Visualization of SpoVAEa protein dynamics in dormant spores of *Bacillus cereus* and dynamic changes in their germinosomes and SpoVAEa during germination

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Abstract

Bacillus cereus spores, like most *Bacillus* spores, can survive for years depending on their specific structure, and germinate when their surroundings become suitable. Spore germination proteins play an important role in the initiation of germination. Because germinated spores lose the extreme resistance of the dormant state, more information related to the function of germination proteins could be useful to develop new strategies to control *B. cereus* spores. Prior work has shown that: i) the channel protein SpoVAEa exhibits high frequency movement in the outer leaflet of the inner membrane (IM) in dormant spores of *B. subtilis*; ii) the dynamics of germinosome formation in developing spores of *B. cereus* indicate that the formation of germinosome foci is slower than foci formation of germinant receptor GerR and scaffold protein GerD. However, the dynamics of movement of SpoVAEa in *B. cereus* spores, and the complete behavior of the germinosome in germinated spores of *B. cereus* are still unclear. In this study, we found that the SpoVAEa fluorescent foci in dormant spores of *B. cereus* redistribute at a lower frequency than in *B. subtilis*, and likely colocalize with GerD in dormant spores. Our results further indicate that: i) overexpression of GerR(A-C-B)-SGFP2 and SpoVAEa-SGFP2 with GerD-mScarlet-I from a plasmid leads to more heterogeneity and lower efficiency of spore germination in *B. cereus*; ii), germinosome foci composed of GerR(A-C-B)-SGFP2 and GerD-mScarlet-I were lost prior to the phase transition in germination; and iii) GerD-mScarlet-I foci spread out but continued to exist beyond the phase transition of *B. cereus* spores.

Keywords: *Bacillus cereus*, spores, SpoVAEa, GerR, GerD, germination

Introduction

32

33   *Bacillus cereus* is a Gram-positive, rod-shaped, spore forming bacterium found in soil. The vegetative
34   cells of *B. cereus* can form endospores under harsh environmental conditions, and spores are
35   capable of surviving for years due to spore-specific features [1]. These specific properties also lead to
36   major challenges to food safety once *B. cereus* contaminates foods, for example dairy products, rice,
37   and chilled foods [2,3]. The major specific structural features of spores include the spore core
38   containing chromosomal DNA, the inner membrane (IM) where germinant receptors (GRs) are located
39   along with the germinosome scaffold protein GerD and channels for CaDPA, the germ cell wall with a
40   thin peptidoglycan layer, the cortex with a thick peptidoglycan layer, the outer membrane, the
41   proteinaceous coat, and finally the exosporium in some *Bacillus* species, including *B. cereus* [4].

42   Dormant spores can initiate germination and outgrow into vegetative cells when GRs sense nutrients
43   in the environment, such as amino acids, inosine, and sugars. Additionally, previous work indicates
44   that GerD acts as a scaffold protein in localizing GRs in the *B. subtilis* spore IM [5,6], and the *B.*
45   *cereus* GerR GR has also been shown to interact with GerD in the dormant spore IM [7]. Another
46   important group of spore germination proteins are the SpoVA proteins encoded by the *spoVA* operon
47   which constitute a CaDPA channel in the IM of bacterial spores. The SpoVA proteins in spores of *B.*
48   *subtilis* are SpoVAA, SpoVAB, SpoVAC, SpoVAD, SpoVAEb, SpoVAEa, and SpoVAF and are made
49   only in developing spores [8]. The SpoVA proteins in spores of *B. cereus* are SpoVAA (BC_4070),
50   SpoVAB (BC_4069), SpoVAC (BC_4068), SpoVAD (BC_4067), SpoVAEb (BC_4066), SpoVAEa
51   (BC_4065) and SpoVAF (BC_4064) encoded in one operon, and SpoVAC (BC_5147), SpoVAD
52   (BC_5148), and SpoVAE (BC_5149) in another operon. Previous work suggests that SpoVAEa of *B.*
53   *subtilis* spores, a soluble protein on the outer surface of the spore IM, plays a role in the
54   communication with the germinant binding GRs. It is thus possible that SpoVAEa of *B. subtilis* spores
55   could stimulate the opening of the SpoVA channel, thereby allowing CaDPA release [9,10]. Recent
56   work in our lab has shown that *B. subtilis* SpoVAEa fused to GFP and expressed on the chromosome
57   in dormant spores exhibits random high frequency movement in the spore IM [11]. However, there is
58   only a limited understanding of the location and physical state of SpoVAEa in dormant spores of the
59   food pathogen *B. cereus*, nor whether SpoVAEa and GerD proteins colocalize at least transiently.

60   The spore germination process in Bacilli and Clostridia has been reviewed in the past years [12–14].
61   Initially germinants bind to GRs, followed by large-scale release of monovalent cations and then
62   CaDPA release mediated by the SpoVA proteins [15,16]. The kinetics and heterogeneity of spore
63   germination triggered by L-alanine have been analyzed giving the frequency distribution at both the
64   population level and in individual spores of *B. cereus* strain T using phase-contrast and fluorescence
65   microscopy [17–19]. Previous work suggests that GerR(A-C-B) is localized in the spore IM using
66   fluorescent reporter protein fusions and the membrane dye FM 4-64, and is primarily responsible for
67   L-alanine germination of *B. cereus* spores [7,20,21]. Recent work also showed that in the IM
68   germinosome, GerR(A-C-B) and GerD could be visualized using fluorescent reporter proteins and this
69   work suggested that the formation of germinosome foci could be significantly slower than the

70 formation of GerR(A-C-B)-SGFP2 and GerD-mScarlet-I foci, with significant heterogeneity in
71 formation of germinosomes foci [7]. However, little information is known about the overall changes or
72 behavior of germination proteins during the germination process of *B. cereus* spores.

73 The strongly enhanced green fluorescent protein (SGFP2) and mScarlet-I have been successfully
74 used in the visualization of spore germination proteins GerR(A-C-B) and GerD in spores of *B. cereus*
75 ATCC 14579 using low-copy number plasmid expression vectors [7,20]. In this work, we aimed to
76 visualize the movement of SpoVAEa with SGFP2 in dormant spores of *B. cereus* ATCC 14579 using
77 fluorescence microscopy, and to analyze the fluorescence distribution by the changes, either up or
78 down, in the full width at half maximum (FWHM) of the fluorescence. Additionally, the phase contrast
79 intensity and fluorescence changes of germinosome foci formed by GerR(A-C-B)-SGFP2 and GerD-
80 mScarlet-I, and SpoVAEa-SGFP2 and GerD-mScarlet-I were tracked by a time-lapse microscope
81 equipped with phase-contrast and fluorescence analysis options. In the present study, we found that
82 SpoVAEa-SGFP2 foci exhibited random movements in the IM, and likely colocalized with GerD-
83 mScarlet-I in dormant spores of *B. cereus*. The results also suggested that expression of GerR(A-C-B)
84 and SpoVAEa proteins with GerD affected germination efficiency and led to slower and more
85 heterogeneous spore germination in *B. cereus*. Upon addition of germinant, germinosome FRET foci
86 were lost and GerR(A-C-B)-SGFP2 as well as GerD-mScarlet-I germinosome foci decreased
87 significantly in intensity. Yet some GerR(A-C-B)-SGFP2 foci and GerD-mScarlet-I foci continued to
88 exist and apparently colocalize also after phase transition, suggesting that germinosome complexes
89 may persist beyond the germination event. Finally, loss of SpoVAEa-SGFP2 foci occurred upon
90 phase transition, while spread of GerD-mScarlet-I foci continued beyond phase-transition.

