



HAL
open science

Early expression of capsule during *Bacillus anthracis* germination

Solène Fastenackels, Michèle Mock, Jean-Nicolas Tournier, Pierre L Goossens

► **To cite this version:**

Solène Fastenackels, Michèle Mock, Jean-Nicolas Tournier, Pierre L Goossens. Early expression of capsule during *Bacillus anthracis* germination. *Research in Microbiology*, 2023, 174 (6), pp.104054. 10.1016/j.resmic.2023.104054 . hal-04077748

HAL Id: hal-04077748

<https://hal.science/hal-04077748v1>

Submitted on 21 Apr 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Journal Pre-proof



Early expression of capsule during *Bacillus anthracis* germination

Solène Fastenackels, Michèle Mock, Jean-Nicolas Tournier, Pierre L. Goossens

PII: S0923-2508(23)00029-3

DOI: <https://doi.org/10.1016/j.resmic.2023.104054>

Reference: RESMIC 104054

To appear in: *Research in Microbiology*

Received Date: 28 October 2022

Revised Date: 10 March 2023

Accepted Date: 15 March 2023

Please cite this article as: S. Fastenackels, M. Mock, J.-N. Tournier, P.L. Goossens, Early expression of capsule during *Bacillus anthracis* germination, *Research in Microbiology*, <https://doi.org/10.1016/j.resmic.2023.104054>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2023 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.

1 **Early expression of capsule during *Bacillus anthracis* germination**

2

3

4 Solène Fastenackels^a, Michèle Mock^b, Jean-Nicolas Tournier^c, Pierre L. Goossens^{d*}

5

6

7 **Affiliations :**

8 ^a Laboratory “Immune Microenvironment and Immunotherapy”, INSERM U1135, Centre
9 d’Immunologie et des Maladies Infectieuses Paris (CIMI-Paris), Paris, France10 ^b Institut Pasteur, c/o Yersinia Unit, 26 rue du Docteur Roux, 75724 Paris cedex 1511 ^c IRBA, Div.DNRBC/D.2MI, 1, place Général Valérie André, 91223 Brétigny/Orge12 ^d Institut Pasteur, Yersinia Unit, 26 rue du Docteur Roux, 75724 Paris cedex 15

13

14

15

16

17

18

19

20

21

22

23

24 ^a solene.fastenackels@sorbonne-universite.fr25 ^b michele.mock@pasteur.fr26 ^c jntournier@gmail.com27 ^{d*} pierre.goossens@pasteur.fr Correspondance and reprints

28

29 **Abstract**

30 *Bacillus anthracis* is a spore-forming bacterium that produces two major virulence
31 factors, a tripartite toxin with two enzymatic toxic activities and a pseudo-proteic capsule.
32 One of the main described functions of the poly-gamma-D-glutamate capsule is to enable *B.*
33 *anthracis* bacilli to escape phagocytosis. Thus, kinetics of expression of the capsule filaments
34 at the surface of the emerging bacillus during germination is an important step for the
35 protection of the nascent bacilli. In this study, through immunofluorescence and electron
36 microscopic approaches, we show the emergence of the capsule through a significant surface
37 of the exosporium in the vast majority of the germinating spores, with co-detection of BclA
38 and capsular material. This suggests that, due to an early capsule expression, the extracellular
39 life of *B. anthracis* might occur earlier than previously thought, once germination is triggered.
40 This raises the prospect that an anti-capsular vaccine may play a protective role at the initial
41 stage of infection by opsonisation of the nascent encapsulated bacilli before their emergence
42 from the exosporium.

43

44

45 **Keywords**46 *Bacillus anthracis*; spore; capsule; germination

47 **1. Introduction**

48 The virulence of *Bacillus anthracis*, the etiological agent of anthrax, relies
49 predominantly on two main factors, a tripartite toxin with two enzymatic activities (lethal and
50 edema factors, metalloprotease and adenylycyclase activities respectively) and a pseudoproteic
51 capsule made of poly-gamma-D-Glutamic acid (PDGA)[1] The capsule protects the bacteria
52 from phagocytosis, is poorly immunogenic [2] and potentially disruptive for the immune
53 response [3, 4]; it enables the bacteria to bind to the liver endothelium [5] and plays a role in
54 sequestration inside pulmonary capillaries at the terminal phase of the infection [6].

55 *B. anthracis* is a spore-forming bacterium. The spore is a specialised cell form that
56 remains dormant and survives for many years, formed of concentric shells surrounding the
57 core that contains the bacterial genetic material: a membrane: a peptidoglycan layer - the
58 cortex: a protein multilayer –the coat: and the exosporium –the outermost structure of the
59 spore [7-9]. The exosporium is composed of a basal layer and an external hair-like nap
60 formed by a single collagen-like glycoprotein called BclA [10, 11]). Upon germination there
61 is an influx of water into the spore, swelling of the spore core, dismantling of the cortex and
62 coat leading to resumption of metabolic activity and cell growth [7-9].

63 Early expression of the toxin genes has been shown [12], as soon as 15 min after
64 germination triggering for mRNA detection. This suggests a potential early role for them in
65 subverting the host defense. Temporal expression of the capsule is also critical to enable
66 protection of the nascent bacilli. How the capsule emerges from a germinating spore that is
67 surrounded by the exosporium and the coat layers is thus of importance.

