Bovine serum albumin promotes reactivation of viable but non-culturable *Mycobacterium tuberculosis* via activation of protein kinase-dependent cell division processes

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12

Abstract

Objective: *Mycobacterium tuberculosis* (Mtb) H37Ra strain has been reported to rapidly enter the viable but non-culturable (VBNC) state following treatment with an NADH oxidase inhibitor (diphenyleneiodonium [DPI]) and to be reactivated by fetal bovine serum (FBS). However, the mechanism underlying FBS-induced reactivation is unclear. We tried to reveal the mechanism of FBS-induced reactivation using *M. tuberculosis* H37Rv.

Methods: First, we evaluated the effect of DPI on culturability, viability and changes of acid-fastness toward H37Rv. Secondly, we measured the reactivation-promoting effects of human serum albumin, egg-white albumin and antioxidative agents in DPI-induced VBNC cells. We also inhibited adenylyl cyclase and protein kinase which is the downstream of adenylyl cyclase to evaluate the influence to reactivation capacity of bovine serum albumin (BSA).

Results: DPI treatment induced VBNC state in H37Rv, resulting in a high proportion of viable cells but a low proportion of culturable cells, loss of acid-fastness and lipid-accumulation. Not only FBS but also BSA alone could reactivate H37Rv. Contrary to our expectation, only human serum albumin had a similar restorative effect to BSA. The inhibition of adenylyl cyclase by SQ22536 did not have a significant effect on reactivation; however, the inhibition of mycobacterial protein kinase by H89 and staurosporine strongly suppressed the BSA-induced reactivation.

Conclusion: DPI-induced VBNC Mtb cells may be reactivated via the activation of protein kinase-dependent cell division processes through interaction with BSA.

Introduction

Mycobacterium tuberculosis (Mtb) is known to as one of the intracellular parasitic bacteria that can survive inside host macrophages. It develops a latent phenotype, dormancy, due to various stresses such as host immune response, oxygen depletion, nutritional starvation, *etc.*

37 The dormant Mtb cells can persist inside the host and cause latent tuberculosis infection (LTBI),
38 which seeks reactivation. Lowering the risk of Mtb reactivation in LTBI patients is considered
39 to be one of the important strategies to reduce the incidence of TB (1). Dormancy phenomenon
40 in non-sporulating bacteria have described as two major states: the viable but non-culturable
41 (VBNC) state and bacterial persistence(2). VBNC cells are the nonculturable population that
42 can be induced by exposure to stress. In addition, they are unable to grown on nutrient media
43 even after removal of stress and require much longer time or specific stimulus for reactivation
44 (2, 3). On the other hand, persistent cells are stochastically arising cells within growing cultures
45 that is tolerant to stress including high-dose antibiotics, and they are also growth-arrested.
46 However, they are typically able to grow on nutrient media following after the removal of stress
47 with short period (2).

48 Many pathogenic bacteria, including Mtb, can enter into the VBNC state, and it is considered
49 to be their natural state in the environment including human body (4). Various *in vitro* models,
50 such as models of hypoxia, nutritional starvation (including potassium-deficiency), a lipid-rich
51 environment, and other multiple stresses are reported to induce a VBNC state in Mtb cells (5–
52 9). A deep understanding of the mechanisms of reactivation is necessary for the prevention of
53 active tuberculosis.

54 One of the major hypotheses regarding the mechanism underlying the formation of VBNC
55 bacteria involves oxidative damage generated by harsh external conditions (10). Thus, some
56 studies have reported that the antioxidation during culture is one of the key mechanisms
57 through which VBNC bacteria are reactivated (11, 12). Some mycobacterial culture media
58 contain bovine serum albumin (BSA) and BSA on mycobacterial culture has been considered
59 to be growth-supporting agent that is involved in detoxification by the absorption of free fatty
60 acid and other growth-suppressing agents that are spontaneously generated during culturing
61 (13–15). For this reason, the mycobacterial culture media that are most frequently used at the
62 present time are BSA-containing systems, such as Middlebrook 7H series or Dubos medium.

63 However, few studies have focused on the effect of such media on VBNC cells.

64 Mukamolova *et al*. revealed that among patients with active TB, there is certain
65 subpopulation for whom sputum is not culturable in conventional medium, but which becomes
66 to be culturable in the presence of resuscitation promoting factor (Rpf), which is a
67 peptidoglycan-hydrolyzing protein that is highly conserved in actinobacteria (16). Rpf
68 reactivates non-culturable Mtb cells both *in vitro* and *in vivo* (17), suggesting the presence of
69 VBNC Mtb cells in clinical specimens.

70 Previously, NADH oxidase inhibitor diphenyleneiodonium chloride (DPI) was reported to
71 simply and rapidly induce a VBNC state in the Mtb H37Ra strain, and incubation with fetal
72 bovine serum, which is a common supplement for mammalian cell cultures, could facilitate the
73 reactivation of VBNC cells, while incubation with OADC (oleate-albumin-dextrose-catalase)
74 supplementation could not (18). This report gave us an important clue for constructing a simple
75 and rapid assay system to induce and reactivate VBNC Mtb cells. However, when we tried to
76 apply this system to H37Rv, we happened to find that DPI-induced VBNC cells that could be
77 reactivated by not only fetal bovine serum but also by the addition of BSA—this was observed
78 for both H37Rv and H37Ra (internal data). Then, we hypothesized that BSA would act as a
79 reactivation-promoting agent toward DPI-induced VBNC cells.

80 One possibility of the reactivation-promoting mechanism of BSA is its antioxidative
81 property, which has been well-studied (19). Another possibility is that the cellular processes
82 involving cyclic AMP, an important second messenger of the cell. Shleeva *et al*. showed that
83 the presence of high amounts of cAMP provided by adenylyl cyclase is also essential for
84 reactivation of *M. smegmatis* and Mtb from the VBNC state (20, 21). Notably, overexpression
85 of Rv2212 gene, which encodes adenylyl cyclase in Mtb (22), significantly affects both entry
86 into and reactivation from the VBNC state (21).

87 Understanding the detailed mechanism of reactivation from the VBNC state is crucial to
88 reduce the risk of reactivation of Mtb cells in LTBI patients. In this study, we examined the

89 effects of antioxidative agents and the inhibition of adenylyl cyclase to reveal the roles of these
90 factors in the reactivation of VBNC Mtb cells.

91 Materials and Methods

92 Bacterial strains, growth media and culture conditions

93 This work was carried out using *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and
94 H37Ra (ATCC 25177). Both cells were cultured for 3–5 days in a 60 mL glycol-modified
95 polyethylene terephthalate (PETG) bottle containing 20 mL Dubos broth (2.5 g/L Na_2HPO_4,
96 2.0 g/L L-asparagine, 1.0 g/L KH_2PO_4, 0.5 g/L pancreatic digest of casein, 0.2 g/L Tween 80,
97 0.5 mg/mL $CaCl_2$•$2H_2O$, 0.1 mg/mL $CuSO_4$, 0.1 mg/mL $ZnSO_4$•$7H_2O$, 0.05 g/L ferric
98 ammonium citrate, 0.01g $MgSO_4$•$7H_2O$) with 5% (v/v) glycerol, and 10% (v/v) ADC
99 supplementation (5.0 g bovine serum albumin fraction V (BSA), 2.0 g dextrose, 0.003 g
100 catalase in 100 mL distilled water) at 37˚C in an orbital shaker (100 rpm) until the OD_{600}
101 reached up to 0.35. All procedures were performed at a BSL-3 facility. For further analysis, we
102 used commercially available BSA Cohn fraction V (Merck, Darmstadt, Germany), unless
103 otherwise stated. In addition, we used molecular biology grade Tween 80 (Merck product
104 number P5188-100ML) without any predilution.

105 Counting the number of culturable cells

106 The cultured bacterial suspension was serially diluted 10-fold in phosphate buffered saline (pH
107 6.8) with 0.1%(w/v) Tween 80 (PBS-T) and 25 μL of each diluent was inoculated onto a
108 Middlebrook 7H10 agar plate supplemented with oleic-albumin-dextrose-catalase (OADC)
109 (Becton Dickinson, Sparks, MD) in duplication. Colonies were counted after at least 3 weeks'
110 incubation at 37˚C under 5% CO_2. The limit of detection was determined to be 20 CFU/mL
111 because of the diluent factor.

