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Heat Tolerance Mechanisms of an Exceptional Strain of *Escherichia coli*

by

Aaron M. Pleitner

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To my parents, Mark and Gayle,

who have given up everything to give me everything.

Abstract

The mechanism of heat resistance in an exceptionally resistant strain of *E. coli* was investigated. *E. coli* AW1.7 was compared to a heat sensitive strain, *E. coli* GGG10. The heat resistance of both strains was increased by inclusion of NaCl, but only *E. coli* AW1.7 exhibited a consistent heat increase to resistance when growth in NaCl concentrations ranging from 2 to 6%. The quantification of cytoplasmic solutes in *E. coli* and the determination of thermal ribosome and protein denaturation demonstrated that ribosomes are more stable in *E. coli* AW1.7 than in *E. coli* GGG10. Ribosome stability and heat resistance corresponded to the accumulation of compatible solutes. Differences in protein denaturation between the strains were not observed. In conclusion, heat resistance in *E. coli* AW1.7 is dependent on solute transport. Knowledge on the mechanism of heat resistance of *E. coli* will facilitate the design of novel intervention methods to warrant food safety.

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I. Introduction and Literature Review

1.1 *Escherichia coli*

Escherichia coli was first identified as *Bacterium coli commune* in 1885 by Theodor Escherich through isolation from fecal samples. Later to be renamed under the new genus *Escherichia, E. coli* is included in coliform bacteria, belonging to the more distinct fecal coliforms (Janda & Abbott, 2006; Bettelheim, 1996). The food industry uses coliform bacteria as an indicator for overall cleanliness. Coliforms are used as an indication of sanitary conditions of processing facilities, the hygienic quality of raw materials, and the adequacy of thermal treatments. The presence of coliforms, including *E. coli*, in heat-processed foods indicates either process failure or post-processing contamination. In cooked and ready-to-eat products, coliform bacteria are indicator organisms for the presence of pathogens (Harrigan, 1998).

E. coli is a gram-negative, non-sporeforming rod that is generally motile with peritrichous flagella. It is typically a part of the natural microflora within the intestinal tracts of humans and animals. It has a very rapid growth rate both aerobically through respiration and anaerobically through fermentation. Most strains ferment lactose and most do not metabolize citrate, differentiating *E. coli* from other organisms in the *Enterobacteriaceae* family (Adams et al., 2008; Brenner, 1984).

Although most strains of *E. coli* are non-pathogenic, certain strains possess virulence factors. These virotypes are divided into subsections

depending on their specific virulence mechanisms. Enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and enterohaemorrhagic *E. coli* (EHEC) are the major virotypes. The severity of disease is dependent upon the infective dose and the mechanism of pathogenicity that are required to cause illness in the host. The most significant virotype is EHEC which has a low infective dose with possibly bloody diarrhea and hemolytic uremic syndrome with considerable mortality rates (Bhunia et al., 2008).

1.2 Outbreaks of Pathogenic *E. coli*

The prevalence of EHEC has been demonstrated over time through the occurrence of foodborne outbreaks. One of the most common vehicles for *E. coli* O157:H7, the most prevalent strain of EHEC, has been beef products, specifically ground beef. In October of 2009, the Center for Disease Control and Prevention (CDC) and the United States Department of Agriculture (USDA) issued a joint statement for the recall of over 545,000 pounds of commercial ground beef product from Fairbank Farms. Despite the recall, 26 individuals in 8 states had become ill; 19 of the 26 patients were hospitalized and 5 developed HUS, which resulted in 2 deaths (Anonymous, 2009a). Similar results were observed for an outbreak in Washington in 1992 to 1993 (Bell et al., 1994). Beef cattle shed fecal matter containing *E. coli* O157:H7 at constant rates (Carlson et al., 2009; Alonso et al., 2007; Bell et al., 1994). The exterior of the carcass becomes contaminated with fecal matter from the cow during slaughter (McEvoy et al., 2003). The main source of contamination is the animal hide during the dressing of the carcass

(Bell, 1998). Once the integrity of the carcass is breached via cutting, grinding, puncturing, and tenderizing, the meat surfaces become contaminated with *E. coli*. Elder et al. (2000) investigated the prevalence of EHEC, specifically *E. coli* O157:H7, within feedlots and processing conditions and found that 28 and 11% of fecal and hide samples, respectively, were contaminated with *E. coli* O157:H7 along with 17% of post-processing samples. Outbreaks related to fresh produce have become more significant in recent years. An example of this is an outbreak that occurred in 2006 related to fresh spinach, which, at the time, was a novel food vehicle for this pathogen. In this outbreak, 199 individuals reported illness in 26 states; 102 patients were hospitalized of which 31 developed HUS; 3 people would eventually die from consumption of contaminated spinach (Anonymous, 2006). The contamination of *E. coli* O157:H7 on spinach greens was traced to fecal runoff, fertilizer from beef cattle manure and wild pigs. As spinach grows close to the ground, pathogens are easily transmissed from ground to plant. Spinach and beef have not been the only products related to outbreaks caused by *E. coli* O157:H7 linked to fecal contamination.

1.3 Heat Resistance of *E. coli*

1.3.1 General Heat Resistance

Traditionally, the application of high temperatures in beef processing facilities is the most commonly used intervention method to reduce the incidence of *E. coli* on carcasses. Such methods include steam and hot water rinses as pasteurization steps (Corantin et al., 2005; Minihan et al., 2003; Nutsch et al., 1997; Phebus et al., 1997). Exceptional heat resistance in *E. coli* and related

organisms questions the efficacy of these intervention methods in providing a safe product to the consumer (Ahmed et al., 1995; Blackburn et al., 1997; Dlusskaya et al., 2011; Murphy, et al., 2004).

Heat resistance is highly variable for *E. coli*. *E. coli* K-12 is relatively heat sensitive with a D_{60}-value of 0.1 to 0.3 min (Chung et al., 2007; Jin et al., 2008; Dlusskaya et al., 2011). Other strains include: *E. coli* DM18.3; 380-94; ATCC 25922 with D_{60}-values of 15, 6.7 and 4.2 min, respectively (Dlusskaya et al., 2011; Riordan et al., 2000; Pereira et al., 2006). *E. coli* LMM1020 and LMM1030, two extremely pressure resistant strains of *E. coli*, exhibited D_{60}-values of 2.26 and 1.71 min (Hauben et al., 1997).

1.3.2 Environmental Conditions and Heat Resistance

Intrinsic factors of food products, including increased concentration of salt and sugar, lowered water activity and pH, impact heat resistance. Acid adapted cultures of *E. coli* exhibit higher heat resistance in pepperoni (Riordan et al., 2000). An increase in heat resistance has been observed for *E. coli* and *Salmonella* spp. in osmotically stressed situations (Blackburn et al., 1997), in products with increased lipid content (Ahmed et al., 1995) and in situations of nutrient limitation (Jenkins et al., 1988). Outbreaks have occurred from consumption of products in which these conditions allowed bacteria to remain viable. This includes: dry, fermented sausage with *E. coli* O157:H7 (CDC 1995; MacDonald et al., 2004), dried, powdered infant formula with *Cronobacter*

sakazakii (Drudy et al., 2006), peanut and peanut butter with *Salmonella* spp. (Anonymous, 2009b; CDC 2007).

1.3.3 *E. coli* strain AW1.7

E. coli AW1.7 is an exceptionally heat resistant bacterium that was isolated from a beef carcass following the application of steam and lactic acid washes in a commercial processing facility (Aslam et al., 2004). *E. coli* AW1.7 has an exceptionally high D_{60}-value of 71 min compared to that of *E. coli* K-12 with a value of 0.1 to 0.3 min (Dlusskaya et al., 2011). The application of high temperature in the form of steam and other stressors including low pH from lactic acid washes may have selected for such an exceptional heat resistance. Ruan et al. (2011) compared gene expression of *E. coli* AW1.7 to a strain of *E. coli* isolated prior to the introduction of current intervention methods, *E. coli* GGG10. *E. coli* AW1.7 overexpressed *nmpC* and other genes coding for membrane proteins responsible for solute transport. Comparison of gene expression for the two strains following exposure to sublethal temperatures, provided evidence that *E. coli* GGG10 overexpressed of *dnaK* (Ruan et al., 2011). The lack of overexpression of *dnaK* in *E. coli* AW1.7 rules out the explanation of an overactive heat shock response for this strain. Fatty acid analysis revealed that *E. coli* AW1.7 possess a higher concentration of saturated fatty acids and C17:0 and C19:0 cyclopropane fatty acids in the outer membrane compared to *E. coli* GGG10. A shift in saturated fatty acid composition as well as increased solute accumulation through porin activity have both been found to contribute to increased stress resistance (Ruan et al., 2011; Alvarez-Ordoñez et al., 2008).

1.4 Ribosomal Integrity under High Temperature

Ribosomes are thought to be a primary target of thermal inactivation. The ribosome is made up of 30 and 50S subunits forming the 70S complex. This complex is necessary for protein synthesis (reviewed with further information in Pace, 1973; Steitz, 2008). In *Staphylococcus aureus*, thermal inactivation correlated to ribosome degradation when the 30S subunit degraded at a lower temperature compared to that of the 50S subunit (Allwood & Russel, 1967; Rosenthal & Iandolo, 1970). Purified ribosomes, harvested from *E. coli*, degraded at the same temperatures to that required for inactivation of the cell (Tal, 1969). The claim that ribosomal integrity coincides with viability is supported by Tolker-Nielsen & Molin (1996). The intracellular content of ribosomal RNA was greatly decreased upon thermal inactivation of *Salmonella enterica* serovar Typhimurium and the supply of magnesium or the restriction of carbon increased thermal resistance and congruently increased ribosomal integrity.

1.5 Heat Shock Response and Heat Resistance

An increase in heat resistance can also be attained by exposure of bacteria to sublethal heat. Juneja et al. (1998) observed a 1.5-fold increase in D_{60}-value for *E. coli* in beef gravy and ground beef following heating to 46°C. Similar increases in heat resistance following the application of sublethal heat to a meat system have been observed for *Listeria monocytogenes* (Farber & Brown, 1990) and *Salmonella* spp. (Mackey & Derrick, 1987). An increase in heat resistance

following exposure to sublethal temperature has been termed the "heat shock response" and is attributable to the synthesis of heat shock proteins. These proteins work as chaperones to allow the correct folding of intracellular proteins and ribosomes. The heat shock response aids in resistance to other stressors. An increase in pressure resistance for *E. coli* was followed by the overexpression of heat shock proteins (Aertsen et al., 2004). Sublethal levels of pressure induced the production of heat shock proteins (Welch et al., 1993). The link between resistance to high temperature and high pressure emphasizes the importance of protein folding for cellular viability through the role of chaperone proteins.

1.6 Sigma Factor Activity

The adaptation of bacteria to high stress environments of osmotic, oxidative, pH, starvation, and sub-lethal temperatures involves expression of specific gene sets transcribed through specific sigma factors. A sigma factor is a required component of RNA polymerase that aids in transcription of DNA by recognition of promoters. RNA polymerase is made up of two components. One component is a complex of α, β, and β' subunits which are differentiated by specific molecular weights. The other component is the sigma factor, σ (Traverse & Burgess, 1969). Burgess et al. observed in 1969 that DNA transcription of specific segments was diminished upon separation of the RNA polymerase complex. Full binding and transcription activity was restored by the addition of the σ factor to the RNA polymerase subunit. This group proposed that because the σ factor specifically binds to promoters, multiple different σ factors may exist for specific gene sets important to environmental scenarios. Further investigation

proved the validity of this concept. Bacteria have multiple σ factors whose role in transcription is dependent upon certain environmental cues. *E. coli* has been a model for the majority of investigations of gram negative bacteria and specific σ factor activity. The activity of the three common subunits of RNA polymerase (α, β, and β') is consistent; however, multiple sigma factors allow for the varying recognition of transcription sites. In addition, the relative binding affinities of the seven different σ factors varies (Maeda et al., 2000). *E. coli* possess seven specific sigma factors as outlined in Table 1.

Table 1. Sigma factors of *E. coli* and their functions

Sigma Factor Function	Identification	Secondary Nomenclature	Reference
General housekeeping	σ^{70}	*rpoD*	Burgess et al., 1969
General stress, stationary phase	$\sigma^{38}(\sigma^{S})$	*rpoS*	Lange & Hengge-Aronis, 1991b., Mulvey & Loewen, 1989
Nitrogen metabolism	$\sigma^{54}(\sigma^{N})$	*rpoN*	Castaño & Bastarrachea,1984, Garcia et al., 1977
Flagella development	σ^{28}	*fliA*	Arnosti & Chamberlin, 1988
Heat shock	$\sigma^{32}(\sigma^{H})$	*rpoH*	Erickson et al., 1987
Additional heat shock, cytoplasmic modification	$\sigma^{24}(\sigma^{E})$	*rpoE*	Erickson & Gross, 1989, Mecsas et al., 1993
Ferric citrate uptake	σ^{fecI}	*fecI*	Ochs et al., 1996

The means of identification of each sigma factor follows two trends. The first being the Greek symbol for σ followed by the molecular weight (Da) for the molecule. The second option represents the set of genes to be transcribed (rpo stands for RNA polymerase and fli for flagella) (Clark, 2005).

1.6.1 Activity of *rpoD*

Initial work in the field of sigma factor and RNA polymerase specificity focused on the characteristics of σ^{70}, which is responsible for the general housekeeping genes including cell maintenance, replication, and metabolism. Activity of σ^{70} occurs during the exponential and stationary phases of growth.

The concentration of σ^{70} is 2 to 3 times that of σ^{28}, σ^{N}, and σ^{S}, the sigma factors responsible for stress response activity to harsh environments. There is no measurable σ^{S} present during the exponential phase of growth, but an increase in concentration to about a third σ^{70} that occurs during entrance into the stationary phase (Jishage et al., 1996).

