Identification of genes regulated by cold temperature – a study of *mdtJI* in *E. coli* O157:H7

A thesis submitted in partial fulfilment
of the requirements for the degree
of
Masters of Science
in Biological Sciences
at
The University of Waikato
by

Shelley Urlich

The University of Waikato
2009

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Abstract

E. coli O157:H7, a serious food-borne pathogen, is capable of adapting to distinctly different environments, ranging from the ruminant, to soil and water. Critical to the success of this pathogen, is the ability to adapt rapidly to changes in the environment. These changes rarely occur in isolation and bacteria, through regulatory networks, can respond to multiple challenges simultaneously, often through master regulators. Understanding the adaptive process of *E. coli* O157:H7, particularly in response to cold temperatures, is vital for elucidating the pathogens ability to persist during food processing. Of major concern to the meat industry, is the ability of *E. coli* O157:H7 to survive multiple hurdle intervention strategies that include both chilling and freezing.

The aim of this project was to identify genes involved in the cold shock response of *E. coli* O157:H7 when exposed to refrigeration temperatures (4, 0, -1.5°C). We hypothesized that *E. coli* O157:H7 is able to withstand chill temperatures by up-regulating genes that allow survival in unfavorable conditions, for example, when the cell is expelled from the ruminant host, into soil or water environments. It is likely that *E. coli* O157:H7, utilizing similar adaptive mechanisms can withstand prolonged periods at refrigeration temperatures. Furthermore, we speculated that quorum sensing (QS) has overtime become integrated into these adaptive pathways, potentially forming an integrated component of the *E. coli* O157:H7 adaptive stress response, including the cold shock response.

A number of genes were identified as being up-regulated in *E. coli* O157:H7 during incubation at 4°C on meat. Of these, four were of particular interest, as they had been previously linked to cell survival processes: *slp* (carbon starvation lipoprotein), *hslJ* (heat inducible protein), *mdtI* (multidrug efflux pump) and *mdtJ* (multidrug efflux pump). RT-PCR data showed that *slp* and the *mdtJI* complex are expressed more at refrigeration temperatures than at 37°C while *hslJ* expression was greatest at 37°C. *mdtJI* was selected for further analysis, because *mdtJI* was the only gene that was not expressed at 37°C when grown on BHI media, plus

these genes had, at the initiation of this project, not been annotated or assigned function. Using a *luxCDABE* promoter reporter, real time analysis of the effect of temperature downshift on *mdtJI* expression was confirmed. Furthermore, expression was demonstrated to increase at high cell density at 37°C, suggesting a regulatory connection to quorum sensing. This coupled with the finding that 400nt upstream of the *mdtJI* promoter was a gene encoding a transporter of AI-2, a QS autoinducer, suggested a link to the LuxS/AI-2 QS regulatory network. Data presented here was unable to confirm a regulatory link to AI-2 itself but it did reveal a link to LuxS.

In conclusion, data presented in this thesis has confirmed that *mdtJI* is involved in the adaptive response, specifically adaptation to cold temperatures in *E. coli* O157:H7, and possibly, to growth cessation. The influence of LuxS on *mdtJI* expression in *E. coli* O157:H7 is most likely to be through metabolic activity, rather than via a QS mechanism.

Acknowledgements

I wish to acknowledge a number of people for their assistance throughout the duration of this study. Let's face it; this thesis was a community effort.

A heartfelt 'thank you' goes to my supervisors, Dr. Ray Cursons, at the University of Waikato, and Dr. Helen Withers at AgResearch. Ray, your laid back style and expertise shall never cease to amaze me, and your guidance, from the beginning, has been thoroughly appreciated. Helen, thank you for taking me under your wing, and once again, dragging me over the finish line. Your seemingly tireless efforts and unfailing support will not be forgotten.

Thank you Gale, for your proof reading expertise, and allowing me time to study, while working for your team. Cheers Ben, for letting me bend your ear on a daily basis, I promise to return the favour. Nick, John, Guill and Yi, thank you for your moral support, and seeking out the odd reference or two on my behalf. To the lovely ladies in D2, your support and encouragement is greatly appreciated. Cheryl Ward and Vicki Smith, you have both been a life line, keeping me up to date with university requirements and thesis protocol. Fiona, you're a formatting legend, Neil you're a stats genius, and Ron, your help with illustrations has been incredible. Emma, thanks for the rescue effort, and cheers to Stefan for reminding me there will be a party when I'm done. A big thanks to the rest of my friends and co-workers, who provided their ears and glasses of wine for my troubles. The chocolate cake is in the oven, and the champagne is on its way.

Thank you to Mum and Dad, for trying to understand, and when you didn't, at least pretending to. Thanks particularly to Dad, for the extra technical help and words of wisdom.

Finally, a very special 'thank you' hug goes to my amazing husband, Tony. Without your love, extra effort around the house, superb culinary skills and never ending support, none of this would have been possible. You are more appreciated than you can possibly imagine.

Table of contents

List of Tables

List of Figures

Abbreviations

AB	Autoinducer Bioassay Media
A/E lesion	Attaching/Effacing lesion (Also, AE)
AHL	*N*-acyl homoserine lactone
AI-2	Autoinducer-2
AI-3	Autoinducer-3
Amp	Ampicillin
ANOVA	Analysis of variance
APC	Aerobic Plate Count
ATCC	American Type Culture Collection
AU	Arbitrary Unit
aw	Water activity
BHI	Brain Heart Infusion
bp	Base pair
Carb	Carbenicillin (also Cb)
Cb	Carbenicillin (also Carb)
CDC	Centers for disease control
cfu	Colony forming units
cm	Centimeters
CM	Conditioned medium
CM+	Conditioned medium made from AI-2 producing bacterial strains
CM-	Conditioned medium made from bacteria that are incapable of AI-2 manufacture
CSP	Cold shock protein
°C	Degrees celsius
dATP	Deoxyadenosine triphosphate
DAEC	Diffusely Adherent *E. coli*
dCTP	Deoxycytidine triphosphate
DEPC	Deionised diethylpyrocarbonate treated water (nuclease free)
dGTP	Deoxyguanosine triphosphate

dH_20	Deionised Water
diam.	Diameter
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates mix of dATP, dCTP, dGTP and dTTP
DPD	4,5-dihydroxy-2,3 -pentanedione
dTTP	Deoxythimidine triphosphate
EAEC	Enteroaggregative *E. coli*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
E. coli	*Escherichia coli*
GFP	Green fluorescent protein
g	grams
GUD	Glucuronidase
HACCP	Hazard analysis and critical control point
HeLa	Immortal cell line derived from cancer cells taken from Henrietta lacks
HUS	Haemolytic Uremic Syndrome
Km	Kanamycin (also kan)
kan	Kanamycin (also Km)
L	Litre
LA	Luria Bertani Agar
LB	Luria Bertani Broth
Lcfu	Luminescence per colony forming unit
LEE	Locus of enterocyte effacement
MA	Marine Agar
µl	microlitre
µg	microgram
mg	milligram

ml	milliliter
mM	millimolar
Min	Minute
MRD	Maximum Recovery Diluent
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
nt	Nucleotide
NZRM	New Zealand Reference Culture Collection (NZRCC), Medical section
NCTC	National Collection of Type Cultures
OD	Optical density
PCR	Polymerase Chain Reaction
PCA	Plate Count Agar
pg	picagram
pmol	picamole
PNP	Polynucleotide phosphorylase
QS	Quorum sensing
RAP-PCR	Random arbitrarily primed polymerase chain reaction
RLU	Relative luminescence unit
RT	Room Temperature
RT-PCR	Reverse Transcription (or Transcriptase) Polymerase Chain Reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sec	Seconds
SAH	*S*-adenosyl-homocysteine
SAM	*S*-adenosyl-methionine
SRH	*S*-ribosyl-homocysteine
STEC	Shiga toxin producing *E. coli*
Stx	Shiga toxin
TBE	Tris-borate-EDTA buffer
Tir	Translocated intimin receptor
TSA	Tryptic Soy Agar

TTP	Thrombotic thrombocytopenic purpura
TTTS	Type three secretion system
U	Unit
VHBA	*Vibrio harveyi* bioluminescence assay
VT	Verocytotoxin
VTEC	Verocytotoxin producing *E. coli*

Chapter 1: Introduction

1.1 *Escherichia coli*

Escherichia coli (*E. coli*) is a gram negative, facultatively anaerobic bacterium, belonging to the Family Enterobacteriaeceae. Commensal *E. coli* are usually harmless, and typically colonise the mucosal layer of mammalian and avian gastro-intestinal tract (Caprioli *et al.*, 2005). However, they can become opportunistic pathogens in cases where the intestinal lumen is breached, or if the host is immunocompromised (Nataro & Kaper, 1998). Furthermore, numerous pathogenic strains of *E. coli*, differentiated by pathotypes and serotypes, have evolved virulence factors that enable them to cause a broad range of diseases in humans (Kaper *et al.*, 2004). Such diseases include urinary tract infections, sepsis and severe diarrhoea. Well-described categories of diarrhoea causing (diarrhoeagenic) *E. coli* include, Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Diffusely adherent *E. coli* (DAEC) and Enterohaemorrhagic *E. coli* (EHEC). Of specific interest, are the Shiga toxin producing EHEC strains (STEC), also known as Verocytotoxin producing *E. coli* (VTEC), of which the most notorious member, associated with the majority of diseases within this group, is *E. coli* O157:H7.

1.2 *E. coli* O157:H7

E. coli O157:H7 is a highly adapted, pathogenic variant of *E. coli that* is part of a genetically distinctive STEC group of EHEC that can produce shiga toxins (Stxs). This virulent O157 strain is of public health concern due to the low infectious dose required to cause food poisoning and the organism's ability to produce stx, which can cause severe, and potentially fatal, diseases such as haemorrhagic diarrhea and haemolytic uremic syndrome (HUS). Furthermore, *E. coli* O157:H7 is capable of persisting in distinctly different environments, ranging from the large intestine of mammals and birds, to soil and water. The robust and adaptive nature of *E. coli* O157:H7 is of particular concern to the food and meat industry. Not only does *E. coli* O157 typically reside in ruminant animals, particularly cattle,

which providing a potential transmission route into the human food chain, but *E. coli* O157 is capable of withstanding many of the current intervention practices, such as chemical washes and chilling procedures, designed to reduce or eliminate pathogens.

E. coli O157:H7 strains can produce two different Shiga-like toxins, Stx1 and Stx2 (Mainil & Daube, 2005). Reports by Karmali (1989, 2005) describe the virulence and pathogenicity of *E. coli* O157:H7 as being the combination of Shiga-toxin production and attaching and effacing (AE) adherence to the bowel. However, there is some confusion over the nomenclature of EHEC, and whether the toxins are named Shiga-, or Vero-toxins. Nataro and Kaper (1998) refer to this confusion as "parallel nomenclature", which is due to simultaneous research occurring as a result of *E. coli* outbreaks in the early 1980s. As a consequence, there are numerous references to both Shiga-like toxin producing *E. coli* (STEC) and Verocytotoxin, or Vero-toxin producing *E. coli* (VTEC). Historically, VTEC were so named for their ability to produce toxins that were cytotoxic to Vero cells (Konowalchuk *et al.*, 1977). In 1982, O'Brien *et al.*, observed that *E. coli* isolates from HUS and haemorrhagic colitis (HC) outbreaks produced two cytotoxins, which had similar cytotoxic effects on HeLa cells as the toxins produced by *Shigella dysenteriae* type 1. These toxins were subsequently named Shigella-like toxin 1 and 2 (SLT-I and SLT-II) (O' Brien & Holmes, 1987). Concurrently, Verotoxin research revealed that two distinct forms of VT existed, which were named VT1 and VT2 (Scotland *et al.*, 1985). VT1 and VT2 were subsequently found to be identical to SLTI and SLTII (O' Brien & Holmes, 1987). SLT-I has been shown, by cloning and sequencing, to have >99% homology with the Shiga toxin (Stx) produced by *S. dysenteriae* (Strockbine *et al.*, 1988). Henceforth, for the purpose of this thesis, the toxins produced by *E. coli* O157:H7 will be referred to only as Shiga, or Shiga-like toxins Stx1 and Stx2. Additionally, Shiga-toxin producing *E. coli* will be referred to as STEC.

1.3 Pathogenicity and Virulence

Sequencing has shown that the genomes of *E. coli* O157:H7 strains, EDL 993 (Hayashi *et al.*, 2001) and Sakai (Perna *et al.*, 2001) are approximately 5.5Mb

compared with the smaller 4.5 Mb genome of K-12 *E. coli* strain MG1655. While these three strains share a common core of 4.1Mb, the genomes of O157 strains are approximately 1.34 Mb larger than K-12 genomes (Hayashi *et al.*, 2001; Karmali *et al.*, 2009; Perna *et al.*, 2001). Much of this extra genome is likely to have been acquired by horizontal gene transfer (Hacker & Carniel, 2001; Hacker & Kaper, 2000), and is comprised of mobile gene cassettes, known as O-islands (Karmali *et al*., 2003), or pathogenicity islands (PAI) (Jores *et al.*, 2004). Many of these cassettes are associated with bacteriophages, and contain the virulence genes which are attributed with the virulence and pathogenicity of *E. coli* O157:H7, for example the phage encoding Stx (Reid *et al.*, 2000; Wick *et al.*, 2005). It is suggested that *E. coli* O157:H7 evolved from the non-toxigenic EPEC strain O55:H7, through the acquisition of the bacteriophages encoding the Stxs (Reid *et al.*, 2000; Wick *et al.*, 2005), the acquisition of the O157 antigen and the large pO157 plasmid (Feng *et al.*, 1998; Wick *et al.*, 2005). Additionally, octamer-based genome sequencing has shown two distinct lineages (I and II) of GUD- and SOR- *E. coli* O157:H7 strains (Kim *et al*; 1989 and 2001). One lineage developed through acquisition of the stx1-encoding phage, but lost both beta- glucuronidase (GUD) and sorbitol fermentation (Sor-) properties. The second lineage, lost motility and retained both SOR and GUD. The strains most commonly associated with human disease are those deriving from lineage I. (Kim *et al*., 2001)

The virulence and pathogenicity of *E. coli* O157:H7 is considered to be largely due to the organism's ability to produce Shiga-toxins, Stx1 and Stx2 encoded by bacteriophages (Karch *et al.*, 2005; Spears *et al.*, 2006). The induction of phage expression, and therefore the induction of Stx expression is an important factor in disease caused by *E. coli* O157:H7 Stx2 is more toxic to endothelial cells than Stx1(Karmali, 2004). Shiga toxins pass through the epithelial layer of the intestine, and exacerbate the microvascular endothelial cells that line the blood vessels of the colon, which leads to the bloody diarrhea associated with EHEC infection (Kaper *et al*., 2004; Karch *et al*., 2005; Spears *et al*., 2006). These toxins are responsible for the development of Haemolytic Uremic Syndrome (HUS), due to their action on the endothelial cells of the glomeruli of the kidney (M Bielaszewska & Karch, 2005). However, production of Shiga toxins alone is not

enough to cause the disease associated with EHEC. Adherence and subsequent colonization of the intestinal mucosa is also a key determinant of EHEC virulence (Caprioli *et al*., 2005; Paton & Paton, 1998).

The mechanism, by which *E. coli* O157:H7 adheres to the mucosal lining of the intestine, and subsequently induces lesions **(Figure 1.1)** is known as attaching and effacing (A/E) (Kaper *et al*., 2004). A/E lesions are typified by effacement of microvilli, and intimate adherence of the bacterial cell to the epithelial membrane. Following adhesion, actin is accumulated beneath the bacteria and cytoskeletal changes occur (Karmali *et al.*, 2009; Nataro & Kaper, 1998). The genes associated with A/E adhesion are located on a large PAI known as the Locus of Enterocyte Effacement (LEE) (Jores *et al.*, 2004). The LEE locus is comprised of 5 operons which are all essential for the establishment of intimate adherence of EHEC (Caprioli *et al.*, 2005; Naylor *et al.*, 2005). These include genes encoding intimin, the translocated intimin receptor (Tir) and the type III secretion system (TTSS) components, EspA, B and D (Caprioli *et al*., 2005). Intimin is a bacterial adhesin encoded by *eae*, which mediates intimate attachment of *E. coli* O157:H7 to eukaryotic cells. Tir is an effector protein that binds intimin following insertion into the epithelial cell membrane (Naylor *et al*., 2005). It is the interaction of Intimin with Tir that results in intimate attachment of the bacteria to the host cell, ultimately resulting in the pedestal formation. TTSS is utilised to inject several effector proteins, including Tir, into the endothelial cells, which then subvert the cellular functions of the host, for the benefit of the pathogen.

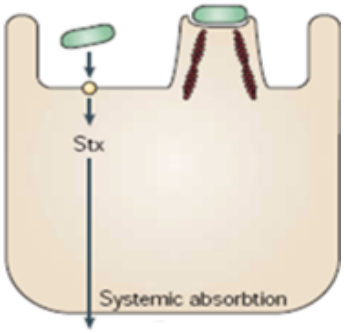


Figure 1.1 Pathogenic Schema for E. coli O157:H7

E. coli O157:H7 adheres to the epithelial cells of the colon, and induces attaching and effacing lesions. The Shiga toxin (Stx) is transported from the bacteria through to the host blood stream, to the kidney where it causes potentially fatal disease. Adapted from Kaper *et al*., (2004), Page 124.

1.4 Symptoms of *E. coli* O157:H7 infection

E. coli O157:H7 food poisoning is normally a result of infection following colonization of the host's intestines. The infectious dose for *E. coli* O157:H7 is very low, with reports of disease caused by as few as 5-500 organisms (Karmali, 1989, 2005), and generally below 100 organisms (Tilden *et al*., 1996). Symptoms of *E. coli* O157:H7 infection range from mild diarrhea to haemorrhagic colitis characterised by haemorrhagic diarrhea and severe abdominal cramps. The severest symptoms usually occur in children under the age of five, or in the elderly or the immune-compromised. These symptoms result from Haemolytic Uremic Syndrome (Robson, 2000), and Thrombotic Thrombocytopenic Purpura (TTP) (Karch *et al*., 2005). Symptoms of HUS and TTP include abnormal bleeding, acute kidney failure, seizures, and coma and can lead to death. HUS develops in approximately 5-10% of children with haemorrhagic colitis caused by *E. coli* O157:H7 (Robson, 2000), and of these, 3-5% cases will result in death (Nataro & Kaper, 1998). Many survivors experience long term complications (Robson, 2000).

1.5 Reservoirs, modes of transmission and significance in the food industry

E. coli O157:H7 is transmitted by faecal-oral route. The intestine of warm-blooded animals is the primary niche (Savageau, 1983), however its survival in both soil and water environments has been well documented (Vital *et al.*, 2008). Transmission routes **(Figure 1.2)** for *E. coli* O157:H7 include contaminated food, particularly of animal origin; contact with live animals carrying *E. coli* O157:H7; or through person-to-person contact following human infection (Karch *et al*., 2005; Michino *et al*., 1998).

1.5.1 Animals as reservoirs for *E. coli* O157:H7

A wide range of animals can act as reservoirs for *E. coli* O157:H7. Studies indicate that STECS in general are more prevalent in ruminants than other animals (Hussein, 2007; Karmali, 1989; Mainil & Daube, 2005) with beef and dairy cattle most often implicated in *E. coli* O157:H7-associated illness in humans (Hussein, 2007). Other reservoirs include sheep (Chapman *et al.*, 1997), goats (M.

Bielaszewska et al., 1997), horses, dogs, deer, (Karch *et al*., 2005), pigs, rabbits, poultry, (Leclercq & Mahillon, 2003; Naylor *et al*., 2005), and house-flies (Sasaki *et al.*, 2000).

1.5.2 Ruminants as principal reservoirs for *E. coli* O157:H7

Ruminants, particularly cattle, are the principal reservoirs for *E. coli* O157:H7 (Karch *et al*., 2005), where the organism colonizes the recto-anal junction (Rice *et al.*, 2003). Importantly, although strains of *E. coli* O157:H7 isolated from cattle have been implicated in human disease, the majority of these strains are not associated with disease in cattle.

Between 2002 and 2005, the prevalence of *E. coli* O157:H7 in beef cattle was estimated to be between 10 and 28% in North America. with similar levels estimated in Europe for beef and dairy cattle between 1999-2007 (Karmali *et al*., 2009). Recent research in New Zealand looking at prevalence on three Waikato dairy farms, revealed overall that 77% of adult dairy cows sampled, were positive for *E. coli* O157:H7(H Withers *et al.*, 2009). However prevalence levels can vary depending on numerous factors which may include the diet of livestock (Hancock *et al.*, 2001), pasture management or animal husbandry practices (H Withers *et al*., 2009), seasonal variation (Chapman *et al.*, 1997; Ogden *et al.*, 2004), all of which alter the shedding rate and therefore the detectability of *E. coli* O157:H7. Furthermore variations in sampling and enumeration techniques make it difficult to draw accurate comparisons (La Ragione *et al.*, 2009).

Generally, transmission from ruminants to humans is through the consumption of foods of ruminant origin, such as undercooked beef patties and unpasteurised milk and cheeses (Karmali *et al*., 2009). However, transmission can occur by direct contact with live animals including cattle and calves (Ellis-Iversen *et al.*, 2009; Karch *et al.*, 2005), which can occur on farm or at petting zoos (Caprioli *et al*., 2005). Interestingly, *E. coli* O157:H7 has been isolated from farm workers that have not presented with symptoms of *E. coli* O157:H7 disease (Caprioli *et al*., 2005), which suggests that farm workers may develop a carrier state, and have immunity to *E. coli* O157:H7.

1.5.3 Other Sources for *E. coli* O157:H7

There are numerous animals other than ruminants suggested as a source for *E. coli* O157:H7. However, there is little evidence presented to date, that proves that non-ruminants are as important as ruminants with regard to *E. coli* O157:H7 transmission (Naylor *et al*., 2005).

Dispersal of animal manure can occur throughout the environment (Caprioli *et al*., 2005) and as a result, secondary fomites, such as farming equipment, water, fruits, vegetables and people may become vehicles for *E. coli* O157:H7 transmission. Water, either consumed (Caprioli *et al*., 2005) or used for recreational purposes such as swimming, has been implicated in large outbreaks of *E. coli* O157:H7 (Holme, 2003; Karch *et al*., 2005; Vital *et al*., 2008). Fruit and vegetables linked to outbreaks or sporadic infections of *E. coli* O157:H7 include alfalfa, radish, broccoli and mung bean seed (Bari *et al.*, 2009); iceberg lettuce (Taormina *et al*., 2009); apple cider and unpasteurised fruit juices (Besser *et al*., 1993; Caprioli *et al*., 2005).

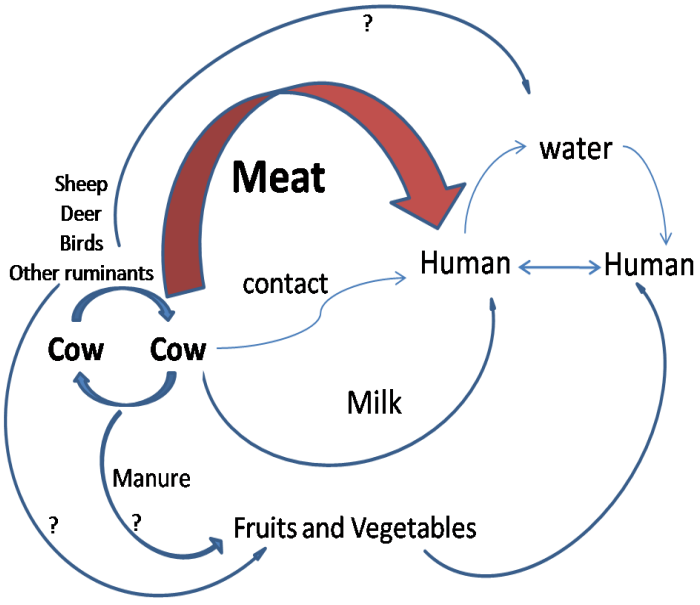


Figure 1.2. Current understanding regarding the potential modes of transmission for *E. coli* O157:H7.

Adapted with permission from Tauxe (1998). Chapter 45, page 448.

1.6 Significance of *E. coli* O157:H7 in the food and meat industry

E. coli O157:H7 has become one of the most significant and recognised food-borne pathogens known (Meng & Doyle, 1998), with cases being reported worldwide. According to a summary of notifiable diseases, reported by the United States (US) Centers for Disease control and Prevention (CDC, 2008), approximately one case of *E. coli* O157:H7 infection has occurred per 100 000 people in the US. In New Zealand, the incidence of *E. coli* O157:H7 has increased from 80 confirmed isolates in 2006, to 120 confirmed isolates in 2008 (ESR, 2008). It is possible that this increase is the result of improved screening and isolation methods for this pathogen.

A review by Stopforth *et al.,* (2007) indicated that in the US alone, a total of US$0.7 billion was lost due to medical costs, loss of productivity and premature death resulting from *E. coli* O157:H7 infections. Most outbreaks were food-borne in origin. Furthermore, current trends in food processing result in the rapid distribution of immense quantities of potentially contaminated products, over large geographic areas in relatively short time periods (Karmali *et al*., 2009), which creates the potential for large scale outbreaks to occur world-wide. In the US, *E. coli* O157:H7 has been declared an adulterant in consumables, hence pre- and post production interventions designed to eliminate *E. coli* O157:H7 from food are critical to food processing industries (Hussein, 2007).

Over the last 20 years, many *E. coli* O157:H7 outbreaks were attributed to the consumption of undercooked, or improperly pasteurized beef and dairy products, and are therefore considered to be the key vehicles by which *E. coli* O157:H7 can enter the food chain (Karch *et al*., 2005). Understanding the relationship between this pathogen and its primary reservoir, namely cattle, has become important to both the dairy and beef industry. Hence most studies have focused on beef and dairy cattle (Hussein, 2007).

The chief source for *E. coli* O157:H7 contamination of meat, is faecal material present on cattle hides (Meng & Doyle, 1998). Beef carcasses are normally contaminated with *E. coli* O157:H7 during the removal of the hide or during

evisceration (Hussein, 2007). A correlation does exist between carcass hygiene and the level of intestinal infection, faecal excretion (Mainil & Daube, 2005), and prevalence of *E. coli* O157:H7 on hides (Elder *et al*., 2000). Mainil and Daube (2005) proposed that if 30% of beef carcasses were positive for the presence of *E. coli* O157:H7, at some point in the slaughter process, a further 7% of the remaining carcasses could become contaminated. Importantly, because *E. coli* O157:H7 is a microbe which is well adapted to a transient life-style, it follows that secondary contamination can occur throughout the entire food chain. Secondary contamination can occur despite attempts by food manufacturers to implement critical control methods to reduce pathogen proliferation.

Operating under the Hazard Analysis and Critical Control Point (HACCP) programme published in 1996 (Hulebak & Scholsser, 2001), meat processors employ various technologies and intervention strategies to reduce pathogens, improve overall microbial quality of carcasses and comply with food safety regulations set by the United States Department of Agriculture – Food Safety and Inspection Service (Edwards & Fung, 2006; Stopforth *et al*., 2007). Standard practices include spot cleaning by knife trimming or steam vacuum (Edwards & Fung, 2006), carcass washing with hot or cold water (Sofos & Smith, 1998), refrigeration or freezing, and acid or antimicrobial sprays and carcass washes (Stopforth *et al.*, 2007; Stopforth *et al.*, 2004). Use of combinations of these intervention strategies is referred to as multiple hurdle interventions ((Karmali *et al*., 2009; Koohmaraie *et al*., 2007). One of the most important strategies employed to reduce microbial growth is the controlled reduction of carcass temperature, through processes such as spray chilling, refrigeration (Lovatt, 2004) and freezing (Dickson, 1991; Lenahan *et al*., 2009; Stopforth *et al*., 2004).

Although HACCP strategies have been shown to reduce general microbial contamination, (Carlson *et al.*, 2008; Hardin *et al.*, 1995), there is concern that *E. coli* O157:H7 will adapt, survive and eventually proliferate in the niche environments created by these practices (Samelis *et al.*, 2005; Stopforth *et al.*, 2007). For example it has been demonstrated that *E. coli* O157:H7 can grow at carcass chill temperatures (Edwards & Fung, 2006; Stopforth *et al*., 2004).

Survival studies performed by Dykes *et al*., (2001) on preservatively packaged (vacuum and carbon dioxide) primal beef cuts, established that numbers of *E. coli* O157:H7 did not change significantly over a period of 6 weeks at -1.5°C, nor after a further 2 weeks at 4°C. Additionally, while studies have shown that freezing can cause damage to cells (Meng & Doyle, 1998), *E. coli* O157:H7 can survive in ground beef which has initially been stored at -80°C and then held at -20°C for up to 9 months. Furthermore, in three different freezing profiles tested by Dykes *et al*., (2006) where beef trim samples, starting at the initial temperature of 25°C, were frozen over different time frames (32, 40 and 44 hours) to a final temperature of -23°C, no significant changes in numbers of *E. coli* O157:H7 occurred. In the same study however, the authors suggested that a slight but significant decrease in the pathogen number was seen if the freezing profiles started at 12°C rather than 25°C.

E. coli O157:H7 is tolerant of acidic environments, (Oh *et al*., 2008). This tolerance enables *E. coli* O157:H7 to survive in foods, such as salami, which traditionally rely on acidic or low pH conditions to inactivate pathogens (Buchanan & Edelson, 1996; Leyer *et al.*, 1995; Meng & Doyle, 1998). Studies by Bracket *et al*., (1994) and Conner *et al*., (1997) demonstrated that organic acid solutions sprayed on beef carcasses are relatively ineffectual against *E. coli* O157:H7. Furthermore, *E. coli* O157:H7 can overcome a number of the components of multiple hurdle interventions, where adaptation to one intervention, such as acid sprays, may bring about increased adaptation to others with the process. For example, acid adaptation of *E. coli* O157:H7, has been shown to increase the survival for up to 14 days in acid-wash stored at 4°C and 10°C (Samelis *et al.*, 2002).

Non-meat examples of *E. coli* O157:H7 resistance to intervention strategies include low pH products such as mayonnaise and yoghurt (Meng & Doyle, 1998); acidic fruit juices, such as apple juice (Caprioli *et al*., 2005; McDowell & Sheridan, 2001). In addition *E. coli* O157:H7 survives better in reduced water activity (aw) foods such as dry rice cereal at refrigeration temperatures (Beuchat, 1996).

Overall, studies suggest that *E. coli* O157:H7 can adapt to stresses, targeted at its reduction or elimination. The potential exists for these pathogens to establish niches within food processing environments, from which future cross contamination can occur.

1.7 Adaptive stress responses in *E. coli* O157:H7

E. coli O157:H7 is capable of living in complex environments, both within the gut of an animal host, and in the external environment. Such surroundings undergo constant chemical and physical changes, and to survive *E. coli* O157:H7 must respond and adapt rapidly to these changes. Using coordinated regulatory networks, *E. coli* O157:H7 senses environmental stressors and expresses proteins critical counteract the negative impacts such changes may have (Giuliodori *et al.*, 2007). Stimuli that *E. coli* O157:H7 would need to respond to rapidly include changes in, pH, oxygen concentration, nutrient availability and shifts in temperature (Wick & Egli, 2004).

Overall, the general stress response for bacteria involves a cascade of changes in gene expression and protein activity, which enable bacteria to survive rapidly changing environmental conditions (Giuliodori *et al*., 2007). Nucleic acids (DNA and RNA), proteins and small effector molecules are all involved in the regulation and coordination of bacterial stress responses (Wick & Egli, 2004). The general stress response for *E. coli* is mediated by the sigma factor, RpoS (σ^S) (Wick & Egli, 2004). In *E. coli*, RpoS activity is regulated at the level of proteolysis, protein activity, transcription and translation (Hengge, 2008). Various triggers, such as entry into stationary phase, acid pH, oxidative stress, heat and cold shock can trigger the induction of σ^S (Wick & Egli, 2004). According to Hengge (2008), approximately 10% of the *E. coli* genome (almost 500 genes), are under some form of RpoS control, either directly or indirectly. Using *E. coli* K-12, White-Zeigler *et al*., (2008) used microarrays to demonstrate that 297 genes, approximately 7% of the genome, have increased expression at 23°C compared with 37°C. Many of those genes are regulated by RpoS, supporting the theory that in the environment, low temperature is a primary trigger for the general stress response.

1.7.1 Cold Shock response

Rapid temperature down-shift (e.g., from 37°C to below 20°C) also known as cold shock (Giuliodori *et al.*, 2004; Giuliodori *et al.*, 2007), is one of the major environmental stresses that *E. coli* O157:H7 must adapt to. Such transitions in temperature would occur for example when the organism is shed from the colon of a warm-blooded animal into soil or water (Phadtare *et al.*, 2000). Specific information regarding the cold shock response in *E. coli* O157:H7 itself is limited with most information derived from research using lab-adapted *E. coli* K-12 strains.

Generally, a reduction in temperature brings about physical changes in the bacterial cell, which have a negative effect on cell functioning. Cell membrane fluidity tends to decrease (Phadtare *et al*., 2000), protein folding becomes less efficient, ribosome functions are stalled and secondary nucleic acid structures stabilise, which reduces the efficiency of mRNA translation and transcription (Wick & Egli, 2004). All of these ultimately result in the arrest cell growth (Phadtare, 2004). Adaptation to these new unfavourable conditions, is required in order for *E. coli* O157:H7 to survive (Wick & Egli, 2004), adapt to, and depending on temperature, eventually resume growth (Jones *et al*., 1987).

The strategy employed by *E. coli,* to counteract the effects brought about by temperature downshift, is complex, and lasts approximately 4 hours (Giuliodori *et al*., 2004). The process largely involves the induction of cold shock genes from the CspA family which act to regulate the expression of proteins required for adaptation (Wick & Egli, 2004). During this time, a set of at least 25 to 27 cold-shock proteins is selectively expressed (Giuliodori *et al.*, 2004) which include nucleic acid-binding proteins involved in the numerous cellular processes such as translation, transcription, DNA replication and supercoiling, RNA degradation, and ribosome maturation. Bulk synthesis of proteins is also repressed.

There are at least five cold shock inducible members of the cold shock protein family of *E. coli* - CspA, the major cold shock protein (Al-Fageeh & Smales, 2006; Wick & Egli, 2004), CspB, CspE, CspG and CspI (Giuliodori *et al*., 2007).

Members of the CspA family are capable of substituting for each other during adaptation to cold temperature (Phadtare & Inouye, 2004). Although there are approximately nine proteins in *E. coli* showing high homology to CspA (Wick & Egli, 2004), not all are inducible by cold shock (Giuliodori *et al*., 2007) Al-Fageeh & Smales (2006) suggest three mechanisms that increase synthesis of the key CSPs: the presence of *cis*-elements in mRNAs that enhance translation during cold-shock; *trans*-acting factors which form part of the translational mechanisms of cold shocked cells, and target cold-shock mRNAs; and modification of translational machinery in order to bring about selective mRNA translation. Some cold shock proteins enable ribosomes to become cold-adapted ribosomes **(Figure 1.3),** which enables the cell to restore translation of bulk mRNA (Wick & Egli, 2004).

The major cold shock protein CspA, is an RNA-binding protein, which is thought to improve translation by specifically binding to single stranded non-duplexed RNA regions, preventing the formation of mRNA secondary structures (Al-Fageeh & Smales, 2006). Levels of CspA , the major cold shock protein, increase to more than 10% of the cell's total protein following temperature downshift, due to stabilisation of *cspA* mRNA. Al-Fageeh & Smales (2006), suggested that CspA is only synthesized upon cold-shock exposure, however Brandi *et al*., (1999) demonstrated that *cspA* is expressed under non-stress conditions, and that transcription of *cspA* is regulated by Fis and H-NS DNA-binding proteins. Furthermore Brandi *et al*., (1999) showed that CspA is involved in its own feedback auto-repression. CspA acts as a transcriptional activator and a transcription anti-terminator (Wick & Egli, 2004) leading to the induction of the additional cold shock proteins. CspA becomes unstable, following the adaptation of *E. coli* cells to cold temperature (Goldenberg *et al.*, 1996), and the mRNA is selectively degraded by the polynucleotide phosphorylase enzyme PNP (Wick & Egli, 2004).

Initiation of translation, or protein synthesis, is a key determinant in the growth and survival of a cell. Bacteria require initiation factors (IFs), IF1, IF2 and IF3 for this. After a down-shift in temperature, the number of ribosomes decrease, and

the level of IFs increase, resulting in an imbalance in the ratio of IFs to ribosomes (Giangrossi *et al.*, 2007). This ratio imbalance creates a cold-shock induced translational bias (Giuliodori *et al.*, 2004), causing translation of cold-shock mRNAs at low temperature. Using the *E. coli* strain MRE600, Giangrossi *et al*., (2007) studied the regulation of *infA* gene, which encodes IF1, as a model for pre- and post-cold-shock regulation. The results of that study showed that within the first few hours of cold adaptation, due to *de novo* transcription and translation of *infA*, IF1 increased three-fold. But this increase varies, depending on the basal level of the transcript at the time of stress. Upon cold-shock, activation of transcription from the promoter (P1) is brought about by the induction of cold-shock protein CspA, and the reduced repression of H-NS (transcriptional regulator). Interestingly, Guilodori *et al*., (2004) also demonstrate that if the basal level of *infA* is low at the time of cold-shock, then transcription of *infA* is brought about by P1 only. Conversely, if cell density is high, and hence the basal level of *infA* is also high, then cold-shock transcription involves 2 promoters, P1 and P2, albeit P1 is still the main cold shock promoter (Giuliodori *et al.*, 2004).

Another factor that may be important for gene regulation in bacteria undergoing cold-shock is DNA supercoiling. DNA supercoiling can effect DNA transcription and hence gene expression (Drlica, 1992). Negative DNA supercoiling has been shown to increase after a temperature downshift resulting in decrease RNA polymerase DNA interaction (Phadtare, 2004). It has also been suggested chaperones are important to cold tolerance and adaptation in bacterial cell systems. By using a series of strains in which key cold adaptation genes were deleted, Strocchi *et al*., (2006) were able to show that the inability of *E. coli* to grow at 4°C was due to the inactivation of GroELS chaperonins. The lack of GroELS prevented proteins such as Dps, ClpB, DnaK and RpsB from refolding and regaining function at low temperatures.

While many of the cold shock processes are now understood, the cold shock response system is possibly one of the most complex bacterial response systems (Phadtare & Inouye, 2004) and it is likely, particularly in the case of *E. coli*

O157:H7 which has a massive genome, that many more genes and systems remain to be elucidated.

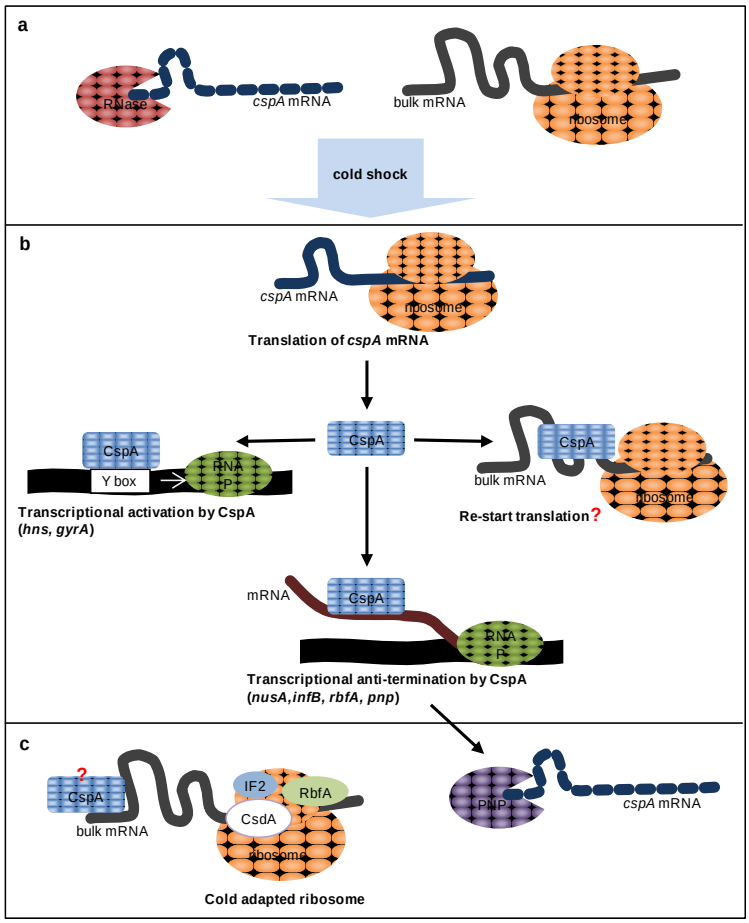


Figure 1.3 The cold shock response. Adapted from Wick and Egli (2004) Page 21.

a) Following cold shock, un-dapted ribosomes are unable to translate mRNA. An exception is mRNA for the major cold shock protein CspA (and probably others: CspB, CspI, CspG, CsdA, RbfA). *cspA* mRNA is stabilized at low temperatures, which increases the levels of cellular CspA.

b) CspA functions as a transcriptional activator and a transcription antiterminator. Further cold shock proteins are induced. CspA might also operate as a chaperone for mRNA, possibly resolving secondary structures which will then enable bulk mRNA to be translated. The ribosome is changed to a cold-adapted ribosome by some of the cold shock proteins, restoring the cells ability to translate bulk mRNA.

c) After acclimation, cold shock proteins are reduced to a basal level. A negative feedback loop, mediated by PNP, degrades mRNAs of cold shock proteins. CspA negatively regulates its own synthesis. The Dashed line represents degradation of mRNA by RNase activity or by PNP.