91 **Results**

92 **Movement of SpoVAEa foci in dormant spore of *B. cereus***

93 Previous studies showed that full width at half maximum (FWHM) can be used to quantitate
94 fluorescence distribution of spore proteins [11]. In this study, we used dormant spores of *B. cereus*
95 strain 014 expressing the fusion protein SpoVAEa-SGFP2 from a plasmid to observe the movement
96 of SpoVAEa-SGFP2 foci. The percent changes of FWHM in 100 frames of an individual *B. cereus*
97 spore during 5 sec was presented as up (positive percentage) or down (negative percentage)
98 compared to the first frame. It was noted that there were different random movements in *B. cereus*
99 spores 1, 2, 3 and 4 (Figure 1.A). The percent changes of FWHM in *B. subtilis* exhibited a wider
100 boundary and higher frequency changes, either up or down, compared to *B. cereus* spore 2 (Figure
101 1.B). This result suggested that the SpoVAEa-SGFP2 foci in spores of *B. cereus* and *B. subtilis* both
102 moved, but behaved somewhat differently. In particular, SpoVAEa fluorescent foci of *B. subtilis*
103 spores redistribute at a higher frequency than those of *B. cereus.* This difference may be caused by
104 the fact that we look at different species with a different protein complement and with genomic
105 expression in *B. subtilis* versus expression from a plasmid in *B. cereus.*
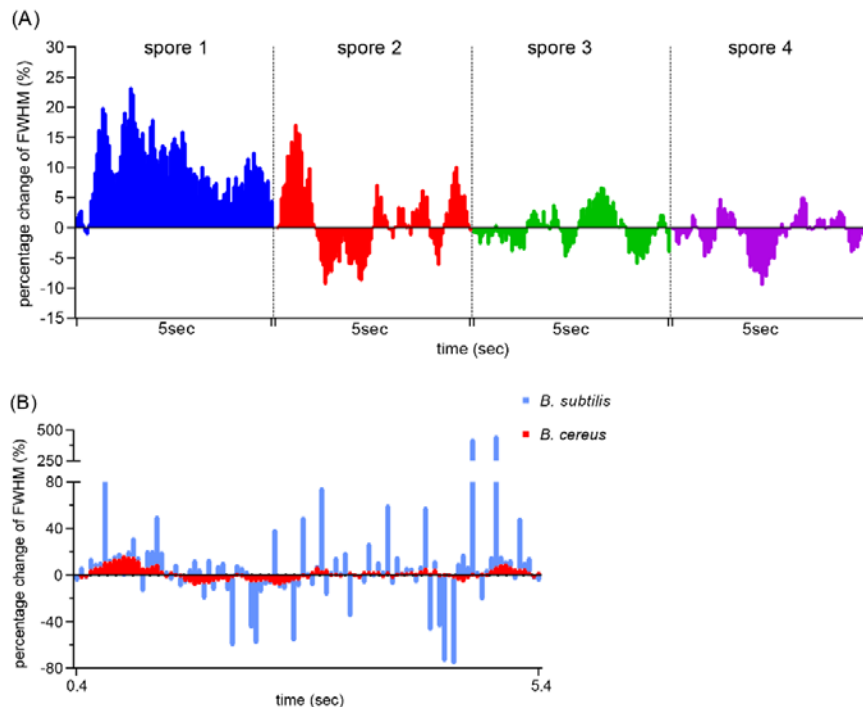
Figure 1. Comparison of the movement of SpoVAEa-SGFP2 foci in dormant spores of *B. cereus* and *B. subtilis*. Panel A, the percent changes of FWHM in individual spores 1, 2, 3 and 4 of *B. cereus*. Panel B, the percent changes of FWHM in a *B. cereus* spore 2 (red square) and a *B. subtilis* spore (blue square). The positive and negative percentages of columns indicate the up and down of the tendency of fluorescence FWHM distribution.

SpoVAEa-SGFP2 levels are enhanced by GerD expression in recombinant *B. cereus* spores

Our recent work showed that GerR(A-C-B) and GerD foci are present and colocalized in dormant spores of *B. cereus* [20]. The fusion protein SpoVAEa-SGFP2 was expressed alone in spores of *B. cereus* strain 014, and expressed together with GerD-mScarlet-I in spores of strain 015 in this work. The fluorescence intensity of SGFP2 in strains 014 and 015 are as expected both higher than those of wild type *B. cereus* cells without recombinant protein. However, it is notable that the fluorescence in *B. cereus* strain 015 looks brighter than in strain 014 (Figure 2.A). When the fluorescence level in the spores of recombinant strains 014 and 015 were compared, 53% of the 014 spores and 43% of the 015 spores gave a 2~3 fold higher fluorescence levels compared to the wild type. Furthermore, levels of recombinant spores with 3-fold higher fluorescence level than wild type spores were higher in strain 015 spores than 014 spores (Figure 2.B). These results indicate that SpoVAEa-SGFP2 can be successfully expressed from a plasmid in two recombinant *B. cereus* strains 014 and 015 and leads to 1 to 6 fold higher fluorescence levels than the wild-type in the SGFP2 channel. Moreover, when SpoVAEa-SGFP2 and GerD-mScarlet-I are expressed from the same plasmid, GerD possibly contributes to the stability of SpoVAEa, or possibly enhances the expression of SpoVAEa.

