Escherichia coli O157; Prevalence, Survival, and Stress Responses During Prolonged Heat and Cold Shocks

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By

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ABSTRACT

Escherichia coli O157 is a food borne pathogen of increasing public health concern worldwide. Cattle have been implicated as the primary reservoir of *E. coli* O157. The fact that the livestock industry has rapidly expanded in Saskatchewan makes it imperative to have a clear scientific understanding of the prevalence of *E. coli* O157 in this province as well as its survival in soil under ambient conditions.

Longitudinal and point studies were employed to determine the prevalence of *E. coli* O157 among Saskatchewan's cattle. During a 2-year period, 23 feedlot and cattle operations were examined and an overall prevalence of 15.6% was reported. The most important finding was that the prevalence rates were highly dependent on cattle density. All *E. coli* O157 isolates obtained from this study were characterized by using multiplex PCR, RAPD fingerprinting, a Vero cell cytotoxicity assay and antibiotic susceptibility tests. This characterization revealed a surprisingly highly virulent and heterogenous population of *E. coli* O157 isolates.

Subsequently, the survival characteristics of *E. coli* O157:H7 ATCC 43894 in sterile soil and manure-amended soil microcosms, as well as *in situ* under ambient environmental conditions were examined. Findings from this work indicated that desiccation had the most lethal effect on cell viability, whereas nutritionally-rich soils significantly increased survival times of the pathogen population.

A final study was designed to examine the survival strategy of hyper- and hypothermally adapted *E. coli* O157 cells exposed to high and low temperatures, with specific focus on the role of RpoS. Using wild type and its *rpoS* null allele *E. coli* O157 strains, in combination with 2D PAGE, It was found that both heat and cold post-acclimation stimulons consisted of two large sub-groups: (i) stress proteins, and (ii)

housekeeping proteins. Comparative proteomic analyses revealed that the GroEL/S chaperonin complex and Pnp ribonuclease played a crucial role in growth resumption during high and low temperatures, respectively. Notably, RpoS had no control over key stress proteins in either stress stimulon. RpoS, however, showed a significantly more pronounced role during cold temperatures, where it was seen to regulate key proteins involved in homoeoviscous adaptation as well as various housekeeping proteins of both stress stimulons.

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1. GENERAL INTRODUCTION

Mammals are generally colonized by *Escherichia coli* at birth and these normally harmless bacteria become part of their intestinal community for the rest of their lives. Under certain circumstances, *E. coli* can produce disease in animals and humans. The most common disease caused by *E. coli* is gastroenteritis. Based on virulence mechanisms or diagnostic features of this pathogen, *E. coli* has been divided into five major groups (Janda and Abbott, 1998). The enterohemorrhagic group (EHEC), a group that has attracted the most attention world wide, is distinguished from the other virulence groups by the production of extracellular cytolytic toxins called verocytotoxins, or Shiga-like toxins, first described by Konowalchuk *et al*. (1977). The enterohemorrhagic strains of *E. coli* are also defined by the presence of specific virulence factors: all strains produce hemolysin (Shmidt *et al*., 1994) and many produce intimin, a 97-kDa attachment and effacement protein (Louie *et al*., 1993).

In 1982, two outbreaks of bloody diarrhea occurred in Oregon and Michigan (USA) (Riley *et al*., 1983), and another in Ottawa (Canada) (Stewart *et al*., 1983), leading to the recognition of a new EHEC serotype*, E. coli* O157:H7. *Escherichia coli* O157:H7 is currently the most common EHEC strain in many regions of the world (Armstrong *et al*., 1996). According to the Public Health Agency of Canada (2005), the majority (95%) of pathogenic *E. coli* isolates from human cases were serovar O157. A critical element in the emergence of *E. coli* O157:H7 as a foodborne pathogen was the evolution of acid resistance, an attribute that promotes survival in acidic environment

and results in efficient transmission with a low infective dose. Enterohemorrhagic *E. coli* strains, especially strain O157, have a low infectious dose of 1 to 100 CFU (Paton *et al*., 1996). *Escherichia coli* O157:H7 causes a wide spectrum of clinical symptoms, including haemorrhagic colitis (bloody diarrhea), haemolytic-uremic syndrome (HUS), non-bloody diarrhea and thrombotic thrombocytopaenic purpura (TTP) (Su and Brandt, 1995). Haemorrhagic colitis is the most common symptom associated with *E. coli* O157:H7, typically appearing 1 to 5 days after ingestion with most patients recovering within 10 days. In some cases, infection may result in life-threatening complications, such as HUS and TTP. After oral consumption, *E. coli* O157 enters the intestines where it attaches to the intestinal mucosa and produces Shiga-like toxins (stx1/stx2). These toxins cause the walls of the intestines to become porous, allowing further toxin to enter the bloodstream and induce the clinical manifestation known as HUS. The toxin subsequently damage red blood cells and blood transfusions are required in more than 70% of HUS cases. In addition, kidney damage occurs from which about 50% of HUS patients suffer acute kidney failure and require dialysis (Siegler, 1995).

It is well documented that the primary reservoir of *E. coli* O157 in the environment is cattle, although other farm animals, including sheep and pigs, have also been shown to be carriers of the pathogen (Wallace *et al*., 1997). Faecal excretion rates of *E. coli* O157 from infected cattle typically range from 10^2 to 10^6 CFU per g (Wang *et al*., 1996). The faecal excretion of *E. coli* O157 by cattle appears to be seasonal, with excretion rates being highest in spring and late summer (Shere *et al*., 1998). *Escherichia coli* O157:H7 can remain viable in non-aerated cattle manure for greater than 12 months and for approximately 2 months in aerated manure piles (Kudva *et al*., 1998). The

behavior of the pathogen in different soil types and the influence of environmental conditions and management strategies on the pathogen remains largely unknown.

Infections of humans with the *E. coli* O157:H7 serotype occurs most frequently as food-borne outbreaks. In 1993, *E. coli* O157:H7 became indelibly linked with the Jack-in-the-Box hamburger chain in the U. S. Northwest after an outbreak killed four and sickened some 700, an outbreak that directly or indirectly led to dramatic changes in meat inspection in the U.S., including much higher levels of public accountability. Food safety has since become a presidential-level priority. Traditionally, industry and regulators have depended on spot-checks of manufacturing conditions and random sampling of final products to ensure safe food. This approach, however, tends to be reactive, rather than preventive. In 1998, the U.S. Department of Agriculture implemented a new system known as Hazard Analysis and Critical Control Point, or HACCP.

Implimentation of the Hazard Analysis and Critical Control Point system led to the following changes:

(i) Federally mandated microbial testing of meat for *E. coli* O157:H7 to provide control of this food pathogen prior to entry into food stream,

(ii) Federal and state laws requiring health providers and public health agencies to report *E. coli* O157:H7-related illnesses to aid in quick identification of when and where problems are occurring,

(iii) Federal and state regulations mandating cooking temperatures of at least 150°F for ground beef and/or heat treatments sufficient to destroy *E. coli* O157:H7, and

(iv) Mandatory food-safety training for all food service workers or any person preparing or serving food to the public.

Most major U.S. food companies already use HACCP in their manufacturing processes, and this approach is increasingly being adopted in other countries, including Canada. Recently, an outbreak of *E. coli* O157:H7 that occurred in Walkerton, Ontario, where seven people were killed and some 2500 sickened, demonstrated the importance of water, and associated manure-contaminated soils, as a significant vector of transmission of this pathonen to municipal drinking water. It also highlighted the need for improved government regulation of the production and delivery of safe food and water.

1.1. Study rationale

The frequency of occurrence of *E. coli* O157 in cattle housed in Saskatchewan feedlots is presently poorly understood. In order to assess health risks associated with Saskatchewan cattle production, as well as to design strategies for controlling the spread of this pathogen, province-specific information is required. Accordingly, the prevalence rate of *E. coli* O157 in Saskatchewan, the sensitivity and reliability of two different techniques designed to detect *E. coli* O157 in naturally-infected samples was determined. These experiments are described in the first two studies of my thesis (chapters 2 and 3). Chapter 4 examined how different soil conditions influenced the survival of *E. coli* O157 under *in vitro* and *in situ* conditions. Lastly, the 5th chapter characterized the general stress response of *E. coli* O157 to prolonged cold and heat shocks, and also delineated the role of the RpoS sigma factor during these stress situations.

These studies were based on the following hypothesis and technical objectives:

1.2. Hypotheses

1. The genetic relatedness of *E. coli* O157 strains isolated from Saskatchewan feedlots will reflect the geographic location of the feedlots from which the isolates were obtained,
2. Increased soil temperature will result in decreased numbers of viable *E. coli* O157 over time, and
3. RpoS, as a general stress response factor, will have a significant role in a prolonged heat and cold shocks response of *E. coli* O157.

1.3. Technical objectives

1. To conduct a province-wide survey of the prevalence of *E. coli* O157 in cattle housed in Saskatchewan feedlots, and to characterize isolates in terms of their biochemical and molecular traits,
2. To elucidate the effects of temperature, time, soil type, and competing microflora on the survival of *E. coli* O157,
3. To evaluate culture-based techniques for the enhanced isolation of *E. coli* O157 from manure samples, and
4. To examine the general stress response of *E. coli* O157 and functional role of RpoS under prolonged heat and cold shocks by comparing the physiological and proteomic state of wild-type and *rpoS* null mutant *E. coli* O157 strains.

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2. LITERATURE REVIEW

2.1. Historical perspectives on the species, *Escherichia coli*

In 1885, a German physician named Theodor Escherich, using aseptic techniques learned from Robert Koch, was able to isolate several microorganisms from the gastrointestinal contents of infants, one of which he named *Bacterium coli commune* (Sussman, 1985). By 1919, Castellani and Chalmers recognized that *Bacterium coli* could not satisfy the biochemical and morphologic criteria for inclusion within this genus (Bettelheim, 1991). To honor Escherich's contributions to this area of study, the genus *Escherichia* was proposed by the same authors. To the present day, the genus *Escherichia* consists of five species: *E. coli*, *E. hermannii*, *E. fergusonii*, *E. vulneris* and *E. blattae* (Scheutz and Strockbine, 2001).

2.2. Morphology and metabolism of *E. coli*

Escherichia coli are straight, cylindrical, Gram-negative rods with rounded ends that are 1.1-1.5 μm in diameter and 2.0-6.0 μm in length. This organism occurs singly or in pairs and can be motile or nonmotile. Motile *E. coli* possess 5 to 10 peritrichously-distributed flagella per cell. The flagellar filament is 20 nm in diameter and up to 20 μm long. It is made of subunits of a single protein, flagellin, which is encoded by the *fliC* gene. Unlike *Salmonella*, most *E. coli* strains have only one flagellin gene and do not undergo phase variation. The flagellin possesses antigenic features and according to Orskov and Orskov (1984), fifty-three antigenically distinct types of flagellin exist. Using the electron microscope, Lawn *et al*. (1977) revealed differences in surface

structure of antigenically distinct filaments, indicating the importance of flagellar morphology in antibody-antigen reactions. Flagellar, or H, antigens make up the third main group of serotyping antigens in *E. coli*, after O and K. In addition to flagella, most strains of *E. coli* have fimbriae (pili) extending from the bacterial surface in great numbers. For instance, *E*. coli K-12 cells each contain 100-500 type 1 fimbriae, with a diameter of 7 nm and a length of 0.2-2.0 μm. So far, more than 30 different fimbriae have been described in *E. coli*, with the common presence of more than one type at a time. The main role of fimbriae is attachment to the intestinal mucosal surface as well as to various inflammatory cells. They bind to oligomannoside-containing glycoproteins present on mucosal surfaces, including the Tamm-Horsfall glycoprotein (Orskov *et al*., 1980), and lamin, a glycoprotein present in basement membranes (Kukkonen *et al*., 1993). A rigid cell wall known as the outer membrane (OM) surrounds *E. coli* cells. The outer membrane consists of three covalently-linked domains: (1) lipid A, (2) the core region of phosphorylated non-repeating oligosaccharides and (3) the immunogenic O antigen polymer of repeating oligosaccharides (1-40 units). Structurally, the OM is organized as two opposing phospholipid-protein leaflets. Its inner leaflet consists of ordinary phospholipids, but these are replaced in the outer leaflet by lipopolysaccharide (LPS). LPS consists of lipid A which can produce fever and a shock syndrome called Gram-negative shock or endotoxic shock. Proteins called porins, or matrix proteins, form pores through the outer membrane that make it possible for hydrophilic solute molecules of molecular weight less than 800 to diffuse through it and into the periplasm. In addition to the porins, *E. coli* possess the most abundant outer membrane protein called Braun's, or murein, lipoprotein, the function of which is to attach the murein layer, a single covalently-bonded molecule, to the outer membrane of the wall. The

murein sacculus is responsible for the shape of the cell and is also vital for its integrity. Internal to the murein is the periplasm, a space containing concentrated solutions of proteins and oligosaccharides. The proteins in the periplasmic space consist of enzymes with hydrolytic functions, sometimes antibiotic-inactivating enzymes, different binding proteins with roles in stress response and active transport of solutes into the cell. The cytoplasmic membrane is the boundary between the cytoplasm and OM, and structurally is a bileaflet like most other biological membranes with an exception that it possesses a very high percentage of proteins (up to 70% of its weight).

E. coli is a facultative anaerobe that has both a respiratory and fermentative type of metabolism. Cells produce strong acids and usually gas during the fermentation of D-glucose. Most strains of *E. coli* ferment lactose rapidly and produce indole. These, and other, biochemical reactions are sufficient to differentiate *E. coli* from other species.

2.3. Pathogenic groups of *E. coli*

Escherichia coli has been suspected to cause diarrheal syndromes in humans since the beginning of modern bacteriology (1890-1910). However, it took the antigenic typing scheme of Kauffmann coupled with epidemiologic surveys of *E. coli*-associated infantile diarrhea in the 1940s and 1950s, to unequivocally establish this species' enteropathogenic capability (Levine, 1987). In recent years, the number of types of *E. coli* associated with gastroenteritis has greatly expanded and includes six major pathogenic classes: – enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), Shiga toxin-producing (STEC), the enteroadherent *E. coli* (EAEC) and a sixth, newly-described agent (see Table 2.1.).

Table 2.1. Pathogenic groups of *E. coli* associated with gastroenteritis.

Current terminology	Acronym	Prior Designations/Inclusive Groups
Enteropathogenic *E. coli*	EPEC	
Enterotoxigenic *E. coli*	ETEC	
Enteroinvasive *E. coli*	EIEC	
Shiga toxin-producing *E. coli*	STEC	Enterohemorrhagic *E. coli* (EHEC), verocytotoxin-producing *E. coli* (VTEC), Shiga-like toxin-producing *E. coli* (SLTEC)
Enteroadherent *E. coli*	EAEC	Enteroaggregative *E. coli* (EAggEC), diffuse adhering *E. coli* (DAEC)
"Reputed agents"	-	Cytotoxic necrotizing factor (CNF)-producing *E. coli*, cytolethal distending toxin (CLDT)-producing *E. coli*

From: The Enterobacteria; Janda and Abbot, 1998.

The terminology for EPEC, ETEC and EIEC has remained unchanged for many years, implying that these three enterovirulent groups were discovered a long time ago and their pathogenicity and epidemiology have been well-characterized. On the other hand, STEC is more recently discovered and it has undergone an evolution in terminology. These organisms were reclassified according to toxin type rather than disease manifestation (hemorrhagic colitis). The main features of the currently recognized groups associated with gastroenteritis are listed in Table 2. 2

Table 2.2. Major chracteristics of diarrheagenic *E. coli* groups

Group	Disease Produced	Nature of Diarrhea		Frequency	Pathogenic mechanism[a]
		Early	Late		
EPEC	Enteritis	Watery	Watery	+	Adherence
ETEC	Enteritis	Watery	Watery	++	Toxin(s)
EIEC	Dysentery	Watery	Bloody	+	Invasion
STEC	Colitis	Watery	Bloody	+++	Toxin(s)
EAEC	Enteritis	Watery	Watery	Unknown	Adherence

From: The Enterobacteria; Janda and Abbot, 1998.

[a]Virulence mechanism or diagnostic feature of each pathogenic group.
+++, common; ++, occasionally isolated; + rare.

All five groups of *E. coli* initially cause a secretory type of gastroenteritis, but only EIEC and STEC are capable of producing bloody diarrhea in the later stages of infection, a major feature that distinguishes them from other enterovirulent *E. coli* groups. It is important to note that only STEC, among all pathogenic groups of *E. coli*, possess a potent cytolytic toxin almost identical to that produced by *Shigella dysenteriae*. Other notable clinical differences among these pathogenic groups of *E. coli* are listed in Table 2.3

Table 2.2. Clinical properties of *E. coli* groups associated with gastroenteritis

Group	Infectios Dose (cfu)	Incubation Period (h)	Duration of Illness (d)		Attack Rate (%)	Presence in Stool	
			Mean	Range		Mucus	Blood
EPEC	10^6-10^{10}	9-19	5	3-14	33-72	+	-
EIEC	≥10^8	<24	2-4	1-12	33-56	+	+
ETEC	10^8-10^{10}	3-166	4-7	1-53	16-77	-	-
STEC	50-700	24-336	4-5	6-9	28-75	-	+
EAEC	~10^{10}	14-46	5	3->14	?	-	-

From: The Enterobacteria; Janda and Abbot, 1998.

2.4. Shiga toxin-producing *E. coli* (STEC) – *E. coli* O157:H7

In 1982 two outbreaks of bloody diarrhea in Oregon and Michigan (USA) (Riley *et al*., 1983) and another in Ottawa (ON Canada) (Stewart *et al*., 1983) led to the recognition of a new pathogenic serotype*, E. coli* O157:H7. *Escherichia coli* O157:H7 is the archetypal serotype for a series of *E. coli* strains capable of producing at least two distinct extracellular cytolytic Shiga-like toxins (Stx1 and Stx2), first described by Konowalchuk *et al*. (1977). This pathogen had previously been isolated from one case of bloody diarrhea in California in 1975. In Canada, the first isolation of *E. coli* O157:H7 was made in 1978 and by the end of 1981, five strains of this serotype were identified. Shiga toxin-producing *E. coli* strains (STEC) are also defined by the presence of specific

virulence factors; almost all strains produce hemolysin and many produce intimin (Schmidt *et al*., 1994), a 97-kDa attachment and effacement protein (Louie *et al*., 1993). Today, numerous serotypes represent the STEC group (see Table 2.4.).

Table 2.3. Serotypes of Shiga toxin-producing *E. coli* (STEC)

Health category and related isolates							
Caused hemolytic uremic syndrome[1]				**Caused other illnesses[2]**			
O2:H5	O2:H6	O2:H7	O2:H29	O1:H2	O1:H20	O2:H27	O8:H$^-$
O5:H$^-$[3]	O6:H$^-$	O8:H2	O8:H19	O8:HUT	O15:H$^-$	O20:H7	O22:H16
O8:H21	O20:H19	O22:H8	O25:H2	O22:H$^-$	O26:H2	O26:H21	O26:H32
O26:H11	O26:H$^-$	O26:HUT[4]	O45:H2	O28:H$^-$	O39:H8	O45:H$^-$	O70:H11
O49:H$^-$	O55:H$^-$	O84:H$^-$	O86:H$^-$	O74:H$^-$	O75:H8	O76:H7	O77:H18
O91:H10	O91:H21	O91:H$^-$	O98:H$^-$	O82:H8	O84:H2	O88:H$^-$	O91:H14
O103:H2	O103:H$^-$	O105:H18	O105ac:H18[5]	O91:HUT	O103:H25	O104:H7	O112:H21
O111:H8	O111:H$^-$	O112ac:H19	O113:H21	O113:H4	O113:H7	O113:H$^-$	O117:H7
O118:H16	O118:H$^-$	O119:H2	O119:H6	O117:H19	O119:H$^-$	O126:H20	O128ab:H8
O121:H19	O125:H$^-$	O128:H2	O128ab:H2[6]	O128:H12	O128:HUT	O132:H$^-$	O141:H$^-$
O145:H25	O145:H28	O145:H$^-$	O146:H21	O146:H28	O146:H$^-$	O150:H$^-$	O156:H25
O153:H25	O154:H$^-$	O157:H7	O157:H$^-$	O163:H$^-$	O171:H2	OR:H19	OR:HUT
O161:H$^-$	O163:H19	O165:H25	O165:H$^-$	OUT:H1	OUT:H4	OUT:H7	OUT:H8
O171:H$^-$	O172:H$^-$	O174:H2[7]	O174:H21	OUT:H10	OUT:H14	OUT:H16	OUT:H18
O174:H$^-$	O177:H$^-$	OR:H4	OR:H25	OUT:H19	OUT:H21	OUT:H28	OUT:H33
OR:H$^-$	OUT:H2[8]	OUT:H11	OUT:H25	OUT:H41	OUT:HUT		
OUT:H$^-$							

Modified from Hussein, 2007.

[1] The STEC serotypes were isolated from humans suffering from hemolytic uremic syndrome (WHO, 1998; Blanco *et al*., 2003).
[2] The STEC serotypes were isolated from humans suffering from a wide range of illnesses such as mild diarrhea, bloody diarrhea, abdominal pain, ulcerative colitis, hemorrhagic colitis and thrombotic thrombocytopenic purpura (WHO, 1998; Blanco *et al*., 2003).
[3] A nonmotile strain.
[4] An untypeable H antigen.
[5] Certain antigenic relationships are represented by 'a' a common factor and 'c' a specific factor (Lior, 1994).
[6] Certain antigenic relationships are represented by 'a' a common factor and 'b' a specific factor (Lior, 1994).
[7] A rough O antigen.
[8] An untypeable O antigen.

Since their discovery as etiologic agents of hemorrhagic colitis in 1982, the clinical importance of *E. coli* O157:H7 and STEC has grown rapidly. To illustrate this fact, the U.S. Department of Health and Human Services (1994) constructed a logarithmic time line describing significant epidemiological events involving *E. coli* O157:H7. In little more than a decade, the notoriety of *E. coli* O157:H7 had undergone a major transformation from that of a newly described agent of diarrheal disease (1982) to the leading bacterial cause of bloody diarrhea in the United States in 1992 (see Figure 2.1.; Janda and Abbott, 1998). *Escherichia coli* O157:H7 is the most common STEC serotype in many regions of the world (Armstrong *et al*., 1996). It has been estimated that *E. coli* O157:H7 cause 73,000 illnesses annually in the United States and non-O157 STEC cause 37,000 illnesses and that 91 deaths occur each year in the USA (Mead *et al*., 1999). In Canada, according to the Public Health Agency of Canada (2005), the majority (95%) of pathogenic *E. coli* isolates from human cases were serovar O157. *Escherichia coli* O157 has the ability to cause both sporadic and large outbreaks. The city of Sakai in Japan experienced the largest outbreak of O157 STEC ever recorded in July 1996, which was part of several outbreaks that summer with an estimated 8000 cases and 6 deaths (Infectious Disease Surveillance Center, Japan, 1997). From an epidemiological point of view it is interesting that STEC infections in humans seem to be different worldwide. *Escherichia coli* O157:H7 is the predominant serotype of STEC in the United States, Canada, the United Kingdom and Japan, but in continental Europe, Australia and Latin America non-O157:H7 STEC serotypes are much more common than *E. coli* O157:H7 (O'Brien and Kaper,1998).

Escherichia coli O157:H7 is serologically determined by two antigens. The somatic O157 antigen reflects the nature of the phospholipid-polysaccharide complex

and the order in which it occurs in the repeating units of the polysaccharide chain. The flagellar H7 antigen is determined by the nature of the repeating flagellar subunits, flagellin. *Escherichia coli* O157:H7 cause a wide spectrum of clinical symptoms including haemorrhagic colitis (bloody diarrhea), haemolytic-uremic syndrome (HUS), non bloody diarrhea and thrombotic thrombocytopaenic purpura (TTP) (Su and Brandt, 1995).

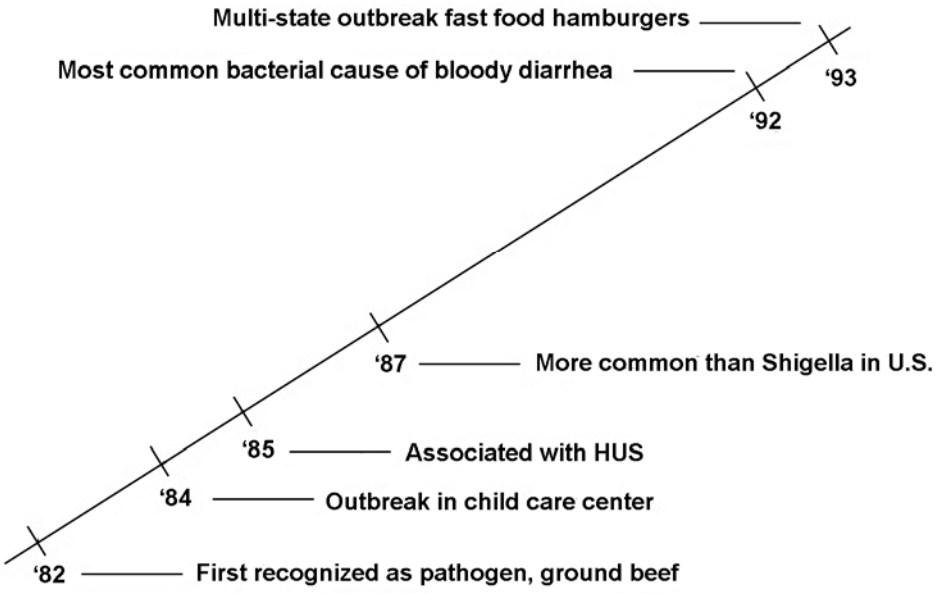

Figure 2.1. Time line of major clinical and epidemiologic events associated with *E. coli* O157:H7 infections. (from: Addressing Emerging Infectious Disease Threats, Atlanta 1994).

Hemorrhagic colitis is the most common symptom associated with *E. coli* O157:H7, typically appearing 1 to 5 days after ingestion with most patients recovering within 10 days. In some cases, infection may result in life-threatening complications such as HUS and TTP. In addition, kidney damage occurs from which about 50% of HUS patients suffer acute kidney failure and require dialysis (Siegler, 1995). The

treatment of individual patients with STEC infections is mainly based on plasma exchange in order to reduce the concentration of Stx1 and/or Stx2 in a patient's body. The use of antibiotic therapy is questionable. It has been reported that the use of antibiotics in STEC infections may increase the risk of developing HUS (Carter *et al*., 1987; Cimolai *et al*., 1990), as antibiotic-mediated cell lysis would actually increase the amount of free Stx in the gut lumen available for absorption. Two *in vitro* studies have shown that treatment of O157:H7 STEC with subinhibitory concentrations of antibiotics resulted in significant increases (up to 50-fold) in the amount of free Stx in the culture medium. According to Cordovez *et al*. (1999), a high rate of antibiotic resistance exists among STEC, and so empirical treatment with an inappropriate drug might confer a selective advantage on the STEC over other members of the gut flora and cause over-growth. Future prospects for treatment and prevention of STEC infection may include binding or neutralization of Stx (Armstrong *et al*., 1991), as well immunization against STEC disease (Bosworth *et al*., 1996).

2.5. Pathogenesis

2.5.1 Acid resistance

The importance of gastric secretions as an early line of defense against enteric pathogens was recognized for a nearly century, with the primary bactericidal barrier being acid dependent (Peterson *et al.*, 1989). Clearly, the infectious dose of different enteric pathogens corresponds to their relative abilities to withstand acid. The oral infectious doses of *Vibrio cholerae,* non-typhi *Salmonella* spp. and *Shigella flexneri* are approximately 10^9, 10^5 and 10^2 cells, respectively (Blaser and Newman, 1982). These doses agree with the relative levels of acid resistance of the bacteria, with *V. cholerae* being the least resistant and *S. flexneri* being the most resistant. Shiga toxin-producing

E. coli strains, especially serotype O157, have a low infectious dose of 1 to 100 CFU (Griffin *et al.*, 1994; Paton *et al.,* 1996), similar to those of *Shigella* spp. A critical element in the emergence of *E. coli* O157:H7 as a foodborne pathogen was the evolution of acid resistance, an attribute that promotes survival in acidic environments and results in efficient transmission with a low infective dose. For instance, successful colonization of the intestine by this organism requires transient survival through the stomach at pH values of 1 to 2 (fasting) or 2 to 7 (during feeding) (Dressman *et al.*, 1990). Waterman and Small (1996) found that 76% of 38 *E. coli* O157:H7 strains had greater than 10% survival after a 2 hour exposure to pH 2.5. Another study revealed that under laboratory conditions, the percentage survival in synthetic gastric fluid (pH 1.5) for *E. coli* O157:H7 exceeded 95% for 1 hour acid exposures of stationary phase cultures (Arnold and Kaspar, 1995). Although the authors observed some variation among *E. coli* O157:H7 strains, the average survival was greater than that observed in other pathogenic groups of *E. coli* and greatly exceeded nonpathogenic controls. The findings of Leyer *et al*. (1995) suggested that *E. coli* O157:H7 cells exposed to acidic conditions (pH 5.0) for short periods were better able to survive in acidic food than unexposed cells. This phenomenon, named acid adaptation or acid habituation, requires active metabolism during incubation (Foster, 1991).

Escherichia coli in general have multiple genetic systems that respond to physical and chemical challenges and that confer resistance to low and lethal pH. The σ^s (or RpoS) subunit of RNA polymerase is a master regulator of the general stress response in *E. coli*. While nearly absent in rapidly growing cells, σ^s is strongly induced during entry into stationary phase and/or many other stress conditions, and is essential for the expression of multiple stress responses (Hengge-Aronis, 2002). Detailed studies

of acid resistance mechanisms in *E. coli* O157:H7 have revealed three systems that can protect cells against pH 2 to 2.5 (Hersh *et al*., 1996; Lin *et al.*, 1995). The first is a glucose repressed system (acid-induced oxidative system) induced in Luria-Bertani broth that is dependent on the alternative sigma factor σ^s, encoded by the gene *rpoS.* Consistent with glucose repression, this system has also proven to be dependent in many situations on the cyclic AMP receptor proteins (Castanie-Cornet *et al*., 1999). The other two clearly defined systems are: i) a glutamate – dependent system, and ii) an acid – induced arginine – dependent system. The glutamate – dependent system is induced in response to low environmental pH and upon entry into stationary phase and consists of two glutamate decarboxylase isoforms, GadA and GadB, and a glutamate γ-aminobutyrate antiporter GadC (Smith *et al*., 1992; Hersh *et al*., 1996). GadA and GadB catalyse the decarboxylation of glutamate to γ-aminobutyrate by replacing the α-carboxyl group with a proton. The decarboxylation product is exported via the GadC antiporter (Hansen *et al*., 2005). Additional protection from acid comes from the periplasmic protein HdeA and the isomer HdeB that have been suggested to have a chaperone-like function, suppressing the aggregation of periplasmic proteins under extreme acidic conditions (Gajiwala and Burley, 2000; Merrell and Camilli, 2002). Acid-induced acid resistance factors also include envelope proteins such as OsmY (Maurer *et al*., 2005), and redox modulators such as Tpx (Stancik *et al*., 2002; Tucker *et al*., 2002). A study conducted by Chang and Cronan (1999) revealed that cyclopropane fatty acid (CFA) plays a major role in protection of *E. coli* from acid shock. According to the same authors, an explanation of the CFA acid protective mechanism can be found in "the bilayer permeability model" (Paula *et al*., 1996), where proton permeability in lipid bilayers is inversely proportional to bilayer thickness. Formation of CFA is post-

synthetic modification where unsaturated fatty acids are converted to CFA, which consequently increase bilayer thickness and lead to acid resistance.

2.5.2 Adherence mechanisms

Escherichia coli O157:H7, after exposure to the harsh conditions of the stomach, must become established in the gut by adhering to intestinal epithelial cells. The principal sites of *E. coli* O157:H7 colonization in humans are the colon and the distal small intestine (Paton and Paton, 1998). It appears that different *E. coli* O157:H7 strains express different adherence phenotypes to epithelial cells. Sherman *et al*. (1987) reported marked quantitative differences (up to 250-fold) in the adherence of five *E. coli* O157:H7 strains to both HEp-2 (human laryngeal epithelioma) and Henle 407 (human colonic carcinoma) cell lines. Some strains of *E. coli* O157 adhered in a diffuse fashion, with bacteria being distributed evenly over the surface of the epithelial cells (diffuse adherence [DA]). Other strains formed tight clusters at a limited number of sites on the epithelial surface (localized adherence [LA]). Interestingly, Sherman and colleagues (1987) in the same study showed that a given strain did not exhibit the same pattern of adherence on both cell lines. At present, the best characterized *E. coli* O157:H7 adherence phenotype is intimate or attaching and effacing (A/E) adherence. The process of A/E lesion formation involves an initial phase of DA or LA adherence followed by loss of enterocyte microvilli and intimate attachment of the bacterium to the cell surface. All of the genes necessary for generation of A/E lesions are encoded on a pathogenicity island called the Locus of Enterocyte Effacement (LEE) (Elliot *et al*., 1998). The LEE contains *sep* and *esc* genes encoding a type III secretion system (Jarvis *et al*., 1995) which plays a critical role in the intimate attachment process by providing a specialized conduit for secretion of the effector proteins from the bacterium into the host cell.

