
# An investigation of the regulation and physiological role of *Listeria monocytogenes* extracellular polymer

By

Ho Ting Lawrence Wong

Thesis submitted to the University of Nottingham
for the Degree of Doctor of Philosophy
September 2012

**Abstract**

It was shown that *Listeria monocytogenes* cells grown in a defined minimal, MCDB202, showed enhanced extracellular polymeric substances production compared to BHI. On the other hand, it was reported that in *L. monocytogenes luxS* mutant, AI-2 reduction and biofilm enhancement were seen. It is hypotheses that there could be a linkage between the AI-2 signaling system and the EPS formation. The expression of EPS could be induced by the reduction in AI-2.

The main aim of the research is to study this EPS formation in minimal media, how is it linked to AI-2 production, the function of the EPS as well as to figure out the linkage between EPS formation with cap genes found in *Listeria* genome.

It was shown that MCDB202 have caused an increase in surface hydrophobicity of the cells. However, cells grown in the defined media did not induced better attachment and biofilm formation towards hydrophobic surfaces. And cells grown in MCDB202 were shown less capable to infect eukaryotic cells in the cell invasion assay. On the other hand, AI-2 production was shown to be relative lower in *Listeria* cell grown in minimal media (MCDB202) than rich media (BHI). Bioinformatics study has shown that only capA homologues, but no capBCDE homologues, were found in *Listeria* genome. However, the bioinformatics works have shown that the capA homologues are unlikely to be contributing the EPS seen produced in *Listeria monocytogenes*. This was further supported in the expression assay that the two genes were not highly expressed in MCDB media.

# Acknowledgements

I would like to take the opportunity here to give thanks to all the people who helped me during my study in Nottingham.

To begin with, I would like to sincerely thank my first supervisor Dr. Cath Rees and my second supervisor Dr. Phil Hill, who have given me great supervision and guidance with tremendous patience, and also unlimited support as well as countless encouragement that made my study possible. Special thanks must also be given to all other staff within the department.

I would also like to give my special thanks to Dave Fowler, Lorraine Gillet and Niki for their assistance and help in lab and materials preparation. Special thanks must be given to Wendy Fielder for her help in my tissue culture work that contribute quite a lot in my work. I would also like to give thanks to all the researchers in the laboratory especially, Steve Sun, Tania Perehinec and Ogueri Nwaiwu, who had helped me a lot throughout the time in the laboratory.

Lastly, and most importantly, I wish to thank my parents who have given me unlimited support and encouragement since I was born and also throughout my education. I am forever indebt of their love.

# Contents

**Chapter 2**

**Chapter 3**



**Chapter 4**

# List of Figures

# Lists of Tables

# Abbreviations

| | |
|---|---|
| **AI** | Autoinducer |
| **ATCC** | American type culture collection |
| **BHI** | Brain Heart Infusion |
| **bp** | base pair |
| **cfu** | Colony forming unit |
| **Caco-2** | Human colonic adenocarcinoma cells |
| **°C** | Degrees Celsius |
| **CV** | Crystal violet |
| **DNA** | Deoxyribonucleic acid |
| **DPD** | 4,5-dihydroxy- 2,3-pentanedione |
| **EPS** | Extracellular polymeric substance |
| **g** | gram |
| **GFP** | Green fluorescent protein |
| **HCL** | hydrochloric acid |
| **kb** | kilobase |
| **Lux** | Luciferase |
| **M** | Molar |
| **MATH** | Microbial attachment to hydrocarbons |
| **MOI** | Multiplicity infection |
| **mol** | Mole |
| **MRD** | Maximum recovery Diluent |
| **OD** | Optical density |
| **P/S** | Penicillin and streptomycin |
| **PCR** | Polymerase chain reaction |
| **QS** | Quorum sensing |
| **RLU** | Relative light unit |
| **RO** | Reverse osmosis |
| **RPM** | Revolutions per minute |
| **RNA** | Ribonucleic acid |
| **RTE** | Ready to Eat |
| **SDW** | Stile Distillated Water |
| **UV** | Ultraviolet |
| **V** | Volts |
| **w/v** | Weight for volume |

# Chapter 1

# Introduction

## 1.1 The Genus of *Listeria* and the Species *Listeria monocytogenes*

*Listeria monocytogenes* is a very common food borne pathogen which was first described as large mononuclear leucocytosis in 1926 during investigation of a rabbit infection and was first named as *Bacterium monocytogenes* (Murray *et al.*, 1926). It was then renamed as *Listeria monocytogenes* in 1940, in honour of the British surgeon Lord Joseph Lister, and it was felt that this was particularly appropriate since the organism had been identified in medical samples (Pirie, 1940). Within the genus of *Listeria* are nine co-related species, *L. innocua*, *L. ivanovii*, *L.grayi*, *L. welshimeri*, *L. seeligeri* and *L. monocytogenes* and three new discovered *Listeria* species know as *Listeria marthii, Listeria rocourtiae* and *Listeria weihenstephanensis* (Graves *et al.*, 2010; Leclercq *et al.*, 2010, Lang Halter *et al.*, 2012). *L. monocytogenes* and *L. ivanovii* and are also the most well studied species since they have been shown to be potentially pathogenic to humans and animals, which has provided a main driver for scientific studies (Leclercq *et al.*, 2010, Graves *et al.*, 2010, Cossart, 2011).

## 1.2 Morphology and characteristics

*L. monocytogenes* are Gram-positive, non-spore forming bacilli that are ubiquitous in the environment. Despite being Gram-positive in nature, some cells, commonly in old cultures, do lose the ability to retain the Gram stain. Physically, they are regular rods with blunt ends which can occur singly or in groups. They may also arrange in short chains, forming characteristic "V" or "Y" chains formation, and is some time described as resembling Chinese characters. Each single rod cell has the size of approximate 0.4-0.5μm in width and 1-2μm in length, depending on the nutrient supply and age of cells

(Gandhi and Chikindas, 2007).Under severe stress conditions, it was observed that *Listeria* cells can undergo changes in cell morphology (Isom *et al.*, 1995). For instance research groups studying *Listeria* physiology under alkaline conditions showed that *L. monocytogenes* 10403S showed a morphology change, forming filamentous or elongated chains under MHB (Mueller-Hinton broth)at pH9 (Giotis *et al.*, 2007). Staining showed that the filaments were multi-nucleated, with nucleoids spaced along the length of the atypical cells. In buffered media, the time of exposure to alkaline conditions was associated with increases in the frequency and length of filaments. In the non-buffered medium, longer exposure was associated with gradual decline in length and the frequency of the filaments indicating that pH condition also induces a change in cell morphology. Filamentation has also been observed when *Listeria* strains LO28 and Scott A cells are grown in high salt and acidic conditions. For instance when LO28 cells were grown in TSB-YE adjusted to pH 5 supplemented with 5% NaCl, they form remarkable long filaments of 2-5μmwhen viewed by SEM (Bereksi *et al.*, 2002).

*L. monocytogenes* will adapt to a wide range of temperatures for growth, from as low as 1°C to 45°C, with 37°C being the optimum temperature (Gandhi and Chikindas, 2007). The bacterium can also survive in a very wide range of cold temperature at as low as -12°C (in a matrix which does not freeze). The bacteria undergo adaptations when they are grown at these different temperatures. The most well studied of these is that *L. monocytogenes* are motile with one to five peritrichous flagella when cultured at 20-28°C, but do not produced flagellar filaments when grown at 30-37 °C.

*Listeria* are naturally found in soil, water, plants, and also the digestive

system of many animals including human beings (Seeliger and Jones, 1986). One factor that contributes to this is that *L. monocytogenes* is also able to survive in various pH conditions, ranging from pH 4.6-9.2. *L. monocytogenes* is also able to survive at minimal water activity level of about 0.9 and also able to grow in NaCl concentrations of up to 10%, making it a very able to survive even in highly preserved food (Gandhi and Chikindas, 2007).

### 1.3 *Listeria monocytogenes* and the food industry

Food borne pathogens have long been a great problem worldwide for food industries and *L. monocytogenes* is one of the major concerns for most food companies. Although *L. monocytogenes* was known as a mammalian pathogen for over 80 years, they were only identified as a food borne pathogens in the 1980s (Farber and Peterkin, 1991). Its success in causing food borne illness is greatly related to the characteristics and survival nature of the species, including survival under a wide range of pH and temperature conditions as described above. As they are very widespread in the environment, contamination of food by *Listeria* is very common. Water, animal feeds, soil and even air can act as vectors for transmission.

Refrigeration is the most common and effective method to extend food shelf life. Since *L. monocytogenes* are psychrophiles, their ability to grow at low temperature results in serious food contamination problems even in well-chilled environments. Very few food borne bacteria are able to grow in such cold conditions. With a lack of competitors, *Listeria* become the predominant organism in cold environments and can also grow in many different chilled foods.

As a facultative anaerobe, *L. monocytogenes* are also able to grow in the absence of oxygen, making it possible for them to grow even in vacuum packed products as well as in food preserved in liquid. With these abilities, *L. monocytogenes* can be found in a wide range of foods, from fresh to frozen meats, raw to cooked foods, seasoned or fermented foods, as well as fruit and vegetables (Walker *et al.*, 1990, Schlech *et al.*, 1983).A UK survey completed between 2006-2007 found that the of *L. monocytogenes* could be isolated from a variety of foods with the prevalence in sandwiches being 7.0%, meat 3.7-4.2%, salads 3.8% and in cured ham 2.1%. The presence of the organism in all these foods are indications of poor control measures during in food production and are alarming figures for the public (Little *et al.*, 2009).

## *1.4* Epidemiology and Pathogenesis

### 1.4.1 Listeriosis

Clinical infection of humans or animals by *Listeria* is termed listeriosis. The main transmission route of *Listeria* to human is primarily known to be food borne, although less commonly it is seen to be directly transmitted via skin contact or wounds. A recent case of direct transmission was seen in an medicalimplantation of a prosthetic knee device caused by contamination of *Listeria* on device, after which *Listeria* were found to persist for 2 years in the patient and before they were cured by antibiotic treatment (Kleemann *et al.*, 2009).

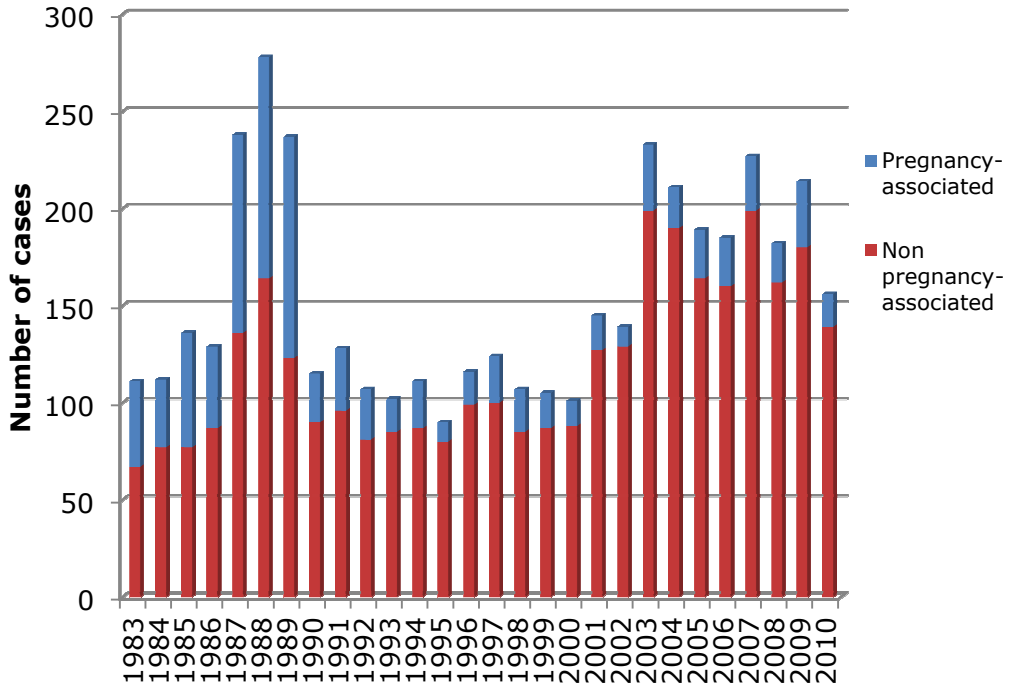
*L. monocytogenes* are sub-grouped into different serotypes based on cells surface somatic (O) and flagellar (H) antigens. All 13 serotypes of *L.monocytogenes* are able to cause human listeriosis, but it has been shown the serotypes 1/2a, 1/2b and 4b most commonly cause human infection

(Gellin *et al.*, 1991, Cossart, 2011). Within the United Kingdom serotype 4b is responsible for most reported cases of human infections (Mook *et al.*, 2011). There is an average of about 3–5 cases of listeriosis per year per million population in most developed countries (Goulet *et al.*, 2008), and there have been about 100-250 cases of listeriosis in England and Wales per year in the past decades (Fig. 1.1; (Mook *et al.*, 2011)).

In the UK hospital–acquired listeriosis is relative common, due to the lowered immune system of health-impaired patients. Reports have shown that between 1999-2007, over 70% of UK hospital-acquired cases of *Listeria* infection were related to consumption of contaminated sandwiches from the hospital menu. In 2007 several thousand sandwiches were withdrawn from hospitals around across London due to the discovery of over 100cfug$^{-1}$ of *L. monocytogenes* in samples tested and this action prevented a large hospital–acquired listeriosis outbreak (Shetty *et al.*, 2009, Little *et al.*, 2012).

There has also been an increase in incidence of listeriosis due to increased consumption of contaminated ready-to-eat (RTE) foods.RTE foods provide a highly nutritious environment for these bacteria and are consumed without the need for complete reheating to kill bacteria, allowing *L. monocytogenes* be a great 'RTE food invader', resulting in rising levels of concern with respect to food safety. One listeriosis outbreak associated with RTE food occurred in Canada in August 2008 which is known as the Maple Leaf Foods incident. It was one of the largest outbreaks of listeriosis in Canadian food history. It was reported that 57 cases of listeriosis occurred which caused 23 deaths. It was later traced back to the contamination of RTE deli meat sold across the country (Farber *et al.*, 2011).

**Figure 1.1: Human cases of *L. monocytogenes* in England and Wales 1983-2010**




Data from the Health Protection Agency Centre for Infections showing human causes of *L.monocytogenes* incidents in England and Wales from 1983 to 2010. Data shows that the increase incidence since 2000 has been primarily in the non-pregnancy associated group.

http://www.hpa.org.uk/hpr/archives/2011/news1311.htm

Although cases of human infection are quite rare, listeriosis results in a relative high mortality rate in infected patients (about 20-40%), particularly in individuals who are immuno-compromised which includes new born babies, patients with long term underlying illness, pregnant women and the elderly. *L.monocytogenes* infection also results in a very high proportion of diagnosed cases being admitted to hospital since it may cause a number of serious health problems which need medical treatment. Early stage symptoms of *L.monocytogenes* infection of humans are flu-like, including headache, muscle pain, chills and also (more rarely) diarrhoea or gastroenteritis. Although it is usually self-limiting in healthy individuals, these symptoms usually attract very low attention and this can result in a delay in treatment, leading to serious disease in some immuno-compromised individuals such as septicaemia, meningitis and even death (Leclercq *et al.*, 2010).Great care must be given to these listeriosis patients. Current treatment is mainly based on the use of ampicillin alone or in combination with gentamicin or other antibiotics which were found to be quite effective against listeriosis (Temple and Nahata, 2000).Unlike the situation for toxigenic *Escherichia coli* 0157, which also produces high levels of mortality, there is no evidence that antibiotic treatments can lead to the induction of toxin production that can contribute to more severe disease (Serna and Boedeker, 2008). However some of the strains of *L. monocytogenes* most often associated with human disease have recently been shown to produce a peptide haemolytic and cytotoxic factor called Listeriolysin S which can be induced by oxidative stress (Cotter *et al.*, 2008).

### 1.4.2 Mechanism of intracellular pathogenic life cycle of *Listeria monocytogenes*

As suggested in section 1.4.1, *Listeria* commonly enter humans after ingestion of contaminated food or sometimes via direct transmission in wounds. In foodborne transmission, after intake into the GI system, there are mainly two mechanisms by which *L.monocytogenes* can enter into the host across intestinal mucosa. The first is the direct invasion of enterocytes lining the GI tract leading to infection of the intestinal cells, that requires ligand-receptor interaction to occur. The second mechanism is phagocytosis by the M cells. This entry pathway is rather unspecific and requires no receptors. After getting into host, the pathogen translocate via lymph nodes and blood streams. The liver and spleen would then be the first target organ. The Listeria multiply actively in fast speed until controlled by cell mediated immune response. unrestricted proliferation of Listeria cells would occurs in immuno-compromised patients, which will further spread to certain secondary target organs, such as the CNS system and the gravid uterus. This would then cause serious illness including meningitis or abortions, causing high mortality rate.

After becoming systemic, *Listeria* invasion of host cells comprises four stages; *Listeria* infection begins with the internalisation of the bacteria into the host cells (summarised in Fig. 1.2). This process can be passive, by the natural phagocytogesis of phagoctyic cells, or active due to the induced uptake of the bacteria by non-phagocytic cells which is triggered by a numbers of *L. monocytogenes*-specific factors (Beauregard *et al.*, 1997). In the active uptake process, the first step of the induced uptake is induced by one of two internalin proteins, InlA and InlB (Bierne *et al.*, 2007). InlA is a surface

protein with an LPXTG motif that is covalent anchored to peptidoglycan whereas InlB interacts non-covalently with lipiteichoic acid. InlA attaches to human adherent junction E-cadherin protein, which is known to be involved in intercellular adhesion, and InlB interacts with Met which is a tyroysine kinase and a receptor for hepatocyte growth factor (HGF) (Shen *et al.*, 2000, Mengaud *et al.*, 1996). After initial adhesion on the host cell surface, the bacteria induces a "zipper" uptake mechanism which involves the endocytic protein clathrin. It was shown that actin and septin are centrally implicated in *Listeria* uptake into the host cell (Veiga and Cossart, 2005) and this allows the cell to move inwards into the host cell. The binding of nlA and InlB proteins to their corresponding receptor causes receptor ubiquitination and lead to recruitment of clathrin and causes a series of rearrangement of cortical cytoskeleton of host cell and induces pathogen uptake into the host (Ireton, 2007, Bonazzi and Cossart, 2006). The binding of InlA and InlB proteins to the receptor induces the assembly of a multi-component signalling platform leading to activation of key cellular pathways such as the phosphatidlylinositol 3-kinases and the mitogen-activated protein kinase pathway. However, the connection between the downstream cascade of the pathway and invasion is not clear (Gaillard *et al.*, 1991, Seveau *et al.*, 2007, Stavru *et al.*, 2011).

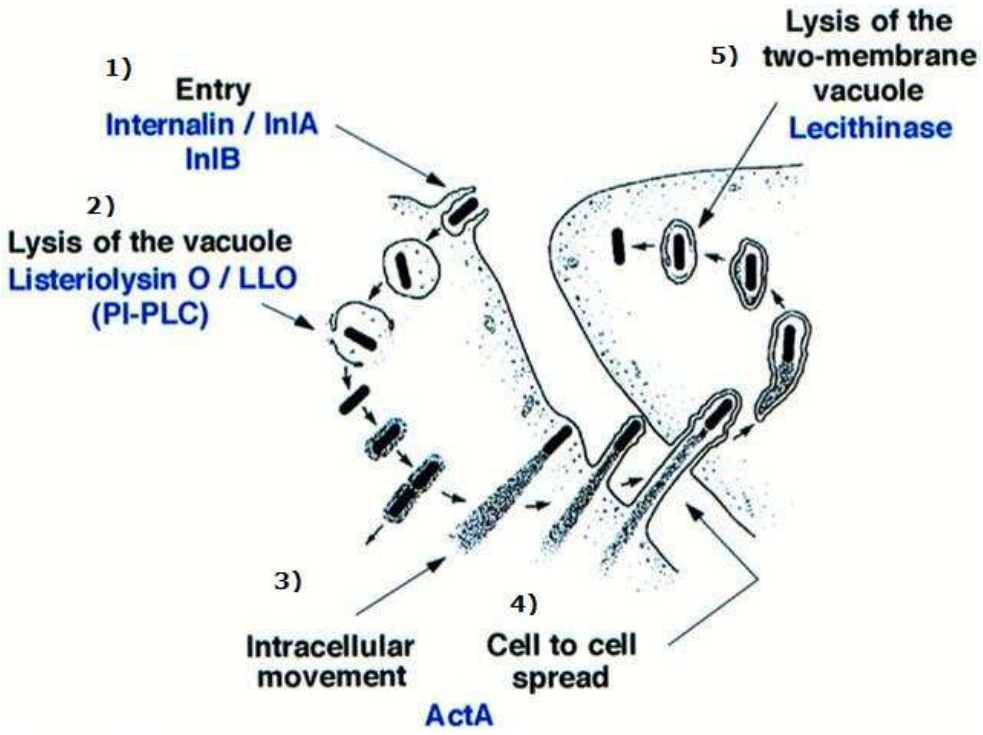
After the internalization of cells, *L. monocytogenes* is surrounded in a membrane-bound vacuoles formed during the phagocytic uptake of cells. *Listeria* escapes from the membrane-bound vacuoles by secreting a pore-forming cytolysin called listeriolysin O (LLO). Pore formation is induced by oilgomerization of cholesterol-associated monomers of LLO that insert into the membrane bilayer. One host factor, GILT, was shown to activate LLO by recognizing its essential cysteine, which then promotes its pore-forming

activity to allow efficient escape of the bacterium from the vacuole. This was demonstrated by showing that GILT knockout mice had increased resistance to *Listeria* infection (Maric *et al.*, 2001). Once free in the cytoplasm, bacteria need to change their metabolism to adapt the intracellular condition. A hexose phosphate transporter (Hpt) is activated which encodes the sugar uptake system for glucose-phosphate which has been shown to be essential for *Listeria* to grow intracellularly (Chico-Calero *et al.*, 2002). The Hpt system is regulated by the PrfA regulator (see section 1.4.3) which also controls the expression of a number of major virulence genes including LLO. With the adaptation to the intracellular condition, *L. monocytogenes* replicates efficiently in the host cytosol and an increase in the number of internalised bacterial is seen (Vazquez-Boland *et al.*, 2001, Schnupf and Portnoy, 2007).

After bacterial cell division has occurred, *Listeria* infection is also characterized by the process of cell-to-cell spread. This is achieved by the action of the ActA protein which is critical for both actin-based intra- and inter-cellular motility. This protein mimics the host cell WASP protein and recruits components of the host cell cytoskeleton and a scaffold of actin filaments is built which propels the bacterium through the host cytoplasm. This can be visualised following acting staining of cells as the so called 'comet tail' structure (Cossart, 2000, Goldberg, 2001). Actin-based motility occurs randomly and can propel the cells towards the host cell membrane. As it is further propelled outwards, a pseudopodium is formed which is engulfed by an adjacent cell and the process of cell-to-cell spread is initiated (Kocks *et al.*, 1993, Gouin *et al.*, 2005).

After engulfment by the second cell, the bacterium is located inside a double membrane-bound vacuole. The *Listeria* cells then escape from the secondary vacuole by lysis vacuoles by the action of LLO, now with a second phospholipase, PlcB (lecithinase). Once they are released into the host cytoplasm a new pathogenic cycle of replication, actin polymerisation and spreading of bacteria to adjacent host cells occurs (Tilney and Portnoy, 1989, Stavru *et al.*, 2011). The *Listeria* cell-to-cell spread mechanism has also been shown to play an important role in crossing materno-foetal and blood-brain interfaces (Robbins *et al.*, 2010).

**Figure 1.2: Schematic representation of the cell infectious process by *Listeria monocytogenes***




The figure shows a summary of the major steps of the infection of *L. monocytogenes* into host cells from 1. Entry, 2.Lysis of vacuoles 3.Intracellularmovement 4.Cell-to-cell spread and 5.Lysis of the double membrane. The corresponding virulence factors associated with each step are indicated in blue (Tilney and Portnoy, 1989).

### 1.4.3 Regulation of the virulence factors

Most of the virulence factors involved in the *Listeria* infectious life cycle are regulated by a transcriptional pleiotropic regulatory factor (PrfA: see Fig. 1.3). Deletion mutants of *prfA* were shown to be non-pathogenic, highlighting its important role in regulating the virulence genes (Park *et al.*, 1992, Milenbachs *et al.*, 1997, Scortti *et al.*, 2007). The PrfA protein is a 237-residue 27 kDa protein, structurally related to enterobacterial regulator Crp (cAMP receptor protein or Cap). It was shown that PrfA exist in two functional states, the native weakly activated state and a highly active state after conformation (Ripio *et al.*, 1996). When cells are in the natural environment, PrfA exists in a low–activity state. The PrfA proteins become activated when bacteria are inside the host cells and levels of the protein also increase in response to changes in temperature and nutrients conditions, resulting in increased expression of the virulence factors in the regulon (Renzoni *et al.*, 1999).

The PrfA regulon system consists mainly of two main parts. First are the direct regulated genes, which is the core PrfA virulon. The first specific 9.3kb pregulon to be characterised, known as the LIPI-1 (*Listeria* pathogenicity island 1),contains the *prfA* gene itself and also 10virulence factors including *actA*, *hly*, *plcA*, *plcB* and *mpl* (a protein required for the processing of both ActA and PlcB; O'Neil *et al*., 2009). The PrfA protein is also known to regulate the *inlAB* operon as well as *inlC* locus. This is known to be the core PrfA direct regulated genes (Milohanic *et al.*, 2003). These genes were shown to be highly regulated by PrfA and were shown to have strong induction during intracellular infection and down-regulated during ex vivo growth. PrfA was shown to bind to a *PrfA* box with a canonical sequence TTAACANNTGTTAA

located at -14 position of the transcription start point of virulence (Kreft and Vázquez-Boland, 2001).

Indirect control of as many as 145 additional genes of EGD cells has also been demonstrated as PrfA associated expression by the use transcriptomic profiling technique. This indirect regulation pathway encodes proteins with various functions including enzymes, stress response, transport etc and only a few of these genes were found to have a putative PrfA box in the promoter region. One of the examples of it was the *bsh* gene that encodes the bile salt hydrolase in pathogenicity of *Listeria* cells (Dussurget *et al.*, 2002). They showed that there is a perfect palindromic sequence found 133bp upstream the bsh start codon which were shown to have only two mismatch with the PrfA box , suggesting it is a PrfA box regulated system.

The regulation of these systems is determined by the concentration of activated PrfA protein in cells, which in turn is controlled in different ways. Firstly, it was found that the *prfA* gene itself is regulated by two promoter ($P1_{prfA}$ and $P2_{prfA}$) located upstream the *prfA* gene. $P1_{prfA}$ directs a low level synthesis of *prfA* mRNA during normal growth condition. The 5'untranslated region of these transcripts form a secondary structure which acts as a temperature sensor. At low temperature (<30°C) the secondary structure prevents ribosomes binding and prevents initiation of protein translation. When the temperature rises to 37°C this causes a disruptions of mRNA secondary structure and allows translation of proteins to begin (Johansson *et al.*, 2002). These regulations allow cells to activate the *prfA* system under intracellular conditions. $P2_{prfA}$ is regulated by the SigB protein which is the stress response regulator. It was shown to be activated in stationary phase of

growth or under stress conditions and when induced leads to production of a shorter transcript that lacks the long 5' UTR and therefore translation from this transcript is not temperature regulated(Rauch *et al.*, 2005).

The third prompter that contributes to the production of PrfA protein is the *plcA* promoter, which contains a *prfA* box and is located in the *plcA* gene which is upstream the *prfA* gene. This provides feedback loop transcriptionally regulated expression of *prfA* from the *plcA-prfA* transcript. This allows a significant increase in the levels of the PrfA protein when the levels of activated PrfA in the cells are required. A disruption of *plcA-prfA* read-through transcript results in a *prfA* deletion phenotype, and complete avirulence of the mutated cells (Camilli *et al.*, 1993), presumably because levels of activated PrfA within the cells are not sufficient to induce expression of all of all the PrfA-controlled genes.

This relates to the third level of *prfA* control which is achieved through variation in the *prfA* boxes. Some boxes were shown to have variations in the sequence and bind PrfA protein less strongly. These require more PrfA within the cell to give the same level of activation as the promoters which contain perfect *prfA* boxes. This was originally described in $P_{act}$, which has one mismatch and $P_{inlAb}$ which as two mismatches (Bohne *et al.*, 1996). Hence it is seen that the PrfA system is highly regulated by a complex multi-layered system, indicating its importance in *L.monocytogenes* controlling expression of its virulence genes. This suggests that expression of the PrfA regulon is an adaptive event, with some cost to the cells being incurred if the genes are expressed at times when they are not required.

**Figure 1.3: The PrfA regulon**

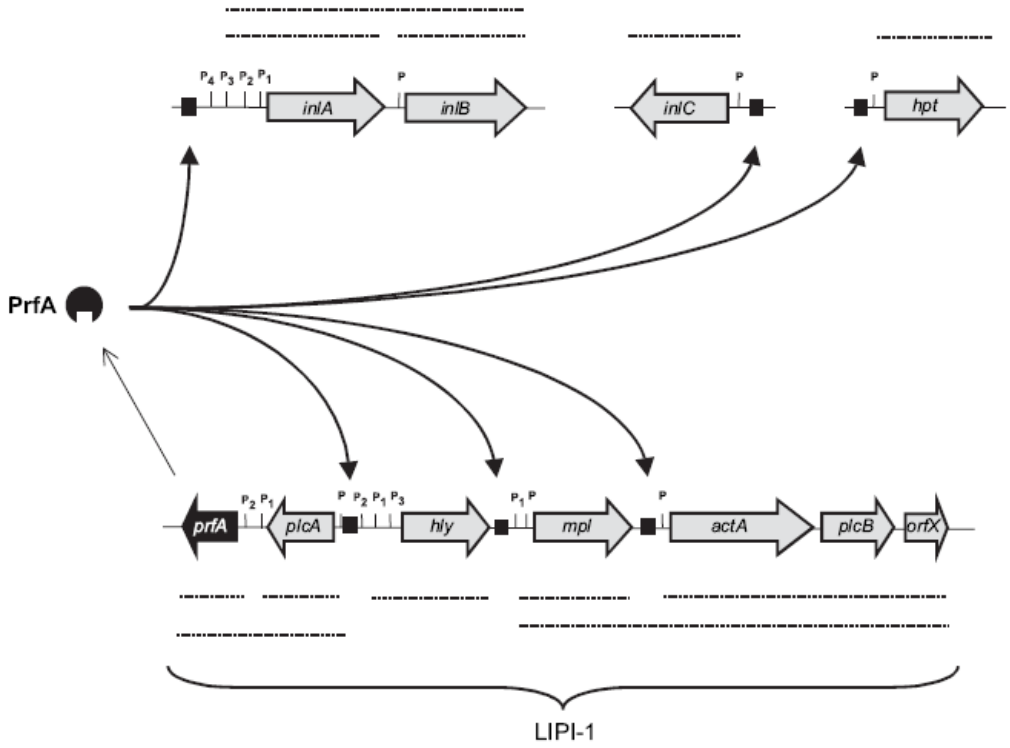



Diagram showing a summary of the PrfA regulation system. PrfA regulates the transcription of the *Listeria* pathogenicity island-1, *inlAB*, *inlC* and the *hp*t operons. Black boxes are indication of the location of the PrfA boxes with "P" indicating the promoter locations and the position and length of the transcripts produced is shown as in dotted lines. (Cossart and Lecuit, 1998, Scortti *et al.*, 2007)

### 1.5.1 Biofilms

When contamination of product with food borne pathogens has been associated with cross-contamination in food factories, there has often been a link made to biofilms formed on food production surfaces. Biofilms occur everywhere and they are defined as the microbial community that attaches to a surface that is then enclosed in hydrated extracellular polymeric substances which are known collectively as glycocalyx (Sandasi *et al.*, 2008). Biofilms are highly organised and are most often multi-species in nature, and the composition and structure varies between species and with the metabolic status of the cells. The biofilm structure is also greatly affected by many extrinsic factors such as temperature, pH, or many other growth conditions. Cells growing in biofilm have been shown to have different cellular physiology to planktonic cells. For instance, Trémoulet *et al.* (2002) studying the proteomic patterns of *L.monocytogenes* grown in biofilm or in planktonic mode, with statistical analysis of 2-D gels, have shown that the expression of at least 30 proteins was significantly affected following the change of condition from planktonic culture to biofilm cultures, including flagellin protein, superoxide dismutase and 30S ribosomal protein S2.

Biofilms acts as barriers which were shown to give protection to cells against a wide range of harmful substances, such as surfactants, antibiotics or detergents and also towards environmental stress like dehydration and heat (Watnick and Kolter, 2000). Belessi et al., (2011) studying the efficiency of different sanitation methods for treatment of *L. monocytogenes* biofilms have shown that the survival of the cells within the biofilm was dependent on the type, concentration and application time of the disinfectant used, as well as the physiological state of cells. This is one of the main concerns for food

production, as in this example microbes were shown to survive treatments used as part of common cleaning procedures established in laboratory tests using planktonic cells. The failure to fully inactivate biofilm cells would then increase the chance of cross-contamination from processing plants to food products which is a great food safety concern (Farber and Peterkin, 1991).

### 1.5.2 Formation of bacterial biofilm

Different stages have been identified in the development of a biofilm. Firstly, surface condition influences whether cells attach to a surface, whether it is a biotic or abiotic surface. Surfaces are conditioned by adsorption of organic and inorganic compounds that can act as nutrients or can just increase the ability of bacterial cells to adhere to a surface. This was shown for *Listeria* in an attachment experiment using conditioned or unconditioned surface, where the conditioning was achieved by submerging stainless steel surfaces in media before rinsing with PBS. Results shows that *Listeria* attached better to surfaces conditioned with RSM (reconstituted skim milk) with 1% sucrose than to a surface conditioned by exposure to TSB (Hood and Zottola, 1997). However it was also reported that skim milk conditioning reduces *Listeria* attachment to stainless steel (Barnes *et al.*, 1999). From this it is clear that the type of food materials of a food production line may have an effect on the biofilm formation on food production surfaces but that the effects of these are not fully understood.

The next step requires transportation of bacteria from the bulk fluid to the surface. This can occur due to cell motility, diffusion, sedimentation or natural Brownian motion of cells. Once the bacteria approach to the surface, initial attachment occurs. Attachment can be classified into reversible and

irreversible attachment. Reversible attachment is achieved through weak forces that may result in an equilibrium distribution of adhering and suspended cells, whereas irreversible attachment is a strong bond that is mediated either by proteins or expolymer. It was found in *Listeria* that irreversible attachment occurs at the very early stage of attachment (less than 5 min), suggesting a very fast transition from reversible to irreversible attachment once attachment occurred (Ute *et al.*, 2005).

Once attached to the surface the cells can grow and divide, so that the cell number starts to increase and microcolonies start for form on the surface. Further growth and maturation then leads to the formation of a thick, complex, well-built biofilm, often referred to as the mature biofilm. Cells will then start to detach from the mature biofilm by an actively regulated process, usually termed dispersion or dissolution, so that the released cells can colonise new environments. At this point the biofilm cycle will start over again when these dispersed cells attach to a new surface (Toole *et al.*, 2000, Abee *et al.*, 2011, Watnick and Kolter, 2000, Mclandsborough *et al.*, 2006).

**Figure 1.4: Diagram summarising the process of microbial biofilm formation**

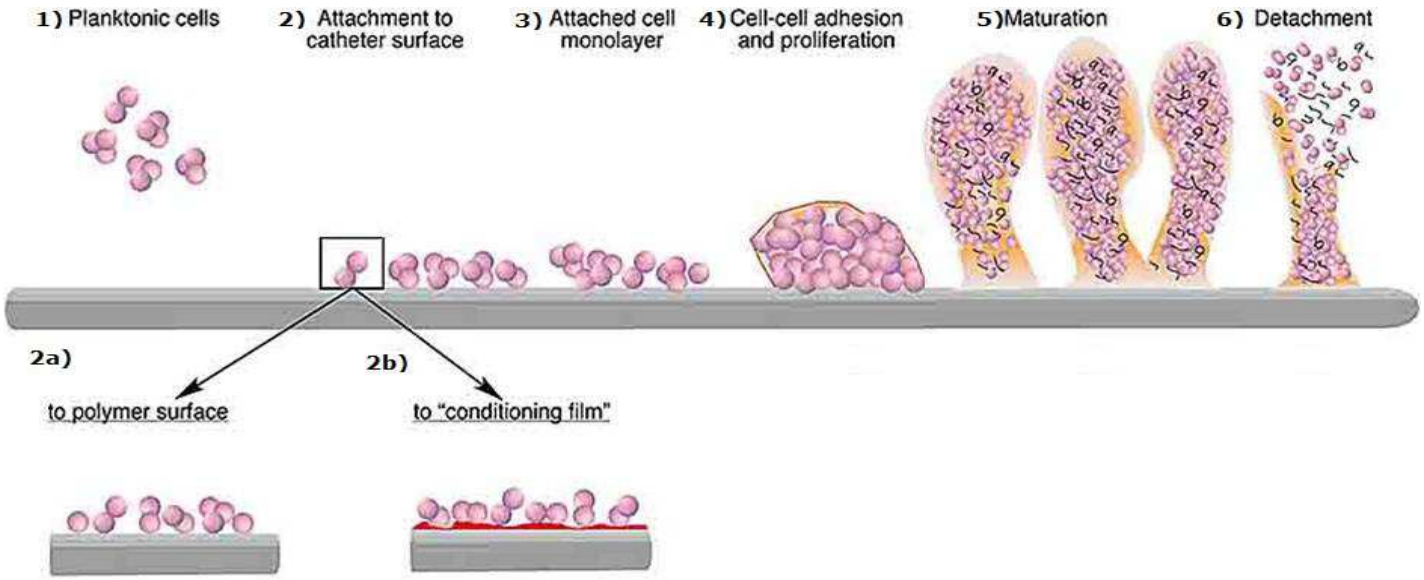



Diagram showing the steps in bacterial biofilm formation. From left to right as shown, 1) Planktonic cells dispersed in liquid, 2) cells attached to either a) normal surface or b) conditioned surface, 3) a monolayer of cells form on surface, 4) cells proliferate and interact, 5) biofilm maturation and 6) cell detachment to restart cycle. Diagram taken from Otto (2004)

### 1.5.3 Cell attachment and hydrophobicity

Clearly cell attachment is the early critical step of biofilm formation and therefore the ability of cells to attach to a particular surface has a great effect on biofilm formation. Many factors such as cell surface properties, surface conditioning, surface roughness and growth medium have been shown to greatly affect cell attachment ability (Palmer *et al.*, 2007). However, of these, one of the most important cell surface properties found to influence the attachment of bacteria cells to a surface is hydrophobicity. It has been shown in many cases that cells with higher surface hydrophobicity are better in adhesion to surfaces such as stainless steel or polystyrene and hence enhance biofilm formation (Poimenidou *et al.*, 2009). This is also the case in *L. monocytogenes* binding to surfaces such as PVC or glass (Takahashi *et al.*, 2010, Di Bonaventura *et al.*, 2008). *Listeria* cell surface hydrophobicity was shown to vary between strains, and has also been shown to be affected by different environmental conditions during growth, such as temperature or salt concentration (Briandet *et al.*, 1999). It is not surprising that the change in environmental condition is strongly linked to bacteria surface properties. One example of this in *L. monocytogenes* is the well characterised temperature regulation of PrfA (introduced earlier) which in turn induces the production of surface proteins which may causes a change in cell surface properties of cells under different environmental conditions (Scortti *et al.*, 2007).

Measurement of microbial cell surface hydrophobicity can be achieved using a wide range of techniques, for instance hydrophobic interaction chromatography (HIC) or contact angle measurement (CAM)

(Gallardo-Moreno *et al.*, 2011). One of the simplest methods used to estimate cell hydrophobicity is the microbial adhesion to hydrocarbons (MATH) assay which measures cell hydrophobicity by estimating the affinity of cells for different hydrocarbon solvents as an indication of the likelihood of cell being able to attach to hydrophobic substances. In this assay, the higher the number of cells that attach to (or partition into) the hydrophobic solvent, the higher the cell hydrophobicity is determined to be (Geertsema-Doornbusch *et al.*, 1993, Rosenberg, 2006).

*Listeria* is able to colonize and build biofilms on a wild range of surfaces. For instance it was shown in the work of Chavant *et al.*(2002) that *L. monocytogenes* biofilms on hydrophilic and hydrophobic surfaces formed differently at various growth temperatures. It have shown that at 20°C and 37°C, three dimensional biofilm structures were seen on both surfaces, however significant detachment were only seen from PTFE biofilms grown at 37°C. At 8°C a minor biofilm was seen on stainless steel surfaces but not on PTFE. This indicates that the nature of the attachment surface and the growth temperature have great influence on biofilm formation (Chavant *et al.*, 2002).

It was previously shown that flagellae are critical for *L. monocytogenes* initial attachment to stainless steel. Vatanyoopaisarn *et al.*(2000) showed that a *Listeria* flagella mutant at 22°C showed a 10-fold lower attachment ability than that of wild type cells. Under conditions that repressed flagella production (37°C), mutant and wild type strains showed no significant difference in attachment. From this they suggested that it is the flagella, but not the motility *per se* that were important for the early attachment (Vatanyoopaisarn *et al.*, 2000). However in contrast, a later study showed

that both flagellum-minus and paralyzed-flagellum mutants were both defective in cell attachment and biofilm formation at the early stage and suggested that it is the flagellar motility that is critical for initial attachment and biofilm formation of *L. monocytogenes* (Lemon *et al.*, 2007). The difference in the methodology in control of motility in the two publications, paralyzed-flagellum mutant vs. temperature control, may have caused the difference in the results obtained. In a more recent paper, it was shown that high salt concentrations (11% NaCl) caused a decrease in the adhesion ability of *Listeria* to surfaces, and this was shown to be correlated to the repression of flagella expression (Caly *et al.*, 2009). All these results indicate that flagella motility is in some way critical in the attachment and biofilm development by *L. monocytogenes*. However the specific role of such motility on attachment and biofilm formation is still uncertain.

### 1.5.4 Biofilms and *Listeria monocytogenes*

*Listeria* has been shown to be very persistent in the food process environment, and this has been related to its ability to form biofilms. It has been shown that *Listeria* biofilm can exist in a food processing environment for up to 10 years, especially in some difficult to access sites in the food production premises and equipment. Carpentier and Cerf (2011) recently reviewed the various published studies of persistent strains and concluded that *Listeria* in biofilms can be shown to be more resistant to disinfectants and sanitizing agents compared to planktonic cells, and this is attributed to the present of a surrounding matrix which provides protection and makes the bacterium even harder to remove from food processing environment. They also concluded that it had not been demonstrated that these persistent cells had a particular difference in physiology which made them intrinsically more

resistant towards stresses and chemicals. For example, one experiment reported had taken "persistent" strains and normal strains and compared their tolerance to Quaternary Ammonium Compounds(QAC) and it has shown that the persistent strain did not show better resistance to the disinfectant than that seen in laboratory adapted strains (Lundén *et al.*, 2003). Another publication has reported studying the attachment ability of persistent strains to surfaces where it was shown that the persistent strains were not better able to attach than the laboratory adapted strains on a long term basis (Lund *et al.*, 2000). From these reports it is clear that the enhancement in resistance to stress is based on the protection provided by the matrix of the biofilm. The review also concluded that some persistent strains occurred due their location in some harbourage site or niches, such as sharp turns in pipes or hard to access surfaces, which were therefore not well cleaned and it was this fact that made them "persist" in the food production line rather than an intrinsic property of the cell (Carpentier and Cerf, 2011).

However other researchers have focussed on the effect of strain variation on biofilm formation. Kalmokoff *et al*. (2001) studied the adsorption, attachment and biofilm formation of various *L. monocytogenes* strains and showed that there is great difference in attachment ability among different *Listeria* strains, and they found that there was no correlation between the adsorption ability and the serotype of the *Listeria* strain (Kalmokoff *et al.*, 2001). However another group studying biofilms showed that in *Listeria* Division II strains, 1/2a and 1/2 c serotypes, had an increased ability to form in biofilms. However these two serotypes are not commonly known to cause foodborne outbreaks of listeriosis suggesting that even if this is true, this does not translate into a significant risk for food safety. They also compared the biofilm formation ability of persistent and non-persistent strains of *Listeria*

isolated from bulk tank milk samples, showing that the persistent strains has a better biofilm than the non-persistent isolated, but in this case there was no significant correlations between this and the serotype of the strains tested (Borucki *et al.*, 2003).

In the natural environment, multispecies biofilms commonly occur. One of the studies working on the attachment of mixed culture of *Listeria* with other common food pathogens, *Flavobacterium,* has shown that there is enhancement in cell attachment to stainless steel comparing to a monoculture, and this work has also shown that *Listeria* cells are able to survive longer in a mixed culture (Bremer *et al.*, 2001). This suggests that the effect goes beyond the simple fact that the *Flavobacterium* in some way conditions the surface, facilitating the attachment of *Listeria*. Another experiment studying interactions of different resident microorganisms and *Listeria* in biofilms also showed that the biofilm formation ability of *Listeria* can be greatly affected by the co-existing microorganisms commonly found in food production lines, but that the majority of these reduced biofilm formation (Carpentier and Chassaing, 2004). For example, they found that 16 out of 29 bacterial cell types tested caused a reduction in *Listeria* biofilm formation and only four bacterial strains (*K. varians* CCL 73, *S. capitis* CCL 54, *S. maltophilia* CCL 47, and *C. testosteroni*) resulted in a positive enhancement of *Listeria* biofilm formation. In the real food processing environment it is more common to find a mixed culture biofilm (Costerton *et al.*, 1995), suggesting that the situation in real environments may be much more complicated than those tested to date under laboratory conditions.

It has been reported that *L.monocytogenes* produces different biofilm structures in the presence and absence of nutrient flow. Under static

conditions, the biofilms were shown to be less organized and formed into only a few multilayers. In contrast, flow conditions produced highly organized microcolonies in ball shapes which were surrounded by a network of knitted chains, giving bigger bio-volume and biofilm thickness (Rieu *et al.*, 2008). It was later shown that two genes, *recA* and *yneA*, which are both involved in the SOS responses in *Listeria* cells, were linked to the formation of these knitted biofilm structures. Mutants of these genes lost the ability to form these knitted chains seen when using the flow conditions. The SOS response is a conserved pathway that activates under stress conditions and this suggested there could be a link between genes induced by the SOS response and the formation of these knitted biofilm under the nutrient flow condition (Van Der Veen and Abee, 2010). This may suggest that there is a possibility that flow conditions may be perceived as a stress condition and may induce the SOS response of *Listeria* and hence causes the change in biofilm morphology.

A recent paper has also shown that extracellular DNA (eDNA) is needed for both initial attachment and early biofilm formation of *L.monocytogenes*. DNaseI treatment of *Listeria* cultures resulted in dispersal of biofilm structures under both static or flow conditions and also resulted in a reduction in cell adhesion. However, the function and the origin of these extracellular DNA is still unclear (Harmsen *et al.*, 2010).

### 1.6.1 Quorum sensing

Quorum sensing (QS) is described as the process where individual bacterial cells, or populations of bacteria, communicate with the use of signalling molecules (Fuqua and Winans, 1994, Rickard *et al.*, 2006). It was a great

discovery in the microbial world that bacteria are able to communicate with each other and produce physiological or behavioural changes in response to these chemicals. Significantly it was shown that expression of various virulence genes or stress response genes were regulated by quorum sensing in many bacteria (Smith *et al.*, 2004). *Vibrio harveyi* and *Vibrio fischeri* are both Gram-negative, free-living, marine bacteria and in the most classical example of quorum sensing, produce bioluminescence in response to the cell density of the population. It was shown that the cell density-dependent response is controlled by a regulation system based on the production and detection of certain small molecules which are now known as autoinducers (Bassler *et al.*, 1997).There are two main type of QS system among bacteria which are the LuxIR-type system found in Gram-negative bacteria and the two-component QS system in Gram-positive cells.

