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► To cite this version:

Vladimir Bidnenko, Arnaud Chastanet, Christine Péchoux, Yulia Redko-Hamel, Olivier Pellegrini, et al.. Complex sporulation-specific expression of transcription termination factor Rho highlights its involvement in *Bacillus subtilis* cell differentiation. 2023. hal-04328824

HAL Id: hal-04328824

<https://hal.science/hal-04328824v1>

Preprint submitted on 7 Dec 2023

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1 **Complex sporulation-specific expression of transcription termination factor Rho**
2 **highlights its involvement in *Bacillus subtilis* cell differentiation.**

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32 **Abstract**

33

34 Transcription termination factor Rho controls pervasive, mainly antisense, transcription
35 initiated at cryptic signals or resulting from read-through at weak terminators in various
36 bacterial species. In *Bacillus subtilis*, Rho is intricately involved in the regulation of
37 phenomena associated with the adaptation to stationary phase and cell differentiation including
38 the ultimate survival program of sporulation.

39 While knockout or overexpression of the *rho* gene alters global transcription and modifies cell
40 physiology, in wild-type *B. subtilis* cells, the reduction of Rho levels during the transition to
41 stationary phase is necessary for both initiation and implementation of the sporulation program.
42 However, the mechanisms that govern Rho expression throughout the cell cycle remain largely
43 unknown.

44 Here, we demonstrate that, besides the previously identified vegetative SigA-dependent
45 promoter active during exponential growth, two distinct mechanisms ensure a spatiotemporal
46 expression of the *rho* gene during sporulation. In the mother cell of the sporangium, *rho*
47 expression occurs through the read-through transcription initiated at the distal SigH-dependent
48 and Spo0A~P-regulated promoter of the *spo0F* gene. In the forespore, *rho* is transcribed from
49 a genuine promoter recognized by the alternative sigma factor SigF. These regulatory elements
50 compensate for the inactivation of SigA-dependent *rho* expression at the end of exponential
51 growth and allow the critical “refueling” of Rho protein in both compartments of the
52 sporangium. We show that altering *rho* expression in the mother cell or in the forespore affects
53 differently the properties and the morphology of mature spores. Moreover, spores formed in the
54 absence of Rho are impaired in their ability to revive under favorable growth conditions,
55 exhibiting accelerated germination and slow outgrowth. Finally, we show that optimal
56 outgrowth of the wild-type spores requires the expression of *rho* during spore maturation and
57 additionally after spore germination.

58

59

60 **Introduction**

61

62 A growing understanding of the importance of transcription termination in the regulation of
63 gene expression in bacteria has stimulated extensive analysis of the proteins that control this
64 universal step in decoding genetic information (Peters et al., 2011; Ray-Soni et al., 2016; Kriner

65 et al., 2016; Turnbough, 2019; Mandell et al., 2022a). Among them is transcription termination
66 factor Rho, an ATP-dependent RNA helicase-translocase that improves the termination
67 efficiency of many intrinsic terminators and is essential for termination at bona-fide Rho-
68 dependent terminators (Roberts, 1969; Quirk et al., 1996; Peters et al., 2009; Hao et al., 2021;
69 Mandell et al., 2022b). Over the last years, considerable advances have been made in unveiling
70 the molecular mechanism of Rho-dependent termination (Song et al., 2022; Molodtsov et al.,
71 2023; Murayama et al., 2023; Rashid and Berger, 2023).

72 Transcriptome analyses of *rho* knockout and conditionally deficient mutants of various bacteria
73 have established the essential role of Rho in controlling pervasive, mainly non-coding and
74 antisense, transcription that originates from cryptic initiation signals or from read-through of
75 transcription terminators (Nicolas et al., 2012, Peters et al., 2012; Mäder et al., 2016; Botella et
76 al, 2017). By controlling transcription genome-wide, Rho directly and indirectly influences
77 various aspects of cellular physiology in bacteria living in diverse habitats (Bidnenko et al.,
78 2017, 2023; Botella et al., 2017; Nagel et al., 2018; Trzilova et al., 2020; Lin et al.; 2021;
79 Kryptou et al., 2023). It is notable that Rho is involved in the regulation of phenomena
80 associated with stationary phase, including stress survival, cell fate determination, antibiotic
81 sensitivity, host colonization and virulence in different bacteria (Lee and Helmann, 2014; Liu
82 et al., 2016; Hafeezunnisa et al., Bidnenko et al., 2017, 2023; Nagel et al., 2018; Trzilova et al.
83 2020; Lin et al., 2021; Figueroa-Bossi et al., 2022).

84 Recently, we provided evidence that Rho is intricately involved in the control of cellular
85 adaptation to stationary phase and in cell-fate decision-making, in particular, the decision to
86 sporulate, in the Gram-positive bacterium *Bacillus subtilis* (Bidnenko et al., 2017, 2023). The
87 involvement of Rho in the sporulation process was also demonstrated in *Clostridioides difficile*
88 and *Bacillus thuringiensis* (Trzilova et al., 2020; Lin et al.; 2021).

89 Sporulation is a complex developmental program that transforms a vegetative bacterial cell into
90 a highly resistant dormant spore, thus ensuring the survival of bacteria under adverse
91 environmental conditions and their dissemination within different ecological niches (de Hoon
92 et al., 2010; Swick et al., 2016; Galperin et al., 2022). A hallmark of sporulation in *B. subtilis*
93 is the asymmetric division of a differentiating cell in two unequal parts: a forespore, which
94 further develops into the spore, and a mother cell, which engulfs the forespore, nourishes it,
95 ensures the synthesis of spore protective layers, and finally lyses to release the mature spore.
96 Proper spore morphogenesis is essential for spore resistance to external damages and its
97 conversion back into a growing cell under favorable conditions through sequential processes of

98 germination and outgrowth (Setlow, 2003; Setlow et al., 2017; Setlow and Christie, 2023;
99 Segev et al., 2013; Abhyankar et al., 2016; Boone and Driks, 2016; Mutlu et al., 2018; 2020).
100 In *B. subtilis*, sporulation is primarily controlled at the level of transcription initiation by the
101 master regulator Spo0A, whose activity depends on phosphorylation mediated by a multi-
102 component phosphorelay, the transition phase-specific sigma factor SigH and a cascade of
103 sigma factors (SigF, SigE, SigG and SigK) that sequentially drive temporally- and spatially-
104 defined transcriptional programs of spore morphogenesis. Sporulation is initiated at a threshold
105 level of Spo0A~P, which activates the expression of *sigH* and several sporulation genes,
106 including *sigF* and *sigE* encoding the inactive forms of sigma factors. Upon completion of the
107 asymmetric septum, activation of SigF in the forespore is required for subsequent activation of
108 SigE in the mother cell. After a complete engulfment of the forespore, the compartment-specific
109 programs of gene expression are further continued by SigG in the forespore and SigK in the
110 mother cell (for comprehensive reviews see Errington, 1993; Stragier and Losick, 1996; Piggot
111 and Hilbert, 2004; Higgins and Dworkin, 2012). The relevant regulons of each sporulation-
112 specific sigma factor, as well as other genes involved in sporulation, were determined using the
113 combination of experimental and bioinformatics approaches (Eichenberger et al., 2003, 2004;
114 Steil et al., 2005; Wang et al., 2006; de Hoon et al., 2010; Overkamp et al., 2015; Meeske et al.,
115 2016; Galperin et al., 2022).

116 We have previously shown that deletion of the *rho* gene in *B. subtilis* prevents intragenic
117 termination of the *kinB* transcript, which encodes the sensor kinase KinB, one of the main
118 kinases feeding phosphate into the Spo0A phosphorelay. This leads to the increased expression
119 of KinB and, consequently, to rapid accumulation of active Spo0A~P to a threshold level that
120 triggers sporulation. Thus, Rho inactivation stimulates sporulation in *B. subtilis* indicating a
121 regulatory role of Rho-mediated transcription termination within the cell fate decision-making
122 network centered on Spo0A~P (Bidnenko et al., 2017). Conversely, maintaining *rho* expression
123 at a stably elevated level throughout *B. subtilis* exponential growth and stationary phase alters
124 the early steps of adaptive reprogramming of cellular transcription, prevents the activation of
125 Spo0A and, additionally, inhibits some late, yet non-identified, sporulation events, with the
126 overall result of blocking the formation of spores (Bidnenko et al., 2023).

127 Comparative transcriptome and proteome analyses have revealed a decrease in the levels of *rho*
128 mRNA and Rho protein during the transition to stationary phase in wild-type *B. subtilis* cells
129 (Nicolas et al., 2012; Bidnenko et al., 2017; 2023). Considering that Rho negatively affected
130 sporulation in our analyses, a decrease in Rho levels appears necessary for both initiation and

131 implementation of the sporulation program. This in turn suggests that *rho* expression during *B.*
132 *subtilis* growth and stationary phase is subject to reliable and timely regulation.

133 The only mechanism known to date for regulating *rho* expression is transcriptional attenuation
134 at the Rho-dependent terminator(s) located within the leader region of the *rho* transcript and
135 consequent premature transcription termination (Ingham et al., 1999). Similar to *B. subtilis*,
136 transcription of the *rho* gene was shown to be autogenously regulated in *Escherichia coli*,
137 *Salmonella* and *Caulobacter crescentus* (Barik et al., 1985; Matsumoto et al., 1986; Italiani et
138 al., 2005; Silva et al., 2019). In *Salmonella*, *rho* autoregulation is counteracted by the small
139 noncoding RNA SraL, which prevents the Rho-mediated termination by direct binding the 5'-
140 UTR of *rho* mRNA (Silva et al., 2019).

141 In the present study, we aimed to gain further insights into *rho* regulation in *B. subtilis* by
142 analyzing the kinetics of *rho* expression at various stages of cellular growth and differentiation.
143 Quite unexpectedly, we found that the expression of the *rho* gene is specifically induced early
144 during sporulation. This counterintuitive finding has focused our subsequent analysis on
145 understanding the mechanism of the sporulation-specific expression of *rho* and its biological
146 significance.

147 We describe two distinct mechanisms controlling *rho* expression in each compartment of the
148 sporulating cells: read-through transcription from the upstream SigH-dependent promoter in the
149 mother cell, and a genuine SigF-dependent *rho* promoter active in the forespore. The latter
150 feature allows us to classify *rho* as a novel member of the SigF regulon of *B. subtilis*. We
151 provide evidence that altering the spatiotemporal expression of *rho* affects spore resistance
152 properties and morphology, in particular, the structure of spore coat. Moreover, spores formed
153 in the absence of Rho are impaired in their ability to revive under favorable growth conditions,
154 exhibiting an accelerated germination and a slow outgrowth. Finally, we show that the optimal
155 rate of spore outgrowth depends on the synthesis of Rho during spore formation and *de novo*
156 after germination.

157

158 **Results**

159

160 **Rho expression is specifically induced during sporulation.**

161 To analyze a real-time expression of the *rho* gene, we constructed a scarless reporter system, in
162 which the luciferase gene *luc* of firefly *Photinus pyralis* was fused to the *rho* promoter (P_{rho}) at

163 the position of the *rho* start codon. The P_{*rho*}-*luc* fusion was similarly located in the *rho* locus of
164 the chromosome in the wild type *B. subtilis* strain BSB1 and the *rho* deletion mutant RM
165 (Bidnenko et al., 2017). The former strain (hereinafter WT) kept an active copy of the *rho* gene
166 due to duplication of the *rho* locus during the construction. By comparing luciferase activity in
167 WT and RM strains under different growth conditions, we sought to assess Rho autoregulation
168 and possibly identify other regulatory factors.

169 In WT cells grown in rich medium LB, the expression of *rho* was mainly limited to the
170 exponential growth phase, characterized by two peaks of luciferase activity at OD₆₀₀ ~0.15 and
171 ~0.4 (Fig 1A). In stationary phase, *rho* expression remained at a basal level. A low-level
172 expression of *rho* and its down-regulation during the transition to stationary phase were
173 reported previously (Ingham et al., 1999; Nicolas et al., 2012; Bidnenko et al., 2017; 2023).
174 The inactivation of Rho in RM cells did not alter the kinetics of *rho* expression, but rather
175 increased its level approximately threefold, suggesting a release from autoregulation (Fig 1A).
176 WT cells grown in the sporulation-promoting Difco Sporulation Medium (DSM) showed a
177 different *rho* expression kinetics, characterized by the additional peak of luciferase activity in
178 stationary phase (Fig 1B). The reactivation of *rho* expression coincided in time with the
179 induction of the sporulation-specific *spoIIAA-AB-sigF* operon, monitored in a separate strain
180 using the P_{*spoIIAA*}-*luc* transcriptional fusion (Fig 1B; Bidnenko et al, 2017). The similarity in
181 timing suggested that *rho* expression during stationary phase in DSM might be linked to
182 sporulation.

183 In RM cells grown in DSM, luciferase activity increased ~7-fold compared to WT cells during
184 exponential growth, but only ~1.5-fold in stationary phase. This suggests that *rho* expression in
185 stationary phase is not subject to significant autoregulation. Curiously, however, the stationary
186 phase-specific expression of *rho* was activated considerably earlier in RM than in WT cells (Fig
187 1C). This effect was reminiscent of the accelerated expression of *spo0A* gene in the sporulating
188 RM cells caused by the up-regulation of Spo0A phosphorelay (Bidnenko et al., 2017).
189 Therefore, we compared the expression of *rho* and *spo0A* genes in WT and RM cells grown in
190 DSM using the P_{*rho*}-*luc* and P_{*spo0A*}-*luc* fusions (Mirouze et al., 2012; Bidnenko et al., 2017). In
191 both strains, *rho* expression in stationary phase timely followed *spo0A* and, in RM cells, the
192 maximal expression levels of both genes was reached ~1.5 hour earlier than in WT (Fig 1D).
193 This observation additionally argued for the sporulation-specific expression of *rho* as a function
194 of Spo0A~P activity.

195 To get more insights into the sporulation-specific expression of *rho*, we analyzed the P_{*rho*}-*luc*
196 activity in *B. subtilis* sporulation mutants. First, we considered *spo0A* and *sigH* mutations,

197 which block initiation of sporulation by inactivating Spo0A and the transition phase-specific
198 sigma factor SigH, respectively. Neither *spo0A* nor *sigH* mutations affected P_{rho} -*luc* activity
199 during exponential growth in DSM, but both specifically blocked it in stationary phase, thus
200 confirming that *rho* expression at this stage is dependent on sporulation-specific factors (Fig
201 1E).

202 Next, we assessed the involvement of the alternative SigF and SigE factors controlling early
203 gene expression in the forespore and mother cell, respectively. The kinetics of P_{rho} -*luc* activity
204 in *sigF* and *sigE* mutants was rather similar to WT cells. However, the stationary phase-specific
205 luminescence appeared slightly lower in the *sigF* background and, in both mutants, remained
206 high after reaching a maximal level (Fig 1F). These results indicated some deregulating effects
207 of *sigF* and *sigE* mutations and suggested that *rho* is expressed in both compartments of
208 sporulating cells.

209 Considering that the known SigA-controlled P_{rho} promoter is mainly active during exponential
210 growth (Fig 1A), we sought for additional regulatory factors of *rho* expression during
211 sporulation.

212

213 **The read-through transcription from the upstream SigH-controlled promoter** 214 **contributes to *rho* expression during sporulation.**

215 The *rho* gene is transcribed from the cognate P_{rho} promoter together with the upstream non-
216 coding S1436 element and the downstream *rpmE* gene in a transcript of ~1.9 kb (Quirk et al.,
217 1991; Ingham et al., 1999; Nicolas et al., 2012). Additionally, the genome-wide transcriptome
218 analyses have revealed several longer read-through transcripts at the *rho* locus, which initiate
219 at the upstream *spo0F* and *fbaA-ywjH* promoters and bypass the intrinsic terminators of the
220 *ywjH* and *glpX* genes (Nicolas et al., 2012; Mandell et al., 2022b; Fig 2A).

221 We asked whether read-through transcription could play a role in the expression of *rho*, in
222 particular during sporulation, as *spo0F* gene is known to be transcribed from alternative SigA-
223 and SigH-dependent promoters, with the latter also being regulated by Spo0A~P (Lewandoski
224 et al., 1986; Predich et al., 1992; Strauch et al., 1993; Asayama et al., 1995). To this end, we
225 sought to enhance transcription termination upstream of the *rho* gene and inserted a DNA
226 fragment containing three intrinsic transcription terminators after the stop codon of the *glpX*
227 gene (hereafter referred 3Ter; Fig 2B; Vagner et al., 1998; Bidnenko et al., 2017). To assess
228 read-through transcription at the *rho* locus and the termination efficiency of the 3Ter insertion,
229 we analyzed *rho*-specific transcripts in WT, *sigH* and 3TER mutant cells by Northern blot (Fig

230 2C). Cells were grown in DSM and sampled during exponential growth (OD₆₀₀ 0.5), transition
231 phase (OD₆₀₀ 1.0) and in stationary phase, at OD₆₀₀ 1.8. In WT and *sigH* mutant cells, we
232 observed several *rho*-specific RNA species, the smallest and most abundant of which
233 corresponded in size to the 1.9 kb *rho-rpmE* mRNA, and the largest (~7 kb) to transcripts
234 initiated at the *fbaA* and/or *spo0F* promoters. Other RNAs of ~4-5 kb in size cannot be assigned
235 to any defined promoter and may result from ribonuclease processing of the larger transcripts.
236 During transition to stationary phase, the larger *rho*-specific RNAs accumulated in WT cells,
237 but gradually disappeared in *sigH* mutant, in accordance with the notion that *spo0F*
238 transcription in stationary phase depends on SigH activity (Fig 2C). Notably, none of the larger
239 *rho*-specific transcripts were detectable in 3TER cells (Fig 2C) confirming that they do indeed
240 originate from upstream of the inserted terminators. All together, these results show an efficient
241 read-through transcription of the *rho* gene, mainly initiated at the *spo0F* promoter.

