Electron microscopy reveals unique spore-like nano forms of *Bacillus cereus*

Sumanta Ghosh[£,1], Biprashekhar Chakraborty[£#,1], Shreya Ghosh[1], Sandip Dey[1], Chiranjit Biswas[1], Rukhsana Chowdhury[2], Krishnananda Chattopadhyay[1,*], Jayati Sengupta[1,*]

[1]Structural Biology and Bioinformatics Division, CSIR-Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Kolkata 700032, India

[2] Infectious Diseases and Immunology Division, CSIR-Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Kolkata 700032, India

[£]**Contributed equally**

***Corresponding Authors: Krishnananda Chattopadhyay (krish@iicb.res.in) & Jayati Sengupta (jayati@iicb.res.in).**

[#]**Current Address:** NPDF Fellow, Department of Biophysics, Molecular Biology and Bioinformatics, Rajabazar Science College, University of Calcutta, India

Running Title: Visualization of bacterial nano-forms

Abstract

Endospore formation under environmental stress conditions is a well-established phenomenon for members of bacterial phylum Firmicutes, among which the most well studied ones belong to genus *Bacillus* and *Clostridium*. So far, known sizes of the spores are all larger than 500 nm. Nano-forms of bacteria have been reported but the notion still remains controversial.

In this study, we provide visual evidences of living nano-entities (named here as 'nano-spores') formed by a bacterial species *Bacillus cereus* under prolonged stress, which are capable of escaping though standard sterile filtration procedure. The existence of nano-forms of bacteria was initially identified in a yeast ribosome preparation. We further demonstrate the transformation of the 'nano-spores' into mature cells upon nutrient supply. Our study not only demonstrates the ability of bacteria to get transformed into yet-unknown form in order to survive under harsh environment, but also brings to light the existence of the smallest possible form of life.

Introduction

Large environmental fluctuations induce transformation of a bacterial cell into its 'starvation form'[1], which eventually gets converted into the'endospore' [2,3]. Bacterial endospores are considered the most resistant living form exhibiting incredible resistance to environmental extremities [4-7]. Under favorable growth condition, endospores germinate to vegetative forms eventually converting to mature bacterial cells [8].

Although rod-shaped bacteria were initially identified as spore-forming species[9], with emerging evidences it appears that a wide range of bacteria are capable of sporulation [10-12]. Interestingly, it has been reported recently that in human microbiota, the spore-forming bacteria were significantly more diverse than the non-spore-forming bacteria[13]. While sporogenesis typically culminates in the production of a single endospore, there is evidence that some bacteria have evolved the means of producing more than one endospore per cell [14].

Based on recent structural studies of the endospore architecture of gram negative as well as gram positive bacteria [15,16], it has been hypothesized that a spore is the common ancestor of all bacteria[17]. Emerging lines of evidence suggests that a wide range of bacteria are capable of sporulation[13,14]. The reported sizes of the endospores differ considerably[4,18]. Typically, mature spores are 0.8–1.2 μm in length and have either a dense spherical or ellipsoidal shape. The existence of nano form of life has been noted [1,6,7,19], which has initiated a long-standing debate [11,20,21].

Here, we provide evidences that *Bacillus cereus* can exist as nano scale structures (sizes of ~20-100 nm) which we have termed here as 'nano-spores' under prolonged stress. Interestingly, the nano-structures were first identified (and characterized as a form of bacterial species closely related to *Bacillus cereus*) in a yeast ribosome preparation. In marked contrast to previous reports on 'nanobes'[7,19], the 'nano-spores' that we have identified were obtained by subjecting *Bacillus cereus* cells to osmotic stress. The maturation

70 of the nano scale structures back to mature bacterial cells was also tracked by exposing them
71 to a nutrient rich environment.
72
73 **Results**
74 ***Identification of ultra-small spore-like structures camouflaged in purified ribosomes***
75 Electron microscopic visualization of a preparation of yeast ribosomes revealing conspicuous
76 small spherical structures (20-50nm) along with the ribosome particles attracted our attention
77 (Fig. 1A). Intriguingly, when the yeast ribosomal preparation was incubated at 37°C with
78 shaking for about 3 days, formation of larger particles was indicated by dynamic light
79 scattering (DLS) analysis (Fig.S1A). Cell-like entities were also detected by atomic force
80 microscopy (AFM) (Fig.S1C). Cryo-electron microscopy (cryo-EM) imaging revealed the
81 presence of virtually transparent cell-like structures (0.6-1 μm) with double membranes
82 (Fig.1B-D). These structures share striking morphological resemblance with the ultra-small
83 bacterial cells recently identified[22]. Nano-scale spherical entities were also detected at the
84 vicinity of the cell-like structures (Fig.1B-D insets). It must be emphasized that these cell-
85 like particles were obtained only after incubation at 37°C, and they were not detected in the
86 initial ribosomal preparation in spite of extensive search through several TEM grids.
87 DLS analysis of ribosome samples at different time points of incubation indicated gradual
88 increase in size of the particles (Fig.S2E). Same samples, when visualized by cryo-EM,
89 showed that the small spherical structures initially observed (Fig.1) grew in size with time to
90 form cell-like structures (Fig.2A,C,E; Fig.S2A-S2D). It was further observed that addition of
91 an intrinsically disordered protein during incubation expedited the enlargement of the cell-
92 like structures (Fig. 2B,D,F; Fig. S1B, S2F). Interestingly, concomitant reduction in the
93 number of ribosomal particles was observed over time (Fig. 2A,B).

We speculated that, during incubation at 37°C, ribosomal RNAs and proteins got disintegrated and acted as the nutrient source for the nano-scale structures to grow in size into larger entities (Fig. 2C,E). To confirm this assumption, we incubated the ribosome preparation with Alexa488 fluorophore-tagged α-synuclein protein (see Materials and Methods). While α-synuclein alone formed fibrillar structures after 48 hours (Fig. 3A), no fibrils could be detected when incubated in the presence of ribosome. In contrast, the fluorophore was found by confocal microscopy to be inside the cell-like structures (Fig. 3B) clearly suggesting that the protein molecules were engulfed by the nano-structures. Further, a RNA specific dye F22 [23] was applied to the ribosome preparation following 60 hours of incubation; subsequent confocal microscopy revealed the presence of the dye within the cell-like structures (Fig. 3C; Fig. S3A-C), which co-localizes with the protein (Fig. 3D,E) , suggesting the presence of nucleotides within the cell.

Nano-scale spore-like structures identified in the ribosome preparation are living entities

The purified yeast 80S ribosome was spotted directly on Luria agar plates and incubated overnight at 37°C, along with a plate in which only the ribosome storage buffer was spotted as control. Unlike the control one (Fig.S4A), the plate with purified yeast 80S ribosome (Fig. S4B) showed distinct bacterial colonies confirming the nano-scale, spore-like structures, observed initially in the ribosomal preparation to be living entities. Considering the ability of the nano-scale spherical structures to convert into mature bacteria, we termed these nanobodies the 'nano-spores'. However, in contrast to the conventional dense structures known for bacterial endospore, the 'nano-spores' detected here were almost transparent in nature (shown in Fig. 1).

We also performed confocal imaging of the ribosome sample, which was incubated with Alexa488 fluorophore-labelled α-synuclein protein (Fig. 3F) for 18 hrs using DNA-specific

119 Hoechst dye. The images revealed the presence of the DNA-specific dye within the cell-like
120 structures (Fig. 3G, Fig. S3D-F) co-localized with the protein (Fig. 3H,I), confirming the cell-
121 like structures to be living entities. Further, DNA content analysis of the same sample at
122 zero (0) hr and 18 hr of incubation, respectively, by flow cytometry revealed an increase in
123 DNA content with time (Fig. S3G & S3H), suggesting that the 'nano-spores' are probably
124 preparing themselves for germination into vegetative form.

125 Phylogenetic analysis of the universally conserved region of the16SrRNA gene, amplified
126 from genomic DNA extracted from these bacterial cells, suggested that they belonged to the
127 genus *Bacillus* (most related to *Bacillus cereus*; Fig. 4). However, the sizes of the cells were
128 found to be much smaller (1-2µm) compared to the reported size of *Bacillus* and resembled
129 the morphology of the forms of large spores or vegetative cells[24]. Conceivably, the *Bacillus*
130 'nano-spores' got co-purified with the yeast 80S ribosomes by virtue of their comparable
131 sizes (~40-50 nm). Notably, association between *Bacillus* and yeast has been reported
132 earlier[25]. Particularly, *B. cereus* has been shown to be capable of establishing parasitic or
133 symbiotic association with fungi.[26,27].

