Effect of Heat Shock on the Thermotolerance and Protein Composition of Yersinia enterocolitica in Brain Heart Infusion Broth and Ground Pork

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ABSTRACT

The optimum conditions required to induce a heat-shock response in Yersinia enterocolitica in brain heart infusion (BHI) broth were determined. The production of heat-shock proteins and the increased thermotolerance of heat-shocked Yersinia cells in ground pork when exposed to higher temperatures was also examined. Heat shocking Y. enterocolitica cells at 45°C for 60 min consistently resulted in an increased number of survivors to a subsequent treatment of 55 or 60°C in BHI broth when compared with non-heat-shocked controls. D values at 55°C were calculated as 7.7 and 2.0 min and at 60°C as 1.6 and 1.2 min for heat-shocked and control cells, respectively. After examination of heat-shocked cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two distinct heat-shock proteins with molecular masses of 70.5 and 58.0 kDa were observed that were not present in the control. Evaluation of heat-shocked and control cell survival in ground pork revealed D55 values of 15.6 and 6.5 min and D60 values of 6.7 and 1.7 min, respectively. The results indicate that prior heat shock can induce increased resistance in Y. enterocolitica in ground pork to higher heat treatments. Survival of Yersinia enterocolitica in cooked meat due to the phenomenon of the heat-shock response can become a cause of concern regarding microbiological food safety.

Key words: Yersinia, heat shock, thermotolerance, heat-shock proteins

Although Yersinia enterocolitica was first isolated in 1939 by Schleifstein and Coleman (21), it has emerged into prominence only over the last two decades. This recognition can be attributed to its implication as the causative organism of several foodborne illness outbreaks. Y. enterocolitica infections, though quite common in Europe and Japan, have only recently been associated with outbreaks in the U.S. (2, 6, 22, 25, 26). Milk has been the source of infection in many of these outbreaks and O:8 the major serotype. Besides milk, the pathogen has also been isolated from pork, beef, poultry, vegetables, tofu, and other miscellaneous prepared food products. Y. enterocolitica has been increasingly isolated from the tongues and tonsils of swine. Yersiniosis in humans following raw pork consumption has led to the implication of swine as the major source of human infections (9, 27).

Symptoms of yersiniosis include gastroenteritis with severe abdominal pain that is often termed pseudoappendicitis. Other complications associated with the illness are arthritis, erythema nodosum, septicemia, and abscess formations on skin and internal organs (24).

When some organisms are exposed to elevated sublethal temperatures, they respond by synthesizing a group of proteins known as heat-shock proteins (hsp) or stress proteins (3, 14, 19). This phenomenon is commonly called the heat-shock response. The heat-shocked cells, when subjected to further higher temperatures, are more thermostolerant than non-heat-shocked cells. Also, the synthesis and degradation of hsps seem to coincide with the development and decay of thermotolerance (13). It has therefore been assumed that the heat-shock response is a protective mechanism for the survival of cells under stress.

The heat-shock response and thermotolerance have been studied in bacteria chiefly by using laboratory broth cultures. The significance in foods has only been studied in the last few years. Mackey and Derrick (16) showed that heat-shocked Salmonella thompson was more resistant with a significantly increased number of survivors when exposed to a heat treatment of 54°C for 1 h. The heat-shocking period appears to be an important factor in increasing the thermotolerance. Results from the studies conducted by Knabel et al. (11) and Farber and Brown (5) clearly demonstrate that heat-shocking for longer periods of time increases the heat resistance of Listeria monocytogenes.

So far, most of the studies on heat-shocked microorganisms in food products have been focused mainly on Listeria spp. and Salmonella spp. Heat sensitivity of Y. enterocolitica has been studied in milk (4, 7, 28) and beef (8); however, the phenomenon of the heat-shock response and thermotolerance of this organism in meat or other food products has not been examined. The ability of Y. enterocolitica to grow at...
refrigeration temperatures and under vacuum further underlines the necessity of the present study.

The objectives were therefore (i) to determine the time-temperature combination that will produce the maximum thermotolerance in *Y. enterocolitica* in brain heart infusion (BHI) broth, (ii) to determine whether production of stress proteins occurs in heat-shocked cells, and (iii) to determine the survival of heat-shocked *Yersinia* cells when inoculated into uncured ground pork and exposed to higher temperatures.