1.7.2 Other stress responses

Other continuously changing environmental cues that *E. coli* O157:H7 responds to include osmolarity, nutrients, oxygen availability, pH, and chemicals including quorum sensing molecules. The most well defined responses to such stressors include the envelope stress response, which is in response to changes in the extracytoplasmic space of gram negative bacteria (Wick & Egli, 2004); the stringent response, which reduces cellular protein synthesis when substrates for protein synthesis are limited (Wick & Egli, 2004); and the acid stress resistant systems, including the acid induced oxidative system, a glutamate-dependent system and the arginine-dependent system (Meng & Doyle, 1998). The acid stress response is of particular importance in EHEC, as it is the ability of EHEC to survive gastric acidity which has been correlated to the low infectious dose required to cause disease in humans (Gorden and Small, 1993).

Many of the adaptive stress responses can be effective against other types of stressors. Studies have shown that a variety of stresses have similar effects on the molecular activity of cells (Wick & Egli, 2004), and stress mechanisms often appear to be linked to each other. Thus, the adaptation to one stress can lead to resistance to another.

Furthermore, upon transition from the animal host to the external environment, *E. coli* O157:H7 faces changes in population density and composition. Concentrations of *E. coli* in its principal habitat can vary between 10^5 and 10^8 colony forming units (cfu) per gram of faeces, with much lower numbers reported in soil and water (Vital *et al.*, 2008). Considering that the population density can change, it follows that changes in the chemical signals to which the organism responds to will occur. Bacteria are known to monitor population density, and modify their cellular processes accordingly (H. Withers *et al.*, 2001). The process in which bacteria monitor population density through chemical signaling is known as quorum sensing (QS). It has been suggested that QS is intimately involved with stress and starvation mediated cellular activity in *E. coli* (DeLisa *et al.*, 2001).

1.8 Quorum Sensing

Quorum sensing (QS) is a term used to describe a complex process in which bacteria secrete and utilise small diffusible signaling molecules, known as autoinducers, in order to communicate cell-to-cell in a density dependent manner (J. M. Henke & B. L. Bassler, 2004; Schauder *et al.*, 2001; Zhang *et al.*, 2008). Bacteria are capable of monitoring their environment by detecting concentrations of autoinducers (E. P. Greenberg *et al.*, 1979; H. Withers *et al.*, 2001), and subsequently altering gene expression and regulation of a number of cellular functions. Altered cellular functions, include expression of virulence factors, swarming behavior, antibiotic production, formation of biofilms (Rezzonico & Duffy, 2008). QS has also been implicated in the stress response (DeLisa *et al.*, 2001). Henke and Bassler (2004) suggested that this form of chemical communication can occur, not only between bacterial species (inter-species), but also between the bacteria and the host, (inter-kingdom communication). Walters and Sperandio (2006), point out that the potential for quorum sensing to occur between the bacteria and host, in which the organisms can coordinate adaptive responses, should not be surprising, considering the diversity and high concentration of bacteria that reside in the gastrointestinal tract (10^{11}-10^{12} bacterial cells/ml). However, while quorum sensing is very popular, an alternative hypothesis for the function for autoinducers was suggested (Redfield, 2002),. This phenomenon, called diffusion sensing (Redfield, 2002), uses autoinducers to sense the dynamics of their immediate environment, and not necessarily the population density alone (Redfield, 2002. Although the opinion has thus far been overlooked by many researchers, Turovskiy *et al.*, in a critical review of the quorum sensing phenomenon (2007) suggested that perceptions may eventually shift more towards an environmental sensing theory.

1.8.1 QS In Gram negative bacteria

Originally, QS was used to describe the regulation of bioluminescence in the gram negative marine symbiont *Vibrio fischeri* (*V. fischeri*) (Nealson *et al.*, 1970; Walters & Sperandio, 2006). At high cell density, bioluminescence occurs in these marine bacteria due to the expression of luciferase (**Figure 1.4A**), which is regulated by two proteins, LuxI and LuxR (Winans & Bassler, 2002). LuxI

synthesizes acylated homoserine lactone (AHL) (Walters & Sperandio, 2006), also known as autoinducer-1 (AI-1) (B Bassler *et al.*, 1997). LuxR detects and binds the AHL (3-oxo-C6-HSL). This complex binds to the *lux* promoter inducing expression of luciferase (Stevens *et al.*, 1994). Following the discovery of this first quorum sensing pathway, homologues of LuxR-LuxI have been identified in over 70 gram negative species (Henke & Bassler, 2004). So far, 12 AHL autoinducers (AI-1) have been identified, differing only on the AHL-acyl side chain moiety (Rezzonico & Duffy, 2008; Steindler & Venturi, 2007). Moreover, five further QS systems, have been identified along with their respective QS molecules (Yoon & Sofos, 2008a). These molecules include: AHL/AI-1, associated with gram negative bacteria (Bassler *et al.*, 1997); Autoinducer 2 (AI-2), a furanosyl borate diester, also found in gram negative and positive bacteria; a newly described Autoinducer 3 (AI-3), which is an aromatic compound (Walters & Sperandio, 2006) that may be involved in cross-communication between bacteria and host cells (Sperandio *et al.*, 2003); *Pseudomonas* quinolone signal family (PQS) (Dubern & Diggle, 2008); and for gram positives only, the AI-peptides (Miller & Bassler, 2001; Yoon & Sofos, 2008a).

Some gram negative bacteria utilise more than one QS system. *Vibrio harveyi* (*V. harveyi*) has two interlinked QS systems **(Figure 1.4B)**, in which AI-1 is synthesized by LuxM, and detected by the sensor LuxN, while AI-2 is produced by LuxS and detected by LuxPQ (B Bassler *et al.*, 1997; Mok *et al.*, 2003). LuxS converts *S*-ribosyl-homocysteine (SRH) into homocysteine releasing 4,5-dihydroxy-2,3-pentanedione (DPD), which cyclises to yield the AI-2 molecule (Schauder *et al.*, 2001). Exogenous AI-2 is subsequently detected by the sensor kinase LuxPQ (Rezzonico & Duffy, 2008). Both AHL and AI-2 QS systems are partially connected and share the same transduction pathway, through LuxU (signal relay protein) to LuxO (terminal response regulator) (Reading & Sperandio, 2006). The LuxS/AI-2 system, is currently recognised as the most widely spread bacterial QS system and has been identified in numerous bacterial species including *E. coli* O15:H7 (M. G. Surette *et al.*, 1999).

Unlike AI-1/AHLs, which are highly specific and for the most part only used for intra-species communication (Zhang *et al*., 2008), LuxS and its synthase AI-2, are highly conserved among numerous bacterial species, (Bassler, 2002; Soni *et al.*, 2008). Hence, AI-2 has been suggested to be a universal bacterial signal molecule, likened to a form of bacterial Esperanto (Turovskiy *et al.*, 2007; Winans, 2002), and not surprisingly this molecule has commanded much interest. Before the AI-2 QS system was fully characterized, Bassler *et al*., (1997) constructed a *V. harveyi* reporter strain, named BB170 (Δ*luxN*), allowing detection of the signaling molecule in spent media. This AI-2 biosensor detection system has been widely used for the detection of AI-2 production by different bacterial species.

1.8.2 LuxS/AI-2 QS in *E. coli* O157:H7

Although AHL detection occurs in *E. coli* O157:H7, it has not been shown to secrete AHLs. Like *Salmonella enterica*, *E. coli* species have a LuxR homologue known as SdiA (Wang *et al.*, 1991), but no homologues for LuxI have been identified (Subramoni and Venturi, 2009). However, AHLs have been shown to influence gene expression in an SdiA-dependent manner in *E. coli* K-12 (van Houdt *et al*, 2006). LuxS/AI-2 and possibly AI-3 are also active in *E. coli* O157:H7 (Schauder *et al.*, 2001; Walters & Sperandio, 2006).

Several studies, involving the comparison of LuxS mutants with wild type strains, have described diverse roles for LuxS in *E. coli*. For example, LuxS has been shown to control virulence determinants such as motility and biofilm formation (Rezzonico & Duffy, 2008), and the regulation of LEE-encoded type III secretion system (TTSS) and flagella expression (Walters & Sperandio, 2006). Interestingly, while LuxS was linked to the expression of LEE genes (Sperandio *et al.*, 1999), it was later suggested that it was not AI-2 that was responsible, but AI-3 (Reading & Sperandio, 2006). Although AI-3 synthesis is not directly attributable to LuxS activity, the metabolic effect on cellular activities of disturbing the methyl cycle resulted in decreased levels of AI-3 (Walters et al, 2006). Complementation using *Pseudomonas aeruginosa* (*P. aeruginosa*) *S*-adenosylhomocysteine hydrolase (SAH) that synthesizes homocysteine directly

from SAH in the luxS mutant restored AI-3 but not AI-2 synthesis (Walters et al, 2006).

Regulation of *E. coli* LuxS and AI-2 production is influenced by a variety of environmental cues such as the presence of certain carbon sources, pH and osmolarity (DeLisa *et al.*, 2001; M. G. Surette *et al.*, 1999). There is accumulating evidence to support the theory that AI-2 regulation may be used for channeling conditions of stress into the QS- dependent regulatory circuit. For example, *E. coli* conditioned medium (CM) contains a factor, later named AI-2, that stimulates expression of *rpoS* (σ^S) (DeLisa *et al*., 2001). Furthermore, stimuli that are known to induce *rpoS* responses, such as heat shock and 4% ethanol, have been shown to reduce AI-2 levels, albeit the reduction was then followed by oscillating increases and decreases of AI-2 levels before finally stabilizing. (Winzer *et al.*, 2003). Searches in literature, for a potential role of QS, specifically in cold stress responses, have proven scarce. However, AI-2 has been shown to be produced in foods such as milk and chicken broth by *E. coli* O157:H7 at various storage temperatures, including 4°C (Yoon & Sofos, 2008b). DeLisa *et al*., (2001) suggest that autoinduction is linked to the stress response for abnormal protein formation. Because cold temperature can effect protein formation, it could be speculated that QS might be involved in the regulation of the cold stress response.

There is, however, some ongoing debate regarding the primary role of LuxS, and AI-2. Apart from in *V. harveyi*, it is been suggested that LuxS is more involved in metabolism than QS, because it is plays an integral role as an enzyme in the activated methyl cycle, where it recycles *S*-adenosylmethionine (SAM) (Winzer *et al*., 2003). Furthermore, in *V. harveyi*, only the signal itself, and not the AI-2 molecule, is transduced inside the cell, whereas in *E. coli*, AI-2 is phosphorylated inside the cell, where it purportedly interacts with LsrR which can act as a regulator of gene expression by repressing the *lsr*-operon. It has thus been suggested that AI-2 is actually being released as a waste product and then being reused as a metabolite (Rezzonico & Duffy, 2008), rather than being at true signaling molecule.

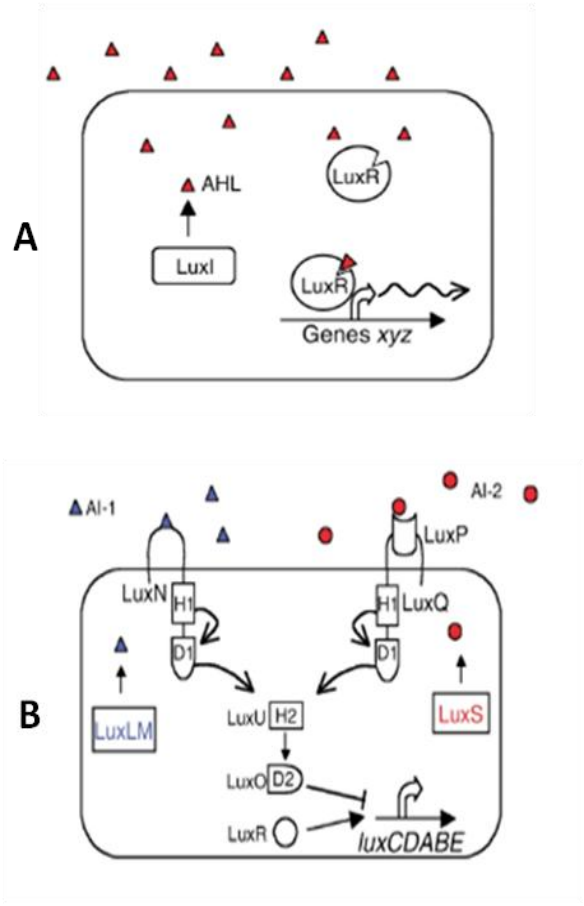


Figure 1.4. Quorum-sensing systems in Gram negative bacteria.

A) LuxI-like proteins produce AHLs (red triangles). AHLs diffuse across the cell membrane and into the surrounding environment, increasing in concentration in proportion to the population density. Autoinducers are bound by LuxR-type proteins, which bind specific promoter DNA elements and activate transcription of target genes (xyz).

B) Quorum sensing in *V. harveyi*. Two interlinked QS systems produce and detect AI-1 (AHL) and AI-2. AI-1 is produced by LuxLM, and detected by the sensor LuxN, while AI-2 is produced by LuxS and detected by sensor LuxPQ. Both systems share the same transduction pathway through LuxU (signal relay protein) to LuxO (terminal response regulator). In the absence of autoinducers, the sensors autophosphorylate. Phosphate is sequentially transferred to LuxU, then LuxO. Phospho-LuxO represses *luxCDABE* transcription. Binding of Autoinducers by LuxN and LuxPQ dephosphorylates LuxU and LuxO. Dephosphorylation of LuxO alleviates *luxCDABE r*epression. The transcriptional regulator, LuxR (not the same as LuxR in figure A) is required for *luxCDABE* expression.

Adapted from Federle and Bassler (2003) pg 1292

1.9 Statement of Hypothesis

Understanding the adaptive process of *E. coli* O157:H7 at cold temperatures could provide answers regarding the pathogens ability to survive and persist during food production, which often involves multiple interventions targeted to the elimination of this pathogen. One key intervention is chilling, particularly in the meat industry.

Despite the fact that many genes and regulatory systems, have been identified in literature (Giuliodori *et al*., 2007; Turovskiy *et al*., 2007; Wick & Egli, 2004) that enable *E. coli* O157:H7 to adapt to its complex and ever changing milieu. Between 30 to 50% of the genome for this pathogen is made of hypothetical proteins for which no function has yet been assigned (Hayashi *et al.*, 2001). Additionally, an area requiring further exploration is the highly conserved LuxS gene (B. L. Bassler, 2002; Soni *et al.*, 2008) and the AI-2 sensing molecule. Since QS has been implicated in the regulation of a number of cellular processes, it could be speculated that QS is not only important to the survival of *E. coli* O157:H7, but potentially contributes to the organism's ability to adapt to environmental changes, including temperature change.

The overall hypothesis for this study is that *E. coli* O157:H7 is able to withstand chilled temperature by up-regulating genes, many of which may be currently assigned that allow this organism to persist and survive in unfavorable conditions. It is further speculated that QS transitions have overtime become integrated in these stress response pathways, potentially forming an integrated component of the *E. coli* O157:H7 adaptive stress response, including the cold shock response.

The main aim for this project is to identify genes differentially expressed in response to refrigeration temperatures, to confirm their link to temperature down shifts and to identify other factors which might influence their expression. Methods such as: Random Arbitrarily primed polymerase chain reaction (RAP-PCR) (Rivera-Marrero *et al.*, 1998); Reverse Transcription PCR (RT-PCR) (Fislage *et al.*, 1997), and biological sensors such as *luxCDABE* bioluminescent

reporters (Winson *et al*., 1998) will be used to monitor gene expression upon temperature down-shift.

One additional factor that will be analysed in this project is QS, particularly the LuxS/AI-2 system. *luxCDABE* reporters will be used in conjunction with *luxS* deletion strain of *E. coli* O157:H7, CLEN34. Expression of genes in parental and *luxS*$^{-}$ strains will be assessed using growth studies and light reporter assays. AI-2 in the form of CM will be complimented back to the *luxS*- *E. coli* O157:H7 strains, and monitored to assess any effect AI-2 may have on the expression genes, identified in the course of this research.

Chapter 2: Materials and Methods

2.1 Bacterial strains

E. coli strains used throughout this study are listed in **Table 2.1**. Likewise, *V. harveyi* strains are listed in **Table 2.2**.

Table 2.1. ***Escherichia coli***

Strain	**Genotype**	**Source/Reference**
NZRM 3614 (NCTC 12900)	O157:H7 Verotoxin negative (VT-)	New Zealand Reference Culture collection
CLEN34	NZRM 3614 *lux*S::Kan	Katy Enfield
MG1655	$F^-\lambda^-$	Jensen (1993)
DH5α	Φ80δ*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17(r_k^-, m_k^+) *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF V*169), *luxS*	Hanahan (1983)
One Shot™ TOP10	F^- *mcrA*, Δ(*mrr-hsd*RMS-*mcrBC*), Φ80*lacZ*ΔM15, Δ*lacX*74, *recA*1, *araD*139, Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL*, (*Str*^{R}) *endA*1, *nupG*	Invitrogen

Table 2.2. ***Vibrio harveyi***

Strain	**Genotype**	**Source/Reference**
BB120 (ATCC BAA-1116)	Wild Type	Bassler *et al*. (1997)
BB170 (ATCC BAA-1117)	*luxN*::Tn*5*:Kan	Bassler *et al*. (1993)
MM32 (ATCC BAA-1121)	*luxN*::Cm, *luxS*::Tn*5*:Kan	Miller *et al*. (2004)

2.2 Plasmids

Plasmids utilised in this study are listed in **Table 2.3**.

Table 2.3. Plasmids

Plasmid	Description	Source/Reference
pCR2.1	TA cloning vector, ColE1 origin, Amp^R, Kan^R	Invitrogen
pSB377	Promoterless *luxCDABE*, Amp^R	Winson *et al*., (1998)
pWU1	pCR2.1 with *mdtJI* promoter region cloned into MCS	This study
pWU2	pSB377 *mdtJI*::*luxCDABE* promoter reporter – *mdtJI* promoter region inserted on an *Eco*R1 – *Sna*B1 fragment from pWU1	This study

2.3 Media and Antibiotics

2.3.1 Luria-Bertani Broth (LB)

LB, consisting of tryptone (10g/l) (Bacto™, BD), yeast extract (5g/l) (Bacto™, BD) and NaCl (5g/l) (BDH), was prepared in dH_2O. The pH was adjusted to 7.4 prior to autoclaving.

2.3.2 Luria-Bertani Agar (LA)

LA was prepared as LB with the addition of agar at15g/l, (Bacto™, BD). The pH was adjusted to 7.4 prior to autoclaving.

2.3.3 Brain Heart Infusion Broth (BHI broth)

BHI broth (Bacto™, BD) was prepared according to manufacturer's instructions.

2.3.4 Brain Heart Infusion Agar (BHI agar)

BHI agar was prepared as BHI broth with the addition of agar at 15g/L (Bacto™, BD).

2.3.5 Plate Count Agar (PCA)

PCA (Difco, BD) was prepared according to manufacturer's instructions or purchased as poured plates (Fort Richard).

2.3.6 Marine Agar (MA)

Marine agar was made from Marine broth (Difco™, BD) which was prepared according to manufacturer's instructions, with the addition of agar at 15g/L (Bacto™, BD).

2.3.7 Autoinducer Bioassay Media (AB)

AB medium was based on that described by Greenberg *et al*. (1979). AB was composed of 0.3M NaCl, 0.05M $MgSO_4$ (Sigma) and 0.2% (w/v) Vitamin-Free Casamino Acids (Bacto™, Difco) in dH_2O. Before autoclaving, pH was adjusted to 7.5. Prior to use, the autoclaved base medium was supplemented with filter sterilized 1% (v/v) 1M Potassium Phosphate buffer (1M K_2HPO_4 and 1M KH_2PO_4; BDH) (pH7), 1% (v/v) 0.1M L-arginine (SERVA) and 2% (v/v) 50% Glycerol (BDH).

2.3.8 Maximum Recovery Diluent (MRD)

MRD (Difco™, BH) was prepared according to manufacturer's instructions.

2.3.9 Preparation of Meat plates

Meat plates were prepared by aseptically cutting circular disks from beef steaks and transferring them to sterile petri dishes **(Figure 2.1 A-F)**. Beef was purchased as whole, untreated striploin from a local abattoir and processed within 12 hours post-slaughter. The outer layer of striploin was doused in 70% ethanol and flamed, before the entire surface was seared with a hot plate. The hot plate was heated for at least 2 minutes over a Bunsen flame before use. After placing on a sterile cutting board, the outer layer of striploin was removed aseptically with a scalpel and forceps. Striploins were portioned into steaks (0.5 cm thick), and disks were cut from the meat using a circular cutting die (35 mm diam.). To obtain uniformity of plates, meat disks were aseptically transferred to petri dishes (35, 0/10 mm; Griener Bio-one) and pressed with a pressing tool. Aseptic technique was followed throughout the procedure, and all tools and surfaces that were in contact with the meat, were sterilized before use. The pressing tool was designed by the author of this study and both cutting and pressing implements were manufactured by Brian Atkins (AgResearch, MIRINZ Site Services). Meat

plates were stored at -1.5°C for less than 48 hours before use, and pH measured from two representative plates.

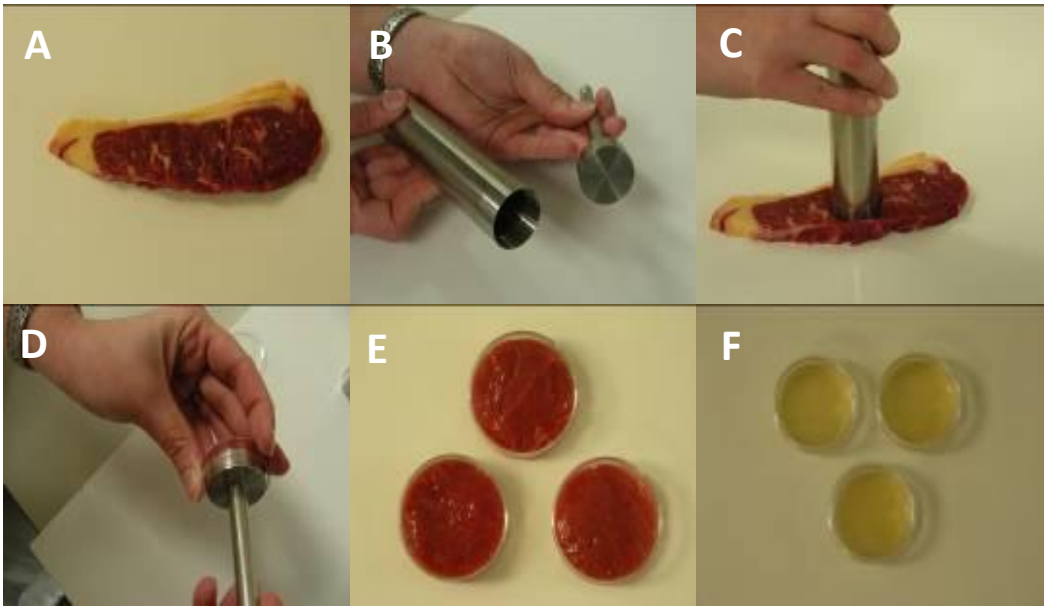


Figure 2.1. Use of sterile cutting and pressing implements for manufacture of meat plates.

A) Striploin steak; B) Cutting and pressing implements; C) Use of cutting implement; D) Pressing meat disk into petri dish E) Final meat plate product; F) BHI control agar plates.

2.3.10 Antibiotics

Antibiotics were added as required at the following final concentrations:

Kanamycin (Sigma) - 25µg/ml

Carbenicillin (Fluka) - 50µg/ml

Ampicillin (Sigma) - 50µg/ml

2.4 Bacterial Growth Conditions

All bacteria were maintained as frozen stocks in LB with 25% glycerol at -80°C. Strain-appropriate antibiotics were added to media, as required.

2.4.1 Standard *E. coli* Growth Conditions

Unless stated otherwise, all *E. coli* cultures were grown at 37°C. *E. coli* was grown for 24 hours on LA plates from frozen stocks. 2-3 freshly isolated colonies were used to inoculate overnight LB or BHI broths for each experiment. Broth cultures were grown with shaking at 37°C at 200rpm.

2.4.2 Standard *V. harveyi* growth conditions

V. harveyi cultures were grown at 30°C. *V. harveyi* was grown for 48 hours on Marine agar plates from frozen stocks. AB medium was inoculated with 2-3 freshly isolated colonies and incubated for 14-24 hours with shaking at 200rpm.

2.5 DNA Isolation and Amplification

2.5.1 Genomic DNA Isolation

Genomic DNA was isolated from bacteria grown on culture plates. A loopful of bacterial cells, was suspended in 200µl of PrepMan™ Ultra Sample Preparation Reagent (Applied Biosystems). The suspension was heated for 10 minutes at 100°C and centrifuged (eppendorf miniSPIN) for 3 minutes at 16000xg to pellet cells. Supernatants were transferred to a fresh micro-centrifuge tube, and cell pellets discarded. DNA was stored at -20°C until required.

2.5.2 Plasmid isolation and purification

Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen Ltd), following the manufacturers protocol.

2.5.3 Polymerase Chain Reaction (PCR)

2.5.3.1 Primers

Primers used for PCR and sequencing are listed in **Table 2.4**.

Table 2.4. Primers

Primer	**Sequence 5´-3´**	**Features**
LuxCRseq	TTGGCAGGTAAACACTA	Sequencing primer: specifically for pSB377 insert:*luxC* junction confirmation
Z2593F	TTCATCAGGCAAGTTTCAC	
Z2593R	CCTTTAGTGCGCTTTCTCAG	
Z2594F	TAACCCGGCAATTTTCATC	
Z2594R	TACCGGTACGCTGTCTATG	
ZPROM1	GT**GAATTC**ATGGTCATCAAAATCGACACTGC	*Eco*R I
ZPROM2	GTG**TACGTA**TTCTCCTGCAAGAGAATTATTTTAA	*Sna*B I
23SFOR	AAAATTAGCGGATGACTTG	
23SREV	TATTAACCTGTTTCCCATC	
hslJ1	AATCCGCCAGAAATCAGC	
hslJ2	CACTTGTGCACCTTCTTTC	
slp1	TGACAAAAGGTGGCACTCATA	
slp2	ACGGTAATACAGCGATTTCT	

2.5.3.2 PCR conditions

PCR conditions used throughout this study for each of the primer pair are summarized in **Table 2.5**.

Table 2.5. PCR conditions and the expected product size

Gene	**Primer Pair**	**Product size (nt)**	**Annealing**	**Extension**
23S	23SF/23SR	645	48°C, 1 min	72°C, 1 min
mdtI	z2594F/z2594R	191	52°C, 30 sec	72°C, 45 sec
mdtJ	z2593F/z2594R	228	52°C, 30 sec	72°C, 45 sec
hslJ	hslJ1/hslJ2	197	52°C, 30 sec	72°C, 45 sec
slp	slp1/slp2	225	54°C, 30sec	72°C, 45sec
***mdtJI* promoter**	ZPROM1/ZPROM2	620	55°C, 1 min	72°C, 1 min

2.5.3.3 PCR protocol

For a 50µl PCR reaction, the following components were combined: 5µl of10x Mg^{2+}-free PCR buffer (Invitrogen); 1.5µl 50mM $MgCl_2$ (Invitrogen); 1µl 10mM dNTP mix (2.5mM each of dATP, dGTP, dCTP and dTTP; Invitrogen); 0.5µl of 10mM Sense primer; 0.5µl of 10mM Antisense primer, 0.5µl of Taq DNA polymerase (5U/µl; Roche), 2µl of DNA template and nuclease-free water (Ambion) to a final volume of 50µl. The reaction was heated to 94°C for 2 minutes to denature the DNA. A total of 25-30 cycles were performed using annealing and extension conditions specific for each primer pair **(Table 2.5).** A final extension step was included (72°C for 7 minutes).

2.5.4 Agarose Gel Electrophoresis

Gels were prepared as 1% (w/v) agarose (GIBCO, BRL) in 0.5x TBE (Tris-borate-EDTA) buffer , (10xTBE buffer stock; Invitrogen). Ethidium bromide (BIORAD) was added to the gel to a final concentration of 0.25µg/ml. Gel loading buffer (GLB; 50% glycerol, 1mM EDTA and 0.4% Bromophenol Blue) was added to the samples prior to loading. DNA was electrophoresed as described by Sambrook *et al.,* (1998). Visualisation of the DNA was by exposure to UV light and the image captured by Gel Doc 1000 and Quantity One imaging software version 4.5.2 (BIORAD). To determine the PCR product sizes, a1Kb plus DNA molecular weight marker (1.0µg/µl; Invitrogen) was used.

2.5.5 Gel extraction

DNA bands were excised from agarose gels and DNA extracted using the Qiagen gel extraction kit (Qiagen Ltd) according to the manufacturer's protocol.

2.6 DNA manipulation

2.6.1 Restriction digests

The following restriction enzymes were used to digest DNA:

*Eco*R I 5′-G$^{\downarrow}$AATTC-3′ (Invitrogen)

*Sna*B I (Eco1051) 5′-TAC$^{\downarrow}$GTA-3′ (Fermentas)

The manufacturer's protocol was followed for each restriction digest according to the enzyme used. Briefly, 2-10µl of plasmid DNA was mixed with 2µl of 10x

buffer, 5U enzyme and sterile distilled water to a volume of 20µl in a sterile 1.5ml eppendorf tube. The content of the tube was briefly spun prior to incubation at 37°C for 2 hours.

2.6.2 PCR product Topoisomerase-mediated ligations

For cloning of PCR products generated using recombinant Taq DNA polymerase, TOPO TA Cloning® kit (Invitrogen) topoisomerase reactions were used. Briefly, 1µl of vector, 1µl of salt solution was mixed with 2-4µl of PCR product. Sterile distilled nuclease-free water was added to achieve a final volume of 6µl. The mixture was left at room temperature for a minimum of 5 minutes. The mixture was either used immediately for transformation or stored at -20°C.

2.6.3 T4 DNA ligase-mediated ligations

Ligation reactions of restriction digested fragments into plasmid vectors were carried out using T4 DNA ligase. Ligations reactions were carried out in a final volume of 10µl, containing 1µl of 10xT4 DNA ligase buffer, 2-5µl of digested purified vector, 2 µl of insert, 1U of T4 DNA ligase and an appropriate volume of sterile distilled nuclease-free water. Reactions were incubated at 16°C overnight in a PCR thermocycler (TC-512; Techne) or (PTC-1000; MJ Research, Inc.). Ligation reactions were purified using a PCR Purification Kit (Qiagen) prior to transformation.

2.7 Bacterial transformation

2.7.1 Chemical Transformations of *E. coli* O157:H7

30ml LB was inoculated with 300µl of an overnight culture, and grown for 2-3 hours. At mid-exponential phase cells were harvested by centrifugation at 4200 rpm for 5 min in a refrigerated bench-top centrifuge (Eppendorf 5417R) and resuspended in 500µl chilled polyethylene glycol (PEG; Promega) 8000 in 50mM $CaCl_2$. Cells were left on ice for a minimum of 30 minutes before 2-10µl of plasmid DNA was added to 100µl of cells. Cells and DNA were mixed gently and left on ice for 30 minutes. Cells were heat shocked at 42°C for 2 minutes. 250µl of S.O.C. (Invitrogen) was added and cells were allowed to recover at 37°C for 60

minutes with shaking. The cells were plated onto LB agar containing the appropriate antibiotics and incubated at 37°C overnight.

2.7.2 Transformation of commercial TOP10 cells

2-4µl of ligated DNA was added to chemically competent TOP10 cells (Invitrogen), mixed gently and left on ice for 30 minutes. Cells were heat shocked at 42°C for 1 minute and returned to ice for 5 minutes. 250µl of S.O.C medium (Invitrogen) was added. Cells were left to recover at 37°C for 30-60 minutes with shaking. The cells were plated onto LB agar containing the appropriate antibiotics and incubated at 37°C overnight.

2.8 DNA sequencing

Sequencing was performed at the Waikato DNA sequencing facility by the Canterbury sequencing and genotyping unit. Resulting sequences were analysed using the GenBank Basic Local Alignment Search Tool (BLAST) available from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/sites/entrez).

2.9 RNA isolation and detection

2.9.1 Fixing of cells for RNA isolation

Cells were simultaneously washed from plates and fixed, by gently pipetting a 5:1 volume of 95% (v/v) ethanol: 5% (v/v) acid phenol (Gibco BRL) solution over the culture plates. Pipette action was used, to wash and remove the bacteria-phenol mixture from the plates. The mixture was transferred to a sterile 2ml micro-centrifuge tube. The cell mixture was inverted and flash frozen in a dry ice and methanol bath. The fixed samples were stored at -80°C.

2.9.2 RNA isolation

Total RNA was isolated from fixed, frozen bacterial cells using the TRIzol® Max™ Bacterial RNA Isolation Kit (Invitrogen). Samples were simultaneously thawed and centrifuged in a bench-top centrifuge (Heraeus Multifuge 3 S-R) at 6000xg (4°C) for 5 minutes. All subsequent centrifugation steps were carried out at 4°C. Supernatants were discarded and the cell pellet resuspended in 200µl of Max Bacterial Enhancement Reagent, preheated to 95°C. The suspension was

incubated for 4 minutes at 95°C before adding 1ml of TRIzol® Reagent. The lysate suspension was mixed by inversion and incubated at room temperature for a further 5 minutes before the addition of 200µl of cold chloroform (Ajax Chemicals) for phase separation. The chloroform-lysate suspensions were mixed vigorously by hand for 15 seconds, incubated at room temperature for 2-3 minutes and centrifuged at 12000xg for 15 minutes. The upper aqueous phase containing the RNA was removed, and transferred to a sterile 2ml eppendorf tube. RNA was precipitated by the addition of 500µl of cold isopropanol (Propan-2-ol; BIOLAB). The resulting mixture was incubated at RT for 10 minutes before centrifugation at 15000xg for a further 10 minutes. Supernatants were discarded, the RNA pellet resuspended in 1ml of 75% ethanol and centrifuged at 7500xg for 5 minutes. Supernatants were discarded and the RNA pellet was dried at room temperature. RNA pellets were resuspended 50µl RNAse-free H_2O (Ambion) containing 1U/µl RNAsin® Plus RNase Inhibitor (Promega). RNA samples were stored at -80°C.

2.9.3 DNAse I treatment of RNA samples

To remove any DNA, the RNA samples were treated with RQ1 RNase-Free DNase (Promega). 5µl of RNA was combined with 1µl of RNAse free DNAse 10x reaction buffer and 4µl of DNase I. Reactions were incubated at 37°C for 30 minutes before the addition RQ1 DNase Stop Solution. DNAse I was inactivated by incubation at 65°C for 10 minutes.

2.9.4 Measurement of RNA concentration and purity

RNA was measured at 260 and 280nm using a nanophotometer (IMPLEN, TLS). The manufacturer's instructions for RNA measurement were followed.

2.9.5 RNA detection by ReverseTranscription Polymerase Chain Reaction (RT-PCR)

2.9.5.1 First-strand cDNA synthesis

For a 20µl first-strand cDNA synthesis reaction, the following components were added to a sterile nuclease-free micro-centrifuge tube: 2pmol primer; 5µl of DNase I-treated RNA preparation (10pg to 5µg of total bacterial RNA); 1µl of 10mM dNTP mix (10mM each dATP, dGTP, dCTP and dTTP; Invitrogen);

nuclease-free DEPC H_20 (Qiagen) to a final volume of 13µl. The mixture was heated to 65°C for 5-minutes and incubated on ice for at least 1minute. The contents were collected by a brief centrifugation before the addition of 4µl 5X First-Strand Buffer (250mM Tris-HCl, pH8.3, 375mM KCl, 15mM $MgCl_2$), 1ul of 0.1M DTT and 1µl of SuperScript™III Reverse Transcriptase (200U/µl; Invitrogen). The reaction was mixed by gentle pipetting and incubated for 30-60 minutes at 55°C. To inactivate the enzyme the reaction was heated to 70°C for 15-minutes. The resulting cDNA was used as a template for second strand synthesis and amplification by PCR. Reagent blanks were included to control for DNA contamination and non-specific reactions.

2.9.5.2 Second strand synthesis

Second strand synthesis was performed following the standard PCR protocol **(Section 2.5.3.3)** using 2µl of cDNA. RT-PCR products were separated and visualised according to the gel electrophoresis protocol **(Section 2.5.4).**

2.9.6 Densitometry

Size and band intensity of RT-PCR products were measured and compared **(Figure 2.2)** using the Alliance UVIBAND (TLS), software version 12.11. Data were expressed as arbitrary units (AU) of intensity. These were calculated as the sum of intensities in the defined area. The density of a spot is calculated from its volume and is made of the sum of all pixel intensities composing the spot. Spot density is dependent on the number of pixels inside the area of a spot, and the intensity of these points ($V = \sum ni\ li$). Fold inductions were calculated compared with band intensity of products at 37°C (AU of sample/AU of sample at 37°C). Ratios to 23SrRNA were calculated (AU of 23S at test temperature/AU of 23S at 37°C), such that band intensity at 37°C equals 1. These values were used to adjust for any differences in RNA concentrations, used in the RT-PCR reactions.

