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

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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

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

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

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

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

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

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

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

Materials and Methods

Bacterial strains, growth media and culture conditions

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

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

Counting the number of culturable cells

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

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

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

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

Induction of the VBNC cells

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

Cell viability assay

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

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

Auramine-O/Nile Red dual staining

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

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

Ziehl-Neelsen staining

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

Airyscan super-resolution microscopy

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

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

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

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

Statistical analysis

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

Results

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

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

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

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

Next, we confirmed that DPI treatment could induce a VBNC state in the H37Rv strain, in addition to H37Ra, which was previously reported (Yeware et al., 2019). As shown in Fig. 2(A), the numbers of culturable cells of untreated control population and DPI-treated population were significantly different: $4.43 \pm 1.43 \times 10^8$ CFU/mL for the untreated control population and $4.64 \pm 6.10 \times 10^3$ CFU/mL for DPI-treated population. Fig. 2(B) shows that the CFDA-positive cells in the untreated control and DPI-treated populations were 81.8 ± 11.4% and 65.9 ± 11.8%, respectively. These results suggested that the majority of DPI-treated H37Rv cells were alive, while their culturability was significantly reduced ($p = 0.033$). Representative images of CFDA/EB dual-stained cells of the untreated control and DPI-treated population also showed that there was little difference in the proportions of CFDA-positive and EB-positive cells. (Fig. 2[C])

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

In addition, we confirmed that DPI could successfully induce a VBNC state in H37Rv as well as H37Ra. The mechanism underlying the effect of DPI is considered to involve the 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

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

Author Contributions

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

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Conflict of Interest Statement

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

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Figure legends

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

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

(B) Representative images of Mtb growth captured at the end of incubation.

Figure 2. Viability analyses of DPI-treated Mtb

(A) Number of culturable cells after DPI treatment. The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Asterisk indicates a statistically significant difference (*$p < 0.05$, Welch's *t*-test). Data represent the mean ± SD from three independent experiments.

(B) Percentages of esterase active cells after DPI treatment. Around 850 cells were directly counted under 100× objective lens. Data represent the mean ± SD from three independent experiments.

(C) Merged fluorescence micrographs of Mtb H37Rv cells stained with carboxyfluorescein diacetate (CFDA) and ethidium bromide (EtBr). Cells stained with CFDA (green) are esterase-positive; those stained with EtBr (red) are membrane-damaged. Images are representative images from three individual experiments. Scale bar: 2μm

(D) Percentage of acid-fast-positive cells (open column) or acid-fast-negative but lipid-accumulation-positive cells (gray column). Approximately 1,100 cells were directly counted under 100× objective lens. Data represent the mean ± SD from four

independent experiments. Asterisk indicates significant difference (***$p < 0.005$, Welch's *t*-test).

(E) Merged fluorescence micrographs of Mtb H37Rv cells stained with auramine-O and Nile Red. Cells stained with auramine-O (green) are acid-fast-positive; those stained with Nile Red (red) are acid-fast-negative but lipid accumulation-positive. Images are representative images from three individual experiments. Scale bar: 2μm

(F) Magnified views of Airyscan confocal super-resolution microscopy observation of three different Mtb cells obtained from DPI-treated culture. Only acid-fast-positive cells (left), both acid-fast-positive and lipid accumulation-positive cells (center, counted as acid-fast-positive) and acid-fast-negative cells with only lipid accumulation-positive cells (right). Dual-positive cells probably represent the transition to a non-culturable state. Indicated foci with arrowheads represent a neutral lipid body. Scale bar: 1 μm

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

(A) Number of culturable cells at the indicated days of incubation, without supplementation or those cells supplemented with 2% FBS or 10% OADC in the presence (closed circle) or absence (open circle) of 0.1% BSA. The CFU/mL values were determined by plating cells onto 7H10 plates in duplicate. Data represent the mean ± SD from three independent experiments. U.D. means under the limit of detection (20 CFU/mL).

(B) Representative images of regrowth under the indicated conditions captured at the end of incubation. None, FBS, and OADC represent no supplementation, 2% FBS and 10% OADC supplementation, respectively.

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.

