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Title : **Visualization of BrdU-labeled DNA in cyanobacterial cells by Hilbert Differential Contrast Transmission Electron Microscopy**

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Running title: BrdU-labeled cyanobacterial DNA visualized by HDC TEM

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Summary:

We have attempted to observe the native shape of DNA in rapidly frozen whole cyanobacterial cells through 5-bromo-2-deoxyuridine (BrdU) incorporation and visualization with a Hilbert Differential Contrast Transmission Electron Microscopy (HDC TEM). The incorporation of BrdU into the DNA of *Synechococcus elongatus* PCC 7942 was confirmed with fluorescently labeled anti-BrdU antibodies and through EDX analysis of ultrathin sections. HDC TEM observed cells that had incorporated BrdU into their DNA exhibited electron dense areas at the location corresponding to fluorescently labeled BrdU. Since various strings and strands were observed in high contrast with the HDC TEM, we conclude that the method promises to allow us to identify and understand bulk structural changes of the *in vivo* DNA and the nucleoid through observation at high resolution.

Introduction:

Rapid freezing is currently the most promising method for preserving the native state of cellular ultrastructure. In our previous work, we have studied ice-embedded whole cyanobacterial cells and their components using zero-loss energy-filtered cryo TEM at intermediate accelerating voltage (Kaneko *et al*., 2005; Kaneko *et al*., 2006).

Others have studied similar material using this technique (Ting *et al*., 2007; Iancu *et al*., 2007). However in our case, we use Hilbert Differential Contrast Transmission Electron Microscopy (HDC TEM), a mode of in-focus phase-contrast imaging (Nagayama, 2005; Nagayama & Danev, 2008) which produces structural images similar to the differential interference contrast (DIC) images of light microscopy. This is accomplished by inserting a half-plane phase plate at the back focal plane of the objective lens (Danev *et al.*, 2002). The most obvious advantage of this technique is that it provides much higher image contrast than conventional underfocus phase-contrast imaging. The identification of thylakoid membranes (T), carboxysomes (C) and lipid droplets (L) in the HDC TEM image of a cyanobacterial cell (Fig 1A) can be confirmed by comparison with a conventionally prepared ultra-thin sectioned image (Fig 1B). There are however striking differences between these two images. First, the rapidly frozen whole cyanobacterial cell (observed by HDC TEM) is surrounded by smooth cell walls, which appear in high contrast even without staining, instead of the typical ragged cell walls in the conventionally prepared TEM image. Second, a large spherical polyphosphate body (P) with associated structures is prominently observed in the HDC TEM image of the rapidly frozen whole cyanobacterial cell. Some of these associated structures can be observed by staining in conventionally prepared TEM

images, but large polyphosphate bodies contract considerably during specimen preparation and shrunken polyphosphate bodies with a diameter bigger than the thickness of the ultrathin sections tend to get detached during sectioning, resulting in obvious holes in the sections. Third, while the cytoplasm is packed with various particles, filaments, and strands in the HDC TEM image of the rapidly frozen whole cyanobacterial cell, in the conventionally prepared specimen there are many empty spaces in the cytoplasm where distinct DNA strands of either fine or bulky form remain (as also seen in Hobot *et al*., 1985). It is clear that the combination of vitreous freezing and cryo HDC TEM imaging allows visualization of cellular structures and components in close to the living state and with high contrast. It is thus especially interesting to observe DNA in the cyanobacterial cell with this combination of techniques. We attempted to impart further contrast to DNA by use of 5-bromo-2-deoxyuridine (BrdU). BrdU has been widely used in cell biological research to label newly synthesized DNA *in vivo*. BrdU-labeled DNA has been detected immunohistochemically by light microscopy as well as by electron microscopy (Ngwenya *et al*., 2005). We examined ice embedded cyanobacterial cells cultured in BrdU containing media by zero-loss energy-filtered 300 kV HDC TEM. During different stages of cell activity, including cell elongation and cell division, the bulk

shape of the DNA and the nucleoid morphology are expected to change considerably. We hope that this technique will allow us to study these changes in close to native state in intact cells.

