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Injury and recovery of *Escherichia coli* ATCC25922 cells treated by high hydrostatic pressure at 400–600 MPa

Keitarou Kimura,* Kazuya Morimatsu,§ Takashi Inaoka, and Kazutaka Yamamoto

Food Research Institute, National Agriculture and Food Research Organization (NFRI-NARO), 2-1-12 Kannodai, Tsukuba, Ibaraki 305-8642, Japan

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Escherichia coli **cells were inactivated by high hydrostatic pressure (HHP) at 400–600 MPa and their recovery under various conditions was evaluated by colony counting and flow cytometer (FCM) analyses. The lag time in colony formation and an improved recovery of cells under less oxidative conditions (pyruvate addition to the medium and incubation in anaerobic conditions) were observed for HHP treated cells, which indicated that a significant portion of cells were injured and recovered during incubation after HHP treatment. The lag time for colony formation varied, which suggested a wave of resuscitation and recovered cells may multiply before other injured cells complete resuscitation. The recovery process was monitored by FCM: The FCM profile of cells stained using propidium iodide and SYTO9 indicated that while the majority of cells died just after HHP treatment, the staining pattern of possibly injured cells displayed a specific spectrum that gradually became consistent with that of the dead cell population and a living cell population simultaneously appeared. Pyruvate addition to the medium not only enhanced viability of HHP treated cells, but also reduced the lethal effect of HHP. These observations suggested that the degree of damage by HHP may differ cell-by-cell, and oxidative stress may continue after HHP treatment. Pyruvate addition to the recovery medium enhanced viability of *E. coli* cells inactivated by HHP treatment in tomato juice as well.**

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[**Key words:** High hydrostatic pressure; *Escherichia coli*; Flow cytometer; Injury and recovery; Pyruvate]

High hydrostatic pressure (HHP) treatment is a non thermal process applied in the food industry to inactivate bacteria with minimal loss of quality (1–5). HHP treatment above 300 MPa is usually applied to inactivate *Escherichia coli* cells. A significant portion of bacterial cells treated by different means of stress, including HHP treatment, heating, drying, starving, low pH, low temperature, and freezing–thawing, will become injured (6–8). Injured bacterial cells do not form colonies on agar plates when assessed via routine monitoring of food hygiene, however, they are not completely killed by these processes and may recover and proliferate once more. Therefore, injured bacteria can be a source of potential risk of food poisoning during food preservation and storage, which has been studied for decades (6,9–16). The injury and recovery of non-pathogenic *E. coli* cells are also important because *E. coli* is an indicator bacterium preferentially used in hygiene assessments (6,9,17).

Lines of experimental results have indicated that membrane damage by HHP treatment led to reactive oxygen species (ROS) production in injured cells, which would eventually impact their viability (6,13). Aersten et al. (14) reported oxidative stress in *E. coli* cells treated by HHP at 300–400 MPa. Particularly, *E. coli* mutants lacking genes responsible for the management of ROS were more sensitive to HHP treatment than parental cells, and incubation under anaerobic conditions improved survival of HHP-treated cells. HHP treatments at 400–600 MPa are commonly used in the food industry, but limited information is available for the dynamics of injury and recovery of cells in this range.

We previously reported that viable *E. coli* colonies appeared in samples treated via HHP at 400–600 MPa when incubated at 25°C after treatment but not at 37°C. This observation implied that cellular damage and recovery after HHP treatment were dependent upon incubation temperature (18). However, the recovery and enumeration of proliferated cells were not clearly demonstrated in this study, and the physiological state of HHP-treated *E. coli* cells is unknown.

It is difficult to detect injured bacteria since they do not grow on conventional agar media. However, several methods have been proposed to detect and enumerate injured cells (19–22): differences between colony forming units (CFUs) on a standard selectable medium and those observed on a non-selectable media and/or under restorative conditions, an expanded lag phase time, and a comparison of CFUs with the viable cell number estimated by cell staining with specific fluorescent dyes. Among fluorescent dyes, propidium iodide (PI) and SYTO9 are mainly used to distinguish between viable and dead cells (live/dead staining). To note, a selective qPCR technique coupled with propidium monoazide, which inhibits DNA amplification of membrane-damaged injured cells, was recently proposed (19,23,24).

