Bovine serum albumin as a resuscitation promoting factor for viable but non-culturable *Mycobacterium tuberculosis* via the activation of protein kinase A-dependent cellular processes

Yuta Morishige[1*], Yoshiro Murase[1], Kinuyo Chikamatsu[1], Akio Aono[1], Yuriko Igarashi[1], Yoshiko Shimomura[1], Makiko Hosoya[1], Keisuke Kamada[1], Hiroyuki Yamada[1], Akiko Takaki[1], and Satoshi Mitarai[1,2]

[1]Department of Mycobacterial Reference and Research, The Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Kiyose, Tokyo, Japan.

[2]Department of Basic Mycobacteriology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan.

*** Correspondence:**

Yuta Morishige

ymorishige@jata.or.jp

Keywords: VBNC, Mycobacterium, Albumin, Resuscitation, Dormancy

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 resuscitated by fetal bovine serum (FBS). However, the mechanism underlying FBS-induced resuscitation is currently unclear. We tried to reveal the underlying mechanism of FBS-induced resuscitation using *M. tuberculosis* H37Rv. Methods: First, we evaluated the effect of DPI on culturability, viability and changes of cellular

phenotypes toward H37Rv. Secondly, we measured the resuscitation-promoting effects of human serum albumin, egg-white albumin, N-acetyl-*L*-cysteine, and D-mannitol in DPI-induced VBNC cells, as antioxidative agents have been reported to be key molecules for resuscitation of other microbes. We also evaluated the effect of inhibition of cAMP production and protein kinase A on BSA-induced resuscitation.

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

Conclusion: DPI-induced VBNC Mtb cells may be resuscitated via the activation of protein kinase A-dependent 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, mainly due to various stresses from host immunity. The dormant Mtb cells can persist inside the host and cause latent tuberculosis infection (LTBI), which seeks reactivation. Lowering the risk of Mtb reactivation in LTBI patients is considered to be one of the important strategies to reduce the incidence of TB (World Health Organization, 2020). Dormancy in non-sporulating bacteria that can regain culturability by certain stimuli is referred to as the viable but non-culturable (VBNC) state, which is first reported in *Escherichia coli* and *Vibrio cholerae* (Xu et al., 1982). Many bacteria, including Mtb, can enter into the VBNC state, and it is considered to be their natural state in the environment (Oliver, 2010). Various *in vitro* models, such as models of

44 hypoxia, nutritional starvation (including potassium-deficiency), a lipid-rich environment, and
45 other multiple stresses are reported to to induce a dormant state in Mtb cells (Wayne and Hayes,
46 1996; Betts et al., 2002; Deb et al., 2009; Salina et al., 2014; Aguilar-Ayala et al., 2018). A
47 detailed understanding of the mechanisms of reactivation is necessary for the prevention of
48 active tuberculosis.

49 One of the major hypotheses regarding the mechanism underlying the formation of VBNC
50 bacteria involves oxidative damage generated by harsh external conditions (Desnues et al.,
51 2003). Thus, some studies have reported that the detoxification of oxidative stress during
52 culture is one of the key mechanisms through which VBNC bacteria are resuscitated (Mizunoe
53 et al., 2000; Imazaki and Nakaho, 2009). Some mycobacterial culture media contain bovine
54 serum albumin (BSA) and BSA on mycobacterial culture has been considered to be growth-
55 supporting agent that is involved in detoxification by the absorption of free fatty acid and other
56 growth-arresting agents that are spontaneously generated during culturing (Davis and Dubos,
57 1947; Dubos, 1947; Lynn et al., 1979). For this reason, the mycobacterial culture media that
58 are most frequently used at the present time are protein-containing systems, such as
59 Middlebrook 7H series or Dubos medium. However, few studies have focused on the effect of
60 such media on VBNC cells.

61 Mukamolova et al. revealed that among patients with active TB, there is certain
62 subpopulation for whom sputum is not culturable in conventional medium, but which becomes
63 to be culturable in the presence of resuscitation promoting factor (Rpf), which is a
64 peptidoglycan-hydrolyzing protein that is highly conserved in actinobacteria (Mukamolova et
65 al., 2006). Rpf reactivates non-culturable Mtb cells both *in vitro* and *in vivo* (Mukamolova et
66 al., 2010), suggesting the presence of VBNC Mtb cells in clinical specimens.

67 Previously, NADH oxidase inhibitor diphenyleneiodonium chloride (DPI) was reported to
68 simply and rapidly induce a VBNC state in the Mtb H37Ra strain, and incubation with fetal
69 bovine serum, which is a common supplement for mammalian cell cultures, could facilitate the

70 resuscitation of VBNC cells, while incubation with OADC (oleate-albumin-dextrose-catalase)
71 supplementation could not (Yeware et al., 2019). This report gave us an important clue for
72 constructing a simple and rapid assay system to induce and resuscitate VBNC Mtb cells.
73 However, when we tried to apply this system to H37Rv, we happened to find that DPI-induced
74 VBNC cells that could be resuscitated by not only fetal bovine serum but also by the addition
75 of BSA—this was observed for both H37Rv and H37Ra (internal data). Then, we hypothesized
76 that BSA would act as a resuscitation-promoting factor toward DPI-induced VBNC cells.

77 To support this hypothesis, there are some reports showing that the removal of oxidative
78 stress is one of the key mechanisms of resuscitation from the VBNC state (Dong et al., 2020).
79 The antioxidative effect of serum albumin has been well-studied (Roche et al., 2008).
80 Accordingly, it seems easy to hypothesize that the resuscitation-promoting effect and
81 antioxidative property of albumin may have a close relationship. Another possibility is that the
82 cellular processes involving cyclic AMP, an important second messenger of the cell. Shleeva
83 et al. showed that the presence of high amounts of cAMP provided by adenylyl cyclase is also
84 essential for resuscitation of *M. smegmatis* and Mtb from a dormant state (Shleeva et al., 2013,
85 2017). Notably, Mtb *Rv2212*, the major gene that encodes adenylyl cyclase (Knapp et al., 2015),
86 its overexpression, significantly affects entry into the dormant state, while its overexpression
87 causes resuscitation (Shleeva et al., 2017).

88 Understanding the detailed mechanism of resuscitation from the VBNC state is crucial to
89 reduce the risk of reactivation of Mtb cells in LTBI patients. In this study, we examined the
90 effects of antioxidative agents and the inhibition of adenylyl cyclase to reveal the roles of these
91 factors in the resuscitation of VBNC Mtb cells.

92 Materials and Methods

93 Bacterial strains, growth media and culture conditions

94 This work was carried out using *Mycobacterium tuberculosis* H37Rv (ATCC 27294) and

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

106 **Counting the number of culturable cells**

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

113 **Effect of BSA on the growth of Mtb cells in Dubos medium**

114 Ten milliliters of log phase culture of Mtb H37Rv that reached an OD_{600} of up to 0.35 in Dubos
115 medium, as described previously, was sedimented at 3,000 ×*g* for 10 minutes at 4˚C and the
116 supernatant was discarded. The sediment was washed twice with fresh Dubos broth and
117 resuspended with 10 mL of Dubos broth. The suspension was diluted to 1:10 with Dubos broth
118 with or without the addition of BSA solution (5.0 g BSA fraction V in 100 mL distilled water)
119 at the final concentration of 0.1% (w/v) BSA. The diluted suspension was dispensed into 24-

120 well plates (Sarstedt, Nümbrecht, Germany) in 1.5 mL increments and sealed with gas-
121 permeable film (Breathe-Easy, Diversified Biotech Inc, Dedham, MA, United States). Cultures
122 were incubated for 5, 10, 15 and 20 days at 37˚C under 5% CO_2. At each time-point, the number
123 of culturable cells was measured as described above.

124 **Induction of the VBNC cells**

125 Induction of the VBNC cells were performed according to the previous report (Yeware et al.,
126 2019) with some modification. In short, the log phase culture of Mtb H37Rv or H37Ra that
127 reached an OD_{600} of up to 0.35 in Dubos medium, as previously described, was evenly divided
128 into 30 mL PETG bottles and 5 mg/mL diphenyleneiodonium chloride (DPI) in dimethyl
129 sulfoxide (DMSO) solution was directly added at the final concentration of 4 μg/mL with
130 immediate agitation. The same volume of DMSO was added to the untreated control sample.
131 Cultures were further incubated for 24 h at 37˚C without shaking. After incubation, their
132 culturability and viability were measured as described above.