**MATERIALS AND METHODS**

**Bacterial culture**

*Yersinia enterocolitica* serotype O:8 (ATCC 27729) was obtained from the American Type Culture Collection in Rockville, MD. The stock culture was maintained in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) containing 15% glycerol at −50°C. Subcultures were maintained in BHI broth at 25°C for experimental purposes.

**Determination of optimum heat-shock conditions**

One milliliter of log-phase *Y. enterocolitica* cells grown at 25°C was inoculated into a tube containing 5.0 ml of fresh BHI broth prewarmed at 40, 45, 48, or 50°C (heat-shock temperatures) in a circulating waterbath (Model 730, Fisher Scientific, Pittsburgh, PA). The fluid levels in the tubes were maintained well below the water level in the bath to ensure uniform heating. The come-up time was recorded using a thermocouple (Omega Engineering Inc., Stanford, CT) attached to a datalogger (Model LI-1000, LI-COR, Lincoln, NE) and calculated as an average of 2.9 min. On reaching one of the heat-shock temperatures, the cells were heat-shocked for either 5, 10, 15, 30, 45, or 60 min. The heat-shocked cells were then immediately subjected to a heat treatment of either 55 or 60°C. The subsequent heating at 55 or 60°C was considered to be the heat treatment. Samples (1 ml) were then drawn at regular intervals and plated on BHI agar after making the appropriate serial dilutions in 0.1% peptone solution. Non-heat-shocked cells exposed to only the higher heat treatments were taken as the control. Colonies were enumerated after incubation of the plates at 30°C for 48 h.

**Determination of heat shock protein production**

A 6-h culture of *Y. enterocolitica* with 10⁸ cells per ml was prepared in 10 ml of BHI broth at 25°C. One-milliliter samples were placed in microcentrifuge tubes and centrifuged at 15,000 × g for 2 min. The supernatant was decanted and the cell pellets were washed three times with M9 medium (Difco) containing 0.05% (wt/vol) Casamino acids and L-proline (Sigma Chemical Co., St. Louis, MO). Washed cells were resuspended in 0.5 ml of the modified M9 medium with supplements (Casamino acids and L-proline) and were heat-shocked for up to 60 min at 45°C. The samples took an average of 1.0 min to reach the higher temperature. Non-heat-shocked cells were used as controls. At timed intervals, the cell suspensions were pulse-labeled with 5 μl of trans-[³⁵S]methionine for 1.0 min and then chased for 1.0 min with 0.5 ml of modified M9 medium containing an additional 400 μg of L-methionine per ml. Protein synthesis was stopped by the addition of 0.1 ml of ice-cold chloramphenicol (Sigma) (2.5 mg/ml of ethanol) and the sample tubes were immediately transferred to an ice bath. Samples thus prepared were stored at −20°C until used. Ten-microliter samples were loaded on gels and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (12) (14% acrylamide gels, room temperature, constant 200V). Low-molecular-mass protein markers (Bio-Rad Laboratories, Richmond, CA) containing up to a 97.4 kDa protein were used as standards. Gels were stained with Coomassie blue, and the labeled polypeptides were detected by autoradiography of the dried gels on X-Omat films (Eastman Kodak, Rochester, NY).

**Determination of the survival of heat-shocked cells after processing in uncured ground pork**

Pork was purchased from the Iowa State University meat-processing plant for the entire study. Microbial counts in the uninoculated meat were ca. 10⁵ colony-forming units (CFU)/g. Samples were also plated on cefsulodin-irgasan-novobiocin (CIN) agar (Difco) and subsequently biochemical tests were performed to determine the presence of any *Y. enterocolitica* in the uninoculated samples. None were detected. On the basis of the results of the first experiment, *Y. enterocolitica* cells were heat shocked in BHI broth at 45°C for 60 min. Non-heat-shocked cells were taken as controls. The heat-shocked and non-heat-shocked cells were then inoculated separately into ground pork (0.2 ml/25 g). The inocula were mixed thoroughly into the meat for 4 min with a stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, OH) so that the meat contained approximately 10⁷ cells per g of either heat-shocked or non-heat-shocked cells. The meat was then divided into 25-g portions in plastic pouches (0.400, Tekmar Company, Cincinnati, OH), spread uniformly in a thin layer, and sealed under air. The meat samples were then exposed to either 55 or 60°C in a circulating waterbath. Random samples were removed at regular intervals, serially diluted in 0.1% peptone solutions, and plated on BHI and CIN agars. Colonies were enumerated after incubation as previously mentioned.

