INACTIVATION MECHANISMS OF ALTERNATIVE FOOD PROCESSES ON
ESCHERICHIA COLI O157:H7

DISSERTATION

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Application of high pressure (HP) in food processing results in a high quality and safe product with minimal impact on its nutritional and organoleptic attributes. This novel technology is currently being utilized within the food industry and much research is being conducted to optimize the technology while confirming its efficacy.

*Escherichia coli* O157:H7 is a well studied foodborne pathogen capable of causing diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. The importance of eliminating *E. coli* O157:H7 from food systems, especially considering its high degree of virulence and resistance to environmental stresses, substantiates the need to understand the physiological resistance of this foodborne pathogen to emerging food preservation methods. The purpose of this study is to elucidate the physiological mechanisms of processing resistance of *E. coli* O157:H7. Therefore, resistance of *E. coli* to HP and other alternative food processing technologies, such as pulsed electric field, gamma radiation, ultraviolet radiation, antibiotics, and combination treatments involving food-grade additives, were studied. Inactivation mechanisms were investigated using molecular biology techniques including DNA microarrays and knockout mutants, and quantitative viability assessment methods.

The results of this research highlighted the importance of one of the most speculated concepts in microbial inactivation mechanisms, the disruption of intracellular
redox homeostasis. Groups of genes involved in redox homeostasis or protection against oxidative stress, such as thiol-disulfide redox systems, Fe-S cluster assembly proteins, stress related DNA binding proteins, sigma factors, and other miscellaneous genes were found involved in the mechanism of inactivation of *E. coli* by HP and combinations of HP and tert-butylhydroquinone. The origin or phage content of *E. coli* O157 exhibited a correlation to processing resistance. Lastly, multiple rounds of HP treatments subsequently protected *E. coli* O157:H7 against a variety of deleterious factors, demonstrating adaptability of the pathogen to stress. The results of this research will provide food processors the knowledge necessary to optimize processing conditions in order to produce a safe food product, devoid of *E. coli* O157:H7.
Dedicated to my family
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CHAPTER 1

LITERATURE REVIEW

1.1 *Escherichia coli O157:H7*, an emerging foodborne pathogen

1.1.1 Historical perspective

In 1982, *E. coli* O157:H7 was first acknowledged as a pathogen during the investigation of a hemorrhagic colitis outbreak (Riley et al. 1983). However, the bacterium was not recognized as a significant and ominous pathogen until it was linked to an outbreak involving undercooked beef from a hamburger fast-food restaurant chain in 1993 (Bell et al. 1994; Rangel et al. 2005). By 2000, reporting of *E. coli* O157 presence in stool specimens from patients exhibiting hemorrhagic colitis or hemolytic uremic syndrome (HUS) was mandatory in 48 states (Rangel et al. 2005). In the United States, it is estimated that 73,480 illnesses from *E. coli* occur each year, resulting in an estimated 2,168 hospitalizations and 61 deaths (Mead et al. 1999).

Reported outbreaks are responsible only for a minority of *E. coli* cases, and outbreak investigations provide great insight into *E. coli* O157 epidemiology (Rangel et al. 2005). Between 1982 and 2002, a total of 350 outbreaks were reported from 49 states, accounting for 8,598 *E. coli* O157-associated illnesses (Rangel et al. 2005). These outbreaks were responsible for 1,493 (17.4%) hospitalizations, 354 (4.1%) cases of HUS, and 40 (0.5%) deaths (Rangel et al. 2005). The most prevalent transmission route for the pathogen was food (52%), specifically ground beef (41%), fresh produce (21%), other
forms of beef (6%), and dairy products (4%) (Rangel et al. 2005). A majority of the outbreaks (89%) occurred between May and November (Rangel et al. 2005), likely due to the warmer weather. Geographically, Minnesota reported the most (43) single-state outbreaks, followed by Washington (27), New York (22), California (18), and Oregon (18) (Rangel et al. 2005). The recent increase in \textit{E. coli} O157 outbreaks may be due to the heightened awareness of the disease, improved diagnostics, increased \textit{E. coli} O157 testing, and improved molecular subtyping.

Between 2006 and 2007, the FDA has noted four major outbreaks of \textit{E. coli} O157 (CDC 2009). In Fall 2006, baby spinach, which was processed and packaged in San Juan Bautista, California, contained \textit{E. coli} O157 and was responsible for 205 illnesses and 3 deaths. Some of the implicated bags of spinach were linked to environmental contamination in a specific field, with possible sources of contamination ranging from the incursion of wild pigs, to the exposure of surface waterways to feces. The actual source of contamination was never determined. In Winter 2006, lettuce served at a large Mexican fast food chain was implicated in an \textit{E. coli} O157 outbreak, resulting in 71 illnesses and leading to 53 hospitalizations, and 8 cases of HUS. Because the outbreak was linked to restaurant locations in four northeastern states, it is likely that contamination of the lettuce occurred prior to reaching the restaurants, although testing has not confirmed the presence of \textit{E. coli} O157 in the lettuce samples. In Fall 2007, the USDA issued a notice about a recall of 21.7 million pounds of frozen beef patties due to \textit{E. coli} O157. The 40 cases of infection spread over 8 states and were responsible for 21 hospitalizations and 2 cases of HUS. In Fall 2007, a food industry giant voluntarily recalled pepperoni pizzas due to the association between product consumption and \textit{E. coli}
O157 infection. Over 20 isolates of *E. coli* O157 have been collected from ill persons in 10 states, with 8 hospitalizations, and 4 cases of HUS reported. The source of contamination remained unknown.

There have been recent advancements in food processing technologies, particularly those that serve as alternatives to heat and are capable of improving the safety of the food supply. These emerging technologies make use of novel antimicrobial agents such as high pressure (HP), pulsed electric fields (PEF), gamma (γ) radiation, and ozone. There is a need to develop these technologies and confirm their efficacy against pathogens’ adaptability to stress and resistance to processing. Addressing these research needs will help minimize disease outbreaks due to serious foodborne pathogens, such as *E. coli* O157.

### 1.1.2 Reservoir

As previously mentioned, the most common food groups to contain *E. coli* O157 are ground beef, fresh produce, other forms of beef, and dairy products, in descending order (Rangel et al. 2005). Thus, it is not surprising that the main reservoir of *E. coli* O157 is cattle (Lira et al. 2004). Although, the farm is most likely the location where the source of contamination should be controlled, the food industry needs to develop new strategies to eliminate this pathogen before it reaches consumers.

*E. coli* O157 is widely distributed throughout bovine populations, where it is a benign commensal of the gastrointestinal tract. In the past two decades, *E. coli* O157 has become increasingly more resistant to many antibiotics (Galland et al. 2001). Acid tolerant *E. coli* O157 has been found in a variety of foods such as raw milk, raw milk
cheese curds, unpasteurized apple cider and juice, lettuce, salads, grapes, alfalfa sprouts, coleslaw, and melons (CDC 2009; Mead and Griffin 1998; Mead et al. 1999; Rangel et al. 2005). Drinking or irrigation water are other possible reservoirs for enterohemorrhagic E. coli (EHEC) (Stevenson et al. 2004).

1.1.3 Human (Q93 phage) versus bovine (Q21 phage) E. coli O157:H7

Most alternative food processes are predicted to induce SOS responses in foodborne microorganisms. Previous studies have identified differences between bovine- and human-origin E. coli O157 isolates with respect to their environmental survivability. Bovine-origin E. coli O157 strains were found to be more resistant to environmental stresses. Differences in survivability of bovine- and human-origin E. coli were observed during drying on concrete (Avery and Buncic 2003). Therefore, determining the differences in processing resistance of E. coli O157 due to environmental origin would be of interest to food manufacturers.

Food processes often cause microbial DNA damage, which could initiate a chain of genetic reactions and enable microorganisms to resist or adapt to the applied stress. One of these reactions includes the RecA/LexA system. RecA is a cellular protein that plays a significant role in DNA repair (Takahashi et al. 1996). It is involved not only in homologous recombination in E. coli, but also in regulation of the activity and synthesis of DNA-repair proteins (SOS induction) and aids in mutational repair.

RecA functions by binding to single-stranded DNA following DNA damage, and forming a helical polymer around the DNA (Takahashi et al. 1996). This nucleofilament interacts with the LexA repressor and stimulates its autocleavage. This action of RecA
increases the synthesis of DNA repair enzymes, including RecA itself (Takahashi et al. 1996). In addition, RecA is responsible for prophage induction. Any treatment that upregulates recA, including DNA-damaging UV irradiation or γ-rays as well as a generalized cellular SOS response, will impact prophage induction and subsequent cell lysis. The transcription antiterminator, Q protein, governs the late-phage gene expression required for virion production and cell lysis by changing the transcription complex initiated at a late promoter, $p_R'$ (Plunkett et al. 1999). The toxins of EHEC are encoded by lysogenic bacteriophages (Smith et al. 1983; O'Brien et al. 1984; Strockbine et al. 1986; O’Brien et al. 1989; Plunkett et al. 1999). The Shiga toxin 2 genes are part of a Q-dependent late transcript, which would be expressed only during lytic growth of the phage.

*E. coli* O157 isolates of human origin that encode the Q933 allele of this gene are more effective at preventing RNA polymerase termination than those isolates encoding the Q21 allele (Guo et al. 1990) that is commonly found in isolates of bovine origin (LeJeune et al. 2004). Thus, Q933-encoding prophages can more readily express the late-phage genes required for cell lysis.

The differences in strains of *E. coli* O157:H7 from human origins versus bovine origins are not well understood. A study demonstrated that there were physiological and biochemical differences between strains from human and bovine origin. However, the strains did not appear to have any traits that may be advantageous in the bovine or human environment (Durso et al. 2004). Another study analyzed human and animal isolates originating from Australia. The Australian population has a low rate of infection by *E. coli* O157:H7; however, these organisms exist in the animal populations. These
researchers found that all isolates contained the virulence genes associated with infection, thus, other factors must be responsible for the low rate of human infection in Australia (Fegan and Desmarchelier 2002).

1.2 Characteristics of E. coli O157:H7

1.2.1 General characteristics

E. coli O157 is a Gram-negative, facultative anaerobic (i.e., possesses both respiratory and fermentative metabolic pathways) rod. Most E. coli O157 strains do not ferment sorbitol and do not contain the enzyme β-glucuronidase (Holt et al. 1994). The optimal growth temperature for E. coli O157 is 37°C (Holt et al. 1994), but it can survive as low as 7°C and as high as 46°C. E. coli O157 prefers a neutral pH (6.0-7.0), but it can survive in food at a pH range of 3.7-4.4 (Weagant et al. 1994). Most E. coli O157 strains ferment glucose and some ferment lactose, producing acid and gas, and are typically oxidase-negative, indole-positive, and urease negative, and when motile, produce peritrichous flagella (Holt et al. 1994). These biochemical characteristics are well-utilized in the identification of the pathogen. In addition, rapid molecular and serological techniques are used in detection methods. However, there are a number of E. coli O157 strains that may have physiological differences (Leclercq et al. 2001). The unique characteristics of E. coli O157, such as acid and cold tolerance, make monitoring this foodborne pathogen critical within the food industry.
1.2.2 Serotyping

There are over 700 antigenic types (serotypes) of *E. coli* which are recognized based on the O (somatic), H (flagellar), and K (capsular) antigens (Paton and Paton 1998; Paton et al. 2001). There are over 200 O antigens. Flagellar proteins are less heterogeneous than the carbohydrate side chains that make up the O groups, thus, considerably fewer antigenic types exist (over 30). The O antigens are composed of lipopolysaccharide complexes, which are part of *E. coli* cell envelope. It is the immunogenicity of the polysaccharide repeating units which gives the O antigens their specificity. Some of the enterohemorrhagic *E. coli* serotypes include O157:H7, O26:H11, O103:H2, O111:NM, O121:H9, and O145:NM (Paton and Paton 1998; Kim and Kim 2004). The H antigen represents the different flagellin present as part of the flagellar structure. The K antigen (or the capsular antigen) is mainly an acidic polysaccharide. In general, the K antigen is left out when serotyping *E. coli*.

1.2.3 Virotyping

Enterohemorrhagic *E. coli* O157 contains the *eaeA* gene which is responsible for the production of attachment-effacement lesions. Enterohemorrhagic *E. coli* carries a 60-MDa plasmid that encodes fimbriae that mediate attachment to culture cells (Tzipori et al. 1987; Paton and Paton 1998). In contrast to enteropathogenic *E. coli* (EPEC), EHEC strains affect the large intestine and produce Shiga toxins. Enterohemorrhagic *E. coli* does not invade mucosal cells as readily as *Shigella*, but produces a toxin that is virtually identical to the Shiga toxin. This toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of these strains to cause HUS.
(Paton and Paton 1998). The Shiga toxin is phage-encoded and its production is enhanced by iron deficiency. EHEC has genetic markers composed of virulence-associated genes, such as *stx* for Shiga toxins 1 and 2, *ehx* for enterohemolysin, and *eae* for intimin, an outer membrane protein involved in intestinal colonization.

### 1.3 *E. coli* O157:H7 pathogenesis

#### 1.3.1 Overview

*E. coli* O157 is particularly important in the food industry because it has a low infectious dose. Serotypes of EHEC, particularly *E. coli* O157:H7, cause bloody diarrhea and no fever, a syndrome known as hemorrhagic colitis (HC). Some of the HC symptoms include severe abdominal cramps with watery diarrhea, which typically becomes bloody within 24 h (O’Brien et al. 1983; Riley et al. 1983; Riley 1987). The diarrhea usually lasts 1 to 8 days. Approximately 5% of people with HC develop a severe complication known as HUS. Symptoms of HUS include anemia (characterized by fatigue, weakness, and light-headedness) caused by the destruction of red blood cells (hemolytic anemia), a low platelet count (thrombocytopenia), and sudden kidney failure. Some people with HUS also develop complications of nerve or brain damage, leading to seizures or strokes. These complications typically develop in the second week of illness and may be preceded by increasing fever. HUS is more likely to occur in children younger than 5 years and in the elderly. Even without HUS and its complications, HC may cause death in older people (Blackall and Marques 2004). HUS is associated with Shiga toxin producing enteric bacteria, such as *E. coli* O157:H7. The production of the
Shiga toxin is key for many of the pathological features of EHEC infection (Paton and Paton 1998; Lathem et al. 2004).

### 1.3.2 Adherence

Adherence is a key virulence determinant involved in EHEC pathogenesis (Paton and Paton 1998; Tatsuno et al. 2000; Paton et al. 2001; Tatsuno et al. 2003; Diez-Gonzalez and Karaibrahimoglu 2004; Kim and Kim 2004). To produce its pathological effects, EHEC need to survive intestinal stresses, adhere to intestinal epithelial cells of the large intestine, and thus, colonize the human gut. Acid resistance of EHEC is an important factor that allows it to survive digestive stresses and colonize the intestinal tract (Diez-Gonzalez and Karaibrahimoglu 2004). Acid resistance of EHEC involves the *rpoS* gene, which encodes a stationary-phase sigma factor (Robey et al. 2001).

After surviving the harsh environment of the stomach, the pathogen must adhere to the intestinal epithelial cells. It is theorized that the colon and perhaps the distal small intestine are the principle sites of EHEC colonization in humans. Among EHEC strains, heterogeneity exists in adherence, and this may reflect differences in pathogenic mechanisms (Robey et al. 2001; Nagano et al. 2003). There has been controversial data about whether pO157 plays a significant role in adherence of EHEC to the colonic epithelium (Paton and Paton 1998).

Some strains of *E. coli* O157 are capable of causing attaching and effacing (A/E) adherence lesions on enterocytes. These A/E lesions involve ultrastructural changes, including loss of enterocyte microvilli and intimate attachment of the bacterium to the cell surface. Beneath the adherent bacteria, there is accumulation of cytoskeletal
components, resulting in the formation of pedestals (Abe et al. 2002). EHEC strains that display the A/E phenotype have a pathogenicity island homologue called Locus for Enterocyte Effacement (LEE) similar to that of EPEC (Elliott et al. 1998). The LEE homolog contains a copy of \textit{eaeA}, Tir (Translocated intimin receptor) homolog (Frankel and Phillips 2008), as well as the Type III secretion system (Devinney et al. 2001). Studies have shown that intimin (\textit{eaeA}), which is an outer membrane protein, is essential for the generation of cytoskeletal rearrangements in HEp-2 cells \textit{in vitro} (Donnenberg et al. 1993; Louie et al. 1993). In addition, \textit{eaeA}-negative mutants lost the capacity to adhere intimately to the colonic epithelium of piglets. Intimin plays a role in the formation of attaching and effacing (A/E) lesions and intestinal colonization (Abe et al. 2002).

There are other bacterial elements that control the adherence to host intestinal epithelium. However, bacterial factors other than intimin required for colonization are not well defined. It has been reported that OmpA may act as an adhesion factor for EHEC. In addition, a ToxB homolog encoded by the pO157 plasmid may promote the bacterial adherence to cultured epithelial cells by an unknown mechanism (Tatsuno et al. 2003). \textit{E. coli} O157:H7 has the ability to express thin aggregative fimbriae, known as curli, on the cell surface (Kim and Kim 2004). Curli allow EHEC O157:H7 to aggregate in large numbers and adhere to HEp-2 cells. This adherence is also affected by alteration of the outer membrane integrity (Kim and Kim 2004). These properties were reconstituted by transformation with a plasmid carrying the \textit{eaeA} gene (Abe et al. 2002). The presence of \textit{eaeA} in \textit{E. coli} of animal origin is most commonly associated with known human-virulent strains such as those belonging to serogroups O157, O26, and
O111. However, there are several EHEC isolates that do not contain the eaeA gene. These strains may produce other virulence factors that compensate for the absence of intimin. Other factors involved in adherence include fimbriae, OMPs, and lipopolysaccharide (LPS).

1.3.3 Shiga toxin

Shiga toxin consists of a single enzymatically active A subunit and multiple B subunits (Paton and Paton 1998; Jones et al. 2000). The Shiga toxin is capable of translocating across the intestinal epithelial cells without apparent cellular disruption via a transcellular pathway. After crossing the epithelial barrier and entering the bloodstream, Shiga toxin targets tissues expressing the appropriate glycolipid receptor, globotriaosylceramide (Gb3). Shiga toxin sensitive cells contain the toxin receptor, Gb3, and sodium butyrate appears to play a role in sensitizing cells to Shiga toxins (Louise et al. 1995). Cows lack the Gb3 receptor, which explains their insusceptibility to the harsh disease of EHEC.

Once toxins bind to Gb3, internalization follows with transport to the trans-Golgi network. After Shiga toxin enters the host cells, the toxin binds to the 60S ribosomes and inhibits peptide chain elongation and protein synthesis, thus leading to cell death (Shimada et al. 1999). The B subunits form pentamers in association with a single A subunit and are responsible for the binding of the toxin to the neutral glycolipid receptors. Shiga toxin 2 seems to be more significant than Shiga toxin 1 in the etiology of HC and HUS.
High levels of Gb₃ are found in the human kidney, particularly in the cortical region, the principle site of renal lesions in patients with HUS (Jones et al. 2000). Erythrocytes (RBCs) contain Gb₄ receptor. The susceptibility of a given cell type to Shiga toxin is not determined solely by its Gb₃ content. Recent studies suggest that the Gb₃ lipid moiety has a significant influence on the interaction between the Gb₃ oligosaccharide head group and the toxin (Kiarash et al. 1994). Other putative virulence factors include enterohemolysin, serine protease (EspP), and a heat-stable enterotoxin.

Foods are processed, commonly by heat, to eliminate pathogenic microorganisms and protect consumers against potential health hazards. Conventional processing technologies ensure food safety but the resulting product is inferior in quality, when compared to the fresh counterpart. Food processors are considering several alternative technologies, in lieu of thermal treatments. It is critical that researchers confirm the efficacy of these alternative technologies, particularly against emerging highly-pathogenic bacteria such as *E. coli* O157 and *L. monocytogenes*.

1.4 Alternative non-thermal technologies

Alternatives to traditional thermal preservation methods of food are being developed in response to consumer demand for “fresh-like” and “healthy” foods. These alternatives include non-thermal technologies such as radiation, pulsed electric fields, high-intensity light pulses, and HP processing. These emerging technologies inactivate microorganisms at lower temperatures than typical heat treatments, thereby minimizing undesirable changes in food quality (Singh and Yousef 2001). Foods processed using heat undergo irreversible changes in color, flavor, and texture. Excessive heat may
degrade food quality by denaturing proteins, breaking emulsions, destroying vitamins, and drying out food (Potter and Hotchkiss 1995). HP processing is a promising heat-alternative treatment which has the potential to meet the demands for high quality foods that are microbiologically safe; this technology is the focus of our study.

1.4.1 Advantages and disadvantages of HP processing

The lethal effects of pressure against microorganisms is driven by the Le Chatelier principle, in which pressure favors any physical changes or chemical reactions associated with a net volume decrease. In biological systems, the important volume-decreasing reactions include protein denaturation, gelation, hydrophobic interactions, and phase changes in lipids (Erkmen and Doğan 2004). Therefore, the pressure-induced changes in the cell differ from those caused by heat (Balny et al. 2002). For example, the structures of pressure-denatured proteins differ from those denatured by heat (Hummer et al. 1998). HP primarily affects noncovalent intermolecular associations such as hydrophobic and hydrogen bond interactions. This leads to conformational changes of protein secondary or tertiary structures. However, small molecules are generally less affected than macromolecules, so that low-molecular weight flavor compounds in foods remain unchanged after pressurization, with quality advantages in some products (Gould 2001).