68 In this study, we addressed the kinetics of capsule expression during *B. anthracis*
69 germination. We observed early detection of capsular material as soon as 15 min after
70 germination triggering. Capsular material was detected in the majority of spores as emerging
71 all around the external layers of the spore.

72 2. Materials and methods

73

74 2.1 Bacterial growth

75 The *B. anthracis* strains used were the encapsulated non-toxinogenic RPG1 strain
76 derived from the RPLC2 strain after transduction of the pXO2 of the ATCC 4229 strain [13],
77 the parental non-encapsulated RPLC2 strain [14] and the Δ -*bclA* RPG1-derived strain
78 (constructed as described previously [10]); the RPLC2 strain is derived from the Sterne strain
79 with one point mutation in the *lef* gene and two point mutations in the *cya* gene leading to
80 enzymatically inactive toxins. To synchronise spore germination, spores (1×10^7 mL⁻¹)
81 were shortly incubated in BHI for 3 min at 37° [13]. This incubation step triggered
82 germination in more than 90% of the spores, allowing the analysis of a homogeneous
83 germinating spore population, as germination was less efficiently triggered in the minimum
84 culture R-medium. After centrifugation (13,000 rpm, 2 min), the germinating spores were
85 resuspended in R-medium with 0.6% sodium bicarbonate [15] and incubated at 37 °C in a 5%
86 CO₂ atmosphere; this growth condition mimics the *in vivo* environment and ensures
87 coordinated *in vitro* expression of the capsule and the toxins. At various timepoints, bacteria
88 were collected, washed in Dulbecco's Phosphate Buffered Saline with MgCl₂ and CaCl₂
89 (Sigma, ref D8662) and then analysed by immunofluorescence or ultrastructural studies (see
90 below).

91 For the *in vivo* approach, 1×10^8 germinated spores obtained after the BHI incubation step
92 were injected intraperitoneally in female outbred Swiss OF1 mice (weight, 22–24 g; Charles
93 River, L'Arbresle, France). One hour later, the animals were euthanased, a peritoneal lavage
94 was performed with 10 mL cold Dulbecco's Phosphate Buffered Saline without MgCl₂ and
95 CaCl₂ (Sigma, ref D8537); the suspension of bacterial and peritoneal cells were then
96 centrifuged in PBS with MgCl₂ and CaCl₂ (1200rpm, 5 min) to pellet the majority of
97 peritoneal cells, the supernatant was then centrifuged (4000rpm, 8mn) to pellet the bacterial
98 suspension. After resuspension in PBS with MgCl₂ and CaCl₂, the bacteria were processed for
99 immuno-staining. Animals were housed in the Institut Pasteur animal facilities, licensed by
100 the French Ministry of Agriculture and in compliance with European regulations. All animal
101 experiments were conducted according to European Union guidelines
102 https://ec.europa.eu/environment/chemicals/lab_animals/3r/key_resources/portals_en.htm
103 and were approved by the animal care and use committee at the Institut Pasteur.

104

105 2.2 Immunofluorescence and ultrastructural studies

106 The germinated spores were fixed in 4% paraformaldehyde for 15 min, deposited on
107 poly-L-lysine-coated (PLL) glass coverslips as described previously [16]. In brief, coverslips
108 were sequentially incubated for 1 hour at room temperature with appropriate dilutions of the
109 primary antibodies and of the species-specific secondary fluorochrome conjugates in PBS
110 0.25% gelatin. Each incubation was followed by three washes of 10 minutes with PBS. In
111 some experiments, the host cell nuclei were stained with 100 nM Topro-3 in the Vectashield
112 mounting medium.

113 The exosporium protein BclA was detected with the mouse monoclonal antibody
114 4B7G12 developed in the laboratory, revealed by an Alexa488-labelled donkey anti-mouse
115 secondary polyclonal antiserum. For the exosporium of the Δ -*bclA* RPG1 strain, devoid of the
116 BclA protein, primary labelling was performed with a murine polyclonal anti-exosporium
117 antiserum raised against Δ -*bclA* spores developed in the laboratory.

118 The PDGA capsular material was detected with a polyclonal rabbit immune serum
119 revealed by an Alexa594-labelled goat anti-rabbit polyclonal antiserum as previously
120 described [17]. No cross reaction between the various antibodies was observed (data not
121 shown). Confocal microscopy was performed on a Leica SP5 confocal microscope using a
122 63x, oil immersion objective. Image deconvolution was performed as already described [18].

123 Quantification of the spore populations exhibiting distinct patterns of BclA and
124 PGDA-labelling was performed at various times of the germination kinetics and the
125 vegetative cells were excluded (Table 1).

126 Transmission electron microscopy was performed on dendritic cells (DC) at 1h of
127 infection with RPG1 and RPLC2 spores as previously described [19]. In brief, DC were
128 infected with the spores and analysed after 30 min. Cells were fixed for 3 h in 3%
129 glutaraldehyde in 0.1 M phosphate buffer and postfixated for 1 h with 1% OsO₄ in 0.1 M
130 cacodylate buffer. The pellet was embedded in epoxy resin and sectioned for transmission
131 electron microscopy.

