to the stationary phase in WT).

1552

1553 **Fig 5. Graphical summary of differential sense and antisense expression (DE) in Rho⁺**
1554 **and $\Delta\rho$ across strands and growth phases.** DE compared to WT (q-value \leq 0.05 and
1555 $|\log_2\text{FC}|\geq 1$) is shown for the antisense (**A**) and sense (**B**) strands of the 4,292 AL009126.3-
1556 annotated genes. Barplot representation of the numbers of DE genes: numbers reported above
1557 each bar correspond to the total of DE genes and, between parentheses, to the subset exhibiting
1558 a minimal expression of $\log_2(\text{fpkm}+5) \geq 5$ in one of the two compared genetic backgrounds.
1559 Heatmap highlighting overlaps between these sets of DE genes: up-regulated genes in yellow,
1560 down-regulated genes in blue, other genes in gray. Left-side of each heatmap: average-link
1561 hierarchical clustering tree based on pairwise distance between genes (L1-norm after encoding
1562 down-regulation and up-regulation as -1 and 1, 0 otherwise).

1563

1564 **Fig 6. Differential expression of the ComK, AbrB, CodY and stringent response regulons**
1565 **in Rho⁺ strain across exponential growth and stationary phase.** Expression changes for the
1566 each gene from the ComK regulon (**A** and **B**), AbrB regulon (**C** and **D**), CodY and the stringent
1567 response regulon (**E** and **F**) by comparing *B. subtilis* Rho⁺ and WT strains under conditions of
1568 exponential growth (A, C, E) and stationary phase (B, D, F). Each point (red for the ComK
1569 regulon, green for the AbrB regulon, blue for the CodY regulon, yellow for the stringent
1570 response regulon, and gray for genes outside of the mentioned regulons) represents one of the
1571 4,292 AL009126.3-annotated genes. Gene names mentioned in the text are indicated.

1572

1573 **Fig 7. Morphology and long-term survival of *B. subtilis* Rho⁺ cells.** Microscopy images (**A**)
1574 and cell length measurement (**B**) of *B. subtilis* WT and Rho⁺ under conditions of exponential
1575 growth and stationary phase. (A) Phase contrast (upper panel) and fluorescence (lower panel)
1576 images of WT and Rho⁺ cells stained with Nile Red at OD₆₀₀ 0.2 and 1.6. Scale bars
1577 correspond to 6 μm. (B) The post-acquisition treatment of the images and determination of the
1578 mean cell lengths was as described in Materials and Methods. Statistical significance was
1579 estimated with a nested t-test, performed with Prism 9 (GraphPad Software, LLC). Note that
1580 the plot show the pooled values of the replicates. *P*-values are displayed as follows: **** =
1581 $P < 0.0001$; *** = $0.0001 < P < 0.001$; ** = $0.001 < P < 0.01$; * = $0.01 < P < 0.05$; ns = $P > 0.05$; ns, non
1582 significant ($p > 1.0$) using a nested t-test. (C) Level of ppGpp under stationary phase. *B. subtilis*
1583 BsB1 WT and Rho⁺ cells were grown in MS medium supplemented with 0.5% (w/v) of
1584 casamino acids to the stationary phase (OD₆₀₀ 1.5). The ppGpp levels were assessed as
1585 described in Materials and Methods. Plotted are the mean values and SD from three independent
1586 experiments. **, $p \leq 0.01$ using a two-tailed t-test. (D) Effect of Rho on long-term survival of
1587 *B. subtilis*. *B. subtilis* WT and Rho⁺ cells were grown in LB medium at 37°C with vigorous
1588 shaking during 48 hours. At the specified growth time, cells were plated on LB plates, and cell
1589 survival in cultures was assessed by the number of viable cells forming colonies (CFU) after
1590 18 hours of incubation at 37°C. Plotted are the average values from three independent
1591 experiments each incorporating three biological replicas of each strain. ***, $p \leq 0.001$ using a
1592 two-tailed t-test.

1593

1594 **Fig 8. Adaptation of *B. subtilis* Rho⁺ to a sudden nutrient downshift.** (A) Rho⁺ cells exhibit
1595 phenotypic amino acid auxotrophy. *B. subtilis* WT, Rho⁺ and Rho⁺_{Q146Stop} cells growing
1596 exponentially (OD₆₀₀ 0.5) in liquid S7 medium containing 0.5% (w/v) of casamino acids (CAA)
1597 at 37°C were spotted in serial dilutions on MS agar plates supplemented or not with CAA
1598 (0.5%). Plates were incubated at 37°C during 18h before imaging. (B) Decreasing of GTP
1599 level rescues the auxotrophic phenotype of Rho⁺ cells. *B. subtilis* WT, Rho⁺ and their respective
1600 derivative strains carrying *guaBS121F* and *guaBT139I* mutations were cultivated in liquid S7
1601 medium containing 0.5% (w/v) of CAA and tested for the ability to grow in the absence of
1602 CAA as in (A). (C) Unlike (p)ppGpp⁰ cells, Rho⁺ strain resists to mild nutrient limitations.
1603 Isogenic WT, (p)ppGpp⁰ and Rho⁺ strains were grown in liquid MS medium containing 0.5%
1604 (w/v) of CAA and spotted in serial dilutions on MS agar plates supplemented either with 0.5%
1605 (w/v) of CAA or with 0.05mg/ml of each of the eight amino acids (valine, isoleucine, leucine,
1606 threonine, histidine, arginine, tryptophan and methionine). Plates were incubated at 37°C during
1607 18h before imaging. In (A, B and C), the experiments were reproduced at least three times
1608 and the representative results are shown. (D, E and F) Rho⁺ cells exhibit altered resistance to
1609 arginine hydroxamate (RHX). (D) Isogenic *B. subtilis* WT, (p)ppGpp⁰ and Rho⁺ strains were
1610 grown to the middle exponential phase (OD₆₀₀ 0.5), treated with 500 µg/ml of RHX for 40
1611 min and plated on LB agar plates. Plates were incubated for 18 h at 37°C before counting viable
1612 cells that formed colonies. Strain survival upon sudden amino acid starvation induced by RHX
1613 was estimated as the percentage of viable cells after and before the treatment. Plotted are the
1614 average values and SD from three independent experiments incorporating three biological
1615 replicas of each strain. **, P ≤ 0.005 using a two-tailed t-test. (E) Increase of ppGpp level
1616 following sudden amino acid starvation. *B. subtilis* WT and Rho⁺ cells were grown in MS
1617 medium supplemented with 0.5% (w/v) of CAA to the middle exponential phase (OD₆₀₀ 0.5)
1618 and treated or not with 500 µg/ml of RHX. Cells were harvested 20 min after addition of RHX,
1619 and ppGpp levels were assessed as described (Materials and Methods). Plotted are the average
1620 values and SD from three independent experiments. ****, p ≤ 0.0001; ***, p ≤ 0.001; **, p
1621 ≤ 0.01; ns, non-significant (p > 1.0) using two-tailed t-test. (F) Growth defect of Rho⁺ strain in
1622 the presence of RHX. *B. subtilis* WT and Rho⁺ strains were cultivated in LB medium without
1623 or with RHX added at concentrations 50 and 100µg/ml in a 96-well microplate. Growth of the
1624 cultures was monitored by OD₆₀₀ measurement at the five-minute intervals using a microplate
1625 reader. Plotted curves are the average OD reads of two independent cultures of each strain