HP is currently used in lieu of thermal pasteurization to process jam, jelly, shellfish, oysters, guacamole, fruit juices, salsa, rice products, fish meal kits, poultry products, and sliced ready-to-eat meats (Considine et al. 2008). The main advantages of HP processing include an instant and isostatic (uniform) distribution of pressure
throughout the food, and consequently, microbial lethality is independent of food size and geometry. In contrast, heat transfer is limited by intrinsic properties of foods and thus, frequently leads to size reduction of the products (San Martin et al. 2002). HP can be applied at ambient temperature, thereby preserving food quality and nutrition while improving energy efficiency. In addition, no deformation of solid foods occurs because the pressure is applied isostatically (Thakur and Nelson 1998; San Martin et al. 2002).

Food enzymes and bacterial spores are very resistant to the pressures commonly used in food processing (Patterson et al. 1995; Smelt 1998); therefore, this technology would be most feasible for refrigerated food products. Although extreme pressure treatments may inactivate enzymes, the sensory properties of foods, such as texture, physical appearance and functionality, can be adversely affected (Knorr 1993). Pressure treatment remains costly, since the equipment suitable for food use is specialized and the capital equipment cost is relatively high (Patterson 2005). This limits application of HP technology to high-value products in the food industry (Smelt 1998).

1.4.2 Microbial inactivation by high-pressure processing

In general, pressures ranging from 300 to 600 MPa can inactivate most pathogenic and spoilage vegetative bacteria, yeasts, and molds (Smelt 1998). Although there are many exceptions, bacterial spores are generally much more resistant to HP than are vegetative cells. In addition, Gram-negative bacteria and exponential-phase cells are more sensitive than Gram-positive bacteria and stationary-phase cells, respectively (Cheftel 1995; Mackey et al. 1995). Viability of pathogenic bacteria decreases
appreciably as the level of pressure and temperature increases (Alpas et al. 2000), though
great variation in HP sensitivity exists among strains.

1.4.3 Combination treatments

HP may be combined with other preservation methods to increase its efficacy and
commercial feasibility. Existing and potential food additives, such as phenolics,
bacteriocins, and potassium sorbate, have been tested in combination with HP (Karatzas
et al. 2001). In addition, changes in treatment temperature and product pH may increase
effectiveness of HP treatment.

Combining phenolic food-grade ingredients, such as tert-butylhydroquinone
(TBHQ), with HP was successful in eradicating pressure-resistant microorganisms in
food. Three strains of *L. monocytogenes* (Scott-A, OSY-8578, and OSY-328) were grown
to the stationary phase in tryptose broth, resuspended in phosphate buffer, and treated
with 100 ppm of phenolic compounds and 400 MPa for 5 min (Chung et al. 2005; Vurma
et al. 2006). Although the phenolic compounds alone were not lethal to the *L.
monocytogenes* strains, the phenolic additives significantly enhanced the lethal effects of
pressure (*p* < 0.0001). Karatzas et al. (2001) also reported a synergistic action between
carvacrol and HP against *L. monocytogenes* Scott A in buffer and in low-fat milk.

Treatments involving combinations of hydrostatic pressure, time, temperature,
and pediocin AcH (an antimicrobial peptide) were evaluated against *E. coli* O157:H7
(Kalchayanand et al. 1998). Cell death increased as pressure, time, or temperature
increased. *E. coli* O157:H7 was found to be the most pressure-resistant when compared
to *Salmonella* Typhimurium, *Serratia liquefaciens*, and *Pseudomonas fluorescens*. Pediocin AcH was found to enhance the effectiveness of HP.

Another study explored the effect of HP (615 MPa) in combination with low temperature (15ºC) on various strains of *E. coli* O157:H7 in grapefruit, orange, apple, and carrot juice (Teo et al. 2001). An *E. coli* three-strain cocktail (SEA13B88, ATCC 43895, and 932) was pressure treated for 2 min and was found to be most pressure resistant in apple juice (0.41 log reduction), but most sensitive in grapefruit juice (8 log reduction). No injured survivors were detected in grapefruit or carrot juice under similar treatment conditions. This suggests that a low-temperature, HP treatment may be sufficient to inactivate *E. coli* O157:H7. However, this is highly dependent on the food matrix.

Alpas et al. (2000) investigated various interactions between HP and pressurization temperature, time, and pH, on death and injury of *E. coli* O157:H7. Viability loss increased as the level of pressure and temperature increased. *E. coli* O157:H7 showed an 8-log cycle reduction when pressurized at 345 MPa at 50ºC. Pressurization in the presence of citric or lactic acid increased the inactivation by an additional 1.2-3.9 log cycles. Therefore, mild heat and acidity increase the effectiveness of HP processing. Pagan et al. (2001) also found that pressure damaged *E. coli* O157:H7 cells are more acid sensitive than native cells. It was suggested that acid exposure may contribute to the loss of protective or repair functions. These results are most applicable to the production of liquid foods with low pH.
1.4.4 Strain variability

Variation in strain sensitivity to a certain process is an important consideration when designing processing technologies. These technologies should be effective against the most resistant strain of the microorganism of concern.

Microorganisms vary considerably in resistance to pressure. This variability is commonly observed not only among genera and species, but also among strains of the same species (Patterson et al. 1995; Alpas et al. 1999; Benito et al. 1999; Tay et al. 2003; Malone et al. 2006). Differences in pressure resistance were observed among strains of both Gram-positive and Gram-negative pathogens including *L. monocytogenes* (Alpas et al. 1999; Tay et al. 2003), *Staphylococcus aureus* (Alpas et al. 1999), *Salmonella* spp. (Alpas et al. 1999), and *E. coli* O157:H7 (Alpas et al. 1999; Benito et al. 1999; Malone et al. 2006). The variation in pressure resistance among bacterial strains is largely demonstrated when the cells are in stationary phase of growth. Bacterial strains that are in exponential phase tend to exhibit similar pressure sensitivity profiles (Benito et al. 1999; Pagan and Mackey 2000; Mañas and Mackey 2004). In addition, Alpas et al. (1999) indicated that differences in pressure resistance among strains exist mainly at lower temperature (25°C) and not at 50°C. Therefore, for any given strain, pressure resistance is influenced by the physiological state of the cells (Mañas and Mackey 2004).

1.4.5 Tailing

Pressure-processed food is typically treated in the range of 200 to 800 MPa, for a few minutes, in order to kill microorganisms of concern in that particular food. Pressure lethality of microorganisms rarely obeys first-order kinetics, and often results in a
microbial inactivation pattern in which a small fraction of the microbial population remains viable, even after prolonged processing. These “survivor tails” give rise to practical problems if manufacturers are considering the development of processes to commercially sterilize food or eliminate pathogens.

Heinz and Knorr (1996) observed the characteristic tailing of the inactivation curves for every pressure-temperature combination tested when vegetative cells of *Bacillus subtilis* were pressurized at 200-450 MPa and 20-40°C. These tailing effects were also shown during inactivation of *Lactobacillus plantarum* treated at 250 MPa (Wouters et al. 1998) and *Salmonella* Typhimurium at 340 MPa (Metrick et al. 1989). Excessive pressure treatment, such as 800 MPa at 30°C for 5 min, failed to eliminate the tailing phenomenon of a barotolerant *L. monocytogenes* strain (Tay et al. 2003). However, these studies indicated that the tailing effect is more pronounced with milder pressure treatments.

Tailing behavior of microorganisms is also observed in pressure-treated food media. Chen and Hoover (2003) noted tailing in all survivor curves of *L. monocytogenes* Scott A treated with HP at four temperatures (22, 40, 45, and 50°C) and two pressure levels (400 and 500 MPa) in ultra-high temperature processed whole milk. The biphasic pattern of inactivation curves, i.e., initial rapid inactivation followed by a slow inactivation phase, also resulted when *E. coli* was pressurized at 250-400 MPa and 5°C in liquid whole egg (Lee et al. 2001). Therefore, the HP-induced tailing phenomenon is commonly exhibited among a variety of microorganisms and under a range of pressure treatment conditions.
Several hypotheses, including clumping, stress adaptation, and genetic heterogeneity, are proposed as possible causes of the tailing phenomenon during microbial inactivation. Researchers suggest that tailing is due to phenotype variations in the resistance of individual cells of the same population (Metrick et al. 1989; Hauben et al. 1997; Karatzas and Bennik 2002; Noma et al. 2006). Metrick et al. (1989) observed the existence of a relatively pressure-resistant sub-population upon pressurizing Salmonella Typhimurium. However, when isolates derived from this apparent barotolerant subfraction were cultured, no significant differences in pressure resistance, compared to the original culture, were observed (Metrick et al. 1989). In contrast, other researchers observed higher barotolerance for isolates from the tailing portion of E. coli MG1655 (Hauben et al. 1997), L. monocytogenes Scott A (Karatzas and Bennik 2002), and E. coli O157:H7 (Noma et al. 2006), compared to that of the original culture. These studies suggest that the elevated level of barotolerance observed in the isolates may be due to the presence of a genetically pressure-resistant subpopulation. This pressure resistance is a stable phenotype rather than a short-lived adaptation.

As previously mentioned, stress adaptation may be a contributor to the phenomenon of “tailing”. If a microorganism is subjected to a sublethal stress and subsequently exhibits a greater resistance to that same stress, this microorganism has developed a stress adaptation. An E. coli O157:H7 culture exposed to peroxide showed a substantial increase in peroxidative tolerance compared to a negative control, i.e., a culture not exposed to the peroxide-based sanitizer (Zook et al. 2001). Extremely pressure-resistant strains of E. coli O157:H7 have been generated by multiple rounds of exposure to HP followed by the selection of survivors (Hauben et al. 1997).
Understanding tailing, stress resistance, and adaptation of a microorganism to food processes is critical to creating safe processes and high quality food products.

**1.4.6 Stress response**

Welch et al. (1993) reported that several heat-shock and cold-shock proteins were induced in *E. coli* treated with sublethal HP (55 MPa), suggesting a direct role of the stress response in HP resistance. Aertsen et al. (2004) also indicated that the gene expression of the heat-shock regulon (*dnaK, lon, clpPX*) was increased after pressurization (75-150 MPa) of *E. coli*, and that the induction of heat-shock genes resulted in barotolerance. In addition, heat-shock mediated pressure resistance was also reported in *Saccharomyces cerevisiae* (Iwahashi et al. 2001). Similarly, elevated levels of cold-shock proteins were observed upon pressurization of *L. monocytogenes* (Wemekamp-Kamphuis et al. 2002). Correspondingly, this cold-shock response was related to the increased pressure resistance of *L. monocytogenes* (Wemekamp-Kamphuis et al. 2002) and *Staphylococcus aureus* (Noma and Hayakawa 2003).

Higher barotolerance of stationary-phase cells is partly correlated to RpoS, a major sigma factor during stationary phase that controls the expression of genes responding to starvation and cellular stresses (Lacour and Landini 2004) in *E. coli* (Robey et al. 2001; Aertsen et al. 2004). Considering these findings, the bacterial stress response that is induced by pressurization may increase cell barotolerance.
1.4.7 Cross protection

A phenomenon known as “cross protection” occurs when a microbial population is subjected to an initial environmental stress, which is subsequently replaced with an unrelated stress, and the resulting microbial population displays increased resistance compared to the original unstressed population (Rowe and Kirk 1999). This phenomenon is critical to the food industry because most food processing conditions are developed with the assumption that healthy microorganisms represent the most resilient bacterial population. The concept of cross protection calls this assumption into question.

In a food manufacturing facility, bacteria are exposed to a variety of environmental stresses which may inadvertently result in a cross-protected population. A study of *E. coli* O157:H7 exposed to commercially available alkaline cleaners and subsequent heat and sanitizers demonstrated cross-protective responses (Sharma and Beuchat 2004). Wild-type cells of *E. coli* O157:H7 that survived treatment with alkaline cleaners containing sodium hydroxide and sodium hypochlorite at 4°C and 23°C had increased thermal tolerance compared to cells exposed to peptone or a cleaner containing ethylene glycol monobutyl ether (Sharma and Beuchat 2004). Certain stresses, such as acid, starvation, and cold, affect the post-stress behavior of *E. coli* O157:H7 (Leenanon and Drake 2001). For example, resistance of *E. coli* O157:H7 to freeze-thaw and heat was enhanced after acid adaptation and starvation. Likewise, if *E. coli* O157:H7 is exposed to a cold stress, heat resistance of the bacterium decreases, while freeze-thaw resistance increases. Heat and freeze-thaw resistance of an *rpoS* mutant increases only after acid adaptation. The cross-protective response has been shown to be primarily mediated by the *rpoS* gene (Rowe and Kirk 1999). *E. coli* O157:H7 developed a cross-
protective response to salt when exposed to a medium with low pH (Garren et al. 1998; Rowe and Kirk 1999).

With the introduction of new or alternative processing technologies, such as HP, into manufacturing environments, the potential for bacteria to acquire cross-protective characteristics exists. This may result in the survival of a residual *E. coli* O157:H7 population containing cross-protective qualities; such a population may be resistant to current plant sanitation procedures, thus creating a food safety threat. Therefore, physiological studies are required to develop a comprehensive picture of the bacterial cell physiological responses to stress. With this information under consideration, processing parameters can be established to minimize cross-protective responses and thus, ensure a safe food product. Lastly, since antibiotic resistance of pathogens is a world-wide concern today, it is necessary to determine whether there may be a cross-protective response to antibiotics after being exposed to an alternative food processing technologies, such as HP.

### 1.4.8 Mechanism of inactivation

The mechanism of microbial inactivation by HP remains in question; however, studies suggest that HP inactivation involves numerous targets in the cell. The changes induced by pressure treatment of microbial cells include alterations in cell morphology and membrane, and alterations in proteins, ribosomes, and the genetic material of microorganisms (Malone et al. 2002; Kaletunç et al. 2004; Mañas and Mackey 2004). Although these changes may not lead directly to cell death, noticeable physical disruption of the cell membrane, such as the formation of vesicles, engrossment of areas in the
membrane, and invaginations toward the cytoplasm, was observed after the pressurization of *E. coli* cells at 100-200 MPa (Mañas and Mackey 2004). These researchers, however, mentioned that these alterations in cell membrane were noticeable in exponential phase, not in stationary-phase cells, inferring a difference in membrane compressibility between those growth phases of cells. Beney et al. (1997) also indicated that pressure (> 200 MPa) resulted in structural perturbations of the phospholipid membrane vesicles and this occurred due to the difference in compressibility between the membrane and the water in the cell cytosol. According to these authors, compressible membrane vesicles produce blebs upon decompression, as a result of losing some of their aqueous content during pressurization, consequently leading to an excess of membrane material in relation to the remaining aqueous content. Vesicles containing cholesterol, which have less compressible membranes, produced a smaller volume decrease and did not form blebs upon decompression (Beney et al. 1997). In addition, similar morphological changes were induced after pressure treatment of various bacterial cells. Membrane invagination was observed in *L. monocytogenes* (Mackey et al. 1994), *Salmonella Typhimurium* (Tholozan et al. 2000), and *Lactococcus lactis* (Malone et al. 2002), and formation of bud scars and surface blisters was noted also in *L. monocytogenes* (Ritz et al. 2001) and *Leuconostoc mesenteroides* (Kaletunç et al. 2004) after pressurization.

Many researchers agree that membrane damage is one of the causes of bacterial inactivation by pressure (Pagán and Mackey 2000; Gänzle and Vogel 2001; Ritz et al. 2001; Russell 2002; Mañas and Mackey 2004). The phase transition of membrane lipids from liquid crystalline to gel, which is typically observed following a temperature downshift, was reported after HP treatment (MacDonald 1993). Kato and Hayashi (1999)
also suggested that HP induces phase transition of natural membranes, which leads to decrease in membrane fluidity, and this may result in breakage of the membrane. Although phase transition of membrane lipids is not necessarily lethal to bacteria, it has been demonstrated that membrane fluidity is linked to increased pressure resistance (Casadei et al. 2002). Similarly, Braganza and Worcester (1986) indicated that HP increases the packing density of membrane lipids and induces phase separations due to differences in compressibility between lipids and proteins in cell membrane.

Studies also indicated that HP targets some essential membrane-bound enzymes (Wouters et al. 1998; Ulmer et al. 2000, 2002). These researchers found that membrane-bound enzymes including HorA (ATP-dependent multidrug resistant transporter), LmrP (proton motive force-dependent transport enzyme), and F_{0}F_{1} ATPase (proton-translocating ATPase) were inactivated when \textit{Lb. plantarum} and \textit{Lc. lactis} cells were treated with pressure. In earlier observations, Silvius and McElhaney (1980) found that membrane-bound ATPase is inactivated when boundary lipids of \textit{Acholeplasma laidlawii} B undergo a phase transition from liquid-crystalline to gel state, and this lipid-phase transition is also induced by the pressure as mentioned earlier. In addition, gel electrophoresis analysis of membrane proteins of \textit{Salmonella} Typhimurium revealed that outer membrane protein profiles were markedly modified after pressure treatment at 350-600 MPa (Ritz et al. 2000).

Loss of membrane integrity was directly related to the pressure-induced death of \textit{E. coli} cells in exponential phase, whereas membranes in stationary-phase cells remained physically intact even in dead cells, demonstrating transient permeability of the membrane (Pagán and Mackey 2000; Mañas and Mackey 2004). Similarly, reversible
outer membrane damage was also observed in *E. coli* cells in other studies (Hauben et al. 1996; Gänzle and Vogel 2001).

Other factors also have been proposed for pressure-induced cell death, especially in the case of stationary-phase cells. Mañas and Mackey (2004) reported nucleoid condensation and protein aggregation inside the cytoplasm of *E. coli* cells. Furthermore, changes in ribosomal conformation were correlated with the pressure-caused loss of cell viability in *E. coli* (Niven et al. 1999) and *L. mesenteroides* (Kaletunç et al. 2004).

Lastly, HP induced an SOS response in *E. coli* (Aertsen et al. 2004). In a study using *E. coli* and DNA microarray analysis, Ishii et al. (2005) reported that heat- and cold- stress responses were induced simultaneously by elevated pressure and that gene expression adjusts to such environmental changes through regulation by several DNA-binding proteins. Thus, the H-NS protein is thought to be a possible regulator for cell adaptation to HP stress. Furthermore, a recent report demonstrated that HP induces a unique oxidative stress (Aertson et al. 2004). Despite these multiple studies, the mechanism of microbial inactivation or resistance to HP is not clear. This study is an attempt to investigate in depth the physiological response of *E. coli* O157:H7 to HP.

1.5 Summary

The importance of eliminating *E. coli* O157:H7 from food systems, especially considering its high degree of virulence and resistance to environmental stresses, substantiates the need to understand the physiological resistance of this foodborne pathogen to emerging food preservation methods. Currently, the mechanism of inactivating enterohemorrhagic *E. coli* by HP is not well understood. Therefore, the
The purpose of this study is to elucidate the genetic mechanisms of processing resistance of \textit{E. coli} O157:H7. The viability and resistance of \textit{E. coli} to alternative food processing technologies, including pulsed electric field, $\gamma$ radiation, ultraviolet radiation, and combination treatments involving food grade additives, were also studied. The results of this research will provide food processors with the knowledge necessary to optimize processing conditions in order to produce safer food products.

1.6 References


CHAPTER 2

GENES OF ESCHERICHIA COLI O157:H7 INVOLVED IN HIGH PRESSURE RESISTANCE

2.1 Abstract

Seventeen *Escherichia coli* O157:H7 strains were treated with high pressure (HP) at 500 MPa and 23±2°C for 1 min. This treatment inactivated 0.6 to 3.4 log CFU/ml, depending on the strain. Diversity of these strains was confirmed by pulsed-field gel electrophoresis (PFGE) analysis, and there was no apparent association between PFGE banding pattern and pressure resistance. The pressure-resistant *E. coli* O157:H7 EC-88 (0.6 log decrease) and pressure-sensitive ATCC 35150 (3.4 log decrease) were treated with a sub-lethal pressure (100 MPa for 15 min at 23±2°C) and subjected to DNA microarray analysis using *E. coli* K12 antisense gene chip. HP affected the transcription of many genes involved in a variety of intracellular mechanisms of EC-88, including the stress response, the thiol-disulfide redox system, Fe-S cluster assembly, and spontaneous mutation. Twenty-four *E. coli* isogenic pairs with mutations in the genes regulated by the pressure treatment were treated with lethal pressures at 400 MPa and 23±2°C for 5 min. The barotolerance of the mutants relative to that of the wild-type strains helped to explain the results obtained by DNA microarray analysis. This study is the first report to demonstrate that the expression of Fe-S cluster assembly proteins and the fumarate nitrate reductase regulator (FNR) decrease the resistance to pressure, while sigma factor (RpoE),
lipoprotein (NlpI), thioredoxin (TrxA), thioredoxin reductase (TrxB), trehalose synthesis (OtsA), and DNA-binding protein (Dps) promote barotolerance.