132 Scanning electron microscopy was performed on 45 min-germinated spores bound to
133 PLL-coated coverslips; after overnight fixation in 2.5 % glutaraldehyde in 0.1 M sodium
134 cacodylate buffer at 4 °C, and incubation with 1/100 diluted in BHI (brain heart infusion)
135 anti-PDGA specific rabbit polyclonal antibodies and anti-BclA mouse monoclonal antibodies
136 as described above and then incubated with a 15 nm immuno-gold conjugate (British Biocell
137 International) coupled with an anti-rabbit goat antibody, and of 10 nm immuno-gold
138 conjugate (British Biocell International) coupled with an anti-mouse goat antibody. Finally, a
139 negative staining was performed with phosphotungstic acid 2 % in water.

140 Of note, all image acquisitions were carried out on PLL-coated supports. This
141 originated from the observation that carbon-coated grids preferentially bound spores that do
142 not present capsular material at the surface in contrast to PLL-coated grids. Indeed, only 5 %
143 of the spore population was positive for anti-capsular material labelling on carbon-coated
144 grids versus 61 % for the PLL-coated grids, as assessed through immunofluorescence
145 detection as described above. Such approach is thus critical to avoid misrepresentation of a
146 particular subpopulation.

Journal Pre-proof

147 3. Results

148 The presence of PDGA capsular material and the BclA protein - an abundant protein
149 of the spore surface - was detected through immuno-fluorescence labelling with specific
150 antibodies (Fig. 1&2); quantitative analysis of both fluorescence signal topographic
151 distribution was performed on the samples (Table 1 built on kinetics as shown in Fig. 1&2).

152 At 30 and 45 min (Fig. 1), many germinating spores of the encapsulated RPG1 strain
153 were labelled with both anti-capsular material and anti-BclA antibodies; co-detection of
154 capsular material and BclA was observed around the whole, or greater part of, spore surface,
155 in 67 % of the bacterial population at 30 min (forms 5+6 in Table 1). This proportion for the
156 forms 5+6 was still around 57 % at 60 min (Table 1); at this timepoint, 29 % of the bacterial
157 cells (form 8 in Table 1) were labelled as encapsulated bacteria that were devoid of anti-BclA
158 labelling. This population (form 8 in Table 1) increased, as expected, to reach 76 % of the
159 labelled bacteria at 120 min, with a few remnants of anti-BclA labelled exosporium; the
160 remaining bacterial cells were anti-BclA labelled spores that did not undergo the germination
161 step (Fig. 1 and form 1 in Table 1). Interestingly, a particular topographic distribution of
162 fluorescence was observed in 8 % of the germinating spores at 30 min; capsular material was
163 observed as dots on some locations of the BclA layer (form 2 in Table 1).

164 Triple labelling (Topro-3 for DNA, and anti-BclA and anti-capsular material labelling)
165 was performed at 45 min, at a time point when the majority of the germinating spores were
166 labelled for BclA and capsule (Fig. 1, time point 45 min); all germinating spores harboured
167 DNA and thus were not empty exosporium shells. Using mouse anti-exosporium polyclonal
168 antibodies, similar co-detection of capsular material and the exosporium layer at 45 min was
169 also observed with spores from a strain deleted of the *bclA* gene (Fig 1).

170 Raising the question of the timepoint of initial detection of the anti-capsular material
171 labelling, we observed that capsular material could be detected as early as 15 min of
172 incubation in R medium 0.6% sodium bicarbonate in a 5% CO₂ atmosphere (Fig 2).
173 Quantitatively, this fluorescence signal was detected in 60% of the germinating spores (forms
174 4+5+6 in Table1). No capsular material labelling was detected immediately after the 3min-
175 germination step in BHI, while all spores were labelled with the anti-BclA antibody (Table 1
176 and Fig. 2).

177 To confirm that this phenomenon also occurs *in vivo*, the germinated spores were
178 inoculated in mice into the peritoneum cavity and bacterial growth allowed to develop *in vivo*;
179 the bacterial cells were recovered after 1h of growth and immunolabelled as above (Fig. 1,

180 uttermost right panel). Similar co-detection of capsular material and BclA around the bacterial
181 surface was observed.

182 Analysis of the fluorescent images at 30 and 45 min suggested that the capsular
183 material was detected outside the BclA layer (Fig. 1). To analyse this particular aspect in
184 more detail, two complementary approaches were developed, confocal microscopy and image
185 deconvolution (Fig. 3a top and bottom panels respectively). Both approaches strongly
186 suggested the capsular material was localised outside the BclA layer. Image deconvolution
187 also suggested that the capsular material was distributed as dots around the surface (Fig. 3a
188 bottom panel).

189 To confirm this observation, an ultrastructural approach was followed, through
190 electron microscopy (Fig 3b and c). Transmission electron microscopy without labelling
191 showed deposition of electron dense material as dots around the most external surface of the
192 encapsulated germinating RPG1 spores inside the hair-like BclA layer (Fig 3b, left), while
193 this was not observed with the parental non-encapsulated RPLC2 strain (Fig 3b, right).

194 We then used a specific qualitative analysis through scanning electron microscopy
195 with labelling by immunogold co-detection of antibodies directed against capsular material
196 (15 nm beads) and BclA (10 nm beads). We were able to observe spores that presented small
197 packs of labelled capsular material that emerge in various locations of the labelled
198 exosporium (Fig. 3c).

199 4. Discussion

200 In this study, we addressed the kinetics of capsule expression and topographic
201 distribution during germination of *B. anthracis* spores, using immunofluorescence and
202 electron microscopy approaches. We show co-detection of both antigens peaking at 30-45
203 min after germination triggering.