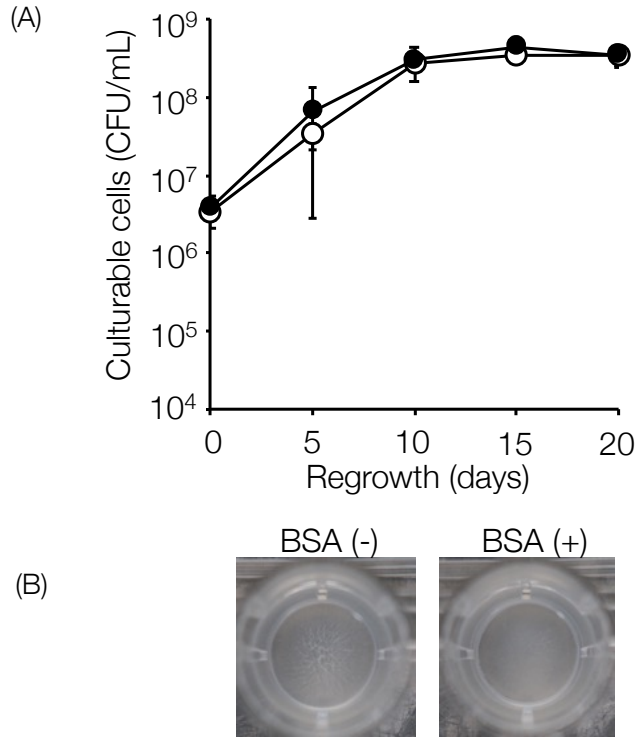
756 Figure 6. Schematic representation of the mechanism of BSA-induced resuscitation of Mtb.
757 Although the detailed mechanism of interaction between BSA and PknA is not yet clear, BSA
758 may— in coordination with PknB—activate PknA and positively regulate target proteins that
759 are involved in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



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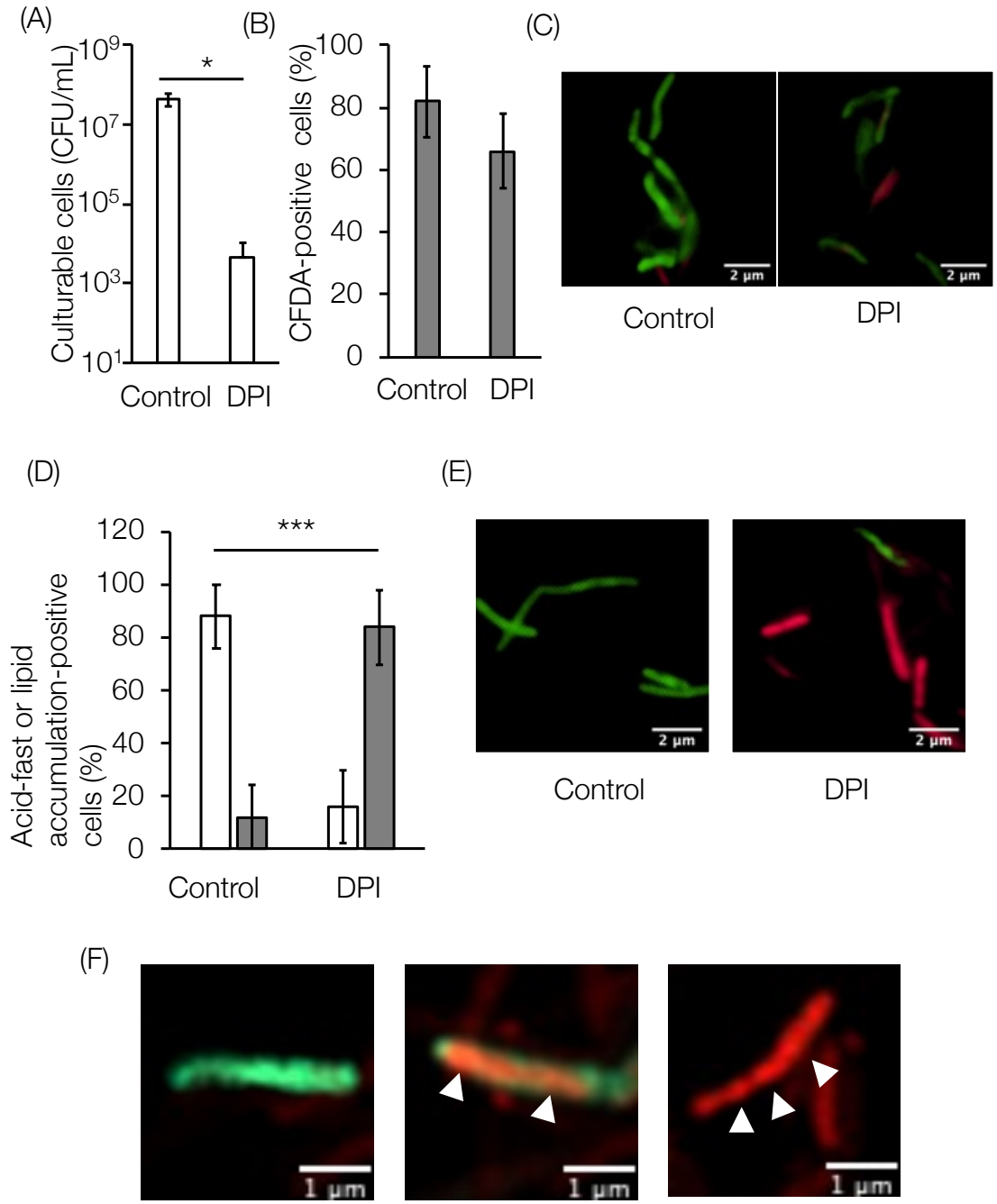
773 Figure 2.



