University of Alberta

Identification and characterization of an extremely heat tolerant *Escherichia coli* from a beef processing facility.

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall, 2008

Your file Votre référence
ISBN: 978-0-494-47204-0
Our file Notre référence
ISBN: 978-0-494-47204-0

In loving memory of Claudia Vassilievna Vassilieva,

my high school teacher, who always supported my crazy idea to become a biologist.

ABSTRACT

The heat resistance of *E. coli* that survived decontamination procedures in a meat processing facility was evaluated. Strains of *E. coli* used included 34 isolates from the slaughter plant environment (SP); 7 isolates from live cattle (LC); strain GGG10 that was isolated from beef carcass prior to the introduction of steam treatment as an industrial practice; *E. coli* K12; and ATCC 25922. These strains were grouped into cocktails of 4-5 strains each differing in their RAPD patterns for subsequent identification. Meat samples were subjected to steam and subsequent lactic acid treatments on meat. These treatments reduced cell counts of *E. coli* strain cocktails by 90 to 99%. D_{60}-values of strains from live cattle, strain GGG10 and collection strains were consistent with literature data (0.1 to 2.5 min); however, D_{60} values of SP strains were up to 100 fold higher and ranged between 14 and 71 min.

ACKNOWLEDGEMENTS

I am grateful to many people who have helped me to get to this point:

To my husband Konstantin and my daughter Kira for their loving support and especially for all long hours of babysitting little Theodore.

To Dr. Lynn McMullen for her calm professionalism and for being not only my supervisor but a role model.

To Dr. Michael Gänzle for treating me as a colleague rather than a student and for all the joy I had arguing with him about experiment design and results.

To Dr. Linda Chui for scrupulous review of my thesis and very useful critical comments.

To Dr. Alexey Atrazhev for his expertise and advice in molecular biology.

To Dana Pierce for being my friend and a partner in crime.

To Linda Ho, who made many of my days much more cheerful.

And to all those people who helped me to become a better professional and a better person.

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1. INTRODUCTION AND REVIEW OF THE LITERATURE.

Illnesses caused by pathogenic *Escherichia coli* are one of the major concern to public health and to the food industry of many countries. The US Center for Disease Control and Prevention estimates that 73,000 cases of illness caused by Shiga toxin producing *E. coli* (STEC) are reported annually in the US. This results in 2000 hospitalizations and 60 deaths (Frenzen *et al.*, 2005). The Public Health Agency of Canada reported 31 cases of confirmed STEC infections in its 2005-2006 Annual Report, which showed a sharp increase in numbers when compared to recent years (11 cases in 2004). In 2007, the Canadian Food Inspection Agency reported 45 confirmed cases of *E. coli* infection in three-month period from July to September (Anonymous, 2007 a). In May of 2006 an outbreak with a total of 18 cases, associated with consumption of beef donairs from a local restaurant chain, was reported by the Capital Health Region, Edmonton, Alberta (Honish *et al.*, 2007; Currie, *et al.* 2007). On July 6, 2007 the Canadian Food Inspection Agency and Canada Safeway issued Health Hazard Alert and recalled beef products contaminated with *E. coli* O157:H7 in Alberta and British Columbia (Anonymous, 2007 b). In 2007 more than 30 million pounds of beef products were recalled in the US due to contamination with *E. coli* O157:H7, resulting in massive financial losses for the meat industry (Shin, 2007).

In Canada HACCP-based food safety programs were developed and implemented (Rajic *et al.*, 2007), but despite all efforts pathogenic *E. coli* remains a serious food safety issue.

1.2. Biology of the organism.

1.2.1 *E. coli* morphology and systematics.

First described as "Bacterium coli commune" in 1885 by Pediatrician Theodor von Escherich of Children's Hospital in Munich, Germany, *Escherichia coli* (Migula 1915; Castellani and Chalmers 1919) is a gram negative non-spore forming straight rod, motile with peritrichous flagella, and occurs as single cells or diplobacilli under microscope.

It is able to grow rapidly on simple carbon sources both aerobically (respiratory metabolism) and anaerobically (fermentation), though citrate cannot be used. The majority of *E. coli* are able to ferment lactose with acid and gas production. It possesses catalase (Brenner, 1984), cytochrome *bo* ubiquinol oxidase (Matsushita *et al.*, 1984; Bolgiano *et al.,* 1993), but does not synthesise detectable amounts of cytochrome *c* under aerobic growth conditions (Thöny-Meyer *et al.*, 1995).

Some strains of *E. coli* have urease activity, which was first described in clinical isolates from urinary tract infection (Wachsmuth *et al.*, 1979). Genes for *ure* are described from both plasmid and genomic DNA (Wachsmuth *et al.*, 1979; Heimer et al. 2002; Collins and Falkow, 1990).

By modern taxonomy (Euzéby, 2008) *E. coli* is classified as follows:

Domain – *Bacteria*

Phylum – *Proteobacteria*

Class – *Gammaproteobacteria*

Order – *Enterobacteriales*

Family – *Enterobacteriaceae*

Genus – *Escherichia*

The majority of *E. coli* strains are nonpathogenic commensals of intestinal tract of humans and other mammals. The normal cell count of *E. coli* in the intestinal tract of healthy human adult is 10^7 to 10^8 CFU per 1 ml of bowel content, which is a small proportion of the total bacterial count of 10^{11} to 10^{12} CFU/ml (Bondarenko *et al.,* 2003). Some strains of commensal *E. coli* are able to produce colicins, which suppress growth of pathogenic strains of *E. coli*. Commensal *E. coli* also assist in hydrolysis of lactose, participate in synthesis of vitamin K and group B vitamins, and are able to stimulate systemic humoral and local immunity (Vorobiev and Pack, 1998). Phylogenetic studies indicate that *E. coli* is closely related to *Shigella* and *Salmonella*, which are recognised as pathogens of warm-blooded vertebrates. Similarities in 16S rRNA genes led some authors to conclude that *E. coli* and *Shigella* spp. are ecovars of the same species (Pupo *et al.,* 1997, 2000; Lawrence and Ochman, 1998). Fukushima *et al.* (2002) demonstrated equally high similarities based on the DNA gyrase (*gyr*B) gene, and Paradis *et al.* (2005) found close relatedness of *Escherichia*, *Shigella*, and *Salmonella* based on genes encoding elongation factor Tu and F-ATPase β-subunit. A group of researchers at Osaka University constructed a phylogenetic tree for 22 strains of *E. coli* based on comparative genomic hybridization (CGH) microarray analysis, which was consistent with conserved genes sequence data as well as multilocus enzyme electrophoresis (Fukiya *et al.,* 2004).

1.2.2 Pathogenicity of enterohaemorrhagic *E. coli.*

Six pathotypes of *E. coli* are associated with intestinal infections (Nataro and Kaper, 1998; Kaper *et al.* 2004): enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC), diffusely adherent (DAEC), and enterohaemorrhagic (EHEC). The latter group belongs to a group of Shiga-toxin producing *E. coli* (STEC). EHEC are now considered to be the most significant group of pathogenic *E. coli* in North America, that are associated with beef.

Pathotypes (Kaper *et al.*, 2004) of *E. coli*, that cause illness in humans and other animals are very diverse. These organisms can cause enteric diseases, ranging from mild to severe diarrhoea, and haemolytic uraemic syndrome (HUS). Acquisition of new genetic material from other bacteria is recognized to be a very important, if not the leading mechanism by which pathogenic strains of *E. coli* evolve (Whittam et al., 1988; Schmidt and Hensel, 2004). EHEC must possess certain virulence factors: verotoxin and intimin. Two verotoxins, encoded by *Stx*1 and *Stx*2 genes, render direct toxic effects on renal cells as well as induction of cytokines resulting in renal inflammation (Andreoli *et al.,* 2002). Intimin-α is encoded by *eae* gene, which is located on the LEE Pathogenicity Island. It plays a crucial role in adhesion of the pathogen to intestinal epithelium (Donnenberg and Whittam, 2001; Kaper *et al.*, 2004). The genome of *E. coli* O157:H7 Sakai is 859kb larger than that of *E. coli* K-12 and bacteriophages play a predominant role in the lateral transfer of the Shiga-toxin genes (Acheson *et al.*, 1998; Hayashi *et al.*, 2001; Ohnishi *et al.*, 2001, Brüssow *et al.*, 2004). Other virulence factors are located on pathogenicity islands, plasmids or transposons and transferred by conjugation (Kaper *et al.,* 2004). *E. coli* O157:H7 evolved from EPEC O55:H7 via acquisition of s*tx* genes and

subsequent serotype change due to structural changes in its lipopolysaccharide (Feng *et al.*, 1998). Genomes of *E. coli* O157:H7 are rapidly diverging and radiating into new niches as the pathogen disseminates (Wick *et al.*, 2005).

EHEC was first recognized as human pathogen after several outbreaks in 1982 in the United States (Riley *et al.*, 1983). Symptoms of the illness include severe abdominal cramps, diarrhea with blood and in most severe cases – HUS and thrombotic thrombocytopenic purpura. In many cases a serotype of *E. coli* – O157:H7 rare at that time was isolated. Later the same organism was isolated from food that people consumed prior to becoming ill. These new isolates produced two distinct toxins, closely related to the toxin produced by *Shigella* (Strockbine *et al.*, 1986). Proteins Stx1 and Stx 2 share 55% amino acid homology (Kaper *et al.*, 2004). Both toxins had severe and irreversible cytopatic effect on African monkey kidney cells. These toxins have synonymous names including shiga-like (Stx) or verotoxin (VT). By the late 1980's *E. coli* O157:H7 became a very important foodborne pathogen, that caused more than 1500 illnesses a year in Canada (Woodward et al., 2002) and now *E. coli* O157:H7 is the most frequent serotype of EHEC that causes human illness in North America.

Cattle are known to be the main reservoir of *E. coli* O157:H7 (Montenegro *et al.*, 1990; Griffin and Tauxe, 1991; Karmali, 1989) and incidence of shedding *E. coli* O157:H7 by cattle is higher during the warm period of the year (Chapman *et al.*, 1997; Heuvelink *et al.*, 1998). A higher incidence of *E. coli* O 157:H7 isolation has been reported for younger animals (Hancock *et al.*, 1994).

In the slaughter process beef carcasses become contaminated during dehiding (Barkocy-Gallagher *et al.,* 2001). *E. coli* O157:H7 was isolated from 3.7% of beef, 1.5% of pork, 1.5% of poultry, and 2.0% of lamb samples obtained from the retail market place, which indicated that the organism was associated with food of animal origin (Doyle and Schoeni, 1987). In recent years the incidence of *E. coli* O157:H7 infections associated with food products other than meat has increased. In 1996, an outbreak associated with consumption of apple juice in Connecticut (Hilborn *et al.,* 2000) and a multistate outbreak caused by tainted lettuce (Hilborn *et al.,* 1999) occurred. In 2002, an outbreak of hemorrhagic colitis caused by consumption of hard cheese made from unpasteurised milk was described in Edmonton (Honish *et al.*, 2005).

Most outbreaks of hemorrhagic colitis are caused by pathogens not associated with food, but the fewer number of foodborne outbreaks involve larger numbers of people. Liu *et al.* (2007) reported higher numbers of sporadic cases of *E. coli* O157:H7 infection compared with the number of outbreak cases in Ontario in a 10 year period. Sporadic cases occur predominantly during summer months and are thought to be caused by recreational activities (Bruneau *et al.*, 2004). Another major cause of outbreaks can be drinking water contaminated with bovine faeces (Hrudey, 2002). These outbreaks involve the highest number of people (Rangel *et al.,* 2005). The incidence of *E. coli* O157:H7 in the watershed increases significantly when heavy rain causes an increased flow in rivers (Cooley *et al.*, 2007). Cases of person-to-person or animal-to-person transmission of the infection are also a concern (Galanis *et al.,* 2003; Waguri *et al.,* 2007). EHEC are also described as being able to loose shiga-toxins (Stx1 or Stx2 or both) during infectious

process in humans, therefore making clinical diagnostics more difficult and less able to track the origin of the pathogen (Bielaszewska *et al.*, 2007 a, b).

Serotypes of *E. coli* other than O157:H7 are not often reported as causative agents of haemorrhagic colitis in Canada; however, spread of non-O157 EHEC or other verotoxigenic bacterial species pathogenic for humans, is a real possibility (Paton and Paton, 1996; Grotiuz *et al.,* 2006; Renter *et al.*, 2007) . Renter *at al.* (2004) showed that prevalence of verotoxigenic *E. coli* O26:H11 and O111:H8 in feces of feedlot cattle in Alberta was much higher than that for O157:H7 (80% and 20% versus 8%). So, occurrence of non-O157 serotypes of verotoxigenic *E. coli* in the food supply in North America and in Canada in particular is not question "if", but "when".

It is important to remember that not all strains of *E. coli* O157:H7 possess the verotoxin genes and, therefore, do not belong to EHEC group. And many EHEC belong to serotypes other than O157:H7. Recent practices to test food products for serological *E. coli* O157:H7 does not satisfy the requirement to protect human health effectively, because there might be a false alarm or a missed danger.

1.3. Methods for detection of *E. coli* in food.

1.3.1 Culture methods.

Generic *E. coli* is an indicator organism for the presence of the pathogenic strains of this species in foods. Methods for cultural analysis for generic *E. coli* in foods have been developed by Health Canada. Approved methods include the most probable number (MPN) method (Chistensen *et al.*, 2002), hydrophobic grid-membrane filter (HGMP)

method (Peterkin *et al.*, 2001), direct plating (DP) method (Szabo and Todd, 1997), and enumeration with 3M™ PETRIFILM™ *E. coli* count plates (Warburton, 2001). All of these methods are based on isolation and phenotypic confirmation of *E. coli*. Standard MPN methods require 8 to 12 days to complete, whereas DP requires about 30 h. The relative disadvantage of the DP method is the inability to detect *E. coli* that are unable to produce indole from tryptophan and the possibility to identify indole and lactose positive enterobacteria such, as *Klebsiella oxytoca, Kluyvera ascorbata,* and some strains of *Citrobacter koseri* as *E. coli* (Garrity *et al.*, 2005).

Cultural analysis for *E. coli* O157:H7 is based on the inability of this serotype to ferment sorbitol (Sherman *et al.*, 1987) and the absence of β-glucuronidase (Krishnan *et al.*, 1987). The organism is usually separated from foods by immunobead separation and plated onto McConkey agar with sorbitol. The advantage of this method is the possibility of isolation of a pure culture for further characterisation. The disadvantage is the long time required for enrichment, isolation, and confirmation. Some strains of *E. coli* O157 serogroup are able to ferment sorbitol and may go undetected with this assay (Ammon *et al.*, 1999; Karch and Bielaszewska, 2001; Eklund *et al.*, 2006).

The optimal incubation temperature for *E.coli* O157:H7 is a subject for discussion. Some authors suggest 42°C as optimal incubation temperature for *E. coli* O157:H7 (Blais *et al*, 1997; Heuvelink *et al.*, 1998) for isolation of the organism, but Raghubeer and Matches (1990), Doyle and Shoeni (1984), and Gonthier *et al.* (2001) indicate that the temperature range for growth of *E. coli* O157:H7 is lower than that of other fecal coliforms and therefore this pathogen can be missed with standard enumeration procedures used for foods and water. It is very likely that all of the authors are right and

optimal growth temperature for *E. coli* O157:H7 is strain dependent. Recently a new procedure, which combines elevated incubation temperature and antimicrobial agents, an antibiotic of cephalosporine group and potassium tellurite, has been proposed (Dogan *et al.*, 2003). Church *et al.* (2007) found that chromogenic BBL CHROMagar O157 (Becton Dickinson) was able to differentiate *E. coli* O157:H7 on the primary isolation plate much more reliably than on Sorbitol-MacConkey agar as it gives distinct mauve coloured colonies while growth of generic *E. coli* is inhibited.

1.3.2 Immunological methods.

These methods are based on the interaction of specific antibodies with surface antigens (O) or verotoxins. These methods are quite sensitive, specific (Feldsine *et al.*, 1997), and easy to perform. Examples include lateral flow device for immunoprecipitation like VIP EHEC (Bio Control Inc.), and Duopath Verotoxins (Merc). The ELISA-based VIDAS system (Bio Mereux) is fully automated (Vernozy-Rozand *et al.*, 1997) and allows immunoseparation of the target organism.

1.3.3 Molecular methods.

Polymerase chain reaction (PCR) targets specific genes, and is highly specific and sensitive. For identification of *E. coli* in foods Health Canada approved a protocol for a semiquantitative real-time PCR (Shaw and Bosley, 2005) that is sensitive and highly species-specific for *E. coli*. This method does not differentiate between generic *E. coli* and EHEC. Primers based on *stx* genes are often used for detection of VTEC (Eklund *et al.*, 2002; Gannon *et al.*, 1992). Multiplex PCR amplifies two or more genes simultaneously in one reaction. For detection and simultaneous characterisation of shiga

toxin-producing *E. coli,* Paton and Paton (1998) created a very elegant multiplex PCR assay that included both shiga toxins, enterohemolysin and intimin. Later Müller *et al.* (2006) developed multiplex PCR for EPEC, atypical EPEC and VTEC, that targeted both shiga toxin genes (*stx*1 and *stx*2), the genes for the bundle-forming pilus operon (*bfp*, encoding type IV pili) and the "pedestal" forming protein gene (*esc*V). In 2007, the same research group published a novel single-step multiplex PCR that targets 13 genes simultaneously, and identifies the pathotype of *E. coli.* The large number of target genes could cause difficulties in interpretation of results.

Real-time PCR, based on fluorescence detection of O157 genes is one of the best methods of rapid analysis of food products. It has been shown effective in detection of EHEC in beef (Lett *et al.*, 1995; Sharma, 2002; McKillip *et al.*, 2004). The only drawback of this technique is the cost of the equipment and reagents (Sharma, 2002). Randomly amplified polymorphic DNA PCR (RAPD), PCR based on amplification of repetitive sequences interspersed in bacterial genome (REP-PCR), and multiplex PCR are very useful and affordable tools for genotyping *E. coli* and for epidemiological purposes (Vidovic and Korber, 2006; Foxman *et al.*, 2005; Pacheco *et al.*, 2001). Pulsed-field gel electrophoresis (PFGE) is a "gold standard" for *E. coli* genotyping, though it is time consuming and usually performed in specialised laboratories (Ribot *et al.*, 2006)

1.4. Control of *E. coli* on meat.

Food safety programs in Canada are based on Codex Alimentarius HACCP principles (Codex Alimentarius Commission, 2001) and regulated by Health Canada and Canadian Food Inspection Agency. In 1996 Health Canada directed the food industry to adopt

HACCP principles. The On-Farm Food Safety Program "The Quality Starts Here", initiated in 1995 by the Canadian Cattlemen's Association, involves 90,000 beef producers nationwide (Rajic *et al.*, 2007). The Food Safety Enhancement Program (FSEP) became mandatory in all federal inspected meat processing facilities in 2005. If the results of regular microbiological tests consistently fall outside operational standards and after a month surveillance period they fail to improve, a meat facility can lose FSEP recognition and plant operations can be ceased (Rajic *et al.*, 2007).

