Enhancement of enterohemorrhagic *Escherichia coli* O157:H7 stress tolerance via pre-heating

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Abstract. Enterohemorrhagic *Escherichia coli* O157:H7 infection causes several hundred cases of food poisoning every year in Japan. In severe cases, this type of food poisoning can be fatal. In the present study, we examined the induction of HSP70 in *E. coli* O157:H7 cells at various temperatures and the thermotolerance of *E. coli* O157:H7 cells alone and in contaminated food following pre-heating. We evaluated the possibility that thermotolerance by *E. coli* O157:H7 increases the likelihood of food poisoning. *E. coli* O157:H7 cells were heated at 43-51˚C, and the survival rate was examined. The temperature of highest induction of HSP70 was used as the pre-heating temperature. We measured the thermotolerance of *E. coli* O157:H7 cells following pre-heating as the survival after heating at 53˚C (lethal temperature). Additionally, we evaluated the thermotolerance of *E. coli* O157:H7 cells in ground beef following pre-heating. Heating at 47˚C for 30 min caused the highest induction of HSP70 and this temperature was selected as the pre-heating temperature. The survival rate was significantly higher for 0-90 min compared to that in cultures incubated at 53˚C without pre-heating indicating thermotolerance. Additionally, in ground beef, thermotolerance in *E. coli* O157:H7 cells was induced by pre-heating. We showed that *E. coli* O157:H7 cells acquired thermotolerance after pre-heating, which significantly increased survival after a lethal temperature, and increased the likelihood of food poisoning.

Introduction

In 1982, there was an outbreak of peculiar colitis with a bloody diarrhea caused by food poisoning after consumption of hamburger meat in Oregon and Michigan in the US. *Escherichia coli* serotype O157:H7 was identified as the pathogenic cause of the food poisoning after its isolation from the patient feces (1).

The first case of infection in Japan occurred at a school in Urawa, Saitama in 1990 (2). At that time, 2 kindergarteners died of hemolytic uremic syndrome (HUS). The first large-scale and widely known group outbreak of *E. coli* O157:H7 in our country occurred in a primary school in Sakai, Osaka in July 1996 (3). Three people among 7,936 infected patients died as a result of infection with *E. coli* O157:H7 (3).

Afterwards, it was believed that infection with *E. coli* O157:H7 would cease to occur, yet several hundred food poisoning cases due to *E. coli* O157:H7 occur each year in Japan according to the Ministry of Health, Labour and Welfare (4).

It is well known that *E. coli* O157:H7 cells are highly infectious and can cause infection with a very small number of bacteria (5). The progression of *E. coli* O157:H7 infection includes intense abdominal pain and diarrhea after the latency period of 3-5 days on average and eventually develops characteristic bloody diarrhea. It is accompanied by HUS and encephalopathy at times. Food poisoning is most likely to be fatal in infants and the elderly; therefore, it has a significant social impact.

Cells of all types, from *E. coli* to yeast and mammals, induce heat shock proteins (HSPs) as a defense mechanism in response to various stresses including heat stress (6). HSPs are biophylaxis- and stress-protective factors that repair stress-induced injury (6). HSP70 is the best-known HSP, and its *E. coli* homolog is known as DnaK (7).

We previously investigated HSP70 which was induced by mild hyperthermia applied in advance to the whole body (pre-mild hyperthermia, preconditioning), and its effect on repair proteins was investigated to assess its protective effect against various stresses (8-10). Our studies demonstrated that HSP70 induced by mild hyperthermia (pre-heating) is expected to defend against second stresses (stress tolerance).

It is well known that HSP70 is induced by various stresses in *E. coli* O157:H7 cells as well as other strains. It is possible that *E. coli* O157:H7 cells acquire stress tolerance by the induction of HSP70 as a result of insufficient heating during cooking or high temperature in the summer (pre-heating by mild heat stress), which increases the survival rate after secondary heat stress and the resulting toxicity.