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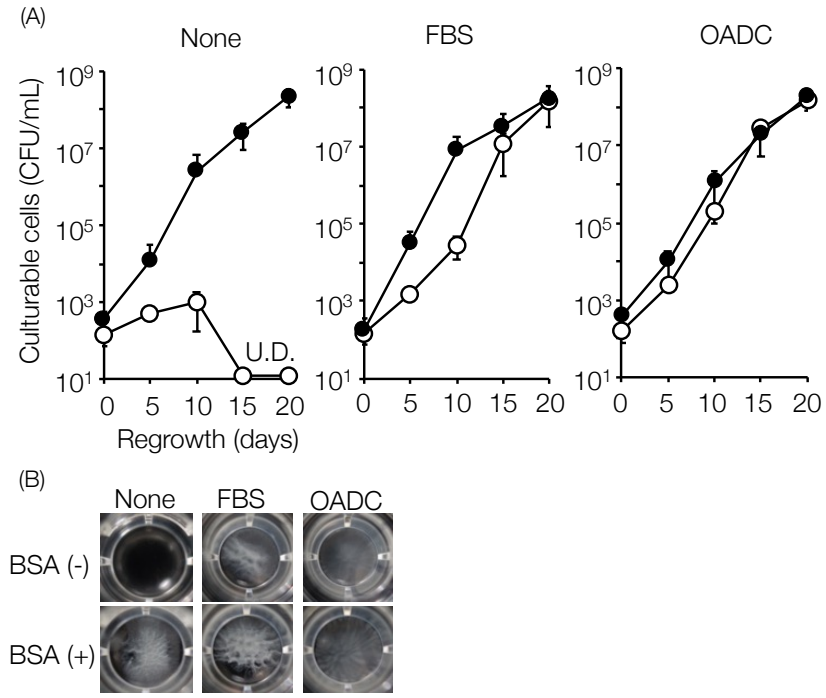

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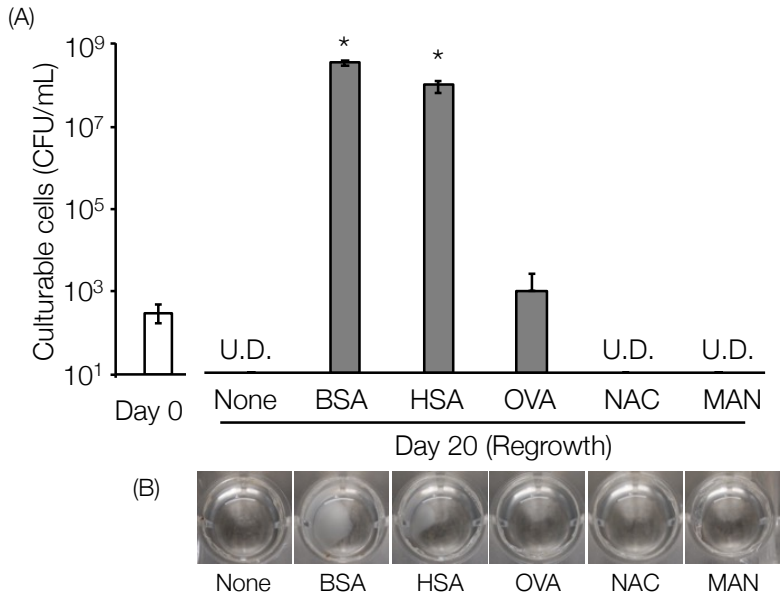

