Effect of heat treatment, irradiation, and storage conditions on the survival, growth, and virulence of heat-shocked *Yersinia enterocolitica* in ground pork

by

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GENERAL INTRODUCTION

A combination of three factors namely: cause of several outbreaks, increased production of ready-to-eat processed food products, and the exorbitantly high costs for treatment of even minor illnesses have made the public more aware of microbiological food safety than ever before. This awareness has led to the rediscovering by the scientific community of certain microorganisms as possible causes of foodborne infections. *Yersinia enterocolitica* and *Listeria monocytogenes* are two typical examples where concrete evidence has been produced regarding their foodborne transmission. Unfortunately, there are several aspects of these organisms that remain to be explored. An attempt has therefore been made in this study to further understand certain behavioral characteristics of *Y. enterocolitica* that can become useful in food processing.

Y. enterocolitica is a cold-tolerant foodborne pathogen. Due to certain important biochemical characteristics it is classified under the family *Enterobacteriaceae*. The organism has been isolated from food products like milk, tofu, vegetables, pork, beef, and poultry. Swine have been incriminated as the major reservoir for human infections. There are two major reasons for this: (1) Studies have shown that healthy pigs can become carriers of this pathogen, and (2) The incidence of yersiniosis is high following raw or undercooked pork consumption. "Yersiniosis" is the disease caused by *Y. enterocolitica*. It causes gastroenteritis with the patient exhibiting symptoms like diarrhea, low fever, and severe abdominal pain. Post-infection manifestations include arthritis and erythema nodosum.

Some of the pathogenic serotypes are O:3, O:9, O:5,27, and O:8. The first three are

common in Europe, whereas O:8 has been the cause of several outbreaks in the US. Serotyping has therefore been used successfully to a certain extent in epidemiological surveys to detect the pathogenic isolates. However, due to the diverse distribution of *Yersinia* in nature and the presence of both virulent and avirulent strains, mere isolation followed by serotyping is insufficient to determine its significance. The problem is further complicated by the changing patterns in the geographical distribution of the pathogenic serotypes over the last decade. Also, the unavailability of antisera for serological typing has led to the development of several laboratory tests that can be conducted routinely to determine the virulence.

Although *Y. enterocolitica* is temperature-sensitive and can be eliminated by heating at 60°C for several minutes, it has the ability to become thermotolerant. Exposure of organisms to sublethal temperatures cause induction of a set of proteins called "heat shock" or "stress" proteins. This phenomenon is termed the "heat shock response". The heat-shocked cells become thermotolerant and are able to survive subsequent higher temperatures. Slow heating of meat to a final internal temperature can result in a higher number of thermotolerant microbial cells due to induction of the heat shock response. Farber and Brown (1990) have suggested that the slower the temperature increase in the meat, the larger the increase in heat resistance or thermotolerance of bacteria.

Another step in processing that is being frequently utilized in the meat industry is packaging under vacuum or modified atmosphere. Retention of freshness and extension of shelf life are the two important goals that can be partly achieved by this technique. However, storage at low refrigeration temperatures cannot be compensated and is an essential

requirement along with packaging. These processes inhibit the aerobic microbial flora that are otherwise the earliest indicators of spoilage. *Yersinia* is not only a facultative anaerobe but also a psychrotroph. Inhibition of competitive spoilage flora by storing under low temperatures and various anaerobic atmospheres can further enhance growth of *Yersinia* . The extended periods of storage can then become ideal for the growth of this pathogen to high infectious levels.

The conventional methods of meat processing like heating, canning, and freezing, although efficient in eliminating pathogens, have one major drawback in common. All these methods change the sensory qualities of the product and especially, of raw meat. The process of irradiation is one of the solutions to this problem. The benefits of irradiating food are several. With the ever increasing demand for microbiologically safe foods, especially for the growing numbers of immunocompromised and the elderly in the population, irradiation like the process of pasteurization, will shortly become not only the method of choice but simply indispensable. However, like heating, irradiation sensitivity of microorganisms varies and needs to be determined if radiation is to be used effectively.

The focus of this research has therefore been to determine the effect of heat shock on thermotolerance of *Y. enterocolitica*, and on survival to irradiation, and to investigate the growth kinetics and virulence of heat-shocked *Yersinia* in pork stored under different packaging atmospheres.

The specific objectives of this study were:

1. To determine the temperature/time combination needed to induce heat shock response

followed by maximum thermotolerance in *Y. enterocolitica* in brain heart infusion broth.

2. To determine the production of stress proteins by the heat-shocked cells.
3. To determine the ability of heat-shocked cells to survive subsequent higher heat treatments and irradiation in ground pork.
4. To determine the effect of storage atmospheres and temperatures on the survival and growth of heat-shocked cells following heat treatment.
5. To determine virulence of the survivor cells.

Dissertation Organization

This dissertation includes three manuscripts to be submitted to the Journal of Food Protection. A general introduction followed by a literature review precedes the first manuscript and a general conclusion of the research follows the third manuscript. References cited in the general introduction, literature review, and general conclusion follows the general conclusion.

LITERATURE REVIEW

Yersinia enterocolitica

Introduction

The bacterium *Yersinia enterocolitica* along with its history, growth requirements, virulence, outbreaks, disease symptoms and sources of infection has been reviewed in detail by several authors (Swaminathan *et al.*, 1982; Schiemann, 1989; Cover and Aber, 1989; Stern and Pierson, 1979; Kapperud, 1991; Doyle and Cliver, 1990; Schofield, 1992). The important characteristics relevant to the present research have been compiled together in this section so as to give the reader a concise but clear understanding of this organism.

History

Y. enterocolitica was first isolated in 1939 by Schleifstein and Coleman from human infections in the New York State Department of Health (Schiemann, 1989). The organism was originally called "*Bacterium enterocoliticum*" and was renamed "*Yersinia enterocolitica*" by Frederiksen in 1964 (Swaminathan *et al.*, 1982). Although *Y. enterocolitica* was first isolated in the United States, the incidence of infection and outbreaks were higher in several European countries, Canada, and Japan. Only in the last two decades, has this organism resurfaced in the US, causing several outbreaks.

Classification, morphology, and growth characteristics

In Bergey's manual (Bercovier and Mollaret, 1984) *Y. enterocolitica* is classified under the family, *Enterobacteriaceae*. Accordingly, like the other members of this family, *Y. enterocolitica* also exhibits the typical characteristics. *Y. enterocolitica* is a Gram negative,

nonsporeforming, coccobacillus. It is motile at temperatures below 30°C, but nonmotile at 37°C, oxidase negative, catalase positive, reduces nitrate to nitrite, and is a facultative anaerobe (Bercovier and Mollaret, 1984). Besides these general characteristics, *Y. enterocolitica* can be differentiated from other "*Yersinia*-like" organisms by certain biochemical and serological reactions. The biochemical tests that help in classification and identification of closely related *Yersinia* spp. including *Y. enterocolitica* are: fermentation of salicin, xylose, lactose, trehalose, sorbitol, sucrose, sorbose, and rhamnose, presence of lecithinase, ornithine decarboxylase, β-galactosidase activity, and utilization of esculin, indole, and nitrate (Schiemann, 1989). The species that are closely related to *Y. enterocolitica* and are nonpathogenic are, *Y. intermedia, Y. frederiksenii, Y. kristensenii,* and *Y. aldovae.* The other two important pathogenic species in the genus *Yersinia* include *Y. pestis* and *Y. pseudotuberculosis.*

Serotyping not only helps in distinguishing *Y. enterocolitica* from *Y. pseudotuberculosis* and other species, but has also been found to be an important tool in detecting the virulent human isolates. At present, approximately 57 serotypes, both pathogenic and nonpathogenic, have been isolated (Schiemann, 1989). Only some serotypes have been consistently isolated from human infections. These human serotypes appear to have a very similar and stable biochemical profile. The heat stable somatic "O" antigen is useful in classification of the different serotypes. The global distribution of the various pathogenic serotypes has also been studied. O:3, O:9, and O:5,27 are distributed mainly in Europe and Asia. In the US, O:4,32, O:20, O:8, O:13a,13b, O:18, and O:21 have been isolated from

humans (Schiemann, 1989).

Y. enterocolitica is temperature-dependent and certain specific characteristics of the organism are associated with temperature. Although the optimum temperature for growth is 25-29°C (Bercovier and Mollaret, 1984), several workers have demonstrated its uninhibited growth at refrigeration temperatures, resulting in its classification as a psychrotroph. Increased growth of the bacterium at cold storage temperatures is known to occur in milk, beef, and pork (Budu-Amoako *et al.*, 1993; Hanna *et al.*, 1979; Lee, 1977; Amin and Draughon, 1987). This property of psychrotrophism has also been utilized for the successful isolation of *Y. enterocolitica* from food and feces (cold enrichment). On the other hand, growth at higher temperatures (35-37°C), even on nonselective media requires supplementation with calcium (Bercovier and Mollaret, 1984). This calcium-dependency of the organism at 37°C has been exploited by certain workers (Gemski *et al.*, 1980; Prpic *et al.*, 1983; Bhaduri *et al.*, 1991) for developing tests for virulence. However, *Yersinia* is temperature-sensitive and can be eliminated from food at temperatures above 60°C (Hanna *et al.*, 1979; Ibrahim and Mac Rae, 1991).

Effects of heat treatment

The sensitivity of *Y. enterocolitica* to heat has been studied in food products like milk and beef. In the study conducted by Hanna *et al.* (1977a), heat resistance of *Y. enterocolitica* varied in different strains. Although all strains examined were killed at temperatures above 60°C in milk, the ATCC strains were more resistant than the isolates from vacuum-packaged meat. In another study conducted by the same authors (Hanna *et al.*, 1977b), it was found that

heating beef to an internal temperature of 60 to 62°C was sufficient to eliminate *Yersinia*. The heat resistance of several pathogenic and nonpathogenic serotypes suspended in whole or skim milk was examined by Toora *et al.* (1992). All were highly sensitive and none survived high-temperature short-time pasteurization (71.8°C for 18 secs.). The $D_{62.8}$ values were 10.53 and 10.35 secs. in skim and whole milk, respectively (Toora *et al.*, 1992). The heat resistance of *Y. enterocolitica* in raw milk was also studied by D'Aoust *et al.* (1988). In their study, raw milk was inoculated with *Yersinia*, both pathogenic and nonpathogenic serotypes, and heat treated at 60 to 72°C for a minimum holding time of 16.2 secs. All the serotypes were found to be highly sensitive and none were detected at temperatures above 63°C.

Sublethal injury of *Y. enterocolitica* following heat treatment was studied by Restaino *et al.* (1980). They showed that serotypes O:3, O:8, and O:17 sustained sublethal injury upon heat treatment at 47°C for 12 to 70 mins. *Y. enterocolitica* serotype O:17, a nonpathogenic serotype was reported to be more sensitive to sublethal heat treatments than the other two pathogenic serotypes. The extent of injury was found to be dependent on the type of the suspending menstruum. The injury of serotype O:17 was higher when the suspending medium was phosphate buffer versus pork infusion, brain heart infusion broth, or nonfat-dry-milk (Restaino *et al.*, 1980).

In conclusion, *Yersinia* is a heat sensitive organism, and does not survive temperatures above 60°C for several minutes. However, the sensitivity of *Yersinia* varies among strains. In most of the studies, the pathogenic serotypes were found to be more heat resistant than the nonpathogenic environmental strains.

Yersiniosis

By definition, the disease caused by *Y. enterocolitica* is termed "Yersiniosis" (Doyle and Cliver, 1990). The clinical manifestations of the disease have been reviewed in detail by Cover and Aber (1989). The incubation period of *Y. enterocolitica* enterocolitis ranges from 1 to 11 days. The organism causes gastroenteritis with typical symptoms in younger children (Tacket *et al.*, 1985). The symptoms include diarrhea, low fever, and abdominal pain. Extreme pain in the lower right quadrant of the abdomen has usually been misdiagnosed as appendicitis (pseudoappendicitis), leading to surgery (Schiemann, 1989). The pseudoappendicular syndrome is more common in older children and young adults (Cover and Aber, 1989). Post-infection manifestations include arthritis and erythema nodosum. Most of the cases are self-limiting and complete recovery can occur within 5 to 14 days of infection. Sometimes there may be complications like bacteremia, inflammation of the small intestine and colon, peritonitis, and intestinal perforation. Extraintestinal infections include pharyngitis, abscess formations on the skin, osteomyelitis, lymphadenitis, urinary tract infections, lung abscess, and pneumonia, to name a few (Cover and Aber, 1989). The extraintestinal infections appear to be more common in adults than in children (Tacket *et al.*, 1985).

Epidemiology

Epidemiologically, *Y. enterocolitica* strains have been classified into two broad categories. Strains isolated from humans and outbreaks are classified under the term "clinical" or "typical". Isolates from raw food materials, water, animals, and soil are termed "environmental" strains. (Stern and Pierson, 1979; Lee, 1977).

Y. enterocolitica has been isolated from food products like milk, vegetables, pork, beef, and poultry. This has led to the implication of food as the major source of infection. Further, food-related outbreaks in the US, have confirmed this view (Schiemann, 1989). Although milk has been associated with several outbreaks in the US (Tacket *et al.*, 1984; Black *et al.*, 1978), some have been caused by direct or indirect consumption of contaminated water (Tacket *et al.*, 1985; Shayegani *et al.*, 1983). Unlike the US, pigs have been incriminated as the major source of infections and the cause of several outbreaks in Europe (Kapperud, 1991). *Yersinia* has been isolated from tongues, tonsils, and feces of swine (Toma and Deidrick, 1975; Kotula and Sharar, 1993; Mafu *et al.*, 1989; DeBoer and Nouws, 1991). A high percentage of healthy Danish pigs were reported to be carriers of the pathogenic serotype O:3 (Andersen, 1988). Carrier pigs are known to shed the organism regularly in their feces. Prevalence of unhygienic conditions during rearing and breeding may have contributed to the development of healthy pigs becoming carriers of this pathogen. Contamination of pork can then occur during slaughter and evisceration processes. Finally, consumption of raw or undercooked pork and pork products can result in infection of humans. The high incidence of *Y. enterocolitica* infections in Belgium has been attributed to the ingestion of raw pork (Tauxe *et al.*, 1987). Besides food products, person-to-person transmission of *Y. enterocolitica* infection has been documented by Toivanen *et al.* (1973).

The changing of the predominant pathogenic serotype in a given geographical area adds another dimension to the problem. Several earlier outbreaks in the US were caused by the serotype O:8 (Table 1). However, more recent surveys, both in the Eastern and Western

Coasts of the US have revealed that, O:3 has become the major serotype (Shayegani *et al.*, 1981; Bottone, 1983; Bissett *et al.*, 1990).

Isolation and identification

In a survey conducted by Bissett *et al.* (1990) over a period of 11 years (1978-89) in California, a dramatic increase in recovery of *Yersinia* was observed in the years 1984-89. There may be two major reasons for this: (a) social changes and (b) development of newer isolation procedures. The social changes include, higher consumption rate of ethnic foods, development of newer processing and packaging techniques, and consumption of raw or undercooked foods for the so called "natural organoleptic and nutritional qualities". However, awareness of the organism as a foodborne hazard, and developing newer, more sensitive culture media are probably the chief reasons for the increased isolation of this pathogen.