2.2 Introduction

*Escherichia coli* O157:H7 has emerged in the past two decades as a significant foodborne pathogen. This bacterium causes hemorrhagic colitis and hemolytic-uremic syndrome, which may lead to renal failure and death (Paton and Paton 1998). Most *E. coli* O157:H7 infections occur through consumption of contaminated food and water. Ground beef, milk, apple juice, produce, and foods that have been stored, cooked, or handled improperly are potential transmission sources of *E. coli* O157:H7. The incidence of this pathogen in many food sources and its low infectious dose are causes of major concern among food processors and regulatory agencies.

Non-conventional technologies, such as those utilizing high pressure (HP), have been introduced as alternatives to heat and other traditional food preservation methods. These alternative technologies are potentially useful in producing safe food without adversely affecting its sensory properties or its freshness attributes. Currently, HP is used commercially to process fruit juices, purees, guacamole, desserts, sauces, oysters, rice dishes, and packaged cured ham (San Martin et al. 2002). One of the challenges associated with HP technology is that different strains of pathogenic bacteria, such as *E. coli* O157:H7, exhibit great variability in resistance to the treatment (Benito et al. 1999).

Several research groups investigated the mechanism of bacterial lethality by HP. According to a recent study, HP induces oxidative stress and the SOS response in *E. coli* (Aertsen et al. 2004a; Aertsen et al. 2005). The loss of *rpoS*, a gene that codes for the
The cell membrane is believed to be the prime target for HP; however, the nature of membrane damage and its relation to cell death may depend on the species and phase of growth (Pagan and Mackey 2000). Recently, *E. coli* was grown at 30 and 50 MPa, and the transcriptional effect of these pressures was analyzed using a DNA microarray (Ishii et al. 2005). According to that report, heat and cold stress responses were induced simultaneously by the elevated pressures. In addition, an *hns* mutant exhibited increased pressure sensitivity. The *hns* gene encodes a histone-like nucleoid structuring protein (H-NS) which preferentially binds to curved DNA sequences, thus contributing to DNA compactness, acts as a versatile transcriptional repressor, and plays a role as an expression modulator of some genes post transcriptionally by affecting mRNA stability and the efficiency of translation (Schröder and Wagner 2002). Therefore, H-NS protein is a likely transcriptional regulator for genes related to the adaptation of *E. coli* to pressure.

In the current study, DNA microarray analysis was carried out to reveal the genes involved in the resistance of *E. coli* O157:H7 to pressure. The gene transcriptional profiles of pressure-treated (sublethally) and untreated bacteria were analyzed to elucidate the potential physiological response of this pathogen to pressure treatment.
Some of the results of the DNA microarray analysis were confirmed by assessing the pressure resistance (i.e., barotolerance) of selected *E. coli* knockout mutants.

### 2.3 Materials and methods

#### 2.3.1 Strains and sample preparation

Strains of *E. coli* O157:H7 were kindly provided by J. LeJeune, Ohio State University, Wooster, Ohio. These are FRIK-526, FRIK-528, FRIK-579, CL-56, EC-84, EC-88, EC-91, EC-92, EC-96, EC-100, EC-103, 93-001, and O-2191831. Additionally, *E. coli* O157:H7 ATCC 35150 and *E. coli* O157:H7 ATCC 43889 were used in this study. For comparative purposes, *E. coli* K12 (a non-pathogenic strain) and O157:H12 (a non-toxigenic strain, Richter International, Inc., Columbus, Ohio) were investigated. Strains EC-84, EC-88, EC-91, EC-96, EC-100, and EC-103 were isolated from cattle. The remaining pathogenic strains were originally isolated from human patients involved in disease outbreaks. For the study comparing isogenic pairs, twenty-four non-pathogenic *E. coli* mutants and their wild-type counterparts were kindly provided by the sources listed in Table 1. All strains were grown from a 0.1% inoculum in tryptose broth (TB; BD Difco, Sparks, Md.) at 35°C for 18 h. All cultures were transferred at least twice before experimentation.
<table>
<thead>
<tr>
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<th>Genotype /Description</th>
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<td>ybdQ⁺</td>
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Table 1. List of mutant and wild-type strains of non-pathogenic *E. coli* tested in this study.
Table 1 continued

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<td>W3110 tna2 ΔlacU169, G0</td>
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2.3.2 Barotolerance of pathogenic and non-pathogenic *E. coli* strains

*E. coli* strains, including O157:H7, O157:H12, and K12, were grown in 10 ml TB to stationary phase (~$1 \times 10^9$ CFU/ml, or an $A_{600}$ of ~0.9), washed in sterile phosphate buffer (12.5 mM, pH 7.2), and then resuspended in 10 ml phosphate buffer. Each sample was then aseptically transferred to a sterile stomacher bag (Fisher Scientific, Pittsburgh, Pa.), vacuum packaged, and the bag was heat sealed. The sample was pressure treated at 500 MPa and 23±2°C for 1 min in a HP processor (Quintus® QFP-6; Flow Pressure Systems, Kent, WA). The initial temperature of the pressure-transmitting fluid was controlled to account for compression heating (3 to 4°C/100 MPa). An untreated control was held at 23±2°C and atmospheric pressure (0.1 MPa) while each treated sample was being pressurized. After pressure treatment, samples were serially diluted using 0.1% peptone water, plated on tryptose agar plates, and incubated at 35°C for 48 h to enumerate the viable count. Similarly, barotolerance of the wild-type strains and mutants of non-pathogenic *E. coli* was determined; however, stationary-phase cultures were directly pressurized in TB at 400 MPa and 23±2°C for 5 min.

2.3.3 Statistical analysis of barotolerance results

For the barotolerance study of the pathogenic strains, the log survivor ratio was calculated as $\log \frac{N}{N_0}$, where $N$ is the CFU/ml of treated sample and $N_0$ is the initial (before treatment) CFU/ml. Pressure resistance of the isogenic non-pathogenic *E. coli* pairs was evaluated by calculating the differential log survivor ratio (DLSR), as follows: $\log (N/N_0)$ of mutant – $\log (N/N_0)$ of wild type, where $N$ is the CFU/ml of treated sample and $N_0$ is the initial (before treatment) CFU/ml. A positive DLSR indicates a
barotolerance that is greater for the mutant than for the wild type, and a negative DLSR indicates the opposite. Data shown are the results of at least three independent replications. Means were compared and standard deviations were calculated using commercial statistical programs (JMPin® [SAS Institute Inc., Cary, N.C.] and SigmaPlot®, version 9.0 [SPSS Inc., Chicago, Ill.]).

2.3.4 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as reported by Jenkins et al. (2002). Extracted DNAs were digested with XbaI (Invitrogen, Carlsbad, CA).

2.3.5 Culture preparation for DNA microarray analysis

*E. coli* O157:H7 EC-88 (pressure-resistant) and *E. coli* O157:H7 ATCC 35150 (pressure-sensitive) were cultured, and cell suspensions were prepared as indicated earlier. The bags of cell suspensions were pressure treated at 100 MPa and 23±2°C for 15 min. The untreated control suspensions were kept at atmospheric pressure (0.1 MPa) and 23±2°C during pressure processing of the treated suspensions.

2.3.6 RNA isolation

Total RNAs were isolated from control and pressure-treated *E. coli* O157:H7 cells by use of a commercial kit (Trizol® method; Invitrogen, Carlsbad, CA) as described by the manufacturer. Briefly, a pressure-treated or untreated *E. coli* O157:H7 cell suspension was centrifuged (10,000 × g for 10 min at 25°C), and 1 ml of Trizol reagent was added to the cell pellet. Trizol-treated cells were kept on ice, glass beads (~0.1 g; 0.1-mm
diameter) were added to the cell suspension, and the suspension was subjected to shaking for 10 s at maximum speed (Mini-Bead Beater™; Bio-Spec Products, Inc., Bartlesville, OK). The cell extract was recovered after centrifugation (10,000 × g for 10 min). RNAs were extracted with chloroform, precipitated with ethanol, and resuspended in diethyl pyrocarbonate-treated water. The RNA preparations were purified further using clean-up columns (RNeasy®; Qiagen Inc., Valencia, CA). RNase-free DNaseI was added to the columns to eliminate DNA contamination. The purity of the RNAs was determined by measuring the absorbance ratio A₂₆₀/A₂₈₀. In addition, each RNA sample was subjected to electrophoresis on a formaldehyde gel to confirm the integrity of the 16S and 23S rRNAs.

2.3.7 DNA microarray analysis

Total RNAs of pressure-treated and control samples were subjected to DNA microarray analysis using *E. coli* K-12 antisense gene chip (Affymetrix, Inc., Santa Clara, CA). The time between the end of pressure treatment and the addition of the Trizol reagent to the treated cells was approximately 15 min. After the pressure treatment and RNA isolation, cDNAs were synthesized according to the protocol supplied by Affymetrix and described by Soupene et al. (2003). The resulting cDNAs were purified with a PCR purification kit (Qiagen, Inc.). The cDNAs (1.5 μg) were then fragmented with DNase I, and the 3’ termini of the fragments were labeled with biotin-ddUTP, using a terminal deoxynucleotide transferase. The hybridization of the fragmented, biotinylated cDNAs to an *E. coli* K12 antisense gene chip and the washing, staining, and scanning of the chip were carried out as described by the chip supplier (Affymetrix Expression
Analysis Technical Manual). The arrays were scanned with a Gene Chip Scanner 3000 (Affymetrix, Santa Clara, CA) at the Heart and Lung Research Institute, Microarray-Genetics Core Laboratory (The Ohio State University, Columbus, OH).

2.3.8 DNA microarray data analysis

Fluorescence intensities of the hybridized chips and numerous quality control parameters were stored in a database. Image analyses and normalization were done using commercial and web-based software (Gene Chip Operating Software v. 1.1 [Affymetrix] and Gene Publisher [http://www.cbs.dtu.dk/services/GenePublisher/]) (Knudsen et al. 2003). The quality of the images was inspected, and images were set up properly (gridding, segmentation, intensity extraction, and background correction) as recommended by the Heart and Lung Institute DNA Microarray Core staff (The Ohio State University, Columbus, OH). Inspection ensured that images were free from defects (e.g., amorphous blobs or masking). Fluorescence intensities for each chip were normalized by applying a single scaling factor of a target intensity of 1,500, which assumes the total amount of mRNA in a cell is constant. Before performing statistical analysis, a filtering process was applied to narrow the gene pool analyzed. The selection criteria to filter out noncritical genes were as follows: (i) all intergenic regions were eliminated; (ii) genes with absent calls were eliminated; (iii) average signal intensities and log_{10} changes [log (treated mean intensity/control mean intensity)] were calculated from a minimum of three independent samples of the filtered genes, and genes for which the log change (x) was in the range of \(-0.15<x<0.15\) were eliminated. The final filtered genes were statistically evaluated for differential expression levels. Therefore, average
signal intensities for each gene in the control and treated samples were determined and compared statistically using the t-test (Excel; Microsoft Inc., Redmond, WA). A volcano plot was constructed to test the relationship between $P$ values and expression changes. The Benjamini and Hochberg false discovery rate (FDR) was applied to correct for multiple testing (Benjamini and Hochberg 1995). These differentially expressed genes were identified and their sequences were examined for homology with genes of known function by using public domain software programs, including GenProtEC, Pubmed (http://www.ncbi.nlm.nih.gov), and Affymetrix Inquery (Affymetrix, Inc.). Certain genes linked to the physiological process of interest were identified.

2.4 Results

2.4.1 Barotolerance of *E. coli* O157 strains

The barotolerance of *E. coli* strains, including O157:H7, O157:H12, and K-12 strains, was determined by comparing viabilities before and directly after a 1 min lethal treatment at 500 MPa and 23±2°C (Figure 1). *E. coli* ATCC 35150 was the least resistant to HP, with a 3.4 log reduction, while EC-88 was one of the most resistant strains, with only a 0.6 log reduction ($P < 0.05$).
Figure 1. LSR \( \{\log_{10} (N/N_0)\} \) of E. coli strains that were grown to stationary phase at 35°C, washed and resuspended in phosphate buffer, and treated for 1 min at 500 MPa and 23°C. Error bars represent ±1 S. D. \((n \geq 3)\).
2.4.2 Genotyping by pulsed-field gel electrophoresis

The sixteen *E. coli* O157 strains and *E. coli* K-12 were compared by PFGE analysis. The genetic similarities among these strains, as judged by the PFGE banding patterns, ranged from 34 to 85% (Appendix A). There was no apparent association between pressure resistance and genetic similarity. *E. coli* O157:H7 EC-88 and ATCC 35150, which were very different in barotolerance, also produced diverse PFGE banding patterns. Therefore, these two strains were further analyzed for gene transcription in response to HP, using DNA microarray analysis.

2.4.3 Gene transcriptional analysis after pressure treatment

More than 100 genes responded to sublethal pressure treatment of stationary-phase, barotolerant *E. coli* O157:H7 EC-88; however, only 36 genes passed the Benjamini and Hochberg corrections for multiple testing (Benjamini and Hochberg 1995). Responses of genes with corrected *P* values of <0.05 were considered significant (Tables 2 and 3). In addition, genes with regulatory or functional similarity were analyzed, despite their marginal significance (marginal values were corrected *P* values of ≥0.05 to <0.10). The sublethal pressure treatment (100 MPa for 15 min at 23±2°C) was sufficient to induce a pressure stress response, but not so severe as to inactivate the cells or prevent transcription.

The DNA-binding protein genes *uspA*, *yiiT*, *ybdQ*, *ynaF*, and *dps* were significantly down-regulated, by 1.8-, 2.0-, 2.1-, 2.4-, and 2.5-fold, respectively (Table 2). In contrast, *yecG* had no change in transcription. Transcription of the stress response genes *hslV*, *ibpA*, *pphA*, and *cspA* significantly increased, by 1.5-, 1.9-, 2.0-, and 3.9-fold,
respectively, due to the pressure treatment (Table 3); however, \textit{ibpB} was not affected. In addition, genes involved in the thiol-disulfide redox system, \textit{trxA}, \textit{grxA}, and \textit{nrdH}, were marginally up-regulated, by 1.4-, 1.4-, and 1.6-fold, respectively, while \textit{nrdI} was significantly up-regulated (1.5-fold). In contrast, \textit{trxB}, \textit{grxB}, \textit{grxC}, \textit{nrdE}, and \textit{nrdF} showed no evident change in transcription. The Fe-S-related genes \textit{fnr} and \textit{iscR} were significantly up-regulated, by 1.5- and 1.8-fold, respectively, while \textit{sufS} was significantly down-regulated, by 1.9-fold. Interestingly, the entire \textit{suf} operon (\textit{sufABCDSE}) appeared to be down-regulated, while \textit{iscU}, \textit{hscA}, and \textit{fdx} had no significant change. The transcription of genes involved in spontaneous mutation (\textit{dinB} and \textit{yafP}) did not change in response to the pressure treatment. However, \textit{yafO} and \textit{yafN}, which are also involved in spontaneous mutation, were significantly up-regulated (1.5-fold) and marginally up-regulated (1.6-fold), respectively. Miscellaneous genes, such as \textit{nlpI} and \textit{rbsD}, were significantly up-regulated, by 1.9- and 4.0-fold, respectively, while \textit{rpoE}, \textit{hns}, and \textit{otsB} were marginally up-regulated, by 1.5-, 1.5-, and 2.1-fold, respectively. On the other hand, \textit{eno} and \textit{yfiD} were significantly down-regulated, by 2.0- and 2.2-fold, respectively, and \textit{rpoS} and \textit{stpA} showed no change. Additional miscellaneous genes were significantly up-regulated; these are \textit{yebF}, \textit{yibF}, \textit{yafQ}, \textit{ycfJ}, \textit{rpsT}, \textit{infra}, \textit{rpsU}, \textit{rhoL}, and \textit{glyY}. The sublethal pressure treatment significantly down-regulated the following additional miscellaneous genes: \textit{ygjR}, \textit{ygcO}, \textit{rbn}, \textit{yniA}, \textit{ycbB}, \textit{ynhG}, \textit{yneA}, \textit{yccJ}, and \textit{ychH}.

The pressure-sensitive strain ATCC 35150 also showed transcriptional changes in response to the pressure treatment similar to those of EC-88. However, after performing the Benjamini and Hochberg FDR method for multiple testing, no significant changes (corrected \textit{P} values of <0.05) in the expression of ATCC 35150 genes in response to the
pressure treatment were confirmed (data not shown). The raw DNA microarray data for both strains are available at The Ohio State University Food Safety Laboratory website (http://www.fst.osu.edu/foodsafetlab/index.htm).
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<th>Gene and functional category&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>0.0083</td>
<td>0.0495</td>
<td>1.9</td>
</tr>
<tr>
<td>ybdQ</td>
<td>Universal stress protein, flavoprotein (UP12)</td>
<td>0.00074</td>
<td>0.022</td>
<td>2.1</td>
</tr>
<tr>
<td>ynaE</td>
<td>Putative electron transfer flavoprotein (UP03)</td>
<td>0.029</td>
<td>0.095</td>
<td>2.4</td>
</tr>
<tr>
<td>Dps</td>
<td>Stress response DNA-binding protein</td>
<td>0.0073</td>
<td>0.047</td>
<td>2.5</td>
</tr>
<tr>
<td>yecG</td>
<td>Putative electron transfer protein (UspC)</td>
<td>0.84</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td>pphB</td>
<td>Serine/threonine-specific protein phosphatase 2</td>
<td>0.674</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td>ibpB</td>
<td>Small heat shock protein</td>
<td>0.98</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Thiol-disulfide redox system</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trxB</td>
<td>Thioredoxin reductase, FAD/NAD(P) binding</td>
<td>0.70</td>
<td>*</td>
<td>1.1</td>
</tr>
<tr>
<td>grxB</td>
<td>Glutaredoxin 2</td>
<td>0.15</td>
<td>*</td>
<td>1.2</td>
</tr>
<tr>
<td>grxC</td>
<td>Glutaredoxin 3</td>
<td>0.95</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Fe-S cluster status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sufS</td>
<td>Selenocysteine lyase, PLP-dependent</td>
<td>0.0028</td>
<td>0.034</td>
<td>1.9</td>
</tr>
<tr>
<td>sufA</td>
<td>Fe-S assembly protein</td>
<td>0.18</td>
<td>*</td>
<td>1.3</td>
</tr>
<tr>
<td>sufB</td>
<td>Putative transport protein associated with Fe-S cluster assembly</td>
<td>0.12</td>
<td>*</td>
<td>1.3</td>
</tr>
<tr>
<td>sufC</td>
<td>Putative transport protein associated with Fe-S cluster assembly</td>
<td>0.18</td>
<td>*</td>
<td>1.3</td>
</tr>
<tr>
<td>sufD</td>
<td>Required for stability of Fe-S component of FhuF protein</td>
<td>0.18</td>
<td>*</td>
<td>1.3</td>
</tr>
<tr>
<td>sufE</td>
<td>Stimulator of SufS activity</td>
<td>0.13</td>
<td>*</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Spontaneous mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dinB</td>
<td>Error-prone DNA polymerase IV</td>
<td>0.97</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td>yafP</td>
<td>Conserved protein with possible role in spontaneous mutation</td>
<td>0.45</td>
<td>*</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eno</td>
<td>Enolase</td>
<td>0.00090</td>
<td>0.020</td>
<td>2.0</td>
</tr>
<tr>
<td>yfiD</td>
<td>Putative formate acetyltransferase</td>
<td>0.0093</td>
<td>0.049</td>
<td>2.2</td>
</tr>
<tr>
<td>rpoS</td>
<td>Sigma S factor of RNA polymerase, major sigma factor during stationary phase</td>
<td>0.26</td>
<td>*</td>
<td>1.1</td>
</tr>
<tr>
<td>sfpA</td>
<td>DNA-bending protein with chaperone activity</td>
<td>0.40</td>
<td>*</td>
<td>1.1</td>
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<tr>
<td>ygiR</td>
<td>Putative NAD(P)-binding dehydrogenase</td>
<td>0.0088</td>
<td>0.049</td>
<td>1.4</td>
</tr>
<tr>
<td>ygcO</td>
<td>Putative 4Fe-4S ferrodoxin-type protein</td>
<td>0.0098</td>
<td>0.0499</td>
<td>1.4</td>
</tr>
<tr>
<td>Rbn</td>
<td>tRNA processing exoribonuclease BN</td>
<td>0.0071</td>
<td>0.047</td>
<td>1.5</td>
</tr>
<tr>
<td>ymiA</td>
<td>Protein kinase-like</td>
<td>0.0043</td>
<td>0.043</td>
<td>1.9</td>
</tr>
<tr>
<td>ycbB</td>
<td>Carboxypeptidase</td>
<td>0.0049</td>
<td>0.044</td>
<td>2.2</td>
</tr>
<tr>
<td>ynhG</td>
<td>ATP synthase subunit</td>
<td>0.0085</td>
<td>0.049</td>
<td>2.3</td>
</tr>
<tr>
<td>yneA</td>
<td>Sugar transport protein (ABC superfamily, periplasmic binding protein)</td>
<td>0.0062</td>
<td>0.044</td>
<td>2.3</td>
</tr>
<tr>
<td>yccJ</td>
<td>Unknown CDS</td>
<td>0.0036</td>
<td>0.038</td>
<td>3.0</td>
</tr>
<tr>
<td>ychH</td>
<td>Involved in peptidyl tRNA hydrolase activity</td>
<td>0.000082</td>
<td>0.015</td>
<td>3.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> *, genes did not pass the filtering process.