133 **Cell viability assay**

134 Mtb cell viability was measured using esterase activity and membrane integrity of the cells
135 using dual staining with 10 mg/mL carboxyfluorescein diacetate (CFDA, Dojindo, Kumamoto,
136 Japan) and ethidium bromide (EB, Dojindo). Briefly, 500 μL of DPI-treated or untreated Mtb
137 culture was sedimented by centrifugation at 15,000 ×*g* for 5 minutes at 4˚C and washed twice
138 with the same volume of PBS-T. The washed bacterial sediment was resuspended in 200 μL of
139 PBS-T with CFDA and EB incubated at room temperature (RT) for 30 minutes in shade. The
140 final concentration of CFDA and EB were 60 μg/mL and 2 μg/mL, respectively. Stained Mtb
141 cells were washed twice with PBS-T and fixed with 3.7% formaldehyde solution at RT for at
142 least 2 h. After fixation, 10 μL of bacterial suspension was smeared on three APS-coated 8-
143 well slides (Matsunami glass, Osaka, Japan) and air-dried. Then the slides were mounted with
144 DPX new non aqueous mounting medium (Merck, Darmstadt, Germany) and a 150 μm-thick

145 cover glass (Matsunami glass, Osaka, Japan) prior to the microscopic observation. Specimens
146 were examined with a BX53 fluorescence microscope (Olympus, Tokyo, Japan) at a
147 magnification of 100× for green (esterase-active with intact membrane; live) or red (esterase-
148 negative with damaged membrane; dead) fluorescing cells. The excitation source for CFDA
149 and EB was generated from a mercury-arc lamp (blue beam, 470–495 nm; green beam, 530–
150 550nm) using a corresponding band-pass filter. The green-fluorescence emission from the
151 CFDA-positive cells was collected through a 510–550 nm band-pass filter. The red-
152 fluorescence emission from EB-positive cells was collected through a 575 nm long-pass filter.
153 At least 10 random fields were observed for each sample and the images (TIFF format) were
154 analyzed using the Fiji/ImageJ software program (Schindelin et al., 2012). The live/dead ratio
155 was calculated by direct counting of green or red fluorescing cells.

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

157 Loss of acid-fastness and accumulation of neutral lipids are distinctive features of dormant
158 mycobacteria (Garton et al., 2002). To detect the phenotype, fluorescent acid-fast staining with
159 auramine-O and neutral lipid staining with Nile Red (9-dimethylamino-5H-benzo-α-
160 phenoxadine-5-one) were performed using a previously described method (Deb et al., 2009)
161 with some modification. Briefly, 10 μL of DPI-treated or untreated culture were smeared onto
162 an APS-coated slide (Matsunami glass, Osaka, Japan) and air-dried. Then, the slide was heat-
163 fixed and cooled down to room temperature before staining. The smear was flooded with
164 fluorochrome staining solution (10 μg/mL auramine-O in 5% [w/v] phenol solution) and
165 incubated at room temperature for 20 min in shade. Excessive dye was removed using 3%
166 hydrochloric acid ethanol for 15 minutes. Next, the smear was covered with 10 μg/mL Nile
167 Red in ethanol and incubated at room temperature for 15 minutes. Finally, the smear was
168 counterstained with 0.1% (w/v) potassium permanganate solution with 1 minute for
169 counterstaining. Stained slides were air-dried and mounted as described previously.

170 Fluorescence microscopy was performed at 100× magnification for green (acid-fastness
171 positive, lipid accumulation negative; active) or red (acid-fastness negative, lipid accumulation
172 positive; dormant) fluorescing cells. The excitation source for auramine-O and Nile Red was
173 generated from a mercury-arc lamp (blue beam, 470–495 nm; green beam, 530–550 nm) using
174 a corresponding band-pass filter. The green-fluorescence emission from auramine-O-positive
175 cells was collected through a 510–550 nm band-pass filter. The red-fluorescence emission from
176 Nile Red-positive cells was collected through 575 nm long-pass filter. At least 5 random fields
177 were observed for each sample and images (TIFF format) were analyzed by the Fiji/ImageJ
178 software program and the proportion of acid-fast-positive cells and lipid-accumulation-positive
179 cells was calculated by direct counting of green or red fluorescing cells.

180 **Ziehl-Neelsen staining**

181 Ziehl-Neelsen staining was also performed to confirm the loss of acid-fastness resulting in the
182 VBNC state by DPI treatment, according to the standard method. Stained slides were air-dried
183 and observed by light microscope under 100x magnification with an oil immersion lens (BX53,
184 Olympus, Tokyo, Japan).

185 **Airyscan super-resolution microscopy**

186 Detailed images of accumulated lipids were also obtained by a confocal laser-scanning
187 microscope (LSM900 with Airyscan 2, Carl Zeiss Microscopy, Jena, Germany). Briefly, culture
188 slides of DPI-treated cells dual stained with auramine-O and Nile Red, as described above,
189 were observed at 63x magnification with oil immersion lens. The excitation source of
190 auramine-O and Nile Red was 488 nm (blue beam) and 563 nm (green beam), from a solid-
191 state laser unit. The green-fluorescence emission from auramine-O and red fluorescence from
192 Nile Red were sequentially collected through a variable dichroic beamsplitter and an Airyscan
193 2 detector. Super-resolution images were processed with the ZEN Blue software program (Carl
194 Zeiss, Jena, Germany).

195 **Resuscitation assay**

196 The resuscitation assay was performed as described elsewhere (Yeware et al., 2019) with some
197 modification. DPI-treated culture was sedimented by centrifugation at 3,000 x*g* for 10 minutes
198 at 4˚C and washed twice with fresh Dubos broth, then resuspended in Dubos broth. The
199 bacterial suspension was diluted to 1:10 with fresh Dubos medium with or without 0.1% (w/v)
200 BSA. For both BSA-containing and BSA-free series, OADC supplementation (Becton
201 Dickinson, Sparks, MD), fetal bovine serum (FBS; Moregate BioTech, Bulimba, Queensland,
202 Australia), or sodium pyruvate (PA; Merck) was added. The final concentration of each reagent
203 was as follows: 10% (v/v) for OADC supplementation, 2% (v/v) for FBS, and 3 mM for PA.
204 Then, the diluted suspension was dispensed into 24-well plates in 1.5 mL increments and sealed
205 with gas-permeable film. Cultures were incubated for 5, 10, 15 and 20 days at 37˚C under 5%
206 CO_2. At each time-point, the number of culturable cells was measured as described previously.

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

209 To determine whether the resuscitation-promoting effect of BSA is its antioxidative property,
210 we measured the resuscitation-promoting activity of other albumins and antioxidative agents
211 toward DPI-treated Mtb cells. Washed DPI-treated cells were resuspended with fresh Dubos
212 broth without BSA and the suspension was diluted to 1:10 with Dubos broth containing BSA,
213 human serum albumin (HSA), ovalbumin (OVA), N-acetyl-L-cysteine (NAC) or D-mannitol
214 (MAN). The final concentration of each reagent was 0.1% (w/v) for BSA, HSA and OVA, 500
215 μM for NAC, and 50 mM for MAN, respectively. Then, the diluted suspension was dispensed
216 into 24-well plates in 1.5 mL increments and sealed with gas-permeable film. The plate was
217 incubated for 20 days at 37˚C under 5% CO_2. At the end of incubation, the number of colonies
218 grown was measured as described previously.

219 **Evaluation of the resuscitation-promoting effect of fatty acid and globulin-free BSA**

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

227 **Effects of adenylyl cyclase or protein kinase A on BSA-induced resuscitation**

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

234 **Statistical analysis**

235 The statistical analysis was performed with R (R Core Team, 2021) using the EZR on R
236 package (Kanda, 2013). P values of < 0.05 were considered statistically significant.

237 **Results**

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

240 We first analyzed the different effects of BSA-containing and BSA-free Dubos medium on
241 the growth of Mtb H37Rv cells. As shown in Fig. 1(A), there was little difference in the rate
242 of growth between the two conditions. Fig. 1(B) also shows the sufficient growth of cells. It

243 seemed that the cells may have been more aggregative without BSA, however, the aggregation
244 was easily homogenized by pipetting and there was no significant difference in the OD_{600} value
245 (data not shown). These results suggest that Mtb could grow normally regardless of the albumin
246 component.

