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I am submitting herewith a dissertation written by Faith Critzer entitled "Transcription Analysis of *Escherichia coli* O157:H7 Exposed to Sodium Benzoate." 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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Transcription Analysis of *Escherichia coli* O157:H7 Exposed to Sodium Benzoate

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

Faith Critzer
May 2008

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Abstract

Advances in microbial genetics have allowed discovery and assignment of function for many genes. High-throughput transcription analysis can be conducted for foodborne pathogens to give insight into mechanisms of adaptation and survival in adverse conditions. With heightened knowledge of gene expression in these conditions, steps can be taken to counteract adaptive mechanisms and inhibit growth or survival of foodborne pathogens.

Sodium benzoate is a food antimicrobial that is commonly used in beverages and fruit juices. A study was conducted to determine the gene expression of *Escherichia coli* O157:H7 when exposed to sodium benzoate.

First, a qualitative study to determine transcription of *marA*, *stx1*, and *eaeA* was undertaken using real-time reverse transcriptase polymerase chain reaction (rt-RT-PCR). Expression of the *mar* operon causes increased antimicrobial resistance in bacterial pathogens. Shiga toxin 1 (Stx1) is a well described verotoxin produced by enterohemorrhagic *E. coli* (EHEC), and EaeA, or intimin, helps establish *E. coli* O157:H7 in the intestinal tract. For *marA* and *stx1*, rt-RT-PCR products were detected at a 1-log greater dilution in sodium benzoate treated cells, indicating a greater level of transcription in these cells.

Next, a microarray study was conducted to determine transcription of *E. coli* O157:H7 when exposed to 0.5% sodium benzoate. Results indicate that the phosphate specific transport (Pst) system was rapidly (within 5 min) up-regulated in response to

sodium benzoate. This system is essential for supplying phosphate used in synthesizing compounds such as ATP, phospholipids, and proteins. Research with *Mycobacterium smegmatis* also shows that this system can serve as an efflux pump.

The urease operon was also shown to be up-regulated in *E. coli* O157:H7 after 60 min of exposure to sodium benzoate. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide, and is one mechanism by which microorganisms survive in acidic environments. In this study, exposure of *E. coli* O157:H7 to sodium benzoate at neutral pH showed increase in transcription of the entire urease operon.

These data indicate that *stx1* and *marA* genes as well as the Pst system and urease operon could play a role in pathogen virulence and survival when treated with sodium benzoate.

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Chapter 1

Review of Current Literature

Escherichia coli O157:H7

E. coli O157:H7 is a member of the enterohemorrhagic *E. coli* (EHEC) and causes hemorrhagic colitis in humans. The symptoms of an *E. coli* O157:H7 infection generally include severe abdominal cramps and diarrhea (often times bloody), which can lead to death. In children under the age of five, infection can lead to a complication known as hemolytic uremic syndrome (HUS), resulting in red blood cell destruction, renal failure, and central nervous system complications *(99)*. *E. coli* O157:H7 can cause disease at a low infectious dose (10 - 100 cells) *(86)*. This can be attributed to the tolerance of this bacterium to low pH, which allows passage through the stomach and colonization in the intestinal tract *(86)*. *E. coli* O157:H7 has been linked as the causative agent in outbreaks associated with unpasteurized apple and orange juice, and experimental data demonstrate the tolerance of *E. coli* O157:H7 to acidic environments *(15, 24, 88, 112)*.

While it is very hard to quantify the impact foodborne illnesses have in the United States, it has been estimated that foodborne disease causes 76 million illnesses annually and approximately 5,000 fatalities *(83)*. There were 16,614 laboratory-confirmed cases of foodborne illness from FoodNet participants in 2005 (~15% of the United States population), and *E. coli* O157:H7 was the causative agent in 2.8% of these cases *(8)*. The CDC estimates that there are 73,000 cases of *E. coli* O157:H7 annually, leading to an estimated 2,100 hospitalizations in the United States *(20)*. It has been estimated that the cost of EHEC infections was $405 million dollars in 2003 *(42)*.

The well-described virulence factors of *E. coli* O157:H7 include *stx1*, *stx2*, and *eaeA (64, 108)*. Shiga toxins are composed of two subunits. The B-subunit binds glycolipids in the host, and the A-subunit inhibits protein synthesis via disruption of ribosomal RNA *(1)*. Shiga-toxins may cause hemorrhagic colitis, hemolytic uremic syndrome (HUS), thrombocytopenia, hemolytic anemia, and renal failure *(63)*. The gene *eaeA* in enterohemorrhagic *E. coli* produces intimin, a 97-kDa protein that plays a role in attachment onto and effacement of microvilli of the small intestine *(34, 41, 71, 82, 118)*.

Antibiotic resistance patterns of *Escherichia coli* O157

The use of antimicrobial agents in food animals can promote antibiotic resistance among both target pathogens and normal microflora. This includes foodborne pathogens such as *E. coli* O157:H7, for which cattle are a known reservoir. Of 236 clinical *E. coli* O157:H7 isolates collected in Washington State between 1984-1991, 5.6% were resistant to streptomycin, sulfisoxazole and tetracycline *(65)*. Among *E. coli* O157:H7 clinical isolates from England and Wales in 1997, 23% were resistant to one antibiotic and 2% were resistant to multiple antibiotics *(117)*. Of the latter, 73% were resistant to streptomycin, sulfonamides and tetracyclines and 14% were resistant to sulfonamides and tetracyclines. Meng and others *(87)* noted a similar antibiotic trend with resistant strains of *E. coli* O157:H7 isolated from humans, animals, and foods from 1984-1993. Seventy percent of the resistant isolates were resistant to streptomycin, tetracycline, and sulfisoxazole *(87)*. However, 19% of these isolates were resistant to

three or more antibiotics *(87)*. The highest rate of antibiotic resistance was seen among cattle isolates.

One hundred and seventy *E. coli* O157:H7 human isolates were received by the Centers for Disease Control and Prevention (CDC) in 2003 *(19)*. Of these, 10.8, 5.1, 3.2, and 2.5% of the isolates were resistant to one, two, three and four antibiotics, respectively *(19)*. Tetracycline, sulfamethoxazole, and ampicillin were the antibiotics to which resistance was the greatest, 5.7, 3.8, and 3.2%, respectively. *E. coli* O157:H7 isolates resistant to at least two Clinical and Laboratory Standards Institute antibiotic subclasses increased from 3.8% in 2002 to 5.1% in 2003 *(19)*. Streptomycin, sulfisoxazole, and tetracycline are not routinely used to treat diarrhea in North America, but selective pressure through the use of antibiotics in animals may select for populations of zooinotic pathogens, such as *E. coli* O157:H7 *(65)*. The mechanisms leading to the observed multiple antibiotic phenotypes in *E. coli* O157:H7 are also unclear and warrant increased attention.

Antibiotic therapy of *E. coli* O157 infections

Children treated with trimethoprim-sulfamethoxazole at a Montreal pediatric hospital from 1989-1990 for *E. coli* O157:H7 enteritis showed no difference in the length of shedding in stool, duration of symptoms, or the occurrence of hemolytic-uremic syndrome (HUS) compared to control patients *(104)*. However, patients treated with antibiotics in that study were started on an antibiotic regimen after isolation of *E. coli* O157:H7 in their stools, rather late in the course of patient illness. The use of

trimethoprim-sulfamethoxazole also did not increase the risk of developing HUS, although this was a relatively small population *(104)*.

In a prospective cohort study conducted among children infected with *E. coli* O157:H7, nine patients were treated with antibiotics and 62 were not; five children from each group developed HUS, 56 and 8%, respectively (P<0.001) *(123)*. Of the five children who received antibiotics and developed HUS, two received trimethoprim-sulfamethoxazole, and three were administered cephalosporins. Other significant factors were initial white blood cell count (P=0.02), with risk increasing with white blood cell count. Time between the onset of diarrhea to the day on which the initial stool culture was obtained also significantly affected outcome (P=0.008); risk of developing HUS was inversely proportional to the number of days from onset of diarrhea *(123)*. This indicates that a rapid onset of symptoms with increased white blood cell counts gave greater risk for HUS regardless of antibiotic treatment.

Three strains of *E. coli* O157:H7 (one producing Stx1, one producing Stx2, and one producing Stx1 and Stx2) were studied for Shiga-like toxin (Stx) production when exposed to sub-inhibitory levels of antibiotics *(53)*. Trimethoprim and cotrimoxazole (trimethoprim and sulfamethoxazole) stimulated Stx production in all three strains. However, the 11 other antibiotics tested had varied effects on the release of Stx in the three strains studied, and emphasized the differences in virulence factors that can be seen among strains of the same pathogen.

A survey of physicians who treated the 163 case *E.* coli O157:H7 outbreak in Osaka, Japan reported that 95.9% of the patients treated were given an antimicrobial

agent, and fosfomycin was the most commonly prescribed *(126)*. *In vitro* assays demonstrated a 7-fold increase in Stx1 production with treatment of sub-inhibitory concentrations of fosfomycin, while none of the antibiotics tested caused an increase in Stx2 production *(126)*. *In vivo* treatment of mice infected with *E. coli* O157:H7 with ciprofloxacin caused increased Stx2 production that was not observed in mice treated with fosfomycin *(128)*.

In another murine model, only treatment with trimethoprim-sulfamethoxazole, caused an increase in Stx levels *(68)*. The time of trimethoprim-sulfamethoxazole treatment influenced the outcome of mortality. When antibiotic intervention was initiated one day post-infection, Stx levels in blood and stool was similar to other antibiotic treatments from which no lethality was observed. However, when the trimethoprim-sulfamethoxazole treatment was started three days post-infection, Stx levels in blood and stool were increased above that of the control and resulted in increased mortality *(68)*.

Intrinsic mechanisms of antibiotic/antimicrobial resistance

Golding and Matthews*(51)* described an intrinsic mechanism in *E. coli* O157:H7 that results in multiple antibiotic resistance due to a mutation in the multiple antibiotic resistance (*mar*) operon. Of 52 chloramphenicol-resistant *E. coli* O157:H7 mutants, half were clinically resistant to tetracycline, and resistance at levels below clinical resistance to tetracycline, nalidixic acid, and ciprofloxacin was also seen in isolates *(51)*. The *mar* operon has also been shown to contribute to multiple antibiotic resistance in *Salmonella*

Enteritidis isolates resistant to 25 ppm chlorine, 500 ppm acetic acid, 10,000 ppm sodium benzoate, or 10,000 ppm sodium nitrite. A single exposure to acetic acid, sodium benzoate, or nitrite induced a two- to four-fold increase in resistance to tetracycline *(103)*. Isolates resistant to 25 ppm chlorine showed increased resistance to tetracycyline, chloramphenicol, and nalidixic acid, with the greatest resistance to tetracycline and chloramphenicol *(103)*. In both studies, susceptibility to the tested antibiotics returned to that of the wild-type with complementation of a functional *marR*, a component of the *marRAB* operon, suggesting that this operon was partly responsible for the observed antibiotic resistance *(51, 103)*.

Multiple antibiotic resistance (mar) operon

Microorganisms have adapted various intrinsic mechanisms of survival in stressful environments. Multiple antibiotic resistance attributed to chromosomal mutations in the *mar* operon was first described in *Escherichia coli* K12 when selecting for low levels of tetracycline or chloramphenicol resistance *(49)*. The susceptibility of these mutants was decreased for the selected agent as well as other structurally unrelated antibiotics such as penicillins, cephalosporins, puromycin, nalidixic acid, and rifampin *(49, 50)*. When in contact with the selective agent, resistance was induced to high levels; however, this resistance was lost when grown in the absence of the inducing agent for more than 100 generations *(49)*. Resistances to the selected compounds were greatly reduced or reversed by insertion of the composite transposon, Tn5, into the *E. coli* chromosome near 34 min (1,636.7 kb), designated *marA (50)*. Tn5-inactivated *mar*

mutants produced a truncated message of 0.7 kb, which reverses the Mar phenotype *(54)*. When Tn5 was excised from *marA*::Tn5 tetracycline sensitive mutants, the multiple antibiotic resistance phenotype returned at a rate of 10^{-8} to 10^{-7} *(50)*. A Mar phenotype has been observed in Gram-negative bacteria with clinical resistance to quinolones *(122)*.

The *mar*-loci have been sequenced in *E. coli* K-12 and *Samonella enterica* serovar Typhimurium DT104 and are present in *Shigella*, *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Hafnia*, indicating that this operon is highly conserved among enteric microorganisms *(25, 29, 67)*. MarA has been found to alter expression of more than 60 chromosomal genes of *E. coli* K-12 *(13)*. The expression of MarA altered the transcription of outer membrane proteins (e.g., OmpF, OmpX, TolC) and showed continued overlap between the *marRAB* and *soxRS* regulons that respond to antibiotic and oxidative stress.

Structure of the* mar *operon

The chromosomally mediated *mar* phenotype was found in mutants that constitutively express the *marRAB* operon *(26)*. The *mar* locus consists of two divergently positioned transcriptional units that flank *marO, marC and marRAB (26, 27)*. The *marO* gene contains two promoters for transcription of MarC and MarRAB ($Pmar_I$ and $Pmar_{II}$, respectively) *(13)*. MarC is theorized to be a putative integral inner membrane protein with unknown function *(13)*. The proteins produced by the *marRAB* operon are: MarR, a repressor*(26, 111)*, MarA, an activator that is associated with

multiple antibiotic resistance *(47, 50)*, and MarB, for which the function is not yet known *(80)*.

Increased expression of the *marRAB* operon will arise from mutations in *marO* or *marR* or from inactivation of MarR when exposed to inducing agents such as salicylate *(3, 4, 27)*. Overexpression of *marA* from a multicopy plasmid resulted in a Mar phenotype in strains where the *mar* locus was deleted *(47)*.

marR

When *marRAB* is not induced, MarR negatively regulates the transcription of MarA by binding to the operator, *marO (111)*. MarR has been found to form oligomers at two sites within *marO in vitro (2, 80)*. Site I begins downstream of the -35 transcriptional start signal for RNA polymerase (RNAP) binding and includes 4 bp of the -10 hexamer *(80)*. Site II begins 13 bp away from site I and ends before the transcription start signal of *marR (80)*. Site II has about 80% homology with site I, and site I alone was able to repress the *mar* operon *(80)*. Further research demonstrated that MarR was able to repress the promoter by a factor of 4.3 with site I in a native form, but *marO* was repressed by a factor of 20 when both sites were in their functional form *(81)*. MarR bound to site I could interfere with the binding of RNAP to the -35 and -10 transcription start signals, thus blocking transcription of *marRAB*. MarR binding at site II may interfere with the formation of an open complex necessary for transcription *(81)*.

The crystalline structure of MarR reveals that it functions as a dimer, and each monomer has a region of two α-helices and three β-sheets giving rise to a winged helix

DNA binding domain *(2, 5)*. Footprinting studies have suggested that MarR is bound to DNA as a dimer since about 20 bp in both strands of DNA in sites I and II of *marO* were protected from digestion by DNAse *(80)*. The 20 bp binding site of *marO* is centered on a palindromic sequence TTGCCnnGGCAA (where n = any nucleotide), and *in vitro* when TTGCC was transversed, all DNA binding ability was lost *(81)*. When the two winged binding domains (Mar-R and Mar-C) were compared among known MarR proteins, Mar-R was found to be highly conserved while functional regions of Mar-C were not *(125)*. These unconserved regions may arise from strain specific differences in *marO* for which the repressor must be able to recognize *(125)*.

marA

The accumulation of an activator, e.g. MarA, is the primary factor that controls the expression of mar phenotype *(13, 54)*. Of the > 60 chromosomal genes whose expression is altered by MarA, approximately 80% increased in transcription and the remainder were down-regulated *(13)*. MarA is in the AraC/XylS family of transcriptional regulators that have a helix-turn-helix DNA binding motif and are positive transcriptional regulators *(45, 46)*. The structurally related transcriptional activators SoxS and RobA are also members of the AraC/XylS family and were first associated with response to superoxide stress and organic solvents. MarA and Rob share 41 and 55% sequence identity with SoxS, the smallest of the proteins in this trio. To different extents, all three proteins regulate genes in the *mar/sox/rob* regulons. The members of this family share a conserved region composed of 99 amino acids (carboxy terminus MarA and SoxS;

amino terminus Rob) that bind DNA as monomers in the promoter region and stimulate RNAP contact and subsequent transcription *(39, 46)*.

The region of DNA that MarA binds to is a 20 bp degenerate sequence know as a 'marbox' that does not indicate a preference for MarA, SoxS or RobA *(77, 78)*. The consensus sequence defined by Martin and others*(77)* (AYnGCACnnWnnRYYAAAYn; where R = G/A, W = A/T, Y = C/T, and n = any nucleotide) is either found upstream (class I) or overlapping (class II) the -35 signal for RNAP *(78)*. The location and orientation of the marbox from the -35 and -10 signals for RNAP has been found to be specific for the promoter *(77)*. The class I promoters (e.g. *mar*, *acrAB,* and *fpr*) interact with the α-carboxy terminal domain (α CDT) of RNAP, and their marbox is located in a reverse or backward orientation *(60, 61)*. In the backward orientation, MarA binds with its amino terminus proximal to the RNAP signal*(77)*. *zwf* and *nfsA* are class I promoters that do not follow this pattern; their marbox is in a forward orientation and closer to the -35 RNAP signal *(59, 100)*. Class II promoters (e.g. *fumC*, *inaA*, *sodA* and *micF*) do not require interaction with α CDT, and their marbox is in the forward orientation and overlaps the -35 RNAP hexamer *(78)*.

Multiple marboxes have been identified for some promoters (e.g. *micF* and *zwf*), that lie farther upstream of the -35 RNAP signal, but most transcriptional activation for *zwf* and *micF* is attributed to the marbox located closest to the promoter *(38, 39, 69)*. It has been demonstrated *in vitro* that SoxS and MarA can utilize the same marbox sequence to activate *zwf* transcription (glucose 6-phosphate dehydrogenase) *(60)*.

Induction of the* mar *operon

Expression of *marA* can be induced by exposure to low levels of tetracycline, chloramphenicol *(49)*, salicylates *(27, 109)* , organic solvents *(11)*, benzoate *(103, 109)*, chlorine, nitrite, acetic acid *(103)*, pine oil *(90)*, bile salts *(105)*, spices, and various food extracts *(107)*. Phenolic compounds, such as salicylate, induce transcription of the *mar* operon by binding to the repressor, MarR *(79)*. Deoxycholate, a bile salt, has also been found to bind to MarR also preventing binding to the *mar* operator region *(105)*. Once the repressor can no longer bind to the operator region, transcription of *marAB* is induced.

Constitutive expression of the *marRAB* operon may give mutants the ability to initially survive treatment with antibiotics and allow low-level resistance to many antimicrobials until secondary mutations arise giving increased resistance to a specific agent *(26)*. Bacterial strains expressing a *mar* phenotype were found to be sensitive to household disinfectants containing hydrogen peroxide, hypochlorite, alkyl dimethyl benzyl ammonium chloride (a quaternary amine), or chloroxylenol (a phenol) *(90)*. Increased levels of MarA, SoxS, or RobA resulted in an increase in resistance to many hydrophobic antibiotics, but expression of the *mar/sox/rob* regulon was not found to cause resistance to hydrophilic aminoglycosides *(11)*.