204 Topographically, the capsular material was detected in the majority of the germinating
205 spores around the whole surface of the exosporium. This strongly suggests that the external
206 spore layers were fissured and the capsular material synthesised by the nascent bacilli
207 emerged through these fissures and breaks. Indeed, dots of capsular material could be detected
208 around the BclA layer in 8% of the germinating spores at 30 min (form 2 in Table 1). This
209 phenomenon was also observed for *in vivo* infection.

210 Our data thus strongly suggest that *B. anthracis* behaves similarly to *B. atrophaeus*
211 and *B. subtilis*, where fissures and breaks have been observed in the coat during germination.
212 [20, 21]. Interestingly, John Ezzell reported a similar finding many years ago [22]; this
213 observation was exploited as a means for *B. anthracis* spore identification through this
214 phenomenon of ‘spore encapsulation’. Of note, we could also observe bacterial cells where
215 the nascent bacillus emerged from the spore coat and exosporium layers only at one end,
216 leaving behind an empty shell. This was in accordance with previously published data
217 reporting that, during spore germination and outgrowth, the outgrowing cell escapes from its
218 exosporium shell by popping through a region of the exosporium that could not be stained by
219 antibodies to the spore specific alanine racemase [23].

220 Toxins and capsule are expressed in a coordinated manner, at the genetic and
221 molecular levels [24, 25]. Our study shows that capsular material could be detected as soon as
222 15 min. PA mRNA has been reported as early as 15 min after germination, thus highlighting
223 the precocity of toxin production [26]. Similarly, lethal and edema factors were detected in
224 the plasma of mice as early as one hour after intranasal inoculation [27]. Two consequences
225 of this early capsular material expression and distribution on the surface of the germinating
226 spores may be envisioned.

227 (i) The lifestyle of *B. anthracis*, an extracellular pathogen, at this early stage is
228 considered to follow two different initial scenarios at the portal of entry into the infected host
229 - an extracellular or an intracellular step - the physiological importance of each one depending
230 on the route of entry. On one hand, during a cutaneous infection, *B. anthracis* spores
231 germinate very efficiently in the extracellular tissues [28]. Furthermore, as reported in
232 inhalational and gastrointestinal infections, as soon as a lesion is present, efficient spore

233 germination occurs at the lesion site [29]. On the other hand, an intracellular pathway has
234 been described, playing a role in the crossing of the respiratory or intestinal epithelial barriers
235 during inhalational and gastrointestinal infections [30-32]; spores are engulfed and, through
236 trafficking, emerge from the cells on the other side of the epithelium. Our study thus suggests
237 that, in both scenarios, early expression of capsular material around the germinating spore
238 would help protect the emerging nascent bacilli at a crucial timepoint when *B. anthracis* is
239 susceptible to the host innate defenses, as it is converting from the resistant dormant spore to
240 the virulent toxinogenic encapsulated bacillus. Combined with early expression of toxins [26,
241 27] this would help subvert the host defenses.

242 (ii) a second outcome of such early capsular material expression on the germinating spores is
243 that they may become a target for a pre-existing anti-capsular vaccine immune response.
244 Current veterinary vaccines rely on live attenuated spores of the toxinogenic non-encapsulated
245 Sterne strain, while human vaccines are mainly based on the common toxin component
246 protective antigen (PA) [33]. However these PA-based vaccines for human use are protective
247 only in some specific animal models [34]. To enhance their efficacy, other components have
248 been added experimentally, especially for protection against inhalational anthrax. Addition of
249 inactivated spores as a source of spore antigens notably increases the efficiency of an anthrax
250 vaccine in the mouse model [14, 35]. Addition of spore or bacilli components, or more
251 specifically of capsular material, have been considered and tested [17, 36, 37]; in particular,
252 the group of Arthur Friedlander has further demonstrated the role of anti-capsule immunity in
253 vaccine development [38, 39]. Our data suggest that the approaches of adjoining capsular
254 material to the current anthrax human vaccines would thus be beneficial earlier than
255 considered; opsonisation of the nascent encapsulated bacilli, even before emergence from the
256 exosporium, would play a protective role at the initial stage of infection.

257 **Acknowledgments**

258

259 We would like to express our thanks to Tam Mignot for the deconvolution analysis, Evelyne
260 Tosi-Couture (Pasteur Institute) and Guy Brochier (IRBA) for their expertise in ultrastructural
261 analysis, and Jean-Philippe Corre for his contributions and discussions.

262

263 **Conflict of interest**

264 All authors have no conflict of interest

Journal Pre-proof

265 **References**

266

267 [1] Mock M, Fouet A. Anthrax. Annual review of microbiology 2001;55:647-71.

