Characterization of Heat Resistant *Escherichia coli* Isolates Associated with Human Infection

by

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) is a bacterial pathogen associated with foodborne diarrheal disease. Infection with STEC presents as a mild, watery diarrhea to hemorrhagic colitis and can progress to the life-threatening complication, hemolytic uremic syndrome. Cattle are recognized as a primary reservoir of STEC and beef products are a major vehicle for STEC infection. STEC is considered a heat sensitive pathogen and the food processing industry heavily relies on thermal inactivation processes to eliminate bacterial contaminants. However, non-pathogenic *E*. *coli* isolated from environmental sources have been found to survive heat exposure at temperatures of 60°C and above. These isolates possess the locus of heat resistance (LHR), which confers resistance to thermal, osmotic, and oxidative stress. It is unknown if heat resistant *E*. *coli*, specifically STEC, exist and if they have been involved in human disease. It is hypothesized that the LHR in clinical *E*. *coli* isolates facilitates the increased survival of pathogenic strains against heat inactivation measures in food processing and consumer cooking practices, and can be a contributing factor in human foodborne infection in Alberta, Canada.

This thesis presents the novel identification and characterization of 3 clinical *E*. *coli* isolates, two being STEC, which possess the LHR. Three real-time quantitative polymerase chain reaction assays were developed and validated for the LHR with heat resistant, environmental *E*. *coli* isolate AW1.7. These assays allow for rapid screening of *E*. *coli* for the LHR from foodborne outbreak investigations and food processing plants.

To investigate the threat of heat resistant *E*. *coli* in the food processing industry and human consumption, the isolates were characterized for their ability to survive heat exposure at 60°C and 71°C in liquid culture media for the calculation of decimal reduction times (D-values) and in ground beef. D_{60}-values of heat resistant isolates all exceeded 10.20 minutes with one isolate's D_{60}-

values ranging from 20.46 to 72.47 minutes in the presence of increasing osmotic stress. Literature on D_{71}-values of foodborne pathogens is limited and the level of heat resistance mediated by the LHR at 71°C is unknown. It was determined that heat resistant isolates possessed elevated D_{71}-values compared to heat sensitive *E. coli* but to a far lesser extent than at 60°C. At temperatures of 71°C and above, it is suspected that the LHR is less effective at mediating turnover of misfolded and denatured proteins than at 60°C. Cell reductions of heat resistant isolates in ground beef patties grilled to 60°C and 71°C were 2.84 and 4.95 log colony forming units (CFU)/mL, respectively, compared to reductions of 6.08 log CFU/mL and greater in heat sensitive *E. coli*.

Despite the numerous thermal inactivation measures used in food processing plants to eliminate foodborne pathogens, *E. coli* biofilms remain a persistent source of contamination. Using an in-house, two-component apparatus, biofilm formation by the 3 clinical isolates was compared with 3 environmental isolates. All isolates harboured the LHR. Optimum conditions for biofilm formation in each of the isolates were determined by manipulating inoculum size, nutrient concentration, and temperature conditions. One out of the 3 clinical, heat resistant isolates was capable of forming biofilms whereas all 3 of the environmental isolates formed biofilms, suggesting that the LHR does not contribute to biofilm formation.

To elucidate the function of the components of the LHR in regards to their contributions to heat resistance, genetic and proteomic analyses of the LHR were conducted. Whole genome sequencing revealed that the LHR sequences in isolates AW1.7, 111, 128, and 8354 were 98.3% similar and that all of the clinical isolates encoded for a larger variant of the LHR compared to the LHR in *E. coli* AW1.7. Constitutive expression of novel Clp protease ClpK, encoded on open reading frame 3 of the LHR, was identified in all heat resistant isolates. However, transgenic strains that expressed ClpK without the entire LHR did not survive heat exposure at 60°C.

In conclusion, this research describes the emergence of multi-stress tolerant *E. coli* implicated in human gastrointestinal disease as a novel food safety risk. The heightened survivability of heat resistant *E. coli* facilitates their evasion from elimination along multiple stages of the farm-to-fork continuum and consequentially increases their potential to cause human foodborne infection.

Preface

This thesis is an original work by Angela Munnmen Ma. Work from Chapters 2 and 3 have been published in peer-reviewed journals. The publication details and individual contributions from all authors are listed below.

Chapter 2 of this thesis has been published as: Ma A, Chui L. Identification of heat resistant *Escherichia coli* by qPCR for the locus of heat resistance. J Microbiol Methods. 2016 Dec 22; 133:87–9. I was responsible for the experimental design, data collection and analysis, and manuscript composition. Dr. Linda Chui was the supervisory author and was involved with concept formation and manuscript editing.

Chapter 3 of this thesis has been published as: Ma A, Glassman H, Chui L. Characterization of *Escherichia coli* possessing the locus of heat resistance isolated from human cases of acute gastroenteritis. Food Microbiol. 2020 Jun; 88(December 2019):103400. I was responsible for experimental design, data collection and analysis, and the manuscript composition. Dr. Heather Glassman contributed to data collection of section 3.2.2. Heat resistance in LB broth and manuscript editing. Dr. Linda Chui was the supervisory author and was involved with manuscript editing.

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Table of Contents

Abstract .... **ii**

Preface .... **v**

Acknowledgements .... **vi**

Table of Contents .... **viii**

List of Tables .... **xii**

List of Figures .... **xiii**

List of Abbreviations .... **xiv**

Chapter 1 Introduction **1**

1.1. *Escherichia coli* **2**

1.1.2. Pathotypes 3

1.1.2.1 Uropathogenic *Escherichia coli* (UPEC) 4

1.1.2.2. Sepsis-associated *Escherichia coli* (SEPEC) 5

1.1.2.3. Neonatal meningitis *Escherichia coli* (NMEC) 6

1.1.2.4. Enterohemorrhagic *Escherichia coli* (EHEC) 6

1.1.2.5. Enteropathogenic *Escherichia coli* (EPEC) 9

1.1.2.6. Enteroinvasive *Escherichia coli* (EIEC)/*Shigella* 10

1.1.2.7. Enterotoxigenic *Escherichia coli* (ETEC) 11

1.1.2.8. Enteroaggregative *Escherichia coli* (EAEC) 12

1.1.2.9. Diffusely adherent *Escherichia coli* (DAEC) 13

1.2. Foodborne *Escherichia coli* infection **16**

1.2.1. 1993 outbreak of *E. coli* O157:H7 17

1.2.2. 2011 outbreak of *E. coli* O104:H4 18

1.2.3. 2012 outbreak of *E. coli* O157:H7 19

1.2.4. Diagnostic laboratory identification 20

1.3. Pathogen elimination methods used in food processing **24**

1.3.1. Thermal inactivation processes 24

1.3.2. Non-thermal pathogen elimination methods 26

1.4. Locus of heat resistance **27**

1.4.1. Identification in *E. coli* 29

1.4.2. Distribution across beta- and gamma- Proteobacteria 30
1.5. Rationale for thesis research 31
1.5.1. Overall thesis hypothesis 32
1.5.2. Specific thesis objectives 33
1.6. References 34
Chapter 2 Development of a real-time quantitative polymerase chain reaction (qPCR) assay for detection of the locus of heat resistance in clinical *Escherichia coli isolates* 58
2.1. Introduction 59
2.2. Materials and Methods 60
2.2.1. Bacterial isolates and genomic DNA extraction 60
2.2.2. qPCR assay design for detection of the LHR 61
2.2.3. Evaluation of qPCR assays as per MIQE guidelines 64
2.2.4. Detection of the LHR in clinical *E. coli* isolates 66
2.2.5. Screening for phenotypic heat resistance 66
2.3. Results 67
2.3.1. Validation of qPCR assays for the LHR 67
2.3.2. Heat resistant clinical *E. coli* isolates 69
2.4. Discussion 70
2.5. References 74
Chapter 3 Characterization of *Escherichia coli* possessing the locus of heat resistance isolated from human cases of acute gastroenteritis 79
3.1. Introduction 80
3.2. Materials and Methods 81
3.2.1. Isolate identification and bacterial growth conditions 81
3.2.2. Heat resistance in LB broth 82
3.2.3. Light and transmission electron microscopy analyses 82
3.2.4. Heat resistance in ground beef 83
3.2.5. Protein mass fingerprinting of heat resistant isolates 85
3.2.6. Statistical analysis 85
3.3. Results 86
3.3.1. Virulence profiles of *E. coli* isolates 86

3.3.2. D-values of heat resistant *E. coli* ... 86
3.3.3. Cellular morphological changes of heat resistant *E. coli* pre- and post- heat treatment ... 90
3.3.4. Survival of heat resistant *E. coli* in ground beef patties after grilling ... 93
3.3.5. Characterization of protein expression in heat resistant *E. coli* ... 95
3.4. Discussion ... 97
3.5. References ... 102
Chapter 4 Determination of biofilm formation in heat resistant *Escherichia coli* isolates 107
4.1. Introduction ... 108
4.2. Materials and Methods ... 110
4.2.1. Bacterial isolates and growth conditions ... 110
4.2.2. Detection of biofilm formation by crystal violet staining ... 110
4.2.3. Determination of optimal biofilm formation conditions ... 113
4.2.4. Genomic DNA isolation, whole genome sequencing, and analysis for biofilm-associated genes ... 113
4.2.5. Motility status ... 117
4.2.6. Statistical analysis ... 117
4.3. Results ... 118
4.3.1. Virulence characteristics and motility of *E. coli* isolates ... 118
4.3.2. Optimization of the two-component apparatus for *E. coli* ... 120
4.3.3. Determination of optimal biofilm formation conditions ... 122
4.3.4. Genetic analysis of biofilm formation-associated genes ... 125
4.4. Discussion ... 126
4.5. References ... 131
Chapter 5 Genomic and proteomic analyses of the locus of heat resistance in *Escherichia coli* isolates ... 138
5.1. Introduction ... 139
5.2. Materials and Methods ... 141
5.2.1. Bacterial isolates and whole genome sequencing ... 141
5.2.2. Genetic analysis of the locus of heat resistance and heat shock- and heat resistance-related genes ... 141

5.2.3. Cloning of ORF 3 from *E. coli* AW1.7 145
5.2.4. Characterization of heat resistance in transformants 151
5.3. Results 152
5.3.1. Genetic characterization of the locus of heat resistance 152
5.3.2. Genetic analysis of heat shock- and heat resistance- related genes 158
5.3.3. Generation of ORF 3 clones 158
5.4. Discussion 165
5.5. References 171
Chapter 6 Discussion and future directions 180
6.1. Discussion 181
6.2. Significance 188
6.3. Future Directions 189
6.4. References 192
Bibliography 198
Appendices 238

List of Tables

Table 1.1. Characteristics of *Escherichia coli* pathotypes 14
Table 2.1. Oligonucleotides used in qPCR assays for detection of the locus of heat resistance . 63
Table 2.2. Specificity panel tested with qPCR assays detecting the locus of heat resistance...... 65
Table 3.1. D-values of *Escherichia coli* isolates at 60°C and 71°C.. 89
Table 4.1. Biofilm formation-associated genes... 115
Table 4.2. Genetic determination of biofilm formation-associated genes in heat resistant *Escherichia coli* 125
Table 5.1. Heat shock- and heat resistance- associated genes and predicted functions............ 143
Table 5.2. Oligonucleotides used in cloning of ORF 3 147
Table 5.3. Sequence homology of open reading frames in the locus of heat resistance in heat resistant *Escherichia coli* isolates 155

List of Figures

Figure 1.1. Recommendations for the detection of Shiga toxin-producing *Escherichia coli* in stool specimens 21
Figure 1.2. Schematic representation of the locus of heat resistance 29
Figure 2.1. Calibration curves of qPCR assays 68
Figure 3.1. Survival curves of *Escherichia coli* isolates 88
Figure 3.2. Colony morphology of heat resistant *Escherichia coli* isolate 111 91
Figure 3.3. Transmission electron micrographs of *Escherichia coli* isolates 92
Figure 3.4. Survival of *Escherichia coli* in ground beef patties 94
Figure 3.5. sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates of *Escherichia coli* 96
Figure 4.1. Two-component apparatus for detecting biofilm formation 112
Figure 4.2. Triphenyltetrazolium Chloride motility test 119
Figure 4.3. Biofilm formation of environmental and clinical *Escherichia coli* isolates 121
Figure 4.4. Optimal conditions for biofilm formation of environmental and clinical *Escherichia coli* isolates 124
Figure 5.1. Dendrogram of the locus of heat resistance in *Escherichia coli* isolates 153
Figure 5.2. Alignment of the locus of heat resistance in *Escherichia coli* isolates 157
Figure 5.3. Agarose gel electrophoresis of blunt-end polymerase chain reaction products of open reading frame 3 159
Figure 5.4. Consensus sequence of open reading frame 3 in *Escherichia coli* transformants ... 162
Figure 5.5. sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates of *Escherichia coli* transformants 163
Figure 5.6. I-TASSER protein modeling of ClpK protein 164

List of Abbreviations

°C	Degree Celsius
AAF	Aggregative adhesion fimbriae
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
APC	Aerobic plate count
BAP	Blood agar plate
C-score	Confidence score
CDC	Centers for Disease Control and Prevention
CF	Colonization factors
CFIA	Canadian Food Inspection Agency
CFU	Colony forming units
CNF	Cytotoxic necrotizing factor
CIDT	Culture independent diagnostic test
CPHLN	Canadian Public Health Laboratory Network
Cq	Quantification cycle
D-value	Decimal reduction time-values
DAEC	Diffusely adherent *Escherichia coli*
DAF	Decay-accelerating factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EAST	Enteroaggregative *Escherichia coli* heat-stable toxin

EHEC	Enterohemorrhagic *Escherichia coli*
EIA	Enzyme immunoassay
EIEC	Enteroinvasive *Escherichia coli*/*Shigella*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended-spectrum beta-lactamase
ETEC	Enterotoxigenic *Escherichia coli*
FoodNet	Foodborne Diseases Active Surveillance Network
Gb3	Globotriaosylceramide
GI	Genomic island
HIV	Human immunodeficiency virus
HPP	High pressure processing
HSD	Honest significance differences
HSP	Heat shock protein
HUS	Hemolytic uremic syndrome
I-TASSER	Iterative Threading ASSEmbly Refinement
LB	Luria Bertani
LEE	Locus of enterocyte effacement
LHR	Locus of heat resistance
LOD	Limit of detection
LT	Heat-labile enterotoxin
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

MIQE	Minimum Information for Publication of Quantitative Real-time Polymerase Chain Reaction Experiments
MLVA	Multiple locus variable-number tandem repeat analysis
mTSB	Modified tryptic soy broth
NAAT	Nucleic acid amplification test
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NMEC	Neonatal meningitis *Escherichia coli*
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pEAF	*Escherichia coli* adherence factor plasmid
PFGE	Pulsed-field gel electrophoresis
PGA	Poly-N-acetyl-D-glucosamine
PMA	Propidium monoazide
qPCR	Real-time quantitative polymerase chain reaction
R^2	Coefficient of determination
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
SAT	Secreted autotransporter toxin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEPEC	Sepsis-associated *Escherichia coli*

ShET	*Shigella* enterotoxin
SIM	Sulfide, Indole, Motility
SMA	Standard methods agar
SMAC	Sorbitol-MacConkey
ST	Heat-stable enterotoxin
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
T3SS	Type III secretion system
TAGC	The Applied Genomics Core
TEM	Transmission electron microscopy
TSB	Tryptic soy broth
TTC	Triphenyltetrazolium Chloride
UPEC	Uropathogenic *Escherichia coli*
USDA FSIS	United States Department of Agriculture Food Safety and Inspection Service
UTI	Urinary tract infection
UV	Ultra-violet
VBNC	Viable but non-culturable
VTEC	Verotoxin-producing *Escherichia coli*

Chapter 1

Introduction

1.1. *Escherichia coli*

In 1884, German-Austrian pediatrician Theodor Escherich isolated the Gram negative, rod-shaped bacteria *Escherichia coli*, originally named *Bacterium coli*, from stools of infants (1,2). *E. coli* is non-spore forming and facultative anaerobic member of the *Enterobacteriaceae* family that is typically found as normal microflora in the human gastrointestinal tract (3). Together with other *Enterobacteriaceae*, *E. coli* is one of the first microorganisms to colonize the gastrointestinal tract of humans within a few hours after birth due to its capacity to grow in the presence or absence of oxygen (4). Commensal *E. coli* strains rarely cause human disease and instead provide a barrier to prevent colonization of the gastrointestinal tract with pathogens (5). They are also involved in nutrient degradation and metabolism, and help to develop and maintain the host immune response. Since its discovery, *E. coli* has been extensively studied and has become the model organism for biotechnology due to its ability to survive harsh conditions, versatility, and ease to manipulate (6).

The core genome of *E. coli* is estimated to contain approximately 1700 genes (7) whereas the pan-genome has been reported to range from 16,000 to 45,000 gene clusters (8), illustrating the phenomenal genomic diversity and plasticity of *E. coli* (9). Furthermore, *E. coli* are highly adapted to survive in diverse environments through the acquisition of foreign genetic material and this ability has significantly contributed to the evolution of *E. coli*. Horizontal gene transfer facilitates the movement of deoxyribonucleic acid (DNA) between prokaryotic cells through transformation, transduction, and conjugation (10). While transformation involves the uptake of free DNA from the environment into the genome, transduction and conjugation are mediated by mobile genetic elements on bacteriophages, and plasmids and genomic islands, respectively (11). Genetic material encoded on mobile genetic elements can then be either integrated into the chromosome or exist through self-replication (12). This uptake of accessory genes including virulence factors,

pathogenicity islands, and antibiotic resistance mechanisms results in the frequent emergence of new pathogenic strains and is reflected in the drastic difference in genome sizes from their commensal counterparts (6,12,13).

1.1.2. Pathotypes

Pathogenic *E. coli* have historically been associated with 4 main types of clinical infection in humans including urinary tract infection (UTI), sepsis, meningitis, and diarrheal diseases (14). Based on the clinical presentation of the infection in the host and shared virulence factors that the strains possess, pathogenic *E. coli* are classified into 9 categories also known as pathotypes, and a summary of their characteristics is shown in Table 1.1. Currently, there are 3 extraintestinal pathotypes and 6 intestinal pathotypes defined. Of the extraintestinal pathotypes, they include uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC), and neonatal meningitis *E. coli* (NMEC) (15). Extraintestinal pathotypes are reported to be closely phylogenetically related and share virulence factors with avian pathogenic *E. coli*, the primary and secondary pathogen of chickens and other avian species (16,17).

The 6 intestinal *E. coli* pathotypes are enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC)/*Shigella*, enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (12). With the exception of EIEC, most intestinal *E. coli* strains remain extracellular and do not invade and replicate within host cells. Due to the plasticity of the *E. coli* genome, diarrheagenic *E. coli* strains have evolved to share quite a few of the genotypic and phenotypic traits that define each pathotype, making it increasingly difficult to clearly categorize strains according to the existing pathotype framework (18). Nonetheless, classification of pathogenic *E. coli* strains to their most suitable pathotype provides a strong fundamental base on which to further study the organism.

1.1.2.1 Uropathogenic *Escherichia coli* (UPEC)

UPEC is a common etiologic agent of UTI in humans and in complicated cases, it can lead to cystitis, pyelonephritis, bacteremia, and septicemia (19). In most cases of uncomplicated UTI, community acquisition and colonization of the perineal region with UPEC occurs first, followed by access to the urinary tract (20). Colonization and ascending infection, two attributes that define pathogenesis of UPEC are achieved by virulence factors that mediate adhesion and motility. Adhesion of UPEC to the renal tubule utilizes fimbriae such as Type 1 and P fimbria. Considering that UPEC must adhere to various surfaces in the process of establishing infection, strains belonging to this pathotype encode for a higher number of fimbrial gene clusters compared to other pathotypes and commensal strains (21). Motility is equally important as adhesion in UPEC in order for the pathogen to ascend from the urethra to the bladder. Flagella have been demonstrated as the primary mediator of motility for UPEC and are necessary for the organism to colonize the bladder and form intracellular bacterial communities (22). Once colonization is established, UPEC express a number of toxins including hemolysin, cytotoxic necrotizing factor, and secreted auto-transporter toxin that are involved in pore formation, immune dysregulation, and vacuolation of renal tubule cells, respectively (23–27). Altogether, the toxins secreted by UPEC lead to significant tissue damage while also orchestrating a reduced host inflammatory response. UPEC also can persist as multicellular communities in the form of biofilms (28) and produce capsules, allowing the organism to evade the host immune response through molecular mimicry (29). In order for UPEC to express a number of its virulence factors, it must acquire iron from the host and it utilizes siderophores, iron transporters, and outer-membrane heme receptors to achieve this function (20). Despite UPEC being highly adapted to effectively cause UTI, treatment options are plentiful; numerous classes of antibiotics including β-lactams, tetracyclines, aminoglycosides, and

quinolones have proven to be effective in eliminating UPEC-associated infections (30). Interestingly, cranberry consumption has been reported to show prophylactic effects against UTI through the anti-adhesive effects of its phenolic compounds, thus preventing UPEC from adhering to bladder cells (31).

1.1.2.2. Sepsis-associated *Escherichia coli* (SEPEC)

SEPEC is the second extraintestinal *E. coli* pathotype and is aptly named for the disease that it causes, sepsis. Septicemic *E. coli* infections in adults typically occur as a result of complications in established UTI or invasive medical procedures (32). In neonates, SEPEC is the second leading cause of early-onset sepsis after Group B *Streptococcus* (33). Chorioamnionitis, intrapartum fever, preterm delivery, prolonged membrane rupture, and very low birth weights have been found to increase the risk of SEPEC early-onset sepsis in neonates (34). Compared to UPEC, the molecular virulence profile of SEPEC has not been intensively characterized and SEPEC strains have been reported to be substantially more heterogeneous (35). However, this pathotype does share a number of virulence factors with UPEC and the remaining extraintestinal pathotype NMEC, including the polysaccharide capsule, type 1 fimbriae, cytotoxic necrotizing factor, and siderophores (36,37). Furthermore, SEPEC is similar to UPEC in that it also encodes for virulence genes that play a role in biofilm formation (15). The importance of horizontal gene transfer is not lost on SEPEC, as many of the virulence factors the pathotype possesses are acquired through plasmids and genomic islands and are important for colonization and invasion of the organism through the host tissue to reach the bloodstream (38). Extensive use of intrapartum antibiotics has been met with criticism in regards to potentially increasing the incidence of antibiotic resistant SEPEC in cases of early-onset sepsis (39). In particular, an increase in ampicillin and gentamicin

resistance has been observed in SEPEC strains isolated from neonatal blood and cerebral spinal fluid (33).

1.1.2.3. Neonatal meningitis *Escherichia coli* (NMEC)

Expanding the gamut of extraintestinal diseases that *E. coli* are capable of causing, NMEC invades the meninges of infants and is responsible for two predominant forms of neuronal injury – necrotic cortical injury and apoptotic hippocampal injury (40,41). NMEC-associated mortality is reported to range from 15-40%, and incidence of early-onset meningitis from NMEC infection has been gradually increasing as rates of infection by Gram positive organisms are decreasing (3,42). Pathogenesis of NMEC infection requires firstly a high level of bacteremia followed by the binding of the organism to the blood-brain barrier through fimbriae and outer membrane proteins (43). The organism then invades the brain microvascular endothelial cells by secreting virulence factors such as cytotoxic necrotizing factor and rearranging host cell actin to survive intracellularly before reaching the central nervous system. Like SEPEC, the polysaccharide capsule is critical for survival of NMEC as well. In addition to its antiphagocytic properties, the K1 capsule also prevents lysosomal fusion of the vacuole that the organism is localized in during its period of invasion of the brain microvascular endothelial cells. Greater than 50% of the genes found in NMEC strains are not present in commensal *E. coli* strains and several of its virulence factors are encoded on plasmids that are shared with the other extraintestinal pathotypes (44).

1.1.2.4. Enterohemorrhagic *Escherichia coli* (EHEC)

Among the intestinal pathotypes, EHEC is of greatest concern in public health and food safety due to its ability to cause significant morbidity and mortality in human infection as a foodborne pathogen. A notable feature of EHEC is that it is of zoonotic origin (45). Despite rarely

causing disease in animals, ruminants are the main natural reservoir for EHEC and interaction with cattle or consumption of contaminated beef is frequently implicated in human cases of EHEC infection (46). Transmission of EHEC occurs by direct interaction with ruminants or other colonized animals, ingestion of food or water contaminated with fecal matter harbouring EHEC, and secondary person-to-person spread by infected individuals (47). EHEC was first described as an emerging foodborne disease in Canada during the 1980s (48,49) and has since been implicated in cases of sporadic disease and outbreaks worldwide (50). It is estimated that there are approximately 2.8 million cases of EHEC infection annually across the globe (51). EHEC are comprised of a subset of *E. coli* strains, most notably belonging to the serogroups O157, O26, O111, O103, O121, O45, and O145, that produce potent cytotoxins historically known as verotoxins due to their toxic effects on African green monkey kidney (Vero) cells (52). Verotoxin-producing *E. coli* (VTEC) are also synonymously referred to as Shiga toxin-producing *E. coli* (STEC) due to the resemblance in the biological activity of the cytotoxins with those produced by *Shigella dysenteriae* (53). The cytotoxins have thus been commonly referred to as Shiga toxins 1 and 2 (Stx1/Stx2) despite being different proteins in the two organisms. EHEC are comprised of VTEC/STEC strains that are specifically associated with human gastrointestinal disease. The Shiga toxins are encoded on an inducible, lysogenic, lambdoid bacteriophage that are integrated into the EHEC chromosome during transduction (53). Composed of 1 A subunit and 5 identical B subunits, the Shiga toxins are AB_5 cytotoxins that bind to a host cell glycosphingolipid, globotriaosylceramide (Gb3) (54). Upon binding of the B subunits to Gb3, the A subunit is endocytosed and activated by cleavage of a disulfide bridge to act on eukaryotic RNA. Removal of an adenine residue from host cell 28S rRNA on the 60S subunit of the ribosome results in a pro-inflammatory and pro-apoptotic ribotoxic stress response (55). Interestingly, Gb3 is not found in

ruminants, thus animals can serve as asymptomatic carriers of EHEC. Although the hallmark of this pathotype is the presence of Stx1 and/or Stx2, pathogenesis in EHEC infection is not achieved solely by the Shiga toxins. EHEC must firstly attach to and colonize the human gastrointestinal tract, notably the distal ileum and colon by attaching to host epithelial cells (12). Attachment and effacement of host epithelial cells has been well characterized in EHEC that bear the locus of enterocyte effacement (LEE). The LEE is a 35 kilobase (kb) genomic island that encodes for proteins involved in the loss of intestinal microvilli and induction of a pedestal of polymerized actin that forms beneath and around the bacterial cell, enabling the secretion of effector proteins that cause cytotoxicity, cytoskeleton reorganization, and electrolyte balance into the host cell (56). While the LEE is a strong indicator of EHEC pathogenicity, LEE-negative EHEC strains have also been reported (57). Such strains use other adhesins not encoded on the LEE to attach to epithelial cells and are still capable of causing disease (58,59).

In regards to clinical presentation of EHEC infection, afflicted individuals typically present with a watery diarrhea to hemorrhagic colitis that can persist from days to weeks depending on the infectious dose that was ingested and the immune status of the individual (60). Additional symptoms also include abdominal pain, fever and/or vomiting (61,62). Despite the self-limiting nature of EHEC, infection can progress to the life-threatening complication known as hemolytic uremic syndrome (HUS), hence the emphasis on rapid diagnosis, treatment, and surveillance of EHEC in public health. HUS is characterized by acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia (63) with the potential for chronic renal disease, neurological disorders, hypertension, and cardiovascular disease despite recovery (64,65). Progression of EHEC infection to HUS is a result of binding and activation of the Shiga toxins to Gb3 receptors expressed in the kidneys and tissues of different organ systems (66). Prevention of HUS

development in pediatric cases of EHEC infection is critical because children possess more Gb3 receptors than adults, increasing their risk for development of HUS (63). Treatment for EHEC infection in uncomplicated cases is supportive oral rehydration without the need for antibiotics. However, in the instance of HUS, the choice to administer antibiotics is controversial despite the severity of the disease. Fluoroquinolone use to treat HUS has been reported to cause induction of the lambdoid bacteriophage encoding the Shiga toxins, leading to increased toxin production and poorer patient outcomes (67). In efforts to explore other methods of care, provision of intravenous hydration therapy has been indicated to be a promising treatment option to prevent HUS development in pediatric cases of EHEC infection (68).

1.1.2.5. Enteropathogenic *Escherichia coli* (EPEC)

The key distinctive feature to the EPEC pathotype in the course of diarrheal disease is the formation of attaching and effacing lesions on the surface of intestinal epithelial cells (12). Like EHEC, most strains belonging to the EPEC classification also possess the LEE. However, EPEC strains can be distinguished from EHEC by the lack of Shiga toxins. For this reason, diarrheal disease in cases of EPEC infection is expectedly milder and typically presents as subclinical to a self-limiting, watery diarrhea (69). Also unlike EHEC, EPEC and the remaining 5 intestinal pathotypes cannot be directly traced to a zoonotic source of transmission. Infection with EPEC occurs via fecal-oral ingestion of the organism from contaminated surfaces, liquids, and person-to-person contact (70). EPEC are currently further classified into 2 categories, typical EPEC (tEPEC) and atypical EPEC (aEPEC) on the basis of the presence or absence of the *E. coli* adherence factor plasmid (pEAF), respectively (71). The pEAF encodes for type IV bundle forming pilus and a transcriptional activator for the LEE, both of which are important virulence factors of EPEC (72,73). Pathogenesis of EPEC largely relies on the LEE and the effector proteins

encoded by it that are secreted into the host cell via the type III secretion system (T3SS). High incidences of infantile and pediatric infections are linked to tEPEC in developing countries (74) but in adults, infection rarely occurs due to either the development of immunity or the loss of EPEC receptors with age (75). aEPEC infection presents expectedly as a less severe, non-dehydrating, and non-inflammatory diarrhea due to the lack of the pEAF but persists for a longer duration than tEPEC infection (76).

1.1.2.6. Enteroinvasive *Escherichia coli* (EIEC)/*Shigella*

Both EIEC and *Shigella* spp. are the etiologic agents of bacillary dysentery, which is characterized by a bloody, mucoid diarrhea with fever and abdominal cramps (77). On the spectrum of disease severity caused by the intestinal pathotypes, EIEC/*Shigella* is associated with poorer patient outcomes due to the risk of developing life-threatening complications that include hypoglycemia, septicemia, HUS, and toxic megacolon (78). Like EPEC, the burden of disease for EIEC/*Shigella* infection in low-income countries is high and has been shown to cause significant morbidity and mortality in children >5 years of age (79). EIEC/*Shigella* targets the colon and rectal mucosa and is the only intestinal pathotype that mediates infection by intracellular invasion. EIEC/*Shigella* first reaches the basolateral side of intestinal epithelial cells by translocating through M cells (77). The bacterial cell is then phagocytosed by macrophages only to trigger cell death and be released into the lamina propria. From there, the organism is free to invade adjacent intestinal epithelial cells from the basolateral side using actin-based motility. Disruption of intestinal epithelial integrity and secretion of *Shigella* enterotoxins 1 and/or 2 (ShET-1/ShET-2) are responsible for the initial presentation of mild diarrhea from infected individuals. Severe cases of EIEC/*Shigella* infection are attributed to secretion of Shiga toxins, whose biological activity is identical to that of the EHEC Shiga toxins. Acquisition of the invasion plasmid pINV and *Shigella*

pathogenicity islands through horizontal gene transfer distinguishes EIEC from commensal *E. coli* strains (3,80) and is responsible for much of EIEC/*Shigella* virulence. Over 100 genes are encoded on pINV and among them is the T3SS, which translocates effector proteins into the host cell to subvert host cell processes for lateral spread and suppress the host immune response (80,81). Phylogenetic studies have identified *Shigella* spp. to be closely related to *E. coli*, rendering a separate genus classification for the organism to be redundant (82,83). Despite the genetic similarities between the two genera, it is interesting to note that EIEC strains are less virulent than *Shigella* spp. and causes a milder gastrointestinal disease (84).

1.1.2.7. Enterotoxigenic *Escherichia coli* (ETEC)

Best known for causing traveler's diarrhea in adults, ETEC is endemic in developing countries and is a common cause of diarrhea in children (85). Clinical presentation of ETEC infection consists of a watery diarrhea with abdominal pain, nausea, and vomiting after ingestion of contaminated food or water with a bacterial load of 10^6 to 10^{10} organisms (12). ETEC pathogenicity is largely attributed to 3 main virulence determinants, a group of colonization factors (CFs), a heat-labile enterotoxin (LT), and a heat-stable enterotoxin (ST). Over 20 characterized CFs mediate attachment of the organism to the small intestine and are comprised of fimbrial, helical, fibrillar, and afimbrial protein adhesins (86). Most CFs are encoded on plasmids and have been shown to exhibit differential specificity to host cell receptors (87,88). LT is an enterotoxin that is further categorized into either type I or type II LTs based on differences between their genetic properties and biological activity (89). Like the Shiga toxins, LT is an AB_5 enterotoxin that activates upon binding of the B subunit to its receptor ganglioside GM1, which is present on the apical surface of intestinal epithelial cells. Upon cleavage of the A subunit once inside the epithelial cell, it acts on the Gsα protein to ultimately stimulate opening of chlorine channels that

secrete electrolytes and water into the lumen. The watery diarrhea observed in infected individuals is largely owing to LT-I, which shares approximately 80% sequence identity with the *Vibrio cholerae* cholera toxin whereas LT-II has not been reported to be associated with fluid accumulation (90). The second enterotoxin involved in ETEC pathogenesis is ST, which is relatively smaller than LT (91). ST binds to the enzyme guanylyl cyclase that is present on the surface of intestinal epithelial cells and through activation of this receptor, chlorine is secreted leading to dehydration of the host cell. In addition, ST further impairs fluid absorption by inhibiting the cell's sodium/hydrogen (Na^+/H^+) exchanger. Like the other intestinal pathotypes, treatment of ETEC infection mainly relies on oral rehydration therapy although antibiotics such as fluoroquinolones can also be administered to shorten the duration of infection in individuals with traveller's diarrhea (85,92).

