Heat treatment is a simple technique for releasing polyphosphate (polyP) from microbial cells. We investigated the ultrastructural alterations of polyP granules (PPGs) in response to heat treatment at 70 °C using a polyP-accumulating phoU mutant of Escherichia coli as a model organism. Electron microscopic and energy-dispersive X-ray analysis suggested concurrent occurrence of PPG degradation and polyP release in the cytoplasm. PolyP is probably released by cytoplasmic extrusion through breaks in the cell envelope.

Key words: polyphosphate; polyphosphate granules; heat treatment; electron microscopy; energy-dispersive X-ray analysis

Phosphorus (P) is an essential constituent of all living organisms. Global reserves of high-quality rock phosphate are limited, and they are being consumed rapidly.1,2) On the other hand, increased input of inorganic phosphate (Pi) into lakes, bays, and other surface waters can cause nuisance phytoplankton growth.3) Considerable attention has been paid to an efficient means of Pi removal from wastewater.4–6) Phosphorus is an essential constituent of all living organisms. Global reserves of high-quality rock phosphate are limited, and they are being consumed rapidly.7,8) We have proposed a simple technique for releasing polyP from sludge microorganisms, hampering further accumulation of high-quality rock phosphate by bacteria.9) In enhanced biological phosphorus removal (EBPR) processes, sludge microorganisms accumulate high levels of polyphosphate (polyP), a linear polymer of P, linked by high-energy phosphoanhydride bonds.10) We have proposed a simple technique for recovering polyP in a reusable form from waste sludges.11,12) We have proposed a simple technique for recovering polyP in a reusable form from waste sludges.13) We have shown that PPGs have a limiting membrane and possess an enzymatic mechanism for acidification.14) We have observed that a phoU mutant strain of Escherichia coli accumulated numerous PPGs under Pi-sufficient conditions, although the parental strain MG1655 did not form visible PPGs.15) In an effort to understand the mechanism of heat-induced leakage of polyP from bacteria, we examined ultrastructural alterations of PPGs in heated cells of the E. coli phoU mutant using electron microscopy and energy-dispersive X-ray (EDAX) analysis. E. coli phoU mutant strain was described previously.16) E. coli cells were grown overnight in 2×YT medium with shaking at 37 °C. Cells grown overnight were inoculated into 200 ml of MOPS (morpholinepropanesulfonic acid) medium containing 2 mM Pi and cultivated for 4 h to allow them to accumulate high levels of polyP. Then they were harvested by centrifugation at 4,000 × g for 5 min and resuspended in 200 ml of distilled water. The cell suspension was heated at 70 °C using a temperature-controlled water bath. Pi, polyP, and total P were determined by methods described previously.17)

A scanning electron microscope (SEM, S-5200, Hitachi, Tokyo) was used to take microscopic pictures of heat-damaged bacterial cells. A heated suspension of E. coli cells was centrifuged at 4,000 × g for 5 min. The pellet was first fixed with 4% w/w glutaraldehyde-0.2 M cacodylate buffer (pH 7.2) for 1 h, and then with 5% w/w osmic acid-0.2 M cacodylate buffer (pH 7.2) for 1.5 h. The samples were dehydrated in a graded ethanol series and then coated with osmium (HPC-1S hollow cathode plasma CVD, Vacuum Device, Mito, Japan). For thin sections, resin-embedded samples were cut with a glass knife on a Reichert-Nissei ultramicrotome, mounted on carbon-coated copper grids (Nisshin EM, Tokyo), and poststained with uranyl acetate and lead citrate. Transmission electron microscopic (TEM) analysis was performed with either a JEOL 100CX (accelerating voltage 80 kV) or a Hitachi H-7650 (80 kV) electron microscope.

Unstained cells were also examined for the presence of electron-dense granules by TEM, as described previously.18) Cells were harvested by centrifugation, suspended in distilled water, and then placed on carbon-coated copper grids. The drops containing the bacterial cells were drained off with filter paper, and the
preparations were completely air dried. Electron microscopy was performed with a Hitachi H-800 electron microscope using an accelerating voltage of 100 kV. EDAX analysis was performed using unstained cells or unstained thin sections. The unstained samples were coated with carbon (JEE-420 carbon coater, JEOL, Tokyo) and analyzed using a Horiba EMAX-7000 (accelerating voltage 100 kV) or a Horiba EMAX EX-250 (80 kV) energy-dispersive microanalyzer.

The *E. coli* phoU mutant accumulated high levels of polyP (approximately 400 nmol of P<sub>i</sub> residues/mg of protein) when the cells were grown in MOPS medium containing 2 mM P<sub>i</sub>. The levels of polyP were at least 400-fold higher than the levels in the wild-type strain MG1655. When a bacterial suspension was heated at 70 °C for 60 min, approximately 90% of intracellular polyP and P<sub>i</sub> was released into the liquid phase. PolyP release continued even after P<sub>i</sub> leakage ceased under 15 min of heating (data not shown). This indicates that polyP was not completely degraded to P<sub>i</sub> when released from the heated cells. TEM analysis of unstained samples revealed that the *E. coli* phoU mutant cells had an enlongate shape and contained PPGs of various sizes in the cytoplasm (Fig. 1A). EDAX analysis confirmed that high levels of P were present in cell areas containing the electron-dense granules. After heating of the bacteria at 70 °C, the electron-dense spots became smears, suggesting the degradation of PPGs in