Further, LEE possesses the *eae* gene encoding an adhesin called intimin, a 934-amino acid product that is responsible for the intimate attachment of the bacteria to the host cell (Jerse and Kaper, 1991), and the *espABD* genes that encode proteins secreted by the type III secretion system with EspA having the role of forming a filamentous-like structure that facilitates translocation of effector proteins (Kenny *et al*., 1996). EspB and EspD, which have hemolytic activity, play roles as pore-forming molecules that provide a portal for the EspA secretion tube to breach the host cell membrane (Warawa *et al*., 1999). The Locus of Enterocyte Effacement also contains the *tir* gene, which encodes the translocated intimin receptor. After the translocation of Tir protein from the *E. coli* O157:H7 cell to the host cell cytoplasm via the EspA tube, it localizes to the plasma membrane of the host cell where it serves as the receptor for attachment of the bacterium (DeVinney *et al*., 1999). In addition to serving as a receptor for intimin, the 78-kDa plasma membrane-bound Tir coordinates remodeling of the host cell cytoskeleton by binding to the host cell protein Nck (Gruenheid *et al*., 2001) further leading to actin polymerization and increase of its apparent size to 90 kDa generating formations named "pedestals". The intimin –Tir interaction is crucial to the pathogenesis of *E. coli* O157:H7 and strains lacking either gene are avirulent in animal models (Marches *et al*., 2000; Dean-Nystrom *et al*., 1998). The Locus of Enterocyte Effacement in *E. coli* O157:H7 also possesses the *ler* gene (LEE-encoded regulator) which encodes a positive regulator of LEE genes (Mellies *et al*., 1999). The majority of the LEE genes are organized into five major polycistronic operons named *LEE1* through *LEE4* and *tir* (Elliot *et al*., 1999). Transcription of these operons is coupled to at least two different regulatory systems. The Ler protein encoded by the first gene in the *LEE1* operon activitates expression of the *LEE2*, *LEE3* and *tir* operons in a regulatory cascade

(Mellies *et al*., 1999). A quorum-sensing molecule, whose synthesis is encoded by the *luxS* gene, appears to regulate expression of these operons. According to Sperandio and co-workers (1999), *LEE1* and *LEE2* are directly activated by the quorum sensing system, while *LEE3* and *tir* are indirectly activated via the enhanced levels of Ler produced from the *LEE1* transcripts.

2.5.3 Shiga toxins and their role in pathogenesis

Synthesis and secretion of the Shiga toxins is the most potent virulence feature of *E. coli* O157:H7. The toxin name is derived from the prototype Shiga toxin from *Shigella dysenteriae*. Not only *E. coli* and *S. dysenteriae* can produce Shiga toxins; *Citrobacter freundii, Aeromonas hydrophila, Aeromonas caviae* and *Enterobacter cloacae* have been reported to be able to express these toxins as well (Paton and Paton, 1998). Shiga toxins all belong to the AB family of protein toxins, which share a similar three dimensional structure of a pentameric binding (B) component, composed of five identical StxB subunits, and a one monomeric enzymatically-active part (A) (Sandvig, 2001). Two main types of toxins, Stx1 and Stx2, are produced by *E. coli* O157:H7. Furthermore, several Stx2 variants have been found, including Stx2c, Stx2d, Stx2e and Stx2f, which range from 99-63 % identity in the A subunit and 95-75 % identity in the B subunit of the Stx2 toxin (Schmidt *et al*., 2000). The molecular weight of the intact toxin is about 70 kD, with the A subunit of about 32 kD and each B subunit of 7.7 kD (Paton and Paton, 1998). The binding pentamers of Shiga toxins are specific for the glycolipid receptor, globotriosyl ceramide (Gb_3) (Lindberg *et al*., 1987), and further serve to deliver the catalytic subunit to the appropriate cellular compartment. One variant toxin, Stx_2e binds to the Gb_4 receptor (Sandving, 2001). The importance of the B-pentamer-Gb_3 interaction is clearly illustrated by the fact that all cells which are susceptible to

STX express Gb_3 on their cell surface, whereas cells that do not express Gb_3 are resistant to the toxin (Weinstein *et al*., 1989). The A chain of STX has two domains that can be separated proteolytically with trypsin: an enzymatically active A1 domain and an A2 domain that serves a structural role in tethering the A1 chain to the B pentamer (Saxena *et al*., 1989).

After toxin synthesis and secretion, they are transported by transcytosis through the lumen and spread by hemotogenous routes to their primary target organs, the kidney and central nervous system tissue (Wadolkowski *et al*., 1990). All these organs possess numerous Gb_3 receptors on their surface. After toxin-receptor binding, the toxin is transported via retrograde transport to the Golgi apparatus and endoplasmatic reticulum (ER) (Sandving and van Deurs, 1996). During this process, the A subunit is nicked by a membrane-bound protease known as furin, generating a catalytically-active a 27 kD active N-terminal A1 fragment and a 4 kD C-terminal A2 fragment, which remains linked by a disulfide bond. The disulfide bond is subsequently reduced, thereby releasing the active A1 component. The released A1 fragment has RNA N-glycosidase activity and removes one adenine from adenosine in position 4324 from the 5' terminus in 28S ribosomal RNA, thereby inhibiting binding of amino-acyl-tRNA to the 60S ribosomal subunit resulting in inhibition of protein synthesis and cell death by apoptosis (Yoshida *et al*., 1999). In addition to their function as cytotoxins, Shiga toxins cause tissue necrosis in the intestine (Thorpe *et al*., 2001). Although both Stx1 and Stx2 share similarity in structure and mechanism of action, a couple of epidemiological studies suggest that Stx2 is more toxic than Stx1, and has a higher propensity to cause HUS than does Stx1 (Boerlin *et al*., 1999; Tesh *et al*., 1993). One possible explanation for this is that the level of transcription of stx_2 *in vivo* is higher than that of stx_1. Transcription of

stx$_1$ is known to be iron repressible *in vitro* and its promoter region includes a recognition site for the *fur* gene product (Weinstein *et al*., 1988). On the other hand, *stx*$_2$ promoter activity is unaffected by osmolarity, pH, oxygen tension, acetate, iron level and carbon source (Muhldorfer *et al*., 1996), factors that ultimately result in a higher synthesis and concentration of Stx2 over Stx1 *in vivo*.

2.5.4 Putative accessory virulence factors

Almost all *E. coli* O157:H7 strains harbor a large 60-65 MDa plasmid (Johnson *et al*., 1983), designated as a "virulence plasmid" (pO157), which plays an important role in virulence of this organism. The large plasmid of O157 encodes the EHEC-hemolysin (Ehx), a novel hemolytic phenotype which is distinct from that associated with the *E. coli* alpha-hemolysin (Schmidt *et al*., 1995). In contrast to alpha-hemolysin, Ehx can be detected on blood agar plates containing washed sheep erythrocytes supplemented with Ca^{2+}. The zones of hemolysis on these plates are smaller and more turbid than those caused by alpha-hemolysin and require over night incubation before they become visible (Beutin, 1991). The hemolysin gene is encoded in a polycistronic operon containing four open reading frames designated EHEC-*hlyCABD* (Schmidt *et al*., 1995). Within this operon, *hlyA* presents the structural gene for the hemolysin, which is synthesized as an inactive precursor with a predicted size of 107 kD and converted to its active form by the product of the *hlyC* gene (Paton and Paton, 1998). The secretion of HlyA is signal peptide-independent and mediated by both a specific membrane translocator system encoded by the *hlyB* and *hlyD* genes (Welch, 1991). A role for Ehx in the pathogenesis of diarrheal disease has not been clearly demonstrated. According to Law and Kelly (1995) the Ehx may contribute to pathogenesis by enzymatically releasing hemoglobin which provides a source of iron, thereby enhancing the growth of

E. coli O157:H7 in the gut. Another putative virulence factor encoded on pO157 is a novel catalase-peroxidase, KatP (Braunder *et al*., 1997). The *katP* gene encodes a 1300-amino acid protein, which is subsequently subjected to N- and C-terminal processing during secretion. The mature form has an apparent size of 104 kD, (Paton and Paton, 1998). There are several lines of evidence suggesting a virulent nature of the KatP protein. Brunder *et al*. (1997) found that KatP is able to cleave human coagulation factor V, leading to exacerbation of hemorrhagic disease. Another virulent feature of KatP is expressing the cytotoxic effect on Vero cells (Djafari *et al*., 1997). In addition, this organism possesses a small 6.6 kb plasmid which encodes a gene that can synthesize colicin D (Bradley *et al*., 1991). Last on the list of *E. coli* O157:H7 virulence factors is a 39-amino-acid enterotoxin, encoded by the *astA* gene that contributes to the pathogenesis of watery diarrhea commonly seen during the early stages of *E. coli* O157:H7 infection (Savarino *et al*., 1991).

2.6. Diagnosis

The low infectious dose (Willshaw *et al*., 1994) in combination with the nature of HUS disease, where the typical clinical signs may only become apparent a week or more following the onset of gastrointestinal symptoms, can complicate diagnosis as the numbers of *E. coli* O157:H7 at this time may be very low (Paton and Paton, 1998). Thus, there is an imperative need to develop very sensitive diagnostic tests for this organism. Diagnostic procedures are based on both detection of Shiga toxins or their genes in different samples as well as the isolation of *E. coli* O157:H7. Although information on the presence of *E. coli* O157:H7 cells can be obtained by molecular analysis of mixed cultures, isolation of *E. coli* O157:H7 is considered as the definitive diagnostic procedure. While isolation of this pathogen may have limited clinical

application, it is of great importance from an epidemiological point of view, as isolation permits additional characterization typing of this organism that may help in an outbreak setting.

In the initial report where *E. coli* O157:H7 was associated with food-borne outbreaks of hemorrhagic colitis, Wells *et al*. (1983) used direct plating of fecal material onto MacConkey agar from human patients in order to isolate this organism. The authors revealed that *E. coli* O157:H7 was not able to ferment D-sorbitol within seven days of incubation, whereas previous reports had indicated that 93% of all *E. coli* of human origin did so. Two years later, Farmer and Davis (1985) exploited this feature to differentiate *E. coli* O157:H7 from other *E. coli* by directly plating samples onto MacConkey base plus 1% D-sorbitol (SMAC). Chapman *et al*. (1991) improved the isolation rate of *E. coli* O157:H7 by supplementing SMAC with 0.05 μg/mL cefixime to inhibit mainly *Proteus* spp. Furthermore, Zadik *et al*. (1993) reported more improvements in recovery rates of this organism by adding potassium tellurite at a concentration of 2.5 μg/mL to create CT-SMAC. Potassium tellurite inhibits *Providencia* spp. and *Aeromonas* spp., both of which are prevalent in the feces of humans and cattle, while *E. coli* O157:H7 strains carry a pathogenicity island termed TAI (tellurite resistance and adherence-conferring island) that encodes for tellurite resistance (Tarr *et al*., 2000). At present, CT-SMAC agar remains the standard media for the isolation of *E. coli* O157:H7 strains.

Sanderson *et al*. (1995) revealed that enrichment of bovine feces in tryptic soy broth (TSB) containing cefixime (0.05 μg/mL) and vancomycin (40 μg/mL) prior to plating on CT-SMAC significantly increased the sensitivity of *E. coli* O157:H7 detection in bovine feces. Generally, the enrichment procedure has a dual purpose: i) to

selectively increase numbers of target organisms by suppressing the growth of background microorganisms, and ii) to dilute inhibitors for downstream procedures that are normally present in fecal samples. It is well known that bilirubin and bile salts, which are present at high concentrations in fecal samples, inhibit *Taq* polymerase and can lead to false-negative results. Inhibitors of *Taq* polymerase are also present in meat samples. For both feces and food samples, the sensitivity of PCR assays is significantly increased if template DNA is extracted from broth enrichment cultures (Gannon *et al*., 1992).

In an attempt to improve the sensitivity, a company named Dynal from Norway developed immunomagnetic separation (IMS) techniques for the isolation of *E. coli* O157:H7 from low-abundance specimens. The procedure involves coating magnetic beads with anti-*E. coli* O157 antibodies and mixing them with a suspension of incubated selective enrichment broth and specimen. The beads and bound bacteria are trapped in a magnetic field (magnetic separation), and after a couple of washing steps the bead-bacteria complex are released and plated onto CT-SMAC agar. Chapman *et al*. (1994) found that IMS was 100 times more sensitive than direct culture plating on either cefixime-rhamnose SMAC or CT-SMAC for isolation of *E. coli* O157 from bovine fecal samples. In a recent study performed by Vidovic *et al*. (2007), it was shown that IMS may be highly variable in the recovery rate of naturally-occurring bovine fecal sample *E. coli* O157 isolates. The variations in the recovery rates of IMS may be due to the high sensitivity of particular isolates to antibiotics that were present in the enrichment broth, implying the importance of appropriate selection of antibiotics during enrichment steps.

In the initial investigation in which *E. coli* O157:H7 was associated with hemorrhagic colitis, isolates were serotyped by standard procedures (Wells *et al*., 1983).

In the late 1980s, latex agglutination reagents for detection of *E. coli* O157 and H7 became available commercially and were shown to be a rapid and economical alternative to tube agglutination (Chapman, 1989).

Polymerase chain reaction (PCR) has been used both to confirm the identity of *E. coli* O157:H7 isolates and to directly detect the organism in suspected samples. Among many PCR protocols designed to identify STEC organisms, Paton and Paton (1998b) developed a multiplex PCR that detected *eaeA*, *ehx* and all known subtypes of Shiga toxins. In several studies it was reported that existing DNA sequence polymorphisms in particular stx_2-related genes may significantly reduce the efficiency of primer annealing, with large effects on the sensitivity of the PCR reaction (Gannon *et al*., 1990). Primers designed for *stx* genes must be selected from the conserved domains of the gene to avoid regions where sequence heterogeneity has been reported.

Tissue culture cytotoxicity assays play an important role in detecting *E. coli* O157 and other STEC organisms in samples where isolation of these organisms is difficult. Konowalchuk *et al*. (1977) first observed that Vero cells possess profound sensitivity to STX and to the present time this cell line remains the "gold standard" for confirmation of the cytotoxicity of putative STX-producing organisms. Vero cells possess a high concentration of both Gb3 and Gb4 receptors on the surface of their plasma membranes, which makes this cell line ideal to detect all known STX variants. The assay involves treatment of Vero cells at the confluent stage with sterile culture extracts and observing cells for a cytopathic effect after 48 to 72 h of incubation (Karmali, 1989). Karmali *et al*. (1985) reported that treating mixed fecal cultures with the antibiotic polymyxin B, to release cell-associated STX, improved the sensitivity of the Vero cell assay.

2.7. Ecology of *E. coli* O157:H7

The primary habitat of *E. coli* O157:H7 is the gastro – intestinal tract, principally the bowel of mammals and birds. When found in nature, either in soil or water, it is derived from its primary habitat, usually by fecal contamination. Cattle are one of the most important hosts and sources of *E. coli* O157:H7. Matthews *et al*. (1997) have shown that 1 to 4% of UK cattle herds are infected with *E. coli* O157:H7. Another study also carried out in the UK showed that the regional incidence of this pathogen in cattle may be as large as 16% (Chapman *et al*., 1997). According to the National Animal Health Monitoring System (2001), prevalence of this pathogen in U.S.A feedlot cattle ranged from 10-23%. Two recent studies performed in Western Canada found approximately 15% of feedlot cattle carrying *E. coli* O157:H7 (LeJeune *et al*. 2004; Vidovic and Korber, 2006). The prevalence of fecal shedding of *E. coli* O157:H7 appears to vary by age, with higher prevalence reported in younger animals in field studies (Hancock *et al*., 1994). Longitudinal studies have shown that individual animals shed *E. coli* O157:H7 transiently (Zhao *et al*., 1995; Shere *et al*., 1998). Studies in cattle have also documented a seasonal pattern to fecal shedding, with the two highest

execretion rates being in spring and in late summer (Shere *et al*., 1998). The period of high excretion rate in cattle has been shown to correlate with the start of the peak cases of human infection (Wallace *et al*., 2000). At present, it is not known whether a direct link exists between seasonal patterns in cattle and humans. Using molecular typing, it has been found that multiple subtypes of *E. coli* O157:H7 may be present within a farm at a single point in time, and that some subtypes appear to be unique to a specific herd or feedlot (Rice *et al*., 1999; Vidovic and Korber, 2006). The subtypes found in a herd may change over time (Faith *et al*., 1996). This diversity may be caused by mutation events or introduction of subtypes that briefly exist in a cattle operation but fail to be maintained within a new environment. Many animals in addition to cattle shed *E. coli* O157:H7, including domestic sheep, goats, horses, dogs and reindeer as well as wild deer, birds and rabbits (Hancock 1998). A single study carried out in the UK showed that fecal material from between 1 to 3% of birds was contaminated by the pathogen (Wallace *et al*., 1997). The finding of identical genetic strains of *E. coli* O157:H7 in isolates from a human case of illness, deer jerky consumed by the patient and the source carcass for the jerky illustrates that wildlife may be a direct source of infection (Keene *et al*., 1997).

Possible routes of entry of *E. coli* O157:H7 into the environment include the direct deposition of feces by pastured cattle, fertilization of agriculture land with manure, and leaching of bacteria from fertilized soil into water. *Escherichia coli* O157:H7 appears to have unique growth and survival characteristics which enable it to survive under a wide range of environmental conditions. Wang *et al*. (1996) found that *E. coli* O157:H7 may replicate in bovine feces at 22°C and 37°C and survive for up to eight weeks at 5°C. Another study performed by Kudva *et al*., (1998) revealed that *E.*

coli O157:H7 can remain viable in non-aerated cattle manure for 12 months and for approximately 2 months in aerated manure. Survival rates of *E. coli* O157 are greatly reduced in cattle manure slurry compared to cattle manure (Maule, 1997); the main reason for this decline are changes in the chemical slurry composition (accumulation of NH_4 and organic acids, reduction in oxygen and available carbon). Although *E. coli* O157:H7 do not naturally belong to the microbial community of aquatic systems, this pathogen has been identified in various types of cattle water sources, including troughs, ponds, tanks and creeks (Sargeant *et al*., 2000; LeJeune *et al*., 2001). According to Wang and Doyle (1998), *E. coli* O157:H7 can survive for up to 90 days in river water, with persistence highly-dependent upon water source and temperature. In bottled water this pathogen may survive as long as 300 days (Warburton *et al*., 1998).

2.8. Sigma s factor and its role in stress response of *E. coli* O157:H7

2.8.1 Background

Different unfavorable environmental conditions trigger a bacterial expression of an appropriate set of genes in order to survive. External stimuli cause exchange of sigma subunits in the RNA polymerase (RNAP) complex and gene expression is adjusted to newly emerged needs. The RNA polymerase of *E. coli* is composed of the core enzyme (including α_2, β and β' with the catalytic activity of RNA polymerization), and one of seven different species of σ subunit (see Table 2.5.). Sigma species may clearly recognize distinguishable gene promoters thereby activating distinct gene sets, known as regulons. Most housekeeping genes expressed during exponential-phase growth are transcribed by the holoenzyme containing the *rpoD* gene product, while the holoenzyme σ^S is essential for transcription of certain stationary-phase specific genes (Lange and

Hennge-Aronis, 1991). The σ^S or RpoS subunit is considered the general stress sigma factor. It is strongly induced when a cell is in stationary phase or in exponential phase exposed to various stress conditions, including: ultra-violet radiation, hyperosmolarity, pH downshift, and non-optimal high or low temperature regimes (Hengge-Aronis 2000). The σ^S increase is often accompanied by a reduction or cessation of growth and provides cells with the ability to survive the actual stress as well as additional stresses not yet encountered, ("cross-protection"). The dual nature of the σ^S stress response is opposite to other specific stress responses, which are triggered by a single stress signal and result in the induction of proteins that allow cells to overcome only a specific stress.

Table 2.4. Sigma subunits of *E. coli*

Sigma subunit	**Protein size (aa)**	**Kd(nM) (RNAP-σ)**	**Intracellular concentration** (molecules/cell)	**Genes under the control of each sigma**
RpoD	613	0.26	700	Growth related genes (~1000)
RpoN	477	1.55	110	Nitrogen regulated genes (~15)
RpoS	330	4.26	<1	Stationary phase/stress response (~140)
RpoH	284	1.24	<10	Heat shock/stress response (~40)
RpoF	239	0.74	370	Flagella-chemotaxis genes (~40)
RpoE	202	2.43	<10	Extreme heat shock/extracytoplasmic genes (~5)
Fecl	173	1.73	<1	Ferric citrate transport (~5)

The sigma factors of *E. coli* are listed in their RNAP dissociation constant (K_d) determined by core enzyme binding in mixed reconstitution experiments (Lange and Hennge-Aronis, 1991).

The *rpoS* gene occurs in the γ branch of the proteobacteria, a group of Gram negative bacteria that includes many human pathogen species and also some beneficial organisms. With minor variations, the general function of σ^S in these bacteria appears to be similar to that in *E. coli*. The synthesis and accumulation of σ^S are controlled at multiple levels, including transcription, translation, proteolysis and activity.

2.8.2 Regulation of rpoS transcription

Analysis of *rpoS* transcription using different experimental approaches revealed that several promoters are involved in *rpoS* transcription. Two closely-spaced and relatively weak promoters (*nlpD*p1 and *nlpD*p2) located upstream of the *nlpD* gene (Lange *et al*., 1995) encode a lipoprotein of unknown function (Lange and Hengge-Aronis, 1994), and generate a bicistronic *nlpD-rpoS* message. The major *rpoS* promoter (*rpoS*p) is located within the *nlpD* gene and produces a monocistronic *rpoS* transcript with a 5' untranslated leader region of 567 nucleotides (Takayanagi *et al*., 1994). The operon promoters *nlpD*p1 and *nlpD*p2 are not regulated by growth phase, whereas *rpoS*p is subject to stationary-phase induction in Luria broth-grown cells (Lange *et al*., 1995). The global transcription regulation of *rpoS* is mostly influenced by the catabolite repressor protein (CRP), guanosine tetraphosphate (ppGpp) and inorganic polyphosphate (poly-P).

Many bacteria use catabolite repression as a way to use the most energetically favorable carbon source available. Low catabolite concentration in *E. coli* triggers formation of cyclic AMP (cAMP) via catalytic activity of membrane-associated adenylate cyclase (encoded by the *cya* gene) (Neidhardt, 1996). Higher concentrations of cAMP subsequently cause binding and activation of CRP leading to transcriptional activation or repression of the *rpoS* gene, dependant on the growth phase of organism. Performing experiments with *cya* (encoding adenylate cyclase) or *crp* (encoding the cAMP protein receptor) mutants, it was found that σ^S levels and the activities of an *rpoS*::*lacZ* fusion were high in exponential phase, implying the repressive nature of cAMP-CRP on *rpoS* transcription. Unpublished results from Scheller and Hengge-Aronis however indicate that cAMP-CRP positively control transcription of *rpoS* during entry into stationary phase. The major *rpoS* promoter is flanked by two putative cAMP-

CRP binding sites which most likely have opposite roles in *rpoS* gene transcription. The upstream location of the cAMP-CRP binding site is similar to that of the *lac* promoter, and corresponds to the classical activator position (Busby and Ebright, 1999), while the second cAMP-CRP box is positioned downstream from the transcriptional start site, and has an inhibitory function.

Inorganic polyphosphate is present in most microorganisms and often accumulates during stationary phase or other stress conditions (Kornberg *et al*., 1999). During exponential growth of *E. coli*, the concentration of inorganic phosphate follows the scheme Pi>PPi>poly-P (Neidhardt, 1996). The importance of poly-P for *E. coli* exposed to different stress conditions (Crooke *et al*., 1994) is linked in part to *rpoS* transcription. Polyphosphate accumulation is positively affected by ppGpp, which seems to inhibit the *ppx*-encoded exopolyphosphatase, an enzyme that degrades polyphosphate. In *E. coli*, ppGpp increases in response to amino acid limitation as well as carbon, nitrogen and phosphorus starvation. Transcriptional *rpoS*::*lacZ* fusion experiments revealed a positive role of ppGpp in *rpoS* transcription (Lange *et al*., 1995), where artificial induction of ppGpp in exponential phase caused an approximate 50-fold increase of RpoS (Brown *et al*., 2002). Due to the pleiotropic nature of ppGpp, its mechanism of action remains mostly unresolved. According to Lange *et al*. (1995), ppGpp does not specifically target promoters involved in *rpoS* transcription but rather transcriptional elongation or transcript stability via altered levels of inorganic polyphosphate (Shiba *et al*., 1997).

2.8.3 Regulation of *rpoS* translation

Using translational *rpoS*::*lacZ* fusions it was found that *rpoS* translation is

stimulated by certain stress conditions, including: hyperosmolarity (Lange and Hengge-Aronis, 1994b), low temperature (Sledjeski *et al*., 1996) and low pH (Kampmann and Hengge-Aronis, unpublished data) as well as entry into stationary phase (Lange and Hengge-Aronis, 1994b). The most important elements in control of *rpoS* translation include secondary structure of *rpoS* mRNA, Hfq and H-NS proteins and small regulatory RNAs.

Monocistronic transcripts originating from the main promoter of *rpoS* carry an unusually long nontranslated 5' region (leader region) which plays a crucial role in translation of *rpoS* mRNA. Approximately 340 nucleotides at the 5' end of an rpoS transcript fold into a very stable and complex cruciform-type structure with further downstream folding of the translational initiation region (TIR) into two less-stable structures. The first structure contains a large hairpin, including the Shine-Dalgarno sequence with no function in *rpoS* translation control, whereas the second structure located near the Shine-Dalgarno sequence is partially complementary to an "internal antisense" region located further upstream (Hengge-Aronis, 2002). At present, it is believed that the topology of TIR plays an important role in the control of *rpoS* translation by changing access to the ribosome during different growth/stress conditions. During optimal growth conditions, TIR is poorly accessible to the ribosome, while under certain stress conditions the secondary structure of rpoS mRNA undergoes changes that permit TIR access to the ribosome, with subsequent initiation of translation (Hengge-Aronis, 2002).

Almost 40 years ago, Franze de Fernandez *et al*. (1968) identified the Hfq protein (host factor I [HF-I]), a 11.2 kDa protein essential for replication of the phage Qβ RNA. The exact molecular function of Hfq in *rpoS* translation still remains

unknown. There are several hypotheses regarding possible action of Hfq protein in *rpoS* translation. Epistasis experiments led to the conclusion that Hfq was directly involved in translation initiation (Muffler *et al*., 1996). A key feature of Hfq is the possession of at least two RNA binding domains that prefer adenosine-uracil rich sequences (Vytrytska *et al*., 1998) leading to high binding affinity to several sites in a large 5' *rpoS* transcript (Cunning *et al*., 1998). By binding to several positions of *rpoS* mRNA, Hfq may stabilize the transcript's secondary structure thereby providing a structural condition for additional activators of *rpoS* translation. Another possible explanation is that Hfq acts like a "platform" bound to *rpoS* mRNA, and thus recruiting additional factors involved in *rpoS* translational control (Hengge-Aronis, 2002). Sledjeski and coworkers (2001) found that Hfq may control *rpoS* translation through another regulatory RNA species, in particular a small regulatory DsrA RNA molecule. A hypothetical model of Hfq-DsrA action includes first binding of Hfq to *rpoS* mRNA which recruits DsrA into a ternary complex that would sequentially lead to alteration of the secondary structure of *rpoS* transcript and initiation of *rpoS* mRNA translation (Hengge-Aronis, 2002). Possibly, the pleiotropic nature of Hfq protein implies a multifunctional role in the *rpoS* translational process.

Histone-like protein H-NS primarily function in nucleoid organization (form a large nucleoprotein complex) and in gene regulation as a repressor (Zhang *et al*., 1996). The homodimer form of H-NS specifically binds to curved regions of DNA, thereby blocking transcription initiation of more than 100 genes that are involved in environmental adaptation (Hommais *et al*., 2001). Experiments with H-NS-deficient mutants have demonstrated a H-NS repression of the RpoS post-transcriptional mechanism, where increased σ^S levels in exponential phase were similar to those

reached by the wild-type in stationary phase (Barth *et al*., 1995). At present, the mechanism by which H-NS down-regulates *rpoS* translation is still unclear.

Several small regulatory RNAs control *rpoS* translation in *E. coli*, mostly via fine-tuning functions. Two RNAs, DsrA and RprA, promote *rpoS* translation, while OxyS acts as a repressor. DsrA is an 87-nucleotide RNA that folds into a three-stem-loop structure (Sladjeski and Gottesman, 1995). The main function of DsrA in *E. coli* physiology may be thermoregulation. Although it plays a minor role for *rpoS* translation in cells grown at 37 or 42°C, it becomes the major factor at ≤30°C (Sledjeski *et al*., 1996). The essence of *rpoS* induction at low temperature lies in enhanced transcription of *dsrA* and six-fold increase in transcript stability under low temperature conditions (Repoila and Gotssman, 2001). It is believed that the stem-loop 1 of DsrA binds to the 5' untranslated leader sequence of the *rpoS* transcript, leading to an increase translation rate via opening the secondary structure of *rpoS* mRNA (Majdalani *et al*., 1998). Sladjeski and coworkers (2001) reported that Hfq protein binds to DsrA causing stabilization and changes in its secondary structure that finally lead to association with *rpoS* mRNA.

RprA is a small RNA involved in *rpoS* translation. Transcription of *rprA* is controlled by the phosphorelay system that regulates capsular polysaccharide synthesis genes, RcsB / RcsC, in which RscC is a transmembrane sensor and RcsB is the response regulator (Majdalani *et al*., 2001). The mechanism by which RprA stimulates *rpoS* mRNA translation is unknown.

The small 109-nucleotide untranslated RNA named OxyS plays a repressive role in *rpoS* transcription (Altuvia *et al*., 1997). It is believed that OxyS binds to Hfq with an A-rich 26 bp linker region and prevents Hfq from activating RpoS in response to oxidative stress (Zhang *et al*., 1998).

2.8.4 Regulation of σ^S proteolysis

Other regulatory pathways under RpoS control are proteolytic degradation in exponentially growing cells (Schweder *et al*., 1996), and inhibition of proteolysis triggered by high osmolarity (Muffler *et al*., 1996b), low pH (Lee *et al*., 1995) temperature (Repoila and Gottesman, 2001) and carbon starvation (Takayanagi *et al*., 1994). According to Lange and Hengge-Aronis (1994) the half-life of RpoS in exponential growing cells is approximately 1.4 minutes, while upon entry into stationary phase the half-life of the same protein increases to 16.5 minutes or even more under osmolarity stress (45 minutes).

Several lines of evidence suggest that a barrel-shaped ClpXP processive protease is a key regulator of RpoS degradation (Zhou *et al*., 2001). Two six-subunit rings identifed as ClpX act as a chaperone discriminating and unfolding proteins to the inner chamber consisting of two seven-subunit rings of ClpP where proteolysis takes place (Kim *et al*., 2000). Mutations in either the *clpP* or *clpX* genes result in stabilization of σ^S (Schweder *et al*., 1996). Pratt and Silhavy (1996) demonstrated both *in vivo* and *in vitro* that σ^S cannot be recognized by ClpXP alone. The two-component regulatory RssB protein is required for recognition of RpoS by the ClpXP complex (Bearson *et al*., 1996). Activity of the RssB is modulated by phosphorylation of a conserved aspartyl residue in the N-terminal of the protein, which results in direct interaction with RpoS (Becker *et al*., 1999). Acetyl phosphate is a phosphate donor that triggers RssB activity and results in a proteolytic rate of RpoS (Bouche *et al*., 1998). Mutation in RssB has been shown to lead to a ten-fold increase of RpoS level during exponential phase, implying the degradable nature of RssB in σ^S physiology (Muffler *et al*., 1996c).

2.8.5 Regulation of σ^S activity

In *in vivo* or *in vitro* experiments, more than 100 promoters have been identified that may be recognized by RNAP containing σ^S, with no consensus promoter sequence (Ishihama, 2000). In addition, a number of σ^D-dependent promoters can be transcribed *in vitro* by the $E\sigma^S$ holoenzyme (Hiratsu *et al*., 1995). The current view of this "sigma selectivity paradox" (Weber *et al*., 2005) is that the $E\sigma^S$ holoenzyme recognizes a specific DNA conformation such as bent DNA regions (Espinosa-Urgel and Tormo, 1993). Another explanation for the sigma selectivity paradox is that $E\sigma^S$ and $E\sigma^D$ holoenzymes use very similar promoters but that minor differences in the extended -10 region (Becker and Hengge-Aronis, 2001) may shift the preference towards one or the other holoenzyme. The most recent findings suggest that interaction between the $E\sigma^S$ holoenzyme and its target promoters is more complex. Changes in the cytoplasmic composition (increased level of trehalose and potassium glutamate) result in decreased superhelicity of template DNA that further enhances transcription by $E\sigma^S$ (Ishihama, 2000). The overall findings lead to the conclusion that a variety of sigma S promoter sequences may be recognized under different specific conditions or in the presence of a specific factor.

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BRIEF INTRODUCTION TO CHAPTER 3

This study was designed to provide fundamental information on the prevalence of *E. coli* O157 in cattle housed across Saskatchewan. To achive this goal, a two-year study was conducted, where in total 23 feed lots were screened for the presence of *E. coli* O157. Accordingly, two approaches were employed: (i) a longitudinal study, where 3 feedlot-cattle operations were examined over a 10-month period, and (ii) a point study, where 20 feedlots were examined once. In addition, all isolates obtained from the longitudinal and point prevalence studies were characterized in terms of their genetic relatedness, antimicrobial susceptibility, virulence profiles, and cytotoxicity.