SoxRS

The SoxR is constitutively expressed and contains binuclear iron-sulfur clusters *(6, 57, 124)*. When the iron-sulfur clusters are in an oxidized state, *SoxS* expression is

dramatically increased compared to when these clusters are in a reduced form *(58)*. SoxR binds equally well to the promoter of SoxS in a reduced or oxidized state, and the oxidized state is thought to aid in the open-complex formation of RNA polymerase *(33)*.

Like MarA, SoxS activates transcription of a number of genes in response to superoxide and antibiotic stress *(6, 58, 69, 70)*. Li and Demple *(69)* demonstrated that purified SoxS bound near the promoter region of genes under control of the *soxRS* regulon (e.g. *micF, nfo, zwf,* and *sodA*) and increased the transcription of these genes. SoxS was shown to bind *in vitro* to a region adjacent to or overlapping the -35 RNAP signal of *zwf, sodA, nfo, micF,* and *fumC (38)*. The superoxide generating herbicide, paraquat, induced *soxS* expression and caused transcription of *sodA* and *micF (102)*. Disruption of *sodA* caused increased sensitivity to cells treated with paraquat *(102)*.

Rob

SoxS and MarA are very similar to the amino terminus of the Rob protein that is known to bind to the right arm of the origin of replication, *oriC (10, 113)*. Rob is located at 99.8 min on the *E. coli* K-12 chromosome and also has a helix-turn-helix motif found in MarA and SoxS *(113)*. Unlike MarA and SoxS, Rob is constitutively expressed to fulfill an unknown purpose. Rob has been reported at levels of 5000 and 1000 molecules per cell for stationary and log phase cells, respectively *(62, 113)*.

Overexpression of Rob resulted in increased resistance to multiple antibiotics and superoxide generating agents *(10)*. Rob activated the transcription of *zwf, fumC, micF, nfo* and *sodA* by binding to the marbox region and interacted with the promoters

of the affected genes similarly to SoxS and MarA *(61)*. Rob had the same affinity for binding *micF* promoter as *oriC* and bound more weakly to the promoters of *sodA, nfo* and *zwf (10)*.

Overlaping Regulation by MarA, SoxS and RobA

Although MarA, SoxS and RobA can regulate the same promoters that respond to multiple antibiotic and superoxide resistance, each protein activates promoters to different extents. The marbox is very important in activator discrimination of promoters. Martin and others *(78)* found that differences in the marboxes versus the core promoters were the principal factors involved in activator discrimination.

Martin and others *(78)* demonstrated that overproduction of SoxS in *marRAB* and *soxRS* deleted strains led to greater superoxide resistance than overproduction of MarA; however, both MarA and SoxS were equally effective in promoting antibiotic resistance. MarA was also shown to increase transcription of seven proteins that were unaffected by SoxS *(52)*. Levels of organic solvent tolerance were reduced in wild type and Mar mutants when only SoxS was present *(119)*. Overproduction of SoxS causes greater superoxide and antibiotic resistance than does overproduction of RobA *(10)*. Overexpression of *SoxS* results in greater transcription of *fumC, sodA, and inaA* when compared to *RobA*, and RobA does not stimulate transcription of *zwf (10)*.

When the marbox from the *mar* promoter was substituted for the native *zwf* marbox, MarA transcriptional activation increased relative to that of SoxS *(78)*. When the *micF* promoter marbox was replaced for that of *fpr,* SoxS transcriptional activation

was increased compared to that of MarA *(78)*. The position of the marbox relative to the promoter (i.e., class I versus class II) has not been shown to be a discriminating factor for activators *(78)*. Promoter discrimination allows for specialized transcriptional activation of MarA, SoxS and RobA. Minute differences between these activators can result in different affinities and interactions with promoter marboxes resulting in varied regulation of many genes within the *mar/sox/rob* regulon.

Genes induced by **Mar, Sox, and Rob**

acrAB

AcrAB is part of a multi-drug efflux system, AcrAB-TolC. AcrA is a periplasmic lipoprotein that spans the periplasmic space between the inner membrane, where its amino terminus is anchored, and outer membrane proteins *(48)*. AcrA is also a member of the membrane-fusion protein (MFP) family *(48)*. AcrB is a putative pump and a member of the resistance-nodulation-division (RND) family *(48)*.

AcrAB expression is under control of MarA and AcrR *(72, 96)*. In both wild-type and Mar mutants, where the *acrAB* locus was deleted, hyper-susceptibility to antibiotics and organic solvents occurred to the same extent *(90, 96, 119)*. Active efflux of harmful compounds has been shown to be a resistance mechanism that results in multiple antibiotic resistance *(14)*. AcrAB production causes an intrinsic level of resistance that is increased in *mar* mutants *(73, 96)*.

tolC

TolC is an outer membrane porin that is part of the AcrAB-TolC efflux system *(40)*. TolC has been shown to be positively regulated by MarA, SoxS, and RobA *(9, 13)*. Increased resistance to organic solvents has been attributed to increased levels of the TolC outer membrane protein and the AcrA inner membrane protein *(9)*. A defective *tolC* gene results in increased sensitivity to organic solvents, and TolC is required for the expression of the Mar phenotype *(9, 40)*.

micF

micF encodes an antisense RNA regulator of outer membrane protein F (OmpF), which serves as a passage for hydrophilic substances *(95)*. The mRNA product of *micF* has a long sequence that is complementary to the 5' end region of *ompF* mRNA. OmpF and OmpC are the two major outer membrane porins of *E. coli* K-12 *(95)*. Limiting OmpF has been shown to occur in *E. coli* K-12 expressing a Mar phenotype, and cells devoid of a *micF* locus were unable to cause a reduction in OmpF *(13, 28)*. MarA has been shown to increase expression of *micF*, causing a decrease in *ompF* mRNA that is available to undergo translation *(13, 28)*.

zwf, fumC, and sodA

Many proteins are involved in protection of the cell from oxidative stress and are positively controlled by the *mar/sox/rob* regulons *(60, 61, 69, 93, 102)*. *zwf* (glucose 6-phosphate dehydrogenase) generates NADPH that supplies reducing capability for

antioxidant enzymes (e.g., glutathione reductase and alkylhydroperoxide reductase) *(32, 43)*. *fumC* is heat-stable fumarase that replaces superoxide sensitive fumarases A and B that are inactivated by superoxide radicals *(32, 43)*. *sodA* (manganese superoxide dismutase) scavanges $O_2^{\cdot -}$ (superoxide) and hydrogen peroxide, thereby retarding oxidative damage *(32)*.

inaA

InaA is a weak-acid inducible protein, and the membrane-permeant acids that induce *inaA* have the ability to lower the internal pH of the cell or act as uncouplers of oxidative phosphorylation *(106)*. Non-dissociating analogs of benzoate and salicylate (benzyl and salicyl alcohol) were unable to induce expression of *inaA (120).* *inaA* maps to 48.6 min on the *E. coli* K-12 chromosome and has been shown to be positively regulated by SoxS and MarA *(110, 120)*. Expression of *MarA* is responsible for 20-50% of the basal expression of *inaA*, and *mar* deleted strains contained 30-50% less InaA when compared to *mar*$^+$ strains induced with paraquat *(110)*.

nfsA

nfsA (formerly *mdaA*) encodes a major oxygen-insensitive nitroreductase that is a flavoprotein in *Escherichia coli* which catalyzes the reduction of nitroaromatic and nitroheterocyclic compounds by the transfer of two electrons from NADPH *(100)*. *nsfA* has been mapped to 19.2 min on the *E. coli* K-12 chromosome *(127)*. *nsfA* has been shown to be expressed under positive regulation of MarA and SoxS *(13, 31, 70)*. A gene

of unknown function, *ybjC*, is expressed by MarA and SoxS in a similar manner, and these two genes may form an operon *(13, 31)*. The marbox for *nfsA* is a class I promoter in forward orientation *(100)*.

Escherichia coli O157:H7 Urease Operon

Escherichia coli O157:H7 is known to produce urea when degrading arginine to putrescine *(91, 92)*. Urease (EC 3.5.1.5) catalyzes the reaction of urea into two molecules of ammonia and carbon dioxide *(89)*. The liberated ammonia results in increased pH of the surrounding environment. *Escherichia coli* O157:H7 EDL933 genomic sequence contains an O-island with two urease gene clusters (*ure*DABCEFG) *(101)*. Urease is composed of three structural proteins, UreA, UreB, and UreC, as well as four accessory proteins, UreD, UreE, UreF, and UreG *(89)*. This enzyme could give enterohemorrhagic *E. coli* (EHEC) the ability to neutralize acidic environments and allow for survival in acidic food products as well as support survival in the gastrointestinal tract.

When studying the presence of the urease gene clusters in *E. coli* O157:H7 isolates, it was found that among serotype O157:H7 isolates (*n*= 59), 98% contained all genes in the urease operon*(44)*. In contrast, none of the genes in the urease operon were present in sorbitol-fermenting O157:NM (non-motile) isolates (*n*=82) *(44)*. However, among O157:H7 isolates found to have the urease operon, only one was found to have urease activity when evaluated with Christensen's agar or with the API 20E system; in this isolate, elevated levels of ammonia were detected *(44)*. When

evaluating nine isolates of serotype O55:H7, from which *E. coli* O157:H7 has been theorized to have diverged, none contained genes in the urease operon *(44)*.

Similarly, Orth *et al. (97)* studied the distribution of *ureC* among 202 EHEC that encompassed 61 serotypes. It was found that all O157:H7 and O157:NM (*n*=72) that displayed the typical non-sorbitol fermenting phenotype possessed the *ure*C gene *(97)*. However, all sorbitol fermenting O157:NM isolates (*n*=8) were negative for *ure*C *(97)*. All isolates containing *ureC* were also found to contain all genes in the urease operon *(97)*. When the *ureC* isolates were evaluated for urease production in urease degradation broth, only 3.4% showed urease activity *(97)*. The nucleotide sequence of all urease genes present in ten O157:H7 isolates studied were found to have ≥ 99% homology *(97)*. These data indicate that the urease operon was acquired by O157:H7 relatively recently and possibly through horizontal gene transfer or gene loss since both O55:H7 and sorbitol fermenting O157:NM strains lack the urease gene cluster, and although many *E. coli* O157:H7 isolates contain the urease operon, it is not commonly expressed under normal culture conditions.

Nakano et al. *(94)* examined urease production by *E. coli* O157:H7 Sakai in various culture media, pH, temperatures, and nutrients via ammonia production without any success at stimulating urease production. When further examining 120 EHEC isolates for urease activity, two isolates were identified as being urease positive *(94)*. All isolates were determined to have a urease operon of similar size (4940 bp), and the nucleotide sequence was similar for a urease positive strain compared to the Sakai strain except for a slightly longer open reading frame in the *ureD* gene of the urease

positive isolate *(94)*. It was revealed that the Sakai strain as well as all urease negative EHEC isolates examined (*n*=158) had a premature stop codon for *ureD* compared to that of the urease negative isolate. When the *ureD* gene of a urease positive EHEC was transformed into the Sakai strain urease production was detected *(94)*. These data indicate that the premature stop codon in UreD plays a role in urease production *in vitro*.

When attempting to stimulate urease production in *E. coli* O157:H7 strain EDL933, urease could not be detected using Christensen's agar, enzymatic assays, or immunoblotting techniques targeting UreC and UreD *(56)*. However, when non-pathogenic *E. coli* DH5α was transformed with the EHEC *ure* gene cluster, urease was detected via enzymatic activity *(56)*. This demonstrated that the urease genes present in the EDL933 strain were functional, but failed to be expressed under multiple culture conditions. It is interesting that the *ure*D nucleotide sequence in strain EDL933 is identical to Sakai *(94)*. When Nakano et al.*(94)* transformed the Sakai *ure*D gene into *E. coli* DH5α, urease was detected. The authors believe that the amber suppressor phenotype of DH5α played a role in read-through of the premature stop codon. Heimer *et al. (56)* determined that a trans-acting factor was inhibiting urease transcription in EHEC. These authors also identified three Fur-like (ferric-uptake regulator) recognition sites upstream of *ureA* and *ureD (56)*. In this study, urease production was detected in two other EHEC strains (IN1 and MO28), and a Δ*fur* background greatly diminished the urease activity of these strains *(56)*. This research indicated that Fur, as well as other

unidentified trans-acting factors, plays a role in regulating the expression of urease in EHEC.

Phosphate Specific Transport (Pst) of *E. coli*

The acquisition of phosphorus is essential for bacteria to supply energy and synthesize DNA, RNA, lipopolysaccharides, and phospholipids. Microorganisms must transport inorganic phosphorus (P_i) into the cytoplasm in order to survive. The first step in this process for *E. coli* is to transport organic and inorganic forms of phosphorus across the cell wall. In *E. coli* there are two common pore-forming proteins, OmpC and OmpF. However, under phosphate limited situations, a third anion binding porin, PhoE, is expressed in order to sequester phosphorus into the periplasm *(98)*.

The Pst system is in the ATP binding cassette (ABC) superfamily of transporters and is one mechanism of transporting P_i across the cell membrane. This system is known to be expressed when P_i concentration is below 0.001 M *(116)*. This system has a high affinity for binding of phosphate and is composed of four proteins, PstS, PstC, PstA, and PstB *(116)*. The gene *pstS* (formerly *phoS*) encodes a phosphate binding protein that binds P_i in the periplasmic space where it can be transported across the cell membrane *(84, 85, 121)*. PstC and PstA are transmembrane proteins that form a passage across the cell membrane (30), while PstB catalyzes the movement of phosphorus into the cytoplasm by hydrolysis of ATP (22).

PhoR and PhoB regulate the Pho regulon, for which the Pst system is a member. PhoR is a transmembrane signal transduction protein that senses low levels of P_i in the

periplasm. PhoR has an extracellular sensory domain and an intracellular signaling domain*(114)*. Under phosphate limited conditions, PhoR undergoes autophosphorylation and acts as a histidine kinase that phosphorylates PhoB *(75, 76)*. Once PhoB is activated, it will begin to stimulate the Pho regulon by binding to the 'Pho box' consensus sequence stimulating transcription of *pstS* *(74, 75)*. In addition to *pstSCAB*, PhoB also regulates the expression of PhoA, which is a periplasmic alkaline phosphatase that will free P_i from phosphate containing compounds so it is available for uptake into the cytoplasm. PhoU is a 21 KDa cytoplasmic protein that is necessary for repression of the Pho regulon *(7, 76)*. Repression occurs when PhoU acts to promote dephosphorylation of PhoB, thus inactivating this protein *(114)*.

A role other than phosphate transport has been determined for the Pst system in *Mycobacterium smegmatis*. A *M. smegmatis* strain which was resistant to ciprofloxacin was found to over-express the Pst operon when grown in sufficient quantities of phosphorus (12, 16, 21). The *pstB* gene was also shown to have increased amplification on the chromosome of the *M. smegmatis* ciprofloxacin resistant strain(21). When the *pstB* gene was mutated, the minimum inhibitory concentration of *M. smegmatis* to ciprofloxacin was greatly reduced (12). It was also demonstrated that the ciprofloxacin resistant strain had a much greater efflux of this fluoroquinolone from the cell compared to that of the wild type or *pstB* mutant (12). The fluoroquinolone resistant *M. smegmatis* strain showed increased phosphate uptake that was diminished along with ciprofloxacin resistance when *pstB* was mutated (16). These results indicate that

the Pst system serves as an efflux mechanism of fluoroquinolone for *M. smegmatis* in addition to the traditional role as a high-affinity transporter of phosphorus.

Sodium Benzoate

Sodium benzoate is a food additive commonly used in acidic beverages due to its relatively low pKa (4.2) and increased solubility (compared to benzoic acid). This compound is generally recognized as safe and may be added at concentrations up to 0.1% in foods (21 CFR 184.1733).

Proposed Mechanisms of Microbial and Fungal Inhibition with Weak Acids

A general mechanism of inhibition for weak acids starts with diffusion of the undissociated compound through the plasma membrane by passive diffusion. Once inside the cell, the higher pH causes dissociation of the acid and lower intracellular pH_i and inhibition of membrane-bound transport *(18)*. Eklund *(35)* determined that uptake inhibition contributed to growth inhibition, but did not seem to completely explain this phenomenon. Eklund *(37)* found that ΔpH (pH difference) was eliminated by sorbic acid as well as methyl and butyl paraben, whereas the membrane potential ($\Delta\psi$) was maintained *(37)*. This indicates more than simple inhibition of the proton motive force, since the membrane potential maintained would allow for uptake of essential compounds for cellular maintenance.

It has been determined that dissociated weak acids also contribute slightly to growth inhibition, which cannot be explained by the effects of pH_i *(36)*. Sorbic acid,

sorbic alcohol, and sorbic aldehyde have been found to cause similar levels of inhibition, demonstrating a mechanism of inhibition other than a weak acid effect *(115)*.

Bracey et al. *(17)* found that pH_i was not altered in *Saccharomyces cerevisiae* exposed to sorbic acid. However, a higher ADP/ATP ratio was found, indicating that the cell was expending energy to maintain pH_i *(17)*, and this expenditure of ATP was theorized to decrease cellular growth. Membrane disruption has also been attributed as a mechanism of inhibition by weak acids *(17, 115)*. Krebs and et al. *(66)* observed a reduction in phosphofructokinase activity in S*accharomyces cerevisiae* when exposed to benzoate *(66)*. The disruption of glycolysis was thought to be caused by a decrease in pH_i that decreased the enzymatic activity of phosphofructokinase.Nitrogen starvation and benzoic acid treatment inhibit growth of *Saccharomyces cerevisiae*. However, when used in combination, they are cytocidal *(55)*. It was found that benzoic acid inhibited the break-down of cytosolic compounds to form amino acids and nucleotides necessary for growth and survival *(55)*.

Some microorganisms contain pathways to degrade weak acids. Benzoate is degraded in microorganisms via the β-ketoadipate pathway resulting in acyl CoA and succinic acid *(23)*. These degradation pathways serve as innate responses to weak acid resistance.

The following chapters of this dissertation will discuss the transcriptional response of *Escherichia coli* O157:H7 when exposed to sodium benzoate. This will help determine some of the primary mechanisms by which *E. coli* responds and survives this stressful environment. It is essential to identify and understand these mechanisms, so

that methods can be developed by the food industry to inhibit or control the survival of this pathogen in foods.