The overall ability of bacteria to react to changing environments and other stimuli requires a change in overall gene transcription. This ability has been termed global control. Each of the sigma factors belongs to its own specific global control system(s) (Wheelis, 2008). When harsh factors are applied during food production and storage, the important global control systems and their sigma factors are σ^{S} and σ^{H}. These are used to react to osmotic stress, high acid, reduced available water, starvation, high pressures, and high and low temperatures.

1.6.2 Activity of *rpoH*

The ability of *E. coli* to adapt to high temperatures has been linked to σ^H (Straus et al., 1987). This sigma factor is responsible for the production of heat shock proteins which are a subset of chaperone proteins. The mechanism of these chaperone proteins is to properly navigate the refolding of intracellular proteins that have become denatured while exposed to high temperatures. This is attained through the consumption of ATP to remove any abnormal hydrophobic interactions that occur within the partially denatured protein. If denatured proteins are beyond repair, the heat shock proteins will recruit proteases to degrade the protein to use its components and to eliminate any toxicity from the degraded protein (Snyder & Champness, 2007; Birge, 2006). The initiation of a heat shock response occurs at sub-lethal temperature in growing bacteria. The major group of heat shock proteins is DnaK, DnaJ, and GroE. These proteins form the DnaK complex that stabilizes proteins but also negatively regulates levels of σ^H. When temperature is increased, the levels of σ^H and the DnaK complex increase and subsequently allow a higher thermal stability of cellular components. When misfolded proteins that require refolding or degradation are not present, the DnaK complex accumulates in the cell. This complex will bind to σ^H, restricting its activity and recycling it for other cellular activities. When the temperature decreases, the levels of σ^H need to be decreased to allow σ^{70} to accumulate. This allows for more efficient growth by the bacterial population. (Birge, 2006; Gamer et al., 1992; Straus et al. 1990; Tilly et al., 1983).

1.6.3 Activity of *rpoS*

Presence and activity of σ^S is induced on the initiation of the stationary phase of growth (Jishage et al., 1996). Western blot analysis determined the presence of σ^S in stationary phase cells but not in cells in the exponential phase of growth. The lack of σ^S infers the lack of a stress response for a cellular population whose main concern is growth rather than survival (Notley-McRobb et al., 2002; Cheville et al., 1996). High σ^S levels induced a shift towards survival in response to nutrient restriction or high stress conditions. The σ^S is responsible for specific phenotypic characteristics to resist such conditions. A general review and compilation of over 50 genes related to the activity of σ^S and stress response are provided by Hengge-Aronis, 2000 and Loewen et al., 1998. Some of the major resistances attributed to σ^S include:

a. Osmotic resistance through trehalose accumulation (Hengge-Aronis et al., 1993; reviewed in Strøm & Kaasen, 1993)
b. Acid resistance (Castanie-Cornet et al., 1999)
c. Resistance to ethanol and oxidative stress (Farewell et al., 1998; Stohmeier et al., 1999)
d. Virulence factors and efficiencies (Beltrametti et al., 1999; review in Dong & Schellhorn, 2010)
e. Morphological cellular size reduction (Lange & Hengge-Aronis, 1991a.)

1.7 Initial Osmotic Stress Mechanism

Under normal environmental conditions, *E. coli* will possess a positive turgor pressure due to a high intracellular osmolarity compared to that of the environment. This positive pressure provides structure to the cell by applying pressure to a malleable cell membrane. In response to osmotic stress, *E. coli* engage specific mechanisms to balance a high turgor pressure. When water activity is decreased in the environment, compared to the interior of a bacterial cell, the cell must maintain its integrity and level of hydration to survive. To balance the cytoplasmic osmotic pressure, bacteria, including *E. coli*, generally accumulate compatible solutes. A compatible solute, or osmolyte, is a compound or ion that is either accumulated from the environment or produced intracellularly to protect the cell. This group of compounds includes sugars, polyols, free amino acids, and derivatives from such groups (Kempf &Bremmer, 1998; Brown & Simpson, 1972). The name "compatible solute" is used because these compounds can be accumulated without interfering with the structure of cellular components, membrane stability, DNA replication, DNA-protein interactions, metabolic machinery and other such processes (Strange & Yancey, 1994). Glycine betaine, proline and trehalose have been found to be effective in osmoprotection for prokaryotic cells, specifically *E. coli* (Landfald & Strøm, 1986; Styrvold & Strøm, 1991; Perroud & LeRudulier, 1985; Record et al., 1998).

1.7.1 Porin Formation

The uptake of compatible solutes in *E. coli* starts with their passive movement through the outer membrane. This is accomplished through pores formed by porins OmpC and OmpF. The formation of porins is dependent on the sensing of osmotic stress. The transcription of *ompC* and *ompF* is regulated by a two component regulatory system. *EnvZ* and *OmpR*, sensor and regulator genes, respectively, sense the osmotic levels of the environment and regulate the level of transcription of porins (Hall & Silhavey, 1979; Hall & Silhavy, 1981). In low osmotic conditions, OmpF is present in increased amounts, whereas when high osmotic conditions are applied, OmpC is more prevalent. OmpF is larger in diameter which allows the movement of molecules that are essential for growth and cell proliferation. A stressful environment forces a cell to switch from growth to survival and OmpC becomes more prevalent. The smaller size of this porin allows movement of compatible solutes but restricts the movement of inhibitory and smaller hydrophobic compounds with larger molecular sizes. The regulation of *ompC* and *ompF* transcription on entry into the stationary phase is controlled by σ^S. On initiation of high/low temperatures and increased/decreased osmotic stress, *ompC* and *ompF* are increased and decreased, respectively. This allows the cell to limit movement of solutes during high temperatures and osmotic stress with OmpC or increase flow of molecules in low temperature or low osmotic stress with OmpF (Pratt & Silhavy, 1995).

1.7.2 Initial Regulation with Potassium Ion and Glutamate Accumulation

The initial reaction of the cell to a sudden change in osmolarity is to accumulate potassium ions (K^+). Accumulation of K^+ compensates for a negative turgor pressure on the cell and is accomplished through an uptake of the ions using multiple nonspecific pores in the outer membrane. The Kdp, Trk, and Kup systems in the inner-membrane transport K^+ (Epstein et al., 1993). Kdp has a high affinity for potassium, which scavenges for the ion if availability is limited (Stumpe et al., 1996). Trk uses ATP to drive a symport system, importing K^+ and exporting protons (Stewart et al., 1985). Kup is a less utilized system under a singular stress; however, enhanced induction occurs during low pH, high osmotic stress conditions. Kup transports K^+ under acidic conditions allowing for cross resistance capabilities (Trchounian & Kobayashi, 1999).

The accumulation of K^+ results in an excess of positively charged ions, which is detrimental to general cell function, organelle integrity and enzyme activity. The positive charge is neutralized by the accumulation of negatively charged glutamate. Synthesis of this compound occurs through either the tandem activity of glutamine synthetase and glutamate synthase, or glutamate dehydrogenase (Meers & Tempest, 1970; Miller & Stadtman, 1972; Yan et al., 1996). Glutamate is ideal to combat excessive K^+ accumulation due to its anionic nature and the ability of the cell to generate glutamate when ammonia and nitrogen are limited in the environment (Measures, 1975; Yan et al., 1996).

1.8 Stable Osmotic Status Balance

1.8.1 Transport Systems and Biosynthesis of Compatible Solutes

Following the initial accumulation of K^+ and glutamate equilibration, the cell will switch to a more practical, long-term mechanism that relies on the accumulation of compatible solutes. Multiple different transport systems actively transport compatible solutes. The ProU transport system is induced by transcription of σ^{70}, most commonly during the exponential phase of growth in high salt environments. This is initiated when the cell senses the presence of K^+-glutamate at as low as 50 μM. Induction of *proU* leads to the accumulation of compatible solutes in *E. coli*, as well as other members of the *Enterobacteriaceae* family (Higgins et al., 1987; Sutherland et al., 1986). The *proU* operon consists of three genes, *proV*, *proW* and *proX*. The first two account for the production of two membrane-associate proteins. The third gene encodes for a periplasmic binding protein. The *proU* operon codes for an ABC transporter that accumulates glycine betaine, proline betaine, proline, carnitine, choline, dimethylpropionate, homotyrobetaine, and γ-butyrobetaine (Csonka 1989; Csonka & Hanson, 1991).

ProP is a secondary transport system that controls movement of solutes into the cytoplasm differently than that of ProU. The transcription of *proP* is regulated by two different promoter sites. The first, P1, is similar in function to *proU* but is only able to deal with minute challenges in osmotic stress and nutrient restriction. The second promoter, P2, is downstream of the P1 transcription site and is regulated during the stationary phase by σ^S (Mellies et al., 1995). ProP accumulates proline, glycine betaine and other osmoprotectants,

through an ion symport at the expense of the proton motive force (Milner et al., 1988; Culham et al., 1993; Kempf & Bremer, 1998).

A third system accumulates choline and converts this precursor to glycine betaine. The *bet* operon produces a secondary transport system, similar to the ProP transport system. This system relies on the proton motive force and the high affinity transport protein BetT to import choline. A membrane bound choline dehydrogenase, encoded by *betA,* oxidizes intracellular choline to glycine betaine aldehyde. Glycine betaine aldehyde is converted by BetB to glycine betaine (Landfald & Strøm, 1986). Although the *bet* operon has a simplistic design, the necessity of available choline and oxygen for choline dehydrogenase creates a challenge. Activity of *betIAB* is regulated through the repressor gene *betI*. The recognition of an aerobic environment is attributable to the two component system of *ArcB* and *ArcA* (Lamark et al., 1996; Iuchi & Lin, 1988; Iuchi et al., 1989).

The transport of compatible solutes from the environment effectively alleviates changes to turgor pressure. However, the systems are dependent on the availability of compatible solutes in the environment. Another option used by *E. coli* and other bacteria is to synthesize compatible solutes endogenously. *E. coli* produce trehalose intracellularly to reduce osmotic pressure. The *ots* operon (*otsA* and *otsB* genes) is transcribed by σ^S. Trehalose synthase (OtsA) uses glucose-6-phosphate and UDP-glucose to generate the intermediate trehalose-6-phosphate. A phosphatase (OtsB) converts trehalose-6-phosphate to trehalose (Glæver et al., 1988; Styrvold & Strøm, 1991). Trehalose aids in the

equilibration of internal and external osmotic pressures. After osmotic pressure is relieved, trehalose can be metabolized as a carbon source. While under osmotic stress and the active transcription of the *ots* system, *treA* is transcribed leading to the production of trehalase located in the periplasm and thus does not interfere with the cytoplasmic accumulation of trehalose. Trehalase is used to breakdown the accumulated trehalose to two glucose molecules, which can then be metabolized by the cell as a carbon source for energy (Styrvold & Strøm,1991, and Strøm & Kaasen, 1993). An additional method of trehalose utilization has been proposed by Gutierrez et al., 1989. *E. coli* K12 that lacks *treA* were unable to grow on media with trehalose and high salt. These workers proposed that trehalase hydrolyzes available trehalose to two glucose molecules that are modified to glucose-6-phosphate by the phosphotransferase mediated uptake system. In doing so, the cell is provided with additional substrate to produce trehalose intracellularly.

1.9 Cross Protective Nature of Stress Responses

Following introduction of a sublethal condition, specific responses to one stress increase survival against other stressful conditions. The most obvious of these protective mechanisms are the multiple capabilities of compatible solutes. Such osmoprotectants also are thermoprotectants and baroprotectants. Intracellular trehalose accumulation increases thermal resistance. In limiting the intracellular trehalose concentration through a reduction of the activity of *otsA* and *otsB* genes, a drastic decrease in thermotolerance is observed (Hengge-Aronis et al., 1991). Trehalose and other sugars replace the water/lipid

interactions of lipid membranes. This is attained by providing an alternative hydrogen bond interaction with the –OH group of the sugar to the phosphate head group of the lipid membrane (Crowe et al., 1984). The application of trehalose in a model phospholipid system in dry conditions provided evidence that the variation in head group orientation of the phosphate group was more stable and led to an increase in disorder. This disorder and stability of the head group led to a more stable liquid-crystalline phase to form (Lee et al., 1986).

The development of protection against other stressors including high pressure and cold temperatures following exposure to osmotic stress has also been shown. As in high temperatures, the accumulation of compatible solutes is the stabilizing factor. The application of osmotic stress to *Lactococcus lactis* with sucrose and NaCl was congruent with an increase in resistance to high pressures compared to control samples without applied osmotic stress. Additionally, lactose and an unknown compatible solute were identified as being accumulated in conditions of osmotic stress (Molina-Höppner et al., 2004). Increased resistance to high pressures was also observed in *L. monocytogenes* and *E. coli* O157:H7 when present in milk compared to poultry meat and a phosphate buffer (Patterson et al. 1995). The presence of sugars in the milk promoted solute transport and accumulation more than that of a poultry meat or buffer system supporting the claims of increased osmotic resistance carrying over to high pressure resistance. An increased resistance to low temperatures correlates with exposure of bacteria to osmotic stress and the accumulation of compatible solutes as well. The concentration of glycine betaine of *L. monocytogenes* was increased

following the application of 4°C and/or 8% NaCl. When both salt and cold temperature were combined, intracellular concentrations were even higher. Additionally, the growth rate at 4°C with glycine betaine present in the growth media was twice that of the control without additional glycine betaine, supporting the beneficial nature of compatible solutes towards cryoprotection (Ko et al., 1994). *E. coli* strains with the ability to produce trehalose intracellularly survived much longer than *E. coli* mutants lacking *otsAB* activity when incubated at 4°C. Such increased viability was congruent with higher intracellular trehalose levels of the strains able to induce *otsAB*. In this instance, the production of trehalose is induced without the presence of osmotic stress but in the presence of cold temperatures, both upon transfer to 16 and 4°C from optimal growth temperatures with increased resistance congruent with trehalose concentrations (Kandror et al., 2002). Such observations provide evidence that bacteria use compatible solutes for resistance to harsh conditions other than osmotic stress.