3. PREVALENCE OF *ESCHERICHIA COLI* O157 IN SASKATCHEWAN CATTLE: CHARACTERIZATION OF ISOLATES USING RAPD-PCR, ANTIBIOTIC RESISTANCE PROFILES, AND PATHOGENICITY DETERMINANTS

Abstract

The prevalence of *Escherichia coli* O157 associated with feedlot cattle in Saskatchewan was determined over a 10 month longitudinal study (3 feedlots) and a point prevalence study (20 feedlots). The prevalence of *E. coli* O157 at the three different sites in the horizontal study varied from 2.5-45%. The point prevalence of *E. coli* O157 among Saskatchewan cattle from 20 different feedlots ranged from 0% to a high of 57%. A statistically significant (P = 0.003) positive correlation was determined to exist between the density of cattle and the *E. coli* O157 prevalence rate. A significant correlation (P = 0.006) was also found between *E. coli* O157 percent prevalence and the number of cattle housed: capacity ratio. All 194 *E.* coli O157 isolates obtained were highly virulent, and RAPD PCR analysis revealed the isolates grouped into 39 different *E. coli* O157 subtypes, most of which were indigenous to specific feedlots. Two of the most predominant subtypes were detected in 11 different feedlots and formed distinct clusters in two geographic regions in the province. Antimicrobial susceptibility testing of the *E. coli* O157 isolates revealed that 10 were multidrug resistant, 73 and 5 of which were resistant to sulfisoxazole and tetracycline, respectively.

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3.1. Introduction

In 1982 two outbreaks of bloody diarrhea in Oregon and Michigan (41) and another in Ottawa (47) led to recognition of a new pathogenic serotype, *Escherichia coli* O157:H7. Since its discovery as an etiologic agent of hemorrhagic colitis in 1982, the clinical importance of *E. coli* O157:H7 has escalated rapidly. In little more than a decade, recognition of *E. coli* O157:H7 underwent a major transformation from that of a newly described agent of diarrheal disease (1982) to the leading bacterial cause of bloody diarrhea in United States in 1992 (18).

The pathogenicity of *E. coli* O157:H7 is associated with a powerful combination of different virulence factors. Several inducible mechanisms of acid resistance (23, 24) potentially contribute to the low infectious dose of this organism (1 to 100 CFU; 16, 36), and its survival in the harsh environment of the stomach. In addition, *E. coli* O157:H7 efficiently adhere to the intestine epithelial cells causing attaching and effacing lesions (AE) (46). The host cells undergo dramatic changes, losing microvilli on the cell surface, followed by the tight attachment of cells to the epithelial surface and deformation of cell cytoskeletal structure resulting in the formation of pedestals (29). A cluster of genes involved in the generation of the AE lesions are chromosomally encoded on a "pathogenicity island" named the Locus of Enterocyte Effacement (28). This pathogenicity island also encodes the *eae* gene which is responsible for the generation of an outer membrane protein called intimin necessary for attachment to enterocytes. Undoubtedly, the most potent virulence feature of *E. coli* O157:H7 is the

ability to produce Shiga toxins (Stx1 and/or Stx2 and variants), a family of unique, heterodimeric protein toxins (32) that cause a wide spectrum of clinical symptoms including hemorrhagic colitis (bloody diarrhea), life-threatening complications such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (48).

Ruminants, especially cattle, have been implicated as the primary reservoir of *E. coli* O157:H7 that infect humans. The fecal excretion of this organism by cattle appears to be seasonal, with excretion rates highest in spring and late summer (17, 45). Human infection with *E. coli* O157:H7 has been associated with various transmission routes, including: direct exposure to infected animals (40), person-to-person passage (4) and mostly by consumption of contaminated foods and waters (5, 10, 20). From an epidemiological point of view it is interesting that Shiga toxin-producing *Escherichia coli* (STEC) infections in humans seem to be different worldwide. *Escherichia coli* O157:H7 is the predominant serotype of STEC in the United States, Canada, the United Kingdom and Japan but in continental Europe, Australia and Latin America non-O157:H7 serotypes are much more common than *E. coli* O157:H7 (33). Incidence of *E. coli* O157:H7 disease have risen in Canada. According to the Public Health Agency of Canada (2005) the majority (95%) of pathogenic *E. coli* isolates from human cases were serovar O157.

As a consequence of the fast growth of the cattle industry in Western Canada, the health risk from *E. coli* O157:H7 has increased, especially among residents of rural communities. There is virtually no information on the occurrence of *E. coli* O157 in Saskatchewan cattle herds and its presence in adjacent environments. This study was conducted to determine the frequency of occurrence of *E. coli* O157 in Saskatchewan cattle and environmental samples (pristine soil and those soils treated with cattle

manure, water from dugouts) by combination of a longitudinal (time course) and a point prevalence (multiple feedlots analyzed at approximately the same time) studies over a two year period. This study also sought to characterize the *E. coli* O157 isolates obtained in terms of their virulence profiles, antibiotic susceptibilities, cytotoxicity potential and RAPD genomic profiling. RAPD DNA banding patterns, in conjunction with virulence and antibiotic resistance patterns, may assist in the epidemiologic tracing of *E. coli* O157 isolates of medical concern.

3.2. Materials and methods

3.2.1 Longitudinal prevalence study

A longitudinal study was designed to examine the prevalence of *E. coli* O157 over a ten-month period at two feedlots (randomly given the identifiers L and V) and a cow-calf operation (WB). At each sampling time, twenty cattle fecal samples were randomly collected from the pen floor from each facility (for a total of 400 fecal samples). An additional 15 environmental samples, consisting of soil samples from fields treated with manure as well as water from dugouts and natural runoff, were collected per sampling period (for a total of 165 environmental samples).

3.2.2 Point prevalence study

The point prevalence study targeted a number of feedlots which ranged in size from 80 to 5,800 head of cattle. Twenty Saskatchewan feedlots were sampled once during the summer of 2004 (from late June 2004 to the end of September 2004). Forty fecal samples were collected from each feedlot. The percent prevalence of *E. coli* O157 in the point and longitudinal (above) studies was calculated as: [(number of samples positive of *E. coli* O157 / total number of samples tested in a feedlot) x 100].

3.2.3 Collection of samples

Over a two year period 1,200 bovine fecal and 165 environmental samples were collected from 23 different cattle facilities as part of both longitudinal and point prevalence studies. All bovine fecal samples were obtained from freshly defecated feces. Approximately 40 g of feces was aseptically collected per sample using a sterile tongue depressor (Baxter Diagnostics Inc. USA) and transferred to a sterile 80 ml plastic container (Starplex, Canada). Typically, 2 to 6 randomly chosen samples were obtained per pen so that each pen in multi-pen feedlots was screened for *E. coli* O157. Samples from lagoons, dugouts and soil were sampled directly using a sterile 80 ml plastic container. All samples were stored in a cooler with frozen cold-packs, transported to the laboratory, and tested within 2-6 hours of collection. All environmental and bovine fecal samples were processed in parallel using cultural methods with and without immunomagnetic separation.

3.2.4 Immunomagnetic separation (IMS)

Samples (1 g or 1 ml) were aseptically added to 10 ml of Buffered Peptone Water (Becton Dickinson and Company, USA) supplemented with cefixime (0.5 mg L^{-1}; Rhone-Poulenc Rorer, Canada), vancomycin (8 mg L^{-1}; Sigma-Aldrich Chemie, Germany) and cefsuludin (10 mg L^{-1}; Sigma-Aldrich Chemie) (7). After homogenization of samples, the suspensions were incubated at 37˚C for 6-8 h without agitation. IMS was performed according to the manufacturer's instructions using a BeadRetriever (Dynal Biotech ASA, Oslo, Norway). One hundred microliters of the concentrated bead-bacteria complex was plated onto sorbitol MacConkey (SMACct; Becton Dickinson and Company) agar plates supplemented with cefixime (0.5 mg L^{-1}) and potassium tellurite (2.5 mg L^{-1}, Oxoid LTD, Hampshire, UK). After 24 h of incubation at 37˚C, discrete

gray non-sorbitol fermenting colonies were selected and tested by seroagglutination using O157 latex reagent (Oxoid LTD). All sero-positive isolates were saved in 10% glycerol at -70°C for further analysis.

3.2.5 Cultural technique

Samples (1 g or 1 ml) were aseptically added to 10 ml of modified EC broth (Becton Dickinson) containing novobiocin (20 mg L^{-1}, Sigma Chemical Co.) vortexed, and then incubated at 37˚C for 18-24 h. After incubation, a 1 ml aliquot of each enrichment culture was serially diluted to 10^{-5} in sterile 0.85% saline to facilitate the recovery of isolates. One hundred microliters of the 10^{-3}, 10^{-4} and 10^{-5} dilutions were spread plated in duplicate onto SMACct agar and incubated at 37˚C overnight. Each non-sorbitol fermenting colony with characteristic morphology for *E. coli* O157 was serologically tested for the somatic O157 antigen and sero-positive isolates were saved as described above.

3.2.6 Multiplex PCR

All presumptive *E. coli* O157-positive isolates were tested for the presence of four virulence determinants, including *stx*$_1$, *stx*$_2$, *eaeA*, and *hlyA*. Template DNA for multiplex PCR and RAPD fingerprinting were prepared from isolates that were grown at 37˚C for 24 h in Lauryl Tryptose broth (Becton Dickinson) with agitation. After incubation, the optical density of the culture was standardized at 600 nm by diluting with broth. Aliquots (400 µl) of the stationary-phase culture with an optical density value of 0.6 were centrifuged and total DNA was extracted according to the manufacture's instruction using a DNeasy Tissue kit (QIAGEN Inc. Mississauga, Canada). For the

longitudinal study, multiplex PCR was performed as described by Fagan *et al.* (12). *Escherichia coli* O157:H7 ATCC 43894 (which possesses the above virulence determinants) and *Pseudomonas fluorescens* strain cc848406E were used as positive and negative controls, respectively. In a recent study (11), it was shown that the Fagan PCR assay was unreliable for the detection of stx_{2d} -positive STEC strains. Thus, the multiplex PCR protocol by Paton and Paton (35), which can identify all known subtypes of Shiga toxins, was used during the analysis of isolates obtained from the point prevalence study.

3.2.7 Genotyping of *E. coli* O157 isolates

In preliminary experiments, four decamer primers were tested against a panel of 15 different *E. coli* O157 isolates for the random amplification of polymorphic DNA: GEN 15001 (5'-GTGCAATGAG-3') (39), 1283 (5'-GCGATCCCCA-3') (15), 1290 (5'-GTGGATGCGA-3') (34) and 1254 (5'-CCGCAGCCAA-3') (1, 2, 26). Primer 1283 provided the greatest discriminatory ability among the *E. coli* O157 test isolates, and was therefore used in this study. To further validate the reproducibility of this primer and procedure, three colonies of the same *E. coli* O157 isolate were amplified by primer 1283 in three independent PCR reactions. The band patterns of the three colonies were consistent both among each of the colonies as well as within the triplicate PCR reactions. Preparation of DNA templates for RADP PCR was described above. PCR was carried out in 50 µl reaction volumes containing 5 µl of DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 250 µM of each dNTP, 0.8 µM of primer and 2 units of *Taq* polymerase. PCR amplification was performed in a Techne TC-412 thermal cycler, as follows: 45 cycles at 94˚C for 1 min, 36˚C for 1 min and 72˚C for 2 min,

followed by an elongation step of 72˚C for 5 min. Amplified products were then electrophoresed at 90 V for 2 h in 1.5% (w/v) agarose gels containing 0.125 μg ml^{-1} ethidium bromide. A 1 Kb Plus DNA ladder was used as a molecular weight marker.

3.2.8 Vero cell cytotoxicity assay

The cytotoxicities of the *E. coli* O157 isolates were determined using the method previously described by Roberts *et al*. (42). Cytopathic effects of the *E. coli* O157 isolates were examined using a Zeiss inverted microscope (200X).

3.2.9 Antibiotic susceptibility testing

Escherichia coli O157 isolates obtained from the point prevalence study (n=131) were examined for antibiotic resistance using Sensititre CMV1AGNF plates (TREK Diagnostic Systems, Cleveland, OH) that contained 17 antimicrobial agents dosed in 96 wells at appropriate dilutions, as specified by NARMS (National Antimicrobial Resistance Monitoring System) of the CDC. Each well of the Sensititre microtiter plate was inoculated according to the instructions of the manufacturer followed by incubation at 37˚C for 24 h. The minimal inhibitory concentration (MIC) was manually determined for each isolate as the lowest concentration of each antibiotic that inhibited visible growth. The MIC breakpoints were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) M100 (30) and M31 (31).

3.2.10 Statistical analysis

Data were analyzed with SAS (SAS Inst-Inc, Cary, NC) by using multiple regression to determine the dependence of prevalence of *E. coli* O157 on size of feedlots, density of cattle in pens, and ratio between housed cattle : feedlot capacity.

Each predictor (size of feedlots, density of cattle in pens, and ratio between housed cattle : feedlot capacity) was also evaluated using stepwise regression. The analysis of DNA patterns obtained using RAPD PCR was performed by using the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc, Exeter Software, Setauket, N.Y.). Similarities between RAPD patterns were determined based on the Dice similarity coefficient. The resulting similarities in the matrix were further processed by employing the unweighted par – group method with arithmetic averages (UPGMA) to create a dendrogram that graphed the genetic relatedness between *E. coli* O157 isolates.

3.3. Results

3.3.1 Prevalence of *E. coli* O157:

3.3.1.1. (i) Longitudinal study

A total of 565 fecal bovine and environmental samples were collected over a 10 month period. Out of 400 fecal samples, *E. coli* O157 was found in 57 samples (14.2 %). At feedlots L and V, *E. coli* O157 isolates were detected during every visit, and the frequency of detection fluctuated from 10-40%, and 30-65%, respectively. The overall prevalence of *E. coli* O157 for feedlots L and V over the 10 month period were 22.5 and 45%, respectively. *Escherichia coli* O157 was found at the cow-calf operation WB only in August, resulting in an overall prevalence of 1.4%. The occurrence of *E. coli* O157 at all three cattle facilities over the ten collection periods are summarized in **Table 3.1**. Out of 165 environmental samples, none were found to be positive for *E. coli* O157.

Table 3.1. Table summarizing the characteristics of feedlots examined, and the prevalence (%) of *E. coli* O157 detected at each facility.

Feedlot	Diet	Capacity	Number of cattle	Housed cattle /capacity	Cattle density (# head / 100 m^2)	Prevalence %
Z	Concentrate - fed	2000	1000	0.50	4.9	22.5
T	Concentrate - fed	6000	5000	0.83	4.8	10
P	Concentrate - fed	3500	1600	0.45	6.5	42.5
B	Concentrate - fed	8000	3000	0.37	6.7	30
G	Concentrate - fed	3000	200	0.06	3.6	0
S	Concentrate - fed	3500	3500	1	7.4	57.5
M	Concentrate - fed	4000	4000	1	5.8	55
W	Concentrate - fed	3500	1500	0.42	4.3	5
HM	Concentrate - fed	7500	5800	0.77	5.4	40
PP	Pasture - fed	1600	80	ND	ND	0
MT	Concentrate - fed	4000	2500	0.62	5.9	32.5
MO	Pasture - fed	800	70	ND	ND	0
K	Concentrate - fed	3400	1300	0.38	5	15
ML	Pasture - fed	1000	120	ND	ND	0
SC	Concentrate - fed	2500	420	0.16	4.7	0
H	Concentrate - fed	700	100	0.14	3	0
WE	Concentrate - fed	3600	1300	0.36	6.5	17.5
TK	Concentrate - fed	2200	1000	0.45	4.3	0
SB	Pasture - fed	3000	80	ND	ND	0
I	Pasture - fed	1500	500	ND	ND	2.5
L[a]	Concentrate- fed	28000	15000	0.53	5.6	22.5
V[a]	Concentrate- fed	17000	12000	0.70	5.8	45
WB[a]	Pasture - fed	1200	300	ND	ND	1.4

[a] superscripts refer to feedlots examined during the longitudinal study.
[a]WB refers to a cattle research station. All remaining different letter designations refer to different feedlots.
[b] ND, not determined.

3.3.1.2. (ii) Point study

The prevalence of *E. coli* O157 in feedlots located throughout Saskatchewan are summarized in **Table 3.1. and Figure 3.1**. A total of 800 samples were collected from 20 feedlots during this study. Out of 20 feedlots, twelve were found positive for *E. coli* O157 within which the prevalence of *E. coli* O157 ranged from 2.5 to 57.5%. A significant positive correlation (P=0.003) was observed between the prevalence rate of *E. coli* O157 and density of cattle in pens (**Figure 3.2.;** constructed using data obtained from both point prevalence and longitudinal studies**)**. There was another statistically

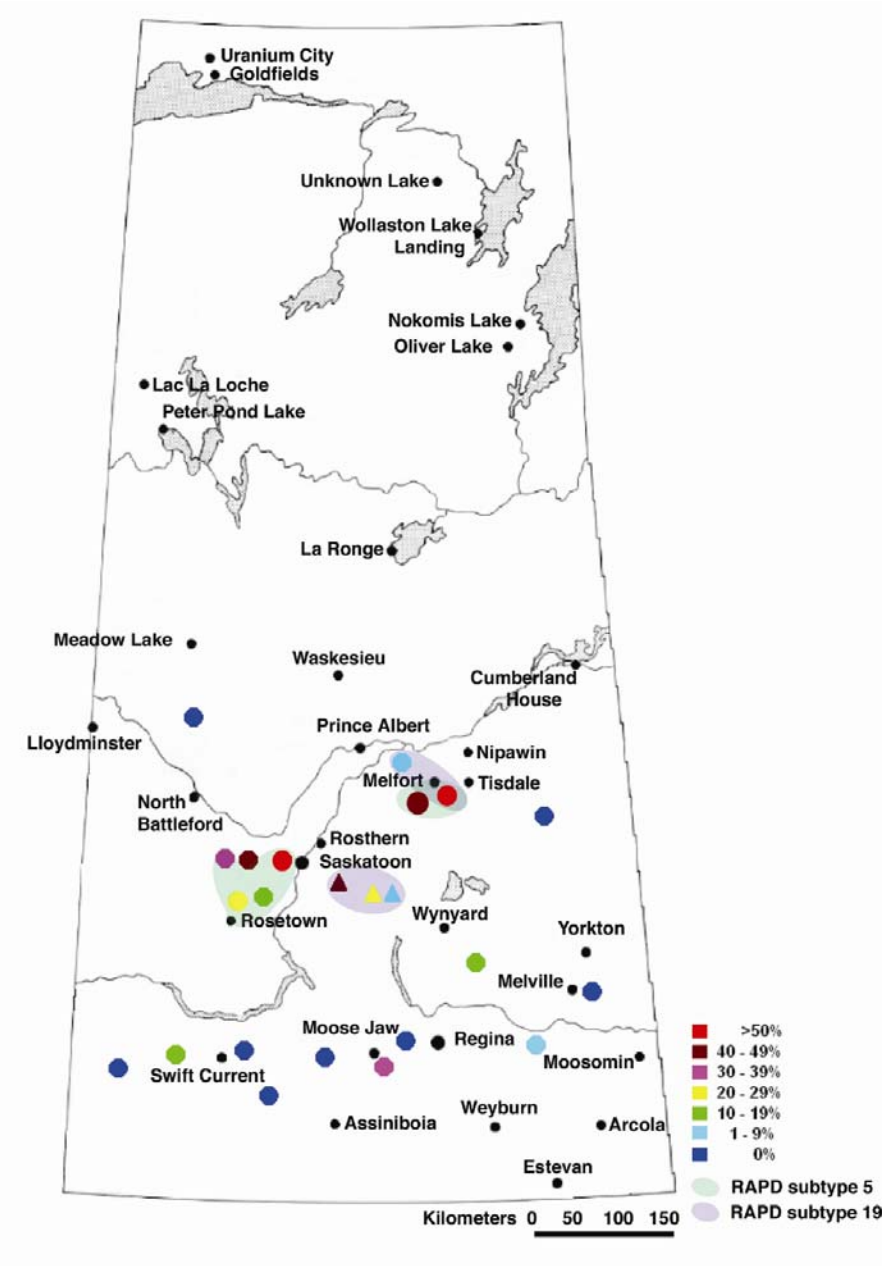

Figure 3.1. Map of Saskatchewan showing the prevalence of *E. coli* O157 detected (see color-coded legend) at feedlots/farms examined over a 2-year period. Triangles correspond to feedlots/farms examined during the longitudinal study, whereas circles represent feedlots examined during the point prevalence study. Note also colored shaded regions showing the geographic location of feedlots where *E. coli* O157 RAPD subtypes 5 and 19 were detected.

significant correlation (P=0.006) found between the prevalence rate and a number of lots occupied by cattle within a feedlot (expressed as the ratio between the housed cattle: feedlot capacity; **Table 3.1.**).

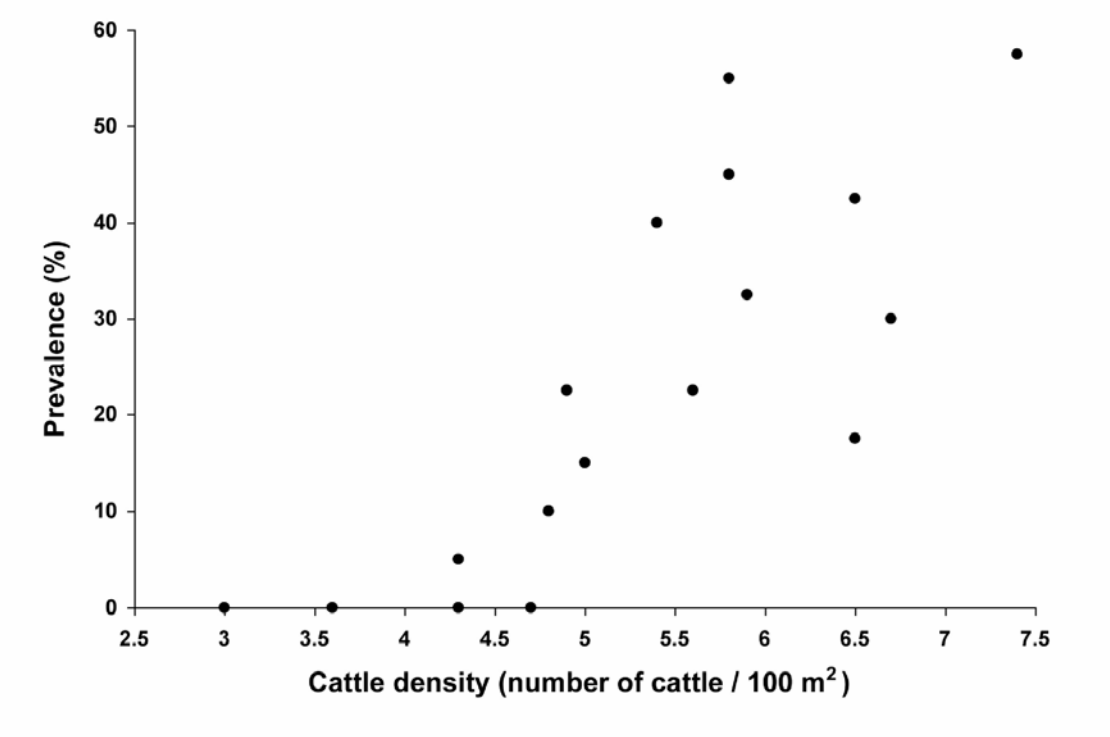

Figure 3.2. Relationship between the prevalence of *E. coli* O157 and the density of cattle (number of cattle:100 m^2) housed at various feedlots (statistically significant at P=0.003).

3.3.2. Virulence profile of *E. coli* O157 isolates

Multiplex PCR confirmed that all 194 serological positive isolates (from both longitudinal and point studies) were indeed *E. coli* O157. These 194 *E. coli* O157 isolates exhibited five different virulence profiles. The majority of the isolates (161 or 82.9%) possessed all four virulence-associated genes (stx_1, stx_2, *eaeA* and *hlyA*). The second most predominant virulence profile was a combination of stx_2, *eaeA* and *hlyA* genes, with 22, or 11.4%, of the isolates having these genes. Six isolates (3.1%) possessed stx_1, *eaeA* and *hlyA* genes and 4 isolates (2.1%) were determined to have stx_1 and *hlyA* virulence markers. A single isolate (0.5%) had a unique virulence profile with only the *hlyA* gene being present. Out of the 194 *E. coli* O157 isolates obtained from across Saskatchewan over the two year study, 193 (99.4%) possessed at least one Shiga

toxin gene and 187 (96.4%) had a combination of *eaeA* and stx_1 and or stx_2, indicating a very high virulence potential of these isolates for humans.

3.3.3 Cytotoxicity

The cytotoxicity effects of all 194 *E. coli* O157 isolates on Vero cells were observed and scaled from a value of 0 (no effect) to 10 (greatest effect), and compared to the reference strain *E. coli* O157 ATCC 43894, known to possess all four virulence determinants. All isolates from this study expressed cytotoxicity that ranged from 5.1 to 10. The majority of isolates (n = 107 or 55%) expressed very high toxicity values of 9.1-10; two other groups (n = 39 or 20% and n = 36 or 19%) had slightly lower cytotoxicities, ranging 8.1-9.0 and 7.1-8.0, respectively. Interestingly, the lowest toxicity (5.1-6.0) was observed for isolate L-9 (longitudinal study), an isolate that possessed both Shiga toxins (data not shown). In contrast, isolate L-12, which possessed only the *hlyA* gene, had a somewhat greater toxic effect (7.1-8.0) (data not shown).

3.3.4 RAPD-PCR

The panel of *E. coli* O157 isolates from the longitudinal and point studies were genetically characterized using the RAPD-PCR technique. All isolates were grouped into 3 major clusters that encompassed 39 different subtypes or RAPD-PCR patterns. Amplification reactions using primer 1283 produced a set of 18 DNA fragments ranging from 450 to 4,000 bp in length. Generally, all isolates displayed a basic total DNA banding pattern; two doublets migrated at 520-560 bp and 650-700 bp, respectively, one triplet at 1,400, 1,500, 1,600 bp and a final band at 2,900 bp. Genetic similarity between the *E. coli* O157 isolates ranged from 0.76 to 1.00. Fourteen isolates from 9 feedlots (B,

HM, M, P, MT, WE, K, V and L) exhibited fourteen distinct genetic patterns, whereas eleven other subtypes (3, 10, 12, 14, 18, 25, 29, 32, 33, 34 and 35) consisted of groups of isolates that were endemic for their feedlots. The two most predominant subtypes, 5 and 19, were detected at six feedlots each, of which eleven feedlots formed four distinct geographic clusters (each < 50 km in diameter) in central and north central Saskatchewan (**Figure 3.1.**).

3.3.5 Antibiotic susceptibility

The antibiotic resistance patterns of the 131 isolates from the point prevalence study are shown in **Table 3.2**. Most of the 85 isolates (65%) were resistant to at least one antibiotic, whereas 46 isolates (35%) were sensitive to all 17 antibiotics tested. No resistance was observed for amikacin, ampicillin, amoxycillin/clavulanic acid, ceftriaxone, ciprofloxacin, cefoxitin, gentamicin, kanamycin, nalidixic acid, trimethoprim/sulfamethoxazole, and ceftiofur. The antibiotic for which resistance was most frequently observed was sulfisoxazole (61%) followed by tetracycline (12%). The prevalence rate of resistance to chloramphenicol and streptomycin among the *E. coli* O157 isolates was 2.3%. Two patterns of multidrug resistance, involving: i) chloramphenicol, sulfisoxazole, streptomycin, and tetracycline, and ii) sulfisoxazole and tetracycline, were observed in 3 (2.3%) and 8 (6%) isolates, respectively (**Table 3.2.**).

Table 3.2. Summary table showing characteristics (isolate number, isolation method, virulence profile, RAPD pattern, antibiotic resistance, and cytotoxicity) of *E. coli* O157 isolates obtained from point study.

Feedlot	Isolate	Isolation method		Virulence profile				RAPD pattern	Antibiotic resistance	Cytotoxicity
		IMS	Cultural	*stx1*	*stx2*	*hly*A	*eae*A			
Z	Z-6	+		+	+	+	+	1	Sulfisoxazole	9.1 - 10
Z	Z-8	+		+	+	+	+	3	Sulfisoxazole	8.1 - 9.0
Z	Z-11	+	+	+	+	+	+	1	Sulfisoxazole	9.1 - 10
Z	Z-14	+		+	+	+	+	1	Sulfisoxazole	8.1 - 9.0
Z	Z-19	+		+	+	+	+	3	Sulfisoxazole	8.1 - 9.0
Z	Z-27	+	+	+	+	+	+	5	Sulfisoxazole	9.1 - 10
Z	Z-30	+	+	+	+	+	+	3	Sulfisoxazole	7.1 - 8.0
Z	Z-37	+		+	+	+	+	3	Sulfisoxazole	7.1 - 8.0
Z	Z-38		+	+	+	+	+	5	Sulfisoxazole	8.1 - 9.0
T	T-16	+		+	+	+	+	1	Susceptible	9.1 - 10
T	T-20[a]	+								
T	T-22		+	+	+	+	+	1	Tetracycline	8.1 - 9.0
T	T-33		+	+	+	+	+	5	Sulfisoxazole	8.1 - 9.0
T	T-36	+	+	+	+	+	+	5	Sulfisoxazole	9.1 - 10
B	B-1	+		+	+	+	+	5	Susceptible	7.1 - 8.0
B	B-3	+	+	+	+	+	+	5	Tetracycline	8.1 - 9.0
B	B-4	+		+	+	+	+	5	Tetracycline	9.1 - 10
B	B-16	+		+	+	+	+	4	Tetracycline	9.1 - 10
B	B-19	+	+	+	+	+	+	2	Tetracycline	9.1 - 10
B	B-22	+		+	+	+	+	6	Susceptible	8.1 - 9.0
B	B-23	+		+	+	+	+	6	Susceptible	7.1 - 8.0
B	B-27	+		+	+	+	+	6	Susceptible	9.1 - 10
B	B-30	+			+	+	+	6	Sulfisoxazole	9.1 - 10
B	B-33	+	+	+	+	+	+	6	Sulfisoxazole	9.1 - 10
B	B-36	+	+	+	+	+	+	6	Sulfisoxazole; Tetracycline	9.1 - 10
B	B-38	+		+	+	+	+	6	Sulfisoxazole; Tetracycline	7.1 - 8.0
P	P-2	+			+	+	+	6	Susceptible	7.1 - 8.0
P	P-3	+		+	+	+	+	6	Sulfisoxazole	9.1 - 10
P	P-4	+	+		+	+	+	16	Susceptible	9.1 - 10
P	P-5	+		+	+	+	+	38	Sulfisoxazole	9.1 - 10
P	P-7	+		+	+	+	+	6	Sulfisoxazole	9.1 - 10
P	P-9	+		+	+	+	+	18	Sulfisoxazole	8.1 - 9.0
P	P-11	+	+		+	+	+	17	Sulfisoxazole	9.1 - 10
P	P-13	+		+	+	+	+	18	Sulfisoxazole	9.1 - 10
P	P-16	+			+	+	+	17	Susceptible	6.1 - 7.0
P	P-21	+	+	+	+	+	+	18	Sulfisoxazole	8.1 - 9.0
P	P-26	+			+	+	+	17	Susceptible	9.1 - 10
P	P-27	+			+	+	+	17	Sulfisoxazole	9.1 - 10
P	P-28	+	+		+	+	+	17	Sulfisoxazole	9.1 - 10
P	P-29	+		+	+	+	+	18	Sulfisoxazole	9.1 - 10
P	P-36	+		+	+	+	+	18	Sulfisoxazole	9.1 - 10
P	P-37	+		+	+	+	+	18	Sulfisoxazole	9.1 - 10
P	P-40	+			+	+	+	17	Sulfisoxazole	9.1 - 10
S	S-1[a]	+	+							
S	S-4	+	+	+		+	+	5	Susceptible	9.1 - 10
S	S-5	+	+	+	+	+	+	5	Susceptible	9.1 - 10

Table 3.2. Continued

Feedlot	Isolate	Isolation method		Virulence profile				RAPD pattern	Antibiotic resistance	Cytotoxicity
		IMS	Cultural	*stx1*	*stx2*	*hlyA*	*eaeA*			
S	S-6	+	+		+	+	+	13	Chloramphenicol; Tetracycline; Streptomycin; Sulfisoxazole	7.1 - 8.0
S	S-7		+	+		+	+	5	Susceptible	7.1 - 8.0
S	S-8	+	+	+	+	+	+	5	Susceptible	6.1 - 7.0
S	S-10	+	+	+	+	+	+	5	Susceptible	6.1 - 7.0
S	S-11		+	+	+	+	+	5	Susceptible	7.1 - 8.0
S	S-14	+	+	+	+	+	+	5	Susceptible	6.1 - 7.0
S	S-16	+	+	+	+	+	+	5	Susceptible	6.1 - 7.0
S	S-19		+	+	+	+	+	5	Susceptible	7.1 - 8.0
S	S-26	+	+	+	+	+	+	7	Susceptible	8.1 - 9.0
S	S-27	+		+	+	+	+	5	Susceptible	9.1 - 10
S	S-28		+	+		+	+	5	Susceptible	7.1 - 8.0
S	S-30		+	+	+	+	+	5	Sulfisoxazole	7.1 - 8.0
S	S-32	+	+	+	+	+	+	5	Sulfisoxazole	8.1 - 9.0
S	S-33	+	+	+	+	+	+	5	Susceptible	7.1 - 8.0
S	S-34		+	+	+	+	+	5	Susceptible	9.1 - 10
S	S-37	+	+	+	+	+	+	7	Susceptible	8.1 - 9.0
S	S-38		+		+	+	+	13	Chloramphenicol; Tetracycline; Streptomycin; Sulfisoxazole	8.1 - 9.0
S	S-39		+	+		+	+	5	Sulfisoxazole	8.1 - 9.0
S	S-40	+	+	+		+	+	5	Sulfisoxazole	7.1 - 8.0
M	M-2		+	+	+	+	+	7	Susceptible	9.1 - 10
M	M-4	+	+		+	+	+	14	Susceptible	9.1 - 10
M	M-5	+			+	+	+	14	Susceptible	8.1 - 9.0
M	M-6	+	+	+		+	+	5	Sulfisoxazole	9.1 - 10
M	M-8		+		+	+	+	5	Chloramphenicol; Tetracycline; Streptomycin; Sulfisoxazole	9.1 - 10
M	M-9		+		+	+	+	5	Susceptible	9.1 - 10
M	M-13		+		+	+	+	7	Susceptible	9.1 - 10
M	M-14	+			+	+	+	5	Sulfisoxazole	7.1 - 8.0
M	M-15		+		+	+	+	10	Susceptible	9.1 - 10
M	M-16		+		+	+	+	7	Susceptible	6.1 - 7.0
M	M-17	+		+	+	+	+	10	Sulfisoxazole	9.1 - 10
M	M-19		+	+	+	+	+	11	Sulfisoxazole	9.1 - 10
M	M-21		+	+	+	+	+	9	Sulfisoxazole	9.1 - 10
M	M-22	+	+	+	+	+	+	19	Susceptible	9.1 - 10
M	M-23	+		+	+	+	+	39	Susceptible	8.1 - 9.0
M	M-26		+	+	+	+	+	19	Sulfisoxazole	7.1 - 8.0
M	M-27	+	+		+	+	+	14	Sulfisoxazole	8.1 - 9.0
M	M-29		+		+	+	+	19	Sulfisoxazole	8.1 - 9.0
M	M-31		+	+	+	+	+	10	Sulfisoxazole	9.1 - 10
M	M-33		+		+	+	+	11	Susceptible	9.1 - 10
M	M-34	+	+		+	+	+	15	Susceptible	9.1 - 10
M	M-40	+		+	+	+	+	11	Sulfisoxazole	7.1 - 8.0