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Chapter 2

Transcription Analysis of stx1, marA, and eaeA Genes in *Escherichia coli* O157:H7 Exposed to Sodium Benzoate

Abstract

Expression of the multiple antibiotic resistance (*mar*) operon causes increased antimicrobial resistance in bacterial pathogens. The activator of this operon, MarA, can alter expression of >60 genes in *E. coli* K12. However, data are lacking concerning the expression of virulence and resistance genes when foodborne pathogens are exposed to antimicrobial agents. This study was conducted to determine transcription of *marA* (*mar* activator), *stx1A* (Shiga toxin 1), and *eaeA* (intimin) genes of *E. coli* O157:H7 EDL933 as affected by sodium benzoate. *E. coli* O157:H7 was grown in Luria-Bertani broth (LB) containing 0 (control) and 1% sodium benzoate at 37°C for 24 h, and total RNA was extracted. Primers were designed for *hemX* (209 bp; housekeeping gene), *marA* (261 bp), and *eaeA* (223 bp) genes; previously reported primers were used for *stx1*. Ten-fold dilutions of RNA were used in a real-time one-step reverse-transcriptase polymerase chain reaction (rt-RT-PCR) to determine transcription levels. All experiments were conducted in triplicate and product detection was validated by gel electrophoresis. For *marA* and *stx1*, rt-RT-PCR products were detected at a 1-log greater dilution in sodium benzoate treated cells than control cells, although cell numbers for each were similar (7.28 and 7.57 log CFU/ml, respectively). This indicates a greater (albeit slight) level of their transcription in the treated as compared to control cells. No difference in expression in the *eaeA* gene was observed. HemX is a putative uroporphyrinogen III methylase. The *hemX* gene had the same level of expression in control and treated cells, validating *hemX* as an appropriate housekeeping marker. These data indicate that *stx1*

and *marA* genes could play a role in pathogen virulence and survival when treated with sodium benzoate, while *eaeA* expression is not altered. Understanding adaptations of *E. coli* O157:H7 during antimicrobial exposure is essential to better understand and implement methods to inhibit or control survival of this pathogen in foods.

Introduction

The impact of foodborne illnesses are very hard to quantify. However, it has been estimated that foodborne diseases cause 76 million illnesses and 325,000 hospitalizations in the U.S. annually (29). *Escherichia coli* O157:H7 alone causes an estimated 73,000 cases of foodborne illness annually with a 0.29% hospitalization rate (29). The Foodborne Diseases Active Surveillance Network (FoodNet) of the Centers for Disease Control and Prevention (CDC) Emerging Infections Program collects data from 10 states, which represents approximately 15% of the United States population. In 2005, 16,614 cases of foodborne illness were reported, and *E. coli* O157 was linked as the causative agent in 2.8% of these cases (41).

Understanding survival of *E. coli* O157:H7 in the environment during exposure to stressful conditions is necessary in order to apply efficacious decontamination and control strategies. Microorganisms manifest various intrinsic mechanisms for survival in stressful environments. Multiple antibiotic resistance attributed to chromosomal mutations in the *mar* operon (multiple antibiotic resistance) was first described in *Escherichia coli* K12 when selecting for low levels of tetracycline or chloramphenicol resistance (17). The *mar*-loci are present in *E. coli* K-12, *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter*, *Enterobacter*, and *Hafnia*, indicating that this operon is highly conserved among enteric microorganisms *(7, 10, 24)*.

The chromosomally mediated mar phenotype was found in mutants that constitutively express the *marRAB* operon *(8)*. The *mar* locus consists of two divergently

positioned transcriptional units that flank *marO: marC and marRAB.* The proteins produced by the *marRAB* operon are: MarR, a repressor *(8, 38)*; MarA, an activator that is associated with multiple antibiotic resistance (16, 18); and MarB, for which the function is not yet known (27).

The accumulation of MarA is the primary factor that controls expression of the mar phenotype (3, 19). MarA has been found to alter expression of more than 60 chromosomal genes; approximately 80% of these increased in transcription, and the remainder were down-regulated in *E. coli* K-12 (3). The expression of MarA altered the transcription of outer membrane proteins (e.g., OmpF, OmpX, OmpC) and increased expression of multi-drug efflux systems. MarA transcriptional regulator has a region composed of 99 amino acids that bind DNA as monomers in the promoter region and stimulate RNA polymerase contact and subsequent transcription (13, 15).

Constitutive expression of the *marRAB* operon may give microorganisms the ability to initially survive treatment with antibiotics and allow low-level resistance to many antimicrobials until secondary mutations arise giving increased resistance to a specific agent *(8)*. Also, the increase in ability of the organism to survive may increase its virulence and/or vice versa.

The well-described virulence factors of *E. coli* O157:H7 include *stx1* and *eaeA* (22, 36). Shiga toxin, *stx1*, is composed of two subunits. The B-subunit binds glycolipids in the host, and the A-subunit inhibits protein synthesis via disruption of ribosomal RNA (1). Shiga-toxins may cause hemorrhagic colitis, hemolytic uremic syndrome (HUS), thrombocytopenia, hemolytic anemia, and renal failure (21). The gene *eaeA* in

enterohemorrhagic *E. coli* produces intimin, a 97-kDa protein that plays a role in attachment onto and effacement of microvilli of the intestine (11, 14, 26, 28, 40).

Sodium benzoate is a food additive commonly used in acidic beverages due to its relatively low pka (4.2) and increased solubility (compared to benzoic acid). This compound is generally recognized as safe and may be added at concentrations up to 0.1% in foods (21CFR184.1733). A general mechanism of microbial inhibition for weak-acids, such as benzoic acid, starts with passive diffusion of the undissociated compound through the plasma membrane. Once inside the cell, the neutral internal pH causes dissociation of the acid, reduction in intracellular pH_i, and inhibition of membrane-bound transport (5). *E. coli* O157:H7 has been linked as the causative agent in outbreaks associated with unpasteurized apple and orange juice, and experimental data demonstrate the tolerance of *E. coli* O157:H7 to acidic environments (4, 6, 30, 39).

Expression of the marA gene and the transcriptional response of two virulence factors, *stx1* and *eaeA*, were investigated to determine the effects of sodium benzoate exposure. It was the goal of this research to compare expression of *stx1*, *eaeA*, and *marA* genes of control *E. coli* O157:H7 (untreated) and *E. coli* O157:H7 strain EDL933 exposed to 1% sodium benzoate.

Materials and Methods

Growth of *Escherichia coli* O157:H7

Escherichia coli O157:H7 strain EDL 933 (genome fully sequenced), obtained from the National Food Safety and Toxicology Center at Michigan State University, was used for this study. Cultures were grown in Luria-Bertani broth (LB; Difco, Becton Dickinson Microbiology Systems; Sparks, MD) containing 0 (control) or 1% sodium benzoate (Fisher Scientific; Fair Lawn, NJ) at 37˚C for 24 h, and transferred a minimum of three successive times at 24-h intervals before use. Cell counts were determined by serial dilutions of the overnight culture and plating on LB agar.

RNA Extraction

The RNeasy® mini kit (Qiagen; Valencia, CA) procedure was used to extract total RNA. Briefly, 1 ml of a 24-h culture was combined with 2 ml RNAprotect® bacteria reagent (Qiagen; Valencia, CA) and incubated for 5 min prior to centrifugation at 10,000 x *g* (4˚C) for 10 min. The cell pellet was combined with lysozyme (3 mg/ml; Sigma Aldrich; St. Louis, MO) in 100 µl 1x Tris-EDTA buffer (TE; Promega; Madison, WI) and incubated for 5 min with frequent agitation. The remainder of the extraction steps were followed according to the manufacturer's instructions and yielded 30 µl total RNA in DNase-RNase free water that was stored at -80˚C. RNA quantity (A_{260}) and purity (A_{260}/A_{280}; A_{260}/A_{230}) were determined using a Nanodrop ND-1000 spectrophotometer

(Nanodrop Technologies; Wilmington, DE, USA). Extracted RNA from all samples was diluted as appropriate to obtain equal concentrations for each treatment (35 ng/µl).

Nucleic Acid Amplification

Real-time one-step reverse-transcriptase PCR (rt-RT-PCR) was performed on serially-diluted RNA extracts. SuperScript™ III Platinum® SYBR® green one-step rt-RT-PCR kit reagents (Invitrogen; Carlsbad, CA) were utilized in 50 µl reactions (5 µl serially-diluted RNA, 25 µl SYBR® green reaction mix, 2 µl BSA, and 1 µl SuperScript™ III reverse transcriptase/Platinum® *Taq* Mix, 0.05 µg forward and reverse primers for the target gene, and the remainder RNase-DNAse free water). Primer sequences (Table 2.1) were developed for *marA*, *eaeA*, and *hemX* using Beacon Designer 4.0 software (Premier Biosoft International; Palo Alto, CA), and previously reported primers were utilized for *stx1* (20). All tables and figures are found in an appendix located at the end of the chapter. A Bio-Rad iCycler iQ™ multi-color real-time PCR detection system was used to conduct rt-RT-PCR using the following parameters: 1) conversion of mRNA to cDNA, 35 min at 50˚C; 2) denature reverse-transcriptase, 2 min at 94˚C; 3) denature template, 45 sec at 94˚C; annealing of primers, 45 sec with appropriate temperature (Table 2.1); and extension with *Taq* polymerase, 45 sec at 72˚C; repeated 50 times; 4) final extension, 7 min at 72˚C. All reactions were verified by gel electrophoresis, and all RT-PCR experiments were conducted in triplicate for each RNA sample.

Results and Discussion

Transcription of *marA* and *stx1* was 1-log greater in *E. coli* O157:H7 grown in the presence of 1% sodium benzoate as compared with the control, although cell numbers for each were similar when plated on LB agar (7.28 and 7.57 log CFU/ml, respectively). Table 2.2 lists the average cycle threshold (Ct) value and the limit of detection for each target transcript under treatment and control conditions. As an example of rt-RT-PCR results, output for *marA* is shown in Figure 2.1. Melt temperature analysis of rt-RT-PCR revealed that a single, specific product was produced for each target gene transcript (Figure 2.2). Gel electrophoresis confirmed rt-RT-PCR results revealing amplicons of the appropriate size, where *marA* and *stx1* products were visualized at a 1-log higher dilution in cells grown in 1% sodium benzoate (Figure 2.3) than in control. Both control and treated cells had similar transcription levels for *eaeA*. Under these conditions, *hemX* was validated as an appropriate housekeeping gene as it was expressed at similar levels in both treatments. It was equally important to design primers for the housekeeping gene that could be utilized at multiple annealing temperatures. Each primer set was optimized for the desired gene target as could be determined from the melt temperature analysis showing single peaks (Figure 2.2).

Expression of *marA* can be induced by exposure to benzoate (33, 37), chlorine, nitrite, acetic acid (33), pine oil (31), bile salts (34), spices and various food extracts (35), low levels of tetracycline and chloramphenicol (17), salicylates *(9, 37)*, and organic solvents (2). Salicylic acid and deoxycholate have been shown to induce transcription of

the *mar* operon by binding to the repressor, MarR (27, 34). Once the repressor can no longer bind to the operator region, transcription of *marAB* is induced. This mechanism may play a role in the observed increase in transcription of *marA* when exposed to sodium benzoate.

The *stx1*gene is encoded by a cryptic prophage, CP-933V, in *E. coli* O157:H7 strain EDL933 (32). Shiga-toxin production can be stimulated by the SOS response or growth under limited iron conditions (23, 32, 42). The effect of macrolide and quinolone antibiotics has been reported to increase Shiga-toxin production via phage induction associated with a bacterial SOS response or phage-induced cell lysis (23, 43). However, information on the response of *stx1* to food antimicrobials is lacking. Leenanon et al. (25) described an increase in production of *stx-2* mRNA for *E. coli* O157:H7 following acid adaptation or starvation. When the gene product of *stx-2* was monitored with *lacZ* fusions, there was no increase in β-galactosidase activity (25). This could be due to an increased sensitivity of RT-PCR assays when compared to β-galactosidase activity, or an indication that post-transcription control of toxin production may be involved (25). Therefore, it is important to understand that our data are only indicative of increased transcription of *stx1*, which may not lead to a subsequent increase in translation of Stx-1 or release of this toxin from the bacterial cell. Further work by Elhanafi et al. (2004) on cold and cold-acid stress of *E. coli* O157:H7 showed no effect on translation of Stx2, HylA, or EaeA production, but HylA and EaeA production were stimulated in an acidic environment (12). Our data also do not show any increase in *eae*A gene expression between treated and untreated cells. This could indicate that once intimate attachment

onto microvilli occurs, increase in *eae*A expression is probably not critical for the organism to grow and survive. However, it is likely that the level of expression of the Shiga-toxins, and subsequent translation and toxin release, play critical roles in causing pathogenic effects in the host, increasing the virulence and survival of the pathogen.

Conclusions

These data indicate that *stx1* and *marA* genes could play a role in *E. coli* O157:H7 virulence and enhanced survival if cells are exposed to sodium benzoate, while *eaeA* transcription was not altered. It is very important to understand the genetic response of pathogens when exposed to antimicrobials in order to assess risk and minimize illness from foodborne pathogens. While RT-PCR reactions can give insight to specific gene expression responses, microarray technologies give researchers the ability to look at the transcription response of all known genes for a microorganism. Our future goals include transcriptome analysis of *E. coli* O57:H7 treated with sodium benzoate using microarrays to obtain further insights into gene expression and gene regulation. Understanding gene regulation by validating microarray results will aid in mapping out metabolic pathways and mechanisms of bacterial survival and adaptations to stress. This ultimately will lead to improvements in design and implementation of appropriate control and mitigation strategies and minimizing foodborne disease outbreaks.

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Appendix

Table 2.1. Primers for rt-RT-PCR assays to determine mRNA expression of *stx1*, *marA*, and *eaeA* in *E. coli* O157:H7 when exposed to 0 and 1% sodium benzoate.

Primer	**Sequence (5'→3')**	**Annealing temp (˚C)**	**Product *Tm* (˚C)**	**Product size (bp)**
stx1F	GTGGCATTAATACTGAATTGTCATCA	65	83.5	108
stx1R	GCGTAATCCCACGGACTCTTC			
marAF	CGAGGACAACCTGGAATCAC	59	86	261
marAR	TGCGGCGGAACATCAAAG			
eaeAF	GGCATGAGTCATACAATAAGAAAG	61	84	223
eaeAR	ATCGTCACCAGAGGAATCG			
hemXF	GAAAATATTCGCTCTCGCCTGCTGG	-[1]	88	209
hemXR	TGGCTTTGCAGGGTTTCCGG			

[1] Primer set for the housekeeping gene, *hemX*, was utilized in all rt-RT-PCR reactions using the optimal annealing temperature for each specific gene target.

Table 2.2. Average cycle threshold (Ct) and limit of detection for *marA*, *stx1*, *eaeA*, and *hemX* rt-RT-PCR product of mRNA from 0 and 1% sodium benzoate-treated *E. coli* O157:H7.

	0% sodium benzoate		**1% sodium benzoate**	
Target gene	**Average[1] Ct value ± S.D.**	**Limit of detection**	**Average Ct value ± S.D.**	**Limit of detection**
marA	34.1 ± 0.6	10^{-6}	36.1 ± 0.75	10^{-7}
stx1	40.0 ± 0.25	10^{-5}	40.2 ± 0.7	10^{-6}
eaeA	36.2 ± 0.6	10^{-6}	35.5 ± 0.46	10^{-6}
hemX	28.4 ± 0.33	10^{-3}	27.3 ± 0.36	10^{-3}

[1] Minimum of three replicates; S.D. = one standard deviation.

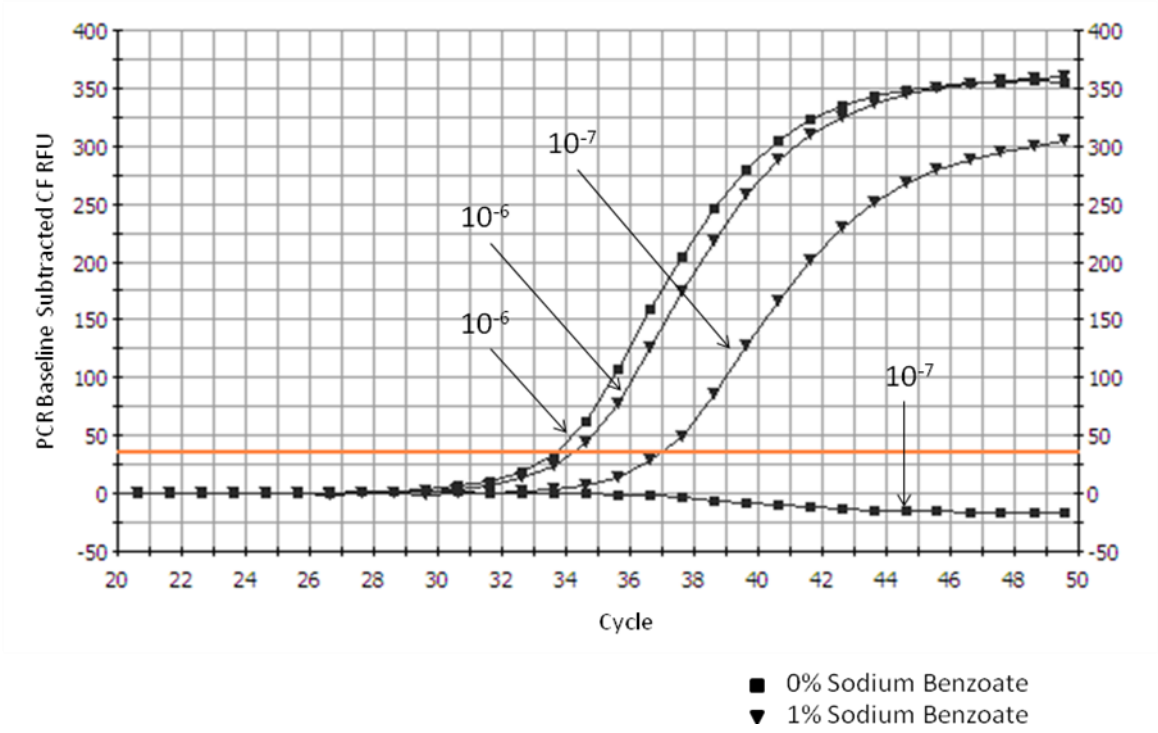

Figure 2.1. An example analysis of RT-PCR with *marA* mRNA showing the limit of detection and Ct values for 0 and 1% sodium benzoate-treated *E. coli* O157:H7.