A baseline study conducted by USDA in 1996 showed that aerobic plate counts were $4.7x10^2$ CFU/cm^2 on carcasses and $7.9x10^3$ CFU/g in ground beef. Levels of *E. coli* were 35 CFU/cm^2 on carcasses and 54 CFU/g in ground beef (Huffman, 2002). To help control the presence of pathogenic *E. coli* on beef, the industry has implemented a number of carcass decontamination interventions.

1.5 Methods of antimicrobial interventions.

1.5.1 Physical interventions.

Heat treatments are the most widely used decontamination interventions in beef processing. There are three major methods of application: hot water rinses, steam pasteurisation and steam vacuuming. Their effectiveness, a 3 to 4 log decrease in cell counts in early studies (Kotula *et al.*,1974, cited by Sheridan, 2004), is now questioned. Phebus *et al.* (1997) reported a 3-log decrease of bacterial counts on beef carcasses after steam pasteurisation, and Morgan *et al.* (1996) showed that steam pasteurisation decreased cell counts more than 2 logs. Minihan *et al.* (2003) reported only a 0.3 to 0.5 log decrease of total cell counts on naturally contaminated beef carcasses after steam

pasteurisation. Hot water washes and steam pasteurisations tend to give similar results. In experiments of Castillo *et al.* (1998) the number of *E. coli* O157:H7 inoculated on the surface of beef in a faecal mass decreased 4 logs after a wash with water at 85°C. Chilling carcasses after heat treatment augments the effect (Sheridan, 2004). It is not clear if this variability over the years of the effectiveness of heat treatment was caused by increased resistance of microorganisms to heat or was due to improved slaughter practices, which lead to less initial contamination. It is difficult to compare studies as the details of the treatments varied.

High hydrostatic pressure (HHP) treatments showed good effectiveness on reduction of bacterial counts in foods like milk (Chen *et al.*, 2006), apple and orange juice (Noma *et al.*, 2004). But it is costly and its application to beef carcasses is questionable. Pressure higher than 500 MPa eliminates *E. coli* but it also causes damage to muscle tissues and ferric haemoprotein denaturation, leading to loss of quality (Lawrie and Ledward, 2006). Another method, irradiation, is quite effective for food decontamination, but consumers do not accept it (Erickson and Doyle, 2007; Hugas *et al.*, 2002). Podolak *et al.* (2006) studied effect of hydrodynamic pressure on the survival of *Escherichia coli* O157:H7 in ground beef and reported a reduction of cell counts up to 90%. This method raises questions about safe usage of explosives in commercial beef processing facilities. Other physical intervention methods, like UV light, ultrasound or pulse-electric field are either not very effective or simply impractical in meat processing (Erickson and Doyle, 2007).

1.5.2 Chemical interventions.

Organic acids, predominantly lactic acid, are used widely for beef carcass decontamination in North America and considered to be an important critical control point. The European Union prohibits use of any agents other than potable water. Effectiveness of acid treatment alone varies in different studies. Prasai *et al.* (1991) demonstrated a one to two log reduction of Viable Cell Counts (VCC) on beef during in-plant trials with 1% lactic acid. Four years later Avens *et al.* (1995) found that treatment with acetic acid was ineffective in their 61 week trial in abattoir. In recent experiments with *E. coli* O157:H7 inoculated on the surface of beef carcasses, effectiveness of 2% lactic acid varied from 1.5 to 5 logs CFU/cm^2 , and 4% acid in combination with high pressure water wash decreased the number of the pathogen up to 7 logs CFU/cm^2 (Stopforth *et al.*, 2004; Bosilevac *et al.*, 2006). Mustapha *et al.* (2002) and Lim and Mustafa (2003) observed total inhibition of *E. coli* O157:H7 (up to 7.29 log CFU/cm^2) on vacuum packaged beef, that was sprayed with 2% polylactic acid with molecular weights of 240, 360, or an equal mixture of both. They suggested that polylactic acid has a prolonged antimicrobial activity as it releases free lactic acid for an extended length of time. Acetic acid in 5% concentration gave a 2 log reduction (Cutter and Siragusa, 1994) or was found to be ineffective (Avens *et al.*, 1996). Smulders and Greer in their 1998 review summarized that the decrease in bacterial numbers on surface of beef varied from 0.3 to 2 logs CFU/cm^2 in experiments with organic acid treatments only. Dormedy *et al.* (2000) validated 2% lactic acid as an effective critical control point ($p < 0.05$). In Canada the use of lactic acid treatment of beef carcasses in combination with steam or hot water

treatment was approved as an efficient, safe and cost effective method of decontamination (Smulders and Greer, 1998).

Of other chemical agents tested in decontamination experiments, 0.1% chlorhexidine reduced cell counts of *E. coli* O157:H7 over 5 log CFU/cm^2 (Delazari *et al.*, 1998). Though chlorhexidine reduces *E. coli* O157:H7 numbers in the experiments and is a broad-spectrum antimicrobial agent, its use in commercial beef processing facilities is questionable due to potential toxic effects (Patel *et al.*, 2006; Faria *et al.*, 2007).Ozone treatments were also found to be ineffective and the reduction of cell counts was not significantly different from those achieved with water wash only (Castillo *et al.*, 2003). Cetylpyridinium chloride had the highest anti-*E. coli* O157:H7 effect in the research of Stopforth *et al.* (2004). It can also increase risk of selection of spoilage organisms like *Pseudomonas* spp. with subsequent contamination of the product due to inherent resistance of these bacteria to quaternary ammonia based compounds (Kolker *et al.*, 1982).

It has been stated that decontamination interventions are most effective when used in combination (Leistner, 1994; Kanatt *et al.*, 2002). Hurdle technology is based on simultaneous disturbance of different homeostasis mechanisms of microorganisms (Leistner, 1992; Leistner, 2002), and it has been demonstrated to be effective during slaughter process for beef decontamination (Hardin *et al.*, 1995; Graves Delmore *et al.*, 1998; Castillo *et al.*, 1998)

1.6 Objectives of the research.

Despite all precautions and decontamination interventions currently employed by beef producers, the presence of pathogenic *E. coli* on beef remains a very serious problem in the meat industry. To achieve the main goal of finding an effective and reliable method to control *E. coli* in beef carcasses, the objectives of this research were to:

1. determine of the ability of generic *E. coli* to survive decontamination interventions currently used by the beef processing industry;

2. investigate possible causative mechanisms of heat resistance of strains of *E. coli*.

2.MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions.

Bacterial strains used in this study are listed in Table I. Sixty four strains of *E. coli* were received from Agriculture and Agri-Food Canada, Lacombe Research Centre, Alberta. They were isolated from a commercial beef slaughter plant where cattle is processed at a rate of 250 head/h (Aslam *et al.,* 2004). Two strains of *E. coli* (FUA 1050 and FUA 1051) from vagina of live cows, strain *E. coli* GGG10 (isolated from a commercial slaughter plant 20 years ago, prior to the commercial use of decontamination interventions), *E. coli* O157:H7 ATCC 43889, *E. coli* O157:H7 ATCC 43890, *E. coli* K-12, and *E. coli* ATCC 25922 were obtained from the University of Alberta Food Microbiology Laboratory collection. Ten strains of *E. coli* were isolated from rectal samples of live cows in the U of A experimental farm (FUA 1040, FUA 1041, FUA 1042, FUA 1043, FUA 1044, FUA 1045, FUA 1046, FUA 1047, FUA 1048, FUA 1049). Two strains of *Shigella* (*S. dysenteriae* A188 and *S. flexneri* A62) were provided by Dr. Linda Chui, Alberta Provincial Laboratory for Public Health.

Bacterial cultures were stored at -80°C in glycerol. Bacterial stocks were prepared as follows: cells from 10 ml of an overnight culture grown in Luria Bertani (LB) broth (Difco™, Becton Dickinson, Sparks, MD, USA) were harvested by centrifugation for 10 min at 6800 x *g*, the supernatant was discarded and the cell biomass was resuspended in 1 ml of fresh LB broth and added to 0.5 ml of 50% glycerol in a cryotube. Prior to the experiments cultures were subcultured at least twice on mEndo agar LES (Endo agar) (Difco™, BD) and on LB agar (LB broth + 15 g/l of agar) to resuscitate the cells.

Table I. Bacteria used in the research.

Source	Strain	Identified as *E. coli* on Endo agar	Identified as *E. coli* on TSI agar	Identified as *E. coli* by PCR
Slaughter plant environment	MB 1.1	+	+	+
	MB 1.2	+	+	N/T*
	MB 1.3	+	+	+
	MB 1.4	+	+	N/T
	MB 1.5	+	+	+
	MB 1.6	+	+	N/T
	MB 1.7	+	+	N/T
	MB 1.8	-	-	+
	MB 2.1	+	+	+
	MB 2.4	+	+	N/T
	MB 3.3	+	+	+
	MB 3.4	+	+	+
	MB 3.5	+	+	N/T
	MB 4.6	+	+	+
	AW 1.1	+	+	N/T
	AW 1.2	+	+	+
	AW 1.3	-	-	+
	AW 1.5	+	+	N/T
	AW 1.6	+	+	N/T
	AW 1.7	+	+	+
	AW 1.8	+	+	N/T
	MB 8.6	-	-	N/T
	MB 10.1	+	+	+
	GM 9.1	+	+	+
	GM 9.3	-	-	-**
	GM 9.5	+	+	+
	GM 9.8	+	+	+
	GM 11.1	-	-	-***
	GM 11.2	+	+	N/T
	GM 11.3	+	+	+
	GM 11.4	+	+	N/T
	GM 11.5	+	+	+
	GM 11.6	+	+	+
	GM 11.9	+	+	+
	GM 12.6	+	+	+
	GM 14.1	+	+	+
	GM 14.3	+	+	+
	GM 14.4	+	+	N/T
	GM 14.5	+	+	+
	AW 12.2	+	+	+
	AW 14.1	+	+	+
	AW 15.1	N/G****	N/G	N/T
	GM 16.1	+	+	+
	GM 16.2	+	+	N/T

Table I. (continued)

	GM 16.3	+	+	+
Slaughter plant	GM 16.4	+	+	N/T
environment	GM 16.5	+	+	N/T
	GM 16.6	+	+	+
	GM 16.7.1	+	+	N/T
	GM 16.7.2	-	-	+
	GM 16.8	+	+	N/T
	GM 18.1	+	+	N/T
	GM 18.2	+	+	+
	GM 18.3	+	+	N/T
	GM 18.4	+	+	+
	GM 18.5	+	+	N/T
	GM 18.6	+	+	+
	MB 16.5	+	+	N/T
	MB 16.6	+	+	+
	MB 16.7	+	+	N/T
	GM 17.1	+	+	N/T
	DM 19.1.1	-	-	N/T
	DM 19.1.2	-	-	-*****
	DM 19.2.1	+	+	N/T
	DM 19.2.2	-	-	N/T
	DM 19.7.1	-	-	N/T
	DM 19.7.2	+	+	+
	DM 18.3	+	+	+
Live cows (rectum)	FUA 1040	+	+	+
	FUA 1041	+	+	+
	FUA 1042	+	+	+
	FUA 1043	+	+	+
	FUA 1044	+	+	+
	FUA 1045	+	+	+
	FUA 1046	+	+	+
	FUA 1047	+	+	+
	FUA 1048	+	+	+
	FUA 1049	+	+	+
Live cows (vagina)	FUA 1050	+	+	+
	FUA 1051	+	+	+
E. coli Top 10		N/T	N/T	+
E. coli GGG10		+	N/T	N/T
E. coli O157:H7 ATCC43889		+	N/T	N/T
E. coli O157:H7 ATCC43890		+	N/T	N/T
E. coli K-12		+	+	+
E. coli ATCC25922		+	+	+
Shigella sonnei ATCC 25931		-	-	-
Shigella flexneri A62		-	-	-
Shigella dysenteriae A188		-	-	+

Strain labels of slaughter plant isolates: MB - manufactured beef, AW - carcass after washing, GM - ground meat, DM - drive mechanisms (belts, rollers, *etc.*). FUA - strain collection of the Laboratory of Food Microbiology of the University of Alberta. *N/T - not tested; **-*Enterobacter* sp.; ***-*E. cloacae*;****N/G - no growth; *****- *Pantoea agglomerans*

All experiments were performed with cultures in the stationary phase only. Unless otherwise stated, cultures were grown overnight in 50 ml of LB broth placed in 250 ml flasks at 37°C and 120 rpm.

2.2 Culture media and phenotypic confirmation of *E. coli.*

For primary isolation and confirmation of the purity of *E. coli*, cultures were streaked onto Endo agar and single colonies were subcultured at least twice to check colony morphology and confirm the purity of the isolates. Colonies with specific metallic shine were described as *E. coli.* Triple Sugar Iron agar (BBL™, BD) was used for further phenotypic characterization of strains.

LB Broth (Difco™, BD) and LB agar were used for growing pure cultures. LB broth was used in determination of thermal resistance and biofilm formation experiments. LB agar and Endo agar were also used to enumerate *E. coli* in intervention experiments.

For analysis of hemolytic activity *E. coli* isolates were plated onto Columbia blood agar (BBL™, BD) with 5% sheep blood and incubated overnight at 37°C.

2.3. Genotyping methods.

2.3.1 Template DNA extraction.

Total genomic DNA was extracted from each pure culture and used for all PCR-based methods. A single colony of each strain was inoculated into 10 ml of LB broth and incubated overnight at 37°C with shaking at 120 rpm. Cells from 1 ml of culture were harvested (6800 x *g* for 7 min) and DNA was isolated using DNeasy Tissue Kit (Qiagen,

Mississauga, ON, Canada) according to the manufacturer's instructions. Presence of DNA was confirmed by electrophoresis in a 1.5% agarose (Invitrogen, Carlsbad, CA,USA) gel with 1x TBE buffer (108 g Tris Base, 55 g Boric acid, 9.3 g Na EDTA, 10 L Distilled water)(80V, 25 min) with subsequent staining with ethidium bromide (BioRad, Missisauga, ON). A 1 Kb+ DNA Ladder (Invitrogen) was used as a size marker. Samples of DNA were aliquoted and stored at -20°C.

2.3.2 Gene specific PCR.

To confirm species identity of the strains of presumptive *E. coli*, PCR with three pairs of primers was performed. One pair of primers was based on the universal stress protein gene (*usp*A) (Chen and Griffiths, 1998), and two pairs of primers targeted hypervariable regions of the *E. coli* 16S rRNA gene (Sabat *et al.*, 2000) (Table II). Primers were obtained from the Integrated DNA Technologies of the Molecular Biology Service Unit of the University of Alberta. For each PCR analysis, 50 µl of the reaction mixture contained: 5 µl of 10x reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 1.25 U of Taq polymerase (all reagents from Invitrogen), 30 pMol of each primer, sterilized MQ water, and 1 µl of template DNA.

The thermal cycling program for *usp*A primers was carried out as follows: heated at 94°C for 5 min; amplified for 30 cycles as follows: 94°C for 2 min, 70°C for 1 min, 72°C for 1 min; 72°C for 7 min. A GeneAmp 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA), was used for all PCR reactions.

Table II. List of primers used in the research

Name	Sequence	Annealing temperature, °C	product size, bp	Reference
uspA	F 5' CCG ATA CGC TGC CAA TCA GT	70	884	Chen and
	R 5' ACG CAG ACC GTA GGC CAG AT			Griffiths, 1998
ECA75F	F 5' GGA AGA AGC TTG CTT CTT TGC	50	544	Sabat *et al.*, 2000
	TGA C			
ECR619R	R 5' AGC CCG GGG ATT TCA CAT CTG			
	ACT TA			
ECP79F	F 5' GAA GCT TGC TTC TTT GCT	54	541	Sabat *et al.*, 2000
ECR620R	R 5' GAG CCC GGG GAT TTC ACA T			
616F	5'-AGAGTTTGATYMTGGCTCAG	52		Ehrmann *et al.*,
630R	5'-CAKAAAGGAGGTGATCC			2003
RpoH2F	5'-ATTCCTACATCCGGGCAGCTAACG	70	771	This study.
RpoH2R	5'-TTTTTTCATCGCGTTCTTTTCCAGC			
M-13 core	5' GAG GGT GGC GGT TCT	35; 50, 45, 53		Vogel *et al.*,
				2000a
DAF 4	5' CGG CAG CGC C	35; 45		Vogel *et al.*,
				2000b
1254	5' CCG CAG CCA A	36; 50		Aslam *et al.*, 2003
BOX A1R	5' CTA CGG CAA GGC GAC GCT GAC G	66		Goldberg *et al.*,
				2006
VT1	5'-ACACTGGATGATCTCAGTGG	55	614	Gannon *et al.*,
VT2	5'-CTGAATCCCCCTCCATTATG			1992.
VT3	5'-CCATGACAACGGACAGCAGTT	55	779	
VT4	5'-CCTGTCAACTGAGCACTTTG			

The thermal cycling program for ECA75F -ECR 619R primers was carried out as follows: primary denaturation at 94°C for 2 min; 40 cycles: denaturation at 94°C for 45 sec, annealing/extension at 72°C for 45 sec; final extension at 72°C for 10 min.

The thermal cycling program for ECP79F-ECR620R was optimized with a focus on annealing temperature and number of cycles. The optimal conditions included an initial heating step at 94°C for 5 min, followed by 35 cycles of: 94°C for 45 s, 54°C for 45 s, 72°C for 1.5 min, final extention at 72°C for 5 min. Analysis for shiga toxin genes *stx*1 and *stx*2 was carried out as described by Gannon *et al.*(1992): heating step at 94°C for 5 min, followed by 35 cycles of: 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, final extention at 72°C for 7 min.