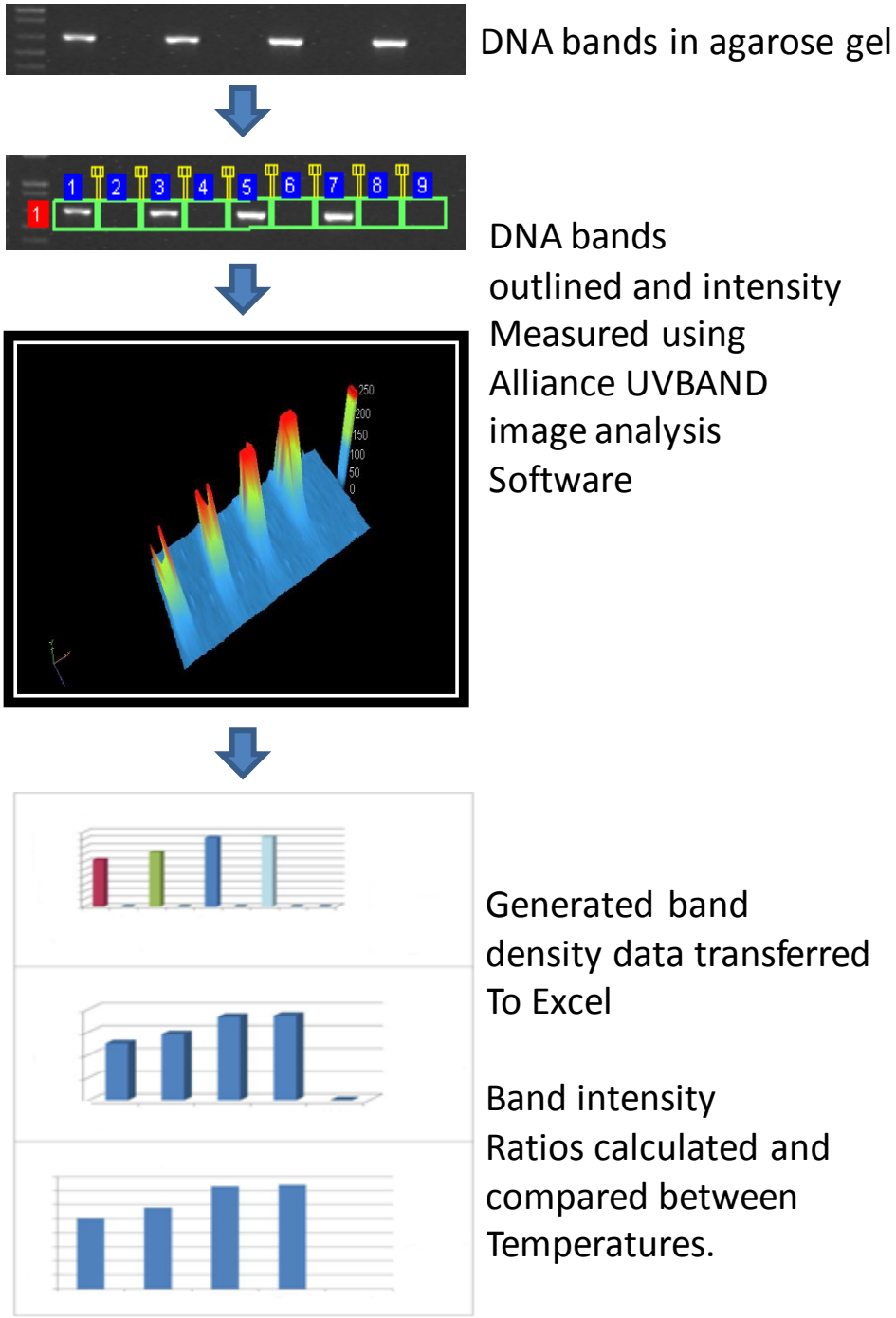


Figure 2.2. Flow diagram outlining the basic method for densitometry/Band intensity quantification using Alliance UVIBAND (TLS) software version 12.11

2.10 *E. coli* Growth experiments

E. coli growth experiments in liquid cultures were carried out in volumes of 10mls or more. A single pure colony was inoculated by sterile loop, into 5ml of broth and grown overnight at 37°C, with shaking at 200rpm. Overnight cultures were grown for 14-17 hours to an absorbance of between 5 and 6 at OD_{600} (IMPLEN,

TLS). Overnight cultures were diluted 1:1000 in fresh media and incubated at the required test temperature. For temperature down-shift experiments, cultures were grown post-dilution for one or two hours at 37°C, before being shifted directly to the test temperature. Samples were taken at specified time intervals for absorbance measurements (OD_{600}) and/or cfu/ml by aerobic plate counting (APC). For APCs, a series of 10-fold dilutions were performed in fresh sterile media, tempered to the same temperature as the culture to be sampled. Duplicate agar plates (LA, PCA or BHI agar) were spread with 100µl of each dilution and incubated at 37°C for 24 hours. Cfus were enumerated by colony counting under magnification and cfu/ml calculated. Non-inoculated controls were incubated to confirm media sterility. Variations to this method are indicated in the results section.

2.11 *E. coli* temperature down-shift for RNA isolation

2.11.1 On BHI agar plates

E. coli O157:H7 was inoculated into BHI broth and grown at 37°C for 18 hours to 10^8 cfu/ml. 1ml was transferred directly to the surface of replicate BHI agar plates (35mm diam.) that had been tempered to 37°C. Inoculated plates were incubated for 3 hours at 37°C before being shifted directly to the required temperatures (4, 0 and –1.5°C) for a further 3 hours. One set of plates was left at 37°C for comparison. Upon completion of the incubation, cells were fixed by addition of a 5% acidic phenol/95% ethanol solution. Cells were harvested and flash frozen using a dry ice/methanol mix **(Section 2.9.1).** Non-inoculated controls for all media were incubated to determine sterility. To determine the inoculum level, APCs were performed as per *E. coli* growth experiments **(Section 2.10)**.

2.11.2 On Meat Plates

Temperature down-shift experiments on meat plates, were performed as per the BHI agar experiments **(Section 2.11.1)**, including controls. One set of BHI agar plates was also incorporated at 37°C to confirm culture viability.

2.12 *V. harveyi* Bioluminescence Assay (VHBA)

2.12.1 Preparation of Conditioned Media (CM)

V. harveyi BB120 was grown in AB at 30°C for 14 hours to an absorbance of 1.5 at OD_{600}. *E. coli* cultures were grown in LB at 37°C, either overnight, to a specific time or OD_{600} as outlined in the results section. Cultures were centrifuged at 10000 rpm for 10 minutes (Heraeus Multifuge 3 S-R) to remove bacterial cells. Supernatants were filter sterilized using 0.22µm filters (Millex GP). CM was frozen at -20°C until required for use.

2.12.2 VHBA using *V. harveyi* BB170

The BB170 VHBA was adapted from methods by Greenberg *et al*., (1979), Bassler *et al*., (1997) and Surrette and Bassler (1998). *V. harveyi* strain BB170, was grown in AB at 30°C with shaking at 200rpm to an absorbance between 1.5 and 1.6 at OD_{600} and diluted 1:1000 in fresh AB. Test strain CMs were added to a final volume of 10% (v/v) to the diluted BB170 cultures and incubated at 30°C with shaking at 200rpm. Hourly samples of 100µl aliquots were dispensed into a 96-well microtitre plate (Greiner Bio-one). Light emission was measured using a POLARstar Galaxy Luminometer (BMG) and FLUOstar Galaxy software version 4.31.0.

2.12.3 VHBA using *V. harveyi* MM32

The MM32 VHBA was adapted from Miller *et al*. (2004). *V. harveyi* strain MM32 was grown in AB at 30°C with shaking at 200rpm for 14-hours to an absorbance of 1.7 at OD_{600} and diluted 1:5000 in fresh AB. Test strain CM was added to a final volume of 10% (v/v) to the diluted MM32 cultures and incubated at 30°C with shaking at 200rpm. Hourly samples were taken and measured as per the BB170 VHBA **(Section 2.12.2).**

2.13 Bioluminescence *luxCDABE* promoter reporter assays

Bioluminescence was used as a measure of promoter activity. Promoters were cloned upstream of the *luxCDABE* operon in pSB377 **(Section 3.2.1)**. Conditions for growth are varied according to the specific experimental conditions being tested and are outlined in the appropriate result sections. In all cases, duplicate

100µl aliquots were removed from the cultures and placed into a 96-well micro-titre plate. Bioluminescence was measured using a luminometer (POLARstar Galaxy; BMG) and analysed using FLUOstar Galaxy software version 4.31.0. At each time-point optical density was measured at 600nm, to assess culture growth. Where appropriate aerobic plate counts were performed to assess the number of cells (cfu/ml). Bioluminescence was reported as relative light units (RLU). Adjustment to optical density and to cell number were made as appropriate (RLU/OD_{600} and Lcfu (luminescence/cfu/ml) respectively).

2.15 Statistical Analyses

All growth curves, luminescent reporter and bioluminescent assays were carried out in either duplicate or triplicate. Statistical analysis was carried out using appropriate methods including: means, ratios, standard deviation, standard error of the mean, t-tests and pairwise analysis of variance (ANOVAs) via Microsoft Excel. Multiple pairwise comparisons were carried out using Genstat. Statistical significance was defined as $p < 0.05$ or a fold-difference of ≥ 2.0.

Chapter 3: Results

Temperature dependent gene expression in *E. coli* O157:H7

3.1 Identifying *E. coli* O157:H7 genes differentially expressed at refrigeration temperatures.

Studies have shown that *E. coli* O157:H7 is exceptionally resistant to external stress factors, including changes in temperature (Dykes *et al.*, 2001; Oh *et al.*, 2008; J.D. Stopforth *et al.*, 2007; Vital *et al.*, 2008). The organism's ability to adapt to and survive cold temperature is of particular importance since cold temperature is one of the major intervention strategies used in the control of food pathogens. Differential expression of genes, in *E. coli* O157:H7 exposed to cold temperatures on a meat surface, was previously observed (Li, personal communication, 2007) using Random Arbitrarily Primed Polymerase Chain Reactions (RAP-PCR). This chapter describes the identification of four differentially expressed *E. coli* O157:H7 genes associated with cold temperature, the confirmation of their expression by directed reverse transcription PCR (RT-PCR) and the use of plasmid reporters for direct monitoring of their expression.

3.1.1 Identification of Differentially expressed genes associated with changes in temperature by RAP-PCR.

A number of the differentially expressed products, previously identified by RAP-PCR (Li, personal communication, 2007) were sequenced and analysed as part of this study. RAP-PCR products were cloned into pCR2.1. Ligated DNA was transformed into TOP10 cells and transformants were selected using LA containing 50 µg/ml kanamycin. Resulting clones were re-streaked for purity. Plasmid DNA was prepared and sequenced. The identity of the sequences was made using the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/sites/entrez) **(Table 3.1)**. A range of RNA-derived products were identified, including sequences from *Bos taurus*, and *E. coli*

O157:H7, as well as sequences which had no match in the online database. RAP-PCR products 2991, 2981, 265A and 295A were derived from RNAs encoded by *mdtI, mdtJI, hslJ* and *slp* **respectively (Figure 3.1, 3.2, 3.3 and 3.4; Table 3.2)**. Raw sequence BLAST analysis of 295A revealed a sequence match to *slp* (216nt with 98% identity) **(Figure 3.1).** *slp* encodes an outermembrane lipoprotein which is expressed during carbon starvation (White-Ziegler *et al.*, 2008). Raw BLAST sequence analysis of 265A revealed a sequence match with *hslJ* (457nt with 99% identity) **(Figure 3.2).** *hslJ* encodes a stress response protein that through conceptual translation and protein identity to the MG1655 HslJ protein was identified as a heat shock-inducible protein (GenBank: AAG56381.1). Further studies have revealed roles for HslJ in novobiocin resistance and the regulation of virulence and transmission (Dowd & Ishizaki, 2006; Lilic *et al.*, 2003). Raw sequence BLAST analysis of 2991revealed a sequence match of 203 nt to *mdtI* (316nt) with 98% identity **(Figure 3.3).** Analysis of 2981 also aligned to *mdtI* sequence (115nt with 98% identity) but the majority of the fragment matched to *mdtJ* (291nt with 95% identity) **(Figure 3.4).** This result suggested that these two genes are transcribed as a single RNA transcript. Interestingly, *mdtJ* and *mtdI* lie next to each other, with *mtdJ* terminating within the coding sequence of *mdtI* by 10nt, and forming a two-gene operon driven from a putative promoter located at the 29 nt upstream of *mtdJ* **(Figure 3.5, 3.6; Tables 3.1, 3.2).** These genes encode a putative multi-drug transporter, which belongs to the small multidrug resistance (SMR) family, and are reported to transport small molecules such as spermidine, nalidixic acid, sodium dodecyl sulfate (SDS), deoxycholate and fosfomycin (Higashi *et al.*, 2008; Nishino & Yamaguchi, 2001). Approximately, 310nt downstream of these genes lies a divergent promoter for *tqsA* **(Figure 3.5, 3.6)**. *tqsA* has been reported to encode a transporter of the quorum-sensing molecule, AI-2 (Herzberg *et al.*, 2006).

Although *mdtI, mdtJ slp* and *hslJ* were observed as being differentially expressed at cold temperature on meat by RAP-PCR, further analysis was required for confirmation of these findings.

Table 3.1. Identification of RAP-PCR products

Fragment ID	Sequence Match*	Function
213A	Vector + unknown	
295A	***slp***	**Carbon starvation associated lipoprotein**
2924A	Vector + unknown	
2991A	***mdtI***	**Multidrug transporter/small molecule**
27A	Vector + unknown	
215B	Vector + unknown	
266A	*hchA* (*yedU*)	Chaperone involved in heat shock
2981A	***mdtJI***	**Multidrug transporter/small molecule**
226A	*yodB*	Putative cytochrome
234	*Bos taurus* seq.	Dihydropyrimidine dehydrogenase
2022	¥pO157 L7011	Plasmid Stabilisation Factor (RelE/ParE)
2035A	*Bos taurus* seq.	Mitochondrian
2401	Vector + unknown	
2913B	Vector + unknown	
2922	16SRNA partial	
29122B	*yeaJ*	Diguanylate cyclase (GGDEF) domain protein
29135	Z3271	Hypothetical protein
243	¥pO157 *espP*	Extracellular serine protease
2024B	Z5897	Hypothetical protein
265A	***hslJ***	**Heat shock protein**

*NC 002655 *E. coli* O157:H7 EDL933

¥AF074613 *E. coli* O157:H7 pO157

Genes identified for further analysis are indicated in bold.

Table 3.2. Genes of interest.

Products	Sequence Coordinates*	Tag Locus*	Gene Id.	NCBI accession
mdtI	c2347907-2348223	z2593	961546	NP_288034
mdtJ	c2348236-2348588	z2594	961548	NP_288035
slp	4454268-4454867	z4908	961185	NP_290077
hslJ	2109817-2190460	z2330	961015	NP_287767

*Accession number: NC 002655 *Escherichia coli* O157:H7 EDL993

A.

```
NAAGTGCGGT TAACAGCGGC GTCTAAACCA CTGTCGNCTA CACCCCGTCC CTCGTCCCGT
CGGGCCCGGG TTGGGCCCCC CGGGTCCTCG CCGTCGTTGT TAGAAATCGC TGTATTACCG
TTGGATAGCT ATGCGAAGCC TGATATTGAA GCCAACTATC TGGGCCGACT GCTCGCCAGA
CAAAGCGGCT TCCTTGATCC AGTGAACTAT CGTAATCACT TTGTATACCA TCCTCGGCAC
CATTCAGGGT GAACAACCTG GCTTTATCAA TAAAGTCCCG TATAACTATC CTGGAAGTGA
ATATGTCAGG
```

B.

```
          gb|AE005174.2| Escherichia coli O157:H7 EDL933, complete genome
          Length=5528445
          Features in this part of subject sequence:
             outer membrane protein induced after carbon starvation
          Score =  374 bits (202),  Expect = 2e-100
          Identities = 212/216 (98%), Gaps = 3/216 (1%)
          Strand=Plus/Plus

Query  95       CGTTGTTAGAAATCGCTGTATTACCGTTGGATAGCTATGCGAAGCCTGATATTGAAGCCA  154
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4454506  CGTTGTTAGAAATCGCTGTATTACCGTTGGATAGCTATGCGAAGCCTGATATTGAAGCCA  4454565

Query  155      ACTATCTGGGCCGACTGCTCGCCAGACAAAGCGGCTTCCTTGATCCAGTGAACTATCGTA  214
                |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4454566  ACTATCAGGGCCGACTGCTCGCCAGACAAAGCGGCTTCCTTGATCCAGTGAACTATCGTA  4454625

Query  215      ATCACTTTGTATACCATCCTCGGCACCATTCAGGGTGAACAACCTGGCTTTATCAATAAA  274
                |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4454626  ATCACTTTGT-TACCATCCTCGGCACCATTCAGGGTGAACAACCTGGCTTTATCAATAAA  4454684

Query  275      GTCCCGTATAACTATCCTGGAAGTGAATATGTCAGG  310
                |||||||||||| |||||||||||||||| ||||
Sbjct  4454685  GTCCCGTATAACT-TCCTGGAAGTGAATATG-CAGG  4454718
```

C.

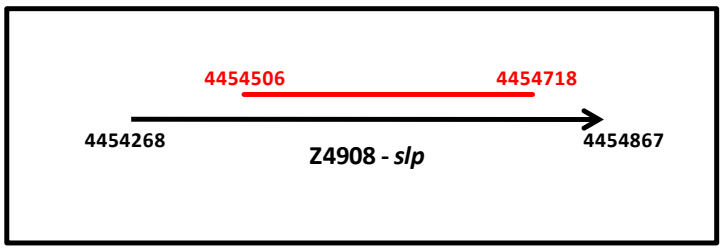


Figure 3.1. Sequence detection from sequencing of RAP-PCR products

A. Raw sequence obtained from sequencing clone 295A.
B. BLAST sequence analysis result.
C. Schematic diagram showing the alignment of the RAP-PCR fragment to the identified gene.

A.

```
AGCAGCGTTT  TGGGTAGGGT  GTGCCGCTTT  ATTGTTGTCG  GCGTGTAGTC
GTGAGCCTGT  TCAGCAGGCG  ACTGCGGCGC  ACGTAGCGCC  AGGTTTAAAA
GCGTCGATGT  CCAGTAGTGG  AGAAGCAAAT  TGTGCAATGA  TCGGCGGTTC
GCTTTCTGTT  GCCCGTCAAC  TGGATGGTAC  GGCGATTGGG  ATGTGTGCAT
TACCCAACGG  CAAACGCTGT  AGCGAACAGT  CACTTGCCGC  CGGGAGCTGT
GGCAGCTATT  AATTCATTAA  ATCCGCCAGC  TTATAAGTTA  ATGTCTGTTT
CGCGGTCGCC  AGCGTTAACT  GGTTCGCGGT  CAGATCCACT  TGTGCACCTT
CTTTCAGCAT  TTCGCTAATG  GTGTTATCGA  GTTCATTAAG  CTGCGGGTTA
GCGCACATCA  TACGGGTCAT  TGCCAGCCCT  TTGGCTGTCA  GTTCACCATT
AGACAGTCTG  AAGCTTAAGT  GGCAAATTCG  TATAAACCTA  CAGGAGTA
```

B.

```
          gb|AE005174.2|  Escherichia coli O157:H7 EDL933, complete genome
          Length=5528445
          Features in this part of subject sequence:
           heat shock protein hslJ
          Score =  839 bits (454),  Expect = 0.0
           Identities = 456/457 (99%), Gaps = 0/457 (0%)
          Strand=Plus/Minus

Query  1        AGCAGCGTTTTGGGTAGGGTGTGCCGCTTTATTGTTGTCGGCGTGTAGTCGTGAGCCTGT  60
                |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  2109916  AGCAGCGTTTTGGGTAGGGTGTGCCGCTTTATTGTTGTCGGCGTGTAGTAGTGAGCCTGT  2109857

Query  61       TCAGCAGGCGACTGCGGCGCACGTAGCGCCAGGTTTAAAAGCGTCGATGTCCAGTAGTGG  120
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2109856  TCAGCAGGCGACTGCGGCGCACGTAGCGCCAGGTTTAAAAGCGTCGATGTCCAGTAGTGG  2109797

Query  121      AGAAGCAAATTGTGCAATGATCGGCGGTTCGCTTTCTGTTGCCCGTCAACTGGATGGTAC  180
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2109796  AGAAGCAAATTGTGCAATGATCGGCGGTTCGCTTTCTGTTGCCCGTCAACTGGATGGTAC  2109737

Query  181      GGCGATTGGGATGTGTGCATTACCCAACGGCAAACGCTGTAGCGAACAGTCACTTGCCGC  240
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2109736  GGCGATTGGGATGTGTGCATTACCCAACGGCAAACGCTGTAGCGAACAGTCACTTGCCGC  2109677

Query  241      CGGGAGCTGTGGCAGCTATTAATTCATTAAATCCGCCAGCTTATAAGTTAATGTCTGTTT  300
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2109676  CGGGAGCTGTGGCAGCTATTAATTCATTAAATCCGCCAGCTTATAAGTTAATGTCTGTTT  2109617

Query  301      CGCGGTCGCCAGCGTTAACTGGTTCGCGGTCAGATCCACTTGTGCACCTTCTTTCAGCAT  360
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2109616  CGCGGTCGCCAGCGTTAACTGGTTCGCGGTCAGATCCACTTGTGCACCTTCTTTCAGCAT  2109557

Query  361      TTCGCTAATGGTGTTATCGAGTTCATTAAGCTGCGGGTTAGCGCACATCATACGGGTCAT  420
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2109556  TTCGCTAATGGTGTTATCGAGTTCATTAAGCTGCGGGTTAGCGCACATCATACGGGTCAT  2109497

Query  421      TGCCAGCCCTTTGGCTGTCAGTTCACCATTAGACAGT  457
                |||||||||||||||||||||||||||||||||||||
Sbjct  2109496  TGCCAGCCCTTTGGCTGTCAGTTCACCATTAGACAGT  2109460
```

C.

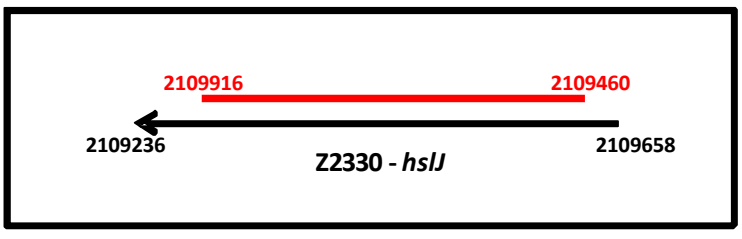


Figure 3.2. Sequence detection from sequencing of RAP-PCR products

A) Raw sequence obtained from sequencing clone 265A. B) BLAST sequence analysis result and C) Schematic diagram showing the alignment of the RAP-PCR fragment to the identified gene.

A.

```
AGATATCTGC CGCGTTAAAC GCGGCAGTCA TAAACACTGG CGTCGTTANN GCCATCCGAT
ACGGGTGCTG GCTGGCATCG TGCTGGATCG TGCTACGTCT GAATGAGGTC GCGCATGGCT
GCTCTCCCTG CGGCGGTGCT GGCTGCCTTT AGTGCGCTTT CTCAGGCCGT TAAAGGGATC
GACTTGTCTG TCGCTTATGC ATTGTGGGGC GGGTTTGGTA TTGCCGCCAC GTTAGCCGCA
GGTTGGATCT TGTTTGGTCA ACGGTTAAAT CGTNACAGGC TGGATTGGCC TGGTCTTGCT
GTATGGCTGG AATGATCATG GTGCAAGCTT AAGGGCGAAT TCGTTTAACC TGCAGGACTA
GTCCCTTTAG TGAGGGTTAA TTCTGAGCTT GGCGTAATCA TGGTCATAGC TGTTTCCTGT
GTGAAATTGT TATCCGCTCA CAAATTCCAA CACAACATAC GAGCCGGAAG CATAAAGTGT
AAAGCCTGGG GTGCCTAATG AGTGAGCT
```

B.

```
          gi|56384585|gb|AE005174.2|  Escherichia coli O157:H7 EDL933, complete
         genome
          Length=5528445
          Features in this part of subject sequence:   possible chaperone
          Score =  353 bits (178),  Expect = 4e-94
           Identities = 201/205 (98%), Gaps = 3/205 (1%)
           Strand=Plus/Minus

Query  120      TGCTCTCCCTG-CGGCGGTGCTGGCTGCCTTTAGTGCGCTTTCTCAGGCCGTTAAAGGGA  178
                ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2348121  TGCTCTCCCTGGCGGCGGTGCTGGCTGCCTTTAGTGCGCTTTCTCAGGCCGTTAAAGGGA  2348062

Query  179      TCGACTTGTCTGTCGCTTATGCATTGTGGGGCGGGTTTGGTATTGCCGCCACGTTAGCCG  238
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2348061  TCGACTTGTCTGTCGCTTATGCATTGTGGGGCGGGTTTGGTATTGCCGCCACGTTAGCCG  2348002

Query  239      CAGGTTGGATCTTGTTTGGTCAACGGTTAAATCGTNACAGGCTGGATTGGCCTGGTCTTG  298
                |||||||||||||||||||||||||||||||| | ||||||||||||||||||||||||
Sbjct  2348001  CAGGTTGGATCTTGTTTGGTCAACGGTTAAATCGTAA-AGGCTGGATTGGCCTGGTCTTG  2347943

Query  299      CTGTATGGCTGGAATGATCATGGTG  323
                |||| ||||||||||||||||||||
Sbjct  2347942  CTGT-TGGCTGGAATGATCATGGTG  2347919
```

C.

Figure 3.3. Sequence detection from sequencing of RAP-PCR products

A) Raw sequence obtained from sequencing clone 2991A. B) BLAST sequence analysis result and C) Schematic diagram showing the alignment of the RAP-PCR fragment to the identified gene.

A.

```
AANATAAGCT NGCTGTCGTC TACAACGCCG GCAGTNNATA TAACTACTAG GCCTGTCTNC
TTACCATTGC CCATCCGCGG GTGCTTGACC ACTTACTTTG GTCGCTGATA GCGAATAAAT
ATCTCTTGCG TGAGAAGTGA CAATGTATAT ATTGGATATT AAGTGTCTTG GCTATTTGCT
ACAGAAATTA CCTGGTACGC TGTCTATGAA ATGGGCGAGC GTCAGTGATG GAAATGGCGG
CTTTATTTTA ATGCTGGTGA TGATTTCTCT GTCGTATATA TTTCTCTCTT TCGCTGTTAA
AAAAATAGCC TTAGTGTGTG GCTTATGCGC TGTGNGGAAG TGTATCGNGT ATTTCTATTT
ATTACCTTGT TTAGCGTTTT TGTTATTCTG ACGAAAGTTT ATCGCTGATG AAAATTTGCC
TGGGTTAACC ACCCTGGTCG CCGGGATAGT TGTTTGATCA AATCAGTGTA CCCTGTAAAA
TGCTGCTGTC AAACCTGAAC TGGAGTGTGA ACCATGGCTG CAGTTTGAAT GGGTTCACTG
CCTGCCTGGC TTGGCATTGG CAAATCTGTG CTGGAAATCG TTGCTAACTG TCTTTTTGAA
ATTTTC
```

B.

```
          gi|56384585|gb|AE005174.2|  Escherichia coli O157:H7 EDL933, complete
         genome
          Length=5528445
           Features in this part of subject sequence:
           possible chaperone
           Score =  339 bits (171),  Expect = 7e-90
           Identities = 277/291 (95%), Gaps = 12/291 (4%)
           Strand=Plus/Minus

Query  176      TTGCTACAGAAATTACCTGGTACGCTGTCTATGAAATGGGCGAGCGTCAGTGATGGAAAT  235
                ||||||||||||||||| ||||||||||||||||||||||||||||||||||| ||||||
Sbjct  2348554  TTGCTACAGAAATTACC-GGTACGCTGTCTATGAAATGGGCGAGCGTCAGTGAGGGAAAT  2348496

Query  236      GGCGGCTTTATTTTAATGCTGGTGATGATTTCTCTGTCGTATATATTTCTCTCTTTCGCT  295
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2348495  GGCGGCTTTATTTTAATGCTGGTGATGATTTCTCTGTCGTATATATTTCTCTCTTTCGCT  2348436

Query  296      GTTAAAAAAATAGCCTTAGTGTGTGGCTTATGCGCTGTGNGGAAGTGTATCGNGTATTTC  355
                |||||||||||||||||| |||||||||||||||||| ||||| |||||| ||||||
Sbjct  2348435  GTTAAAAAAATAGCCTTAG-GTGTGGCTTATGCGCTGTG-GGAAG-GTATCG-GTATTT-  2348381

Query  356      TATTTATTACCTTGTTTAGCGTTTTTGTTATTCTGACGAAAGTTTATCGCTGATGAAAAT  415
                |||||||||||||||||||| |||||||||| |||||||||||||||||||||||||
Sbjct  2348380  TATTTATTACCTTGTTTAGCG-TTTTGTTATTC-GACGAAAGTTTATCGCTGATGAAAA-  2348324

Query  416      TTGCCTGGGTTAACCACCCTGGTCGCCGGGATAGTTGTTTGATCAAATCAG  466
                ||||| |||||||||||||||||||||||||||   ||||||||||||||
Sbjct  2348323  TTGCC-GGGTTAACCACCCTGGTCGCCGGGATAGT--GTTGATCAAATCAG  2348276

           Features in this part of subject sequence:
           possible chaperone
           possible chaperone
           Score =  103 bits (52),  Expect = 7e-19
           Identities = 108/116 (93%), Gaps = 8/116 (6%)
           Strand=Plus/Minus
Query  491      AAACCTGAACTGGAGTGTGAACCATGGCTGCAGTTTGAATGGGTTCACTGCCTGCCTGGC  550
                ||||||||||||||| |||||||||||| |||||||||||||||||||| ||| |||||||
Sbjct  2348258  AAACCTGAACTGGAG-GTGAACCATGGC-GCAGTTTGAATGGGTTCAC-GCC-GCCTGGC  2348203

Query  551      TTGGCATTGGCAAATCTGTGCTGGAAATCGTTGCTAACTGTCTTTTTGAAATTTTC  606
                | |||||||||||| || |||||||||||||||||||| ||||||||||||||||
Sbjct  2348202  T-GGCATTGGCAA-TC-GTGCTGGAAATCGTTGCTAAC-GTCTTTTTGAAATTTTC  2348151
```

C.

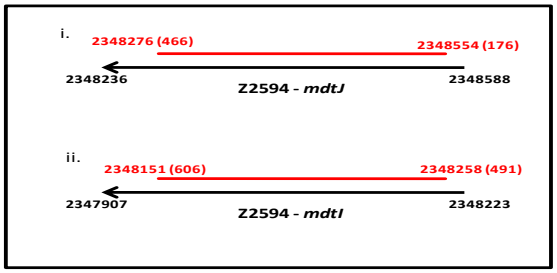


Figure 3.4. Sequence detection from sequencing of RAP-PCR products

A) Raw sequence obtained from sequencing clone 2981A, B) BLAST sequence analysis result. And C) Schematic diagram showing the alignment of the RAP-PCR fragment to the identified gene.

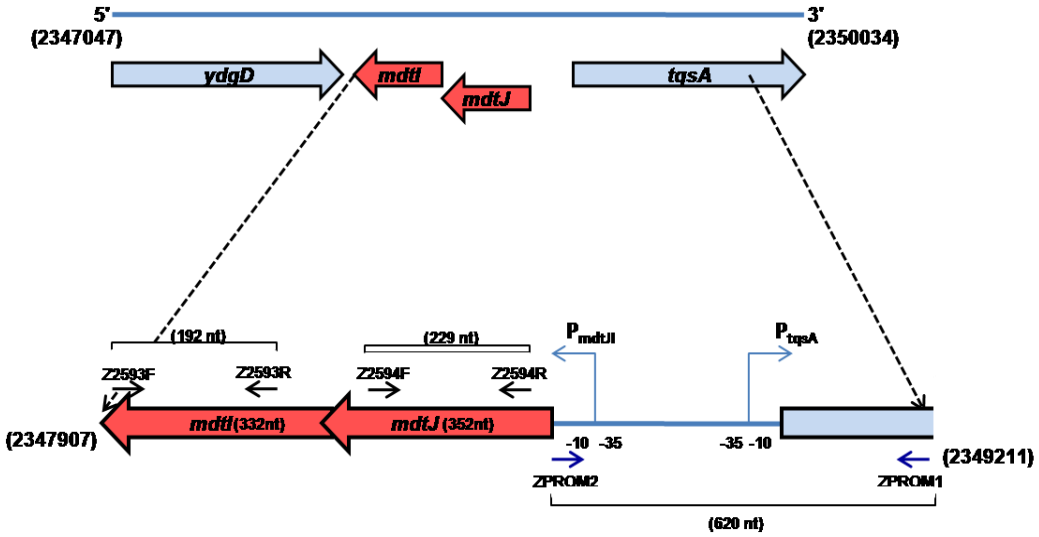


Figure 3.5. Schematic alignment of *mdtJI* genes in *E. coli* O157:H7 EDL933

Showing the location of the promoters and genes within this region. Primers used in this study are indicated by the arrows and the size of product is indicated in brackets. Sequence orientation and coordinates for EDL933 (Accession Number: NC 002655 Escherichia coli O157:H7 EDL993) are shown in brackets under the upper blue line.

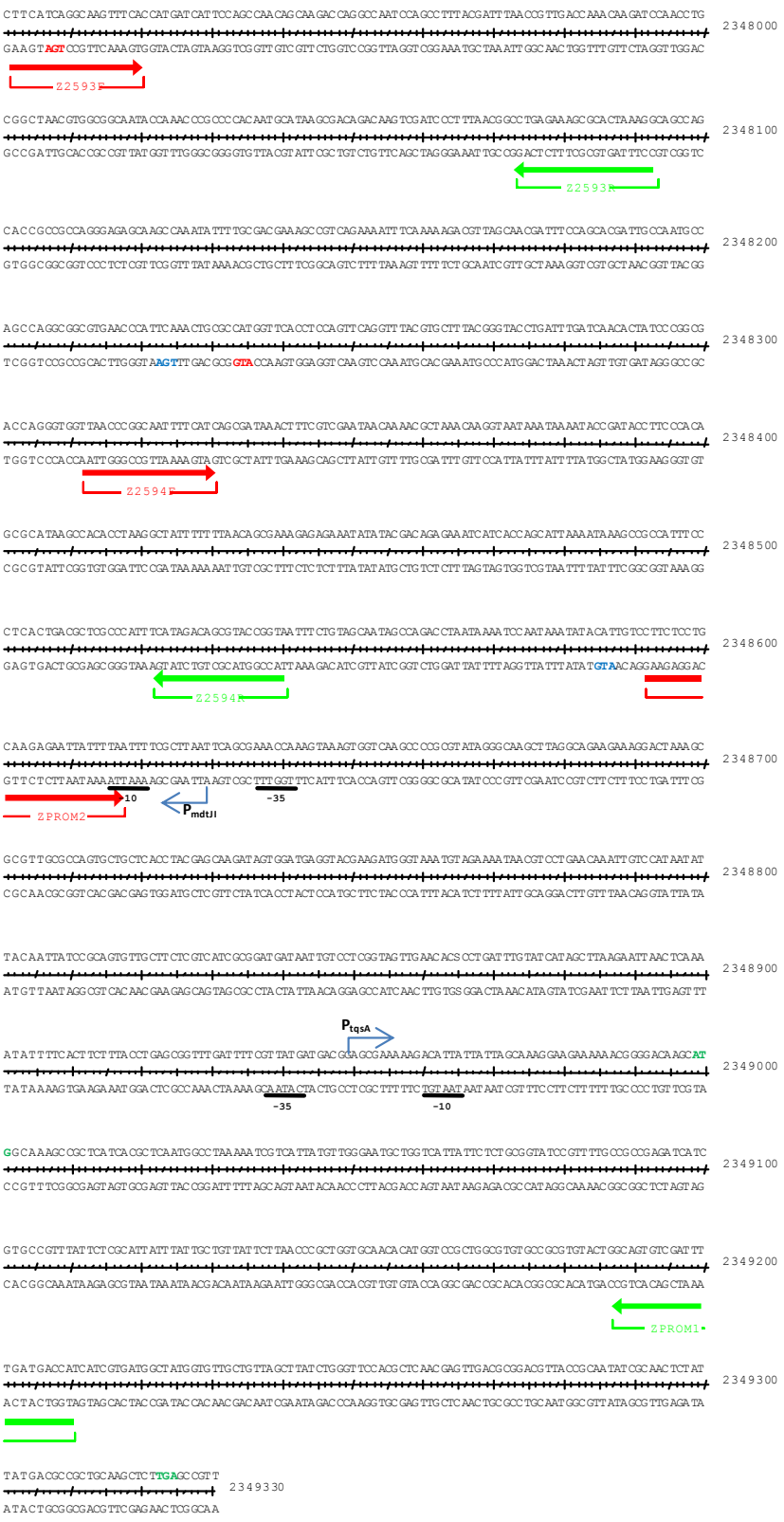


Figure 3.6. *E. coli* O157:H7 *mdtJI* and *tqsA* sequence with primer sites indicated.
Start and stop codons are indicated in bold (*mdtJ* –blue*; mdtI* –red; *tqsA* –black). Putative promoters are indicated, with the -10 and -35 sites underlined. The sequence coordinates are from NC 002665.

3.1.2 Confirmation of altered gene expression associated with cold temperature exposure using RT-PCR

Specific analysis of mRNA expression can provide information regarding the transcriptional activity within a bacterial population. Gene-specific RT-PCR was the first strategy employed to monitor gene expression after *E. coli* O157:H7 had been exposed to a down-shift from optimal growth temperature to refrigeration temperatures.

To detect specific mRNA produced in response to cold temperature, *E. coli* cells were exposed to a rapid down-shift in temperature (cold shock) prior to RNA isolation. RT-PCR was performed to detect the presence of specific mRNAs and confirm that the genes identified by RAP-PCR **(Section 3.1.1)** were expressed in response to cold temperature.

3.1.2.1 Temperature down-shift on BHI agar plates

Although the identified genes were originally isolated from experiments conducted on meat, the initial conformation cold-shock experiments were carried out using BHI broth and agar. BHI is a rich medium that has similarities with meat, but provides a more consistent matrix where potential experimental variables are more easily controlled.

E. coli O157:H7 was inoculated into BHI broth and grown at 37°C for 18 hours, to a cell density of 10^8 cfu/ml. The final cell density was confirmed by Aerobic plate count (APC) at 37°C. 1ml of culture was transferred to the surface of 37°C-tempered replicate BHI agar plates (35mm diam.). Non-inoculated controls for all media were incubated to determine media sterility. Inoculated plates were incubated for 3 hours at 37°C to allow the culture to stabilise, before being down-shifted to the required test temperatures of 4, 0 and -1.5°C. The stabilisation period was used to ensure that observed changes in gene expression were, in fact, induced by the temperature change, and not induced by the shift from liquid BHI to solid BHI agar. Plates were incubated for a further 3 hours at the new temperatures. As a control for gene expression at the optimal growth temperature, one set of plates remained at 37°C. Upon completion of the incubation period,

cells were fixed by the addition of 5% acidic phenol/95% ethanol fixative solution, harvested and flash frozen using a dry ice/methanol bath.

Following fixation, total bacterial RNA was isolated, and RT-PCR performed using specific primers and protocols designed for detection of MdtI, MdtJ, Slp, HslJ and 23SrRNA RNAs **(Table 2.4).** PCR products were mixed at a ratio of 5µl:1µl with gel loading buffer (glb), and loaded onto 1% (w/v) agarose gels. Gel electrophoresis was performed as described by Sambrook *et al.*, (1998). Visualisation of DNA was by exposure to UV light and the image captured by Gel Doc 1000 and Quantity One imaging software version 4.5.2 (BIORAD). To determine the PCR product sizes, a 1Kb plus (Invitrogen) DNA molecular weight marker was used.

RT-PCR with gene specific primers for 23SrRNA, MdtI, MdtJ, Slp, and HslJ yielded amplicons of expected sizes: 23S (645 bp); MdtI (191bp); MdtJ (225.bp); Slp (225 bp) and HslJ (197 bp). Control reactions, which did not contain SSIII reverse transcriptase, did not yield any PCR products **(Figure 3.7)**. 23SrRNA, a stable RNA, used as a control for RNA preparation efficiency and normalization, yielded product at all temperatures tested (37, 4, 0 and -1.5°C). Similarly, PCR products were generated with *slp*-specific primers indicating that *slp* mRNA was expressed at all four temperatures, however, less *slp* mRNA was present at 37°C than at the colder temperatures. *hslJ* mRNA was also present in cultures incubated at all temperatures, however more HslJ product was generated at 37°C than at the colder temperatures. *mdtI* and *mdtJ* mRNA was only detected by RT-PCR in cultures held at the three cold temperatures, 4, 0 and -1.5°C and not at 37°C.

To clearly define the differences in gene expression at the different temperatures, the intensity of the RT-PCR bands was quantified using UVIBAND (TLS), software version 12.11 **(Figure 3.8)**. Data are expressed as arbitrary units of intensity. Ratios to 23SrRNA were calculated to normalize RNA loading.

Quantification of the intensities of the 23SrRNA RT-PCR products revealed that there were differences in RNA loading for each of the temperatures **(Figure 3.8)**.

The differences in 23SrRNA were calculated and reported as relative band intensity: Relative band intensities = Arbitrary units (AU) of test samples/AU of 37°C samples. Ratios of RT-PCR rRNA loading at 37°C:4°C, 37°C:0°C and 37: -1.5°C were calculated as 1:1.16; 1:1.46 and 1:1.48 respectively **(Figure 3.9)**. These 23SrRNA product-loading ratios were used for normalization of the Slp, HslJ, MdtI and MdtJ RT-PCR products.

Densitometry performed on MdtI PCR products **(Figure 3.10 A)** revealed that no MdtI was expressed at 37°C. However *mdtI* mRNA was expressed at refrigeration temperatures (124-152AU), with 124-152 times more expression at cold temperature than at 37°C (4°C = 152:1; 0°C = 124:1; -1.5°C = 127:1). A similar trend was observed for *mdtJ* expression **(Figure 3.10B)**, although the ratios were much higher. *mdtJ* was expressed between 30849 -41539 times more at refrigeration temperatures compared with 37°C (4°C= 36695:1; 0°C = 30849:1; -1.5°C= 41539:1). The high ratios observed were potentially an artifact arising from high gel exposure, combined with non-detection of *mdtJ* mRNA at 37°C. The differences in exposure are clearly apparent when comparing the intensities for the molecular weight marker (1Kb plus) across all gels **(Figure 3.7).** Band intensity quantification of the Slp RT-PCR products **(Figure 3.10C)** revealed that while *slp* was expressed at 37°C, expression was up-regulated by 3.8-4.7 times at refrigeration temperatures (4°C = 4.68:1; 0°C = 3.91:1 and -1.5°C = 3.80:1). Conversely, *hslJ* **(Figure 3.10D)** was expressed approximately twice as much at 37°C than at 4 and -1.5°C, and three times as much at 0°C. (4°C = 0.43:1; 0°C = 0.30:1; -1.5°C = 0.52:1).