1.1.2.8. Enteroaggregative *Escherichia coli* (EAEC)

Much like ETEC, EAEC infection presents in two forms, as a watery diarrhea in children of low-income, developing countries and as a self-limiting traveller's diarrhea in adults from developed countries (93). Cases of coinfection of EAEC and human immunodeficiency virus (HIV) has been reported to be a distinguishing feature of EAEC that other pathotypes do not share and is a contributing factor to the syndrome "slim disease" in HIV-infected individuals who present with persistent diarrhea and muscle wasting (94,95). EAEC pathogenesis is not dissimilar from that of the other intestinal pathotypes despite the heterogeneity between strains; EAEC causes diarrheal disease by firstly adhering to intestinal epithelial cells, increasing mucous production on the host cell surface and forming biofilms and then secretes toxins that results in intestinal inflammation (96). EAEC aggregately adhere to intestinal epithelial cells in a distinct stacked-brick pattern that is facilitated by a plasmid-encoded aggregative adherence fimbriae (AAFs) and additional

afimbrial adhesins (97). This same plasmid also harbours several key virulence factors of EAEC that are involved in the spread of EAEC across the intestinal mucosa and in the production and secretion of heat-stable and heat-labile enterotoxins and cytotoxins. Toxin production by EAEC causes microvillus vesiculation, impairment of intestinal tight junctions, and stimulates an inflammatory immune response (98). Treatment of EAEC infection with antibiotics is typically restricted for use in confirmed cases of traveller's diarrhea or in immunocompromised patients (99). Otherwise, oral rehydration therapy is recommended in most cases of infection.

1.1.2.9. Diffusely adherent *Escherichia coli* (DAEC)

The remaining intestinal *E. coli* pathotype is DAEC, which although causes diarrheal disease, utilizes an entirely different adhesion mechanism to attach to intestinal epithelial cells that is not observed in the other 5 pathotypes. DAEC strains encode for the Afa/Dr family of adhesins that bind to brush border-associated decay-accelerating factor (DAF), a highly expressed molecule found on intestinal epithelial cells (100). Afa/Dr adhesins are not specific to DAEC and have been identified in the extraintestinal pathotype UPEC as well. Interestingly, DAEC strains do not possess any genes that encode for secretion systems and pathogenesis relies entirely on the induction of cytoskeleton rearrangements in the host cell that lead to brush border lesions and loss of microvilli (101). Bacterial flagella are another virulence factor that DAEC possess and are highly involved in the stimulation of the host inflammatory response. Individuals with DAEC infection present with a watery diarrhea and persistent cases may be associated with inflammatory bowel disease and Crohn's disease in pediatrics and adults, respectively (102). Oral rehydration therapy is the only method of treatment for DAEC infection currently recommended.

Table 1.1. Characteristics of *Escherichia coli* pathotypes

Pathotype	Site of infection	Disease caused	Virulence factors	Treatment
UPEC	Urinary tract, bladder, kidney	UTI, cystitis, pyelonephritis	Afimbrial adhesins, fimbriae, polysaccharide capsule, α-hemolysin, CNF, SAT	Antibiotics
SEPEC	Bloodstream	Sepsis	Fimbriae, polysaccharide capsule, CNF	Antibiotics
NMEC	Meninges, subarachnoid space, brain vasculature	Meningitis	Fimbriae, K1 capsule, CNF	Antibiotics
EHEC	Distal ileum, colon	Diarrhea, hemorrhagic colitis, hemolytic uremic syndrome	Stx, LEE, hemolysin	Oral rehydration, supportive, intravenous hydration

UTI, urinary tract infection; CNF, cytotoxic necrotizing factor; SAT, secreted autotransporter toxin; Stx, Shiga toxins; LEE, locus of enterocyte effacement; ShET-1/ShET-2, *Shigella* enterotoxin 1 and 2; CFs, colonization factors; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin; AAFs, aggregative adhesion fimbriae; EAST, EAEC heat-stable toxin.

Table 1.1 continued. Characteristics of *Escherichia coli* pathotypes

Pathotype	Site of infection	Disease caused	Virulence factors	Treatment
EPEC	Small intestine	Watery diarrhea	Type IV bundle forming pilus, LEE	Oral rehydration, antibiotics
EIEC/ *Shigella*	Colon	Bacillary dysentery, hemolytic uremic syndrome	ShET-1/ShET-2, Stx	Oral rehydration, antibiotics
ETEC	Small intestine	Watery diarrhea	CFs, LT, ST	Oral rehydration, antibiotics (fluoroquinolones)
EAEC	Colon	Watery diarrhea, traveller's diarrhea	AAFs, flagella, biofilm, EAST	Oral rehydration, antibiotics
DAEC	Intestine (exact location unknown)	Watery diarrhea	Afa/Dr family of adhesins, flagella	Oral rehydration

UTI, urinary tract infection; CNF, cytotoxic necrotizing factor; SAT, secreted autotransporter toxin; Stx, Shiga toxins; LEE, locus of enterocyte effacement; ShET-1/ShET-2, *Shigella* enterotoxin 1 and 2; CFs, colonization factors; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin; AAFs, aggregative adhesion fimbriae; EAST, EAEC heat-stable toxin.

1.2. Foodborne *Escherichia coli* infection

Sporadic cases and outbreaks of *E. coli* have been reported worldwide in both developing and developed countries. In low income, developing countries, outbreaks associated with food and other transmission routes are often the result of inadequate clean water and poor sanitation (61,85). In developed countries, foodborne outbreaks of EPEC, ETEC, EAEC, and DAEC are increasingly rare because access to safe drinking water, sanitation, and hygiene is readily available, thus reducing the circulation of strains belonging to these pathotypes. Consequentially, diagnostic testing and epidemiological tracing of foodborne outbreaks in developed jurisdictions mainly prioritize EHEC and STEC strains due to its zoonotic origin, low infectious dose, and the high rates of morbidity and mortality associated with infection.

Worldwide, STEC is estimated to cause 2,801,000 cases of acute infection annually and 3890 cases of HUS (103). Approximately 730 cases of STEC cases are reported annually in Canada and it is estimated that upwards of 50 unreported cases occur each year (104). The province of Alberta has one of the highest rates of STEC infection in the country with 1547 cases reported over a 10 year period from 2008 through 2018 (105). STEC cases in Alberta occur in both urban centers with high population densities and rural areas where cattle farming is widespread (106). To achieve an inclusive epidemiological understanding of the burden of STEC disease, an essential component is a robust and responsive diagnostic laboratory that can keep up with the evolving nature of STEC. Over the course of history, foodborne outbreaks of STEC have served as important catalysts in developing the algorithm for its identification and surveillance in diagnostic laboratories, improving sampling and inactivation processes in the food processing industry, and advancing STEC research in the fields of diagnostic and food microbiology.

1.2.1. 1993 outbreak of *E. coli* O157:H7

STEC gained worldwide attention in 1993 when an American multistate outbreak of *E. coli* O157:H7 resulted in 732 cases of acute infection of which 151 were hospitalized and 45 developed HUS (107). The outbreak began in early December of 1992; concern was raised when an increased number of pediatric patients at the Children's Hospital and Medical Center in Seattle, Washington all presented with hemorrhagic colitis (108). All cases of infection were determined to originate from consumption of undercooked beef hamburgers sold at the American fast-food chain Jack in the Box. Following investigation, it was discovered that the patty processing protocol of the fast-food chain did not comply with Washington state law, which required a minimum internal temperature of 68.3°C while the Jack in the Box protocol required only 60°C. Specifically, the 46 g patty that was prepared exclusively for the children's menu was found to be associated with pediatric cases of infection but not the larger 114 g patty. All beef products received by Jack in the Box chains in Washington state were recalled and prevented an estimated 800 potential cases of infection (108).

The rapid response to this recall can be attributed to the fact that Washington State was one of the few state health departments that classified *E. coli* O157:H7 as a notifiable disease in 1993. Sweeping reforms to public health and food safety occurred as a result of this outbreak, including the upgrade of *E. coli* O157:H7 as a notifiable disease at all state health departments and the classification of the organism as an adulterant in ground beef (109). The Centers for Disease Control and Prevention (CDC) also recognized the importance of molecular fingerprinting as a tool for further confirmation of clonal relatedness of strains isolated from patients and foods. Specifically, pulsed-field gel electrophoresis (PFGE) was utilized in tracing this outbreak. A modified PFGE protocol along with standardized procedure, equipment, and software for analysis

was established for foodborne outbreak detection, investigation, and surveillance nationwide, which eventually led to the establishment of PulseNet in 1996 (110,111).

1.2.2. 2011 outbreak of *E. coli* O104:H4

In 2011, a European outbreak of *E. coli* O104:H4 associated with fenugreek sprouts that began in Germany resulted in 4,321 cases of foodborne disease (112). Person-to-person transmission facilitated the spread of the strain across 16 countries (113–115). The contaminated seeds originated from Egypt and were imported some time in 2009. However, it is unknown if contamination occurred at the production farm, during transportation, or at the receiving site. Of the 4321 cases of infection, over 900 individuals developed HUS and more than 50 deaths were reported (116,117).

This particular strain of *E. coli* O104:H4 possessed virulence factors that were typical of the EAEC pathotype but surprisingly also encoded for stx_2, a virulence gene commonly associated with EHEC and EIEC/*Shigella*. The identification of Stx2 in this strain has since prompted re-evaluation of the existing pathotype framework and provides compelling evidence for diagnostic laboratories to consider identification of STEC isolates outside of the O157:H7 serotype and top 6 STEC serogroups (O26, O111, O103, O121, O45, and O145) in cases of foodborne infection. Comprehensive identification and surveillance of clinical STEC isolates beyond those belonging to O157 and the top 6 serogroups was not widely conducted in diagnostic laboratories prior to this outbreak. Since the emergence of this O104:H4 strain, diagnostic laboratories have begun considering the value in isolation and surveillance methods for non-top 6 STEC isolates. Doing so would further scientific research in EAEC/STEC hybrid strains and also improve foodborne outbreak detection and investigations where the etiologic agent could not be identified previously (118).

1.2.3. 2012 outbreak of *E. coli* O157:H7

The largest meat recall in Canadian history is attributed to an outbreak of mechanically tenderized beef that was contaminated with *E. coli* O157:H7. In September 2012, the Canadian Food Inspection Agency (CFIA) was notified by the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) of beef trimmings exported by XL Foods Inc. that were positive for *E. coli* O157:H7 (119). Coincidentally, a Canadian secondary beef processor also reported the presence of the organism on beef trimmings that same day. PFGE results for *E. coli* O157:H7 isolated from the beef trimmings were uploaded onto PulseNet USA by the USDA FSIS, prompting a call for all PulseNet Canada participating laboratories to conduct PFGE and search for matches (120). Over a period of weeks, the CFIA began the process of recalling all beef products originating from XL Foods Inc. as public health laboratories sought to identify cases of human infection. However, measures were not implemented early enough to prevent disease and ultimately resulted in 18 cases of acute gastroenteritis limited to Canada (121). No infected individuals developed HUS and 0 deaths were reported. Consumption of undercooked beef steaks, filet mignon used for steak tartare, and lean ground beef was implicated in all cases of foodborne infection from this outbreak. All beef cuts were mechanically tenderized by retailers prior to packaging. The food safety investigation revealed that the beef processing plant was not following proper protocol for microbiological sampling and testing for beef, allowing contaminated beef products to leave the plant. At the retail level, beef products did not have labels indicating that they had been mechanically tenderized but no other deficiencies in their food safety protocols were detected. In instances of consumption of mechanically tenderized beef, the risk for *E. coli* O157:H7 infection increases by 5 fold and may be further magnified if beef is not fully cooked (122).

Beef production is an important industry in Canada and it is estimated that the country exports approximately 597,000 tonnes of beef and cattle valued at $3.7 billion (Canadian dollars) to 56 different countries annually (123). In response to the outbreak, voluntary recall by XL Foods Inc. in partnership with the CFIA led to the disposal of 4000 tonnes of beef and beef products, which represents a minimum of 12,000 head of cattle (119). Understandably so, the economic loss to XL Foods Inc. and the beef production industry was significant. Closure of XL Foods Inc. resulted in the inability for cattle producers to sell their cattle for slaughter and with the loss of confidence in the safety of Canadian beef, import to international partners was halted. Losses to the beef production industry as a result of this outbreak were reported to range from $16 to $27 million Canadian dollars. Corrective actions issued by the CFIA in response to the outbreak included revisions to meat inspection regulations and the requirement for processing plants and retailers to identify all products that are mechanically tenderized and provide a safe cooking instructions label for consumers.

1.2.4. Diagnostic laboratory identification

Rapid and accurate diagnosis of STEC is vital for management of patient treatment and tracing of foodborne outbreaks. While screening for *E. coli* O157:H7 and the top 6 serogroups has been readily adopted in laboratories, testing for other STEC serotypes is not commonly practiced although they may contribute to unreported cases of diarrheal disease (124–126). The 2012 German outbreak of *E. coli* O104:H4 raised concerns of STEC serotypes that are currently not part of diagnostic laboratories' testing algorithm. Hence, guidelines for front line diagnostic laboratories to detect non-O157 STEC in addition to *E. coli* O157:H7 have been developed by the CDC (127,128) and the Canadian Public Health Laboratory Network (CPHLN) (129). Recommended testing algorithms now include culturing for *E. coli* O157:H7 and testing for Shiga

toxins to detect non-O157 STEC from all stools submitted for routine testing from patients with acute gastroenteritis (Figure 1.1).

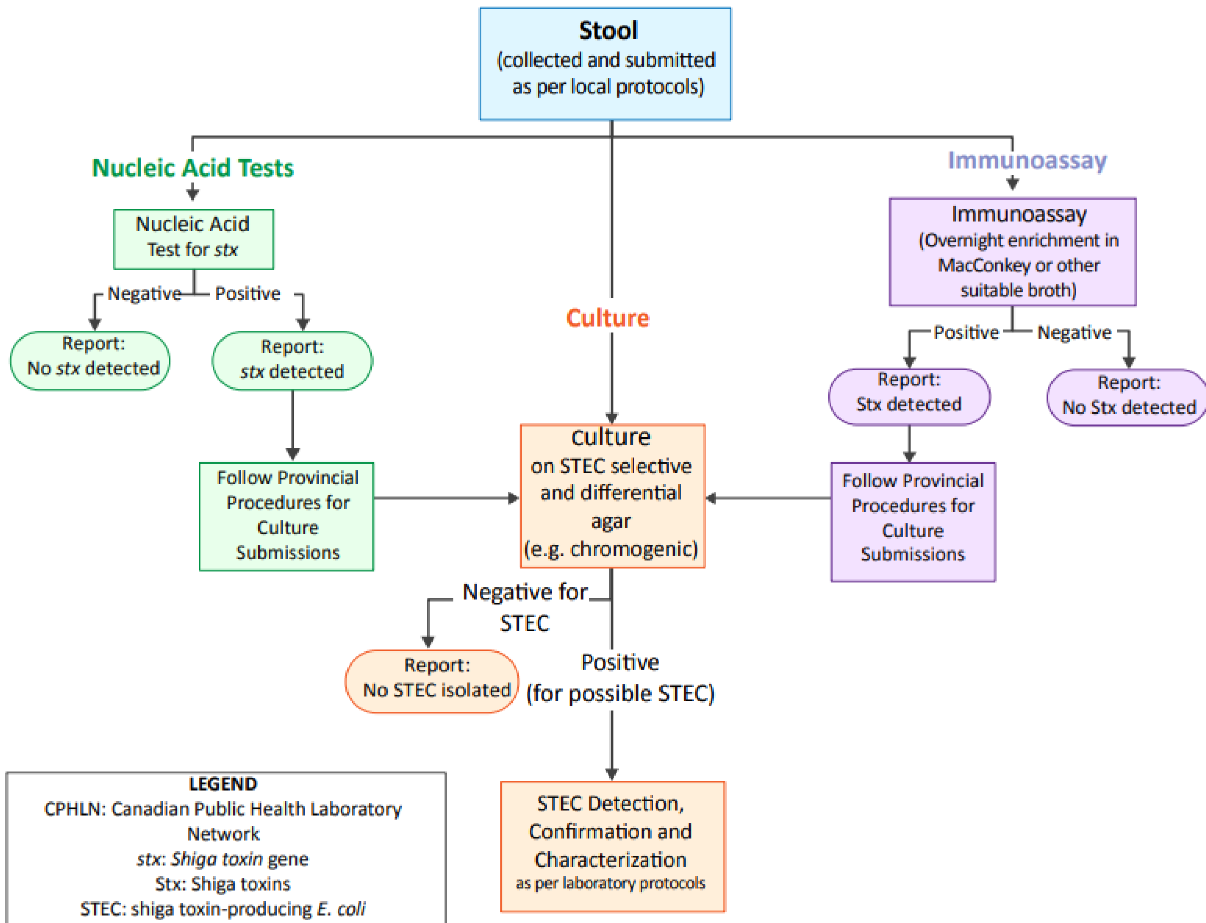

Figure 1.1. Recommendations for the detection of Shiga toxin-producing *Escherichia coli* in stool specimens by the Canadian Public Health Laboratory Network. Image reproduced from reference (129) under Creative Commons Attribution CC BY 4.0 license.

Culture-based testing remains the gold standard for detection of STEC and many other bacterial agents of infection, and advances in differential and selective agars have facilitated easier identification of viable bacteria from patient stool samples. The first iteration of such agars exploited the biochemical trait of most *E. coli* O157:H7 strains' inability to ferment sorbitol (62). Sorbitol-MacConkey (SMAC) agar was developed to select for O157:H7 strains on the basis that non-sorbitol fermenting organisms would produce white colonies on the plates (130). SMAC agar does have limitations however, due to its inability to detect non-O157 STEC and some sorbitol-fermenting O157:H7 strains (131). Differential and selective media options have since expanded; with the introduction of several chromogenic media that incorporate proprietary chromogenic substrates to facilitate detection of O157 and non-O157 STEC strains (132). Although culture media permit determination of viable bacteria in an infection, they do not provide information on whether strains are toxigenic or not.

Culture independent diagnostic tests (CIDTs) are recommended to be included in a diagnostic laboratory's STEC testing algorithm in addition to culturing of the organism on selective and differential media (129). Enzyme immunoassays (EIAs) for the detection of Shiga toxins are one form of CIDTs. Most commercial EIAs detect Stx1 and Stx2 expression using monoclonal antibodies immobilized on membranes that can be used directly with stool (133,134). The second CIDT method for detection of STEC is nucleic acid amplification tests (NAATs) for detection of STEC virulence genes. Since the advent of real-time polymerase chain reaction (PCR) platforms, numerous commercial assays targeting stx_1 and stx_2 and other STEC virulence genes such as the intimin gene, *eae*, and hemolysin gene, *hly*, with the capacity for multiplexing are now available on the market (135). If the expertise and instrumentation is available, in-house developed molecular assays for detection of STEC virulence genes have been shown to be rapid and more

affordable options in comparison to commercial NAATs with superior sensitivities and specificities (126). Furthermore, molecular assays for *stx* subtyping and serotyping permit for an even more comprehensive STEC detection and characterization system (136).

Investigation and surveillance of foodborne STEC is equally important as its detection. Although serotyping is a useful classification system for STEC, a deeper level of typing is required to determine phylogenetic relatedness between strains in foodborne outbreak investigations and surveillance. PulseNet is an international laboratory network founded by the CDC for the detection, investigation, and surveillance of foodborne illnesses and is comprised of public health laboratories across Canada, the United States of America, Europe, Asia Pacific, Africa, Middle East, Latin America, and the Caribbean (137). The ultimate goal is for participating laboratories to upload molecular typing data of bacterial pathogens isolated from cases of foodborne infection to facilitate identification and control of outbreaks internationally. PFGE and multiple locus variable-number tandem repeat analysis (MLVA) are 2 major molecular typing methods used by PulseNet participants to determine the relatedness of isolates through the generation of DNA fingerprints and can be used in combination to improve the resolution of STEC typing (138). With significant advances in whole genome sequencing for pathogen identification and typing, PulseNet seeks to standardize and implement this method for real-time foodborne detection and investigation in the future (139).

The Foodborne Diseases Active Surveillance Network (FoodNet) performs active, population-based surveillance for 8 foodborne bacterial pathogens and *Cyclospora* (140) in Canada and the United States. Both *E. coli* O157:H7 and non-O157 STEC are monitored by FoodNet sentinel sites, providing surveillance data from laboratory surveys and population surveys to estimate the burden of disease and identify new risk factors for foodborne infection (141).

FoodNet collaborates with public health laboratories, epidemiologists, and organizations such as PulseNet and food safety forums to provide the foundation for food safety policy and improve disease surveillance across clinical, food, and environmental sectors.

1.3. Pathogen elimination methods used in food processing

In efforts to reduce the global incidence of foodborne pathogens and facilitate international foods trade, the development and management of robust food safety programs are essential. Ever evolving microbial threats to food safety include increasing rates of antimicrobial resistant bacteria and the growing proportion of enteric viruses and parasites involved in foodborne disease (142). At the level of food processing, monitoring, sampling, and testing of foods for adulterants at each stage of the farm-to-fork-continuum is a core component of food safety programs (143). Food processing plants use a variety of pathogen elimination methods to reduce the microbial load on foods in efforts to ensure that the risk for foodborne disease at the stage of consumption by the consumer is minimal. Among all methods aimed at eliminating foodborne pathogens including bacteria, viruses, and parasites, thermal inactivation is most frequently used and is still one of the most effective methods (144).

1.3.1. Thermal inactivation processes

Thermal inactivation remains the primary method of STEC elimination from animal carcasses, specifically cattle. It is commonly used in the forms of steam pasteurization or spray washes for different animal components and along multiple points of the farm-to-fork continuum. Cattle sold from feedlots for slaughter frequently harbour STEC in their gastrointestinal tract, thus care must be taken to avoid cross-contamination from the carcasses onto final meat products

(145,146). Thermal inactivation measures for STEC in cattle processing ranges in temperature from 15°C to 95°C. Lower temperature heat exposure (15°C to 60°C) is used for antimicrobial wash steps that contain acetic acid, lactic acid, or sodium hydroxide whereas steam pasteurization requires temperatures of 82°C to 95°C to be effective at eliminating STEC on carcasses. Washes are rarely applied at higher temperatures of 70°C and above as this can result in discoloration of the meat and production of aerosols. Thermal inactivation also plays a role in plant sanitation and sterilization through application of chemical sanitizers including chlorine-based compounds, hydrogen peroxide, and peroxyacetic acid on surfaces and equipment at temperatures ranging from 50°C to 82°C (147,148). High temperature exposure alters bacterial cells on multiple levels, disruption of the cell envelope leads to the loss of membrane-associated functions and leakage of cellular contents, denaturation and aggregation of proteins and damage to ribosomes result in the attenuation of protein synthesis, and DNA denaturation causes an increase in the rate of mutations. The accumulation of multiple, simultaneous injuries to the cell consequentially results in cell death (149).

In meat processing, by-product materials including manure, waste feed, and carcass slaughter waste are commonly composted together and used as fertilizer in croplands (150). Foodborne pathogens including STEC have been found to reside within compost, hence their inactivation prior to use of compost is necessary to prevent contamination of crops and agricultural runoff (151,152). The food processing industry relies on thermal inactivation measures at 50°C and 60°C to reduce levels of viable bacteria in compost. However, depending on compost composition and fat content in carcass waste, the effectiveness of thermal inactivation can vary significantly(150).

1.3.2. Non-thermal pathogen elimination methods

Foods that heat treatment cannot be used on such as fresh produce and shellfish, and the rising consumer demand for minimally processed foods have paved the way for development and implementation of non-thermal, alternative food processing methods. Such methods include high pressure processing (HPP), gases, pulsed ultra-violet (UV) light, and ionizing radiation.

High hydrostatic pressure processing and ultra high-pressure processing comprise HPP methods and are commonly used to eliminate *E. coli*, *Salmonella*, *Listeria*, and *Vibrio* in fruits, juices, vegetables, seafood, and sauces (153,154). HPP inhibits protein synthesis and enzyme function, denatures bacterial DNA, and alters cell morphology without impacting the appearance and texture of food, hence the desire for its use (155). However, a major limitation of HPP in elimination of *E. coli* is the high variability in pressure resistance between strains (156).

Use of antimicrobial gases such as ozone and those generated by cold plasma is another non-thermal, alternative food processing method that has garnered attention for their use on fresh produce and efficacy against bacterial biofilms (157,158). Gases show bactericidal effects on both Gram positive and Gram negative organisms by oxidizing proteins and degrading the cell envelope, resulting in leakage of cellular contents (159).

Pulsed UV-light is a novel technology for inactivation of foodborne pathogens on fresh foods (160). UV-light converted from electrical energy stored within a capacitor is released in short bursts or pulses onto the surface of foods, which results in the formation of thymine dimers within the DNA of microorganisms to stop cell replication (161). Pulsed UV-light improves on the established continuous UV light inactivation process, with reports of greater penetration capacity and bacterial reductions when used on fecal matter and small areas (162). Reductions in

foodborne pathogens by pulsed UV-lights have been shown to be comparable or greater than other inactivation methods with the additional benefits of a shorter treatment time compared to thermal inactivation processes.

Ionizing radiation such as gamma (γ) irradiation and electron beam irradiation is known to be the best method for control of foodborne pathogens in raw meat (163) and its use has been shown to be highly effective at reducing bacterial loads, specifically *E. coli*, on beef (164). Additionally, ionizing radiation can be used in combination with heat treatment to reduce the irradiation doses but not compromise food quality (165). Gamma irradiation involves the production of electromagnetic γ-rays by the radioactive isotope Cobalt 60 that mediate swelling and breakage of DNA in microorganisms so that cell replication is halted (166). The mechanism of action of electron beam irradiation is similar to that of gramma irradiation but does not require radioactive isotopes (167).

1.4. Locus of heat resistance

External stressors greatly impact the populations of *E. coli* that can survive and thrive in an environment. Beyond the adaptive mechanisms to survive environmental stress natively found in *E. coli*, acquisition of a 14 kb genomic island known as the locus of heat resistance (LHR) confers additional heat resistance to temperatures of 60°C and above. The LHR was first characterized in *E. coli* AW1.7, a non-pathogenic strain isolated from a beef slaughter plant (168). *E. coli* AW1.7 was reported to not only survive heat inactivation procedures that reflect those used in food processing but to exhibit increased heat resistance in conditions of osmotic stress (169,170). Heat exposure at 60°C for 5 minutes reduced cell counts of stationary phase *E. coli* AW1.7 by less

than 1 log colony forming units/millilitre (CFU/mL) whereas heat sensitive strains that did not possess the LHR were reduced by greater than 7 log CFU/mL (171). In exponentially growing *E. coli* AW1.7, cell counts were reduced by 3 CFU/mL, indicating that although the LHR mediates heat resistance in both exponential and stationary phase cells, it is less effective at earlier growth phases. With the addition of 2%, 4%, and 6% sodium chloride (NaCl) in growth media, heat resistance of *E. coli* AW1.7 was significantly increased through acquisition of compatible solutes such as glucose, trehalose, and glycine betaine (170).

Comprised of 16 open reading frames (ORFs), the LHR is flanked by mobile genetic elements facilitating horizontal transfer (Figure 1.2) (172). It is predicted to encode for proteins involved in heat shock, turnover of misfolded proteins, protection against oxidative stress, and cell envelope maintenance (173). A putative potassium/hydrogen (K^+/H^+) exchanger is among one of the proteins encoded by the LHR and may play a role in the elevated heat resistance observed when isolates are exposed to heat treatment in combination with osmotic stress. In addition to resistance to osmotic stress, the K^+/H^+ exchanger and heat shock proteins encoded by the LHR also confer resistance against oxidative stress by protecting membrane lipids and cytoplasmic proteins when challenged with hydrogen peroxide, sodium hypochlorite, and peroxyacetic acid (174). Cross-talk observed between the ORFs encoded in the cell envelope maintenance component of the LHR and housekeeping genes suggests that the LHR is not self-regulated (173).

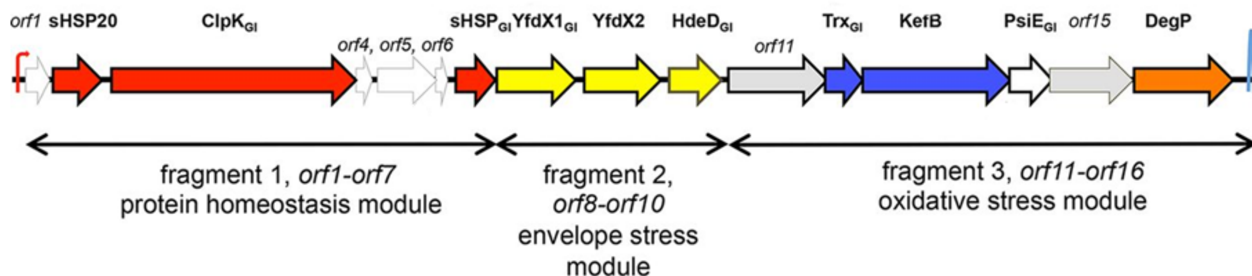

Figure 1.2. Schematic representation of the locus of heat resistance and putative functions encoded by the genes located on the genomic island in heat resistant *Escherichia coli* AW 1.7. Proteins are color coded based on their predicted function: red, heat shock proteins; yellow, hypothetical proteins with a possible relationship to envelope stress; blue, proteins related to oxidative stress; and orange, serine protease DegP that is essential for growth at high temperatures. Genes carry the footnote "GI" (for genomic island) if an orthologue of the same gene is present in genomes of *E. coli*. Open reading frames are numbered if there is no known function associated with the genes; predicted functions of proteins are written above. Image adapted from reference (173) with permission from The American Society for Microbiology.

1.4.1. Identification in *E. coli*

In a study to determine the frequency of LHR-positive *E. coli* circulating in beef processing environments, 4.3% of strains were identified to possess the LHR (171). Bioinformatic analyses have also revealed that approximately 2% of the *E. coli* genome sequences or whole genome shotgun sequences available on the National Center for Biotechnology Information (NCBI) database have the LHR (172). Characterization of the LHR in *E. coli* is largely limited to isolate AW1.7. However, there have been reports of heat resistant *E. coli* strains isolated from raw milk cheese (175), wastewater treatment plants (176), and hospital settings (177). In the heat resistant raw milk cheese isolate, a variant to the 14 kb LHR described by Mercer *et al*. (172) was identified (178). This LHR variant equally confers heat resistance but also contains an additional locus encoding type 3 fimbriae, increasing the length of the LHR to 19 kb. A coverage of 77% and sequence identity of 88% was present in the LHR variant in comparison to its 14 kb counterpart.

Boll *et al*. reported 36% of 90 raw milk cheese *E. coli* isolates were positive for the LHR and it is likely that thermization processes of raw milk at temperatures of 57°C-68°C for the production of cheeses select for the survival of heat resistant strains. Interestingly, Zhi *et al*. identified 59% of 70 wastewater isolates from chlorinated sewage to possess the LHR (176). Considering the LHR also provides protection against oxidative stress, it is possible that the chlorine treatments employed in wastewater sanitation also positively select for heat resistant *E. coli* strains despite the lack of high temperature stress. High temperature treatments for the inactivation of microorganisms are not limited to the food processing industry but also widely used in hospital settings for the cleaning and decontamination of medical devices such as catheters and endoscopes (179). From a collection of 115 extended-spectrum beta-lactamase- (ESBL) producing *E. coli* isolates from a Danish hospital, 3 (2%) heat resistant isolates were identified (180). Heat resistant isolates were cultured from patients with symptomatic urinary tract infections and one isolate was identified to contain a second copy of multiple ORFs from the LHR involved in heat shock. This isolate exhibited increased heat resistance compared to the other 2 heat resistant isolates that only possessed one copy of the ORFs, suggesting an additive effect in heat resistance. Furthermore, deletion of the duplicate copy of the ORFs did not impart heat sensitivity on the isolate. As ESBL-producing *E. coli* continue to rise as a worldwide concern in nosocomial infections (181), the emergence of heat resistant, multidrug resistant *E. coli* further complicates the issue with their ability to survive high temperature decontamination measures.

1.4.2. Distribution across beta- and gamma- Proteobacteria

Characterization studies have identified heat resistant beta (β)- and gamma (γ)-Proteobacteria beyond *E. coli*. Within the *Enterobacteriaceae* family, LHR-positive *Salmonella enterica* (171), *Enterobacter cloacae*, *Klebsiella pneumoniae* (182), and *Cronobacter sakazakii*

(183) have been reported. Heat resistance in *S. enterica* was comparable with *E. coli* AW1.7 but lower levels were observed in heat resistant *E. cloacae* (171). Out of 105 *K. pneumoniae* isolates cultured from bacteremic patients staying in the intensive care unit of a Danish hospital, Bojer *et al*. (182) identified 31 to be heat resistant. The LHR has also been extensively characterized in *C. sakazakii*, an opportunistic pathogen that has been implicated in neonatal infections by means of consuming contaminated powdered infant milk formula. Gajdosova *et al*. (183) analyzed an 18 kb variant of the LHR in *C. sakazakii* that conferred high levels of heat resistance at 58°C heat exposure. It is theorized that the level of thermal protection conferred by the LHR is genus specific, with heat resistance greatest in *E. coli* and *S. enterica* in comparison with other *Enterobacteriaceae* (171). Through bioinformatic analyses, the LHR has also been found in *Yersinia enterocolitica*, *Citrobacter* sp., and *Pseudomonas aeruginosa* (172,178).