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Therefore, we examined the induction of HSP70 in *E. coli* O157:H7 cells by various temperatures and the influence of HSP70 induced by prior heat stress (pre-heating) on *E. coli* O157:H7 cells, and then evaluated the thermotolerance of *E. coli* O157:H7 cells to secondary heat stress. Furthermore, we examined the effect of the pre-heating of *E. coli* O157:H7 cells in food by adding *E. coli* O157:H7 cells to ground beef, since many cases of *E. coli* O157:H7 food poisoning are reported by the Ministry of Health, Labor and Welfare.

Materials and methods

Preparation of the E. coli O157:H7 cell suspension. *E. coli* O157:H7 (IID959) was added to nutrient broth (Eiken Chemical Co., Ltd., Tokyo, Japan), and incubated overnight at 37˚C for 20 h, without shaking (static culture). The culture broth was centrifuged (2150 x g) at room temperature for 10 min. The supernatant was discarded, and the cells were suspended with sterile saline of 1/10 of the culture broth after cells were washed with sterile saline. The number of cells in the suspension was calculated from its optical density at 600 nm (OD_{600}).

Measurement of thermosensitivity at various temperatures. The *E. coli* O157:H7 suspensions were heated at 43, 45, 47, 49, 51 or 53˚C in an incubator. Each heated cell suspension was removed to calculate cell survival at 0, 10, 20, 30, 60, 90, 120 or 180 min after heating. Each cell suspension was placed in a sterile Petri dish (in duplicate) and mixed with desoxycholate agar (Eiken Chemical) by using pour plating techniques. After the Petri dish was incubated at 37˚C for 24 h, the numbers of viable *E. coli* O157:H7 colonies were counted and expressed as colony-forming units (CFUs). The survival rate at each temperature was expressed as a ratio relative to the number of colonies at 0 min (11).

Measurement of HSP70 expression induced by heating at various temperatures. The *E. coli* O157:H7 cell suspensions were heated at 43, 45, 47, 49 or 51˚C in the incubator, and the expression of HSP70 in *E. coli* O157:H7 cells after heating for 0, 30, 60 or 90 min was measured by an enzyme-linked immunosorbent assay (ELISA) as reported by Itoh *et al* (12-14). *E. coli* O157:H7 cells were dissolved in sodium dodecyl sulfate (SDS) to quantify the expression of HSP70 by ELISA.

Effect of pre-heating. The *E. coli* O157:H7 cell suspensions were heated at 47 or 49˚C for 30 min (pre-heating). The pre-heated cell suspensions were incubated at 37˚C for 0, 10, 20, 30, 60, 120 or 180 min without shaking (static culture), and then they were heated at 53˚C for 0, 10, 20, 30, 60, 120 or 180 min. Survival rates were measured and expressed as CFUs.

Effect of pre-heating of E. coli O157:H7 cells in ground beef. The effect of pre-heating of *E. coli* O157:H7 cells in food was examined in ground beef, since there were many food poisoning cases after consumption of ground beef due to *E. coli* O157:H7 food poisoning as reported by the Ministry of Health, Labor and Welfare.

The *E. coli* O157:H7 cell suspension was mixed with E. coli-free ground beef. The ground beef containing *E. coli* O157:H7 cells was heated at 47˚C for 30 min (pre-heating), and incubated at 37˚C for 30 min without shaking (static culture), followed by heating at 53˚C for 0, 30, 60, 90, 120 or 180 min. The survival rates in each heated ground beef sample were measured and expressed as CFUs. The survival rates of pre-heated *E. coli* O157:H7 cells were compared with the samples of *E. coli* O157:H7 in ground beef that were not heated and the *E. coli* O157:H7 cell suspension alone.

