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Growth delay analysis of high-salt injured *Escherichia coli* O157:H7 in fermented soybean paste by real-time PCR and comparison of this method with other estimation methods

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ABSTRACT

The aim of this study was to develop an estimation method for bacterial injury levels of *Escherichia coli* O157:H7 in food products. Bacterial growth was monitored in recovery medium and quantified by real-time PCR to quantify the growth delay phenomenon as growth delay time (GDT). The relationship between GDT and stress levels (salt-concentration: 9, 13, 15, and 17%; stress exposure times:0, 6, 12, 18, and 24 h) was determined. The GDT increased with stress exposure time, and the higher the salt concentration was, the faster the GDT increased. Moreover, bacterial injury levels in 15% NaCl-containing media were estimated by a conventional culture-based method, bacterial membrane destruction assay, NADPH content assay, and intercellular ATP content assay. In these estimations, the result suggested a decrease in bacterial activity and increase in bacterial membrane destruction from 6 to 12 h of exposure, which was consistent with the results of the GDT monitoring method. Furthermore, the estimation of bacterial injury levels in high-salt food (fermented soybean paste) was investigated, and GDT extension was successfully observed until the bacteria completely reached sublethal conditions. Therefore, GDT monitoring by real-time PCR showed the ability to estimate bacterial injury levels along with other existing methods.

1. Introduction

Salt curing of food is an efficacious method for microbiological control that has long been used for food preservation. In general, high salt concentrations will completely kill or inhibit the growth of bacteria that contaminate food products (Masuda, Hara-Kudo, & Kumagai, 1999). However, in 2012, Japan experienced more than one outbreaks of *Escherichia coli* O157:H7 caused by salt-pickled Chinese cabbage (Iijima, Sakamoto, Watahiki, Ohnishi, & Igimi, 2014), which led to 169 hospitalizations and 8 deaths. Contamination from food-manufacturing environments and secondary contamination (Grimme & Dumontet, 2012, pp. 156–159) in food production were assumed to have caused this serious outbreak.

High-salt foods are thought to have high microbial safety levels. However, the assumption that high-salt treatment can prevent outbreaks of foodborne illness due to contaminated products is not completely consistent with the actual conditions. In particular, it has been reported that injured bacteria that have undergone some stress (freezing, drying, heating, etc.) and have become risks in food safety because they cannot recover under certain conditions (for example, in the presence of deoxycholic acid and high salt concentrations) (Morichi, 1972; Ray, 2004; Whesche, Gurtler, Marks, & Ryser, 2009). When manufacturers consider whether foodborne pathogens in food products are alive or dead, the presence of injured bacteria is also recognized as a threat.

To estimate bacterial injury levels, some evaluation methodologies have been developed. A conventional culture-based method is generally used to estimate the number of injured bacteria using the difference in viable colony numbers between cultures grown on selective and non-selective agar (Hara-Kudo et al., 2000). The double agar plate method compares bacterial growth on selective and non-selective agars and then uses the difference in counts to determine the proportion of injured bacteria. However, it is difficult to accurately estimate bacterial injury levels by this method because the components of selective agar can cause additional stress to cells and inhibit the process of recovery from the initial damage (Clark & Ordal, 1969; Smith & Archer, 1988). The growth delay method (Tsuchido, Koike, & Takano, 1989) was also developed for viability assessment of bacterial cells subjected to

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environmental stress; in this method, the multiplication rate of bacteria in recovery medium is monitored by optimal density (OD) measurement. However, this method is hampered by the existence of background microflora. Therefore, this method is not useful for the estimation of injured bacteria in real food materials.

The LIVE/DEAD ® BacLight ™ Bacterial Viability Kit (Molecular Probes, Eugene, USA), which is based on the specific fluorochrome staining of membrane destruction, has been used to assess cell viability (Berny, Hammers, Bosshard, Weilenmann, & Egli, 2007). However, this kit requires optimization of fluorescent dye concentrations because food materials will inhibit the staining of bacterial cells. Furthermore, membrane staining is not suitable for damage level assessment of specific foodborne pathogenic bacteria because of the existence of background microflora. In addition, an ATP-based assay that detects intercellular ATP with firefly luciferase can detect all viable cells and monitor the recovery of injured bacteria for the DNA-based molecular analyses that is isolated directly from the samples (Giovannoni, Britschgi, Moyer, & Field, 1990; Pace, Stahl, Lane, & Olsen, 1985; Yamashoji, Asakawa, Kawasaki, & Kawamoto, 2004). The menadione-catalyzed assay is also a useful method for the measurement of bacterial activity; this method detects intercellular NADPH activity in viable cells (Kawasaki, Musgrove, Murata, Tominaga, & Kawamoto, 2008; Kawasaki, Yamashoji, Asakawa, Kamikado, & Kawamoto, 2007). However, this method is not suitable for the detection of specific target bacteria in food materials because of interference from background microflora and food ingredients.

To develop applications for the estimation of bacterial injury levels, we focused on the growth delay phenomenon in injured bacteria. Kawasaki, Hosotani, Noviyanti, Koseki, and Inatsu (2018), developed a real-time PCR-based monitoring method to estimate the relationship between injury levels of *Salmonella* Enteritidis in ground beef samples subjected to various heat exposure treatments and monitored the growth delay time (GDT). In a previous study, a relationship between GDTs and heat exposure levels was observed; the GDT slope increased as the heat exposure level increased. However, the changes in GDT were not examined by real-time PCR or another bacterial injury estimation method in the previous study.

