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I am submitting herewith a dissertation written by Kristina K. Carter entitled “Gene Expression Analysis of *Escherichia coli* O157:H7 at 10 and 37°C and Under Acidic Conditions Using High Density Oligonucleotide Microarrays.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

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**Gene Expression Analysis of *Escherichia coli* O157:H7 at 10 and 37°C and Under
Acidic Conditions Using High Density Oligonucleotide Microarrays**

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Kristina K. Carter
December, 2007

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Acknowledgements

The author with gratitude and appreciation, wishes to acknowledge the contributions of all those who have given their time, attention, and expertise in order to make this achievement possible. To my committee members, thank you for your guidance and encouragement over the past three years. To the faculty and students of the UT Food Science and Technology Department, thank you for your support and encouragement. I greatly appreciate everything each of you has done for me.

Abstract

This purpose of this investigation was to use DNA microarrays to study gene expression in *E. coli* O157:H7 under refrigerated and acidic conditions. Total RNA from *E. coli* O157:H7 grown to 7 log CFU/mL under control (37 °C, pH 7), refrigerated (10°C, pH 7), acid adapted (37°C, pH 5.5, then lowered to 3.5), and acid shocked (37°C, pH 7, then lowered to 3.5) conditions was extracted using an optimized Qiagen RNeasy procedure. Total RNA was converted to cDNA, labeled, and hybridized onto an Affymetrix GeneChip® *E. coli* Genome 2.0 Array. Results were analyzed using Analysis of Variance (ANOVA) and a t-test with significance set at $p < 0.01$ and an expression cut-off of 2-fold difference in gene expression. Expression of selected genes, including an internal control (*hemX*) was confirmed using real time reverse transcriptase PCR.

Microarray results revealed 293 down-regulated and 375 up-regulated genes. Cold shock genes *cspE*, *cspA*, *cspG*, and *cspH* were down-regulated; *recA* and SOS DNA repair genes *uvrB*, *yebG*, *ruvA* and *B*, *lexA*, and *dinI* were up-regulated. Expression of *fhuA* (outer membrane protein) and *napG* (electron transport) genes was 115- and 70-fold greater under refrigeration. Microarray analysis revealed that genes involved in DNA binding, biosynthesis, iron, transport and cellular metabolism were significantly up-or down-regulated in acid adapted cells. Genes related to iron uptake and transport (*cyo*, *ent*, *fhu*, *fep*, and *feo* operons) were up-regulated under acid shocked conditions. For acid adapted conditions, expression of *cfa* (cyclopropane fatty acid biosynthesis; decreases membrane fluidity and increases acid resistance) and genes involved in the glutamate decarboxylase acid resistant systems were significantly up-regulated. The virulence genes *eae*, *stx1*, and *stx2* were not differentially expressed under either condition.

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CHAPTER I
REVIEW OF CURRENT LITERATURE

Introduction

Each year in the United States, an estimated 76 million people contract a foodborne illness (Mead, 1998). Due to the large number of individuals affected by foodborne illnesses, food safety has become increasingly important. *Escherichia coli* O157:H7 is one of the most widely studied and publicized foodborne pathogens. This organism is estimated to cause 73,000 cases of illness and 63 deaths per year in the United States (CDC, 2006). The public came to know this organism after an outbreak associated with consumption of undercooked ground beef from a fast-food restaurant chain occurred. Although most of the general public associates *E. coli* O157:H7 with undercooked or contaminated ground beef, it has also been associated with other foods including milk, yogurt, dry-fermented sausage, alfalfa sprouts, lettuce, and unpasteurized apple cider (Cody, 1999; Cheville, 1996).

Apple cider, unlike many other food products, is very acidic (pH ~ 3.5) and is processed and held at refrigeration temperatures (4°C). Because of these characteristics (acidity and refrigeration), apple cider was originally thought to be an unlikely vehicle for transmission of *E. coli* O157:H7. Since the first outbreak associated with unpasteurized apple cider occurred in 1991, there have been six additional outbreaks, one involving the death of a 16-month-old girl (Janisiewicz, 1999). Although the FDA requires warning labels to be placed on unpasteurized products, some consumers still prefer the unpasteurized products because of their sensory characteristics.

An important characteristic of *E. coli* O157:H7 is its ability to survive in acidic and refrigerated environments. Contamination of apples can occur when “dropped” apples, apples which have fallen to the ground, are harvested (FDA, 1999). These apples

may harbor fecal matter from cattle, deer, or other mammals where *E. coli* O157:H7 is naturally present in the intestines (Boyce, 1995). The contaminated apples are then transported for processing and pressed, introducing acid to the bacteria which may be present. Following pressing, the product is refrigerated introducing a second stress environment to the bacteria. The ability of foodborne pathogens to exhibit adaptive responses to stressful conditions, such as acid and refrigeration in food systems, has received a lot of attention; however, the genetic mechanisms enabling survival under these conditions are not fully understood. With the entire genome of *E. coli* O157:H7 sequenced, opportunities to study the effects of conditions created during food processing on gene expression are now available.

***Escherichia coli* O157:H7**

History. *E. coli* was discovered in 1885 by a German pediatrician, Theodore Escherich (Donnenberg, 2002). Since, microbiologists have studied this organism individually and as a surrogate for other microorganisms. *E. coli*, a facultative anaerobe, colonizes the intestines of humans and mammals soon after birth and remains until death (Donnenberg, 2002). Although the strain of *E. coli* that is found naturally in our intestines is non-pathogenic, there are multiple pathogenic strains that can cause an array of illnesses ranging in severity from diarrhea to hemolytic-uremic syndrome and even death. The ability of these different strains to cause such diversity in illnesses is attributed to the genetic code of each strain. All of the strains have a similar back-bone to the non-pathogenic *E. coli*, but the pathogenic strains contain virulence factors such as bacteriophages, pathogenic islands, plasmids, and blocks of virulent encoding DNA

(Donnenberg, 2002). *E. coli* O157:H7 is a virulent strain that contains all of the virulence factors specified above.

E. coli O157:H7 first appeared as a pathogen in 1982 when implicated as the cause of two outbreaks of hemorrhagic colitis, with the clinical symptoms of abdominal cramps and bloody stools with little or no fever (Mead, 1998). In 1983, physicians noticed an association between *E. coli* O157:H7 infection and the production of shiga toxins, stx, causing hemolytic uremic syndrome (HUS) (Karmali, 1983). HUS is characterized as acute renal injury, thrombocytopenia and microangiopathic hemolytic anemia and is characteristic in over 90% of all *E. coli* O157:H7 cases (Mead, 1998). The *E. coli* strain known as O157:H7 was the first of many strains to become referred to as enterohemorrhagic *E. coli* (EHEC). The organism expresses the 157th O antigen and the 7th flagellar H antigen (Mead, 1998).

Today, researchers know more about *E. coli* O157:H7 than ever before due to the complete genomic sequencing in 2001. Two different *E. coli* O157:H7 outbreak strains were sequenced by two different research groups: a group from the University of Wisconsin, O157:H7 EDL 933, and a Japanese group from the Kazusa Institute, O157:H7 Sakai (Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001; Donnenberg, 2002). Sequencing of the pathogenic *E. coli* O157:H7 strain allowed for comparison with a non-pathogenic laboratory strain, K-12, which was sequenced in 1997 (Blattner, 1997; Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001).

Both the pathogenic and the non-pathogenic strains of *E. coli* were found to have a circular chromosome ranging in size from 4.5 to nearly 5.6 million basepairs in length, with K-12 the smaller and O157:H7 the larger (Blattner, 1997; Hayashi et al., 2001a;

Hayashi et al., 2001b; Perna et al., 2001;Donnenberg, 2002). The two strains share a 4.1 million basepair core region, or backbone, which has inserts of lineage-specific elements (O or K islands). These islands are DNA segments which attribute to the difference in size and to the pathogenic characteristics that distinguish two strains (Blattner, 1997; Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001; Donnenberg, 2002). There are 177 O-islands present in the EDL 933 strain and 234 K-islands present in the K-12 strain (Blattner, 1997; Donnenberg, 2002; Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001). Of these O and K islands, 146 are found at the same position on the *E. coli* chromosome (Blattner, 1997; Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001; Donnenberg, 2002). In the pathogenic EDL 933 strain, O-islands larger than 5,000 basepairs in length were found to contain genes which may be associated with pathogenic functions (Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001; Donnenberg, 2002). The most significant O-island present in *E. coli* O157:H7 is the Locus of Enterocyte Effacement (LEE) pathogenic island. The LEE island is 35.6 kilo basepairs in length and encodes genes for attachment and effacement as well as for the type III secretion system (TTS) (Perna et al., 2001; Donnenberg, 2002). These O-islands are believed to have been acquired by horizontal gene transfer since they have characteristics of the classical form of horizontal transfer described by Hacker and Kaper in 2000. The classic O-island form contains a large genome region with distinct boundaries defining it from its backbone region, a G+C content that is greater than the genomic average, a tRNA gene that is present within the island as well as an integrase, and transposase and other recombinase genes that can mobilize the island (Hacker and Kaper, 2000; Donnenberg, 2002).

The original *E. coli* K-12 strain from 1922 possessed the bacteriophage lambda as a prophage. This cryptic lambda prophage was incapable of growth production but carried genes capable of complementing and/or rescuing mutants of viable phages (Donnenberg, 2002). Present K-12 strains, MG1655 and W3110, are free of the bacteriophage lambda, but results from genome sequencing of MG1655 revealed eight other recognizable bacteriophages (Blattner et al., 1997; Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001; Donnenberg, 2002). The quantity of bacteriophages present in *E. coli* O157:H7 EDL 933 is twice that of the K-12 strain. Since, some of the 16 bacteriophages in EDL933 are not capable of lytic growth or carry functional bacteriophage genes, they are known as cryptic prophages (CPs) (Blattner et al., 1997; Perna et al., 2001; Donnenberg, 2001). The two shiga toxins, *stx1* and *stx2*, encoded by *E. coli* O157:H7 are encoded in the late regions of lambda bacteriophage. *Stx2* is capable of lytic growth and production of infectious particles (Donnenberg, 2002). *Stx1* is dependent upon transcription of the *stx2* gene and both *stx1* and *stx2* toxin production is coupled with lysis of the bacterium and phage release (Donnenberg, 2002; Perna et al., 2001).

Early studies with *E. coli* K-12 revealed the presence of a single-copy F plasmid and further research demonstrated that plasmids were common in clinical *E. coli* strains as well. The pathogenic *E. coli* O157:H7 strain also carries a large F-like plasmid, pO157, which is 60 MD in length. This plasmid differs from the non-pathogenic *E. coli* plasmid because it carries genes essential for infection (Perna et al., 2001; Donnenberg, 2002). The genes located on the *E. coli* O157:H7 F-plasmid which contribute to infection are: hemolysin, catalase-peroxidase, the bundle forming pilus, and the Type II secretion

system, which is responsible for protein transport across the inner membrane followed by transport across the outer membrane (Perna et al., 2001; Nataro, 1998; Donnenberg, 2002). The exact role of all the pO157 genes in pathogenicity is still unknown.

The complete genomic sequence of *E. coli* O157:H7 has revealed many differences between the pathogenic and non-pathogenic strains. This has led to a greater understanding of the mechanisms by which *E. coli* O157:H7 causes disease in humans.

Mechanisms of Infection. Non-pathogenic *E. coli* is naturally found in the intestines, but the pathogenic strain *E. coli* O157:H7 affects 73,000 people each year causing severe intestinal disease (CDC, 2006). In the past, outbreaks were associated with ingestion of contaminated undercooked ground beef, but in 2006, two major outbreaks occurred: one involving spinach and a second involving a multi-state taco fast food chain. Foods often implicated in *E. coli* O157:H7 outbreaks include: alfalfa sprouts, unpasteurized apple cider, salami, lettuce, cheese-curd, unpasteurized orange juice, and raw milk (CDC, 2006). Other modes of transmission that have also been documented include: un-chlorinated drinking water, recreational swimming areas, and person to person transmission (Boyce et al., 1995; CDC, 2006). The infectious dose necessary to cause an illness is extremely low, requiring ingestion with as few as 10-100 cells (<http://edis.ifas.ufl.edu/SS197>). Once ingested the cells must colonize and adhere to the epithelial lining of the stomach. Following adherence, invasion of the host cells occurs and toxins are produced, resulting in diarrhea.

An extensive amount of research has been done using primate models to observe and understand the mechanisms of enteropathogenic *E. coli* (EPEC) adherence and

colonization within the stomach. It is believed that the adherence and colonization mechanism for *E. coli* O157:H7 is similar to those of EPEC (Nataro and Kaper, 1998).

If small amounts, 10-100 cells of *E. coli* O157:H7, are ingested, the cells that survive the harsh acidic environment of the stomach bind to the follicle epithelium of Peyer's patches within the small bowel before colonizing the colon (Nataro and Kaper, 1998; Kaper and O'Brien, 1998; Proulx et al., 2001; Torres et al., 2005). Quorum sensing, cell-to-cell communication, aids in colonization by regulating the type III secretion (TTS) apparatus, which controls the adhesion of *E. coli* O157:H7 cells to the epithelium (Proulx et al., 2001; Torres, 2005). Attachment and adhesion of *E. coli* O157:H7 occurs by the translocation of the *Esp* genes and the intimin receptor, *tir* or *EspE*, from *E. coli* O157:H7 into the host cell (Nataro and Kaper, 1998; Proulx et al., 2001; Torres et al., 2005). Transfer of the translocated intimin receptor enables binding of intimin from *E. coli* O157:H7 to the intestinal epithelium (Nataro and Kaper, 1998; Proulx, 2001; Torres, 2005). The genes required for attachment, effacement, and lesion formation are located on the LEE island found on the bacterial chromosome (Nataro and Kaper, 1998).

Through quorum sensing mechanisms, *E. coli* O157:H7 senses the presence of large numbers of bacteria in the intestines. They then secrete shiga toxins stx1 and/or stx2, lipopolysaccharides, and other virulence factors into the intestinal lumen, epithelial cells, and endothelial cells (Nataro and Kaper, 1998; Kaper and O'Brien, 1998). It has been reported that shiga toxins are essential for the development of bloody diarrhea and hemorrhagic colitis (Proulx et al., 2001). *E. coli* O157:H7 may possess one or both shiga-toxins: stx1 and/or stx2. The structure for all shiga toxins consists of one A and five B

subunits (Natero and Kaper, 1998; Kaper and O'Brien, 1998; and Mead, 1998). The B subunits are responsible for binding to the glycosphingolipid receptor, Gb₃. After binding of stx to Gb₃, the toxin is endocytosed by the cell, transported to the Golgi apparatus, and then to the endoplasmic reticulum (Natero and Kaper, 1998; Kaper and O'Brien, 1998; and Mead, 1998). The A subunit has N-glycosidase activity, which leads to cell death through inhibition of protein synthesis in any cell that possesses the Gb₃ receptor (Natero and Kaper, 1998; Kaper and O'Brien, 1998; and Mead, 1998).

Shiga toxins produced in the intestines are translocated to the bloodstream. The exact mechanisms by which stx circulates to the bloodstream and its target organisms are not known (Natero and Kaper, 1998; Kaper and O'Brien, 1998). In vitro studies have shown that stx moves across the epithelial cell layer by a transcellular pathway (Kaper and O'Brien, 1998). Stx binds to Gb₃, which is present in high concentration in the renal tissue. Thus, stx has been found to be cytotoxic to renal endothelial cells (Kaper and O'Brien, 1998; Proulx et al, 2001). Stx damages the glomerular endothelial cells leading to narrowing of capillary lumina and occlusion of the glomerular microvasculature with platelets and fibrin (Proulx et al, 2001). This decrease in glomerular filtration is responsible for renal failure that occurs in HUS patients (Natero and Kaper, 1998; Proulx et al., 2001).

The host inflammatory response activated by stx includes: proinflammatory cytokines, such as tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and TNF from the kidney (Kaper and O'Brien, 1998; Proulx et al., 2001). Studies have shown that TNF- α and IL-6 enhance the cytotoxic effects of stx because they induce the expression of Gb₃ and increase binding of stx to endothelial cells (Kaper and O'Brien, 1998; Proulx

et al, 2001). Patients with HUS were found to have elevated levels of IL-6 in their blood and urine and these levels correlated with the severity of their disease (Mead, 1998; Proulx et al., 2001).

Detection Methods. The Centers for Disease Control (CDC) consider *E. coli* O157:H7 the leading cause of foodborne illness and death in the United States because of its pathogenesis and the severity of disease (CDC, 1993). Because of its severity, state and government agencies have developed reliable methods to detect the presence of the organism in contaminated foods and clinical samples.

Physicians who suspect their patient of having a possible *E. coli* O157:H7 infections are encouraged to collect a stool sample. The sample should be collected during the early stages of infection when the bacterium is shedding in large amounts through the feces making detection more likely. The specimen is screened using sorbitol-MacConkey (SMAC) agar and incubated overnight (24 hrs) at 35-37°C. SMAC contains sorbitol, which *E. coli* O157:H7 is unable to ferment, and the antimicrobial reagents cefixime and tellurite, which act to suppress normal flora growth and make detection of *E. coli* O157:H7 easily recognizable. A positive *E. coli* O157:H7 colony appears as a 1-2 mm neutral or gray colony with a smoky center. Testing a fecal sample with SMAC from a suspected patient has been found to be the most cost effective method available. Tarr (1995) estimated the cost of materials to detect *E. coli* O157:H7 to be less than \$1.00 per stool sample plus the labor to review the plate. With such minimal costs, requesting a stool sample can easily eliminate any misdiagnosis. Confirmation of a positive SMAC test is completed by additional selective and differentiation tests such as the MUG assay,

which tests for production of beta-glucuronidase. *E. coli* O157:H7 is unable to produce beta-glucuronidase, so a black compound is generated, whereas a non-O157:H7 strain of *E. coli* can produce beta-glucuronidase and will generate a fluorescent compound with this substrate (Natero and Kaper, 1998).

March and Ratnam (1986) presented a study determining the efficacy of MacConkey agar containing sorbitol as compared to MacConkey agar containing lactose for the detection of *E. coli* O157:H7 in stools. The trial involved 1,000 diarrheal stools over a 20 month period with each cultured on SMAC and MacConkey media. The SMAC medium permitted easy recognition of *E. coli* O157:H7 as colorless non-sorbitol-fermenting colonies, while fecal flora appeared pink. The detection of *E. coli* O157:H7 on SMAC medium had a sensitivity of 100%, a specificity of 85%, and an accuracy of 86%. SMAC medium proved to be a simple, inexpensive, rapid, and reliable means for detecting *E. coli* O157:H7 from stools (March and Ratnam, 1986).

Another confirmation assay tests for the O157 LPS antigen. Various commercial pharmaceutical agencies have developed diagnostic kits to test for this antigen using methods such as Enzyme-Linked Immunosorbent Assay (ELISA), latex reagents, colloidal gold-labeled antibody, and other formats. Currently, the CDC has evaluated three commercial latex reagent kits: two for detecting the O157 antigen (Oxoid Diagnostic Reagents, Pro-Lab Inc.), and one for detecting the H7 antigen (Remel Microbiology Products) (Sowers et al., 1996). A total of 159 strains collected between 1989 and 1993 were tested, including strains that possessed cross-reacting antigens or were not able to ferment sorbitol within 48 hr on SMAC agar (Sowers et al., 1996). When compared to the reference CDC O157 antiserum method, latex kits demonstrated 100%

sensitivity and 100% specificity for identifying the *E. coli* O157 antigen. The Remel H7 latex reagent test had 96% sensitivity and 100% specificity when compared to the reference method (Sowers et al., 1996). This study shows that latex reagent tests produce valid results and are an alternative to standard serological methods for identifying the O157 and the H7 antigens (Sowers et al., 1996).

In addition to antigen detection kits, numerous immunological assays have been developed to detect shiga toxins. To date, the FDA has approved the Premier EHEC Test kit manufactured by Meridian Diagnostics, Inc. This kit uses monoclonal antibodies directed against stx1 and stx2 to capture the antigens. In addition to monoclonal antibodies, it also utilizes polyclonal anti-stx antibodies and horseradish peroxidase for detection. This kit has been tested and found capable of detecting stx antigens from bacterial cultures, food samples, and fresh or frozen stool samples. Clinicians and researchers found this kit to be easy to use, sensitive, specific, reliable, and cost effective (\$864 for 1-94 samples) (Acheson et al., 1994).

The traditional method for detecting *E. coli* O157:H7 from food is very similar to the clinical method of detection from stool samples. In foods, a preliminary enrichment step using EHEC enrichment broth (EEB) is performed at 37°C. This step is necessary since *E. coli* O157:H7 is often present in very low numbers, less than 100 colonies, in foods. Following enrichment, 0.1 milliliters of the appropriately diluted homogenized food samples are plated on tellurite-cefixime-sorbitol MacConkey (TCSMAC) agar. Both EEB and TCSMAC contain anti-microbial reagents which suppress normal flora growth and interference. An *E. coli* O157:H7 colony is colorless or neutral/gray with a smoky center and 1-2 mm in diameter. Confirmation is achieved through biochemical assays that

include: Kovac's reagent, streaking onto Levine's eosin-methylene blue (L-EMB) agar, immunological assays, and testing for O157 LPS antigen or stx antigens.

Through advances in technology, DNA based detection methods have assisted clinicians and researchers in providing rapid, usually less than a week, detection of *E. coli* O157:H7. The most current DNA based detection method is real time polymerase chain reaction (rt-PCR). This method uses DNA primers and probes to detect specific sequences of *E. coli* O157:H7 genes. Currently, many of the primers/probes used for rt-PCR based detection have focused on the virulence genes: *stx1*, *stx2*, *eae*, *ehx*, *uidA*, or *fliC*. This method of detection does have inefficiencies with stool and food samples; specifically, it suffers from problems with inhibitory substances found in the sample that can prevent detection of the organism and detection of low numbers of the organism. Because of these problems, a commercial kit using immunomagnetic separation (IMS) has been designed: Dynabeads anti-*E. coli* O157 by Dynal, Inc. This technique uses magnetic beads coated with antibodies against *E. coli* O157:H7 to isolate the organism from heavily contaminated organic samples such as food or feces. Researchers have been successful using the IMS method with and without pre-enrichment steps in combination with any currently used detection method (Nataro and Kaper, 1998).

Ibekwe and Grieve (2003) applied the real-time PCR method to detect and quantify *E. coli* O157:H7 in pure culture, soil, manure, feces, and waste water with and without an enrichment step. The gene targets used for detection were *stx1*, *stx2*, and *eae*. All samples were inoculated with 8 log CFU/mL of an O157:H7 strain and a non-O157:H7 and then incubated for 0, 2, 8, 16 and 24 hr. The detection limit without enrichment for *E. coli* O157:H7 with rt-PCR was 3 log CFU/mL in pure culture and 4 log

CFU/g in environmental samples. With the enrichment step, the detection limit improved, enabling detection of <10 CFU/g in environmental samples. Real-time PCR assays enable sensitive and rapid quantification of *E. coli* O157:H7 in environmental samples with and without enrichment (Ibekwe and Grieve, 2003).

Fratamico et al. (1995) used multiplex PCR for the detection of *E. coli* O157:H7. For multiplex PCR, primers specific for EHEC *eaeA* gene, conserved sequences of *stx1* and *stx2*, and the 60MDa plasmid were used. The specificity of the assay was evaluated with 61 bacterial strains including 16 *E. coli* O157:H7 strains, 8 *E. coli* O157 (NM and H⁻) strains, *E. coli* strains of other serotypes, *Shewanella putrefaciens* and *Pseudomonas aeruginosa*. The results showed amplification products for the plasmid, *stx*, and *eaeA* genes for all *E. coli* strains of serotype O157:H7, O157:NM, and O157:H⁻. For the other *E. coli* strains, *Shewanella putrefaciens* and *Pseudomonas aeruginosa*, at least one primer set had no amplification indicating the sample was not O157. Multiplex PCR proved to be a useful method in identifying EHEC of serogroup O157 (Fratamico et al, 1995).

Fu et al. (2005) combined immunomagnetic bead separation with rt-PCR for the detection of *E. coli* O157:H7 from a mixture of *E. coli* strains in ground beef samples without enrichment. The primers selected for this study targeted the *eaeA* gene. The results from the immunomagnetic bead separation revealed no cross-reactivity with other strains of *E. coli* and it was specific in capturing *E. coli* O157:H7 from the mixture. The assay had a minimum detection limit of <2 log CFU/mL in buffer and detected 4 log CFU/g of *E. coli* O157:H7 in ground beef. A reduction of meat associated inhibitors was

also seen. A combination of immunomagnetic separation and rt-PCR was effective in detecting *E. coli* O157:H7 from ground beef (Fu et al., 2005).

DeTrana et al. (2005) used a combination of filtration and immunomagnetic bead separation with rt-PCR for the detection of *E. coli* O157:H7 from apple juice. This research focused on the genes targets *stx1*, *stx2*, *uidA*, and *eaeA*. Continued success at quantifying as few as 35 cells/mL of apple cider using quantitative rt-PCR was found. The successful quantification of low numbers of *E. coli* O157:H7 in apple cider without the need for time-consuming enrichment procedures contributed to the significance of the work (DeTrana et al., 2005).

Researchers have learned a great deal about the mechanisms that cause illness and have greatly improved detection methods of *E. coli* O157:H7; however, there are still many un-answered questions. Scientists do not completely understand the mechanisms that enable *E. coli* O157:H7 to survive harsh food processing conditions or allow persistence in the environment for extended lengths of time.

Outbreaks. In 2006, two large, multi-state outbreaks of *E. coli* O157:H7 occurred in the United States. One outbreak was linked to contaminated spinach, while the second was associated with food items from a taco fast food restaurant chain (CDC, 2006). Between 1994 and 1998, there have been over 50 *E. coli* O157:H7 outbreaks (<http://www.cdc.gov/ncidod/dbmd/diseaseinfo/csteec98.htm>). The largest outbreak to date in the United States, which affected 732 individuals and killed four children, involved four states and occurred between December 1992 to January 1993 (Nataro and Kaper, 1998). This outbreak was attributed to contaminated undercooked hamburgers.

Outbreaks of *E. coli* O157:H7 have also been linked to contaminated water sources including recreational water, well water, and municipal water. In Missouri, an outbreak from contaminated municipal water occurred because of an improperly repaired water system allowing un-chlorinated water to be distributed. This outbreak affected 243 individuals and killed four people (Swerdlow et al., 1992).

In addition to ground beef, vegetables, and water, outbreaks have occurred that involved acidic foods such as apple cider, orange juice, and fermented sausages. These foods were previously not considered to be a problem because of their low pH level, less than 4.0 (Cody et al., 1999; Kaper and O'Brien, 1998; Hilborn et al., 2000). The first confirmed outbreak in the United States linked to contaminated, unpasteurized apple cider occurred in Massachusetts in 1991 (Kaper and O'Brien, 1998). This outbreak affected 23 people. Since, there have been numerous outbreaks linked to contaminated unpasteurized apple cider. In 1996, three outbreaks all linked to contaminated unpasteurized apple cider occurred. The largest outbreak that year involved three states with 71 confirmed cases and one death (Cody et al., 1999).

With *E. coli* O157:H7 estimated to cause 73,000 illnesses each year, scientists are searching for methods to reduce these numbers. Although more is known about *E. coli* O157:H7 than ever before, there are still many unanswered questions about the organism. Such questions include: how does it survive freezing and refrigeration temperatures, and what is the key to survival under acidic conditions? With technology moving towards understanding genetic mechanisms involved in pathogenicity in specific environmental conditions, many of these questions may be answered in the near future.

Apple (*Malus domestica*) Cider

The principle difference between apple cider and apple juice is that cider is unpasteurized, whereas apple juice is pasteurized by heating at 68°C for 20 to 30 min or 82°C for 20 to 30 seconds (Downing, 1989).

Records show that apple cider consumption dates as far back as Julius Caesar (Janick, 1996). The Americas were introduced to apple trees from the English settlers and are believed to have begun drinking cider shortly thereafter with apple cider consumption reaching its peak in the US during the mid-nineteenth century (Janick, 1996). Today most people drink pasteurized apple juice; however, there are still a large number of those who prefer unpasteurized apple cider because of its taste and/or texture. Processors who make unpasteurized apple cider are often located in the northern regions of the country (Connecticut, Massachusetts, New York, Oregon and Washington) and process the juice during the fall or cooler months of the year (Janick, 1996). The basic process of unpasteurized apple cider involves: harvesting of apples, receiving the apples at processing facility, chopping and pressing the apples for juice, filtering large particles from the juice, and bottling the juice product for sale to the consumer.

Apples selected for cider production are of a specific apple variety. These apples are often unpalatable or inedible because of the “tannin” content of the juice (King, 2005). In the cider industry, “tannin” refers to the bitter, astringent flavor of the juice from an apple (King, 2005). According to cider producers, this is the key characteristic that gives cider its flavor. Many producers use a blend of juices from specific apple varieties to make the desired cider flavor. The blending of juices combines different levels of brix, acid, tannin, and chemical compounds that are unique to each apple variety

(King, 2005). The Long Ashton Research Station in Bristol, England is recognized as an authority on apple cider research and developed a classification system of apple varieties which can be used in determining juice blend characteristics (King, 2005). The classification system characterized by certain threshold levels of tannin and acidity is presented in Table 1.1 (All tables and figures appear in the appendices for each chapter). There are numerous apple varieties that can be used in juice and cider production to obtain a blend of specific flavors. Table 1.2 lists a few apple varieties that are specific for each blend category.

The acid found in apples, which can vary in concentration depending on the apple variety, is malic acid (chemical structure shown in Figure 1.1). Malic acid, an organic acid, is the result of the synthetic activities of plants. This acid is found naturally in the tissue of apples and is released in the juice of an apple when it is pressed or crushed during processing. This acid is responsible for giving the distinct flavor. The typical pH of an apple juice or cider ranges from 3.3 to 4.1 (Janick, 1996). This low pH level is what originally led scientists to believe that apple juice or cider products would not allow survival of *E. coli* O157:H7.

In 1997, following three large *E. coli* O157:H7 outbreaks the previous year, the Food and Drug Administration (FDA) inspected fresh, unpasteurized apple cider manufacturers to identify possible sources of *E. coli* O157:H7 contamination and to determine methods to reduce future contamination. The inspection findings were published in 1999. The FDA investigated 237 unpasteurized apple cider manufactures in 32 states finding that most were small processors who grew their own apples, producing less than 10,000 gallons of cider per year (53%), and sold the product on site (54%) or to

a local retailer (44%). The harvesting method used by the cider processors revealed that 72% allowed deer grazing or other animals on the orchard or adjacent property. A majority (66%) of the processors used natural rainfall to irrigate the apple orchards, while the remaining processors used irrigated water from municipal or well sources. Of those using irrigated water, 67% of the water supplies were untested. The investigation surveyed the processors about the apples used for processing to determine if they were “dropped apples” (apples from the ground) or harvested from the trees. Thirty-seven percent (82 out of 237) of the processors reported the use of dropped apples, but only those that were wholesome apples or recent drops. Upon receiving the apples, 60% of the processors applied additional sanitary controls. Of the 39% of the processors who washed their apples, 8% used water from a private source that had not been tested for microbial contamination. Prior to chopping and pressing, 84% washed, with 62% of those using a brush wash and 21% using a chlorine based sanitizer wash. Sixteen percent of the processors that did not wash the apples before pressing also did not wash the apples upon receipt. Ninety-one percent of the processors discarded bruised and rotten apples prior to pressing and chopping, while the remaining allowed these types of apples to continue to be processed (FDA Report, 1999).

Equipment and the processing plant also proved to be a likely source of contamination. Thirty-three percent of the processors did not adequately clean the equipment. Forty-one percent had open passageways into the processing area that allowed flies, birds, dogs, and other animals into the processing area (FDA Report, 1999).

The FDA also investigated the microbial quality of the apples and the apple cider. Samples for analysis were collected from five locations: incoming apples, water used for

washing, apples before pressing, finished apple cider before addition of preservatives, and final product with preservatives. The samples were tested for aerobic plate counts (APC), total coliforms (TC), fecal coliforms (FC), and generic *E. coli*, with the last two used as an indicator of fecal contamination. Subsets of samples were tested using the Bacteriological Analytical Manual (BAM) for pathogenic bacteria: *E. coli* O157:H7 and *Salmonella* species. One processing facility had an incoming apple sample which was positive for *Salmonella*, but none of the processing facilities samples were positive for *E. coli* O157:H7. For samples taken from incoming apples, the APC and TC counts were low, while the FC and generic *E. coli* counts were below the level of detection. When testing the water used for washing, half of the processors used water with ≤ 3 CFU/mL. Thirty processors used water with elevated APC and TC, with FC and generic *E. coli* found in 12 of these. Samples of apples before processing had low levels of FC and generic *E. coli*, but slightly elevated levels of APC and TC. Analysis of samples of apple cider with and without preservatives showed that after the addition of preservatives, TC and APC counts decreased while six samples without preservative were positive for FC and generic *E. coli*. Two samples with preservative samples were also positive for FC and generic *E. coli*. The FDA concluded that cider processing neither decreased nor increased coliform levels from the incoming product to the outgoing cider. Further conclusions were that microbial hazards, such as contaminated water, no initial washing step, and/or use of dropped apples that were introduced at the beginning of processing will be carried through to the final product (FDA Report, 1999).

The FDA has since implemented a Hazard Analysis Critical Control Point (HACCP) rule for the juice industry (FDA, 2001). A requirement for a five-log reduction

of pertinent pathogens was also implemented (FDA, 2001). On August 28, 1997 the FDA being requiring a warning statement on all unpasteurized juice beverages stating:

"WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria that can cause serious illness in children, the elderly, and persons with weakened immune systems" (FDA, 1998). In addition the US Apple Association has developed a leaflet (Figure 1.2) about unpasteurized cider to be provided to consumers at retail cider locations.

Researchers have analyzed different mechanisms that have enabled *E. coli* O157:H7 to reside in apple tissue. Cattle, sheep, deer, birds, and many other animals, all of which were found on or near the farms during the FDA inspections, serve as reservoirs for *E. coli* O157:H7, but there may be additional reservoirs. A study by Janisiewicz et al. (1999) assessed the potential of transmission of *E. coli* O157: H7 by fruit flies to fresh cut apple tissue. They used a fluorescent tagged nonpathogenic *E. coli* strain inoculated onto a compost pile of decaying apples and peaches. The fruit flies from the decaying food were placed in a glass chamber along with wounded (bruised, punctured) apples tissue. After 24 hr, the bacterium was detectable in the wounded apples. These results indicate that *E. coli* O157:H7 can be transmitted to the tissue of wounded apples by fruit flies (Janisiewicz et al., 1999).

Researchers have also studied the roles of apple variety, harvesting dates, and source (tree-picked or dropped) of the apple in the growth of *E. coli* O157:H7 in damaged apples. Dingman (2000) assessed these topics in five apple varieties (Golden Delicious, Red Delicious, McIntosh, Macoun and Melrose). In this study, on five weekly visits to a Connecticut farm, two apples of each source and from each of the five varieties were

selected. Following bruising at the lab, through intentional dropping on the floor, one dropped and one tree-picked apple were inoculated by a hypodermic needle with 10^5 *E. coli* O157:H7 cells into the damaged tissue of the apple. The results showed that Golden Delicious, Macoun, Red Delicious, and Melrose varieties were susceptible to the growth of *E. coli* O157:H7 in the damaged tissue. There was no difference in growth of *E. coli* O157:H7 in lab-dropped or tree-picked apples. Date of harvest and source had no change in the effect of *E. coli* O157:H7 growth. *E. coli* O157:H7 did grow on McIntosh apples for two days, but was detected six days after inoculation. McIntosh apples, compared to the Red Delicious apple variety, maintained an 80 fold lower cell count, suggesting the presence of some factor in McIntosh apples that inhibits the growth of *E. coli* O157:H7 (Dingman, 2000).

In addition to studying transmission and effects of apple variety with *E. coli* O157:H7, researchers have also assessed the survival capabilities of *E. coli* O157:H7 in apple cider. Zhao (1993) researched the fate of *E. coli* O157:H7 in unpasteurized apple cider both with and without preservatives at 8 or 25°C. They inoculated 0.5 milliliters of *E. coli* O157:H7, populations at 1.0×10^2 or 1.0×10^5 , into 50 milliliters of apple cider both with and without preservatives. Measurements on the survival of *E. coli* O157:H7 occurred each day for 36 days. The results showed that cider samples stored at 8°C and inoculated with higher *E. coli* O157:H7 populations remained stable for 12 days after inoculation. The bacteria had a higher survival length, 10-31 days, when stored at 8°C compared to 2-3 days when stored at 25°C. The preservative, potassium sorbate, had little effect on reducing the *E. coli* O157:H7 populations, since survival was detected in 8°C samples after 20 days. In cider samples containing sodium benzoate, bacteria were not

detected after 10 days at a storage temperature of 8°C. Results from this study indicate that use of the preservative, sodium benzoate, may increase the safety of apple cider (Zhao, 1993).

Nyquist-Battie et al. (2005) used an antibody-based detection method, sandwich ELISA, for the detection of *E. coli* O157:H7 from acid shocked, acid adapted and apple juice environments. Acid shocked cells were grown at pH 7, centrifuged, and re-suspended in media at pH 4 for 2 hr prior to detection. Acid adapted cells were grown for 18 hr in media containing 1% glucose, a condition that slowly lowers the pH to 4.8 by the end of the time period. The apple juice cells were grown in culture media at pH 7 overnight, and then re-suspended in apple juice without preservatives at 25°C for 7 days without shaking. The results from the sELISA assay showed a reduced fluorescence signal in acid shocked cells at pH 4 compared to cells at the higher pH levels. In addition, the percent live cells, as determined by direct cell counts was reduced significantly ($p < 0.01$) at pH 4 compared to the higher pH. The acid adapted cells were similar in fluorescence signal and percent live cells as the control cells. Cells incubated in apple juice did not affect sELISA detection or fluorescence compared to control cells. This study shows that brief incubation at pH 4 caused reduction in the ability to detect *E. coli* O157:H7 using sELISA. The results also showed that acid adaptation did not affect sELISA detection or viability (Nyquist-Battie et al., 2005).

Reinders et al. (2001) studied the survival of *E. coli* O157:H7 in model apple juice medium with varying concentration of proline and caffeic acid. The results with proline acid showed that at pH of 3.2 and 3.8, *E. coli* O157:H7 survived significantly better with proline than without it. Modified apple juice with proline at any pH (3.2, 3.8,

4.4, and 5.0) was not found to influence the survival of the cells in the stationary phase. The results from caffeic acid revealed that the stationary phase cultures survived better than the log-phase cultures and were detectable after 72 hr. From this study, it was concluded that phenolic acids, such as caffeic acid, may play an important role in the propagation of *E. coli* O157:H7 in apple juice or apple cider.

In the wake of a substantial amount of research confirming that *E. coli* O157:H7 is capable of surviving in an acidic and refrigerated environment such as that of apple cider, researchers have begun questioning the mechanisms that enable the bacterium to survive under these conditions. The environmental conditions such as acid and refrigeration induce stress to the organism and cause the organism to undergo physical and biological changes. With new technologies, researchers can study these physical and biological changes by studying the genes expressed under specific environmental conditions. One way to study gene expression is through microarray analysis.

Microarrays

The 1960's were known as the microbiology era; today, with completion of the human genome project, researchers are calling it the genomic era because of the large number of research projects geared towards studying genes and gene functions. The goal in genomic research is to provide scientists with an inventory of all genes, the genomic equivalent to the Period Table (Lander, 1999). One of the latest methods used to study genes is microarrays. Microarrays have made studying genes and gene functions on the genomic scale possible by making it easy to measure transcripts for every gene simultaneously, provide a direct correlation between the function of a gene and its

expression level, and measure gene expression in a cell to determine the biochemical make up, how regulatory systems operate within the cell, how the cell is built, and what it can do (Brown, 1999).

The technology behind microarrays, DNA blotting, has been used for the last 25 years (Lander, 1999; Stoughton, 2005). DNA blotting methods were first used by Ed Southern who labeled a nucleic acid sequence, hybridized the labeled sequence to a known sequence which was immobilized on a solid surface for detection (Schulze, 2001; Lucchini et al., 2001; Lander, 1999). The idea of depositing multiple nucleic acid sequences, representing different genes, onto a solid surface soon followed and with the use of robotics, microarrays were developed (Lucchini, 2001). Two key innovations made printing of microarrays possible: use of a non-porous solid support glass, and the development of high-density spatial synthesis of oligonucleotide (a short polymer of DNA 2-20 nucleotides in length) (Lander, 1999). A microarray is comprised of a large number of highly ordered, immobilized target sequences attached to a small solid surface about the size of a microscope slide. Microarrays have the advantage of being able to hold a large number of nucleic acid sequences with each corresponding to a different gene, being very small in size, and being sensitive (Stark, 2003). This platform enables the simultaneous measurement of expression levels of thousands of genes in a single assay (Harrington, 2000). Detection of gene expression using microarrays is done by fluorescently labeling DNA which was prepared from messenger RNA, and then measuring the level of fluorescence with a laser scanner. The laser, along with a computer, measures the hybridization intensities that correlate to gene expression levels for each of the genes on the array. There are two basic types of microarrays: spotted

microarrays, or cDNA arrays, and high density oligonucleotide arrays. These two types of microarrays are differentiated by the method used to print the DNA onto the slide (Schulze, 2001).

Spotted Microarrays. Spotted arrays use the complementary DNA (cDNA) product from PCR reactions. These purified PCR products, which are greater than several hundred base pairs in length, are generated from cDNA libraries or clone collections using vector-specific or gene-specific primers and are printed onto the glass slide at defined locations (Schulze, 2001). The cDNA “spots” are typically 100-300 μm in size and spaced equally apart (Schulze, 2001). They are deposited onto solid surfaces either in specified locations by a robot which uses active dispensers that are an inkjet based technology using piezoelectric or solenoid valve delivery, or by passive dispensers, which apply the cDNA solution with a pin (Brewer, 2003). Of the two methods, the passive dispenser method is preferred because a higher density of spots can be achieved using this approach (Brewer, 2003). The spots printed on the glass slide contain a minimum volume of 50 pica-liters of cDNA (Brewer, 2003). Using the spotted method, microarrays will consist of 30,000-80,000 cDNA spots on the surface of the glass slide (Schulze, 2001).

Using a spotted microarray, researchers can compare the gene expression of two biological samples on one chip: control and experimental. The messenger RNA (mRNA) is reverse transcribed into DNA and the samples are then labeled through incorporating the fluorescently tagged nucleotides Cy3 and Cy5 dyes. The labeled DNAs are mixed together then hybridized onto the array. Gene expression levels are determined by

measuring the signal intensities of the fluorophore calculated as ratios (Harrington, 2000).

The numerous benefits to the spotted array include: arraying of un-sequenced clones from cDNA libraries which can be useful in gene discovery, flexibility in the choice of the spotted elements, choice of printing a smaller number of spots for a specific investigation, and are generally cheaper than high density oligonucleotide arrays (Schulze, 2001). As a result, spotted arrays have been the most frequently used arrays in the academic setting (<http://cmgm.stanford.edu/pbrown/mguide/index.html>).

High Density Oligonucleotide Arrays. The high density oligonucleotide arrays are manufactured commercially by two companies: Affymetrix and Agilent. The more popular company is Affymetrix GeneChips[®] (Woo, 2004). On the oligonucleotide arrays, a gene is represented by 15-20 different oligonucleotide sequences between the lengths of 20-25 nucleotides each and include a mismatch (MM) control, which is identical to its perfect match (PM) partners except for a single base difference in a central position (Harrington, 2000). These GeneChips[®] are created by synthesizing tens of thousands of short oligonucleotides onto glass wafers, one nucleotide at a time, through photolithography technology. In manufacturing of photolithographic based microarrays, initially, the solid surface is embedded with linker molecules that have a photo-labile protective group. A mask is placed over the slide and illuminated with light, selectively removing the exposed protective group. The glass wafer microarray is then incubated with a solution containing a particular photo-protected nucleotide which will only couple with the exposed linker molecules. The excess molecules are then removed, another mask

placed, and exposure to light is used to de-protect other areas of the glass wafer. A solution containing a different photo-protected nucleotide is then exposed to the wafer and consequently coupled to the newly exposed areas. The process is repeated for all four nucleotides (A, C, G, T), and the photo-protected groups are replaced with photo-sensitive groups. The process continues onto the next layer. This technology allows oligonucleotides of any code to be constructed, producing an extremely high density microarray (Brewer, 2003; Lipschutz, 1999).

High density oligonucleotide arrays differ from the spotted arrays in the manner in which experimental samples are applied or hybridized to the array. Experimental eukaryotic mRNA samples are converted to biotinylated cRNA from oligo-primed cDNA. Each sample is then hybridized to a separate array. For prokaryotic samples, the mRNA is extracted, converted to cDNA, labeled, and hybridized to the array, with each treatment having a separate array. For example, if a research has both a control and experimental sample, at least two GeneChips[®] will be needed to make comparisons, while only one microarray is needed for a spotted array. Detection of a high density oligonucleotide array is completed through fluorescence scanning of each GeneChip[®] and comparing ratios between chips.

Comparison of Array Platforms. The biggest difference between the two microarray platforms is cost. High density oligonucleotide microarrays are significantly more expensive at approximately \$500 an array (Brewer, 2003). The high price is a result of commercial companies doing all the work rather than the work being done by the researcher. As a result, the high density microarrays are designed to uniquely represent a

specific gene and are able to minimize cross-hybridization between similar gene sequences (Harrington, 2000). The potential for cross-hybridization between genes of similar sequences is high with spotted arrays. For example, out of 4288 open reading frames (ORFs) in *E. coli*, 556 ORFs contain regions of greater than 200 base-pairs sharing a minimum of 50% identity to at least one other ORF in the genome (Harrington, 2000). For a spotted array, these sequence similarities would result in a high level of cross-hybridization between spots; whereas with a high density array with 20 different oligonucleotide sequences and a mismatch (MM) control for each gene, cross-hybridization is minimized (Harrington, 2000). Another benefit to the high-density oligonucleotide array is the flexibility allowed in sample comparisons. Since each sample is hybridized to an individual array, the researcher can perform an experiment between two samples and then follow-up later with a third sample, allowing for comparisons between all three experimental samples.

A major disadvantage to high density arrays is the lack of flexibility in the design of the array. The design choice is designated by the company. Although, this disadvantage may not hold true in the years to come; Affymetrix has begun to allow custom design arrays, but at a high price (Brewer, 2003). Spotted arrays allow for a greater degree of flexibility in the choice of arrayed elements, particularly for the preparation of smaller, customized array for specific investigations. This benefit for the spotted array is also a draw-back because a researcher has to commit to time consuming cDNA handling and carefully select genes before performing the experiment.

Woo et al. (2004) compared the variability in gene expression levels from three different array platforms: cDNA, oligonucleotide, and Affymetrix GeneChip[®]. Total

RNA was extracted from the liver tissue of two types of mice and hybridized onto the arrays. The results showed that all array platforms were comparable, but the Affymetrix GeneChips[®] had a higher precision across technical replicates. The Affymetrix arrays also had the greatest range in the magnitude of expression levels compared to the other platforms. The cDNA arrays showed the smallest range and displayed the poorest precision among the technical replicates (Woo, 2004).

Rogojina et al. (2003) directly compared the results from the Affymetrix arrays to spotted two-color arrays by evaluating the internal consistency within each platform. To compare the results between the two platforms, the intensity values for each were standardized and scaled allowing for direct comparison of the data. The results showed that within the platforms, there was a high level of consistency, but between the platforms there was a dramatic lack of agreement between the data. Because of the lack of agreement, real time reverse transcriptase PCR (rt-RT-PCR) was used to determine which platform was most accurate. Results from this analysis revealed that the gene expression levels were in better agreement with the Affymetrix platform. Rogojina concluded that comparisons between the platforms did not match, with rt-RT-PCR analysis confirming the results from the Affymetrix arrays but not for the two-color arrays, suggesting that confirmation by an alternative method is necessary for microarray analysis (Rogojina, 2003).

There are several different platforms available for microarray analysis, with the spotted two-color and the Affymetrix GeneChip being the most popular among researchers. A number of studies have been conducted to compare the different platforms but none agree as to which platform is best. Researchers should carefully select an array

platform and confirm the results from the arrays using an alternative method such as rt-RT-PCR or blotting.

Application of Microarrays in Food Microbiology. Microarrays are currently being utilized in many areas of scientific research, including food microbiology, for expression profiling, pathogen detection and characterization.

Gene expression profiling is the most common use of DNA microarrays (Sasseti, 2002). The rationale behind gene expression profiling is that bacteria tightly regulate their genes, expressing them only when the gene product is required. Microarrays measure expression by the relative abundance of RNA transcripts in bacteria subjected to different stimuli. Tao et al. (1999) compared the expression level of *Escherichia coli* genes when the organism was grown in M63 minimal media with 0.2% glucose and in rich media, Luria broth containing 0.2% glucose. The media differed in that the minimal media contained glucose as the sole energy source and ammonia as the nitrogen source, while Luria broth contained amino acids as sources of nitrogen, tryptone and yeast extract for macromolecule synthesis with glucose as the energy source. RNA extracted from cells grown in each medium were labeled and hybridized onto two-color-spotted arrays. Results from the arrays showed that cells grown in the rich media had more and significantly higher expression levels of translation genes. Cells grown in the minimal media had elevated expression levels of the genes involved in biosynthesis of amino acids. Virtually 50% of the known RpoS-dependant genes were expressed at significantly higher levels in minimal medium cells than in the rich medium cells. Results from the study show that microarrays are a useful tool to study genomic expression assays and can

be applied to study whole-bacterial-cell physiology for hypothesis generation for a specific study of particular genes (Tao, 1999).

In the past, bacterial pathogens were detected and characterized through a series of biochemical tests that were laborious and required trained personnel. PCR is a rapid, sensitive method with the ability to detect small quantities of a microorganism, but it is limited to the number of primer sets and the length of the PCR product. Alternatively, DNA microarrays provide a means of detecting thousands of specific primer sets, or gene products, simultaneously. With a microarray slide capable of holding 10,000 specific DNA sequences, microarrays are useful tools for detection and characterizing pathogenic bacteria.

Chizhikov et al. (2001) compared gel electrophoresis to oligonucleotide microarray analysis of multiplex PCR products of six genes encoding bacterial virulence factors and antigenic determinants. The microarray was able to detect the bacterial factors in 15 different strains of *Salmonella* spp., *Shigella* spp., and *E. coli*, whereas the gel electrophoresis analysis resulted in unexpected nonspecific amplified products that lead to ambiguous results (Chizhikov, 2001).

Delaquis et al. (2002) designed microarrays with DNA probes specific for virulence factors of *E. coli* O157:H7 genes to differentiate this microorganism from nonpathogenic *E. coli* strains isolated from milk and ground beef samples. In addition, Delaquis et al. also designed a microarray for the detection of *Listeria monocytogenes* from milk samples using 16S-rRNA-based oligonucleotides specific for the pathogen.

Wilson et al. (2002) developed a multi-pathogen identification (MPID) microarray for identification of 18 pathogens: 11 bacteria, 5 RNA viruses, and 2

eukaryotes. This MPID microarray offers an alternative to current methods for high throughput screening of bacterial and viral pathogens.

Hong et al. (2004) developed an oligonucleotide microarray based on the mutation region of 23S-rDNA for the detection of 14 bacterial pathogens isolated from foodborne infections. Hong's group was able to achieve high sensitivity and specificity for 10 pathogenic species, but two species had weak cross-reactivity with *E. coli* and two strains did not result in successful hybridization. This microarray combined when applied with PCR resulted in a detection level of 10 CFU/mL and is useful as a rapid diagnostic test (Hong, 2004).

DNA microarrays also have proven to be a useful tool for further characterization of foodborne isolates such as, *Listeria monocytogenes*, *E. coli* O157:H7, *Salmonella*, *Campylobacter jejuni*, and *Helicobacter pylori* (Kostrzynska, 2006). They have been used to genotype pathogens, describe genetic diversity, for classification based on phylogeny, and to study the epidemiology by identification of polymorphisms of conserved or variable genes among isolates (Kostrzynska, 2006). This information is useful in providing clues to the pathogenicity, evolution, host specificity, virulence, and antibiotic resistance of an isolate.

Genotyping methods, or DNA fingerprinting, usually involve electrophoresis of DNA fragments where identification is based on the banding patterns of the DNA fragments. However, if sequence information is available for an organism, a whole-genome microarray is a useful alternative for genotyping isolates. Dorrell et al. (2001) constructed a whole-genome microarray from a *Campylobacter jejuni* reference strain, hybridized it with 11 different *Campylobacter* strains, and analyzed the array for

differential spotting patterns. Dorrell was able to identify genes in the reference strain that were absent or divergent in some of the other strains tested. These genes include those that encoded for surface structures, acquisition of iron, DNA restriction, and DNA modification. This technique proved to be useful in providing information about genetic diversity and classes of genes that may be dispensable or variable within a species.

Taboada et al. (2004) also used a whole-genome microarray of a *Campylobacter jejuni* strain to compare the genomes of 51 food and clinical isolates from around the world. Results from this study revealed that 350 genes were divergent across multiple *C. jejuni* strains and 249 were unique. This approach enables researchers to identify global trends of gene conservation in *C. jejuni* and enables them to define a group of genes which can be used as markers for the genotyping of *C. jejuni*.

Borucki et al. (2003) developed a mixed-genome DNA microarray to differentiate between related isolates of *L. monocytogenes*. Borucki constructed an array using 600 base pair fragments from a random shotgun library created with 10 different *L. monocytogenes* strains. Experiments confirmed through the probe hybridization patterns that phylogenetic groups could be identified and matched to those that have been previously described and that four probe sequences could differentiate between 50 *L. monocytogenes* strains. Furthermore, several probes from the microarray were sequenced and identified as divergent strains related to virulence and ability to survive of the pathogen. This *L. monocytogenes* mixed-genome DNA microarray proved useful in differentiating between strains, characterizing strains, and identifying potential virulent genes (Borucki, 2003).

Microarrays have astonishing potential for use in food safety because of their ability to discriminate among sequence variation between multiple isolates and their ability to identify the presence or absence of genes. Microarrays are demonstrating they can be flexible and used not only as a tool for gene expression research but also for detection and characterization of foodborne microorganisms (Kostrzynska, 2006).

Gene Expression Responses to Environmental Stresses

Background. Genes are expressed through a multi-step process beginning with the transcription of DNA into messenger RNA, followed by post transcriptional modification and translation into amino acids and proteins. The amount of protein that is made when a specific gene is expressed depends on the developmental stage, the metabolic state, or the physiological state of the cell. Not all of the genes in a cell are used all the time: some will be expressed only during growth, some will be expressed when the cell undergoes damage or death, and some will be expressed under certain environmental conditions. A cell regulates which genes are expressed and the level of expression based on its environmental surroundings. Bacteria cells are constantly encountering many different environments. In the natural environment, bacteria usually have limited nutrients available; however, in the laboratory environment, bacteria are grown in nutrient-rich media and outside stresses are later induced upon the cell and controlled by the researcher. Stress is considered to be any condition that will cause the bacteria to deviate from optimal growth conditions resulting in reduced growth rate (Foster, 2000). Research on bacterial responses to stress, such as acid and refrigeration, has provided scientists with insight into cellular functions such as gene regulation and control.