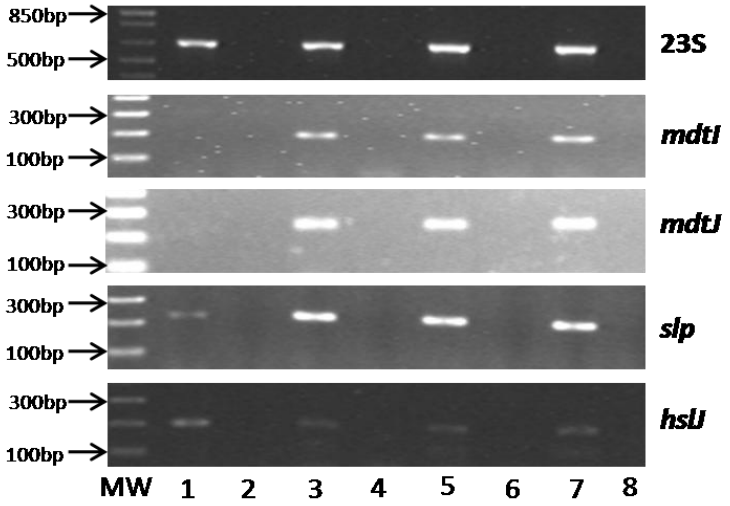


Figure 3.7. RT-PCR products generated from total bacterial RNA preparations isolated from *E. coli* O157:H7 cultures exposed to different temperatures on BHI agar. Products were separated on a 1% agarose gel.

Lane MW, 1Kb plus molecular weight marker; lane 1, RNA isolated from culture grown at 37°C plus RT; lane 2, 37°C product minus RT; lane 3, 4°C product plus RT; lane 4, 4°C product minus RT; lane 5, 0°C product plus RT; lane 6, 0°C product minus RT; lane, -1.5°C product plus RT; lane 8, -1.5°C plus RT.

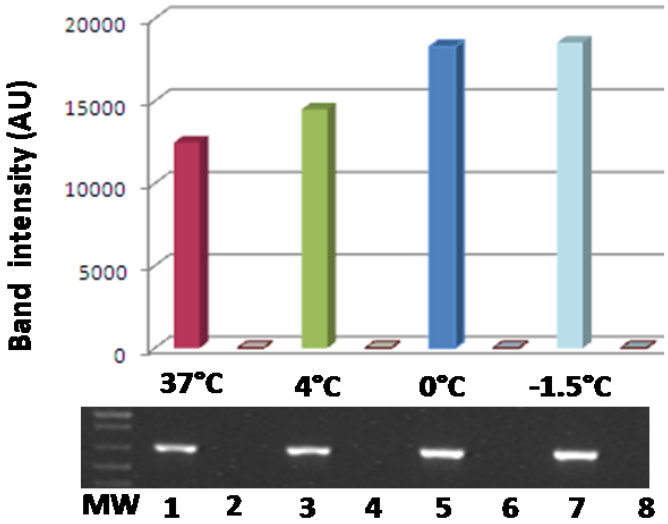


Figure 3.8. Densitometry analysis of band intensity for 23SrRNA RT-PCR product. RNA was isolated from *E. coli* O157:H7 cultures grown at 37°C then incubated at either 37, 4, 0 or -1.5°C.

Lane MW, 1Kb plus molecular weight marker; lane 1, RNA isolated from culture exposed to 37°C plus RT; lane 2, 37°C product minus RT; lane 3, 4°C product plus RT; lane 4, 4°C product minus RT; lane 5, 0°C product plus RT; lane 6, 0°C product minus RT; lane, -1.5°C product plus RT; lane 8, -1.5°C plus RT.
Band intensity values are expressed as Arbitrary Units (AU) generated by analysis with UVIBAND (TLS), software version 12.11.

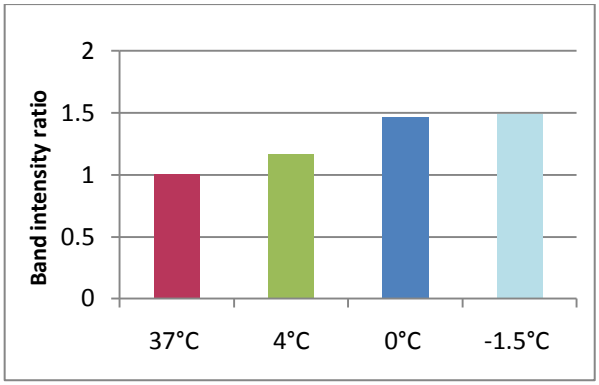


Figure 3.9. Relative band intensity ratios for 23S RT-PCR products.

Ratios of RT-PCR rRNA loading at 37°C:4°C, 37°C:0°C and 37:-1.5°C were calculated as 1:1.16; 1:1.46 and 1:1.48 respectively. These product-loading ratios were used for normalization of the Slp, HslJ, MdtI and MdtJ RT-PCR products.

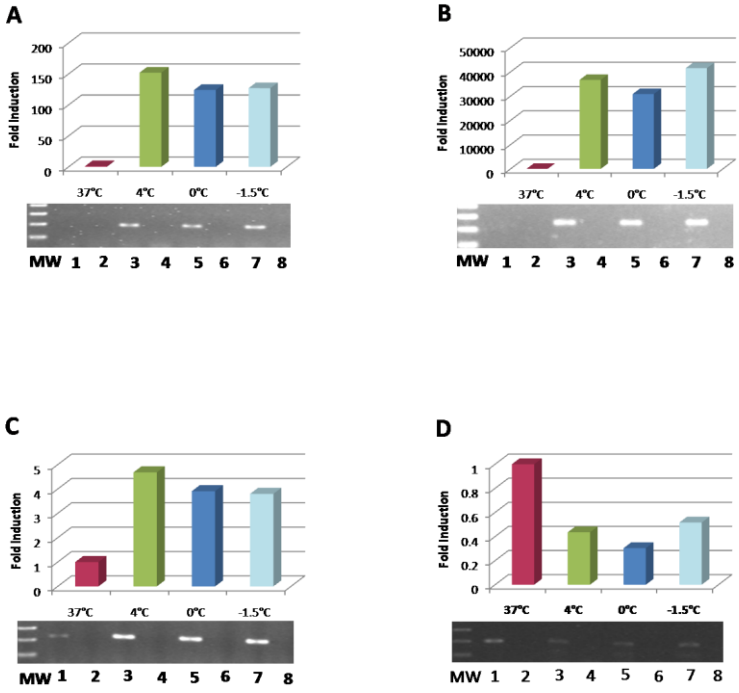


Figure 3.10. RT-PCR densitometry results for expression of *mdtI* (A), *mdtJ* (B), *slp* (C) and *hslJ* (D)

Post-temperature shift from 37°C to 37, 4, 0 and -1.5°C on BHI agar plates. Fold induction of relative expression is the ratio of band intensity (AU) at the test temperature/band intensity (AU) at 37°C. The results were normalized to 23SrRNA loading ratios prior to the calculation of fold induction.
Lane MW, 1Kb plus molecular weight marker; lane 1, RNA isolated from culture exposed to 37°C plus RT; lane 2, 37°C product minus RT; lane 3, 4°C product plus RT; lane 4, 4°C product minus RT; lane 5, 0°C product plus RT; lane 6, 0°C product minus RT; lane, -1.5°C product plus RT; lane 8, -1.5°C plus RT.

Analysis of the expression pattern of the four genes revealed that only *mdtJ* and *mdtI* were switched on at refrigeration temperatures, with no mRNA detected at 37°C. Furthermore, sequence analysis had revealed that these genes formed an operon and are therefore transcriptionally linked. Based on this data it was decided to further investigate the expression of *mdtJI* operon at refrigeration temperatures.

3.1.2.2 Temperature down-shift on meat plates

On BHI agar, *mdtI* and *mdtJ* genes were highly expressed at cold temperatures but were not detected at 37°C. Temperature down-shift experiments on meat, were performed in the same way as those performed on BHI agar, to determine if *mdtJ* and *mdtI* expression was dependent on temperature within a chilled meat context. Experiments were performed on duplicate meat plates, and RT-PCR was carried out seperately for each set of samples.

For both sets of RT-PCR, gene specific primers for 23SrRNA, MdtI and MdtJ yielded amplicons of the expected size. Control reactions which did not contain SSIII reverse transcriptase (RT) did not yield PCR products. 23SrRNA was used as a control for RNA preparation efficiency and normalisation, and yielded product at all temperatures. Similarly MdtI and MdtJ reactions yielded product at all temperatures, although the RT-PCR product bands were fainter in samples exposed to 37°C than those exposed to refrigeration temperatures **(Figure 3.11)**, suggesting up-regulation in expression.

To clearly define the differences in gene expression observed for *mdtI* and *mdtJ* at different temperatures, the intensity of the RT-PCR bands were quantified as for BHI agar experiments **(Figures 3.8-3.10)**. Quantification of the intensities of the 23S RT-PCR products revealed slight differences in RNA loading for each of the temperature samples. Relative band intensities reflected the ratios of rRNA loading and were calculated as Arbitrary units(AU) of test samples/AU of 37°C samples **(Table 3.3)**. These 23SrRNA loading ratios were used for normalisation of the MdtI and MdtJ RT-PCR products.

Average densitometry ratios, calculated for MdtI products **(Figure 3.12 A)** which had been extracted from *E. coli* O157:H7 cells on a meat surface, revealed that *mdtI* was expressed approximately twice as much at refrigeration temperatures compared to 37°C (4°C = 2.17:1; 0°C = 2.08:1; -1.5°C = 2.2:1). Similar increases in expression were observed for *mdtJ*, with 2-3 times more product generated from the samples harvested at the refrigeration temperatures **(Figure 3.12B)** compared with 37°C (4 = 2.90:1, 0 = 2.1:1; -1.5°C = 2.98:1).

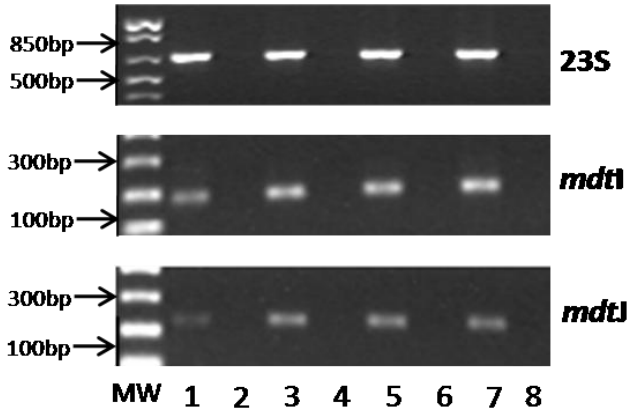


Figure 3.11. RT-PCR products generated from total bacterial RNA preparations isolated from *E. coli* O157:H7 cultures exposed to different temperatures on meat. Products were separated on a 1% agarose gel.

Lane MW, 1Kb plus molecular weight marker; lane 1, RNA isolated from culture grown at 37°C plus RT; lane 2, 37°C product minus RT; lane 3, 4°C product plus RT; lane 4, 4°C product minus RT; lane 5, 0°C product plus RT; lane 6, 0°C product minus RT; lane, -1.5°C product plus RT; lane 8, -1.5°C plus RT.

Table 3.3. Relative band intensities for 23S RT-PCR product*

	37°C	**4°C**	**0°C**	**-1.5°C**
Set A	1.00	1.00	1.08	1.10
Set B	1.00	0.96	1.07	0.87

*Used for normalization of the MdtI and MdtJ RT-PCR products

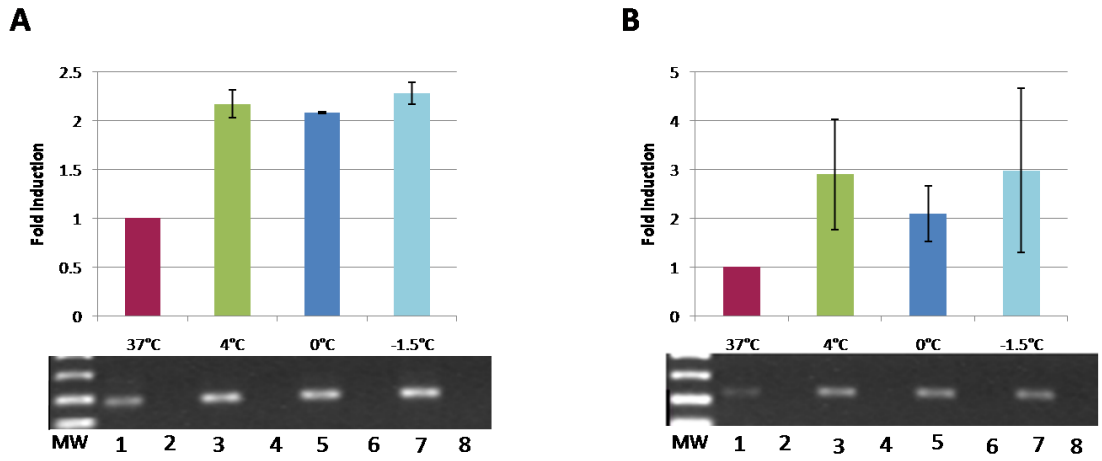


Figure 3.12. RT-PCR densitometry results for expression of *mdtI* (A), and *mdtJ* (B) post-temperature shift from 37°C to 37, 4, 0 and -1.5°C on Meat plates.

Fold induction of relative expression is the ratio of band intensity (AU) over band intensity (AU) at 37°C.
Data shown are the representative of two separate experiments. Average fold inductions calculated for the experiments. Standard Error of the Mean is indicated by error bars.

3.2 Confirmation of mdtJI expression in response to temperature down-shifts using bioluminescent promoter reporters

Expression of *mdtI* and *mdtJ* was up-regulated in *E. coli* O157:H7 on BHI agar and on meat plates that were held at refrigeration temperatures compared to the optimal growth temperature of 37°C. RT-PCR was used to measure the RNA levels present in samples taken at a single time point; 3 hours after temperature downshifts. However, cell recovery difficulties were encountered with meat plates leading to low bacterial RNA recovery. To directly monitor *mdtJI* promoter activity, a *mdtJI*::*luxCDABE* promoter reporter was constructed and transformed into *E. coli* O157:H7. The expression of *mdtJI* in response to both optimal and refrigeration temperatures was monitored over growth.

3.2.1 Construction of bioluminescent *mdtJI* promoter reporter

A promoter reporter plasmid, pSB377, containing a promoterless *luxCDABE* cassette (Winson et al., 1998) was used to construct a bioluminescent reporter plasmid, pWU2 **(Table 2.3)** for *mdtJI* expression **(Figure 3.13).** Briefly, the promoter region, including the 500 bp upstream of the -35 RNA polymerase recognition site was PCR amplified and cloned initially into pCR2.1 to create

pWU1. pWU1 DNA was isolated and confirmed by PCR amplification, as well as by DNA sequencing. The primers used for the original PCR amplification contain endonuclease restriction sites for EcoR 1 and SnaB 1 to facilitate transfer of the mdtJI promoter into the correct position upstream of the *luxCDABE* cassette in pSB377 **(Table 2.4)**. To aid the selection of the correct promoter clone, the mdtJI promoter-pSB377 ligation was initially transformed into chemically competent TOP10 cells. Clone selection was carried out using carbenicillin in the media and via screening of colonies for light production (UVItech, Alliance 4.7; for an example, **see Figure 3.14A-B**). Light-producing colonies were re-streaked for purity, and stable light production confirmed. Plasmid DNA was isolated and sequenced to confirm the insert was correct and that the junction between the promoter and the start site of the *luxC* gene was aligned appropriately. The correct plasmid, pWU2, was transformed into *E. coli* O157:H7. *E. coli* O157:H7 transformants were selected by growth on LA-carbenicillin plates at 37°C and screened for light production **(Figure 3.14A-B)**. Bioluminescent transformants were purified by sub-culturing onto fresh selective medium **(Figure 3.14C)**. The stability of pWU2 in *E. coli* O157:H7 was confirmed by plating from both broth and agar plates and analyzing colonies for the ability to produce bioluminescence. No significant plasmid loss was detected. *mdtJI::luxCDABE* integrity in pWU2 was confirmed by DNA sequencing. *E. coli* O157:H7 pWU2 was used in subsequent light reporter assay experiments.

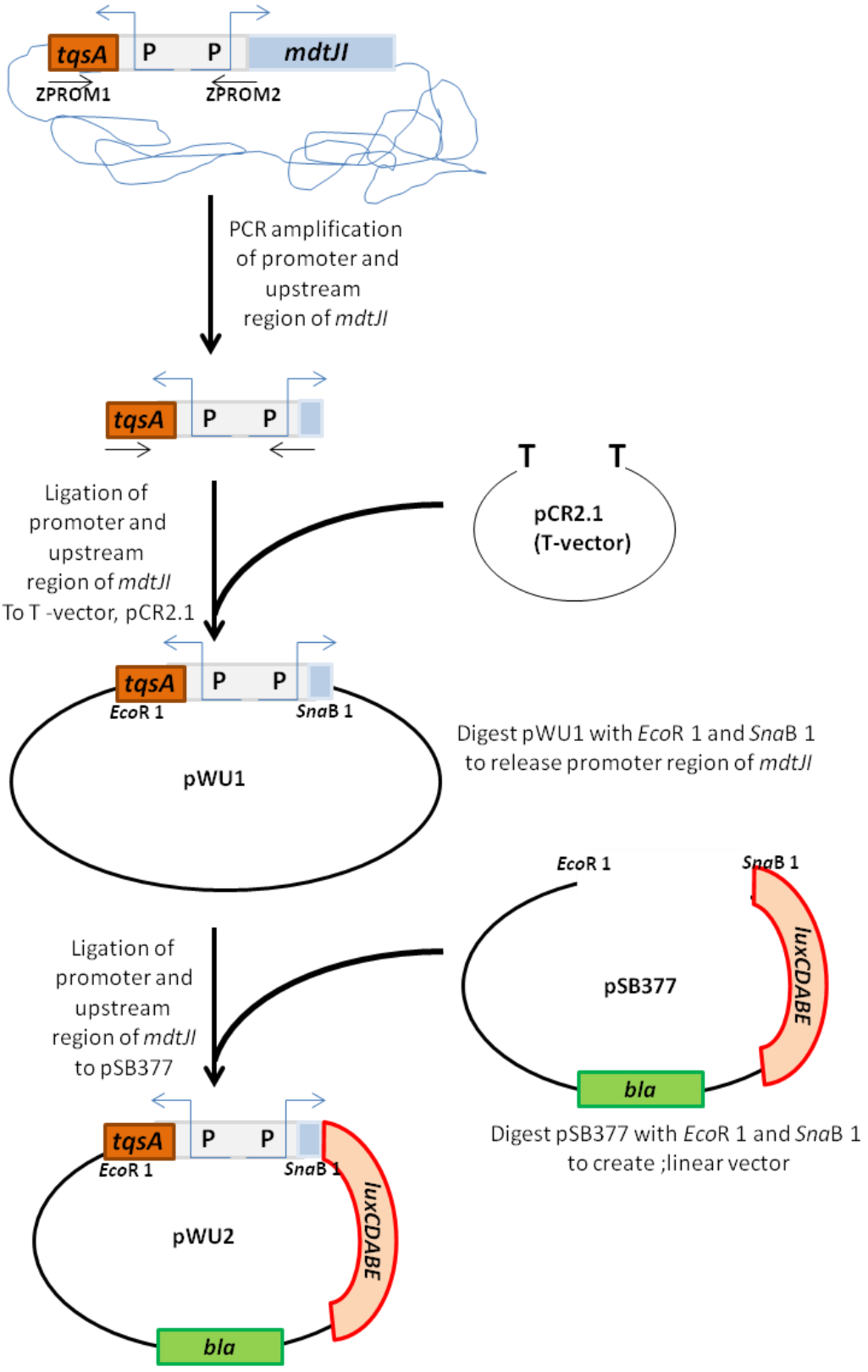


Figure 3.13. Schematic diagram illustrating the construction of pWU2

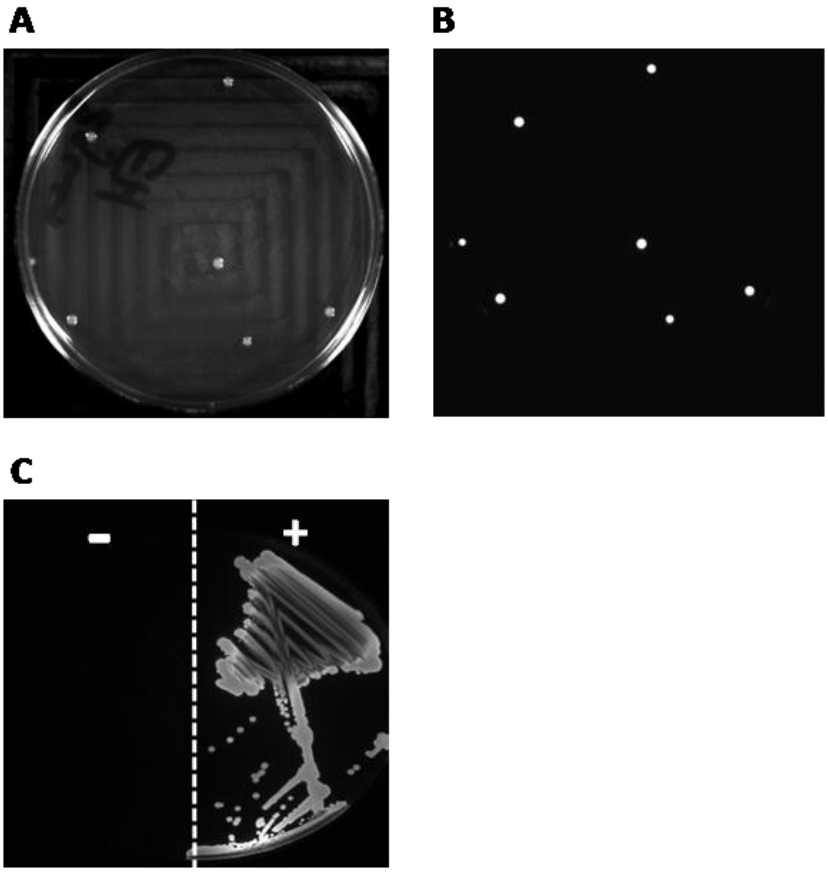


Figure 3.14 Bioluminescent colonies

A) Standard photograph of *E. coli* O157:H7 pWU2 transformants on LA +carbenicillin. Image capture (UVItech Alliance 4.7)
B) Biolumeniscent capture from transformant colonies. Image capture time = 30 seconds (Uvitech Alliance 4.7).
C) Example of purity plate showing Positive (+) bioluminescence from *E. coli* O157:H7 pWU2 and the Negative (-) bioluminescence *E. coli* O157:H7 pSB377.

3.2.2 Does *mdtJI* promoter expression occur at the optimal growth temperature of 37°C?

E. coli O157:H7 pWU2 was grown overnight in LB at 37°Cand subsequently diluted 1:1000 in fresh LB containing carbenicillin to obtain a cell density, of between 4 and 5 x 10^6 cfu/ml ($O.D_{600}$ 0.000-0.006). Cultures were placed at 37°C and incubated with shaking at 200 rpm for 8 hours. Hourly samples were taken to measure cell density and bioluminescence. Cell density was measured using OD_{600} **(Figure 3.15)** and APCs to obtain cfu/ml **(Figure 3.17)**. Exponential growth was attained for the first two hours of incubation **(Figure 3.15)**. Growth rate slowed as the culture transitioned into stationary phase (2 to 4 hours). From 4 to 8 hours no major increase in cell density was observed (3.2 and 6 respectively)

with a generation time of approximately 4 hours. Cell number continued to increase over the 8-hour experiment, with a 100-fold increase in cell number occurring over the first 4 hours of growth. Less than 0.5-fold increase was observed between hours 4-8, in agreement with the culture being in stationary phase.

mdtJI promoter activity was recorded over the course of growth, by monitoring light production, produced by the LuxCDABE proteins expressed from pWU2 **(Figure 3.16)**. Cells in the overnight culture contained a significant amount of LuxCDABE proteins light at T0, after culture dilution was 23445 RLU. From this high level, light emission was observed to decrease over 25-fold during the two hours of growth (T2: 938RLU compared to T0: 23445 RLU), suggesting that *mdtJI* promoter activity was insufficient to maintain this level of bioluminescence output. This decrease in expression continued to occur as the optical density increased exponentially. As cell growth slowed (2-3 hour readings), the level of light emission was observed to plateau and then to increase 10-fold during early stationary phase, suggesting that the promoter had become active during the transition period into stationary phase. At T8 there was a notable decline in expression. This data suggests that *mdtJI* expression is linked not only to temperature changes but also to cellular activity associated with changes in growth rate and therefore may have a broader range of activity.

Bioluminescence per cell (Lcfu/ml) showed that there was a steady decrease in bioluminescence per cell over time, with a final expression of 1.7 times less per cell at T8 (0.00056 Lcfu) than at T0 (0.00096 Lcfu) **(Figure 3.18)**. No increase in *mdtJI* expression was observed per cell at 37°C, regardless of growth phase.

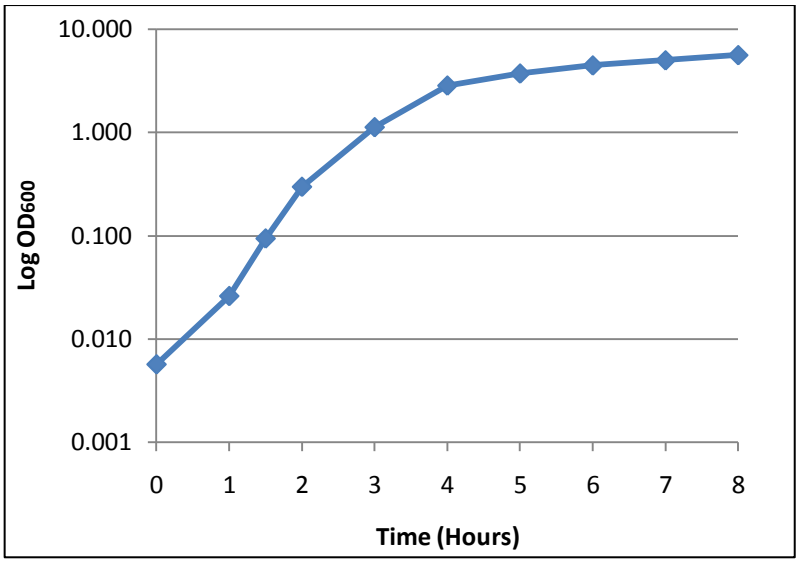


Figure 3.15. Growth of *E. coli* O157:H7 pWU2 at 37°C

Duplicate experiments were performed.
For each experiment, OD_{600} readings were carried out in triplicate.
Results shown are the combined average of duplicate experiments.
SEMs ≤0.036

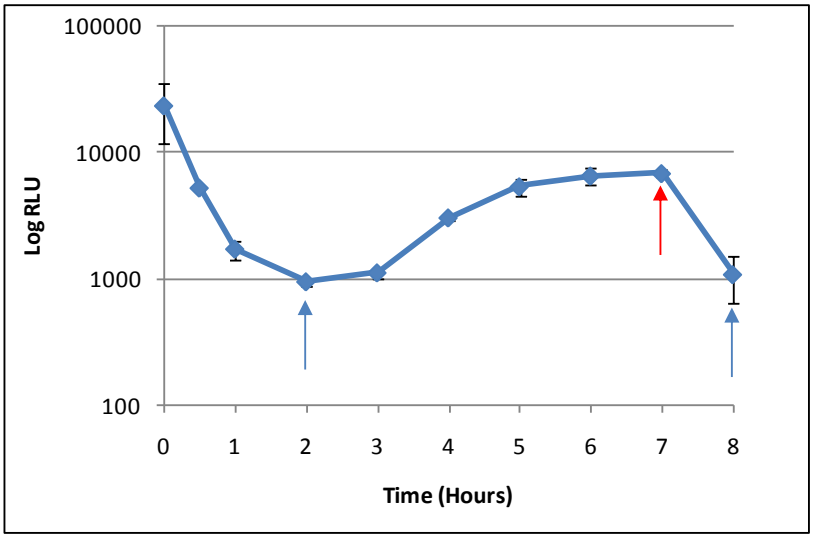


Figure 3.16. Expression of *mdtJI* in LB at 37°C.

Blue arrow indicates lowest level of expression, while the red arrow indicates peak induction

Duplicate experiments were performed

For each experiment, OD_{600} readings were carried out in triplicate, and luminescence readings in duplicate.

Results shown are the combined average of duplicate experiments.

RLU = Relative light units. (Relative light units = light units (AU) / OD_{600})

SEMs are indicated by error bars.

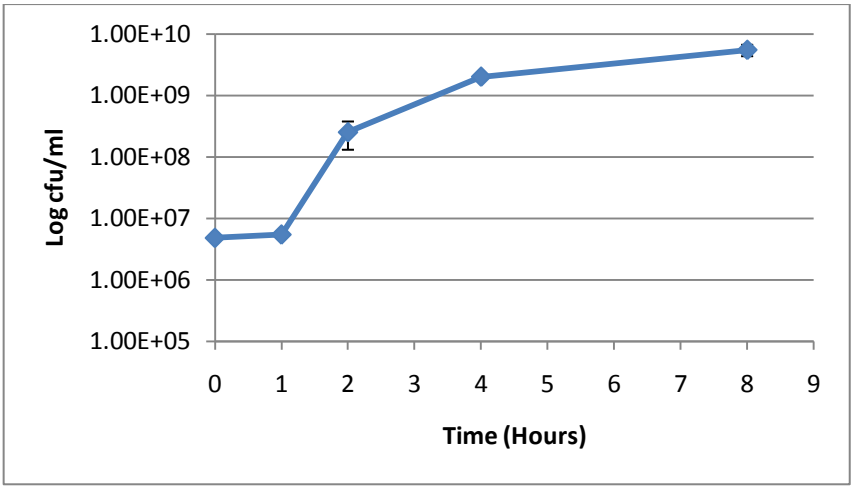


Figure 3.17. Growth of *E. coli* O157:H7 pWU2 at 37°C

Duplicate experiments were performed

In each experiment

For each experiment, OD_{600} readings were carried out in triplicate.

Results shown are the combined average of duplicate experiment

SEMS are indicated by error bars.

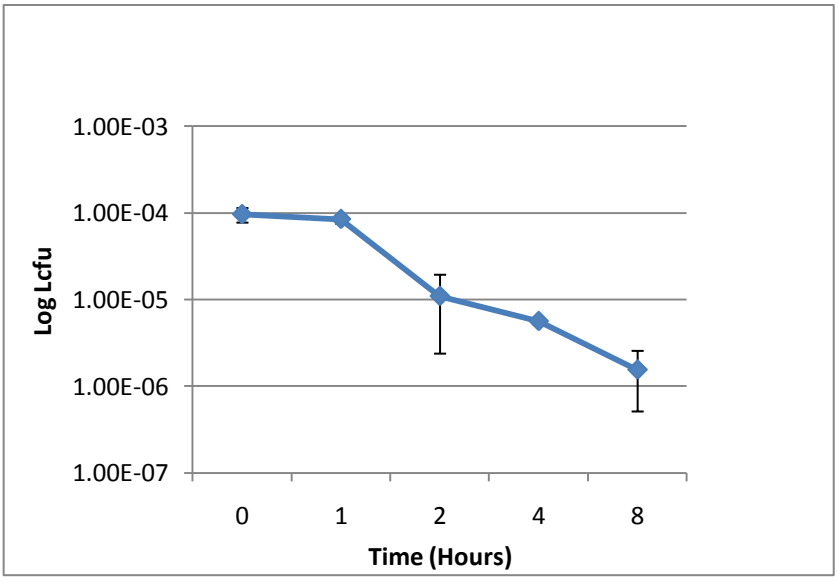


Figure 3.18. Expression of *mdtJI* in LB at 37°C.

Lcfu = Light units expressed per colony forming unit (Light units (AU)/cfu/ml)

Experiments were performed in duplicate.

For each experiment, OD readings were carried out in triplicate and luminescence readings in duplicate.

Results shown are the combined average of duplicate experiments.

SEMs are indicated by error bars.

3.2.3 Temperature downshift from 37°C at hour 1

To determine if *mdtJI* was expressed upon exposure to refrigeration temperatures, downshift experiments were performed using *E. coli* O157:H7 pWU2. *E. coli* O157:H7 pWU2 was grown overnight in LB at 37°Cand subsequently diluted 1:1000 in fresh LB containing carbenicillin to obtain between 4 and 5 x 10^6 cfu/ml ($O.D_{600}$ 0.000-0.006). To allow the residual light level produced by cells during stationary phase to reduce, diluted cells were incubated at 37°C for 1 hour. Following this incubation, at the point of temperature downshift, the culture containing 5-6 x 10^6 cfu/ml ($O.D_{600}$ 0.010-0.040) was divided into three equal aliquots. Two aliquots were exposed to a temperature downshift, one to 7°C and one to 4°C. The third aliquot was returned to 37°C as a control at the optimal growth temperature. Every hour for 8 hours after the temperature shift, duplicate 100µl aliquots were transferred from each culture into a 96-well microtitre plate. Bioluminescence was measured using a luminometer (POLARstar Galaxy; BMG) and analysed using FLUOstar Galaxy software version 4.31.0. At each time point, OD_{600} was measured to assess growth at each temperature **(Figure 3.19)** and to adjust luminescence readings to Relative Luminescent Units (RLU = luminescence/$O.D_{600}$). Additionally, APC's were performed at each time point to assess the number of viable cells in the cultures at each temperature (cfu/ml) **(Figure 3.21)**, and to calculate luminescence per colony forming unit (Lcfu:Lcfu = luminescence/cfu/ml).

After the dilution during the pre-incubation period at 37°C, cell number and the OD continued to rise **(Figure 3.19, 37°C)**. After the shift to 7°C and 4°C, there was no significant increase in either OD or cell number over the remaining period of the experiment **(Figure 3.19, 4&7°C)**.

30 minutes (T0.5) after the downshift in temperature from 37°C to 4 and 7°C, there was a significant difference in *mdtJI* expression ($p = 0.001$-0.002). *mdtJI* expression at 37°C decreased from an average of 23445 RLU at T0, to 5211 RLU at T 0.5, while cultures shifted to 4 and 7°C maintained their expression of *mdtJI* with bioluminescence levels of between 19000-22000 RLU **(Figure 3.20)**. There was no significant difference in expression of *mdtJI* seen at any time between 7°C and at 4°C ($p \geq 0.118$). *mdtJI* expression remained between 11000 – 23000 RLU, for the duration of the experiment following the temperature down-shift. As previously noted, the level of bioluminescence produced, by the culture held at 37°C continued to decrease. T2 showed the greatest point of difference between expression of *mdtJI* at 37°C compared with the cold temperatures. At T2, *mdtJI* is expressing between 14-18 times more at refrigeration temperatures (13029-16445 RLU) than it is at 37°C (938 RLU; $p = 0.000$). The observed peak in *mdtJI* expression during stationary phase at 37°C is still, however, significantly less ($p \leq 0.02$) compared with expression at 4°C (12984 RLU) and 7°C (12439 RLU) respectively. Finally *mdtJI* expression at 37°C, declines to 1069 RLU at T8, which is 11-13 x less than expression at refrigeration temperatures at T8 ($p = 0.000$).

Similar trends in *mdtJI* promoter activity per cell were observed at both 4 and 7°C, with constant Lcfu observed over the duration of the experiment **(Figure 3.22)**. This is in contrast with expression of *mdtJI* per viable cell (cfu/ml) which continued to decline. At T8, expression of *mdtJI* was 10-15 times higher per cell, for cells shifted to 4°C (0.00058 Lcfu) and 7°C (0.00084 Lcfu) compared with those at 37°C.

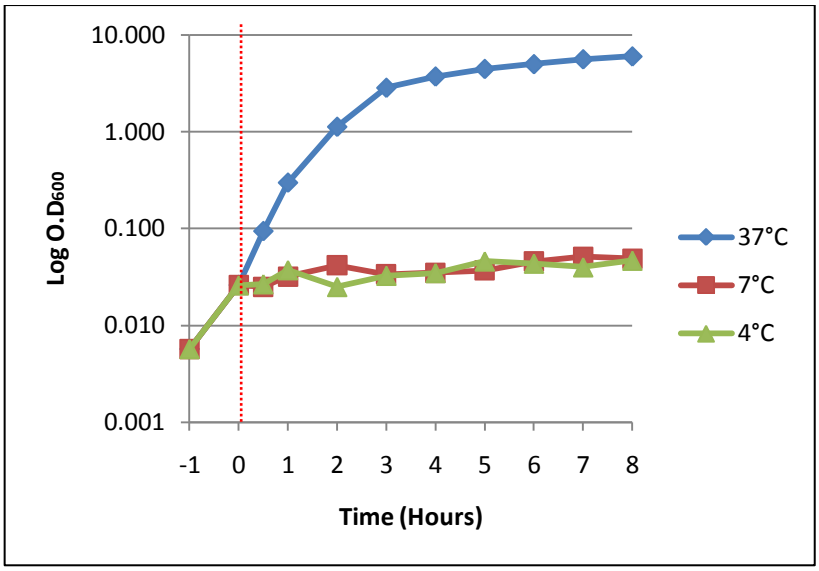


Figure 3.19. Growth of *E. coli* O157:H7 pWU2 following temperature shift from 37°C to 7°C and 4°C

Temperature shift was performed after growth at 37°C for 1 hour
Time of shift occurs at T0, indicated by dashed red line.
Experiments were performed in duplicate
For each experiment OD_{600} readings were performed in triplicate
Results shown are the combined average of duplicate experiments
SEMs for 37°C, 7°C and 4°C were ≤0.036, ≤0.007 and ≤0.007 respectively

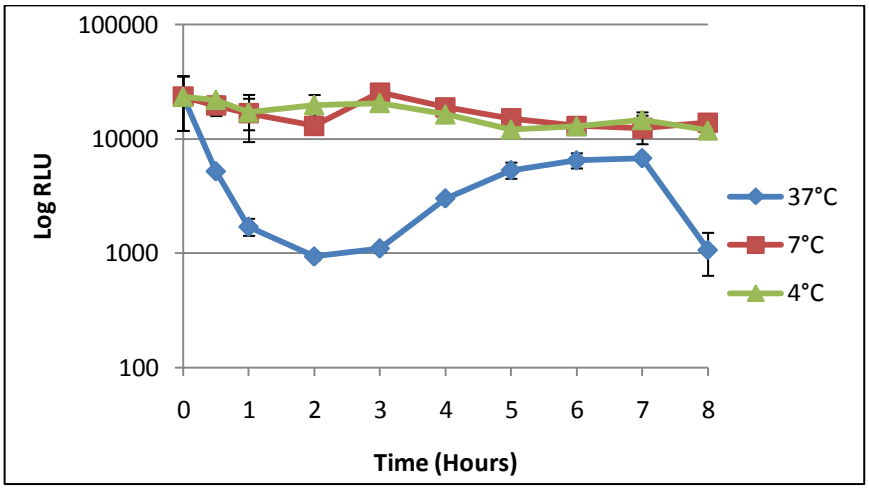


Figure 3.20. Expression of *mdtJI* in LB following temperature shifts from 37°C to 7 and 4°C.

Temperature shift carried out after initial growth at 37°C for 1 hour.
Blue arrow indicates lowest level of expression, while the red arrow indicates peak induction
For each experiment, OD_{600} readings were carried out in triplicate, and luminescence readings in duplicate.. Duplicate experiments were performed
Results shown are the combined average of duplicate experiments.
RLU = Relative light units. (Relative light units = light units (AU) / OD_{600})
SEMs are indicated by error bars
Expression of *mdtJI* in LB at 37°C.