Increased stability was also shown in model protein systems. Sucrose, glucose, sorbitol and glycerol increase the melting temperature (T_m) for egg proteins, ovalbumin, lysozyme, and conalbumin (Back et al., 1979). Similar results were found with regards to the efficacy of glycine betaine and the aggregation of citrate synthase. Following exposure to sub-lethal temperature, an increase in available glycine betaine caused a decrease in enzyme aggregation (Caldas et al., 1999). This indicates an increase in enzyme stability due to a decrease in disorder limiting possible aggregation. The mechanism for such thermal protection is the affinity of the solute for hydrophobic regions of the

protein surface, stabilizing proteins and increasing resistance to aggregation. The hydrophobic regions on protein surfaces are preferentially hydrated with a solute, such as glycerol, limiting the amount of interaction of intermediate structure of partially denatured and unfolding proteins. In doing so, the rate and amount of free energy driving further denaturation is decreased and the affinity for protein-protein aggregation is limited leading to an increase in native protein stabilization (Vagenende, et al., 2009).

1.10 Monitoring Cell Integrity

Confirmation of mechanisms contributing to heat resistance requires understanding of heat sensitive targets, and evaluation of mechanisms known to contribute to heat resistance in *E. coli*. These particularly include heat shock proteins and osmolytes. Experimental methods with the capability to characterize the bacterial response to stress include the investigation of gene expression, synthesis of proteins, and other means to manage stress. However, to understand the role of heat shock proteins, membrane modifications and osmolytes in heat resistance, additional methods to assess cell integrity and protein stability are required. Such methods include Differential Scanning Calorimetry (DSC) and Fourier-Transform Infrared Spectroscopy (FT-IR).

Differential Scanning Calorimetry measures the difference in the rate of heat flow of a sample cell compared to a reference cell due to variations of heat exchange between cells (Höhne et al., 2003). This technique is used to observe the endothermic energy of bacterial cell suspensions providing an indirect

approach to measuring structural degradation. It is possible to determine the point of cellular inactivation. Initially, DSC was used in microbiology to investigate spore activation and germination (Maeda et al., 1974; Maeda et al., 1975; Van Cauwelaert & Verbeke, 1979). This technique was also used to investigate cellular degradation of vegetative cells with increasing temperatures (Mackey et al., 1993; Miles et al., 1986; Verrips & Kwast, 1977). Mackey et al. (1991) identified the cellular components of *E. coli* that were degraded at each endothermic peak on the DSC thermogram. This approach identified the ribosomal subunits and the 70S complex as the most heat sensitive organelles of the cell.

Fourier-Transform Infrared Spectroscopy determines protein structure and conformational changes in the protein. Changes in protein structure are observed by the vibrational energy of bonds forming the secondary structure of a protein, specifically the C=O bond of peptide linkages. Such vibrations are observed between wavelengths of 1600 and 1700 cm^{-1} (Byler & Susi, 1986; Krimm, 1983; reviewed in Kong & Yu, 2007).

Due to the noninvasive nature of FT-IR, this technique has been used for extensive work to differentiate among bacteria. Differences in cellular components and traits have lead to distinctive spectra that are compared among strains and even serotypes, allowing for a means of rapid identification of bacteria (Burgula et al., 2006; Helm et al., 1991; Naumann et al., 1988; Naumann et al., 1991; Oust et al., 2004). Researchers have compared gene expression through microarray and biomolecular composition through FT-IR for *C. jejuni*

(Moen et al, 2005; Oust et al., 2006). Lower incubation temperatures and varying oxygen availability cause a reduction in protein bond intensity while the concentration of poly- and oligosaccharides increase. A down-regulation of the targeted genes for such an increase in carbohydrate concentrations is observed leading to the suggestion that there is either a lag between gene expression and effected carbohydrate and protein composition, or an additional gene set in the group of osmoregulated periplasmic glucans. Such observations support the use of additional techniques to investigate biochemical composition of cells exposed to environmental stress.

1.11 Research Objectives

This research aimed to test the hypothesis that active solute transport leading to compatible solute accumulation and increased cellular organelle stability contribute to the exceptional heat resistance of *E. coli* AW1.7. The specific objectives were:

1) to understand the impact of environmental conditions that may influence the heat resistance of *E. coli* AW1.7;
2) to elucidate the cellular response to stress; and
3) to determine the stability of cellular components during thermal stress.

II. Materials and Methods

2.1 Bacterial strain selection

E. coli AW1.7 and GGG10 were used in this study. *E. coli* AW1.7 is an extremely heat resistant strain that was isolated from a commercial beef processing facility. *E. coli* GGG10 was isolated approximately 20 years ago prior to the introduction of current intervention methods (Aslam et al., 2004; Dlusskaya et al., 2011).

Cultures of *E. coli* were grown in sterile Luria Bertani (LB) broth consisting of 10 g tryptone (BD Bacto^{TM}, Mississauga, Canada), 5 g yeast extract (BD Bacto^{TM}) and 10 g sodium chloride (NaCl) (Fisher Scientific, Ottawa, Canada) unless otherwise noted.

To prepare stock bacterial cultures, strains were incubated overnight at 37°C with agitation at 200 rpm in 10mL of LB broth. The culture was centrifuged for 10 min at 6,800 x *g*, the supernatant was discarded and the cell pellet was resuspended in 1 mL of LB Broth. The cell suspension was added to 0.5 mL of sterile 80% glycerol in a cryotube and placed in a -80°C freezer until further use.

Prior to use in experiments, cell cultures were streaked onto LB agar (LB broth + 15g/L agar) plates and a single colony was streaked onto LB agar plate for resuscitation of bacterial strains. An isolated colony was inoculated into 25 mL of

LB broth in 50 mL conical tubes at 37°C with agitation (200 rpm). Overnight cultures were used for all experiments unless otherwise noted.

2.2 Generation of a heat sensitive derivative of *E. coli* AW1.7

E. coli AW1.7 contains four plasmids (Ruan, unpublished). Classical plasmid curing was used to remove plasmid DNA from the strain (Trevors, 1986). Sodium dodecyl sulfate (SDS) and increased growth temperature were used as curing agents (Mirmomeni et al., 2007; Hill & Carlisle, 1981). To remove a plasmid from *E. coli* AW1.7, the culture was grown overnight in LB broth and cultured as outlined in Figure1.

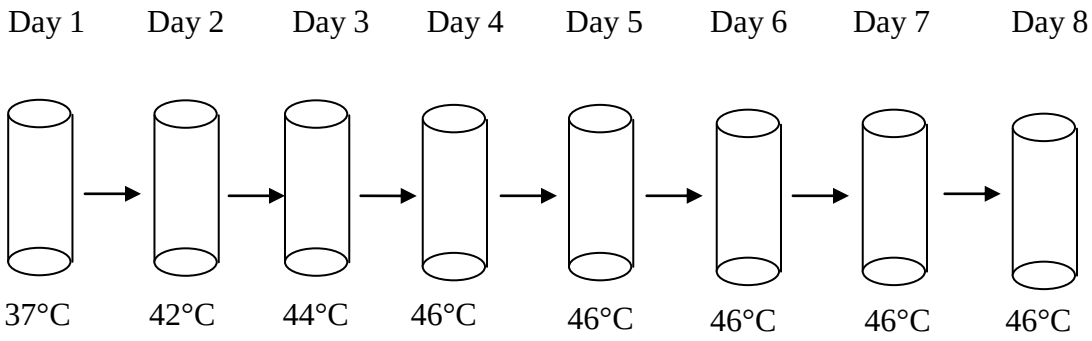

Figure 1. Plasmid curing protocol. A 1:10 dilution was used for each subculture. All media from Day 2 onwards contained 0.1% SDS.

Following each day of incubation, the culture was streaked onto three LB agar plates. After the completion of the curing cycle (a total of 5 days at 46°C with 0.1% SDS), cultures were screened for loss of heat resistance. From each plate, a single colony was inoculated into 300 µL LB broth in a microtiter plate. Cultures from all three growth temperatures, each day of incubation at 46°C and those with and without SDS were used for investigation. Microtiter plate wells

were overlayed with 25 µL of mineral oil and incubated overnight at 37°C. A 100 µL aliquot of each culture was placed in 200 µL PCR tubes and heated at 60°C for 30 min and cooled to 4°C in a PCR thermocycler (GeneAMP PCR System 9700, Applied Biosystems, Streetsville, Canada). Samples were placed on ice until they were plated onto LB agar and incubated at 37°C overnight. The remaining 200 µL of the original 300 µL of sample was held at 4°C for later reference. Frozen stock cultures of the heat sensitive strains were prepared from the reference microtiter plates to be screened for plasmid loss. *E. coli* AW1.7 and GGG10 were used for positive and negative controls for heat resistance and sterile LB broth was used for a negative control.

Plasmid purification was performed using QIAprep Spin Miniprep Kit (Qiagen, Toronto, Canada) according to the manufacturer's instructions. Isolated plasmid DNA was separated on 1.5% agarose (Invitrogen, Carlsbad, CA) gel with 1x TBE buffer (108 g Tris Base, 55 g Boric acid, 9.3g NaEDTA, 10 L Distilled water) with electrophoresis parameters of 80V for 2 hours. The gels were stained with ethidium bromide (BioRad, Mississauga, Canada) and visualized under UV. A 2 Kb+ DNA ladder (Invitrogen) was used as a size marker. Culture from *E. coli* AW1.7 wildtype and four heat sensitive strains were compared.

2.3 Minimum inhibitory concentration (MIC) of NaCl

The MIC method (Andrews, 2001) was used to determine possible differences in the salt tolerance of the strains. A 96 well microtiter plate was filled with 100 µL of LB broth with either 0 or 21% NaCl. To incrementally

adjust the concentration of NaCl in the wells of the microtiter plate, 200 µL of LB broth containing either 21 or 0% NaCl was added to the first well. From this well, 200 µL was carried over to the next well. This action was repeated for the next 10 wells. Each well was inoculated with 10 µL of overnight culture of *E. coli* AW1.7, GGG10, or AW1.7ΔpHR1 resulting in 10^8 CFU/mL per well. The concentrations of NaCl were calculated to be 6.5, 10.7, 13.5, 15.4, 16.7, 17.5, 18.1, 18.5, 18.7, 18.9, 19.0 (0% NaCl LB diluted with 21% NaCl LB) and 12.8, 8.6, 5.8, 3.9, 2.6, 1.8, 1.2, 0.8, 0.6, 0.4, and 0.3 (21% NaCl LB diluted with 0% NaCl LB). Following inoculation, 25 µL of mineral oil was placed in each well to prevent evaporation of media. The microtiter plate was then sealed with parafilm. Plates were incubated at 37°C with an agitation at 200 rpm. Turbidity of each well was assessed at 18, 24, and 48 h. LB broth with and without cultures acted as positive and negative controls, respectively.

2.4 Effects of NaCl and glycine betaine on heat resistance

To determine if NaCl and glycine betaine affect heat resistance, the NaCl concentration of LB broth was adjusted to concentrations of 0, 2, 4, and 6% w/v. Aliquots of 1 mL of an overnight culture of *E. coli* AW1.7, GGG10 or AW1.7ΔpHR1 were placed in 1.5 mL Eppendorf tubes. Samples were submerged in an agitating water bath at 60°C. *E. coli* AW1.7 was exposed to 60°C for 0, 5, 15, and 30 min. *E. coli* GGG10 and AW1.7ΔpHR1 were exposed to 60°C for 0, 5, 10, and 15 min. Following heating, samples were immediately placed on ice. Cell populations were enumerated after surface plating on LB agar and incubation at 37°C for 18 h. Experiments were performed in triplicate.

LB broth containing 6% NaCl with and without 5 mM glycine betaine (Sigma Aldrich, Oakville, Canada) was inoculated and incubated at 37°C overnight (18 h) until stationary phase was reached. Cultures were heated at 60°C for either 30 min (*E. coli* AW1.7) or 15 min (*E. coli* GGG10). Cell counts were determined by surface plating on LB agar following incubation at 37°C for 18 h.

2.5 Effect of *nmpC* activity on heat resistance

The heat resistance of *E. coli* AW1.7 harboring pYFP or pYFP-*yafQ-dinJ* and *E. coli* GGG10 harboring pYFP or pYFP-*nmpC* was determined. The generation of the mutant strains is outlined in previous work (Ruan et al., 2011). Overnight cultures were grown in 50 mL of LB broth in 250 mL flasks at 37°C with agitation (250 rpm). Cultures were exposed to 60°C in a water bath for either 2, 4, 8 and 16 min (*E. coli* AW1.7) or 1, 2 and 3 min (*E. coli* GGG10). Cell counts were determined by surface plating on LB agar following incubation at 37°C for 18 h.

2.6 Effect of heat shock response on heat resistance

To determine if exposure to sublethal temperature would increase heat resistance, a heat shock response was induced in *E. coli*. *E. coli* AW1.7; GGG10; and AW1.7ΔpHR were grown at 37°C overnight in LB broth. Overnight cultures (100µL) were placed in a 200µL PCR tube and heated in a PCR thermocycler (GeneAMp PCR System 9700). An additional 100 µL was placed on ice to serve as a control. A heat shock response was induced by heating for 15 min at 50°C, then cells were held for 15 min at 37°C prior to storage at 4°C for no more than

15 min until further analysis. Cell counts were obtained pre- and post-sublethal temperature abuse to ensure no cell reduction occurred at 50°C and that initial cell concentrations were similar prior to lethal temperature treatment. Cells with and without sublethal thermal treatment were heated in the thermocycler to 60°C for either 40 min (*E. coli* AW1.7) or 6 min (*E. coli* AW1.7ΔpHR1 and GGG10). Samples were immediately placed on ice. Cells were plated onto LB agar and incubated at 37°C overnight. Experiments were performed in triplicate.