2.3.3 Random Amplified Polymorphic DNA analysis.

Three primers were chosen for RAPD analysis (See Table II). For the 1254 decamer primer PCR amplification was performed as follows: 2 cycles at 94°C for 5 min, 36°C for 5 min, 72°C for 5 min; 10 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 2 min; 20 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; and extension at 72°C for 7 min. For the DAF 4 primer, the amplification protocol was optimized and the following protocol gave the optimal result: 94°C for 3 min; 3 cycles at 94°C for 5 min, at 35°C for 5 min, at 72°C for 5 min; 32 cycles at 94°C for 30 s, at 45°C for 2 min, at 72°C for 3 min; final extension at 72°C for 7 min. For the M13 - core primer the PCR reactions were carried out as follows: 94°C for 3 min; 3 cycles at 94°C for 3 min, at 32°C for 5 min, at

72°C for 5 min; 35 cycles at 94°C for 30 s, at 45°C (50°C or 53°C) for 2 min, at 72°C for 3 min; final extension at 72°C for 7 min.

To confirm stability of the DNA and reproducibility of RAPD patterns, PCR amplification was performed with DNA from the same strains isolated at two different times six month apart after several culture passages. All RAPD PCR amplifications were performed in the same thermocycler machine to enhance reproducibility of RAPD patterns. For RAPD PCR reactions *GoTaq* Flexi DNA polymerase (Promega, Madison, WI, USA) was used as it yielded RAPD patterns of much higher quality compared to Invitrogen *Taq* Polymerase (Fig. 1). For the *GoTaq* polymerase 50 µl of master mix

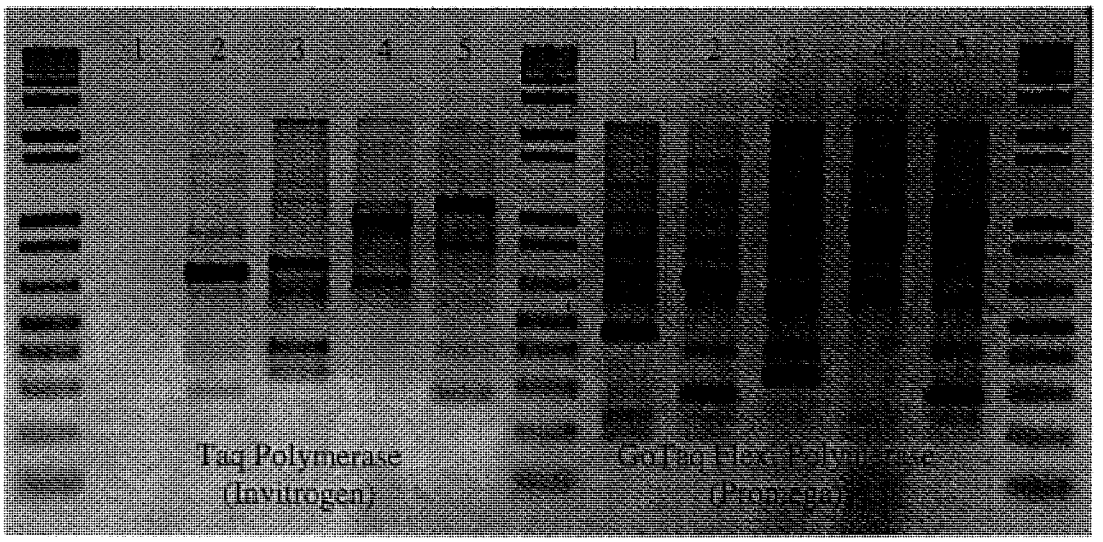

Fig. 1. RAPD patterns with the DAF4 primer obtained in simultaneous PCR with either Taq polymerase (Invitrogen) or GoTaq polymerase (Promega). Lanes: 1 - MB 2.1; 2 - AW 1.7; 3 - GM 11.5; 4 - GM 18.6; 5 - DM 18.3.

contained 1 µl of template DNA, 10 µl of 5x buffer, 3 µl (1.5 mM) of $MgCl_2$, 1 µl (200 µM) of dNTPs, 1.5 µl (150 pMol) of primer, 0.25 µl (1.25 U) of *GoTaq* Flexi DNA

polymerase, and 33.25 µl of MQ H_2O. Amplified DNA fragments were separated in 1.5% agarose gels (80V for 2 hours) and stained with ethidium bromide for 30 min.

Gels were scanned and digitally recorded. After conversion and normalization with Adobe PhotoShop Elements 2.0, patterns were downloaded into the pattern analysis software package BioNumerics™ 3.0 (Applied Maths, Kortrijk, Belgium) for image processing. Pearson correlation dendrogram comparisons were calculated by the software and were based on the number and position of bands. Patterns with similarity of 90% or higher were considered to belong to the same strain.

2.3.4 Repetitive element PCR.

Repetitive element PCR (Rep - PCR) is based on amplification of repetitive DNA sequences in the bacterial chromosome. Rep - PCR reactions were carried out in 25 µl of master mix, containing 2.5 µl (1x) buffer, 1 µl (1mM) $MgCl_2$, 0.2 µl (1 U) *Taq* Polymerase, 0.5 µl (100 mM each) dNTPs (Invitrogen), 0.5 µl (50 pMol) of BOX A1R primer (Goldberg et al., 2006; see Table II), 1µl of template DNA, and 19.3 µl of sterilized MQ water. Separation of amplified fragments was carried out the same way as described for RAPD PCR.

2.3.5 Sequencing.

PCR for sequencing purposes was performed in total volume of 150 µl (3 x 50 µl). The reaction mixture (50 µl) contained: 1x *Pfu* buffer (*Pfu* Turbo polymerase, Stratagene, La Jolla, CA, USA), primers (30 pMol each), 200 mM each of dNTPs, 0.3 µl of Taq/Pfu Polymerases mixture (4:1), 1 µl of template DNA, and MQ water to 50 µl. PCR products

were purified using QIAquick PCR Purification Kit (Qiagen) and sent to the DNA Core Services Lab (Dept. of Biochemistry, University of Alberta) for sequencing.

Primers used for sequencing are listed in Table II. For 16S rDNA sequencing, primers 616F and 630R were used. Primers RpoH2F and RpoH2R were designed with GeneTool 2.0 (BioTools Inc., Edmonton, AB, Canada).

2.3.6 Sequence analysis.

Chromatograms of all sequences were verified and sequences were corrected if needed using GeneTool 2.0. Gene sequences of the 16S rRNA were analyzed with Basic Local Alignment Search Tool (BLAST http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and bacterial isolates were identified to the species level. Sequences of *rpo*H gene were aligned and compared with CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Nucleotide sequences were translated into protein sequences with ExPASy Translate Tool (http://us.expasy.org/tools/dna.html) and then aligned and compared with CLUSTALW2.

2.4 Survival of *E. coli* after decontamination interventions.

Based on comparisons of RAPD patterns, a total of 6 cocktails of strains of *E. coli* were created. Each cocktail contained 5 strains of *E. coli* with easily distinguishable RAPD patterns using DAF4 as the primer.

To create cocktails, strains were incubated overnight in LB broth at 37°C with shaking at 120 rpm. The OD_{600} of each culture was measured (Ultrospec 100 PRO, Biochrom Ltd., Cambridge, UK) and a proportional aliquot of each strain was calculated to obtain

equal density of cells for each strain in a cocktail. To confirm recovery of all strains, proportional aliquots of strains were mixed together, then 100 µl of the cocktail was serially diluted 1:10 to 10^{-6} in sterile buffered peptone water (1 g peptone, 1 ml of 0.15 M NaOH, 1L H_20) and plated onto LB agar. After 18 h of incubation DNA from randomly chosen colonies was isolated and RAPD PCR was performed as described above. Experiments were run in duplicate. If all strains are present in the cocktail in equal cell counts, the likelihood of not picking a specific strain is 4/5. After 13 consecutive isolations, the likelihood of not picking a specific strain was $(4/5)^{13}$ or 0.055 (5.5%). Twenty six isolates were obtained from each series of the experiment: 13 from each LB agar and Endo agar.

In vivo experiments were conducted as shown in Fig. 2. Beef samples (4.9 cm^2) were prepared as described by Pierce (2008). Experiments were carried out as follows: 100 µl of a cocktail of *E. coli* was spread onto the surface of an aseptically prepared meat sample (Pierce, 2008) and allowed to dry at room temperature for 15 min. Samples were steamed at 98°C for either 8 or 15 s, cooled for 1 min and sprayed with 2 or 4.5% (v/v) lactic acid. Samples were held at room temperature for 5 min and then stomached (Stomacher Lab-Blender 400, Seward, Brinkman Instruments, Worthing, UK) for 1 min in 90 ml of buffered peptone water and kept on ice less than 15 min before making 1:10 serial dilutions in buffered peptone water and plating. Control samples were not subjected to steam or lactic acid treatments and were kept on ice prior plating.

Samples were plated onto LB agar to count all surviving cells and Endo agar to count cells that were not sublethally injured. Experiments were run in quadruplicate. Inoculated

agar plates were incubated overnight at 37°C. Statistical analysis was performed with Sigma Plot software by using Student's t-test.

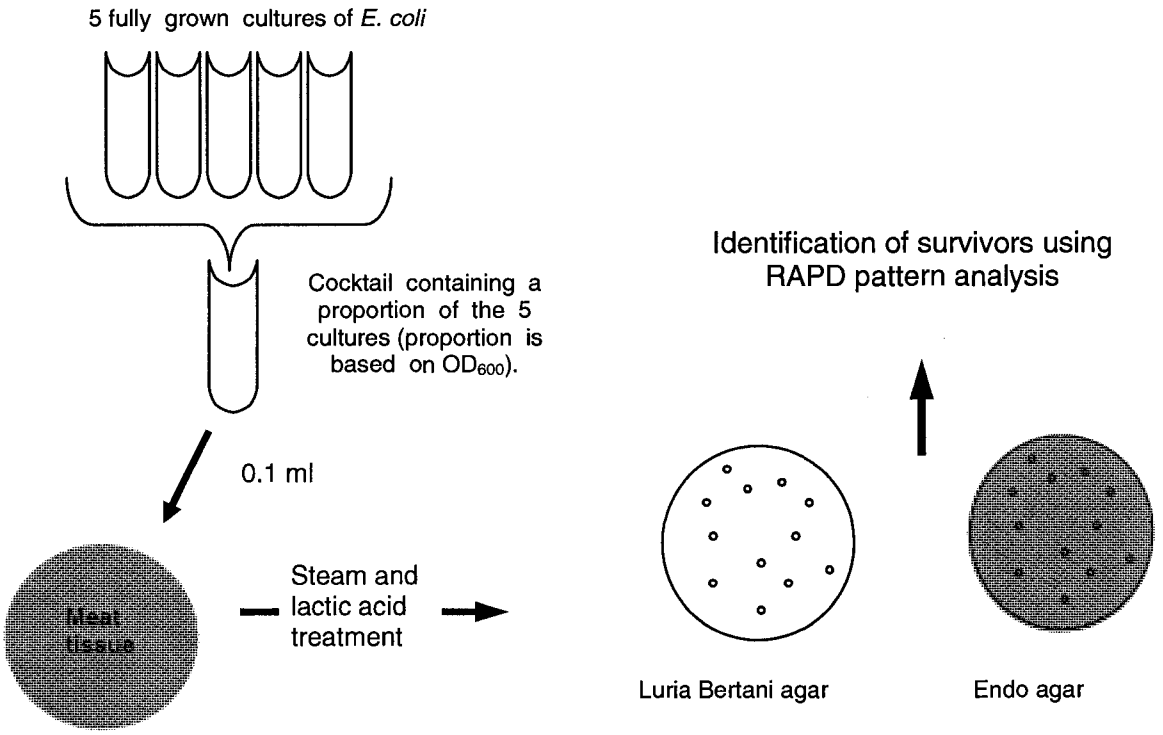

Fig. 2. Flow diagram of the experiment to determine the survival of *E. coli* exposed to steam and lactic acid treatments.

2.5 Thermal inactivation of *E. coli* on meat.

2.5.1 Primary evaluation of thermal resistance.

The goal of this experiment was to choose a heat susceptible and a heat resistant strain out of 10 strains of *E. coli* (MB 2.1; MB 3.4; AW 1.3; AW 1.7; GM 11.5; GM 11.9; GM 16.7.2; GM 18.4; GM 18.6; DM 18.3) for further experiments. Three 200 µl aliquots of overnight culture for each strain were placed into 1.5 ml Eppendorf tubes (Thermo Fisher Scientific, Edmonton, AB) and heated in dry bath incubator (Thermo Fisher Scientific) at

58°C. Eppendorf tubes were removed after 5, 15, and 30 min of incubation and kept on ice before plating. Serial dilutions in buffered peptone water were plated in triplicate onto LB agar and Endo agar, plates were incubated at 37°C for 18 h and cell counts were calculated. Experiments were run in duplicate.

2.5.2 Effect of heat on survival of *E. coli* strains AW 1.7 and DM 18.3 on lean meat, fat, and in liquid media.

Two strains of *E. coli* AW1.7 and DM18.3 were chosen from the previous experiment as the most heat resistant and one of the most heat susceptible strains among slaughter plant isolates, respectively.

For heat treatment on tissue, overnight cultures of each strain were inoculated onto four pieces of aseptically prepared lean and four pieces of aseptically prepared adipose tissues for each strain. Meat tissue samples were prepared as described by Pierce (2008). Each piece of meat was inoculated with 100 µl of the culture and air dried at room temperature for 15 min prior to heating. For heat treatment in liquid media, 1 ml of culture (four samples for each strain) was centrifuged at 10,600 x *g* for 10 min. The supernatant was discarded and pellet was resuspended in an equivalent volume of fresh LB broth or Meat Extract. Meat extract was prepared as follows: 1 part of ground beef + 2 parts of distilled water (w/v) was stirred overnight on magnetic stirrer at 4°C, centrifuged at 6,800 x *g* for 10 minutes, and the supernatant was filter sterilized through a 0.22 micrometer syringe filter (Fisher Scientific). All meat, fat and liquid samples were placed into sterile bags (Nasco Whirl Pak, Fort Atkinson, WI, USA) and the air was squeezed out prior to closing with metal tabs. Bags were fully submerged in a water bath

at 60 °C. Control samples were not subjected to heat (0 min). Samples were removed after 5, 15 and 30 min and kept on ice before plating as described above. The experiment was run in duplicate and all samples were plated in triplicate.

2.5.3 Determination of D and Z values.

To determine the D_{60} of strains of *E. coli*, overnight cultures of *E. coli* AW 1.7, DM 18.3, K-12, GGG10, FUA 1041, and FUA 1044, grown in LB broth, were aliquoted into sterile bags (1 ml of culture per bag per each sampling time) and exposed to heat in hot water bath as described in the previous experiment. Samples were kept in ice prior plating. Serial dilutions were plated in triplicate. The experiment was run in duplicate.

For *E. coli* AW1.7, D_{65} and D_{70} were determined. Heat survival curves were plotted and linear regressions were calculated by Microsoft Excel 7.0. All correlation coefficients were > 0.9. D-values were calculated as negative reciprocals of the slopes (D=-1/a).

The Z-value of *E. coli* AW1.7 was estimated from the thermal destruction curve (D-value versus temperature) in a logarithmic scale. The regression line was calculated by Microsoft Excel 7.0 ($r^2 = 0.91$).

2.6. Expression of stress response proteins.

To determine the levels of expression of heat stress proteins DnaK, σ^E and σ^{32} in *E. coli* AW 1.7 and GGG10 after heat stress, Western blot analysis (Cseke et al., 2004) was performed.

For this experiment 50 ml each of overnight cultures were centrifuged at 6,800 x *g* for 10 min. Approximately 45 ml of the supernatant was placed into sterile 100 ml flasks with a magnetic stirrer. The flasks were submerged into a water bath at 37°C (control) or 46°C. The pellet of bacterial biomass was resuspended in the remaining supernatant in the same centrifuge tube and kept at 37°C with shaking until required temperature of supernatant in flasks was reached (about 40 minutes). Once the supernatant reached the required temperature, bacterial suspensions were added to the prewarmed supernatant. Samples were removed after 10 sec, 5 and 15 min. For each sample, 10 µl of the bacterial suspension was immediately added to 50 µl of SDS-PAGE loading buffer (2.5 ml 1M Tris buffer, pH 6.8; 3 ml 20% sodium dodecyl sulfate (SDS); 2ml glycerol; 50 mg bromophenol; distilled water to make 9 ml; 1 ml β-mercaptoethanol, added *ex tempore*) and the sample was denatured at 95°C for 7 min. Another 100 µl was placed into an Eppendorf tube for subsequent OD_{600} measurement and kept on ice prior the measurement. Samples were loaded into 10% SDS-PAGE gel (Resolving gel: 4.9 ml distilled water; 2.5 ml 40% bis-acrylamide; 2.5 ml 1.5M Tris buffer, pH 8.8; 0.1 ml 10% SDS. Stacking gel: 2.45 ml distilled water; 1.25 ml 40% bis-acrylamide; 1.25 ml 0.5 Tris buffer, pH 6.8; 0.05 ml 10% SDS. ammonium persulfate (50 µl of 10% solution) and 7 µl of tetramethylethylenediamine (TEMED) were added to initiate polymerization) and the amount of sample was chosen to represent equal cell density of bacterial suspension. It was calculated based on OD_{600}. Recombinant σ^E protein (Neoclone, Madison, WI, USA) was used as a positive control. Proteins in gels were visualized by staining in Coumassie Blue (0.3% Coumassi Brilliant Blue 250; 50% methanol; 10% acetic acid; 40% distilled water). Electrophoresis was carried out with a Mini Gel system (BioRad) at 110V for

approximately 40 min. Electrode buffer was prepared as follows: 30.3 g Tris base, 144.0 g glycine, 10.0g SDS, distilled water was added to make 10 L.

After electrophoretic separation, proteins were transferred from the gel onto a nitrocellulose membrane using the following protocol:

1. Nitrocellulose membrane (BioRad) was presoaked in Transfer buffer (Tris Base - 5.8 g, glycine - 2.9 g, methanol – 200 ml, distilled water – 800 ml) for 2 to 10 min

2. A "sandwich" of a piece of filter paper (BioRad), membrane and the gel was assembled in a container filled with transfer buffer. After putting the membrane onto the gel it was squeezed with a piece of plastic pipette or special roller to remove all air bubbles and the membrane was allowed to stick to the gel. A second piece of filter paper was put on the membrane and rolling was repeated.