134 We examined the cultured bacterial cells under electron microscope. Remarkable network
135 of pili-like structures, known to form in growing bacteria in order to exchange their genetic
136 material, was observed (Fig. 5A,B) indicating its ability to rapidly grow under favourable
137 growth conditions. In some views it appeared that the cells were embedded within a glutinous
138 sack (Fig. S5A, B), which seemed to be a self-produced matrix resembling bio film
139 architectures. Bio film formation has been reported earlier for various *Bacillus*
140 species[4,12,18,28].

141 In line with an earlier observation[24], the *Bacillus* strain in this study was found to be
142 completely resistant to a number of antibiotics, like ampicillin and kanamycin, whereas a

143 relatively high concentration of chloramphenicol (~70-140 μg/ml) and tetracycline partially
144 inhibited bacterial growth (Fig. S5C, D).
145
146 ***Efficient spore forming ability of the bacterial species***
147 Bacterial cells are known to form highly resistant endospores during unfavourable
148 environmental conditions. Hence, it may be assumed that mature bacterial cells (Fig. 5A, B),
149 which were obtained from the 'nano-spores', can also be transformed to spores again,
150 provided necessary external stress is applied. To verify this hypothesis, the cells were
151 submerged in a solution of absolute ethanol and ammonium acetate (salt-ethanol treatment)
152 and kept at -80°C (see online Methods). After overnight incubation, a precipitate was
153 detected upon centrifugation, which was found to be shrunken cells when visualized under
154 electron microscope (Fig. 5C). Unusual fibrillar structures were detected in the supernatant
155 (Fig. 5D; Fig. S5E). Interestingly, a previous high-resolution AFM study reported that spore
156 coat is composed of fibrillar structures [29]. Other studies also claimed that the amyloid fibrils
157 form protective coat for the bacterial cells[30]. It is quite possible that under stress, amyloid-
158 like fibrils (seen in supernatant) were released from the cell surface. It should be noted that
159 *E. coli* cells lysed completely within overnight incubation when subjected to similar
160 treatment (Fig. S5F).
161 The salt-ethanol treatment procedure consisted of multiple steps, which are described in
162 detail in Fig. 6. Formation of the 'nano-spores' (~20-100 nm) was detected eventually in the
163 fourth day supernatant when the sample was centrifuged (Fig. 5E). We also detected the
164 presence of some particles, less than 200nm in size, containing unusual structural features
165 (Fig. S6). The 'nano-spores' thus obtained (Fig. 5E) reverted back to vegetative forms when
166 grown overnight in LB broth or in LB agar plate at 37°C (Fig. 5F;Fig. 6; Fig. S4C). 16S
167 rRNA sequence phylogenetic analysis of these cells once again confirmed that this species

168 belonged to the genus *Bacillus*. The whole cycle has been schematically represented in Fig.
169 S7.

170 Thus, we tracked the complete cycle of transformation of the bacterium, from 'nano-
171 spores' (initially observed in our ribosomal preparation) to vegetative cells, and then back to
172 'nano-spores' by applying prolonged osmotic stress, which can again grow to mature cells
173 (Fig. S7), confirming that the 'nano-spore' identified in this study are living entities.

174

175 ***Bacillus cereus cells are capable of forming nano-spores under osmotic stress:***

176 Phylogenetic analysis indicated *Bacillus cereus* is the closest neighbour of the 'nano-spore'-
177 forming species identified in this study. Hence, we subjected *Bacillus cereus* $M^1{}_{16}$ strain
178 (Fig.7A) to similar salt-ethanol stress as mentioned earlier (Fig. 6). On the fourth day
179 following salt-ethanol treatment (as mentioned previously in Fig. 6), both pellet (P) and
180 supernatant (S) fraction were filtered through 0.22 µm filter (called fP and fS, respectively,
181 henceforth) and subsequently viewed using cryo-EM. Nano-scale structures, very similar to
182 those observed in purified yeast 80S ribosome were visualized in both supernatant (fS) and
183 pellet (fP) (following removal of larger particles by filtration) fractions (Fig. 7B; 7E). More
184 importantly, when the 'fS' fraction thus obtained was spotted on LB agar plate and incubated
185 overnight at 37°C, bacterial colonies were observed (Fig. S4D), suggesting the nano-scale
186 structures to be living entities.

187 It has been reported that supernatants of growing bacteria (containing muropeptides
188 derived from the peptidoglycan fragments released by the bacteria) induce germination in
189 dormant bacterial spores via a specific pathway[31]. All gram negative bacteria, as well as
190 spore forming gram positive bacterial species, are known to release peptide fragments which
191 can induce spore germination[31]. To see the effect of muropeptides released by *B. cereus* cells
192 over the 'nano-spores' of the same species, we incubated both the 'fP' and 'fS' fractions in

193 the supernatant obtained after harvesting an overnight *B. cereus* culture. Indeed, faster
194 maturation of nano-structures was observed in the supernatant of *B. cereus* culture compared
195 to its growth in sterile LB media, which is apparently due to the presence of muropeptides in
196 the *B. cereus* culture media. Interestingly, cryo-EM images revealed a difference in
197 appearance of the matured cell-like structures formed from the 'fP' and 'fS' fractions in
198 sterile media (Fig. 7C) and *B. cereus* culture media (Fig.7D). While the cell-like structures in
199 sterile media appeared dense, the structures formed in *B. cereus* culture media were more
200 transparent, having striking similarity with those observed following incubation of yeast
201 ribosome preparation at 37°C.
202
203 ***Characterization of B. cereus nano-spores:***
204 Next, as we set out to track the maturation process of the *B. cereus* 'nano-spores' into mature
205 cells, the 'nano-spores' were incubated in LB broth at 37°C with shaking (allows the
206 enlargement of nano-forms as seen previously). Aliquots were taken at regular time intervals
207 and visualized under phase-contrast microscope. Germination of a bacterial spore into a cell
208 is known to be accompanied by a change in the refractility of the spore. As a result, spore
209 loses its brightness, which makes the germinated cell appear dark under phase contrast
210 microscope compared to an ungerminated spore[32,33]. Nano-structures are not visible in light
211 microscope. Aliquots taken at an earlier stage of incubation (~14hr) revealed the presence of
212 shining white (bright) particles (Fig 7F), while aliquots taken at a later stage (~22hr) revealed
213 the presence of grey to black (dark) rod shaped particles (Fig 7G), similar to the B.cereus
214 cells (Fig. 7A). Interestingly, an aliquot taken from an intermediate point (~18hr) revealed
215 the presence of both bright and dark particles (Fig S4E & S4F), albeit segregated in separate
216 microscopic fields. Our observations imply that maturation of *B.cereus* nano-structures
217 occurs with an initial enlargement into the known forms of endospores (500-1000nm)

218 followed by their germination into vegetative cells. It has been suggested earlier that a
219 dormant but mature spore stores all the components necessary for germination[34]. Thus, the
220 initial enlargement phase of the nano-structures (observed in our study) prior to germination
221 is presumably required for getting themselves ready for germination.

222 The spores are known to be resistant to environmental extremities and some
223 superdormant spores have also been reported having elevated level of resistance[35,36]. To test
224 whether the nano-structures have similar kind of resistance, the nano-structures isolated
225 following osmotic stress and an earlier stage (~14hr) aliquot from the previous experiment
226 were kept in boiling water for half an hour and then streaked onto LB agar plate. Bacterial
227 colony formation on a plate upon overnight incubation at 37 ^{o}C (Fig. 7H; 7I & 7J) is the
228 evidence of a highly resistant nature of the nano-structures.

229

230 **Discussion**

231 Existence of nano bacteria was claimed in the late 90's [7,19]. However, this claim has
232 particularly become controversial when it was found that non-organic materials, like calcium
233 and phosphate ions, can hijack proteins from cell culture media to grow like nanobacteria. It
234 was argued [21] that the so called 'nanobes' are not 'living units', and could simply be
235 fragmented portions of larger cells. We, for the first time, not only identified nano-scale,
236 spore-like structures possessing fascinating characteristics but also provided unambiguous
237 evidence that the structures are 'living units'. Further, we discovered an unprecedented
238 characteristic of *Bacillus cereus*, namely, to form nano-scale structures, and it probably won't
239 be too ambitious to suggest that many spore forming bacteria likely have the ability to form
240 similar nano-structures.

241 The nano-size living entities we first identified were co-purified with the ribosome. To
242 confirm that, all ingredients required for ribosome purification~~s~~ were thoroughly checked to

243 rule out any contamination of mature bacterial cells. Although we filtered the ribosome
244 preparation through 0.22 micron filter prior to apply on agar plate, one far-fetched possibility
245 was that the fully grown bacterial cells were co-purified with the yeast ribosome and got
246 transformed to spores during storage (along with the ribosome) at -80°C. To examine that,
247 we kept cultured bacterial cells (grown from the purified ribosome preparation) at -80°C in
248 the same way as the ribosomes are preserved. The cells remained the same in size and
249 morphology even after two weeks, ruling out this possibility.