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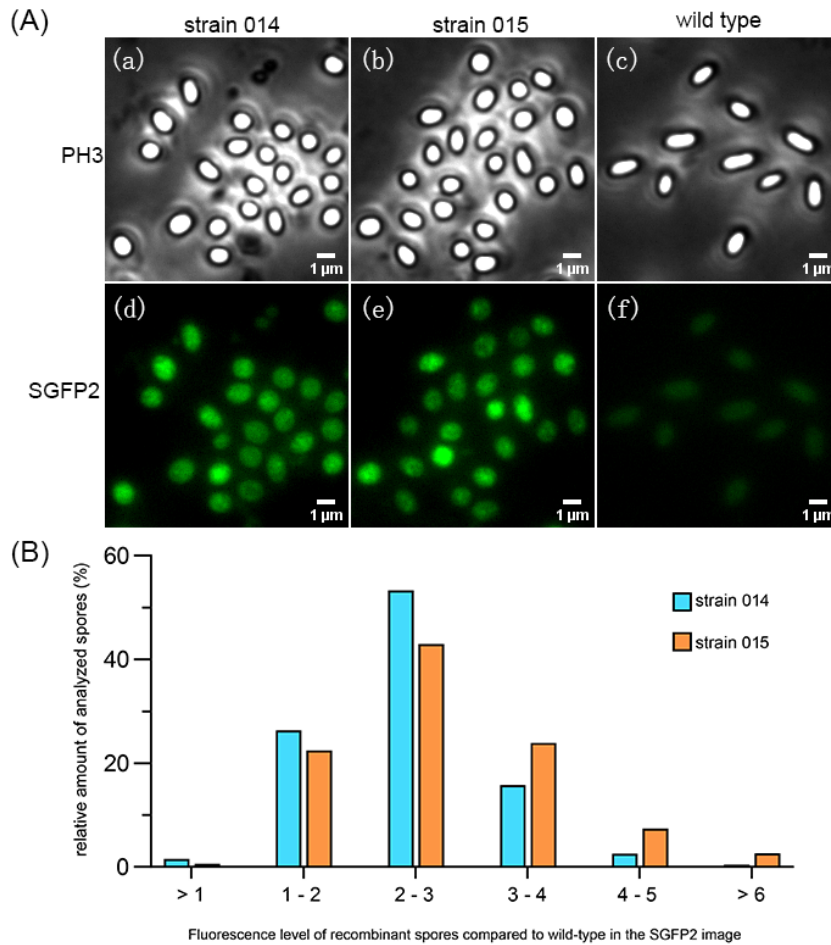
Figure 2. Visualization and comparison of SpoVAEa-SGFP2 fusion protein fluorescence in dormant spores of *B. cereus* strains 014 and 015. Panel A, dormant spores of *B. cereus* strains 014, 015 and wild type were visualized: (a), (b), and (c) in the PH3 channel, or (d), (e), and (f) in the SGFP2 fluorescence channel. PH3, phase contrast. Panel B, fluorescence level in the SGFP2 channel in spores of strains 014 and 015 compared to wild type. The number of analyzed spores of *B. cereus* strain 014, 015 and wild type were 1564, 2901, and 916, respectively.

Colocalization analysis between SpoVAEa and GerD proteins

The next aim was to study the possible interaction of SpoVAEa-SGFP2 and GerD-mScarlet-I in dormant spores of *B. cereus*. The spectrum of SGFP2 and mScarlet-I have an overlapping region, that may produce a larger Pearson's coefficient, which is a commonly used colocalization indicator, [7,22,23]. To reduce any effects of GerD-mScarlet-I itself, spores of *B. cereus* strain 007 expressing GerD-mScarlet-I was used as a control. The analysis (Fig. 3) showed that the Pearson's coefficient of channels SGFP2 and mScarlet-I in spores of *B. cereus* strain 015 was significantly higher than those in the control. This result indicated that there is likely colocalization between the SpoVAEa-SGFP2 and GerD-mScarlet-I proteins in *B. cereus* spores.
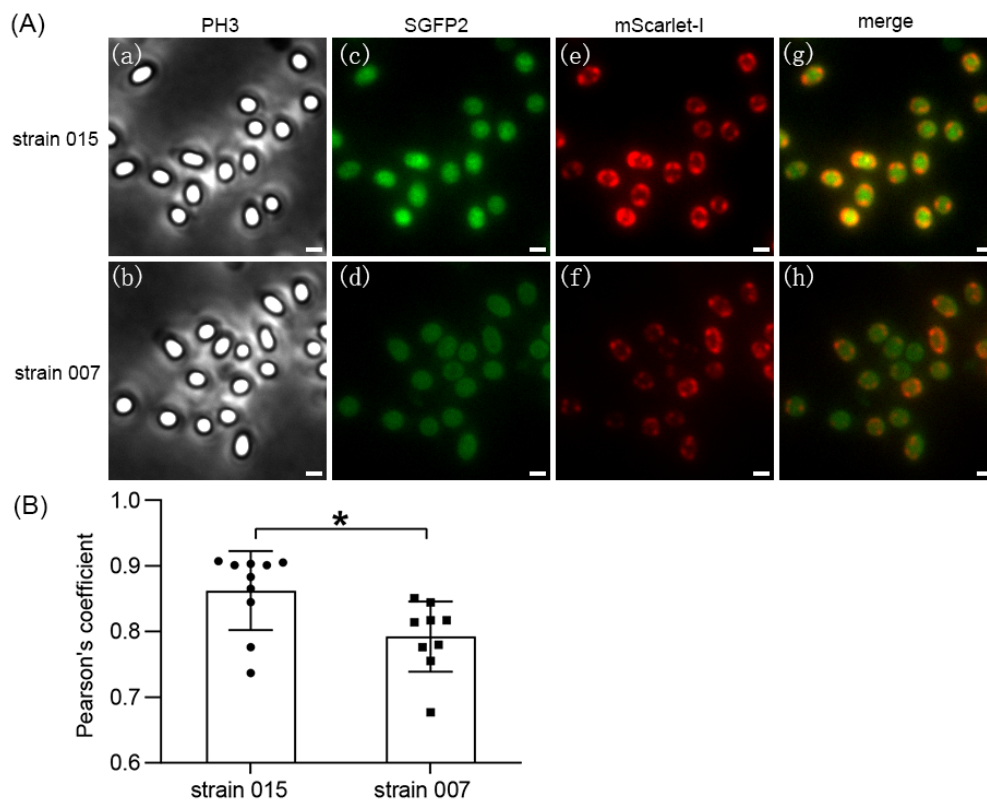
Figure 3. Co-localization analysis between SpoVAEa and GerD proteins in dormant spores of *B. cereus* strain 015. Panel A, visualization of *B. cereus* strain 015 and 007 spores: (a) and (b) Phase-contrast channel (PH3); (c) and (d), SGFP2 channel; (e) and (f), mScarlet-I channel; (g) and (h), merged image of SGFP2 and mScarlet-I channels. The scale bar is 1 μm. Panel B, Pearson's coefficient between SGFP2 and mScarlet-I channels. Data are shown as a mean with SD. *, $P < 0.05$.