268 [2] Fouet A. The surface of *Bacillus anthracis*. Molecular aspects of medicine 2009;30:374-
269 85.270 [3] Jelacic TM, Ribot WJ, Chua J, Boyer AE, Woolfitt AR, Barr JR, et al. Human Innate
271 Immune Cells Respond Differentially to Poly-gamma-Glutamic Acid Polymers from *Bacillus*
272 *anthracis* and Nonpathogenic *Bacillus* Species. J Immunol 2020;204:1263-73.273 [4] Jelacic TM, Ribot WJ, Tobery SA, Chabot DJ, Friedlander AM. Poly-gamma-Glutamic
274 Acid Encapsulation of *Bacillus anthracis* Inhibits Human Dendritic Cell Responses.
275 ImmunoHorizons 2021;5:81-9.276 [5] Piris-Gimenez A, Corre JP, Jouvion G, Candela T, Khun H, Goossens PL. Encapsulated
277 *Bacillus anthracis* interacts closely with liver endothelium. The Journal of infectious diseases
278 2009;200:1381-9.279 [6] Jouvion G, Corre JP, Khun H, Moya-Nilges M, Roux P, Latroche C, et al. Physical
280 Sequestration of *Bacillus anthracis* in the Pulmonary Capillaries in Terminal Infection. The
281 Journal of infectious diseases 2016;214:281-7.282 [7] Driks A. The *Bacillus anthracis* spore. Molecular aspects of medicine 2009;30:368-73.

283 [8] Moir A. How do spores germinate? Journal of applied microbiology 2006;101:526-30.

284 [9] Setlow P. Spore germination. Current opinion in microbiology 2003;6:550-6.

285 [10] Sylvestre P, Couture-Tosi E, Mock M. A collagen-like surface glycoprotein is a
286 structural component of the *Bacillus anthracis* exosporium. Molecular microbiology
287 2002;45:169-78.288 [11] Sylvestre P, Couture-Tosi E, Mock M. Polymorphism in the collagen-like region of the
289 *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. Journal of
290 bacteriology 2003;185:1555-63.291 [12] Cote CK, Welkos SL. Anthrax Toxins in Context of *Bacillus anthracis* Spores and Spore
292 Germination. Toxins 2015;7:3167-78.293 [13] Gimenez AP, Wu YZ, Paya M, Delclaux C, Touqui L, Goossens PL. High bactericidal
294 efficiency of type IIa phospholipase A2 against *Bacillus anthracis* and inhibition of its
295 secretion by the lethal toxin. J Immunol 2004;173:521-30.296 [14] Brossier F, Levy M, Mock M. Anthrax spores make an essential contribution to vaccine
297 efficacy. Infection and immunity 2002;70:661-4.

- 298 [15] Ristroph JD, Ivins BE. Elaboration of *Bacillus anthracis* antigens in a new, defined
299 culture medium. *Infection and immunity* 1983;39:483-6.
- 300 [16] Prina E, Abdi SZ, Lebastard M, Perret E, Winter N, Antoine JC. Dendritic cells as host
301 cells for the promastigote and amastigote stages of *Leishmania amazonensis*: the role of
302 opsonins in parasite uptake and dendritic cell maturation. *Journal of cell science*
303 2004;117:315-25.
- 304 [17] Candela T, Dumetz F, Tosi-Couture E, Mock M, Goossens PL, Fouet A. Cell-wall
305 preparation containing poly-gamma-D-glutamate covalently linked to peptidoglycan, a
306 straightforward extractable molecule, protects mice against experimental anthrax infection.
307 *Vaccine* 2012;31:171-5.
- 308 [18] Mignot T, Merlie JP, Jr., Zusman DR. Regulated pole-to-pole oscillations of a bacterial
309 gliding motility protein. *Science (New York, N.Y)* 2005;310:855-7.
- 310 [19] Tournier JN, Quesnel-Hellmann A, Mathieu J, Montecucco C, Tang WJ, Mock M, et al.
311 Anthrax edema toxin cooperates with lethal toxin to impair cytokine secretion during
312 infection of dendritic cells. *J Immunol* 2005;174:4934-41.
- 313 [20] Plomp M, Leighton TJ, Wheeler KE, Hill HD, Malkin AJ. In vitro high-resolution
314 structural dynamics of single germinating bacterial spores. *Proceedings of the National*
315 *Academy of Sciences of the United States of America* 2007;104:9644-9.
- 316 [21] Santo LY, Doi RH. Ultrastructural analysis during germination and outgrowth of
317 *Bacillus subtilis* spores. *Journal of bacteriology* 1974;120:475-81.
- 318 [22] Ezzell JW, Abshire TG. Encapsulation of *Bacillus anthracis* spores and spore
319 identification. *Salisbury Medical Bulletin* 1996;87S:42.
- 320 [23] Steichen CT, Kearney JF, Turnbough CL, Jr. Non-uniform assembly of the *Bacillus*
321 *anthracis* exosporium and a bottle cap model for spore germination and outgrowth. *Molecular*
322 *microbiology* 2007;64:359-67.
- 323 [24] Fouet A, Mock M. Regulatory networks for virulence and persistence of *Bacillus*
324 *anthracis*. *Current opinion in microbiology* 2006;9:160-6.
- 325 [25] Koehler TM. *Bacillus anthracis* physiology and genetics. *Molecular aspects of medicine*
326 2009;30:386-96.
- 327 [26] Cote CK, Rossi CA, Kang AS, Morrow PR, Lee JS, Welkos SL. The detection of
328 protective antigen (PA) associated with spores of *Bacillus anthracis* and the effects of anti-PA
329 antibodies on spore germination and macrophage interactions. *Microbial pathogenesis*
330 2005;38:209-25.