3. After the sandwich was assembled, it was transferred from the assembly container onto the cassette, making sure the gel faced the black side of the cassette.

4. A Protein Transfer Unit (BioRad) was assembled as described by the manufacturer, filled with transfer buffer, and a magnetic stirrer was added.

5. Proteins were transferred overnight from the gel to the membrane at 30V at 4°C with constant slow stirring.

After transfer the edges of the membrane were trimmed to the size of the gel. The gel was discarded and membrane was rinsed in PBST buffer (PBS + 0.1% Tween 20) three

times for 7 min. Phosphate buffered saline (PBS) was prepared as follows: 10.9 g Na_2HPO_4 (anhydrous); 3.2 g NaH_2PO_4 (anhydrous); 90 g NaCl; 10 L distilled water; pH adjusted to 7.2.

After rinsing, the membranes were blocked in 5% solution of skim milk in PBST for 1 h at room temperature to prevent nonspecific binding of antibodies to the membrane.

The milk solution was discarded and primary anti-DnaK antibodies (EMD Chemicals, PA, USA) or anti-σ^E antibodies 1RE53 (Neoclone) were added. Antibodies were diluted (1:3000 for DnaK and 1:1000 for σ^E) in 5% milk-PBST (blocking) solution. The membranes were incubated for 2 h at room temperature and washed three times for 7 min in PBST. The secondary Goat-antimouse (Invitrogen) antibody was diluted 1:5000 in blocking solution and added to the membranes. Membranes were incubated for 1 h and washed three times for 7 min in PBST.

Membranes were developed with two developing solutions, mixed in equal proportions (Solution 1: 1 ml of 250 mM luminol in DMSO; 0.44 ml of 90 mM p-coumaric acid in DMSO, 10 ml of 1M Tris-HCl pH 8.5; distilled water up to 100 ml. Solution 2: 64 µl of 30% hydrogen peroxide; 10 ml of 1 M Tris-HCl pH 8.5; distilled water up to 100 ml) for 2 min. Images were taken on X-ray film (Fuji, Japan).

If needed, membranes were stripped with a special buffer (0.2 M glycine; 0.1% SDS; 300 ml distilled water; pH adjusted to 2.2 with HCl; Tween 20 was added *ex tempore* to the final concentration of 1%) for at least 2 h and blotted again.

2.7 Ability of *E. coli* to form biofilms.

E. coli AW1.7, GGG10, and K-12 were inoculated into test tubes with LB broth containing stainless steel coupons, prepared as described by Kovacevic (2007). Tubes were incubated at 37°C with shaking for 72 h. Every 24 h 9 ml of LB broth was removed and replaced with a fresh portion of LB broth. After incubation stainless steel coupons were placed into small Petri dishes and gently rinsed with filter sterilized 0.9% saline 3 times for 1 min.

Coupons were stained with LIVE/DEAD Bac Light Bacterial Viability Kit (Invitrogen), which contains the green-fluorescent SYTO® 9 stain and the red-fluorescent propidium iodide stain. Coupons were exposed to the stain for 15 min (approx. 100 µl of dye per coupon) and gently rinsed in saline. The coupons were examined under a fluorescent microscope (Carl Zeiss Inc., Germany) using Axio-Vision 4.6 software (Carl Zeiss) for data capture.

3. RESULTS

3.1 Confirmation of identity of strains of *E. coli.*

Of the 64 cultures of presumptive *E. coli* received from Agriculture and Agri-Food Canada, 59 were pure, one did not grow, and 4 cultures contained more than one strain. After streaking to check the purity of the cultures a total of 67 strains were isolated. Based on observation of growth on Endo and TSI agars, 57 of the strains were identified as *E. coli*. Nine cultures were identified as non - *E. coli* enterobacteria as they fermented glucose and were lactose and sucrose negative on TSI agar. Four strains were Gram-negative and unable to ferment glucose; further identification of these strains was not performed.

Strains of *E. coli* isolated from rectal samples of cows were grown on Endo agar. Ten isolates that fermented lactose and had a characteristic green shine were inoculated into TSI agar, where they gave a typical response for *E. coli*. They were confirmed to be *E. coli* by PCR with species specific primers.

The main problem of identification of generic *E. coli* is the differentiation between *Escherichia coli* and *Shigella* species. Many researchers have shown that *Shigella* spp. and *E. coli* to belong to the same species based on their DNA relatedness (Paradis *et al.*, 2005; Lan and Reeves, 2002; Lai *et al.,* 1998; Fukushima *et al.*, 2002; Goris *et al.*, 2007). To ensure that the strains used in this study were not *Shigella* spp., PCR amplification was carried out with three pairs of species-specific primers. PCR reactions with primers ECA75F and ECR619R were not sensitive enough to differentiate the presumptive *E. coli* from *Shigella* spp., as the positive control did not give a band (Fig. 3a). Primers based on

*usp*A yielded double bands (Fig. 3b) and did not distinguish between *E. coli* Top 10 and *S. sonnei* ATCC 25931. The primers ECP79F-ECR620R did not yield bands in case of *S. sonnei* and *S. flexneri*, but gave a band when DNA from *S. dysenteriae* was used as a template (Fig. 4). Humans are known to be the only reservoir for *Shigella* (Niyogi, 2005; Sureshbabu, 2006). In this study all strains were isolated from non-human sources, therefore all PCR positive strains were considered to be *E. coli*. Three out of six strains biochemically identified as non - *E. coli* also gave negative results with *usp*A based primers. The other three strains were positive and were later confirmed as *E. coli* by sequencing of 16S rDNA.

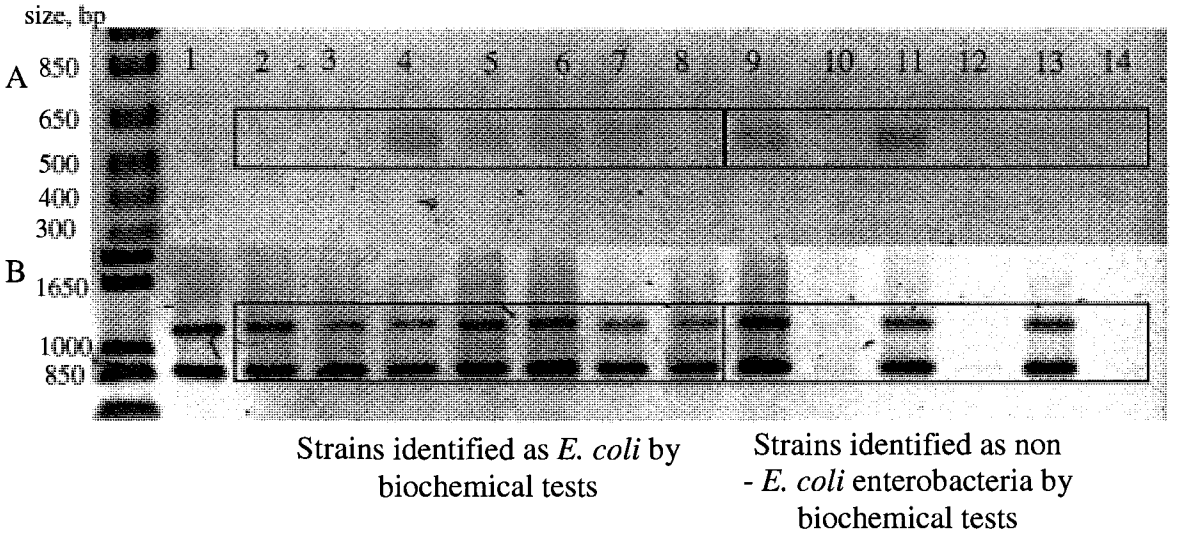

Fig. 3. Results of species specific PCR. A - with ECF75-ECR619 primers. Expected size of PCR product - 544 base pairs. B - with *usp*A - based pair of primers. . Expected size of PCR product - 844 base pairs. DNA from the following strains was used as a template: 1- *E. coli* Top10; 2 - *S. sonnei* ATCC 25931; 3 - GM 9.1; 4 - GM 11.3; 5 - MB 2.1; 6 - MB 3.4; 7 - MB 16.6; 8 - DM 18.3; 9 - MB 1.8; 10 - DM 19.1.2; 11 - GM 16.7.2; 12 - GM 9.3; 13 - AW 1.3; 14 - GM 11.1.

Sequencing of 16S rDNA of 10 strains (Lanes 5 to 14 on Fig. 4) confirmed the results of PCR. Strains MB 2.1, MB 3.4, MB 16.6, DM 18.3, MB 1.8, GM 16.7.2 and AW 1.3 were identified as *E. coli,* strain DM 19.1.2 was identified as *Pantoea agglomerans*,

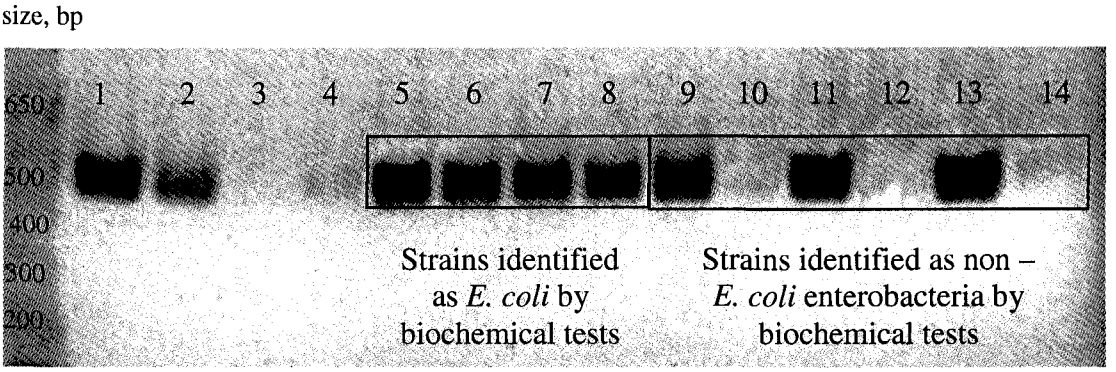

Fig. 4. Results of species specific PCR with ECF79-ECR620 primers. Expected size of the PCR product – 541 base pairs. DNA from the following strains was used as a template: 1 – *E. coli* ATCC 25922; 2 – *S. dysenteriae* A 188; 3 – *S. flexneri* A 62; 4 – *S. sonnei* ATCC 25931; 5 – *E. coli* MB 2.1; 6 – *E. coli* MB 3.4; 7 - *E. coli* MB 16.6; 8 - *E. coli* DM 18.3; 9 - *E. coli* MB 1.8, 10 - *E. coli* DM 19.1.2; 11 - *E. coli* GM 16.7.2; 12 - *E. coli* GM 9.3; 13 - *E. coli* AW 1.3; 14 - *E. coli* GM 11.1.

strain GM 9.3 was identified as *Enterobacter* sp., and strain GM 11.1 was identified as *Enterobacter cloacae*. A total of 50 strains (44 identified as *E. coli* and 6 identified as enterobacteria other than *E. coli* by phenotype based methods), were tested with ECP79F-ECR620R primers, of which 47 were positive (Table I). Strains of *E. coli* isolated from live cows were also tested for verotoxin genes *stx*1 and *stx*2. One strain (FUA1041) was positive for *stx*2 (Fig 5). None of these strains demonstrated haemolysis on 5% sheep blood agar (Data not shown).

3.2 RAPD analysis.

RAPD analysis of strains of *E. coli* was a useful tool in this study to monitor survival of individual strains of *E. coli* on meat inoculated with a cocktail of strains in intervention treatments. It was highly discriminative and reproducible with the primer DAF4 and reproducible but less discriminative with the primer 1254. As shown in Fig. 6, the RAPD patterns obtained with DAF4 were the same for DNA isolated in June, 2006 and kept frozen at -20°C until analysis 6 month later and for DNA isolated in November, 2006

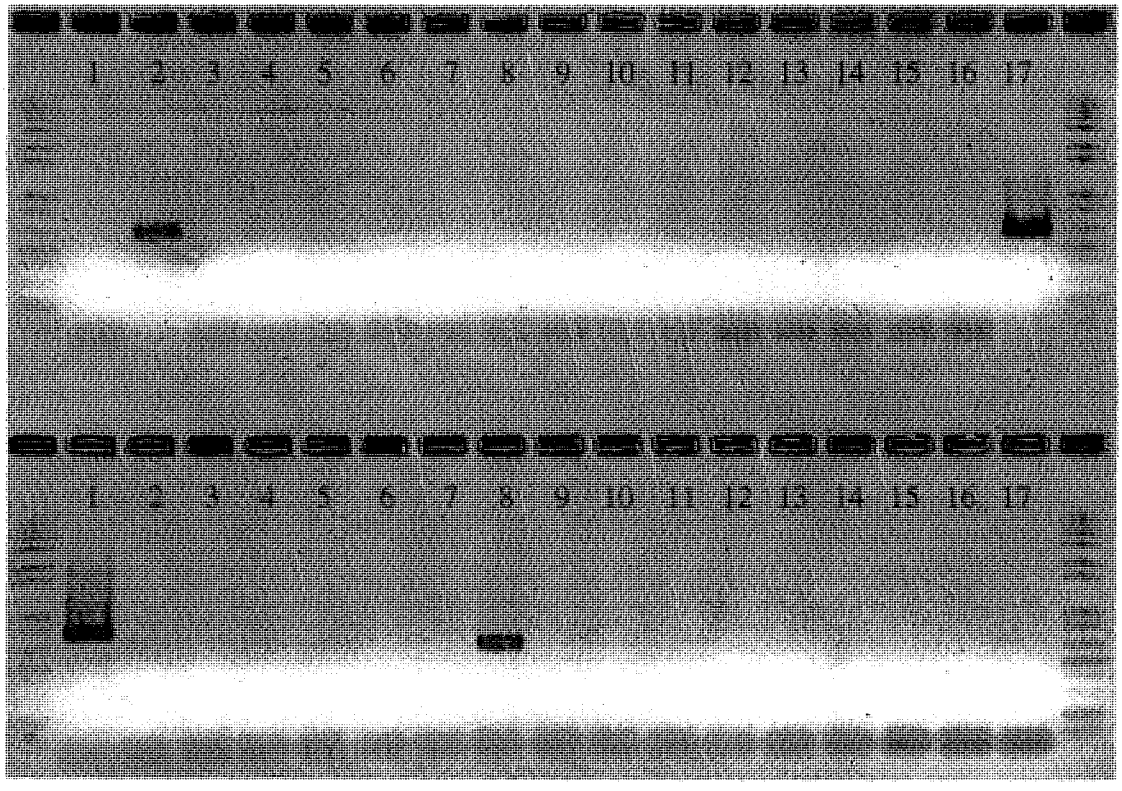

Fig. 5. Results of PCR analysis for *stx*1 (A) and *stx*2 (B). The size of the PCR product for *stx*1 is 614 bp and for *stx*2 is 779 bp. Lanes: 1 - *E. coli* O157:H7 ATCC 43889 (positive control for *stx*2); 2 - *E. coli* O157:H7 ATCC 43890 (positive control for *stx1*); 3 - *E. coli* AW 1.7; 4 - *E. coli* GGG10; 5 - *E. coli* FUA 1050; 6 - *E. coli* FUA 1051; 7 - *E. coli* FUA 1040; 8 - *E. coli* FUA 1041; 9 - *E. coli* FUA 1042; 10 - *E. coli* FUA 1043; 11 - *E. coli* FUA 1044; 12 - *E. coli* FUA 1045; 13 - *E. coli* FUA 1046; 14 - *E. coli* FUA 1047; 15 - *E. coli* FUA 1048; 16 - *E. coli* FUA 1049; 17 - *S. dysenteriae* A188.

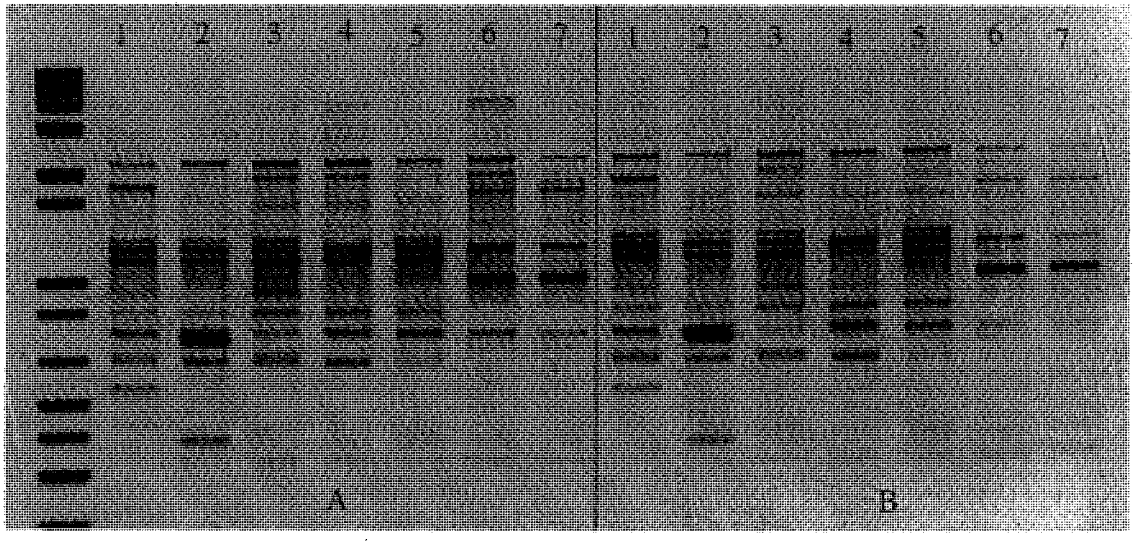

Fig. 6 Reproducibility of RAPD patterns, obtained with DNA isolated in June 2006 and kept at -20°C (A) and in November 2006 after numerous passages of *E. coli* cultures (B). Primer DAF4. Lanes: 1 - AW 1.3; 2 - GM 16.7.2; 3 - MB 1.8; 4 - GM 11.9; 5 - MB 16.6; 6 - MB 3.4; 7 - GM 18.4.

after several passages of the same strains. The primer M-13 core did not yield any satisfactory results in this study (data not shown).