1.5. Rationale for thesis research

The burden of foodborne STEC infection extends its reach across public health and the food processing industry. STEC comprise the most pathogenic intestinal *E. coli* strains, with the capacity to cause significant morbidity and mortality in infected individuals. In both sporadic and outbreak cases of foodborne STEC infection, consumption of undercooked ground beef or other beef products is reported to be a leading cause of infection. Thermal inactivation processes utilized in the food processing industry and consumer guidelines for safe cooking temperatures have historically been believed to be sufficient for eliminating STEC on beef products. However, diverse consumer preferences for consumption of beef provide opportunities for STEC to cause disease. Mechanical tenderization and undercooking of beef to achieve an enhanced texture and

flavour increase the chances for STEC to contaminate sterile tissue and evade inactivation during cooking processes. Efforts to minimize the risk of beef-associated foodborne STEC infection include continual implementation and improvements to food safety programs at processing plants and increasing consumer awareness and education at retail sites.

A novel threat to food safety is the emergence of heat resistant *E. coli* and other Proteobacteria that possess the LHR. Heat resistant *E. coli* have been identified circulating environmental niches relevant to human foodborne disease, such as beef slaughter plants, water systems, and dairy processing plants. No heat resistant *E. coli* isolates have been associated with acute gastrointestinal infection thus far, raising the question of whether heat resistant, pathogenic strains, specifically STEC, exist. If STEC were to acquire the LHR, current thermal inactivation processes used for the elimination of STEC on beef and other foods may be insufficient and potentially select for horizontal transfer of the LHR between strains. In order to comprehensively evaluate the risk of heat resistant *E. coli* on foodborne disease, it is necessary to determine if heat resistant STEC have been involved in human infection.

1.5.1. Overall thesis hypothesis

The presence of the locus of heat resistance in clinical *E. coli* isolates facilitates the increased survival of pathogenic strains against heat inactivation measures in food processing and consumer cooking practices, and can be a contributing factor in human foodborne infection in Alberta, Canada.

1.5.2. Specific thesis objectives

This thesis seeks to elucidate the contribution of the locus of heat resistance in human cases of acute gastrointestinal *E. coli* infection and consists of 4 specific research aims:

1. To develop a real-time quantitative polymerase chain reaction (qPCR) assay for detection of the locus of heat resistance in *E. coli* isolates from clinical samples in Alberta submitted to Alberta Precision Laboratories – Provincial Laboratory from 2009 through 2014 (**Chapter 2**).

2. To characterize the heat resistance of *E. coli* isolates exposed to 60°C and 71°C heat inactivation treatment under conditions of increasing osmotic stress for determination of decimal reduction time-values (D-values) in culture media and when inoculated in a food matrix of ground beef and grilled (**Chapter 3**).

3. To determine if heat resistant *E. coli* isolates produce biofilm in response to nutrient depletion, cell concentration manipulation, and different incubation temperatures. (**Chapter 4**).

4. To investigate the composition and function of the locus of heat resistance in the clinical, heat resistant *E. coli* isolates through genomic and proteomic analyses (**Chapter 5**).

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Chapter 2

Development of a real-time quantitative polymerase chain reaction (qPCR) assay for detection of the locus of heat resistance in clinical *Escherichia coli* isolates[*]

[*] A portion of this chapter has been published as Ma A, Chui L. Identification of heat resistant *Escherichia coli* by qPCR for the locus of heat resistance. J Microbiol Methods. 2016 Dec 22; 133:87–9.

2.1. Introduction

The bacterium *Escherichia coli* largely exists as a harmless commensal organism in the gastrointestinal tract of humans and other warm-blooded mammals (1). However, acquisition of a diverse array of virulence factors enable specific *E. coli* strains to become pathogenic (2). Pathogenic *E. coli* are capable of causing a wide spectrum of human diseases including diarrhea, urinary tract infection, sepsis, and meningitis (3). Foodborne infection by intestinal pathogenic *E. coli*, particularly those by Shiga toxin-producing *E. coli* (STEC) is a major public health concern. STEC is capable of causing gastrointestinal illness ranging from diarrhea to hemorrhagic colitis (4,5), and can progress to the life-threatening complication known as hemolytic uremic syndrome (6,7). STEC is the sole *E. coli* subtype of zoonotic origin, with cattle being a major reservoir (8). Ingestion of contaminated, undercooked beef that does not reach an internal temperature of 71.1°C as recommended by Health Canada (9) and the United States Department of Agriculture Food Safety and Inspection Service (USDA FSIS) (10) remains a significant cause of STEC-associated outbreaks (11,12). In 2012, an *E. coli* O157:H7 outbreak originating from contaminated, tenderized steak resulted in the largest recall of beef products in Canada, illustrating the devastating social and economic burden of STEC (13).

Non-pathogenic environmental strains of heat resistant *E. coli* surviving inactivation procedures at 71°C have been identified (14,15); if pathogenic strains were to acquire this trait, they may become a new source of human STEC infection. Heat resistance is attributed to a 14 kb genomic island termed the locus of heat resistance (LHR), which contains 16 open reading frames (ORFs) predicted to encode proteins associated with heat shock, cell envelope maintenance, and turnover of misfolded proteins (16). Current methods for identification of the LHR include phenotypic and genetic assays. Phenotypic determination of the LHR typically involves exposure

of bacterial cultures to temperatures of 60°C and above for select time intervals followed by spread plating of heated cultures onto solid agar media. Subsequent enumeration of colonies that grow after overnight incubation facilitates identification of heat resistant strains. Phenotypic heat resistance screening can be achieved through the use of water baths (14) and hot plates (17). Current methods identifying the LHR through genetic analysis include whole genome sequencing and end point PCR (16).

The identification of heat resistant pathogenic *E. coli* could pose a threat to food safety and raises the question of its contribution to human infection. However, a rapid and robust genetic method for identification of heat resistant *E. coli* has yet to be developed. A qPCR assay for detection of the LHR in *E. coli* would be a beneficial tool for use in microbial interventions and foodborne outbreak investigations in food safety and public health sectors, respectively. In this chapter, the performance of 3 qPCR assays using hydrolysis probes developed to identify heat resistant clinical *E. coli* isolates were evaluated.

2.2. Materials and Methods

2.2.1. Bacterial isolates and genomic DNA extraction*

A total of 613 clinical *E. coli* isolates consisting of O157 strains (n=574) and non-O157 strains (n=39) (18) collected between 2009 through 2014 in Alberta were included in this research project. To validate the qPCR assays, *E. coli* isolate AW1.7 was grown on sheep blood agar plates (BAP) (Dalynn Biologicals, Calgary, AB, Canada) from frozen skim milk stock and its DNA was extracted using the MagaZorb DNA mini-prep kit (Promega Corporation, Madison, WI, USA).

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* *E. coli* isolate AW1.7 supplied by Dr. Lynn McMullen and Dr. Michael Gänzle.

The KingFisher mL Purification System (Thermo Fisher Scientific, Waltham, MA, USA) was used to automate the procedure. In brief, colonies were swept from the BAP using a sterile cotton swab and inoculated into 12 mmol/L Tris to establish a cell suspension at an optical density (OD) at 600 nm of 0.5 (Microscan Turbidity Meter, Siemens, Oakville, ON, Canada). A 500 µL aliquot of the cell suspension was washed once by centrifugation for 2 minutes at 13,000 × g and re-suspended in 200 µL of 12 mmol/L Tris. Twenty µL of PK solution and 200 µL of lysis buffer were added to the cell pellet and the mixture was incubated at 60°C for 10 minutes on a heat block (VWR Scientific Standard Heatblock; VWR International, Edmonton, AB, Canada). Binding, washing, and elution steps were completed by the KingFisher mL Purification System. The lysed sample was added to 500 µL of binding buffer and 20 µL of MagaZorb reagent in a KingFisher tube. After binding, the sample was washed (2x) with 1 mL of wash buffer and 200 µL of genomic DNA was eluted with 10 mmol/L Tris. All DNA extracts of *E. coli* AW1.7 were quantified with the Qubit 4 Fluorometer (Invitrogen, Burlington, ON, Canada). DNA extracts for screening of clinical *E. coli* isolates were obtained by inoculating a single colony grown on BAP into 200 µL rapid lysis buffer (100 mmol/l NaCl; 10 mmol/l Tris-HCl, pH 8.3; 1 mmol/l EDTA, pH 9.0; 1% Triton X-100), which was boiled for 15 minutes and then centrifuged (13,000 × g) for 15 minutes (19). The supernatant was used as the DNA template.

2.2.2. qPCR assay design for detection of the LHR

Primers and probes were designed against the LHR identified in heat resistant *E. coli* AW1.7, whole genome shotgun sequence LDYJ00000000 (14.673 kb in size) using the Integrated DNA Technologies PrimerQuest Tool. Horizontal transfer of the LHR via adjacent putative transposases has been suggested as the most likely method of transmission (16); hence, ORFs 3, 8, and 11 were chosen as amplification targets to facilitate coverage across the whole locus. Targets and sequences

of each primer and probe are indicated in Table 2.1. Five µL of template DNA was added to a PCR cocktail consisting of 1x TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA), 5 µmol/L of primers and probe, and PCR grade water for a final volume of 20 µL. *E. coli* AW1.7 and water were included as positive and no template negative controls, respectively, for all assays. PCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, Burlington, ON, Canada) with the following amplification conditions: 95°C for 20 seconds, 40 cycles of 95°C for 3 seconds, and 60°C for 30 seconds.

Table 2.1. Oligonucleotides used in qPCR assays for detection of the locus of heat resistance

Target	Forward primer, reverse primer, and probe sequence*	Coordinates	Amplicon size (bp)
ORF 3	F: 5'-CCATTCTTATGTCGGTCCAGAG-3'	2035-2056	128
	R: 5'-CCACCTTGCTGACCTGTT-3'	2145-2162	
	P: 5'-[6-FAM]-ATTTCCTGA-[ZEN]-TTGGTCTGGCCGAGG-[IABkFQ]-3'	2058-2081	
ORF 8	F: 5'-TCGGTAAAGAAAGCGGTCAAG-3'	6316-6336	123
	R: 5'-CATCGGAAGGTTGTCGGTTT-3'	6419-6438	
	P: 5'-[6-FAM]-TTGTCTCGG-[ZEN]-GAGTTGTTGGGTGAT-[IABkFQ]-3'	6337-6360	
ORF 11	F: 5'-GAAGCGATTGTCCGAGCTAAG-3'	9137-9157	112
	R: 5'-TGCTTGCCACTTCGTTATCC-3'	9229-9248	
	P: 5'-[FAM]-TTTGAAGCA-[ZEN]-TCTGTTCGCCGTCCT-[IABkFQ]-3'	9205-9228	

* 6-FAM, 5' 6-carboxyfluorescein; ZEN, ZEN™ Internal Quencher; IABkFQ, 3' Iowa Black® Dark FQ.

2.2.3. Evaluation of qPCR assays as per MIQE guidelines

Each of the 3 qPCR assays were validated according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (20). Analytical sensitivity expressed as the limit of detection (LOD), calibration curves, and PCR efficiencies were determined for all assays with *E. coli* AW1.7. The LOD for each assay was determined using extracted DNA from 10-fold cell dilutions of *E. coli* AW1.7. The neat concentration was established with a cell suspension adjusted to an OD of 0.5 at 600 nm (Microscan Turbidity Meter, Siemens, Oakville, ON, Canada) using 12 mmol/L Tris. A 100 µL aliquot of each dilution was simultaneously plated onto Luria Bertani (LB) plates for colony enumeration following overnight incubation at 37°C. The LOD was considered to be the minimum number of CFU corresponding to 3 consecutive positive amplification results by each of the PCR assays. To generate the calibration curves for each qPCR assay and calculate their respective PCR efficiencies, the copy number for *E. coli* AW1.7 was determined using the following formula:

$$\frac{[amount\ of\ DNA\ (ng)] \times (6.0221 \times 10^{23} molecules/mole)}{(length\ of\ dsDNA\ amplicon \times 660\ g/mole) \times (1 \times 10^{9}\ ng/g)} = copy\ number$$

After determining the copy number for the neat concentration of *E. coli* AW1.7 DNA, the extract was serially diluted 10-fold with 10 mmol/L Tris to obtain dilutions ranging from 10^7 to 10^1 copy number/reaction. The mean Cq values obtained for each dilution were plotted against copy number/reaction to generate calibration curves and calculate the PCR efficiencies. Calculation of LOD and PCR efficiencies were tested in triplicate over 3 different runs for each assay. Specificity of the assays was also determined by testing each qPCR assay against a panel of Gram positive and Gram negative organisms used for validation of all in-house developed genetic assays (Table

2.2). DNA extracts of each organism in the specificity panel were tested for the presence of LHR with the qPCR assays in triplicate.

Table 2.2. Specificity panel tested with qPCR assays detecting the locus of heat resistance

Gram positive	Gram negative
Staphylococcus aureus (ATCC 25923)	*Proteus mirabilis* (ATCC 43071)
Staphylococcus epidermidis (ATCC 49134)	*Proteus vulgaris* (ATCC 13315)
Staphylococcus saprophyticus (ATCC BAA-750)	*Yersinia enterocolitica* (ATCC 9610)
Micrococcus luteus (ATCC 49732)	*Salmonella enterica* ser. Typhimurium (ATCC 14028)
Enterococcus faecalis (ATCC 29212)	*Serratia marcescens* (ATCC 8100)
	Shigella sonnei (clinical isolate A79)
	Klebsiella pneumoniae (ATCC 13883)
	Enterobacter cloacae (ATCC 13047)
	Pseudomonas aeruginosa (ATCC 27853)

2.2.4. Detection of the LHR in clinical *E. coli* isolates

Following validation of the qPCR assays with *E. coli* AW1.7, DNA extracts of the 613 clinical *E. coli* isolates were screened for the LHR with the qPCR assays. A sample was considered a true positive if it produced amplification curves for all 3 targets. Each sample was tested in triplicates on 3 different runs. A true negative was defined as yielding no amplification for each target.

2.2.5. Screening for phenotypic heat resistance*

All *E. coli* isolates that tested positive with the qPCR assays were screened for phenotypic heat resistance through exposure to 60°C heat shock using a water bath (Thermo Haake DC10-W19/B; Thermo Fisher Scientific, Waltham, MA, USA). Two 200 µL aliquots of overnight culture grown in LB broth were washed and re-suspended in 60°C pre-heated LB broth. Aliquots were heated for 90 minutes and 120 minutes and then chilled for 2 minutes in an ice-water bath. A 100 µL aliquot was plated onto LB plates for overnight incubation at 37°C. Isolates with ≥50 colonies on LB plates proceeded to screening at 71°C heat shock using a similar protocol: 200 µL aliquots were retrieved every minute from the water bath over a 5 minute incubation period and chilled before plating. Unheated controls were included in both experiments. *E. coli* AW1.7 was used as the positive control.

* Alan Poon, Raisa Kassam, and Dr. Heather Glassman tested a proportion of *E. coli* isolates for phenotypic heat resistance.

2.3. Results

2.3.1. Validation of qPCR assays for the LHR

Calibration curves and PCR efficiencies calculated from the slopes of each curve were determined for each of the qPCR assays (Figure 2.1). From the calibration curves, coefficients of determination (R^2) and amplification efficiencies for ORFs 3, 8 and 11 were 0.998 and 0.80, 0.999 and 1.04, and 1.000 and 1.07, respectively. From the lowest serial dilution of the *E. coli* AW1.7 cell suspension that corresponded to positive amplification in triplicate over 3 days, the LOD for all assays was calculated to be as low as 3 CFU/reaction. Successful amplification was also observed with single colony genomic DNA extraction.

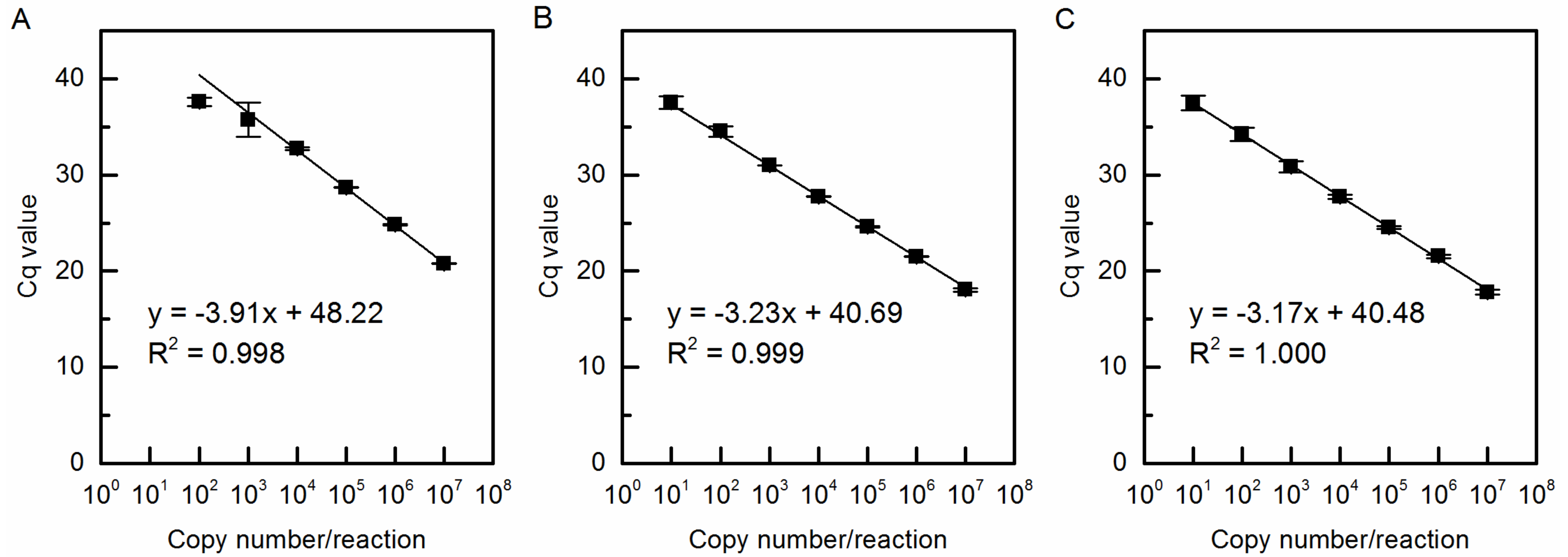

Figure 2.1. Calibration curves of *Escherichia coli* AW1.7 for qPCR assays targeting ORFs 3 (A), 8 (B), and 11 (C) of the locus of heat resistance.

Testing of the qPCR assays against the specificity panel revealed no amplification in all Gram positive organisms. In *Enterobacter cloacae* ATCC 13047, amplification for ORF 3 was detected but no amplification curves were observed for the qPCR assays targeting ORFs 8 and 11. Presence of the amplicon sequence generated by the qPCR assay for ORF 3 in *E. cloacae* ATCC 13047 (NCBI reference sequence: NC_014121.1) was confirmed with the NCBI Blast Server with a coverage of 100% and 98.44% identity. No matches were obtained for the primer and probe sequences of ORFs 8 and 11 against the *E. cloacae* ATCC 13047 genome when searched with the NCBI Blast server. When queried against the entire LHR sequence of *E. coli* AW1.7 from which the qPCR assays were designed, 30% coverage with 97.46% identity in *E. cloacae* ATCC 13047 genome was indicated. qPCR assays for ORFs 8 and 11 also produced amplification in *Pseudomonas aeruginosa* ATCC 27853. No amplification curve was detected for the qPCR assay targeting ORF 3. Again, coverage for the amplicon sequences and the LHR in the *P. aeruginosa* ATCC 27853 genome (NCBI Reference Sequence: NZ_CP011857.1) was confirmed with the NCBI Blast Server. No matches were obtained for the ORF 3 primer and probe sequences. However, 100% coverage and 100% identity for the ORF 8 amplicon sequence and 100% coverage and 99.11% identity for the ORF 11 amplicon sequence were reported. Interestingly, when queried against the LHR sequence of *E. coli* AW1.7, a coverage of 96% and 95.32% identity for *P. aeruginosa* ATCC 27853 was reported. All other Gram negative organisms in the specificity panel failed to amplify with the qPCR assays.

2.3.2. Heat resistant clinical *E. coli* isolates

Of the 613 clinical *E. coli* isolates tested with the developed qPCR assays, 3 (0.5%) were positive for the presence of the LHR (isolate accession numbers of 111, 128, and 8354). Isolates 111 and 128 also possessed virulence factors reflective of STEC including Shiga toxins and the

eae gene, which encodes for the intimin protein. LHR-positive isolates survived both 90 minutes and 120 minutes heat exposure at 60°C as indicated by growth on the LB plates. Phenotypic heat resistance in LHR-positive isolates was also observed in the isolates that advanced to screening at 71°C heat exposure; Isolates AW1.7 and 111 were recovered after 3 minutes of heat treatment at 71°C but isolates 128 and 8354 only survived up to 2 minutes with no growth on the LB plates at the 4 and 5 minute time intervals. All remaining isolates that were negative for the qPCR assays also did not survive heat resistance screening at the 2 temperatures, supporting a sensitivity and specificity of 100% for detection of heat resistant *E. coli*.

2.4. Discussion

The qPCR assays described in this chapter are a useful screening method for rapid detection of heat resistant *E. coli* and the protocol can be adopted for use in food and environmental laboratories equipped for nucleic acid testing. Current genetic methods for identification of heat resistant *E. coli* utilize end-point PCR and whole genome sequencing. However, there are limitations to these approaches. End-point PCR requires agarose gel electrophoresis for analysis (16), thus increasing turn-around time and requiring more manual labor as compared to the qPCR assays. Advantages of qPCR over end-point PCR also include greater sensitivity through the use of fluorescence-based detection and the ability to discriminate gene copies across a wider dynamic range (21). The qPCR assays I developed in this study utilized hydrolysis probes, but it is also possible to adapt the assays to use fluorescent dyes such as SYBR Green I, as illustrated by Yang *et al*. (22). SYBR Green I binds to the minor groove of dsDNA and emits fluorescence 1000× greater than when it is free in solution (23). As the amount of dsDNA increases as a result of

amplification within the reaction tube, the fluorescent signal generated from SYBR Green I directly increases (24). Substitution of SYBR Green I in the assays offers advantages including cost benefits since probe design and synthesis are not required and minimal modifications to the existing amplification conditions. The main limitation to consider before use of SYBR Green I in qPCR assays is the potential for nonspecific amplification products such as primer-dimers falsely elevating the fluorescence detected in the reaction (25–27). Thus, it is imperative that highly specific primers are designed to their target sequences. A post-PCR dissociation curve should be analyzed when SYBR Green I is utilized in the assay to confirm that the fluorescence generated is a result of the intended target amplification and not from nonspecific amplification products. Furthermore, extensive validation of assays employing SYBR Green I is typically required to account for mutations in the target sequence that may alter the temperature of the dissociation curve. Even with as low as a single nucleotide variation in the amplified region, the melting temperature of the sequence can shift by 1.5°C ± 0.5°C, thus producing a different dissociation curve from the wild type (28). Should laboratories choose to use SYBR Green I in their qPCR assays, sequence mutations and their effects on the dissociation curve need to be evaluated prior to implementation of the assays in order to prevent potential false negative results. In regards to using whole genome sequencing in a microbiological setting, this technology offers numerous advantages including high throughput and large data acquisition for genetic analyses such as detection of the LHR (29). However, there are limitations to consider with the adoption of whole genome sequencing. Not all laboratories are equipped with expensive instrumentation, technical expertise, data storage, and bioinformatics for analysis and interpretation.

Adoption of the qPCR assays as a screening method for detection of the LHR prior to phenotypic determination of heat resistant *E. coli* offers benefits in cost and turn-around time.

Strong evidence supports heat resistance at temperatures of 60°C and above is linked to the presence of the LHR (16,30). Thus, firstly screening isolates of interest for the LHR followed by phenotypic confirmation of heat resistance in LHR-positive isolates would be the most logical approach. Despite the cost of the instrumentation required for qPCR being higher than for phenotypic testing, the turn-around time and manual labor required is significantly reduced. Furthermore, substitution of hydrolysis probes in favor of SYBR Green I would additionally reduce costs associated with materials (24,31). If isolates were to all be phenotypically tested for heat resistance as opposed to screened by qPCR, the turn-around time for results would require 3 days opposed to 2 hours.

The focus of this thesis is on the characterization of heat resistant *E. coli* and accordingly, the qPCR assays I developed were validated against *E. coli* and non-*E. coli* strains. However, LHR sequences have also been identified in other Gram negative organisms including *Yersinia enterocolitica*, *Cronobacter sakazakii*, *E. cloacae*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *P. aeruginosa* (32–34). Among the organisms in the specificity panel that the qPCR assays were tested against, amplification was detected in *E. cloacae* ATCC 13047 and *P. aeruginosa* ATCC 27853. Out of the 3 targets, no amplification of ORF 3 was detected in *P. aeruginosa* ATCC 27853 although 96% coverage and 95.32% identity for the LHR was reported. Sequence variations in the LHR in different species may account for the lack of amplification in the qPCR assay targeting ORF 3. Phylogenetic analysis for the LHR by Boll *et al*. with a heat resistant *E. coli* strain isolated from Swiss raw milk cheese (35) also confirmed the presence of the LHR in *P. aeruginosa* ATCC 27853 among other *Pseudomonas* spp. (36). Compared to the 96% coverage of the LHR in *P. aeruginosa* ATCC 27853, the 30% coverage of the LHR in the genome of *E. cloacae* ATCC 13047 was significantly lower, suggesting that amplification by the qPCR assay for ORF 3 but not

for ORFs 8 and 11 may be a result of genetic similarity in the target sequence as opposed to partial acquisition of the LHR by horizontal transfer, from which can be elucidated by genomic analysis. Additional studies to determine if *E. cloacae* ATCC 13047 and *P. aeruginosa* ATCC 27853 are phenotypically heat resistant would also provide further explanations to the data presented in this chapter. In agreement with the results presented in this chapter, the LHR has not been identified in Gram-positive organisms by other researchers in the field.

From the collection of clinical pathogenic *E. coli* isolates included in this study, only a small proportion were identified to possess the LHR. However, heat resistant *E. coli* has the potential to be exceptionally problematic in non-intact and mechanically tenderized meats where surface contaminants can be inoculated deep into internal tissue, as demonstrated in previous outbreaks associated with tenderized beef (13,37). Cooking methods such as sous-vide cooking where the temperature does not exceed 60°C also present an optimal environment for heat resistant *E. coli* to remain in viable numbers sufficient to cause disease. The qPCR assays discussed in this chapter can be a valuable tool for human foodborne illness investigations where identification of heat resistant *E. coli* can uncover new sources of infection.

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Chapter 3

Characterization of *Escherichia coli* possessing the locus of heat resistance isolated from human cases of acute gastroenteritis*

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3.1. Introduction

Shiga-toxin producing *Escherichia coli* (STEC) accounts for a significant proportion of foodborne diseases in Canada. Symptoms of STEC infection include diarrhea and hemorrhagic colitis (1), which can potentially escalate to hemolytic uremic syndrome (HUS). HUS is characterized by renal insufficiency, microangiopathic hemolytic anemia, and thrombocytopenia (2). Outbreaks associated with *E. coli* serogroup O157 are most commonly reported and are associated with a variety of food products including fresh fruits and vegetables, dairy products, and meat (3). Cattle are a major reservoir of STEC (4) and frequently implicated in foodborne disease as a result of consumption of undercooked, contaminated beef (5). In 2012, an outbreak linked to mechanically tenderized beef contaminated with *E. coli* O157:H7 originating from Alberta, Canada became the largest food recall in Canadian history (6). All clinical cases in this outbreak were due to consumption of undercooked, contaminated beef. As a result of the outbreak, public confidence on the reliability of food safety practices weakened and the cattle industry similarly suffered significant economic setbacks (6).

The locus of heat resistance (LHR) may confer exceptional heat resistance to members of the *Enterobacteriaceae* family, with many being foodborne pathogens (7). Comprised of 16 open reading frames (ORF), putative functions of the LHR include protein homeostasis related to heat shock, cell envelope maintenance, and protection against oxidative stress (8). ORF 3 of the LHR encodes for a novel Clp ATPase, ClpK, and is the main contributor to the observed heat resistance in LHR-positive Gram negative bacteria (9). The LHR is transferrable via putative transposases that flank the operon (8) and has been identified on plasmids and in the chromosome. Heat resistant *E. coli* possessing LHR-encoding plasmids also carry genes for multi-drug resistance and biofilm production (10,11) and threatens the potential emergence of multi-stress resistant *E. coli*. LHR-

positive strains of *E. coli* identified to date are capable of withstanding heat exposure at 60°C for 30 minutes with minimal cell reduction (12–14). In most reports of heat resistant *E. coli*, strains are of food or environmental origin and lack the Shiga toxin genes and the locus of enterocyte effacement, which are virulence factors that play a major role in causing foodborne disease (13,15,16).

In the previous chapter, 613 clinical *E. coli* isolates were screened for the presence of the LHR and 3 were identified (17). All of the isolates survived phenotypic heat resistance screening at 60°C for 120 minutes. As the first report of LHR-positive *E. coli* related to human illness, it highlights the potential for pathogenic, heat resistant *E. coli* in the food processing industry and public health. The objective of this part of the study was to characterize the clinical LHR-positive isolates previously identified for their heat resistance at 60°C and 71°C in both broth medium and ground beef, and to investigate protein expression by the LHR.

3.2. Materials and Methods

3.2.1. Isolate identification and bacterial growth conditions

Three heat resistant, clinical *E. coli* isolates with accession numbers 111, 128, and 8354, from a collection of 613 isolates were identified by qPCR and 60°C heat shock as previously described in Chapter 2 (17). For all experiments, heat resistant, environmental isolate AW1.7 and heat sensitive, clinical isolate 126 served as positive and negative controls, respectively. All isolates were retrieved from frozen skim milk stocks and streaked onto sheep blood agar plates (BAP) (Dalynn Biologicals, Calgary, AB, Canada) and incubated for 24 hours at 37°C. Single colonies from the BAP were inoculated into 10 mL of Luria Bertani (LB) broth (Becton Dickinson,

Mississauga, ON, Canada) and incubated at 37°C for 24 hours with agitation at 225 rpm (MaxQ 2506 Reciprocating Shaker; Thermo Fisher Scientific, Waltham, MA, USA) prior to all experiments. For experiments to determine the decimal reduction times (D-values) at 60°C and 71°C and protein mass fingerprinting of each isolate, LB broth was supplemented with an additional 2% (20 g/L) and 4% (40 g/L) NaCl (EMD Chemicals Inc., Gibbstown, NJ, USA).

3.2.2. Heat resistance in LB broth

Survival curves and D-values at 60°C and 71°C (D_{60}- and D_{71}-values) were determined for each isolate in LB broths containing 0, 2, and 4% additional NaCl. Stationary phase cultures were first adjusted to an optical density (OD) of 0.5 at 600 nm (Microscan Turbidity Meter, Siemens, Oakville, ON, Canada). For both temperatures, 200 µL aliquots of culture were washed and re-suspended in preheated LB broth (60°C or 71°C) prior to being immediately placed in a water bath for each specific sampling time. Aliquots were removed at various intervals over a 30 minute incubation at 60°C and a 5 minute incubation at 71°C, respectively. Following heat treatment, aliquots were chilled in ice water for 2 minutes and serially diluted 1/10 for spread plating on LB plates (Becton Dickinson, Mississauga, ON, Canada) in triplicates. Resultant colonies after 24 hours incubation at 37°C were enumerated and D-values were calculated from the slope of linear regression of semi-logarithmic plots.

3.2.3. Light and transmission electron microscopy analyses[*]

Isolates AW1.7, 126, 111, 128, and 8354 were cultured overnight in LB broth and aliquoted into 1 mL samples. Samples were washed and re-suspended in preheated LB broth and exposed to heat treatment at 60°C for 60 minutes. Methanol-fixed smears of each isolate were made and Gram

[*] Arlene Oatway at the University of Alberta Advanced Microscopy Facility sectioned and provided imaging assistance of transmission electron microscopy samples.

stained. Isolates for transmission electron microscopy (TEM) analysis were similarly heated for 60 minutes at 60°C with the adjustment of aliquoting the 1 mL samples in triplicates. Unheated controls were also prepared for each isolate in the same manner. Triplicates were pooled together to generate cell pellets and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde overnight. Samples were then washed with 0.1 mol/L phosphate buffer for 10 minutes (3x) and post-fixed with 1% osmium tetroxide for 1 hour. All samples were subjected to a subsequent buffer wash (3x) and dehydrated through a graded ethanol series (50%, 70%, 90%, and 100%). To ensure removal of all water, two 100% ethanol washes were included before suspending the pellet in a 1:1 ratio of ethanol and Spurr low viscosity resin for 2 h. Following incubation, samples were pelleted and re-suspended with 100% Spurr resin for 2 hours (3x). Lastly, samples were pelleted and transferred to BEEM capsules for curing of Spurr resin in a 70°C oven overnight. Ultra-thin sections were cut using the Ultracut E Reichert Jung Ultramicrotome and stained with uranyl acetate and lead citrate. Imaging was conducted using the Morgagni 268 transmission electron microscope (Thermo Fisher Scientific FEI, Hillsboro, OR, USA) with a Gatan Orius CCD Camera and Gatan DigitalMicrograph software (version 1.81.78). All TEM processing and imaging was conducted at the University of Alberta Advanced Microscopy Facility.