**Statistical analyses**

The heat treatments (55 and 60°C) of heat-shocked and control *Yersinia* cells, both in BHI broth and ground pork, were performed in triplicate. The D values were calculated as the reciprocal of the slope obtained by using linear regression analyses of the data. Significant differences between the regressions were determined by an analysis of covariance.

**RESULTS AND DISCUSSION**

**Optimum conditions of heat shock**

When exposed to higher temperatures (55 and 60°C), an increased number of survivors of heat-shocked *Y. enterocolitica* was observed under all the time-temperature combinations used, when compared with controls (data not shown). However, the initial studies showed that the difference in the number of survivors between the heat-shocked and control samples after heat treatment at 55 or 60°C was less than 1 log unit. Also, repeated experiments did not give consistent results. Heat shocking at 45°C for 60 min with a further heat treatment at 55°C consistently resulted in a higher number of survivors when compared with controls (Fig. 1). Preliminary experiments showed that heat shocking at 45°C for 60 min in BHI broth without exposure to a higher heat treatment did not cause any significant decrease in cell count (data not shown). The heat-shocked samples (45°C for 60 min) had 4.9 ± 0.3 and 4.2 ± 0.4 log number of survivors after a heat treatment of 55°C for 15 and 20 min, respectively. No
Survivors were present in the non-heat-shocked control samples after the same periods of heat treatment. There was a significant difference in $D_{55}$ values (Table 1), with heat-shocked cells having a $D$ value as high as four times those of controls.

The optimum temperature for heat shocking $Y$. enterocolitica was similar to those for other mesophilic bacteria. Lindquist (14) has pointed out that for mesophilic bacteria, temperatures between 45 and 50°C are optimum for development of the heat-shock response and thermotolerance. Increased resistance by heat-shocked $E$. coli O157:H7 to a higher heat treatment of 55°C was also observed by Murano and Pierson (18). Similar results were also obtained by Farber and Brown (5) working with $Listeria monocytogenes$ in a sausage mix. The authors indicated that thermotolerance of $Listeria$ cells increases with increasing heat-shock time. Knabel et al. (11) demonstrated that heat shocking $L$. monocytogenes at 43°C for 30 to 60 min increased thermostolerance, compared with that of cells heat shocked for only 5 min at the same temperature. Heat shocking $Y$. enterocolitica at 45°C for 60 min in BHI broth was then used for subsequent experiments in pork samples.

**TABLE 1.** $D$ values of heat-shocked and non-heat-shocked control $Y$. enterocolitica in BHI broth and ground pork at different heat-treatment temperatures

<table>
<thead>
<tr>
<th>Temp. ($^\circ$C)</th>
<th>BHI</th>
<th>Pork</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heat-shocked</td>
<td>Control</td>
</tr>
<tr>
<td>55</td>
<td>7.7A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0B</td>
</tr>
<tr>
<td>60</td>
<td>1.6A</td>
<td>1.2A</td>
</tr>
</tbody>
</table>

<sup>a</sup> Different letters within each temperature and for either BHI or pork denote significant difference at the 0.05 level.

**Gel electrophoresis of heat-shock proteins**

Thermotolerance of heat-shocked bacteria has usually been associated with the production of heat-shock or stress proteins (5, 11, 16, 18). This finding prompted us to determine whether heat shocking would also result in the production of stress proteins by $Y$. enterocolitica. Two distinct heat-shock proteins with molecular masses of 70.5 and 58.0 kDa were observed (Fig. 2). These proteins were either not produced or produced in undetectable amounts by the non-heat-shocked cells. Production of heat-shock proteins was almost immediate after exposure to the sublethal temperature of 45°C. Initially, there appeared to be an increased synthesis of both heat-shock and normal proteins, but over time, reduction in synthesis of normal proteins and continued synthesis of heat-shock proteins was noted. Similar observations have been made by Neidhardt et al. (19), who observed that the rate of synthesis of heat-shock proteins accelerates within seconds to rates many times higher than their pre shift rates and then declines to a steady state, characteristic of the elevated temperature. The same authors have also noted that a shift to 43 to 47°C results in the production of large amounts of heat-shock proteins and that the rate of synthesis of non-heat-shock proteins decreases.