In this study, we performed population analyses of *E. coli* cells treated at 400–600 MPa to distinguish between live and dead cells and to demonstrate cellular recovery and proliferation. The lag time

* Corresponding author. Tel.: +81 29 838 8094; fax: +81 29 838 7996.
E-mail address: keitarou@affrc.go.jp (K. Kimura).
§ Present address: Department of Food Production Science, Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama 790-8566, Japan.

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in colony formation of HHP treated cells was monitored daily, and time-dependent changes of the live/dead staining pattern was observed using a flow cytometer (FCM) and PI and SYTO9. In addition, viable cells were enumerated under aerobic and less oxidative conditions, the latter achieved via pyruvate addition to medium and incubation under anaerobic conditions. Furthermore, we inoculated tomato juice with *E. coli* cells and assessed survival after HHP treatment under oxidative and less oxidative conditions at 25°C and 37°C.

MATERIALS AND METHODS

***E. coli* strain and media** *E. coli* strain ATCC25922 was purchased from the American Tissue Culture Collection (Manassas, VA, USA). For colony counting, cells serially diluted in PBS buffer [0.02% (w/v) KH_2PO_4, 0.29% (w/v) $Na_2HPO_4 \cdot 2H_2O$, 0.02% (w/v) KCl, and 0.8% (w/v) NaCl, pH 7.4–7.5] were spread on LB plates containing 1.0% (w/v) peptone (Bacto Tryptone, Becton, Dickinson and Company, Parks, MD, USA), 0.5% (w/v) yeast extract (Becton Dickinson), 1.0% (w/v) NaCl (Wako Pure Chemical Industries, Osaka, Japan), and 1.5% (w/v) agar (Nacalai Tesque, Kyoto, Japan). If necessary, sodium pyruvate (Wako Pure Chemical) was added to the LB medium. AnaeroPack-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan) was used to incubate agar plates under anaerobic conditions to evaluate the contribution of atmospheric oxygen. LB agar plates were kept under anaerobic conditions for 3 d, and the plates were then withdrawn from the anaerobic chamber for colony counting and incubated for an additional 3 d under aerobic atmospheric conditions. Other chemicals were purchased from Wako Pure Chemical.

HHP treatment *E. coli* cells (0.1 mL of pre-culture) were inoculated in 100 mL LB liquid medium in a 500-mL flask with baffles and propagated for 18 h at 37°C with vigorous shaking (130 rpm). Cells were harvested by centrifugation with a swing rotor (2600 ×*g*, 12 min, 15°C). Cells were then suspended in 40 mL PBS buffer, centrifuged again, and resuspended in 200 mL PBS buffer. The optical density (OD_{670}) of the final cell suspension was in the range of 2.1–2.3. One unit of OD_{670} × mL corresponded to $8.4 \times 10^8 \pm 5.4 \times 10^7$ cells (CFU). The cell suspension (approximately 200 mL) was packed in a sterile retort pouch (R-1625H, Meiwa packs, Osaka, Japan), sealed, and then transferred into the cylinder vessel (diameter: 60 mm × 200 mm) of an HHP machine (Dr. CHEF, Kobe Steel, Kobe, Japan), which was filled with water and maintained at 25°C by heating and cooling units. The hydrostatic pressure inside the vessel was increased at a rate of 200 MPa/min and kept at 400, 500, and 600 MPa for 10 min, then decreased to atmospheric pressure at 200 MPa/min. The temperature inside the vessel transiently drifted during the pressing and depressing processes (28.0°C ± 0.3°C–20.7°C ± 0.4°C, 28.3°C ± 0.4°C–19.3°C ± 0.5°C, and 28.8°C ± 0.2°C–18.0°C ± 0.5°C for 400-, 500-, and 600-MPa treatments, respectively). After HHP treatment, cells were appropriately diluted in PBS buffer and were spread on LB agar plates and incubated for 6 d at 37°C and 25°C; colonies were observed daily. For the tomato juice experiments, tomato juice from concentrated tomatoes (Kagome, Nagoya, Japan) was used to suspend *E. coli* cells in the final step of sample preparation. *E. coli* cells pouched in parallel without further treatment (living cells) and those heated at 90°C for 30 min in a water bath (dead cells) were used as controls.

The HHP treatment was repeated in biological triplicates, and the colony count was duplicated for each experiment. Means ± standard error (SE) were calculated from individual results (n = 3). ND (not detected) indicates that the number of colonies was less than 10 (CFU/OD_{670}·mL).