3.2.4. Heat resistance in ground beef*

Lean (15% fat) ground beef obtained from a local processing plant was divided into 200 g portions and stored at -20°C until used in grilling experiments. The aerobic plate count (APC) of un-inoculated ground beef was first determined by removing 25 g and mixing it with 225 mL of 1% buffered peptone water (BD, Mississauga, ON, Canada) in a sterile Whirl-Pak homogenizer blender filter bag (Nasco, Fort Atkinson, WI, USA) using a Stomacher Lab-Blender 400 (Seward,

* Laboratory facilities and equipment for grilling experiments were provided by Dr. Lynn McMullen.

Brinkman Instruments, Worthing UK). From the liquid portion of the meat slurry, 100 µL was plated onto Standard Methods Agar plates (SMA) (Dalynn Biologicals, Calgary, AB, Canada) and chromogenic UriSelect4 (Bio-Rad Laboratories, Mississauga, ON, Canada) plates. Seven mL of overnight culture (adjusted to 0.5 OD at 600 nm for a concentration of 8 log CFU/mL) was inoculated into the remaining 175 g ground beef and the mixture was massaged for 2 minutes for even distribution. From this mixture, a 25 g portion was removed to determine the bacterial cell count before grilling by mixing it with 225 mL of modified tryptic soy broth (mTSB) (Dalynn Biologicals, Calgary, AB, Canada) and EHEC supplement (Dalynn Biologicals, Calgary, AB, Canada) in the stomacher. The slurry was serially diluted 1/10 and 100 µL of the appropriate dilution was plated onto UriSelect4 plates. The remaining 150 g ground beef was formed into a patty with a diameter of 11.5 mm using a Single Hamburger Press (Weston Brand Pragotrade, Strongsville, OH, USA) and cooked on a preheated grill (Cuisinart, Woodbridge, ON, Canada) until the geometric center reached the temperature of 60°C. The internal temperature of the patty was monitored using a Tinytag View 2 Data Logger (Gemini Data Loggers, West Vancouver, BC, Canada). Upon reaching the target temperature, the patty was removed and sectioned into four 25 g samples. Each sample was mixed with 225 mL of iced mTSB and EHEC supplement in the stomacher bag and 1/10 serial dilutions were spread onto UriSelect4 plates. All samples were plated in triplicates and incubated for 24 hours at 37°C for cell counts. Experiments were repeated for all isolates with the internal target temperature of the patties increased to 71°C.

3.2.5. Protein mass fingerprinting of heat resistant isolates

Protein gel electrophoresis was conducted on each isolate using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Isolates were first cultured in LB broths of 0, 2, and 4% NaCl and then adjusted to 0.5 OD at 600 nm. From each culture, 1 mL was aliquoted into microfuge tubes in triplicates. Two sets of the aliquots were exposed to heat treatment in a water bath at 60°C and 37°C for 60 minutes, respectively. The remaining set of aliquots remained at room temperature for the incubation duration as controls. Following heat treatment, the aliquots were washed and re-suspended in 100 µl of sample loading buffer (2x Laemmli Sample Buffer, Bio-Rad Laboratories, Mississauga, ON, Canada; β-mercaptoethanol, Sigma-Aldrich, Oakville, ON, Canada) for denaturation at 95°C for 5 minutes on a heat block (VWR Scientific Standard Heatblock; VWR International, Edmonton, AB, Canada). Denatured samples were pelleted and 2.5 µL of each sample was loaded onto a 4-12% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Mississauga, ON, Canada). Precision Plus Protein Kaleidoscope Standard (Bio-Rad Laboratories, Mississauga, ON, Canada) served as the protein standard. Electrophoresis was performed in Tris-glycine-SDS running buffer (Bio-Rad Laboratories, Mississauga, ON, Canada) at 200 volts for 30 minutes. Empty wells were loaded with 10 µL of 1x sample buffer. Gels were stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories, Mississauga, ON, Canada) according to manufacturer's guidelines. Following staining, protein bands of interest were excised and submitted for in-gel digestion with trypsin and protein mass fingerprinting at the Alberta Proteomics and Mass Spectrometry Facility.

3.2.6. Statistical analysis

The means of at least 3 independent experiments were determined. D-values for each isolate across 0%, 2%, and 4% NaCl concentrations and at each NaCl concentration between

isolates were compared by analysis of variance (ANOVA); *post hoc* pairwise comparisons were conducted using Tukey's honest significance differences (Tukey's HSD) method. Cell counts recovered in uncooked and cooked (60°C and 71°C) patties within isolates were similarly compared by ANOVA with *post hoc* pairwise comparisons using Tukey's HSD method. Cell counts of cooked patties (60°C and 71°C) between clinical isolates and *E. coli* AW1.7 were compared using a Two-Sample t-Test. Statistical calculations were performed using OriginPro 2016 (OriginLab, Northampton, MA, USA). A 95% significance (P = 0.05) was used for all analyses.

3.3. Results

3.3.1. Virulence profiles of *E. coli* isolates

All clinical isolates tested for heat resistance were previously characterized for the presence or absence of STEC virulence factors, including stx_1, stx_2, and *eae*, by qPCR (18). Of the 3 clinical, heat resistant isolates identified, their serotypes and virulence profiles were determined as follows: isolate 111 – serotype ONT:H25 and positive for stx_1 and *eae* and negative for stx_2, isolate 128 – serotype O11:H25 and positive for stx_1 and negative for stx_2 and *eae*, and isolate 8354 – serotype O157:H7 and negative for stx_1, stx_2, and *eae*.

3.3.2. D-values of heat resistant *E. coli*

To evaluate heat resistance in clinical *E. coli* isolates, a preliminary screening was conducted on all isolates upon exposure to 60°C heat treatment (17). Following incubation of isolates for 60 and 120 minute intervals in the water bath, no cell recovery by culture was observed except for heat resistant control AW1.7 and isolates 111, 128, and 8354. Confluent growth on LB plates was seen in all heat resistant isolates up to the 120 minute interval. To determine D-values

of the clinical isolates, survival curves at 60 and 71°C were plotted and compared to heat resistant control *E. coli* isolate AW1.7 and heat sensitive, clinical isolate 126 (Figure 3.1). From the slopes of the linear regressions plotted, D_{60}- and D_{71}-values for each of the isolates in the presence of 0, 2, and 4% NaCl were calculated (Table 3.1). D_{60}-values of heat sensitive control 126 were comparable to those reported in literature (15). All heat resistant isolates possessing the LHR exhibited considerably higher D_{60}-values than the heat sensitive control. The addition of 4% NaCl significantly increased D_{60}-values for clinical isolates 128 and 8354 compared to D_{60}-values at 0% and 2% NaCl but not for clinical isolate 111. There were significant variations in D_{60}-values at each salt concentration between all isolates but no distinguishable trends were observed. At 71°C, heat resistant isolates also possessed elevated D-values compared to the heat sensitive control. D_{71}-values of clinical isolate 128 were significantly increased in the presence of 4% NaCl but were decreased in isolate 8354.

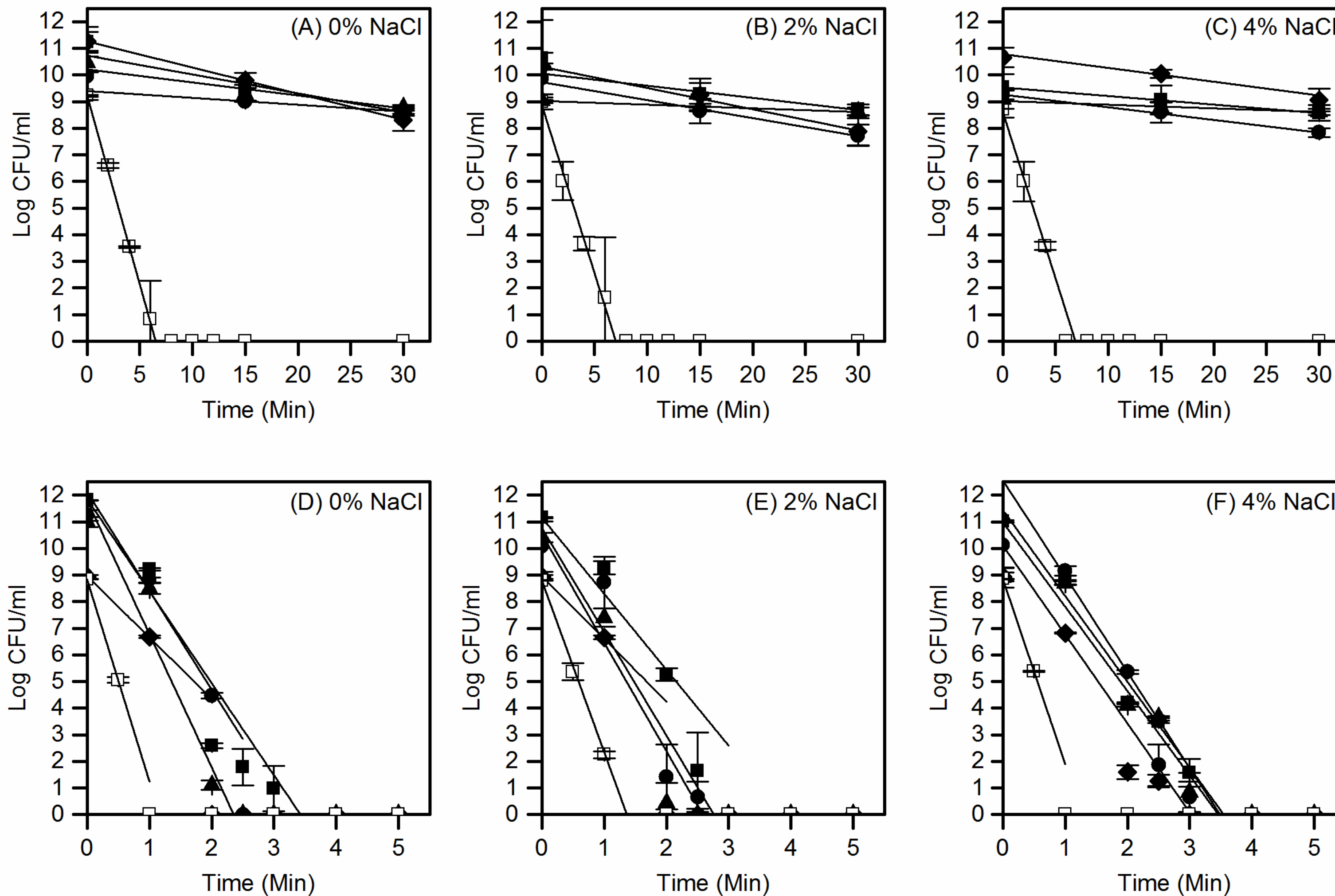

Figure 3.1. Survival curves of *Escherichia coli* isolates exposed to 60°C (A-C) and 71°C (D-F) heat shock in conditions of increasing osmotic stress. D_{60}- and D_{71}-values were calculated from the slope of linear regressions of semi-logarithmic plots using mean ± standard deviation of triplicate experiments. *E. coli* isolates: (■) positive control AW1.7, (●) clinical isolate 111, (▲) clinical isolate 128, (◆) clinical isolate 8354, (□) negative control 126.

Table 3.1. D-values of *Escherichia coli* isolates at 60°C and 71°C

Isolate	D_{60}-value (minutes)			D_{71}-value (minutes)		
	0% NaCl	2% NaCl	4% NaCl	0% NaCl	2% NaCl	4% NaCl
AW1.7	14.07$^{a\ 1}$	21.92$^{a\ 2,3}$	31.04^{5,6}	0.29$^{a\ 1}$	0.35$^{a\ 2}$	0.32$^{a\ 4,5,6}$
126	0.70^{b}	0.79^{b}	0.80^{b}	0.13^{b}	0.16^{b}	0.14^{b}
111	38.73	14.80$^{c\ 2,4}$	21.02$^{c\ 5,7}$	0.27$^{c\ 1}$	0.26$^{c\ 2,3}$	0.28$^{c\ 4,7,8}$
128	20.46	72.08^{d}	72.47^{d}	0.20^{d}	0.25$^{d\ 3}$	0.31^{5,7,9}
8354	10.20$^{e\ 1}$	12.53$^{e\ 3,4}$	19.32 ^{6,7}	0.43^{e}	0.42^{e}	0.30^{6,8,9}

D-values for an isolate between salt concentrations with the same lowercase letters are not significantly different ($P \geq 0.05$). D-values at each salt concentration between isolates with the same numbers are not significantly different ($P \geq 0.05$).

3.3.3. Cellular morphological changes of heat resistant *E. coli* pre- and post- heat treatment

A change in colony morphology of heat resistant isolates incubated at 60°C was also noted on their respective LB plates (Figure 3.2, A to C). Between 0 to 45 minutes of heat exposure, only large colonies were observed on the agar plates; whereas from 45 to 60 minutes, a smaller variant was seen as well. Beyond 60 minutes of heat exposure, only small colonies were found. Gram stains of large and small colonies obtained following heat treatment at 60°C for 0 and 60 minutes revealed no changes in the shape or length of individual bacillus (Figure 3.2, D to F). This shift in colony morphology was similarly detected at 71°C heat exposure, although for a shorter duration of time. Re-inoculation of large and small colonies from 0, 45, and 60 minute time points of heat treatment into fresh LB broth for determination of the LHR by qPCR and heat shock at 60°C was conducted on all isolates. Results from qPCR and heat shock indicated that all subcultures still possessed the LHR and both variants of colonies were again observed between the same time intervals, regardless of the initial colony size that was inoculated. Despite the lack of cellular changes observed by Gram stain, analysis by TEM revealed significant findings in heat-treated cells. Unheated cells displayed smooth surfaces and the presence of a typical electron-dense cytoplasm (Figure 3.3 A). Following heat treatment at 60°C for 60 minutes, heat resistant isolates developed filaments in the mid-region of the cells as presented in Figure 3.3 C to F (indicated by the arrow in C). Cell envelopes of heat resistant isolates showed no disruptions and cytoplasm density and cell size remained unchanged. In contrast to this, cells of heat sensitive control 126 displayed significant wrinkling of the cell envelope (indicated by the uncolored arrow in Figure 3.3 B), filament development, lightening of the cytoplasm, and leakage of cell contents (indicated by the black arrow in Figure 3.3 B). Additionally, cell fragments were abundant when imaging was conducted, indicative of cell death.

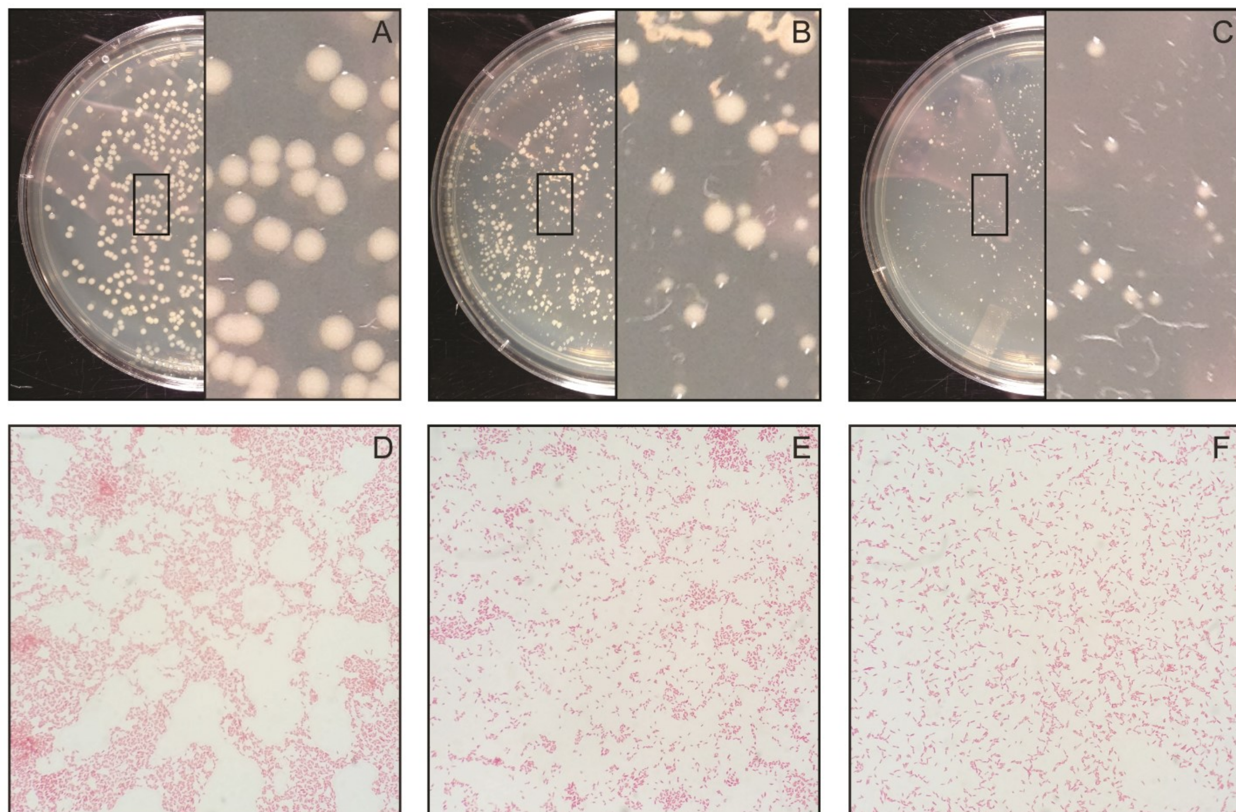

Figure 3.2. Colony morphology of heat resistant *Escherichia coli* clinical isolate 111 on Luria Bertani plates following heat exposure at 60°C for 0 minutes (A), 45 minutes (B), and 60 minutes (C). Gram stains of isolate 111 smeared from large colonies at 0 minutes (D), large colonies at 60 minutes (E), and small colonies at 60 minutes (F) heat exposure.

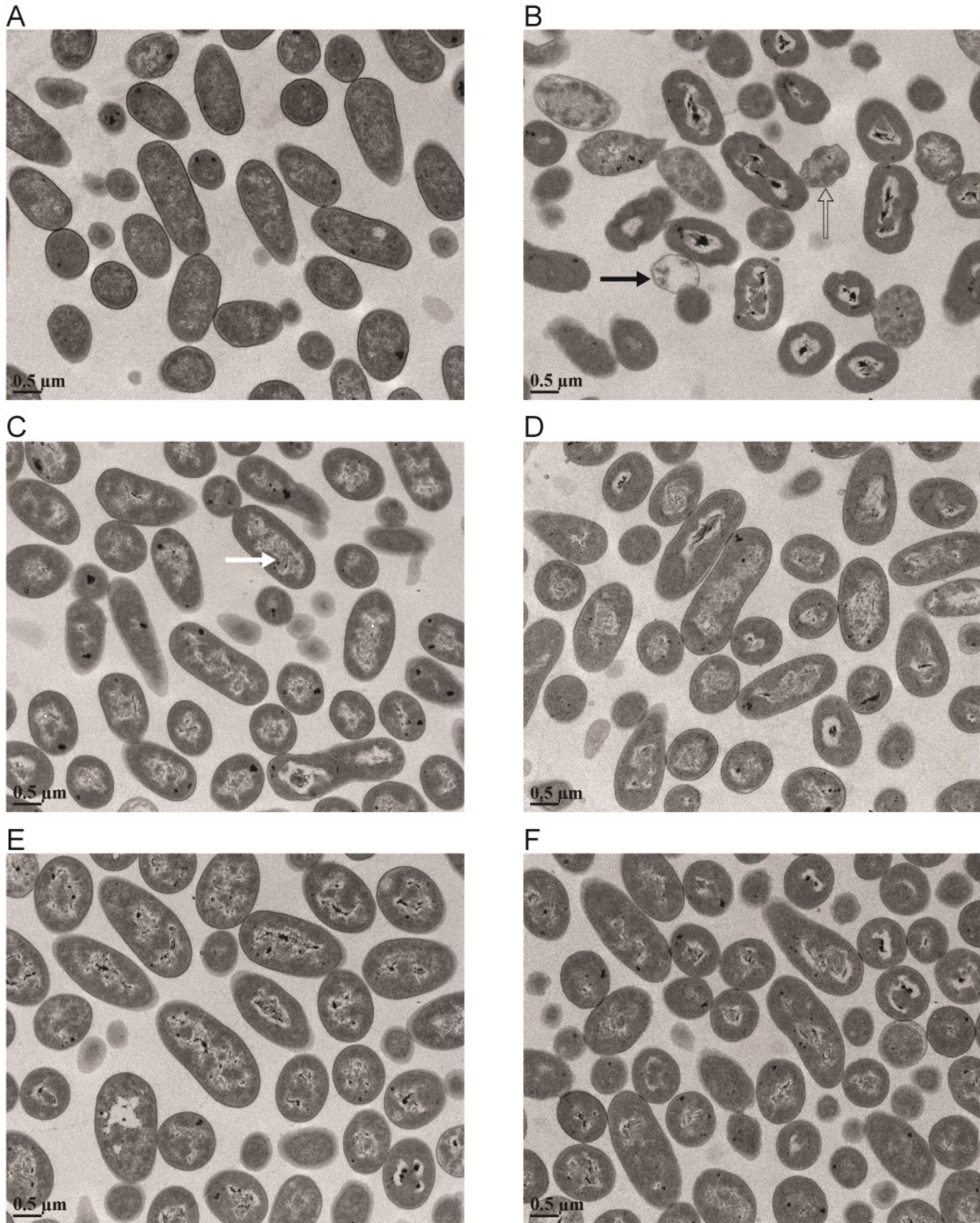

Figure 3.3. Transmission electron micrographs of *Escherichia coli* before (A) and after (B to F) heat exposure at 60°C for 60 minutes. *E. coli* isolates: heat resistant control isolate AW1.7 (A), heat sensitive control isolate 126 (B), AW1.7 (C), 111 (D), 128 (E), and 8354 (F). Uncolored arrow, wrinkling of cell envelope; black arrow, leakage of cell contents; white arrow, filament development.

3.3.4. Survival of heat resistant *E. coli* in ground beef patties after grilling

Heat resistance of isolates in food was investigated by grilling inoculated ground beef patties to an internal temperature of 60°C and 71°C, corresponding to medium rare and well done beef as per Health Canada's internal cooking temperatures guide (19). In Canada, an internal temperature of 71°C is recommended for safe consumption of ground beef. However, lower cooking temperatures for ground beef (medium rare to well done: range of 45 °C to 98.9°C) are accepted internationally and the final cook temperature is not always measured before serving (20). Total cell counts in un-inoculated ground beef were less than 2000 CFU/mL and 900 CFU/mL on SMA and UriSelect4 plates, respectively. No *E. coli*, which would produce pink colonies due to the activity of β-galactosidase on the chromogenic components of the UriSelect4 plates, was identified on UriSelect4 plates in all un-inoculated ground beef samplings. Patties were grilled for an average of 3.26 minutes and 2.86 minutes to reach an internal temperature of 71.14°C and 59.97°C, respectively. Cell counts of heat sensitive control 126 were reduced by greater than 6 orders of magnitude at both temperatures (Figure 3.4) whereas reductions of 2.13-2.84 log CFU/mL and 4.55-4.95 log CFU/mL were obtained for the clinical, heat resistant isolates from patties grilled to 60 and 71°C, respectively. Interestingly, greater cell reductions in the grilled patties were observed for heat resistant control AW1.7 compared to the clinical isolates despite the survival curves in LB broth being similar. Reductions of 4.37 log CFU/mL at 60°C and 5.74 log CFU/mL at 71°C were obtained. No significant difference between grilling temperatures on cell recovery was observed for heat sensitive control 126. On the other hand, survival observed from cell counts after grilling at 60°C was significantly greater than at 71°C for all heat resistant isolates. Mean difference in survival of heat resistant, clinical isolates was significantly greater than AW1.7 at 60°C (111: 2.71 log CFU/mL, 128: 1.78 log CFU/mL, 8354: 1.97 log CFU/mL; $P < 0.0001$ for

all comparisons) and 71°C (111: 1.25 log CFU/mL $P = 0.004$; 128: 1.39 log CFU/mL, $P = 0.002$; 8354: 1.16 log CFU/mL, $P = 0.004$).

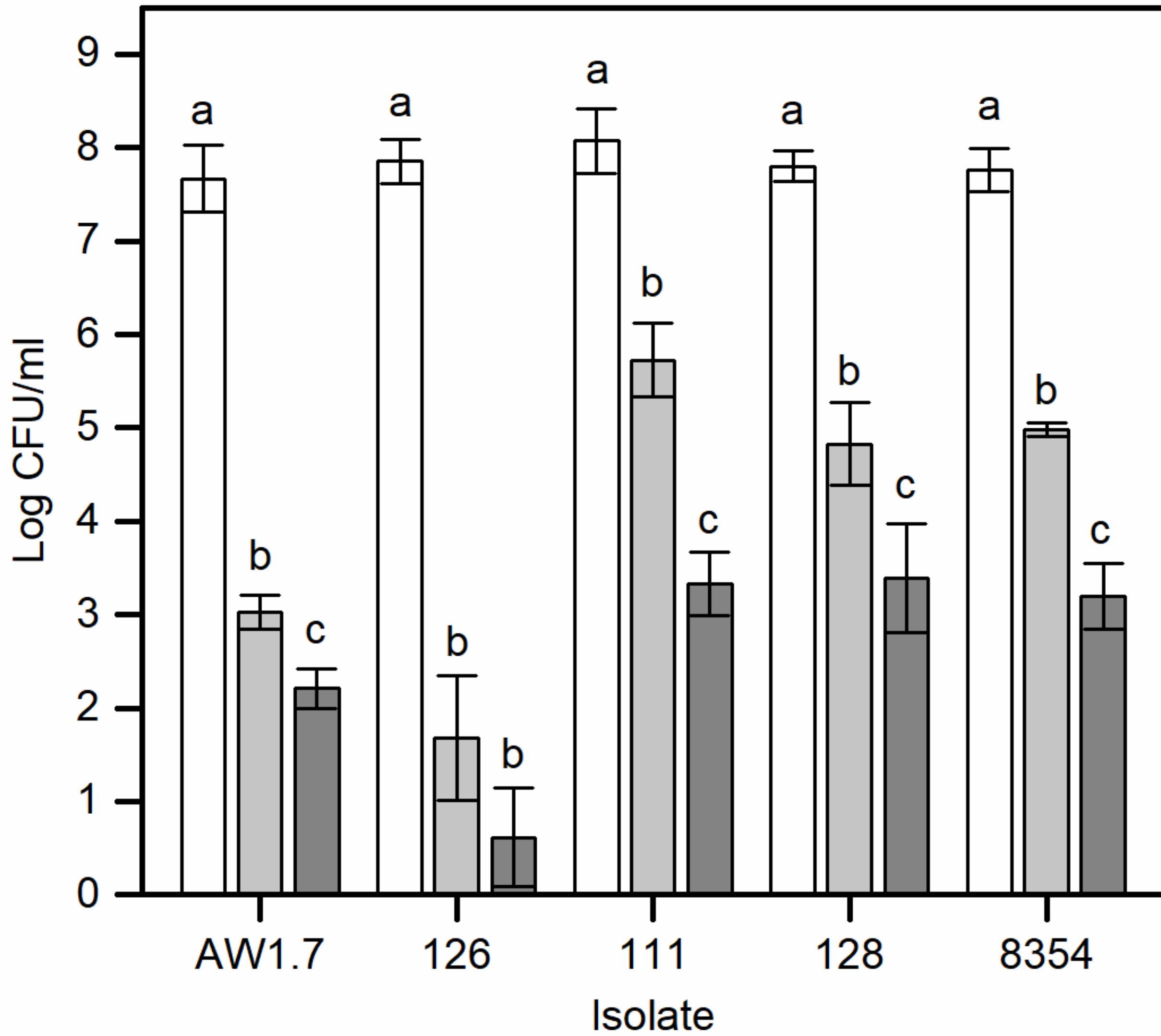

Figure 3.4. Survival of *Escherichia coli* in ground beef patties grilled to an internal temperature of 60 and 71°C. White bars, recovery from uncooked portions; light grey bars, recovery from portions cooked to 60°C; dark grey bars, recovery from portions cooked to 71°C. Means within an isolate with different letters significantly differ ($P < 0.05$).

3.3.5. Characterization of protein expression in heat resistant *E. coli*

To investigate the expression of the LHR in heat resistant *E. coli*, gel electrophoresis using SDS-PAGE and protein mass fingerprinting were conducted. Isolates were cultured in LB broths of 0, 2, and 4% NaCl 24 hours prior to denaturation of whole cell lysates with and without heat treatment to identify if protein expression by the LHR was constitutive or inducible. Protein bands visualized that were exclusive to heat resistant isolates (Figure 3.5) were excised and analyzed by Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF) mass spectrometry. A 99.7 kDa ClpK homolog, composed of 905 amino acids, first identified in heat resistant *Klebsiella pneumoniae* (9) was identified in all heat resistant *E. coli* isolates. Protein gel profiles were identical for each isolate regardless of the presence of NaCl in culture media and heat treatment (Appendix A and B), indicating constitutive expression of ClpK in isolates possessing the LHR.

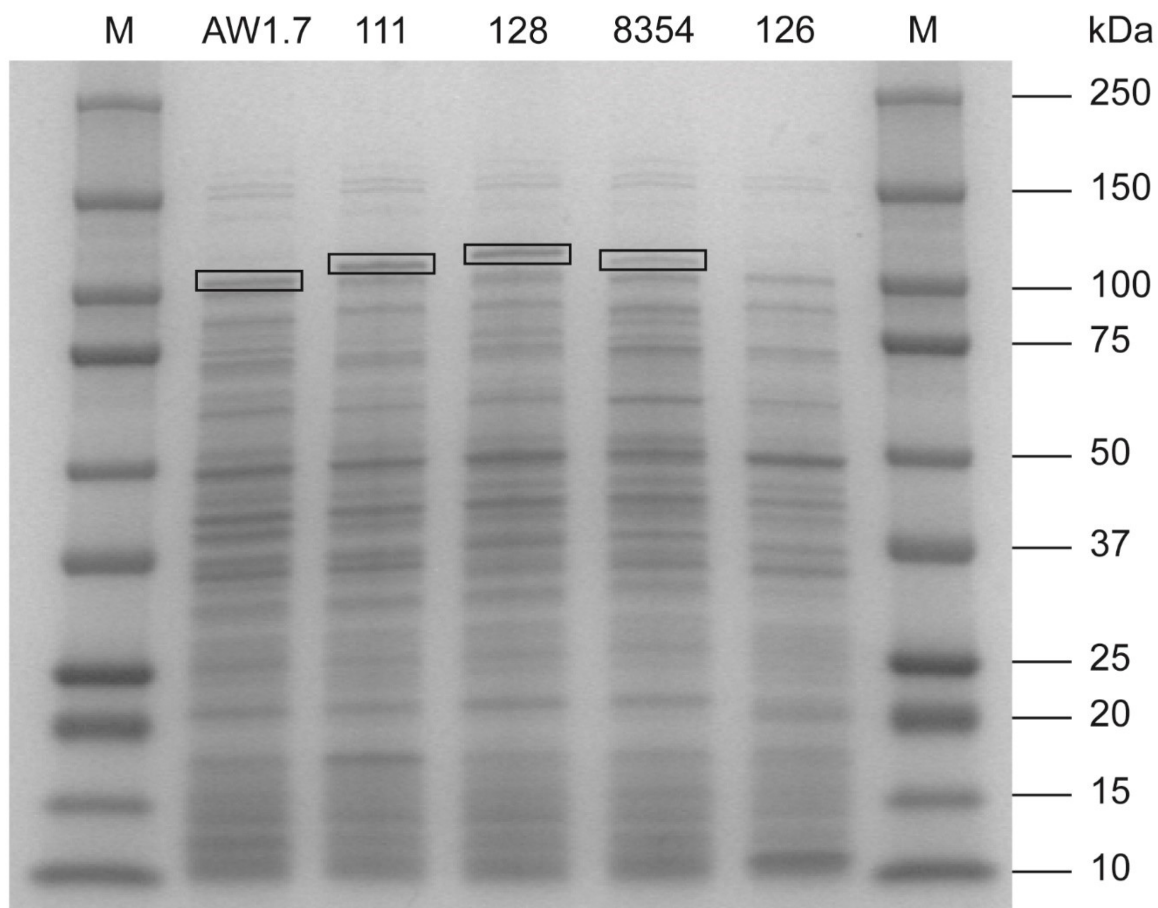

Figure 3.5. sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates of *Escherichia coli* cultured in LB broth with 0% additional NaCl. M, Bio Rad Precision Plus Protein Standard. Black boxes, additional protein band containing the ClpK homolog observed in heat resistant isolates. Indicated bands of interest were excised and digested for mass spectrometry protein identification by the Alberta Proteomics and Mass Spectrometry Facility.

