THE EFFECT OF HEAT SHOCK, GROWTH ATMOSPHERE, AND RECOVERY ATMOSPHERE ON THE SURVIVAL OF *Escherichia coli* 0157:H7 TO HEAT

by

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(ABSTRACT)

E. coli 0157:H7 is an important foodborne pathogen, responsible for several outbreaks of hemorrhagic colitis where improperly cooked hamburger meat was thought to be the vehicle. Various time/temperature combinations were used to determine the optimum conditions of heat shock which would result in the greatest number of survivors to a 55°C heat treatment. The optimum conditions were 42°C for 5 minutes and were used throughout the study.

Heat shock of aerobically grown cells resulted in an increase in the mean D value after a 55°C heat treatment by a factor of 2.1 over nonheat-shocked controls. Heat shock of anaerobically grown cells also resulted in a significant increase in mean D value over nonheat-shocked controls. Anaerobic growth itself resulted in an increase in the ability of the cells to survive the 55°C heat treatment when compared with aerobically grown

cells. Both heat-shocked and anaerobically grown cells contained a protein corresponding to a sigma^{32} subunit of RNA polymerase which has been identified as the 71,000 dalton heat shock protein characteristic of E. coli cells.

Anaerobic plating resulted in a significant increase in the mean D values of both aerobically grown and anaerobically grown cells. The largest increase in mean D values was observed in aerobically grown nonheat-shocked cells, which increased by a factor of 2.3 when plated anaerobically instead of aerobically. The activities of catalase and superoxide dismutase in aerobically grown and anaerobically grown cells were studied to determine the reason why anaerobic plating enhanced recovery of cells. The activities of both enzymes were eliminated after heat treatment at 55°C for 20 minutes, regardless of whether the cells were heat-shocked or not.

The ability of heat shock and anaerobic growth to protect the cells from a subsequent heat treatment was tested by measuring the rate of release of cell materials during heating at 55°C. Heat-shocking and anaerobic growth resulted in even faster release of cell materials during heating than controls, suggesting that neither of

these stresses protected the cells against the effects of heat.

The effect of heat shock on cell injury was studied. Heat shock of aerobically grown cells resulted in the greatest difference in log number of cells between cells plated in nonselective medium vs. selective medium. Thus, more cells were injured if heat-shocked than if not heat-shocked. Heat-shocking of anaerobically grown cells also resulted in more injured cells than nonheat-shocked controls.

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INTRODUCTION

Escherichia coli 0157:H7 is a gram negative, facultative anaerobe considered to be the causative agent of hemorrhagic colitis in humans (95). This disease is characterized by severe abdominal pain, grossly bloody diarrhea, and little or no fever (38). Studies in the United States and Canada have shown that the organism can also cause two other serious conditions, hemolytic uremic syndrome and thrombocytopenic purpura (111). E. coli 0157:H7 has become a cause for concern to health officials due to the increase in reported cases over the last decade (46). Elderly patients are at the highest risk of infection with this pathogen, which not always manifests itself as a simple case of diarrhea. Out of 136 cases in four nursing home outbreaks from 1982 to 1986, 25 resulted in death of the patients (16, 100, 47).

The vehicle of infection in most cases of hemorrhagic colitis has been identified as cooked hamburger meat (100, 95), with only one outbreak being attributed to consumption of raw milk (83). It is assumed that incidences of hemorrhagic colitis are due to undercooking or to post-cooking contamination of the food rather than to survival of the organism to the cooking

procedures. This is because E. coli 0157:H7 is not considered to be a heat resistant organism, exhibiting poor growth in the temperature range generally used for recovery of other E. coli serotypes from foods (22).

When either whole organisms or individual cells are shifted to temperatures slightly above their normal range for growth, their resistance to a subsequent heat treatment is greatly increased (67). This phenomenon, known as the heat shock response, has been observed in cultured mammalian cells, yeast, fungi, and in bacteria such as E. coli and the foodborne pathogen Salmonella typhimurium (106, 130, 71). This increased heat resistance of microorganisms due to heat shock has been given recent attention by food scientists due to its possible effect on pathogens in foods (3). In their study on Salmonella thompson in eggs, Mackey and Derrick showed that a 54°C incubation for 1 hour results in a 5 $\log_{10}$ reduction in the number of nonheat-shocked survivors, whereas the same treatment reduces the number of heat-shocked cells by only 1 $\log_{10}$ (70).

Coupled to the potential for survival of heat-shocked cells, is the concern that the use of anaerobic environments for packaging of thermally processed foods may improve the ability of surviving pathogenic bacteria

to recover from injuries received during processing (9). Such anaerobic conditions are used in the storage of food products as well as in the preparation of foods ranging from pasta to beef patties by a process known as "sous-vide" (French for "under vacuum"). In this process, products are cooked under vacuum at low heat for a relatively long time, which preserves the flavor of the product better than conventional methods that employ high heat processing (28). Foods prepared by the sous-vide process are also stored under anaerobic conditions, a procedure aimed at retarding spoilage by aerobic microorganisms.

Storage of foods under anaerobic conditions and the role of such an environment in the ability of cells to recover from heat has been studied in various bacterial systems. In studies on the effect of aeration on the recovery of heated Salmonella typhimurium, Gomez and Sinskey found that more cells could be recovered after heating when incubated under a nitrogen atmosphere than under air (34). Knabel et al. found that Listeria monocytogenes cells could be recovered in greater number after pasteurization if the recovery was done under strict anaerobic conditions (60).

The objectives of this study were to:

1) determine whether heat shock and/or anaerobic growth of E. coli 0157:H7 cells improved the ability of the cells to recover from a subsequent heat treatment
2) determine whether anaerobic plating improves the ability of E. coli 0157:H7 cells to recover from a heat treatment
3) determine whether heat shock and/or anaerobic growth of E. coli 0157:H7 cells elicit the synthesis of stress proteins
4) determine the effect of heat shock and/or anaerobic growth on injury of E. coli 0157:H7 cells after heating
5) determine whether the increase in survival to a subsequent heat treatment after heat shock and/or anaerobic growth of the cells can be attributed to a protective effect

REVIEW OF THE LITERATURE

I. *E. coli* 0157:H7

A. General Characteristics

Escherichia coli 0157:H7 is a facultatively anaerobic, gram negative rod, which was identified as the causative agent of hemorrhagic colitis in humans (95). Unlike other *E. coli* serotypes, the organism is not invasive, as tested by the Sereny test, and does not ferment sorbitol. It is also nonhemolytic on sheep- or rabbit-blood agar (128). Like most *E. coli* strains, *E. coli* 0157:H7 grows rapidly at 37°C, with a generation time of 0.49 hours in Trypticase Soy Broth (22). However, unlike most *E. coli* strains, it does not grow well at 44 to 45.5°C, a range used in procedures that detect fecal coliforms in foods (30, 127).

The organism produces a cytotoxin which has been shown to induce irreversible cytopathic changes in Vero cells within 3 days (57). The nature of the toxin has been found to be Shiga-like. O'Brien et al. showed that isolates from cases of hemorrhagic colitis in the United States are neutralized by Shiga toxin antiserum prepared in rabbits using *Shigella dysenteriae* (81). In addition,

toxin from E. coli 0157:H7 was demonstrated to be immunologically indistinguishable from Shiga toxin by the Ouchterlony immunodiffusion analysis (80). More recently, the same investigators have shown that the Shiga-like toxin produced by E. coli 0157:H7 is actually phage-mediated. O'Brien and his co-workers, using a group of phages isolated from strains that cause hemorrhagic colitis, were able to mediate Shiga-toxin production in E. coli K-12, a normally nontoxigenic strain (79).

A rapid fluorogenic assay, known as the MUG test, has been developed for E. coli and is used extensively to differentiate this organism from other coliforms (26). The compound 4-methylumbelliferone glucuronide (MUG) is hydrolyzed by glucuronidase, an enzyme present in most E. coli strains, to yield a fluorogenic product. Doyle tested 8 strains of E. coli 0157:H7 by the MUG assay and found that seven were negative (22). In addition, in a study involving ground beef patties inoculated with E. coli 0157:H7, and subsequently stored at -20°C, it was shown that this organism is able to survive well in ground beef during frozen storage for as long as 9 months, a property that is not commonly found in nonpathogenic E. coli strains (22).

Several methods of identification of E. coli 0157:H7 are currently under investigation. Harris et al. developed a special medium containing sorbitol in a purple agar base which has proven to be succesful in the isolation of sorbitol negative organisms (43). More recently, Gubash et al. found that bromthymol blue inhibits the growth of E. coli 0157:H7 and they suggest that this inhibition could be used as a presumptive test to identify hemorrhagic E. coli isolates in laboratories that do not have serotyping capabilities (40). Several laboratories are currently using monoclonal antibodies to E. coli 0157 polysaccharide antigen and to H7 flagellar antigen in an enzyme-linked immunosorbent assay to detect this organism in food samples (87).

B. Infections Caused by the Organism

Infection with Escherichia coli 0157:H7 was first identified as the cause of hemorrhagic colitis in two outbreaks in 1982 when 47 people developed the disease after eating hamburgers contaminated with the organism (95). The symptoms of hemorrhagic colitis include severe abdominal cramps and watery diarrhea. This is followed by a grossly bloody stool (84). Table 1 contains

TABLE 1

Characteristics of Patients in Major Outbreaks of *Escherichia coli* 0157:H7 Infections[a]

Date	Location	Population	Suspected Source	No. of Patients	% With Diarrhea	% With Cramps	% With Vomiting	% With Fever	% With HUS or TTP[b]	% Hospitalized	% Dead
1982	Oregon	Community	ground beef	26	100	100	44	8	0	73	0
1982	Michigan	Community	ground beef	21	100	100	61	6	0	67	0
1982	Ontario	Nursing Home	unknown	31	65	32	10	--	--	13	3
1984	Nebraska	Nursing Home	ground beef	34	56	74	26	18	3	41	12
1984	N. Carolina	Day Care Ctr	unknown	36	31	64	44	42	8	--	0
1985	Ontario	Nursing Home	sandwich meal	73[c]	[e]	--	--	15[f]	22[f]	--	35[f]
1986	Ontario	Kindergarten	raw milk	46[d]	--	--	--	7[g]	7[g]	--	--
1986	Alberta	Nursing Home	ground beef	16	100	100	--	--	--	--	6
1986	Washington	Community	ground beef	37	97	100	56	29	11	46	5

[a] Adapted from Reference 84.

[b] Hemolytic Uremic Syndrome or Thrombocytopenic Purpura.

[c] 55 were residents and 18 were staff members.

[d] 42 were children and 4 were adults.

[e] 75% of infected residents and 28% of infected staff members had bloody diarrhea.

[f] Percent of residents only.

[g] Percent of children only.

NOTE: Where data is not given, it is because of insufficient information from the source.

epidemiological data on the major outbreaks of E. coli 0157:H7 infections from 1982 to 1986 (38). As can be seen from this information, bloody diarrhea is the most common symptom, followed by cramps and vomiting. Fever occurs but it is usually not high. In the Washington State outbreak, for instance, the temperature was >38°C in only 7 of 23 patients with bloody diarrhea.

Some patients experience nonbloody diarrhea, and these usually have less severe illness than those with bloody stools. In one study, the duration of diarrhea, the number of stools per day, and the proportion of patients with abdominal cramps, vomiting, and fever were all lower in patients with nonbloody diarrhea than in those with the typical bloody stool (111).

Other patients infected with E. coli 0157:H7 experience no symptoms at all. In a nursing home outbreak, the organism was isolated from a nurses' aide who was asymptomatic (47). In another outbreak, E. coli 0157:H7 was isolated from an asymptomatic person who ate hamburger meat contaminated with the organism (100).

Hemorrhagic colitis can also lead to complications which can endanger the patient's life. The most common of these are hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (38).

Hemolytic uremic syndrome is the most common cause of acute renal failure in children and infants. As a result of the disease, renal lesions occur and are characterized by vacuolation and necrosis of the proximal convoluted tubular cells in the kidney (8). Karmali et al. have shown that there is a correlation between the incidence of hemorrhagic colitis and hemolytic uremic syndrome (57). They found that 73% of sporadic cases of HUS had evidence of infection with E. coli 0157:H7, indicating an association between the organism and the onset of HUS.

The Washington State outbreak of hemorrhagic colitis clearly showed that both hemolytic uremic syndrome and thrombotic thrombocytopenic purpura can result from infection with E. coli 0157:H7 (see Table 1). Hemolytic uremic syndrome and TPP are medical terms which are used interchangeably by the medical community. Thrombocytopenic purpura is usually diagnosed in the case when adults are involved whereas HUS is the term given to the same condition in adults and younger patients (29). Risk factors for these complications are not known. However, it has been suggested that patients at the extremes of age are at a greater risk of developing hemolytic uremic syndrome or thrombotic thrombocytopenic purpura (87). In the Oregon and Michigan State

outbreaks, where the median age of patients was 28 and 17 years, respectively, no cases of HUS or TPP were reported. However, in the Nebraska outbreak where the median age was 86 years, 3% of the patients developed HUS or TPP (see Table 1). The risk of death is also strongly related to age, with no fatalities in the first community outbreaks, in which children and yound adults predominated. In contrast, as many as 35% of all patients in the Ontario nursing home outbreak died as a result of infection with E. coli 0157:H7 (see Table 1). Therefore, even though hemorrhagic colitis is not life-threatening, concern about outbreaks of this disease exist due to the potential for death in the very young and the very old.

As far as therapy is concerned, no evidence to date shows that antimicrobials are beneficial. In a nursing home outbreak, patients who died were more likely to have received antimicrobial therapy than those who lived (16).

C. Isolation From Food Sources

Escherichia coli 0157:H7 has been linked to consumption of gound beef, primarily through

epidemiological studies (95, 30, 84). It has been isolated from a frozen ground beef patty obtained from a meat processor that supplied beef patties to restaurants associated with outbreaks in Oregon and Michigan in 1982 (128). More recently, Doyle and Schoeni have isolated E. coli 0157:H7 from other sources such as pork, poultry, and lamb, suggesting that foods of animal origin are an important source of infection (21). In support of this, Beery et al. demonstrated that this organism can colonize the ceca of chickens and be excreted in the feces for several months (7). In addition, E. coli 0157:H7 has been implicated in an outbreak of hemorrhagic colitis that occurred as a result of drinking raw milk at a dairy. In this case, kindergarten children were affected and the organism was isolated from the feces of an asymptomatic child (83).

There is no doubt that E. coli 0157:H7 is prevalent in meats and there should be no doubt of the risk of infection from improperly cooked meats to which the general public may be exposed. In view of this, one may ask whether the current guidelines for cooking meat products is adequate for the destruction of this organism. The Food Safety and Inspection Service of the United States Department of Agriculture (FSIS of the

USDA) proposed control limits of temperature and time combinations in the December 27, 1988 issue of the Federal Register for preparing cooked, uncured meat patties (see Table 2) (88). These combinations of times and temperatures represent those necessary to achieve a 7-D log cycle reduction, based upon a study by Goodfellow and Brown on Salmonella (35). In this proposal, an internal temperature of 160°F (71.1°C) must be achieved before the product can be considered fully-cooked.

Log cycle reduction values are used to express the extent to which a food must be processed in order to render it safe for consumption. It is based on the D value, or the time needed to destroy 90% of all organisms. Thus, a D_{55} value of 5 minutes for Salmonella is the time it will take to destroy 90% of all Salmonella organisms in the food when processed at 55°C. The log cycle reduction value is the process necessary to reduce the population of organisms to a certain number. For example, a 12-D log cycle reduction represents a process necessary to reduce the bacterial population to 10^{-12} cells (113). Also, it can be expressed as the process that will cause a reduction of the number of organisms present by log intervals. Thus, a 1-D process will destroy 90% of the organisms, 2-D will destroy 99%, 3-D

TABLE 2

Temperature/Time Processing Combinations Proposed by Food Safety Inspection Service for Cooking Uncured Meat Patties (December 27, 1988 Proposal)[a]

Minimum Internal Temperature		Minimum Processing Time After Minimum Temperature is Reached
Deg. F	Deg. C	Time (minutes)
130	54.4	121
131	55.0	97
132	55.6	77
133	56.1	62
134	56.7	47
135	57.2	37
136	57.8	32
137	58.4	24
138	58.9	19
139	59.5	15
140	60.0	12
141	60.6	10
142	61.1	8
143	61.7	6
144	62.2	5
145	62.8	4
146	63.3	3
148	64.4	2
151	66.1	1
154	67.8	[b]
160	71.1	[c]

[a] From Reference 88.

[b] 30 seconds.

[c] Instantly.

will destroy 99.9%, 4-D will destroy 99.99%, and so on.

The FSIS proposal, based upon the study by Goodfellow and Brown, was withdrawn in the June 5, 1990 issue of the Federal Register as a result of comments received from the food industry (45). According to the FSIS, a majority of the comments opposed requiring internal processing temperatures of 160°F (71.1°C) for fully-cooked patties. They stated that the industry currently uses 145°F (62.8°C), which they believe to be adequate. Furthermore, they state that such a high temperature as 160°F would result in significant changes in the taste, texture, and yield of the product. In addition, several commenters suggested that a 7-D log cycle reduction process is too stringent and that a 4-D process is sufficient. As a result of these comments, the FSIS concluded that it would require a 5-D process for Salmonella as the standard for fully-cooked patties, instead of a 7-D process. In addition, instead of requiring that the fully cooked product reach an internal temperature of 160°F (71.1°C), the agency proposed to require that the product be cooked "until juices run clear". However, the agency increased the minimum temperature it would require for cooking the patties from 54.4°C with 121 minutes of dwell time to 66.1°C for 41

seconds (see Table 3).