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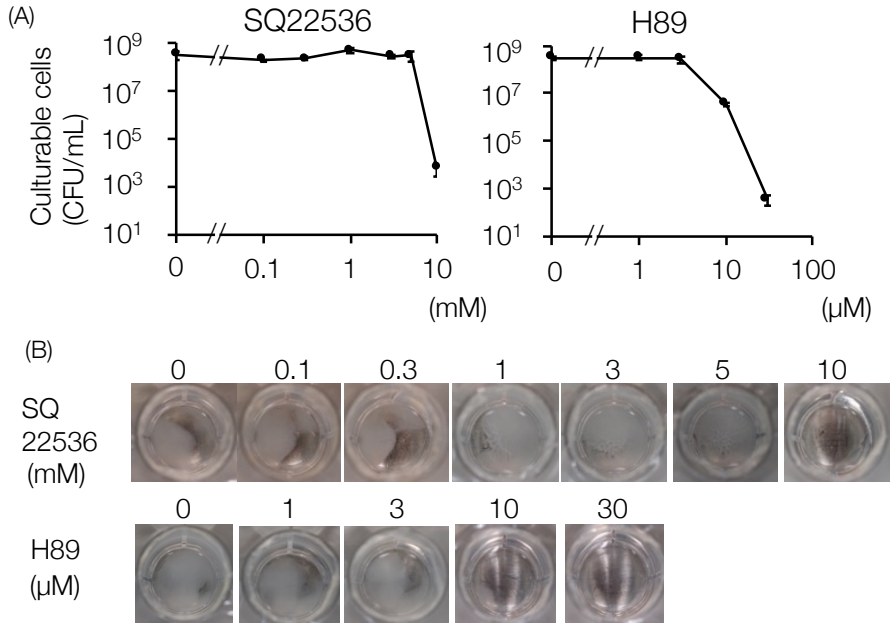

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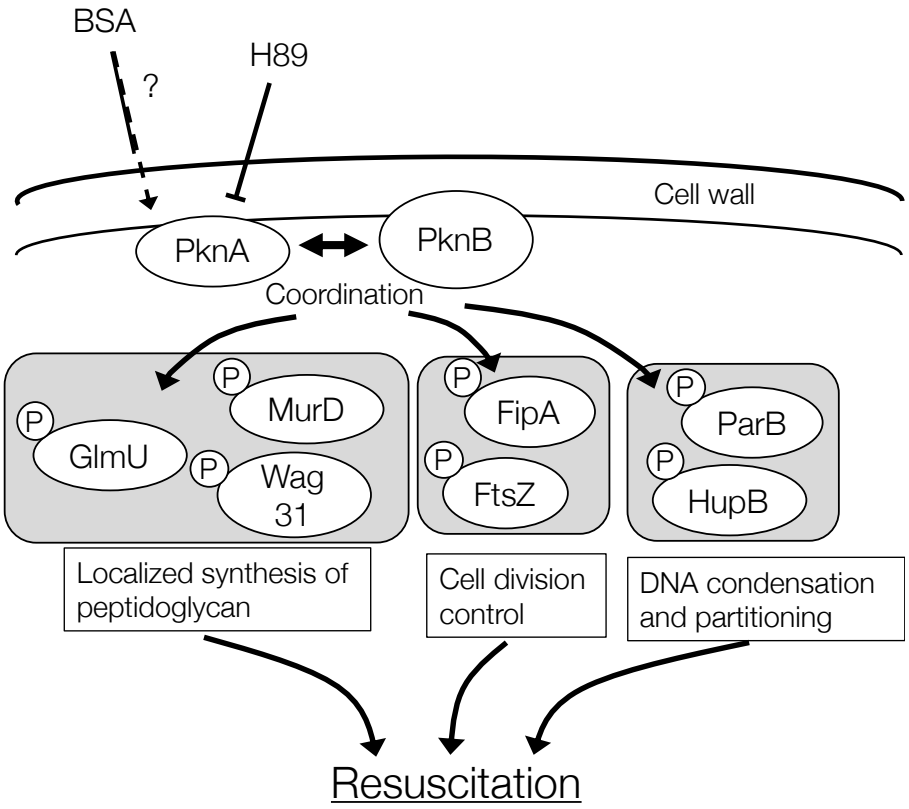
786 Figure 6.



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