<sup>b</sup> The genes were grouped according to Ecocyc (2005).

Table 2. Genes that were down-regulated in response to HP (100 MPa for 15 min at 23±2°C) in *E. coli* O157:H7 EC-88.
<table>
<thead>
<tr>
<th>Gene and functional category(^b)</th>
<th>Relevant function</th>
<th>(P) value (t test)</th>
<th>Corrected (P) value (FDR)(^a)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress response</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hslV</em></td>
<td>Peptidase component of the HslUV protease</td>
<td>0.0014</td>
<td>0.023</td>
<td>1.5</td>
</tr>
<tr>
<td><em>ibpA</em></td>
<td>Small heat shock protein</td>
<td>0.0053</td>
<td>0.043</td>
<td>1.9</td>
</tr>
<tr>
<td><em>pphA</em></td>
<td>Serine/threonine-specific protein phosphatase 1, signals protein misfolding</td>
<td>0.000094</td>
<td>0.0084</td>
<td>2.0</td>
</tr>
<tr>
<td><em>cspA</em></td>
<td>Major cold shock protein 7.4, transcription antiterminator of <em>hns</em>, ssDNA-binding property</td>
<td>0.0011</td>
<td>0.020</td>
<td>3.9</td>
</tr>
<tr>
<td>Thiol-disulfide redox system</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>trxA</em></td>
<td>Thioredoxin 1, redox factor, carrier protein</td>
<td>0.039</td>
<td>0.0991</td>
<td>1.4</td>
</tr>
<tr>
<td><em>grxA</em></td>
<td>Glutaredoxin 1</td>
<td>0.011</td>
<td>0.051</td>
<td>1.4</td>
</tr>
<tr>
<td><em>trxC</em></td>
<td>Thioredoxin 2</td>
<td>0.083</td>
<td>0.14</td>
<td>2.2</td>
</tr>
<tr>
<td><em>nrdI</em></td>
<td>Stimulates ribonucleotide reduction</td>
<td>0.0089</td>
<td>0.048</td>
<td>1.5</td>
</tr>
<tr>
<td><em>nrdH</em></td>
<td>Glutaredoxin-like protein</td>
<td>0.036</td>
<td>0.0995</td>
<td>1.6</td>
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<tr>
<td><em>nrdE</em></td>
<td></td>
<td>0.64</td>
<td>*</td>
<td>1.1</td>
</tr>
<tr>
<td><em>nrdF</em></td>
<td></td>
<td>0.72</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe-S cluster status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fnr</em></td>
<td>Transcriptional regulator of aerobic, anaerobic respiration, osmotic balance (cAMP-binding family)</td>
<td>0.0053</td>
<td>0.045</td>
<td>1.5</td>
</tr>
<tr>
<td><em>iscR</em></td>
<td>Repressor of the <em>iscRSUA</em> operon, involved in assembly of Fe-S clusters</td>
<td>0.0054</td>
<td>0.042</td>
<td>1.8</td>
</tr>
<tr>
<td><em>iscU</em></td>
<td>Fe-S cluster template protein</td>
<td>0.10</td>
<td>*</td>
<td>1.2</td>
</tr>
<tr>
<td><em>hscA</em></td>
<td>Chaperone (Hsp70 family), involved in assembly of Fe-S clusters</td>
<td>0.98</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Fdx</em></td>
<td>[2Fe-2S] ferredoxin, electron carrier protein, involved in assembly of Fe-S clusters</td>
<td>0.89</td>
<td>*</td>
<td>1.0</td>
</tr>
<tr>
<td>Spontaneous mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>yafO</em></td>
<td>Conserved protein with possible role in spontaneous mutation</td>
<td>0.0044</td>
<td>0.042</td>
<td>1.5</td>
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<tr>
<td><em>yafN</em></td>
<td>Conserved protein with possible role in spontaneous mutation</td>
<td>0.022</td>
<td>0.082</td>
<td>1.6</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>nlpI</em></td>
<td>NlpI lipoprotein</td>
<td>0.00062</td>
<td>0.028</td>
<td>1.9</td>
</tr>
<tr>
<td><em>rbsD</em></td>
<td>Membrane-associated component of high-affinity D-ribose transport system, membrane</td>
<td>0.0023</td>
<td>0.034</td>
<td>4.0</td>
</tr>
<tr>
<td><em>rpoE</em></td>
<td>Sigma E factor of RNA polymerase, response to periplasmic stress</td>
<td>0.039</td>
<td>0.098</td>
<td>1.5</td>
</tr>
<tr>
<td><em>Hns</em></td>
<td>Transcriptional regulator, DNA-binding protein HLP-II, increase DNA thermal stability</td>
<td>0.49</td>
<td>0.49</td>
<td>1.5</td>
</tr>
<tr>
<td><em>otsB</em></td>
<td>Trehalose-6-phosphate phosphatase, osmoregulation</td>
<td>0.028</td>
<td>0.097</td>
<td>2.1</td>
</tr>
<tr>
<td><em>yebF</em></td>
<td>Conserved protein</td>
<td>0.0011</td>
<td>0.023</td>
<td>1.4</td>
</tr>
<tr>
<td><em>yibF</em></td>
<td>Putative glutathione S-transferase enzyme</td>
<td>0.00062</td>
<td>0.022</td>
<td>1.5</td>
</tr>
<tr>
<td><em>yafQ</em></td>
<td>Conserved protein</td>
<td>0.0058</td>
<td>0.043</td>
<td>1.6</td>
</tr>
<tr>
<td><em>ycfJ</em></td>
<td>Membrane protein</td>
<td>0.0077</td>
<td>0.047</td>
<td>1.6</td>
</tr>
<tr>
<td><em>rpsT</em></td>
<td>30S ribosomal subunit protein S20</td>
<td>0.0062</td>
<td>0.043</td>
<td>1.6</td>
</tr>
<tr>
<td><em>infA</em></td>
<td>Protein chain initiation factor IF-1</td>
<td>0.0025</td>
<td>0.032</td>
<td>1.8</td>
</tr>
<tr>
<td><em>rpsU</em></td>
<td>30S ribosomal subunit protein S21</td>
<td>0.0023</td>
<td>0.032</td>
<td>2.1</td>
</tr>
<tr>
<td><em>rhoL</em></td>
<td><em>rho</em> operon leader peptide</td>
<td>0.0031</td>
<td>0.035</td>
<td>3.4</td>
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<tr>
<td><em>glyY</em></td>
<td>Glycine tRNA3</td>
<td>0.00038</td>
<td>0.023</td>
<td>5.8</td>
</tr>
</tbody>
</table>

\(^a\) *: genes did not pass the filtering process.
\(^b\) The genes were grouped according to Ecocyc (2005).

Table 3. Genes that were up-regulated in response to HP (100 MPa for 15 min at 23±2°C) in *E. coli* O157:H7 EC-88.
2.4.4 Barotolerance of *E. coli* mutants

The barotolerance levels of available isogenic *E. coli* K-12 pairs covering various genotypic backgrounds were compared after pressure treatment at 400 MPa for 5 min. Barotolerance was quantitatively expressed as the DLSR, as described earlier (Figure 2). The 400-MPa treatment allowed the most discrimination between the isogenic pairs. The *dps*, *trxA*, *trxB*, *otsA*, *nlpI*-B, *rpoS*, *rpoE*, and *nlpI* A mutants were significantly (*P*<0.05) more sensitive to HP than their wild-type counterparts, with DLSRs of -1.7, -1.6, -0.8, -0.5, -1.1, -1.2, -2.9, and -3.0, respectively. Individually, the *hns* and *stpA* mutants showed no differences in barotolerance compared to their wild-type counterparts; however, an *hns stpA* double mutant became much more pressure sensitive than the wild type, with a DLSR of -3.0. The *fdx*, *iscU*, *hscA*, *sufABCDSE*, and *fnr* mutants were more resistant to HP than their wild-type counterparts; the corresponding DLSRs were 2.0, 2.2, 2.2, 2.2, and 3.0.
Figure 2. DLSR for *E. coli* isogenic pairs (mutants and corresponding wild-type strains) that were grown to stationary phase and pressure treated at 400 MPa for 5 min at 23±2°C. The DLSR equals log \( \frac{N}{N_0} \) mutant – log \( \frac{N}{N_0} \) wild type, where \( N \) is the CFU/ml of treated sample and \( N_0 \) is the initial (before treatment) CFU/ml. Positive DLSRs indicate barotolerance levels that are greater for the mutant than for the wild type, and error bars represent 1 standard deviation. *, the mutant was significantly different (\( P < 0.05 \)) from its wild-type counterpart.
2.5 Discussion

The current study demonstrated considerable variability among *E. coli* O157:H7 strains in response to HP (Figure 1). Strain variability has been reported frequently when pathogens were treated with minimal or non-thermal preservation methods. A genetic typing technique using PFGE did not discriminate properly between pressure-resistant and pressure-sensitive *E. coli* O157:H7 strains (data not shown). Similarly, a previous study demonstrated variability among *Listeria monocytogenes* strains in the response to a pulsed electric field treatment, but strain resistance to a pulsed electric field was not associated with the PFGE pattern (Lado and Yousef 2003).

Food-borne pathogenic and spoilage microorganisms are commonly more processing resistant in the stationary than in the exponential phase of growth; therefore, stationary-phase cells were analyzed in this study. Transcriptional analysis using DNA microarray methodology revealed considerable differences between barotolerant (EC-88) and barosensitive (ATCC 35150) *E. coli* O157:H7 strains. More than 100 genes responded to the sublethal pressure treatment, particularly in the pressure-resistant strain EC-88. Assessing the barotolerance of *E. coli* strains having mutations in genes believed important to pressure resistance helped us to interpret the results of the microarray analysis. However, discrepancies between the results obtained by the microarray analysis and those for the mutant barotolerance test were inevitable; the former measures only the transcriptional response to sublethal pressure, whereas the latter represents the outcome of the entire gene expression process as the result of a lethal pressure treatment. This discussion will be restricted to *E. coli* EC-88, the pressure-resistant strain that demonstrated significant transcriptional changes when treated with pressure at 100 MPa.
for 15 min. Genes responding to pressure were grouped according to primary function, but overlap between these groups was not avoidable. *E. coli* O157:H7 is genetically unstable due to its many prophages (Hayashi et al. 2001). Therefore, an *E. coli* K-12 gene chip is suitable for measuring the conserved genes of *E. coli* O157:H7. However, *E. coli* O157:H7 prophages may be related to pressure resistance, and responses of their genes were not measured in this study. Although the sensitive strain (ATCC 35150) did not produce any significant results with the DNA microarray analysis, it is still important that many of the same genes as those in the resistant strain were up- or down-regulated, just not at such pronounced levels, including those of the thiol-disulfide redox system.

### 2.5.1 Stress-response genes

When *E. coli* is subjected to conditions that arrest its growth (e.g., starvation) or exposed to DNA-damaging agents (e.g., UV), genes for universal stress proteins are expressed. The well-characterized universal stress protein A (UspA) and its paralogues (UspC, UspD, and UspE) are coordinately regulated in response to DNA damage (Gustavsson et al. 2002). This UspA family includes UP03 (encoded by *ynaF*) and UP12 (encoded by *ybdQ*); the latter is a putative substrate of the molecular chaperone GroEL (Bochkareva et al. 2002). Treatment of *E. coli* O157:H7 with HP down-regulated the UspA family genes 1.8- to 2.4-fold, but the expression of *yecG*, which codes for the universal stress protein C (EcoCyc 2005), was not altered (Table 2). A *ybdQ* mutant was similar to the parent strain in pressure sensitivity (Figure 2).

HP down-regulated *dps* by 2.5-fold (Table 2). This gene encodes Dps, a DNA-binding protein produced by starved cells which plays a major role in protecting bacterial
DNA from reactive oxygen species (ROS) (Almiron et al. 1992). Dps restricts iron uptake and sequesters intracellular iron during H\textsubscript{2}O\textsubscript{2} stress to protect the cell from the Fenton reaction (Park et al. 2005). Dps is regulated by the \textit{rpoS} gene product and integration host factor (IHF [a DNA-bending protein]) in the stationary phase and by OxyR/IHF during exponential phase (Altuvia et al. 1994). HP may have down-regulated \textit{dps} by altering the conformation of the DNA, inhibiting IHF’s ability to facilitate the binding of RpoS to the \textit{dps} promoter. Other IHF-regulated genes were affected by pressure treatment, as discussed below. Krzyzaniak et al. (1996) reported DNA conformational changes in low-salt buffer when the solution was treated with HP at 600 MPa. Other researchers observed a condensation of the \textit{E. coli} nucleoid in response to HP at 200 MPa for 8 min (Mañas and Mackey 2004). Reducing the transcription of \textit{dps} in response to pressure may allow the chromosomal DNA to renature to its protective state without interruption by abundant DNA-binding proteins. It is likely that the Dps reserve of the cell is sufficient for reconfiguring the supercoiled protective state of the DNA. The \textit{dps} mutant was significantly more sensitive to HP than its wild-type counterpart (Figure 2). This finding indicates that the lack of Dps sensitizes \textit{E. coli} to the pressure treatment, probably by exposing DNA to conformational changes or by exposing the cells to oxidative damage, perhaps via the Fenton reaction. As stated earlier, HP has been shown to induce oxidative stress (Aertsen et al. 2005).

Heat-shock proteins (Hsp’s) are important in the bacterial stress response. Many of these proteins are molecular chaperones that bind to nascent, misfolded, or damaged polypeptides and assist them in reaching a native conformation (Hartl and Hayer-Hartl 2002). Chaperones of \textit{E. coli} include Hsp60 (GroEL), Hsp70 (DnaK), Hsp100, and the
small heat-shock proteins IbpA and IbpB. The latter category is believed to assist in the refolding of denatured proteins in the presence of other chaperones (Veinger et al. 1998). The \textit{pphA} gene encodes a serine/threonine-specific protein phosphatase that induces the accumulation of heat-shock proteins by signaling protein misfolding (Missiakas and Raina 1997). The pressure treatment increased the transcription of \textit{ibpA} and \textit{pphA} approximately two-fold (Table 3). Transcription of \textit{ibpB} did not change in response to the sublethal pressure treatment (Table 2). The \textit{ibpAB} mutant was slightly sensitive to pressure, but it was not statistically different from its wild-type counterpart in barotolerance (Figure 2). HP may cause protein denaturation and aggregation in the bacterial cell. According to Mañas and Mackey (2004), cytoplasmic proteins in \textit{E. coli} are aggregated in response to pressure treatment. Increased transcription of some heat-shock protein genes (Table 3) may represent the cell response to protein denaturation by pressure, and the accumulation of these gene products may aid in repairing pressure damage. Although the barotolerance test of the \textit{ibpAB} double mutant did not indicate increased pressure sensitivity, recent studies showed that heat shock cross-protected \textit{E. coli} against pressure (Aertsen et al. 2004b) and that HP induces eleven heat-shock proteins in \textit{E. coli} (Welch et al. 1993).

The cold-shock protein CspA is an RNA chaperone which destabilizes RNA secondary structures formed at low temperature, thus making them susceptible to ribonucleases (Jiang et al. 1997). Such a function may be crucial for efficient translation of mRNAs at low temperatures and may also have an effect on transcription. In addition, CspA induces the transcription of a histone-like nucleoid structuring protein (H-NS) (Bae et al. 2000), which controls the expression of many genes regulated by environmental
parameters such as pH, temperature, and osmolarity (Atlung and Ingmer 1997). Treating *E. coli* O157:H7 with 100 MPa up-regulated *cspA* 3.9-fold (Table 3). Experiments using reverse transcription-real-time PCR confirmed the up-regulation of *cspA* in response to the pressure treatment (data not shown). The pressure treatment may have affected RNA in a fashion similar to that caused by cold shock, causing increased transcription of *cspA*. Interestingly, there was no significant difference in sensitivity to HP between the *cspA* mutant and its wild-type counterpart (Figure 2). HP has been shown to induce four cold shock proteins (Welch et al. 1993). Furthermore, *E. coli* is currently known to have nine CspA homologues (Phadtare and Inouye 2004).

### 2.5.2 Thiol-disulfide redox system

*E. coli* contains two thioredoxins (Trx1 and Trx2) and three glutaredoxins (Grx1, Grx2, and Grx3). These are small proteins with two redox-active cysteine thiols, which, by thiol-disulfide (SH/S-S) interchange, reduce acceptor disulfides in the cell’s key proteins (Ortenberg et al. 2004). An example of the activity of these cytoplasmic thioredoxins is the reduction of periplasmic DsbC through membrane-bound DsbD, where the reduced DsbC can then be used in disulfide bond isomerization and proper protein folding in the periplasm (Beckwith 2003). Recently, it was shown that the thioredoxin system has chaperone properties that do not involve cysteine residues (Kern et al. 2003). The three most effective cytoplasmic disulfide-reducing proteins of *E. coli* are Trx1 (*trxA*), Trx2 (*trxC*), and Grx1 (*grxA*) (Stewart et al. 1998). The sublethal pressure treatment marginally up-regulated *trxA* and *grxA* transcription (1.4-fold) (Table 3). However, there was no change in transcription of *trxB*, *grxB*, and *grxC* in response to
pressure. Transcripts of the rhoL gene, encoding the rho operon leader peptide, which is located immediately downstream of trxA (EcoCyc 2005), was significantly increased in response to pressure, by 3.4-fold.

*E. coli* has a ribonucleotide reductase encoded by the nrdHIEF operon, and expression of this operon is triggered in response to oxidative stress. The nrdHIEF operon is up-regulated when different chemical oxidants (e.g., H$_2$O$_2$ and paraquat) are added or when major antioxidant defenses are lost (Monje-Casas et al. 2001). It was suggested that an enhanced ribonucleotide reductase may protect DNA against reactive oxygen species escaping from the antioxidant defenses. HP treatment significantly and marginally up-regulated nrdI and nrdH, respectively. In fact, it appeared that the entire nrd operon was up-regulated due to pressure treatment.

Both trxA and trxB mutants were significantly more sensitive to HP than were their wild-type counterparts (Figure 2). The thioredoxin reductase (trxB) can reduce Trx1 in the cytosol. The reduced state of Trx1 can aid in disulfide isomerization to correctly fold a misoxidized protein (Rietsch et al. 1996). HP promoted β-lactoglobulin aggregation through thiol-disulfide interchange reactions (Funtenberger et al. 1997). A study investigating the effects of pressure on the hydrophobicity and interactions of -SH groups of myofibrillar proteins showed that the number of free –SH groups grew with increasing pressure and treatment time (Chapleau et al. 2002). We propose that HP denatures proteins in a way that increases the accessibility of –SH or S-S to catalytic agents in the cell cytosol. According to Åslund and Beckwith (1999), “disulfide stress” occurs when unwanted disulfide bonds are generated in microbial cells, which will ultimately impact the activity of the redox proteins, and thus, the cell redox homeostasis.
In response to HP, *E. coli* up-regulated the thiol-disulfide redox system in a manner that increased barotolerance, perhaps by facilitating proper protein folding and/or maintaining redox homeostasis.

### 2.5.3 Iron-sulfur cluster

Iron-sulfur (Fe-S) clusters are prosthetic groups commonly found in proteins that participate in oxidation-reduction reactions. The FNR protein of *E. coli* is required for transcriptional regulation of many anaerobic metabolism genes in response to oxygen availability (Salmon et al. 2003). Under anaerobic conditions, FNR is active and contains a [4Fe-4S] cluster. In the presence of oxygen, [4Fe-4S]-FNR is converted to [2Fe-2S]-FNR, resulting in monomerization and inactivation of FNR as a gene regulator. Prolonged exposure to oxygen converts [2Fe-2S]-FNR to apoFNR, an FNR that is devoid of Fe-S clusters (Achebach et al. 2005). The assembly of Fe-S clusters in *E. coli* involves two independent systems, namely ISC, encoded by *iscSUA-hscBA-fdx*, and SUF, encoded by *sufABCDSE* (Tokumoto et al. 2004). Unlike the ISC system, the SUF system is required for Fe-S cluster assembly under iron starvation conditions and perhaps to provide a shielded pathway for donating sulfane sulphur to Fe-S clusters (Outten et al. 2004). An *E. coli* mutant in which the entire *isc* operon was deleted grew very poorly and had a marked decrease in Fe-S protein activity compared to the wild type, while the deletion of the entire *suf* operon caused no severe phenotypic effects (Takahashi and Tokumoto 2002). When both operons were deleted the cells were not viable. However, the overproduction of *suf* operon products suppressed the defects in the *isc*-deleted strain.
The sublethal pressure treatment significantly down-regulated $suf/S$, and the entire $suf$ operon appeared to be down-regulated (Table 2). Additionally, the $sufABCDSE$ mutant was significantly more barotolerant than its wild type (Figure 2). The $suf$ operon is believed to be regulated by two mechanisms. When the ferric uptake regulation protein Fur binds to Fe, it represses the $suf$ operon. It is likely that pressure treatment increases the availability of iron ions in the cell, thus repressing the $suf$ operon, or at least one of its genes. Some Fe-S clusters are sensitive to environmental stresses, and the release of Fe from these proteins under deleterious conditions (e.g., pressure) may cause further cell damage via the Fenton reaction. In addition, these findings may indicate that pressure treatment enhances oxidative reactions, probably through modifying macromolecules and exposing sites (such as thiol-disulfide groups) which react quickly with these oxidative species. With oxygen solubility increasing under HP, it is also likely that pressure treatment sensitizes proteins containing Fe-S clusters to oxygen, causing the destruction of the Fe-S clusters and thus releasing Fe, which is disadvantageous to the cell. The second mechanism of regulating $suf$ involves IHF, a DNA-bending protein, along with OxyR; both are required for $suf$ activation. Similar to the $dps$ regulation proposed earlier, pressure may cause physical alterations to the promoter region of the $suf$ operon impeding proper binding of OxyR and IHF.