1626 grown in triplicates at each condition. The analysis was performed three times; the results of a
1627 representative experiment are presented.

1628

1629 **Fig 9. Sensitivity of *B. subtilis* Rho⁺ to fatty acid starvation and heat stress.** Growth curves
1630 (A) and viability (B) of *B. subtilis* WT (blue lines), its isogenic (p)ppGpp⁰ (green lines) and
1631 Rho⁺ (red lines) strains non-treated (solid lines; circles) or starved for fatty acids by treatment
1632 with cerulenin (+ C; double lines; triangles). Cells were grown in LB at 37°C to OD₆₀₀ 0.1, and
1633 cerulenin was added to the halves of cultures to final concentration 10µg/ml. Cell survival was
1634 estimated by plating bacterial cultures at the indicated time on LB agar and counting of CFUs
1635 after 18 h of incubation at 37°C. Each data point in B is the mean of at least three counts. (C)
1636 Colony formation of *B. subtilis* WT and its isogenic (p)ppGpp⁰, Rho⁺ and Rho⁺_{Q146Stop} (Sup 1)
1637 strains at 37°C and 55°C. Cell cultures growing exponentially in LB medium at 37°C were
1638 spotted in serial dilutions (from 0 to 10⁻⁵) on LB agar plates and incubated at 37°C or at 55°C
1639 for 18 h before imaging.

1640

1641

1642 **Supporting Information**

1643

1644 **S1 Fig. Graphical representation of the sporulation-proficient suppressor mutations in**
1645 **Rho from *B. subtilis* Rho⁺ strain.** The primary sequence of *B. subtilis* Rho subunit is shown
1646 (NP_391589.2). The major motifs (as in D'Heygère *et al.*, 2015) are boxed and highlighted in
1647 grey. The identified amino acid substitutions are marked in red.

1648

1649 **S2 Fig. *B. subtilis* Rho⁺ exhibits competence–negative phenotype.** Transformability of *B.*
1650 *subtilis* WT (blue lines) and Rho⁺ (red lines) strains by the plasmid pIL253 (closed circles) and
1651 homologous genomic DNA (open circles). Competence induction and transformation were
1652 performed as described in Materials and Methods and Fig 3B. The experiment included three
1653 biological replicas of each strain and was reproduced twice. The results of a representative
1654 experiment are presented. The data are independent from Fig 3B.

1655

1656 **S3 Fig. Rho⁺ strain differs from *spo0A* mutant in the activation of *comK*.** Kinetics of
1657 luciferase expression in *B. subtilis* WT (blue lines), Rho⁺ (red lines) and *spo0A* (gray lines)

1658 mutant cells bearing the $P_{comK-luc}$ transcription fusion and grown in competence-inducing
1659 medium as described in Materials and Methods. For each strain, plotted are the mean values of
1660 luminescence readings corrected for OD from four independent cultures analyzed
1661 simultaneously. The double-lined curves depict characteristic growth kinetics of cells measured
1662 by OD₆₀₀. The experiment was reproduced three times and is independent from Fig 2C. The
1663 data from a representative experiment are presented.

1664

1665 **S4 Fig. CodY inactivation does not restore *comK* expression in Rho⁺ cells.** Kinetics of
1666 luciferase expression from the $P_{comK-luc}$ transcription fusion in *B. subtilis* WT (blue open
1667 squares), Rho⁺ (red open circles) cells and their respective *codY* mutants (fill-in blue and red
1668 symbols) grown in competence-inducing medium. For each strain, plotted are the mean values
1669 of luminescence readings corrected for OD from four independent cultures analyzed
1670 simultaneously. Characteristic growth kinetics of WT and Rho⁺ cells and of their *codY*
1671 derivatives are depicted by double- and single-lined curves, respectively. Presented are the
1672 results of two independent experiments performed using freshly prepared media.

1673

1674 **S5 Fig. Genome wide effects of Rho over-production on the *B. subtilis* transcriptome**
1675 **during exponential growth and stationary phase in rich medium.** Transcriptome changes in
1676 the antisense (A and B) and sense (C and D) strands during exponential growth (A and C) and
1677 stationary phase (B and D), respectively. Each point represents one of the 4,292 AL009126.3-
1678 annotated genes. Coordinates on x- and y-axes correspond to the normalized expression level
1679 (average of log₂(fpkm+5) over biological replicates) measured with RNAseq in *B. subtilis* WT
1680 and Rho⁺, respectively. Background colors of the points indicate TRs whose transcription level
1681 is strongly up-regulated (yellow) or down-regulated (blue) in the Rho⁺ vs. WT comparison
1682 made in *B. subtilis* by RNAseq.