Materials and Methods:

Synechococcus elongatus PCC 7942 was cultivated in liquid BG-11 medium under continuous light (60 μE・$m^{-2}s^{-1}$) at 30°C. The cells were transferred to fresh media every 7 days and the second day culture was used for BrdU incorporation. BrdU (Sigma) was added to the medium to a final concentration of 10 μM. After 2,12, or 24 h of culture in BrdU containing media, cells were collected by centrifugation and rinsed in 0.2 M sucrose solution three times.

For preparation of ice embedded cyanobacteria, the collected cells were dropped on a copper grid coated with carbon film. After removing excess liquid carefully with the tip of a filter paper, the sample was rapidly frozen in liquid ethane using a LEICA rapid-freezing device (LEICA EM CPC). The grid with ice-embedded cells was transferred to the specimen chamber of the TEM using a cryo-transfer system. The specimen chamber was cooled with liquid helium. For observation, a JEOL JEM-3100FFC electron microscope was operated at 300 kV accelerating voltage, in

zero-loss energy-filtered mode, with the HDC phase plate inserted into the back focal plane of the objective lens.

For conventional chemical fixation, the cells were fixed in 2% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.0) for 2 h at room temperature and in a refrigerator overnight. After rinsing in buffer, the cells were postfixed in 2% OsO_4 in buffer for 2 h at room temperature. They were then dehydrated in an acetone series and embedded in Spurr's resin. Ultrathin sections (ca. 90 nm in thickness estimated from the silver-gold interference color) were cut with a diamond knife on a Sorvall MT2-B ultramicrotome. After staining with uranyl acetate and lead citrate, sections were observed with a Hitachi H-7500 TEM at an accelerating voltage of 100 kV.

For Energy Dispersive X-ray (EDX) analysis, post fixation with OsO_4 was omitted from the chemical fixation. Ultrathin sections (ca. 120 nm in thickness, estimated from gold interference color) were made from dehydrated and resin embedded samples and mounted on copper grids. They were analyzed with a JEOL JEM-2100F equipped with a JEOL JED-2300T EDX system.

For DAPI staining of DNA, 2 μl of DAPI solution (10 μg/ml) was added to 20 μl of sample. After 10 min incubation in the dark, the specimens were observed with a Nikon fluorescent microscope under UV illumination.

For detection of BrdU incorporation, FITC conjugated monoclonal antibodies against BrdU (BrdU-Proliferation Marker supplied by abcam, ab7384) was applied following the manufacture's protocol. Stained cells were observed with an Olympus fluorescent microscope equipped with a DP-50 CCD camera.

Results and Discussion:

After rapidly growing cells were cultured in BrdU containing media for 24 h, incorporation of BrdU was confirmed by fluorescent microscopy using FITC labeled anti-BrdU antibodies (Fig 2B). Incorporation of BrdU was detected often at the positions corresponding to DAPI stained DNA (Fig 2A). When cells which had not been incubated with BrdU were treated with FITC labeled anti-BrdU antibodies for control, no intense fluorescence was observed in the cells (Fig 2C). It is known that the *Synechococcus elongatus* PCC 7942 cell contains 2 to 5 copies of its genome (Mori *et al*., 1996). The observed localization of BrdU or DAPI stained DNA may thus reflect the localization of each genome. Incorporation of BrdU was further confirmed by EDX analysis of ultrathin sections. Localization of Br and P at a small polyphosphate body and a nearby DNA-like structure was confirmed by line analysis (Fig 3). Localization of Br at polyphosphate bodies may be due to the attachment of

DNA fibrils to the body since close association of the fibrils and polyphosphate bodies are often observed (Fig 1B). Indeed, these fibrils were stained intensely with osmium amine-SO_2 which is specific for DNA (data not shown).