Are these proposed guidelines adequate for the destruction of E. coli 0157:H7? An answer to this question was submitted by Doyle and Schoeni in their study on the survival of E. coli 0157:H7 to various temperatures in ground beef. These investigators found that this organism is not heat resistant. In fact, it is more heat sensitive than salmonellae, with a D value at 62.8°C for E. coli 0157:H7 of 24 seconds and of 36-42 seconds for Salmonella spp. (22, 35). From this study, it appears that the time/temperature combinations proposed by the FSIS in preparing pre-cooked meat patties are more than adequate. According to Table 2, a temperature of 54.4°C for 121 minutes is suggested as adequate cooking temperature and time for processing uncured ground beef patties. From the study by Doyle and Schoeni, the $D_{54.4}$ for E. coli 0157:H7 is 39.8 minutes. Therefore, 54.4°C for 121 minutes should result in about a 3-D log cycle reduction. This appears to be adequate for the destruction of this organism under normal conditions. However, this may not be sufficient for two reasons. First, a reduction in the range of 5-D to 10-D, instead of 3-D, is considered standard when prevention of economically important spoilage is considered, and even

Table 3

Temperature/Time Processing Combinations Proposed by Food Safety Inspection Service for Cooking Uncured Meat Patties (June 9, 1990 Proposal)[a]

Minimum Internal Temperature		Minimum Processing Time After Minimum Temperature is Reached	
Deg. F	Deg. C	Time(min.)	Time(sec.)
151	66.1	0.68	41
152	66.7	0.54	32
153	67.2	0.43	26
154	67.8	0.34	20
155	68.3	0.27	16
156	68.9	0.22	13
157 or >	69.4 or >	0.17	10

[a] From Reference 45.

higher log cycle reductions are recommended if the organisms of concern are pathogens (113). Second, E. coli 0157:H7 could become more heat resistant if certain circumstances were to exist. Such conditions are the subject of the next topic of review, that of the heat shock response.

II. The Heat Shock Response

A. General Characteristics

When individual cells or whole organisms are exposed to temperatures slightly above their normal physiological level, they synthesize a number of proteins known as heat-shock proteins (HSPs). Accompanying this synthesis of HSPs is the de novo ability of the cells to withstand exposure to heat treatments that would otherwise be lethal to the cells (106). This increase in the heat resistance after heat shock is known as the heat shock response (67). The response has been studied mainly in eucaryotic systems. Some of the studies with procaryotes include an investigation of Bacillus species which demonstrated that exposure of vegetative cells to a mild heat stress, or heat shock, results in an increase in the

ability of this organism to tolerate subsequent exposure to high levels of heat (112). Similar studies have been conducted with Staphylococcus aureus (53), Salmonella typhimurium (71), Bacteroides fragilis (36), and Escherichia coli, strain K-12 (120, 115).

The induction of HSPs is very rapid, and the temperature at which HSP synthesis occurs varies from organism to organism. In E. coli K-12 it occurs at 45 to 50°C when the cells are grown at 37°C, and at 42 to 45°C when the cells are grown at 30°C. It is usually observed at 10 to 15°C above the growth temperature of the cells (131).

B. Heat Shock Proteins

At least 17 proteins have been classified as HSPs in E. coli after analysis by two-dimensional gel electrophoresis of heat-shocked cells. Their molecular weight ranges from 10,000 to 94,000 (78). Table 4 contains a listing of the heat shock proteins of E. coli, along with the gene that is thought to code for the synthesis of each one.

The major HSP and the one which has attracted the most attention is the DnaK protein, coded by the E. coli

TABLE 4

The Heat Shock Proteins of Escherichia coli[a]

Number	Designation	Mol.Wt.	Name	Gene
1	B25.3	25,300	--	htpA
2	B56.5	62,883	GroEL	groEL
3	B66.0	69,121	DnaK	dnaK
4	B83.0	70,263	sigma	rpoD
5	C14.7	14,700	--	htpE
6	C15.4	10,670	GroES	groES
7	C62.5	62,500	--	htpG
8	D33.4	33,400	--	htpH
9	D48.5	48,500	--	htpI
10	D60.5	60,500	lysyl-tRNA synthetase	lysU
11	F10.1	10,100	--	htpK
12	F21.5	21,500	--	htpL
13	F84.1	84,100	--	htpM
14	G13.5	13,500	--	htpN
15	G21.0	21,000	--	htpO
16	H94.0	94,000	Lon, La	lon
17	H26.5	26,500	DnaJ	dnaJ

[a] Adapted from Reference 78.

dnaK gene. It was originally thought to be required for replication of bacteriophage lambda (114). Synthesis of this protein occurs even prior to heat shock. Its concentration increases after heat shock and then declines after about 20 minutes. The protein has been shown to have weak ATPase activity (134). The DnaK protein has recently been shown to actually modulate the heat shock response. The presence of this protein actually results in inhibition of the synthesis of other heat shock proteins (117).

In addition to the DnaK protein, the most abundant HSPs are those coded by the groEL and groES genes. They also have weak ATPase activity and are thought to be essential for the formation of many phages (48). The GroES and GroEL proteins are thought to be necessary for growth. In experiments with heat sensitive mutants, it was observed that these mutants were unable to synthesize RNA and DNA at high temperatures (124).

The rest of the HSPs are believed to be present in much smaller quantities in the cell than either the GroEL, GroES, or the DnaK proteins. This is perhaps because the latter proteins are present in the cell even before heat shocking while the former HSPs are synthesized only as part of the heat shock response. Of

these less abundant HSPs, protein B83.0 is known as the sigma factor which aids RNA polymerase in recognizing the promoter for the synthesis of the other HSPs (17). This protein is thought to control the synthesis of the other HSPs in E. coli, as evidenced by the decline in the synthesis of HSPs by changes in the rate of synthesis of protein B83.0 (39).

Protein D60.5 is thought to be a subunit of tRNA synthetase (121). This enzyme is involved, among other things, in the synthesis of various phosphorylated derivatives of adenosine (122). This fact has become of increasing importance due to recent findings that such phosphorylated nucleotides are involved in the induction of the heat shock response (65).

Protein H26.5 is the product of the dnaJ gene, and is necessary in DNA replication of phages. It is the only E. coli heat shock protein that is entirely associated with the cell envelope (101).

The last of the proteins whose function is identified is H94.0, which is the product of the lon gene cluster. It is believed that the Lon proteins have protease activity, which serves to degrade cellular proteins that are damaged by the heat and thus speed up the repair process (64).

Most of the genes coding for HSPs are arranged in clusters in the bacterial genome. The groE genes, for instance, are part of the same operon, located at 94 minutes on the circular 100-minute E. coli chromosome (116). The genes coding for DnaK and DnaJ proteins form an operon at 0.5 minutes (101). The gene for the sigma factor (rpoD) is located within an operon at 67 minutes. This operon also encodes for one of the ribosomal proteins and for DNA primase (11).

Induction of the heat shock response involves the synthesis of all 17 HSPs at the transcription level, as seen by experiments in which HSPs synthesis was delayed by the RNA synthesis inhibitor rifampin (130). Induction can be detected less than 15 seconds after a shift from 30 to 42°C in E. coli (130).

C. HSPs and Thermotolerance

There is a great deal of evidence which points to the production of HSPs as the necessary step in the induction of thermotolerance in cells. HSPs are indeed considered to be the central element in the phenomenon. Agents such as ethanol, which induce HSP production in some organisms, also induce thermotolerance (123). In addition, the kinetics of thermotolerance have been found

to strongly correlate with the kinetics of HSP production, with a maximum in the ability to tolerate heat correlating well with the accumulation of HSPs in the cell (66). Conversely, a decrease in thermotolerance has been shown to coincinde with degradation of HSPs in various organisms. When heat-shocked cells are incubated at normal growth temperatures, they lose their thermotolerance gradually. This loss in the ability to survive extreme temperatures has been shown to correlate with the loss of HSPs by the cells (63). In addition, studies involving mutants unable to tolerate heat stress reveal that these organisms are defective in their ability to synthesize HSPs (97).

In spite of all the interest in the synthesis of HSPs, the two most important questions remain unanswered: what triggers the synthesis of these proteins, and what is the function of the proteins. To answer the first question, several investigators have found that heat is not the only unducer of the synthesis of HSPs. Morgan et al. have shown that exposure of Salmonella typhimurium to various stresses, such as H_2O_2, ethanol, nalidixic acid, and anaerobiosis results in the synthesis of stress proteins, some of which overlap with those proteins produced after heat shock (77). In addition, Spector et

al. found that Salmonella typhimurium cells that are grown anaerobically are able to produce HSPs, suggesting that anaerobic growth itself may be considered a stress for the cell, and thus an inducer of HSPs synthesis (110).

In answering the second question, investigators have been able to elucidate several functions of some of the HSPs. These include: having ATPase activity (134), being involved in RNA splicing (133), and in forming large aggregates that bind to DNA and thus protect it from damage by heat (56).

From these results, more questions arise: is the function of heat shock proteins one of protection or do they play a role in the recovery and repair of the cell after the heat stress? Furthermore, are other stresses, such as oxidative stress and starvation, able to render the cell more resistant to heat? To answer these questions, one must consider the effect of heat on the injury and recovery of microorganisms, as well as the effect of oxygen on the cell. These subjects are discussed in the next sections of this review.

III. Injury

A. Introduction

Many of the physical treatments used in food processing are designed to kill or decrease the number of pathogenic and spoilage microorganisms. If the treatment is not severe enough, the surviving population of organisms may be "injured". These injured cells may escape detection because they are not able to grow in the selective medium that is often used to measure the survival of microorganisms after a heat process. There is potential for health hazard to the consumer of processed foods since injured organisms are capable of repair and of subsequent growth. For these reasons, it is important to understand what causes cell injury, and what mechanisms are involved in the repair process.

Injury is defined as the loss of a characteristic ability by a microorganism due to exposure of the organism to a sublethal environmental stress (54). It is usually detected by testing the inability of the cells to grow in a medium containing a selective agent, when compared to the ability to grow in a non-selective, enriched medium. The injured cells in a given population will retain their ability to grow in the absence of a

selective agent, such as sodium deoxycholate, salt, or some other selective agent, while not being able to proliferate in its presence.

There are many examples of environmental stresses that have the potential to damage bacterial cells when applied sublethally. The most common is the use of elevated temperatures (13, 98). Other forms of stress, which have been studied within the context of cell injury, are: freezing (89, 125), irradiation (19), and the use of antimicrobials (105).

The organism Escherichia coli has been studied in terms of its susceptibility to injury following one or more of the stresses mentioned above (99). In the case of chemical injury, E. coli expresses this damage by losing its tolerance to salt (108). Sublethal freezing causes susceptibility to surface-active agents and to endogenous lysozyme (91). When heat is used to injure the cells, the damage is mainly in single-strand breaks to the DNA (107). In short, there are many cellular changes induced by stress, and these vary depending on the microorganism in question, as well as on the type of stress that causes the injury.

B. Sites of Damage

In general terms, all forms of injury which have been studied to date involve membrane damage with loss of internal components, and increased sensitivity to chemicals. In this section, a summary of the most common targets of injury will be presented, as they have been studied in E. coli as well as other bacteria.

Freezing of cells causes the organisms to release enzymes from the periplasmic space. This causes autodigestion, as enzymes such as lysozyme attack the peptidoglycan moiety of the cell wall. This results in the organism becoming sensitive to the action of bile salts and chelators like EDTA (73).

Injury to the cell membrane has been detected in E. coli as a result of freezing and thawing (14). Such damage is easily seen by measuring the loss of 260- and 280-nm absorbing material from the cell after injury. Release of lipopolysaccharide material has also been observed after heating E. coli, and is attributed to membrane damage (50). This damage to membrane lipids has also been reported in Salmonella typhimurium (118).

Damage to ribosomes and other nucleic acid material is another manifestation of injury to bacterial cells. Damage to the 30S ribosomal particle of E. coli as a

result of heat stress has been reported by Weiss and Tal (126). In 1972, Sedwick and Bridges reported the existence of single-strand breaks in the DNA of E. coli as a consequence of heating (107). In addition, Alur and Gomez also showed damage to DNA in the form of single-strand breaks, but this time as a result of injury by freezing and thawing the cells (1).

Finally, one of the most common types of damage to the cell is that which affects the molecules, such as enzymes, which are used in the cell's metabolic processes. The effect of heating on enzymes has always been attributed to their denaturation which results in a loss of activity (129). This phenomenon has been studied mainly in Staphylococcus aureus, where a decrease in the activity of certain enzymes of glucose metabolism has been noted (10).

C. Repair

From the above information, it is evident that cell injury results in various cellular modifications, most of which have been identified only in general terms. Regardless of the damage, it is important to realize that, with injury, comes an attempt by the cell to recover from the injury through a repair process.

Repair, also termed resuscitation, of the cells involves resumption of certain cellular activities or functions essential to normal growth and environmental resistance. The cellular modifications, which the organism undergoes when it is injured, are reversed and any losses are restored to the normal state. All this occurs during a period of time begining right after injury, and just preceding cell division. The period of repair is often seen as an extended lag phase of growth that is longer than that of normal cells (85).

Ribosomes that are degraded during injury have been found to be regenerated after the cells are allowed to recover (54). In a study on the recovery of Staphylococcus aureus after heating, it was found that the cells actually used the damaged 50S subunit as a source of protein for new ribosome synthesis and assembly (98).

During recovery after membrane damage, many investigators have noted the synthesis of new phospholipids. Salmonella typhimurium was shown to incorporate ^{14}C from glucose-U-^{14}C into lipid during recovery after a heat treatment (86). Protein synthesis has been found to be essential in the repair of frozen cells as well as heat-injured ones (90, 109). However,

protein synthesis may not be necessary for recovery of all cells. In their work with Yersinia enterocolitica, Restaino et al. found that chloramphenicol, a protein synthesis inhibitor, had no effect in reducing the rate of recovery of heat-injured cells. However, in the same study, these investigators found that RNA synthesis was required for repair, and that DNA and cell wall synthesis were not necessary (94). These results are supported by earlier work by Iandolo and Ordal which showed that almost all of the synthesis taking place during repair of S. aureus after injury was devoted to RNA synthesis (54).

In spite of the difference of opinion regarding which cell components are necessary for repair, it appears that all cells studied so far depend on ATP for the repair process to begin (89, 91).

IV. Oxygen Metabolism and Toxicity

A. Respiration and Fermentation in E. coli

Escherichia coli 0157:H7 is a facultatively anaerobic organism, able to grow in both the presence or absence of oxygen. This means that it is capable of coupling catabolic reactions with anabolic reactions by

either respiration or fermentation. When oxygen is present, the organism uses it as a terminal electron acceptor in what is commonly known as aerobic respiration. If other electron acceptors are present, such as nitrate, then the organism is said to undergo anaerobic respiration. In either of these respiratory mechanisms, ATP is produced by oxidative phosphorylation coupled to an electron transport system (41). In the absence of terminal electron acceptors, fermentation takes place and energy obtained from substrate-level phosphorylations.

There are other differences that can be found in cells grown aerobically versus those grown under anaerobic conditions. Cells grown in the presence of oxygen contain mainly type b cytochromes. The main b-type cytochrome is cytochrome o, which absorbs light at a wavelength around 560 nm. As the avilability of oxygen decreases during shift to an anaerobic environment, cytochromes d and a_1 appear and are used in anaerobic respiration when a terminal electron acceptor such as fumarate or nitrate is present (93).

Along with changes in the cytochrome content of the cell, are changes in the enzymes which are used for metabolism. The enzyme nitrate reductase, for instance,

is induced by the presence of nitrate and the abscence of oxygen in the medium. E. coli utilizes this compound as terminal electron acceptor in the respiratory chain by a dissimilatory nitrate reduction reaction (41). In E. coli, nitrate is reduced to nitrite and water, and yields as many ATPs as oxygen when the latter is present and used as electron acceptor (55). As mentioned before, when there are no terminal electron acceptors present under anaerobic conditions, the organism derives its energy from fermentation of whatever carbon source is available, usually glucose.

During aerobic growth, the cell utilizes the carbon source through the enzymes of the hexose monophosphate pathway of metabolism. This is evidenced by the presence of high levels of activity of the enzyme 6-phosphogluconate dehydrogenase, which is responsible for the formation of ribulose-5-phosphate in this pathway. Coupled to the induction of this enzyme, aerobic growth results in the inhibition of the synthesis of the enzyme phosphofructokinase, which is an integral component of the Embden Meyerhoff Parnas (EMP) pathway used in the fermentation of sugars by microorganisms (92). Most of the energy in the form of ATP is then derived during respiratory catabolism as a result of the tricarboxylic

acid (TCA) cycle reactions yielding reducing equivalents as well as ATP.

During the shift from aerobic growth to anaerobiosis, many changes take place. The pathways of carbon source utilization are changed. The consumption of glucose is achieved primarily by the EMP pathway, rather than by the hexose monophosphate pathway. Pyruvate is metabolized in a different way. The pyruvate dehydrogenase complex, which serves as the gateway to the TCA cycle during respiratory catabolism, is repressed and inhibited (42). Pyruvate is metabolized instead by pyruvate formate-lyase, which results in the formation of acetyl CoA without reduction of NAD (61). The respiratory chain is inactivated during fermentation, with ATP synthesis being derived from substrate-level phosphorylations. All of the alterations in carbon metabolism during the switch from aerobic to anaerobic growth, and viceversa, are accomplished by modulating enzyme activity as well as by induction/repression controls (102).