Table 3.2. Continued

Feedlot	Isolate	Isolation method		Virulence profile				RAPD pattern	Antibiotic resistance	Cytotoxicity
		IMS	Cultural	*stx1*	*stx2*	*hlyA*	*eaeA*			
W	W-12	+	+	+	+	+	+	19	Sulfisoxazole	7.1 - 8.0
W	W-37	+	+	+	+	+	+	19	Sulfisoxazole	9.1 - 10
HM	HM-3	+	+	+	+	+	+	12	Sulfisoxazole	7.1 - 8.0
HM	HM-6		+	+	+	+	+	11	Susceptible	6.1 - 7.0
HM	HM-8		+	+	+	+	+	11	Susceptible	8.1 - 9.0
HM	HM-11		+	+	+	+	+	11	Sulfisoxazole	8.1 - 9.0
HM	HM-13	+		+	+	+	+	11	Sulfisoxazole	9.1 - 10
HM	HM-14	+	+	+	+	+	+	11	Sulfisoxazole	8.1 - 9.0
HM	HM-19	+	+	+	+	+	+	11	Sulfisoxazole	9.1 - 10
HM	HM-20	+	+	+	+	+	+	11	Sulfisoxazole	7.1 - 8.0
HM	HM-22	+	+	+	+	+	+	11	Sulfisoxazole	8.1 - 9.0
HM	HM-23	+		+	+	+	+	11	Sulfisoxazole	9.1 - 10
HM	HM-25	+	+	+	+	+	+	5	Sulfisoxazole	9.1 - 10
HM	HM-26	+		+	+	+	+	12	Sulfisoxazole	9.1 - 10
HM	HM-27	+	+	+	+	+	+	8	Sulfisoxazole	8.1 - 9.0
HM	HM-28		+	+	+	+	+	12	Sulfisoxazole	8.1 - 9.0
HM	HM-34	+		+	+	+	+	12	Sulfisoxazole	7.1 - 8.0
HM	HM-35	+	+	+	+	+	+	12	Sulfisoxazole	9.1 - 10
MT	MT-2	+	+	+	+	+	+	23	Sulfisoxazole	9.1 - 10
MT	MT-3	+	+	+	+	+	+	25	Sulfisoxazole	9.1 – 10
MT	MT-4	+		+	+	+	+	25	Sulfisoxazole	9.1 - 10
MT	MT-6	+		+	+	+	+	25	Sulfisoxazole	9.1 - 10
MT	MT-7	+		+	+	+	+	25	Susceptible	9.1 - 10
MT	MT-9	+		+	+	+	+	26	Susceptible	9.1 - 10
MT	MT-17	+	+	+	+	+	+	26	Susceptible	9.1 - 10
MT	MT-18	+	+	+	+	+	+	25	Sulfisoxazole	9.1 - 10
MT	MT-21	+	+	+	+	+	+	17	Susceptible	9.1 - 10
MT	MT-25		+	+	+	+	+	2	Susceptible	9.1 - 10
MT	MT-30	+	+	+	+	+	+	25	Susceptible	9.1 - 10
MT	MT-34		+	+	+	+	+	25	Sulfisoxazole	9.1 - 10
MT	MT-38	+	+	+	+	+	+	25	Susceptible	9.1 - 10
K	K-4	+		+	+	+	+	28	Tetracycline; Sulfisoxazole	9.1 - 10
K	K-5	+		+	+	+	+	29	Tetracycline; Sulfisoxazole	9.1 - 10
K	K-7	+		+	+	+	+	29	Tetracycline; Sulfisoxazole	9.1 - 10
K	K-11	+	+	+	+	+	+	29	Tetracycline; Sulfisoxazole	9.1 - 10
K	K-12	+		+	+	+	+	29	Tetracycline; Sulfisoxazole	9.1 - 10
K	K-28	+		+	+	+	+	29	Tetracycline; Sulfisoxazole	8.1 - 9.0
WE	WE-4	+		+	+	+	+	26	Sulfisoxazole	8.1 - 9.0
WE	WE-14	+	+	+	+	+	+	26	Sulfisoxazole	9.1 - 10
WE	WE-18		+	+	+	+	+	26	Sulfisoxazole	9.1 - 10
WE	WE-23	+	+	+	+	+	+	19	Sulfisoxazole	9.1 - 10
WE	WE-27		+	+	+	+	+	26	Sulfisoxazole	9.1 - 10
WE	WE-28		+	+	+	+	+	26	Sulfisoxazole	9.1 - 10
WE	WE-29	+		+	+	+	+	24	Sulfisoxazole	9.1 - 10
I	I-31	+	+	+	+	+	+	17	Susceptible	9.1 - 10

[a] *E. coli* O157 isolates that could not be subcultured after initial recovery.

3.4. Discussion

The combined prevalence rate of *E. coli* O157 (15.6%) for the longitudinal and point prevalence studies was slightly higher than that of a recent study conducted in Canada (22). However, it is generally difficult, and sometimes impossible, to extrapolate results obtained from different studies due to a large variety of screening procedures for *E. coli* O157 survey. As reported by Sanderson *et al*. (43), using large-volume (10-g) fecal samples increased the detection sensitivity by 5.4% over cotton-tipped swabbing of fecal samples. To increase the survey sensitivity, we tested two small fecal samples per sampling unit in parallel by using two different enrichment steps with and without IMS. Our combined approach (which involved using both cultural and cultural-IMS methods) increased the sensitivity of *E. coli* O157 detection in the longitudinal and point studies by 19.3% and 25.2%, respectively, over the use of culture-IMS alone. The results from this study confirmed recently findings of Pearce *et al*. (38) who noted a marked improvement in the sensitivity of detection via testing multiple small fecal samples per fecal sampling unit.

The frequency of occurrence of the *E. coli* O157 among surveyed feedlots showed high variability, ranging from 0 to 57.5%, without any geographic distribution pattern (**Figure 3.1.).** Two determinants were found to have an important impact on the prevalence of *E. coli* O157 in Saskatchewan feedlots. Statistically, the most significant correlation (**P = 0.003**) was observed between density of cattle in pens (**Figure 3.2.)** and the prevalence rate of *E. coli* O157. The second statistically significant correlation (**P = 0.006**) was found between the ratio between the housed cattle: feedlot capacity and the prevalence of the pathogen (**Table 3.1.)**. The effects of these two densities on the prevalence of *E. coli* O157 among feedlot cattle may be illustrated by an example. The feedlots "S" and "M", which operated at full capacity with 13.6 and 17.3 m^2 / head,

resulted in the highest prevalence rates (57.5 and 55%), respectively. In contrast, feedlots "H" and "G", which operated at 14 and 6% of capacity (33.2 and 27.8 m^2 / head), respectively, resulted in no detectable *E. coli* O157 samples. Performing stepwise regression statistical analyses, we found that the percentage of variance (R^2) in the prevalence explained by the effect of cattle density in pens alone was 62.6%, whereas the percentage of variance in the prevalence explained by both densities was 78.7%. These results suggest that both densities had an additive effect on spreading of pathogen among feedlot cattle. The high pen density increases contact between infected and non-infected animals as well as poses a stressful condition for cattle, resulting in a higher shedding rate of the pathogen. Another density-related factor (the housed cattle : feedlot capacity) most likely accelerates dissemination of *E. coli* O157 between pens via some other mechanism (air dust, runoff, etc.). To avoid diet effect on the analyses of the prevalence distribution rates, all results obtained from pasture fed cattle were not statistically processed.

Results from multiplex PCR analyses indicate that the majority of *E. coli* O157 isolates obtained from this study likely represent a significant public health risk. Several authors have previously pointed out that EHEC isolates able to produce functional stx_2 toxin and intimin are strongly associated with human morbidity (6, 14, 36). Out of 194 *E. coli* O157 isolates recovered from Saskatchewan cattle, 94% (n = 183) harbored both *eaeA* and stx_2 genes, two of the most potent EHEC virulence determinants. Two recent studies carried out in Australia (13) and at three large Midwestern beef processing plants in the USA (3) reported a prevalence of 56% and 64%, respectively, among the *E. coli* O157 isolates for both stx_1 and stx_2, whereas in the present study, 83% of the isolates

carried the same combination of toxins. These results suggest that the virulence potential of isolates from Saskatchewan cattle herds is relatively high.

A Vero cell assay confirmed this assumption; all *E. coli* O157 isolates expressed a strong cytopathic effect against Vero cells. We further observed a high cytotoxicity, ranging from 7.1-8.0, for one stx_1/stx_2 negative isolate (data not shown). This paradoxical situation has been observed elsewhere (19, 27) and suggests that pathogenic mechanisms of *E. coli* O157 are not completely understood.

Based on genetic diversity that arose from the *E. coli* O157 isolates, we classified Saskatchewan's feedlots into 3 categories. The first category represented feedlots having an indigenous population of *E. coli* O157 where no genetically divergent isolates were observed to be present. The RAPD-PCR patterns of *E. coli* O157 isolates recovered from the K feedlot displayed only two unique genetic subtypes (subtypes 28 and 29; **Table 3.2.**) indicating their endemic character. The majority of feedlots (Z, HM, P, S, L and V) were classified into a second category, where genetically more or less unrelated isolates were introduced and combined with a group of endemic isolates. It is possible that genetically more related isolates evolved from an existing endemic population. The third observed category of feedlots were those with high numbers of genetically unrelated *E. coli* O157 subtypes, implying that genetically diverse isolates originated from different ancestors (21) and were introduced into the feedlot, most probably by incoming cattle. Feedlot M, which included 9 different genetic subtypes (7, 14, 5, 10, 11, 9, 19, 39 and 15) that were found in all three clusters, provides an example of this.

The use of antibiotics in food producing animals for growth promotion, chemotherapy and prophylaxis is linked to the emergence and dissemination of

antibiotic resistance genes (49). Although Shiga toxin producing *E. coli* infections do not involve the use of antimicrobial therapy (8, 50), *E. coli* O157 may easily acquire drug resistance genes in their natural habitat and transfer them to other organisms of clinical relevance. Herein we provide insight of antibiotic profiles of *E. coli* O157 isolates collected over a considerable geographical area that comprised a cattle population originating from across Canada and the USA. The most prevalent antibiotic determinant was sulfisoxazole resistance. Our RAPD-PCR results suggest that resistance to this drug was widely distributed among 22 different subtypes of *E. coli* O157 found in all three genetic clusters. The ubiquitous dissemination of sulfisoxazole resistance among genetically-diverse *E. coli* O157 isolates clearly suggests the convergent acquisition of drug resistance genes by these different isolates, rather than the clonal spread of a sulfisoxazole-resistant isolate across this geographic region. The high rate of resistance to sulfisoxazole may be attributed to the long history of sulfa drug use (since the mid-1930s) in the treatment of infectious diseases. Furthermore, the *sul* gene that encodes dihydropteroate (sulfonamide inhibitor) has been found within the conserved segments of several types of integrons (9, 25), implying the effective flux of *sul* in the microbial population. The prevalence rate of tetracycline resistance (12%) in *E. coli* O157 isolates from this study was similar to findings of another study carried out in the USA (44). Based on examination of 361 *E. coli* O157 isolates recovered from humans, cattle, swine and food, Schroeder *et al.* (44), observed 9 % resistance to tetracycline in STEC O157 organisms. Three isolates obtained during this study (S-6, S-38 and M-8) were found in two separate feedlots with multi-antibiotic resistance to chloramphenicol, tetracycline, streptomycin and sulfisoxazole. The same virulence profiles and multidrug resistance traits of these organisms could indicate epidemic spread of a multidrug

resistant organism through herds in this region. Based on genetic typing, isolates S-6 and S-38 comprised a distinct genetic subtype (subtype 13), confirming the above-mentioned indication. In contrast, isolate M-8 was genetically different from isolates S-6 and S-38. Interestingly, isolate M-8 had the same RAPD-PCR pattern (subtype 5) as most isolates from the feedlot S, indicating a possible ancestral source of multidrug resistance determinants for all three isolates. Most of the surveyed feedlots used the ionophore antibiotic, Rumensin™ (monensin; personal communication). Monensin inhibits Gram-positive microflora that tend to proliferate under concentrate (high starch) diets, and because of its general mode of action (at the level of the cell membrane), acquired mechanisms of resistance doubtful. The use of monensin was not likely to have contributed to any of the antibiotic resistance profiles observed in this study.

In conclusion, this study clearly demonstrates a high impact of cattle density, and ratio between housed cattle : feedlot capacity on the prevalence rate of *E. coli* O157, and thus may serve as a baseline for risk assessment modeling and reducing the prevalence rate of *E. coli* O157 among cattle in feedlots. Furthermore, in addition to providing current insight into the antibiotic susceptibility of *E. coli* O157 isolates originating from across Canada and the USA, this work also illustrates how combining antibiotic resistance and genetic profiles provides a more comprehensive basis for comparing the relatedness and dissemination of pathogenic (resistant) *E. coli* strains.

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BRIEF INTRODUCTION TO CHAPTER 4

Research in this chapter was performed to determine the sensitivity and reliability of two different techniques for detecting *E. coli* O157 from naturally infected samples. The two techniques, (i) buffered peptone water containing vancomycin, cefsulodin and cefixime followed by immunomagnetic separation (IMS-VCC), and (ii) modified *Escherichia coli* broth supplemented with novobiocin, both followed by culturing on cefixime tellurite sorbitol McConkey agar plates, were employed in parallel during a two-year study. Furthermore, when the results of this study were examined in combination with the genetic clustering of isolates data from Chapter 3, new information on the high fluctuation rate of the sensitivity of the IMS-VCC technique was revealed.

4. SENSITIVITY OF TWO TECHNIQUES TO DETECT *ESCHERICHIA COLI* O157 IN NATURALLY-INFECTED BOVINE FAECAL SAMPLES

Abstract

The aim of this study was to investigate the sensitivity and reliability of two techniques commonly used for the isolation of *Escherichia coli* O157: i) buffered peptone water (BPW) containing vancomycin, cefsulodin and cefixime followed by immunomagnetic separation (IMS-VCC) and ii) modified *Escherichia coli* (EC) broth supplemented with novobiocin (m ECn), both followed by culturing on cefixime tellurite sorbitol McConkey (ctSMAC) agar plates. Over a two-year period, 24 feedlots/farms located over a large geographical area (~600 x 450 km) were screened for the presence of *Escherichia coli* O157. A total of 194 *E. coli* O157 isolates were identified; 151 (77.4%) using IMS-VCC and 108 (55.4%) using m ECn. The recovery rates of IMS-VCC varied from 100 to 47%, whereas for m ECn ranged from 100 to 16%. All isolates were grouped, using randomly amplified polymorphic DNA – polymerase chain reaction (RAPD-PCR), into 3 major clusters that comprised 39 different subtypes, providing evidence of considerable genetic heterogeneity.

The results from this study revealed false negatives in IMS-VCC technique, most probably due to the high genetic diversity of environmental *E. coli* O157 isolates and antibiotic sensitivity. Using only IMS-VCC as a method for detection may result in significant underestimation of the pathogen. Performing two different enrichment steps in parallel can lead to markedly improved recovery rates of *E. coli* O157 isolates from naturally-infected samples.

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4.1. Introduction

Escherichia coli O157:H7 has emerged as a zoonotic pathogen of increasing public health concern. The low infectious dose (Willshaw *et al*., 1994) and a wide range of clinical manifestations, including life – threatening hemolytic uremic syndrome (HUS) (Griffin *et al*., 1991; Karmali *et al*., 1985) make human infections of particular concern. Ruminants, especially cattle, have been implicated as the primary reservoir of *E. coli* O157:H7 (Chapman *et al*., 1993; Wells *et al*., 1991) from where it can enter the environment and the food chain. Jones (1999) and Meng and Doyle (1998) underlined that *E. coli* O157:H7 outbreaks have been attributed to a diverse range of sources, including swimming pools, water supplies, raw vegetables and more commonly beef products. In view of the low infectious dose and the potential severity of infections caused by *E. coli* O157:H7, it is imperative that sensitive methods are employed in detection of the organism in its natural reservoir and the food chain in order to reduce human exposure to the pathogen as well as to establish food safety controls.

The application of immunomagnetic separation (IMS) has increased the rate of *E. coli* O157 isolation from human specimens (Karch *et al*., 1996), food (Ogden *et al*., 2001) and bovine feces (Heuvelink *et al*., 1998; Sanderson *et al*., 1995). Heuvelink *et al.* (1998) showed that use of modified *E. coli* broth containing novobiocin (20 mg/liter) (m ECn) followed by immunomagnetic separation (IMS) technique resulted in a sevenfold increase in the rate of isolation of *E. coli* O157 from adult cattle (naturally colonized by *E. coli* O157) compared with the recovery rate after plating onto cefixime tellurite

sorbitol McConkey (ctSMAC) following selective enrichment (m ECn). In contrast, Sanderson *et al*. (1995) found that use of tryptic soy broth supplemented with cefixime (50 ng/ml) and vancomycin (40 µg/ml) (TSBcv) in conjunction with IMS was only slightly more sensitive (79%) than broth enrichment (TSBcv) followed by plating (71%) for samples from calves orally inoculated with *E. coli* O157:H7.

This study was designed, in part, to examine the sensitivity of IMS-VCC (vancomycin, cefsulodin, and cefixime) and modified *Escherichia coli* broth supplemented with novobiocin (m ECn) followed by plate culture. The different methods were compared during a longitudinal and large-scale point prevalence study that involved the analysis of >1400 samples of water, soil, manure slurry and cattle feces collected over a two year period. In addition, to investigate the relationship between genetic traits of *E. coli* O157 isolates and recovery method, we characterized all isolates by using randomly amplified polymorphic DNA PCR (RAPD-PCR).

4.2. Material and methods

4.2.1 Study design and collection of samples

The longitudinal study was performed at four cattle facilities over a ten-month period. A total of 610 bovine fecal and environmental samples were obtained from November 2002 to the end of August 2003. The point prevalence study was conducted during the summer of 2004 (from late June to the end of September). This study extended the sampling base by an additional 880 bovine fecal samples collected from 20 different feedlots. Approximately 40 g of freshly defecated feces was aseptically collected using a sterile tongue depressor (Baxter Diagnostics Inc. USA) and transferred to a sterile 80 ml plastic container (Starplex, Canada). Samples from lagoons, dugouts and soil were sampled directly using a sterile 80 ml plastic container. All samples were

stored in a cooler with frozen cold-packs, transported to the laboratory, and tested within 2-6 h of collection.

4.2.2 Sensitivity of enrichment broth-cultural methods

To determine the most sensitive enrichment broth for the cultural method, we conducted a preliminary experiment where three common enrichment broths for *E. coli* O157 were tested. A set of vials which contained 31 g. of non-sterile sieved (3 mm) silty clay loam soil (60% water capacity) were spiked with serial 10-fold decreasing inocula of *E. coli* O157:H7 ATCC 43894 cells ranging in concentration from 1.7 x 10^7 to 1.7 x 10^1 cfu/ml. The spiked soils were mixed thoroughly and then incubated overnight at room temperature. Enumeration of *E. coli* O157:H7 cells in the soil microcosms was performed by adding 50 mL of sterile water to 31 g. of spiked soil followed by ~ 2 min. of vigorous shaking until a homogeneous soil/liquid suspension was obtained. After soil homogenization,1 ml of well-mixed suspension of water and spiked soil was added to: i) 9 ml of plain Trypticase Soy Broth (Difco), ii) 9 ml of Trypticase Soy Broth supplemented with cefixime (50 ng ml^{-1}) and vancomycin (40 µg ml^{-1}; Sigma Chemical Co., St. Louis, Mo.) (Sanderson *et al*., 1995), and iii) 9 ml of mEC broth (Difco Laboratories) with novobiocin 20 µg ml^{-1} (Sigma Chemical Co.) (McDonough *et al*., 2000). After overnight incubation at 37°C, 0.1 ml of the 10^3, 10^4 and 10^5 dilutions of the above enrichment cultures were spread plated in triplicate onto 150 mm sorbitol MacConkey plates (Becton Dickinson and Company) containing cefixime (0.5 mg L^{-1}) and potassium tellurite (2.5 mg L^{-1}) (ctSMAC). After 24 h of incubation at 37˚C, discrete gray non-sorbitol fermenting colonies with a black point in the center were selected as suspect *E. coli* O157.

4.2.3 Immunomagnetic separation (IMS) – cultural technique

Samples (1 g or 1 ml) were aseptically added to 10 ml of Buffered Peptone Water (BPW) (Becton Dickinson and Company, USA) supplemented with cefixime (0.5 mg L^{-1}; Rhone – Poulenc Rorer, Canada), vancomycin (8 mg L^{-1}; Sigma – Aldrich Chemie, Germany) and cefsulodin (10 mg L^{-1}; Sigma – Aldrich Chemie) (VCC) (Chapman *et al*., 1994). After samples were homogenized, the suspensions were incubated at 37˚C for 6 – 8 h without agitation. IMS was then performed using a BeadRetriever (Dynal Biotech ASA, Oslo, Norway) by adding 1 ml of the BPW-VCC enrichment culture to 20 µl of anti-O157 immunomagnetic beads (Dynal Biotech ASA) followed by incubation, mixing and 2x washing of the immobilized immunomagnetic beads in 1 ml of PBS containing 0.05% Tween 20 (Sigma Chemical Co. St. Louis, USA). After the last wash step, the immunomagnetic beads were released into a final tube containing 100 µl of wash buffer (PBS/0.05% Tween 20). From the concentrated bead – bacteria complex, 100 µl was then gently resuspended and plated onto ctSMAC agar plates, as outlined in the previous section. After 24 h of incubation at 37˚C, discrete gray non-sorbitol fermenting colonies were selected as suspect *E. coli* O157.

4.2.4 m ECn cultural technique

From each sample, 1 g or 1 ml, was aseptically suspended in 10 ml of m ECn broth, vortexed, and then incubated at 37˚C for 18 – 24 h without agitation. After incubation, a 1 ml aliquot of enrichment culture was serially diluted to 10^{-5} in sterile 0.85% saline. From the 10^{-3}, 10^{-4} and 10^{-5} dilutions, 100 µl was plated in duplicate onto ctSMAC agar and incubated at 37˚C overnight. Non-sorbitol fermenting colonies with characteristic morphology and appearance of *E. coli* O157 were serologically tested for somatic O157 antigen, as described in the following section.

4.2.5 *E. coli* O157 confirmation

Up to 10 suspect *E. coli* O157 colonies from each ctSMAC plate were tested by using O157 latex reagent (Oxoid LTD). All sero-positive isolates obtained from the longitudinal and point prevalence studies were then tested by multiplex PCR for the presence of four virulence determinants (*stx*$_1$, *stx*$_2$, *eaeA*, and *hlyA*) using assays described by Fagan *et al*. (1999) and Paton and Paton (1998).

4.2.6 Genomic fingerprint analyses

The discriminatory ability and stability of randomly amplified polymorphic DNA (RAPD) patterns were tested in a preliminary study against a panel of 15 different *E. coli* O157 isolates with four decamer primers: GEN 15001 (5'-GTGCAATGAG-3') (Radu *et al*., 2001), 1283 (5'-GCGATCCCCA-3') (Galland *et al*., 2001), 1290 (5'-GTGGATGCGA-3') (Pacheco *et al*., 1997) and 1254 (5'-CCGCAGCCAA-3') (Akopyanz *et al*., 1992; Aslam *et al*., 2003; Makino *et al*., 1994). Primer 1283 showed the greatest stability and discriminatory ability among the *E. coli* O157 test isolates, and was therefore used in this study. The RAPD analysis of *E. coli* O157 isolates was performed using the method previously described by Vidovic and Korber (2006).

4.2.7 Statistical analysis

The analysis of DNA patterns obtained using RAPD PCR was performed by using the Numerical Taxonomy and Multivariate Analysis System (NTSYSpc, Exeter Software, Setauket, N.Y.). Similarities between RAPD patterns were determined based on the Dice similarity coefficient. The resulting similarities in the matrix were further processed by employing the unweighted par – group method with arithmetic averages

(UPGMA) to create a dendrogram that graphed the genetic relatedness between *E. coli* O157 isolates.

4.3. Results

The efficiency of the three enrichment broths to recover *E. coli* O157:H7 from spiked soil samples are shown in **Table 4.1**. The m ECn was determined to be the most sensitive of the broth-based enrichment methods, and thus was selected to compare with the IMS-VCC method.

Table 4.1. Recovery of spiked *E. coli* O157 from soil by using three different enrichment procedures.

	Detection of *E. coli* O157 with the following enrichment broths:		
Spiked soil cfu g^{-1}	TSB[a]	TSB + cefixime and vancomycin	m EC + novobiocin
540000	+	+	+
54000	+	+	+
5400	+	+	+
540	+	+	+
54	+	+	+
5		+	+
0.5			+
0.05			

[a] TSB = trypticase soy broth

During the longitudinal study, 57 *E. coli* O157 isolates were obtained from the 445 bovine fecal samples using both the m ECn and IMS-VCC enrichment and isolation methods, resulting in an overall prevalence rate of 12.8%. Of these 57 isolates, 47 (82.4%) were detectable by IMS in conjunction with culturing, whereas 20 (35%) isolates were recoverable by the m ECn followed by culturing on ctSMAC agar. During the longitudinal study, 165 environmental samples were also screened for *E. coli* O157; none were found to be positive for *E. coli* O157 by either method.

During the point prevalence study, a total 138 *E. coli* O157 isolates were recovered from 880 bovine fecal samples using both methods, resulting in an overall prevalence rate of 15.7%. These 138 isolates were obtained from 15 different feedlots located over a large geographical area (~600 x 450 km). The IMS-VCC enrichment accounted for the recovery of 104 (75.3%) of these 138 *E. coli* O157 isolates, whereas the m ECn enrichment method followed by ctSMAC plating accounted for 88 (63.7%) of the total *E. coli* O157 isolates obtained. The recovery rates (the number of isolates obtained using a particular enrichment/isolation method/total number of isolates obtained using both enrichment/isolation methods) of *E. coli* O157 using IMS-VCC enrichment fluctuated from 100 to 45 % among these fifteen feedlots, whereas for enrichment using m EC broth with novobiocin the recovery rates ranged from 100 to 16%. An overall recovery of *E. coli* O157 isolates for IMS-VCC enrichment followed by ctSMAC was 77.4% of the total number of *E. coli* O157-positive fecal samples identified during this study, compared to m ECn which accounted for the recovery of 55.4% of samples (**Figure 4.1.)**.

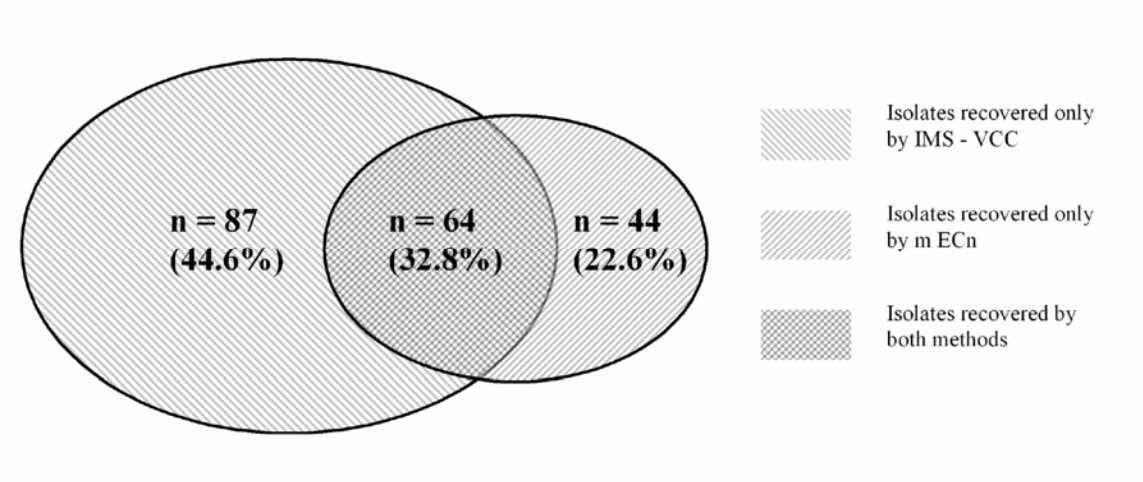

Figure 4.1. Overall comparison of the sensitivity of IMS-VCC and m ECn enrichment procedures followed by culturing on SMACct agar plates to isolate *E. coli* O157 from naturally infected bovine feces.

All 194 isolates of *E. coli* O157 obtained from the longitudinal and point studies were grouped into 3 major clusters **(Figure 4.2.)** consisting of 39 different subtypes or RAPD PCR patterns **(Figure 4.3.)**. Amplification reactions using decamer primer 1283 produced a set of 18 DNA fragments ranging from 450 to 4, 000 bp in length. Genetic similarity between the *E. coli* O157 isolates ranged from 0.81 to 1.00. Based on genetic relatedness and dissemination of pathogen, RAPD typing revealed the existence of two groups of *E. coli* O157 isolates. The first group of isolates was characterized by their unique genetic subtypes (for example, subtype 28 and 29), indicating their endemic nature. In this case, these two highly related sub-types made up a single type (95% similarity) on the dendrogram shown in figure 2, which were only detected on one feedlot. The second group of isolates (representing the more common situation) was widespread among surveyed feedlots, implying transient introduction. For example, the two most predominant subtypes, 5 and 19, were detected at six feedlots each in central and north-central Saskatchewan.

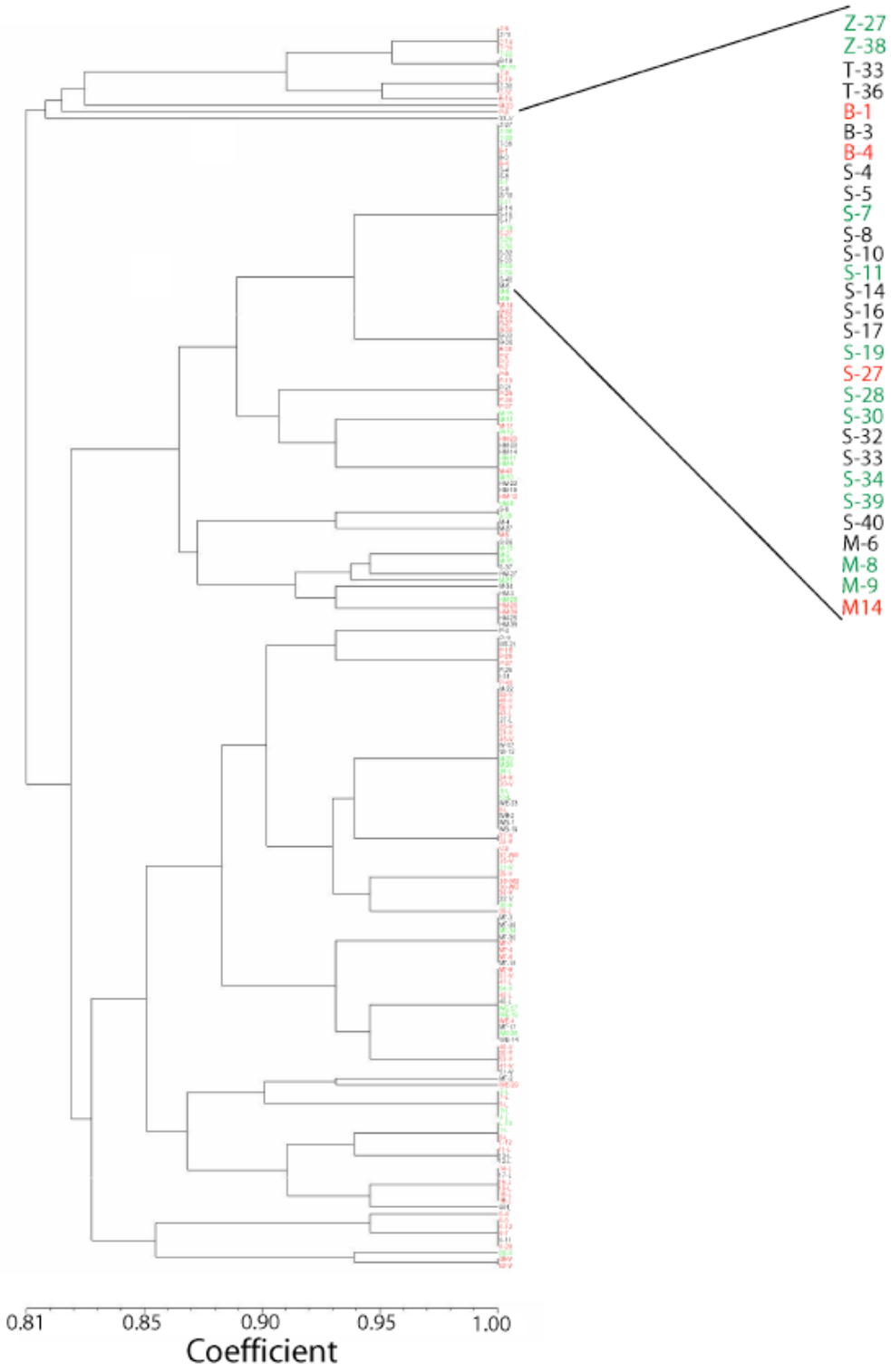

Figure 4.2 Relatedness of 194 isolates of *E. coli* O157 from the longitudinal and point studies. The dendogram was created by using the Dice coefficient and UPGMA analysis. The numbers on the scale at the bottom of the dendogram indicate the degree of similarity between *E. coli* O157 isolates. Colour coded dendogram showing: (●) isolates that were captured by both methods, (●) isolates captured only by IMS and (●) isolates captured only by cultural method. Blowout section shows details of genetic subtype 5.