Enrichment media include simple buffer solutions, modified Rappaport broth, and bile-oxalate sorbose broth. Incubation at 4°C for 14 to 21 days further enhances the growth of this organism due to its psychrotrophic nature (Schiemann, 1989). The property of *Y. enterocolitica* to tolerate bile salts, certain dyes, and antimicrobials like novobiocin and irgasan (2, 4, 4'-trichloro-2-hydroxydiphenyl ether) has been used in developing selective media for its isolation. Plating media include Salmonella-Shigella (SS), Xylose-Lysine-Deoxycholate (XLD), Deoxycholate-Citrate-Lactose (DCL), MacConkey, Hektoen, Bismuth Sulfite (BS), and Lactose-Saccharose-Urea (LSU) agars (Schiemann, 1989).

Table 1. Major outbreaks in the US.

Year	Location	Vehicle	Serotype	References
1981-82	Washington State	Tofu	O:8	Tacket *et al.*, 1985
1972	N. Carolina	sick puppies?	O:8	Gutman *et al.*, 1973
1981	Sullivan county, New York	Powdered milk, chow mein	O:8, O:34	Shayegani *et al.*, 1983
1982	Tennessee, Arkansas, Mississippi	Pasteurized milk?	O:13, O:18	Tacket *et al.*, 1984
1976	Oneida county, New York	chocolate milk	O:8	Black *et al.*, 1978

An exceptionally excellent medium for growing *Y. enterocolitica* from food or clinical samples, is the Cefsulodin-Irgasan-Novobiocin (CIN) agar. The CIN agar was first prepared by Schiemann in 1979 (Schiemann, 1979). The medium is not only highly selective for *Y. enterocolitica*, but also provides unique colony morphology that is very easy to detect (Head *et al.*, 1982; Harmon *et al.*, 1983). The colonies on this medium are 0.5 to 1.0 mm. in diameter, slightly raised, smooth, and with a distinctive transparent border ("bullseye" appearance).

Biochemically, for identification, the typical strains are usually lactose negative, urease positive, and citrate negative. On Triple-Sugar-Iron (TSI) agar, *Y. enterocolitica* produces either an acid butt and slant or acid butt and alkaline slant without H_2S and little or no gas (Cover and Aber, 1989). Development of gene probes has also enabled the rapid detection of this pathogen when present either in pure form (culture) or in food and clinical specimens

(Kapperud *et al.*, 1993; Miliotis *et al.*, 1989).

Virulence Characteristics of *Yersinia enterocolitica*

Introduction

As *Yersinia enterocolitica* is commonly found in food and environmental samples, isolation and identification should be followed by detection of virulence to determine its significance. Especially because, unlike *Salmonella* where all strains are pathogenic, strain-to-strain variation in pathogenicity occurs in *Y. enterocolitica*. A given strain is classified either pathogenic or nonpathogenic on the basis of its occurrence in an outbreak, ability to invade tissue cells, and ability to induce disease when injected into experimental animals (Farmer III *et al.*, 1992). The overall expression of virulence in *Yersinia* appears to be based on a combination of several factors. The important among them being, presence of a 42-48 Mda plasmid, production of outer membrane proteins, siderophore production, and many other phenotypic characteristics encoded by genes in the bacterial chromosome. The requirement of both plasmid and chromosomal gene sequence for the complete expression of virulence has been clearly stated by Kwaga and Iversen (1992). Therefore, several *in vitro* tests that can routinely be performed in a laboratory have been developed to detect virulence in *Yersinia*. Although, workers who have developed these tests have differences in naming any one test as superior (considering sensitivity and specificity), they all agree that no single test is sufficient to determine the virulence of a given isolate.

Virulence mechanism

Broadly, the virulence of any given enteric pathogen can be divided into two

categories: (a) Invasiveness, and (b) Toxin production. Regarding *Yersinia*, investigations have been conducted under both the above mentioned categories.

Invasiveness. Tissue culture studies have shown that pathogenic strains exhibit adhesion and invasion of eukaryotic cells. However, unlike *Shigella*, *Y. enterocolitica* do not multiply intracellularly in tissue cultures (Schiemann, 1989). Some investigators claim that both adherence and invasion are chromosome mediated, while others are of the opinion that, only invasion is chromosomal and adherence on the other hand, is plasmid mediated (Schiemann, 1989). Miller and Falkow (1988) have demonstrated that, two genetic loci, *inv* and *ail*, present on the chromosome confer the property of invasiveness in pathogenic *Yersinia*. Further, Pierson and Falkow (1990) have shown that pathogenic strains carry both *inv* and *ail* genes that are functional, while the environmental strains carry only a nonfunctional *inv* gene. In the latter, *ail* gene is absent.

Rabbit inoculation studies have indicated that, pathogenesis is probably due to invasion of the epithelial cells of the intestinal mucosa (Une and Zen-Yoji, 1979). Further, these workers also showed that the virulent cells are not capable of multiplying both inside the cells and in the lamina propria. They also speculated that the organisms may be transferred to other lymph follicles via blood and lymph streams. In the same study, avirulent cells did not exhibit invasion of epithelial cells and were rapidly excreted from the gut. In conclusion, Une and Zen-Yoji strongly attribute pathogenicity of *Yersinia* to its ability to invade host cells.

Toxin production. *Y. enterocolitica* is known to produce a heat-stable enterotoxin (YeST) with a molecular weight of 9,000 - 9,700 daltons. Also, the YeST can survive 121°C

for 30 mins., and resist protease and lipase activities (Jay, 1992; Boyce *et al.*, 1979). The action of *Yersinia* toxin is similar to that of *Escherichia coli* enterotoxin (Schiemann, 1989). The YeST stimulates guanylate cyclase and the cAMP response, but not adenylate cyclase in the intestine (Jay, 1992). Production of YeST *in vitro* has been demonstrated by using either rabbit ileal loop or suckling mouse assay (Schiemann, 1989). However, the importance of YeST in pathogenicity has been questioned. Several clinical isolates were examined by Feeley *et al.* (1979). They found that all the isolates examined produced the toxin at 25°C, but were negative at 36°C. They therefore concluded that, pathogenesis due to toxin production in the intestine (approx. 37°C) is very unlikely. On the other hand, Delor *et al.* (1990) isolated the gene required to produce the YeST. They then prepared probes to detect the gene sequence in other *Yersinia* isolates. The workers found that all pathogenic strains, and none of the nonpathogenic strains, contained the YeST gene. They have therefore suggested that YeST may probably be involved in pathogenesis. The involvment of enterotoxin in pathogenesis has also been questioned by Schiemann and Devenish (1982). They found a high correlation between pathogenicity, HeLa cell invasion, and certain biochemical characteristics. However, their results indicated that even environmental strains produced the heat-stable enterotoxin.

Another aspect of toxin production that needs to be addressed, especially concerning food, is its resistance to high temperatures. Feeley *et al.* (1979) have mentioned the possibility of preformed *Yersinia* toxin in food. On the same lines, they suggested that, although *Y. enterocolitica* does not produce enterotoxin at 37°C, the production of preformed toxin in food can lead to foodborne intoxication. Also, due to the organism's psychrotrophic nature, it

has the capacity to grow at storage temperatures to sufficiently high numbers and produce enough toxin. Boyce *et al.* (1979) have shown that the enterotoxin is stable at 121°C for 30 mins. and also at temperatures of 4 or -40°C for at least 5 months. It is also, not only acid-stable in food but can retain its activity against gastric acidity (Kapperud, 1991). Very few studies have been done regarding any food product becoming a potential source of the preformed toxin. Production of toxin in milk was determined by Walker and Gilmour (1990). In their experiment, toxin production was observed only when the milk was stored at 25°C for 48 h and was absent in milk stored at 4°C for up to 7 days. Similar results have been obtained by Francis *et al.* (1980). They indicated that production of YeST is lower at 4°C versus 25°C. Also, the time required is >12 days when stored at 4°C. Production of toxin in meat is yet to be studied. The information gathered up to this point in the literature suggests that intoxication by preformed YeST in food is unlikely to be a significant public health problem.

In conclusion, pathogenesis of *Y. enterocolitica* definitely involves invasion of intestinal cells. However, toxin production may or may not be involved in pathogenesis. More research needs to be done to answer the question of toxicity by *Y. enterocolitica.*

Virulence markers

Virulent *Y. enterocolitica* is generally associated with certain phenotypic characteristics. These characteristics are determined either by the plasmid or chromosomal genes. Although plasmids of various sizes may be present in the organism, a 42-48 Mda plasmid has been usually associated with virulence. The plasmid and chromosomal mediated virulent characteristics are given in Table 2. Details of the individual virulent tests can be

Table 2. Plasmid and chromosomal mediated virulent characteristics of *Y. enterocolitica.*

Virulence characteristics	References
Plasmid mediated	
Calcium dependency	Gemski *et al.*, 1980
Sereny-like conjunctivitis test	Aulisio *et al.*, 1983; Mors *et al.*, 1980
Peroral inoculation of mice	Aulisio *et al.*, 1983
Intraperitoneal inoculation of suckling mice	Aulisio *et al.*, 1983
Auto-agglutination assay	Lee *et al.*, 1981
Lethality for gerbils	Portnoy *et al.*, 1981
Detachment of HEp-2 cell monolayer	Portnoy *et al.*, 1981
Production of V and W antigens	Carter *et al.*, 1980
Congo red uptake assay	Prpic *et al.*, 1983; Bhaduri *et al.*, 1991
Resistance to human serum	Pai *et al.*, 1982
Crystal violet binding assay	Bhaduri *et al.*, 1987
Chromosomal mediated	
HeLa cell invasion	Lee *et al.*, 1977
Heat-stable enterotoxin production	Mors *et al.*, 1980
HEp-2 cell invasion	Kay *et al.*, 1983
Siderophore production	Heesemann, 1987
Salicin fermentation	Schiemann *et al.*, 1982
Esculin hydrolysis	Schiemann *et al.*, 1982
Pyrazinamidase activity	kandolo *et al.*, 1985
Prod. of iron-regulated outer membrane proteins	Carniel *et al.*, 1987

obtained from the given references.

Besides plasmid and chromosomal characteristics, biotyping and serotyping have also been used in determining virulence. Most of the biochemical reactions that are useful in determining virulence are stable for a given strain. Also, the biochemical reactions are temperature dependent and incubation at the temperatures mentioned in the literature is essential. Table 3 lists some of the biochemical characteristics of *Y. enterocolitica* that are

Table 3. Biochemical recognition of pathogenic *Y. enterocolitica*[a]

Biochemical test	Temperature (°C)	Reaction
Cefsulodin-Irgasan-Novobiocin agar	32	Colony morphology
Kligler iron agar	35	K/A-[b]
Christensen's urea agar	35	+
Sucrose	25	+
Rhamnose	25	-
Raffinose	25	-
Melibiose	25	-
α-methylglucoside	25	-
Simmon's citrate	25	-
Salicin	35	-
Esculin	25	-

[a] Table adapted from the review "*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*" by Schiemann, 1989.

[b] alkaline slant and acid butt or alkaline slant and alkaline butt

useful in determining virulence.

Isolates, usually from patients, if found to belong to serotypes O:3, O:9, O:5,27, and O:8 can be considered as probable pathogens (Schiemann, 1989). Most of these serotypes appear to have similar biochemical reactions. However, some strains under these serotypes may be nonpathogenic and biochemically different. Also, the use of serotyping is rather limited for routine laboratory testing due to the difficulty in obtaining the antisera. However, serotyping is an important tool for epidemiological surveys and during outbreaks.

Correlation of virulence markers

Not all the virulent characteristics are exhibited by any one virulent strain. For example, some strains may possess a 42 to 48 Mda plasmid, but may still remain nonpathogenic (Schiemann, 1989; Doyle and Cliver, 1990). Tests that are plasmid mediated

can become negative due to loss of the plasmid. Autoagglutination, congo red assay, crystal violet uptake, and calcium dependency are tests commonly performed to determine plasmid mediated virulence. All these tests can become negative with the loss of the plasmid. Besides, some of these tests are also difficult to interpret (Kay *et al.*, 1983). The size of the virulent plasmid and whether more than one plasmid is present in *Y. enterocolitica* is not clear. Most of the articles in the literature state that, the size of the virulent plasmid ranges from 42 to 48 Mda. In their study, Kay *et al.* (1982) demonstrated the presence of two plasmids, both of which are implicated with virulence. The molecular weights of the two plasmids identified were 42 and 82 Mda. Low molecular weight plasmids (3-36 Mda) have also been isolated from *Yersinia* (Lee *et al.*, 1981). Prpic *et al.* (1983) mentions that, molecular weight of virulent plasmids may vary in different strains. The authors therefore suggest that isolation of a plasmid alone is insufficient to determine virulence.

Besides variance in size, the plasmids are also highly unstable and are easily lost either during storage, growth at 37°C, or repeated subcultures as in a laboratory (Schiemann, 1989; Kwaga and Iversen, 1992). Examining the fecal samples from patients suffering from yersiniosis, Noble *et al.* (1987) showed that not all isolates had the virulent plasmid. In the same study, the authors concluded that biochemical reactions like salicin fermentation, esculin hydrolysis, and pyrazinamidase activity are stable and more useful in determining virulence. Finally, plasmids of similar size have also been isolated from nonpathogenic species of *Yersinia* and strains of *Y. enterocolitica* (Schiemann, 1989; Doyle and Cliver, 1990). In spite of knowing all this information regarding the plasmid, there are several workers who still

associate virulence of *Yersinia* with presence or absence of the plasmid. DNA probes have therefore been developed to detect the plasmid directly from food or other clinical samples (Miliotis *et al.*, 1989; Koeppel *et al.*, 1993; Kapperud *et al.*, 1993; Robins-Browne *et al.*, 1989).

Unlike the plasmid, the second school of thought is the association of virulence with HeLa or HEp-2 cell invasion and lethality to mice. Lee *et al.* (1977) first demonstrated the ability of virulent *Y. enterocolitica* to invade HeLa cells. They showed that the property of invasion is stable and even old stock cultures of several clinical isolates remained positive. After comparing several phenotypic virulence characteristics, Aulisio *et al.* (1983) concluded that, any strain that is HeLa cell invasive should be considered a potential pathogen. Kay *et al.* (1983) examined hundred clinical isolates of *Y. enterocolitica* obtained by the CDC (Centers for Disease Control) over a period of ten years (1970-80). Phenotypic characteristics that were both plasmid and chromosome mediated were examined. Their results indicated that mouse lethality and tissue invasiveness (both chromosome mediated) are the most useful in predicting virulence. Similar conclusions like Kay *et al.* (1983) were also drawn by Kwaga and Iversen (1992). Mors and Pai (1980) also showed that HeLa cell invasion correlated well with diarrheal symptoms in patients suffering from yersiniosis.

Although HeLa cell invasion and mouse lethality are effective in determining virulence, they are cumbersome, expensive, and the facilities may not be available in all laboratories for routine examination. Lee *et al.* (1977) and Schiemann and Devenish (1982) demonstrated that, isolates that were HeLa cell invasive were also negative for salicin fermentation and

esculin hydrolysis. Relationship between tissue invasiveness, pyrazinamidase activity, salicin fermentation, and esculin hydrolysis was also determined by Farmer III *et al.* (1992). Their results indicated that, pyrazinamidase test exhibited 95% sensitivity and 92% specificity when compared to tissue invasiveness. The salicin and esculin tests had 100% sensitivity and 92% specificity with tissue invasiveness; and the correlation between salicin and esculin tests was 100%. Complete agreement between salicin fermentation, esculin hydrolysis, and pyrazinamidase activity was found by Noble *et al.* (1987) when examining clinical isolates.

