Cytoplasmic Acidification May Occur in High-Pressure Carbon Dioxide-Treated Escherichia coli K12

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While studying the mechanism by which high-pressure carbon dioxide treatment (HCT) inactivates bacteria, we found that the efficiency of DNA recovery via phenol extraction was extraordinarily low from E. coli K12 cells that had been subjected to HCT. DAPI staining of the treated cells, however, revealed that nuclear DNA was present. Most DNA from the cells subjected to HCT was probably caught in the denatured protein layer during phenol extraction. The efficiency of DNA recovery from proteinase-treated crude extracts from cells subjected to HCT was high. Crude extracts of E. coli K12 cells that had not undergone HCT were intentionally acidified with acetic acid to pH 5.2 to cause acidic coagulation of cytoplasmic proteins. The efficiency of DNA recovery from the acidified extracts was low. These results suggest that in cells subjected to HCT, cytoplasmic pH is reduced to around pH 5.2, and that nuclear DNA becomes entangled in coagulated cytoplasmic proteins. Acidification of the cytoplasm might be the primary mechanism by which HCT inactivates bacteria.

Key words: cytoplasmic acidification; Escherichia coli K12; high-pressure carbon dioxide

Heat treatment induces several changes in cells, including alteration in the appearance of the cell, leakage of low-molecular-weight materials, protein coagulation, and DNA and RNA breakdown.¹¹ In the present study, we investigated the effect of HCT on nucleic acids in Escherichia coli K12. For comparison, heat treatment and high hydrostatic pressure treatment were also used. Bacterial cells can be killed by hydrostatic pressures of 100 to 500 MPa.¹¹,¹²

In the course of this study, we found that the efficiency of DNA recovery by phenol extraction was extraordinarily low from E. coli K12 cells that had undergone HCT. We attempted to determine the mechanism underlying this phenomenon in an effort to gain a better understanding of how microorganisms are inactivated by HCT.

Materials and Methods

Bacteria. Escherichia coli K12 IAM1264 was obtained from the Institute of Molecular and Cellular Biosciences, The University of Tokyo (Tokyo). The strain was grown overnight at 37 °C with shaking in 10 ml of LB broth (1% trypton (Difco Inc., Michigan, USA), 0.5% yeast extract (Difco), 0.5% yeast extract (Difco), 1% NaCl, pH 7.2) in an L-tube. The fully grown cells were subjected to HCT.

High-pressure carbon dioxide treatment. Cells were collected by centrifugation at 8,000 rpm for 10 min; the pellet was washed twice with 1/15 M phosphate buffer (pH 6.98), and then suspended in that buffer. Cell suspensions (10 ml) were poured into sterile stainless steel test tubes (104 × 17 mm in diameter). The test tubes

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were placed into a prototype pressurization apparatus (AKICO Co., Tokyo, Japan) and the cell suspensions were pressurized with CO$_2$ at 4 MPa at 35°C for 1 min. Approximately 30 sec was needed to achieve a treatment pressure of 4 MPa, and the decompression time was approximately 30 s. The temperature around the pressurized cells was regulated using a thermocontrolled heater.

**High hydrostatic pressure treatment.** Cell suspensions were sealed in sterile screw-capped plastic tubes (5 ml capacity; Greiner Labortechnik Co., Ltd., Frickenhausen, Germany) that were pressurized with a prototype pressurization apparatus (model HPV-80C20-S; Sugino Machine Ltd., Toyama, Japan). The time needed to achieve the treatment pressure was approximately 60 s for 200 MPa. The decompression time was approximately 10 s. The temperature of the pressure cell was regulated with a thermocontrolled water bath. Cell suspensions were treated at 275 MPa, 35°C for 1 min.

**Heat treatment.** Cell suspensions (2 ml) were transferred into glass test tubes (10 x 100 mm), which were then immersed in a water bath equilibrated at 56°C for 1 min.

**Measurement of surviving cells.** The number of surviving cells was determined by plating 100 μl of appropriately diluted samples onto nutrient agar (Difco) plates. Colonies were counted after incubation at 37°C for 24 h.

**Microscopic observations.** Samples were observed and photographed with an Olympus BX60 fluorescence microscope equipped with a UPlanFI oil immersion lens (Olympus, Tokyo, Japan) and a PM-C35DX camera (Olympus). DAPI fluorescence was observed with a modified protocol described elsewhere.14,15) A cell suspension was sonicated with a Bioruptor sonicator (Cosmo Bio Co., Ltd., Tokyo, Japan). Fifty 30-s sessions of sonication at 60-s intervals were used. The pH of the sonicated cell extracts was adjusted to 5.2 using acetic acid (Wako Pure Chemical Industries, Ltd.). Nucleic acids were extracted from these acidified cell extracts by a modified protocol described elsewhere.14,15) Nucleic acid extraction including proteinase treatment was performed according to a modified protocol described elsewhere.16)

**Acidification of E. coli crude extract.** Stationary phase cells were collected by centrifugation at 8,000 rpm for 10 min at 5°C, and then the pellet was washed twice with 1/15 M phosphate buffer (pH 6.98) and resuspended in 10 ml of Tris–HCl buffer (pH 7.3). The cell suspension was sonicated with a Bioruptor sonicator (Cosmo Bio Co., Ltd., Tokyo, Japan). Five 30-s sessions of sonication at 60-s intervals were used. The pH of the sonicated cell extracts was adjusted to 5.2 using acetic acid (Wako Pure Chemical Industries, Ltd.). Nucleic acids were extracted from these acidified cell extracts by a modified protocol described elsewhere.14,15)

**Statistical analysis.** All experiments were repeated 3 times, and one sample was used per experiment. The data presented are the means of three replicate experiments. Significant differences were determined using Student’s t test (P < 0.05).