2.7 Identification and quantification of compatible solutes

To determine the intracellular levels of compatible solutes in response to increasing osmotic stress, *E. coli* AW1.7 and GGG10 were grown to stationary phase in LB broth (overnight 18 h) and subcultured (1%) into LB broth with 0, 2, and 6% NaCl. Cells were incubated at 37°C with agitation (200 rpm). When cultures reached the beginning of stationary phase, triplicate samples were taken from each culture, plated onto LB agar and incubated at 37°C for enumeration. Cell counts for *E. coli* AW1.7 were $1.1\pm0.2x10^9$, $9.3\pm0.6x10^8$, and $9.1\pm0.5x10^8$ CFU/mL, for 0, 2, and 6% NaCl respectively. Cell counts for *E. coli* GGG10 were $1.2\pm0.1x10^9$, $9.7\pm0.2x10^8$, and $9.5\pm0.2x10^8$ CFU/mL, for 0, 2, and 6% NaCl, respectively.

Cells were centrifuged at 5311 x *g* for 10 min and the supernatant was discarded. The cell pellet was resuspended in 10 mL of growth media, centrifuged and the cell pellet was resuspended in 10 mL growth media for an

additional cell wash. Final cell pellets were frozen in liquid nitrogen. Triplicate sample sets were prepared and analyzed.

Cell pellets were analyzed for compatible solutes by the Metabolomics Innovation Centre (TMIC). A Varian 500 MHz NMR with cold probe was used for metabolite determination. Further sample preparation and analysis followed the procedure of Maharjan & Ferenci (2003).

Statistical analysis was performed using PROC GLM of SAS (v. 9.2). Differentiation between means was done using Duncan's Multiple Range test.

2.8 Ribosome denaturation of *E. coli* during thermal treatment

The ability of the *E. coli* strains to protect cellular integrity was investigated using differential scanning calorimetry (DSC). Experiments were carried out in the laboratory of Prof. Dr. Roland Winter at the Technische Universität Dortmund, in Dortmund, Germany. A VP-DSC MicroCalorimeter (Microcal; Northampton, MA) was used to measure the overall latent heat of the sample in order to determine the point of ribosomal degradation in the form of endothermic energy.

Cultures of *E. coli* AW1.7, GGG10 and AW1.7ΔpHR1 were grown overnight in LB broth with 0 or 4% NaCl added to the media. Cells were centrifuged at 5311 x *g* for 10 min at 4°C. The supernatant was removed and the cell pellet was resuspended in 3 mL of sterile media used for growth. The resuspended culture was portioned into 1.5 mL Eppendorf tubes, centrifuged at 6300 x *g* for 5 min, and the supernatant was removed with the cell pellet

resuspended in sterile media used for growth. Centrifugation was repeated for a total of three cell washes. Cell pellets were frozen in liquid nitrogen for overnight shipment on dry ice to Dortmund, Germany.

The effect of flash freezing on cell viability was investigated. Following freezing in liquid nitrogen and overnight storage at -80°C, no significant decrease in cell counts was observed, and no difference in heat resistance between fresh and frozen cultures was observed.

Samples were stored at -70°C. Thawed cell pellets were resuspended in 1 mL of 10 mM Tris-HCl buffer, pH 7.3 [1.211g/L Tris Base (Sigma) in 1 L of distilled water and pH adjusted with HCl]. The cell suspension was centrifuged at 6731 x *g* for 5 min, the supernatant removed, and the cells were resuspended in 1 mL of fresh Tris buffer. The cell suspension (0.5 mL) was loaded into the DSC sample cell and the same volume of Tris buffer was loaded into a reference cell for comparison. The scan rate of the DSC was 1.5°C/min (90°C/hr) with a pre-scan equilibrium at 20°C for 15 min. Two baseline scans were performed prior to experimental scanning and a secondary experimental scan was performed to identify any re-occurring thermal peaks due to reversible phase changes. Peaks were identified using Origin Pro 7.5 software (OriginLab, Thermo Fisher Scientific, Waltham, USA).

Following scanning, all thermograms were normalized by the dry weight of each individual cell suspension. Dry weights were obtained by freeze drying

the cells (Christ Alpha 1-2 LD plus freeze drier, SciQuip Ltd, Merrington, UK). DSC experiments were performed in duplicate.

2.9 Protein denaturation during heating of *E. coli*

The folding and unfolding of all intracellular proteins within *E. coli* AW1.7; GGG10; and AW1.7ΔpHR1 was measured using FT-IR coupled with incremental temperature shifting. Experiments were carried out in the laboratory of Prof. Dr. Roland Winter at the Technische Universität Dortmund, in Dortmund, Germany. A Nicolet 5700 FT-IR (Thermo Fisher Scientific, Waltham, USA), coupled with a liquid nitrogen cooled MCT detector (HgCdTe) was used for analysis. Sample preparation was identical as outlined for DSC measurement to the point of thawing.

Strains *E. coli* AW1.7, GGG10, AW1.7ΔpHR1 grown in LB broth with 2% NaCl were selected for investigation. Once thawed, cell pellets were resuspended in 300 µL of Deuterium Oxide (D_2O) (Merck). The cell suspension was centrifuged at 6731 x *g* for 5 min. The supernatant was removed and the cell wash was repeated to replace H_2O with D_2O. After the second centrifugation, the supernatant was removed and the cell pellet was resuspended in 100 µL of D_2O. A 40 µL volume of the cell concentrate was placed between two CaF_2 plates for FT-IR analysis. Prior to sample loading and analysis, a background scan was performed on the chamber atmosphere to remove any background noise. The sample was loaded into the FT-IR stage and a baseline scan was taken for reference purposes. The sample was attached to an external water bath for temperature control. Starting at an initial temperature of 25°C, the temperature

increased every 10 min by 2°C to reach a final temperature of 79°C. During scanning, a total of 256 scans were taken and averaged for a final spectrum at each temperature. Spectra were measured and recorded using the Omnic Professional software (Thermo Scientific). The initial background scan was subtracted from each scan to remove possible contamination of CO_2 from the FT-IR spectra.

Spectra analysis was performed using Grams/AI 8.0 software (Thermo Fisher Scientific). Each set of spectra was normalized using the initial baseline scan as a reference. The second derivative was calculated from the normalized peak data for further analysis. Spectra analysis was performed from wavelength areas 1600 to 1700 cm^{-1} with a resolution of $2cm^{-1}$. Table 2 represents the specified wavelengths chosen to target the amide I band of secondary protein structures (Kong & Yu, 2007 and Byler & Susi, 1986).

Table 2. Secondary protein structures assigned from the Amide I band

Wavelength frequency	**Protein structure**
1618 ± 2	Intermolecular β-sheet
1637 ± 2	Intramolecular β-sheet
1645 ± 2	Random turn
1653 ± 2	α-helix
1683 ± 2	β-turn

Peak absorption maximum as well as overall peak area were calculated and plotted over the temperature range studied using Grams/AI 8.0 and Origin 7.5 software.

III. Results

3.1 Generation of a heat sensitive derivative of *E. coli* AW1.7

E. coli AW1.7 contains four plasmids whose role in conferring the exceptional heat resistance to this strain is unknown. Classical plasmid curing techniques of increased incubation temperature and SDS were chosen to force plasmid loss. Figure 2 shows DNA isolated from *E. coli* AW1.7; GGG10 and a heat sensitive derivative of *E. coli* AW1.7 following plasmid curing. Incubation at 46°C with 0.1% SDS for 3 and 4 days resulted in creation of 7 and 10 heat sensitive isolates out of a total of 12 culture samples, respectively. After the fifth day of incubation at 46°C, 12 out of 12 isolates were heat sensitive. Plasmid isolation demonstrated the loss of the largest plasmid (ca. 10 kb) in all heat sensitive isolates of *E. coli* AW1.7, designated pHR1. A distinct band of DNA was observed in the wildtype strain (lane 1) but was not present in the derivative strain subjected to plasmid curing (lane 3). Upon confirming this, the new variant strain was titled *E. coli* AW1.7ΔpHR1 with the removed plasmid named pHR1.

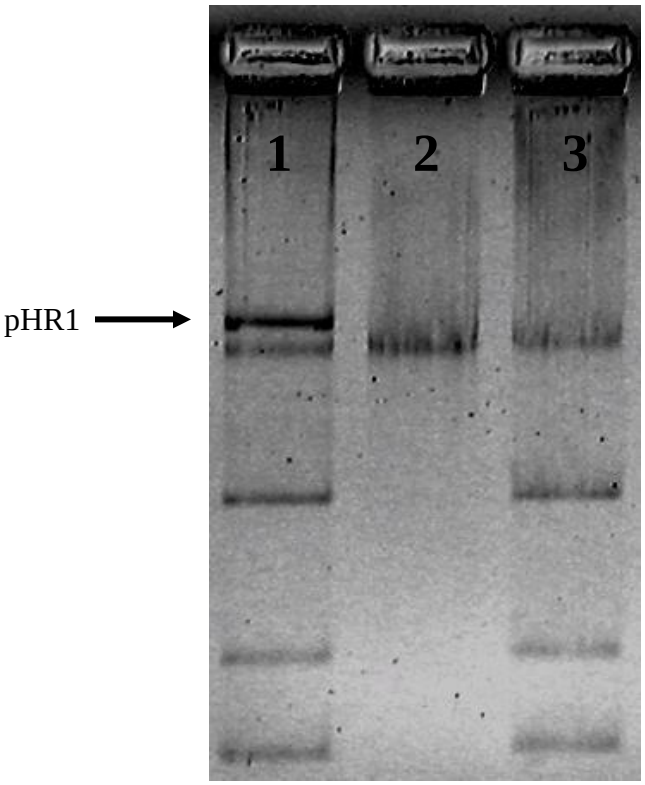

Figure 2. Agarose gel following electrophoresis of DNA isolated from wildtype *E. coli* (AW1.7: lane 1, and GGG10 lane 2) and *E. coli* AW1.7 after plasmid curing experiments (AW1.7ΔpHR1: lane 3).

3.2 Minimum inhibitory concentration (MIC) of NaCl

The resistance of the three strains of *E. coli* to increased osmotic stress was investigated. Using the MIC method, overnight cultures were exposed to LB with NaCl concentrations ranging from 0% to 19% NaCl. Comparable growth occurred for all three strains in all conditions up to a NaCl concentration of 6.5%. A NaCl concentration of 8.6% inhibited the growth of all three strains (data not shown).

3.3 Effects of NaCl and glycine betaine on heat resistance

Heat resistance in *E. coli* AW1.7 was linked to solute transport (Ruan et al., 2011); therefore, the effect of NaCl on heat resistance was determined. Figure 3 shows the cell counts of the three strains following treatment at 60°C in media containing different NaCl concentrations. Heat treatment for 30 min at 60°C reduced cell counts of *E. coli* AW1.7 by approximately 4 logs when no NaCl was present. A 1 to 1.6 log reduction occurred when NaCl (2, 4 and 6%) was present. Heating *E. coli* GGG10 for 10 min at 60°C reduced counts below the detection limit of $5x10^2$ CFU/mL in 0% NaCl and log reductions of 6, 2.6 and 3.4 were observed when cultures were heated in 2, 4 and 6% NaCl LB broth, respectively. Counts of *E. coli* AW1.7ΔpHR1 fell below the detection limit when NaCl was not added and population reductions of log 4.3, 1.7, and 2.4 were observed when cultures were heated in for 2, 4 and 6% NaCl LB broth, respectively. The heat resistance of *E. coli* AW1.7 is thus dependent on NaCl concentration. Cellular inactivation over high temperature was comparably high and similar in the range of 2 to 6% NaCl. A drastic decrease in cell population was observed at 0%.

The two heat sensitive strains showed the largest increase in heat resistance in 4% NaCl and, to a lesser extent, in 6% NaCl as well. However, at 2% NaCl, an initial heat resistance is observed but then drastically decreased. This study is the first to report the rate of cellular inactivation for *E. coli* AW1.7ΔpHR1 at high temperature.