112 Effect of BSA on the growth of Mtb cells in Dubos medium

113 Ten milliliters of log phase culture of Mtb H37Rv that reached an OD_{600} of up to 0.35 in Dubos
114 medium, as described previously, was sedimented at 3,000 ×*g* for 10 minutes at 4˚C and the
115 supernatant was discarded. The sediment was washed twice with fresh Dubos broth and
116 resuspended with 10 mL of Dubos broth. The suspension was diluted to 1:10 with Dubos broth
117 with or without the addition of BSA solution (5.0 g BSA fraction V in 100 mL distilled water)
118 at the final concentration of 0.1% (w/v) BSA. The diluted suspension was dispensed into 24-
119 well plates (Sarstedt, Nümbrecht, Germany) in 1.5 mL increments and sealed with gas-
120 permeable film (Breathe-Easy, Diversified Biotech Inc, Dedham, MA, United States). Cultures
121 were incubated for 5, 10, 15 and 20 days at 37˚C under 5% CO_2. At each time-point, the number
122 of culturable cells was measured as described above.

123 **Induction of the VBNC cells**

124 Induction of the VBNC cells were performed according to the previous report (18) with some
125 modification. In short, the log phase culture of Mtb H37Rv or H37Ra that reached an OD_{600} of
126 up to 0.35 in Dubos medium, as previously described, was evenly divided into 30 mL PETG
127 bottles and 5 mg/mL diphenyleneiodonium chloride (DPI) in dimethyl sulfoxide (DMSO)
128 solution was directly added at the final concentration of 4 μg/mL with immediate agitation.
129 The same volume of DMSO was added to the untreated control sample. Cultures were further
130 incubated for 24 h at 37˚C without shaking. After incubation, their culturability and viability
131 were measured as described above.

132 **Cell viability assay**

133 Mtb cell viability was measured using esterase activity and membrane integrity of the cells
134 using dual staining with 10 mg/mL carboxyfluorescein diacetate (CFDA, Dojindo, Kumamoto,
135 Japan) and ethidium bromide (EtBr, Dojindo, Kumamoto, Japan). Briefly, 500 μL of DPI-
136 treated or untreated Mtb culture was sedimented by centrifugation at 15,000 ×*g* for 5 minutes
137 at 4˚C and washed twice with the same volume of PBS-T. The washed bacterial sediment was

138 resuspended in 200 µL of PBS-T with CFDA and EtBr incubated at room temperature (RT) for
139 30 minutes in shade. The final concentration of CFDA and EtBr were 60 µg/mL and 2 µg/mL,
140 respectively. The stained Mtb cells were washed twice with PBS-T and fixed with 3.7%
141 formaldehyde solution at RT for at least 2 h. After fixation, 10 µL of bacterial suspension was
142 smeared on three APS-coated 8-well slides (Matsunami glass, Osaka, Japan) and air-dried.
143 Then the slides were mounted with DPX new non aqueous mounting medium (Merck,
144 Darmstadt, Germany) and a 150 µm-thick cover glass (Matsunami glass, Osaka, Japan) prior
145 to the microscopic observation. Specimens were examined with a BX53 fluorescence
146 microscope (Olympus, Tokyo, Japan) at a magnification of 100× for green (esterase-active with
147 intact membrane; live) or red (esterase-negative with damaged membrane; dead) fluorescing
148 cells. The excitation source for CFDA and EtBr was generated from a mercury-arc lamp (blue
149 beam, 470–495 nm; green beam, 530–550nm) using a corresponding band-pass filter. The
150 green-fluorescence emission from the CFDA-positive cells was collected through a 510–550
151 nm band-pass filter. The red-fluorescence emission from EtBr-positive cells was collected
152 through a 575 nm long-pass filter. At least 10 random fields were observed for each sample and
153 the images (TIFF format) were analyzed using the Fiji/ImageJ software program (23). The
154 live/dead ratio was calculated by direct counting of green or red fluorescing cells.

155 **Auramine-O/Nile Red dual staining**

156 Loss of acid-fastness and accumulation of neutral lipids are distinctive features of VBNC
157 mycobacteria (24). To detect the phenotype, fluorescent acid-fast staining with auramine-O and
158 neutral lipid staining with Nile Red (9-dimethylamino-5H-benzo-α-phenoxadine-5-one) were
159 performed using a previously described method (9) with some modification. Briefly, 10 µL of
160 DPI-treated or untreated culture were smeared onto an APS-coated slide (Matsunami glass,
161 Osaka, Japan) and air-dried. Then, the slide was heat-fixed and cooled down to room
162 temperature before staining. The smear was flooded with fluorochrome staining solution (10

163 μg/mL auramine-O in 5% [w/v] phenol solution) and incubated at room temperature for 20 min
164 in shade. Excessive dye was removed using 3% hydrochloric acid ethanol for 15 minutes. Next,
165 the smear was covered with 10 μg/mL Nile Red in ethanol and incubated at room temperature
166 for 15 minutes. Finally, the smear was counterstained with 0.1% (w/v) potassium permanganate
167 solution with 1 minute. Stained slides were air-dried and mounted as described previously.
168 Fluorescence microscopy was performed at 100× magnification for green (acid-fastness
169 positive, lipid accumulation negative; active) or red (acid-fastness negative, lipid accumulation
170 positive; VBNC) fluorescing cells. The excitation source for auramine-O and Nile Red was
171 generated from a mercury-arc lamp (blue beam, 470–495 nm; green beam, 530–550 nm) using
172 a corresponding band-pass filter. The green-fluorescence emission from auramine-O-positive
173 cells was collected through a 510–550 nm band-pass filter. The red-fluorescence emission from
174 Nile Red-positive cells was collected through 575 nm long-pass filter. At least 5 random fields
175 were observed for each sample and images (TIFF format) were analyzed by the Fiji/ImageJ
176 software program (23) and the proportion of acid-fast-positive cells and lipid-accumulation-
177 positive cells was calculated by direct counting of green or red fluorescing cells.

178 **Ziehl-Neelsen staining**

179 Ziehl-Neelsen staining was also performed to confirm the loss of acid-fastness resulting in the
180 VBNC state by DPI treatment, according to the standard method.(25) The slide was observed
181 by light microscope under 100x magnification with an oil immersion lens (BX53, Olympus,
182 Tokyo, Japan).

183 **Confocal super-resolution microscopy**

184 Detailed images of accumulated lipids were also obtained by a confocal laser-scanning
185 microscope (LSM900 with Airyscan 2, Carl Zeiss Microscopy, Jena, Germany). Briefly, culture
186 slides of DPI-treated cells dual stained with auramine-O and Nile Red, as described above,
187 were observed at 63x magnification with oil immersion lens. The excitation source of

188 auramine-O and Nile Red was 488 nm (blue beam) and 563 nm (green beam), from a solid-
189 state laser unit. The green-fluorescence emission from auramine-O and red fluorescence from
190 Nile Red were sequentially collected through a variable dichroic beamsplitter and an Airyscan
191 2 detector. Super-resolution images were processed with the ZEN Blue software program (Carl
192 Zeiss, Jena, Germany).

193 **VBNC reactivation assay**

194 The VBNC reactivation assay was performed as described elsewhere (18) with some
195 modification. DPI-treated culture was sedimented by centrifugation at 3,000 x*g* for 10 minutes
196 at 4˚C and washed twice with fresh Dubos broth, then resuspended in ten-milliliters of Dubos
197 broth. The bacterial suspension was diluted to 1:10 with fresh Dubos medium with or without
198 0.1% (w/v) BSA. For both BSA-containing and BSA-free series, OADC supplementation
199 (Becton Dickinson, Sparks, MD), fetal bovine serum (FBS; Moregate BioTech, Bulimba,
200 Queensland, Australia), or sodium pyruvate (PA; Merck) was added. The final concentration
201 of each reagent was as follows: 10% (v/v) for OADC supplementation, 2% (v/v) for FBS, and
202 3 mM for PA. Then, the diluted suspension was dispensed into 24-well plates in 1.5 mL
203 increments and sealed with gas-permeable film. Cultures were incubated for 5, 10, 15 and 20
204 days at 37˚C under 5% CO_2. At each time-point, the number of culturable cells was measured
205 as described previously.