As stated earlier, FNR is an oxygen sensor that regulates genes involved in anaerobic respiration. Although the Fe-S cluster in active FNR is degraded by exposure to oxygen, a previous study showed that the level of FNR itself did not differ significantly between anaerobic and aerobic conditions (Trageser et al. 1990). In the current study, $fnr$ was significantly up-regulated in response to HP treatment (Table 3).
According to Trageser et al. (1990), proteolysis of FNR occurs by a protease which is located outside the cytoplasmic membrane or activated upon disruption of the membrane. Therefore, it is likely that pressure treatment disrupted the cell membrane, thus exposing FNR to proteolysis. Since fnr is self-regulated, the proteolysis of FNR would up-regulate transcription of the gene. A strain with a mutation in fnr was much more resistant to HP than its wild type (Figure 2). As suggested earlier, Fe-S clusters in some proteins (e.g., FNR) are likely targets for HP, and the release of Fe by this treatment is disadvantageous to the cell. In addition, FNR may regulate genes that affect barotolerance, perhaps those encoding Fe-S-related proteins.

The ISC system (encoded by iscSUA-hscBA-fdx) is the primary manager of Fe-S cluster assembly in *E. coli* (Tokumoto et al. 2004). Pressure treatment significantly up-regulated iscR (Table 3), a gene involved in repressing the isc operon. Compared to the wild type, the iscR mutant was not significantly more sensitive to HP. Individual mutations in iscS, iscU, hscB, hscA, and fdx caused changes in the growth rate, nutritional requirements, and Fe-S enzyme activities, with the iscS mutant showing the most severe phenotypic changes (Tokumoto and Takahashi 2001). The iscU, hscB, hscA, and fdx mutants had identical phenotypic changes, while the iscR mutant displayed no phenotypic differences from the wild type (Tokumoto and Takahashi 2001). Consistent with our hypothesis on the contribution of Fe-S clusters to pressure sensitivity, the iscU, hscA, and fdx mutants were significantly more resistant to HP than were their wild-type counterparts (Figure 2). In summary, this research suggests that HP affects Fe-S proteins, Fe-S cluster assembly proteins, the Fe-S cluster status, and/or Fe availability, all of which affect cell redox homeostasis.
2.5.4 Genes involved in spontaneous mutation

The *dinB* operon (*dinB*-*yafN*-*yafP*) is known to be involved in spontaneous mutation, while the exact role of each *yaf*-encoded protein is unclear. However, a polar *dinB* (error-prone DNA polymerase IV) mutation caused a decrease in spontaneous mutation, whereas a nonpolar substitution and nonpolar deletion allele of *dinB* did not (McKenzie et al. 2003). Therefore, *yafN*, *yafO*, and *yafP* are partly or wholly responsible for phenotypes in spontaneous mutation, translesion synthesis, and adaptive mutation. HP-induced spontaneous mutation may result in the development of pressure-resistant *E. coli* strains. Extremely pressure-resistant strains have been generated by multiple rounds of exposure to HP followed by selection of survivors (Hauben et al. 1997). The current research shows that *yafO* and *yafN* are significantly and marginally up-regulated due to pressure, respectively, while *dinB* and *yafP* transcripts showed no significant change. The *yafN*-*yafP* mutant was similar to its wild type in pressure resistance.

2.5.5 Miscellaneous Genes

The current study showed no statistical differences in the expression of *hns*, a transcriptional dual-regulator gene, and *stpA*, which codes for an H-NS-like protein, in response to sublethal pressure treatment (Tables 2 and 3). Similarly, *hns* and *stpA* mutants were not significantly different in pressure resistance from their wild-type counterparts. However, when both genes were deleted, the resulting double mutant was highly pressure sensitive (Figure 2). Therefore, H-NS and StpA can independently contribute to barotolerance, and the basal level of either one of these DNA-binding proteins may regulate genes involved in barotolerance. According to Ishii et al. (2005), an
*E. coli* *hns* mutant was more sensitive to HP than its wild-type counterpart. In conclusion, *hns*, independently or in combination with *stpA*, is critical for *E. coli* barotolerance.

Sigma E (*rpoE*), a periplasmic stress sigma factor, controls the folding of polypeptides in the bacterial envelope and the biosynthesis/transport of lipopolysaccharide. The conditions that cause unfolding of polypeptides are signaled by RseA and RseB proteins (Dartigalongue et al. 2001). The NlpI protein is a recently discovered lipoprotein, and its exact role in the cell is under investigation. Ohara et al. (1999) indicated that NlpI is possibly involved in cell division. In response to the sublethal pressure treatment, *rpoE* and *nlpI* were marginally and significantly up-regulated, respectively (Table 3). In addition, *rpoE* and *nlpI* mutants were significantly more sensitive to HP than their wild-type counterparts. This suggests that HP induces periplasmic stress by denaturing polypeptides involved in the synthesis of the bacterial envelope. Interestingly, a deep-sea bacterium, *Photobacterium* sp. strain SS9, contains an *rpoE*-like locus that controls membrane protein synthesis and growth at HP (Chi and Bartlett 1995). Lastly, it is plausible that NlpI is involved in the repair of cell injury caused by HP treatment.

According to Robey et al. (2001), the stationary-phase sigma factor RpoS is involved in the resistance of *E. coli* O157:H7 to HP. The current study confirms this finding; an *rpoS* mutant was more sensitive to pressure than its wild-type counterpart (Figure 2). Synthesis of trehalose may protect the bacterial cell against heat, cold, and osmotic stresses and oxygen radicals (Kandror et al. 2002). The *otsAB* genes are involved in trehalose synthesis (EcoCyc 2005). Sublethal pressure treatment marginally up-
regulated otsB (Table 3), and the otsA mutant was more sensitive to HP than its wild-type counterpart (Figure 2). Since E. coli O157:H7 was not exposed to excessive heat or cold temperatures in this study, the changes in the expression of the trehalose synthesis genes suggest that the HP treatment induces osmotic and/or oxidative stress. Lastly, HP significantly up-regulated rbsD, a ribose mutarotase gene, and glyY, a glycine tRNA3 gene, by 4.0- and 5.8-fold, respectively. However, the rbsD mutant was similar to its wild-type counterpart in barotolerance. Up-regulation of rbsD and glyY in response to HP is interesting, but the role of these genes in resistance of E. coli O157:H7 to pressure is not well understood.

2.6 Conclusion

Alternative food preservation technologies such as HP are promising, but the wide variation in resistance among bacterial strains should be addressed to ensure successful implementation of these technologies in the food industry. HP had a profound effect on the transcriptional profile of E. coli O157:H7. This study is the first to propose that stress-related DNA-binding proteins, protein aggregation, the thiol-disulfide redox system, and Fe-S cluster status greatly influence the barotolerance in E. coli O157:H7. In addition, this is the first report to suggest that the dinB operon may be involved in the pressure resistance. The importance of both stpA and hns as well as nlpI, rpoE, and otsAB in HP resistance is proposed in this study. Future research in this laboratory will investigate the detailed mechanisms involved in the barotolerance of E. coli O157:H7 and other food-borne pathogens.
2.7 References


CHAPTER 3

PROPOSED MECHANISM OF INACTIVATING \textit{ESCHERICHIA COLI} O157:H7 BY HIGH PRESSURE IN COMBINATION WITH \textit{TERT}-BUTYLHYDROQUINONE

3.1 Abstract

The aim of this study was to investigate the mechanisms of lethality enhancement when \textit{Escherichia coli} O157:H7, and selected \textit{E. coli} mutants, were exposed to \textit{tert}-butylhydroquinone (TBHQ) during high pressure (HP) treatment. \textit{E. coli} O157:H7 EDL-933, and fourteen \textit{E. coli} K12 strains with mutations in selected genes, were treated with dimethyl sulfoxide solution of TBHQ (15-30 ppm), and processed with HP (400 MPa, 23 ± 2°C for 5 min). Treatment of wild-type \textit{E. coli} strains with HP alone inactivated 2.4-3.7 log CFU/ml, whereas presence of TBHQ increased HP lethality by 1.1-6.2 log CFU/ml; TBHQ without pressure was minimally lethal (0-0.6 log reduction). Response of \textit{E. coli} K12 mutants to these treatments suggests that iron-sulfur cluster-containing proteins ([Fe-S]-proteins), particularly those related to the sulfur mobilization (SUF system), nitrate metabolism, and intracellular redox potential, are critical for the HP-TBHQ synergy against \textit{E. coli}. Mutations in genes maintaining redox homeostasis and anaerobic metabolism were associated with HP-TBHQ resistance. The redox cycling activity of cellular [Fe-S]-proteins may oxidize TBHQ, potentially leading to the generation of bactericidal reactive oxygen species. A mechanism is proposed for the enhanced lethality
of HP by TBHQ against *E. coli* O157:H7. The results may benefit food processors using HP-based preservation, and biologists interested in piezophilic microorganisms.

3.2 Introduction

*Escherichia coli* O157:H7 causes serious food-transmitted diseases and the pathogen typically has a low infectious dose (Paton and Paton 1998). This bacterium tends to be unstable genetically (Hayashi et al. 2001), and it may adapt readily to various environmental or processing stresses (Hauben et al. 1998; Zook et al. 2001). The pathogen may develop resistance to food preservation methods, particularly those relying on high pressure (HP) and other emerging technologies (Lado and Yousef 2002). Exposure to multiple pressure cycles developed *E. coli* O157:H7 strains with abnormal resistance to this new preservation method (Hauben et al. 1998). Therefore, scientific and industrial groups are in search of agents that synergistically enhance the efficacy of emerging technologies against adaptable and processing-resistant pathogens; safety of “food of the future” depends on the success of these efforts.

The mechanism of microbial inactivation by HP is not fully understood, but several studies suggest that the treatment targets the bacterial membrane. Phase transition, from liquid crystalline to gel, of membrane lipids was reported after HP treatment (MacDonald 1993). It has also been demonstrated that cells with a relatively fluid membrane, i.e., with a high degree of unsaturation, are relatively resistant to pressure (Casadei et al. 2002). Membrane damage after pressurization has been reported repeatedly, as indicated by enhanced uptake of vital dyes, loss of protein, RNA, and osmotic responsiveness, and physical perturbations of the cell envelope, especially for
exponential-phase cells (Pagán and Mackey 2000; Mañas and Mackey 2004). Additional HP cell-damaging events such as ribosome conformational changes, protein denaturation, and enzyme inactivation, have also been described (Mackey et al. 1994; Niven et al. 1999; Mañas and Mackey 2004). Recently, Malone et al. (2006) reported that HP altered the transcription of many genes involved in a variety of intracellular mechanisms including the stress response, thiol-disulfide redox system, iron-sulfur cluster ([Fe-S]) assembly, and spontaneous mutation.

Increasing the efficacy of HP against pressure-resistant foodborne pathogens requires the use of combination treatments. The HP-additive combination should have beneficial applications in food preservation. Phenolic compounds, which are commonly used to protect food against lipid oxidation (Shahidi 2000), may increase the antimicrobial efficacy of HP in combined treatments (Mackey et al. 1995). Several research groups have shown that phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and TBHQ may function as antimicrobial agents against pathogens including *Staphylococcus aureus*, *Salmonella Typhimurium* (Davidson et al. 1981), *Listeria monocytogenes* (Yousef et al. 1991), and *E. coli* O157:H7 (Ogunrinola et al. 1996). According to recent studies in this laboratory, addition of TBHQ to cells, in suspension or inoculated in food, increased the lethality of HP against *L. monocytogenes* (Chung et al. 2005; Vurma et al. 2006). Although TBHQ may act as a pro-oxidant in some biological systems (Kagawa et al. 1993), generation of tert-butylquinone (TBQ), through oxidation of TBHQ, in food is not a concern since this compound is likely to be reduced back to TBHQ when ingested (Anon 2004). A Joint
FAO/WHO Expert Committee re-evaluated the toxicity of TBHQ, and asserted its safety as a food additive (WHO 1998; Anon 2004).

Phenolic compounds such as TBHQ or BHA are metabolized in vivo, potentially altering cell redox homeostasis (Tajima et al. 1991; Williams et al. 2004). It is hypothesized in the current study that TBHQ is internalized in E. coli during the pressure treatment and the additive is oxidized by the pressure-altered cell. Products of this oxidation enhance the lethality of the pressure treatment and thus HP and TBHQ act synergistically against the bacterium. Therefore, the objectives of this study were to assess the enhancement in pressure lethality by TBHQ treatment, against E. coli O157:H7, and to elucidate the mechanism of enhanced lethality on the basis of the hypotheses just described. E. coli mutants defective in genes involved in maintaining redox homeostasis and anaerobic metabolism were evaluated for their association with HP-TBHQ resistance.

3.3 Materials and methods

3.3.1 Bacterial strains

E. coli O157:H7 EDL-933 and fourteen E. coli K12 knockout mutants and their wild-type counterparts, covering various genotypic backgrounds, were tested. Sources and description of these mutants are included in Table 4. All strains were grown from a 0.1% inoculum in tryptose broth (TB; Becton, Dickinson and Co., Sparks, MD, USA) at 35°C for 18 h. All cultures were transferred at least twice before experimentation.
### DNA binding proteins

<table>
<thead>
<tr>
<th>Allele</th>
<th>Strain</th>
<th>Genotype</th>
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<th>Source</th>
</tr>
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<tr>
<td>ybdQ†</td>
<td>BW25113</td>
<td>lacI&lt;sup&gt;+&lt;/sup&gt; rpsD&lt;sup&gt;T16&lt;/sup&gt; ΔlacZ&lt;sup&gt;W16&lt;/sup&gt; hsdR514 ΔaraBAD&lt;sub&gt;MMM3&lt;/sub&gt; ΔrhaBAD&lt;sub&gt;OT28&lt;/sub&gt;</td>
<td>YbdQ (or UspG); universal stress protein of the UspA family.</td>
<td>Bochkareva et al. 2002</td>
</tr>
<tr>
<td>ybdQ mutant</td>
<td>-</td>
<td>BW25113 ΔybdQ&lt;sup&gt;+&lt;/sup&gt;:&lt;sup&gt;km&lt;/sup&gt;</td>
<td>Dps complex; stationary-phase nucleoid component that sequesters iron and protects DNA from damage.</td>
<td>Almiron et al. 1992</td>
</tr>
<tr>
<td>dps†</td>
<td>ZK126</td>
<td>W3110 tna2 ΔlacU169</td>
<td>Fumarate nitrate reductase transcriptional regulator (FNR); stimulates the transcription of genes required for anaerobic respiration, and represses those for aerobic growth.</td>
<td>Wyborn et al. 2002</td>
</tr>
<tr>
<td>dps mutant</td>
<td>SF2080</td>
<td>ZK126 &lt;sup&gt;dps&lt;/sup&gt;:&lt;sup&gt;kan&lt;/sup&gt;</td>
<td>Fumarate nitrate reductase transcriptional regulator (FNR); stimulates the transcription of genes required for anaerobic respiration, and represses those for aerobic growth.</td>
<td>Wyborn et al. 2002</td>
</tr>
<tr>
<td>fnr†</td>
<td>MG1655</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; iihG&lt;sup&gt;+&lt;/sup&gt; rfb-50 rph-1</td>
<td>Fumarate nitrate reductase transcriptional regulator (FNR); stimulates the transcription of genes required for anaerobic respiration, and represses those for aerobic growth.</td>
<td>Wyborn et al. 2002</td>
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<td>fnr mutant</td>
<td>JRG5390</td>
<td>MG1655 Δfrn::&lt;sup&gt;cm&lt;/sup&gt;</td>
<td>Fumarate nitrate reductase transcriptional regulator (FNR); stimulates the transcription of genes required for anaerobic respiration, and represses those for aerobic growth.</td>
<td>Wyborn et al. 2002</td>
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### Fe-S cluster status

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<td>iscU&lt;sup&gt;+&lt;/sup&gt; hscA&lt;sup&gt;+&lt;/sup&gt; fdx&lt;sup&gt;+&lt;/sup&gt; suf&lt;sub&gt;ABCDSE&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>MG1655</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; Wild Type</td>
<td>Iron-sulfur cluster biosynthesis and assembly proteins.iscU; scaffold protein involved in iron-sulfur cluster assembly.HscA; chaperone, member of Hsp70 protein family.Fdx; ferredoxin that may serve as an intermediate site for [Fe-S] assembly.SUF; system involved in iron-sulfur cluster synthesis under oxidative stress and iron limitation.</td>
<td>Djaman et al. 2004</td>
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<td>iscU mutant</td>
<td>OD110</td>
<td>MG1655 ΔiscU110::&lt;sup&gt;cm&lt;/sup&gt;</td>
<td>Iron-sulfur cluster biosynthesis and assembly proteins.iscU; scaffold protein involved in iron-sulfur cluster assembly.</td>
<td>Djaman et al. 2004</td>
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<td>hscA mutant</td>
<td>OD114</td>
<td>MG1655 ΔhscA12::&lt;sup&gt;cm&lt;/sup&gt;</td>
<td>Iron-sulfur cluster biosynthesis and assembly proteins.iscU; scaffold protein involved in iron-sulfur cluster assembly.</td>
<td>Djaman et al. 2004</td>
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<td>fdx mutant</td>
<td>OD115</td>
<td>MG1655 Δfxd-115::&lt;sup&gt;kan&lt;/sup&gt;</td>
<td>Iron-sulfur cluster biosynthesis and assembly proteins.iscU; scaffold protein involved in iron-sulfur cluster assembly.</td>
<td>Djaman et al. 2004</td>
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<td>suf&lt;sub&gt;ABCDSE&lt;/sub&gt; mutant</td>
<td>WO19</td>
<td>Δsuf&lt;sub&gt;ABCDSE19&lt;/sub&gt;::kan</td>
<td>Iron-sulfur cluster biosynthesis and assembly proteins.iscU; scaffold protein involved in iron-sulfur cluster assembly.</td>
<td>Djaman et al. 2004</td>
</tr>
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<td>iscR†</td>
<td>MG1655</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; iihG&lt;sup&gt;+&lt;/sup&gt; rfb-50 rph-1</td>
<td>IscR; transcriptional regulator that negatively regulates the expression of the iscRSUA operon.</td>
<td>Schwartz et al. 2001</td>
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<tr>
<td>iscR mutant</td>
<td>PK4854</td>
<td>MG1655 ΔiscR</td>
<td>IscR; transcriptional regulator that negatively regulates the expression of the iscRSUA operon.</td>
<td>Schwartz et al. 2001</td>
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### Nitrate Utilization

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<td>RK4353</td>
<td>ΔMacU169 araD139 rpsL gyrA non</td>
<td>Nar; membrane-bound nitrate reductase.</td>
<td>Stewart and MacGregor 1982</td>
</tr>
<tr>
<td>nar mutant</td>
<td>JCB4041</td>
<td>RK4353, ΔnarZ::Δ, ΔnarG::Δ, ΔnarL::Tn10</td>
<td>Nar; membrane-bound nitrate reductase.</td>
<td>Stewart and MacGregor 1982</td>
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<td>nar napF mutant</td>
<td>JCB4042</td>
<td>JCB4041, ΔnapF</td>
<td>Nar; membrane-bound nitrate reductase.</td>
<td>Brondijk et al. 2002</td>
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<td>nar napGH mutant</td>
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<td>Nar; membrane-bound nitrate reductase.</td>
<td>Brondijk et al. 2002</td>
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<td>nar napFGH mutant</td>
<td>JCB4044</td>
<td>JCB4041, ΔnapF, ΔnapGH</td>
<td>Nar; membrane-bound nitrate reductase.</td>
<td>Brondijk et al. 2002</td>
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<td>nar napC mutant</td>
<td>JCB4045</td>
<td>JCB4041, ΔnapC</td>
<td>Nar; membrane-bound nitrate reductase.</td>
<td>Brondijk et al. 2002</td>
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### Thiol-disulfide redox system

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<tr>
<td>trxA†</td>
<td>GJ1427</td>
<td>thi rha nagA lacZ trkA405 kdp-200::[Δlacr(Ap)]</td>
<td>Thioredoxin; small electron-transfer protein which contains a cysteine disulfide/dithiol active site.</td>
<td>Sardesai and Gowrishankar 2001</td>
</tr>
<tr>
<td>trxA mutant</td>
<td>GJ1426</td>
<td>GJ1427 ΔtrxA</td>
<td>Thioredoxin; small electron-transfer protein which contains a cysteine disulfide/dithiol active site.</td>
<td>Sardesai and Gowrishankar 2001</td>
</tr>
</tbody>
</table>

Table 4. Wild-type and mutant strains of *E. coli* K12 tested in this study.
3.3.2 TBHQ preparation

TBHQ was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Solutions were prepared by dissolving TBHQ in dimethyl sulfoxide (DMSO; 99.9% spectrophotometric grade, Sigma), and 100 µl of this stock solution was added to 900 µl cell suspension (i.e., 10%, vol/vol) to achieve a final TBHQ concentration of 15 or 30 ppm. In most experiments involving *E. coli* O157:H7 EDL-933 and *E. coli* mutant strains, 15 ppm TBHQ were used. For testing the *E. coli* strains defective in nitrate metabolism, 30 ppm TBHQ was used. Differing concentrations of TBHQ allowed for optimal discrimination between the strains.