Global Stress Regulator. The identification of a global gene regulator in response to any stress has only recently occurred due to the emergence of molecular technology. *E. coli* has long been the “model organism” for molecular microbiology studies because of its simplicity as an organism and ability to grow quickly under laboratory conditions. With this organism, researchers observed that bacterial cells responded to alteration in their environment by activating groups of genes which were under the control of a common regulatory protein, or a global gene regulator. Studies ultimately showed that this global gene regulator is affected by metabolic growth, cell division and cellular responses to environmental surroundings. The global regulatory, σ^s , a product of the *rpoS* gene, is expressed in response to reduction or cessation of growth, and in response to many stresses such as high osmolarity, deviation from optimal temperatures and acidic environments (Foster, 2000; Dodd, 2002).

The *rpoS* gene is part of the *nlpD-rpoS* operon and its product, σ^s , has more than 50 genes under its control (Loewen, 1998; Foster, 2000). Hengge-Aronis et al. found two genes, *otsAB* and *treA*, to be under the control of the *rpoS* gene product and are involved in providing the bacteria with resistance to heat and osmotic stress (Hengge-Aronis et al., 1991). Loewen and Hengge-Aronis et al. (1994) found that the *rpoS* gene, as well as *katEG* and *xthA*, are involved in oxidative stress. Schellhorn, Stones, Gentry, Lange, and Hengge-Aronis all concluded that transcription of *rpoS* increases at least two-fold as a result of osmotic shock, acid shock, and starvation induced environmental stresses (Schellhorn and Stones, 1992; Gentry et al., 1993; Lange and Hengge-Aronis, 1994).

The induction of *rpoS* is not the same under all stress conditions. Studies have shown that certain stress conditions induce the global regulator gene but may also induce specific independent genes (Foster, 2000; Dodd, 2002). Induction of *rpoS* is crucial for long-term survival and adaptation of the cell, but reversal of *rpoS* expression and its down-stream targets can occur at anytime if the environmental conditions improve. This unique ability provides *E. coli* as well as other gram negative organisms with adaptive flexibility to many environmental conditions.

RpoS is regulated at multiple levels and with different stresses affecting the levels of transcription, translation and σ^s proteolysis (Foster, 2000). For example, in rapidly growing cells not subjected to any stresses, σ^s is scarcely detectable, but in potentially lethal stress conditions requiring the most rapid response, σ^s proteolysis levels are effected and in gradually deteriorating environmental conditions, transcription and translation levels of *rpoS* are affected (Foster, 2000).

In addition to controlling over 50 genes, *rpoS* also plays a role in expression of virulence genes. Researchers have shown that *Salmonella rpoS* mutants have reduced virulence (Wilmes-Riesenberg et al., 1997; Coynault et al., 1996; Fang et al., 1992). The *spv* gene in *Salmonella*, which is encoded by the virulence plasmid, is required for growth in lymphoid organs. In order for the *spv* operon to be turned-on, expression of the global regulator, *rpoS*, is required and in addition a local protein activator SpvR must be produced (Heiskanen et al., 1994; Kowarz et al., 1994).

Salmonella is not the only gram negative pathogenic organism that uses *rpoS* as a regulator for virulence genes. Pathogenic *E. coli* strains also require expression of *rpoS*.

In pathogenic *E. coli*, genes encoded for the type III secretion system, those required for adhesion, are under the control of the *rpoS* product, σ^S (Beltrametti et al., 1999).

Response to Acid Stress. Outbreaks have shown that *E. coli* O157:H7 can survive and cause disease from acidic foods. In 1980, based on epidemiological evidence, apple cider was linked to an outbreak of *E. coli* O157:H7 in Canada (Steele et al., 1982). Apple cider was also found to be the vehicle of transmission in an outbreak of *E. coli* O157:H7 in Massachusetts in 1991 (Zhao et al., 1993). In 1996, three outbreaks involving *E. coli* O157:H7 and apple cider occurred (CDC, 1996 and Whitmore, 1996). In 1997, the FDA recalled several hundreds of gallons of apple cider because *E. coli* O157:H7 was detected in the juice (FDA, 1999). In 1999, apple cider was again linked as the etiological agent for 17 cases of *E. coli* O157:H7 (CDC, 1999).

Researchers have demonstrated the survival capabilities of *E. coli* O157:H7 in acidic foods. Zhao et al. (1993) showed that *E. coli* O157:H7 grew and persisted in apple cider with a pH between 3.6 and 4.0. Brackett et al. (1994) showed that sprays of acetic, citric, and lactic acid at a temperature of 55°C did not reduce *E. coli* O157:H7 populations on raw beef surfaces. Cutter and Siragusa (1994) reported that washes of beef carcass with an organic acid did not completely inactivate *E. coli* O157:H7. Conner and Kotrola (1995) tested growth and survival of *E. coli* O157:H7 with six different organic acids (acetic, pH 5.2; citric, pH 4.0; lactic, pH 4.7; malic, pH 4.0, mandelic, pH 5.0; and tartaric, pH 4.1) held for 56 days at 25, 10 and 4°C. The results indicated that *E. coli* O157:H7 has the ability to survive in acidic conditions, pH $\geq$ 4.0, but survival is affected by the type of acid and temperature (Conner, 1995).

The studies above show the acid tolerance of un-adapted *E. coli* O157:H7 cells. In some gram-negative organisms, such as *Salmonella Typhimurium* and *E. coli* O157:H7, the mechanisms used for acid adaptation are distinctly different from those of acid tolerance or acid shock. Acid adaptation is the process where cells are grown in a slightly acidic environment (pH 4.5-6.0) where cells are allowed to adapt and/or grow under mildly acidic environments. Acid tolerance or shock is the ability of cells grown at neutral pH (pH 7.0) to survive at very low pH, typically less than 3.0.

Leyer et al. (1995) adapted *E. coli* O157:H7 cells at a pH of 5.0 prior to subjecting the cells to lactic acid, pH 3.85; fermented meat, pH 5.6; shredded hard salami, pH 5.0; and apple cider without preservatives, pH 3.42. For lactic acid, acid-adapted cells had a population decline of 100-fold after 60 min of exposure to lactic acid. The non-adapted cells were not detectable after a 60 minute exposure, indicating a 7-log loss of viability. In the fermented meat after 7 hr of incubation, the number of non-adapted cells decreased from 22,000 cells per gram to 680 viable cells per gram, while the adapted cells only declined from 23,000 to 14,000 cells per gram. In the salami, adapted cells survived much better than the non-adapted cells, with 1,200 viable adapted cells per gram being detected as compared to 160 viable non-adapted cells per gram. In the apple cider, non-adapted cells died off very rapidly and were not detectable after 28 hr; however, the adapted cells after 28 hr had only decreased 10-fold from a 5-log CFU/mL initial volume and were detected after 81 hr at ~ 60 CFU/mL. The results from this experiment showed that *E. coli* does have an acid-adapted response, which is different from that of acid tolerance or acid shock, and the acid-adapted response

enhances the survival of *E. coli* O157:H7 in the presence of lactic acid, fermented sausage and apple cider (Leyer et al., 1995).

The tactics used by *E. coli* and *Salmonella* to combat the biological effects of acid stress include inducing a system to alkalinize internal pH, changes in the proteome, protection of the cytoplasm, and protection of the periplasm. Enteric organisms attempt to maintain an internal pH between 7.6 and 7.8, but can survive with an internal pH of 5.0 (Doyle et al., 2006). *E. coli* and *Salmonella* grow in conditions between pH of 5 and 8.5, but if allowed to adapt to acidic environments first, can survive at pH as low as 2.5 (Foster, 2000). In order to maintain a stable internal pH, pH gradient pumps are used to move protons into the cell at an alkaline pH or extrude them at an acidic pH. These gradient pumps are composed of potassium-proton antiporters and sodium-proton antiporters. A shift to an acidic environment activates the K⁺/proton antiporter, resulting in alkalization of the cytoplasm; a shift to an alkaline environment results in acidification of the cytoplasm by activation of the Na⁺/proton antiporter (Brey et al, 1979).

The log and stationary phase acid response are different for both *Salmonella* and *E. coli*. Cells growing exponentially at pH of 7.7 that are shocked by acid, pH of 4.5, induce genes related to the alternate sigma factor encoded by *rpoS* and *PhoP*, a two-component signal transduction system, and the *Fur* gene an iron regulator (Foster, 2000). Induction of these genes in-turn induces the acid shock proteins enabling acid tolerance for log phase cells. Cells in the stationary phase are already expressing sigma factor related genes prior to introducing acid to the cell's environment and therefore already possess general stress resistance. Upon introducing acid to the environment of the

stationary phase cells, *OmpR*, an osmolarity response regulator, is transcribed enabling the cells to have an enhanced acid tolerance. In addition to proton gradient pumps and different log and stationary phase acid survival mechanisms in *E. coli*, three stationary phase acid resistance systems have been identified: glucose repressed system, glutamate decarboxylase system, and arginine decarboxylase system.

The glucose repressed system is present in both *E. coli* strains and in *Shigella flexneri* (Foster, 2000). The system is capable of protecting the cells to a pH of 2.5 in minimal media, but requires activation by a brief exposure to glutamate prior to lowering the pH (Castanie-Cornet, 1999). As the name indicates, the system represses glucose, but is dependent on two regulators the *rpoS* and *Crp*, a cyclic AMP receptor protein (Castanie-Cornet, 2001; Foster, 2000, Castanie-Cornet, 1999).

The glutamate decarboxylase system (GAD) relies on exogenously supplied glutamate for protection during acidic shock challenge, pH 2.0, in minimal media (Castanie-Cornet, 1999; Foster, 2000; Masuda and Church, 2003). The GAD system is composed of three genes: *gadA*, *gadB*, and *gadC*. *gadA* and *gadB* encode isozymes of glutamate decarboxylase which catalyzes the conversion of glutamate to γ -amino butyric acid (GABA) and *gadC* located downstream from *gadB* encodes a glutamate- γ -amino butyric acid antiporter. This system provides acid resistance to the cell by the consumption of intracellular protons which leak across the cell membrane during extreme acid conditions. After consumption of the proton, *gadC* transports the decarboxylated product back out of the cell, maintain a near-neutral intracellular pH (Small and Waterman, 1998; Foster, 2000).

The third acid resistance system in *E.coli* is the arginine decarboxylase system and is not present in *Shigella* species (Foster, 2000). This system, like the glutamate counterpart, also requires an amino acid. The arginine decarboxylase system converts intracellular arginine to agmatine consuming a proton in the process (Foster, 2000). The decarboxylase enzyme is encoded by *adiA* and the antiporter for this system is not known (Stim and Bennett, 1993; Foster, 2000) *adiA* is expressed under anaerobic growth conditions in complex media at a low pH. *adiA* is thought to be regulated by *cysB* or *adiY*. *CysB* is not known to be involved in sensing pH, but *adiY* is homogenously similar to the *AraC* class of genes which are known as regulatory genes.

Lin et al. (1996) performed a study to determine the survival characteristics of enterohemorrhagic *E. coli* during acid stress and compared these results with that of freshly isolated commensal isolates and laboratory *E. coli* strains. The study used 10 *E. coli* O157:H7 strains, 3 commensal isolates, and 3 laboratory strains to determine their ability to survive at 37°C and at pH of 3.0 when using hydrochloric acid to lower the pH. The study attempted to determine the survival capabilities and the contributions made by three previously characterized acid resistance systems: oxidative system, glutamate and arginine dependant systems. In addition, the study assessed the survival capabilities and the contributions of the three resistance systems when the strains of *E. coli* were adapted, pH 4.0 or 4.4, first. The results from this study showed that acid resistance of enterohemorrhagic *E. coli* is far superior to that of a laboratory strain when challenged in LB at pH 2.0. The arginine system provided more protection in the EHEC strains than in commensal strains, but the glutamate system provided better protection and was equally effective regardless of the strain type (Lin et al., 1996).

Large et al. (2005) performed a similar study comparing the differences between the acid resistance mechanisms in shiga toxin producing *E. coli* and quantified the survival rates for each. For the study, 30 *E. coli* O157:H7 strains, 18 O26:H11 strains, 4 O11:H8 strains and 14 O121:H19 strains were used. A modified version of Lin's method was used for measuring acid resistance. To test the acid resistance systems, bacterial cultures were initially grown in LB, pH 7.0 for 24 hr. This was followed by inoculation of the cells for 22 hr into media specific for the acid resistance system being tested. For the glutamate system, cell were grown in LB with 0.4% glucose, while cultures were grown in LB pH 5.5 with 0.1M morpholineethanesulfonic for the oxidative system, and, brain heart infusion broth with 0.6% glucose was use for the arginine system. Following the 22 hr incubation, the cells was transferred a third and final time into minimal essential medium (MEM) containing 0.4% glucose at a pH of 2.0 or 2.5. Samples were plated and enumerated at 0, 2, and 6 hr. The results from the study showed that the STEC strains were not significantly more acid resistant than commensal strains, but that the glutamate system provided the best protection with less than 0.1 log reduction in CFU/mL per hr under extreme acidic condition, pH 2.0. The study concluded that *E. coli* O157:H7 can survive in low pH using the three acid resistance mechanisms described, but there is no evidence that *E. coli* O157:H7 has greater resistance in any individual system compared to that of other shiga toxin producing strains. *E. coli* O157:H7 strains may use multiple mechanisms yet to be identified in combination to achieve a higher acid resistance level compared to that of other bacteria (Large et al., 2005).

Castanie-Cornet et al. (1999) did an in-depth investigation on the GAD system. Castanie-Corent examined whether one or both of the decarboxylase genes are expressed

under acidic conditions, how each gene is regulated in response to pH and growth conditions, whether both isoforms contribute to acid resistance and the role of *rpoS* in the GAD system. The study used various *E. coli* K-12 strains with the necessary genes for the three acid resistance systems (*gadA*, *gadB*, *gadC*, *adiA*, *ropS*, *cysB*, *crp*, and *cya*) present or mutated out. Castanie-Corent et al. assessed the percent survival of K-12 with the various mutants for the three acid resistance systems. The results of this experiment showed that *gadC* is required for the organisms to survive using the GAD acid resistance system at pH 2.5 and that a *gadC* mutation affects only the putative antiporter system which exchanges extracellular glutamate for intracellular GABA. *AdiA* and *cysB* are both required for the arginine decarboxylase system at pH 2.5 and *cysB* is a regulator for the arginine dependent system. *RpoS* plays a larger role in the glucose dependant system than in the arginine and glutamate decarboxylase systems. The second experiment assessed the role of the two isoforms in the GAD complex. Suicide mutagenesis was used in this study to generate mutations in the *gadA*, *gadB* and *gadC* genes. The results indicated that a *gadB* mutation affects acid resistance making the organism acid sensitive. Both isoforms, the *gadA* and the *gadBC* operon, function in the glutamate-dependent acid resistance at pH 2.5. Next, Castanie-Corent et al. examined the difference between stationary and exponential phase glutamate-dependent acid resistance. The results revealed that exponentially grown cells at pH of 7.0 possessed very small amounts of *gadA* and *gadB*, but exponentially grown cells at pH of 5.5 strongly induced *gadA* and *gadB*. Stationary phase cells grown at pH 7.0 induced the GAD complex, but *gadB* was present at higher levels than *gadA*. Stationary phase cells in combination with an acidic pH had much higher levels of GAD expression compared to stationary phase cell with a neutral pH.

The role of *rpoS* in the GAD complex was determined by using a K-12 strain with *rpoS* and a strain with an *rpoS* mutation and inducing the glutamate-dependant acid resistance. Castanie-Cornet et al. noticed that the product of the *rpoS* gene, σ^S , was required for expression of both *gadA* and *gadB* in stationary phase cells at pH 7.0, but was not required for expression of either gene in exponentially grown cells at pH of 5.0. These results suggest that there are two regulators for the GAD complex, one regulator is *rpoS* and the second is unknown.

Research has shown that the glutamate and arginine decarboxylase systems are capable of providing protection to *E. coli* under an acid pH. In addition to these two systems, there are other decarboxylase systems, lysine and ornithine, that do not provide protection against acid for *E. coli* (Foster, 2000). Each of these decarboxylase systems has an optimum pH, with the GAD system capable of handling the most acidic pH (Foster, 2000). The genetic involvement of acid resistance is an extremely complex phenomenon. Advancement has been made in identifying some of the genes and proteins involved, but there is still a great deal of un-answered questions about additional genes, proteins, and mechanisms involved in the regulation of pH. With the advances in molecular technology, these aspects of communication between microorganisms and their environment can be discovered.

Response to Cold Stress. Although refrigeration and freezing are methods used in the food industry to preserve perishable foods, many foodborne pathogens, such as *E. coli* O157:H7, can survive under both conditions for extended storage periods. Refrigeration and freezing temperatures preclude growth of *E. coli* O157:H7 and induces stress on the

organism (Uyttendaele et al., 2001). Most microorganisms respond to stress by producing a “stress response,” which can enable the organism to survive and enhance its tolerance to subsequent stresses (Rowe and Kirk, 1999; Tetteh and Beuchat, 2003). Researchers studying response proteins that are synthesized under cold environmental conditions (cold shock) have primarily focused their studies on non-pathogenic strains of *E. coli*. However, since *E. coli* O157:H7 is known to tolerate cold storage in foods for extended periods, there is need to understand the role of cold shock proteins in survival of this pathogen during cold storage.

Cold shock is induced in an organism when the temperature falls below 21°C, or below the physiological growth temperature range. When this temperature shift occurs, cell growth is stopped and can, in some cases, resume after approximately 4 hr. Within the 4 hr time period, the cell undergoes extensive changes in order to survive. In *E. coli*, protein and nucleic acid production are suppressed, and the fatty acid composition and physiochemical state of the cell membrane changes. After an extended incubation period, 28 new proteins are synthesized that are not synthesized during normal growth conditions. One of the new proteins synthesized is the CspA protein, which can reach up to 13% of the total protein content in an *E. coli* cell after cold shock has been induced and maintained. This protein plays a significant role in enabling *E. coli* to survive at freezing temperatures and grow under refrigeration conditions, < 4°C.

Increasing research has begun to answer questions regarding the role of cold shock proteins (csp) in survival of microorganisms during cooling and freezing, but this information is rather lacking when compared to the depth of research compiled for heat shock proteins. To a greater extent, there are fewer data regarding cold shock proteins

and their response in pathogenic microorganisms. Cold shocked *E. coli* O157:H7 survived significantly better ($P<0.05$) in whole egg, milk, and tryptic soy agar after 28 days of freezing at -20°C (Bollman et al., 2001). The role of csp expression and subsequent bacterial survival should be further investigated using pertinent pathogens for specific foods in order to understand the impact of stress response of these microorganisms. It has also been demonstrated that *E. coli* O157:H7 is more tolerant of freezing at -18°C than non-O157 *E. coli* strains (Grzadkowska and Griffiths, 2001), further justifying the need for research with this specific pathogen to determine the basis for its enhanced survival as compared to that of its non-pathogenic relative. CspA can be induced after a temperature shift from 37°C to 30°C , and CspB and CspG are only expressed after a temperature decrease below 20°C (Phadtare et al., 2000). *E. coli* grown at 37°C under conditions in which protein synthesis had been inhibited with kanamycin and chloramphenicol showed induction of CspA, CspB, and CspG 30 min after cold shock at 15°C (Etchegaray and Inouye, 1999). However, data are lacking regarding the expression of these and other cold shock proteins in a food matrix with pathogens.

Uttendaele et al. (2001) assessed the survival of a mixture of *E. coli* O157:H7 in ground beef subjected to refrigeration and freezing and the survival of a mixture of *E. coli* O157:H7 in fermented sausage held at 7°C and 22°C . Results indicated that *E. coli* O157:H7 was able to survive in ground beef at 7°C for 11 days and at -18°C for 35 days with only a 1 log reducing during the storage period. At both 7°C and 22°C a gradual reduction, 2-2.5 log reduction, of *E. coli* O157:H7 was seen in fermented sausage over the 35 day storage period. Uttendaele concludes that *E. coli* O157:H7 will remain a hazard even if the food product is held at low or freezing temperatures.

Abdula-Raouf et al. (1993) determined the survival of *E. coli* O157:H7 in ground beef salad containing 40% mayonnaise (pH 5.4 - 6.7) at refrigerated, 5°C, temperatures. The study found that there was no change from the 5 log CFU/mL viable population inoculated onto the product after 24 hr. These results indicate that *E. coli* O157:H7 can survive without reduction under refrigerated and mildly acidic conditions.

Hsin-Yi and Chou (2001) studied the effects of acid adaptation and temperature on the survival of *E. coli* O157:H7 in acidic fruit juices. Two *E. coli* O157:H7 strains were adapted at pH 5.0 in tryptic soy broth for 4 hr prior to inoculation into juices. Mango (pH 3.2) and asparagus (pH 3.6) juice were inoculated with 6 log CFU/mL acid adapted or non-adapted *E. coli* O157:H7 cells and incubated at 7°C and 25°C, with sampling every 24 hr. The results found no difference in the survival of acid adapted and non-adapted *E. coli* O157:H7 in asparagus juice at 7°C. In the mango juice, regardless of adaptation, the viable population declined faster at 25°C than at 7°C. Survival, regardless of acid adaptation, was greater at 7°C than at 25°C. These results demonstrated that both non-adapted and adapted *E. coli* O157:H7 cells are capable of surviving in acidic juice at refrigerated temperatures (Hsin-Yi and Chou, 2001).

Low temperature storage is one of the most important parameters used in the food industry to regulate microorganisms. With the ability of *E. coli* O157:H7 to survive under these conditions, a greater understanding of the mechanisms used to survive and adapt to cold temperatures is needed.

Specific Aims

The specific aims of this research project are to assess the capabilities of microarray analysis in determining gene expression of *E. coli* O157:H7 under environmental conditions similar to those used in the food industry to prevent bacterial growth. Studies have shown that *E. coli* O157:H7 can survive in acidic environments and low temperatures through use of a global regulatory gene, *rpoS*, which regulates additional genes necessary for survival under stressed environmental conditions (Cheng and Kasper, 1998; Diez-Gonzales et al., 1998; Tsai and Ingham, 1997; Jarvis and Russell, 2001). Further investigations are needed to determine the full extent of gene involvement under these environmental conditions. Our goal is to determine which genes are expressed under environmental conditions similar to those used in the food industry and how these conditions affect the survival capability and virulence of *E. coli* O157:H7.

Hypothesis

Environmental conditions play a significant role in gene expression of *E. coli* O157:H7 and affect the virulence of the organism.

Objectives

- 1. To develop and optimize a protocol for removing *Escherichia coli* O157:H7 EDL 933 total RNA for microarray analysis from refrigerated (10°C) and acidic environmental conditions (3.4 M malic acid will be used to lower the pH).**

- 2. To compare gene expression in *Escherichia coli* O157:H7 EDL 933 under refrigerated (10°C) environmental conditions using high density oligonucleotide microarrays.**
- 3. To compare gene expression in *Escherichia coli* O157:H7 EDL 933 under acidic environments (pH 3.5) using high density oligonucleotide microarrays; and compare the expression level upon introduction to acid of select genes at 0 minutes, 5 minutes, 15 and 60 minutes.**

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Appendix

Table 1.1: Apple Variety Classification System for Blending

Category	Tannin Level (g/mL)	Malic Acid Level (g/mL)	Tannin/acid Combination
Sweet (SW)	< 0.2	< 0.45	Low tannin / low acid
Bittersweet (BSW)	> 0.2	< 0.45	High tannin / low acid
Sharps (SH)	< 0.2	> 0.45	Low tannin / high acid
Bittersharps (BSH)	> 0.2	> 0.45	High tannin / high acid

(King, 2005)

Table 1.2: Apple Varieties Specific to Blend Category

Sweet	Bittersweet	Sharps	Bittersharps
Apples for Cider: Berkeley Pippin Court Royal Pomme Gris Sweet Alford Woodbine	Apples for Cider: Ashton Brown Jersey Brown Snout Cow Jersey Gilpin Jersey	Apples for Cider: Yellow Styre Brown's Apple Winter Stubbard York Imperial Ponsford	Apples for Cider: Worcester Pearmain Foxwhelp Stoke Red Dufflin
Standard Apples: Baldwin McIntosh Golden Russet	Standard Apples: Lindel Newtown Pippin Red Astrakhan	Standard Apples: Crimson King Rhode Island Winesap	Standard Apples: Martha Crabapples Dolgo

(King, 2005)

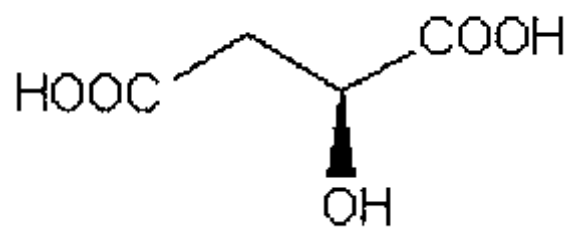


Figure 1.1: Chemical structure of malic acid (L-form)

A Special Message to Our Special Customers

You love apple cider – that delicious, full-bodied beverage with that unmistakable caramel color. Cider has been enjoyed by Americans beginning with this country's first settlers, when cider was a staple of the colonial diet. And fresh apple cider fits right into today's healthy lifestyles, because it is 100% fruit juice, sweetened only by Mother Nature, and is fat-free and cholesterol-free.

Outbreaks of *E. coli* O157:H7 infection due to contaminated fresh (that is unpasteurized) apple cider have been far fewer than those due to some other foods. Healthy adults don't usually experience severe effects. However, children, the elderly and persons with weakened immune systems are at greater risk of complications from illness caused by this bacteria. These persons should take precautions to protect themselves from foods that might carry this bacteria.

The federal Centers for Disease Control and Prevention advise that concerned consumers can reduce their risk of infection by first boiling fresh cider or drinking pasteurized cider or juice. Bringing unpasteurized cider just to a boil is adequate.

The fresh cider industry is expanding efforts to protect their products and customers from contamination by harmful *E. coli* O157:H7 bacteria. You can be assured the industry is working hard to make sure that cider remains a healthful drink.

Producers, Retailers, Consumers.

Figure 1.2: Leaflet message produced by the US Apple Association and provided to consumers at apple cider retail outlets. (FDA, 1999)

CHAPTER II

OPTIMIZATION OF TOTAL RNA EXTRACTION FROM *ESCHERICHIA COLI* O157:H7 EDL 933 FOR MICROARRAY ANALYSIS

Abstract

In this work, we describe an optimized protocol for total RNA extraction from *Escherichia coli* O157:H7 for microarray analysis. Two common extraction methods (RNeasy[®] Mini Kit [Qiagen] a TRIzol[®] Reagent [Invitrogen]) were evaluated on four treated sample groups of *E. coli* O157:H7 grown to 7 log CFU/mL in Luria-Bertani broth as follows: control (pH 7; 37°C), refrigeration (pH 7; 10°C), acid shocked (pH 7 for 6 hr at 37°C; then pH reduced to 3.5 for up to 60 min), and acid adapted (pH 5 for 12 hr at 37°C; then pH reduced to 3.5 for up to 60 min). Cultures were centrifuged; total RNA was extracted initially according to manufacturers' protocols and then modified as necessary. Total RNA quality and quantity were determined spectrophotometrically (expressed as absorbance) using the 260/280 absorbance ratio; organic carryover (impurities) was determined using the 260/230 absorbance ratio. Our results demonstrated that a 260/280 ratio of 1.8 to 2.0 for extracted total RNA was optimal for microarray analysis. Lower ratios indicate that extracted total RNA is not pure, resulting in poor microarray hybridization; 260/230 ratios < 1.8 indicate that organic compounds from the extraction are carried over. Extracting using TRIzol[®] reagent yielded low levels of RNA, with a 260/230 ratio consistently less than 1.8 from all sample groups. The RNeasy[®] Mini Kit extracted optimal total RNA levels with optimal 260/280 and 260/230 ratios from control and refrigeration groups, but no total RNA was extracted from the acid shocked or acid adapted groups. Microscopic and culture analyses revealed that neither acid shocked nor acid adapted cells were intact or viable after extraction. Modifying the extraction protocol to include RNA stabilizing reagents, increased lysozyme concentration, increased extraction incubation time, and cell lysis columns was

necessary to provide optimal total RNA yield for microarray analysis. These results demonstrate the need for optimization of total RNA extraction methods when culturing conditions deviate from optimal.

Introduction

Many of the latest detection methods for microorganisms incorporate molecular technologies such as polymerase chain reaction (PCR), real time PCR (rt-PCR), blotting (Northern, Southern, Western), and microarrays. All of these require extraction of nucleic acid from the organism of interest. Warburg and Christian (1942) reported that a spectrophotometric absorbance ratio at 280 and 260 nm can be used to determine nucleic acid contamination during protein purification. Proteins are measured spectrophotometrically at 280 nm, and nucleic acids are detected at 260 nm. As a result, molecular biologists now use the absorbance ratio 260/280 nm for determining the quantity and quality of nucleic acid extracted from a sample (Wilfinger, 1997). It is generally agreed by most molecular biologists that the 260/280 absorbance ratio is a useful measurement for determining the quantity of nucleic acid in a sample, but the reliability of the ratio for determining the quality, or purity, is questionable. Historically, a 260/280 ratio of 1.8 for DNA or 2.0 for RNA has been used to indicate sample purity, but research has shown that pH, the presence of salts, or even a change in the water used can greatly affect the absorbance ratio (Wilfinger, 1997). Since a slight change in environmental surroundings can affect the absorbance ratio at 260/280, it obviously cannot be used as a sole indicator of purity of the sample. Molecular biologists agree that the 260/280 ratio is necessary because it provides the researcher with useful information about proteins and nucleic acid in a sample. A low 260/280 ratio indicates that there may be insufficient nucleic acid present or that too much DNA or protein remains in the extract. Nucleic acids are measured at 260 nm; the difference between an RNA sample and a DNA sample is only about 0.2. Extracted DNA should have a 260/280 absorbance

ratio of 1.8, whereas the ratio for RNA should approach 2.0. This slight difference is because RNA contains more uracil, and DNA has more thymine (Manchester, 1995). Many nucleic acid extraction and purification methods employ the use of organic chemicals; these chemicals can occasionally be carried-over and present in the solution along with the extracted nucleic acid. These chemicals, such as ethanol, strongly absorb light at wavelengths less than 260 nm. An uninformed researcher using only the 260/280 absorbance ratio may believe that the sample contains no chemical carry-over; therefore, has a pure nucleic acid extract. For this reason, Huberman (1995) reported that it is essential, when evaluating nucleic acids, to take a measurement at a shorter wavelength (e.g., 230 nm) as well as at 260 and 280 nm. RNA purity is determined by the 260/230 ratio. A 260/230 absorbance ratio > 1.8 indicates good quality of total RNA; ratios < 1.8 are indicative of poor quality owing to carryover of organic compounds.

The use of microarrays as a method for detecting gene expression has only been around for the last several years. Microarrays (a.k.a., gene chip, DNA chip, biochip) enable the study of thousands of genes, simultaneously, on a single “chip.” Microarrays are fabricated by high speed robotics on substrates such as glass slides, about the size of a microscope slide, and imprinted with thousands of genes that provide scientists a systematic way to survey DNA and RNA variation of an organism. Use of microarrays in food microbiology is still in its infancy, but with more and more interest in learning about gene expression in foodborne microorganisms, microarrays are becoming more widely used. A necessity for microarray analysis is for total RNA to be of adequate purity or quality (Ojaniemi et al., 2003). It is also important to ensure that a sufficient volume of RNA is available for microarray analysis (Ojaniemi et al., 2003). For microarray analysis,

it is essential that the RNA extraction method is capable of yielding good quantity, quality and purity of RNA.

There are many different extraction methods commercially available, with variations in their techniques and chemistry. The most popular extraction methods are kit based, such as the RNeasy[®] Mini Kit (Qiagen; Valencia, CA) or a single-step extraction methods, such as the TRIzol[®] Reagent extraction method (Invitrogen; Carlsbad, CA). These two methods differ in the manner in which cells are lysed to release RNA and the method used to capture RNA for purification. The TRIzol[®] extraction method uses a phenol-guanidinium mixture for cell lysis, chloroform is added, and a centrifugation step is carried out to separate RNA from DNA and other-cellular material. RNA is then precipitated with isopropanol, washed with ethanol, and- re-suspended in RNase free water (Invitrogen product guide). The Qiagen RNeasy[®] Mini Kit uses lysozyme and buffers containing guanidine thiocyanate (GITC) and ethylenediaminetetraacetic acid (EDTA) to lyse the cells. Lysed cells are passed through, by centrifugal force, a column which binds RNA but allows other material to pass through. The RNA bound to the column is washed with a buffer solution and with ethanol. After washing, RNA is re-suspended in RNase free water (Qiagen, 2001). **This study was conducted to evaluate two RNA extraction methods, TRIzol[®] and the Qiagen RNeasy[®] Mini Kit, on four different environmental sample groups, and to optimize them, as necessary, for use with future microarray analysis.**

Materials and Methods

Cell Cultures. *Escherichia coli* O157:H7 EDL 933 (genome fully sequenced), obtained from the National Food Safety and Toxicology Center at Michigan State University, was used for this study. *E. coli* O157:H7 EDL 933 was cultured at 37°C in TSB (pH 7.0) for 24 hr, transferred into Luria-Bertani broth (LB, pH 7.0) cultured for 24 hr at 37°C, and transferred a minimum of three successive times at 24 hr intervals before use. Four treatment groups were cultured in LB, in triplicate, to 7 log CFU/mL (late exponential phase) as follows: control (pH 7; 37°C), refrigeration (pH 7; 10°C), acid shocked (pH 7 for 6 hr at 37°C; then pH reduced to 3.5 for up to 60 min), and acid adapted (pH 5 for 12 hr at 37°C; then pH reduced to 3.5 for up to 60 min); pH was adjusted with 3.4 M malic acid (King, 2005; Argos Chemicals, NJ). Growth curves were determined for each of the four treatments under the appropriate temperature and pH conditions (Figures 2.1-2.3). Inoculated broth cultures (50 mL) were incubated at 10 or 37°C, and samples were taken at selected intervals for the duration necessary to achieve populations of 7 log CFU/mL. After determining the incubation time necessary to obtain 7 log CFU/mL, culturing was repeated, and at the end of each appropriate incubation period, 5-10 1-mL aliquots of each culture were collected (in triplicate), and centrifuged at 14,000 x *g* in preparation for extraction of total RNA.

Total RNA Extractions. Total RNA was extracted using two commercial extraction protocols, the RNeasy[®] Mini Kit (Qiagen) and TRIzol[®] Reagent (Invitrogen) extraction according to the manufacturer's instructions. Because of poor purity, quality, and quantity of RNA extracted using TRIzol[®], this method was abandoned. The Qiagen

method, which yielded good purity, quality, and quantity of total RNA from control and refrigeration cells, was modified to improve RNA extraction from acid shocked and acid adapted cells. Modification consisted of addition of an RNA stabilizing reagent prior to cell lysis, increasing the lysozyme concentration from 0.4 to 3 $\mu\text{g}/\mu\text{L}$, increasing the incubation time from 5 to 10 min, and incorporation of a cell shredder column. Prior to extraction of total RNA, an RNA stabilizing reagent (RNAprotect Bacteria Reagent; Qiagen, Valencia, CA) was added at a 2:1 ratio (e.g., 1200 μL stabilizing reagent/600 μL suspended cells), the suspension was vortexed, incubated for 5 min (room temperature), then centrifuged at 14000 x g for 10 min. The supernatant was decanted, and the pellet was re-suspended in Tris-EDTA buffer (TE; 10 mM Tris and 1mM EDTA) buffer containing lysozyme (3 $\mu\text{g}/\mu\text{L}$, increased from 0.4 $\mu\text{g}/\mu\text{L}$ in the original protocol). The suspension was incubated at room temperature for 10 min (increased from 5 min for the original protocol). After incubation, 350 μL of RTL buffer (Qiagen) containing guanidine thiocyanate, (GDTA, provided with kit) was added and the entire volume was passed through a cell-lysate homogenizer column (Qiasredder) by centrifugation at 11,000 x g for 2 min. The remainder of the protocol was followed without adaptation from the manufacturer's recommendations. 250 μL of ethanol was added to the lysate and mixed by pipetting. The entire sample was transferred to the RNeasy mini column and centrifuge at > 8,000 x g for 15 sec. Flow through was discarded, 700 μL of buffer RW1 (Qiagen, provided with kit) was added to the column and centrifuged at > 8,000 x g for 15 sec. Flow through was discarded 500 μL of buffer RPE (Qiagen, provided with kit) was added to the column, centrifuged at > 8,000 x g for 15 sec and flow through was discarded. Another 500 μL of buffer RPE was added to the column, centrifuged at >

8,000 x *g* for 2 min and flow through was discarded. Samples were centrifuged at > 8,000 x *g* for 2 min prior to adding 30 μ L of RNase-free water (repeated twice) for a final elute volume of ~60 μ L.

Absorbance Measurements. RNA quantity and quality were determined spectrophotometrically (expressed as absorbance) using the 260/280 absorbance ratio; organic carryover (impurities) was determined using the 260/230 absorbance ratio. Absorbance readings and ratios were measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Statistical Analysis. Using the 260/230 absorbance ratio data, a completely randomized design with factorial comparison of treatments (SAS, v. 9.1, Carry, NC) was used to determine differences ($p < 0.05$) in extraction protocols.

Results and Discussion

This study evaluated two extraction methods for downstream microarray analysis. Results of this study revealed that total RNA extractions using the TRIzol[®] method were inferior to the Qiagen RNeasy Mini Kit method. The Qiagen method, without any modification, was determined to be adequate for extraction of total RNA from the control and refrigerated *E. coli* O157:H7 cells (Figure 2.4; Table 2.2). The Qiagen method yielded substantially greater quality, purity and quantity (Table 2.2) compared to the TRIzol method. However, the unmodified Qiagen method yielded unsatisfactory

extraction quantity and purity of total RNA from acid shocked and acid adapted *E. coli* O157:H7 cells (Figure 2.4).

The poor quantity ($260/280 < 1.8$) and purity ($260/230 < 1.8$) of total RNA extracted using the TRIzol method (Figure 2.4; Table 2.2) indicates high carryover of non-RNA material, such as proteins and organic chemicals, which interfere with subsequent analyses including reverse transcriptase and hybridization onto microarrays slides. The TRIzol[®] method uses differential centrifugation to separate nucleic acids from the reagent and cellular material, whereas the Qiagen method incorporates a filter that binds RNA for subsequent wash steps and then releases it upon the addition of water. Since RNA is bound to the filter, this allows for easy washing and purification of the RNA as opposed to removing the supernatant by decanting by a pipette. In addition, the methods differ in the mechanism used to lyse the cell. The TRIzol[®] method relies on a single step addition of the TRIzol[®] Reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, followed by homogenization to lyse the cells. Alternatively, the Qiagen method uses lysozyme for hydrolysis of the peptidoglycan, in combination with 1x TE buffer followed by the addition of a second buffer, RTL (included in the kit), which contains guanidine thiocyanate, for total cell lysis. The main purpose of EDTA is to bind divalent cations, Mg^{+2} , Mn^{+2} and/or Ca^{+2} , which are required for enzymatic function (Barton, 2005). The removal of divalent cations by EDTA results in the destabilization of the lipopolysaccharide monolayer of the membrane (Neidhardt, 1996). Several studies have incorporated the use of EDTA and Tris. Leive (1965) found that *E. coli* briefly treated with EDTA in Tris buffer release one-half of the lipopolysaccharide membrane from the outer membrane of the cell and made the cells more sensitive to

hydrophobic agents (Neidhardt, 1996). Another study incorporated EDTA, Tris, and 20% sucrose followed by dilution in water to release proteins from the periplasm. In this procedure, EDTA and Tris weakened the outer membranes, the periplasm filled with the sucrose solution, and upon dilution in water, cells ruptured due to the massive influx of water caused by the osmotic pressure difference (Neidhardt, 1996). Tris, a primary amine, acts by weakening the tight association between lipopolysaccharide molecules through replacement of cations bound to the lipopolysaccharide layer and through steric hindrance (Neidhardt, 1996). The Qiagen method also provided better purity of total RNA possibly as a result of the filter which captured the RNA for washing and purification, thereby minimizing chemical carry-over. On the other hand, the final step in the TRIzol[®] method prior to re-suspending the RNA in water was to wash the RNA pellet with ethanol and air-dry the samples. RNA extracted using the TRIzol[®] method was consistently contaminated with chemical carry-over, as indicated by the low 230/260 ratios. Although, the samples were dried until no visible liquid was detected, residual ethanol was most likely the cause for the unsatisfactory 230/260 ratios. The use of a vacuum centrifuge may have improved ethanol removal (Gouffon, personal communication).

Both extraction methods were incapable of providing sufficient quantity or purity (260/280 and 260/230 respectively) of total RNA from acid adapted or acid shocked *E. coli* O157:H7 cells. Extraction of RNA from these cells followed incubation for one hr at pH 3.5. Microscopic and cultural analyses revealed that acid shocked and acid adapted cells were intact and viable after the lysis step recommended by the manufacturer (Table

2.1). Without modifications to these protocols, *E. coli* O157:H7 retained viability, typically with less than a 1-log reduction in population (Table 2.1).

In the present study, acid cells were more resistant to total RNA extraction methods compared to the other treatments, suggesting that exposure to acidic conditions resulted in cellular modifications that conferred greater resistance to cell lysis. The characteristic Gram-negative outer membrane contains phospholipids, lipopolysaccharides, and lipoproteins, and its structure is maintained by hydrophobic and hydrophilic boundaries. The ability to survive in acidic environments is attributed to the high concentrations of bound lipid in the cell wall compared to that under optimal conditions (Barton, 2005). Brown et al. (1997) found that the membrane composition of acid adapted *E. coli* had more cyclopropane derivatives and fatty acids present as compared with strains that had not been exposed to low pH (Brown et al., 1997). *Yersinia enterocolitica* was also found to produce increased proportions of cyclopropane and saturated fatty acids when grown under mildly acidic conditions (Bodnaruk and Golden, 1996). Research has revealed that during acid habituation of *E. coli*, a proportion of the mono-unsaturated fatty acids were either converted to cyclopropane fatty acids or were replaced by saturated fatty acids (Brown et al., 1997). In addition, cells with higher levels of intrinsic acid tolerance had high levels of cyclopropane fatty acid compared with strains with low acid tolerance, suggesting that cyclopropane fatty acids may play a role in cell protection at low pH (Brown et al., 1997).

Changes in membrane composition under acidic conditions also had an adverse effect on detection of *E. coli* O157:H7 from low pH environments (Nyquist-Battie et al., 2005). These researchers hypothesized that reduced detection was due to changes in gene

expression that altered the expression of surface antigens necessary for the antibody-based detection method they used (Nyquist-Battie et al., 2005). Microarray analysis could reveal the genes that are involved in enhanced survival of *E. coli* O157:H7 under acidic conditions.

Our modifications to the Qiagen method (addition of RNA stabilizer, increased incubation time [from 5 to 10 min], increased lysozyme concentration [from 0.4 to 3 µg/µl], and use of cell shredder columns) significantly ($p < 0.05$) improved RNA purity (Figure. 2.5A-D) from acid shocked and acid adapted cells to acceptable levels. Various combinations of these modifications, however, were unsatisfactory. For example, use of cell shredders without the increase of lysozyme, incubation time or addition of the bacterial stabilizing reagent was unsatisfactory; each individual modification alone was unsatisfactory but in combination proved effective for extraction of total RNA from acid treated cells. Modifying the extraction protocol to include RNA stabilizing reagents, increased lysozyme concentration, increased extraction incubation time, and cell shredders was necessary to provide optimal total RNA yield for microarray analysis. These results demonstrate the need for optimization of total RNA extraction methods when culturing conditions deviate from optimal. Failure to do so can adversely affect the quality and quantity of extracted RNA.

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Appendix

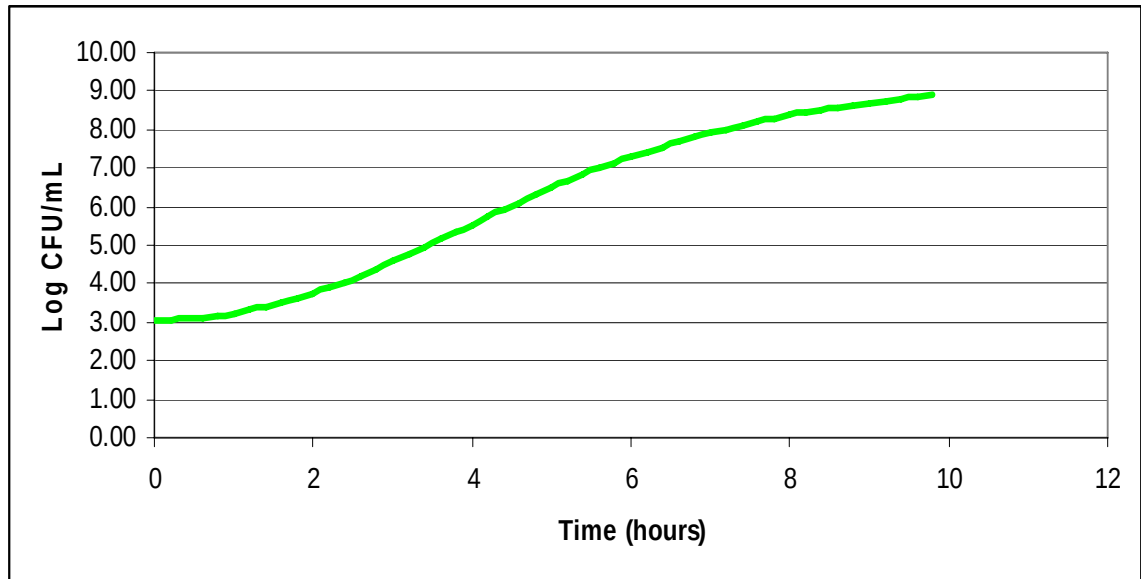


Figure 2.1: Growth curve for *E. coli* O157:H7 EDL 933 incubated at 37°C (control).

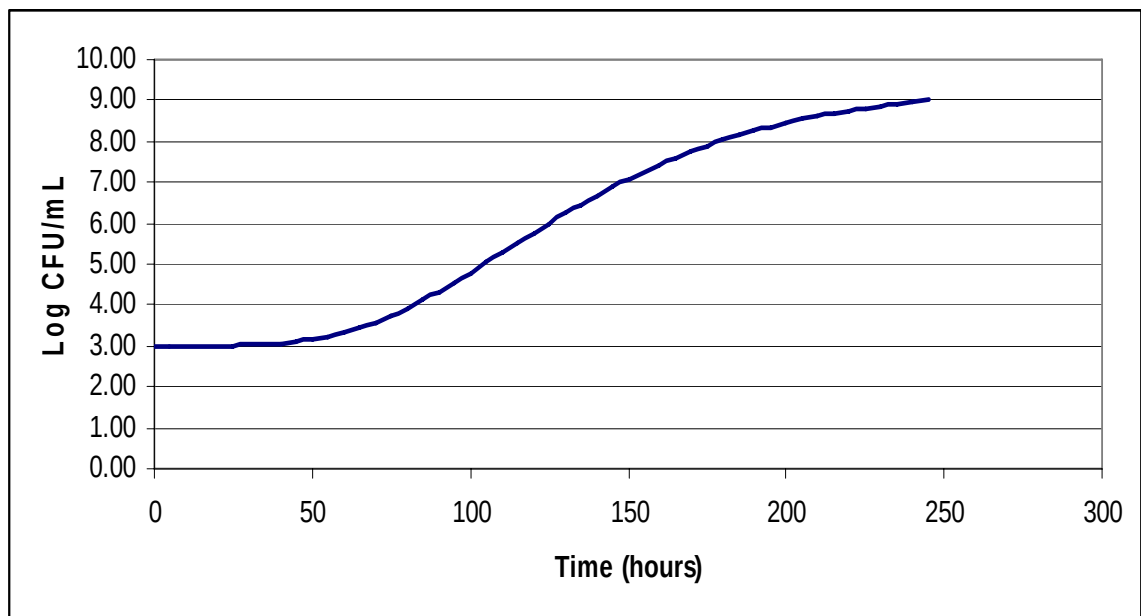


Figure 2.2: Growth curve for *E. coli* O157:H7 EDL 933 incubated at 10°C.

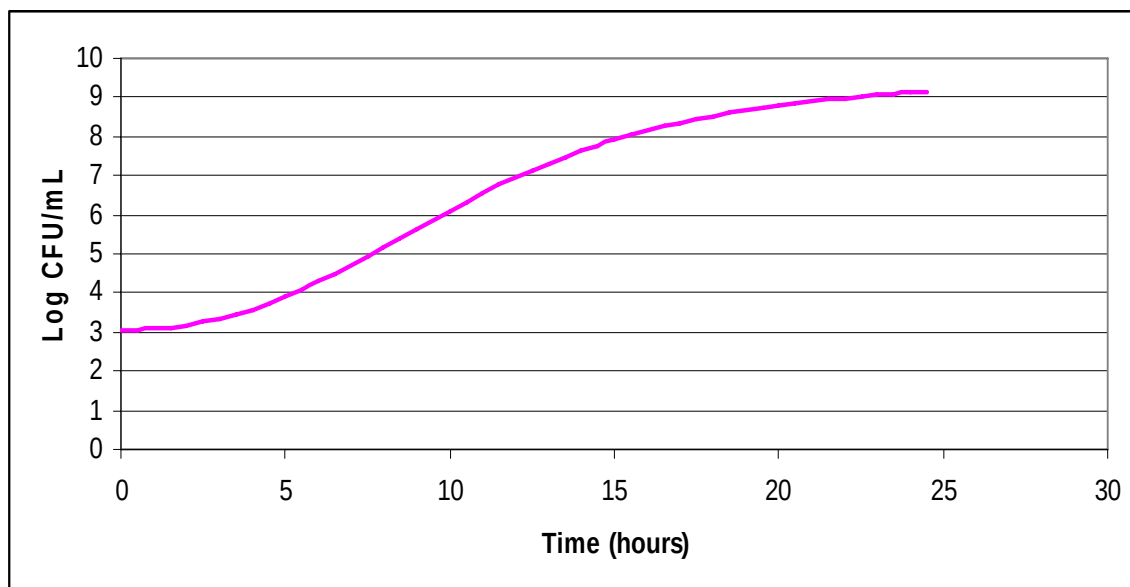


Figure 2.3: Growth curve for *E. coli* O157:H7 EDL 933 adapted to pH 5.5.

Table 2.1: *E. coli* O157:H7 EDL 933 population recovered after the Qiagen lysis step in which modifications were made.

Lysis protocol ¹	Population recovered (log CFU/mL) from:	
	Acid shocked cells	Acid adapted cells
Initial volume	6.5	7.07
L-0.4, I5	4.3	5.225
SR, L-0.04, I5	- ²	-
L-0.04, I5, CS	-	-
SR, L-0.04, I5, CS	-	-
L-0.04, I10	4.3375	6.685
SR, L-0.04, I10	-	-
L-0.04, I10, CS	-	-
SR, L-0.04, I10, CS	-	-
L-3, I5	3.82	6.7475
SR, L-0.04, I5	-	-
L-0.04, I5, CS	-	-
SR, L-0.04, I5, CS	-	-
L-3.0, I10	3.615	6.2975
SR, L-3.0, I10	-	-
L-3.0, I10, CS	-	-
SR, L-3.0, I10, CS	-	-

¹ L= lysozyme at 0.4 or 3 µg/µl; I= incubation time of 5 or 10 min; SR=stabilizing reagent; CS=cell shredder.

² Not detected.

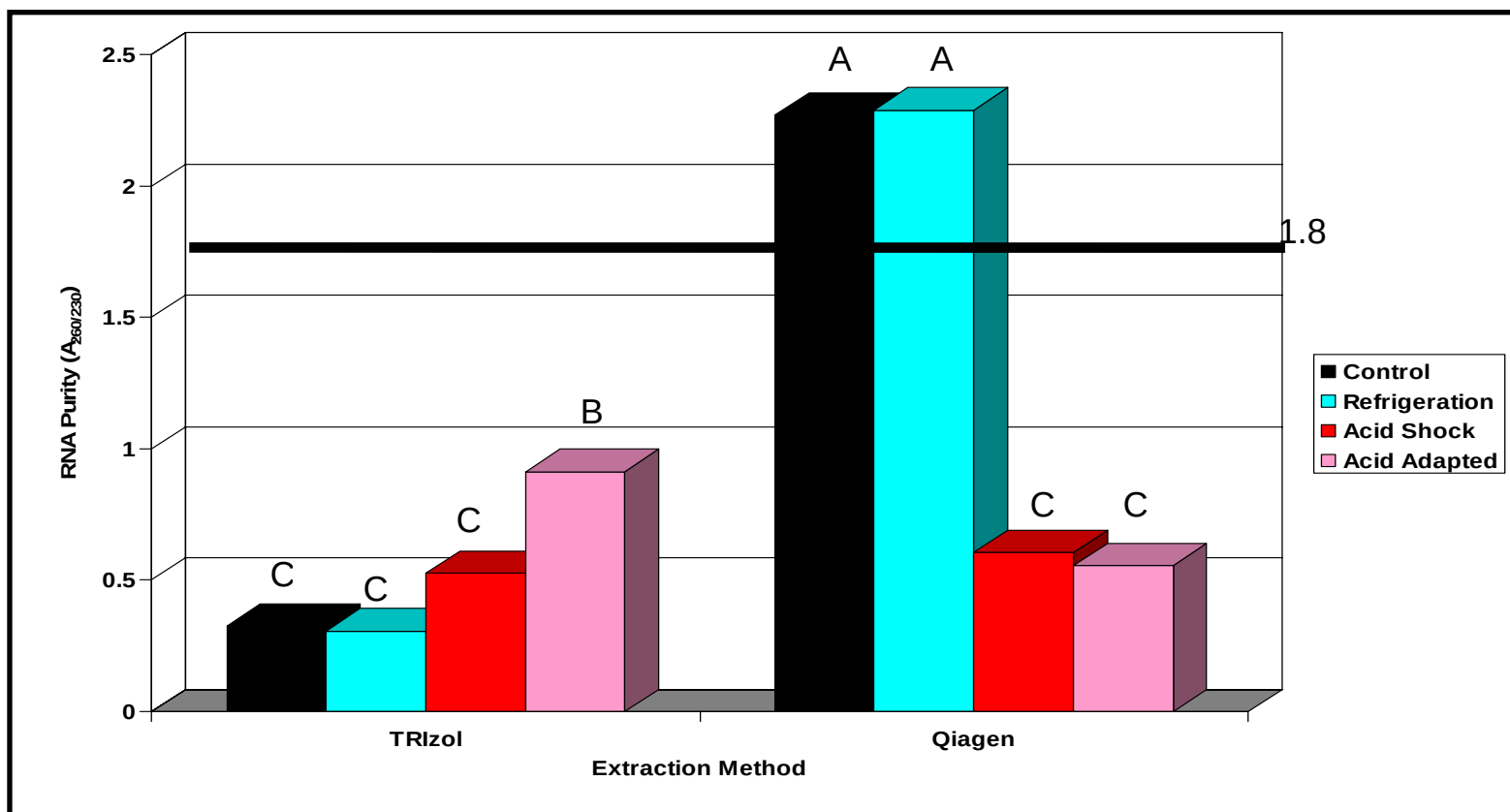


Figure 2.4: Comparison of TRIzol[®] and Qiagen extraction protocols for purity of RNA.
Letters above bars that are different indicates significant difference ($P < 0.05$).