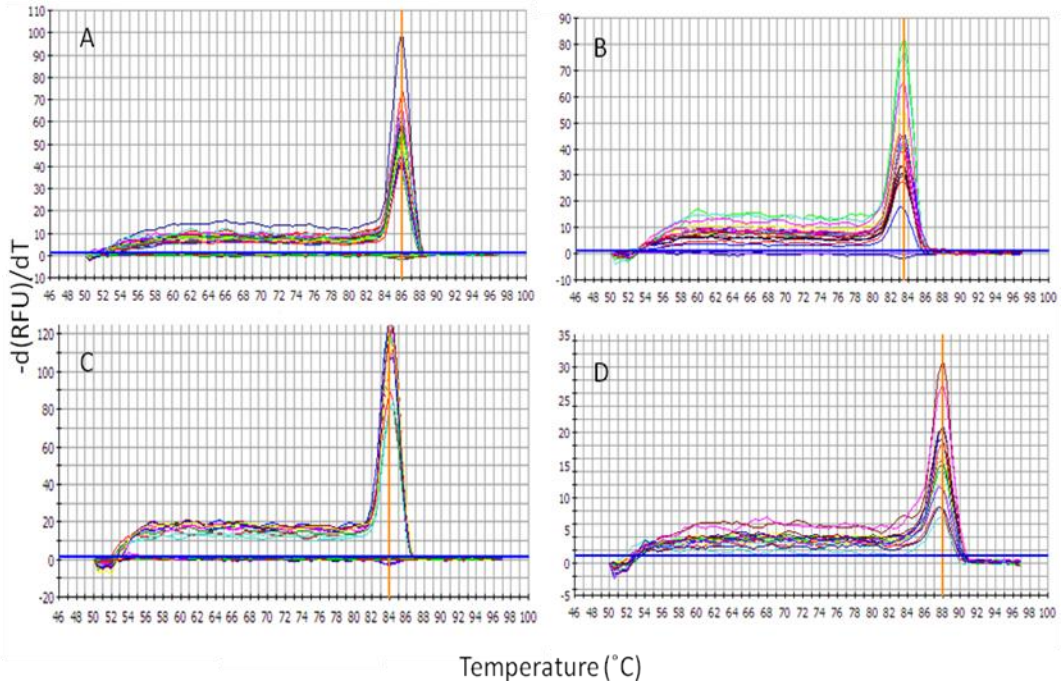

Figure 2.2. Melt temperature analysis of rt-RT-PCR products for A) *marA*, B) *stx1*, C) *eaeA*, and D) *hemX*.

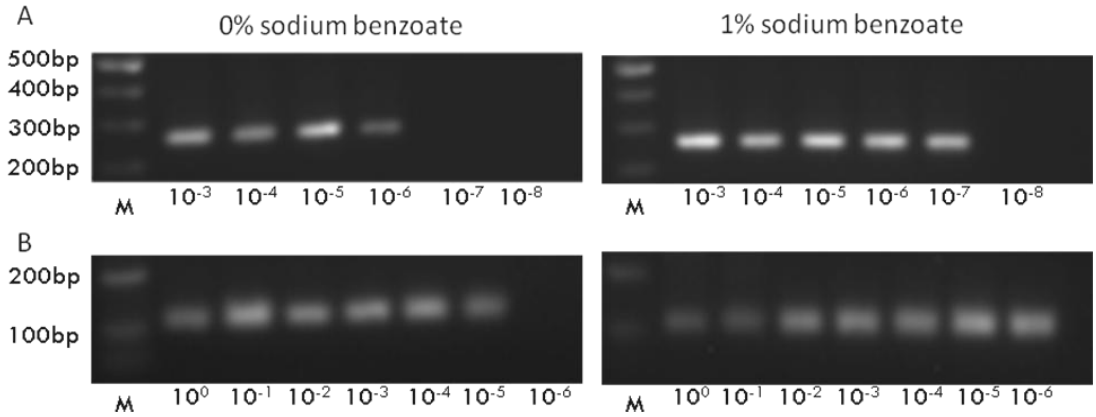

Figure 2.3. Agarose gel electrophoresis validation of A) *marA* and B) *stx1* rt-RT-PCR products (from three replicates) to verify a 10-fold increase in transcription of these genes when treated with sodium benzoate. M 100 bp marker

Chapter 3

Increased Transcription of the Phosphate Specific Transport (Pst) System of *Escherichia coli* O157:H7 Exposed to 0.5% Sodium Benzoate Determined with High-density Oligonucleotide Microarrays and Reverse-transcriptase Polymerase Chain Reaction

Abstract

Sodium benzoate is a widely used food antimicrobial in drinks and fruit juices. A microarray study was conducted to determine the transcriptional response of *Escherichia coli* O157:H7 to 0.5% (w/v) sodium benzoate, and the phosphate specific transport (Pst) system was found to be highly up-regulated. The Pst system is composed of four subunits: PstS, a periplasmic phosphate binding protein; PstA and PstC, involved in formation of the transmembrane portion of the Pst system; and PstB, an ATPase. This system transports inorganic phosphate into bacterial cells under phosphate limited conditions. Stationary phase *E. coli* O157:H7 grown in 150 ml Luria-Bertani broth (LB) was exposed to 0 (control) and 0.5% sodium benzoate. Each treatment was duplicated, sampled at 0 (immediately after exposure), 5, 15, 30, and 60 min. Total RNA was extracted and analyzed with *E. coli* 2.0 Gene Chips. Significant ontology categories affected by sodium benzoate exposure were determined with JProGO software.

Increased expression of the Pst system was observed after 5 min of exposure to sodium benzoate; *pstS*, *pstA*, *pstB*, and *pstC* genes were up-regulated >2-fold (linear scale) at 5, 15, 30, and 60 min. Increased expression of several other efflux systems, such as AcrAB-TolC, was also observed.

The Pst system may act as an efflux pump under these stress-adapted conditions, as well as increase transport of phosphorus to aid in DNA, RNA, ATP, and phospholipid production. Understanding adaptations of *E. coli* O157:H7 under antimicrobial exposure is essential to better understand and implement methods to inhibit or control its survival in foods.

Introduction

E. coli O157:H7 is a member of the enterohemorrhagic *E. coli* (EHEC) and causes hemorrhagic colitis in humans. The symptoms of an *E. coli* O157:H7 infection generally include severe abdominal cramps and diarrhea (often times bloody), which can lead to death. In children under the age of five, infection can lead to a complication known as hemolytic uremic syndrome (HUS), resulting in red blood cell destruction, renal failure, and central nervous system complications *(43)*. *E. coli* O157:H7 can cause disease at a low infectious dose (10 - 100 cells) *(39)*. This can be attributed to the tolerance of this bacterium to low pH, which allows passage through the stomach and colonization in the intestinal tract *(39)*. *E. coli* O157:H7 has been linked as the causative agent in outbreaks associated with unpasteurized apple and orange juice, and experimental data demonstrate the tolerance of *E. coli* O157:H7 to acidic environments *(5, 13, 40, 47)*.

While it is very hard to quantify the impact foodborne illnesses have in the United States, it has been estimated that foodborne disease causes 76 million illnesses annually and approximately 5,000 fatalities *(36)*. There were 16,614 laboratory-confirmed cases of foodborne illness from FoodNet participants in 2005 (about 15% of the United States population), and *E. coli* O157:H7 was the causative agent in 2.8% of these cases (3). The Centers for Disease Control and Prevention estimates that there are 73,000 cases of *E. coli* O157:H7 illness annually, leading to an estimated 2,100 hospitalizations in the United States (9). It was estimated that the cost of EHEC infections was $405 million dollars in 2003 *(22)*.

The well-described virulence factors of *E. coli* O157:H7 include *stx1*, *stx2*, and *eaeA (27, 45)*. Shiga toxins are composed of two subunits. The B-subunit binds glycolipids in the host, and the A-subunit inhibits protein synthesis via disruption of ribosomal RNA (1). Shiga-toxins also cause hemorrhagic colitis, hemolytic uremic syndrome (HUS), thrombocytopenia, hemolytic anemia, and renal failure *(26)*. The gene *eaeA* in enterohemorrhagic *E. coli* produces intimin, a 97-kDa protein that plays a role in attachment onto and effacement of microvilli of the intestine *(16, 21, 31, 35, 51)*.

Sodium benzoate is a food additive commonly used in acidic beverages due to its relatively low pKa (4.2) and increased solubility (compared to benzoic acid). This compound is generally recognized as safe and may be added at concentrations up to 0.1% in foods (21CFR184.1733). Benzoate is degraded in microorganisms via the β-ketoadipate pathway resulting in acyl CoA and succinic acid (12). These degradation pathways serve as innate responses to weak acid resistance.

A general mechanism of inhibition for weak-acids starts with diffusion of the undissociated compound through the cell membrane by passive diffusion. Once inside the cell, the higher pH causes dissociation of the acid and lower intracellular pH_i and inhibition of membrane-bound transport (8). Eklund *(17)* determined that uptake inhibition contributed to growth inhibition, but did not seem to completely explain this phenomenon. Eklund *(20)* found that ΔpH (pH difference) was eliminated by sorbic acid as well as methyl and butyl paraben, whereas the membrane potential ($\Delta\psi$) was maintained *(19)*. This indicates more than simple inhibition of the proton motive force, since the membrane potential maintained would allow for uptake of essential

compounds for cellular maintenance. It has been determined that dissociated weak acids also contribute a small portion to growth inhibition, which cannot be explained by the effects of pH_i*(18)*. Sorbic acid, sorbic alcohol and sorbic aldehyde have been found to cause similar levels of inhibition, demonstrating a mechanism of inhibition other than a weak acid effect *(49)*. Bracey and others *(7)* found that pH_i was not altered in *Saccharomyces cerevisiae* exposed to sorbic acid. However, a higher ADP/ATP ratio was found indicating that the cell was expending energy to maintain pH_i (7), and this expenditure of ATP was theorized to decrease cellular growth.

Membrane disruption has also been attributed as a mechanism of inactivation by sorbic acid *(7, 49)*. Krebs and *et al. (29)* observed a reduction in phosphofructokinase activity in *Saccharomyces cerevisiae* exposed to sorbic acid *(29)*. The disruption of glycolysis was thought to be caused by a decrease in pH_i that decreased the enzymatic activity of phosphofructokinase. Nitrogen starvation and benzoic acid treatment inhibit growth of *Saccharomyces cerevisiae*. However, when used in combination, they are cytocidal *(23)*. It was found that benzoic acid inhibited the break-down of cytosolic compounds to form amino acids and nucleotides necessary for growth and survival *(23)*.

Phosphate Specific Transport (Pst) of *Escherichia coli*

The acquisition of phosphorus is essential for bacteria to supply energy and synthesize DNA, RNA, lipopolysaccharides, and phospholipids. Microorganisms must transport inorganic phosphorus (P_i) into the cytoplasm to survive. The first step in this process for *E. coli* is to transport organic and inorganic forms of phosphorus across the

cell wall. In *E. coli,* there are two common pore-forming proteins, OmpC and OmpF. However, under phosphate limited situations, a third anion binding porin, PhoE, is expressed in order to sequester phosphorus in the periplasm *(41)*.

The Pst system is in the ATP binding cassette (ABC) superfamily of transporters and is one mechanism of transporting P_i across the cell membrane. This system is known to be expressed when P_i concentration is below 0.001 M *(50)*. The Pst system has a high affinity for binding of phosphate and is composed of four proteins, PstS, PstC, PstA, PstB, and PhoU *(50)*. The mechanism by which phosphate is transported across the cell membrane is shown in Figure 3.1. All tables and figures are found in an appendix located at the end of the chapter. The gene *pstS* (formerly *phoS*) encodes a phosphate binding protein that binds P_i in the periplasmic space where it can be transported across the cell membrane *(37, 38, 52)*. PstC and PstA are transmembrane proteins that form a passage across the cell membrane (14), while PstB catalyzes the movement of phosphorus into the cytoplasm by hydrolysis of ATP (11).

PhoR and PhoB regulate the Pho regulon, of which the Pst system is a member. PhoR is a transmembrane signal transduction protein that senses low levels of P_i in the periplasm. PhoR has an extracellular sensory domain and an intracellular signaling domain *(48)*. The mechanism by which phosphate concentration is sensed by PhoR is shown in figure 3.2. Under phosphate limited conditions, PhoR undergoes autophosphorylation and acts as a histidine kinase that phosphorylates PhoB (Figure 3.2) *(33, 34)*. Once PhoB is activated, it stimulates the Pho regulon by binding to the 'Pho box' consensus sequence stimulating transcription of *pstS* *(32, 33)*. In addition to

pstSCAB-phoU, PhoB also regulates the expression of PhoA, which is a periplasmic alkaline phosphatase that frees P_i from phosphate containing compounds so it is available for uptake into the cytoplasm. PhoU is a 21 kDa cytoplasmic protein that is necessary for repression of the Pho regulon *(2, 34)*. PhoU acts with phosphate-bound PstS, PstA, PstB, and PstC to form a repressor complex keeping PhoR in the dephosphorlyated state (Figure 3.3). PhoU also acts to promote dephosphorylation of PhoB, thus inactivating this protein *(48)*.

A role other than phosphate transport has been determined for the Pst system in *Mycobacterium smegmatis*. A ciprofloxacin-resistant strain of *M. smegmatis* strain was found to over-express the Pst operon when grown in sufficient quantities of phosphorus (4, 6, 10). The *pstB* gene was also shown to have increased amplification on the chromosome of the ciprofloxacin-resistant strain of *M. smegmatis* (10). When the *pstB* gene was mutated, the minimum inhibitory concentration of ciprofloxacin for *M. smegmatis* was greatly reduced (4). It was also demonstrated that the ciprofloxacin resistant strain had a much greater efflux of this fluoroquinolone from the cell compared to that of the wild type or *pstB* mutant (4). The fluoroquinolone resistant *M. smegmatis* strain showed increased phosphate uptake that was diminished along with ciprofloxacin resistance when *pstB* was mutated (6). These results indicate that the Pst system serves as an efflux mechanism of ciprofloxacin for *M. smegmatis* in addition to the traditional role as a high-affinity transporter of phosphorus.

Materials and Methods

Escherichia coli O157:H7

Escherichia coli O157:H7 strain EDL 933, obtained from the National Food Safety and Toxicology Center at Michigan State University, was used for this study. Cultures were grown in 125 ml Luria-Bertani broth (LB; Difco, Becton Dickinson Microbiology Systems; Franklin Lakes, NJ) at 37˚C for 24 h, and transferred a minimum of three successive times at 24-h intervals before use. The entire volume of stationary-phase cells were centrifuged at 8000 x *g* for 10 min at 4˚C. The cell pellet was resuspended in LB containing 0 (control) or 0.5% (w/v) sodium benzoate (Fisher Scientific; Fair Lawn, NJ). At every sampling time, cell counts were determined by surface plating on Sorbitol MacConkey Agar (SMAC; Difco, Becton Dickinson Microbiology Systems; Franklin Lakes, NJ.

Experimental Design

A completely randomized design with repeated measures was used to study the effects of sodium benzoate exposure on *E. coli* O157:H7. The control (0%) and treatment (0.5%) sodium benzoate conditions were replicated once and sampled over five time points [0 (immediately after exposure), 5, 15, 30, and 60 min].

Total RNA Isolation

The RNeasy Mini Purification Kit (Qiagen; Valencia, CA) was used to extract total RNA from *E. coli* O157:H7 cultures. 1.2 ml RNA*later* Stabilization Reagent (Qiagen;

Valencia, CA) was combined with 0.6 ml *E. coli* O157:H7. RNA*later* immediately stabilizes RNA to help safeguard against degradation of mRNA. After 10 min at room temperature, the mixture was centrifuged at 8000 x *g* for 10 min under refrigeration (Eppendorf 5417C; New York, NY). The supernatant was decanted and the bacterial pellet was stored at -70˚C until further processed (approx. 2-10 hr).

100 µl tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, ph 8.0; Promega; Madison, WI) containing lysozyme (3 mg/ml; Sigma-Aldrich; St. Louis, MO) was added to each cell pellet. The pellet was thoroughly mixed and incubated at room temperature for 10 min. This allowed for disruption of the cell wall and membrane to allow for extraction of RNA. 350 µl buffer RLT was combined with the tris-EDTA/lysozyme cell suspension to further lyse the cells. Buffer RLT contains guanidine thiocyanate which aids in denaturing proteins, effectively inactivating ribonucleases to preserve RNA quality. The total volume was combined with 250 µl of 200 proof ethanol (Aaper Alcohol; Shelbyville, KY) to solubilize the cell lysate, and the entire sample was transferred to the RNeasy Mini spin column in a collection tube and centrifuged at 8000 *x g* for 15 sec at room temperature (Sorvall Biofuge Pico; Kendro, Germany). The silica-based membrane efficiently binds up to 100 µg of RNA longer than 200 bases. The collection tube and eluate were discarded, and the spin column was placed in a new collection tube. 700 µl buffer RW1 was applied to the column, centrifuged, and flow-through was discarded as described previously. RW1 also contains guanidine thiocyanate to help disrupt protein structure and ethanol to solubilize and remove other cellular material. Twice, 500 µl of buffer RPE was applied to the spin column allowing 5 min prior to centrifugation at 8000

x g for 15 sec. Ethanol also is a constituent of RPE and serves a primary role of removing residual salts from previous buffers. The spin column was placed in a 1.5 ml collection tube that was used for long-term storage. In order to elute RNA from the spin column, 30 µl RNase-free water was applied directly to the membrane and centrifuged at 8000 *x g* for 15 sec. Ten RNA samples were extracted for each treatment and replicate (40/ time point). The ten samples were pooled and re-aliquoted to remove variation in RNA concentration from differing RNA extraction efficiencies. The RNA eluate was stored at -80˚C until evaluated for quantity, quality, and purity.

Evaluation of RNA Quality, Quantity, and Purity

The NanoDrop spectrophotometer (NanoDrop Technologies; Wilimington, DE) was utilized to determine absorbance of samples at 230, 260, and 280 nm. RNA concentration is determined by absorbance at 260 nm (1 absorbance unit=40 µg/ml). The A_{260} / A_{230} and A_{260} / A_{280} ratios indicate contamination (e.g. proteins, ethanol, salts) and should fall between 1.8 to 2.1. Lower ratios may interfere with down-stream processing of RNA for microarrays and RT-PCR.

In addition to spectrophotometric analysis, RNA samples were also analyzed for quality using the Agilent 2100 electrophoresis bioanalyzer (Agilent Technologies; Santa Clara, CA). This analysis allows for visualization of 23S and 16S rRNA bands, assuring that there is no degradation of RNA and the absence of chromosomal DNA.

High Density Oligonucleotide Microarray Analysis

cDNA Synthesis

The Affymetrix protocol for GeneChip prokaryotic processing was followed for this study. *In vitro* synthesized polyadenylated transcripts for *Bacillus subtilis* (Affymetrix; Santa Clara, CA) were diluted 1 x $10^{-2.5}$ and spiked-into the primer annealing master mix at a volume of 2 µl. This "spike-in" is evaluated in the final processing of the microarray as a means of monitoring the assay sensitivity and performance from the beginning to final stages. Ten micrograms of total RNA was added to the primer annealing master mix (final concentration 0.33µg/µl). Ten microliters of random primers (Invitrogen; Carlsbad, CA) at a concentration of 75 ng/µl (final concentration 25 ng/µl) and nuclease-free water (Ambion; Austin, TX) were added to bring the entire master mix volume to 30 µl. The following conditions were utilized to anneal random primers: 70˚C for 10 min; 25˚C for 10 min; chill to 4˚C (PCR Sprint Thermal Cycler; Thermo Fisher Scientific; Waltham, MA).