3.4. Discussion

Heat inactivation remains a highly effective method of pathogen reduction and elimination in food processing. The discovery of the LHR and various heat resistant *Enterobacteriaceae* (8-10) stresses the importance that deeper understanding of the mechanisms mediating this heat resistance phenotype and methods for identification of heat resistant pathogens in the farm-to-fork continuum are necessary. Heat resistant *E. coli* have been successfully identified in numerous environmental and food sources including wastewater (21), cattle transport trailers and slaughterhouses (15,22), and cheese (13) by using the LHR as a genetic predictor. Despite the increase in recent literature documenting the identification of heat resistant *E. coli*, the predicted prevalence of the LHR in pathogenic and non-pathogenic *E. coli* remains at 2% (8). Heat resistant, clinical isolates have been scarcely identified with the exception of two isolates described in 2017 by Boll *et al.* (10), one being an ESBL-producer and the other an enteroaggregative *E. coli* strain (13). In this chapter, we characterize the first documentation of heat resistant, clinical *E. coli* isolates obtained from patients experiencing acute gastroenteritis.

Each of the heat resistant, clinical isolates were investigated for their survival in liquid growth media for the determination of D_{60}- and D_{71}-values, and in a food matrix of ground beef, respectively. Significant increases in D_{60}-values were observed in heat resistant isolates compared to the heat sensitive control, in agreement with other heat resistant *Enterobacteriaceae* described in literature. The LHR is predicted to encode for a number of ion-exchangers and proteases that may be involved in the survival of heat resistant cells under conditions of osmotic stress (8). Enhanced survival of LHR-positive isolates in highly osmotic environments has been documented in *E. coli* (23) and *Cronobacter sakazakii* (24). Furthermore, Mercer *et al*. documented increased expression of genes comprising the LHR from isolates cultured in growth medium supplemented

with NaCl (7). In addition to the LHR, the accumulation of compatible solutes such as glycine betaine and proline in heat resistant isolates has been reported to provide thermal stability of ribosomes at high temperatures (23,25). The survival curves and D_{60}-values presented in this study provide further support for the hypothesis of interplay between heat and osmotic stress in LHR-positive isolates and its role in multi-stress resistance. However, it is without a doubt that numerous factors contribute to the enhanced survivability of *E. coli* to heat and osmotic stress, suggesting an in-depth exploration into this topic is necessary to fully understand the involvement of each mechanism.

Survival at 71°C in heat resistant isolates has been largely unexplored. In this study, the D_{71}-values obtained indicate that slight thermal protection is conferred by the LHR but to a far lesser extent than at 60°C. It is likely that at temperatures of 71°C and above, the putative proteins of the LHR involved in turnover of misfolded and denatured proteins can no longer provide the protection offered at 60°C. At both 60 and 71°C, significantly higher D_{60}-values for heat resistant isolate 128 in 2 and 4% NaCl in comparison with the other isolates were observed. We speculate that the addition of an second LHR variant recently reported in a clinical ESBL-producing *E. coli* isolate (10) may be present and functionally active in isolate 128 and thus confers heightened heat resistance. It is possible that highly heat resistant isolates possess 2 copies of the LHR, with one encoded on the chromosome and the other on a plasmid.

Heat resistance of the isolates after 60°C heat shock was unchanged regardless of the duration of heat exposure and colony morphology seen on the LB plates. These observations suggest that the smaller variant growing after 45 minutes of heat exposure was not the emergence of a second population of *E. coli* but the result of cell death and subsequent reduction in colony size as heat treatment progressed. This hypothesis is supported by the fact that only small colonies

grew after 60 minutes heat treatment, indicating that there were not enough viable cells to form large colonies by that point. Gram stains of isolates immediately following heat treatment at 60°C did not display any morphological changes in the cells. However, the level of magnification with light microscopy greatly limits the ability to discern cellular changes, hence the need for TEM analysis. TEM imaging revealed significant changes in heat-treated cells that have not been previously reported. The transition of bacteria to cell wall deficient L-forms in response to external stressors such as lethal heat treatment has been proposed in both Gram-positive and Gram-negative bacteria, including *E. coli* (26). Polymorphic L-forms no longer retain their rigid cell walls, yet are able to continue cell division (27) and are considered an adaptive strategy for bacterial survival (28). Heat resistant isolates characterized in this study did not transition to L-forms and retained their cell walls despite heat treatment for extended periods of time, which may be attributed to the predicted function of ORFs 8 to 10 of the LHR in cell envelope maintenance (8). The presence of filaments in the cytoplasm of heat resistant isolates was the only difference observed between unheated and heat-treated cells. Feliciano *et al*. reported filament development in *E. coli* treated with neutral electrolyzed water and organic acid sanitizers (29) that showed similar cellular morphology as observed in this study. Similarly, incubation of *E. coli* with formic and propionic acids has resulted in the formation of filaments in response to DNA damage (30). Thermal death of *E. coli* is strongly associated with ribosomal melting that occurs between 40°C to 85°C and peaks at 75.1°C (31,32). As a result of irreversible ribosomal unfolding and denaturation, protein synthesis is halted and cell death ultimately occurs. In contrast to this, DNA denaturation peaks at 94.8°C, occurring at temperatures well above those necessary for cell inactivation and is partially reversible up to 125°C (32). In the case of LHR-positive, heat resistant isolates, enhanced heat shock resistance and turnover of misfolded proteins is provided through ORFs 1 to 7 (7) and may

explain the preservation of ribosomal function and subsequent recovery on LB plates that was not observed with heat sensitive control 126. Since the LHR does not provide protection against DNA denaturation, the presence of filaments in heat resistant isolates is not surprising. Further investigations on the findings observed in the transmission electron micrographs and the use of differential scanning calorimetry may provide greater insight on the function of the LHR in heat resistant *E. coli*.

To investigate the threat of heat resistant *E. coli* in food consumption, grilling experiments using lean ground beef spiked with cultures of heat resistant *E. coli* were conducted. As expected, heat resistant isolates survived significantly more than their heat sensitive counterpart. At 71°C, the recommended minimum internal temperature for ground beef according to Health Canada (19), heat resistant isolates were not fully eliminated (cell recovery of 2.21-3.39 log CFU/mL from an initial inoculum of 8 log CFU/mL). Furthermore, cooking ground beef to 71°C is not agreed upon globally. Burger patties grilled to a doneness of medium rare are accepted and on occasion preferred by consumers. Survival of clinical, heat resistant isolates after grilling was observed to be significantly greater than the environmental control isolate AW1.7 at both temperatures, suggesting that these pathogenic strains may be more adept at reaching the final stage of the farm-to-fork continuum and consequently causing human foodborne illness.

Using protein gel electrophoresis and peptide mass fingerprinting, ClpK, encoded by ORF 3 of the LHR, was identified in all heat resistant isolates. Expression of ClpK was observed to be constitutive regardless of heat and osmotic stress, suggesting that the LHR confers a baseline level of heat resistance independent of environmental stimuli. ClpK has been reported to be the largest contributor towards heat resistance in LHR-positive isolates, where its deletion renders isolates heat sensitive and insertion of the *clpK* gene into heat sensitive strains confers significant tolerance

to high temperatures (33,34). Interestingly, Mercer *et al*. reported that transfer of the entire LHR is necessary to confer heat resistance in derivative *E. coli* strains when exposed to high temperatures (7). It is suggested that downstream ORFs 8 to 10, which are predicted to encode for proteins involved in envelope stress maintenance, together with ClpK are necessary to confer heat resistance in *E. coli*. The LHR is not regulated in the same manner in all *E. coli* strains and what distinguishes them from other heat resistant *Enterobacteriaceae* that require only ClpK remains to be elucidated.

In this study I documented and characterized heat resistant *E. coli* causing acute gastroenteritis in humans. In addition to *E. coli* serotype O157:H7, the province of Alberta also identifies non-O157 top six (O26, O45, O103, O111, O121, and O145) and non-top six STEC serogroups. Such comprehensive surveillance of *E. coli* is not practiced across Canada and consequentially, heat resistant *E. coli* from uncommon serogroups may not be detected from clinical cases of acute gastroenteritis if only serotype O157:H7 and the non-O157 top six serogroups are routinely identified. The heightened survivability of heat resistant *E. coli* suggests that the organism may be capable of surviving pathogen intervention processes in food processing and cooking and environments such as these may select for LHR-positive strains. Consumer preference for tender and flavourful food attained through low temperature cooking methods such as sous vide or undercooking red meats present optimal conditions for heat resistant pathogens to persist and potentially cause human illness. The extent of involvement of heat resistant *E. coli* in foodborne illness is unknown and screening for LHR-positive isolates in multiple stages of food production may provide an estimation of its threat on food safety.

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Chapter 4

Determination of biofilm formation in heat resistant *Escherichia coli* isolates

4.1. Introduction

Escherichia coli is ubiquitous as normal flora in humans and animals (1). However, acquisition of virulence factors allows some *E. coli* strains to become pathogenic and consequentially, become etiological agents for a multitude of human infections. Of the pathogenic *E. coli*, pathotypes can be classified into two categories, intestinal and extraintestinal, based on virulence factors the strains possess and their corresponding clinical presentations in humans (2). Intestinal *E. coli* pathotypes include enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), and diffuse-adhering *E. coli* (DAEC) that typically present as diarrheal disease in human infection. Urinary tract infections, meningitis, and sepsis are commonly attributed to infection by extraintestinal *E. coli* pathotypes including uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-associated *E. coli* (SEPEC), respectively.

Biofilms are microbial aggregates living as a community through secretion of an extracellular polymeric matrix composed of exopolysaccharides, proteins, and DNA (3). Such communities can persist on abiotic and biotic surfaces for extended periods of time due to their high resistance to disinfectants and antimicrobials. *E. coli* biofilms are responsible for a plethora of nosocomial, device-related infections and are a persistent source of contamination in the environment (4,5). The ability of *E. coli* biofilms to withstand harsh conditions is achieved by quorum sensing, the chemical signalling pathway in which bacterial cells communicate with each other through secretion of autoinducer substances to mediate biofilm formation and maturation, and secretion of virulence factors (3). EAEC and UPEC pathotypes have been thoroughly investigated for their biofilm forming potential (6); however, research in biofilm formation in other pathotypes, including EHEC requires further investigation. EHEC is a zoonotic agent of foodborne

illness and cattle can serve as a natural reservoir for the pathotype (7). Routes of EHEC transmission include consumption of contaminated food or water, direct contact with animals, and person-to-person spread (8). In conditions of poor sanitation in food processing, EHEC biofilms on equipment and surfaces can be a source of persistent contamination on carcasses and food products (9). Processing plants routinely incorporate high temperature disinfectant solutions of 50°C into their sanitation procedures to remove visible grime and also eliminate bacterial biofilms (10,11). However, the circulation of EHEC strains possessing the locus of heat resistance (LHR), which confers exceptional thermotolerance to temperatures of 60°C and above (12), may be problematic in food processing environments. Biofilm formation in both EHEC and heat resistant *E. coli* strains has not been well explored, but the presence of the organism may serve as a substantial threat to food safety (8,13). Reports of heat resistant, clinical isolates have shown resistance to heat and osmotic stress at temperatures of 60°C and 71°C (14,15). These temperatures are comparable to those used in the food processing industry's heat inactivation procedures and reflective of temperatures recommended for safe consumer cooking.

The objectives of this chapter were to comparatively detect biofilm formation in clinical and environmental *E. coli* isolates possessing the locus of heat resistance using an in-house two-component apparatus and characterize their genetic profiles in respect to biofilm formation-associated genes.

4.2. Materials and Methods

4.2.1. Bacterial isolates and growth conditions*

Heat resistant *E. coli* isolates included in this chapter originated from both environmental and clinical sources; 3 clinical isolates previously identified as 111, 128, and 8354 were from human cases of acute gastroenteritis (15). Environmental isolate AW1.7 originated from a local cattle slaughter plant (16) and isolates 53 and 63 were obtained from a municipal wastewater treatment plant (17). All of these isolates were recovered from frozen skim milk stocks and streaked onto sheep blood agar plates (BAP) (Dalynn Biologicals, Calgary, AB, Canada) and incubated for 24 hours at 37°C for use in subsequent experiments.

4.2.2. Detection of biofilm formation by crystal violet staining

Biofilm formation was detected using an in-house two-component apparatus (Figure 4.1 A) as described below. Sterile sticks are taped onto the longitudinal sides of a 96 well flat bottom clear polystyrene microplate (Corning; Millipore Sigma, Milwaukee, WI, USA) in which a 96 well PCR plate (MicroAmp; Thermo Fisher Scientific, Waltham, MA, USA) is laid on top. The pegs of the PCR plate are submerged into the wells of the flat bottom microplate inoculated with bacterial culture. The top and bottom components of the apparatus are sealed together with tape and the entire structure is incubated. Biofilm formation develops on the pegs of the PCR plate instead of forming in the flat bottom microplate as shown in conventional biofilm assays. Following incubation, the PCR plate is immersed in two 96 well round bottom microplates with crystal violet and ethanol for staining and de-staining, respectively.

* *E. coli* isolates 53 and 63 supplied by Alberta Precision Laboratories – Provincial Laboratory Environmental department.

Detection of biofilm formation for the 6 heat resistant *E. coli* isolates was achieved by inoculating a single colony from each BAP culture into 10 mL of Luria Bertani (LB) broth (Becton Dickinson, Mississauga, ON, Canada) followed by incubation at 37°C for 24 hours with agitation. Stationary phase cultures of the isolates were adjusted to an optical density (OD) at 600 nm of 0.5 (Microscan Turbidity Meter, Siemens, Oakville, ON, Canada) from which 1 mL aliquots were washed and re-suspended with phosphate buffered saline (pH 7.0). For each isolate that biofilm formation was to be determined, the wells of the flat bottom microplate were filled with 140 µL of LB broth in triplicate. To these wells, 10 µL of the aliquots of bacterial cells were added with saline used as a blank. Sterile sticks were taped onto the tissue culture plate and the PCR plate was laid on top prior to sealing the entire apparatus with additional tape. The apparatus was stored in a sealed container lined with damp paper towels to prevent evaporation over a 24 hour incubation period at 4°C. Following incubation, the PCR plate was disassembled from the apparatus and washed in 200 mL of Milli-Q water for 30 seconds with light agitation by hand (4x). The washed PCR plate was then turned with the pegs facing upwards to remove excess Milli-Q water and dried for 10 minutes. To a 96 well round bottom clear polystyrene microplate (Corning; Millipore Sigma, Milwaukee, WI, USA), 200 µL of crystal violet (Millipore Sigma, Milwaukee, WI, USA) diluted to 1% with Milli-Q water was added to each well corresponding with the wells of the flat bottom microplate. The PCR plate was laid on top of the round bottom microplate so that the pegs were submerged in the crystal violet solution for 30 minutes at 24°C. Following staining, the PCR plate was again washed in 200 mL of Milli-Q water with light agitation for 30 seconds (4x) and dried for 10 minutes (Figure 4.1 B). Lastly, a second round bottom microplate was prepared with 150 µL of 95% ethanol per well for de-staining of the pegs. The PCR plate was immersed in the round bottom microplate for 30 minutes at 4°C during which any crystal violet adhering to the pegs

dissolved into the ethanol. Absorbance of crystal violet was measured at 595 nm using a SpectraMax 190 Microplate Reader (Molecular Devices LLC, San Jose, CA, USA) and SoftMax Pro software. Screening for biofilm formation in LB broth was repeated for all isolates at incubation temperatures of 24°C and 37°C.

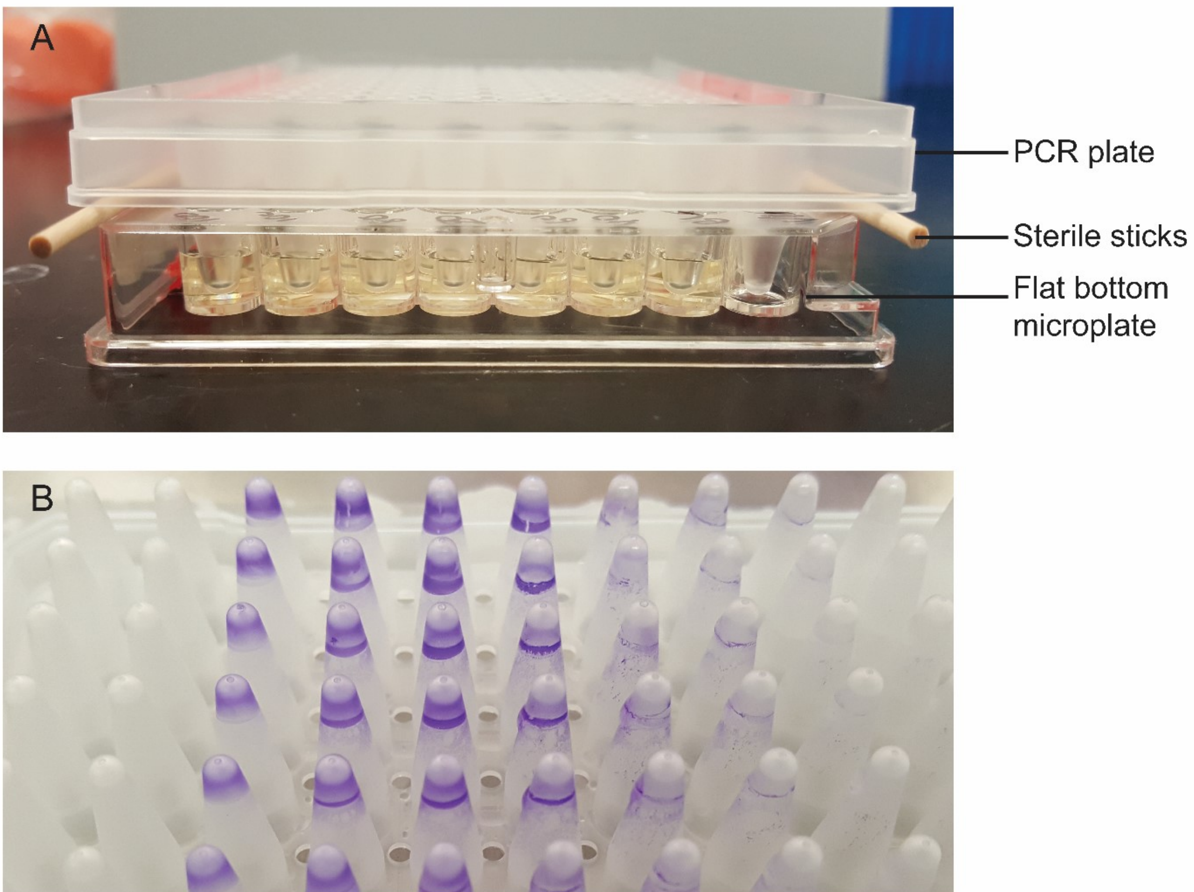

Figure 4.1. Two-component apparatus for detecting biofilm formation (A). The pegs of the PCR plate are submerged into wells of the flat bottom microplate containing bacterial cells. The PCR plate rests on top of 2 sterile sticks to prevent the pegs from direct contact with the bottom of the microplate. Biofilms form on the pegs that are subsequently stained with 1% crystal violet (B).

4.2.3. Determination of optimal biofilm formation conditions

After initial screening for biofilm formation in LB broth, experimentation to determine the optimal conditions such as inoculum size, nutrient concentration, and temperature conditions for biofilms were performed for each isolate using the two-component apparatus. Modifications for this experimental procedure required serial dilutions of LB broth with Milli-Q water to obtain concentrations from 100% to 10% in decreasing intervals of 10%. After adjustment to an OD of 0.5 at 600 nm, stationary phase cultures were washed and then serially diluted with saline to obtain cell concentrations ranging from 8 log CFU/mL to 1 CFU/mL over 5 dilutions. The flat bottom microplate was subsequently inoculated with 140 µL of LB broth dilutions and 10 µL of inoculum dilutions of cells according to a plate map; lastly a PCR plate was overlaid on top of the microplate as described above. Incubation conditions, staining and de-staining procedures, and measurement of absorbance were performed as previously described.

4.2.4. Genomic DNA isolation, whole genome sequencing, and analysis for biofilm-associated genes[*]

Genomic DNA of heat resistant *E. coli* isolates AW1.7, 111, 128, and 8354 was extracted from overnight cultures grown on BAP using the MagaZorb DNA mini-prep kit (Promega Corporation, Madison, WI, USA). The Qubit 4 Fluorometer (Invitrogen, Burlington, ON, Canada) was used to determine the quality and quantity of DNA. Sequencing libraries were prepared using the Nextera XT kit (Illumina Inc., San Diego, USA). Whole genome sequencing was performed using the Illumina MiSeq platform (Illumina Inc., San Diego, USA) according to the manufacturer's instructions. MiSeq sequencing runs were performed with paired-end 250-

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[*] The Applied Genomics Core (TAGC) at the University of Alberta conducted whole genome sequencing of *E. coli* isolates AW1.7, 111, and 128. Alberta Precision Laboratories – Provincial Laboratory Bacterial Typing Unit conducted whole genome sequencing of *E. coli* isolate 8534. Dr. Shuai Zhi assembled all genomes.

nucleotide reads. Trimmomatic Version 0.38 (18) was used to trim the low-quality reads of each genome with the following parameters: SLIDINGWINDOW=4:15, LEADING=3, TRAILING=3 MINLEN=36. *De novo* assembly was performed using SPAdes Version 3.9.1 (19) with '--careful' and '-k 21,33,55,77' options. Presence or absence of biofilm formation-associated genes (Table 4.1) in each of the isolates was identified using the NCBI BLAST server.

Table 4.1. Biofilm formation-associated genes

Gene	Gene ID	Description	References
csgB	947391	Minor curlin subunit	(40)
csgA	949055	Major curlin subunit	(41)
csgC	945623	Inhibitor of CsgA amyloid formation	(42)
csgD	949119	DNA-binding transcriptional dual regulator of genes involved in curli assembly, transport, and structure components	(41,43)
csgE	945711	Specificity factor in the CsgG mediated outer membrane translocation of the curli subunits	(34)
csgF	945622	Acts in conjunction with CsgB to initiate curli subunit polymerisation	(34)
csgG	945619	Forms the secretion channel for curli subunits, providing stability to CsgA and CsgB during assembly	(41)
hha	945098	Represses the transcription of fimbrial genes	(44)
bcsA	948053	Catalytic subunit of cellulose synthase	(35)
bcsB	948045	Cellulose synthase periplasmic subunit	(35)
pgaC	945606	Poly-N-acetyl-D-glucosamine (PGA) synthase subunit mediating translocation and/or docking of PGA to the cell surface	(36)
papC	7152342	Export and assembly of pili subunits across the outer membrane needed for formation of P fimbriae	(45)
agn43	946540	Autoaggregation factor promoting adhesion	(46,47)

Table 4.1 continued. Biofilm formation-associated genes

Gene	Gene ID	Description	References
fimA	948838	Major subunit of type 1 fimbriae	(48)
fimB	948832	Recombinase regulating type 1 fimbriae production	(49)
fimE	948836	Recombinase regulating type 1 fimbriae production	(49)
mrkA	8569608	Major subunit of plasmid encoded type 3 fimbriae	(50,51)
mrkB	8569607	Type 3 fimbriae chaperone involved in assembly and anchorage of fimbrial filaments	(51,52)
mrkC	8569606	Type 3 fimbriae usher involved in assembly and anchorage of fimbrial filaments	(51,52)
mrkD	8569605	Adhesin subunit of type 3 fimbriae	(50,51)
mrkF	8569604	Stabilizes intact type 3 fimbriae	(50,51)

4.2.5. Motility status

Presence or absence of the *fliC* gene as a genetic marker for motility was determined for isolates AW1.7, 111, 128, and 8354 using the NCBI BLAST server against their respective genomes. Phenotypic expression for flagellin was confirmed for all isolates including environmental isolates 53 and 63 with 2 motility tests, Triphenyltetrazolium Chloride (TTC) medium (Dalynn Biologicals, Calgary, AB, Canada) and Sulfide, Indole, Motility (SIM) medium (Dalynn Biologicals, Calgary, AB, Canada). Isolated colonies from overnight cultures were inoculated from the BAP into the motility test media by stabbing the center to a depth of approximately 1.5 inches. The test media were incubated overnight at 37°C. Extension from the stab line for growth as visualized by turbidity or cloudiness, and diffusion of formazan in the case of TTC medium were considered positive indicators for motility. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as a positive and negative control, respectively.

4.2.6. Statistical analysis

The means of at least 3 independent experiments were determined in order to optimize the experimental procedure of the two-component apparatus for each isolate at all incubation temperatures. Biofilm formation between environmental and clinical isolates at each incubation temperature was compared using a Two-Sample t-Test with OriginPro 2016 (OriginLab, Northampton, MA, USA). A 95% significance ($P = 0.05$) was used. The means of at least 3 independent experiments were determined for all serial dilutions of inoculum and LB broth for each isolate when determining optimal biofilm formation conditions.

4.3. Results

4.3.1. Virulence characteristics and motility of *E. coli* isolates

Characterization of the clinical isolates for key virulence factors of the EHEC pathotype including stx_1, stx_2, and *eae*, was previously described in Chapter 3. Isolate 111 was positive for stx_1 and *eae* and negative for stx_2, isolate 128 was positive for stx_1 and negative for stx_2 and *eae*, and isolate 8354 was negative for stx_1, stx_2, and *eae*. Despite clinical isolate 8354 lacking the genetic predictors of EHEC, it was the sole etiologic agent identified from a case of acute gastroenteritis. Expectedly, environmental isolates AW1.7, 53, and 63 were negative for EHEC virulence factors including stx_1, stx_2, and *eae*. Isolates AW1.7, 111, 128, and 8354 all possessed the *fliC* gene encoding for flagellin. All isolates were positive for phenotypic motility as confirmed by TTC and SIM media. Interestingly, motility was not the same for all isolates when observed by TTC medium (Figure 4.2). Isolates AW1.7, 53, 63, and 111 showed less diffusion of formazan compared to isolates 128 and 8354 as observed by the spread of pigment from the stab line. All isolates showed a diffuse zone of growth from the stab line in SIM medium.

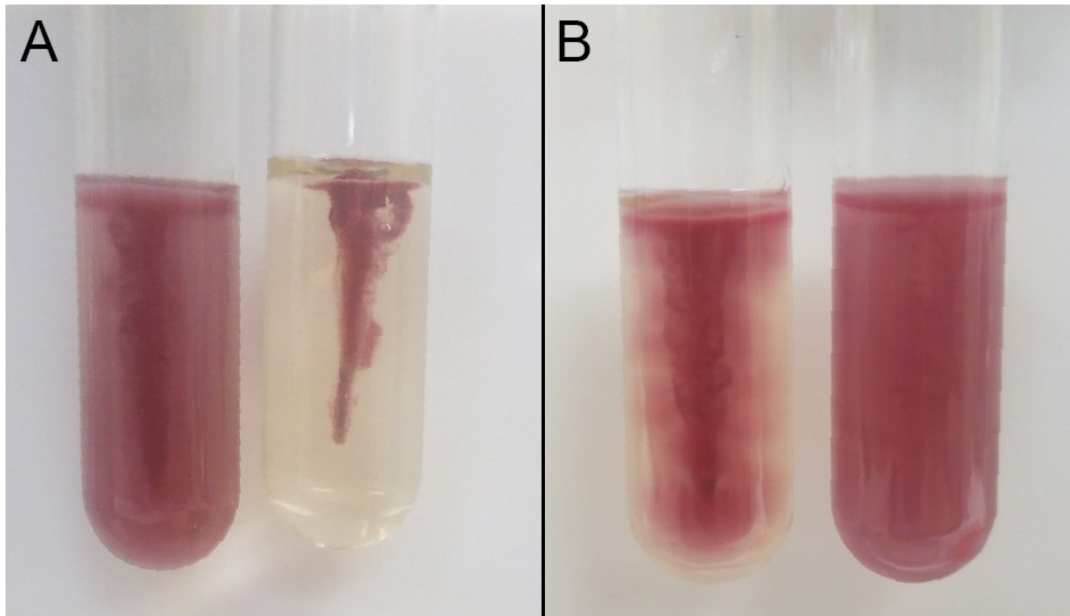

Figure 4.2. Triphenyltetrazolium Chloride (TTC) motility test media of (A) positive control *Escherichia coli* ATCC 25922 (left) and negative control *Klebsiella pneumoniae* ATCC 700603 (right) and (B) heat resistant isolates AW1.7 (left) and 128 (right). Results from TTC media of isolates 53, 63, and 111 were identical to isolate AW1.7 and isolate 8354 was identical to isolate 128.

4.3.2. Optimization of the two-component apparatus for *E. coli*

Initial screening for biofilm formation using bacterial cultures standardized to 8 log CFU/mL revealed biofilm formation in all environmental isolates and clinical isolate 111 at temperatures of 24°C and 37°C but not at 4°C (Figure 4.3). No biofilm formation was detected from clinical isolates 128 and 8354 at all incubation temperatures. At an incubation temperature of 37°C, environmental isolates showed increased biofilm formation compared to clinical isolates whereas at 24°C, biofilm formation in both groups were similar. No statistical differences in biofilm formation were observed between environmental and clinical isolates at all incubation temperatures. Neither inoculum size nor nutrient concentration was manipulated at this stage in order to optimize the experimental procedure for the two-component apparatus.

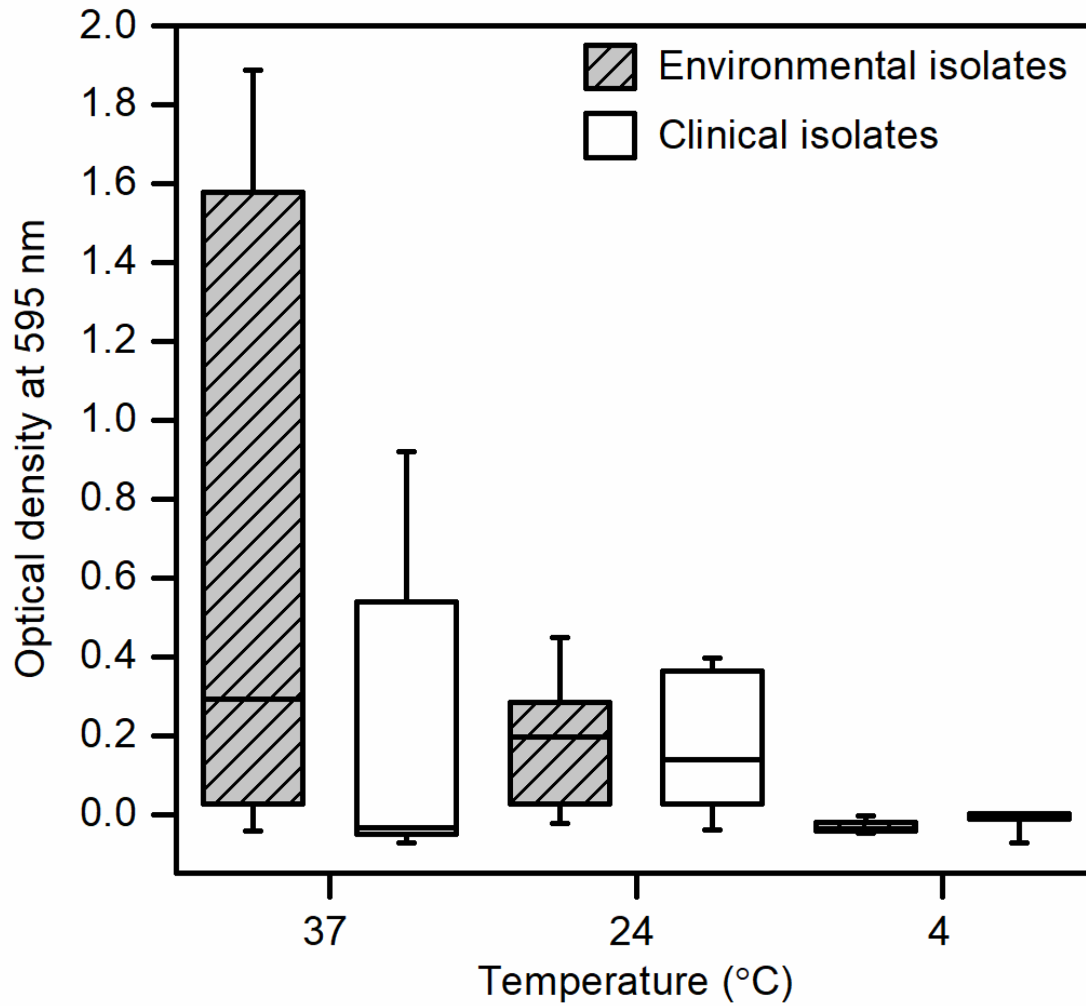

Figure 4.3. Biofilm formation of environmental and clinical *Escherichia coli* isolates in 100% LB broth and 8 log CFU/mL inoculum size incubated at temperatures of 37°C, 24°C, and 4°C for optimization of the two-component apparatus. Data presented as means ± standard deviations of triplicate experiments.

4.3.3. Determination of optimal biofilm formation conditions

To determine the optimal biofilm formation conditions for each isolate, manipulation of inoculum size and nutrient concentration was incorporated through serial dilutions of each variable. Again, similar observations of no biofilm formation were obtained at 4°C for all isolates regardless of inoculum size and nutrient concentration. For the 4 isolates that were capable of forming biofilms at 24°C and 37°C, conditions yielding maximum biofilm formation were not identical within isolates at different incubation temperatures or between isolates at the same temperatures (Figure 4.4). Among the isolates, environmental isolate AW1.7 formed the most biofilm at 24°C and 37°C temperatures as measured by absorbance of crystal violet at 595 nm. In addition, biofilm formation of AW1.7 was evidently different between the two temperatures, with maximum formation in 40% LB broth and 6 log CFU/mL inoculum, and 100% LB broth and 8 log CFU/mL inoculum at 24°C and 37°C, respectively. Environmental isolate 53 was capable of forming biofilms at 24°C in concentrations of 30% to 90% LB broth at all cell concentrations with the exception of 8 log CFU/mL and maximum formation was observed at 60% LB broth and 4 log CFU/mL. Biofilm formation in isolate 53 was interestingly quite different at 37°C, with maximum formation at 50% LB broth for most cell concentrations. Furthermore, biofilm formation at this temperature was undetectable or weak until a nutrient concentration of 40% LB broth was reached. Isolate 63, also of environmental origin, was capable of forming biofilms at both 24°C and 37°C. At 24°C, biofilm formation was linear, with maximum formation observed at 90% and 100% LB broth at all cell concentrations. Similar to isolate 53, at 37°C, isolate 63 was capable of forming biofilms only in specific conditions; LB broth concentrations of 0% to 20% did not support biofilm formation at any inoculum sizes. Optimal conditions for maximum biofilm formation in isolate 63 at 37°C were observed at 40% LB broth and 1 log CFU/mL, and 50% LB broth and 8 log CFU/mL.