242 Northern analysis also provided information about P_{*rho*} functioning. The levels of the 1.9 kb *rho*
243 transcript decreased in stationary WT cells, in line with the down-regulation of the SigA-
244 dependent P_{*rho*} at this stage (Fig 2C; Nicolas et al., 2012), and became barely detectable in the
245 stationary *sigH* mutant cells (Fig 2C). However, the dramatic decrease in the abundance of this
246 short transcript in the *sigH* mutant was not due to the inhibition of read-through transcription
247 from P_{*spo0F*} promoter, as the amount of the 1.9 kb *rho* transcript remained high in the stationary
248 3TER cells (Fig 2C).

249 To better understand the expression of *rho* in the absence of read-through transcription, we
250 analyzed the activity of P_{*rho*}-*luc* fusion in the 3TER strain and its sporulation-deficient
251 derivatives. In 3TER P_{*rho*}-*luc* cells grown in DSM, luciferase activity was identical to WT P_{*rho*}-
252 *luc* during exponential growth, but decreased ~3-fold in stationary phase, highlighting the role
253 of read-through transcription in the *rho* expression at this stage (Fig 2D). Intriguingly, the
254 residual luminescence in the stationary 3TER P_{*rho*}-*luc* cells was blocked by *spo0A* and *sigH*
255 (Fig 2E) and partially inhibited by *sigF* inactivation, while mutation of *sigE* had no effect (Fig
256 2F). All together, these results indicated that the *glpX-rho* intergenic region contains additional
257 structural factor(s) of *rho* expression active during sporulation and likely dependent on SigF.

258

259 **The sporulation-specific expression of *rho* is compartmentalized.**

260 Since SigF is primarily associated with the forespore, this led us to investigate potential
261 compartmentalization of *rho* expression during sporulation. To this end, we constructed a P_{*rho*}-
262 *gfp* fusion expressing green fluorescent protein GFP at the *rho* chromosomal locus of WT cells

263 and analyzed its activity during sporulation at a single-cell level by fluorescence microscopy.
264 We observed an increase of fluorescence intensity in the forespores of the sporulating WT P_{rho} -
265 *gfp* cells immediately after asymmetric division (S1 Fig and Fig 3). This observation further
266 suggested a particular role of SigF in the sporulation-specific expression of *rho*. Following it,
267 we analyzed the activity of P_{rho} -*gfp* fusion in *sigF* and *sigE* mutant cells.
268 Absence of SigE disables the SigE-dependent inhibitors of septation, therefore provoking the
269 formation of asymmetric septa at both poles of sporulating cells (Illing and Errington, 1991;
270 Setlow et al., 1991; Lewis et al., 1994; Eichenberger et al., 2001). Because activation of SigE
271 in the mother cell sequentially depends on SigF activity in the forespore (Londono-Vallejo &
272 Stragier, 1995; and references therein), both *sigE* and *sigF* mutants display a similar disporic
273 cell phenotype. Such disporic cells were readily seen in P_{rho} -*gfp sigF* and P_{rho} -*gfp sigE*
274 sporulating cultures, in addition to cells containing one asymmetric septum. The mother cells
275 of both *sigF* and *sigE* sporangia maintained GFP signal at a level similar to that of WT,
276 indicating that *rho* expression is SigE-independent (Fig 3). In contrast, while the forespore-like
277 structures showed an increased fluorescence in *sigE* mutant similarly to the WT forespores,
278 their brightness was significantly diminished in *sigF* sporangia (Fig 3). Together with the results
279 from the previous section, this observation indicated that, in forespores, *rho* is expressed from
280 a promoter located in the *glpX-rho* intergenic region and dependent, directly or indirectly, on
281 SigF.

282

283 **The 5'-UTR of *rho* contains a genuine SigF-dependent promoter active in forespores.**

284 Previous analyses have identified a *rho* promoter that matches the SigA-binding sequence
285 consensus ($^{sigA}P_{rho}$) 293-320 bp upstream the *rho* start codon (Quirk et al., 1993; Ingham et al.,
286 1999; Fig 4A). Recent reassessment of *B. subtilis* promoters using the unsupervised sequence
287 clustering algorithm has classified $^{sigA}P_{rho}$ to the M16 cluster of SigA-dependent promoters,
288 which are characterized by an extended 3'-terminal G-rich -10 box and a variable -35 box with
289 the conserved TTG stretch (Nicolas et al., 2012; Fig 4B). Looking for alternative *rho*
290 expression signals, we analyzed the *glpX-rho* intergenic region for the presence of the SigF-
291 binding consensus -35 (GYATA) and -10 (GGnnAnAHTR) sequences, where Y is C or T; H is
292 A or C or T; R is A or G; and n is any nucleotide (Wang et al., 2006). Such features were found
293 within the $^{sigA}P_{rho}$ sequence itself (Fig 4C). Within the -10 box of $^{sigA}P_{rho}$, we identified
294 GGTA AAAATA sequence perfectly matching the SigF -10 consensus and, 14 bp upstream, a
295 5-nucleotide GAATA sequence differing from the SigF -35 consensus by one nucleotide. Of

296 note, the same -35 sequence is present in the SigF-regulated promoters of *yuiC*, *yabT*, *yjbA*,
297 *ypfB* and *ythC* genes (Wang et al., 2006). Moreover, the 14-bp spacer between -35 and -10
298 sequences is highly A/T-rich that is characteristic of most SigF-dependent promoters (Wang et
299 al., 2006).

300 To prove that the identified sequences constitute a novel SigF-dependent *rho* promoter
301 (hereafter $\text{sig}^{\text{F}}\text{P}_{rho}$), we proceeded to site-directed mutagenesis of the $\text{P}_{rho-gfp}$ fusion, anticipating
302 that altering the SigF-binding sequences would specifically affect GFP expression in the
303 forespores. Because of a strong overlap between the -10 sequences of $\text{sig}^{\text{A}}\text{P}_{rho}$ and putative
304 $\text{sig}^{\text{F}}\text{P}_{rho}$, we targeted the -35 GAATA sequence of the latter and replaced the invariant T
305 nucleotide (Amaya et al., 2001; Wang et al., 2006) with A (*mF-35T/A P_{rho-gfp}*) or C (*mF-35T/C*
306 *P_{rho-gfp}*) (Fig 4C). Next, we compared the activity of the mutant fusions with the original one
307 (WT *P_{rho-gfp}*) and the one preceded by the transcriptional terminators (3TER *P_{rho-gfp}*). Cells
308 were set to sporulate in DSM medium, and the fluorescence levels in predivisional cells and
309 two compartments of sporangia were quantified at the time of a maximal SigF activity. As we
310 observed earlier (Fig 3), the sporulating WT *P_{rho-gfp}* cells showed a higher level of fluorescence
311 in the forespores than in the mother cells; in the latter, *gfp* expression appeared similar to
312 predivisional cells confirming our hypothesis that *rho* expression in the mother cell is SigE-
313 independent (Fig 4D and 4E). A similar pattern of the brighter forespores was observed in 3TER
314 *P_{rho-gfp}* cells, even though the fluorescence was globally lower probably due to the inhibition
315 of the read-through transcription by the 3Ter insert (Fig 4D and 4E).

316 In contrast, and as expected, both *mF-35T/A* and *mF-35T/C* mutations of *P_{rho-gfp}* specifically
317 inhibited the burst of fluorescence in the forespores, similarly to *sigF* mutation (Fig 4D and
318 4E, and Fig 3). At present, it is unclear why *mF-35T/A P_{rho-gfp}* mutant strain displayed a higher
319 basal fluorescence (Fig 4E). Nevertheless, these results clearly indicate that the forespore-
320 specific expression of *rho* depends on the alternative promoter $\text{sig}^{\text{F}}\text{P}_{rho}$.

321 This conclusion was further supported by the analysis of the mutated *P_{rho-luc}* fusion: both point
322 mutations *mF-35T/A* and *mF-35T/C* efficiently inhibited the sporulation-specific luciferase
323 activity in 3TER cells (S2 Fig).

324 Overall, our results indicate that, during growth and sporulation, the expression of *rho* gene
325 relies on three distinct features: (i) a SigA-dependent *rho* promoter that is active during
326 exponential growth, (ii) read-through transcription from the upstream SigH-dependent *spo0F*
327 promoter during early stationary phase and in the mother cells of sporangia, and (iii) a SigF-
328 dependent *rho* promoter active in the forespores.

329

330 **Differential expression of Rho affects spore properties and morphology.**

331 The revealed spatiotemporal expression of *rho* suggested that besides modulation of Spo0A
332 activity at the onset of sporulation (Bidnenko et al., 2017), Rho can be involved in the
333 subsequent steps of spore development. To address this hypothesis, we used a set of five strains
334 differentially expressing Rho during sporulation, namely: the wild type (WT), a strain blocked
335 for the *rho* read-through transcription (3TER), their derivatives inactivated for *rho* expression
336 in the forespore by *mF-35T/A* mutation of $\text{sig}^{\text{F}}\text{P}_{rho}$ (WT-mT/A and 3TER-mT/A), and a mutant
337 deleted for *rho* (RM).

338 Initially, we compared sporulation capacities of the five strains. To this end, we induced
339 sporulation by the resuspension method and assessed asymmetric division of individual cells
340 and formation of the heat-resistant spores at the initial and final stages of sporulation,
341 respectively. Among the five strains, RM cells showed a highest rate of asymmetric division
342 corroborating our previous data on the accelerated sporulation of Δrho mutant, most probably
343 due to a more efficient activation of Spo0A (S3 Fig; Bidnenko et al., 2017). Interestingly, the
344 proportion of cells with asymmetric septum was also higher in 3TER and 3TER-mT/A strains
345 compared to WT or WT-mT/A mutant (S3 Fig) suggesting a regulatory crosstalk between
346 Spo0A~P activating read-through transcription of *rho* and Rho modulating activity of the
347 Spo0A phosphorelay. However, while RM formed the heat-resistant spores considerably faster
348 and with maximal yield, other mutants resembled more WT cells (S3 Fig). Therefore, only the
349 complete inactivation of Rho significantly affected sporulation dynamics.

350 Next, we let cells to sporulate in DSM for 24 hours, during which more than 80 percent of cells
351 in each strain formed spores, purified the mature spores, and compared some of their damage
352 resistance properties. It should be noted, however, that most resistance phenotypes of spores
353 are determined by multifactorial mechanisms, what makes their dissection difficult (reviewed
354 in Setlow, 2006; Setlow and Christie, 2023). All spores differentially expressing *rho* were
355 similarly resistant to lysozyme, which targets the cortex peptidoglycan (reviewed in Henriques
356 and Moran, 2007), and became similarly sensitive to it after chemical removal of the coat (S4
357 Fig). This indicates that the permeability of spore coat to lysozyme was not detectably affected
358 by altered expression of *rho*. Likewise, all five types of spores showed similar levels of wet-
359 heat resistance, a complex spore phenotype determined by factors expressed in both spore
360 compartments (S4 Fig). At the same time, unlike the others, RM spores contained higher
361 (~35%) levels of dipicolinic acid (DPA) (Fig 5A), an important factor of the heat-resistance

362 synthesized in the mother cell, but accumulated in the spore core (reviewed in Nicholson et al.,
363 2000; Setlow, 2016). Moreover, the mutant RM and also WT-mT/A and 3TER-mT/A spores,
364 formed in the absence of the forespore-specific expression of *rho*, appeared more sensitive to
365 ultraviolet (UV) light than WT and 3TER spores (Fig 5B). This effect was spore-specific, as
366 vegetative RM and WT cells were equally resistant to UV radiation (S5 Fig). The UV-resistance
367 of mature spores is mainly determined by the α/β small acid-soluble proteins (SASPs) and
368 photoproduct lyase SplB synthesized in the forespores (Mason and Setlow, 1986; Pedraza-
369 Reyes et al., 1994; Setlow, 2007). Based on our results, it is possible that Rho expressed from
370 SigF-dependent promoter is involved in the regulation of some of these UV-resistance factors.
371 Finally, we analyzed the ultra-structure of the purified spores by transmission electron
372 microscopy, which revealed significant differences between the spores in the structure of their
373 coats (Fig 6). In accordance with numerous previous studies (reviewed in Henriques & Moran,
374 2007; McKenney et al., 2013; Driks and Eichenberger, 2016), WT spores exhibited a regular
375 surface layer composed of a lamellar inner coat and a striated thick electron-dense outer coat.
376 Two coats were tightly attached to each other in a large majority (over 92 percent) of WT spores
377 or occasionally separated across a limited area (Fig 6). In contrast, RM spores contained a
378 lamellar inner coat similar to WT spores, but their outer coat remarkably lost electron-density
379 and striated structure and appeared half thinner compared to WT spores (respectively, 36.2 +/-
380 0.0091 nm and 74.2 +/- 0.021 nm, as measured for 100 spores of each strain). Moreover, above
381 80 percent of RM spores showed an extensive detachment of inner and outer coats, often along
382 the entire spore perimeter (Fig 6). Spores differentially expressing *rho* exhibited intermediate
383 ranges of structural alterations of their coats. The outer coat of 3TER spores appeared less
384 striated and low electron-dense compared to WT, therefore resembling the RM coat. Moreover,
385 the outermost layer often peeled off the 3TER spore surface. However, we did not observe
386 3TER spores with the separated inner and outer coats (Fig 6). In WT-mT/A spores, the outer
387 coat was electron-dense and striated like a WT layer, but detached from the inner coat in ~30
388 percent of spores, although across less extended areas than in RM spores (Fig 6). Finally, 3TER-
389 mT/A spores contained a low structured outer coat locally separated from the inner coat in ~30
390 percent of spores (Fig 6). By combining these features, 3TER-mT/A spores appear most similar
391 to RM spores, which seems in line with the absence of sporulation-specific *rho* expression in
392 both mutant strains. We conclude that altering expression of *rho* during sporulation in the
393 mother cell (3TER) or in the forespore (mT/A) induces specific defects of spore morphology,
394 which are accentuated by the entire loss of the Rho activity in RM strain.

396 **Rho mediates spore germination and outgrowth.**

397 The ability to resume vegetative growth in favorable nutrient conditions, referred to as spore
398 revival, is a basic spore property and spore quality indicator (Henriques and Moran, 2007;
399 Setlow, 2013; Sinai et al, 2015; Ramírez-Guadiana et al., 2017a; Christie and Setlow, 2020).
400 We compared the revival abilities of spores differentially expressing Rho by evaluating the
401 optical density (OD₆₀₀) of spore suspensions after the induction of germination. Rapid
402 rehydration and structural changes of the germinating spores alter their optical properties
403 resulting in a decrease in OD₆₀₀. The OD₆₀₀ of reviving spores remains stable during the post-
404 germination phase of metabolic resumption and molecular reorganization, designated the
405 “ripening period”, before increasing later during spore outgrowth, when the emerging cells
406 start to grow and divide (Segev et al., 2013; Rosenberg et al., 2015). For simplicity, we
407 consider the outgrowth period as a time needed for a spore culture to restore its initial OD₆₀₀
408 after germination.

409 After the addition of the germinant L-alanine, RM spores lost OD₆₀₀ faster than the others, thus
410 showing the highest germination rate (Fig 7A). Similarly, RM spores germinated more
411 efficiently with the germinants AGFK (a mixture of L-asparagine, glucose, fructose and
412 potassium chloride) and L-valine recognized by other germinant receptors than L-alanine (S6
413 Fig). Other spores germinated similarly to WT with all three germinants (Fig 7A and S6 Fig).
414 In contrast to the rapid germination, the outgrowth of RM spores in a defined minimal medium
415 was significantly delayed compared to other spores (Fig 7B). At the same time, the viability of
416 RM spores during germination was not decreased compared to WT spores (S7 Fig) and
417 therefore could not explain the outgrowth delay. As for other spores, the enrichment of the
418 growth medium by casamino acids (0.5%) increased the outgrowth rate of RM spores, which,
419 nevertheless, remained below the WT level (Fig 7C). These results indicate that *rho* inactivation
420 imposes stricter requirements on the spore outgrowth.

421 We asked whether Rho activity is needed exclusively during spore morphogenesis for the
422 formation of an appropriate molecular basis for spore revival in the future, or for a correct
423 metabolic resumption during spore outgrowth. To answer this question, we placed the *rho* gene
424 under the control of the IPTG-inducible promoter P_{spac}, which allowed us to selectively control
425 *rho* expression during sporulation and/or spore outgrowth. By immunoblot analysis of P_{spac}-
426 *rho* cells using the Rho^{Bs}-specific antiserum, we established that the induction of P_{spac}-*rho* by
427 IPTG (100μM) determines a near-natural level of Rho protein (Materials and Methods; S8 Fig).
428 Following this, we allowed P_{spac}-*rho* cells to sporulate in the presence or absence of IPTG
429 100μM, purified the mature spores from the IPTG-induced and non-induced cultures and

430 compared the revival of two types of $P_{\text{spac}}\text{-rho}$ spores in the conditions where *rho* expression
431 was omitted or *de novo* induced by IPTG.