The RNA/primer master mix was centrifuged at 5000 x *g* for 1 min at room temperature. The SuperScript first-strand synthesis system (Invitrogen; Carlsbad, CA) reagents and SUPERase-In (Applied Biosystems; Foster City, CA) were combined with the 30 µl RNA/primer master mix as described in Table 3.1. The cDNA synthesis conditions were: 25˚C for 10 min; 37˚C for 60 min; 42˚C for 60 min; 70˚C for 10 min; chill to 4˚C. Remaining RNA was degraded with the addition of 20 µl/rxn of 1N NaOH and incubated

at 65˚C for 30 min. The reaction was neutralized with the addition of 20 µl/rxn of 1N HCl.

Purification and Quantitation of cDNA

The MinElute PCR Purification Kit (Qiagen; Valencia, CA) was utilized to clean up the cDNA synthesis product prior to further microarray processing. Five volumes of buffer PB were added to the cDNA product, placed on a MinElute spin column, and centrifuged at room temperature at 5000 x *g* for 1 min. Eluate was discarded and 750 µl of buffer PE was applied to the column and centrifuged as described above. Residual ethanol from the PE buffer was allowed to evaporate from the spin column prior to eluting cDNA with 12 µl buffer EB. The resulting purified product was analyzed as previously described with the NanoDrop spectrophotometer to determine cDNA yield.

cDNA Fragmentation

Purified cDNA (3 µg) was combined with 10X One-Phor-All buffer (GE Healthcare; Buckinghamshire, England), DNAse I (0.6 U/µl; GE Healthcare; Buckinghamshire, England), and nuclease-free water as described in Table 3.2. The reaction was held at 37˚C for 10 min followed by 98˚C for 10 min to inactivate DNAse I.

Terminal Labeling

The GeneChip DNA Labeling Reagent (Affymetrix; Santa Clara , CA) was used to label the 3' termini of the fragmented cDNA products with biotin. Volumes of reagents utilized in this reaction are listed in Table 3.3. The reaction was held at 37˚C for 60 min followed by addition of 2 µl of 0.5M EDTA (Invitrogen; Carlsbad, CA) to arrest the

labeling reaction by inactivating the terminal deoxynucleotidyl transferase (Promega; Madison, WI).

cDNA Target Hybiridization

The biotin labeled, fragmented cDNA was added to the hybridization master mix. Table 3.4 describes the reagents utilized in this reaction. The B2 control oligo acts as a hybridization control and allows for alignment of the GeneChip microarray in the analysis software. Herring sperm DNA was used as a blocking agent during hybridization to prevent improper annealing of cDNA fragments with probes.

The *E. coli* Genome 2.0 Array (Affymetrix; Santa Clara, CA) was allowed to equilibrate to room temperature. This GeneChip allows detection of transcripts from *E. coli* K12 (MG1655) as well as a uropathogenic strain (CFT073) and two enterohemorrhagic strains of the serotype O157:H7 (EDL933 and Sakai). 200 µl of the hybridization solution was added to the GeneChip and the array was placed in the hybridization oven (Affymetrix; Santa Clara, CA) at 45˚C, 60 RPM for 16 hr. The hybridization solution was then removed and replaced with 80 µl of non-stringent wash buffer (wash buffer A; 6x SSPE, 0.01% Tween-20) and the GeneChips were stored at 4˚C until washed and stained.

GeneChip Washing, Staining, and Scanning

The GeneChip Operating Software (GCOS; Affymetrix; Santa Clara, CA) controlled the operation of the Fluidics Station 450 (Affymetrix; Santa Clara, CA). Streptavidin phycoerythrin (SAPE; Table 3.5) was loaded into the first and third 1.5 ml staining vial in

the fluidics station, while the second stain vial contained an antibody solution mix (Table 3.6). The Mini_prok2v1_450 fluidics station protocol (Table 3.7) was followed for washing and staining hybridized biotin labeled cDNA fragments. The GeneChip Scanner 3000 (Affymetrix; Santa Clara, CA) and GCOS were used to produce .cel files which contained the fluorescence intensity data for a probe. Each target sequence contains 11 25-oligomer probes that are spread throughout the entire sequence (perfect match) as well as 11 25-oligomer probes that match the target with the exception of the 13th nucleotide which is a mismatch to the desired target sequence and indicates non-specific binding (mismatch). Fluorescence data for these 22 probes are combined into a probe set for further analysis.

Statistical Analysis of Microarray Data

The R programming environment (v 2.5.1) was used for all microarray data analysis. First, GCOS generated CEL files, CDF file, and probe file were uploaded into the GCRMA package (v 2.0.0) *(54)*. This program normalizes microarray data by using Affymetrix probe sequence affinities based on the specific probe sequence in addition to normalization from background noise also used in robust multiarray average (RMA) analysis. Following normalization with the GCRMA package, the intensities for probe sets were compared among treated and control cells across all time points using the microarray analysis of variance package (MAANOVA; v 1.4.1) *(53)*. Next, the positive false discovery rate (pFDR; q-value) was determined to differentiate genes which were significantly regulated in response to exposure of sodium benzoate (qvalue; v 1.10.0)

(15). A subset of genes which were differentially regulated ($q < 0.05$) were determined and used for further analysis.

Selection of genes for validation with real-time one-step reverse-transcriptase PCR (rt-RT-PCR)

Three tools were used to analyze microarray results so a subset of significantly regulated genes could be selected for further validation with rt-RT-PCR. The Pathway Tools Omics Viewer (SRI International; Menlo Park, CA) was used to visualize metabolic, transport, and signaling pathways in addition to proteins integral to the membrane and periplasm specific to *E. coli* O157:H7 *(42)*. The output would link through pathway tools to EcO157Cyc (SRI International; Menlo Park, CA) and give further insight to pathways, molecular interactions, proteins, and genes. However, the database specific to *E. coli* O157:H7 was computationally generated with very little curation based on findings in current literature. When the pathways of interest were homologous to *E. coli* K-12, the EcoCyc database which receives extensive literature based curation was utilized to gain further insight to functional and regulatory information for a pathway of interest *(28)*.

A tool was also used to analyze the gene products that were significantly regulated with respect to their biological processes, cellular components, and molecular functions. These ontology terms were analyzed with the Java Tool for the Functional Analysis of Prokaryotic Microarray Data using the Gene Ontology (JProGO; Braunschweig, Germany) *(46)*. Wilcoxon's nonparametric test was used to determine statistically significant ontology nodes using an FDR multiple hypothesis correction

(p<0.05). These outputs allowed for selection of genes which would undergo further validation of expression using rt-RT-PCR.

Selection of housekeeping genes for standardization of rt-RT-PCR

Secondary validation of microarray data was achieved with the Pfaffl method which incorporates the efficiency of a PCR reaction in addition to the difference in Ct values between the treated and control samples *(44)*. The equation below shows how the ratio is calculated to determine increased or decreased levels of target mRNA when comparing the control and treated sample. This difference is also standardized with one or more reference or housekeeping genes which are expressed at the same level between the control and treatment.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{Ct}_{\text{target}}(control\text{-}sample)}}{(E_{\text{reference}})^{\Delta \text{Ct}_{\text{reference}}(control\text{-}sample)}}$$

From the microarray data, genes were selected which had a highly non-significant q-value (q>0.98). Genes were excluded if their function was not known, or if there was a high amount of variability between replicate microarrays. rt-RT-PCR was used to validate these genes as appropriate housekeeping markers for microarray validation. Each sample was run in triplicate and there were a total of six cycle threshold values for each treatment within each time point. A completely randomized design with repeated measures was used to assure there was no difference in cycle threshold values within each time point (p > 0.05; SAS v9.1; Cary, NC). This

methodology helped to assure that there was no difference in mRNA expression between treatments.

Nucleic Acid Amplification

rt-RT-PCR was performed on RNA extracts as a means of microarray validation. SuperScript™ III Platinum® SYBR® green one-step rt-RT-PCR kit reagents (Invitrogen; Carlsbad, CA) were utilized in 25 µl reactions (2.5 µl serially-diluted RNA, 12.5 µl SYBR® green reaction mix, 1 µl BSA, and 0.5 µl SuperScript™ III reverse transcriptase/Platinum® *Taq* Mix, 0.025 µg forward and reverse primers for the target gene, and the remainder RNase-DNAse free water). Primer sequences (Table 3.8) were developed using Beacon Designer 4.0 software (Premier Biosoft International; Palo Alto, CA). The Bio-Rad My iQ™ single color real-time PCR detection system (Hercules, CA) was used to conduct rt-RT-PCR using the following parameters: 1) conversion of mRNA to cDNA, 35 min at 50˚C; 2) denature reverse-transcriptase, 2 min at 94˚C; 3) denature template, 30 sec at 94˚C; annealing of primers, 45 sec at 55˚C; and extension with *Taq* polymerase, 30 sec at 72˚C; repeated 40 times; 4) final extension, 5 min at 72˚C; 5) melt curve . All rt-RT-PCR experiments were conducted in triplicate for each RNA sample and a negative control was included for each assay.

Results and Discussion

Microarray analysis revealed a large number of genes that were significantly regulated in response to 0.5% sodium benzoate. As shown in Table 3.9, the number of

genes which were differentially regulated increased with exposure time, and after 60 minutes of exposure to sodium benzoate 1707 genes were found to be differentially expressed ($q \leq 0.05$).

Ontology analysis allowed for insight to genes which responded to sodium benzoate exposure based on biological process, molecular functions, or cellular components. Genes categorized into biological processes were the greatest to respond across all time points (Table 3.10). There were a large number of genes associated with biological process nodes which responded quickly to sodium benzoate exposure (0 and 5 min). Most of these nodes were associated with metabolic activities such as protein biosynthesis. Genes associated with the ribosome and RNA binding were also found for these early time point. Genes involved in protein translation respond very early to the stimulus of sodium benzoate.

There was a rapid decline in significant ontology categories at 15 min, but an increase was observed for 30 and 60 min. At 15 min the biological process nodes represented genes that responded to unfolded proteins and alcohol metabolism. Significant cellular component nodes were found for genes involved with the cell membrane. Genes were also found to be regulated that responded to the molecular functions of carbohydrate transport and nickel ion binding.

Genes associated with molecular functions and cellular components had a greater response to sodium benzoate after 30 and 60 min of exposure. Molecular function nodes which had increased expression at these time points pertained to RNA and ATP binding activity as well as carbohydrate transport. The significant cellular

component nodes at these times were oriented around the ribosome and products which were integral to the membrane or part of the inner membrane. Biological process nodes associated with a response to chemical and antibiotic stimuli were found after 15 min of exposure and continued until 60 min. Many of these genes are components of efflux pumps as well as transcription regulators, such as *marA,* the multiple antibiotic resistance regulon activator.

Phosphate transport was one biological process node which was significantly regulated at 30 and 60 min. This insight spurred a closer look at this finding. Increased expression after only 5 min was found for all genes associated with the high affinity phosphate transport system (*pstSCAB-phoU*) when *E.coli* was exposed to sodium benzoate. Therefore, validation of the genes integral to the pst system as well as the transcriptional regulators (*phoRB*) for this operon was conducted using the Pfaffl method.

The glutamine synthetase adenyltransferase (*glnE*) gene catalyzes the addition of AMP to a subunit of glutamine synthetase. PspF is a σ^{54} dependent transcriptional activator that is cis-acting on the phage-shock protein (psp) operon and has been found to be constitutively expressed *(24, 25)*. No significant differences were found in Ct values for these genes across treatments within time points ($p > 0.05$). Therefore, *glnE* and *pspF* were utilized as the housekeeping genes by which rt-RT-PCR assays were normalized in the Pfaffl method.

The $\log_2$ fold-change between 0.5% sodium benzoate treated *E. coli* O157:H7 cells and that of the control conditions is shown in Table 3.11. Microarray generated

values for the *pstSCAB* and *phoBR* genes in the Pho regulon indicate at least a 2.8-fold induction (linear-scale) when *E. coli* O157:H7 was exposed to sodium benzoate. When these values were validated with rt-RT-PCR, *phoB* and *phoR* transcription levels were not as high as indicated by microarray analysis. The microarray results indicated an 11- to 17-fold increase in transcription of *phoB*, and at least a 34-fold induction of *phoR* when assessed on a linear scale. However, when determining transcript levels with rt-RT-PCR, the highest induction observed was a 5.2-fold increase for *phoB* at 5 min. This indicates that for these two transcripts, there was increased probe fluorescence seen in the microarray analysis. This could be attributed to non-specific binding of cDNA to probe sets in the microarray assay, which would arbitrarily increase the observed fluorescence. Also, typically the Affymetrix prokaryotic microarrays are only exposed once with phycoerythrin, but in this study all arrays were exposed twice to this fluorophore. This could also increase the level of fluorescence, but since all arrays were processed in the same manner this result should have been negated when determining the ratio of the control to treated conditions. Also, when comparing the induction levels for *pstSCAB* observed in microarray and rt-RT-PCR, very similar results were obtained (Table 3.11). This support non-specific binding to the *phoBR* probes in the Affymetrix *E. coli* 2.0 microarrays, thereby artificially inflating the fluorescence values obtained. This stresses the importance of validating microarray results with a secondary method such as rt-RT-PCR or Northern hybridization. Nevertheless, increased transcription was observed for *pstSCAB* and *phoBR* when *E. coli* O157:H7 was exposed to 0.5% sodium benzoate (Table

3.11). The Ct values and standard deviations obtained from the validation of transcription levels with rt-RT-PCR are listed in Table 3.12.

PhoU was shown to have increased transcription across all time points (Table 3. 13). However, these values were not validated with rt-RT-PCR, and like *phoBR,* may have arbitrarily high fold-changes detected with microarrays. The polyanion porin, PhoE, was not found to be differentially regulated at any time point in the microarray study. This suggests that transcription of this gene is not stimulated under these growth conditions. If phosphate was limited, transcription of this gene should have increased to help sequester phosphate and other organic molecules that bound phosphorus from the growth medium.

The *pstSCAB-phoU* and *phoBR* genes may have increased transcription to serve as an efflux pump in addition to phosphate transport. Although this phenomenon has not been documented in *E. coli*, it has recently been determined for *Mycobacterium smegmatis (4, 6)*. Several components of other multidrug efflux pumps were also shown to have increased transcription in response to exposure with 0.5% sodium benzoate (Table 3.14). Although there are several different systems for transporting these compounds from the cytoplasm, only one porin (TolC) is used to span the outer membrane. In this study, *tolC* transcription was not observed to be increased while the proteins that span the cell membrane and periplasm, such as those cited in Table 3.12, were shown to have increased expression.

These results indicate that there is an increased need for phosphate when *E. coli* O157:H7 is exposed to sodium benzoate. Therefore, if phosphate could be limited, *E.*

coli O157:H7 could have decreased ability to adapt and survive this stressful environment. If phosphate is depleted, the microorganism's ability to repair DNA and synthesize mRNA would be hampered. Protein biosynthesis would also be negatively influenced that would in turn reduce enzymatic activity. The cell would also have lowered ability to produce lipopolysaccharides and phospholipids in the cell membrane. Most importantly, the energy supply of the cell would be greatly impacted under restriction of intracellular phosphate.

The pst system has been found to be a virulence factor in *E. coli* O78, causes airsacculitis and septicemia in poultry. When the pst system was mutated, the infectivity of this strain was attenuated in an avian model and fewer isolates were obtained from infected tissues *(30)*. The mutated strain was also less able to survive acid-shock treatment (pH 3.0) when compared to the wild-type and had a two-fold lower MIC for polymixin *(30)*. Although these results were found in an extraintestinal *E. coli*, they indicate that the pst system plays a large role in the ability of *E. coli* O78 to survive and cause illness. This observation has also been made in *Proteus mirabilis,* which causes complicated urinary tract infections. When the *pstS* and *pstA* genes were inactivated with Tn5 insertions, the Pho regulon was constitutively expressed. Although the mutant cells grew and survived in urine and growth media *in vitro,* they did not cause urinary tract infections in a murine model. When these mutants were complemented with functional *pstS* and *pstA*, the ability to cause urinary tract infections was restored to that of the wild-type. Both of these examples demonstrate the importance of the pst system in survival and pathogenicity of microorganisms.

Conclusions

The pst system is up-regulated in response to sodium benzoate in *E. coli* O157:H7. This system would give *E. coli* O157:H7 the ability to recharge intracellular phosphate supplies to rapidly produce several compounds such as ATP, nucleic acids, and phospholipids in response to this adverse environment. This system has been shown to play a role in virulence and pathogenicity in other microorganisms and is part of the adaption of *E. coli* O157:H7 to weak acids. In addition to the known role as a high-affinity transport system for phosphate, the pst system may also serve as an efflux pump allowing removal of benzoate.

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Appendix

Table 3.1. cDNA synthesis master mix components.

Reagent	Volume per reaction (µl)
5X First-strand cDNA Buffer	12
100 mM DDT	6
10 mM dNTP Mix	3
SUPERase-In (20 U/µl)	1.5
Superscript II (200 U/µl)	7.5
RNA/primer Hybridization	30

Table 3.2. cDNA fragmentation master mix components.

Reagent	Volume per reaction (µl)
10x One-Phor-All Buffer	2
cDNA	15
DNAse I	1.8
Nuclease Free Water	1.2

Table 3.3. Terminal labeling of cDNA fragments with biotin master mix components.

Reagent	**Volume per reaction (µl)**
5x Reaction Buffer	10
GeneChip DNA Labeling Reagent, 7.5mM	2
Terminal Deoxynucleotidyl Transferase	2
Nuclease Free Water	16
Fragmented cDNA Product	20

Table 3.4. Biotin labeled cDNA hybridization master mix components.

Reagent	**Volume per reaction (µl)**
2x Hybirdization Buffer[1]	40
3 nM B2 Control Oligo	1.3
10 mg/ml Herring Sperm DNA	0.8
50 mg/ml BSA	0.8
100% DMSO	6.2
Nuclease Free Water	5.9
Fragmented and Labeled cDNA Product	25

[1] 200 mMMES, 2M [Na^+], 20 mM EDTA, 0.01% Tween-20

Table 3.5. Streptavidin phycoerythrin (SAPE) staining solution components.

Reagent	**Volume per reaction (µl)**	**Final concentration**
2x MES stain buffer	600	1x
50 mg/ml BSA	48	2 mg/ml
1 mg/ml streptavidin phycoerythrin	12	10 µg/ml
Nuclease free water	540	

Table 3.6. Antibody solution mix components.

Reagent	**Volume per reaction (µl)**	**Final concentration**
2x MES Stain Buffer	300	1x
50 mg/ml BSA	24	2 mg/ml
10 mg/ml Goat IgG	6	0.1 mg/ml
0.5 mg/ml Anti-streptavidin Antibody (goat), Biotinylated	6	5 µg/ml
Nuclease Free Water	540	

Table 3.7. Mini_prok2v1_450 fluidics station program.