247 **II. DPI treatment could induce a VBNC state in Mtb H37Rv**

248 Next, we confirmed that DPI treatment could induce a VBNC state in the H37Rv strain, in
249 addition to H37Ra, which was previously reported (Yeware et al., 2019). As shown in Fig.
250 2(A), the numbers of culturable cells of untreated control population and DPI-treated
251 population were significantly different: 4.43 ± 1.43 $x10^8$ CFU/mL for the untreated control
252 population and 4.64 ± 6.10 $x10^3$ CFU/mL for DPI-treated population. Fig. 2(B) shows that the
253 CFDA-positive cells in the untreated control and DPI-treated populations were 81.8 ± 11.4%
254 and 65.9 ± 11.8%, respectively. These results suggested that the majority of DPI-treated H37Rv
255 cells were alive, while their culturability was significantly reduced ($p = 0.033$). Representative
256 images of CFDA/EB dual-stained cells of the untreated control and DPI-treated population also
257 showed that there was little difference in the proportions of CFDA-positive and EB-positive
258 cells. (Fig. 2[C])

259 Auramine-O/Nile Red dual staining showed the reduction of acid-fastness and the
260 accumulation of the neutral lipid, triacylglycerol, which are common morphological features
261 of dormant Mtb cells.(Garton et al., 2002; Bhatt et al., 2007; Deb et al., 2009; Kapoor et al.,
262 2013) As shown in Fig. 2(D), the proportion of acid-fast-negative/lipid accumulation-positive
263 cells was 84.0 ± 14.0% in the DPI-treated population, and 12.1 ± 11.9% in the untreated control
264 population. Representative images of auramine-O/Nile Red dual-stained cells of the untreated
265 control and DPI-treated populations also showed that there were distinctive differences in the
266 proportions of acid-fast-positive cells and lipid-accumulation-positive cells (Fig. 2[E]). The
267 loss of acid-fastness was also confirmed by Ziehl-Neelsen staining; the majority of DPI-treated

268 population were found to have lost their acid-fastness (Suppl. Fig. 1). Interestingly, Airyscan
269 super-resolution microscopy revealed that were some DPI-treated cells that were both acid-
270 fast-positive and neutral lipid stain-positive (Fig. 2[F]). This could also reveal the distribution
271 of the lipid body in the cell as some foci of relatively strong signals of Nile Red, suggesting
272 that the transition from culturable to VBNC state. These results indicated that DPI treatment
273 successfully induced the VBNC state in H37Rv as well as in H37Ra.

274 III. The effects of BSA, FBS, OADC and sodium pyruvate on the resuscitation of 275 DPI-treated Mtb cells

276 As described elsewhere, Yeware et al. first reported that the resuscitation of DPI-induced
277 VBNC Mtb cells was only achieved by incubation with FBS; OADC could not facilitate
278 resuscitation. As shown in Fig. 3(A) and (B), our results partially support their study with some
279 differences; although the culturability of DPI-treated H37Rv cells was regained by incubation
280 with FBS-supplemented medium, OADC-supplemented medium could also induce
281 culturability. Surprisingly, the addition of albumin alone into the medium could also induce
282 reactivation. The addition of FBS in BSA-free Dubos medium slightly reduced the regrowth
283 rate; however, cells were successfully resuscitated at the end of incubation with or without FBS.
284 These phenomena were also observed in H37Ra (Suppl. Fig 2). Thus, we used the H37Rv strain
285 for the further analyses in this study.

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

289 IV. The resuscitation-promoting effect of albumin is not due to its antioxidative 290 property or the supplementation of fatty acid from BSA.

291 As shown in Fig. 4(A) and (B), the resuscitation-promoting effect of albumin was specific
292 to bovine and human serum albumin. Ovalbumin did not show a resuscitation-promoting effect
293 but maintained the number of culturable cells in this system. Furthermore, N-acetyl-*L*-cysteine
294 (antioxidative agent) and D-mannitol (free radical scavenger) did not show any such effects.
295 At the end of incubation, there were no detectable culturable cells in either the NAC-treated or
296 MAN-treated cells.

297 We also checked whether the purity of albumin affects the promotion of resuscitation using
298 ethanol and heat-treated albumin and confirmed that there was no significant difference in
299 resuscitation-promoting effects of normal, ethanol-fractionated albumin, and fatty acid-free
300 albumin (Suppl. Fig 4). These results suggest that impurities of commercially available
301 albumin including fatty acids do not affect the regrowth of DPI-treated Mtb.

302 **V. The resuscitation-promoting effect of albumin is canceled by treatment with**
303 **protein kinase A inhibitor.**

304 As shown in Fig. 5(A) and (B), SQ22536, adenylyl cyclase inhibitor, only suppressed
305 resuscitation at a very high concentration (10 mM) in this study. However, H89, a protein
306 kinase A inhibitor, suppressed regrowth in a dose-responsive manner. In detail, incubation with
307 10 μM or 30 μM H89 resulted in a CFU/mL value for $3.42 \times 10^6 \pm 7.45 \times 10^5$ CFU/mL and
308 $3.63 \times 10^2 \pm 1.70 \times 10^2$ CFU/mL, while incubation without H89 resulted in $3.00 \times 10^8 \pm 5.07 \times$
309 10^7 CFU/mL. We should note that neither SQ22536 nor H89 caused a drastic reduction of the
310 growth of intact MTB cells. (Suppl. Fig. 5A and 5B). These results suggest that protein kinase
311 A might mainly be involved with the resuscitation of VBNC Mtb cells triggered by BSA and
312 the contribution of adenylyl cyclase may be smaller than that of protein kinase A.

313 **Discussion**

314 In this study, we demonstrated that the resuscitation of DPI-induced VBNC Mtb cells could
315 be facilitated not only by incubation with FBS but also by incubation with albumin-containing
316 supplements like OADC supplementation or BSA alone. We also showed that the resuscitation-
317 promoting effect of albumin may not be due to its antioxidative property. Furthermore, we
318 suggested that the BSA-induced resuscitation may occur via the activation of protein kinase A-
319 dependent processes.

320 Firstly, we found that DPI-induced VBNC could facilitate resuscitation not only by
321 incubation with FBS but also with OADC supplementation and BSA alone, suggesting albumin
322 might act as a resuscitation-promoting agent in both H37Rv and H37Ra (Fig .3 and Suppl. Fig.
323 2). These findings were contrary to the findings of our previous study. We considered the
324 reason for the difference may be due to contaminant in Tween 80. As described previously, the
325 purity of Tween 80 seemed to be important for the growth of Mtb cells. We also confirmed that
326 the resuscitation was not facilitated by the use of prediluted Tween 80 for the preparation of
327 Dubos medium (internal data) for either H37Rv and H37Ra. Thus, our results suggested that
328 the key component of resuscitation-promoting agent in FBS might be serum albumin, and
329 further analyses were performed using H37Rv. We also tested this effect in Wayne's hypoxic
330 culture, which is widely used for inducing a dormant state of Mtb, and found that there was no
331 significant difference according to the presence or absence of BSA (data not shown).

332 Pyruvate, which is known to act as resuscitation promoting agent for both gram-negative
333 bacteria, such as *Eshcerichia, Legionella, Vibrio* and gram-positive bacteria such as
334 *Staphylococcus* (Pasquaroli et al.; Asakura et al., 2005, 2007; Ducret et al., 2014), did not show
335 any resuscitation-promoting effect in our experiment (Suppl. Fig.3). Vilhena et al. reported that
336 their proteomic analysis of VBNC *E. coli* cells during resuscitation revealed that enzymes
337 involved in pyruvate metabolism, pyruvate formate-lyase-activating protein (PflA),
338 phosphoenolpyruvate carboxykinase (PckA) and lactate dehydrogenase (LldD), were
339 significantly upregulated (Vilhena et al., 2019), while Wagley et al. also reported that

340 VPA1499, an LldD homologue of Vibrio parahaemolyticus, was significantly upregulated in
341 VBNC *V. parahaemolyticus* and that depletion of LldD led to quicker entry to the VBNC state
342 (Wagley et al., 2021). Interestingly, they showed that the addition of pyruvate, the product of
343 lactate oxidation, to LldD-depleted mutant restored the wild-type characteristics of cells in the
344 VBNC state. Their studies might give some crew of the lack of resuscitation-promoting effect
345 toward mycobacterial VBNC. Since Mtb does not have a catalytic center of pyruvate formate-
346 lyase (PflB), pyruvate might not be able to act as resuscitation-promoting agent, although
347 pyruvate enhances the growth of Mtb and *M. bovis* both intracellular and extracellular growth
348 (Schaefer, 1952; Osada-Oka et al., 2019).