B. Oxygen Toxicity

One aspect about aerobic vs. anaerobic metabolism that needs to be considered is the way that cells grown

under either condition are able to deal with oxygen toxicity. Some of the oxygen that is consumed by the cells is converted spontaneously, or by enzymatic action, to several active oxygen species such as superoxide anion ($O_2^{\cdot -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$).

When oxygen accepts a single electron, it is converted to the superoxide anion, or to its protonated form, the perhydroxyl radical ($^{\cdot}HO_2$) (25). The superoxide anion can react as a reductant or an oxidant, resulting in the formation of oxygen in the former, and hydrogen peroxide in the latter case. Superoxide anion is thought to exert its toxic effects on cellular components by its involvement in the formation of hydrogen peroxide, and not on its own. Some investigators have proposed that the superoxide radical is actually inocuous because, in some cases, the use of superoxide dismutase has failed to alter toxicity (104).

Superoxide is converted to hydrogen peroxide in a dismutation reaction that occurs either spontaneously or as a result of the enzyme superoxide dismutase (SOD). Fridovich and others have calculated the rate of enzymic dismutation of superoxide radical and found it to be 10^6-times greater than that of spontaneous dismutation (31).

There are three distinct types of superoxide dismutases which can be distinguished by their metal content. Most procaryotes produce a superoxide dismutase with either iron or manganese as the prosthetic group, while eucaryotes have a copper-zinc cytoplasmic enzyme and a manganese-containing mitochondrial enzyme (5). Gregory et al. discovered that E. coli possesses an iron-containing SOD located in the periplasmic space and a manganese-containing enzyme located in the cytoplasm. The former constitutes an essential defense against exogenous superoxide radicals while the latter SOD constitutes a defense against endogenous superoxide radicals (37). Hydrogen peroxide is produced by the action of SOD on superoxide radicals. It is broken down to H_2O and O_2 by the enzyme catalase. This compound can also be broken down in the presence of a reducing agent, such as cytochrome c, and the enzyme peroxidase to form water (103).

It is easy to see why the enzymes SOD and catalase are so important for the survival of the organism. Without them, H_2O_2 would accumulate during aerobic respiration. In microorganisms that lack these enzymes, growth is arrested and death ensues (23). Damage done by oxygen radicals in the absence of catalase and SOD is by

the peroxidation of lipids, by the lysis of some cell types, and by the destruction of certain enzymes (58, 59).

For a long time it was believed that strictly anaerobic bacteria, as well as facultative anaerobes grown under anaerobic conditions, were devoid of SOD and catalase. This view changed when SOD activity was discovered in two clostridial species by Hewitt and Morris, and in other anaerobes by Fridovich (49, 32). E. coli has been found to have catalase specific activities of 46 U/mg of protein when grown aerobically and of 11.0 U/mg protein when grown anaerobically. The activity of SOD was found to be 33.4 and 15.3 U/mg protein for aerobically- and anaerobically-grown cells, respectively (96).

Both catalase and superoxide dismutase are thermolabile enzymes. Amin and Olson found that staphylococcal catalase activity decreased 10- to 20-fold faster at 54.4°C than at 37.8°C (2). Dallmier and Martin found that SOD specific activity of Listeria monocytogenes decreased quickly when heated to 55°C (18).

It is evident that heat has a detrimental effect on both of these enzymes. It is, therefore, relevant to ask whether cells which have been exposed to heat are able to

survive aerobic environments in view of the fact that their main weapons against the effects of toxic oxygen radicals are inactivated after heating. If cells are indeed unable to grow under these conditions, can the detrimental effect of oxygen on these cells be eliminated by the addition of certain compounds to the medium in order to enhance their recovery after heating? Both of these questions have been examined by various laboratories within the context of injury and resuscitation.

C. Oxygen Radical Scavengers and Recovery

Dallmier and Martin studied the susceptibility of L. monocytogenes to hydrogen peroxide after heat treatment. They found that incubation of the cells at temperatures above 55°C resulted in an increase in the susceptibility of the organism to 100mM H_2O_2 (18). Similar results were reported by Yoshpe-purer and Henis on the susceptibility of E. coli to 0.034% H_2O_2 after heating (132).

Other investigators have opted to study the phenomenon of inactivation of catalase and SOD after heating by supplementing the medium, in which the heated cells are recovered, with oxygen radical scavengers. In this way, they could determine whether the inability of

the cells to grow under oxygen-rich conditions is really due to their lack of protection against toxic oxygen radicals. Flowers et al. tested the effect of adding catalase to the recovery medium of Staphylococcus aureus cells previously heat-stressed. They found an increase in the colony counts of up to 1000-fold when catalase was added to various recovery media over catalase-free preparations (27). Similar results were obtained by Mackey and Derrick with Salmonella typhimurium, where not only catalase, but pyruvate was also used to enhance the recovery of heat-injured cells (72). The effect of adding sodium pyruvate to the medium is ascribed to its ability to degrade H_2O_2, rather than through supplementation of a required nutrient (4).

More recently, McDonald and co-workers tested various compounds on their ability to enhance recovery of E. coli after freezing or heating. Among the compounds tested were sodium pyruvate, catalase, thioglycolate, dimethyl sulfoxide, sodium metabisulfite, ethoxyquin, n-propyl gallate, alpha-tocopherol, ascorbic acid, and 3,3'-thiodipropionic acid. Thioglycolate was used for its ability to block formation of H_2O_2, whereas dimethyl sulfoxide and sodium metabisulfite were used for their ability to scavenge superoxide radicals (15, 75, 51).

The rest of the compounds are considered to be antioxidants (68). Of all the compounds added to the medium, sodium pyruvate and 3,3'-thiodipropionic acid resulted in the largest increase in the recovery of the cells after exposure to 57°C for 12 minutes (76).

If the recovery of heat-stressed cells is impaired by their inability to prevent damage by oxygen radicals, it follows that anaerobic recovery of the cells may result in a high recovery of the cells due to the absence of such radicals under these conditions. Gomez and Sinskey have shown that S. typhimurium cells, which had been heated to 50°C for 15 minutes, recovered their ability to grow in the presence of nitrogen better than in the presence of air (34). In addition, Knabel et al. found that enumeration of heat-stressed L. monocytogenes under strict anaerobic conditions resulted in significantly higher number of survivors than when recovered aerobically (60).

V. Summary and Experimental Rationale

There have been several reported outbreaks of hemorrhagic colitis in humans in recent years. Even though much research has been devoted to identifiying the

causative agent of this disease, as well as to its mechanism of action, not much has been done on its ability to survive processing temperatures. Most investigators believe that the outbreaks have been caused by consumption of inappropriately cooked ground beef, as opposed to survival of the organism to adequate heating conditions. The results of studies presented in this review indicate that E. coli 0157:H7 is not particularly heat resistant, and that processing protocols used today should be effective in destroying it.

The ability of microorganisms to become more heat resistant to a processing temperature following a mild heat stress has been verified in many bacteria, including some food-borne pathogens such as Listeria and Salmonella. It is possible that E. coli 0157:H7 may also be able to survive normal processing conditions if its ability to recover from a heat treatment is improved by heat shock. For this reason, the effect of heat shock on the ability of E. coli 0157H7 cells to survive a subsequent heat treatment was investigated.

Other forms of stress, such as starvation, gamma-irradiation, and treatment with ethanol have resulted in increasing the ability of organisms to withstand a subsequent heat treatment. Some investigators have even

found that lack of oxygen, or anaerobiosis, can also result in an increased ability of microorganisms to survive other stresses. The effect of anaerobic growth on the heat resistance of E. coli 0157:H7 was studied in order to determine whether it results in an increased ability for the organism to survive a subsequent heat treatment.

It is recognized that not all the organisms subjected to a heat process are all destroyed but that a large number actually survive the process and are merely injured. Such injured microorganisms present a threat to the safety of the food product since many have been shown to be capable of repairing themselves after the heat treatment. The repair process often results in the organisms being able to proliferate in the food. Recovery conditions can play a role in the number of injured organisms that are able to recover from the heat treatment. Studies on aerobic vs. anaerobic recovery of thermally-processed cells have led many investigators to believe that anaerobic recovery can result in higher numbers of injured microorganisms being recovered than aerobic recovery. This is thought to be due to a lack of detoxifying enzymes in injured cells, which would make it harder for the cells to repair themselves in the presence

of oxygen and, consequently, in the presence of oxygen-derived toxic radicals. In this study, we sought to determine the degree to which E. coli 0157:H7 heat-shocked and control cells are injured following a heat process, as well as the ability to repair themselves in aerobic vs. anaerobic conditions. In addition, the ability of heat-shocked and control cells to grow in the presence of toxic oxygen radicals before and after heat treatment was investigated.

MATERIALS AND METHODS

I. Bacterial Strain

Escherichia coli serotype 0157:H7 was obtained from American Type Culture Collection in Rockville, Maryland as catalogue number ATCC 35150. This strain was originally isolated by Riley et al. from feces of a patient suffering from hemorrhagic colitis during an outbreak in Michigan in 1982 (95).

II. Bacteriological Media

Trypticase Soy Broth (TSB) and Trypticase Soy Agar (TSA), both in powder form, were obtained from BBL Becton Dickinson in Cockeysville, Maryland. Aerobic TSB tubes and TSA plates were prepared by dissolving the powder in distilled H_2O, and autoclaving at 121°C for 20 minutes as described by the manufacturer. For culturing the organism anaerobically, media were prepared according to the methods of Holdeman et al. described in the Anaerobe Laboratory Manual published by Virginia Polytechnic Institute and State University (52).

To prepare pre-reduced anaerobic TSB tubes, the powdered medium was weighed and placed in an Erlenmeyer flask for boiling, along with the appropriate amount of

distilled water. Resazurin was then added to a concentration of 0.008% (Allied Chemical, Morristown, N.J.). This is an Eh indicator which turns pink under aerobic conditions and colorless under anaerobic conditions. The medium was then boiled evenly and thoroughly in order to drive off oxygen and to partially reduce the ingredients. To prevent the medium from boiling over, the flask was fitted with a removable glass chimney. Boiling was performed until resazurin turned from pink to colorless. At this time, the contents of the flask were immediately flushed with anaerobic-grade oxygen-free CO_2 gas and cooled in an ice bath to room temperature. Cysteine hydrochloride was then added as a reducing agent to further reduce the medium (Sigma Chemical, St. Louis, MO). Using 8N NaOH (or 5N HCl), the pH was adjusted to 7.0 while CO_2 was continuously being bubbled into the medium. After the desired pH was reached, the carbon dioxide was replaced by oxygen-free nitrogen gas. The TSB was then dispensed into tubes that were being flushed with nitrogen gas. These were then stoppered and autoclaved at 121°C for 20 minutes.

For making TSA roll tubes, TSB was prepared as described above and dispensed in 10-ml aliquots into glass roll tubes (Bellco Glass, Inc., Vineland, N.J.)

containing enough granulated agar (BBL Becton Dickinson, Cockeysville, MD) to achieve a 2.5% agar concentration per tube. The tubes were then stoppered and autoclaved as described above.

In studies on the recovery of injured E. coli cells, Violet Red Bile (VRB) Agar has been used as the selective medium (44). In this study, a modified Violet Red Bile Agar (MVRB) was used. The medium consisted of 0.75% yeast extract (BBL Becton Dickinson, Cockeysville, MD), 0.7% peptone (Difco Laboratories, Detroit, MI), 0.226% bile salts No. 3 (Difco), 1.5% lactose (Difco), 0.5% NaCl (Mallinckrodt, Inc., Paris, KY), and 1.5% granulated agar in distilled water. This formula is exactly the same as that for VRB agar, except that there is no crystal violet or neutral red in it. These ingredients were excluded because their presence would otherwise mask the color of resazurin, which is pink under aerobic conditions. Since these dyes impart a deep purple color to the medium, it would be impossible to be certain whether cultures grown anaerobically have remained anaerobic throughout the period of incubation. All MVRB ingredients were dissolved by boiling in distilled water, and autoclaved as described above. To prepare anaerobic MVRB agar, the procedure described above for the preparation of

anaerobic TSA roll tubes was followed.

Dilution blanks consisting of 0.1% peptone were used throughout the studies. Anaerobic blanks also contained resazurin and cysteine hydrochloride, and were prepared and dispensed as per the method of Holdeman et al. as described above for TSB tubes (52).

III. Maintenance of Anaerobiosis

All culture inoculations, as well as transfers from anaerobic media to dilution blanks, and all inoculations into anaerobic roll tube media were performed under strict anaerobic conditions using the V.P.I. Anaerobic Culture System (Bellco Glass, Inc., Vineland, N.J.) as described by Holdeman et al. (52).

All heat-shocking and heat-treatment procedures requiring the maintenance of anaerobiosis were performed using sterile 10-ml volume borosilicate glass screw thread vials (Kimble) fitted with open tops and Teflon-faced silicone septa (Wheaton, Mellville, NY).

IV. Heat Shocking Procedure

E. coli 0157:H7 cells were grown either aerobically in TSB or anaerobically in pre-reduced TSB at 30°C for 6 hours, or until mid logarithmic growth. One-ml of either

culture was injected into vials containing 5.0 ml of aerobic or anaerobic TSB, pre-warmed at 42°C in a Haake Model FS2 circulating water bath, equipped with a thermoregulator (Haake Instruments, Inc., Rochelle Park, N.J.). Samples were injected using a sterile 3.0 ml syringe fitted with a 25 gauge needle (Becton-Dickinson, Rutherford, N.J.). The come-up time, or time it takes for the temperature to reach 42°C after addition of the 1.0 ml of cells, was measured using a thermocouple connected to a datalogger (Campbell 21X Micrologger, Campbell Scientific, Inc.). The come-up time was determined in triplicate and the mean was 45 seconds.

The cells were heat-shocked for 5 minutes by immersing the vials in the 42°C circulating water bath. Immediately after heat shock, cultures were subjected to a 55°C heat treatment as described below.

V. Heat Treatment Procedure

Cells that were previously heat-shocked, or nonheat-shocked controls were injected in 1.0-ml aliquots into vials containing 5.0 ml of either aerobic or anaerobic TSB, pre-warmed at 55°C in a Lauda Model WB-20/R circulating water bath (Brinkman Instruments, Inc., Westbury, N.Y.). The come-up time after addition of the

1.0 ml of cells was determined to be 30 seconds, using a thermocouple as described above. The inoculated vials were immersed in the 55°C water bath and kept at this temperature for 60 minutes. Samples were removed at 20-minute intervals from each vial using a sterile syringe, and immediately cooled in ice.

Aerobic and anaerobic heat-shocked and control samples to be cultured aerobically were serially diluted in aerobic peptone dilution blanks, and spread plated onto aerobic media (either TSA or MVRB, or both). The plates were incubated at 37°C for 24 hours and colonies were counted using a Quebec colony counter model 3325 (American Optical Corp., Buffalo, N.Y.).

Aerobic and anaerobic heat-shocked or control samples to be cultured anaerobically were serially diluted in anaerobic pre-reduced peptone dilution blanks using the V.P.I. Anaerobic Culture System as described previously. Diluted samples were inoculated into molten anaerobic roll tubes containing either TSA or MVRB, tempered at 47°C. The inoculated roll tubes were immediately spun in a Bellco Roll Tube Spinner (Bellco Glass, Inc., Vineland, N.J.) for 5 minutes at room temperature, or until the agar had solidified. The tubes were then incubated at 37°C for 24 hours and the colonies

were examined and counted using an AO dissecting microscope model 568 (American Optical Corp., Buffalo, N.Y.).

VI. Assay for Measuring Catalase Levels

Cultures were grown either aerobically or anaerobically in TSB at 30°C for 6 hours. Heat-shocked and control cultures were either heat treated at 55°C for 20 minutes or left unheated, and were then tested for catalase activity by the method of Beers and Sizer (6). This method is based on the degradation of hydrogen peroxide by cellular catalase, which is measured as a decrease in absorbance at 240 nm in the presence of the enzyme with time. Hydrogen peroxide absorbs strongly at 240 nm (molar extinction coefficient = 43,600). Therefore, if a suspension contains catalase, there will be a decrease in absorbance at 240 nm due to the breakdown of hydrogen peroxide by the following equation:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Cultures were centrifuged in an IEM Centra-M microcentrifuge (International Equipment Co., Needham, MA.) for 2 minutes, and washed 3X with 50 mM KPO_4 buffer (pH 7.0). Cells were treated with toluene in order to

make the cells "leaky" and thereby make the treated cells fully permeable to hydrogen peroxide. Toluene treatment involved the addition of 2.0 ml of toluene to 4.0 ml of cells. The suspensions were gently shaken for 1 minute. The upper toluene layer was removed and the treated cell suspensions were assayed immediately. The assay was done at room temperature using a Spectronic 1001 split-beam spectrophotometer (Milton Roy Co., Rochester, N.Y.). The reaction mixture consisted of 0.5 ml of cell extract + 1.5 ml of 50 mM KPO_4 buffer in a quartz cuvette fitted with a stir bar. The contents were thoroughly mixed by a magnetic stirrer, and this mixture was used as the blank to zero the instrument. The assay was started by adding 1.0 ml of a 55 mM hydrogen peroxide solution, which was prepared by mixing 0.25 ml of 30% H_2O_2 (Sigma Chemical Co., St. Louis, MO) with 39.75 ml of the potassium phosphate buffer. Thorough mixing of the cuvette contents was carried out for 10 seconds, as measured by a timer/stopwatch (Fisher Scientific, Springfield, N.J.). Immediately after mixing, the absorbance at 240 nm was recorded with time, using the stopwatch. Each assay was performed in triplicate. The specific activity reported was based upon an average of three assays, and was expressed as units of activity per

mg of protein. Protein concentration was measured by the biuret method, as modified by Ohnishi and Barr (82), using the Sigma microprotein determination kit (Sigma Chemical Co., St. Louis, MO). Supernatant fluid taken from toluene-treated cells was boiled for 15 minutes and used as a negative control.