Overexpression of GerR and SpoVAEa with GerD affects *B. cereus* spore germination

Our recent work suggested that there is a significant kinetic heterogeneity in the formation of a germinosome in developing spores of *B. cereus* [7]. In this study, the initiation time of germination (named germX) is presented as the difference in spores of strain 006 expressing GerR(A-C-B)-SGFP2 and strain 014 expressing SpoVAEa-SGFP2 compared to wild type. When GerR(A-C-B)-SGFP2 and SpoVAEa-SGFP2 with GerD-mScarlet-I expressed from plasmid in *B. cereus* strains F06 and 015 respectively, germX exhibited greater heterogeneity than spores of *B. cereus* strains 006 and 014 (Figure 4). In particular, spores of *B. cereus* strain 007 expressing GerD-mScarlet-I alone from plasmid exhibited a very different germX of individual spores (Figure 4). Our result indicated that overexpression of GerD-mScarlet-I from plasmid in *B. cereus* led to more heterogeneity of spore germination.

Previous work has shown that expression of the GerA GR controlled by the strong forespore-specific *sspB* promoter in the *B. subtilis* genome can significantly increase the rate of germination triggered by L-alanine [24]. Our results showed that spores of all different *B. cereus* strains started to germinate by

162   1, 10 and >20 min, namely groups germX_1, germX_10, and germX_>20. Most spores were in group

163   germX_1, with 81.2%, 82.9%, 48.5%, 97.7%, 74.7%, and 99.8% of spores of *B. cereus* strains F06,

164   006, 015, 014, 007 and wild type, respectively (Figure 5). Overexpression of GerR(A-C-B) with or

165   without GerD in strains F06 and 006 led to significantly lower germination efficiency compared to wild

166   type spores, with effects in F06 spores slightly greater than in 006 spores. The results also showed

167   that overexpression of SpoVAEa alone in spores of strain 014 had no effect on germination efficiency

168   compared to wild type. However, expression of SpoVAEa with GerD in spores of *B. cereus* strain 015

169   significantly ($P < 0.0001$) slowed germination compared to wild type spores (Figure 5). Importantly,

170   whether GerD was expressed alone from a plasmid in *B. cereus* strain 007 or along with GerR in

171   strain F06 or SpoVAEa in strain 015, the overexpression of GerD led to a significantly lower

172   germination efficiency compared to wild type spores (Figure 5). Overall, our results indicate that

173   overexpression of the GerR GR and SpoVAEa with GerD from plasmids affects the germination

174   efficiency of *B. cereus* spores.
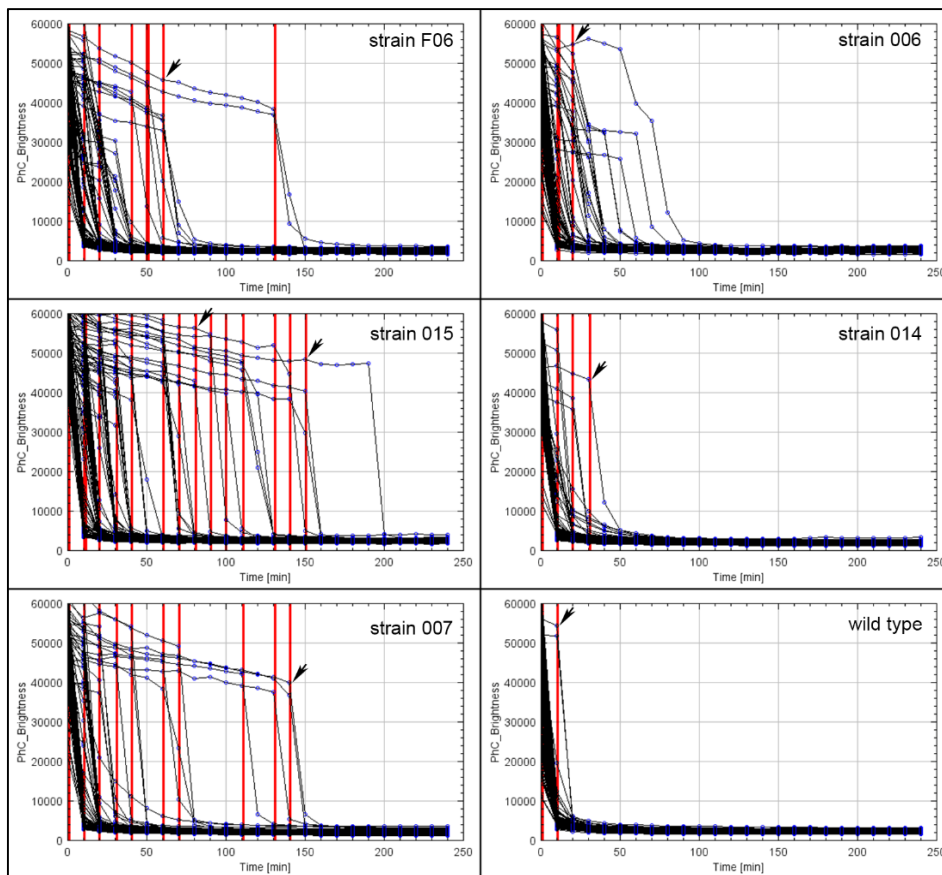
175

176   **Figure 4.** The phase plots show the germinated spores of *B. cereus* strains F06, 006, 015, 014, 007 and wild

177   type. The red lines and black arrows indicate the initiation time of spore germination, termed germX. Each black

178   line indicates the change of phase-contrast intensity in an individual spore during germination. The numbers of

179   analyzed spores of *B. cereus* strains F06, 006, 015, 014, 007 and wild type were 108, 92, 122, 233, 107 and 265,

180   respectively.
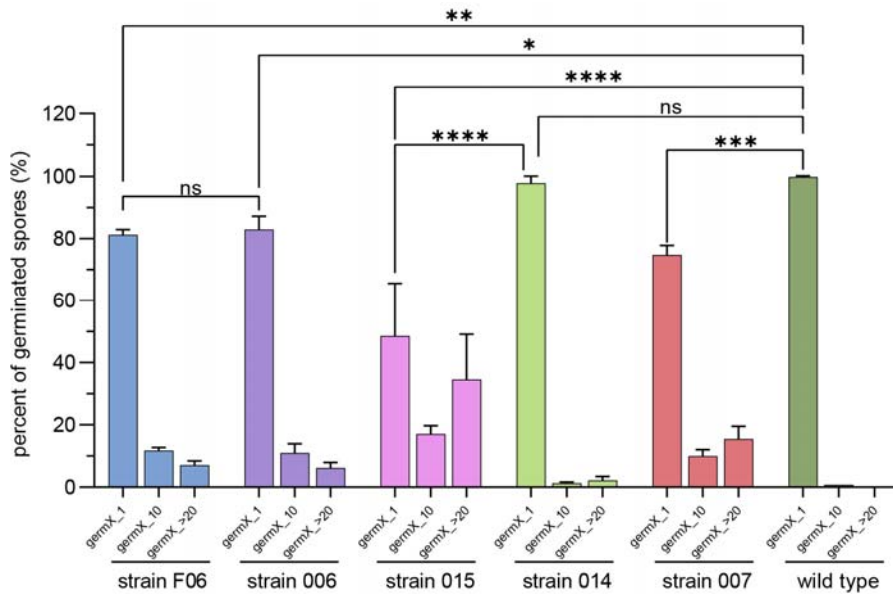
Figure 5. Germination of spores of *B. cereus* strains F06, 006, 015, 014, 007 and wild type were analyzed based on the different germX times: germX_1, initiation time of germination at ≤1 min; germX_10, initiation time of germination at <10 min; germX_>20, initiation time of germination more than 20 min. Data are shown as the mean with SD and are averages of three independent experiments. The numbers of analyzed germinated spores of different *B. cereus* strains are listed in Table S2. ns, not significant; *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$; ****, $P < 0.0001$.