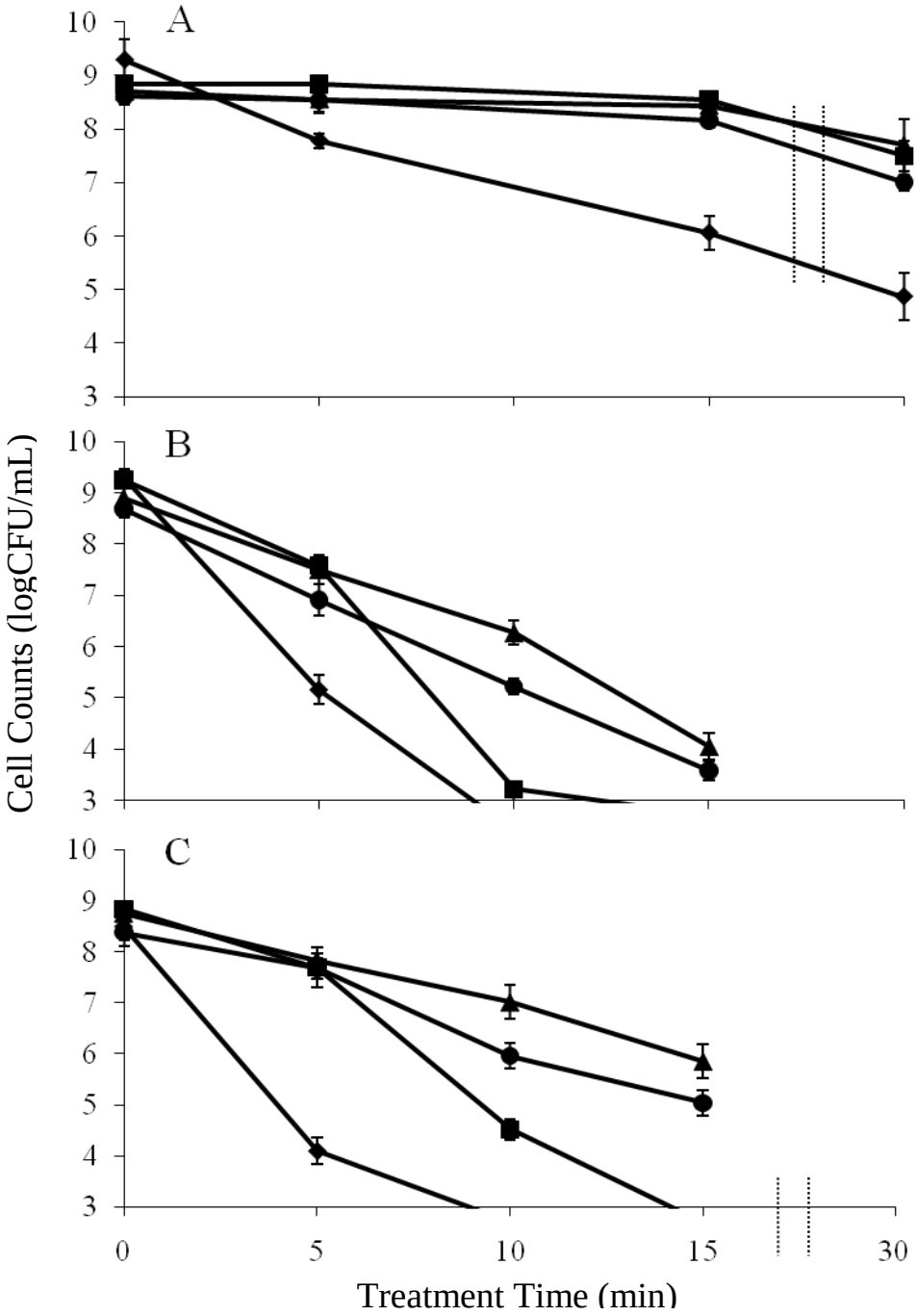

Figure 3. Cell counts of *E. coli* following treatment at 60°C in LB broth with different NaCl concentrations. Figure A: *E. coli* AW1.7; Figure B: *E. coli* GGG10; Figure C: *E. coli* AW1.7ΔpHR1. Symbol sets: ♦ for 0% NaCl, ■ for 2% NaCl, ▲ for 4% NaCl, and ● for 6% NaCl. Symbols crossing the *x* axis indicate cell counts below the detection limit of 2.7 log CFU/ml. n = 3.

In comparison to *E. coli* GGG10, *E. coli* AW1.7ΔpHR1 is still 1 to 2 logs higher following thermal treatment for 15 min.

To determine if additional compatible solutes could further increase the observed heat resistance, 5 mM glycine betaine was added to LB broth with 6% NaCl. No difference was observed in the heat resistance when glycine betaine was added (data not shown).

3.4 Effect of *nmpC* on heat resistance

The role of *yafQ-dinJ* and *nmpC* in the heat resistance of *E. coli* was determined by expression in *E. coli* AW1.7 and GGG10, respectively. Figure 4 shows the cell counts of the *E. coli* strains containing pYFP following exposure to 60°C for up to 8 min. The heat resistance of *E. coli* AW1.7pYFP-*yafQ-dinJ* was not different from that of the control strain, *E. coli* AW1.7pYFP. However, the survival of *E. coli* GGG10pYFP-*nmpC* at 60°C was significantly improved compared to that of control strain *E. coli* GGG10pYFP. Cell counts were consistently higher throughout treatment and differed by about 3 and 2 log CFU/mL after 1 and 2 min of treatment; cell counts for both strains were below the detection limit after 3 min.

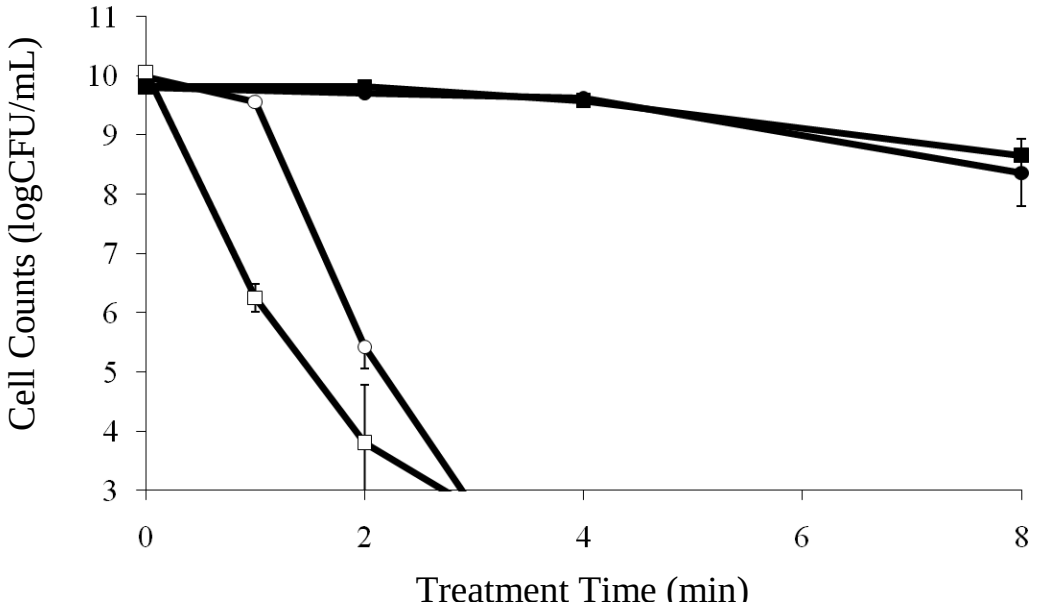

Figure 4. Cell counts of *E. coli* AW1.7 expressing *yafQ-dinJ* and *E. coli* GGG10 expressing *nmpC* after heating at 60°C. Symbols: *E. coli* GGG10 (□) and *E. coli* AW1.7 (■) control strains containing only pYFP, *E. coli* GGG10pYFP-*nmpC* (○) or *E. coli* AW1.7pYFP-*yafQ-dinJ* (●). Symbols crossing the *x* axis indicate cell counts below the detection limit of 2.7 log CFU/ml. n = 3.

3.5 Effect of heat shock response on heat resistance

E. coli AW1.7 does not overexpress heat shock proteins in response to exposure to 50°C (Ruan et al., 2011). To determine the effect of sublethal heat exposure on overall heat resistance, the three strains were exposed to 50°C for 15 min, followed by 37°C for 15 min and storage at 4°C prior to heat treatment. Exposure to sublethal temperatures was followed by thermal treatment at 60°C for 40 min (*E. coli* AW1.7) and 6 min (*E. coli* GGG10; AW1.7ΔpHR1). Figure 5 shows the reduction in cell counts following heat exposure with and without exposure to sublethal temperatures. Exposure to heat shock did not influence the survival of *E. coli* AW1.7. An increase in heat resistance was observed following

exposure to sublethal temperatures for *E. coli* GGG10 and AW1.7ΔpHR1, the two heat sensitive strains.

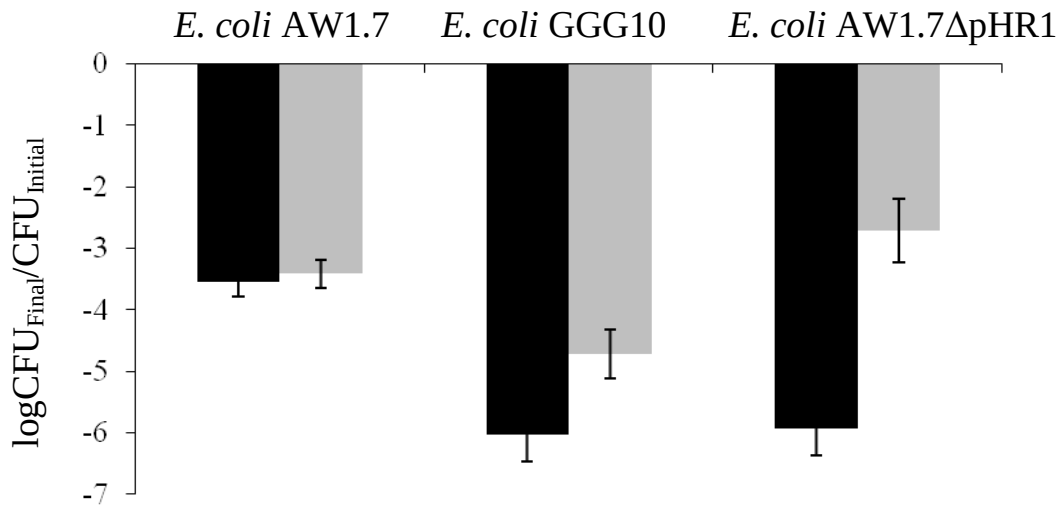

Figure 5. Reduction in cell counts following heat treatment at 60°C with and without sublethal heat shock. Black bars: control samples without heat shock treatment. Grey bars: samples following heat shock treatment at 50°C for 15 min *E. coli* AW1.7 was heated for 40 min while *E. coli* GGG10 and AW1.7ΔpHR1 were heated for 6 min n = 3.

3.5 Identification and quantitation of compatible solutes

Nuclear Magnetic Resonance was used to quantify compatible solutes accumulated by *E. coli* AW1.7 and GGG10 during the stationary phase of growth. This study aimed to perform two functions: to determine if there were any differences in the types and quantity of compatible solutes accumulated by the two strains and, to present a thorough listing of potential compatible solutes.

Table 3 reports the concentrations of the compatible solutes present in the two strains grown in LB broth with 0, 2, and 6% NaCl. The concentration of solutes accumulated in *E. coli* AW1.7 that were significantly different at the three

NaCl concentrations included 4-aminobutyrate, 5,6-dihydrouracil, alanine, aspartate, betaine, glycine, hypoxanthine, isoleucine, leucine, proline, succinate, threonine, trehalose, and valine. For *E. coli* GGG10 at the three NaCl concentrations, solutes that had significantly different concentrations included: 4-aminobutyrate, aspartate, glycine, hypoxanthine and succinate.

The concentration of a number of compounds accumulated by *E. coli* AW1.7 grown in 0% NaCl were significantly different from the concentrations detected when the strain was grown in the presence of NaCl; moreover, solute accumulation in *E. coli* AW1.7 was more active compared to *E. coli* GGG10 grown in 0% NaCl. These included arginine, glutamate, lactate, lysine, phenylalanine, pyroglutamate, tryptophan, tyrosine, and valine within the strain. The concentration of 4-aminobutyrate, 5,6-dihydrouracil, alanine, arginine, betaine, glutamate, glycine, hypoxanthine, isoleucine, lactate, leucine, lysine, methionine, phenylalanine, proline, pyroglutamate, succinate, threonine, trehalose, tryptophan, tyrosine, and valine were higher in *E. coli* AW1.7 compared to that found in *E. coli* GGG10. The concentration of solutes that were significantly different at 2% NaCl between the two strains included 4-aminobutyrate, aspartate, lysine, pyroglutamate, and succinate. Pyroglutamate and succinate were higher in *E. coli* GGG10 while the concentration of the others was higher in *E. coli* AW1.7.

Table 3. Mean concentration of intracellular solutes in *E. coli* AW1.7 and GGG10 grown in LB broth with 0, 2, and 6% NaCl.

Intracellular Compatible Solute concentrations (µM)												
	E. coli AW1.7						*E. coli* GGG10					
	0%NaCl LB Broth		2%NaCl LB Broth		6%NaCl LB Broth		0%NaCl LB Broth		2%NaCl LB Broth		6%NaCl LB Broth	
Compatible Solute	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
4-Aminobutyrate	124^{Aa}	5	282^{Ab}	36	199^{Ac}	23	69^{Ba}	9	56^{Bb}	12	206^{Ab}	26
5,6-Dihydrouracil	199^{Aa}	11	98^{Ab}	14	30^{Ac}	6	126^{Ba}	13	119^{Aa}	16	33^{Ab}	6
Alanine	175^{Aa}	113	816^{Ab}	126	583^{Ac}	68	962^{Ba}	180	1011^{Aa}	50	583^{Ab}	13
Arginine	838^{Aa}	123	470^{Ab}	57	513^{Ab}	37	422^{Ba}	48	412^{Aa}	20	401^{Ba}	37
Aspartate	127^{Aa}	13	47^{Ab}	4	0^{Ac}	0	109^{Aa}	17	77^{Bb}	6	0^{Ac}	0
Betaine	323^{Aa}	14	3399^{Ab}	204	1978^{Ac}	198	132^{Ba}	29	3374^{Ab}	39	2272^{Ac}	128
Glucose	0^{Aa}	0	0^{Aa}	0	1256^{Ab}	94	0^{Aa}	0	0^{Aa}	0	740^{Bb}	21
Glutamate	2113^{Aa}	100	609^{Ab}	78	518^{Ab}	82	821^{Ba}	134	943^{Aa}	64	430^{Ab}	26
Glycine	855^{Aa}	41	383^{Ab}	56	272^{Ac}	37	463^{Ba}	59	578^{Ab}	38	285^{Ac}	23
Hypoxanthine	276^{Aa}	1	156^{Ab}	40	92^{Ac}	8	48^{Ba}	10	249^{Ab}	20	109^{Ac}	10
Isoleucine	922^{Aa}	27	370^{Ab}	39	288^{Ac}	34	473^{Ba}	44	460^{Aa}	31	286^{Ab}	6

a. Means in the same row with the same superscript are not significantly different (P>0.05)
b. Lowercase superscripts designate differences among NaCl concentrations for each strain
c. Uppercase superscripts designate differences between strains for each NaCl concentration

Table 3. cont.

