Electron microscopy reveals unique nano forms of bacterial spores

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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 > 500 nm. Nano-forms of bacteria have been reported but the notion still remains controversial.

In this study we provide direct evidence of living nano-entities (termed here as 'nano-spores') formed by a bacterial species of genus *Bacillus* under prolonged stress, which are capable of escaping though standard sterile filtration procedure. We further demonstrate the transformation of the nano-spores to mature forms upon nutrient supply and again conversion of mature forms to nano-spores under salt-ethanol stress. Our study not only unfurls the ability of bacteria to rapidly get transformed into yet-unknown spore forms in order to survive under harsh environment, but also brings to light the existence of smallest possible form of life.

Large environmental fluctuations induce transformation of a bacterial cell into its‘starvation form’(*1*), which eventually gets converted into the most resistant living form, the‘endospore’(*2*). Based on recent structural studies of the endospore architecture of gram negative as well as gram positive bacteria(*3, 4*), it has been hypothesized that a spore is the common ancestor of all bacteria(*5*). Emerging evidences suggest that a wide range of bacteria are capable of sporulation(*6, 7*). The reported sizes of the endospores differ considerably(*8*). 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, 9-11*), which has initiated a long-standing debate(*12-14*).

Here, we, for the first time, provide convincing evidence of the presence of living nano-scale transparent structures (termed here as nano-spores) in yeast ribosome preparation (sizes of ~20-50 nm). In sharp contrast to previous reports on ‘nanobes’(*9, 10*), we not only identified nano-spores and tracked their maturation, but also induced nano-spore formation from mature bacterial cells under stress. Moreover, the nano-spores thus formed could be converted to mature bacterial cells when nutrients were supplied.

Electron microscopic visualization of a preparation of yeast ribosomes revealing conspicuous small spherical structures (20-50nm) along with the ribosome particles attracted our curious attention (Fig.1A). Intriguingly, when the yeast ribosomal preparation was incubated at 37^{o}C with shaking, there was an indication of the formation of larger particles (Fig. S1).Electron microscopy revealed the presence of virtually transparent cell-like structures (600 nm-1 μm)with double membranes (Fig.1B-D, Fig.S2A-D). These structures sharedstriking morphological resemblance with the ultra-small cells recently identified(*15*). Nano-scale spherical entities were also detected at the vicinity of the cell-like structures (Fig.1B-D insets). It must be emphasized that these cell-like particles were obtained only after

incubation at 37^{o}C, and they were not detected in the initial ribosomal preparation in spite of extensive search through several TEM grids.

Ribosome samples at different time points of incubation were visualized by TEM. We found small spherical structures initially detected (Fig.1), grew in size as the incubation progressed (Fig. 2A,C,E; Fig.S2E). Concomitant reduction in the number of ribosomal particles was observed (Fig. 2). Presumably, upon incubation at 37^{o}C, ribosomal RNAs and proteins disintegrated, which provided nutrients for the nano-scale structures to grow in size into larger entities (Fig.2C,E). It was further observed that the presence of a disordered protein as an additional nutrient source expedited the maturation of the cell-like structures (Fig.2B, D, F; Fig.S2F).

The purified yeast 80S was spotted directly on Luria agar plates and incubated overnight at 37^{o}C. Distinct bacterial colonies were observed confirming that the nano-scale, spore-like structures, observed initially in the ribosomal preparation (Fig.1A), were living entities. We termed these nano-scale spherical structures as ‘nano-spores’. However, in contrast to the conventional dense structures known for bacterial endospore, the nano-spores detected here were almost transparent in nature (Fig. 1, 2). It should be noted here that the ribosome storage buffer was also spotted on Luria agar as control but no bacterial growth was observed after similar incubation. Phylogenetic analysis of the universally conserved region of the16SrRNA gene amplified from genomic DNA extracted from these bacterial cells suggested that they belonged to the genus *Bacillus* (most related to *Bacillus cereus*; Fig.S3). However, the sizes of the cells were found to be much smaller (1-2µm) compared to the reported size of *Bacillus* and resembled the morphology of the forms of large spores or vegetative cells(*16*). Conceivably, the *Bacillus* nano-spores got co-purified with the yeast 80S ribosomes by virtue of their comparable sizes (~40-50 nm). Notably, association between *Bacillus* and yeast has been reported earlier(*17*).