3.3.3 Pressure treatment

All strains were grown to stationary phase (~1.0 x 10^9 CFU/ml) in 10 ml TB. Portions of the culture (900 µl) and TBHQ solution (100 µl) were aseptically transferred to a small sterile stomacher bag (Fisher Scientific, Pittsburgh, PA, USA) and the bags were sealed using a vacuum sealer (Vacmaster, Kansas City, MO, USA). Control treatments included cultures that were not treated, or treated with DMSO, TBHQ in DMSO, or DMSO in combination with HP. Samples were pressurized at 400 MPa (23 ± 2°C holding temperature) for 5 min in a HP processor (Quintus® QFP-6, Flow Pressure Systems, Kent, WA, USA). The initial temperature of the pressure transmitting fluid was controlled to account for the compression heating (3 to 4°C /100 MPa). An untreated control was held at 25°C and atmospheric pressure (0.1 MPa) while each treated sample was being pressure processed. After pressure treatments, samples were serially-diluted
using 0.1% peptone water, plated on tryptose agar (TA; Becton, Dickinson and Co.), and incubated at 35°C for 48 h to enumerate the survivors.

3.3.4 Inactivation of *E. coli* O157:H7 by HP-TBHQ in the presence of water-soluble reducing agents

Oxidation of TBHQ by *E. coli* during the pressure treatment is a proposed mechanism for the enhancement in lethality of HP by TBHQ. Therefore, selected reducing agents were added to cell suspensions and changes in cell sensitization to pressure (by TBHQ) were measured. Glutathione, ascorbate, and cysteine (Sigma) were dissolved individually in sterile deionized water. Cystine (Sigma), the oxidized form of cysteine, was also used. Each solution was added to an overnight culture of *E. coli* O157:H7 EDL-933 (final concentration was 30 ppm) and the mixtures were treated with TBHQ (15 ppm) and HP (400 MPa and 23 ± 2°C for 5 min) combination. Pressure treatments were executed as indicated earlier.

3.3.5 Treating *trxA* mutant, maintained under anaerobic or aerobic environments

The thioredoxin-deficient mutant, *E. coli* trxA, and its wild type were grown, prepared for treatment, and enumerated under aerobic or anaerobic conditions. Anaerobic conditions were achieved using an anaerobic chamber (Forma Scientific, Marietta, OH, USA). The mutant and the wild type were treated with TBHQ (15 ppm), HP (400 MPa and 23 ± 2°C for 5 min) or their combination.
3.3.6 Quantifying bacterial response to treatments

Initial and treatment-surviving populations of *E. coli* were determined using spread-plating on TA. Inoculated plates were incubated at 35°C for 24 h, and colonies were enumerated. Reduction in bacterial population was quantified as log survivor ratio (LSR), calculated as log \( \frac{N}{N_0} \), where \( N \) is CFU/ml in treated sample and \( N_0 \) is initial (before treatment) CFU/ml. A larger absolute value of LSR corresponds to a greater sensitivity of the microorganism to the applied treatment. For the isogenic *E. coli* pairs, the differential log survivor ratio (DLSR = LSR mutant – LSR wild type) was calculated. A positive DLSR indicates that pressure resistance is greater for the mutant than the wild type, and a negative value indicates the opposite.

3.3.7 Statistical analysis

Data were analyzed using t-test and analysis of variance. Means of LSR or DLSR were compared using Tukey’s HSD post-hoc analysis with a 5% significance level (\( P < 0.05 \)). Data analyses were carried out using commercial statistical programs (JMPin®, SAS Institute Inc., Cary, NC, USA; SigmaPlot® version 9.0; SPSS Inc., Chicago, IL, USA).
3.4 Results

3.4.1 Assessing synergy between HP and TBHQ, and pressure-sensitization of \textit{E. coli} by the additive

The parameters, LSR and DLSR, were used to quantify the responses of individual \textit{E. coli} strains and isogenic pairs, respectively, to treatments with HP, TBHQ or their combinations, as described in the "Materials and methods" section. Negative LSR is indicative of microbial inactivation, and the absolute value of this parameter, i.e., [LSR], equals the decrease in \textit{E. coli} log CFU/ml in response to the treatment. Therefore, [LSR] will be used interchangeably with the term “lethality” throughout this study. Measured LSRs for \textit{E. coli} wild-type and mutant strains, after various treatments, are shown in Table 5.
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* Values with the same letter within each cell are not statistically different ($\alpha = 0.05$).

Table 5. LSRs of selected *E. coli* K12 isogenic pair strains after treatment with HP (400 MPa for 5 min at 23 ± 2°C) or combination of HP, DMSO (10% vol/vol) and TBHQ (15 ppm or 30 ppm). The absolute LSR equals the decrease in log CFU/ml. Statistics comparison of means (SAS Institute Inc., Cary, NC, USA) was done within each cell of three treatments for each wild-type and mutant strain.
Synergy between HP and TBHQ may be judged by the greater lethality of the HP-TBHQ combination, compared with the sum of lethalities resulting from the application of HP and TBHQ treatments individually. Since TBHQ alone, at the levels used in this study, had little or no effect on *E. coli* (0 to 0.6 log CFU/ml), the synergy is assessed as the increase in lethality (i.e., increase in [LSR]) when cells were treated with HP-TBHQ, compared with HP alone (Table 5). However, this synergy depended largely on the use of DMSO as a solvent for TBHQ. This solvent is widely used for the delivery of hydrophobic compounds in biological systems (Yu and Quinn 1998). There were generally small, but occasionally significant, increases in the lethality caused by HP-DMSO combination, compared to the HP treatment alone (Figure 3 and Table 5). However, lethality owing to HP-DMSO was considerably less than that resulting from the HP-(DMSO/TBHQ) combination. As TBHQ alone exhibited no lethality to *E. coli*, the synergy just described was attributed to the sensitization of the pathogen to HP by TBHQ. Throughout this study, treatments that include TBHQ implies that the additive was dissolved in DMSO; however, for simplicity, the solvent will not be explicitly listed as being part of these combined treatments, unless such reference is warranted.
Figure 3. The LSR of stationary-phase *E. coli* O157:H7 EDL-933 when treated with HP (400 MPa for 5 min at 23°C), or combinations of HP and TBHQ (15 ppm), with or without 30 ppm glutathione, ascorbate, cysteine, or cystine. The additive TBHQ was dissolved in dimethyl sulfoxide (DMSO) and the solvent was used in control treatments. The “HP-Reducing Agent” bar represents the average LSR for the control treatments involving HP in combination with 30 ppm reducing agent (glutathione, ascorbate, or cysteine) or cystine. LSR = log [(CFU/ml in treated sample)/(CFU/ml in untreated sample)]. Error bars represent -1 standard deviation (n ≥ 3). Values with the same letter are not statistically different (α = 0.05).
3.4.2 Sensitivity of *E. coli* O157:H7 EDL-933 to HP and TBHQ, and the protective effect of water-soluble reducing agents

*E. coli* O157:H7 EDL-933 is one of the most well characterized enterohemorrhagic *E. coli* strains. It was originally isolated, in 1982, from ground beef linked to multi-state disease outbreak in the U.S. More recently, the genome of the EDL strain was sequenced (Perna et al. 2001). Therefore, EDL-933 often has been used as a reference strain for *E. coli* O157:H7 and it was tested, in this study, for sensitivity to HP in the presence of TBHQ.

Treating *E. coli* O157:H7 EDL-933 with HP (400 MPa, at 23°C, for 5 min) decreased the pathogen population by 2.4 log (Figure 3). Presence of 30 ppm of selected water-soluble reducing agents (glutathione, ascorbate, or cysteine) or cystine, the oxidized form of cysteine, did not alter the resistance of the strain to pressure. However, 15 ppm TBHQ (dissolved in DMSO) significantly increased the lethality of pressure against the pathogen by 6.0 log CFU/ml (Figure 3). Considering that TBHQ at 15 ppm was not lethal to this pathogen (data not shown), the sensitization of the bacterium to HP by TBHQ is apparent.

Some water-soluble reducing agents diminished the lethality of the HP-TBHQ combination (Figure 3). Presence of glutathione or cysteine during the HP-TBHQ treatment caused less lethality (-5.2 and -4.1 LSR, respectively) compared to HP-TBHQ combination treatment without these agents (-8.4 LSR). Therefore, addition of these two reducing agents significantly protected the cells from the HP-TBHQ treatment. There was no significant ($P \geq 0.05$) change in LSR when HP-TBHQ treatment was carried out in the presence or absence of ascorbate (Figure 3). Similarly, cystine did not alter HP-TBHQ
lethality and this was judged by insignificant ($P \geq 0.05$) change in LSR when the combination treatment was done with or without cystine (Figure 3).

### 3.4.3 Mutations in genes encoding DNA-binding proteins

*E. coli* K12 strains with mutations in *fnr* (a gene encoding fumarate nitrate reductase regulator), *dps* (encodes stress response DNA-binding protein), and *ybdQ* (encodes universal stress protein), and their wild-type counterparts (Table 4), were treated with HP or HP-TBHQ. Similar to other strains investigated in this study, wild types and corresponding *fnr* and *dps* mutants were significantly sensitized to HP when TBHQ was added to the cell suspension prior to the pressure treatment (Table 5). Significant sensitization to pressure by DMSO was observed in the wild type, but not in the *fnr* mutant strain, whereas DMSO sensitized the *dps* mutant, but not its wild-type counterpart, to the pressure treatment (Table 5). Contribution of mutations in *fnr* and *dps* to pressure resistance or sensitivity (as indicated by positive or negative DLSR, respectively) is shown in Figure 4. Significant increases in resistance to pressure (2.4 DLSR) and pressure-TBHQ combination (3.1 DLSR) with a mutation in *fnr* were observed. *E. coli* with a mutation in *dps* was more sensitive to both HP and HP-TBHQ treatments than its wild-type counterpart (Table 5). Similarly, Figure 4 shows that a mutation in *dps* significantly sensitized *E. coli* to HP (-1.5 DLSR) and HP-TBHQ combination (-3.6 DLSR). However, mutation in *ybdQ* did not alter the resistance of *E. coli* to HP or HP-TBHQ treatments, and the corresponding DLSR were 0.05 and 0.20, respectively (Figure 4).
Figure 4. DLSR for *E. coli* isogenic pairs (*fnr*, *ybdQ*, and *dps* mutants and corresponding wild types) that were grown to stationary phase and exposed to HP (400 MPa for 5 min at 23 ± 2°C) or combination of HP and TBHQ (15 ppm). DLSR equals \([\log (N/N_0)_{\text{mutant}} - \log (N/N_0)_{\text{wild type}}]\), where \(N\) is the CFU/ml of treated sample and \(N_0\) is the initial (before treatment) CFU/ml. Positive DLSR indicates a barotolerance that is greater for the mutant than the wild type, and error bars represent -1 standard deviation. A bar labeled with a letter indicates that the mutant was significantly different \((P < 0.05)\) than its wild-type counterpart. Bars with the same letter, within a mutant, are not statistically different \((\alpha = 0.05)\).
3.4.4 Iron-sulfur cluster assembly mutants

*E. coli* K12 with mutations in genes involved in maintaining iron-sulfur clusters (ISC system) and sulfur mobilization (SUF system), and the corresponding wild-type strains (Table 4) were compared for resistance to pressure and HP-TBHQ combination. When treated with pressure only, *E. coli* K12 *iscU*, *hscA*, *fdx*, and *sufABCDSE* mutants showed greater resistance to pressure than their wild-type counterparts (Table 5), with significant (*P* <0.05) DLSRs of 1.9, 1.7, 1.9, and 2.0, respectively (Figure 5). However, upon treatment with HP-TBHQ combination, *iscU*, *hscA*, and *fdx* mutants were more sensitive than their wild-type counterparts (DLSRs of -0.94, -0.95, and -0.5), while the *sufABCDSE* mutant was significantly more resistant than its wild type (4.4 DLSR). No differences in lethality between *iscR* isogenic pairs were observed when treated with HP or HP-TBHQ.
Figure 5. DLSR for *E. coli* isogenic pairs (Fe-S cluster assembly mutants, *iscU*, *hscA*, *fdx*, *sufABCDSE*, and *iscR*, and corresponding wild types) that were grown to stationary phase and exposed to HP (400 MPa for 5 min at 23 ± 2°C) or combination of HP and TBHQ (15 ppm). DLSR equals $\log \left( \frac{N}{N_0} \right)_{\text{mutant}} - \log \left( \frac{N}{N_0} \right)_{\text{wild type}}$, where $N$ is the CFU/ml of treated sample and $N_0$ is the initial (before treatment) CFU/ml. Positive DLSR indicates a barotolerance that is greater for the mutant than the wild type, and error bars represent -1 standard deviation. A bar labeled with a letter indicates that the mutant was significantly different ($P < 0.05$) than its wild-type counterpart. Bars with the same letter, within a mutant, are not statistically different ($\alpha = 0.05$).
3.4.5 Nitrate metabolism mutants

Five *E. coli* K12 mutants (*nar, nar napF, nar napGH, nar napFGH, and nar napC*) involved in nitrate metabolism (Table 4) were treated with HP or HP-TBHQ, and their resistance to these treatments was compared. Among the strains tested in this category, only those with mutations in *nar napF* and *nar napFGH* were significantly more resistant to HP alone than their wild-type counterparts; DLSRs were 1.1 and 1.3, respectively (Figure 6). When treated with HP-TBHQ, all the five mutants were significantly more resistant to the combination treatment than their wild-type counterparts, with DLSR ranging from 1.9 to 3.5.
Figure 6. DLSR for *E. coli* isogenic pairs (nitrate utilization mutants, and corresponding wild types) that were grown to stationary phase and exposed to HP (400 MPa for 5 min at 23 ± 2°C) or combination of HP and TBHQ (30 ppm). DLSR equals \[ \log \left( \frac{N}{N_0} \right)_{\text{mutant}} - \log \left( \frac{N}{N_0} \right)_{\text{wild type}} \], where \( N \) is the CFU/ml of treated sample and \( N_0 \) is the initial (before treatment) CFU/ml. Positive DLSR indicates a barotolerance that is greater for the mutant than the wild type, and error bars represent -1 standard deviation. A bar labeled with a letter indicates that the mutant was significantly different \( (P < 0.05) \) than its wild-type counterpart. Bars with the same letter, within a mutant, are not statistically different \( (\alpha = 0.05) \).
3.4.6 Treating \textit{trxA} mutant under anaerobic or aerobic environment

\textit{E. coli} K12 with a mutation in \textit{trxA}, a gene encoding thioredoxin (a small protein involved in thiol-redox processes), and the corresponding wild-type strain (Table 4) were maintained under aerobic or anaerobic conditions and tested for resistance to HP or HP-TBHQ combination. When the strain pair was treated with HP only, the \textit{trxA} mutant was significantly more pressure-sensitive than its wild-type counterpart; DLSRs were -1.4 and -0.68 for cultures maintained under aerobic and anaerobic conditions, respectively (Figure 7). However, during the HP-TBHQ combination treatment, the aerobically-maintained \textit{trxA} mutant was significantly more resistant than its wild type (2.5 DLSR). Under anaerobic conditions, however, the \textit{trxA} mutant showed no significant difference in HP-TBHQ inactivation, compared to its wild type (0.05 DLSR).
Figure 7. DLSR for *E. coli trxA* mutant and its corresponding wild type that were grown to stationary phase, and exposed to HP (400 MPa for 5 min at 23 ± 2°C) or combination of HP and TBHQ (15 ppm), in the presence or absence of air. DLSR equals \(\log \left( \frac{N}{N_0} \right)_{\text{mutant}} - \log \left( \frac{N}{N_0} \right)_{\text{wild type}}\), where \(N\) is the CFU/ml of treated sample and \(N_0\) is the initial (before treatment) CFU/ml. Positive DLSR indicates a barotolerance that is greater for the mutant than the wild type, and error bars represent ±1 standard deviation. A bar labeled with a letter indicates that the mutant was significantly different \((P < 0.05)\) than its wild-type counterpart. Bars with the same letter, within a condition, are not statistically different \(\alpha = 0.05\).
3.5 Discussion

Antimicrobial property of phenolic antioxidants (e.g., BHA, BHT, and TBHQ) is well documented (Davidson et al. 1981; Yousef et al. 1991; Ogunrinola et al. 1996). According to a recent report, several phenolic antioxidants sensitized *L. monocytogenes* to HP treatment (Vurma et al. 2006). Similarly, the current study shows that TBHQ and HP combination treatment synergistically inactivated *E. coli* O157:H7 EDL-933. Phenolic antioxidants are used as food additives to prevent lipid oxidation owing to their ability to scavenge oxidative-free radicals (Bors and Saran 1987). However, phenolic compounds acting as antioxidants to lipid often act as pro-oxidant to other molecules such as DNA and protein (Laughton et al. 1989). Considering the antioxidant properties of TBHQ, the presence of this compound during pressure treatment may have altered the cell metabolic pathways governing redox homeostasis. Therefore, the mechanism of sensitizing *E. coli* to pressure by TBHQ was investigated by using mutants that are defective in genes involved in maintaining redox homeostasis and anaerobic metabolism.

Incorporation of the hydrophobic TBHQ into the aqueous cell suspension was made possible by dissolving the additive into DMSO. Consequently, DMSO-based control treatments were included in this study. The solvent occasionally sensitized *E. coli* strains to HP, but this effect was generally much smaller than that attributed to TBHQ (Table 5). Pressure treatment favors transition of membrane phospholipids from liquid crystalline to gel state (MacDonald 1993; Kato and Hayashi 1999; Winter 2001), which may contribute to bacterial lethality. A low concentration of DMSO stabilizes the bilayer gel phase (Yamashita et al. 2000). Therefore, transition of the cell membrane to gel phase
by HP and stabilization of this gel by DMSO may explain the slight bacterial lethality enhancement when cells are pressure treated in the presence of DMSO.

### 3.5.1 Contribution of genes involved in anaerobic respiration

The FNR protein serves as a transcriptional factor under anaerobic growth conditions (Kiley and Beinert 1999; EcoCyc 2005). This protein contains an oxygen-sensitive iron-sulfur cluster ([Fe-S]) that functions as a sensor of anaerobiosis. Increased exposure to oxygen disrupts the [4Fe-4S] complex and converts it to the iron-free apoFNR (Achebach et al. 2005). In *E. coli*, FNR positively regulates many genes that encode enzymes for the anaerobic reduction of alternate terminal electron acceptors such as nitrate, fumarate, and DMSO (Kiley and Beinert 1999). Accordingly, nitrate, fumarate, and DMSO reductases are lacking in *fnr* mutants, such as the one used in this study (EcoCyc 2005).

A mutation in *fnr* increased the resistance of *E. coli* to pressure (Figure 4), which is consistent with the previously reported results from this laboratory (Malone et al. 2006). In addition, the *fnr* mutant was more resistant to HP-DMSO than its wild-type strain (Table 5). The solvent, DMSO, may serve as a terminal electron acceptor in anaerobic respiration (EcoCyc 2005). Previous research in this laboratory suggests that some [Fe-S]-proteins are sensitive to pressure (Malone et al. 2006), and it may be hypothesized that DMSO reductase is similarly pressure-sensitive. These findings may explain the greater sensitivity of the wild-type strain to HP-DMSO and HP-(DMSO/TBHQ) combinations, compared with the *fnr* mutant strain (Table 5). However,
to prove this hypothesis, strains with mutations in \textit{dmsABC} should be tested in future investigations.

Release of iron ions in cytosol, from the pressure-damaged [Fe-S]-proteins, would be detrimental to the cells, particularly those pretreated with TBHQ. Activation of the TBHQ by Cu$^{2+}$ results in the formation of TBQ, semiquinone anion radical, and reactive oxygen species (ROS); the latter may participate in oxidative damage of DNA both \textit{in vitro} and \textit{in vivo} (Li and Trush 1993; Li et al. 2002). Similarly, release of iron ions into cell cytosol under pressure, as suggested by Malone et al. (2006), may have activated TBHQ, leading to oxidative damage of the cell components via the Fenton Reaction, and causing the sensitization to pressure (Beckwith 2003; Green and Paget 2004).