250 Our results indicated that the nano-structures possess some of the spore characteristics.
251 However, they may be considered as yet-another 'starvation form' of bacteria. It may be
252 argued that nano-structures of this kind would be incapable of packaging essential biological
253 macromolecules inside its small volume. It has been a matter of dispute whether
254 macromolecules are essential for spore germination[8,33,34,37-40]. The prevailing view was that
255 the spores can go on to germination without the need of any macromolecular synthesis, which
256 starts only after germination, as the germinated spore gets converted into a growing cell [8,34].
257 However, this idea was challenged by Sinai *et.al* arguing that protein synthesis is necessary
258 for spore germination[33]. Although the debate is still ongoing [37,39], it is now believed that
259 storage of the bio-macromolecules is not essential for germination, and that these molecules
260 can be generated rapidly just before germination [39]. Our observation of the enlargement of
261 the 'nano-spore' size, as well as an increase in DNA content prior to germination supports
262 this notion.

263 It is evident from our study that 'nano-spores', by virtue of their size similarity with
264 ribosomes, can get purified together with the organelles. However, it is not clear whether
265 those are engulfed inside the yeast cells or if they remain attached to the cell surface.
266 Nevertheless, the ribosome preparation protocol eliminates cell membrane and other debris at
267 the very first step, and, thus, we suspect that the 'nano-spores' likely hide inside the yeast

268 cells. Apparently, other microbes outnumber them in normal environment and, hence, they
269 prefer to stay in 'nano-spore' form unless favourable condition is available[8]. The small size
270 of the spores, which would make a typical sterile filtration process ineffective, and their
271 persistence even after salt-alcohol treatment, requires serious attention, e.g., in case of
272 hospital-acquired antibiotic resistance and microbial survival in extraterrestrial space.
273
274 **Methods:**
275 **Ribosome Purification:**
276 A diploid wild type strain MATaα derived by combining two haploid strains, 8534-10A
277 (MATa, leu2, ura3, his4) and 6460-8D (MATα, met3), of *S. cerevisiae* was grown overnight
278 in Yeast-Peptone-Dextrose (Y.P.D.) complete medium at 30°C. After around 18 hours the
279 cells were harvested at 6000g for 10 min at 4°C. The cell pellet was re-suspended in
280 ribosome buffer (100mM KOAc, 20mM HEPES-KOH, pH 7.6, 10mM Mg $(OAc)_2$, 1mg/ml
281 heparin, 2mM DTT, 0.5mM PMSF) and lysed by passaging twice through French pressure
282 cell (16000 psig at first and then 18000psig). Lysate was centrifuged at 17000g for 30 mins
283 at 4°C. Supernatant was then subjected to ultra-centrifugation at 350000g for 1hr at 4°C. The
284 pellet obtained after centrifugation was re-suspended in minimum volume of ribosome buffer
285 and the volume was made up to18ml by adding high salt buffer (ribosome buffer plus 500
286 mM KCl) and kept on ice for 60 min with gentle stirring at regular intervals. This was
287 followed by centrifugation at 16000g for 10 min at 4°C for 3 to 4 times until no pellet could
288 be visible. The supernatant was layered over a sucrose cushion and centrifuged at 350000g
289 for 1 hr at 4°C. Pure yeast 80S ribosome obtained as pellet was re-suspended in storage
290 buffer (10mM Tris-HCl (pH 7.5), 12.5mM $Mg(OAc)_2$, 80mM KCl, 5mM 2-Mercaptoethanol,
291 0.5mM PMSF) and stored at 80°C.

292 Considering ribosome purification protocol, it is quite unlikely for mature bacterial cells
293 (more that 10 fold differences in their sizes) to be co-purified with ribosome. Instead, it is
294 more likely that the bacterial spores might have co-purified with the yeast 80S ribosome
295 probably by virtue of their comparable sizes (yeast ribosomes ~40-50 nm).

296 **Transmission Electron Microscopy:**

297 Electron microscopy images were obtained with a Tecnai^{TM} G2 Spirit transmission electron
298 microscope at an acceleration voltage of 120 kV. A drop of the sample solution (4 µl) was
299 spotted on a carbon-coated copper grid. The grid was dried carefully, keeping it in a vacuum
300 desiccator for 24 hr and was finally examined under the electron microscope to study the
301 ultra-structural features of the sample (bacterial cells/spores) at different conditions.

302 **Cryo-electron Microscopy (Cryo-EM):**

303 Cryo-grids were prepared with standard protocol[41]. Ribosome preparation following
304 incubation at different time points of incubation was spotted on glow-discharged lacey carbon
305 copper grid and plunge-frozen in liquid ethane using Vitrobot (FEI Company). Grids were
306 transferred to a cryo-holder and visualized in Tecnai POLARA (FEI Company) equipped
307 with 300 kV field emission gun (FEG) at low electron dose. Images were collected at
308 82600× magnification (~5 µm under focus). Images were captured on an Eagle 4k × 4k CCD
309 camera (FEI Company) with a final pixel size of 1.86 Å.

310 **Atomic Force Microscopy (AFM):**

311 AFM was performed using a Pico Plus 5500 AFM (Agilent Technologies, USA) with a Piezo
312 scanner having a maximum range of 9 µm. After incubating for 44 hours, 10 ul of newly
313 formed cells was deposited on a freshly cleaved muscovite ruby mica sheet (ASTM V1grade
314 ruby mica from MICAFAB, Chennai). After 30 minutes the sample was dried with a vacuum
315 drier. The cantilever resonance frequency was 150−300 kHz. The images (256 pixels × 256
316 pixels) were captured using a scan size of between 0.5 and 8 µm at a scan speed of 0.5 lines/s.

317 The length, height, and width of protein fibrils were measured manually using PicoView1.10
318 software (Agilent Technologies, USA).

319 **Dynamic Light Scattering (DLS):**

320 The Malvern Zetasizer Nano ZS DLS system (Malvern Instruments Ltd., UK) was used to
321 perform all DLS measurements. This system is equipped with a 633 nm He−Ne laser and an
322 APD detector configured to collect backscattered light at 173°. The measurement sample
323 was allowed to equilibrate for 120 s at 20°C prior to the measurements. Fifteen separate runs,
324 each of 10s duration were averaged for each DLS measurement. DLS data were analyzed
325 using Malvern Zetasizer 6.12 software. The reported mean particle hydrodynamic radii (R_H)
326 were calculated using the intensity based particle size distributions.

327 **Confocal Microscopy:**

328 For the assessment of protein consumption inside the bacillus cells, we fluorescently labelled
329 both protein and ribosome with specific dyes. The protein α-synuclein was labelled with
330 Alexa fluor 488 C_5 maleimide (alexa488) using standard protocol [42]. After labelling we did
331 vigorous dialysis followed by size exclusion chromatography to remove all the free dyes in
332 solution. The labelling efficiency of α-synuclein (α-syn) was 20%. Mature fibrils are formed
333 after agitation of 200nm labelled α-syn (Total concentration of α-syn is 200μM). Excitation
334 and emission wavelength of alexa488 is 488nm and 520nm (blue fluorescence) respectively.
335 Existing RNA was labelled by F-22 RNA dye following published procedure [23]. Excitation
336 and emission wave length of F-22 dye is 548nm and 620nm (red fluorescence) respectively.
337 For DNA identification, cells were labelled with Hoescht 33342 (Thermo Fisher Scientific).
338 The excitation and emission wavelength is 361 nm and 497 nm. After labeling, the cells were
339 observed under a Leica DMI6000 B inverted microscope equipped with Plan Apo 100X/1.40
340 oil objective (Leica TCS SP8 confocal system) and Leica DMI6000 B inverted microscope
341 equipped with Plan Apo 100X/1.40 oil objective (Leica TCS SP8 confocal system).

342 **DNA Count:**

343 For DNA content in solution with increasing hours of incubation we stained the cells at 0 and
344 18 hours with DNA specific Hoescht 33342 dye and quantified the DNA count using BD
345 LSRFortessa FACS instrument. Fluorescence intensity of Hoescht dye was measured at indo-
346 violet channel with excitation wavelength 355 and emission range 425-475nm. The data were
347 analysed by BD FACS Diva software.

348 **Salt-ethanol treatment on the bacterial cells:**

349 Ribosome preparation was directly applied on LB agar and kept at 37°C. Upon overnight
350 incubation slimy bacterial colonies were observed. The bacterial colonies were re-suspended
351 in LB broth and incubated at 37°C with shaking. Log phase cells were harvested and re-
352 suspended in 20mM sodium phosphate (pH 7.5) and then half the volume of 7.5M
353 ammonium acetate was added to it. This was followed by addition of double the total volume
354 of absolute ethanol and kept at-80°C. Similar treatment was carried with *Bacillus cereus*
355 cells.