We examined the cultured bacterial cells under electron microscope. Remarkable network of pili formation in order to exchange of genetic materials was observed (Fig.3A, B) indicating its ability to rapidly grow under favourable conditions. In some views it appeared that the cells were embedded within a glutinous sack (Fig. S4A, B), which seemed to be a self-produced matrix. Bio film formation has been reported earlier for various *Bacillus* species(*18-21*). In line with an earlier observation(*16*), the *Bacillus* strain in this study was found to be completely resistant to antibiotics, like ampicillin and kanamycin, whereas, a relatively high concentration of chloramphenicol (~70-140 µg/ml) and tetracycline partially inhibited bacterial growth (Fig.S4C, D).

Bacterial cells are known to form highly resistant endospores during unfavourable environmental conditions. Hence, it may be assumed that mature bacterial cells (Fig.3A, B), which were obtained from the nano-spores (Fig. 1), should also be transformed to spores again, provided necessary external stress is applied. To verify this hypothesis, the cells were submerged in a solution of absolute ethanol and ammonium acetate (salt-ethanol treatment) and kept at -80^{o}C (see online Methods). After overnight incubation, a precipitate was detected upon centrifugation which was found to be shrunken cells when visualized under electron microscope (Fig.3C).Unusual fibrillar structures were detected in the supernatant (Fig.3D; Fig.S4E). Interestingly, a previous high-resolution AFM study reported that spore coat is composed of fibrillar structures(*22*). Other studies also claimed that the amyloid fibrils form protective coat for the bacterial cells(*23*). It is quite possible that under stress amyloid-like fibrils (seen in supernatant) were released from the cell surface. It should be noted that *E. coli* cells lysed completely within overnight when subjected to similar treatment (Fig.S4F).

The salt-ethanol treatment procedure consisted of multiple steps, which are described in detail in Fig.S5. Formation of the nano-spores (~20-80 nm) was detected eventually in the

fourth day supernatant when the sample was centrifuged (Fig.3E). We also detected the presence of some particles, less than 200nm in size; containing unusual structural features (see Fig. S6).

The nano-spores thus obtained could be reverted back to vegetative forms when grown overnight in LB broth or in agar plate at 37^{o}C (Fig. 3F). 16S rRNA sequence phylogenetic analysis of those cells confirmed that this species belonged to the genus *Bacillus*. Thus, we tracked the complete cycle of transformation of the bacterium from nano-spores (initially observed in our ribosomal preparation) to vegetative cells, back to nano-spores by applying prolonged osmotic stress, which can again grow to mature cells (Fig.4) in favourable environment, confirming that the 'nano-spore' identified in this study are living entities.

Concluding remarks

Existence of nano bacteria was claimed in late 90's (*9*, *10*), although their implications as a living organism have never been established. This issue has particularly become more controversial when it was found that non-organic materials, like calcium and phosphate ions, have been found to hijack proteins from cell culture media to grow like nanobacteria. It was argued(*13*) that the so called 'nanobes' are not 'living units', and could simply be fragmented portions of larger cells. We, for the first time, not only have identified nano-scale spore-like structures but also provided unambiguous evidence that the structures we identified are 'living units'.

Although the bacterium likely belongs to a known species of the genus *Bacillus*, in the present study we have identified unprecedented characteristics of this species, particularly, the rapid sporulation process and fascinating characteristics of the nano-spores.

It is evident from our study that nano-spores, by virtue of their size similarity with ribosome, can get purified together with the organelle. However, it is not clear though

whether the spores are engulfed inside the yeast cells or do they remain attached to the cell surface. Apparently other microbes outnumber them in normal environment and hence they prefer to stay in spore form unless favourable condition is available(*24*). The small size of the spores, which would make a typical sterile filtration process ineffective, and their persistence even after salt-alcohol treatment requires serious attention, for example, in case of hospital-acquired antibiotic resistance and microbial survival in extraterrestrial space.

Author contributions

SG and BC performed experiments to monitor bacterial transformation to spore and spore to mature bacteria, 16S rRNA preparation for sequencing. SD performed antibiotic experiments. RC analyzed the 16S rRNA gene sequencing data and shared expertise to critically check the results. CB performed TEM and cryo-TEM surveys, KC and JS conceived the project, supervised research and JS, KC and RC wrote the paper with inputs from SG, BC.