The LuxIR system of Gram-negative bacteria is a rather direct system, with LuxI producing the Acyl Homoserine Lactone family of molecules (AHLs) and these diffuse out of cells. The concentration of this signal increases when cell number increases and it then diffuses back into the cell and binds to the cytoplasmic regulator LuxR. This complex then binds to sequences in the upstream of the *luxCDABE* operon and activates gene expression. The LuxR-AHL complex will also activate the expression of *luxI* to induce more autoinducer production, and hence creates a positive feedback for a fast transition for adaptation to high cell density environments. AHL autoinducers are rather unique among species suggesting it is more likely it is used for intra-species communication rather than wider bacterial communication (Williams, 2007).

The two-component system of Gram-positive bacteria consists of a membrane-bound histidine kinase receptor and a cognate cytoplasmic response regulator. The cells produce modified oligopeptides as the signalling molecules and when these are recognised by the cells, intrinsic autophosphorylation activity of a membrane bounding receptor is stimulated. This transfers a phosphate group to a cognate response regulator, which will then function as a DNA binding transcription factors to control gene expression (Pestova *et al.*, 1996, Peterson *et al.*, 2000).

As studies on *V. harveyi* progressed, it was shown to have a hybrid of these two QS systems. In this organism it is now known that there are three main groups of autoinducers (HAI-1, AI-2 and CAI-1) which are regulated by three Lux systems (see Fig. 1.5). HAI-1 is produced by the autoinducer synthase, LuxM and is detected by the LuxN histidine kinases. The HAI-1 system functions as the LuxI-type protein originally identified in the Gram-negative system. AI-2 molecules are synthesized by enzyme LuxS and detected via the complex of LuxP (a periplasmic protein) and LuxQ (a Histidine kinase). CAI-1 is produced by CqsA synthase and is detected by CqsS histidine kinase.

The LuxN, LuxPQ and CqaS act as both kinases and phosphatases and regulate the activity of LuxU and LuxO regulators in responses to external autoinducer levels (Ng and Bassler, 2009). Under low cell density conditions, the kinase activities become predominant and this results in the phosphorylation of a histidine residue. Phosphates from the three receptors are transferred to a single phosphotransfer protein LuxU which then further transfers it to LuxO. LuxO is a functional transcriptional activator once it is phosphorylated and activates the transcription of five regulatory RNAs (Qrr1-5 sRNAs). The target for the Qrrs RNAs is the mRNA that encodes the

LuxR protein, the transcription regulator of *luxCDABE* operon. Under low cell density (LCD; Fig. 1.5) conditions, LuxR is not produced and hence *lux* expression is not active and no bioluminescence is produced by the cells.

When cell density increases (High Cell Density; HCD, Fig. 1.5), there is an increase in autoinducer concentration in the surrounding environment, and autoinducer molecules will bind to their cognate receptors. This will switch the receptors from kinases to phosphatases. This causes a reversion of phosphate flow and causes a dephosphorylation of LuxO. Under these conditions QrrsRNAs would not be made and LuxR synthesis is not interrupted, and hence expression of the *lux* operon is induced and bioluminescence is produced. It was also seen that QS in *V. harveyi* regulates metalloprotease production as well as represses TypeIII secretion systems (Ng and Bassler, 2009), suggesting the wider importance of QS in the species.

**Figure1.5: The Quorum sensing systems in *Vibrio***

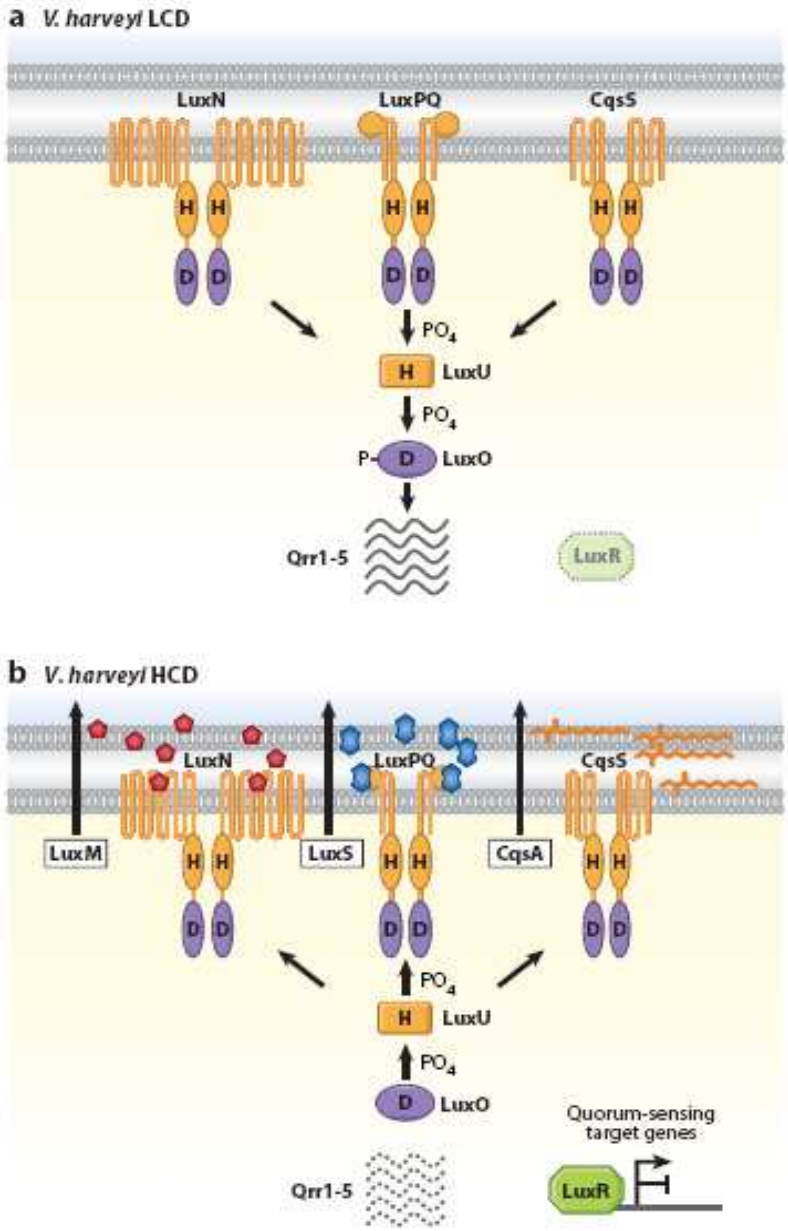


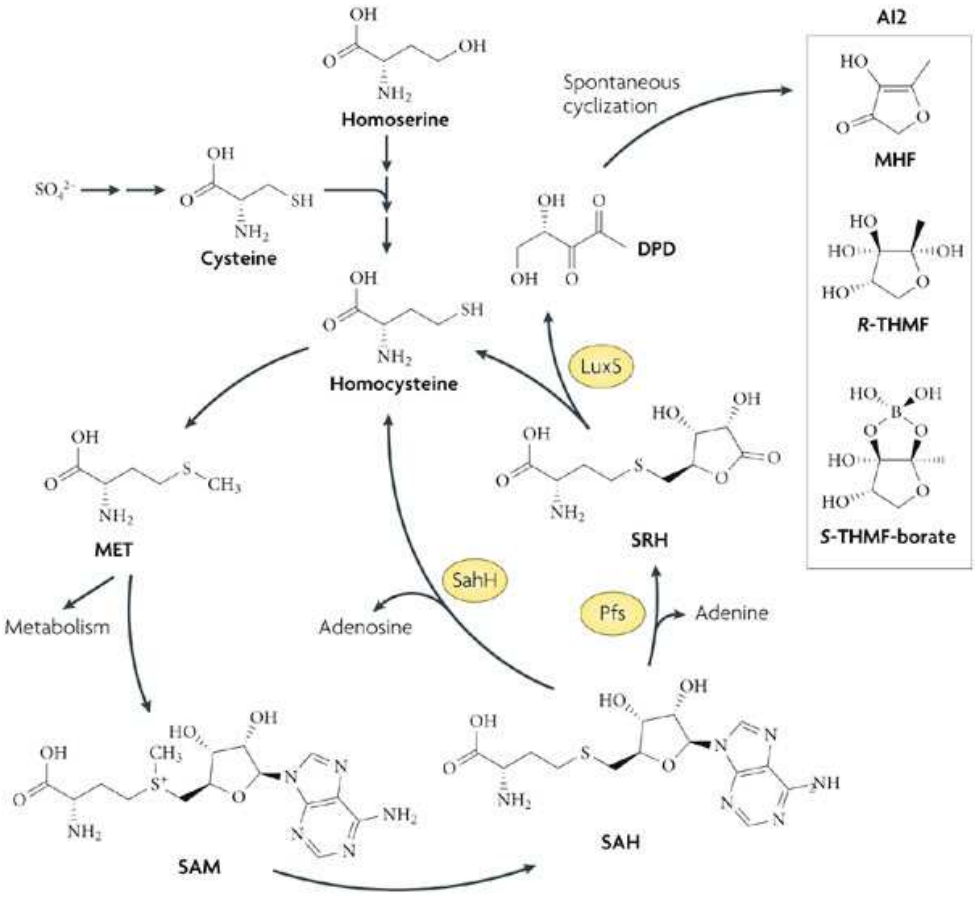

Diagram of the HAI-1, CAI-1 and AI-2 QS systems in *Vibrio* under *a*) low cell density (LCD) and b) high cell density (HCD) conditions. At LCD, a phosphate flows downwards to LuxO and causes transcription of Qrr1-5 sRNAs to interrupt LuxR synthesis. At HCD, phosphate flow is reversed. Production of Qrrl-5 sRNAs is inhibited, and LuxR is produced to induce Lux operon expression (Ng and Bassler, 2009).

Bassler *et al*. (1997) have been studying the cross-species induction of bioluminescence from *Vibrio* with the use of mutant strains that can act as biosensors. It allows the measurement of AI-2 molecule production by different species and is known as the AI-2 bioassay. The strains used are termed BB170 (AI-1 sensor$^-$) and BB886 (sensor AI-2$^-$). Using these biosensors and samples of cell-free cultures prepared from various bacteria species, it was found that AI-1 molecules are specific to *Vibrio* species, and therefore the it is suggested that this is the intra-species communicating system, whereas AI-2 system (detected by BB170) was found to respond to wide range of Gram-positive and Gram-negative bacteria and is therefore more likely to be used for inter-species communication. This communication system was also found in *L. monocytogenes* (Bassler *et al.*, 1997, Ng and Bassler, 2009).

### 1.6.2 AI-2 and LuxS

To date AI-2 (autoinducer-2) is known to be produced by over 100 species of bacteria (Hardie and Heurlier, 2008, Ng and Bassler, 2009). The production of AI-2 originates from the activated methyl cycle (AMC) which is the metabolic pathway required for methionine biosynthesis. It is started by the methyltransferase-catalysed cleavage of the methyl group of SAM. This produces S-adenosylhomocysteine (SAH) as a toxic by-product of the metabolic pathway. A Pfs nucleosidase will cleave the adenine from SAH to a non-toxic form, S-ribosylhomocysteine (SRH). A by-pass metabolism converts SRH to 4,5-dihydroxy- 2,3-pentanedione (DPD) and homocysteine with the help of the autoinducer synthase enzyme LuxS (Hardie and Heurlier, 2008). LuxS proteins are found to be highly conserved across many different types of bacteria, indicating that they may have a common function in these different species (Bassler *et al.*, 1997). DPD is the precursor for AI-2 synthesis (Fig.1.6), and the AI-2 that are produced are most often mixtures of similar molecules. These are then released from the cells and the extracellular level is therefore dependent on population density (Hardie and Heurlier, 2008)

**Figure 1.6: The SAM cycle and the formation of AI-2**




The diagram showed the SAM cycle and the metabolism of AI-2 production.It started by the methyltransferase catalysed the cleavage of the methyl group of SAM to give SAH. A Pfs nucleosidase will cleave the adenine from SAH to a non-toxic form S-ribosylhomocysteine (SRH). A by-pass metabolism converts SRH to 4,5-dihydroxy- 2,3-pentanedione (DPD) and homocysteine catalysed by LuxS. DPD acts as a precursor of AI-2. (Hardie and Heurlier, 2008)

### 1.6.4 Quorum Sensing and Biofilm Formation

The regulation of the process of biofilm formation is unclear in many ways, and it would be useful to have a better understanding of this mechanisms for medical, environmental and food protection applications. It has been suggested that quorum sensing is involved in the establishment and growth of biofilms of many bacteria, with increases in the levels of signalling molecules occurring due to accumulation in the biofilm matrix. This causes a change in physiological state of bacterial cells during the different stages of bacterial biofilm formation (Asad and Opal, 2008).

However, in *L. monocytogenes* no direct and specific evidence has been produced to support the apparent correlation between quorum sensing and biofilm formation. Various groups have been working on *luxS* mutants of different bacteria, including *L. monocytogenes (Lyon et al., 2001, Schneider et al., 2002).* A number of studies have shown that biofilm formation is highly linked with LuxS and AI-2 production in *Listeria*. Sela *et al.* (2006) working with a *luxS* mutant of *L. monocytogenes*, showed that detectable levels of AI-2 were diminished greatly showing that this gene was responsible for producing an AI-2 molecule. They also showed that the *luxS* mutants were able to build up thicker and denser biofilms and hence making cells more firmly attached to surfaces, and therefore more difficult to remove. From these observations they concluded that AI-2 inhibits biofilm formation in *L. monocytogenes,* which is in turn controlled by LuxS. However, addition of exogenous DPD did not restore the original biofilm phenotype and the reason for this is still unclear (Sela *et al.*, 2006). It was later shown in a study of biofilm formation by *luxS* mutants of *S. oralis* and *S. gordonii* that AI-2 complementation only restored wild type biofilm formation under a particular

low concentration (1nM AI-2). This suggests that there could be a chance that the concentration of AI-2 is a critical factor in the regulation system (Challan Belval *et al.*, 2006).

In contrast, other research groups have identified a reduction in biofilm formation by *luxS* mutants of other species such as *Bacillus subtilis*, *Streptococcus mutans* and *Salmonella typhimurium* (Lombardia *et al.*, 2006, De Keersmaecker *et al* 2005; Merritt *et al.*, 2003). In other cases, such as that found in *E. coli* and *S. aureus*, *luxS* mutants showed no changes in biofilm formation compared to the wild type strains (Doherty *et al.*, 2006; Beloin *et al.*, 2006). However, uniformity is low between these data sets, as different methodologies were used by different research groups in most cases. Differences in methodologies were seen in the choice of media, time of biofilm incubation, flow conditions, and all these are factors that may cause physiological changes to bacteria. In addition different methods of biofilm assessment were used and this makes direct comparison between the different publications rather hard.

From all the evidence above, it is clear that the AI-2 signalling is important for different aspects of cell physiology among different species. With the limited knowledge and techniques available on the AI-2 system at the present, it is hard to draw strict conclusion from the experiments described, but it is clear that more understanding of the AI-2 system of *Listeria* is required to try and answer some of the current conflicting ideas reported in the literature.

## 1.7 Extracellular polymeric substances (EPS)

### 1.7.1 General background

Unfortunately "EPS" has been used by different research group as abbreviation for "extracellular polysaccharides", "exopolysaccharides, and "extracellular polymeric substances" or "exopolymeric substances". This makes the literature sometime confusing, and sometimes makes it difficult to understand exactly what is being studied. Extracellular polymeric substances (EPS) are organic macromolecules produced by microbes and are formed by polymerization of one or more similar building blocks or different repeating units (Wingender *et al.*, 1999). However EPS may also contain non-polymeric substituent as components such as organic groups acetyl, succinyl and inorganic groups such as sulphate. Great variations in structure and forms of EPS have been described in different bacteria, including compounds or mixtures of polysaccharides, amino acids, nucleic acids, and even lipids (Liu and Fang, 2002, Platt *et al.*, 1985, Azeredo *et al.*, 1999). So in this thesis, EPS is used for "extracellular polymeric substance", which was suggested by Wingender (1999) as a more comprehensive and general term to use in the study of EPS. This would also suit better in EPS study of *L. monocytogenes* as the exact content and composition of the extracellular substance is still unclear.

### 1.7.2 Formation of EPS

EPS, by definition, is located at the cell surface of, or outside and detached from, bacteria and can fill the space between cells existing in a colony. EPS may be produced in the results of different cellular process, but are produced through specific biosynthetic pathways and are then exported and actively

translocated to the cell surface. It can also be produced by spontaneous liberation of integral cellular components, which is commonly seen in Gram-negative bacteria and different mechanisms to produce EPS are adopted by different bacterial species (Wingender *et al.*, 1999, Nielsen *et al.*, 1997, Flemming *et al.*, 2007, Marvasi *et al.*, 2010).

### 1.7.3 Function and importance of EPS

The presence of EPS has been shown to cause alternation in the surface properties of bacterial cells, such as zeta potential and cell hydrophobicity (Tsuneda *et al.*, 2003). It was shown that EPS participates in the formation of microbial aggregates, bridging and binding of cells, in the formation of biofloc in a culture media and in biofilm formation on surfaces (Mcswain *et al.*, 2005, Neu and Marshall, 1990). Tsuneda *et al*. (2003) have been studying the effect of EPS on attachment of 27 heterotrophic bacterial strains isolated from wastewater. They compared the attachment ability and zeta potential of EPS-rich and EPS-poor bacteria strains, with or without the addition of the supplements hexose and pentose to the growth media. They have shown that when EPS production is low, cell adhesion on solid surfaces is inhibited by the *electrostatic interaction,* and when EPS production is profuse, cell adhesion is enhanced by *polymeric interactions.* It has been suggested that EPS enhances cell adhesion to surfaces by polymeric interaction which can reduce the attachment inhibition of electrostatic interaction (Tsuneda *et al.*, 2003, Allison and Sutherland, 1987). EPS can also serve as a structural component of a microbial biofilm (Cammarota and Sant'anna, 1998, Costerton *et al.*, 1995). It was shown that *B. subtilis* produces extracellular EPS during biofilm formation. Mutations on EPS production genes have shown severe effect on biofilm formation, suggesting EPS is important to biofilm formation and

structure (Branda *et al.*, 2006).

In addition EPS is known to be a virulence factor in the infectious process, in this case protecting the bacterial cell from host defense system and also enhancing attachment to host cells (Roberts, 1996, Costerton *et al.*, 1999). EPS has also been seen to give protection against biocides to cells in natural environments by acting as a barrier (Stewart and William Costerton, 2001, Costerton *et al.*, 1981). In an antibiotic sensitivity assay of *Pseudomonas aeruginosa* in an extracellular polysaccharide matrix, it was shown that the minimal inhibitory concentration and minimal bactericidal concentration of antibiotics to bacteria surrounded by EPS may be up to 100–1000-fold higher compared with that for planktonic bacteria (Høiby *et al.*, 2010). The EPS can also help with water retention to prevent desiccation of cells under water stress condition. It was shown that bacterial exopolysaccharide in biofilms holds water like sponge and hydrogel to reduce evaporation rate (Flemming *et al.*, 2007). Hence it is clear that the presence of EPS on the surface of bacterial cells has great biological importance for bacterial survival and virulence.

### 1.7.4 Poly-gamma-glutamate

One of the most common examples of non-polysaccharide EPS is the poly-gamma glutamate (PGA) produced by the *Bacillus* species (Candela and Fouet, 2006). It is a polyamide produced by amide linkage in the polymer backbone. Poly-γ-glutamate (PGA) are produced by a number of bacteria, most of which are Gram-positive. It was first described in *Bacillus anthracis*, which were shown to form capsules composed solely of PGA, which in turn was shown to be a virulence factor for this organism (Bajaj and Singhal, 2011). PGA capsules were also found to provide protection of *B. anthracis*

against phage attack. This indicates the biological roles of PGA are quite diverse.

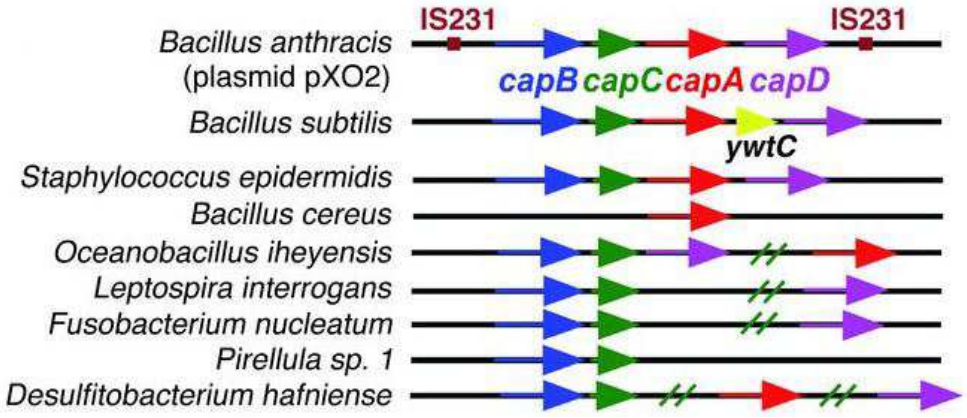
The genes associated with PGA formation have been identified, in a number of organisms and two nomenclatures have been adopted, being the *cap* (capsule) and the *pgs* (polyglutamate synthase). The classification of genes as either *cap* or *pgs* depends on the role identified for the PGA. The term *cap* is used when capsule formation is seen, in other words, the PGA is surface attached and forms a defined capsule layer surrounding the cell. The term *pgs* is used when the PGA is found to be released from the cell surface to form a more diffuse slime layer (Marvasi *et al.*, 2010). Since the *cap* genes are associated with *B. anthracis* virulence, these have been particularly well studied. The *cap* operon consists of 5 core genes, *capABCDE*. The genes *capB and capC* are known to encode the PGA synthase, whereas *capA and capE* are required for transport of the PGA out of the cell cytoplasm. The gene *capD* encodes the glutamyl-transpeptidase, which has a main role in PGA anchorage. The exact role of each of these is still under investigation (Candela and Fouet, 2006).

### 1.7.5 Distribution of *Cap* genes in bacteria

The organisation and complement of *cap* genes have not always been found to be the same in the different bacteria studied to date and only some species contain a full set of the cap biosynthetic genes (Kocianova *et al.*, 2005). In some organisms, such as *Bacillus cereus,* only the *capA* gene is present (Fig. 1.8) which is theoretically not sufficient for PGA production as CapA is only known to function as a transporter in the PGA synthesis system. However these bacteria have been shown to produce an EPS material (Vilain *et al.*, 2009). In this case either there is a separate gene for EPS formation that has

not been identified, or CapA may still act as an EPS formation gene in these situations.

**Figure1.7: Distributionof *cap* gene in various bacteria**




The diagram shows the orientation of *cap* genes in different species. It shows the *capBCAD* operon of *B. anthracis* compared to other species having different orientation of the *cap* genes or even *cap* genes found at separated locations (Kocianova *et al.*, 2005).

In *L. monocytogenes* an isolated *capA* homologue has also been reported by Begley *et al.*(2002) working on bile resistance of *L. monocytogenes*. They identified random transposon mutants of *L. monocytogenes* strain *LO28* with altered resistance towards bile. It was found that a one of these transposon mutations was located in a *capA* gene homologue (*lmo0516*). This indicates that *lmo0516* may be is responsible for a product that either detoxifies the bile or for producing a protective substance against bile, which could be EPS. The protective effect of EPS against bile was seen in *Bifidobacterium* where is was shown that cells with EPS had a better resistance to bile and low pH, suggesting the function of EPS against the antimicrobial action of bile (Alp

and Aslim, 2010). It was also seen in *Lactobacillus* that bile was shown to causes increase in hydrophobicity of cells and also increase in biofilm formation, which suggest that EPS production may be an adaptive cell response towards exposure to bile (Burns *et al.*, 2011). Interestingly another research group reported that the expression levels of *lmo0516* were enhanced during cell invasion process (Camejo *et al.*, 2009), suggesting the possibility that *lmo0516* may be associated with the of virulence or intracellular adaptation genes.

### 1.7.6 Evidence for EPS production in *L.monocytogenes*

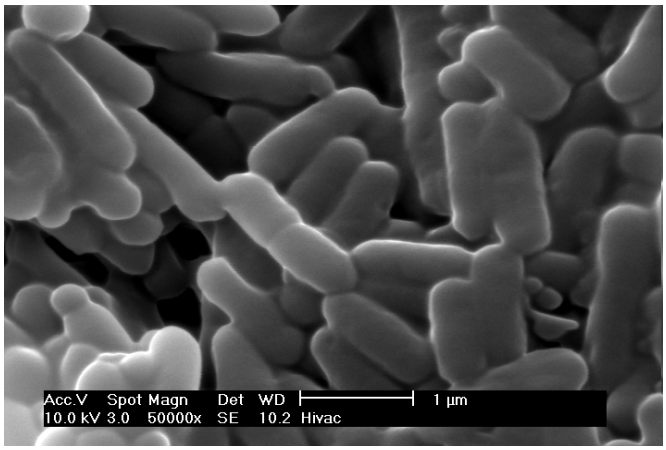
It has long been suggested that *L. monocytogenes* do not produce EPS capsules. However one particular old paper from Smith and Metzger did indicate that a *Listeria* capsule could be seen in TEM images. However, this finding was later challenged on the grounds of the quality of the images (Smith and Metzger, 1962, Edwards and Stevens, 1963). In our research group we have recently identified certain extracellular polymeric substance which was thought to be similar to PGA (Poly-γ-glutamate) because it gave a characteristic appearance after staining with Giemsa stain which is used for the identification of *B. anthracis* capsules (Nwaiwu, 2010). It was also shown that *Listeria* grown in a minimal medium (D10 and MCDB202) produced more capsule-like material than those grown in a rich media (BHI) when imaged using SEM (Fig. 1.7). From this image it can been seen that *Listeria* cells grown in BHI appears as separate, single rods showing little evidence of EPS formation, whereas the cells grown in minimal media are surrounded by a visible coating of EPS, and also are surrounded by strings-like structure spanning between cells. This work provided the first conclusive evidence of the production of EPS by *Listeria*. Although this is contradictory to many previous studies of *Listeria* cell surfaces, most of the experiments described

were performed under rich nutrient conditions and SEM images were usually taken of rapidly growing cells.

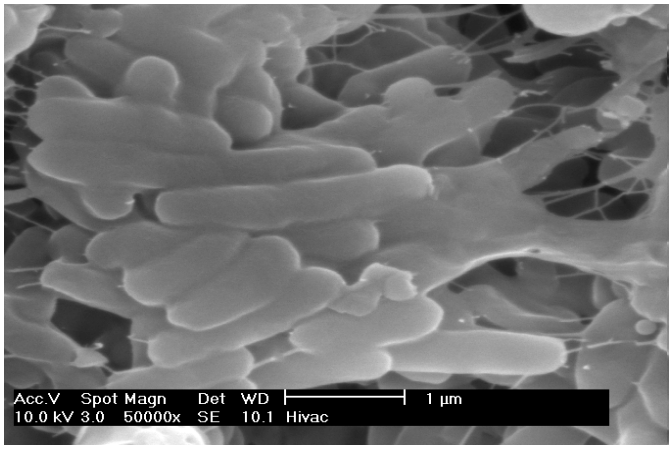
It was found from the genome sequences that no known homologues of the polysaccharide biosynthesis genes were present in *Listeria* species, suggesting it is very unlikely that the EPS detected is sugar based in nature. With the discovery of *capA* homologue *lmo0516* in *L. monocytogenes* cells, and the similarity in the staining properties of *Listeria* EPS and PGA, it was thought that there could be a linkage between the presence of these *capA* genes homologues and the EPS formation seen in *Listeria* and this idea formed the basis of this study.

**Figure 1.8: Evidence of EPS production by *L. monocytogenes***

A)




B)




SEM images of *L. monocytogenes* cells after growing in (a) BHI or (b) MCDB202 liquid culture in testtube at 37°C overnight. Cells grown in defined media shows a coated structure with more string like structures present connecting the cells (Nwaiwu, 2010).

## 1.8 Aims and Objectives

This research project was initiated on the evidence gained for the production of an extracellular polymeric substance by *Listeria* by a previous PhD student in the research group. The main focus of this work would be to try and understand the physiological role and genetic basis of EPS formation.

One of the first aims was to have a better understand what conditions induce EPS production and this would further lead on to determine if there is a linkage between Quorum sensing and the EPS formation.

A second aim was to test if the extracellular polymeric substance would cause a change in cell surface properties and cell physiology.

Finally, given the apparent similarity in the staining of PGA and the *Listeria* EPS, and the presence of a *capA* gene homologue in the *Listeria* genome, it was decided to investigate whether there is a linkage between the EPS production and the *capA* homologues by taking both a bioinformatics approach and investigating levels of gene expression.

# Chapter 2

# Materials and Methods

## 2.1 Media or agar

Media and solutions were prepared with laboratory fitted reverse osmosis water (RO water), which was then sterilized by autoclaving at 121 °C and 15 p.s.i for 20 min or filtered (Minisart, High Flow) depending on media characteristics.

### 2.1.1 Brain Heart infusion (BHI) broth

BHI powder (Oxoid) was added to RO water (37 g to 1 L) and autoclaved.

### 2.1.2 Brain Heart infusion (BHI) Agar

BHI powder (Oxoid) was added to RO water (37 g to 1 L), supplemented with agar powder (Oxoid) at 1.5 % (w/v) and autoclaved. It was then poured into Petri dishes with a volume of approximately 25 ml per plate.

### 2.1.3 BHI-Sucrose broth

BHI powder (Oxoid) was added to RO water (37 g to 1 L) and supplemented with 0.5 M sucrose (BDH) autoclaved at 10 psi for 15 min.

### 2.1.4 Luria Broth (LB)

15 g Formulated LB powder (Fisher Scientific Bioreagents) was added to RO water and was autoclaved.

### 2.1.5 Luria Broth (LB) agar

15 g Formulated LB powder (Fisher Scientific Bioreagents) was added to RO water and supplemented with agar powder (Oxoid) at 1.5 % (w/v) was autoclaved. It was then poured into Petri dishes with a volume of approximately 25 ml per plate.

### 2.1.6 Autoinducer Bioassay (AB) Media

17.5 g of NaCl, 6.02 g of $MgSO_4$ and 2 g of vitamin-free casamino acids were added to 1 L of RO water for form the media base. It was then adjusted to pH 7.5 with KOH and autoclaved. Filter sterilized 1 M of phosphate buffer (pH7; 10 ml), filter sterilized 0.1M L-arginine (10 ml) and autoclaved 50 % (v/v) glycerol (20 ml) were added to 160 ml of the media base. The media is used within a week.

### 2.1.7 2X YT Medium

Tryptone (16 g; Fisher Scientific), yeast extract (10 g; Fisher Scientific) and NaCl (5 g; Fisher Scientific) were dissolved in 1 L of RO water and the broth adjusted to pH 7.2 before sterilising by autoclaving.

### 2.1.8 Dulbecco's Modified Eagle Medium (DMEM)

Pre-prepared DMEM media (Gibco) was supplemented with 10 % (v/v) Fetal Bovine Serum (Gibco), non-essential amino acids (Gibco)( 100X - Glycine 750 mg/L, L-Alanine 890 mg/L, L-Asparagine1320 mg/L,L-Aspartic acid 1330 mg/L, L-Glutamic Acid mg/L, 1740, L-Serine 1050, L-Proline1150 mg/L) The media was prepared either with or without Penicillin/Streptomycin (Gibco) at 50$\mu g ml^{-1}$

### 2.1.9 Chick Fibroblast Basal Media 202 (MCDB 202)

MCDB 202 is a defined media and was prepared as described by Chavant *et al*. (2002) by dissolving 9.877 g of MCDB 202 media (US Biological) in 1 L of RO water with addition of 1 % (w/v) yeast nitrogen base without amino acid (Difco) and 3.6 g of glucose (Fisher Scientific). The solution was then filter sterilized using a 0.2 µm pore size filter (Minisart).

Table 2.1 Components of commercial MCD B202

**Chick Fibroblast Basal Medium MCDB 202**

| Inorganic Salts: | mg $L^{-1}$ |
|---|---|
| Ammonium Metavandate | 0.000585 |
| Ammonium Molybdate | 0.0012359 |
| Calcium Chloride•2H20 | 22 |
| Cupric Sulfate •5H20 | 0.0002497 |
| Ferric Nitrate•9H20 | 1.39 |
| Magnesium Sulfate | 180.57 |
| Manganese Sulfate•5H2O | 0.0001205 |
| Nickel Chloride Hexahydrate | $1.1885X10^{-6}$ |
| Potassium Chloride | 186.25 |
| Potassium Phosphate Mono | 68.05 |
| Sodium Chloride | 7183.2 |
| Sodium Metasilicate•9H2O | 0.1421 |
| Sodium Phosphate Dibasic | 71.05 |
| Sodium Selenite | 0.00789 |
| Stannous Chloride Dihydrate | 0.000001128 |
| Zinc Sulfate•7H2O | 0.02875 |

| Amino Acids: | mg $L^{-1}$ |
|---|---|
| L-Alanine | 8.9 |
| L-Arginine | 52.26 |
| L-Asparagine | 132.1 |
| L-Aspartic Acid | 13.31 |
| L-Cysteine | 24.44 |
| L-Glutamic Acid | 14.71 |
| L-Glutamine | 146 |
| Glycine | 7.51 |
| L-Histidine | 15.52 |
| L-Isoleucine | 13.12 |
| L-Leucine | 39.36 |
| L-Lysine | 29.24 |

| Amino Acids (Cont.): | mg $L^{-1}$ |
|---|---|
| L-Methionine | 4.48 |
| L-Phenylalanine | 4.96 |
| L-Proline | 5.76 |
| L-Serine | 31.53 |
| L-Threonine | 35.73 |
| L-Tryptophan | 6.13 |
| L-Tyrosine | 9.06 |
| L-Valine | 35.16 |

| Vitamins: | mg $L^{-1}$ |
|---|---|
| Biotin | 0.00733 |
| Choline Chloride | 13.96 |
| Vitamin B12 | 0.1355 |
| Folinic Acid, Calcium | 0.0006016 |
| Myo-Inositol | 18.02 |
| Nicotinamide | 6.1 |
| D-Pantothenic Acid•Ca | 0.2383 |
| Pyridoxine•HCl | 0.0616 |
| Riboflavin | 0.1129 |
| Thiamine•HCl | 0.337 |

| Other: | mg $L^{-1}$ |
|---|---|
| Adenine | 0.135 |
| Thymidine | 0.07266 |
| D-Glucose | 1440 |
| HEPES Free Acid | 7149 |
| Linoleic Acid | 0.0561 |
| Lipoic Acid | 0.00206 |
| Phenol Red, Sodium | 1.242 |
| Putrescine•2HCl | 0.0001611 |
| Sodium Pyruvate | 55 |

## 2.2 Buffers and Solutions

### 2.2.1 TAE (Tris-acetate-EDTA) buffer

50 X TAE buffer was prepared with 242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL of 500 mM EDTA (pH 8.0) solution in 1L RO water.

### 2.2.2 Phosphate Buffered saline (PBS)

One Phosphate Buffered saline tablet (Sigma) was dissolved in 100 ml RO water. The solution was then autoclaved and stored at room temperature.

### 2.2.3 HEPES/Sucrose Solution

HEPES (0.238g; Sigma) was dissolved in 1 L RO water and supplemented with 0.5 M sucrose (BDH) and autoclaved at 10 psi for 15 min. The final concentration of the components was 1 mM Hepes, 0.5 M sucrose.

### 2.2.4 RF1

Rubidium chloride (12.1 g; Fisher Scientific), manganese chloride (9.895 g of Fisher Scientific), potassium acetate (2.944 g; Fisher Scientific), calcium chloride (1.47 g Fisher Scientific) and 150 g of glycerol (Courtin and Warner Ltd) were dissolved in 1 L of RO water and adjusted to pH 5.8. The solution was filter sterilized using a Minisart 0.2 µm filter.

### 2.2.5 RF2

MOPS (1.05 g; Fisher Scientific Bioreagents), rubidium chloride (0.6g; Fisher Scientific), Calcium Chloride (5.51 g; Fisher Scientific) and glycerol (75 g; Courtin and Warner Ltd) were dissolved in 500 ml of RO water and filter sterilized using a Minisart 0.2 µm filter.

### 2.2.6 MATH NaCl Buffer

NaCl (8.76 g; Fisher Scientific) was added to 1 L of RO water to give a final concentration of 0.15 M and was sterilised by autoclaving.

### 2.2.7 Maximum Recovery Diluent (MRD)

9.5 g of formulated MRD powder (1g peptone, 8.5g NaCl, Oxoid) was added to 1 L of RO water and was autoclaved.

### 2.2.8 Triton-X

Triton-X-100 (50 mg; Sigma-Aldrich) was dissolved in 10 ml of RO water making up to a concentration of 0.5 % (w/v). The solution was filter sterilized using a Minisart 0.2 µm filter

### 2.2.9 SOC

20g Bacto Tryptone,5g Bacto Yeast Extract 2ml of 5M NaCl.2.5ml of 1M KCl.10ml of 1M $MgCl_2$ 10ml of 1M $MgSO_4$ 20ml of 1M glucose, were dissolved in 1L of distilled $H_2O$ and autoclaved.

### 2.2.10 Lambda Buffer

6mM Tris-HCl (pH 7.6), 10 mM MgSO4 and 0.05% (w/v) Gelatin were dissolved in RO water and autoclaved.

## 2.3 Preparation of Antibiotics and antimicrobial solutions

### 2.3.1 Antibiotics

Antibiotics were prepared by dissolving the desiccated antibiotics in the solvent at a stock concentration. They were filter sterilised using a Minisart 0.2 µm filter and stored at -20 °C for further use.

Table 2.2 Antibiotics used in thesis

| **Antibiotics** | **Solvent** | **Stock Conc.** |
|---|---|---|
| Ampicillin | RO water | 50 mg $ml^{-1}$ |
| Erythromycin | 70% ethanol | 50 mg $ml^{-1}$ |
| Kanamycin | 70% ethanol | 75 mg $ml^{-1}$ |

### 2.3.2 Nisin

A stock solution of Nisin (Danisco, Aplin and Barrett) was prepared at 10 mg $ml^{-1}$ by dissolving in 0.002 M HCL and buffered to pH 3.0 with 1 M NaOH. The solution was then filter sterilized using a Minisart 0.2 µm filter and stored at -20 °C for further use.

### 2.3.3 Lysozyme

A stock lysozyme solution (Sigma) was prepared in RO water at a concentration of 0.5 g $ml^{-1}$ and was then filter sterilised using a Minisart 0.2 µm filter. The solution was stored at -20 °C for further dilution and use.

### 2.3.4 Bovine Bile

Bovine Bile (3 g; Sigma-Aldrich) was dissolved in 10 ml of RO water and autoclaved to make 30 % (w/v) Bile solution.

## 2.4 Bacterial Stains

**Table 2.3 Bacterial Stains used in this study**

| Strain | Description | Reference |
|---|---|---|
| *Listeria monocytogenes* EGD | Serotype 1/2a, Clinical strain | (Murray *et al.*, 1926) |
| *Listeria monocytogenes* ATCC 23074 | Serotype 4b,Clinical strain | American Type Culture Collection |
| *Listeria monocytogenes* 10403S | Serotype 1/2a, Clinical strain | (Bishop and Hinrichs, 1987) |
| *Listeria monocytogenes* 00054-0305 | Serotype 1/2b, Vegetables sources | Vatanyoopaisarn, UoN thesis |
| *Listeria innocua* ATCC 11994 | Serotype 6a | American Type Culture Collection |
| *Vibrio harveyi* BB170 | AI-1 sensor mutant (Only responds to AI-2) | (Bassler *et al.*, 1993) |
| *Vibrio harveyi* BB120 | Common wild type (sensor 1+ sensor 2+) | (Bassler *et al.*, 1993) |
| *Escherichia coli* TOP10 | For making Chemically Competent *E. coli* | (Sternglanz *et al.*, 1981) |
| *Escherichia coli* MDS | For making Electro-Competent *E. coli* | (G. Posfai *et al.,* 2006) |

## 2.5 Cultivation and storage of bacteria strains

Bacterial cells were streaked over BHI (For *Listeria*) or LB (For *Vibrios* and *Escherichia*) agar plate and were incubated at 37 °C for 24 h. This was then stored at 4 °C as a source of viable bacterial cells. To maintain cells line, the bacteria cells were sub-cultured every 2-3 weeks. Long term storage of strain was achieved by picking single colonies from a plate culture and transferring to a Microbank tube (Pro-lab Diagnostics). After shaking, the liquid within the tube was removed and the tube was stored at -80 °C as a long term stock. To regrow cells from Microbank tubes, a single bead was taken and spread onto a BHI agar plate and incubated at 37 °C overnight to produce fresh colonies.

## 2.6 Human Tissues and phage

Table 2.4 Human Tissues and phage used in this research

| **Cell line** | **Description** |
|---|---|
| Caco-2 | Human colonic carcinoma cell line |
| **Phage** | **Description** |
| A511 | Phage that infects *Listeria monocytogenes* (Loessner, 1991) |

## 2.7 Plasmids used in the thesis

Table 2.5 Plasmids original present

| **Plasmid** | **Description** | **Reference** |
|---|---|---|
| pSB3008 | pDEST-pUNK1 with PBS10::gfp3::luxABCDE:: term | Gaddipati, University of Nottingham |
| pUNK1 | Gram positive shuttle vector with OriE1 from pUC18 and pAMβ1 as origin of replication for Gram positive EmR | Gaddipati, University of Nottingham |
| pDONORP4-P1R | To clone attB4 and att B1 flanking PCR products | Invitrogen Cat. No. 12537-023 |
| pDONOR221-lux | Entry clone with LuxABCDE | Invitrogen Cat. No. 12537-023 |
| pDONORPR-P3 term | Entry clone with terminator | Gaddipati, University of Nottingham |

## 2.8 Plasmids generated in this thesis

Table 2.6 Plasmids generated and their descriptions

| **Name** | **Description** |
|---|---|
| p0516upentry | pDONORP4-P1R linked to 1kb upstream lmo0516 (Entry clone) |
| p0017upentry | pDONORP4-P1R linked to 1kb upstream lmo0017 (Entry clone) |
| pLMO0516up | pDEST-pUNK1 with 1kb up stream lmo0516 ::luxABCDE:: term (Expression clone) |
| pLMO0017up | pDEST-pUNK1 with 1kb up stream lmo0017 ::luxABCDE:: term (Expression clone) |

## 2.9 Primer used in this thesis

Table 2.7 Primers sequences for PCR reactions

| Name | Sequences (5'-3') | Target nt sequence |
|---|---|---|
| 0017U-Pr-attB4F | GGG GAC AAC TTT GTA TAG AAA AGT TGC GAT AGA CTT CCA GAC ATC TTT TGG ATT AC | NC_003210.1 22426 - 22397 |
| 0017U-Pr-attB1R | GGG GAC TGC TTT TTT GTA CAA ACT TGT TTT TCT CCT CCT AAA TTA AAA AGT TAT CTA ATT CTA TCA G | NC_003210.1 21427 - 21467 |
| 0516U-Pr-attB4F | GGG GAC AAC TTT GTA TAG AAA AGT TGT GGG CTA GTT TTC AAT TTA TCT GGG TTT TTA TTT TG | NC_003210.1 19246 - 192311 |
| 0516U-Pr-attB1R | GGG GAC TGC TTT TTT GTA CAA ACT TGC TAG ATA TCC TCC GTA GTT CCT TTT TCT CTA AGT ATA G | NC_003210.1 191347 - 191384 |

NC - NCBI Reference Sequence Database

## 2.10 MATH assay

The assay was originated from Rosenberg *et al*. (1980). Cells were grown in 10 ml of MCDB 202 or BHI overnight at 37 °C. Cultures were centrifuged (10 min, 5000g) and the cells washed by resuspending in 0.15 M NaCl. Finally the

cells were recovered by centrifugation and cells were resuspended in 0.15 NaCl to absorbance of approximately 1. The absorbances ($A_{400nm}$) were recorded as $A_0$. A sample (3 ml) was transferred into a 15 ml test tube. Different volumes of N-octane (150, 250, 400 or 800 µl) were added to the test tubes and triplicate samples of each were prepared. The tubes were vortexed for 90 s and allowed to stand for 15 min to allow the phases to separate. A sample (1 ml) of the lower layer was taken and the absorbance ($A_{400nm)}$ recorded as A.

### 2.11 CV biofilm assay

The CV biofilm assay was described by O' Toole and Kolter in 1998. *Listeria* cells were allowed to grow in LB media overnight at 37 °C. Cultures were centrifuged at 5000*g* for 10 min and the cells washed by resuspending in MCDB202 or BHI. Cells were then diluted into the media to be tested. A sample (200 µl) was transferred to each well (8 wells for each set). The plates were placed at 37 °C for 24, 48, and 72 h. The media was removed by aspiration from the plate, and wells were washed with PBS. To each well 200 µl of 0.1% (W/V) crystal violet solution was added and allowed to stand for 15 min. CV solutions were removed and the wells were washed with 200 µl PBS 3 times. Absolute ethanol (200 µl) was added to each well and then the Absorbance at 600 nm was measured using a Micro-titre plate reader (Tecan Genios Pro multifunctional detector).

### 2.12 CV attachment assay

*Listeria* cells were grown in the media to be tested overnight at 37 °C. Samples (200 µl) of the cultures were transferred to each well (8 wells for each set). The plates were placed at 37 °C for 0, 1, 2, 4, 6 and 24 h. After this the media was removed from the plate by aspiration, and wells were washed

with 250 µl PBS once. Crystal violet (200 µl of 0.1 % (w/v) solution) were added to the wells for 15 min. CV solutions were removed and the wells were washed with 250 µl PBS 3 times. Absolute ethanol (200 µl) was added to each well and then the Absorbance at 600 nm was measured using a Micro-titre plate reader (Tecan Genios Pro multifunctional detector).

## 2.13 AI-2 assays

### 2.13.1 *Listeria* AI-2 Assay

The AI-2 bioassay was first described by Bassler *et al.* (1997). To use this to detect AI-2 produced by *Listeria,* cells and strain *V. harveyi* BB120 were grown in 10 ml of either BHI or MCDB 202 broth overnight at 37 °C. The cultures were centrifuged and the supernatant filtered using a Minisart 0.2 µm filter. The supernatant samples were stored at -20 °C. The *V. harveyi* reporter BB 170 was grown in 10 ml of AB medium over night at 37 °C. A sample (0.1 ml) of this culture was used to inoculate 100 ml of AB medium. A 180 µl sample of this diluted culture of *V. harveyi* reporter BB 170 and 20 µl of each supernatant were added into individual wells of a 96-well microtitre plate. A sample of *V. harveyi* strain BB120 supernatant was used as a positive control and uninoculated media was used as a negative control. All tests were done in triplicate. The $OD_{600nm}$ of samples and the bioluminescence produced by the reporter were measured with microtitre plate reader (Tecan Genios Pro multifunctional detector) at a 30 min interval for 12 h. The level of AI-2 detected was determined relative to the positive and negative control results.

### 2.13.2 Measurement of DPD using *Vibrio harveyi* bioassay

The *V. harveyi* reporter BB 170 was grown in 10 ml of AB medium over night at 37 °C. 0.1 ml of the culture was used to inoculate 100 ml of AB medium. A sample (180 µl) of this diluted reporter culture was added into individual

wells of a 96-well microtitre plate. A sample (20 µl) of DPD at various concentrations, or supernatant samples, were added to the wells. Media with no DPD supplement was used as a negative control. All tests were performed in triplicate. The absorbance ($OD_{600nm}$) and the bioluminescence was measured with the microtitre plate reader (Tecan Genios Pro multifunctional detector) at a 30 min intervals for 12 h at 30 °C.