432 We observed that spores, which were formed in the absence of Rho ($P_{\text{spac}}\text{-rho}^{(-\text{IPTG})}$), exhibited
433 an accelerated germination like RM spores, while those that expressed *rho* during
434 morphogenesis ($P_{\text{spac}}\text{-rho}^{(+\text{IPTG})}$), germinated as WT (Fig 8A). Addition of IPTG together with
435 the germinant did not modify germination kinetics of either spore type (Fig 8B). The two types
436 of spores also behaved differently during outgrowth. In the absence of IPTG, the outgrowth of
437 $P_{\text{spac}}\text{-rho}^{(-\text{IPTG})}$ spores was identical to RM spores, while $P_{\text{spac}}\text{-rho}^{(+\text{IPTG})}$ spores grew out at an
438 intermediate rate between WT and RM (Fig 8C). Interestingly, *de novo* induction of *rho*
439 expression by IPTG had a small but reproducible stimulatory effect on the outgrowth rates, so
440 that $P_{\text{spac}}\text{-rho}^{(+\text{IPTG})}$ spores were able to “catch up” with the outgrowth of WT spores (Fig 8D).
441 IPTG had no effect on the germination or outgrowth of WT and RM spores.

442 From these experiments, we conclude that normal spore revival requires expression of *rho*
443 during spore morphogenesis and, although to a lesser extent, *de novo* during spore outgrowth.

444

445

446 **Discussion**

447

448 The main achievement of the present analysis is the revelation of spatiotemporal expression of
449 the termination factor Rho during sporulation in *B. subtilis* via two distinct mechanisms: read-
450 through transcription initiated at a distal promoter controlled by SigH in the mother cells, and
451 transcription from the authentic SigF-dependent *rho* promoter in the forespores. These
452 regulatory elements compensate for the inactivation of SigA-dependent *rho* expression at the
453 end of exponential growth and allow the “refueling” of Rho protein in both compartments of
454 the sporangium.

455 Our results also show that maintaining Rho levels during sporulation is necessary for the
456 formation of spores with normal morphology, resistance properties and the ability to return to
457 a vegetative state under favorable environmental conditions.

458

459 **Compartmentalized expression of *rho* during sporulation.**

460 The sporulation-specific read-through transcription of *rho* is initiated by the activation of the
461 SigH-dependent promoter of *spo0F* gene encoding the phosphotransferase Spo0F of the Spo0A
462 phosphorelay. The *spo0F* expression is regulated by Spo0A~P and constitutes one of the

463 positive feedback loops of Spo0A~P at the onset of sporulation (Lewandoski et al., 1986;
464 Predich et al., 1992; Strauch et al., 1993; Asayama et al., 1995; Chastanet et al., 2010).
465 Previously, we have established that Rho negatively regulates the expression of some
466 components of the Spo0A phosphorelay, like the sensor kinase KinB (Bidnenko et al., 2017).
467 Therefore, correct modulation of Spo0A~P activity at the initial steps of *B. subtilis*
468 differentiation requires the decrease in the *rho* expression levels (Bidnenko et al., 2017). As we
469 show here, this decrease is partially due to the silencing of the SigA-dependent *rho* promoter
470 by yet unknown mechanism. It appears that alongside a self-reinforcing loop involving Spo0F,
471 Spo0A~P simultaneously activates the Rho-mediated regulatory circuit, which can be viewed
472 as a negative feedback loop of Spo0A~P. This regulatory function of Rho could be particularly
473 important to fine-tune Spo0A~P activity during initiation of sporulation and in the mother cells,
474 where Spo0A~P remains functional after asymmetric division (Fujita and Losick, 2003). Our
475 data indicate that the mother cell-specific sigma factor SigE is not involved in the expression
476 of *rho*. Nevertheless, inhibition of the *rho* read-through transcription results in the structural
477 changes of spore coat, whose assembly depends on the activity of the SigE- and SigK-controlled
478 genes (Eichenberger et al., 2003; Henriques and Moran, 2007; Driks and Eichenberger, 2016).
479 This emphasizes an extended activity of Rho during the mother cell gene expression program.
480 Identification of the alternative forespore-specific *rho* promoter matching the SigF-binding
481 consensus allows us to classify *rho* as a member of the SigF regulon. Interestingly, an earlier
482 analysis of forespore gene expression has detected the up-regulation of *rho* in sporulating *sigF*⁺
483 *B. subtilis* cells (see Supplemental Table1 in Wang et al., 2006). However, *rho* was not induced
484 by the artificial expression of SigF during exponential growth in LB, a second criterion used
485 for gene classification to the SigF regulon (Wang et al., 2006). It is possible that, in that analysis,
486 the untimely activated SigF failed to compete with the abundant vegetative SigA for binding to
487 the overlapping cognate *rho* promoters. The silencing of the SigA-dependent *rho* promoter
488 might facilitate SigF binding. However, the mechanism of this silencing is unclear, and despite
489 some discrepancies between the reports, SigA remains active in the forespores (Lord et al.,
490 1999; Fujita, 2000; Li et Piggot, 2001; Marquis et al., 2008; Riley et al., 2018; 2021). It is also
491 notable that the *rho* gene is located within a chromosomal cluster of SigF-dependent genes
492 activated the first during the forespore formation (Steil et al., 2005; Wang et al., 2006), what
493 pinpoints its early expression. At the same time, our data indicate the role of Rho in the
494 resistance of spores to ultraviolet radiation, the main factors of which, the α/β -type small acid-
495 soluble spore proteins (SASPs) and the spore photoproduct lyase SplB, are controlled by the
496 late forespore-specific sigma factor SigG (Mason and Setlow, 1986; Pedraza-Reyes et al., 1994;

497 Setlow, 2001). Therefore, like the read-through transcription in the mother cells, the SigF-
498 mediated *rho* expression appears to ensure Rho activity during the entire forespore-specific
499 gene expression program. It can also mediate the packaging of Rho protein in mature spores,
500 where it has been recurrently identified by mass spectrometric analysis (Swarge et al., 2020; Tu
501 et al, 2020).

502

503 **Rho involvement in the spatiotemporal regulation of spore development.**

504 Two spore phenotypes, a decreased UV-resistance and a structurally altered outer coat, resulting
505 from the complete inactivation of *rho*, or following the inhibition of *rho* expression in the
506 forespores and in the mother cells, respectively, highlight the importance of the
507 compartmentalized Rho-mediated transcription termination for spore development. Our
508 previous transcriptome analysis of RM cells in stationary phase revealed disordered and
509 untimely expression of many sporulation genes via their extensive sense or antisense read-
510 through transcription or, more rarely, unscheduled activation of promoters (Bidnenko et al.,
511 2023). The relevant gene expression changes are compiled in S1 Table and can be visualized at
512 http://genoscapist.migale.inrae.fr/seb_rho/ (Bidnenko et al., 2023; Dérozier et al., 2021). We
513 posit that at least some of the sporulation genes abnormally expressed in the stationary RM cells
514 are subject to Rho-mediated regulation during sporulation. At the same time, we are aware that
515 most of potential sporulation-specific targets of Rho are not yet expressed in stationary phase.
516 For instance, the stationary phase transcriptome of RM cells does not provide an obvious
517 explanation for the decreased UV-resistance of spores differentially expressing *rho*. Indeed, the
518 observed upregulation of several *ssp* genes in the stationary RM cells, in particular *sspC*, is
519 contrary to what one could expect if spores become sensitive to UV. It is therefore likely that
520 the effect of Rho in stationary phase and in sporulating cells is different for some genes.

521 The existing transcriptome data probably provide the most straightforward mechanistic hints
522 on the involvement of Rho in the regulation of spore coat morphogenesis. The prominent coat
523 defects in RM and 3TER-mT/A spores resemble those induced by the inactivation of
524 morphogenetic proteins CotO, CotH or CotG, or auxiliary proteins of coat assembly, superoxide
525 dismutase SodA and holin YwcE (McPherson et al., 2005; Zilhão et al., 1999; Isticato et al.,
526 2020; Freitas et al., 2020; Henriques et al., 1998; Real et al., 2005; Henriques and Moran, 2007).
527 All of the abovementioned mutant spores exhibit a disordered, low electron-dense and thinner
528 outer coat frequently detached from the inner layer. The protein kinase CotH and its target
529 protein CotG were recently identified as the main morphogenetic factors determining the

530 regular structure of spore coat (Nguyen et al, 2016; Freitas et al., 2020; Isticato et al., 2020; Di
531 Gregorio Barletta et al., 2022). It has been shown that phosphorylated CotG assembles in the
532 coat, while its non-phosphorylated form sequesters other coat proteins in the mother cell
533 cytoplasm (Di Gregorio Barletta et al., 2022).

534 Interestingly, the expression levels of *cotG* are strongly upregulated in RM cells, due to the
535 transcription upshift at, or close to, the SigK-dependent *cotG* promoter and the read-through
536 transcription initiated at the upstream *alsR* gene (Nicolas et al., 2012; Bidnenko et al., 2023; S1
537 Table). The same read-through transcript appears antisense to the oppositely oriented
538 neighboring genes *cotB* and *cotH*. We suggest that Rho participates in the control of the
539 transcription of the *cotB-cotH-cotG* gene cluster during sporulation and that inactivation of the
540 *rho* expression in the mother cells modifies the CotG-CotH protein balance important for
541 correct assembly of CotG, thus leading to spore coat defects. Work is in progress to test this
542 hypothesis.

543 Alongside the shared phenotypes linked to the inhibition of spatiotemporal expression of *rho*,
544 RM spores are specifically characterized by the increased levels of DPA content. The synthesis
545 of DPA and its transfer to the core across the spore membranes involves the protein products
546 of both mother cell- and forespore-specific genes (Daniel and Errington, 1993; Ramírez-
547 Guadiana et al., 2017b; Gao et al., 2022). None of the genes involved in the DPA metabolism
548 are subject to unscheduled activation in RM cells in stationary phase. However, we detected
549 strong induction of a defined SigA-dependent antisense promoter located within the SigG-
550 regulated *spoVAA-spoVAB-spoVAC-spoVAD-spoVAEB-spoVAEA-spoVAF-lysA* operon,
551 whose products are essential for DPA uptake into the core and release during germination
552 (Tovar-Rojo et al., 2002; Gao et al., 2022; Nicolas et al., 2012; Bidnenko et al, 2023). In the
553 wild type *B. subtilis* cells, this antisense promoter is weakly activated under some stress
554 conditions and late in sporulation (Nicolas et al., 2012). Remarkably, in RM cells, the generated
555 ~5 kb-long antisense transcript extends across *spoVAA*, *spoVAB*, *spoVAC* genes and the *dacF*-
556 *spoIIAA-spoIIAB-sigF* operon. It remains to be established whether this antisense transcript
557 affects the expression of the genes on the opposite strand during RM sporulation.

558

559 **Rho involvement in spore revival.**

560 RM spores also differ from their siblings by their impaired revival capacity, showing a rapid
561 germination followed by slow outgrowth. The rate of germination in response to different
562 nutrient stimuli is mainly determined by the levels of corresponding germinant receptors in the
563 spore membrane (Cabrera-Martinez et al., 2003; Ghosh et al., 2012; Chen et al., 2014). The

564 RM stationary phase transcriptome showed unscheduled up-regulation of the SigG-controlled
565 *gerBA-gerBB-gerBC* operon encoding the AGFK germinant receptor GerB (Corfe et al., 1994)
566 and the SigK-controlled *gerPA-gerPB-gerPC-gerPD-gerPE-gerPF* operon, whose products
567 facilitate the passage of nutrient germinants across the spore coat (Behravan et al., 2000).
568 Although these features suggest that Rho controls both operons during sporulation, they seem
569 insufficient to explain the rapid germination of RM spores in response to L-alanine or L-valine.
570 Among the *gerAA-gerAB-gerAC* genes encoding the L-alanine germinant receptor GerA
571 (Feavers et al, 1990; Amon et al., 2022, and references therein), only *gerAA* appears up-
572 regulated ~3-fold in the stationary RM cells.

573 Interestingly, Rudner and colleagues identified *rho* among the genes, whose inactivation by
574 Mariner transposon results in a premature germination of the developing spores triggered by
575 the GerA receptor, probably in response to some undefined defects of spore envelope (Meeske
576 et al., 2016; Ramírez-Guadiana et al., 2017). The authors report that GerA activity results in a
577 decreased sporulation efficiency of the *rho::Mariner* mutant, which is opposite to our recurrent
578 observation of a highly efficient sporulation in the *rho*-deleted RM cells. Nevertheless, the
579 established genetic interaction between Rho and GerA proteins may be relevant to the
580 germination phenotype of the mature RM spores, whose coat is severely altered. Moreover, the
581 structure of RM spore coat suggests a lower level of coat protein cross-linking (Henriques and
582 Moran, 2007), which can facilitate spore germination, as previously observed (Abhyankar et
583 al., 2015). The germination rate of RM spores might be also influenced by high levels of DPA,
584 a potent non-nutrient germinant that stimulates cortex hydrolysis by CwlJ hydrolase both
585 directly and during nutrient-induced germination (Paidhungat and Setlow, 2000; Paidhungat et
586 al., 2001).

587 More generally, spore germination is a complex highly heterogeneous process influenced by
588 multiple factors, including, among others, the metabolic activity of cells during sporulation and
589 the duration of spore formation (Segev et al. 2012; Bressuire-Isoard et al., 2018; Mutlu et al,
590 2018, 2020; Riley et al., 2021; Rao et al., 2022). From this viewpoint, a more synchronous and
591 rapid sporulation in RM population (Bidnenko et al., 2017, and this study) might result in a
592 more homogeneous molecular composition of spores and, consequently, in a decreased
593 heterogeneity of spore germination.

594 At the same time, the absence of Rho negatively affects the outgrowth of the germinated RM
595 spores even in a nutrient-enriched medium. Therefore, *rho* inactivation imposes more stringent
596 requirements for spore outgrowth, which in itself is more exacting than vegetative growth
597 (O'Brien and Campbell, 1957). Taken together, a rapid germination and a slow outgrowth of

598 RM spores suggest that, in the wild type background, Rho determines the fitness of reviving
599 spores.

600 Using a controlled *rho* expression system, we showed that the normal rate of spore outgrowth
601 requires *rho* expression both during spore formation and after spore germination. During
602 sporulation, Rho would ensure a proper composition of a spore molecular cargo required for
603 spore revival, which also includes Rho protein itself (Mutlu et al, 2018; Rao et al., 2022; Swarge
604 et al., 2020). After germination, Rho would be required in the ripening period, to control a
605 proper termination of the actively resuming transcription, and later, during implementation of
606 the transcription program of vegetative growth. This is consistent with the differential gene
607 expression analysis of the reviving *B. subtilis* spores, which detected the upshifts in *rho*
608 expression shortly after germination and at the end of outgrowth (Swarge et al., 2020). Most
609 probably, the resumption of *rho* expression during outgrowth reflects the reactivation of SigA-
610 dependent *rho* promoter.

611 To conclude, this and our previous analyses trace the activity of Rho through each stage of the
612 complex program of *B. subtilis* sporulation, from the regulation of Spo0A~P activation at the
613 onset of sporulation, through the subsequent morphogenesis of spores and up to the modulation
614 of spore revival. This highlights the existence of specific targets for Rho-mediated transcription
615 termination within the regulon of each alternative Sigma factor, which certainly include,
616 alongside the known coding genes, a particular set of non-coding and anti-sense transcripts.
617 Appropriate regulation of these targets requires in turn an accurate time- and spatial-specific
618 regulation of the *rho* expression, through mechanisms we describe. Overall, our results
619 strengthen our vision of Rho as a built-in regulatory module of *B. subtilis* cell differentiation.

620

621

622 **Materials and Methods**

623

624 **Bacterial strains and growth conditions**

625 All strains used in the analysis originate from *B. subtilis* 168 *trp*⁺ strain BSB1 (Nicolas et al.,
626 2012) and are listed in S2 Table. The *Escherichia coli* TG1 strain was used for construction of
627 intermediate plasmids. Cells were routinely grown in liquid or solidified rich LB medium at
628 37°C. Standard protocols were used for transformation of *E. coli* and *B. subtilis* competent cells
629 (Harwood and Cutting, 1990). Sporulation of *B. subtilis* cells was induced by the method of
630 nutrient exhaustion in supplemented Difco sporulation medium (DSM; Difco) (Schaeffer et al.,

631 1965) or by the resuspension method (Harwood and Cutting, 1990), as detailed in (Bidnenko et
632 al., 2023). Optical density of the bacterial cultures was measured with NovaspecII Visible
633 Spectrophotometer, Pharmacia Biotech. When required, antibiotics were added at following
634 concentrations: 0.5 μg per ml of erythromycin, 3 μg per ml of phleomycin, 100 μg per ml of
635 spectinomycin, and 5 μg per ml of chloramphenicol to *B. subtilis* cells; and 100 μg per ml of
636 ampicillin or 20 μg per ml of kanamycin to the plasmid-containing *E. coli* cells. For the
637 induction of P_{spac} -*rho* fusion, IPTG (isopropyl- β -D-1-thiogalactopyranoside) inducer was
638 added to cells and spores to final concentration 100 μM .