VII. Assay for Measuring Superoxide Dismutase Levels

The procedure used to measure SOD activity was the xanthine oxidase-cytochrome c assay of McCord and Fridovich (74). In this assay, superoxide radicals are generated by the action of xanthine oxidase, a flavoprotein. Xanthine oxidase oxidizes xanthine and the reduced flavoprotein subsequently reduces O_2 to O_2^-. The superoxide radicals (O_2^-) in turn reduce cytochrome c to the ferrous form which has a red color. This superoxide-dependent reduction of cytochrome c results in an increase in absorbance at 550 nm which can be monitored spectrophotometrically. Superoxide dismutase inhibits the reduction of cytochrome c, since it scavenges superoxide radicals before they can reduce cytochrome c. One unit of SOD activity is defined as that which will inhibit the standard assay by 50%.

In order to calibrate the standard assay, sufficient

xanthine oxidase was added to the reaction mixture to obtain a rate of cytochrome c reduction of 0.025 Absorbance units/minute. The standard assay consisted of 0.15 ml of 0.001 M xanthine, 0.30 ml of 0.1 mM cytochrome c, 0.005 ml of 0.03 M NaCN, 1.7 ml SOD buffer, and 0.05 ml xanthine oxidase (Sigma Chemical Co., St. Louis, MO).

Cells were centrifuged for 2 minutes in a microcentrifuge as described for the catalase assay, and were washed 3X in SOD buffer. This buffer consisted of 50 mM KPO_4 buffer (pH 7.8) containing 1.0 mM EDTA. After washing, the cells were resuspended in 1.0 ml SOD buffer and 0.5 ml of toluene was added. The suspension was mixed thoroughly for 1 minute and the toluene layer was carefully removed. The treated cell suspension was added to the standard assay. The reactions were started by adding xanthine oxidase to the cuvette after all other ingredients had been added. Protein concentration was determined as described in the catalase assay. The percent inhibition of the standard assay by SOD was obtained. Units of activity of SOD were derived from a standard curve of SOD activity vs. percent inhibition of xanthine oxidase, and were expressed as units per mg of protein.

VIII. Susceptibility to H_2O_2

Cells were grown aerobically or anaerobically in TSB at 30°C for 6 hours. Heat shocking was performed, and heat-shocked and control cells were either heated at 55°C for 20 minutes or left unheated. Cells were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 0.03% reagent grade H_2O_2, for 0, 15, or 30 minutes at 37°C. Appropriate dilutions in 0.1% peptone were made and plated aerobically in TSA plates. The plates were incubated at 37°C for 24 hours and colonies counted. The concentration of hydrogen peroxide used was the same as that used by Yoshpe and Henis in their experiments on the susceptibility of E. coli to hydrogen peroxide (132). They found that hydrogen peroxide concentrations between 0.025 and 0.05% were desirable since, at higher levels, catalase is inhibited in E. coli cells.

IX. Measurement of Release of Cellular Material

This assay is based on the method of Russell and Harries (99). It is a measure of the degree of injury to the cell membrane due to heat. Following sublethal heat stress, the injured cells become osmotically sensitive and begin to leak soluble, low-molecular weight precursors into the extracellular environment. These

compounds absorb at a wavelength of 260 nm and have been identified as RNA and RNA-derived nucleotides (12).

Cells were grown aerobically or anaerobically in TSB and heat-shocked as described previously. Non heat-shocked controls were also prepared. Cells were then heat treated at 55°C for 0, 20, 40, 60, or 80 minutes, chilled immediately, and the absorbance at 260 nm was recorded using the spectrophotometer described previously. Distilled water was used as the blank.

X. SDS PAGE of Cell-free Extracts

Cells were grown aerobically or anaerobically in 100 ml of aerobic or pre-reduced TSB flasks for 6 hours at 30°C in a controlled environment incubator shaker (New Brunswick Scientific Co., Inc., Edison, N.J.). Ten mls. of the log-phase cells were inoculated into flasks containing either aerobic or anaerobic TSB pre-warmed at 42°C for heat-shocking. Cells were centrifuged at 9,100 x g for 20 minutes at 4°C. Cells were washed 3X by centrifugation using 50 mM potassium phosphate buffer (pH 7.0) and resuspended in 20 mls of the buffer. The cell suspensions were lysed using a French pressure cell apparatus set at 1,600 psi (American Instrument Co., Silverspring, MD). After lysis, the suspension was

centrifuged at 9,100 x g for 20 minutes to remove whole cells. Protein concentration was determined by the modified biuret method as described previously. The concentration was adjusted to 250 ug/ml in all samples. The supernatant fraction was then stored at -20°C until used.

Cell-free extract proteins were separated by SDS Poyacrylamide Gel Electrophoresis (PAGE) based on the method of Laemmli (62). The cell extracts were suspended in equal parts of sample buffer. This buffer contained 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-beta-mercaptoethanol, and 0.001% bromphenol blue in 0.0625 M Tris-HCl buffer (pH 6.8). All chemicals were obtained from Sigma Chemical Co., St. Louis, MO. The cell extracts in sample buffer were boiled for 2 minutes and cooled in ice. Samples were then loaded onto a 2" x 2" 7.5% polyacrylamide homogenous Phast Gel, and run with the Pharmacia Phast System (Pharmacia LKB Biotechnology, Uppsala, Sweden). This system consists of a double-gel electrophoresis unit equipped with a built-in power source and staining chamber. The gel was run at 250 V and at 10°C. Staining was done using the Sigma silver stain kit (Sigma Chemical Co., St. Louis, MO), adapted for use with the Phast System^{r}.

SDS PAGE molecular weight standards ranging from 97,400 to 14,400 MW (Bio-Rad Laboratories, Richmond, CA) were run in the same gel as the samples. A calibration curve was generated by plotting the molecular weight of the standards vs. the distance migrated from the interface of the stacking and separating gels in centimeters (Figure 1). From this curve, the molecular weights of the sample protein bands were obtained.

XI. Western Dot Blot of Cell Extracts

Any protein bands present in heat-shocked samples which are not present in controls are considered to be heat shock proteins by definition. However, in order to have supportive evidence of whether the proteins present in each of the cell extracts tested in this study were indeed heat shock proteins, monoclonal antibodies specific for one of the major heat shock proteins were reacted with cell extracts using the Western Dot Blot procedure. This procedure is based on the affinity of antibodies for proteins that may be present in the samples. The proteins are loaded onto a membrane, such as nitrocellulose, and incubated with antibodies specific for the protein in question. In our study, we used monoclonal antibodies developed in mice. The membrane is

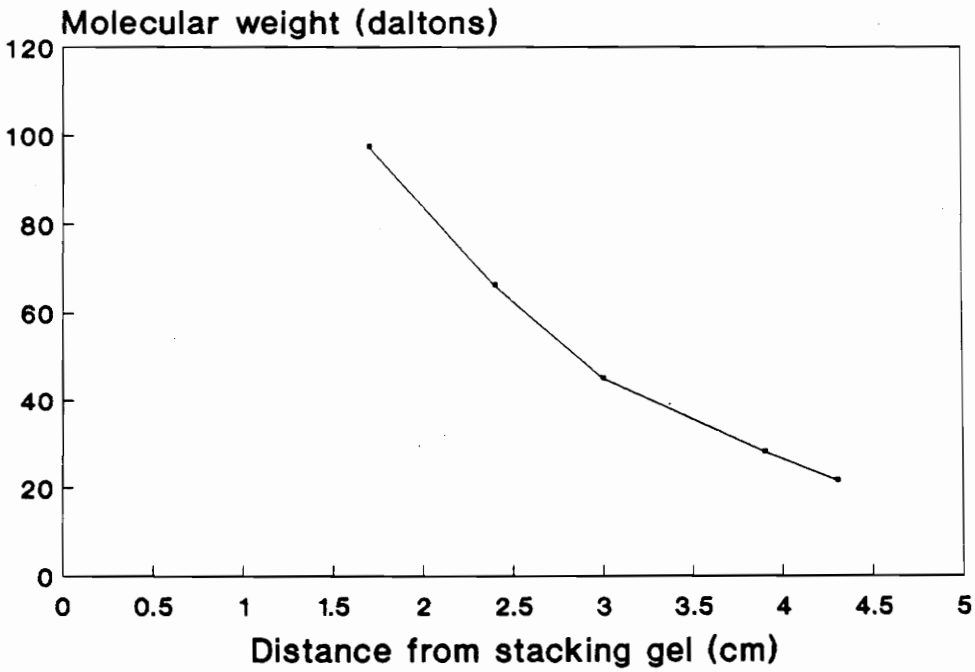


Figure 1: Standard Curve of Molecular Weight Markers.

then exposed to anti-mouse antibodies developed in rabbits to which an enzyme has ben chemically linked. After adequate incubation, the nitrocellulose membrane is soaked in a solution containing the substrate for the enzyme. The ensuing reaction results in the development of a visible color everywhere on the membrane where the enzyme-linked antibodies are bound. Since the enzyme-linked antibodies are specific for the mouse monoclonal antibodies, which in turn are specific for the heat shock protein being sought, any bands where the color is detected represent bands of the heat shock protein in question. Cell-free extracts in SDS ample buffer were applied onto nitrocellulose membrane paper, 0.45 um (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) in 5 ul aliquots per spot and allowed to dry. The nitrocellulose was soaked in washing buffer containing 3.0% gelatin for 15 minutes with gentle agitation. The washing buffer consisted of 20 mM Tris and 0.5 M NaCl (ph 7.2). This was followed by soaking twice in washing buffer for 5 minutes each time. The nitrocellulose was then soaked for 60 minutes in a 1:1000 dilution of mouse monoclonal antibodies in washing buffer. The antibodies were specific for the 70,000 M.W. E. coli heat shock protein considered to be the sigma^{32} subunit of RNA

polymerase. These antibodies were a generous gift of Scott A. Lesley and Nancy E. Thompson from the McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin.

After the incubation in monoclonal antibodies, the paper was washed twice in washing buffer and then soaked for 60 minutes in a 1:2000 dilution of goat anti-mouse antiserum conjugated with the enzyme horseradish peroxidase (Kirkegaard & Perry, Gaithersburg, MD). The paper was washed twice in washing buffer and then soaked in substrate solution for 15 minutes, containing the substrate for the enzyme horseradish peroxidase used to bind to the monoclonal antibodies. The substrate solution consisted of 16.7 ml of washing buffer containing 10 ul of 30% H_2O_2 to which 10 mg of 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) in 3.3 ml of ice-cold methanol were added immediately before soaking the nitrocellulose in it. The reaction was stopped by soaking the paper in washing buffer after 15 minutes, or until the development of purple color was observed.

XII. Western Blot of Electrophoretically Transferred Cell Proteins Separated by SDS PAGE

Cell extracts in SDS sample buffer were loaded onto

a 7.5% SDS PAGE gel and run to separate the proteins as described previously, using the Pharmacia Phast System. In order to determine if any of the protein bands obtained after the separation corresponded to the 70,000 M.W. sigma^{32} subunit of RNA polymerase. The following was performed: the proteins were separated by SDS PAGE and then were transferred onto nitrocellulose membrane by electroblotting. A Western Blot using monoclonal antibodies specific for this protein as per the method of Towbin et al. was then performed (119).

This method is based on the detection of a particular protein among the protein bands present in a sample by antibodies specific for that particular protein. The difference between electrophoretic transfer and dot blot is that the proteins in the sample have been separated in order to determine which protein band binds to the antibodies used. However, when proteins are separated by electrophoresis, the proteins are trapped in the matrix of the gel which makes it physically impossible for the antibodies specific for any of the proteins to reach and bind to them. For this reason, the proteins are transferred from the gel onto nitrocellulose. On this surface, the proteins are easily accessible to the antibodies.

Briefly, the procedure used was as follows: a piece of nitrocellulose membrane paper (0.45 um) was cut to the same size of the gel and placed immediately on the unstained gel after protein separation was completed. The gel and nitrocellulose membrane were placed on filter papers soaked in transfer buffer and the proteins were electrophoretically transferred onto the nitrocellulose membrane by using the Phast Transfer Semi-dry Electrophoretic System (Pharmacia LKB Biotechnology, Uppsala, Sweden), which is an attachment to the Pharmacia Phast System used in the separation procedure described above.

The protein transfer was performed at 20V and at 15°C and run for 15 minutes. At that time, the membrane was carefully separated from the gel. The membrane was then treated as described in the Western Dot Blot procedure described previously using monoclonal antibodies developed in mice, specific for the 70,000 M.W. heat shock protein, and the commercially available enzyme-linked goat anti-mouse antibodies.

RESULTS

I. Optimization of Heat Shock Parameters

Aerobically and anaerobically grown E. coli 0157:H7 cells were heat-shocked at various time/temperature combinations in order to determine the following:

1) whether the heat shock response could be induced in this organism
2) the optimal conditions of heat shock time and temperature that would result in the highest difference in survival to a subsequent heat treatment between heat-shocked and nonheat-shocked cells

Cells grown aerobically and heat-shocked at 42°C for 5 minutes resulted in a higher mean log number of survivors to a 55°C heat treatment than cells heat- shocked at any other time and temperature combinations (Table 5, Figure 2). In fact, heat shocking aerobically grown cells at 42°C resulted in the highest mean log number of survivors at 5 to 15 minutes, when compared to other temperatures tested (Figure 2).

Cells grown anaerobically also resulted in the highest mean log number of survivors to a subsequent 55°C

TABLE 5

Effect of Time and Temperature Combinations of Heat Shock on Survival of Aerobic and Anaerobic E. coli 0157:H7 Cells To a Subsequent Heat Treatment[1]

Heat Shock Temp (°C)	Heat Shock Time (min.)	Mean Log # Survivors(A)[2]	Mean Log # Survivors(AN)[3]
32	0	5.0^{b}	5.1^{b}
	5	6.2^{e}	5.0^{b}
	10	5.5^{c}	5.1^{b}
	15	5.0^{b}	5.1^{b}
34	0	5.0^{b}	5.0^{b}
	5	6.5^{f}	5.3^{c}
	10	5.9^{de}	5.4^{c}
	15	4.3^{a}	5.2^{bc}
42	0	5.0^{b}	5.0^{b}
	5	$\mathbf{7.5^{h}}$	5.8^{d}
	10	6.8^{g}	5.4^{c}
	15	5.4^{c}	5.0^{b}
45	0	5.0^{b}	4.9^{b}
	5	6.6^{fg}	5.3^{c}
	10	5.9^{d}	5.0^{b}
	15	4.2^{a}	4.1^{a}

Means with different letters within a column are significantly different ($p \leq 0.05$).

[1] Heat treatment was at 55°C for 20 minutes.

[2] Aerobically-grown cells in Trypticase Soy Agar.

[3] Anaerobically-grown cells in Trypticase Soy Agar.

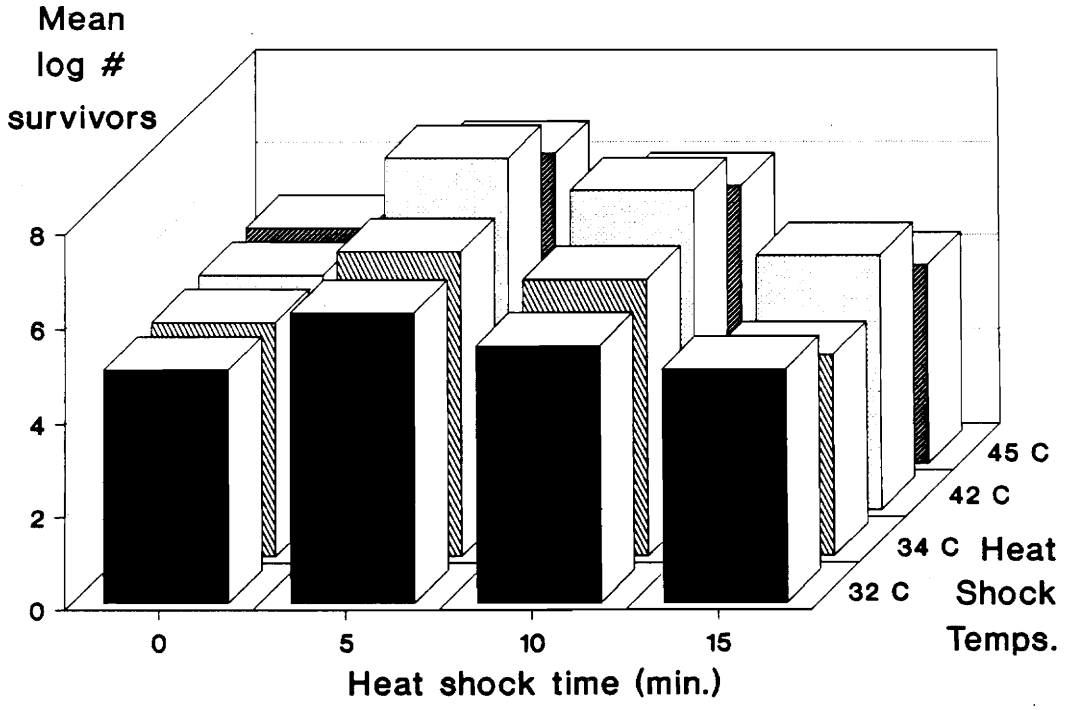


Figure 2: Effect of Heat Shock Time and Temperature on Survival of Aerobically grown E. coli 0157:H7 Cells After Heating at 55°C for 20 Minutes.

heat treatment when heat-shocked at 42°C for 5 minutes (Table 5). However, compared to aerobically grown cells, heat-shocking at any time/temperature combination resulted in significantly lower mean log number of survivors when cells were grown anaerobically. The optimal time/temperature combination for both aerobically and anaerobically grown cells was 42°C for 5 minutes. Therefore, these conditions were used throughout the rest of the study whenever heat-shocking of aerobically grown or anaerobically grown cells was performed.