Step	Temperature (˚C)	Description
Post Hyb Wash #1	30	10 cycles of 2 mixes/cycle with Wash Buffer A
Post Hyb Wash #2	50	4 cycles of 15 mixes/cycle with Wash Buffer B
1st Stain	35	Stain the probe array for 5 min in SAPE
2nd Stain	35	Stain the probe array for 5 min in Antibody Solution Mix
3rd Stain	35	Stain the probe array for 5 min in SAPE
Final Wash	35	5 cycles of 4 mixes/cycle with Wash Buffer A

Table 3.8. Primers for rt-RT-PCR assays to determine mRNA expression of *pstS*, *pstC*, *pstA*, *pstB*, *phoB*, *phoR*, *glnE*, and *pspF* in *E. coli* O157:H7 when exposed to 0 and 0.5% sodium benzoate.

Primer	Sequence (5'→3')	Product Tm (˚C)	Product size (bp)
pstSF	TGACACTTACCAGAAAGAAACC	86	233
pstSR	TACCATCCAGCACCAGTTC		
pstCF	GCCTTTGGTATCGGTATCC	85	179
pstCR	TTGGTGAACGGAAGAACG		
pstAF	GGATTTATCTGGCGGAATATGG	86.5	229
pstAR	TACGGCACCAGTTTCAGC		
pstBF	CTTCTACTACGGCAAATTCC	87	379
pstBR	GGTTTCGTTCCACAATGC		
phoBF	CGAAGCGGAAGATTATGACAG	83.5	158
phoBR	TCAACATCACCACTGGAATATC		
phoRF	TCTGGTGTAACGGTCTGG	85.5	200
phoRR	CAACTGTTTGTGGGTATAAGG		
glnEF	GGATGGCTTCGTCTATCG	88	361
glnER	GAGCTGGAACACCTGAAC		
pspFF	TCTGCTGGATTCCGAACTG	86.5	226
pspFR	GCATACCAACCGCACATTC		

Table 3.9. Number of differentially regulated genes over time (q<0.05) when *E. coli* O157:H7 is exposed to 0.5% sodium benzoate

Time (min)	**Number of genes differentially regulated**
0	270
5	458
15	564
30	795
60	1707

Table 3.10. Number of significant ontology nodes within biological process, cellular components, and molecular function as determined with JProGO.

Time	Biological Process	Cellular Components	Molecular Function	p-value
0	51	8	11	5.1077E-4
5	57	0	3	5.1851E-4
15	4	2	6	1.0249E-4
30	14	10	13	2.7975E-4
60	32	15	26	6.3335E-4

Table 3.11. Increased transcription of *pstS*, *pstC*, *pstA*, *pstB*, *phoB*, and *phoR* mRNA in *E. coli* O157:H7 when exposed to 0.5% sodium benzoate and analyzed with high density oligonucleotide microarrays and validated with rt-RT-PCR.

Target mRNA	**Time**	**Microarray log_2 fold-change**	**Microarray q-value**	**rt-RT-PCR log_2 fold-change**
pstS	0	-0.02	0.14[1]	Nd[2]
	5	2.4	9.65 E-6	2.8
	15	2.7	4.41 E-6	2.7
	30	2.6	5.14 E-6	2.5
	60	2.5	6.83 E-6	3.3
pstC	0	-0.7	0.01	nd
	5	2.8	6.9 E-6	1.8
	15	2.9	9.49 E-6	1.7
	30	2.8	7.42 E-6	1.8
	60	3.4	5.17 E-6	1.9
pstA	0	-1.14	0.02	nd
	5	2.3	2.29 E-5	2.9
	15	2.5	1.47 E-5	3.1
	30	2.5	1.62 E-5	3.3
	60	2.4	1.86 E-5	3.1
pstB	0	-1.3	0.04	nd
	5	1.5	0.0003	2.1
	15	2.0	7.33 E-5	2.0
	30	1.9	9.25 E-5	2.2

Table 3.11. Continued

Target mRNA	Time	Microarray log_2 fold-change	Microarray q-value	rt-RT-PCR log_2 fold-change
pstB	60	2.0	7.01 E-5	2.5
phoB	0	1.9	0.01	nd
	5	3.8	2.29 E-5	2.4
	15	3.5	1.8 E-5	2.1
	30	4.1	1.4 E-5	1.7
	60	3.8	2.19 E-5	2.2
phoR	0	2.3	0.001	nd
	5	5.1	1.53 E-5	2.0
	15	5.7	8.46 E-6	2.0
	30	6.0	5.11 E-6	1.6
	60	5.3	1.32 E-5	1.9

[1] q-value for differentially regulated genes ≤0.05.
[2]Not determined

Table 3.12. Average cycle threshold (Ct) values (n=6) of rt-RT-PCR for *pstS*, *pstC*, *pstA*, *pstB*, *phoB*, and *phoR* mRNA in *E. coli* O157:H7 when exposed to 0 and 0.5% sodium benzoate.

		0% Sodium Benzoate		**0.5% Sodium Benzoate**	
Target mRNA	**Time**	**Ct**	**Std. Deviation**	**Ct**	**Std. Deviation**
pstS	5	13.93	0.42	10.78	0.91
	15	13.88	0.22	10.47	0.38
	30	15.28	0.47	10.72	0.48
	60	14.41	0.38	10.91	0.17
pstC	5	15.99	0.3	14.01	0.15
	15	15.49	0.3	13.25	0.14
	30	15.25	0.48	12.97	0.43
	60	15.19	0.3	13.2	0.38
pstA	5	12.96	0.24	10.40	0.31
	15	13.15	0.33	10.25	0.18
	30	13.33	0.46	10.23	0.18
	60	13.5	0.32	10.91	0.36
pstB	5	13.27	0.25	10.76	0.53
	15	13.08	0.41	10.33	0.14
	30	12.84	0.5	9.84	0.17
	60	13.29	0.29	10.46	0.4
phoB	5	14.42	0.65	11.72	0.49
	15	14.5	0.32	11.8	0.28

Table 3.12. Continued

Target mRNA	Time	0% Sodium Benzoate Ct	0% Sodium Benzoate Std. Deviation	0.5% Sodium Benzoate Ct	0.5% Sodium Benzoate Std. Deviation
phoB	30	14.85	0.54	11.19	0.17
	60	14.54	0.27	12.02	0.27
phoR	5	14.27	0.49	11.65	1.79
	15	14.24	0.27	11.24	0.1
	30	15.18	0.58	11.14	0.23
	60	13.87	0.17	11.6	0.38

Table 3.13. Transcription of *phoU* mRNA in *E. coli* O157:H7 when exposed to 0.5% sodium benzoate and analyzed with high density oligonucleotide microarrays.

Target mRNA	**Time (min)**	**Microarray $\log_2$ fold-change**	**Microarray q-value**
phoU	0	-1.7	0.019
	5	1.3	0.0043
	15	2.1	0.00041
	30	1.7	0.0013
	60	1.6	0.0015

Table 3.14. Log_2 fold changes obtained with microarray studies to determine transcription of genes involved in multidrug efflux in *E. coli* O157:H7 when exposed to 0.5% sodium benzoate.

		Time (min)				
Target mRNA	**Efflux pump**	**0**	**5**	**15**	**30**	**60**
acrA	AcrAB-TolC	0.1[1]	0.2	0.6	1.4	1.8
		(0.59[2])	(0.43)	(0.07)	(4.49E-6)	(1.14E-6)
acrB		-0.4	0.1	0.5	1.3	2.2
		(0.33)	(0.52)	(0.36)	(1.68E-5)	(1.97E-6)
tolC		0.5	0.6	0.7	0.8	0.6
		(0.72)	(0.23)	(0.58)	(0.38)	(0.84)
acrD	AcrAD-TolC	0.3	0.3	0.3	1.3	2.1
		(0.15)	(0.37)	(0.22)	(2.17E-5)	(7.62E-6)
emrA	EmrAB-TolC	2.9	2.9	3.6	4.7	3.7
		(1.22E-6)	(1.34E-6)	(6.11E-7)	(3.01E-7)	(5.48E-7)
emrB		2.7	3.4	4.9	5.7	5.0
		(3.77E-5)	(1.26E-5)	(6.18E-6)	(1.12E-6)	(2.05E-6)

[1]Log_2 fold change (q-value).
[2]Q-value for differentially regulated genes ≤0.05.

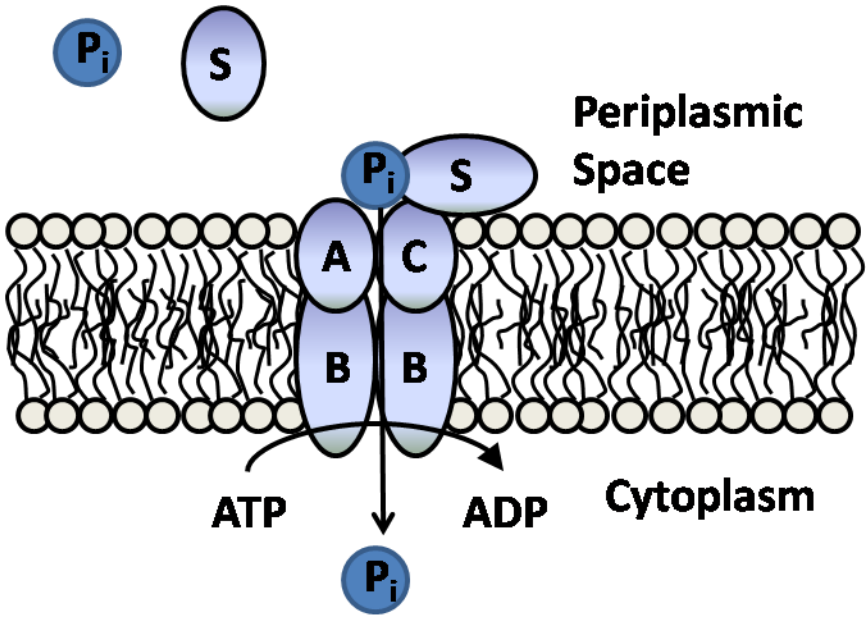

Figure 3.1. Diagram of the phosphate specific transport (Pst) system of *E. coli* O157:H7. This system actively transports phosphate across the cytoplasmic membrane under P_i starvation conditions (Pi= inorganic phosphate; S=PstS protein that binds phosphate in the periplasmic space; A and C are PstA and PstC transmembrane proteins; B= PstB protein that is the ATP binding domain).

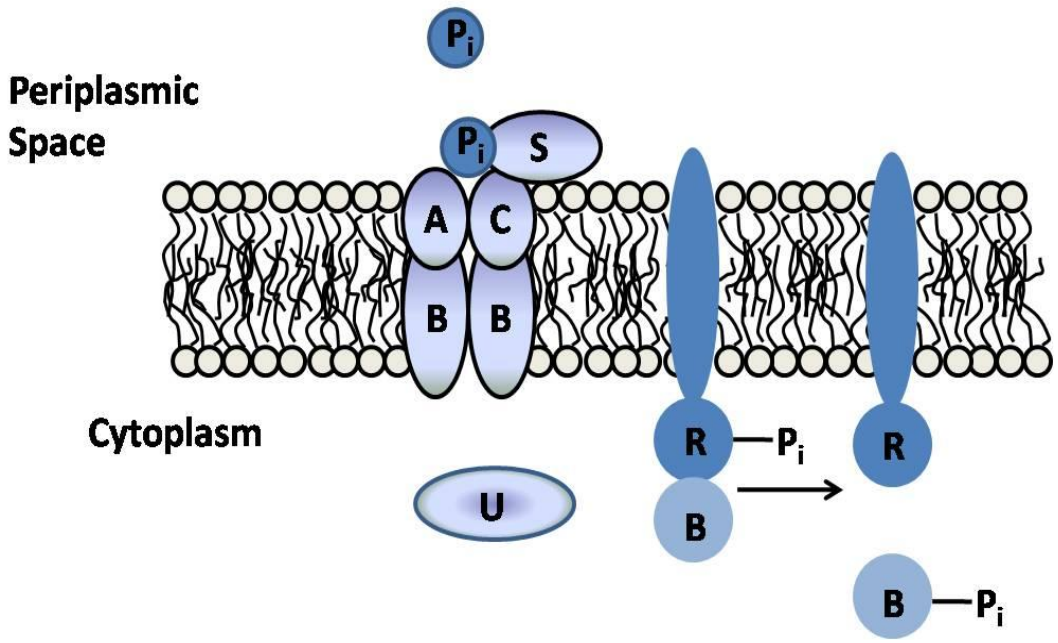

Figure 3.2. Under phosphate limited conditions, PhoU is removed from the repressor complex and PhoR undergoes autophosphorylation. Once this occurs, PhoR acts as a histidine kinase and phosphorylate PhoB activates the Pho regulon by binding Pho boxes and stimulating RNAP contact.

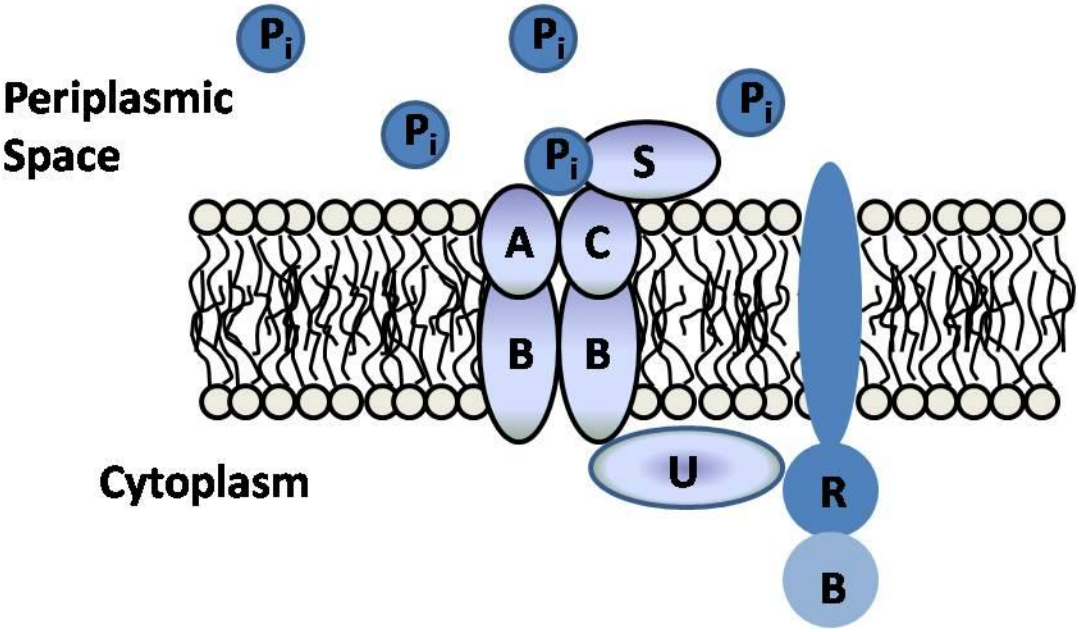

Figure 3.3. Negative regulation of Pho operon by repressor complex comprised of P_i bound PstS, PstABC, PhoU, and PhoR. By this mechanism PhoR is maintained in the dephosphorylated repressor form, negatively regulating the Pho operon (Pi= inorganic phosphate; S=PstS protein that binds phosphate in the periplasmic space; A and C are PstA and PstC transmembrane proteins; B= PstB protein that is the ATP binding domain; U=PhoU negative regulator; R=PhoR transmembrane sensor protein; B=PhoB phosphate limited response regulator).

Chapter 4

Increased Transcription of the Urease Operon of *Escherichia coli* O157:H7 Exposed to 0.5% Sodium Benzoate Determined with High-density Oligonucleotide Microarrays and Reverse-transcriptase Polymerase Chain Reaction.

Abstract

Sodium benzoate is a widely used food antimicrobial in drinks and fruit juices. Understanding gene regulation in *E. coli* O157:H7 under antimicrobial stress is critical to enable the design of appropriate control strategies. High-throughput novel technologies such as microarrays enable the study and characterization of gene expression. Microarrays were used to determine the transcriptional response of *E. coli* O157:H7 after exposure to 0.5% sodium benzoate.

Stationary phase *E. coli* O157:H7 grown in 150 ml Luria-Bertani broth (LB) was exposed to 0 (control) and 0.5% sodium benzoate. Each treatment was duplicated, sampled at 0 (immediately after exposure), 5, 15, 30, and 60 min. Total RNA was extracted and analyzed with *E. coli* 2.0 Gene Chips.Differentially regulated genes (positive-false discovery rate < 0.05) were determined using the R-MAANOVA (microarray analysis of variance) package.

Microarray analysis revealed that all genes in the urease operon were up-regulated > 2-fold (linear-scale) after 60 min exposure to sodium benzoate. The urease operon is composed of three structural genes, *ureA*, *ureB*, and *ureC*, and four accessory genes, *ureD*, *ureE*, *ureF*, and *ureG*.

Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide, and is one mechanism by which microorganisms survive in acidic environments. Ammonium ions accumulate in the surrounding environment, effectively mediating a rise in pH. In this study, exposure of *E. coli* O157:H7 to sodium benzoate in a neutral pH environment

resulted in an increase in transcription of the entire urease operon. Understanding adaptations of *E. coli* O157:H7 after exposure to antimicrobials is essential to better understand and implement methods to inhibit or control its survival in foods.

Introduction

E. coli O157:H7 is a member of the enterohemorrhagic *E. coli* (EHEC) and causes hemorrhagic colitis in humans. The symptoms of an *E. coli* O157:H7 infection generally include severe abdominal cramps and diarrhea (often times bloody), which can lead to death. In children under the age of five, infection can lead to a complication known as hemolytic uremic syndrome (HUS), resulting in red blood cell destruction, renal failure, and central nervous system complications *(37)*. *E. coli* O157:H7 can cause disease at a low infectious dose (10 - 100 cells) *(29)*. This can be attributed to the tolerance of this bacterium to low pH, which allows passage through the stomach and colonization in the intestinal tract *(29)*. *E. coli* O157:H7 has been linked as the causative agent in outbreaks associated with unpasteurized apple and orange juice, and experimental data demonstrate the tolerance of *E. coli* O157:H7 to acidic environments *(3, 7, 30, 42)*.

While it is very hard to quantify the impact foodborne illnesses have in the United States, it has been estimated that foodborne disease causes 76 million illnesses annually and approximately 5,000 fatalities *(28)*. There were 16,614 laboratory-confirmed cases of foodborne illness from FoodNet participants in 2005 (~15% of the United States population), and *E. coli* O157:H7 was the causative agent in 2.8% of these cases *(2)*. The CDC estimates that there are 73,000 cases of *E. coli* O157:H7 annually, leading to an estimated 2,100 hospitalizations in the United States *(6)*. It has been estimated that the cost of EHEC infections was $405 million dollars in 2003 *(16)*.

The well-described virulence factors of *E. coli* O157:H7 include *stx1*, *stx2*, and *eaeA (23, 40)*. Shiga toxins are composed of two subunits. The B-subunit binds glycolipids in the host, and the A-subunit inhibits protein synthesis via disruption of ribosomal RNA *(1)*. Shiga-toxins may cause hemorrhagic colitis, hemolytic uremic syndrome (HUS), thrombocytopenia, hemolytic anemia, and renal failure *(22)*. The gene *eaeA* in enterohemorrhagic *E. coli* produces intimin, a 97-kDa protein that plays a role in attachment onto and effacement of microvilli of the small intestine *(10, 15, 26, 27, 46)*.