639

640 **Strains and plasmids construction**

641 All intermediate plasmids were constructed using Q5 High Fidelity DNA Polymerase and DNA
642 modification enzymes purchased from New England Biolabs. Transcriptional fusion of the
643 luciferase gene *luc* and the *rho* promoter (P_{rho} -*luc*) was constructed as follows. The
644 oligonucleotide pairs glpXBam/veb738 were used to amplify a \sim 1 Kb fragment of *B. subtilis*
645 chromosome located directly upstream the *rho* start codon. The 5' part of the *luc* gene was
646 amplified from the plasmid pUC18cm-Luc (Mirouze et al., 2012) using oligonucleotides
647 *lucintrev* and veb739 complementary to veb738. Two DNA fragments were joined by PCR
648 using the primers glpXBam and *lucintrev*, and the resulting DNA fragment was cloned at
649 pUC18cm-Luc using BamHI and BstBI endonucleases and T4 DNA ligase. The resulting
650 plasmid pBRL862 was integrated by single crossover at the *rho* chromosomal locus of BSB1
651 (WT) and BRL1 (Δ *rho*) cells using selection for chloramphenicol-resistance. By this event, the
652 *luc* gene was placed under all natural regulatory signals of the *rho* expression. In the WT
653 background, the replaced intact copy of *rho* gene remained active under the control of own
654 promoter.

655 Insertion of three intrinsic transcription terminators (3TER) upstream the chromosomal P_{rho} -*luc*
656 fusion was performed as follows. The 3TER DNA fragment amplified from pMutin4 plasmid
657 using oligonucleotides veb734 and veb735 was end-joined with the DNA fragments amplified
658 from pBRL862 using the pairs of oligonucleotides glpXBam/veb798 (5'-complementary to
659 veb734) and *lucintrev*/veb797 (5'-complementary to veb735). The primers glpXBam and
660 *lucintrev* were used in the joining reaction. The PCR product was cut by EcoRV and BstBI
661 endonucleases and cloned at a similarly cut and gel-purified pBRL862 plasmid. The resulting
662 plasmid pBRL1107 was integrated at the *rho* locus of BSB1 chromosome as above. The created

663 3TER- P_{rho} -*luc* transcriptional fusion contains intact 5'-UTR of *rho* and the 3TER insertion
664 immediately after the *glpX* stop codon.

665 Transcriptional fusion between the *gfp* gene, encoding Green Fluorescent Protein, and the *rho*
666 promoter (P_{rho} -*gfp*) was constructed similarly to P_{rho} -*luc*. The oligonucleotides *glpXBam* and
667 *veb742* were used for amplification of the *rho* moiety from BSB1 chromosome, and the *gfp*
668 gene was amplified from the plasmid pCVO119 using the primers *veb741* and *veb740*
669 complementary to *veb742*. The primers *glpXBam* and *veb741* were used for joining PCR, and
670 the resulting DNA fragment was cloned in pCVO119 plasmid using *BamHI* and *NcoI*
671 endonucleases and T4 DNA ligase. The resulting plasmid pBRL893 was integrated by single
672 crossover at the *rho* locus of the BSB1 chromosome using selection for spectinomycin
673 resistance.

674 To construct the 3TER- P_{rho} -*gfp* transcriptional fusion, the DNA fragment containing 3TER and
675 the *rho* 5'-UTR was amplified from pBRL1107 plasmid using the primers *glpXBam* and
676 *veb742* and fused to the *gfp* gene as described above for pBRL893. The product of joining PCR
677 was cut by *EcoRV* and *NcoI* endonucleases and cloned at a similarly cut and gel-purified
678 pBRL893. The resulting pBRL1150 plasmid was inserted in the BSB1 chromosome as above.

679 Site-directed mutagenesis of the Sigma F-dependent *rho* promoter of the P_{rho} -*luc* and P_{rho} -*gfp*
680 transcriptional fusions was performed as follows. The plasmids pBRL893 and pBRL1107 were
681 entirely amplified using the pairs of side-by-side oligonucleotides *veb803/veb802*, containing
682 a 5'-terminal C nucleotide to introduce the point mutation *mF-35T/C*, and *veb803/veb805*, with
683 a 5'-terminal A for the point mutation *mF-35T/A*. The PCR products were phosphorylated using
684 T4 polynucleotide kinase, self-ligated and treated with *DpnI* endonuclease to remove the
685 template DNA prior transformation in *E. coli* cells. The resulting mutated derivatives of the
686 plasmids pBRL893 (pBRL1141 and pBRL1164) and pBRL1107 (pBRL1116 and pBRL1162)
687 were controlled by sequencing and inserted into the *B. subtilis* BSB1 chromosome as above to
688 produce the mutated fusions *mF-35T/C P_{rho}-gfp* and *mF-35T/A P_{rho}-gfp* and 3TER *mF-35T/C*
689 P_{rho} -*luc* and 3TER *mF-35T/A P_{rho}-luc*, respectively.

690 Structural modifications of the *rho* expression unit at natural locus were performed by the
691 method for allelic replacement using a shuttle vector pMAD (Arnaud et al., 2004).

692 To insert 3TER transcription terminators at the *rho* locus, two DNA fragments amplified from
693 the BSB1 chromosome with the pairs of oligonucleotides *veb795/veb796* and *veb797/veb798*
694 were end-joined to the 3TER DNA fragment (see above) by PCR using the primers *veb795* and
695 *veb796*. The product of joining PCR was cut with *BamHI* and *EcoRI* endonucleases and cloned
696 in the pMAD vector (Arnaud et al., 2004). The resulting plasmid pBRL1108 was integrated

697 into the *rho* locus of the BSB1 chromosome with selection of the erythromycin-resistant
698 transformants at 37°C non-permissive for plasmid replication. Single transformants were
699 propagated in LB without antibiotic at 30°C to induce the loss of the vector and plated at LB-
700 plates at 37°C. The erythromycin-sensitive clones were selected among the grown colonies and
701 tested by PCR for the presence of the 3TER insertion using oligonucleotides veb734 and
702 veb735, and the selected clones were controlled for structural integrity of the *glpX-3TER-rho*
703 region by sequencing.

704 The point mutation *mF-35T/A* was introduced in the *rho* locus of BSB1 and BRL1130 (3TER)
705 strains as follows. Two DNA fragments were amplified from pBRL1164 plasmid and BSB1
706 chromosome by PCR with the respective pairs of oligonucleotides veb796/veb808 and
707 veb806/veb807, complementary to veb808. The fragments were joined by PCR using veb796
708 and veb806 as primers, and the resulting product was cloned at pMAD vector using BamHI and
709 EcoRI endonucleases and T4 DNA ligase. The resulting plasmid was transformed into BSB1
710 cells and the transformants were processed as described above to select the point mutant WT-
711 mT/A. The mutant strain 3TER-mT/A was constructed in a similar way using the plasmid
712 pBRL1162 as a template in the first PCR and WT-mT/A strain as a recipient for transformation.
713 To construct the IPTG-controlled system for *rho* expression, the DNA fragment amplified from
714 BSB1 chromosome by PCR with the oligonucleotides veb806 and veb880 was digested by
715 EcoRI and BamHI endonucleases and clones at pMUTIN4 vector (Vagner et al., 1998). The
716 resulting plasmid was transformed in BSB1 cells with selection to erythromycin resistance.
717 For the purification of the *B. subtilis* Rho protein, *rho* gene was amplified by PCR with
718 oligonucleotides veb596 and veb599, treated by NdeI and SalI endonucleases and cloned at the
719 expression vector pET28a (Novagen) allowing expression of Rho with N-terminal hexa-
720 histidine tag (Ingham *et al.*, 1999). The resulting plasmid pETRho was transferred to *E. coli*
721 strain BL21-CodonPlus (DE3)-RIL (Stratagene).

722 **Luciferase assay**

723 Analysis of promoters' activity using luciferase fusions was performed as described previously
724 (Mirouze *et al.*, 2011) with minor modifications detailed in (Bidnenko et al., 2023). Cells were
725 grown in LB medium to mid-exponential phase (OD₆₀₀ 0,4-0,5), cultures were centrifuged and
726 resuspended to OD 1.0 in fresh LB or DSM, to follow expression of the fusions during growth
727 or sporulation, respectively. The pre-cultures were next diluted in respective media to OD₆₀₀
728 0.025. The starter cultures were distributed by 200µl in a 96-well black plate (Corning, USA)
729 and Xenolight D-luciferin K-salt (Perkin, USA) was added to each well to a final concentration

730 of 1.5 mg/ml. The cultures were grown under strong agitation at 37°C and analyzed in Synergy
731 2 Multi-mode microplate reader (BioTek Instruments). Relative Luminescence Units (RLU)
732 and OD₆₀₀ were measured at 5 min intervals. Each fusion-containing strain was analyzed at
733 least three times. Each experiment included four independent cultures of each strain.

734

735 **Epifluorescence microscopy and image processing**

736 For all microscopic observations, cells were mounted on a 2% agarose pad and topped with a
737 coverslip. Bacteria were imaged with an inverted microscope (Nikon Ti-E) equipped with an
738 iLas2 laser coupling system from Roper Scientific (150 mW, 488nm and 50 mW, 561 nm), a
739 100× oil immersion phase objective, an ORCA-R2 camera (Hamamatsu) and controlled by the
740 MetaMorph software package (v 7.8; Molecular Devices, LLC). The post-acquisition treatment
741 of the images was done with the Fiji software (van Ooij et al., 2004; Schindelin et al., 2012).
742 To determine the frequency of cells entering into sporulation, cultures were sampled three hours
743 after the induction of sporulation by the resuspension method (Harwood and Cutting, 1990).
744 Sampled cells were mixed with the lipophilic fluorescent dye Mitotracker Red (10 µg/ml final
745 concentration) prior to microscopic observation. Asymmetric septa were manually counted in
746 two independent replicas (N > 450 per strain and per replica).

747 To assess *rho* expression during sporulation using GFP as a reporter, cells bearing the non-
748 modified or the point-mutated P_{rho} -*gfp* transcriptional fusions were induced for sporulation by
749 the resuspension method and sampled as above. Sampled cells were mixed with the lipophilic
750 fluorescent dyes Nile Red (10 µg/ml final concentration) prior to microscopic observation. To
751 measure the fluorescence intensity in the different compartments, circular areas of a constant
752 0.45 µm diameter were drawn in the center of individual compartments (forespores, mother
753 cells, or predivisional cells) using images showing membrane-labelled cells, and recorded as a
754 list of ROI. ROIs were subsequently applied over the corresponding image displaying the GFP
755 signal, and the average fluorescence over each ROI recorded. Background fluorescence,
756 determined as the average fluorescence from ROIs of identical areas spread over the field
757 (outside cells), was subtracted to give the final fluorescence intensity of individual
758 compartments. Counting was performed in at least two fields of view and for a minimum of
759 800 cell compartments per strain, in two independent experiments. Plotted values are average
760 fluorescence intensities from pooled replicas, thus standard deviations account for cell to cell
761 variability across all fields and replicas.

762

763 **RNA preparation and Northern blotting**

764 Total RNA was extracted from *B. subtilis* cultures at OD₆₀₀ indicated in the text by the glass
765 beads method (Bechhofer et al., 2008). For Northern blotting, 5 µg of RNA were separated on
766 1% agarose-formaldehyde (2.2M) gel in MOPS (50mM)/EDTA (1mM) buffer (pH 7.0) and
767 transferred to Hybond N+ membrane (GE-Healthcare) as described previously (Redko et al.,
768 2013). Membrane pre-hybridization and the *rho* RNA detection by hybridization with the *rho*-
769 specific $\alpha^{32}\text{P}$ -labelled riboprobe was performed as detailed in (Gilet et al., 2020). The riboprobe
770 was synthesized by T7 RNA polymerase in the presence of [α -³²P]UTP at the template of the
771 purified PCR fragment obtained with oligonucleotides YRH1 and YRH2 (S3 Table). The
772 reverse PCR primer YRH1 contained at the 5'-end the T7 RNA polymerase promoter sequence
773 (TAATACGACTCACTATA).

774

775 **Sporulation assay**

776 Cells were induced for sporulation by the resuspension method, and the cultures were analyzed
777 for the presence of spores starting from six hours after resuspension. The percentage of spores
778 in a sample was calculated as proportion of viable cells after heating at 70°C for 15 min to the
779 total number of cells as described in (Bidnenko et al., 2023).

780

781 **Spore purification and treatments**

782 *B. subtilis* cells growing exponentially in LB were suspended in DSM at OD₆₀₀ 0.05 and
783 cultured at 37°C with aeration for 24 hours. Spores were purified by sequential rounds of
784 intensive washing in ice-cold water and centrifugation over 3 days as described by Nicholson
785 and Setlow in (Harwood and Cutting, 1990). The samples of the purified spores were controlled
786 for the absence of cells by the heat-resistance test as described above and stored at 4°C in water
787 at OD₆₀₀ > 10. The purity of standard spore samples was above 95 percent.

788 For electron microscopy analysis, spores were additionally purified on Nicodenz (Axis-Shield,
789 United Kingdom) density gradients. Spores were suspended in 1 ml of 20 % Nicodenz solution,
790 layered on 50 % Nicodenz (15 ml) in the centrifuge tubes and centrifuged at 14,000 x g for 30
791 min at 10°C. The pelleted pure spores were washed 5 times in ice-cold water to remove traces
792 of Nicodenz and kept at 4°C before analysis.

793 Prior the assays of spore resistance phenotypes and germination, the purified spores were
794 suspended in 10 mM Tris HCl (pH 8.0) at OD₆₀₀ 1.0 and activated by heating at 70°C for 30
795 min and subsequent cooling in ice for 20 min. The activated spores were used in the assays
796 within 1 hour.

797 Chemical removal of spore coats was performed according to (Riesenman and Nicholson,
798 2000). Spores were suspended in decoating solution (50 mM Tris base, 8 M urea, 50 mM
799 dithiothreitol, 1% sodium dodecyl sulfate; pH 10.0) at OD₆₀₀ 5.0 and incubated at 60°C for 90
800 min with vortexing. After the treatment, spores were intensively washed in STE buffer (150
801 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA; pH 8.0) as described.

802

803 **Transmission electron microscopy**

804 Purified spores were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2)
805 for 1 h at room temperature. Samples were contrasted with 0.5% Oolong tea extract in
806 cacodylate buffer and postfixed with 1% osmium tetroxide containing 1.5% potassium
807 cyanoferrate. The samples were dehydrated and embedded in Epon (Delta Microscopies,
808 France), as described (Theodorou et al., 2019). Thin sections (70 nm) were collected onto 200-
809 mesh copper grids and counterstained with lead citrate. Grids were examined with a Hitachi
810 HT7700 electron microscope operated at 80 kV, and images were acquired with a charge-
811 coupled device camera (Advanced Microcopy Techniques; facilities were located on the
812 MIMA2 platform [INRAE, Jouy-en-Josas, France;
813 <https://doi.org/10.15454/1.5572348210007727E12>]). The post-acquisition treatment of the images
814 and measurement of the thickness of the spore coat were performed using the Fiji software
815 (Schindelin et al., 2012).

816

817 **Spore UV resistance assay**

818 The activated spores were suspended in Tris-HCl (pH 8.0) at OD₆₀₀ 0.5, pipetted in triplicates
819 (50 µl) in the wells of 48-well sterile microtiter plates (Evergreen Scientific) and irradiated by
820 ultraviolet (254 nm) light using Stratalinker 2400 UV Crosslinker (Stratagene) at the J/m² doses
821 indicated in the text (one plate per UV dose). The irradiated spore suspensions were plated in
822 sequential dilutions at LB plates and the colonies were counted after 24 hours of incubation at
823 37 °C. The UV- resistance was determined as a ratio of the colony-forming units in the
824 irradiated and non-irradiated samples of spores. Five experiments were performed with two
825 independent sets of spores differentially expressing Rho.

826 To determine the effect of *rho* inactivation on the UV resistance of vegetative cells, WT and
827 RM cultures in the late exponential phase (~10⁸ cells/ml) were plated on LB plates and
828 irradiated with the increasing doses of UV (254 nm) light using a Stratalinker 2400 UV

829 Crosslinker (Stratagene). The UV-resistance of cells was estimated as above. Three
830 independent experiments were performed.

831

832 **Dipicolinic acid (DPA) assay**

833 The DPA content of spores was analyzed according to the protocol of Nicholson and Setlow
834 (in Harwood and Cutting, 1990).