Intracellular Compatible Solute Concentration (µM)												
	E. coli AW1.7						*E. coli* GGG10					
	0%NaCl LB Broth		2%NaCl LB Broth		6%NaCl LB Broth		0%NaCl LB Broth		2%NaCl LB Broth		6%NaCl LB Broth	
Compatible Solute	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lactate	125^{Aa}	23	39^{Ab}	9	74^{Ab}	14	22^{Ba}	4	29^{Aa}	8	202^{Bb}	26
Leucine	2033^{Aa}	38	855^{Ab}	129	628^{Ac}	40	895^{Ba}	60	941^{Aa}	53	671^{Ab}	11
Lysine	1225^{Aa}	149	750^{Ab}	76	569^{Ab}	58	491^{Ba}	50	407^{Ba}	27	376^{Aa}	84
Methionine	568^{Aa}	26	250^{Ab}	32	201^{Ab}	25	293^{Ba}	26	308^{Aa}	23	207^{Ab}	7
Phenylalanine	1265^{Aa}	82	569^{Ab}	44	485^{Ab}	51	626^{Ba}	62	648^{Aa}	50	442^{Ab}	35
Proline	663^{Aa}	41	448^{Ab}	39	227^{Ac}	22	514^{Ba}	58	647^{Aa}	85	215^{Ab}	29
Pyroglutamate	1449^{Aa}	85	528^{Ab}	62	479^{Ab}	56	704^{Ba}	81	706^{Ba}	23	483^{Ab}	33
Succinate	1944^{Aa}	30	843^{Ab}	181	476^{Ac}	47	982^{Ba}	82	1514^{Bb}	86	470^{Ac}	5
Threonine	403^{Aa}	80	32^{Ab}	7	202^{Ac}	19	29^{Ba}	3	48^{Aa}	12	185^{Ab}	16
Trehalose	103^{Aa}	10	0^{Ab}	0	210^{Ac}	58	0^{Ba}	0	0^{Aa}	0	43^{Ab}	38
Tryptophan	285^{Aa}	10	141^{Ab}	36	129^{Ab}	17	121^{Ba}	14	113^{Aa}	20	119^{Aa}	4
Tyrosine	391^{Aa}	32	161^{Ab}	26	133^{Ab}	14	207^{Ba}	19	185^{Aa}	23	136^{Ab}	6
Valine	1185^{Aa}	48	513^{Ab}	50	378^{Ac}	55	656^{Ba}	92	606^{Aa}	35	370^{Ab}	5

a. Means in the same row with the same superscript are not significantly different ($P>0.05$)
b. Lowercase superscripts designate differences among NaCl concentrations for each strain
c. Uppercase superscripts designate differences between strains for each NaCl concentration

Figure 6 summarizes the trends for accumulation of nitrogenous compounds (alanine, arginine, aspartate, glutamate, glycine, hypoxanthine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, pyroglutamate, threonine, tryptophan, tyrosine, and valine), betaine, succinate and sugars (glucose and trehalose). When cells were grown in LB broth with 0% NaCl, the total concentration of nitrogenous compounds detected in *E. coli* AW1.7 was twice the concentration found in *E. coli* GGG10; however, when grown in LB broth with 2 or 6% NaCl, the concentration of the nitrogenous compounds was similar. In both strains, the accumulation of betaine occurred when cultures were grown in the presence of NaCl; however, the amount of betaine accumulated when cultures were grown in LB broth containing 6% NaCl was lower than that for culture grown in 2% NaCl. The concentration of succinate varied between strains. *E. coli* AW1.7 accumulate the largest concentration of succinate at 0% NaCl conditions while *E. coli* GGG10 increased accumulation at 2% NaCl to a point higher than that of *E. coli* AW1.7 at 2% NaCl. There was no difference between the cultures with succinate accumulation when strains were grown in the presence of 6% NaCl. The total concentration of intracellular sugars was low when strains were grown in 0 or 2% NaCl but when strains were grown in 6% NaCl they both accumulated significantly higher concentrations of sugars. *E. coli* AW1.7 was found to accumulate twice the concentration of sugars than that of *E. coli* GGG10 at 6% NaCl.

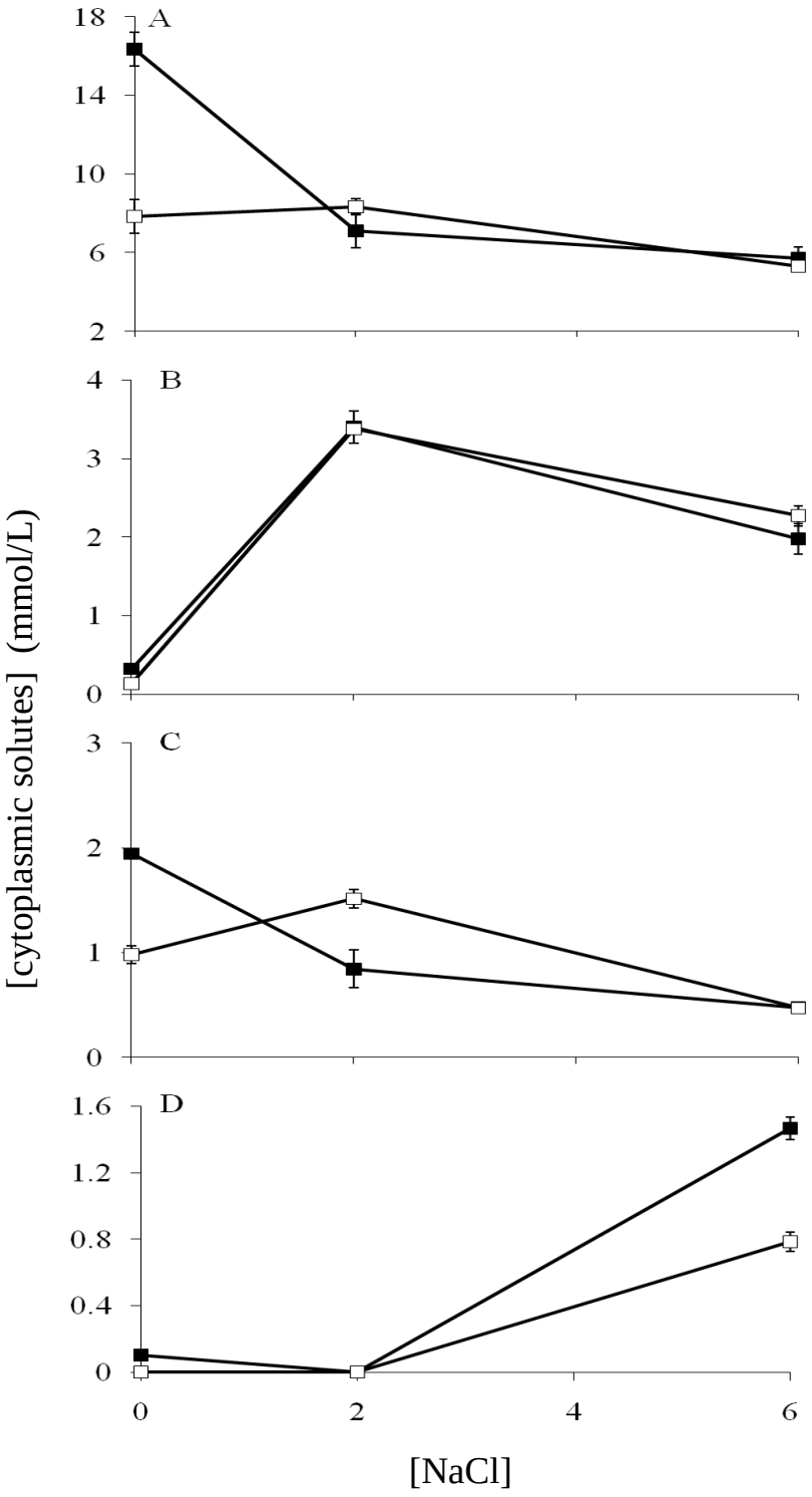

Figure 6. Total concentration of groups of compatible solutes in *E. coli* AW1.7 (■) and *E. coli* GGG10 (□) grown in LB broth with different NaCl concentrations. Panels: A: nitrogenous compounds, B: betaine, C: succinate, D: sugars. n=3.

3.6 Ribosome denaturation of *E. coli* during incremental thermal treatment

Differential scanning calorimetry was used to identify changes in endothermic energy during heating to determine the point of cellular degradation over incremental temperature increase. The DSC measures endothermic events during heating. When bacterial cells with different D-values are analyzed with DSC, a shift in the initial peaks of the thermogram is observed (Miles et al., 1986; Mohácsi-Farkas et al., 1999; Nguyen et al., 2006). A delay or positive shift in peak occurrence is correlative to a higher D-value comparative to bacteria with a lower D-value. The addition of osmotic stress also causes a positive shift in the initial peaks in the DSC thermogram of cells of *Listeria monocytogenes* (Anderson et al., 1991).

The DSC thermograms illustrate multiple peaks over a temperature range of 20 to 110°C. Figure 7 is a sample thermogram for *E. coli* AW1.7 grown in 0 and 4% NaCl LB broth. Table 4 is a summary of the denaturation temperature of each peak in the thermograms for all three strains at 0 and 4% NaCl. The use of Tris-HCl buffer instead of LB broth led to a relief in osmotic stress due to a lack of NaCl in the buffer that cells were suspended in for analysis. LB broth and NaCl alone caused too much interference in the thermogram and thus neither could be used in the suspension medium for analysis (data not shown). The alleviation of osmotic stress could have minimized the differences in the temperature of denaturation of the ribosomes when cells were grown with or without NaCl.

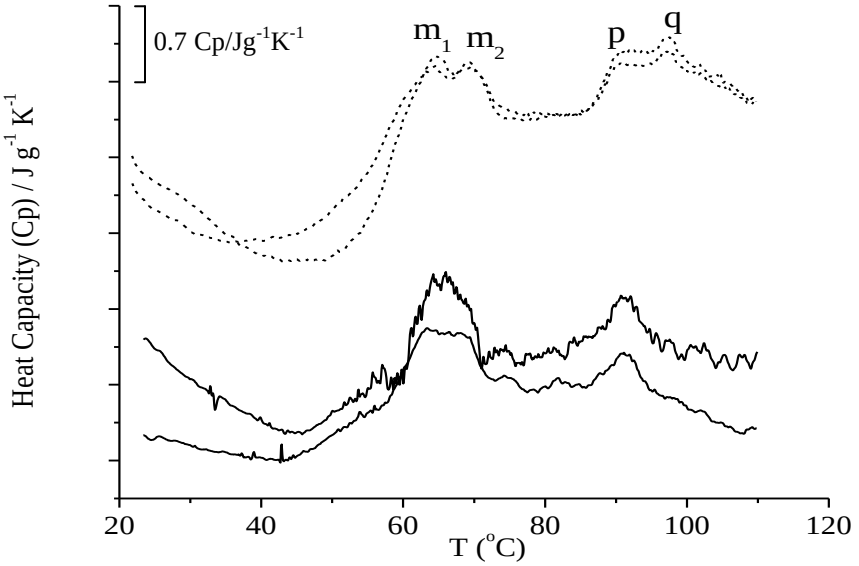

Figure 7. DSC thermogram for *E. coli* AW1.7 at 0 (——) and 4% NaCl (- - - -). Duplicate analysis presented.

Table 4. Denaturation temperatures of cell components[a] of strains of *E. coli* determined by DSC. n = 2

		Endothermic Peak Location (Temp)(°C)[c]							
		m_1		m_2		p		q	
Strain	[NaCl]	Mean	±[c]	mean	±	mean	±	mean	±
AW1.7	0	63.7	0.8	66.1	0.2	90.9	0.2	ND[b]	ND
	4	64.3	0.4	69.2	0.3	90.9	0.3	97.5	0.0
GGG10	0	60.7	0.3	70.5	1.9	90.2	0.1	99.3	0.1
	4	59.9	0.2	71.4	0.2	89.2	0.1	96.9	0.1
AW1.7ΔpHR1	0	62.9	0.0	67.1	0.2	90.4	0.2	ND	ND
	4	61.1	0.9	71.7	0.2	89.9	0.0	97.2	0.2

a. Designation of peaks is in accordance with that of Mackey et al., 1991 (m_1 represents ribosomal subunit 30S and m_2 represents ribosomal subunit 50S and the 70S complex, p represents cellular DNA, and q represents the melting of cell envelope components).
b. ND indicates that a peak was not detected in the temperature range where an endothermic event was expected.
c. ± is representative of the range around the mean value.

No significant shift was observed for peaks p and q, which represent the degradation of DNA and the cell envelope, respectively. However, when the

NaCl concentration was increased, an obvious shift in the temperature point of the m_2 peak for each strain was apparent. There was a positive shift of 3.1°C for *E. coli* AW1.7, 0.9°C for *E. coli* GGG10, and 4.6°C for *E. coli* AW1.7ΔpHR1 when salt concentration was changed from 0 to 4% NaCl. Comparison of DSC data for a single strain revealed that there was no significant shift in the m_1 peak.

When comparing between strains, a difference in the ribosome melting temperature was observed for the location of the m_1 peak. At 4% NaCl, the m_1 peak for *E. coli* AW1.7 was 3.2°C and 4.4°C higher than that of *E. coli* AW1.7ΔpHR1 and GGG10, respectively. Similar increases were observed for strains grown in 0% NaCl. There was no significant difference in the temperature of the m_2 peak, among strains, grown under the same conditions.

