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Heat resistance of *Salmonella* Enteritidis under prolonged exposure to acid-salt combined stress and subsequent refrigeration

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ABSTRACT

Salmonella Enteritidis is a major foodborne pathogen exposed to various environmental and preservation stresses in the food chain. Because adaptive responses of viable bacterial cells in the presence of sublethal stress can induce cross-protection against different stresses, we investigated the heat resistance of *Salmonella* Enteritidis at 60 °C under prolonged exposure to acid-salt combined stress and subsequent refrigeration. *Salmonella* Enteritidis was grown in tryptic soy broth at four pH values (4.5, 5.4, 6.4, and 7.3) and four NaCl concentrations (0%, 1%, 2%, and 3%) at 37 °C for 24 h and then incubated at 4 °C for 0, 1, 4, or 7 days. For 0 and 1 day-refrigerated cultures, previous adaptation to single stresses (acid or salt stress) increased the heat resistance of *Salmonella* Enteritidis, resulting in increased D-values, whereas the combination of acid and salt stress reduced heat tolerance; acid stress played a more critical role in mediating this effect than salt concentration. To elucidate the related mechanisms, the expression levels of heat shock sigma factors (*rpoH*) and heat shock genes (*dnaK* and *groEL*) were analyzed and found to be associated with the heat resistance of *Salmonella* Enteritidis. The refrigeration period was negatively correlated ($P < 0.01$) with the D-value ($r = -0.505$) and with the transcript levels of *rpoH* ($r = -0.654$), *dnaK* ($r = -0.652$), and *groEL* ($r = -0.645$). Our findings demonstrated that acid-salt combined preservation techniques and subsequent refrigeration may prevent *S.* Enteritidis survival in heat-pasteurized food products caused by cross-protection of acid or salt adapted cells.

1. Introduction

Foodborne pathogens encounter various environmental and preservation stresses as “hurdles”, such as acid, salt, and cold stress in food chains (Tan et al., 2010). Acidification is an important preservative method that inhibits the growth and survival of microorganisms (Álvarez-Ordóñez et al., 2009; Kumar and Kumar, 2003). In particular, a low pH environment is found in fermented and acidic foods, such as cheese, juices, and sausage (Álvarez-Ordóñez et al., 2008; Bacon et al., 2003; Mazzotta, 2001). The use of organic acids to decontaminate food surfaces also provides acid stresses to existing microorganisms (Bacon et al., 2003; Singh et al., 2010). Salt is another important ingredient used in the food industry due to its positive effects on water binding, texture, viscosity, flavor, and food safety (Lee et al., 2012). Generally, sodium chloride (NaCl) is used as a salting source (Lee et al., 2012). In addition to salt, refrigeration is the most widely used intervention method to inhibit or inactivate pathogenic microorganisms in food products (Álvarez-Ordóñez et al., 2009; McMeechan et al., 2007; Yang et al., 2014b). In food industry, hurdle technology, commonly referring to combined application of such preservative methods, has been used to decrease losses of nutritional and sensory quality of foods by reducing stress level of each process (Chen et al., 2017; Di Pasqua et al., 2013; Ngnitcho et al., 2017). Moreover, hurdle technology exhibits synergistic effects due to different mechanisms of stresses involved in the inhibition or inactivation of foodborne pathogens (Ngnitcho et al., 2017).

However, survival in the presence of such environmental or preservation stresses may induce adaptive responses, which can influence the resistance of microorganisms in harsher environments than the experienced stress (Álvarez-Ordóñez et al., 2008; Kumar and Kumar, 2003; Tosun and Gönül, 2003). In addition, adaptive responses can also induce cross-protection, which enhances the resistance of pathogens to different stresses (Kumar and Kumar, 2003; Tosun and Gönül, 2003). Especially, many previous studies have demonstrated that microorganisms show changes in their heat resistance after exposure to acid, osmotic, and cold stress (Álvarez-Ordóñez et al., 2009; Yang et al., 2014a; Yang et al., 2014b). Because heat treatment is a common method used to eliminate foodborne pathogens, such phenomena are of great interest in food preservation involving many hurdles (Álvarez-Ordóñez et al., 2009).