Of the 3 clinical, heat resistant isolates, only isolate 111 was a biofilm former. Isolate 111 formed biofilms at all inoculum sizes at 24°C incubation but maximum formation was observed only up to 80% LB broth. In addition, biofilm formation was substantially lower at an inoculum size of 8 log CFU/mL regardless of LB broth concentration compared to the other dilutions. Interestingly, at 37°C, only an inoculum of 8 log CFU/mL of isolate 111 had biofilm formation. All other cell dilutions of isolate 111 were unable to form any biofilm across all LB broth concentrations.

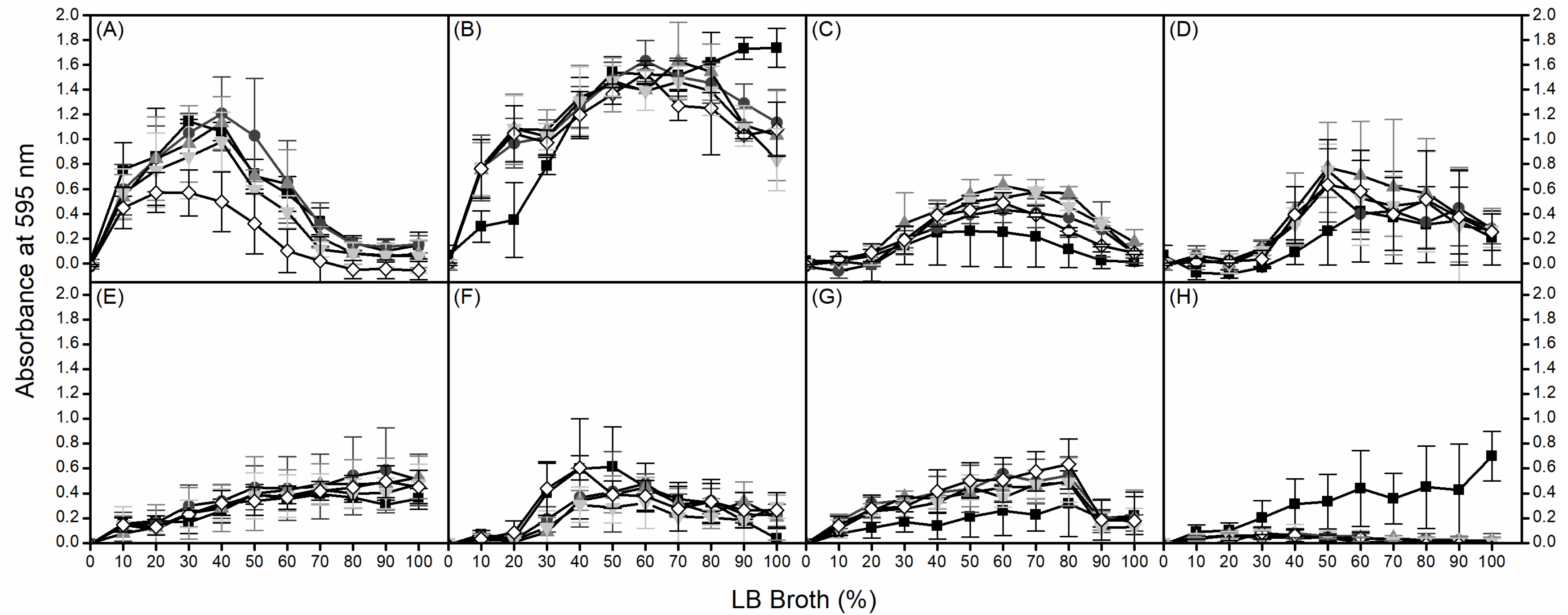

Figure 4.4. Biofilm formation in heat resistant *Escherichia coli* environmental isolates AW1.7 at 24°C (A) and 37°C (B); 53 at 24°C (C) and 37°C (D); 63 at 24°C (E) and 37°C (F); and clinical isolate 111 at 24°C (G) and 37°C (H) under conditions of inoculum size and nutrient concentration manipulation. Bacterial inoculum sizes: (■) 8 log CFU/mL, (●) 6 log CFU/mL, (▲) 4 log CFU/mL, (▼) 2 log CFU/mL, (◇) 1 CFU/mL. Data presented as means ± standard deviations of triplicate experiments.

4.3.4. Genetic analysis of biofilm formation-associated genes

Genome sequence data was used to determine the presence or absence of known biofilm formation-associated genes for isolates AW1.7, 111, 128, and 8354 (Table 4.2). Whole genome sequencing data of environmental isolates 53 and 63 were not available for our analysis. Of the isolates that whole genome sequencing data were obtained, all isolates possessed the *csgBAC* and *csgDEFG* operons but lacked the *mrkABCDF* operon, which encodes for curli and type 3 fimbriae, respectively. Interestingly, despite isolates AW1.7 and 8354 both possessing the same genetic profile for biofilm formation-associated genes, only the former was capable of forming biofilm. Clinical isolate 111 possessed the most biofilm formation-associated genes that were of interest, yet was substantially weaker at forming biofilm than isolate AW1.7.

Table 4.2. Genetic determination of biofilm formation-associated genes in heat resistant *Escherichia coli*

	Gene*								
Isolate	*hha*	*bcsA*	*bcsB*	*pgaC*	*papC*	*Agn43*	*fimA*	*fimB*	*fimE*
AW1.7	+	+	+	+	-	-	-	-	-
111	+	+	+	+	-	-	+	+	+
128	+	-	-	+	-	-	+	+	+
8354	+	+	+	+	-	-	-	-	-

*All genes comprising the *csgBAC* and *csgDEFG* operons were present in the isolates. All genes comprising the *mrkABCDF* operon were absent in the isolates.

4.4. Discussion

Biofilm formation using the in-house two-component apparatus was investigated in 3 clinical, heat resistant *E. coli* isolates. Heat resistant *E. coli* isolates AW1.7, 53, and 63 obtained from the environment served as a comparison of a different isolation source and the latter 2 isolates were included as positive controls for the assay. All 3 environmental isolates were capable of forming biofilms whereas biofilm formation was detected only in clinical isolate 111. Furthermore, isolate AW1.7 was most proficient at biofilm formation at 37°C compared to isolates 53 and 63. This difference may be attributed to their respective sources of isolation. External stressors significantly influence an organism's potential for biofilm formation. Isolate AW1.7 originated from a local cattle slaughter plant and may be highly adapted to temperatures of 37°C and above that reflect the gastrointestinal tract of ruminants (20) and thermal inactivation processes used in beef processing. On the other hand, isolates 53 and 63 were obtained from municipal wastewater, which ranges in temperatures between 4°C and 20°C (17). Although these isolates were unable to form biofilms at 4°C, biofilm formation was detected 24°C and 37°C. Likely, *E. coli* strains that persist in environmental settings are more proficient at forming biofilms as a result of adaptation to various stress conditions as compared to the clinical isolates (21,22).

Conventional biofilm procedures measure total biofilm production on the bottom of a microplate well following incubation by staining with crystal violet and measuring the absorbance. However, a disadvantage to this method is that matrix components and dead cells might settle at the bottom of the well and uptake the stain, which may lead to a potential over-estimation of biofilm formation (23). The in-house two-component apparatus detects biofilm formation on the underside of the PCR plate, eliminating any over-estimation as a result of inadequate washing of the microplate wells in the conventional method. Various factors such as inoculum size and

concentration of nutrient media can be manipulated and added to the same microplate for evaluation of their effect on biofilm formation. Furthermore, the two-component apparatus utilizes PCR plates made of polypropylene but the material of the pegs on which the biofilms form can easily be substituted or coated with an array of different materials or substances to facilitate biofilm formation in fastidious organisms or to assess susceptibility to antimicrobials and disinfectants. Resemblances are shared between the two-component apparatus discussed in this chapter and the commercially available MBEC Assay (Innovotech Inc., Edmonton, AB, Canada), formerly the Calgary Biofilm Device (24). With limited research funding available, an in-house assembled two-component apparatus can be used to test for biofilm formation in a variety of bacteria. The two-component apparatus has previously been used to investigate biofilm formation in *Listeria monocytogenes* (data not published) but has not yet been evaluated for *E. coli*. This work highlights that the two-component apparatus can be used to determine biofilm formation in both Gram positive and Gram negative organisms.

Current pathogen intervention steps for cattle carcasses in food processing plants include hot water, steam pasteurization, and antimicrobial solutions applied at high temperatures (10,11). During such processes, buildup of mud and feces from contaminated hides on processing equipment may occur. Poor plant sanitation can allow for biofilm formation on these surfaces and become a source of contamination (9). Many processing plants incorporate high temperatures into their cleaning procedures but limited research has been conducted thus far on whether heat resistance contributes to increased survival of *E. coli* in biofilms (25). Furthermore, previous studies on the effect of environmental stressors on EHEC found it unlikely that strains could possess virulence genes for human infection and phenotypes for evading pathogen intervention measures in the food processing industry (26). However, clinical isolate 111 that possesses Shiga

toxin, the LHR, and the biofilm forming phenotype was characterized in this work, indicating that pathogenic *E. coli* strains with heightened survival traits are in circulation and may represent a significant food safety threat. Future studies on assessing the survival of biofilms formed by heat resistant *E. coli* after exposure to high temperatures reflective of those used in food processing may provide insight on its persistence in food processing plants.

The genes involved in biofilm formation and those related to quorum sensing belong to a highly complex field of study that is ever expanding. This chapter presents insightful genetic analysis of genes associated with biofilm formation in *E. coli*, particularly STEC. Active motility through flagellin has previously been described as a requirement for biofilm formation in *E. coli* (27,28) in order to overcome the repulsive electrostatic and hydrodynamic forces in a liquid environment (29). However, non-motile *E. coli* K-12 and EAEC strains have been reported to form biofilms by overexpression of surface adhesins to compensate for the lack of force-generating movements provided by flagellin (30,31). All heat resistant isolates were motile, fulfilling the first prerequisite for biofilm formation. The locus of enterocyte effacement (LEE) is a pathogenicity island that defines the EHEC pathotype and plays a vital role in the development of attaching and effacing lesions, and adherence to intestinal epithelial cells during pathogenesis (32). Despite the 3 clinical, heat resistant isolates originating from cases of acute gastroenteritis that would likely be indicative of EHEC infection, only isolate 111 was positive for the LEE, as determined by detection of the *eae* gene. The adhesin intimin is encoded by the *eae* gene, one of 41 open reading frames in the LEE (33). Regulation of the LEE is mediated by quorum sensing, specifically by the *luxR* homolog *sdiA* (3,9), and the SdiA protein has been proposed to be involved in biofilm formation in *E. coli*. It is speculated that regulation of the LEE through SdiA may also be reflective

of the isolate's ability to form biofilms and is supported by the findings in this chapter. Isolate 111 was the only clinical isolate that formed biofilms and also possessed the LEE.

Since use of isolates 53 and 63 was limited to detection of biofilm formation only, it is unknown if they possess the biofilm formation-associated genes discussed below. Curli and cellulose are two commonly studied phenotypic markers of biofilm formation as they are major components of the biofilm matrix (34,35). Of the 4 heat resistant *E. coli* isolates that were sequenced, the *csgBAC* and *csgDEFG* operons that encode for curli were present and with the exception of clinical isolate 128; the *bcsA* and *bcsB* genes, which encode for cellulose, were identified in all remaining isolates. However, biofilm formation was only detected in isolates AW1.7 and 111, indicating that genetic and/or phenotypic characterization of curli and cellulose does not correspond with biofilm forming potential and should not be used as the sole methods to investigate biofilm formation. Both biofilm forming isolates AW1.7 and 111 possess the *pgaC* gene but lack the *papC* and *Agn43* genes. *pgaC* encodes for the synthesis of PGA polymer, which plays a role in biofilm adhesion (36). However, PGA is not the only adhesion factor in biofilm formation as LPS, curli, fimbriae, and pili are also involved in this process. Understandably, all clinical isolates were negative for the *papC* and *Agn43* genes that encode for adhesion factors P pili and autoaggregation factor antigen 43, respectively. P pili are commonly found in UPEC organisms as they are critical adhesion factors for the pathogenesis of ascending urinary tract infections (37). Autoaggregation factor antigen 43 has been reported in high prevalence in UPEC, EAEC, and SEPEC pathotypes but not in EHEC (6,38). Thus, it is not surprising that the clinical isolates of diarrheal origins would not possess these two genes. Interestingly, members of the *fim* operon, *fimA*, *fimB*, and *fimE* were detected in isolates 111 and 128 but not in AW1.7 or 8354. Type 1 fimbriae are proteinaceous filamentous adhesins that are important for attachment to abiotic

surfaces (39). Its prevalence has been reported to be high in UPEC, EPEC, and SEPEC pathotypes but similarly has not been investigated thoroughly in EHEC (6). It is possible that type 1 fimbriae may not play a significant role in biofilm formation in EHEC pathotypes, as reflected in the data presented in this chapter. The last biofilm formation-associated genes of interest were those comprising the *mrk* operon. Encoding for type 3 fimbriae, the operon was identified as an additional member in the LHR in a heat resistant *E*. *coli* strain isolated from raw milk cheese (25). The *mrk* operon is commonly present in *Klebsiella pneumoniae* but rarely reported in *E*. *coli*. The lack of the *mrk* operon suggests that the LHR present in the heat resistant isolates likely originates from a different source than the one identified by Marti *et al*. (25).

By using the two-component apparatus in this study, I was able to confirm biofilm formation in *E*. *coli* isolates from environmental and clinical sources and provide additional evidence that environmental strains demonstrate a greater ability at forming biofilms. The combination of biofilm forming potential of a strain along with genetic analysis of related genes allows for deeper investigation in understanding the correlation between biofilm formation and the presence of possible gene requirements. Further studies are necessary to determine the prevalence of virulent, multi-stress tolerant *E*. *coli* strains and its impact on food safety and public health.

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Chapter 5

Genomic and proteomic analyses of the locus of heat resistance in *Escherichia coli* isolates

5.1. Introduction

Infectious *Escherichia coli* exist as intestinal and extraintestinal pathogens in regards to causing human infection. Of the 6 *E. coli* pathotypes implicated in intestinal disease, they possess a complex combination of virulence factors distinct to each pathotype that manifest in different clinical presentations (1). A variety of factors place the enterohemorrhagic *E. coli* (EHEC) pathotype at the forefront of clinical relevance, including the replication of the organism in the gastrointestinal tract of asymptomatic ruminants, a low infectious dose, and its potential to cause chronic and life-threatening complications in infected individuals (2,3). Foodborne outbreaks of EHEC continue to be of concern to the food processing industry and public health system despite robust pathogen inactivation processes in place and improvements in laboratory detection of cases (4,5). As of 2017, the annual incidence of EHEC infection per 100,000 people in Canada is 3.13 (6). Contaminated foods such as beef (7), pork (8), flour, and cheese products (9) serve as known vehicles of EHEC infection and practices must be in place at various levels of food processing to prevent the organism from reaching the consumer.

The plasticity of the *E. coli* genome allows for genetic elements to be readily acquired through various means of horizontal transfer, examples including the key virulence factors in EHEC pathogenicity Shiga toxins 1 and/or 2 and the locus of enterocyte effacement (LEE), which are obtained through infection by bacteriophage and uptake of genomic islands, respectively. Recent emergence of members of the *Enterobacteriaceae* family including *Klebsiella pneumoniae* (10), *Salmonella enterica*, *Enterobacter* spp. (11), *Cronobacter sakazakii* (12), and *Escherichia coli* (13–15) that have acquired a genomic island termed the locus of heat resistance (LHR), raises concerns of highly heat resistant organisms circulating in the environment. The LHR is reported to confer heat resistance at temperatures above 60°C in all strains that have acquired it and

bioinformatic analysis suggests its presence in β- and γ- proteobacteria as well (13). Putative proteins encoded by the LHR play a role in heat shock and protein homeostasis, envelope stress, and oxidative stress that are expressed and regulated separately from the heat shock response mediated by alternative sigma factor RpoH (11). Food processing plants heavily rely on thermal intervention processes such as steam pasteurization and hot water washes to reduce the bacterial load on food products (16) and predictably, have become environmental niches for the circulation of heat resistant *E. coli*. Although heat resistant strains possessing the LHR can be generalized to withstand heat exposure above 60°C, differences in D_{60}-values warrant genetic analysis of the LHR and their respective genomes to elucidate the cause. Well documented observations about the LHR that are applicable to all heat resistant organisms thus far include the presence of flanking mobile genetic elements, encoding of putative proteins involved in heat shock, cell envelope maintenance, and turnover of misfolded proteins, and a high nucleotide identity of >99% in most of the shared open reading frames (ORFs) (13). However, there are a number of distinguishing features specific to the LHR of different genera and strains. The LHR in heat resistant *K. pneumoniae* and *C. sakazakii* is located on a plasmid (10,12) whereas it has been found in both the chromosome and on plasmids in heat resistant *E. coli* (13,17). Unequal levels of heat resistance have also been observed in transgenic strains generated from cloning experiments with homologous ORFs from the LHR in heat resistant organisms of different genera (12,13,18). Insertion of ORF 3 from the LHR of heat resistant *K. pneumoniae*, which encodes for novel Clp chaperone ClpK, has been reported to be sufficient for expression of phenotypic heat resistance in transgenic strains (18) but this finding has yet to be confirmed in other heat resistant organisms.

With the novel identification of heat resistant *E. coli* from human cases of acute gastroenteritis, it is important to characterize the genetic elements mediating heat resistance in

each of the isolates. To achieve this, comparative genetic analysis on the LHR and other heat shock and heat resistance mechanisms in the 3 heat resistant, clinical isolates was conducted. The second aim of this chapter was to investigate the contribution of ORF 3 of the LHR in *E. coli* isolate AW1.7 on heat resistance through generation of a transgenic strain.

5.2. Materials and Methods

5.2.1. Bacterial isolates and whole genome sequencing

Heat resistant *E. coli* isolates AW1.7, 111, 128, and 8354, and heat sensitive isolate 126 recovered from skim milk stocks were streaked onto sheep blood agar plates (BAP) (Dalynn Biologicals, Calgary, AB, Canada) and incubated for 24 hours at 37°C prior to extraction of genomic DNA with the MagaZorb DNA mini-prep kit (Promega Corporation, Madison, WI, USA). Whole genome sequencing and genome assembly were completed as previously described in Chapter 4.

5.2.2. Genetic analysis of the locus of heat resistance and heat shock- and heat resistance-related genes*

A dendrogram was generated from multiple sequence alignment of the LHR sequences of the 4 heat resistant isolates (open gap penalty, 100%; unit gap penalty, 0%) using BioNumerics version 6.01 software (Applied Maths, Austin, TX, USA). ORFs of the LHR in each of the heat resistant isolates were predicted and annotated by GeneMark.hmm for prokaryotes (19) and analyzed for sequence homology by pairwise alignment against the ORFs of AW1.7. Mobile

* Vincent Li and Theodore Chiu provided for technical assistance with genetic analysis of the locus of heat resistance.

genetic elements in the LHR were identified with ISfinder (20). Putative promoters for transcription of the LHR were predicted using CNNPromoter_b (21) with requirements of a prediction score above 0.9 (range of 0 to 1.0) and coordinates indicating the promoter was upstream of an ORF and not within it. Lastly, rho-independent terminators were identified using ARNold (22). Presence or absence of heat shock- and resistance-associated genes (Table 5.1) in addition to those encoded by the LHR were investigated in all 5 isolates by searching the National Center for Biotechnology Information (NCBI) BLAST server against the assembled genomes.

Table 5.1. Heat shock- and heat resistance- associated genes and predicted functions

Gene	Gene ID	Encoded protein description	References
rpoH	947970	RNA polymerase sigma factor RpoH (σ^{32}) that controls the heat shock response during log phase growth	(23)
DnaK	944750	70 kDa ATP-dependent heat shock chaperone involved in folding of polypeptide chains, turnover of misfolded proteins, and protein secretion	(24–26)
DnaJ	944753	40 kDa heat shock co-chaperone modulating the substrate binding and ATPase activity of DnaK	(27)
GrpE	947097	Nucleotide exchange factor regulating the DnaK-substrate association/dissociation cycle through turnover of ATP	(28,29)
GroEL	948665	60 kDa chaperonin involved in prevention and refolding of misfolded proteins, and folding of polypeptide chains	(30)
GroES	13699849	10 kDa co-chaperonin that binds to GroEL in the presence of ATP to assist protein folding	(31,32)
ibpA	948200	ATP-independent small heat shock protein that with IbpB associates with aggregated proteins to stabilize and protect them from irreversible denaturation and proteolysis	(33,34)
ibpB	948192	Small heat shock protein that works together with IbpA	(33–35)
htpG	945099	90 kDa ATP-dependent heat shock chaperone involved in protein folding and interacts with the DnaK-DnaJ-GrpE chaperone system	(36)

Table 5.1 continued. Heat shock- and heat resistance- associated genes and predicted functions

Gene	Gene ID	Encoded protein description	References
clpA	945764	ATP-dependent Clp chaperone subunit of the ClpAP serine protease complex	(37)
clpX	945083	ATP-dependent Clp chaperone subunit of the ClpXP serine protease complex	(37)
clpP	945082	ATP-dependent Clp serine protease that together with ClpA or ClpX degrades unfolded and misfolded proteins	(38)
clpB	947077	Chaperone that disaggregates and reactivates strongly aggregated proteins in cooperation with the DnaK-DnaJ-GrpE chaperone system	(39,40)

5.2.3. Cloning of ORF 3 from *E. coli* AW1.7[*]

To investigate the contribution of ClpK that is encoded by ORF 3 on heat resistance conferred by the LHR, a plasmid containing ORF 3 from *E. coli* AW1.7 was constructed and cloned into One Shot OmniMAX 2 T1 Chemically Competent *E. coli* using the TOPO XL-2 Complete PCR Cloning Kit (Invitrogen, Burlington, ON, Canada). All primer and probe sequences with the exception of T3 and T7 primers (purchased from the University of Alberta The Applied Genomics Core) were designed using the Integrated DNA Technologies PrimerQuest Tool and are indicated in Table 5.2.

Genomic DNA of AW1.7 was extracted using the QIAGEN DNeasy Blood and Tissue Kit (Toronto, ON, Canada) following the manufacturer's instructions with slight modifications to the procedure: From an overnight culture grown in 2 mL of tryptic soy broth (Becton Dickinson, Mississauga, ON, Canada), 1 mL of cells were harvested by centrifugation at 5000 × g for 10 minutes in a 1.5 mL microfuge tube (Eppendorf Centrifuge 5425; Thermo Fisher Scientific, Waltham, MA, USA). The cell pellet was resuspended in 180 µL of Buffer ATL and 20 µL of Proteinase K was added. The tube was mixed thoroughly by vortex and then incubated at 56°C on a heat block (VWR Scientific Standard Heatblock; VWR International, Edmonton, AB, Canada) for 10 minutes. Afterwards, 10 mg/mL DNase-free RNase A (QIAGEN; Toronto, ON, Canada) was added to the tube, which was incubated at 37°C for 30 minutes. The tube was vortexed for 15 seconds and 200 µL of Buffer AL and 200 µL of 100% ethanol were added. Contents of the tube were transferred into a DNeasy Mini spin column that was centrifuged at 6000 × g for 1 minute. All flow through was discarded and 500 µL of Buffer AW1 was added to the column. The column

[*] Dr. Michael Bording-Jorgensen provided experimental assistance with cloning of open reading frame 3.

was again spun down at 6000 × g for 1 minute and flow through was removed. Five hundred µL of Buffer AW2 was added to the column and spun down at 20,000 × g for 3 minutes to dry the DNeasy membrane. Genomic DNA was eluted with 200 µL of Buffer EB by incubating the column at room temperature for 1 minute followed by centrifugation at 6000 × g for 1 minute. Quality and quantity of the extracted genomic DNA was determined using the Qubit 4 Fluorometer (Invitrogen, Burlington, ON, Canada).

Table 5.2. Oligonucleotides used in cloning of ORF 3

Primer set	Function	Sequence (5'-3')
Cloning	Amplification of ORF 3 for TOPO XL-2 cloning	F: ATGGCCAGAAAACAATGCCA R: CCATCAAGATGCGTCGCT
ORF 3	qPCR detection of ORF 3	F: CCATTCTTATGTCGGTCCAGAG R: CCACCTTGCTGACCTGTT P: [6-FAM]-ATTTCCTGA-[ZEN]-TTGGTCTGGCCGAGG-[IABkFQ]
T3	Sequencing primer for T3 RNA polymerase promoter in plasmid	F: AATTAACCCTCACTAAAGGG
T7R	Sequencing primer for T7 RNA polymerase promoter in plasmid	R: GCTAGTTATTGCTCAGCGG
Sanger F1-4	Sequencing primers to concatenate fragments for consensus sequence	F: AGATCGAGTCTGAGGCCAAG F: CGCTGAACGAGTACCAGAAATAC F: AACGAGTTGACGGTGGAAG F: GGATTTCACCAACACCATCATC
Sanger R1-4	Sequencing primers to concatenate fragments for consensus sequence	R: GTCTACAACATCCTGCTGCA R: CTACCTTCCTGTTTCTGGGC R: CGTATTCAAACCGATGATGGCG R: CCATTCTTATGTCGGTCCAGAG

To produce blunt-end PCR products of ORF 3, 2 µL of genomic DNA correlating to a concentration of ~35 ng was added to a PCR cocktail consisting of Platinum SuperFi Green PCR Master Mix (2X), 10 µmol/L of forward and reverse cloning primers, and PCR grade water for a final volume of 50 µL. In a second PCR reaction tube, a modified version of the PCR cocktail was made with the addition of 10 µL of SuperFi GC Enhancer (5X) to account for the GC content of ORF 3 exceeding 65%. PCR was performed on both tubes using the Eppendorf Mastercycler pro S (Thermo Fisher Scientific; Waltham, MA, USA) with the following amplification conditions: 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, 58°C for 10 seconds, 72°C for 1.5 minutes, and a final extension at 72°C for 5 minutes. A 7 kb control included in the TOPO XL-2 Complete PCR Cloning Kit was prepared according to manufacturer's instructions and amplified simultaneously with the ORF 3 PCR reaction to ensure the validity of the PCR reaction. Agarose gel electrophoresis of 2 µL of each PCR product on a 0.8% agarose gel (Ultrapure Agarose; Invitrogen, Burlington, ON, Canada) was performed at 145 volts for 55 minutes to verify their respective size and quality. TrackIt 1 Kb Plus DNA Ladder (Invitrogen, Burlington, ON, Canada) served as the DNA standard.

All following steps of cloning were performed following the manufacturer's instructions without modifications to the procedure. First, the ORF 3 PCR product produced from the PCR reaction containing SuperFi GC Enhancer (5X) was purified using the provided PureLink Quick Gel Extraction and PCR Purification Combo Kit. In brief, 50 µL of the PCR product was column purified using the PureLink Clean-up Spin Column by firstly diluting it with 200 µL of Binding Buffer that was subsequently spun down at 10,000 × g for 1 minute at room temperature. All flow through was discarded and 650 µL of Wash Buffer was added to the column. The column was again centrifuged at 10,000 × g for 1 minute at room temperature and the flow through was

discarded. The column was centrifuged at maximum speed for 3 minutes at room temperature to remove any residual wash buffer and the purified PCR product was eluted in 50 µL of sterile, nuclease-free water (pH>7.0) by incubating the column at room temperature for 1 minute followed by centrifugation at 10,000 × g for 1 minute. The purified PCR product was quantified using the Qubit 4 Fluorometer and subsequently diluted in a 1:10 ratio with sterile, nuclease-free water.

TOPO cloning of the ORF 3 insert into the pCR-XL-2-TOPO Vector was performed using 1.4 µL of the 1:10 diluted purified PCR product corresponding to a 1:1 molar ratio of insert to vector as determined by the following calculations:

$$\frac{length\ of\ insert\ (bp)}{length\ of\ vector\ (3965\ bp)} \times 10\ ng\ of\ vector = ng\ of\ insert\ needed\ for\ 1:1\ molar\ ratio$$

$$\frac{2853\ bp\ insert}{3956\ bp\ vector} \times 10\ ng\ of\ vector = 7.21\ ng\ of\ insert\ needed$$

$$\frac{7.21\ ng\ of\ insert\ needed}{5.20\ ng\ of\ 1:10\ diluted\ PCR\ product} = 1.39\ \mu L\ of\ insert\ needed$$

To 1.4 µL of purified PCR product, 1 µL of pCR-XL-2-TOPO Vector, and 1 µL of Salt Solution (provided in the TOPO XL-2 Complete PCR Cloning Kit) was added to 2.6 µL of sterile, nuclease-free water for a final volume of 6 µL. The reaction tube was gently mixed and centrifuged prior to incubating for 30 minutes at room temperature and then placed on ice for transformation. Two µL of the cloning reaction was added to a thawed vial of One Shot OmniMAX 2 T1 Chemically Competent *E. coli* and gently mixed. Concurrently, a transformation control was prepared using 1 µL of the provided pUC19 control plasmid that was added to a second vial of One Shot OmniMAX 2 T1 Chemically Competent *E. coli*. Both vials were incubated on ice for 30 minutes and then heat shocked for 30 seconds in a 42°C water bath (Thermo Haake DC10-W19/B;

Thermo Fisher Scientific, Waltham, MA, USA). Immediately after, the vials were returned to ice for 2 minutes to which 250 µL of SOC medium was then added. The vials were incubated at 37°C for 1 hour with horizontal agitation at 225 rpm (MaxQ 2506 Reciprocating Shaker; Thermo Fisher Scientific, Waltham, MA, USA). Transformed cells were spread onto pre-warmed (37°C) LB plates (Becton Dickinson, Mississauga, ON, Canada) containing 50 µg/mL kanamycin (Gibco Kanamycin Sulfate; Thermo Fisher Scientific, Grand Island, NY USA) and 1 mmol/L isopropyl β- d-1-thiogalactopyranoside (IPTG) (Invitrogen, Burlington, ON, Canada) or 100 µg/mL ampicillin (Gibco Ampicillin, sodium salt, irradiated; Thermo Fisher Scientific, Grand Island, NY USA) and 1 mmol/L IPTG at volumes of 50 µL and 100 µL. Plates were incubated at 37°C overnight. The following day, an isolated colony from the pU19 control transformation was subcultured onto a BAP and inoculated into skim milk and frozen for storage. From the plates that were spread with the ORF 3 transformation, 48 isolated colonies were randomly selected and subcultured. Genomic DNA from the 48 transformants was extracted with 200 µL rapid lysis buffer (100 mmol/L NaCl; 10 mmol/L Tris-HCl, pH 8.3; 1 mmol/L EDTA, pH 9.0; 1% Triton X-100) and analyzed for ORF 3 using the qPCR assay previously described in Chapter 2.

To confirm the insertion and orientation of ORF 3, 3 randomly chosen transformants (identifiers: 3, 10, 22) all positive for ORF 3 by qPCR, were amplified for the insert using the ORF 3 TOPO cloning primers following the same PCR reaction and cycling conditions as described above for sequencing. To 10 µL of PCR product, 3.2 pmol/µL of T3 primer, T7 primer, TOPO cloning forward and reverse primer, and 8 additional sequencing primers (forward and reverse) were added. The samples were sequenced by Sanger sequencing with the addition of 5% dimethyl sulfoxide (DMSO) to account for their high GC content at The Applied Genomics Core (TAGC) at the University of Alberta. Fragments were analyzed for quality and concatenated to generate

consensus sequences using BioEdit (version 7.0.5.3) (41). Protein gel electrophoresis was also conducted on the 3 transformants using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Overnight cultures grown in LB broth were adjusted to an optical density of 0.5 at 600 nm (Microscan Turbidity Meter, Siemens; Oakville, ON, Canada) and prepared for electrophoresis as previously described in Chapter 3 with the modification of no heat treatment to the aliquots. Any protein bands that resolved at 100 kDa were excised and submitted for in-gel digestion with trypsin and protein mass fingerprinting at the Alberta Proteomics and Mass Spectrometry Facility. ClpK protein structure was predicted using the sequence obtained from the protein mass fingerprinting of the transformants with the highest score (NCBI reference sequence: WP_004152116.1) with the Iterative Threading ASSEmbly Refinement (I-TASSER) server (42–44). Transmembrane topology and signal peptides of the ClpK sequence were predicted with Phobius (45)

5.2.4. Characterization of heat resistance in transformants

All 48 transformants were screened for phenotypic heat resistance upon exposure to 60°C heat shock similar to previously described in Chapter 2. Two 200 µL aliquots of overnight cultures grown in LB broth were washed and re-suspended in 60°C pre-heated LB broth. One aliquot was not heated and immediately placed in an ice-water bath for 2 minutes while the second aliquot was heated in a water bath for 10 minutes and then chilled in the ice-water bath for 2 minutes. From each aliquot, 100 µL was spread plated onto LB plates and incubated overnight at 37°C. Environmental *E. coli* isolate AW1.7 and clinical isolate 126 served as a positive and negative control, respectively. Growth on LB plates from heated aliquots were indicative of heat resistance. Using the same experimental procedure, the pUC19 transformant was also screened for heat

resistance to confirm that the One Shot OmniMAX 2 T1 Chemically Competent *E. coli* did not possess native heat resistance.