Live/dead staining and analysis by FCM The HHP-treated cell suspensions were redeposited in sterile glass bottles at 25°C for the FCM analyses. HHP-treated cells were appropriately diluted in PBS buffer (OD_{670} = 0.1) and stained with a mixture of PI (final concentration: 30 μM) and SYTO9 (final concentration: 5 μM) in the dark for 15 min using a LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Thermo Fisher Scientific, Waltham, MA, USA). Cell samples were then subjected to a flow cytometer (EC800 Cell Analyzer, Sony, Tokyo, Japan) equipped with three lasers (488 nm, 405 nm, and 642 nm) and two filters (525/50 BP and 595/50 BP) to detect SYTO9 and PI emission. PI and SYTO9 fluorescence was detected with detectors, FL5 and FL2, respectively, and intensity of the fluorescence was expressed as a logarithmic arbitrary unit. Distribution of 25,000 cells was analyzed in biological triplicates by installed software (Sony, Tokyo, Japan). Heat inactivated dead cells (90°C, 30 min) and living cells (no HHP treatment) were used as references to set regions of dead, living, and the injured cells. FCM observations were performed daily, and cell samples were simultaneously subjected to colony counting on LB agar plates.

Dissolved oxygen levels A handheld water quality meter equipped with a dissolved oxygen (DO) tip (D-55, Horiba, Kyoto, Japan) was used to measure DO levels. The meter was calibrated based on the atmospheric oxygen level.

RESULTS

Inactivation of *E. coli* ATCC25922 cells by HHP treatment We first examined the dose–inactivation curve (100–600 MPa) of *E. coli* strain ATCC25922 (Fig. 1). The CFU logarithmic reduction was more than 6 for 400 MPa and 500 MPa, and viable cells were not detected after HHP treatment at 600 MPa (<10 CFU/OD_{670}·mL). Cells were not sufficiently inactivated below 200 MPa (Fig. 1).

HHP-treated cells were spread on LB agar plates and incubated simultaneously at 37°C and 25°C. CFUs were monitored daily (Fig. 2). As a consequence, much more colonies were apparent at 25°C than at 37°C for samples treated at 400 MPa and 500 MPa.

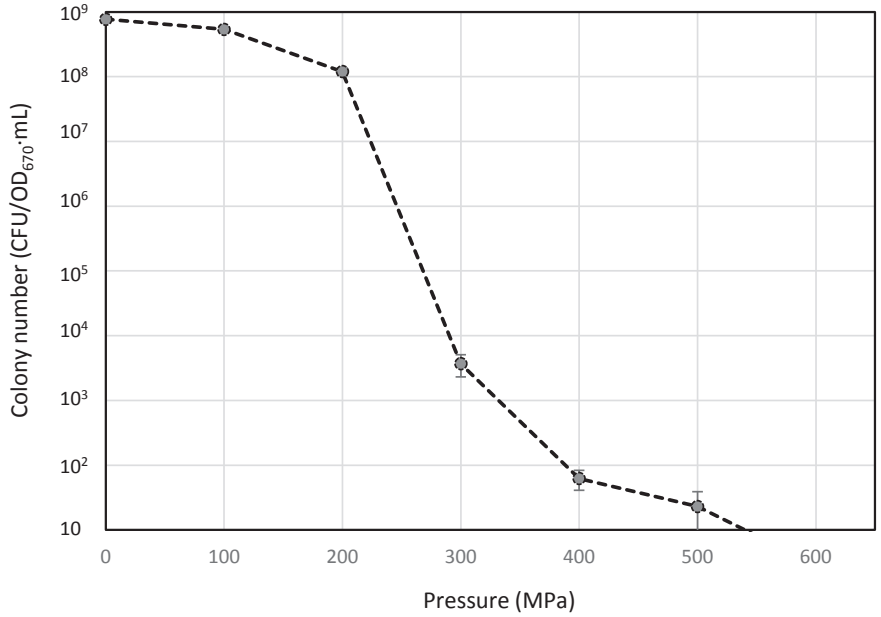

FIG. 1. Inactivation of *E. coli* ATCC25922 cells by HHP treatment. *E. coli* cells were treated by HHP, 100–600 MPa, at 25°C for 10 min, as described in Materials and methods. Cells were appropriately diluted and spread on LB agar plates and incubated at 37°C for 48 h. Experiments were repeated in biological triplicates, and the colony number was expressed as a $\log_{10}$ value ± SE (n = 3). The initial colony number (no HHP treatment) was $8.4 \times 10^8 \pm 5.4 \times 10^7$ (CFU/OD_{670} mL).

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