835 **Spore germination and outgrowth assay**

836 Spore germination and outgrowth assays were performed in MS medium (10.8 g l⁻¹ of K₂HPO₄,
837 6 g l⁻¹ of KH₂PO₄, 1 g l⁻¹ of C₆H₅Na₃O₇·2H₂O, 0.2 g l⁻¹ of MgSO₄·7H₂O, and 2 g l⁻¹ of K₂SO₄)
838 supplemented with 0.5% glucose, 0.01% L-tryptophan, 0.1% glutamate, 0.1 mM of FeCl₃
839 citrate, 0.1 mM of CaCl₂, 1 mM of MgSO₄ and trace elements (0.001 g l⁻¹ of MnCl₂ 4H₂O,
840 0.0017 g l⁻¹ of ZnCl₂, 0.00043 g l⁻¹ of CuCl₂· 2H₂O, 0.0006 g l⁻¹ of CoCl₂ 6H₂O and 0.0006 g
841 l⁻¹ of Na₂MoO₄· 2H₂O). The stock solutions (100mM) of L-alanine and L-valine germinants
842 were prepared in 10 mM Tris-HCl buffer (pH 8.0) containing 10mM D-glucose and 100mM
843 KCl. The stock solution of AGFK germinant mixture contained 100 mM L-asparagine, 10mM
844 D-glucose, 10 mM D-fructose and 100mM KCl in 10 mM Tris-HCl buffer (pH 8.0). The
845 activated spores were diluted to OD₆₀₀ 0.2 in the cold MS medium and distributed by 135 µl in
846 the 96-well plate. Spore suspensions were simultaneously induced for germination by adding
847 15µl of a stock germinant solution, incubated under strong agitation at 37°C and monitored for
848 OD₆₀₀ at 2 min intervals in Synergy 2 Multi-mode microplate reader (BioTek Instruments). It
849 usually took ~1 min between germinant addition and the first OD₆₀₀ reading. When needed, the
850 stock solution of the germinant L-alanine was supplemented with 1mM IPTG or 5% casamino
851 acids to get their final concentrations in spore suspensions 100µM and 0.5%, respectively.
852 Three independently prepared sets of spores were used in the analysis. The assays were
853 performed with each set of spores at least twice and included up to six replicas of each spore
854 suspension in the same plate.

855 **Purification of the *B. subtilis* Rho protein for antibody preparation**

856 *E. coli* BL21-CodonPlus (DE3)-RIL cells containing pETRho were grown to an OD₆₀₀ of 0.2
857 in a 400 ml culture (2YT medium) at 16°C, and Rho expression was induced by the addition of
858 0.5 mM IPTG with continued growth overnight. The culture was harvested, pelleted and frozen
859 at -80°C until further use. The frozen cells were resuspended in 10 ml of lysis buffer at 4°C
860 containing 20 mM Tris-HCl pH 9.0, 100 mM Na₂HPO₄, 0.3 M NaCl, 10% glycerol and 0.1%

861 Triton X-100, to which were added 10 mg/ml DNase I and an EDTA-free anti-protease tablet
862 (Roche). The suspension was passed twice through a French press (20 000 p.s.i.) and the lysate
863 centrifuged for 30 min at 15 000 g. Imidazole-HCl (pH 8.0) was added to the supernatant to
864 give a final concentration of 1 mM and the resultant suspension was applied to a 1 ml Ni²⁺-
865 NTA column (Qiagen). The resin was then washed sequentially with 10 ml of lysis buffer
866 (without Triton X-100), 10 ml of buffer containing 20 mM Tris-HCl pH 9.0, 0.3 M NaCl,
867 20 mM Imidazole and, finally, 10 ml 20 mM Tris-HCl pH 9.0, 0.3 M NaCl and 250 mM
868 Imidazole. *B. subtilis* Rho was eluted with 20 mM Tris-HCl pH 9.0, 100 mM Na₂HPO₄, 0.3 M
869 NaCl and 500 mM Imidazole as 1ml fractions into collection tubes pre-filled with 3 ml elution
870 buffer lacking Imidazole, to immediately dilute the sample 4-fold and avoid precipitation. The
871 protein peak was determined initially by measuring the protein concentration (Bio-Rad) in the
872 different fractions. Peak fractions were pooled and dialyzed over-night in buffer containing
873 20 mM Tris-HCl pH 9.0, 100 mM Na₂HPO₄, 0.3 M NaCl, 10% glycerol. Protein purity was
874 verified by SDS-PAGE analysis and estimated at >95%. A sample of purified *B. subtilis* Rho
875 protein at 1.1 mg/ml in storage buffer was filtered through 5µm, 0.5µm and then 0.2µm
876 Acrodisc filters before rabbit immunization to generate custom anti-Rho polyclonal antibodies
877 through the commercial '87-day anti-antigen classical' program of Eurogentec (Belgium). The
878 low solubility of *B. subtilis* Rho in buffers suitable for immobilization on affinity
879 chromatography columns prevented further purification of the anti-Rho antibodies from the
880 crude sera. The best anti-Rho serum aliquot was selected from the immunization program
881 aliquots by Western blotting with purified *B. subtilis* Rho and *B. subtilis* cell extract samples.

882

883 **Western blotting**

884 The crude cell extracts were prepared using Vibracell 72408 sonicator (Bioblock scientific).
885 Bradford assay was used to determine total protein concentration in each extract. Equal amounts
886 of total proteins were separated by SDS-PAGE (10% polyacrylamide). After the run, proteins
887 were transferred to Hybond PVDF membrane (GE Healthcare Amersham, Germany), and the
888 transfer quality was evaluated by staining the membrane with Ponceau S (Sigma-Aldrich). The
889 Rho protein was visualized by hybridization with antiserum against *B. subtilis* Rho (Eurogentec,
890 Belgium; dilution 1:5,000) and the secondary peroxidase-coupled anti-rabbit IgG antibodies
891 A0545 (Sigma-Aldrich; dilution 1:20,000).

892

893

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895

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1243 **FIGURE LEGENDS**

1244 **Fig 1. Rho is specifically expressed during *B. subtilis* sporulation.**

1245 (A) Rho expression in vegetative cells is limited to the exponential growth phase and
1246 autoregulated. Kinetics of luciferase activity in *B. subtilis* WT P_{rho} -*luc* (empty circles) and RM
1247 P_{rho} -*luc* (filled-in circles) cells grown in rich medium LB. In this and other panels, the plain
1248 dotted and solid lines represent growth curves of the Rho-proficient strains and *rho*-deletion
1249 mutant (RM), respectively, measured by optical density OD₆₀₀. (B) In the sporulation-inducing

1250 conditions, *rho* expression is additionally activated in stationary phase. Comparative analysis
1251 of the luciferase activity in WT P_{rho} -*luc* cells grown in LB (brown circles) and in sporulation
1252 medium DSM (blue circles). Activation of luciferase expression from the early sporulation
1253 promoter *spoIIA* in the control WT P_{spoIIA} -*luc* cells grown in DSM (grey squares) marks the
1254 initiation of sporulation. (C) In the sporulation-inducing conditions, the autoregulation of Rho
1255 is weakened in stationary phase. Kinetics of luciferase activity in *B. subtilis* WT P_{rho} -*luc* (empty
1256 blue circles) and RM P_{rho} -*luc* (filled-in blue circles) cells grown in sporulation medium DSM.
1257 (D) Rho expression in the sporulation-inducing conditions correlates with the activation of
1258 Spo0A. Comparative analysis of luciferase expression from P_{rho} -*luc* (blue circles) and P_{spo0A} -
1259 *luc* (pink triangles) transcriptional fusions in WT (empty symbols) and RM (filled-in symbols)
1260 cells grown in DSM. (E and F) Rho expression in stationary phase depends on the initiation of
1261 sporulation. Kinetics of luciferase expression from P_{rho} -*luc* transcriptional fusions in WT cells
1262 (blue circles) and the sporulation mutants (E): *spo0A* (orange circles) and *sigH* (grey circles);
1263 and (F): *sigF* (red circles) and *sigE* (green circles). Measurements were taken every 5 minutes
1264 after cells inoculation in media at optical density $OD_{600} \sim 0.025$ (time point 0). For each strain,
1265 plotted are the mean values of luminescence readings corrected for OD from four independent
1266 cultures analyzed simultaneously. Each panel presents the simultaneously collected data. The
1267 data in (C) and (D) and in (E) and (F) were obtained in two independent experiments. Each
1268 strain and growth condition was tested at least three times. The results from the representative
1269 experiment are presented.

1270 **Fig 2. Role of the read-through transcription in the *rho* expression during sporulation.** (A)
1271 Schematic representation of gene organization and transcription within the *rho* locus of *B.*
1272 *subtilis* chromosome. Arrow-shaped and flat rectangles indicate protein-coding genes and non-
1273 coding RNA S-segments (Nicolas et al., 2012), respectively. Curved arrow-ended lines
1274 represent promoters; their regulatory sigma factors are indicated. Straight dot-ended lines
1275 represent intrinsic terminators. The underlying lines indicate the transcripts of the *rho* gene,
1276 which were identified in (Nicolas et al., 2012); the transcript initiated at the known *rho*
1277 promoter is bolded. The approximate size of the transcripts is shown. Small black rectangle
1278 schematizes the 5' *rho*-specific riboprobe used in the Northern blot. (B) Cartoon of the insertion
1279 of three intrinsic terminators at the end of *glpX* gene in the 3TER strain. (C) Northern blot
1280 analysis of the *rho*-specific transcripts in the wild type (WT), and the mutant *sigH* and 3TER
1281 strains during sporulation. Cells were grown in DSM and sampled during exponential growth
1282 (OD_{600} 0.5), the transition to stationary phase (OD_{600} 1.0) and in stationary phase (OD_{600} 1.8).

1283 Total RNA was extracted, processed and hybridized with the *rho*-specific riboprobe as
1284 described in Materials and Methods. *Upper panel*. The *rho*-specific transcripts visualized by
1285 Northern blotting. The arrows indicate the approximate size range of the transcripts. *Bottom*
1286 *panel*. Ribosomal 16S and 25S RNAs stained with ethidium bromide were used to control the
1287 equilibrium of the loaded RNA samples. (D) Suppression of the read-through transcription
1288 inhibits *rho* expression in stationary phase. Kinetics of luciferase activity in *B. subtilis* WT P_{rho} -
1289 *luc* (blue circles) and 3TER P_{rho} -*luc* (violet triangles) cells grown in the sporulation-inducing
1290 DSM. (E and F) In the absence of read-through transcription, the residual expression of *rho* in
1291 stationary phase still depends on sporulation. Kinetics of luciferase expression in 3TER P_{rho} -
1292 *luc* strain (violet triangles) and its sporulation mutants (E): *spo0A* (orange triangles) and *sigH*
1293 (grey triangles); and (F): *sigF* (red triangles) and *sigE* (green triangles) grown in DSM. The
1294 data in (E) and (F) were obtained in the same experiment and are independent from (D). The
1295 data were collected and processed as described in Fig 1. The experiments were reproduced at
1296 least three times. The results from the representative experiment are presented.

1297 **Fig 3. The expression of *rho* in the forespore compartment of sporangium depends on**
1298 **Sigma F.**

1299 The WT P_{rho} -*gfp* strain and its $\Delta sigF$ and $\Delta sigE$ mutant derivatives were induced for sporulation
1300 by the resuspension method as described in Materials and Methods. Cells were sampled three
1301 hours after resuspension in the nutrient-poor SM medium and observed by phase contrast
1302 microscopy, and by epifluorescence illumination of GFP or membrane-affine dye in two
1303 independent replicas. The right-hand cartoon depicts spatial GFP-mediated fluorescence in cells
1304 with asymmetric septa.

1305 **Fig 4. Forespore-specific expression of *rho* depends on the activity of a genuine SigF-**
1306 **dependent promoter.**

1307 (A and B) Sequence and structural elements of the SigA-dependent *rho* promoter reported by:
1308 (A; Ingham et al., 1999) and (B; Nicolas et al., 2012). The -35 and -10 boxes are bolded and
1309 colored in blue (A) and green (B). In (B), the upper blue lines indicate the relative position of
1310 the *rho* promoter identified by (Ingham et al., 1999). (C) Sequence and structural elements
1311 (bolded and colored in red) of putative SigF-dependent *rho* promoter identified in this analysis
1312 as matching consensus sequences recognized by SigF-containing RNA polymerase (Wang et
1313 al., 2006). The upper green lines indicate the relative position of the SigA-dependent *rho*
1314 promoter identified by (Nicolas et al., 2012). The asterisk indicates a conserved thymine

1315 nucleotide in the -35 sequence of the SigF-dependent promoter subjected to mutagenesis. (D
1316 and E) Mutations of the SigF-dependent *rho* promoter specifically inhibit *rho* expression in the
1317 forespores. The wild type (WT) and 3TER cells bearing the non-modified P_{*rho*}-*gfp* fusion and
1318 WT P_{*rho*}-*gfp* cells containing the indicated single-nucleotide mutations of putative SigF-
1319 dependent *rho* promoter were induced for sporulation and analyzed for GFP-mediated
1320 fluorescence as described in Materials and Methods and Fig 3. (D) Micrographs of typical cells
1321 observed by phase contrast, epifluorescence illumination of GFP or membrane-affine dye. (E)
1322 Average fluorescence intensities determined in predivisional cells and each compartments of
1323 sporangia in each strain. Fluorescence intensity was determined as described in Materials and
1324 Methods in two independent experiments. Bars represent standard deviation from the mean
1325 values.

1326

1327 **Fig 5. The alteration of the spatiotemporal expression of *rho* affects the resistance**
1328 **properties of mature spores.**

1329 Spores produced by WT and 3TER cells expressing *rho* from the non-modified promoters, their
1330 mutant derivatives WT-mT/A and 3TER-mT/A inactivated for *rho* expression in the forespore
1331 and RM cells expressing no Rho were analyzed for the levels of dipicolinic acid (A) and the
1332 resistance to ultraviolet irradiation (B) as described in Materials and Methods. (A) Spore DPA
1333 contents are normalized to the wild type level. The assay was reproduced ten times with two
1334 independently prepared sets of five spores. Plotted are mean values from all measurements.
1335 Statistical significance was estimated with a two-tailed t-test. The displayed p-value is as
1336 follows: *** $p \leq 0.001$. (B) UV test was reproduced five times with one set of spores. The bars
1337 represent standard deviation from the mean values.

1338

1339 **Fig 6. The alteration of the spatiotemporal expression of *rho* affects the morphology of**
1340 **spores.**

1341 Thin section transmission electron micrographs of spores produced by cells differentially
1342 expressing *rho*. White arrows indicate a thinner and less electron-dense outer coat in RM, 3TER
1343 and 3TER-mT/A spores, which did not express *rho* in the forespores during maturation. Black
1344 arrows indicate the detachment of outer and inner coats in RM, WT-mT/A and 3TER-mT/A
1345 which did not express *rho* in the mother cells.

1346

1347 **Fig 7. Rho activity determines the revival properties of spores.**

1348 Spores produced by WT (blue triangles), RM (red squares), 3TER (brown crosses), WT-mT/A
1349 (orange circles) and 3TER-mT/A (violet diamonds) cells were induced by the germinant L-
1350 alanine (10mM) and analyzed for germination (A) and outgrowth in the nutrient-poor MS
1351 medium (B) as described in Materials and Methods. (C) WT (filled-in blue triangles) and RM
1352 (filled-in red squares) spores were compared for outgrowth in the nutrient-replenished MS
1353 medium containing 0.5% casamino acids. The experiments were performed at least twice with
1354 three independent sets of spores. Each experiment included up to six replicas of individual
1355 suspensions of spores. The results of the representative experiment are plotted. The bars
1356 represent standard deviation from the mean values.

1357

1358 **Fig 8. The wild-type rate of spore outgrowth requires the expression of *rho* during spore**
1359 **morphogenesis and *de novo* after germination.**

1360 *B. subtilis* $P_{spac-rho}$ cells expressing Rho protein from the IPTG-inducible promoter were
1361 induced to sporulate in DSM in the absence (brown symbols) or presence (violet symbols) of
1362 IPTG 100 μ M. The mature spores were purified and analyzed for germination with L-alanine
1363 (A and B) and outgrowth in MS medium (C and D) in the absence (A, B and C; circles) or
1364 presence of IPTG 100mM (B and D; diamonds). The WT (bleu triangles) and RM (red squares)
1365 spores produced simultaneously with $P_{spac-rho}$ spores were used to compare the germination in
1366 (A) and outgrowth in (C and D). Of note, *rho* induction by IPTG (B): had no effect on the
1367 germination rates of the both types of $P_{spac-rho}$ spores, but (D): accelerated the outgrowth of
1368 spores expressing *rho* during morphogenesis (violet diamonds) to the WT level. The
1369 experiments were performed at least twice with two independent samples of the IPTG-induced
1370 or non-induced $P_{spac-rho}$ spores and included up to six replicas of individual suspensions of
1371 spores The results of the representative experiment are plotted. The bars represent standard
1372 deviation from the mean values.

1373

1374

1375 **Supporting information**

1376

1377 **S1 Fig. Induction of *Prho-gfp* expression in the forespore follows the progression of the**
1378 **engulfment process.**

1379 Montage of fluorescent microscopy images (upper row: green channel; lower row: red channel)
1380 of individual wild type cells bearing $P_{rho-gfp}$ transcriptional fusion during the growth in DSM.
1381 Cells were labeled with the red fluorescent Nile-Red dye and observed 2 hours after entering

1382 stationary phase (T2). Images are displayed from left to right following the progression of the
1383 sporulation process, from predivisional to fully engulfed prespore state (red channel), to show
1384 an increase of the fluorescence levels (green channel) in the prespore.