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25. Acknowledgements

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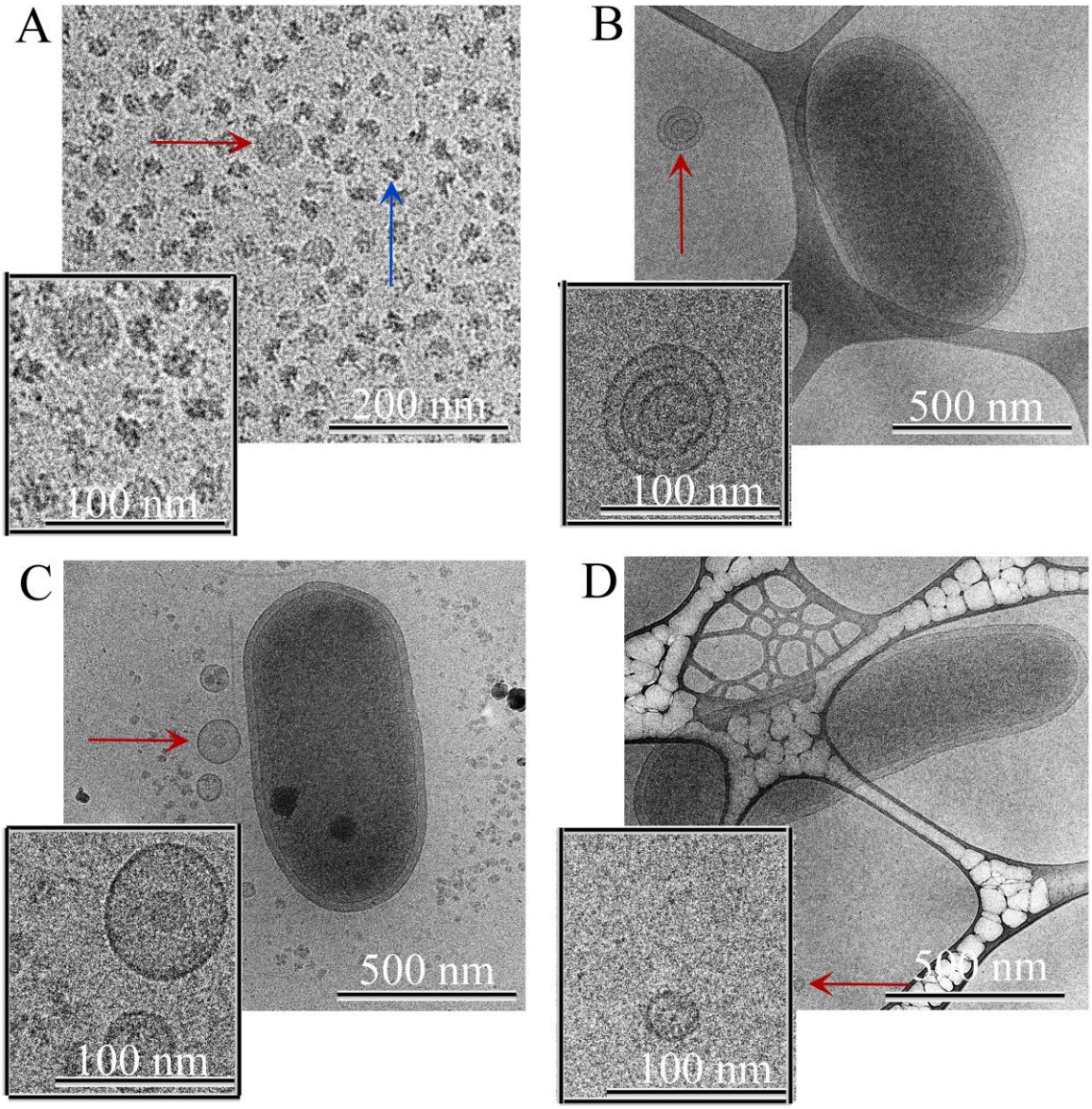

Figure 1: Cell-like structures observed in ribosome preparation. (A) A cryo-TEM image shows small spherical structures (marked with red arrow) along with ribosome particles (blue arrow) purified from yeast cells. (B-D) Large transparent cell-like structures (200 nm -1.5 µm) were detected in cryo-TEM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