From all the evidence put forward so far by various workers, conclusions like those of Riley and Toma (1989) can be drawn as follows:

1. Salicin/esculin (+) and pyrazinamidase (+) = nonpathogenic
2. Salicin/esculin (-) and pyrazinamidase (-) = pathogenic
3. Salicin/esculin (-) and pyrazinamidase (+) = nonpathogenic

If these three tests appear to be inconclusive on routine examination, then the isolate should be examined for tissue invasiveness and lethality for mice.

As salicin fermentation, esculin hydrolysis, and pyrazinamidase tests were performed in this study (Papers II and III), brief descriptions of the individual tests follow.

Salicin fermentation. Salicin is a β-glucoside. The test involves examination of the isolate to produce β-glucosidase. The medium used is heart infusion broth containing 1% salicin and another 1% of Andrade's indicator (all autoclaved at 121°C for 8 mins.). The isolate is inoculated into the medium (tubes) and incubated at 37°C for 24 h. The pale brown medium turns bright pink if the test is positive. Pathogenic strains are salicin negative (no pink

color).

Esculin hydrolysis. Like salicin, esculin is also a β-glucoside and the test involves detection of β-glucosidase production. The isolate is inoculated onto bile-esculin agar (Difco Laboratories, Detroit, MI) and incubated at 25°C for 48 h. A positive test is one where the colonies are surrounded by a black halo. Pathogenic strains are esculin negative (no black halos).

Pyrazinamidase activity. The principle of this test involves production of pyrazine-carboxylamidase or pyrazinamidase by the organism. As described by Kandolo and Wauters (1985), hydrolysis of pyrazinamide in the medium by pyrazinamidase results in the production of pyrazinoic acid. Further, addition of ferrous solution changes the pyrazinoic acid to a brownish pink color. Slants of pyrazinamide agar are prepared by adding pyrazinamide to trypticase soy agar with yeast extract followed by autoclaving at 121°C for 15 mins. (Kandolo and Wauters, 1985). The slants are incubated at 25°C for 48 h after inoculation. One ml. of freshly prepared 1% ferrous ammonium sulfate solution is then poured over the slant. A positive pyrazinamidase reaction is indicated by a pink to brown color developing on the slant within 15 mins. Pathogenic *Yersinia* strains are pyrazinamidase negative (slants remain colorless).

The Heat Shock Response

Introduction

When some organisms are exposed to elevated temperatures, they respond by synthesizing a group of proteins known as heat shock proteins (hsps) or stress proteins (Burdon,1986; Lindquist, 1986; Neidhardt *et al.*, 1984). These proteins are highly conserved

in evolution. The response is commonly known as the heat shock response. This phenomenon has been found to be present in both prokaryotes and eukaryotes and has been observed from eubacteria to archaebacteria as well as in plants and animals (Lindquist, 1986). The synthesis of these proteins can also be induced by other stresses such as aerobic or anaerobic growth (oxidative stress), irradiation (UV rays), chemicals (ethanol), antibiotics (nalidixic acid), and also by viral infection (Lindquist, 1986; Neidhardt *et al.*, 1984; Spector *et al.*, 1986). The principal function of the hsps appears to be that of protection of the organism against a variety of stress conditions, especially heat. These proteins may also be involved in normal cell growth and development (Lindquist, 1986).

Much of the knowledge of the hsps comes from experiments that have been performed in eukaryotes such as *Drosophila* and mammalian cells. In bacteria, the heat shock response has been studied mainly in various *E. coli* strains (Grossman *et al.*, 1987; Lemaux *et al.*, 1978; Neidhardt *et al.*, 1984; Taura *et al.*, 1989; Zylicz *et al.*, 1983), as well as in *Bacillus* species (Arnosti *et al.*, 1986; Streips and Polio, 1985), *Staphylococcus aureus* (Batish *et al.*, 1989) and *Salmonella typhimurium* (Spector *et al.*, 1986).

General characteristics

The temperature required to induce the synthesis of hsps varies with different organisms. Within each organism there appears to be no sharp threshold temperature that must be achieved to evoke the heat shock response. For example, *E. coli* strains that can grow over a broad temperature range produce hsps when the incubation temperature is shifted from 28 to 33°C or 36°C; the high temperature is still below the optimum temperature of 37°C

(Lemaux *et al.*, 1978). When *E. coli* are grown at 30 to 37°C, they start synthesizing the hsps when the temperature is raised to 42 to 45°C (Lindquist, 1986; Neidhardt *et al.*, 1984). It has been concluded that, in general, for all organisms that grow over a broad range of temperatures, the maximum response is usually achieved at 10 to 15°C above the optimum growth temperature. In organisms that grow over a more restricted range, maximum response occurs at about 5°C above the optimum (Lindquist, 1986).

The heat shock response appears to be transient in most organisms at moderate temperatures lasting for about 20 minutes. The response is sustained at higher temperatures until eventually the cells begin to die (Lindquist, 1986; Yamamori *et al.*, 1978). When exposed to an elevated temperature (heat shock), the rate of synthesis of hsps accelerates within seconds to rates many times above their preshift rates. It then declines to a steady state, characteristic of the elevated temperature (Neidhardt *et al.*, 1984).

In general, a shift from low (10-30°C) to elevated temperatures (35-43°C) induces increased synthesis of both normal cellular proteins and hsps. Temperature shifts to 43-47°C result in production of large amounts of hsps and the rate of synthesis of nonheat shock proteins decreases. Above 47°C, hsps are exclusively synthesized and this synthesis appears to continue as long as the cells survive and continue protein synthesis (Neidhardt *et al.*, 1984).

Heat shock proteins

The hsps can be visualized by SDS-polyacrylamide gel electrophoresis and by Western blot techniques. In the latter method, the hsps are electrophoretically separated and transferred to nitrocellulose paper. The proteins are then detected by using tagged monoclonal antibodies

(Straus *et al.*, 1987). About 17 hsps have been identified in *E. coli*, with molecular weights ranging from 10 to 94 kDa (Neidhardt *et al.*, 1984). The most important among them being σ^{32}, DnaK, GroEL, GroES, Lon, DnaJ, and LysU proteins.

Mechanism of induction

Studies in *E. coli* indicate that the heat shock response is controlled primarily at the level of transcription (Lindquist, 1986; Neidhardt *et al.*, 1984). The repression of hsps synthesis is also controlled at the transcriptional level (Yamamori and Yura, 1980).

One of the hypotheses for induction is that most of the inducers cause DNA and protein damage in the bacterial cells. Increased amounts of the abnormal proteins in the cells then activate the *htpR* gene (Munro and Pelham, 1985). The σ^{32} produced from this gene activation competes with σ^{70} for the RNA core polymerase. At elevated temperatures, more of σ^{32} is produced and therefore the amount of σ^{32}-bound polymerase increases. The σ^{32} specifically recognizes promoters of hsps genes in preference to those for normal proteins or nonheat shock genes (Piper, 1987). The activated heat shock genes then synthesize hsps, and the relative amount of nonheat shock proteins decreases (Grossman *et al.*, 1987). Grossman *et al.* (1987) suggested that the DnaK protein either directly or indirectly represses translation of *htpR* (*rpoH*) mRNA. This results in a decrease of σ^{32} synthesis and a rapid decline in intracellular level of σ^{32}. As a consequence, the rate of synthesis of hsps decreases. Taura *et al.* (1989) suggested that a factor may be present that inactivates and dissociates σ^{32} from the core RNA polymerase, or a factor that directly binds to the control regions of the hsps genes.

It is also possible that, one or several hsps themselves could act as the negative regulators at lower temperatures.

Functions of heat shock proteins

The specific functions of hsps are not yet understood, but their synthesis at high temperatures suggests that they are in some way involved in protection of the cells at the elevated temperatures. The GroEL and DnaK proteins have been found to be associated with ATP and have a weak ATPase activity (Neidhardt *et al.*, 1984). The GroEL, DnaK, and DnaJ proteins also participate in phage replication (Neidhardt *et al.*, 1984). Unfolding of chromosomes occur at high temperatures (50-52°C) and may lead to loss of viability of the cells. Large amounts of the GroEL protein have been found associated with the unfolded chromosome, thereby preventing further unfolding (Neidhardt *et al.*, 1984). The σ^{32} factor controls the heat shock response. At elevated temperatures σ^{32} is produced in large amounts, that then binds to RNA polymerase making the latter more sensitive to the promoters of hsps genes (Grossman *et al.*, 1987). The DnaK protein serves as the negative control of the heat shock response (Neidhardt *et al.*, 1984). The Ion protein has protease activity and degrades abnormal and denatured proteins that would have otherwise formed aggregates and become toxic to the cells (Neidhardt *et al.*, 1984). Finally, the LysU protein has lysyl-tRNA synthetase activity and activates lysine and tRNA for protein synthesis (Neidhardt *et al.*, 1984).

Heat shock response and thermotolerance

Besides production of hsps, the heat-shocked cells also appear to be more thermotolerant to lethal temperatures (Knabel *et al.*, 1990; Mackey and Derrick, 1987;

Murano and Pierson, 1992). Thermotolerance of an organism can be defined as its ability to survive otherwise lethal temperatures. For example, >50°C can be considered lethal temperatures for mesophilic bacteria whose optimum temperature is usually between 30 to 37°C.

Experiments have revealed that, production of hsps and development of thermotolerance occur simultaneously when bacterial cells are exposed to sublethal temperatures. Therefore it has been postulated that hsps must be in some way involved in protecting the cells against heat and in development of thermotolerance. Also, decay in thermotolerance appears to coincide with the degradation of hsps. This relationship has been used to suggest a further conformation of the protective function of hsps.

In the meat industry, meats that are heated up slowly to a final internal temperature may contain microbial cells with an increased heat resistance due to the heat shock response. Farber and Brown (1990) suggested that, the slower the temperature increase in the meat, the larger the increase in heat resistance or thermotolerance. There are also several situations both in industry and in homes when the food may be subjected to temperature abuse. For example, meats left on warming trays before being given a final reheating could possibly acquire an enhanced thermotolerant microbial population. Packaging, especially under an anaerobic environment can further help in survival and growth of these heat-shocked thermotolerant cells.

Application of Vacuum and Modified Atmospheres in Meat Packaging

Introduction

Increasing the shelf life and maintaining the fresh quality of the product are the primary goals of packaging meat and poultry under vacuum or other modified atmospheres (MA). Although modified atmosphere packaging (MAP) was used in the 1930's for shipment of fresh beef from New Zealand and Australia, renewed interest in this technology and its increased use was seen only after 1981 (Farber, 1991). Marks and Spencer in the UK in 1981 introduced a wide range of meat products packaged under MA. This was probably due to the higher demand by consumers for fresh, chilled, preservative-free products instead of canned and frozen products (Farber, 1991).

Two important factors that affect microbial growth in meat or other food products are temperature and packaging atmosphere. The predominant flora in the meat is also chiefly determined by these two factors. Lambert *et al.* (1991) have reviewed in detail the microorganisms that can be found in meat stored under various packaging atmospheres and temperatures. According to their review (Lambert *et al.*, 1991), *Pseudomonas* grows rapidly under aerobic conditions at both refrigeration and higher temperatures. *Brochothrix thermosphacta* becomes dominant when the storage temperature is increased and microaerophilic conditions prevail. However, lactic acid bacteria are the predominant flora when the meat is stored under anaerobic conditions at refrigeration temperatures. Also, most members of the family *Enterobacteriaceae* are able to grow at temperatures above 8°C. In earlier studies of MAP, the primary concern was the possible growth of *Clostridium*

botulinum that did not grow at low temperatures, but could grow if the product was temperature abused. However, the increased knowledge of psychrotrophic foodborne pathogens like *Listeria*, *Yersinia*, and *Aeromonas* has led to new safety concerns (Farber, 1991). The major reason being that, packaging under MA and storage at refrigeration temperatures significantly increases the shelf life of the product. Under these conditions and given the extended period of time, the psychrotrophic pathogens will have sufficient time to grow and multiply to dangerous levels without any significant changes in the sensory qualities of the product (Farber, 1991).

Growth of organisms in meat can be effectively controlled by employing the "hurdle concept" (Lambert *et al.*, 1991). According to this concept, several hurdles or "inhibitory factors" can be used in combination during processing and storage of meat. The final effect is synergistic with reduced risk of developing resistant organisms to any one or all factors. This method also reduces the undesirable side-effects of any single factor if used alone usually at high levels. Hence, packaging under vacuum and other gases is being increasingly employed in industry as an essential part of processing and transportation of meat.

Gases used and their advantages

"Modified atmosphere" and "controlled atmosphere" are the two terms commonly used in packaging. The term "modified atmosphere" indicates packaging a product with a certain mixture of gases only in the beginning and is not altered during storage. On the other hand, "controlled atmosphere" (CA) indicates maintaining the required mixture and concentration of gases throughout storage (Genigeorgis, 1985). Vacuum packaging (VP) is

also considered a type of MAP as the environment around the product is modified by the application of vacuum. However, for the sake of identifying vacuum versus other gases, VP and MAP are considered as separate entities in this section. MAP is generally preferred over CAP mainly due to reduced cost and labor.

Packaging of meat is primarily done either under vacuum or MA. For MAP, either a single gas or a mixture of gases are used. Oxygen, carbon dioxide, and nitrogen are the three gases most commonly used in varied concentrations.

The advantages of vacuum packaging of meat during cold storage include, reduction in weight loss due to dehydration, preservation of muscle color, aging within the package, elimination of external contamination, and prolonging shelf life (Lambert *et al.*, 1991). When meat is packed in gas impermeable pouches, a rapid increase in the CO_2 occurs in the headspace of the package due to microbial and tissue respiration (Silliker and Wolfe, 1980). The authors have also pointed out that, within 4 h the concentration of CO_2 reaches 10-20% and the maximum level achieved is usually around 30% on storage. The critical role of CO_2 in vacuum packaged products is also emphasized by Enfors *et al.* (1979). From their studies Enfors *et al.* (1979) have speculated that, shelf life extension of vacuum packaged pork is chiefly due to the accumulation of CO_2 and not due to reduced O_2 tension. Besides CO_2 activity, the reduced microbial growth may also partly be due to the production of antimicrobials by lactic acid bacteria that can grow under vacuum (Lambert *et al.*, 1991). A major limiting factor of vacuum packaged wholesale cuts is discoloration due to formation of metmyoglobin. Secondly, vacuum packaging produces mechanical strain on products

(Lambert *et al.*, 1991). This can lead to exudation of meat and puncture of packages if bones are present. Using of "bonegard" between the bone and the film is being used as one of the solutions to prevent package puncture (Lambert *et al.*, 1991).