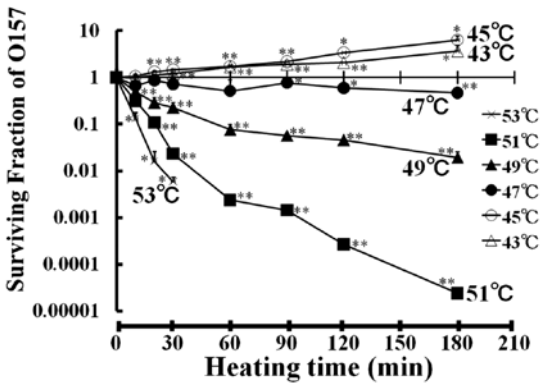

Figure 1. *E. coli* O157:H7 suspensions were heated at 43, 45, 47, 49, 51 or 53˚C for 0, 10, 20, 30, 60, 90, 120 or 180 min. The survival rate at each temperature was expressed as a ratio relative to that at 0 min. Date are expressed as the means ± SD. $^{*}P<0.05$, $^{**}P<0.01$ compared with the number of colonies at 0 min.

Results

Measurement of thermosensitivity. The thermosensitivity of *E. coli* O157:H7 cells after incubation at various temperatures (43, 45, 47, 49, 51 or 53˚C) for 0-180 min is shown in Fig. 1. The number of *E. coli* O157:H7 colonies increased significantly after heating at 43 and 45˚C which are the optimum temperatures for culturing *E. coli* O157:H7 cells, and decreased significantly after 90 min of heating at 47˚C (heat stress). In addition, after heating at 49˚C, the survival of *E. coli* O157:H7 cells decreased significantly after 10 min of heating and declined to 1% of the baseline value after 180 min of heating. Furthermore, heating at a higher temperature, 51˚C, decreased the survival of *E. coli* O157:H7 cells significantly after 10 min, and no colonies were observed after heating for 180 min. Viable *E. coli* O157:H7 cells were not found after heating at 53˚C for 60 min.

Expression of HSP70 after heat stress. The expression of HSP70 in *E. coli* O157:H7 cells after heating at 43-51˚C is shown in Fig. 2. *E. coli* O157:H7 cells did not express HSP70 after heating at 43 and 45˚C for 0-180 min. The expression of HSP70 in *E. coli* O157:H7 cells was increased significantly by heating at 47˚C for 30-90 min, and the highest HSP70 expression occurred after 60 min. HSP70 expression after heating at 49˚C was lower than that after heating at 47˚C, but HSP70 expression in *E. coli* O157:H7 cells was significantly induced by heating at 49˚C. HSP70 expression was not induced by heating at 51˚C.

The expression of HSP70 was maximal after heating at 47˚C for 60 min; however, the standard deviation at that time was large. There was no significant difference in the expression of HSP70 after incubation for 60 and 30 min. In addition, the

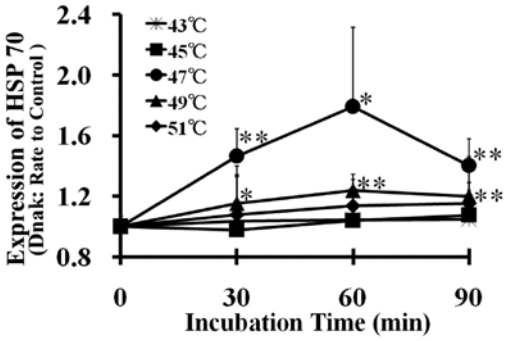

Figure 2. *E. coli* O157:H7 cell suspensions were heated at 43, 45, 47, 49 or 51˚C, and HSP70 expression in O157:H7 cells after heating for 0, 30, 60 or 90 min was measured by ELISA. Date are expressed as the means ± SD. *P<0.05, **P<0.01 compared with the initial dose.