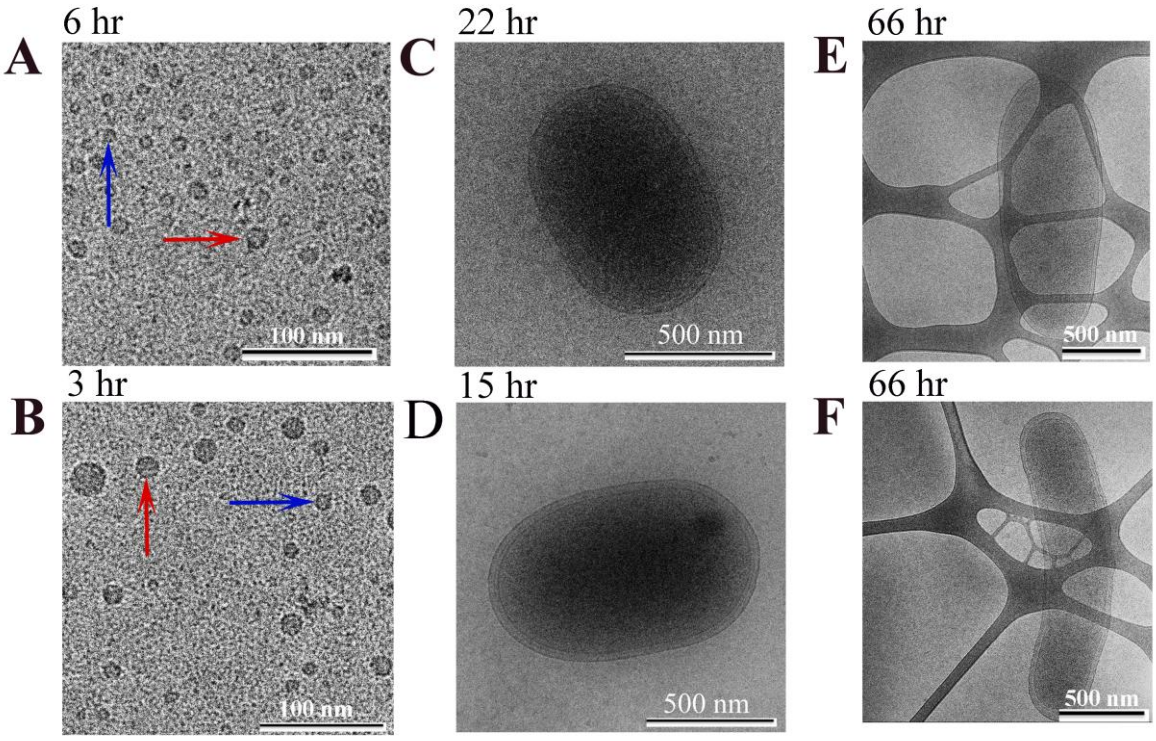

Figure 2: Tracking periodic changes of non-ribosomal particles in ribosome preparation. (A,C,E) Cryo-TEM images show the presence of nano-scale spherical structures (marked with red arrow) along with ribosome (blue arrow) after 6 hours of incubation of ribosomes at 37 ^{o}C. The size of the small nano-scale structures (red arrows) increased with time, simultaneously the number of ribosome particles decreased. (B, D. F) The process described above was found to speed up in the presence of added intrinsically disordered protein, like alpha synuclein. (C) Large structures (500-800 nm) were observed after 22 hours of incubation of ribosomes, whereas, (D) particles of similar size were formed within 15 hours in the presence of externally added protein. At 66 hours, transparent cell-like structures formed in ribosome preparation (E). In presence of the protein (F) bigger size particles formed at similar time points.