Dynamics of germinosome behavior upon germination triggered by L-alanine in *B. cereus* spores

Our recent study suggested that the formation of FRET foci of GerR(A-C-B)-SGFP2 and GerD-mScarlet-I could be significantly slower than the foci in SGFP2 and mScarlet-I channels in *B. cereus* spores [7]. In this work, we aimed to track the dynamic changes of germinosome FRET foci upon germination triggered by L-alanine in spores of *B. cereus* strain F06 expressing GerR(A-C-B)-SGFP2 and GerD-mScarlet-I fusion proteins from a plasmid. The phase-contrast channel (PH3) recorded the transition between a phase-bright individual spore to a phase-dark spore at 1 min and 10 min of the germination time in groups germX_1 and germX_10, respectively (Figure 6.A, Table S3). Upon the phase transition in the germX_1 group, the intensity of germinosome FRET foci at 10 min was significantly lower than at the beginning (0 min), as well as that of GerR(A-C-B)-SGFP2 and GerD-mScarlet-I foci (Figure 6.B, Table S3). In addition, the intensity of the FRET foci of germX_10 spores at 30 min was significantly lower compared to 20 min, that is, the intensity drop occurred always after phase transition (Figure 6.B, Table S3). In addition, any remaining GerD-mScarlet-I foci became diffuse in germinated spores of groups germX_1 and germX_10 (Figure 6.B). These results indicate that the germinosome FRET foci in spores of *B. cereus* were largely lost shortly after the phase transition. Some GerR(A-C-B)-SGFP2 foci and GerD-mScarlet-I foci continued to exist and apparently colocalize also after phase transition, suggesting that germinosome complexes may persist beyond the germination event.
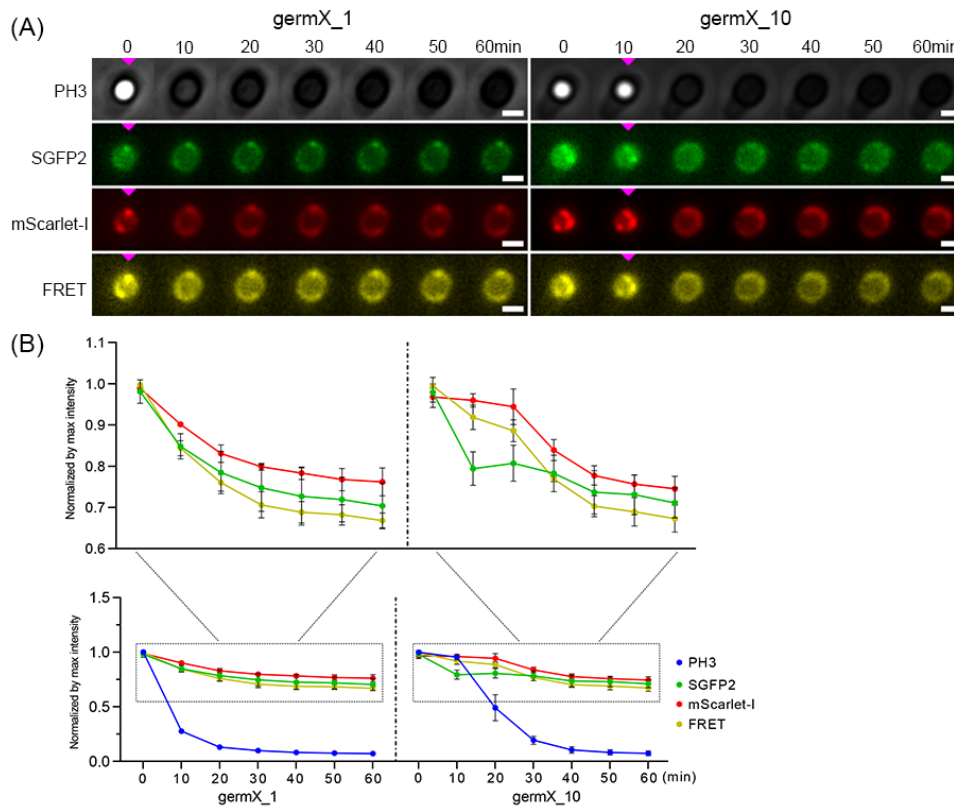
Figure 6. Dynamic changes in germinosome foci upon germination of *B. cereus* strain F06 spores. Panel A, visualization of the changes oinGeR-SGFP2, GerD-mScarlet-I and germinosome foci at 10 min intervals over 60 min. The left column is PH3, SGFP2, mScarlet-I and FRET channels of group germX_1. The right column is PH3, SGFP2, mScarlet-I and FRET channels of the germX_10 group. PH3, phase-contrast. The pink triangles indicate the initiation of germination. The scale bar is 1 μm. Panel B, the line charts of PH3, SGFP2, mScarlet-I and FRET channels. Left column, germX_1 group; right column, germX_10 group. Data are shown as the mean with SD and are averages of three independent experiments. The number of analyzed germinated spores of *B. cereus* strains F06 is listed in Table S2. The statistical analysis of the later time points in line charts in comparation to the previous one are listed in Table S3.

Dynamics of SpoVAEa and GerD proteins during germination of *B. cereus* spores triggered by L-alanine

In this work, recombinant spores of *B. cereus* strain 015 expressing SpoVAEa-SGFP2 and GerD-mScarlet-I fusion proteins from a plasmid were used to visualize the dynamic changes of SpoVAEa and GerD upon germination initiated by L-alanine. The results showed that the phase-contrast intensity of germinated spores at 10 min in group germX_1 was very greatly decreased compared to that of phase-bright spores at 0 min. In the group germX_10, the phase transition occurred and the phase-contrast intensity of phase-dark germinated spores at 20 min was greatly decreased compared to that at 10 min (Figure 7, Table S4). The fluorescence intensity in the SGFP2 or mScarlet-I channel of germinated spores at 10 min in group germX_1 was decreased compared to that of phase-bright spores at 0 min, but this reduction was not significant ($P > 0.05$). The results showed that the

228   SpoVAEa-SGFP2 foci were lost, and overall spore green fluorescence intensity dropped upon

229   initiation of germination. The same is true for GerD-mScarlet-I, although, in accordance with our

230   results described in Figure 6, some foci continued to exist beyond the phase transition albeit at less

231   fluorescent intensity.