3.7 Protein denaturation during heating of *E. coli*

To determine any differences in protein orientation and confirmation over an increasing temperature range, FT-IR was used to capture secondary structures of intracellular proteins. The amide I' region of protein bands was specifically targeted in the wavelength range of 1600 cm^{-1} to 1700 cm^{-1}. The vibrational force of C=O bands being stretched is observed. The frequency of such band stretching is dependent on the secondary structures of proteins. As temperature increases, folded proteins become unfolded, lose their native structure, and form agglomerated structures. Figure 8 presents an example FT-IR spectra and second derivative spectra. These spectra represent intracellular protein orientation and respective denaturation over increasing temperature for *E. coli* AW1.7. As temperature increases, α-helix and intramolecular β-sheet structures, at

wavelengths 1654 cm^{-1} and 1637 cm^{-1} respectively, decrease. Concomitantly, disordered structures of intermolecular β-sheet and β-turn structures, at wavelengths 1618 cm^{-1} and 1683 cm^{-1} respectively, increase.

No difference was observed in protein denaturation for the three strains under investigation. Figure 9 represents the alterations of α-helix, β-sheet (inter- and intramolecular) and β-turns in the form of a sigmoidal plot over the temperature range specified. These plots were generated through mapping the intensity of each peak presented in Figure 8B. Overall, the T_m of intracellular protein was estimated to be 60°C. The temperature range at which protein denaturation initiated and continued at the most rapid rate is in accordance with that of lethal temperatures for *E. coli*.

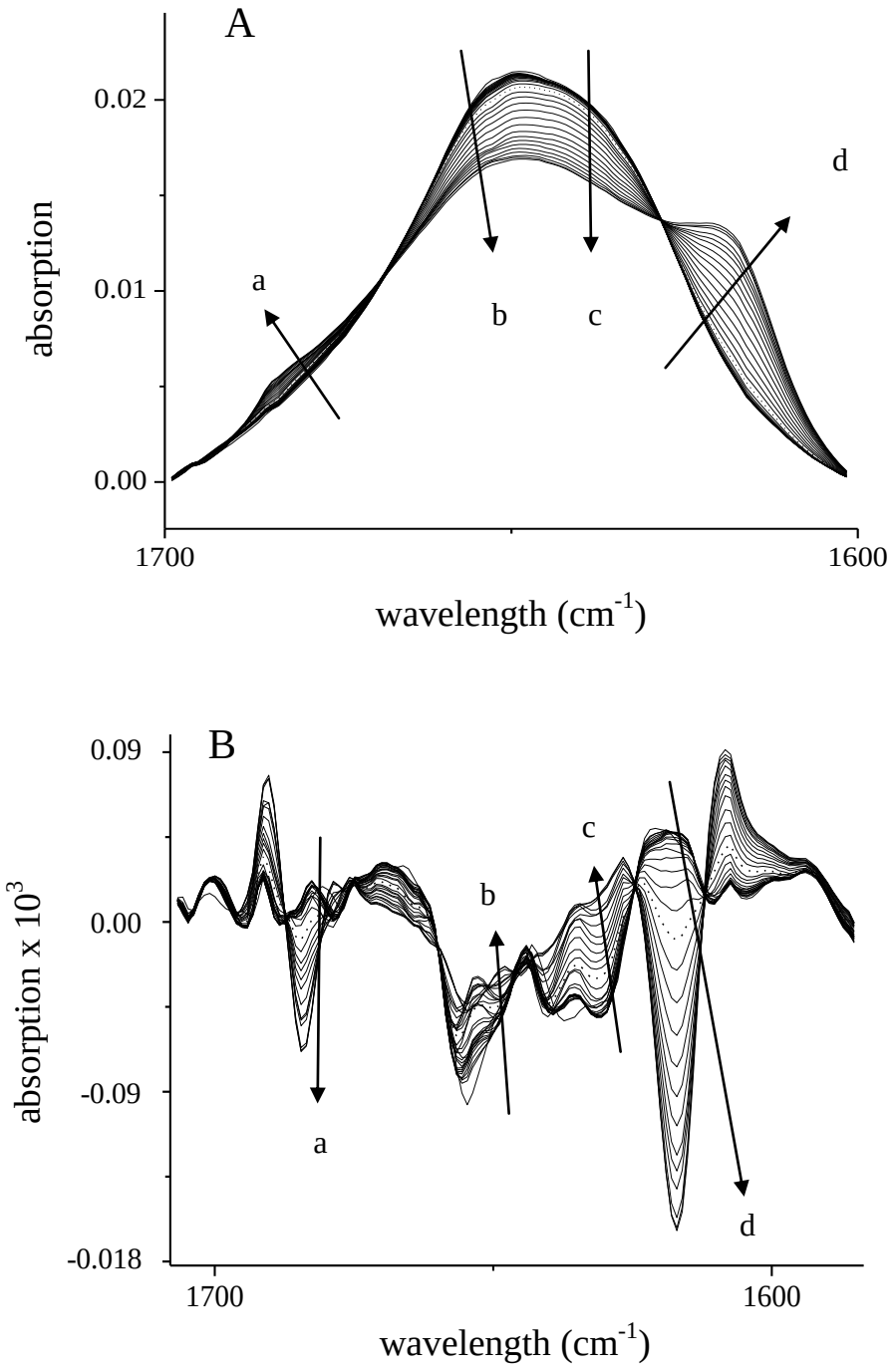

Figure 8. Original (A) and second derivative (B) FT-IR spectra for *E. coli* AW1.7; direction of arrow indicates increasing temperature from 25 to 79°C. Symbol a: wavelength 1683 for β-turns; b: α-helix at 1654; c: intramolecular β-sheet at 1637 respectively; d: aggregated intermolecular β-sheet at 1618.

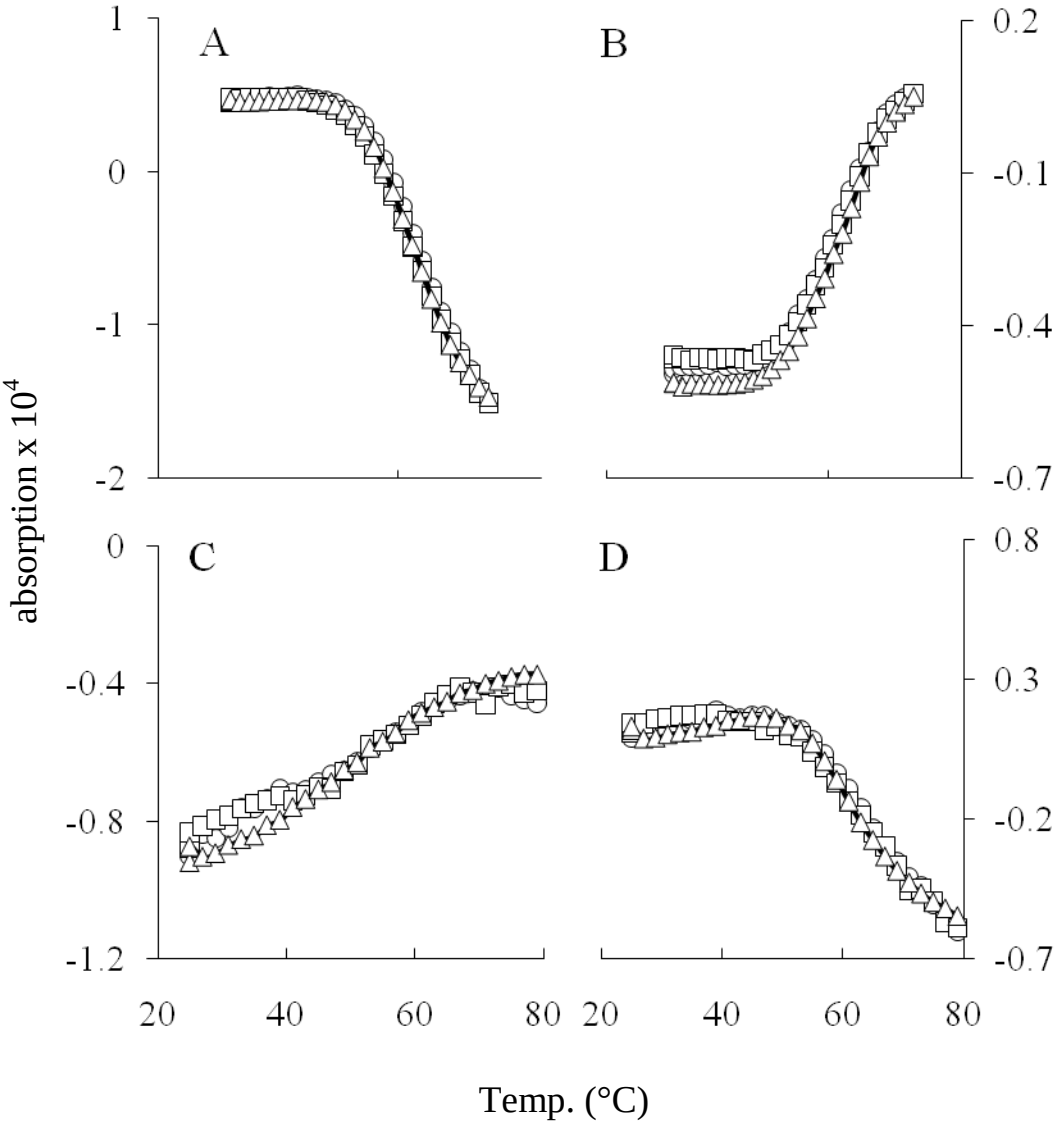

Figure 9. Sigmoidal plots for protein orientations with increasing temperature measured through FT-IR analysis. Figure A: intermolecular β sheet, figure B: intramolecular β sheet, figure C: α helix, and figure D: β turns. ❍: *E. coli* AW1.7; ❑: *E. coli* GGG10; Δ: *E. coli* AW1.7 ΔpHR1.

IV. Discussion

Intervention methods used by the meat industry aim to lower the overall microbial load of food products, to ensure the safety of the consumer and to extend the storage life of products. The most common intervention method utilized in beef carcass preparation is the application of high temperature. Steam and hot water pasteurization processes are effective measures of reducing the numbers of microorganisms on carcasses (Corantin et al., 2005; Minihan et al., 2003; Nutsch et al., 1997; Phebus et al., 1997). The resistance to heat is attributable to the intrinsic nature of a food product and the exceptional heat resistance of some bacteria emphasizes the need to understand the mechanisms behind the exceptional heat resistance of some bacteria.

E. coli AW1.7 is exceptionally heat resistant (Dlusskaya et al., 2011) and varies in the physiological and genetic characteristics from a heat sensitive strain of *E. coli* (Ruan et al., 2011). The aim of this study was to further elucidate the mechanism of heat resistance of *E. coli* AW1.7 and the effects of osmotic stress on heat resistance.

Initial work indicated that plasmid DNA in *E. coli* AW1.7 confers the exceptional heat resistance. The combination of elevated growth temperatures with SDS effectively cures plasmids in both gram negative and positive bacteria (Sonstein & Baldwin, 1972; Hill & Carlisle, 1981). SDS, an anionic detergent, is thought to target the integrity of the outer membrane and pili structures requiring cells to expend more energy on maintaining cell stability rather than plasmid replication, which will slow generation times (Salisbury et al., 1972; Tomoeda et

al., 1968). At a high growth temperature, a discrepancy occurs in the growth rate between cells with and without the demand for generation of additional DNA, making the loss of a non-essential plasmid beneficial (May et al., 1964). The loss of heat resistance in *E. coli* AW1.7ΔpHR1 occurred over a period of 5 d at the highest temperature and SDS concentration used. The occurrence of few heat sensitive cultures after 3 d, followed by the complete loss of heat resistance after 5 d, supports the theory that there are variations in growth rate and alteration of cellular machinery over multiple generations. The plasmid, pHR1, was lost in tandem with the overall loss of heat resistance in *E. coli* AW1.7ΔpHR1 in 12 different cultures in duplicate trials (this study; Ruan 2010). This supports the claim that the largest plasmid in *E. coli* AW1.7 is responsible for the majority of the heat resistance in *E. coli* AW1.7.

The heat resistance of *E. coli* AW1.7 was compared to *E. coli* AW1.7ΔpHR1 and *E. coli* GGG10. Varying concentrations of NaCl were used to impose osmotic stress on the cultures. A response to osmotic stress can increase the overall heat resistance of bacteria due to cross protection. An increase in heat resistance for *Salmonella* spp. and *E. coli* strains is observed when cells are exposed to increased concentrations of NaCl and sugars (Blackburn et al., 1997; Mattick et al., 2001; Dega et al., 1972; Goepfert et al., 1970). Similar trends in increased heat resistance with increased osmotic stress have been reported in other bacteria (Calhoun & Frazier, 1966; Verrips & Kwast, 1977; Juneja & Eblen, 1999).

The heat resistance of *E. coli* AW1.7 was increased when NaCl was added to the growth medium. The increase in heat resistance for this strain was consistent across the three concentrations of NaCl used in the experiments. However, the heat resistance of the heat sensitive strains was highest at 4% NaCl and lower at 2 and 6%. The differential effect of 2 and 6% NaCl on the heat resistance in heat sensitive strains of *E. coli* implies that *E. coli* AW1.7 responds differently to osmotic stress. Baird-Parker et al. (1970) reported variations in D-values for *Salmonella* spp. with NaCl, glycerol and sucrose. The D_{60}-value of *Salmonella enterica* serovar Bedford 286 peaked at an a_w of 0.96 while *Salmonella enterica* serovar Bedford 2315 portrayed comparable D_{60}-value from a_w 0.96 to 0.92. The different heat resistance of different strains of *Salmonella* Bedford at decreasing a_w is similar to the differences in peak heat resistance for *E. coli* AW1.7, GGG10 and AW1.7ΔpHR1. Cross protection by osmotic stress against lethal heat stress is variable among strains of bacteria. An additional possibility to explain the difference in heat resistance trends at different NaCl concentrations is that the heat sensitive strains were more sensitive to 6% NaCl. However, MIC data provided evidence that the inhibitory concentration of NaCl for all three *E. coli* strains is between 6.5 and 8.5% NaCl and that there is no drastic difference in resistance to osmotic stress due to increased salinity for *E. coli* AW1.7, GGG10, and AW1.7ΔpHR1.