356 Salt-ethanol treated cell suspension was subjected to centrifugation at 13000 rpm at different
357 time points yielding pellet and supernatant fractions. The pellet obtained was further re-
358 suspended in sodium phosphate buffer (pH 7.5) for further investigations. Notably, when
359 similar treatment was applied overnight to *E. coli* cells (control bacterial species) no pellet
360 was detected upon centrifugation and only cell debris was observed in the supernatant. The
361 'nano-spores' were observed at different steps of treatment in both supernatant and pellet
362 fraction. Such fractions containing 'nano-spores' were filtered through 0.22 um filter
363 followed by ultracentrifugation at 350000 g (the speed at which yeast 80S ribosome is
364 precipitated) to precipitate the nano-spores.

365 **Determination of spore character of the nano-spores:**

366 Spore suspensions fS and fP (200 µl) were added to 1 ml growth media and incubated at 37°C
367 with shaking. At specific time intervals aliquots were taken for heat-treatment and
368 microscopy.

369 **Phase-contrast microscopy:**

370 Samples were air dried, heat or mathol fixed on glass slides and visualized by positive phase
371 contrast microscopy under 100X oil-immersion objective lens, phase-3 position in an upright
372 bright field-phase contrast microscope (DM-2500, Leica Microsystems, Germany). The
373 viewer was unaware of the expected outcome of the respective stages of the samples to avoid
374 any biasness. An additional 2.5 digital zoom factor was incorporated for higher
375 magnification.

376

377 **Acknowledgements**

378 This work was supported by CSIR-Indian Institute of Chemical Biology, Kolkata, India,
379 CSIR Network project 'UNSEEN' and SERB, DST (India) sponsored projects. SG and SD
380 acknowledge UGC, India, for senior research fellowship. We would like to thank Prof.
381 Syamal Roy for helpful discussions. We also thank Dr. Anirban Kundu for helping in
382 phylogenetic analysis, Mr. Diptodeep Sarkar Mr. Binayak Pal, and Mr. Somnath Mazumder
383 for technical help with microscopy, Mr. Tanmoy Dalui for FACS measurement, Prof. Young-
384 Tae Chang, Department of Chemistry, National University of Singapore, for gifting us the
385 RNA specific F22 dye and Dr. Animesh Naskar for gifting us the *Bacillus cereus* strain.

386

387 **Author contributions**

388 SG(1) and BC performed experiments to monitor bacterial transformation to spore and spore
389 to mature bacteria, 16S rRNA preparation for sequencing. SG(2) assisted SG(1) and BC to
390 perform experiments with *B. cereus* cells. SD performed antibiotic experiments. RC

391 analyzed the 16S rRNA gene sequencing data and shared expertise to critically check the
392 results. CB performed TEM and cryo-TEM surveys, KC and JS conceived the project,
393 supervised research and JS, BC and KC wrote the paper with inputs from SG(1).

394

395 **Conflict of interest**

396 The authors declare that they have no conflict of interest.

397

398 **References:**

399 1. Velimirov, b. Nanobacteria, ultramicrobacteria and starvation forms: A search for the
400 smallest metabolizing bacterium. *Microbes and Environments* **16**, 67-77 (2001).
401 2. Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J. & Setlow, P. Resistance of Bacillus
402 endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev*
403 **64**, 548-72 (2000).
404 3. Nanjundiah, V. The smallest form of life yet? *J Biosci* **25**, 9-10 (2000).
405 4. Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. & Kolter, R. Sticking together: building a
406 biofilm the Bacillus subtilis way. *Nat Rev Microbiol* **11**, 157-68 (2013).
407 5. Dring, G.J. & Gould, G.W. Reimposition of heat-resistant on germinated spores of Bacillus
408 cereus by osmotic manipulation. *Biochem Biophys Res Commun* **66**, 202-8 (1975).
409 6. Miller, V.M. et al. Evidence of nanobacterial-like structures in calcified human arteries and
410 cardiac valves. *Am J Physiol Heart Circ Physiol* **287**, H1115-24 (2004).
411 7. Uwins, P.J.R., Webb, R.I. & Taylor, A.P. Novel nano-organisms from Australian sandstones.
412 *American Mineralogist* **83**, 1541-1550 (1998).
413 8. Setlow, P. Spore germination. *Curr Opin Microbiol* **6**, 550-6 (2003).
414 9. Doi, Y., Lee, B.-S., Iriye, R., Tabata, S. & Tateishi, K. . Dominantly growing bacteria in
415 malodorless domestic sewage treatment tanks and their biochemical characteristics. *J*
416 *Antibact Antifungal Agents* **26**, 53–63 (1998).
417 10. Ajithkumar, B., Ajithkumar, V.P., Iriye, R., Doi, Y. & Sakai, T. Spore-forming Serratia
418 marcescens subsp. sakuensis subsp. nov., isolated from a domestic wastewater treatment
419 tank. *Int J Syst Evol Microbiol* **53**, 253-8 (2003).
420 11. Cisar, J.O. et al. An alternative interpretation of nanobacteria-induced biomineralization.
421 *Proc Natl Acad Sci U S A* **97**, 11511-5 (2000).
422 12. Auger, S. et al. Biofilm formation and cell surface properties among pathogenic and
423 nonpathogenic strains of the Bacillus cereus group. *Appl Environ Microbiol* **75**, 6616-8
424 (2009).
425 13. Browne, H.P. et al. Culturing of 'unculturable' human microbiota reveals novel taxa and
426 extensive sporulation. *Nature* **533**, 543-6 (2016).
427 14. Hutchison, E.A., Miller, D.A. & Angert, E.R. Sporulation in Bacteria: Beyond the Standard
428 Model. *Microbiol Spectr* **2**(2014).
429 15. Tocheva, E.I. et al. Peptidoglycan remodeling and conversion of an inner membrane into an
430 outer membrane during sporulation. *Cell* **146**, 799-812 (2011).
431 16. Tocheva, E.I. et al. Peptidoglycan transformations during Bacillus subtilis sporulation. *Mol*
432 *Microbiol* **88**, 673-86 (2013).

433 17. Tocheva, E.I., Ortega, D.R. & Jensen, G.J. Sporulation, bacterial cell envelopes and the origin
434 of life. *Nat Rev Microbiol* **14**, 535-542 (2016).
435 18. Carrera, M., Zandomeni, R.O., Fitzgibbon, J. & Sagripanti, J.L. Difference between the spore
436 sizes of Bacillus anthracis and other Bacillus species. *J Appl Microbiol* **102**, 303-12 (2007).
437 19. Kajander, E.O. & Ciftcioglu, N. Nanobacteria: an alternative mechanism for pathogenic intra-
438 and extracellular calcification and stone formation. *Proc Natl Acad Sci U S A* **95**, 8274-9
439 (1998).
440 20. Urbano, P. & Urbano, F. Nanobacteria: facts or fancies? *PLoS Pathog* **3**, e55 (2007).
441 21. Young, J.D. & Martel, J. The rise and fall of nanobacteria. *Sci Am* **302**, 52-9 (2010).
442 22. Luef, B. et al. Diverse uncultivated ultra-small bacterial cells in groundwater. *Nat Commun* **6**,
443 6372 (2015).
444 23. Li, Q. et al. RNA-selective, live cell imaging probes for studying nuclear structure and
445 function. *Chem Biol* **13**, 615-23 (2006).
446 24. Adimpong, D.B. et al. Antimicrobial susceptibility of Bacillus strains isolated from primary
447 starters for African traditional bread production and characterization of the bacitracin
448 operon and bacitracin biosynthesis. *Appl Environ Microbiol* **78**, 7903-14 (2012).
449 25. Frey-Klett, P. et al. Bacterial-fungal interactions: hyphens between agricultural, clinical,
450 environmental, and food microbiologists. *Microbiol Mol Biol Rev* **75**, 583-609 (2011).
451 26. Ivanova, N. et al. Genome sequence of Bacillus cereus and comparative analysis with Bacillus
452 anthracis. *Nature* **423**, 87-91 (2003).
453 27. Huang, C.J., Wang, T.K., Chung, S.C. & Chen, C.Y. Identification of an antifungal chitinase
454 from a potential biocontrol agent, Bacillus cereus 28-9. *J Biochem Mol Biol* **38**, 82-8 (2005).
455 28. Morikawa, M. et al. Biofilm formation by a Bacillus subtilis strain that produces gamma-
456 polyglutamate. *Microbiology* **152**, 2801-7 (2006).
457 29. Plomp, M., Leighton, T.J., Wheeler, K.E., Hill, H.D. & Malkin, A.J. In vitro high-resolution
458 structural dynamics of single germinating bacterial spores. *Proc Natl Acad Sci U S A* **104**,
459 9644-9 (2007).
460 30. Gebbink, M.F., Claessen, D., Bouma, B., Dijkhuizen, L. & Wosten, H.A. Amyloids--a functional
461 coat for microorganisms. *Nat Rev Microbiol* **3**, 333-41 (2005).
462 31. Shah, I.M., Laaberki, M.H., Popham, D.L. & Dworkin, J. A eukaryotic-like Ser/Thr kinase
463 signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell* **135**, 486-96
464 (2008).
465 32. Kong, L., Zhang, P., Setlow, P. & Li, Y.Q. Characterization of bacterial spore germination using
466 integrated phase contrast microscopy, Raman spectroscopy, and optical tweezers. *Anal*
467 *Chem* **82**, 3840-7 (2010).
468 33. Sinai, L., Rosenberg, A., Smith, Y., Segev, E. & Ben-Yehuda, S. The molecular timeline of a
469 reviving bacterial spore. *Mol Cell* **57**, 695-707 (2015).
470 34. Moir, A. How do spores germinate? *J Appl Microbiol* **101**, 526-30 (2006).
471 35. Ghosh, S., Zhang, P., Li, Y.Q. & Setlow, P. Superdormant spores of Bacillus species have
472 elevated wet-heat resistance and temperature requirements for heat activation. *J Bacteriol*
473 **191**, 5584-91 (2009).
474 36. Ghosh, S. & Setlow, P. The preparation, germination properties and stability of
475 superdormant spores of Bacillus cereus. *J Appl Microbiol* **108**, 582-90 (2010).
476 37. Korza, G., Setlow, B., Rao, L., Li, Q. & Setlow, P. Changes in Bacillus Spore Small Molecules,
477 rRNA, Germination, and Outgrowth after Extended Sublethal Exposure to Various
478 Temperatures: Evidence that Protein Synthesis Is Not Essential for Spore Germination. *J*
479 *Bacteriol* **198**, 3254-3264 (2016).
480 38. Setlow, P. Summer meeting 201--when the sleepers wake: the germination of spores of
481 Bacillus species. *J Appl Microbiol* **115**, 1251-68 (2013).