II. Effect of Heat Shock on Survival of *E. coli* 0157:H7 Cells

A) Cells Grown Aerobically and Recovered Aerobically

Aerobically grown *E. coli* 0157:H7 cells were heat-shocked at the optimal conditions described above, and subjected to a subsequent 55°C heat treatment. Cultures were then plated aerobically in Trypticase Soy Agar to determine the number of survivors to the heat treatment. The mean D value of aerobically grown cells previously heat-shocked was significantly different from nonheat-shocked aerobically grown cells (Table 6, Figure 3). In fact, the mean D value of heat-shocked cells was 2.1 times higher than that for nonheat-shocked controls

TABLE 6

Mean D Values at 55°C of *E. coli* 0157:H7 Cells Recovered in Selective or Nonselective Media as Affected by Heat Shock, Growth Atmosphere, and Recovery Atmosphere After Heating[1]

Growth Atmosph.[2]	Recovery Atmosph.[2]	Heat Shock[3]	D values Nonselec.[4]	D values Selec.[5]
A	A	NO	8.0^{b}	6.7^{a}
A	A	YES	16.8^{f}	10.1^{c}
A	AN	NO	18.3^{g}	12.5^{de}
A	AN	YES	22.2^{h}	12.5^{de}
AN	AN	NO	11.1^{d}	8.3^{b}
AN	AN	YES	16.7^{f}	8.0^{b}
AN	A	NO	12.5^{de}	10.0^{c}
AN	A	YES	14.3^{e}	13.3^{e}

Means within a column followed by a different letter are significantly different ($p \leq 0.05$).

[1] Heating was at 55°C.

[2] Growth was at 30°C in aerobic (A) or anaerobic (AN) Trypticase Soy Broth. Recovery was at 37°C in aerobic (A) or anaerobic (AN) media (selective or non selective).

[3] Cells were either heat shocked at 42°C for 5 minutes (Yes), or not heat-shocked (No).

[4] Non selective medium was Trypticase Soy Agar.

[5] Selective medium was Modified Violet Red Bile Agar.

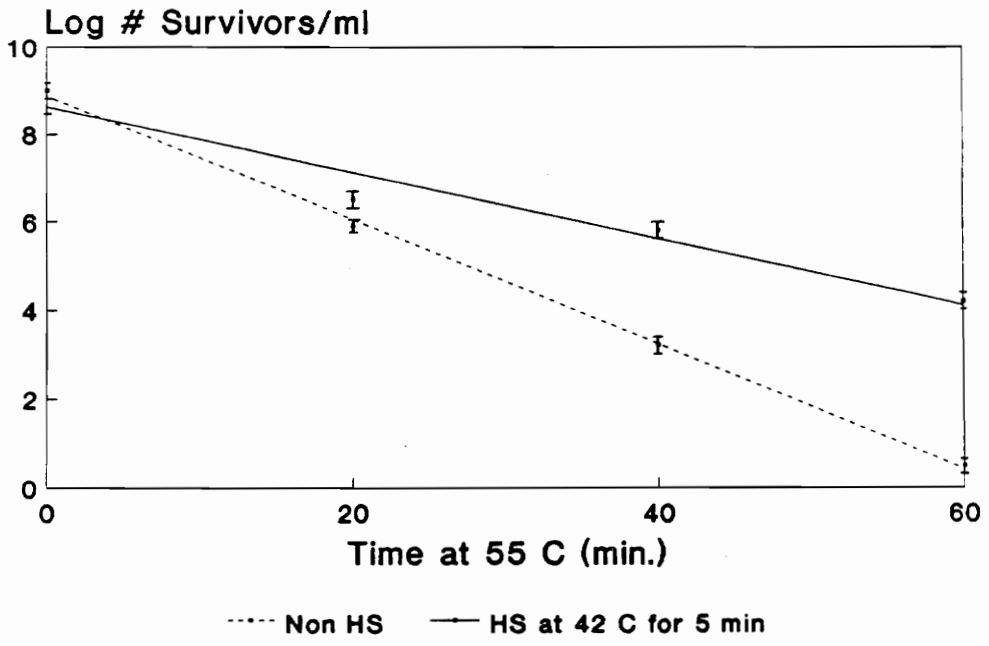


Figure 3: Effect of Heat Shock (HS) on Survival of Aerobically grown E. coli 0157:H7 Cells Plated in Aerobic Trypticase Soy Agar.

(16.8/8.0 = 2.1).

B) Cells Grown Anaerobically and Recovered Anaerobically

Anaerobically grown E. coli 0157:H7 cells were heat-shocked and subjected to a subsequent 55°C heat treatment. The cells were then plated in anaerobic Trypticase Soy Agar roll tubes. Heat shock of anaerobically grown cells resulted in a significantly higher mean D value than anaerobically grown cells not previously heat-shocked (Table 6, Figure 4). The mean D value for heat-shocked anaerobically grown cells was 1.5 times greater than the value for nonheat-shocked controls (16.7/11.1 = 1.5). This difference between heat-shocked and nonheat-shocked anaerobically grown cells, although significant, was not as large as the difference between the mean D value of aerobically grown heat-shocked and nonheat-shocked cells.

When comparing aerobically grown cells recovered aerobically and anaerobically grown cells recovered anaerobically, it is evident that nonheat-shocked anaerobically grown cells resulted in a higher number of survivors to the 55°C heat treatment than nonheat-shocked aerobically grown cells, with mean D values of 11.1 and 8.0 respectively (Table 6, Figure 3, Figure 4). In

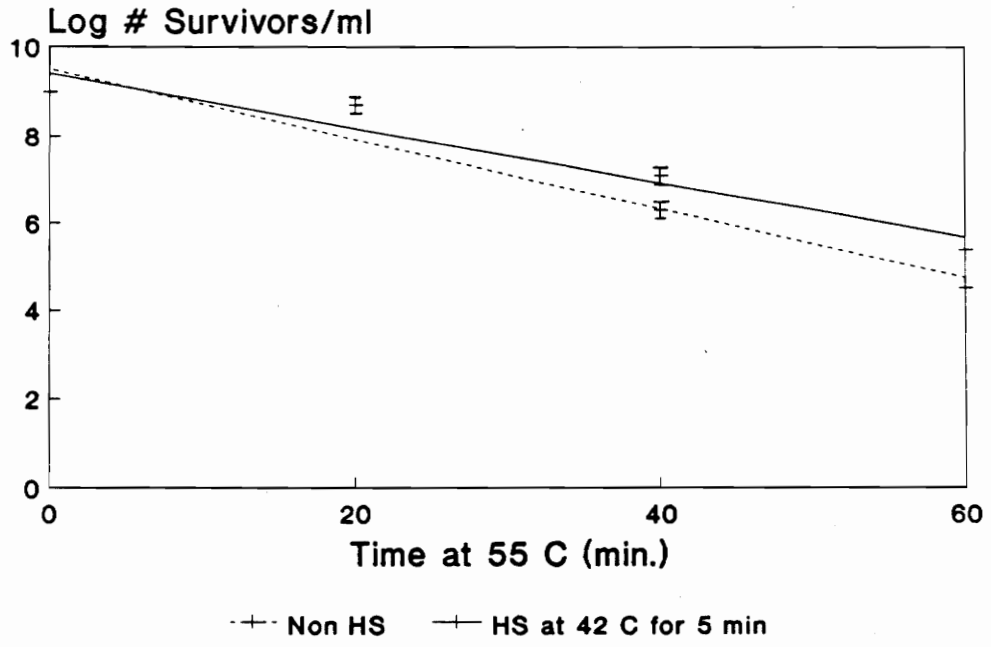


Figure 4: Effect of Heat Shock (HS) on Survival of Anaerobically grown E. coli 0157:H7 Cells Plated in Anaerobic Trypticase Soy Agar.

comparing heat-shocked aerobically grown and heat-shocked anaerobically grown cells, there was no significant difference in the mean D values, with aerobically grown heat-shocked cells recovered aerobically having a mean D value of 16.8 and anaerobically grown heat-shocked cells recovered anaerobically having a mean D value of 16.7 (Table 6).

III. Effect of Plating Atmosphere on Recovery of *E. coli* 0157:H7 Cells

A) Cells Grown Aerobically and Recovered Anaerobically

Aerobically grown *E. coli* 0157:H7 cells were heat-shocked, subjected to a 55°C heat treatment, and plated in anaerobic Trypticase Soy Agar roll tubes. Heat shocking resulted in a higher mean D value of aerobically grown cells recovered anaerobically when compared with nonheat-shocked controls (Table 6). In fact, heat-shocked cells had a mean D value 1.2 times higher than nonheat-shocked controls (22.2/18.3 = 1.2). In comparing mean D values of aerobically grown cells recovered aerobically vs. aerobically grown cells recovered anaerobically, the following can be seen: aerobically grown nonheat-shocked cells recovered anaerobically resulted in higher mean D values than aerobically grown

nonheat-shocked cells recovered aerobically (Table 6, Figure 5). In fact, the mean D value of aerobically grown nonheat-shocked cells recovered anaerobically was 2.3 times higher than the mean D value of aerobically grown nonheat-shocked cells recovered aerobically.

In addition, the mean D value of aerobically grown heat-shocked cells recovered anaerobicaly was 1.3 times higher than that of aerobically grown heat-shocked cells recovered aerobically (22.2/16.8 = 1.3). In fact, aerobically grown heat-shocked cells recovered anaerobically resulted in the highest mean D value of all heat-shocked or nonheat-shocked cells tested, regardless of growth or recovery atmosphere (Table 6). Therefore, recovery atmosphere of aerobically grown cells, whether heat-shocked or not, resulted in a significant increase in the mean D value of survivors to a 55°C heat treatment.

B) Cells Grown Anaerobically and Recovered Aerobically

Anaerobically grown E. coli 0157:H7 cells were heat-shocked, subjected to a 55°C heat treatment, and plated in aerobic Trypticase Soy Agar plates. Heat shocking did not result in a significant difference in the mean D value of anaerobic cells recovered

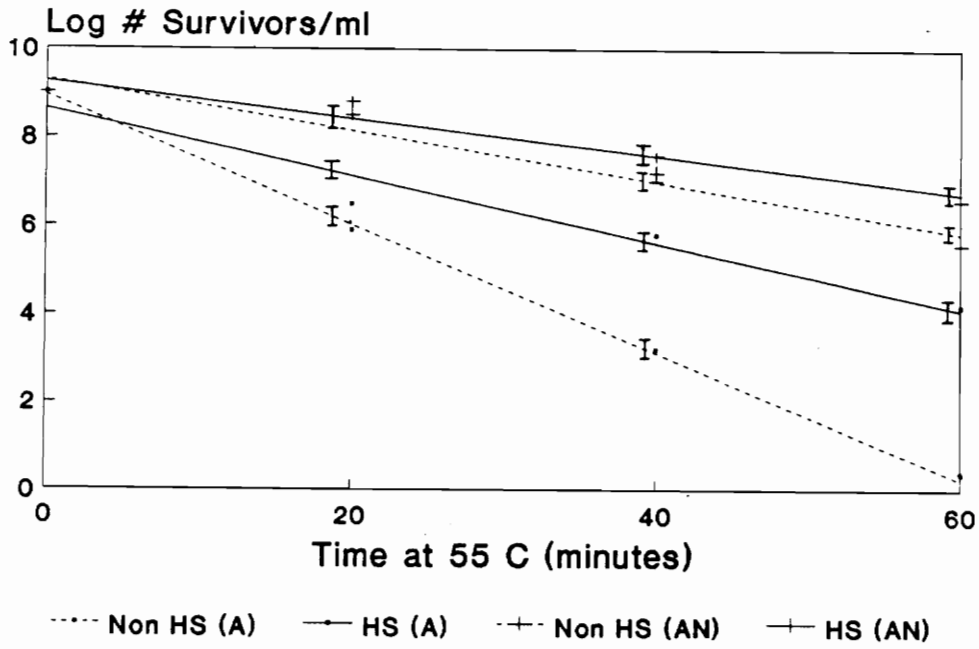


Figure 5: Effect of Plating Atmosphere on Recovery of Heat-Shocked (HS) vs. Control (Non HS) Aerobically grown E. coli 0157:H7 Cells. Cells Were Plated in Aerobic (A) or Anaerobic (AN) Trypticase Soy Agar.

aerobically, with heat-shocked cells having a mean D value of 14.3 and nonheat-shocked cells having a value of 12.5 (Table 6, Figure 6). There was no significant difference in the mean D value of anaerobically grown nonheat-shocked cells recovered anaerobically and the mean D value of anaerobically grown cells recovered aerobically, with the former resulting in a mean D value of 11.1 and the latter in a mean D value of 12.5 (Table 6). However, there was a significant difference between the mean D value of anaerobically grown heat-shocked cells recovered anaerobically and anaerobically grown heat-shocked cels recovered aerobically, with the former resulting in a mean D value of 16.7 and the latter resulting in a mean D value of 14.3 (Table 6).

Therefore, recovery atmosphere of anaerobically grown cells resulted in a significant difference between cells recovered anaerobically and those recovered aerobically only when the cells were previously heat-shocked.

IV. Effect of Heat Shock and Plating Atmosphere on Injury of *E. coli* 0157:H7 Cells

A) Cells Grown Aerobically

Aerobically grown *E. coli* 0157:H7 cells were heat-shocked, subjected to a 55°C heat treatment, and

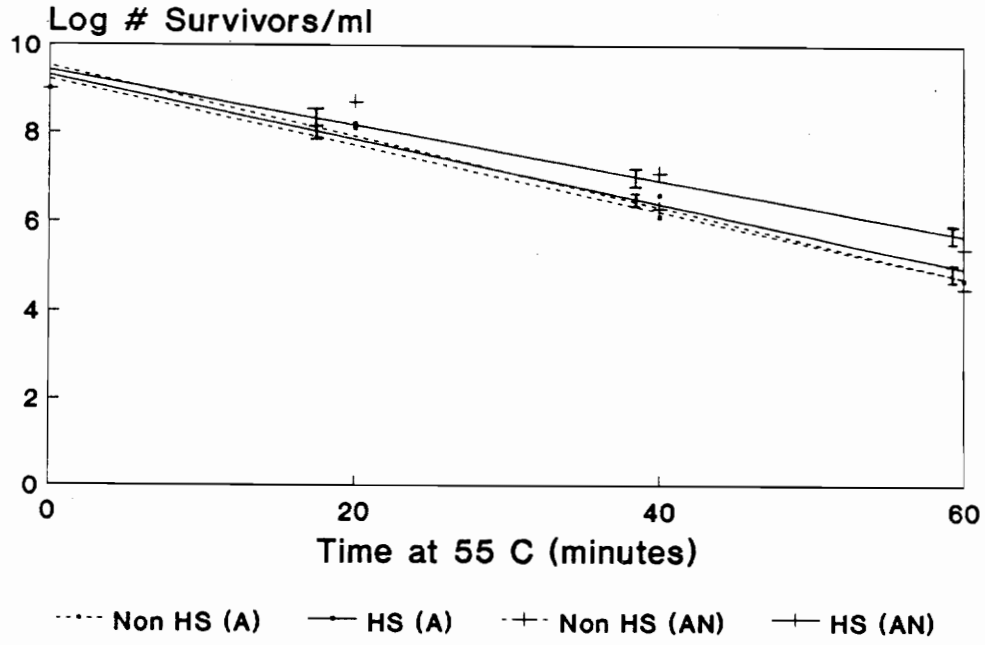


Figure 6: Effect of Plating Atmosphere on Recovery of Heat-Shocked (HS) vs. Control (Non HS) Anaerobically grown E. coli 0157:H7 Cells. Cells Were Plated in Aerobic (A) or Anaerobic (AN) Trypticase Soy Agar.

recovered on aerobic or anaerobic Trypticase Soy Agar (nonselective medium), and in aerobic or anaerobic Modified Violet Red Bile Agar (selective medium). Heat shocking of aerobically grown cels resulted in the largest significant difference between mean D values of cells plated on selective medium vs. cells plated on nonselective medium. This is seen by a mean D value of 10.1 in selective medium vs. 16.8 in nonselective. That heat-shocking resulted in the greatest difference in D value between selective and nonselective media was observed, regardless of whether the media were aerobic or anaerobic (Table 6, Figure 7, Figure 9). However, among heat-shocked aerobically grown cells, the largest difference between mean D values in selective vs. nonselective media for aerobically grown heat-shocked cells was obtained when the cells were recovered anaerobically, with a mean D value in nonselective medium 1.8 times larger than the mean D value in the selective medium. The lowest difference between mean D values of aerobically grown cells plated in selective vs. nonselective medium was observed when the cells were not heat-shocked and were recovered in aerobic media (Table 6).