Abbreviations: NaCl, sodium chloride; TSB, tryptic soy broth; TSA, tryptic soy agar; PBS, phosphate-buffered saline; PCR, polymerase chain reaction

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To elucidate the mechanisms of bacterial heat resistance, most studies have focused on the roles of heat shock genes and proteins in response to environmental changes (Álvarez-Ordóñez et al., 2009; Di Pasqua et al., 2013; Yang et al., 2014b). The *dnaK* gene is involved in refolding or destruction of proteins and prevents the formation of inclusion bodies by reducing aggregation and promotion of proteolysis of misfolded proteins (Hasan and Shimizu, 2008; Singh et al., 2007). Additionally, the *groEL* gene functions in protein transit between soluble and insoluble protein fractions and participates in disaggregation and inclusion body formation (Hasan and Shimizu, 2008). Heat shock genes require specific recognition by RNA polymerase associated with the alternative sigma factors, such as σ^{32} (*rpoH*), σ^{24} (*rpoE*), and σ^{38} (*rpoS*) (Hasan and Shimizu, 2008; Tomoyasu et al., 2003; Yang et al., 2014a). In particular, the *rpoH* gene provides protection against cytoplasmic thermal stress by regulating the transcription of heat shock genes (Guisbert et al., 2004; Hasan and Shimizu, 2008; Yang et al., 2014b).

Salmonella spp. are a leading cause of foodborne diseases worldwide, causing a variety of illnesses, including typhoid fever, gastroenteritis, and septicemia (Álvarez-Ordóñez et al., 2009; Álvarez-Ordóñez et al., 2008; Tan et al., 2010). Among various *Salmonella* serovars, *Salmonella* Enteritidis is the most common serovar responsible for human salmonellosis worldwide (Yang et al., 2014a; Yang et al., 2014b). *S*. Enteritidis outbreaks are typically linked to consumption of contaminated foods, including eggs, poultry, ground beef, and vegetables which require the hurdle technology to ensure the pathogen eliminated or rendered harmless in the final products (Álvarez-Ordóñez et al., 2008; Yang et al., 2014a; Yang et al., 2014b). Although the responses of *Salmonella* spp. to several stresses, including heat, acid, cold, and salt, have been studied in the past few decades (Yang et al., 2014b), most of these studies have employed single factors (treatment temperature, pH, or water activity) (Manas et al., 2003). Other studies have focused on the effects of short-term exposure of *Salmonella* spp. to environmental stresses on subsequent bacterial survival during heat treatments and the potential mechanisms mediating such stress responses (Yang et al., 2014a).

In this study, we aimed to evaluate the subsequent thermal resistance of *S*. Enteritidis following its prolonged exposure to acid-salt combined stress followed by refrigeration. We calculated D-values (the time required for a 1 log or one decimal reduction in the microorganisms) of *S*. Enteritidis under lethal heat conditions (60 °C). Additionally, the transcript levels of heat shock sigma factors (*rpoH*) and major heat shock genes (*dnaK* and *groEL*) were investigated to determine whether these genes were involved in *S*. Enteritidis stress responses during growth under different conditions or with heat treatment.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. Enteritidis 106 (serotype D1[1, 9, 12 g, m:1, 7]; human-origin strain) was used in the present study. This strain was maintained on tryptic soy agar (TSA; Difco, Detroit, MI, USA) at 4 °C and subcultured once a month until used. Single colonies were grown in 10 mL tryptic soy broth (TSB; Difco) at 37 °C for 24 h. Subsequently, 50 μL culture was transferred to 10 mL TSB at four pH values (7.3, 6.4, 5.4, and 4.5) and four NaCl levels (0%, 1%, 2%, and 3% w/v) resulting in 16 experimental combinations. Addition of NaCl was performed prior to sterilization of basal medium (TSB), which already contained 0.5% w/v NaCl. Notably, in the present study, all NaCl concentrations are described according to the externally added NaCl; therefore, TSB without additional salt was considered as 0% NaCl. pH adjustment was performed using 1 N HCl after autoclaving (121 °C for 15 min). All cultures were then incubated at 37 °C for 24 h, and cold adaptation involved maintaining cultures at 4 °C for 1, 4, or 7 days. The non-refrigerated (0 day) cultures and short-period (1 day), medium-period (4 days), and long-period (7 days) refrigerated cultures were used to determine heat resistance and gene expression levels. Preliminary studies confirmed that in all cases, cell viability remained constant throughout the cold storage period.