349 Our results showed that the resuscitation promoting effect of albumin might be structure-
350 specific because ovalbumin (OVA) did not facilitate resuscitation, but bovine (BSA) and
351 human serum albumin (HSA) did. Focusing on their amino acid sequences, BSA and HSA
352 share 76% sequence homology (Huang et al., 2004); however, BSA and OVA do not have
353 sequence homology. One of the considerable roles of albumin may be as an antioxidative agent
354 due to its cysteine residues with free thiols (Roche et al., 2008; Rombouts et al., 2015). BSA
355 and HSA have only one cysteine residue with free thiol, while OVA has four cysteines with
356 free thiols (Broersen et al., 2006). It therefore seems to be more reductive than BSA and HSA.
357 Our results were paradoxical in relation to this hypothesis; however, Davis and Dubos showed
358 the differential growth-promoting effect of albumin toward Mtb. Their study supplied some
359 interesting clues for our present study; ovalbumin does not show a growth-promoting effect,
360 while bovine and human serum albumin showed the effect (Davis and Dubos, 1947). Moreover,
361 no antioxidative agents, such as N-acetyl-*L*-cysteine and D-mannitol, showed any resuscitative
362 effects (Fig. 4). We also examined catalase, a component of OADC supplementation, and its
363 resuscitation promoting effect toward VBNC *Corynebacterium, Lactobacillus, Salmonella,*
364 *Ralstonia* and *Vibrio* (Kong et al., 2004, 2014; Senoh et al., 2015; Liu et al., 2017; Morishige
365 et al., 2017; Hamabata et al., 2021); no resuscitative effects were observed (data not shown).

366 These results suggest that the effect of albumin may be due to complex functions rather than
367 its antioxidative property.

368 Serum albumin is known to act as a good carrier of many kinds of biologically active
369 substances, including free fatty acid. In general, BSA is added to medium for mycobacterial
370 culture as a carrier of growth-supporting agents, including fatty acids. Shleeva et al. showed
371 that several types of unsaturated free fatty acid, such as oleic, linoleic, and arachidonic acids,
372 can promote the resuscitation of VBNC *M. smegmatis* in modified Sauton's medium and oleic
373 acid also works for Mtb (Shleeva et al., 2013). In this study, we should note that impurities of
374 albumin did not affect resuscitation. Although the underlying mechanism is still unclear, fatty
375 acid and globulin-free BSA and BSA Cohn fraction V did not show any difference toward
376 resuscitation of DPI-treated Mtb (Suppl. Fig. 4).

377 The assay of inhibition of resuscitation by SQ22536 and H89 gave us an important clue for
378 understanding resuscitation. Shleeva et al. showed that the presence of high amounts of cAMP
379 provided by adenylyl cyclase is essential for resuscitation from a dormant state in *M. smegmatis*
380 and *M. tuberculosis* (Shleeva et al., 2013, 2017). Especially in *M. tuberculosis*, the
381 overexpression of *Rv2212*, the major gene that encodes adenylyl cyclase (Knapp et al., 2015),
382 significantly affects both entry into the dormant state and resuscitation (Shleeva et al., 2017).
383 In the present study, we could suppress the resuscitation of DPI-induced VBNC cells by
384 inhibiting adenylyl cyclase only with a very high concentration of SQ22536. Although the
385 underlying mechanism is unclear, adenylyl cyclase might not play a key role in resuscitation
386 from a DPI-induced VBNC state. However, the inhibition of protein kinase A by H89 clearly
387 suppressed resuscitation. Mtb has the eukaryotic-type Ser/Thr protein kinase PknA, which
388 regulates major cell processes, regulates several types of proteins involved with mycobacterial
389 cell division and morphogenesis via phosphorylation of such proteins in coordination with
390 protein kinase B (PknB; Manuse et al., 2016; Carette et al., 2018). For example,
391 phosphorylation of GlmU (*Rv1018c*) and MurD (*Rv2155c*), Wag31 (*Rv2145c*), HupB

392 (*Rv2986c*) and ParB (*Rv3917c*), influence the production and cross-linking of peptidoglycan
393 precursors (Thakur and Chakraborti, 2008; Parikh et al., 2009; Gee et al., 2012; Jagtap et al.,
394 2012), the spatial localization of peptidoglycan synthesis (Kang et al., 2008), DNA
395 condensation and partitioning (Gupta et al., 2014; Baronian et al., 2015), respectively. It should
396 be noted that the phosphorylation of FtsZ and FipA by PknA controls cell division under
397 oxidative stress (Thakur and Chakraborti, 2006; Sureka et al., 2010). Our study suggested that
398 the inhibition of PknA by H89 seems to critically affect several important cellular processes,
399 followed by resuscitation (Fig. 6). To confirm the essentiality of PknA and other involved
400 proteins toward resuscitation from this DPI-induced VBNC state by albumin, further studies
401 using experiments such as knock-down of genes using the CRISPR interference (CRISPRi)
402 technique (Rock et al., 2017), because such proteins—with the exception of FipA (*Rv0019c*)—
403 are known to be essential for *in vitro* growth (Sassetti et al., 2003; Griffin et al., 2011; DeJesus
404 et al., 2017; Minato et al., 2019); thus, manipulating their coding genes is not permissible.

405 We also confirmed that the absence of BSA in Dubos medium did not show any significant
406 effect on the growth of Mtb (Fig.1[A]). This may be due to the purity of Tween 80 because
407 Dubos et al. previously reported that the presence of unpurified Tween 80 may inhibit growth
408 due to release of free oleic acid (Davis and Dubos, 1947). Our result was in good concordance
409 with that study. We also confirmed that the use of prediluted Tween 80 (20% w/v) in BSA-free
410 Dubos medium clearly arrested the growth of Mtb (internal data). The concentration of free
411 fatty acid in the medium with prediluted Tween 80 was approximately 80 μM. On the other
412 hand, the concentration in the medium with non-prediluted Tween 80 was only approximately
413 7 μM (unpublished data), suggesting that excessive concentrations of fatty acid are toxic for
414 Mtb (Dubos, 1950).

415 In addition, we confirmed that DPI could successfully induce a VBNC state in H37Rv as
416 well as H37Ra. The mechanism underlying the effect of DPI is considered to involve the
417 inhibitory effect of NADH oxidase, which results in the inhibition of the electron transport

system of Mtb. This may induce a hypoxic state in the liquid culture, resulting in significant decrease of culturability (Fig.2[A]). However, viability was highly retained (65.9%), suggesting that majority of the cells transitioned to VBNC state (Fig. 2[B]). Additionally, transition to VBNC state was also confirmed by auramine-O and Nile Red staining. The loss of acid-fastness and the accumulation of neutral lipids are distinctive features of dormant mycobacterial cells, and can be detected by acid-fast staining and neutral lipid staining. Our data also demonstrated that the majority of DPI-treated Mtb H37Rv cells were stained with Nile Red alone (Fig. 2[D]). Airyscan super-resolution microscopy showed the presence of cells that were stained with auramine-O and which contained an intracellular lipid body stained with Nile Red (Fig. 2[F]), which may indicate that the cells were on the way to the dormant state from an actively growing state, with drastic alteration of the lipid metabolism.

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

Conclusion

Our data showed that the presence of BSA in Dubos medium could trigger the resuscitation of the DPI-induced VBNC state in both H37Rv and H37Ra strains. The resuscitation-promoting effect of other kinds of albumins and antioxidative agents, including N-acetyl-*L*-cysteine and D-mannitol was tested; however, only human serum albumin showed a resuscitation-promoting effect. The results of the assay of resuscitation using SQ22536 and

443 H89 demonstrated that the inhibition of protein kinase A suppressed resuscitation to a much
444 greater extent than adenylyl cyclase, indicating the presence of a protein kinase A-dependent
445 resuscitation-promoting pathway.

446 **Author Contributions**

447 YMori, YMura and SM conceived and designed the experiments. YMori performed
448 experiments. YMura, KC, AA, YI, YS, MH, KK, HY, AT, and SM assisted in the experiments.
449 YMori acquired data and YMori and YMura analyzed data. YMori performed the statistical
450 analysis. YMori and SM wrote the manuscript. All authors contributed to the article and
451 approved the submitted version.

452 **Funding**

453 This work was supported by Research Program on Emerging and Re-emerging Infectious
454 Diseases of Japan Agency for Medical Research and Development (AMED) and Grant-in-Aid
455 for Early-Career Scientists of Japan Society for the Promotion of Science (JSPS) KAKENHI.
456 Grant Numbers are JP21fk0108090 and 21K15442, respectively.

457 **Acknowledgments**

458 Authors thank K. Suenaga and H. Sakuma (Carl Zeiss Co., Ltd., Tokyo, Japan) for their kind
459 assistance in obtaining super-resolution images. Authors also thank Dr. A. Osugi (The Research
460 Institute of Tuberculosis, Japan Anti-Tuberculosis Association) for valuable discussions.
461 Authors also thank Dr. Brian Quinn (Japan Medical Communication. Inc.) for the help in the
462 preparation of this manuscript.

463 **Conflict of Interest Statement**

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

References

Aguilar-Ayala, D. A., Cnockaert, M., Vandamme, P., Palomino, J. C., Martin, A., and Gonzalez-Y-Merchand, J. (2018). Antimicrobial activity against *Mycobacterium tuberculosis* under in vitro lipid-rich dormancy conditions. *J. Med. Microbiol.* 67, 282–285. doi:10.1099/jmm.0.000681.