Sodium benzoate is a food additive commonly used in acidic beverages due to its relatively low pKa (4.2) and increased solubility (compared to benzoic acid). This compound is generally recognized as safe and may be added at concentrations up to 0.1% in foods (21 CFR 184.1733).

A general mechanism of inhibition for weak-acids starts with diffusion of the undissociated compound through the cell membrane by passive diffusion. Once inside the cell, the higher pH causes dissociation of the acid and lower intracellular pH_i and inhibition of membrane-bound transport *(5)*. Eklund *(11)* determined that uptake inhibition contributed to growth inhibition, but did not seem to completely explain this phenomenon. Eklund *(14)* found that ΔpH (pH difference) was eliminated by sorbic acid as well as methyl and butyl paraben, whereas the membrane potential ($\Delta\psi$) was maintained *(13)*. This indicates more than simple inhibition of the proton motive force, since the membrane potential maintained would allow for uptake of essential compounds for cellular maintenance. It has been determined that dissociated weak acids also contribute a small portion to growth inhibition, which cannot be explained by

the effects of pH_i *(12)*. Sorbic acid, sorbic alcohol and sorbic aldehyde have been found to cause similar levels of inhibition, demonstrating a mechanism of inhibition other than a weak acid effect *(45)*. Bracey and others *(4)* found that pH_i was not altered in *Saccharomyces cerevisiae* exposed to sorbic acid. However, a higher ADP/ATP ratio was found indicating that the cell was expending energy to maintain pH_i *(4)*, and this expenditure of ATP was theorized to decrease cellular growth.

Membrane disruption has also been attributed as a mechanism of inactivation by sorbic acid *(4, 45)*. Krebs and *et al. (25)* observed a reduction in phosphofructokinase activity in *Saccharomyces cerevisiae* exposed to sorbic acid *(25)*. The disruption of glycolysis was thought to be caused by a decrease in pH_i that decreased the enzymatic activity of phosphofructokinase. Nitrogen starvation and benzoic acid treatment inhibit growth of *Saccharomyces cerevisiae*. However, when used in combination, they are cytocidal *(18)*. It was found that benzoic acid inhibited the break-down of cytosolic compounds to form amino acids and nucleotides necessary for growth and survival *(18)*.

Escherichia coli O157:H7 Urease Operon

Escherichia coli O157:H7 is known to produce urea when degrading arginine to putrescine *(32, 33)*. Urease (EC 3.5.1.5) catalyzes the reaction of urea into two molecules of ammonia and carbon dioxide *(31)*. The liberated ammonia results in increased pH of the surrounding environment. *Escherichia coli* O157:H7 EDL933 genomic sequence contains an O-island with two urease gene clusters (*ure*DABCEFG) *(38)*. Urease is composed of three structural proteins, UreA, UreB, and UreC, as well as

four accessory proteins, UreD, UreE, UreF, and UreG *(31)*. This enzyme could give enterohemorrhagic *E. coli* (EHEC) the ability to neutralize acidic environments and allow for survival in acidic food products as well as support survival in the gastrointestinal tract.

When studying the presence of the urease gene clusters in *E. coli* O157:H7 isolates, it was found that among serotype O157:H7 isolates (*n*= 59), 98% contained all genes in the urease operon*(17)*. In contrast, none of the genes in the urease operon were present in sorbitol-fermenting O157:NM (non-motile) isolates (*n*=82) *(17)*. However, among O157:H7 isolates found to have the urease operon, only one was found to have urease activity when evaluated with Christensen's agar or with the API 20E system; in this isolate, elevated levels of ammonia could be were detected *(17)*. When evaluating nine isolates of serotype O55:H7, from which *E. coli* O157:H7 has been theorized to have diverged, none contained genes in the urease operon *(17)*.

Similarly, Orth *et al.(35)* studied the distribution of *ureC* among 202 EHEC that encompassed 61 serotypes. It was found that all O157:H7 and O157:NM (*n*=72) that displayed the typical non-sorbitol fermenting phenotype possessed the *ure*C gene *(35)*. However, all sorbitol fermenting O157:NM isolates (*n*=8) were negative for *ure*C *(35)*. All isolates containing *ureC* were also found to contain all genes in the urease operon*(35)*. When the *ureC* isolates were evaluated for urease production in urease degradation broth, only 3.4% showed urease activity *(35)*. The nucleotide sequence of all urease genes present in ten O157:H7 isolates studied were found to have ≥ 99% homology *(35)*. These data indicate that the urease operon was acquired by O157:H7

relatively recently and possibly through horizontal gene transfer or gene loss since both O55:H7 and sorbitol fermenting O157:NM strains lack the urease gene cluster, and although many *E. coli* O157:H7 isolates contain the urease operon, it is not commonly expressed under normal culture conditions.

Nakano et al.*(34)* examined urease production by *E. coli* O157:H7 Sakai in various culture media, pH, temperatures, and nutrients via ammonia production without any success at stimulating urease production. When further examining 120 EHEC isolates for urease activity, two isolates were identified as being urease positive *(34)*. All isolates were determined to have a urease operon of similar size (4940 bp), and the nucleotide sequence was similar for a urease positive strain compared to the Sakai strain except for a slightly longer open reading frame in the *ureD* gene of the urease positive isolate *(34)*. It was revealed that the Sakai strain as well as all urease negative EHEC isolates examined (*n*=158) had a premature stop codon for *ureD* compared to that of the urease negative isolate. When the *ureD* gene of a urease positive EHEC was transformed into the Sakai strain urease production was detected*(34)*. These data indicate that the premature stop codon in UreD plays a role in urease production *in vitro*.

When attempting to stimulate urease production in *E. coli* O157:H7 strain EDL933, urease could not be detected using Christensen's agar, enzymatic assays, or immunoblotting techniques targeting UreC and UreD *(19)*. However, when non-pathogenic *E. coli* DH5α was transformed with the EHEC *ure* gene cluster, urease was detected via enzymatic activity *(19)*. This demonstrated that the urease genes present in the EDL933 strain were functional, but failed to be expressed under multiple culture

conditions. It is interesting that the *ure*D nucleotide sequence in strain EDL933 is identical to Sakai *(34)*. When Nakano et al.*(34)* transformed the Sakai *ure*D gene into *E. coli* DH5α. urease was detected. The authors believe that the amber suppressor phenotype of DH5α played a role in read-through of the premature stop codon. Heimer *et al.(19)* determined that a trans-acting factor was inhibiting urease transcription in EHEC. These authors also identified three Fur-like (ferric-uptake regulator) recognition sites upstream of *ureA* and *ureD (19)*. In this study, urease production was detected in two other EHEC strains (IN1 and MO28), and a Δ*fur* background greatly diminished the urease activity of these strains *(19)*. This research indicated that Fur, as well as other unidentified trans-acting factors, plays a role in regulating the expression of urease in EHEC.

Materials and Methods

Escherichia coli O157:H7

Escherichia coli O157:H7 strain EDL 933, obtained from the National Food Safety and Toxicology Center at Michigan State University, was used for this study. Cultures were grown in 125 ml Luria-Bertani broth (LB; Difco, Becton Dickinson Microbiology Systems; Franklin Lakes, NJ) at 37˚C for 24 h, and transferred a minimum of three successive times at 24-h intervals before use. The entire volume of stationary-phase cells were centrifuged at 8000 x *g* for 10 min at 4˚C. The cell pellet was resuspended in LB containing 0 (control) or 0.5% (w/v) sodium benzoate (Fisher Scientific; Fair Lawn,

NJ). At every sampling time, cell counts were determined by surface plating on sorbitol MacConkey Agar (SMAC; Difco, Becton Dickinson Microbiology Systems; Franklin Lakes, NJ).

Experimental Design

A completely randomized design with repeated measures was utilized to study the effects of exposuring *E. coli* O157:H7 to sodium benzoate. The control (0%) and treatment (0.5%) sodium benzoate conditions were replicated once and sampled over five time points [0 (immediately after exposure), 5, 15, 30, and 60 min].

Total RNA Isolation

The RNeasy Mini Purification Kit (Qiagen; Valencia, CA) was utilized to extract total RNA from *E. coli* O157:H7. 1.2 ml RNA*later* Stabilization Reagent (Qiagen; Valencia, CA) was combined with 0.6 ml *E. coli* O157:H7. RNA*later* immediately stabilizes RNA to help safeguard against degradation of mRNA. After 10 min at room temperature, the mixture was centrifuged at 8000 x *g* for 10 min under refrigeration (Eppendorf 5417C; New York, NY). The supernatant was decanted and the bacterial pellet was stored at -70˚C until further processed (approx. 2-10 hr).

100 µl tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, ph 8.0; Promega; Madison, WI) containing lysozyme (3 mg/ml; Sigma-Aldrich; St. Louis, MO) was added to each cell pellet. The pellet was thoroughly mixed and incubated at room temperature for 10 min. This disrupts the cell wall and membrane and allows for extraction of RNA. 350 µl of

buffer RLT was combined with the tris-EDTA suspension to further lyse bacterial cells. Buffer RLT contains guanidine thiocyanate which aids in denaturing proteins and effectively inactivates ribonucleases to preserve RNA quality. The total volume was combined with 250 µl of 200 proof ethanol (Aaper Alcohol; Shelbyville, KY) to solubilize the cell lysate, and the entire sample was transferred to the RNeasy Mini spin column in a collection tube and centrifuged at 8000 *x g* for 15 sec at room temperature (Sorvall Biofuge Pico; Kendro, Germany). The silica-based membrane efficiently binds up to 100 µg of RNA longer than 200 bases. The collection tube and eluate were discarded and the spin column was placed in a new collection tube. 700 µl of buffer RW1 was applied to the column, centrifuged, and flow-through was discarded as described previously. RW1 also contains guanidine thiocyanate to help disrupt protein structure and ethanol to solubilize and remove other cellular material. Twice, 500 µl of buffer RPE was applied to the spin column allowing 5 min prior to centrifugation at 8000 *x g* for 15 sec. Ethanol also is a constituent of RPE and serves a primary role of removing residual salts from previous buffers. The spin column was placed in a 1.5 ml collection tube that was used for long-term storage. In order to elute RNA from the spin column, 30 µl of RNase-free water was applied directly to the membrane and centrifuged at 8000 *x g* for 15 sec. Ten RNA samples were extracted for each treatment and replicate (40/time point). The ten samples were pooled and divided into aliquots to remove variation in RNA concentration from differing RNA extraction efficiencies. The RNA eluate was stored at -80˚C until evaluated for quantity, quality, and purity.

Evaluation of RNA Quality, Quantity, and Purity

The NanoDrop spectrophotometer (NanoDrop Technologies; Wilimington, DE) was utilized to determine absorbance of samples at 230, 260, and 280 nm. RNA concentration is determined by absorbance at 260 nm (1 absorbance unit=40 µg/ml). The A_{260} / A_{280} and A_{260} / A_{230} ratios indicate contamination (e.g. proteins, ethanol, salts) and should fall between 1.8 to 2.1. Lower ratios may interfere with down-stream processing of RNA for microarrays and RT-PCR.

In addition to spectrophotometric analysis, RNA samples were also analyzed for quality using the Agilent 2100 electrophoresis bioanalyzer (Agilent Technologies; Santa Clara, CA). This analysis allows for visualization of 23S and 16S rRNA bands, assuring that there is no degradation of RNA and the absence of chromosomal DNA.

High Density Oligonucleotide Microarray Analysis

cDNA Synthesis

The Affymetrix protocol for GeneChip prokaryotic processing was followed for this study. *In vitro* synthesized polyadenylated transcripts for *Bacillus subtilis* (Affymetrix; Santa Clara, CA) were diluted 1 x $10^{-2.5}$ and spiked-into the primer annealing master mix at a volume of 2µl. This “spike-in” is evaluated in the final processing of the microarray as a means of monitoring the assay sensitivity and performance from the beginning to final stages. Ten micrograms of total RNA was added to the primer annealing mastermix (final concentration 0.33µg/µl). Ten microliters of random primers

(Invitrogen; Carlsbad, CA) at a concentration of 75 ng/µl (final concentration 25ng/µl) and nuclease-free water (Ambion; Austin, TX) were added to bring the entire master mix volume to 30 µl. The following conditions were utilized to anneal random primers: 70˚C for 10 min; 25˚C for 10 min; chill to 4˚C (PCR Sprint Thermal Cycler; Thermo Fisher Scientific; Waltham, MA)

The RNA/primer master mix was centrifuged at 5000 x *g* for 1 min at room temperature. The SuperScript first-strand synthesis system (Invitrogen; Carlsbad, CA) reagents and SUPERase-In (Applied Biosystems; Foster City, CA) were combined with the 30µl RNA/primer master mix as described in Table 4.1. All tables and figures are found in an appendix located at the end of the chapter. The cDNA synthesis conditions were: 25˚C for 10 min; 37˚C for 60 min; 42˚C for 60 min; 70˚C for 10 min; chill to 4˚C. Remaining RNA was degraded with the addition of 20µl/rxn of 1N NaOH and incubated at 65˚C for 30 min. The reaction was neutralized with the addition of 20µl/rxn of 1N HCl.

Purification and Quantitation of cDNA

The MinElute PCR Purification Kit (Qiagen; Valencia, CA) was utilized to clean up the cDNA synthesis product prior to further microarray processing. Five volumes of buffer PB were added to the cDNA product, placed on a MinElute spin column, and centrifuged at room temperature at 5000 x *g* for 1 min. Eluate was discarded and 750 µl of buffer PE was applied to the column and centrifuged as described above. Residual ethanol from the buffer PE was allowed to evaporate from the spin column prior to

eluting cDNA with 12 µl buffer EB. The resulting purified product was analyzed as previously described with the NanoDrop spectrophotometer to determine cDNA yield.

cDNA Fragmentation

Purified cDNA (3µg) was combined with 10X One-Phor-All buffer (GE Healthcare; Buckinghamshire, England), DNAse I (0.6 U/µl; GE Healthcare; Buckinghamshire, England), and nuclease-free water as described in Table 4.2. The reaction was held at 37˚C for 10 min followed by 98˚C for 10 min to inactivate DNAse I.

Terminal Labeling

The GeneChip DNA Labeling Reagent (Affymetrix; Santa Clara , CA) was used to label the 3' termini of the fragmented cDNA products with biotin. Volumes of reagents utilized in this reaction are listed in Table 4.3. The reaction was held at 37˚C for 60 min followed by addition of 2µl of 0.5M EDTA (Invitrogen; Carlsbad, CA) to arrest the labeling reaction by inactivating the terminal deoxynucleotidyl transferase (Promega; Madison, WI) .

cDNA Target Hybiridization

The biotin labeled, fragmented cDNA was added to the hybridization master mix. Table 4.4 describes the reagents utilized in this reaction. The B2 control oligo acts as a hybridization control and allows for alignment of the GeneChip microarray in the analysis software. Herring sperm DNA was used as a blocking agent during hybridization to prevent improper annealing of cDNA fragments with probes.

The *E. coli* Genome 2.0 Array (Affymetrix; Santa Clara, CA) was allowed to equilibrate to room temperature. This GeneChip allows the detection of transcripts from *E. coli* K12 (MG1655) as well as a uropathogenic strain (CFT073) and two enterohemorrhagic strains of the serotype O157:H7 (EDL933 and Sakai). 200 µl of the hybridization solution was added to the GeneChip and the array was placed in the hybridization oven (Affymetrix; Santa Clara, CA) at 45˚C, 60 RPM for 16 hr. The hybridization solution was then removed and replaced with 80 µl of non-stringent wash buffer (wash buffer A; 6x SSPE, 0.01% Tween-20) and the GeneChips were stored at 4˚C until washed and stained.

GeneChip Washing, Staining, and Scanning

The GeneChip Operating Software (GCOS; Affymetrix; Santa Clara, CA) controlled the operation of the Fluidics Station 450 (Affymetrix; Santa Clara, CA). Streptavidin phycoerythrin (SAPE; Table 4.5) was loaded into the first and third 1.5 ml staining vial in the fluidics station, while the second stain vial contained an antibody solution mix (Table 4.6). The Mini_prok2v1_450 fluidics station protocol (Table 4.7) was followed for washing and staining hybridized biotin labeled cDNA fragments. The GeneChip Scanner 3000 (Affymetrix; Santa Clara, CA) and GCOS were used to produce .cel files which contained the fluorescence intensity data for a probe. Each target sequence contains 11 25-oligomer probes that are spread throughout the entire sequence (perfect match) as well as 11 25-oligomer probes that match the target with the exception of the 13th nucleotide which is a mismatch to the desired target sequence and indicates non-

specific binding (mismatch). Fluorescence data for these 22 probes are combined into a probe set for further analysis.

Statistical Analysis of Microarray Data

The R programming environment (v 2.5.1) was used for all microarray data analysis. First, GCOS generated CEL files, CDF file, and probe file were uploaded into the GCRMA package (v 2.0.0) *(48)*. This program normalizes microarray data by using Affymetrix probe sequence affinities based on the specific probe sequence in addition to normalization from background noise also used in robust multiarray average (RMA) analysis. Following normalization with the GCRMA package, the intensities for probe sets were compared among treated and control cells across all time points using the microarray analysis of variance package (MAANOVA; v 1.4.1) *(47)*. Next, the positive false discovery rate (pFDR; q-value) was determined to differentiate genes which were significantly regulated in response to exposure of sodium benzoate (q-value; v 1.10.0) *(9)*. A subset of genes which were differentially regulated ($q < 0.05$) were determined and used for further analysis.

Selection of genes for validation with Real-time one-step reverse-transcriptase PCR (rt-RT-PCR)

Three tools were used to analyze microarray results so a subset of significantly regulated genes could be selected for further validation with rt-RT-PCR. The Pathway Tools Omics Viewer (SRI International; Menlo Park, CA) was used to visualize metabolic,

transport, and signaling pathways in addition to proteins integral to the membrane and periplasm specific to *E. coli* O157:H7 *(36)*. The output would link through pathway tools to BioCyc (SRI International; Menlo Park, CA) and give further insight to pathways, molecular interactions, proteins, and genes. However, the database specific to *E. coli* O157:H7 was computationally generated with very little curation based on findings in current literature. When the pathways of interest were homologous to *E. coli* K-12, the EcoCyc database which receives extensive literature based curation was utilized to gain further insight to functional and regulatory information for a pathway of interest *(24)*.