206 **Evaluation of the reactivation-promoting effect of human serum albumin, egg-white**
207 **albumin (ovalbumin) and antioxidative agents**

208 To determine whether the reactivation-promoting effect of BSA is its antioxidative property,
209 we measured the reactivation-promoting activity of other albumins and antioxidative agents
210 toward DPI-treated Mtb cells. Washed DPI-treated cells were resuspended with fresh Dubos
211 broth without BSA and the suspension was diluted to 1:10 with Dubos broth containing BSA,
212 human serum albumin (HSA), ovalbumin (OVA), N-acetyl-*L*-cysteine (NAC) or D-mannitol

213 (MAN). The final concentration of each reagent was 0.1% (w/v) for BSA, HSA and OVA, 500
214 μM for NAC, and 50 mM for MAN, respectively. Then, the diluted suspension was dispensed
215 into 24-well plates in 1.5 mL increments and sealed with gas-permeable film. The plate was
216 incubated for 20 days at 37˚C under 5% CO_2. At the end of incubation, the number of colonies
217 grown was measured as described previously.

218 **Evaluation of the reactivation-promoting effect of fatty acid and globulin-free BSA**

219 To determine the effect of BSA Cohn fraction V contaminants, we measured the reactivation-
220 promoting activity of fatty acid and globulin-free BSA toward DPI-treated VBNC Mtb cells.
221 Washed DPI-treated cells were resuspended with fresh Dubos broth without BSA and the
222 suspension was diluted to 1:10 with Dubos broth containing BSA Cohn fraction V or fatty acid
223 and globulin-free BSA. Fatty acid and globulin-free BSA were acquired from Merck. The final
224 concentration of both BSAs was 0.1% (w/v). The number of culturable cells was measured as
225 described previously.

226 **Effects of adenylyl cyclase or protein kinase on BSA-induced reactivation**

227 Washed DPI-treated VBNC Mtb cells were resuspended with fresh Dubos broth and the
228 suspension was diluted to 1:10 with Dubos broth with 0.1% (w/v) BSA containing adenylyl
229 cyclase inhibitor SQ22536 (Merck, Darmstadt, Germany) or protein kinase inhibitor H89
230 (Abcam, Cambridge, United Kingdom) or staurosporine (Merck). The final concentration of
231 each inhibitor was as follows: 0.1 to 10 mM for SQ22536, 1 to 30 μM for H89 and 1 to10 μM
232 for staurosporine. Then, the number of culturable cells were measured as described previously.

233 **Molecular docking simulation**

234 Molecular docking simulation was performed by using AutoDock Vina software, an open-
235 source molecular docking program designed by the Scripps Research Institute (26, 27). 3D
236 structures of receptor proteins, PknA and Rv2212, were obtained from Protein Data Bank (28)

237 and AlphaFold Database (29, 30), respectively. PDB ID for PknA was 4OW8 (31) and
238 AlphaFold Database identifier for Rv2212 was AF-P9WMU7-F1. We removed water
239 molecules from proteins, added polar hydrogen and charge, and adjusting X-Y-Z coordinates
240 and grid size for optimizing molecular docking. 3D structures of ligand molecules, H89,
241 SQ22536 and ATP were obtained from PubChem Database. PubChem CID of each ligand were
242 449241, 5270 and 5957, respectively. Graphical processing was done by Chimera software (32).

243 **Statistical analysis**

244 The statistical analysis was performed with R (33) using the EZR on R package (34). *P* values
245 of < 0.05 were considered statistically significant.

246 **Results**

247 **I. BSA is not essential for the growth of MTB in Dubos medium with purified non-**
248 **prediluted Tween 80**

249 Figure 1 shows the different effects of BSA-containing and BSA-free Dubos medium on the
250 growth of Mtb H37Rv. As shown in Fig. 1(A), there was no significant difference in the rate
251 of growth between the two conditions. Fig. 1(B) also shows the sufficient growth of cells. The
252 cells were more aggregative without BSA, however, the aggregation was easily dispersed by
253 pipetting and there was no significant difference in the OD_{600} value. The albumin component
254 was inessential for Mtb growth.

255 **II. DPI treatment induced a VBNC state in Mtb H37Rv**

256 Figure 2 shows the DPI treatment results for the induction of VBNC state in H37Rv and
257 H37Ra (18). As shown in Fig. 2(A), the numbers of culturable cells of untreated control
258 population and DPI-treated population were significantly different: $4.43 \pm 1.43 \times 10^8$ CFU/mL

259 for the untreated control population and $4.64 \pm 6.10 \times 10^3$ CFU/mL for DPI-treated population.
260 Fig. 2(B) shows that the CFDA-positive cells in the untreated control and DPI-treated
261 populations were 81.8 ± 11.4% and 65.9 ± 11.8%, respectively. These results suggested that
262 the majority of DPI-treated H37Rv cells were alive ($p = 0.169$), while their culturability was
263 significantly reduced ($p = 0.033$). Representative images of CFDA/EtBr dual-stained cells of
264 the untreated control and DPI-treated population also showed that there was little difference in
265 the proportions of CFDA-positive and EtBr-positive cells. (Fig. 2[C])

266 Auramine-O/Nile Red dual staining showed the reduction of acid-fastness and the
267 accumulation of the neutral lipid. As shown in Fig. 2(D), the proportion of acid-fast-
268 negative/lipid accumulation-positive cells was 84.0 ± 14.0% in the DPI-treated population, and
269 12.1 ± 11.9% in the untreated control population ($p < 0.01$). Representative images of
270 auramine-O/Nile Red dual-stained cells of the untreated control and DPI-treated populations
271 also showed that there were distinctive differences in the proportions of acid-fast-positive cells
272 and lipid-accumulation-positive cells (Fig. 2[E]). The loss of acid-fastness was also confirmed
273 by Ziehl-Neelsen staining; the majority of DPI-treated population were found to have lost their
274 acid-fastness (Suppl. Fig. 1). Interestingly, Airyscan microscopy revealed some DPI-treated
275 cells were both acid-fast stain-positive and neutral lipid stain-positive (Fig. 2[F]). These results
276 indicated that DPI treatment induced the VBNC state in H37Rv as well as in H37Ra.

277 III. The effects of BSA, FBS, OADC and sodium pyruvate on the reactivation of DPI-
278 treated VBNC Mtb cells

279 As shown in Fig. 3(A) and (B), our results partially support the previous study (18) with
280 some differences; although the culturability of DPI-treated H37Rv cells was restored by
281 incubation with FBS-supplemented medium, OADC-supplemented medium could also induce
282 culturability. The addition of albumin alone into the medium could also induce reactivation.
283 The addition of FBS in BSA-free Dubos medium slightly reduced the regrowth rate; however,

284 cells were successfully reactivated at the end of incubation with or without FBS. These
285 phenomena were also observed in H37Ra (Suppl. Fig 2). Thus, we used the H37Rv strain for
286 the further analyses in this study.

287 Incubation with sodium pyruvate, which was reported to have a reactivation-promoting
288 effect on VBNC cells elsewhere, led to transient regrowth by day 15 and a slight reduction at
289 day 20. (Suppl. Fig. 3[A] and [B]).

290 We also confirmed that the presence of small population of intact Mtb cells (10^3 CFU/mL)
291 could grow normally in BSA-free Dubos medium, suggesting that the reactivation might not
292 be due to the presence of small number of culturable cells after DPI-treatment (data not shown).

293 IV. The antioxidative property or the fatty acid from BSA did not promote 294 reactivation.

295 As shown in Fig. 4(A) and (B), the reactivation-promoting effect of BSA was specific to
296 bovine and human serum albumin. Ovalbumin did not show a reactivation-promoting effect
297 but maintained the number of culturable cells in this system. NAC (antioxidative agent) and
298 D-mannitol (free radical scavenger) did not show any reactivation capacity.