Asakura, H., Igimi, S., Kawamoto, K., Yamamoto, S., and Makino, S.-I. (2005). Role of *in vivo* passage on the environmental adaptation of enterohemorrhagic *Escherichia coli* O157:H7: cross-induction of the viable but nonculturable state by osmotic and oxidative stresses. *FEMS Microbiol. Lett.* 253, 243–9. doi:10.1016/j.femsle.2005.09.039.

Asakura, H., Ishiwa, A., Arakawa, E., Makino, S., Okada, Y., Yamamoto, S., et al. (2007). Gene expression profile of *Vibrio cholerae* in the cold stress-induced viable but non-culturable state. *Environ. Microbiol.* 9, 869–79. doi:10.1111/j.1462-2920.2006.01206.x.

Baronian, G., Ginda, K., Berry, L., Cohen-Gonsaud, M., Zakrzewska-Czerwińska, J., Jakimowicz, D., et al. (2015). Phosphorylation of *Mycobacterium tuberculosis* ParB participates in regulating the ParABS chromosome segregation system. *PLoS One* 10, e0119907. doi:10.1371/journal.pone.0119907.

Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., and Duncan, K. (2002). Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol. Microbiol.* 43, 717–31. doi:10.1046/j.1365-2958.2002.02779.x.

Bhatt, A., Fujiwara, N., Bhatt, K., Gurcha, S. S., Kremer, L., Chen, B., et al. (2007). Deletion of kasB in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proc. Natl. Acad. Sci. U. S. A.* 104, 5157–

489 62. doi:10.1073/pnas.0608654104.

490 Broersen, K., Van Teeffelen, A. M. M., Vries, A., Voragen, A. G. J., Hamer, R. J., and De
491 Jongh, H. H. J. (2006). Do sulfhydryl groups affect aggregation and gelation properties
492 of ovalbumin? *J. Agric. Food Chem.* 54, 5166–74. doi:10.1021/jf0601923.

493 Carette, X., Platig, J., Young, D. C., Helmel, M., Young, A. T., Wang, Z., et al. (2018).
494 Multisystem Analysis of *Mycobacterium tuberculosis* Reveals Kinase-Dependent
495 Remodeling of the Pathogen-Environment Interface. *MBio* 9. doi:10.1128/mBio.02333-
496 17.

497 Davis, B. D., and Dubos, R. J. (1947). THE BINDING OF FATTY ACIDS BY SERUM
498 ALBUMIN, A PROTECTIVE GROWTH FACTOR IN BACTERIOLOGICAL
499 MEDIA. *J. Exp. Med.* 86, 215–228. doi:10.1084/jem.86.3.215.

500 Deb, C., Lee, C. M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., et al. (2009).
501 A novel in vitro multiple-stress dormancy model for *Mycobacterium tuberculosis*
502 generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One* 4, e6077.
503 doi:10.1371/journal.pone.0006077.

504 DeJesus, M. A., Gerrick, E. R., Xu, W., Park, S. W., Long, J. E., Boutte, C. C., et al. (2017).
505 Comprehensive Essentiality Analysis of the *Mycobacterium tuberculosis* Genome via
506 Saturating Transposon Mutagenesis. *MBio* 8. doi:10.1128/mBio.02133-16.

507 Desnues, B., Cuny, C., Grégori, G., Dukan, S., Aguilaniu, H., and Nyström, T. (2003).
508 Differential oxidative damage and expression of stress defence regulons in culturable
509 and non-culturable *Escherichia coli* cells. *EMBO Rep.* 4, 400–404.
510 doi:10.1038/SJ.EMBOR.EMBOR799.

511 Dong, K., Pan, H., Yang, D., Rao, L., Zhao, L., Wang, Y., et al. (2020). Induction, detection,
512 formation, and resuscitation of viable but non-culturable state microorganisms. *Compr.*
513 *Rev. Food Sci. Food Saf.* 19, 149–183. doi:10.1111/1541-4337.12513.

514 Dubos, R. J. (1947). THE EFFECT OF LIPIDS AND SERUM ALBUMIN ON

515 BACTERIAL GROWTH. *J. Exp. Med.* 85, 9–22. doi:10.1084/jem.85.1.9.

516 Dubos, R. J. (1950). THE EFFECT OF ORGANIC ACIDS ON MAMMALIAN TUBERCLE
517 BACILLI. *J. Exp. Med.* 92, 319–332. doi:10.1084/JEM.92.4.319.

518 Ducret, A., Chabalier, M., and Dukan, S. (2014). Characterization and resuscitation of "non-
519 culturable" cells of *Legionella pneumophila*. *BMC Microbiol.* 14, 3. doi:10.1186/1471-
520 2180-14-3.

521 Garton, N. J., Christensen, H., Minnikin, D. E., Adegbola, R. A., and Barer, M. R. (2002).
522 Intracellular lipophilic inclusions of mycobacteria in vitro and in sputum. *Microbiology*
523 148, 2951–2958. doi:10.1099/00221287-148-10-2951.

524 Gee, C. L., Papavinasasundaram, K. G., Blair, S. R., Baer, C. E., Falick, A. M., King, D. S.,
525 et al. (2012). A phosphorylated pseudokinase complex controls cell wall synthesis in
526 mycobacteria. *Sci. Signal.* 5, ra7. doi:10.1126/scisignal.2002525.

527 Griffin, J. E., Gawronski, J. D., Dejesus, M. A., Ioerger, T. R., Akerley, B. J., and Sassetti, C.
528 M. (2011). High-resolution phenotypic profiling defines genes essential for
529 mycobacterial growth and cholesterol catabolism. *PLoS Pathog.* 7, e1002251.
530 doi:10.1371/journal.ppat.1002251.

531 Gupta, M., Sajid, A., Sharma, K., Ghosh, S., Arora, G., Singh, R., et al. (2014). HupB, a
532 nucleoid-associated protein of *Mycobacterium tuberculosis*, is modified by
533 serine/threonine protein kinases in vivo. *J. Bacteriol.* 196, 2646–57.
534 doi:10.1128/JB.01625-14.

535 Hamabata, T., Senoh, M., Iwaki, M., Nishiyama, A., Yamamoto, A., and Shibayama, K.
536 (2021). Induction and Resuscitation of Viable but Nonculturable *Corynebacterium*
537 *diphtheriae*. *Microorganisms* 9. doi:10.3390/microorganisms9050927.

538 Huang, B. X., Kim, H. Y., and Dass, C. (2004). Probing three-dimensional structure of
539 bovine serum albumin by chemical cross-linking and mass spectrometry. *J. Am. Soc.*
540 *Mass Spectrom.* 15, 1237–1247. doi:10.1016/J.JASMS.2004.05.004.

541 Imazaki, I., and Nakaho, K. (2009). Temperature-upshift-mediated revival from the sodium-
542 pyruvate-recoverable viable but nonculturable state induced by low temperature in
543 *Ralstonia solanacearum*: linear regression analysis. *J. Gen. Plant Pathol. 2009 753* 75,
544 213–226. doi:10.1007/S10327-009-0166-0.
545 Jagtap, P. K. A., Soni, V., Vithani, N., Jhingan, G. D., Bais, V. S., Nandicoori, V. K., et al.
546 (2012). Substrate-bound crystal structures reveal features unique to *Mycobacterium*
547 *tuberculosis* N-acetyl-glucosamine 1-phosphate uridyltransferase and a catalytic
548 mechanism for acetyl transfer. *J. Biol. Chem.* 287, 39524–37.
549 doi:10.1074/jbc.M112.390765.
550 Kanda, Y. (2013). Investigation of the freely available easy-to-use software "EZR" for
551 medical statistics. *Bone Marrow Transplant.* 48, 452–8. doi:10.1038/bmt.2012.244.
552 Kang, C.-M., Nyayapathy, S., Lee, J.-Y., Suh, J.-W., and Husson, R. N. (2008). Wag31, a
553 homologue of the cell division protein DivIVA, regulates growth, morphology and polar
554 cell wall synthesis in mycobacteria. *Microbiology* 154, 725–735.
555 doi:10.1099/mic.0.2007/014076-0.
556 Kapoor, N., Pawar, S., Sirakova, T. D., Deb, C., Warren, W. L., and Kolattukudy, P. E.
557 (2013). Human granuloma in vitro model, for TB dormancy and resuscitation. *PLoS One*
558 8, e53657. doi:10.1371/journal.pone.0053657.
559 Knapp, G. S., Lyubetskaya, A., Peterson, M. W., Gomes, A. L. C., Ma, Z., Galagan, J. E., et
560 al. (2015). Role of intragenic binding of cAMP responsive protein (CRP) in regulation
561 of the succinate dehydrogenase genes Rv0249c-Rv0247c in TB complex mycobacteria.
562 *Nucleic Acids Res.* 43, 5377–93. doi:10.1093/nar/gkv420.
563 Kong, H. G., Bae, J. Y., Lee, H. J., Joo, H. J., Jung, E. J., Chung, E., et al. (2014). Induction
564 of the viable but nonculturable state of *Ralstonia solanacearum* by low temperature in
565 the soil microcosm and its resuscitation by catalase. *PLoS One* 9, e109792.
566 doi:10.1371/journal.pone.0109792.