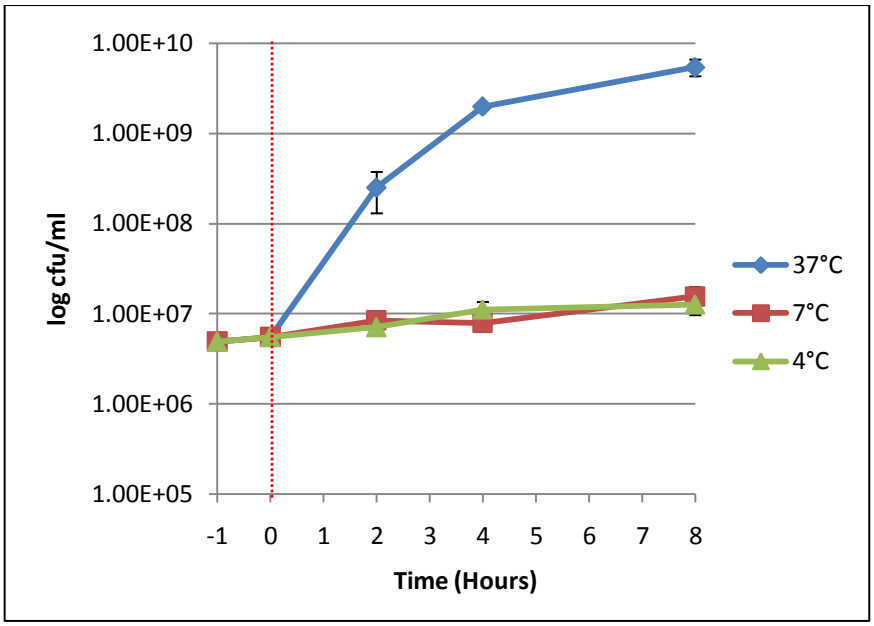


Figure 3.21 Growth *E. coli* O157:H7 pWU2 following temperature shift from 37°C to 7 and 4°C.

Temperature down-shift to 7 and 4°C was performed after growth at 37°C for hour 1. Time of shift is indicated by dashed red line.
Experiments were performed in duplicate.
For each experiment, OD_{600} readings were performed in duplicate
Results shown are the combined average of duplicate experiments
SEMs are indicated by error bars

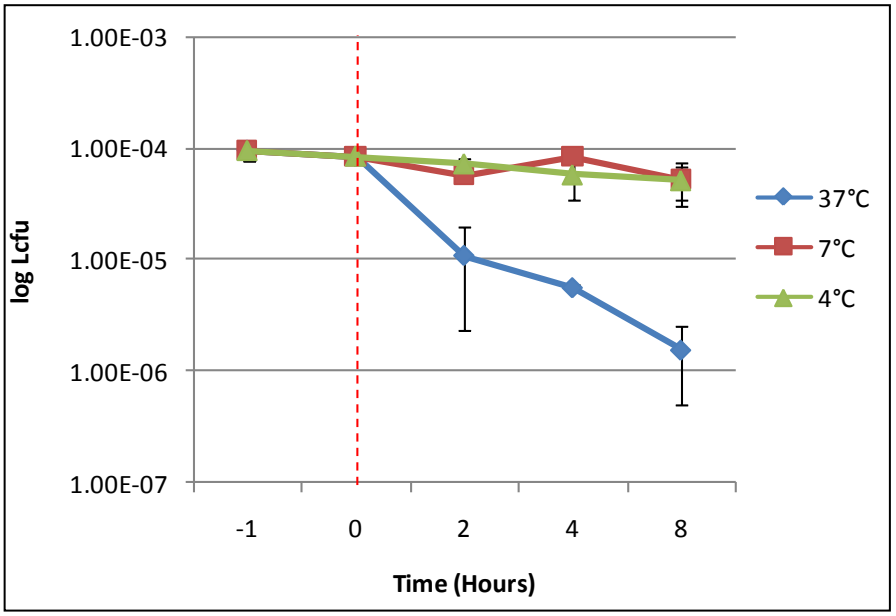


Figure 3.22. Expression of *mdtJI* in LB following temperature shifts from 37°C to 7 and 4°C.

Temperature shift carried out after initial growth at 37°C for 1 hour.
Lcfu = Light units expressed per colony forming unit (Light units (AU)/cfu/ml)
Experiments were performed in duplicate.
For each experiment $OD6_{00}$ readings were performed in triplicate and luminescence readings in duplicate.
Results shown are the combined average of duplicate experiments
SEMs are indicated by error bars.

3.2.4 Temperature shift from Hour 2

To allow residual bioluminescence levels, produced by the culture during stationary phase to reduce further, diluted cells were pre-incubated at 37°C for 2 hours instead of 1 hour, before the temperature downshift. Following this incubation, at the point of temperature downshift, the culture (average $O.D_{600}$ ~0.260) was divided into four equal aliquots. Three aliquots were exposed to a temperature downshift to: 7°C, 4°C and -1.5°C. -1.5°C was included in these experiments since this is recognized as the (optimal long-term chill storage temperature for meat. One aliquot was returned to 37°C as a control for *mdtJI* promoter activity at the optimal growth temperature. Cultures were sampled and bioluminescence measured hourly for 3 hours after the temperature shift. At each time point OD_{600} was measured **(Figure 3.23)**.

After the dilution, during the pre-incubation at 37°C, the OD continued to rise **(Figure 3.23)**. Following the shift to 7°C, 4°C and -1.5°C there was no significant increase in OD over the remaining period of the experiment. Growth continued at 37°C for a further hour, before slowing as the culture transitioned into stationary phase.

One hour (T1) after the downshift in temperature from 37°C to 7, 4 and –1.5°C, there was a significant difference in *mdtJI* expression ($p = 0.004$, 0.007 and 0.000 respectively). In line with previous observations, *mdtJI* expression at 37°C decreased, with a 3-fold reduction (2.86) from an average of 1831 RLU at T0, to 640 RLU at T1, while cultures shifted to 7, 4 and -1.5°C maintained their expression of *mdtJI* between 1568-2195 RLU **(Figure 3.24)**. No significant difference in bioluminescence output was observed between the cultures held at any of refrigeration temperatures, *mdtJI* expression remained between 1407-2194 RLU, for the duration of the experiment following temperature down-shift. However, at 37°C, following the initial 3-fold reduction of *mdtJI* expression observed at T1, expression remained constant (~ 632 RLU) until T2, then increased to 1665 RLU by T3. At 37°C, there is no significant difference in *mdtJI* expression at T3 compared with T0 ($p = 0.774$). When the temperature shift is carried out after 2 hours at 37°C, the greatest point of difference in expression

between at 37°C and cold temperatures occurred at T1 post temperature shift. At T1, *mdtJI* is expressing between 2.5-3.5 times more at refrigeration temperatures (1568-2194 RLU) than it is at 37°C (640 RLU; $p \leq 0.0007$). The observed rise in expression at 37°C for hour 3, coincides with the transition from exponential growth to stationary phase.

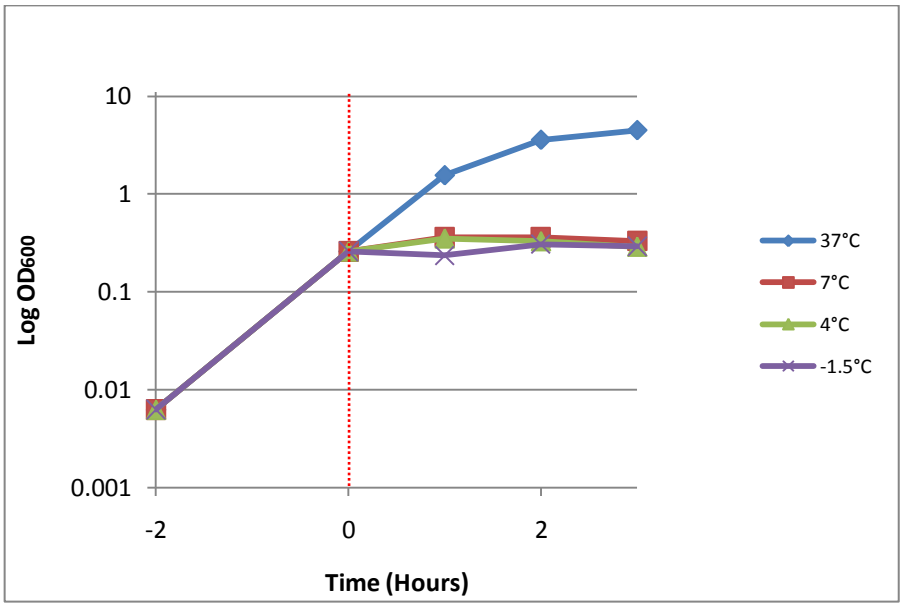


Figure 3.23. Growth of *E. coli* O157:H7 pWU2 following temperature shift from 37°C to 7, 4 and -1.5°C.

Temperature shifts were performed after 2 hours growth at 37°C, as indicated by the dashed red line.

Experiments were performed in duplicate.

For each experiment, OD_{600} readings were performed in triplicate.

Results shown are the combined average of duplicate experiments.

SEMs for 37, 7, 4 and -1.5°C were ≤0.082, ≤0.054, ≤0.052 and ≤0.012 respectively

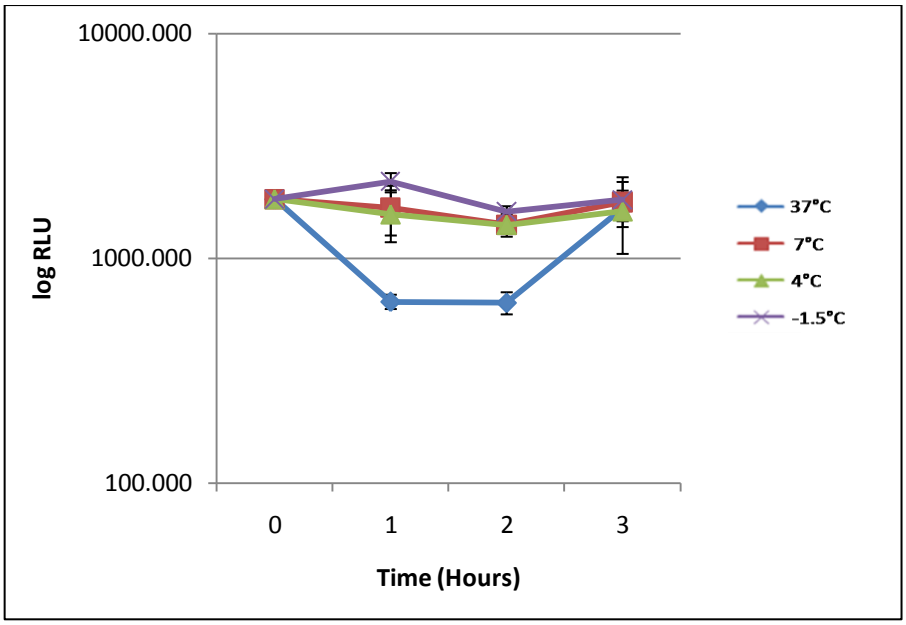


Figure 3.24. Expression of *mdtJI* in LB for 3 hours following temperature shifts from 37°C to 7°C, 4°C. and -1.5°C

Temperature shifts were performed after initial growth at 37°C for 2 hours, results shown are from time of shift (T0).

Results shown are the combined average of duplicate experiments.

RLU = Relative light units. (Relative light units = light units (AU) / OD_{600})

SEMs are indicated by error bars

Expression of *mdtJI* in LB at 37°C.

Blue arrow indicates lowest level of expression, while the red arrow indicates peak induction

Duplicate experiments were performed

3.3 Conclusions

In this chapter, a number of genes were identified as being expressed when *Escherichia coli* O157:H7 was exposed to refrigeration temperatures **(Table 3.1)**. From these, four genes were selected for further study **(Table 3.2)**. Although all four genes were regulated in a temperature dependent manner, only *mdtJ* and *mdtI* were expressed at refrigeration temperatures but not at 37°C when grown on BHI. However, further analysis revealed that when grown on meat, *mdtJI* was indeed expressed at 37°C but to a lesser degree than at 4°C **(Figures 3.11 & 3.12)**. Transcription of *mdtJ* and *mdtI* arises from a single promoter. To monitor expression the promoter plus an additional 400nt upstream were cloned into pSB377 to create a bioluminescent promoter reporter, pWU2 **(Figure 3. 13)**. Using this reporter, we were able to determine that *mdtJI* expression was indeed

temperature dependent with less light, and therefore less promoter activity, in cultures incubated at 37°C compared to those at refrigeration temperatures **(Figures 3.20, 3.22 & 3.24)**. Curiously, we also observed increased light output from *Escherichia coli* O157:H7 cultures during early stationary phase **(Figures 3.16 & 3.20)**. This may explain why overnight cultures grown at 37°C were observed to produce light, which decreased exponentially on culture dilution and initiation of growth.

In conclusion, data presented here has confirmed that *mdtJI* is involved in the adaptive response, specifically adaptation to cold temperatures in *E. coli* O157:H7, and possibly, to growth cessation which occurs during entry into stationary phase. In Chapter 4 we will explore the role of culture density in the regulation of *mdtJI* expression.

Chapter 4: Results

The influence of quorum sensing on cold adaptation in *E. coli* O157:H7

Sequence analysis established that the *mdtJI* operon is in close proximity to *tqsA* (also known as *ydgG*) **(Section 3.1.1; Figure 3.5)**. TqsA is a putative transporter of the QS signal, AI-2, generated by LuxS (Herzberg *et al.*, 2006). TqsA is a transmembrane protein which either exports AI-2 out of the cell, or inhibits the uptake of AI-2 (Herzberg *et al*., 2006). There are approximately 400nt between the divergent promoters of *mdtJI* and *tqsA*. The close proximity of these promoters suggests that they may influence each other's activity. In light of this information, we hypothesized that quorum sensing and AI-2 may influence expression of MdtJI. If *mdtJI* transcription is influenced by temperature down-shift, as well as the presence of AI-2, it is possible that AI-2 and/or LuxS, through its role in central metabolism, maybe involved in the cold adaptation response.

In addition, an increase in the expression of the *mdtJI* promoter was observed that coincided with the transition of *E. coli* O157:H7 pWU2 into stationary phase. This result suggests that high cell density and/or growth phase may also stimulate the expression of *mdtJI*. This relationship with culture density further supports the hypothesis that QS and AI-2 may influence expression of *mdtJI*.

To elucidate the role of quorum sensing and AI-2 in the adaptation of *E. coli* O157:H7 to cold temperature, a VHBA assay was used to detect when during growth exogenous AI-2 was produced.

4.1 Detection of AI-2 using the *V. harveyi* AI-2 Bioluminescence Assay (VHBA).

LuxS is required for the production of AI-2 in both *V. harveyi* and *E. coli*. Standard assay methods (VHBA), for the detection of AI-2 in culture supernatants have been developed. These assays incorporate a *V. harveyi* sensor strain that emits light in the presence of AI-2. There are two different VHBA sensor strains, BB170 (M. Surette & Bassler, 1998) and MM32 (S. T. Miller *et al.*, 2004) that can be used to detect exogenous AI-2 in conditioned medium (CM).

4.1.1 Using the sensor strain *V. harveyi* BB170

One sensor strain often used for VHBA is BB170. This strain has a mutation in the *luxN* gene, which renders it incapable of sensing and responding to the AHL (AI-1) signal molecule whilst retaining the ability to respond to AI-2. BB170 does harbor an intact *luxS* gene however, and is able to produce endogenous AI-2 during mid to late exponential phase. Hence, the endpoint of the experiment is determined by monitoring the point at which the maximum light emission differential occurs.

V. harveyi BB170 was grown in AB at 30°C with shaking to an absorbance between 1.5 and 1.6 at OD_{600} and subsequently diluted 1:1000 in fresh AB. 10% CM (v/v) prepared from wild type *V. harveyi* BB120 (*luxS*$^+$) was added to the diluted *V. harveyi* BB170 culture and incubated at 30°C with shaking. 100µl aliquots were dispensed into a 96-well microtitre plate at hourly intervals, and light emission measured.

Basal light emission was observed from BB170 which had either 10% (v/v) sterile LB or AB added **(Figure 4.1)**, with the lowest level occurring at 4 hours. After 4 hours the level of light increased exponentially. Higher levels of light were emitted from cultures mixed with 10% CM (v/v) made from BB120 during the first 4 hours of the experiment. The highest light emission differential occurs at this point. For VHBAs, which incorporate BB170 as the sensor strain, this would be the optimal time at which CMs from test strains can be assessed for AI-2. This background luminescence makes this assay difficult to work with, as the point of

highest light emission differential is variable for each experiment, and has to be determined for each sample. One possible solution to this would be to use a sensor strain that does not contain LuxS and therefore cannot produce AI-2. MM32 offers this alternative.

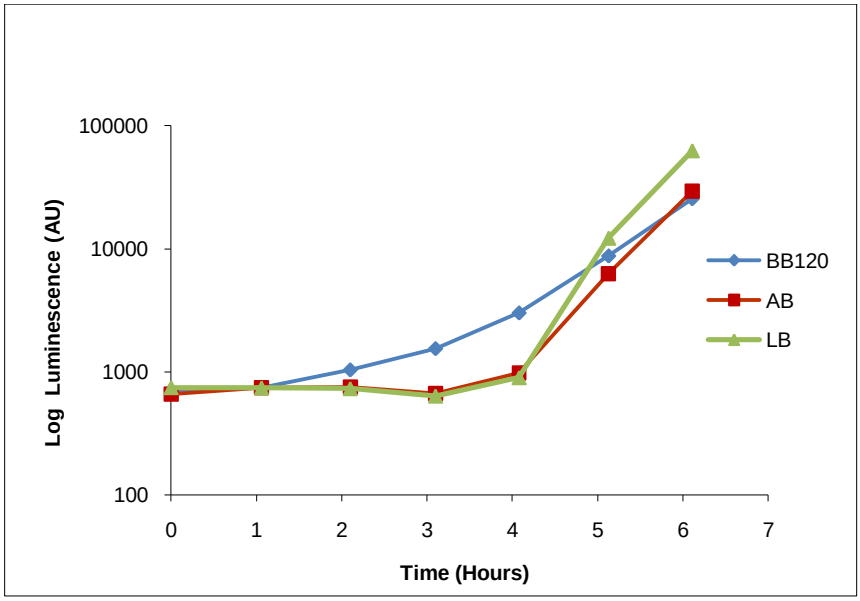


Figure 4.1. VHBA measuring the presence of AI-2 in media using the sensor strain, BB170
Quantity of light emitted (AU) by BB170. This strain will eventually produce an endogenous AI-2 signal upon 10% (v/v) addition of a variety of media. LB and AB are culture media that have not been exposed to bacteria, whereas BB120 is preconditioned medium (CM). BB120 culture was grown to an OD_{600} of 1.5-1.6. 3-4 hours is the time point at which the greatest effect of addition of CM on light emission is seen.
AU = Arbitrary Units

4.1.2 Using the sensor strain *V. harveyi* MM32

V. harveyi sensor strain MM32 was chosen as an AI-2 reporter because it lacks both the LuxN receptor needed to respond to AHL, and the LuxS enzyme needed to synthesize DPD, the precursor of AI-2 (S. T. Miller *et al.*, 2004). Hence, MM32 cannot produce AI-2, (Pereira *et al.*, 2008) and therefore should be ideal for detecting the presence of exogenous AI-2 present in conditioned medium (CM).

A VHBA was performed using MM32 to determine the detection profile *V. harveyi* MM32 was grown as per BB170, but to a final OD_{600} of 1.7 and subsequently diluted 1:5000 into fresh AB. Test strain CM was added at a final concentration of 10% (v/v). Incubation, sampling and light measurement was the same as for VHBA using BB170, however hourly samples were measured for 9 hours.

Where AI-2 was present, MM32 produced an exponential induction of bioluminescence **(Figure 4.2)**, which occurred after 6 hours of incubation. All control CMs, which did not contain AI-2, did not induce luminescence from the MM32 sensor strain at any stage during the assay. Based on this data, an assay window of between 6 to 8 hours can be used for determining the presence of AI-2 in culture supernatants.

Although the MM32 results appear cleaner, with no background interference caused by the presence of endogenous AI-2 which occurs with BB170, a trade off exists since the MM32 assay takes up to 8 hours to complete. For this study, the additional time required for the MM32 assay was acceptable, and VHBA's using MM32 as the sensor strain to detect the presence of AI-2 were used to determine AI-2 levels in CM.

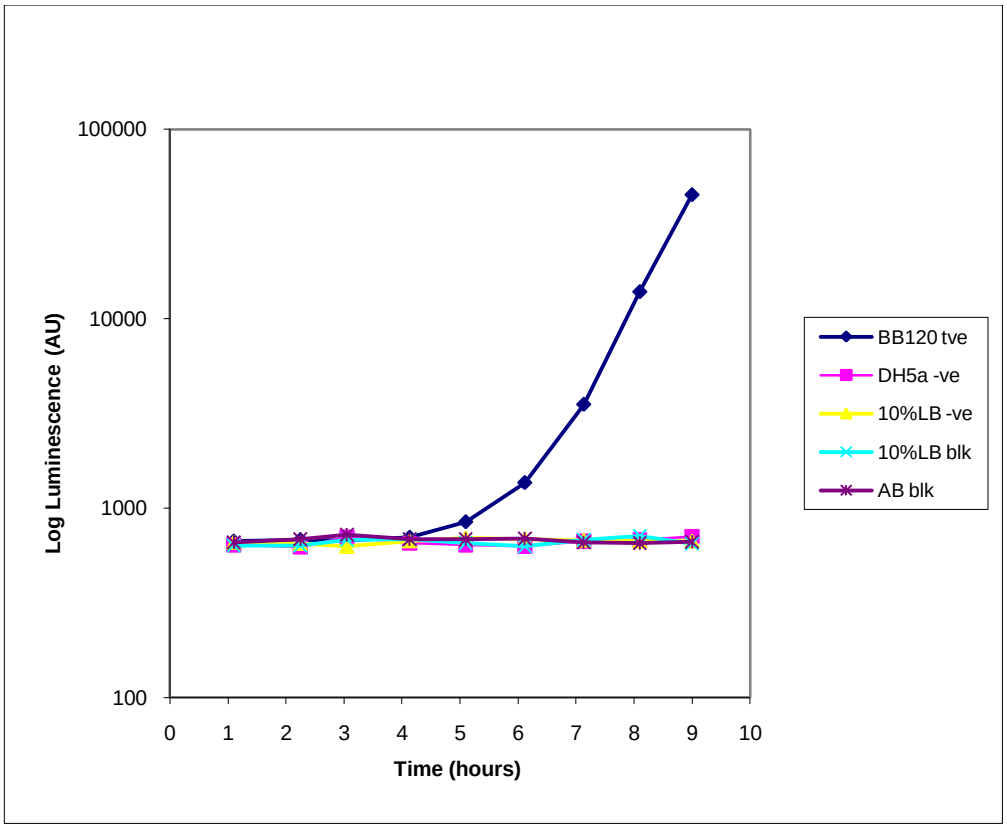


Figure 4.2. VHBA measuring the presence of AI-2 in media using the sensor strain, MM32

Quantity of light emitted by MM32. This strain has no endogenous AI-2 signal upon 10% (v/v) addition of a variety of media. LB and AB are culture media that have not been exposed to bacteria whereas BB120 is a positive AI-2 preconditioned medium (CM). BB120 culture was grown to an OD_{600} of 1.7. For future VHBAs using MM32, 7-8 hours will be the time point at which luminescence is recorded.

BB120 = 10% conditioned media, exposed to growth of BB120 positive AI-2 producing strain.

DH5a –ve = 10% conditioned media, exposed to growth of DH5α, strain that does not produce. AI-2. 10% LB –ve = 10% fresh LB only added to MM32.

LB blk = LB media only blank, without BB120 or MM32.

AB blk = AB media only blank, without MM32

AU = Arbitrary Units

4.2 AI-2 production by *E. coli* O157:H7 during growth at optimal temperature (37°C).

A number of studies have indicated that *E. coli* O157:H7 can produce AI-2 (Sperandio *et al.*, 1998; Sperandio *et al.*, 1999) however no data has been published profiling AI-2 expression during growth at 37°C. To confirm the optimal point in growth to prepare AI-2 containing conditioned media, *E. coli* O157:H7 and CLEN34 were inoculated into LB and grown at 37°C with shaking for 12 hours **(Figure 4.3)**. Samples were harvested hourly, and CM prepared by centrifugation followed by filter sterilisation to remove any remaining bacterial cells. VHBAs using MM32 were performed for each CM sample. Data has been normalized such that light emission measurements are expressed as fold-induction compared with the negative control sample produced from CLEN34 cultures.

AI-2 levels in CM increased 20-fold during exponential growth (T0 – T4); **(Figure 4.3 and 4.4; T0-T4)**. AI-2 levels were highest in early stationary phase between hours 4-8, where levels of AI-2 had increased approximately 108 times (~111.34 RLU) compared with T0 (1.03 RLU). The level of AI-2 in the CM reduced at T10, dropping from 111.34 at hour 8, to 13 RLU at hour 10, and this level was maintained in the final sampling at T12. No light was produced by MM32 in response to any of the CLEN34 *luxS*$^{-}$ negative control CM samples (data not shown). A representative CLEN34 CM sample taken at T6, when AI-2 levels would be maximal in the parental strain, is shown **(Figure 4.4. Sample - neg LuxS^{-})**. In addition, light was not produced in the presence of DH5α CM (*luxS*$^{-}$), or LB medium alone **(Figure 4.4. Sample - neg DH5 and blk respectively)**. MM32 did produce light in response to CM made from BB120 **(Figure 4.4 Sample - pos)**. Based on this data, CM was harvested during early stationary phase, between hours 4-8, for use in subsequent experiments requiring the addition of exogenous AI-2.

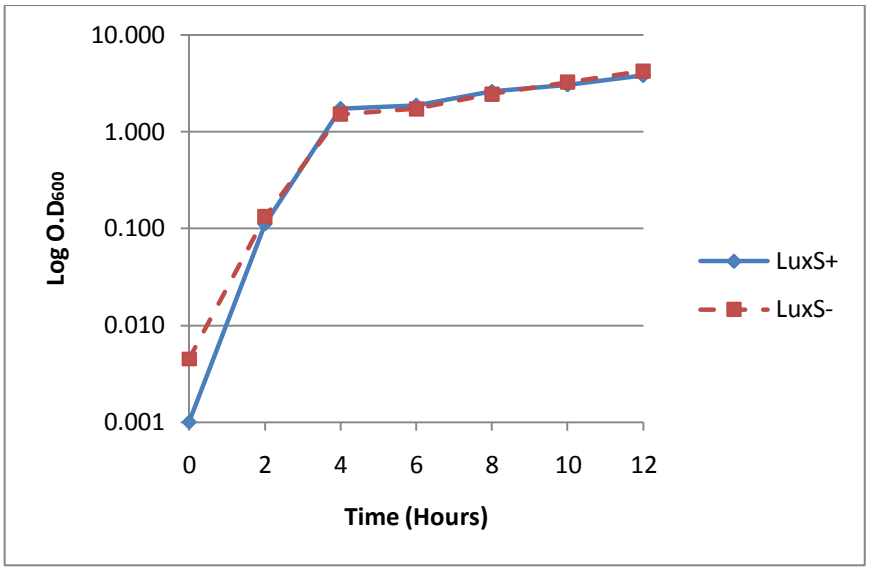


Figure 4.3. Growth of *E. coli* O157:H7 (LuxS$^+$) and CLEN34 (LuxS$^-$) in LB at 37°C

Experiments were performed in duplicate

For each experiment, OD_{600} readings were performed in triplicate.

Results shown are the combined average of duplicate experiments.

Standard Error of the Mean (SEM) for OD_{600} was ≤0.106 for LuxS$^+$ and ≤ 0.093 for LuxS$^-$

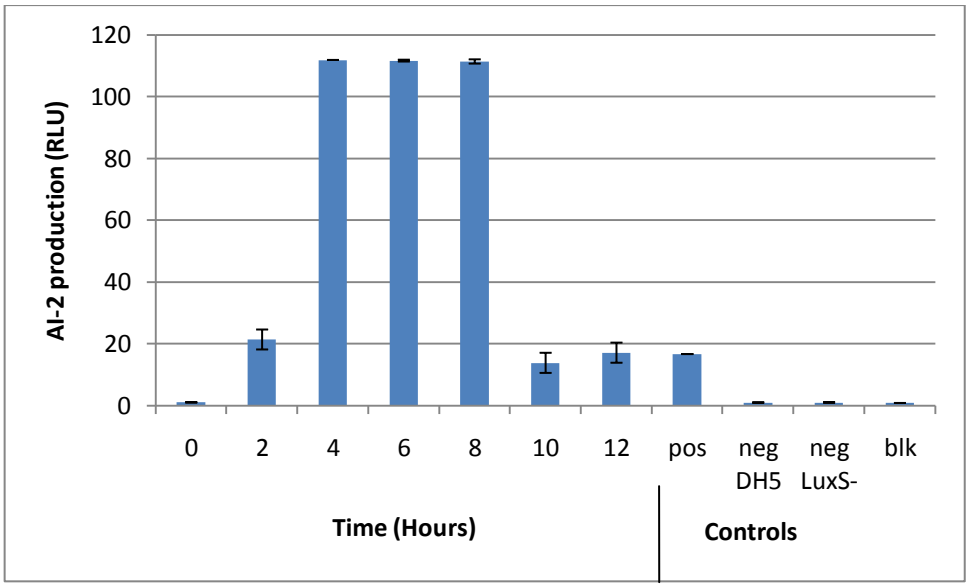


Figure 4.4. AI-2 production by *E. coli* O157:H7 at 37°C

RLU = Luminescence produced by MM32 in presence of 10% AI-2 containing CM/Luminescence produced by MM32 in response to 10% CM without AI-2 (LB grown with LuxS$^-$ culture).

Duplicate independent experiments were performed.

SEMs are indicated by Error bars.

pos = AI-2 positive CM, exposed to growth of V. *harveyi* BB120.

Neg DH5 and Neg luxS$^-$ = AI-2 negative CM, exposed to growth of LuxS$^-$ strains DH5α and CLEN34. The representative LuxS$^-$ CM shown, was exposed to growth of CLEN34 for 6 hours.

4.3 Effect of temperature downshift on AI-2 expression in *E. coli* O157:H7

To determine whether or not AI-2 is produced when *E. coli* O157:H7 is exposed to refrigeration temperatures; temperature down-shift experiments were performed. Two refrigeration temperatures were selected: 4 and -1.5°C.

E. coli O157:H7 was grown overnight (14-17 hours) with shaking in LB at 37°C to an OD_{600} of 1.5-1.6. Overnight cultures were diluted in fresh LB. Diluted cells were incubated at 37°C for 2 hours prior to the temperature down-shift. Samples were harvested hourly, both before and after the temperature down-shift, and tested for the presence of AI-2. AI-2 data is reported as the ratio (or fold induction) of RLU induced by 10% (v/v) test culture CM to RLU induced by 10% (v/v) control BB120 CM. Absorbance at OD_{600} was measured to determine growth phase of the culture. Luminescence was induced by the addition of the control BB120 CM to confirm that the assay was working appropriately (see **Figure 4.4. Sample - pos)**. No light was produced in response to either of the negative CM controls: DH5α or CLEN34 **(see Figure 4.4. Sample - neg DH5 and neg LuxS$^-$ respectively)**.

Immediately prior to the temperature shift (T2) from 37°C to 4°C **(Figure 4.5)**, *E. coli* O157:H7 CM induced 0.650-fold lighter than BB120. CM harvested 1 hour later at T3, from *E. coli* O157:H7 culture at 37°C, produced 2.4-fold more light than BB120. . This data suggests that exogenous AI-2 production continued to increase during the first three hours of growth at 37°C. CM harvested from cultures incubated at 4°C, showed only a slight but statistically insignificant fold increase in the level of bioluminescence compared to that observed prior to the temperature shift (0. 992 compared to 0.656 respectively). These findings suggest that AI-2 levels are not increasing upon exposure to 4°C but remaining at a level consistent with cell density. There was approximately 4-fold less relative light produced in response to CM harvested from 4°C, than that from 37°C, which is consistent with the observed increase in culture density which occurred at 37°C. CM harvested from 37°C after 4 hours (T4), resulted in only a 1.4-fold light induction, approximately half that observed at T3 (T3 = 2.4, T4 = 1.4) suggesting

that AI-2 levels in the CM had diminished by approximately 2-fold, after 4 hours growth at 37°C. No significant change was observed for light induction from CM harvested from 4°C, between T3 (0.992) and T4 (0.932) suggesting that AI-2 levels did not increase or decrease in the CM maintained at 4°C.

Light production induced by cultures shifted to -1.5°C in LB, showed similar trends in AI-2 levels as those seen for 4°C **(Figure 4.6)**.

These temperature downshift assays were repeated using BHI media to determine whether the nutritional status of the culture influenced AI-2 production. Similar trends in fold induction of light were observed for CMs harvested from cultures grown in BHI as LB at the three test temperatures: 37°C, 4°C and -1.5°C; with a significant increase in AI-2 levels occurring at 37°C. However, in BHI medium, luminescence in response to CM harvested from 37°C at T3 and T4, did not decrease but remained steady (2.2/2.2 and 1.6/1.2; **Figure 4.7 and Figure 4.8 respectively)**. While similar trends showing a constant level of AI-2 production in line with that observed at the point of downshift to refrigeration temperatures were observed, the difference in ratios between AI-2 produced at 37°C compared with the cold temperatures was greater **(Figure 4.5 and 4.6; LB and 4.7 and 4.8; BHI).**

This data suggests that while AI-2 is produced during growth at 37°C, significant AI-2 levels are not produced upon exposure to the refrigeration temperatures, 4 or -1.5°C. AI-2 production is maintained at a level in line with the OD of the culture at transfer.

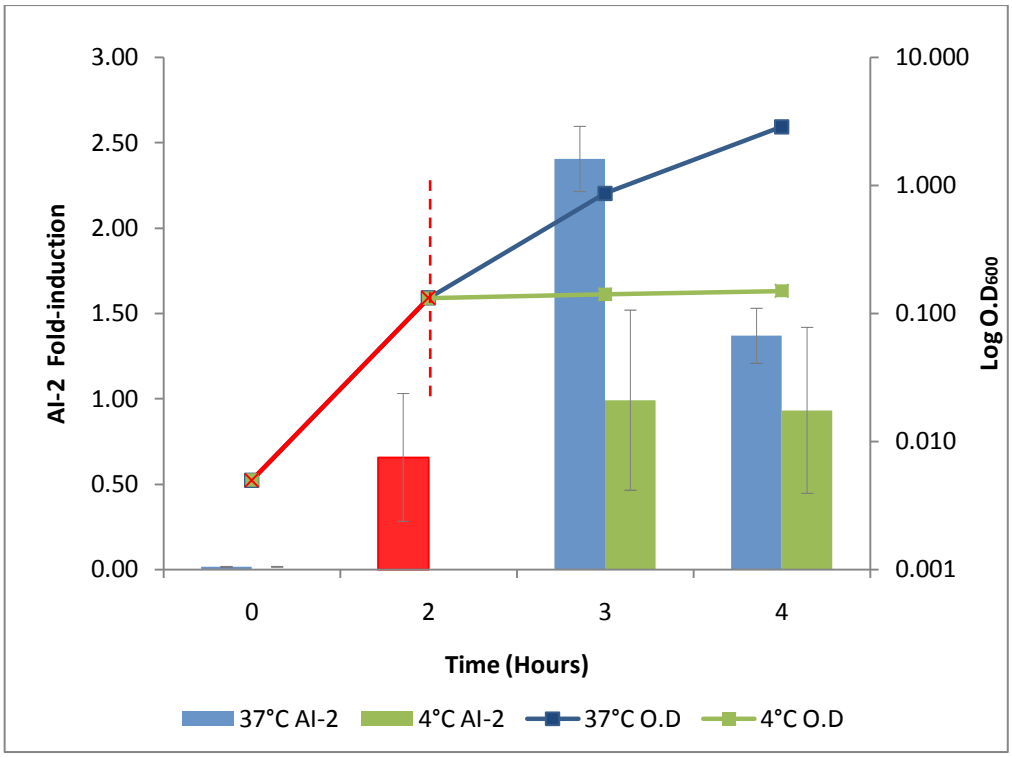


Figure 4.5. AI-2 production in LB upon temperature down-shift from 37°C to 4°C

Time of temperature shift (T2) is indicated by a dashed red line

AI-2 fold induction = Light emitted by MM32 in response to test CM divided by RLU emitted by MM32 in response to positive CM control (BB120).

Duplicate experiments were performed

OD_{600} readings were performed in triplicate and Luminescence readings in duplicate.

Results shown are the average of the duplicate experiments.

SEMs are indicated by error bars.

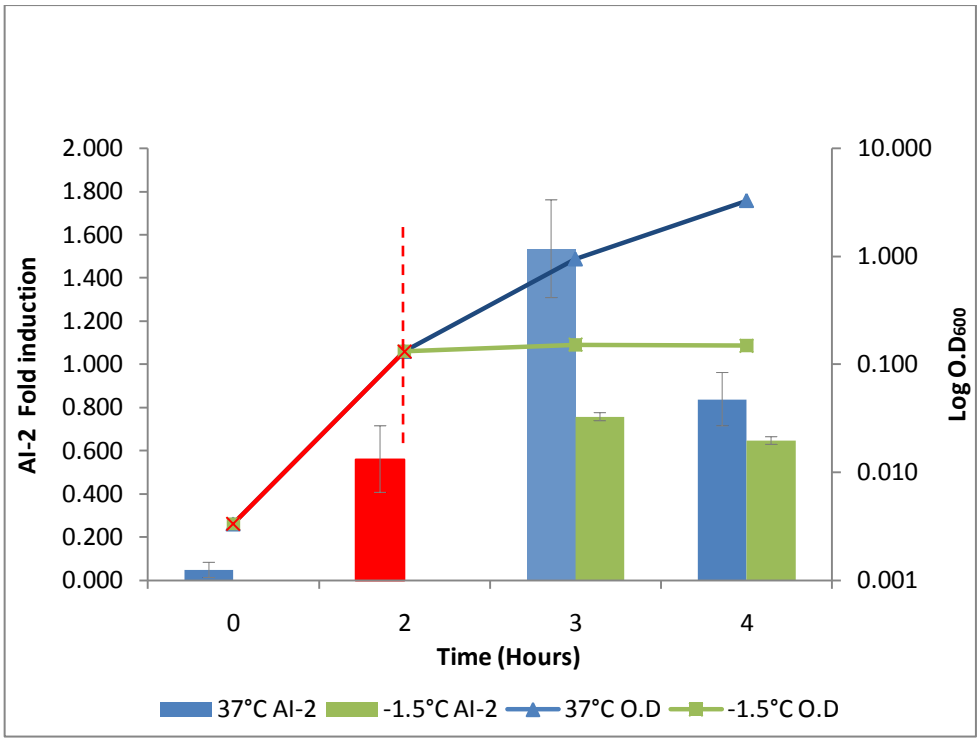


Figure 4.6. AI-2 production in LB upon temperature down-shift from 37°C to -1.5°C
Time of temperature shift (T2) is indicated by a dashed red line
AI-2 fold induction = Light emitted by MM32 in response to test CM divided by RLU emitted by MM32 in response to positive CM control (BB120).
Duplicate experiments were performed
OD_{600} readings were performed in triplicate and Luminescence readings in duplicate.
Results shown are the average of the duplicated experiments.
SEMs are indicated by error bars.

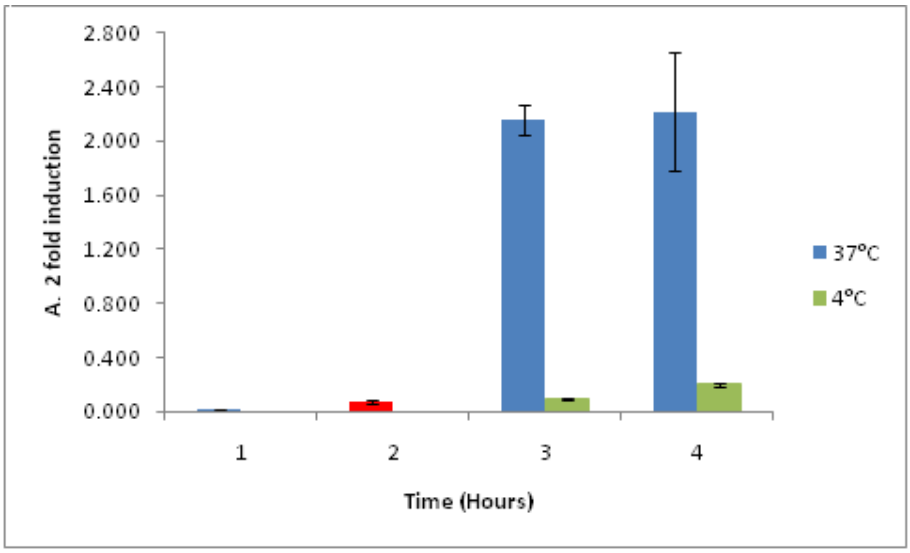


Figure 4.7. AI-2 production in BHI upon temperature down-shift from 37°C to 4°C

Time of temperature shift (T2) is indicated in red.
AI-2 fold induction = Light emitted by MM32 in response to test CM divided by RLU emitted by MM32 in response to positive CM control (BB120).
Duplicate experiments were performed.
For each experiment, luminescent readings were carried out in duplicate.
Results shown are the average of the duplicated experiments.
SEMs are indicated by error bars.