Among all the gases used in packaging, CO_2 is the most effective in retarding microbial growth. This is achieved by extending the lag phase and increasing the generation time of microorganisms (Genigeorgis, 1985). However, the inhibitory effect of CO_2 is dramatically reduced if cells have already entered the log phase during CO_2 application (Genigeorgis, 1985). The bacteriostatic effect of CO_2 is also reduced with increasing storage temperature (Farber, 1991). The concerns that have been raised regarding CO_2 packaging are: (1) Possible enhanced growth of *C. botulinum*, (2) Probably stimulate germination of clostridial spores, (3) Inhibits normal aerobic spoilage flora that are indicators of early spoilage, and (4) Potential for temperature abuse. All these concerns can be overcome if CO_2 packaging is used as only one of several steps employed in processing to eliminate foodborne pathogens. As mentioned earlier, application of the hurdle concept can reduce the potential risks of CO_2 packaging, making this technique all the more beneficial.

The functions of O_2 include, enhancing the red color of meat, increasing odor shelf life, retaining the oxygenated form of myoglobin, preventing irreversible conversion of myoglobin to metmyoglobin, and inhibiting growth of certain psychrotrophic spoilage organisms like *Moraxella* and *Acinetobacter* at high concentrations (Genigeorgis, 1985). Although O_2 stimulates growth of aerobic bacteria, it is useful in inhibiting strict anaerobic pathogens like *C. botulinum* (Farber, 1991). Nitrogen by itself has no antibacterial qualities. However, mere

displacement of O_2 by N_2 in the package can result in decreased O_2 content thereby increasing shelf life and reducing oxidative rancidity (Lambert *et al.*, 1991). N_2 is also useful as a filler gas to prevent pack collapse in MAP (Farber, 1991).

Effects of vacuum and MAP on bacteria

Daniels *et al.* (1985) have reviewed in detail the theories regarding the mechanisms by which CO_2 exerts its bacteriostatic and/or bactericidal effects on microorganisms. The probable mechanisms can be summarized as follows: (1) CO_2 has the capacity to easily penetrate the cell membranes of bacteria and thereby affect not only the chemical processes in the cell membrane but also inside the cells, (2) By lowering the internal pH of the cell by acidification and affecting the metabolic activities of the cell, and (3) May have deleterious effects on certain enzyme systems in the cell.

The effects of vacuum and MAP on normal flora and on specific pathogens have been studied by several workers. The optimal concentration of CO_2 that is effective in inhibiting meat spoilage bacteria is in the range of 40 to 60% (Farber, 1991). Generally, lower concentrations (<40 to 60%) are ineffective whereas higher concentrations (>40 to 60%) do not contribute to any further inhibition. Also, higher concentrations can cause bleaching or discoloration of meat, especially beef (Farber, 1991). Studies also show an inverse relationship between temperature and the bacteriostatic effect of CO_2. Lowering the storage temperature greatly enhances the inhibitory effect of CO_2. Overall, CO_2 is inhibitory to molds, yeasts, and aerobic spoilage bacteria. Lactic acid bacteria and anaerobes are generally unaffected by CO_2 (Genigeorgis, 1985). Lactobacilli are highly resistant and have the capacity to grow even

under 100% CO_2 concentration (Lambert *et al.*, 1991). High levels of CO_2 have also very little effect on facultative anaerobic pathogens like *Salmonella* spp., *S. aureus*, *Campylobacter*, *Y. enterocolitica*, and *L. monocytogenes* (Lambert *et al.*, 1991). The probable enhanced growth of these pathogens under high CO_2 tension has also been speculated (Lambert *et al.*, 1991).

The prevalent microbial flora on fresh pork packed under elevated levels of CO_2 and stored at -1, 4.4, and 10°C was examined by McMullen and Stiles (1993). They found that aeromonads, lactic acid bacteria, and members of the family *Enterobacteriaceae* are capable of growth at 10°C storage. However, at 4.4 and -1°C, the major flora consisted of aeromonads, *B. thermosphacta*, and lactic acid bacteria (McMullen and Stiles, 1993). Effects of prolonged storage at -1.5°C under CO_2 was also examined by Greer *et al.* (1993). They isolated only lactic acid bacteria from samples stored up to 24 weeks. Christopher *et al.* (1979) studied the microbiology of pork packaged in various gas atmospheres. Pork roasts were packed either under vacuum or various gas mixtures (CO_2, O_2, N_2, CO) and stored for up to 35 days at 1 to 3°C. The concentrations of CO_2 varied from 20 to 51% and that of O_2 from 25 to 100% (Christopher *et al.*, 1979). However, unlike other similar studies, these workers found that the psychrotrophic and lactobacilli counts were not significantly different when stored under the various atmospheres. Effect of CO_2 on respiration and growth rates of several spoilage bacteria was examined by Gill and Tan (1980). Their studies indicated that both respiration and growth rates of *Pseudomonas*, *Alteromonas putrefaciens*, and *Y. enterocolitica* are lowered by higher CO_2 concentrations. In the same study, *Enterobacter* and *Microbacterium thermosphactum* were unaffected by CO_2 whereas inhibition of

Acinetobacter continued to increase with increasing concentration of CO_2.

Effect of 80% CO_2 versus air on survival and growth of both pathogens and spoilage bacteria in chicken was determined by Baker *et al.* (1986). In their study, chicken meat was inoculated with either *Pseudomonas fragi*, *S. typhimurium*, *S. aureus*, or *C. perfringens* and stored at 2, 7, and 13°C under CO_2 or air. The authors concluded that, although less growth was observed in CO_2 packed chicken, temperature played an important role in retarding growth (Baker *et al.*, 1986). Effects of vacuum and CO_2 on growth of *L. monocytogenes* have been studied by Gill and Reichel (1989), Manu-Tawaiah *et al.* (1993), and Hudson *et al.* (1994). In all the three studies, CO_2 was found to be effective in inhibiting *L. monocytogenes.* Gill and Reichel (1989) and Hudson *et al.* (1994) have drawn similar conclusions. According to them, CO_2 is more effective than vacuum packaging in inhibiting *L. monocytogenes* especially when the temperature of storage is between 0 and 5°C. However, they differ in their results regarding vacuum packaging. According to Gill and Reichel (1989), vacuum packaging is adequate for inhibition if the storage temperature is below 0°C. On the other hand, Hudson *et al.* (1994) have demonstrated growth of *Listeria*, *Aeromonas*, and *Yersinia* at both -1.5 and 3°C under vacuum.

Effects of vacuum and MAP on *Y. enterocolitica*

Several studies have been performed to determine the effects of vacuum and MAP on the growth of *Y. enterocolitica* over storage (Gill and Reichel, 1989; Manu-Tawaiah *et al.*, 1993; Nielsen and Zeuthen, 1984; Zee *et al.*, 1984; Kleinlein and Untermann, 1990; Hudson *et al.*, 1994; Rowe, 1988). All these studies indicate that *Yersinia* is comparatively more

resistant than other psychrotrophic pathogens like *L. monocytogenes* and *A. hydrophila* to CO_2. Growth of *Yersinia* occurs even at 0°C under vacuum and the organism is able to compete with other spoilage flora at refrigeration temperatures (Gill and Reichel, 1989; Manu-Tawaiah *et al.*, 1993). Concentrations of CO_2 up to 40% are insufficient to inhibit *Yersinia* at temperatures above 2°C in meat (Manu-Tawaiah *et al.*, 1993; Hudson *et al.*, 1994). On the contrary, studies by Kleinlein and Untermann (1990) indicate that growth of pathogenic serotypes of *Yersinia* can be inhibited by competitive background flora. In their study, serotypes O:3 and O:9 (pathogenic) were inhibited by normal spoilage flora when stored at temperatures of 1 to 15°C under 20% CO_2 in beef (Kleinlein and Untermann, 1990). Nielsen and Zeuthen (1984) examined the effect of a combination of temperature, vacuum, and salt (3.8%) on the growth of *Yersinia* in bologna. Their results showed that, *Yersinia* can grow uninhibited under those conditions even at 5°C.

In conclusion, using of vacuum and MA in packaging of meat is beneficial when compared to air. Nevertheless, storing the packed samples at temperatures near 0°C is essential and should not be compensated. Storage below 0°C under vacuum or MA can completely inhibit or reduce growth of several pathogens including *Yersinia* and also spoilage bacteria, thereby increasing the shelf life and quality of meat products.

Irradiation

Introduction

Irradiation can be used as a substitute for many traditional methods of eliminating pathogens and spoilage organisms in food without altering the sensory qualities. The

beginnings of food irradiation can be traced back to 1895 when von Roentgen discovered X-rays followed closely by Becquerel in 1896 with the discovery of radioactivity. In 1905, Liebur, Appleby, and Banks for the first time proposed the use of ionizing radiation to kill bacteria in food (Josephson, 1983). Use of irradiation in food began in earnest in 1953 when the US Army started programs on food irradiation (Josephson, 1983). It included studies in various aspects of irradiation such as radappertization of beef, using of smaller doses to eliminate spoilage and pathogenic bacteria, and also, toxicological studies on irradiated foods. In the same year, President Eisenhower enunciated the "atoms for peace" program to the United Nations (Josephson, 1983). The availability of ^{60}Co and ^{137}Cs at reasonable prices after World War II, along with the development of suitable machinery for their use, enhanced the rapid development of food irradiation. The next major step was in 1958 when an amendment was made to the Food, Drug and Cosmetic Act, legally defining ionizing radiation as a new food additive (Josephson, 1983). Since July 1985, the Food and Drug Administration (FDA) has permitted irradiation of fresh, non-heat processed pork within a dose range of 0.3 to 1.0 kGy. This permission for gamma irradiation was given primarily for elimination of *Trichinella spiralis* (Engel *et al.*, 1988). Although FDA authorizes the use of irradiation, the Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA) is involved in regulations and control, safety, and labelling of meat and poultry products (Engel *et al.*, 1988).

Definitions, units, and sources of energy

The terms "radiation", "irradiation", and "ionizing radiation" are clearly defined by

Radomyski *et al.* (1994). By their definitions, radiation is a physical phenomenon in which energy travels through space or matter; irradiation is the process of applying radiation energy to a product; and ionizing radiation is when electrically charged particles or ions are used. Low (0.75 - 2.5 kGy), medium (2.5 - 10 kGy), and high (30 - 40 kGy) doses are usually defined by the terms radurization, radicidation, and radappertization, respectively (Jay, 1992). Radiations important in food preservation include, ultraviolet rays, X-rays, gamma rays, and microwaves (Jay, 1992). The ionizing radiations have wavelengths of 2000 Å or less and are classified as α particles, β rays, γ rays, X-rays, and cosmic rays (Jay, 1992). Details of these individual radiations and their uses have been reviewed elsewhere.

The types of ionizing radiations commonly used in food are: (1) Gamma rays from the radionuclides ^{60}Co and ^{137}Cs, (2) X-rays, and (3) Electrons generated from machine sources operated at or below an energy level of 10 MeV (Million electron Volts). The radiation dose absorbed is described by the unit "Gray". One Gray (Gy) unit is equal to 100 rads or 1 Joule/kg., and 1 kGy equals 10^5 rads.

Advantages and disadvantages

The advantages of irradiation are several and definitely outweigh the disadvantages or concerns. Some of the advantages are: (1) Economical benefits are much higher than the initial cost of irradiating a product, (2) Requires less energy for radicidation when compared to freezing and canning for elimination of microorganisms and increasing the shelf life, (3) The product can be packaged before irradiation and this eliminates the possibilities of cross contamination and/or recontamination, (4) Irradiation can replace chemical fumigation to

control insect manifestation commonly found in dried and fresh fruits, flour and cereals, (5) The size of the product can vary enormously, *e.g.* from sliced deli meats to crates of potatoes or 50 - 100 lb. sacks of flour, (6) Irradiation can not only retain the volatiles in spices, but also reduce the microbial load and replace the chemical fumigants, (7) Can inhibit sprouting of tubers and bulbs and extend storage life, and (8) Irradiation can either completely replace or reduce the amount of food additives used. *e.g.* the amount of nitrite in bacon, ham, and other ready-to-eat products (Anonymous, 1983; Kampelmacher, 1983).

The concerns that have been raised against irradiation and wholesomeness of food products are: (1) Probable induction of radioactivity in the food, (2) Elimination of known pathogens and development of new radioresistant strains, (3) Production of toxic radiolytic substances in the food, and (4) Loss of nutrients (Anonymous, 1983). Animal feeding studies for mutagenicity, teratogenicity, and nutrient deficiency have shown that these concerns are unfounded (Anonymous, 1983). Josephson *et al.* (1978) in their review have concluded that, low dose irradiation (<10 kGy) of food products cause minimal loss of nutrients and are comparable to those which occur when conventional methods of cooking are used. Irradiation in the frozen state and in the absence of oxygen and light can further reduce the changes in lipids and vitamins (Josephson *et al.*, 1978; Skala *et al.*, 1987). The amount of the radiolytic compounds produced are in very minute quantities even at high doses and the animal studies did not show any harmful effects (Brynjolfsson, 1985). Also, the so-called "radiolytic products" are not only produced during conventional cooking but are also found in natural, nonirradiated foods. For example, benzene is naturally found in fish, eggs, vegetables, and

dairy products (Brynjolfsson, 1985).

No evidence has been reported regarding enhanced pathogenicity in known pathogens or development of pathogenic characteristics in nonpathogens (Farkas, 1989). Occasional changes in shape, growth response, sensitivity to selective agents, and changes in biochemical characteristics are only temporary and the organisms change back to their original character after resuscitation in a nonselective medium or in a food product (Farkas, 1989). Resistance to irradiation is slight unlike antibiotic or heat resistance and develops only after repeated exposure to irradiation followed by subculturing of the survivors (Maxcy, 1982). As per the guidelines of the World Health Organization (WHO), a total absorbed dose of only up to 10 kGy is allowed if repeated irradiation of food is to be done (Maxcy, 1982). This prevents the development of irradiation resistant strains. Also, repeated irradiation and subculturing weakens the bacterial cells, making them not only less infective but also more exacting in their growth requirements (Maxcy, 1982). In conclusion, development of a radiation resistant strain, its rapid growth, and becoming a potential hazard are highly unlikely.

Effects of irradiation on micro-organisms

Although the exact events and mechanisms that occur in a bacterial cell following exposure to irradiation are not completely understood, certain speculations have been made. These include alteration of cell membrane affecting transportation of nutrients, deleterious effects on enzymes, fragmentation of DNA, reduction of phosphorylation affecting energy metabolism, and finally, changes in DNA that in turn affects normal cell activity and reproduction (Urbain, 1986). Sufficient doses invariably lead to death of the organism.

Production of free radicals during irradiation, chiefly by the dissociation of water exerts damaging "indirect" effect on bacterial constituents (Huhtanen *et al.*, 1989). The oxygen molecule has unpaired electrons and can easily react with the free radicals. This prevents radical recombination, thereby initiating a chain reaction in producing increased number of free radicals. Therefore, oxygen enhances the indirect action of radiation and causes lowering of the D value (Urbain, 1986).

Besides the water content (a_w) of the product and the presence of oxygen during irradiation, certain other factors also influence the resistance of an organism. These include, the type and number of the microbial species, the growth phase of the cells, irradiation dose rate, temperature during irradiation, and the pH and presence of preservatives in the product. In general, lower pH and higher temperature during irradiation reduces the resistance of the microoganism. As far as sensitivity of microorganisms are concerned, the Gram negative vegetative bacteria are highly sensitive to irradiation, followed by Gram positive bacteria. Spores, viruses, and enzymes are highly resistant (Farkas, 1989). The most radiation resistant bacteria found in beef and pork belong to the *Moraxella/Acinetobacter* group. Although these bacteria can survive fairly high irradiation doses, they are unable to grow and multiply in fresh meat as their requirement for water is very high and is above that of meat (Maxcy, 1982). Prolonged storage of irradiated ground pork under vacuum at 5°C resulted in a predominant growth of lactobacilli and coryneforms (Ehioba *et al.*, 1988). The D values of some important foodborne pathogens are given in Table 4. From this table it is apparent that a dose of 3 kGy is sufficient to eliminate most of these pathogens.