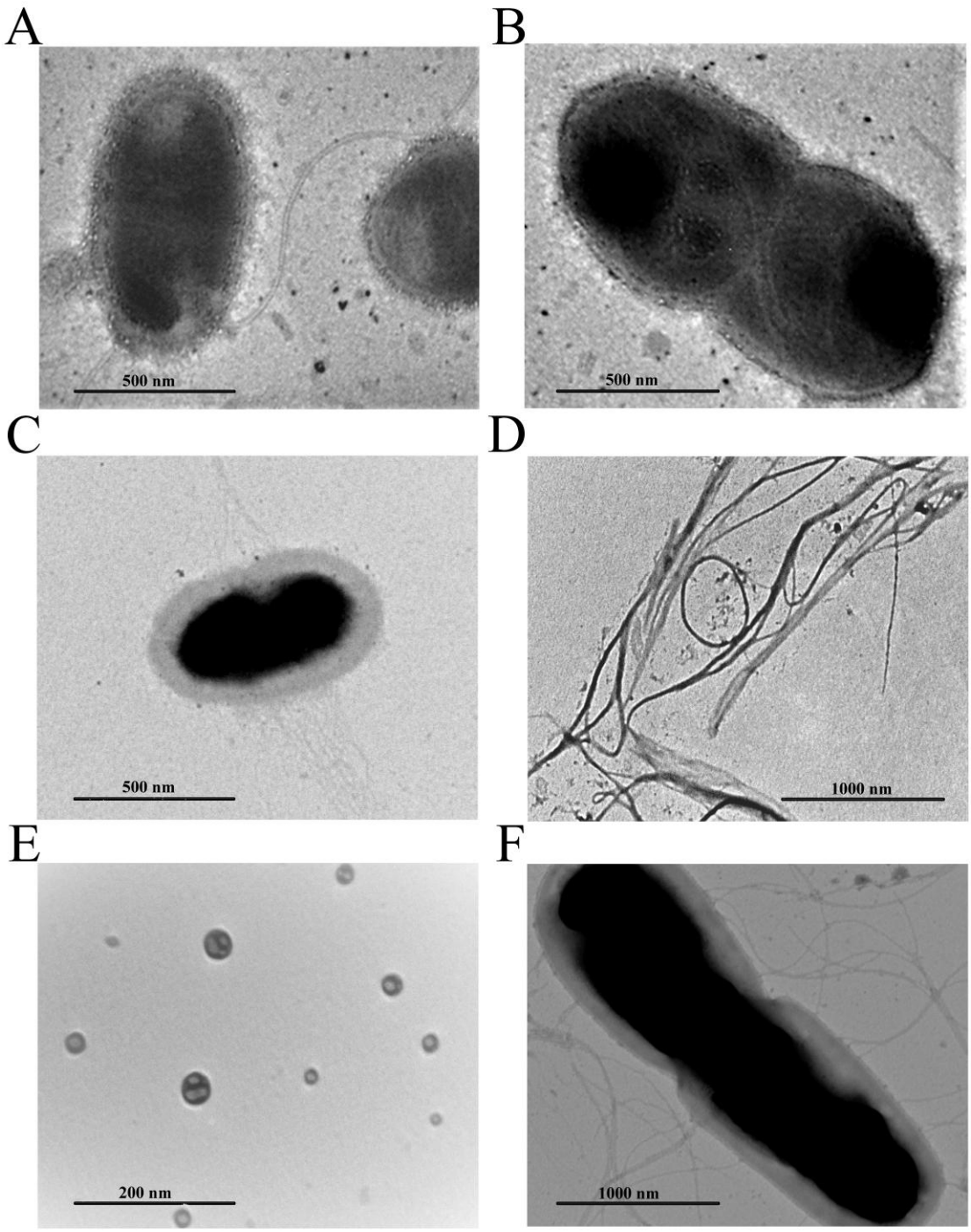

Figure 3: Confirmation of the presence of living entities: TEM images show (A, B) vegetative forms of bacterial cell when Yeast ribosome preparation was cultured in Luria Broth (LB). Following overnight ethanol treatment precipitate was found at the bottom of the tube. The sample was subjected to centrifugation and (C) shrunken cells with fibrils coming off the structure were observed in the pellet, whereas (D) supernatant was full of fibrillar structures. (E) Nano-sized (20-50 nm) spherical structures (termed here as nono-spores) appeared in the supernatant upon prolonged salt-ethanol treatment and subsequent centrifugation. (F) TEM image shows growth of bacterial cells when the nano-spores (formed under salt-ethanol stress) were allowed to grow in LB.