1683

1684 **S6 Fig. Differential expression of the ComK, CodY and stringent response regulons in *B.***
1685 ***subtilis* Δrho strain.** Expression changes for each gene from the ComK regulon (A, B), CodY
1686 and the stringent response regulons (C, D) by comparing *B. subtilis* Δrho and WT strains under
1687 exponential growth (A and C) and stationary phase (B and D) conditions. Each point (red for
1688 the ComK regulon, blue for the CodY regulon, yellow for the stringent response regulon, and
1689 gray for genes outside of the mentioned regulons) represents one of the 4,292 AL009126.3-
1690 annotated genes.

1691

1692 **S7 Fig. Lowering GTP levels does not rescue thermo-sensitive phenotype of Rho⁺ strain.**

1693 *B. subtilis* WT, Rho⁺ cells and their respective *guaB* S121F and *guaB* T139I mutants were
1694 grown in LB medium at 37°C to mid exponential phase (OD₆₀₀ 0.5), spotted in serial dilutions
1695 on LB agar plates and incubated at 37°C and 55°C for 18 hours. The experiment was reproduced
1696 at least three times and the representative results are shown.

1697

1698 **S8 Fig. Thermo-sensitivity of Rho⁺ strain is not due to a low level of *comGA* expression,
1699 as *comGA* mutant resists high temperature.** *B. subtilis* WT, its isogenic *comK* and *comGA*

1700 mutants and Rho⁺ cells growing exponentially (OD₆₀₀ 0.5) in LB medium were streaked on LB
1701 agar plates and incubated at 37°C and 55°C for 18 hours before imaging. One representative
1702 experiment out of three conducted is shown.

1703

1704 **S1 Table Sporulation proficient and thermoresistant suppressors of Rho⁺ strain.**

1705 **S2 Table Differential expression analysis of Rho⁺ and $\Delta\rho$ vs. WT *B. subtilis* BsB1 cells.**

1706 **S3 Table Comparison between sets of DE genes and *SubtiWiki* regulons.**

1707 **S4 Table. Strains and plasmids used in this study.**

1708 **S5 Table. Oligonucleotides used for strains construction.**

1709

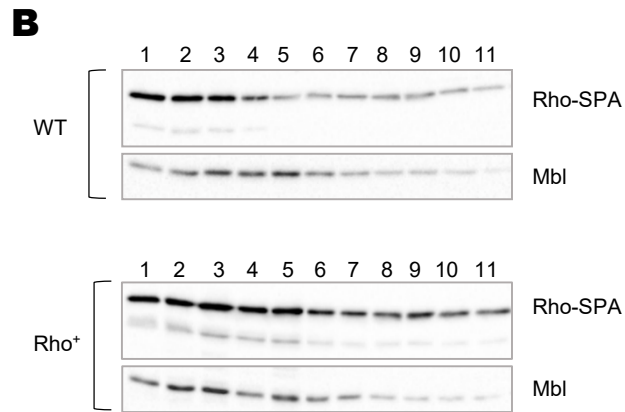
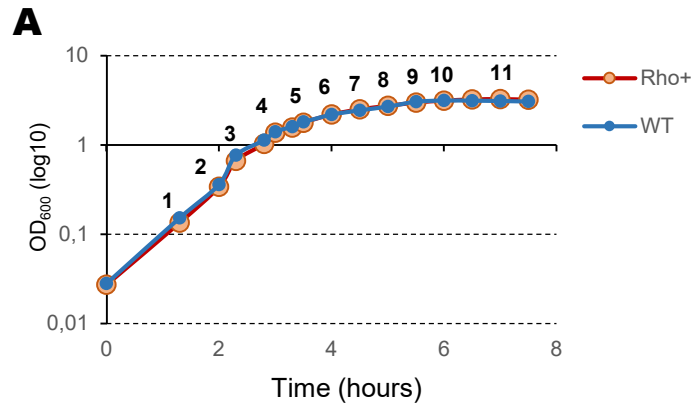