The RAPD patterns of the strains of *E. coli* were analyzed with BioNumerics 3.0 software and a dendrogram was constructed. Similarity among all of the RAPD patterns obtained with the DAF4 primer of the *E. coli* isolates was less than 90% (Fig. 7a). This level of similarity was used to discriminate between strains of *E. coli*. The patterns obtained with the 1254 primer were divided into 4 clusters of isolates with the pattern similarity more than 90% among each cluster (Fig. 7b). Based on these data, cocktails of strains with distinctive DAF4 RAPD patterns were created for intervention experiments (a total of 6 cocktails). RAPD primer DAF 4 was used to identify survivors in all intervention experiments. If needed, primer 1254 was used as a secondary primer to distinguish between isolates with similar DAF4 patterns.

The attempt to use REP-PCR for subtyping of *E. coli* isolates was not as successful as RAPD. REP-PCR did not yield patterns of acceptable quality for strain identification (Fig. 8).

3.3 Survival of *E. coli* after intervention treatments on meat.

The first series of experiments were conducted under conditions simulating those used in slaughter plant practices: 8 sec steam and subsequent spray with 2% lactic acid (Gill and Bryant, 2000). The decrease in cell counts observed was 0.8 to 1.5 log. All strains in the cocktails survived the interventions (Fig. 9 and Table III). The same results were obtained when samples were first treated with lactic acid and then with steam (data not shown). Since these treatments did not reduce cell counts of four cocktails that consisted

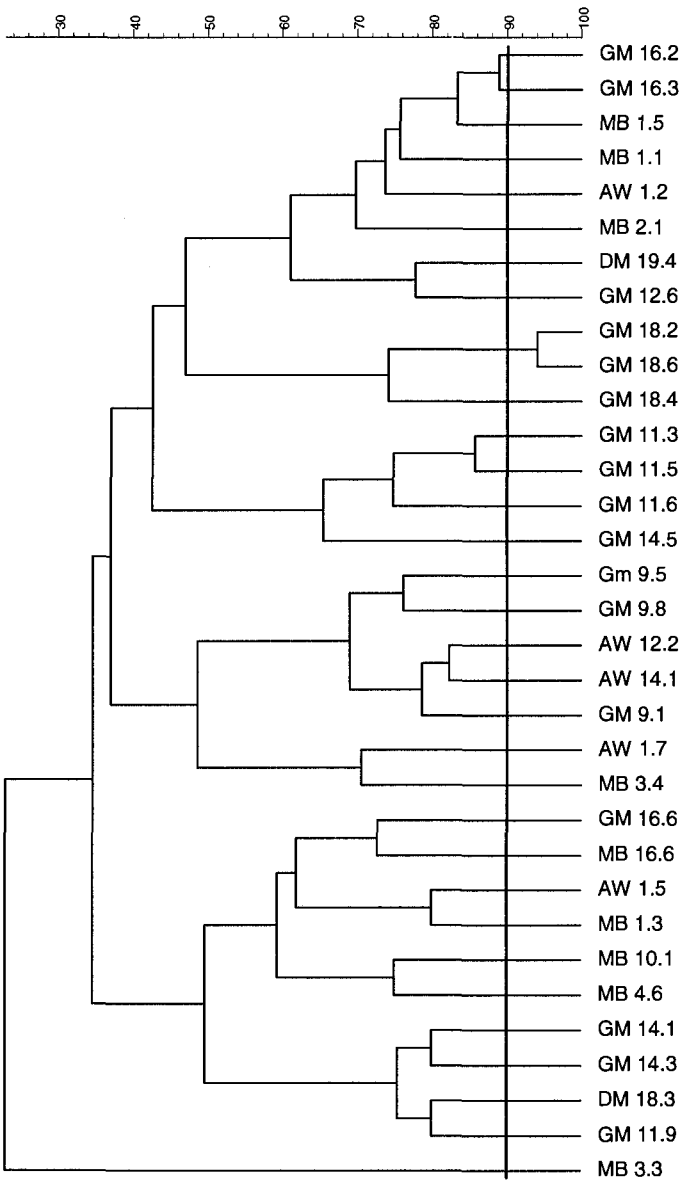

Fig. 7a. Pearson correlation comparison of *E. coli* strains based on their DAF4 RAPD patterns. Similarity of independent patterns obtained for the same strain were 90% or higher. Therefore, isolates with similarity less than 90% were considered to belong to different strains.

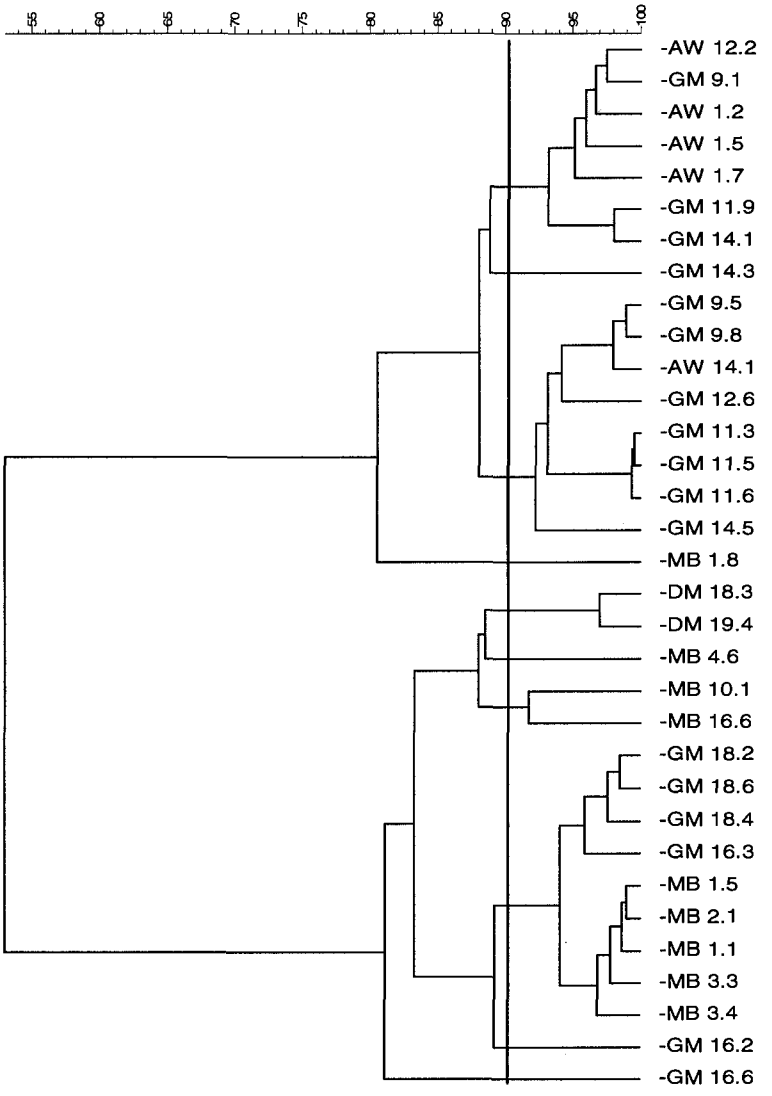

Fig. 7b. Pearson correlation comparison of *E. coli* strains based on their RAPD 1254 patterns. Similarity of independent patterns obtained for the same strain were 90% or higher. Therefore, isolates with similarity less than 90% were considered to belong to different strains.

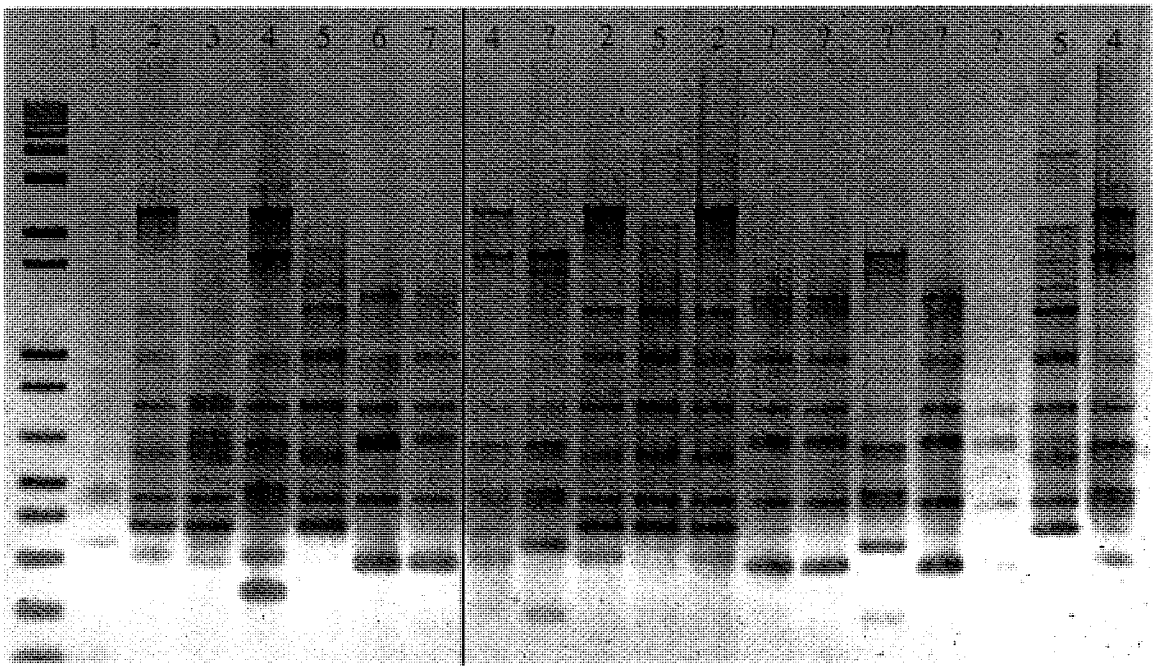

Fig. 8. Subtyping of *E. coli* strains by REP-PCR (lanes 1-7 on the left) and identification of strains isolated from the strain cocktail (on the right). Lanes: 1 - AW 1.3; 2 - GM 16.7.2; 3 - MB 1.8; 4 - GM 11.9; 5 - MB 16.6; 6 - MB 3.4; 7 - GM 18.4.

of slaughter plant isolates of *E. coli* on lean meat samples, intensity of the treatment was increased. Cocktails #1 and #2 were chosen for further experiments, where steaming for 15 sec and subsequent spray with 4.5% lactic acid were applied. Cell counts of cocktails #1 and #2 decreased about 1 log. Sublethal injury was not observed, as cell counts on LB agar (where all survivors will grow) and on Endo agar (where sublethally injured cells do not grow) were the same. All strains in cocktails #1 and #2 survived the interventions (Fig. 10 and Table III). Cocktails #5 and #6, which consisted of isolates from live cows and collection strains were subjected to the same intervention treatment the cell counts decreased approximately 2 logs. No sublethal injury was detected as no statistically confident difference between cell counts on LB agar and cell counts on Endo agar was determined (Fig. 11). Only one strain *E. coli* ATCC 25922, did not survive the

Table III. Incidence of recovery of strains of *E. coli* recovered from lean meat tissue after steam and lactic acid treatments.

Cocktail #	Strains in the cocktail	Treatment: 8" steam and 2% lactic acid — Incidence of recovery of each of the strains		Cocktail #	Strains in the cocktail	Treatment: 15" steam and 4.5% lactic acid — Incidence of recovery of each of the strains	
		LB agar (n=13)	Endo agar (n=13)			LB agar (n=13)	Endo agar (n=13)
#1	MB 3.4	2	2	#1	MB 3.4	3	1
	AW 1.3	2	3		AW 1.3	3	5
	GM 11.9	4	5		GM 11.9	2	1
	GM 16.7.2	2	1		GM 16.7.2	1	2
	GM 18.4	3	1		GM 18.4	4	4
#2	MB 2.1	2	1	#2	MB 2.1	1	2
	AW 1.7	1	4		AW 1.7	1	5
	GM 11.5	1	3		GM 11.5	1	1
	GM 18.6	4	2		GM 18.6	6	4
	GM 18.3	5	2		GM 18.3	4	1
#3	GM 9.5	3	1	#5[a]	ATCC	0	0
	GM 11.3	5	4		25922		
	GM 12.6	1	2		GGG10	2	3
	GM 16.2	2	1		FUA 1050	6	5
	GM 16.6	2	5		FUA 1051	3	3
#4	MB 1.3	1	4	#6	FUA 1040	3	1
	MB 3.3	2	2		FUA 1041	3	4
	MB 10.1	3	4		FUA 1044	1	1
	GM 9.8	3	2		FUA 1045	2	4
	AW 12.2	4	1		FUA 1048	4	3

[a]) for cocktail #5 n=11

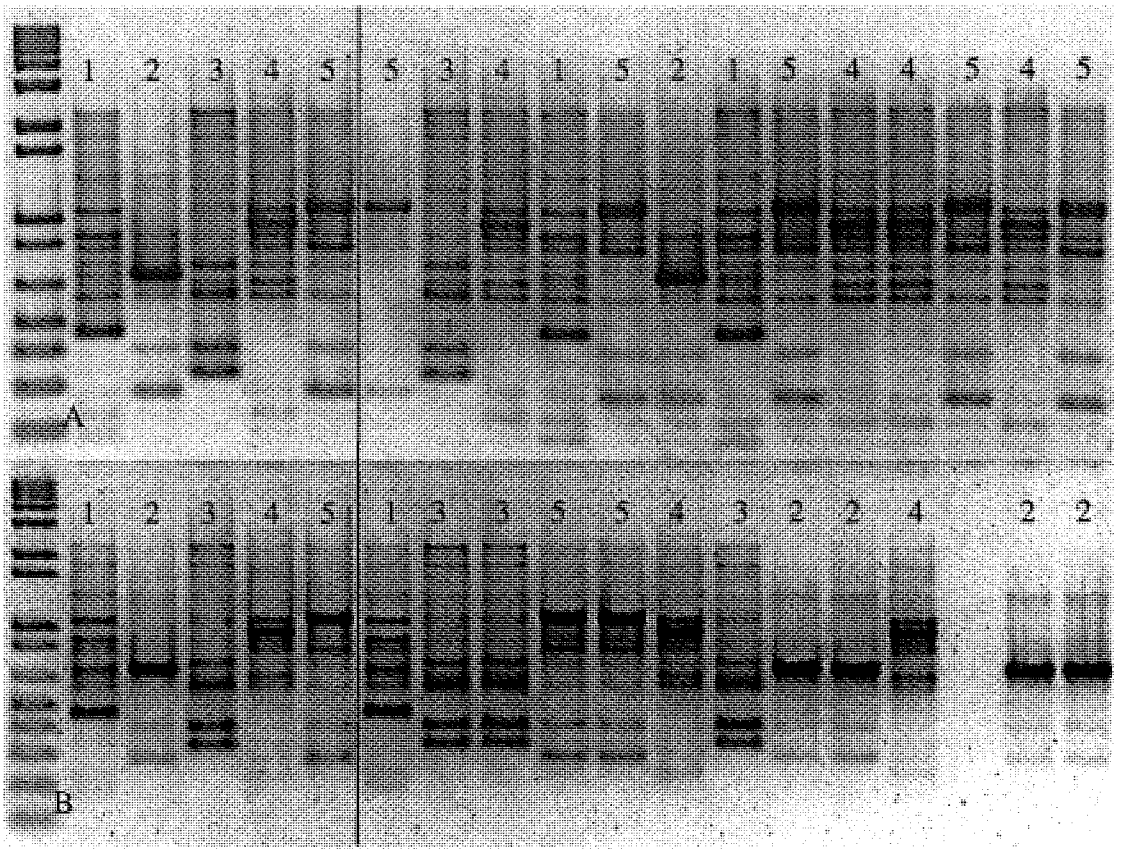

Fig. 9. RAPD patterns of *E. coli* strains recovered from cocktail #2 inoculated onto meat and subjected to 8" steam and 2% lactic acid treatment. Patterns were obtained with the DAF4 primer. Lanes 1 - 5, reference strains: 1 - MB 2.1; 2 - AW 1.7; 3 - GM 11.5; 4 - GM 18.6; 5 - DM 18.3. Isolates recovered after the intervention treatments from LB agar (Panel A) or Endo agar (Panel B) are depicted on the right side.

intervention (Table III). Reduction of cell counts was higher ($p \leq 0.075$) in cocktails of isolates obtained from live animals, compared to cocktails of strains obtained from the slaughter plant. No significant difference was found between these two types of cocktails composed of strains from a slaughter plant or from live animals (Fig. 11).