### 2.14 Lysozyme resistance assay

Cells were grown overnight in 5 ml MCDB 202 or BHI broth. Cells were collected by centrifugation 5000g for 10 min and resuspended in 5ml PBS. The samples were diluted to approximate $10^7$ cfu $ml^{-1}$ and the actual cell count determined by serial dilution and viable count on LB agar. Lysozyme was added to a final concentration of 50 mg $ml^{-1}$. A sample (1 ml) was transferred in to 7 individual Eppendorf tubes. The tubes were placed in the incubator at 37 °C. One tube of each sample was taken out at 30 min intervals. The tube was centrifuged 13000g for 2 min and the cells resuspended in 1 ml MRD. The number of cells surviving was determined by serial dilution and viable count on BHI agar. The experiment was performed in triplicate.

### 2.15 Nisin treatment assay

Cells were grown overnight in 5 ml MCDB 202 or BHI. Cells were collected by centrifugation 5000*g* for 10 min and resuspended in 5ml PBS. The samples were diluted to approximate $10^7$ cfu $ml^{-1}$ and the actual cell count determined by serial dilution and viable count on BHI agar. Nisin was added at three different final concentration (see Chapter 4 for details). A sample (1 ml) of each treatment was transferred in to 7 individual Eppendorf tubes. The tubes were placed in the incubator at 37 °C. One tube of each sample was taken out

30 min intervals. The tube was centrifuged 13000*g* for 2 min and resuspended in 1 ml MRD. The number of cells surviving was determined by serial dilution and viable count on BHI agar. The experiment was performed in triplicate.

## 2.16 Bile treatment assay

Cells were grown overnight in 5 ml MCDB202 or BHI. Cells were collected by centrifugation at 5000g for 10 min and resuspended in 5ml PBS containing 30 % (w/v) Bovine Bile (Sigma). Samples were incubated in 37 °C for 5 min and then the cells were recovered by centrifugation at 13000g for 2 min and washed with 5ml PBS. The number of cells surviving was determined by serial dilution and viable count on BHI agar using the Miles Misra technique. The experiment was performed in triplicate.

## 2.17 Tissue culture invasion assay

### 2.17.1 Culturing of Caco-2 monolayer cells

Caco-2 cells were cultured in DMEM without phenol red supplemented with 10 % (v/v) heat inactivated foetal bovine serum and 0.1 mg $ml^{-1}$ penicillin and 0.1 mg $ml^{-1}$ streptomycin at 37 °C under humid conditions in a 75 $cm^2$ tissue culture flask in a $CO_2$ incubator. The cell lines were maintained by changing medium every 48-72 h.

### 2.17.2 Cell line maintenance

The Caco-2 cells were grown as described in section 2.17. When the cell reached about 80 % confluence, they cells were passaged by trypsinisation. This was achieved by removing the media from the flask and the cell monolayer were washed with 10 ml PBS 3 times. The PBS was removed, 2 ml of 1% trypsin (Gibco) was added into the flasks and incubated at the $CO_2$

incubator for 3 min. After this the excess trpysin solution was removed by aspiration, and the cells were detached from the flask surface by gentle tapping on the flask. Fresh DMEM media (10 ml) was added to the flask and the cells were disaggregated by repeated pipetting to give a single cell suspension. Half of the cell suspension was transferred into a new tissue culture flask. To both the old and new flask, DMEM media was added to a final volume of 15 ml and the cultures returned to the incubator for further cell culture.

### 2.17.3 Cell line preparation

Caco-2 cells were allowed to grow in a 75 $cm^2$ tissue culture flask to 80 % confluence. Caco-2 cells were trypsinized as described in section 2.17.2 and resuspended in 50 ml DMEM supplement with 10% (v/v) foetal bovine serum and NEAA with antibiotics. Cells were seeded on to a coated, 6-well, clear, flat bottomed tissue culture plate and incubated at 37 °C in 5% $CO_2$ for 72 h. The Caco-2 cells were washed with 50ml of PBS and incubated in 50ml DMEM with 10 % (v/v) foetal bovine serum and NEAA without antibiotics and further incubated at 37 °C in 5% $CO_2$ for 24 h. The cells were washed and incubated with 50ml fresh DMEM with 10 % (v/v) foetal bovine serum and NEAA with antibiotics for a further 1 h before invasion assays were performed.

### 2.17.4 Preparation of *Listeria* cell inoculants

*Listeria* cells were grown overnight in 10 ml MCDB 202 or BHI. They were recovered by centrifugation at 5000g for 10 min and resuspended in 10 ml PBS. They were then diluted to an approximate cell density of $10^8$ cfu $ml^{-1}$.

### 2.17.5 Cell invasion assay

The cell invasion was done as described by (Gaillard *et al.*, 1987) but

Lysozyme was used rather than gentamycin treatment to inactivate *Listeria* that did not penetrate the eukaryotic cells (Gaddipati thesis ref). The Caco-2 cells were infected with *Listeria* cells at an initial M.O.I. of approximately 100 bacteria per cell. The plates were incubated for 30 min at 37 °C in 5 % $CO_2$. Wells were then washed with PBS then 1 ml of lysozyme (50 mg $ml^{-1}$) was added to the wells to inactivate any external *Listeria* cells and samples incubated for 20 min at 37 °C. The wells were then washed 3 times with 2ml PBS to remove any remaining external *Listeria* cells. Finally The Caco-2 cells were lysed by adding 1 ml of 0.5 % (w/v) triton-X100 to the wells and incubating at 4 °C for 15 min. The viable bacterial count was determined by serial dilution and viable count on BHI plates using the Miles Misra technique.

## 2.18 Bacteriophage sensitivity assay

2.18.1 Production of bacteriophage

A sample (20 ml) of a *L. monocytogenes* EGD overnight liquid culture grown in MCDB202 and BHI was diluted to $OD_{600nm} \approx 0.05$. Phage (A511) were added at a M.O.I of 10 and incubated at 37 °C in orbital shaker. Growth was monitored spectrophotometrically at 600nm (CECIL CE 2021) every hour until lysis occurred, and the liquid lysate stored at 5 °C overnight. Bacteriophage were enumerated using Miles and Misra technique by diluting the bacteriophage in 10-fold steps in lambda buffer. To form the lawns for the phage titration, 100 µl of host strain was added to 5 ml of molten soft top agar (BHI soft agar) and this was poured over a BHI agar plate and allowed to set. The agar surface was then inoculated with three 10 µl drops of each of the phage dilutions and the plates incubated at 30 °C for 18 – 24 h. The numbers of plaques were counted and the phage titre determined.

2.18.2 Preparation of Tea extract

Loose-leaf tea (7 g Gunpowder Leaf Tea) was added to RO water and boiled for 10 min to produce a 7 % (w/v) tea infusion. The infusion was filtered with cellulose filter paper (Whatman International Ltd.) and the resulting tea infusion were autoclaved and stored at 4 °C.

2.18.3 Phage infection assay

*Listeria* cells EGD were grown in 5 ml of MCDB or BHI overnight at 37 ºC. The cells were recovered by centrifugation 5000g at for 10 min and then resuspended in 5 ml lambda buffer to a final cell density of $10^7$ cfu $ml^{-1}$. A511 phage were added to the sample at an M.O.I. = 10 and the sample mixed in a rotating shaker (Grant Scientific) at 60 rpm for 3 min. The sample was then incubated statically at 37 ºC for 1 h to allow phage infection. Every 10 min, a 1 ml sample was removed taken and to this was added 1 ml tea extract (section 2.18.2) and this was incubated for 15 min at room temperature. Serial dilutions of the samples were then prepared and the bacteriophage titre determined (as section 2.18.1). Experiments were performed in triplicate.

## 2.19 DNA methods

2.19.1 Simple Extraction of DNA for PCR

*L. monocytogenes* were grown overnight on LB plates. One colony was picked and transferred into 100 µl of sterile RO water. The samples were heated at 95 °C for 5 min in a heating block. After gentle shaking, the samples were centrifuged at 13,000 x g for 5 min at room temperature. The supernatant from this was used as a template DNA for PCR.

### 2.19.2 Polymerase chain reaction

For PCR reactions a total volume of 20 µl was generally used. Within this template DNA represented 1.5 µl of the volume and 10 µl of KOD Hot start DNA polymerase master mix. Generally a volume of 0.6 µl of each primer was used (see relevant results chapters for details of primer concentrations). PCR reactions were made up to volume using RO water. A Techne PCR thermal cycler was used (for PCR conditions see relevant results chapters). The polymerases were activated by setting the first step at 95 °C for 2 min. After 30 complete cycles, the samples were cooled and held at 10 °C.

### 2.19.3 Extraction of PCR product

Extraction of PCR products from gels was performed using Zymoclean™ Gel DNA Recovery Kit. The part of the gel with the target band was cut out of the agarose gel and transferred into am Eppendorf microcentrifuge tube. Three gel volumes of ADB buffer was added to the tube and this was incubated at 40 °C for 10 min until the gel was completely dissolved. The solution was transferred into a Zymo-spin column and onto a collection tube. It was then centrifuged at 13,000*g* for 40 s. Wash buffer (200 µl) was used to wash through the tube 3 times. The column was transferred onto a new micro-centrifuge tube and 8 µl of RO water added before it was centrifuged for 30 s at 13000*g* to elute the purifed DNA. DNA samples were stored at -80 °C.

### **2.19.4 MultiSite Gateway System (Invitrogen)**

#### 2.19.4.1 BP Reaction for cloning PCR products

BP recombination reactions were performed by adding 1 µl of PCR product (100 fmoles) to 1 µl of pDONR vector plasmid (200 ng). To this were added 4

µl of SDW and 2 µl of BP Clonase reaction enzyme mix (Invitrogen) and the sample mixed by gentle vortexing. The reaction mixtures were incubated at 25 °C overnight. After this 0.5 µl of Proteinase K was added to the sample and the DNA used for transformation of bacteria host strains.

#### 2.19.4.2 LR reactions for creating expression clones

LR recombination reactions were performed by mixing 2 µl of each entry clone and 2 µl of pDESTR4R3 destination vector and 1 µl of the LR ClonaseII enzyme mix (Invitrogen) in an Eppendorf tube and mixing the components by brief vortexing. The reaction mixture was incubated at 25 °C overnight and the samples dialysed with 0.025 µm dialysis filter against distilled water before being electroporated into bacterial host strains.

### 2.19.5 Minipreparation of plasmid DNA

The extraction of plasmids from *E. coli* was performed using a Miniprep kit (Zymo Research, Zyppy™ Plasmid Miniprep Kit). Cells from an overnight cell *E. coli* culture in LB media (3 ml) collected by centrifugation at 13000*g* for 1 min to make 600µl high cell density culture into a micro-centrifuge tube in LB. 7X lysis buffer (100 µl) was added to the cell suspension and mixed with gentle shaking. Within two minutes, 350 µl of cold Neutralization Buffer were added to the sample and mixed. The tubes were centrifuged at 15,000*g* for 4 min. The supernatant was transferred into a Zymo-spin IIN column and then onto a collection tube. This was then centrifuged at 13000g for 15 s to remove the flow-through. The column was washed with 200 µl of Endo-wash Buffer followed by 400 µl of Zyppy wash buffer. Finally 30 µl of the Zyppy Elution Buffer was added to the column and the column was transferred on to a centrifuged tube. Plasmid DNA was eluted into the tube by 15 s centrifugation at 13000*g*. The DNA was then stored at -80 °C for further use.

### 2.19.6 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis. Agarose was dissolved in TAE buffer at a concentration of 0.8 % (w/v) by heating in a microwave oven at 500 W for 2 min. The agarose was allowed to cool to hand hot and Ethidium bromide added to a concentration of 0.4 µg $ml^{-1}$. The agarose was poured into electrophoresis trays, combs inserted and allowed to set by further cooling at room temperature. The gel was then submerged in TAE buffer in an electrophoresis tank. DNA samples and a molecular weight ladder were mixed with loading dye (NEB) before loading into the wells. The samples were electrophoresed on the gel at 70-85 V for 1-2 h. The bands were visualised under UV using a Light Imager (Bio-Rad).

### 2.19.7 Restriction Digestion of DNA

DNA was digested using restriction enzymes generally by adding restriction enzyme (at 10 Units) and 2 µl of the corresponding restriction buffer were added to the DNA sample in a total volume of 20 µl and incubated at the 37°C overnight. The restricted DNA was analysed by agarose gel electrophoresis for DNA size analysis.

## 2.20 Preparation of competent cells

2.20.1 Preparation of *E. coli* Hanahan competent cells for chemical transformation (Hanahan, 1983)

*E. coli* TOP10 were grown in 20 ml of SOC media overnight at 37 °C with shaking at 150 rpm. A sample (2.5 ml) of the overnight culture was used to inoculate 250 ml of 2X YT medium. The cells were allowed to grown to an $OD_{600nm}$ = 0.5. The Cells were pelleted at 7000g for 10 min at 4 °C. The cells were resuspended in 83 ml of RF1 (section 2.2.4) and incubated on ice for 1 h. Cells were pelleted again at 7000g for 10 min at 4°C. The sample was resuspended in 20 ml of RF2 (section 2.2.5) and further incubated for 15 min on ice. Samples (100 µl) of the cells were transferred into micro-centrifuge tubes and store at -80 °C for further use.

2.20.2 Preparation of *E. coli* competent cells for electroporation

*E. coli* MDS cells were grown overnight in 20 ml of LB media at 37 °C with shaking. A sample (10 ml) of overnight culture was used to inoculate 1 L of LB broth. The cells were allowed to grow with shaking at 37 °C to $OD_{600nm}$ of approximate 0.5-0.8. The cells were collected by centrifugation at 7000 x g for 10 min at 4 °C, and were resuspended in 1 L of cold, sterile RO water. Cells were then centrifuged again at 7000*g* for 10 min and resuspended to 500 ml cold sterile RO water. Cells were again pelleted and resuspended in a final volume of 20 ml of sterile 10 % (w/v) glycerol in RO water and stored at -80°C.

### 2.20.3 Preparation of *Listeria* competent cells for electroporation

This was performed according to the method described by (Park and Stewart, 1990). *L. monocytogenes* cells were grown overnight in 15 ml of BHI/0.5 M sucrose media at 37 °C with shaking. A sample (10 ml) of the overnight culture was used to inoculate 500 ml of BHI/0.5M sucrose media. The cells were allowed to grow with shaking at 37°C to $OD_{600nm}$ of approximately 0.2. Penicillin was then added to a concentration of 10 µg $ml^{-1}$, and were incubated for further 2 h. The cells were collected by centrifugation at 7000g for 10 min at 4 °C in a Beckman JS-21 centrifuge using a JA10 rotor, and were resuspended in 500 ml of 1 mM Hepes, 0.5 M sucrose. Cells were then pelleted again and resuspended in 250 ml 1 mM Hepes, 0.5 M sucrose. Cells were again pelleted and resuspended in a final volume of 1.25 ml of 1 mM Hepes, 0.5 M sucrose with the addition of 10 % (w/v) glycerol and stored at -80 °C.

## 2.21 Bacteria cell Transformation

### 2.21.1 Chemical transformation

DNA (approx. 100 ng) was added to 100 µl of Hanahan competent cells (section 2.20.1). The mixture was incubated on ice for 30 min. and then placed in a 42 °C water bath for 60 s before being placed immediately on ice for 2 min. Then 900 µl of LB broth was added to the sample and it was further incubated at 37 °C for 60 min with shaking. The cells were plated onto plated onto LB agar with selective antibiotics (see relevant results chapters for details) and incubated at 37 °C overnight.

### 2.21.2 Electroporation of *E .coli* cells

DNA samples were dialysed for 20 min using a 0.025 µm drop dialysis filter (Bio-Lab) floating on sterile RO water. *E. coli* electrocompetent cells stored at -80 °C (section 2.20.3) were thawed on ice. The dialysed DNA samples were added to the cells. The mixture was transferred into a cold electroporation cuvette and this was then placed into the gene pulsar apparatus set at 25 µF, 2.5 kV, 200 W for electroporation. Immediately after the pulse, the samples was transferred into 1 ml of LB media and incubated for 1 h at 37 °C. The samples were plated onto LB agar with selective antibiotics (see relevant results chapters for details) and incubated at 37 °C overnight.

### 2.21.3 Electroporation of *Listeria* cells

DNA samples were dialysed for 20 min using a 0.025 µm drop dialysis filter (BioLab) floating on sterile RO water. *Listeria* competent cells stored at -80 °C (section 2.20.3) were thawed on ice. The dialysed DNA samples were added to the cells and the mixture was transferred into a cold electroporation cuvette. It was then placed into the gene pulsar apparatus set at 25 µF, 2.5 kV, 200 W for electroporation. Immediately after the pulse, the samples was transferred into 1 ml of LB media and incubated for 1 h at 37 °C. The samples were plated onto LB agar with selective antibiotics (see relevant results chapters for details) and incubated at 37 °C overnight.

## 2.22 Analysis of promoter expression by bioluminescence

*Listeria* cells were grown overnight in BHI or MCDB 202 broth. Five samples (200 µl) were transferred into individual wells of a 96 well microtitre plate. The plates were allowed to incubate in a microtitre plate reader (Tecan Genios Pro multifunctional detector) and $OD_{600nm}$ and bioluminescence measurements taken for 12 h at 30 min intervals.

# Chapter 3

# The Effect of Minimal Media on *Listeria* Cell Hydrophobicity, Cell Attachment and Biofilm Formation

## 3.1 Introduction

Previous work by this research group has shown that *L. monocytogenes* cells grown in defined media MCDB 202 produce a certain cell surface material that forms a capsule-like structure which was not seen when cells were grown in BHI media using the same incubation conditions (Nwaiwu, 2010). This raised the question of whether this change in cell surface structure may lead to changes in cell surface properties. One of the most common cell surface properties studied in bacterial cell physiology is cell hydrophobicity. Hydrophobicity of cells is one of the main factors contributing the likelihood of cells attaching to various surfaces and, hence, also determines the efficiency of biofilm development (Takahashi *et al.*, 2010). In this chapter an investigation was performed to determine the effect of growing *L. monocytogenes* in defined (MCDB202) or rich media (BHI) on cell hydrophobicity, cell attachment and biofilm formation.

## 3.2 Cell hydrophobicity

### 3.2.1 Identifying changes in cell hydrophobicity

Cell hydrophobicity can be estimated by the use of the microbial attachment to hydrocarbons assay (MATH assay). In this study the MATH assay was based on the method described by Rosenberg *et al.* (1980). Cells were grown in the different test media, which were a defined media (MCDB 202) and a rich media (BHI) and then the hydrophobicity of the cells determined. Hydrophobicity was estimated by calculating the percentage of affinity using the equation:

$$\% \text{ affinity} = (A_0\text{-}A)/A_0 \text{ X } 100\%$$

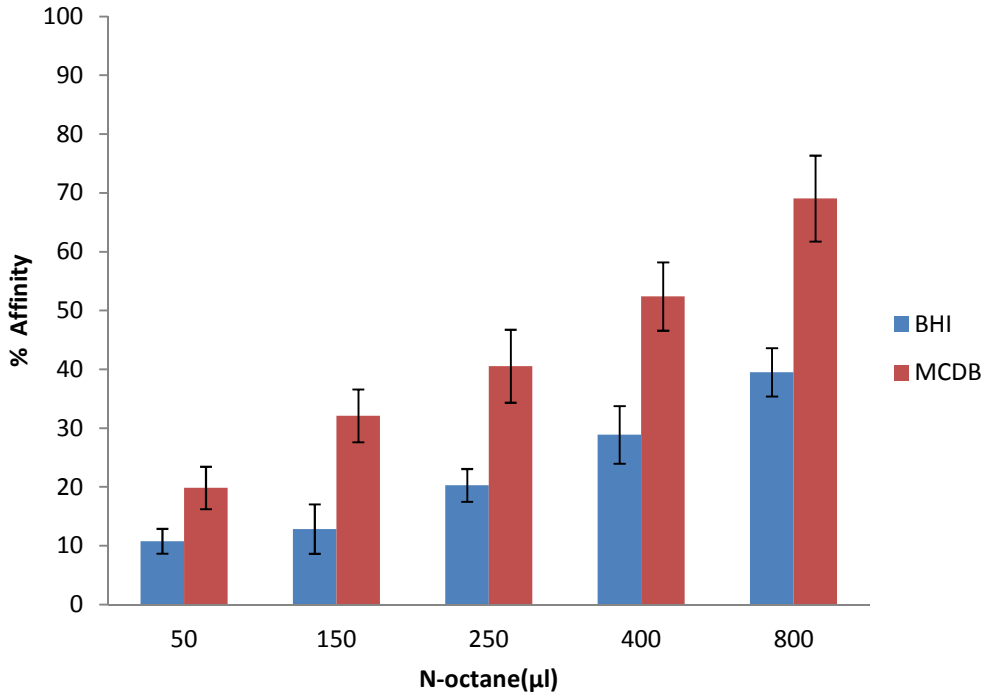
$A_{0:}$ Intial absorbance before adding hydrocarbons

A: Final absorbance

A high percentage affinity value indicates that the cells are more hydrophobic and a low affinity percentage affinity value indicated that the cells are less hydrophobic. As cell hydrophobicity is the measure of the amount of bacteria cells partitioning into the hydrocarbon phase of the mixture, different volumes of hydrocarbon may influence the results obtained and different published methods for this assay recommend the use of different volumes of solvent. To have more confidence in the results obtained, instead of using a single (250µl) volume of hydrocarbon as in the previous used protocol, different volume of hydrocarbon (ranging from 50-800µl) was added to the samples to measure cell partitioning.

The first hydrophobicity tests were performed using *L. monocytogenes* EGD strain (serotype 1/2a) as it is a very common strain used in research, which has been well characterized. From the result (Fig. 3.1), it is clear that for all 5 tested hydrocarbon volumes, *Listeria* EGD cells grown in the defined minimal media had a higher hydrophobicity than those grown in the rich media, and showed that this result was not affected by the volume of the solvent used. However as the volume of hydrocarbon increased, the percentage affinity value for both cells grown in BHI and MCDB 202 media increased, resulting in an affinity of nearly 70% for cells grown in MCDB 202 and 40% for those cells grown in BHI when 800µl of the hydrocarbon was used. This is possibly due to the increased hydrocarbon volume preventing saturation of the solvent system and therefore favouring cell partitioning. Alternatively this may be due to a more even distribution of the solvent throughout the emulsion formed during vortexing, resulting in cells having more opportunity to associate with the hydrocarbon droplets. The conclusion from this work was that the absolute percentage of attachment determined using this method is not that significant, but the relative percentage of attachment of different samples could be used to indicate differences in cell surface properties.

**Figure 3.1: Determining cell hydrophobicity of *Listeria* EGD using a modified MATH assay**




Cells were grown in MCDB 202 or BHI broth at 37 °C overnight. Based on the method described by Rosenberg *et al.* (1980), cultures were centrifuged and resuspended in 150 mM NaCl to $OD_{400nm}$ of approximately 1.0. A sample (3 ml) of each cell suspension was vortexed for 120 s with 50, 150, 250, 400, 800µl of N-octane and allowed to stand 15 min. A sample was taken from the lower aqueous layer and the $OD_{400nm}$ value determined. Data presented represents the results from three independent cultures and for each experiment triplicate samples were taken. Error bars represents the stand deviation calculated.

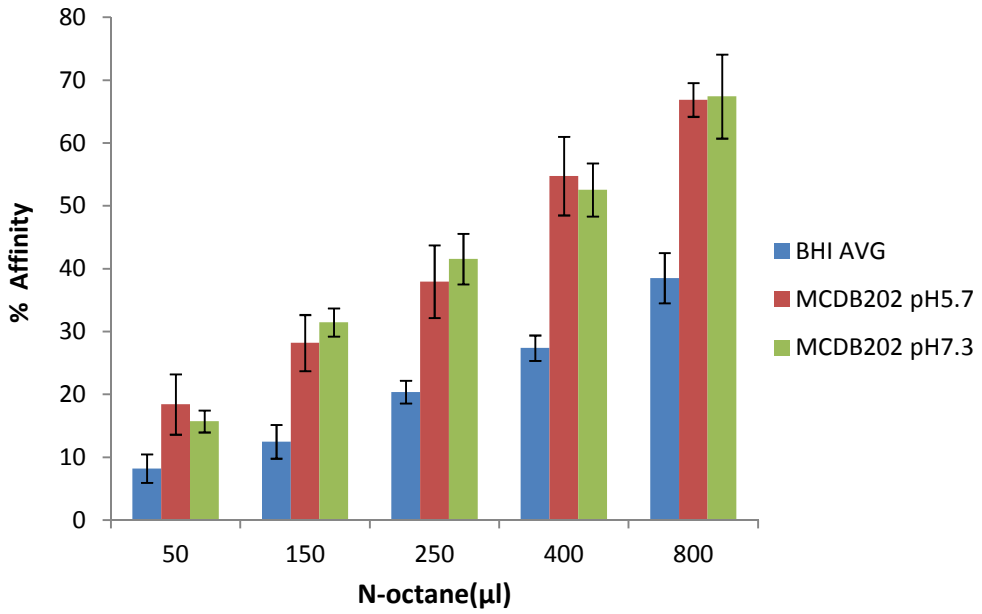
% affinity = (Ao-A)/Ao X 100%

It is clear from the results that the *L. monocytogenes* EGD cells grown in the defined media showed an increase in cell hydrophobicity compared to cells grown in BHI broth, indicating that a detectable change occurred on the EGD cell surface, which may be related to the formation of the extracellular polymeric substance observed on the cell surface.

### 3.2.2 The effect of medium pH on hydrophobicity

The MCDB 202 media used for these experiments had a lower pH (pH 5.7) than that of BHI (pH 7.2) which may be a factor contributing the change in cell hydrophobicity. To answer this question, the experiment presented in figure 3.2 was repeated using a pH-modified MCDB 202 to culture the *Listeria* cells. From the results (Fig. 3.2) there were no differences between the hydrophobicity of the samples grown in MCDB 202 pH 5.3 and MCDB 202 pH 7.2, and both sets of cells were clearly more hydrophobic than those grown in BHI broth. This indicates that the enhancement in cell hydrophobicity seen when cells were grown in MCDB 202 was not caused by the difference in pH of the two media.

**Figure 3.2: Effect of pH on cell hydrophobicity of *Listeria* EGD cells grown in MCDB 202**




Cells were grown in MCDB 202 at pH 5.7 and 7.2 or in BHI broth. Based on the method described by Rosenberg *et al.* (1980), cultures were centrifuged and resuspended in 150 mM NaCl to $OD_{400nm}$ of approximately 1.0. A sample (3 ml) of each cell suspension was vortexed for 120 s with 250 µl of N-octane and allowed to stand 15 min. A sample was taken from the lower aqueous layer and the $OD_{400nm}$ value determined. Data presented represent the results from three independent cultures and for each experiment triplicate samples were taken.

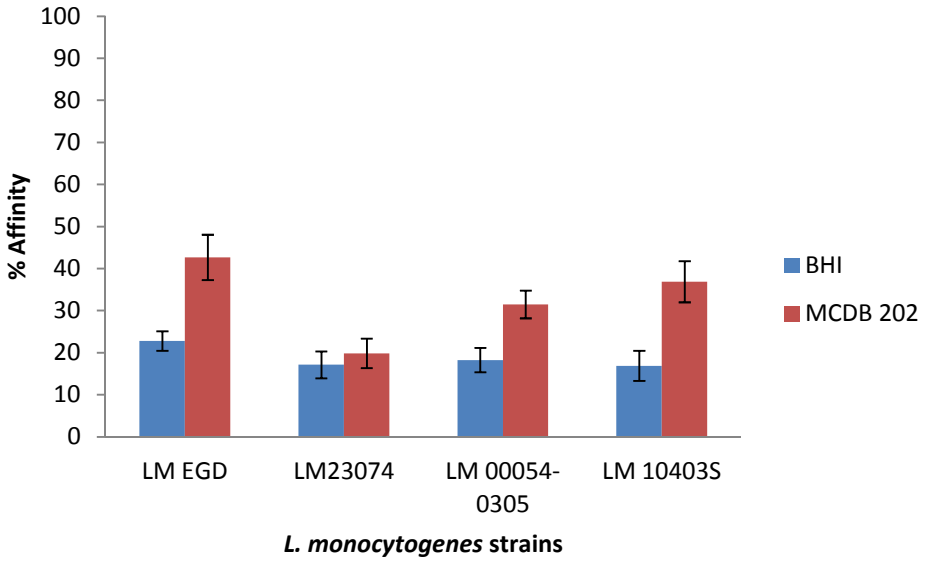
% affinity = (Ao-A)/Ao X 100%

### 3.2.3 Hydrophobicity of different *Listeria* strains

To have a better understanding of whether these changes in hydrophobicity were strain specific, three more strains of *L. monocytogene*s were also tested. These included another well characterized clinical strain LM 10403S (serotype 1/2a), and the ATCC strain LM 23074 (serotype 4b), which is also a clinical isolate and represents the other major serotype associated with human disease. One environmental strain, LM 00054-0305 (serotype1/2b) was also tested as a representative of an organism that has not been cultured in the laboratory for long periods of time. As the results from the last experiment showed that the differences in hydrophobicity could be detected irrespective of the solvent volume, a volume of 250 µl of N-octane was used as described by Rosenberg *et al.*(1980) and experiments were performed in triplicate.

From the results (Fig. 3.3), three out of four *Listeria* strains tested showed a higher hydrophobicity when they were grown in the defined media, MCDB 202, than when grown in BHI. The *Listeria* cells grown in BHI had a hydrophobicity scores ranging from 15% to 20% whereas cells grown in MCDB 202 had percentage affinity values ranging from 20% to 40%. One of the strains tested, LM 23074 showed a smaller difference in the MATH assay results indicating that the level of change in cell surface hydrophobicity following growth in MCDB 202 may vary among different *L. monocytogenes* strains, but that the effect was not strain specific. Also the level of hydrophobicity of the environmental isolate was not different to that of the isolates that had been cultured in the laboratory for a long period of time. This suggests that this may not be a phenotype that is dependent on selection by environmental pressure but is a more intrinsic property of the cell.

**Figure 3.3: Effect of different growth media on cell surface hydrophobicity of *Listeria* strains.**




4 Strains of *L*isteria cells were grown in MCDB 202 or BHI broth. Based on the method described by Rosenberg *et al.* (1980), cultures were centrifuged and resuspended in 150 mM NaCl to $OD_{400nm}$ of approximately 1.0. A sample (3 ml) of each cell suspension was vortexed for 120 s with 250 µl of N-octane and allowed to stand 15 min. A sample was taken from the lower aqueous layer and the $OD_{400nm}$ value determined. Data presented represents the results from three independent cultures and for each experiment triplicate samples were taken.

% affinity = (Ao-A)/Ao X 100%

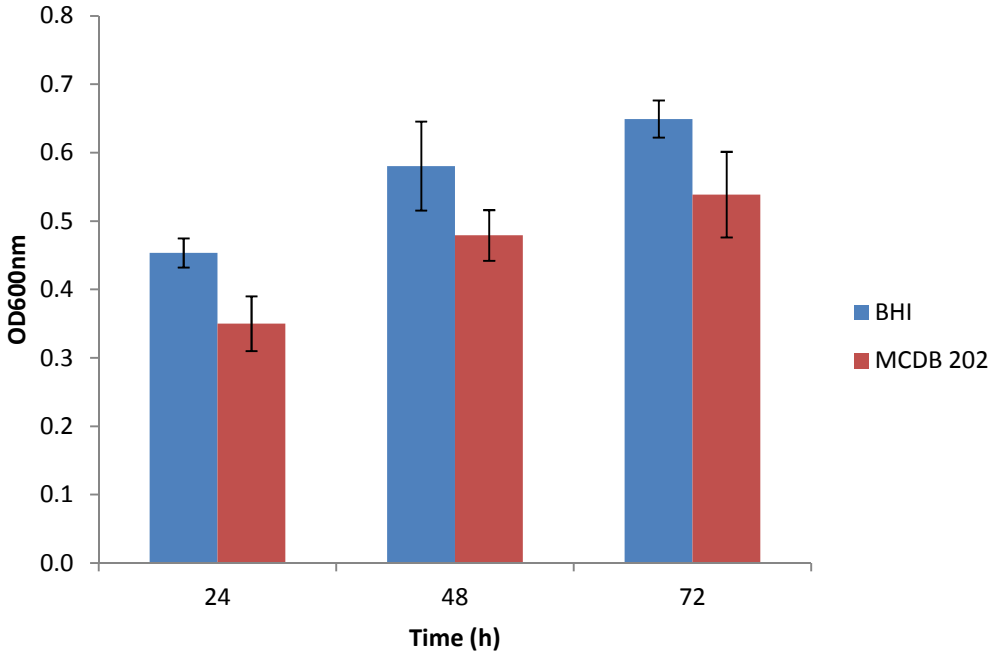
### 3.3 Biofilm assay

In previous studies of cellular hydrophobicity, Takahashi *et al.*(2010) studied 24 different *Listeria* strains by testing their ability to form biofilms, attach to PVC as well as using the MATH assay to assess hydrophobicity. They have shown that there is a good correlation between initial attachment of cells, biofilm formation and hydrophobicity of bacteria strains. The researchers have suggested that an increase in hydrophobicity of cells may have give rise to an increase in initial attachment ability and hence have give changes to the level of biofilm formation (Takahashi *et al.*, 2010). It has also been reported that *Listeria* cells grown in the defined media produces more biofilm than cells grown in BHI broth (Chavant *et al.*, 2002), and - according to our results - this could be explained because production of the extracellular polymeric substance was being induced. Hence to investigate the effect of minimal media on the ability of *Listeria* to produce a biofilm, a Crystal Violet (CV) biofilm assay was performed (Djordjevic *et al.*, 2002) which uses a dye to non-specifically stain biofilm material attached to the surface of the culture vessel after the broth culture has been removed.

To perform the experiment EGD cells were grown statically in a 96-well microtitre plate for 24, 48 and 72 h in the different media being tested to allow a biofilm to form on the surface of the wells. In this case MCDB202 adjusted to pH 7.2 was used to rule out any effects caused by a difference in pH of the media. After incubation, the culture was removed, and the wells were washed using PBS to removed non-attached material and crystal violet was then used to stain the remaining biofilm materials on the well surface. The amount of stain was measured by solubilising the dye in ethanol and then determining the optical density of the sample at 600nm (section 2.11).

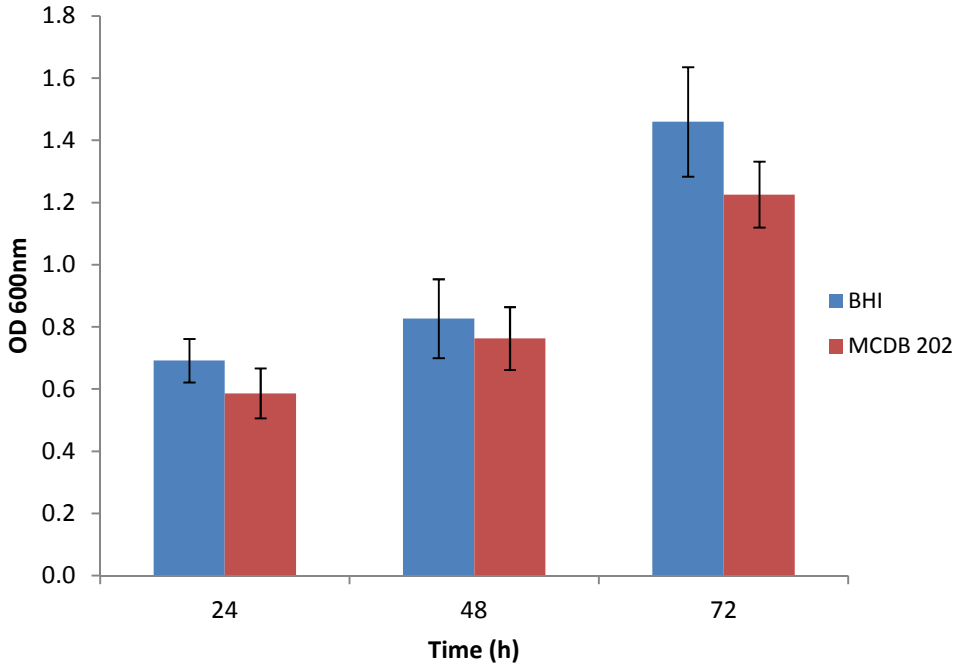
One problem that existed was that the cells grew very slowly in the minimal media, and therefore being able to directly compare the biofilm results for samples grown in different media at the same time was difficult to achieve. To address this problem cells were first grown overnight in a media other than BHI and MCDB202, the LB broth (it is a common media used in laboratory other than BHI. For *Listeria* this is not nutrient limiting, but growth rates are slower than those achieved in BHI). The cells were then recovered by centrifugation and resuspended into the test media to inoculate the microtitre plates. Since the cells were incubated for a minimum of 24 h, this would allow sufficient time for the cells to adapt to the test media during the incubation period and therefore any differences in the ability to attach to the surface of the well would be apparent. It was felt that by inoculating the cells at a high OD ($A_{600nm}$=0.8) would help remove any effects due to the different growth rates of *Listeria* in MCDB202 and BHI (Fig. 3.5). However since most published protocols suggest inoculating cultures at a low OD and allowing the cells to grow to high OD during the experiment, a low inoculum was also used (Fig. 3.4).

**Figure 3.4: Effect of minimal media on biofilm production of *Listeria monocytogenes* (Low density inoculation)**




*Listeria* cells were grown in 5 ml LB media overnight. Cells were diluted into the two media to be tested at $OD_{600nm}$ of 0.3. 200 µl of the sample were transferred to each well. The plates were placed at 37 °C for 24, 48, and 72 h. The media were removed from the plate, and wells were washed with 200µl of PBS for three times (section 2.11). To stain the biofilm material, 200 µl of 0.1% (w/v) crystal violet (CV) were added to the wells for 15 min. CV solutions were removed and the wells were washed with PBS three times. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 600 nm. The experiment was performed in triplicate. Error indicates the standard deviated calculated.

**Figure 3.5: Effect of minimal media on biofilm production of *Listeria monocytogenes* (High density inoculation)**

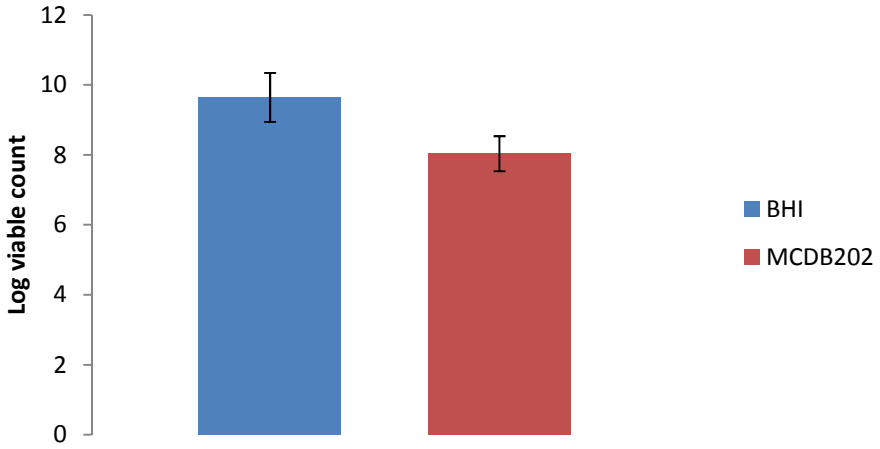



*Listeria* cells were grown in 5 ml LB media overnight. Cells were diluted into the two media to be tested at $OD_{600nm}$ of 0.8. 200 µl of the sample were transferred to each well. The plates were placed at 37 °C for 24, 48, and 72 h. The media were removed from the plate, and wells were washed with 200µl of PBS for three times (section 2.11). To stain the biofilm material, 200 µl of 0.1% (w/v) crystal violet (CV) were added to the wells for 15 min. CV solutions were removed and the wells were washed with PBS 3 times. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 600 nm. The experiment was performed in triplicate.

*Listeria* cells grown in MCDB 202 did not show an increase in biofilm formation in the CV assay when using either a high or a low inoculation level (Fig. 3.4 and 3.5), and hence the increased hydrophobicity of cells grown in MCDB 202 did not seem to enhanced biofilm formation as postulated. Indeed it was seen that the amount of biofilm formed was highest when cells were grown in BHI after three days of incubation (Fig. 3.5).

When comparing the two level of inoculation used (Figs. 3.4 and 3.5), a higher cell inoculation into both media resulted in more material being attached to the surfaces at all sampling time points. The samples using low inoculation produced CV $A_{600nm}$ readings of 0.35-0.65 whereas those using the high inoculation gave CV $A_{600nm}$ readings of 0.6-1.4. This suggested that the level of inoculation has the greatest effect on biofilm formation. Hence the viable cell count of the two cultures in the microtitre plate wells were also measure at the third day of incubation (Fig. 3.6). Due to a lower nutrient content in MCDB 202, the final viable cell count in these culture were about one $\log_{10}$ lower than those grown in BHI broth. This may be contributing the higher amount of biofilm material detected in the BHI sample after 72 h. However, from these results it was hard to come to a conclusion about the biofilm potential of the samples relative to the viable cell count.

**Figure 3.6: The difference in viable count of *Listeria* cells in MCDB 202 and BHI sample of Biofilm assay**




*Listeria* cells were grown in 5 ml LB media overnight. Cells were diluted into the two media to be tested at $OD_{600nm}$ of 0.8. 200 µl of the sample were transferred to each well. The plates were placed at 37 °C for 72 h. The viable count of the samples was measure by serial dilution and plating out on BHI agar plate. The results represent the average of triplicate data and standard deviation is represented in error bars.

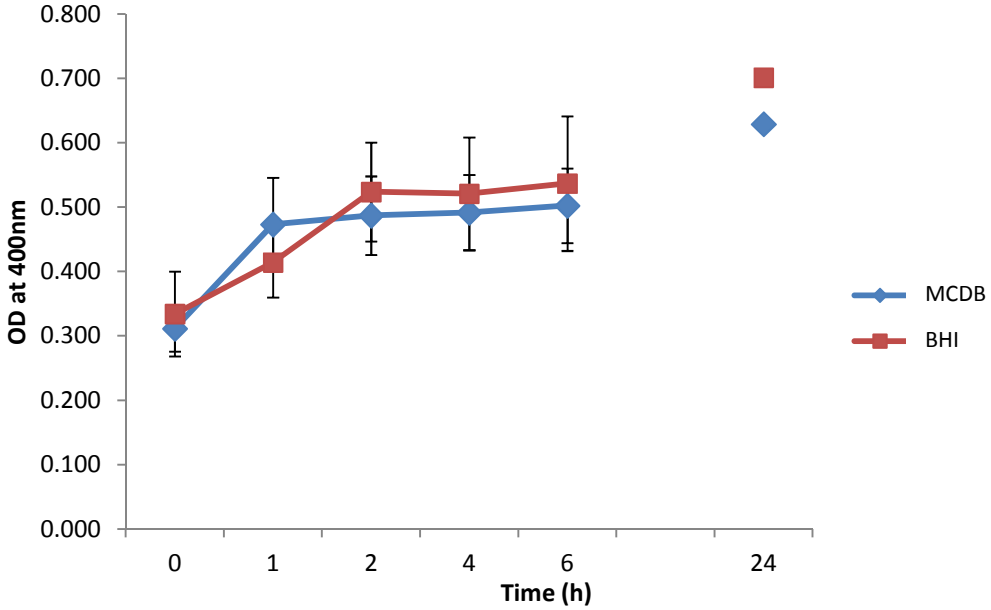
### 3.4 Initial attachment assay

Apart from biofilm formation, cell hydrophobicity is also greatly related to cell initial attachment to surfaces. Initial attachment describes the ability of cells to adhere to surfaces within a short period of time. More precisely the initial attachment indicates the efficiency with cells attach to a surface and therefore give a higher chance that a biofilm will form as attachment is the first step of cell biofilm formation. This cell property can be tested by an attachment assay which uses the same principles as the biofilm assay, using crystal violet to stain the cells attached to wells. However, the time of incubation is reduced (6 hours) which is aimed to measure only the ability of the cells to initially attach to the surfaces before biofilm is formed (Vatanyoopaisarn *et al*., 2000).

This protocol was suggested by Vatanyoopaisarn et al. (2000) who were studying the difference in attachment ability of wildtype *Listeria* and flagella mutants. In their study it was shown that *Listeria* cell attachment is not affected by motility but by the presence or absence of flagella. In this study the attachment test method was based on the assay used by Vatanyoopaisarn *et al.* (2000) but modified to allow differences in cell attachment levels caused by growing the cells in the two difference culture media to be determined. This was done by growing the *Listeria* EGD cells in BHI or MCDB 202 overnight at 37°C prior to performing the attachment assay.

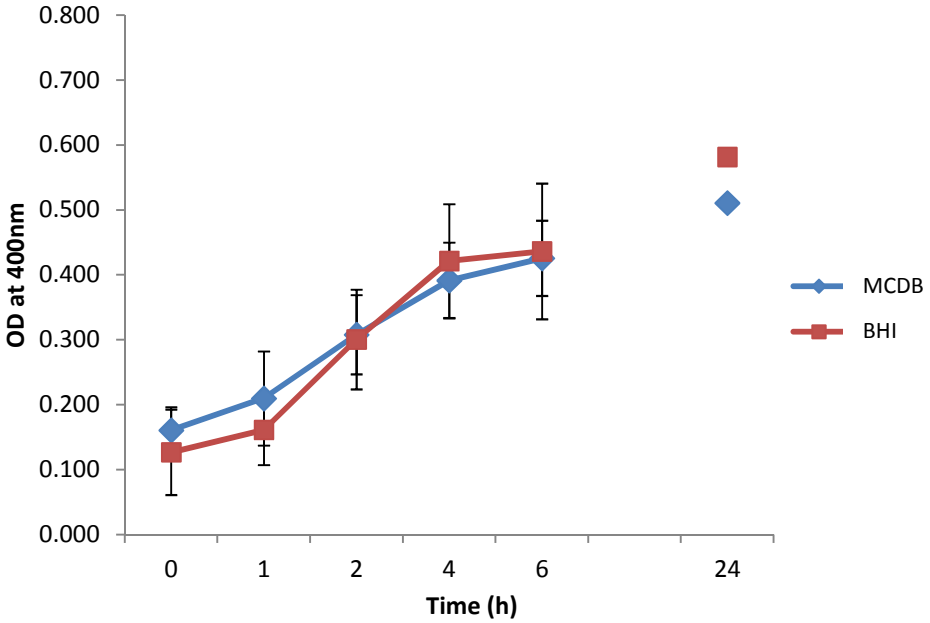
From the results of the attachment test, it was seen that the cells grown in BHI and MCDB 202 give quite similar attachment levels in the first 6 h of the test, so the increase in hydrophobicity of *Listeria* cells grown in MCDB 202 did not enhance cell attachment. Using both high and low inoculation levels (Figs. 3.7 and 3.8), there a gradual increase in attachment overtime was seen, however, when using a higher inoculum the attachment level reaches a plateau earlier (2-3 h) that when using a low inoculums (4-5 h). So again, it is obvious that inoculation level (or cell density of the culture) has an observable effect on cell attachment results and no difference in the ability of *Listeria* to attach to a surface that could be attributed to growth in the different media was detected.