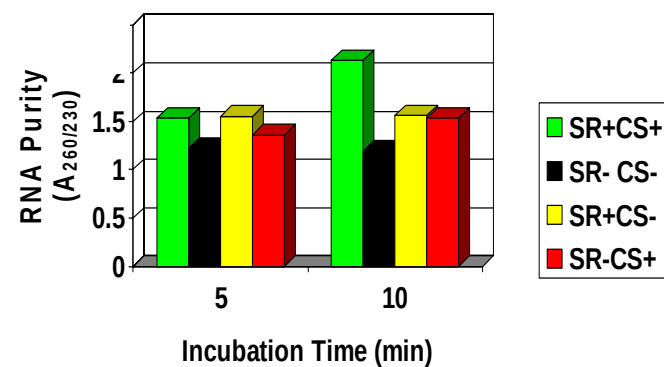
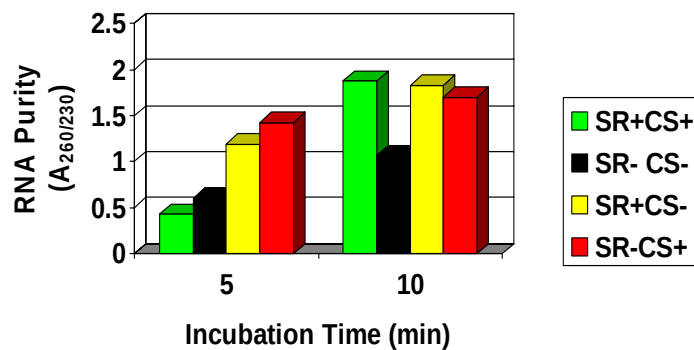
Table 2.2 Absorbance ratios for total RNA extracted from *E. coli* O157:H7 using unmodified TRIzol and Qiagen protocols.

Treatment	Absorbance ratio ¹			
	260/280 ²		260/230 ³	
	TRIzol	Qiagen	TRIzol	Qiagen
Control	1.54	2.03	0.63	1.89
Acid adapted	1.58	1.61	0.25	0.58
Acid shocked	1.60	1.52	0.44	0.61
Refrigeration (10°C)	1.47	2.03	0.33	1.87

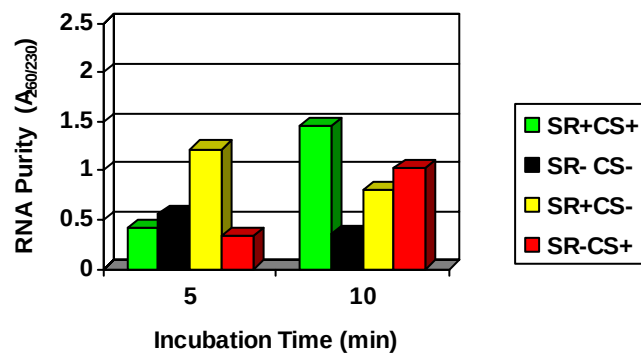
¹ Mean ratio, n=5.

² Indicator of quantity of RNA (ideal is 1.8 – 2.0).

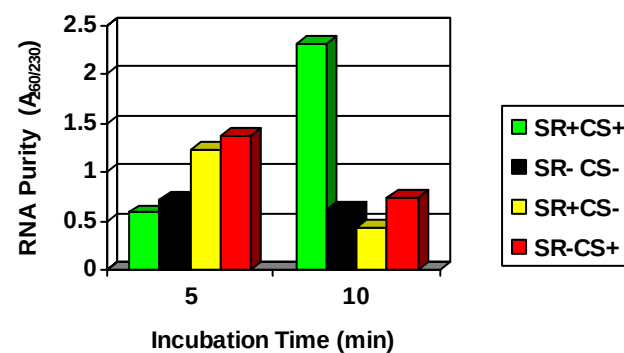
³ Indicator of purity of RNA (ideal is > 1.8).



C



D



* Top row of graphs – acid shock cells; Bottom row of graphs – acid adapted cells; Green bar-with stabilizing reagent and with cell shredder; Black bar- without stabilizing reagent and without cell shredder; Yellow bar-with stabilizing reagent, without cell shredder; Red bar-without stabilizing reagent, with cell shredder.

Figure 2.5A -D: Comparison of Qiagen modification steps for acid adapted and acid shocked cells

CHAPTER III

COMPARISON OF GENE EXPRESSION IN *ESCHERICHIA COLI* O157:H7 EDL

933 AT 10 AND 37°C USING HIGH-DENSITY OGLIONUCLEOTIDE

MICROARRAYS

Abstract

Gene expression analysis of *E. coli* O157:H7 EDL 933 cultured at 10 and 37°C was assessed using high density oligonucleotide microarrays. Cells were grown to the 7 log CFU/mL (3 reps) at 10 (refrigerated) and 37°C (control) followed by optimized total RNA extraction and hybridization onto Affymetrix GeneChip® *E. coli* Genome 2.0 Array, and confirmation by real-time reverse-transcriptase PCR (rt-RT-PCR). Microarray results ($p < 0.001$) revealed 293 down-regulated and 375 up-regulated genes. Cold shock genes, *cspE*, *cspA*, *cspG*, and *cspH* were down-regulated; *recA* and SOS DNA repair genes, *uvrB*, *yebG*, *ruvA* and *B*, *lexA*, and *dinI* were up-regulated. Expression of *fhuA* (an outer membrane protein) and *napG* (ferredoxin-type protein involved in electron transport) genes was 115- and 70-fold greater under refrigeration as compared with controls; their expression, along with *hemX* (methylase-type gene; an internal control), *ompT* and *gltJ* (both negatively expressed) was confirmed by rt-RT-PCR. Results from this study indicate that SOS DNA repair, outer membrane, and electron transport genes were utilized for survival under refrigeration conditions. These data should aid in the design of inactivation methods for *E. coli* O157:H7 in foods.

Introduction

Escherichia coli O157:H7 was first identified as a pathogen in 1982 after causing two outbreaks of hemorrhagic colitis (Mead, 1998). Since 1982, there have been numerous *E. coli* O157:H7 outbreaks linked to various contaminated foods. In 2006, there were two multi-state outbreaks involving spinach and food items from a taco fast food restaurant chain (CDC, 2006a; CDC, 2006b). Other foods implicated in outbreaks include lettuce, ground beef, fermented sausage, milk, yogurt, orange juice, and apple cider (Doyle, 2001). Each of these foods is stored under refrigeration (4°C). Refrigeration is one of the most commonly used methods for preserving foods because of its ability to cause metabolic injury and inactivation of microorganisms in the food. Studies have shown that *E. coli* O157:H7 growth is inhibited at temperatures < 6°C, but the organism survives well under refrigeration environments (Uyttendaele, 2001).

The cellular response to unfavorable environmental conditions involves the synthesis of new proteins in the early stages of exposure to any stress followed by increase in synthesis of some proteins and the decline in synthesis of proteins not required for survival (Golovlev, 2003). In non-pathogenic *E. coli*, cold shock response is induced when the temperature falls below 20°C. Within the first four hours following a temperature decline, cell growth is arrested, synthesis of most proteins and nucleic acids is suppressed, and the fatty acid composition and the physiochemical state of the cell membrane changes (Golovlev, 2003). Cells respond by synthesizing up to 28 cold shock proteins not found during normal growth conditions. Among these 28 proteins is the CspA protein, which can increase to 13% of the total protein content in the cell (Golovlev, 2003). During the first four hours following the decrease in temperature,

synthesis of CspA increases 100-fold (Golovlev, 2003). Because of the increased in synthesis and the high concentration of CspA, this protein may be a general regulator of cellular response to cold shock. In addition to the synthesis of new proteins at temperatures below 20°C, DNA responds with an increase in super-coiling and a decrease in ribosomal translation efficiency (Storz, 2000).

A growing/popular trend in molecular biology is the use of microarrays (gene chips). Microarrays are fabricated by high-speed robotics on glass slides (typically) with probes [nucleic acid sequences (e.g., DNA, oligonucleotides)] of known identity. Microarrays can contain thousands of single-stranded DNA sequences, specific to individual genes arranged in a grid pattern. They can be used to determine expression or identification of gene sequences of target organisms based upon complementary binding with the probes to target sequences. As such, an experiment using a single microarray can provide information on thousands of genes simultaneously (Shi, 2002).

Most of the research with bacteria in cold environments has focused around non-pathogenic *E. coli*. There are significant differences between the non-pathogenic *E. coli* and the pathogenic *E. coli* O157:H7; the pathogenic strain encodes 1632 proteins that are not present in non-pathogenic strains, and 131 proteins are thought to have virulence-related functions. The differences between the two strains became apparent once the complete genome of *E. coli* O157:H7 was fully sequenced in 2001 (Perna et al., 2001; Hayashi et al., 2001a; Hayashi et al., 2001b). This has opened new doors for research opportunities using molecular tools. **The purpose of this investigation was to study gene expression *E. coli* O157:H7 EDL 933 during late logarithmic phase cultures grown at 10 and 37°C.** Understanding the regulation of the genes involved in cold stress

responses will aid in the development of effective control and preventive strategies for *E. coli* O157:H7 in foods.

Materials and Methods

Cell Cultures. *Escherichia coli* O157:H7 EDL 933 (genome fully sequenced), obtained from the National Food Safety and Toxicology Center at Michigan State University, was used for this study. *E. coli* O157:H7 EDL 933 was cultured at 37°C in TSB (pH 7.0) for 24 hr, transferred into Luria-Bertani broth (LB, pH 7.0) and cultured for 24 hr at 37°C, and transferred a minimum of three successive times at 24 hr intervals before use. A 24 hr LB culture of *E. coli* O157:H7 was diluted to 4 log CFU/mL; 5 mL was inoculated in 45 mL of LB (pH 7.0) (initial concentration 3 log CFU/mL) and incubated at 37°C for 6 hr (control) and 10°C for 148 hr (refrigeration) to achieve late log phase populations of 7 log CFU/mL.

RNA Extraction. Total RNA from *E. coli* O157:H7 EDL 933 cells grown at 37°C (control) and 10°C (refrigeration) were extracted using an optimized Qiagen RNeasy[®] Mini Kit (Qiagen, Valencia, CA). From each of the three replicates, three 600 µL were removed, transferred to sterile, RNA/DNA-free collection tubes containing 1200 µL of bacterial stabilizing reagent (Qiagen), vortexed, incubated for 5 min, and centrifuged 14,000 x *g* for 10 min at 4°C. The cell pellet was re-suspended in 100 µL of TE buffer (10 mM Tris and 1 mM EDTA) (Promega, Madison, WI) containing 0.4 µg/µL lysozyme (Sigma, St. Louis, MI) and incubated for 5 min. Following incubation, 350 µL of RTL buffer containing guanidine thiocyanate (supplied with the kit) was added. The remainder

of the protocol was followed according to the manufacturer's instructions. 250 μ L of ethanol was added to the lysate and mixed by pipetting. The entire sample was transferred to the RNeasy mini column and centrifuge at $> 8,000 \times g$ for 15 sec. Flow through was discarded, 700 μ L of buffer RW1 (Qiagen, provided with kit) was added to the column and centrifuged at $> 8,000 \times g$ for 15 sec. Flow through was discarded 500 μ L of buffer RPE (Qiagen, provided with kit) was added to the column, centrifuged at $> 8,000 \times g$ for 15 sec and flow through was discarded. Another 500 μ L of buffer RPE was added to the column, centrifuged at $> 8,000 \times g$ for 2 min and flow through was discarded. Samples were centrifuged at $> 8,000 \times g$ for 2 min prior to adding 30 μ L of RNase-free water (repeated twice) for a final elute volume of $\sim 60 \mu$ L.

Absorbance Measurements. The three extracted RNA samples from each of the three replicates were combined, yielding three composite replicates, and measured spectrophotometrically. Absorbance readings (230nm, 260nm, and 280nm) and ratios (260/230 and 260/280) were measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was also used to analyze quality of RNA prior to microarray analysis.

Microarrays. Affymetrix GeneChip[®] *E. coli* Genome 2.0 Array containing approximately 20,000 genes for four *E. coli* strains (K-12, O157:H7 ELD 933, O157:H7 Sakai, and uropathogenic CFT073) was used. Extracted total RNA was converted to

cDNA, hybridized, washed, stained, and scanned according to the manufacturer's instructions.

cDNA Synthesis and Labeling. For cDNA synthesis, 10 µg total RNA was reverse transcribed using the SuperScript II system for first strand cDNA synthesis (Life Technologies, (Rockville, MD). For each the reaction, 10 µL of 75 ng/µL random primers were mixed with RNA samples and heated to 70°C for 10 min. After cooling to 25°C for 10 min, the reaction buffer was added according to the manufacturer's instructions. Samples were incubated at 25°C for 10 min, 37°C for 60 min, and 42°C for 60 min. After 10 min of the 42°C cycle, 200 U/µL SuperScript II was added to the reaction and incubated for the final 50 min. SuperScriptII was heat inactivated at 70°C for 10 min, and the mixture was cooled to 4°C. Non-converted RNA was removed by adding 20 µL of 1N NaOH, incubating at 65°C for 30 min, and then adding 20 µL of 1N HCl. cDNA was purified using the MinElute PCR Purification Kit (Qiagen), quantified based on absorption at 260 nm, and then fragmented using a partial DNase I digest. For up to 5 µg isolated cDNA, 0.6 U/µg DNase I (Amersham Biosciences, Piscataway, NJ) was added and incubated for 10 min at 37°C followed by inactivation of DNaseI at 98°C for 10 min. The fragmented cDNA was 3' labeled for 60 min at 37°C using GeneChip DNA Labeling Reagent (Affymetrix) and terminal deoxynucleotidyl transferase (Promega). The reaction was stopped with the addition of 0.5M EDTA. The fragmented and 3'-end-labeled cDNA was added to the hybridization solution without further purification.

Array Hybridization, Washing, and Scanning. The hybridization solution contained 100 mM MES (Sigma Aldrich), 1 M NaCl, 20 mM EDTA, and 0.01% Tween 20 (pH 6.6; referred to hereafter as 1x MES). In addition, the solution contained 10 mg/mL herring sperm DNA (Promega), 50 mg/mL bovine serum albumin (BSA) and 3 nM control Oligo B2 (Affymetrix). The 55 μ L of solution was combined with the fragmented and 3'-end-labeled cDNA sample and transferred into the array cartridge. Hybridization was carried out at 45°C for 16 hr with mixing on a rotary mixer at 60 rpm. Following hybridization, the sample solution was removed and the array was washed and stained per manufacturer instructions (Affymetrix, Inc). To enhance the signals, 1 mg/mL streptavidin phycoerythrin and 50 mg/mL BSA in 2x MES was used as the first staining solution. After removal of the streptavidin solution, an antibody mix containing 10 mg/mL goat IgG, 0.5 mg/mL biotin bound anti-streptavidin antibody, and 50 mg/mL BSA in 2x MES was added as the second stain. Nucleic acid was fluorescently labeled by incubation with 10 μ g/mL streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA) and 2 mg/mL BSA in 1x MES, third stain solution. The arrays were read at 570 nm with a resolution of 3 μ m using a confocal laser scanner (Affymetrix).

Statistical Analysis. Following scanning, the image was visually inspected for abnormalities and artifacts. A quality report was generated containing the scale factor, background, percent genes called present, and the internal/external controls. After the initial image quality check, data was generated using the GeneChip® Operating System (GCOS) (Affymetrix). The data were analyzed using Iobion's Array Assist (Stratagene, LaJolla, CA), which analyzes for potential outliers, then performs statistical analysis. The

three replicates were averaged and an analysis of variance ANOVA ($p < 0.01$) was performed to compare treatments. The Bonferroni correction factor was used due to multiple comparisons made, and each gene is considered an independent statistical event.

Microarray Confirmation. After data analysis, a housekeeping gene that was expressed at a statistically similar level across all treatments was selected. Additionally, four statistically significant genes that were up- or down-regulated by at least 50-fold (Table 3.1) were selected, confirmed, and validated by real-time reverse transcriptase polymerase chain reaction (rt-RT-PCR) using the BioRad iCycler with Superscript III Platinum SYBR[®] Green One-step qRT-PCR kit (Invitrogen). Primers for each gene were designed using Beacon Designer 4 software (Premier BioSoft Int., Palo Alto, CA), and thorough BLAST searches were conducted to avoid non-specific binding or cross-reactivity (Table 3.1). The rt-RT-PCR assay for each primer pair was optimized (Tables 3.2-3.4). A concentration of 250 ng/ul of total RNA from each treatment was used for rt-RT-PCR analysis. Gene expression was validated by rt-RT-PCR using a 10-fold dilution series to determine differences in expression. RT-PCR products were evaluated by rt-RT-PCR threshold cycle (C_t) values, and by gel electrophoresis; specificity was determined using melt curve analysis. The dilution series was performed in triplicate and used to confirm differences in expression level observed with microarrays. Mean C_t values for each replicate were reported.

Results and Discussion

Microarrays. Results from microarray analysis revealed a total of 668 genes significantly ($p < 0.01$) up- or down-regulated at least 2-fold as compared to controls. Of these, 375 genes were positively expressed under refrigeration conditions relative to controls and 293 genes were negatively expressed as compared with controls (Figure 3.1).

The genes were clustered according to biological function using DAVID Bioinformatics (Dennis et al., 2003; Douglas et al., 2003). Of the 668 genes, those having a biological function involving flagellum were most significant (enrichment score >30), and all were positively expressed (<http://web.utk.edu/~dgolden/microarrays/refrig-flagella.pdf>). The enrichment score is an indicator of the involvement of a gene group in association with the total genes in the study; the higher the score the more important/involved the genes are in the study. The following operons ($p \leq 0.01$; ± 2 -fold) were identified within the flagellum cluster: *flgAMN*, *flgBCDEFGHI*, *flgKL*, *flhDC*, *flhBA*, *fliAZY*, *fliDST*, *fliFGHIJK*, *fliMNOPQR*, *motABcheAW*, and *cheRBYZ* (<http://www.cib.nig.ac.jp/dda/taitoh/ecoli.operon.html>).

E. coli monitors its surrounding environment through different transmembrane chemoreceptors. The complex regulation of chemotaxis and flagellum is well documented (Vatanyoopaisarn et al., 2000; Landini and Zehnder, 2002; Leathart and Gally, 1998). In *E. coli*, the genes related to flagellum are organized into 15 operons with the master regulator, *flhDC*, at the pinnacle of hierarchical control of chemotaxis and flagellum gene expression (Neidhardt, 1996). The products of *flhDC* operon are necessary for expression of all other flagellum genes (Neidhardt, 1996). The *flhDC*

operon activates transcription of class II promoters. Class II genes encode proteins for the basal body and hook of the flagellum as well as for *fliA* (Polen et al., 2003). *FliA* is necessary for transcription of class III genes which encode *fliM* and proteins needed for flagellar assembly, motor activity (*motABcheAW* operon) and chemotaxis (*cheRBYZ* operon) (Polen et al., 2003). Full expression of *flhDC*, and thus all the flagellum genes, requires cAMP-catabolite activator protein (CRP), the carbon storage regulator (Csr), the nucleoid-associated-protein (H-NS), heat shock proteins (DnaK, DnaJ, and GrpE), regulators of phosphatidylethanolamine biosynthesis (Pss and Psd) and the polyphosphate and the quorum-sensing regulator (QseBC) (Polen et al., 2003). Researchers have shown that a mutation in any of these regulators results in limited or no transcription of the master flagellum regulator, *flhDC*, thereby decreasing synthesis of flagella with a subsequent decrease in motility of cells in the population (Sperandio et al., 2002; Shi et al., 1993; Shi et al., 1992; Soutourina et al., 1999).

Under adaptation to cold environments, researchers have observed negative regulation of *fliC*, resulting in the inability to form flagella (Vatanyoopaisarn et al., 2000; Landini and Zehnder, 2002), but proteins responsible for fimbrial adhesion (fimF, G, and H) were transcribed (Leathart and Gally, 1998). Fimbriae allow bacteria to adhere to specific surfaces (Vatanyoopaisarn et al., 2000). Landini and Zehnder (2002) reported that during growth of *E. coli* under a limited O₂ supply, the *fliC* gene showed reduced levels of expression. On the other hand, Soutourina and Bertin (2003) demonstrated that *fliC* is under-expressed under oxygen limiting conditions. Our results indicate that flagellar genes are expressed under refrigeration adapted environments. This could be in response to a chemotaxis mechanism that causes cells to move away from the cold

environment or move toward other cells and form clusters for survival; or due to the growth stage of the cells, late logarithmic phase.

Genes involving iron had an enrichment score of 14.28

(<http://web.utk.edu/~dgolden/microarrays/refrig-iron.pdf>). Of the genes in the iron cluster, the following operons were up-regulated: *napAGHBC*, responsible for electron transfer of ferredoxin-type proteins and cytochrome c-type proteins; *fhuACDB*, involved in ferrichrome-iron transport; *entCEBA*, enterobactin biosynthesis; and *fepDGC*, ferric enterobactin uptake. (<http://www.cib.nig.ac.jp/dda/taitoh/ecoli.operon.html>). Iron is a necessary mineral for bacteria, and bacteria often encounter environments where iron is limited. Bacteria require a few million iron atoms per cell for the production of cytochromes, iron-proteins and Fe-S clusters present in redox proteins (Barton, 2005). In this study, *E. coli* O157:H7 EDL 933 was grown aerobically; therefore iron uptake will be discussed as it pertains to aerobic environments. Under aerobic conditions, Fe^{3+} is insoluble in the environment and bacteria produce chelating agents, known as siderophores that sequester and mobilize Fe^{3+} . Siderophores, which are specific for a given species, have a six-coordinate octahedral complex with Fe^{3+} held in the center of the molecular arrangement (Barton, 2005). Bacterial siderophores are grouped according to the type of ligand that holds Fe^{3+} , with the most common ligand being hydroxamates (Figure 3.2) (Barton, 2005). *E. coli*, *Salmonella*, and *Shigella* spp. have hydroxamate siderophores (Barton, 2005). The binding of Fe^{3+} to the siderophore must be greater than the solubility constant for the insoluble iron compounds (Barton, 2005). Under stress conditions, *E. coli* produces enterobactin, also known as enterochelin, which acquires iron from the environment. Of the iron transport systems found in *E. coli*, the tonB

transport system is among the most widely studied. The following genes are associated with the tonB iron transport system and were significantly up-regulated in this study: *fep*, responsible for ferric-enterobactin uptake; *ent*, encodes for biosynthesis; *fhu*, encodes for iron transport into the cell; and *tonB*, responsible for synthesizing the tonB protein which is required for activation of porins in the outer membrane. Because of the high binding affinity for Fe^{3+} , enterobactin acquires iron from the environment (Litwin and Calderwood, 1993). The ferri-enterobactin complex moves through the outer membranes by way of a porin produced by *fhuA*, *fhuE*, or *fepA* (Figure 3.3). The energy required for opening the channel comes from the tonB protein, which spans the plasma membrane, periplasm, and the outer membrane (Litwin and Calderwood, 1993). Once the ferri-enterobactin is in the periplasm, it interacts with *fepB* or *fhuD* which are specific binding proteins for ferri-enterobactin (Litwin and Calderwood, 1993). *FepB* or *fhuD* proteins are part of an ABC transporter which delivers ferri-enterobactin to the cytoplasm through the use of dual transmembrane proteins of *fepC* or *fhuC* (Litwin and Calderwood, 1993). The energy required for this transport system is derived from ATP hydrolysis. Once in the cytoplasm, ferri-enterobactin complex is degraded by a specific esterase to release Fe^{3+} (Schultz-Hauser et al., 1992; Barton, 2005). Enterobactin is degraded to dihydroxybenzoic acid (DBA) which is released from the cell as an end product (Litwin and Calderwood, 1993). In *E. coli*, the release of DBA does not result in an energy drain, since fewer than a million atoms of iron are required for the growth of a single cell (Litwin and Calderwood, 1993). Fe^{3+} is reduced to Fe^{2+} by NAD(P)H-driven ferric reductase, and this reduced iron form is used for synthesis of cytochromes and proteins with Fe-S centers (Barton, 2005). This unique transport system which binds and

transports Fe^{3+} into the cells is regulated by *fur* and was found to be the same mechanism for the transport of heme (Foster and Hall, 1992; Storz, 2000). In *E. coli*, *fur* functions as a repressor that utilizes Fe^{2+} as a cofactor to bind specific promoter iron-regulated genes, resulting in the transcriptional repression of these genes in iron-rich environments (Thompson et al., 2002). Previous research has shown that iron transport is a widespread mechanism utilized under various environmental conditions. In the present study, iron transport was a significant factor in *E. coli* O157:H7 incubated under refrigeration environments.

The *feoAB* genes were also positively expressed, indicating that a second iron transport system was utilized under refrigeration. The *feoAB* operon is responsible for ferrous (Fe^{2+}) iron transport (Kammler et al., 1993). The operon is activated by *fnr* and also regulated by *fur* (Kammler et al., 1993). The transport of ferrous iron into the cell is driven by ATP hydrolysis (Kammler et al., 1993). This ferrous transport system is believed to be important during survival of *E. coli* in the intestines (Stojiljkovic et al., 1994). Stojiljkovic et al. (1994) used a mouse model to determine the effectiveness of ferrous iron transport and colonization in the intestine of mice. They found that *feo* mutants were less effective at colonizing mouse intestines than *feo+* strains of *E. coli* suggesting the involvement of this iron transport system for survival and colonization within the intestines (Stojiljkovic et al., 1994).

E. coli expresses three nitrate reductases; nitrate reductases A and Z are membrane bound and reduce nitrate in the cytoplasm. The third nitrate reductase, nap, is located in the periplasm (Wang et al., 1999). Nap is a high-affinity, non-energy-conserving enzyme that is expressed at low nitrate concentrations (Wang et al., 1999).

Nitrate reductase activity, which has been identified in a variety of bacteria, is the product of four genes, *napD*, *A*, *B*, and *C*. These genes appear to be essential for nitrate reductase enzymatic activity in all bacteria tested thus far. The nap complex in *E. coli* is encoded by the *napFDAGHBC* operon (<http://www.cib.nig.ac.jp/dda/taitoh/ecoli.operon.html>).

NapAB is part of a catalytic complex found in the periplasm of the cell and is believed to receive electrons from the quinol pool by way of the membrane-bound cytochrome *NapC* (Potter and Cole, 1999). The quinol pool is an electron pool fed by substrate-specific dehydrogenases (Potter and Cole, 1999). *NapF*, *G*, and *H* are hypothesized to synthesize iron-sulfur proteins and are believed to be involved in electron transfer; however, none of them have been found to be essential for nap activity in *E. coli* (Potter and Cole, 1999). *NapF*, *G*, and *H* are believed to contribute to maintenance of the maximal rate of nitrate reduction, but they are not essential for electron transfer from the quinol pool to the nap complex (Potter and Cole, 1999).

In many bacteria, periplasmic nitrate reductases are encoded by a set of five to seven genes that are found on a single operon. The genes most commonly found on the *nap* operon are *napA*, *napB*, *napC*, and *napD* (Brondijk et al., 2002). The *NapA* protein is the catalyst and contains the molybdopterin guanine dinucleotide cofactor that is the site of nitrate reduction (Brondijk et al., 2002). It is located in the periplasm where it forms a complex with *NapB*, the electron donor to *napA* (Potter and Cole 1999). The *napAB* protein complex is believed to receive electrons from quinol dehydrogenase, *napC*, located in the cytoplasmic membrane (Brondijk et al., 2002). The fourth *nap* protein, *napD*, is also found in the cytoplasm, and research has demonstrated its involvement in the post-translational activity of *napA* (Potter and Cole, 1999). In addition to these four

proteins, additional proteins are encoded by the *nap* operon in different bacterial species. Different combinations of *napE*, *F*, *G*, *H* and *K* are found in different species and are generally not essential for *nap* activity (Brondijk et al., 2002).

Metabolism function was also found to be of importance based upon the clustering data, enrichment score 5.73 (<http://web.utk.edu/~dgolden/microarrays/refrig-metab.pdf>). During stress, it is necessary for microorganisms to maintain the physiology and operation of the cell for survival. This is accomplished, in part, by maintaining structural integrity of cellular components such as proteins, membranes, and ribosomes, and to keep important systems in the cell, such as those responsible for protein synthesis functioning. Temperature is an important parameter that regulates the activity of a microorganism. Growth of microorganisms at temperatures below optimal changes their metabolic processes. Reduced temperatures cause delays in enzyme activity, metabolic imbalance, reduced ion transport and diffusion and cessation of growth (Olson and Nottingham, 1980; Wiebe, 1992). Russell et al. (1995) reported that decreased temperature results in an extension of the lag phase, reduced growth rate, decrease in cell volume and an inhibition of DNA, RNA and protein synthesis. These effects of cold temperature incubation were also observed in this study. From the growth curve data (Chapter 2, Figures 2.1 and 2.2), the lag phase was extended from approximately 1 hr for control cells to nearly 50 hr under refrigerated conditions. The growth rate at 10°C was substantially reduced, and it required culturing for 150 hr to reach the 7 log CFU/mL target population. Additionally, inhibition of DNA, RNA and protein synthesis was indicated by the large number of SOS and DNA repair genes that were up-regulated.

The last significantly expressed cluster involved membrane-related genes (<http://web.utk.edu/~dgolden/microarrays/refrig-memb.pdf>). This was also the largest group of clustered genes (enrichment score 3.76). Nearly 25% of the genes up- or down-regulated under refrigeration conditions were related to membrane function. The cell membrane plays a major role in protecting the cell from the outside environment. In addition, the membrane must also allow for the effective transport of necessary molecules into the cytoplasm to keep the cell alive (Wiebe, 1992). Within the cell membrane, phospholipids molecules are arranged in the form of a bilayer with the polar head at the intracellular and extracellular surfaces (Neidhart et al., 1990). These groups allow interaction with the aqueous phases on the inside and outside of the cell. The fatty acid acyl chains are stacked in parallel at right angles to the plane of the membrane, with the terminal methyl group situated in the interior of the bilayer (Albert et al., 1994). It is documented that microorganisms adjust their membrane lipid composition in response to changes in temperature (Brown and Minnikin, 1973; Russell, 1984; Tsuchiya et al., 1987). Generally, during low-temperature growth, the fluidity of the membrane is altered as a result of changes in the membrane fatty acid composition of phospholipids and glycolipids (Russell et al., 1995). In order to maintain cellular function, the membrane lipid bilayers need to remain fluid in order for membrane proteins to continue pumping ions, taking up nutrients and performing respiration (Berry and Foegeding, 1997). Therefore, it is essential that the membrane lipids are in a liquid crystalline state (Berry and Foegeding, 1997). When the temperature is decreased, cellular fluid becomes gel-like, thereby preventing proteins from functioning normally. In order to return to a fluid-like state, changes in the structure of the fatty acid chain must occur (Barton, 2005).

Under cold temperatures, unsaturation of fatty acid chains occurs, increasing the fluidity of the membrane (Beales, 2004). Unsaturated fatty acid groups create more instability to the membrane than saturated chains (Beales, 2004). Unsaturated fatty acid adaptation occurs rapidly in the membrane through the action of desaturase enzymes (Beales, 2004). Additionally, in response to decreases in temperature, the average fatty acid chain length may be shortened, in-turn increasing the fluidity of the cell membrane because there are fewer carbon-carbon interactions between neighboring side chains (Russell, 1990). An increase in the amount and/or kind of branched fatty acids may also occur, and a reduction in the proportion of cyclic fatty acids can occur, thus causing an increase in mono-unsaturated straight chain fatty acids, as seen in *Salmonella* (Russell, 1984). This transformation of the membrane in order to maintain fluidity enables the cell to continue regulating essential cellular activities, including solute transport systems and essential membrane bound enzymes (Russell et al., 1995).

The effects of reduced temperature on the membrane fatty acid composition of *L. monocytogenes* has been studied extensively (Russell et al., 1995). The main change observed when the temperature is reduced below optimal is an increase in the proportion of C_{15:0} at the expense of C_{17:0} fatty acids. The shortening in fatty acid chain length lowers the melting temperature and aids in maintaining membrane fluidity at the decreased temperature (Russell et al., 1995). At reduced temperatures, *E. coli* and *Yersinia enterocolitica*, also increase synthesis of unsaturated fatty acids at the expense of saturated fatty acids (Marr and Ingraham 1962; Abbas and Card, 1980; Goverde et al., 1994). In this study, the large number of membrane function genes that were up-regulated

under refrigeration is consistent with previous studies indicating changes in the membrane.

The outer membrane protein, ompT, of *E. coli* is a serine protease (now classified as an aspartyl protease) and is a member of the omptin family of proteins (White et al., 1999; Leytus et al., 1981). Leytus et al. (1981) demonstrated that ompT catalyzes the activation of plasminogen to plasmin, a function that is physiologically significant for the virulence of *Y. pestis* and for several clinical *E. coli* isolates (White et al., 1999; Leytus et al., 1981). The endoprotease produced by ompT is a stress induced and increases its expression level in response to heat shock (Okuno et al., 2004). Sugimura and Nishihara (1988) demonstrated that protease activity in the cells is greater at 37°C than at 30°C and expression *ompT* is higher in the late logarithmic and stationary phases than in the logarithmic phase (White et al., 1999; Okuno et al., 2004). Baneyx (1999) showed through mutation analysis that the *ompT* gene is non-essential; the deletion had no effect on the growth of *E. coli* (White et al., 1999). In our study, ompT was down-regulated, confirming that protease activity is decrease at lower temperatures and indicating that these pathogenesis-related functions were not active under refrigeration.

E. coli has two primary pathways for glutamate synthesis. The glutamate synthase pathway is essential for synthesis of glutamate at low ammonium concentrations and for regulation of the glutamine pool (Helling, 1994). The glutamate dehydrogenase pathway (GDH) catalyzes the reduction of 2-oxoglutarate to glutamate (Helling, 1994). The glutamate synthase pathway fixes ammonia into organic molecules, such as glutamine, which can then be made into glutamate, and this pathway is active when the ammonia concentration is too high (Helling, 1994). In *E. coli*, there are four protein transporters

responsible for the uptake and transport of glutamate (GltIJKL, GltP, GltS, and GadC). Of these, GltIJKL accounts for approximately 15% of the total transport velocity of glutamate (Schellenberg and Furlong, 1977). The GltIJKL glutamate/aspartate transporter is a component of the ATP binding cassette (ABC) transporter superfamily (Linton and Higgins, 1998). Sequence resemblance with known ABC transporter components suggests that GltJK is the membrane components, GltL is the ABC transporter protein and GltI is the periplasmic-binding protein of the GltIJKL glutamate ABC transporter system (Urbanowski et al., 2000; Linton and Higgins, 1998). Expression of *gltI* was found to be greatly reduced when *E. coli* K-12 strain was grown in rich medium (Kahane et al., 1976). Expression of *gltI* is also reduced when *E. coli* is grown with glucose rather than succinate (Willis et al., 1975). This reduction in *gltI* expression in-turn reduces the expression of *gltJKL*, since *gltI* transcribes the periplasmic binding protein for the *gltJKL* transporter. In our study, *E. coli* O157:H7 was grown in LB broth, which contains glucose. This could account for the negative regulation of the *gltIJKL* transport system that was observed.

The most intriguing results of this study were from the clusters of SOS and DNA repair genes, and those known to be involved in cold shock (<http://web.utk.edu/~dgolden/microarrays/refrig-iron.pdf>). Most (19 of 24) of the DNA repair and SOS genes were up-regulated, but all the cold shock genes were down-regulated. This is likely due to the fact that *E. coli* O157:H7 was in the logarithmic phase of growth at the point of total RNA extraction. Therefore, the cold shock genes were already expressed during the lag phase and turned off while DNA repair genes were utilized. Jones et al. (1992) reported that when *E. coli* was subjected to temperatures of

10°C during lag phase, proteins involved in transcription and translation are still synthesized. This is opposite of what occurs when growth is prevented by other conditions, i.e. the synthesis of proteins greatly declines.

Mizushima et al. (1997) determined that subjecting *E. coli* to cold shock increased negative supercoiling of DNA, leading to increased expression of DNA repair genes. DNA is naturally negatively supercoiled, but in response to various environmental factors, e.g., high osmolarity and heat shock, supercoiling of DNA has been reported to increase (Higgins et al., 1988; Mizushima et al., 1997).

Genes related to bacteriophage function were also clustered (<http://web.utk.edu/~dgolden/microarrays/refrig-phage.pdf>). These were all up-regulated; the shiga toxin genes, *stx1* or *stx2*, were significantly ($p \leq 0.01$) expressed but fell below the 2-fold cut-off set for the data. Buncic and Avery (1998) found that cold storage in nutritious media did not affect shiga toxin production, but shiga toxin production was increased during subsequent growth following cold storage (Buncic and Avery, 1998). Elhanafi et al. (2004) reported cold or cold and acid stress had no effect on shiga toxin II production. In this cluster, a single gene, *fliN*, related to virulence function (type III secretion system) was identified as up-regulated (3.90-fold).

rt-RT-PCR Confirmation. Based on the microarray data, four genes were selected for confirmation using real time reverse transcriptase PCR (Table 3.1). Two genes, *ompT* and *gltJ*, are related to membrane function (the largest group of genes affected); two genes, *napG* and *fhuA*, are involved in iron transport. Additionally, *hemX* was selected as

the housekeeping gene because it was similarly expressed (i.e., not significantly up- or down-regulated) under both conditions (Figure 3.4; Table 3.4; Figures 3.5 & 3.7).

The genes involved in iron function, *napG* and *fhuA*, were up-regulated. As such, the refrigeration dilution series would be expected to extend farther than the control dilution series for both genes. However, the dilution series for *napG* (Figure 3.4; Table 3.5; Figures 3.8 & 3.10) did not confirm this. It should be noted that, due to a power failure, total RNA which was used in the microarrays analysis was lost and new, re-extracted total RNA was used for the remaining rt-RT-PCR confirmation studies. One rt-RT-PCR run was accomplished with the original RNA, and a + 2-log greater expression level was noted for refrigeration cells as compared with control cells. *NapE, F, G, H* and *K* genes are found in a variety of bacteria and are predicted to encode for iron-sulfur proteins. However, they are not essential for nap activity. Since *napG* is not essential, this may explain why no difference in expression was observed upon our subsequent extraction and analysis. *NapC* was up-regulated (6.64-fold) and is essential for nap activity, and would have been a better choice for rt-RT-PCR confirmation.

Up-regulation of *fhuA*, which is involved in membrane and iron function, was confirmed by rt-RT-PCR; *fhuA* was detected at a 1-log greater dilution in refrigerated cells than in control cells (Figure 3.4; Table 3.6; Figures 3.11 & 3.13).

Based upon microarray analysis, *ompT* and *gltJ* were down-regulated under refrigeration conditions. Both were genes are involved in membrane function. *OmpT* is an outer membrane protein involved in proteolysis and catalyzes the activation of plasminogen to plasmin (White et al., 1999); *gltJ*, an ABC superfamily membrane protein, is part of the *gltJKL-sroC* operon and involved in glutamate/aspartate transport in

the inner membrane (Kahane et al., 1976). Results from rt-RT-PCR analysis for both of the genes confirm the microarray data. A -1-log difference was detected in *ompT* expression in refrigerated cells compared with control cells (Figure 3.4; Table 3.7; Figures 3.14 & 3.16). Similarly, a -1.5-log difference in *gltJ* expression in refrigerated cells was observed (Figure 3.4; Table 3.8; Figures 3.17 & 3.19).

Conclusions

From the clustering results, we found significant involvement of membrane, metabolism, flagella, and iron genes. Information on the stress response in *E. coli* O157:H7 at reduced temperatures will help the food industry understand how this organism behaves in food products and how the extent of the stress responses is affected by pre-storage temperatures. While perishable foods are stored at temperatures below that used in this study (10°C), the temperature we used is commonly found in processing areas where minimizing microbial growth is critical. As such, pre-exposure of *E. coli* O157:H7 to cold during processing could predispose this pathogen to enhanced survival during subsequent cold storage. A greater understanding of the mechanisms of cold adaptation may offer insight into the development of strategies for controlling growth and survival of *E. coli* O157:H7 in chilled and frozen foods.

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Appendix

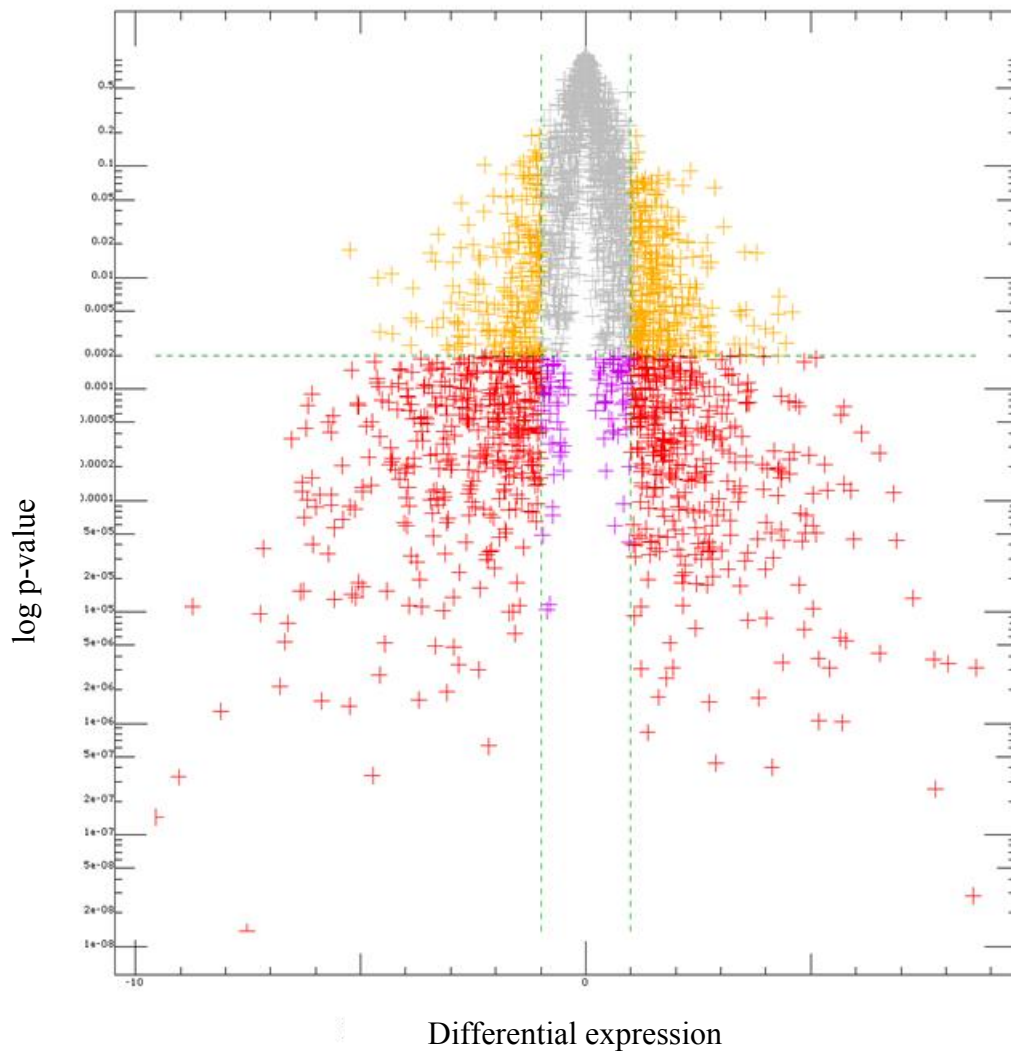
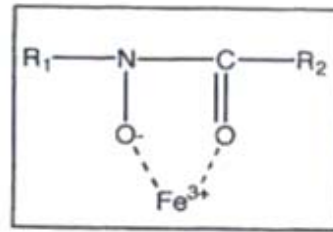


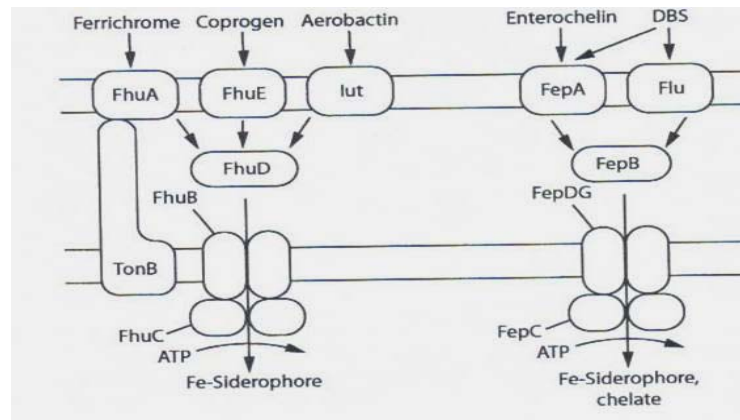
Figure 3.1: Plot of p-values vs. differential expression (fold change) of genes from *E. coli* O157:H7 control vs. refrigeration treatments.

(b) Hydroxamate



(Barton, 2005)

Figure 3.2: Hydroxamate bacterial siderophores



(Barton, 2005)

Figure 3.3: FhuA-TonB complex

Table 3.1: Primer sequences for genes selected for rt-RT-PCR confirmation.

Gene	Primer sequence	Product length
<i>hemX</i>	Forward: GAAAATATTCGCTCTCGCCTGCTGG	209 bp
	Reverse: TGGCTTTGCAGGGTTTCCGG	
<i>napG</i>	Forward: GTCAGCGAAACCTCAAATGG	217 bp
	Reverse: GGTGTCGTAAGGGCAAGC	
<i>fhuA</i>	Forward: ACAGTGTGCGGCATTAGC	208 bp
	Reverse: AGAGTCGTCGTAACCAAACC	
<i>ompT</i>	Forward: TTATCTAGCCGAAGAAGGAG	231 bp
	Reverse: AGTTGTGTATCAGGGTGTC	
<i>gltJ</i>	Forward: TTCCTTTCATCCATGCTCTG	201 bp
	Reverse: ATCATCTCTGAGGTCATCGG	

Table 3.2: rt-RT-PCR program for *napG* and *fhuA*.

Cycle #	Temperature - time	Replicates
1	50°C - 35 minutes	1
2	94°C - 2 minutes	1
	94°C - 45 seconds	
3	50°C - 45 seconds	50
	72°C - 45 seconds	
4	72°C - 7 minutes	1
5	50°C in 0.5°C increments every 10 seconds	96
6	4°C	Hold

Table 3.3: rt-RT-PCR program for *ompT* and *gltJ*

Cycle #	Temperature - time	Replicates
1	50°C - 35 minutes	1
2	94°C - 2 minutes	1
	94°C - 45 seconds	
3	53°C - 45 seconds	50
	72°C - 45 seconds	
4	72°C - 7 minutes	1
5	50°C in 0.5°C increments every 10 seconds	96
6	4°C	Hold

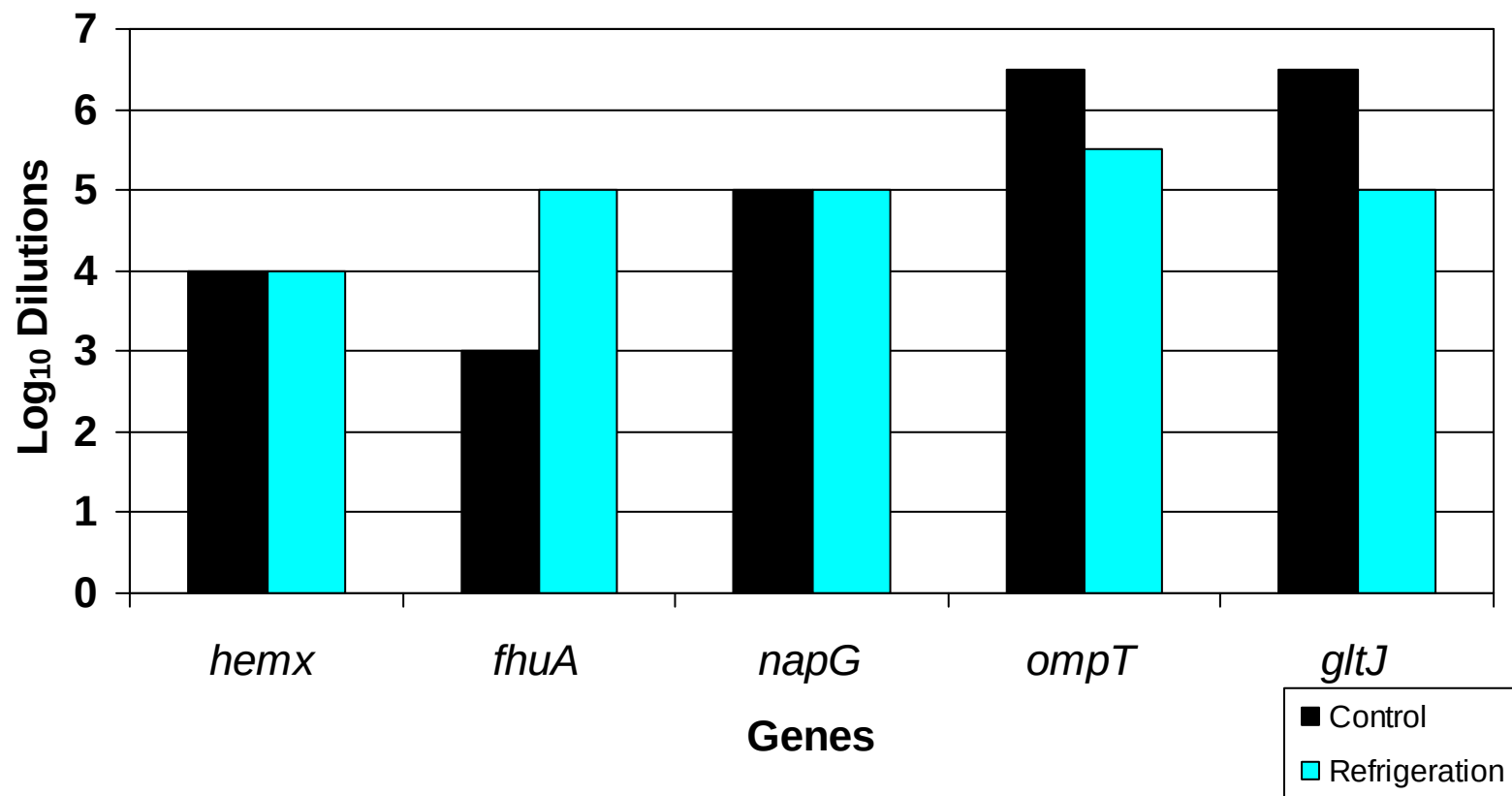


Figure 3.4: Differences in expression level of refrigeration genes

Table 3.4: C_t values for *hemX* in control and refrigerated *E. coli* O157:H7.

Treatment	Dilution			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Control	19.49 ± 1.09	22.92 ± 1.65	26.33 ± 1.72	29.44 ± 1.83
Refrigeration	18.73 ± 1.09	21.70 ± 0.63	25.10 ± 0.76	28.06 ± 0.80

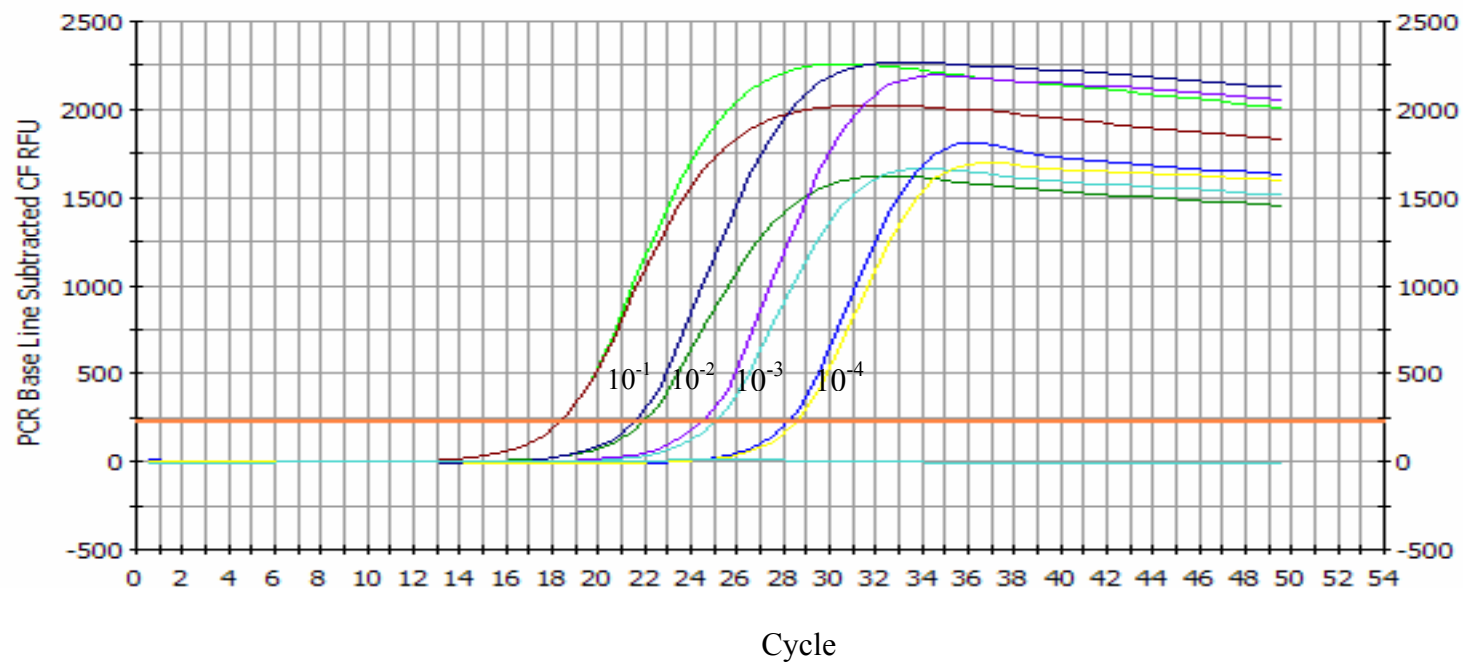


Figure 3.5: C_t curve for *hemX*

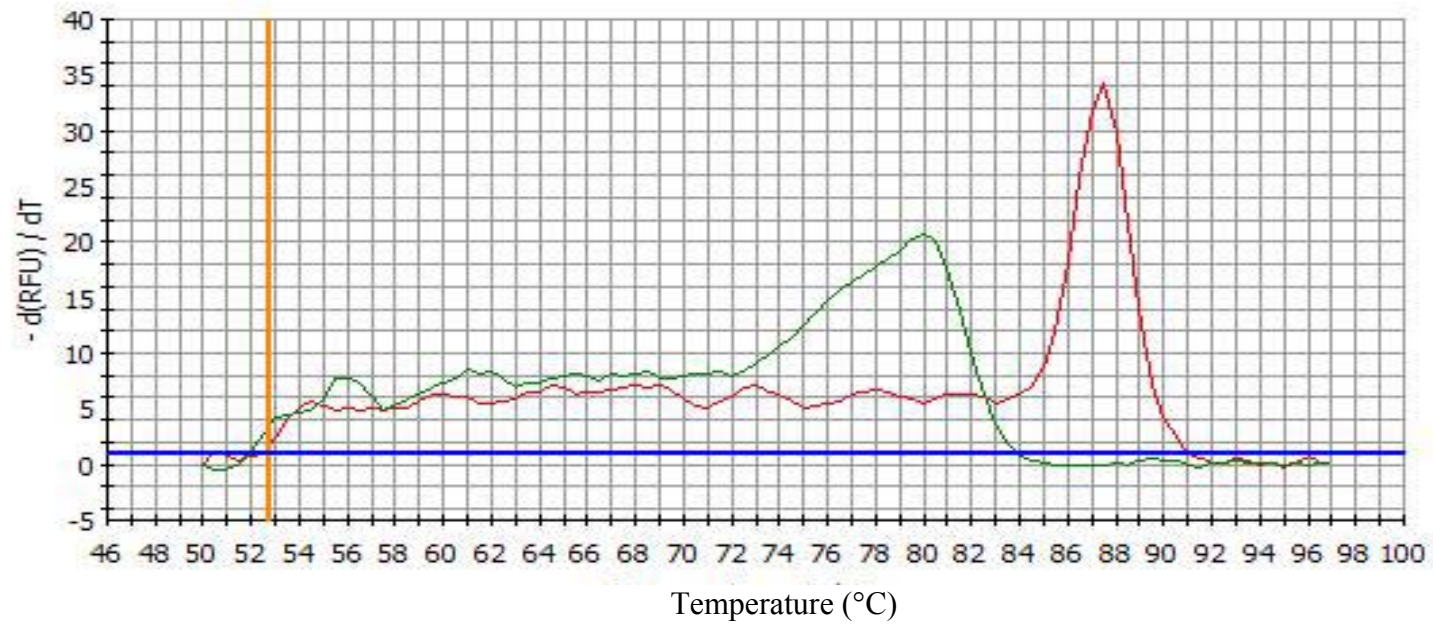


Figure 3.6: T_m graph for *hemX*

* T_m values for *hemX*, 87.5°C (red); *hemX* negative (green)

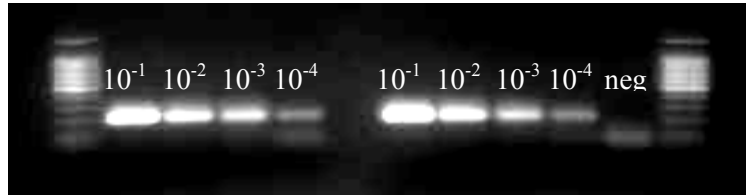


Figure 3.7: Control and refrigeration *hemX* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lane 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: empty; Lane 7: Refrig 10^{-1} ; Lane 8: Refrig 10^{-2} ; Lane 9: Refrig 10^{-3} ; Lane 10: Refrig 10^{-4} ; Lane 11: negative; Lane 12: 100bp DNA marker.