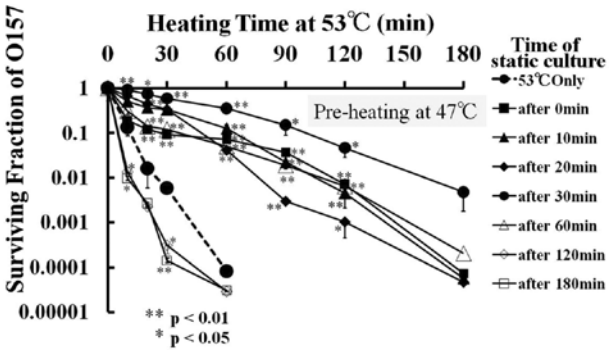

Figure 3. *E. coli* O157:H7 cell suspensions were heated at 47˚C for 30 min (pre-heating). The pre-heated cell suspensions were incubated at 37˚C for 0, 10, 20, 30, 60, 120 or 180 min, and then they were heated at 53˚C for 0, 10, 20, 30, 60, 120 or 180 min again. The survival rate at each incubation time was expressed as a ratio relative to that at 0 min. Date are expressed as the means ± SD. *P<0.05, **P<0.01 compared with cultures that were not pre-heated.

cell cycle of *E. coli* O157:H7 required approximately 30 min to complete. Therefore, we decided that heating for 30 min at 47˚C was the best condition for pre-heating *E. coli* O157:H7 cells to induce HSP70. In addition, we determined the effect of pre-heating at 49˚C for 30 min, as the expression of HSP70 after heating at 49˚C was not substantially higher than that after heating at 47˚C, but the increase in HSP70 expression was significant.

Effect of thermotolerance by pre-heating. Fig. 3 shows the effect of thermotolerance in *E. coli* O157:H7 cells with HSP70 induced by pre-heating at 47˚C for 30 min. The survival rates of *E. coli* O157:H7 cells heated at 53˚C after pre-heating at 47˚C for 30 min were significantly higher than those after heating at 53˚C without pre-heating, excluding the 120 and 180 min static cultures at 37˚C after pre-heating at 47˚C for 30 min. This prevention of the secondary heat stress at 53˚C (second stress; high temperature) by pre-heating at 47˚C (first stress; mild temperature) indicated thermotolerance. In particular, the increased survival rate (thermotolerance) of static cultures that were incubated for 30 min at 37˚C after pre-heating at 47˚C for 30 min was the highest (Fig. 3), and the survival rate after heating at 53˚C for 180 min after pre-heating was 1%

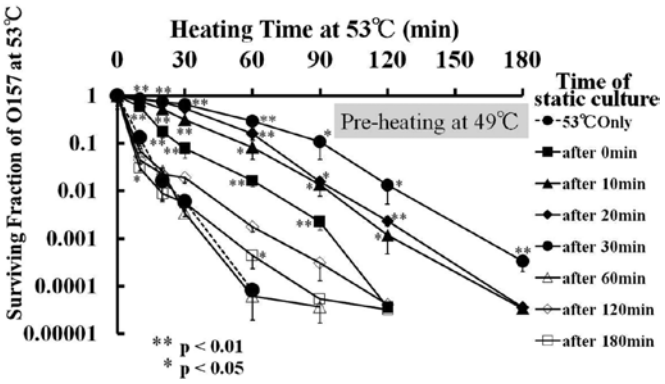

Figure 4. E. coli O157:H7 cell suspensions were heated at 49˚C for 30 min (pre-heating). The pre-heated cell suspensions were incubated at 37˚C for 0, 10, 20, 30, 60, 120 or 180 min, and then they were heated at 53˚C for 0, 10, 20, 30, 60, 120 or 180 min. The survival rate at each incubation time was expressed as a ratio relative to that at 0 min. Date are presented as the means ± SD. *P<0.05, **P<0.01 compared with cultures that were not pre-heated.