When rapidly frozen BrdU incorporating cells were observed by HDC TEM, electron dense areas (areas in the specimen that more strongly scatter electrons) appeared in certain portions of the cells (Fig 4C). Electron dense areas were also clearly observed when imaged at high underfocus without a phase plate (Fig 4B). In order to confirm that the elevated electron density is in fact due to the BrdU incorporation, the cells incubated with BrdU for 2 h and observed at high underfocus without phase plate are presented in Fig 4A. Except for the polyphosphate body, no electron dense area was obvious in cells incubated for only 2 h. Figure 4C shows the same cells as Fig 4B with HDC TEM imaging. Detailed ultrastructure is greatly enhanced by the effect of the phase plate.

Incorporation of BrdU successfully conferred electron density in ice embedded whole cyanobacterial cells observed with conventional high-underfocus cryo-TEM imaging as well as with HDC TEM, the location of which in both cases closely agreed with fluorescently labeled BrdU. The increase of electron density in the chromosomal DNA area in HDC TEM images was estimated based on the difference in image

intensity between the chromosomal area and the adjacent cytoplasm. Taking the average of 6 cells each, we found that the increase of electron density in the chromosomal DNA area was approximately 30 % in cells incubated for 12 h, and 45 % in cells incubated for 24 h in HDC TEM images. Previous attempts at visualization of BrdU-DNA in thick samples were unsuccessful (Burkholder & Wang, 1978). It is likely that the reasons why BrdU containing DNA could be recognized through its electron density alone in this study are the absence of staining (except for BrdU itself) and the higher accelerating voltage of 300 kV.

Since the typical duplication time of this species under optimal growing conditions is less than 12 h (Mori *et al*., 1996), it is likely that almost all cells in the culture are incorporating BrdU after 24 h in non-synchronized cultures. Some of the genome would undergo two cycles of duplication within this period, possibly resulting in bifilar BrdU incorporation. Depending on the duplicating cycle of each chromosomal DNA, the amount of BrdU contained should be different. It is known that the chromosomal DNA of the species consists of ca. 2700 kbp and about half of it is Adenine-Thymine (AT). Assuming the maximal BrdU incorporation rate of 100%, one can estimate that as many as 1.3 million Br atoms are localized in bifilar BrdU-labeled DNA forming a cloud of increased electron density. The estimated value of Br would be double, if

subsequently duplicated DNA stayed in situ before separating.

In higher magnification, electron dense strands are hardly visible with conventional high-underfocus cryo-TEM in electron dense areas of the cell (Fig 5A). Further detailed ultrastructure could be visualized in the electron dense areas by applying the phase plate (Fig 5B). The electron dense strands observed in these areas are possibly composed of BrdU-labeled DNA. Although numerous similar strands can be observed in HDC TEM images of cells without BrdU labeling, such prominent electron dense strands were not observed (Fig 1A, Fig 5C). Similar DNA conformations can be observed in conventionally prepared ultrathin sections of the cells without BrdU labeling (Fig 1B, 5D) in which fibrils are exhibited by electron dense staining such as uranium and lead. In order to fully confirm DNA strands in HDC TEM images, application of tomography or examination of vitreously frozen seections (Al-Amoudi *et al.*, 2004) will be necessary. Cryo electron tomography has been successfully applied to whole cells of cyanobacteria (Ting *et al.*, 2007) and to isolated carboxysomes (Iancu *et al.*, 2007) for reconstruction of the three dimensional architecture close to the living state. The advantage of cryo TEM is that it not only exhibits ultrastructure close to the living state, but also retains all the *in vivo* molecular constituents. HDC TEM imaging greatly increases the contrast of cryo-TEM

specimens, revealing structures that could easily be missed with conventional high-underfocus imaging. By combining this technique with BrdU labeling of DNA, it may be possible to follow *in vivo* events of DNA synthesis, and bulk structural change of DNA and nucleoid during various cell activities including stress responses (Asadulghani *et al*., 2004; Nitta *et al*., 2005) at high resolution.