**Figure 3.7: The effect of defined media on Cell attachment level of *Listeria* cells without dilution**




*Listeria* cells were allowed to grow BHI or MCDB 202 overnight at 37°C. 200µl of the undiluted sample from overnight culture were transferred to individual wells. The plates were placed at 37°C for 0, 1, 2, 4, 6 and 24 h. The media was removed from the plate, and wells were washed with 200 µl PBS (section 2.12). Crystal violet (CV; 200 µl of 0.1% (w/v) solution) were added to the wells for 15 min. CV solutions were removed and the wells were washed with PBS three times. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 400 nm. Error bars indicates the standard deviation calculated.

**Figure 3.8 The effect of defined media on Cell attachment level of *Listeria* cells (innocula $A_{600nm}$= 0.6)**




*Listeria* cells were allowed to grow BHI or MCDB 202 overnight at 37°C. 200µl of the diluted sample of overnight culture (OD at 0.6) were transferred to individual wells. The plates were placed at 37°C for 0, 1, 2, 4, 6 and 24 h. The media were removed from the plate, and wells were washedwith200 µl of PBS (section 2.12). Crystal violet (CV; 200 µl of 0.1% w/v solution) were added to the wells for 15 min. CV solutions were removed and the wells were washed with PBS three times. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 400 nm. Error bars indicates the standard deviation calculated.

### 3.5 Attachment to hydrophobic microtitre plates

In the initial experiments in this chapter, the change in media was shown to cause a detectable change in *L. monocytogenes* cell hydrophobicity. However no effect was seen on cell attachment and biofilm formation when cells were grown in the same minimal media. It was suspected that there could be some unknown factor accounting for these unexpected results, such as the nature of the surface that was being used for the assay. The CV assay has been used to measure the biofilm potential of many bacteria that produce extracellular polymers composed of sugars or amino acids but these do not increase cell hydrophobicity. Therefore it was possible that this assay was not appropriate to monitor changes in adhesion due to the increased hydrophobicity.

To examine this, the biofilm and attachment assay were repeated with another type of microtitre plate. Immuno 96 micro well plate (NUNC) is titre plate in which the well surface is coated with a specialize hydrophobic substance known as *PolySorp,* and hence may be a better surface to detect changes in the attachment of the *Listeria* cells grown in minimal media with an increased hydrophobicity, as they would be more likely to attach to these hydrophobic well surfaces.

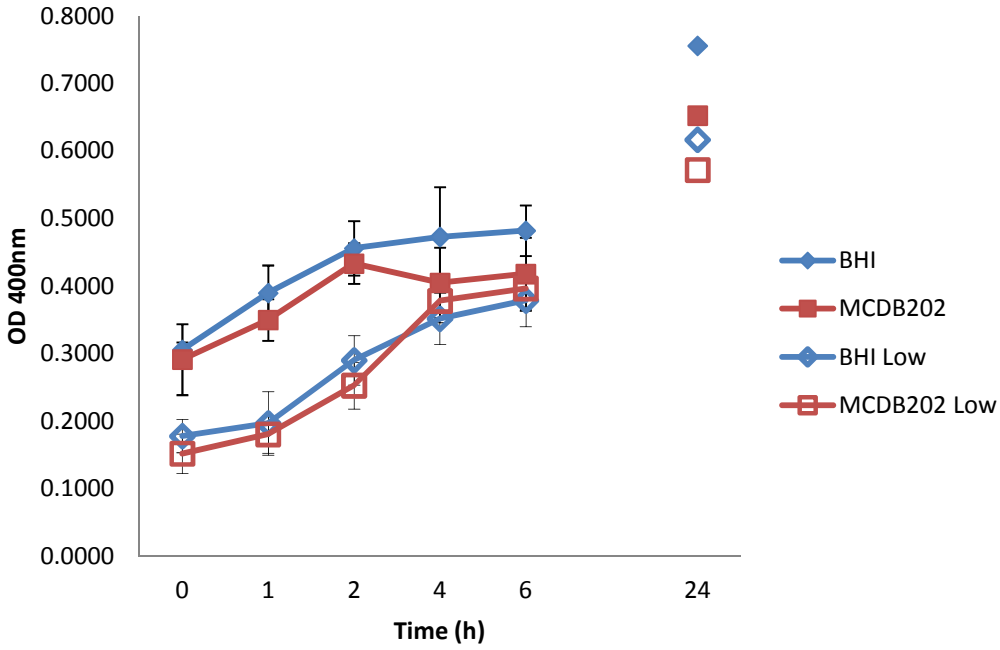
To perform both the attachment and biofilm assay experiment *L. monocytogenes* EGD cells were grown statically in a 96-well ImmunoPolySorp plate for 6 or 24, 48 or 72 h in the different media being tested to allow cells to attach or – during the later stages of incubation -biofilm to form on the surface of the wells. After incubation, the culture was removed and the wells washed using PBS to removed non-attached material and then crystal violet used to stain the remaining material on the well

surface (section 2.11). The amount of stain was measured by solubilising the dye in ethanol and then determining the absorbance of the sample at 600nm.

The results of the attachment test (Fig. 3.9) and biofilm assays (Fig 3.10) on the PolySorpsurface were similar to those recorded using normal microtitre plates in sections 3.3 and 3.4. The cells grown in BHI and MCDB 202 produced quite similar attachment levels in the first 6 h of the test and these levels were very similar those achieved using standard microtitre plates. Similarly, the biofilm formation assay using the hydrophobic plates showed that *Listeria* grown in BHI produced much higher amounts of biofilm material than cells grown in MCDB 202, which again was a similar result to that achieved using standard microtitre plates. This indicated that the more hydrophobic surface did not favour the attachment of the more hydrophobic *Listeria* cells grown in MCDB 202 and that the changes in the surface properties of the *Listeria* cells do not affect its ability to bind to the two types of microtitre plate used.

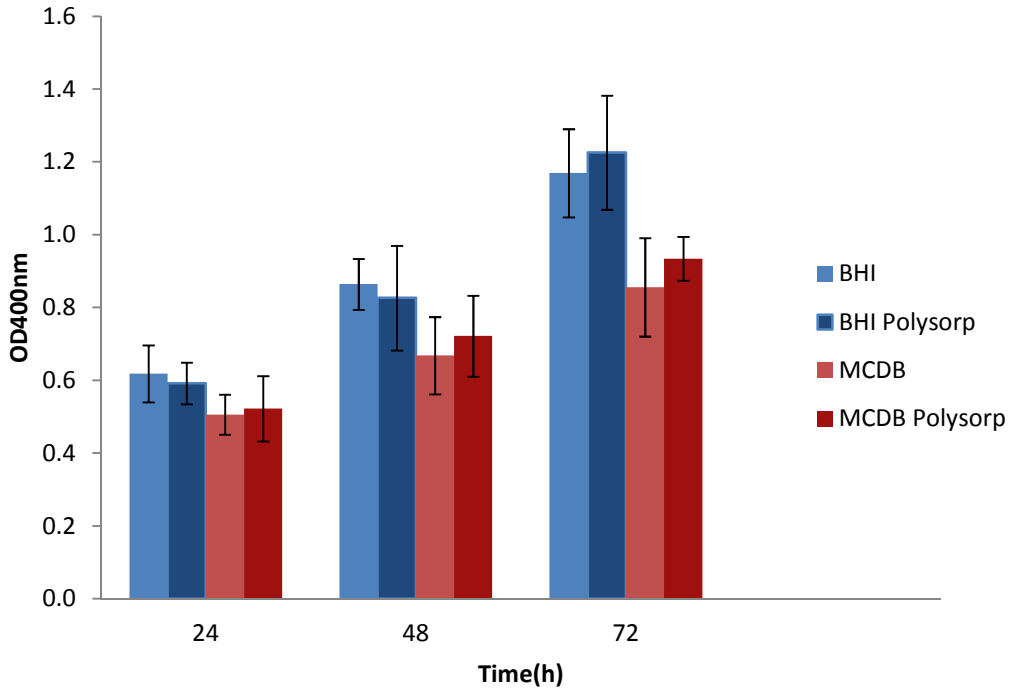
Standard polystyrene microtitre plates are also hydrophobic in nature, but the PolySorp surface has a higher hydrophobicity and is recommended for work with more hydrophobic molecules. However using this did not give rise to a better attachment. This may be suggesting that as long as the surfaces are hydrophobic, the level in hydrophobicity on surfaces may not produce significant effects on cell attachment and biofilm formation. Perhaps, if any differences do exist, these microtitre plate assays are not sensitive enough to detect differences in the levels of cell binding.

**Figure 3.9: The effect of hydrophobic surfaces on cell attachment level of *Listeria* cells grown in BHI and MCDB 202**




*Listeria* cells were allowed to grow BHI or MCDB 202overnight at 37°C. 200µl of the undiluted overnight culture (High inoculation) or the diluted samples ($OD_{600nm}$ at 0.6 –Low inoculation) were transferred to individual well of an Immuno PolySorp plate. The plates were placed at 37°C for 0, 1, 2, 4, 6 or24 h. The media were removed from the plate, and wells were washed with 200µl of PBS three times (section 2.12). Crystal violet (CV; 200 µl of 0.1% solution) was added to the wells for 15 min. CV solutions were removed and the wells were washed with PBS three times. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 400 nm. Error bars indicates the standard deviation calculated.

**Figure 3.10: The effect of hydrophobic surfaces on Biofilm formation level of *Listeria* cells grown in BHI and MCDB 202**



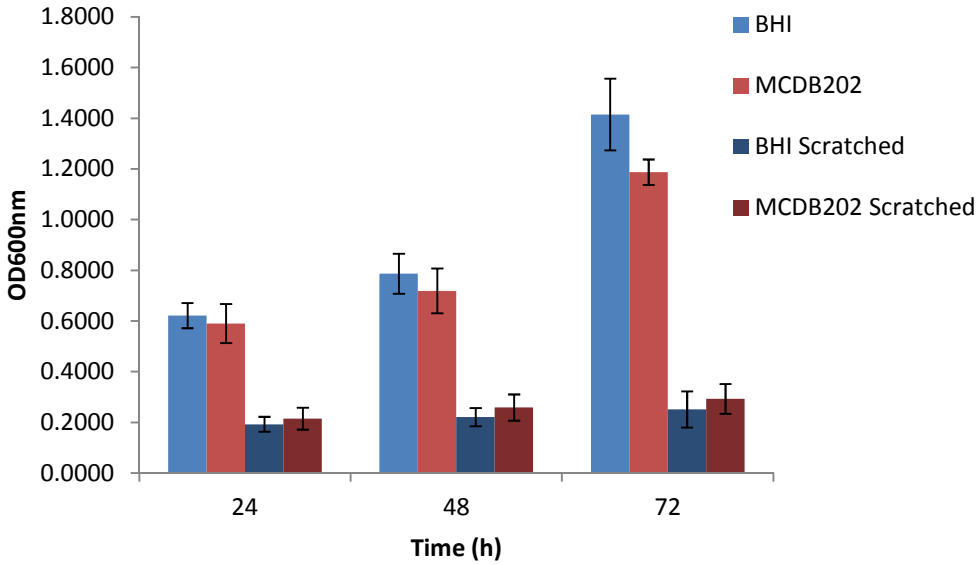

*Listeria* EGD cells were grown in 5 ml LB media overnight. Cells were diluted into the two media to be tested at $A_{600nm}$of 0.8. 200 µl of the sample were transferred to each well of Immunopolysorp plate (BHI Polysorp and MCDB polysorp, respectively) as well as into the wells of a standard normal microtitre plate (BHI and MCDB) as a control. The plates were placed at 37 °C for 24, 48, and 72 h. The media were removed from the plate, and wells were washed with 200µl of PBS three times (section 2.11). Crystal violet (CV; 200 µl of 0.1% solution) was added to the wells for 15 min. CV solutions were removed and the wells were washed with PBS three times. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 400 nm. Each test condition was performed in triplicate. Error bars indicates the standard deviation calculated.

### 3.6 Vertical surface attachment test

During the biofilm assay described in section 3.3, it was noticed that before dissolving the crystal violet in ethanol there appeared to be a difference in the pattern of crystal violet stain on wells containing MCDB202 and BHI cultures. In wells containing *Listeria* grown in BHI the crystal violet was mostly seen to be settled at the bottom of the well whereas only a very light stain was seen at the bottom of the MCDB202 culture wells. However once the stain was solubilised the amount of stain measured was not as different as expected from this visual observation. One reason for this could be that the distribution of the attached material was very different in the two samples, with the material in the MCDB sample being more dispersed and less on the bottom.

To test this, the experiment was repeated but after the first PBS wash, and before the ethanol was added, the CV stain at the bottom of all well was scratched off using a small scraper to remove all the visible CV stain on the bottom of the well . The wells were then further washed with PBS twice. The aim of this was to try and assess the level of biofilm formation on the vertical surfaces of the well alone, excluding the material that sediment at the bottom of the wells.

**Figure 3.11: Vertical attachment of *Listeria* cells grown in MCDB 202 and BHI broth**




*Listeria* EGD cells were grown in 5 ml LB media overnight. Cells were diluted into the two media to be tested at $OD_{600nm}$ of 0.8. Samples (200 µl)were transferred to each well of ImmunoPolySorp plate. The plates were placed at 37 °C for 24, 48, and 72 h. The media was removed from the wells, and these were washed with PBS. Crystal violet (CV; 200 µl of 0.1% solution) was added to the wells for 15 min. CV solutions were removed and the wells were washed once with 200 µl of PBS. The CV stains at the bottom of the well were removed by scratching off the crystal violet stains seen on the bottom of the well with a fine scraper. The wells were then further washed with 200 µl of PBS twice. Absolute ethanol (200 µl) was added to the wells and absorbance measured at 600 nm. Each measurement was performed in triplicate. Error bars indicates the standard deviation calculated.

From the results presented in Figure 3.11 it is clear that most of the CV stain in both samples was settled at the bottom of the wells, since the overall CV values dropped dramatically when this area of staining was removed prior to solubilisation. One possibility to explain this is cell settlement results in enhanced biofilm formation at base of well and indicates that most biofilm is formed at the bottom of the wells rather than vertical well surfaces.

Considering the vertical attachment level of the samples with cells in MCDB 202 and BHI, they were actually quite comparable in level, giving an absorbance value of about 0.2, indicating the ability to attach to vertical surface were very similar in cells grown in the two media. This was different from the results of the normal CV biofilm assay, highlighting that the higher biofilm level seen in cells grown in BHI were possibly caused by a higher cell mass settling at the bottom of the well and perhaps indicating either that the cells grown in MCDB 202 were remaining in suspension longer than those grown in BHI. However this could also just reflect the fact that a lower cell number were in the well.

### 3.7 Discussion

From the result, it can be seen that growth in one specific defined media does cause changes in the *L. monocytogenes* cells resulting in an increase in cell hydrophobicity. However, from the results using the attachment assay and biofilm assays, there is no evidence showing that cells grown in MCDB 202 have better biofilm or cell attachment capabilities compared to cells grown in BHI Broth, even when the cells were tested using a specialized hydrophobic surface. However it was also seen that the most biofilm in the BHI culture was formed where the cells formed as sediment in the bottom of wells and that there were no observable difference in the vertical attachment seen in the two culture media. However since it was likely that the culture density in MCDB 202 was lower than the BHI cultures, this could indicate that the cells were better able to attach to the surface of the wells.

In this chapter, the main finding was the enhanced cell hydrophobicity seen when the *Listeria* cells were grown in MCDB 202, which provides a link to the EPS production observed microscopically and suggests that producing this material changes the cell surface hydrophobicity. However, in other organisms EPS has also been shown to enhance attachment and hence increase biofilm formation (Flemming *et al.*, 2007, Wingender *et al.*, 1999) and cells having higher surface hydrophobicity have been shown to have better attachment (Takahashi *et al.*, 2010). However this correlation was not seen in our experiments.

In the MATH assay, it was shown that increasing the amount of N-octane increases the affinity of cell attaching to hydrocarbon. This was also reported by Rosenberg (2006). He also pointed out another consideration in the MATH

assay, suggesting that cells may form a "girdle" around the oil and water interface instead of partitioning completely into the hydrocarbon phase due to their amphipathic nature (Rosenberg, 2006). This idea is supported by microscopic pictures from other researchers in this group showing cells concentrated at the surface of oil droplets following partitioning into solvents in what is described as a cream layer (Nwaiwu, 2010).

Briandet *et al*. (1999) studied the hydrophobicity change of *Listeria* Scott A cells grown in different media. They showed that a supplement of 7.5 g glucose or 1N lactic acid per litre of TSYE medium resulted in a nearly two-fold increase in affinity to hydrocarbons in the MATH assay. However, the reason to for this was not stated. As lactic acid is a by-product in fermentative metabolism in *Listeria*, it is not clear if this would affect the result in our experiment. On the other hand, in the preparation of MCDB 202 media, 3.6g of glucose is added as a supplement per litre of the preparation. However glucose is also present in BHI broth at $2gL^{-1}$. It was not tested whether the slightly higher glucose content present in the MCDB 202 media causes the increase in hydrophobicity in the cells, but this seems very unlikely, especially as growth in more limited in MCDB 202 compared to BHI. Since we are comparing hydrophobicity of cells in MCDB 202 and BHI broth, which have such a great difference in the content, it is hard to identify the role of specific components in this way.

Mafu *et al.* (1991) investigated the physicochemical forces involving the adhesion of *L. monocytogenes* to surfaces. The research group used 22 various *L. monocytogenes* strains and compared the relative surface hydrophobicity with the salt aggregation test, which is another way to test for cell hydrophobicity under salt solvent. They showed that a decrease in the pH

of the medium caused an increase in cell hydrophobicity which was possibly due to a change in electrostatic forces. However, here the role of pH was investigated and it was shown that the enhanced cell hydrophobicity was not caused by the lower pH of the MCDB 202 media used in the early stages of the work.

When this work was initiated it was expected that the higher hydrophobicity of cells grown in MCDB 202 would induce better attachment ability, and hence increase biofilm formation. In the attachment assay, the level of attachment in both cell samples was similar in the first few hours and in the biofilm assay, it was shown that cells grown in BHI produced more but this seemed mostly influenced by cell number in the wells. Cell attachment and biofilm formation are affected by many individual factors, such as nutrient content and the nature of the attaching surface. The lack of difference seen in these studies may be a combination effect of different factors, in particular the difference in cell mass achieved in the two tested media. Although the initial inoculation level can be standardized, cell growth rate in the two media were quite different, causing a variation in the cell number of the two tested media at the end of the assay incubation period. However it is very difficult to eliminate this problem in the experiment design. It is important to consider that there is such a cell mass variation among experimental samples.

Some workers have shown that cell attachment may be independent of the cell density (Mai and Conner, 2007) but the evidence from this work suggests that for *Listeria* this is not the case. Other workers have shown that biofilm formation is greatly affected by nutrient availability (Stepanović *et al.*, 2004, Kim and Frank, 1995, Stoodley *et al.*, 1998, Andrew, 2005). Despite the similarity in glucose levels in the two media the growth rate indicates that

MCDB 202 is nutrient limiting for *Listeria* and this seems to be a crucial factor in induction of the EPS.

It was shown in work of Tresse *et al*. (2006) that the adhesion capability of *L. monocytogenes* was greatly reduced when culturing cells at pH5 rather than a more normal pH 7 condition. However contradictory results were reported by other research groups. Smoot and Pierson (1998) studied the cells adhered to rubber in sterile phosphate buffer conditions at various pH values from pH4 to 9. They showed that the levels of attached cells achieved were lower when attachment occurred under alkaline conditions. However in this case rather than pH it was noted that the bacterial growth rate was more important than the different pH of the two media. This finding means that different results between studies may be mainly due to different experimental approaches, including growth media which cause changes in factors such as growth that are more important than the pH of the environment.

The material of the surface is also known to influence cell attachment and biofilm formation of cells. It was shown that bacteria are more likely to attach to a more hydrophobic material (Sinde and Carballo, 2000). However, research has also shown that *Listeria* was better able to attach to stainless steel (hydrophilic) than PVC surfaces (Chavant *et al.*, 2002). To rule out the role of EPS in surface attachment, more studies are needed to determine the kinetics of attachment and biofilm formation of *Listeria* grown in MCDB 202 to different surfaces. Further experiments could be done using glass or stainless steel surfaces which have different hydrophobic nature than PVC. However time was not available in this study to fully answer these questions, especially as this was not the main focus of the work.

# Chapter 4

# Investigating the biological role of *Listeria* EPS

## 4.1 Introduction

In Chapter 3, the effect of the EPS on biofilm formation, attachment and cell hydrophobicity was investigated and, although a change in surface hydrophobicity was detected, no biological role was identified. In order to provide a deeper understanding of the effect of this capsule-like extracellular substance on the properties of the cell, some further aspects of cell biology were studied.

It has been shown that the biofilm matrix provides a degree of protection for the cells towards many harmful substances, such as antibiotics, antimicrobial substances and disinfectants (Costerton *et al.*, 1995, Watnick and Kolter, 2000). It is also the case that many bacterial capsules have an involvement in the invasion process (Sahly *et al.*, 2000, Campos *et al.*, 2004, Roberts, 1996). So the work presented in this chapter was aimed to determine if the capsule-like layer identified on *Listeria* confers protection to the cells against antimicrobial challenges.

## 4.2 Bile salt

Bile is found in the GI tract of mammals where it helps emulsify the fats in the food to aid better digestion, and it also acts as a protective defense mechanism against the invasion of external pathogens present in food (Begley *et al.*, 2005). A number of bacteria have been shown to have resistance or tolerance towards bile. This was seen in *L. monocytogenes*, which has been shown to be able to infect the gallbladder (bile storage organ) (Sleator *et al.*, 2009, Dowd *et al.*, 2011). It was also found that a deletion mutant of a *capA* gene (*Bacillus* PGA capsule synthesis gene) homologue *lmo0516* in *Listeria* has impaired the resistance towards bile (Begley *et al.*,

2002). It would be logical, if bile resistance was linked to capsule formation, that this gene had a role in capsule biosynthesis. So an investigation was performed to determine whether growing the *Listeria* cells in conditions known to induce capsule production (minimal media) had an effect on the bile resistance of wild type *L. monocytogenes* cells.

To do this the cells were treated with bile and then the survival rates determined. The method used was based on that described by Begley *et al*. (2002). *L. monocytogenes* EGD cells were grown overnight in 5ml MCDB202 or BHI. Cells were then centrifuged and resuspended in PBS with 30% (w/v) Bovine Bile. Samples were incubated in 37 °C for 5 min and then the cells collected by centrifugation and washed with PBS to remove the remaining traces of the Bovine Bile (see section 2.16). The number of surviving cells was determined by serial dilution using the Miles Misra technique.

From the results presented in Table 4.1 it is clear that cells grown in BHI had a better resistance towards bile salts than did the cells grown in the defined media. This indicates that there is no enhanced bile protection seen in the cell grown in MCDB202. Since the *Listeria capA* homologue mutant was reported to be more sensitive to Bile salts, if this gene was linked to EPS formation, it would be expected that the cells grown in MCDB 202 would have shown increased resistance to the bile salts. Indeed the cells grown in MCBD 202 seemed to be more sensitive to bile salts by at least a factor of 10. This may be possible due to the limited nutrients.

**Table 4.1: Effect of Bile salt treatment on *L. monocytogenes* survival**

| Growth medium | % survival |
|---|---|
| BHI | 0.12 ± 0.040 |
| MCDB202 | 0.0045 ± 0.0015 |

*L. monocytogenes* EDG cells were grown overnight in 5ml MCDB202 or BHI. Cells were then centrifuged and resuspended in PBS with 30% Bovine Bile (Sigma). Samples were incubated in 37 °C for 5 min and then the cells collected by centrifugation and washed with PBS to remove the remaining traces of the Bovine Bile (section 2.16). The cell counts were determined by serial dilution using the Miles Misra technique. The percentages of survival were calculated as the % of cells in the bile-treated sample relative to the untreated control samples. The experiment was repeated for 3 times. Error was calculated as standard deviation.

To complete this study, the *lmo0516* mutant strain was requested from the research group that described it, but unfortunately it was reported that the strain could not be recovered from frozen culture (Dr C. Gahan, University College Cork, Ireland, pers. comm). This indicates that perhaps cell physiology was generally adversely affected by this mutation which resulted in its bile sensitivity, rather than being related to a specific phenotype such as EPS production.

### 4.3 Effect of EPS on Nisin and Lysozyme sensitivity

Nisin is an antibacterial substance in the form of short peptide comprising 34 amino acid residues, some of which are post-translationally modified to form lanthionine structures (Cheigh and Pyun, 2005). It is produced by bulk fermentation using *Lactococcus lactis*. It targets Lipid II, a precursor required in cell wall formation, to form a complex and is then inserted into the cell cytoplasmic membrane. This forms ion channels or pores, which results in dissipation of the membrane potential of bacteria cells (Stevens *et al.*, 1991, Bruno *et al.*, 1992, Chu *et al.*, 2010).

Lysozyme is another common preservative used in the food industry. It is also naturally found in mammal secretions, such as human tears or milk. It is an enzyme that attacks the peptidoglycan of Gram-positive bacteria by hydrolyzing the 1,4-beta-linked glycosidic bond between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of the cell wall components (Hughey and Johnson, 1987).

Nisin resistance has been reported in *L. monocytogenes* for decades, and researchers have already suggested the possibility of *Listeria* resistance arising in nisin preserved food (Davies and Adams, 1994, Delves-Broughton *et al.*, 1996). *Listeria* cells resistant to nisin were seen to have alterations in both the cytoplasmic membrane and the cell wall (Crandall and Montville, 1998). They have shown that the nisin-resistant cells could continue grow in the presence of nisin. This suggests that the use of a combination of preservatives should be used to reduce the chance of *Listeria* nisin resistance arising. In contrast, a recent publication has shown that *Listeria* has a certain amount of natural intrinsic resistance to low concentrations of

lysozyme (Boneca *et al.*, 2007) but lysozyme was still found to be quite effective towards *Listeria*. Hence it is often suggested that lysozyme and nisin be used together to achieve the best control of *L. monocytogenes*.

As both of these chemicals require intimate contact with the cell surface to have an effect, experiments were performed to test if the EPS seen when *Listeria* grown in minimal media provides protection against these two antimicrobial chemicals.

### 4.3.1 Effect of EPS on Nisin sensitivity

To do this *L. monocytogenes* EGD cells were grown overnight in either 5ml MCDB202 or BHI. The cells were collected by centrifugation and resuspended in PBS (section 2.15). The samples were diluted to approximately $10^7$ cfu $ml^{-1}$, with the actual cell count being determined by serial dilution and viable count. Nisin was added at three different final concentrations (2.5, 0.25 and 0.125 mg $ml^{-1}$), which were those used in a previous study of nisin resistance in *Listeria* cells by Davies and Adams (1994). The cells were incubated at 37°C and then samples taken every 30 min. The cells were recovered by centrifugation and resuspended in MRD to help dilute out the antimicrobial agent. The cell counts were determined by serial dilution and viable cell count.

From the results presented in Figure 4.1 and Table 4.2, it was seen that the concentration of nisin used affected the rate of *Listeria* killing. At the highest concentration there was no difference between the cells irrespective of the growth media used. However at lower concentration, those cells grown in MCDB202 were more sensitive to nisin than cells grown in BHI media,

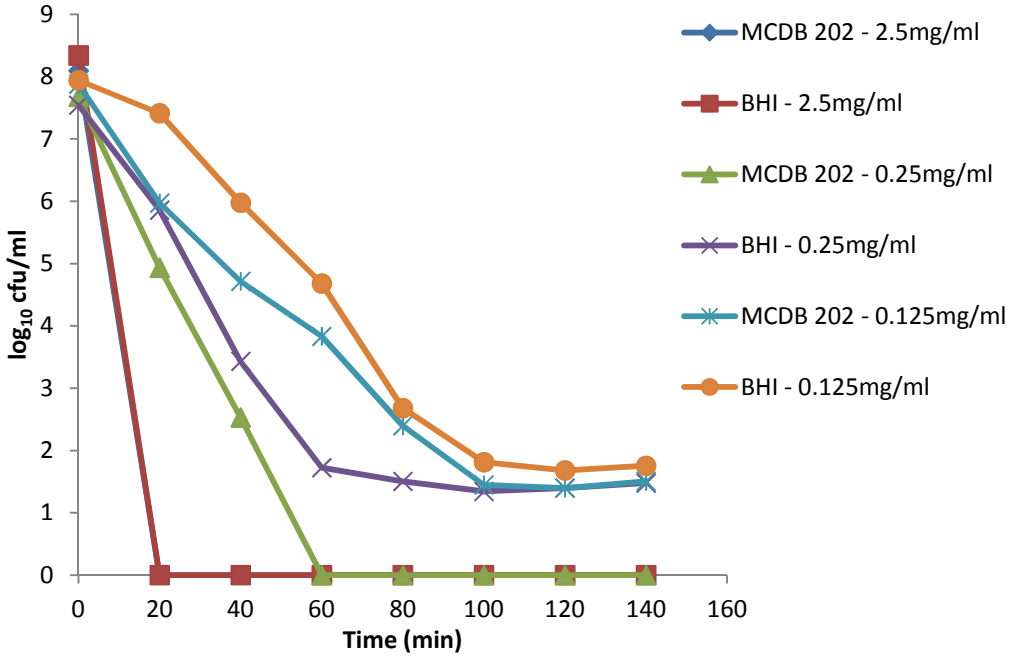
indicated by the higher residual population found when a concentration of 0.25 mg $ml^{-1}$ lysozyme was used, and a slightly faster death rate over the 1$^{st}$ hour of the experiment when 0.125 mg $ml^{-1}$ lysozyme was used. At the lowest nisin concentration, about nearly 2 $log_{10}$ cells survived after the nisin treatment. However, when the cells were treated with high nisin concentration, no *Listeria* survived. This indicates that either the nisin concentration was a sub-lethal challenge that the cell could tolerate as it could repair the damage being inflicted, or the nisin had not reached a threshold concentration that was sufficient to kill all the cells. However the results suggest that the extracellular substance formed on the surface of cells grown in MCDB202 does not give additional protection to the *Listeria* cells against that action of nisin.

**Table 4.2: D-values and survival rate of *L. monocytogenes* cells treated with Nisin**

| Growth media/treatment | D value (min) | Survival (log value) |
|---|---|---|
| BHI broth - 0.25 mg $ml^{-1}$nisin | 9.52 | 1.505 |
| MCDB 202 broth - 0.25 mg $ml^{-1}$ nisin | 8.33 | - |
| BHI broth - 0.125 mg $ml^{-1}$nisin | 20.05 | 1.755 |
| MCDB 202 broth - 0.125 mg $ml^{-1}$nisin | 17.78 | 1.477 |

D-value was calculated as the time to allow one $log_{10}$ reduction in cell count. This was calculated with the slope of the curve in the units of min at the early stage of killing (time points between 20-60min). The survival counts were measured as the cell counts become steady at the later stage of treatment (time points between 100-140min). The experiment was done three times.

**Figure 4.1: The effect of minimal media on *Listeria* sensitivity towards Nisin**



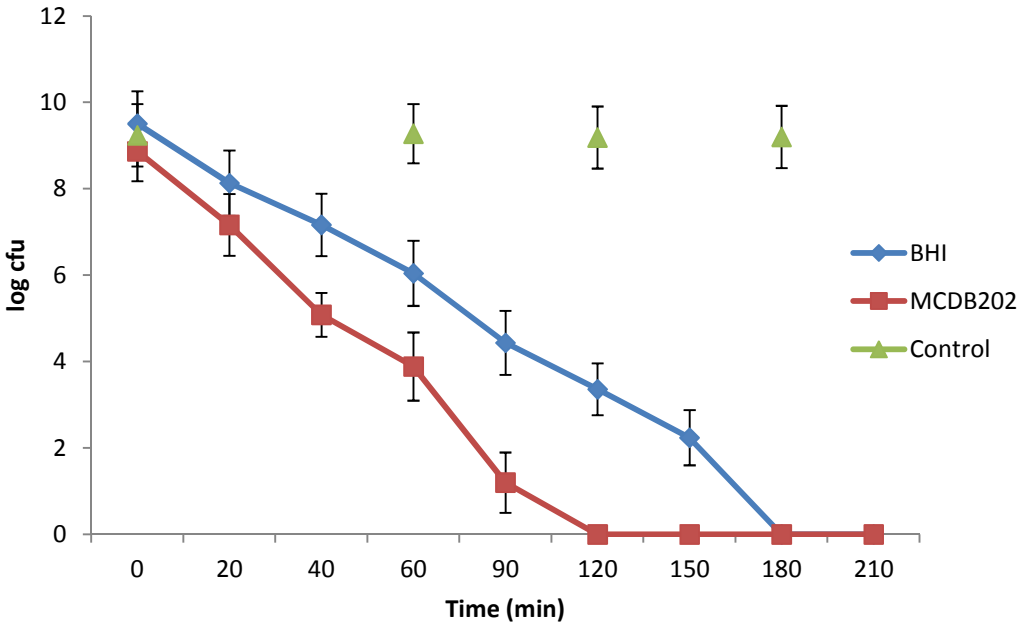

*L. monocytogenes* EGD was grown overnight in either 5ml MCDB202 or BHI broth, then cells were collected by centrifuging and resuspended in 5 ml PBS. The samples were diluted to approximate $10^7$cfuml$^{-1}$ and the actual cell count being determined by serial dilution and viable count. Nisin was added to the samples at t=0 at three different final concentrations (2.5, 0.25 and 0.125 mg ml$^{-1}$). The cells were incubated at 37°C and then samples taken every 30min. The cells were recovered by centrifugation 5000g for 10 min and resuspended in 5 ml MRD to help dilute out the antimicrobial agents. The cell counts were determined by serial dilution and viable cell count in LB agar. The legend of the graph indicate the cell culture media and the concentration of nisin added to each of the samples.

### 4.3.2 Effect of EPS on lysozyme resistance

To investigate the effect of EPS production on lysozyme treatment, cells were grown overnight in 5 ml MCDB202 or BHI broth, then recovered by centrifugation and resuspended in PBS. The samples were diluted to approximate $10^7$ cfu $ml^{-1}$ and the cell count determined using serial dilution and viable count. Lysozyme was added to the cells to a final concentration of 50 mg $ml^{-1}$ which was the concentration reported by Gaddipati (2007) to be effective at killing *Listeria* cells during tissue culture invasion experiments. The cells were then incubated at 37°C and samples were taken every 30 min. The cells were recovered by centrifugation and resuspended in MRD to wash out the lysozyme. The survival rate was determined by measuring the viable count (see section 2.14).

In contrast to nisin, lysozyme was found to be quite effective at killing *Listeria* since no residual population of resistant cells was seen. Again, from the graph (Fig. 4.2) it can be seen that cells grown in MCDB202 were more sensitive towards lysozyme than cells grown in BHI media since the D-value recorded (Table 4.3) was lower for these cells. Therefore it seems that the extracellular substance formed on the surface of cells grown in MCDB202 does not provide protection of the *Listeria* cells towards lysozyme.

**Figure 4.2: The effect of EPS on *Listeria* sensitivity towards lysozyme**




*L. monocytogenes* EGD was grown overnight in 5ml MCDB202 or BHI broth. Cells were centrifuged at 5000*g* for 10 min and resuspended in 5 ml PBS. The samples were diluted to approximately $10^7$cfu $ml^{-1}$ with the cell count estimated by viable count. Lysozyme was added to a final concentration of 50mg$ml^{-1}$. A sample (1ml) of each cell suspension was removed every 30min. The cells were recovered by centrifugation at 3000*g* for 5 min and resuspended in MRD (5 ml) with no lysozyme added. The cell survival was determined by viable count on LB agar. The experiment was performed in triplicate. The error bars indicate standard deviation calculated

**Table 4.3: D-value of *Listeria monocytogenes* EGD cells treated with lysozyme**

| Growth media | D-value (min) |
|---|---|
| BHI broth | 20.0 |
| MCDB 202 broth | 13.8 |

The D-values were estimated as the time taken to give one $\log_{10}$ reduction in cell count during treatment.

Therefore, no evidence was uncovered of a role for the EPS in the protection of *Listeria* against challenge with any of the antimicrobial agents tested (i.e. bile salts, nisin or lysozyme). Similarly, no evidence was found from the results presented in Chapter 3 that it plays a role in the adhesion of these cells to surfaces. Hence it must be assumed that the EPS has some other biological function. The next tests described were performed to see if the EPS played a role in either evading virus infection or whether production of EPS is significant during infection of eukaryotic cells.

## 4.4 Effect of culturing L. monocytogenes in MCDB202 on Phage infection

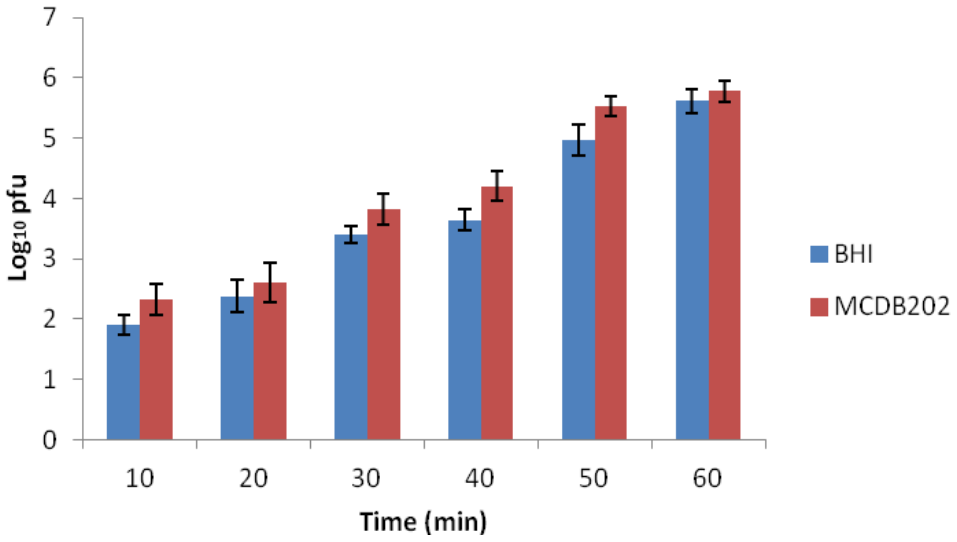
Bacteriophages are viruses that infect bacteria. They attach on to the bacterial cell surface and infect the cells with various mechanisms (Young, 1992). Many *Listeria* phages have been isolated and characterized and because of their natural abundance in nature, phage infection is one of the most common challenges faced by bacterial cells living in the natural environment. Capsules have been shown to give protection to bacterial cells against phage infection by blocking access to surface receptors required for the first stage of phage binding to the host cell (Bernheimer and Tiraby, 1976, Hyman and Abedon, 2010). Hence another possible role for the *Listeria* EPS is to protect the cells again phage infection. Hence the effect of EPS production on phage infection of *Listeria* cells was also investigated.

To monitor the absorption rate of phage to a cell surface, experimenters normally use an antibody to specifically inactivate any phages that have not infected the cell. However such an antibody was not available. Hence a modification of a new phage-based detection method for *Listeria* (El-Emam and Rees, University of Nottingham, unpublished) was used to enable the phage adsorption kinetics to be followed. This assay uses tea extracts such as those described by de Siqueira *et al*. (1996) to chemically inactivate the bacteriophage rather than using an antibody. The phage used is the well characterized broad host range lytic phage A511 (Guenther *et al.*, 2009).

The aim of the experiment was to determine the infection rate of *Listeria* phage into *L. monocytogenes* cells grown in BHI or MCDB202 media. This was done by growing the cells in the two media overnight at 37 °C. The culture was then diluted to an approximate cell density of $10^7$cfu $ml^{-1}$. Phage A511 was added to the sample at an M.O.I. of 10. Samples were taken every 10 min and treated with tea extract (section 2.18.2) for 15 min to inactivate any phage that had not entered the host cells. The samples were then diluted and samples of each dilution were plated on to a *Listeria* lawn. The plates were incubated in 30°C overnight and then the numbers of plaques (representing individually infected cells in the original sample) were counted.

The result in Figure 4.3 shows that although with a slightly higher infection rate seen in cells grown in MCDB202, statistical tests indicated that the results were not significantly different (p-value > 0.05). Indeed the fact that the infection rates did not show great different in both conditions suggests that the phages infection were not affected by growing the cells in MCDB 202.

**Figure 4.3: Effect of EPS on phage infection**




The *L. monocytogenes* EGD cells were grown in MCDB202 or BHI broth overnight, and then the cells were pelleted at 5000g for 10 min and resuspended into lambda buffer to an approximate cell density of $10^7$cfu $ml^{-1}$. Phage A511 was added to the sample at M.O.I. of 10 (multiplicity of infection; 10 phage particles per cell). Every 10 min, samples (1 ml) were taken and treated with tea extract (section 2.18.2) for 15 min to inactivate any phage that had not entered the host cells. The samples were then diluted to a series of 10 fold dilution and samples of each dilution were plated on to a *Listeria* lawn (see section 2.18.3). The plates were incubated in 30°C overnight and then the numbers of plaques (representing individually infected cells in the original sample) were counted.

## 4.5 Cell invasion assay

Camejo *et al*. (Camejo *et al.*, 2009) published a study that showed that the expression level of several *L. monocytogenes* genes, including *lmo0516,* was increased during infection of mouse spleen. As there was a possibility that this gene was linked to capsule formation, it was decided to investigate whether EPS formation has an effect on cell invasion.

To determine this, *L. monocytogenes* EGD was again grown in the two media, BHI and MCDB202, and then these cells were used to infect the human cell line Caco-2 (see section 2.17). These infection assays are normally carried out using gentamycin to ensure that cells that do not become internalized are killed. However previous work has shown that there are problems with this method as the antibiotic can enter the host cells and kill the internalized bacteria, leading to an underestimate of cell numbers (Drevets *et al.*, 1994). As had been previously used successfully in cell invasion assay by other workers in the research group (Gaddipati, 2007), and lysozyme was shown to have a good activity against the *L. monocytogenes* cells in section 4.3, it was used as the antimicrobial agent in the invasion assay in this study. Cells that are able to invade the Caco-2 cells will be protected against the activity of the lysozyme. To determine how many cells have been able to infect the host cells, these are lysed, the internalised *Listeria* cells are released and the number of released cells is used to estimate the efficiency of cell invasion.

The cell invasion was performed as described by Gaillard (Gaillard *et al.*, 1987). Caco-2 cells were allowed to grow to 80% confluence and transferred to 6 well titre plates (section 2.17.3). *Listeria* cells were grown overnight in

MCDB202 and BHI and then they were pelleted and resuspended in PBS. The Caco-2 cells were infected with *Listeria* cells with an initial M.O.I. of 100 bacteria per cell. The plates were incubated for 30 min at 37 °C to allow internalisation. Any remaining external cells were removed, and the wells were washed with PBS before lysozyme (50 mg $ml^{-1}$) was added to kill the external remaining *Listeria* cells (section 2.17.5). The Caco-2 cells were lysed to release the internalised Listeria cells. The viable bacteria counts were determined and the percentage invasion calculated relative to the original cell count. Percentage of invasion of MCDB 202 cells and percentage of BHI cells invasion ratio (%M/%B) was also estimated to give comparison on the invasion level of MCDB202 cells and BHI cells.

The invasion assay (Table 4.4) showed that *L. monocytogenes* EGD cells grown in BHI were more invasive than those grown in MCDB202, and on average were 1.75-fold better at becoming internalised. In other words, cells grown in MCDB 202 medium were less able to infect eukaryotic cells. Given the day to day variation seen in these result, the fact that the relative infection ratios (%B/%M) were consistent among the three sets of data suggests that this is a robust conclusion. This indicates that there could be changes in surface properties that is detrimental to the ability of the cells to attach and/or invade the eukaryotic cells.

**Table 4.4: The effect of EPS production on the cell invasion assay**

| | % Invasion | | Ratio |
|---|---|---|---|
| | MCDB202 | BHI | M%/B% |
| Set 1 | 0.034 | 0.051 | 0.67 |
| Set 2 | 0.037 | 0.061 | 0.60 |
| Set 3 | 0.024 | 0.050 | 0.47 |
| **Average** | **0.032** | **0.054** | **0.58** |

The Caco-2 cells (section 2.17.3) were infected with *L. monocytogenes* EGD using an initial M.O.I. of 100 bacteria per cell. The plates were incubated for 30 min at 37 °C in 5% $CO_2$ and then the wells were washed with PBS. 1ml of lysozyme (50 mg $ml^{-1}$) was added to the wells and samples incubated for 20 min. The wells were washed again 3 times with PBS and then the Caco-2 cells were lysed by adding 1 ml of 0.5% Triton- X100 to the wells and incubating at 4°C for 15 min. (see section 2.17.5). The viable count was determined using the Miles Misra technique on LB agar. The % of invasion was calculated as the % of the recovered cell against the original cell count. %M/%B is the percentage of infection of MCDB202 cells relative to BHI samples.

It has been reported in the literature that bacterial capsules, such as polysaccharide or polyglutamate capsules, act as a physical barrier that prevents recognition and engulfment by non-specific phagocytes (Urban *et al.*, 2006, Wilson *et al.*, 2002). In the case of *Listeria* infection, the Inl proteins have to come into contact with the specific host cell surface receptor to induce up take and infection of non-professional cells, and any physical barrier may also hinder Inl protein contact with these receptors. Hence it is possible that the EPS capsule formed by the *L. monocytogenes* cells grown in MCDB 202 would have hindered the internalizing of the *Listeria* cells, making the cells less infectious toward eukaryotic cells.

## 4.6 Discussion

The idea of the testing the effect of EPS protecting the cells against bile treatment or having a role in cell invasion was first initiated by the report showing that *lmo0516* mutation were shown to have reduction in bile resistance and also *lmo0516* were over expressed in cell invasion process (Camejo *et al.*, 2009, Begley *et al.*, 2002). In this chapter, it was shown that *L. monocytogenes* cells grown in MCDB202 were generally more sensitive to bile, nisin and lysozyme treatments as well as phage infection showing that the EPS produced does not act as a simple physical barrier protecting the cells. However cells grown in MCDB202 did seem to be physically weaker than cells grown in BHI. The difference in nutrient content among the two media may affect the growth and formation of the cell wall of the bacteria cells. It was seen also in *Staphylococcus aureus* that cells grown in long term starvation will show adaptation to nutrient condition, and will differentiate into smaller and weaker cells. It may also be the case for *Listeria* (Watson *et al., 1998)*.

It was also seen that mutation in pgdA, responsible for wall-associated De-N-acetylase, may not be lethal in normal condition of Listeria cells but cells were shown to be more sensitive towards autolysis inducing agents such as EDTA CAMP etc. suggesting that alternation of cell wall formation and structure may affect cell sensitivity towards bactericidal agents (Popowska *et al.*, 2009). In *Listeria innocua*, acid stress causes a change in the lipid composition of cell wall, with a decrease in anteiso fatty acid content, and also causes a change in sensitive towards killing agents, quaternary ammonium compound. This was further linked to an increase of cell hydrophobicity. All these suggest that media condition is greatly related to bacteria cell wall composition and hence cell strength and cell properties (Moorman *et al.*, 2008).

Results from the bile resistance assays suggested that the bile-resistance of *lmo0516* may not be linked to the EPS formation, although to formally prove this conclusion it would be necessary to reconstruct the same *capA* mutation described by Begley *et al.* (2002) since the original mutant was not recoverable. However the cells also displayed a reduced ability to infect eukaryotic cells and if *lmo0516* is up-regulated during cell invasion, this result does not seem to be consistent with the idea that *capA* is linked to EPS biosynthesis. However in the literature it is still not clear how the *lmo0516* is responsible for bile tolerance or why it is up-regulated following cell invasion as the initial reports by Begley *et al.* (2002) and Camejo *et al.* (2009) describing these phenomena have not been followed up.