Among the anaerobic respiration genes tested in this study, the \textit{napF} mutant in particular was resistant to HP and exhibited even greater resistance to HP-TBHQ combination-treatment, compared with its wild type (Figure 6). These findings may suggest that the [Fe-S] in NapF are particularly sensitive to HP. NapF is a ferredoxin-type protein that does not appear to be involved in the electron transfer from menaquinol or ubiquinol to Nap (Brondijk et al. 2002). Proposed disassembly of the [3Fe-4S] in NapF by pressure and release of iron ions in the cytosol should be detrimental to the cell, particularly in the presence of TBHQ, as described earlier.

3.5.2 Role of genes encoding iron-sulfur cluster assembly proteins

Two systems in \textit{E. coli} are involved in maintaining [Fe-S] in the cell, ISC (iron sulfur cluster) and SUF (sulfur mobilization) (EcoCyc 2005). The ISC proteins, encoded by \textit{iscRSUA-hscBA-fdx}, are thought to be involved in the general biosynthesis pathway
(housekeeping) for numerous [Fe-S]-proteins (Frazzon and Dean 2001), whereas the SUF system, encoded by sufABCDSE, plays a lesser role in constructing [Fe-S] under normal growth conditions (Fontecave et al. 2005). It has been suggested that the SUF system is adapted to synthesize [Fe-S] when iron or sulfur metabolism is disrupted by iron starvation or oxidative stress (Lee et al. 2004; Outten et al. 2004). Furthermore, the SUF system is regulated by Fur (Fe metabolism) and OxyR (oxidative stress regulator), whereas the ISC system is regulated by the [Fe-S] repressor, IscR (EcoCyc 2005). It is likely that the SUF system is particularly important for the assembly of [Fe-S]-proteins that pertain to oxidative stress or redox homeostasis.

Consistent with an earlier observation (Malone et al. 2006), a mutation in the gene encoding IscR did not affect E. coli barotolerance (Figure 5). Schwartz et al. (2001) found minimal growth defects with an iscR deletion strain. Furthermore, an iscR mutant had normal levels of [Fe-S]-proteins (Tokumoto and Takahashi 2001). Frazzon and Dean (2001) suggested that [Fe-S]-proteins establish biosynthetic homeostasis by effectively competing with IscR for the available clusters. Therefore, IscR receives [Fe-S] only when the capacity to produce these clusters exceeds the demand. These observations suggested that a deletion of iscR does not cause much of an adverse effect in the cell. Consistent with these earlier reports, iscR mutant and wild-type strains were similar in tolerance to HP and HP-TBHQ combination treatments (Figure 5).

Presence or activity of [Fe-S], or associated proteins, generally decreases barotolerance of E. coli (Malone et al. 2006). Therefore, E. coli strains with mutations in the ISC system (iscU, hscA, fdx) and suf were relatively pressure resistant, compared to their wild-type counterparts (Figure 5). Interestingly, iscU, hscA, and fdx mutants were
sensitive to HP-TBHQ, while the *suf* mutant was considerably more resistant to the combination treatment, when compared to their wild-type counterparts (Figure 5). Tokumoto and Takahashi (2001) demonstrated that a deletion of one component among IscU, HscA, and Fdx proteins caused the loss of function of the remaining proteins, suggesting that each gene in the ISC system is important for the activity of [Fe-S]-proteins. Although not tested in this study, an *iscS* mutant had the most profound effect on decreased [Fe-S]-protein activity, when compared with the other genes in the ISC system (Schwartz et al. 2000).

Considering that the ISC system is primarily maintaining the overall [Fe-S] status in the cell, we hypothesize that the SUF system is mostly involved in constructing [Fe-S] for a specific subset of [Fe-S]-proteins that may be related to redox homeostasis and/or pressure sensitivity. Although some redundancy was observed between SUF and ISC systems in the [Fe-S] assembly (EcoCyc 2005), substrate affinity during the assembly of specific [Fe-S]-proteins may favor the involvement of the SUF more than the ISC system. Therefore, mutants defective in the ISC system have less of the active housekeeping [Fe-S]-proteins, but have normal levels of activity for SUF-related [Fe-S]-proteins, which make the cells more susceptible to the HP-TBHQ inactivation. On the other hand, the SUF mutant would have normal levels of activity for the housekeeping [Fe-S]-proteins, but would have negligible activity from the SUF-related [Fe-S]-proteins; these differences in specific [Fe-S]-protein activities may have led to the differences seen between the SUF and ISC systems in response to HP-TBHQ combination treatment (Figure 5). Further studies are needed to determine if the activity of certain [Fe-S]-
proteins, which may interact with the high redox cycling compound, TBHQ, are diminished in the suf mutant.

3.5.3 Involvement of thiol-disulfide redox system and intracellular redox balance

Thioredoxin-1 (coded by trxA) is a primary component of the thiol-disulfide redox system in E. coli. It is a small (~12 kDa) electron-transfer protein that oxidizes thiol groups or reduces disulfide bonds of proteins, depending on its redox state, and it can also act as a molecular chaperone (EcoCyc 2005). Therefore, these thioredoxins maintain the reductive intracellular redox potential of the cell (Holmgren 1989). The redox potential of E. coli thioredoxin is low (-270 mV at pH 7); thus, the protein may reduce disulfide bonds that form spontaneously in cytoplasmic proteins when E. coli is grown aerobically (Prinz et al. 1997).

A recent study demonstrated that HP induces endogenous intracellular oxidative stress in E. coli MG1655 by generating disulfide bonds in a leaderless alkaline phosphatase (Aertsen et al. 2005). Growth of E. coli aerobically creates a relatively high redox potential in the periplasm and cytosol (George and Peck 1998). This turns off anaerobic respiration owing to the disassembly of the oxygen sensing, [Fe-S]-clusters (EcoCyc 2005). Under these conditions, induction of thioredoxin synthesis is crucial to protect other proteins from oxidative damage by ROS (Green and Paget 2004). Treating aerobically-grown cells with pressure probably speeds up the destruction of a subset of [Fe-S], and thus increases the cytosol redox potential. Proteins may become mis-oxidized during HP treatment (Malone et al. 2006), in which case, TrxA is needed for cell
recovery from this disulfide stress (Åslund and Beckwith 1999; Beckwith 2003). Therefore, the chaperone properties of TrxA may be required for the recovery from the adverse effect of the pressure treatment. Absence of thioredoxin-1 in these cells is detrimental and thus, the \textit{trxA} mutant was sensitive to the pressure treatment, compared with its wild type (Figure 7). OxyR was shown to be constitutively expressed in a \textit{trxA} mutant (Åslund et al. 1999); this would help the cell cope with the oxidative damage that may have resulted from the metabolism of TBHQ. In agreement with this analysis, results of the current study show that under aerobic conditions, the \textit{trxA} mutant is actually more resistant to the HP-TBHQ combination treatment than its wild type, while under anaerobic conditions there were no significant differences (Figure 7). This again supports the notion that the differences in intracellular redox potential and the assembly of specific [Fe-S], relate to the increased sensitivity to HP-TBHQ combination treatment. In addition, these results show that aerobic and anaerobic shifts in environmental conditions play a role in the HP-TBHQ inactivation.

Maintaining the intracellular redox balance is critical in both HP and HP-TBHQ resistance. Addition of the water-soluble reducing agents glutathione and cysteine significantly diminished the lethality of the HP-TBHQ combination treatment against \textit{E. coli} O157:H7 (Figure 3). Glutathione and cysteine are strong reducing agents that protect the cells against oxidative damage or redox stress (Williamson et al. 1982; Chesney et al. 1996). Glutathione and cysteine probably neutralize the ROS that were catalytically created by the uncontrolled or derailed metabolic redox cycling of TBHQ. These findings suggest that TBHQ increases oxidative damage mediated by HP.
3.5.4 Miscellaneous DNA-binding proteins genes

In stationary-phase cells, Dps organizes the chromosome into a highly ordered complex (Ceci et al. 2004). The Dps protein protects DNA against oxidation by scavenging free iron and thus preventing the Fenton reaction. Mutation in *dps* sensitizes bacteria to pressure, as well as multiple other stresses (Nair and Finkel 2004; Malone et al. 2006). In this study, the *dps* mutant was significantly more sensitive to HP-TBHQ treatment than to HP alone (Table 5). This observation was also confirmed in Figure 4, which shows that the DLSR value for HP-TBHQ combination treatment was lower than that for HP treatment. These results further support the notion that release of iron ions, Fenton reaction, and DNA damage contribute to the TBHQ-assisted HP inactivation of *E. coli*. It is not obvious why a mutation in *ybdQ* (which encodes a universal stress protein) did not alter sensitivity to the HP-TBHQ combination-treatment (Figure 4).

3.6 Conclusion

This study proposes sensitizing mechanisms for HP treatment by TBHQ against *E. coli*. The findings suggest that [Fe-S]-proteins, particularly those related to nitrate metabolism and the intracellular redox potential, critically enhance lethality of *E. coli* by HP-TBHQ combination. The redox-cycling capability of TBHQ may lead to the generation of substrates for the Fenton reaction, or the production of phenoxy radicals (ROS); these reactive products could ultimately lead to DNA or cell-membrane damage. This study may help food processors develop effective and commercially viable HP-based preservation methods. The study may also benefit biologists interested in pressure-tolerant organisms and deep-sea life.
3.7 References


CHAPTER 4

ASSOCIATION OF PROPHAGE ANTITERMINATOR Q ALLELES AND SUSCEPTIBILITY TO FOOD-PROCESSING TREATMENTS APPLIED TO *ESCHERICHIA COLI* O157 IN LABORATORY MEDIA

4.1 Abstract

Resistance of *Escherichia coli* O157 to inactivation by high-pressure (HP) processing, heat, and UV and gamma radiation was associated with the allele of the prophage-encoded antiterminator Q gene present upstream of the Shiga-toxin gene *stx*₂. Increased processing may be required to kill certain strains of *E. coli* O157, and the choice of strains used as surrogate markers for processing efficiency is critical.

4.2 Introduction

Shiga-toxin producing *Escherichia coli* O157 causes an estimated 70,000 cases of human illness each year in the United States (MMWR 2004). Because of its low infective dose, reducing contamination in foods and water to extremely low levels is critical (Teunis et al. 2004). In addition to heat, HP, UV, and gamma (γ) radiation are also used to enhance the safety of foods, especially in foods that will be consumed without further processing such as fruit juices (Brinez et al. 2006; Buchanan et al. 1998; Oteiza et al. 2005). Although cattle are presumed to be the primary source of *E. coli* O157 contamination of the food supply (LeJeune and Wetzel 2007), not all bovine-origin
isolates are equal in their ability to produce Shiga toxin, the production of which is tightly linked with bacteriophage-mediated bacterial lysis. Given the potential for many decontamination processes to induce prophages and result in phage-mediated bacterial lysis, the present study was undertaken to compare processing resistance among *E. coli* O157 isolates having defined prophage antiterminator genetic elements.

### 4.3 Materials and methods

For bacterial strains and sample preparation, 23 strains of *E. coli* O157:H7 and one *E. coli* O157:H12 OSU-1 were examined for processing resistance (Table 6). Strains of bovine and human origin were selected based on the antiterminator Q allele present upstream of the *stx*₂ gene (LeJeune et al. 2004). All strains were grown from a 0.1% inoculum in Tryptose broth and incubation at 35°C for 18 h. All cultures were transferred at least twice before experimentation. All strains were grown to stationary phase (10⁹ CFU/ml) in tryptose broth (Difco, Becton Dickinson, Sparks, MD) before treatment. Each isolate was tested with each treatment three times.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Q Allele</th>
<th>Origin</th>
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<tbody>
<tr>
<td>ATCC 43889</td>
<td>933</td>
<td>Human</td>
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<tr>
<td>OSY-636.01</td>
<td>933</td>
<td>Meat</td>
</tr>
<tr>
<td>OSY-636.02</td>
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<td>Meat</td>
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<tr>
<td>ATCC 35150</td>
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<td>EC 103</td>
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</table>

Table 6. Isolates of *E. coli* O157 containing either the Q<sub>933</sub> (human) or Q<sub>21</sub> (bovine) allele.
The HP treatment was followed according to Malone et al. (2003), with a few modifications. Stationary-phase cells \((10^9 \text{ CFU/ml})\) in five ml of tryptose broth (Difco, Becton Dickinson) were aseptically transferred to sterile stomacher bags (11.4 by 22.9 cm; Fisher Scientific, Pittsburgh, PA), vacuum packaged, and the bags were heat sealed. The sample was pressure treated at 500 MPa for 1 min at 23±2°C, in a Quintus® high-pressure food processor (Flow Pressure Systems, Kent, WA). The initial temperature of the pressure transmitting fluid (one part distilled water to one part Houghto-Safe 620 TY; Houghton International, Valley Forge, PA) was controlled to account for compression heating. An untreated control was left at 23±2°C at atmospheric pressure (0.1 MPa) while each treated sample was being pressurized. After pressure treatment, the treated and control samples were serially diluted using 0.1% peptone water, plated on tryptose agar plates, and incubated at 35°C for 48 h to enumerate the viable count.

For the UV treatment, \(10^{-2}, 10^{-4}, \text{ and } 10^{-6}\) dilutions of stationary-phase *E. coli* O157 cultures were made in 0.1% peptone water. One hundred microliters of each dilution was surface plated on 100-mm tryptose agar plates. Plates were kept covered 15 min at 37°C before exposure to UV. The UV lamp (254 nm, 15 W; G15T8 General Electric, Cleveland, OH) was vertically adjusted to a distance from the Petri plate that resulted in 400 \(\mu\)W/cm\(^2\) light intensity and stabilized (15 min). The UV intensity was monitored with a 254-nm radiometer probe (model UVS-25, Ultraviolet Products, San Gabriel, CA). The plates containing *E. coli* O157 were uncovered and UV treated (400 \(\mu\)W/cm\(^2\)) for 5 s. Untreated plates were maintained at 37°C, but not exposed to UV irradiation. Both treated and control plates were incubated for 48 h at 35°C and enumerated.
For the $\gamma$ radiation treatment, stationary-phase cells ($10^9$ CFU/ml) in tryptose broth (Difco, Becton Dickinson) were aseptically transferred to a sterile stomacher bag (11.4 by 22.9 cm; Fisher Scientific), vacuum packaged, and the bag was heat sealed. Then, the sample bags were placed in a cobalt-60 $\gamma$ radiation source at the Ohio State University Nuclear Reactor Laboratory (Columbus, OH) at a dose of 0.7 kGy. This 4000-Ci source had a dose rate of 1.40 kGy/h. The temperature of the shielding pool, known as the Bulk Shielding Facility, was approximately 21±3°C. Therefore, the control samples were left at 21±3°C while the treated sample was $\gamma$ irradiated. After $\gamma$ radiation, the treated and control samples were serially diluted using 0.1% peptone water, plated on tryptose agar plates, and incubated at 35°C for 48 h to enumerate the viable count.

For the heat treatment, 1 ml of stationary-phase ($10^9$ CFU/ml) culture was dispensed in a microcentrifuge tube and placed in a 55°C water bath for 8 min. A control sample was left at 21±3°C for 8 min. After the heat treatment, serial dilutions of the control and heat-treated samples were aseptically prepared, using 0.1% peptone water. The samples were transferred to tryptose agar plates to enumerate viable cells.

For the statistical analysis, the log reduction ratio was calculated as $\log N_o/N$, where $N_o$ is the population count in the untreated control specimen, and $N$ is the count in the treatment sample. The average log reduction ratio of isolates encoding the Q933 allele (human strains) was compared with that of isolates encoding only Q21 (bovine strains) and O157:H12 OSU-1, for each treatment, using analysis of variance, followed by Tukey’s Honestly Significant Difference test ($P$ value<0.05, $n\geq3$) (JMP IN, SAS Institute, Cary, NC).
4.4 Results and discussion

Isolates encoding the Q933 allele were more sensitive to all processing treatments than were isolates encoding the Q21 allele (Figure 8). In addition, the stx-negative strain (O157:H12 OSU-1) was more resistant to UV and γ radiation—but not heat or pressure—than were isolates encoding the Q933 or Q21 genetic marker.
Figure 8. Reduction ratios ($\log_{10} (N_0/N)$) of 12 *E. coli* O157:H7 isolates with the Q$_{933}$ allele, 11 isolates containing the Q$_{21}$ allele, and a non-lysogenic strain, O157:H12 OSU-1. Error bars represent standard deviation, where each strain was tested three times. Values with the same letter are not statistically different ($\alpha = 0.05$). Comparisons were only made within each treatment.
Processing variability among isolates of *E. coli* O157 has been reported earlier (Alpas et al. 1999; Whiting and Golden 2002). The SOS response and treatments such as UV and γ radiation that up-regulate *recA*, initiate cell lysis among lysogens (Kim and Oh 2000). Aertsen et al. (2005) recently reported prophage induction in EDL933 strains was not dependent upon DNA damage, but rather a RecA-independent trigger of the SOS response involving the endonuclease Mrr. Prophage is induced by the SOS response. The antiterminator Q gene governs late-phage gene expression required for virion production and cell lysis (Wagner et al. 2001). *E. coli* O157 isolates of human origin that encode the *Q*₉₃₃ allele of this gene are more effective at preventing RNA polymerase termination than are those isolates encoding the *Q*₂₁ allele that is commonly found in isolates of bovine origin (LeJeune et al. 2004). Thus, *Q*₉₃₃-encoding prophages can more readily express the late-phage genes required for cell lysis. Given that survival patterns of foodborne pathogens are dependent on intrinsic characteristics of the substrate, as well as the initial concentration of the inoculum, caution should be used when extrapolating the results of these studies to naturally contaminated foods. Additional challenge studies using applicable food products should be performed.

Compared to *Q*₉₃₃ isolates, *Q*₂₁ isolates were more resistant to all treatments tested. Based on these results, we hypothesize that some food-processing treatments induce phage-mediated lysis, which also contributes to post-processing lethality. Those isolates with more easily inducible phages (i.e., the human isolates) were consistently more susceptible to all treatments tested. Similar to the results obtained in our experiments, previous studies have identified differences between bovine- and human-origin *E. coli* O157 isolates with respect to their environmental survival (Avery and
Buncic 2003). The tendency of Q$_{21}$-encoding isolates to be more resistant to antimicrobial factors leads to many questions. Although the strains more commonly associated with human disease (LeJeune et al. 2004) were more susceptible to the decontamination treatments, the importance of viable Shiga toxin-encoding phages that presumably remain in food is unknown. These phages may infect and lysogenize other *E. coli*, either in the food or the host gastrointestinal tract, potentially resulting in Shiga-toxin-related diseases (Gamage et al. 2004; James et al. 2001; Schmidt et al. 1999). However, from these studies it is not completely evident that it is the Q alleles themselves that govern susceptibility to the treatments tested, or whether this gene is simply a marker for one or more other genetic traits shared by Q$_{21}$-encoding isolates; the association we report should be considered when designing food processing operations.

4.5 References


CHAPTER 5

Escherichia coli O157:H7 Barotolerance and Cross-Protection Generated by Multiple Rounds of High Pressure Treatments

5.1 Abstract

Escherichia coli O157:H7 EDL-933 is a well studied foodborne pathogen that is capable of causing diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Control of such a pathogen using emerging processing technologies (e.g., high pressure [HP]) should be carefully assessed. The goal of this study is to determine if E. coli O157:H7-acquired resistance to HP makes the pathogen more tolerant of other antimicrobial treatments. Barotolerant E. coli O157:H7 mutants, OSY-MBM, OSY-ASM, and OSY-MAM, were generated by exposing the wild-type strain, EDL-933, to repeated treatments with HP. These E. coli O157:H7 strains were exposed to pulsed electric field (PEF; 25 kV/cm for 144 µs), gamma (γ) radiation (0.7 kGy), ultraviolet (UV) radiation (400 µW/cm² for 5 s), heat (55°C for 8 min), and alkaline conditions (0.025 M KOH or NaOH for 30 min) to determine if cross-protection was induced by HP. Additionally, the minimum inhibitory concentrations (MICs) of a variety of antibiotics, namely kanamycin, ciprofloxacin, streptomycin, gentamycin, chloramphenicol, tetracycline, nalidixic acid, and ampicillin, were determined for the wild-type and mutant strains. The barotolerant mutant strains, OSY-MBM and OSY-ASM, were more resistant
to PEF, \( \gamma \) and UV radiation than was the wild-type strain, EDL-933. Pressure resistance
did not render the strains resistant to heat or alkaline treatment. Interestingly, HP altered
the antimicrobial susceptibility of the barotolerant mutants. These results suggest that
inadequate use of HP can produce processing resistant mutants and may alter their
susceptibility to antibiotics.

5.2 Introduction

Emergence of processing-resistant strains among foodborne pathogens is a major
concern to the food industry as well as to public health. Repeated exposure of pathogens
to a sublethal antimicrobial process may select for subpopulations that are resistant to
lethal levels of this process as well as to other non-related antimicrobial treatments
(Rowe and Kirk 1999).