The genetic and physiological traits of *E. coli* AW1.7 are described in previous work by Ruan et al. (2011). The over expression of *nmpC, potH hisM* and *yicM*, four genes related to porin formation and transport proteins, occurred in

E. coli AW1.7 compared to *E. coli* GGG10 (Ruan et al., 2011). An increase in porin formation may allow an increase in solute accumulation and explains the variations of heat resistance at altered NaCl concentrations (Pichereau et al., 2000). The comparison of the heat resistance of isogenic *E. coli* strains GGG10pYFP and GGG10pYFP-*nmpC* clearly demonstrates a contribution of NmpC to heat resistance. The expression of *nmpC* in *E. coli* GGG10 led to an increase in resistance to 60°C. However, *E. coli* GGG10pYFP-*nmpC* remained substantially more sensitive to heat than *E. coli* AW1.7, demonstrating that expression of NmpC alone explains only a part of the heat resistance and that other factors make a more substantial contribution. It is presumed that *nmpC* is still active in *E. coli* AW1.7ΔpHR1 and may contribute to the slightly higher resistance to heat than that of the wildtype *E. coli* GGG10, a strain that does not express NmpC.

The accumulation of compatible solutes was induced through osmotic pressure with the goal to provide further evidence that compatible solutes increase heat resistance (Roeßler & Müller, 2001). In the current study, LB broth provided adequate resources for osmotic resistance and a cross resistance for increased temperatures as no difference was observed in inactivation at 60°C with additional glycine betaine provided. NMR analysis of cytoplasmic solutes supported the hypothesis that porin activity and the role of solute transport contribute to the heat resistance for *E. coli* AW1.7. When *E. coli* AW1.7 was grown in broth with no additional NaCl, the amino acid concentrations were roughly double that of the control strain. When grown in broth with 6% NaCl

added, the intracellular levels of sugars including trehalose and glucose in *E. coli* AW1.7 were double that of *E. coli* GGG10. However, at all three NaCl concentrations, the concentration of glycine betaine was not significantly different between strains. Previous investigations identified glycine betaine, proline, and trehalose accumulation on induction of osmotic stress and introduction into stationary phase for *E. coli* (Landfald & Strøm, 1986; Larsen et al., 1987; and Perroud & Rudulier, 1985) and other bacteria (Glaasker et al., 1998; Holtmann & Bremer, 2004; and Kets et al., 1997). Additional amino acids compete with the uptake of glycine betaine, the most commonly found, abundant and effective accumulated compatible solute. Perroud & Le Redulier (1985) tracked a decrease in uptake of radioactively labeled glycine betaine when *E. coli* was provided with dimethylglycine, β-alanine betaine, proline, proline betaine, and pipecolate betaine. The presence of choline and glycine betaine aldehyde increased osmotic resistance; however, these two compounds are precursors for glycine betaine formation and, overall, increase final intracellular concentration of glycine betaine. The enzyme activity of choline dehydrogenase and glycine betaine-aldehyde dehydrogenase peaks around 0.4 to 0.6 M NaCl (between 2 and 4% NaCl w/v) and steadily decreases (Landfald & Strøm, 1986). The rate of glycine betaine uptake is the highest when the growth media for *L. monocytogenes* contains 4% NaCl, with rate of uptake at 2% being higher than that at 6% NaCl (Ko et al., 1994). A decrease in enzyme activity and in rate of betaine uptake at high saline conditions supports the observation in this study of decreased intracellular concentrations of glycine betaine when cells were grown

in a medium containing 6% NaCl compared to that concentration when grown in a medium containing 2% NaCl.

Larsen et al. (1987) reported that in addition to the uptake of glycine betaine, proline betaine was found to be comparable in accumulated intracellular concentrations at high osmotic pressures. Following restriction of the two betaine types, trehalose, the main sugar, and glutamic and aspartic acids, the two main amino acids, are accumulated in the cytoplasm to restore growth at high osmotic pressures (Larsen et al., 1987). In the current study, the decrease in amino acid accumulation when *E. coli* were grown in LB broth containing 0 to 2% added NaCl can be attributed to the preference for accumulation of betaine over amino acids to sustain growth and viability in high saline environments. The restriction of glycine betaine in growth media coincides with an increase in expression of the *ots* operon, corresponding to an increase in trehalose accumulation (Glæver et al., 1988). The decrease in glycine betaine uptake and precursor enzyme activity in saline conditions above 4% NaCl reported by Landfald & Strøm (1986) coincides with a decrease in betaine concentration at 6% NaCl in the current study. Along with this, the increase in trehalose generation when glycine betaine is not available explains why intracellular sugar accumulation was only observed at 6% NaCl in the current study (Glæver et al., 1988). The levels of betaine were similar in strains at both NaCl concentrations but the concentration of intracellular sugars was double in cells of *E. coli* AW1.7 grown with 6% NaCl added to the medium. A decrease in heat resistance was observed when *E. coli* GGG10 was grown in media containing 6% NaCl.

However, *E. coli* AW1.7 maintained a consistent resistance to heat when grown with NaCl concentrations ranging from 2 to 6%. This strain accumulated twice as much trehalose than *E. coli* GGG10, and additionally accumulated glucose (this study). This observation coincides with the claim of Welsh & Herbert (1999) that trehalose is a more effective thermoprotectant than betaine.

The role of compatible solutes in increased cellular integrity at high temperatures is supported through the DSC analysis of cellular components. An increase in the NaCl concentration from 0 to 4% in the growth media led to the delay of the peak that corresponds to the 50S and 70S ribosomal subunit and complex, respectively. The highest rate of reduction of cell populations of *L. monocytogenes* and *Campylobacter* spp. corresponds to a point on the thermogram adjacent to the peak maxima for ribosomal degradation (Anderson et al., 1991; and Nguyen et al., 2006). This observation implies that the temperature at which cell viability is compromised corresponds to the degradation of ribosomes. A comparison of strain specific D-values reveals a correlation to population destruction rate and the m_1 peak location. Dlusskaya et al. (2011) reported a D_{60}-value for *E. coli* GGG10 to be 0.65 ± 0.1 min and a comparable D_{65}-value for *E. coli* AW1.7 to be 1.1 ± 0.1 min, indicating similar population reduction at 60 and 65°C between the two strains. Similarly, the location of the m_1 peak occurs around 60°C for *E. coli* GGG10 and 64°C for *E. coli* AW1.7. This observation provides insight into the rate of cell population destruction and ribosome destruction.

In agreement with Anderson et al. (1991) and Nguyen et al. (2006), other research groups observed a shift in the position of the initial peaks that represent the ribosomal structure for bacteria of different heat resistance (Lee & Kaletunç, 2002; Mackey et al., 1993; Miles et al., 1986; Mohácsi-Farkas et al., 1999). This is in accordance with the results of the current study were the m_1 peak, representative of the 30S subunit, shifted to a higher temperature for *E. coli* AW1.7 as compared to that observed for the heat sensitive strains. The thermogram peak values reported in this study are lower than those compared to previous studies on *E. coli* (Mackey et al., 1991; Mackey et al., 1993; Lee and Kaletunç, 2002). This is due in part to a slower rate of heating used in this study, however, leading to less lag time between temperature and endothermic energy readings. Conventional methods have used a concentrated cell pellet for sample types while in this study the cells were resuspended in Tris-HCl buffer and treated in suspension.

FT-IR analysis of temperature-induced protein denaturation led to the conclusion that the exceptional heat resistance in *E. coli* AW1.7 is not attributable to variations in protein stability. Specifically in *E. coli*, FT-IR analysis after application of adverse environments led to the observation of statistically different orientation and strength of α-helical bonds supporting the claim that a change in structure and folding orientation of intracellular proteins was altered under stress conditions. An alteration in the fatty acid profile of the cellular membrane occurred in all conditions and ethanol and low temperatures resulted in adaptation of carbohydrate content (Moen et al., 2009). The

investigation of other bacteria including *C. jejuni* (Moen et al., 2005) and *Lactococcus lactis* (Kilimann et al., 2006) supported the ability of FT-IR to differentiate within strains grown under different adverse conditions.

Protein and organelle stability was also investigated in terms of heat shock response (this study) and expression of genes for chaperone proteins (Ruan et al., 2011). *E. coli* AW1.7 was not more resistant to high temperature when exposed to sublethal temperatures prior to heating. The heat resistance of *E. coli* GGG10 and *E. coli* AW1.7ΔpHR1 was increased by 1 and 3 logs, respectively, for cultures exposed to sublethal temperatures. The lack of heat shock response in the wildtype *E. coli* AW1.7 and the presence of one in *E. coli* GGG10 correspond to the overexpression of *dnaK*, *rpsK*, *rpsC*, *rplD*, *rpmC*, and *rpsG* in *E. coli* GGG10 relative to *E. coli* AW1.7 following sublethal heat shock (Ruan et al., 2011). This suggests an increase in ribosome stability and, in more general terms, a heat shock response. *E. coli* AW1.7ΔpHR1 had a heat shock response and also demonstrated higher efficiency of such a response compared to *E. coli* GGG10. The presence of a heat shock response in the derivative strain suggests that the plasmid pHR1 may be responsible for silencing the response in the wildtype strain.

In this study, the comparison of protective effects of osmotic sublethal heat stress on *E. coli* suggest that osmotic stress is more protective than heat shock in the ability of these organisms to survive high temperatures. This conclusion is generally in agreement with literature data. Table 5 summarizes the increase in heat resistance attained following exposure to either sublethal high

temperatures or osmotic stress for various bacteria. The application of heat shock allowed for, at most, a 10 min increase in D-values. Osmotic stress results in an increase in D-values as high as 120 min. In the current study, the final cell population of the *E. coli* strains were reduced 1 to 2 logs more than that of the heat shocked cultures. In other studies, *E. coli* O157:H7 was aided in resistance to heat more by osmotic stress than by heat shock (Kauer et al., 1998) while the only drastic increase in heat resistance provided by a heat shock was for *Salmonella* Typhimurium (Mackey & Derrick, 1986), which may be an outlier or a more efficient strain in terms of a heat shock. Overall, the response to osmotic pressures and the resultant solute transport provides a more effective cross protective mechanism to increased temperatures compared to that of bacteria exposed to slightly increased environmental temperatures and the resultant heat shock protein mediation on cell stability.

Table 5. Summary of the change in heat resistance contributed by either a heat shock response or osmotic stress[a]

Heat Shock Response			Osmotic Stress		
Bacteria	Heat Resistance	Reference	Bacteria	Heat Resistance	Reference
E. coli O157:H7	No difference 60°C; 1 min	Kauer et al., 1998	*E. coli* O157:H7	2.5 log increase (55°C; 5 min)[b]	Kauer et al., 1998
E. coli O157:H7	3 log increase 60°C; 10 min	Juneja et al., 1998	*E. coli* O157:H7	80 to 120 min increase $D_{62.5°C}$-value[b]	Blackburn et al., 1997
Salmonella Typhimurium	5 log increase 59°C; 2.5 min	Mackey & Derrick, 1986	*E. coli*	42.6 min increase $D_{57°C}$- value[c]	Goepfert, et al., 1970
Salmonella Typhimurium	1.4 min increase D-value$_{57.5°C}$	Bunning et al., 1990	*Salmonella* spp.	25-94 min increase $D_{57.5°C}$-value[c]	Goepfert, et al., 1970
Listeria monocytogenes	10 min increase $D_{60°C}$-value	Ágoston et al., 2010	*Listeria monocytogenes*	4 log increase (60°C; 1 min)[c]	Fernández, et al., 2007

a. Heat resistance represented as either D-value (time and temperature specified) or a comparison of overall log reduction where the comparison is the difference between the final cell count (log CFU/mL) and the specified time, temperature and osmotic stress propagator
b. NaCl used for application of osmotic stress
c. Sucrose used for application of osmotic stress

Table 5 cont.[a]

Heat Shock Response			Osmotic Stress		
Bacteria	Heat Resistance	Reference	Bacteria	Heat Resistance	Reference
E. coli AW1.7	No difference 60°C; 40min	This study	*E. coli* AW1.7	3 log increase 60°C; 30 min[b]	This study
E. coli AW1.7ΔpHR1	3 log increase 60°C; 6 min	This study	*E. coli* AW1.7ΔpHR1	4 log increase 60°C; 5 min[b]	This study
E. coli GGG10	1.5 log increase 60°C; 6 min	This study	*E. coli* GGG10	2.5 log increase 60°C; 5 min[b]	This study

a. Heat resistance represented as either D-value (time and temperature specified) or a comparison of overall log reduction where the comparison is the difference between the final cell count (log CFU/mL) and the specified time, temperature and osmotic stress propagator
b. NaCl used for application of osmotic stress
c. Sucrose used for application of osmotic stress

In conclusion, this study provided insight into the mechanism of the exceptional heat resistance of *E. coli* AW1.7. With the aid of increased solute transport and protective mechanisms aimed towards the protection of ribosomal integrity and overall cellular viability, this otherwise generic strain of *E. coli* has the ability to resist high temperatures for prolonged periods of time. Further understanding of the genetic determinants of the exceptional heat resistance may be elucidated through further study of the role of the plasmid, pHR1, and the study of the newly formed derivative strain, *E. coli* AW1.7ΔpHR1. Additional work is necessary to understand the molecular and physiological basis for the differences in the heat resistance of the wildtype and derivative strain of *E. coli* AW1.7.

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