2.2. Determination of heat resistance of S. Enteritidis

The resistance of stress-adapted and unadapted cells to heat inactivation at 60 °C was determined. One milliliter of culture was centrifuged at 15,770*g* for 3 min and washed with 1 mL phosphate-buffered saline (PBS; pH 7.4). The cell suspensions were added to 9 mL TSB, resulting in 10^8–10^9 CFU/mL. Fifty microliters of inoculated TSB was injected into a 1.1-mm diameter capillary tube (Kimble Chase, Vineland, NJ, USA) using a micropipette, and the capillary tubes were sealed using Cha-seal tube sealing compound (Kimble Chase), which is typically used to seal hematocrit capillary blood collection tubes before centrifugation. The capillary tubes were submerged into a water bath set to 60 °C. Each 50 μL of unheated cell suspension was serially diluted in PBS and plated in duplicate on TSA to confirm initial populations. After corresponding heating times (0, 40, 80, and 120 s), capillary tubes were immediately immersed in an ice water bath (maximum 1 h) until microbiological analysis. The ends of the capillary tubes were crushed using a capillary tube cutter to obtain a heated cell suspension using a micropipette. The population of surviving microorganisms was evaluated by dilution of heated cell suspensions in 450 μL PBS, and the serial dilutions were spread onto TSA plates in duplicate. Plates were incubated for 24 h at 37 °C. All treatment conditions were evaluated in triplicate.

2.3. RNA sample preparation, cDNA synthesis, and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from each bacterial culture using the NucliSENS easyMAG system (bioMérieux, Marcy l'Etoile, France) and RNase-free DNase I (TaKaRa Bio, Inc., Shiga, Japan), according to the manufacturers' instructions. cDNA was then prepared by reverse transcription (PrimeScript RT Reagent Kit; TaKaRa). Quantitative real-time PCR was performed using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) to evaluate the transcript levels of *rpoH*, *dnaK*, *groEL*, and 16S rRNA (reference gene). All primers used in this study are listed in Table 1. Relative expression was assayed with 10 μL SYBR premix Ex Taq (TaKaRa), 0.4 μL forward primer, 0.4 μL reverse primer, 2 μL cDNA, 0.4 μL Rox Reference Dye (TaKaRa), and 6.8 μL deionized sterile distilled water (Bioneer, Daejeon, Korea). The thermocycler conditions used were as follows: 30 s at 95 °C, followed by 40 cycles of incubation at 95 °C for 5 s and 55 °C for 34 s. The relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Statistical analysis

Survival curves (the population of surviving microorganisms [log cfu] versus heating time [s]) were plotted for 64 experimental

Table 1
Primers for heat shock genes used for real-time PCR in this study.

Gene	Sequence (5′ to 3′)	Product size (bp)	Reference
16S rRNA	F: CAGAAGAAGCACCGGCTAAC R: GACTCAAGCCTGCCAGTTTC	167	Yang et al., 2014a
rpoH	F: GTTTCCTTCGCCGTACACTG R: CCACCATTTCAACCTCATCC	169	Yang et al., 2014a
dnaK	F: CGATTATGGATGGAACGCAGG R: GGCTGACCAACCAGAGTT	180	Fong and Wang, 2016
groEL	F: GACCTGAAAGGCCAGAACGA R: GCGCAGAACGGGTAACTTTG	229	Tomoyasu et al., 2003