4.4. Discussion

In the current study, large numbers of samples (bovine feces, soil etc.) collected from 24 feedlots/farms and their surrounding areas were tested in parallel using IMS-VCC and m ECn enrichment procedures followed by culturing on ctSMAC agar plates. BPW-VCC (conventional IMS broth) followed by IMS is a widely-used enrichment procedure and recommended by the U. S. Food and Drug Administration for detection of *E. coli* O157; it is generally considered to be the "gold standard" approach. Alternatively, m ECn enrichment is a less-expensive, alternative technique that has seen use in several studies (Okrend *et al*. 1990; Faith *et al*. 1996; Heuvelink *et al*. 1998).

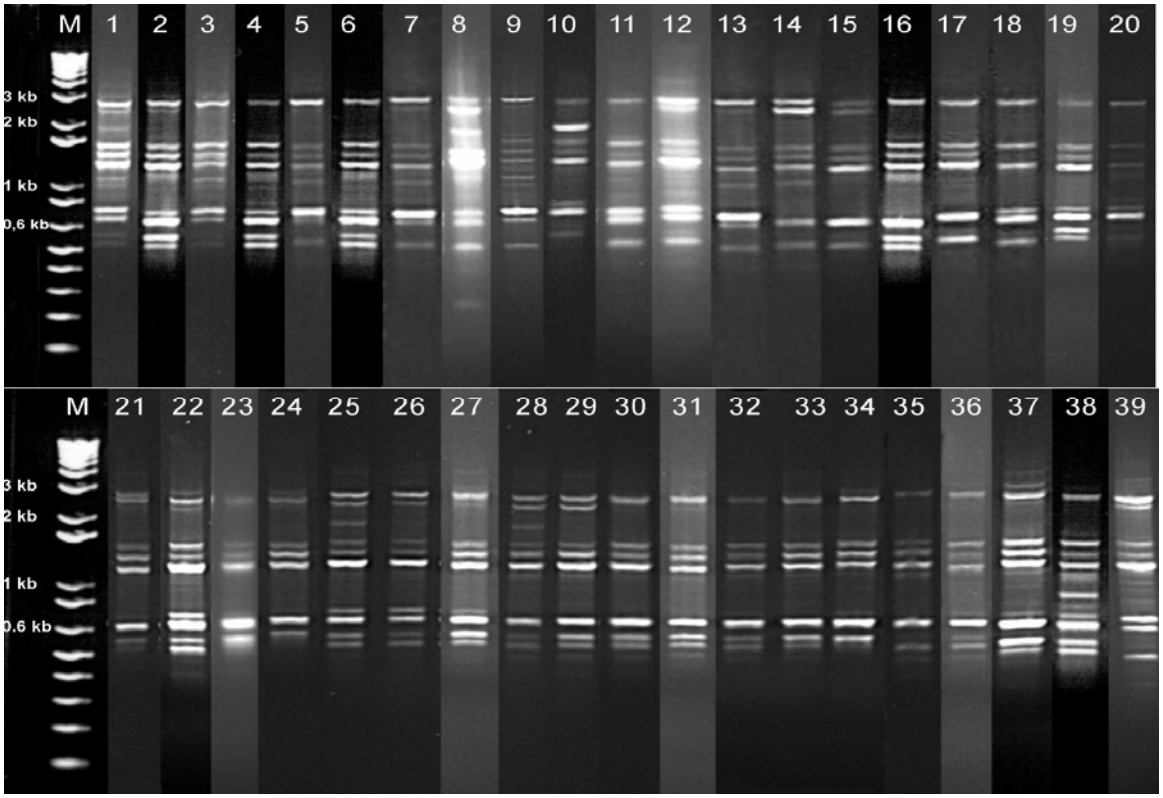

Figure 4.3 RAPD profiles of *E. coli* O157 isolates obtained from the central – southern region of Saskatchewan over two year period. Different RAPD patterns shown in the figure represent 39 genetic subtypes (the numbers above lanes indicating number of genetic subtype). Lane M contains the 1 kb Plus DNA ladder molecular weight marker.

Using a large sampling base of naturally-infected cattle, the current study confirmed that IMS-VCC enrichment followed by ctSMAC plating was a more sensitive recovery technique. However, the ability of IMS-VCC to recover *E. coli* O157 isolates from bovine feces was highly variable among certain feedlots included in our survey. For example, the two feedlot operations designated "S" and "M" (**Table 4.2.)** housed a high proportion of infected cattle. IMS-VCC enrichment and isolation was able to detect 65 and 45% of the total number of *E. coli* O157 isolates determined to be present at these feedlots, respectively; whereas, m ECn enrichment recovered 96 and 77% of the total isolates, respectively. This inconsistent performance of IMS-VCC was unlikely to be due to differences in pathogen distribution within the fecal samples, as the inocula for both techniques were taken from the same portion of fecal sample. Furthermore, this paradoxical result occurred despite the fact the m ECn method required a dilution step prior to plating where either 10 or 1 µl of enrichment culture was tested in order to facilitate the recovery of isolates, compared to the use of 1000 µl of IMS-VCC enrichment broth prior to recovery with beads. Genomic fingerprinting analysis of *E. coli* O157 isolates recovered from feedlots "S" and "M" revealed the heterogeneous nature of these isolates, consisting of 10 different subtypes **(Table 4.2.),** with the most predominant *E. coli* O157 isolate being subtype 5 **(Figure 4.3.)** which accounted for 50 % (22/44) of the total population. Furthermore, it was observed that the subtype 5 isolates were more efficiently recovered (90.9 %) using m ECn compared to IMS-VCC (59.0 %). Failure of IMS-VCC to actually recover the majority of *E. coli* O157 isolates at particular feedlots was most likely associated with the inhibitory effects of vancomycin, cefixime and cefsulodin (VCC) on the most prevalent *E. coli* O157 isolate subtype 5 found at these two feedlots. These findings suggest a "chink in the gold-

Table 4.2 Summary of characterization of *E. coli* O157 isolates obtained from the two feedlot operations "S " and "M".

Feedlot	Isolate	Isolation method		Virulence profile				RAPD pattern
		IMS	Cultural	*stx*1	*stx*2	*hly*A	*eae*A	
S	S-1[a]	+	+					
S	S-4	+	+	+		+	+	5
S	S-5	+	+	+	+	+	+	5
S	S-6	+	+		+	+	+	13
S	S-7		+	+		+	+	5
S	S-8	+	+	+	+	+	+	5
S	S-10	+	+	+	+	+	+	5
S	S-11		+	+	+	+	+	5
S	S-14	+	+	+	+	+	+	5
S	S-16	+	+	+	+	+	+	5
S	S-17	+	+	+	+	+	+	5
S	S-19		+	+	+	+	+	5
S	S-26	+	+	+	+	+	+	7
S	S-27	+		+	+	+	+	5
S	S-28		+	+		+	+	5
S	S-30		+	+	+	+	+	5
S	S-32	+	+	+	+	+	+	5
S	S-33	+	+	+	+	+	+	5
S	S-34		+	+	+	+	+	5
S	S-37	+	+	+	+	+	+	7
S	S-38		+		+	+	+	13
S	S-39		+	+		+	+	5
S	S-40	+	+	+		+	+	5
M	M-2		+	+	+	+	+	7
M	M-4	+	+		+	+	+	14
M	M-5	+			+	+	+	14
M	M-6	+	+	+		+	+	5
M	M-8		+		+	+	+	5
M	M-9		+		+	+	+	5
M	M-13		+		+	+	+	7
M	M-14	+			+	+	+	5
M	M-15		+		+	+	+	10
M	M-16		+		+	+	+	7
M	M-17	+		+	+	+	+	10
M	M-19		+	+	+	+	+	11
M	M-21		+	+	+	+	+	9
M	M-22	+	+	+	+	+	+	19
M	M-23	+		+	+	+	+	39
M	M-26		+	+	+	+	+	19
M	M-27	+	+		+	+	+	14
M	M-29		+		+	+	+	19
M	M-31		+	+	+	+	+	10
M	M-33		+		+	+	+	11
M	M-34	+	+		+	+	+	15
M	M-40	+		+	+	+	+	11

[a] Isolate of *E. coli* O157 that could not be subcultured after first recovery.

standard armour" of IMS-VCC, and revealed a complicating factor for the identification of *E. coli* O157 from naturally-contaminated samples due to the high genetic diversity of this organism and possible different sensitivities to particular antibiotics used during enrichment. Our combined approach which involved using both mECn – ctSMAC and IMS-VCC methods increased the sensitivities of *E. coli* O157 detection in the longitudinal and point studies by 19.3 and 25.2 %, respectively, over the use of the IMS-VCC alone. Ogden *et al*. (2001) found that the presence of cefixime and cefsulodin in the enrichment media may potentially be inhibitory to some strains of *E. coli* O157, supporting our assumption that the IMS-VCC "false negative" results seen during our study may have occurred because of strong antibiotic inhibition. In another study, Foster *et al.* (2003) using 721 cattle faecal samples from 43 farms, compared the sensitivity of BPW-VCC and BPW without antibiotics (BPW-WOA) prior to IMS. The authors clearly demonstrated that BPW-WOA was the superior pre-enrichment medium for the isolation of *E. coli* O157 from bovine faeces. In addition, they reported poor performance of the BPW-VCC in recovering the most common *E. coli* O157 isolate (phage type 21/28) in cattle faeces. This finding is of particular concern as phage type 21/28 has been most frequently associated with human *E. coli* O157 cases in the UK. Hepburn *et al*. (2002) used soils spiked with a cocktail of four atoxigenic *E. coli* O157 strains to compare the recovery efficacy of using BPW and BPW supplemented with vancomycin (with or without one quarter strength cefixime and cefsulodin). The authors determined that BPW supplemented with vancomycin (with or without one quarter strength cefixime and cefsulodin) was superior in terms of recovery compared to BPW with the full strength vancomycin, cefixime and cefsulodin.

In conclusion, this study provides evidence that the high genetic diversity of naturally-occurring *E. coli* O157 isolates have differing responses to the enrichment conditions in IMS-VCC and m ECn. The application of two different enrichment steps in parallel may lead to significant improvements in *E. coli* O157 detection sensitivity.

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BRIEF INTRODUCTION TO CHAPTER 5

In order to facilitate the interpretation of the prevalence data reported in chapter 3, an examination of the factors that affect microbial survival was needed. Accordingly, experiments were designed to qualitatively and quantitatively determine the biotic and abiotic factors that influenced the survival of *E. coli* O157 in soil environments. To achive this goal, *E. coli* O157:H7 bacteria were incubated over time in two types of soil microcosms: (i) sterile soil under controlled conditions of moisture, sterility, and temperature, and (ii) cattle manure amended soil under controlled conditions of moisture and temperature. In addition, the survival of this organism was examined *in situ* during the growing season. The *in situ* experiments included the survival of *E. coli* O157:H7 in manure amended soil and plain soil, both with and without drainage of naturally precipitated water.

Since there has been virtually no data on the prevalence and survival of *E. coli* O157 in Saskatchewan, the analysis obtained from these studies have fundamental research and applied value.

5. EFFECT OF SOIL COMPOSITION, TEMPERATURE, INDIGENOUS MICROFLORA AND ENVIRONMENTAL CONDITIONS ON THE SURVIVAL OF *ESCHERICHIA COLI* O157:H7

Abstract

The survival of *Escherichia coli* O157:H7 in replicate soil microcosms was quantified in two types of silty clay loam soil (high-carbon and low-carbon) under either sterile or non-sterile conditions. Microcosms were held at -21°C, 4°C, and 22°C under constant soil moisture content. Differences existed ($P<0.05$) in survival of *E. coli* O157:H7 in low- and high-carbon soil at all temperatures indicating an important role of soil composition on the survival of this pathogen. The highest death rate of *E. coli* O157:H7 in sterile soil occurred in the low-carbon soil at 4°C, whereas in non-sterile soil the highest death rate was observed in the low-carbon soil at 22°C. These results suggest that the most lethal effects on *E. coli* O157:H7 in the sterile system occurred via the synergy of nutrient limitation and cold stress; whereas, in the non-sterile system lethality was due to inhibition by indigenous soil microorganisms and starvation. Results obtained from an *in situ* field survival experiment demonstrated the apparent sensitivity of *E. coli* O157:H7 cells to dehydration, information that may be used to reduce environmental spread of this pathogen as well as formulate appropriate waste management strategies.

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5.1 Introduction

Human infection with *E. coli* O157:H7 has been associated with various transmission routes including direct exposure to infected animals (Renwick *et al*. 1993), person-to-person passage (Belongia *et al*. 1993) and most typically by the consumption of contaminated undercooked ground beef (Armstrong *et al*. 1996; Doyle *et al*. 1997). Serious outbreaks or sporadic *E. coli* O157 infections could potentially originate with ready-to-eat products contaminated by raw vegetables or fruits previously exposed (directly or indirectly) to animal manure (Fukushima *et al*. 1997; Islam *et al*. 2004; Mohle-Boetani *et al*. 2001). For example, in 1996 a large outbreak of *E. coli* O157 took place in Japan among elementary school children where over 6,000 and 100 cases of hemorrhagic colitis and hemolytic-uremic syndrome were reported, respectively (Fukushima *et al*. 1997). The source of infection was traced to radish sprouts that most likely had contact with contaminated manure. The major transmission routes of *E. coli* O157 from agriculture lands into surface or ground waters occurs via the application of manure as a soil amendment, defecation of grazing cattle and sheep, and the improper storage of manure on soil (Fenlon et al. 2000; Guselle and Olson 2004).

Although *E. coli* O157:H7 associated outbreaks of gastrointestinal illness continue to attract worldwide attention (most recently linked with fresh spinach produced in California), the survival of the pathogen in soil and the influence of environmental conditions on *E. coli* O157:H7 remains poorly understood. The study conducted by Kudva *et al*. (1998) revealed that *E. coli* O157:H7 is able to survive for

more than 1 year in a non-aerated ovine manure pile exposed to environmental conditions. Jiang and colleagues (2002) investigated the survival of *E. coli* O157:H7 in manure-amended autoclaved and unautoclaved soil held at 5°, 15° and 21°C. The authors found that the pathogen population declined more rapidly in manure-amended unautoclaved soil under the same conditions due to antagonistic interactions with indigenous soil microorganism and were therefore unable to observe an influence of abiotic factors on the survival of *E. coli* O157:H7.

The objective of this study was to isolate the effect of abiotic factors (soil composition and temperature), as well as a combination of abiotic and biotic (indigenous soil and manure microorganisms) factors and adverse environmental conditions, on the survival of *E. coli* O157:H7 in soil. Accordingly, we used sieved-packed soil microcosms with high- and low-carbon sterile silty clay loam soils as well as the same unautoclaved soils amended with fresh bovine manure to assess how soil composition, temperature and indigenous soil microorganisms affected survival of *E. coli* O157:H7. Lastly, the influence of environmental conditions on the survival of the pathogen was investigated *in situ,* using clay loam soil with and without manure and drainage.

5.2 Materials and methods

5.2.1. Inoculum preparation

Escherichia coli O157:H7 ATCC 43894 was used as the test organism in these studies. Inocula were prepared from stock cultures kept in 10 % (vol/vol) glycerol at −70°C by streaking onto Tryptic Soy agar (EMD Chemicals Inc., Germany), followed by incubation at 37°C for 24 hours. Three well-isolated colonies were inoculated into 50 mL of tryptic soy broth and incubated overnight at 37°C with constant agitation (150 rpm). After incubation, 50 mL of the culture was centrifuged (4000 x g for 10 min.) and

washed three times with sterile reverse osmosis grade (RO) water. Cells were resuspended in RO and adjusted to a 0.5 McFarland standard (~1.5 x 10^8 cells mL^{-1}). Cell density of the inoculum was determined in triplicate by serial dilutions and spread plating on Tryptic Soy agar, followed by incubation at 37°C for 24 hours.

5.2.2 Manure

All bovine fecal materials used in these studies were obtained from a dairy farm located at the University of Saskatchewan. Fresh feces from healthy cattle with no *E. coli* O157 history were collected aseptically in sterile plastic bags. Feces were tested once again for the presence of *E. coli* O157 by the methods described elsewhere (Vidovic and Korber 2006). Fecal material was transported to the laboratory immediately and used within 2 hours of collection.

5.2.3 Autoclaved soil microcosms

The soil used in these studies was a silty clay loam (Laura, Saskatchewan) collected from the surface (5 cm depth) and subsurface (approximately 30 cm depth). Soils obtained from different depths were distinct in their chemical compositions; the surface soil had 21.87 organic carbon g. / 1000 g. (hereafter referred to as high-carbon soil); 1.98 total nitrogen g. / 1000 g., and 0.345 total sulfur g. / 1000 g., whereas the subsurface soil had 10.03 organic carbon g. / 1000 g. (hereafter referred to as low-carbon soil) 1.01 total nitrogen g. / 1000 g., and 0.00 total sulfur g. / 1000 g. Both soils were separately passed through a 3 mm pore diameter sieve and autoclaved at 121°C for 30 minutes on three successive days. Aliquots of 25 g. of each sterile soil type were aseptically transferred into sterile 5 x 7.5 cm (80 mL) polypropylene vials (Starplex, Canada). A 1 mL aliquot of *E. coli* O157:H7, diluted to result in ~1.5 x 10^6 cfu per g. of

soil, was then added to each vial. Inoculated soils were mixed thoroughly using a sterile wooden stick and then the appropriate amount of sterile (RO) water was added to each vial to adjust the soil water moisture to 60 % holding capacity (Cassel and Nielsen 1990). Soil microcosms were then capped, placed in the biohazard bags and stored in the dark at -21°C, 4°C and 22°C.

5.2.4 Unautoclaved manure-amended soil microcosms

High- and low-carbon silty clay loam soils (as described above) were used in the manure-amended unautoclaved soil microcosm study. Soils were sieved through a 3 mm pore diameter screen and 25 g. aliquots placed into sterile 80 mL plastic containers. Fresh unautoclaved bovine manure (0.57 g. or 2.3 % w/w) was incorporated into each 25 g. of soil, which was equivalent to a typical bovine manure application rate (30 tonnes / ha.) used in Canada (Schoenau and Assefa 2004). The *E. coli* O157:H7 inoculum was diluted to 8.8 x 10^5 cfu mL^{-1} and then 1 mL of this suspension was added to 25 g. soil. The final concentration of *E. coli* O157:H7 in each inoculated soil microcosm was 2.7 x 10^4 cfu g^{-1}, a concentration chosen to simulate the maximum possible number of *E. coli* O157:H7 in the soil through contact with naturally-contaminated bovine manure (Robinson et al. 2004). The inoculated soils were mixed thoroughly using sterile wooden sticks, and then sterile (RO) water was added to obtain 60% of the soil water holding capacity. Soil microcosms were capped and stored under the same conditions as described above (-21°C, 4°C and 22°C).

5.2.5 In situ E. coli O157:H7 survival assay

The soil used for the *in situ E. coli* O157:H7 survival assay was a clay loam excavated from a field located in Alvena, Saskatchewan, with a known history of

agriculture use. The texture of this soil was determined to contain 43.6% silt, 39.9% clay and 16.5% sand with an average value of 16.6 g. / 1000 g. soil of organic carbon. The soil was sieved as described above and then 11 kg aliquots were transferred to eight sterile polypropylene trays (54x43x13 cm). Four approaches were employed for studying the survival of *E. coli* O157:H7 under field conditions. In the first approach, 200 mL of the *E. coli* O157:H7 suspension ($7x10^7$ cfu mL^{-1}) was inoculated into duplicate trays containing 11 kg of soil, with one of the trays being perforated to allow water drainage. The inoculum was incrementally incorporated into the soils by repeated cycles of wetting followed by mixing. In the second approach, manure was added to soil incubated in two trays (with and without drainage), followed by mixing and incorporation of 200 mL of the *E. coli* O157:H7 suspension ($7x10^7$ cfu mL^{-1}). Manure application rates were the same as described above. As a control, the distribution of *E. coli* O157:H7 in soil containers following inoculation were determined by taking twelve random 10 g. samples from each tray (6 each from the soil surface and 6 from the bottom), and then serially diluting and plating onto 150 mm Sorbitol MacConkey plates (Becton Dickinson and Company) containing cefixime (0.5 mg L^{-1}) and potassium tellurite (2.5 mg L^{-1}) (ctSMAC). Plates were incubated at 37°C for 24 hours. Additional controls included manure-amended soil as well as *E. coli* O157:H7- free soil (the last two approaches), both incubated with and without drainage. All eight test systems were immediately transported to the field site where they were incubated under field conditions. These experiments were initiated in May 2004 and sampled over a four month period to assess survival of *E. coli* O157:H7 in soil during the growing season. During sampling, soil moisture was qualitatively assessed with respect to the presence of visible zones of moisture or drying, or whether standing water (or a visible water table)

was evident. Experiments were conducted in an enclosed area inside of a no-trespassing area of the farm; the University's biosafety office provided warning signs and labels that were displayed at the study site during the study.

5.2.6 Sampling and enumeration of *E. coli* O157:H7, coliforms and heterotrophs

Viable *E. coli* O157:H7 cell counts in the autoclaved and unautoclaved manure-amended soil microcosms were determined at day 0 (post-inoculation), days 4 and 8 and then on a biweekly basis. Enumeration of viable *E. coli* O157:H7 cells for the *in situ* assay was carried out at 0, 4, 7, and 13 days post-inoculation, and thereafter every ten days over a four month period. Enumeration of *E. coli* O157:H7 cells in the microcosms was performed by adding 50 mL of sterile water to 25 g. of soil followed by ~ 2 min. of shaking until soil was completely resuspended. A 1 mL aliquot of the soil suspension was then serially diluted (1:10) in saline and 0.1 mL of the appropriate dilutions were spread plated in triplicate onto ctSMAC agar plates, and incubated for 24 hours at 37˚C. After incubation, discrete gray non-sorbitol fermenting colonies with a black point in the center were randomly selected and tested for the presence of the somatic O157 antigen by the O157 latex reagent (Oxoid LTD). In addition, *E. coli* O157:H7 cell counts in sterile (autoclaved) soil were performed by the pour plating method using MacConkey agar (Becton Dickinson and Company) when cell numbers declined to ~ 1 x 10^1 cfu g^{-1}. All lactose-positive colonies were counted. Enumeration of total heterotrophic and total coliform bacteria were performed in triplicate by spread plating a 0.1 mL aliquot of an appropriate dilution onto R2A (Difco) and MacConkey agar plates followed by incubation at 20˚C for 5 days and at 37˚C for 24 hours, respectively. Cell counts for the *in situ* survival assay were performed by aseptically collecting six 10 g. soil samples from each of the eight treatments (three each from the surface and the bottom at the

same x-y coordinates). For each sampling period, a total of 48 samples were collected, placed in a cooler with frozen cold-packs, transported to the laboratory and processed within 2-3 hours of collection, as described above.

5.2.7 Statistical analyses

All data used in statistical analyses with sterile and unautoclaved amended soils were averages of triplicate measurements, expressed as $\log_{10}$ cfu per g. of soil for each treatment x time combination. This approach resulted in only a single value for each combination of factors but avoided pseudo-replication. Results were analyzed as a 2 (soil sterility) × 2 (soil carbon level) × 2 (linear or quadratic effect of incubation temperature) × 2 (linear or quadratic effect of incubation time) factorial treatment structure in a completely randomized experimental design using the mixed models procedure of SAS (SAS Institute, Inc., Cary, NC). Having only a single value for each combination of treatments would normally result in inadequate replication for evaluation of treatment effects and interactions. However, limiting the evaluation for effect of incubation time to linear or quadratic effects avoids this limitation with the added advantages of simplifying results interpretation and allowing comparisons to be made across soil sterility treatments (autoclaved versus unautoclaved soils) where sampling times were staggered. Additionally, for illustrative purposes, within a given sterility × carbon level × temperature combination, the linear or quadratic effect of incubation time on viable bacterial counts were determined using the regression procedure of SAS. To explain treatment interactions, simple effect comparisons were made amongst parameters from regression equations evaluating the linear effect of incubation time using unpaired T-tests. Measurements of *E. coli* O157:H7 *in situ* survival were

replicated six times (each in triplicate) for each soil treatment. An average of six readings with standard error bars was reported.

5.3 Results

5.3.1 Survival *E. coli* O157:H7 in sterile soils

In order to isolate abiotic effects on the survival of *E. coli* O157:H7 in soil systems, we controlled environment parameters (temperature, sterility, soil moisture and known soil composition) so that viability/survival of cells would only reflect soil composition and temperature. Initial plate counts of *E. coli* O157:H7 for high-and low-carbon soils were 1.5 x 10^6 cfu g^{-1} and 1.6 x 10^6 cfu g^{-1}, respectively. After 390 days of incubation at -21°C, only a slight decline in the number of viable *E. coli* O157:H7 cells was observed, resulting in a death rate of 0.0013 $\log_{10}$ cfu g^{-1} per day and 0.0009 $\log_{10}$ cfu g^{-1} per day, respectively (**Fig. 5.1.A**). The number of *E. coli* O157:H7 cells present in high- and low-carbon sterile soils held at 4°C decreased slightly for both soils over the first 138 days of incubation (**Fig. 5.1.B**). After this slow die off period, *E. coli* O157:H7 cells incubated in the sterile low-carbon soil entered a rapid death phase (biweekly mortality rate = 0.43 $\log_{10}$ g^{-1}) such that no viable cells remained following day 306. In contrast, the number of viable *E. coli* O157:H7 cells in the high-carbon soils decreased slowly (biweekly mortality rate = 0.08 $\log_{10}$ g^{-1}) until the end of the experiment (390

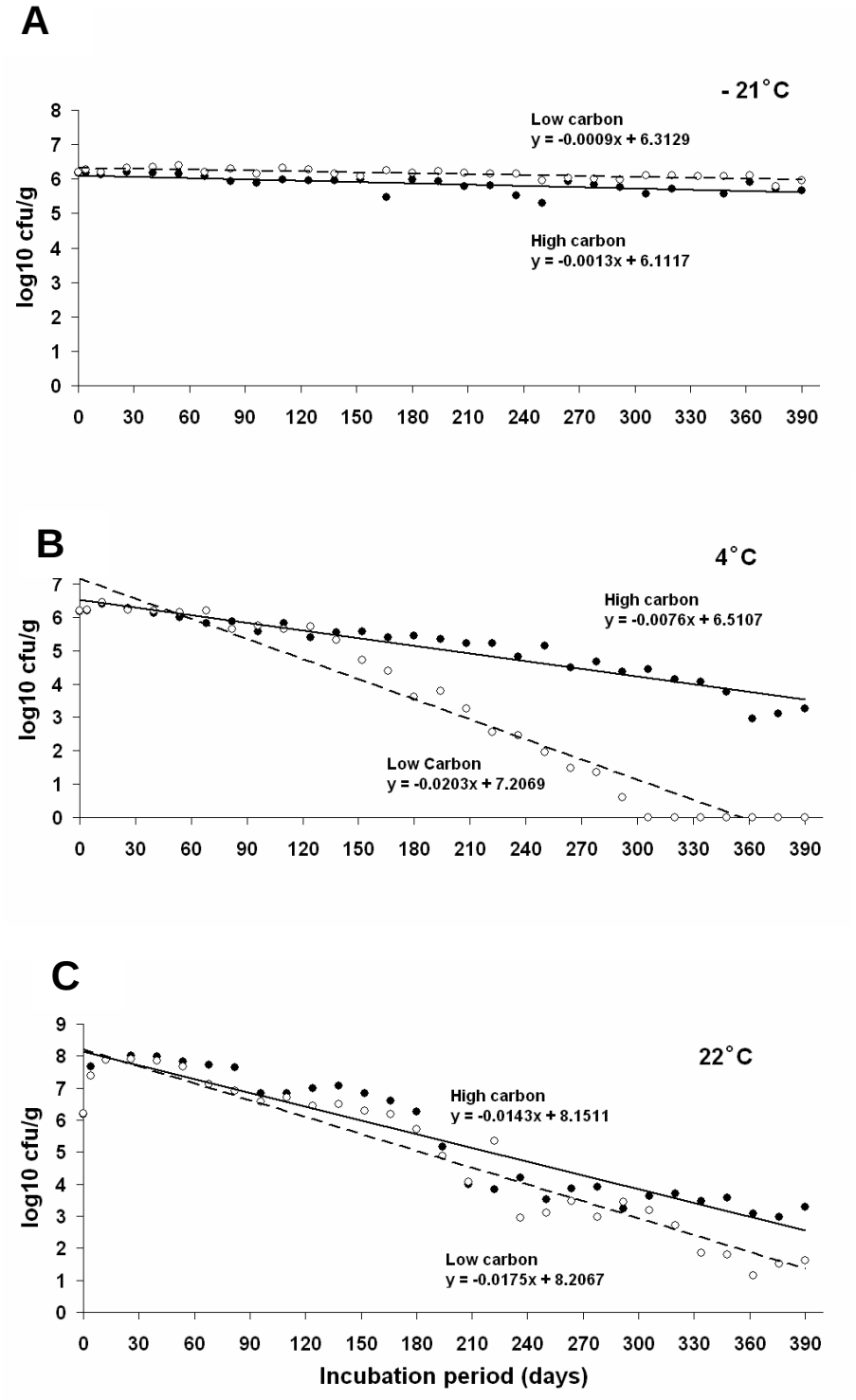

Figure 5.1 Survival of *E. coli* O157:H7 in autoclaved high-carbon soil (●) and low-carbon soil (○) microcosms. Viable counts of *E. coli* O157:H7 were determined in triplicate by using the non-enrichment culture spread and pour plating methods. Values are averaged and expressed as $\log_{10}$ cfu per gram**.**

days), at which time 1.8 x 10^3 cfu g^{-1} still remained. The mortality rate was higher ($P<0.01$) in the low-carbon soil with a decline rate of 0.0203 $\log_{10}$ cfu g^{-1} per day compared to a decline rate of 0.0076 $\log_{10}$ cfu g^{-1} per day in the high-carbon soil (**Fig. 5.1.B)**. The survival pattern of *E. coli* O157:H7 incubated at 22°C was significantly different from that at 4°C (**Fig. 5.1.C**). The number of *E. coli* O157:H7 cells in both soil types incubated at 22°C increased over the first 26 days by 2 logs, and then declined over the next 152 days to approximately initial cell (T = 0 days) concentration levels (~1.6 x 10^6 cfu g^{-1}) (**Fig. 5.1.C**). Following day 180, bacteria held in both soils at 22°C entered a sharp decline phase resulting in a decrease in viable cell number of 3 $\log_{10}$ g^{-1} over 70 days. After this period, the number of viable cells remained relatively constant in the high-carbon soil but continued to decline in the low-carbon soil.