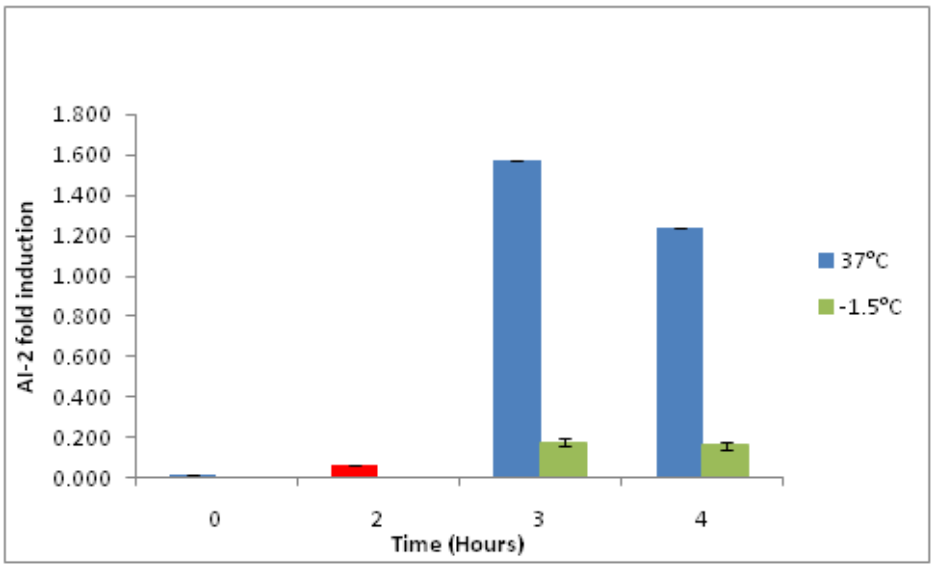


Figure 4.8. AI-2 production in BHI upon temperature down-shift from 37°C to -1.5°C

Time of temperature shift (T2) is indicated in red
AI-2 fold induction = Light emitted by MM32 in response to test CM divided by RLU emitted by MM32 in response to positive CM control (BB120).
Duplicate experiments were performed.
For each experiment, luminescence was read in duplicate.
Results shown are the average of the duplicated experiments.
SEMs are indicated by error bars.

4.4 Does LuxS influence the survival of *E. coli* O157:H7 at refrigeration temps?

Data presented here has shown that AI-2 production does not change within the first 2 hours following exposure to refrigeration temperatures **(Section 4.3)**. However, the question remains as to whether the metabolic role of LuxS, within the activated methyl cycle, has any influence over growth and survival of *E. coli* O157:H7, following exposure to refrigeration temperatures.

To determine if the presence of LuxS affects survival of this pathogen at refrigeration temperatures, experiments were performed, in which the growth of *E. coli* O157:H7 (*luxS*$^+$), was compared to CLEN34 (*luxS*$^-$) following a temperature down-shift from 37°C to the refrigeration temperatures of 4 and -1.5°C.

Both *E. coli* cultures were grown for 14-17 hours in LB, with shaking to OD_{600} 5 – 6. Cultures were diluted in fresh LB and grown for 1 hour before samples were split and a temperature shift from 37°C to 4°C was performed. Samples were taken hourly for absorbance measurements (OD_{600}), Aerobic plate counts (APC) were performed every 2 hours to enumerate viable colonies and establish survival curves (cfu/ml).

Both *luxS*$^+$ and *luxS*$^-$ cultures grew exponentially **(Figure 4.9)** for the first 4 hours of growth, with generation times of 21 and 18 minutes respectively. This difference in growth rate resulted in the optical density of the *luxS*- culture being higher at the point of temperature shift. No significant increase in growth was observed for either culture, from hour 5 to hour 9 (T4-T8) as both cultures entered into stationary phase (stationary phase generation time equaled 216 minutes). Both cultures ceased exponential growth immediately upon exposure to 4°C. *E. coli* O157:H7 OD levels remained between 0.025 and 0.047 for 8 hours following temperature down-shift. Likewise, CLEN34 maintained OD levels (0.068 to 0.107) throughout the same time period. To determine if there was any significant difference in survival for cultures with or without *luxS* when exposed to 4°C and to allow for the difference in optical density at the point of downshift,

calculations were performed for each time point, in order to assess growth over time. Growth over time equals OD at a chosen time point, divided by OD at the time of shift (T0). No significant difference between *E. coli* O157:H7 cultures were observed at any time point during the experiment (37°C, $p \geq 2.57$; 4°C, $p \geq 0.10$).

Growth curves constructed using cfu/ml **(Figure 4.10)** confirmed the results observed using absorbance (OD_{600}). No significant difference in cell number was observed for both *luxS*$^{+}$ or *luxS*$^{-}$ cultures at 37°C or upon exposure to 4°C (37°C $p \geq 0.823$; 4°C, $p \geq 0.074$).

A second temperature shift experiment was performed, to determine (a) whether allowing cultures to grow for longer prior to the temperature down-shift, and (b) whether the refrigeration temperature used, would have any effect on the growth or survival for either culture. The temperature shift was therefore performed after 2 hours exponential growth at 37°C, and cultures were shifted to both 4°C and -1.5°C. Since similar results were observed previously for OD and APC, only OD was used to measure growth **(Figure 4.11)**. OD_{600} was monitored hourly for 3 hours post temperature shift.

After 2 hours of exponential growth at 37°C, both *E. coli* O157:H7 and CLEN34 behaved in the same way, when exposed to 4 and -1.5°C **(Figure 4.10)**. Growth was arrested upon exposure to either of the refrigeration temperatures for both cultures, in a manner similar to that observed at 4°C in the previous experiment **(Figure 4.8)**. There was no significant difference in growth or survival at 4 or -1.5°C for 3 hours following exposure to these temperatures.

This data, suggests that LuxS is not involved in the survival of *E. coli* O157:H7 during short term exposure to refrigeration temperatures.

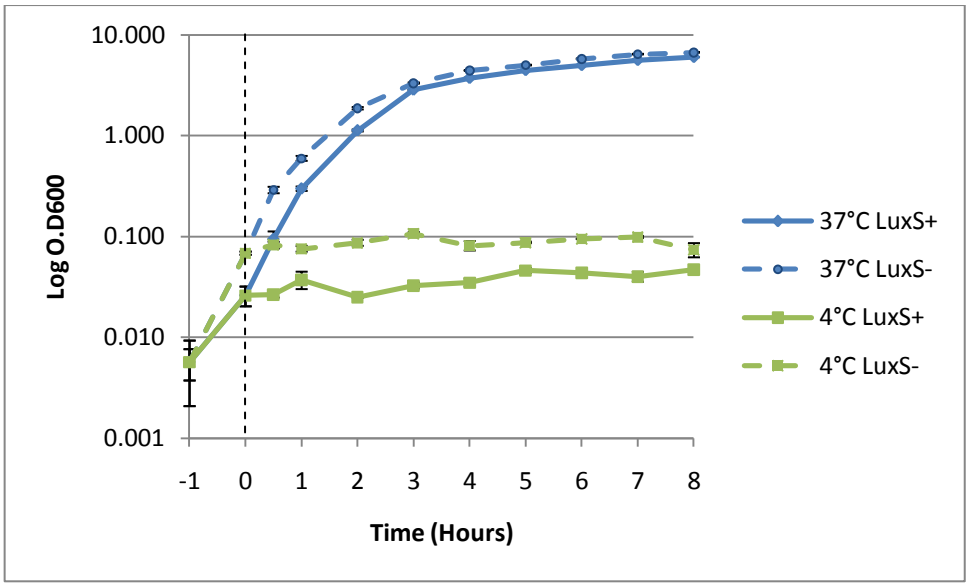


Figure 4.9. Growth of *E. coli* O157:H7 parental strain (LuxS$^+$) and CLEN34 (LuxS$^-$) following a temperature down-shift from 37°C to 4°C.

Temperature shift was performed after initial growth at 37°C for 1 hour, and is indicated by a dashed black line.
Duplicate experiments were performed.
For each experiment, OD_{600} readings were carried out in triplicate.
Results shown are the combined average of duplicate experiments.
SEMs are indicated by error bars.

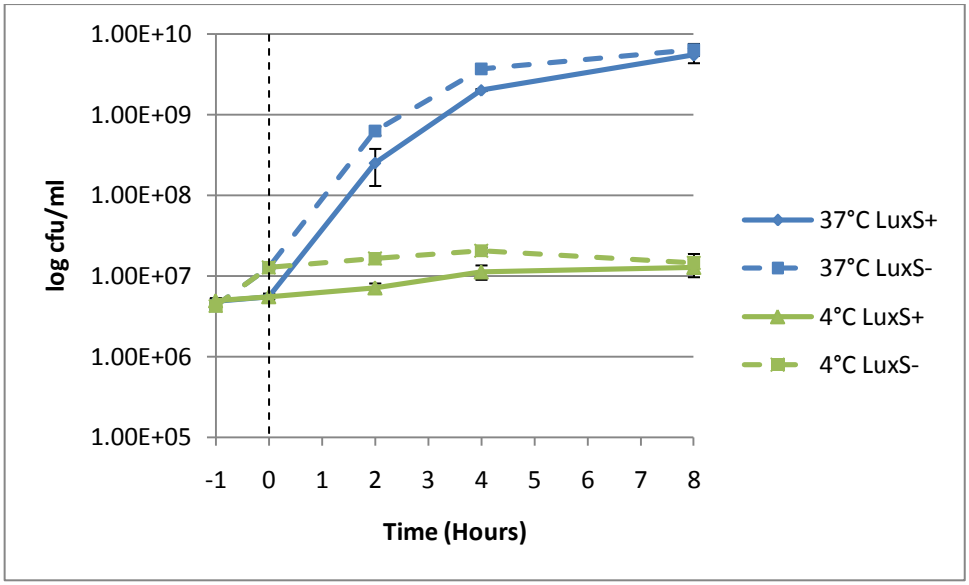


Figure 4.10. Growth of *E. coli* O157:H7 parental strain (LuxS$^+$) and CLEN34 (LuxS$^-$) following a temperature down-shift from 37°C to 4°C.

Temperature shift was performed after initial growth at 37°C for 1 hour, and is indicated by dashed black line.
Duplicate experiments were performed.
For each experiment, APCs were performed in duplicate.
Results shown are the combined average of duplicate experiments.
SEMs are indicated by error bars.

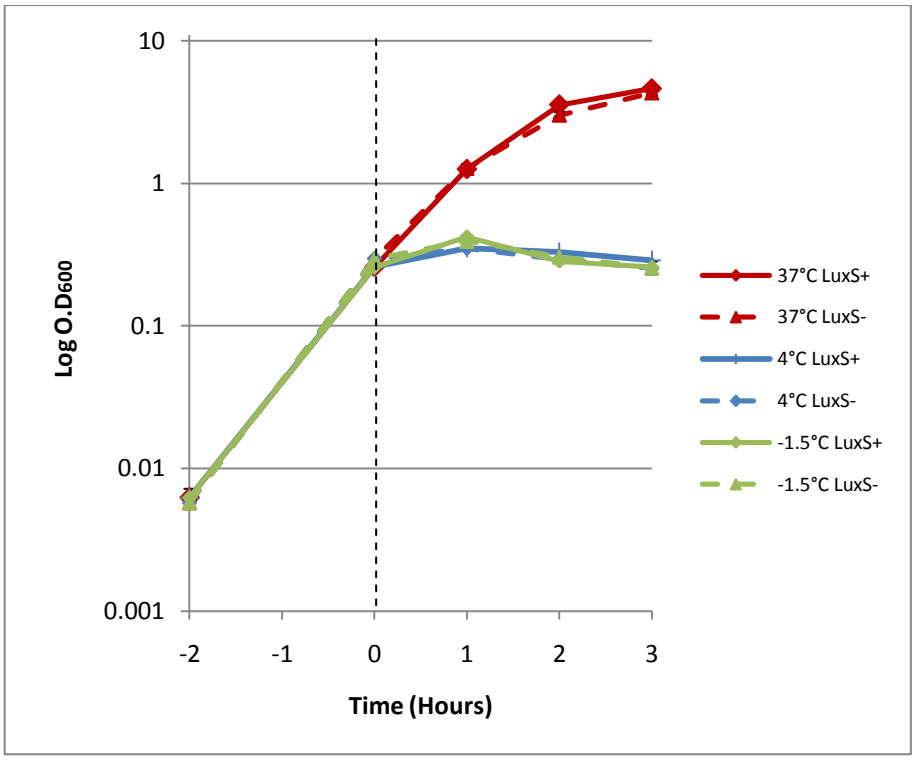


Figure 4.11. Growth of *E. coli* O157:H7 parental strain (LuxS$^+$) and CLEN34 (LuxS$^-$) , following a temperature down-shift from 37°C to 4°C and -1.5°C.

Temperature shift was performed 2 hours after growth at 37°C, and is indicated by dashed black line.

Duplicate experiments were performed.

For each experiment, OD readings were performed in triplicate.

Results shown are the combined average of duplicate experiments.

SEMs: 37°C LuxS$^+$, ≤0.082; 37°C LuxS$^-$, ≤0.161; 4°C LuxS$^+$, ≤0.052; 4°C LuxS$^-$, ≤0.066; -1.5°C LuxS$^+$, ≤0.012; -1.5°C LuxS$^-$, ≤0.036.

4.5 Does LuxS or AI-2 affect the expression of *mdtJI*?

To determine whether QS, specifically the LuxS/AI-2, is involved in the regulation of *mdtJI* expression, pWU2 was transformed into CLEN34 (*luxS*$^-$).

The parental and *luxS*$^-$ strains containing the *mdtJI* reporter were grown in LB at 37°C, with shaking for 9 hours. Light reporter assays were performed hourly as previously described **(Section 3.2.2)**, with both APCs and OD_{600} measurements performed to ascertain growth, and population density.

4.5.1 Effect of *luxS* on the expression of *mdtJI* at 37°C (optimal growth)

It was previously noted that *mdtJI* expression decreased during exponential growth at 37°C in the parental strain of *E. coli* O157:H7 **(Figures 3.16 & 3.20)** and increased during early stationary phase **(Figure 3.16 & 3.20)**.

In the absence of *luxS*, the expression of *mdtJI* was observed to continuously decrease at 37°C, for the duration of the experiment **(Figure 4.12, 37°C LuxS$^-$)**. Specifically, *mdtJI* expression in CLEN34, underwent a 25-fold reduction from 6498 RLU at T0, to 258 RLU at T8. Unlike the parental strain, no increase in expression was observed during early stationary phase. The most significant difference between the parental strain and the *luxS* mutant, was observed at hour 4, when *mdtJI* expression in CLEN34 was 4 times less than that observed for *E. coli* O157:H7 (753 RLU and 3019 RLU respectively). This RLU data suggests that LuxS has an influence on the *mdtJI* expression at 37°C.

Conversely, *mdtJI* expression results related to viable cell number showed a different outcome. Data using Lcfu **(Figure 4.13)** showed that *mdtJI* expression at 37°C, continuously decreased in both strains, for the duration of the experiment (T0 to T8). *mdtJI* expression decreased approximately 56-fold, from $8.47x10^{-5}$ to $1.50x10^{-6}$ Lcfu in the parental *E. coli* O157:H7, while expression in CLEN4 decreased approximately 117-fold from 3.51×10^{-4} to 3.00×10^{-7} Lcfu. Initial observations suggested that *mdtJI* expression at 37°C is always lower in the absence of *luxS* **(Figure 4.13).** Statistically, there is a very significant difference between the parental strain and CLEN34 at T4 (p= 0.000). This data supports the positive role of *luxS* in the expression of *mdtJI* at 37°C. However, the differences observed are potentially due to CLEN34 having grown slightly faster within the first hour at 37°C, (T-1 to T0), creating an initial difference in cell numbers by T0 (*luxS*$^+$, 5.5×10^6 cfu/ml; *luxS*$^-$, 1.27×10^7 cfu/ml). In addition, *mdtJI* expression in both strains, was slightly, yet significantly different (p = 0.034) from the start (*luxS*$^+$,$8.47x10^{-5}$ Lcfu ; *luxS*$^-$, $3.51x10^{-5}$ Lcfu). To address this, further statistical analysis was carried out to determine the change in expression of *mdtJI* over time (T1-8/T0) for both strains. Results of this analysis, showed no significant difference in the expression of *mdtJI*, between the parental and mutant strains at any time during growth at 37°C ($p \geq 0.070$).

4.5.2 Effect of *luxS* on the expression of *mdtJI* at refrigeration temperatures

It has been established for the parental strain **(Section 3.2.3; Figures 3.20 & 3.22)** that *mdtJI* expression remains constant following exposure to refrigeration temperatures.

To determine if LuxS influences the expression of *mdtJI* at 4°C, temperature down-shift experiments were performed using CLEN34 pWU2. RLU results, showed that *mdtJI* expression remained relatively constant, ranging between 11907 to 23445 RLU for 8 hours following exposure to 4°C **(Figure 4.12, 4°C).** Similar results were seen for Lcfu **(Figure 4.13, 4°C).** No significant differences in the expression of *mdtJI* were observed at any time, between the parental strain and the *luxS*$^{-}$deletion strain. This was confirmed using both RLU ($p \geq 0.421$) and Lcfu ($p \geq 0.183$) as units of luminescent measurement. Similar results were observed for temperature down-shift experiments performed at -1.5°C **(Figure 4.14).** Based on this data, LuxS does not influence the expression of *mdtJI* upon exposure to refrigeration temperatures.

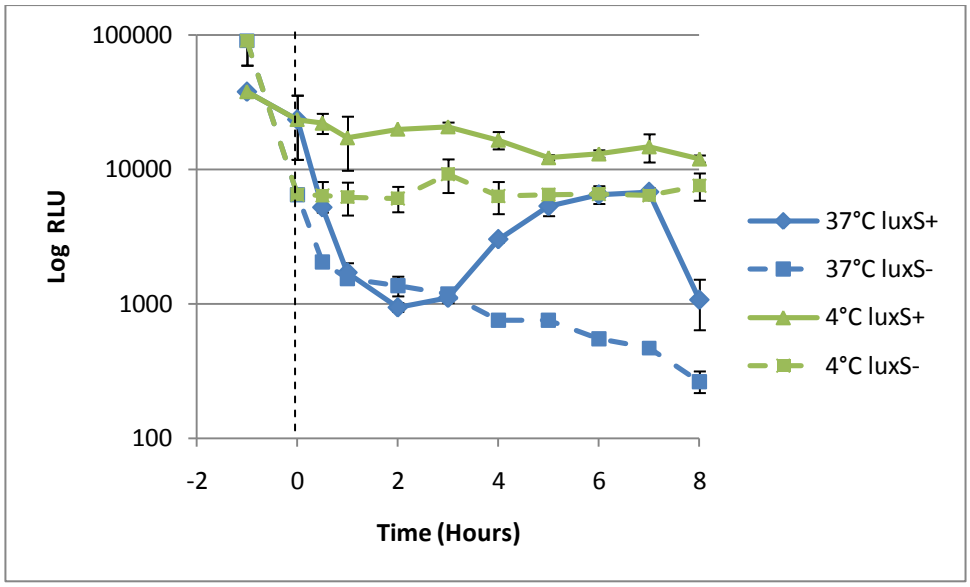


Figure 4.12. Expression of *mdtJI* in *E. coli* O157:H7 parental strain (LuxS$^+$) and CLEN34 (LuxS$^-$) at 37°C and upon exposure to 4°C.

Temperature shift was performed 2 hours after growth at 37°C, and is indicated by dashed black line.
RLU = Relative light units. (Relative light units = light units (AU) divided by O.D)
Experiments were performed in duplicate. For each experiment, OD readings were carried out in triplicate and luminescence readings in duplicate.
Results shown are the combined average of duplicate experiments.
SEMs are indicated by error bars.

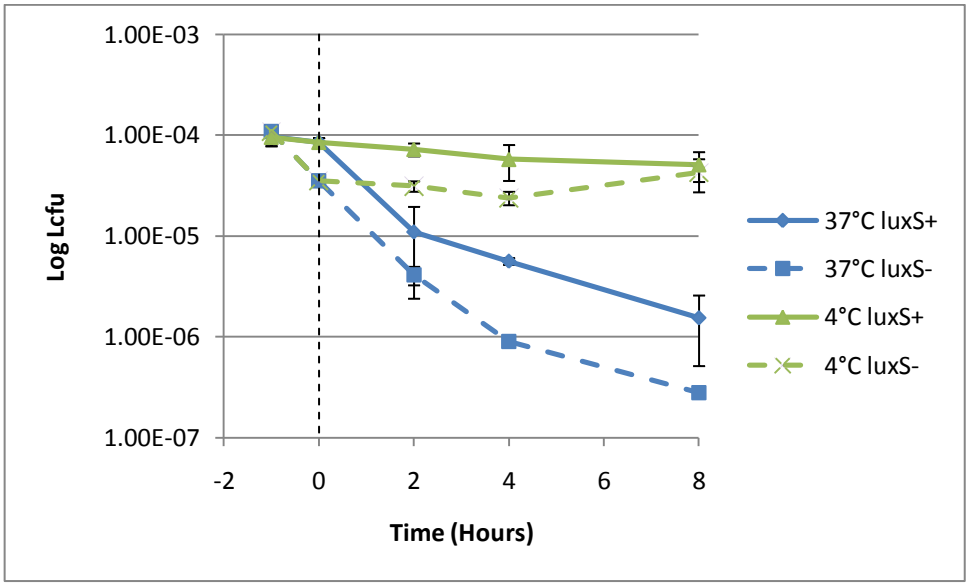


Figure 4.13. Expression of *mdtJI* in *E. coli* O157:H7 (*luxS*$^+$) and CLEN34 (*luxS*$^-$) at 37°C and upon exposure to 4°C.

Lcfu = Luminescence per colony forming unit (cfu). Lcfu = light units (AU) divided by cfu/ml
Experiments were performed in duplicate.
For each experiment, APCs were performed in duplicate, to obtain cfu/ml. Luminescence was also measured in duplicate. Results shown are the combined average of duplicate experiments.
Standard Error of the mean (SEM) is indicated by error bars.
Dashed black line indicates time of temperature shift

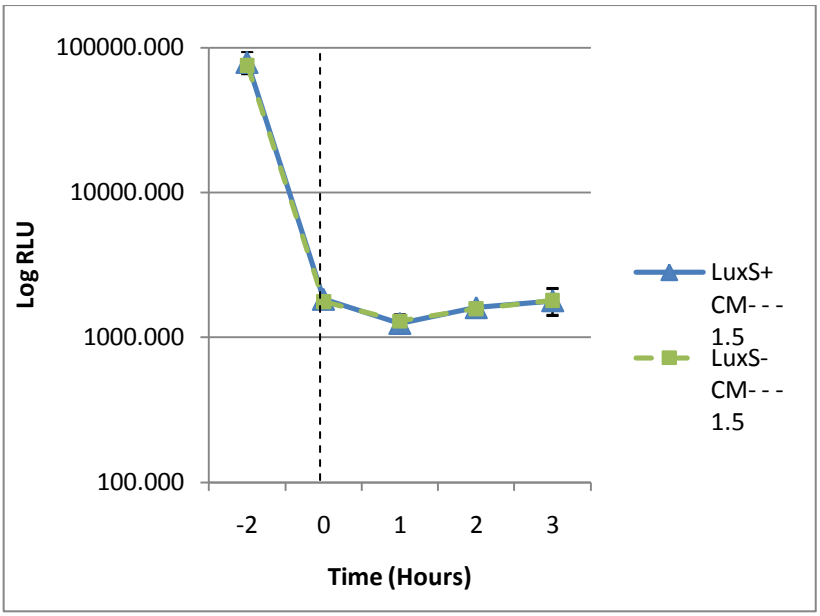


Figure 4.14. Expression of *mdtJI* in *E. coli* O157:H7 parental strain (LuxS$^+$) and CLEN34 (LuxS$^-$) following exposure to -1.5°C

RLU = Relative light units. (Relative light units = light units (AU) divided by O.D)
Experiments were performed in duplicate.
For each experiment, OD readings were carried out in triplicate. Luminescence measurements were performed in duplicate.
Results shown are the combined average of duplicate experiments.
Standard Error of the mean (SEM) is indicated by error bars.
Dashed black line indicates time of temperature shift

4.6 Is AI-2 and QS responsible for the differential expression of *mdtJI* at 37°C?

The only observable affect of LuxS on *mdtJI* expression occurred at 37°C, using RLU as the unit of measurement for luminescence. The question remained, as to whether this affect was due to AI-2 and QS, or due to the metabolic activities of LuxS. Hence AI-2 complementation studies using conditioned media were performed at 37°C.

To determine whether the addition of exogenous AI-2 was able to restore *mdtJI* expression at 37°C, 10% (v/v) AI-2 positive CM (CM+) and AI-2 negative (CM-) were added to parental and *luxS* mutant cultures at T5. A culture the *E. coli* O157:H7 parental strain was left untampered at 37°C, without the addition of CM (Neat), as a control for the dilution effects that may have occurred upon addition of CM.

mdtJI expression for both strains at 37°C **(Figure 4.15),** showed the same trends as previously described **(Figures 3.16 & 4.12)** despite the presence of exogenous AI-2 from T5 onwards. The addition of CM containing AI-2 had no significant affect on the expression of *mdtJI* in CLEN34 (*luxS*$^{-}$). There was no significant difference in the expression of *mdtJI* in CLEN34 grown with CM+ or with CM-, either at the time of addition (T5) or for the 3 hours following ($p \geq 0.156$). This data suggests that under these conditions, exogenous AI-2 is unable to complement the absence of LuxS and it is likely that the observed effect may be metabolic rather than QS-based.

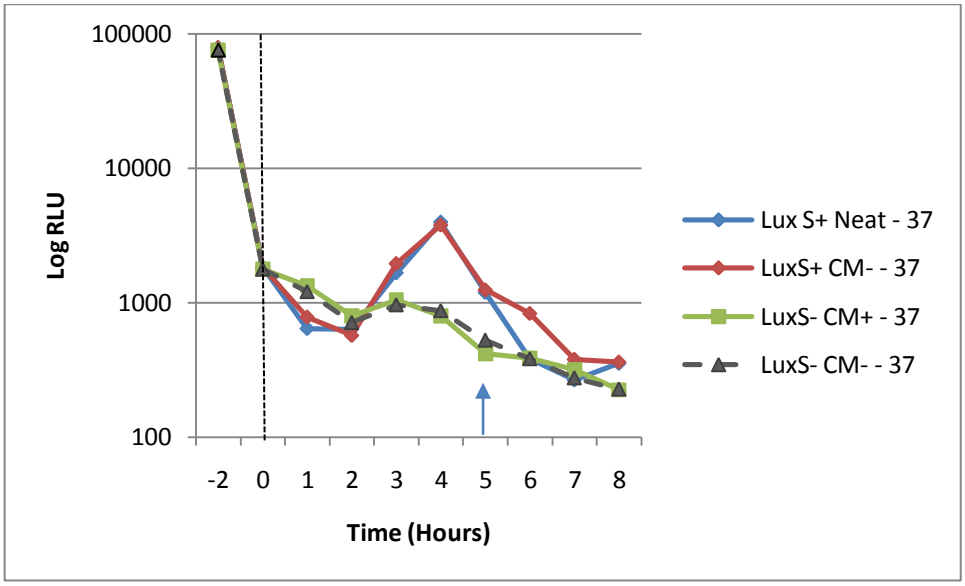


Figure 4.15. Effect of the addition of exogenous AI-2 (CM) on the expression of *mdtJI* in *E coli* O157:H7 (LuxS^{+}) and CLEN34 (LuxS^{-}) grown at 37°C.

RLU = Relative light units. (Relative light units = light units (AU) divided by O.D)

Experiments were performed in duplicate. For each experiment, OD measurements were performed in triplicate and luminescent readings were carried out in duplicate.

Results shown represent the combined average of duplicate experiments.

Blue arrow indicates time of CM+ and CM- addition.

Dashed black line indicates time of temperature shift.

LuxS+ Neat = E. coli O157:H7 Parental strain grown in LB with no addition of CM

LuxS+ CM- = E. coli O157:H7 Parental strain grown in LB, with the addition of CM+ (AI-2+)

LuxS- CM+ = CLEN34 grown in LB, with the addition of CM+

LuxS- CM- = CLEN34 grown in LB with the addition of CM- (AI-2-)

4.7 Conclusions

In Chapter 3, we determined that *mdtJI* expression occurred in *E. coli* O157:H7 not only at refrigeration temperatures but also during stationary phase at 37°C **(Figure 3.16)**. This observation lead us to hypothesize that population density might influence expression of *mdtJI*. Further supporting this hypothesis was the observation that 400nt upstream of the mdtJI operon promoter, was the promoter for *tqsA* (**Figure 3.6**), a transporter for the QS signal molecule, AI-2. *E. coli* O157:H7 does produce AI-2 during early stationary phase growth at 37°C **(Figure 4.4)**. In addition, AI-2 was recovered in CM from cultures held at refrigeration temperatures; the level of AI-2 reflected the culture density at the time of the shift **(Figure 4.5, 4.6)**. Viability of *E. coli* O157:H7 cells lacking a functional *luxS* gene was found to be the similar to the parental strain at refrigeration temperatures **(Figure 4.9, 4.10)**. Using pWU2 to report *mdtJI* expression, we determined that LuxS but not AI-2 affected *mdtJI* expression at 37°C **(Figure 4.15)**. No significant difference in *mdtJI* expression was observed at refrigeration temperatures for cells containing *luxS* compared to those without **(Figure 4.12, 4.13)**.

Based on the data presented here, the influence of LuxS on *mdtJI* expression in *E. coli* O157:H7 is most likely to be through metabolic activity, rather than QS activity.

Chapter 5: Discussion

E. coli O157:H7 is a major food-borne pathogen, mainly associated with the consumption of undercooked beef products. Using co-ordinate regulatory networks, and expressing specific stress related proteins, this pathogen is capable of surviving and adapting to a wide range of environments. For example, reservoirs for *E. coli* O157:H7 range from the intestine of warm blooded animals, particularly cattle, to soil and water. Rapid adaptation to these changeable environments, including temperature down-shift, is critical to the survival of this pathogen. Understanding the adaptive processes of *E. coli* O157:H7, including the pathogens ability to survive the cold temperatures associated with refrigeration, is of particular importance to the food and meat industry.

The initial intention of this study was to identify genes or operons, within *E. coli* O157:H7, involved in adaptation to cold temperatures and ultimately their long term survival during refrigeration. This ability to survive has major implications for the meat industry, where the presence of *E. coli* O157:H7 can lead to product rejection. Although extensive studies have been conducted on survival under stress, most have used the related lab-adapted *E. coli* K-12 strain. *E. coli* O157:H7 has a very large genome, with an additional 1.3Mb of DNA compared to *E. coli* K-12, much of which has yet to be assigned function. Numerous studies have demonstrated that *E. coli* O157:H7 is extremely hardy, capable of surviving in what would otherwise be considered hostile environments for an enteric organism. It is not therefore, unrealistic to expect that novel genes may be identified, within the *E. coli* O157:H7 genome that have yet to be associated with cold shock and adaptation to temperatures less than optimal for growth.

RAP-PCR (Li, personal communication, 2007) revealed that a number of genes in *E. coli* O157:H7, were differentially expressed at cold temperatures, within a meat context. Sequencing analysis revealed that a wide range of functionally different genes were expressed under these conditions **(Table 3.1)**. A range of gene types

were identified, and from those *slp*, *hslJ*, *mdtJ* and *mdtI* were selected for further analysis **(Table 3.2)**. Surprisingly, these genes had been reported in literature as being involved in the adaptive processes for environmental stress conditions other than cold temperature.

hsIJ encodes HsIJ, a heat shock inducible protein. In addition to temperature response this protein has been linked with novobiocin resistance, the regulation of virulence and transmission (Dowd & Ishizaki, 2006; Lilic *et al.*, 2003). In this study, we observed a 2-fold increase in expression at 37°C compared to 4, 0 or -1.5°C **(Figures 3.7 *hslJ* and 3.10 D)**, which is consistent with its role in heat shock as reported in literature (Dowd & Ishizaki, 2006; Lilic et al., 2003). It is unclear at this stage why *hslJ* expression appeared to increase in the preliminary cold shock experiments, but this may be an artifact of the RAP-PCR methodology.

In contrast, *slp* was also expressed at 37°C, but showed a 3.4-4.7-fold increase in expression at refrigeration temperatures **(Figures 3.7 *slp* and 3.10 C)**. *slp* encodes a lipoprotein, located in the outer membrane, which is induced upon carbon starvation (starvation lipoprotein) (White-Ziegler *et al*., 2008). Along with 7% of the *E. coli* K-12 genome, *slp* expression was shown to be higher at 23°C compared with 37°C. Of those 7%, 40% of genes, including *slp,* were regulated by RpoS, a major regulator of stress gene expression (White-Ziegler et al., 2008). While these studies utilised parallel cultures grown at 23 and 37°C, with a 14°C temperature difference, rather than temperature down-shifts, their findings do support those reported in this study.

The final set of genes examined, are reported to form a membrane-associated complex which functions as a putative multi-drug transporter. The MdtJ/MdtI complex is reported to transport nalidixic acid, sodium dodecyl sulfate (SDS), deoxycholate and fosfomycin (Higashi *et al.*, 2008; Nishino & Yamaguchi, 2001) and spermidine (Higashi et al., 2008). Detailed analysis of the RAP-PCR products revealed that *mdtJ* and *mdtI* are transcribed as a single mRNA molecule. Examination of the genome sequence confirmed that the genes form a discrete

operon. Preliminary experiments carried out using BHI agar confirmed the RAP-PCR result, demonstrating that both genes are expressed at refrigeration temperatures **(Figures 3.7 *mdtI* & *mdtJ* and 3.10 A&B)**. *mdtI* and *mdtJ* genes were chosen for further study, because they were the only genes examined in the preliminary experiments of this study, to be detected at cold temperature, and not at the optimal growth temperature (37°C). Furthermore, no temperature-dependent regulation has thus far been reported.

Interestingly, in contrast to *E. coli* O157:H7 grown on BHI agar, *mdtJ* and *mdtI* mRNA was expressed in cells cultured on a meat surface at 37°C **(Figures 3.11 and 3.12)**. The meat substrate is far more complex than BHI medium, and it is likely that there are a number of factors, present in the meat matrix, which could have activated the *mdtJI* promoter, for example, spermidine. Spermidine (SPD), as well as spermine (SPM) and putrescine (PUT), are dietary polyamines which occur naturally in meat and are essential for normal cell growth (Higashi *et al.*, 2008; Kalac, 2006). Usually, PUT, SPD and SPM can be found in fresh meat at levels of, <2, <5 and 20-40mg/kg respectively (Kalac, 2006). Immediately post-slaughter, high levels of spermine and spermidine can be detected in red meat, although spermine is the most prevalent polyamine at approximately 70% of the total amine levels (Kalac, 2006). Spermidine is also prevalent at 4°C in beef product. This polyamine is important in cellular metabolism since it converts *S*-adenosylmethionine (SAM) to spermine, which is an essential growth factor for some bacteria (Kalac, 2006). However, an over-accumulation of spermidine can be toxic in *E. coli*, inhibiting protein synthesis by binding to and precipitating RNA polymerase, thereby inhibiting growth. Two options available to deal with excess spermidine in the cell are metabolism by acetylation, via spermidine acetyltransferase (Carper *et al.*, 1991; Limsuwun & Jones, 2000) and active excretion. The MdtJI complex is involved in the excretion of spermidine from *E. coli* cells (Higashi et al., 2008). This may explain why *mdtJI* was expressed in bacteria, located on a meat surface at 37°C, but not on BHI agar.

Nonetheless, *mdtJ* and *mdtI* were expressed more at cold temperature than at 37°C in the meat matrix. This data suggests that temperature is an important regulator

of *mdtJI* expression. Studies have reported, that alternative spermidine reduction systems, such as acetylation catalysed by spermidine acetylase, are increased in response to the exposure of *E. coli* to 7°C (Limsuwun & Jones, 2000). Limsuwun and Jones (2000) determined that spermidine acetyltransferase is required to prevent spermidine toxicity at low temperatures, in *E. coli*. Data presented here also showed that mdtJI expressed more at 7°C compared with 37°C **(Figure 3.20)**. Furthermore, there was no significant difference in the expression of *mdtJI* at 7°C compared to 4°C. It would appear that cold temperature increases the need for the bacterial cell to reduce spermidine concentrations, which may be in part due to the need to release any additional protein inhibition, resulting from the presence of spermidine, which could impair adaptation processes.

BHI medium was used for the *mdtJI* expression studies because BHI bares nutritional similarities to meat, but is a more consistent and defined bacteriological medium. However, it was demonstrated in this study that results can differ when using the more complex matrix of meat. These findings highlight the care that must be taken when extrapolating conclusions from results observed using standard laboratory media, to the more complex environment of food and meat.

The cold temperatures chosen for this study reflect preservation and refrigeration temperatures used for domestic purposes, such as in the home or in supermarket displays (4°C and 0°C), and chill storage temperatures relevant to the meat industry (-1.5°C). 7°C was included in these studies for completion as there are a number of studies which use this as the lower temperature. Furthermore, 7°C can be used to represent chill temperature abuse scenarios, which can create problems in the preservation of meat and other fresh produce. No significant difference in the expression of *mdtI* or *mdtJ*, was observed between any of the cold temperatures used, suggesting that the degree of downshift may be more important than the final temperature per se. The reduction threshold required to stimulate *mdtJI* expression, has not been defined in this study, but the degree of down-shift in temperature is likely to be less than 30°C. It is important to realize, of course, that this response to refrigeration temperatures is brought about by

evolutionary responses to temperature down-shifts within the environment, and not to refrigeration itself.

E. coli O157:H7 growth ceases upon exposure to 4 or -1.5°C **(Figures 3.19, 3.21, 3.23, 4.5, 4.6, 4.9, 4.10 & 4.11)**. However, a whole cascade of gene expression will be activated as part of the cold shock response (Phadtare, 2004). How to capture the change in mRNA profiles for the bacterial populations at the different temperatures and compare them is a major issue; each method has its drawbacks. In this study we chose to carry out the initial confirmation analysis using RT-PCR of single points, before and after the temperature shift. This was complemented with the use of bioluminescence promoter reporters which provided expression data over time. The use of these reporters eliminates experimental problems associated with RNA extraction, amplification efficiencies, normalization of sample concentration and gel loading. Use of appropriate experimental controls is paramount as is sample normalization using a stable RNA, in this case 23SrRNA, to overcome these issues. A number of alternative methods to RT-PCR are available to directly measure mRNA concentrations but each has limitations. Methods include real time or quantitative RT-PCR (qRT-PCR), quantitative northern analysis and microarrays. In recent years a number of plasmid borne *gfp* and *luxS* promoter fusion reporters have been developed and used to monitor *in situ* promoter expression, giving real time data on promoter activity. For this study, an *mdtJI* promoter::*luxCDABE* plasmid-based reporter was constructed.

Interestingly, the overnight cultures of *E. coli* O157:H7 pWU2 showed high levels of light emission suggesting that *mtdJI* was expressed during established stationary phase. Upon culture dilution, light emission was observed to decrease steadily during exponential growth at 37°C with a 25-fold drop in expression. Curiously, light emission was observed to increase and subsequently decrease during the early stages of stationary phase **(Figure 3.16).**

Traditionally, light emission has been reported as RLU with normalization to culture optical density. To determine light production per viable cell, APCs were used to calculate the viable cell number present. Conversely to the OD related

data, light emission and therefore *mdtJI* expression continuously decreased, up to 56-fold for the duration of the experiment at 37°C. No increase in expression, transient or otherwise, was observed during early stationary phase. The reason for this difference is unclear, but it does highlight differences in methods used for analysing gene expression by population. There are a few reasons why this difference may exist. Firstly, the Lcfu data was based on 2 hourly samples as opposed to the hourly intervals used for OD measurements. It is therefore possible that the increase in light expression, seen upon transition into stationary phase using RLU, was missed using Lcfu, merely due to the timeframe in which the sample was taken. However, if this were the case, it would be likely that some indication of change in gene expression would have been seen. Alternatively, cells within the population may not be expressing a uniform level of MdtJI and light. It is likely that expression within these sub-populations and the size of the population is dynamic and therefore, expression per cell may vary over time. It is not possible to determine from the methods used and the data presented here, if these sub-populations exist. These results have highlighted the need to look at how individual cells behave within a population. Flow cytometry would allow individual cell light emissions to be measured to determine the amount of *mdtJI* expression per cell, and determine whether sub-populations do exist. However, the first step would be to confirm the Lcfu results, by performing APCs more frequently.