5.3. Results

5.3.1. Genetic characterization of the locus of heat resistance

The genomes of the 4 heat resistant *E. coli* isolates, of which 3 were identified from acute gastrointestinal infections, were sequenced and analyzed to compare their respective LHRs. LHR sequences of each isolate were determined by searching the genomes against the LHR sequence of *E. coli* AW1.7 whole genome shotgun sequence LDYJ00000000 with NCBI Blast. LHR sequences were identified in the genomes of *E. coli* AW1.7, 111, 128, and 8354, whereas it was absent in heat sensitive control isolate 126. For all of the heat resistant *E. coli* isolates, the LHR was chromosomally encoded as opposed to located on a plasmid. Whole genome sequencing confirmed the results from the qPCR assays and phenotypic heat resistance characterization studies discussed in previous chapters. Sequence size of the LHR in each of the isolates differed; AW1.7 was 14,671 bp, 111 was 15,633 bp, 128 was 15,667 bp, and 8354 was 16,910 bp long. A dendrogram generated from the multiple sequence alignment of the LHRs in the 4 heat resistant isolates revealed that the LHRs were 98.3% similar (Figure 5.1). Clinical isolates 111 and 8354 were located on a divergent branch from *E. coli* AW1.7 whereas the LHR of isolate 128 was more similar to isolate AW1.7.

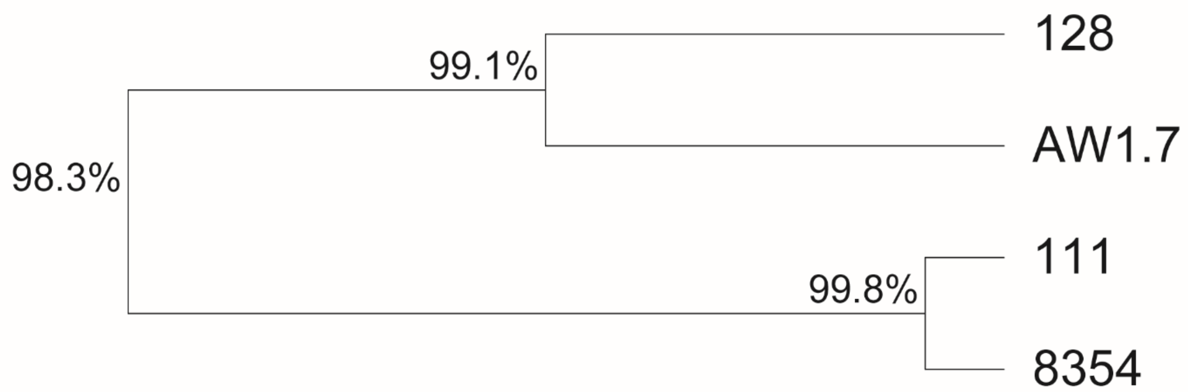

Figure 5.1. Dendrogram with percent similarity between branches generated from multiple sequence alignment of the locus of heat resistance in heat resistant *Escherichia coli* isolates AW1.7, 111, 128, and 8354.

ORFs in the LHR were predicted and annotated for each of the heat resistant isolates. There were 16 putative ORFs predicted in *E. coli* AW1.7, 15 ORFs in isolate 111 and 128, and 18 ORFs in isolate 8354. Pairwise alignment of the predicted ORFs for the clinical isolates compared to those in AW1.7 are indicated in Table 5.3. With the exception of ORF 3, all ORFs in isolate AW1.7 that are also present in the clinical isolates were the same size (bp). In all clinical isolates, ORF 5 encoding an ATP-dependent zinc metalloprotease FtsH (UniProtKB database accession: P0AAI3) was identified (Figure 5.2). This ORF was absent in isolate AW1.7 but it possessed ORFs 6 and 7 that encode for the N-terminal and C-terminal fragments of the same metalloprotease, in its place. Three additional ORFs were identified in isolate 8354 that were not present in any of the other heat resistant isolates. Addition of ORFs 16, 17, and 18 increased the size of the LHR in isolate 8354 by approximately 1,240 bp. ORF 16 was predicted to encode for a zinc binding metalloendopeptidase HtpX homolog (UniProtKB database accession: B8FG65), whereas for ORF 17 no proteins of significant similarity could be predicted on the Blast server against the UniProtKB database. ORF 18 was predicted to be a second copy of ORF 15, which encodes for PsiE, a cell membrane protein involved in the cellular response to phosphate starvation (UniProtKB database accession: P0A7C8).

Table 5.3. Sequence homology of open reading frames in the locus of heat resistance in heat resistant *Escherichia coli* isolates

Open reading frame (ORF)	Isolate			
	Size of ORF (bp)	Percent identity of each ORF relative to AW1.7 unless absent (%)		
	AW1.7	111	128	8354
1	282	98.6	99.3	98.6
2	570	99.8	99.8	99.7
3*	2895	98.8	97.8	98.8
4	192	99.7	99.7	99.2
5†	Absent	1728 bp	99.9	99.8
6	687	Absent	Absent	Absent
7	141	Absent	Absent	Absent
8	459	98.3	99.4	99.4
9	915	99.5	99.7	99.6
10	888	99.3	99.2	99.2
11	612	98	98	98

*Size of ORF 3 in isolates 111, 128, and 8354 differed from isolate AW1.7 as follows: 2928 bp, 2961 bp, 2928 bp, respectively.

†ORF 5 encoding an ATP-dependent zinc metalloprotease FtsH not present in isolate AW1.7. Percent pairwise alignment was determined relative to isolate 111. Size of ORF 5 in isolates 128 and 8354: 1728 bp and 1731 bp, respectively.

‡A second copy of ORF 15 encoding the *psiE* gene present in isolate 8354 as ORF 18.

Table 5.3 continued. Sequence homology of open reading frames in the locus of heat resistance in heat resistant *Escherichia coli* isolates

Open reading frame (ORF)	Isolate			
	Size of ORF (bp)	Percent identity of each ORF relative to AW1.7 unless absent (%)		
	AW1.7	111	128	8354
12	1167	99.3	99.4	99.1
13	441	99.3	98.9	99.3
14	1716	99.5	99.6	99.6
15	498	99.8	99.8	99.6
16	Absent	Absent	Absent	459 bp
17	Absent	Absent	Absent	294 bp
18[‡]	Absent	Absent	Absent	498 bp
19	927	99.2	99.2	99.2
20	1152	99.7	99.7	99.8

*Size of ORF 3 in isolates 111, 128, and 8354 differed from isolate AW1.7 as follows: 2928 bp, 2961 bp, 2928 bp, respectively.

[†]ORF 5 encoding an ATP-dependent zinc metalloprotease FtsH not present in isolate AW1.7. Percent pairwise alignment was determined relative to isolate 111. Size of ORF 5 in isolates 128 and 8354: 1728 bp and 1731 bp, respectively.

[‡]A second copy of ORF 15 encoding the *psiE* gene present in isolate 8354 as ORF 18.

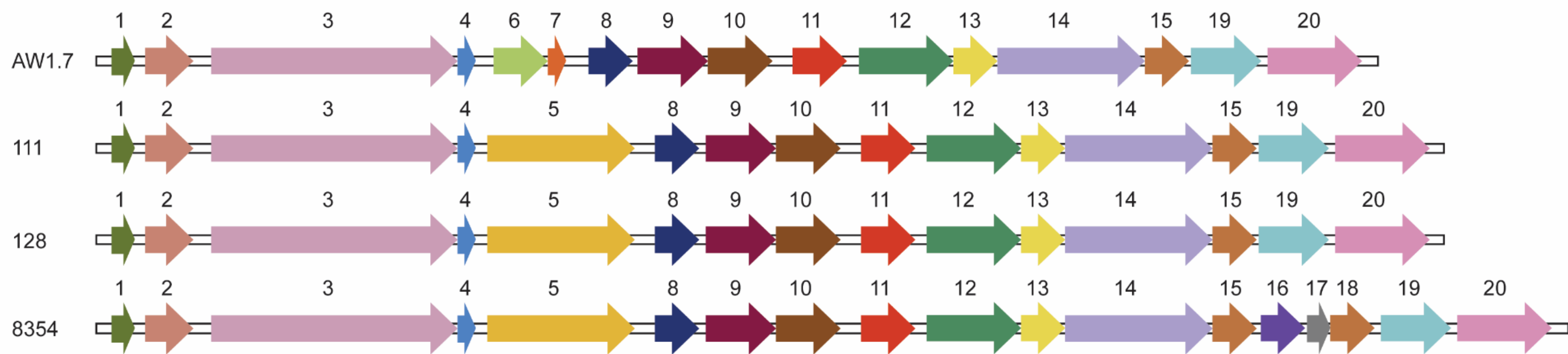

Figure 5.2. Alignment of the locus of heat resistance in *Escherichia coli* isolates AW1.7, 111, 128, and 8354. Homologous open reading frames are shown in the same color.

In all heat resistant isolates, flanking mobile genetic elements were identified. IS5 family transposases were predicted on the 5' and 3' ends of the LHR in each isolate. Two putative promoters for transcription of the LHR in isolate AW1.7 were identified. Promoters that bind to envelope stress transcription regulator EvgA and primary sigma factor RpoD were predicted upstream of ORF 1 and ORF 12, respectively. In the LHR of the clinical, heat resistant isolates 111, 128, and 8354, there were 3 putative promoters found. Like isolate AW1.7, promoters that interact with EvgA and RpoD were predicted but an additional putative promoter upstream of ORF 11 was also identified. It was indeterminable if this promoter interacted with ArgR, ArgR2, or Ihf. A single rho-independent terminator was identified in the LHR of all heat resistant isolates.

5.3.2. Genetic analysis of heat shock- and heat resistance- related genes

Heat shock- and heat resistance- related genes as indicated in Table 5.1 were searched against the 4 heat resistant *E. coli* isolates and heat sensitive isolate 126 using the NCBI Blast server. In both heat resistant and heat sensitive isolates, all genes were present, confirming that heat shock- and heat resistance- related genes in *E. coli* that are not members of the LHR do not mediate heat resistance at temperatures above 60°C.

5.3.3. Generation of ORF 3 clones

ORF 3 from heat resistant *E. coli* isolate AW1.7 was cloned into heat sensitive *E. coli* to determine if heat resistance is conferred by Clp chaperone ClpK using The TOPO Cloning methodology. Production of blunt-end PCR products of ORF 3 was achieved with the addition of SuperFi GC Enhancer (5X) into the PCR reaction tube (Figure 5.3) and TOPO Cloning was performed using the TOPO XL-2 Complete PCR Cloning Kit.

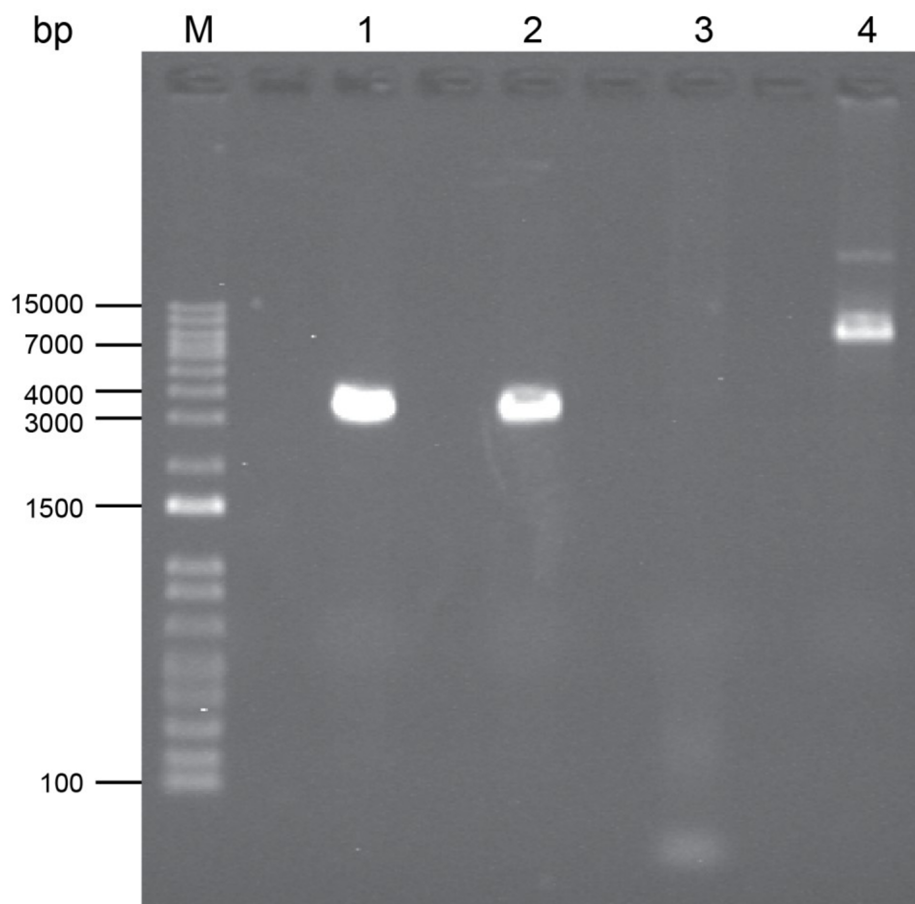

Figure 5.3. 0.8% agarose gel electrophoresis of blunt-end PCR products of ORF 3 from genomic DNA of *Escherichia coli* AW1.7. M, TrackIt 1 Kb Plus DNA Ladder; 1, PCR product with SuperFi GC Enhancer (5X); 2, 1:10 dilution of PCR product with SuperFi GC Enhancer (5X); 3, PCR product without SuperFi GC Enhancer (5X); 4, TOPO XL-2 7 kb control PCR product.

Successful transformation of One Shot OmniMAX 2 T1 Chemically Competent *E. coli* with the ORF 3 recombinant and the pUC19 control plasmid was observed by growth on the LB plates. Of the 48 ORF 3 transformants randomly chosen for subsequent analysis, 46 (95.8%) were positive for ORF 3 by qPCR (Appendix C). To identify transformants with ORF 3 correctly inserted in the plasmid, it was decided that transformants would be arbitrarily selected for Sanger sequencing in groups of 3 until a transformant with an ORF 3 insert in the correct orientation was found. The first group of transformants to be sequenced consisted of transformants 3, 10, and 22. Cq values for ORF 3 by the qPCR assay for these 3 transformants were 9.84, 12.25, and 10.37, respectively. Consensus sequences for ORF 3 in the correct orientation were identified in transformants 10 and 22 (Figure 5.4) but not transformant 3. Pairwise sequence alignments of ORF 3 in transformants 10 and 22 against the ORF 3 sequence in *E. coli* AW1.7 was determined to be 99.24% and 99.37%, respectively.

5'-GCAGGTTTAAACGAATTCGCCCTTATGGCCAGAAAACAATGCCAGGTCTGCGGCCAACCC
GCCACCGTGCGGGTGGAAGCCAATCTCAACGGTCGTCACAGCACCATGCTGTTGTGCGACGAC
CATTACCGGCAATTAGTGCGCCAGCAAAAGCGCACCGTTTCGCCGCTGGAAGCCTTGTTCGGT
TCGCGCAGCGGCCTGTTCGAGGACTTCCTCGGCAGTGACTTCTTCCGCATCGGCGACGACGCG
ACGCCAGTTGCCGCCGATACCGATGACGTGGTCGATGCCTCGTTTGGCGAGCCCGCCGCCGCA
GGTTCGGGTGCGCCGCGCCGTCGCGGCAGTGGGCTGGCCAGCCGCATCAGCGAACAGTCGGAA
GCCTTGTTGCAGGAGGCCGCCAAACACGCTGCCGAATTTGGCCGCTCCGAGGTGGATACCGAA
CATCTGCTGCTGGCGCTGGCCGACAGCGACGTGGTCAAGACCATCCTGGGTCAGTTCAAGATC
AAGGTCGATGACCTCAAGCGGCAGATCGAGTCTGAGGCCAAGCGCGGGGACAAGCCCTTCGAG
GGCGAGATCGGCGTGTCGCCGCGCGTGAAGGATGCGCTCAGCCGCGCCTTCGTGGCCTCCAAT
GAACTCGGCCATTCTTATGTCGGTCCAGAGCATTTCCTGATTGGTCTGGCCGAGGAAGGCGAA
GGGCTGGCCGCCAACCTGCTGCGCCGCTACGGCCTGACGCCGCAGGCGCTGCGCCAACAGGTC
AGCAAGGTGGTCGGCAAGGGCGCCGAGGATGGACGCGCTGAGACGCCGACCAACACGCCAGAA
CTCGACAAGTACTCGCGCGACCTGACCAAGATGGCGCGCGACGGCAAGCTCGACCCGGTGATC
GGCCGCGCGCAGGAGATCGAAACCACCATCGAGGTGCTGGCCCGGCGCAAGAAGAACAACCCG
GTGCTGATCGGCGAGCCGGGTGTGGGCAAGACCGCCATCGTCGAAGGGCTGGCGCAGCGCATG
GTGGCGGGTGAAGTGCCCGAGACCTTGCGCGACAAGCGCCTGGTGGAACTCAACATCAACGCC
ATGGTGGCCGGCGCCAAATATCGCGGCGAGTTCGAGGAGCGCGTGCAGAAGGTGCTGAAGGAG
GTGACCGAGCACCAGGGCGAGCTGATTTTGTTCATCGACGAGGTGCACACCATCGTCGGTGCC
GGCCAGGGCGGTGGCGAAGGCGGGCTGGACGTGGCCAACGTATTCAAACCGATGATGGCGCGC
GGTGAACTCAACCTGATCGGCGCCACGACGCTGAACGAGTACCAGAAATACATCGAGAAGGAT
GCCGCACTGGAGCGGCGCTTCCAGCCGGTGACGGTGCCCGAGCCGACGGTGGCCCAGACCATC
ATGATTCTGCGCGGCCTGCGCGACACCTTCGAGGCGCACCACAAGGTCAGCATCTCCGAGGAC
GCGATCATCGCGGCGGCCGAGTTGTCCGACCGCTACATCACGGCGCGCTTCCTGCCGGACAAG
GCGATCGACCTGCTCGACCAGGCGGCCGCGCGCGTGAAGCTGTCGGCCACGGCCCGGCCGGTG
GCCGTGCAGGAGCTGGAGTCCGAACTGCACCAGCTGCGGCGTGAACAGGATTACGTGGCCGCG
CGCAAGCAGTACGACCAGGCCGCCGAGCTCGGCAAGCGCATCGAAGCCAAGGAGGCCGAGCTC
AAGAAGCTCGTCGAGAACTGGGAGCGGGAGCGGGCCTCCGGCAGTGCAGAGGTCAAGGCGGAA
CACGTGGCGCAGATCGTCTCGCGCCTGACCGGCATCCCGGTCAACGAGTTGACGGTGGAAGAG
CGCGAAAAGCTGCTGCACTTGGAACAACGGCTGCACGAGCGCCTGGTGGGACAAGACGAGGCG
GTCCGTGCCGTGGCCGATGCCGTGCGGCTGTCCCGCGCCGGCCTGCGCGAAGGCAGCAAACCG
GTGGCTACCTTCCTGTTTCTGGGCCCGACCGGGGTGGGCAAGACCGAGCTCGCCAAGGCATTG
GCCGAATCGATCTACGGCGATGAGCACGCCCTGTTGCGCATCGACATGTCGGAATATGGCGAA
CGCCATACCGTGGCGCGGCTGGTGGGCGCGCCTCCGGGCTATGTCGGTTACGACGAAGGCGGT
CAGCTCACCGAGAAGGTGCGGCGCAAGCCCTACAGCGTGCTGCTGCTCGACGAGATCGAGAAG
GCACACCCTGACGTCTACAACATCCTGCTGCAAGTGTTCGACGACGGTCGCCTCACCGACGGC
AAGGGCCGGGTGGTGGATTTCACCAACACCATCATCATCGCCACGTCCAACCTGGGTTCGGAC
ATCATCCAGCGACGGCTGAAGGCGCGTGGGGCGGCCGACGAGGAGTACGAAAAGACCAAGGCC
GAGGTCATGGACGTGCTGCGCGGCCACTTCCGGCCCGAGTTCCTCAACCGCATCGACGAGATC
ATCGTCTTCCATGCGCTGGGTAAGGAGGAGATCCGCCACATCGTCGGCCTGCAGCTCGATCGC
GTGGCGCGCAGCGCCGCCAGCCAGGGCGTGACGCTCACTTTCGATCAGACGCTGATCGACCAT
TTCGCGGAAGAAGGCTACAAACCCGAGTTCGGTGCGCGTGAACTCAAGCGCCTGATCCGCAGC
GAGCTGGAAACTGCGCTGGCGCGCGAGATGCTCGGTGGCGGCATCGGCAAGGGCGATCACGCC
AGCGCACGCTGGGACGACAAGGCCGAACGCGTGGTGTTCGAACGCAAAGAGCCACTGCAGACC
CCGGCCGAGCCGGAGCAGCCGGATGCCGCGAAGGCGACCGAGACGCCGCACGGCGACGCTGGC
AAAGGCTCGCGCAAGAAGAAGTCGGCGAGCGACGCATCTTGATGAAGGGCGAATTCGCGGCCG
CTAAATTCAATTCGCCCTATAGTGAGTCGTATTACAA-3'

Figure 5.4. Consensus sequence from One Shot OmniMAX 2 T1 Chemically Competent *Escherichia coli* transformants 10 and 22 of open reading frame (ORF) 3 encoding ClpK. Nucleotides are formatted based on their corresponding sequence source or primer sequences: boxed, TOPO XL-2 plasmid sequences flanking 5' and 3' ends of the ORF 3 insert; dark green, TOPO XL-2 forward cloning primer; light green, TOPO XL-2 reverse cloning primer; underlined, ORF 3 qPCR forward primer; orange, ORF 3 qPCR probe; red, ORF 3 qPCR reverse primer; pink, Sanger sequencing forward primers (n=4); blue, Sanger sequencing reverse primers (n=4).

In addition to sequencing, protein gel electrophoresis and protein mass fingerprinting were conducted for the 3 transformants. In transformants 10 and 22, a potential doublet at approximately 100 kDa was observed (Figure 5.5). However, these 2 bands lacked the resolution of the doublet in *E. coli* AW1.7 at the same molecular weight. Bands with a molecular weight of 100 kDa in transformants 3, 10, and 22 were excised and identified by protein mass fingerprinting. From the protein database, the ClpK protease (NCBI reference sequence: WP_004152116.1) was identified in transformants 10 and 22 but not in transformant 3 as suspected. Using I-TASSER, the tertiary protein structure of ClpK (Figure 5.6 A) was predicted to consist of helixes, strands, and coils with a confidence score (C-score) of -1.50 (range -5 to 2). Against the Protein Data Bank (PDB) library, the tertiary structure of ClpK had the closest structural similarity to *Saccharomyces cerevisiae* heat shock protein 104 (hsp104): casein complex, middle domain conformation (PDB ID: 5vy9A) (Figure 5.6 B) with a similarity score (TM-score) of 0.818 (range of 0 to 1). A coverage of 82.7% in the aligned regions of ClpK to *S. cerevisiae* hsp104: casein complex, middle domain conformation was calculated. A ligand binding complex for adenosine diphosphate (ADP) in ClpK was predicted with a C-score of 0.54 (range of 0 to 1) (Figure 5.6 C) and no enzyme active sites were identified. Using Phobius, no membrane helices were predicted to be present in ClpK.

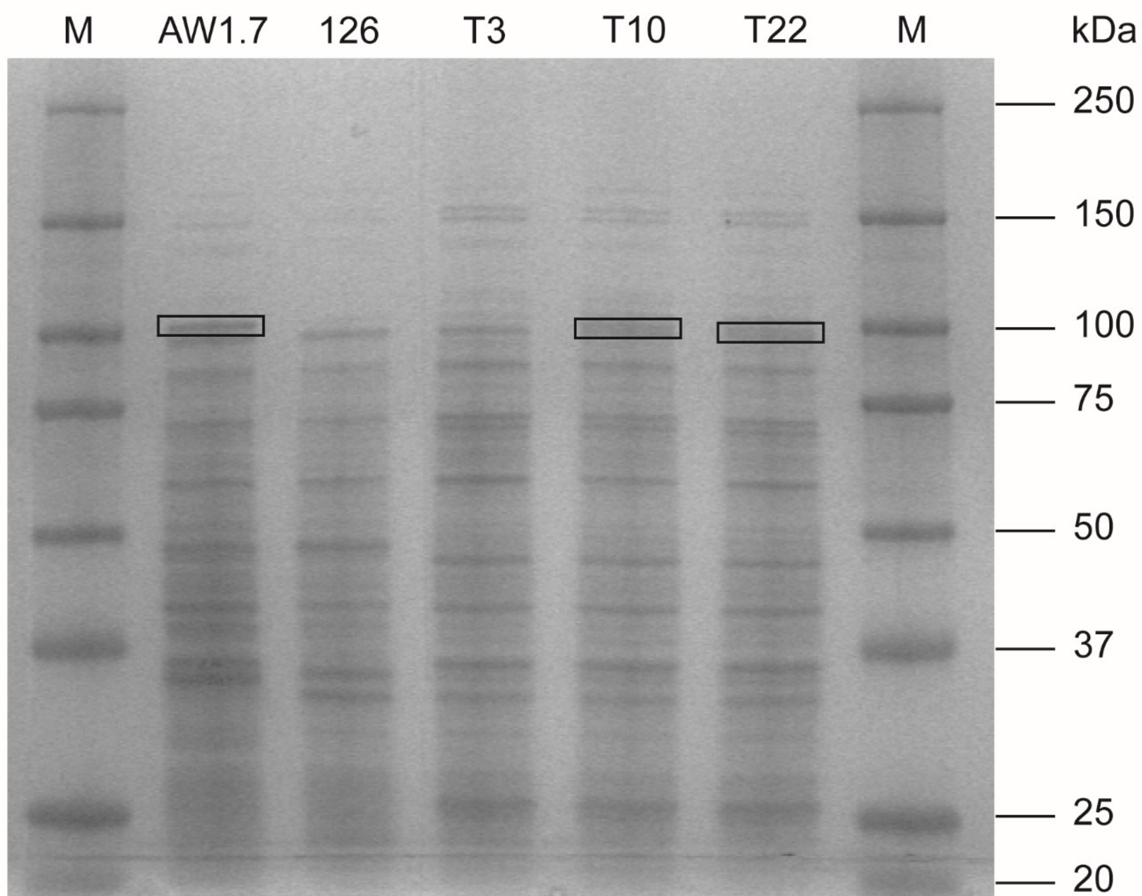

Figure 5.5. sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates of *Escherichia coli* cultured in LB broth with 0% additional NaCl. M, Bio Rad Precision Plus Protein Standard; T3, transformant 3; T10, transformant 10; T22, transformant 22. All bands observed at 100 kDa from transformants 3, 10, and 22 were excised and digested for mass spectrometry protein identification by the Alberta Proteomics and Mass Spectrometry Facility.

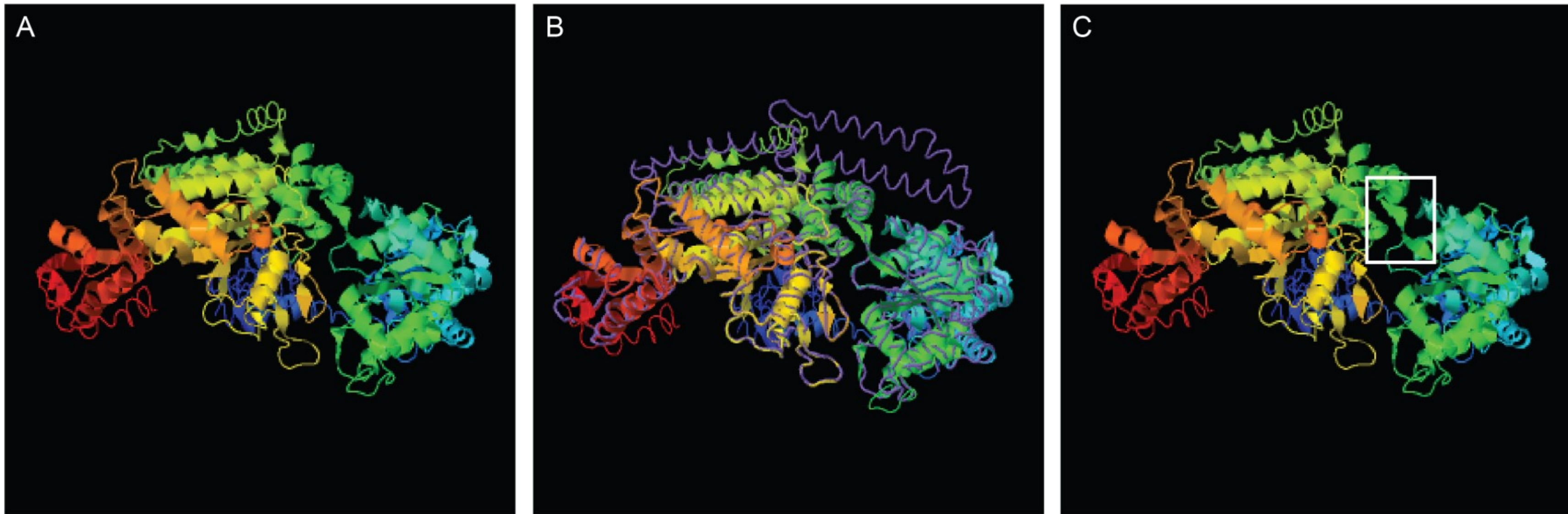

Figure 5.6. I-TASSER protein modeling of ClpK protein (NCBI reference sequence: WP_004152116.1) encoded by open reading frame 3 in transformants 10 and 22. (A), predicted tertiary structure of ClpK model; (B), structural alignment and superposition of predicted ClpK model with protein of closest structural similarity from the Protein Data Bank library, *Saccharomyces cerevisiae* heat shock protein 104 (hsp104):casein complex, middle domain-conformation. Purple trace, *S. cerevisiae* hsp104 protein model; (C), white rectangle, predicted binding area for adenosine diphosphate in predicted ClpK model.

Phenotypic heat resistance at 60°C was screened in all 48 ORF 3 transformants and the pUC19 plasmid control transformant with *E. coli* isolates AW1.7 and 126 serving as the positive and negative controls, respectively. All isolates grew from the unheated aliquots but recovery was only observed in heat resistant *E. coli* isolate AW1.7 after heat exposure for 10, 15, and 30 minutes at 60°C. No inherent heat resistance was observed in the One Shot OmniMAX 2 T1 Chemically Competent *E. coli* with the pUC19 plasmid nor was any conferred by ORF 3 in the transformants.

5.4. Discussion

The emergence of heat resistant *E. coli* involved in human illnesses brings forth concerns of its risk to the food processing industry and public health. Through acquisition of the LHR, foodborne pathogens can survive thermal inactivation measures at temperatures of 60°C and above. Human enteric infection with *E. coli* is largely attributed to the EHEC pathotype, which already possesses virulence factors such as Shiga toxins and the LEE to cause serious illnesses. Horizontal transfer of the LHR into EHEC can result in the survival of these strains at multiple points of the farm-to-fork continuum where they would typically be eliminated through lethal heat processes, resulting in the potential for greater incidence of acute gastrointestinal disease.