Fig 1

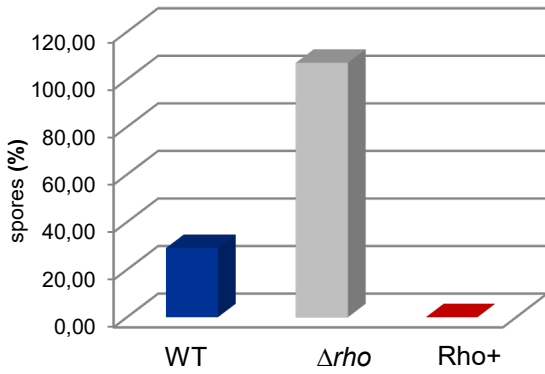
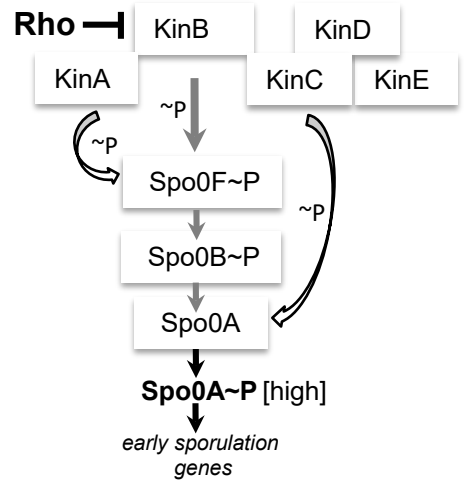
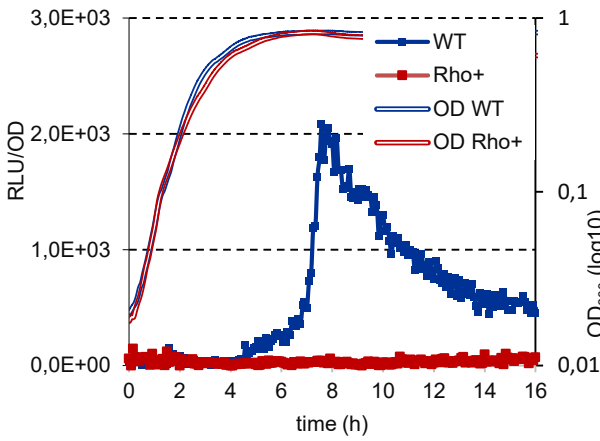
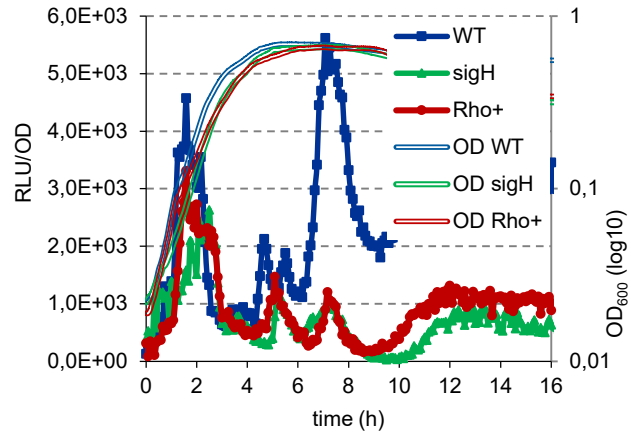
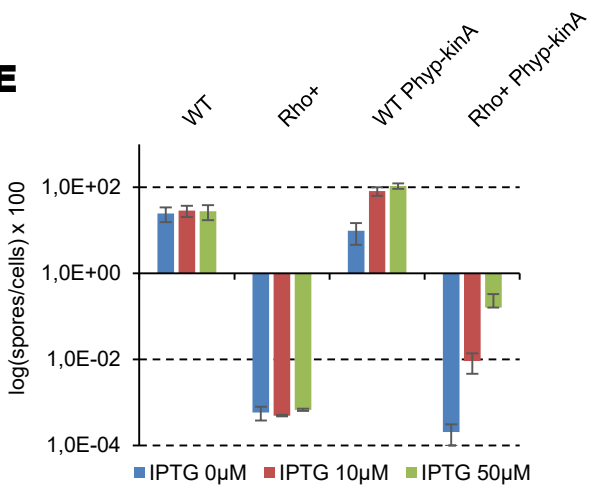
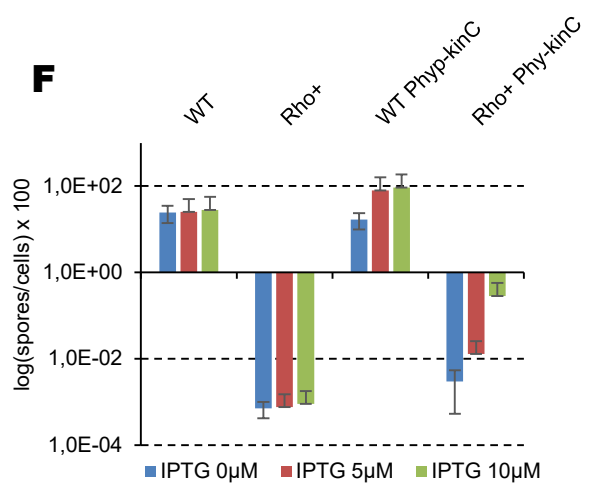
A**B****C****D****E****F**