5.3.2 Survival *E. coli* O157:H7 in unautoclaved manure-amended soils – influence of biotic effects

To determine additional effect of indigenous soil and manure microorganisms, we used unautoclaved high-and low-carbon soil amended with bovine manure incubated under the same conditions as described above. The number of viable *E. coli* O157:H7 cells incubated at -21°C in both the manure-amended high- and low-carbon soils declined over time **(Fig. 5.2.A, B**); whereas, in the autoclaved soils held at -21°C, *E. coli* O157:H7 numbers showed almost no decline. The lethal effect at -21°C was greater in the low-carbon soil (0.0158 $\log_{10}$ cfu g^{-1} per day), where *E. coli* O157:H7 became undetectable after 220 days; whereas, in the high-carbon soil *E. coli* O157:H7 numbers declined to 2 x 10^2 cfu g^{-1} (0.0084 $\log_{10}$ cfu g^{-1} per day). The survival curves of total

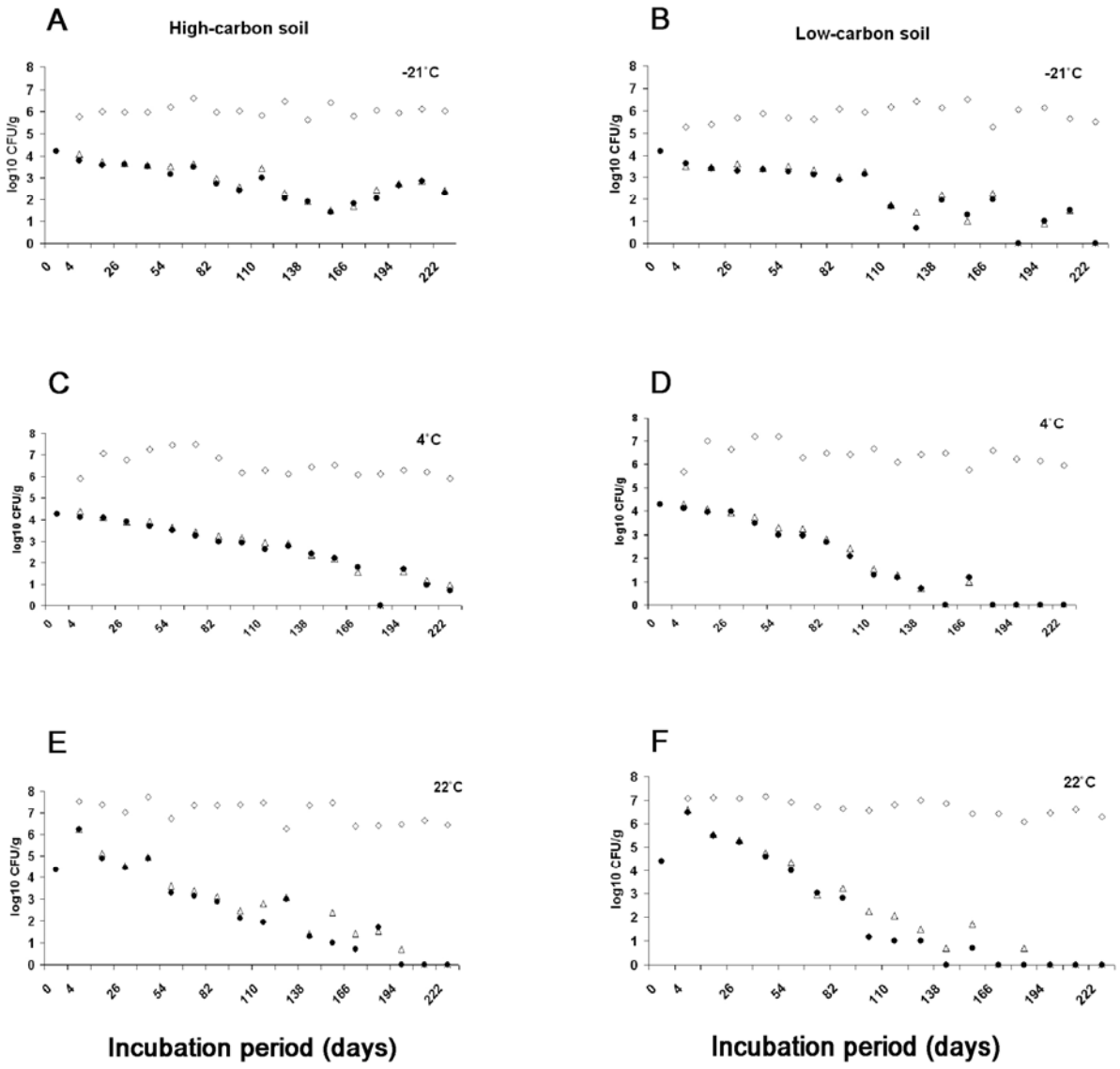

Figure 5.2 Persistence of *E. coli* O157:H7 (●), total coliforms (Δ), and total heterotrophs (◇) in unautoclaved high-carbon and low-carbon soils supplemented with 2.3 % (w/w) fresh bovine manure. Viable cell counts of *E. coli* O157:H7, total coliforms, and total heterotrophs were determined in triplicate by using the non-enrichment culture spread plating method. Plate count values are expressed as $\log_{10}$ cfu per gram.

coliforms introduced into the soils by bovine feces were almost identical to that of *E. coli* O157:H7 (**Fig. 5.2.A, B**). However, indigenous soil microorganisms remained constant at ~1 x 10^6 cfu g^{-1} over the duration of the experiment. In both unautoclaved, high- and low-carbon manure-amended soils held at 4°C, the number of coliform and *E.*

coli O157:H7 bacteria decreased by ~1 log g^{-1} after 70 days, while heterotrophic counts increased by 1.5 logs g^{-1} over the same period (**Fig. 5.2.C, D**). *Escherichia coli* O157:H7 cells incubated at 4° C in the low-carbon soil died off more rapidly compared with those incubated in the high-carbon soil. The sharp decrease in the number *E. coli* O157:H7 cells in the low-carbon soil resulted in no organisms being detected by day 154 (**Fig. 5.2.D**). In contrast, 2 x 10^2 cfu g^{-1} *E. coli* O157:H7 cells were found in the high-carbon soil by the completion of the experiment. Once again, the concentration of indigenous soil microorganisms in both types of soils held at 4˚C was determined to remain constant at ~ 5 x 10^6 cfu g^{-1} while *E. coli* O157:H7 and coliforms both declined at the same rate. The survival pattern of *E. coli* O157:H7 incubated at 22˚C in both types of manure-amended soils was similar to the pattern observed in the autoclaved soil held at the same temperature. A distinct characteristic of the survival curve for *E. coli* O157:H7 cells at 22˚C in both the autoclaved and unautoclaved manure-amended soils was an initial 2 log g^{-1} increase in cell number (**Fig. 5.1.C and 5.2.E, F**). However, the time during which *E. coli* O157:H7 cells proliferated in the unautoclaved manure-amended soil was much shorter (4 days) compared to the sterile soil (26 days). After this initial cell growth period, *E. coli* O157:H7 cells entered a die-off phase in both manure-amended soil types, with a higher mortality rate seen in the low-carbon soil **(Fig. 5.2.E, F)**. Lethality of *E. coli* O157:H7 in non-sterile soils at all incubation temperatures was higher ($P<0.01$) in the low-carbon soil than in the high-carbon soil**.** The ability of *E. coli* O157:H7 to survive in unautoclaved soil treated with cattle manure was reduced ($P<0.01$) at all temperatures compared to survival in autoclaved soil. For non-sterile soils, the effect of temperature on the rate of population decline was linear and showed

the greatest rate of decline ($P<0.01$) for viable *E. coli* O157:H7 cells in the low-carbon soil incubated at 22°C (0.037 $\log_{10}$ cfu g^{-1} per day) **(Fig. 5.2.F)**.

5.3.3 *In situ* survival of *E. coli* O157:H7

Escherichia coli O157:H7 was applied to test soil systems in relatively high numbers, ranging from 1.9 x 10^5 to 7.1 x 10^5 cfu g^{-1}, as determined by immediately enumerating cell numbers following inoculation (day 0). Climatic conditions during the experiment (the 2004 growing season) were compiled from the Environment Canada website (http://www.climate.weatheroffice.ec.gc.ca) for total precipitation (mm) and mean temperature (C°), respectively, as follows: May 17.2 / 12.6°C, June 58 / 13°C, July 20.6 / 16.6°C, August 68.4 / 13.9°C and September 33.6 / 10.9°C. Considerable rainfall over the first part of the study period resulted in visible standing water or a water table in all of the undrained microcosms (which remained for 34-43 days), versus the drained microcosms, where surface soil drying was evident after 24 days and subsurface drying by 34 days. Three distinct phases of *E. coli* O157:H7 survival were observed in all soil treatments (manure-amended and plain soil under drained and undrained conditions). The first phase was characterized by high persistence, but for differing lengths of time, in drained compared to undrained soils. *Escherichia coli* O157:H7 cell numbers remained constant (~ 10^4 – 10^5 cfu g^{-1}) in drained manure-amended and plain soil over the first 23 days; whereas, in the same type of undrained soil, *E. coli* O157:H7 cell numbers remained constant for 43 days (**Fig. 5.3.**). After the high persistence phase, *E. coli* O157:H7 entered a sharp decline phase where the number of viable cells decreased by 2.1 and 1.6 $\log_{10}$ cfu g^{-1} in drained manure-amended and plain soil, respectively, over a 10 day period (**Fig. 5.3.**).

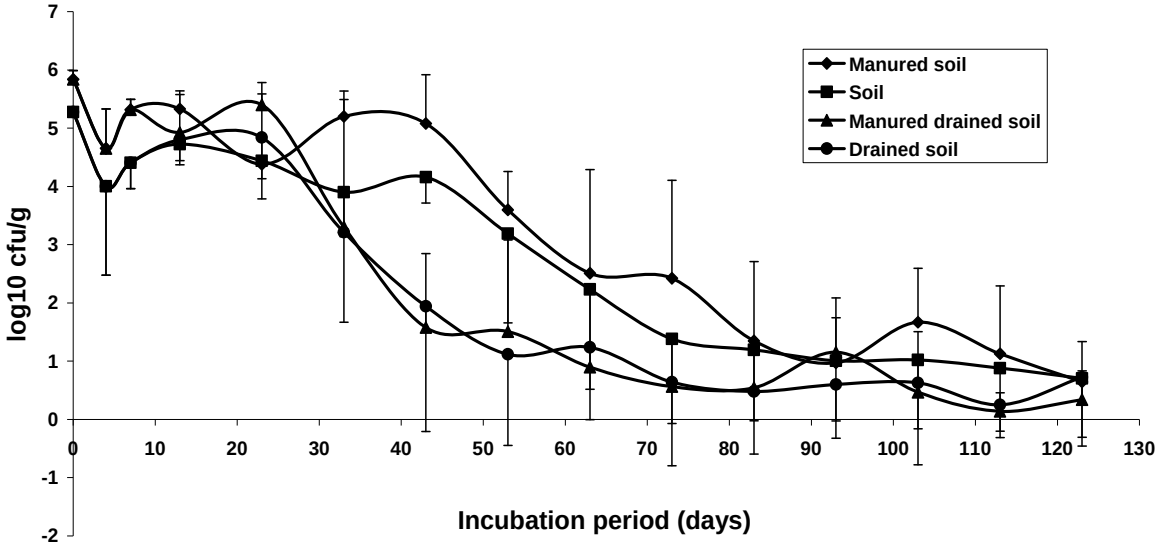

Figure 5.3 Survival of *E. coli* O157:H7 in manure-amended drained soil (▲), manure-amended undrained soil (♦), drained soil (●), and undrained soil (■) incubated under ambient field conditions during the growing season. Each point is the mean of six sample values (three from the surface and three from the bottom). The error bar represents the standard deviation between the six sample readings.

The high rate of *E. coli* O157:H7 cell death initially occurred in the upper surface layers (from 0 - 5 cm) of the soil (**Table 5.1.**). Despite the strong surface lethality, high numbers of viable cells persisted in subsurface soil layers, resulting in high overall standard deviation values for viable *E. co*li O157:H7 cell numbers (**Fig. 5.3. and Table 5.1.**). Death of the pathogen was observed in drained soils over the next twenty days, resulting in a further 2 $\log_{10}$ g^{-1} decline. A similar high death rate phase was observed in undrained manure-amended and plain soils, reducing cell numbers by 2.8 $\log s_{10}$ g^{-1} in both types of soil over the same period of time (**Fig 5.3.**). The third phase of pathogen survival was characterized by a slow die-off of cells. For example, viable cell counts in drained manure-treated and plain soil declined over 70 days by 1.1 and 0.4 $\log_{10}$ g^{-1}, respectively (**Fig. 5.3.**). Similar slow, lethal effects were observed in undrained soils, but were seen to be of a shorter duration (**Fig. 5.3.**).

5.4 Discussion

Findings from these experiments clearly demonstrated a great effect of soil nutrient composition and temperature on pathogen survival. While incubation at -21°C had a protective effect on *E. coli* O157:H7 cells in both types of autoclaved soils, incubation at 4°C, in combination with the low-carbon soil, resulted in the greatest lethal effect on the pathogen. An explanation for this significantly-higher mortality rate may include multiple stress factors existing in the low-carbon soil at low temperature. For instance, the low-carbon soil, which contained 1% organic carbon, 0.1 % total nitrogen and 0.000 % total sulfur was nutrient-limiting when compared with the high-carbon soil (2.1 % organic carbon, 0.198 % total nitrogen and 0.034 % total sulfur). In addition, 4°C is markedly different from the temperature of the natural gastrointestinal that *E. coli* O157:H7 inhabits (37°C), and thus poses a significant temperature stress for this organism (Jones et al., 1987). The combination of nutrient-limitation and cold stress most likely act synergistically, resulting in the highest mortality rate against *E. coli* O157:H7 cells. Nonetheless, results from this study revealed that *E. coli* O157:H7 may remain viable over 390 days in the autoclaved high-carbon soil incubated at either 4°C or 22°C.

Despite increasing the nutritional value of unautoclaved soil by addition of cattle manure (2.3 % by weight), the death rate of *E. coli* O157:H7 remained higher ($P<0.01$) than in autoclaved soil. Heterotrophic cell numbers remained high over time (~1 x 10^6 cfu g^{-1}) whereas coliforms and *E. coli* O157:H7 sharply declined over the same period. Roszak and Colwell (1987) found that more than 90% of viable naturally-occurring soil bacteria are not culturable and therefore have remained undetected, implying that *E. coli* O157:H7 likely faced much stronger competition in soil than indicated by the total

heterotrophic cell count. In contrast to the situation in autoclaved soil, a greater lethal effect on this pathogen was observed at 22°C than at 4°C in both unautoclaved high- and low-carbon soils (**Fig. 5.2.C, D, E** and **F**). Results from this study confirm the earlier findings of Jiang *et al*. (2002) who observed that the antagonistic effect of indigenous soil and manure microorganisms to *E. coli* O157:H7 was expressed more greatly at 21°C than at 5°C. In addition, we observed that soil composition in the unautoclaved manure-amended soil still played a role ($P<0.01$) in the survival of the pathogen, with greater cell death occurring in the nutrient-limited soils. It is noteworthy that lethal effects of indigenous soil microbes against *E. coli* O157:H7 were also observed at -21° C for both unautoclaved high- and low-carbon soils, an effect that was absent in the pure culture (sterile soil) condition.

One of the most informative approaches for studying the survival of *E. coli* O157:H7 in soils is to release known numbers of the organism onto a test plot exposed to environmental oscillations, and then directly sample over time. A trend common to the survival of *E. coli* O157:H7 in all treatments exposed to the environmental conditions was a 30 day period of high mortality (**Fig. 5.3.**). The most important observation was that these high mortality rate periods could be correlated with soil drying (**Table 5.1.**), suggesting that desiccation had a significant impact on *E. coli* O157:H7 survival.

Table 5.1. *In situ* survival of *E. coli* O157:H7 cells sampled from the surface and bottom soil layers over the most lethal period (June 10 to August 20).

Date	Incubation period (days)	Mortality rates (log cfu/10day)								Mean temperature (°C)	Total precipitation (mm)
		Drained				Undrained					
		Soil		Manure-amended soil		Soil		Manure-amended soil			
		Surface	Bottom	Surface	Bottom	Surface	Bottom	Surface	Bottom		
June 10-20	13-23	0.01	+ 0.08[c]	+ 0.97[c]	0.02	0.5[e]	0.07[e]	1.64[e]	0.26[e]	11.9	44.6
June 21-30	24-33	2.0[d]	1.24	4.12[d]	0.07	0.71	0.35[e]	+ 1.47[c]	+ 0.17[ce]	13.9	1.6
July 01-10	34-43	2.4[d]	0.14[d]	0.74[d]	2.69[d]	+ 0.51[c]	0	0.5	+ 0.25[ce]	14.3	3.6
July 11-20	44-53	+ 0.3[cd]	1.94[d]	+ 0.33[cd]	0.47[d]	1.65[d]	0.28	1.6[d]	1.35	18	2.6
July 21-30	54-63	0.3[d]	+ 0.54[cd]	0.79[d]	0.42[d]	1.44[d]	0.47	1.7[d]	0.47	15.7	14.4
July 31-August 10	64-73	0[bd]	1.19[d]	0[bd]	0.52[d]	0.65[d]	1.05[d]	0.46[d]	+ 0.29[cd]	16	16.8
Aug. 11-20	74-83	0[bd]	0.31[d]	0[bd]	0.17[d]	0[bd]	0.37[d]	+ 0.01[cd]	2.17[d]	13.8	9

[a] Each value is an average of three independent samples performed in triplicate.
[b] No detection of *E. coli* O157:H7.
+ [c] Numbers of *E. coli* O157:H7 cfu g^{-1} increase.
[d] Soil samples that visibly appeared dry.
[e] Standing water, or a sub-surface water table, present.

Dehydration was qualitatively observed to initially occur more rapidly (by days 24-33) on the surface of drained soils, and along with factors like solar irradiation and increasing salt concentration, caused increased *E. coli* O157:H7 cell lethality (**Table 5.1.**).

The process of soil dehydration continued over the next twenty days, further causing a high decline in cell viability. It has been documented that *E. coli* O157:H7 may leach through soil (Gagliardi and Karns 2000) and therefore the sharp decline of viable cells in drained soils may also have been due to cell leaching. Artz *et al.* (2005) have shown that leaching of *E. coli* O157:H7 through a soil matrix with a bulk density of 1.15 g cm^{-3} was very low, demonstrating a clear effect of bulk density on leachability of *E. coli* O157:H7 in repacked core experiments. Soil used in this *in situ* assay was a clay loam, and clay particles generally impart a fine texture and a heavy nature to soil (England *et al*. 1993) suggesting a low leaching potential. Parallel experiments performed with undrained soils, however, revealed the same pattern of lethality on *E. coli* O157 survival triggered by soil dehydration (after 44-53 days of incubation; **Table 5.1.**). Accordingly, in undrained manure-amended and plain soil where *E. coli* O157:H7 cells were not subject to leaching by rainfall, a sharp decline in viable cell number (2.8 $\log_{10}$ g^{-1} over 30 days) was observed, suggesting a strong lethal effect of dryness on the pathogen. Losses of *E. coli* O157:H7 cell viability in drained soils were more rapid than in undrained soils (**Fig. 5.3.**), indicating the existence of a strong lethal effect caused by more intensive and complete desiccation of drained soil and also partial cell leaching.

In conclusion, the present study revealed that soil composition played an important role on *E. coli* O157:H7 survival indicating that nutritionally-rich soil, in combination with moisture, may significantly extend the viability of *E. coli* O157:H7 in

soil environments. Considered together with competition by indigenous microbes and soil temperature, nutrient levels may have a considerable effect on *E. coli* O157:H7 survival. Lastly, there seemingly was an overriding influence of desiccation on the overall persistence of *E. coli* O157:H7 in soil environments. These results provide evidence that within soil types found in Saskatchewan, the survival of this pathogen is strongly influenced by moisture and soil nutrients and thus these factors should be a consideration in any management strategy.

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BRIEF INTRODUCTION TO CHAPTER 6

This chapter represents a natural extension of the previous studies that, in part, examined the survival of *E. coli* O157:H7 in soils under various nutritional, temperature and environmental regimes. In this chapter, a more detailed investigation was undertaken to determine the underlying role of the global stress response of *E. coli* O157 to prolonged heat and cold shocks, and also to characterize the regulatory role of RpoS sigma factor during these stress situations. Comparative proteomic analyses of the hypo- and hyperthermally adapted wild-type and *rpoS* mutant *E. coli* O157 strains allowed the identification and description of crucial proteins involved in growth resumption during extreme temperature conditions.

6. GLOBAL STRESS RESPONSE OF *ESCHERICHIA COLI* O157 AND THE ROLE OF RPOS SIGMA FACTOR DURING PROLONGED HEAT AND COLD SHOCKS

Abstract

Escherichia coli O157, a mesophile that primarily inhabits the gastro-intestinal tract, responds to temperature down or up-shifts with transient expression of adaptive proteins that allow survival of the organism under unfavorable temperature conditions followed by growth resumption. The purpose of this study was to identify heat and cold adaptive proteins induced during transition from growth arrest to growth resumption with specific focus on the role of the RpoS sigma factor. Using the comparative proteomic analysis of hypo- and hyper-thermally adapted wild type and *rpoS* null mutant *E. coli* O157 cells, we identified 56 protein spots that underwent significantly different expression upon temperature shock or *rpoS* mutation. All identified proteins of cold and heat post-acclimation stimulons fell into two large sub-groups: i) stress proteins and ii) housekeeping proteins. Proteomic analysis revealed that GroEL/S chaperonin complex plays a crucial role in growth resumption during elevated temperature, and also confirms an essential role of Pnp ribonuclease in transition from growth arrest to cell division in hypothermally adapted cells. In addition, we found that RpoS has no control over the key stress proteins in either stress stimulons. However, RpoS was shown to regulate the expression of a key protein involved in homoeoviscous adaptation, as well as various proteins involved in central metabolic pathways of both stress stimulons.

6.1. Introduction

Escherichia coli O157:H7 is a human pathogen known worldwide as a cause of life-threatening illnesses that include: haemolytic uraemic syndrome (Riley, 1983) and thrombotic thrombocytopaenic purpura (Su and Brandt, 1995). The majority of infections with *E. coli* O157:H7 are the result of food contamination, commonly undercooked red meat. A critical element in the emergence of *E. coli* O157:H7 as a foodborne pathogen was lateral gene transfer that resulted in over 1380 new genes being encoded in strain-specific clusters that are absent in the non-pathogenic strain *E. coli* K-12 (Perna *et al*., 2001). It is important to note that many clusters have no obvious role in virulence, but may confer strain-specific abilities to survive in different stress conditions (Perna *et al*., 2001), as already shown in a case of acid stress (Arnold and Kaspar, 1995) where the survival of *E. coli* O157:H7 cells greatly exceeded non-pathogenic *E. coli* controls.

Changing between different unfavorable environmental conditions trigger bacterial responses by replacing the sigma subunit in the RNA polymerase (RNAP) causing a shift in protein synthesis, typically towards different stress proteins. *Escherichia coli* possess seven sigma (σ) species that bind RNAP and then subsequently direct transcription initiation by recognizing promoters of specific groups of genes. During exponential growth, most of the cell's housekeeping genes are transcribed by the holoenzyme containing σ^D (RpoD), while upon entry into stationary phase the holoenzyme σ^S (RpoS) is essential for transcription of certain stationary-phase specific

genes (Lange and Hennge-Aronis, 1991). The synthesis of RpoS is also induced when *E. coli* cells are exposed to various stress conditions, for example, high osmolarity, starvation, low pH and non-optimal high or low temperature. By using genome-wide expression analysis, more than 480 genes of the *E. coli* genome have been identified to be under regulatory control of σ^S, with a set of 140 core genes being expressed under all stress conditions tested (Weber *et al*., 2005). The expression of the σ^S-dependent core genes follows the entry into stationary phase and provides the cells with the ability to survive stationary phase stress, as well as additional stresses not yet encountered, a phenomenon known as "cross-protection". In contrast to the core genes, the majority of the σ^S-controlled genes may be recognized by the σ^S subunit only under specific environmental conditions or in the presence of specific factors (Ishihama, 2000), allowing cells to respond in a highly precise manner. The dual nature of the σ^S stress response is opposite to other specific stress responses, which are triggered by a single stress signal and result only in the induction of highly-specific proteins designed to overcome a specific stress situation. The RpoS subunit of RNAP is therefore considered to be a master regulator of the general stress response in the γ branch of the proteobacteria, a group of Gram-negative bacteria that includes many human pathogen species such as *E. coli* O157 and also some beneficial organisms.

In the present study, the role of the σ^S was investigated under a range of temperature stress conditions in enterohaemorrhagic *E. coli* O157. The objective of this study was to determine the impact of σ^S on the survival of *E. coli* O157 under cold and heat stress conditions, as well as to reveal key stress proteins that may be different from those in non-pathogenic *E. coli*.

6.2 Experimental procedures

6.2.1 Preliminary study

A preliminary study was performed to determine mutations at the *rpoS* locus in 48 *E. coli* O157 strains that were isolated from bovine feces during the summer of 2004. Each strain possesses different genetic signatures based on random amplification of polymorphic DNA and virulence profiles (Vidovic and Korber, 2006). *Escherichia coli* O157 strains were streaked onto Luria-Bertani (LB) agar from stock cultures kept in 10% (vol/vol) glycerol at -70°C, and then incubated at 37°C for 24 hours. After incubation, the panel of *E. coli* O157 isolates was tested for glycogen synthesis, as described below in the glycogen assay.

6.2.2 Bacterial strains, plasmids and growth conditions

Escherichia coli K-12 strain DH5α was used as the host for the recombinant plasmids pSV1, pSV2 and pSV4, while *E. coli* SM10 λpir was used to maintain the recombinant plasmid pSV3. Wild type *E. coli* O157 strain B-1 possesses four virulence-associated genes (stx_1, stx_2, *hlyA* and *eaeA*) and expresses a relatively high cytotoxicity effect of 7.1-8.0, as determined by polymerase chain reaction (PCR) analysis and the Vero cells toxicity assay, respectively (Vidovic and Korber, 2006). A wild type nalidixic acid-resistant *E. coli* O157 B-1 is a spontaneous mutant obtained in our laboratory. Plasmids pUC19 (Vieira and Messing, 1982), pRE112 (Edwards *et al*., 1998) and pACYC184 (Chang and Cohen, 1978) were used as a cloning vectors. Plasmid pBSL130 (Alexeyev *et al*., 1995) was used as a source for the streptomycin resistance gene cassette. The bacterial strains were grown at 37° or 30°C in LB broth. Growth media were supplemented with chloramphenicol (30 μg ml^{-1}), ampicillin (100 μg ml^{-1}),

nalidixic acid (75 μg ml^{-1}) and streptomycin (35 μg ml^{-1}) for maintenance of recombinant plasmids and selection of the bacterial strains, as required.

6.2.3 General recombinant DNA techniques

Plasmid DNA and DNA fragments from agarose gels were purified using a commercial PCR purification kit (Qiagen). All PCR amplifications were performed using Phusion high fidelity DNA polymerase (New England BioLabs, Pickering, Canada) according to manufacturer's instructions. Restriction enzymes, T4 DNA ligase and alkaline phosphatase were purchased from New England BioLabs and Invitrogen (Burlington, Canada), respectively. Standard DNA cloning procedures were carried out as described elsewhere (Sambrook *et al*., 1999).

6.2.4 Construction of the *rpoS* insertion null allele

The *rpoS* gene was amplified by PCR from the *E. coli* O157-B1 strain using the forward primer (5'-*GTT* GGA TCC CCC CCG GGA TCA CGG GTA GGA GCC ACC TT-3') and the reverse primer (5'-*GAT* GAA TTC TCC CCC CGG GGG GAT TAC TCG CGG AAC AGC GCT TCA G-3'), clamps shown in italics, the *Eco*RI and *Bam*HI sites are underlined, respectively. The 1204 bp PCR product was cloned into the pUC19 vector, yielding the recombinant plasmid pSV1. The *rpoS* gene was confirmed by sequencing and then interrupted by insertion of the streptomycin resistance (Str^r) gene cassette at a unique *Eag*I site (position 760 bp of the coding sequence), generating pSV2. A 2.5 kb *rpoS*::str fragment was amplified from pSV2 using the primers RPOS3F (5'-*GGC* GGT ACC CCC CCG GGA TCA CGG GTA GGA GCC ACC TT-3') and RPOS4R (5'-*GGC* GAG CTC TCC CCC CGG GGG GAT TAC TCG CGG AAC AGC GCT TCA G-3') which introduced the restriction sites *Kpn*I and *Sac*I, respectively. This

PCR product was then double-digested with *Kpn*I and *Sac*I restriction enzymes and then ligated into the corresponding sites of the suicide vector pRE112 (Cm^r), resulting in plasmid pSV3. The recombinant plasmid pSV3 was introduced into *E. coli* SM10 λpir (Miller and Mekalanos, 1988) and transformants were selected on LB agar plates containing both streptomycin and chloramphenicol, resulting in strain SV519. Strain SV519 then served as the donor during conjugation with the nalidixic acid-resistant wild type *E. coli* O157 B-1 strain. The resulting exoconjugants were selected on LB agar plates containing nalidixic acid, chloramphenicol and streptomycin, yielding strain SV520 which was then plated onto modified LB agar (no NaCl) containing 5% sucrose and incubated overnight at 30° C. Sucrose-resistant colonies were screened for sensitivity to chloramphenicol due to the loss of the suicide vector. The chloramphenicol-sensitive streptomycin-resistant transconjugants represent the second homologous recombination event where the wild-type *rpoS* gene of *E. coli* O157 B-1 was replaced with the interrupted *rpoS* gene, resulting in strain SV521 with a null *rpoS* allele. The genotype of strain SV521 was confirmed by both PCR analysis and DNA sequencing.

6.2.5 Glycogen assay

To confirm the RpoS status, *E. coli* O157 *rpoS* null mutants (~50 strains) obtained as described above, were tested for glycogen production, a trait known to be under control of RpoS (Hengge-Aronis and Fisher, 1992; Wei *et al.*, 2000). Both *E. coli* O157 B-1 wild-type and putative *rpoS*$^-$ mutant strains were streaked on LB agar plates and incubated overnight at 37°C. After incubation, plates were left at 4°C for 24 h before they were flooded with concentrated iodine. Glycogen-deficient *rpoS*$^-$ mutants

gave a negative staining reaction (white colonies), whereas the wild-type glycogen-excess strains generated a positive staining reaction (dark brown colonies).

6.2.6 Complementation study

A ~1.2 kb DNA fragment containing an intact promotorless *rpoS* gene with its ribosome binding site was amplified by PCR with the following primers; RPOS5F (5'-*GTT* GGA TCC AAG GCC AGC CTC GCT TGA GAC TG -3') and RPOS6F (5'-*CTC* GTC GAC AAG GGA TCA CGG GTA GGA GCC ACC TT -3') to which *Bam*HI and *Sal*I sites were introduced (as underlined), respectively. Genomic DNA from the *E. coli* O157 B-1 strain was used as a template. The amplified *rpoS* gene was cloned downstream of the tet promoter into the corresponding *Bam*HI and *Sal*I sites of pACYC184, resulting in plasmid pSV4. The correct insertion and orientation of the *rpoS* gene in pSV4 was confirmed by double digestion and DNA sequencing of pSV4, respectively. Both the *E. coli* O157-B1 wild type (positive control) and its *rpoS* null mutant strains were transformed with the recombinant plasmid pSV4. The complemented strains were grown overnight in LB broth supplemented with chloramphenicol, then washed twice with sterile water and further used, as described below.

6.2.7 Stress Assays

Stress assays were performed using overnight cultures of the wild type *E. coli* O157 B-1 and its *rpoS* null mutant that had been grown in LB at 37 °C with constant shaking ~190 r.p.m. The seed cultures were diluted 1/100 in 50 mL of freshly prepared LB and grown at 37 °C to an optical density at 600 nm of 0.4 (early mid-exponential growth phase). Flasks containing cultures reaching their mid-exponential phase were

immediately transferred to a water bath15 °C, a treatment hereafter referred to as cold stress and 45 °C, hereafter referred to as heat stress; flasks were maintained with constant shaking at 190 r.p.m. (Brunswick RF-10 Frigidflow bath circulator; temperature ± 0.2 °C). Cells were incubated for an additional 6 hours. Viable cell counts were performed by tenfold serial dilutions in pre-cooled or pre-warmed 0.9% NaCl at time zero and then at every hour of the incubation. Aliquots of 0.1 mL were plated on LB agar in triplicate and then incubated at 37 °C for 24 hours.

6.2.8 Two-dimensional gel electrophoresis

Wild type *E. coli* O157 B-1 and *rpoS* mutant strains SV521 were grown overnight in LB at 37 °C with constant agitation (~190 rpm). Cultures were diluted 1:100 into fresh LB and then propagated at 37 °C to the mid-exponential phase (OD_{600} – 0.4). Both wild type and *rpoS* mutant strains were transferred at mid-exponential phase to 15 °C (cold stress), 45 °C (heat stress) and 37 °C (control) conditions and then held at these temperatures for 60 minutes. Total protein was extracted from 10 mL of culture by centrifugation (14000 rpm for 3 minutes) followed by extraction of the resuspended cell pellets in 1 mL of lysis buffer (9.8 M urea, 4% CHAPS, 2% IPG buffer pH 3-10, 1.5 mM phenylmethylsulfonylfluoride) at room temperature for 2 hours with occasional vortexing. The cell-lysis solution was then centrifuged at 12000 r.p.m. for 5 minutes. The resulting supernatant was resuspended in 3 x volume of ice-cold acetone, mixed vigorously and then incubated overnight at -21 °C to precipitate proteins. The precipitated protein was isolated by centrifugation at 4000 r.p.m. for 15 minutes and then resuspended in 300 µl of protein rehydration solution (9.8 M urea, 4% CHAPS, 2% IPG buffer pH 3-10, 100 mM DL-Dithiothreitol [DDT]) following incubation at room

temperature for 1 hour with repeated vortexing. Protein concentration was determined by using a dye-binding assay (Bradford, 1976) and a protein assay kit (Bio-Rad Laboratories, Hercules, CA). The total protein from wild-type and *rpoS* mutant strains was then analysed by high-resolution 2D PAGE, using Immobiline DryStrips (pI 4.0 to 7.0) and a Multiphor II electrophoresis unit (GE Healthcare Bio-Sciences Little Chalfont, UK). First dimension electrophoresis was performed by dehydration of the immobilized pH gradient strip with 130 µl of rehydration solution (9.8 M urea, 2% CHAPS, 2% IPG buffer pH 3-10,100 mM DDT, 0.005% bromophenol blue) containing 10 µg of protein at room temperature for ~20 hours. Isoelectric focusing was performed at 200 V for 1 min., 0-3500 V running time 2800 kVh, and 0-3500 V running time 5200 kVh, using a Multiphor II electrophoresis unit. Before running the second dimension, the strips were equilibrated for 15 minutes sequentially in two equilibration buffers (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS and 0.002% bromophenol blue) with addition of 100 mg DDT in the first buffer and 250 mg of iodoacetamide in the second buffer per 10 mL of equilibration buffer. Second dimension electrophoresis was carried out by placing the equilibrated strips in the well of 14% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel, overlaying the strip with 0.5% agarose and running at 100 V for 2 hours. After electrophoresis, the gels were silver stained following the manufacturer's (GE Healthcare Bio-Sciences) instructions. The stained gels were scanned on an Epson 1200C scanner with a transparency adapter as 8-bit greyscale 300-dot/in. images. Quantitatively-different proteins that were expressed within the wild-type in all three temperature treatments, and also between the wild-type and *rpoS* mutant strains within the same temperature treatment, were analysed by using Phoretix 2D version 2004 software (Nonlinear Dynamics Ltd., Newcastle upon Tyne, United

Kingdom). A protein spot volume that increased by ≥ 2-fold was considered as up-regulation; while a protein spot volume that decreased by ≥ 2-fold was interpreted as down-regulation.