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Figure Legends:

Fig 1 (A) A 300 kV HDC TEM image of an ice embedded whole cyanobacterial cell, not incubated with BrdU (near focus).

(B) A 100 kV conventional TEM image of a chemically fixed, resin embedded , sectioned cell without incubation with BrdU. The sections were stained with uranyl acetate and lead citrate, which conferred electron density to strands assumed to be DNA.

C: carboxysomes, L: lipid droplets, P: polyphosphate body (in A) or its residual hole (in B), T: thylakoid membranes, arrows: DNA fibers. Bars = 100 nm.

Fig 2 (A) Fluorescent microscope image of DAPI stained cyanobacterial cells. Autofluorescence of chlorophyll under UV irradiation exhibits red color in cells .

(B) Fluorescent microscope image of cyanobacterial cells cultured with BrdU for 24 h and incubated with FITC conjugated anti BrdU antibody.

(C) Fluorescent microscope image of cyanobacterial cells cultured without BrdU and incubated with FITC conjugated anti BrdU antibody.

Bars = 1 μm.

Fig 3 EDX line analysis of an ultrathin section of cyanobacteria cells cultured with BrdU for 24 h and chemically fixed without osmium. Blue line: phosphorus,

orange line: bromine. Bar = 100 nm.

Fig 4 (A) A 300 kV conventional TEM image of an ice embedded whole cyanobacterial cell incubated with BrdU for 2 h (≈15 μm defocus).

(B) A 300 kV conventional TEM image of an ice embedded whole cyanobacterial cell incubated with BrdU for 24 h (≈15 μm defocus).

(C) A 300 kV HDC TEM image of the same ice embedded whole cyanobacterial cell incubated with BrdU for 24 h (near focus).

Bars = 100 nm.

Fig 5 (A) A 300 kV conventional TEM image of an ice embedded whole cyanobacterial cell incubated with BrdU for 24 h (≈15 μm defocus). A portion of Fig 4B in higher magnification.

(B) A 300 kV HDC TEM image of an ice embedded whole cyanobacterial cell incubated with BrdU for 24 h (near focus). A portion of Fig 4C in higher magnification. This is the same area as shown in Fig 5A. Arrows: putative labeled DNA.

(C) A 300 kV HDC TEM image of an ice embedded whole cyanobacterial cell not

incubated with BrdU (near focus).

(D)A 100 kV conventional TEM image of a chemically fixed, resin embedded, sectioned cell not incubated with BrdU. The sections were stained with uranyl acetate and lead citrate which conferred electron density to strands assumed to be DNA.

Bars = 100 nm.