Fig 2

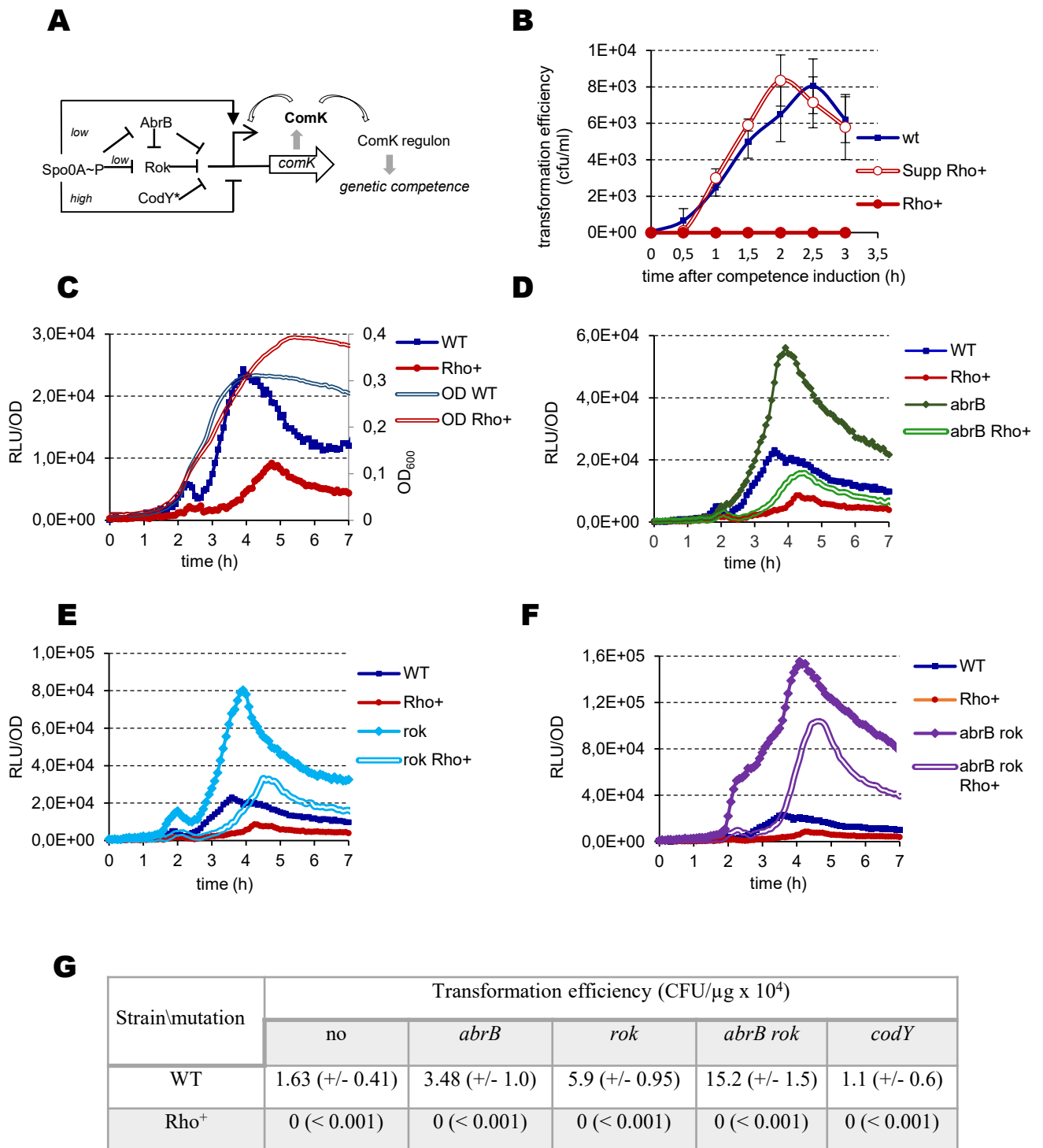


Fig 3

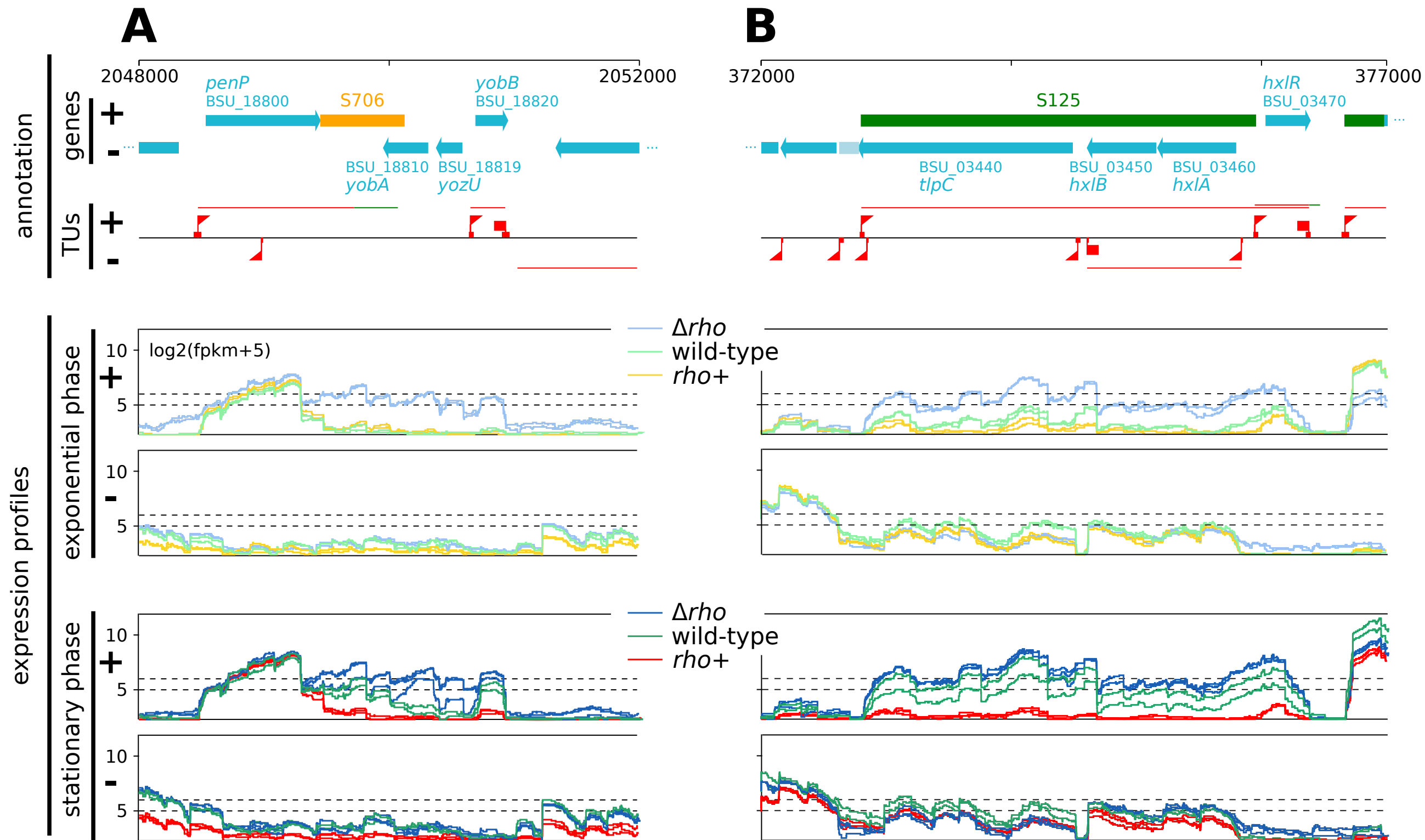
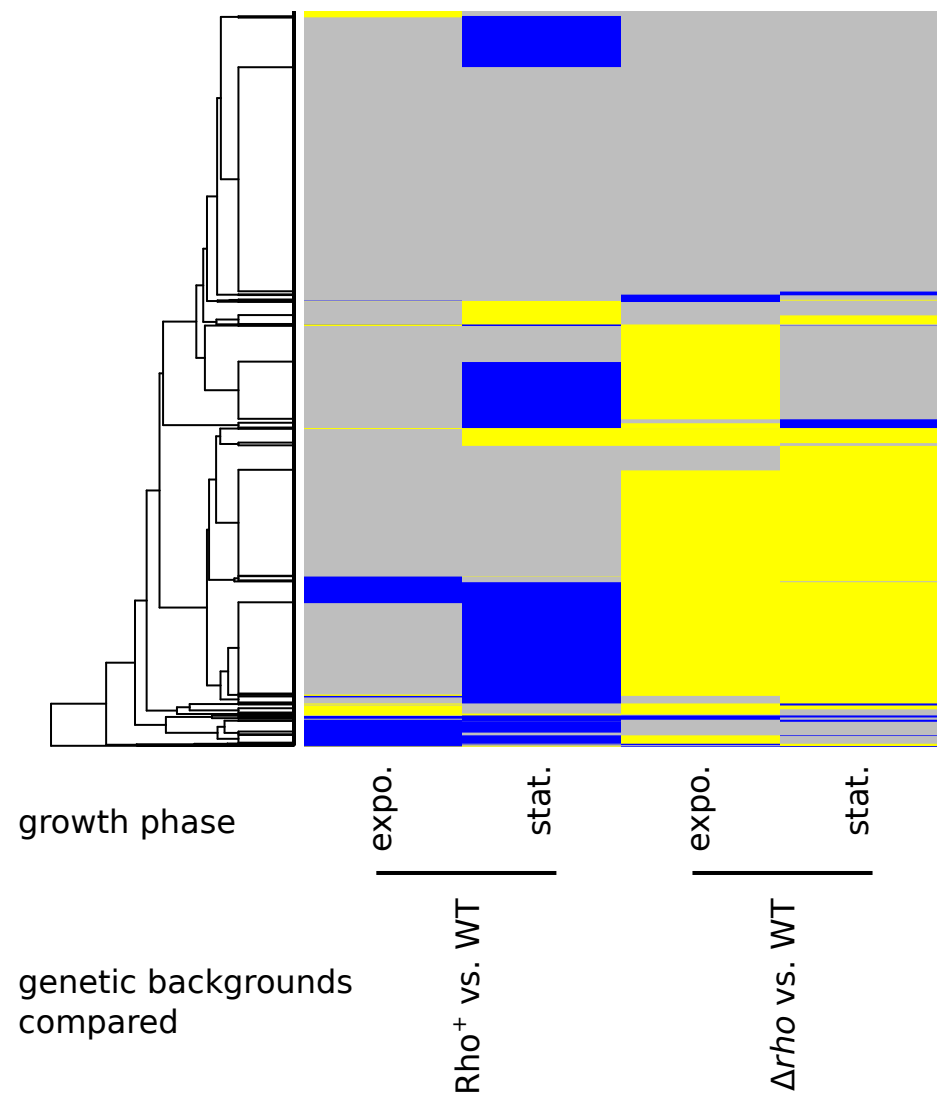