482 39. Sinai, L. & Ben-Yehuda, S. Commentary: Changes in Bacillus Spore Small Molecules, rRNA,
483 Germination, and Outgrowth after Extended Sublethal Exposure to Various Temperatures:
484 Evidence that Protein Synthesis Is Not Essential for Spore Germination. *Front Microbiol* **7**,
485 2043 (2016).
486 40. Vinter, V. Symposium on bacterial spores: V. Germination and outgrowth: effect of
487 inhibitors. *J Appl Bacteriol* **33**, 50-9 (1970).
488 41. Grassucci, R.A., Taylor, D.J. & Frank, J. Preparation of macromolecular complexes for cryo-
489 electron microscopy. *Nat Protoc* **2**, 3239-46 (2007).
490 42. Joshi, N. et al. Attenuation of the early events of alpha-synuclein aggregation: a fluorescence
491 correlation spectroscopy and laser scanning microscopy study in the presence of surface-
492 coated Fe3O4 nanoparticles. *Langmuir* **31**, 1469-78 (2015).

493

494 **Figure Legends:**

495

496 **Figure 1: Cell-like structures observed in ribosome preparation.** (A) A cryo-TEM image
497 shows small spherical structures (marked with red arrow) along with ribosome particles (blue
498 arrow) purified from yeast cells. (B-D) Large transparent cell-like structures (200 nm -1.5
499 μm) were detected in cryo-TEM when 80S ribosome preparation was incubated at 37°C for
500 up to 72 hours. Nano-scale structures (red arrows) were observed in the vicinity of the large
501 structures. Insets of A-D show close up views of nano-scale structures. Notably, ribosome
502 particles were found to disappear with time as the time of incubation progressed.

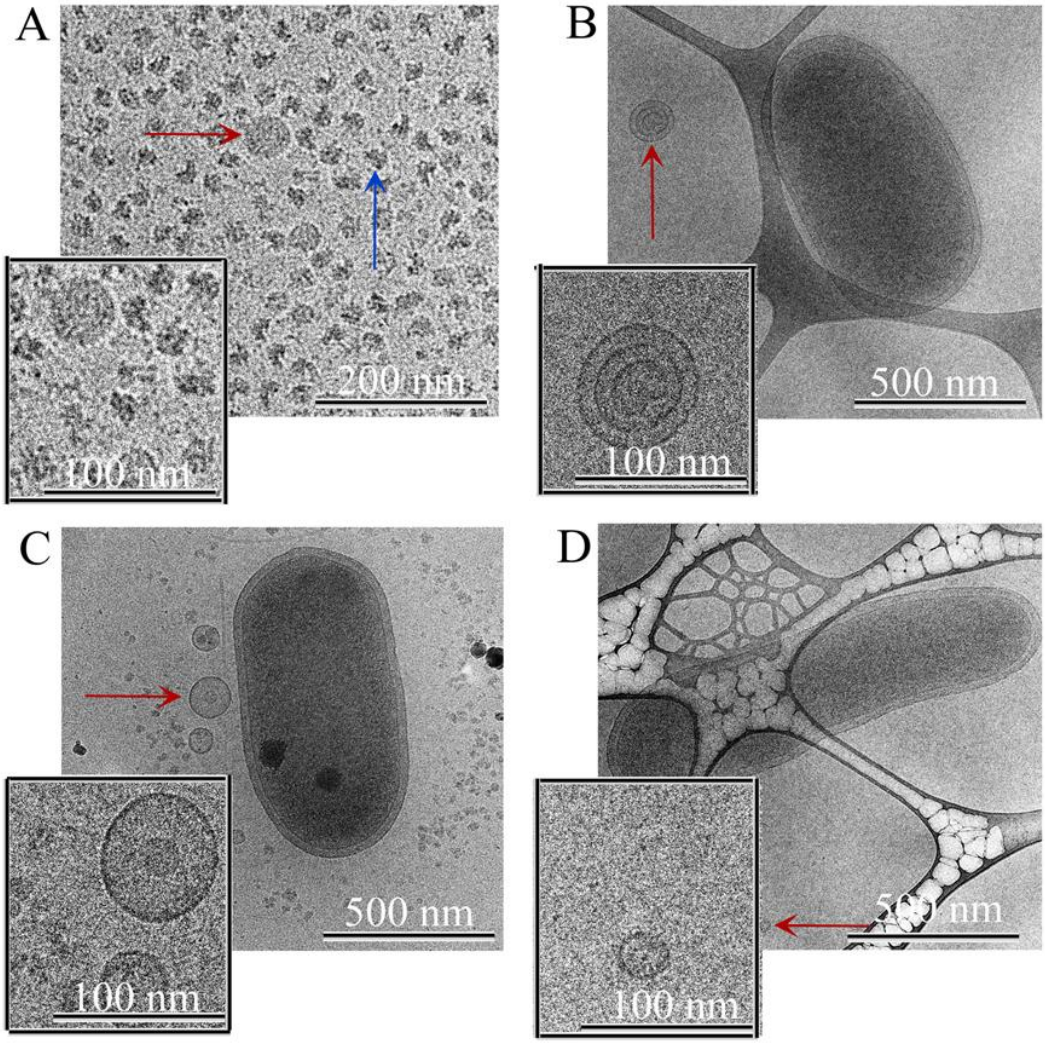
503

504 **Figure 2: Tracking periodic changes of non-ribosomal particles in ribosome**
505 **preparation.** (A,C,E) Cryo-TEM images show the presence of nano-scale spherical
506 structures (marked with red arrow) along with the ribosomes (blue arrow) after 6 hours of
507 incubation of ribosomes at 37 ^{o}C. As the size of the small nano-scale structures (red arrows)
508 increased with time, the number of ribosome particles simultaneously decreased. (B, D. F)
509 The process described above was found to speed up in the presence of added intrinsically
510 disordered protein, like alpha synuclein. (C) Large structures (500-800 nm) were observed
511 after 22 hours of incubation of ribosomes, whereas, (D) particles of similar size were formed