Table 3.5: C_t values for *napG*

Treatment	Dilution				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Control	20.15 ± 0.64	23.70 ± 0.42	27.75 ± 0.38	31.48 ± 1.28	33.4 ± 1.06
Refrigeration	19.5 ± 1.27	23.3 ± 0.99	27.10 ± 0.85	31.23 ± 0.79	33.8 ± 2.23

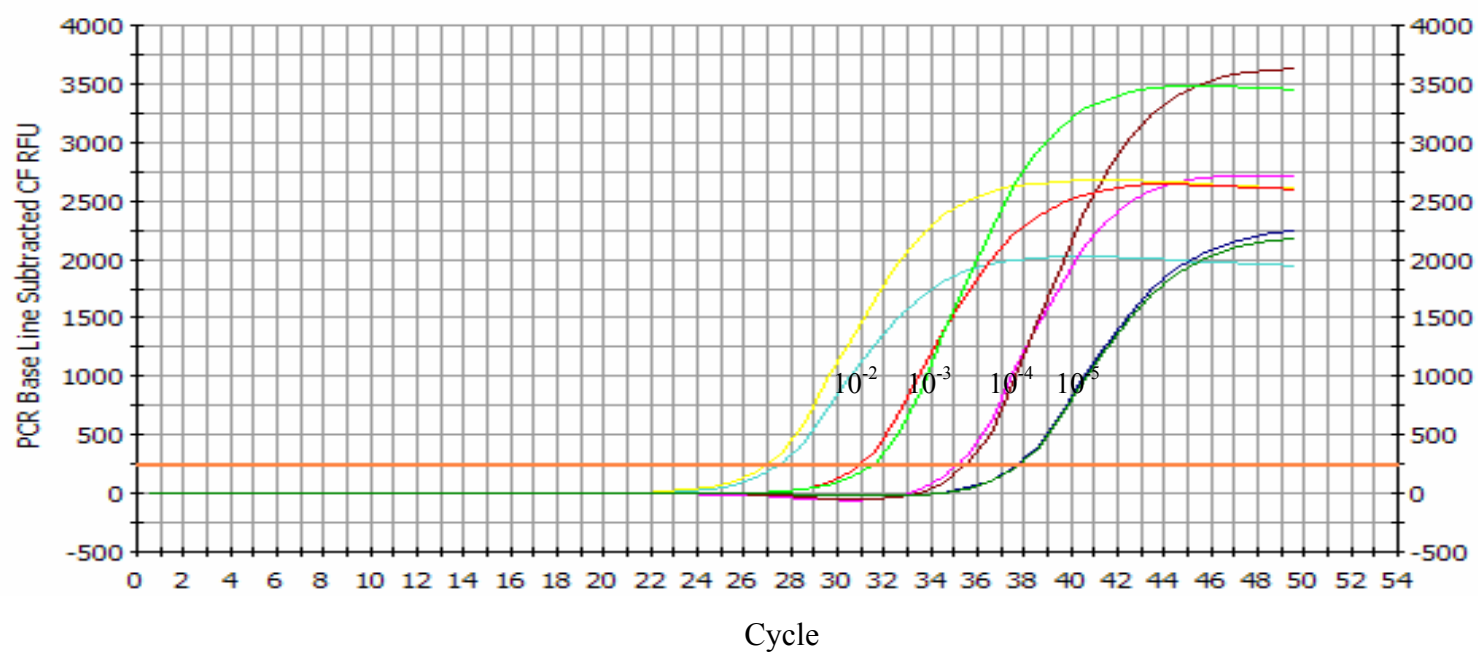


Figure 3.8: C_t curves for *napG*

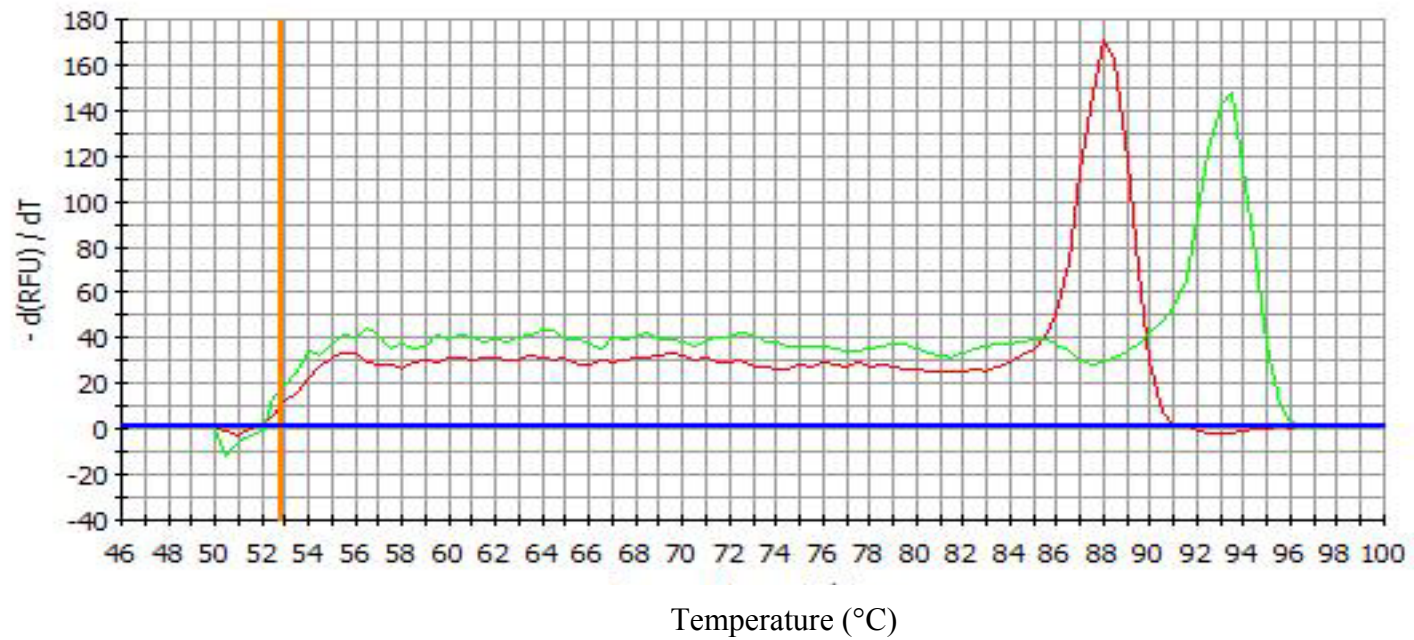


Figure 3.9: T_m curve for *napG*

*Red: T_m for hemX, 88°C; Green: T_m for *napG*, 93°C

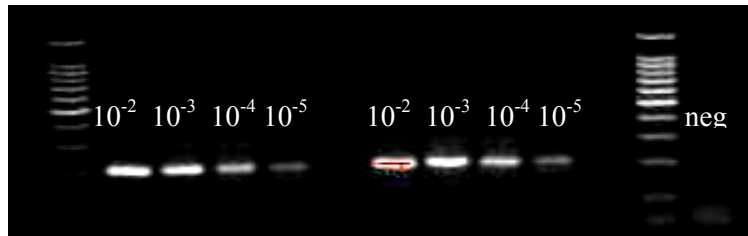


Figure 3.10: Gel of *napG* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lane 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: empty; Lane 7: Refrig 10^{-2} ; Lane 8: Refrig 10^{-3} ; Lane 9: Refrig 10^{-4} ; Lane 10: Refrig 10^{-5} ; Lane 11: empty; Lane 12: 100bp DNA marker; Lane 13: negative.

Table 3.6: Average C_t values for *fhuA*

Treatment	Dilution			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Control	19.7	23 ± 0.35	26.27 ± 0.98	
Refrigeration	18.8	21.57 ± 0.35	25.87 ± 1.53	25.97 ± 0.50

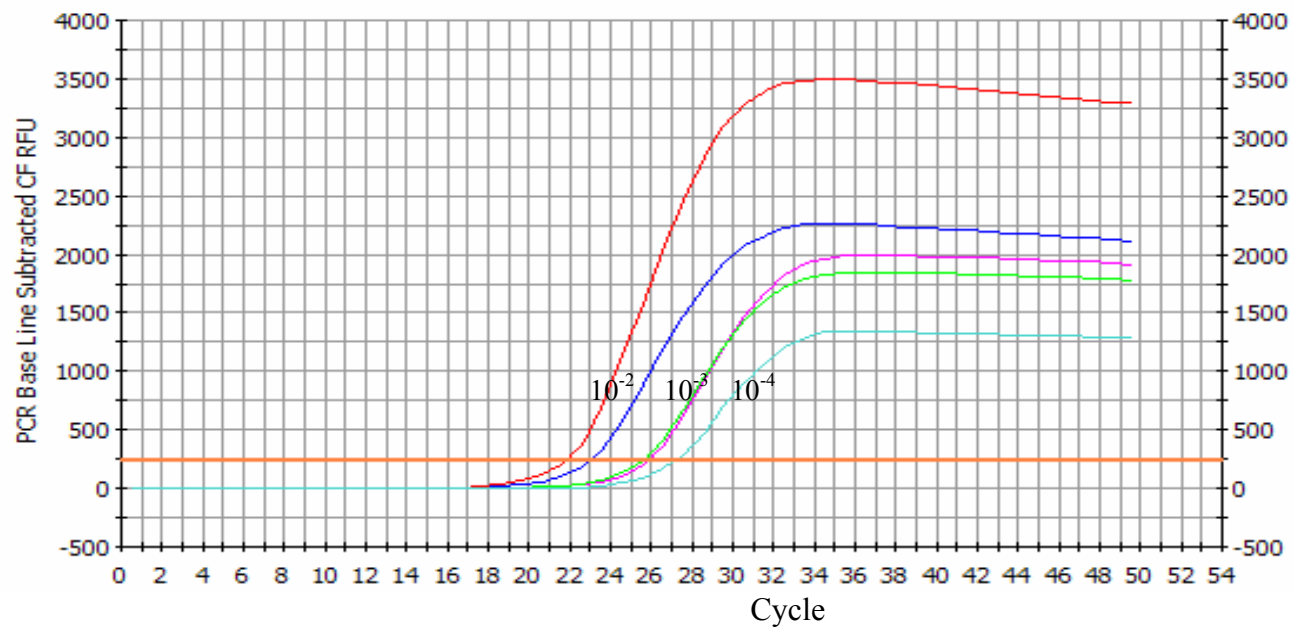


Figure 3.11: C_t curves for *fhuA* dilutions

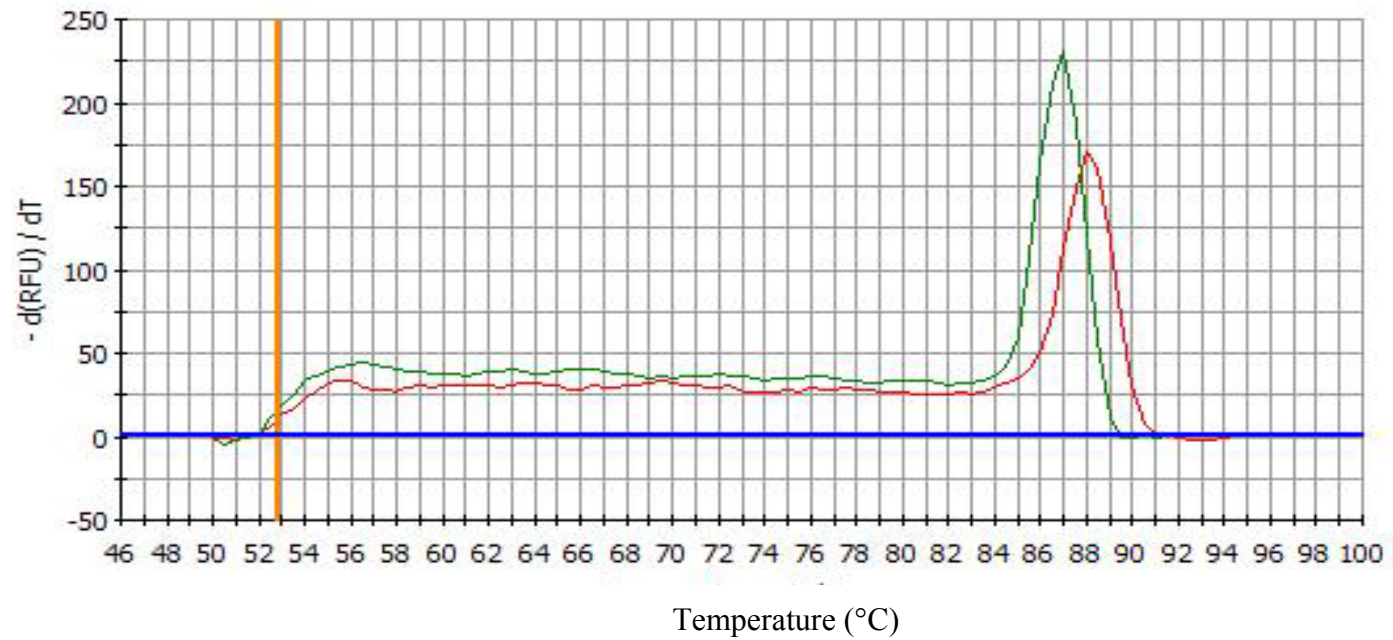


Figure 3.12: T_m curve for *fhuA*

*Red: T_m for *hemX*, 88°C; Green: T_m for *fhuA*, 87°C



Figure 3.13: Gel of *fhuA* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lanes 4-9: empty; Lane 10: 100bp DNA marker; Lane 11: Refrig 10^{-2} ; Lane 12: Refrig 10^{-3} ; Lane 13: Refrig 10^{-4} ; Lane 14-18: empty; Lane 19: negative; Lane 20: 100bp DNA marker.

Table 3.7: Average Ct values for *ompT*

Treatment	Dilution							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	$10^{-5.5}$	10^{-6}	$10^{-6.5}$
Control	17.33 ± 0.40	21.33 ± 0.21	25.27 ± 0.15	28.9 ± 0.78	29.2 ± 0.10		32.67 ± 0.32	33.73 ± 0.32
Refrigeration	18.23 ± 0.49	21.43 ± 0.35	25.6 ± 0.10	28.9 ± 0.75	32.23 ± 0.21	33.23 ± 0.49		

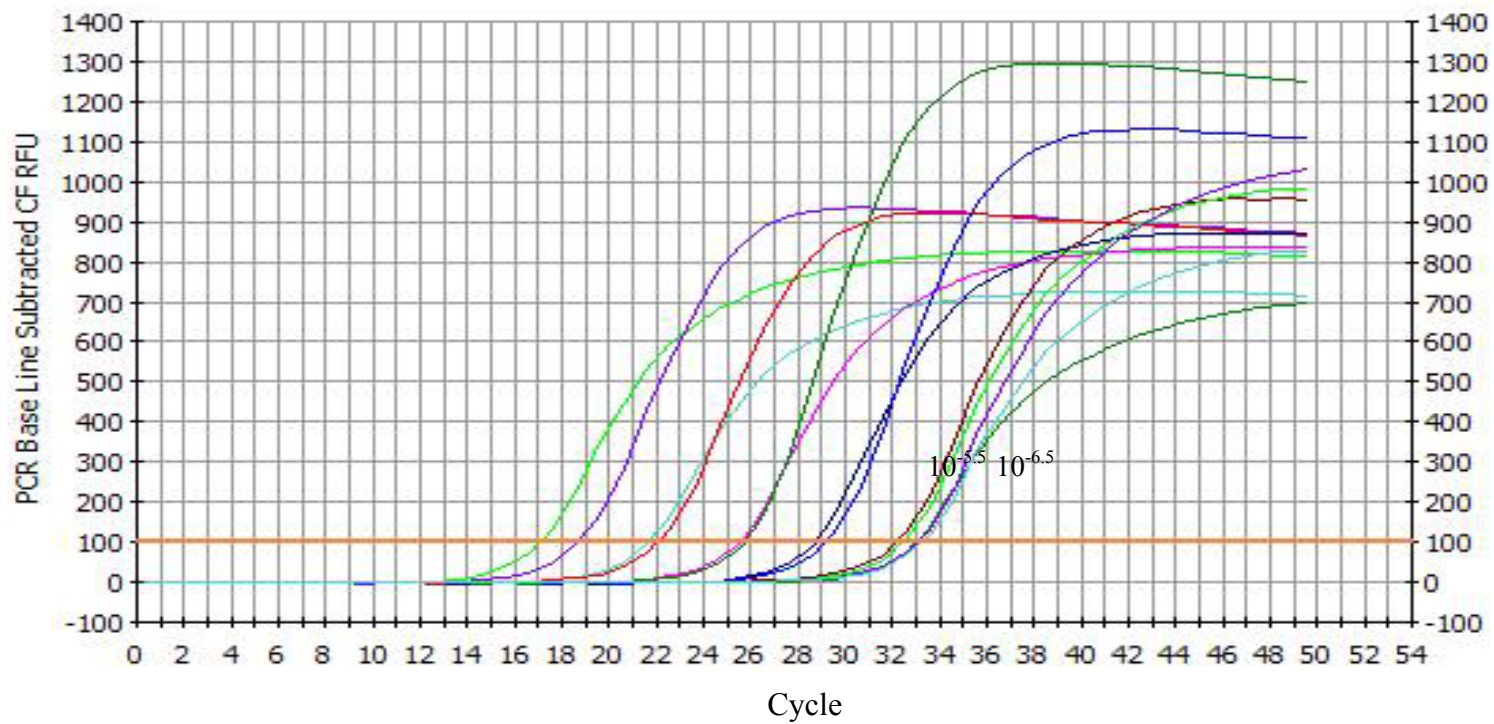


Figure 2.14: C_t curves for *ompT* dilutions

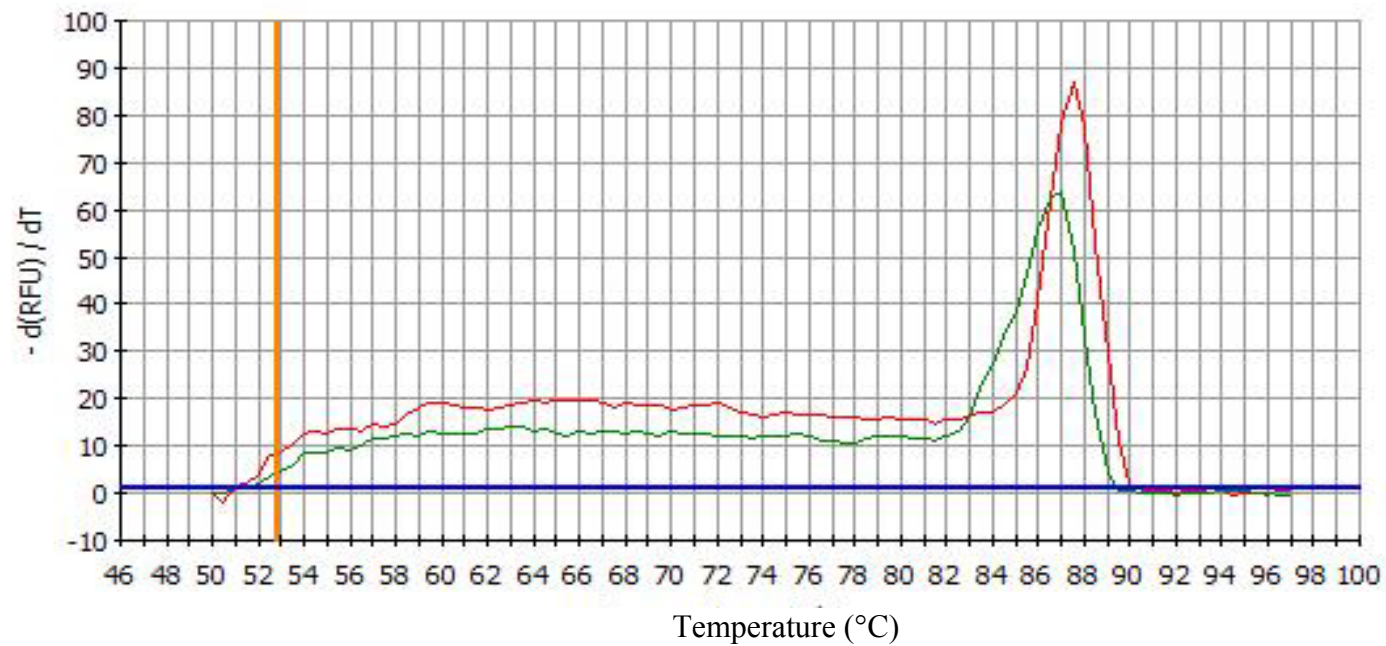


Figure 3.15: T_m curve for *ompT*
 *Red: *hemX* T_m , 88°C; Green: *ompT* T_m , 87°C

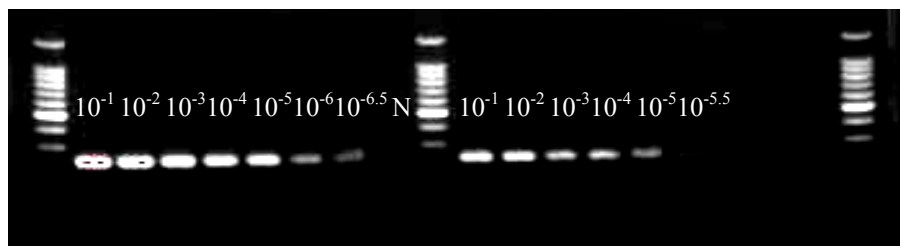


Figure 3.16: Gel of *ompT* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lanes 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: Control 10^{-5} ; Lane 7: Control 10^{-6} ; Lane 8: Control $10^{-6.5}$; Lane 9: negative; Lane 10: 100bp DNA marker; Lane 11: Refrig 10^{-1} ; Lane 12: Refrig 10^{-2} ; Lane 13: Refrig 10^{-3} ; Lane 14: Refrig 10^{-4} ; Lane 15: Refrig 10^{-5} ; Lane 16: Refrig $10^{-5.5}$; Lane 17-19: empty; Lane 20: 100bp DNA marker.

Table 3.8: Average C_t values for *gltJ*

Treatment	Dilution							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	$10^{-4.5}$	10^{-5}	10^{-6}	$10^{-6.5}$
Control	20.57 ± 0.29	24.2 ± 0.70	25.87 ± 0.93	27.9 ± 1.11		28.43 ± 0.32	30.73 ± 0.50	32.8 ± 0.9
Refrigeration	19.97 ± 0.31	22.33 ± 1.10	26.77 ± 0.70	30 ± 0.87	31.13 ± 0.68	33.63 ± 0.35		

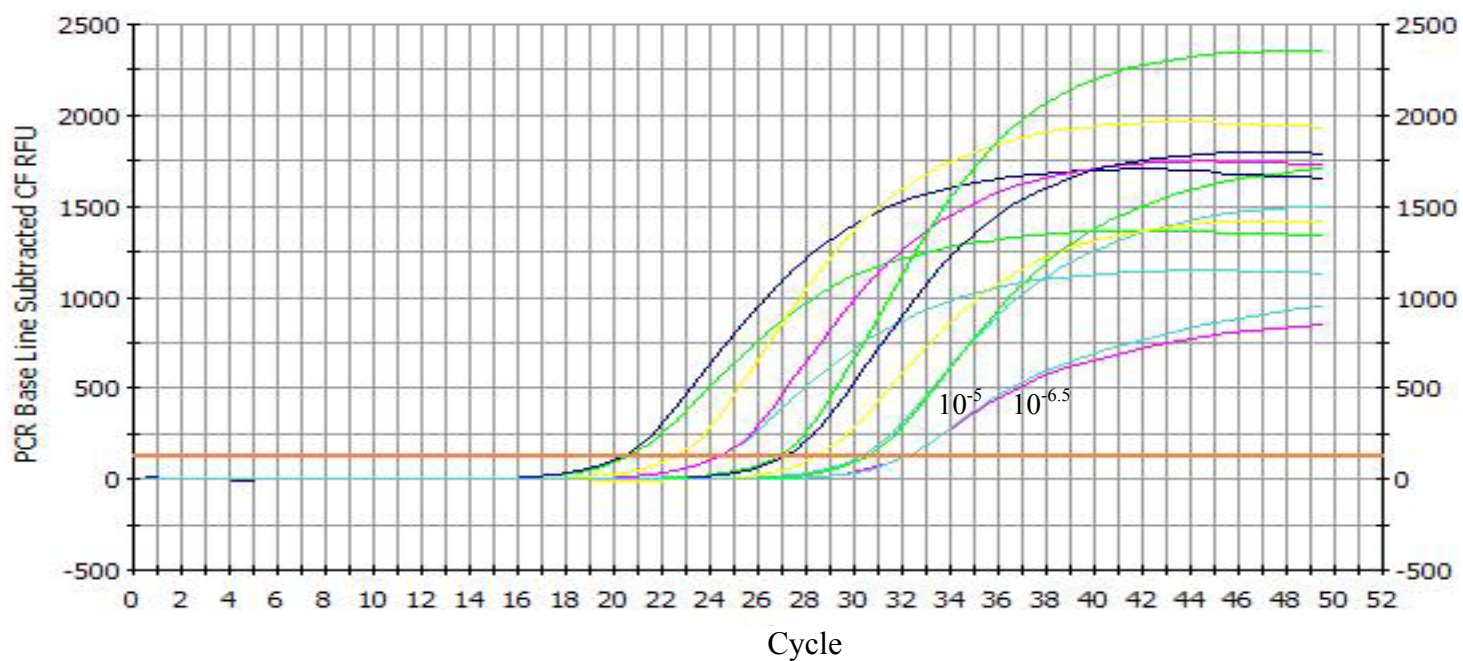


Figure 3.17: C_t curves for *gltJ* dilutions

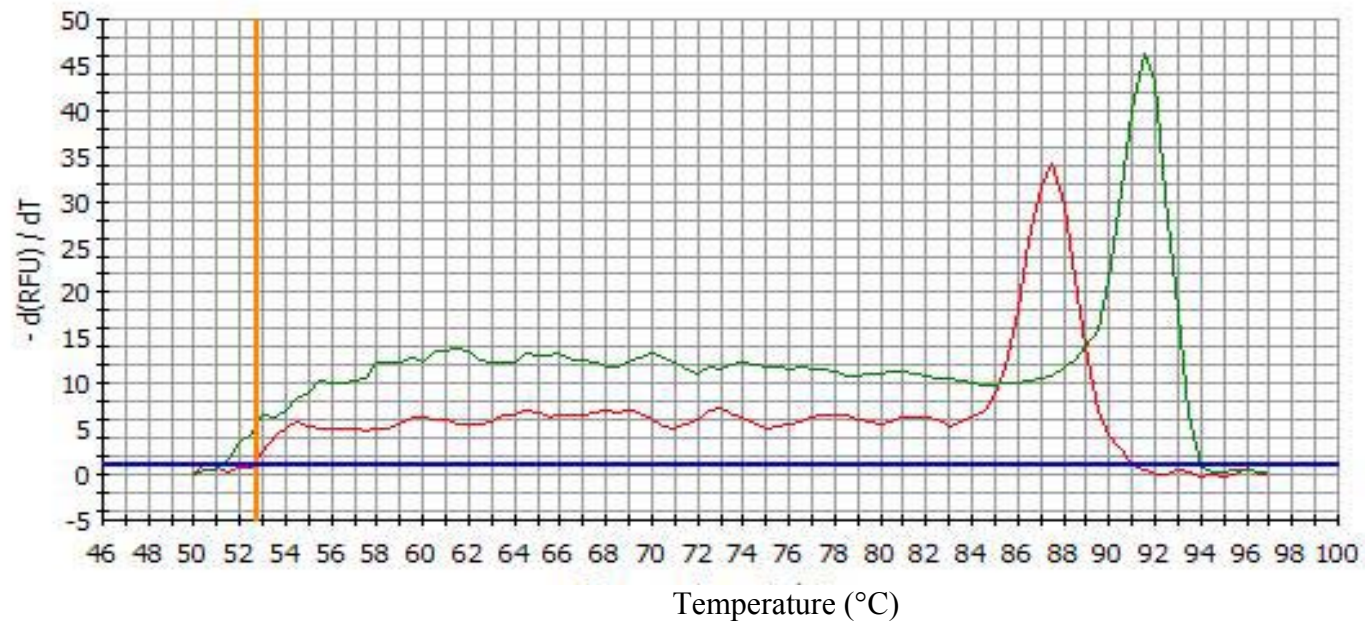


Figure 3.18: T_m curves for *gltJ*

*Red: T_m for *hemX*, 88°C; Green: T_m for *gltJ*, 92°C

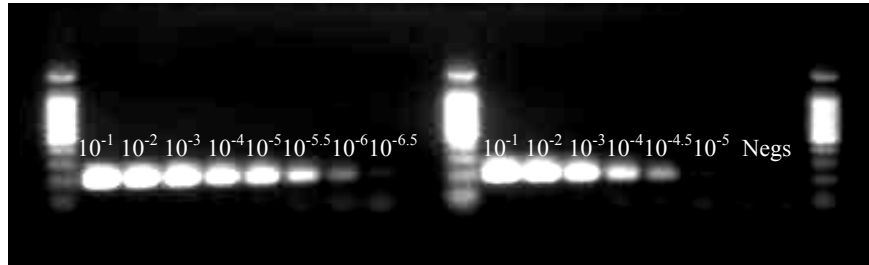


Figure 3.19: Gel of *gltJ* dilutions

*Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lane 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: Control 10^{-5} ; Lane 7: Control $10^{-5.5}$; Lane 8: Control 10^{-6} ; Lane 9: Control $10^{-6.5}$; Lane 10: empty; Lane 11: 100bp DNA marker; Lane 12: Refrig 10^{-1} ; Lane 13: Refrig 10^{-2} ; Lane 14: Refrig 10^{-3} ; Lane 15: Refrig 10^{-4} ; Lane 16: Refrig $10^{-4.5}$; Lane 17: Refrig 10^{-5} ; Lane 18 and 19: negatives; Lane 20: 100bp DNA marker.

CHAPTER IV

COMPARISON OF GENE EXPRESSION IN *ESCHERICHIA COLI* O157:H7 EDL

933 UNDER ACIDIC ENVIRONMENTAL CONDITIONS USING HIGH-

DENSITY OGLIGONUCLEOTIDE MICROARRAYS

Abstract

The purpose of this study was to use microarrays to study gene expression of *E. coli* O157:H7 under acidic conditions commonly associated with apple cider and apple cider processing, to determine its mechanisms of survival and adaptation, and to determine the role of gene expression. *E. coli* O157:H7 EDL 933 was grown to 7 log CFU/mL in Luria-Bertani broth as follows: control (pH 7; 37°C); acid shocked (pH 7 for 6 hr at 37°C, then pH reduced to 3.5 for up to 60 min); and acid adapted (pH 5 for 12 hr at 37°C, then pH reduced to 3.5 for up to 60 min). Total RNA was extracted using an optimized extraction method. RNA was converted to DNA (cDNA), labeled, and hybridized onto Affymetrix GeneChip® *E. coli* Genome 2.0 Array slides. Results from the microarrays were analyzed using GC-RMA, ANOVA and a t-test to compare treatments, significance set at $p < 0.01$ and a cut-off of 2-fold difference in gene expression. The results from the microarray analysis for acid shocked cells indicated that genes related to iron, electron transport, and metabolism were significantly up- or down-regulated ($p \leq 0.01$). Genes involved in DNA binding, biosynthesis, iron, transport and cellular metabolism were significantly up-or down-regulated ($p \leq 0.01$) in acid adapted cells. Genes related to iron uptake and transport (*cyo*, *ent*, *fhu*, *fep*, and *feo* operons) were very important under acid shocked conditions, all positively expressed. For acid adapted conditions, expression of the cyclopropane fatty acid biosynthesis gene, *cfa*, and genes involved in the glutamate decarboxylase acid resistant systems was significantly up-regulated.

Introduction

Escherichia coli O157:H7 is one of the most widely studied and publicized foodborne pathogens. This organism causes an estimated 73,000 illnesses and 63 deaths per year in the United States (CDC, 2005). Although most of the general public associates *E. coli* O157:H7 with undercooked or contaminated ground beef, other foods implicated in outbreaks include milk, yogurt, dry-fermented sausage, alfalfa sprouts, lettuce, spinach, other produce items, and unpasteurized apple cider (Cody, 1999; Cheville, 1996). Apple cider is acidic (pH ~ 3.5) and is typically processed and held at 4°C. Because of these characteristics (acidity and refrigeration), apple cider was originally thought to be an unlikely vehicle for *E. coli* O157:H7 transmission. Since the first outbreak associated with apple cider in 1991, there have been six outbreaks associated with unpasteurized apple cider, one of which resulted in the death of a 16-month-old girl (Janisiewicz, 1999). Apples are thought to become contaminated by cattle feces (Boyce, 1995) and harvested from the ground instead of from the trees (FDA, 1999). Although the FDA recommends consumption of pasteurized cider, some consumers still prefer the unpasteurized product because of its flavor.

The ability of *E. coli* O157:H7 to survive under acidic conditions is a concern for both food processors and clinicians, since organic acids are commonly used in food to inhibit its growth and survival. Acid is also one of the first barriers employed by the body to defend against *E. coli* O157:H7 infections (Hill, 1995). Organic acids are commonly found in some foods, such as apple cider, they may be added as preservatives in other foods and/or they may be present as a result of microbial fermentation (Hill, 1995). Most pathogenic bacteria cease to grow at pH < 4.5, but some can survive at even lower pH

(Hill, 1995). Acid adaptation is the process where cells are grown in a slightly acidic environment (pH 4.5-6.0) (cells allowed to adapt and/or grow under mildly acidic environments), acid tolerance is the ability of cells to survive at a very low pH, typically 2.5 and acid shock is the induction of cells into and acid environments pH<4.0 from a neutral pH environment.

The physiological changes involved in acid response are not completely understood, but this adaptive phenomenon can aid in the survival of pathogenic bacteria in acidic foods. The tactics identified by *E. coli* and *Salmonella* to combat the biological effects of acid stress include: inducing a system to neutralize internal pH and changes in protein abundance, location, and state in the cytoplasm and periplasm (Lopez-Campistrous et al., 2005). *E. coli* and *Salmonella* can grow over a pH range of 5 to 8.5, but if allowed to adapt to acidic environments first, cells can survive at pH as low as 2.5 (Foster, 2000). Enteric organisms attempt to maintain an internal pH between 7.6 and 7.8, but can survive with an internal pH as low as 5.0 (Doyle et al., 2006). In order to maintain a stable internal pH, gradient pumps are used to move protons into the cell at alkaline pH or exclude them at acidic pH. A shift to an acidic environment activates the K⁺/proton antiporter, resulting in alkalinization of the cytoplasm; a shift to an alkaline environment results in acidification of the cytoplasm (Brey et al., 1979).

Three stationary phase acid resistance systems have been identified in *E. coli*: glucose repressed system, glutamate decarboxylase system, and arginine decarboxylase system. The glucose repressed system, also known as the oxidative system, is present in *E. coli* and *Shigella flexneri* (Storz, 2000). The system is capable of protecting the cells at pH as low as 2.5 in minimal media, but requires activation by brief exposure to glutamate

prior to lowering pH (Castanie-Cornet, 1999). As the name indicates, the system is repressed by glucose metabolism, but is dependent on two regulators, the *rpoS* and *Crp*, a cyclic AMP receptor protein (Castanie-Cornet, 2001; Storz, 2000, Castanie-Cornet, 1999).

The glutamate decarboxylase system (GAD), also present in *E. coli* and *Shigella*, relies on external sources of glutamate for protection during acidic shock challenge at pH 2.0 in minimal media (Castanie-Cornet, 1999; Storz, 2000; Masuda and Church, 2003). The GAD system is composed of three genes: *gadA*, *gadB*, and *gadC*. Genes *gadA* and *gadB* encode isoenzymes of glutamate decarboxylase which catalyses the conversion of glutamate to γ -amino butyric acid (GABA); *gadC*, located downstream from *gadB*, encodes a glutamate- γ -amino butyric acid antiporter. This system provides acid resistance to the cell by the consumption of intracellular protons that leak across the cell membrane during extreme acid conditions. After consumption of the proton, *gadC* transports the decarboxylated product out of the cell, thereby maintaining a near-neutral intracellular pH (Small and Waterman, 1998; Storz, 2000).

The third acid resistance system in *E. coli*, and not present in *Shigella*, is the arginine decarboxylase system (Storz, 2000). This system, like its glutamate counterpart, also requires an amino acid, in this case arginine. The arginine decarboxylase system converts intracellular arginine to agmatine, consuming a proton in the process (Storz, 2000). The decarboxylase enzyme is encoded by *adiA* and the antiporter for this system is not known (Stim and Bennett, 1993; Storz, 2000). *adiA* is expressed under anaerobic growth conditions in complex media at a low pH and is thought to be regulated by *CysB* or *adiY*. *CysB* is not known to be involved in sensing pH, but *adiY* is homologously

similar to the regulatory *AraC* (the activator protein of the L-arabinose operon *araBAD*) class of genes.

The glutamate (GAD) and arginine decarboxylase systems are capable of providing protection to *E. coli* at acidic pH, each having an optimum pH, with the GAD system capable of handling the most acidic pH (Storz, 2000). Scientists have only begun to scratch the surface of the genetic involvement of bacterial acid resistance, an extremely complex phenomenon. In addition to the above described and identified acid tolerance systems, it has been hypothesized that *E. coli* O157:H7 also expresses its virulence genes in acid environments.

While research has primarily focused on the genes involved in the three identified acid resistance systems, further studies using microarrays will allow for the identification and elucidation of genes that play a key role in survival, virulence and pathogenesis. Advancements have been made in identifying some of the genes and proteins involved, but there is still a great deal of unanswered questions about additional genes, proteins and mechanisms involved in the regulation of pH, particularly for *E. coli* O157:H7. With advances in molecular technology, these aspects of communication between microorganisms and their environment, mechanisms of survival and the expression of virulence genes can be investigated. It is known that *E. coli* O157:H7 can survive and grow under acidic conditions; however adaptation to acid by *E. coli* O157:H7 can significantly enhance its survival in acidic foods (Conner, 1995; Leyer, 1995). The study of gene expression under acidic and cold environmental conditions, such as those employed during food processing and storage, will aid in the elucidation of mechanisms and biochemical pathways utilized by this pathogen during growth and survival under

these unfavorable conditions. This in turn will allow for better effective inactivation and control strategies to be implemented.

E. coli is a widely studied organism, yet many facets of this bacterium are not fully understood (Donnenberg, 2002). The availability of the complete genome sequence of non-pathogenic *E. coli* K-12 in 1997 and the complete genome of *E. coli* O157:H7 in 2001 have provided a better understanding of the differences between non-pathogenic and pathogenic serotypes (Blattner, 1997; Donnenberg, 2002; Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001). One of the major differences between *E. coli* O157:H7 and K-12 is the size of the *E. coli* O157:H7 chromosome, being 5.4 Mb in size and 859 Kb larger than K-12 (Hayashi et al., 2001a; Hayashi et al., 2001b; Perna et al., 2001). Both serotypes have a 4.1 Mb conserved region serving as the backbone of the *E. coli* chromosome. An approximately 1.4 Mb sequence is specific to *E. coli* O157:H7. The *E. coli* O157:H7 chromosome encodes about 1632 proteins that are not present in the K-12 serotype, and at least 131 of these proteins presumably have virulence-related functions (Hayashi et al., 2001a; Hayashi et al., 2001b). Most of the genomic research that has been done with *E. coli* has focused around the K-12 serotype. However, with the availability of the genome sequence of *E. coli* O157:H7, new avenues of research can be pursued to gain new insights and better understand the physiology of this pathogenic serotype.

Functional genomics is one of the most effective approaches for increasing knowledge at the molecular level of metabolic and adaptive mechanisms in whole cells (Kuipers, 1999). Genomics of foodborne pathogens can enable the identification of biochemical and enzymatic changes involved in stress response. In turn, insight into the

development of novel preservation and pathogen control strategies can be gained. The metabolic pathways that contribute to growth and survival of pathogens in food processing environments, as well as, virulent genes expressed under these conditions can be studied and elucidated using microarrays (Abee et al., 2004).

Microarrays, a novel technology widely used in clinical settings, have recently been used to study foodborne pathogens. Microarrays have the capability of measuring thousands of genes simultaneously making the study of genes and gene functions on the genomic scale possible (Harrington, 2000). This highly sensitive technology provides a direct correlation between the function of a gene and its expression level, measures gene expression in a cell to determine its biochemical composition, helps determine operation of regulatory systems, and deduces the cell composition and its capabilities (Brown, 1999).

The technology behind microarrays, DNA blotting, has been used for the last 25 years (Lander, 1999; Stoughton, 2005). DNA blots were first used by Ed Southern, who labeled nucleic acid sequences, then hybridized them onto a solid surface for detection (Schulze, 2001; Lucchini et al., 2001; Lander 1999). The idea of depositing multiple nucleic acid sequences, representing different genes, onto a solid surface soon followed and with the use of robotics, microarrays were developed (Lucchini, 2001). A microarray is comprised of a large number of highly ordered immobilized nucleic acid sequences, each corresponding to a different gene, attached to a small solid surface the about the size of a microscope slide (Stark, 2003). The arrays are hybridized to labeled complementary DNA (cDNA), which is prepared from target messenger RNA, and then detected (e.g., by laser scanning for fluorescently labeled cDNA).

Microarrays are utilized in many areas of scientific research, including food microbiology, where they are currently being used for expression profiling, pathogen detection and characterization. Gene expression profiling is the most common use of DNA microarrays (Sasseti, 2002). The rationale behind gene expression profiling is that bacteria tightly regulate their genes, expressing them only when the gene product is required. Microarrays have extraordinary potential for use in food safety because of their ability to discriminate between multiple isolates and their ability to identify the presence or absence of genes. They also are used for detection and characterization of foodborne microorganisms (Kostrzynska, 2006).

A noteworthy property of *E. coli* O157:H7 is its ability to survive in acidic and cold environments. During processing of foods, this pathogen often is exposed to acidic and refrigeration environments, developing adaptive responses to the environment. With the entire genome of *E. coli* O157:H7 sequenced, opportunities to study genes under specific environmental conditions are now available.

This purpose of this study was to use microarrays to study gene expression of *E. coli* O157:H7 exposed to acidic conditions commonly associated with food processing and storage and to determine the mechanisms of adaptation and survival of this pathogen. This will allow for the development of appropriate novel control and inactivation strategies (chemical or physical) for better control of *E. coli* O157:H7 in foods.

Materials and Methods

Cell Cultures. *E. coli* O157:H7 EDL 933 was used for this study and was obtained from the National Food Safety and Toxicology Center at Michigan State University. *E. coli* O157:H7 EDL 933 was cultured at 37°C in TSB (pH 7.0) for 24 hr, transferred into Luria-Bertani broth (LB, pH 7.0) cultured for 24 hr at 37°C, and transferred a minimum of three successive times at 24-hr intervals before use. After transfers, 5 mL (3 log CFU/mL) was transferred into 45 mL of pH 5.0 LB (for acid adapted) and pH 7.0 LB (for acid shock and control); pH adjusted were made with 3.4 M malic acid (determined from King, 2005). Cultures were incubated at 37°C as follows: control, 6.5 hr; acid shock, 6.5 hr; and acid adapted, 12.5 hr. These incubations were used to achieve cell populations, for each treatment, of 7-log CFU/mL. Acid adapted and acid shocked cultures were centrifuged at 5,000 x *g* for 10 min. The supernatant was removed, and cells were resuspended in 50 mL of pH 3.5 LB for 60 min. This process was replicated three times.

RNA Extraction. Total RNA was extracted using an optimized Qiagen RNeasy mini kit (Qiagen, Valencia, CA) extraction method. From each of the three replicates and four time points (0, 5, 15, and 60 min), three 600 µl samples were removed, transferred to sterile, RNA/DNA-free collection tubes containing 1200 µl of bacterial stabilizing reagent, vortexed, incubated for 5 min at 25°C, and centrifuged at 14,000 x *g* for 10 min at 4°C. Cell pellets were resuspended in 100 µl of TE buffer (10 mM Tris and 1 mM EDTA) (Promega) containing 3.0 mg/µL lysozyme and incubated for 10 min. After incubation, 350 µL of RTL buffer (Qiagen) containing guanidine thiocyanate, (GDTA,

provided with kit) was added and the entire volume was passed through a cell shredder (Qias shredder) by centrifugation at $11,000 \times g$ for 2 min. The remainder of the protocol was followed without adaptation from the manufacturer's recommendations. 250 μL of ethanol was added to the lysate and mixed by pipetting. The entire sample was transferred to the RNeasy mini column and centrifuge at $> 8,000 \times g$ for 15 sec. Flow through was discarded, 700 μL of buffer RW1 (Qiagen, provided with kit) was added to the column and centrifuged at $> 8,000 \times g$ for 15 sec. Flow through was discarded 500 μL of buffer RPE (Qiagen, provided with kit) was added to the column, centrifuged at $> 8,000 \times g$ for 15 sec and flow through was discarded. Another 500 μL of buffer RPE was added to the column, centrifuged at $> 8,000 \times g$ for 2 min and flow through was discarded. Samples were centrifuged at $> 8,000 \times g$ for 2 min prior to adding 30 μL of RNase-free water (repeated twice) for a final elute volume of $\sim 60 \mu\text{L}$.

Absorbance Measurements. The three extracted RNA samples (for each time point) from each of the replicates were combined, providing three pooled replicates, and measured spectrophotometrically. Absorbency readings (230, 260, and 280 nm) and ratios (260/230 and 260/280 nm) were measured using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) was also used to analyze the quality of RNA prior to microarray analysis.

Microarrays. Affymetrix GeneChip[®] *E. coli* Genome 2.0 Array containing approximately 20,000 genes for four *E. coli* strains (K-12, O157:H7 ELD 933, O157:H7

Sakai, and uropathogenic CFT073) was used. RNA from each of the three control, acid shocked, and acid adapted replicates was converted to cDNA, hybridized, washed, stained, and scanned according to manufacturer's protocol.

cDNA Synthesis and Labeling. For cDNA synthesis, total RNA (10 µg) from each of the 9 samples (control, acid shocked, acid adapted, in triplicate) was reverse transcribed using the SuperScript II system for first strand cDNA synthesis from Life Technologies (Rockville, MD). For each reaction, 10 µL of 75 ng/µL random primers were mixed with the RNA samples and heated to 70°C for 10 min. After cooling to 25°C for 10 min, the reaction buffer was added according to the manufacturer's recommendations. Samples were then incubated at 25°C for 10 min, 37°C for 60 min, 42°C for 10 min; 200 U/µL SuperScript II was added per sample to the reaction, and samples were incubated for an additional 50 min at 42°C. SuperScript II was heat inactivated at 70°C for 10 min, and the mixture was cooled to 4°C. RNA was removed using 20 µL of 1N NaOH, incubation at 65°C for 30 min, and 20 µL of 1N HCl. cDNA was purified with the MinElute PCR Purification Kit (Qiagen) according to manufacturer's protocol. The cDNA was quantified based on absorption at 260 nm and then fragmented using a partial DNase I digest. For up to 5 µg isolated cDNA, 0.6 U/µg DNase I (Amersham Biosciences, Piscataway, NJ) was added and incubated for 10 min at 37°C followed by inactivation of DNase I at 98°C for 10 min. The fragmented cDNA was 3'-end –labeled for 60 min at 37°C using GeneChip DNA Labeling Reagent (Affymetrix, Santa Clara, CA) and terminal deoxynucleotidyl transferase (Promega, Madison, WI). The reaction was stopped with the addition of 0.5 M EDTA. The

fragmented and 3'-end-labeled cDNA was added to the hybridization solution without further purification.

Array Hybridization, Washing and Scanning. The hybridization solution (pH 6.6; referred to hereafter as 1x MES) contained 100 mM MES (Sigma Aldrich, St. Louis, MO), 1 M NaCl, 20 mM EDTA, and 0.01% Tween 20. In addition, the solution contained 10 mg/mL herring sperm DNA (Promega), 50 mg/mL bovine serum albumin (BSA) and 3 nM control Oligo B2 (Affymetrix). The 55 μ L of solution was combined with the fragmented and 3'-end-labeled cDNA sample. Sample solution mixes were pipetted into the array cartridge. Hybridization was carried out at 45°C for 16 hr with mixing on a rotary mixer at 60 rpm. Following hybridization, the sample solution was removed and the array was washed and stained as recommended in the technical manual (Affymetrix, Inc). To enhance the signals, 1 mg/mL streptavidin phycoerythrin and 50 mg/mL BSA in 2x MES was used as the first staining solution. After removal of the streptavidin solution, an antibody mix containing 10 mg/mL goat IgG, 0.5 mg/mL biotin bound anti-streptavidin antibody, and 50 mg/mL BSA in 2x MES was added as the second stain. Nucleic acid was fluorescently labeled by incubation with 10 μ g/mL streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA) and 2 mg/mL BSA in 1x MES, third stain solution. The arrays were read at 570 nm with a resolution of 3 μ m using a confocal laser scanner (Affymetrix).

Statistical Analysis. After scanning, the image was visually inspected for abnormalities and artifacts. A quality report was generated containing the scale factor,

background, percentage of genes identified as present, and the internal/external controls. After the initial image quality check, data were generated using the GeneChip[®] Operating System (GCOS) (Affymetrix) which produced the following files: DAT, original scanned image; CEL, intensity view of the GeneChip; and CHP, data extracted from analyzing the CEL file. These files were then imported into Iobion's Array Assist (Agilent Technologies-Stratagene) which allows for analysis of the Affymetrix GeneChip[®] data using the algorithms GC-RMA. This software was used to determine potential outliers, then to perform statistical analysis. The three replicates were averaged and an analysis of variance ($p < 0.01$) was performed followed by a t-test to compare individual treatments (e.g. Control vs. Acid adapted; Control vs. Acid shock; Acid adapted vs. Acid shock). The Bonferroni correction factor was used due to multiple comparisons being made and each gene was considered an independent statistical event.

Microarray Confirmation. After data analysis, a housekeeping gene was selected that was expressed at a similar level across all treatments (statistically similar). Four genes that were significantly expressed ($p \leq 0.01$) and up- or down- regulated at ≥ 50 -fold (Tables 4.1) were selected, confirmed and validated by a real-time reverse transcriptase polymerase chain reaction (rt-RT-PCR) assay using an iCycler real-time PCR machine (BioRad, Hercules, CA) with a Superscript III Platinum Sybr green One-step qRT-PCR kit (Invitrogen, Santa Clara, CA). Primers for each gene were designed using Beacon Designer 4 software, and thorough BLAST searches were conducted to avoid non-specific binding or cross-reactivity. The rt-RT-PCR assay for each primer pair was then optimized (Tables 4.2-4.5). A concentration of 250 ng/ μ L of RNA from each treatment

was used for rt-RT-PCR analysis. Gene expression was validated by rt-RT-PCR using a 10-fold dilution series to determine differences in expression. rt-RT-PCR products were evaluated by rt- RT-PCR threshold cycle (C_t) values and by gel electrophoresis, and specificity was determined using melt curve analysis and melt temperatures (T_m). The dilution series were performed in triplicate and used to confirm differences in expression level determined by microarray analysis. The C_t values were reported as means of three replicates.

Results and Discussion

Acid Adapted Microarrays. Results from the acid adapted microarrays revealed that a total of 949 genes were significantly expressed ($p < 0.01$) at a cut-off of ± 2 -fold or greater. Of these, 497 genes were positively expressed (up-regulated), i.e., expressed at a higher level in acid adapted cells than in control cells; 452 genes were negatively expressed (negatively expressed), i.e., expressed at lower levels in acid adapted cells as compared with control cells (Figure 4.1).