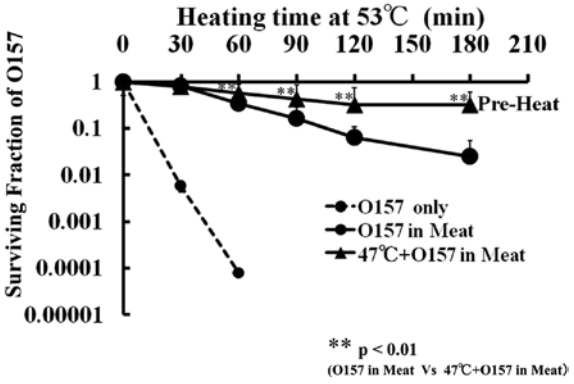

Figure 5. Ground beef containing *E. coli* O157:H7 cells was heated at 47˚C for 30 min (pre-heating) and incubated at 37˚C for 30 min, and then it was heated at 53˚C for 0, 30, 60, 90, 120 or 180 min. The survival rate of pre-heated *E. coli* O157:H7 cells was compared with that of cells that were not pre-heated in ground beef and in cell suspensions alone. Date are expressed as the means ± SD. *P<0.05, **P<0.01 compared with cultures that were not pre-heated.

of the baseline survival rate. The thermotolerance induced by pre-heating at 47˚C for 30 min was shown in the order of 30, 10, 0, 20 and 60 min of static culture at 37˚C.

The effect of thermotolerance induced by pre-heating at 49˚C for 30 min is shown in Fig. 4. Similar to the pre-heating at 47˚C for 30 min, the survival rate of *E. coli* O157:H7 cells heated to 53˚C after pre-heating at 49˚C for 30 min was higher than that after heating at 53˚C without this pre-heating. The thermotolerance induced by pre-heating at 49˚C for 30 min was induced significantly after 30, 20, 10 and 0 min of static culture at 37˚C. The effect of thermotolerance induced by pre-heating at 49˚C for 30 min was lower than that induced by heating at 47˚C.

Effect of thermotolerance in E. coli O157:H7 cells in ground beef induced by pre-heating. The effect of thermotolerance in *E. coli* O157:H7 cells induced by pre-heating at 47˚C for 30 min was measured in ground beef (Fig. 5). All *E. coli* O157:H7 cells were killed by heating at 53˚C for 60 min. The survival rate of *E. coli* O157:H7 cells that were mixed with ground beef

after heating at 53˚C was increased significantly regardless of pre-heating. However, the survival rate of *E. coli* O157:H7 cells after heating at 53˚C in ground beef was increased significantly by pre-heating at 47˚C for 30 min before heating at 53˚C. The thermotolerance of *E. col*i O157:H7 cells in meat, which was measured as the survival rate of these cells in meat by heating at 53˚C for 180 min after pre-heating at 47˚C for 30 min, was 31.9% (almost all *E. coli* O157:H7 cells were alive). The effect of thermotolerance in *E. coli* O157:H7 cells by pre-heating was confirmed in food.

Discussion

There are many reports in the fields of molecular biology and the food microbiology which present various viewpoints regarding the stress response (15,16). Hernandez *et al* reported on the stress response of *Staphylococcus aureus* (17), but there are few reports on the stress response and tolerance of bacteria that cause food poisoning (18). In particular, there are only a few reports of the thermotolerance of enterohemorrhagic *E. coli* (19). We examined the stress tolerance induced in *E. coli* O157:H7 cells by various stresses, particularly heat stress (pre-heating), as there are many cases of infection with *E. coli* O157:H7 cells, which have a large social toll.

E. coli cells activate a stress response to heat stress and induce HSPs to protect themselves from heat stress induced injury when they are cultured under stress temperatures but not high temperatures that cause complete cell death (20). HSPs that are induced by environmental stresses, such as heat stress, repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins, and inhibit protein aggregation, which protects cells from detrimental environmental stresses (21).

Various HSPs are induced by the response to heat stress in *E. coli*. In addition, molecular chaperones, such as DnaK (HSP70), DnaJ (HSP40), GrpE (HSP70 cofactor), GroEL (HSP60), GroES (HSP10), IbpA (HSP104), and IbpB (HSP110), and proteases such as Lon, ClpP, and ClpB, are essential for protein synthesis and protein homeostasis in cells (22). The expression mechanism is thought to occur as follows. Transcription and translation of the *rpoh* gene, which is the structural gene of σ^{32} factor, increases with heat stress. It then combines with a RNA polymerase core enzyme by being replaced by σ^{70} factor, the heat shock protein gene with a promoter recognized by σ^{32} during heat stress, which was functional during the prevous logarithmic growth phase (23).