# Chapter 5

# Effect of growth media on

# *Listeria monocytogenes* AI-2 production

# and

# the effect of DPD on

# *Listeria monocytogenes*

# cell physiology

## 5.1 Introduction

One of the common research areas in microbial biofilm formation is a link to quorum sensing (Hardie and Heurlier, 2008). Quorum sensing (QS) is described as the process where bacteria - individuals or populations - communicate by the use of certain signalling molecules produced by the cell (Rickard *et al.*, 2006). A wide range of studies have shown that biofilm formation is directly linked with the gene *luxS* and autoinducer-2 (AI-2) production. Various groups have been working on different *luxS* mutant bacteria and the AI-2 signalling system, including in *L. monocytogenes*. Sela *et al.* (1996) showed that detectable AI-2 activity was diminished in a *luxS* mutant of *L. monocytogenes*, demonstrating that the signalling molecule is produced by this organism. In this work they also showed that the *luxS* mutants were able to build up thicker and denser biofilms and hence were better able to attach to surfaces. However exogenous AI-2 was not able to restore the phenotype of the deletion mutant, indicating that the regulation of this phenotype may be complex (Sela *et al.*, 2006). Despite this, these results showed that there is a link between AI-2, LuxS and biofilm formation in *L. monocytogenes*.

Our observations concerning the prouction of EPS material when *Listeria* is grown in minimal media. Chavant *et al.* (2002) demonstrated that *L. monocytogenes* LO28 cells grown in MCDB202 were better able to produce biofilm than in rich medium. Taken with the observation that biofilm production is related to the production of AI-2, these two results could be related and may provide an insight into the basis of the quorum sensing regulation of biofilm formation through changes in cell metabolism, and AI-2

production by LuxS, when cells are grown in minimal media rather than rich media. So the aim of the work presented in this chapter was to test whether the level of AI-2 production changed when cells were grown in defined media compared to levels produced in a nutrient-rich media (BHI). This was followed by a number of experiments using artificial AI-2, studying its effects on cell growth, biofilm formation and hydrophobicity.

## 5.2 AI-2 Bioassay

The AI-2 bioassay was introduced by Bassler *et al.* (1997). The basis of the assay is the use of the marine, bioluminescent bacterium *Vibrio harveyi* as a bio-reporter. This has been used as a reporter to detect the level of AI-2 in solutions or media (Bassler *et al.*, 1993, Bassler *et al.*, 1997, Turovskiy and Chikindas, 2006). *V. harveyi* BB120 is a wild type strain which produces both AI-1 and AI-2 signalling molecules. *V. harveyi* BB170 is an AI-1 sensor mutant used as a reporter in the bioassay that is only able to produce bioluminescence in response to exogenous AI-2. During the bioassay, samples of supernatant or media to be tested are added to the cultures of the reporter strain *V. harveyi* BB170. The light production by the *V. harveyi* gives an estimation of the relative AI-2 levels present in the test sample.

**Figure 5.1: Diagram of autoinducer bioassay with the use of *Vibrio harveyi* BB170 reporter**

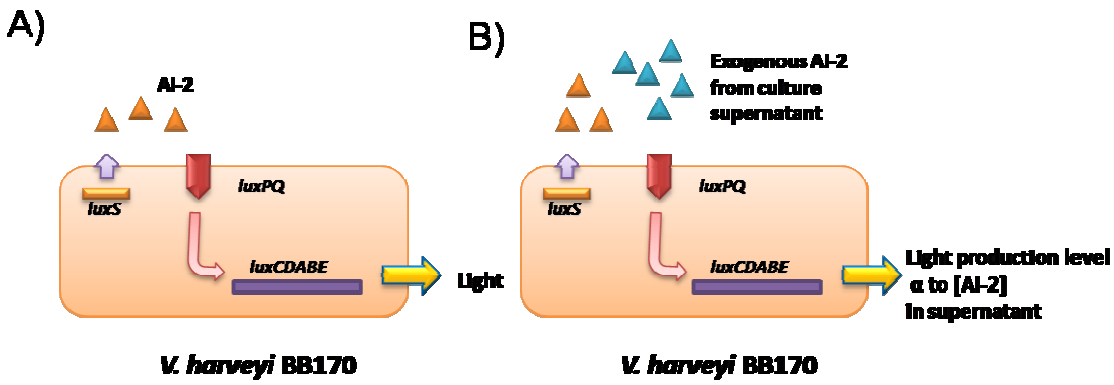



Diagram shows the basic principle of the autoinducer bioassay. Panel A shows the AI-2 induction mechanism of *V. harveyi* without addition of exogenous AI-2. AI-2 is produced by LuxS and is exported out of the cell as an autoinducer signalling molecule. This is detected by LuxPQ, the AI-2 sensor. Panel B shows the situation that occurs when exogenous AI-2 is added during the bioassay. As the concentration of AI-2 in the culture increases, the induction of bioluminescence also increases. The relative increase in AI-2 provides an estimation of the relative amount of exogenous AI-2 added to the culture of reporter bacteria.

### 5.2.1 Establishing the reporter strain assay

Initially the bioluminescence response of the reporter strain *V. harveyi* BB170 was tested using different concentration of natural AI-2 produced by bacteria. This was done by preparing samples of *V. harveyi* BB120 culture supernatant which is known to contain levels ofAI-2 detectable by the reporter (Bassler *et al*., 1993). The supernatant samples were diluted and added to a culture of the reporter strain, and then the light output monitored over time (Fig.5.2).

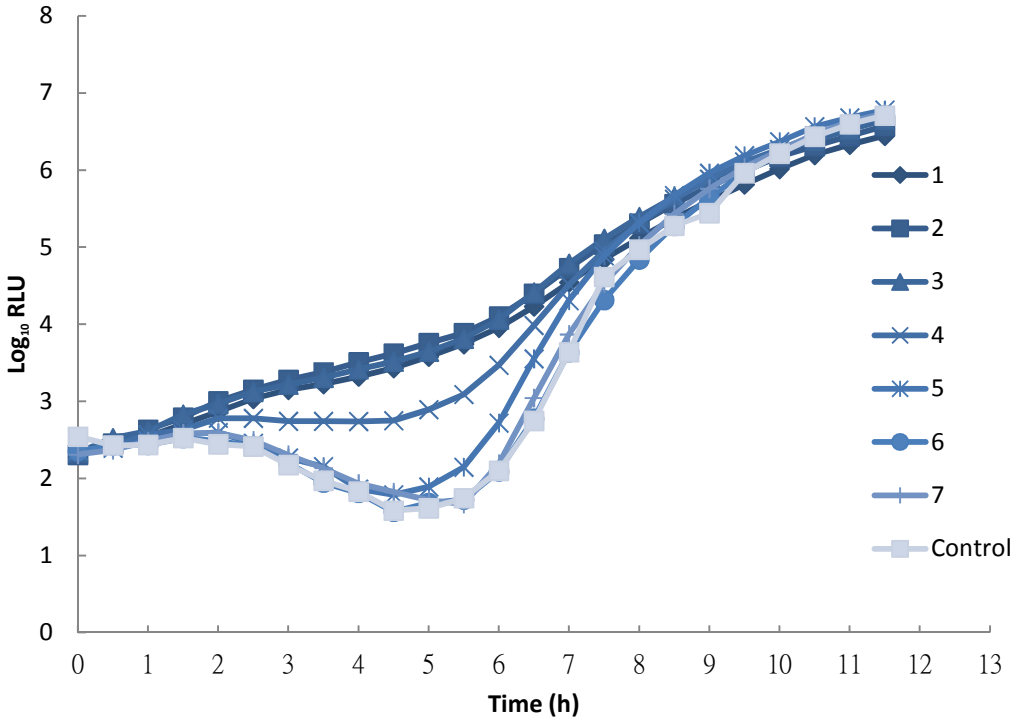
The results showed that the reporter produced bioluminescence in response to the AI-2 present in the supernatant from *V. harveyi* BB120 as expected. However, instead of giving a very clear difference in light levels when different concentrations of culture supernatant were used, the curves produced did not indicate a proportional response. The three most concentrated samples merged to at an upper activation level and the three most diluted samples merged together at a lower activation level. It is clear that the concentration of AI-2 is critical for the induction level of bioluminescence operon in the reporter strain but some limitations of the assay became apparent in that either very high or very low concentrations of AI-2 are not easily quantified. It was also clear that the differences in light level were only clearly visible during the first 8 h of the assay and therefore this was used as the key measurement time point for future experiments.

An increase in bioluminescence in the first 2-3 h was seen giving a small peak in light output. This may be the response of the BB170 strain to AI-2 present in the previous overnight culture and also the external AI-2 in the supernatant samples. After this small peak, the bioluminescence level dropped to give a low point in the curve at about 4 h. After this time, a sharp

increase in bioluminescence after 7-8 h of incubation was seen even when uninoculated media was used. Since BB170 is still able to produce AI-2, as the cell mass of reporter cells increases, the AI-2 concentration also increases in the assay.

From the data shown in figure 5.2, the point at which the bioluminescence of the negative control is the lowest is taken to be the point where most AI-2 from the previous BB170 overnight culture has been used up and before much new AI-2 has been produced. This is the point used as a reference for comparing the amount of exogenous AI-2 present in different culture supernatants.

**Figure 5.2 Response of *V. harveyi* reporter strain to different dilutions of wildtype *V. harveyi* BB120 supernatant**




The experiment was performed as described by Bassler *et al.* (1993). *V.harveyi* BB120 were grown in LB media overnight. The cultures were centrifuged and the culture supernatant filtered sterilized. 10-fold serial dilutions were performed to achieve various dilutions of AI-2 media from BB120 culture. As the initial concentration was unknown, the labels indicate the number of 10-fold dilutions of the original sample performed (i.e. 1 = $10^{-1}$ dilution). The *V.harveyi* reporter BB 170 was grown in AB medium overnight and then 0.1 ml of this culture used to inoculate 100 ml of AB medium. A sample (180 µl) of this diluted culture was mixed with a 20µl sample of each supernatant dilution in individual wells of a 96-well plate. Uninoculated broth was used as negative control. The optical density (600nm) and the bioluminescence produced by the reporter were measured using a microtitre plate reader (Tecan) at a 30 min interval for 12 h (further dilutions were prepared but these are not shown).

### 5.2.2 AI-2 production by *L. monocytogenes* grown in different media

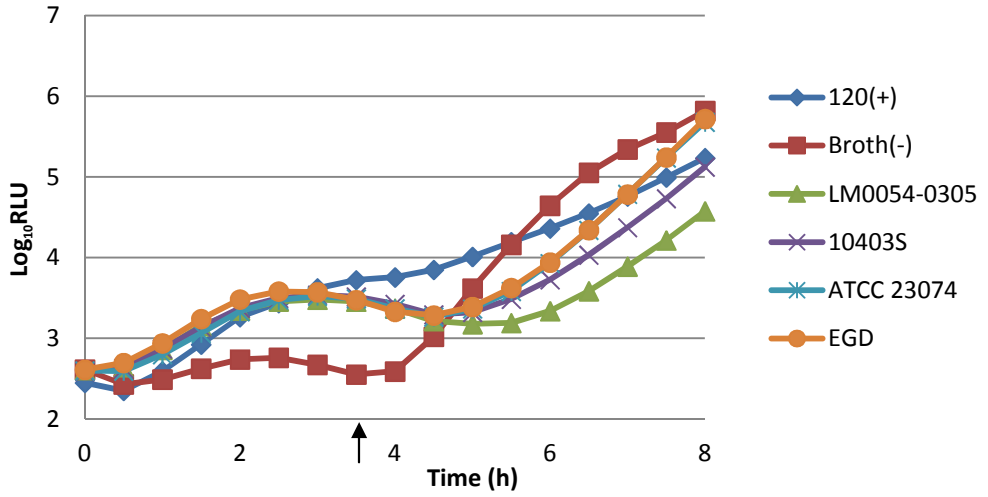
After testing the reporter, the AI-2 bioassay was performed to try and determine the relative AI-2 levels produced by *Listeria* cells grown in different media. Four different *L. Monocytogenes* strains (Table 5.1) were grown overnight at 37°C in the two media to be tested, MCDB202 and BHI. The cell culture supernatants were filtered sterilized and were added to the reporter culture. Culture supernatant from *V. harveyi* strain BB120 was used as a positive control whereas an uninoculated media was used as a negative control.

*L. monocytogenes* 10403S, EGD, ATCC23074 (refer to Table 2.4) were chosen because they are strains that have been well studied in research and to represent the two major serotypes associated with human disease. *L. monocytogenes* 00054-0305 was included as a representative of an environmental strain.

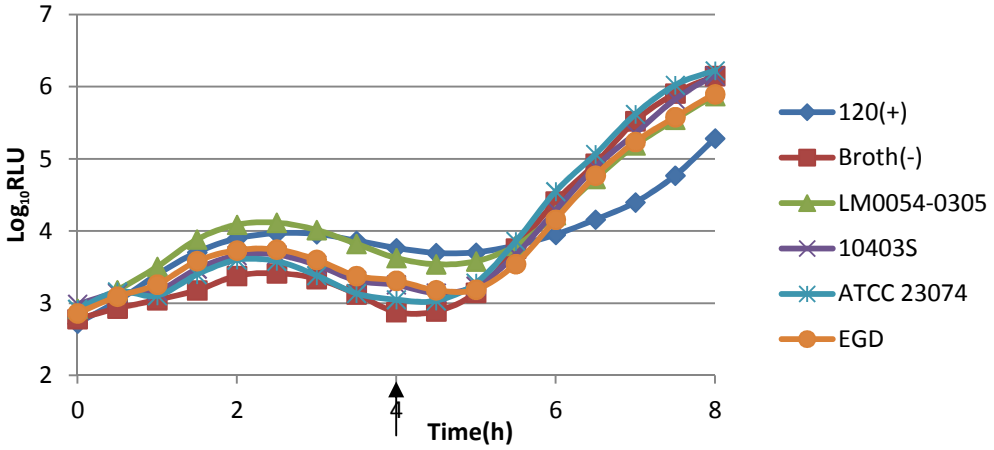
From the results (Fig. 5.3), the reporter strain BB170 produced similar patterns of bioluminescence when either the *Listeria* culture supernatants or wild type *V. harveyi* culture supernatant were added. Comparison of the results of these experiments shown in figure 5.3 showed that three out of the four *L. monocytogenes* strains tested (LM10403S, ATCC23074 and EGD) were found to produce relatively much lower levels of detectable AI-2 when they were grown in the defined media (MCDB202) compared to when they were grown in the rich media (BHI). All samples, including the positive control, showed reduction in AI-2 activity when the cells were grown in MCDB202 media. However one of the strains, LM00054-0305, showed different results and for this strain AI-2 production was less affected by the media. This is seen from the graph in Panel A where the curves for the *Listeria* supernatants all lie closer to the positive control line whereas in Panel B they lie closer to the negative control except for the LM00054-0305.The curve for LM00054-0305 samples lies close to the positive control in both graphs.

**Figure 5.3: Detection of AI-2 in cultures of *Listeria* grown in BHI and MCDB202**

A)




B)



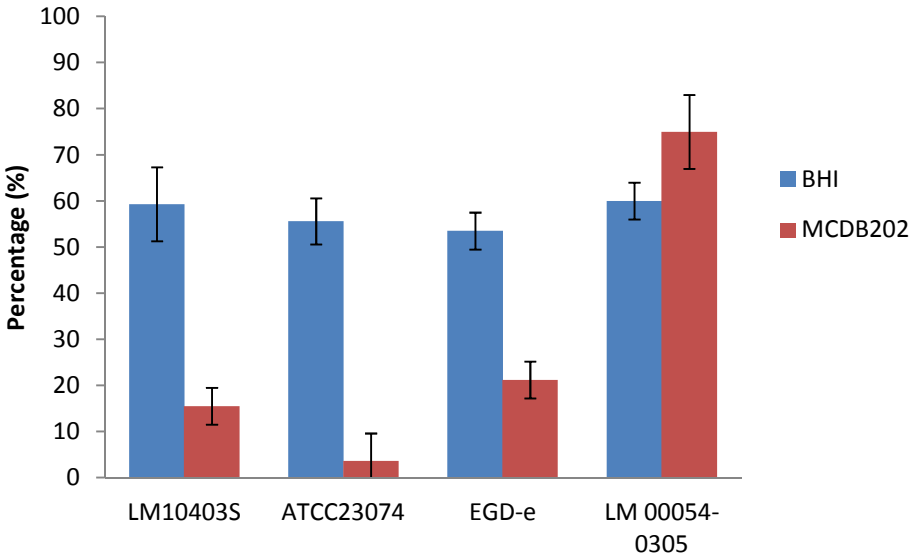

*Listeria* strains and BB120 were grown in BHI or MCDB202 overnight. The cultures were centrifuged and the supernatant filtered sterilised (Section 2.13). *V. harveyi* BB 170 was prepared as described in Figure 5.1. Supernatant from *V. harveyi* BB120 was used as a positive control (120+)and uninoculated media as a negative control (Broth (-)). The $OD_{600nm}$ and bioluminescence were measured at 30 min intervals for 12 h. Panel A shows the results of the BHI culture supernatants and Panel B the results of the MCDB202 culture supernatants. Arrows indicates the lowest point of the negative control. Experiment was done in 8 replicates and mean values were displayed

The relative levels of induction of the reporter strain when culture supernatants from which strain tested were found to be higher when the cells were grown in the MCDB202 media rather than in BHI. This LM00054-0305 strain is a factory isolate that was isolated from vegetables whereas the other three tested strains are clinical isolates. It was thus possible that this strain that comes from a very different environment did have a very a different pattern of AI-2 production.

In accordance with other published works using this assay system, the level of AI-2 in a sample can be determined relative to the light output achieved for the positive and negative controls after the low point of the bioluminescence induction curves (Bassler et al, 1993). Hence to try and simplify the data, the level of AI-2 production by *L. monocytogenes* grown in BHI and MCDB202 were analysed in this way by determining the relative levels of AI-2 production compared to the results at one time point (4 h) for the positive and negative control samples.

Using this analysis (Fig. 5.4) it was clear that three out of the four *L. monocytogenes* strains tested (10403S, ATCC23074 and EGD) produced much lower levels of AI-2 when they were grown in the minimal media (MCDB202) compared to when they were grown in the rich media (BHI). The patterns of AI-2 production induction in those three strains were quite consistent, being much lower in MCDB 202 media. In contrast LM00054-0305 showed a slight increase in the relative AI-2 production when it was grown in the defined media compared to that in BHI. This suggested that LM00054-0305 AI-2 production was not affected by the change in media.

**Figure 5.4: Relative levels of AI-2 production by *L.monocytogenes* grown in BHI and MCDB202**




The results of the 4h time point the graphs in Figure 5.3 were determined. The amount of bioluminescence of each of the samples was calculated as a percentage of the value of the positive control and the negative control to give the relative bioluminescence level of each sample in terms of percentage. Error bars indicates the standard deviation.

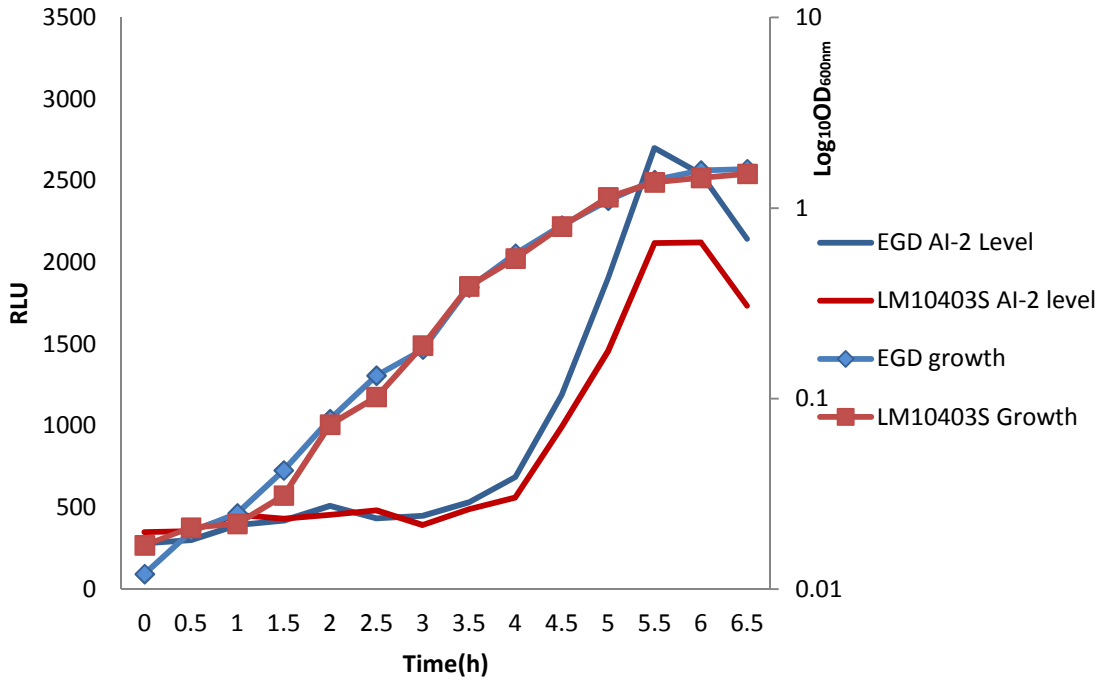
Percentage = (Data-Negative control) / (Positive - Negative control) X100%

### 5.2.3 Correlation between growth of *Listeria* and AI-2 production

Autoinducers were first discovered as a quorum sensing molecules, which are linked to cell number and hence related to growth of a culture. In other words, the level of AI-2 produced by a culture is dependent on growth phase. In order to look more closely at the relationship between AI-2 levels produced by *Listeria* at different stage of growth, the two well studied clinical strains of *L.monocytogenes*, EGD and LM10403S, were used. Culture supernatants were taken at different time points during the growth of each of the cultures. The supernatant cultures were prepared (section 2.13.1) and added to the individual sets of reporters as above. The amount of AI-2 present determined using the AI-2 bioassay by the bioluminescence level at the 4h time point. A graph of this data was then plotted against the time point for comparison with growth.

From the results (Fig. 5.5), it is clear that the two *Listeria* strains produced similar levels of growth and also patterns of AI-2 production. The AI-2 levels were quite low and steady for the first 4 h of growth before starting to increase at the early stationary phase of growth. The AI-2 levels reached a peak when cells started to enter stationary phase after 5.5 h of growth and after this the AI-2 level dropped. AI-2 activity was limited for stationary phase sample indicated by the low bioluminescence, which suggested that cells may be not producing AI-2 under high cell density condition.

**Figure 5.5: Correlation between growth of *Listeria* and AI-2 production**




*Listeria* cells were inoculated in the 10ml of BHI and growth ($OD_{600nm}$) monitored over time. Samples were taken every 30 min and centrifuged to remove the cells. The supernatant was filtered with Minisart 0.2 µm filter. The reporter strain BB170 was prepared as described in Figure 5.1 and 180µl of this diluted culture mixed with 20 µl of each culture supernatant sample in individual wells of a 96-well microtitre plate. Uninoculated media was used as a negative control (data not shown). All assays were performed in triplicate and the bioluminescence produced by the reporter was measured at 30 min intervals for 6.5 h.

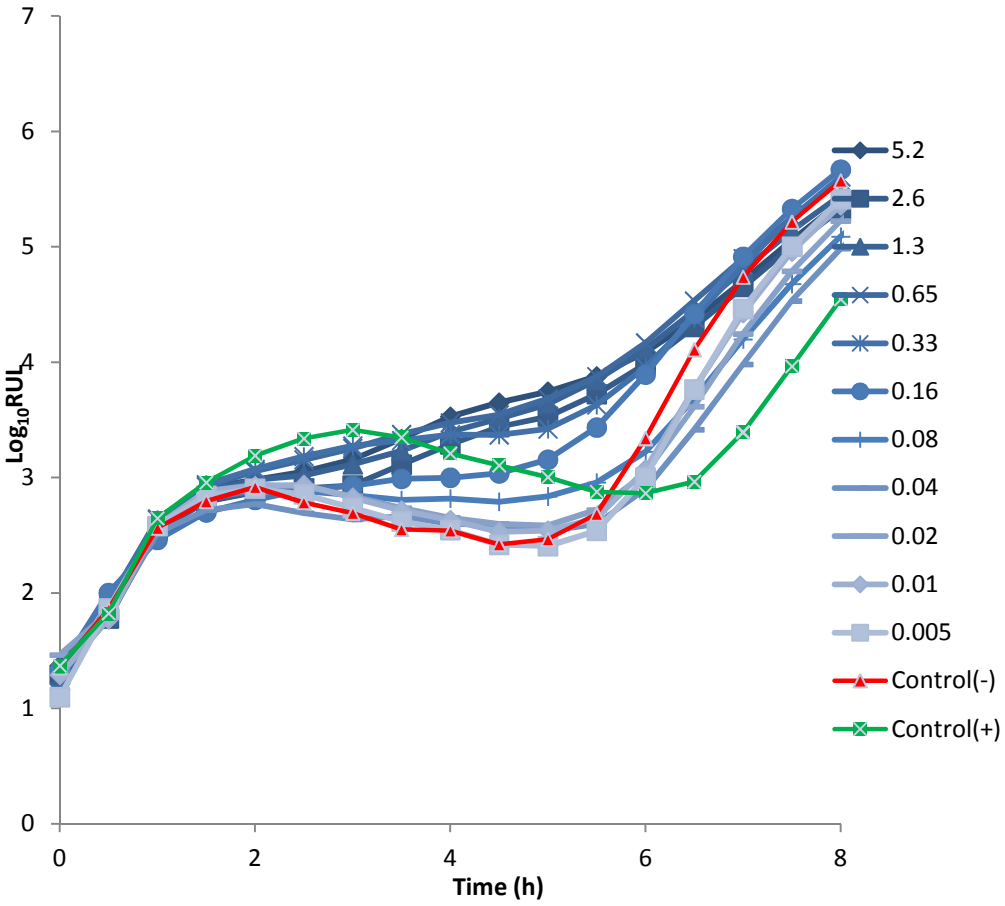
## 5.3 Establishing DPD with BB170 reporter

The autoinducer AI-2 is a mixture of unstable molecules which it is not possible to extract and isolate. It is produced by a unknown metabolic reaction from the precursor DPD, 4,5-dihydroxy-2,3-pentanedione. It was suggested previously that DPD would naturally form AI-2, so that DPD may be used in place of purified AI-2 in experimental studies of gene control (Lowery *et al.*, 2008, Rickard *et al.*, 2006). Hence it was proposed to use DPD to investigate a potential role of AI-2 in EPS production. However, before using DPD for these experiments on *L. monocytogenes* cells, it was important to determine if synthesized DPD could activate the *V. harveyi* biosensor, indicating that it was able to form the AI-2 molecule.

### 5.3.1 Detection of DPD using the *V. harveyi* biosensor

To test whether DPD can activate the AI-2 the reporter strain, *V. harveyi* BB170was again used. Artificial DPD was serially diluted in 2-fold steps, and samples of each dilution added to the reporter strain. Bioluminescence levels were measured over time and water (the diluents for the DPD) was used as a negative control. So that the levels of induction achieved by the DPD samples could be compared with a positive control, filtered BB120 culture supernatant was used.

**Figure 5.6: Response of *V. harveyi* reporter strain to DPD**



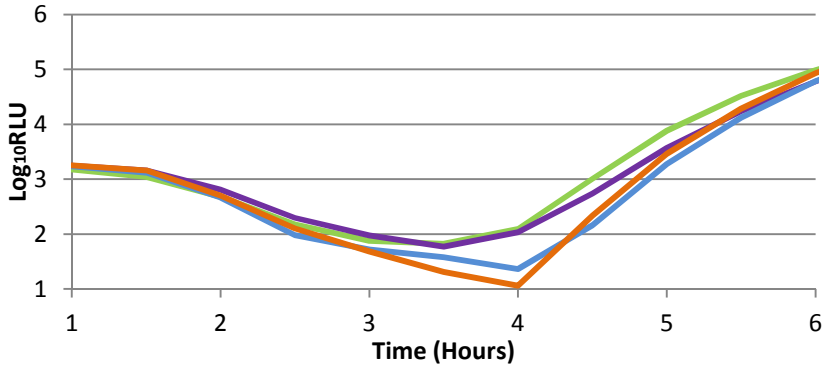

A 2-fold serial dilution of DPD was prepared in SDW. *V. harveyi* BB170 was grown overnight in 10 ml of AB medium then 0.1 ml of the culture were used to inoculate into 100 ml of AB medium. A sample (180µl) of this diluted BB170 culture was mixed with and 20µl of each of the DPD samples in individual wells of a 96-well microtitre plate. All experiments were performed in triplicate. The bioluminescence produced by the reporter was measured using a microtitre plate reader (Tecan) at a 30 min interval for 8h. The added DPD concentrations (mg $ml^{-1}$) from high to low, are shown in dark to light blue.

From the graph above (Fig. 5.6), it can be seen that the bioluminescence output of the reporter strain BB170 was related to the concentration of the DPD added to the culture. This shows that the addition of the exogenous DPD was able to activate the reporter strain, indicating that cells response to exogenous DPD works in the same way as AI-2. As before, the curves at the two extreme concentrations did not produce a proportional response. This suggested that there could be saturation of the reporter system at the high concentrations of DPD (seen for the top few concentrations of DPD), so that an increase in DPD concentration will not give an increase in bioluminescence level. On the other hand very low activation was seen using the low DPD concentration. The level of bioluminescence produced at the lowest concentration sample was very close to the negative control (pure water) suggesting there is a threshold concentration required to achieve activation of the reporter system.

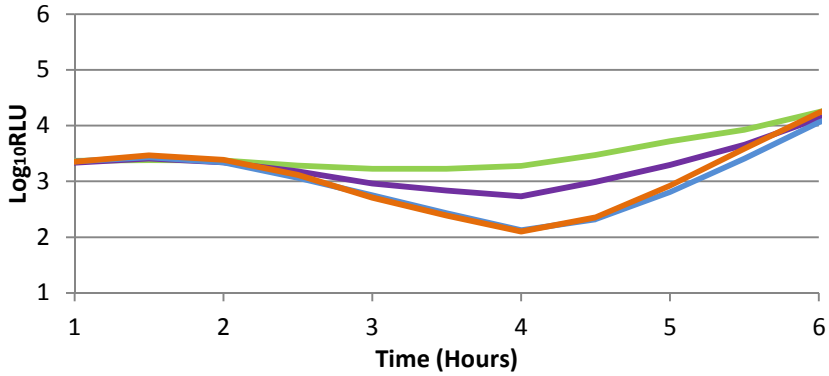
Since we wished to rule out the effects of adding the *Listeria* growth media to the reporter strain (the composition of the AB media is very different to that of BHI or MCDB202), the assay was repeated but this time the DPD was diluted in each of the *Listeria* growth media (Fig. 5.7).

**Figure5.7: Response of *V. harveyi* reporter strain to DPD diluted in different *Listeria* growth media**

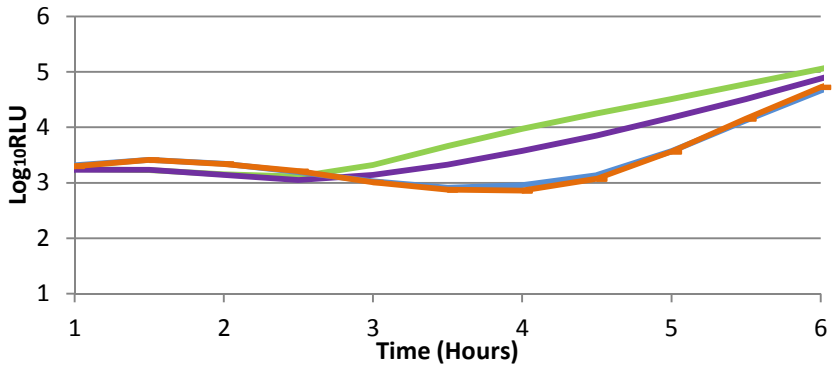
A)




B)




C)



DPD was diluted to 5 mg $ml^{-1}$ ( )، 0.05mg/$ml^{-1}$( )or 0.0005mg/$ml^{-1}$( ) in panel a) BHI, panel b) MCDB202 and panel c) RO water. The *V. harveyi* report BB170 was grown overnight in 10 ml of AB medium then 0.1 ml of the culture was used to inoculate into 100 ml of AB medium. A sample (180µl) of this diluted BB170 culture was mixed with and 20µl of each of the DPD samples in individual wells of a 96-well microtitre plate. All experiments were performed in triplicate. The bioluminescence produced by the reporter was measured with the microtitre plate reader (Tecan)at a 30 min intervals for 10 h. Either pure media or pure RO water alone were used as negative controls ( ). Time point between 6-10 h were not shown due for clarity.

Again, the level of bioluminescence produced was seen to be related to the amount of DPD added to the samples (Fig. 5.7) and, as before, an increase in the amount of the exogenous DPD added induced the reporter strain BB170 to produce more light, indicating that the cells do respond to exogenous DPD in the same way as AI-2 and that this is not affected by the presence of other media components in the DPD sample. An effect on light production of DPD was seen in each of the experiments between 2-6 h. The shapes of the different curves before and after this period were approximately the same for the individual samples.

This fits the previous results suggesting *V.harveyi* BB170 responds to the external level of AI-2. When we compare the activation of light in the three media, the light production with the lowest DPD concentration (0.0005) was minimal in all three media. A difference in the curve shape was seen at the higher concentrations DPD (5 and 0.05) when the results for water and MCDB202 are compared to the result seen for BHI, where much less activation occurred. This shows that BHI suppresses the overall level of light and the maximum level of induction seems to be reduced relative to the other two samples

### 5.3.2 Comparison of biosensor response towards artificial DPD and *Listeria* AI-2

It was shown in section 5.2 that 3 out of 4 *Listeria* strains (*L. monocytogenes* EGD, ATCC 23074 and LM 10403S) grown in BHI produced more AI-2. It would be useful if DPD could be used to estimate the actual amount of AI-2 in a sample. To do this, the bioassay reporter must give the same signal in response to DPD and AI-2. The DPD and AI-2 from a culture of *L. monocytogenes EGD* was tested to see if they active the biosensor in the same way. This was done by making samples of DPD diluted in BHI, MCDB202 and water to a final concentration of 0.5 mg $ml^{-1}$. Samples were then incubated with a 180 µl sample of the *V. harveyi* BB 170 reporter strain. Spent supernatant from *L. monocytogenes EGD* grown in BHI were also tested. The spent culture supernatant samples were prepared by taking supernatant from the *Listeria* culture after 6 h of incubation and filter sterilized. A 6 hour culture was used because it was seen in figure 5.5 that the AI-2 level was maximal at that growth phase.

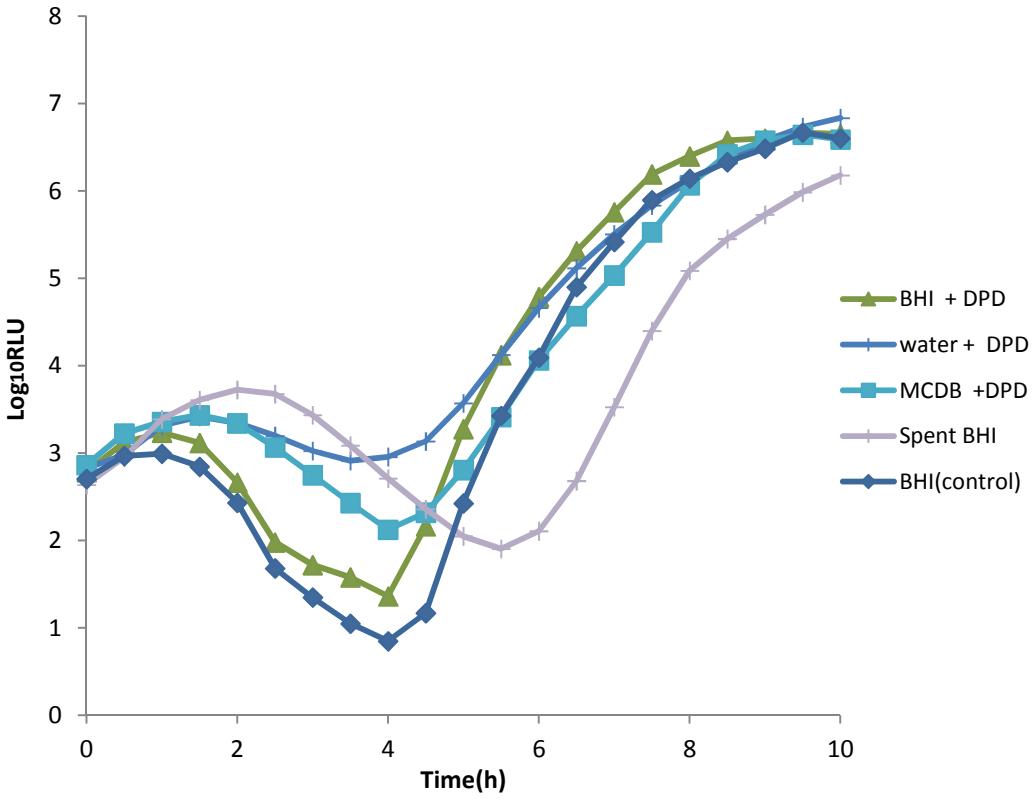
When comparing the induction of bioluminescence from *V. harveyi* BB 170 by the synthesized DPD in different diluents, it is clear that the level of response was greatest when the DPD was diluted in water (Fig. 5.7). This difference may be caused by adding different amounts of nutrient to the reporter culture which is grown in a minimal media so that the induction in response to the AI-2 molecules is detectable.

It was also observed that the reporter did not respond in the same way to the AI-2 produced in the spent supernatant from the *Listeria* culture (Fig. 5.8). For the three DPD samples, irrespective of the diluents used, the shapes of the curves were more or less similar, just giving a variation in the height of

the activation peak. However the result in the spent BHI culture produced a higher and wider increase in the activation peak at about 5.5 h, which was about 2 h after the activation peak seen when DPD samples were used. Interestingly the negative control (BHI broth) curve is more like the shape seen for the DPD samples than that seen using spent BHI medium. This suggests that some other compounds produced by the *Listeria* cells is affecting the expression of the reporter genes in BB170 which could be the present of AI-2 in supernatant. The obvious effect of the nutritional content on the reporter gene signal suggests that it is also important to look at the effect of growth of the biosensor when samples containing different growth media are added to it, as this may affect growth of the reporter and therefore the production of bioluminescence, which changes during the growth of BB170.

Although a difference in time of activation of bioluminescent production in seen in the *in vitro* synthesized DPD, it can be seen that cells do respond to the molecules. Hence DPD was then used for further experiments to investigate its effect on *L. monocytogenes* cells. However it should be noted that even though only a very low concentration of the different media is used (1 in 10 dilution; 20µl of the supernatant and 180µl of AB media), the different media samples did show an observable effect on bioluminescence production by the reporter suggesting mild variation in growth environment may cause changes in reporter physiology. This needs be taken into account in interpretation of other experiments.

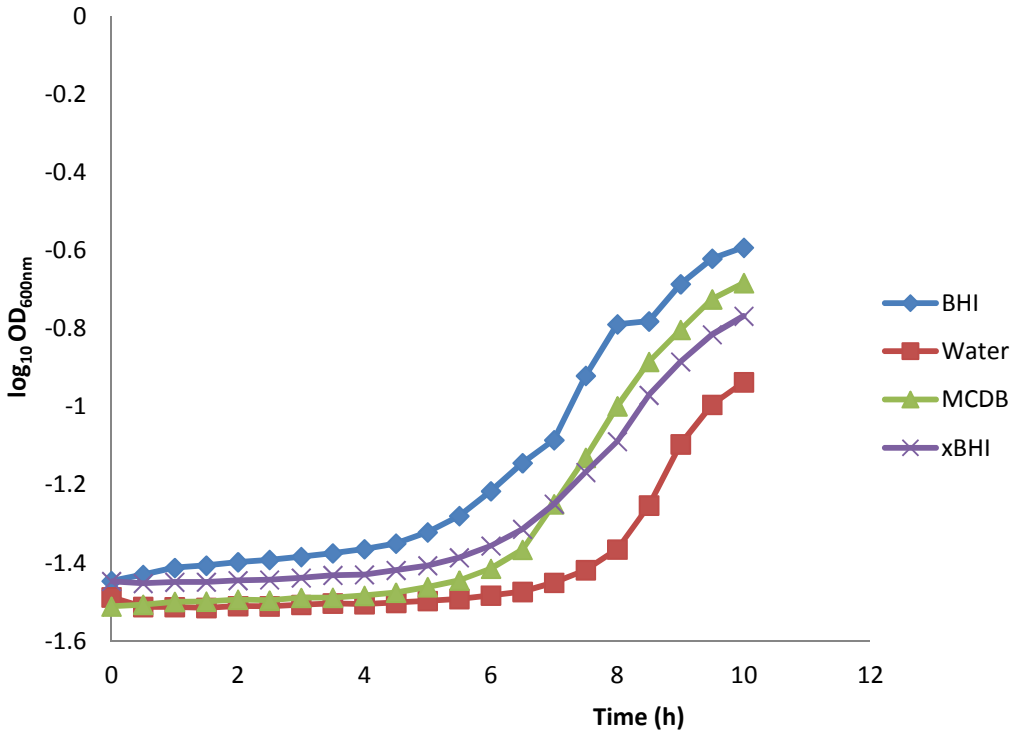
**Figure 5.8: Response of *V. harveyi* reporter strain BB170 to DPD or AI-2 produced by *Listeria***




The *V. harveyi* report BB 170 was grown overnight in 10 ml of AB medium. Cells were recovered from a 0.1 ml sample of the culture by centrifugation and were used to inoculate into 100 ml of AB medium. 180 µl of this diluted reporter culture was added into individual wells of a 96-well plate and incubated with samples (20µl) of different DPD solution or *L. monocytogenes EGD* supernatant samples (section 2.13.2). The bioluminescence produced by the reporter was measured with the Tecan at a 30 min interval for 10 h.

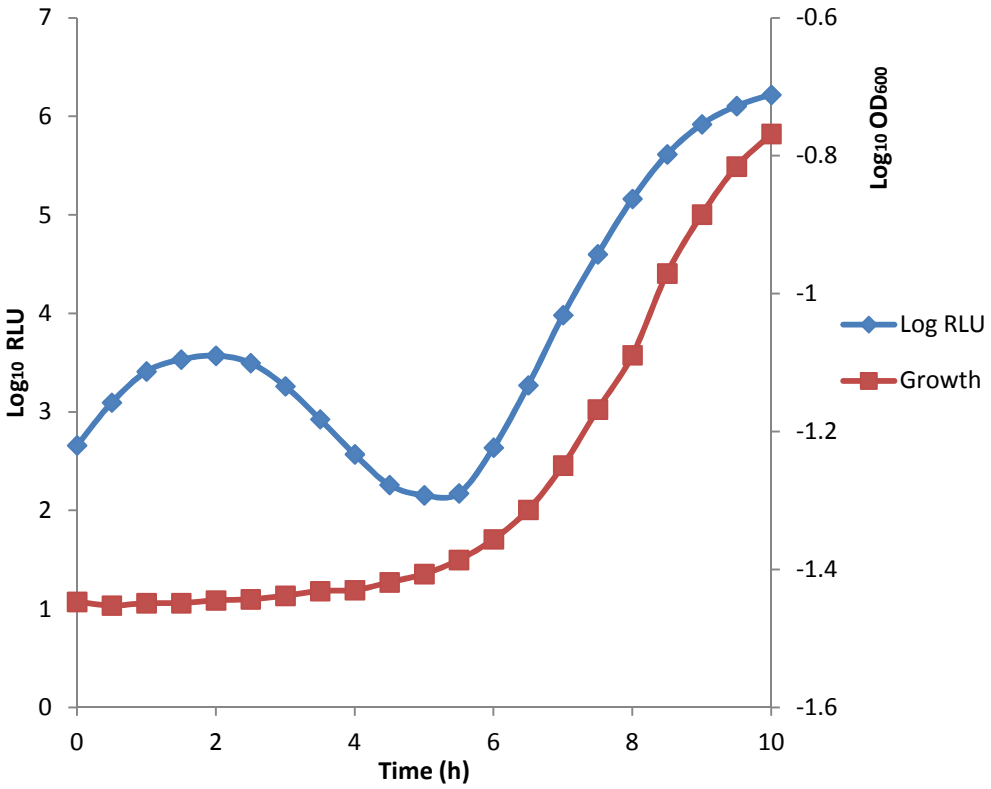
Figure 5.9 shows the growth pattern of the *Vibrio* cells during the experiment presented in Figure 5.8 and it can be seen that adding these different supplements did cause a variation in growth. However, the cells remained in lag phase for the first 5 h of the experiment for all samples, indicating that cell growth was minimal at the time the peak of activity was determined in Figure 5.8 for the DPD samples, indicating that growth was unlikely to affect these results. However the results for the spent BHI sample are less clear. The curve of the growth and light output for the spent BHI sample alone is presented in figure 5.10. It showed a wider peak and with the lowest point of the RLU was seen at the time point of 5.5 hour, where the growth has already started.

**Figure 5.9: The effect of addition of different test samples on the growth of *V. harveyi* BB170**




The *V. harveyi* report BB 170 were grown in 10 ml of AB medium overnight. 0.1 ml of the culture was used to inoculate into 100 ml of AB medium. Samples (180µl) of this diluted reporter culture was added into individual wells of a 96-well plate and samples (20µl) of water, BHI broth, MCDB202 broth or *Listeria* culture supernatant (xBHI) were added into individual wells of a 96-well plate. Growth was monitored using $OD_{600nm}$readings using the Tecan at a 30 min interval for 12 h. Data were mean value for 3 replicates of experiment

**Figure 5.10: Growth and light output from *V. harveyi* BB 170 in response to *Listeria* BHI culture supernatant**




The *V. harveyi* report BB 170 was grown overnight in 10 ml of AB medium. Cells were recovered from a 0.1 ml sample of the culture by centrifugation and were used to inoculate into 100 ml of AB medium. 180µl of this diluted reporter culture was added into individual wells of a 96-well plate and incubated with samples (20µl) of *L. monocytogenes EGD* supernatant samples (section 2.13.2). The bioluminescence and OD produced by the reporter was measured with the Tecan at a 30 min interval for 10 h

## 5.4 DPD complementation experiments

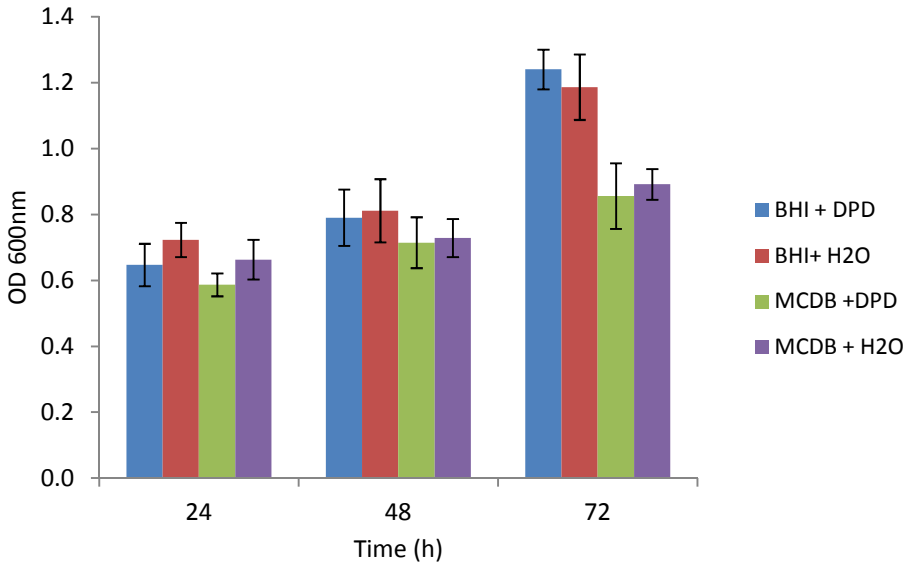
Although differences were found in the response of the biosensor to *Listeria* AI-2 and DPD, there was evidence that an active molecule that could be detected by the biosensor strain BB170 was being produced from the DPD when this molecule spontaneously breaks down. In a *luxS* mutant of *L. monocytogenes*, it was shown that AI-2 levels were diminished and biofilm formation was enhanced (Sela *et al.*, 2006). It was also reported that added exogenous DPD did not restore the mutant to a wild type phenotype (Sela *et al.*, 2006) and the research group concluded that AI-2 may not have a direct effect on biofilm formation. The AI-2 biosensor assay has also been used to show that the increased biofilm seen when cells were grown in a nutrient-limited media also corresponded to a reduction in AI-2 level. Previous work has shown that exogenous DPD had no effect on *Listeria* biofilm formation when cells were grown in Tryptone Soy Broth (Challan Belval *et al.*, 2006), a nutrient rich media. Since it was shown in section 5.2 that cells grown in MCDB202 media resulted in lower levels of AI-2 production by *Listeria*, the following experiments were aimed to add DPD to counter the effect of the reduced AI-2 when the cells were grown in minimal media to see if it is able to restore the phenotype (e.g increase in hydrophobicity seen in chapter 3) of the cell.