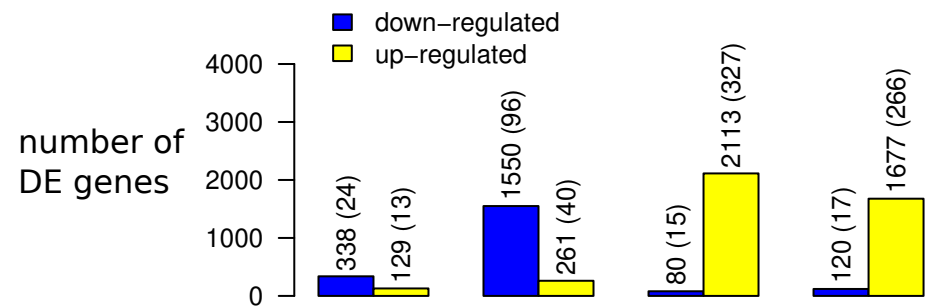
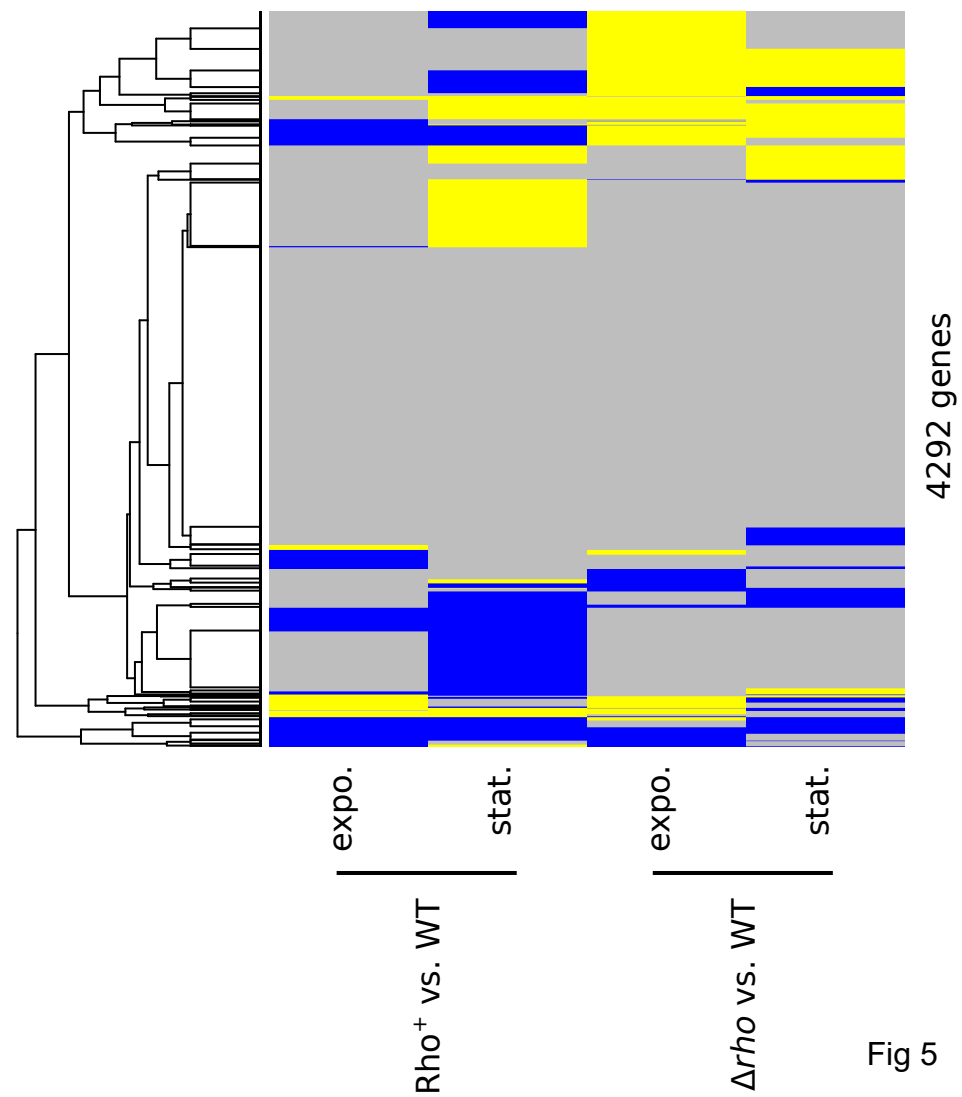
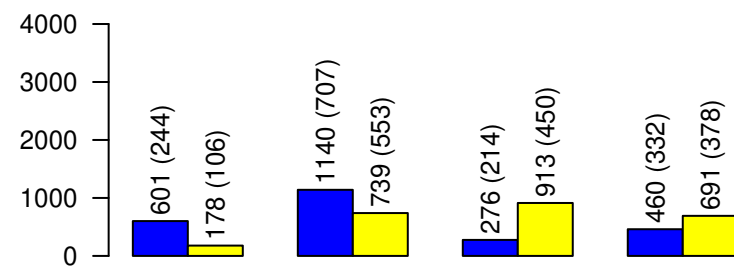