567 Kong, I.-S., Bates, T. C., HÃ¼lsmann, A., Hassan, H., Smith, B. E., and Oliver, J. D. (2004).
568 Role of catalase and oxyR in the viable but nonculturable state of *Vibrio vulnificus.*
569 *FEMS Microbiol. Ecol.* 50, 133–142. doi:10.1016/j.femsec.2004.06.004.
570 Liu, J., Li, L., Peters, B. M., Li, B., Chen, L., Deng, Y., et al. (2017). The viable but
571 nonculturable state induction and genomic analyses of *Lactobacillus casei* BM-
572 LC14617, a beer-spoilage bacterium. *Microbiologyopen* 6. doi:10.1002/mbo3.506.
573 Lynn, M., Wilson, A. R., and Solotorovsky, M. (1979). Role of bovine serum albumin in the
574 nutrition of *Mycobacterium tuberculosis. Appl. Environ. Microbiol.* 38, 806–810.
575 doi:10.1128/aem.38.5.806-810.1979.
576 Manuse, S., Fleurie, A., Zucchini, L., Lesterlin, C., and Grangeasse, C. (2016). Role of
577 eukaryotic-like serine/threonine kinases in bacterial cell division and morphogenesis.
578 *FEMS Microbiol. Rev.* 40, 41–56. doi:10.1093/femsre/fuv041.
579 Minato, Y., Gohl, D. M., Thiede, J. M., Chacón, J. M., Harcombe, W. R., Maruyama, F., et
580 al. (2019). Genomewide Assessment of *Mycobacterium tuberculosis* Conditionally
581 Essential Metabolic Pathways. *mSystems* 4. doi:10.1128/mSystems.00070-19.
582 Mizunoe, Y., Wai, S. N., Ishikawa, T., Takade, A., and Yoshida, S. (2000). Resuscitation of
583 viable but nonculturable cells of *Vibrio parahaemolyticus* induced at low temperature
584 under starvation. *FEMS Microbiol. Lett.* 186, 115–120. doi:10.1111/J.1574-
585 6968.2000.TB09091.X.
586 Morishige, Y., Koike, A., Tamura-Ueyama, A., and Amano, F. (2017). Induction of viable
587 but nonculturable *Salmonella* in exponentially grown cells by exposure to a low-
588 humidity environment and their resuscitation by catalase. *J. Food Prot.* 80, 288–294.
589 doi:10.4315/0362-028X.JFP-16-183.
590 Mukamolova, G. V., Turapov, O., Malkin, J., Woltmann, G., and Barer, M. R. (2010).
591 Resuscitation-promoting factors reveal an occult population of tubercle bacilli in
592 sputum. *Am. J. Respir. Crit. Care Med.* 181, 174–180. doi:10.1164/rccm.200905-

593 0661OC.

594 Mukamolova, G. V, Murzin, A. G., Salina, E. G., Demina, G. R., Kell, D. B., Kaprelyants, A.
595 S., et al. (2006). Muralytic activity of *Micrococcus luteus* Rpf and its relationship to
596 physiological activity in promoting bacterial growth and resuscitation. *Mol. Microbiol.*
597 59, 84–98. doi:10.1111/j.1365-2958.2005.04930.x.

598 Oliver, J. D. (2010). Recent findings on the viable but nonculturable state in pathogenic
599 bacteria. *FEMS Microbiol. Rev.* 34, 415–425. doi:10.1111/j.1574-6976.2009.00200.x.

600 Osada-Oka, M., Goda, N., Saiga, H., Yamamoto, M., Takeda, K., Ozeki, Y., et al. (2019).
601 Metabolic adaptation to glycolysis is a basic defense mechanism of macrophages for
602 *Mycobacterium tuberculosis* infection. *Int. Immunol.* 31, 781–793.
603 doi:10.1093/INTIMM/DXZ048.

604 Parikh, A., Verma, S. K., Khan, S., Prakash, B., and Nandicoori, V. K. (2009). PknB-
605 mediated phosphorylation of a novel substrate, N-acetylglucosamine-1-phosphate
606 uridyltransferase, modulates its acetyltransferase activity. *J. Mol. Biol.* 386, 451–64.
607 doi:10.1016/j.jmb.2008.12.031.

608 Pasquaroli, S., Zandri, G., Vignaroli, C., Vuotto, C., Donelli, G., and Biavasco, F. Antibiotic
609 pressure can induce the viable but non-culturable state in *Staphylococcus aureus*
610 growing in biofilms. doi:10.1093/jac/dkt086.

611 R Core Team (2021). R: A Language and Environment for Statistical Computing. Available
612 at: https://www.r-project.org.

613 Roche, M., Rondeau, P., Singh, N. R., Tarnus, E., and Bourdon, E. (2008). The antioxidant
614 properties of serum albumin. *FEBS Lett.* 582, 1783–1787.
615 doi:10.1016/J.FEBSLET.2008.04.057.

616 Rock, J. M., Hopkins, F. F., Chavez, A., Diallo, M., Chase, M. R., Gerrick, E. R., et al.
617 (2017). Programmable transcriptional repression in mycobacteria using an orthogonal
618 CRISPR interference platform. *Nat. Microbiol.* 2, 16274.

619 doi:10.1038/nmicrobiol.2016.274.

620 Rombouts, I., Lagrain, B., Scherf, K. A., Lambrecht, M. A., Koehler, P., and Delcour, J. A.
621 (2015). Formation and reshuffling of disulfide bonds in bovine serum albumin
622 demonstrated using tandem mass spectrometry with collision-induced and electron-
623 transfer dissociation. *Sci. Rep.* 5, 12210. doi:10.1038/srep12210.

624 Salina, E. G., Waddell, S. J., Hoffmann, N., Rosenkrands, I., Butcher, P. D., and Kaprelyants,
625 A. S. (2014). Potassium availability triggers *Mycobacterium tuberculosis* transition to,
626 and resuscitation from, non-culturable (dormant) states. *Open Biol.* 4.
627 doi:10.1098/rsob.140106.

628 Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003). Genes required for mycobacterial
629 growth defined by high density mutagenesis. *Mol. Microbiol.* 48, 77–84.
630 doi:10.1046/j.1365-2958.2003.03425.x.

631 Schaefer, W. B. (1952). GROWTH REQUIREMENTS OF DYSGONIC AND EUGONIC
632 STRAINS OF *MYCOBACTERIUM TUBERCULOSIS VAR. BOVIS*. *J. Exp. Med.* 96,
633 207–219. doi:10.1084/JEM.96.3.207.

634 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al.
635 (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods 2012*
636 *97* 9, 676–682. doi:10.1038/nmeth.2019.

637 Senoh, M., Hamabata, T., and Takeda, Y. (2015). A factor converting viable but
638 nonculturable *Vibrio cholerae* to a culturable state in eukaryotic cells is a human
639 catalase. *Microbiologyopen* 4, 589–96. doi:10.1002/mbo3.264.

640 Shleeva, M., Goncharenko, A., Kudykina, Y., Young, D., Young, M., and Kaprelyants, A.
641 (2013). Cyclic amp-dependent resuscitation of dormant mycobacteria by exogenous free
642 fatty acids. *PLoS One* 8, e82914. doi:10.1371/journal.pone.0082914.