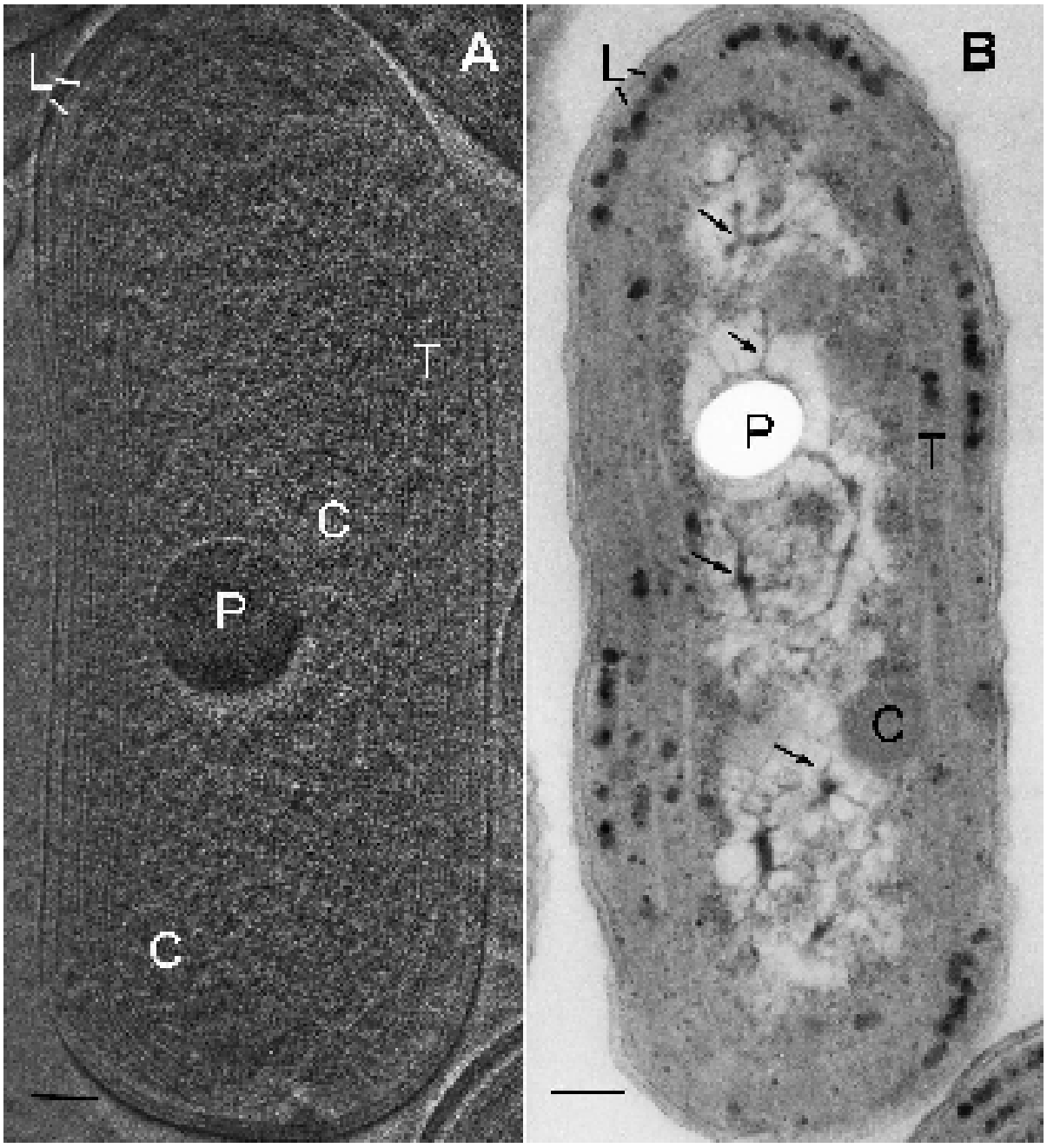

Fig.1