Genes were clustered according to biological function using DAVID Bioinformatics (Dennis et al., 2003; Douglas et al., 2003). Of the 949 genes that were significantly ($p \leq 0.01$) expressed, those having a biological function related to cellular biosynthesis were most significant, with an enrichment score > 10 . Enrichment score is an indicator of the involvement of a gene group relative to the total genes in the study; the higher the enrichment score, the more important/involved the genes are in the study. Of the 130 genes involved in biosynthesis ($p \leq 0.01$, ≤ -2 -fold or ≥ 2 -fold change in expression), 25 genes were up-regulated and the remaining 105 were down-regulated

(<http://web.utk.edu/~dgolden/microarrays/aa-biosynth.pdf>). The most significantly down-regulated genes were those involved in purine or pyrimidine biosynthesis. Purines and pyrimidines make up the two groups of nitrogenous bases of nucleic acids, specifically deoxyribonucleotides and ribonucleotides, which serve as the foundation for the genetic code (Snyder and Champness, 2003). This level of down-regulation of purines indicates that new DNA or RNA was not being synthesized or synthesized at a greatly reduced rate.

Also identified in the biosynthesis cluster was the *cfa* gene, up-regulated 29.05-fold. *Cfa* functions to synthesize cyclopropane fatty acids. Up-regulation of *cfa* after exposure to acid indicates that the fatty-acid composition of the cell membrane was affected under acid adapted conditions. Cyclopropane fatty acid formation increases in many bacteria as they enter the stationary phase of growth. Because of the role of cyclopropane fatty acids in acid resistance, exponential phase cells are more sensitive to acid than stationary phase cells (Chang and Cronan, 1999). Chang and Cronan (1999) reported that exposure of *E. coli* to moderately acidic conditions resulted in a substantial increase in *cfa* transcription.

In *Clostridium acetobutylicum*, a decrease in the ratio of unsaturated to saturated fatty acids coupled with an increase in the amount of cyclopropane fatty acids was observed in low pH environments (LePage et al., 1987). This change in fatty acid composition decreases the fluidity of the membranes, thereby protecting the cell from low external pH. When grown at pH 5, *Yersinia enterocolitica* increased the proportions of cyclopropane and saturated fatty acids and decreased the level of unsaturated fatty acids compared to cells grown at pH 9 (Bodnaruk and Golden, 1996). Brown et al. (1997)

found a similar effect with *E. coli*. The membrane composition of acid-adapted *E. coli* had more cyclopropane fatty acids present compared to cells not exposed to acidic conditions (Brown et al., 1997). Brown et al. (1997) also found that during acid habituation of *E. coli*, a proportion of the mono-unsaturated fatty acids were either converted to cyclopropane fatty acids or replaced by saturated fatty acids, and that *E. coli* cells with high levels of acid tolerance had high levels of cyclopropane fatty acids compared with strains of low tolerance (Brown et al., 1997).

In addition to genes involved in cellular biosynthesis, genes involved in cellular metabolism (enrichment score, 5.25) were also differentially expressed (<http://web.utk.edu/~dgolden/microarrays/aa-metab.pdf>). Results revealed that 120 genes involved in metabolism were significantly up- or down regulated. Among this cluster of genes, *gadA*, *gadB*, *gadX*, *xasA*, *adiA*, and *adiY* genes were up-regulated; these genes are involved in two different acid responses systems. The *gadAB* genes are involved in the glutamate acid resistance system, and the *adiA* gene is involved in the arginine acid resistance system. Both of these acid resistance systems are decarboxylase acid resistance systems. Gale and Epps (1942) found relatively high activities for arginine, ornithine, lysine, and histidine decarboxylases (glutamate decarboxylase was not identified until the early 1990's) in cultures grown in acidic media, whereas amino acid deaminases were high in alkaline media. Therefore, decarboxylases and deaminases neutralize the medium under acid and alkaline conditions, respectively (Gale and Epps, 1942).

These amino-acid dependent, acid resistance systems are composed of dedicated pairs of amino acid decarboxylases and antiporters. The decarboxylase/antiporter-dependent acid resistant systems have relatively simple modes of action involving three

genes for both systems. The glutamate system utilizes *gadA*, *gadB*, and *gadC*, but is subjected to multiple levels of control. To date, 11 regulator proteins are known to affect the induction of the glutamate system (Ma et al., 2003; Castanie-Cornet et al., 2001). The central activator of the GAD system is *gadE*, of the LuxR-family, formerly known as *yhiE*. *gadE* binds to a 20 bp sequence called the *gad* box located 63 bp upstream from the transcriptional start sites of *gadA* and *gadBC* (Ma et al., 2003; Castanie-Cornet et al., 2001). The *gad* box and the *gadE* activator are essential for expression of both *gadA* and *gadBC*, and therefore acid resistance. The role of the other 10 regulators varies with growth phase and media composition. At pH 5.5 and during exponential growth, the *gadA/BC* genes can be expressed; otherwise, the *gadA/BC* genes are not expressed until the culture reaches the stationary phase of growth (Masuda and Church, 2003; Ma et al., 2004). Regardless of when the genes are activated, once induced, the system provides acid resistance at pH as low as 2.5 (Masuda and Church, 2003; Ma et al., 2004). The first *gadE* activation circuit includes the membrane-bound sensor, kinase *EvgS*, the response regulator, *EvgA*, and an *AraC*-like regulator, *YdeO* (Masuda and Church, 2003; Ma et al., 2004). *YdeO* activates transcription of *gadE*, and *EvgAS* can also activate *gadE* transcription and repress transcription of *ydeO* (Masuda and Church, 2003; Ma et al., 2004). All three of these regulators were negatively expressed in our study. These regulators bind to DNA sequences upstream of *gadE*, and all three were found to not be involved in *gad* regulation during stationary phase (Masuda and Church, 2003).

A second activation circuit includes *CRP*, a cyclic-AMP receptor protein, *rpoS*, and two *araC*-like regulators, *gadX* and *gadW*. This activation circuit is what appears to have occurred in our study. Our data indicate that *crp* and *gadX* were involved in

expression of the *gadA/BC* complex due to their up-regulation, (2.35- and 4.03-fold increase, respectively). The two *araC* regulators, *gadX* and *gadW*, are located downstream of *gadA* and are transcribed by an independent promoter *rpoS*, σ^{38} (Ma et al., 2002). *gadX* and *gadW* directly activate transcription of *gadE* when cells are grown in LB under mildly acidic conditions, and therefore, trigger *gadA/BC* induction. *GadX* and *gadW* can co-active the *gadA/BC* complex, and these two activators also act separately in the regulation of the GAD system. *GadX* can repress *gadW* but still activate *gadA/BC*; *gadW* can repress *rpoS*, σ^{38} , preventing activation of *gadX* (DeBiase et al., 1999; Ma et al., 2002). The tightly controlled regulation of *gadX* and *gadW* is most apparent during exponential growth in complex media under mildly acidic conditions (Ma et al., 2002). Cyclic AMP and *crp* have a very predominate role in the regulation of the *gad* complex. Growth under acidic conditions reduces the concentration of cAMP in the cell, which initiates a cascade that increases expression of *rpoS*; in turn, σ^{38} increases the synthesis of *gadX* (Lange and Hengge-Aronis, 1994; Ma et al., 2003). This increase in *gadX* stimulates transcription of *gadE* and the decarboxylase genes and down-regulates *gadW* (Lange and Hengge-Aronis, 1994; Ma et al., 2003). Also identified as being up-regulated in the metabolism cluster were the *adiA* (129.92-fold increase) and *adiY* (10.95-fold increase) genes. The *adiA* and *adiY* genes are part of the arginine dependent decarboxylase acid resistance system. This system has not been as thoroughly researched as the *gad* system but is probably equally as complex. The *adiA* gene is located upstream of *adiC*, and these two genes are separated by the *adiY* gene (Stim-Herndon et al., 1996). Over-expression of *adiY* has been shown to activate *adiA* and *adiC*, but mutations of *adiY*

do not appear to affect *adiA* or *adiC*. The role of *adiY* in response to environmental changes has not been identified.

Tramonti et al. (2002) identified *yhiM*, *yhiN*, *ybaS*, *yhiF*, *slp*, *hdeD*, *hdeA*, and *hdeB* as *gadX* dependent, acid inducible genes during log phase growth. Six of these genes were also up- or down-regulated in our study (*yhiM*, +103.74; *yhiN*, -5.67; *ybaS*, +13.90; *slp*, +7.01; *hdeA*, +2.03; and *hdeD*, +2.43). Tramonti et al. (2002) further identified that the *gadE* and *hdeABD* operons were not present in *Salmonella* and hypothesized that these genes may have been acquired through evolution and represent an acid-fitness island in *E. coli* (Tramonti et al., 2002).

The positive expression of genes related to the GAD acid resistance system in our study indicates that this system was involved in acid resistance of our acid adapted *E. coli* O157:H7 cells. Since these cells were grown under mildly acidic conditions (pH 5.5), there was likely a reduced concentration of cyclic AMP initiating the expression of *rpoS* and *gadX*, which stimulated transcription of *gadE* and *gadA/BC*.

In this study, 164 genes related to membrane function (enrichment score, 3.74) were significantly up- or down-regulated (± 2 -fold) (<http://web.utk.edu/~dgolden/microarrays/aa-memb.pdf>). *xasA* was identified among this cluster because it is a trans-membrane amino acid antiporter thought to be involved in the glutamate decarboxylase system (Hersh et al., 1996). Hersh et al., (1996) designated *xasA* for extreme acid sensitive gene, possible a regulator gene which senses acidic environments. In this study, a *xasA* mutant strain grown at pH 7 to 8 showed 100-fold poorer survival in acid than the parent strain containing *xasA*. In *Shigella flexneri*, *xasA* was found to be a homolog of *gadC*, in which *xasA* appears to encode a permease for the

decarboxylated product of *gadB* (Hersh et al., 1996). These results suggest that *xasA* participates in a glutamate decarboxylase alkalization cycle to protect *E. coli* from cytoplasmic acidification (Hersh et al., 1996).

E. coli has two primary pathways for glutamate synthesis. The glutamate synthase pathway is essential for synthesis of glutamate at low ammonium concentrations and for regulation of the glutamine pool (Helling, 1994). The glutamate dehydrogenase pathway (GDH) catalyzes the reduction of 2-oxoglutarate to glutamate (Helling, 1994). The glutamate synthase pathway fixes ammonia into organic molecules, such as glutamine, which can then be made into glutamate, and this pathway is active when the ammonia concentration is too high (Helling, 1994). In *E. coli*, there are four protein transporters responsible for the uptake and transport of glutamate (GltIJKL, GltP, GltS, and GadC). Of these, GltIJKL accounts for approximately 15% of the total transport velocity of glutamate (Schellenberg et al., 1977). The GltIJKL glutamate/aspartate transporter is a component of the ATP binding cassette (ABC) transporter superfamily (Linton et al., 1998). Sequence resemblance with known ABC transporter components suggests that GltJK is the membrane components, GltL is the ABC transporter protein, and GltI is the periplasmic-binding protein of the GltIJKL glutamate ABC transporter system (Urbanowski et al., 2000; Linton et al., 1998). Expression of *gltI* was found to be greatly reduced when *E. coli* K-12 strain was grown in rich medium (Kahane et al., 1976). Expression of *gltI* is also reduced when *E. coli* is grown with glucose rather than succinate (Willis et al., 1975). This reduction in *gltI* expression in-turn reduces the expression of *gltJKL*, since *gltI* transcribes the periplasmic binding protein for the *gltJKL* transporter. In our study, *E. coli* O157:H7 was grown in LB broth, which contains

glucose. This could account for the negative regulation of the *gltIJKL* transport system that was observed. The glutamate decarboxylase system (GAD) was utilized in acid adapted cells. As such, the uptake of glutamate may have occurred through the GAD system such that the *gltIJKL* system was not needed, and therefore, down regulated.

In the membrane cluster group, *appC* (+119.56) and *appB* (+243.88) genes were also significantly up-regulated. These genes also appeared with the iron cluster (enrichment score, 2.23). *AppBC* genes are involved in oxygen reductase and iron binding during the growth phase, anaerobiosis, and phosphate starvation (Dassa et al., 1991). The *app* genes have 60% homology with cytochrome genes, *cydA* and *cydB*, which perform the terminal step in the electron transport chain, reducing oxygen to water (Dassa et al., 1991). The *appCB-appA* operon is under the control of the transcriptional activator, *appY* (Atlung et al., 1997). This operon is induced when cells enter the stationary phase of growth and, when carbon or phosphate is limited (Atlung et al., 1997). In our study, induction of *appBC* by *appY* appears to be activated by pH as well. AppY protein is also involved in anaerobic induction of the *hya* operon (also up-regulated in our study), which encodes hydrogenase 1 and is located immediately upstream from the *appCBA* operon (Atlung et al., 1997). AppY protein regulates *appA* synthesis by activating transcription from the *appCp* promoter (Atlung et al., 1997). Genetic analysis of the *appCp* promoter region indicates that upstream sequences are required for its activation. This suggests that this upstream sequence has a specific binding site for AppY initiating transcription of *hya* operon and *appBC* (Atlung et al., 1997).

Of significance in the acid adapted microarray study was the regulation of the genes involved in the glutamate acid resistance system. Although there are 11 regulatory proteins that are capable of inducing this system, the only genes found to be significantly involved under our experimental conditions were *gadX*, *gadA*, *gadB*, *crp*, and two genes from another acid resistance system, the arginine decarboxylase system. Although we were unable to determine if these were being utilized prior to lowering the pH from 5.5 to 3.5, we can conclude that two decarboxylase systems were being utilization by acid adapted *E. coli* O157:H7, with the GAD system predominating. In addition to the decarboxylase systems, expression of the *cfa* gene was observed, indicating that cyclopropane fatty acid synthesis was increased in response to acid challenge.

Acid Shocked Microarrays. Results of the acid shocked microarray study reveal that 461 genes were significantly ($p \leq 0.01$) up- or down-regulated (at least 2.0-fold) (Figure 4.2). Of these, 263 were down-regulated, and 198 were up-regulated (Figure 4.2). Clustering analysis using DAVID bioinformatics (Dennis et al., 2003; Douglas et al., 2003) revealed 114 genes involved in transport (enrichment score 11.9) (<http://web.utk.edu/~dgolden/microarrays/as-transport.pdf>), 109 genes involved in membrane function (enrichment score 2.69) (<http://web.utk.edu/~dgolden/microarrays/as-memb.pdf>), approximately 60 genes involved in each, metabolism (enrichment score 4.85) (<http://web.utk.edu/~dgolden/microarrays/as-metab.pdf>) and biosynthesis (enrichment score 1.90) (<http://web.utk.edu/~dgolden/microarrays/as-biosynth.pdf>) and 55 genes involved in iron function (enrichment score 9.83) (<http://web.utk.edu/~dgolden/microarrays/as-iron.pdf>).

Genes related to iron binding, uptake, and transport (*fhuACBD*, *entCEBA*, *fepDGC*, *feoAB*, *tonB* and *cirA*) and cytochrome O oxidase (*cyoANCDE*) were positively expressed. Genes related to hydrogenase -1, -2, and -3 (*hya*, *hyb* and *hyc* operons, respectively) were negatively expressed in the transport, membrane and iron clusters; *gadB* and *xasA* genes, both of which are involved in the glutamate acid resistance system, were negatively expressed, but two genes related to arginine, one which synthesized arginine (*argS*) and an arginine repressor (*argR*), were positively expressed.

Positively expressed operons that are involved in iron activity were: *fhuACDB*, ferrichrome-iron transport; *entCEBA*, enterobactin biosynthesis; *fepDGC*, ferric enterobactin uptake; *tonB*, energy transducer of iron; and *feoAB*, iron binding (<http://www.cib.nig.ac.jp/dda/taitoh/ecoli.operon.html>). Iron is a necessary mineral for bacteria and is frequently limited in the surrounding environment. Bacteria require several million iron atoms per cell for the production of cytochromes, iron-proteins, and the Fe-S cluster present in redox proteins (Barton, 2005). In our study, *E. coli* O157:H7 was grown under aerobic conditions. Therefore iron uptake will be discussed only as it pertains to aerobic environments. Under aerobic conditions, Fe^{3+} is insoluble in the environment and bacteria produce chelating agents, i.e., siderophores, which sequester and mobilize Fe^{3+} . Siderophores, which are specific for a given bacterial species, have a six-coordinate octahedral complex with Fe^{3+} held in the center of the molecular arrangement (Barton, 2005; Chung et al., 2006). Bacterial siderophores are grouped according to the type of ligand that binds Fe^{3+} , with hydroxamates being the most common (Figure 4.3) (Barton, 2005; Chung et al., 2006). *E. coli*, *Salmonella*, and *Shigella* sp. all utilize hydroxamate siderophores (Chung et al., 2006). The binding affinity

of Fe^{3+} to the siderophore must be greater than the solubility constant for the insoluble iron compounds (Chung et al., 2006). Under iron stress conditions, *E. coli* produces enterobactin, also known as enterochelin, which acquires iron from the environment. The gene clusters responsible for iron transport and activity in *E. coli* include: *fep* genes responsible for ferric enterobactin uptake, *ent* genes, which encode for biosynthesis and *fhu* genes, which encode for transport of Fe^{3+} into cells and regulation of tonB protein required for activation of porins in the outer membrane for transport (Chung et al., 2006). Owing to the high binding affinity for Fe^{3+} , enterobactin acquires iron from the environment (Litwin and Calderwood, 1993). The ferri-enterobactin complex moves through the outer membrane using a porin produced by *fhuA*, *fhuE*, or *fepA* (Figure 4.4) (Barton, 2005; Neidhart, 1996; Litwin and Calderwood, 1993). The energy required for opening the channel comes from the tonB protein, which spans the plasma membrane, periplasm, and the outer membrane (Barton, 2005; Neidhardt, 1996). Once the ferri-enterobactin complex is in the periplasm, it interacts with *fepB* or *fhuD* which are specific binding proteins for ferri-enterobactin (Litwin and Calderwood, 1993). *FepB* and *fhuD* proteins are part of an ABC transporter which delivers ferri-enterobactin to the cytoplasm through the use of dual trans-membrane proteins (Barton, 2005; Neidhardt, 1996). The energy required for this transport is derived from ATP hydrolysis (Neidhardt, 1996). Once in the cytoplasm, ferri-enterobactin is degraded by a specific esterase to release Fe^{3+} (Barton, 2005; Neidhardt, 1996). Enterochelin is degraded to dihydroxybenzoic acid (DHBA) which is released from the cell as an end product (Barton, 2005; Neidhardt, 1996). In *E. coli*, the release of DHBA does not amount to energy depletion since less than a million atoms of iron are required for the growth of a

single cell (Barton, 2005). Fe^{3+} is reduced to Fe^{2+} by NAD(P)H-driven ferric reductase, and this reduced iron form is used for synthesis of cytochromes and proteins with Fe-S centers (Barton, 2005). The tonB ferrichrome transport system, which binds and transports Fe^{3+} into the cells, is regulated by *fur* (Storz, 2000). TonB ferrichrome transport system is also responsible for the transport of heme (Foster and Hall, 1992; Storz, 2000). In *E. coli* *fur* functions as a repressor utilizing Fe^{2+} to bind to specific iron-regulatory genes, resulting in the transcriptional repression of these genes under iron supplied environments (Thompson et al., 2002).

The *feoAB* operon was also positively expressed under acid shocked conditions. The genes of this operon are responsible for the ferrous (Fe^{2+}) iron transport system (Kammler et al., 1993). The operon, like the tonB complex, is activated by *fnr* and regulated by *fur* (Kammler et al., 1993). The transport of Fe^{2+} into the cell is driven by ATP hydrolysis (Kammler et al., 1993). This ferrous transport system is believed to be important during survival of *E. coli* in the intestines. Stojiljkovic et al. (1993) used a mouse model to determine the effectiveness of ferrous iron transport and colonization in the intestine of mice. They found that *feo* mutants were less effective at colonizing mice intestines than *feo*⁺ strains of *E. coli*, suggesting the involvement of this iron transport system for survival and colonization within the intestines (Stojiljkovic et al., 1993).

Positively expressed in every cluster were the cytochrome O oxidase genes of the *cyoABCDE* operon. Under aerobic respiration, *E. coli* contains two terminal oxidases, cytochrome O and cytochrome d (Cotter et al., 1990). Both catalyze the oxidation of ubiquinol-8 and the reduction of oxygen to water (Cotter et al., 1990). The genes related to cytochrome O oxidase (*cyoABCDE*) were positively expressed in acid shocked *E. coli*

O157:H7. The *cyo* operon can be activated by *arcA*, *gadE*, or *CRP* and is repressed by *fnr* or *fur* (Cotter et al., 1990). The enzymes encoded by the *cyo* operon are bound to the membrane. The cytochrome O oxidase enzyme complex contains two protoheme IX groups (b555 and b562) and two copper atoms per group (Cotter et al., 1990). Previous studies have shown that the cytochrome O oxidase complex dominate under oxygen-rich growth conditions, whereas, cytochrome d oxidase enzymes accumulate under oxygen limiting conditions and upon entering the stationary phase (Rice and Hempfling, 1978). Based on these conditions and our data, cytochrome oxidase complexes appear to have role in survival under stress conditions. Cotter et al. (1990) determined how the *cyo* operon was regulated in response to pH. These researchers used media with an adjusted pH ranging from 5.3 to 7.5 and determined that expression of the *cyo* operon increased as pH increased. In our study, expression of the *cyo* operon was low under acidic conditions, confirming the role of acid in *cyo* expression.

Results of our study revealed that genes related to hydrogenase function (*hya*, *hyb*, and *hyc* operons) were negatively expressed and identified in the transport, membrane and iron clusters. The *hya* and *hyb* operons, composed of genes that synthesize uptake hydrogenase enzymes, were down-regulated under acid shock conditions. During anaerobic growth, *E. coli* is capable of synthesizing two uptake hydrogenase isoenzymes, Hyd1 and Hyd2, both of which catalyze the oxidation of hydrogen gas (Sawers and Boxer, 1986). Hyd1 and Hyd2 are alpha-beta-heterodimers comprised of a small subunit and a larger nickel-containing catalytic unit (Sawers and Boxer, 1986). They are localized in the inner membrane of *E. coli* facing the periplasmic space (Rodrigue et al., 1996). The genes encoding Hyd1 small and large subunits are *hyaA* and *hyaB*, respectively; the genes

which encode Hyd2 small and large subunits are *hybA* and *hybC*, respectively (Menon et al., 1994; Menon et al., 1990). The remaining genes in the *hya* and *hyb* operons (*hyaDEF* and *hybFG*) are necessary for post-transcriptional alterations of the emerging structural subunits (Menon et al., 1994; Menon et al., 1991). Induction of *hyb* expression requires the Fnr protein (Sawers et al., 1985), whereas induction of *hya* requires the sigma factor and *rpoS* and the transcription regulators *arcA* and *appY* (Bronsted and Atlung, 1994). King and Przybyla (1999) under anaerobic conditions evaluated expression of *hya* and *hyb* under acidic (pH 5.5) and alkaline (pH 8.5) environments and found: *hya* expression was increased at pH 5.5 in both complex and minimal media. Similarly, *hyb* expression reached maximal levels in alkaline medium and was diminished under acidic conditions (King and Przybyla, 1999). In addition, other researchers have found that H₂ uptake activity of Hyd2 is greater than that of Hyd1 (Atlung et al., 1992; Sawers and Boxer, 1986). The pH optimum for Hyd1 ranges for 6.0 to 8.0, whereas Hyd2 has an optimum at pH 8.0 (Atlung et al., 1992; Sawers and Boxer, 1986). In our study, *E. coli* O157:H7 was incubated for 1 hr at pH 3.5, and all genes involved in the *hya* and *hyb* operons were negatively expressed. The differences seen in our study and that of King and Przybyla (1999) may be attributed to our use of aerobic and greater acidity (pH 3.5). Since synthesis of Hyd1 and Hyd2 occurs during anaerobic conditions, these hydrogenase isoenzymes likely were not synthesized in our study, and therefore were negatively expressed. Furthermore, the *hya* operon was positively expressed in our acid adapted studies, suggesting that expression of *hya* operon is favored under mildly acidic conditions or when cells are pre-adapted to a mildly acid environment before exposure to greater acidity.

A third hydrogenase operon, *hyc*, was also identified as being negatively expressed as a result of acid shock. *E. coli* catalyzes the cleavage of intracellular formate to H₂ and CO₂ this carried out by the enzyme formate hydrogenlyase, FHL (Self, 2001). This complex includes a formate dehydrogenase isoenzyme, FDH-H, encoded by the *fdhF* operon, a hydrogenase isoenzyme, HYD3, encoded by the *hyc* operon, and intermediate electron carriers, also encoded by the *hyc* operon (Self et al., 2001). FHL is produced only under anaerobic environments and in the absence of an alternate terminal electron acceptor, such as nitrate (Self and Shanmugan, 2000). Reduced levels of *hyc* expression result from a reduction of intracellular formate preventing FHL from produce H₂ and CO₂ (Self et al., 2001).

GadB, *evgS*, *evgA*, and *xasA* genes, all part of the glutamate acid resistance system, were negatively expressed under acid shocked conditions. *GadB* in conjunction with *gadA* encode isoenzymes of glutamate decarboxylase which catalyze the conversion of glutamate to γ -amino butyric acid (GABA). *EvgS* is a membrane-bound sensor and *evgA* is the response regulator for *evgS*; both genes activate *gadE* transcription, in-turn activating the glutamate decarboxylase system (Masuda and Church, 2003; Ma et al., 2004). The fact that these genes were negatively expressed in our study indicates that the glutamate decarboxylase acid resistance system was not utilized under acid shock conditions.

Two genes, *argR* and *argS*, both involved in arginine biosynthesis, were positively regulated under acid shocked conditions. *ArgS*, responsible for the synthesis of arginine, is a member of the family of Class I aminoacyl tRNA synthetases, which read the genetic code by covalently linking amino acids to their specific tRNA molecules (Lim

et al., 1989). The reaction is driven by ATP hydrolysis (Tian, 1994). ArgR is the regulator of *argS*; ArgR is a protein that negatively regulates arginine biosynthesis and the *carAB* operon (Lim et al., 1989; Stirling et al., 1988; Tian et al., 1994). The expression of these genes indicates the synthesis of arginine by *argS*, but positive expression of *argR* indicates repression, possibly by feedback inhibition of *argS* as well as all other genes responsible for arginine biosynthesis (*argECBH*).

Acid Adapted vs. Acid Shocked Microarray Study. Gene expression comparisons were made between the acid adapted and acid shocked microarrays. The results revealed 779 genes that were significantly up- or down-regulated ($p < 0.01$; at least 2-fold) (Figure 4.5); 433 genes were negatively expressed (positively regulated/expressed under acid adapted conditions; Figure 4.5), and 346 genes were positively expressed (positively regulated/expressed under acid shocked conditions; Figure 4.5). This study revealed that genes related to iron uptake and transport (*cyo*, *ent*, *fhu*, *fep*, and *feo* operons) were very important under acid shocked conditions, all positively expressed. For acid adapted conditions, expression of the cyclopropane fatty acid biosynthesis gene, *cfa*, and genes involved in the glutamate decarboxylase acid resistant systems was significantly up-regulated.

For our study, acid adapted *E. coli* O157:H7 cells were grown and adapted to a mildly acidic environment, pH 5.5, prior to introducing the cells to a more acidic environment, pH 3.5; acid shocked *E. coli* O157:H7 cells were grown initially at a pH 7.0, then re-suspending in LB broth at pH 3.5. Our results indicate these two conditions induce different gene expression patterns necessary for survival of *E. coli* O157:H7 EDL

933. Acid adapted cells do not require increased protein synthesis, as indicated by the down- regulation of genes involved in purine or pyrimidine biosynthesis. Instead, they rely upon physiologically controlling pH by the glutamate decarboxylase acid resistance system.

Acid shocked cells had a very different gene expression pattern with a large number of genes involved in iron uptake and transport being significantly up-regulated. Iron is essential for growth, and a shift from high iron concentration to low iron concentration would serve as an important regulatory signal to the cell. Iron solubility is a function of pH. Therefore, it is reasonable to hypothesize that cells indirectly sense acid in terms of iron availability (Hall and Foster, 1996). A study by Hall and Foster (1996) showed that iron availability alone will not trigger an acid tolerance response in *Salmonella* Typhimurium, but that the cells must also experience low pH. Because of the insolubility of iron, microorganisms have evolved a number of mechanisms for the acquisition of adequate iron from the environment. These mechanisms are believed to be closely linked to bacterial virulence (Litwin and Calderwood, 1993). In our study, the virulence genes were expressed, but their expression was not significantly affected by acidic conditions (i.e., below the 2-fold cut off). Other researchers have found similar results. Elhanafi et al., (2004) show that growth in acidic media resulted in increased expression of the *eaeA* (attaching and effacing) and *hlyA* (hemolysin) gene but did not impact the expression of *stx* genes. Muhldorfer et al., (1996) reported related results; pH did not increase the production of shiga toxins. Yuk and Marshall (2005) showed the shiga toxin production was dependant on the organic acid used but was not dependant on the pH levels. Most notably, Duffy et al., (2000) reported that *E. coli* O157:H7 cells

grown under mildly acidic conditions, pH 5.6 had a lower levels of shiga toxin produced than cells grown at neutral pH, 7.4.

Transcriptional regulation of genes in *E. coli* by iron is mediated by the protein product of the *fur* locus, which acts as a co-repressor with ferrous iron by the sequence-specific protein-DNA interaction at the promoter regions of the *fur*-regulated genes. The genes identified in this study as being up-regulated under acid shocked conditions are regulated by *fur*. Several investigators have identified an “iron box” for binding of the *fur* repressor. Iron concentration regulates gene expression in *E. coli* because the *fur*-binding site is located near or within the promoter of the iron-regulated genes. Fe^{2+} acts as a co-repressor so that at sufficient intracellular concentrations of iron the Fur-iron complex binds DNA at the iron box and prevents transcription of the iron-regulated gene (Litwin and Calderwood, 1993).

rt-RT-PCR Confirmation. Confirmation of the microarrays was carried-out using rt-RT-PCR. Four genes were selected from the acid adapted microarray results (*xasA*, +241.48; *appC*, +119.56; *gadB*, +31.46; and *gltJ*, -101.28) and four genes were selected from the acid shocked microarrays (*cyoE*, +143.16; *fhuA*, +102.72; *gadB*, -56.26; *gltJ*, -46.55) for analysis (Table 4.1). Additionally, *hemX* was selected as the housekeeping gene because it was similarly expressed (i.e., not significantly up- or down-regulated) under all treatment conditions (Table 4.6; Figures 4.6, 4.7, 4.10-4.12).

The rt-RT-PCR results for the acid adapted treatment confirm that of the microarrays. There was a 4.5-log difference in expression of *xasA* between control (endpoint 10^{-4}) and the acid adapted (endpoint $10^{-8.5}$) cells (Table 4.11; Figures 4.6, 4.23-

4.25). A 2-log difference in expression of *appC* was observed between control (endpoint 10^{-5}) and acid adapted (endpoint 10^{-7}) cells (Table 4.12; Figures 4.6, 4.26-4.28). For *gadB*, after holding in acidic media for 60 min, a 1-log difference in expression was observed (control endpoint $10^{-6.5}$; acid adapted endpoint $10^{-7.5}$) (Table 4.13; Figures 4.6, 4.29, 4.30 & 4.33). After 60 min in acidic LB, there was a 2-log difference in expression of *gltJ*, which was down-regulated (control endpoint $10^{-6.5}$; acid adapted endpoint $10^{-4.5}$) (Table 4.17; Figures 4.6, 4.46, 4.47 & 4.50).

For the acid shocked treatment, the rt-RT-PCR results also confirm the microarray data. A 2-log difference between control (endpoint 10^{-3}) and acid shocked cells (endpoint 10^{-5}) was noted for *fhuA* expression (Table 4.9; Figures 4.7, 4.17 & 4.19). A 1.5-log difference in expression was detected for *cyoE* (control endpoint $10^{-6.5}$; acid shocked endpoint 10^{-8}) (Table 4.10; Figures 4.7, 4.20 & 4.22). A $\frac{1}{2}$ -log difference (after 60 min) was observed for *gadB* (control endpoint $10^{-6.5}$; acid shocked endpoint 10^{-6}) (Table 4.13; Figures 4.7, 4.29, 4.31 & 4.33), and a 2-log difference was seen in *gltJ* expression (60 min) (control endpoint $10^{-6.5}$; acid shocked $10^{-4.5}$) (Table 4.17; Figures 4.7, 4.46, 4.48 & 4.50).

Time Point Analysis. Two genes, *gadB* and *gltJ*, which were significant ($p < 0.01$) and differentially expressed when comparing against the control for both acid adapted and acid shocked cells were further evaluated upon introduction into acidic LB (pH 3.5), from either pH 5.5 or 7.0 following 0, 5, 15 and 60 minute incubation, pH 3.5. Results for *gadB* with acid adapted cells at the different time points indicate that the glutamate decarboxylase system was utilized at time zero (i.e., immediately after transferring cells

to pH 3.5 LB (control endpoint $10^{-6.5}$; acid adapted endpoint $10^{-7.5}$) (Table 4.14; Figures 4.8, 4.34, 4.35 & 4.37). At 5 min, *gadB* was further induced showing a 1-log increase in expression between the 0 (endpoint $10^{-7.5}$) and 5 min (endpoint $10^{-8.5}$) holding times (Table 4.15; Figures 4.8, 4.38, 4.39 & 4.41); Expression of *gadB* in control cells remained the same for all time points (endpoint $10^{-6.5}$) (Figure 4.8). After 15 min, *gadB* expression was still elevated as compared to 0 min, but expression was lower than that observed after 5 min (Table 4.16; Figures 4.8, 4.42, 4.43 & 4.46). Differences in expression of *gadB* were as follows: acid adapted endpoint at 0 min was $10^{-7.5}$, endpoint at 5 min was $10^{-8.5}$, and endpoint at 15 min was 10^{-8} (Figure 4.8). At 60 min, *gadB* expression returned to the level originally observed (i.e., at the 0 min time point) endpoint $10^{-7.5}$ (Figure 4.8).

The differences in expression of *gadB* for acid adapted *E. coli* O157:H7 over the four time points indicates that the glutamate decarboxylase system was being utilized at 0 min. Because the system was already on at 0 min, it was probably also on while the cells were growing at pH 5.5 in LB. Once the pH was lowered to 3.5, expression of *gadB* increased by 1-log, this is indicated by an increase in the endpoint differences between 0 and 5 min (Figure 4.8). At 15 min the expression level was still elevated compared to 0 and 60 min, indicating that the decarboxylase system continued to consume protons in order to maintain a stable intracellular pH.

Expression of *gadB* in acid shocked cells at the different time points indicates that the glutamate decarboxylase system was under reduced utilization at all time points as compared to control and acid adapted cells. All time points for acid shocked cells had an endpoint of 10^{-6} (Tables 4.13-4.16; Figures 4.8). The endpoints for the acid shocked time

series was lower than that of the control acid adapted cells indicating down-regulation of *gadB*. Since there was no difference detected across the times, other mechanisms must have been utilized in order for the cell to maintain a stable intracellular pH. As noted with the acid shocked microarray data, a significant number of iron transport genes were expressed as opposed to an acid resistance system.

The *gltJ* gene was also analyzed after holding acid adapted and acid shocked *E. coli* O157:H7 in pH 3.5 LB broth for 0, 5, 15, and 60 min (Figure 4.9). Results from the microarray analysis and confirmation after 60 min indicate that the *gltJ* gene was down-regulated for both treatments (endpoint $10^{-4.5}$) as compared with the control (endpoint $10^{-6.5}$) (Table 4.17; Figures 4.9, 4.46, 4.47, 4.48 & 4.50). At 0 min, *gltJ* was expressed at higher levels in acid shocked cells (endpoint $10^{-7.5}$) (Table 4.18; Figures 4.9, 4.53 & 4.54) as compared to control cells (endpoint $10^{-6.5}$) (Table 4.18; Figures 4.9, 4.51 & 4.54), but expression in adapted cells (endpoint $10^{-4.5}$) was lower than in controls (Table 4.18; Figures 4.9, 4.52 & 4.54). However, after holding for 5-60 min, expression of *gltJ* was lower in acid adapted and acid shocked cells (endpoint $10^{-4.5}$) than in controls (endpoint $10^{-6.5}$) (Figure 4.9). The decrease in expression of *gltJ* after holding in pH 3.5 LB indicates that the gene was not essential for survival under the acidic conditions used in this study, and as a result, was repressed. *E. coli* contains four transporters for the uptake of glutamate (*GltIJKL*, *GltP*, *GltS*, and *GadC*). The *GltIJKL* operon accounts for approximately 15% of the total transport velocity of glutamate (Schellenberg et al., 1977). The reduced expression in *gltJ* during holding suggests that another of these four transporters was being utilized. In the acid adapted studies, *gadB* was positively expressed, and is likely the transporter utilized instead of *gltJ*. For the acid shocked cells,

the *gadA/BC* operon was negatively expressed, so uptake of glutamate occurred during the first 5 min; no other transporter was identified as positively expressed.

Conclusions

Results of this study indicate that acid adapted and acid shocked *E. coli* O157:H7 utilize different genes for survival at low pH. Acid adapted cells utilize the glutamate decarboxylase acid resistance system and possibly the arginine acid resistance system. Acid adapted cells also synthesize cyclopropane fatty acids, and as a result, manifest greater expression of the *cfa* gene. On the other hand, a substantial number of iron transport genes are up-regulated in acid shocked cells. It is known that iron solubility is a function of pH, but the availability of iron alone will not trigger an acid response. Our data indicate that iron plays an important role in survival of acid shocked *E. coli* O157:H7. None of the virulence genes (*eae*, *stx1*, or *stx2*) were significantly, differentially expressed. The shiga toxin genes were significantly ($p > 0.01$) up-regulated, but the difference in expression was fell below the 2-fold increase that was established as the minimum level of consideration for this study.

Acid adapted and acid shocked cells had genes from the glutamate synthase operon that were down-regulated after holding at pH 3.5 for 60 min. Time point analysis revealed that the *gltJ* gene was up-regulated in acid shocked cells immediately upon placing cells in pH 3.5 LB, well before the cells adjusted to the low pH. These data suggest that glutamate synthesis did not occur as a result of this pathway at low pH.

This study provides information that is useful for determining gene regulation in acid adapted and acid shocked *E. coli* O157:H7 during late exponential growth. Further

research is needed to determine the role of iron genes in acid shocked cells as compared to acid adapted cells to determine when these genes become expressed and to determine what mechanism(s) responsible for activation of virulence genes.

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Appendix

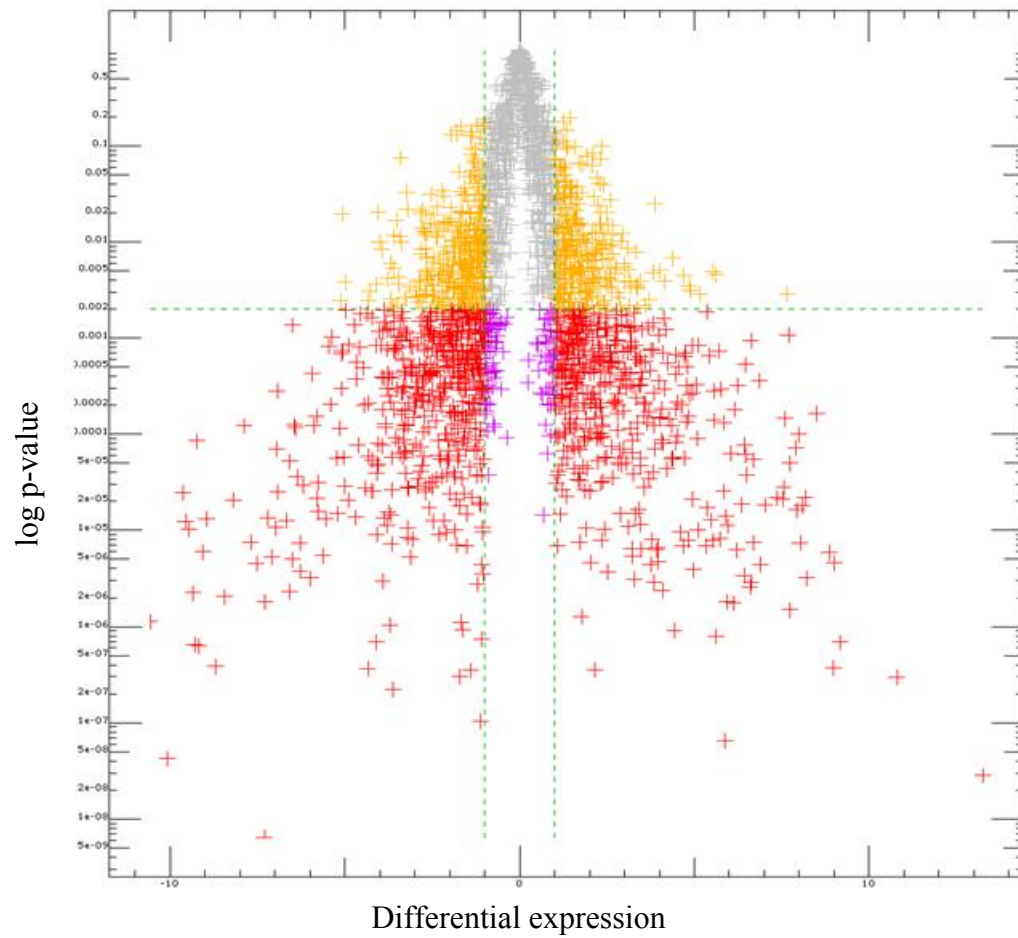


Figure 4.1: Plot of p-values vs. differential expression (fold change) of genes from *E. coli* O157:H7 control vs. acid adapted treatments.

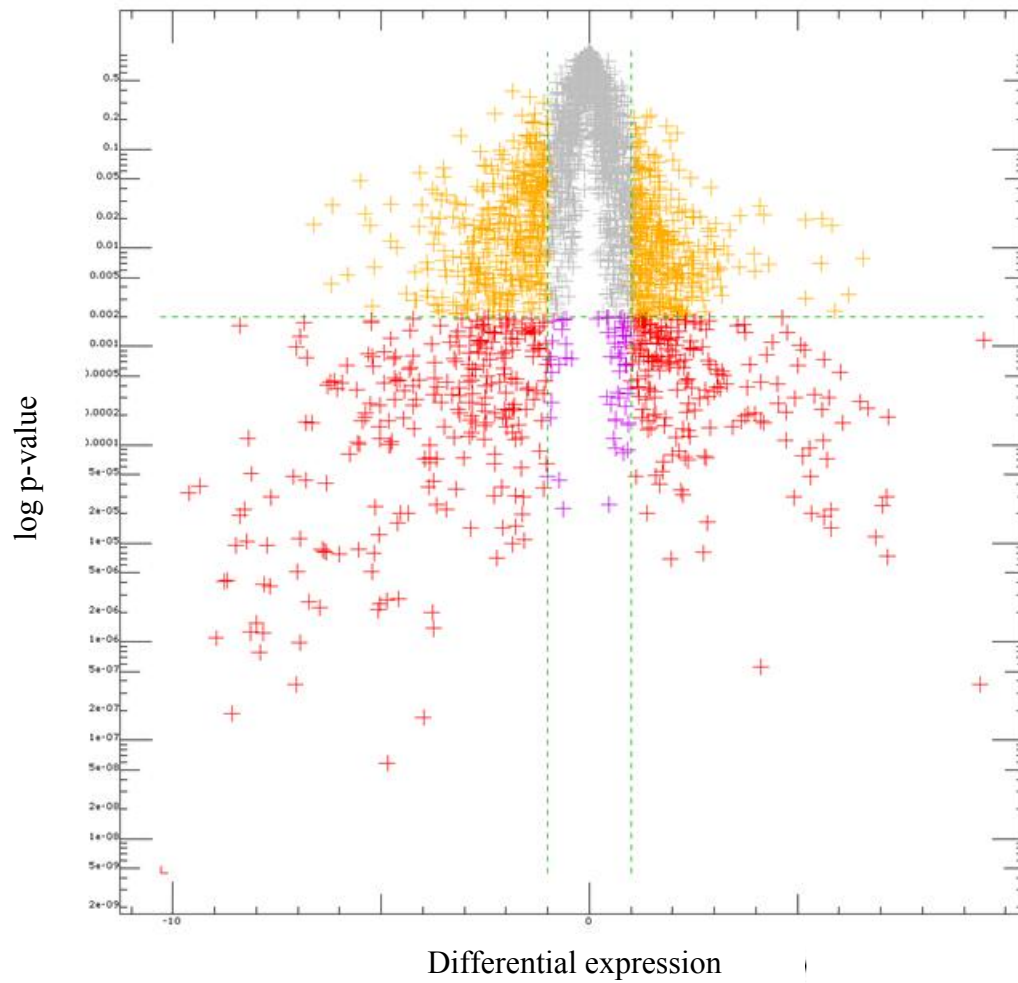
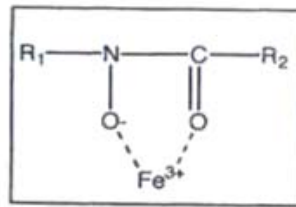


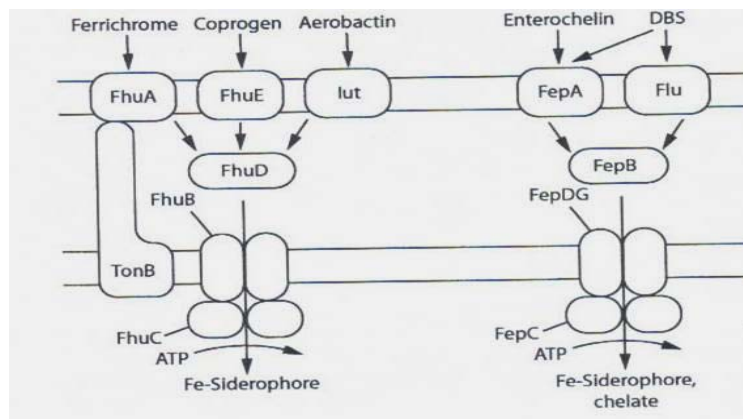
Figure 4.2: Plot of p-values vs. differential expression (fold change) of genes from *E. coli* O157:H7 control vs. acid shocked treatments.

(b) Hydroxamate



(Barton, 2005)

Figure 4.3: Structure of hydroxamate bacterial siderophores



(Barton, 2005)

Figure 4.4: FhuA-TonB complex

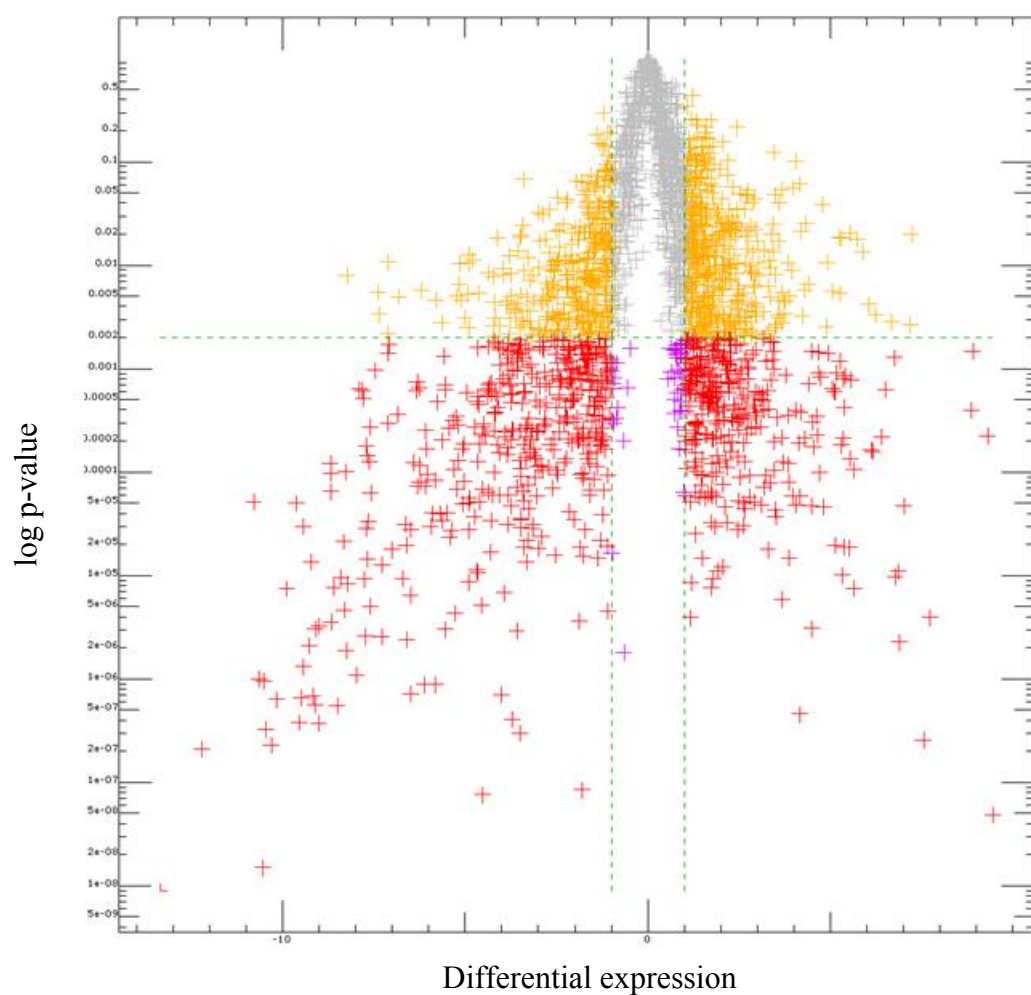


Figure 4.5: Plot of p-values vs. differential expression (fold change) of genes from *E. coli* O157:H7 acid shock vs. acid adapt treatments.