Fig 4

A antisense strand



B sense strand



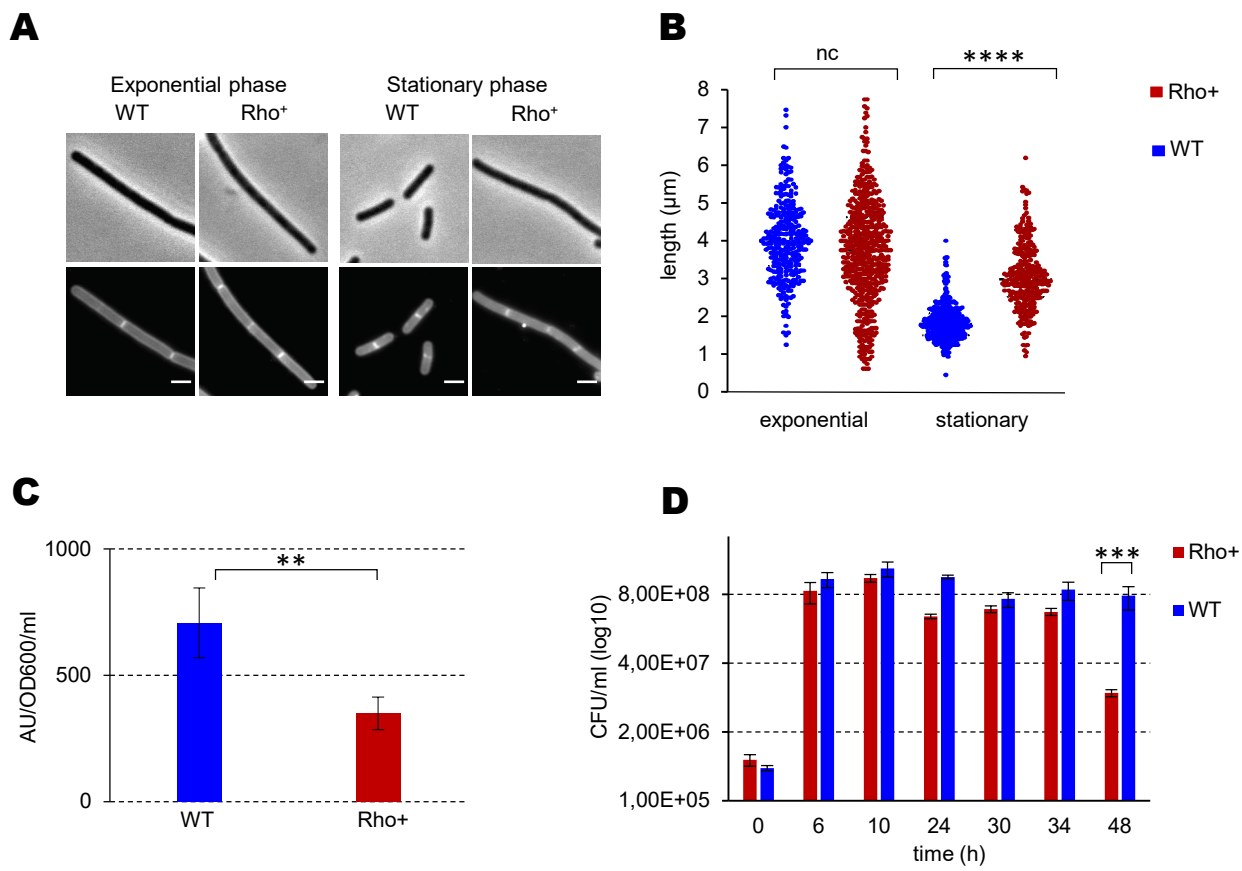


Fig 7