6.2.9 Protein identification

Protein spots were excised from the gel, and digested with trypsin according to the protocols for the MassPrep robotic workstation (Waters-Micromass, Manchester, United Kingdom). Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was carried out with a capLC liquid chromatograph inter-faced to a Q-ToF Ultima Global hybrid tandem mass spectrometer fitted with a Z-spray nanoelectrospray ion source (Waters-Micromass, Manchester, United Kingdom). The LC-MS/MS data were processed with ProteinLynx software (Waters-Micromass) and then screened against the NCBInr, MSDB and Swiss-Prot/TrEMBL protein databases using Mascot Search (Matrix Science Ltd., London, United Kingdom).

6.2.10 Fatty acid profile analysis

Fatty acid methyl esters (FAME) analysis was conducted as described elsewhere (Annous *et al*., 1997) using 40 mg of washed cell pellets that had been exposed to different temperature treatments, as described above. A Hewlett Packard 5890 series 2 gas-liquid chromatograph (Hewlett Packard, Avondale, Pa.) equipped with a flame ionization detector and a 25 m x 0.2 mm methyl phenyl silicone fused silica capillary column (Ultra 2; Hewlett Packard) was used for identification of FAMEs. Results were automatically integrated by the Hewlett Packard 3365 series II ChemStation software version A.03.21, and FAMEs were identified with the MIDI Microbial Identification Software (Sherlock TSBA Library version 4.1; Microbial ID, Inc., Newark, Del.).

6.2.11 Experimental replication

Data from all experiments, including control treatments (37 ˚C) for both the wild type and the *rpoS* null mutant, represent the averages of at least three independent experiments. The differential expression of each protein spot of interest was determined from averaged spot volumes from four gels independently replicated, with a maximum variation of 30% in spot volume between the gels.

6.3 Results and discussion

6.3.1 Preliminary study and confirmation of *rpoS* mutant status

The high polymorphism rate of the *rpoS* locus from enterohemorrhagic *E. coli* strains may easily result in a non-functional RpoS sigma factor (Waterman and Small, 1996). To ensure that the *E. coli* O157 test-strain used in these studies possessed a functional *rpoS* sigma factor, 48 genetically-different *E. coli* O157 isolates from our laboratory collection were screened for their ability to synthesize glycogen (Notley-McRobb *et al*., 2002), as glycogen synthesis is under the direct control of RpoS (Hengge-Aronis and Fischer, 1992). Among all tested strains, the most pronounced glycogen production was exhibited by *E. coli* O157 strain B-1 (data not shown), and therefore it was chosen for further experimenation. As shown in the **Fig. 6.1.**, the glycogen synthesis in the *E. coli* O157 *rpoS* null mutant (white colonies indicate the absence of glycogen) was aborted due to a dysfunctional RpoS sigma factor, while the wild type strain was shown to be glycogen-positive (dark colonies).

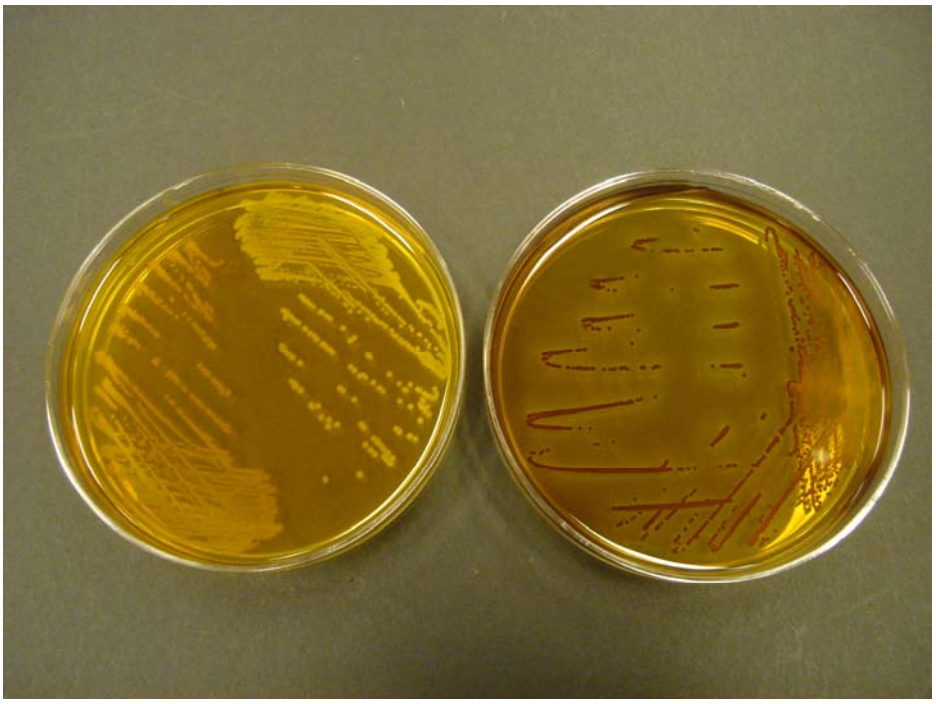

Figure 6.1. Glycogen phenotypes of wild-type *E. coli* B-1 strain and *rpoS* null mutant SV521 strains reveal a functional status of RpoS sigma factor. Colonies were grown overnight at 37˚C and then stored at 4˚C for another 24 h. Intracellular glycogen in colonies was stained with iodine, resulting in two phenotypes: (i) dark brown colonies indicate glycogen-contaning cells with a functional σ^S (wild type) and (ii) white colonies indicate glycogen-deficient cells with non-functional σ^S (mutant).

6.3.2 Response to cold~heat stress

Cultures of *E. coli* O157 B-1 wild type and its *rpoS* mutant were exponentially grown at 37˚C and then subjected to temperature shifts to 15˚ and 45˚C. The growth curves shown in **Fig. 6.2.** indicate that both heat and cold shock had a more substantial effect on the viability of the *rpoS* mutant than on that of the wild type strain. Furthermore, the *rpoS* mutant showed greater sensitivity to the cold stress than to the heat stress; results are presented in panels **A** and **B** of **Fig. 6.2**.

To determine whether the reduced viability of the mutant was caused by a dysfunctional *rpoS* gene or was due to polar effects on downstream genes, the plasmid pSV4 containing the intact *rpoS* coding sequence was introduced into *E. coli* O157 B-1 wild type and *rpoS* mutant strains for complementation study. **Figure 6.2.C** clearly

indicates that all changes in the viability of the mutant were due to loss of *rpoS* function, as cold tolerance was completely restored in the complemented mutant compared to that of the complemented wild type strain. It is important to point out that introduction of the low-copy pSV4 plasmid (~15 copies per a cell) will normally result in reduced growth rates of transformed cells and consequently only transformed strains were comparable in our stress assay. The growth curves of the wild type and its *rpoS* null mutant strains showed two distinct phases at both temperature extremes: (i) the first hour of incubation was characterized by growth arrest, (ii) after an hour of acclimation, the growth phase was restored, indicating a new phase in the stress response of *E. coli* O157. The main focus of this study was the post-acclimation phase, which started after approximately one hour of incubation at both extremes of temperatures; therefore, one hour of incubation was chosen as the sampling time for subsequent experiments (see **Fig. 6.2.A** and **B**).

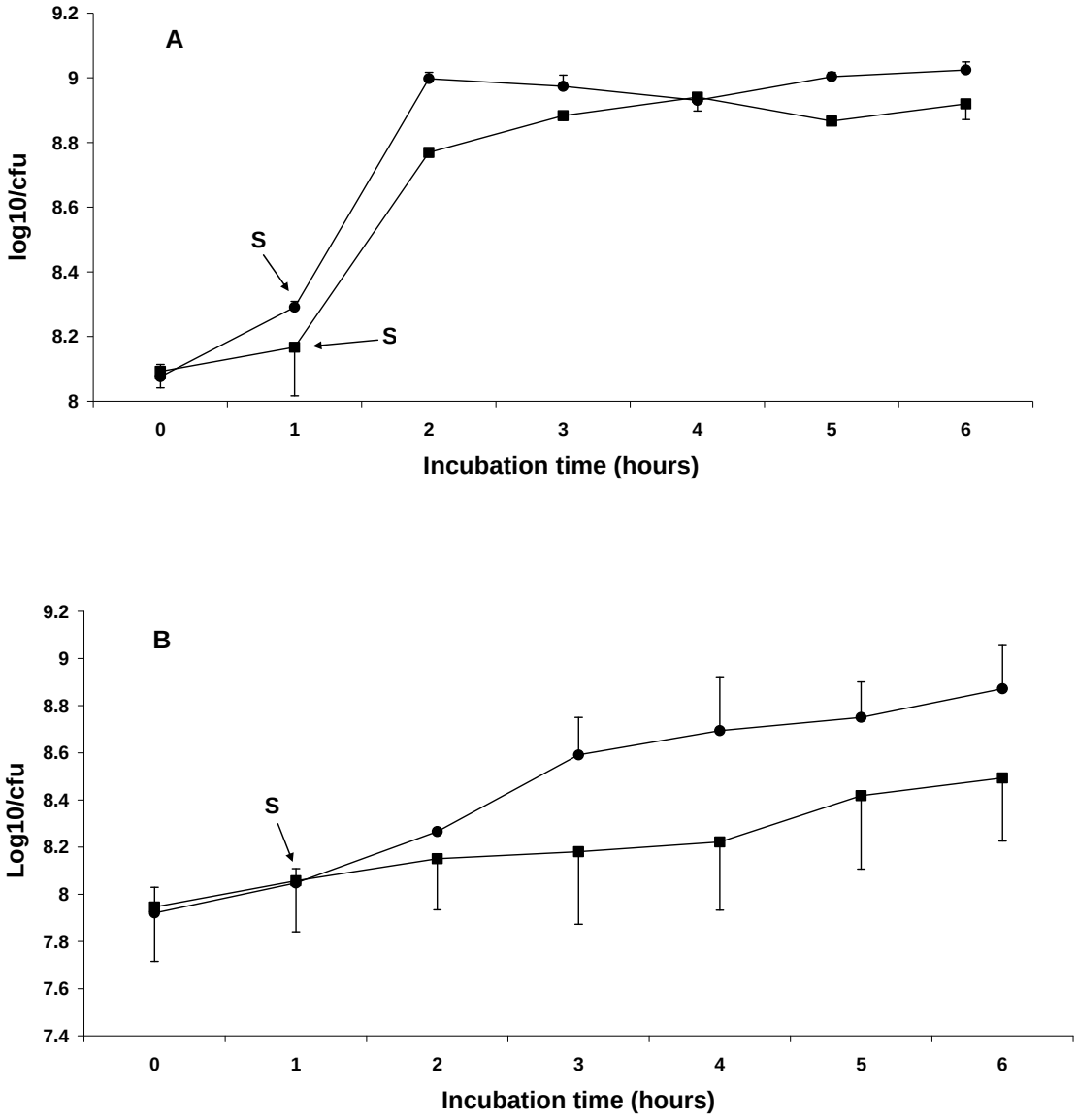

Figure 6.2. Stress growth assays and complementation study. (A) Heat-stress growth assay. (B) Cold-stress growth assay. Symbols: ● strain B-1 (wild type) and ■ strain SV521 (*rpoS* ::*str*). All strains were grown to early mid-exponential phase in LB broth and then subjected to different temperature shocks. Viable counts of strains were determined in triplicate for each experimental reading; the error bar represents the standard deviation between three experimental readings. Sampling time (S) for the proteome and fatty acid analysis is shown by arrows. (C) Complementation study. The experiment was carried out as in A and B except that two additional strains were used. Symbols: ♦ strain B-1 (wild type), ■ strain SV521 (*rpoS* ::*str*), ▲ the wild type complemented with *rpoS* in plasmid pSV4 (positive control) and ● the *rpoS* mutant complemented with *rpoS* in plasmid pSV4.

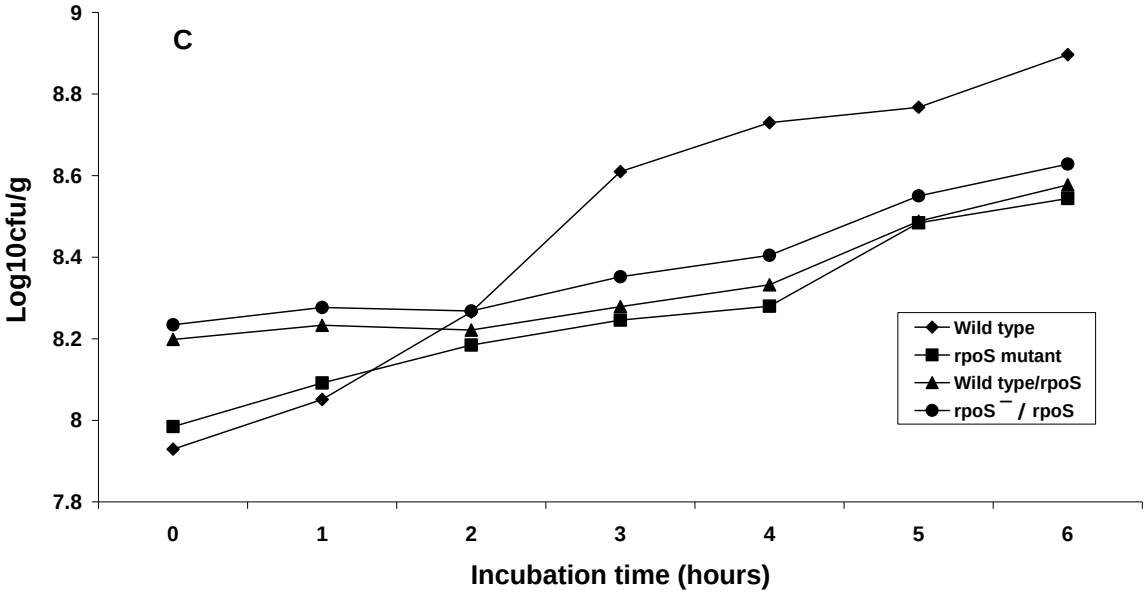

Figure 6.2. Continued

6.3.3 Global protein analysis

With an aim to specifically identify cold and heat-stress induced proteins of *E. coli* O157 that are regulated by RpoS, we performed a comparative analysis of the total proteome of wild type and mutant strains that have been grown under the same heat or cold stress conditions. Furthermore, to elucidate a total heat and cold stress stimulon of *E. coli* O157, we compared the total proteome of the wild type grown at different temperature stress conditions to the proteome of the same organism grown at 37°C. Mid-exponential phase cultures exposed to different temperature regimes were used, as imposed stress at this growth phase results in induction of proteins specific to these stress situations with no induction of cross-protective proteins. Proteins were separated by 2D PAGE across a pH range of 4-7 and proteins induced ≥2-fold based on six master gel image analysis were identified. In total, 67 spots from all six gels (**Fig. 6.3.**) were found to satisfy the ≥2-fold up- or down- regulation requirements of which 56 spots were identified using LC-MS/MS (**Tables 6.1.** and **6.2.**). The 56 identified proteins were

classified into nine categories based on their function: (i) adaptation to stress, (ii) global regulatory function, (iii) metabolism and transport, (iv) energy production and conversion (v) amino acid metabolism, (vi) nucleotides and phosphate metabolism, (vii) lipid metabolism, (viii) motility and (ix) unknown function.

6.3.4 Response to heat post-acclimation phase

6.3.4.1 RpoS-independent proteins

One hallmark of the heat stress response in *E. coli* is its transient nature (Zhao et al., 2005); the early phase is characterized by growth arrest followed by restoration of growth. Our results reflect a state of hyperthermally-adapted cells entering into a phase of restored growth. The RpoS-independent stress proteins that were identifed (**Table 6.2.**) fell into three main classes: (i) molecular chaperones / protease (GroEL, GroES, HchA and serine protease), (ii) DNA/RNA stabilizing enzymes (elongation factor EF-Tu, 50S ribosomal protein L25), and (iii) an anti-oxidative protein (a probable peroxidase).

Among the RpoS-independent heat stimulon, the most dramatic induction was observed for the chaperonin GroEL and its co-chaperonin GroES (Spots 9, 10 and 2). Both chaperonins form a fully functional 1:1 GroEL-GroES complex that is essential in protein folding under all cell conditions (Horwich *et al*., 1993).

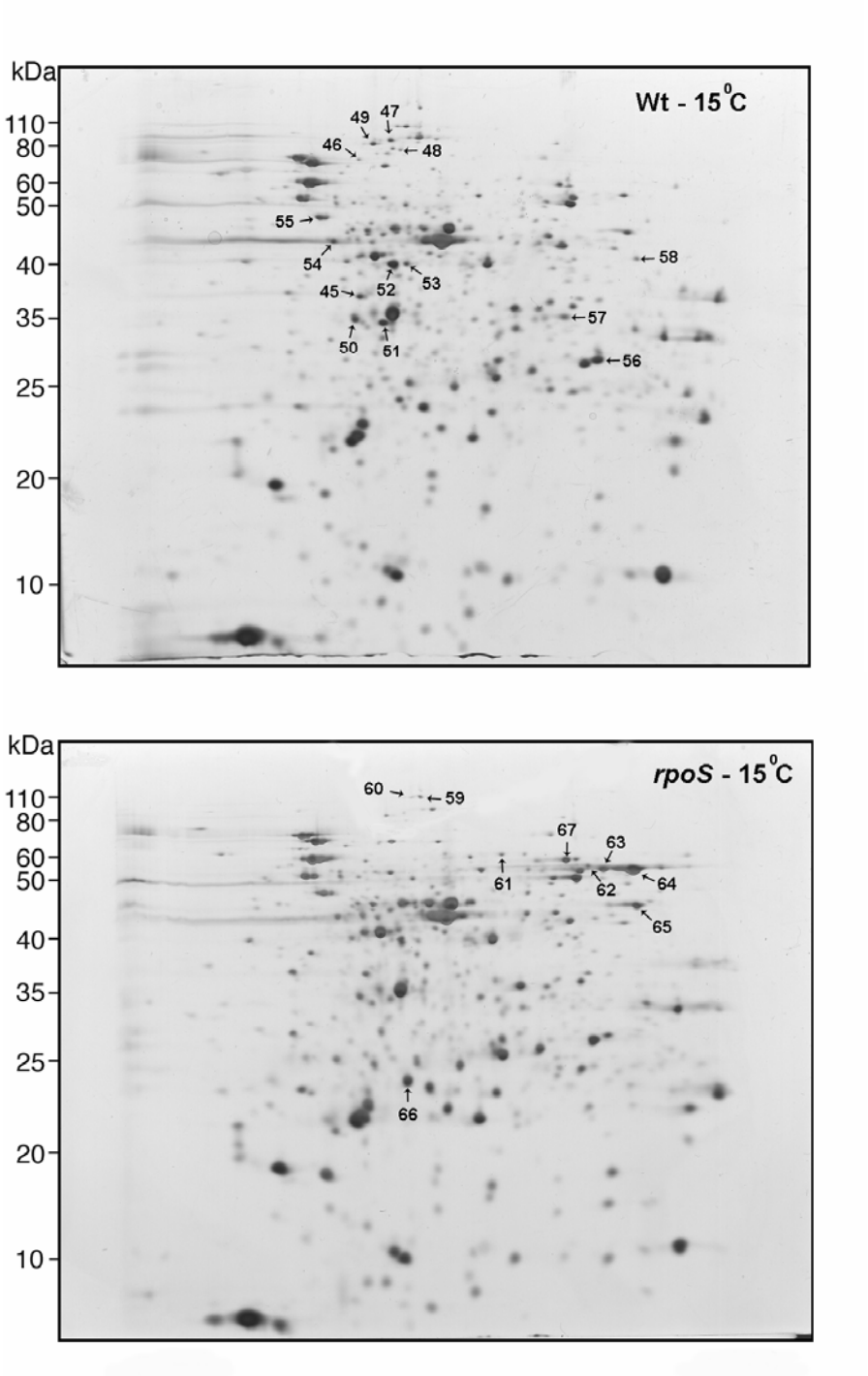

Figure 6.3. Comparative 2-D PAGE proteome analysis of *E. coli* O157 B-1 strain (wild type) and *rpoS* null mutant SV521 strain designed to reveal proteins induced at transition from the growth arrest to resumption of growth (see sampling time at Fig. 6.2.) during cold and heat shock. Also a control was included for both organisms (non-stressful condition i.e. 37°C). Four independent experiments were carried out for each strain/temperature condition, resulting in 24 electrophoregrams that were processed by Phoretix 2D software. One representative electrophoregram is presented for each strain/temperature condition. Spots of indentifed proteins are indicated by arrows and presented in Tables 6.1. and 6.2.

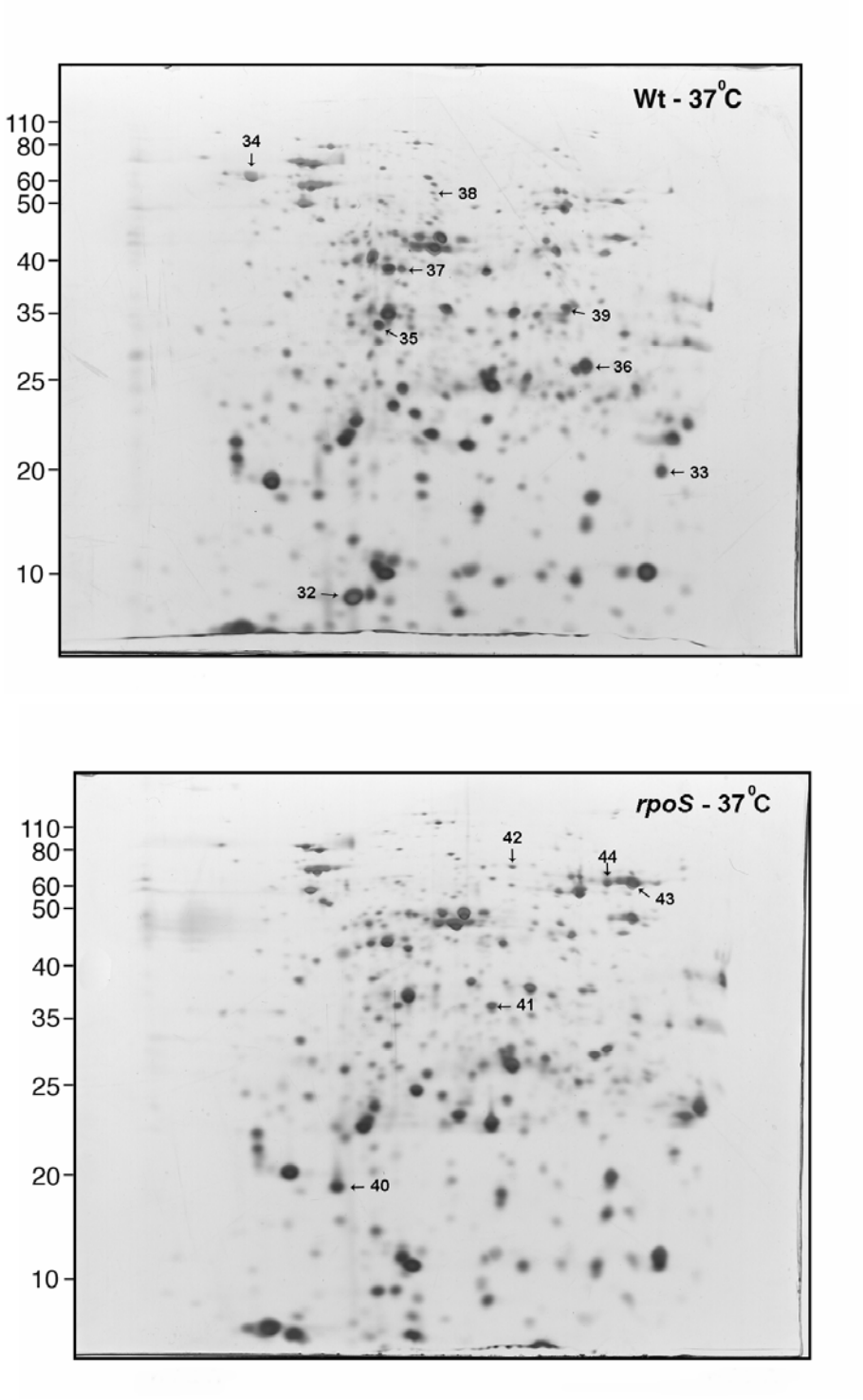

Figure 6.3. Continued

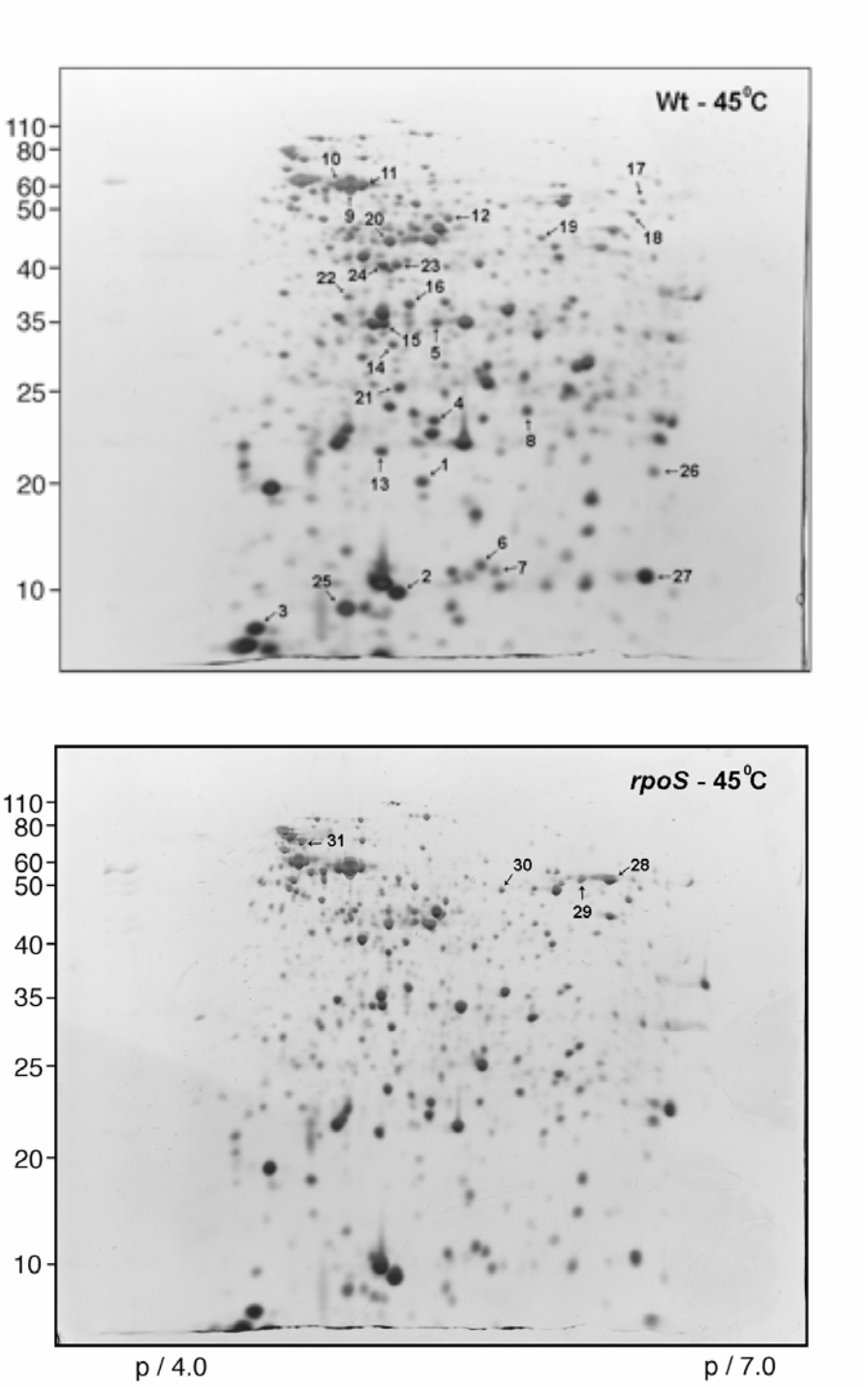

Figure 6.3. Continued

Table 6.1. List of proteins found to be RpoS-dependent.

Name	Function	Accession name	Spot	p/ the.	MW the.	Induction of proteins		Funct. category[a]
						wt	rpoS	
Proteins under positive/negative control of σ^S at 45°C								
OsmY	Osmotic adaptation	gi/15804947	1	5.9	21.0	4.2	/	AS
SspA	Stringent starvation protein A	gi/15803763	21	5.2	24.3	2.6	/	GR;AS
TalB	Chain A, transaldolase B	gi/1941982	22	5.1	35.2	2.3	/	AM
GlpQ	Glycerophosphodiester phosphodiesterase	gi/15802788	23	5.4	40.8	3.2	/	MT
GlpQ	Glycerophosphodiester phosphodiesterase	gi/15802788	24	5.4	40.8	3.5	/	MT
YfiD	Putative formate acetyltransferase	gi/15803106	25	5.1	14.2	2.5	/	E
YdjA	Hypothetical protein Nitroreductase-like family	gi/15802176	26	6.3	20.0	2.0	/	E
Rp1I	50S ribosomal protein L9	gi/15804792	27	6.2	15.7	2.1	/	GR
GuaB	IMP dehydrogenase	gi/15803032	28	5.9	54.5	/	4.4	NPM
GuaB	IMP dehydrogenase	gi/15803032	29	5.9	54.5	/	3.8	NPM
Proteins under positive/negative control of σ^S at 37°C								
YfiD	Putative formate acetyltransferase	gi/15803106	32	5.1	14.2	3.3	/	E
YdjA	Hypothetical protein Nitroreductase-like family	gi/15802176	33	6.3	20.0	2.6	/	E
RbsB	D-ribose periplasmic binding protein	gi/15804351	36	6.8	30.9	3.2	/	MT
GlpQ	Glycerophosphodiester phosphodiesterase	gi/15802788	37	5.4	40.8	6.1	/	MT
FliC	Flagellar biosynthesis	gi/15802358	34	4.7	59.9	Pres-ent	/	M
ManX	PTS system mannose-specific EIIAB component	gi/15802230	39	5.7	35.0	2.6	/	MT
MglB	Galactose-binding transport protein	gi/15802706	35	5.6	35.7	2.6	/	MT
Tpx	Thiol peroxidase	gi./15801846	40	4.7	17.8	/	4.0	AS
HchA	Chaperone Hsp31	gi/15802400	41	5.5	31.8	/	2.8	AS
PurH	Phosphoribosylaminoimid azolecarboxamide formyltransferase, IMP cyclohydrolase	gi/15804597	42	5.5	57.3	/	Pres-ent	NPM
GuaB	IMP dehydrogenase	gi/15803032	43	5.9	54.6	/	6.5	NPM
GuaB	IMP dehydrogenase	gi/15803032	44	5.9	54.6	/	10.1	NPM

Table 6.1. Continued

Name	Function	Accession name	Spot	p/ the.	MW the.	Induction of proteins		Funct. category[a]
						wt	rpoS	
Proteins under positive/negative control of σ^S at 15°C								
MalE	Maltodextrin-binding protein	gi/15804627	52	5.5	43.3	3.5	/	MT
GlpQ	Glycerophosphodiester phosphodiesterase	gi/15802788	53	5.4	40.8	18.2	/	MT
FusA	Elongation factor G	gi/15803853	47	5.2	77.5	3.1	/	GR
FusA	Elongation factor G	gi/15803853	46	5.2	77.5	Present	/	GR
AtpA	ATP synthase subunit alpha	gi/15804334	58	5.8	55.2	Present	/	E
RbsB	D-ribose periplasmic binding protein	gi/15804351	56	6.8	30.9	4.2	/	T
FabD	Malonyl CoA-acyl carrier protein transacylase	gi/15801209	51	5.0	32.4	6.8	/	LM
FabD	Malonyl CoA-acyl carrier protein transacylase	gi/15801209	50	5.0	32.4	3.2	/	LM
MalM	Periplasmic protein of mal regulon	gi/15834274	57	7.7	31.9	Present	/	U
PurH	Phosphoribosylaminoimidazolecarboxamide formyltransferase, IMP cyclohydrolase	gi/15804597	61	5.5	57.3	/	3.8	NPM
GuaB	IMP dehydrogenase	gi/15803032	62	5.9	54.5	/	3.4	NPM
GuaB	IMP dehydrogenase	gi/15803032	63	5.9	54.5	/	4.6	NPM
GuaB	IMP dehydrogenase	gi/15803032	64	5.9	54.5	/	4.2	NPM
GlyA	Serine hydroxymethyltransferase	gi/15803076	65	6.0	45.3	/	2.2	AM
GuaB	IMP dehydrogenase	gi/15803032	67	5.9	54.5	/	2.8	NPM
RpiA	Ribose-5-phosphate isomerase A	gi/15803449	66	5.2	22.8	/	2.1	NPM

Table 6.2. List of proteins found to be RpoS-independent

Name	Function	Accession name	Spot	p/ the.	MW the.	Induction of proteins		Functio. category[a]
						45°C	Control 37°C	
Proteins induced in the wild type at 45°C								
GroES	Chaperonin	gi/15599582	2	5.1	10.4	Present	/	AS
	Probable peroxidase	gi/15598725	4		21.9	Present	/	AS
HchA	Chaperone Hsp31	gi/15802400	5	5.5	31.2	6.7	/	AS
Ndk	Nucleoside diphosphate kinase	gi/1353658	6	5.4	15.5	Present	/	NPM
RplY	50S ribosomal protein L25	gi/15599866	8	5.8	22.0	Present	/	GR; AS
GroEL	Chaperonin HSP60 family	gi/15599581	9	4.8	57.3	Present	/	AS
GroEL	Chaperonin HSP60 family	gi/576779	10	4.8	57.3	Present	/	AS
GroEL	Chaperonin HSP60 family	gi/576779	11	4.8	57.3	Present	/	AS
ArcA	Arginine deiminase	gi/42543632	12	5.7	45.9	Present	/	AM
MglB	Galactose-binding transport protein	gi/15802706	15	5.7	35.7	Present	/	MT
YahI	Carbamate kinase-like protein	gi/15600366	16	5.7	33.9	5.8	/	E
GuaB	IMP dehydrogenase	gi/15598965	17	6.0	51.9	Present	/	NPM
AlgY	Serine protease	gi/15595963	18	7.7	50.3	Present	/	AS
FabF	Putative beta-ketoacyl-ACP synthase	gi/15801212	19	5.2	44.1	Present	/	LM
TufA	Elongation factor Tu	gi/15599461	20	5.3	43.3	Present	/	GR; AS
Proteins induced in the wild type at 15°C						15°C	37°C	
FusA	Elongation factor G	gi/15803853	47	5.2	77.6	Present	/	GR; AS
Pnp	Polynucleotide phosphorylase	gi/15803706	49	5.3	79.8	3.6	/	GR; AS
AtpD	ATP synthase subunit beta	gi/15804332	55	4.9	50.3	5.8	/	E

[a] Proteins were categorized as follows: AS, adaptation to stress; GR, global regulation; MT, metabolism and transport; E, energy production and conservation; AM, amino acid production; NPM, nucleotides and phosphate metabolism; LM, lipid metabolism; M, motility; U, unknown function.