Table 4.1: Primer sequences for select genes

Gene	Primer sequence	Product size
<i>hemX</i>	Forward: GAAAATATTCGCTCTCGCCTGCTGG Reverse: TGGCTTTGCAGGGTTTCCGG	209 bp
<i>appC</i>	Forward: CCTCGCCACTCACTCATTAG Reverse: ATCAGCATCACCAGTAGCAG	336 bp
<i>cyoE</i>	Forward: GTAACGAAACCAGGCATCATC Reverse: AAACCACAGCAGCATAAAGC	285 bp
<i>fhuA</i>	Forward: ACAGTGTGCGGCATTAGC Reverse: AGAGTCGTCGTAACCAAACC	208 bp
<i>gadB</i>	Forward: ACGGATTTAAGGTCGGAACACTAC Reverse: CTGACGAGCGTTGCCATC	156 bp
<i>gltJ</i>	Forward: TTTCCTTTCATCCATGCTCTG Reverse: ATCATCTCTGAGGTCATCGG	201 bp
<i>xasA</i>	Forward: TTTGAGTTATATGGGCGTAGAAGC Reverse: TGGGACATCAGAACGGTAAAGG	210 bp

Table 4.2: rt-RT-PCR program for *fhuA*

Cycle #	Temperature - time	Replicates
1	50°C - 35 minutes	1
2	94°C - 2 minutes	1
3	94°C - 45 seconds	50
	50°C - 45 seconds	
	72°C - 45 seconds	
4	72°C - 7 minutes	1
5	50°C in 0.5°C every 10 seconds	96
6	4°C	hold

Table 4.3: rt-RT-PCR program for *cyoE*, *gadB* and *gltJ*

Cycle #	Temperature - time	Replicates
1	50°C - 35 minutes	1
2	94°C - 2 minutes	1
3	94°C - 45 seconds	50
	53°C - 45 seconds	
	72°C - 45 seconds	
4	72°C - 7 minutes	1
5	50°C in 0.5°C every 10 seconds	96
6	4°C	hold

Table 4.4: rt-RT-PCR program for *xasA*

Cycle #	Temperature - time	Replicates
1	50°C - 35 minutes	1
2	94°C - 2 minutes	1
3	94°C - 45 seconds	50
	57°C - 45 seconds	
	72°C - 45 seconds	
4	72°C - 7 minutes	1
5	50°C in 0.5°C every 10 seconds	96
6	4°C	Hold

Table 4.5: rt-RT-PCR program for *appC*

Cycle #	Temperature - time	Replicates
1	50°C - 35 minutes	1
2	94°C - 2 minutes	1
3	94°C - 45 seconds	50
	54°C - 45 seconds	
	72°C - 45 seconds	
4	72°C - 7 minutes	1
5	50°C in 0.5°C every 10 seconds	96
6	4°C	hold

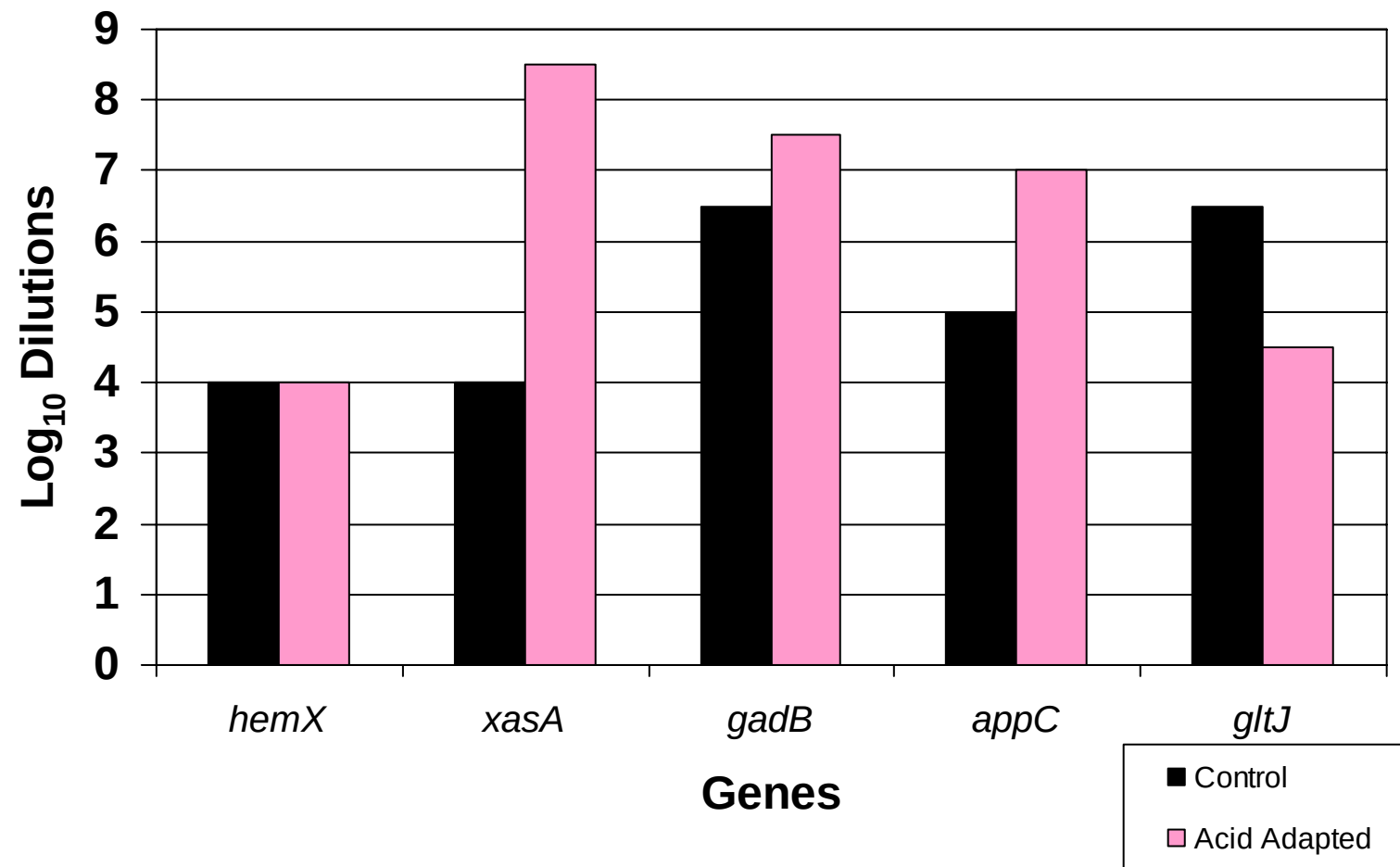


Figure 4.6: Differential expression levels of acid adapted genes

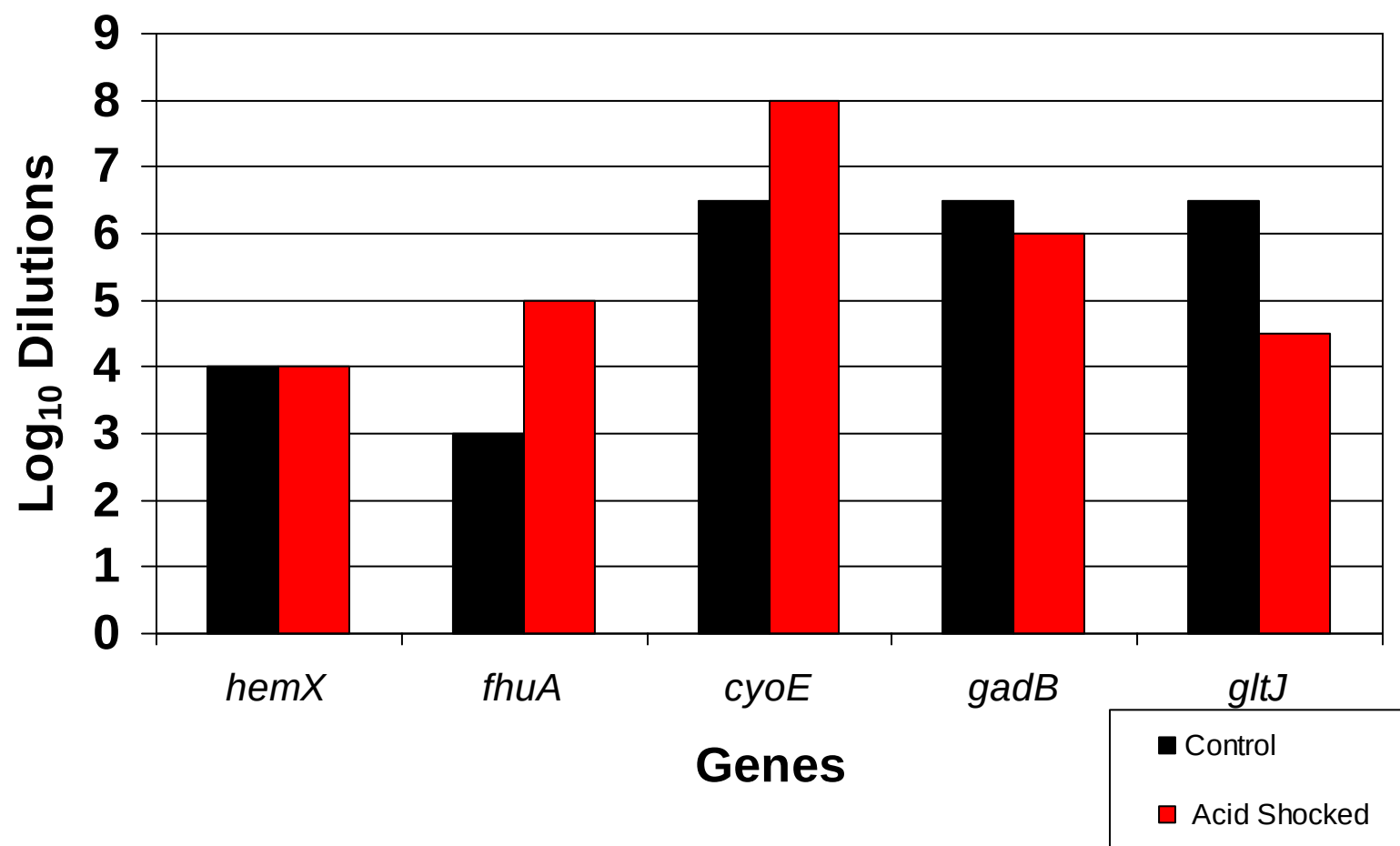


Figure 4.7: Differential expression levels of acid shocked genes

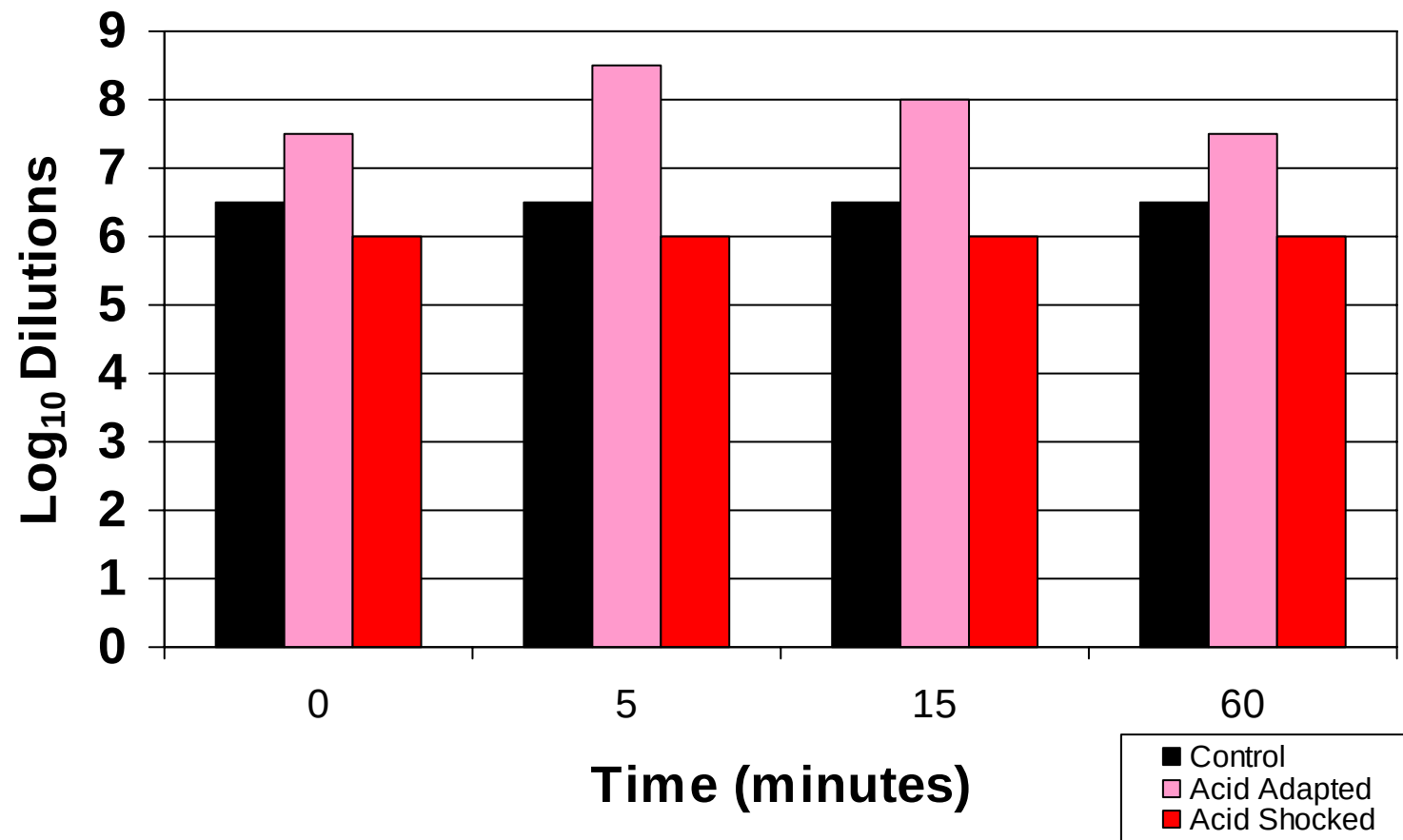


Figure 4.8: Differential expression levels of *gadB* at 0, 5, 15 and 60 minutes

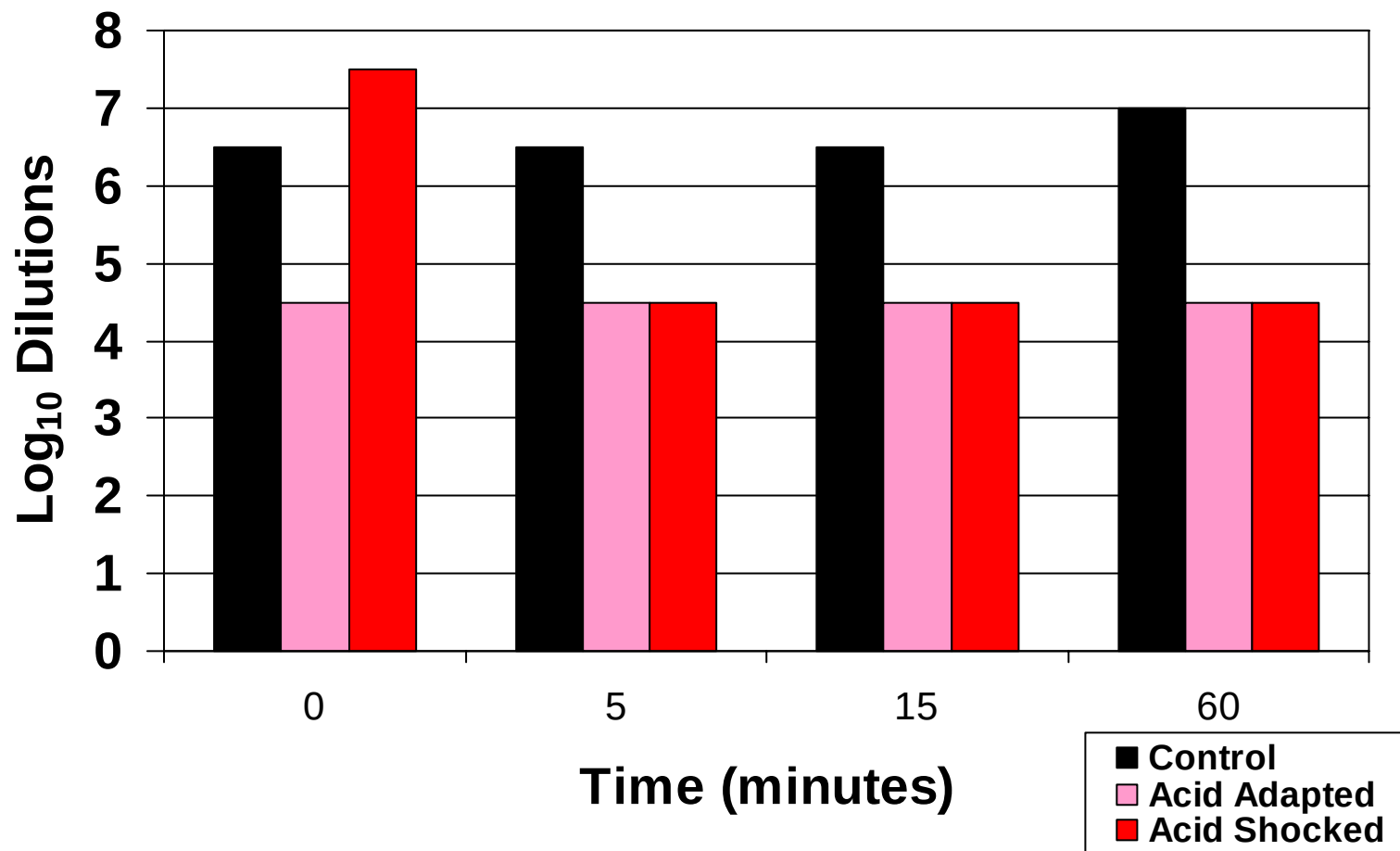


Figure 4.9: Differential expression levels of *gltJ* at 0, 5, 15 and 60 minutes

Table 4.6: Average C_t values for <i>hemX</i> (housekeeping gene)				
Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Control	19.49 ± 1.09	22.92 ± 1.65	26.33 ± 1.72	29.44 ± 1.83
Acid shocked	18.03 ± 0.48	21.36 ± 0.45	25.43 ± 1.05	28.28 ± 1.51
Acid adapted	17.98 ± 0.98	21.33 ± 1.10	24.89 ± 1.34	28.23 ± 0.10

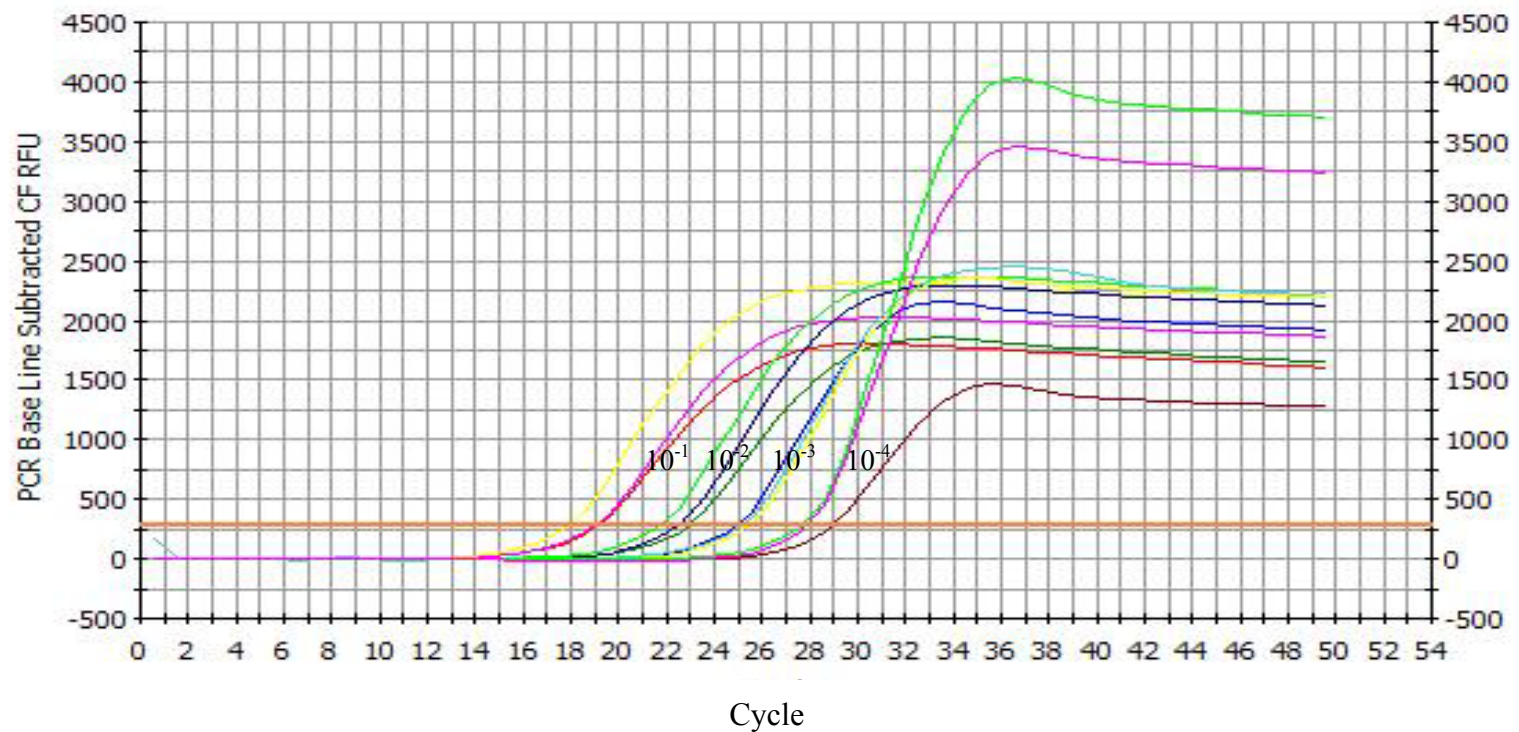


Figure 4.10: C_t curves for *hemX*

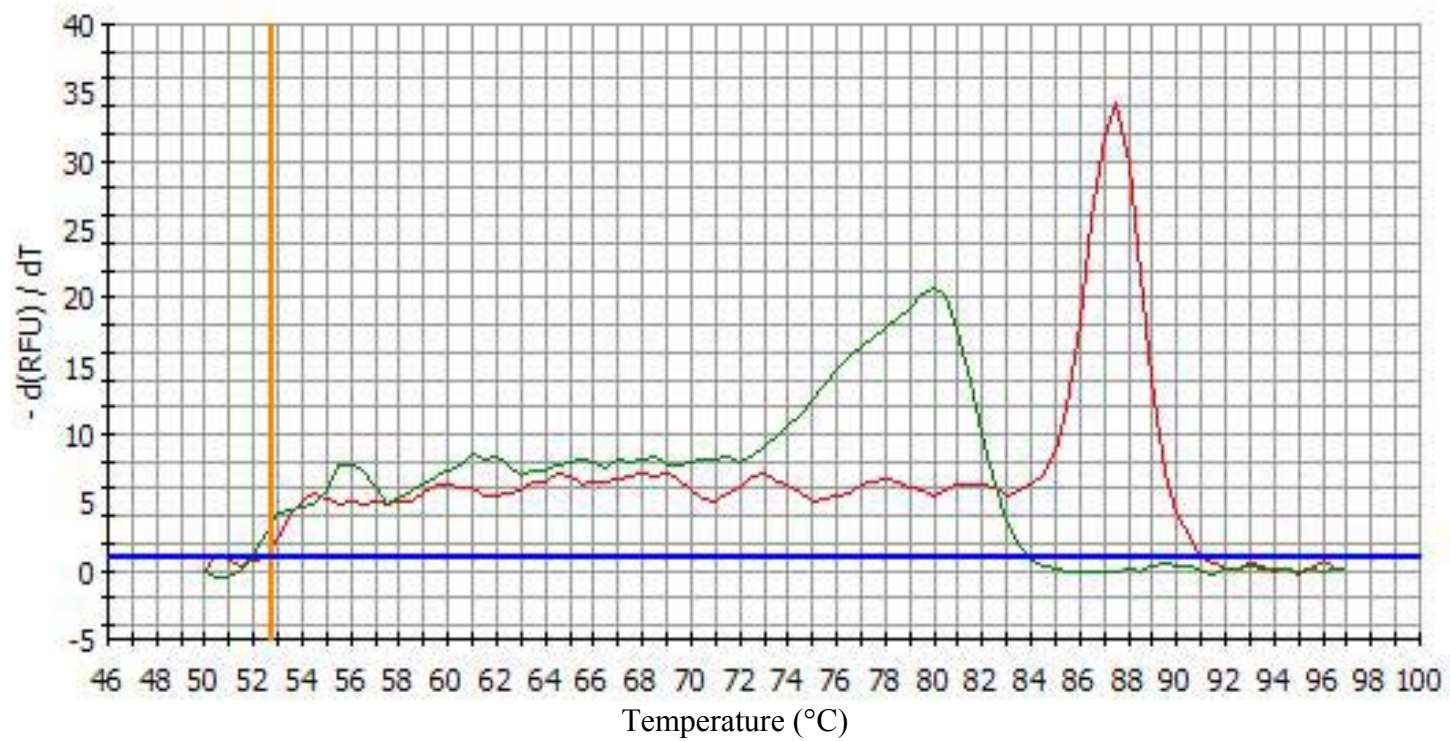


Figure 4.11: T_m graph for *hemX*

*Red: T_m values for *hemX*, 88°C; Green: *hemX* negative

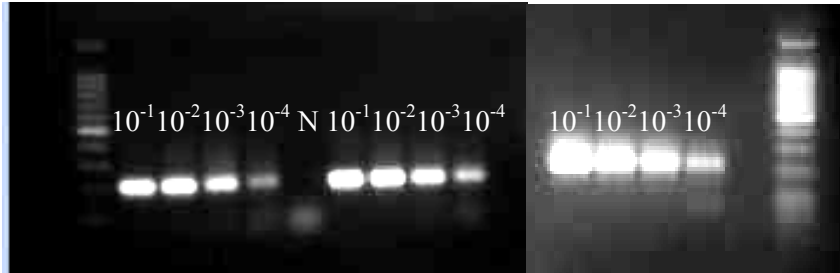


Figure 4.12: Gel of *hemX* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lane 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: negative; Lane 7: Acid Shocked 10^{-1} ; Lane 8: Acid Shocked 10^{-2} ; Lane 9: Acid Shocked 10^{-3} ; Lane 10: Acid Shocked 10^{-4} ; Lane 11: empty; Lane 12: Acid Adapted 10^{-1} ; Lane 13: Acid Adapted 10^{-2} ; Lane 14: Acid Adapted 10^{-3} ; Lane 15: Acid Adapted 10^{-4} ; Lane 16: empty; Lane 17: 100bp DNA marker.

Table 4.7: Average Ct values for *hemX* under acid adapted conditions at 0, 5, 15 minutes

Time (min)	10^{-1}	10^{-2}	10^{-3}	10^{-4}
0	18.30 ± 0.71	22.55 ± 1.20	25.4 ± 0.56	28.55 ± 0.49
5	17.3 ± 1.10	22.1 ± 0.79	27.16 ± 1.11	30.63 ± 1.64
15	17.4 ± 0.43	24.0 ± 1.21	28.5 ± 1.81	30.05 ± 0.65

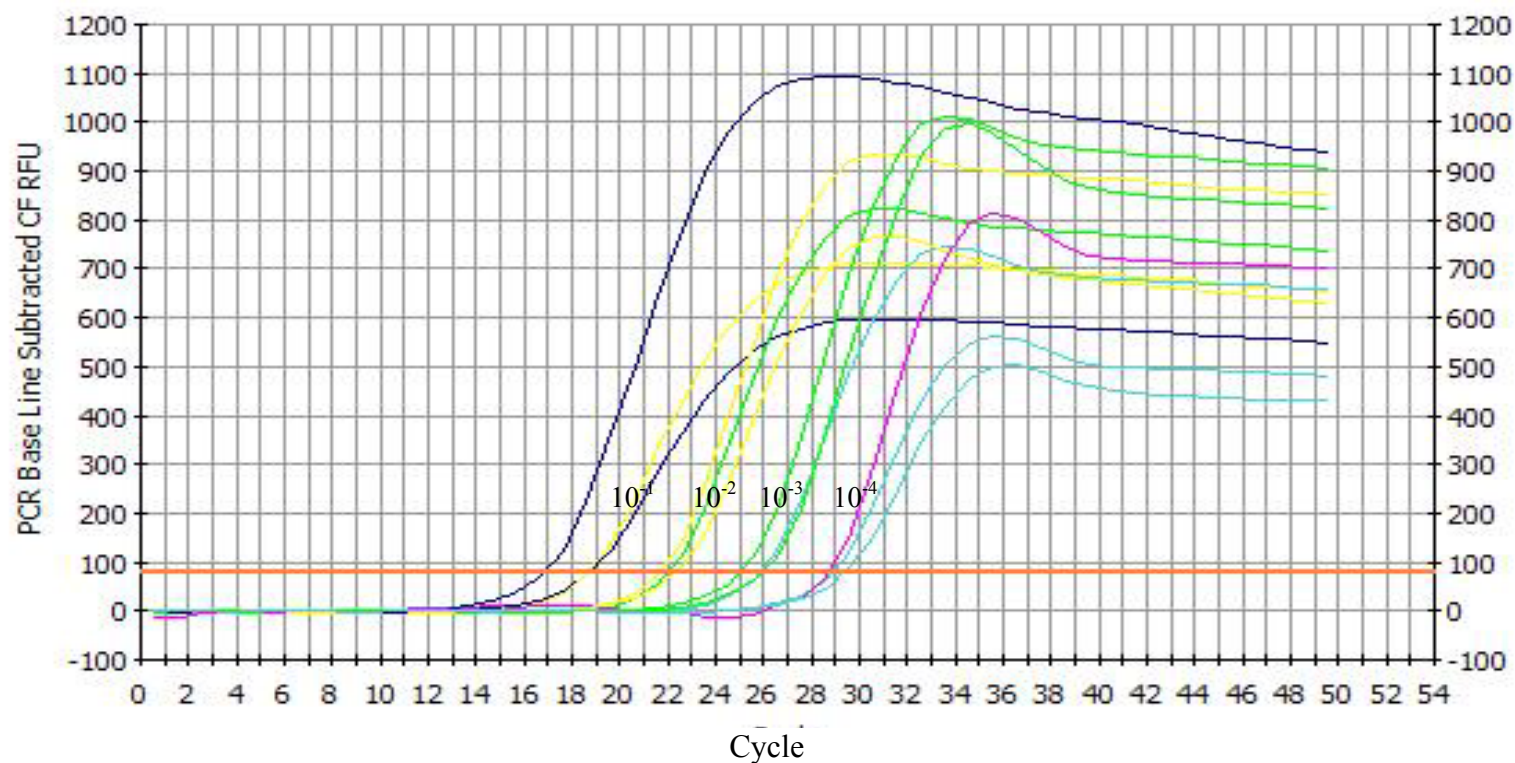


Figure 4.13: Acid adapted *hemX* C_t curves for time points 0, 5, and 15 minutes

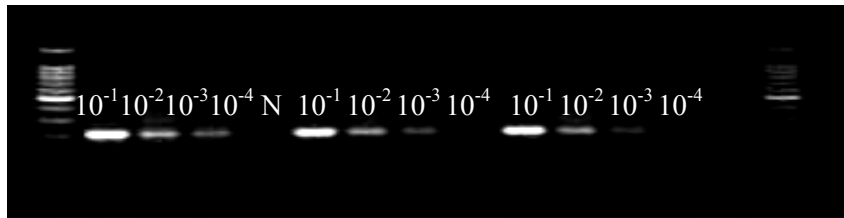


Figure 4.14: Gel of acid adapted *hemX* dilutions at 0, 5, and 15 minutes

*Lane 1: 100bp DNA marker; Lane 2: acid adapted 0 min 10^{-1} ; Lane 3: acid adapted 0 min 10^{-2} ; Lane 4: acid adapted 0 min 10^{-3} ; Lane 5: acid adapted 0 min 10^{-4} ; Lane 6: acid adapted 5 min 10^{-1} ; Lane 7: acid adapted 5 min 10^{-2} ; Lane 8: acid adapted 5 min 10^{-3} ; Lane 9: acid adapted 5 min 10^{-4} ; Lane 10: acid adapted 15 min 10^{-1} ; Lane 11: acid adapted 15 min 10^{-2} ; Lane 12: acid adapted 15 min 10^{-3} ; Lane 13 acid adapted 15 min 10^{-4} ; Lane 14: empty; Lane 15: negative; Lane 16: 100bp marker.

Table 4.8: Average C_t values for *hemX* under acid shocked conditions at 0, 5, 15 minutes

Time (min)	10^{-1}	10^{-2}	10^{-3}	10^{-4}
0	19.17 ± 1.02	23.93 ± 1.11	27.63 ± 1.36	29.33 ± 0.76
5	19.33 ± 1.27	24.10 ± 2.03	28.77 ± 2.14	30.53 ± 2.15
15	18.10 ± 1.16	23.9 ± 0.85	27.20 ± 1.3	29.20 ± 1.50

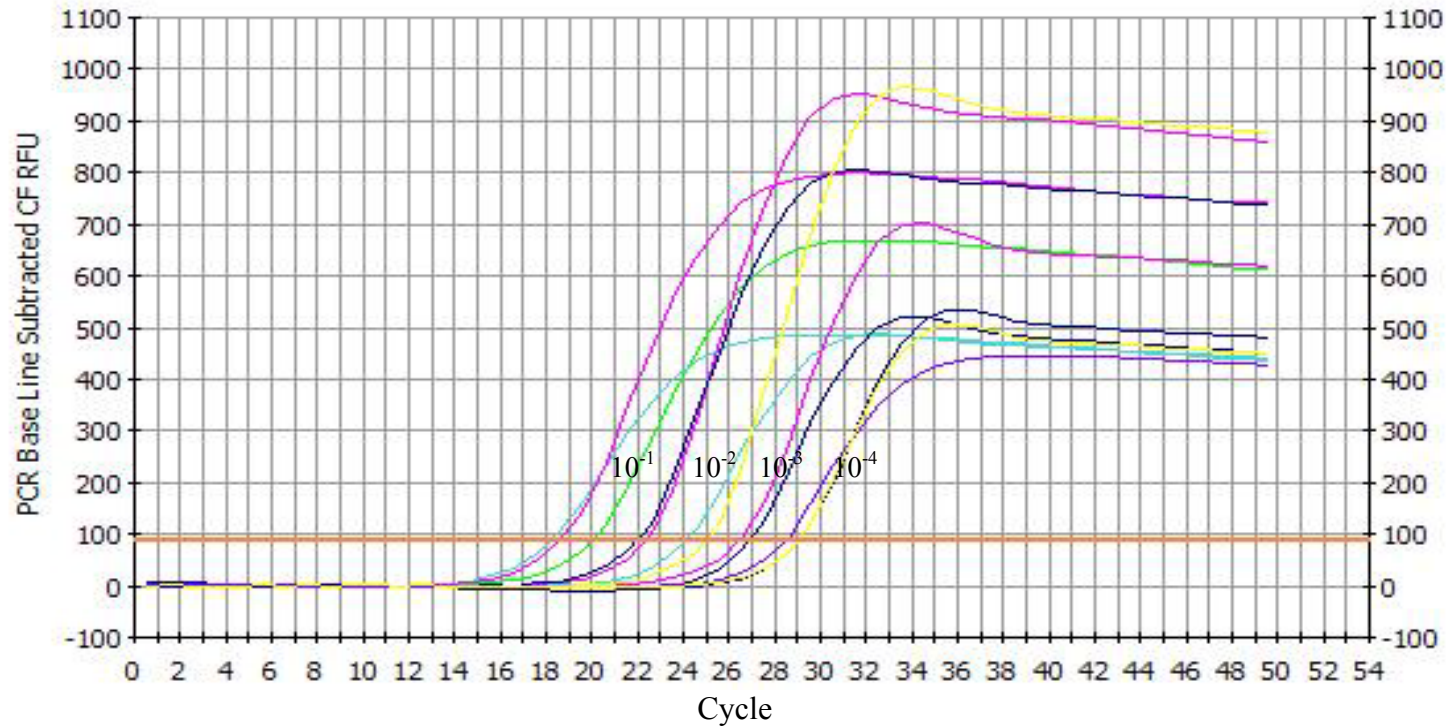


Figure 4.15: Acid shocked *hemX* C_t curves for time points 0, 5, and 15 minutes

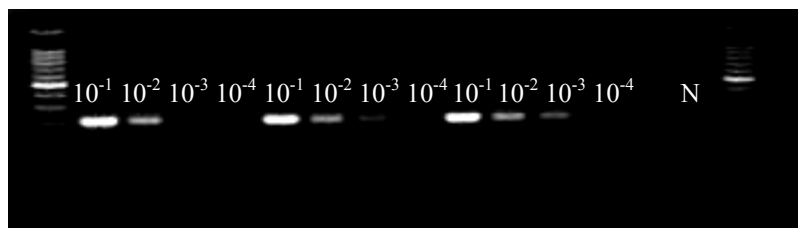


Figure 4.16: Gel of acid shocked *hemX* dilutions at 0, 5, and 15 minutes

*Lane 1: 100bp DNA marker; Lane 2: acid shocked 0 min 10^{-1} ; Lane 3: acid shocked 0 min 10^{-2} ; Lane 4: acid shocked 0 min 10^{-3} ; Lane 5: acid shocked 0 min 10^{-4} ; Lane 6: acid shocked 5 min 10^{-1} ; Lane 7: acid shocked 5 min 10^{-2} ; Lane 8: acid shocked 5 min 10^{-3} ; Lane 9: acid shocked 5 min 10^{-4} ; Lane 10: acid shocked 15 min 10^{-1} ; Lane 11: acid shocked 15 min 10^{-2} ; Lane 12: acid shocked 15 min 10^{-3} ; Lane 13 acid shocked 15 min 10^{-4} ; Lane 14: empty; Lane 15: negative; Lane 16: 100bp marker.

Table 4.9: Average C_t values for <i>fhuA</i>					
Treatment	10^{-2}	10^{-3}	10^{-4}	$10^{-4.5}$	10^{-5}
Control	19 ± 0.35	23.27 ± 0.98			
Acid shocked	18.23 ± 0.35	22.97 ± 0.35	24.53 ± 0.64	26.4 ± 0.5	28.3 ± 0.23

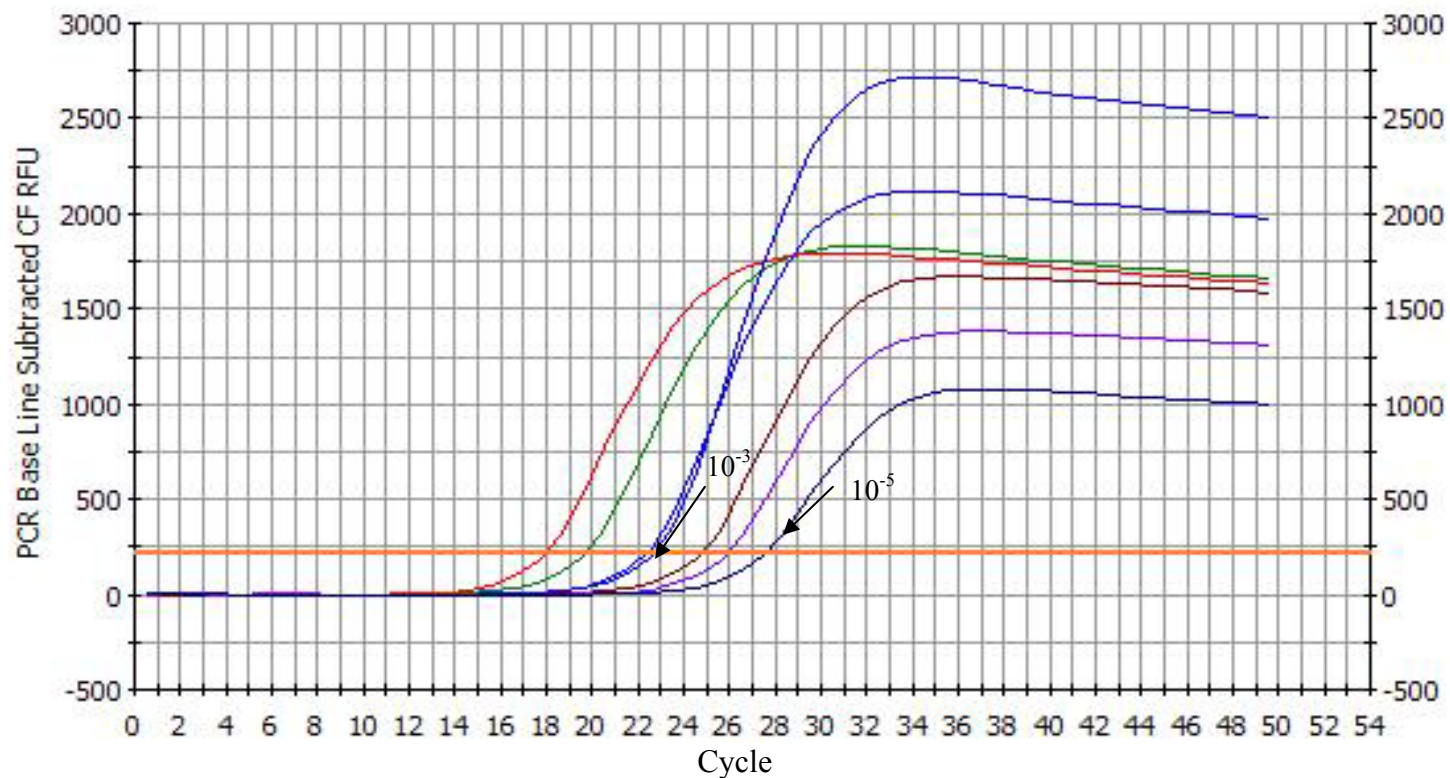


Figure 4.17: C_t curves for control and acid shocked *fhuA* dilutions

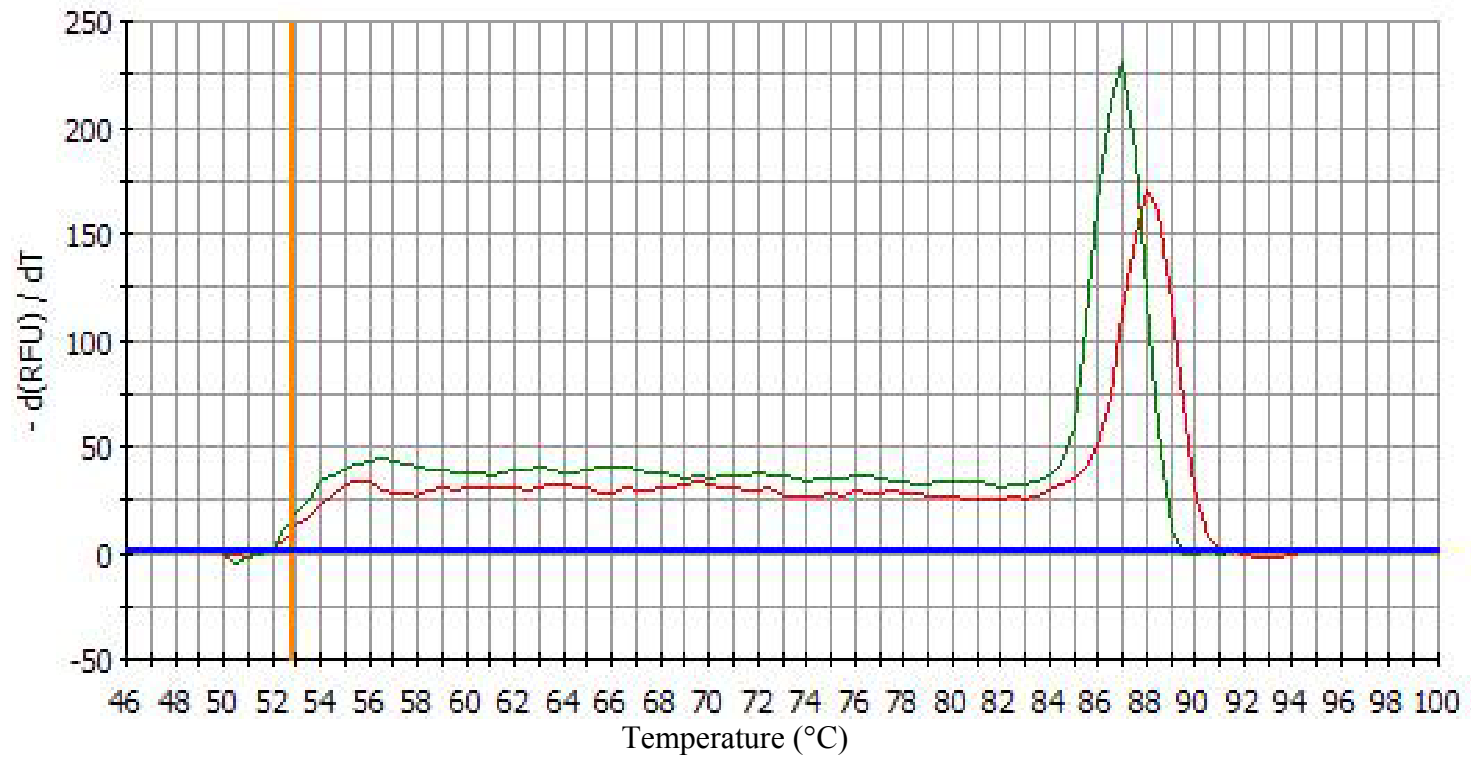


Figure 4.18: T_m curve for *fhuA*

*Red: T_m for *hemX*, 88°C; Green: T_m for *fhuA*, 87°C

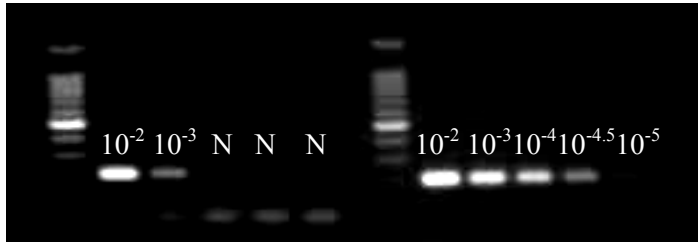


Figure 4.19: Gel of *fhuA* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lane 4-6: negatives; Lane 7: 100bp DNA marker; Lane 8: Acid Shocked 10^{-2} ; Lane 9: Acid Shocked 10^{-3} ; Lane 10: Acid Shocked 10^{-4} ; Lane 11: Acid Shocked $10^{-4.5}$; Lane 12: Acid Shocked 10^{-5} ; Lane 13: empty.

Table 4.10: Average C_t values for <i>cyoE</i>									
Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$10^{-6.5}$	10^{-7}	$10^{-7.5}$	10^{-8}
Control	18.43 ± 0.15	21.9 ± 0.56	25.5 ± 0.85	28.73 ± 0.42	33.57 ± 0.61	38.2 ± 1.31			
Acid shocked	15.43 ± 0.72	18.9 ± 0.26	23.1 ± 0.6	27.67 ± 0.06	31.96 ± 0.21		35.53 ± 0.35	38.23 ± 0.21	40.2 ± 0.36

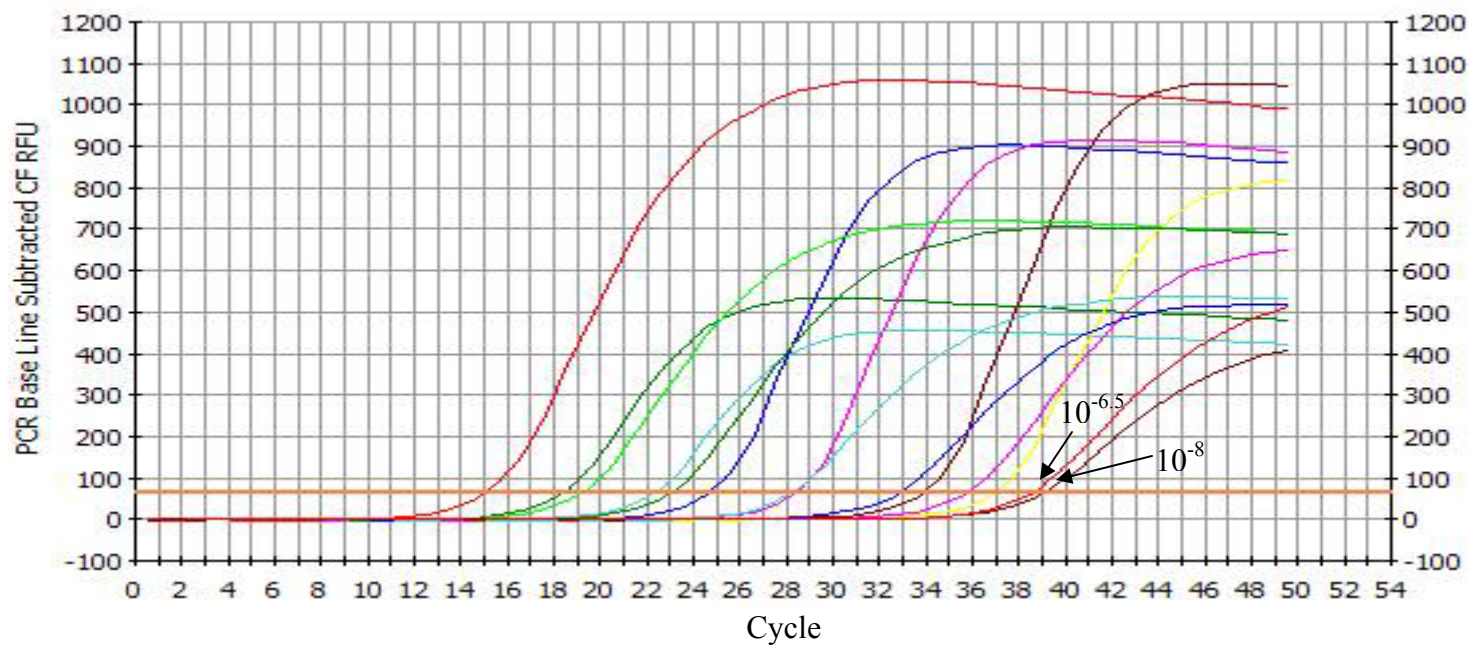


Figure 4.20: C_t curves for *cyoE* dilutions

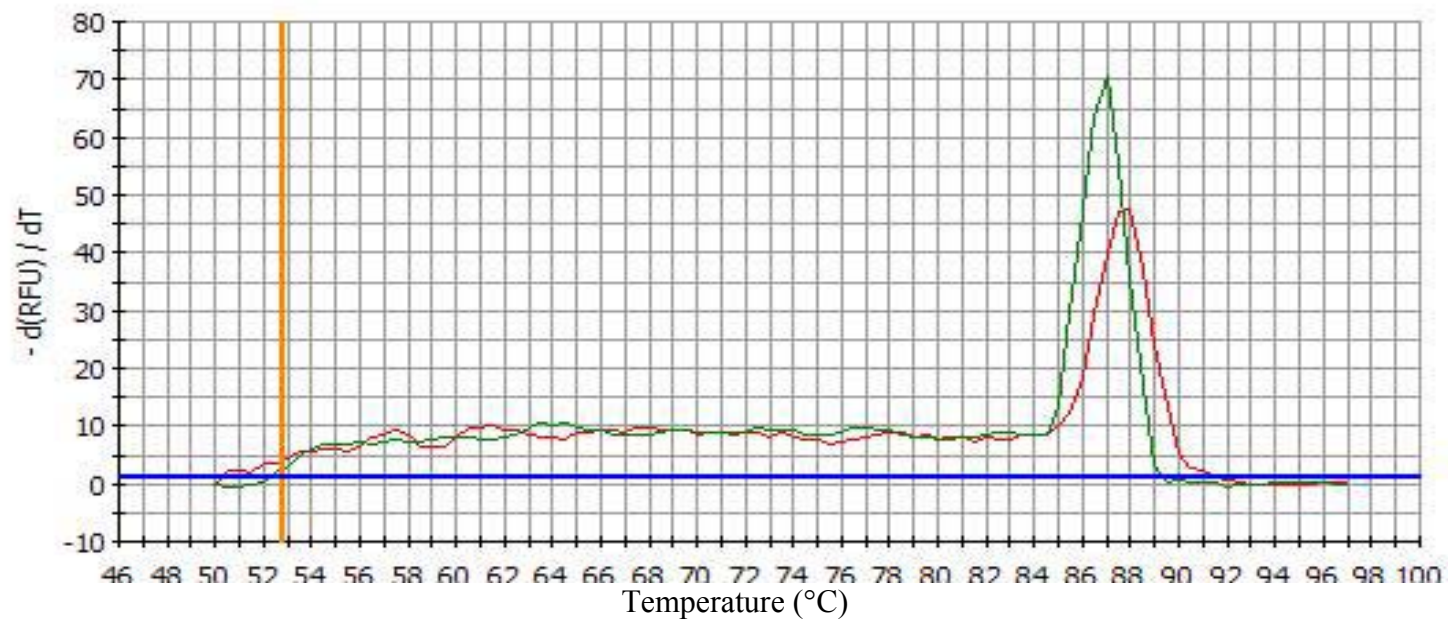


Figure 4.21: T_m curve for *cyoE*

*Red: T_m for *hemX*, 88°C; Green: T_m for *cyoE*, 87°C

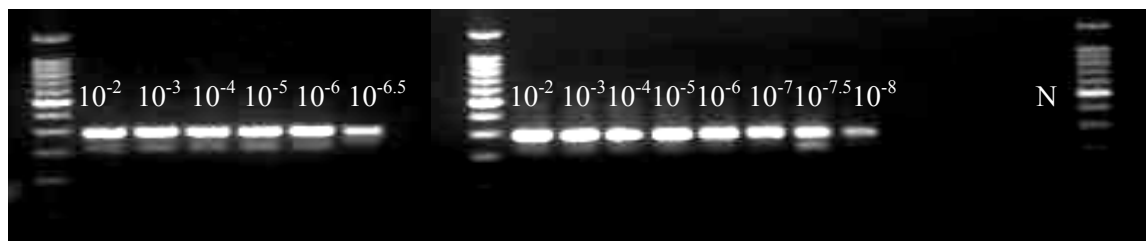


Figure 4.22: Gel of *cyoE* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lane 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: Control 10^{-6} ; Lane 7: Control $10^{-6.5}$; Lane 8: empty Lane 9: 100bp DNA marker; Lane 10: Acid Shocked 10^{-2} ; Lane 11: Acid Shocked 10^{-3} ; Lane 12: Acid Shocked 10^{-4} ; Lane 13: Acid Shocked 10^{-5} ; Lane 14: Acid Shocked 10^{-6} ; Lane 15: Acid Shocked 10^{-7} ; Lane 16: Acid Shocked $10^{-7.5}$; Lane 17: Acid Shocked 10^{-8} ; Lanes 18-20: empty; Lane 21: negative; Lane 22: 100bp DNA marker.

Table 4.11: Average C_t values of <i>xasA</i>										
Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	$10^{-7.5}$	10^{-8}	$10^{-8.5}$
Control		$23.32 \pm$	$26.53 \pm$							
	20 ± 0.01	0.29	0.83	30 ± 1.09						
Acid	$13.23 \pm$	$16.6 \pm$	$20.38 \pm$	$24.13 \pm$	$28.02 \pm$	$29.9 \pm$	$34.13 \pm$	$35.7 \pm$	$38 \pm$	$38.03 \pm$
adapted	0.47	0.26	0.39	0.25	0.43	1.08	1.08	1.83	0.91	0.49

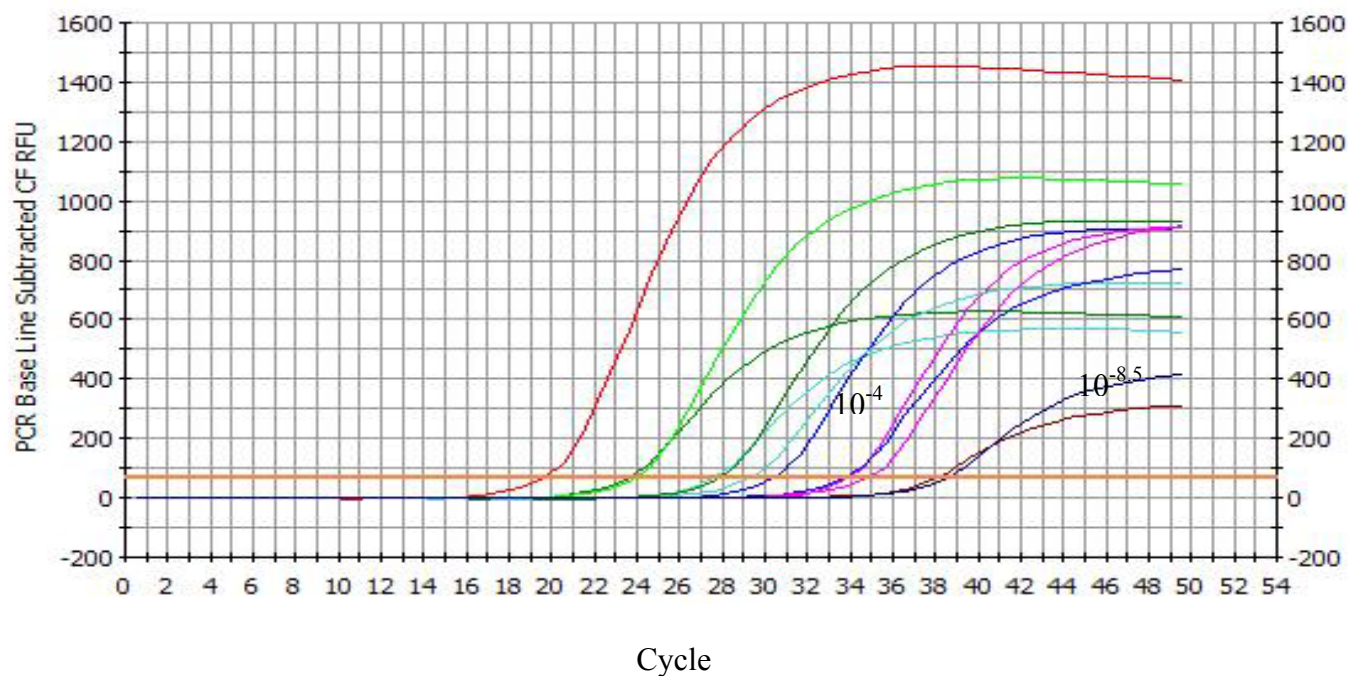


Figure 4.23: C_t curves for *xasA* dilutions

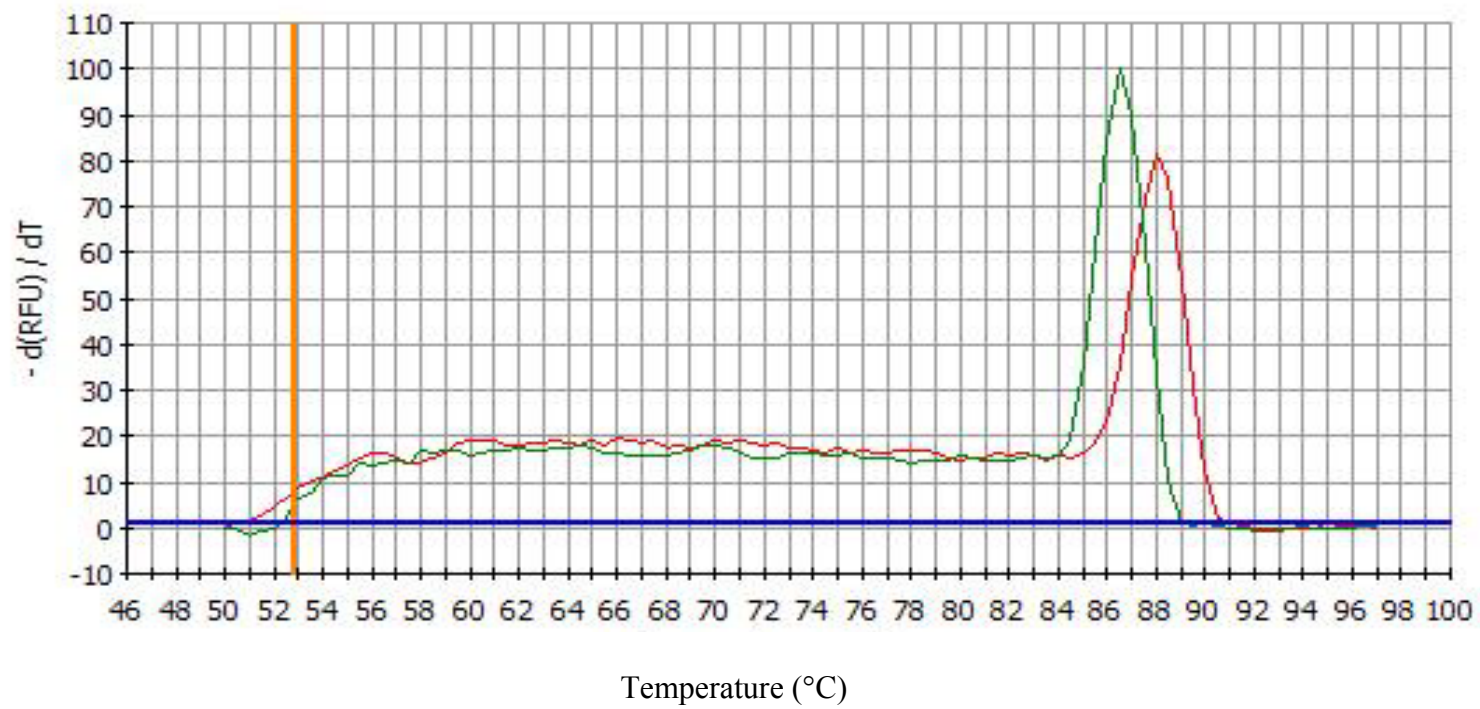


Figure 4.24: T_m curve for *xasA*
 *Red: T_m for *hemX*, 88°C; Green: T_m for *xasA*, 86.5°C

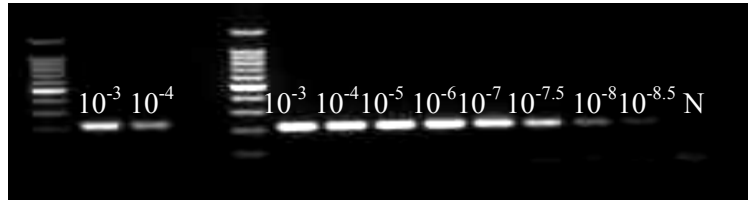


Figure 4.25: Gel of *xasA* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-3} ; Lane 3: Control 10^{-4} ; Lane 4: empty; Lane 5: 100bp DNA marker; Lane 6: Acid Adapted 10^{-3} ; Lane 7: Acid Adapted 10^{-4} ; Lane 8: Acid Adapted 10^{-5} ; Lane 9: Acid Adapted 10^{-6} ; Lane 10: Acid Adapted 10^{-7} ; Lane 11: Acid Adapted $10^{-7.5}$; Lane 12: Acid Adapted 10^{-8} ; Lane 13: Acid Adapted $10^{-8.5}$; Lane 14: negative.