1385

1386 **S2 Fig. Inactivation of the SigF-dependent *rho* promoter inhibits sporulation-specific**
1387 **expression of luciferase in 3TER P_{rho} -*luc* cells.**

1388 The mutational analysis of the SigF-dependent promoter of *rho* using luciferase was performed
1389 in the 3TER genetic background to limit the interference of the read-through transcription.
1390 3TER cells bearing a non-modified (violet triangles) or the point-mutated *mF-35T/A* (green
1391 triangles) and *mF-35T/C* (orange triangles) P_{rho} -*luc* fusions were set to sporulate in DSM and
1392 analyzed for luciferase activity as described in Fig 2. Of note, the mutation *mF-35T/A*
1393 specifically decreases luciferase expression during sporulation, while the mutation *mF-35T/C*
1394 has a global inhibitory effect (scaled differently in the graph). Nevertheless, the sporulation-
1395 specific luminescence in 3TER *mF-35T/C* P_{rho} -*luc* is affected to a similar extent as in 3TER
1396 *mF-35T/A* P_{rho} -*luc*.

1397

1398 **S3 Fig. Complete inactivation of *rho* has a maximal effect on sporulation efficiency. (A)**

1399 Cells were induced for sporulation by the resuspension method, sampled three hours after
1400 resuspension, colored with lipid affine Mito-tracker red and analyzed by fluorescence
1401 microscopy for the presence of asymmetric septum. The proportion of septum-containing cells
1402 was determined by manual counting, in at least three fields of view and for a minimum of 600
1403 cells per strain and per replica. Plotted are average proportions of septum-containing cells from
1404 three experimental replica. The bars represent standard deviation from the mean values. (B and
1405 C) After resuspension, cells were continuously propagated in a poor SM medium and analyzed
1406 for the formation of heat-resistant spores at the indicated time (B) and overnight (20 hours) (C),
1407 as described in Materials and Methods. Plotted are average proportions of the heat-resistant
1408 spores from three independent experiments, two of which were performed in a prolongation of
1409 microscopy analyses in (A). The bars represent standard deviation from the mean values.

1410

1411 **S4 Fig. Alteration of *rho* expression does not significantly affect the resistance of spores**
1412 **to heat and lysozyme.**

1413 The purified spores of the wild type strain (WT), *rho*-deletion mutant (RM) and strains altered
1414 for *rho* expression in the mother cell (3TER) or in the forespore (WT-mT/A) or in the two
1415 compartments of sporangia (3TER-mT/A) were analyzed for the resistance to lysozyme (A and

1416 B) and heat (C). (A) Spores were activated as described in Materials and Methods, suspended
1417 in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) containing 10 mg/ml lysozyme (Fluka) at
1418 OD₆₀₀ 0.2 and incubated at 37°C for 1 hour. (B) Spores were chemically treated to remove
1419 spore coats as described in Materials and Methods. Decoated spores were suspended in 10mM
1420 Tris-HCl (pH 8.0) at OD₆₀₀ 0.2 and treated with lysozyme (10 µg/ml, final concentration) for
1421 10 and 20 minutes at 37°C. In (A) and (B), spore viability after lysozyme treatment was
1422 established by plating spores from the treated and untreated samples at LB agar plates. (C) The
1423 activated spores were heated at 90°C for the indicated time, plated at LB agar plates and
1424 incubated at 37°C for 24 hours. The heat resistance of spores was calculated as a proportion of
1425 viable colony-forming spores in heated and unheated samples. The same set of spores was used
1426 in all experiments; spore samples in (B) originate from two independent decoating procedures.
1427 The experiments were reproduced four times, and the established average values are presented
1428 with the standard deviations of the mean (bars).

1429

1430 **S5 Fig. Rho inactivation does not affect UV-resistance of vegetative cells.**

1431 The wild type (WT, blue line) and *Δrho* (RM, red line) cells were grown in LB to OD₆₀₀ 0.5,
1432 plated at LB-agar plates in serial dilutions and irradiated by ultraviolet light at the indicated
1433 doses using the UV crosslinker as described in Materials and Methods. Cells were scored after
1434 24h of incubation at 37°C. The experiment was reproduced twice with three culture samples
1435 of each strain. Plotted are the mean values from two experiments.

1436

1437 **S6 Fig. Absence of Rho accelerates spore germination with different nutrient germinators.**

1438 Spores produced by WT (blue triangles), RM (red squares), 3TER (brown crosses), WT-mT/A
1439 (orange circles) and 3TER-mT/A (violet diamonds) cells were induced for germination by (A)
1440 the germinant AGFK (10 mM L-asparagine, 1mM D-glucose, 10mM KCl and 10 mM D-
1441 fructose, final concentrations) or (B) L-valine (10mM). The experiments were performed twice
1442 with two independent sets of spores. Each experiment included up to six replicas of individual
1443 suspensions of spores. The results of the representative experiment are plotted. The bars
1444 represent standard deviation of the mean values.

1445

1446 **S7 Fig. Absence of Rho does not affect viability of germinating spores.**

1447 The wild type WT (blue) and *rho*-minus RM (red) spore suspensions at OD₆₀₀ 0.1 were
1448 activated and induced for germination by 10mM L-alanine as described in Materials and
1449 Methods. Spore viability during germination was established by plating of spores in sequential

1450 dilutions at LB-agar plates at the indicated time after germinant addition. Plates were incubated
 1451 at 37°C for 24h before the colonies counting. The experiment was reproduced twice with two
 1452 independently prepared spore samples and included four replicas of each spore suspension. The
 1453 results of the representative experiment are plotted. The bars represent standard deviation from
 1454 the mean values.

1455

1456 **S8 Fig. Functional analysis of $P_{spac-rho}$ transcriptional fusion.**

1457

1458 *B. subtilis* cells expressing *rho* from the IPTG-inducible promoter ($P_{spac-rho}$) were incubated in
 1459 LB medium in the presence of the indicated concentrations of IPTG to OD₆₀₀ 0.6 and analyzed
 1460 for the levels of Rho protein in comparison with wild-type cells (WT) by Western blot with the
 1461 specific anti-Rho^{Bs} anti-serum as described in Materials and Methods. Protein equilibrium
 1462 between the loaded samples was controlled by Bradford assay and visualization of Mbl protein
 1463 by anti-Mbl antibodies as described in (Bidnenko et al., 2023).

1464

1465 **S1 Table. Differential expression analysis of genes from the “Sporulation” lifestyle**
 1466 **category in stationary Δrho vs. stationary WT *B. subtilis* cells (compiled from S3 Table,**
 1467 **Bidnenko et al., 2023).**

1468 **S2 Table. Strains and plasmids used in the analysis.**

1469 **S3 Table. Oligonucleotides used for strains construction.**

1470

1471

1472 **S2 Table. Strains and plasmids used in this study.**

Strains	Genotype (resistance)	Source or reference
BSB1	<i>B. subtilis</i> 168 <i>trp</i> ⁺	Nicolas <i>et al.</i> , 2012
BRL1	BSB1 $\Delta rho::phleo$ (<i>phleo</i> ^R)	Bidnenko <i>et al.</i> , 2017
BRL111	BSB1 $P_{spoIIA-luc}$ (<i>Cm</i> ^R)	Bidnenko <i>et al.</i> , 2017
BRL116	BSB1 $P_{spo0A-luc}$ (<i>Cm</i> ^R)	Bidnenko <i>et al.</i> , 2017
BRL118	$\Delta rho::phleo P_{spo0A-luc}$ (<i>Cm</i> ^R)	Bidnenko <i>et al.</i> , 2017
BRL871	BSB1 $P_{rho-luc}$ (<i>Cm</i> ^R)	This study
BRL880	$\Delta rho::phleo P_{rho-luc}$ (<i>phleo</i> ^R <i>Cm</i> ^R)	This study
ABS549	PY79 $\Delta spo0A::tet$ (<i>Tet</i> ^R)	Chastanet <i>et al.</i> , 2010
BKK00980	<i>B. subtilis</i> 168 <i>trpC2 sigH::km</i> (<i>Km</i> ^R)	Koo <i>et al.</i> , 2017
BRL877	BSB1 $spo0A::tet P_{rho-luc}$ (<i>Tet</i> ^R <i>Cm</i> ^R)	This study
BRL898	BSB1 $sigH::km P_{rho-luc}$ (<i>Km</i> ^R <i>Cm</i> ^R)	This study
RL1265	$sigF::km (spoIIAC\Delta::km)$	Fawcett <i>et al.</i> , 2000

EU8701	<i>sigE::erm (spoIIIGBA::erm)</i>	Kenney and Moran, 1987
MO1780	<i>sigE::tet (spoIIIGABA::tet)</i>	Guérout-Fleury <i>et al.</i> , 1996
BRL878	BSB1 <i>sigE::erm P_{rho}-luc</i> (Em ^R Cm ^R)	This study
BRL879	BSB1 <i>sigF::km P_{rho}-luc</i> (Km ^R Cm ^R)	This study
BRL894	BSB1 <i>P_{rho}-gfp</i> (Sp ^R)	This study
BRL956	BSB1 <i>sigE::tet P_{rho}-gfp</i> (Tet ^R Sp ^R)	This study
BRL957	BSB1 <i>sigF::km P_{rho}-gfp</i> (Km ^R Sp ^R)	This study
BRL1111	3TER <i>P_{rho}-luc</i> (Cm ^R)	This study
BRL1119	3TER <i>-35mT/C P_{rho}-luc</i> (Cm ^R)	This study
BRL1165	3TER <i>-35mT/A P_{rho}-luc</i> (Cm ^R)	This study
BRL1159	3TER <i>P_{rho}-gfp</i> (Sp ^R)	This study
BRL1144	BSB1 <i>-35mT/C P_{rho}-gfp</i> (Sp ^R)	This study
BRL1172	BSB1 <i>-35mT/A P_{rho}-gfp</i> (Sp ^R)	This study
BRL1175	3TER <i>spo0A::tet P_{rho}-luc</i> (Tet ^R Cm ^R)	This study
BRL1176	3TER <i>sigH::km P_{rho}-luc</i> (Km ^R Cm ^R)	This study
BRL1177	3TER <i>sigE::tet P_{rho}-luc</i> (Tet ^R Cm ^R)	This study
BRL1178	3TER <i>sigF::km P_{rho}-luc</i> (Km ^R Cm ^R)	This study
BRL1130	3TER	This study
BRL1185	BSB1 <i>-35mT/A</i>	This study
BRL1194	3TER <i>-35mT/A</i>	This study
BRL1300	BSB1 <i>rho.ΩP_{spac}-rho</i> (Em ^R)	This study
Plasmids		
pUC18Cm-luc	Cm ^R , Amp ^R	Mirouze <i>et al.</i> , 2011
pCVO119	Sp ^R Amp ^R	Van Ooij <i>et al.</i> , 2004
pMAD	Em ^R , Amp ^R	Arnaud <i>et al.</i> , 2004
pMUTIN4	Em ^R	Vagner <i>et al.</i> , 1998
pET28a	Km ^R	Novogen
pBRL862	Cm ^R , Amp ^R ; used to construct BRL871 (<i>P_{rho}-luc</i>)	This study
pBRL893	Sp ^R Amp ^R ; used to construct BRL894 (<i>P_{rho}-gfp</i>)	This study
pBRL1107	Cm ^R , Amp ^R ; used to construct BRL1111 (3TER <i>P_{rho}-luc</i>)	This study
pBRL1150	Sp ^R Amp ^R ; used to construct BRL1159 (3TER <i>P_{rho}-gfp</i>)	This study
pBRL1116	Cm ^R , Amp ^R ; used to construct BRL1119 (3TER <i>-35mT/C P_{rho}-luc</i>)	This study
pBRL1162	Cm ^R , Amp ^R ; used to construct BRL1165 (3TER <i>-35mT/A P_{rho}-luc</i>)	This study
pBRL1141	Sp ^R Amp ^R ; used to construct BRL1144 (<i>-35mT/C P_{rho}-gfp</i>)	This study
pBRL1164	Sp ^R Amp ^R ; used to construct BRL1172 (<i>-35mT/A P_{rho}-gfp</i>)	This study
pBRL1108	Em ^R , Amp ^R ; used to construct BRL1130 (3TER)	This study
pBRL1174	Em ^R , Amp ^R ; used to construct BRL1185 (BSB1 <i>-35mT/A</i>)	This study
pBRL1180	Em ^R , Amp ^R ; used to construct BRL1194	This study

	(3TER -35mT/A)	
pMutin4rho	Em ^R , Amp ^R ; used to construct BRL1300 (rhoΩP _{spac} -rho)	This study
pETRho	Km ^R ; pET28a-rho	This study

1473

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1494 encodes a sporulation-essential sigma factor in Bacillus subtilis. *Journal of*
1495 *bacteriology*, 169(7), 3329-3339.

1496

1497 **S3 Table. Oligonucleotides used for strains construction**

Oligonucleotide	Sequence (5' -> 3')
glpXBam *	CACGCTTCTGgATcCTCCTGACATG
veb738 **	TATGTTTTTGGCGTCTTCCATAAAAACACCACGCTTTTC
lucintrev	CAATCAGAGTGCTTTTGGCGAAG

veb739	ATGGAAGACGCCAAAAACATA
veb734	CTAGACCCGGGGATCTCTG
veb735	TCTAGAGCAACGTTCTTGC
veb797 **	<u>GCAAGAACGTTGCTCTAGACAAACAGAAAAAGCGGGTG</u>
veb798 **	<u>CAGAGATCCCCGGGTCTAGTTATGGACGGATTACAAGATTGG</u>
veb742 **	<u>GTTCTTCTCCTTTACTCATAAAAAACACCACGCTTTTC</u>
veb741	<i>acgctcgac</i> TTATTTGTATAGTTCATCCATG
veb740	ATGAGTAAAGGAGAAGAAC
veb802 ***	<u>cAATTTTTT</u> GAAATAAGGTAAAAATAAAG
veb803	TTCAACTGAAATAAGGTTTTGAC
veb805 ***	<u>aAATTTTTT</u> GAAATAAGGTAAAAATAAAG
veb795	CTTGG <i>Atcc</i> TAGTTGATCGGTCTC
veb796	TGGA <i>Att</i> CGCTGACGGTTATC
veb808	CTCTGAACATGTCATCTGCTGC
veb806	TGA <i>g</i> GATCCGGATAAAGTGGCG
veb807	GCAGCAGATGACATGTTTCAGAG
veb880	GGGTGGAATTCTTACCGATAGAAG
veb596	<i>cgcctcgac</i> TTACCTTCTTGCAGATGATAG
veb599	<i>gatccat</i> ATGAAAGACGTATCTATTTCTC
YRH1****	<u>CTAATACGACTCACTATAGGAGACAGGAATCCGAAACCTTCAG</u>
YRH2	TCTCCTATTACAGCAAACCTGAC

1498

1499 * Here and thereafter, the modified nucleotides are in small characters and the recognition
1500 sites of the endonucleases are in italics.

1501 ** Sequences complementary to other oligonucleotides are underlined

1502 *** The 5'-terminal nucleotides creating the mutations are bolded and underlined.

1503 **** T7 promoter sequence is underlined.