*Escherichia coli* O157:H7 is a foodborne pathogen that may cause diarrhea,
hemorrhagic colitis and hemolytic uremic syndrome (Paton and Paton 1998; Ryu and
Beuchat 1998). The bacterium shows genetic instability and rapid adaptation to stresses
(Hayashi et al. 2001; Iuchi and Lin 1993; Ryu and Beuchat 1999; Zook et al. 2001).
Several studies showed that strains of *E. coli* O157:H7 have diverse processing resistance
(Alpas et al. 1999; Malone et al. 2006).

High pressure (HP) is currently utilized to manufacture safe food while preserving
its nutritional and organoleptic properties (Berg and Bartles 2004). HP processing is
currently used in lieu of thermal pasteurization to process jam, jelly, shellfish, oysters,
guacamole, fruit juices, salsa, rice products, fish meal kits, poultry products, and sliced
ready-to-eat meats (Considine et al. 2008). Concerns associated with the use of HP
technology in food processing include high initial equipment costs and the potential decrease in process efficacy due to adaptation of the pathogen to the process or emergence of barotolerant strains (Hauben et al. 1998). Research is being conducted to resolve these issues. However, little is known about the potential for induction of multiple-processing resistant *E. coli* O157:H7 upon repeated exposure to HP.

In this study, the well-characterized *E. coli* O157:H7 EDL-933 strain was repeatedly treated with HP and the selection for pressure-resistant mutants was monitored. The objective is to determine if repeated exposure to pressure develops mutants that are not only barotolerant but also exceptionally resistant to additional antimicrobial processes.

5.3 Materials and methods

5.3.1 Bacterial culture

*E. coli* O157:H7 EDL-933 was kindly provided by J. LeJeune, Ohio State University, Wooster, OH. The bacterium was grown to stationary phase (~1.0 X 10⁹ CFU/ml) from a 0.1% inoculum in Tryptose broth (TB; BD Difco; Becton, Dickinson and Co., Sparks, MD) and incubation at 35°C for 18 h before treatment.

5.3.2. Isolation of pressure-resistant mutant of *E. coli* O157:H7 EDL 933

Cell suspension of *E. coli* O157:H7 EDL 933 was aseptically transferred to a sterile stomacher bag (Fisher Scientific, PA), vacuum-packaged, and the bag was heat-sealed. The packaged culture was pressure-treated in a HP food processor (Quintus®; Flow Pressure Systems, Kent, WA), and the initial temperature of pressure transmitting
fluid was controlled to account for compression heating. The culture of *E. coli* O157:H7 EDL 933 was sequentially pressure treated at 350 MPa (8 rounds), 400 MPa (8 rounds) and 500 MPa (4 rounds); each treatment was carried out for 1 min at 23±2 °C. After each round of pressurization, treated cells were grown to stationary phase in TB. After the 20th round, treated cells were diluted in 0.1% peptone water and plated on tryptose agar (TA), and plates were incubated at 35°C for 18 h. Five colonies were selected from the countable plates and transferred to TB, and the cell population was grown to the stationary phase. The resulting five cultures were sequentially subjected to 800 MPa at 25±3°C for 1 min (twice), 5 min (twice), and 20 min (once). Aliquots of the five pressure-treated cultures (100 µl each) were dispensed in microcentrifuge tubes, each containing 1 ml of TB. After 18 h incubation at 35°C, growth was observed in three of the five *E. coli* O157:H7 isolates (OSY-MBM, OSY-ASM, and OSY-MAM). These HP resistant isolates were subjected to an additional pressure treatment at 800 MPa for 20 min, and survivors were recovered by plating on TA. The three HP-resistant isolates were confirmed to be *E. coli* O157:H7 by biochemical, serological, and genetic methods, targeting important characteristics including O and H antigens, and virulence factors (procedure and data are not shown). Stock cultures of these HP-resistant mutants was prepared in 40% glycerol and stored at -80°C until use in subsequent experiments.

5.3.3. Susceptibility of *E. coli* mutants to various processes

Inocula of *E. coli* O157:H7 EDL 933 and the pressure-resistant mutants (OSY-MBM, OSY-ASM, and OSY-MAM) were grown overnight in TB to produce stationary-phase population (~1.0 X 10⁹ CFU/ml). The overnight cultures, or dilutions thereof, were
exposed to various lethal processes HP, pulsed electric field (PEF), γ and ultraviolet (UV) radiation, heat, alkali, and antibiotics), and resistance to these processes was measured.

5.3.3.1. HP treatment

Stationary-phase cultures of *E. coli* O157:H7 strains were treated with 600 MPa for 5 min (23±2°C) as stated earlier. An untreated control was held at 23±2°C at atmospheric pressure (0.1 MPa) while each treated sample was being pressurized. HP-treated and control cultures were plated on TA and plates were incubated at 35°C for 48 h.

5.3.3.2. PEF treatment

Susceptibility of *E. coli* O157:H7 strains to PEF was tested in a laboratory-scale PEF processor (OSU-4C; Ohio State University, Columbus), as described earlier (Lado et al. 2004) with modifications. Briefly, 50 ml of the stationary-phase cultures were diluted in 450 ml sterile distilled H₂O, producing a cell suspension containing 10⁷ to 10⁸ CFU/ml. The cells suspension was treated with PEF at 25 kV/cm for 144 µs. The initial treatment temperature (PEF inlet) was set at 21°C. The sample temperature momentarily increased to 45-49°C during treatment. The control samples were held at 21°C and briefly placed in a water bath at 47°C. PEF-treated and control cell suspensions were plated on TA and plates were incubated at 35°C for 48 h.
5.3.3.3. γ radiation treatment

Vacuum-packaged stationary-phase cultures were treated with 0.7 kGy at 21±3°C in a Cobalt-60 γ radiation source at the Ohio State University Nuclear Reactor Laboratory (Columbus, OH). The radiation source provides a dose rate of 1.40 kGy/h. Control cultures were held at 21±3°C while treated samples were γ irradiated. The γ irradiated and control cultures were plated on TA and plates were incubated at 35°C for 48 h.

5.3.3.4. UV treatment

Dilution (10⁻², 10⁻⁴, and 10⁻⁶) of the stationary-phase cultures of E. coli O157:H7 strains were prepared using 0.1% peptone water. Portions (100 ul) of diluted cultures were surface-plated on TA. The UV-lamp (254 nm, 15 Watt, G15T8 General Electric, Co., Cleveland, OH) was vertically adjusted to a distance from the Petri-plate that resulted in 400 µW/cm² light intensity and stabilized for 15 min before use to treat the strains. The UV intensity was monitored with a 254-nm radiometer probe (model UVS-25, Ultraviolet Products, Inc., San Gabriel, CA). After 15 min of spreading, the plates containing E. coli O157 were uncovered and UV-treated (400 µW/cm²) for 5 seconds. Both treated and control plates were directly incubated for 48 h at 35°C and resulting colonies were enumerated.

5.3.3.5. Heat treatment

Aliquots of the stationary-phase cultures were dispensed in microcentrifuge tube and heat treated in a waterbath at 55°C for 8 min. The tubes were totally immersed in the
water. Heat-treated and control cultures were plated on TA and plates were incubated at 35°C for 48 h.

5.3.3.6. Alkali treatment

Susceptibility of *E. coli* O157:H7 strains to high pH values was tested as described by Sharma and Beuchat (2004) with modifications. Briefly, the stationary-phase cultures were centrifuged and resuspended in 0.1% peptone water, and KOH or NaOH solution was added to the cell suspension resulting in 0.025 M. Control treatment was the untreated 0.1% peptone cell suspension. The mixtures were shaken for 30 min at 23°C. pH of the cell suspensions treated with KOH and NaOH were 11.75 and 11.76, respectively. The Dey-Engley (DE) neutralizing broth at pH 6.0 (BBL/Difco) was added to the treated and control samples at 1.2X concentration and the resulting pH was 7.1 and 7.2 for suspensions treated with KOH and NaOH, respectively. Alkali-treated and control cultures were plated on TA and plates were incubated at 35°C for 48 h.

5.3.3.7. Susceptibility to antibiotic

Preparation of the antibiotic plates and determination of the minimum inhibitory concentrations (MICs) of the selected antibiotics against *E. coli* O157:H7 strains were performed as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2005). The medium used was Iso-Sensitest Agar (Oxoid, Inc., Ogdensburg, NY). Kanamycin (0.06-256 mg/L), ciprofloxacin (0.004-128 mg/L), streptomycin (4-128 mg/L), gentamycin (0.03-128 mg/L), chloramphenicol (0.06-256 mg/L), tetracycline (0.06-256 mg/L), nalidixic acid (1-128 mg/L), and ampicillin (0.13-
256 mg/L) were tested. The density of the inoculum was adjusted to 10⁴ CFU/spot on the agar. The spots were allowed to dry at room temperature before inverting the plates for aerobic incubation (35°C for 18 h).

5.3.4. Quantifying bacterial response to treatments

Resistance of *E. coli* O157:H7 strains to HP, PEF, γ radiation, and alkali treatments was quantified as the log reduction. The log reduction is defined as the log \( \frac{N_o}{N} \), where \( N \) is CFU/ml in treated sample and \( N_o \) is initial (before treatment) CFU/ml.

5.3.5. Statistical analysis

Three independent replications, at least, of each experiment were performed. Data were analyzed using t-test or analysis of variance. Means of LSR were compared using Tukey’s Honestly Significant Difference post-hoc analysis with a 5% significance level (\( P < 0.05 \)). Data analyses were carried out using commercial statistical programs (JMPin®, SAS Institute Inc., Cary, NC, USA; SigmaPlot® version 9.0; SPSS Inc., Chicago, IL, USA).

5.4 Results

5.4.1 Response to HP treatment

Treatment of the wild-type and the barotolerant mutant strains of *E. coli* O157:H7 with 600 MPa for 5 min decreased their populations by 1.7 to 5.6 log CFU/ml (Figure 9). According to these findings, *E. coli* O157:H7 mutants OSY-MBM and OSY-ASM were
the most pressure resistant \((P\text{-value} < 0.05)\) with 1.7 and 1.9 log reductions, respectively, while the EDL-933 (wild-type strain) was the most sensitive, exhibiting 5.6 log-reduction. The mutant OSY-MAM (2.8 log reduction) was more resistant to HP than EDL-933, but significantly more pressure sensitive than the mutants OSY-ASM and OSY-MBM.

5.4.2. Response to PEF treatment

Treatment of \textit{E. coli} O157:H7 strains with PEF at 25 kV/cm for 144 \(\mu\)s modestly inactivated their populations by 0.62 to 2.0 log CFU/ml (Figure 9). Resistance to PEF process was significantly \((P\text{-value} < 0.05)\) greater for the \textit{E. coli} O157:H7 strains OSY-ASM and OSY-MBM than it was for EDL-933 and OSY-MAM strains.
Figure 9. Log reduction of *E. coli* O157:H7 EDL-933 and mutant strains tested against six stresses, which include HP, PEF, \(\gamma\) radiation (Gamma), UV, heat, and alkaline (KOH) treatments. Values with the same letter are not statistically different (\(\alpha = 0.05\)). Comparisons were only made within each treatment.

### 5.4.3. Response to \(\gamma\) radiation treatment

A small dose of \(\gamma\) radiation (0.7 kGy) at 21°C decreased the populations of *E. coli* O157:H7 strains by 1.7 to 3.2 log CFU/ml (Figure 9). *E. coli* O157:H7 mutants, OSY-ASM and OSY-MBM, were more radiation resistant than OSY-MAM (1.7, 2.1, and 3.2 log reductions, respectively). The wild-type strain, *E. coli* O157:H7 EDL-933, was more sensitive to \(\gamma\) radiation (2.9 log reduction) than the mutants OSY-ASM and OSY-MBM,
but there was no significant difference between OSY-MAM and EDL-933 in sensitivity to \( \gamma \) radiation.

### 5.4.4. Response to UV treatment

When agar plates, seeded with *E. coli* O157:H7 strains, were exposed to 254-nm UV radiation (400 \( \mu \text{W/cm}^2 \)) for 5 sec, the treatment inactivated 1.7 to 3.8 log CFU/plate (Figure 9). The three *E. coli* O157:H7 barotolerant strains were significantly more UV-resistant than was the wild-type strain (\( P \)-value < 0.05). When the barotolerant *E. coli* O157:H7 strains were compared, OSY-MAM was significantly more UV-sensitive than OSY-MBM and OSY-ASM mutants (\( P \)-value < 0.05).

### 5.4.5. Response to heat treatment

*E. coli* O157:H7 EDL-933 and mutant strains OSY-MBM, OSY-ASM, and OSY-MAM were similar in resistance to the thermal treatment at 55°C for 8 min; log reductions for these strains were 2.1, 1.2, 1.5, and 1.6, respectively (Figure 9).

### 5.4.6. Response to alkaline treatment

When cell suspensions of the barotolerance-variable *E. coli* O157:H7 strains were exposed to pH 11.8 (using KOH) for 30 min, the alkaline treatment inactivated 1.0 to 2.8 log CFU/ml (Figure 9). Two of the pressure resistant mutants, OSY-MBM and OSY-MAM, were significantly (\( P \)-value < 0.05) more sensitive to the alkali treatment than was the pressure-sensitive wild-type strain (EDL-933). Adjusting the pH of cell suspension to
11.8, using NaOH instead of KOH, resulted in similar lethality among the *E. coli* O157:H7 strains.

### 5.4.7 Antibiotic susceptibility

The minimum inhibitory concentrations were measured for eight antibiotics against *E. coli* O157:H7 EDL-933 and the three pressure-resistant mutant strains (Table 7). All strains were similar in their susceptibility to kanamycin, streptomycin, chloramphenicol, and nalidixic acid. Compared with *E. coli* O157:H7 EDL-933, at least two of the three pressure-resistant mutants were resistant to ciprofloxacin, tetracycline, and ampicillin. On the contrary, these mutant strains were either comparable in sensitivity or more sensitive to gentamycin than the wild-type strain.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Minimum Inhibitory Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDL-933</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.03</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>8</td>
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<tr>
<td>Gentamycin</td>
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<tr>
<td>Chloramphenicol</td>
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</tr>
<tr>
<td>Tetracycline</td>
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<td>Nalidixic Acid</td>
<td>8</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>32</td>
</tr>
</tbody>
</table>

Table 7. The antimicrobial resistance of *E. coli* O157:H7 EDL-933 and its isogenic mutants against eight antibiotics.

5.5 Discussion

*E. coli* O157:H7 is an emerging foodborne pathogen, having important characteristics such as low infectious dose, genetic instability, rapid adaptability to environmental stresses, and ability to transfer virulence factors to other microorganisms (Schmidt et al. 1999; Gamage et al. 2003; Gamage et al. 2004). Pressure-resistant strains of *E. coli* O157:H7 have been generated by multiple rounds of exposure to HP (Hauben et al. 1997). Repeated exposure of *E. coli* O157:H7 EDL-933 to pressure resulted in mutants with high barotolerance, suggesting that HP treatment induced or selected for
spontaneous mutations. These barotolerant mutant strains exhibited resistance properties to other antimicrobial processing technologies.

In addition to their resistance to pressure, the OSY-ASM and OSY-MAM mutants were also found to be resistant to PEF treatments and $\gamma$ radiation, but this was not the case for OSY-MAM. The pressure-resistant *E. coli* O157:H7 mutants were relatively resistant to UV radiation, compared to their wild-type counterparts. The three pressure-resistant strains varied in resistance to UV radiation.

Contrary to the findings just discussed, pressure resistance did not coincide with heat resistance. Interestingly, two of the pressure-resistant mutants exhibited alkali sensitivity while the third was similar to the wild-type strain in resistance to the treatment. Exposure to multiple rounds of HP not only created processing resistant mutants of *E. coli* O157:H7, but altered the antibiotic susceptibility profile of each mutant. Similarly, the use of biocides in *Salmonella enterica* and *E. coli* O157:H7 caused cross-resistance to other antimicrobial agents (Braoudaki and Hilton 2004).

There were variations in the antibiotic profile of barotolerant *E. coli* O157:H7 mutants. The barotolerant mutants were more resistant than EDL-933 to ampicillin, an antibiotic that targets the cell wall. The barotolerant strains, OSY-ASM and OSY-MAM, were more resistant to ciprofloxacin than was EDL-933 or OSY-MBM. However, OSY-MBM and OSY-ASM were more resistant to gentamycin and tetracycline than were EDL-933 and OSY-MAM. Ciprofloxacin is a quinolone that targets nucleic acid synthesis whereas gentamycin and tetracycline target protein synthesis. For kanamycin, streptomycin, chloramphenicol, and nalidixic acid, there were no differences in the MICs between EDL-933 and the barotolerant mutants. It is known that antibiotic resistance
involves the uptake and efflux of the antibiotic (Kumar and Schweizer 2005). Since HP effects the cell wall and other intracellular components, efflux and uptake of the antibiotics would most likely be affected leading to differing antibiotic resistant profiles for the barotolerant strains.

In conclusion, this study demonstrated that *E. coli* O157:H7 exposed to multiple rounds of HP exhibited cross protection to a variety of food processes as well as to some antibiotics. Future research is needed to determine if the specific genes involved in pressure resistance also confer resistance to these antimicrobial treatments. Since the parent strain, EDL-933, is well studied and its genome has been sequenced (Hayashi et al. 2001), the mutants created in this study will be useful in future research advancements.

5.6 References


CHAPTER 6

FINAL CONCLUSION

High pressure (HP) has emerged recently as a promising non-thermal food processing technology. The current study addresses the mechanism of inactivation of Escherichia coli O157:H7 by pressure, which untimely helps food processors optimize HP treatments, thereby produce safe products with minimal quality degradation. The approach followed in this study revealed that many genes contribute to the resistance or sensitivity of *E. coli* to pressure, including those associated with redox homeostasis and protection against oxidative stress. The thiol-disulfide redox system, Fe-S cluster assembly proteins, stress-related DNA binding proteins, and sigma factors were all involved in the inactivation mechanism of *E. coli* O157 by HP and by combinations of HP and tert-butylhydroquinone (HP-TBHQ). The origin and phage content of *E. coli* O157:H7 appear also to influence barotolerance. Development of processing resistant *E. coli* O157 through exposure to multiple rounds of HP reinforces the importance of eradicating the pathogen population during the treatment.

The gene transcriptional analysis in the current investigation made it possible to propose mechanisms for the inactivation of *E. coli* by HP, but confirmation of these mechanisms requires additional studies. Detailed proteomic analysis would complement the finding of this study. Proteins expressed in response to pressure treatment may be detected using 2-D gel electrophoresis, or liquid chromatography coupled with mass
spectrometry (LC-MS). Elucidation of the structure of key proteins would require their purification, followed by amino acid sequencing using MS and nuclear magnetic resonance analyses, or other techniques. *E. coli* is a well-studied facultative anaerobe that served as a model bacterium in the current study, but applicability of these findings to other bacteria needs to be explored. Physiological response of other common foodborne pathogens, such as *Salmonella* sp. and *Listeria* sp., to pressure treatment should be considered.

Responsiveness of SUF and ISC systems to stress was reported earlier and observed in the current study, yet the functional differences between these two systems remain unclear. Thus, further research is required to understand the key differences between these two systems. Although these two systems seem to have overlapping functions, it may be proposed that SUF is involved in oxidative stress, whereas ISC serves as a “housekeeping” system for maintaining Fe-S clusters. Strains with mutations in various genes representing the SUF and ISC systems could be systematically exposed to oxidative and HP stresses, and responses of these mutants are monitored.

Importance of Fe-S proteins to barotolerance of *E. coli* was clearly evident in this study. Therefore, a comprehensive list of Fe-S proteins in *E. coli* O157 should be compiled and a logical approach to monitor the contribution of these proteins to pressure resistance should be developed. If a protein is found to be critical to pressure resistance, the corresponding gene should be placed in *E. coli* O157 and have it overexpressed, and changes in barotolerance of the pathogen are monitored. A similar overexpression approach could be achieved for the SUF system, and other genes related to barotolerance, including *hns* and *stpA*.
Altered redox homeostasis is proposed as a mechanism of *E. coli* inactivation by HP treatment. Consequently, aerobic or anaerobic status of food during HP treatment may influence the efficacy of the process against foodborne microorganisms; this concept should be carefully investigated. There is some similarity between the responses of *E. coli* to HP treatment and cold shock. This implies that storage temperature of food could condition the microbial load to become resistant to pressure processing. Therefore, the role of storage temperature and time in adaptation of foodborne microorganisms to pressure processing should be explored. Lastly, further research is needed to understand the physiology of deep sea microorganisms; such studies could help food processors explain the behavior of foodborne microorganisms during pressure treatments.


Bergey’s manual of determinative bacteriology, 9th ed. Lippincott Williams and Wilkins, Baltimore, MD.


Isolation and characterization in mini-Tn5Km2 insertion mutants of enterohemorrhagic Escherichia coli O157:H7 deficient in adherence to Caco-2 cells. Infect. Immun. 68:5943-5952.


Figure 10. Dendogram showing genetic similarity of 17 *E. coli* strains based on macrorestriction with *Xba*I. Lane labels are the strain designations.