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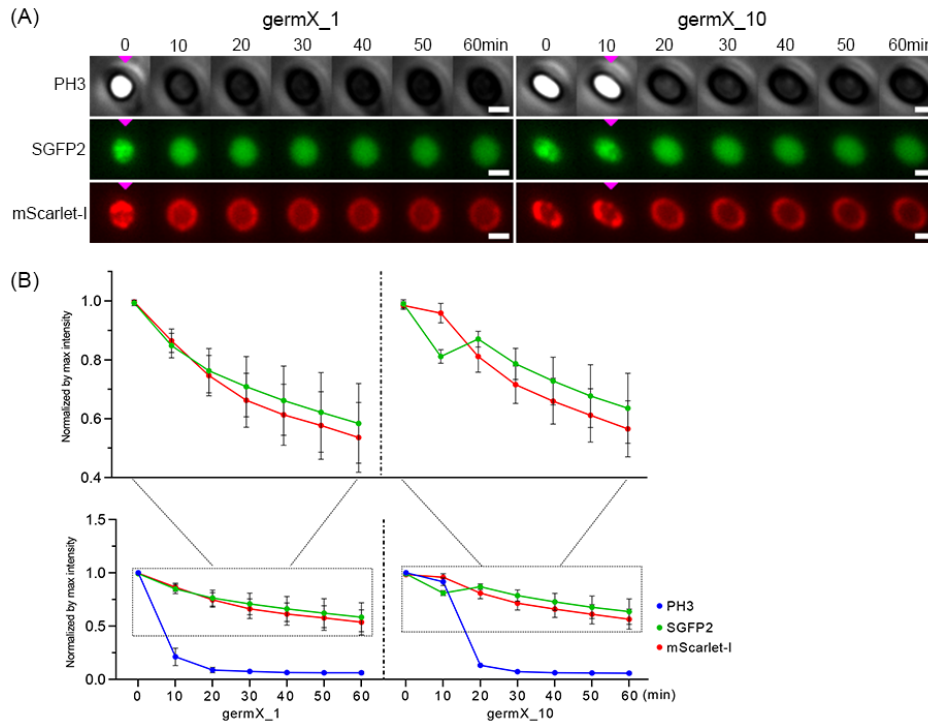
Figure 7. Dynamic changes in SpoVAEa-SGFP2 and GerD-mScarlet-I during germination of *B. cereus* strain 015 spores. Panel A, visualization of changes in SpoVAEa-SGFP2 and GerD-mScarlet-I at 10 min intervals over 60 min. The left column is PH3, SGFP2 and mScarlet-I channels of group germX_1. The right column is PH3, SGFP2 and mScarlet-I channels of the germX_10 group. PH3, phase-contrast. The pink triangles indicate the initiation of germination. The scale bar is 1 μm. Panel B, the line charts of PH3, SGFP2 and mScarlet-I channels. Left column, germX_1 group; right column, germX_10 group. Data are shown as the mean with SD and represent three independent experiments. The numbers of analyzed germinated spore of *B. cereus* strains 015 are listed in Table S2. The statistical analysis of the latter time points in line charts in comparation to the previous one is listed in Table S4.

Discussion

244   *B. cereus* spores, like most *Bacillus* spores, have various resistance characteristics due to spore

245   specific structures, and can restart metabolism only after spore germination has been completed. The

246   nutrient germination of spores is initiated by germinant binding to specific GRs localized in spore's IM,

247   including GerR, GerK, GerG, GerL, GerQ, GerI, and GerS in spores of *B. cereus*, with GerR triggering

248   germination with L-alanine [21,25]. In addition, SpoVAEa is an IM component of the SpoVA protein

249   CaDPA channel and GerD is a scaffold protein playing an important role in germinosome formation

250   and thus spore germination in *B. subtilis* and *B. cereus* [6,7,9]. To extend these latter observations,

251   we have now studied the dynamic changes of SpoVAEa protein in dormant and germinated spores of

252   *B. cereus*, and the kinetic changes in germinosome foci during the germination process.

253   Based on previous observations, the expression level of GerD and SpoVA proteins in *B. subtilis*

254   spores are ~10^2-fold and 10^3-fold higher than those of GRs [26]. Fluorescence microscopy of *B.*

255   *cereus* spores showed clear foci of GerD-mScarlet-I and indistinct foci of SpoVAEa-SGFP2, indicating

256   that a possible reason could be the different level of the two proteins. However, the weaker

257   fluorescent signal of SpoVAEa-SGFP2 than that of GerD-mScarlet-I in our work might be caused by

258   expression of only one subunit of the SpoVA complex, or perhaps SpoVAEa has a lower expression

259   level than other SpoVA proteins [9].

260   In this study, recombinant *B. cereus* spores overexpressing GerR(A-C-B) and SpoVAEa with and

261   without GerD from a plasmid were used to assess the effects of these proteins on germination

262   triggered by L-alanine. Spores expressing GerR(A-C-B) with GerD in group germX_1 exhibited two

263   significant reductions in germination, 0 min versus 10 min and 10 min versus 20 min in the phase-

264   contrast channel, but there was only one significant reduction 0 min versus 10 min with spores

265   expressing GerR(A-C-B) alone (Figure S1, Table S3, Table S5); note similar results found in group

266   germX_10. However, spores overexpressing SpoVAEa with and without GerD both presented only

267   one significant reduction in the phase-contrast channel in groups germX_1 and germX_10 (Figure S1,

268   Table S4, Table S5). These results indicated that overexpression of GerD has an inhibitory effect on

269   spore germination heterogeneity and efficiency, and note that the heterogeneity of spore germination

270   is consistent with previous work [17,19]. Considering initiation of spore nutrient germination, a recent

271   study suggested that in *B. subtilis*, GerAB is responsible for sensing L-alanine, indicating that the

272   function of GerA's B subunit is to detect nutrient germinants [27]. Our recent studies also suggest that

273   there is interaction of GerRB with GerD and with a GR B subunit possibly bound by L-alanine [7,28].

274   Finally, the possible reason for this inhibitory effect can be that GerD might occupy the limited binding

275   site or position on the surface of GerRB when spore germination is triggered by L-alanine.