- 331 [27] Rougeaux C, Becher F, Goossens PL, Tournier JN. Very Early Blood Diffusion of the
332 Active Lethal and Edema Factors of *Bacillus anthracis* After Intranasal Infection. The Journal
333 of infectious diseases 2020;221:660-7.
- 334 [28] Corre JP, Piris-Gimenez A, Moya-Nilges M, Jouvion G, Fouet A, Glomski IJ, et al. In
335 vivo germination of *Bacillus anthracis* spores during murine cutaneous infection. The Journal
336 of infectious diseases 2013;207:450-7.
- 337 [29] Glomski IJ, Piris-Gimenez A, Huerre M, Mock M, Goossens PL. Primary involvement of
338 pharynx and peyer's patch in inhalational and intestinal anthrax. PLoS pathogens 2007;3:e76.
- 339 [30] Goossens PL, Tournier JN. Crossing of the epithelial barriers by *Bacillus anthracis*: the
340 Known and the Unknown. Frontiers in microbiology 2015;6:1122.
- 341 [31] Russell BH, Vasan R, Keene DR, Koehler TM, Xu Y. Potential dissemination of *Bacillus*
342 *anthracis* utilizing human lung epithelial cells. Cellular microbiology 2008;10:945-57.
- 343 [32] Weiner ZP, Glomski IJ. Updating perspectives on the initiation of *Bacillus anthracis*
344 growth and dissemination through its host. Infection and immunity 2012;80:1626-33.
- 345 [33] Tournier JN, Ulrich RG, Quesnel-Hellmann A, Mohamadzadeh M, Stiles BG. Anthrax,
346 toxins and vaccines: a 125-year journey targeting *Bacillus anthracis*. Expert review of anti-
347 infective therapy 2009;7:219-36.
- 348 [34] Goossens P. Animal models of human anthrax: The Quest for the Holy Grail. Mol
349 Aspects Med. 2009;doi:10.1016/j.mam.2009.07.005
- 350 [35] Gauthier YP, Tournier JN, Paucod JC, Corre JP, Mock M, Goossens PL, et al. Efficacy
351 of a vaccine based on protective antigen and killed spores against experimental inhalational
352 anthrax. Infection and immunity 2009;77:1197-207.
- 353 [36] Friedlander AM, Little SF. Advances in the development of next-generation anthrax
354 vaccines. Vaccine 2009;27 Suppl 4:D28-32.
- 355 [37] Manish M, Verma S, Kandari D, Kulshreshtha P, Singh S, Bhatnagar R. Anthrax
356 prevention through vaccine and post-exposure therapy. Expert opinion on biological therapy
357 2020;20:1405-25.
- 358 [38] Chabot DJ, Ribot WJ, Joyce J, Cook J, Hepler R, Nahas D, et al. Protection of rhesus
359 macaques against inhalational anthrax with a *Bacillus anthracis* capsule conjugate vaccine.
360 Vaccine 2016;34:4012-6.
- 361 [39] Chabot DJ, Scorpio A, Tobery SA, Little SF, Norris SL, Friedlander AM. Anthrax
362 capsule vaccine protects against experimental infection. Vaccine 2004;23:43-7.
- 363
- 364

365 **Legends to figures**

366

367 **Figure 1:** Co-detection of capsular material and exosporium during germination of *B.*
368 *anthracis* spores of the encapsulated RPG1 strain and the *bclA*-deleted derived strain (Δ -
369 *bclA*): germinating spores were immuno-labelled 30 and 45 min after germination triggering
370 for 3 min in BHI and further growth in R-medium 0.6% Bicarbonate in 5% CO₂ atmosphere.
371 Representative germinating spores are represented at higher magnification beside groups of
372 bacterial cells for each time points. Red fluorescence for anti-capsular material labelling,
373 green fluorescence for monoclonal anti-BclA or polyclonal anti-exosporium labelling for the
374 Δ -*bclA* strain. Simultaneous DNA labelling (Topro-3, blue fluorescence) is shown at 45 min.
375 For the *in vivo* germinating spores, 1×10^8 germinated RPG1 spores obtained after the 3min-
376 BHI incubation step were inoculated intraperitoneally and recovered one hour later. Images
377 are representative of at least two to three experiments (except for the *in vivo* experiment for
378 ethical reasons).

379

380 **Figure 2:** Kinetics, from 15 min to 2h, of co-detection of capsular material and exosporium
381 BclA during germination of *B. anthracis* spores of the encapsulated RPG1 strain; same
382 growth conditions and immunofluorescence labelling as in Fig.1. The 3-min time point
383 represents the spores after germination triggering in BHI, in conditions where the capsule is
384 not expressed.

385

386 **Figure 3:** Same growth conditions as in Fig.1. (a) Same immunofluorescence labelling as in
387 Fig.1; (top) confocal analysis of germinating spores at 45 min, (bottom) image deconvolution
388 in the same experimental conditions. (b and c) spore germination was studied independently
389 by transmission electron microscopy w/o labelling (JNT's lab) and by scanning electron
390 microscopy with specific labelling of BclA and capsular material (PLG's lab) - (b)
391 Transmission electron microscopic analysis of extracellular RPG1 spores (left) and the non-
392 encapsulated RPLC2 parental strain (right) 1h after dendritic cell infection (see Materials and
393 methods). (c) scanning electron microscopic analysis of immunolabelled RPG1 germinating
394 spores at 45 min (see Fig. 1) ; 10 nm beads for the anti-BclA antibodies, 15 nm beads for the
395 anticapsular material antibodies (see Materials and methods); to facilitate reading of the
396 image is shown in the insert a colored representation of the 10 nm anti-BclA beads (colored in
397 green) and 15 nm anti-capsule beads (colored in red) in a representative given area of the
398 spore.