Table 4. D values of some important foodborne pathogens and spoilage organisms.

Organism	D values (kGy)	Suspending medium	Irradiation temperature (°C)	References
A. hydrophila	0.14 - 0.19	Beef	2	Palumbo *et al.*, 1986
C. jejuni	0.18	Beef	2 - 4	Clavero *et al.*, 1994
E. coli O157:H7	0.24	Beef	2 - 4	Clavero *et al.*, 1994
L. monocytogenes	0.45	Chicken	2 - 4	Huhtanen *et al.*, 1989
Salmonella spp.	0.38 - 0.77	Chicken	2	Thayer *et al.*, 1990
S. aureus	0.36	Chicken	0	Thayer *et al.*, 1992
Y. enterocolitica	0.11	Beef	25	El-Zawahry *et al.*, 1979
C. botulinum (spores)	3.56	Chicken	-30	Anellis *et al.*, 1977
Moraxella phenylpyruvica	0.63 - 0.88	Chicken	-	Patterson, 1988
Acinetobacter calcoaceticus	0.26	Fish protein powder	-	Tsuji, 1983

Effects of irradiation on *Y. enterocolitica*

Y. enterocolitica is highly sensitive to irradiation and a dose of 1 kGy is sufficent to eliminate this pathgen from ground beef (El-Zawahry and Rowley, 1979; Tarkowski *et al.*, 1984). The D values for the pathogenic serotypes O:3, O:5,27, and O:9 were 0.10, 0.16, and 0.21, respectively, when irradiated at room temperature (Tarkowski *et al.*, 1984). Similar values were also obtained by El-Zawahry and Rowley (1979). In their studies, 3 clinical strains were examined for radiation resistance when suspended either in trypticase soy broth or ground beef. The D values were 10.9 krads (0.1 kGy) and 19.5 krads (0.2 kGy), respectively, in the two suspending media (El-Zawahry and Rowley, 1979). Effects of temperature during

irradiation was also examined in the study. The workers found that there was little difference in radiation resistance between 5 and 25°C or between -10 and -30°C. However, the major difference in resistance was between 5 and -10°C. The authors were of the opinion that the cell counts may actually decrease over storage at -20°C and *Yersinia* may not be a health hazard after a low dose of irradiation (El-Zawahry and Rowley, 1979). Further studies on the combined effects of freezing and irradiation were conducted by El-Zawahry and Grecz (1981). *Y. enterocolitica* cells were suspended in phosphate buffer and irradiated after freezing at 0, -18, and -75°C. The speculations were confirmed when the results showed that, freezing alone without irradiation is deleterious leading to increased number of injured cells. Further, they demonstrated that freezing produces a greater extent of cell injury than inactivation, whereas radiation inflicts more inactivation than injury (El-Zawahry and Grecz, 1981). The authors concluded that radiation combined with freezing and in the presence of repair inhibitors like sodium chloride, can completely inhibit or eliminate *Yersinia* in processed foods.

The microflora of pork loins after irradiation at 3.0 kGy and storage under vacuum at 4°C was determined by Lebepe *et al.* (1990). They detected some *Yersinia* spp. (<2 cells/cm^2) only after enrichment. However, *Yersinia* did not grow over storage. Growth of *Yersinia* in ground beef when stored under vacuum at 7°C following irradiation at 0.6 and 2.0 kGy doses was also studied by Fu (1994). In his study, *Yersinia* was not detected following either of the two doses, storage under vacuum, and enrichment before plating. Unlike the study by Lebepe *et al.* (1990), Fu (1994) had inoculated a pathogenic strain of *Y. enterocolitica* (serotype O:8) into ground beef. The difference in results between Fu and Lebepe *et al.* may probably be due

to strain variations in radiation sensitivity. Also, Lebepe *et al.* (1990) had stored the irradiated pork for up to 98 days versus 7 days of storage by Fu (1994). However, in both studies *Y. enterocolitica* was not competitive with the normal flora and did not grow to significant numbers over storage.

In conclusion, although irradiation is useful in eliminating both pathogenic and spoilage bacteria in meat, it should not be used as a substitute for good manufacturing practices (GMP's). With more and more processed ready-to-eat foods becoming an essential part of the consumers diet, irradiation is an invaluable process that cannot be overlooked.

EFFECT OF HEAT SHOCK ON SURVIVAL OF *YERSINIA ENTEROCOLITICA* TO HEATING IN BHI BROTH AND GROUND PORK

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ABSTRACT

The optimum conditions required to induce heat shock response in *Y. enterocolitica* in Brain Heart Infusion (BHI) broth was determined. Production of heat shock proteins and the increased thermotolerance of heat-shocked *Yersinia* in ground pork when exposed to higher temperatures was also examined. Heat shocking of *Y. enterocolitica* cells at 45°C for 60 mins consistently resulted in increased number of survivors to a subsequent treatment of 55 or 60°C in BHI broth when compared to nonheat-shocked controls. D values at 55°C were calculated as 7.7 and 2.0 mins and at 60°C as 1.6 and 1.2 mins for heat-shocked and controls, respectively. After examination of heat-shocked cells by SDS-PAGE, two distinct heat shock proteins with molecular weights of 70.5 and 58.0 kDa were observed that were not present in the control. Evaluation of survival of heat-shocked and control cells in ground pork revealed D_{55} values of 15.6 and 6.5 mins and D_{60} values of 6.7 and 1.7 mins 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* in meat due to the phenomenon of heat

shock response is a cause of concern regarding microbiological food safety.

INTRODUCTION

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 outbreaks. *Y. enterocolitica* infections though quite common in Europe and Japan, have only recently been associated with outbreaks in the US (*2, 25, 6, 22, 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 (*27, 9*).

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 (hsps) or stress proteins (*3, 14, 19*). This phenomenon is commonly called "heat shock response". The heat-shocked cells, when subjected to further higher temperatures, appear to be more thermotolerant when

compared to nonheat-shocked cells. Also, the synthesis and degradation of hsps appear to coincide with development and decay of thermotolerance (*13*). It has therefore been assumed that heat shock response is a protective mechanism for the survival of cells under stress.

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 (*15*) showed that heat-shocked *Salmonella thompson* was more resistant with 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 demonstrates 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 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* 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 ml of log phase *Y. enterocolitica* cells at 25°C was inoculated into a tube containing 5.0 ml of fresh BHI broth prewarmed at either 40, 45, 48 or 50°C 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 mins. On reaching one of the heat shock temperatures, the cells were heat-shocked for either 5, 10, 15, 30, 45 or 60 mins. 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 as heat treatment. One ml samples were then drawn at regular intervals and plated in duplicate on BHI agar after making the appropriate serial dilutions in 0.1% peptone solution. Nonheat-

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 48h.

Determination of heat shock protein production.

A 6 h culture of *Y. enterocolitica* with 10^9 cells/ml was prepared in 10 ml of BHI broth at 25°C. One ml samples were placed in microcentrifuge tubes and centrifuged at 15,000x *g* for 2 mins. The supernatant was decanted and the cell pellets were washed 3x 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) and were heat-shocked for up to 60 mins at 45°C. The samples took an average of 1.0 min to reach the higher temperature. Nonheat-shocked cells were used as controls. At timed intervals, the cell suspensions were pulse-labeled with 5 µl of trans-^{35}S methionine for 1.0 min and chased for another 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 ice-cold chloramphenicol (2.5 mg/ml 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 and separated by SDS-Polyacrylamide gel electrophoresis as described by Laemmli (*12*) by using 14% acrylamide gels at room temperature, and a constant 200V. Low molecular weight protein markers containing up to 97.4 kDa proteins were used as standards (Bio-Rad Laboratories, Richmond, CA). 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.

On the basis of the results of the first experiment, *Y. enterocolitica* cells were heat-shocked in BHI broth at 45°C for 60 mins. Nonheat-shocked cells were taken as controls. The heat-shocked and nonheat-shocked cells were then inoculated separately into ground pork (0.2 ml/25 g). The inocula were mixed thoroughly into the meat for 4 mins by using a stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, OH) so that the meat contained approximately 10^7 cells/g of either heat-shocked or nonheat-shocked cells. The meat was then divided into 25 g portions in plastic pouches (O_2 permeability <2.5 ml/645 cm^2/24 h at 23°C and 0% RH and water vapor transmission of <1.0 g/645 cm^2/24 h at 38°C and 90% RH, Curlon™ 863 Saran, Curwood Inc., Oshkosh, WI), 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 Cefsulodin-Irgasan-Novobiocin (CIN) agars (Difco). Colonies were enumerated after incubation as previously mentioned.

Statistical analyses.

The heat treatments (55 and 60°C) of heat-shocked and control *Yersinia,* 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 analysis of covariance.

RESULTS AND DISCUSSION

Optimum conditions of heat shock.

When exposed to higher temperatures, increased number of survivors of heat-shocked *Y. enterocolitica* were observed under all the time/temperature combinations used, when compared to controls. 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 were less than one log. Also, repeated experiments did not give consistent results. Heat shocking at 45°C for 60 mins with a further heat treatment at 55°C consistently resulted in higher number of survivors when compared to controls (Fig. 1). Preliminary experiments showed that heat shocking at 45°C for 60 mins 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 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 mins, respectively. No survivors were present in the nonheat-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 4x those of controls.

The optimum temperature for heat shocking *Y. enterocolitica* was similar to those of other mesophilic bacteria. Lindquist (*14*) had discussed that for mesophilic bacteria, temperatures between 45 to 50°C were optimum for development of 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* increases with increasing heat shock time. Knabel et al. (*11*) demonstrated that heat-shocking of *L. monocytogenes* at 43°C for 30 to 60 mins showed increased thermotolerance when compared to cells heat-shocked for only 5 mins at the same temperature. Heat-shocking *Y. enterocolitica* at 45°C for 60 mins in BHI broth was then used for subsequent experiments in pork samples.

Gel electrophoresis of stress proteins.

Thermotolerance of heat-shocked bacteria has usually been associated with the production of heat shock or stress proteins (*18, 16, 5, 11*). This 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 weights of 70.5 and 58.0 kDa were observed (Fig. 2). These proteins were either not produced, or produced in very minute undetectable amounts by the nonheat-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-shocked 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 preshift rates and then decline 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 production of large amounts of heat shock proteins and that the rate of synthesis of nonheat shock proteins decreases.

Experimenting with heat-shocked *E. coli* O157:H7, Murano and Pierson (*18*) demonstrated production of a 71 kDa protein corresponding to the σ^{32} subunit of RNA polymerase. The σ^{32} subunit is known to play a pivotal role in regulation and synthesis of other stress proteins (*23*). In our present study, one of the stress proteins detected had a molecular weight of 70.5 kDa and it is possible that it is the same σ^{32} subunit. Heat shock proteins ranging in size from 7.5 to 80 kDa have been observed in *Y. enterocolitica* serotype O:3 (*30*). They have 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 molecular weight of 400-500 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 development of arthritis-like symptoms in humans.

Survival of the heat-shocked cells in ground pork.

Figure 3 compares the survivor 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 by the heat-shocked cells compared to the control when exposed to both 55 and 60°C heat treatments. Heat treatment at 60°C resulted in rapid reduction in the number of *Yersinia*, especially the controls (undetected after 9 mins). 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 increased number of survivors (approx. 1.1 log after 15 mins) in heat-shocked samples compared to the control (Fig. 3).

Death of bacterial cells when 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 σ^{32} subunits (*17*). Further, the increased production of σ^{32} subunits at elevated temperatures promote 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, have also been ascribed as functions of hsps.

Besides production of hsps, 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 temperature enhanced heat resistance of cells to further higher temperatures. The higher heat resistance was shown to coincide with decreased unsaturation of the 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 get saturated at higher temperatures. 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* (16, 10). 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 pathogens in raw meat. The results of the present study indicate that *Yersinia* did exhibit heat shock response and produced hsps. Also, the heat-shocked cells were more thermotolerant and were able to survive low processing temperatures. It is therefore important to bear in mind the possible presence of heat-shocked pathogens such as *Yersinia* when establishing safe heat treatment guidelines for food.

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Table 1. *D values (minutes) of heat-shocked and nonheat shocked control* Yersinia *in BHI broth and ground pork at different heat treatment temperatures.*

Temp. (°C)	BHI		Pork	
	Heat-shocked	Control	Heat-shocked	Control
55	7.7[a]	2.0[b]	15.6[a]	6.5[b]
60	1.6[a]	1.2[a]	6.7[a]	1.7[b]

[a,b] Different superscripts within each temperature and for either BHI or pork denote significant difference at the 0.05 level.

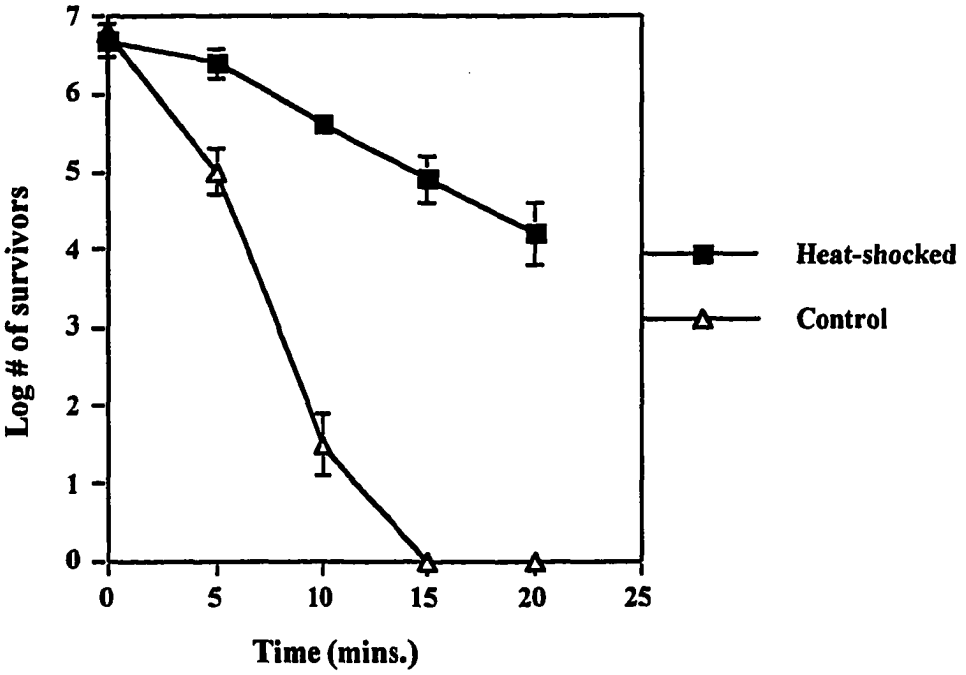

Figure 1. Yersinia enterocolitica *heat-shocked at 45°C for 60 mins. followed by heat treatment at 55°C in BHI broth.*