### 5.4.1 The effect of DPD on Biofilm formation

The CV Biofilm assay was repeated using the protocol described in Section 3.4. However this time DPD was added to the samples to see if it would affect biofilm formation by the *Listeria* cells EGD. Cells were grown in BHI and MCDB202 media with the addition of DPD or SDW as control. Biofilm levels were assessed to see the effect of DPD on the two cells samples.

In this experiment (Fig. 5.11) little difference in biofilm formation was found during the first 48 h of attachment. Consistent with the results presented in Chapter 3, after 72 h more attached material was detected when the cells were grown in BHI. Comparing the results from the biofilm assay in the presence or absence of DPD, no difference was seen in the biofilm level on the microtitre plates, suggesting that the addition of the AI-2 had no significant effect on biofilm formation of *L. monocytogenes* cells grown in either BHI or MCDB202. This agreed with the results of other workers reported in the literature suggesting that AI-2 may not act as a direct signalling molecule controlling biofilm formation.

**Figure 5.11: The effect of DPD on Biofilm formation of *Listeria monocytogenes***




*L. monocytogenes EGD* cells were grown in MCDB202 and BHI media overnight. A sample (1 ml) of the cells were then centrifuged and washed, and then inoculated into 5 ml of the media to be tested (either BHI or MCDB 202). A sample of this (180 µl) was transferred into the wells of a microtitre plate and either 20 µl of DPD (5.2 mg $ml^{-1}$) or water (negative control) added to the wells. The plates were incubated at 37°C for 24, 48 and 72 h. After this time the media was removed from the plate and the wells washed with PBS. CV (200 µl of a 0.1% solution) was added to the wells for 15 min. The CV solution was removed and the wells were washed 3 times with PBS before 200 µl of absolute ethanol was added to the wells. Absorbance was measured at 600 nm using a microtitre plate reader (Tecan). Error bars indicates standard deviation of 8 replicates.

### 5.4.2 The effect of DPD on *Listeria* cell Growth

In the previous experiments presented in Chapter 3 (Section 3.3) it was found that the amount of biofilm formed was related to the cell density of the cultures tested. It was possible that the effects of the added DPD could be masked if this molecule affects the growth of *Listeria* in the different media. Therefore it was also investigated whether AI-2 has an effect on the growth of *L. monocytogenes*. As AI-2 is also produced by the *Listeria* cells during growth, it was decided to determine if AI-2 added in the initial stages of growth of a culture would have any effect on cell growth, especially in minimal media where little AI-2 was being produced.

The growth of *Listeria* cells in both media is shown in figure 5.12. For each of the media, the patterns of cell growth was similar with or without the addition of DPD, including the length of the lag phase, the growth rate in the exponential phase (Table 5.3) and the time of entry to stationary phase. From these results it was concluded that that the addition of DPD and, hence the presence of AI-2, did not have any obvious effect on the growth of *L. monocytogenes* in either media.

**Table 5.1: Growth rate of *Listeria* in exponential phase in BHI or MCDB202 with or without addition of DPD**

| Sample | Growth rate in exponential phase ($h^{-1}$) |
|---|---|
| BHI + water | 0.82 |
| BHI +DPD | 0.85 |
| MCDB202+water | 0.38 |
| MCDB202 +DPD | 0.45 |

Growth rate was calculated with the following formula with time points taken during exponential phase (Reading taken from Time 4h-7h):

$$\text{Specific Growth Rate} = \frac{(\log_{10}N - \log_{10}No) \times 2.303}{T - T_0}$$

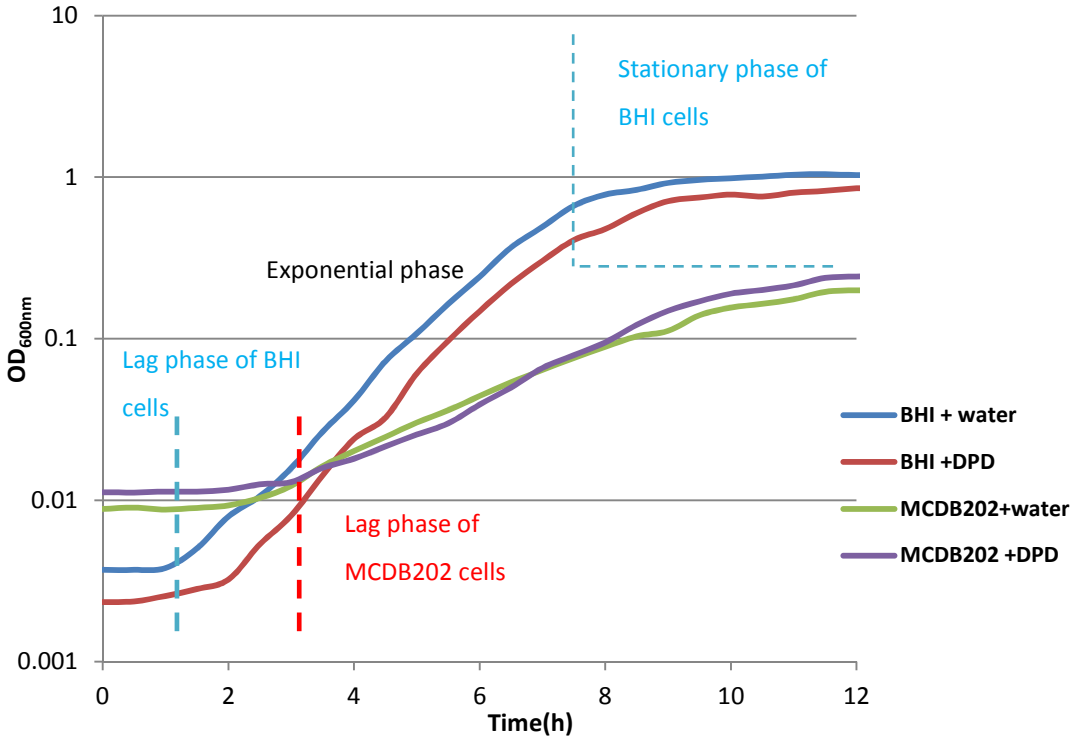
No = $OD_{600nm}$reading at $T_0$ in the early *Exponential* phase

N = $OD_{600nm}$reading at T in the late *Exponential* phase

To = Time of first measurement in the early *Exponential* phase

T = Time of second measurement in the late *Exponential* phase

**Figure 5.12: The effect of DPD on the growth of *L. monocytogenes***



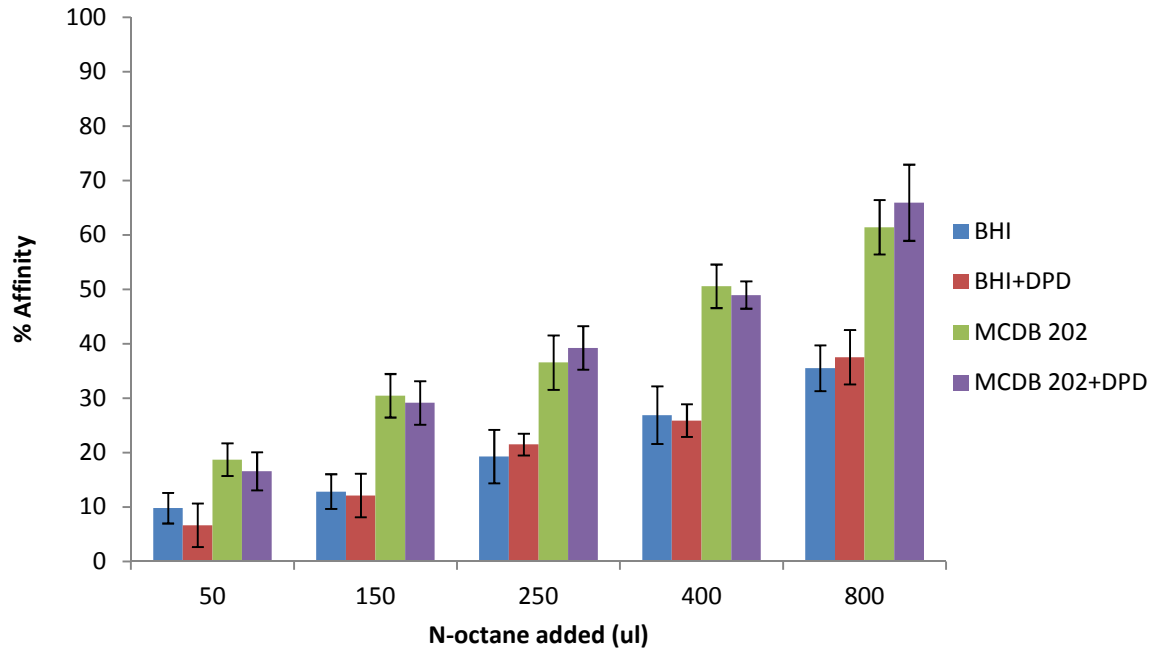

*L. monocytogenes* EGD cells were grownMCDB202 or BHI broth and cultures were grown overnight at 37°C .A sample of this (180µl) was transferred into the wells of a microtitre plate and either 20 µl of DPD (5.2 mg $ml^{-1}$) or water (negative control) added into individual wells of a 96-well plate. The $OD_{600nm}$ of cultures was measured using the microtitre plate reader (Tecan) at a 30 min interval for 12 h. Stationary phase for MCDB 202 cells not reached. Curves showed mean value of 3 replicates.

### 5.4.3 The effect of DPD on *Listeria* cell hydrophobicity

The MATH assay results presented in section 3.2 showed that, *L. monocytogenes* cells grown in MCDB202 are more hydrophobic than those grown in BHI, and also it was shown in this chapter that cells grown in MCDB202 produce less AI-2. Therefore it was investigated whether adding AI-2 to MCDB202 would have an effect on cell hydrophobicity of *L.monocytogenes.*

The hydrophobicity experiment was performed as described in section 3.2. Briefly cells were grown overnight in the two different test media supplemented with DPD to a final concentration of 0.5mgml$^{-1}$). The cell hydrophobicity was then determined using the MATH assay. From the results (Fig. 5.13), addition of DPD to the growth media had no effect on the hydrophobicity of the *Listeria* cells. It is clear that addition of AI-2 to *L. monocytogenes* cells grown in MCDB202 did not reduce the hydrophobicity to the level seen when the cells were grown in BHI, suggesting that there was no relationship between the low AI-2 levels and the surface changes detected when the cells were grown in this media and hence that AI-2 is not acting as a signal molecule controlling this phenotype.

**Figure 5.13: The effect of DPD on the hydrophobicity of *Listeria* grown in MCDB202**




Cells were grown in 9ml of BHI or MCDB202 overnight with addition of 1ml of 5mgml$^{-1}$ DPD (to a final concentration of 0.5 mg ml$^{-1}$) or water. Cultures were centrifuged and cells washed in 0.15 M NaCl. The centrifuged cells were resuspended in 0.15 M NaCl to an absorbance ($OD_{600nm}$) of approximately 1. The absorbance was recorded as Ao. A sample (3 ml) of each culture was transferred in to different test tubes and different volumes of N-octane (150, 250, 400 or 800 µl) added to them. The tubes were vortexed for 90 s and allowed to stand for 15 min. 1 ml of the lower aqueous layer was removed and $OD_{600nm}$ measured. Each test was performed in triplicate.

## 5.5 Discussion

From the results presented in this Chapter, it was found that three out of the four *L. monocytogenes* strains tested showed a reduction in AI-2 production when the cells were grown in a defined media compared to the levels produced in a rich media. It was also shown that AI-2 production in *L. monocytogenes* is growth phase-dependent and the amount produced increases rapidly during the late exponential phase of growth. It was also found that bioluminescence induction in the reporter strain *V. harveyi* BB 170 responded to artificial DPD in a similar way to natural AI-2 produced during cell growth. Finally addition of DPD to cultures to increase levels of AI-2 had no significant effect on *L. monocytogenes* biofilm formation, growth or cell surface hydrophobicity. However the conclusions drawn here were limited by the difficulties encountered standardising the AI-2 bioassay.

AI-2 is known to be a very small and unstable molecule, and very often, produced in only very small amounts, making the detection of AI-2 very difficult using chemical or physical methods, such as HPLC or GC. Hence a bioassay was introduced over 15 years ago and utilises the reporter strain *V. harveyi* BB170, which produces luminescence in response to AI-2. This has been widely adopted in studies of AI-2-controlled gene expression due to the simplicity of the protocol and the fact that it does not require the use of expensive machinery, providing a simple estimation ofAI-2 levels in solution. However questions have been raised about this assay. For instance Vilchez *et al.* (2007) found that the quantification of AI-2 with bioassay is not reliable, due to the non-linear relationship seen between the fold induction values recorded and AI-2 concentration in samples. They also suggested that the detectable range is very narrow (ranging from 0.4 μM to 35 μM). These

concerns were reflected in the results gained in this study when testing serial dilutions of BB120 supernatant (containing natural AI-2) and DPD, which showed that only a narrow range of concentrations of AI-2 were detectable by the BB170 reporter and results for AI-2 levels above or below this range produced identical results.

In addition, the whole concept of this bioassay is actually not as simple as it seems. As it is a bioassay which relies on the use of a living reporter, anything that affects the physiology of the reporter strains also affects the results obtained. It was shown by Vilchez*et al.* (2007) that growth and bioluminescence production by the BB170 reporter in an AI-2 bioassay is greatly affected by trace elements, particularly iron compounds. Similarly Dekeersmaecker and Vanderleyden (2003) studying *Lactobacillus* AI-2 production have shown that the present of glucose in the samples resulted in induction of a bioluminescence signal. They also showed that the low pH in supernatant of *Lactobacillus* also affected bioluminescence production by this reporter. Similar results were seen by Turovskiy and Chikindas (2006), showing that as little as 0.125 g $l^{-1}$ of glucose would stimulate growth of BB170 7-fold and hence affect the reporter results. However, they also suggested that an excess of glucose had an inhibitory effect on bioluminescence production by the reporter strain and that the AI-2 bioassay is better used as a qualitative rather than quantitative method for AI-2 detection, due to high variability of the assay.

Glucose is a readily utilisable carbon source for bacteria, and this was present in the two media being tested. However in the experiments performed here residual levels in the media should have been significantly reduced during cell growth and supernatants from stationary phase cultures are expected to

have limited amounts of glucose remaining and so the effect of glucose on the reporter suggested above may be ruled out. However since the cultures grow to a lower cell density in MCDB 202 than in BHI, more glucose may remain in the stationary phase culture supernatants of cells grown in MCDB 202.

Despite this it was found that the sample being tested did have an effect on the growth rate of the reporter strain. As seen from the results in Figure 5.2, a large increase in the indigenous levels of bioluminescence induction was seen from the reporter strain itself as the cell number increased, so anything that increases the growth of the reporter strain hence also affects the bioluminescence production. So to allow comparison of results when performing these assays it is important to start with similar initial reporter cell densities, and to ensure that the test samples added do not increase growth rate. In addition the bioluminescence induction levels recorded were compared to a relevant control, thus minimizing the effect of the different composition of the supernatants which could affect the results.

Other possible factors affecting the reporter strain could be the presence of excretion products or variation in secreted metabolites found in the culture supernatants of different samples. Hence standardizing the *V. harveyi* BB170 bioassay is difficult due to the potential for interference and numerous other physiological factors that can affect the reporter strain and, as concluded by Turovskiy and Chikindas (2006), the AI-2 bioassay is perhaps better used as a qualitative rather than quantitative method for AI-2 detection, due to high variability of the assay.

From the results obtained here, it was shown that the content of the supernatant used in the bioassay did have a large effect on the AI-2 bioassay result. For instance there was an obvious difference in the pattern of bioluminescence seen when either DPD or *Listeria* culture supernatants were added to the reporter strain. This may have been due to a difference in the form of the signalling molecule being generated or could be the effect of other components in the BHI broth that affected the induction of the reporter gene system. This latter explanation seems more likely since the BHI control sample did not show the same change in the pattern of bioluminescence induction. However even if the bioassay may not be good for quantitative analysis, the results from this study were able to show that most of the *L. monocytogenes* strains tested produced higher levels of AI-2 when grown in BHI than in MCDB202. This is seen from the graphs in Figure 5.3 where the results for the BHI culture supernatant samples clustered closer to the positive control, whereas those for the MCDB202 culture supernatant samples were closer to the negative control line.

Complementation of AI-2 by adding DPD has been used a number of times in investigations of several different bacteria, most often when studying *luxS* mutants which were shown to have defects in AI-2 production. In most of these experiments, exogenous DPD was added to cultures to test if it was possible to restore a wildtype phenotype. For instance Auger *et al.* (2006) shown that the addition of DPD causes a decrease in biofilm formation in a *luxS* mutant of *Bacillus cereus* and that AI-2 promotes cell detachment from a preformed biofilm. They also proved that DPD had no effect on the growth of planktonic *B. cereus* cells. Similarly Rickard *et al*. (2006) have shown that a *luxS* mutant of *Streptococcus oralis* that is unable to produce AI-2 did not exhibit mutualism with *Actinomyces naeslundii* in a dual species biofilm and

generated sparse biofilm with much lower cell mass. Addition of chemically synthesised AI-2 (DPD) was able to restore mutuality growth and biomass in the biofilm. They concluded that AI-2 is linked to interspecies signalling in the case of mutualism. In contrast to these studies, Sela *et al.* (2006) showed that *luxS* mutants of *L. monocytogenes* produce thicker and denser of biofilms. However, in this case addition of exogenous AI-2 did not restore the phenotype and therefore they concluded that the function of LuxS involved in repression of attachment and biofilm formation is unrelated directly to AI-2. Contradiction in results between different studies may be due to the variation between the species being studied, indicating a response could be species-specific, or could be explained by the use of different experimental approaches and methods. Hence it is often difficult to directly compare results from different studies.

In this work, a *luxS* mutant was not used. Instead, *Listeria* cells grown in MCDB 202, which was shown to have reduced AI-2 production and enhanced EPS production, were used as a complementation target. The first complementation tests on biofilm formation were performed in a similar way to those reported by Sela *et al.* (2006), and the results were found to be consistent. This investigation was then extended to investigate effects on growth and hydrophobicity however, no effects were seen. All these results suggest that increased levels of DPD do not have an effect on these particular phenotypes of *L.monocytogenes*.

However before this idea is dismissed altogether, Rickard *et al.* (2006) have also shown that the AI-2 is a concentration-dependent signal. They have determined the optimal concentration of DPD for their oral bacterial system lies between 0.08nM and 0.8nM, which is much lower than the level

detectable by the *V. harveyi BB170* reporter. This is quite a narrow range and not many of the researchers in the field have used such a specific DPD concentration (Rickard *et al.*, 2006). In this study the DPD concentration used for the complementation test (about 0.5 μM) was over 100 times higher than that found to be effective by Rickard and co-workers and this may be why negative results were obtained. However the concentration used in this study was similar to that using in the study of *Bacillus cereus* which did produce a positive result (Auger *et al.*, 2006). This indicates that the effective concentration on AI-2 is species-specific. Hence the only way to find out if there is an effective concentration of AI-2 for the phenotypes being studied here in *Listeria* is to repeat the test on using various concentrations to see if there is any effect to the cells.

It is seen that the reporter strain BB170 produces AI-2 on its own which make it hard to give an exact measurement of the exogenous AI-2. Another reporter strain MM30, which produces no AI-2 (*lux*S::Tn*5*Kan$^{R}$) has been sometime used as negative control in the assay (Surette *et al.*, 1999, Freeman *et al.*, 2000). The construction of a double mutation of both the AI-1 sensor and the *luxS* gene may produce a better reporter characteristic, with bioluminescence only responding to AI-2 and with no indigenous AI-2 being produced by the reporter strain. In other words, reporter would only respond to exogenous AI-2, which fits better for AI-2 Bioassay. However, such a strain has yet to be reported in the literature and BB 170 is accepted as a valid reporter for the qualitative detection of AI-2.

# Chapter 6

# Identification of Potential *Listeria* genes Required for EPS Production using a Bioinformatics Approach

## 6.1 Introduction

From the published analysis of the *L. monocytogenes* genome, it was clear that this organism did not encode any of the genes associated with synthesis of polysaccharide capsular material. Since the ability to make extracellular polymeric substance (EPS) was not strain-specific (Chapter 3), it was unlikely that the genes responsible for this were simply not present in the genome of the two strains for which a full genome sequence is available in the databases.

Due to the fact that the *Listeria* EPS material was identified using a *Bacillus* capsular stain, it was first suspected that there might be a linkage to the polyglutamate EPS produced by *Bacillus* species, encoded by the genes of the *cap* operon. Since *Listeria* is closely related to *Bacillus,* it was decided to investigate whether homologues of these genes were present in the genome and hence may contribute to the formation of the *Listeria* EPS. This was done using a bioinformatics approach, specifically trying to identify any similarity between the capsule genes from *B. anthracis* and the sequences in the *L. monocytogenes* genome.

## 6.2 Direct Blast of *B. anthracis cap* genes in *Listeria* genome

The first step was to search for *cap* gene homologues within the *Listeria* genome at both DNA level and a protein level. This was done using the BLAST Tool found at the NCBI website http:// www.blast.ncbi.nlm.nih.gov/Blast.cgi. No homologues of any of the *cap* genes were found using a nucleotide sequence BLAST search (BLASTn) with the *B. anthracis capA* gene, so protein blast using BLASTp was performed using the *B. anthracis capA* gene protein sequence (Fig. 6.1).

**Figure 6.1: Protein Sequence of Capsule biosynthesis protein**

```
1   MRRKLTFQEK LLIFIKKTKK KNPRYVAIVL PLIAVILIAA TWVQRTEAVA PVKHRENEKL
61  TMTMVGDIMM GRHVKEIVNR YGTDYVFRHV SPYLKNSDYV SGNFEHPVLL EDKKNYQKAD
121 KNIHLSAKEE TVKAVKEAGF TVLNLANNHM TDYGAKGTKD TIKAFKEADL DYVGAGENFK
181 DVKNIVYQNV NGVRVATLGF TDAFVAGAIA TKEQPGSLSM NPDVLLKQIS KAKDPKKGNA
241 DLVVVNTHWG EEYDNKPSPR QEALAKAMVD AGADIIVGHH PHVLQSFDVY KQGIIFYSLG
301 NFVFDQGWTR TKDSALVQYH LRDNGTAILD VVPLNIQEGS PKPVTSALDK NRVYRQLTKD
361 TSKGALWSKK DDKLEIKLNH KHVIEKMKKR EKQEHQDKQE KENQVSVETT T
```

The protein sequence of CapA in *B. anthracis* (str. A2012; http://www.ncbi.nlm.nih.gov/protein/NP_653031.1) was downloaded from NCBI online gene bank. The Protein contains 411 amino acids and the amino acid sequence was used in Blastp to search for homologues in the *Listeria* genome.

Using BLASTp, *B. anthracis* CapA protein homologues were found in many *L. monocytogenes* strains, and two CapA protein homologues (*lmo0017* and *lmo0516*) were found in the genome sequence of *L. monocytogenes* EGD-e. In some cases, only one CapA homologue was present, this includes the strain F2365. In F2365, the *capA* homologue (termed lmof2365-0020) was found at gene location 0020, which is close to the position of *lmo0017* in strain EGD. Protein alignment showed that lmof2365-0020 is more similar to *lmo0017* than the second homologue identified in EGD, *lmo0516*.

The fact that these genes were only found using a protein search and not using a nucleotide search may be due to the variation in codon usage

between the two species (*L. monocytogenes* codon usage; GC%= 38.40; 1st letter GC 49.78% 2nd letter GC 35.79% 3rd letter GC 29.63%; *B. subtiltis* codon usage; GC%= 43.49; 1st letter GC 51.17% 2nd letter GC 35.55% 3rd letter GC 43.75%; source http://www.kazusa.or.jp/codon/) and indicates that the genes have not been recently acquired by *Listeria*. However no matches with a similar level of identity were found in any of the *Listeria* genome sequences for the other genes in the *B. anthracis cap* operon, namely *capB, capC, capD and capE* using either a protein or nucleotide BLAST search.

The Blastp outputs are displayed in Table 6.1, showing the score and e values of the Blast sequence results. The score (S) is the scoring of local ungapped alignments which is an indication of how the sequence matches. The higher the score, the more residues that matches between the query and subject sequences. The Expect value (E-value) is the probability that the sequence similarity detected occurs randomly hence it decreases exponentially as the Score (S) of the match increases. The lower the E-value, the more "significant" the match is. The alignment results of the *B. anthracis* CapA protein with the *L. monocytogenes* protein homologues *lmo0017* and *lmo0516* is shown in Figure 6.2.

**Table 6.1**: **BLAST results of *Bacillus* Cap protein in *L. monocytogenes* EGD and F2365genome sequences**

| EGD-e- | | Score | E-value |
|---|---|---|---|
| **CapA ~** | **lmo0017/ lmo0516 Similar to *B. anthracis* CapA protein** | **S: 130/113** | **E: 1e-30/2e-25** |
| CapB~ | lmo1049/ lmo1551/ lmo2694 folC/ Hypothetical protein | S: 28 | E: 2.4 |
| CapC~ | lmo0607 Similar to ABC transporters/ Hypothetical protein | S: 26.6 | E:2.7 |
| CapD~ | lmo2693/ lmo0481/ lmo1153 Hypothetical protein | S: 26 | E:8 |
| CapE~ | lmo1403 DNA mis-match repair protein | S: 23.5 | E: 8 |
| LMOF2365- | | Score | E-value |
| **CapA~** | **Lmof2365-0020 Conserved hypothetical protein** | **S: 131** | **E: 3.2e -31** |
| CapB~ | Lmof2365-1070/ Lmof2365-1572 folC/ moeB | S: 29 | E: 1.2 |
| CapC~ | Lmof2365-0636 ABC transporters/ ATP-binding protein | S: 26 | E: 2.8 |
| CapD~ | Lmof2365-2672 Thymidylate kinase | S: 27.3 | E: 7 |
| CapE~ | Lmof2365-2649 Hypothetical protein | S: 23.1 | E:9.8 |

BLASTp results of *B. anthracis* Cap protein in *L. monocytogenes* EGD and F2365genome sequences using NCBI website BLAST software. Only homologues with high similarity to CapA were found in both strains of *Listeria*. CapB, CapC, CapD and CapE Blast results only gave very low score hits indicating that no similar proteins were present in *Listeria*.

**Figure 6.2: Details of *Listeria* genes identified as *Bacillus* CapA homologues**

a) lmo0017

```
 GENE ID: 984984 lmo0017 | similar to Bacillus anthracis CapA protein
(polyglutamate capsule biosynthesis) [Listeria monocytogenes EGD-e]
(10 or fewer PubMed links)

 Score =  130 bits (326),  Expect = 1e-30, Method: Compositional matrix adjust.
 Identities = 85/248 (34%), Positives = 126/248 (51%), Gaps = 7/248 (3%)

Query  60   LTMTMVGDIMMGRHVKEIVNRYGTDYVFRHVSPYLKNSDYVSGNFEHPVLLEDKK-NYQK  118
            +   GDIM    ++ ++   G DY F +V   LK+SDY   + E  +    KK  YQ+
Sbjct  59   FNIDFTGDIMFDWDLRPVLAEKGMDYPFNNVREELKSSDYTFVDLETAITTRTKKVPYQE  118

Query  119  ADKNIHLSAKEETVKAVKEAGFTVLNLANNHMTDYGAKGTKDTIKAFKEADLDYVGAGEN  178
                + +   ++ A+K AG  ++N++NNH+ DY   G  DT  A +  +L YVGAG+N
Sbjct  119  ----FWIKSDPSSLTALKNAGVDMVNISNNHILDYYEDGLLDTTAALRANNLAYVGAGKN  174

Query  179  FKDVKNIVYQNVNGVRVATLGFTDAFV-AGAIATKEQPGSLSMNPDVLLKQISKAKDPKK  237
              +   +   ++ G +V  + F   F   G IA ++ PG  +     L+++  K +  K
Sbjct  175  EDEAYQLKVADIKGNKVGFMSFCHFFPNTGWIADEDTPGVTNGYDLNLVEEKIKEERAKN  234

Query  238  GNADLVVVNTHWGEEYDNKPSPRQEALAKAMVDAG-ADIIVGHHPHVLQSFDVYKQGIIF  296
             + D +VV  HWG E  N P   Q    K +VD    D IV  HPH LQ F+VYK   I
Sbjct  235  KDIDYMVVYFHWGVEKTNTPVDYQTQYVKKLVDDNLVDAIVASHPHWLQGFEVYKDVPIA  294

Query  297  YSLGNFVF  304
            YSLGNF+F
Sbjct  295  YSLGNFLF  302
```

b) lmo0516

```
 GENE ID: 985267 lmo0516 | similar to Bacillus anthracis encapsulation protein
CapA [Listeria monocytogenes EGD-e] (10 or fewer PubMed links)

 Score =  113 bits (282),  Expect = 2e-25, Method: Compositional matrix adjust.
 Identities = 84/274 (31%), Positives = 120/274 (44%), Gaps = 37/274 (14%)

Query  42   WVQRTEAVAPVKHRENEKLTMTMVGDIMMG------RHVK--EIV--NRYGTDYVFRHVS  91
            W     A      +E   +T+TMVGD   G       H+K   +   N     YV+++
Sbjct  144  WFYAAAATNQQGLKEKGVVTLTMVGDNSFGTYPETPEHLKFDNVFQKNNGNNTYVYKNCL  203

Query  92   PYLKNSDYVSGNFEHPVLLEDKKNYQKADKNI-HLSAKEETVKAVKEAGFTVLNLANNHM  150
            P+ K+ DY   N E         N  KA+  +  + +    V  +  +G    NLANNH
Sbjct  204  PWFKSDDYTIINAESAF-----TNATKAENKMWRIKSDPAHVAFLPASGVDAANLANNHT  258

Query  151  TDYGAKGTKDTIKAFKEADLDYVGAGENFKDVKNIVYQNVNGVRVATLGFTDAFVAGAIA  210
             DY   G  DT+KAFKE ++    A    +        + G++   LG+
Sbjct  259  MDYFQVGYDDTLKAFKENNIPVFNADAPLE-------TTIKGMKTVLLGYD---------  302

Query  211  TKEQPGSLSMNPDVLLKQISKAKDPKKGNADLVVVNTHWGEEYDNKPSPRQEALAKAMVD  270
                +S      L++I K     K    LV+VN HWG EY   P+  Q      A++D
Sbjct  303  -----CRMSQQSPAYLERIVKDVKKYKKEDTLVIVNMHWGVEYRETPTDYQTQFGHAILD  357

Query  271  AGADIIVGHHPHVLQSFDVYKQGIIFYSLGNFVF  304
            AGADII+G HPH L+S + YK   I YS+G+F F
Sbjct  358  AGADIIMGSHPHRLESVEKYKDKYIVYSMGDFAF  391
```

Aligned Blastp result of *B. anthracis* CapA and its homologues in *Listeria* EGD strain from the NCBI online Blast software. Panel A shows the result of lmo0017 and panel B for lmo0516.Both genes showed a high score and

E-value in the alignment with CapA protein of *B. anthracis* indicates a high similarity in protein structure (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The gene location and details of the *Listeria* gene *lmo0017* and *lmo0516* are shown in Table 6.2. Both of the genes are located on the negative strand of the *Listeria* EGD genome. The length of the ORF of *lmo0017* and *lm00516* was 1119bp and 1431bp, respectively, whereas the *B. anthracis* capA gene is 1236bp in length. The full sequence of *lmo0017* and *lmo0516* protein and gene are shown in Appendix I. Figure 6.3 shows the gene location and genes flanking lmo0017 and lmo0516 in the *L. monocytogenes* genome.

**Table 6.2: Gene location and description of *lmo0017* and *lmo0516* in *L. monocytogenes* EGD genome sequence**

| **Gene name** | ***lmo0017*** | ***lmo0516*** |
|---|---|---|
| **Description** | Similar to CapA protein | Hypothetical protein |
| **Location** | 20308-21426 | 549916-551346 |
| **Strand direction** | Negative strand | Negative strand |
| **Length Size** | 1119 bp | 1431bp |

**Figure 6.3: Diagram of Gene location of *lmo0017* and *lmo0516* in *L. monocytogenes* EGD genome sequence**

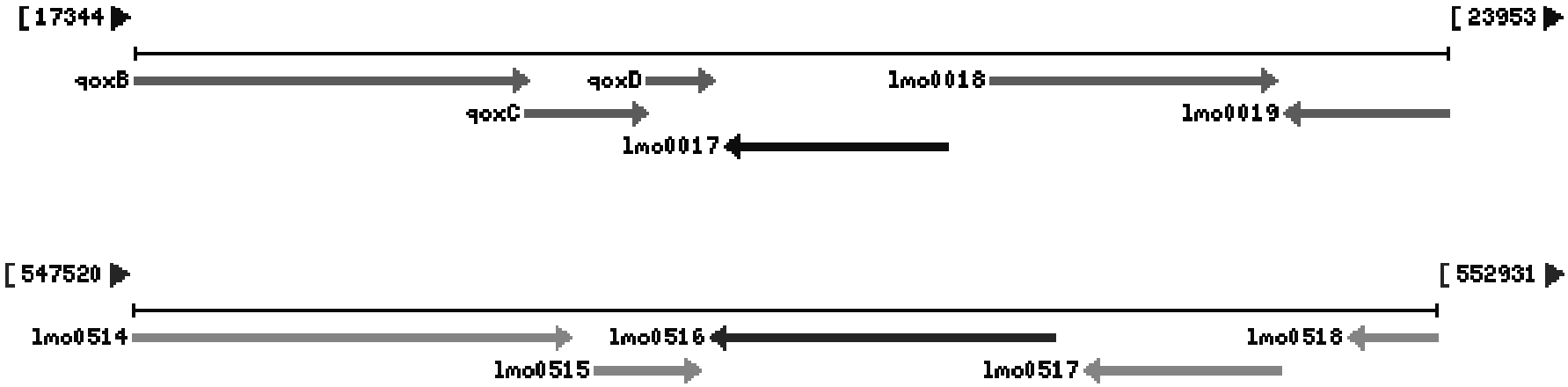



Diagram showing *Listeria* genome context of genes *lmo0017* and *lmo0516* in the *L. monocytogenes* EGD sequence, showing its neighboring genes and their corresponding direction of transcription. Arrows indicate the strand direction.

*lmo0017*: (http://www.ncbi.nlm.nih.gov/gene/984984)

*lmo0516:*(http://www.ncbi.nlm.nih.gov/gene/985267)

### 6.3 Searching for Possible Conserved CapA sequences

To extend the search for possible CapA homologues in the *Listeria* genome, a search for similar proteins from other bacteria species was performed using amino acid sequence of both lmo0017 and lmo0516 as the query with the BLASTp software. This would identify protein sequences with high similarity to lmo0017 and lmo0516 from other bacteria. These output sequences were then multiple aligned to search for conserved sequence among these similar protein homologues (Fig. 6.4 and 6.5). If a core conserved sequence could be identified by this alignment, this motif could be used to try and identify more degenerate versions of the *capA* gene present in the genome that might be responsible for production of the *Listeria* EPS.

**Figure 6.4: Multiple sequence alignment of lmo0017 homologues**


Alignment were generated using ClustalW2 software. Individual colours represent amino acids with similar properties. Grey boxes represent conserved sequences. Sequences used for the alignment were (1) *L. monocytogenes* lmo0017; (2) *Geobacter metallireducens* Capsule biosynthesis protein; (3) *Bacillus thuringiensis* Capsule biosynthesis protein CapA; (4)*Paenibacillus* Bacterial capsule synthesis protein; (5) *Bacillus subtilis* Capsule biosynthesis protein; (6) *Myxococcusxanthus* Conserved hypothetical protein.

**Figure 6.5: Multiple sequence alignment of lmo0516 homologues**


Alignment were generated using ClustalW2 software. Individual colours represent amino acids with similar properties. Grey boxes represent conserved sequences. Sequences used for the alignment were (1) *L. monocytogenes* lmo0516; (2) *Anoxybacillus flavithermus*poly-gamma-glutamate biosynthesis enzyme; (3) *Natranaerobius thermophilus* poly-gamma-glutamate biosynthesis enzyme; (4) *Clostridium botulinum* Encapsulation protein CapA; (5) *Clostridium difficile* Putative lipoprotein; (6) *Geobacter metallireducens* Capsule biosynthesis protein.

From the above two alignments, it is seen that for both proteins, there are conserved residues found among the two group of sequences. Interestingly the motifs identified are not identical, showing that there is degeneracy between the two gene homologues in *Listeria*. For instance the motif in lmo0017 at position 138 is NNH-$(X)_2$–DYY–$(X)_6$–DT whereas the equivalent motif at position 255 in *lmo0561* is NNH-$(X)_2$–D–$(X)_4$–G–$(X)_2$-DT. Similarly motif at *lmo0017* position 275 $(X)_2$-H-(X)-P-(X)-V-$(X)_4$-E-(X)-Y were shown to be similar to that of lmo0516 position 263 G-(X)-HPH-$(X)_5$-Y Motif in lmo0017 254 YSLGNF-(X)-F were similar to that of *lmo0516* position 384 YS-(X)-G-(X)-F-(X)-F. Notably, for each of these conserved regions, the sequence in *lmo0017* and *lmo0516* were usually the least alike within the group. There could be a chance that the conserved motifs in these two proteins have already been modified due to evolution.

The three conserved motifs were used to search the NCBI database using the BLAST programme to see if the conserved sequences were present in genes with known function. Among the 3 conserved sequences identified by this search, the conserved sequence found was shown to appear in many CapA, Encapsulation or polyglutamate syntheses proteins from a wide range of bacterial species. This indicates the gene could have evolutionary linkage to PGA synthesis genes, and this may be its role in *Listeria* (See Appendix II).

## 6.4 Searching for possible *capBCDE* genes in the *Listeria* genome

In *Bacillus*, the *cap* operon consists of five genes. The genes *capA* and *capE* are required for PGA transport, whereas *capC* and *capD* are required for PGA synthesis and *capD* was found to be the glutamyl-transpeptidase that is required for PGA anchorage (Candela and Fouet, 2006). As CapA is known to be required only for transportation of the PGA, so the presence of the remaining 4 genes, *capB, capC, capD, capE*, are also required to produce PGA. However, *capBCDE* homologues were not found by direct gene and protein BLAST searches as shown above. So the bioinformatics approach used to identify the conserved motifs in CapA was then used to look for motifs conserved in known possible CapBCDE proteins with the idea that these conserved sequences may then be used as target to identify possible gene homologues in the *Listeria* genome. However, after the search for conserved sequence among different Cap*BCDE* from various bacteria, no sequences with high similarity results were found, indicating there is a low chance of finding possible *capBCDE* genes or proteins within the *Listeria* genome. (see Appendix III)

## 6.5 Studying location and neighbouring genes of *capA* homologues

The bioinformatic approach had only identified homologues of the *capA* gene sequences that did not appear to form part of a PGA biosynthetic operon. So the question then remained what was the role of these orphan genes. Further studies of the genetic context of other *capA* homologues was showed that in the genome of another species of *Bacillus,* for instance *B. cereus*, isolated *capA* gene homologous have also been identified which are not associated with other *cap* biosynthetic operon genes (Han *et al.*, 2006) and - like the

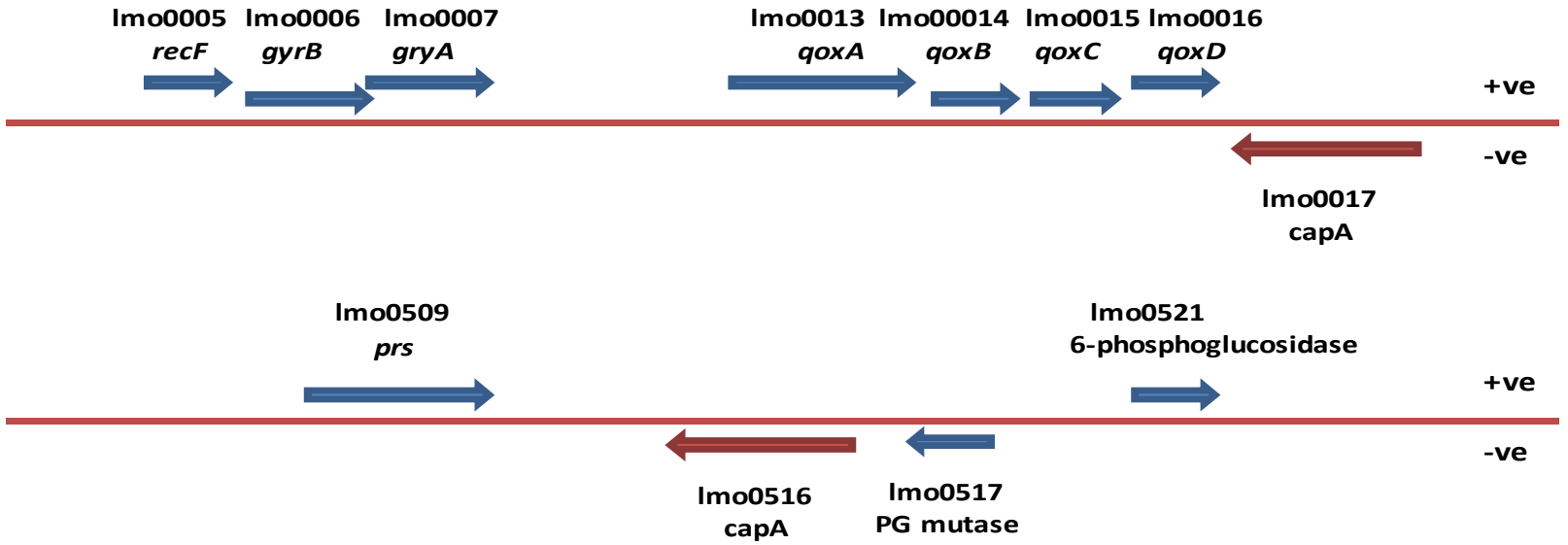
situation in *Listeria* - these genes are located in the chromosome rather than on a plasmid as is the case for the *cap* operon in *B. anthracis*. Hence in terms of gene location and operon context, the *Listeria capA* homologues are more similar to those of *B. cereus* than those of *B. anthracis* (see Appendix IV). Since the *capA* homologues found in both *L. monocytogenes* and *B. cereus shared* this similarity, an analysis was undertaken to determine if any information about the role of these orphan genes could be gained by comparing the genes linked to them in the chromosome.

To initiate this investigation, 1kb of sequence upstream and downstream of *lmo0017* and *lmo0516* were used to BLAST against the whole *B. cereus and B. anthracis* genome. The results for the *B. cereus* were felt to be particularly relevant due to the fact that this organism also had an orphan *capA* gene. Only the *lmo0017* 1kb downstream sequence showed any sequence matches, but this was only in one single gene homologue at position 985361 to 985874 in the *B. cereus* E33L genome.However, further analysis around that region of the *B. cereus* genome identified showed no similarity to a *capA* gene. As perhaps was expected, the results of the comparison of with the *B. anthracis* genome identified no sequences similar to either the upstream or downstream regions of *lmo0017* or *lmo0516*.

Since a direct search of genome sequence did not reveal any similarities, it was then decided to search for neighbouring genes of the *Listeria capA* homologues according to function to see if the *capA* from *Listeria* have any linkage with the genes associated with *capA* in *B. anthracis* or *B. cereus*. The neighbouring genes of *lmo0017* and *lmo0516* with known function were selected and these are shown in Figure 6.6 and the result of the search based on the function of these is shown in Table 6.3.

Homologues of some of the genes neighbouring the two *Listeria capA* homologues were found in the *B.* cereus genome sequence, however the locations of these were not nearby the *B. cereus capA* orphan genes (Table 6.3). Similarly, homologues of the genes associated with the *Listeria capA* genes were also found on the *B. anthracis* genome sequence, but again these were not associated with the *capA* gene located in the plasmid, indicating a low chance of a functional relationship. These also suggested that the orphan CapA homologues in *Listeria* are unlikely to be genes that have been acquired in an evolutionary process because it is very unlikely that this one gene would have been acquired without any of the neighbouring genes.

**Figure 6.6: The neighbouring genes of the *Listeria capA* homologues (only showing those found in *Bacillus* genome)**




The neighbouring genes of the *Listeria capA* homologues present in *Bacillus* genome (according to function). The gene *Lmo0017* and *lmo0516* were presented in red and the neighbouring genes in blue. Arrow direction represents the strand that the gene was located. The diagrams of 15 neighbouring gene of *lmo0017* and *lm0516* were simplified that only showing genes present in *Bacillus* genome. There was no known gene upstream of lmo0017 present in the *Bacillus* genome.

**Table 6.3: Location of neighbouring genes of lmo0017 and lmo0516 in *Bacillus* genome**

| *Function of gene identified in Listeria* | **Gene number** | | | |
|---|---|---|---|---|
| | ***Listeria EGD*** | ***B. cereus Q1*** | ***B. cereus ATCC14579*** | ***B. anthracis Ames Ancestor*** |
| *capA* | **lmo0017/0516** | BCQ5115/1856 | BC3308/BC1783 | pXO2_0064 |
| **Genes nearby lmo0017** | | | | |
| *recF* | **lmo0005** | BCQ0004 | BC0004 | GBAA_0004 |
| *gyrB* | **lmo0006** | BCQ0005 | BC0005 | GBAA_0005 |
| *gyrA* | **lmo0007** | BCQ0006 | BC0006 | GBAA_0006 |
| *qoxA* | **lmo0013** | BCQ0772 | BC0698 | GBAA_0703 |
| *qoxB* | **lmo0014** | BCQ0771 | BC0697 | GBAA_0702 |
| *qoxC* | **lmo0015** | BCQ0770 | BC0696 | GBAA_0701 |
| *qoxD* | **lmo0016** | BCQ0669 | BC0695 | GBAA_0700 |
| **Genes nearby lmo0516** | | | | |
| *Prs*[a] | **lmo0509** | BCQ0057 | - | GBAA_0049 |
| Phosphoglyceratemutase | **lmo0517** | BCQ_2058 | BC4971 | GBAA_3545 |
| 6-phospho-beta-glucosidase | **lmo0521** | BCQ_5031 | BC5209 | GBAA_5441 |

[a]this gene is described as similar to phosphoribosyl pyrophosphate synthetase and is not the *prs* gene located at the end of LI-P1 virulence gene region in *L. monocytogenes*

The table shows the function and location of genes surrounding the two *capA* homologues in *L. monocytogenes* and the location of the corresponding genes in three different *Bacillus* species genomes. The genes found neighbouring *capA* homologues were not located near to that in *Bacillus capA*.