Therefore, heat-shocking had an effect of increasing

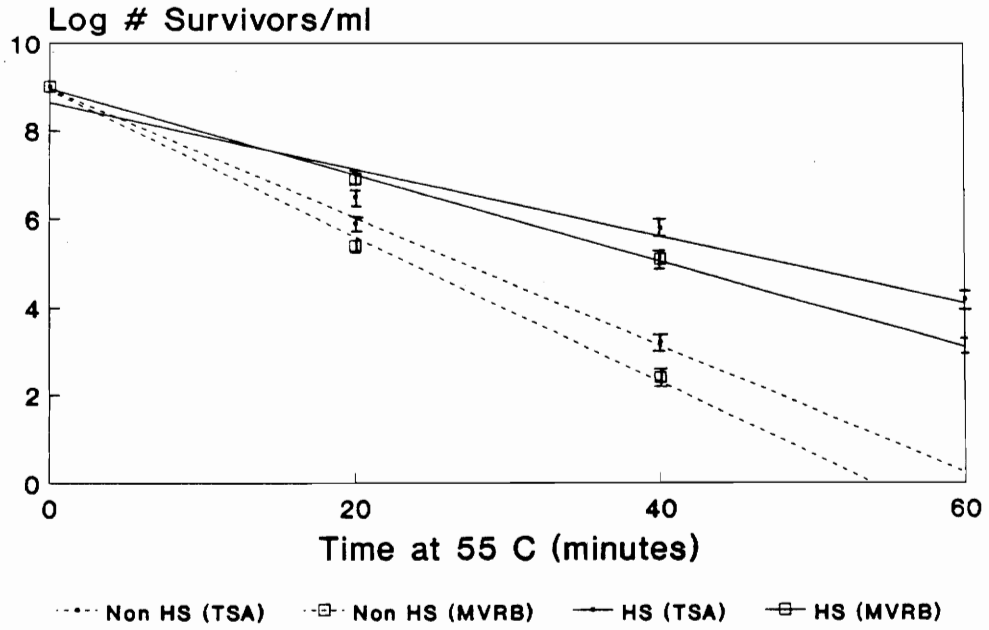


Figure 7: Growth of Heat-Shocked (HS) vs. Control (Non HS) Aerobically grown E. coli 0157:H7 Cells in Aerobic Trypticase Soy Agar (TSA) and Aerobic Modified Violet Red Bile Agar (MVRB).

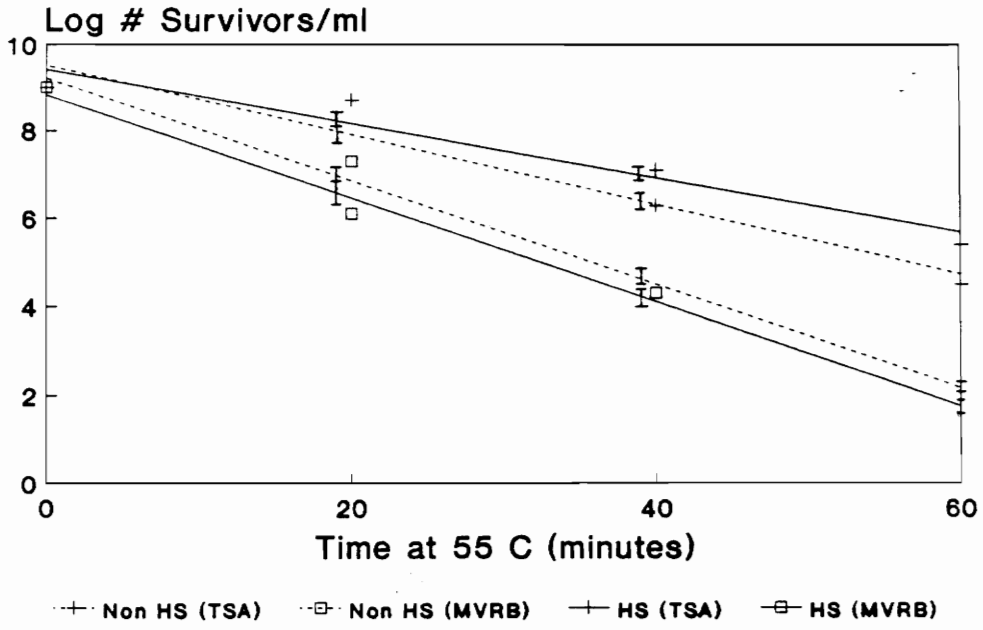


Figure 8: Growth of Heat-Shocked (HS) vs. Control (Non HS) Anaerobically grown E. coli 0157:H7 Cells in Anaerobic Trypticase Soy Agar (TSA) and Anaerobic Modified Violet Red Bile Agar (MVRB).

the number of injured aerobically grown cells when compared to nonheat-shocked aerobically grown cells, regardless of whether the cels were recovered aerobically or anaerobically.

B) Cells Grown Anaerobically

Anaerobically grown E. coli 0157:H7 cells were heat-shocked, subjected to a 55°C heat treatment, and recovered on aerobic or anaerobic nonselective medium and on aerobic or anaerobic selective medium. Heat shocking of anaerobically grown cells resulted in the largest difference in the mean D value of cells plated in selective medium vs. nonselective medium when compared with results obtained for nonheat-shocked anaerobically grown cells only when the cells were recovered in anaerobic media (Table 6, Figure 8, Figure 10). In this case, the mean D value of anaerobically grown heat-shocked cells recovered in anaerobic nonselective medium was 2.1 times higher than the mean D value when the cells were recovered in anaerobic selective medium. In fact, this was the largest difference between selective and nonselectvie media observed regardless of whether the cells were grown aerobically or anaerobically, and whether they were plated in aerobic or anaerobic media

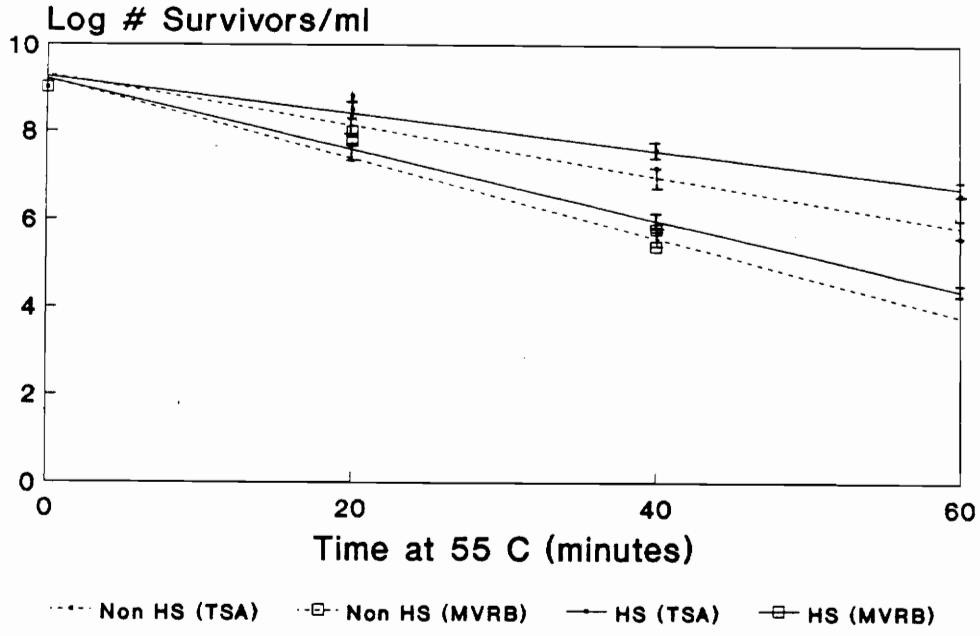


Figure 9: Growth of Heat-Shocked (HS) vs. Control (Non HS) Aerobically grown E. coli 0157:H7 Cells in Anaerobic Trypticase Soy Agar (TSA) and Anaerobic Modified Violet Red Bile Agar (MVRB).

(Table 6). When anaerobically grown heat-shocked cells were recovered in aerobic media, the difference between the mean D values of selective and nonselective media was similar to that of anaerobically grown nonheat-shocked cells recovered in aerobic media (Table 6, Figure 10). The lowest difference between mean D values of cells recovered in selective vs. nonselective media was observed in anaerobically grown heat-shocked cells recovered in anaerobic media. In fact, there was no significant difference in the mean D values of selective and nonselective media of anaerobically grown heat-shocked cells (Table 6).

Therefore, heat shocking had an effect of increasing the number of injured anaerobically grown cells when compared to nonheat-shocked cells only when the cells were recovered anaerobically.

V. Effect of Heat Shock on Catalase and Superoxide Dismutase Activity of Aerobically grown and Anaerobically grown Cells

Catalase and superoxide dismutase activities were measured in aerobically grown and anaerobically grown E. coli 0157:H7 cells before and after heat shock, and before and after a 55°C heat treatment for 20 minutes. Catalase activity was higher in nonheat-shocked

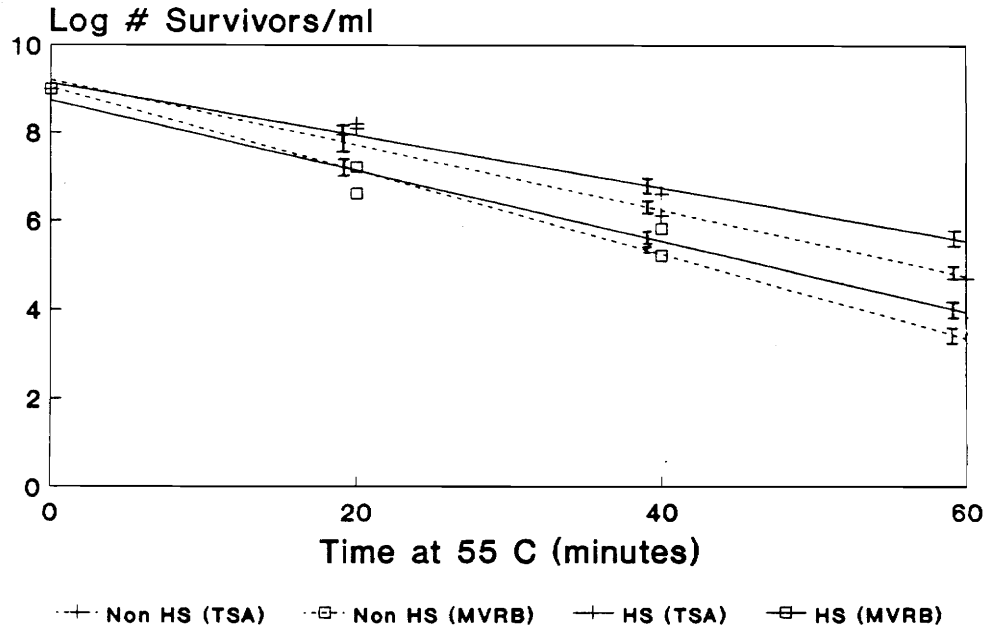


Figure 10: Growth of Heat-Shocked (HS) vs. Control (Non HS) Anaerobically grown E. coli 0157:H7 Cells in Aerobic Trypticase Soy Agar (TSA) and Aerobic Modified Violet Red Bile Agar (MVRB).

aerobically grown cells than in heat-shocked cells when the activity was measured prior to heating at 55°C (Table 7). Catalase activity was also higher in nonheat-shocked anaerobically grown cells than in heat-shocked cells when the activity was measured prior to heating at 55°C. Therefore, heat shocking lowered the activity of catalase in both aerobically grown and anaerobically grown cells. Catalase activity was higher in aerobically grown cells, regardless of whether the cells were heat-shocked or not. This is expected since catalase is known to be present in aerobically grown bacterial cells in higher concentrations than in anaerobicallygrown cells.

Catalase activity was eliminated by heat treatment at 55°C for 20 minutes in aerobically grown heat-shocked and control cells. This was regardless of whether the cells were previously heat-shocked or not (Table 7).

Superoxide dismutase activity was higher in nonheat-shocked aerobically grown cells than in heat-shocked cells prior to heating at 55°C for 20 minutes (Table 8). Superoxide dismutase activity was also higher in nonheat-shocked anaerobically grown cells than in heat-shocked cells prior to heating. Therefore, heat shocking lowered the activity of superoxide dismutase in both aerobically grown and anaerobically grown cells. Superoxide

TABLE 7

Catalase Activity of E. coli 0157:H7 Cells Grown Aerobically or Anaerobically[a]

Treatment[c]	Catalase Activity[b]			
	Aerobically grown		Anaerobically grown	
	Control[d]	H.S.[e]	Control[d]	H.S.[e]
Unheated	46.0(3.0)[f]	33.1(5.0)	22.4(2.0)	11.8(2.0)
Heated	0.0(0.4)	0.0(0.5)	0.0(0.6)	0.0(0.8)

[a] Cells grown in aerobic or anaerobic Trypticase Soy Broth as described in Materials and Methods Section.

[b] Expressed as Units of Activity/mg of protein. Protein concentration measured by Biuret assay.

[c] Cells heated at 55°C for 20 minutes.

[d] Non heat-shocked cells.

[e] Cells heat-shocked at 42°C for 5 minutes.

[f] Numbers in parentheses represent +/- standard deviation.

Superoxide Dismutase Activity of E. coli 0157:H7 Cells Grown Aerobically or Anaerobically[a]

Treatment[c]	SOD Activity[b]			
	Aerobically grown		Anaerobically grown	
	Control[d]	H.S.[e]	Control[d]	H.S.[e]
Unheated	49.3(3.6)[f]	28.3(3.0)	15.7(1.5)	11.5(1.1)
Heated	0.0(0.5)	0.0(0.3)	0.0(0.3)	0.0(0.2)

[a] Cells grown in aerobic or anaerobic Trypticase Soy Broth as described in Materials and Methods Section.

[b] Expressed as Units of Activity/mg of protein. Protein concentration measured by Biuret assay.

[c] Cells heated at 55°C for 20 minutes.

[d] Non heat-shocked cells.

[e] Cells heat-shocked at 42°C for 5 minutes.

[f] Numbers in parentheses represent +/- standard deviation.

dismutase activity was higher in aerobically grown cells than in anaerobically grown cells, regardless of whether the cells were heat-shocked or not. Superoxide dismutase activity was eliminated by heat treatment at 55°C for 20 minutes in all cells tested, regardless of growth atmosphere or whether the cells were heat-shocked or not.

VI. Susceptibility of E. coli 0157:H7 Cells to H_2O_2

A) Aerobically Grown Cells

E. coli 0157:H7 aerobically grown cells were tested for the effect of heat shock on their susceptibility to hydrogen peroxide before and after heat treatment. Exposure of heat-shocked aerobically grown cells to 0.03% H_2O_2 resulted in 63% survivors (Figure 11). Exposure of nonheat-shocked aerobically grown cells to the hydrogen peroxide resulted in 52% survivors. Exposure of aerobically grown heat-shocked cells to hydrogen peroxide after a 55°C heat treatment for 20 minutes resulted in about a 54% survival. This represents a decrease in the percent survivor of heat-shocked cells of approximately 9% due to the heat treatment. Exposure of nonheat-shocked aerobically grown cells to the hydrogen peroxide after the 55°C heat treatment resulted in 25% survivors.

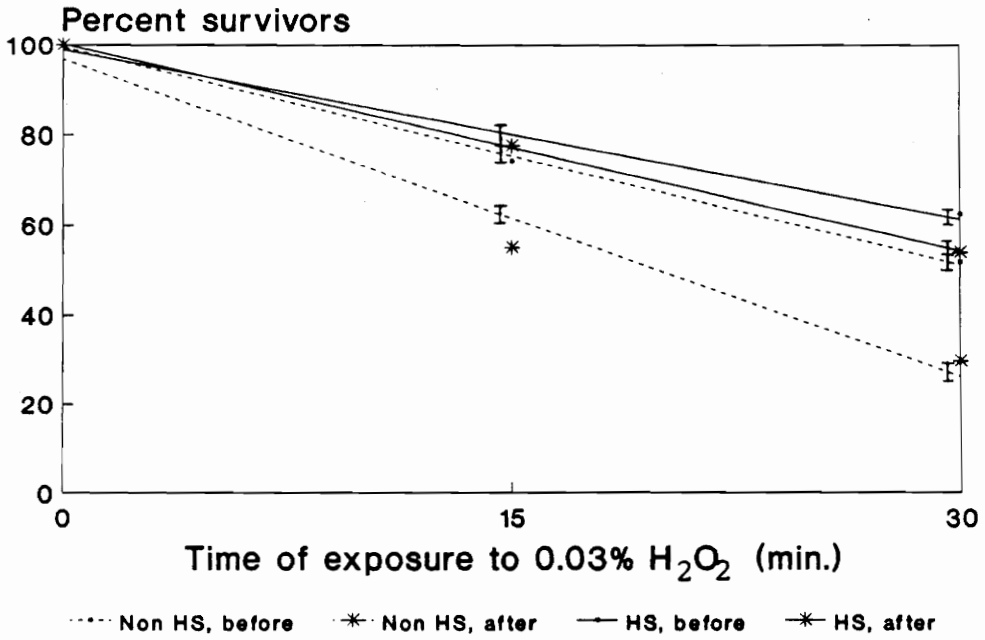


Figure 11: Susceptibility of Heat-Shocked (HS) vs. Control (Non HS) Aerobically grown E. coli 0157:H7 Cells to Hydrogen Peroxide Before and After Heat Treatment at 55°C for 20 Minutes.