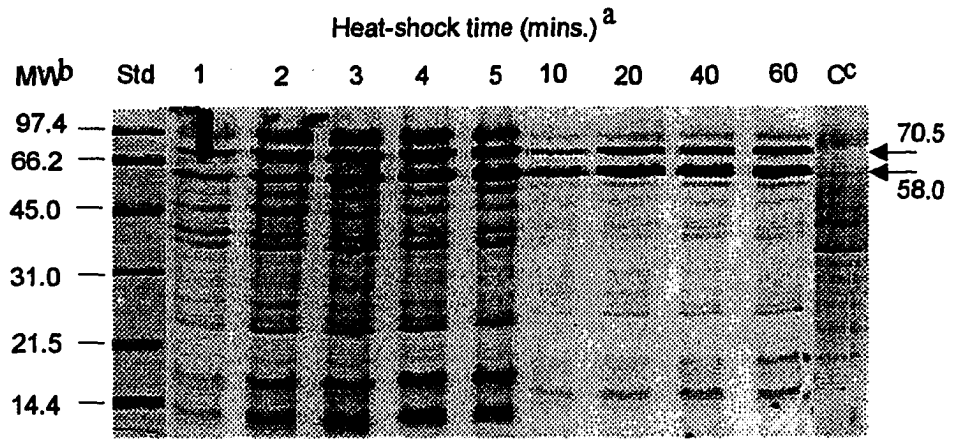

Figure 2. *Autoradiogram of SDS-polyacrylamide gel electrophoretic separation of whole cell proteins before and after heat shock.*

[a] Time of exposure of cells to 45°C in modified M9 medium
[b] Molecular weight of standards
[c] Control sample [nonheat-shocked]

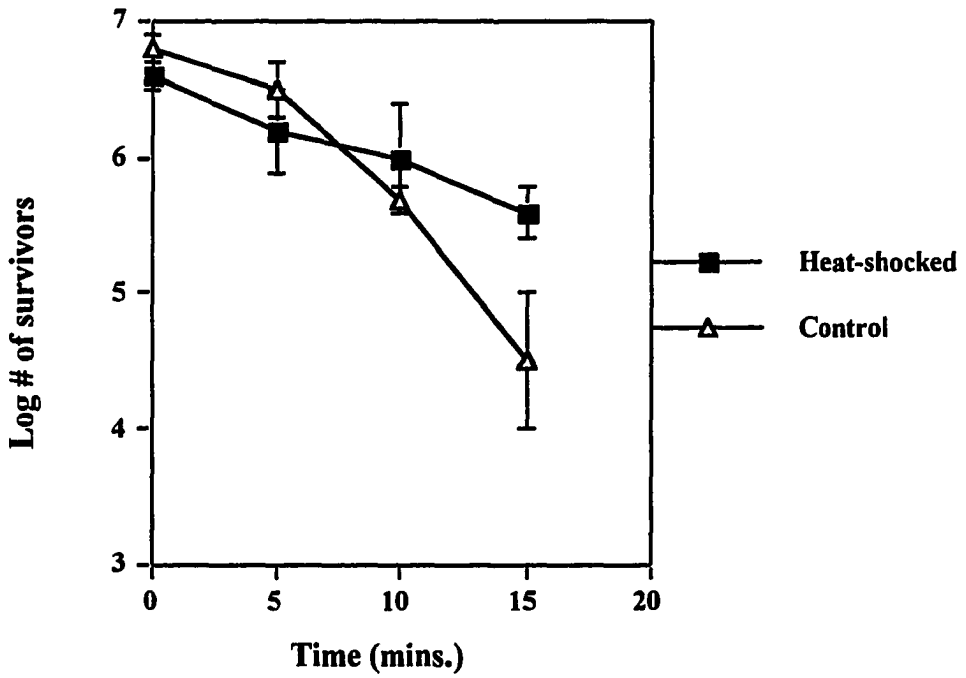

Figure 3. Yersinia enterocolitica *heat-shocked at 45°C for 60 mins. followed by heat treatment at 55°C in ground pork.*

EFFECT OF STORAGE CONDITIONS ON GROWTH OF HEAT-STRESSED *YERSINIA ENTEROCOLITICA* IN GROUND PORK

A paper to be submitted to the Journal of Food Protection

Kalpana Shenoy and Elsa A. Murano

ABSTRACT

Ground pork was inoculated with either heat-shocked or nonheat-shocked control *Yersinia enterocolitica*. After mixing the inocula thoroughly, the meat was divided into 25 g portions in plastic pouches and sealed either under air, vacuum, or modified atmosphere (50% CO_2 + 50% N_2). All the samples were heat treated at 55°C for 15 mins and then stored either at 25 or 4°C. Samples were plated after storage at regular intervals and the growth of *Yersinia* was determined. Survivors were also examined for virulence. Growth of *Y. enterocolitica* with minimal lag periods, for both heat-shocked and control, was observed under all atmospheres and both temperatures. There was no significant difference in growth rates between the heat-shocked and control samples under all the storage atmospheres and temperatures. Also, in both heat-shocked and control samples, *Yersinia* grew rapidly under all atmospheres, and the survivors remained virulent. The results indicated that storage of meat at 4°C whether under vacuum or modified atmosphere was insufficient to inhibit growth of *Y. enterocolitica*, and that prior heat shock had no effect on growth rate or virulence of survivors.

INTRODUCTION

Some of the critical factors that affect safety and shelf life of perishable packaged food products include chemical and nutrient composition of the food, a_w, pH, presence of antimicrobials, the initial number of pathogenic and spoilage flora, storage temperature and, the gaseous environment inside the package (*12, 14*). Controlling one or more of these factors helps in extending the shelf life of the product. As a consequence, the technology of packaging meat under vacuum or modified atmosphere (MA) has gained renewed interest since 1980 (*7*). Numerous sources in the literature have discussed the advantages of packing raw meat and meat products under vacuum or other modified atmospheres (*31, 10, 20, 7*). Among the gases used in modified atmosphere packaging (MAP), the concentration of CO_2 in the headspace of the package appears to be the main limiting factor that is responsible for reducing microbial growth. The mechanism of CO_2 action on bacterial cells has been investigated by several workers and many theories have been put forward. Several of these theories have been reviewed in detail by Daniels et al. (*6*). The inhibitory activities of CO_2 include interference with dehydrogenating and decarboxylating enzymes, changing of the intracellular pH, interference with membrane fluidity, and the toxicity of undissociated carbonic acid.

The phenomenon of the heat shock response and the exhibition of increased thermotolerance by several microorganisms have been extensively studied (*1, 8, 23, 25*). The duration of thermotolerance and growth under anaerobic conditions appears to vary in different heat-shocked organisms. This may be due to variations in species and strains used in the experiments. Different experimental designs and interpretations may also have contributed

to the variation in results. Mackey and Derrick (*22*) showed that the development of thermal resistance by heat-shocked *Salmonella typhimurium* was rapid and lasted for at least 10 h. On the other hand, Farber and Brown (*8*) indicated that heat-shocked *Listeria monocytogenes* retained increased thermotolerance for at least 24 h when stored at 4°C. Further, Knabel et al. (*18*) demonstrated that storage under anaerobic conditions enhanced recovery of heat treated *Listeria monocytogenes* in milk. From all the aforementioned results, we can deduce that heat-shocked organisms have a higher chance of survival when stored under anaerobic conditions. Also, storage at refrigeration temperatures can further enhance the growth of psychrotrophic organisms like *Listeria* and *Yersinia*.

In earlier experiments, we had determined that heating *Y. enterocolitica* in brain heart infusion (BHI) broth at 45°C for 60 mins resulted in the induction of the heat shock response, with two distinct heat shock proteins being detected by SDS-PAGE. Also, inoculation of these heat-shocked cells into ground pork and exposure to higher temperatures resulted in their increased thermotolerance and higher survival. However, the effect of storage atmospheres on recovery of the heat-shocked cells remains to be examined.

Another aspect of heat-shocked *Yersinia* that requires examination is the virulence characteristic. Bhaduri and Turner-Jones (*4*) have reported that *Y. enterocolitica* retained its virulent plasmid when exposed to vacuum or high levels of CO_2 at 28°C for 24 h. Bhaduri et al. (*5*) have also demonstrated the stability of the plasmid and virulence characteristics when *Yersinia* are exposed to elevated temperatures. On the other hand, several other workers have indicated that the virulent plasmid is unstable at temperatures above 37°C (*19, 29*). Batish et

al. (*2*) showed that heat stressed *Staphylococcus aureus* lost its virulence characteristics. However, four to five passages in BHI broth helped the stressed staphylococci to regain virulence.

The present study has therefore been undertaken to determine the effect of storage atmospheres on growth of heat-shocked *Y. enterocolitica* under various temperatures, and to determine virulence of the survivor cells after storage.

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.

Sample preparation, inoculation, packaging, and heat treatment.

One ml of log phase cells of *Y. enterocolitica* grown at 25°C was inoculated into 5.0 ml BHI broth prewarmed at 45°C. The cells were heat-shocked at 45°C for 60 mins. This temperature/time combination for heat-shocking was determined by previous experiments. Cells that were not exposed to 45°C were taken as the nonheat-shocked control. The heat-shocked and nonheat-shocked cells were then inoculated separately into ground pork. The inocula were mixed thoroughly into the meat for 4 mins by using a stomacher (Lab Blender 400, Tekmar Company, Cincinnati, OH). The meat contained a final concentration of approximately 10^7 CFU/g of either heat-shocked or nonheat-shocked cells. In both cases, the

meat was then divided into 25 g portions in plastic pouches (O_2 permeability <2.5 ml/645 cm^2/24h at 38°C and 90% RH, Curlon™ 863 Saran, Curwood Inc., Oshkosh, WI) and sealed either under air, vacuum, or modified atmosphere (Model A300 CVP machine, CVP System, Inc., Downers Grove, IL). The modified atmosphere contained a mixture of 50% CO_2 and 50% N_2. All the meat samples were then exposed to 55°C for 15 mins (heat treatment). The heat treated samples were then stored either at 25 or 4°C. Samples were plated after 0, 1, and 2 days of storage at 25°C and 0, 3, 6, 9, and 12 days of storage at 4°C. At each storage interval, samples were homogenized with 100 ml of 0.1% peptone solution for 2 mins in a stomacher. The homogenates were then serially diluted in 0.1% peptone solution and surface plated on BHI and CIN (Cefsulodin-Irgasan-Novobiocin) agars (Difco).

Virulence tests.

Survivors were confirmed after storage by colony morphology and by biochemical reactions for *Y. enterocolitica*. The colonies were then tested for virulence. Preliminary tests for crystal violet and congo red uptake assays gave negative results for virulence. This may be due to loss of the virulence plasmids during the heat treatment and subculturing procedures. Therefore, tests for plasmid mediated virulence were discontinued and instead, chromosomal mediated virulence tests were performed routinely after every experiment. The survivors were examined for salicin fermentation, esculin hydrolysis (*9, 30, 26*), and pyrazinamidase activity (*15*).

Statistical Analyses.

All the experiments were performed in triplicate and the data were analyzed by

analysis of variance (*28*). Linear regressions were computed for heat-shocked and control samples at a given storage temperature and atmosphere by using the linear portion of the growth curves to predict growth rates. Significant differences in growth rates between heat-shocked and control samples and also between similar samples (either heat-shocked or control) stored under different atmospheres at a given temperature (25 or 4°C) were also determined by analysis of covariance.

RESULTS AND DISCUSSION

Exposure of heat-shocked and control cells in ground pork to a temperature of 55°C for various time intervals showed that, the heat-shocked cells are more thermotolerant or heat resistant than the control (Fig. 1). The D value was calculated as the reciprocal of the slope of the linear regression line drawn through the log number of survivors at different times of exposure at 55°C without storage. The D_{55} values of the heat-shocked and control cells were 15.6 and 6.5 mins, respectively.

During storage, heat-shocked and control *Yersinia* had no detectable lag periods and grew rapidly under all atmospheres and at both temperatures. When stored at 25°C (temperature abuse), both the heat-shocked and control cells grew rapidly and reached approx. 10^9 CFU/g of meat within 2 days. Similar counts were obtained under all the three packaging atmospheres (Fig. 2). Vacuum and modified atmospheres were ineffective in inhibiting the growth of heat-shocked and control cells at 25°C. At the same time, there was no enhanced growth under vacuum or modified atmosphere when compared to air. In other words, there was no significant difference ($p>0.05$) in growth rates between heat-shocked and

control samples, stored under the three atmospheres at 25°C (Table 1). The number of cells increased by approx. 2.4 and 2.9 logs overnight in the heat-shocked and control samples, respectively.

When stored at 4°C, the heat-shocked and control cells required about 9 days to reach approx. 10^8 CFU/g under the different atmospheres (Fig. 3). If shelf life is defined as the time taken for microbial counts to reach approx. 10^7 CFU/g, then simply a storage at a lower temperature, in this case 4°C, is sufficient to increase the shelf life by about 5 days as far as *Y. enterocolitica* is concerned. Comparison of growth rates between heat-shocked and control samples stored at 4°C indicated that there was no significant difference ($p>0.05$) between them when stored under all the three atmospheres (Table 1).

Uninoculated samples were examined for any *Y. enterocolitica* present in the meat. None were detected. This indicates that, there were $<10^2$ CFU/g present in the meat samples at the time of inoculation. The total counts in fresh uninoculated pork were 1.4×10^2 CFU/g. The spoilage flora in the meat identified after heat treatment and storage were *Enterobacter* spp., *Hafnia* spp., micrococci, and *Serratia* spp. Routine examination for virulence (negative for salicin, esculin, and pyrazinamidase tests) after storage indicated that the surviving Yersiniae retained their virulence after heat treatment and storage under the three atmospheric conditions and both temperatures.

According to Lindquist (*21*), the length of time during which increased thermotolerance is exhibited by heat-shocked cells appear to be a function of several factors. Some of them being, heat-shocking temperature, duration of heat shock, previous growth

temperature of the cells, and also the metabolic state of the cells. Like *Listeria monocytogenes* (*8*) and *Salmonella typhimurium* (*22*), the present study indicates that increased thermotolerance and higher survival exhibited by heat-shocked *Y. enterocolitica* is only temporary and probably lasts for a few hours when compared to nonheat-shocked cells. On prolonged storage at refrigeration temperatures and under various packaging atmospheres, growth rates of heat-shocked and nonheat-shocked *Yersinia* are the same (Table 1).

Some workers have shown that production of peroxidase and superoxide dismutase (SOD) enzymes is increased dramatically in heat-shocked cells (*3, 27, 16*). The hypothesis is that these enzymes may partly be responsible for the increased thermotolerance and higher survival exhibited by heat-shocked cells. That being the case in the present experiment, we should have observed significantly increased growth rate in the heat-shocked samples when compared to the control. Further, if this theory was true in the given situation, there would have been decreased growth with a longer lag phase for the nonheat-shocked cells during storage. However, the results indicate otherwise. There is no significant difference in growth rates between heat-shocked and nonheat-shocked cells when stored at 4°C and under all the three atmospheres. Knabel et al. (*18*) have also stated that, catalase and SOD are heat sensitive and are rapidly inactivated at temperatures of 55 and 60°C. Therefore, based on the present evidence we can conclude that SOD, catalase or peroxidase like the heat shock proteins, may only be protective for the heat-shocked cells for a few hours after heat-shocking. However, they do not significantly help the heat-shocked cells to grow at a higher rate over storage.