To complete this analysis a second search was performed the other way round, searching the neighbouring genes found near the orphan *B. cereus capA* genes against the *Listeria* genome sequence (Table 6.4). This was done by identifying the function of the neighbouring genes of the *B. cereus capA* gene and searching for the corresponding genes in the *Listeria genome*. Again no neighbouring genes were found to be shared between the *Bacillus capA and Listeria capA* homologues.

It is clear then that the locations of these genes in the different species varies a lot, and many of the genes associated with *cap*A in *B. cereus* are not found at all in the *Listeria* genome. Again this supports the idea that this gene has not been gained by a horizontal gene acquisition event and that the *capA* homologues are very unlikely to have direct relationship within the locus, which may suggest that they may have been acquired and maintained in their current form by a different evolutionary process.

**Table 6.4: Location of neighbouring genes of *Bacillus CapA* in *Listeria monocytogenes* genome**

| *Gene identified in B. cereus* | **Organism** | | | | |
|---|---|---|---|---|---|
| | ***B.cereus Q1*** | ***B.cereus ATCC14579*** | ***B.anthracis Ames Ancestor*** | ***Listeria EGD*** | ***Listeria str. 4b F2365*** |
| *capA* | BCQ5115/1856 | BC3308/1783 | pXO2_0064 | lmo0017/0516 | lmof020/0545 |
| ilvA | **BCQ1855** | **BC1781** | | lmo1991 | lmof2365_2014 |
| ilvD | **BCQ1854** | **BC1780** | | lmo1983 | lmof2365_2006 |
| ydfK | **BCQ_1857** | - | | N/A | N/A |
| Multidrug resistance protein B | **BCQ_1859** | **BC1786** | | N/A | N/A |
| fabZ | **BCQ_5117** | BC5280 | | lmo2524 | lmof2365_2497 |
| epsC | **BCQ_5113** | BC5276 | | lmo0938 | lmof2365_0959 |
| epsE | **BCQ_5111** | - | | lmo0933 | N/A |
| D-alanyl-D-alanine carboxypeptidase | BCQ_4665 | **BC3307** | | lmo2812 | lmof2365_1883 |
| 3-oxoadipate enol-lactonase | BCQ_3094 | **BC3312** | | N/A | N/A |
| gamma glutamyltranspeptidase | | | **pXO2_0063** | N/A | N/A |
| PagR | | | **pXO2_0069** | N/A | N/A |

N/A = homologue not found in Listeria

The table shows the location of neighbouring gene of CapA in three *Bacillus* species genomes (in BOLD). The corresponding genes in the *Listeria* genome species with the same function and their gene location were also listed in the table.

## 6.6 Protein structure modelling

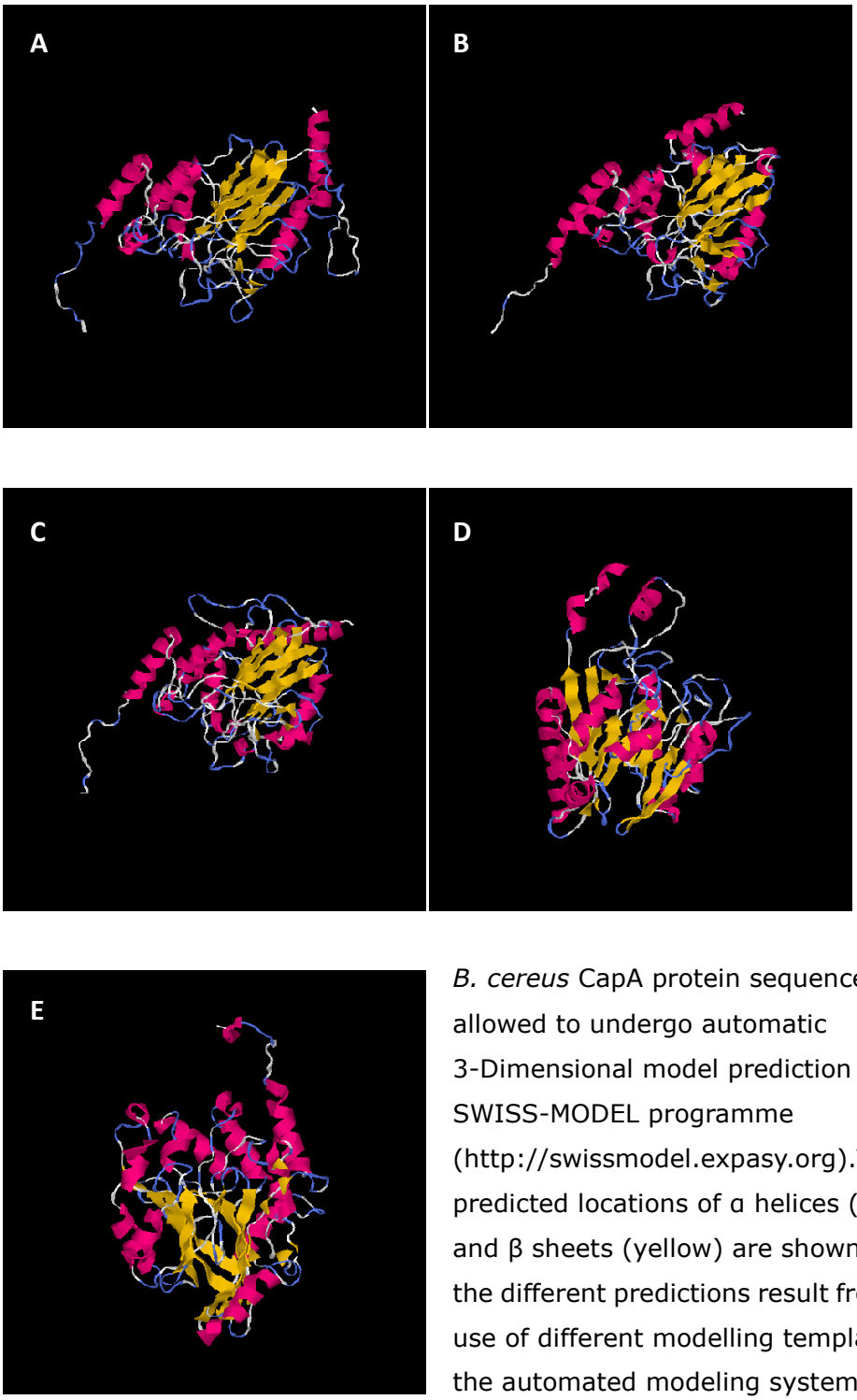
From the protein alignments presented in Figures 6.3 and 6.4 it is clear that, despite the evidence of a diverged role for CapA in *Listeria,* some core functional motifs are retained which suggests that the protein does have a biological function. However it is possible for conserved motifs to be retained while proteins adopt a different biological function. In this case the overall 3-dimensional structure of the protein tends not to be conserved (Whisstock and Lesk, 2003). In order to estimate the similarity between the *Bacillus* CapA proteins and the CapA homologues found in *L. monocytogenes*, the protein sequences were used to generate a predicted 3-dimensional model of the different proteins. Although this may not give a specific determination of structure, this might provide some evidence of the similarity between the target proteins. This was done using the bioinformatics software provided on SWISS-MODEL website (http://swissmodel.expasy.org). The proteins sequences were uploaded and underwent automatic 3-Dimensional model prediction.

The results are presented in in Figures 6.7 – 6.10 and indicated that the CapA protein from *B. cereus* was more similar to the CapA protein from *B. anthracis* in terms of number and location of alpha-helixes and beta sheets whereas lmo0017 and lmo0516 proteins were less similar to *B. anthracis* CapA and showed more variable length and number of sheets and helixes. This result suggested that lmo0017 and lmo0516 proteins may have a different function from those CapA proteins found in *B. anthracis*.

However, in terms of the basic structures presented in the modelling output, it can be seen that all the four proteins are predicted to form a cluster of β sheet surrounded by a certain numbers of alpha-helixes. This is a common

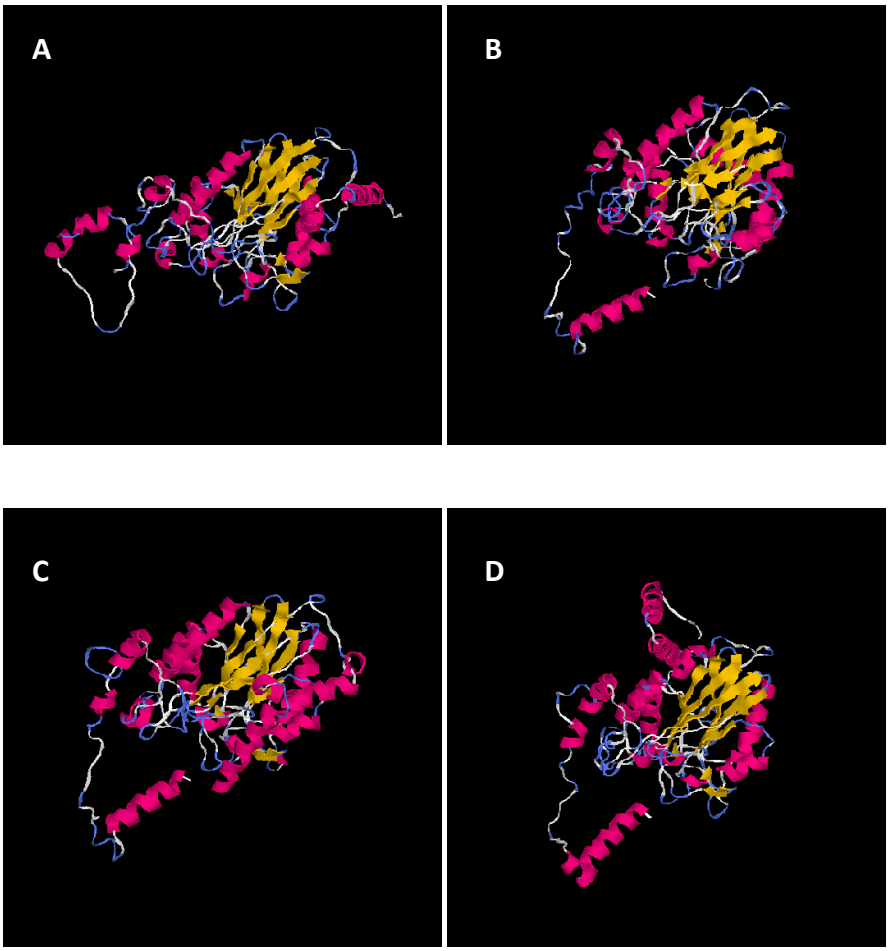
structure found in transport proteins that form channels in the membrane (Eisenberg, 1984). As capsule or PGA has also not been identified in *B. cereus*, suggesting the presence of an orphan CapA homologue may not be linked to PGA synthesis. Even without the whole PGA synthesis operon in *B. cereus* and *L. monocytogenes*, there could be a chance that the CapA homologue may carry similar functions as CapA being a transmembrane transporter of an unknown substance. This is seen by the similarity in protein sequence as well as folding prediction. However, these results are just predicted models based on the protein sequences, and therefore no absolute conclusion can be drawn from these diagrams without additional evidence of protein structure gained from structural analysis of purified protein.

**Figure 6.7: Predicted 3D model of *B. cereus* CapA proteins**



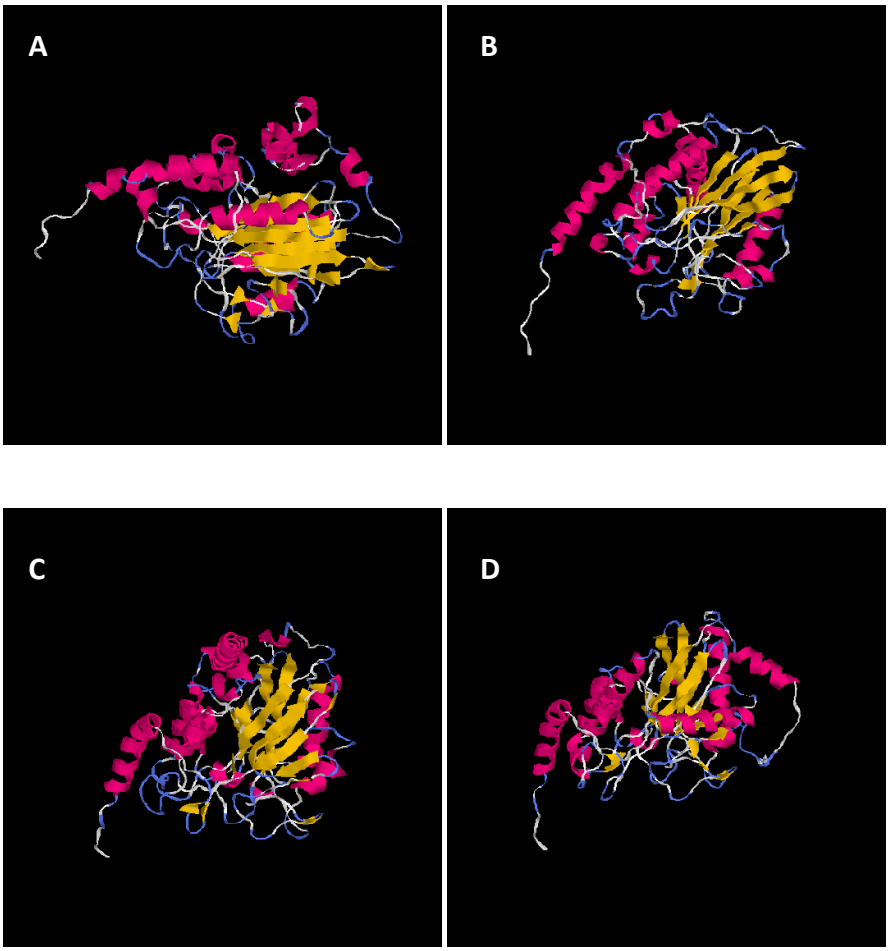

*B. cereus* CapA protein sequence was allowed to undergo automatic 3-Dimensional model prediction using SWISS-MODEL programme (http://swissmodel.expasy.org).The predicted locations of α helices (pink) and β sheets (yellow) are shown, and the different predictions result from the use of different modelling templates in the automated modeling system.

**Figure 6.8: Predicted 3D model of *B. anthracis* CapA protein**



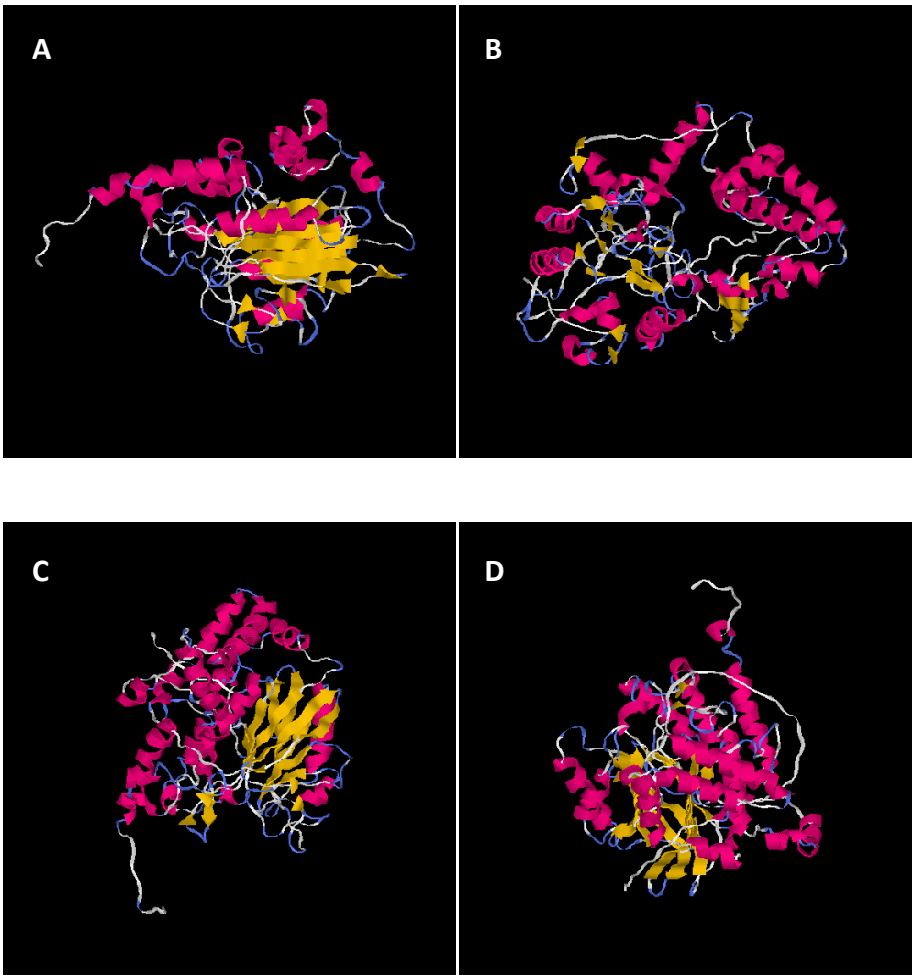

*B. anthracis* CapA protein sequence was allowed to undergo automatic 3-Dimensional model prediction using SWISS-MODEL programme (http://swissmodel.expasy.org).The predicted locations of α helices (pink) and β sheets (yellow) are shown, and the different predictions result from the use of different modelling templates in the automated modeling system.

**Figure 6.9: Predicted 3D model of lmo0017 protein**




*Listeria* lmo0017 protein sequence was allowed to undergo automatic 3-Dimensional model prediction using SWISS-MODEL programme (http://swissmodel.expasy.org).The predicted locations of α helices (pink) and β sheets (yellow) are shown, and the different predictions result from the use of different modelling templates in the automated modeling system.

**Figure 6.10: Predicted 3D model of lmo0516 protein**



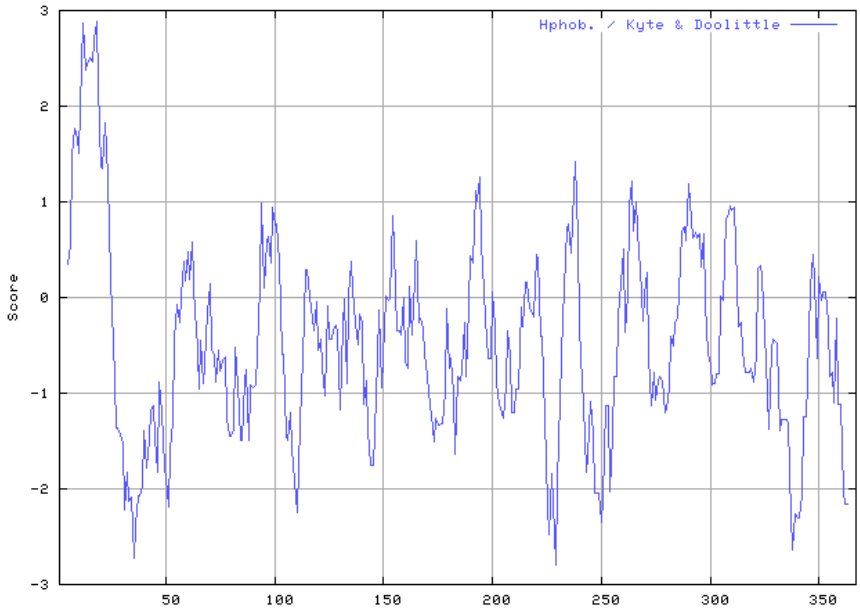

*Listeria* lmo0516 protein sequence was allowed to undergo automatic 3-Dimensional model prediction using SWISS-MODEL programme (http://swissmodel.expasy.org). The predicted locations of α helices (pink) and β sheets (yellow) are shown, and the different predictions result from the use of different modelling templates in the automated modeling system.

## 6.7 Protein sequences Hydrophobicity Profiling

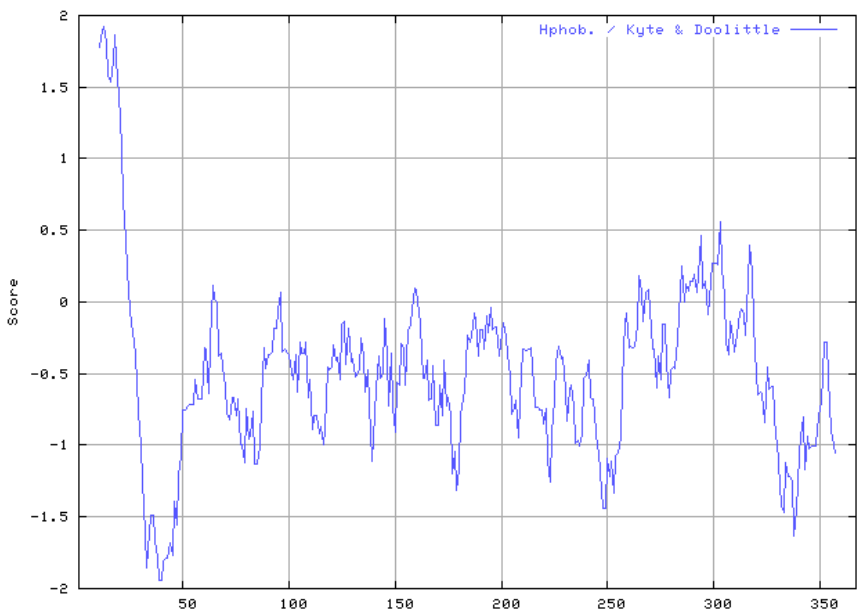
As the results from the protein modelling suggested that the target proteins, lmo0017 and lmo0516, may be transmembrane proteins and therefore the protein sequences were also used to determine the hydrophobicity profile according to Kyte and Doolittle (1982) which provides an estimation of the hydrophobicity of different parts of a protein sequence. Each amino acid is given a score based on its hydrophobicity, with a value of 4.6 for the most hydrophobic and -4.6 for the most hydrophilic. This scoring, when plotted on an X axis that represents the linear order of amino acids in the protein, allows predictions of sequence turning positions, exposed and buried residues and membrane spanning segments of protein to be made. Transmembrane proteins usually contain regions of mostly non-polar residues which interact with the organic layer of the membrane and transmembrane transporters may have characteristic structures containing consecutive helices creating a hydrophilic canal to allow aqueous substances to cross the membrane (Rath and Deber, 2012). When carrying out a hydropathy plot, a window size of 19-21 is used to allow membrane-spanning domains to be identified clearly as those regions with a peak greater than 1.6 on the plot. Proteins with similar function may have similar hydrophobicity profiles even if there is no clear homology revealed from the primary sequence (Eisenberg *et al.*, 1984). If the specific structure of a protein is not known, the hydrophobicity profile is only useful to provide an indication of likely similarity of proteins in terms of the general organisation of hydrophobic domains rather than providing proof of related structure or function. The results of this analysis are presented in Figures 6.11-6.14.

**Figure 6.11: Hydrophobicity profiling of *B. cereus* CapA protein**

A)



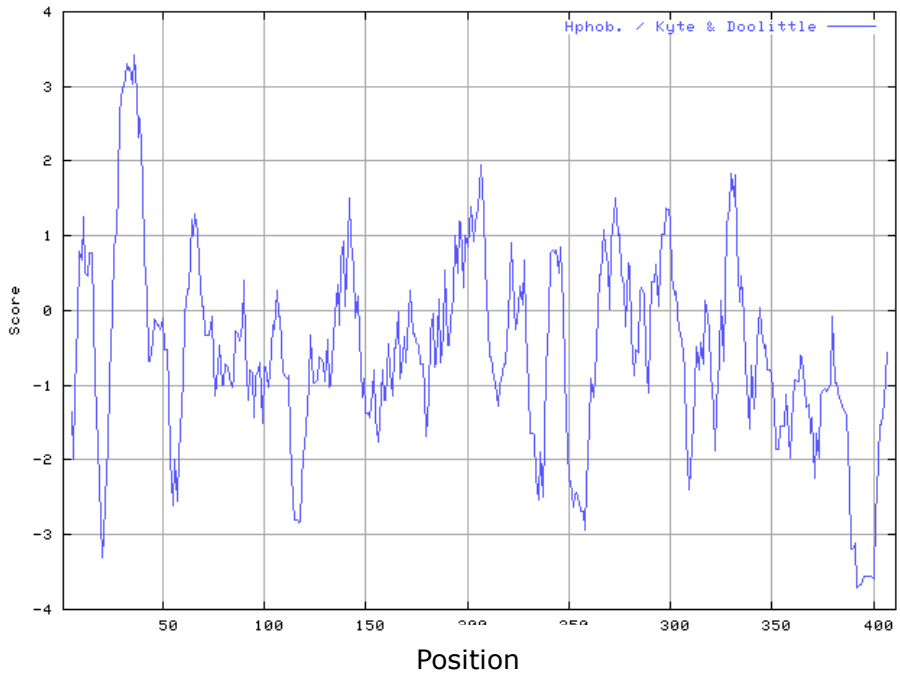

B)



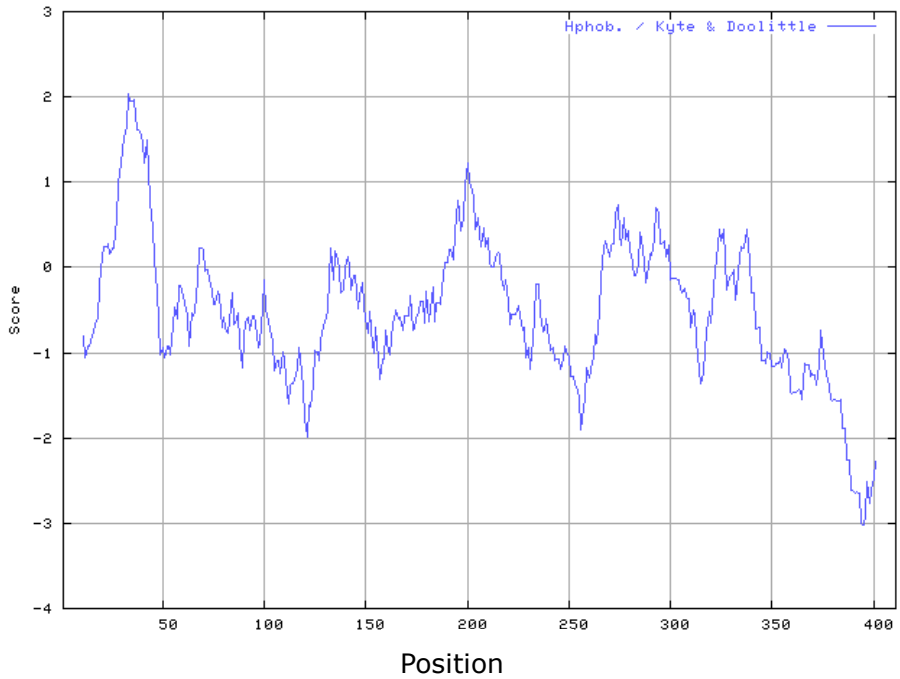

The hydropathy plots for the *B. cereus* CapA protein were generated using Protscale (http://www.expasy.ch/tools/protscale.html). Plots generated using either a 9 window (Panel A) or21 window (Panel B) value according to Kyte and Doolittle (1982). Positive and negative scores represent hydrophobic and hydrophilic regions, respectively.

**Figure 6.12: Hydrophobicity profiling of *B. anthracis* CapA protein**

A)



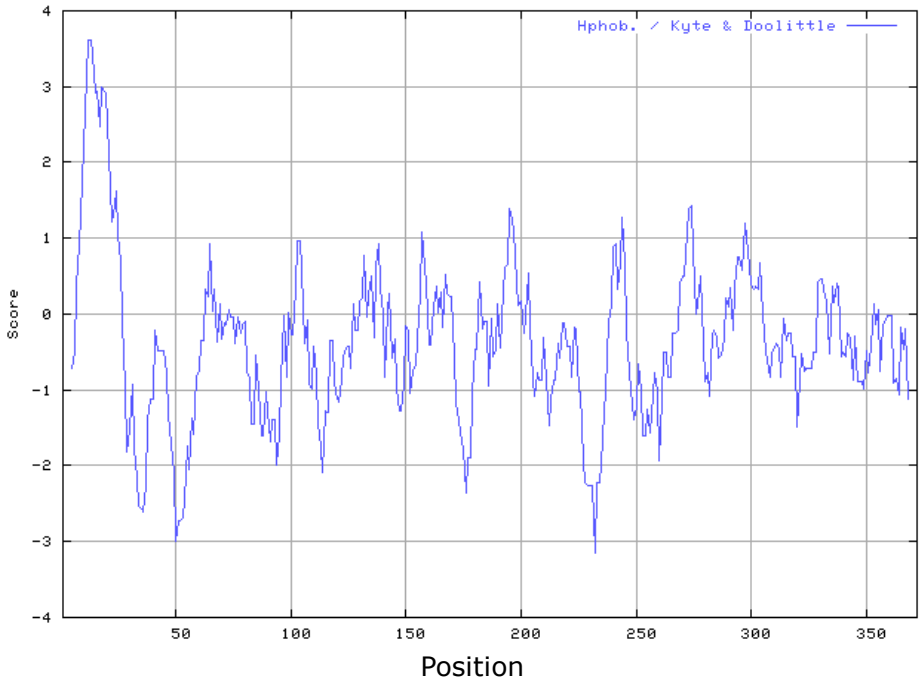

B)



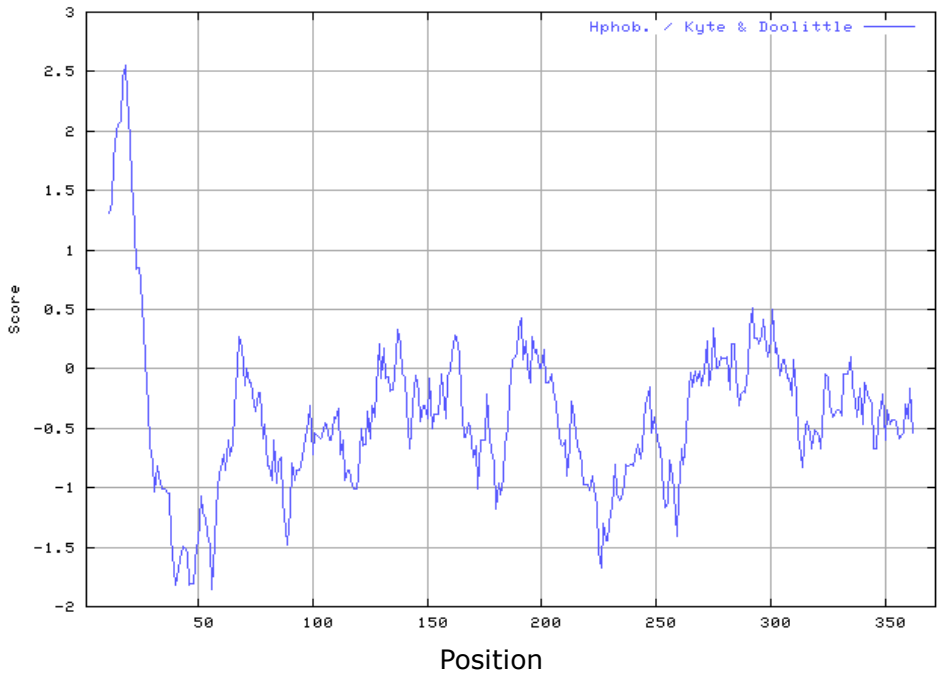

The hydropathy plots for the *B. anthracis* CapA protein were generated using Protscale (http://www.expasy.ch/tools/protscale.html). Plots generated using either a 9 window (Panel A) or 21 window (Panel B) value according to Kyte and Doolittle (1982). Positive and negative scores represent hydrophobic and hydrophilic regions, respectively.

**Figure 6.13: Hydrophobicity scale profiling of lmo0017 protein**

A)_



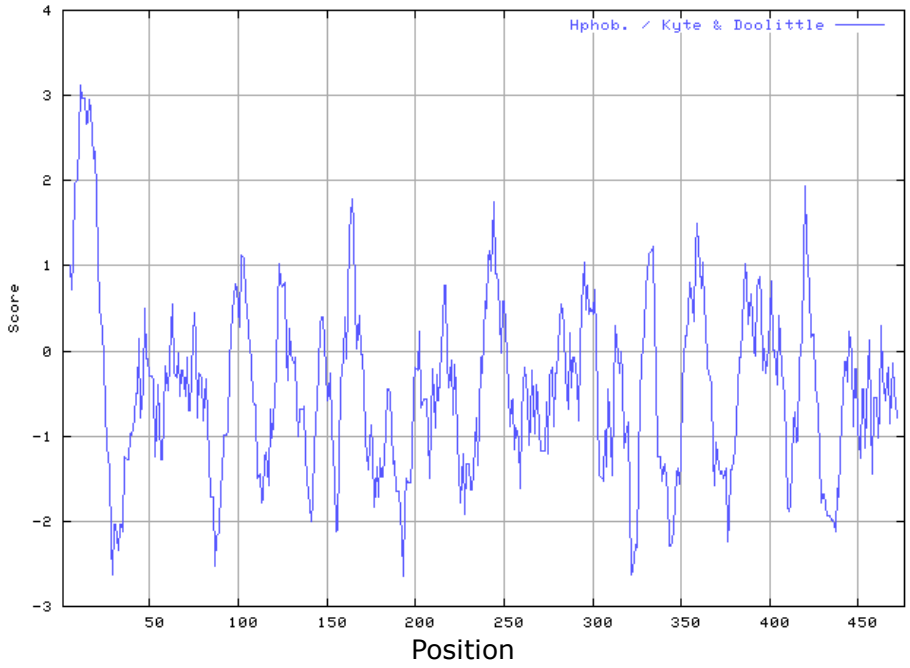

B)



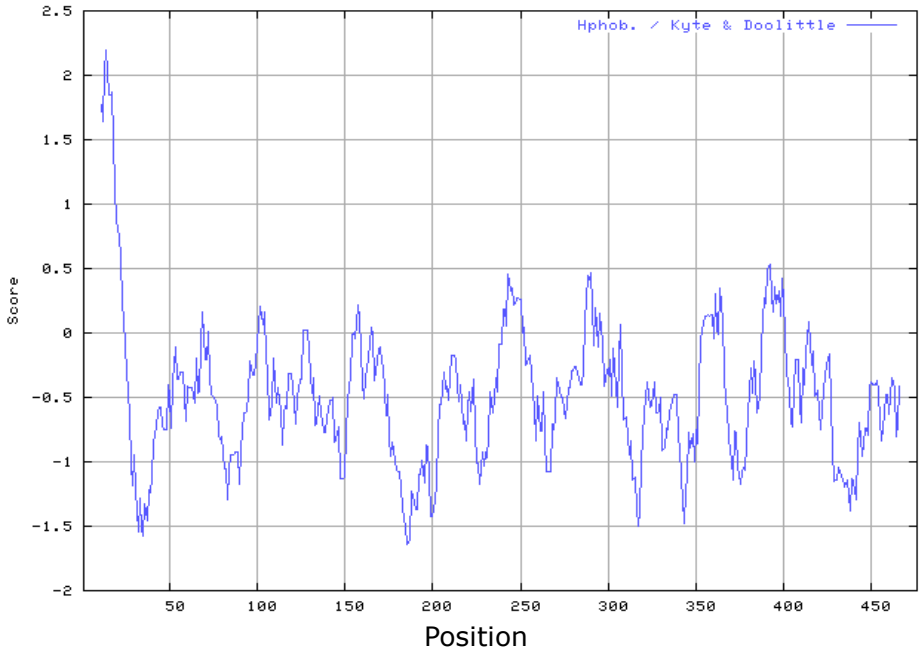

The hydropathy plots for the *L. monocytogenes* lmo0017 protein were generated using Protscale (http://www.expasy.ch/tools/protscale.html). Plots generated using either a 9 window (Panel A) or 21 window (Panel B) value according to Kyte and Doolittle (1982). Positive and negative scores represent hydrophilic and hydrophobic regions, respectively.

**Figure 6.14: Hydrophobicity profiling of lmo0516 protein**

A)




B)




The hydropathy plots for the *L. monocytogenes* lmo0516 protein were generated using Protscale (http://www.expasy.ch/tools/protscale.html). Plots generated using either a 9 window (Panel A) or 21 window (Panel B) value according to Kyte and Doolittle (1982). Positive and negative scores represent hydrophilic and hydrophobic regions, respectively.

Comparison of the 9-windows hydrophobicity profiles of CapA from *B. cereus*, lmo0017 and lmo0516 show that they have a similar range of hydrophobicity scores, ranging from about -1.5 to 1.5, whereas that of the *B. anthracis* CapA protein has a range of -3 to 2 indicating a wider range of hydrophobicity changes and perhaps then a different structure to the three orphan *capA* protein genes.

When the 21 windows plots were compared, no particular peaks greater than 1.6 were seen on any of the plots and the range of fluctuation is much lower in all the four proteins. However it is again obvious that the pattern produced from *B. anthracis* CapA protein sequence is quite different from the other three proteins giving a lower level of fluctuation and a clearer profile. This result also indicates that, in terms of the hydrophobicity scale, the *B.anthracis* CapA is quite different from the other three proteins and therefore perhaps suggests a different physiological function.

## 6.8 Discussion

Overall, using a variety of different bioinformatics approaches, it can be finally confirmed that that the two homologues of the *B.anthracis capA* gene that were identified in *L. monocytogenes* are orphan genes and that no evidence of the biosynthetic genes required for the production of PGA (*capBCDE* gene or *CapBCDE* protein homologues) can be found in the *Listeria* genome. In addition for the orphan genes there seemed to be little evidence for a functional association with other similar proteins since the location of *capA* genes were different in the different species indicating a low chance of evolutionary linkage between these proteins. Three dimensional structure modelling suggested that the homology identified at the sequence level is

likely to produce proteins of similar overall structure to the *B. anthracis* CapA protein but the hydrophobicity profile suggested that perhaps the orphan CapA proteins form a distinct group, and therefore may now represent a protein that has a distinct function.

Bioinformatics analysis is based on the comparison of genomic or proteomic data, and in this case was expanded beyond direct comparison of single gene sequences to ask questions about gene location. In addition modelling tools were used to compare predicted protein structure and the potential characteristics of the protein under study. However bioinformatics can only provide supporting evidence about protein structure or function and can only provide a numeric estimation of how proteins or genes are alike, or to detect the presence or absence of gene homologues in genome. Specific functions and patterns of expressions of genes and proteins cannot be determined by this method and therefore unfortunately the results do not help identify what this different biological role may be.

While this analysis was being carried out, it was discovered by other researchers in the group through chemical analysis of the EPS that the material produced by *Listeria* is not PGA (Nwaiwu, 2010). This result supports the conclusion of the bioinformatics analysis in that the *Listeria* CapA homologues are unlikely to be involved in the transport of PGA since no evidence for any of the other biosynthetic genes required for the production of PGA could be found. However, the results gained here have provided new understanding about the *capA* homologues found in the *Listeria* genome, which now seem to be an interesting conserved orphan gene that exists without the other members of the PGA biosynthetic genes. The hydropathy plots still indicate an overall hydrophilic character and therefore it is rather

not possible that these proteins have transport functions. It is also seen that all four protein showed a hydrophobic N-terminus, which suggested that there is possibility that the protein may be a secreted protein.

The three-dimensional protein structures provided in this study are only computational prediction based on the protein sequences provided (Zhang, 2008). Homology Modeling methods rely on the use of similar known proteins and in this case were only able to determine small fragments of the overall protein structure. Hence it is by no means certain if the results obtained do provide the actual structure of the protein, and this data needs to be confirmed using other physical techniques. Protein structure can be determined by methods such as X-ray crystallography or Nuclear Magnetic Resonance (Schwede *et al.*, 2003). However the proteins from the lmo0017 and lmo0516 genes have not yet been identified or isolated, meaning that such definitive analysis methods are not yet possible to confirm the results obtained. In particular further determination of the protein structure would be needed to investigate whether or not the orphan genes did have a distinct structure to that of the *B. anthracis* CapA protein. However the structure of a protein will clearly affect its function or characteristics, and therefore the fact that these orphan genes do not conserve the same predicted structure as the *B. anthracis* CapA protein is a first indication that they may have a different physiological role.

Amino acid hydrophobicity has great effect on protein structure conformation and hence the functions of a protein. The hydrophobicity plot is sometimes use to estimate the function or location of a protein. However, the hydrophobicity plot is only a reference plot which cannot give direct evidence

of protein structure. In our study, this was used mainly to compare similarities between the different proteins and also to see if the orphan CapA proteins retained the same transmembrane characteristics that have been identified in the *B. anthracis* CapA protein (Eisenberg *et al.*, 1984). As we can see from the plots above, the graphs for the orphan genes are actually rather unclear in comparison to the *B. anthracis* CapA protein without clear indications of hydrophilic regions between the more hydrophobic parts of the protein. As the structure of the protein is not known, and there are no straightforward or defined methods to interpret these plots, the amount of information that can be deduced is very limited.

From the data above it was concluded that the two *Listeria* CapA protein homologues have a low chance of linkage with the production of EPS seen in *L. monocytogenes*. However there have been only a limited number of publications that have described the *capA* genes in *Listeria*. Only one recent report studying the *L. monocytogenes* exoproteome, by the use of secretomic analyses, mentions lmo0017 (Desvaux *et al.*, 2010). They have shown that lmo0017 and lmo0516 proteins were not found on *Listeria* cell surface, and they therefore concluded that the two proteins were not likely to function as PGA synthases. However, they didn't focus on the fact that the *B. anthracis* CapA protein was shown to be a transporter (Marvasi *et al.*, 2010). On the other hand, the *lmo0516* mutant was shown to have impaired bile resistance, suggesting that this CapA homologue may be functioning to protect the cell from external challenge, which may in turn linked with a secreted molecule or surface-located proteins (Begley *et al.*, 2002). Hence there could be a chance that the *Listeria* protein is located in the membrane where it still acts as a transporter, but this was then not detected by exoproteome analysis which only identifies proteins released from the cell.

# Chapter 7

# Expression of the

# *Listeria monocytogenes capA*

# Gene homologues

## 7.1 Introduction

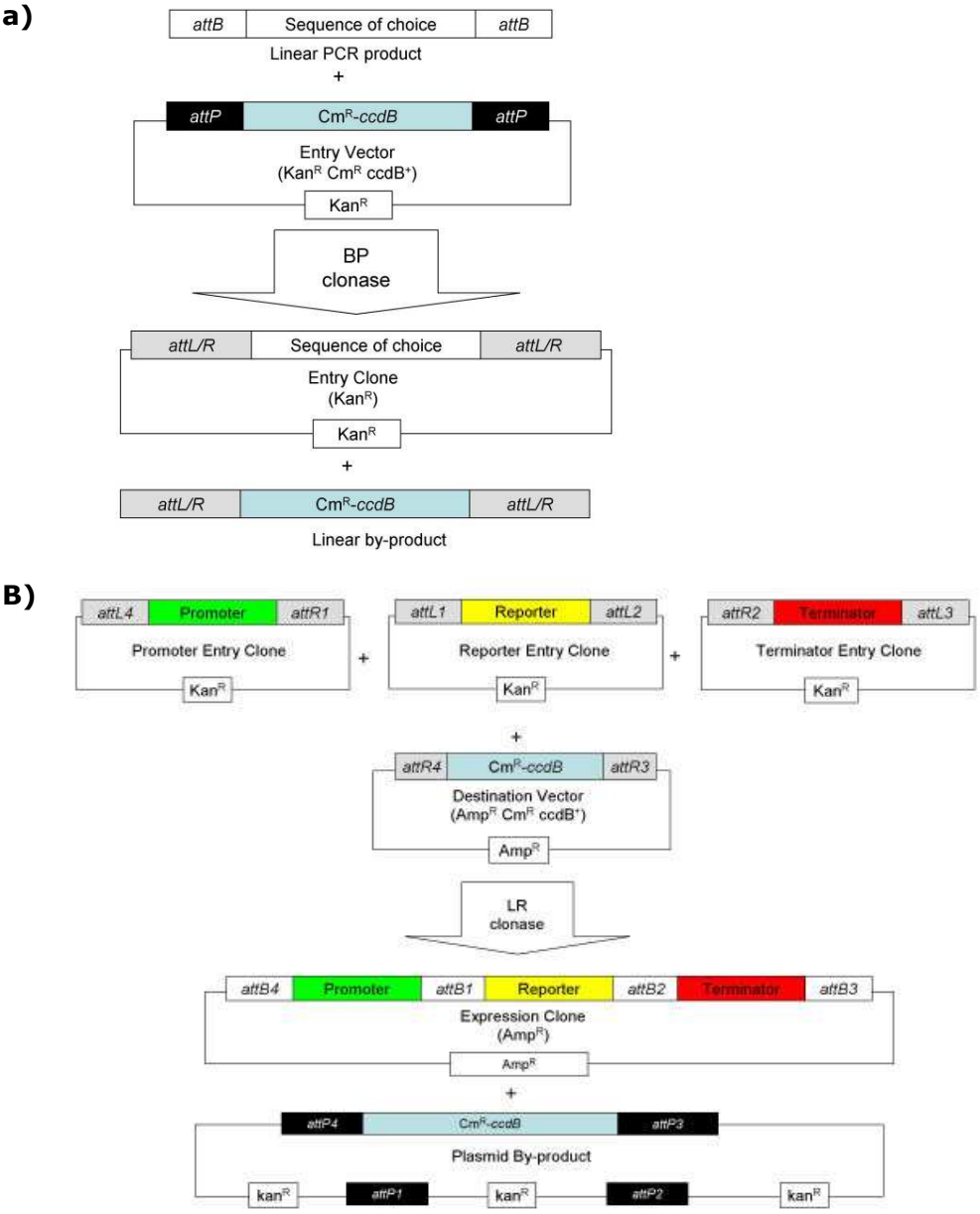
In Chapter 6, lmo0017 and lmo0516 were identified as two CapA protein homologues present in the *L.* monocytogenes genome, and the *capA* gene is known to be required for PGA capsule formation in *Bacillus* species, specifically acting as the central protein of the export complex (Roberts, 1996). Although the bioinformatics analysis carried out could not identify homologues of any of the other *cap* genes, it is possible that the *capA* genes are conserved because they are involved in the synthesis of a different type of surface polymer. To see if there was any evidence of this, this part of the research aimed to determine the expression pattern of the two genes using growth conditions known to up-regulate *Listeria* capsule production. This was done by constructing reporter plasmids containing the promoter regions of the two *Listeria* genes fused to the *lux* operon, so that the level of promoter expression could be monitored by the level of light production. The plasmid construction was performed using the Gateway recombinatorial cloning system described by Perehinec *et al. (2007).*

## 7.2 The Gateway Recombination system

The Gateway recombinatorial cloning system is designed to give rapid and precise construction of plasmids as an alternative to conventional cloning methods that use restriction enzymes and ligation. The system consists of two recombination steps; the first step is termed the BP reaction and this transfers DNA fragments flanked by *attB* sites into a plasmid which contains *attP* sites (called an Entry vector), producing an Entry clone. The *attB* sites are introduced into the DNA fragment to be cloned by being present in the primer used to amplify the DNA sequence. The combination between the PCR product and the Entry vector is catalysed by BP clonase, and results in the desired DNA fragment being flanked by *attL/R* sites (Fig. 7.1a).

The Entry clones are transformed into cells for amplification of the DNA and, after purification, are used in the second recombination called the LR reaction. In this step different Entry clones containing DNA fragments flanked by specific *attL/R* sites are recombined with a Destination vector, which also contains *attL/R* sites, to produce the final expression clone. This recombination reaction is catalysed by LR clonase and produces, in one step, an expression clone in which each of the three Entry DNA fragments are fused together, separated by an *attB* site (Perehinec *et al.*, 2007).

**Figure 7.1: The Gateway Recombination system**




The diagram shows the diagram of the Gateway Recombination system. Panel A shows the BP reaction. The BP recombination transfers DNA fragment flanked by *attB* sites into plasmid containing *attP* sites, which produces the Entry Clone. Panel B shows the LR reaction. The Entry Clones containing the target DNA fragments recombine with a Destination Vector to produce the expression clone according the sequence of the individual *attL/R* sites (Perehinec *et al.*, 2007).