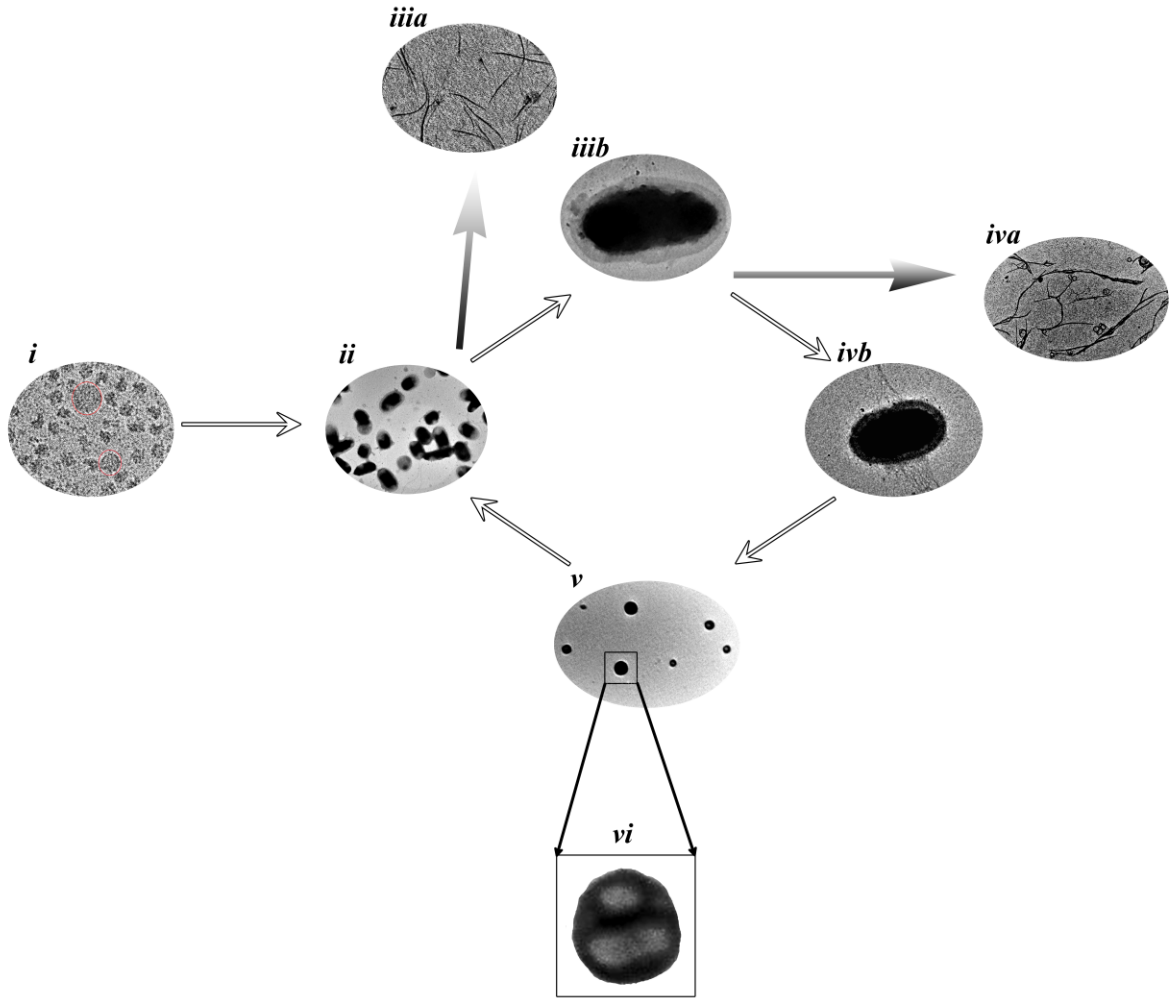

Figure 4: Schematic representation of the life cycle of maturation of the bacterial species. Initially spherical structures were seen in the Yeast ribosome preparation (i). Formation of larger spores/vegetative cells occurred when Yeast ribosomes were added directly to culture media (ii). Upon overnight salt-ethanol stress followed by centrifugation supernatant revealed fibrils (iiia) while shrunken cells were found in pellet (iiib). Continued salt-ethanol stress produced more fibrils in the supernatant (iva) and smaller cells in pellet (ivb). Nano-spores were eventually visible when the supernatant was filtered through 0.22 micron syringe filter following prolonged salt-ethanol stress treatment (v). Close up view of TEM image of a nano spore (vi). The nano-spores (formed under salt-ethanol stress), when cultured in LB, again transformed to bacterial cells (ii).