3.4 Heat resistance of strains of *E. coli*.

Because the steam treatment did not reveal a decrease in numbers of *E. coli* more than one to two orders of magnitude, it was assumed that the heat resistance of strains could be one of the mechanisms of their survival. A preliminary evaluation of the heat tolerance

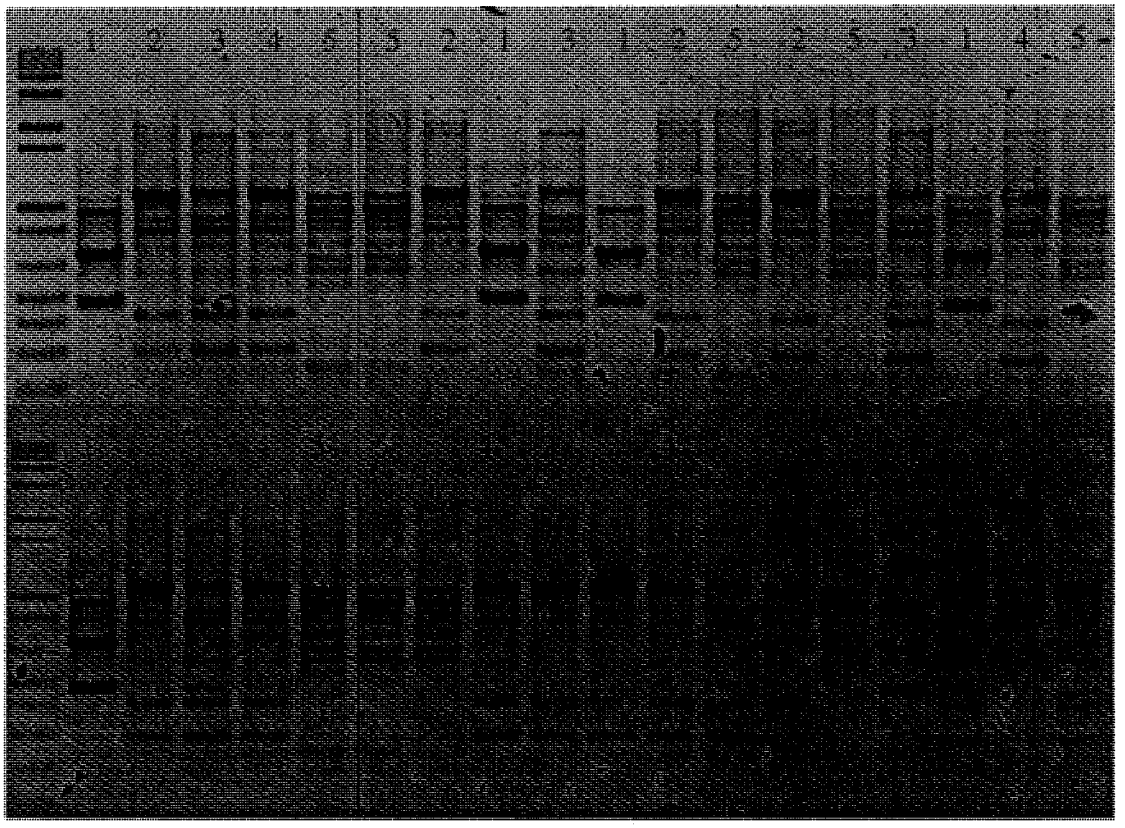

Fig. 10. RAPD patterns of *E. coli* strains recovered from cocktail #1 inoculated onto meat and subjected to 15" steam and 4.5% lactic acid treatment. Patterns were obtained with the DAF4 primer. Lanes 1 - 5, reference strains: 1 - MB 3.4; 2 - AW 1.3; 3 - GM 11.9; 4 - GM 16.7.2; 5 - GM 18.4. Isolates recovered after the intervention treatments from LB agar (Panel A) or Endo agar (Panel B) are depicted on the right side.

of the strains isolated from the slaughter plant showed that their cell counts ranged from $1.2x10^9$ to $1.7x10^7$ CFU/ml after 5 min at 58°C. Two strains of *E. coli* were chosen for further experiments: the most heat resistant one, *E. coli* AW 1.7, and one of the least heat resistant strains, *E. coli* DM 18.3.Their cell counts were $1.8x10^7$ and $4.9x10^4$ CFU/ml, respectively, after 30 min at 58°C. The survival of *E. coli* AW 1.7 and DM 18.3 on lean and adipose tissues, and in liquid media (LB broth and Beef Extract) subjected to 60°C for 30 min is shown in Fig. 12. In this experiment, where meat samples were fully submerged into hot water, no protective effect of the meat tissue was noticed in

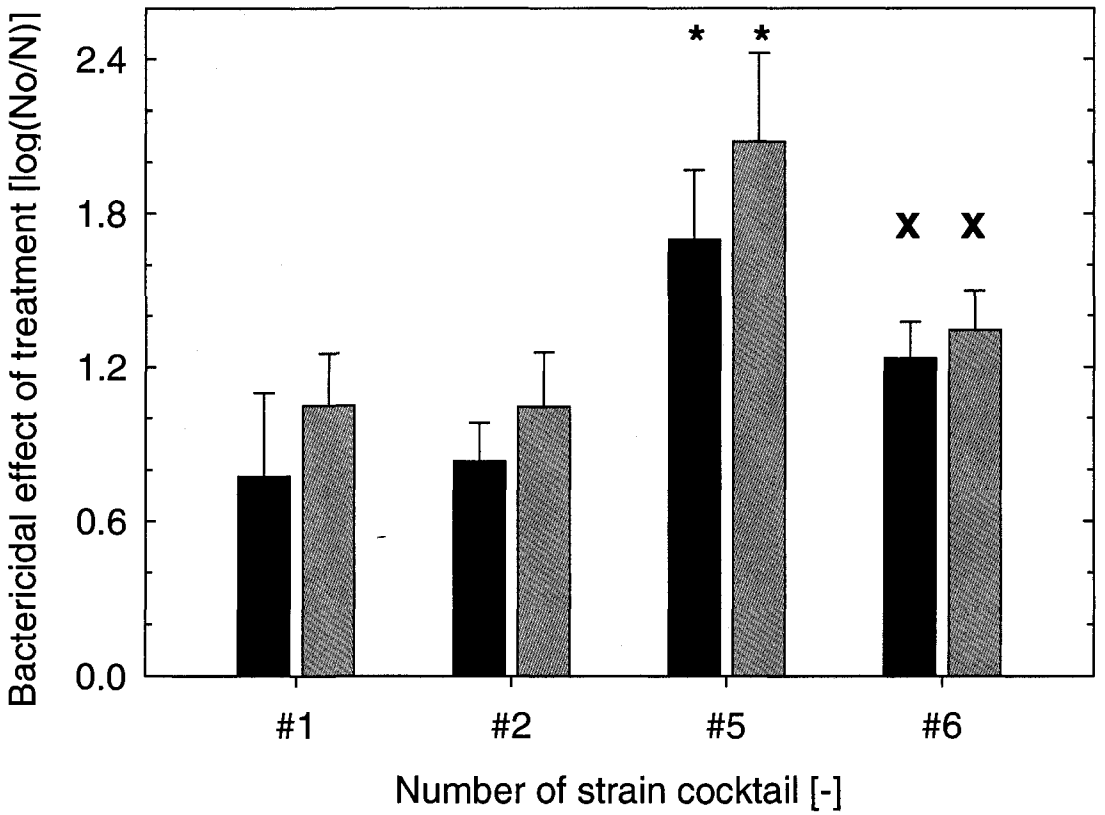

Figure. 11. Bactericidal effect of 15" steam and 4.5% lactic acid treatments on cocktails of *E. coli* inoculated on meat. Cell counts were determined on LB agar (black bars) and Endo agar (grey bars) before and after treatment. Data are the mean ± standard deviation of four independent experiments. * indicates a significant difference from cocktails # 1 and #2 with $p < 0.01$; x indicates a difference from cocktails # 1 and # 2 with $p < 0.075$

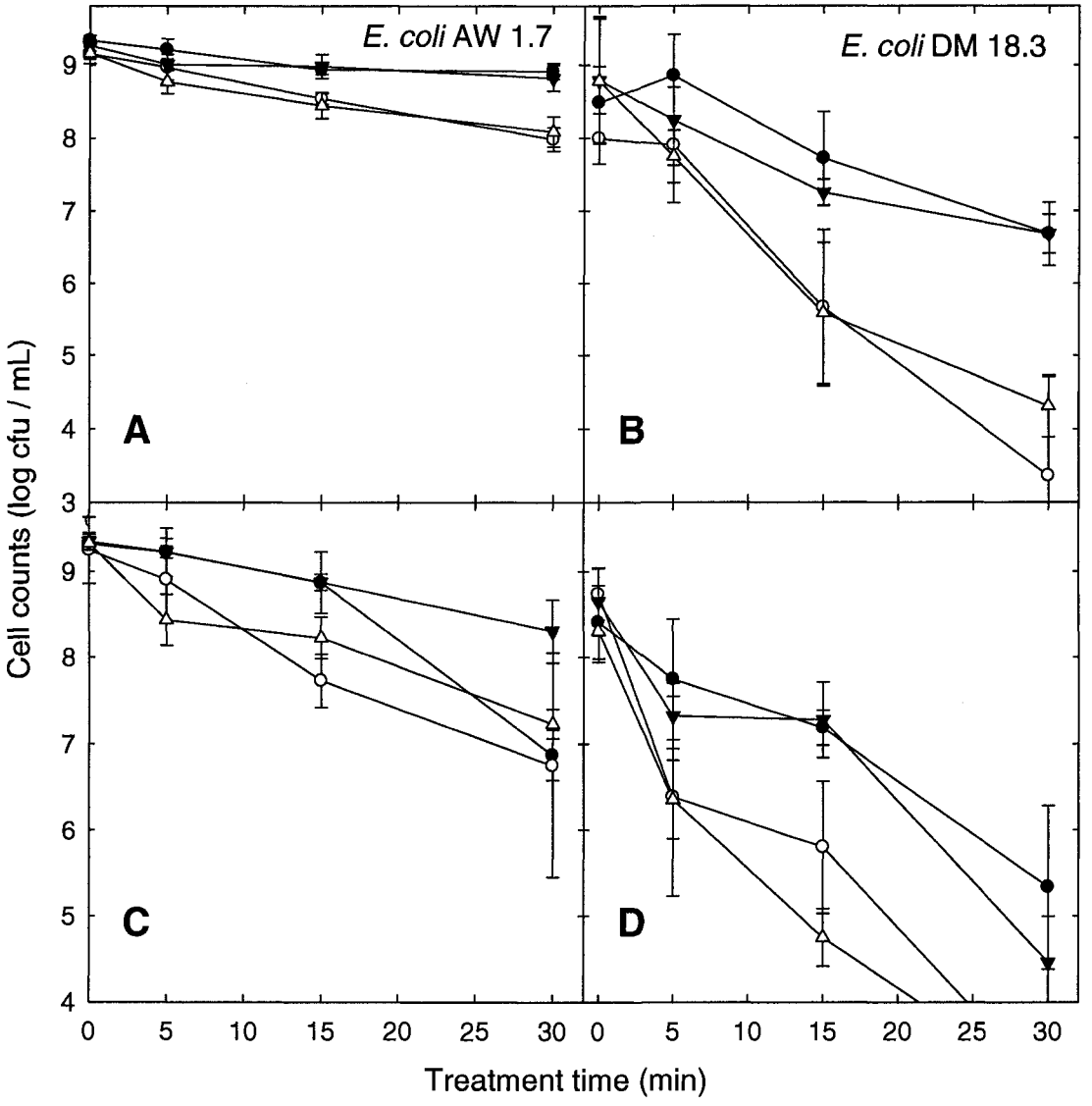

Fig. 12. Cell counts of *E. coli* AW 1.7 and *E. coli* DM 18.3 after heating at 60°C in liquid media (A and B) and on meat tissue (C and D). Data are mean ± standard deviation of six values.

Legend for panels A and B: ● - heated in LB broth, plated onto LB agar; ▼- heated in meat extract, plated onto LB agar; ○ - heated in LB broth, plated onto Endo agar; Δ - heated in meat extract plated onto Endo agar.

Legend for panels C and D: ● - heated on fat tissue, plated onto LB agar; ▼- heated on lean meat tissue, plated onto LB agar; ○ - heated on fat tissue, plated onto Endo agar; Δ - heated on lean meat tissue, plated onto Endo agar.

comparison with results obtained in liquid media. However, both strains seemed to survive better on lean meat than on adipose tissue. No significant amount of sublethal injury was documented for *E. coli* AW 1.7. In case of *E. coli* DM 18.3 99% of cells were sublethally injured and grew on LB agar but not on Endo agar. D_{60}-values of *E. coli* AW 1.7 and *E. coli* DM 18.3 were calculated from data shown on Fig. 12 and varied for different substrates. As shown in Table IV, the time for thermal inactivation of *E coli* AW 1.7 was much higher in LB Broth and Meat Extract than on meat. Survival of *E coli* DM 18.3 was generally less than *E coli* AW 1.7, but the D_{60} of *E coli* DM 18.3 was still

Table IV. Decimal reduction time (D-value) for two strains of *E. coli* isolated from slaughter plant environment, heated in different substrates at 60°C and plated on either LB or Endo agar.

Heating substrate	Plating media	D_{60}	
		E. coli AW 1.7	*E. coli* DM 18.3
Luria Bertani Broth	LB agar	71.4±1.5[a]	14.6±1.7
	Endo agar	25.4±0.1	6.2±1.2
Meat extract	LB agar	86.3±27.5	14.7±3.1
	Endo agar	29.7±3.5	6.7±0.3
Fat	LB agar	12.2±0.7	10.2±1.3
	Endo agar	12.7±4.5	7.3±1.1
Lean meat	LB agar	29.7±8.2	7.9±0.3
	Endo agar	16.2±0.14	7.6±0.8

[a]) Data are mean ± standard deviation of six values (two experiments, each plated in triplicate).

higher in liquid media than on the surface of meat. The D_{60} of *E coli* AW 1.7 on adipose tissue surface is more than two times lower (12.2 min) than when lean tissue was used as the heating substrate (29.7 min). *E coli* DM 18.3 did not show as much difference

between the D_{60} values obtained on lean meat or fat. The Z-value of the most heat resistant strain *E coli* AW 1.7 was 4.2°C (Fig. 13).

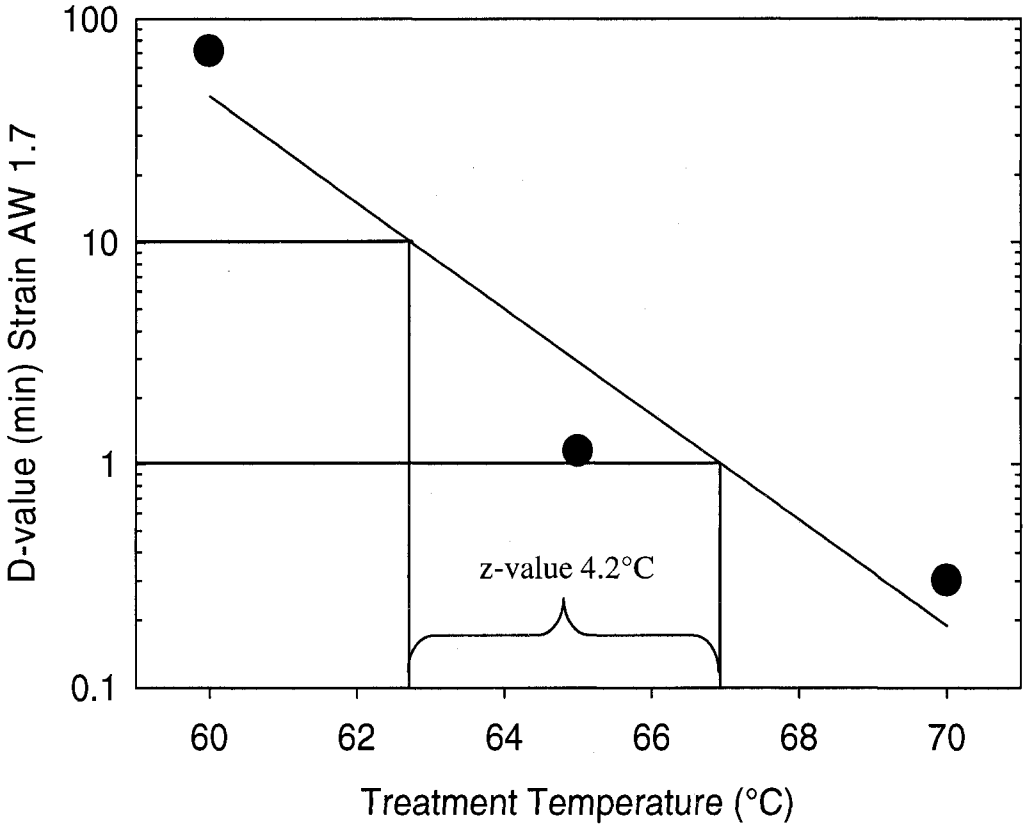

Fig. 13. Plot of the D-values of *E. coli* AW1.7 versus temperature (60, 65 and 70°C) used to calculate the z-value. LB broth was used as the heating substrate.

Survival (CFU/ml) and D_{60}-values of strains obtained from live cows and laboratory collection strains of *E. coli* were much lower when compared to isolates from the slaughter plant (Fig. 14). D_{60}- value of *E. coli* K-12, taken as a reference strain (Chung *et al.*, 2007), was 0.1 min, D_{60} of *E. coli* GGG10 was 0.65 min versus 71.4 minutes of *E. coli* AW 1.7 and 14.6 minutes of *E. coli* DM 18.3 (Table V).

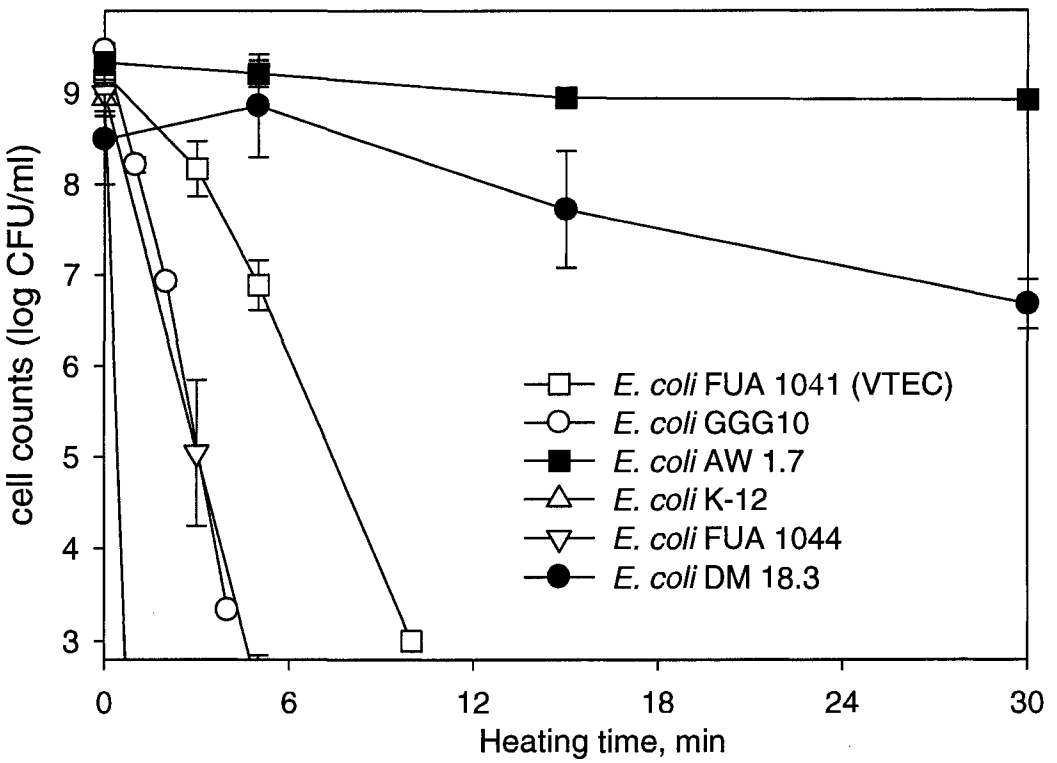

Fig. 14. Survival of strains of *E. coli* isolated from slaughter plant environment (black labels) and control strains (white labels) after heating at 60°C in LB broth.

Table V. D_{60}-values of *E. coli* strains used in the research.