276   Our recent results using FRET-based analysis indicate that there is interaction between GerR(A-C-B)-

277   SGFP2 and GerD-mScarlet-I, moreover, the dynamics of germinosome formation suggest that the

278   formation of foci in the FRET channel could be significantly slower than formation of the GerR(A-C-B)-

279   SGFP2 and GerD-mScarlet-I foci. To further assess this interaction, we observed the changes of

280   germinosome foci upon germination initiated by L-alanine in *B. cereus* spores. In this experiment, the

281   protein FRET pairs, GerR(A-C-B)-SGFP2 and GerD-mScarlet-I, were expressed from a plasmid and

282   driven by their native promoters during sporulation. Possibly consistent with the role of the B subunit

283   of GerA in *B. subtilis*, GerRB may also be responsible for initiating germination with L-alanine in *B.*

284   *cereus* (Figure 6). Once the process of spore germination was initiated, our results showed that some

285   GerD SpoVAEa colocalization likely occurs and germinosome FRET positive foci were lost upon

286   phase transition while some germinosome complexes may continue to exist beyond this time point.

287   Figure 8 shows a hypothetical sequence of events that may occur during spore germination. A note of

288   caution is warranted because the germination proteins were overexpressed from a plasmid and

289 evidently may disrupt the dynamic balance in germination protein assembly in sporulation and
290 germination. Importantly though, all germination proteins studied were expressed from the plasmid
291 under the control of their respective native promoters allowing relative expression differences to be
292 conserved.
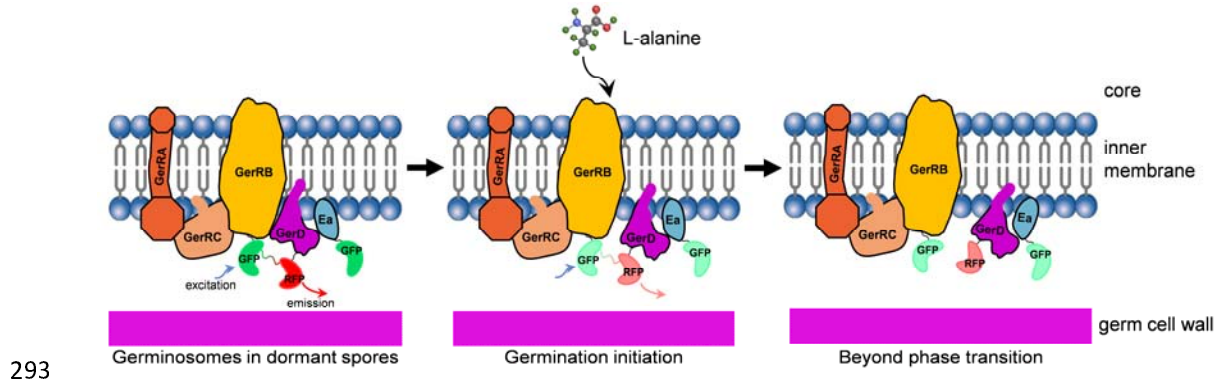
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294 **Figure 8**. The proposed model of germinosome dynamics during germination triggered by L-alanine in *B. cereus*
295 spores. The left most panel: i) FRET positive germinosome formation due to FRET interaction between GerR(A-
296 C-B)-SGFP2 and GerD-mScarlet-I; the darker green-red line between GFP (deep green) and RFP (deep red)
297 indicates the energy transfer path in the FRET event between GerR(A-C-B) and GerD; ii) likely colocalization
298 between SpoVAEa and GerD proteins, albeit transient; GFP - SGFP2; RFP - mScarlet-I; Ea - SpoVAEa. The
299 middle panel: i) FRET positive germinosomes are largely lost upon the phase transition in germination initiation
300 caused by L-alanine; the FRET signal (light green-red line) between GFP (light green) and RFP (light red) has
301 become weak, indicating that FRET events between GerR(A-C-B) and GerD have been gradually lost, consistent
302 with GerD and GerRB-GFP moving apart; ii) the SpoVAEa-SGFP2 and GerD-mScarlet-I fluorescence intensities
303 have decreased upon initiation of germination. The right most panel: i) there are no longer any FRET positive
304 germinosomes, but some GerR(A-C-B)-SGFP2 foci (light green) and GerD-mScarlet-I foci (light red) continue to
305 exist, indicating colocalization of GerR(A-C-B) and GerD after the phase transition; ii) some GerD foci also
306 continue to exist and likely colocalize, perhaps transiently with SpoVAEa.

307 In summary, the SpoVAEa-SGFP2 protein exhibits random movement on the outer surface of spores'
308 IM and a likely transient co-localization with GerD-mScarlet-I in dormant spores of *B. cereus*. Studying
309 spore germination in phase-contrast microscopy suggested that overexpression of GerR(A-C-B)-
310 SGFP2 and SpoVAEa-SGFP2 with GerD-mScarlet-I from a plasmid leads to more heterogeneity and
311 lower efficiency of spore germination in *B. cereus* pointing to the need for future studies to investigate
312 the stoichiometry of the germinosome components in *B. cereus* in more detail. The dynamics of
313 germination showed that germinosome foci composed of GerR(A-C-B)-SGFP2 and GerD-mScarlet-I
314 were lost soon after the phase transition. Further work related to the machinery of spore germination,
315 should likely focus on detailed interaction studies between the SpoVA channel and GerD, or the
316 SpoVA channel and other GRs using site-directed mutagenesis guided by molecular dynamics protein
317 modelling.

318 **Materials and Methods**

319 **Recombinant plasmids and *B. cereus* strains**

320 The recombinant plasmids and *B. cereus* strains used in this study are listed in Table 1. All primers
321 used are listed in Table S1. The recombinant plasmids were constructed as described in our previous
322 studies [7,20]. Briefly, the region of 226 bp located in the upstream region of the *spoVA* operon was
323 considered as the promoter region of gene *spoVAEa* and named PEa. The PEa fragment was
324 inserted into pHT315 between *Kpn* I and *Xba* I sites resulting in plasmid pHT315-PEa. Next, the
325 *spoVAEa* (BC_4065) gene was amplified from genomic DNA of *B. cereus* ATCC 14579 (GenBank:
326 AE016877) using a pair of primers, 315_YW-42 and 315_YW-43. The *SGFP2* gene with stop codons
327 was fused to the 3' end of the *spoVAEa* gene using a two-fusion PCR. The fusion product was
328 inserted into pHT315-PEa between *Xba* I and *Hin*d III sites. The resulted ligation product was
329 transformed into competent *E. coli* cells and selection of positive clones giving plasmid pHT315-f14.
330 The fusion fragment PD-*gerD-mScarlet-I* was amplified from plasmid pHT315-f05 and inserted into
331 pHT315-f14 between *Kpn* I and *Eco*R I sites, giving plasmid pHT315-f15. The correct construction of
332 recombinant plasmids pHT315-f14 and pHT315-f15 was confirmed by sequencing, followed by
333 electroporation into competent *B. cereus* ATCC 14579 cells, and selection and confirmation with
334 colony PCR of an erythromycin-positive single colony.