1504

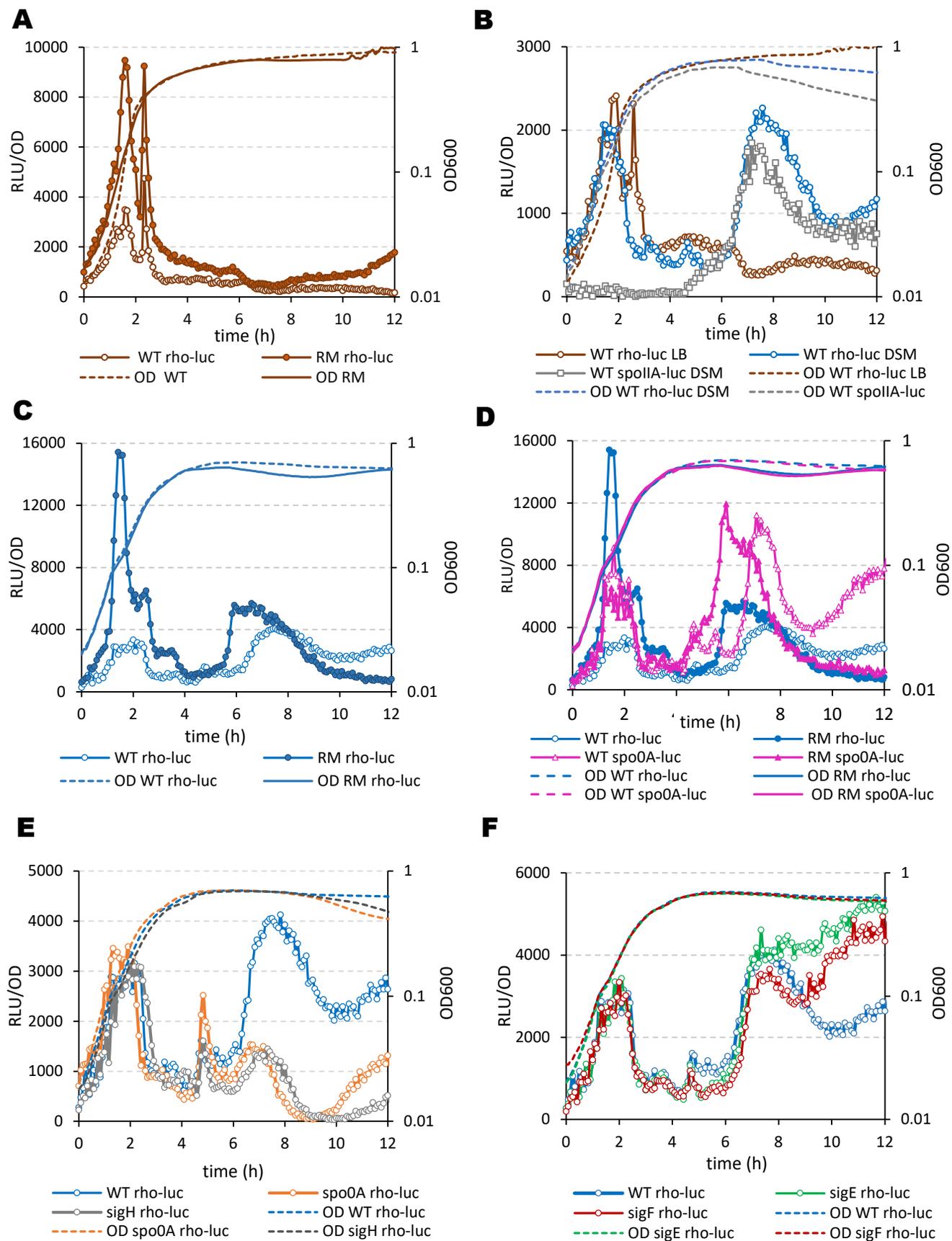


Fig 1

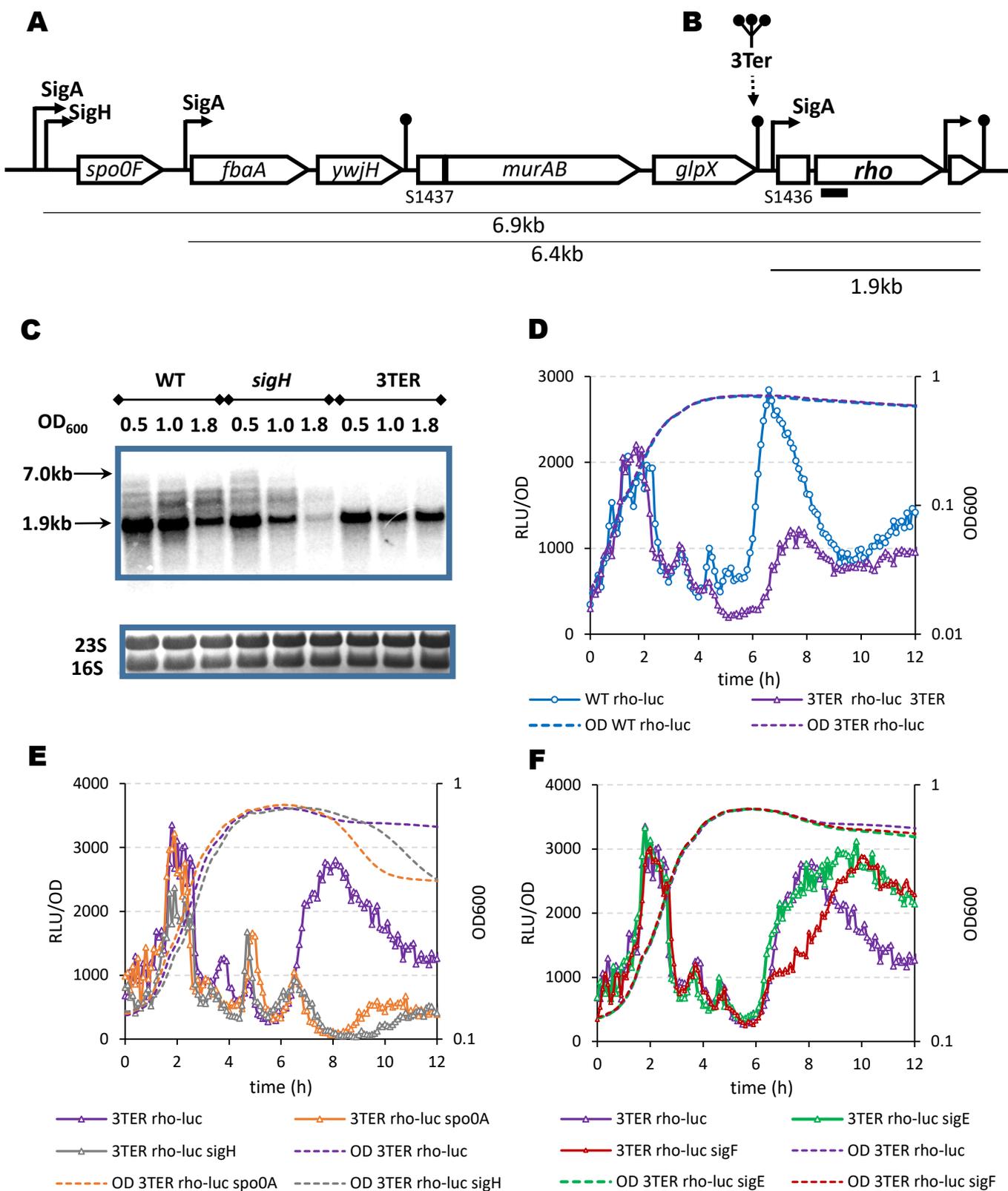


Fig 2

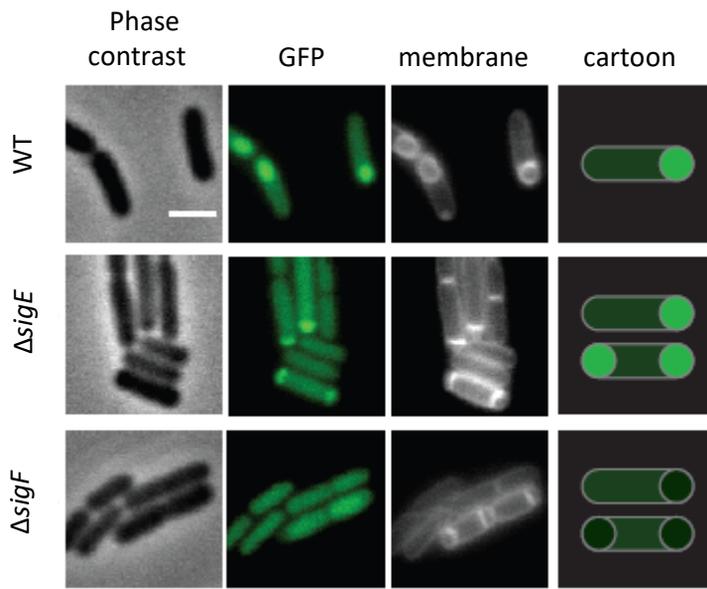


Fig 3

A tcagTGAATaatttttgaataaggTAAAAAtaaagagtgtgc
 -35 -10

B tcAGTTGaataatttttgaaaTAAGGTAAAAATAAAGAGTGctgc
 -35 -10

C tcagttGaATAaatttttgaataaaGGTAAAAATAaagagtgtgc
 -35 -10
 *
 14 bp A/T rich spacer

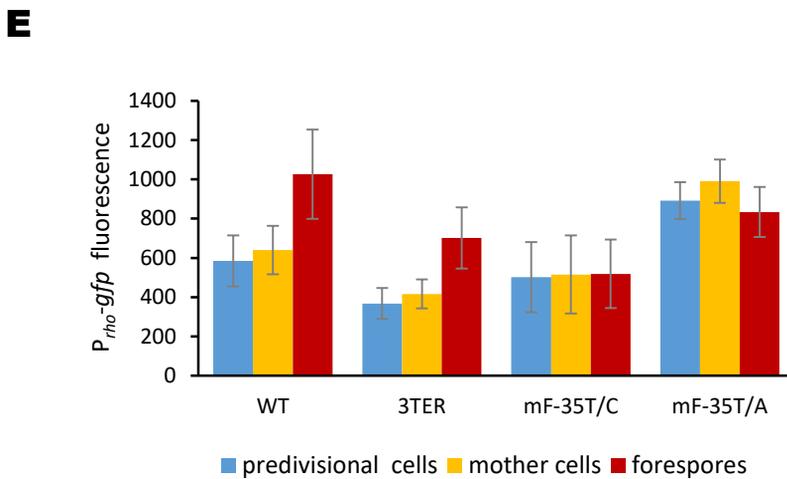
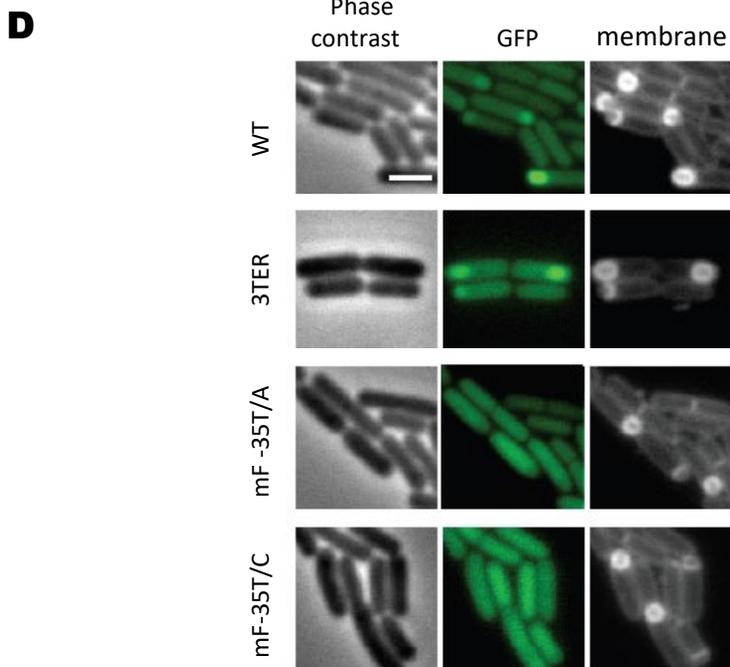


Fig 4

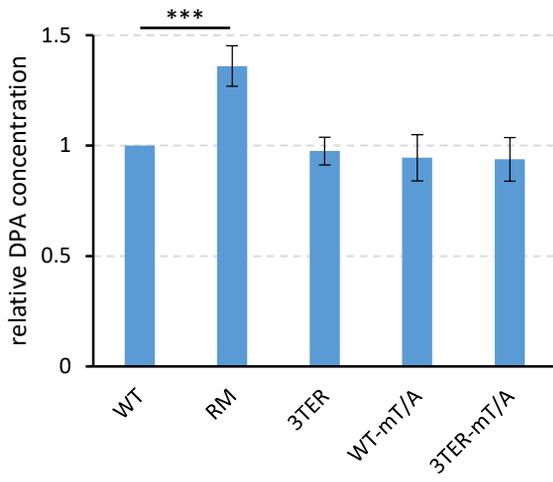
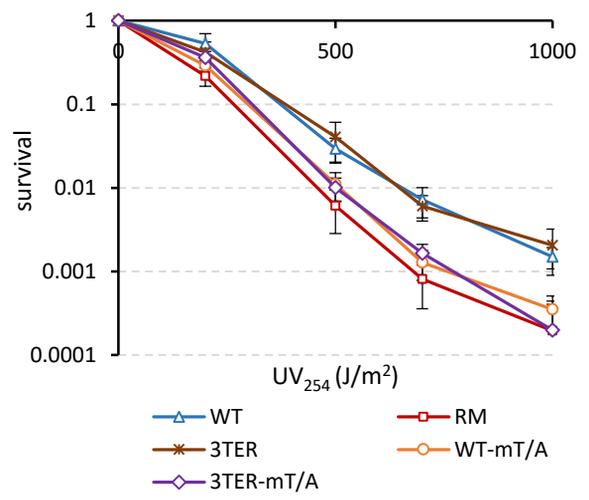
A**B**

Fig 5

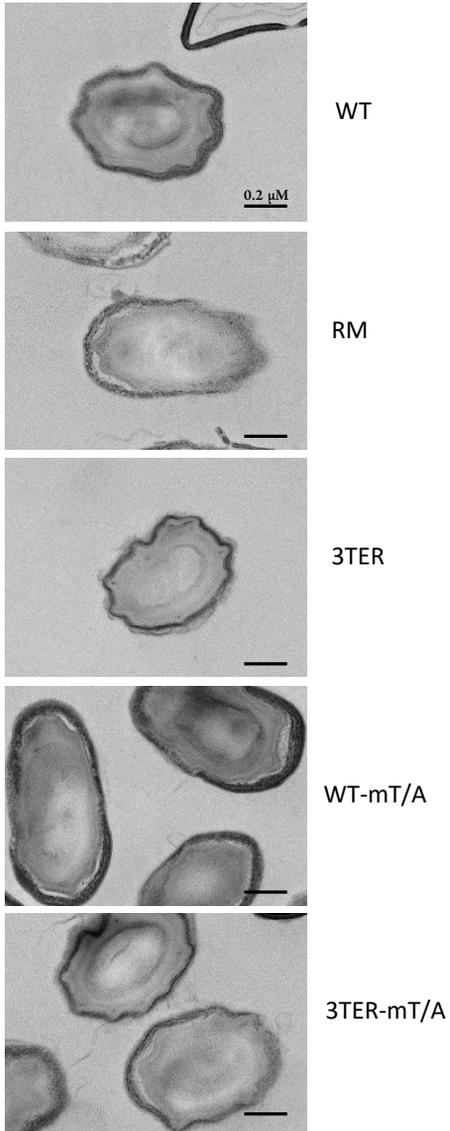


Fig 6

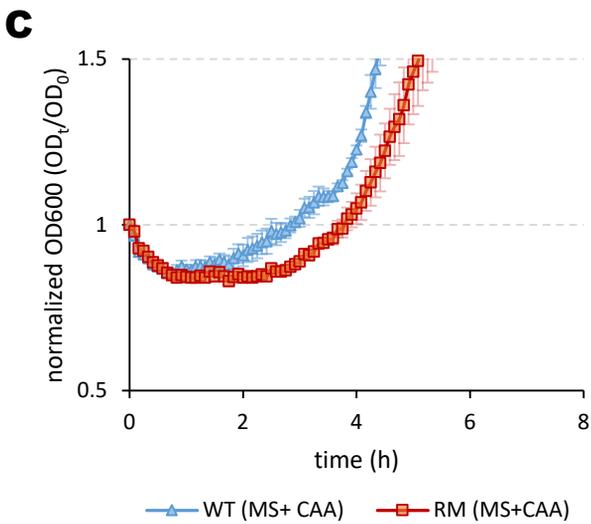
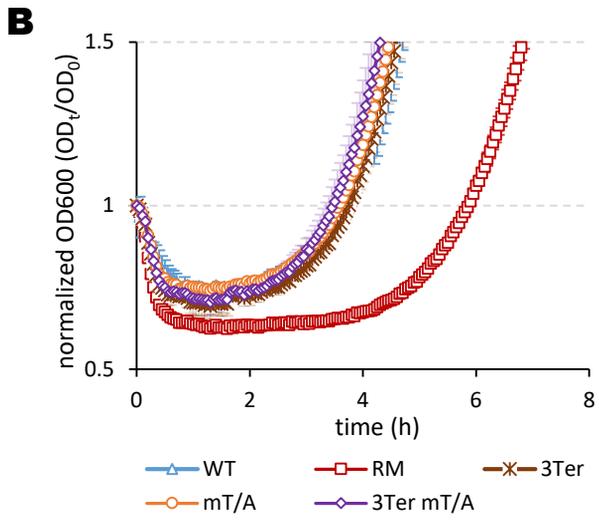
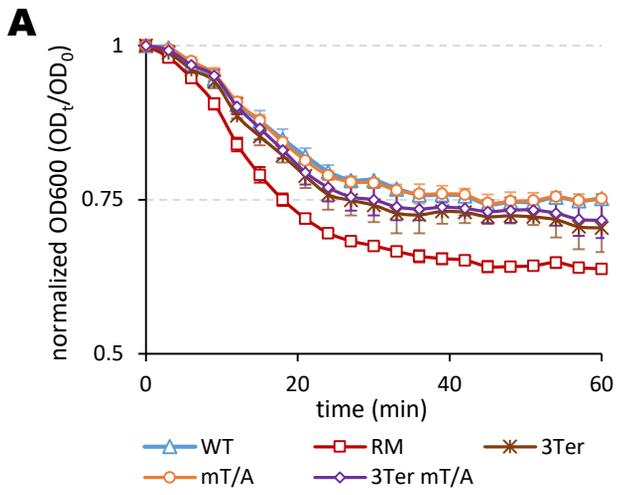