Selection of housekeeping genes for standardization of rt-RT-PCR

Secondary validation of microarray data was achieved with the Pfaffl method which incorporates the efficiency of a PCR reaction in addition to the difference in Ct values between the treated and control samples *(39)*. The equation below shows how the ratio is calculated to determine increased or decreased levels of target mRNA when comparing the control and treated sample. This difference is also standardized with one or more reference or housekeeping genes which are expressed at the same level between the control and treatment.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{Ct}_{\text{target}}(control\text{-}sample)}}{(E_{\text{reference}})^{\Delta \text{Ct}_{\text{reference}}(control\text{-}sample)}}$$

From the microarray data, genes were selected which had a highly non-significant q-value (q>0.98). Genes were excluded if their function was not known, or if there was a high amount of variability between replicate microarrays. rt-RT-PCR was used to validate these genes as appropriate housekeeping markers for microarray validation. Each sample was run in triplicate and there were a total of six cycle threshold values for each treatment within each time point. A completely randomized design with repeated measures was used to assure there was no difference in cycle threshold values within each time point (p>0.05; SAS v9.1; Cary, NC). This methodology helped to assure that there was no difference in mRNA expression between treatments.

Nucleic acid amplification

rt-RT-PCR was performed on RNA extracts as a means of microarray validation. SuperScript™ III Platinum® SYBR® green one-step rt-RT-PCR kit reagents (Invitrogen; Carlsbad, CA) were utilized in 25 µl reactions (2.5 µl serially-diluted RNA, 12.5 µl SYBR® green reaction mix, 1 µl BSA, and 0.5 µl SuperScript™ III reverse transcriptase/Platinum® *Taq* Mix, 0.025 µg forward and reverse primers for the target gene, and the remainder RNase-DNAse free water). Primer sequences (Table 4.8) were developed using Beacon Designer 4.0 software (Premier Biosoft International; Palo Alto, CA). The Bio-Rad My iQ™ single color real-time PCR detection system (Hercules, CA) was used to conduct rt-RT-PCR using the following parameters: 1) conversion of mRNA to cDNA, 35 min at 50˚C; 2) denature reverse-transcriptase, 2 min at 94˚C; 3) denature template, 30 sec at 94˚C; annealing of primers, 45 sec at 55˚C; and extension with *Taq* polymerase, 30 sec at 72˚C;

repeated 40 times; 4) final extension, 5 min at 72˚C; 5) melt curve. All rt-RT-PCR experiments were conducted in triplicate for each RNA sample and a negative control was included for each assay.

Results and Discussion

Microarray analysis revealed a large number of genes that were significantly regulated in response to 0.5% sodium benzoate. As shown in Table 4.9, the number of genes which were differentially regulated increased with exposure time, and after 60 minutes of exposure to sodium benzoate 1707 genes were found to be differentially expressed ($q \leq 0.05$).

Ontology analysis allowed for insight to genes which responded to sodium benzoate exposure based on biological process, molecular functions, or cellular components. Genes categorized into biological processes were the greatest to respond across all time points (Table 4.10). There were a large number of genes associated with biological process nodes which responded quickly to sodium benzoate exposure (0 and 5 min). Most of these nodes were associated with metabolic activities such as protein biosynthesis. Genes associated with the ribosome and RNA binding were also found for these early time point. Genes involved in protein translation respond very early to the stimulus of sodium benzoate.

There was a rapid decline in significant ontology categories at 15 min, but an increase was observed for 30 and 60 min. At 15 min the biological process nodes

represented genes that responded to unfolded proteins and alcohol metabolism. Significant cellular component nodes were found for genes involved with the cell membrane. Genes were also found to be regulated that responded to the molecular functions of carbohydrate transport and nickel ion binding.

Genes associated with molecular functions and cellular components had a greater response to sodium benzoate after 30 and 60 min of exposure. Molecular function nodes which had increased expression at these time points pertained to RNA and ATP binding activity as well as carbohydrate transport. The significant cellular component nodes at these times were oriented around the ribosome and products which were integral to the membrane or part of the inner membrane. Biological process nodes associated with a response to chemical and antibiotic stimuli were found after 15 min of exposure and continued until 60 min. Many of these genes are components of efflux pumps as well as transcription regulators, such as *marA,* the multiple antibiotic resistance regulon activator.

Ontology analysis gives insight to expression of genes that are found in *E. coli* K-12. While there is large amount of overlap between the genomes of K-12 and O157:H7, genes specific to O157:H7, termed O-islands, may contribute to this pathogen's ability to survive adverse environments as well as cause illness *(38)*. The urease operon was one such O-island which was found to have an increase in mRNA transcription when *E. coli* O157:H7 was exposed to sodium benzoate for 60 min. This enzyme could give enterohemorrhagic *E. coli* (EHEC) the ability to neutralize acidic environments and allow for survival in acidic food products as well as support survival in the gastrointestinal

tract. Therefore, further validation of the microarray results for the structural proteins of the urease operon with rt-RT-PCR were conucted.

The glutamine synthetase adenyltransferase (*glnE*) gene catalyzes the addition of AMP to a subunit of glutamine synthetase. PspF is a σ^{54} dependent transcriptional activator that is cis-acting on the phage-shock protein (psp) operon and has been found to be constitutively expressed *(20, 21)*. No significant differences were found in Ct values for these genes across treatments within time points for either gene ($p > 0.05$). Therefore, *glnE* and *pspF* were utilized as the housekeeping genes by which rt-RT-PCR assays were normalized in the Pfaffl method.

The $\log_2$ fold-change between 0.5% sodium benzoate treated *E. coli* O157:H7 cells and that of the control conditions is shown in Table 4.11. The structural genes of the urease operon (*ureA,ureB,* and *ureC*) were validated with rt-RT-PCR. All structural genes had increased expression when cells were exposed to 0.5% sodium benzoate for 60 min (Table 4.11). Expression of *ureA* was slightly higher in the rt-RT-PCR analysis compared to the microarray data (3.1 versus 2.6 $\log_2$ fold-change, respectively). However, comparative fold-change values for *ureB* and *ureC* were slightly lower for the rt-RT-PCR analysis compared to those obtained from microarray analysis (Table 4.11). This indicates that the production of urease may increase in response to sodium benzoate since the transcription of the genes which compose the three subunits of the apourease enzyme are up-regulated.

The accessory genes of the urease operon were not validated with rt-RT-PCR, but the fold-change values from microarray analysis are shown in Table 4.12. They follow a

similar trend in expression to the structural genes of urease. All accessory protein genes have a > 4-fold increase in transcription (linear scale), but like *ureB* and *ureC,* these values may be slightly inflated in the microarray analysis. Urease is a nickel-containing enzyme that requires these accessory proteins to interact with the structural subunits UreA, UreB, and UreC. The homologous proteins of *Klebsiella aerogenes* have been studied to determine the function of urease accessory proteins; UreD, UreF, and UreG interact with the structural subunits while UreE binds intracellular Ni^{2+} *(8, 43, 44)*. All accessory proteins interact to promote the transfer of nickel to the metallocenter of the enzyme which is necessary for urease activity *(8, 19, 43, 44)*.

It was noted when conducting analysis in the Omics Viewer that the nickel transporter proteins of *E. coli* O157:H7 were down-regulated in sodium benzoate treated cells (NikABCDE; Table 4.13). However, this down-regulation was lessened with time and the activator of this protein, Fnr, was increased with exposure time (Table 4.13). Fnr is known to activate the nickel transporter operon in the absence of oxygen since nickel is required during anaerobic growth for NiFe-hydrogenase synthesis *(41)*. However, both cultures were grown in a similar manner with shaking to increase culture aeration. Therefore, this mechanism should not playing a large role in *fnr* transcription. Most likely, the increased transcription of *fnr* observed in this study is in response to cell need for nickel for adequate urease production.

Since the production of functional urease by *E. coli* O157:H7 has been questioned in previous research *(17, 19, 34, 35)*, the maintenance of an alkaline environment in urea broth and agar was studied for further validation of these findings.

First, cells were grown in LB with and without 0.5% sodium benzoate for 24 hr, after which they were surface plated onto urea agar. *E.* coli O157:H7 on urea agar did not produce an alkaline reaction, indicating that urease was not produced under either of these conditions. However, when stationary phase *E. coli* O157:H7 was inoculated into urea broth with and without 0.5% sodium benzoate for 24 hr, an alkaline environment was maintained in urea broth containing 0.5% sodium benzoate, and an acidic environment was produced in the control (Figure 4.1). This indicates that *E. coli* O157:H7 responds rapidly to the stimulus when producing urease, and once this stimulus is removed urease is no longer produced.

When examining *fur* expression in the microarray data, a slight increase in transcription was seen among all time points for sodium benzoate treated cells (data now shown); there was a 1.4-1.8 fold increase (linear scale) in transcription for *fur*. This supports the findings that Fur can help stimulate production of urease, but there is also a secondary trans-acting factor that controls the regulation of this operon *(19)*.

This indicates that sodium benzoate allows *E. coli* O157:H7 to maintain an alkaline environment that is not achieved otherwise. This supports data from microarray and rt-RT-PCR results and indicates that increased transcription of urease mRNA produces a functional enzyme that can help to neutralize acidic environments.

Conclusions

These data support previous research that *E. coli* O157:H7 does not express urease constitutively, but requires stimulation of this operon from the environment. Exposure to sodium benzoate in a neutral pH (6.8) resulted in increased transcription of structural and accessory urease genes. Analysis of urease production in urea broth confirmed that a functional enzyme was produced under these conditions that counteracted the effects of acid production in a culture system. This indicates that research which concluded that urease genes were not functional due to lack of expression may have simply not been applying the appropriate stress to simulate expression of this enzyme. Further research can focus on exposure to sodium benzoate followed by exposure to low pH and further examination of the resulting transcriptome with respect to adaptation mechanisms as well as virulence factors in EHEC. Further studies will be focused on isolating urease structural proteins in sodium benzoate treated cells for further validation of this finding.

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Appendix

Table 4.1. cDNA synthesis master mix components.

Reagent	Volume per reaction (µl)
5X First-strand cDNA Buffer	12
100 mM DDT	6
10 mM dNTP Mix	3
SUPERase-In (20 U/µl)	1.5
Superscript II (200 U/µl)	7.5
RNA/primer Hybiridization	30

Table 4.2. cDNA fragmentation master mix components.

Reagent	Volume per reaction (µl)
10x One-Phor-All Buffer	2
cDNA	15
DNAse I	1.8
Nuclease Free Water	1.2

Table 4.3. Terminal labeling of cDNA fragments with biotin master mix components.

Reagent	Volume per reaction (µl)
5x Reaction Buffer	10
GeneChip DNA Labeling Reagent, 7.5mM	2
Terminal Deoxynucleotidyl Transferase	2
Nuclease Free Water	16
Fragmented cDNA Product	20

Table 4.4. Biotin labeled cDNA hybridization master mix components.

Reagent	Volume per reaction (µl)
2x Hybirdization Buffer[1]	40
3 nM B2 Control Oligo	1.3
10 mg/ml Herring Sperm DNA	0.8
50 mg/ml BSA	0.8
100% DMSO	6.2
Nuclease Free Water	5.9
Fragmented and Labeled cDNA Product	25

[1] 200 mMMES, 2M [Na^+], 20 mM EDTA, 0.01% Tween-20

Table 4.5. Streptavidin phycoerythrin (SAPE) staining solution components.

Reagent	**Volume per Reaction (µl)**	**Final Concentration**
2x MES Stain Buffer	600	1x
50 mg/ml BSA	48	2 mg/ml
1 mg/ml Streptavidin Phycoerythrin	12	10 µg/ml
Nuclease Free Water	540	

Table 4.6. Antibody solution mix components.

Reagent	Volume per reaction (µl)	Final concentration
2x MES Stain Buffer	300	1x
50 mg/ml BSA	24	2 mg/ml
10 mg/ml Goat IgG	6	0.1 mg/ml
0.5 mg/ml Anti-streptavidin Antibody (goat), Biotinylated	6	5 µg/ml
Nuclease Free Water	540	

Table 4.7. Mini_prok2v1_450 fluidics station program.

Step	Temperature (˚C)	Description
Post Hyb Wash #1	30	10 cycles of 2 mixes/cycle with Wash Buffer A
Post Hyb Wash #2	50	4 cycles of 15 mixes/cycle with Wash Buffer B
1st Stain	35	Stain the probe array for 5 min in SAPE
2nd Stain	35	Stain the probe array for 5 min in Antibody Solution Mix
3rd Stain	35	Stain the probe array for 5 min in SAPE
Final Wash	35	15 cycles of 4 mixes/cycle with Wash Buffer A

Table 4. 8. Primers for rt-RT-PCR assays to determine mRNA expression of *ureA*, *ureB*, *ureC*, *glnE*, and *pspF* in *E. coli* O157:H7 when exposed to 0 and 0.5% sodium benzoate.

Primer	**Sequence (5'→3')**	**Product*Tm* (˚C)**	**Product size (bp)**
ureAF	CTTAACTATCCCGAATCC	86.5	138
ureAR	TTCTGGTATGCCTTCC		
ureBF	GCAGTATTATCGTTGAAAATC	83.5	111
ureBR	AGCCTCTTGCCTTCTGG		
ureCF	CGGGTTGCTGGGTAAAG	86.5	328
ureCR	CGGATTGGTTGAGGAAGG		
glnEF	GGATGGCTTCGTCTATCG	88	361
glnER	GAGCTGGAACACCTGAAC		
pspFF	TCTGCTGGATTCCGAACTG	86.5	226
pspFR	GCATACCAACCGCACATTC		

Table 4.9. Number of differentially regulated genes over time (q<0.05) when *E. coli* O157:H7 is exposed to 0.5% sodium benzoate.

Time (min)	**Number of genes differentially regulated**
0	270
5	458
15	564
30	795
60	1707

Table 4.10. Number of significant ontology nodes within biological process, cellular components, and molecular function as determined with JProGO.

Time	Biological Process	Cellular Components	Molecular Function	p-value
0	51	8	11	5.1077E-4
5	57	0	3	5.1851E-4
15	4	2	6	1.0249E-4
30	14	10	13	2.7975E-4
60	32	15	26	6.3335E-4

Table 4. 11. Increased transcription of *ureA, ureB,* and *ureC* mRNA (q<0.05) in *E. coli* O157:H7 when exposed to 0.5% sodium benzoate for 60 min and analyzed with high density oligonucleotide microarrays and validated with rt-RT-PCR.

Target mRNA	**Microarray log_2 fold-change**	**Microarray q-value**	**rt-RT-PCR log_2 fold-change**
ureA	2.3	1.66 E-6	3.1
ureB	2.0	8.41E-7	1.6
ureC	1.4	1.25 E-6	0.7

Table 4. 12. Increased transcription of *ureD, ureE, ureF,* and *ureG* mRNA in *E. coli* O157:H7 when exposed to 0.5% sodium benzoate for 60 min and analyzed with high density oligonucleotide microarrays.

Target mRNA	Microarray $\log_2$ fold-change	Microarray q-value
ureD	2.5	1.92 E-6
ureE	2.1	5.03 E-6
ureF	2.5	3.65 E-6
ureG	2.5	5.08 E-7

Table 4.13. Transcription of *nikA*, *nikB*, *nikC*, *nikD*, *nikE*, *nikR*, and *fnr* mRNA in *E. coli* O157:H7 when exposed to 0.5% sodium benzoate and analyzed with high density oligonucleotide microarrays.

	Microarray $\log_2$ fold-change							
Target mRNA	**5 min**	**q-value**	**15 min**	**q-value**	**30 min**	**q-value**	**60 min**	**q-value**
nikA	-2.8	0.005	-4.3	0.001	-2.8	0.004	-0.5	0.0005
nikB	-2.0	0.003	-4.1	1.5E-5	-2.8	9.56E-5	-1.2	0.004
nikC	-2.3	0.005	-4.6	0.0001	-3.9	0.0003	-1.5	0.02
nikD	-2.1	0.001	-4.3	2.41E-5	-3.6	8.06E-5	-1.1	0.01
nikE	-1.9	0.0006	-3.2	1.61E-5	-2.9	4.77E-5	-1.3	0.003
nikR	-1.8	2.77E-5	-2.8	2.6E-6	-2.6	3.4E-6	-1.0	0.0004
fnr	0.4	0.06	1.1	2.16E-5	1.6	5.6E-6	2.1	1.7E-6

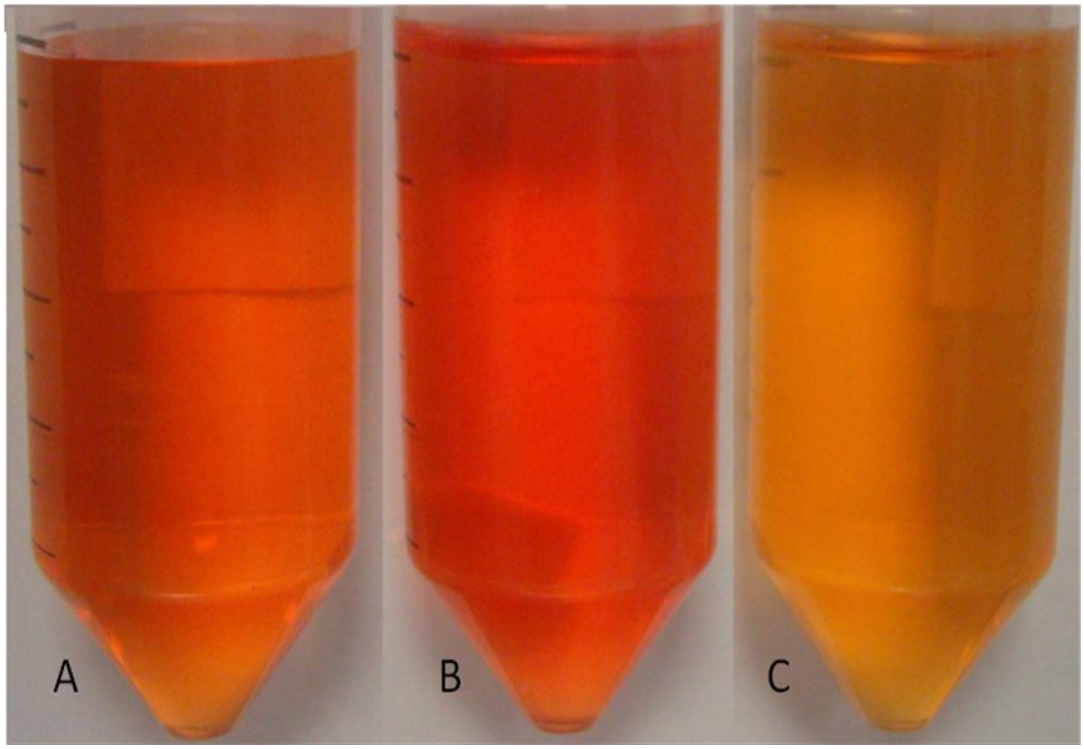

Figure 4.1. Growth of *E. coli* O157:H7 in A) urea broth with B) 0.5% sodium benzoate and C) 0% sodium benzoate. Red color indicates alkaline reaction.

Vita

Faith Johnson Critzer was born in Nashville, Tennessee, on August 4, 1978. Faith grew up in Nolensville, Tennessee and graduated from Page High School in 1997. She continued her education at the University of Tennessee where she earned two B.S. degrees majoring in Animal Science and Food Science and Technology and one M.S. degree in Food Science and Technology with an emphasis in Food Microbiology. Upon completion of her Ph.D., Faith will pursue a career in academia where she can continue to conduct research in the area of food microbiology