Evidence of the LHR conferring heat resistance has been observed in *Enterobacteriaceae* bacteria such as *K. pneumoniae* (10), *C. sakazakii* (12), and most notably, non-pathogenic *E. coli* (13). The identification of heat resistant *E. coli* implicated in human infection is a novel finding and characterization of these isolates at both a genetic and phenotypic level is necessary to gain a comprehensive understanding of the threat these organisms may pose on food safety and the healthcare system. In Chapter 3, differences in the extent of heat resistance conferred by the LHR

in the 4 heat resistant *E. coli* isolates was observed. Analysis of the genomes revealed that all 3 of the clinical, heat resistant isolates possessed at minimum, the full length LHR comprised of 16 ORFs as described in *E. coli* AW1.7 by Mercer *et al*. (13). The LHRs in the clinical, heat resistant isolates were highly similar to that of *E. coli* AW1.7. However, there were notable differences shared among the LHR of the clinical isolates that were not present in the LHR of *E. coli* AW1.7. One key addition in the clinical isolates was the presence of ORF 5, which encodes for an ATP-dependent zinc metalloprotease, FtsH. In *E. coli* AW1.7, this ORF is incomplete and in its place are 2 separate ORFs that encode for only the N- and C- terminals of the metalloprotease. The gene encoding the FtsH metalloprotease is however, present in a 19 kb variant of the LHR identified in a heat resistant *E. coli* raw milk cheese isolate (17). ATP-dependent proteases such as this one are involved in protein quality control and regulation (46) through unfolding, remodeling, and disaggregation of proteins (47). FstH interacts with membrane proteins such as SecY, YccA, and notably RpoH in instances of incomplete or improper assembly of their corresponding complexes (48), but is not unique to the LHR as it is an essential protein for cell viability in *E. coli* (49). SecY and YccA are involved in protein secretion and inhibition of FtsH, respectively, and do not play a role in the response to heat shock. However, RpoH is an alternative RNA polymerase sigma factor that is critical in the regulation of expression of heat shock genes during exponential growth (50,51). RpoH is one of 7 sigma factors in *E. coli*, which enable specific binding of RNA polymerase to gene promoters in response to different environmental stressors (52). In situations of heat shock, intracellular levels of RpoH increase and then associate with RNA polymerase to form a holoenzyme and initiate transcription of heat shock genes to maintain membrane functionality and homeostasis (53,54). RpoH is rapidly degraded after heat shock and transcription of heat shock genes similarly follow suit, facilitating a coordinated response to heat shock only

occurs as necessary (55). Beyond heat shock response, RpoH is also involved in the response to ethanol, basic pH changes, carbon starvation, and osmotic shock (56–59). Although the presence of ORF 5 in the LHR of the clinical isolates suggests involvement in turnover of misfolded proteins and heat shock response, no similarities in D_{60}-values between the clinical isolates distinguished their heat resistance from that of *E. coli* AW1.7. FtsH is not the only protease encoded by the LHR, ORFs 19, and 20 that are present in all isolates also encode for proteases (11) that likely play a role in facilitating heat resistance in the isolates. Interestingly, increased survival of the clinical isolates compared to *E. coli* AW1.7 was observed in ground beef patties that were grilled to an internal temperature of 60°C (Chapter 3). It is possible that the addition of ORF 5 in the clinical isolates may contribute to heightened heat resistance when inoculated in a solid matrix opposed to in liquid culture media. Heat resistant, clinical isolate 8354 possessed 3 additional ORFs in its LHR of which 2 were predicted to encode for a zinc binding metalloendopeptidase HtpX homolog and a second copy of the PsiE protein. In collaboration with FtsH, HtpX is a heat-inducible metalloprotease that functions in elimination of misfolded and incorrectly assembled proteins. However, HtpX unlike FtsH does not possess ATPase activity (60). Transcription of the *psiE* gene is induced in conditions of phosphate, carbon, or nitrogen starvation (61), and may play a role in the multi-stress response conferred by the LHR in addition to heat resistance (62,63). Truncation of the gene encoding PsiE (ORF 15) in *E. coli* AW1.7 did not alter heat resistance (11) and its redundancy in isolate 8354 may similarly contribute little to survival at high temperatures.

Genetic analysis of the LHR in all 4 heat resistant isolates demonstrates that the sequences are highly similar. Multiple sequence alignment confirmed that differences in the LHR between the clinical isolates and *E. coli* AW1.7 are attributed to DNA insertions including ORFs 5, 16, 17 and 18. The third putative promoter predicted upstream of ORF 11 identified in all clinical isolates

is most likely to interact with ArgR, ArgR2, or Ihf. Both ArgR and ArgR2 serve to repress arginine synthesis (64) whereas Ihf functions in genetic recombination, and transcriptional and translational control of DNA (65). It is most plausible that the LHR in the clinical isolates was acquired by horizontal gene transfer from heat resistant *E. coli* as opposed to other genera of the *Enterobacteriaceae* family; the number of putative transposases in heat resistant *E. coli* isolates are limited to 2 whereas upwards of 5 have been reported in heat resistant *K. pneumoniae* and *C. sakazakii* (10,12). Another similarity in the LHR shared between the clinical isolates and *E. coli* AW1.7 that differs from heat resistant bacteria of other genera is that the LHR is encoded on the chromosome instead of being located on a plasmid.

There is substantial evidence that the presence of the LHR can be used as a predictor for heat resistance at temperatures above 60°C in *E. coli* (13,17,66). This hypothesis is further supported by the identification of genes that mediate heat shock and heat resistance at lower temperatures in both heat resistant isolates and heat sensitive isolate 126. The genetic analysis supporting the contribution of the LHR to heat resistance, however, does not completely explain the differences in the D_{60}- and D_{71}- values observed in the heat resistant isolates presented in Chapter 3, suggesting that the extent of heat resistance may be strain specific. Pleitner *et al*. (63) proposed that dynamic interplay between heat and osmotic stress affects heat resistance and ribosome stability in *E. coli* AW1.7 at 60°C through the accumulation of compatible solutes, which may also be the case in the clinical, heat resistant isolates. Future studies to quantify compatible solutes and analyze ribosome denaturation after heat exposure can be employed to explore the intricacies of how phenotypic heat resistance in LHR-positive isolates differ in conditions with and without osmotic stress.

The functional properties of the ORFs encoded by the LHR can be elucidated by the cloning of selected single ORFs or portions of the LHR and determining if heat resistance is conferred. In attempts to clone individual portions of the LHR (ORFs 1 to 8, ORFs 9 to 11, ORFs 12 to 20) from *E. coli* AW1.7 into heat sensitive strains, no resultant heat resistance was expressed (13). Cloning of ORFs 1 to 11 from the LHR of *E. coli* AW1.7 though, did result in heat resistance close to that of wild type *E. coli* AW1.7 (11,66). Similarly, cloning studies of heat resistant *K. pneumoniae* and *C. sakazakii* have also been conducted (12,18). Successful transformation of heat sensitive *E. coli* with ORF 3 and a portion of the LHR encoding ORFs 8 to 11 from heat resistant *K. pneumoniae* and *C. sakazakii*, respectively, resulted in an increase in heat resistance (12,18). However, the levels of heat resistance conferred in the transformants were comparably lower than that of the wild type *K. pneumoniae* and *C. sakazakii*. In efforts to determine if heat resistance can be similarly conferred by ORF 3 from the LHR of *E. coli* as was reported in heat resistant *K. pneumoniae*, a transgenic strain expressing ClpK from *E. coli* AW1.7 was generated. Interestingly, heat resistance was not observed in the transformants despite expression of the ClpK protein. Bojer *et al*. (18) reported that transformants with ORF 3 from heat resistant *K. pneumoniae* only expressed heat resistance if the competent cells chosen encode for the ClpP protease, suggesting that interaction between the 2 Clp proteins is necessary. To account for this prerequisite, the One Shot OmniMAX 2 T1 Chemically Competent *E. coli*, which are *E. coli* DH5α derivatives, in the TOPO XL-2 Complete PCR Cloning Kit were confirmed by the manufacturer to natively encode for ClpP prior to use in the cloning of ORF 3 from heat resistant *E. coli* AW1.7. Thus, despite the presence of ClpK and ClpP in the transformants, their inability to survive lethal heat inactivation may propose that heat resistance mediated by the components of the LHR operates differently between genera.

Current knowledge on heat resistance mediated by the LHR has largely been limited to non-pathogenic strains of *E. coli* and other members of the *Enterobacteriaceae* family. To gain a more comprehensive understanding of the composition and function of the LHR in heat resistant, clinical *E. coli* isolates associated with cases of acute gastroenteritis, whole genome sequencing and cloning were utilized. Genetic analysis suggests that the larger LHR variant found in heat resistant, clinical *E. coli* isolates may be more commonly acquired by virulent strains of the foodborne pathogen as opposed to the 14 kb LHR identified in non-pathogenic, environmental isolate AW1.7. The circulation of heat resistant *E. coli* in the farm-to-fork continuum emphasizes the potential need for improvements to pathogen intervention processes in food processing and increased diligence in consumer cooking behaviours. Going forward, efforts to identify and characterize heat resistant *E. coli* from cases of foodborne infection will allow for more accurate estimates on its potential as an emerging food safety threat.

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Chapter 6

Discussion and future directions

6.1. Discussion

Shiga toxin-producing *Escherichia coli* (STEC) is the causative agent of numerous sporadic foodborne infections and outbreaks worldwide. Gastrointestinal infection with STEC is associated with serious consequences including hemolytic uremic syndrome, especially in pediatric and immunocompromised individuals (1). Despite the significant burden of STEC on public health, treatment options are limited to supportive therapies such as oral rehydration and intravenous hydration (2–4). Furthermore, antibiotics to treat STEC infection have been met with controversy as their administration may induce further production of the Shiga toxins (5). Currently, there is no human vaccine against STEC. Cattle vaccines against *E. coli* O157:H7 have received regulatory approval in Canada and the United States of America but their use has not been adopted by the cattle production industry due to low economic incentive (6). As a result, much of STEC elimination and patient management relies on the interventions in place in the food processing industry and public health, respectively. Due to its zoonotic origin and presence in cattle particularly (7), pathogen elimination processes in the food processing industry are critical for the prevention of STEC from reaching consumers and potentially causing foodborne disease. Thermal inactivation is the primary method to eliminate STEC from contaminated animal carcasses and meat products along multiple stages of the farm-to-fork continuum, with temperatures of different treatments ranging from 15°C to 95°C (8). However, limitations in sampling and testing for adulterants in the food processing industry can occasionally result in STEC reaching the consumer. To manage foodborne STEC infections, rapid and comprehensive laboratory diagnosis and strain characterization, outbreak investigation, and surveillance are important.

The genomic diversity and plasticity of *E. coli* are two key drivers of pathogenicity in this organism (9). Horizontal gene transfer has facilitated the acquisition of a large proportion of virulence factors in *E. coli*, STEC notwithstanding. Both the Shiga toxins and the locus of enterocyte effacement, two important virulence factors of STEC pathogenicity, are acquired from horizontal gene transfer (10,11). Thus, the emergence of STEC possessing new combinations of virulence factors is a continual concern. STEC has generally been considered a heat sensitive pathogen by the food processing industry and the World Health Organization (12). However, the recent emergence of non-pathogenic *E. coli* strains possessing the locus of heat resistance (LHR) is a novel threat to food safety. Acquired by horizontal transfer, the LHR confers heat resistance to temperatures of 60°C and above. The circulation of heat resistant *E. coli* in food processing environments (13,14) necessitates the determination of whether heat resistant STEC exist and are involved in human foodborne infection. This thesis presents the novel identification of clinical STEC isolates possessing the LHR implicated in cases of acute gastroenteritis and provides a detailed genetic and phenotypic investigation of their survival mechanisms upon exposure to various environmental stressors.

Methods to detect heat resistant organisms prior to identification of the LHR were restricted to phenotypic screening by water baths (15) or thermal plates (16). Such methods involve the exposure of bacterial cultures to specific temperatures, typically 60°C and above, for a certain time interval followed by plating of the heated cultures onto solid agar media for enumeration of survivors. However, phenotypic screening for heat resistant organisms is inefficient and labor intensive, especially for large collections of isolates. Upon identification and whole genome sequencing of heat resistant *E. coli* isolate AW1.7, end-point polymerase chain reaction (PCR) assays were developed for detection of the LHR (13). To further improve upon the genetic methods

for identification of LHR-positive *E. coli* in the scope of translational research, I developed 3 quantitative real-time PCR (qPCR) assays to detect the LHR (**Chapter 2**). These 3 assays utilize hydrolysis probes targeting open reading frames (ORFs) 3, 8, and 11 of the LHR and were validated with *E. coli* AW1.7. This work was published according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (17). The qPCR assays offer the benefits of a shorter turn-around-time and ability to screen a larger proportion of isolates at one time compared to the existing end-point PCR assays. Furthermore, I tested the qPCR assays against a specificity panel of common Gram positive and Gram negative pathogens and determined that in addition to *E. coli*, they can also be used to detect the LHR in β- and γ- Proteobacteria. To elucidate if heat resistant *E. coli* have contributed to human infection, 613 clinical *E. coli* isolates submitted to Alberta Precision Laboratories – Provincial Laboratory from 2009 through 2014 were screened with the qPCR assays. Three isolates, two being STEC, were determined to possess the LHR and were confirmed for phenotypic heat resistance by heat treatment with a water bath. Following publication of this study, the qPCR assays have since been utilized by Alberta Agriculture and Forestry, Agriculture and Agri-Food Canada (16,18), and AgResearch Ltd. in New Zealand (data not published) to identify heat resistant *E. coli* in the cattle processing industry. This study is the first publication in the field of heat resistant *Enterobacteriaceae* that identifies *E. coli* isolates harboring both the LHR and Shiga toxins associated with human diarrheal disease.

Following the identification of the 3 clinical, heat resistant *E. coli* isolates, **Chapter 3** aimed to characterize their heat resistance upon exposure to temperatures of 60°C and 71°C. Considering heat inactivation is the primary method to reduce and eliminate STEC in food processing and consumer consumption, determination of decimal reduction times (D-values) for different foodborne pathogens is critical. D-values are a key parameter in food safety that represent

the heating time required to reduce 90% of the existing microbial population at a specific temperature (19). D_{60}-values of *E. coli* are generally reported to be below 1 minute (15), supporting the claim that STEC are heat sensitive. However, the LHR confers exceptionally high levels of heat resistance; D_{60}-values of the heat resistant, clinical isolates all exceeded 10.20 minutes and in 2 of the isolates, was amplified in conditions of increasing osmotic stress. Prior to this study, literature on D_{71}-values for *E. coli* was limited despite 71°C being a commonly recommended safe internal cooking temperature for beef (20). In this study, I determined the D_{60}- and D_{71}- values of the heat resistant, clinical isolates in a liquid medium and further characterized their survival in a food matrix of ground beef to investigate the threat of heat resistant *E. coli* in food consumption (21). Cell reductions of heat resistant isolates in ground beef patties grilled to 60°C and 71°C were 2.84 and 4.95 log colony forming units (CFU)/mL, respectively, compared to reductions of 6.08 log CFU/mL and greater in heat sensitive *E. coli*. Transmission electron microscopy was used to gain a deeper understanding of the LHR in regards to phenotypic and morphological changes that heat resistant isolates undergo during exposure to high temperatures. Transmission electron micrographs revealed that the heat resistant isolates retain their cell envelopes and cytoplasm density following heat treatment at 60°C whereas heat sensitive *E. coli* display significant wrinkling of the cell envelope and leakage of cellular contents, indicative of cell death. These findings confirm the predicted functions encoded by the LHR including protein homeostasis and cell envelope stress maintenance at a phenotypic level (22). Preliminary investigation of protein expression exclusive to LHR-positive isolates is also presented in this study. Constitutive expression of novel Clp chaperone ClpK, encoded by ORF 3 of the LHR, was observed in the heat resistant isolates and laid the groundwork for further proteomic analysis of the LHR that is presented in **Chapter 5** of this thesis. Overall, this study provides new evidence supporting the

hypothesis that the LHR facilitates interplay between heat and osmotic resistance (13,23) and highlights the potential for heat resistant STEC to be a novel threat to food safety.

Despite the effectiveness of thermal inactivation processes, *E. coli* biofilms continue to be a persistent source of contamination in food processing environments (24). Biofilms exhibit high levels of resistance to antimicrobials and disinfectants (25), both of which comprise commonly used compounds for pathogen elimination and sanitation in the food processing industry (26,27). The biofilm forming potential of heat resistant *Enterobacteriaceae* isolated from food processing plants and hospital environments has been explored (16,28,29). However, since this thesis is the first documentation of heat resistant STEC, their capacity to form biofilms is currently unknown. The aims of this study (**Chapter 4**) were to investigate biofilm formation in the 3 heat resistant, clinical *E. coli* isolates by using an in-house, two-component apparatus and characterize the presence or absence of biofilm formation-associated genes in each of the isolates. Biofilms are not regarded as an essential virulence factor for STEC survival and pathogenesis (3), suggesting that the biofilm forming potential of the clinical *E. coli* isolates is likely weaker than that of environmental *E. coli* strains (30). However, if STEC are capable of biofilm formation, it may increase their survival in food processing environments and potentially contribute to its transmission. To investigate this, I included 3 heat resistant, environmental *E. coli* isolates obtained from a food processing plant and wastewater treatment plant to compare biofilm formation between LHR-positive *E. coli* from clinical and environmental sources. After preliminary screening for biofilm formation, I conducted experiments using the two-component apparatus to determine the optimum conditions for biofilm formation in each of the heat resistant isolates by manipulating inoculum size, nutrient concentration, and temperature conditions. Biofilm formation in the heat resistant isolates was detected at temperatures of 24°C and 37°C but not at

4°C. Furthermore, biofilm formation was observed in all 3 environmental isolates but only one clinical isolate despite the overlap in biofilm formation-associated genes encoded by the isolates from both sources. This study provides evidence that regardless of the LHR, environmental isolates are more proficient at biofilm formation than clinical isolates likely as a result of adaptation to various stress conditions they have previously encountered in their respective environmental niches. The two-component apparatus measures biofilm formation through the use of a 96 well PCR plate that is laid on top of a microplate inoculated with bacterial culture. Motile bacteria form biofilms on the pegs of the PCR plate that are submerged in the microplate wells, which are then stained with crystal violet. This method offers the advantage of preventing over-estimation of biofilm formation that can occur with conventional biofilm procedures that measure static biofilm formation on the bottom of a microplate well (31). Furthermore, the two-component apparatus is an affordable alternative to the commercially available MBEC Assay/Calgary Biofilm Device (32) for research groups that seek to determine biofilm formation in Gram positive and Gram negative organisms. In conclusion, this study further characterizes the multi-stress tolerance capabilities of the heat resistant, clinical isolates in conditions related to food processing, signifying that such strains in circulation may be a serious food safety and public health risk.

In **Chapter 3**, the phenotypic characteristics of heat resistant *E. coli* were investigated. However, significant differences in D_{60}-values between the isolates were observed, necessitating genetic analysis of the LHR and their respective genomes (**Chapter 5**). The current consensus is that organisms harbouring the LHR are capable of surviving lethal heat exposure at 60°C but variants of the LHR in different genera and strains have been shown to influence the extent of heat resistance conferred (22,28,33,34). In this study, the two objectives were to characterize the genetic elements mediating heat resistance in each of the 3 clinical, heat resistant isolates and to

investigate the contribution of ORF 3 of the LHR on heat resistance in *E. coli*. To complete the first objective, I conducted comparative genetic analysis of the LHR and other heat shock and heat resistance mechanisms by utilizing whole genome sequencing. The LHR was determined to be chromosomally encoded in all 3 clinical isolates as opposed to located on a plasmid. Interestingly, phylogenetic analysis revealed that the LHR in the clinical isolates are located on a divergent branch from environmental *E. coli* isolate AW1.7, which was also isolated in Alberta. This finding is attributed to differences in the number of putative ORFs encoded by the LHR in the clinical isolates from *E. coli* AW1.7. Comparative genetic analysis also showed that heat resistant and heat sensitive *E. coli* strains possess the same heat shock- and heat resistance- associated genes that mediate the stress response at lower temperatures, indicating that the LHR is the main contributor to heat resistance at temperatures above 60°C. In **Chapter 3**, I published data from protein gel electrophoresis experiments showing that novel Clp chaperone ClpK (encoded by ORF 3 of the LHR) is constitutively expressed in heat resistant *E. coli* isolates. Cloning and proteomic studies on heat resistant *Klebsiella pneumoniae* report that expression of ClpK is all that is required to confer heat resistance at 60°C (29). It is unknown if acquisition of ORF 3 alone from heat resistant *E. coli* is similarly sufficient. Thus, I generated a transgenic *E. coli* strain expressing ClpK from heat resistant *E. coli* AW1.7 and evaluated its phenotypic heat resistance. I concluded that unlike ClpK from heat resistant *K. pneumoniae*, the entirety of the LHR is required to confer heat resistance in the transgenic strains of *E. coli* included in this study, suggesting that components of the LHR operate differently between genera. This study contributes valuable findings on the LHR of heat resistant, clinical *E. coli* isolates circulating in Alberta. ORF 5 is present in the LHRs of the clinical isolates identified in Alberta and in the LHR of heat resistant *E. coli* isolated from

Swiss raw milk cheese (28,35), suggesting the possibility that this variant of the LHR may be circulating in the province.

In conclusion, this thesis expands on the breadth of knowledge in the field of heat resistant *Enterobacteriaceae* by linking heat resistant *E. coli* circulating in the environment with human infection. Heat resistant pathogens in multiple environmental niches have been reported thus far, but with no connection to human disease. The identification of clinical *E. coli* isolates that possess the LHR illustrates the threat they pose on food safety at various points in the farm-to-fork continuum. Further study on the contribution of heat resistant *E. coli* in human foodborne infection may potentially identify new sources of contamination and shortcomings in current pathogen inactivation methods used in the food processing industry.

6.2. Significance

This thesis presents the novel identification of heat resistant, clinical *E. coli* isolates implicated in human diarrheal disease possessing the LHR. Furthermore, 2 of the 3 isolates characterized in this work are STEC, highlighting the potential emergence of multi-stress tolerant, pathogenic *E. coli*. In this work, 3 in-house qPCR assays are described for the rapid detection of the LHR in *E. coli* that vastly improve on the current screening methods for heat resistant organisms. Implementation of these assays in food processing and public health systems can provide a more comprehensive understanding of the threat heat resistant *E. coli* pose on food safety and human foodborne infection. For instance, *E. coli* possessing the LHR can be considered in cases where patients are exposed to well-cooked foods as a potential source of infection. The ability for heat resistant isolates to survive heat exposure at temperatures of 60°C and 71°C in

various matrices suggests they may persist in food processing environments and withstand cooking practices, suggesting re-evaluation of current pathogen elimination methods may be necessary. Biofilm formation in heat resistant *E. coli* further exacerbates their threat on food safety. In addition to their ability to withstand thermal inactivation processes, biofilms comprised of heat resistant *E. coli* may resist antimicrobial and disinfectant treatments used in plant sanitation and become a persistent source of contamination in food processing plants. Pathogenic *E. coli* are constantly evolving to evade human interventions and expand their gamut of virulence factors to cause disease, emphasizing the importance of continual efforts to improve microbiological elimination methods in food safety and diagnostic identification and characterization of foodborne pathogens.

6.3. Future Directions

The genetic and phenotypic features of heat resistance conferred by the LHR are thoroughly investigated in this thesis. One interesting observation discussed in this work is the differences in the D_{60}- and D_{71}- values observed in the heat resistant isolates. Genetic variations in the LHR of the isolates may contribute to influencing the extent of heat resistance between the isolates but each isolate's ability to accumulate compatible solutes during heat stress may also be a factor. Accumulation of compatible solutes was determined to affect heat resistance and ribosome stability in *E. coli* AW1.7 (23), suggesting some form of interplay between heat and osmotic stress at 60°C. It is possible that the clinical, heat resistant isolates accumulate compatible solutes of varying amounts and composition, thus influencing their heat resistance at higher osmotic conditions. Experiments that can provide more insight on how heat and osmotic resistance influence the survival of the clinical isolates after 60°C heat exposure include quantification of

compatible solutes by nuclear magnetic resonance with a cold probe and analysis of ribosome denaturation by differential scanning calorimetry.

In this thesis, biofilm formation in heat resistant *E. coli* was investigated on polypropylene surfaces. Further studies to determine the ability of the isolates to produce biofilm on different surfaces such as polystyrene and stainless steel, both of which are relevant in food processing (36,37), would prove valuable. Polystyrene 96 well plates are available commercially and can be easily adapted for use with the two-component apparatus. However, detection of biofilms on stainless steel is more difficult. Biofilm detection on stainless steel surfaces have historically used coupons that are typically 25×25 mm ± 1 mm in size, making it difficult to obtain reproducible results due to its small surface area. However, stainless steel equipment and surfaces are abundant in food processing plants, justifying the need to explore biofilm formation on this material.

The ability of *E. coli* to enter a viable but non-culturable (VBNC) state has been explored in environmental settings such as river water and in raw produce (38,39). It is unknown if heat exposure at 60°C induces heat resistant *E. coli* to enter a VBNC state, which may further complicate their presence in the food processing industry. Future studies to determine if VBNC bacteria are induced by heat treatment can be achieved by staining and quantifying live and dead *E. coli* using the LIVE/DEAD Baclight assay and fluorescent microscopy. Another method worth considering for differentiation of live and dead *E. coli* is the use of propidium monoazide (PMA) in viability PCR. Similar to how propidium iodide is used in the LIVE/DEAD Baclight assay, PMA is a photo-reactive DNA-binding dye that preferentially binds to double stranded DNA released by dead cells (40,41). Viability PCR utilizes qPCR instrumentation with the additional steps of application of PMA to the cells and activation of the dyes by intense visible light (42). Use of viability PCR to differentiate live and dead STEC has been demonstrated by Jones (42) and

this technology can also be applied to determination of VBNC *E. coli* in heat resistant isolates. The CFU of heat treated isolates can be calculated from viability PCR and spread plating on solid agar media with the difference between the two representing the proportion of VBNC *E. coli*. Benefits of viability PCR include a more rapid and accurate quantification compared to the LIVE/DEAD Baclight assay.

Lastly, this thesis identified 3 (0.5%) heat resistant, clinical *E. coli* isolates from a collection of 613 isolates. Since the current positivity rate for the LHR in clinical isolates is low, it is difficult to claim that heat resistant *E. coli* is an immediate cause of concern in foodborne infection. Routine screening for the LHR in clinical isolates submitted to Alberta Precision Laboratories – Provincial Laboratory can provide a more accurate estimation of the contribution of heat resistant *E. coli* in human infection and whether the rate of horizontal transfer of the LHR is increasing.

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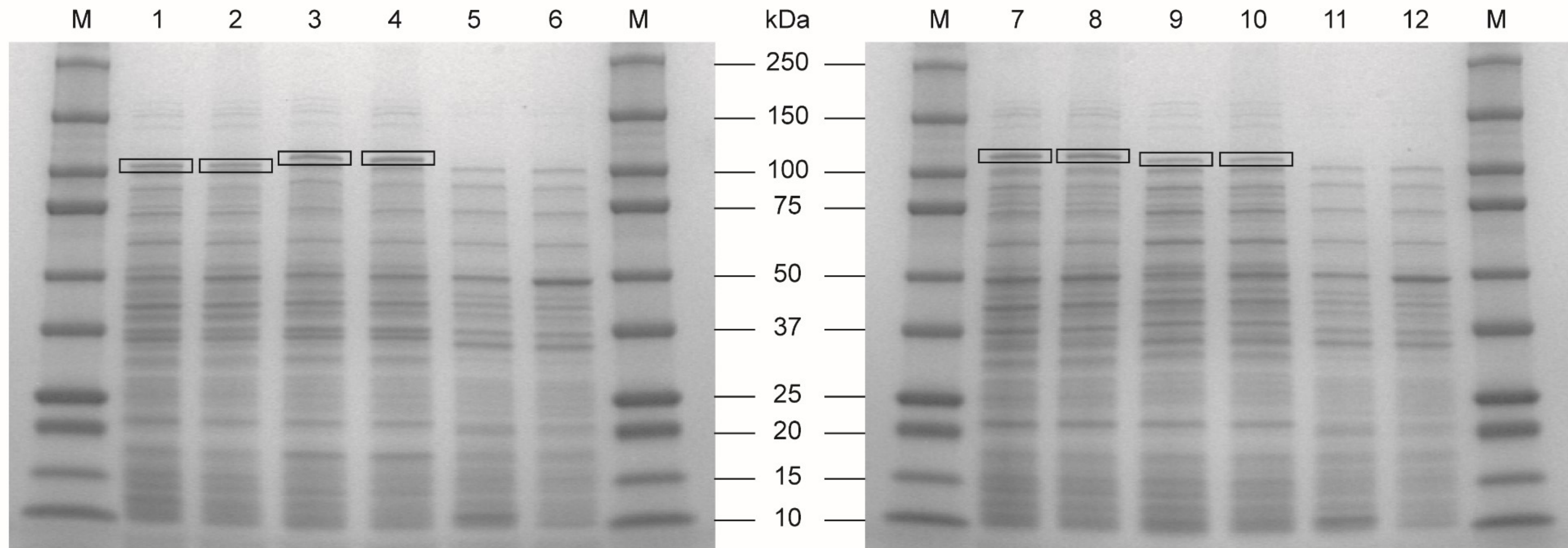
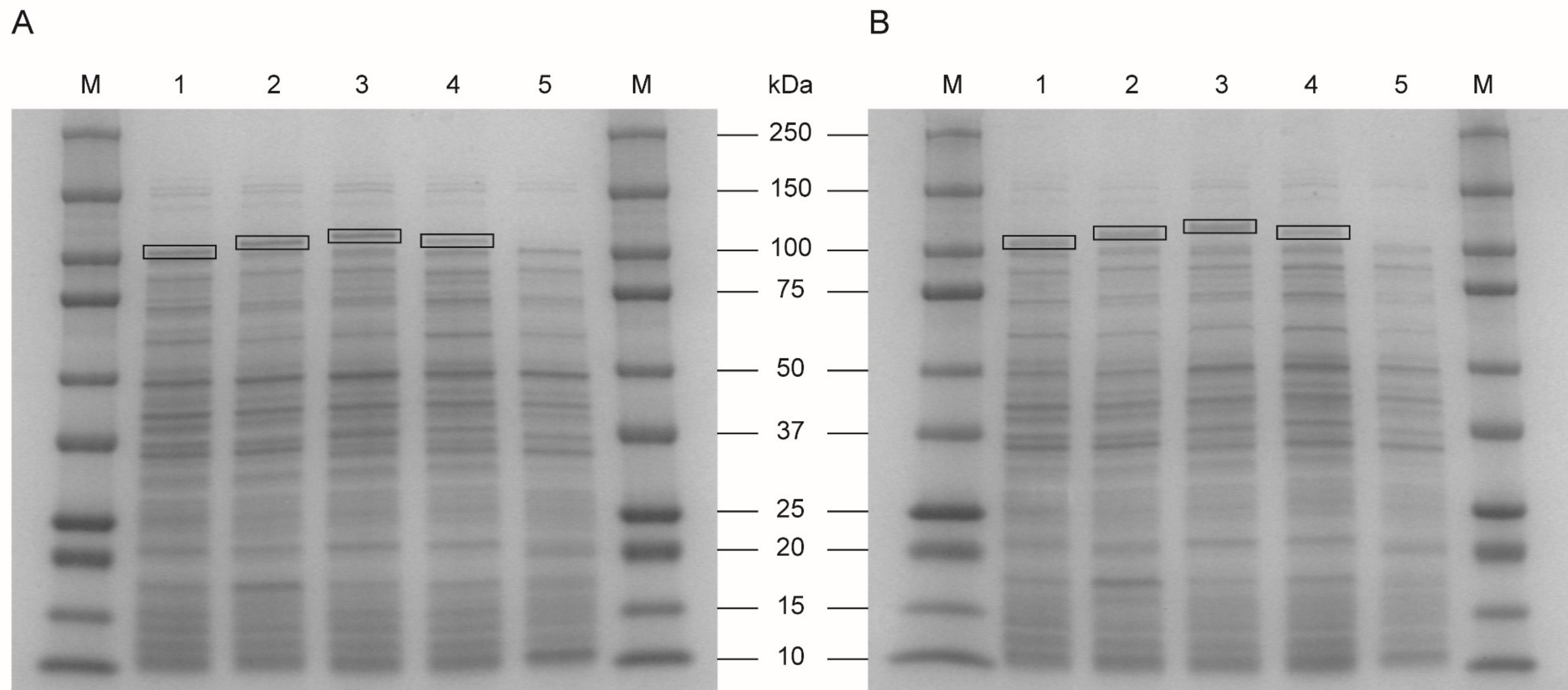
Appendices



Appendix A. **sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates of *Escherichia coli* cultured in LB broth with 0% additional NaCl with heat treatment at 60°C and 37°C for 60 minutes.** M, Bio Rad Precision Plus Protein Standard; 1, AW1.7 after 37°C heat treatment; 2, AW1.7 after 60°C heat treatment; 3, 111 after 37°C heat treatment; 4, 111 after 60°C heat treatment; 5, 126 after 37°C heat treatment; 6, 126 after 60°C heat treatment; 7, 128 after 37°C heat treatment; 8, 128 after 60°C heat treatment; 9, 8354 after 37°C heat treatment; 10, 8354 after 60°C heat treatment; 11, 126 after 37°C heat treatment; 12, 126 after 60°C heat treatment. Black boxes, additional protein band containing the ClpK homolog observed in heat resistant isolates.



Appendix B. **sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates of *Escherichia coli* cultured in LB broth with (A) 2% additional NaCl and (B) 4% additional NaCl.** M, Bio Rad Precision Plus Protein Standard; 1, AW1.7; 2, 111; 3, 128; 4, 8354; 5, 126. Black boxes, additional protein band containing the ClpK homolog observed in heat resistant isolates.

Appendix C. qPCR results of ORF 3 transformants

Isolate	Cq value
Transformant 1	12.33
Transformant 2	11.48
Transformant 3	9.84
Transformant 4	11.28
Transformant 5	10.55
Transformant 6	11.35
Transformant 7	14.22
Transformant 8	13.58
Transformant 9	15.97
Transformant 10	12.25
Transformant 11	12.33
Transformant 12	10.96
Transformant 13	11.97
Transformant 14	31.64
Transformant 15	10.20
Transformant 16	14.36
Transformant 17	13.25
Transformant 18	11.46
Transformant 19	8.22
Transformant 20	10.32
Transformant 21	10.40

Appendix C continued. qPCR results of ORF 3 transformants

Isolate	Cq value
Transformant 22	10.37
Transformant 23	11.29
Transformant 24	11.90
Transformant 25	25.13
Transformant 26	19.26
Transformant 27	14.03
Transformant 28	11.90
Transformant 29	12.32
Transformant 30	10.53
Transformant 31	No amplification
Transformant 32	16.62
Transformant 33	21.64
Transformant 34	17.99
Transformant 35	9.51
Transformant 36	10.78
Transformant 37	16.82
Transformant 38	9.57
Transformant 39	No amplification
Transformant 40	16.65
Transformant 41	14.57
Transformant 42	11.33

Appendix C continued. qPCR results of ORF 3 transformants

Isolate	Cq value
Transformant 43	9.25
Transformant 44	9.21
Transformant 45	8.10
Transformant 46	7.52
Transformant 47	9.69
Transformant 48	11.30
E. coli AW1.7	22.83
No template control	No amplification