643 Shleeva, M. O., Kondratieva, T. K., Demina, G. R., Rubakova, E. I., Goncharenko, A. V.,
644 Apt, A. S., et al. (2017). OVerexpression Of adenylyl cyclase encoded by the

645 *Mycobacterium tuberculosis* Rv2212 gene confers improved fitness, accelerated
646 recovery from dormancy and enhanced virulence in mice. *Front. Cell. Infect. Microbiol.*
647 7, 370. doi:10.3389/fcimb.2017.00370.
648 Sureka, K., Hossain, T., Mukherjee, P., Chatterjee, P., Datta, P., Kundu, M., et al. (2010).
649 Novel role of phosphorylation-dependent interaction between FtsZ and FipA in
650 mycobacterial cell division. *PLoS One* 5, e8590. doi:10.1371/journal.pone.0008590.
651 Thakur, M., and Chakraborti, P. K. (2006). GTPase activity of mycobacterial FtsZ is
652 impaired due to its transphosphorylation by the eukaryotic-type Ser/Thr kinase, PknA. *J.*
653 *Biol. Chem.* 281, 40107–13. doi:10.1074/jbc.M607216200.
654 Thakur, M., and Chakraborti, P. K. (2008). Ability of PknA, a mycobacterial eukaryotic-type
655 serine/threonine kinase, to transphosphorylate MurD, a ligase involved in the process of
656 peptidoglycan biosynthesis. *Biochem. J.* 415, 27–33. doi:10.1042/BJ20080234.
657 Vilhena, C., Kaganovitch, E., Grünberger, A., Motz, M., Forné, I., Kohlheyer, D., et al.
658 (2019). Importance of Pyruvate Sensing and Transport for the Resuscitation of Viable
659 but Nonculturable *Escherichia coli* K-12. *J. Bacteriol.* 201. doi:10.1128/JB.00610-18.
660 Wagley, S., Morcrette, H., Kovacs-Simon, A., Yang, Z. R., Power, A., Tennant, R. K., et al.
661 (2021). Bacterial dormancy: A subpopulation of viable but non-culturable cells
662 demonstrates better fitness for revival. *PLoS Pathog.* 17, e1009194.
663 doi:10.1371/journal.ppat.1009194.
664 Wayne, L. G., and Hayes, L. G. (1996). An in vitro model for sequential study of shiftdown
665 of *Mycobacterium tuberculosis* through two stages of nonreplicating persistence. *Infect.*
666 *Immun.* 64, 2062–2069. doi:10.1128/iai.64.6.2062-2069.1996.
667 World Health Organization (2020). "TB disease burden," in *Global Tuberculosis Report*
668 *2020*, Chapter 4, 23.
669 Xu, H. S., Roberts, N., Singleton, F. L., Attwell, R. W., Grimes, D. J., and Colwell, R. R.
670 (1982). Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in

671 the estuarine and marine environment. *Microb. Ecol.* 8, 313–23.
672 doi:10.1007/BF02010671.
673 Yeware, A., Gample, S., Agrawal, S., and Sarkar, D. (2019). Using diphenyleneiodonium to
674 induce a viable but non-culturable phenotype in *Mycobacterium tuberculosis* and its
675 metabolomics analysis. *PLoS One* 14, e0220628. doi:10.1371/journal.pone.0220628.

676

677

678

679

680

681

682

683

684

685

686

687

688

689

690 **Figure legends**

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

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

698 Figure 2. Viability analyses of DPI-treated Mtb

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

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

727 Figure 3. Resuscitation of DPI-treated Mtb H37Rv cells by FBS and OADC supplementation

728 (A) Number of culturable cells at the indicated days of incubation, without
729 supplementation or those cells supplemented with 2% FBS or 10% OADC in the
730 presence (closed circle) or absence (open circle) of 0.1% BSA. The CFU/mL values
731 were determined by plating cells onto 7H10 plates in duplicate. Data represent the
732 mean ± SD from three independent experiments. U.D. means under the limit of
733 detection (20 CFU/mL).
734 (B) Representative images of regrowth under the indicated conditions captured at the end
735 of incubation. None, FBS, and OADC represent no supplementation, 2% FBS and
736 10% OADC supplementation, respectively.

737 Figure 4. Effect of human serum albumin, ovalbumin and antioxidants on the resuscitation of

738 DPI-treated Mtb.

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

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

750 Figure 5. The effect of SQ22536 or H89 on the resuscitation of DPI-treated Mtb

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

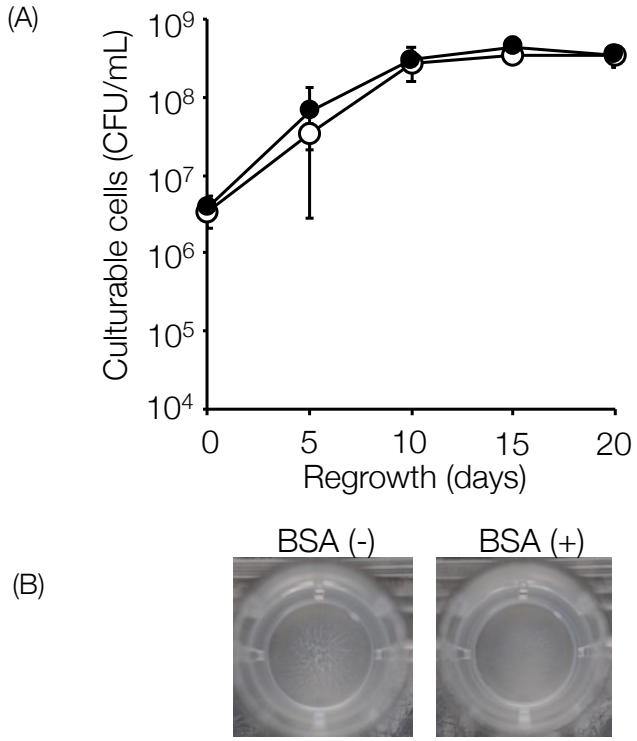
754 The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data
755 represent the mean ± SD from three independent experiments.

756 Figure 6. Schematic representation of the mechanism of BSA-induced resuscitation of Mtb.
757 Although the detailed mechanism of interaction between BSA and PknA is not yet clear, BSA
758 may— in coordination with PknB—activate PknA and positively regulate target proteins that
759 are involved in the localized synthesis of peptidoglycan, cell division control, and DNA

760 condensation and partitioning through their phosphorylation, resulting in the resuscitation of
761 VBNC Mtb cells.