**Results and Discussion**

To investigate the effect of HCT on nucleic acids in *E. coli*, we extracted the total nucleic acids from *E. coli* subjected to HCT (4 MPa, 35°C) by a standard phenol extraction method. For comparison, cells that had undergone heat treatment (HT; 56°C) and high hydrostatic pressure treatment (HPT; 275 MPa, 35°C) were also used (Fig. 1). These three, HCT, HT and HPT, conditions gave the almost same inactivation ratios. The treatment period was 1 min in all cases. Nucleic acids were prepared from these *E. coli* cells that had been subjected to HT and HPT. RNA is known to be degraded in cells subjected to HT,11) however, DNA degradation was not clearly observed. As shown in Fig. 2, the amount of 16S and 23S rRNA was greatly reduced in cells subjected to HT, as expected. In contrast, there was...
no apparent change in the amount of nucleic acids in cells subjected to HPT. Interestingly, the amount of chromosomal DNA in cells subjected to HCT was greatly reduced (Fig. 2). DAPI staining of cells subjected to HCT, however, clearly showed that chromosomal DNA was present (Fig. 3). This indicates that the chromosomal DNA in the cells subjected to HCT was not degraded, but was probably prevented in some way from being extracted.

In phenol extraction, nucleic acids are extracted in the upper water layer, whereas denatured proteins coagulate and localize in the interphase between the water and phenol. We suspect that most of the DNA from the cells subjected to HCT was caught in the denatured protein

![Graph showing Log Numbers of Viable Cells Remaining after Various Treatments.](image)

**Fig. 1.** Log Numbers of Viable Cells Remaining after Various Treatments. The treatment conditions used yielded a similar sterilization ratio for *E. coli* cells. All treatments were applied for 1 min. Error bar showed the standard deviation.

![Electrophoretic analysis of nucleic acid extraction](image)

**Fig. 2.** Effect of Three Different Treatments on Nucleic Acid Extraction from *E. coli*. All treatments were applied for 1 min.

![Photomicrographs showing E. coli cells stained with DAPI before and after HCT](image)

**Fig. 3.** Photomicrographs Showing *E. coli* Cells Stained with DAPI before and after HCT. Bar = 5 μm.
layer during phenol extraction. To determine the effect of the presence of protein in the crude extracts of cells subjected to HCT, crude extracts were treated with proteinase K. As expected, the efficiency of DNA recovery from the protease-treated crude extracts was high (Fig. 4). These observations suggest that formation of complexes of cytoplasmic denatured protein and chromosomal DNA results in low recovery of chromosomal DNA from cells subjected to HCT.

It is possible that acidification of the cytoplasm in microorganisms subjected to HCT induces denaturation of cytoplasmic proteins. Spilimbergo et al. estimated the inner-cellular pH of Bacillus subtilis to be 3.4 in the HCT and to be 5.2 immediately after HCT using a fluorescent dye.17 And it is well known that most cytoplasmic proteins of E. coli coagulate around pH 5.0 to 5.2. Crude extracts of E. coli K12 that had not been subjected to HCT were intentionally acidified to pH 5.2 using acetic acid to cause acidic coagulation of cytoplasmic proteins, and these crude extracts were subjected to phenol extraction. As shown in Fig. 5, the efficiency of DNA recovery from the acidified crude extract was low. These results suggest that HCT causes the pH of the cytoplasm to drop to around pH 5.2, and that nuclear DNA becomes entangled in the coagulated cytoplasmic proteins. The efficiency of DNA recovery via phenol extraction from cells subjected to HCT is expected to be low.

The difference in recovery between rRNA and chromosomal DNA from HCT cells is speculated to depend on molecular sizes (Fig. 2). Molecular size of 23S rRNA was approximate 3,000 bases, and that of degraded chromosomal DNA would be at least 100 kbp. High molecular weight chromosomal DNA would easily be caught in the denatured protein. In fact, enough plasmid DNA could be extracted from E. coli cells subjected to HCT (data not shown). On the other hand, Chomczynski and Sacchi showed that RNA could be extracted separately from DNA by guanidinium thiocyanate-phenol-chloroform extraction at pH 5.2.18

Our data clearly but indirectly indicate that HCT causes cytoplasmic acidification in bacteria. The results of our previous study also strongly suggest that HCT decreased cytoplasmic pH in the yeast Saccharomyces cerevisiae.7 We conclude that HCT decreases cytoplasmic pH in microorganisms, and that this acidification would induce cytoplasmic proteins to coagulate. This situation might result in the inactivation of microorganisms.

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