399 **Table 1:** Quantitative analysis of the topographical distribution of the immunofluorescence
400 during germination of the encapsulated RPG1 strain; a numbered schematic representation of
401 the type of observed immunofluorescence signals is shown in the two left columns. After a
402 3min-step of germination triggering (“0”min), germinated spores were incubated in R
403 medium 0.6% bicarbonate in a 5% CO₂ atmosphere, recovered at various time points
404 thereafter and immunolabelling performed after binding on PLL-coated glass coverslips (see
405 Materials and Methods). Enumeration of the labelled spores is expressed as percentage (n=
406 number of spores enumerated on successive fields). red: anticapsular material labelling, green:
407 anti-BclA labelling.
408



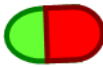


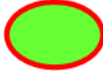


| Form type | Immuno-fluorescence distribution | "0" min (n=103) | 15 min (n=55) | 30 min (n=64) | 60 min (n=59) | 90 min (n=50) | 120 min (n=50) |
|-----------|-------------------------------------------------------------------------------------|-----------------|---------------|---------------|---------------|---------------|----------------|
| 1 |  | 100% | 16 % | 9 % | 10 % | 26 % | 24 % |
| 2 |  | | 24 % | 8 % | | | |
| 3 |  | | | 2 % | | | |
| 4 |  | | 31 % | 12 % | 3 % | 4 % | |
| 5 |  | | 27 % | 31 % | 10 % | 2 % | |
| 6 |  | | 2 % | 36 % | 47 % | 16 % | |
| 7 |  | | | 2 % | | | |
| 8 |  | | | | 29 % | 52 % | 76 % |