512 within 15 hours in the presence of externally added protein. At 66 hours, transparent cell-like
513 structures formed in ribosome preparation (E). In presence of the protein (F) bigger size
514 particles formed at similar time points.
515
516 **Figure 3: Localization of tagged-protein inside the cell-like living entity by confocal**
517 **microscopy.** (A) Confocal imaging showed fibrillar structures formed by the Alexa488
518 tagged α-synuclein (green fluorescence) when incubated at 37°C with shaking. (B)
519 Fluorescence image following incubation of the protein along with yeast 80S ribosome
520 preparation under similar conditions showed no fibrils; instead, some spherical structures of
521 05-1.5 um in size, detected by green fluorescence of Alexa488, co-localized inside. (C)
522 Fluorescence imaging of the same region of interest (ROI) stained with RNA specific F-22
523 dye, also confirmed them as a living entities. (D,E) An overlay of green and red fluorescence
524 and DIC images of ROI showed the green and red fluorescence to be localized within the
525 cell-like structures. (F-I) Using Hoechst stain, fluorescence and DIC imaging showed co-
526 localization of both protein and DNA inside such structures.
527
528 **Figure 4: Phylogenetic identification of the bacteria by 16S rDNA sequencing.** (A)
529 Chromatogram of the *"query sequence"*, obtained from sequencing the 16s rRNA gene
530 segment, amplified using universally conserved primers 27F and 1492R. (B) Nucleotide
531 BLAST output of the *"query sequence"*, showing high sequence identity with different
532 species of *Bacillus* genus. (C) Alignment of the *"query sequence"* with all the 16S rRNA
533 gene sequences (from different *Bacillus* species), showing maximum sequence identity that
534 was done using MEGA7. The phylogenetic tree was then constructed using "neighbor
535 joining method" within MEGA7.
536

537 **Figure 5: Confirmation of the presence of living entities.** TEM images show (A, B)
538 vegetative forms of bacterial cells when yeast ribosome preparation was cultured in Luria
539 Broth (LB). Following overnight ethanol treatment, a precipitate was found at the bottom of
540 the tube. The sample was subjected to centrifugation and (C) shrunken cells with fibrils
541 coming off the structure were observed in the pellet, whereas (D) supernatant was full of
542 fibrillar structures. (E) Nano-sized (20-50 nm) spherical structures (termed here as 'nano-
543 spores') appeared in the supernatant upon prolonged salt-ethanol treatment and subsequent
544 centrifugation. (F) TEM image shows growth of bacterial cells when the 'nano-spores'
545 (formed under salt-ethanol stress) were allowed to grow in LB.
546
547 **Figure 6: Detailed strategy to track nano-spore formation from bacterial cells under**
548 **salt-ethanol stress.** (A) TEM images of bacterial colony re-suspended in buffer.
549 Centrifugation on first day following salt-ethanol treatment (marked as Procedure 1) showed
550 (B) shrunken cells in pellet and (C) fibrillar structures in supernatant. After two days of
551 treatment, (D) shrunken cells along with nano-scale spherical structures in the vicinity of the
552 cells were observed in pellet, and (E) fibrillar structures were observed in supernatant as
553 before. On the fourth day of treatment, upon centrifugation, supernatant and pellet fractions
554 were collected. Supernatant fraction was again filtered through 0.22 micron syringe filter,
555 followed by ultracentrifugation at the same speed used to precipitate yeast ribosome (marked
556 as Procedure 2). (F) Few cells in pellet were seen, while, (G) 'nano-spores' (~20-80 nm)
557 were detected in the supernatant fraction. The size of the cells (seen in pellet) gradually
558 decreased from 1000-1200 nm to 800-1000 nm within 24 hours and further to 500-800 nm in
559 48 hours. Pellet obtained after two days of salt-ethanol treatment was separately subjected to
560 further salt-ethanol treatment (Procedure 1), and on the fourth day supernatant and pellet
561 fractions were collected following centrifugation. (H) 'Nano-spores' in the supernatant

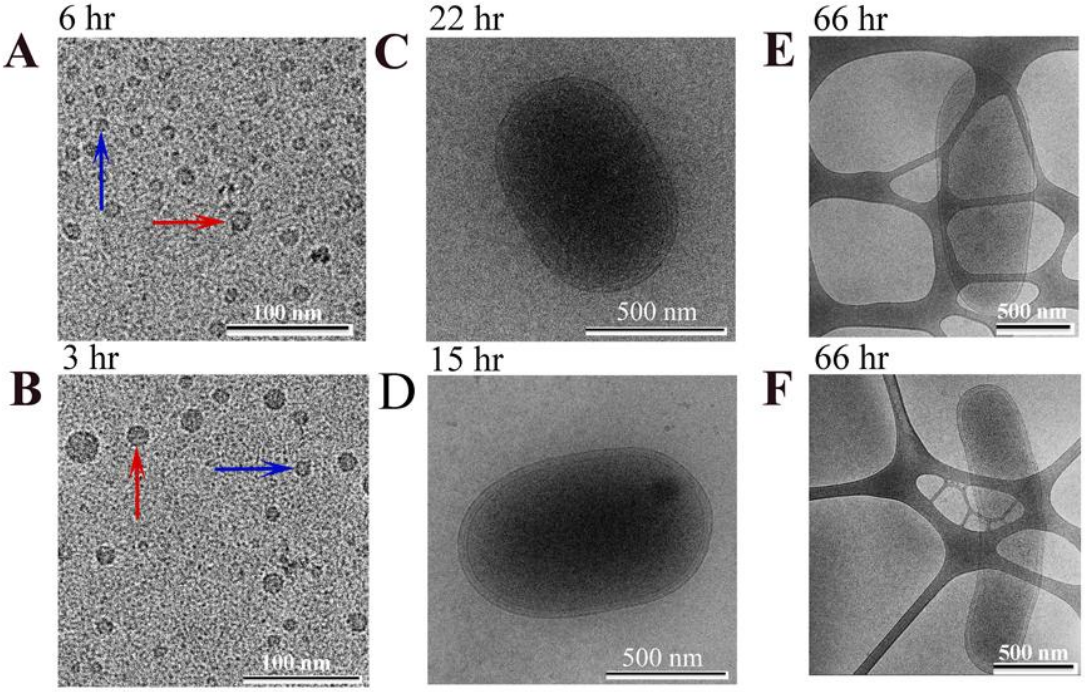
562 (following Procedure 2), and (I) shrunken cells in pellet were visible. The pellet obtained
563 after two days of salt-ethanol treatment when subjected to Procedure 2, also led to the
564 separation of (J) small size spores (30-60 nm) in solution. (K) Cultures of each of these
565 'nano-spores' (marked with red circles) on solid or liquid media, led to the formation of
566 bacterial growth again.
567
568 **Figure 7: Characterisation of *B.cereus* nano-spores**. (A) Mature *B.cereus* cells as seen
569 under phase-contrast microscope. (B) Nano entities (termed as 'nano-spores') observed under
570 Cryo-TEM after subjecting the *B.cereus* cells to osmotic stress. (C) Rod-shaped mature
571 bacilli cell-like structures observed under Cryo-TEM after incubating the 'nano-spores'
572 (observed in (B)) at 37°C with shaking in LB medium. (D) Transparent cell-like structures
573 observed under Cryo-TEM after incubating the 'nano-spores' (observed in (B)) at 37°C with
574 shaking in *B.cereus* minimal culture media. (E) *B.cereus* 'nano-spores' as observed under
575 Cryo-TEM. (F) Ungerminated *B.cereus* spores observed under phase-contrast microscope
576 after incubating the *B.cereus* 'nano-spores' at 37°C with shaking in LB medium for ~14hr.
577 (G) Germinated *B.cereus* spores or cells observed under phase-contrast microscope after
578 incubating the *B.cereus* 'nano-spores' at 37°C with shaking in LB medium for ~22hr. (H)
579 Bacterial colony was observed after overnight incubation at 37°C from *B.cereus* 'nano-
580 spores', as well as from ungerminated *B.cereus* spores (I) following heat treatment, but not
581 from germinated *B.cereus* spores or cells (J).
582