Following exposure to refrigeration temperatures, *mdtJI* expression remained constant for up to 8 hours. This was confirmed using both RLU and Lcfu **(Figures 3.2.0 and 3.22)**. Curiously, the level of expression appeared to be linked to the bacterial concentration at the time of the shift. This data supports the RT-PCR results, which showed that *mdtJI* was expressed more at refrigeration temperatures, than at 37°C. We hypothesized that the MdtJI complex is required at cold temperatures, as well as involved in the overall stress response. This is supported by the expression observed on meat compared to BHI agar and by the increase in expression observed upon entry into stationary phase. Stationary phase presents a number of stress conditions for the organism, including nutrient limitation and toxin build up, which may explain *mdtJI* expression upon transition

to stationary phase. Protein multi-tasking is not unusual in bacteria, a number of proteins have been suggested in literature to be multifunctional, and MdtJI has been shown to increase resistance to nalidixic acid, and combat spermidine toxicity. Stress responses are linked as part of a regulatory network, and it is likely that MdtJI is part of this network.

Plasmid-based *luxCDABE* reporter constructs were used in this study. The plasmid *luxCDABE*::*mdtJI* constructs were useful and allowed real-time visualisation of *mdtJI* expression from the *E. coli* O157:H7. However, some plasmid-*lux* constructs have been reported by Amin-Hanjani *et al*. (1993) to exhibit higher luminescence than those incorporated into the chromosome, most likely due to the higher copy number. Furthermore, antibiotic selection is required to maintain the plasmid. This imparts a metabolic load on the cell that may influence cell activities. However in spite of these limitations, valuable data of a comparative nature, rather than absolute values of total promoter activity, can be collected

To fully capture the regulation of a given promoter, 500nt of sequence upstream of the -35 is cloned in addition to the promoter sequence. This sequence is important because it will contain the binding site for any regulatory proteins or cofactors which will influence the binding efficiency of RNA polymerase to the -35 and -10 recognition sites of the promoter. An extra 500nt of upstream sequence was duly incorporated into *mtdJI*::*luxCDABE* reporter. Interestingly, analysis of this upstream sequence revealed the presence of a divergent promoter, located approximately 400nt upstream of the *mdtJI* promoter **(Figure 3.5)**. The gene transcribed by this promoter encodes *tqsA*, which is implicated in the transport of AI-2, a QS autoinducer molecule (Herzberg *et al.*, 2006).

QS systems have been implicated in the adaptive stress response systems of bacterial populations (DeLisa *et al.*, 2001). Furthermore, it has been suggested that this form of chemical communication, can occur, not only between bacterial species, but also between bacteria and host in inter-kingdom signaling (B. Bassler & Losick, 2006; J. M. Henke & B. L. Bassler, 2004), between the bacteria and the

host. This communication, in which bacteria sense their host, and vice versa is not unexpected, considering the diversity and concentration of bacteria that reside in the host (Walters & Sperandio, 2006), combined with the fact that both micro-organisms and their hosts have co-existed, and co-evolved for millions of years (Hughes & Sperandio, 2008). LuxS, the AI-2 synthase, has been linked to the expression of AI-3, a molecule similar to the neurotransmitter, noradrenaline. The concentrations of neurotransmitters such as noradrenaline are known to rise when the host is under stress. Bacteria can sense these molecules and alter their behavior accordingly. It is interesting that an exporter of AI-2 is situated in such close proximity, and may therefore be transcriptionally linked, to a spermidine and multi-drug transporter, *mdtJI*. The order of genes within a genome is rarely coincidental, with evolution driving genetic organisation. The finding that *mdtJI* and *tqsA* are so close, lead us to hypothesise that QS, specifically AI-2 and LuxS, may be involved in the regulation of *mdtJI* expression.

To test out this hypothesis, pWU2 was transformed into CLEN34, a *luxS* deletion-insertion mutant. There was no significant difference in *mdtJI* expression between the parental or CLEN34 cultures, with regard to the expression of *mdtJI* upon exposure to refrigeration temperatures **(Figures 4.12, 4.13 and 4.14).** However, LuxS/AI-2 did influence expression of *mdtJI* at 37°C in *E. coli* O157:H7. RLU data showed that in the presence of LuxS, *mdtJI* expression decreased during exponential growth and increased again during early stationary phase. In the absence of LuxS however, *mdtJI* expression in CLEN34 was shown to continuously decrease, even during stationary phase. **(Figure 4.12)**. Lcfu data showed a similar decrease in *mdtJI* expression in CLEN34 with an approximately 117-fold reduction. Initial observations suggested that *mdtJI* expression at 37°C was always lower in the absence of *luxS* **(Figure 4.13)**. However, further statistical analysis showed that no significant difference in the expression of *mdtJI* existed between the parental and mutant strains at any time during growth at 37°C ($p \geq 0.070$), suggesting there may be little or no influence of LuxS on *mdtJI* expression at 37°C. These results conflict with RLU results obtained using $OD6_{00}$ as the measurement of cell density, again highlighting the difference seen using these two methods.

There are two ways in which the LuxS may influence the expression of *mdtJI*. One is through metabolism and the other is via the AI-2 QS pathway (Winzer *et al.*, 2003). To ascertain if AI-2 was responsible for the observable effect of LuxS on the expression of *mdtJI*, conditioned medium containing the signal molecule was added to *E. coli* O157:H7 pWU2, and CLEN34 pWU2, growing at 37°C. There was no discernable difference in the expression of *mdtJI* upon the addition of AI-2 positive, or AI-2 negative CM in either strain **(Figure 4.15)**. This suggests that AI-2 does not have an effect on the expression of *mdtJI* and that any effect is likely to be driven metabolically rather than through a QS based mechanism. However, the addition of AI-2 was performed at a single time point, during stationary phase **(Figure 4.15, T5)**. Further work needs to be carried out to confirm whether or not the addition of AI-2, has any influence on the expression of *mdtJI* at other times during growth. Furthermore, AI-2 was added in the form of CM only. Ideally the signal molecule should be added in purified form, as CM can contain compounds other than AI-2 which may have an influence. Unfortunately, this is not commercially available, and it is both costly and time consuming to manufacture. An appropriate negative AI-2 CM, made from CLEN34 (*luxS*$^{-}$ strain) was used as a control for these experiments to minimise any culture-dependent variables, which may be associated with the addition of CM. Since there was no observable affect upon the addition of either AI-2 positive or negative CM, the lack of pure AI-2 was not considered a limiting factor for these experiments.

CLEN34 is lacking the *luxS* gene, which means the expression of the *lsr* gene (LuxS regulated) which encodes the LsR transport system may be disrupted. The LsR transport system internalizes endogenously produced AI-2, as well external AI-2 (Taga *et al.*, 2003; Taga *et al.*, 2001). Therefore, questions remain as to whether the addition of exogenous AI-2 truly had no effect on the *mdtJI* gene **(Figure 4.14)**, or if the lack of response was merely because *luxS*$^{-}$ cells were unable to import the AI-2 molecule. Complementation of CLEN34 with *luxS* would return the metabolic function, but would also return AI-2 production. This would not resolve whether or not the observed effects were metabolic of QS derived. An alternative method, for returning the metabolic function of *luxS*,

without restoring the QS function could be achieved by replacing the missing *luxS* gene with *sahH* (Walters & Sperandio, 2006*)*. *sahH* encodes the enzyme, *S*-adenosylhomocysteine (SAH) hydrolase, and is part of an alternative pathway for the detoxification of SAH.

Importantly, the absence of LuxS had no observable affect on the growth or survival of *E. coli* O157:H7, at 37°C or upon exposure to refrigeration temperatures **(Figures 4.9 and 4.10)**. These findings confirm that the differences seen between the expression of *mdtJI* in the parental strain, and *luxS*$^{-}$ strain, were not merely due to any growth differences, which could have occurred due to the deletion of *luxS*. However, these observations are limited short incubations at refrigeration temperatures. During long term exposure to 4°C, the presence of LuxS does adversely affect the survival of *E. coli* O157:H7 being *luxS*$^{-}$ was advantageous **(Appendix 1)**.

In line with the literature, AI-2 levels increased at 37°C, with increasing OD **(Figures 4.5 and 4.6)**. Additionally, AI-2 levels remained constant, following a temperature down-shift from 37°C to both 4 and -1.5°C, in line with the population density **(Figures 4.5 to 4.8)**. It is not clear, whether the lack of change with respect to exogenous AI-2 is due to a lack of AI-2 production, altered AI-2 transport, impaired AI-2 degradation or a combination of all three scenarios. This data, does suggest however, that AI-2 is unlikely to be involved in the cold temperature response. This theory is further supported by the findings that LuxS, which is responsible for the production of AI-2, has no influence, at least in the short term, over the growth or survival of *E. coli* O157:H7, or the expression of *mdtJI* at refrigeration temperatures.

In summary, the main findings in this study were;

- *slp, hslJ* and the *mdtJI* are differentially expressed in *E. coli* O157:H7 at refrigeration temperatures.
- *mdtJI* is involved adaptive response, specifically adaptation to cold temperatures in *E. coli* O157:H7, and possibly to growth cessation which occurs during entry into stationary phase.

- Although *mdtJI* was situated in close proximity to *tqsA*, LuxS and AI-2 have not been shown in this study to influence expression of *mdtJI* at refrigeration temperatures
- LuxS/A-2 does not affect short term survival of *E. coli* O157:H7 at refrigeration temperatures.
- LuxS but not AI-2, influences *mdtJI* expression at 37°C; most likely metabolically rather than QS based.

Chapter 6: Future directions and recommendations

In light of the potential enormity of this topic, it is important to recognise that the experiments in this thesis were carried out in the manner of a "shot gun" approach, with the intention of identifying areas worthy of further investigation. Most of the experiments, while designed to answer questions, often gave rise to new questions, which only serves to highlight the complexity of studies of this nature. The interactions of bacteria with their environment and the responses they generate are rarely simple, and this study has merely touched on the variables associated with these interactions. Further experiments will need to be carried out in order to confirm these preliminary findings, and further elucidate their meaning. Some of the key experiments and questions for the future are listed below:

What is the role of QS in *mdtJI* expression?

- Metabolic versus QS
  - Metabolic only complementation of the *luxS* mutation using the *P. aeruginosa S*-adenosylhomocysteine hydrolase (*sahH*) gene
  - QS complementation - Addition of purified AI-2
  - Total complementation of the *luxS* mutation with *luxS* to confirm that the effects observed are due to the absence of LuxS rather than to any secondary mutation generated during construction of the deletion strain or subsequent growth
- Timing – is timing of AI-2 addition important in the regulation of *mdtJI* expression?
  - Add AI-2 at different time points during growth at 37°C to determine if a role for AI-2 can be determined.
- *tqsA* - is the location of this gene significant in the regulation of *mdtJI*?

What is the role of *mdtJI* in *E. coli* O157:H7?

Construct an *mdtJI* deletion mutation to investigate the role of *mdtJI* in *E. coli* O157:H7.

Construct a chromosomal-based *mdtJI* promoter reporter to reduce copy number effects in the expression assays.

Investigate further the roles that *mdtJI* has within *E. coli* O157:H7

Stress responses

Multi-drug exporter, including spermidine.

How do these influence survival at cold temperatures? Including on Meat?

mdtJI expression modification

What other stresses influence *mdtJI* expression?

Investigate the discrepancies seen with regards to mdtJI expression at 37°C using RLU and Lcfu data. Extra APC sample points will be included, to confirm if the continuous reduction of mdtJI expression seen at 37°C using Lcfu, was not merely due missing data. Also, flow cytometry exper*iments* can be used to ascertain if there are actually subpopulations of *E. coli* O157:H7 which vary in their expression of mdtJI.

What does effect survival of *E. coli* O157:H7 on meat?

A role for QS?

Development of methods

For VHBA assays - what is the influence of meat and meat extracts on the viability of *V. harveyi* strains? Can the traditional assay be used?

Investigate alternative AI-2 detection assays, or alternative biological sensors.

- *In situ* analysis tools - Use of *luxCDABE*-reporters or *gfp*-reporters technology to monitor survival of *E. coli* O157:H7 at cold temperature on the meat surface

- Other genes?
  - *slp* and *hslJ* were observed as being differentially expressed at 37°C compared with refrigeration temperatures. Use similar experimental strategies as those used and discussed in this thesis to examine their role in survival at cold temperatures on meat.

Bacteria become lab-adapted very quickly and this is known to influence behavior. What is the affect of lab adaptation on the response to stress?

- Using similar assays as those described in this thesis, assess the role of these genes in the cold shock response/survival using strains of *E. coli* O157:H7 that have been recently isolated from meat and the meat processing environment and are not lab-adapted.

What other experimental procedures can be used, or methodologies improved?

- Strengthen the results and conclusions by increasing the number of experimental replicates, thus improving the statistical analysis.
- As discussed earlier, all experimental techniques have limitations and it is important to determine which procedure will generate the data required.
  - RNA analysis
    - quantitative northern analysis
    - Real Time PCR (qRT-PCR) analysis using SBYR Green
    - DNA Microarrays probed using Cy-3 and Cy-5 labeled cDNA populations
  - Growth/expression analysis
    - Biological *gfp*-tagging of bacterial cells for detection in complex media. For example, on the meat surface
    - Chemical fluorescent cell labeling for detection in complex media using, for example, 4´,6-diamidino-2-phenylindole, dihydrochloride (DAPI), a DNA interchelator; carboxyfluorescein diacetate, succinimidyl (CDFA) a membrane bound
    - Flow cytometry to measure individual cells in the population

References

Al-Fageeh, M. B., & Smales, C. M. (2006). Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian systems. *Biochemical Journal, 397*, 247-259.

Amin-Hanjani, S., Meikle, A., Glover, L. A., Prosser, J. I., & Killham, K. (1993). Plasmid and chromosomally encoded luminescence maker systems for detection of *Pseudomonas fluorescens* in soil. *Molecular Ecology, 2*, 47-54.

Bari, M. L., Nei, D., Enomoto, K., Todoriki, S., & Kawamoto, S. (2009). Combination treatments for killing *Escherichia coli* O157:H7 on Alfalfa, Radish, Broccoli, and Mung Bean Seeds. *Journal of Food Protection, 72*, 631-636.

Bassler, B., Greenberg, E., & Stevens, A. (1997). Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *Journal of Bacteriology, 179*, 4043-4045.

Bassler, B., & Losick, R. (2006). Bacterially speaking. *Cell, 125*, 237-246.

Bassler, B., Wright, M., Showalter, R., & Silverman, M. (1993). Intercellular signaling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Molecular Microbiology, 9*, 773-786.

Bassler, B. L. (2002). Small talk: Cell-to-cell communication in bacteria. *Cell, 109*, 421-424.

Besser, R. E., Lett, S. M., Weber, J. T., Doyle, M. P., Barret, T. J., Wells, J. G., *et al*. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *Journal of the American Medical Association, 269*, 2217-2220.

Beuchat, L. (1996). Pathogenic microorganisms associated with fresh produce. *Journal of Food Protection, 59*, 203-216.

Bielaszewska, M., Janda, J., Blahova, K., Minarikova, H., Jikova, E., Karmali, M. A., *et al*. (1997). Human *Escherichia coli* O157:H7 infection associated with the consumption of unpasteurized goat's milk. *Epidemiology and Infection, 119*, 299-305.

Bielaszewska, M., & Karch, H. (2005). Consequences of enterohaemorrhagic *Escherichia coli* infection for the vascular endothelium. *Thrombosis and Haemostasis, 94*, 312-318.

Brackett, R. E., Hao, Y., & Doyle, M. P. (1994). Ineffectiveness of hot acid sprays to decontaminate *Escherichia coli* O157:H7 on beef. *Journal of Food Protection, 57*, 198-203.

Brandi, A., Spurio, R., Gualerzi, C. O., & Pon, C. L. (1999). Massive presence of the *Escherichia coli* 'major cold-shock protein' CspA under non-stress conditions. *The EMBO Journal, 18*, 1653-1659.

Buchanan, R., & Edelson, S. (1996). Culturing enterohaemorrhagic *Escherichia coli* in the presence and absence of glucose as a simple means of evaluating the acid tolerance of stationary-phase cells. *Applied and Environmental Microbiology, 62*, 4009-4013.

Caprioli, A., Morabito, S., Brugere, H., & Oswald, E. (2005). Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Veterinary Research, 36*, 289-311.

Carlson, B. A., Ruby, J., Smith, G. C., Sofos, J. N., Bellinger, G. R., Warren-Serna, W., *et al*. (2008). Comparison of antimicrobial efficacy of multiple beef hide decontamination strategies to reduce levels of *Escherichia coli* O157:H7 and *Salmonella*. *Journal of Food Protection, 71*, 2223-2227.

Carper, S. W., Willis, D. G., Manning, K. A., & Gerner, E. W. (1991). Spermidine acetytation in response to a variety of stresses in *Escherichia coli*. *The Journal of Biological Chemistry, 266*, 12439-12441.

CDC. (2008). Summary of Notifiable Diseases. *Morbidity and Mortality Weekly Report*, from www.cdc.gov/mmwr/summary.html

Chapman, P. A., Siddons, C. A., Cerdan Malo, A. T., & Harkin, M. A. (1997). A 1-year study of *Escherichia coli* O157:H7 in cattle, sheep, pigs and poultry. *Epidemiology and infection, 119*, 245-250.

Conner, D. E., Kotrola, W. B., Mikel, W. B., & Tamblyn, K. C. (1997). Effect of acetic-lactic acid treatments applied to beef trim on populations of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in ground beef. *Journal of Food Protection, 60*, 1560-1563.

DeLisa, M. P., Valdes, J. J., & Bentley, W. E. (2001). Mapping stress-induced changes in autoinducer AI-2 production in chemostat-cultivated *Escherichia coli* K-12. *Journal of Applied Microbiology, 183*, 2918-2928.

Dickson, J. S. (1991). Control of *Salmonella typhimurium*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 on beef in a model spray chilling system. *Journal of Food Science, 56*, 191-193.

Dowd, S. E., & Ishizaki, H. (2006). Microarray based comparison of two *Escherichia coli* O157:H7 lineages. *BMC Microbiology, 6*(30), doi:10.1186/1471-2180-6-30.

Drlica, K. (1992). Control of bacterial DNA supercoiling. *Molecular Microbiology, 6*, 425-433.

Dubern, J., & Diggle, S. P. (2008). Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species. *Molecular Biosystems, 4*, 882-888.

Dykes, G. (2006). Laboratory-based simulation of freezing profiles of beef trim for *Escherichia coli* 0157 survival determinations. *Journal of Microbiological Methods, 64*, 266-274.

Dykes, G., Moorhead, S. M., & Roberts, S. L. (2001). Survival of *Escherichia coli* 0157:H7 and *Salmonella* on chill-stored vacuum or carbon dioxide packaged primal beef cuts. *International Journal of Food Microbiology, 64*, 401-405.

Edwards, J. R., & Fung, D. Y. C. (2006). Prevention and decontamination of *Escherichia coli* O157:H7 on raw beef carcasses in commercial beef abattoirs. *Journal of Rapid Methods and Automation in Microbiology, 14*, 1-95.

Elder, R. O., Keen, J. E., Siragusa, G. R., Barkocy-Gallagher, B. A., Koohmaraie, M., & Laegreid, W. W. (2000). Correlation of enterohaemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences, 97*, 2999-3003.

Ellis-Iversen, J., Cook, A. J. C., Smith, R. P., Pritchard, G. C., & Nielen, M. (2009). Temporal patterns and risk factors for *Escherichia coli* O157 and *Campylobacter* spp. in young cattle. *Journal of Food Protection, 72*, 490-496.

ESR. (2008). Public Health Surveillance. From http://www.surv.esr.cri.nz/enteric_reference/vtec_isolates.php

Federle, M. J., & Bassler, B. L. (2003) Interspecies communication in bacteria. *The Journal of Clinical Investigations, 112*, 1291-1299.

Feng, P., Lampel, K. A., Karch, H., & Whittam, T. S. (1998). Genotypic and phenotypic changes in the emergence of *Escherichia coli* O157:H7. *Journal of Infectious Diseases, 177*, 1750-1753.

Fislage, R., Berceanu, M., Humboldt, Y., Wendt, M., & Oberender, H. (1997). Primer design for prokaryotic differential display RT-PCR. *Nucleic Acids Research, 25*, 1830-1835.

Giangrossi, M., Brandi, A., Giuliodori, A. M., Gualerzi, C. O., & Pon, C. L. (2007). Cold-shock-induced *de novo* transcription and translation of *infA* and role of IF1 during cold adaptation. *Molecular Microbiology, 64*, 807-821.

Giuliodori, A. M., Brandi, A., Gualerzi, C. O., & Pon, C. L. (2004). Preferential translation of cold-shock mRNAs during cold adaptation. *RNA, 10*, 265-276.

Giuliodori, A. M., Gualerzi, C. O., Soto, S., Vila, J., & Tavio, M. M. (2007). Review on bacterial stress topics. *Annals of the New York Academy of Sciences, 1113*, 95-104.

Goldenberg, D., Azar, I., & Oppenheim, A. B. (1996). Differential mRNA stability of the *cspA* gene in the cold-shock response of *Escherichia coli*. *Molecular Microbiology, 19*, 241-248.

Greenberg, E. P., Hastings, J. W., & Ulitzur, S. (1979). Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. *Archives of Microbiology, 120*, 87-91.

Hacker, J., & Carniel, E. (2001). Ecological fitness, genomic islands and bacterial pathogenicity. *EMBO Report, 2*, 376-381.

Hacker, J., & Kaper, J. B. (2000). Pathogenicity islands and the evolution of microbes. *Annual Reviews in Microbiology, 54*, 641-679.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology, 166*, 557.

Hancock, D., Besser, T., Lejeune, J., Davis, M., & Rice, D. (2001). The control of VTEC in the animal reservoir. *International Journal of Food Microbiology, 66*, 71-78.

Hardin, M. D., Acuff, G. R., Lucia, L. M., & Savell, J. W. (1995). Comparison of methods for contamination removal from beef carcass surfaces. *Journal of Food Protection, 58*, 368-374.

Hayashi, T., Makino, K., Ohinishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., *et al*. (2001). Complete genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Research, 8*, 11-22.

Hengge, R. (2008). The two-component network and the general stress sigma factor RpoS (σ^S) in *Escherichia coli*. In R. Utsumi (Ed.), *Bacterial Signal Transduction: Networks and Drug Targets* (pp. 40-53): Landes Bioscience and Springer Science+Business Media.

Henke, J. M., & Bassler, B. L. (2004). Bacterial social engagements. *Trends in Cell Biology, 14*, 648-656.

Henke, J. M., & Bassler, B. L. (2004). Three parallel quorum-sensing systems regulate gene expression in *Vibrio Harveyi*. *Journal of Bacteriology, 186*, 6902-6914.

Herzberg, M., Kaye, I. K., Peti, W., & Wood, T. K. (2006). YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. *Journal of Bacteriology, 188*, 587-598.

Higashi, K., Ishigure, H., Demizu, R., Uemura, T., Nishino, K., Yamaguchi, A., *et al*. (2008). Identification of spermidine excretion protein complex (MdtJI) in *Escherichia coli*. *Journal of Bacteriology, 190*, 872-878.

Holme, R. (2003). Drinking water contamination in Walkerton, Ontario: positive resolutions from a tragic event. *Water Science and Technology, 47*, 1-6.

Hughes, D. T., & Sperandio, V. (2008). Inter-kingdom signaling: communication between bacteria and their hosts. *Nature Reviews Microbiology, 6*, 111-120.

Hulebak, K. L., & Scholsser, W. S. (2001). HACCP history and conceptual overview (Publication from USDA FSIS: http://www.ce.ncsu.edu/risk/pdf/hulebak.pdf (Accessed May 2009).

Hussein, H. S. (2007). Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. *Journal of Animal Science, 85*, E63-E72.

Jensen, K. (1993). The *Escherichia coli* K-12 'wild types' W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. *Journal of Bacteriology, 175*, 3401-3407.

Jones, P. G., VanBogelen, R. A., & Neidhardt, F. C. (1987). Induction of proteins in response to low temperature in *Escherichia coli*. *Journal of Bacteriology, 169*, 2092-2095.

Jores, J., Rumer, L., & Wieler, L. H. (2004). Impact of the locus of enterocyte effacement pathogenicity island on the evolution of pathogenic *Escherichia coli*. *International Journal of Medical Microbiology, 294*, 103-113.

Kalac, P. (2006). Biologically active polyamines in beef, pork and meat products: A review. *Meat Science, 73*, 1-11.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology, 2*, 123-140.

Karch, H., Tarr, P., & Bielaszewska, M. (2005). Enterohaemorrhagic *Escherichia coli* in human medicine. *International Journal of Medical Microbiology, 295*, 405-418.

Karmali, M. A. (1989). Infection by verocytotoxin-producing *Escherichia coli*. *Clinical Microbiology Reviews, 2*, 15-38.

Karmali, M. A. (2004). Prospects for preventing serious systemic toxemic complications of shiga toxin-producing *Escherichia coli* infections using shiga toxin receptor analogues. *Journal of Infectious Diseases, 189*, 355-359.

Karmali, M. A. (2005). Use of comparative genomics as a tool to assess the clinical and public health significance of emerging Shiga toxin-producing *Escherichia coli* serotypes. *Meat Science, 71*, 62-71.

Karmali, M. A., Gannon, V., & Sargeant, J. M. (2009). Verocytotoxin-producing *Escherichia coli* (VTEC). *Veterinary Microbiology*, Epub, April 10.

Karmali, M. A., Mascarenhas, M., Shen, S., Ziebell, K., Johnson, S., Reid-Smith, R., *et al.* (2003). Association of genomic O island 122 of *Escherichia* coli EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. *Journal of Clinical Microbiology, 41*, 4930-4940.

Kim, J., Niefeldt, J., Ju, J., Wise, J., Fegan, N., Desmarhelier, P., *et al.* (2001). Ancestral divergence, genome diversification, and phylogenetic variation in subpopulations of sorbitol-negative, beta-glucuronidase-negative enterohaemorrhagic *Escherichia coli* O157:H7. *Journal of Bacteriology, 183*, 6885-6897.

Konowalchuk, J., Speirs, J. I., & Stavric, S. (1977). Vero response to cytotoxin of *Escherichia coli*. *Infection and Immunity, 18*, 775-779.

Koohmaraie, M., Arthur, T. M., Bosilevac, J. M., Brichta-Harhay, D. M., Kalchayanand, N., Shackleford, S. D., *et al.* (2007). Interventions to reduce/eliminate *Escherichia coli* 0157:H7 in ground beef. *Meat Science, 77*, 90-96.

La Ragione, R. M., Best, A., Woodward, M. J., & Wales, A. D. (2009). *Escherichia coli* O157:H7 colonization in small domestic ruminants. *FEMS Microbiology Reviews, 33*, 394-410.

Leclercq, A., & Mahillon, J. (2003). Farmed rabbits and ducks as vectors for VTEC O157:H7. *The Veterinary Record, 152,* 723-724.

Lenahan, M., Crowley, H., O' Brien, S. B., Byrne, C., Sweeney, T., & Sheridan, J. J. (2009). The potential use of chilling to control the growth of Enterobacteriaceae on porcine carcasses and the incidence of *E. coli* O157:H7 in pigs. *Journal of Applied Microbiology, 106*, 1512-1520.

Leyer, G. J., Wang, L., & Johnson, E. A. (1995). Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. *Applied and Environmental Microbiology, 61*, 3752-3755.

Lilic, M., Jovanovic, M., Jovanovic, G., & Savic, D. J. (2003). Identification of the CysB-regulated gene, *hslJ*, related to the *Escherichia coli* novobiocin resistance phenotype. *FEMS Microbiology Letters, 224*, 239-246.

Limsuwun, K., & Jones, P. G. (2000). Spermidine acetyltransferase is required to prevent spermidine toxicity at low temperatures in *Escherichia coli*. *Journal of Bacteriology, 182*, 5373-5380.

Lovatt, S. (2004). Refrigeration and freezing technology: Principles. In W. Jensen, C. Devine & M. Dikeman (Eds.), *Encyclopaedia of Meat Sciences* (pp. 1131-1137): Oxford:Elsevier.

Mainil, J. G., & Daube, G. (2005). Verotoxigenic *Escherichia coli* from animals, humans and foods: who's who? *Journal of Applied Microbiology, 98*, 1332-1344.

McDowell, D. A., & Sheridan, J. J. (2001). Survival and growth of vero cytotoxin-producing *E. coli* in the environment. In G. Duggy, P. Garvey & D. A. McDowell (Eds.), *Verocytotoxigenic Escherichia coli* (pp. 279-304). Trumbull: Food and Nutrition Press Inc.

Meng, J., & Doyle, M. P. (1998). Microbiology of shiga toxin-producing *Escherichia coli* in foods. In J. B. Kaper & A. D. O' Brien (Eds.), *Escherichia coli O157:H7 and Other Shiga Toxin-Producing E. coli Strains*. Washington, D.C.: ASM Press.

Michino, H., Araki, K., Minami, S., Nakayama, T., Ejima, Y., Hiroe, K., *et al*. (1998). Recent outbreaks of infections caused by *Escherichia coli* O157:H7 in Japan. In J. B. Kaper & A. D. O' Brien (Eds.), *Escherichia coli O157:H7 and Other Shiga Toxin-Producing E. coli Strains*. Washington, DC: ASM press.

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Review of Microbiology, 55*, 165-199.

Miller, S. T., Xavier, K. B., Campagna, S. R., Taga, M. E., Semmelhack, M. F., Bassler, B., *et al*. (2004). *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Molecular Cell, 15*, 677-687.

Mok, K. C., Wingreen, N. S., & Bassler, B. L. (2003). *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *The EMBO Journal, 22*, 870-881.

Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews, 11*, 142-201.

Naylor, S. W., Gally, D. L., & Low, J. C. (2005). Enterohaemorrhagic *E. coli* in veterinary medicine. *International Journal of Veterinary Microbiology, 295*, 419-441.

Nealson, K. H., Platt, T., & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminescent system. *Proceedings of the National Academy of Sciences, 69*, 1073-1076.

Nishino, K., & Yamaguchi, A. (2001). Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. *Journal of Bacteriology, 183*, 5803-5812.

O' Brien, A. D., & Holmes, R. K. (1987). Shiga and shiga-like toxins. *Microbiological Reviews, 51*, 206-220.

O' Brien, A. D., LaVeck, G. D., Thompson, M. R., & Formal, S. B. (1982). Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. *Journal of Infectious Diseases, 146*, 763-769.

Ogden, I. D., MacRae, M., & Strachan, N. J. (2004). Is the prevalence and shedding concentrations of *E. coli* O157:H7 in beef cattle in Scotland seasonal? *FEMS Microbiology Letters, 233*, 297-300.

Oh, D., Pan, Y., Berry, E., Cooley, M., Mandrell, R., & Breidt, F. (2008). *Escherichia coli* O157:H7 strains isolated from environmental sources differ significantly in acetic acid resistance compared with human outbreak strains. *Journal of Food Protection, 72*, 503-509.

Paton, J. C., & Paton, A. W. (1998). Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews, 11*, 450-479.

Pereira, C. S., McAuley, J. R., Taga, M. E., Xavier, K. B., & Miller, S. T. (2008). *Sinorhizobium meliloti*, a bacterium lacking the autocinducer-2 (AI-2) synthase, responds to AI-2 supplied by other bacteria. *Molecular Microbiology, 70*, 1223-1235.

Perna, N. T., Plunkett III, G., Burland, V., Mau, B., Glasner, J. D., Rose, D. J., *et al*. (2001). Genome sequence of enterohoaemorrhagic *Escherichia coli* O157:H7. *Nature, 409*, 529-533.

Phadtare, S. (2004). Recent developments in bacterial cold-shock response. *Current Issues in Molecular Biology, 6*, 125-136.

Phadtare, S., & Inouye, M. (2004). Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-*csp*-deletion strains of *Escherichia coli*. *Journal of Bacteriology, 186*, 7007-7014.

Phadtare, S., Yamanaka, K., & Inouye, M. (2000). The Cold Shock Response. In G. Storz & R. Hengge-Aronis (Eds.), *Bacterial Stress Responses* (pp. 33-45). Washington, D.C.: ASM Press.

Reading, N. C., & Sperandio, V. (2006). Quorum sensing: the many languages of bacteria. *FEMS Microbiology Letters, 254*, 1-11.

Redfield, R. J. (2002). Is quorum sensing a side effect of diffusion sensing? *Trends in Microbiology, 10*, 365-370.

Reid, S. D., Herbelin, C. J., Bumbaugh, A. C., Selander, R. K., & Whittam, T. S. (2000). Parallel evolution of virulence in pathogenic *Escherichia coli*. *Nature, 406*, 64-67.

Rezzonico, F., & Duffy, B. (2008). Lack of genomic evidence of A1-2 receptors suggests a non-quorum sensing role for *luxS* in most bacteria. *BMC Microbiology, 8*(154) doi: 10.1186/1471-2180-8-154.

Rice, D. H., Sheng, H. G., Wynia, S. A., & Hovde, C. J. (2003). Rectoanal mucosal swab is more sensitive than fecal culture and distinguishes *Escherichia coli* O157:H7-colonised cattle and those transiently shedding the same organism. *Journal of Clinical Microbiology, 41*, 4924-4929.

Rivera-Marrero, C. A., Burroughs, M. A., Masse, R. A., Vannberg, F. O., Leimbach, D. L., Roman, J., *et al*. (1998). Identification of genes differentially expressed in *Mycobacterium tuberculosis* by differential display PCR. *Microbial Pathogenesis, 25*, 307-316.

Robson, W. L., (2000). Haemolytic uraemic syndrome. *Paediatric Drugs, 2*, 243-252.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1998). *Molecular Cloning: A Laboratory Manual* (2nd ed.). Cold Spring Harbor, New York: Cold Spring Harbor Press.

Samelis, J., Sofos, J. N., Kendall, P. A., & Smith, G. C. (2002). Effect of acid adaptation on survival of *Escherichia coli* O157:H7 in meat decontamination washing fluids and potential effects of organic acid interventions on the microbial ecology of the meat plant environment. *Journal of Food Protection, 65*, 33-40.

Samelis, J., Sofos, J. N., Kendall, P. A., & Smith, G. C. (2005). Survival or growth of *Escherichia coli*: 0157:H7 in a model system of fresh meat decontamination runoff waste fluids and its resistance to subsequent lactic acid stress. *Applied and Environmental Microbiology*, 71, 6228-6234.

Sasaki, T., Kobayashi, M., & Agui, N. (2000). Epidemiological potential of excretion and regurgitation by *Musca domestica* (Diptera: Muscidae) in the dissemination of *Escherichia coli* O157:H7 to food. *Journal of Medical Entomology, 37*, 945-949.

Savageau, M. A. (1983). *Escherichia coli* habitats, cell types and molecular mechanisms of gene control. *American Naturalist, 122*, 732-744.

Schauder, S., Shokat, K., Surette, M., & Bassler, B. (2001). The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Molecular Microbiology, 41*, 463-476.

Scotland, S. M., Smith, H. R., & Rowe, B. (1985). Two distinct toxins active on Vero cells from *Escherichia coli* O157:H7. *Lancet, 326*, 885-886.

Sofos, J. N., & Smith, G. C. (1998). Nonacid meat decontamination technologies: model studies and commercial applications. *International Journal of Food Microbiology, 44*, 171-188.

Soni, K. A., Lu, L., Jesudhasan, P. R., Hume, M. E., & Pillai, S. D. (2008). Influence of autoinducer-2 (AI-2) and beef sample extracts on *E. coli* O157:H7

survival and gene expression of virulence genes *yadK* and *hha*. *Journal of Food Science, 73*, 135 -139.

Spears, K. J., Roe, A. J., & Gally, D. L. (2006). A comparison of enteropathogenic and enterohaemorrhagic *Escherichia coli* pathogenesis. *FEMS Microbiology Letters, 255*, 187-202.

Sperandio, V., Kaper, J. B., Bortolini, M. R., Neves, B. C., Keller, R., & Trabulsi, L. R. (1998). Characterisation of the locus of enterocyte effacement (LEE) in different enteropathogenic *Escherichia coli* (EPEC) and Shiga-toxin producing *Escherichia coli* (STEC) serotypes. *FEMS Microbiology Letters, 164*, 133-139.

Sperandio, V., Mellies, J., Nguyen, L., Shin, S., & Kaper, J. B. (1999). Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli* O157:H7. *Proceedings of the National Academy of Sciences, 96*, 15196-15201.

Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P., & Kaper, J. B. (2003). Bacteria-host communication: The language of hormones. *Proceedings of the National Academy of Sciences, 100*, 8951-8956.

Steindler, L., & Venturi, V. (2007). Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiology Letters, 266*, 1-9.

Stevens, A. M., Dolan, K. M., & Greenberg, E. P. (1994). Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain and RNA polymerase to the *lux* promoter region. *Proceedings of the National Academy of Sciences, 91*, 12619-12623.

Stopforth, J. D., Skandamis, P. N., Geornaras, I., & Sofos, J. N. (2007). Acid tolerance of acid-adapted and nonacid-adapted *Escherichia coli* 0157:H7 strains in beef decontaminated runoff fluids or on beef tissue. *Food Microbiology, 24*, 530-538.

Stopforth, J. D., Yoon, Y., Belk, K. E., Scanga, J. A., Kendall, P. A., Smith, G. C., *et al*. (2004). Effect of simulated spray chilling with chemical solutions on acid-habituated and non-acid-habituated *Escherichia coli* O157:H7 cells attached to beef carcass tissue. *Journal of Food Protection, 67*, 2099-2106.

Strocchi, M., Ferrer, M., Timmis, K. N., & Golyshin, P. N. (2006). Low temperature-induced systems failure in *Escherichia coli*: Insights from rescue by cold-adapted chaperones. *Proteomics, 6*, 193-206.

Strockbine, N. A., Jackson, M. P., Sung, L. M., Holmes, R. K., & O' Brien, A. D. (1988). Cloning and sequencing of the genes of Shiga toxins from *Shigella dysenteriae* type 1. *Journal of Bacteriology, 170*, 1116-1122.

Surette, M., & Bassler, B. (1998). Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proceedings of the National Academy of Sciences USA, 95*, 7046-7050.

Surette, M. G., Miller, M. B., & Bassler, B. L. (1999). Quorum sensing in *Escherichia coli, Salmonella typhimurium,* and *Vibrio harveyi:* A new family of genes responsible for autoinducer production. *Proceedings of the National Academy of Sciences USA, 96*, 1639-1644.

Taga, M. E., Miller, S. T., & Bassler, B. L. (2003). Lsr-mediated transport and processing of AI-2 in *Salmonella typhimurium*. *Molecular Microbiology, 50*, 1411-1427.

Taga, M. E., Semmelhack, M. F., & Bassler, B. L. (2001). The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Molecular Microbiology, 42*, 777-793.

Taormina, P. J., Beuchat, L. R., Erickson, M. C., Ma, L., Zhang, G., & Doyle, M. P. (2009). Transfer of *Escherichia coli* O157:H7 to iceberg lettuce via simulated field coring. *Journal of Food Protection, 72*, 465-472.

Tauxe, R. V. (1998). Public health perspective on immunoprophylactic strategies for *Escherichia coli* O157:H7: Who or what would be immunized? In J. B. Kaper & A. O' Brien (Eds.), *Escherichia coli O157:H7 and Other Shiga Toxin-Producing E. coli strains* (pp. 445-448). Washington, DC 20005: ASM Press.

Tilden, J. J., Young, W., McNamara, A. M., Custer, C., Boesel, B., Lambert-Fair, M. a., *et al*. (1996). A new route of transmission for *Escherichia coli* infection from dry fermented salami. *American Journal of Public Health, 86*, 1142-1145.

Turovskiy, Y., Kashtanov, D., Paskhover, B., & Chikindas, M. L. (2007). Quorum sensing: Fact, fiction, and everything in between. *Advances in Applied Microbiology 62*, 191-234.

Vital, M., Hammes, F., & Egli, T. (2008). *Escherichia coli* O157 can grow in natural fresh water at low carbon concentrations. *Environmental Microbiology, 10*, 2387-2396.