Fig 7

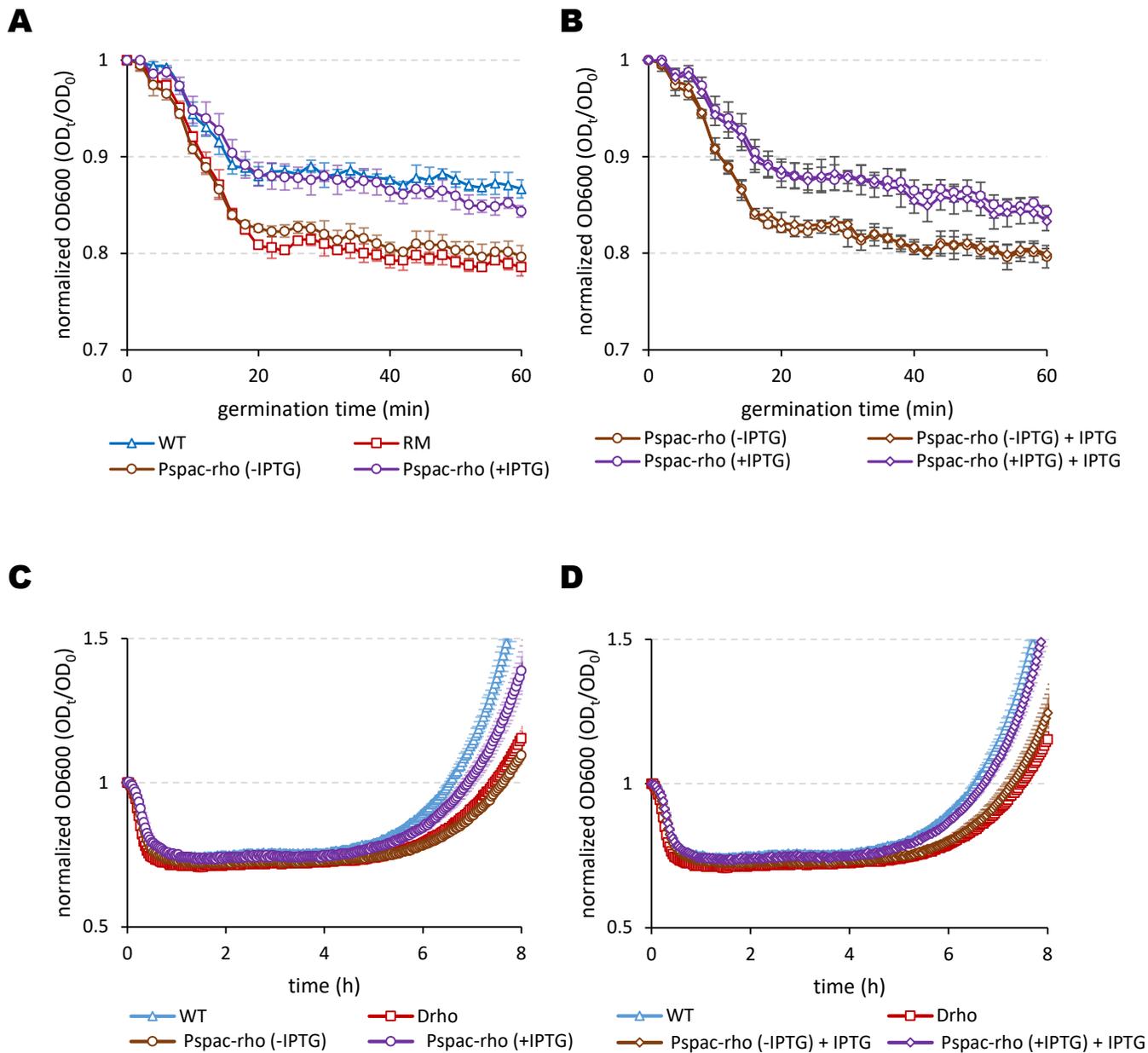
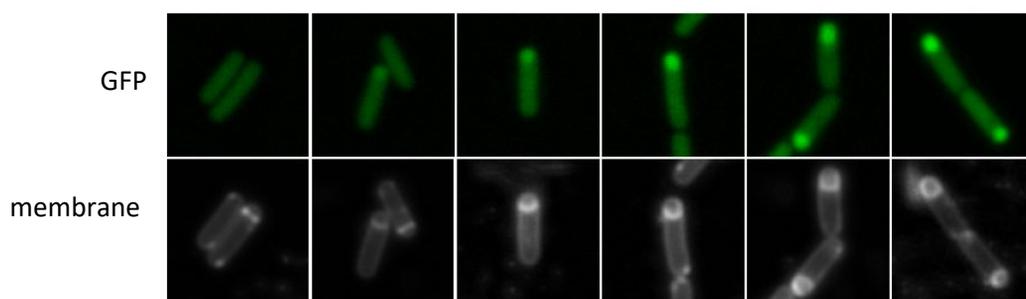
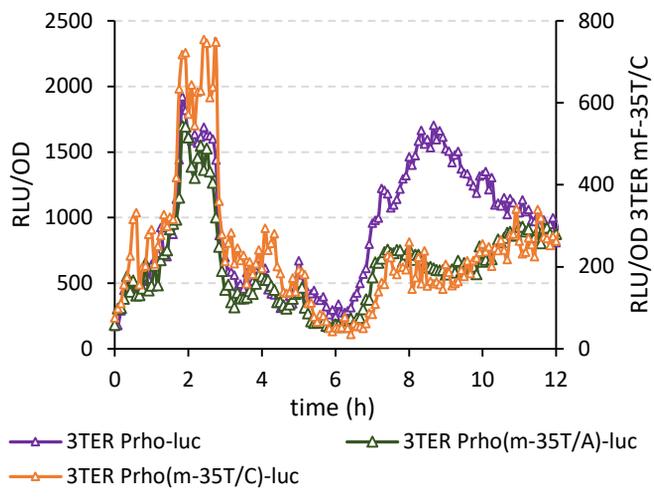


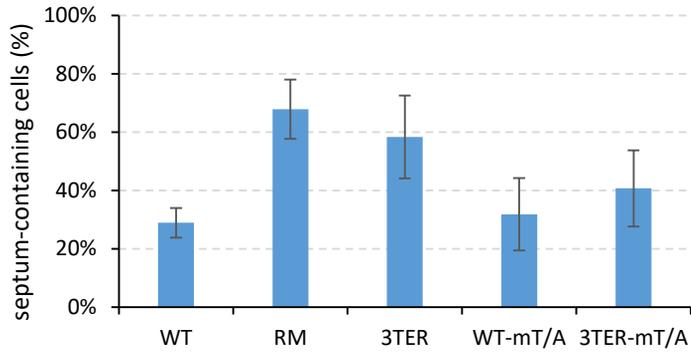
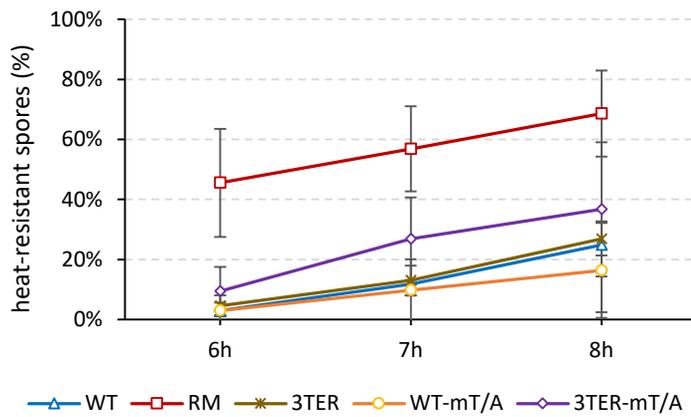
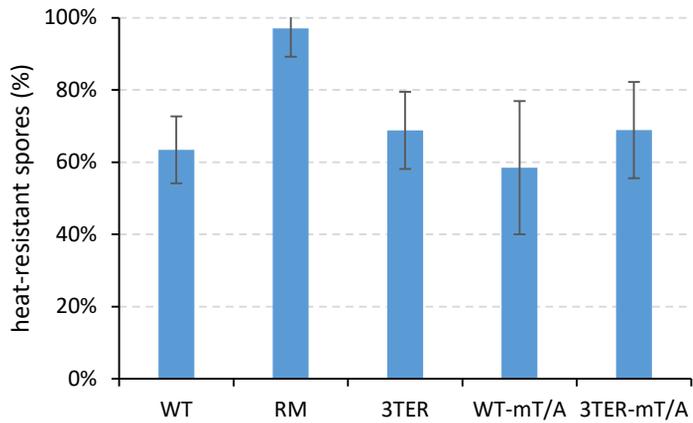
Fig 8



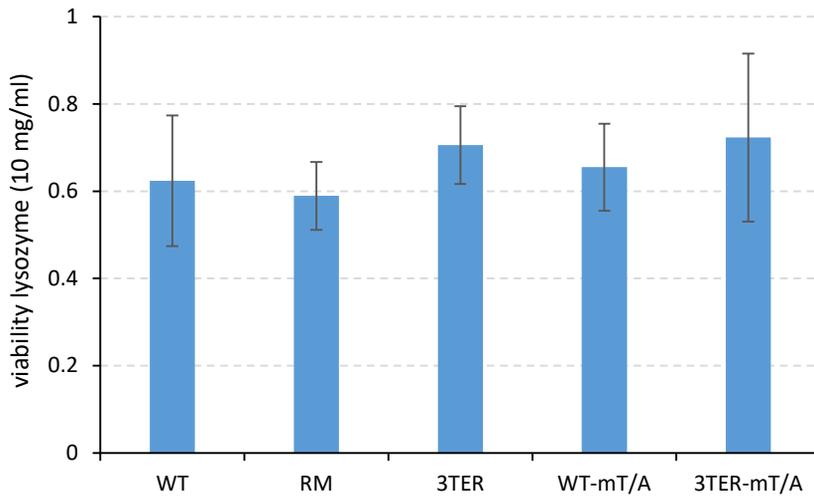
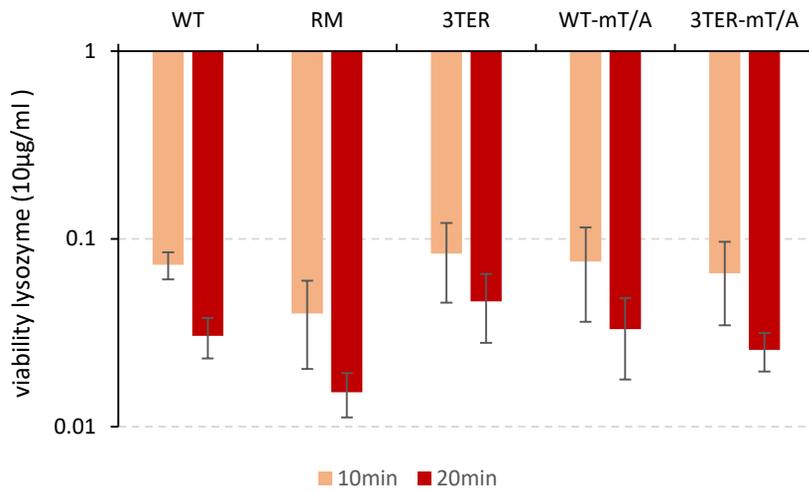
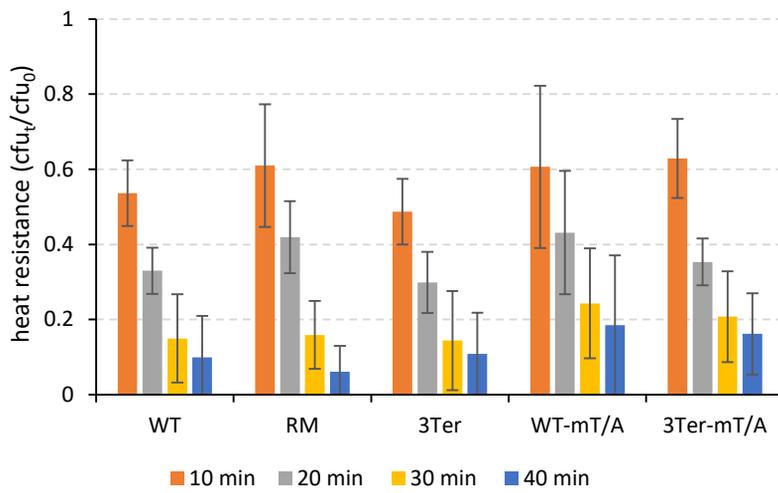
S1 Fig



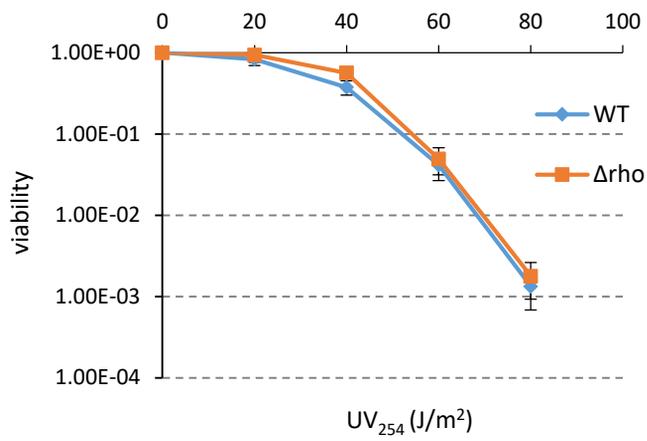
S2 Fig

A**B****C**

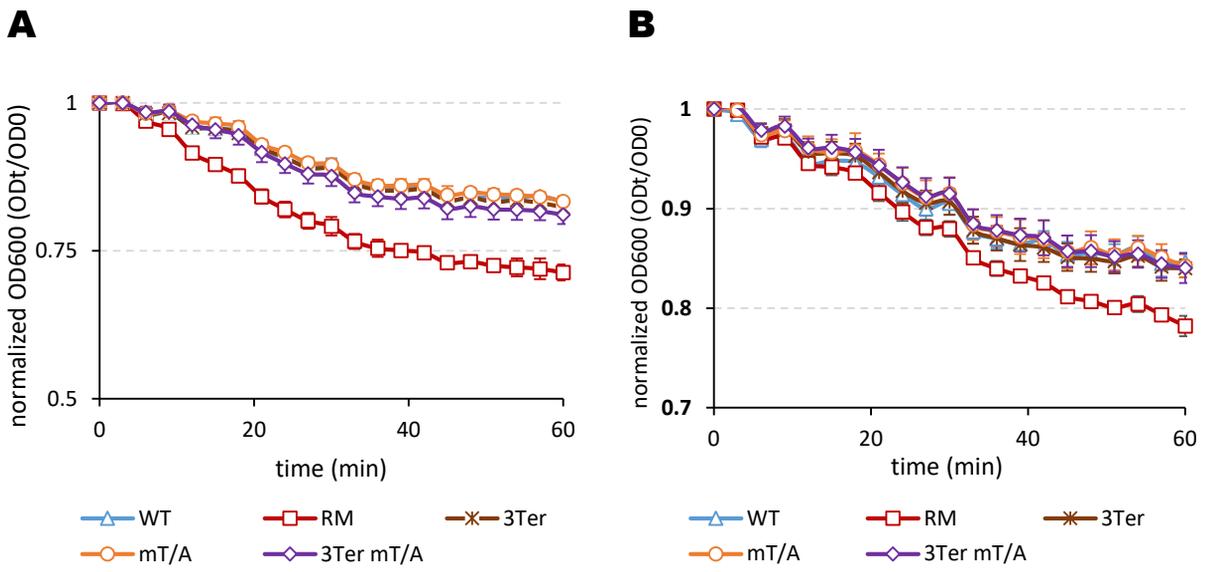
S3 Fig

A**B****C**

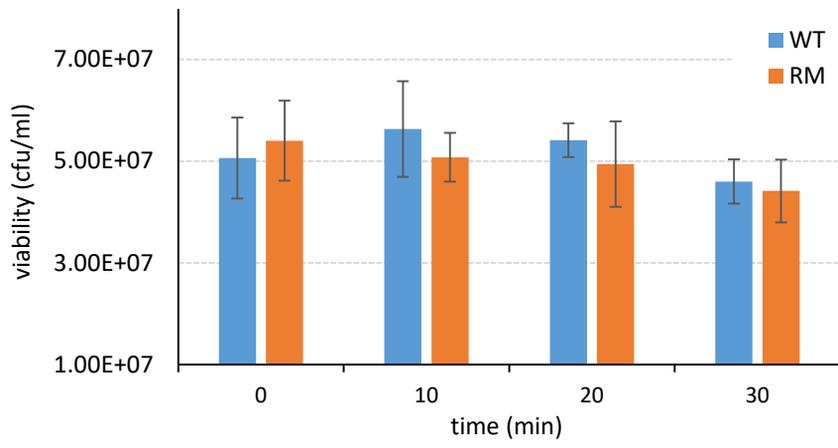
S4 Fig



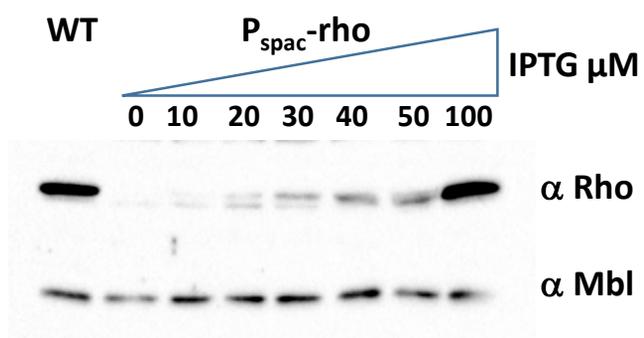
S5 Fig



S6 Fig



S7 Fig



S8 Fig