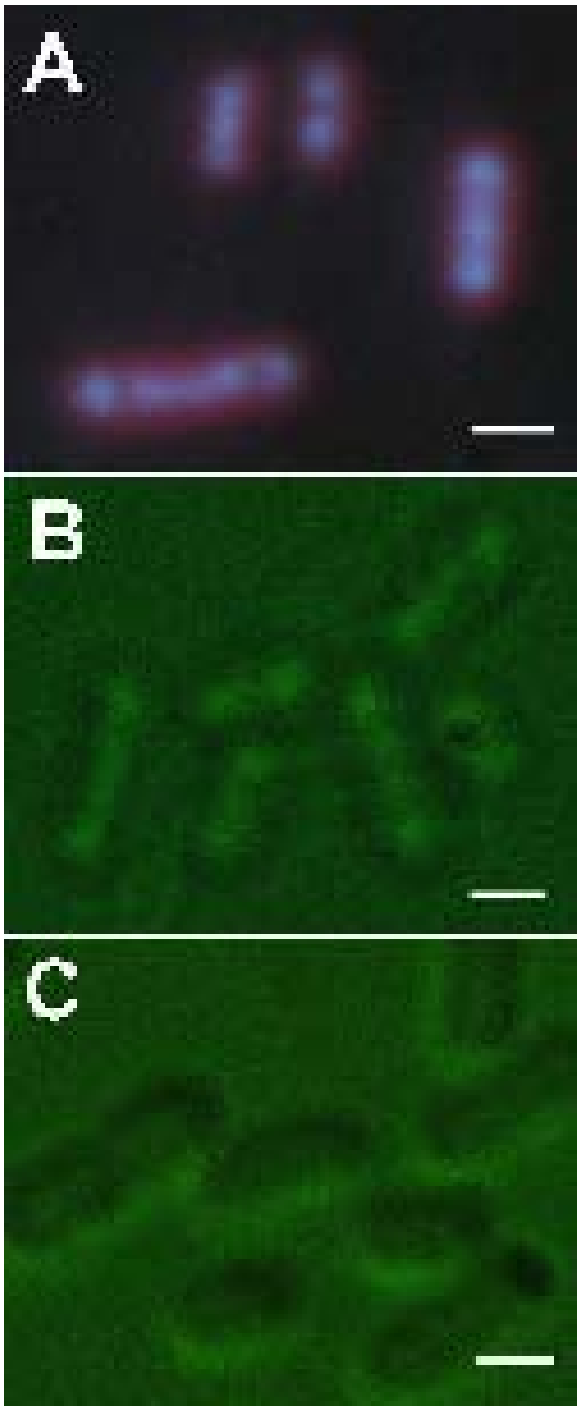

Fig.2

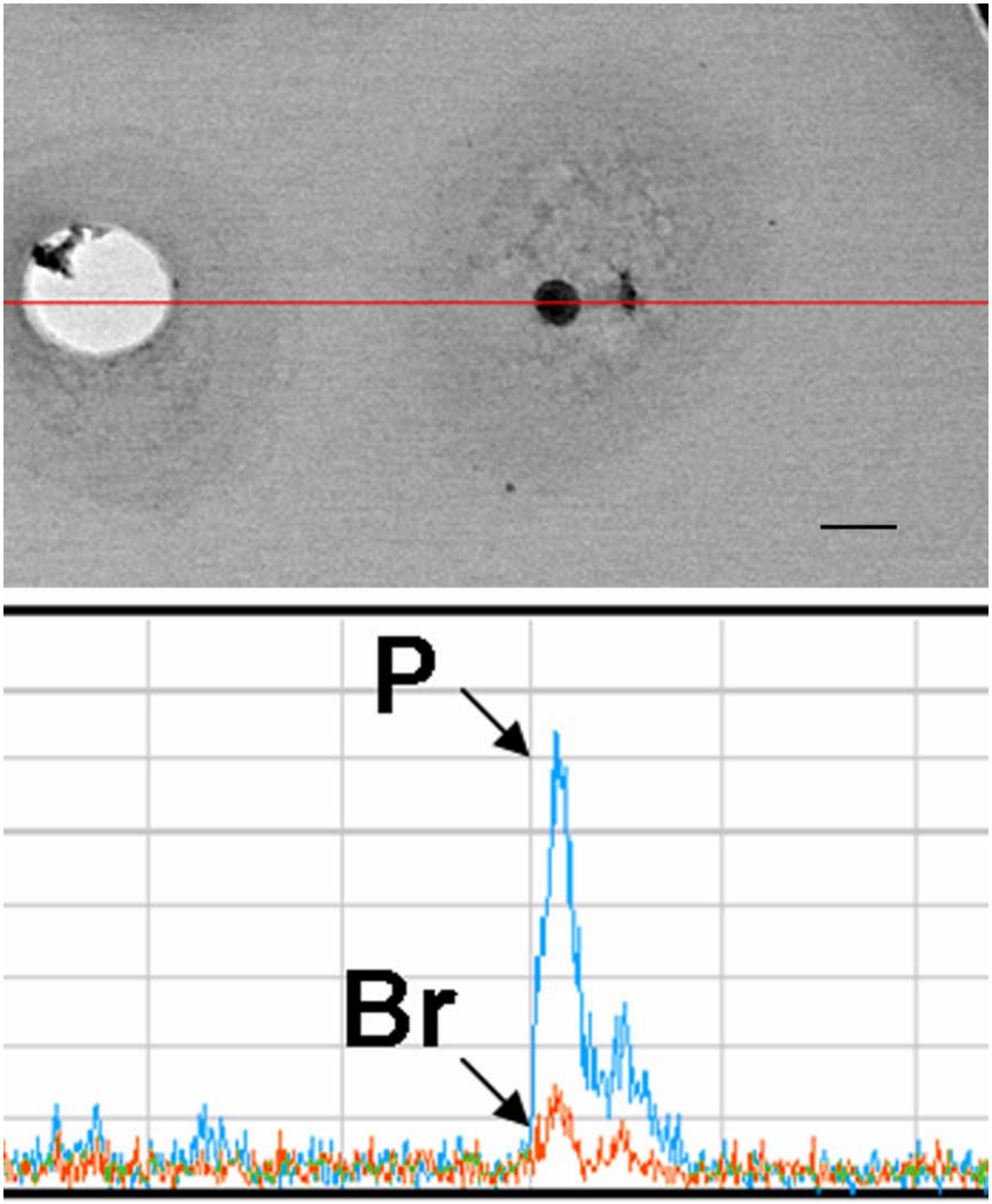