Table 4.12: Average Ct values of <i>appC</i>						
Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Control	19.63 ± 0.55	22.8 ± 0.46	27.1 ± 0.46	30.36 ± 0.5		
Acid adapted	19.63 ± 0.50	23.57 ± 0.23	27.47 ± 0.5	31.13 ± 0.29	32.56 ± 0.93	34.47 ± 2.4

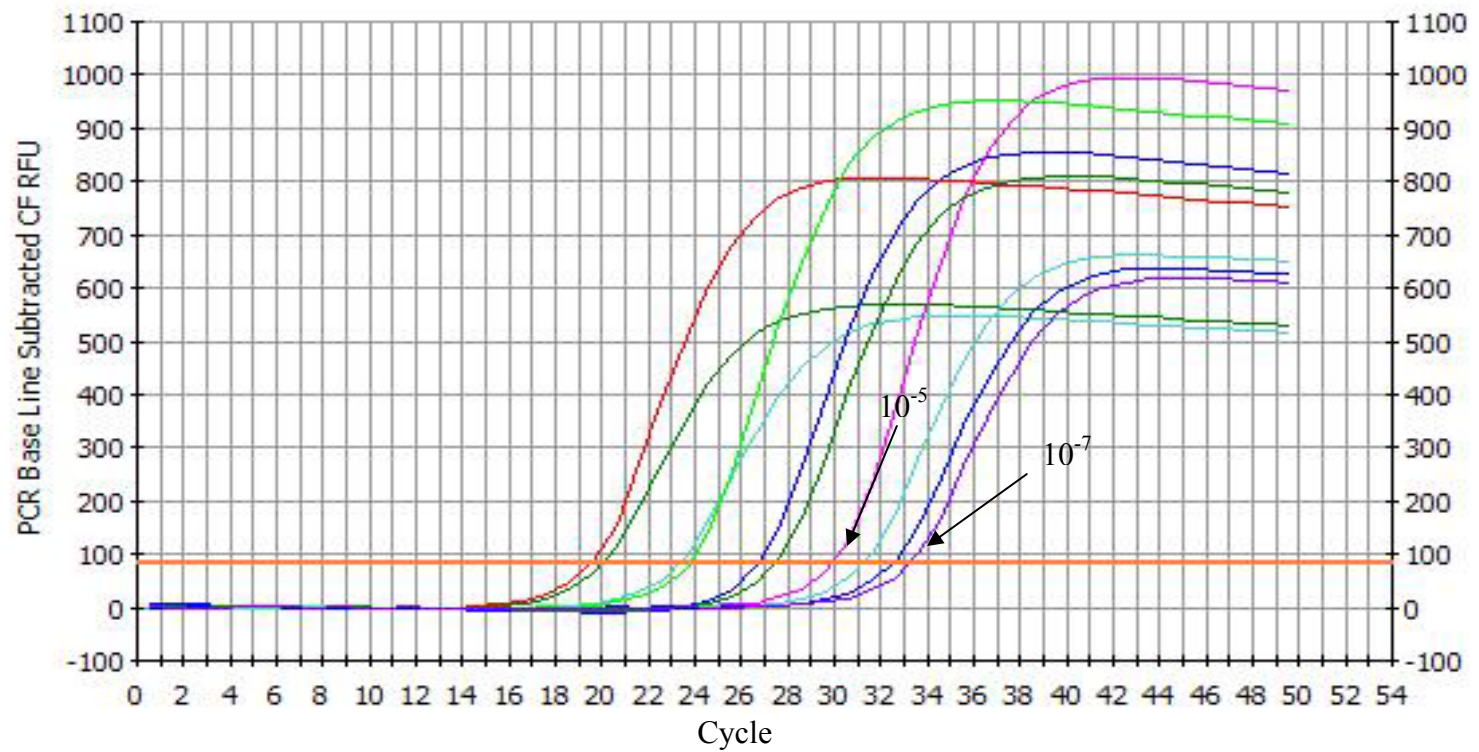


Figure 4.26: C_t curves for *appC* dilutions

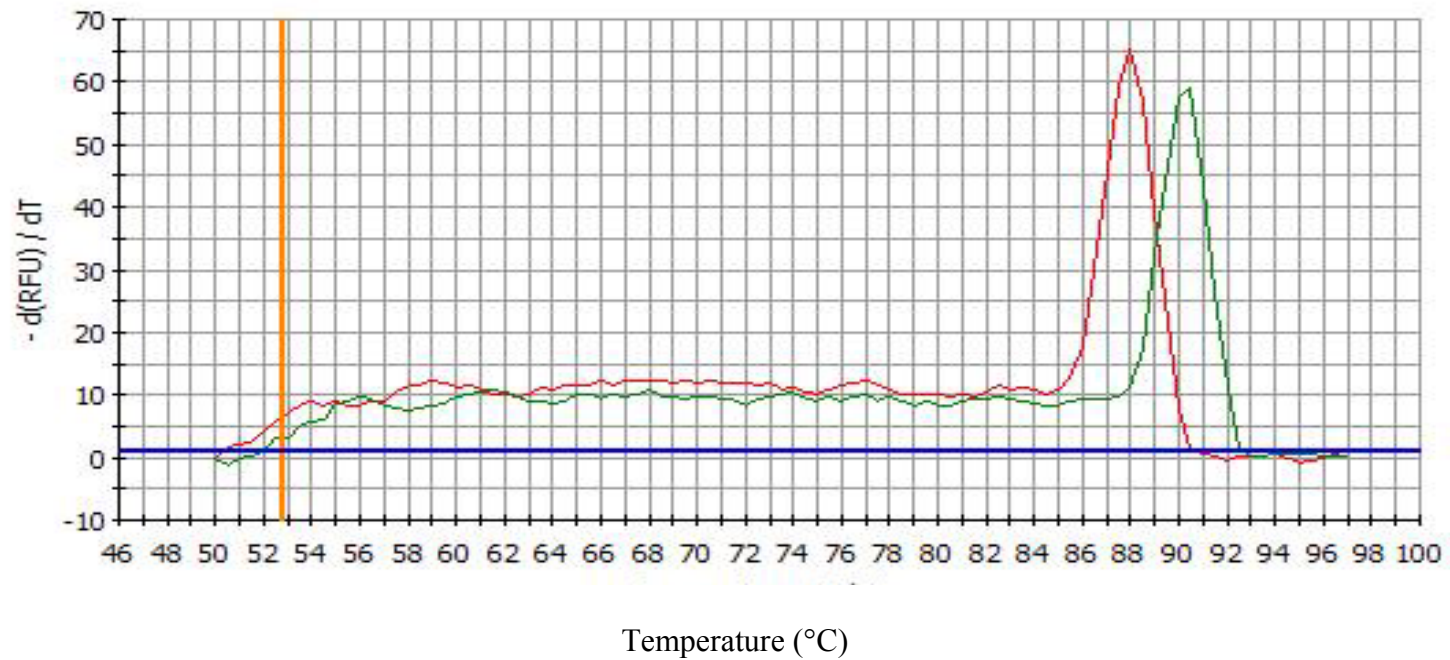


Figure 4.27: T_m curve for *appC*

*Red: T_m for *hemX*, 88°C; Green: T_m for *appC*, 90°C



Figure 4.28: Gel of *appC* dilutions

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lane 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: Negative; Lane 7 and 8: empty; Lane 9: 100bp DNA marker; Lane 10: Acid Adapted 10^{-2} ; Lane 11: Acid Adapted 10^{-3} ; Lane 12: Acid Adapted 10^{-4} ; Lane 13: Acid Adapted 10^{-5} ; Lane 14: Acid Adapted 10^{-6} ; Lane 15: Acid Adapted 10^{-7} .

Table 4.13: Average C_t values for *gadB* at 60 minutes

Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	$10^{-5.5}$	10^{-6}	$10^{-6.5}$	10^{-7}	$10^{-7.5}$
Control	21.5 ± 0.28	25.1 ± 1.91	27.86 ± 2.7	31.91 ± 2.48	35.67 ± 2.3	36.27 ± 2.28	37.94 ± 1.22		
Acid shocked	21.9 ± 0.85	26.4 ± 0.32	29.88 ± 1.07	32.8 ± 0.47	34.1 ± 1.10	36.23 ± 1.18			
Acid adapted	17.16 ± 0.28	22.15 ± 2.30	25.3 ± 1.63	26.95 ± 1.06	28.18 ± 1.45	30.97 ± 1.66	32.15 ± 2.31	34.98 ± 1.49	36.13 ± 1.69

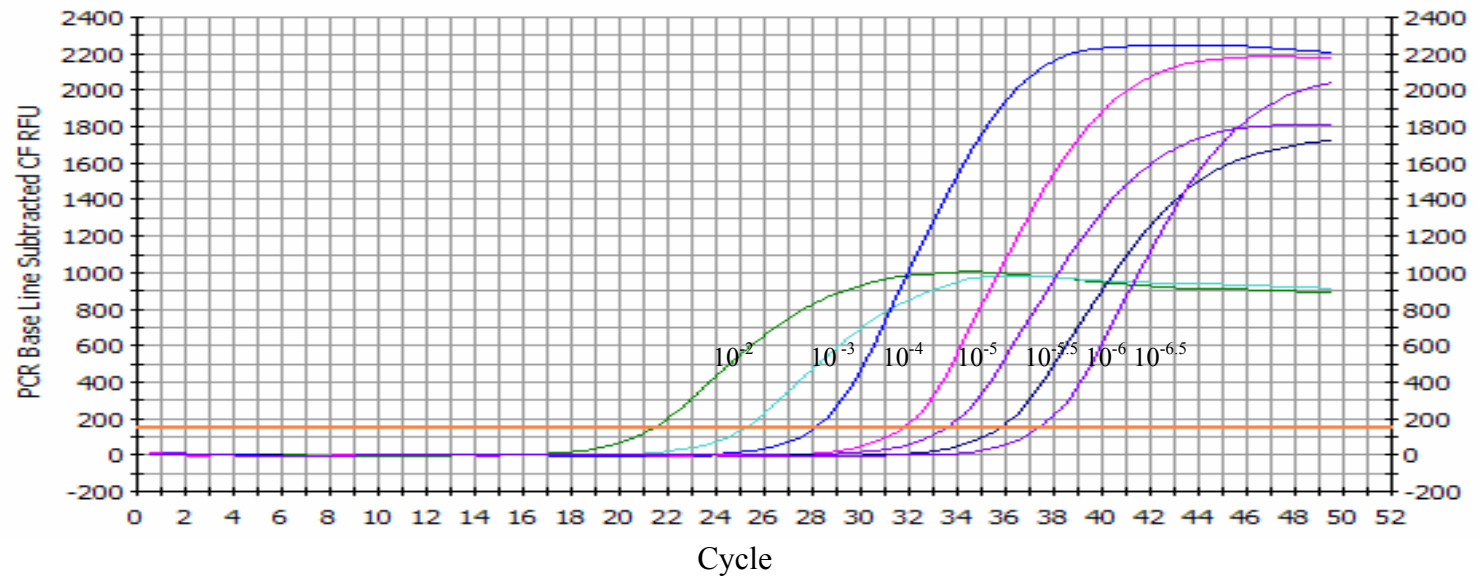


Figure 4.29: C_t curves for control *gadB* dilutions at 60 minutes

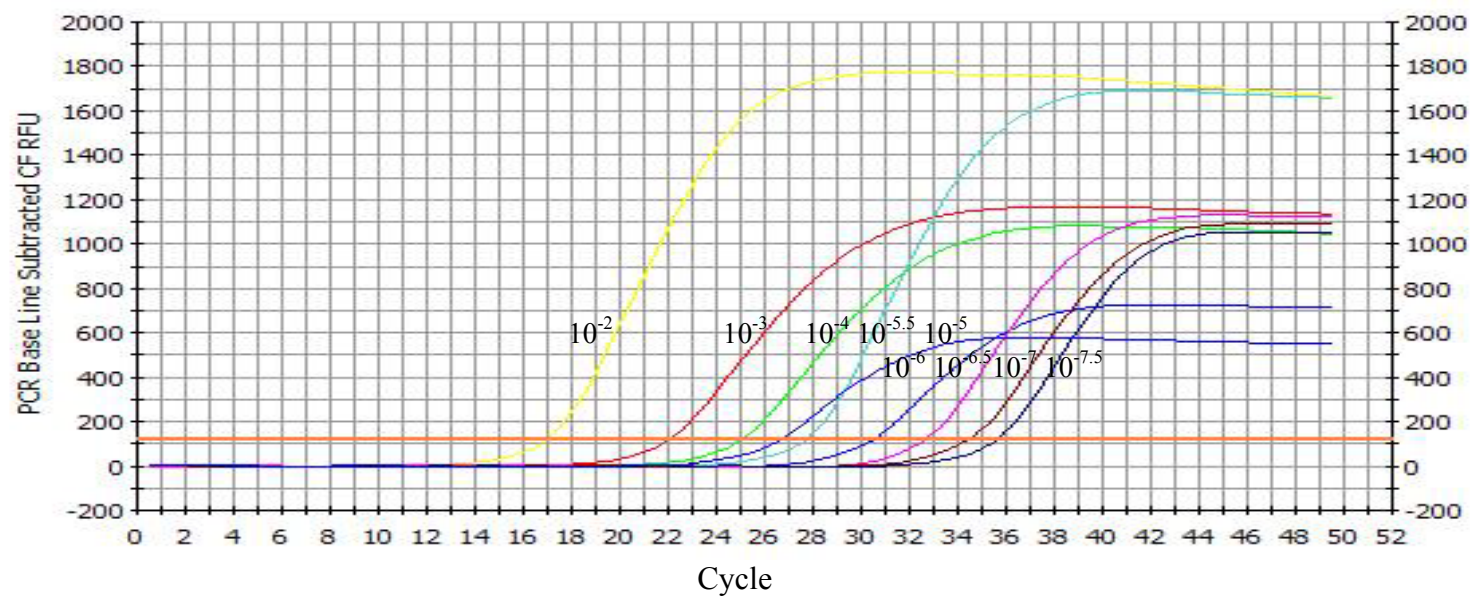


Figure 4.30: Ct curves for acid adapted *gadB* dilutions at 60 minutes

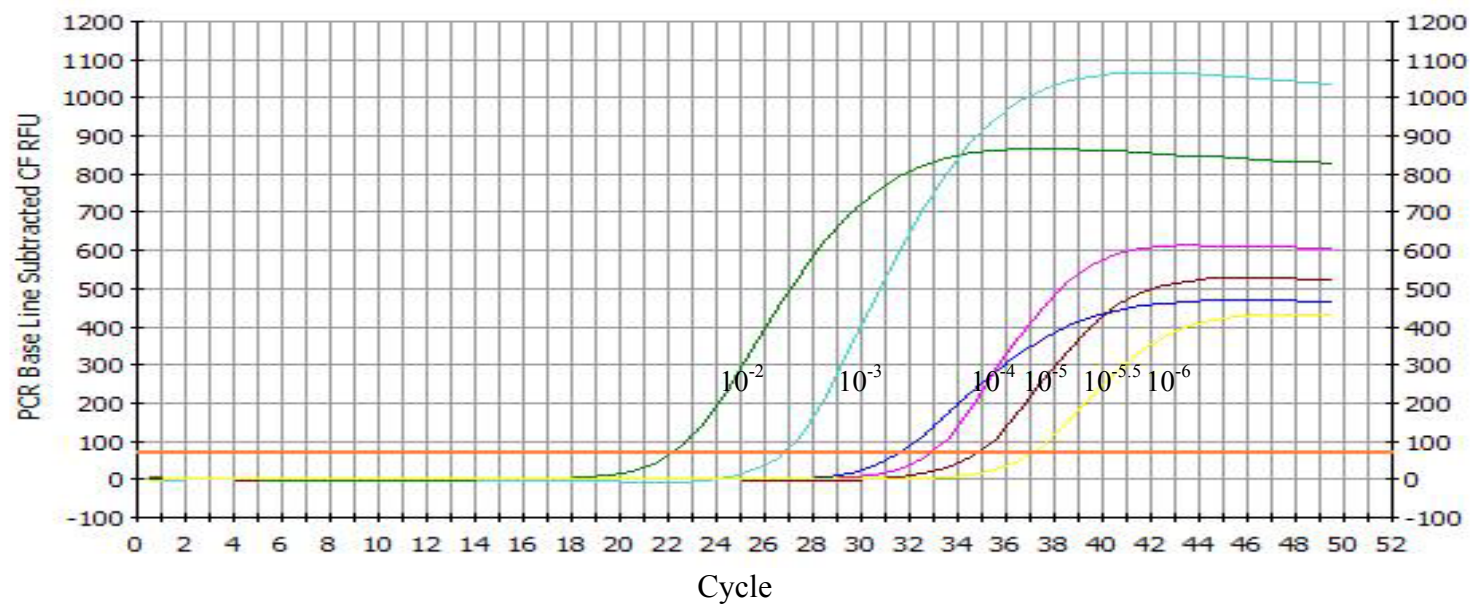
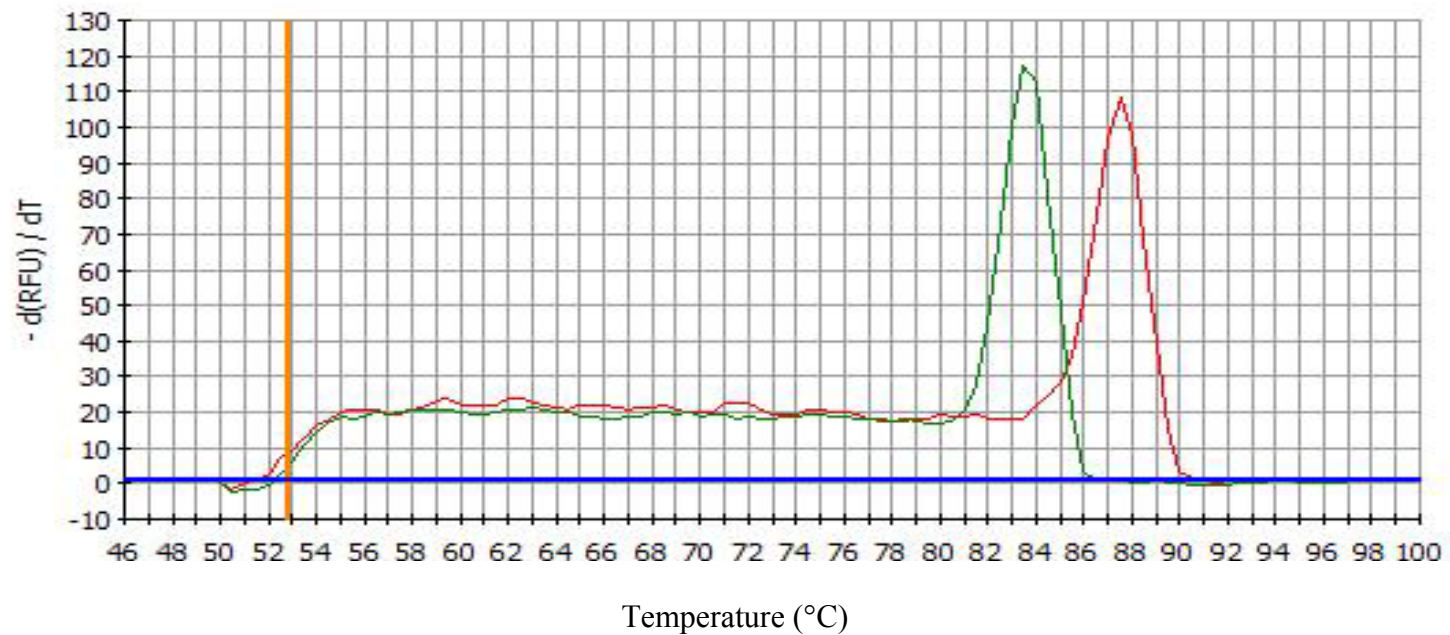


Figure 4.31: C_t curves for acid shocked *gadB* dilutions at 60 minutes



Temperature (°C)
 Figure 4.32: T_m curve for *gadB*
 *Red: T_m for *hemX*, 88°C; Green: T_m for *gadB*, 83°C

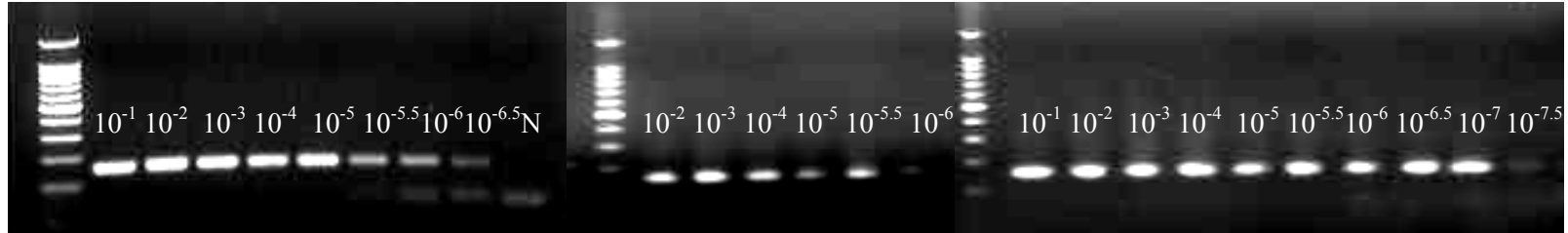


Figure 4.33: Gel of *gadB* dilutions at 60 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lanes 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: Control 10^{-5} ; Lane 7: Control $10^{-5.5}$; Lane 8: Control 10^{-6} ; Lane 9: Control $10^{-6.5}$; Lane 10: negative; Lane 11: 100bp DNA marker; Lane 12: Acid Shocked 10^{-2} ; Lane 12: Acid Shocked 10^{-3} ; Lane 13: Acid Adapted 10^{-4} ; Lane 14: Acid Shocked 10^{-5} ; Lane 15: Acid Shocked $10^{-5.5}$; Lane 16: Acid Shocked 10^{-6} ; Lane 17: 100bp DNA marker; Lane 18: Acid Adapted 10^{-1} ; Lane 19: Acid adapted 10^{-2} ; Lane 20: Acid Adapted 10^{-3} ; Lane 21: Acid Adapted 10^{-4} ; Lane 22: Acid Adapted 10^{-5} ; Lane 23: Acid Adapted $10^{-5.5}$; Lane 24: Acid Adapted 10^{-6} ; Lane 25: Acid Adapted $10^{-6.5}$; Lane 26: Acid Adapted 10^{-7} ; Lane 27: Acid Adapted $10^{-7.5}$; Lane 28: empty.

Table 4.14: Average C_t values for *gadB* at 0 minutes

Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	$10^{-5.5}$	10^{-6}	$10^{-6.5}$	10^{-7}	$10^{-7.5}$
Control	20.5 ± 0.28	22.14 ± 1.91	25.66 ± 2.70	29.1 ± 2.48	31.27 ± 2.3	34.05 ± 1.22	35.27 ± 2.28		
Acid shocked	19.73 ± 0.4	22.37 ± 0.55	25.92 ± 0.1	28.97 ± 0.35	32.3 ± 0.3	34.3 ± 0.17			
Acid adapted	17.17 ± 0.33	22.89 ± 0.1	25.73 ± 2.08	29.18 ± 1.87		32.46 ± 0.52	33.87 ± 0.56	35.8 ± 0.62	36.1 ± 0.38

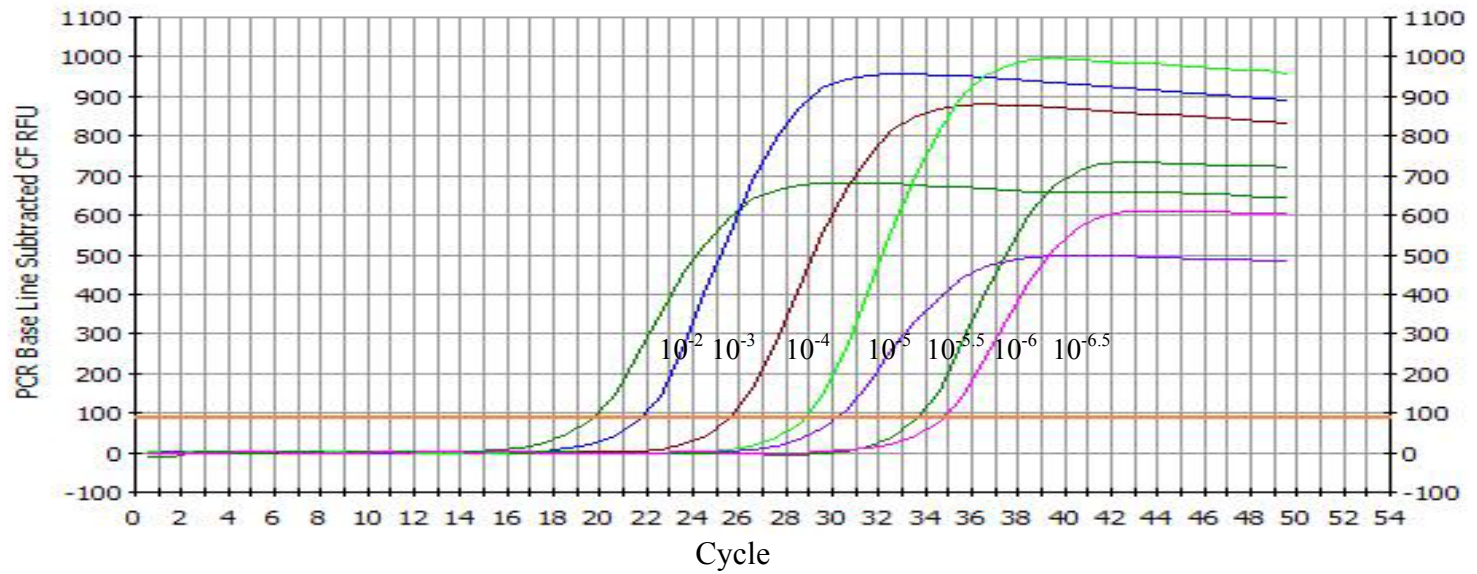


Figure 4.34: C_t curves for control *gadB* dilutions at 0 minutes

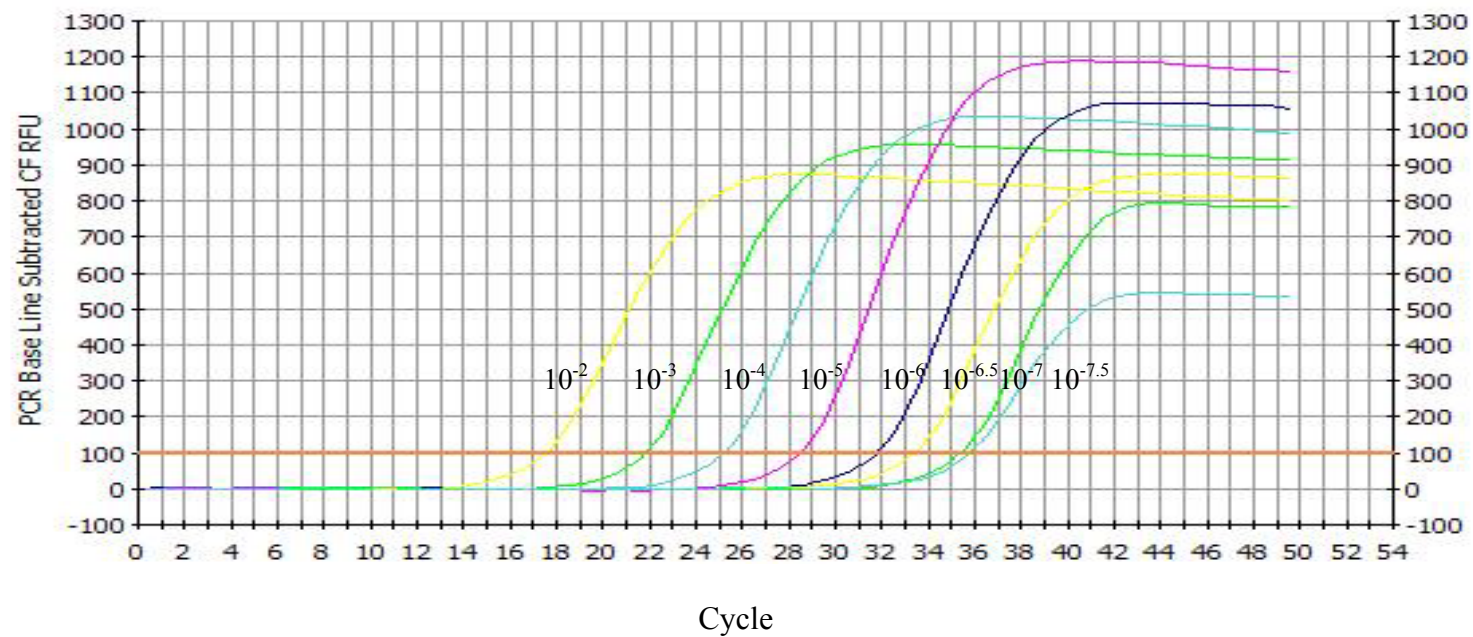


Figure 4.35: C_t curves for acid adapted *gadB* dilutions at 0 minutes

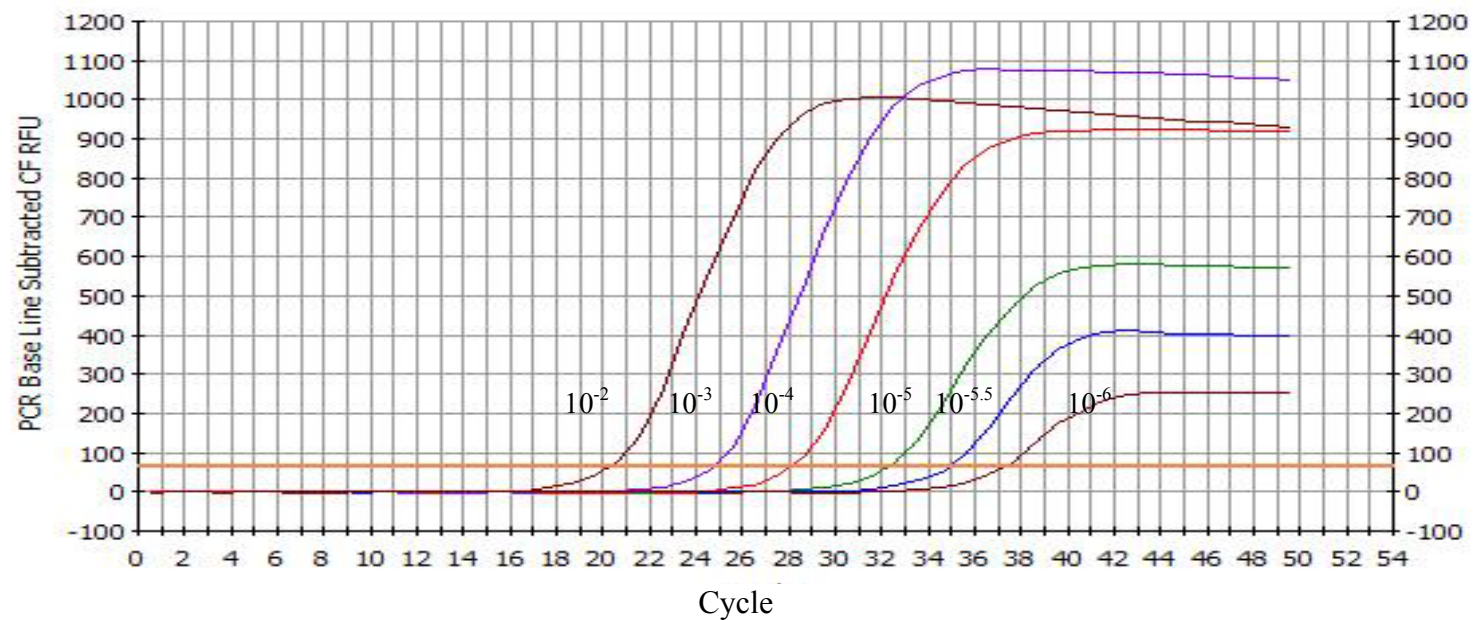


Figure 4.36: C_t curves for acid shocked *gadB* dilutions at 0 minutes

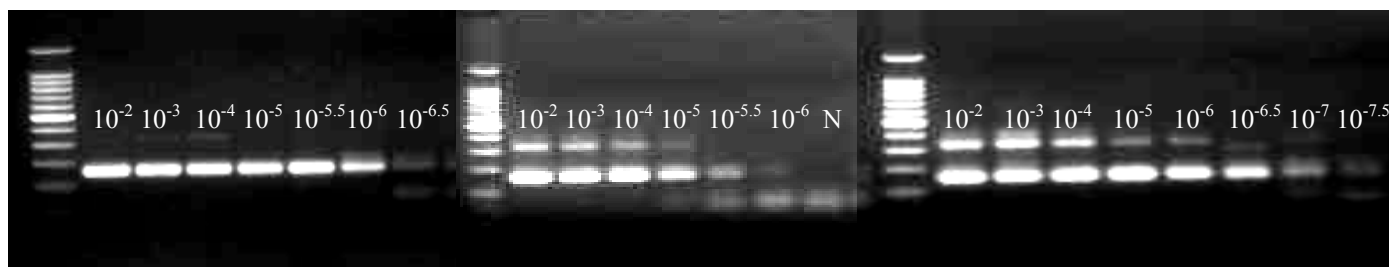


Figure 4.37: Gel of *gadB* dilutions at 0 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lanes 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: Control $10^{-5.5}$; Lane 7: Control 10^{-6} ; Lane 8: Control $10^{-6.5}$; Lane 9: 100bp DNA marker; Lane 10: Acid Shocked 10^{-2} ; Lane 11: Acid Shocked 10^{-3} ; Lane 12: Acid Adapted 10^{-4} ; Lane 13: Acid Shocked 10^{-5} ; Lane 14: Acid Shocked $10^{-5.5}$; Lane 15: Acid Shocked 10^{-6} ; Lane 16: 100bp DNA marker; Lane 17: Acid Adapted 10^{-2} ; Lane 18: Acid adapted 10^{-3} ; Lane 19: Acid Adapted 10^{-4} ; Lane 20: Acid Adapted 10^{-5} ; Lane 21: Acid Adapted 10^{-6} ; Lane 22: Acid Adapted $10^{-6.5}$; Lane 23: Acid Adapted 10^{-7} ; Lane 24: Acid Adapted $10^{-7.5}$.

Table 4.15: Average C_t values for <i>gadB</i> at 5 minutes											
Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	$10^{-5.5}$	10^{-6}	$10^{-6.5}$	10^{-7}	$10^{-7.5}$	10^{-8}	$10^{-8.5}$
Control	19.0 ± 0.14	22.65 ± 0.21	27.0 ± 0.28	30.8 ± 0.42	31.65 ± 0.35	32.85 ± 0.97	34.3 ± 0.14				
Acid shocked	16.6 ± 0.17	21.3 ± 0.95	26.4 ± 0.25	29.97 ± 0.12	30.83 ± 0.57	35.3 ± 0.35					
Acid adapted	13.2 ± 0.26	18.2 ± 0.36	23.07 ± 0.41	26.4 ± 0.71	27.93 ± 0.87	30.7 ± 0.4	31.9 ± 0.34	33.87 ± 0.67	34.9 ± 0.66	36.07 ± 0.06	37.63 ± 0.81

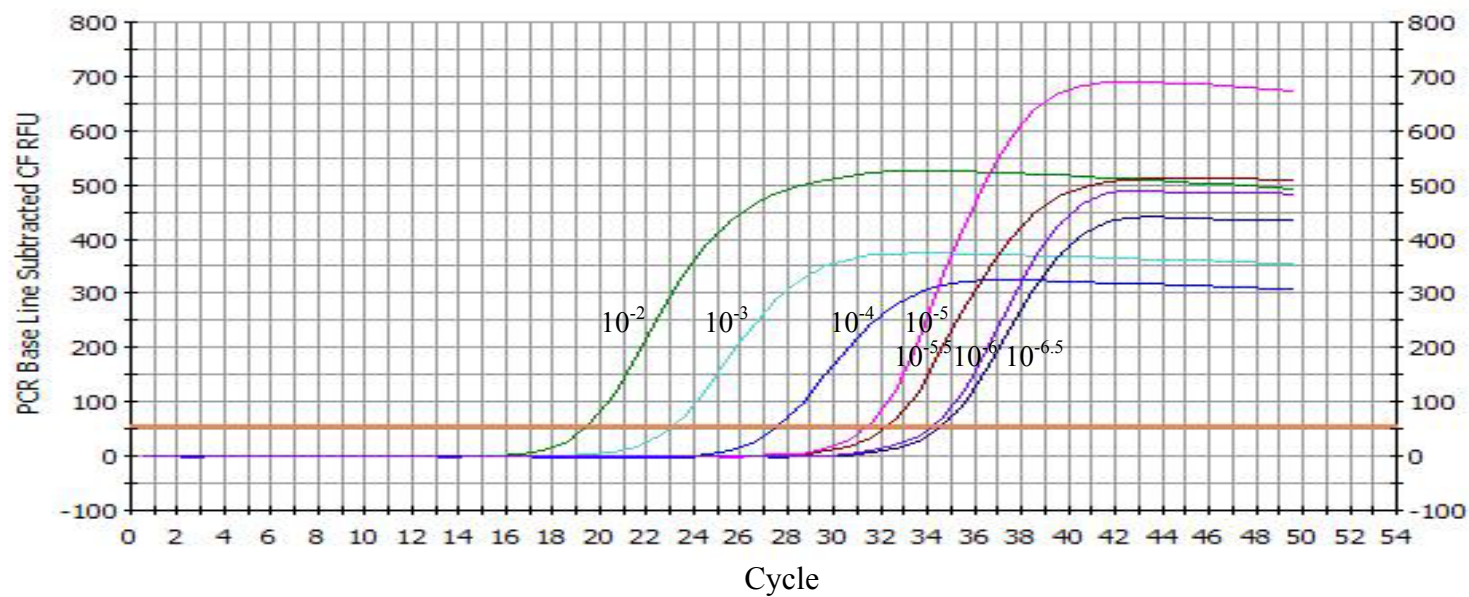


Figure 4.38: C_t curves for control *gadB* at 5 minutes

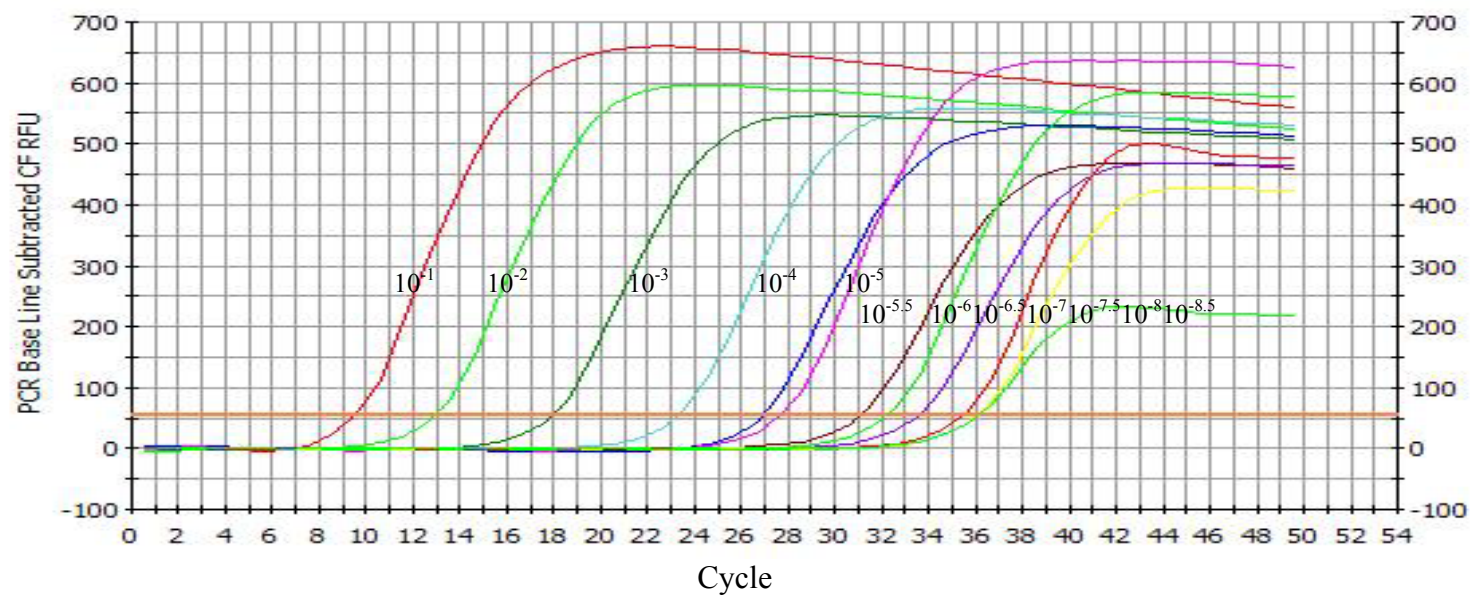


Figure 4.39: C_t curves for acid adapted *gadB* dilutions at 5 minutes

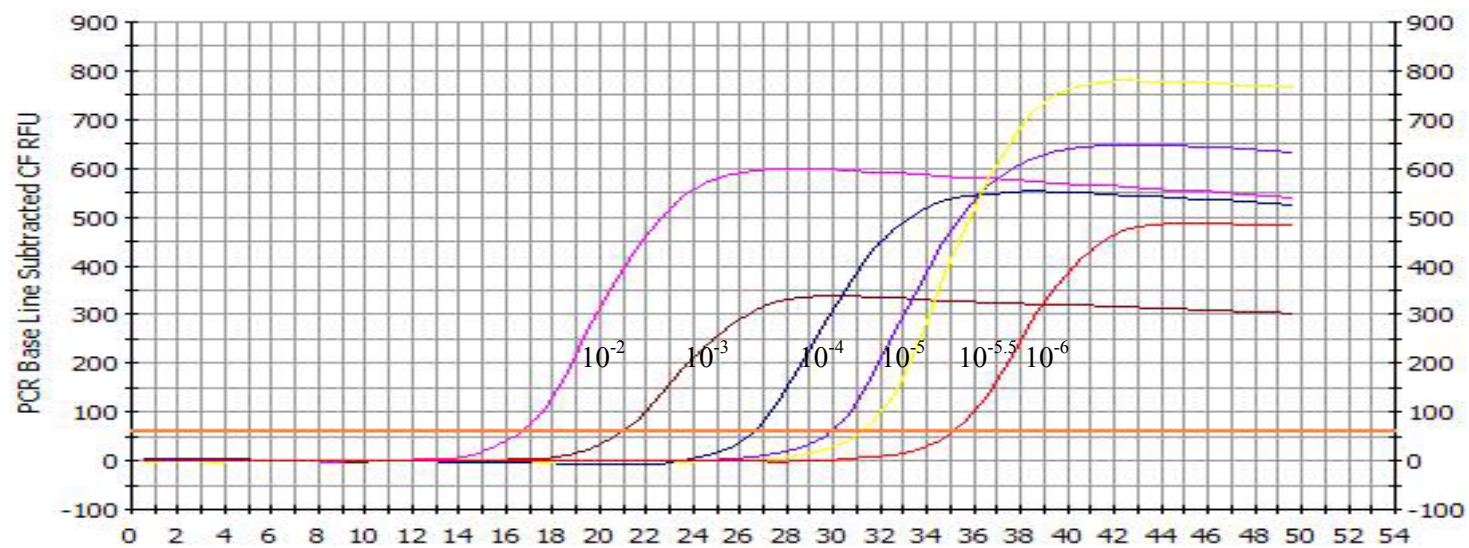


Figure 4.40: C_t curves for acid shocked *gadB* dilutions at 5 minutes

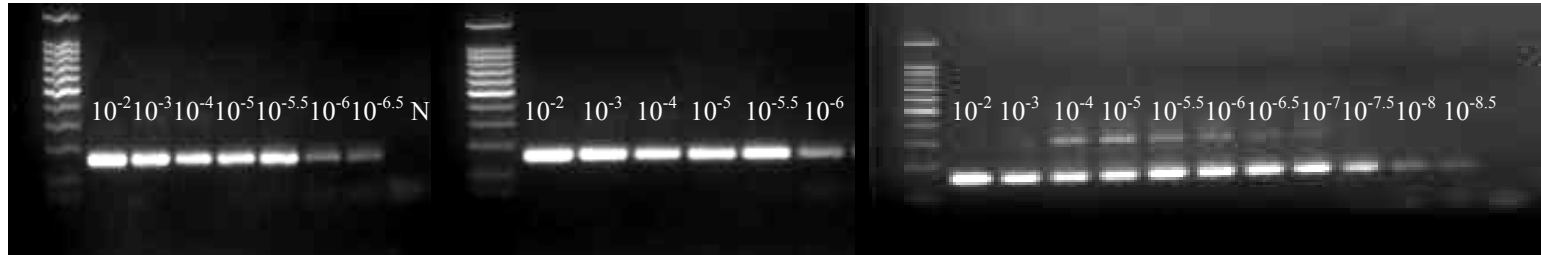


Figure 4.41: Gel of *gadB* dilutions at 5 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lanes 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: Control $10^{-5.5}$; Lane 7: Control 10^{-6} ; Lane 8: Control $10^{-6.5}$; Lane 9: negative; Lane 10: 100bp DNA marker; Lane 11: Acid Shocked 10^{-2} ; Lane 12: Acid Shocked 10^{-3} ; Lane 13: Acid Adapted 10^{-4} ; Lane 14: Acid Shocked 10^{-5} ; Lane 15: Acid Shocked $10^{-5.5}$; Lane 16: Acid Shocked 10^{-6} ; Lane 17: 100bp DNA marker; Lane 18: Acid Adapted 10^{-2} ; Lane 19: Acid adapted 10^{-3} ; Lane 20: Acid Adapted 10^{-4} ; Lane 21: Acid Adapted 10^{-5} ; Lane 22: Acid Adapted $10^{-5.5}$; Lane 23: Acid Adapted 10^{-6} ; Lane 24: Acid Adapted $10^{-6.5}$; Lane 25: Acid Adapted 10^{-7} ; Lane 26: Acid Adapted $10^{-7.5}$; Lane 27: Acid Adapted 10^{-8} ; Lane 28 Acid Adapted $10^{-8.5}$; Lane 29: empty.

Table 4.16: Average C_t values for *gadB* at 15 minutes

Treatment	10^{-2}	10^{-3}	10^{-4}	10^{-5}	$10^{-5.5}$	10^{-6}	$10^{-6.5}$	10^{-7}	$10^{-7.5}$	10^{-8}
Control	19.4 ± 0.42	22.75 ± 0.07	26.07 ± 0.71	30.65 ± 0.64	31.4 ± 0.71	34.4 ± 0.14	35.1 ± 0.28			
Acid shocked	25.53 ± 0.67	30.2 ± 0.46	33.47 ± 0.21	35.1 ± 0.17	36.4 ± 0.20	37.17 ± 0.21				
Acid adapted	12.6 ± 0.40	19.37 ± 0.21	24.3 ± 0.36	28.1 ± 0.37	29.27 ± 0.60	31 ± 0.36	32.5 ± 0.44	33.5 ± 0.35	35.6 ± 0.5	37.13 ± 0.93

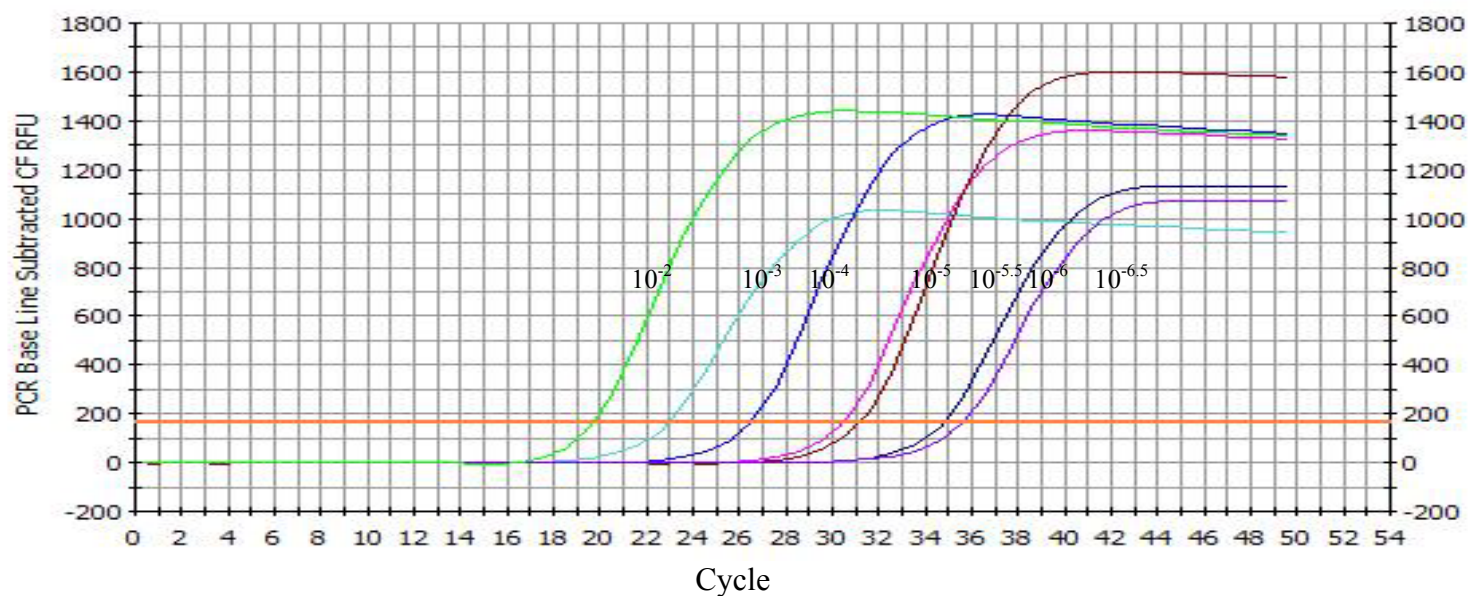


Figure 4.42: C_t curves for control *gadB* dilutions at 15 minutes

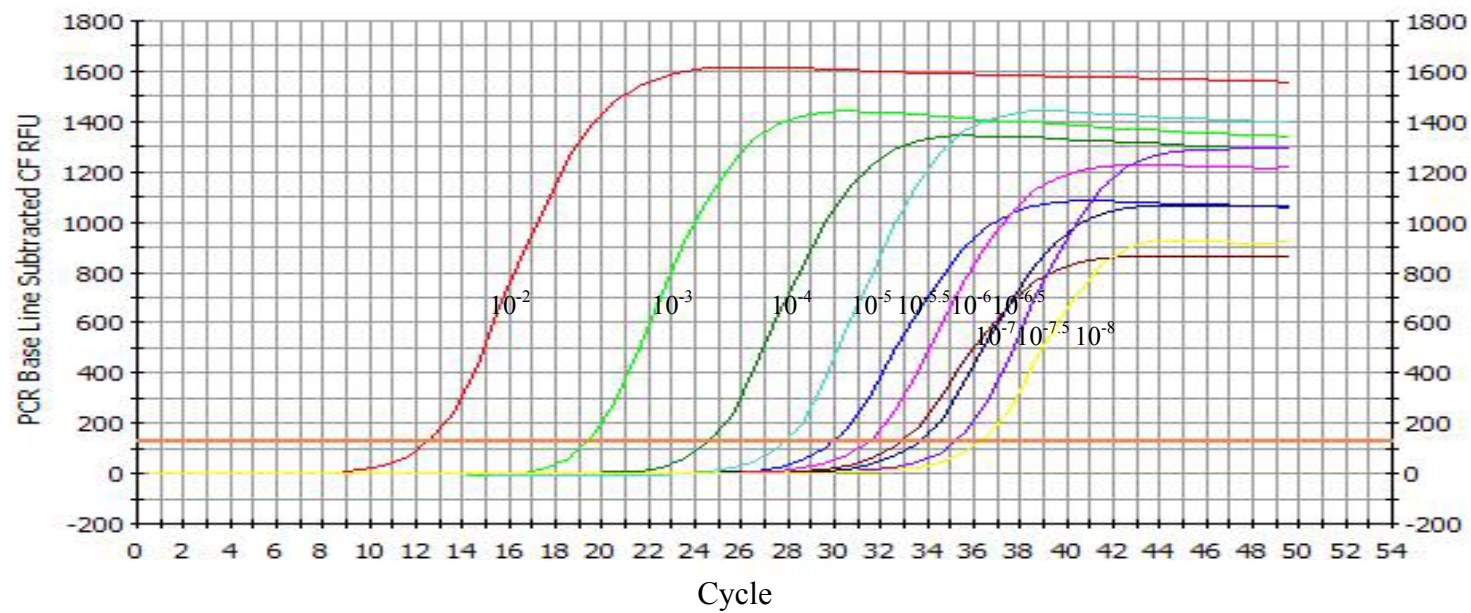


Figure 4.43: C_t curves for acid adapted *gadB* dilutions at 15 minutes

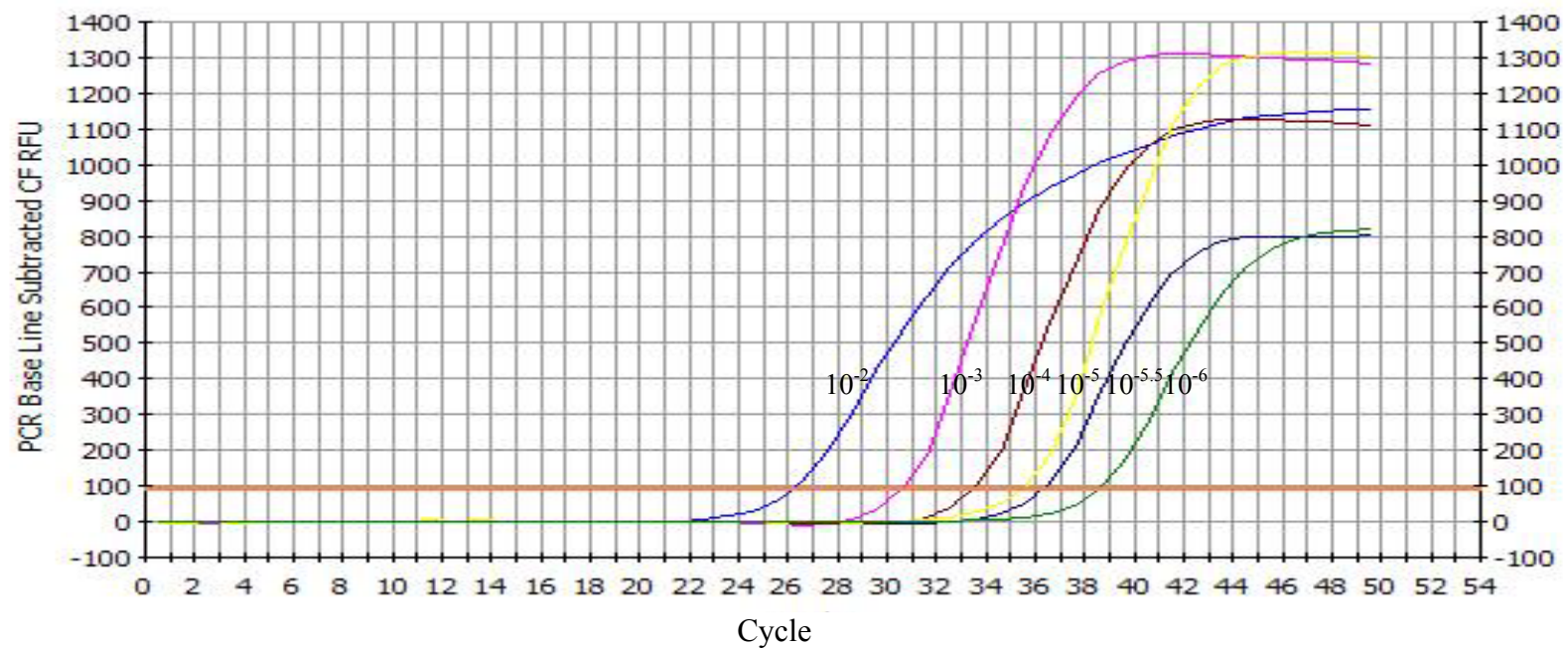


Figure 4.44: C_t curves for acid shocked *gadB* dilutions at 15 minutes

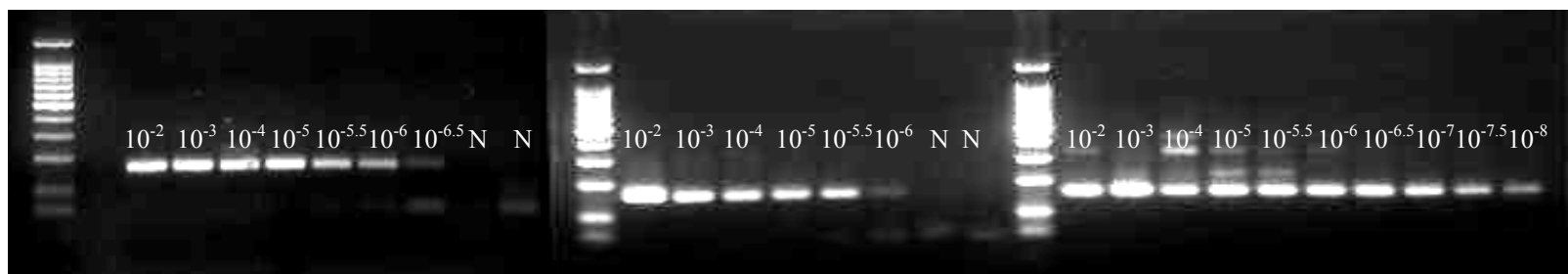


Figure 4.45: Gel of *gadB* dilutions at 15 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lanes 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: Control $10^{-5.5}$; Lane 7: Control 10^{-6} ; Lane 8: Control $10^{-6.5}$; Lane 9 and 10: negative; Lane 11: 100bp DNA marker; Lane 12: Acid Shocked 10^{-2} ; Lane 13: Acid Shocked 10^{-3} ; Lane 14: Acid Shocked 10^{-4} ; Lane 15: Acid Shocked 10^{-5} ; Lane 16: Acid Shocked $10^{-5.5}$; Lane 17 and 18: negatives; Lane 19: 100bp DNA marker; Lane 20: Acid Adapted 10^{-2} ; Lane 21: Acid adapted 10^{-3} ; Lane 22: Acid Adapted 10^{-4} ; Lane 23: Acid Adapted 10^{-5} ; Lane 24: Acid Adapted $10^{-5.5}$; Lane 25: Acid Adapted 10^{-6} ; Lane 26: Acid Adapted $10^{-6.5}$; Lane 27: Acid Adapted 10^{-7} ; Lane 28: Acid Adapted $10^{-7.5}$; Lane 29: Acid Adapted 10^{-8} .