583

584 **Figure 1**

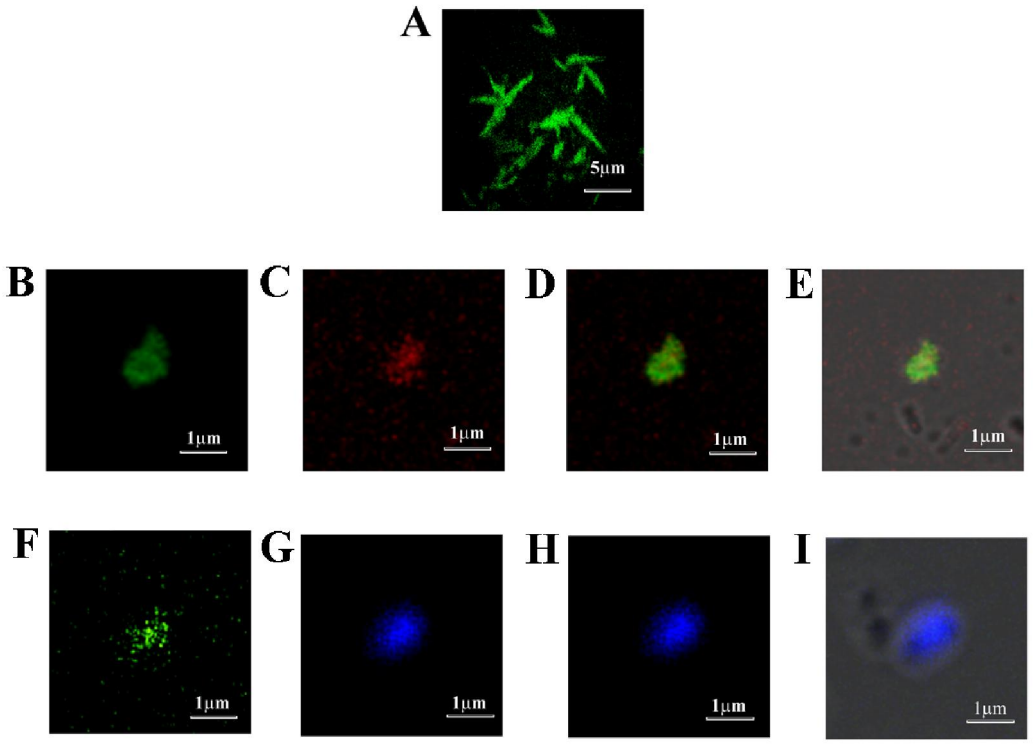
585



586

587 **Figure 2**

588



589

590 **Figure 3**

591

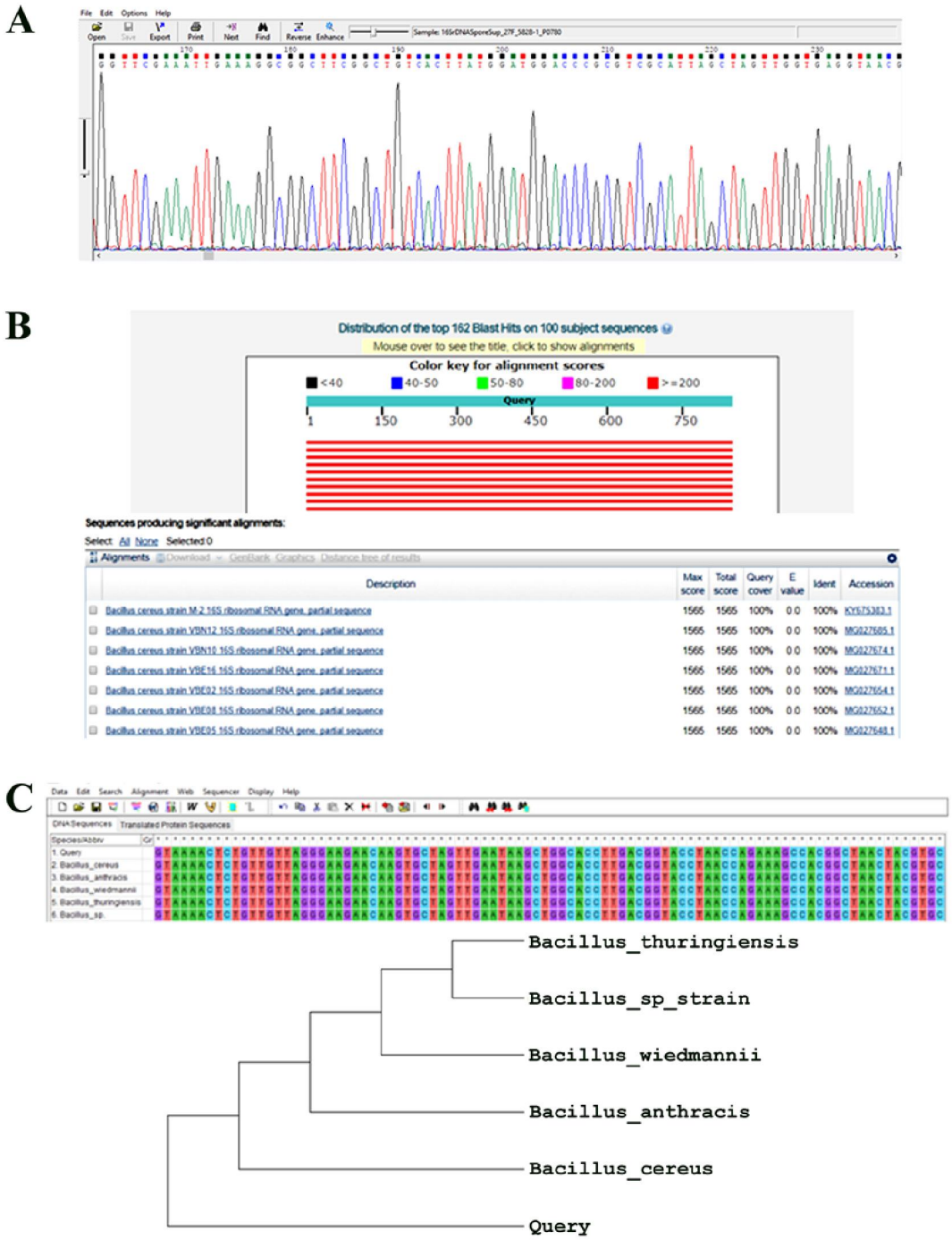

A
B
Distribution of the top 162 Blast Hits on 100 subject sequences
Mouse over to see the title, click to show alignments
Color key for alignment scores
<40
40-50
50-80
80-200
>=200
Query
1
150
300
450
600
750
Sequences producing significant alignments:
Select All None Selected 0
Alignments
Description
Max score
Total score
Query cover
E value
Ident
Accession
C
Bacillus_thuringiensis
Bacillus_sp_strain
Bacillus_wiedmannii
Bacillus_anthracis
Bacillus_cereus
Query