Strain	Source	Time, min
AW 1.7	Slaughter plant (current)	71.4±1.5[a]
DM 18.3	Slaughter plant (current)	14.6±1.7
K-12	Collection	0.11
GGG10	Slaughter plant (1980s)[b]	0.65±0.01
FUA 1041 (VTEC)	Live cow (rectum)	2.2±0.2
FUA 1044	Live cow (rectum)	0.76±0.03

[a]) Data are shown as means ± standard deviation of at least four replicates

[b]) The strain was isolated before the use of steam as an intervention became a commercial practice

3.5 Characterization of *rpo*H gene of heat resistant and heat susceptible *E. coli.*

To find a possible mechanism of such an extreme difference in heat resistance of *E. coli* strains isolated from a slaughter plant, the *rpo*H gene of heat resistant and susceptible strains was sequenced. This gene encodes an alternative sigma factor of RNA polymerase, σ^{32}, which regulates cytoplasm heat shock response of *E. coli.* Alignment of *rpo*H gene sequences did not reveal any relevant differences between the strains (Fig. 15). None of 22 nucleotide substitutions in 697 base pair DNA sequences of the strains lead to any amino acid substitutions in σ^{32} protein (data not shown). Codon Plot analysis of sequences showed no evidence of more effective codon usage by *E. coli* AW 1.7 in protein synthesis (data not shown).