299 We also checked whether the purity of albumin affects the promotion of reactivation using
300 fatty acid and globulin-free BSA and confirmed that there was no significant difference in
301 reactivation-promoting effects (Suppl. Fig 4). These results suggest that commercially
302 available albumin including fatty acids do not affect the reactivation of DPI-treated Mtb.

303 V. The reactivation-promoting effect of albumin is canceled by treatment with 304 eukaryotic protein kinase inhibitor.

305 As shown in Fig. 5(A) and (B), SQ22536, adenylyl cyclase inhibitor, only suppressed
306 reactivation at a very high concentration (10 mM) in this study. However, H89, a eukaryotic
307 protein kinase inhibitor, suppressed reactivation in a dose-responsive manner. In detail,

308 incubation with 10 μM or 30 μM H89 resulted in a CFU/mL value for $3.42 \times 10^6 \pm 7.45 \times 10^5$
309 CFU/mL and $3.63 \pm 1.70 \times 10^2$ CFU/mL, while incubation without H89 resulted in 3.00×10^8
310 $\pm 5.07 \times 10^7$ CFU/mL. We also confirmed that staurosporine, which is known as mycobacterial
311 protein kinase PknB inhibitor (35), suppressed reactivation at 10 μM and resulted in a CFU/mL
312 value for $2.09 \times 10^3 \pm 7.62 \times 10^2$ CFU/mL, while incubation without staurosporine resulted in
313 $3.58 \times 10^8 \pm 5.78 \times 10^7$ CFU/mL (Fig 5[A] and [B]). All inhibitors used in this study did not
314 cause a reduction of the growth of intact MTB cells (Suppl. Fig. 5A and 5B).

315 We also performed molecular docking simulation of these inhibitors toward their
316 considerable targets on Mtb. As shown in Suppl. Table 1, both SQ22536 and H89 have
317 sufficient affinity toward Rv2212 (adenylyl cyclase) and PknA (protein kinase), respectively.

318 **Discussion**

319 In this study, we confirmed that DPI could induce a VBNC state in H37Rv as well as H37Ra.
320 The mechanism underlying the effect of DPI is considered to involve the inhibitory effect of
321 NADH oxidase, which results in the inhibition of the electron transport system of Mtb. This
322 may induce a hypoxic response in the liquid culture, resulting in significant decrease of
323 culturability (Fig.2[A]). However, viability was highly retained (65.9%), suggesting that
324 majority of the cells transformed to VBNC (Fig. 2[B]). Additionally, VBNC transformation
325 was confirmed by auramine-O and Nile Red staining. The loss of acid-fastness and the
326 accumulation of neutral lipids are distinctive features of VBNC mycobacterial cells.

327 Our data also demonstrated that the majority of DPI-treated Mtb H37Rv cells were stained
328 with Nile Red alone (Fig. 2[D]). Airyscan super-resolution microscopy showed the presence of
329 cells that were stained with auramine-O and which contained an intracellular lipid body stained
330 with Nile Red (Fig. 2[F]). This could also reveal the distribution of the lipid body in the cell as
331 some foci of relatively strong signals of Nile Red, suggesting that the transformation from
332 growing state to VBNC, with drastic alteration of the lipid metabolism.

333 Secondly, we found that DPI-induced VBNC could facilitate reactivation not only by
334 incubation with FBS but also with OADC supplementation and BSA alone, suggesting albumin
335 might act as a reactivation-promoting agent in both H37Rv and H37Ra (Fig .3 and Suppl. Fig.
336 2). These findings were contrary to the findings of the previous study, which showed that only
337 FBS could facilitate reactivation (18). We considered the reason for the difference may be due
338 to contaminant in Tween 80, presence of excessive amount of oleic acid. We also confirmed
339 that the reactivation was not facilitated by the use of prediluted Tween 80 for the preparation
340 of Dubos medium (internal data) for both H37Rv and H37Ra. Thus, our results suggested that
341 the key component of reactivation-promoting agent in FBS might be serum albumin, and
342 further analyses were performed using H37Rv.

343 We also tested the reactivation-promoting effect of BSA in Wayne's hypoxic culture, which
344 is widely used for inducing a VBNC of Mtb, and found that there was no significant difference
345 with or without BSA (data not shown).

346 Pyruvate, which is known to act as reactivation promoting agent for both gram-negative
347 bacteria, such as *Escherichia, Legionella, Vibrio* and gram-positive bacteria such as
348 *Staphylococcus* (36–39), did not show any reactivation-promoting effect in our experiment
349 (Suppl. Fig.3). Vilhena *et al.* reported that their proteomic analysis of VBNC *E. coli* cells
350 during reactivation revealed that enzymes involved in pyruvate metabolism, pyruvate formate-
351 lyase-activating protein (PflA), phosphoenolpyruvate carboxykinase (PckA) and lactate
352 dehydrogenase (LldD), were significantly upregulated (40), while Wagley *et al.* also reported
353 that VPA1499, an LldD homologue of Vibrio parahaemolyticus, was significantly upregulated
354 in VBNC *V. parahaemolyticus* and that depletion of LldD led to quicker entry to the VBNC
355 state (41). Interestingly, they showed that the addition of pyruvate, the product of lactate
356 oxidation, to LldD-depleted mutant restored the wild-type characteristics of cells in the VBNC
357 state. Their studies might give some crew of the lack of reactivation-promoting effect toward
358 mycobacterial VBNC. Since Mtb does not have a catalytic center of pyruvate formate-lyase

359 (PflB), pyruvate might not be able to act as reactivation-promoting agent, although pyruvate
360 enhances the growth of Mtb and *M. bovis* both intracellular and extracellular growth by
361 promoting TCA cycle and other energy-producing pathways. (42, 43).

362 Our results showed that the reactivation promoting effect of albumin might be structure-
363 specific because ovalbumin (OVA) did not facilitate reactivation, but bovine (BSA) and human
364 serum albumin (HSA) did. Focusing on their amino acid sequences, BSA and HSA share 76%
365 sequence homology (44); however, BSA and OVA share less sequence homology (16%). One
366 of the considerable roles of albumin may be as an antioxidative agent due to its cysteine
367 residues with free thiols (19, 45). BSA and HSA have only one cysteine residue with free thiol,
368 while OVA has four cysteines with free thiols (46). OVA therefore seems to be more reductive
369 than BSA and HSA. Our results were paradoxical in relation to this hypothesis; however, Davis
370 and Dubos showed the differential growth-promoting effect of albumin toward Mtb. Their
371 study supplied some interesting clues for our present study; ovalbumin does not show a growth-
372 promoting effect, while bovine and human serum albumin showed the effect (15). Moreover,
373 the antioxidative agents used in this study, NAC and D-mannitol, showed any restorative
374 effects (Fig. 4). We also examined catalase, a component of OADC supplementation, and its
375 reactivation promoting effect toward VBNC *Corynebacterium, Lactobacillus, Salmonella,*
376 *Ralstonia* and *Vibrio* (47–52); no restorative effects were observed (data not shown). These
377 results suggest that the effect of albumin may be due to complex functions rather than its
378 antioxidative property.

379 Serum albumin is known to act as a good carrier of many kinds of biologically active
380 substances, including free fatty acid. It is also known as a detoxifier of growth-arresting
381 substances in culture medium. BSA is added to mycobacterial culture medium as both a carrier
382 and detoxifier of growth-supporting and growth-arresting substances, respectively. Shleeva *et*
383 *al*. showed that several types of unsaturated free fatty acid, such as oleic, linoleic, and
384 arachidonic acids, can promote the reactivation of VBNC *M. smegmatis* in modified Sauton's

385 medium and oleic acid also works for Mtb (20). In this study, we should note that impurities
386 of albumin did not affect reactivation. Although the underlying mechanism is still unclear, both
387 fatty acid and globulin-free BSA and BSA Cohn fraction V showed similar reactivation-
388 promoting effect toward DPI-treated Mtb (Suppl. Fig. 4).