Fig.3

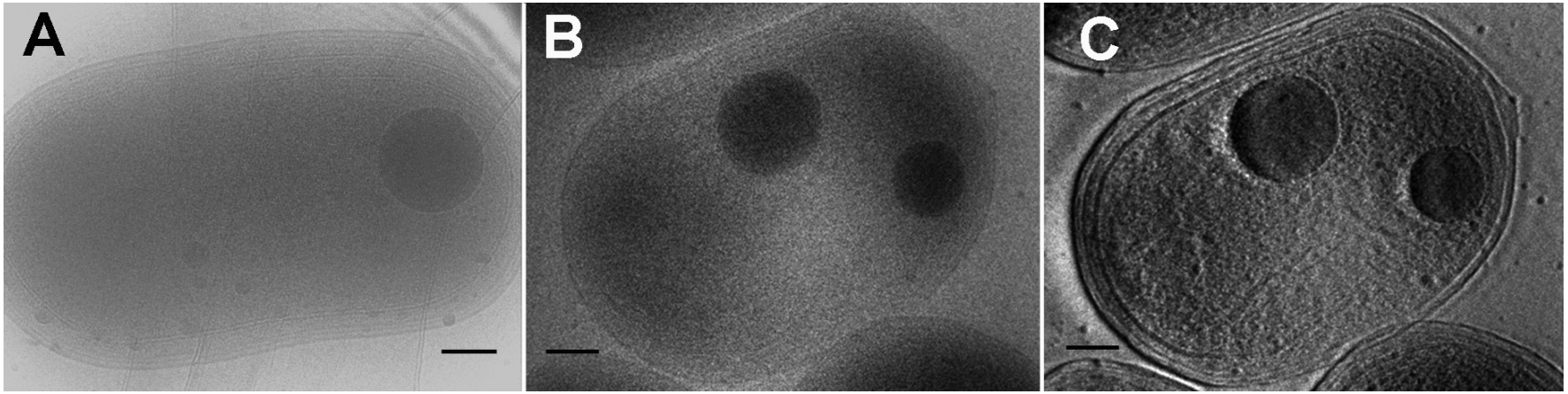