After 12 days of storage at 4°C, there was no difference in counts between the heat-shocked samples stored under the different atmospheres. However, storage at 4°C for 12 days resulted in increased counts of the nonheat-shocked cells under vacuum and modified atmosphere versus air (Fig. 3). A similar trend in growth of *Yersinia* was also observed by Manu-Tawaiah et al. (*24*). This may probably be due to the competitive growth of aerobic spoilage flora. Increased growth of spoilage flora was observed in the present experiment in samples packaged under air versus those packaged under vacuum or modified atmosphere.

The effects of storage temperature and packaging atmospheres on growth during storage have been studied by several workers. Gill and Reichel (*11*) demonstrated that, unlike *Listeria monocytogenes* and *Aeromonas hydrophila*, *Y. enterocolitica* has the ability to grow at 5°C under CO_2. Their results also indicated that *Yersinia* can grow with a minimal lag period under vacuum at temperatures of 0, 2, 5, and 10°C. However, they concluded that storage below 0°C under vacuum or below 2°C under CO_2 should be sufficient to inhibit growth of *Yersinia*. Inhibition of *Yersinia* at 1°C under CO_2 (*17*), and at -1.5 and 3°C under vacuum (*13*) have also been recorded in the literature.

The results of the present experiment are in agreement with those of Gill and Reichel (*11*). Growth of *Yersinia* with minimal lag period was observed in the present experiment when stored at 4°C under air, vacuum, and modified atmosphere. Growth rate below 4°C was not examined. Besides temperature, the inhibitory effect of 50% CO_2 appears to be minimal in the present study. This may be due to the growth phase of the cells. In their review, Lambert et al. (*20*) have mentioned that CO_2 is most effective when the microbial cells are in the lag

phase of growth. Further, the inhibitory effect of CO_2 will dramatically be reduced if the cells have already entered the log phase during application. In our study, we had inoculated log phase heat-shocked and control *Yersinia* into the meat. Therefore, we may not have been able to observe the inhibitory activity of CO_2.

In conclusion, we can state that there is no significant difference in growth rates between heat-shocked and control *Yersinia* when stored at 4 or 25°C, under various packaging atmospheres. The survivors also retained their virulence and were easily detected. *Yersinia* showed the ability to grow rapidly under various packaging atmospheres and at refrigeration temperatures. Based on our results, and those obtained by other workers, we can conclude that the storage temperature and atmosphere (especially the percentage of CO_2 used) have to be determined and carefully applied to meat to prevent outbreaks by this organism.

ACKNOWLEDGMENT

Funding for this research was provided by a special grant of the USDA through the Food Safety Consortium.

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Table 1. *Growth rates (linear regression coefficients)[a] of heat-shocked and control* Y. enterocolitica *inoculated into ground pork and stored at 25 and 4°C.*

Storage temp.	Atmospheres	Heat-shocked	Control
25°C	Air	$1.65^{x,p}$	$2.20^{x,q}$
	Vacuum	$1.75^{x,p}$	$2.15^{x,q}$
	MA[b]	$1.75^{x,p}$	$2.3^{x,q}$
4°C	Air	$0.31^{x,r}$	$0.31^{x,s}$
	Vacuum	$0.28^{x,r}$	$0.40^{x,s}$
	MA	$0.30^{x,r}$	$0.39^{x,s}$

[a] Linear regression coefficents for log growth phase (log numbers/day).

[b] Modified atmosphere (50% CO_2 and 50% N_2).

[x] Values with the same superscript in a row are not significantly different ($p>0.05$).

[p,q,r,s] Values with the same superscript in a column at a given temperature are not significantly different ($p>0.05$).

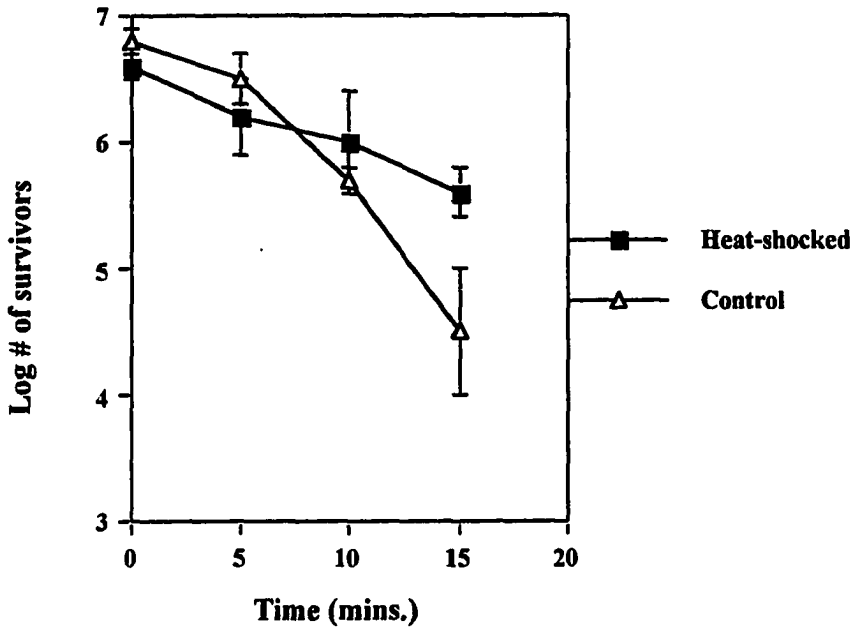

Figure 1. *Survival of heat-shocked and control* Y. enterocolitica *when heated at 55°C in ground pork.*

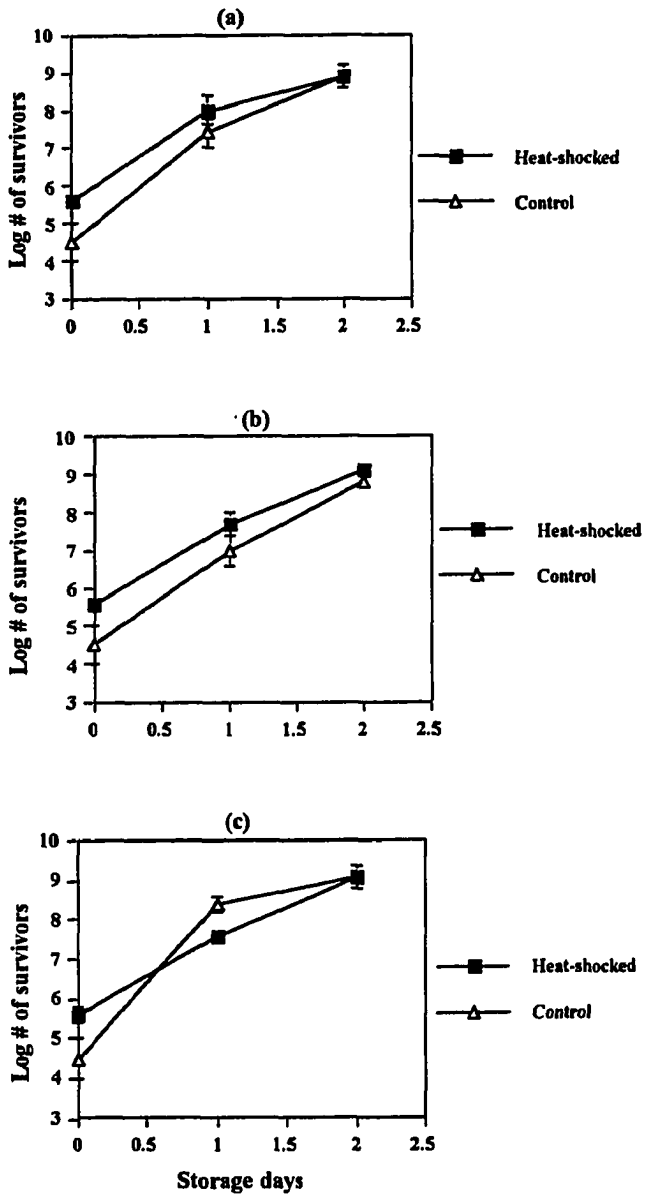

Figure 2. *Growth of heat-shocked and control* Y. enterocolitica *stored at 25°C under (a) air, (b) vacuum, and (c) modified atmosphere.*

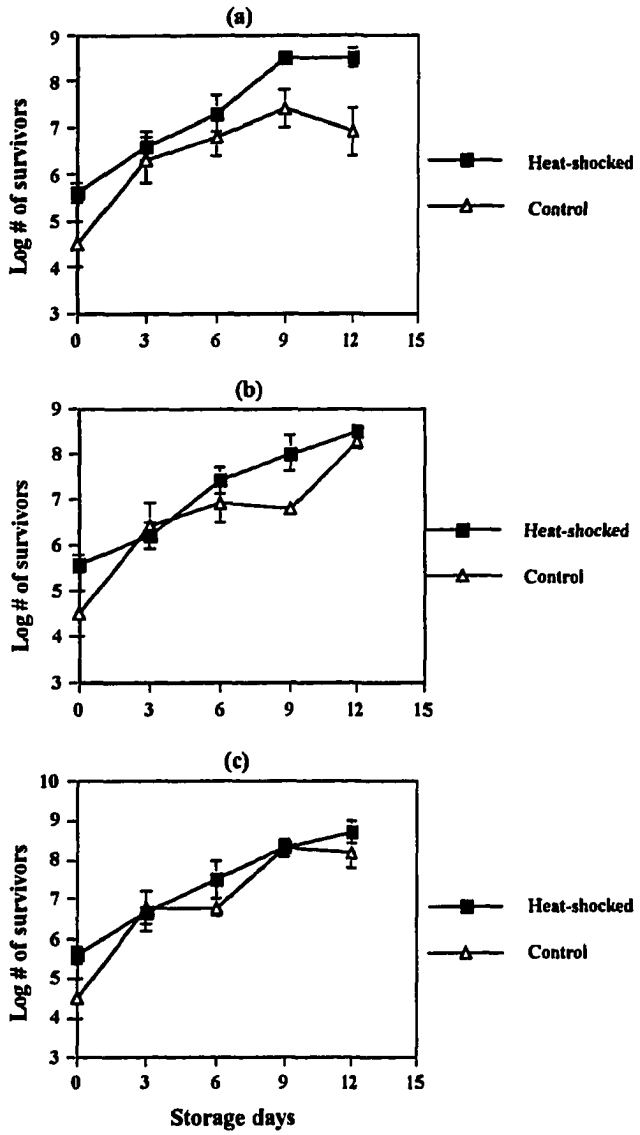

Figure 3. Growth of heat-shocked and control Y. enterocolitica *stored at 4°C under (a) air, (b) vacuum, and (c) modified atmosphere.*

EFFECT OF HEAT SHOCK ON SURVIVAL OF *YERSINIA ENTEROCOLITICA* TO IRRADIATION IN GROUND PORK

A paper to be submitted to the Journal of Food Protection

Kalpana Shenoy and Elsa A. Murano

ABSTRACT

Earlier studies have shown that heat-shocked *Yersinia enterocolitica* (45°C/60 mins) are more resistant to a subsequent heat treatment of 55 or 60°C in ground pork than cells not previously heat-shocked. The present study indicates that, unlike heat treatment, the heat-shocked *Yersinia* were not significantly different in their resistance to irradiation from their nonheat-shocked counterparts in the same suspending medium. Packaging under vacuum did not increase the resistance of this organism to irradiation. The average irradiation D values for the heat-shocked and nonheat-shocked cells under air and vacuum were the same (0.15 kGy). Also, the heat-shocked irradiation survivors exhibited chromosomal mediated virulence characteristics, just as the nonheat-shocked controls.

INTRODUCTION

Irradiation is an alternative for conventional methods of cooking to reduce or eliminate foodborne pathogens and spoilage organisms, thereby increasing the safety and storage stability of the product. It is a process which can substitute several food preservatives,

insecticides, and other chemical additives that are of growing concern to consumers. Compared to heat, irradiation causes fewer changes in nutrient and sensory attributes (*1*). Studies have indicated that irradiation at doses of 1.5 to 3.0 kGy at refrigeration temperatures, are sufficient to destroy both spoilage and pathogenic organisms like *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Salmonella* spp., and *Listeria monocytogenes* (*2, 17, 13, 6*). Irradiation of meat at above freezing temperatures is not only economically practical, but also requires lower doses for eliminating microorganisms (*10*). The sensory changes at doses up to 3.0 kGy at 2-5°C temperature, are minimal (*10*).

Contradictory results have been obtained by workers when irradiating various pathogens suspended in meat packed under air, vacuum or other gases (N_2, CO_2). Some claim that organisms are more sensitive when irradiated under air or oxygen. The reason being, free radicals are formed when irradiated in the presence of oxygen and these free radicals have deleterious effects on the bacterial cells (*11*). Others have argued, and proved by their experiments, that irradiation under vacuum does not increase the resistance of an organism and that, the D values are the same when irradiated either under air or vacuum.

Previous studies have shown that, when *Y. enterocolitica* is heat-shocked at 45°C for 60 mins and then exposed to a heat treatment of 55 or 60°C, increased survivors were present when compared to nonheat-shocked cells. The heat-shocked cells produced stress proteins and appeared to be more thermotolerant. Concerns have been raised regarding radioresistance, detection, and virulence of irradiated microorganisms in food. Considering all the above criteria, the objectives of the present study were (i) To determine D values for heat-

shocked versus nonheat-shocked *Y. enterocolitica* exposed to irradiation, (ii) To determine the effect of packaging atmospheres on resistance during irradiation, and (iii) To determine virulence of the survivor cells.

MATERIALS AND METHODS

Sample preparation, inoculation, and irradiation.

Frozen ground pork was obtained from the Meats Laboratory at Iowa State University. The ground pork in the frozen state was cut into one inch thick slices with an approximate diameter of 2.5 inches. The slices were packed under vacuum and sterilized by irradiating at 30 kGy. The irradiation sterilized pork was stored at -50°C till use.

Y. enterocolitica, serotype O:8 (ATCC 27729, American Type Culture Collection, Rockville, MD) was grown in Brain Heart Infusion (BHI) broth (DIFCO Laboratories, Detroit, MI) at 25°C. One ml each of the log phase cells was inoculated into 2 tubes containing 5.0 ml of fresh BHI broth. The cells in one tube were heat-shocked at 45°C for 60 mins in a waterbath. This temperature and time for heat shocking was determined by earlier studies. The second tube was taken as the nonheat-shocked control.

The sterile frozen ground pork was thawed before inoculation. The heat-shocked and control cells were then inoculated separately into the meat and mixed thoroughly for four minutes using a stomacher (Lab Blender 400, Tekmar Co., Cincinnati, OH). This gave an approximate concentration of 10^7 CFU/g of meat. The meat was then divided into 25g samples in 60x15mm sterile plastic petri plates. This ensured uniform thickness of the samples for irradiation. The petri plates were then packed either under air or vacuum in plastic pouches

(Curlon™ 863 Saran, Curwood Inc., Oshkosh, WI). After packing, the samples were immediately irradiated at the Linear Accelerator facility at Iowa State University.