389 The reactivation inhibition assay by SQ22536, H89 and staurosporine gave us an important
390 clue for understanding the effect of BSA. In the present study, we could suppress the
391 reactivation of DPI-induced VBNC cells by inhibiting adenylyl cyclase only with a very high
392 concentration of SQ22536. Although the underlying mechanism is unclear, adenylyl cyclase
393 may not play a key role in reactivation from a DPI-induced VBNC state, while the inhibition
394 of protein kinase by H89 and staurosporine clearly suppressed reactivation. These results
395 suggest that protein kinase might mainly be involved with the reactivation of VBNC Mtb cells
396 triggered by BSA and the contribution of adenylyl cyclase may be smaller than that of protein
397 kinase.

398 Mtb has the eukaryotic-type Ser/Thr protein kinases PknAB and PknD-L. For example,
399 PknA regulates major cell processes, regulates several types of proteins involved with
400 mycobacterial cell division and morphogenesis via phosphorylation of such proteins in
401 coordination with PknB (Manuse *et al*., 2016; Carette *et al*., 2018); phosphorylation of GlmU
402 (Rv1018c) and MurD (Rv2155c), Wag31 (Rv2145c), HupB (Rv2986c) and ParB (Rv3917c),
403 influence the production and cross-linking of peptidoglycan precursors (55–58), the spatial
404 localization of peptidoglycan synthesis (59), DNA condensation and partitioning (60, 61),
405 respectively. It should be noted that the phosphorylation of FtsZ and FipA by PknA controls
406 cell division under oxidative stress (62, 63). In addition, mycobacterial protein kinase has
407 cyclic AMP-dependent protein kinase domains which is very similar to eukaryotic version,
408 which was shown in PknB (64) and staurosporine, which act as PknB inhibitor, could also
409 inhibit reactivation of DPI-induced VBNC Mtb (Fig. 5).

410 Our study suggested that the inhibition of mycobacterial protein kinase by H89 and

411 staurosporine seems to critically affect several important cellular processes, followed by
412 reactivation (Fig. 6). To confirm the essentiality of mycobacterial protein kinase and other
413 involved proteins toward reactivation from this DPI-induced VBNC state by albumin, further
414 studies using experiments such as knock-down of genes using the CRISPR interference
415 (CRISPRi) technique (65), because such proteins—with the exception of FipA (Rv0019c)—
416 are known to be essential for *in vitro* growth (66–69); thus, manipulating their coding genes is
417 not permissible.

418 **Conclusion**

419 Our data showed that the presence of BSA in Dubos medium is not only for depletion of
420 growth-supressing substances but also could trigger the reactivation of the DPI-induced VBNC
421 state in both H37Rv and H37Ra strains. The reactivation-promoting effect of other kinds of
422 albumins and antioxidative agents, including NAC and D-mannitol was tested; however, only
423 human serum albumin showed a reactivation-promoting effect. The results of the inhibition
424 assay of reactivation using SQ22536, H89 and staurosporine suggests the possibility that the
425 inhibition of mycobacterial protein kinase suppressed reactivation to a much greater extent than
426 adenylyl cyclase, indicating the presence of a reactivation-promoting pathway which is protein
427 kinase-dependent.

428 To the best of our knowledge, few studies have focused on the reactivation-promoting effect
429 of albumin toward VBNC Mtb. Taken together, our findings indicated that the reactivation of
430 MTB from a DPI-induced VBNC state could be obtained by interaction with bovine serum
431 albumin and the mechanism of albumin-induced reactivation may involve the activation of
432 mycobacterial protein kinase, which regulates several important processes of cellular
433 component construction and cell division. Although it remains unclear how albumin interacts
434 with VBNC cells, our study may supply some important clues to understanding the physiology
435 of the mycobacterial VBNC state.

436 **Author Contributions**

437 YMori, YMura and SM conceived and designed the experiments. YMori performed
438 experiments. YMura, KC, AA, YI, YS, MH, KK, HY, AT, and SM assisted in the experiments.
439 YMori acquired data and YMori and YMura analyzed data. YMori performed molecular
440 docking simulation and statistical analysis. YMori and SM wrote the manuscript. All authors
441 contributed to the article and approved the submitted version.

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453 **Conflict of Interest Statement**

454 The authors declare that the research was conducted in the absence of any commercial or
455 financial relationships that could be construed as a potential conflict of interest.

456

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686 **Figure legends**

687 Figure 1. Growth curves of Mtb H37Rv cells in Dubos medium in the presence or absence of
688 BSA

689 (A) Number of culturable cells in Dubos medium in the presence (closed circle) or
690 absence (open circle) of 0.1% BSA at the indicated days of incubation. The CFU/mL
691 values were determined by plating cells onto 7H10 plates in duplicate. Data represent
692 the mean ± SD from three independent experiments.
693 (B) Representative images of Mtb growth captured at the end of incubation.

694 Figure 2. Viability analyses of DPI-treated Mtb

695 (A) Number of culturable cells after DPI treatment. The CFU/mL values were determined
696 by plating cells onto 7H10 plates in duplicate. Asterisk indicates a statistically
697 significant difference (*$p < 0.05$, Welch's *t*-test). Data represent the mean ± SD from
698 three independent experiments.
699 (B) Percentages of esterase active cells after DPI treatment. Around 850 cells were
700 directly counted under 100× objective lens. Data represent the mean ± SD from three
701 independent experiments.
702 (C) Merged fluorescence micrographs of Mtb H37Rv cells stained with
703 carboxyfluorescein diacetate (CFDA) and ethidium bromide (EtBr). Cells stained with
704 CFDA (green) are esterase-positive; those stained with EtBr (red) are membrane-
705 damaged. Images are representative images from three individual experiments. Scale
706 bar: 2μm
707 (D) Percentage of acid-fast-positive cells (open column) or acid-fast-negative but lipid-
708 accumulation-positive cells (gray column). Approximately 1,100 cells were directly
709 counted under 100× objective lens. Data represent the mean ± SD from four

710 independent experiments. Asterisk indicates significant difference (***$p < 0.005$,
711 Welch's *t*-test).
712 (E) Merged fluorescence micrographs of Mtb H37Rv cells stained with auramine-O and
713 Nile Red. Cells stained with auramine-O (green) are acid-fast-positive; those stained
714 with Nile Red (red) are acid-fast-negative but lipid accumulation-positive. Images are
715 representative images from three individual experiments. Scale bar: 2μm
716 (F) Magnified views of Airyscan confocal super-resolution microscopy observation of
717 three different Mtb cells obtained from DPI-treated culture. Only acid-fast-positive
718 cells (left), both acid-fast-positive and lipid accumulation-positive cells (center,
719 counted as acid-fast-positive) and acid-fast-negative cells with only lipid
720 accumulation-positive cells (right). Dual-positive cells probably represent the
721 transition to a non-culturable state. Indicated foci with arrowheads represent a neutral
722 lipid body. Scale bar: 1 μm

723 Figure 3. Reactivation of DPI-treated Mtb H37Rv cells by FBS and OADC supplementation

724 (A) Number of culturable cells at the indicated days of incubation, without
725 supplementation or those cells supplemented with 2% FBS or 10% OADC in the
726 presence (closed circle) or absence (open circle) of 0.1% BSA. The CFU/mL values
727 were determined by plating cells onto 7H10 plates in duplicate. Data represent the
728 mean ± SD from three independent experiments. U.D. means under the limit of
729 detection (20 CFU/mL).
730 (B) Representative images of regrowth under the indicated conditions captured at the end
731 of incubation. None, FBS, and OADC represent no supplementation, 2% FBS and
732 10% OADC supplementation, respectively.

733 Figure 4. Effect of human serum albumin, ovalbumin and antioxidants on the reactivation of

734 DPI-treated Mtb.

735 (A) Number of culturable cells at the end of incubation, supplemented with albumins or
736 antioxidative agents. The CFU/mL values were determined by plating cells onto 7H10
737 plates in duplicate. Data represent the mean ± SD from three independent
738 experiments. Asterisk indicates a statistically significant difference between Day 0
739 and Day 20 by Student's *t*-test. (*$p<0.05$)
740 (B) Representative images of regrowth captured at the end of incubation.

741 Abbreviations:
742 BSA, bovine serum albumin; HSA, human serum albumin; OVA, ovalbumin; NAC, N-
743 acetyl-L-cysteine; MAN, D-mannitol.
744 The final concentrations of each reagent were as follows:
745 BSA, HSA and OVA: 0.1% (w/v), NAC: 500 μM, MAN: 50 mM.