Fig.4

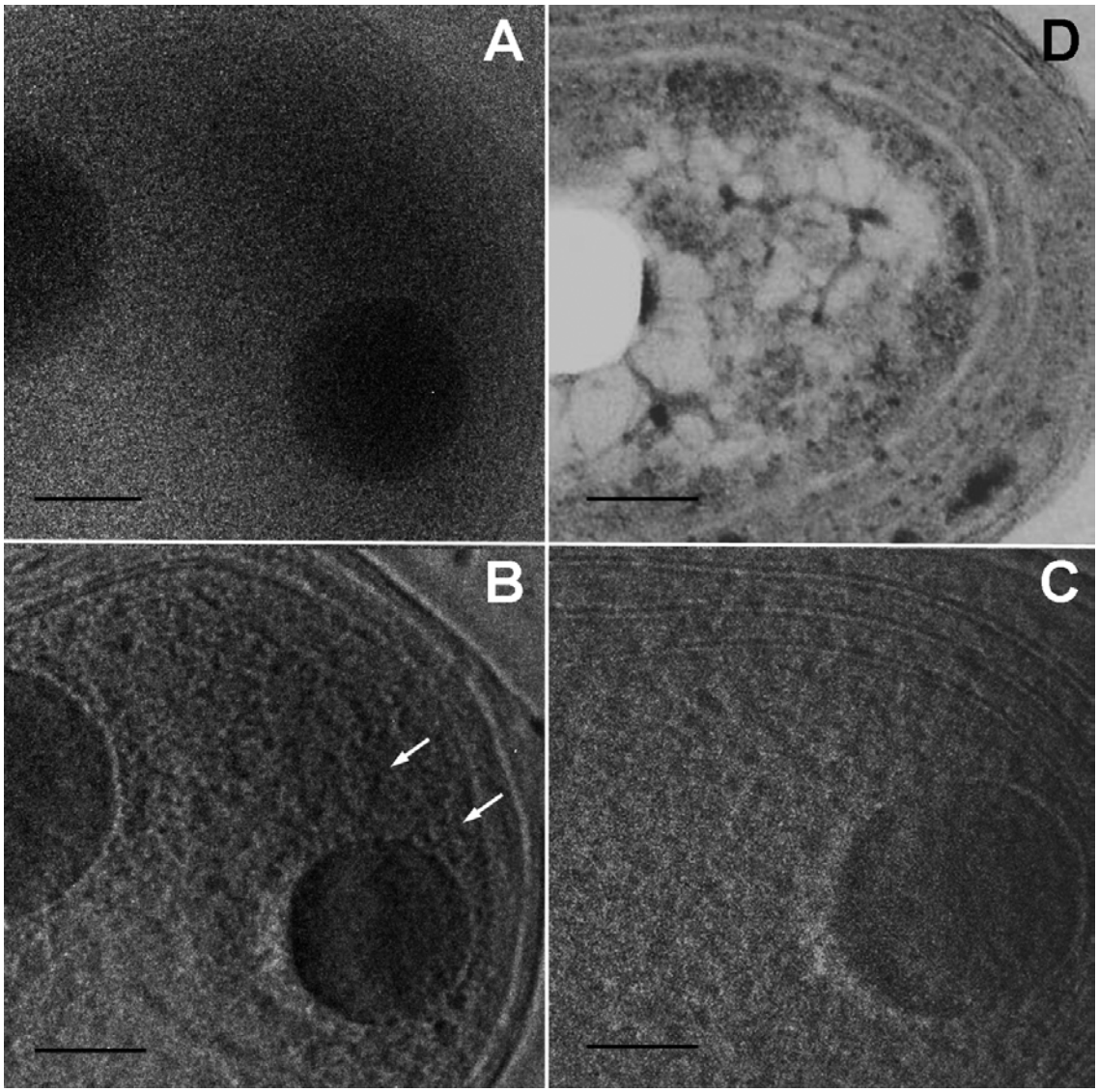

Fig.5