Table 1: Fastenackels et al

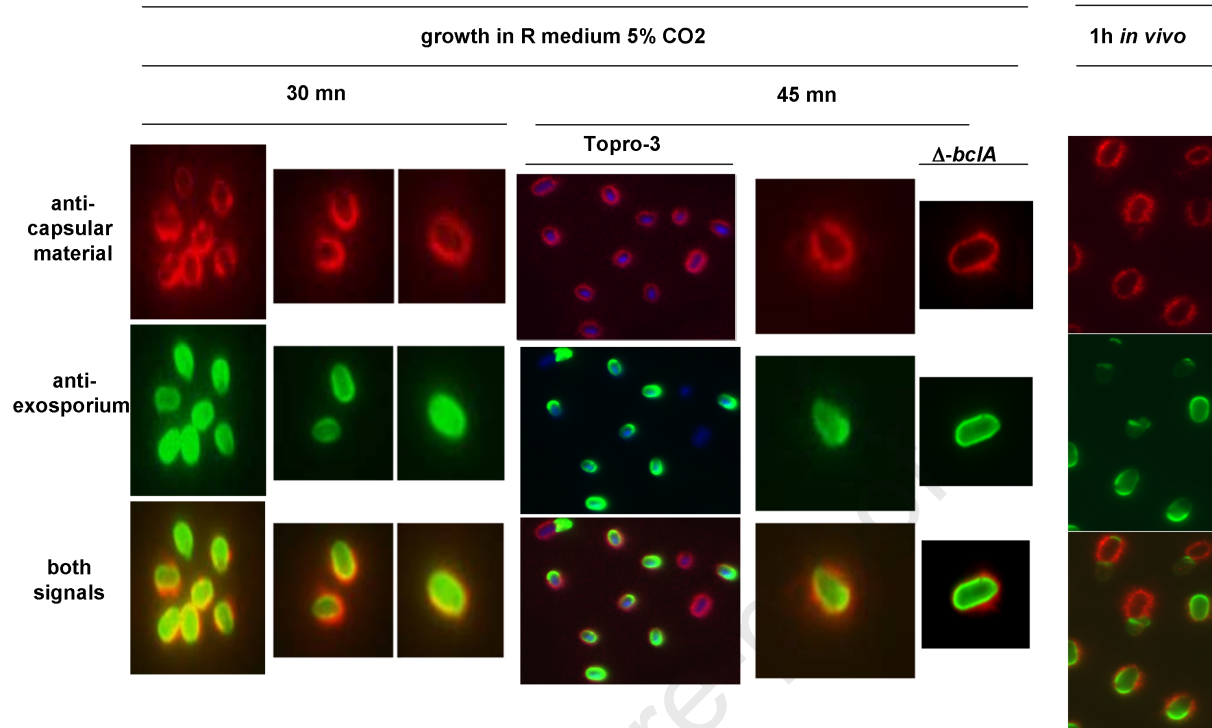


Fig 1: Fastenackels et al

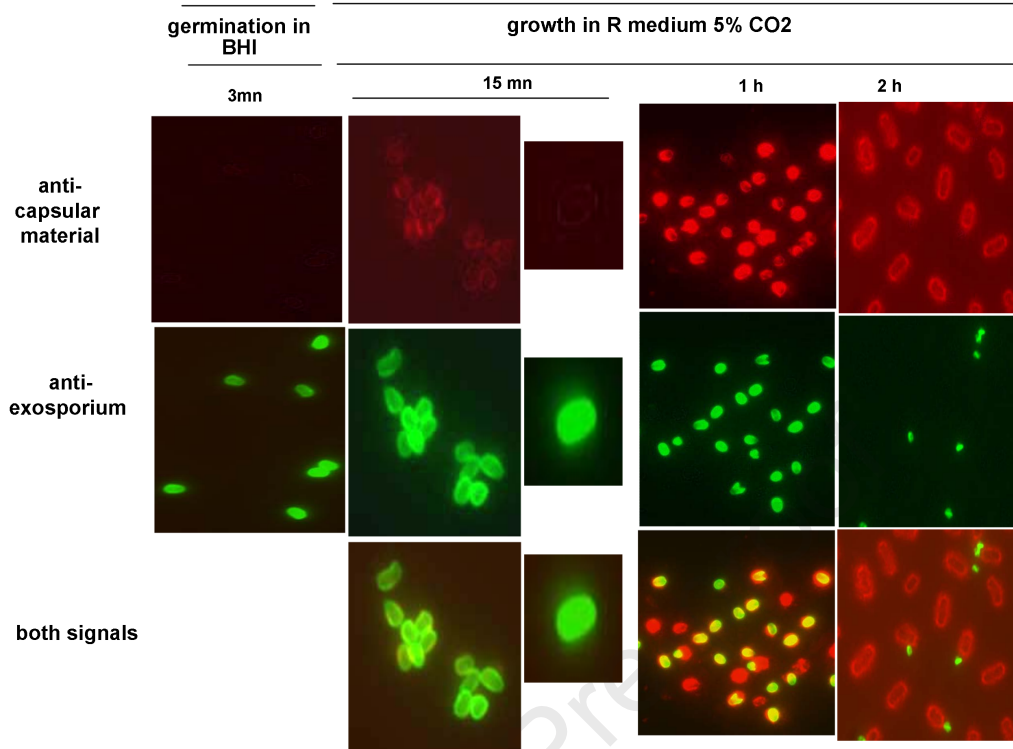


Fig 2: Fastenackels et al

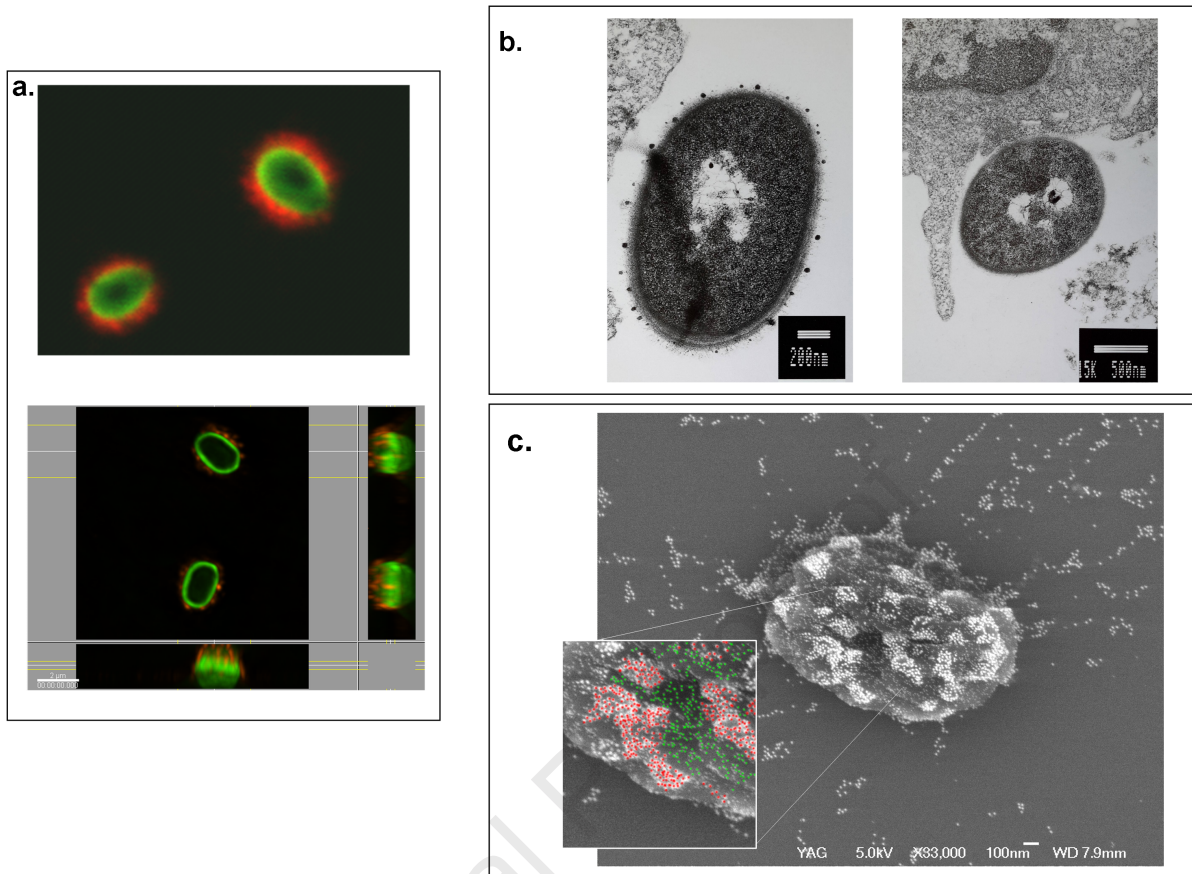


Fig 3: Fastenackels et al