In heat-shocked $E$. coli O157:H7 a 71 kDa protein corresponding to the $\sigma^{32}$ subunit of RNA polymerase is known to play a pivotal role in regulation and synthesis of other stress proteins (18, 23). In the present study, one of the stress proteins detected had a molecular mass of 70.5 kDa, and it is possible that it is the same $\sigma^{32}$ subunit. Heat-shock proteins ranging in size from 7.5 to 80 kDa have been observed in $Y$. enterocolitica serotype O:3 (30). It was also shown that a 60 kDa stress protein corresponds to the GroEL protein of $E$. coli. The GroEL protein has been known to be essential for $E$. coli growth and is also involved in morphogenesis of coliphages (30). It is likely that the 58-kDa stress protein detected in our experiment corresponds to the 60-kDa protein of $Y$. enterocolitica, serotype O:3. The 60-kDa proteins are known to be subunits of a native protein with a molecular mass of 400 to 500 kDa (30). Antibodies to this 60-kDa protein have been found in sera of patients with yersiniosis and detection of these antibodies increases with increased age. Yamaguchi et al. (30) speculate that the 60-kDa stress protein may be involved in the development of arthritis-like symptoms in humans.

**Survival of the heat-shocked cells in ground pork**

Figure 3 compares the survival curves of heat-shocked and control cells when heat treated at 55°C in ground pork. As indicated by the $D$ values (Table 1), increased resistance was observed for the heat-shocked cells compared with the controls when exposed to both 55 and 60°C heat treatments. Heat treatment at 60°C resulted in rapid reduction in the number of $Yersinia$ cells, especially in the controls (undetectable after 9 min). Increased numbers of injured cells were produced in both heat-shocked and control samples at 60°C. Most of the survivors produced only pinpoint colonies after incubation at 30°C for 3 days and were difficult to enumerate. Suspect colonies were tested by Enterotube® and...
confirmed for Yersinia. Heat treatment at 55°C resulted in an increased number of survivors (ca. 1.1 log units more after 15 min) in heat-shocked samples compared with the control (Fig. 3).

The death of bacterial cells exposed to high temperatures is probably due to DNA and protein damage. Accumulation of these abnormal, denatured proteins can become toxic to the cells (20). Studies have shown that the function of hsps includes ATPase activity (31). Some hsps were found to be associated with unfolded chromosomes, preventing their further unfolding and loss of viability (19). It has also been hypothesized that denatured proteins in the bacterial cells induce synthesis of σ32 subunits (17). Further, the increased production of σ32 subunits at elevated temperatures promotes specifically the production of other hsps. Protease activity for the degradation of denatured proteins and catalase activity for eliminating free radicals that are toxic to the cells are functions that have also been ascribed to hsps.

Besides production of hsps, the increased thermotolerance of heat-shocked cells may also be due to saturation of phospholipids in the cell membrane. It was demonstrated by Beuchat and Worthington (1) that higher growth temperatures enhanced the heat resistance of cells to further higher temperatures. The higher heat resistance was shown to coincide with a decreased unsaturation of phospholipids. They suggested that a cytoplasmic membrane containing low unsaturated phospholipids was important in increasing the thermotolerance of a cell. Tsuchiya et al. (29) indicated that four major phospholipids in the cell membrane of Y. enterocolitica do indeed become saturated at higher temperatures. The results of our study show that Yersinia produced hsps rapidly with increase in temperature (45°C). Moreover, the heat-shocked cells became more thermotolerant when exposed to higher temperatures in both BHI and pork. Therefore, production of hsps may be only one of several mechanisms by which heat-shocked cells become more thermotolerant.

Slow cooking of meat (lower heating rates) has been reported to increase the heat resistance of Salmonella typhimurium and Listeria monocytogenes (10, 15). Also, inadvertent preheating can occur during fabrication and processing of raw meat. Washing of carcasses with hot water sprays and packaging of ground pork in shrink tunnels are examples in processing of raw meat where heat shocking of organisms can occur. This can lead to a higher chance of survival of Y. enterocolitica during subsequent cooking, especially of certain meat products that are cooked slowly at lower temperatures and longer periods. The results of the present study indicate that Y. enterocolitica exhibit a heat-shock response and produce hsps. Also, the heat-shocked cells were more thermotolerant at temperatures of 55 and 60°C when compared to non-heat-shocked controls. It is therefore important to keep in mind the possible presence of heat-shocked pathogens such as Yersinia spp. when establishing safe heat-treatment guidelines for food.

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