The radiation source used was a linear-electron-beam accelerator with an energy level of 10 MeV. The dose was controlled by altering the conveyor speed (11.05 to 29.20 ft/ min.) and the power level (1.1 and 2.1 kW). The samples were irradiated at doses of 0, 0.25, 0.5, 0.75, and 1.0 kGy. The actual absorbed doses were measured by placing alanine pellets on the surface and bottom of the samples. An Electron Paramagnetic Resonance (EPR) instrument was used to determine the absorbed dose (Bruker Instruments Inc., Billerica, MA). The temperature of the samples was maintained between 5 and 8°C during irradiation. After irradiation the samples were stomached with 0.1% peptone solution, followed by appropriate serial dilutions and surface plated on Brain Heart Infusion (BHI) agar and Cefsulodin-Irgasan-Novobiocin (CIN) agar (DIFCO). The colonies were enumerated after incubation at 30°C for 48 h.

Virulence tests.

The virulence characteristics of *Y. enterocolitica* are both plasmid and chromosomal mediated. Repeated subculturing as in a laboratory (like in the present experiment) can result in the loss of the plasmid (*8*). Therefore, tests for detection of the virulent plasmid are not very useful. The chromosomal mediated virulent characteristics are stable and were therefore used in this experiment to determine virulence of the survivor cells. The survivors were examined for salicin fermentation, esculin hydrolysis (*4, 14, 12*), and pyrazinamidase activity (*7*). The virulence tests were performed on the survivors after every experiment.

Statistical analyses.

The experiments both under air and vacuum were performed in triplicate. The average values of the log number of survivors were plotted against the irradiation doses. The D value was calculated as the reciprocal of the slope obtained from the linear regression line. At each dose level a 3-way ANOVA was performed to determine significant differences between heat-shocked and nonheat-shocked control samples, under air and vacuum, and on BHI and CIN agars.

RESULTS AND DISCUSSION

Figures 1 and 2 show the log number of survivors over increasing doses of irradiation. From these figures, an average D value of 0.15 kGy was calculated for heat-shocked and nonheat-shocked control cells when irradiated under air and vacuum. There was no significant difference ($p>0.05$) in survival between the heat-shocked and control cells when irradiated either under air or vacuum. On the basis of the D value obtained, we can estimate a 6.7 log reduction by a dose of 1.0 kGy. Statistical analyses indicates that there was no significant difference ($p>0.05$) between BHI and CIN agar counts and both media were equally efficient for growth of the survivors after irradiation. There was approximately up to a 96% recovery of *Yersinia* on CIN when compared to BHI agar. The virulence tests (negative for salicin, esculin, and pyrazinamidase) also indicated that the survivors remained virulent after irradiation.

An irradiation D value of 0.15 kGy for *Y. enterocolitica* indicates that this organism is highly sensitive to irradiation. Similar values for *Yersinia* in ground raw beef were obtained by

Tarkowski et al. (*15*). In their experiment, *Y. enterocolitica* serotypes O:3, O:5,27, and O:9 were irradiated at doses up to 1.0 kGy in ground beef (*15*). The D values were 0.10, 0.16, and 0.21 kGy respectively. Irradiation of 3 clinical strains of *Y.enterocolitica* in trypticase soy broth and ground beef was studied by El-Zawahry and Rowley (*3*). The 3 strains were found to be extremely sensitive to irradiation and had an average D value at 25°C of 0.1 and 0.2 kGy in trypticase soy broth and ground beef, respectively.

Heat-shocked *Yersinia* have been shown to be more resistant than nonheat-shocked cells when exposed to a subsequent higher heat treatment. However, we have shown that irradiation at 1 kGy eliminated heat-shocked and control *Yersinia*. Also, vacuum packaging, which is being increasingly used in industry to prolong shelf life of raw meat and meat products, did not increase the resistance or enhance the survival of the cells after irradiation. Thayer and Boyd (*16, 18*) showed that irradiation of mechanically deboned chicken meat inoculated with *E. coli* O157:H7 or *Salmonella typhimurium* under air and vacuum resulted in no difference in D values. In an experiment conducted by Lebepe et al. (*9*), some *Yersinia* spp. survived irradiation at a dose of 3.0 kGy under vacuum. Low numbers ($<10^2/cm^2$) of *Yersinia* were detected immediately after irradiation. These were isolated only after enrichment in PBS/KOH medium, but they did not grow over storage in pork (*9*). The effect of two irradiation doses (0.8 and 2.0 kGy) on survival and growth of *Y. enterocolitica* in ground beef was examined by Fu (*5*). At both doses of irradiation and storage either under air or vacuum at 7°C for 7 days followed by storage at 25°C for 2 days did not show the presence of any survivors even after enrichment. This further confirms our results regarding the

sensitivity of the organism and its unlikely chances of growth during storage, regardless of the atmosphere of irradiation.

The FDA has already approved the use of irradiation of up to 1 kGy for eliminating *Trichinella spiralis* in pork. *Y. enterocolitica* counts in processed pork under hygienic conditions do not normally exceed 10^2 CFU/g, unless the product has been abused. Therefore, a dose of 1kGy should be sufficient to completely eliminate this pathogen from pork. In conclusion, the results of this experiment indicate that, even though irradiation did not affect the virulence of survivors, irradiation of pork at a dose of 1 kGy was sufficient to eliminate both heat-shocked and nonheat-shocked *Yersinia*. Also, packaging under vacuum did not increase the resistance of this organism to irradiation compared to samples packaged in air.

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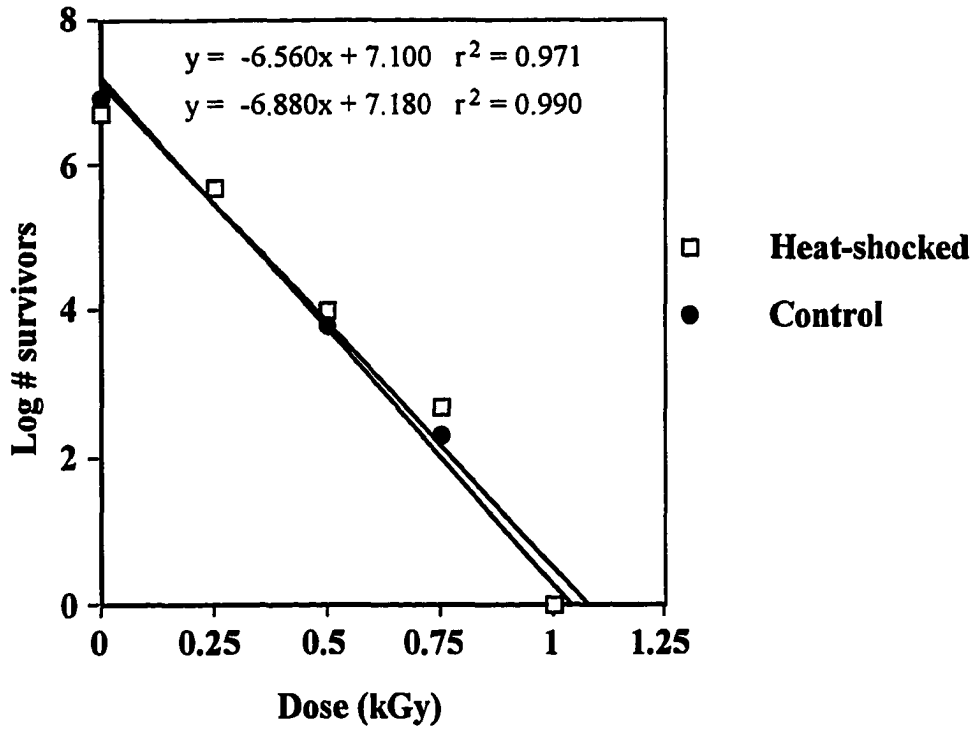

Figure 1. *Irradiation of heat-shocked and control* Yersinia enterocolitica *in ground pork under air.*

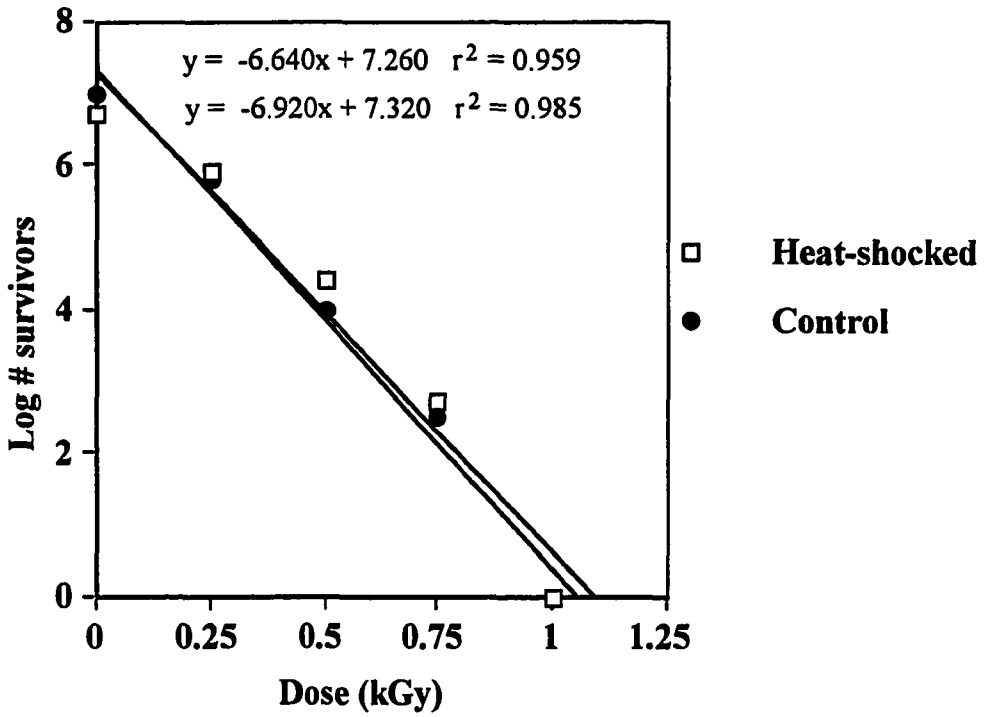

Figure 2. *Irradiation of heat-shocked and control* Yersinia enterocolitica *in ground pork under vacuum.*

GENERAL CONCLUSIONS

Some of the questions asked, for which we sought an answer were: (1) Does *Y. enterocolitica* serotype O:8 exhibit the phenomenon of heat shock response and produce heat shock proteins (hsps) when exposed to sublethal temperatures? (2) If so, are the heat-shocked cells more thermotolerant than nonheat-shocked controls when subjected to subsequent higher temperatures either in brain heart infusion (BHI) broth or pork? (3) Is the growth rate of heat-shocked cells significantly different from the control when stored under various packaging atmospheres and temperatures? (4) Can irradiation be used effectively in eliminating both heat-shocked and control *Yersinia*? (5) Do the survivors of heat treatment or irradiation remain virulent? The experiments performed were useful in answering these questions. The results with brief discussions are summarized as follows:

1. Heat shocking *Y. enterocolitica* at 45°C for 60 mins. in BHI broth with a further heat treatment at 55°C consistently resulted in increased number of survivors when compared to controls. There was a significant difference in D_{55} values, with heat-shocked cells having D values of approximately as high as 4x those of controls.
2. Two distinct heat shock proteins with molecular weights of 70.5 and 58.0 kDa were produced by heat-shocked *Yersinia*. Production of hsps was almost immediate after exposure to the sublethal temperature of 45°C. Initially, there appeared to be an increased synthesis of both heat-shocked and normal proteins, but over time, reduction in synthesis of normal proteins and continued synthesis of hsps was noted. The 70.5 kDa protein isolated is probably the σ^{32} subunit of RNA polymerase. Production of a

71.0 kDa protein corresponding to the σ^{32} subunit has been detected by Murano and Pierson (1992) in *Escherichia coli* O157:H7. A 60 kDa stress protein corresponding to the GroEL protein of *E. coli* has been detected in *Y. enterocolitica* serotype O:3 by Yamaguchi *et al.* (1990). Antibodies to this 60 kDa protein have been found in sera of patients suffering from yersiniosis. Involvement of the 60 kDa protein in development of arthritis-like symptoms have been speculated (Yamaguchi *et al.*, 1990). The 58.0 kDa hsp that was isolated in serotype O:8 is probably the same 60 kDa protein of serotype O:3.

3. The D_{55} and D_{60} values for heat-shocked cells in ground pork were 15.6 and 6.7 mins. and those for controls were 6.5 and 1.7 mins. respectively. These values indicate a significant increase in thermotolerance of heat-shocked cells in ground pork. Slow cooking of certain meat products or the inadvertent heating during fabrication and processing of raw meat can result in production of thermotolerant cells. Therefore, the possible presence of heat-shocked pathogens like *Yersina* has to be kept in mind when establishing heat treatment guidelines.
4. Growth of *Y. enterocolitica* with minimal lag periods, for both heat-shocked and control was observed under all packaging atmospheres and storage temperatures. There was no significant difference in growth rates between the heat-shocked and control samples under the given conditions. Also, in both kinds of samples (heat-shocked and control), *Yersinia* grew rapidly under all atmospheres. All the survivors examined remained virulent. The results indicate that storage of meat at 4°C, even

under vacuum or modified atmosphere was insufficient to inhibit growth of *Y. enterocolitica.*

5. Unlike heat treatment, the heat-shocked *Yersinia* were not significantly different in their resistance to irradiation from their nonheat-shocked counterparts when suspended in pork. Packaging under vacuum did not increase the resistance of this organism to irradiation. An average D value of 0.15 kGy was calculated for both heat-shocked and nonheat-shocked cells under air and vacuum. Cefsulodin-Irgasan-Novobiocin (CIN) agar was efficient in recovery of irradiated *Yersinia* and all the survivors exhibited the virulent characteristics.

Heat shock response and development of thermotolerance of pathogens including *Yersinia* are phenomena that needs to be taken into consideration when establishing temperature guidelines for cooking. *Y. enterocolitica* counts in raw meat processed under strict hygienic conditions do not normally exceed 10^2 CFU/g. Therefore, on the basis of the D values obtained in the present study, heat treatment of meat at 60°C for approx. 14-15 mins should be sufficient to eliminate even heat-shocked *Yersinia* from meat. Storage of meat under vacuum or modified atmosphere should be accompanied by temperatures of not more than 0°C to prevent the growth of *Yersinia.* Irradiation is a technique that can be used effectively to eliminate *Yersinia* and other pathogens, reduce spoilage flora, retain the sensory qualities, and extend the shelf life of food products. Low dose irradiation (up to 3 kGy) is sufficient to eliminate *Yersinia,* both heat-shocked and nonheat-shocked from pork without altering the sensory qualities.

Suggestions for Additional Research

More information is needed regarding *Y. enterocolitica* that can be used in food processing. Some of the studies that would further shed light on this organism are:

1. Detection of more serotypes that are presently unknown having the capacity to cause disease. This should include development of not only newer methods of isolation but that would also determine the virulent nature of the isolate. Sources of infection besides swine, water, and milk also needs to be established.
2. The effects of irradiation on *Yersinia*, whether it causes inactivation or only injury needs to be examined. Also, storage of meat for extended periods of time must be followed by enrichment and detection of survivors. This will verify the contradictory results of certain previous works on the ability of the organism to survive low irradiation doses.
3. Effects of "synthetic" and "natural" preservatives in meat and other food products in inhibiting growth of *Yersinia* are largely unknown and need to be studied.
4. Study on the effect of sanitizers, especially those used for washing of carcasses, to reduce *Y. enterocolitica* counts in meat can become useful in processing.

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