335 Table 1. *B. cereus* strains and plasmids used in this study

Strains	Plasmid Present (+)	Description of Inserted Genes	Sources or references
B. cereus ATCC 14579	wild type	No	Lab stock
strain 014	+pHT315-f14 Ery^r	PEa-*spoVAEa-SGFP2*	This study
strain 015	+pHT315-f15 Ery^r	PEa-*spoVAEa-SGFP2* and PD-*gerD-mScarlet-I*	This study
strain 006	+pHT315-f01 Ery^r	PR-*gerR(A-C-B)-SGFP2*	[7]
strain 007	+pHT315-f05 Ery^r	PD-*gerD-mScarlet-I*	[7]
strain 010	+pHT315-f10 Ery^r	PD-*gerD-SGFP2*	[7]
strain F06	+pHT315-f06 Ery^r	PR-*gerR(A-C-B)-SGFP2* and PD-*gerD-mScarlet-I*	[7]

336 Abbreviations: PEa, promoter of *spoVA* operon; PR, promoter of *gerR* operon; PD, promoter of *gerD*; Ery^r, resistant to
337 erythromycin.

338 **High frequency time-lapse image acquisition and analysis**

339 Dormant spores of *B. cereus* strain 014 were prepared and purified as described in previous work [20].
340 A Nikon Eclipse Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with a sCmos camera
341 (Hamamatsu Flash 4.0 V2, Hamamatsu City, Japan) and wide-field fluorescence components was
342 used to capture 100 frames of 14-bit SGFP2 images (excitation at 488 nm and emission at 535 nm)
343 with an exposure time of 50 ms for each frame and no delay interval. An individual spore located in

344   100 frames was selected, duplicated, and analyzed by the plugin Adrian's FWHM in ImageJ. The

345   percent change of FWHM in the second frame to the hundredth frame relative to the FWHM in the

346   first frame was calculated, and the graph was made by the software of GraphPad Prism version 9.3.

347   **Images of SpoVAEa-SGFP2 expressed in spores of *B. cereus* strains 014 and 015;**

348   **acquisition and analysis**

349   The preparation of *B. cereus* dormant spores and their visualization were carried out as described in

350   our previous work [20]. Spores of *B. cereus* strain 014 expressing SpoVAEa-SGFP2 and spores of *B.*

351   *cereus* strain 015 expressing SpoVAEa-SGFP2 and GerD-mScarlet-I were captured in the phase-

352   contrast and SGFP2 (excitation at 470 nm and emission at 516 nm) channels using a Nikon Eclipse

353   Ti-E microscope. Images were analyzed by the ObjectJ SporeAnalyzer_1c.ojj in Fiji/ImageJ

354   (https://sils.fnwi.uva.nl/bcb/objectj/examples/SporeAnalyzer/MD/SporeAnalyzer.html).

355   **Co-localization assays and data analysis**

356   Dormant spores of *B. cereus* strains 015 and 007 were prepared and purified as described in our

357   previous work [20]. Spores of *B. cereus* strains 015 and 007 were captured in three channels: phase-

358   contrast, SGFP2 (excitation at 470 nm and emission at 516 nm) and mScarlet-I (excitation at 555 nm

359   and emission at 593 nm) using a Nikon Eclipse Ti-E microscope. All acquired images in the co-

360   localization assay were processed with ImageJ. The SGFP2 and mScarlet-I images were used to

361   calculate the co-localization indicator Pearson's coefficient by the plugin JACoP in ImageJ [22].

362   **Germination assays by time-lapse imaging and data processing**

363   Dormant spores of *B. cereus* strains F06, 006, 015, 014, 007 and wild-type spores were prepared and

364   purified as described previously [20]. Microscope slides were prepared as described previously [29].

365   Briefly, a 65 μl size Gene frame with 0.25 mm thickness (Thermofisher Scientific, The Netherlands,

366   Cat. No.: AB0577) was attached on the center of a normal microscope slide. A liquid mixture for an

367   agarose pad was made with a 1:1 mixture of 2x germination buffer (see below) and 2% agarose in a

368   heat block at 55°C. 60 μl of the liquid mixture was pipetted on the area of frame, immediately pressed

369   with another slide and placed at 4°C for at least 20 min to solidify.

370   Dormant spores suspended in ice-cold PBS (pH 7.4) were heat activated for 15 min at 70°C and

371   washed three times with ice-cold PBS (pH 7.4) by centrifugation at 14,300 ×*g* for 15 min at 4°C. The

372   heat-treated spores were suspended in ice-cold germination buffer (50 mM Tris-HCl (pH 7.4), 10 mM

373   NaCl and 100 mM L-alanine) at an OD600 of 15. The spore suspension (1.3 μl) was dropped onto the

374   solid agarose pad, immediately covered by a cover slide (18×18 mm) and was now ready for time-

375   lapse microscopy.

376   A Nikon Eclipse Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with an sCmos camera

377   (Hamamatsu Flash 4.0 V2, Hamamatsu City, Japan), phase-contrast, and wide-field fluorescence

378 components was used to track gemination of *B. cereus* spores for 4 hours with 10 min intervals.
379 Spores of *B. cereus* strain F06 expressing GerR(A-C-B)-SGFP2 and GerD-mScarlet-I were captured
380 by four images, phase-contrast, SGFP2 fluorescence (excitation at 470 nm and emission at 516 nm),
381 mScarlet-I (excitation at 555 nm and emission at 593 nm) and FRET (excitation at 470 nm and
382 emission at 593 nm). Spores of *B. cereus* strain 006 expressing GerR(A-C-B)-SGFP2 and spores of *B.*
383 *cereus* strain 014 expressing SpoVAEa-SGFP2 were captured by phase-contrast and SGFP2 images.
384 Spores of *B. cereus* 015 expressing SpoVAEa-SGFP2 and GerD-mScarlet-I were captured by phase-
385 contrast, SGFP2 and mScarlet-I images. Spores of *B. cereus* 007 expressing GerD-mScarlet-I were
386 captured by phase-contrast and mScarlet-I images.

387 All 16-bit type images taken in germination assays were converted to 32-bit type. Selection and
388 measurement of the area of background in samples without an image were carried out, and
389 background was subtracted by the tool of Process—Math—Subtract in Fiji/ImageJ. The germinated
390 spores were analyzed and various intensities of individual spores measured using the ObjectJ
391 SporeTrackerC_1h.ojj in Fiji/ImageJ
392 (https://sils.fnwi.uva.nl/bcb/objectj/examples/sporetrackerc/MD/SporeTrackerC.html).

Acknowledgments

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