In this study, the growth of *E. coli* O157:H7 injured by high-salt concentration in recovery medium was monitored by real-time PCR to determine the GDT, and the correlation with stress levels was considered (salt concentrations and exposure times). Furthermore, viable bacterial activity (ATP), indicated by membrane destruction levels (LIVE/DEAD ® BacLight ™ Bacterial Viability Kit) and intercellular respiratory metabolism (NADPH), was examined to verify the causal factors of GDT extension under high-salt stress conditions. Finally, for application in real food materials, the GDT extension of *E. coli* O157:H7 inoculated in fermented soybean paste, a high-salt fermented food model, was also monitored.

2. Materials and methods

2.1. Inoculum preparation

In the current study, the wild-type *Escherichia coli* O157:H7 strain CR3 (isolate from bovine feces, stx negative and eae positive) was used (Inatsu, Bari, Kawasaki, Isshiki, & Kawamoto, 2005). *E. coli* O157:H7 CR3 was grown in trypticase soy broth (TSB; BBL; Becton, Dickinson and Company) overnight at 35 °C. This culture was transferred to fresh TSB medium and incubated at 35 °C. The *E. coli* O157:H7 culture was monitored until the early stationary phase. The optical density (Abs 600 nm) of *E. coli* O157:H7 culture was monitored until it reached an OD of 0.80, measured by an automatic OD detector (Bio-Plotter, Toyo-Sokki Co. Ltd., Japan).

2.2. Preparation of media

Trypticase soy agar (TSA; Difco; Becton, Dickinson and Company), deoxycholate agar (DESO, Nissui, Japan), and TSB were prepared following the manufacturer's instructions. High-salt media were prepared at concentrations of 9%, 13%, 15%, and 17% NaCl-TSB (w/v), and the NaCl concentration of each high-salt medium was measured by a salinometer (PAL-SALT, ATAGO, JAPAN) in each experiment. The water activity (a_w) value was determined using an AQUALAB a_w meter (Decagon Devices, Inc., Washington, USA).

2.3. Growth delay analysis of high-salt injured E. coli O157:H7 in high-salt medium models

2.3.1. High-salt stress treatment

The *E. coli* O157:H7culture of early stationary phase (OD 0.80) was diluted (10^6 CFU/mL) with phosphate buffered saline (PBS, NaCl 0.85%, pH 7.5), 50 μL of diluted solution was immediately inoculated into 5 mL of each high-salt medium (initial concentration at stress exposure of approximately 10^4 CFU/mL) and incubated at 35 °C.

2.3.2. Inoculation in recovery medium and recovery monitoring

At selected time intervals, 50 μL of high-salt medium containing injured *E. coli* O157:H7 was transferred to 5 mL of TSB and cultured for recovery at 35 °C. During this recovery incubation step, 100 μL of cultured was taken every 2 h and DNA extraction as described below. This sampling step was repeated for at least 18 h after the recovery inoculation. To construct a standard curve, serial dilutions *E. coli* O157:H7 cultures in PBS were used (10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/mL). A standard curve was constructed for each real-time PCR amplification and analysis. This standard curve was used to obtain quantitative values of the concentration of each DNA template, which were finally converted to *E. coli* O157:H7 bacterial cell concentrations.

2.3.3. DNA extraction

DNA extraction was performed with a Qiagen DNeasy Blood and Tissue Kit (QIAGEN GmbH, Germany). One hundred microliters of recovery medium samples and serial dilutions of *E. coli* O157:H7 cultures for constructing the standard curve were immediately added into 300 μL of guanidine isothiocyanate (with 2% Tween 20, w/vol) and mixed well by vortexing. The bacterial cells were completely lysed, and subsequently, 200 μL of ethanol was added to each sample, and the samples were transferred onto DNA-binding columns. DNA extraction and column washing were performed by following the manufacturer's instruction for the Qiagen DNeasy Blood and Tissue Kit to obtain total DNA. The extracted solutions obtained from this step were used as DNA templates for real-time PCR quantification.

2.4. Real-time PCR conditions

The primers targeted the *E. coli* O157:H7 *eaeA* gene fragment (Sharme, Dean-Nystrom, & Casey, 1999). The detection probe was labeled with FAM (6-carboxyfluorescein) and BHQ1 (Black Hole Quencher). The real-time PCR volume was 25 μL, and the reaction consisted of 2.5 μL of DNA template and 22.5 μL of real-time PCR master mix solution, containing TaqMan gene expression master mix (Applied Biosystems), 200 nM primers (Fwd-primer: 5′-CGCGGATTAG ACTTCGGCTA-3′ and Rev-primer: 5′-CGTTTTGGCACTATTTGCCC-3′), and 100 nM probe (internal probe: 5′- FAM-AACGCCGATACCATTACT TATACCGC-BHQ1-3′). PCR reactions were performed in an ABI Prism 7900 sequence detection system (Applied Biosystems), and the conditions for the real-time PCR assays were as follows: 2 min at 50 °C; initial denaturation at 95 °C for 10 min; and 50 cycles of denaturation for 20 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C.