This represents a decrease in the percent survivors of nonheat-shocked cells of 27% due to the heat treatment. Therefore, the 55°C heat treatment resulted in an increase in the susceptibility of heat-shocked and nonheat-shocked aerobically grown cells to 0.03% hydrogen peroxide. However, heat shocking resulted in more survivors to the hydrogen peroxide when compared to nonheat-shocked cells, regardless of whether the cells were heat treated at 55°C or not.

B) Anaerobically Grown Cells

E. coli 0157:H7 anaerobically grown cells were tested for the effect of heat shock on their susceptibility to hydrogen peroxide before and after heat treatment. Heat shock of anaerobically grown cells not heat treated resulted in 70% survivors after a 30 minute exposure to 0.03% hydrogen peroxide (Figure 12). Nonheat-shocked anaerobically grown cells not heat treated at 55°C resulted in essentially the same percent survivors as heat-shocked anaerobically grown cells after a 30 minute exposure to hydrogen peroxide. When heat-shocked anaerobically grown cells were heated at 55°C for 20 minutes, they resulted in 25% survivors after exposure to hydrogen peroxide for 30 minutes. The same percent

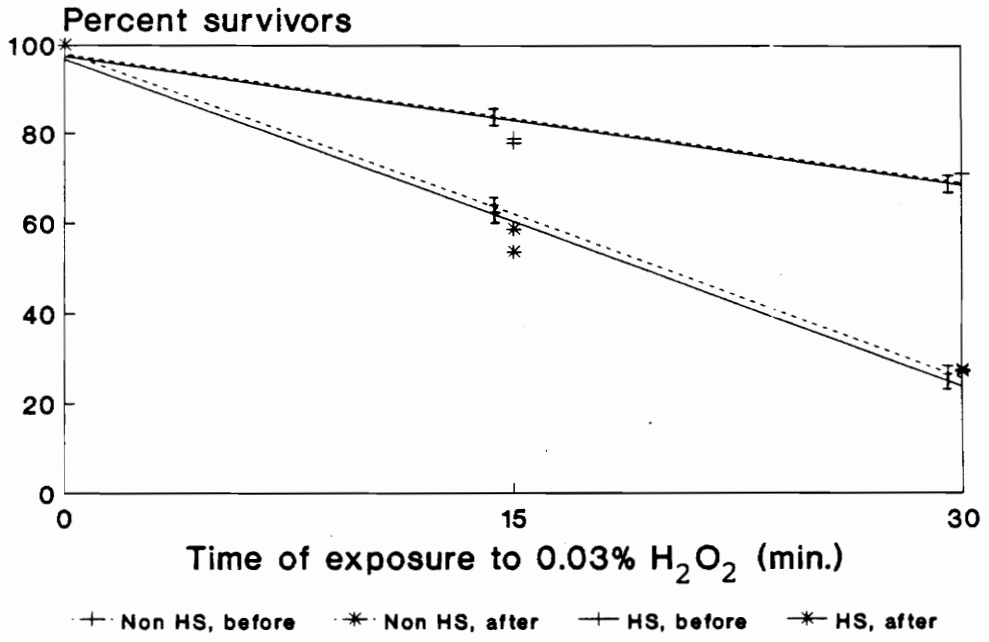


Figure 12: Susceptibility of Heat-Shocked (HS) vs. Control (Non HS) Anaerobically grown E. coli 0157:H7 Cells to Hydrogen Peroxide Before and After Heat Treatment at 55°C for 20 Minutes.

survivors, namely 25%, was observed for anaerobically grown nonheat-shocked heat-treated cells. This represents a decrease in percent survivors of 45%. Therefore, heat resulted in an increase in the susceptibility of heat-shocked and nonheat-shocked anaerobically grown cells to hydrogen peroxide, with heat-shock having no effect in increasing or decreasing the susceptibility of the cells. This is in contrast with the results obtained with aerobically grown cells, where heat-shocked cells resulted in more survivors, and thus a lower susceptibility to hydrogen peroxide, than nonheat-shocked cells.

VII. Rate of Release of Cell Materials

E. coli 0157:H7 aerobically grown and anaerobically grown cells were tested for the effect of heat shock on cell injury after exposure to a 55°C heat treatment. The degree of injury was measured by the release of cell materials after heating, which is indicative of injury to the cell membrane since this would allow leakage of cellular components. Aerobically grown heat-shocked cells released 260 nm-absorbing material at a rate of 0.012 O.D. units per minute during the first 20 minutes of exposure to 55°C heat treatment (Table 9, Figure 13).

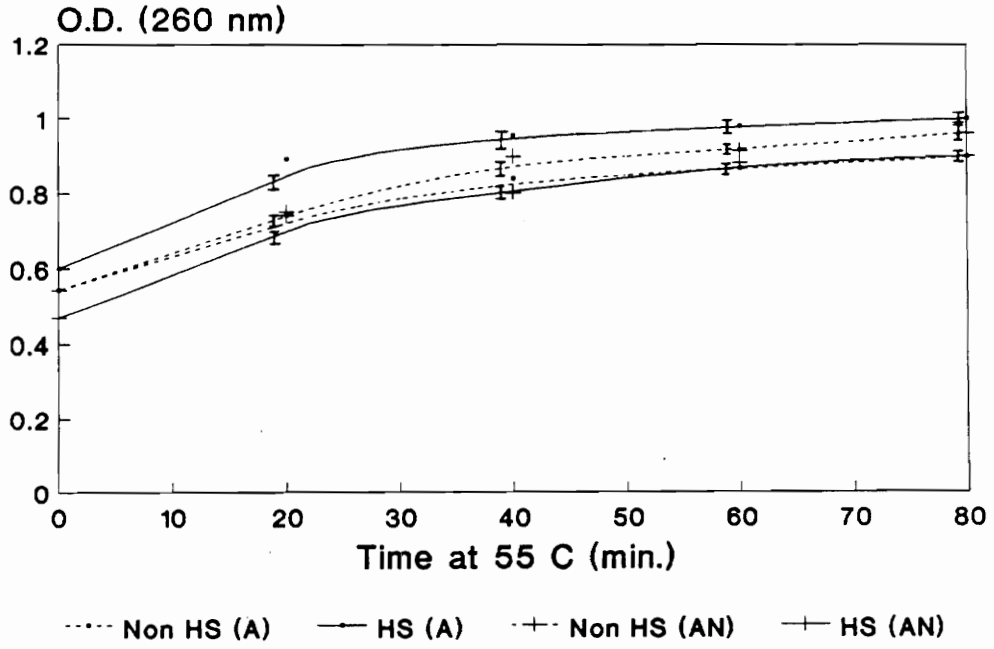


Figure 13: Rate of Release of Cell Components by Heat-Shocked (HS) vs. Control (Non HS) Aerobically (A) and Anaerobically grown (AN) E. coli 0157:H7 Cells After Heating at 55°C.

TABLE 9

Mean Rate of Release of Cellular Material After Heat Treatment from E. coli 0157:H7 Cells Grown Aerobically or Anaerobically[1]

	Rate of Release[2]	
Heat Shock[3]	Aerobically grown	Anaerobically grown
Yes	0.012[a]	0.015[a]
No	0.0075[b]	0.0075[b]

Means followed by a different letter are significantly different ($p \leq 0.05$).

[1] Heat treatment was at 55°C.

[2] Expressed as mean O.D. units (at 260 nm) per minute. Rate was obtained after the first 20 minutes of exposure to heat.

[3] Non heat-shocked cells (No) and heat-shocked cells at 42°C for 5 minutes (Yes).

Aerobically grown nonheat-shocked cells released material at a rate of 0.0075 O.D. units per minute during the first 20 minutes of exposure to 55°C. Therefore, heat shocking increased the rate of release of cellular materials, and thus of injury, over nonheat-shocked cells by 1.6 times (Table 9).

Anaerobically grown heat-shocked cells released 260 nm-absorbing material at a rate of 0.015 O.D. units per minute during the first 20 minutes of exposure to 55°C. This is not significantly different from the results obtained for aerobically grown heat-shocked cells. Anaerobically grown nonheat-shocked cells resulted in a release of material at a rate of 0.0075 O.D. units per minute during the first 20 minutes of exposure to 55°C, which is not significantly different from the rate of release of materials by aerobically grown nonheat-shocked cells.

Therefore, there was no significant difference between the rate of release of aerobically grown or anaerobically grown cells when they were both heat-shocked, or between aerobically grown and anaerobically grown cells when they were both nonheat-shocked. However, there was a significant difference in the rate of release of cellular materials between heat-shocked and

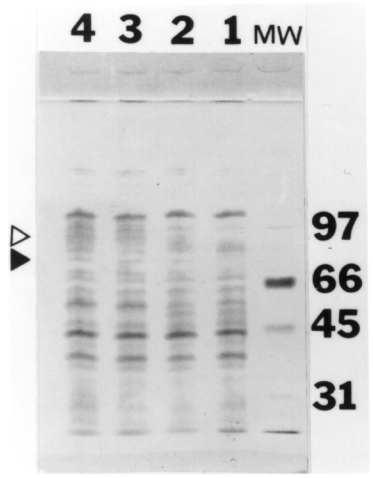
nonheat-shocked cells, whether aerobically grown or anaerobically grown, with heat shock resulting in a higher rate of release of cell materials than nonheat-shocked cells (Table 9, Figure 13).

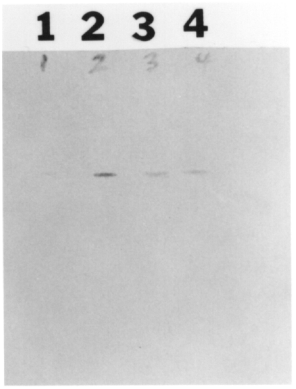
VIII. Effect of Heat Shock on Protein Profile of E. coli 0157:H7 Cells

E. coli 0157:H7 aerobically grown and anaerobically grown cells were heat-shocked and the whole cell proteins were separated by SDS polyacrylamide gel electrophoresis. Figure 14a is a photograph of the actual 7.5% SDS gel that was run to separate the whole cell lysate proteins. There were at least two proteins that were present in the aerobically grown heat-shocked cells that were not present in the aerobically grown nonheat-shocked cells. These proteins were also detected in both anaerobically grown cell samples, whether heat-shocked or not. The first protien had a molecular weight of approximately 71,000 daltons (closed arrowhead, Figure 14a) and the other protein was determined to have a molecular weight of approximately 84,000 daltons (open arrowhead, Figure 14a).

In order to determine whether the 71,000 M.W. protein band corresponded to the sigma^{32} subunit protein of RNA polymerase, which is a stress protein, a Western

(a)





(b)

Figure 14:

(a) 7.5% SDS-Polyacrylamide Electrophoresis Gel Depicting Separation of Whole Cell Lysate Proteins of E. coli 0157:H7 Grown Aerobically or Anaerobically, Before and After Heat Shock. Arrows Indicate Protein Bands Present in Aerobically grown Heat-Shocked Cells and in Anaerobically grown Cells, But Absent in Aerobically grown Non Heat-Shocked Cells (closed arrow: 71,000 M.W. protein, open arrow: 84,000 M.W. protein). Molecular Weights of Markers Are Given in Kilodaltons (from Standard Curve of Molecuar Weight Markers, Figure 1).

(b) Detection of Sigma^{32} Protein in Separated Whole Cell Lysate Proteins. Proteins Were Transferred by Electroblotting onto Nitrocellulose and Reacted With Monoclonal Antibodies Specific for Sigma^{32} Protein.

Cells were grown aerobically and not heat-shocked (lane 1), aerobically and heat-shocked at 42°C for 5 minutes (lane 2), anaerobically and not heat-shocked (lane 3), or anaerobically and heat-shocked at 42°C for 5 minutes (lane 4).

Blot Assay was performed, using monoclonal antibodies specific for this protein, by transferring the separated proteins onto nitrocellulose, and by performing the assay on whole cell protein extracts. Figure 14b is a photograph of the Western Blot performed on the proteins which were electrotransferred from the SDS PAGE gel onto nitrocellulose. One band was detected, corresponding to the 71,000 M.W. protein which was separated by electrophoresis (closed arrowhead, Figure 14a). This band was present in aerobically grown heat-shocked cells as well as in both anaerobically grown cell samples, although it was present in aerobically grown heat-shocked cells in higher concentration than in the others, as observed in the darker band in the Western Blot of transferred proteins (Figure 14b) and in the darker dot in the Western Dot Blot performed using whole cell protein extracts (Figure 15).

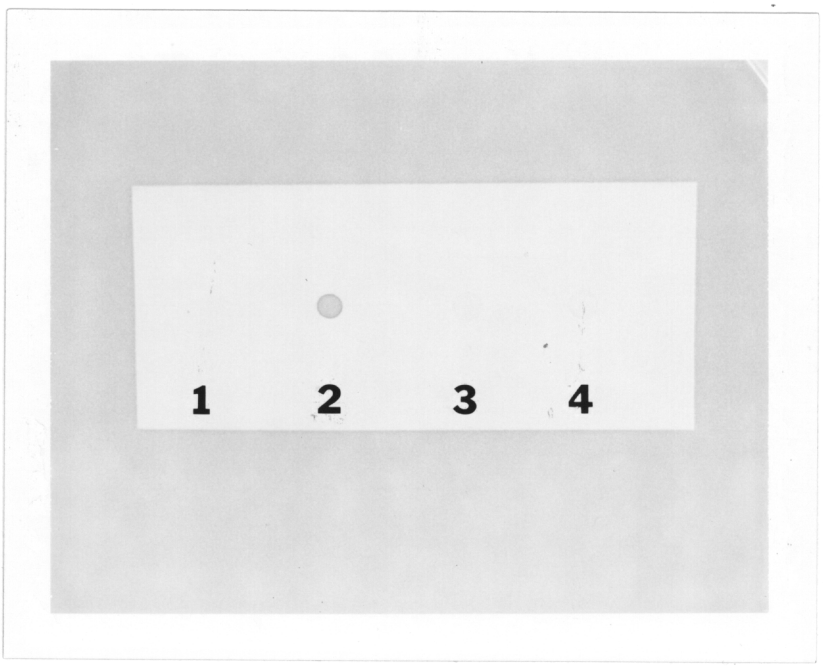


Figure 15: Detection of Sigma^{32} Protein in Whole Cell Lysate Proteins. Proteins Were Directly Loaded onto Nitrocellulose and Reacted With Monoclonal Antibodies Specific for Sigma^{32} Protein.

Cells were grown aerobically and not heat-shocked (1), aerobically and heat-shocked at 42°C for 5 minutes (2), anaerobically and not heat-shocked (3), or anaerobically and heat-shocked at 42°C for 5 minutes (4).

DISCUSSION

The effect of heat shock on the heat resistance of Escherichia coli 0157:H7 bacterial cells was investigated in order to answer the following basic question: can exposure of the cells to an environmental stress increase their ability to survive a 55°C heat treatment? In answering this question, the following objectives were set:

1) to determine whether the environmental stresses of heat shock and/or anaerobic growth improve the ability of E. coli 0157:H7 cells to survive a subsequent heat treatment
2) to determine whether heat shock and/or anaerobic growth elicits the synthesis of stress proteins in E. coli 0157:H7 cells
3) to determine whether anaerobic plating improves the ability of the cells to recover from the heat treatment
4) to determine whether the mechanism responsible for the increase in survival of the cells after heat shock and/or anaerobic growth is one of protection
5) to determine the effect of heat shock and/or anaerobic growth on injury to the cells after

heating

The following definitions of terms used throughout this discussion are presented in order to help the reader: "aerobically grown" refers to cells grown under aerobic conditions in Trypticase Soy Broth; "anaerobically grown" refers to cells grown under strict anaerobic conditions in pre-reduced Trypticase Soy Broth. Cells were either "heat-shocked", meaning that they were subjected to heating at 42°C for 5 minutes, or "nonheat-shocked", which refers to not having been exposed to 42°C for 5 minutes.

Initially, the optimum heat shock conditions of time and temperature were determined. A heat shock at 42°C for 5 minutes resulted in the highest number of survivors to a subsequent heat treatment. Yamamori et al. reported that heat-shocking E. coli cultures at 42°C for 5 to 10 minutes results in the highest number of survivors to a subsequent heat treatment (130). Also, in their heat shock studies on E. coli and Caulobacter crescentus, Gomes et al. found that 5 minutes at 42°C results in a maximum heat shock response for E. coli K-12 (33). These conditions of heat shock were considered optimal for our study and were used in the rest of the experiments which

called for heat-shocking of cells.