Table 4.17: Average C_t values for <i>gltJ</i> at 60 minutes									
Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}	$10^{-4.5}$	10^{-5}	10^{-6}	$10^{-6.5}$	10^{-7}
Control	20.57 ± 0.29	24.2 ± 0.7	25.86 ± 0.9	27.9 ± 1.11		28.93 ± 0.32	30.73 ± 0.50	32.8 ± 0.9	34.72 ± 0.18
Acid shocked	19.73 ± 0.47	23.63 ± 1.27	27.5 ± 0.89	31.4 ± 1.31	32.77 ± 1.19				
Acid adapted	19.17 ± 0.76	22.23 ± 1.07	26.57 ± 1.55	30.83 ± 2.23	31.97 ± 1.55				

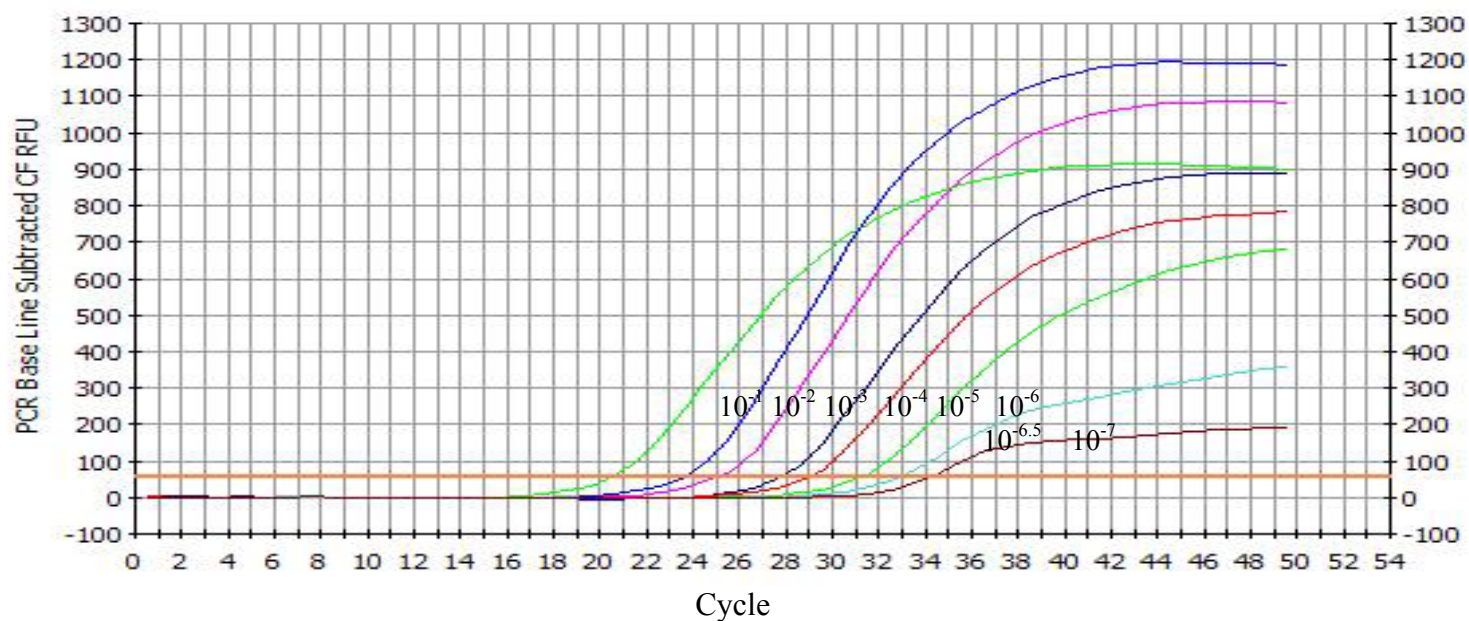


Figure 4.46: C_t curves for control *gltJ* dilutions at 60 minutes

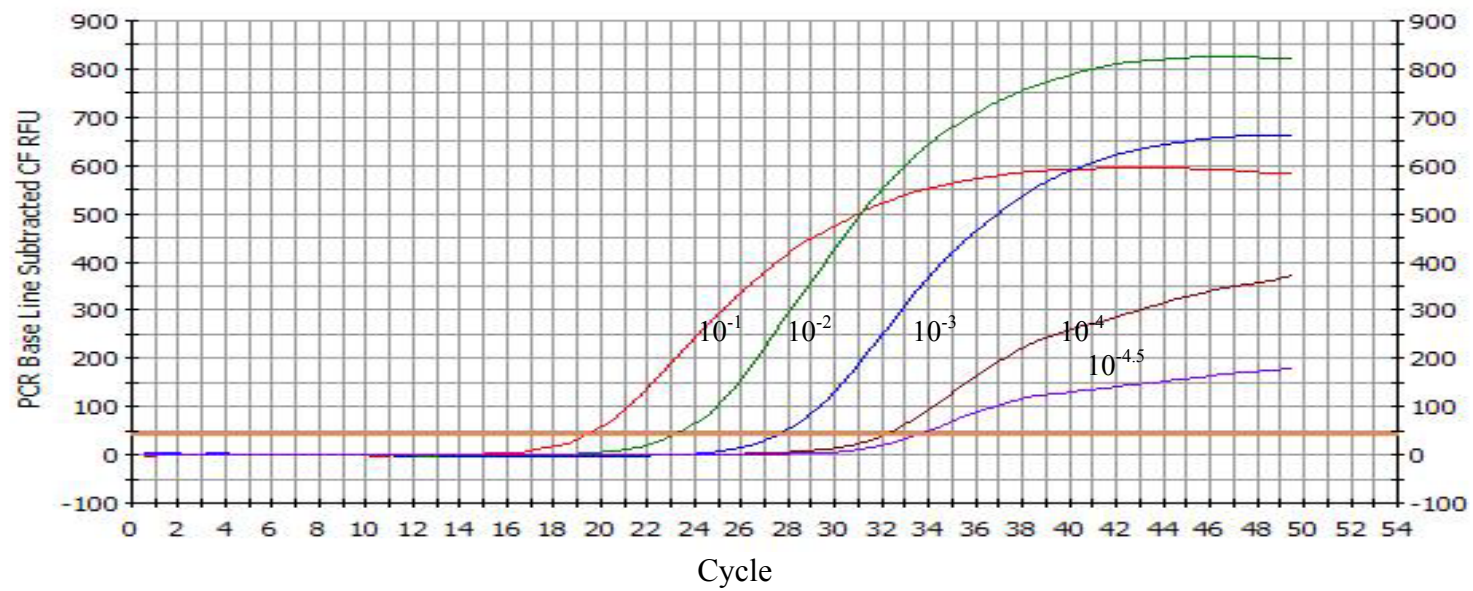


Figure 4.47: C_t curves for acid adapted *gltJ* dilutions at 60 minutes

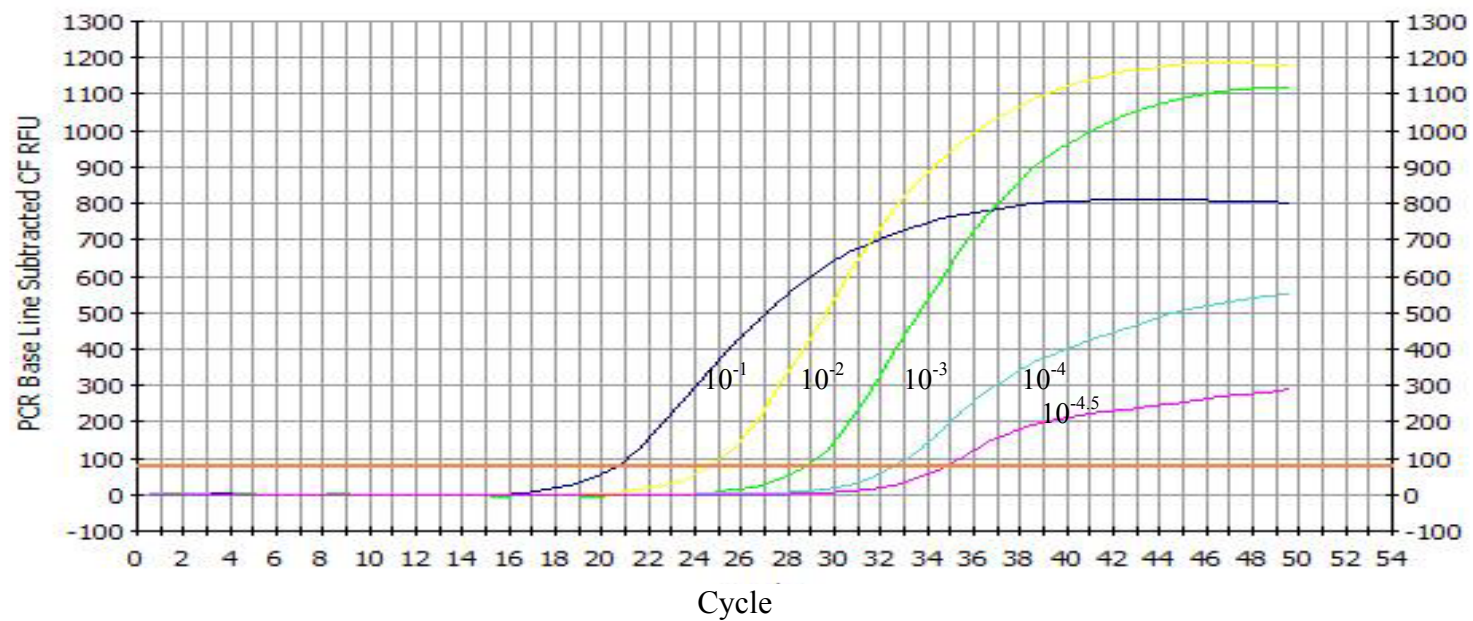


Figure 4.48: C_t curves for acid shocked *gltJ* dilutions at 60 minutes

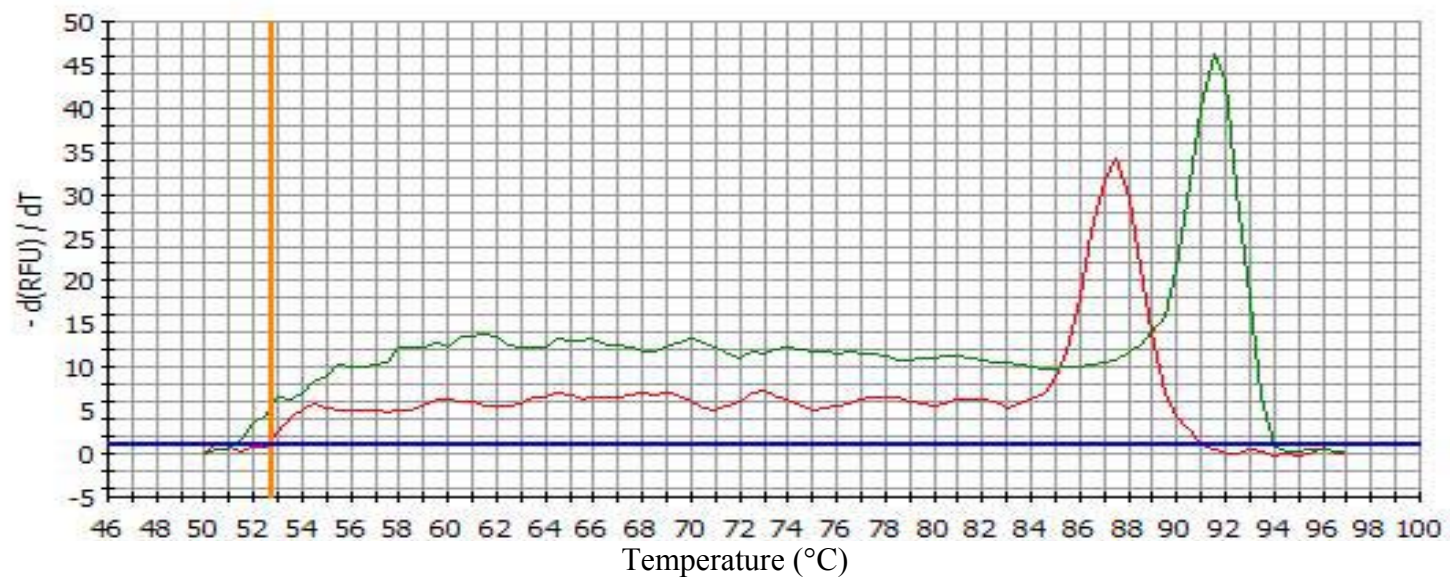


Figure 4.49: T_m curve of *gltJ*

*Red: T_m for *hemX*, 88°C; Green: T_m for *gltJ*, 92°C

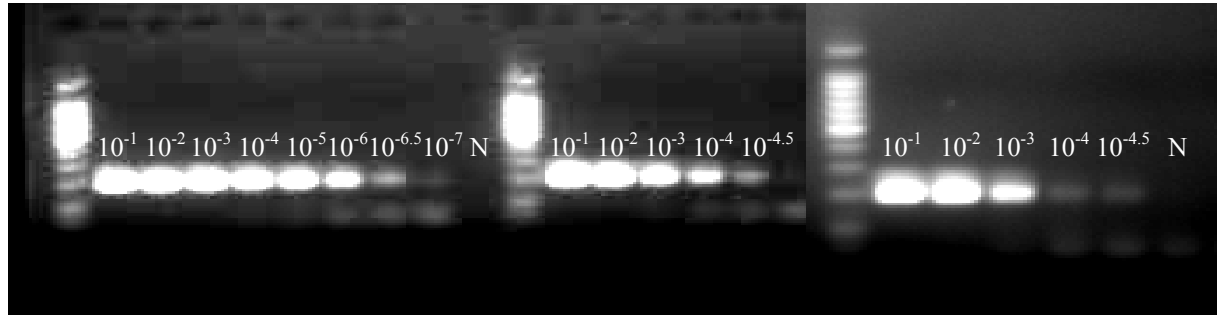


Figure 4.50: Gel of *gltJ* dilutions at 60 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lanes 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: Control 10^{-5} ; Lane 7: Control 10^{-6} ; Lane 8: Control $10^{-6.5}$; Lane 9: Control 10^{-7} ; Lane 10: negative; Lane 11: 100bp DNA marker; Lane 12: Acid Shocked 10^{-1} ; Lane 12: Acid Shocked 10^{-2} ; Lane 13: Acid Adapted 10^{-3} ; Lane 14: Acid Shocked 10^{-4} ; Lane 15: Acid Shocked $10^{-4.5}$; Lane 16: negative; Lane 17: 100bp DNA marker; Lane 18: Acid Adapted 10^{-1} ; Lane 19: Acid adapted 10^{-2} ; Lane 20: Acid Adapted 10^{-3} ; Lane 21: Acid Adapted 10^{-4} ; Lane 22: Acid Adapted $10^{-4.5}$; Lane 23: negative; Lane 24: empty.

Table 4.18: Average Ct values for *gltJ* at 0 minutes

Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}	$10^{-4.5}$	10^{-5}	10^{-6}	$10^{-6.5}$	10^{-7}	$10^{-7.5}$
Control	18.77 ± 0.35	21.33 ± 0.15	25.9 ± 0.17	30.37 ± 0.5		32.83 ± 0.12	33.17 ± 0.55	33.97 ± 0.15		
Acid shocked	14.5 ± 0.28	16.05 ± .07	17.16 ± 0.47	23.37 ± 1.36		28.1 ± 0.20	29.57 ± 0.83	32.7 ± 0.10	32.8 ± 0.53	32.9 ± 0.29
Acid adapted	16.1 ± 0.46	22.6 ± 0.17	28.8 ± 0.95	32.38 ± 0.85	33.57 ± 0.45					

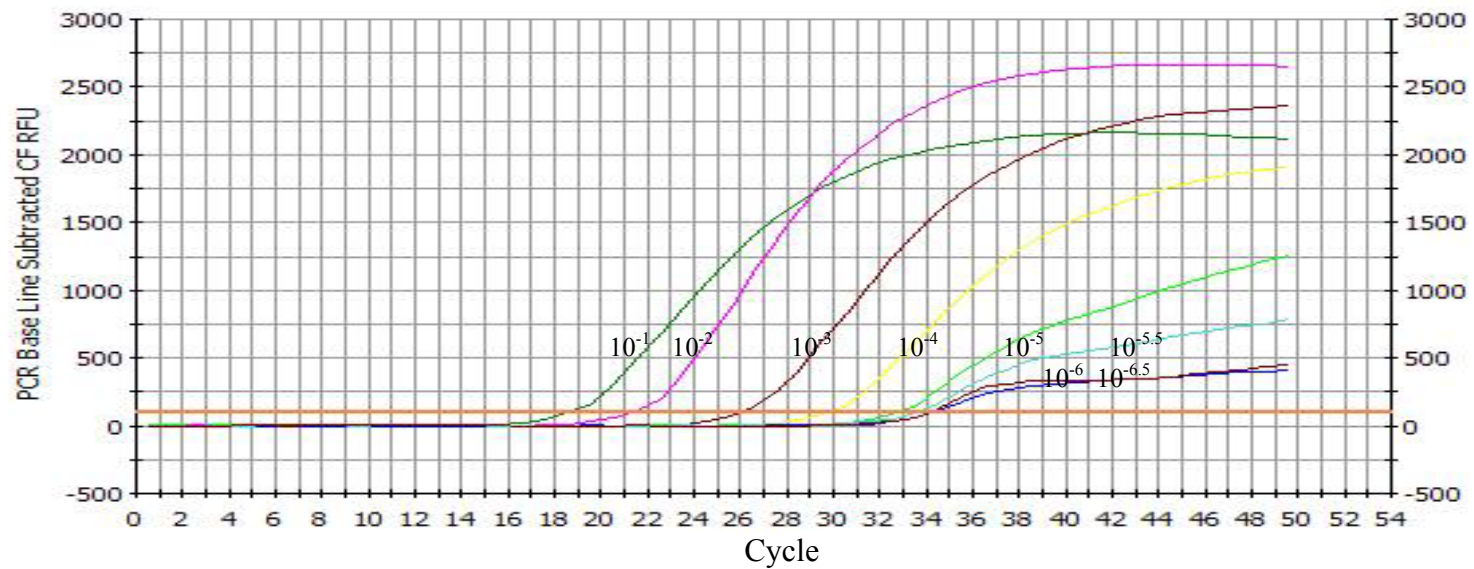


Figure 4.51: C_t curves for control *gltJ* dilutions at 0 minutes

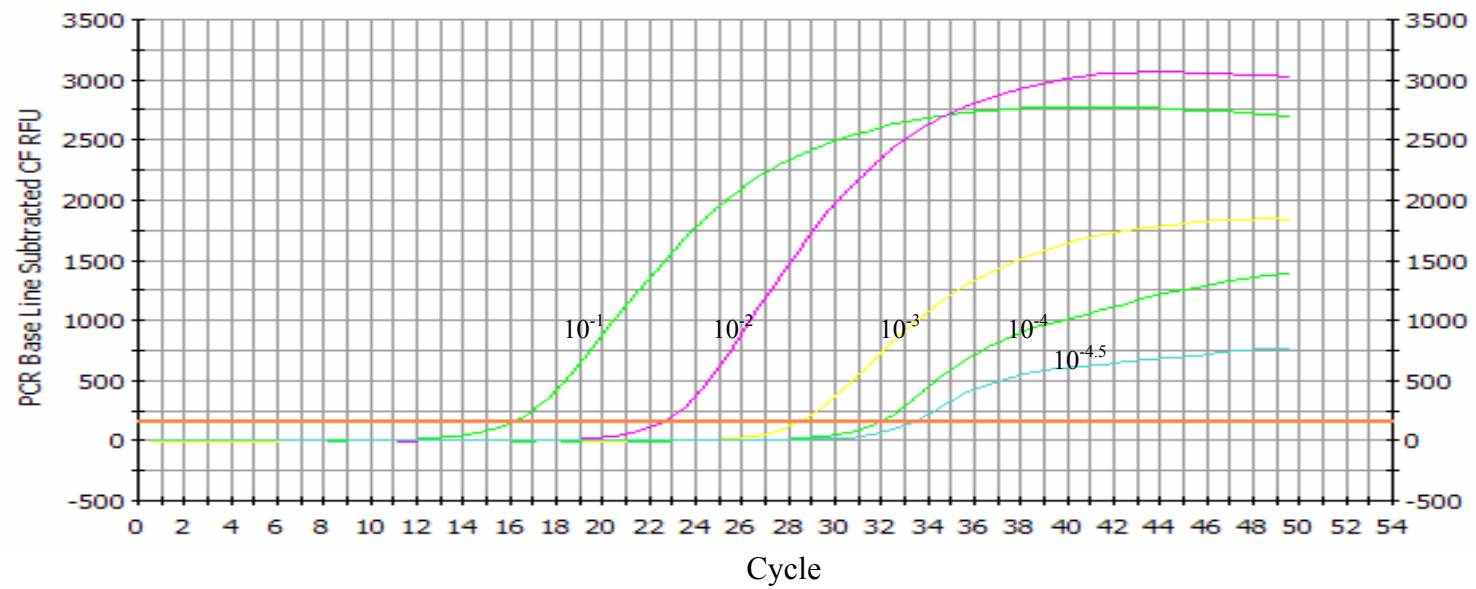


Figure 4.52: C_t curves for acid adapted *gltJ* dilutions at 0 minutes

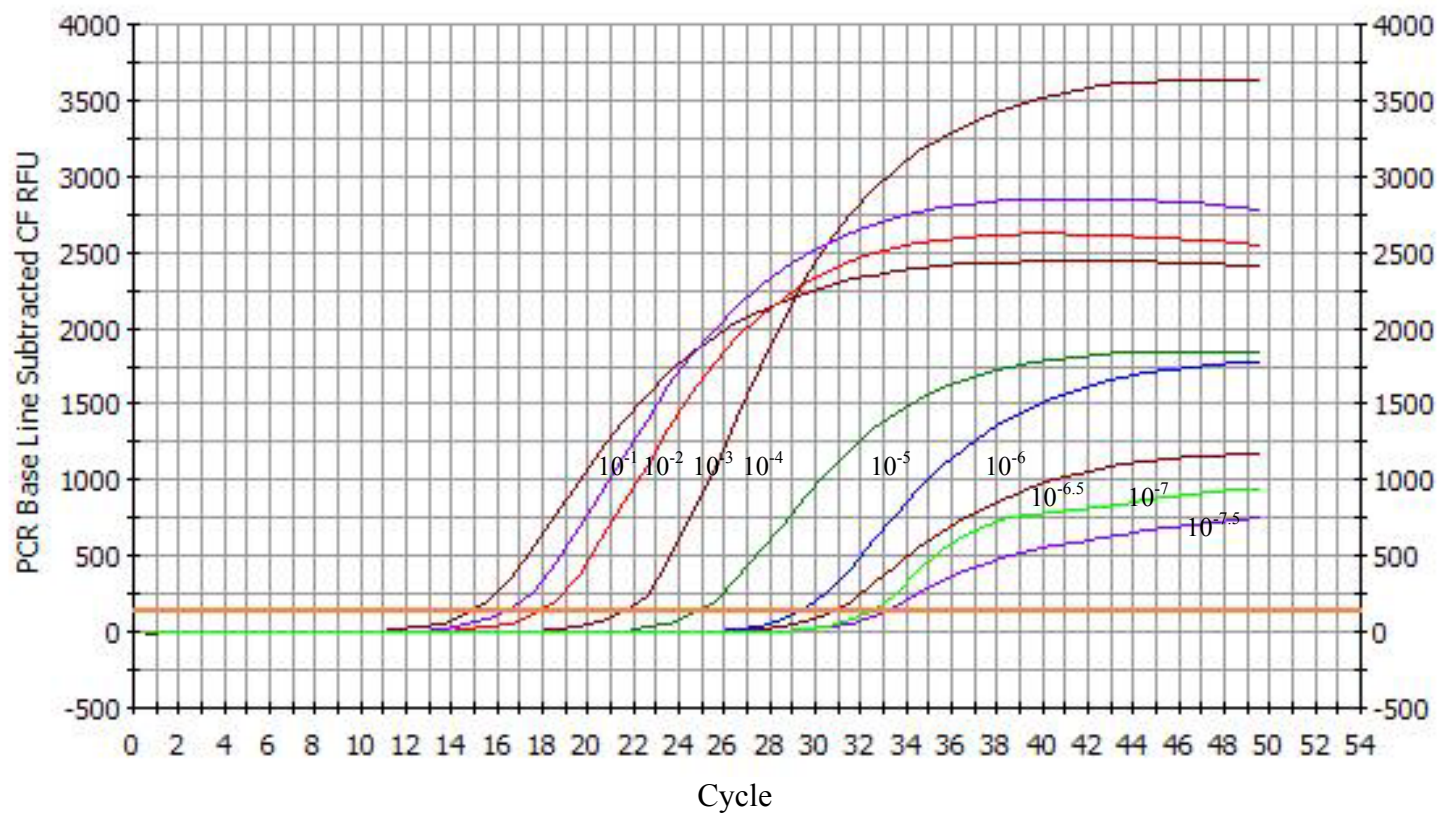


Figure 4.53: C_t curves for acid shocked *gltJ* dilutions at 0 minutes

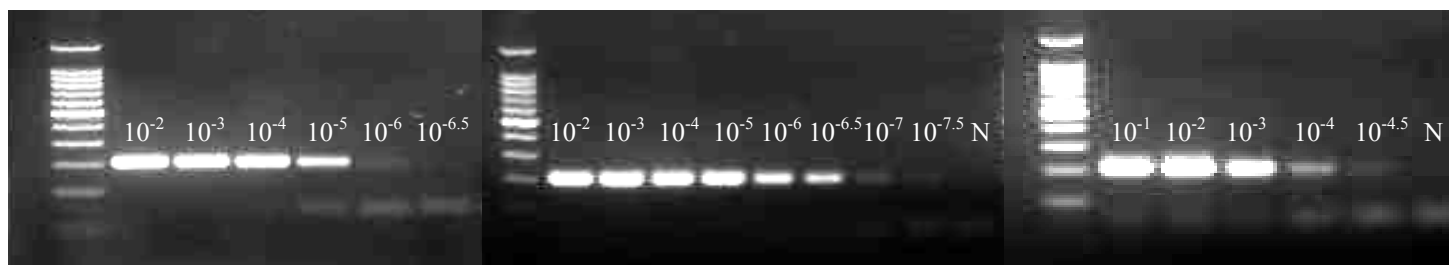


Figure 4.54: Gel of *gltJ* dilutions at 0 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-2} ; Lane 3: Control 10^{-3} ; Lanes 4: Control 10^{-4} ; Lane 5: Control 10^{-5} ; Lane 6: Control 10^{-6} ; Lane 7: Control $10^{-6.5}$ Lane 8: 100bp DNA marker; Lane 9: Acid Shocked 10^{-2} ; Lane 10: Acid Shocked 10^{-3} ; Lane 11: Acid Adapted 10^{-4} ; Lane 12: Acid Shocked 10^{-5} ; Lane 13: Acid Shocked 10^{-6} ; Lane 14: Acid Shocked $10^{-6.5}$; Lane 15: Acid Shocked 10^{-7} ; Lane 16: Acid Shocked $10^{-7.5}$; Lane 17: negative; Lane 18: 100bp DNA marker; Lane 19: Acid Adapted 10^{-1} ; Lane 20: Acid adapted 10^{-2} ; Lane 21: Acid Adapted 10^{-3} ; Lane 22: Acid Adapted 10^{-4} ; Lane 23: Acid Adapted $10^{-4.5}$; Lane 24: negative.

Table 4.18: Average C_t values for <i>gltJ</i> at 5 minutes								
Treatment	10^{-1}	10^{-2}	10^{-3}	10^{-4}	$10^{-4.5}$	10^{-5}	10^{-6}	$10^{-6.5}$
Control	17.73 ± 0.15	20.83 ± 0.29	25.37 ± 0.42	30.2 ± 0.61		32.4 ± 0.5	33.13 ± 0.23	34.33 ± 0.10
Acid shocked	16.7 ± 0.1	20.93 ± 0.11	26.07 ± 0.31	30.57 ± 0.45	31.17 ± 0.25			
Acid adapted	16.63 ± 0.25	21.53 ± 0.49	27.03 ± 0.31	30.15 ± 0.07	31.1 ± 0.57			

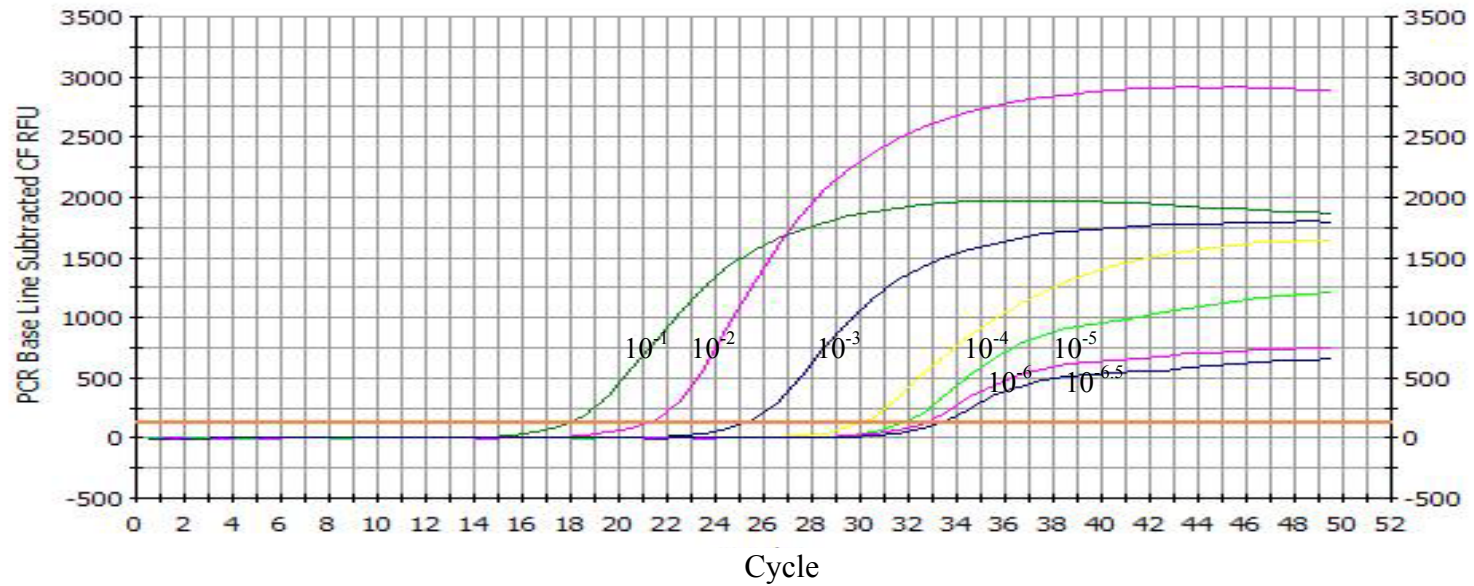


Figure 4.55: C_t curves for control *gltJ* dilutions at 5 minutes

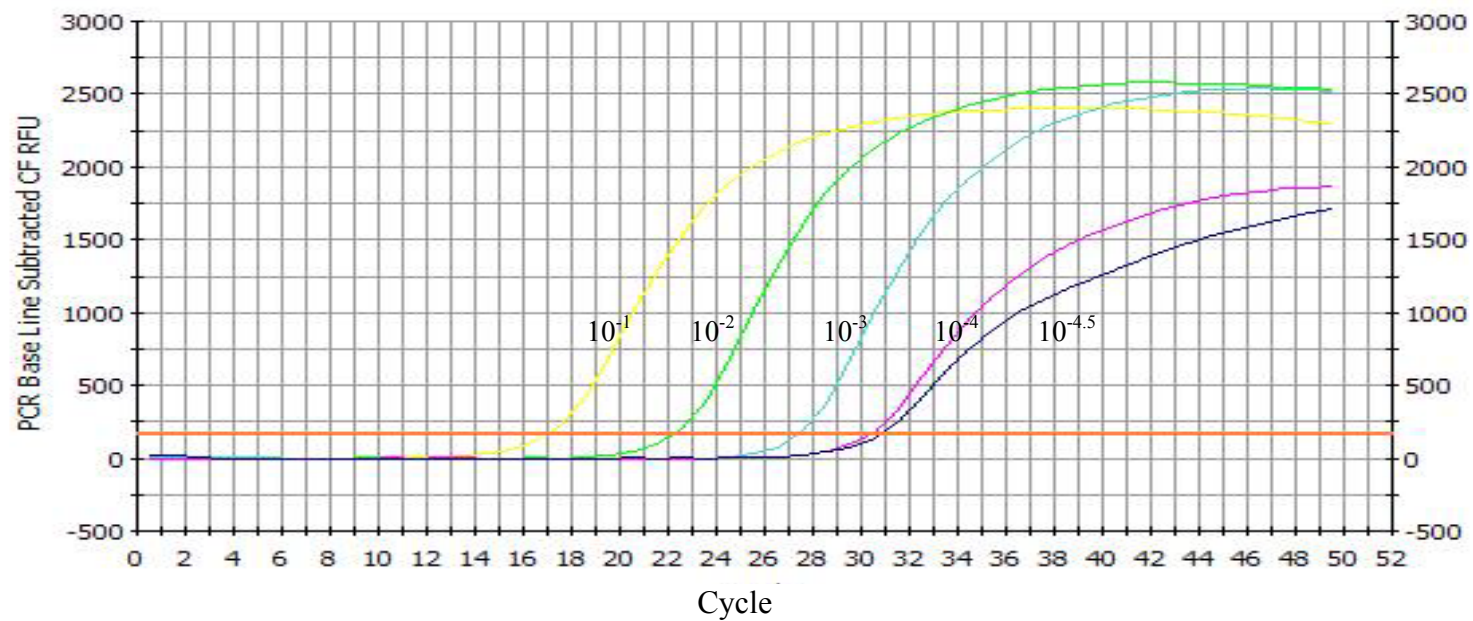


Figure 4.56: C_1 curves of acid adapted *gltJ* dilutions at 5 minutes

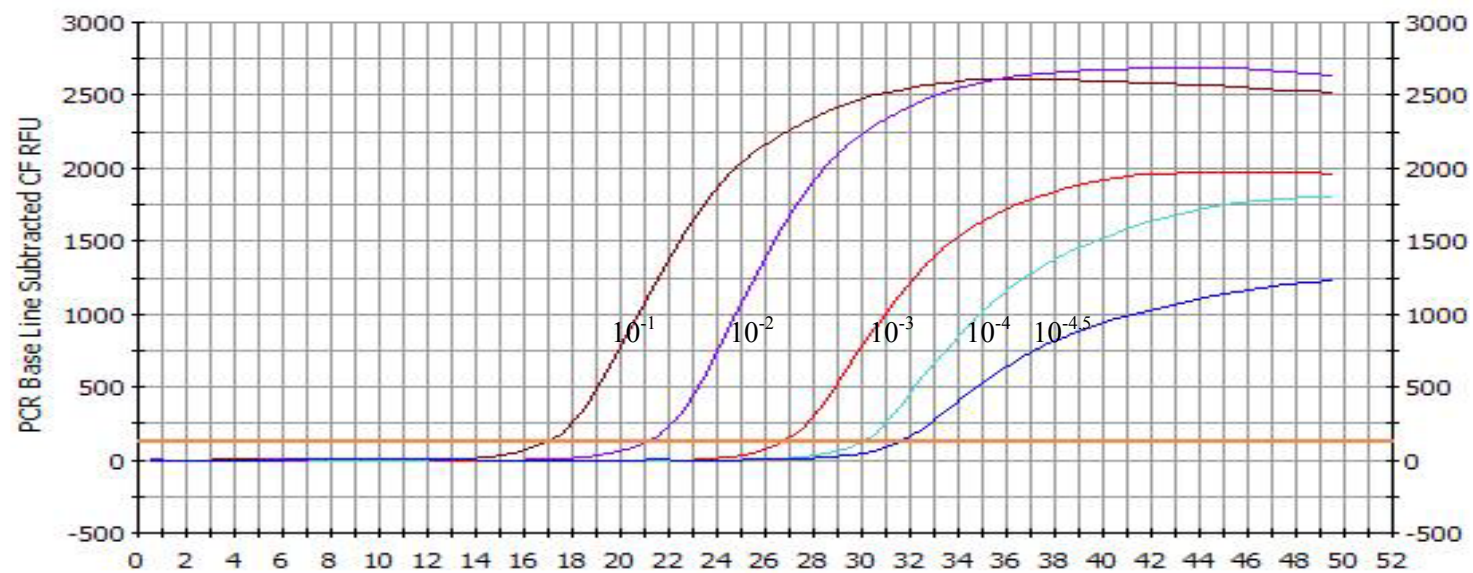


Figure 4.57: C_t curves for acid shocked *gltJ* dilutions at 5 minutes



Figure 4.58: Gel of *gltJ* dilutions at 5 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lanes 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: Control 10^{-5} ; Lane 7: Control 10^{-6} ; Lane 8: Control $10^{-6.5}$; Lane 9: 100bp DNA marker; Lane 10: Acid Shocked 10^{-1} ; Lane 11: Acid Shocked 10^{-2} ; Lane 12: Acid Adapted 10^{-3} ; Lane 13: Acid Shocked 10^{-4} ; Lane 14: Acid Shocked $10^{-4.5}$; Lane 15-19: empty; Lane 20: 100bp DNA marker; Lane 21: Acid Adapted 10^{-1} ; Lane 22: Acid adapted 10^{-2} ; Lane 23: Acid Adapted 10^{-3} ; Lane 24: Acid Adapted 10^{-4} ; Lane 25: Acid Adapted $10^{-4.5}$; Lane 26: empty.

Table 4.19: average C _t values of <i>gltJ</i> at 15 minutes								
Treatment	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ^{-4.5}	10 ⁻⁵	10 ^{-5.5}	10 ⁻⁶
Control	18.07 ± 0.11	20.67 ± 0.21	24.8 ± 0.26	29.33 ± 0.23		32.1 ± 0.3	33.93 ± 0.15	34.05 ± 0.13
Acid shocked	17.17 ± 0.31	21.73 ± 0.06	27.9 ± 0.65	30.87 ± 1.47	32.1 ± 0.52			
Acid adapted	16.75 ± 0.21	22.67 ± 0.35	26.33 ± 0.50	31.6 ± 0.26	32.07 ± 0.31			

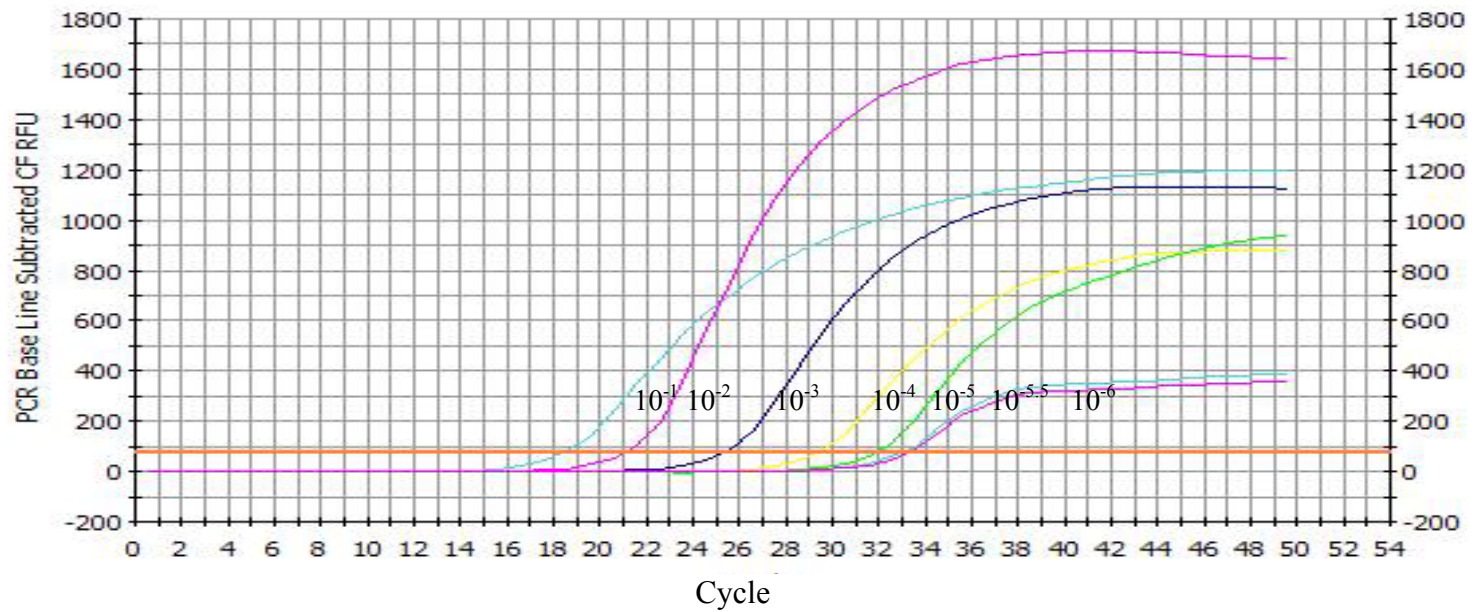


Figure 4.59: C_t curves for control *gltJ* dilutions at 15 minutes

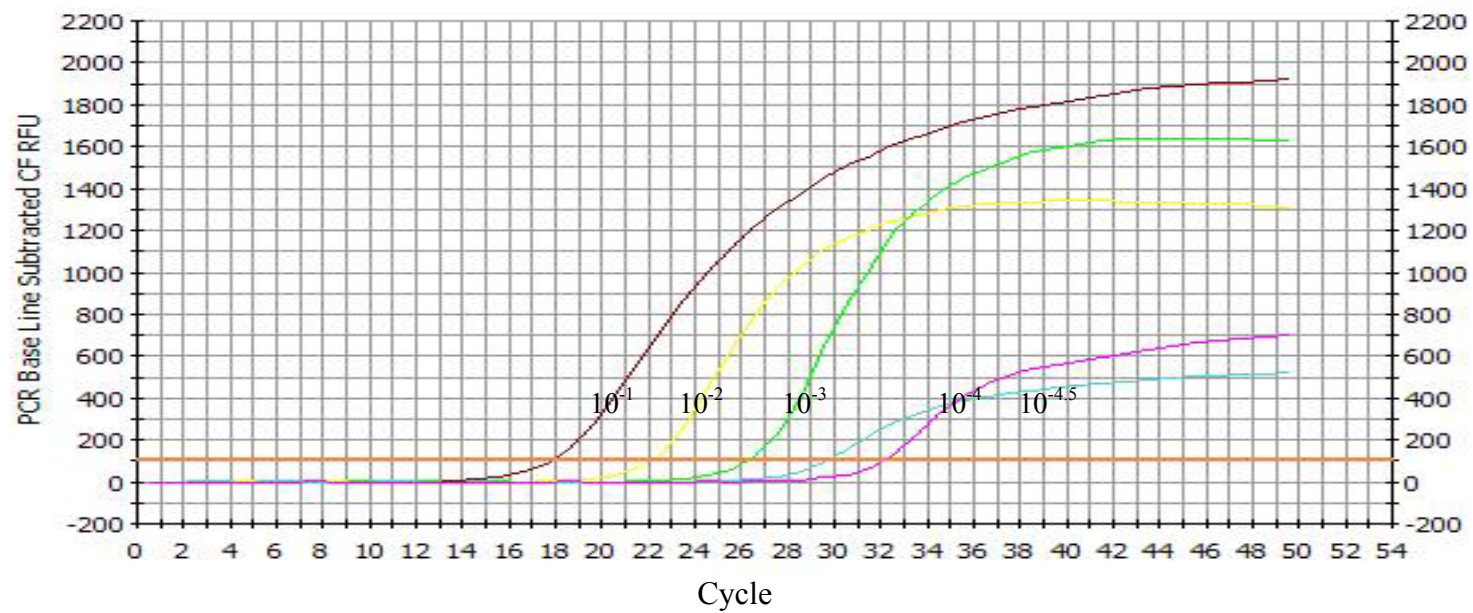


Figure 4.60: C_t curves for acid adapted *gltJ* dilutions at 15 minutes

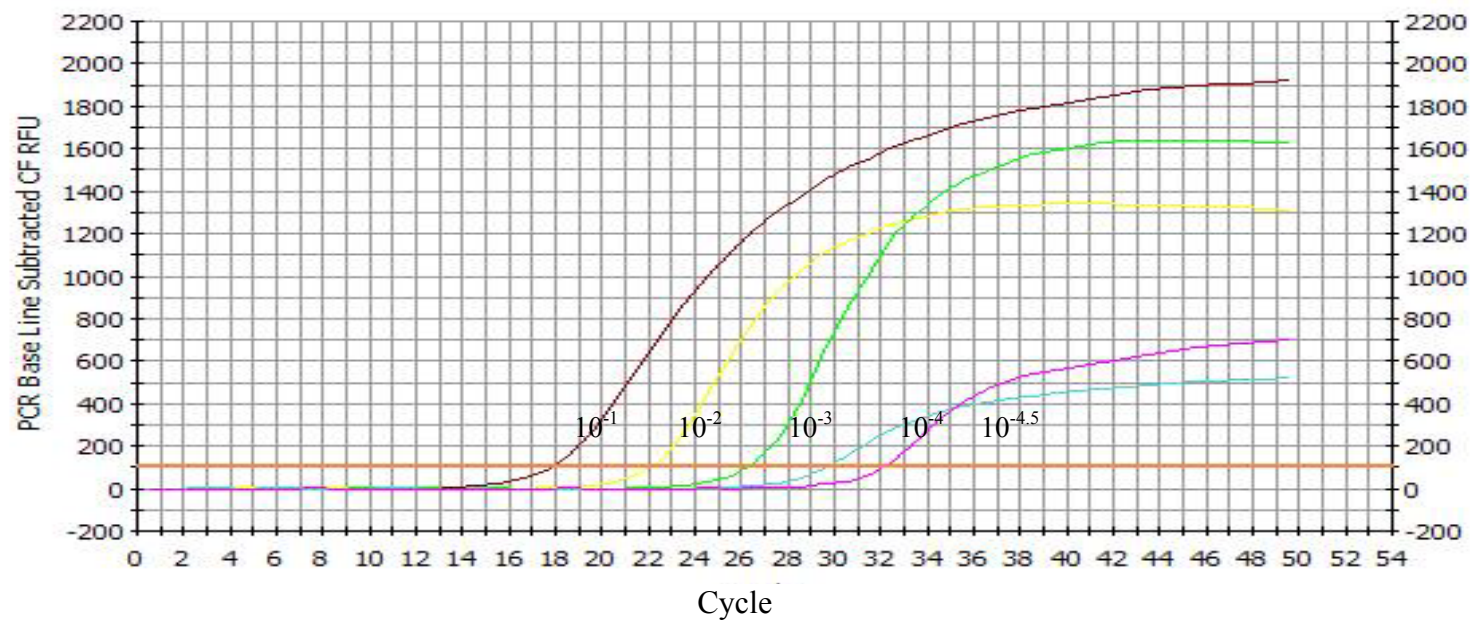


Figure 4.61: C_t curves for acid shocked *gltJ* dilutions at 15 minutes



Figure 4.62: Gel of *gltJ* dilutions at 15 minutes

* Lane 1: 100bp DNA marker; Lane 2: Control 10^{-1} ; Lane 3: Control 10^{-2} ; Lanes 4: Control 10^{-3} ; Lane 5: Control 10^{-4} ; Lane 6: Control 10^{-5} ; Lane 7: Control $10^{-5.5}$; Lane 8: Control 10^{-6} ; Lane 9: negative; Lane 10: 100bp DNA marker; Lane 11: Acid Shocked 10^{-1} ; Lane 12: Acid Shocked 10^{-2} ; Lane 13: Acid Adapted 10^{-3} ; Lane 14: Acid Shocked 10^{-4} ; Lane 15: Acid Shocked $10^{-4.5}$; Lane 16 and 17: negatives; Lane 18 and 19: Empty; Lane 20: 100bp DNA marker; Lane 21: Acid Adapted 10^{-1} ; Lane 22: Acid adapted 10^{-2} ; Lane 23: Acid Adapted 10^{-3} ; Lane 24: Acid Adapted 10^{-4} ; Lane 25: Acid Adapted $10^{-4.5}$; Lane 26: negative.

VITA

Kristina K. Carter was born in the panhandle of Florida on November 10, 1979 to Garrett and Penelope Carter. Kristina, along with her younger brother John G. Carter, grew up in the panhandle of Florida where she later graduated from Choctawhatchee High School in 1997. Following high school, Kristina obtained a Bachelors of Science in Food Science and Human Nutrition from the University of Florida, GO GATORS!! In 2002, Kristina accepted a position with the Florida Department of Health Bureau of Laboratories located in Tampa, FL. In conjunction with working for the DOH, Kristina attended the University of South Florida College of Public Health where she obtained a Master of Science in Public Health specializing in Emerging and Infectious Diseases. In 2004, Kristina began a PhD program at the University of Tennessee and completed her PhD in Food Science and Technology with a minor in Statistics in the Fall of 2007.