Therefore, we first measured the survival rate of *E. coli* O157:H7 cells at temperatures of 43-53˚C (thermal sensitivity) and measured the expression of HSP70 at 43-53˚C (stress response).

Heating at 43 and 45˚C increased the number of *E. coli* O157:H7 cells, thus these were considered the optimum growth temperatures. Heating at 47 and 49˚C decreased the number of *E. coli* O157:H7 cells, thus these were considered nonlethal temperatures (heat stress for *E. coli* O157:H7 cells). Heating at 51 and 53˚C markedly decreased the number of *E. coli* O157:H7 cells, therefore, these were considered lethal temperatures.

HSP70 expression was not induced in *E. coli* O157:H7 cells by heating at 43-45 and 51-53˚C, but its expression was induced by heating at 47-49˚C. However, the expression of HSP70 was maximal after heating at 47˚C for 60 min. In the present study, the following observations were made. i) There was no significant difference in the expression of HSP70 after heating for 30 and 60 min; ii) the standard deviation was large after heating for 60 min; iii) the expression of HSP70 was significantly and stably induced after heating for 30 min with a small standard deviation; and iv) the cell cycle of *E. coli* O157:H7 cells lasted approximately 30 min. Therefore, we selected the heating condition at 47˚C for 30 min as the best pre-heating temperature and time for *E. coli* O157:H7 cells and investigated the survival rate for heating at 53˚C after pre-heating (thermotolerance). In addition, we measured the effect of pre-heating at 49˚C for 30 min, as the increase in the expression of HSP70 was significant ($P<0.05$).

For 0-90 min of static culture after pre-heating, the survival rate of *E. coli* O157:H7 cells heated at 53˚C after pre-heating at 47˚C for 30 min was significantly higher ($P<0.05$) than that when heating at 53˚C without pre-heating, which indicated the development of thermotolerance. In particular, the increase in the survival rate of the static culture after incubation for 30 min at 37˚C after pre-heating at 47˚C for 30 min was the highest, and it decreased after incubation for 20, 10 and 60 min. Thermotolerance was not observed in static cultures incubated for 120 or 180 min at 37˚C after pre-heating at 47˚C for 30 min.

The cell cycle of the *E. coli* O157:H7 cells lasted approximately 30 min; therefore, it is not likely that the heat shock response to induce HSP70 occurred in dividing cells after pre-heating for 30 min at 47˚C. Consequently, we hypothesized that *E. coli* O157:H7 cells acquired transient thermotolerance from pre-heating. After pre-heating at 49˚C, thermotolerance was equally recognized in static cultures incubated for 0-30 min at 37˚C after pre-heating at 49˚C for 30 min; however, this effect was significantly lower ($P<0.05$) than that induced by pre-heating at 47˚C.

In regard to the thermotolerance of *E. coli* O157:H7 cells, changes in their toxigenicity and survival rate are important. When the effect of the aforementioned pre-heating was previously examined, the verotoxin that *E. coli* O157:H7 cells produced was measured by the latex agglutination method (24) simultaneously with measurement of the cell survival rate. However, the verotoxin could not be measured in this experiment because its concentration was below the limit of detection (1-2 ng/ml). The toxic substance of *E. coli* O157:H7 infection is verotoxin (VT1, VT2) (25), which is strikingly similar to Shiga toxin. There are no reports regarding the toxigenicity of verotoxin in *E. coli* O157:H7 cells during heat stress and the stress response. Additionally, intimin (26), which acts as an adhesion molecule at the time of infection, and other factors involved in *E. coli* O157:H7 infections are important subjects for future studies.