In the results presented in this dissertation, heat-shocking increased the D_{55} value of aerobic cells in Trypticase Soy Broth by a factor of 2.1 over nonheat-shocked controls. This is a significant increase in the number of survivors to the 55°C heat treatment when compared with the controls. Knabel et al. reported that heat shocking Listeria monocytogenes cells at 43°C for 30 to 60 minutes results in higher numbers of microorganisms recovered after pasteurization than nonheat-shocked cells (60). Under normal conditions, L. monocytogenes does not survive the minimum high-temperature-short-time pasteurization treatment of 71.1°C for 15 seconds established by the Food and Drug Administration (20). From their results using heat-shocked cells, these authors suggest that if L. monocytogenes is heat-shocked before pasteurization, the organism may be able to survive the process.

In a separate study on L. monocytogenes, Fedio and Jackson found that pre-heating the cells at 48°C for 60 minutes in Trypticase Soy Broth results in a marked increase in the number of survivors to a 60°C heat treatment when compared with controls (24). Mackey and Derrick showed that the number of survivors of Salmonella

typhimurium to a high heat treatment increases substantially over controls when the cells, grown at 37°C, are held at 42 to 48°C before heating (71). The same investigators found that D values for Salmonella thompson cells held at 48°C for 30 minutes prior to heating at 54°C in minced beef increases by a factor of 2.4 (70).

In view of results presented on the heat shock response of E. coli 0157:H7, it is possible that heat-shocking of this organism could result in its survival in ground beef after cooking. According to the Food Safety Inspection Service of the United States Department of Agriculture, beef patties that are cooked at 55°C should be heated for at least 97 minutes in order to render the food safe to eat (88). Doyle and Schoeni reported a D_{55} value for E. coli 0157:H7 in beef patties of 32 minutes. According to the results presented here on the doubling effect that heat-shocking of E. coli 0157:H7 cells in Trypticase Soy Broth had on the number of survivors after heating at 55°, the FSIS procedure may not be sufficient to destroy this organism in ground beef patties.

Heat shock of anaerobically grown E. coli 0157:H7 cells increased the number of survivors to a heat treatment after anaerobic plating, when compared with

nonheat-shocked controls. This increase, however, was not as significant as that seen in aerobically grown heat-shocked cells. It can be concluded that heat-shocking does not have as large of an effect on anaerobically-grown E. coli 0157:H7 cells as on aerobically grown cells. But when D values of anaerobically grown nonheat-shocked cells were compared with D values of aerobically grown nonheat-shocked cells, higher values were obtained for the anaerobically grown controls than for the aerobically grown controls. Thus, anaerobic growth increased the ability of the cells to survive the 55°C heat treatment.

It has been reported that anaerobiosis can be considered a form of stress in some bacterial cells, in that it induces the production of several heat shock proteins. In studies on Salmonella typhimurium, Morgan et al. reported that this organism produces certain stress proteins during anaerobic growth, which overlap with proteins induced by heat shock (77). According to the results presented here, it is evident that not only does heat-shocking E. coli 0157:H7 cells elicit a heat shock response, enabling the cells to survive a heat treatment, but anaerobic growth of the cells also results in an enhanced ability to survive incubation at 55°C. nI

order to fulfill the second objective of the study, namely to investigate the ability of heat-shocked and/or anaerobically grown cells to produce stress proteins, whole cell lysates of aerobically grown and anaerobically grown cells were obtained and the proteins were separated by SDS-PAGE before and after heat shock. Separation of the proteins revealed a 71,000 M.W. protein band which was detected in aerobically grown heat-shocked cells and in anaerobically grown cells, regardless of whether the latter were heat-shocked or not. The protein was not detected in aerobically grown nonheat-shocked controls. From the Western Blot results, this protein correpsonds to the sigma^{32} subunit of RNA polymerase, which has been recognized as a stress protein by various investigators (39). The fact that aerobically grown cells synthesize this protein after heat shock but not before is not surprising, since it has been suggested that this protein is required for the normal expression of heat shock genes (39).

Anaerobically grown cells, both heat-shocked and controls, contained the sigma^{32} protein, but in lesser quantities than aerobically grown heat-shocked samples. This was evident by a fainter band being detected in the Western Blot of proteins extracted from anaerobically

grown cells than from aerobically grown heat-shocked cells. The reason why the concentration of this protein is lower in anaerobically-grown (anaerobically-stressed) cells than in aerobically grown heat-shocked cells is not clear. Given the fact that anaerobic stress seems to affect the heat-shock response, it may be that anaerobiosis induced the production of proteins different from those induced by heat shock, and thus the levels required of the heat stress protein known as sigma^{32} is different in anaerobiosis-stressed cells when compared with heat-stressed cells. Morgan et al. reported that heat stress of S. typhimurium cells induces the production of 13 proteins, while anaerobic stress induces the production of 14 proteins, only 2 of which overlap with the heat-induced proteins (77). It is also possible that there is another sigma subunit, found only in anaerobically grown cells, which is responsible for the synthesis of anaerobically-induced stress proteins. However, such information has not yet been reported in the literature.

It is interesting to note that both heat-shocked and nonheat-shocked anaerobically-grown cells had similar levels of this protein, as judged by the intensity of the Western Blot bands and dots. It is possible that

anaerobiosis supersedes heat shock in the synthesis of stress proteins, inducing the production of anaerobically-induced stress proteins only. This would explain why anaerobically grown E. coli 0157:H7 cells did not respond as well to heat shock as aerobically grown cells.

Another reason why we were unable to detect as much sigma^{32} subunit protein in anaerobically grown cells than in aerobically grown heat-shocked cells may not be due to underproduction of this protein by the former. It may be due to degradation of the protein during production of anaerobically-induced stress proteins. Grossman et al. reported that the sigma^{32} protein is unstable in that changes in its rate of synthesis will result in rapid changes in its concentration within the cell (39). After production of stress proteins as a result of anaerobic growth the concentration of the sigma subunit may decrease due to its instability under anaerobic conditions.

Another protein which was detected in aerobically grown heat-shocked cells and in anaerobically grown heat-shocked and control cells had a molecular weight of 84,000 daltons. This corresponds to a protein which has been found to be produced in S. typhimurium as a result

of heat stress as well as anaerobic stress (77). The function of this protein in E. coli has not been determined, but it is known to have catalase activity in S. typhimurium.

From our results, anaerobic growth constitutes an advantage to the cell in that it enhanced its ability to survive a heat treatment. These results were obtained with cells that were grown anaerobically and plated anaerobically. In addition, heat shock of aerobically grown cells increased the number of survivors to a heat treatment, and these results were obtained with cells that were grown aerobically and plated aerobically. These observations lead one to ask what would be the effects of aerobically plating anaerobically grown cells and vice versa? We sought to investigate this question as part of the third objective of this dissertation.

Aerobic plating of anaerobically grown cells resulted in higher numbers of survivors to a heat treatment than aerobically grown cells. Aerobic plating reduced the number of survivors when the cells were grown aerobically but not when they were grown anaerobically. Heat shock also improved the ability of the cells to survive the heat treatment when plated aerobically, as seen by an increase in the number of survivors when

compared with nonheat-shocked controls. These results support the notion that both anaerobic growth and heat shock correspond to stresses which enable the cells to survive a heat treatment, and which improve their ability to recover aerobically.

Anaerobic plating of both aerobically and anaerobically grown cells enhanced the recovery of the cells by increasing the number of survivors to the heat treatment. Even aerobically grown nonheat-shocked cells, which resulted in the lowest number of survivors after the heat treatment when plated aerobically, increased by virtue of being plated anaerobically. Anaerobic plating of cells somehow enhanced the ability of both aerobically and anaerobically grown cells to recover after a heat treatment.

In general, when bacterial cells are recovered under aerobic conditions after a heat treatment, the number of cells recovered is lower than if recovered under anaerobic conditions (54). This is due to the spontaneous formation of toxic oxygen radicals in aerobic media. If the recovery medium is anaerobic, no oxygen radicals are present. The lack of oxygen radicals like superoxide or hydrogen peroxide in anaerobic media improves the chances of cells to recover since they do

not have to undergo detoxification reactions to eliminate the toxic oxygen radicals. If the cells are exposed to heat, such detoxification could be impossible due to the inactivation of cellular enzymes by heat, which are used to eliminate such radicals.

Several investigators have studied the ability of recovery atmosphere to influence the recovery of heat-treated cells. Knabel et al. reported that strict anaerobic recovery of L. monocytogenes cells results in higher numbers of organisms being recovered after pasteurization when compared with recovery under aerobic conditions (60). McDonald et al. showed that E. coli cells heated at 57°C are recovered aerobically at the same high levels as if recovered under anaerobic conditions only if compounds that degrade hydrogen peroxide are added to the recovery medium (76).

When aerobic and anaerobic E. coli 0157:H7 cells, whether heat-shocked or not, were exposed to a 55°C heat treatment, the activities of both catalase and superoxide dismutase were eliminated after 20 minutes. These enzymes are part of the mechanism which scavenges oxygen radicals in the cell. Since the enzymes were inactivated by the heat in both aerobically and anaerobically grown cells, it stands to reason that anaerobic plating of E.

coli 0157:H7 cells resulted in higher number of survivors than aerobic plating simply because no oxygen radicals were present under these conditions. Having lost the capability to destroy the radicals, the cells were better able to recover anaerobically.

It has long been assumed that the heat shock response serves to protect cells against heat. Heat shock proteins, which are synthesized as a result of heat shock, have been found to associate themselves with DNA in various cell types. The proteins are thought to act by protecting the genetic material by insulating it against heat and other damage (123). As part of the fourth objective of this dissertation, we sought to determine whether the stress response, elicited by heat shock and anaerobic growth, protects E. coli 0157:H7 cells against the effects of heat. Aerobically and anaerobically grown heat-shocked and nonheat-shocked cells were tested for their ability to release cellular material after heating. The theory behind this test is that cells which are protected against heat should not release cellular contents as rapidly as nonprotected cells, due primarily to protection against damage to the cell membrane. Both aerobically grown and anaerobically grown heat-shocked cells released material at a higher

rate than nonheat-shocked controls. Thus, heat shocking and anaerobic growth did not protect the cells against injury. According to the results presented here, heat-shocking and anaerobic growth increased the ability of cells to survive the heat treatment. Since this increase does not seem to be due to a protective effect, it is possible that the stress response of E. coli 0157:H7 cells acts by enhancing the recovery of treated cells and not by protecting them against heat.

The fact that some heat shock proteins associate with DNA after heat shock might be as a step in enhancing the ability of the cells to effect repairs after injury, rather than a protective function prior to injury. Such a function could be ascribed to the DnaJ and DnaK proteins of E. coli, which are known to be involved in RNA and DNA synthesis (78). In addition, the heat shock gene htpR has been shown to influence cell division, a necessary component of any repair mechanism (120). It is possible, then, that the function of stress proteins (heat- and anaerobically-induced) in E. coli 0157:H7 cells is to enhance cellular growth, and so aid the cell in repairing itself after heat treatment, rather than to protect the cell against heat.

Since it was believed that heat-shocking and

anaerobic growth failed to protect E. coli 0157:H7 cells, an investigation into the effect of these stresses on cell injury was conducted. Heat-shocked aerobically grown E. coli 0157:H7 cells were found to be more injured after a 55°C heat treatment than nonheat-shocked controls. This was the case regardless of whether the cells were recovered aerobically or anaerobically. These results agree with the idea that heat-shocking does not protect the cells against injury. However, in somewhat of a contradiction, heat-shocking also resulted in an increase in the total number of survivors to the 55°C heat treatment. One explanation for this may be that heating at 55°C causes the same injuries to cells but heat-shocking may enable the cells to begin repairing themselves faster than if they had not been heat-shocked. According to this theory, nonheat-shocked cells may not able to begin repairing themselves as quickly and thus fall victim to the detrimental effects of heat and die before they are able to begin the recovery process.

Anaerobically grown heat-shocked cells were more injured than nonheat-shocked controls when both were recovered anaerobically. The explanation for this may be for reasons similar to what happens in aerobically grown heat-shocked cells: heat-shocking results in cells being

able to initiate repairs sooner than nonheat-shocked controls. More cells are detected as being injured but in the process of repair, rather than dead. Nonheat-shocked cells, on the other hand, are not able to begin repairing and thus die quickly after the 55°C heat treatment. Heat-shocking of anaerobic cells, then, did not protect them against the heat treatment, but rather enhanced the ability of the cells to begin the repair process.

In summary, the results presented in this dissertation all point to a concern that anaerobic packaging of foods, as well as exposure of the food to a mild heat stress or heat shock, can threaten the ability of a heat process to satisfactorily render the food microbiologically safe for human consumption. Heat shock of E. coli 0157:H7 cells prior to heat treatment at 55°C resulted in an increase in the ability of the organism to recover after the treatment. Furthermore, anaerobic growth of the cells resulted in a significantly higher number of survivors to a heat treatment than aerobic growth without heat shock. Plating atmosphere played a significant role in the recovery of cells, with the greatest number of survivors obtained in aerobically

grown heat-shocked cells recovered anaerobically.

Heat shock of cells in a food product can occur during a slow come-up time in processing the food, such as occurs in "sous vide" processing. Mackey and Derrick reported that a slow rise in the processing temperature of S. typhimurium (0.6°C/minute) resulted in significantly higher number of survivors to the process than a rapid rise (10°C/minute) (69). The authors stressed the importance of whether a particular process could induce the heat shock response in pathogens present in a product.

Even though E. coli 0157:H7 is not considered to be particularly heat resistant under normal conditions, we have shown that if exposed to either a heat shock or to anaerobic stress, it is able to survive a 55°C heat treatment which can lead to the survival of this pathogen in foodstuffs with serious medical consequences. In addition, anaerobic recovery of heat-treated cells resulted in an enhancement of the recovery of aerobically grown cells when compared with cells when recovered aerobically. Anaerobic recovery did not significantly affect anaerobically grown cells, since these cells were always recovered in high numbers due to having been subjected to anaerobic stress during growth.

Anaerobic storage of heat-processed foods is a practice which is prevalent in the meat industry. According to our results, such anaerobic storage can lead to an enhancement of the recovery of E. coli 0157:H7 cells after heat treatment. For this reason, it is recommended that the ability of microorganisms to recover under anaerobic conditions be taken into consideration when determining the shelf-life stability of products packed under such conditions.

Further work needs to be done using this organism, in order to determine the full impact that heat shocking and anaerobic growth and/or recovery can have on processing of foods including ground beef patties. Studies on the effect of heat shock and anaerobic incubation of E. coli 0157:H7 must be carried out in ground beef in order to determine whether a problem really exists. Also, the role of heat shock proteins on the repair of injured organisms should be examined further in order to better understand this phenomenon. In particular, the nature of the relationship between heat stress and anaerobic stress should be studied.

Now that it has been established that heat shock is not the only stress to the cell that can render it more heat resistant, it would be pertinent to determine what

other stresses to which foodborne pathogens are exposed can have the same effect. Exposure to sanitizers, mild extremes of pH, and cold temperatures may enable the cells to withstand not only heat treatment, but other processes designed to prevent spoilage, such as gamma-irradiation and refrigeration. Due to the fact that E. coli 0157:H7 is known to survive freezing temperatures, it would be interesting to determine whether such a stress would enable the organism to grow at refrigerated temperatures. If so, this would present a new cause for concern to packagers of refrigerated prepared food products.

In view of the results presented and discussed here on the effect of heat shock, growth atmosphere, and recovery atmosphere on the survival of E. coli 0157:H7 to heat, it is imperative to establish whether preventive measures can and should be taken so that environmental stresses, such as heat and anaerobiosis, do not become sources for potential pathogens to proliferate even when appropriate food processing procedures are followed.

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CURRICULUM VITAE

Elsa Alina Murano was born in Havana, Cuba in 1959. After living in South and Central America, she moved with her family to Puerto Rico, where she attended elementary and junior high school. At the age of 14 she moved to Miami, Florida where she attended Miami Coral Park Senior High School. After graduation in 1977, Elsa attended Miami-Dade Community College and Florida International University, while working part-time at Kelly Tractor Co. as a secretary. In 1981, she received a Bachelors of Science Degree from Florida International University, where she majored in Biological Sciences.

From 1981 to 1983, Elsa worked in the research laboratory of Dr. Josel Szepsenwol at FIU and presented two abstracts at meetings of the Federation of American Society for Experimental Biology on the tumorigenicity of marijuana derivatives in mice. She also co-authored a paper in 1985 published in Oncology on the development of synovial sarcomas in mice after treatment with cannabinol.

In 1984 she was accepted to the graduate program at the Department of Anaerobic Microbiology at Virginia Polytechnic Institute & State University under the

direction of Dr. Cecil S. Cummins. In 1985, Elsa married Peter S. Murano, a Ph.D. student in the Department of Human Nutrition and Foods. In 1987, she was awarded one of two Fellowships of the American Society for Microbiology Pre-doctoral Minority Student Program. In that year, Elsa earned a Masters in Science Degree on her work on the antitumor activity of Propionibacterium acnes vaccines. Partial results were presented at the 1986 meeting of the American Society for Microbiology, and at the 1987 FASEB National Meeting. The work was published in Cancer Immunology & Immunotherapy in 1988.

After earning an M.S. degree, Elsa was accepted in the Ph.D. program at the Department of Food Science and Technology at Virginia Polytechnic Institute & State University under the direction of Dr. Merle D. Pierson. She defended her Ph.D. on September 5, 1990 and is currently Assistant Professor of Microbiology at Iowa State University, Ames, Iowa, where she resides with her husband, Dr. Peter Murano. Dr. Elsa Murano's research goals are to continue work with the heat shock response of foodborne bacteria, as triggered by chemical, radiation, and other stresses within the context of food safety.