3.6 Western Blot analysis of DnaK, σ^{32}, and σ^{E} proteins.

Another approach to find the cause of such an unusual heat resistance was Western Blot analysis of expressed heat shock proteins. We chose σ^{E} as the bacterial envelope stress response factor, σ^{32} as the cytoplasm stress response factor, and DnaK, a molecular chaperone of Hsp70 class that negatively controls σ^{32} activity. No differences in amount of DnaK or σ^{E} (Fig. 16) were noticed. For σ^{E}, bands were located at 38 to 41 kDa instead of the expected 24 kDa; however, single bands indicate that 1RE53 antibodies gave no cross reaction. The amount of σ^{32} was below detection limit (Data not shown).

```
3-3             GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
AW1.7           GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
k-12ref         GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
K-12            GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
2260-2          GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
GM11.9          GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
DM18.3          GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
GGG10           GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
ATCC25922       GCTGACGAGGAGCGGGCGCTGGCTGAAAAGCTGCATTACCATGGCGATCTGGAAGCAGC 59
                ************************************************************

3-3             TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
AW1.7           TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
k-12ref         TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
K-12            TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
2260-2          TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
GM11.9          TAAAACGCTGATCCTGTCTCACTTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
DM18.3          TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
GGG10           TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
ATCC25922       TAAAACGCTGATCCTGTCTCACCTGCGGTTTGTTGTTCATATTGCTCGTAATTATGCGGG 119
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k-12ref         CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
K-12            CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
2260-2          CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
GM11.9          CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
DM18.3          CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
GGG10           CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
ATCC25922       CTATGGCCTGCCACAGGCGGATTTGATTCAGGAAGGTAACATCGGCCTGATGAAAGCAGT 179
                ************************************************************

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AW1.7           GCGCCGTTTTAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
k-12ref         GCGCCGTTTCAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
K-12            GCGCCGTTTCAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
2260-2          GCGCCGTTTCAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
GM11.9          GCGCCGTTTTAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
DM18.3          GCGCCGTTTTAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
GGG10           GCGCCGTTTCAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
ATCC25922       GCGCCGTTTTAACCCGGAAGTGGGTGTGCGCCTGGTCTCCTTCGCCGTTCACTGGATCAA 239
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k-12ref         AGCAGAGATCCACGAATACGTTCTGCGTAACTGGCGTATCGTCAAAGTTGCGACCACCAA 299
K-12            AGCAGAGATCCACGAATACGTTCTGCGTAACTGGCGTATCGTCAAAGTTGCGACCACCAA 299
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GM11.9          AGCAGAGATCCACGAATACGTCCTGCGTAACTGGCGTATCGTCAAAGTTGCGACCACCAA 299
DM18.3          AGCAGAGATCCACGAATACGTCCTGCGTAACTGGCGTATCGTCAAAGTTGCGACCACCAA 299
GGG10           AGCAGAGATCCACGAATACGTTCTGCGTAACTGGCGTATCGTCAAAGTTGCGACCACCAA 299
ATCC25922       AGCAGAGATCCACGAATACGTTCTGCGTAACTGGCGTATCGTCAAAGTTGCGACCACCAA 299
                ******************** ***************** ********************

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AW1.7           AGCGCAGCGCAAACTGTTCTTCAACCTGCGTAAAACCAAGCAGCGTCTGGGCTGGTTTAA 359
k-12ref         AGCGCAGCGCAAACTGTTCTTCAACCTGCGTAAAACCAAGCAGCGTCTGGGCTGGTTTAA 359
K-12            AGCGCAGCGCAAACTGTTCTTCAACCTGCGTAAAACCAAGCAGCGTCTGGGCTGGTTTAA 359
2260-2          AGCGCAGCGCAAACTGTTCTTCAACCTGCGTAAAACCAAGCAGCGTCTGGGCTGGTTTAA 359
GM11.9          AGCGCAGCGCAAACTGTTCTTCAACCTGCGTAAAACCAAGCAGCGTCTGGGCTGGTTTAA 359
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                ****** ** ************************** ***********************

3-3             CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTTCGTGA 419
AW1.7           CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTTCGTGA 419
k-12ref         CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGA 419
K-12            CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGA 419
2260-2          CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGA 419
GM11.9          CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGA 419
DM18.3          CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGA 419
GGG10           CCAGGATGAAGTCGAAATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTTCGTGA 419
ATCC25922       CCAGGATGAAGTCGAGATGGTGGCCCGTGAACTGGGCGTAACCAGCAAAGACGTACGTGA 419
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3-3             GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
AW1.7           GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
k-12ref         GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
K-12            GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
2260-2          GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
GM11.9          GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
DM18.3          GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
GGG10           GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
ATCC25922       GATGGAATCACGTATGGCGGCACAGGACATGACCTTTGACCTGTCTTCCGACGACGATTC 479
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3-3             CGACAGCCAGCCGATGGCACCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
AW1.7           CGACAGCCAGCCGATGGCACCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
k-12ref         CGACAGCCAGCCGATGGCTCCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
K-12            CGACAGCCAGCCGATGGCTCCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
2260-2          CGACAGCCAGCCGATGGCTCCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
GM11.9          CGACAGCCAACCGATGGCACCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
DM18.3          CGACAGCCAACCGATGGCACCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
GGG10           CGACAGCCAGCCGATGGCACCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
ATCC25922       CGACAGCCAGCCGATGGCTCCGGTGCTCTATCTGCAGGATAAATCATCTAACTTTGCCGA 539
                ********* ******** ****************************************

3-3             CGGCATCGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
AW1.7           CGGCATCGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
k-12ref         CGGCATTGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
K-12            CGGCATTGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
2260-2          CGGCATTGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
GM11.9          CGGCATCGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
DM18.3          CGGCATCGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
GGG10           CGGCATCGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGTCTGACCGACGCGATGCA 599
ATCC25922       CGGTATTGAAGATGATAACTGGGAAGAGCAGGCGGCAAACCGCCTGACCGATGCGATGCA 599
                *** ** ********************************** ******** ********

3-3             AGGTCTGGACGAGCGTAGCCAGGACATCATCCGCGCGCGCTGGCTGGACGAAGACAACAA 659
AW1.7           AGGTCTGGACGAGCGTAGCCAGGACATCATCCGCGCGCGCTGGCTGGACGAAGACAACAA 659
k-12ref         GGGTCTGGACGAACGCAGCCAGGACATCATCCGTGCGCGCTGGCTGGACGAAGACAACAA 659
K-12            GGGTCTGGACGAACGCAGCCAGGACATCATCCGTGCGCGCTGGCTGGACGAAGACAACAA 659
2260-2          GGGTCTGGACGAACGCAGCCAGGACATCATCCGTGCGCGCTGGCTGGACGAAGACAACAA 659
GM11.9          GGGTCTGGACGAACGCAGCCAGGACATCATCCGCGCGCGCTGGCTGGACGAAGACAACAA 659
DM18.3          GGGTCTGGACGAACGCAGCCAGGACATCATCCGCGCGCGCTGGCTGGACGAAGACAACAA 659
GGG10           AGGTCTGGACGAACGCAGCCAGGACATCATCCGCGCGCGCTGGCTGGACGAAGACAACAA 659
ATCC25922       AGGTCTGGACGAGCGTAGTCAGGATATCATCCGCGCGCGCTGGCTGGATGAAGACAACAA 659
                ********** ** ** ***** ******** ************** **********

3-3             GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
AW1.7           GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
k-12ref         GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
K-12            GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
2260-2          GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
GM11.9          GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
DM18.3          GTCCACGTTGCAGGAACTGGCTGACCGTTACGGCGTTT 697
GGG10           GTCCACGTTGCAGGAACTGGCTGACCGTTACGGTGTTT 697
ATCC25922       GTCCACGTTGCAGGAACTGGCTGACCGTTACGGTGTTT 697
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Fig. 15. Comparison of partial sequences of *rpo*H gene of *E. coli*. The gene encodes the alternative sigma factor σ^{32}, which mediates the heat shock response of *E. coli*. Strains analyzed: *E. coli* FUA 1050 isolated from bovine vagina; *E. coli* AW 1.7 isolated from slaughter plant environment; K-12 ref sequence was taken from whole genome database (NCBI Nucleotide, access code U00096); *E. coli* K-12 collection strain; *E. coli* FUA 1041 (VTEC) from bovine feces; *E. coli* GM 11.9 isolated from slaughter plant environment; *E. coli* DM 18.3 isolated from slaughter plant environment; *E. coli* GGG10 isolated from slaughter plant environment 20 years ago; *E. coli* ATCC 25922 collection strain. Translation of the *rpo*H gene sequences into corresponding σ^{32} amino acid sequences shows that all strains produce identical proteins (data not shown), i.e. because of the degenerate genetic code, these minor DNA sequence variations do not alter the protein sequence.

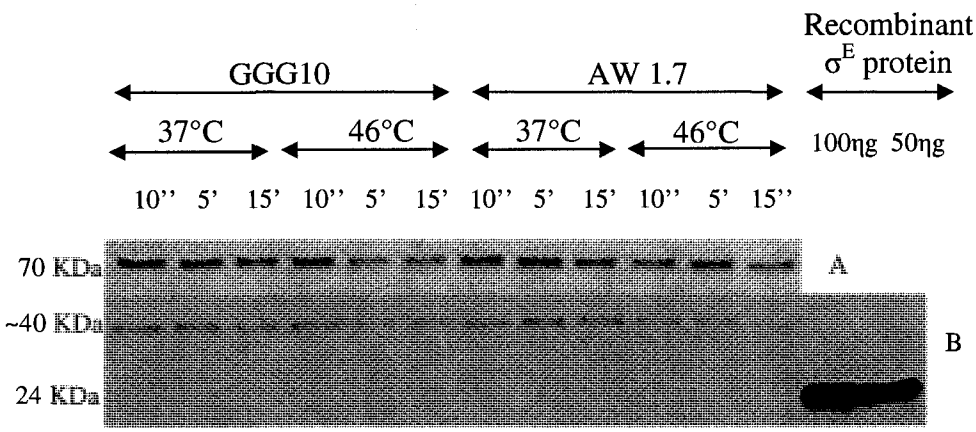

Fig. 16. Western blot analysis of the heat shock proteins, DnaK (A) and σ^E (B), of *E. coli* strains GGG10 and AW 1.7 in crude cell protein extract. Recombinant σ^E was added as a control.

3.7 Biofilm formation by *E. coli* AW 1.7.

E. coli AW 1.7 had noticeable tendency to flocculate in liquid culture. It settles on the bottom of a flask few minutes after being removed from the shaker. It has been shown for other bacteria species, that flocculating strains are more likely to form biofilms as they produce polysaccarides, which promote their adhesion to surfaces (Malik *et al.*, 2003; Flemming *et al.*, 2007). Biofilm formation has also been demonstrated for *E. coli* in anaerobic conditions (Latimer and Poole, 2007).

In this study the coverage of stainless steel coupon surface by *E. coli* AW 1.7 looked no different than that formed by non-flocculating *E. coli* K-12 and GGG10; however, *E. coli* AW 1.7 had a tendency to form microcolonies while the cells of non-flocculating strains were distributed evenly (Fig. 17). Cells of *E. coli* AW 1.7 also had a distinct ovoid shape instead of a normal bacillar shape of control *E. coli* K-12.

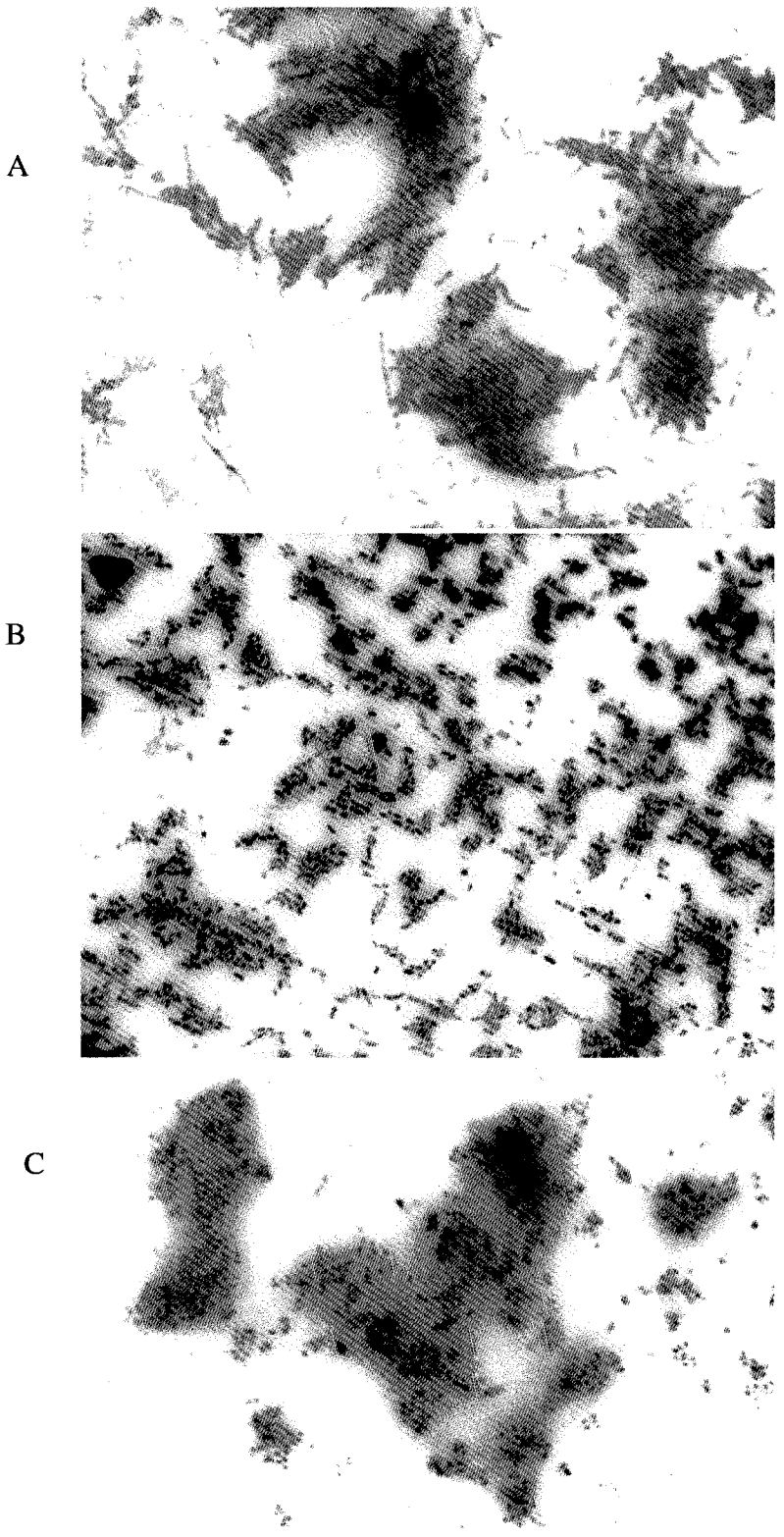

Fig. 17. Cells of *E. coli* strains K-12 (A), GGG10 (B), and AW 1.7 (C) grown on the surface of stainless steel for 72 h and stained with LIVE/DEAD Bac Light Bacterial Viability Kit (Invitrogen)(red/green fluorescence, x1000).

4. DISCUSSION

The presence of generic *E. coli* on meat is an indicator for presence of pathogenic strains of *E. coli,* in particular *E. coli* O157:H7. It also successfully serves as an indicator of the effectiveness of interventions during the slaughter process. Some strains of *E. coli* survive all intervention steps during beef processing (Aslam *et al.*, 2003). The aim of this study was to analyze if these strains of *E. coli* survive all decontamination procedures due to their inherent heat resistance.

In the experiments to evaluate survival of *E. coli* after heat treatment, a reliable tool for accurate identification of strains recovered after heating was needed. For this purpose strains of *E. coli* were grouped into cocktails of five strains, each with notably different RAPD fingerprint patterns. RAPD analysis of strains of *E. coli* had a much greater ability to discriminate between strains of *E. coli* than REP-PCR. REP-PCR has been shown to work well for identification of sources of fecal pollution (Edge, Hill, 2007; Orsi *et al.*, 2007; Dombek *et al.*, 2000; Holloway P, 2001; Meays *et al.*, 2004) but its ability to differentiate between strains of the same origin or serotype was poor (Hahm et al., 2003). In the current study the reproducibility of the REP-PCR patterns did not provide a consistent tool for adequate discrimination between strains. RAPD-PCR with the DAF4 primer resulted in the most discriminative patterns of strains of *E. coli* in this study. This was in accordance with the research results of Vogel *et al.* (2000), who obtained highly discriminative fingerprints of strains of *E. coli* for epidemiological purposes. Other researchers demonstrated irreproducible and unsatisfactory results when this primer was used for RAPD analysis of clinical isolates of *Nocardia* (Kalpoe *et al.*, 2007) and good results for discrimination of isolates of *Acinetobacter baumannii* (AitMhand *et al.*, 2002).

RAPD PCR with the 1254 primer yielded fewer bands than the DAF4 and was less discriminative, but as reproducible as the RAPD patterns generated with DAF4 primer. The composition of the cocktails of *E. coli* was based on the DAF4 RAPD patterns. After 15 s steam and 4.5% lactic acid treatments cell count reductions were 1-2 log CFU/ml and all strains used in the experiment except for *E. coli* ATCC 25922 were recovered. Similar cell count reduction of *E. coli* O157:H7 ATCC 43895 on beef surface, 2 log CFU/cm^2 after 10 s and 3 log CFU/cm^2 after 60s of steam pasteurisation at 99.2°C, was obtained by McCann *et al.* (2006). This might be the result of initial high (10^8 CFU per 5 cm^2 sample in our case) cell concentrations as described for high pressure treatments (Furukawa *et al.*, 2002). Resistance to heat treatment of the strains of *E. coli* isolated from slaughter plant environment was comparable. Laboratory collection control strains were less resistant as the reduction of cell counts was significantly ($p< 0.01$) higher. Among control strains, the culture collection strain *E. coli* ATCC 25922 was the least resistant as it did not survive the interventions. The difference in the reduction of cell counts between cocktails of slaughter plant isolates and control strains cocktails led to the conclusion, that intrinsic strain characteristics may have been responsible in these results rather than the influence of the meat tissue matrix. All of the strains *E. coli* isolated from the abattoir previously had been exposed to all of the interventions during the slaughter process.

The D - values of these strains at 60°C were about 100 times higher than the D-values of strains isolated from live cows, strains isolated from carcasses prior to the use of interventions, and laboratory collection strains, which were used as controls. The D_{60}

values of the control strains were in agreement with those reported in the research literature (Table VI).

Table VI. Reported decimal reduction time at 60°C for strains of *Escherichia coli.*

D_{60} for strains of *E. coli*, min	Strains	Reference:
0.22	K-12	Jin *et al.*, 2008
0.32	K-12	Chung *et al.*, 2007
0.4 - 1.5	O157:H$^-$; Stx$^-$ (LTH 5807)	Weiss and Hammes , 2005
0.47	O157:H7 (204P)	Ahmed *et al.*, 1995
1.9	O157:H7 (EDL-931, A 9218-C1, 45753-35, 933)	Juneja and Marmer , 1999
2	not specified	Mossel *et al.*, 1995
3.2	204P, 505B, 933, 932, F 501, F 585	Murfy *et al.*, 2004
4.2	ATCC 25922	Pereira *et al.*, 2006
6.7	380-94	Riordan *et al.*, 2000

The D_{60} of *E. coli* K-12 in this study was slightly less that that reported by Chung *et al.* (2007) and Jin *et al.* (2008). In this study time needed to reach the required temperature of samples was reduced to a minimum. Strains of *E. coli* with D_{60} values close to 70 min have not been reported to the date. This might be a result of the lack of research on thermal resistance of strains isolated from a food processing environment. In the majority of published research, strains from laboratory collections or clinical isolates (*E. coli* O157:H7) were used. Variations in tolerance to other interventions such as high pressure treatments among different enterobacteria, including strains of *E. coli* has also

reported (Metrick *et al.*, 1989; Tahiri *et al.*, 2006; Erkmen, Doğan, 2004). Margosch et al. (2004) reported high variation in D_{120} for strains of *Clostridium botulinum*. The D-value of their most resistant strain was 1.2 min, six times higher than that of other strains. The authors stated that large numbers of strains need to be studied to obtain reliable data on inactivation of the microorganism to improve food preservation techniques. The results obtained in this study indicate that a large number of strains are not needed for this purpose, but the strains used must be taken from the food processing environment. In this study, such a big difference in thermotolerance between isolates of *E. coli* from slaughter plant and the strains that never underwent any intervention treatments indicates the potential of tremendous selective pressure of interventions on the populations of *E. coli*. This selective pressure may lead to selection and possible accumulation and persistence of extremely heat resistant strains of bacteria in the food processing environment.

The influence of fat content in ground meat on heat tolerance of *E. coli* and *Salmonella* sp. have been well described (Ahmed *et al.*, 1995; Juneja and Embden, 2000). Higher fat content in food matrix provides better protection for bacterial cells, as bacteria are able to transport (Nunn and Simmons, 1978; Maloy *et al.*, 1981; DiRusso and Black, 2004) and incorporate unsaturated fatty acids into their membranes to maintain their optimal fluidity (O'Kelly and Spiers, 1991). In this *study E. coli* AW 1.7 survived better on lean meat tissue (D_{60} = 29.7 min) than on fat (D_{60} = 12.2 min).The D-value of *E. coli* DM 18.3 was still higher on fat than on lean tissue (10.2 min versus 7.9 min). A possible explanation of this fact may be in different initial permeability of cell membranes. Possible involvement of morphogene *bol*A into outer membrane permeability will be discussed later. If oval shaped *E. coli* AW 1.7 has a less permeable outer membrane than

E. coli DM 18.3, in this heat survival experiment it doesn't have enough time either to use fatty acids of fat for protection or to penetrate deeper into the fat tissue. This leads to decreased survival of *E. coli* AW 1.7 on fat versus lean meat tissue. And morphologically normal *E. coli* DM 18.3 survives heat better when applied on fat surface than on lean meat as described in previous research (Maloy *et al.*, 1981; O'Kelly and Spiers, 1991; DiRusso and Black, 2004).

Other pathogenic bacteria associated with fresh meat usually do not have such a devastating impact on their victims as the EHEC with the exception of *Listeria monocytogenes.* Mackey et al. (1990) found D_{60} value of *L. monocytogenes* to be 3.8 min. Sörquist (2003) calculated D_{60} of 1.5 min and 3 min for *L. monocytogenes* and *L. innocua,* respectively. This author also found *Enterococcus faecium* to be the most heat resistant organism (D_{60} = 19.2 min) in experiments with bacterial strains that had not previously been subjected to heat shock. Such a high initial resistance of enterococci to elevated temperatures can lead to even higher survival of enterococci than *E. coli* during beef processing with the potential for possible subsequent transmission of antibiotic resistance genes from animal associated enterococci to human associated strains (Bates *et al.*, 1994; van den Bogaard and Stobberingh, 2000).

To determine the cause of extreme heat resistance of slaughter plant isolates the expression of proteins, shown to confer elevated heat tolerance to *E. coli*, was investigated. Levels of three important heat shock proteins in *E. coli*: DnaK, σ^{32}, and σ^{E} were chosen for analysis.

DnaK is a 70KDa heat shock protein and molecular chaperone that plays an essential role in proper folding of newly synthesized proteins and repairing of misfolded proteins (Lund, 2001; Kerner *et al.*, 2005). It is one of the most abundant proteins of *E. coli* cells (Georgopoulos *et al.,* 1982) and one of the most conserved proteins in nature (Bardwell and Craig, 1986). Its presence is essential under heat and oxidative stress, and starvation leads to elevated levels of DnaK (Rockabrand *et al.*, 1995; Paek and Walker, 1987; Spence *et al.*, 1990). DnaK stabilizes σ^S in response to carbon starvation (Rockabrand *et al.*, 1998). It negatively controls σ^{32} by preventing it from binding to core RNA polymerase (Craig and Gross, 1991; Hartl *et al.*, 1992) and therefore making σ^{32} prone to degradation by FtsH protease (Herman *et al.*, 1995; Tomoyasu *et al.*, 1998).

In this study DnaK was present in significant amounts and the concentration of σ^{32} was below detection limits of Western Blot analysis. No difference in *rpo*H gene sequences that would lead to amino acid substitution in σ^{32} protein was detected. This was in good accordance with the data of Mc Carty *et al.* (1996) and Yura *et al.* (2000), who demonstrated negative control of DnaK on σ^{32}. In this study the difference in the DnaK levels between heat sensitive and heat resistant strains was too low to be detected by the Western Blot method. Aertsen *et al.* (2004) demonstrated in experiments with exponentially growing cultures of pressure resistant strains of *E. coli* that expression of DnaK was 2 to 5 fold higher than that detected in pressure sensitive strains. These authors used an ELISA-based method that is more sensitive than the Western Blot.

A 24 KDa σ^E was first described in 1989 by two independent groups (Erickson and Gross,1989; Wang and Kaguni, 1989) in runoff transcription assays for transcription initiation studies. Originally σ^E was purified from RNA polymerase holoemzyme

(RNAP) as a sigma factor responsible for recognizing and initiation of transcription from the third promoter of *rpo*H - P3. This promoter remains the only functioning one among four *rpo*H promoters after a shift to 50°C. Promoters P1, P4, and P5, recognized by predominant sigma factor σ^{70}, are shut off (Erickson et al., 1987; Gross et al., 1990). The RNAP σ^{E} (E σ^{E}) was also shown to transcribe the promoter of *deg*P, the gene encoding a serine protease, which is involved in periplasmic heat-shock response, chaperone function, and apoptosis (Erickson and Gross, 1989). Dartigalongue *et al.* (2001) found twenty four σ^{E} dependent promoters (Table VII). Expression of σ^{E} is induced by perpilasmic proteins misfolded by various types of stress (Mecsas *et al.*, 1993; Missiakas *et al.*, 1996), overproduction of outer membrane proteins (Rouviere *et al.*, 1995), or accumulation of protein precursors (Wild *et al.*, 1993). Malone *et al.* (2006) reported that the expression of *rpo*E was upregulated 1.5 fold at 100 MPa. The *rpo*E gene encoding the 191 amino acid polypeptide σ^{E}, was also described by two independent groups. Rudd (Rouviere *et al.*, 1995) discovered the gene after comparison of partial sequence of the σ^{E} protein with DNA sequence from the Genebank. Raina *et al.* (1995) used a genetic screen that was designed to isolate *trans*-acting mutations that abolish expression from *rpo*HP3 or *htr*A, a heat shock endoprotease gene. In the only published attempt to evaluate the amount of endogenous σ^{E} protein in crude protein extracts of *E. coli* Bergendahl *et al.* (2003) reported σ^{E} levels in an exponentially growing culture of *E. coli* to be insignificant and below Western Blot detection limits. The data from the present study demonstrate that a substantial amount of σ^{E} protein is present in a stationary phase culture of *E. coli* (Fig. 16). There is no noticeable difference in the σ^{E} expression between heat resistant and heat susceptible strains, probably due to the relatively low sensitivity of Western Blot

Table VII. Genes regulated by σ^E.

Gene name	Function	Reference
*bac*A	Undecaprenol kinase	Rezuchova *et al.*, 2003
*cut*C	Copper sensing	Dartigalongue *et a*l., 2001
*deg*P	Protease	Stauch *et al.*, 1989
*dsb*C	Folding envelope protein	Dartigalongue *et al.*, 2001
*dsb*C	Thiol:disulfide oxidoreductase	Dartigalongue *et al.*, 2001
ecf (*ecf*A-*ecf*M)	Unknown	Dartigalongue *et al.*, 2001
*ecf*A	Lipopolysaccahride biogenesis	Dartigalongue *et al.*, 2001
*ecf*E	Putative carboxypeptidase	Dartigalongue *et al.*, 2001
*fkp*A	Peptidyl prolyl isomerase	Danese *et al.*, 1997
*fus*A *tuf*A *rec*R	Translation elongation factors	Rezuchova *et al.*, 2003
*htr*M	Lipopolysaccaride biosynthesis	Dartigalongue *et al.*, 2001
*ipx*P	Palmitoleoyl transferase	Rezuchova *et al.*, 2003
*lpx*D *lpx*A	Lipid A	Dartigalongue *et al.*, 2001
*mdo*G	oligosaccharide syhthesis	Dartigalongue *et al.*, 2001
*mic*A	Antisense RNA MicA	Udekwu and Wagner, 2007
*nlp*B	Lipoprotein	Dartigalongue *et al.*, 2001
psd	Phosphatidylserine decarboxylase	Rezuchova *et al.*, 2003
*rpo*D	Housekeeping sigma factor	Dartigalongue *et al.*, 2001
*rpo*E	Sigma factor	Raina *et al.*, 1995
*rpo*H	Sigma factor	Wang and Kaguni, 1989
*rse*A *rse*B	Negative regulators of σ^E	Dartigalongue *et al.*, 2001
*rse*C	Positive regulator for σ^E	Dartigalongue *et al.*, 2001
*ryb*B	Small RNA	Thompson *et al.*, 2007
*sbm*A	Putative transport protein	Rezuchova *et al.*, 2003
*six*A	Phosphohistidine phosphatase	Rezuchova *et al.*, 2003
skp	Outer membrane proteins folding	Dartigalongue *et al.*, 2001
*smp*A	Small outer membrane protein	Rezuchova *et al.*, 2003
*sur*A	Folding envelope protein	Dartigalongue *et al.*, 2001
*yba*B	Unknown. DNA repair	Rezuchova *et al.*, 2003
*yea*Y	Putative outer membrane lipoprotein	Rezuchova *et al.*, 2003
*yfe*Y	Unknown	Rezuchova et al., 2003
*yii*S	Unknown	Rezuchova *et al.*, 2003

analysis. Fig. 16 demonstrates that the two-fold difference in the amount of recombinant σ^E loaded in the gel is not visible. Therefore, more sensitive methods, such as DNA microarray analysis, are needed to evaluate alterations in expression of heat shock proteins in highly heat resistant strains, as Western Blot analysis as done in this study is

unable either to confirm or exclude possibility of these alterations. The most unexpected result was that molecular weight of the recombinant and endogenous σ^E protein was different. The molecular weight of endogenous protein was about 40 KDa. The only band present on Western Blots indicates the specificity of the 1RE53 antibody used in the experiments. It may be possible that σ^E protein is predominantly present in the cell as a dimer and amount of monomer is below the detection limits of Western Blot analysis. The possibility that σ^E protein is fused with another protein must also be considered. More research is needed to further characterize the structure and function of endogenous σ^E protein in stationary phase cells of *E. coli*.

Biofilm formation by pathogenic bacteria is a serious problem in the food industry as biofilms are a constant source of food contamination with spoilage and pathogenic bacteria (Boulange-Peterman *et al.*, 1993; Austin and Berferon, 1995; Boulange-Peterman, 1996; Ryu and Bauchat, 2005). Cell surface traits such as adhesin protein (Ag43), pili, curli, flagella, EPS capsules, LPS, teichoic acids, and surface charge can contribute to the attachment (Kumar and Anand, 1998; Beech *et al.*, 1991; Spenceley *et al.*, 1992; Davey and O'Toole, 2000). The autoaggregation protein Ag43 is encoded by the *flu* (*agn*43) gene and is the mediator of flocculation of *E. coli* in broth. It also dramatically enhances biofilm formation (Reisner *et al.*, 2003). Shembri *et al.* (2003) demonstrated that clumping, mediated by Ag43, protects bacteria from antibacterial compounds. Reisner et al. (2006) demonstrated a significant impact of environmental factors on biofilm formation by commensal and pathogenic strains of *E. coli*. Microcolony formation proceeds after irreversible attachment of an organism to the surface (Chmielewski and Frank, 2003). It results from simultaneous aggregation and

growth of the microorganism. *E. coli* AW 1.7 was found to form microcolonies on the stainless steel surface. It also autoaggregates intensively in LB broth. The cells of *E. coli* AW 1.7 grown on a surface of stainless steel look shorter and more round when compared to *E. coli* K-12. The morphogene *bol*A is known to alter the morphology of *E. coli* cells when overexpressed (Aldea *et al.*, 1988). Two promoters govern its expression. The P2 promoter is regulated by σ^{70} and transcribes *bol*A constitutively (Santos *et al.*, 1999). The P1 promoter is under direct control of σ^{S} (Lange and Hengge-Aronis, 1991) and induction of growth phase dependent promoter also transcripts *bol*A (Aldea *et al.*, 1989). Santos *et al.* (2006) demonstrated that the promoter P1, the main promoter for *bol*A morphogene, is triggered in response to all forms of stress, including heat shock. Overexpression of *E. coli bol*A induces biofilm formation, reinforces the integrity of outer membrane and decreases its permeability by creating elevated levels of OmpC porine protein (Freire et al., 2006). No data about the effect of *bol*A overexpression on *E. coli* thermotolerance is available to date.

Mechanisms and the origin of the extremely heat resistant phenotype in *E. coli* remains to be investigated. It is clear that the evolution of such strains takes time and involves various mechanisms. Adaptive mutations and subsequent selection of mutants may play a role in this process. Adaptive mutations are defined as the process by which stresses that are not directly mutagenic, activate mechanisms that cause mutations even in non-growing cells (Roth *et al.*, 2006). Where and how these mutations occur in *E. coli* is unknown. In some cases altering the number of copies of genes in the cell leads to changes in organism physiology. Amplification of a single master transcriptional regulator *evg*A conferred a 10^2 to 10^4 increase in *E. coli* survival at the temperatures from

50 to 54°C (Christ and Chin, 2008). Park *et al.* (2005) knocked out the *ubi*X gene, which is involved in ubiquinone biosynthesis in *E. coli*, and the mutant strain became more heat resistant. Stringent conditions of beef processing interventions might prevent growth of susceptible "parent" strains and allow only the mutants to grow.

So called persisters can also contribute to the amount of surviving cells. Shah *et al.* (2006) reported that about 1% of a bacterial population are persistent cells. They also showed that the gene expression profile of persistent sells is distinct from that observed for cells in the exponential and stationary phase. But persistence in bacteria is just a phenotypic switch, a physiological state, and these cells are not resistant mutants. Balaban *et al.* (2004) found that cultures grown from persistent cells remained sensitive to the same antibiotic. Therefore, persisters which survived decontamination interventions during meat processing or persist on the equipment, are not expected to be extremely heat or acid tolerant, and researchers will continue to isolate intervention survivors with low D values.

To date, persisters and highly stress adapted strains are described for generic *E. coli* (Aertsen *et al.*, 2005; Shah *et al.*, 2006; Smirnova *et al.*, 2007). As generic *E. coli* is an indicator organism for EHEC, it is question of time to find EHEC with similar characteristics. Accumulation of persisters and thermotolerant strains as well as biofilm formation by *E. coli* in meat processing plants pose the danger of product contamination. Strains with D_{60} about 70 min are not fully inactivated during cooking process under current Health Canada guidelines.

This study demonstrates the ability of generic *E. coli* to survive decontamination interventions, currently used by the beef processing industry due to inherent heat resistance of the processing plant isolates. More research is needed to elucidate molecular traits and physiological mechanisms these organisms employ for extreme heat survival.

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