Figure 4

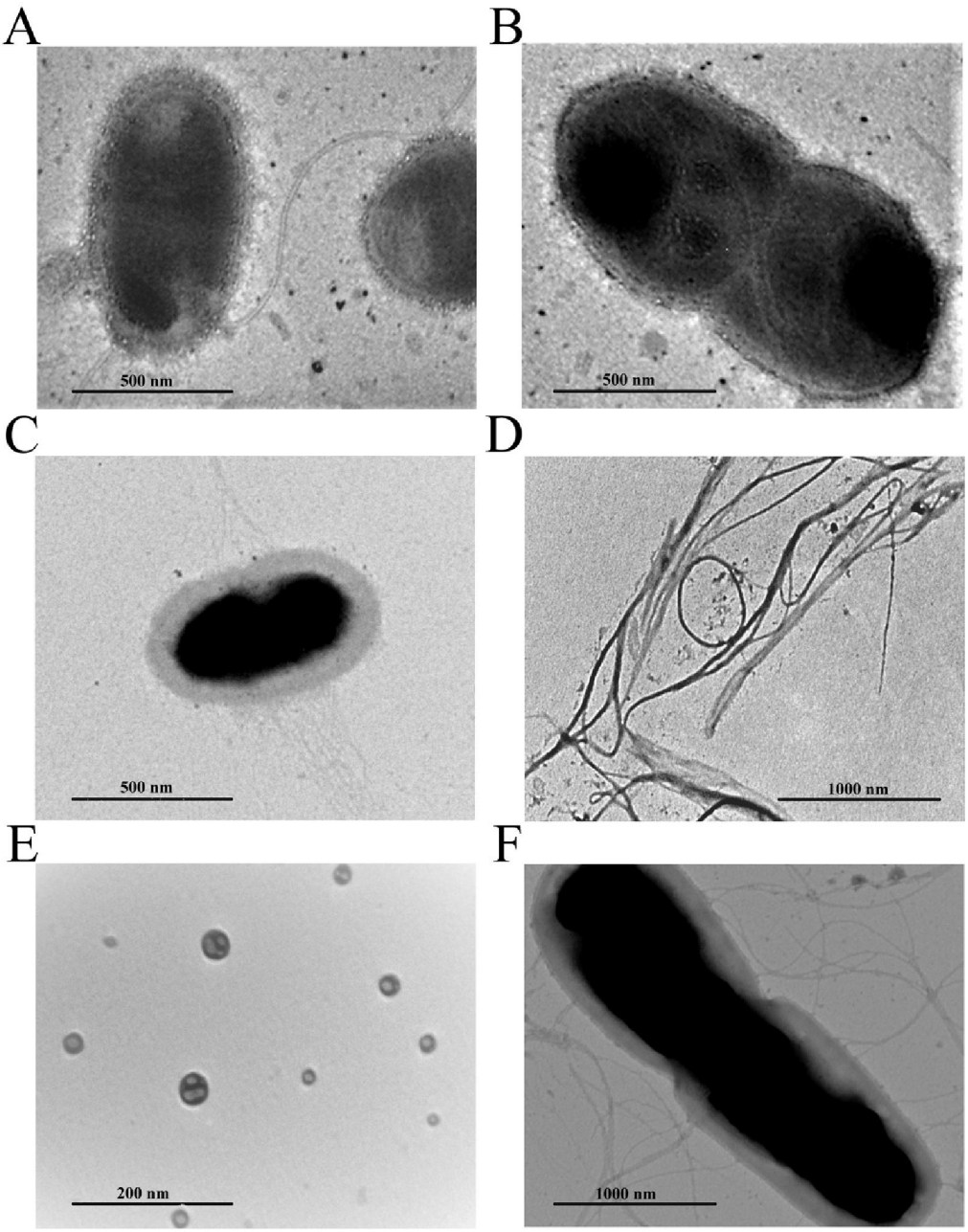


Figure 5

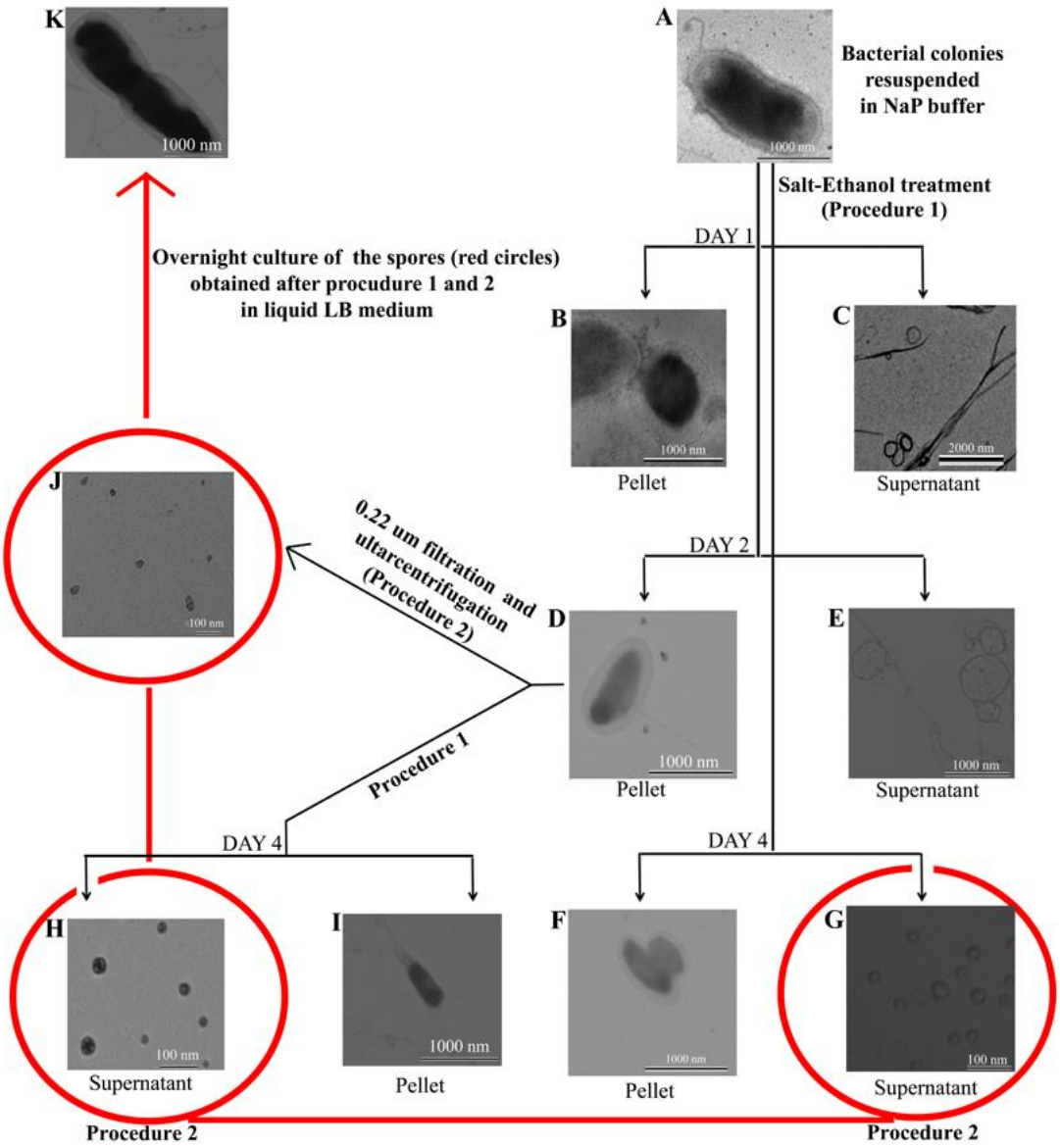

K
1000 nm
A
1000 nm
Bacterial colonies
resuspended
in NaP buffer
Salt-Ethanol treatment
(Procedure 1)
DAY 1
Overnight culture of the spores (red circles)
obtained after procudure 1 and 2
in liquid LB medium
B
1000 nm
Pellet
C
2000 nm
Supernatant
J
100 nm
0.22 um filtration and
ultarcentrifugation
(Procedure 2)
DAY 2
D
1000 nm
Pellet
E
1000 nm
Supernatant
Procedure 1
DAY 4
DAY 4
H
100 nm
Supernatant
I
1000 nm
Pellet
F
1000 nm
Pellet
G
100 nm
Supernatant
Procedure 2
Procedure 2

Figure 6

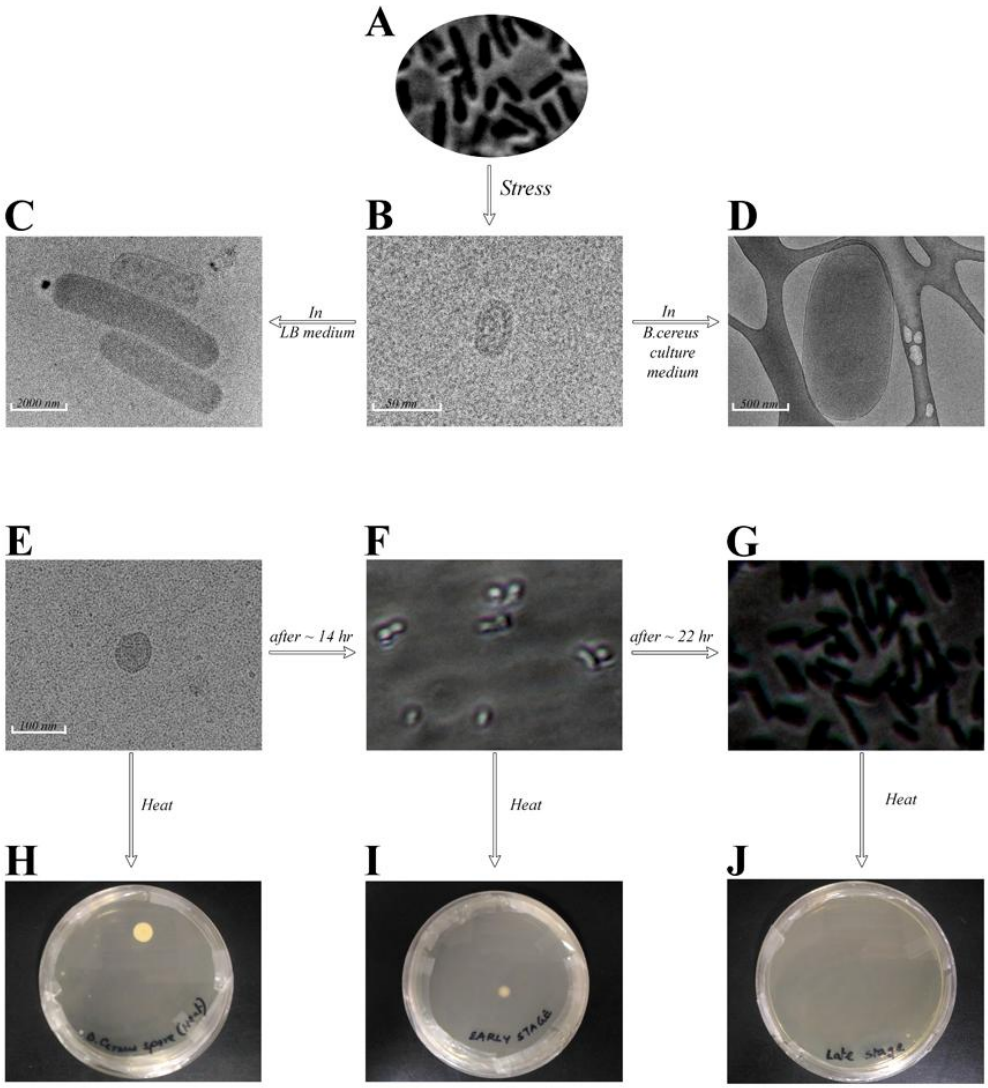

A
Stress
C
B
D
In
LB medium
In
B.cereus
culture
medium
2000 nm
500 nm
500 nm
E
F
G
after ~ 14 hr
after ~ 22 hr
100 nm
Heat
Heat
Heat
H
I
J
EARLY STAGE
Late stage

Figure 7