Walters, M., & Sperandio, V. (2006). Quorum sensing in *Escherichia coli* and *Salmonella*. *International Journal of Medical Microbiology, 296*, 125-131.

Wang, X. D., de Boer, P. A., & Rothfield, L. I. (1991). A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of *Escherichia coli*. *The EMBO Journal, 10*, 3363-3372.

White-Ziegler, C. A., Um, S., Perez, N. M., Berns, A. L., Malhowski, A. J., & Young, S. (2008). Low temperature (23°C) increases expression of biofilm-, cold-shock- and RpoS-dependent genes in *Escherichia coli* K-12. *Microbiology, 154*, 148-166.

Wick, L. M., & Egli, T. (2004). Molecular components of physiological stress responses in *Escherichia coli*. *Advances in Biochemical Engineering/Biotechnology, 89*, 1-45.

Wick, L. M., Qi W., Lacher, D. W., & Whittam, T. S. (2005). Evolution of Genomic Content in the Stepwise Emergence of *Escherichia coli* O157:H7. *Journal of Bacteriology, 187*, 1783-1791.

Winans, S. C. (2002). Bacterial esperanto. *Nature Structural Biology, 83-84*.

Winans, S. C., & Bassler, B. L. (2002). Mob psychology. *Journal of Bacteriology, 184*, 873-883.

Winson, M. K., Swift, S., Hill, P. J., Sims, C. M., Griesmayr, G., Bycroft, B. W., *et al*. (1998). Engineering the *lux*CDABE genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn*5* constructs. *FEMS Microbiology Letters, 163*, 193-202.

Winzer, K., Hardie, K. R., & Williams, P. (2003). LuxS and autoinducer-2: Their contribution to quorum sensing and metabolism in bacteria. *Advances in Applied Microbiology, 53*, 291-396.

Withers, H., Bright, B., Clemens, R., Urlich, S., & Brightwell, G. (2009). Effects of off-pasture management on pathogen carriage within the dairy herd. *Manuscript in preparation.*

Withers, H., Swift, S., & Williams, P. (2001). Quorum sensing as an integral component of gene regulatory networks in gram-negative bacteria. *Current Opinion in Microbiology, 4*, 186-193.

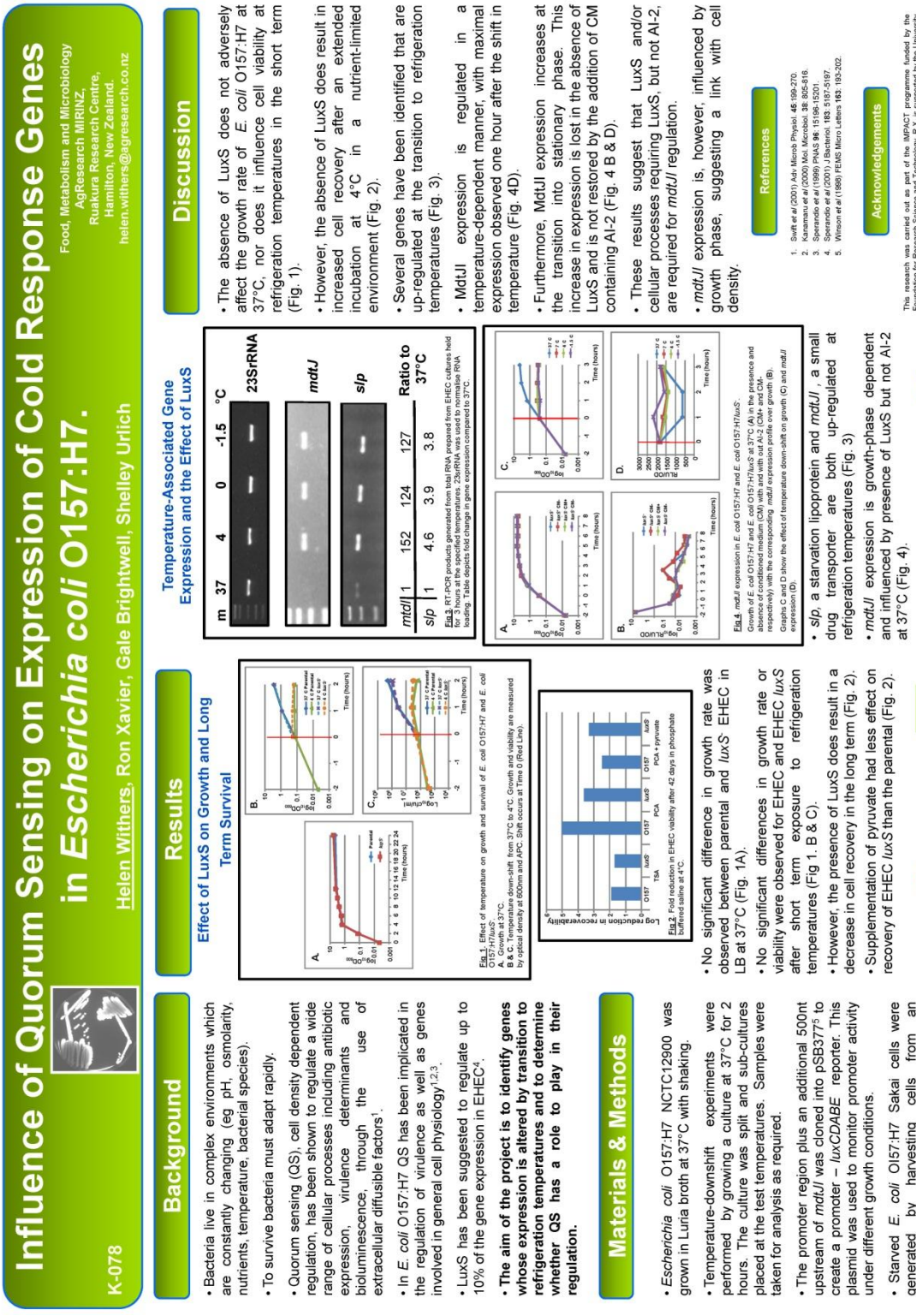
Yoon, Y., & Sofos, J. N. (2008a). Autoinducer-2 activity of gram-negative foodborne pathogenic bacteria and its influence on biofilm formation. *Journal of Food Science, 73*, M140-147.

Yoon, Y., & Sofos, J. N. (2008b). Conditions affecting autoinducer-2 based quorum sensing of *Escherichia coli* 0157:H7 on fresh beef. *Journal of Food Safety, 28*, 587-600.

Zhang, M., Sun, K., & Sun, L. (2008). Regulation of autoinducer 2 production and *luxS* expression in a pathogenic *Edwardsiella tarda* strain. *Microbiology, 154*, 2060-2069.

Appendices

Appendix 1


Influence of Quorum Sensing on Expression of Cold Response Genes in Escherichia coli O157:H7.
K-078
Helen Withers, Ron Xavier, Gale Brightwell, Shelley Urlich
Food, Metabolism and Microbiology
AgResearch MIRINZ,
Ruakura Research Centre,
Hamilton, New Zealand.
helen.withers@agresearch.co.nz
Background
• Bacteria live in complex environments which are constantly changing (eg pH, osmolarity, nutrients, temperature, bacterial species).
• To survive bacteria must adapt rapidly.
• Quorum sensing (QS), cell density dependent regulation, has been shown to regulate a wide range of cellular processes including antibiotic expression, virulence determinants and bioluminescence, through the use of extracellular diffusible factors[1].
• In E. coli O157:H7 QS has been implicated in the regulation of virulence as well as genes involved in general cell physiology[1,2,3].
• LuxS has been suggested to regulate up to 10% of the gene expression in EHEC[4].
• The aim of the project is to identify genes whose expression is altered by transition to refrigeration temperatures and to determine whether QS has a role to play in their regulation.
Materials & Methods
• Escherichia coli O157:H7 NCTC12900 was grown in Luria broth at 37°C with shaking.
• Temperature-downshift experiments were performed by growing a culture at 37°C for 2 hours. The culture was split and sub-cultures placed at the test temperatures. Samples were taken for analysis as required.
• The promoter region plus an additional 500nt upstream of mdtJI was cloned into pSB377[5] to create a promoter – luxCDABE reporter. This plasmid was used to monitor promoter activity under different growth conditions.
• Starved E. coli O157:H7 Sakai cells were generated by harvesting cells from an exponentially growing culture and resuspending them in phosphate buffered saline at 4°C.
Results
Effect of LuxS on Growth and Long Term Survival
Fig.1 Effect of temperature on growth and survival of E. coli O157:H7 and E. coli O157:H7luxS.
A. Growth at 37°C.
B & C. Temperature down-shift from 37°C to 4°C. Growth and viability are measured by optical density at 600nm and APC. Shift occurs at Time 0 (Red Line).
Fig.2 Fold reduction in EHEC viability after 42 days in phosphate buffered saline at 4°C.
• No significant difference in growth rate was observed between parental and luxS- EHEC in LB at 37°C (Fig. 1A).
• No significant differences in growth rate or viability were observed for EHEC and EHEC luxS after short term exposure to refrigeration temperatures (Fig 1. B & C).
• However, the presence of LuxS does result in a decrease in cell recovery in the long term (Fig. 2).
• Supplementation of pyruvate had less effect on recovery of EHEC luxS than the parental (Fig. 2).
Temperature-Associated Gene Expression and the Effect of LuxS
Fig.3 RT-PCR products generated from total RNA prepared from EHEC cultures held for 3 hours at the specified temperatures. 23srRNA was used to normalise RNA loading. Table depicts fold change in gene expression compared to 37°C.
Fig.4 mdtJI expression in E. coli O157:H7 and E. coli O157:H7luxS:
Growth of E. coli O157:H7 and E. coli O157:H7luxS at 37°C (A) in the presence and absence of conditioned medium (CM) with and with out AI-2 (CM+ and CM- respectively) with the corresponding mdtJI expression profile over growth (B).
Graphs C and D show the effect of temperature down-shift on growth (C) and mdtJI expression (D).
• slp, a starvation lipoprotein and mdtJI , a small drug transporter are both up-regulated at refrigeration temperatures (Fig. 3)
• mdtJI expression is growth-phase dependent and influenced by presence of LuxS but not AI-2 at 37°C (Fig. 4).
Discussion
• The absence of LuxS does not adversely affect the growth rate of E. coli O157:H7 at 37°C, nor does it influence cell viability at refrigeration temperatures in the short term (Fig. 1).
• However, the absence of LuxS does result in increased cell recovery after an extended incubation at 4°C in a nutrient-limited environment (Fig. 2).
• Several genes have been identified that are up-regulated at the transition to refrigeration temperatures (Fig. 3).
• MdtJI expression is regulated in a temperature-dependent manner, with maximal expression observed one hour after the shift in temperature (Fig. 4D).
• Furthermore, MdtJI expression increases at the transition into stationary phase. This increase in expression is lost in the absence of LuxS and is not restored by the addition of CM containing AI-2 (Fig. 4 B & D).
• These results suggest that LuxS and/or cellular processes requiring LuxS, but not AI-2, are required for mdtJI regulation.
• mdtJI expression is, however, influenced by growth phase, suggesting a link with cell density.
References
1. Swift et al (2001) Adv Microb Physiol. 45:199-270.
2. Kanamaru et al (2000) Mol. Microbiol. 38: 805-816.
3. Sperandio et al (1999) PNAS 96: 15196-15201.
4. Sperandio et al (2001) J Bacteriol. 183 : 5187-5197.
5. Winson et al (1995) FEMS Micro Letters 163 : 193-202.
Acknowledgements
This research was carried out as part of the IMPACT programme funded by the Foundation for Research Science and Technology. R.X. is supported by the University of Waikato PhD stipend program.
agresearch
Farming, Food and Health. First
Te Ahuwhenua, Te Kai me te Whai Ora, Tuatahi
RESEARCH SCIENCE & TECHNOLOGY

Appendix 2

Approval and BCH numbers for GMC07011 (GMO07/ARR004)

Approval code	Organism	BCH number
GMC001332	Vibrio harveyi (Johnson and Shunk, 1936) Baumann et al., 1981 (GMO07/ARR004)	41290

Approval and BCH numbers for GMD07064 (GMO07/ARR003)

Approval code	Organism	BCH number
GMD004759	Aeromonas hydrophila Chester, 1901; Stanier, 1943 (GMO07/ARR003)	41291
GMD004760	Arcobacter butzleri Kiehlbauch et al., 1991) Vandamme, et al 1992 (GMO07/ARR003)	41292
GMD004761	Arcobacter cibarius Houf et al 2005 (GMO07/ARR003)	41293
GMD004762	Arcobacter cryaerophilus (Neill et al., 1985) Vandamme, et al 1991 (GMO07/ARR003)	41294
GMD004763	Arcobacter skirrowii Vandamme, et al 1992 (GMO07/ARR003)	41295
GMD004764	Bacillus cereus Frankland and Frankland, 1887; Skerman et al., 1980 (GMO07/ARR003)	41296
GMD004765	Brochothrix spp Sneath and Jones, 1976;	41297
GMD004766	Campylobacter coli Doyle, 1948; Veron and Chatelain, 1973 (GMO07/ARR003)	41298
GMD004767	Campylobacter jejuni Jones et al., 1931; Veron and Chatelain, 1973 (GMO07/ARR003)	41299
GMD004768	Clostridium estertheticum Collins et al, 1993 (GMO07/ARR003)	41300
GMD004769	Clostridium perfringens Veillon and Zuber, 1898; Hauduroy et al.,1937 (GMO07/ARR003)	41301
GMD004770	Escherichia coli (Migula 1895) Castellani & Chalmers 1919 (GMO07/ARR003)	41302
GMD004771	Hafnia alvei Møller, 1954 (GMO07/ARR003)	41303
GMD004772	Lactobacillus spp Beijerinck 1901, Skerman et al, 1980 (GMO07/ARR003)	41304
GMD004773	Listeria monocytogenes Murray et al., 1926; Pirie, 1940 (GMO07/ARR003)	41305
GMD004774	Pseudomonas aeruginosa Schroeter, 1872; Migula, 1900 (GMO07/ARR003)	41306
GMD004775	Salmonella enterica Serovar Typhimurium (ex Kauffmann and Edwards, 1952) Le Minor and Popoff, 1987) (GMO07/ARR003)	41307
GMD004776	Salmonella typhi (Schroeter, 1886) Warren and Scott, 1930 (GMO07/ARR003)	41308
GMD004777	Serratia spp Bizio, 1823; Skerman et al., 1980 (GMO07/ARR003)	41309
GMD004778	Shewanella spp MacDonell and Colwell, 1986 (GMO07/ARR003)	41310
GMD004779	Shigella flexneri Castellani and Chalmers, 1919 (GMO07/ARR003)	41311
GMD004780	Staphylococcus aureus Rosenbach, 1884; Lapage et al., 1992 (GMO07/ARR003)	41312
GMD004781	Yersinia enterocolitica Schleifstein and Coleman, 1939; Frederiksen, 1964 GMO07/ARR003)	41313

Appendix 3

ENVIRONMENTAL RISK MANAGEMENT AUTHORITY
ERMA New Zealand
NGĀ KAIWHAKATŪPATO WHAKARARU TAIAO

ER-AF-02-3-IBSC 07/06

Decision form for development in containment of a genetically modified organism by rapid assessment under section 42 or 42A.

This form is a recommended decision template for use by Institutional Biological Safety Committees (IBSCs) exercising authority, delegated from the Environmental Risk Management Authority (ERMA New Zealand), to make decisions on applications to develop low risk genetically modified organisms in containment.

In exercising the delegation, IBSCs are required to demonstrate that they have followed the provisions of the Hazardous Substances and New Organisms (HSNO) Act 1996, the HSNO (Low-Risk Genetic Modification) Regulations 2003 and the HSNO (Methodology) Order 1998. This form and checklist are designed to provide IBSCs with a means of recording that they have followed the processes required by the Act and the Methodology.

The checklist, therefore, serves two purposes: it provides the IBSC with a systematic approach to their deliberation, and it serves as evidence that the IBSC has met the requirements of the Act and the Methodology. Thus it forms part of the decision.

When submitting a decision to ERMA New Zealand, the IBSC should:

1. Send electronic copies of the decision form (Microsoft Word format) and checklist to the following email address:

   IBSC@ermanz.govt.nz

2. Post a signed copy of the completed decision form and checklist along with the application[1] to:

   ERMA New Zealand,
   PO Box 131,
   Wellington
   Attention: Dr Libby Harrison
   Group Manager
   New Organisms

3. Send a copy of the decision to the applicant

[1] In the case of an application update, the IBSC should provide the original application and decision as well as the update form.

Cover page

ER-AF-02-3-IBSC 07/06

Institutional Biological Safety Committee decision form[2] to develop a low-risk genetically modified organism in containment

ERMA Office use only

Application Code:	
Application Approval Code(s):	
BCH Number[3] (if applicable):	

Institutional Biological Safety Committee:	AgResearch-Ruakura
IBSC Institution Code:	GMO07/ARR003
Application type:	<To develop in containment a genetically modified organism under section 40(1)(b) of the Hazardous Substances and New Organisms (HSNO) Act.>
Applicant:	AgResearch
Purpose:	To generate a series of GM strains of bacteria relevant to the food industry that can be used to elucidate mechanisms involved in persistence and survival within this environment.
Date application received:	14 August 2007
Considered by:	BSO, Chair, Community Layperson, Microbial geneticist, Molecular biologists (2)
Consideration date:	20 August 2007

1. Summary of the decision:

The application to develop the following organism(s) is **approved, with controls** having been considered in accordance with the relevant provisions of the Hazardous Substances and New Organisms (HSNO) Act 1996, the Hazardous Substances and New Organisms (Low-Risk Genetic Modification) Regulations 2003, and the HSNO (Methodology) Order 1998.

The application was considered by the IBSC under delegation from the Authority as provided for under section 19(2)(a) of the HSNO Act.

2. Sequence of the consideration

In accordance with sections 42 and 42A of the HSNO Act (rapid assessment), the approach adopted by the IBSC was to identify the circumstances of the genetic modification(s), to evaluate these against the criteria set out in the HSNO (Low-Risk Genetic Modification) Regulations 2003 established under section 41 of the Act, and to consider whether there are any residual risks of significance that require further consideration (if so, see Annex A).

[2] This decision form should be used in conjunction with the checklist.
[3] Biosafety Clearing House record identification number.

3. Organism description Table(s)

The organism description can be specific to individual GMOs or it can encompass a project description[4]. HOWEVER, the organism description needs to CLEARLY describe the full range of GMOs permitted by this approval so ERMA New Zealand can be satisfied that it conforms with the HSNO (Low-Risk Genetic Modification) Regulations 2003. For example: "not low-risk" modifications need to be clearly excluded from the vectors and donor nucleic acids if you are expressing uncharacterised nucleic acid sequences from pathogenic organisms, OR, for example, if using (non-pathogenic) *Escherichia coli* as a host, identify it as the non-pathogenic strains or strains K 12 or B.

The organism(s) for development are:

Name of the host organism:	*Escherichia coli* (non-pathogenic laboratory adapted strains) – Migula, 1895; Castellani and Chalmers, 1919
Specify the category of **host organism** **e.g. Category 1 or 2**[5]	Category 1
What the organism is modified with: Please specify vector and donor DNA	Targeted mutations (deletion mutations, deletion-insertion mutations, systematic truncation mutations and point mutations) to eliminate or regulate protein expression or permitted controlled expression of genes associated with the following microbial activities: - surface adherence - biofilm formation - cell to cell communication (quorum sensing) - persistence and development of viable but non culturable states (eg VNC) - establishment and survival under changing environmental conditions **Vectors** – Standard non-self transmissible *E. coli* cloning and expression vectors (eg pBluescript, pCR-Blunt-II, pCR2.1, pBR322), mobilisable non-replicating vectors (eg pDM4, pCVD441, pKNG101), P1 and P22 bacteriophage vectors, bioluminescent, colourmetric and fluorescent vectors (eg pSB377, pSB401) and inducible protein expression vectors (eg pBAD, pTrc99a, ProEX-HT). Vector DNA will include standard inducible promoters, selectable markers and protein purification tags. **Donor DNA** - will be obtained from food-borne bacteria found in New Zealand food processing plants and food products and may include the following Risk group 1 or 2 organisms: (eg *Yersinia enterocolitica. Clostridium* spp.,

[4] As described in our "Policy documents relating to New Organisms" (ER-PO-NO-01). For more guidance refer to ERMA New Zealand User Guide "*Making an application for Rapid Assessment to Develop in Containment a Project of Low Risk Genetically Modified Organisms*".
[5] According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.

	Bacillus cereus, Campylobacter jejuni, Campylobacter coli, Aeromonas hydrophila, Staphylococcus aureus, Listeria monoytogenes, Escherichia coli (including toxigenic strains such as EHEC), *Arcobacter cryaerophilus, Arcobacter butzleri, Arcobacter cibarius, Arcobacter skirrowii, Shigella flexneri, Hafnia alevi Pseudomonas aeruginosa, Lactobacillus* spp., *Brochothrix* spp., *Serratia* spp., and *Shewanella* spp) Modifications to the donor DNA will be carried out *in vitro* and the modified DNA returned to *E. coli* K-12 (eg DH5-α, TOP10) prior to being transferred to the final recipient. The final recipient of the modified donor DNA will be the same species as the original donor.
Please specify the category of genetic modification e.g. **Category A or B**[6]	Category A
Containment level e.g. PC1/PC2[7]	PC1
Approved/declined	Approved

Name of the host organism:	*Escherichia coli* (non-pathogenic - commensal strains) – Migula, 1895; Castellani and Chalmers, 1919; *Clostridium estertheticum* Collins et al, 1993; *Lactobacillus* spp – Beijerinck 1901, Skerman et al, 1980; *Brochothrix* – Sneath and Jones, 1976; *Shewanella* – MacDonell and Colwell, 1986 *Serratia* spp (non-pathogenic) – Bizio, 1823; Skerman et al., 1980.
Specify the category of **host organism** **e.g. Category 1 or 2**[8]	Category 1
What the organism is modified with: Please specify vector and donor DNA	Targeted mutations (deletion mutations, deletion-insertion mutations, systematic truncation mutations and point mutations) to eliminate or regulate protein expression or permitted controlled expression of genes associated with the following microbial activities: - surface adherence - biofilm formation - cell to cell communication (quorum sensing)

[6] According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.
[7] As in the Australian/New Zealand Standard 2243.3:2002 with modifications referred to in the MAF Biosecurity Authority ERMA NZ Containment Standards.
[8] According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.

	- persistence and development of viable but non culturable states (eg VNC) - establishment and survival under changing environmental conditions **Vectors** – Standard non-self transmissible *E. coli* cloning and expression vectors (eg pBluescript, pCR-Blunt-II, pCR2.1, pBR322), mobilisable non-replicating vectors (eg pDM4, pCVD441, pKNG101), P1 and P22 bacteriophage vectors, bioluminescent, colourmetric and fluorescent vectors (eg pSB377, pSB401) and inducible protein expression vectors (eg pBAD, pTrc99a, ProEX-HT). Vector DNA will include standard inducible promoters, selectable markers and protein purification tags appropriate for use in a given host. **Original Donor DNA** - will be obtained from food-borne bacteria found in New Zealand food processing plants and food products and may include the following Risk group 1 organisms: (eg *Clostridium estertheticum, Escherichia coli* (non pathogenic commensal strains), *Lactobacillus* spp., *Brochothrix* spp., *Serratia* spp. (non-pathogenic), and *Shewanella* spp). Modifications to the donor DNA will be carried out *in vitro* and the modified DNA returned to *E. coli* K-12 (eg DH5-α, TOP10) prior to being transferred to the final recipient. The final recipient of the modified donor DNA will be the same species as the original donor. Where possible bacteria for manipulation have been named to the species level. Several bacteria have been named to genera level where it is unclear exactly which species will be most relevant to investigations within the project. No pathogenic species for humans have been identified within these genera to date.
Please specify the category of genetic modification e.g. **Category A or B**[9]	Category A
Containment level e.g. PC1/PC2[10]	PC1
Approved/declined	Approved

[9] According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.
[10] As in the Australian/New Zealand Standard 2243.3:2002 with modifications referred to in the MAF Biosecurity Authority ERMA NZ Containment Standards.

Name of the host organism:	*Escherichia coli* (pathogenic) – Migula 1895; Castellani and Chalmers, 1919; *Yersinia enterocolitica* - Schleifstein and Coleman, 1939; Frederiksen, 1964; *Salmonella enterica* Serovar Typhimurium – (*ex* Kauffmann and Edwards, 1952) Le Minor and Popoff, 1987); *Salmonella typhi* – (Schroeter, 1886) Warren and Scott, 1930; *Shigella flexneri* - Castellani and Chalmers, 1919; *Arcobacter cryaerophilus* – (Neill *et al.*, 1985) Vandamme, et al 1991 ; *Arcobacter butzleri* – (Kiehlbauch *et al.*, 1991) Vandamme, et al 1992; *Arcobacter cibarius* – Houf et al 2005 ; *Arcobacter skirrowii* - Vandamme, et al 1992; *Hafnia alvei* – Møller, 1954; *Clostridium perfringens* - Veillon and Zuber, 1898; Hauduroy et al.,1937; *Bacillus cereus* - Frankland and Frankland, 1887; Skerman et al., 1980; *Campylobacter jejuni* – Jones et al., 1931; Veron and Chatelain, 1973 ; *Campylobacter coli* – Doyle, 1948; Veron and Chatelain, 1973 ; *Aeromonas hydrophila* - Chester, 1901; Stanier, 1943 ; *Staphylococcus aureus* – Rosenbach, 1884; Lapage et al., 1992; *Listeria monocytogenes* – Murray et al., 1926; Pirie, 1940 ; *Pseudomonas aeruginosa* - Schroeter, 1872; Migula, 1900 ; *Serratia* spp. (pathogenic) – Bizio, 1823; Skerman et al., 1980.
Specify the category of **host organism** **e.g. Category 1 or 2**[11]	Category 2
What the organism is modified with: Please specify vector and donor DNA	Targeted mutations (deletion mutations, deletion-insertion mutations, systematic truncation mutations and point mutations) to eliminate or regulate protein expression or permitted controlled expression of genes associated with the following microbial activities: - surface adherence - biofilm formation - cell to cell communication (quorum sensing) - persistence and development of viable but non culturable states (eg VNC) - establishment and survival under changing environmental conditions **Vectors** – Standard non-self transmissible *E. coli* cloning and

[11] According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.

	expression vectors (eg pBluescript, pCR-Blunt-II, pCR2.1, pBR322), mobilisable non-replicating vectors (eg pDM4, pCVD441, pKNG101), P1 and P22 bacteriophage vectors, bioluminescent, colourmetric and fluorescent vectors (eg pSB377, pSB401) and inducible protein expression vectors (eg pBAD, pTrc99a, ProEX-HT). Vector DNA will include standard inducible promoters, selectable markers and protein purification tags appropriate for use in a given host. **Original Donor DNA** - will be obtained from food-borne bacteria found in New Zealand food processing plants and food products and may include the following Risk group 2 organisms: (eg *Escherichia coli, Yersinia enterocolitica, Salmonella enterica* Serovar Typhimurium, *Salmonella typhi, Shigella flexneri, Arcobacter cryaerophilus, Arcobacter butzleri, Arcobacter cibarius, Arcobacter skirrowii, Hafnia alvei, Clostridium perfringens, Bacillus cereus, Campylobacter jejuni, Campylobacter coli, Aeromonas hydrophila, Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa Serratia* spp. (pathogenic)). Modifications to the donor DNA will be carried out *in vitro* and the modified DNA returned to *E. coli* K-12 (eg DH5-α, TOP10) prior to being transferred to the final recipient. The final recipient of the modified donor DNA will be the same species as the original donor. Where possible bacteria for manipulation have been named to the species level. Bacteria have been named to genera level where it is unclear exactly which species will be most relevant to investigations within the project. These genera do include both pathogenic and non pathogenic species and will handled accordingly. Due to the nature of the study, the following exception will apply: No genes responsible for the production of exotoxins will be cloned or expressed as part of this study.
Please specify the category of genetic modification e.g. **Category A or B**[12]	Category B
Containment level e.g. PC1/PC2[13]	PC2

[12] According to the HSNO (Low-Risk Genetic Modification) Regulations 2003.
[13] As in the Australian/New Zealand Standard 2243.3:2002 with modifications referred to in the MAF Biosecurity Authority ERMA NZ Containment Standards.

Approved/declined	Approved

4. Use of special genetic material

Human Genes or Native introduced flora and fauna:	YES	NO
Does the proposed development use genetic material from native flora and/or fauna; or flora and/or fauna valued by Māori ?		No
Does the proposed development involve human cell lines or human genetic material of Māori whakapapa or origin ?		No
If "YES" to either of the above please clearly record evidence that appropriate Māori consultation has occurred with local iwi regarding this approval (i.e. who was consulted, their status, and the results of the consultation).		

5. Identification and assessment of the significant risks and costs of the organism

Describe any significant (non-negligible) risks identified, along with the Committee's assessment of the risks. Describe and justify any additional controls applied to manage the risks.

The committee discussed the potential risk to the staff working with these organisms and decided the level of training and expertise within the group was adequate to deal with the perceived risk. Documented staff training is based on information contained within the "AgResearch MIRINZ Meat Industry Microbiological Methods - Edition 4 (Micromanual)" and the "AgResearch Ruakura Transitional and Containment Facility Manual - Version 2.2".

6. Containment

Describe the containment system (physical and operational).

All work will be carried out with in the PC2/2+ laboratories in D block, MIRINZ, AgResearch Ltd, Ruakura which conform to and are audited against MAF/ERMA Containment standards AS/NZ Standard 2243.3:2002: Safety in Laboratories: Part 3: Microbiological aspects and containment facilities at PC2 (as detailed in the MIRINZ Quarantine and Containment Manual) and MAF Biosecurity Authority/ERMA New Zealand Standard: Facilities for Microorganisms and Cell Cultures: 2007.
No additional safety precautions beyond those associated with PC2 and PC2+ need be taken for handling of these strains. All biological waste generated during manipulations will be autoclaved within the facility prior to disposal. Biological liquid waste will be disposed of by the addition of an appropriate disinfectant e.g. 10% sodium hypochlorite or will be autoclaved before disposal.

7. Controls

In considering all the matters to be addressed detailed in the Third Schedule Part I "*Containment Controls for Importing, Developing or Field Testing of Genetically Modified Organisms*" of the HSNO Act, this approval is subject to the following controls:

1. The operation, management and construction of the containment facility[14] shall be in accordance with the:
   - The MAF Biosecurity Authority/ERMA New Zealand Standard **Facilities for Microorganisms and Cell Cultures:2007**
   - The Australian/New Zealand Standard 2243.3:2002 Safety in laboratories: Microbiological aspects and containment facilities, at Physical Containment Level 1 (PC1) for category A modifications and Physical Containment Level 2 (PC2) for category B modifications.
2. If for any reason a breach of containment occurs the applicant shall notify the facility Supervisor and ERMA New Zealand immediately the event is noticed (and at least within 24 hours of the breach being detected) and shall immediately implement a contingency plan for the recovery and eradication of any organisms or viable material that has escaped.
3. The Authority or its authorised agent or properly authorised enforcement officers, may inspect the facilities at any reasonable time.

Additional controls

List any additional controls

(1) Genetic material may be transferred to a transitional host (*Escherichia coli*-K12) for manipulation prior to the transfer to the final recipient. The final recipient of modified DNA will be the same species as the donor.
(2) Antibiotic markers will be restricted to those used in commercially available vectors (eg Chloramphenicol, tetracycline, ampillicin and kanamycin). No antibiotic marker will be placed into an organism where that specific antibiotic is a known clinical treatment of an infection caused by that specific organism.
(3) No genes of known exotoxins will be cloned and expressed in this study.
(4) All handling of genetically modified STEC will be carried out in a biological safety cabinet with a PC2 laboratory. Only staff with appropriate experience and training will be used in projects handling these GMOs.

Signed: ..... Date: 24 August 2007
(on behalf of the institution)

Name: Dr. Jimmy Suttie

Position: Chairperson AgResearch-Ruakura IBSC

[14] Containment facility means a facility registered under section 39 of the Biosecurity Act 1993

Checklist

NB- this checklist should be completed by the IBSC, and signed and dated by the Chair of the IBSC and returned to ERMA New Zealand with the decision form.

- Sections referenced in the text below indicate sections of the Hazardous Substance and New Organisms Act 1996
- Clauses referenced in the text below indicate clauses of the Hazardous Substances and New Organisms (Methodology) Order 1998

		Yes/No/ N/A
1	***Legislative criteria for the application***	
1.1	The application was lodged pursuant to section 40(1)(b) of the Act.	Yes
1.2	The application was considered in accordance with section 42 and 42A and matters relevant to the purpose of the Act.	Yes
2	***Consideration of the application***	
2.1	The IBSC holds delegation from the Authority as provided under section 19(2)(a) of the HSNO Act.	Yes
2.2	The purpose is provided for under section 39(1)(a) of the Act i.e. *The development of any genetically modified organism.*	Yes
2.3	Does the IBSC consider the information provided by the applicant is relevant and appropriate to the scale and significance of the risks, costs, and benefits associated with the application (clause 8)?	Yes
2.4	If NO – <please explain>	
2.5	Was any expert advice sought (clause 17)?	No
2.6	If YES – name of the expert(s) and the nature of the advice sought: <text in here>	
2.7	If YES – was the applicant informed (clause 18)?	
3	***Assessment against the criteria for low risk genetic modifications***	
3.1	Is the IBSC satisfied that each of the genetically modified organisms described in the application meet the criteria for a low-risk genetic modification specified in the criteria made under section 41 of the Act, being the HSNO (Low-Risk Genetic Modification) Regulations 2003? <If not, give details>	Yes

4	***Applications involving native flora and fauna***	
4.1	Does the application involve native or valued introduced flora and/or fauna as host organisms or as a source of genetic material? (Please ensure section 4 of decision form is complete.)	No
4	***Applications involving human genetic material or human cells***	
4.2	Does the application use any genetic material or cells obtained directly from human beings?	No
4.3	If YES, has approval from an Ethics Committee been obtained?	n/a
4.4	Does the application involve the use of human cells or human genetic material sourced directly from individuals of Māori whakapapa or origin?	No
4.5	If YES, please record details in section 4 of the decision (who was consulted, their status and the results of the consultation).	
5	***Identification of significant risks***[15]	
5.1	Are there any significant risks or costs to the environment, including the sustainability of all native and valued introduced flora and fauna?	No
5.2	Are there any significant risks to the intrinsic value of ecosystems?	No
5.3	Are there any significant risks or costs to human health, including public health?	No
5.4	Are there any significant risks to Māori and their taonga?	No
5.5	Are there any significant economic risks or costs?	No
5.6	Are there any risks to New Zealand's international obligations, including DNA derived from CITES species or use of CITES species as host organisms?	No
	If YES is checked in any of 5.1-5.6, please list the significant risks identified in section 5 of the decision form and discuss how they were assessed in terms of likelihood and consequence, and what controls were imposed to manage them.[16]	

[15] See Annex A
[16] Clauses 12 and 13 of the Methodology.

6	***Containment of the organisms***	
6.1	Has the IBSC considered the adequacy of containment in accordance with section 42 or 42A, and whether the modification may result in (a) GMO(s) having a greater ability to escape from containment than the unmodified organism(s)? Please record details in section 6 of the decision. Please ensure the containment controls have been specified. Note that controls relevant to the physical containment level set in the Regulations cannot be removed.	Yes
6.2	Are any additional measures proposed because of the particular nature of the organism(s)? If YES, please ensure additional controls are listed on the decision form.	No
6.3	Are there any other matters that may affect the adequacy of containment such as the expected time-frame for the project, and external matters such as the potential for sabotage? If YES, please explain.	No
7	***Decision*** In this section YES confirms approval – if any of the answers to 7.1-7.4 are NO, then the application is declined.	
7.1	The IBSC is satisfied that the application is for one of the purposes specified in section 39(1) of the Act, being section 39(1)(a): *The development of any genetically modified organism*?	Yes
7.2	Based on analysis of the information provided, and having considered the characteristics of the organisms and the modifications and the criteria for low-risk genetic modification detailed in the HSNO (Low-Risk Genetic Modification) Regulations 2003, it is the view of the IBSC that the organism(s) meet the criteria for rapid assessment (as per section 42(2)).	Yes
7.3	The IBSC is satisfied that the proposed containment regime together with any additional controls imposed will adequately contain the organism(s) as required by section 42(2) of the Act.	Yes
7.4	In accordance with clause 36(2)(b) of the Methodology the IBSC records that, in reaching this conclusion, it has applied the relevant criteria from the Methodology.	Yes
7.5	The application for development of a genetically modified organism (detailed) is thus approved, with controls as detailed on the decision document.	

Signed: ...James M Suttie..... Date: 24 August 2007
(on behalf of the institution)

Name: Dr. Jimmy Suttie Position: Chairperson, AgResearch-Ruakura IBSC

Annex A - Guidelines for dealing with significant risks

Significant risks are those risks that the IBSC considers are not negligible (i.e. they require active management beyond the normal requirements of the specified physical containment level). In most circumstances the default controls will be adequate to contain the organism(s), and there will not be any significant residual risks. However, there may be some instances where the IBSC considers that this is not the case and where additional controls should be applied. In these situations the IBSC may choose to present a full assessment of the significant residual risk or contact ERMA New Zealand.

Where the IBSC considers that there are significant risks that require full consideration, then they may decide to use the following approach to assessing these risks, prior to evaluating options for managing or reducing the risks.

If the IBSC deems that the organism(s) cannot be adequately contained or that the risks cannot be reduced to a negligible level by applying additional controls, then the application is not appropriate for rapid assessment and should be declined or referred to ERMA New Zealand.

Assessment of the significant adverse effects (risks and costs) of the organism(s)

Adverse effects (risks and costs) may be grouped into categories reflecting those used in the identification section of the decision e.g., effects on, for example:

- biological and physical environment
- human welfare including health and safety
- social or community conditions
- Māori issues and concerns, and
- economic aspects.

Each adverse effect should be discussed under a separate heading and cross-referenced to the identification section of the decision. Information provided that has been produced for other processes or jurisdictions (in New Zealand or overseas) should be discussed with reference to clause 20.

Example

Outline of the application: The applicant is proposing to modify a plant where full shoots and roots will form, and the pollen is required for scientific analysis. (For this example and simplicity only one risk has been identified, often there will be more than one significant risk).

The following wording provides an example for the Identification and Assessment section of the decision form.

> *The risks and costs assessed were those identified as potentially significant, having regard for those matters set out in clauses 9 and 10 of the Methodology. Risks were considered in terms of the requirements of clause 12 of the Methodology, including especially the*

assessment of consequences and probabilities, the impact of uncertainty and the impact of risk management. Costs were considered in terms of clause 13 of the Methodology.

When going through the checklist, section 4.1 was checked as YES because the IBSC identified a significant risk that the genetically modified organism may cause an adverse effect on the environment if the pollen escaped from containment and was found to hybridise with flora.

The IBSC proceeded to address this significant risk by the following process.

Significant risks, costs and benefits identified for assessment and evaluation were as follows, following clauses 9 and 10 of the Methodology, which incorporate sections 5, 6, and 8 of the HSNO Act. The components of this risk are:

- *the likelihood of escape from containment*
- *the likelihood of hybridisation with valued plant species*
- *the effect should hybridisation occur*

The Committee considered the nature of the potential adverse effect (clause 12(a)), relating to <plant species> hybridising with other <plant species> if they escaped from containment.

The Committee was unable to determine whether hybrids occur between <plant species>, so there is uncertainty over whether hybrids with the naturalised species could occur (clause 12(e)). Since <plant species> already in New Zealand are recorded as uncommon, the Committee considers that it is very unlikely for escaping pollen to land on receptive flowers outside of containment but the effect if it should occur would be minor-moderate, therefore the risk is low (clause 12(b)).

In order to reduce the risk, by reducing the likelihood of escape of pollen, the Committee has proposed an additional control. In the event of initiating flowering, all pollen shall be contained by bagging and seed shall be collected. The containment manual shall be updated to reflect the process for bagging pollen and collecting seed.

The Committee is satisfied that the <plant species> are easily identifiable due to their phenotype, and easily eradicated (clause 12(d)). Eradication procedures include physical removal for small scale infestations or by a range of common soil-applied and plant applied herbicides including Glyphosphate, Diuron, Metribuzin, Simazine, Chlorpropham, DCPA and Trifluralin for larger scale infestations.

The Committee considers that the risk of hybridisation is reduced to negligible by the additional controls on pollen and seed. (clause 12(c)).