The GroEL/S complex promotes folding by binding a wide variety of non-native polypeptides (Hlodan *et al*., 1995), diminishing irreversible aggregation and unfolding kinetically-trapped intermediates (Weissman *et al*., 1994), followed by the release of the polypeptide into the central cavity of the chaperonin complex and its accurate folding

(Mayhew *et al*., 1996). An abundance of the GroEL/S in hyperthermally-adapted cells indicates, in part, an increased level of misfolded proteins, as well as conditions where the native form of proteins is not achievable without assistance of the GroEL/S chaperonin machine. Guisbert *et al*., (2004) showed that the GroEL/S: like heat shock protein (Hsp) DnaK, is involved in the regulation of heat shock sigma factor (RpoH). Overexpression of GroEL/S results in inhibition of RpoH activity *in vivo* with the same efficiency as that of DnaK (Guisbert *et al*., 2004), giving a new functional insight of this chaperonin system. Overexpression of GroEL/S in heat adapted cells results in silencing of the RpoH regulon, which is crucial in the early phase of heat stress response, indicating that the GroEL/S chaperonin machine is a key molecule of the post-acclimation stress phase. Another significant up-regulation was observed in the heat-inducible chaperone A (HchA), resulting in 6.7 and 5.0-fold increases in both wild type- and *rpoS* null mutant-stressed cells compared to that of wild type non-stressed cells (**Table 6.2.**). This thermal pattern of HchA induction (**Fig. 6.4.**) in both genetic backgrounds can be explained by an existence of dual *hchA* promoters, recognizable by the σ^D and σ^S subunits of RNA polymerase (Mujacic and Baneyx, 2006).

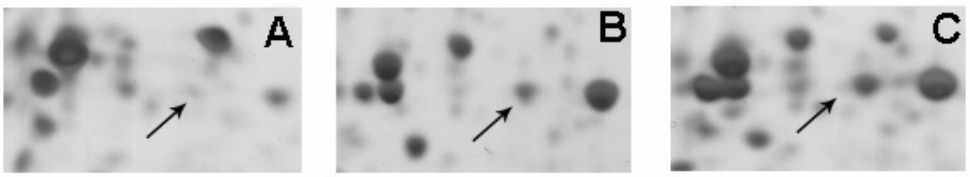

Figure 6.4. Influence of dual promoters, recognized by σ^D and σ^S, on expression of HSP HchA in exponentially grown *E. coli* O157 cells exposed to prolonged heat shock. Arrowheads indicate the level of HchA in enlarged regions of 2-D gels: (A) control-wild type at 37°C, (B) *rpoS* mutant at 45°C and (C) wild type at 45°C. The level of HchA was increased for 5.0-fold and 6.7-fold in *rpoS* mutant and wild type backgrounds, respectively, compared to that in control, indicating a major role of σ^D-dependent promoter in transcription of HchA in *E. coli* O157 cells exposed to prolonged heat shock.

Recent work by Mujacic and Baneyx (2006) showed that *hchA* thermal induction primarily relies on the relief of H-NS-mediated silencing of σ^D-dependent transcription, which is in agreement with our data where significant induction (5.0-fold) of HchA was observed in the *rpoS* null background. The comparative analysis of the *E. coli* O157 proteome allowed the quantitive determination of the contribution of σ^S in thermal induction of HchA; our data suggests that σ^S contributed ~25% (based on fold-increase) of the protein level increase in the wild type background compared to that *rpoS* null mutant, suggesting a minor role of σ^S in the thermal expression of HchA. Induction of HchA, a holding molecular chaperone (holdase) which stabilizes unfolding intermediates (Sastry *et al*., 2002) in heat adapted cells, indicates an increased level of heat damaged proteins. Here it is important to note that the induction of *hchA* is kinetically distinct from that of other heat shock genes, for instance, the level of the Hsp DnaK increases following a 15 minute heat shock but returned to basal levels after 60 minutes of high temperature incubation; whereas, HchA remains elevated even after 60 minutes (Mujacic and Baneyx, 2006). Taken together, our proteomic data revealed that the chaperone machines GroEL/S, in concert with HchA, play a crucial role in coping with prolonged heat shock. While overexpressed GroEL/S in part, silences a subset of the heat stimulon of hyperthermally adapted cells, it alternativly promotes crucial protein folding in times of severe and prolonged thermal stress. The unique HchA chaperone is designed to be stable over longer periods of heat stress, and prevents overloading of the GroEL/S protein folding machinery (Mujacic and Baneyx, 2006).

Furthermore, proteomic analysis showed that the σ^S-independent heat stimulon in hyperthermally-adapted *E. coli* O157 cells was not only confined to stress adaptive proteins (molecular chaperone, RNA/DNA stabilizing enzymes and anti-oxidant protein)

but also included proteins that regulate central metabolic pathways and structural modifications of the cell wall. The induction of proteins involved in central metabolic pathways significantly included: a galactose-binding transport protein, a carbamate kinase-like protein, a nucleoside diphosphate kinase, an IMP dehydrogenase, an arginine deiminase and putative beta-ketoacyl-ACP synthase, enzymes/proteins that correspond to transport, energy production, nucleotides and phosphate metabolism, amino acid synthesis and fatty acid metabolism. Significantly-higher expression of enzymes involved in the central metabolic pathways clearly suggest that growth of stressed cells had become restored; indeed, the growth curves in our stress assay show a sharp increase in growth rate following the sampling time for cellular protein, (**Fig. 6.2.A**), confirming the proteomic results.

6.3.4.2 RpoS-dependent proteins

Using comparative proteomic analysis of both organisms grown under non-stressful conditions, we observed that the *rpoS* mutation had an effect on the proteome of exponentially-growing *E. coli* O157 (control), resulting in two overlaps with heat and cold shock responses (see **Fig. 6.5.A**). The first overlap between the heat shock response and the control response included the up- regulation of the putative formate acetyltransferase YfiD, a hypothetical protein YdjA, and glycerophosphodiester phosphodiesterase GlpQ, as well as down- regulation of IMP dehydrogenase (**Fig. 6.5.B**), clearly suggesting that these proteins are not induced by elevated temperature.

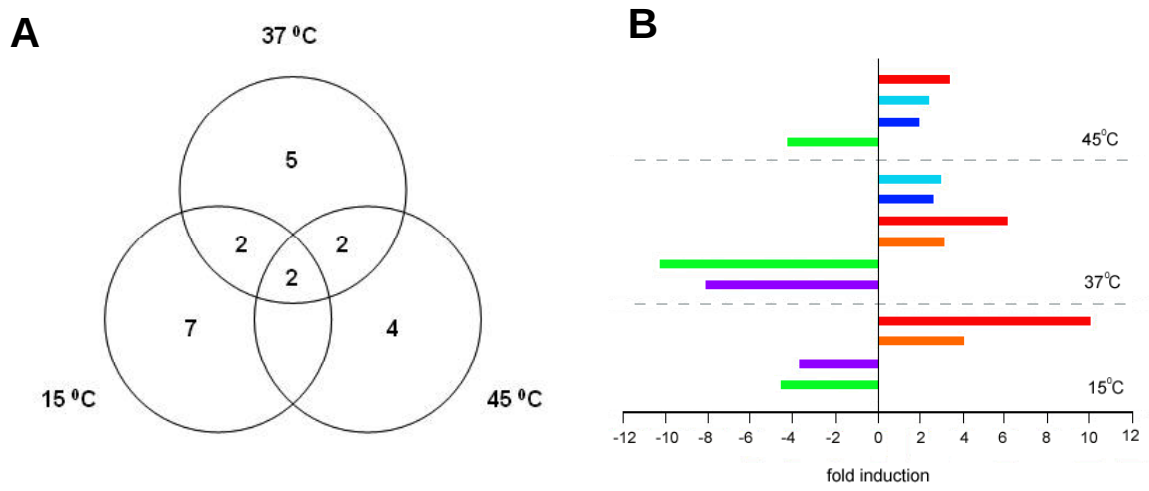

Figure 6.5. (A) The number of RpoS-dependent proteins significantly up- or down-regulated under three temperature conditions are presented in a Venn diagram. (B) Overlaps of heat and cold RpoS-dependent proteins with control. Qualitative and quantitative presentation of color-coded proteins follows: ■ GlpQ, ■ GuaB, ■ Yfid, ■ YdjA, ■ RbsB and ■ PurH.

A study performed by Rahman and colleagues (2006) using DNA microarray and RT-PCR techniques confirms our observation; they found that 72 and 25 genes of exponentially grown *rpoS*-deficient mutant of *E. coli* at 37°C were down- or up-regulated, respectively, implying the existence of an *rpoS* regulatory role during exponential growth despite a lower expression level.

The most pronounced up-regulation (4.2-fold) of an RpoS-dependent protein in response to heat stress was the periplasmic protein encoded by the σ^S-dependent core gene, *osmY* (see **Table 6.1.**). It has been shown that periplasmic OsmY protein is strongly induced by σ^S in specific environmental conditions such as: the stationary phase (Yim *et al*., 1994), hyperosmotic stress (Barron *et al*., 1986) and a low pH (Weber *et al*., 2005), showing its multifunctional nature in stress responses. Our report indicates a protective role of the periplasmic OsmY protein in hyperthermally-adapted *E. coli* O157 cells, adding this protein to the list of proteins in the heat post-acclimation stimulon. The

function of this protein is unknown; however, it is believed that OsmY is a binding protein with possible function in the transport of different osmolytes (Yim and Villarejo, 1992). Clearly, more studies are warranted to elucidate the exact protective mechanisms of the OsmY, which is also known to be involved in coping with various stresses. Another σ^S-dependent protein induced during heat stress was the stringent starvation protein A (SspA), which has dual function in *E. coli*: (i) the transcriptional activation of phage P1 late genes (Hansen *et al*., 2003), and (ii) the repression of histone-like protein (H-NS) (Hansen *et al*., 2005). The nature of SspA induction in *E. coli* O157 exposed to heat stress remains elusive. In this study, it was also shown that a housekeeping protein, TalB, and the 50S ribosomal protein L9, were induced during the heat post-acclimation phase, reflecting a general up-regulation of the non-oxidative branch of the pentose phosphate pathway, as well as further protection of heat damaged RNA-binding proteins, respectively. It is noteworthy that the heat shock protein 31 (HchA), which was up-regulated 1.7-fold in the wild type relative to the *rpoS* mutant, was just under proposed fold-factor of regulation cut-off (>2.0), and therefore it was not included in Table 5.1.

6.3.5 Response to cold post-acclimation phase

6.3.5.1 RpoS-independent proteins

Temperature downshift of exponentially growing *E. coli* O157 culture from 37˚C to 15˚C resulted in rapid growth arrest, a phenomenon known as the acclimation phase, followed by a resumption of exponential growth, but at a slower rate compared to that at 37°C. During the acclimation phase, cold shock proteins (CSPs) essential for cellular adaptation to low temperature are transiently expressed, with a dramatic increase at the beginning of cold acclimation (induction stage) followed by their repression at the end

of this phase. To study the role of σ^S factor in the regulation of proteins expressed at the post-acclimation phase, proteins of crucial importance for cell growth at low temperature, we analyzed the proteome of the control and hypothermally- adapted cells of the wild-type and RpoS deficient mutant (see sampling time in **Fig. 6.2.B**).

Among the RpoS-independent cold stimulon (Table 5.2), polynucleotide phosphorylase (Pnp), a key protein for the resumption of growth of hypothermally-adapted cells, was found to be up-regulated by 3.6-fold. Polynucleotide phosphorylase is a cold shock-inducible bifunctional enzyme with a phosphorolytic 3' to 5' exoribonuclease activity involved in mRNA degradation and 3' terminal oligonucleotide polymerase activity (Bijoy and Kushner 2000). The induction of Pnp at the end of the acclimation phase is in agreement with previous studies where it was shown that Pnp plays a critical role during transition from the acclimation phase to cell growth resumption after cold shock (Yamanaka and Inouye 2001; Neuhaus *et al*., 2000). Cells encountering cold shock initially induce a family of CSPs, with the predominant production of CspA, the major cold-shock protein, making up more than 10% of total cellular protein synthesis (Goldstain *et al*., 1990). After cold adaptation, degradation of CspA mRNAs becomes esssential for the cells, as high expression of CspA prevents translation of bulk mRNAs, thus blocking cell growth (Neuhaus *et al*., 2000). To promote proliferation of hypothermally-adapted cells, Pnp antagonize CspA by the selective degradation of its mRNAs at the stage of repression during the end of acclimation phase (Yamanaka and Inouye 2001), which is in full correlation with our proteomic results. Importantly, it has been reported that the function of Pnp is not only restricted to mRNA turnover, but also extends to cell replication efficiency at low

temperature (Clements *et al*., 2002), emphasizing the importance of this molecule during the cold post-acclimation phase.

Besides expression of the cold shock-inducible Pnp, it was observed a significant induction of the ATP synthase β subunit (5.8-fold) and elongation factor G in an RpoS-independent manner. Induction of these two enzymes corresponded to energy production and protein synthesis, reflecting the entry into a new phase characterized by resumption of growth after cold shock.

6.3.5.2 RpoS-dependent proteins

The second overlap of the proteome (**Fig. 6.5.B**) of cold stress and control included: the D-ribose periplasmic binding protein and glycerophosphodiester phosphodiesterase, as up-regulated proteins, and phosphoribosylaminoimidazole carboxamide formyltransferase, IMP cyclohydrolase and IMP dehydrogenase as down-regulated proteins, indicating that they were not part of the cold stimulon.

The most pronounced induction of an RpoS-dependent protein (6.8-fold) in hypothermally- adapted cells was malonyl CoA-acyl carrier protein (ACP) transacylase (**Table 6.1**), encoded by *fabD*, an enzyme that plays a key role in fatty acid synthesis (FAS) and polyketide synthase (PKS). The malonyl CoA-ACP transacylase catalazyes a thioesterification of malonate from CoA to ACP, providing malonyl-ACP precursors for both PKS and FAS (Florova *et al*., 2002). In both processes, malonyl-ACP functions as a two-carbon donor in the chain elongation process to generate the appropriate product. In type II PKS, this product is elongated without modification of the β carbon, whereas in FAS, the elongation process occurs after the β carbon has been fully reduced (Zhou *et al*., 1999). The PKS and FAS systems use separate ACPs and β ketoacyl ACP synthases

to generate significantly-different products, but use malonyl ACP as a substrate, suggesting that FabD represents a link between the processes of polyketide and fatty acid biosynthesis (Summers *et al*., 1995). It has been reported that low cultivation temperature increases the rigidity of the cell membrane, further compromising cellular diffusion rates and causing cluster formation of integral membranous proteins (Hazel, 1995). The significant induction of FabD in hypothermally-adapted cells may be associated with the production of long-chain fatty acids, a strategy employed to maintain optimal membrane fluidity at low temperatures (known as homeoviscous adaptation) (Sinensky, 1974).

Another set of RpoS-dependent proteins induced at the post-acclimation phase includes the maltodextrin-binding protein, elongation factor G, ATP synthase α–subunit and the periplasmic protein of the mal regulon, proteins involved in transport, synthesis of proteins and production of energy, clearly reflecting the growth restoration of these hypothermally-adapted cells. The proteins serine hydroxymethyltransferase and ribose-5-phosphate isomerase A were also found to be down-regulated, indicating in a change in amino acid synthesis.

6.3.6 Fatty acid analysis

To determine whether significant up-regulation of malonyl CoA-ACP transacylase, the enzyme involved in fatty acid synthesis, had an impact on membrane fluidity, the fatty acid profile of the hypothermally-adapted cells was determined. As malonyl CoA-ACP transacylase showed RpoS dependency, a fatty acid analysis of the wild type and its *rpoS* null mutant was performed.

Comparative lipid analysis revealed that the malonyl CoA-ACP transacylase significantly modified membrane composition, resulting in a shift in lipid production

towards long-chain fatty acids (**Fig. 6.6.**). The concentration of short-chain fatty acids (12:0 and 14:0) in membranes of both organisms was found to be approximately equal, whereas the level of longer-chain fatty acids (16:0) increased by 59% in the wild type compared to the *rpoS* mutant, further confirming the involvement of the malonyl CoA-ACP transacylase in the process of homeoviscous adaptation. Interestingly, a constant peak corresponding to cyclopropane fatty acid (CFA) was observed in the wild type, while the *rpoS* mutant showed a deficiency in CFA production (**Fig. 6.6.**). Formation of CFA is, in part, dependent on the RpoS sigma factor, and is considered to be a conditional and post-synthetic modification of membrane lipid bilayers, which results in an increased membrane fluidity (Chang and Cronan, 1999). This suggests that both FabD and Cfa work in concert to increase membrane fluidity of hypothermally-adapted cells. It has been reported that CFA plays protective roles in other stress conditions: for example, it is induced during the development of acid tolerance response in *E. coli* (Chang and Cronan, 1999) and *Lactococcus lactis* (Budin-Verneuil *et al*., 2007).

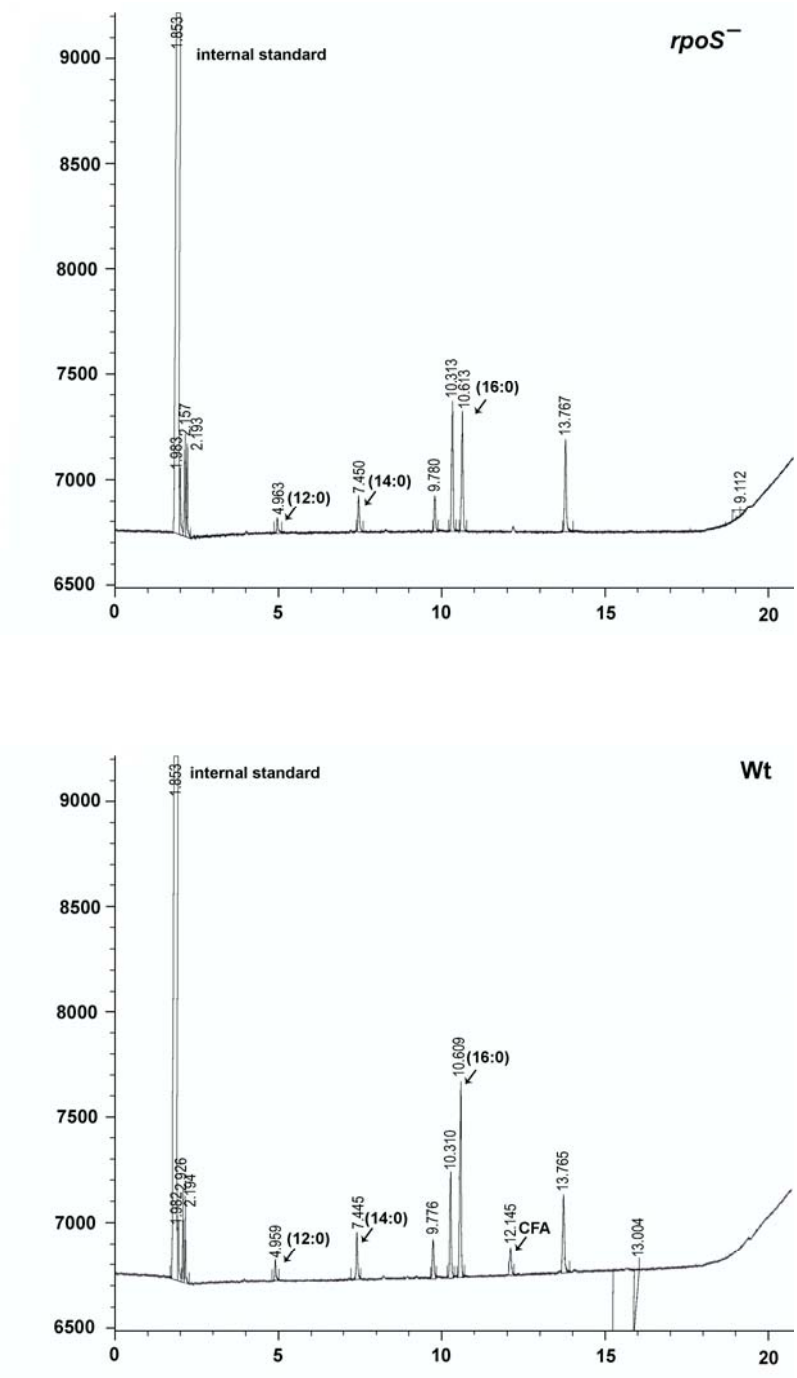

Figure 6.6. Lipid profiles of membranes of the hypothermally-adapted *E. coli* O157 wild type and *rpoS* mutant strains. Tested strains were sampled at the begining of growth resumption during cold shock (see Fig. 6.2.).

6.4 Concluding remarks

It has been shown that both cold and heat shock responses are characterized by their transient nature with an initial growth arrest in the acclimation phase followed by growth resumption in the post-acclimatation phase. Although the heat and cold shock responses in *E. coli* have been extensively studied, little is known about the molecular mechanisms that govern survival, and in particular proliferation of cells exposed to these stress conditions.

This study, using the comparative proteomics approach for hypo- and hyper-thermally-adapted cells, demonstrated that both the cold and heat post-acclimation stimulon consisted of two large sub-groups: i) stress proteins, and ii) housekeeping proteins. In addition, the cold stimulon possessed an enzyme involved in homeoviscous adaptation. Proteomic results also revealed that the GroEL/S molecule plays an essential role in heat stress post-acclimation by silencing the heat shock sigma factor (not a single member of RpoH regulon was identified) and promoting accurate protein folding during prolonged heat shock. A specific ribonuclease, Pnp, was identified, confirming previous findings (Yamanaka and Inouye 2001; Neuhaus *et al*., 2000) which emphasized a critical role for Pnp in cold adaptation. Interestingly, both GroEL/S and Pnp play a similar role in different shock conditions, silencing previously-expressed stress proteins, and therefore may be considered functional homologues. Furthermore, proteomic analysis, backed by fatty acid analysis, clearly indicate an important role of FabD in homeoviscous adaptation during cold-shock.

Through examination of the *rpoS* mutant proteome profile, the role of RpoS sigma factor in adaptation and proliferation of hypo- and hyper-thermally adapted cells was determined. As illustrated in **Fig. 6.7.**, the RpoS sigma factor has no control over the key

stress proteins (GroEL/S and Pnp) in both temperature shock conditions, suggesting a minor role of the general stress regulon in these instances. However, the RpoS sigma factor showed a more pronounced role in cold shock, regulating a key enzyme involved in conditional modification of membrane lipid bilayers of hypothermally-adapted cells. Factor RpoS also exhibited regulatory control over numerous proteins mainly involved in the central metabolic pathways in both stress stimulons, reflecting its specific importance in promoting resources (energy, protein synthesis, etc.) during proliferation of the hypo- and hyper-thermally-adapted cells.

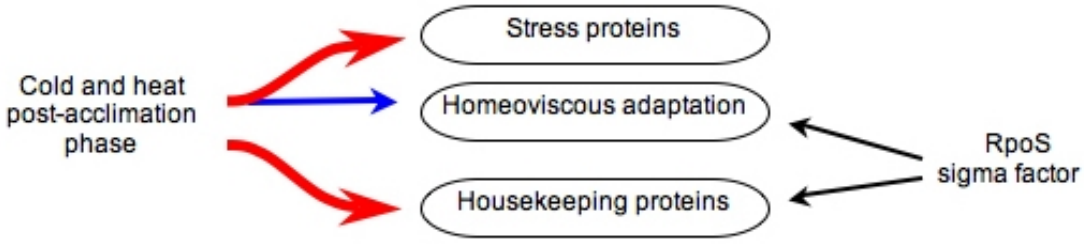

Figure 6.7. Model of cold and heat post-acclimation response and the role of RpoS in regulating these responses. Prolonged heat and cold shocks induce two large groups of stress and housekeeping proteins, marked with red arrows. There is also an additional response in the cold shock marked with blue arrow, indicating proteins involved in homeoviscous adaptation. RpoS showed control over various housekeeping and homeoviscous adaptive proteins, while key stress proteins remained independent from RpoS regulation.

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7. GENERAL DISCUSSION

Escherichia coli O157:H7 is recognized world-wide as a significant food-borne pathogen that poses a serious threat to public health. The low infectious dose and high virulence of *E. coli* O157:H7 make human infections particularly severe. The primary route of *E. coli* O157 infection to humans has been associated with consumption of inadequately cooked minced beef. Ruminants, especially cattle, have been implicated as the primary reservoir of *E. coli* O157:H7 (Chapman *et al*., 1993; Wells *et al*., 1991). This pathogen resides harmlessly in the digestive system of cattle until excretion in animal feces, after which it can enter the environment and the food chain. This has serious implications for the land-based disposal of organic wastes such as cattle manure, cattle slurry, and abattoir waste. As intensive livestock industry expands in Saskatchewan, it is important to have a documented understanding not only of the prevalence of *E. coli* O157, but its survival in native soil and climate conditions. Herein, the most important findings in chapters three, four and five relate to the prevalence and survival of *E. coli* O157 in soil, while in the sixth chapter the focus was shifted towards understanding the molecular response of this pathogen to prolonged cold and heat shocks.

Using a combined longitudinal and point prevalence study approach, where 23 feedlots and cattle operations scattered across Saskatchewan were examined for the presence of *E. coli* O157, several important conclusions were drawn. A salient observation was that cattle density was the most important determinant in *E. coli* O157 prevalence. Statistically-processed data showed that the prevalence of *E. coli* O157 in various feedlots could be explained by the effect of cattle density 78.7% of the time. In addition, characterization of all *E. coli* O157 isolates revealed two important

characteristics: (i) from a clinical point of view, the majority of *E. coli* O157 strains recovered in this study pose a high health threat to humans, as the majority of these strains have an exceptionally-high virulence potential, as determined by multiplex PCR and Vero-cell assay, and (ii) from an ecological point of view, the population of *E. coli* O157 isolates examined during my study consisted of indigenous and transient groups, implying a dynamic nature of existing populations of this human pathogen.

Another important observation was that inconsistent recovery from naturally-contaminated samples using IMS-VCC was related to the high genetic diversity of *E. coli* O157 isolates. Genomic fingerprint analysis of *E. coli* O157 isolates revealed that the high percentage of false-negative results was due to the same isolate, implying the existence of inhibitory effects of antibiotics used in the selective enrichment procedures on certain *E. coli* O157 isolates. Use of two different enrichment broths in parallel resulted in a significant increase (~ 20%) in the sensitivity of *E. coli* O157 detection in longitudinal and point studies over the IMS-VCC use alone.

Using comparative *in situ* assays, where the survival of *E. coli* O157:H7 was examined in manure-amanded and plain soils with and without drainage revealed that desiccation posed the most lethal threat to a population of this pathogen in the soil environment. In addition, it was determined that soil composition played an important role in *E. coli* O157:H7 survival, indicating that nutritionally-rich soils may be protective of *E. coli* O157:H7 populations. The fact that desiccation imposes the most lethal effect on this pathogen and that nutrients from soil may extend the survival times of *E. coli* O157:H7 cells, can serve as baseline data that may be used to predict the survival of this pathogen under a range of environmental conditions.

The final chapter deals with the role of RpoS sigma factor under prolonged heat and cold shock conditions, as well as the general response of *E. coli* O157 to these stressful situations. This chapter was designed to be a focused extension of chapter four, which describes the effect of various nutritional, temperature and environmental conditions on the survival of *E. coli* O157:H7 in soil. In contrast to the fourth chapter, where various conditions were examined without understanding the molecular mechanisms involved in the response to these stresses, in the fifth chapter, the focus was shifted to understanding the molecular mechanisms employed by *E. coli* O157 in coping with prolonged heat and cold shocks. Previous studies related to the heat and cold stress responses were focused on the acclimation phase, a phase characterized by growth arrest. The present work has advanced our understanding of cell proliferation under cold and heat shock by examining acclimatized cells during these stress conditions. The main characteristic of heat and cold post-acclimation phases was growth resumption of stress-adapted organisms and therefore the primary interest was in the global stress response of *E. coli* O157 in transition from growth arrest to growth resumption. As the RpoS sigma factor is known to be a master regulator of the general stress response, special attention was directed at investigation of a possible role of this sigma factor during prolonged heat and cold shocks. A global picture of the *E. coli* O157 response and the regulatory function of RpoS under stress conditions was derived using comparative proteomic 2D PAGE of the wild-type *E.coli* O157 B-1 and *rpoS* null mutant SV521 strains exposed to prolonged heat and cold temperatures. The general stress response to both prolonged heat and cold shocks resulted in a heterologous stimulon, with the two most predominant groups being: (i) stress proteins, and (ii) housekeeping proteins. As reported earlier (Yamanaka and Inouye 2001; Neuhaus *et al*., 2000) and also confirmed here, the Pnp

molecule plays a key role in prolonged cold shock. Ribonuclease Pnp selectively degrades mRNAs of CSP, resulting in their repression. As the overproduction of CSP proteins have deleterious effect on the hypothermally adapted cells, the ribonuclease Pnp modifies the cold stimulon in acclimatized cells and promotes the necessary conditions for synthesis of a new set of proteins which promote proliferation of these cells. A functional homologue of Pnp during prolonged heat stress was also indentifed. Dramatically-high induction of GroEL/S and no induction of the RpoH regulon was demonstrated in these experiments. This result, together with an earlier report (Guisbert *et al*., 2004) which indicated that overexpression of GroEL/S may silence the RpoH regulon, led to the conclusion that the GroEL/S complex modifies the heat stimulon in a transition phase during heat shock when cell-division is again restored. Comparing the proteomes of the wild-type and mutant, It was determined that RpoS has no control over key stress proteins under these stress conditions. RpoS showed a more pronounced role in hypothermally adapted cells than in hyperthermally-adapted cells, as evidenced by regulating key enzymes involved in homeoviscous adaptation. Generally, RpoS showed control over numerous housekeeping proteins under both stress stimulons, suggesting that RpoS has a more significant role in proliferation of hypo- and hyperthermally-adapted cells than in their adaptation during these stress conditions.

8. CONCLUSIONS AND FUTURE DIRECTIONS

1. Cattle density has the most pronounced effect on the prevalence rate of *E. coli* O157,
2. Isolates of *E. coli* O157 from Saskatchewan feedlot cattle have a high virulence potential,
3. The combined employment of two enrichment broths, i) modified *Escherichia coli* broth supplemented with novobiocin and ii) buffered peptone water containing vancomycin, cefsulodin and cefixime, increased the sensitivity of *E. coli* O157 detection by 22%,
4. Physical desiccation poses the most lethal effect on the viability of *E. coli* O157 cells exposed to soil environments,
5. GroEL/S plays a crucial role in growth resumption of *E. coli* O157 cells during prolonged heat shock,
6. FabD is a key enzyme employed in homeviscous adaptation of hypothermal acclimatized *E. coli* O157 cells,
7. RpoS has no control in *E. coli* O157 cells over key proteins in prolonged heat and cold stimulons, and
8. The chaperonin GroEL/S and ribonuclease Pnp may be considered as functional homologues, playing key roles in two different stress stimulons.

The laboratory collection of approximately 200 *E. coli* O157 isolates that were collected from feedlots scattered across Saskatchewan presents a valuable source for further research. Determination of minimal inhibitory concentration of these *E. coli* O157 isolates for several antibiotics that were employed in enrichment broths (novobiocin, vancomycin, cefsulodin and cefixime) may establish a new, more reliable

and sensitive enrichment procedure for the isolation of *E. coli* O157 cells from natural samples.

To the best of the author's knowledge, the study examining the global stress response of *E. coli* O157 to prolonged heat and cold shocks is the first report describing key proteins involved in bacterial proliferation during these stress conditions. This study may have important applied value, especially from a biotechnological point of view. Further research related to bacterial proliferation under extreme temperature conditions may include some important species that have been extensively used in fermentation processes designed to generate various products. Understanding the molecular mechanisms involved in propagation of beneficial microorganisms exposed to prolonged heat or cold shocks may enable advances in genetic engineering leading to significantly higher productivity.

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