746 Figure 5. The effect of SQ22536 or H89 or staurosporine on the reactivation of DPI-treated
747 Mtb

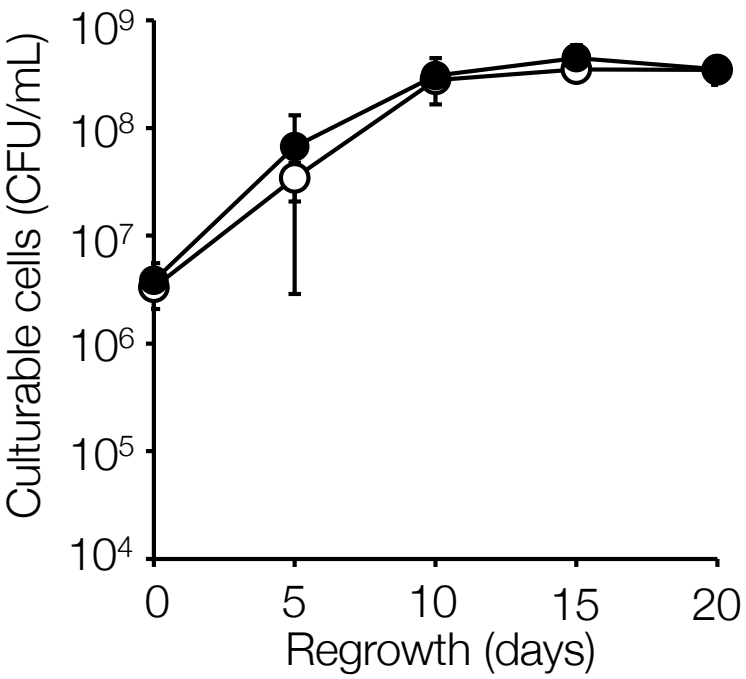
748 (A) Number of culturable cells at the end of incubation, supplemented with 0.1% BSA
749 and SQ22536 or H89 or staurosporine. Inhibitor was added at the indicated
750 concentration.
751 (B) Representative images of regrowth captured at the end of incubation.

752 The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data
753 represent the mean ± SD from three independent experiments.

754 Figure 6. Schematic representation of the mechanism of BSA-induced reactivation of Mtb.
755 Although the detailed mechanism of interaction between BSA and PknA/PknB is not yet

756 clear, BSA may activate PknA/PknB and positively regulate target proteins that are involved
757 in the localized synthesis of peptidoglycan, cell division control, and DNA condensation and
758 partitioning through their phosphorylation, resulting in the reactivation of VBNC Mtb cells.

(A)


Culturable cells (CFU/mL)
10^9
10^8
10^7
10^6
10^5
10^4
0
5
10
15
20
Regrowth (days)

(B)

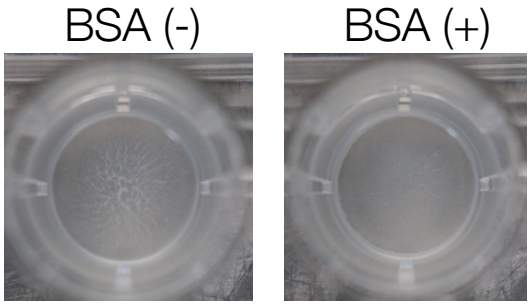

BSA (-)
BSA (+)

Fig 1.

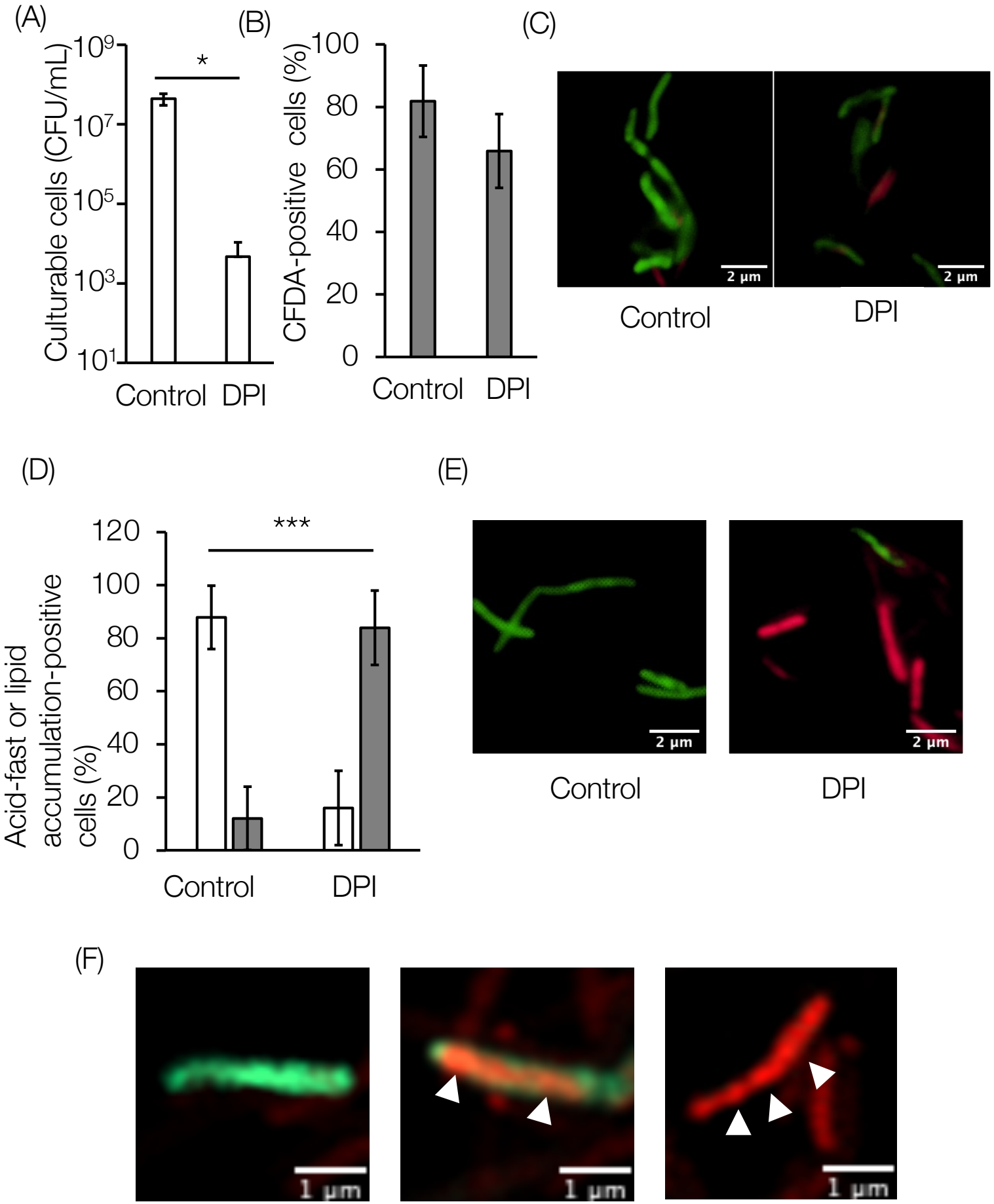

(A)
Culturable cells (CFU/mL)
10^9
10^7
10^5
10^3
10^1
*
Control
DPI
(B)
CFDA-positive cells (%)
100
80
60
40
20
0
Control
DPI
(C)
2 μm
2 μm
Control
DPI
(D)
Acid-fast or lipid accumulation-positive cells (%)
120
100
80
60
40
20
0
***
Control
DPI
(E)
2 μm
2 μm
Control
DPI
(F)
1 μm
1 μm
1 μm

Fig 2.