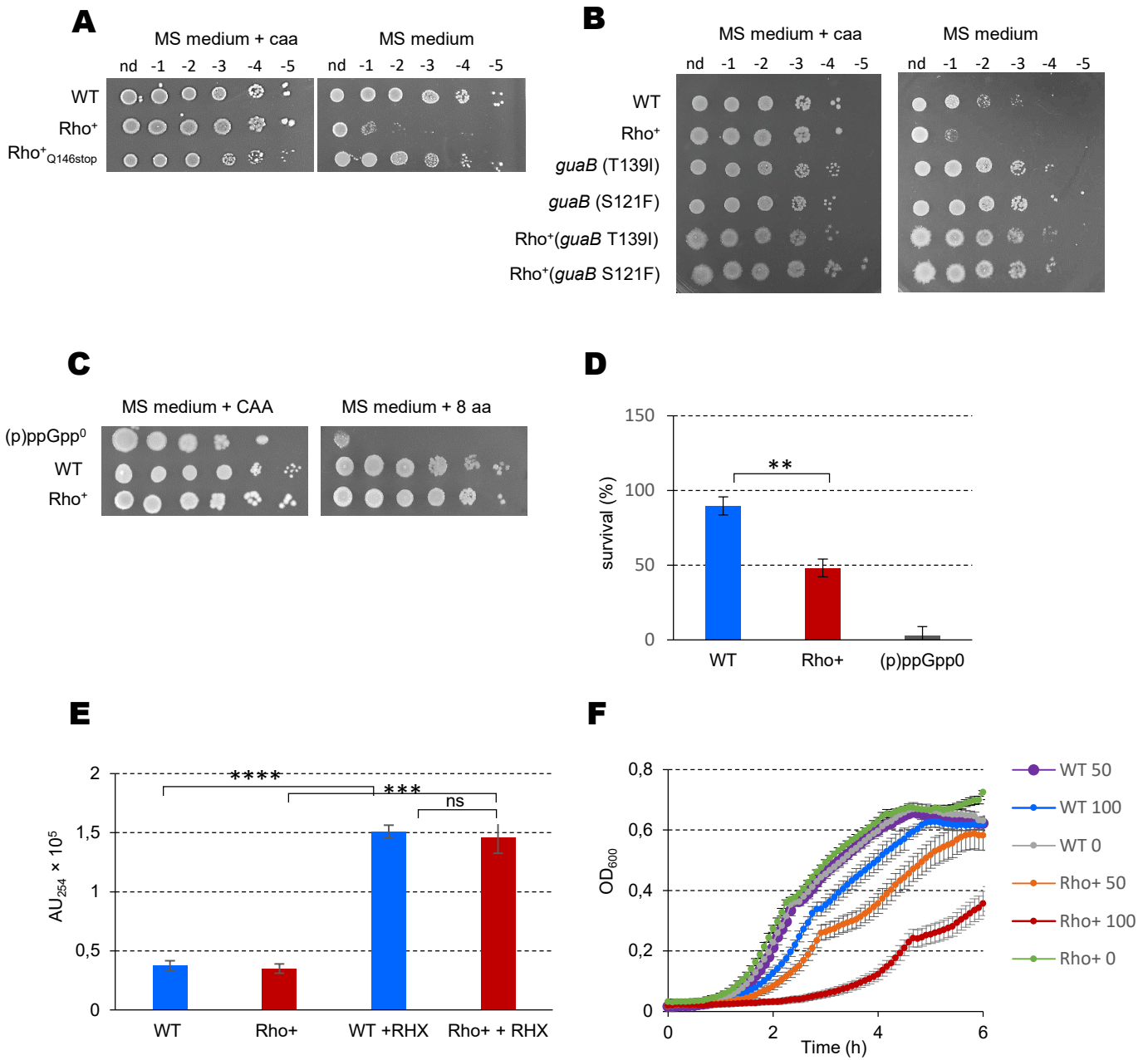


Fig 8

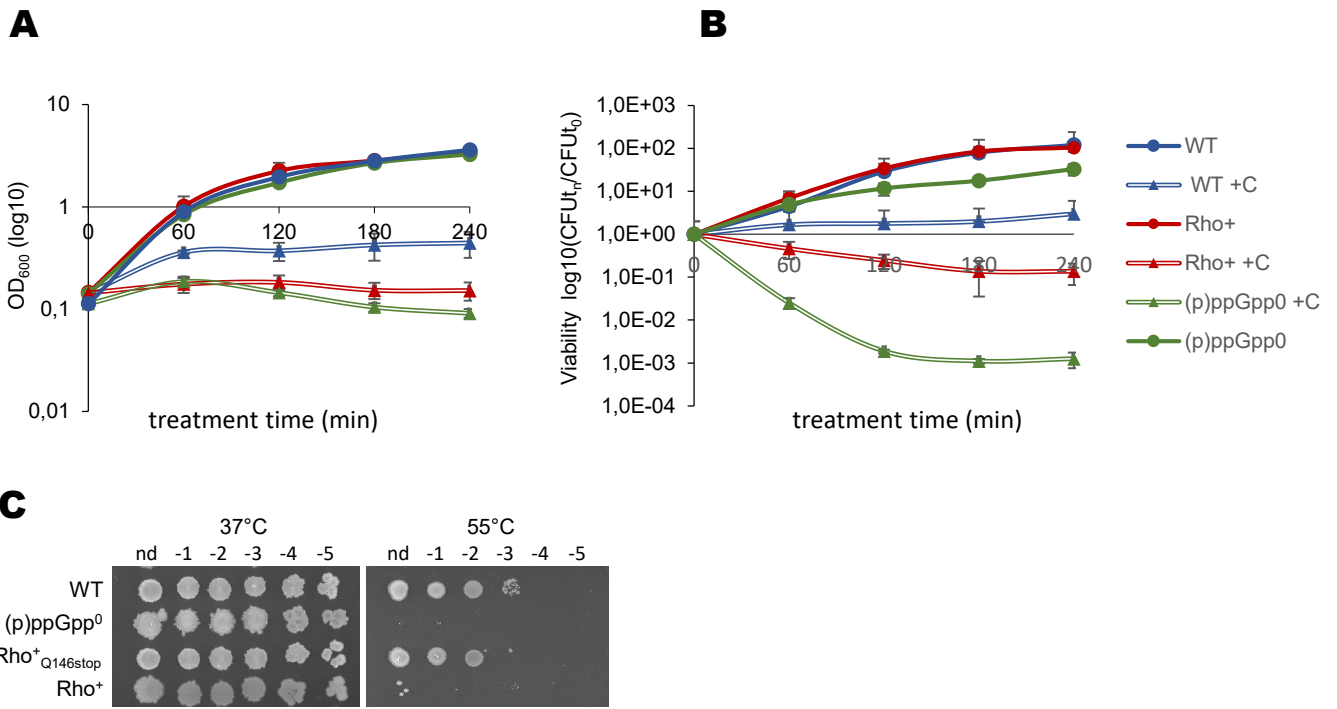


Fig 9