762 **Figures**

763 Figure 1.



764

765

766

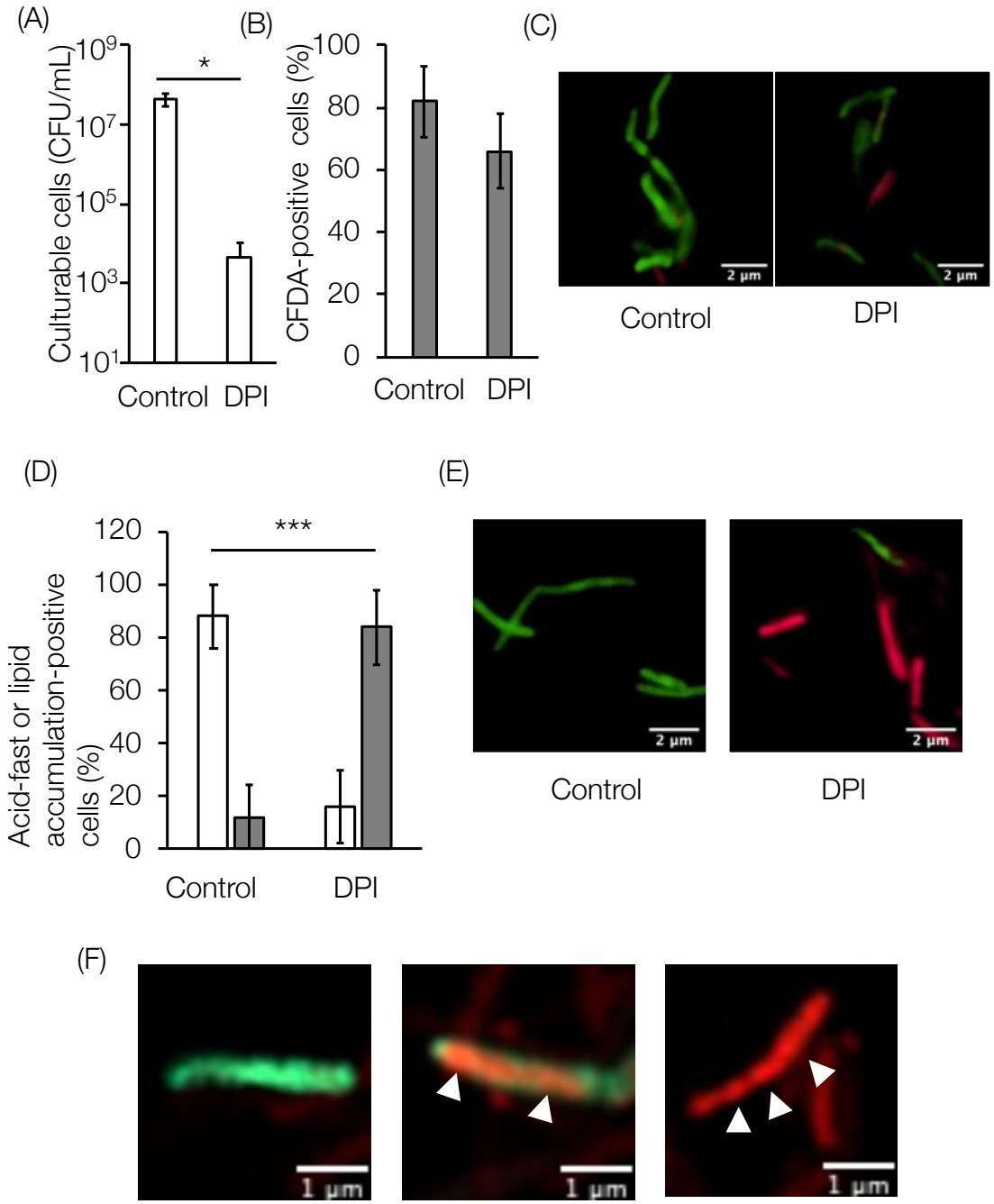
767

768

769

770

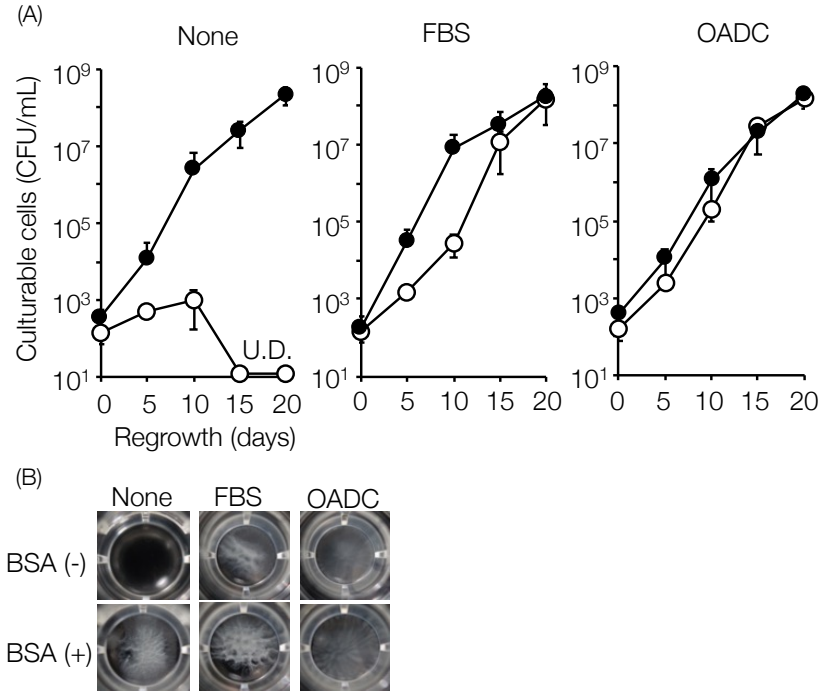
Figure 2.


(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

776

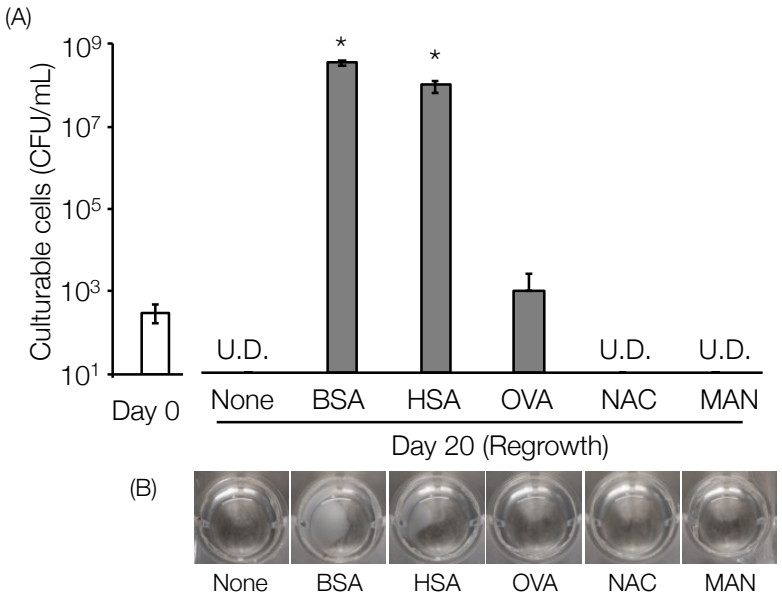
777

778 Figure 3.



779

780 Figure 4.

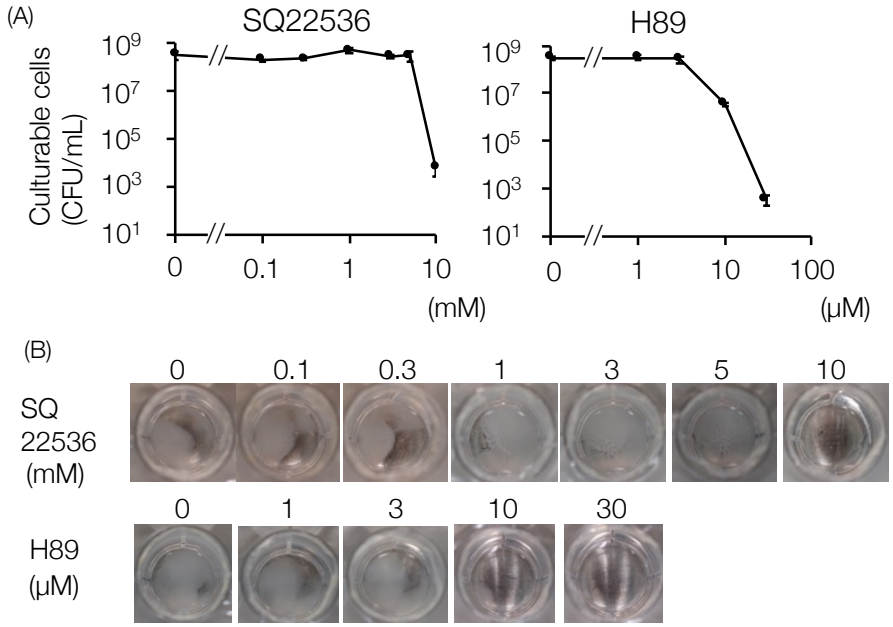


781

782

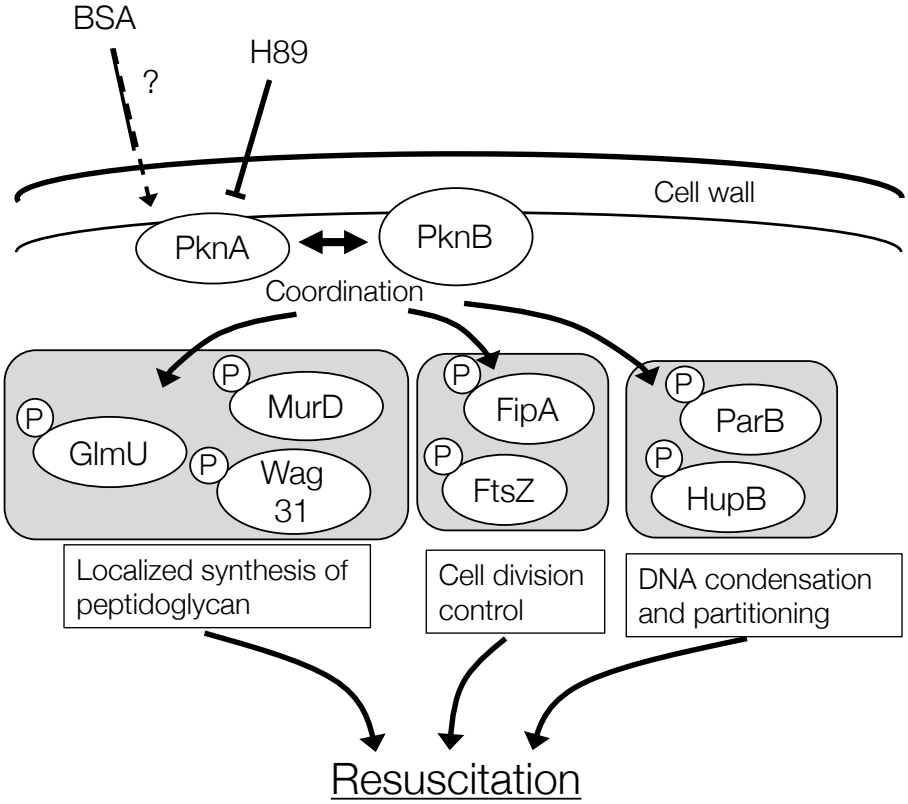
783

784 Figure 5.



785

786 Figure 6.


BSA
?
H89
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
Resuscitation

787