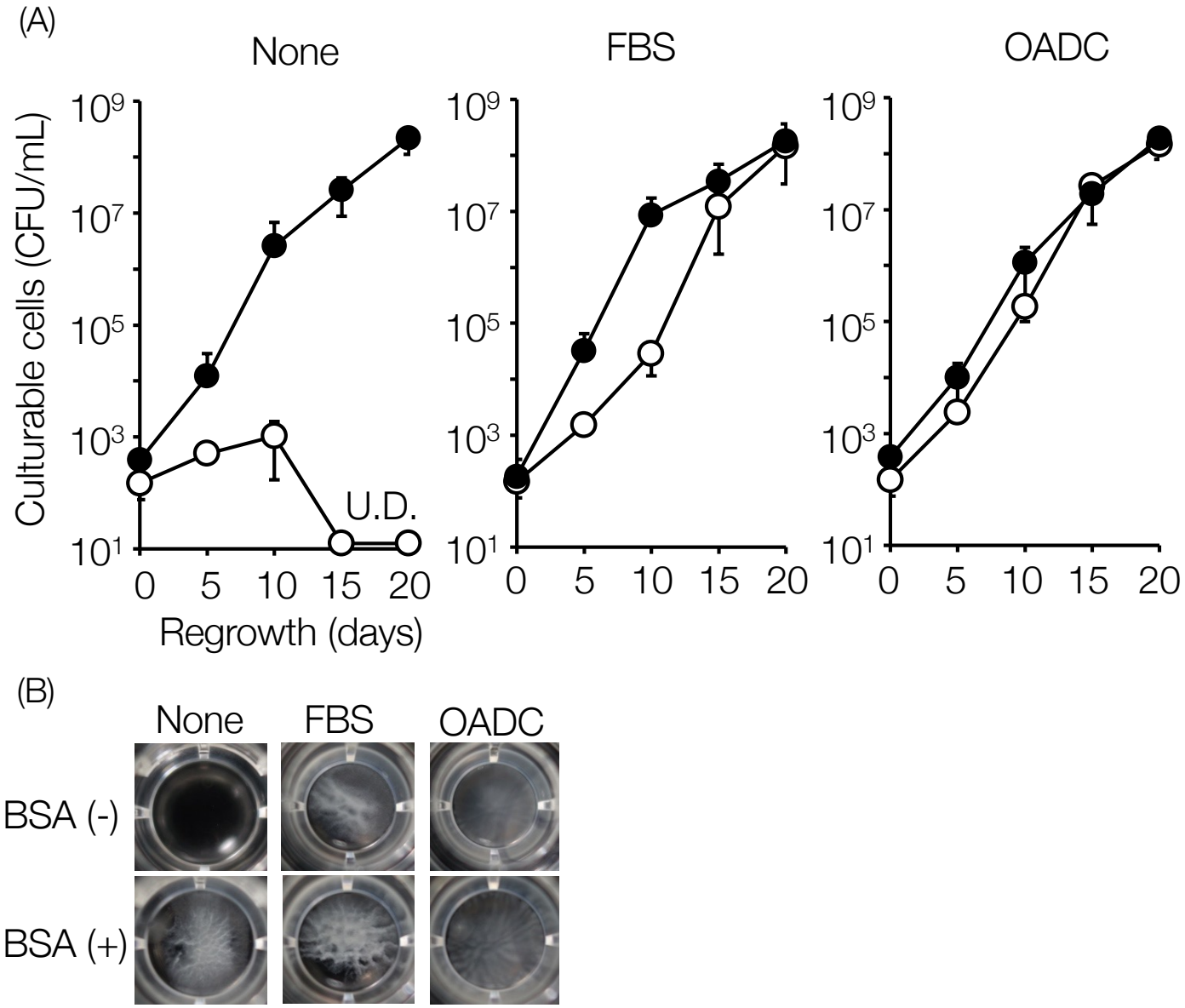

(A)
None
FBS
OADC
Culturable cells (CFU/mL)
10^9
10^7
10^5
10^3
10^1
U.D.
0 5 10 15 20
Regrowth (days)
(B)
None
FBS
OADC
BSA (-)
BSA (+)

Fig 3.

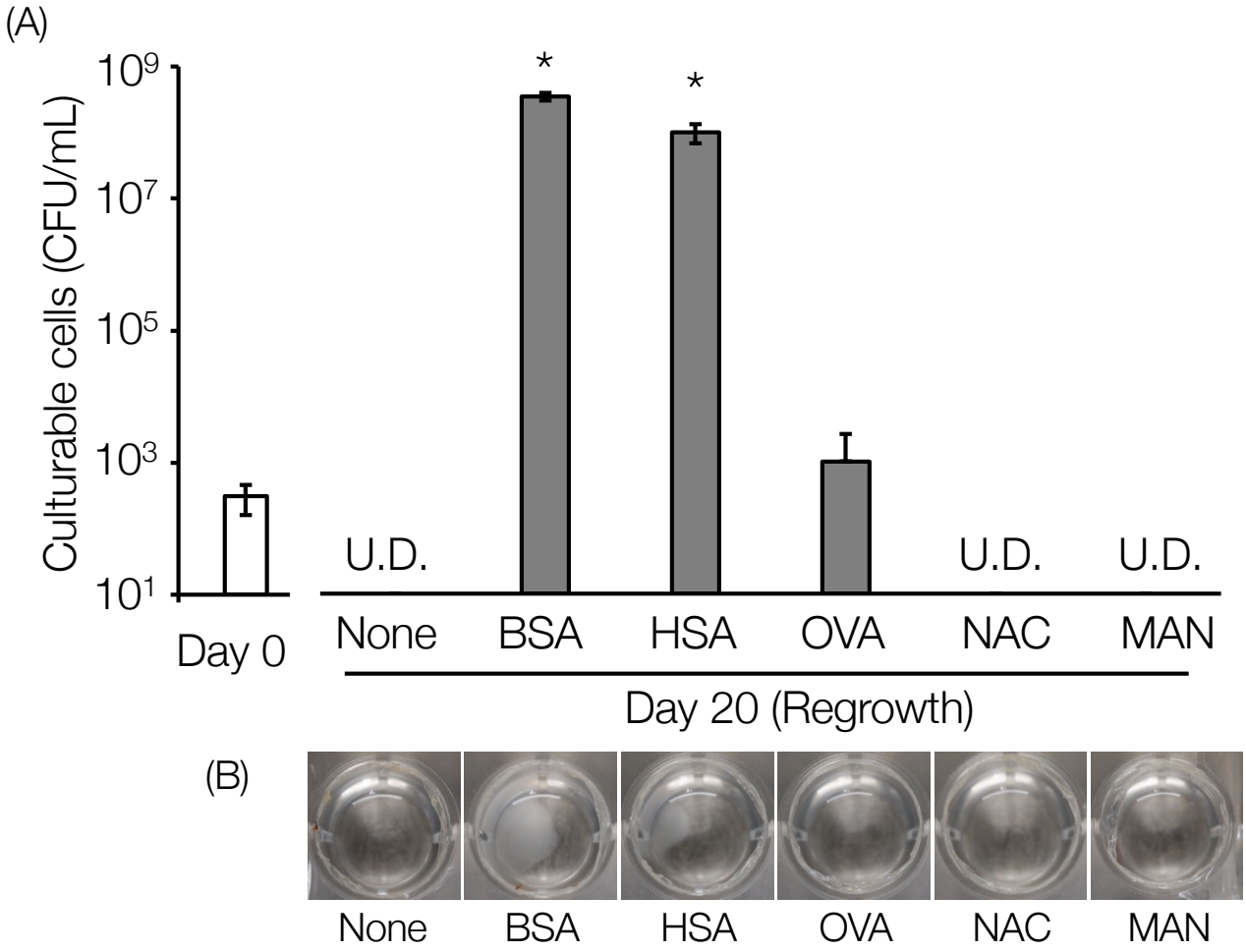
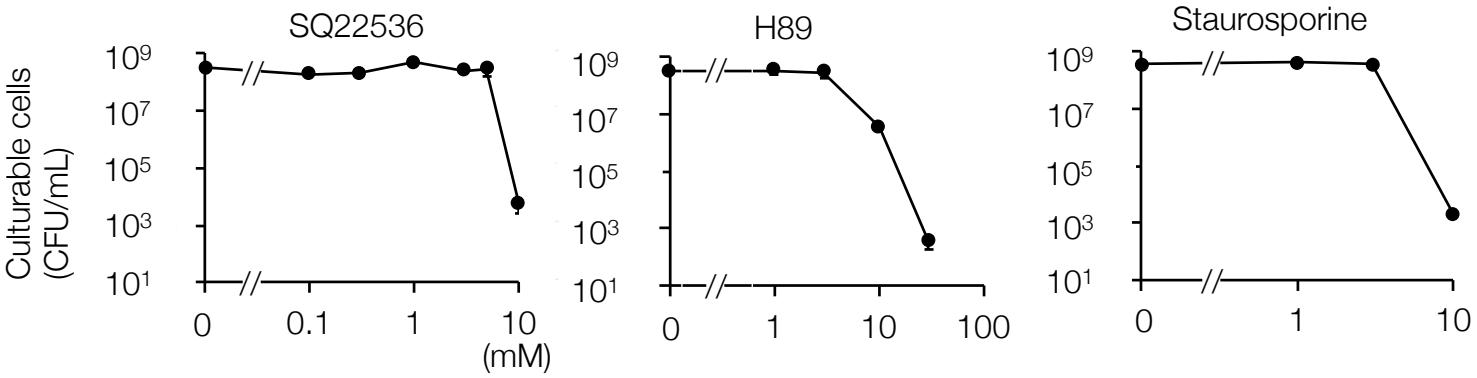