**Fig. 1.** TEM and EDAX Analysis of *E. coli* phoU Mutant Grown in MOPS Medium Containing 2 mM P<sub>i</sub>.

Unstained and unfixed samples were examined for the presence of PPGs before (A) and after (B) heating at 70 °C for 15 min. Different areas (indicated by circles) of the samples were analyzed for chemical composition. T-1, TEM image of an unheated cell; T-2, TEM image of a heated cell; X1, EDAX spectrum of a cytoplasmic area; X-2, EDAX spectrum obtained from an electron-dense body; X-3, EDAX spectrum of a cytoplasmic leakage (visible as a smear near the bacterial cell); X-4, EDAX spectrum of a reference area.
the cytoplasm (Fig. 1B). Cytoplasmic leakage was visible near the heated cells, and considerable amounts of P were detected in the cytoplasmic leakage.

Under the conditions of fixation used, the cells of the *E. coli phoU* mutant were well preserved (Fig. 2A). They generally displayed the appearance of a normal healthy cell (Fig. 2B). When the *E. coli* cells were heated to 70°C, blebs of various sizes appeared on the cell surface (Fig. 2C). The bleb formation was observed immediately after the start of heating, and the blebs were detectable throughout the cell surface. Although considerable irregularity was detected in the shape of bacterial cells, there was little or no evidence of cell lysis. TEM analysis confirmed the formation of surface

**Fig. 2.** SEM (A, C) and TEM (B, D, and E) Images of *E. coli phoU* Mutants before (A, B) and after (C, D, and E) Heat Treatment at 70°C for 15 min.

Solid arrows indicate wall disruption accompanied by cytoplasmic leakage. A black circle shows surface blebs. Arrowheads show intense cytoplasmic aggregates. Open arrows indicate membrane-structured vesicles detached from the cell wall. A SEM image of *E. coli* cells was also taken after heat treatment in the presence of 5 mM Al$_2$(SO$_4$)$_3$ (F).

**Fig. 3.** TEM Images of Unstained *E. coli phoU* Cells.

Cell samples were taken before heating (A) and after 5 min of heating (B). Arrows indicate PPGs before heat treatment. EDAX spectra of areas containing a cell void (CV) and a PPG (indicated by black circles) are shown in C and D respectively.
blebs on the heated E. coli cells (Fig. 2D). Cytoplasmic aggregation was perceivable mainly at the periphery of the heated cells. Additionally, cell leakage accompanying wall disruption, as judged by decreased electron density, was visible, and membrane-structured vesicles, which had been released from the cells, were occasionally observable (Fig. 2E). These observations were in good agreement with previous reports on ultrastructural alteration of wild-type E. coli cells under heat treatment.15–17 We have observed that heat-induced polyP release from sludge microorganisms was strongly inhibited by the presence of metal cations, such as Mg2+, Fe3+, and Al3+.18 As can be seen in Fig. 2F, when E. coli cells were heated to 70 °C in the presence of 5 mM Al2(SO4)3, unshaped aggregates were visible around the blebs on the cell wall. Although the chemical composition of the extracellular aggregates could not be determined, this SEM image suggests that polyP, which had leaked from the heated cell, precipitated with Al3+ onto the cell surface during heat treatment.

Ultrastructural alterations in PPGs in response to heating at 70 °C were examined by TEM and EDAX analysis (Fig. 3). Since osmium interferes with the P line, unstained thin sections were used in EDAX analysis. Before heat treatment, PPGs were randomly distributed in the cytoplasm (Fig. 3A). Occasionally, large PPGs were observable in the untreated cells. At 5 min of heating, large cell voids were formed in the cytoplasm, but no significant P was detectable in the voids (Fig. 3B and C). Obviously, the sizes of the PPGs were significantly smaller than those detected before heating. The averages of the apparent diameters of 20 randomly selected PPGs were 0.98 ± 0.81 nm and 0.38 ± 0.33 nm before and after heat treatment respectively. Occasionally, PPGs were perceivable around the surface of the heated cells. Since the thin sections were unstained, it was not possible to discern a well-defined outline of a bacterial cell. However, as shown in Fig. 2, TEM analysis of stained thin sections revealed the presence of cytoplasmic leakage accompanied by cell-wall disruption. Taking this into account, it is possible that polyP was released from the heated cells through cytoplasmic leakage. It should be noted that the leaked polyP was not fixed in the thin sections. As described above, cell pellets were resin-embedded after they were collected by centrifugation. Nearly all of the leaked materials were removed during sample processing.

In the present study, we examined ultrastructural alterations in PPGs in response to heating of bacteria at 70 °C. It should be noted that activated sludge contains numerous microorganisms with different cell-wall structures. In a previous study, we observed that the overall appearance of sludge microorganisms was not significantly changed by heating.9 This suggests that heat-induced polyP leakage from the sludge microorganisms can be attributed mainly to partial cell-wall disruption but not to cell lysis, similarly to those observed in E. coli phoU mutant cells.

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