It appears that determining whether *E. coli* O157:H7 cells acquire thermotolerance to heat stress is important when they contaminate common food. Therefore, we investigated whether the thermotolerance of *E. coli* O157:H7 cells that were added to ground beef was induced by pre-heating, since there are many food poisoning cases in meat artifacts in Ministry of Health, Labour and Welfare reports. In general, the *E coli* O157:H7 contamination rate in ground beef is 1.1-5.0% (27). There are many reports regarding the heat of sterilization (28,29), but

there are few reports that have examined the tolerance effect of pre-heating (19).

All *E. coli* O157:H7 cells were killed by heating for 60 min at 53˚C. In contrast, the survival rate of *E. coli* O157:H7 cells in ground beef was increased significantly (P<0.01) with or without pre-heating. This discrepancy was most likely due to the differences in thermal conductivity and heat absorbance between the nutrient broth and ground beef as well as the protection of *E. coli* O157:H7 cells from heat by muscle cells and proteins in ground beef.

Additionally, the survival rate of pre-heated *E. coli* O157:H7 cells in ground beef was significantly higher (P<0.01) than that in cells without pre-heating. Even after heating at 53˚C for 180 min, the survival rate of *E. coli* O157:H7 cells was high (31.9%). It was confirmed that thermotolerance in *E. coli* O157:H7 was similarly acquired after pre-heating in ground beef.

Heat stress response repairs stress damage and protects and helps cells to adapt to heat stress. Previously, we reported the protective effects of HSP70 induced by pre-heating against various environmental stresses for laboratory animals and humans, such as the thermotolerance of vascular endothelial cells (30), prevention of gastric ulcer induced by restraint and water-immersion stress in rats (9), prevention of renal failure in mice, protection against radiation damage in the small intestine (8), protection against damage due to tongue burning in mice (8), and defense against human fatigue (10).

For most organisms, the expression of the stress protein HSP70 which protects cells from various stresses is essential for their survival. However, the acquisition of thermotolerance by *E. coli* O157:H7 cells in food may result in severe symptoms or death in humans. Therefore, the proper temperatures for cooking, preservation, and handling foods are important.

When exposed to a secondary stress (extreme stress) after expression of HSP70 induced by an initial stress (mild stress), the survival rate of *E. coli* O157:H7 cells was significantly increased, and effective stress tolerance was noted. In this situation, even when the type of stress was different before (first) and after (second) the acquisition of stress tolerance, stress tolerance was observed (31,32). In conclusion, HSP70 is induced by various stresses including heat stress, and its activation induces tolerance to any further stress. Thus, HSP70 is categorized as a stress protein.

It is known that a synergistic effect can occur when heat and other treatments are combined or used concurrently. However, when there is a static incubation process that permits the activation of the stress response (HSP70) between these two treatments, then a resistance effect (stress tolerance), which is quite different from a synergistic effect, can occur (32). Conversely, the measurement of HSP70 may be effective for determining whether stress tolerance was acquired in the reaction.

When *E. coli* O157:H7 cells express HSP70 in response to environmental stresses, such as improper temperature for food preservation, insufficient cooking heat, or incomplete sterilization, *E. coli* O157:H7 cells acquire stress tolerances, such as thermotolerance. Then, *E. coli* O157:H7 cells become hardier and cannot be sufficiently killed by the sterilization process, by normal cooking temperatures and by disinfection methods, which facilitate food poisoning and nosocomial infections.

In addition, since a similar mechanism is likely to occur in other types of bacteria that cause food poisoning and in other infectious diseases, great care is necessary for proper sterilization and disinfection to prevent food poisoning and nosocomial infections.

In conclusion, we examined the induction of HSP70 in *E. coli* O157:H7 cells at various temperatures and the thermotolerance of *E. coli* O157:H7 cells alone and in contaminated food following pre-heating. We evaluated the possibility that thermotolerance by *E. coli* O157:H7 increases the likelihood of food poisoning.

We showed that *E. coli* O157:H7 cells acquired thermotolerance after pre-heating, which significantly increased survival after a lethal temperature, and increased the likelihood of food poisoning. As a result, proper sterilization and disinfection techniques are important to prevent food poisoning and nosocomial infections.

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