(A)
Culturable cells (CFU/mL)
10^9
10^7
10^5
10^3
10^1
*
*
U.D.
U.D.
U.D.
Day 0
None
BSA
HSA
OVA
NAC
MAN
Day 20 (Regrowth)
(B)
None
BSA
HSA
OVA
NAC
MAN

Fig 4.

(A)


SQ22536
H89
Staurosporine
Culturable cells (CFU/mL)
10^9
10^7
10^5
10^3
10^1
0
0.1
1
10
100
(mM)

(B)

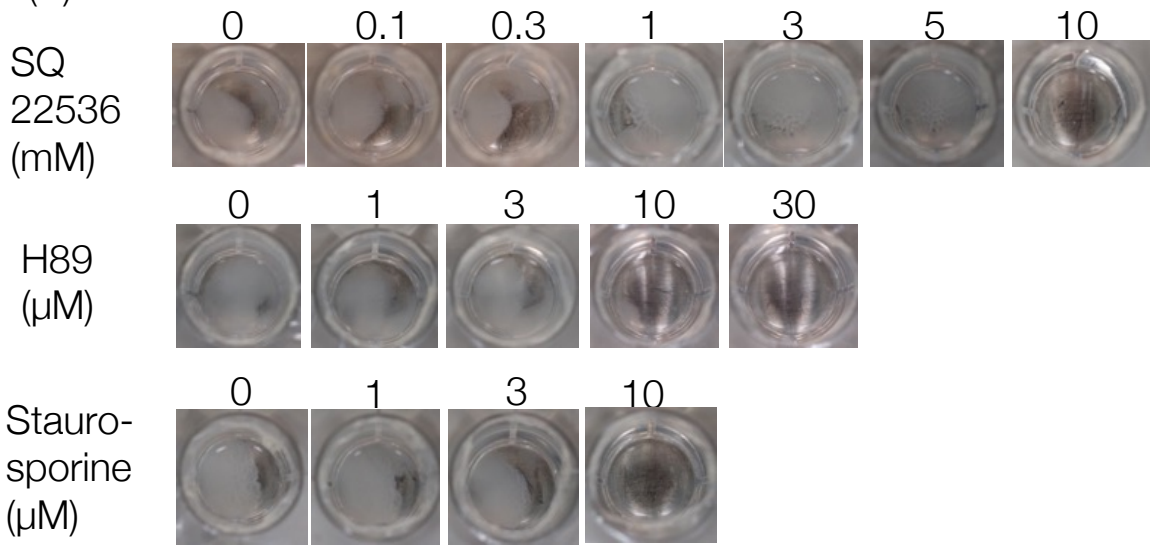

SQ 22536 (mM)
0
0.1
0.3
1
3
5
10
H89 (μM)
0
1
3
10
30
Stauro-sporine (μM)
0
1
3
10

Fig 5.

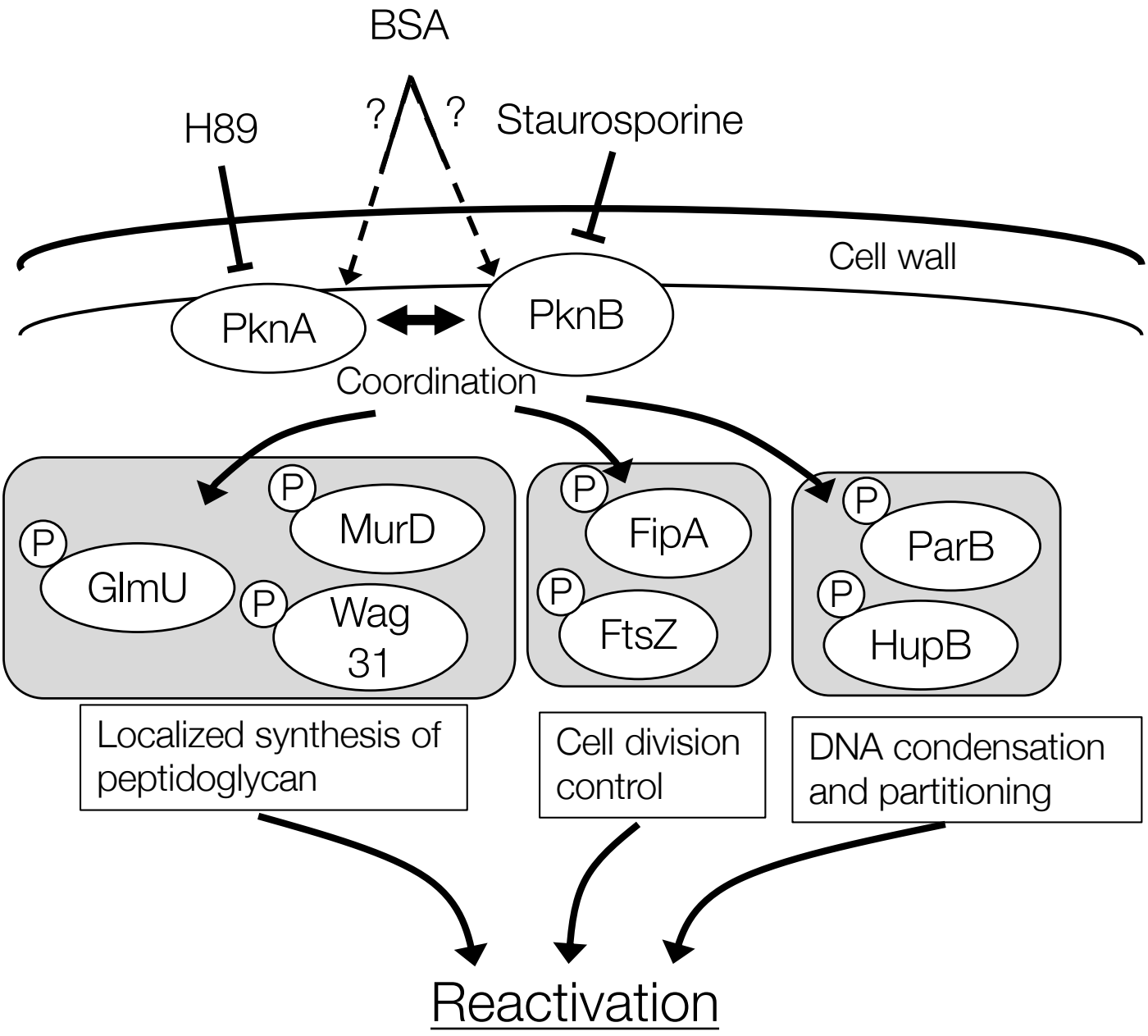

BSA
H89
?
?
Staurosporine
Cell wall
PknA
PknB
Coordination
P
MurD
P
GlmU
P
Wag
31
P
FipA
P
FtsZ
P
ParB
P
HupB
Localized synthesis of peptidoglycan
Cell division control
DNA condensation and partitioning
Reactivation

Fig. 6