## 7.3 Plasmid construction

To construct the reporter plasmids, first the sequences corresponding to a region 1kbp upstream of the two *capA* gene homologues were identified. Primers were designed to the 5′ and 3′ ends of this sequence so that the primers ended with C or G at the 3′ end, to help ensure good initiation of PCR amplification, and the appropriate *att* sequences were incorporated at the 5′ end of the sequences. Primers melting temperatures ($T_m$) were chosen to be approximately 70°C using the simple formula 3(A+T)+4(G+C). For longer primers this is not an accurate determination of $T_m$, but using this rule is a simple way to ensure that the PCR conditions are optimal for both primers designed. The final primer sequences are shown in Table 7.1.

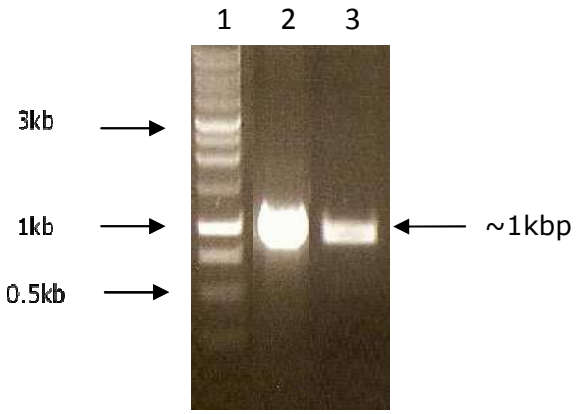
Table 7.1: Primers used for DNA construction

| Name | Sequences(atta sequence in BOLD) | Tm (℃) | Length (b) |
|---|---|---|---|
| 0017U-Pr-attB4F | **GGG GAC AAC TTT GTA TAG AAA AGT TG**C GAT AGA CTT CCA GAC ATC TTT TGG ATT AC | 73.8 | 56 |
| 0017U-Pr-attB1R | **GGG GAC TGC TTT TTT GTA CAA ACT TG**T TTT TCT CCT CCT AAA TTA AAA AGT TAT CTA ATT CTA TCA G | 72.4 | 67 |
| 0516U-Pr-attB4F | **GGG GAC AAC TTT GTA TAG AAA AGT TG**T GGG CTA GTT TTC AAT TTA TCT GGG TTT TTA TTT TG | 70.7 | 62 |
| 0516U-Pr-attB1R | **GGG GAC TGC TTT TTT GTA CAA ACT TG**C TAG ATA TCC TCC GTA GTT CCT TTT TCT CTA AGT ATA G | 69.0 | 64 |

The table shows the primers designed used for PCR amplification of a 1kbp region upstream of the two *Listeria capA* homologues flanked by appropriate *att* sites (indicated by BOLD text). Corresponding target sequences locations were displayed in section 2.9.

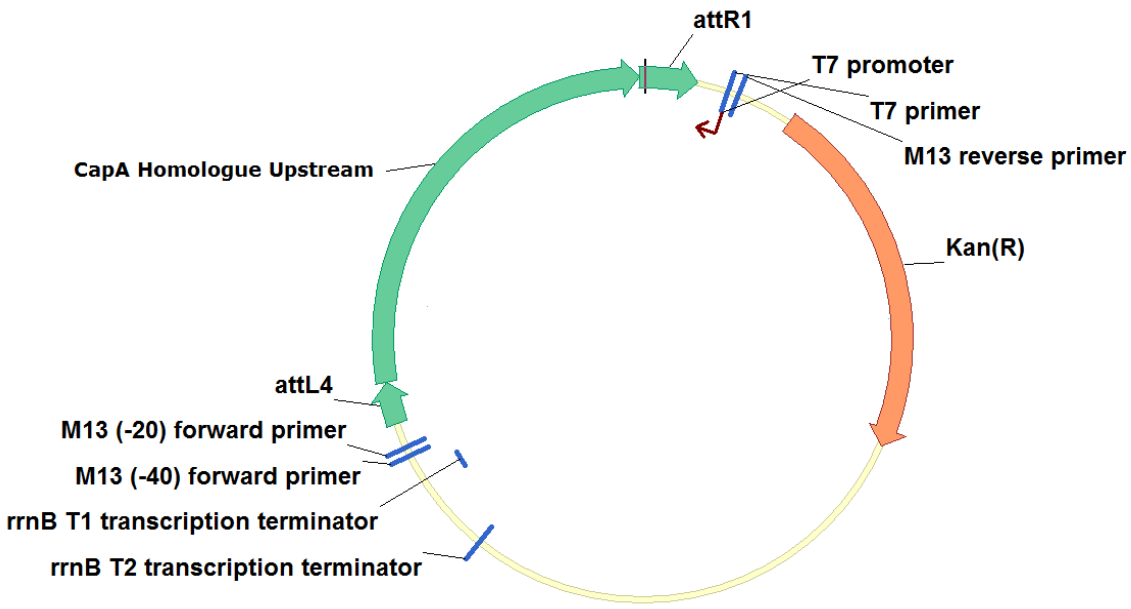
The primers were used to PCR amplify sequences from *L. Monocytogenes* EGD genome. The PCR products were analysed on an agarose gel and in each case the expected DNA band of approximately 1052 bp was produced (Fig. 7.2). The PCR products were then extracted using a Gel DNA Recovery Kit (section 2.19.3). The purified DNA fragments were then used in a BP reaction (section 2.19.4) with the vector plasmid (pDONORP4-P1R) to produce the entry clones p0017upentry and p0516upentry (Fig. 7.2).

**Figure 7.2: Agarose gel of amplified promoter regions of *lmo0017* and *lmo0516***




For the PCR reaction 1.5µl of the *L. monocytogenes* EGD template DNA (section 2.19) was mixed with 0.6µl of each primers, 10µl of KOD Hot start master mix (section 2.19.2) and 0.5µl DNA polymerase. SDW was added to make the final volume of the reaction 20µl in total. PCR was performed using a thermal cycler (Techne), using a hot start cycle at 95°C for 2 min. This was followed 30cycles of 95°C for 20s, 64°C for 12s and 70°C for 30s and a final extension of 70 °C for 15 min. The PCR products were separated on 0.8% (w/v) agarose gel at 75 V for 90 min (section 2.19.6). The bands were visualised under UV Light imager (Bio-Rad). Lane 1 contains the molecular weight markers (1kbp DNA ladder), and lane 2 and 3 contain the amplified 1 kbp promoter regions of *lmo0017* and *lmo0516*, respectively, containing the *attB/P* sequences. Both fragments were expected to be 1052 bp in length.

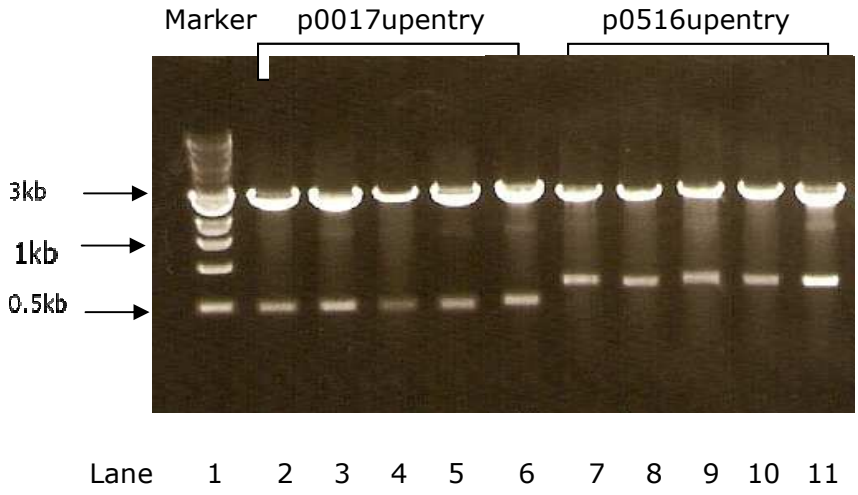
**Figure 7.3: Diagram of Entry clone structure**




The diagram shows the structure of the entry clones, p0516upentry and p0017upentry, which contain the 1 kbp regions upstream of the two *capA* homologues, *lmo0516* and *lmo0017*, respectively, in the Entry vector pDONRP4-P1R. The promoter regions are flanked by the *att*L4 and *att*R1 sites. A Kanamycin [Kan(R)] resistance gene was present for selection. The final plasmid size was 3646 bp.

The entry clones p0516upentry and p0017upentrywere transformed into *E. coli* (section 2.21.1) to allow amplification of the DNA. The plasmids were then extracted using a small scale plasmid extraction kit (section 2.19.5) and the structure of the entry clones confirmed using restriction enzyme digestion. The plasmid p0017upentry was cut with *Eco*RV and gave the expected fragments of 3118bp and 525bp whereas the p0516upentry plasmid was cut with *Eco*RI and gave the expected fragments of 2992bp and 721bp. The results of the restriction digest are shown in Figure 7.4.

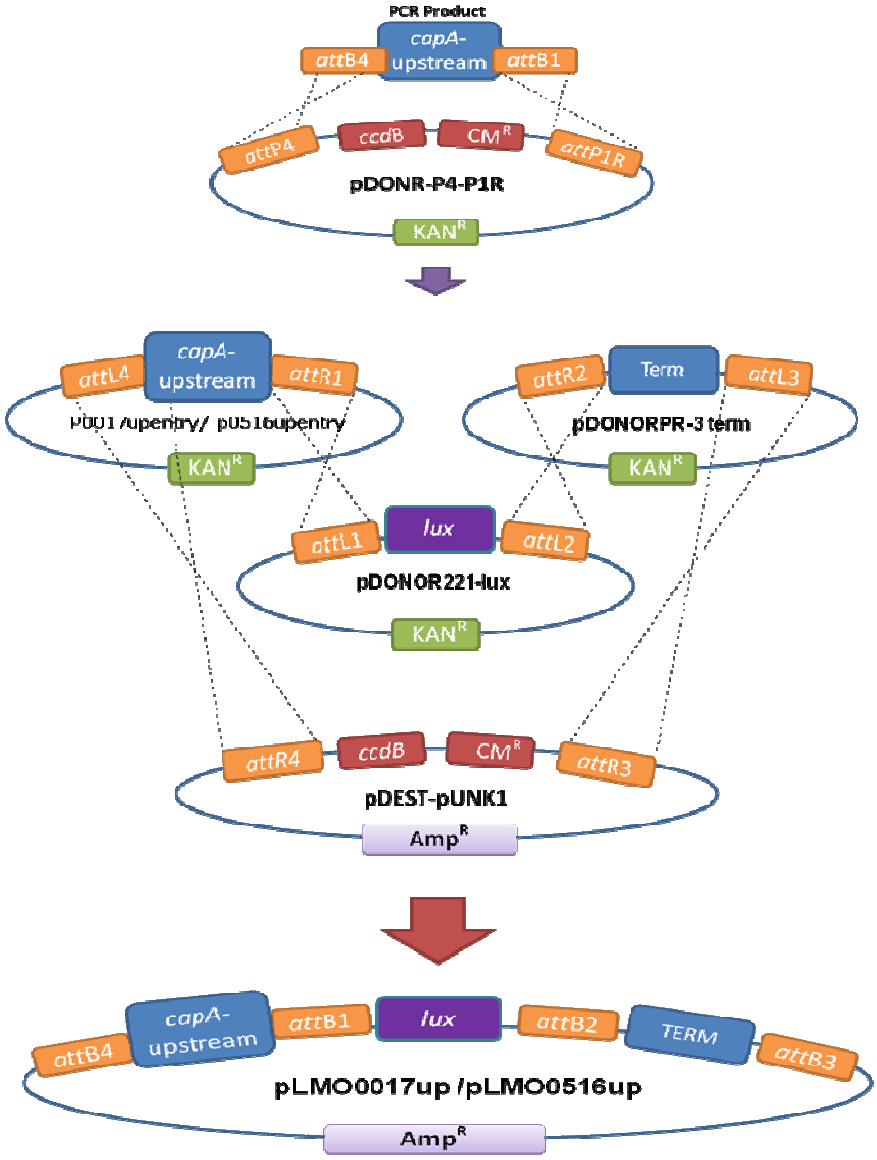
**Figure 7.4: Restriction analysis of entry clones**




The transformed cells were selected on kanamycin agar plates. Colonies were picked and grown overnight in LB media. Plasmids were extracted with miniprep kit (section 2.19.5). The digested DNA was separated on a 0.8% (w/v) agrose gel at 75 V for 90 min and the bands visualised under UV Light imager (Bio-Rad; section 2.19.6). Lane 1 contains the molecular weight markers (1kbp DNA ladder), and in lane 2 to 6 are digested products of independent clones of p0017upentry and in lane 7 to 11 are digested products of p0516upentry.

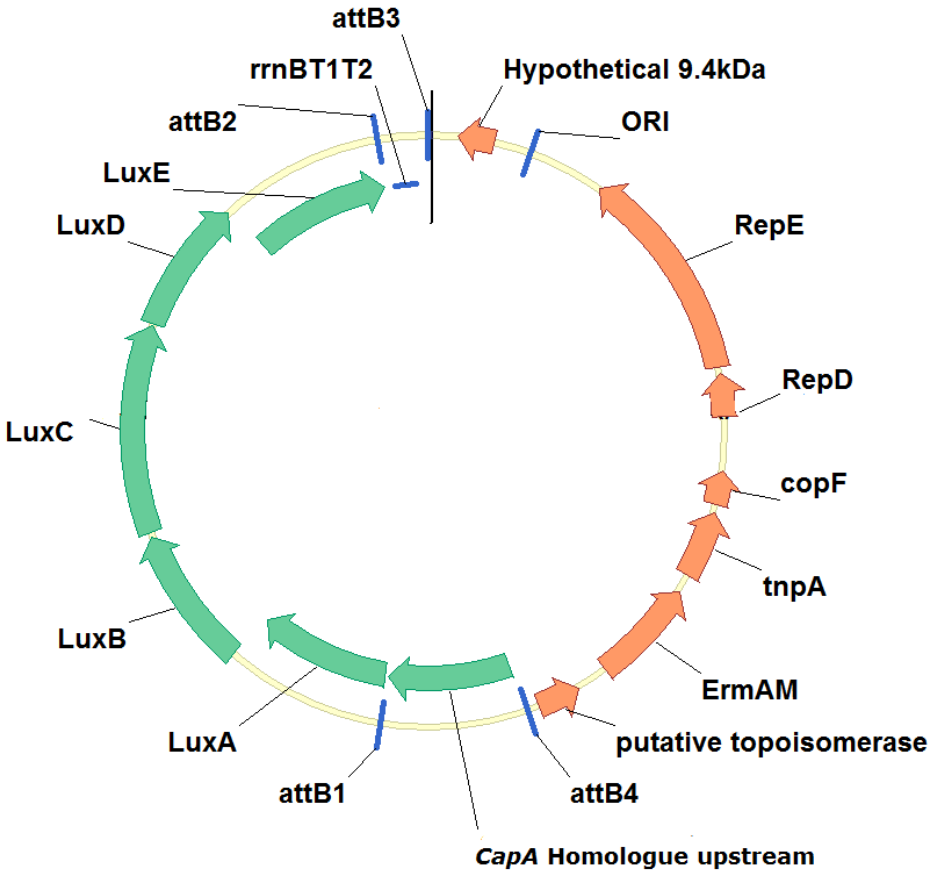
After confirming the plasmid structure was correct, the entry clones were used in an LR reaction that was designed to fuse the promoter regions present in the entry clones p0017upentry and p0516upentry with a *lux* operon. This was achieved by the use of another entry clone containing the *lux* operon (pDONOR221-lux, Gram-positive optimized Entry clone with *gfp::luxABCDE* dual reporter, (Perehinec *et al.*, 2007) so that expression of the *capA* genes could be monitored by studying the bioluminescence production from the *lux* operon. The third entry clone, pDONORPR-3 term, was also used in the LR reaction to introduce a terminator sequence downstream of the *lux* operon in the final reporter construct (Perehinec *et al.*, 2007). These three plasmids were recombined with a Invitrogen gateway system destination vector, pDEST R3-R4E (Perehinec *et al.*, 2007), to form the expression clones termed pLMO0017up and pLMO0516up (Fig. 7.6). The overall schematic is shown in Figure 7.5.

**Figure 7.5: Schematic showing design of recombination cloning pathway**




Summary diagram of the Gateway Recombination system used for the plasmid construction. The entry clones p0017upentry and p0516upentry were formed by recombining the PCR products containing the 1 kbp upstream sequences of the *capA* homologues into pDONR-P4-P1R. These were recombined with a Destination Vector (pDEST-pUNK1) along with Entry Clones containing the *lux* operon (pDONOR221-lux) and a terminator (pDONOR-3term) to give the expression clones pLM0017up and pLM0516up.

**Figure 7.6: Diagram of Expression clones pLMO0017up and pLMO0516up**

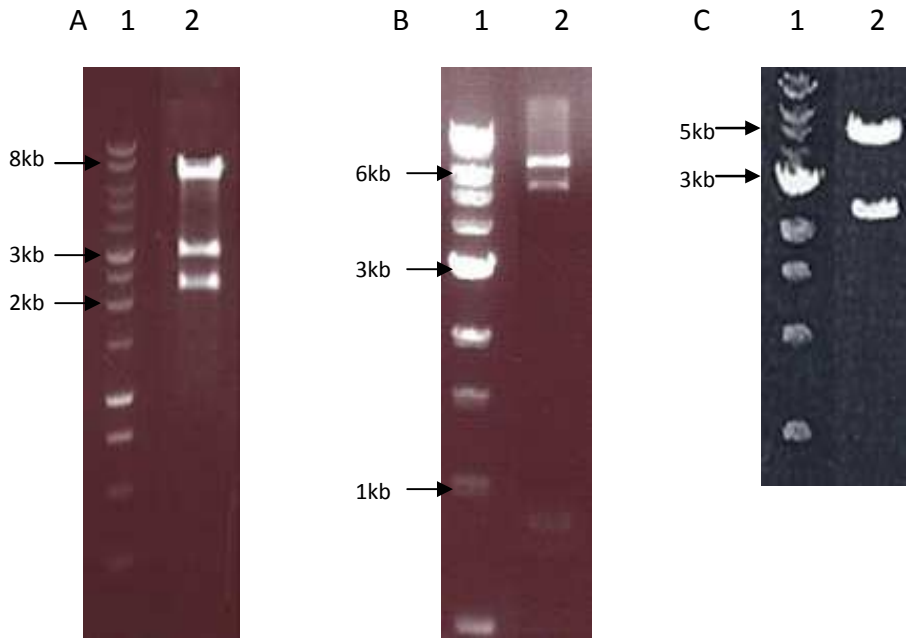



The diagram shows the structure of the expression clone, pLMO0017up and pLMO0516up, produced by the LR reaction between eitherp0017upentry or p0516upentry and pDONOR221-lux, pDONORPR-3 term and pDEST R3-R4E. The final plasmid sizes were 12,645bp.

The expression clones were transformed into *E. coli* cells by electroporation (section 2.21.2) so that the DNA could be amplified before analysis of plasmid structure and further transformation into *Listeria*. The transformed cells were selected on the basis of bioluminescence production. Individual colonies were picked and the plasmids were purified using a small scale DNA plasmid

extraction kit as before (section 2.19.5) and the structure of the expression clones was confirmed by the use of restriction enzymes. In this case the expected band sizes for the pLMO0017up expression plasmid were 5539 bp and 7106 bp, however, the band size produced were different to those expected (Fig. 7.10A). There were three restriction digest products with sizes of approximately 7 kbp, and 2 kbp and 3.5 kbp. It was later found that this was caused by the presence of an extra *Bam*HI site in the *lux* operon which is not present in the plasmid map provided. This was confirmed by restriction of the pDONOR221-lux plasmid using *Bam*HI which confirmed that an extra restriction site existed that was not shown in the plasmid map (Fig. 7.10C). Once this anomaly was resolved, the fragments of the plasmid corresponding to promoter region were found to be correctly inserted into the destination vector

**Figure 7.7: Restriction analysis of Expression clones**

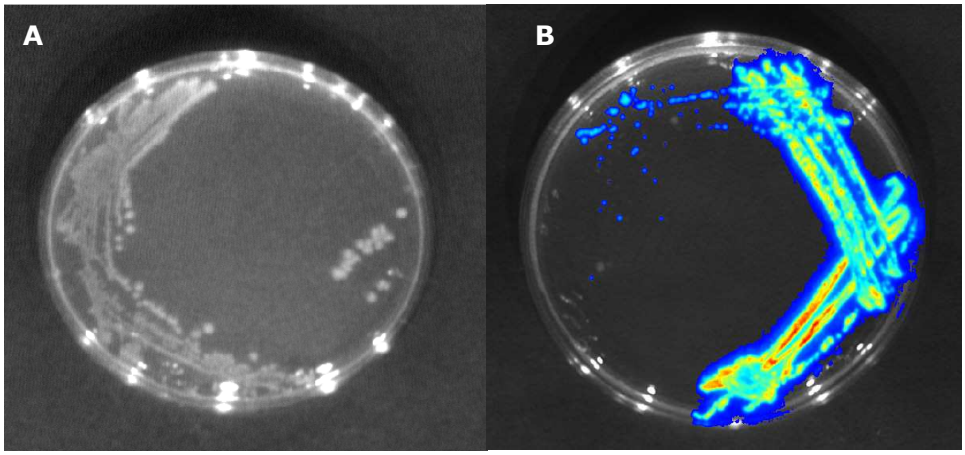



Restriction analysis of the final expression clones in the destination vector. The digested DNA were separated on 0.8% (w/v) agrose gel at 75 V for 90 min (section 2.19.6.) and the bands were visualised under UV Light imager (Bio-Rad). In each panel lane 1 contains the molecular weight markers and lane 2 the restricted plasmid DNA. Panel A) plasmid pLMO0017up cut with *Bam*HI to give bands of approximately 7kbp, and 2 kbp and 3.5 kbp. Panel B) The plasmid pLMO0516upcut with *Eco*RI to give bands of expected length of 6612 bp, 5363 bp and 670 bp. Panel C) *Bam*HI digest of the *lux* operon entry clone, pDONOR221-lux, showing two fragments of approximately 2.5 kbp and 5.5 kbp.

The expression clones were then transformed into *L. monocytogenes* EGD so that the constructs could be used to monitor promoter activity inside its native cell. Cells were transformed using the *Listeria* electrophoresis protocol (section 2.21.3). After transformation only cells transformed with pLMO0516up produced any light (Fig. 7.8A), and no light was detected from cells transformed with pLMO0017up plasmid (Fig. 7.8B).

Since the plasmids were both bioluminescent in *E. coli*, the reason for the lack of light from pLMO0017up was first investigated by extracting the plasmid DNA from the transformed *L. monocytogenes* cells and the structure of the plasmids present in these cells were reconfirmed using restriction analysis as above. The results showed that the plasmids present in *Listeria* were the same as that transformation into the cells (data not shown), indicating that the correct plasmid had been transformed into the cells and this was not the reason the cells were not emitting light.

**Figure 7.8: Light production by *L. monocytogenes* containing reporter plasmids**




*L. monocytogenes* EGD cells transformed with (Panel A) pLMO0017up or (Panel B) pLMO0516up expression clones. Transformed cells were grown on LB agar plate for 10 h and then the plates were examined under bioluminescence imager to record the bioluminescence produced by each of the reporter plasmids. No light was seen from *L. monocytogenes* EGD transformed with pLMO0017up.

The sequence analysis had shown that predicted *lmo0017* is not within an operon structure. Figure 7.9 shows the neighboring genes of *lmo0017* and their corresponding direction of transcription. It shows that *lmo0017* and *lmo0018* are transcribed in different directions and that *lmo0017* is on the negative strand of the genome whereas *lmo0018* is on the positive strand. The gene immediately downstream of *lmo0017*, *lmo0016* (*qoxD*), is also on the opposite strand. This indicates that *lmo0017* is not within an operon, as it presents a single gene, and hence the promoter should lie within 1 kbp of the start of the ORF of *lmo0017*.

## 7.4 Promoter expression studies

### 7.4.1 *L. monocytogenes* (pLMO0017up)

As no light was seen from the *L. monocytogenes* cells transformed with pLMO0017up, it was possible that the promoter was repressed because of the growth media used (BHI agar). As EPS induction was known to occur in liquid culture, the transformed cells were tested for light production in liquid media. To do this *L. monocytogenes* (pLMO0017up) was inoculated in both BHI broth and MCDB202 broth. Then cells were transferred into individual wells of a microtitre plate. The plates were incubated for 12 h at 25°C and 37 °C. Growth was monitored by measuring $OD_{600nm}$ and expression of the *lux* genes by bioluminescence measurements. Untransformed *L. monocytogenes* EGD cells were used as a negative control.

The results (Fig. 7.8) showed that there was no light production by the *L. monocytogenes* (pLMO0017up) cells when grown in either MCBD202 or BHI broth at either 25°C or 37 °C and the light levels produced were similar to those produced by the untransformed *L. monocytogenes* EGD samples and this represented basal levels of light in the system generated by autofluorescence, suggesting that it is not the agar or the incubation time that affects the gene expression.

To confirm that the correct sequence had been inserted into pLMO0017up, the plasmid DNA was sequenced, and the results indicated that the sequence cloned into the plasmid was that expected (see Appendix V). As the sequence was able to function as a promoter in *E. coli,* this suggested that the promoter in the pLMO0017up may not have been active under the test conditions used. With the use of online bioinformatics BPROM software, it was found that the 1kb up-stream region of lmo0017 holds two possible promoter regions (as shown in table 7.3), which confirm that promoter activities should be present. This was seen in the bioluminescence expression of lmo0017 up stream region transformed into *E. coli.* However, the reason that promoter was not seen activated in *Listeria* is unclear. Given the limited time remaining for this work, no further analysis of this promoter construct was carried out.

**Table 7.2 Possible promoter in 1kb upstream of lmo0017**

**Number of predicted promoters: 2**

**A)**

| **Promoter Pos:** | **894** | **LDF-** | **6.93** |
|---|---|---|---|
| -10 box at pos. | 879 (TTTTATGAT) | Score | 70 |
| -35 box at pos. | 862 (TTTAAA) | Score | 41 |

**Oligonucleotides from known TF binding sites**

| | | | |
|---|---|---|---|
| lrp: | ATTTTTTT | at position 857 | Score - 11 |
| lexA: | TTTTTTTA | at position858 | Score - 16 |
| crp: | TCACAATT | at position896 | Score - 10 |
| fnr: | ACAATTTA | at position898 | Score - 6 |

**B)**

| **Promoter Pos:** | **327** | **LDF-** | **5.20** |
|---|---|---|---|
| -10 box at pos. | 312 (ATTTACGAT) | Score | 49 |
| -35 box at pos. | 293 (TTCAAG) | Score | 34 |

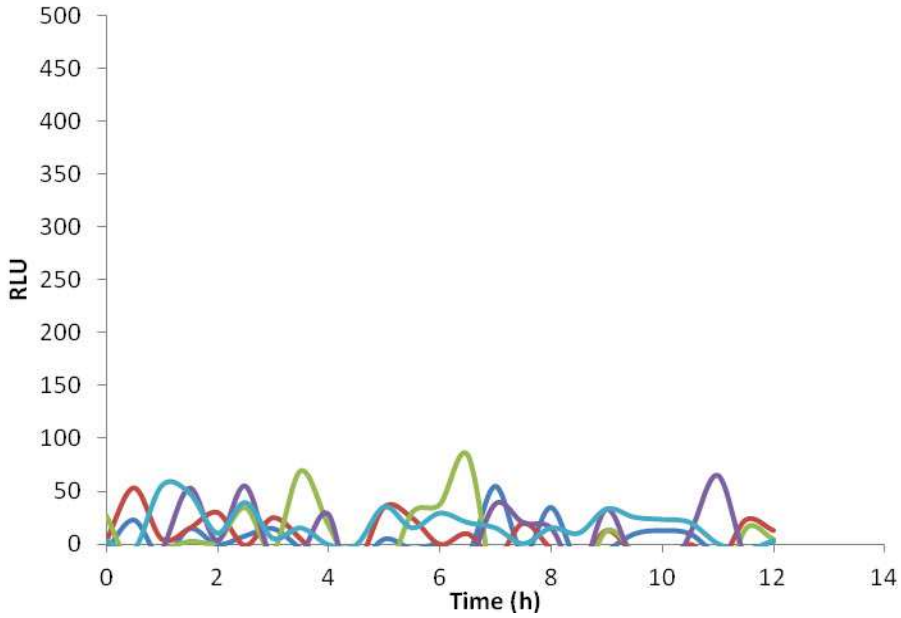
**Oligonucleotides from known TF binding sites**

| | | | |
|---|---|---|---|
| metR: | ATTTTTCC | at position319 | Score - 6 |
| argR2: | CATATTTT | at position333 | Score - 8 |
| lrp: | TATTTTTT | at position335 | Score - 11 |
| fnr: | TTTTTTGA | at position337 | Score - 9 |

The 1kb upstream region of lmo0017 was inputed to the online bioinformatics software, Softberry BPROM (Bacteria promoter prediction software), searching for possible promoter location. Panel A and B showed two possible promoter regions with in the sequence and its corresponding -10 box, -35 box and TF binding sites position.

http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb

**Figure 7.9: Light production by *L. monocytogenes* (pLMO0017up) in different media and at 25°C and 37°C**




*L. monocytogenes* EGD cells transformed with pLMO0017up, and also the untransformed parent strain, were grown overnight in 10ml BHI at 37°C. This was used to inoculated BHI and MCDB202 broth (10 ml) at a 1 in 10 dilution. Samples (200µl) of each strain were transferred to the wells of a microtitre plate and incubated at 25°C or 37°C for 12 h in a Tecan plate luminometer. The bioluminescence levels were measured at 30 min intervals. Results are shown for *L. monocytogenes* (pLMO0017up) grown in BHI at 25°C ( — ) or 37 °C ( — ), *L. monocytogenes* (pLMO0017up) grown in MCDB 202 at 25°C ( — ) or 37 °C ( — ) and untransformed *L. monocytogenes* grown in BHI at 37 °C ( — ).

### 7.4.2 *L. monocytogenes* (pLMO0516up)

Light production by *L. monocytogenes* EGD transformed with pLMO0516up was also monitored in both MDCB 202 and BHI broth as described in section 7.3.1. Samples were incubated for 12 h at 25°C (Figs. 7.12 and 7.13) and 37°C (Figs 7.14 and 7.14). As before, untransformed *L. monocytogenes* EGD wild type strains were used as a negative control. As a positive control, *L. monocytogenes* EGD was transformed with a plasmid containing the *Bacillus subtilis* ribosomal protein subunit S10 promoter fused to the same *lux* operon in the same destination vector as that used to build pLMO0516up (pSB3008; Fig 7.11) was used. This plasmid was available within the research group and the promoter has been found to be highly expressed in *Listeria* (Gaddipati, 2007).

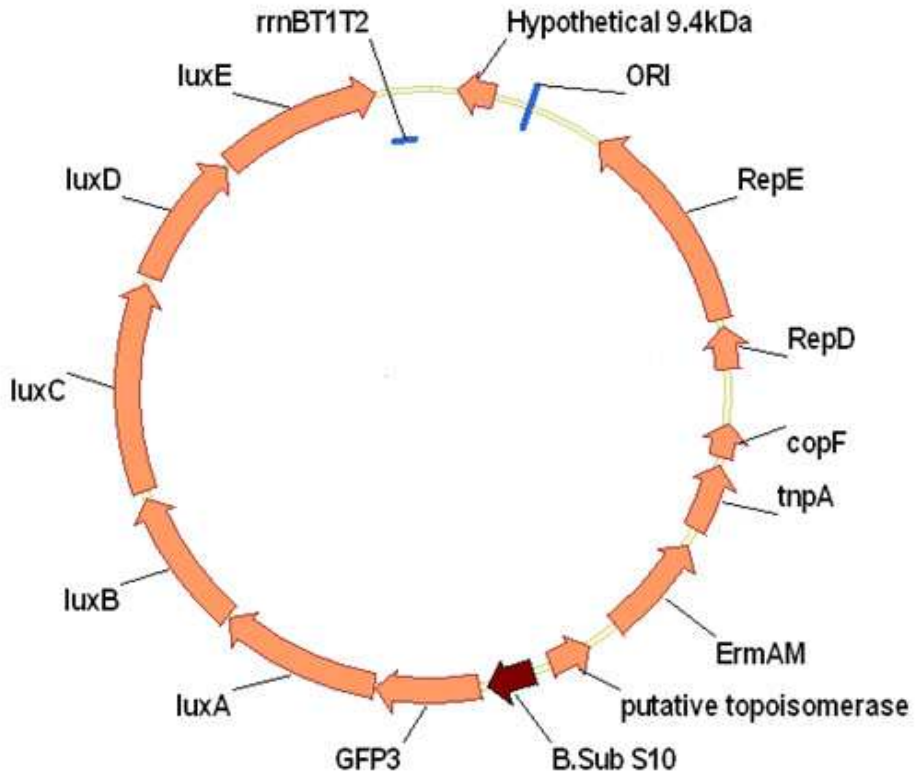
As the untransformed *L. monocytogenes* EGD produced only very low background levels of light (similar to those shown on Fig. 7.9), the results of the negative control samples are not included to allow a better presentation of the data. To allow comparison of the level of expression achieved in the two different media, and to account for the different levels of growth that occurred, the bioluminescence data is presented RLU/$OD_{600nm}$ to adjust for the differences in cell mass in each of the samples. To give an understanding in the effect of inoculation level on the expression of the promoters, two inoculation levels ($OD_{600nm}$ =0.05 and 0.8) were tested in the experiment.

From the four graphs presented in figures 7.11-14 below, it can be seen that the *lmo0516* promoter construct produced a lower expression level of the *lux* operon than did the BS10 promoter in all the cases. However, the expression patterns are quite similar in terms of the period of expression and time of

peak expression. In addition it was seen that the two promoters had a much higher level of expression in BHI than was seen in the more minimal media MCDB202. Comparing the graphs of experiments performed at different growth temperatures, the promoter expression levels were generally higher for cells at the 37°C than those at 25°C, indicating that the expression of both promoters were affected by temperature.

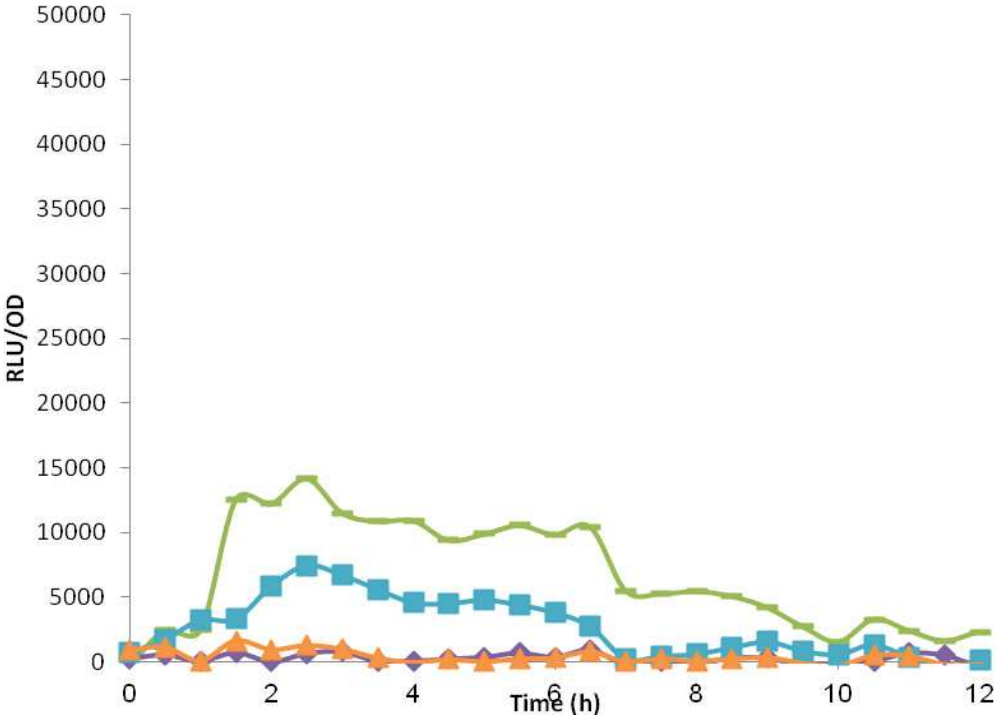
These findings suggested that expression of this *lmo0516 capA* gene homologue was not induced by growth in MCDB 202 and therefore is unlikely to be linked to production of the highly expressed extracellular polymeric substance since the phenotype and gene expression pattern did not correlate. Instead they indicated that the expression levels of the two promoters were controlled by factors such as growth temperature and nutrients provided.

**Figure 7.10: Plasmid map of pSB3008 expression vector**



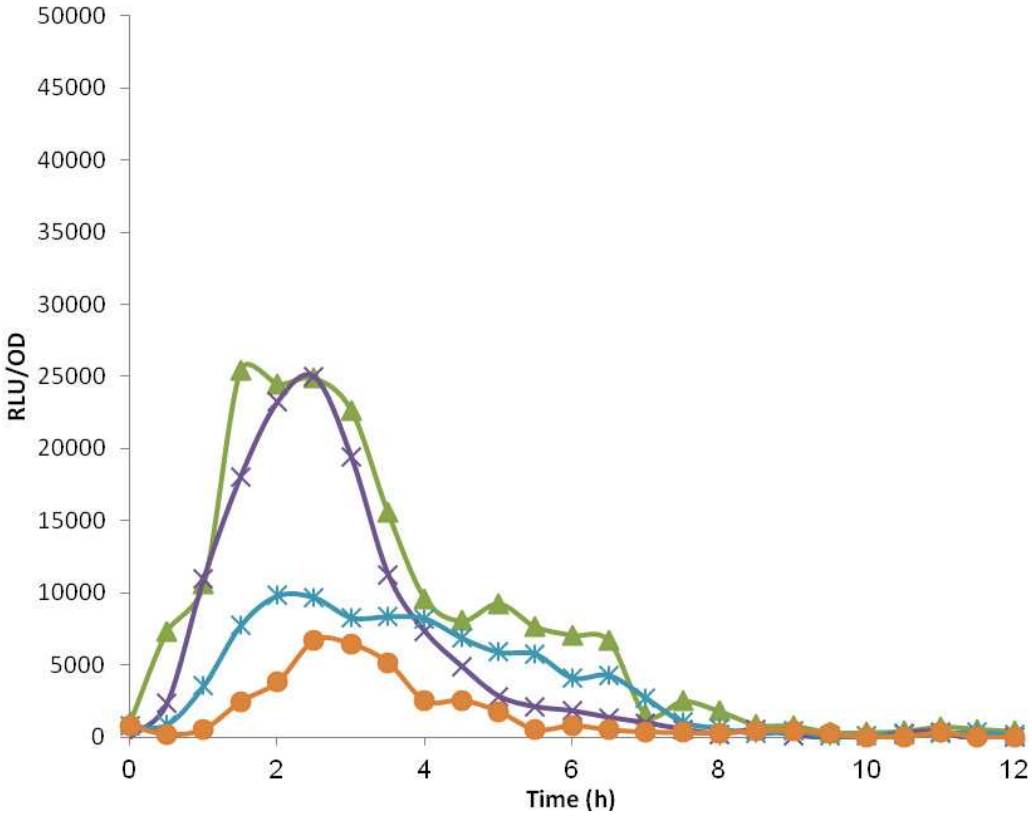

The diagram shows the structure of the plasmid pSB3008 (Gaddipati, 2007). It contains a *Bacillus subtilis* S10 promoter linked to a dual *gfp-lux* operon and a rrnT1T2 terminator, recombined into the pDEST-pUNK1 vector ($P_{Bs10}$:*gfp*:*lux*:Term). The total plasmid size is 12,827bp.

**Figure 7.11: Light production from the pLMO0516up and pSB3008 in *L. monocytogenes* EGD grown in MCDB202 at 25°Cusing two different inoculation levels**



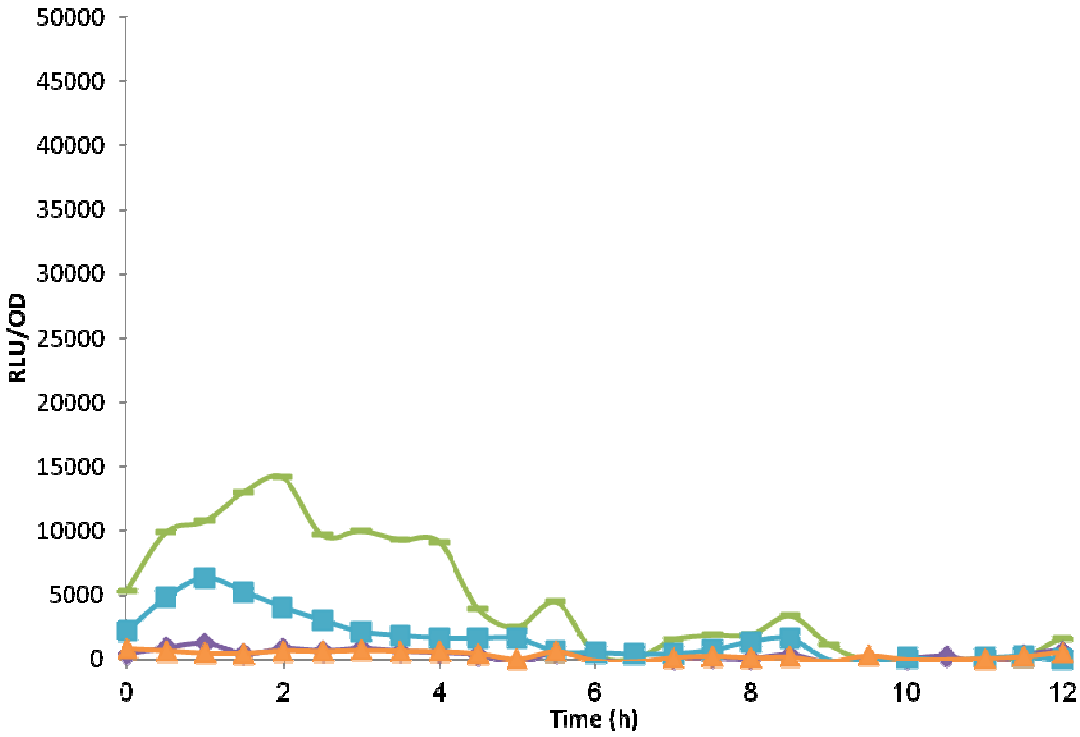

*L. monocytogenes* EGD transformed with pSB3008 orpLMO0516up were grown overnight in 10ml BHI broth at 37°C. The cultures were used to inoculate MCDB202 using two different inoculation levels ($OD_{600nm}$ =0.05 and 0.8). Samples (200µl) of each culture were transferred into the wells of a microtitre plate and the plates incubated for 12 h in a Tecan plate luminometer at 37°C. The $OD_{600nm}$readings and the bioluminescence level were measured at 30 min intervals. Results are shown for *L. monocytogenes* (BS10) grown in MCDB 202 at 25 °C with high ( ) and low ( )inoculation and *L. monocytogenes* (pLMO0017up) at 25 °C with high ( ) and low inoculation( ).

**Figure 7.12: Light production from the pLMO0516up and pSB3008 in *L. monocytogenes* EGD grown in BHI at 25°Cusing two different inoculation levels**



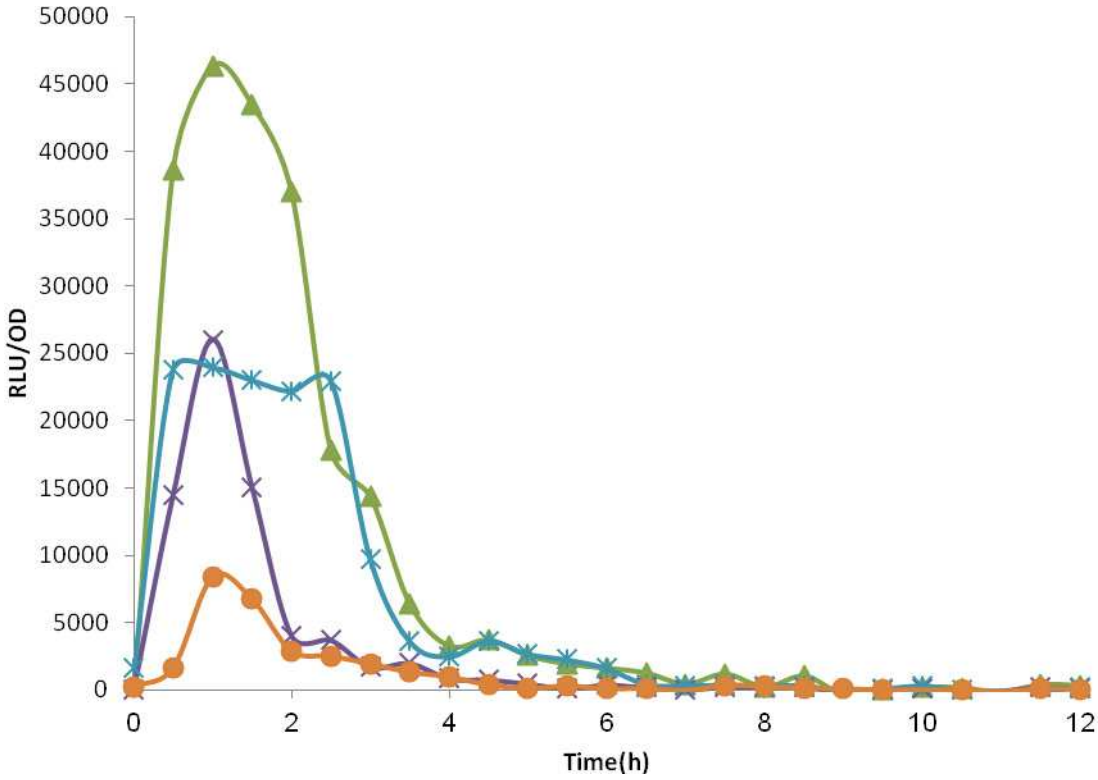

*L. monocytogenes* EGD transformed with pSB3008 orpLMO0516up were grown overnight in 10ml BHI broth at 37°C. The cultures were used to inoculate MCDB202 using two different inoculation levels ($OD_{600nm}$ =0.05 and 0.8). Samples (200µl) of each fresh culture were transferred into the wells of a microtitre plate and the plates incubated for 12 h in a Tecan plate luminometer at 37°C. The $OD_{600nm}$ readings and the bioluminescence levels were measured at 30 min intervals. Results are shown for *L. monocytogenes* (BS10) grown in BHI at 25 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* (pLMO0017up) at 25 °C with high ( ) and low inoculation ( ).

**Figure 7.13: Light production from the pLMO0516up and pSB3008 in *L. monocytogenes* EGD in MCDB202 at 37 °C using two different inoculation levels**




*L. monocytogenes* EGD transformed with pSB3008 orpLMO0516up were grown overnight in 10ml BHI broth at 37°C. The cultures were used to inoculate MCDB202 using two different inoculation levels ($OD_{600nm}$ =0.05 and 0.8). Samples (200µl) of each culture were transferred into the wells of a microtitre plate and the plates incubated for 12 h in a Tecan plate luminometer at 37°C. The $OD_{600nm}$readings and the bioluminescence level were measured at 30 min intervals. Results are shown for *L. monocytogenes* (BS10) grown in MCDB 202 at 37 °C with high ( ) and low ( )inoculation and *L. monocytogenes* (pLMO0017up)at 37 °C with high ( ) and low inoculation( ).

**Figure 7.14: Light production from the pLMO0516up and pSB3008 in *L. monocytogenes* EGD in BHI at 37 °C using two different inoculation levels**

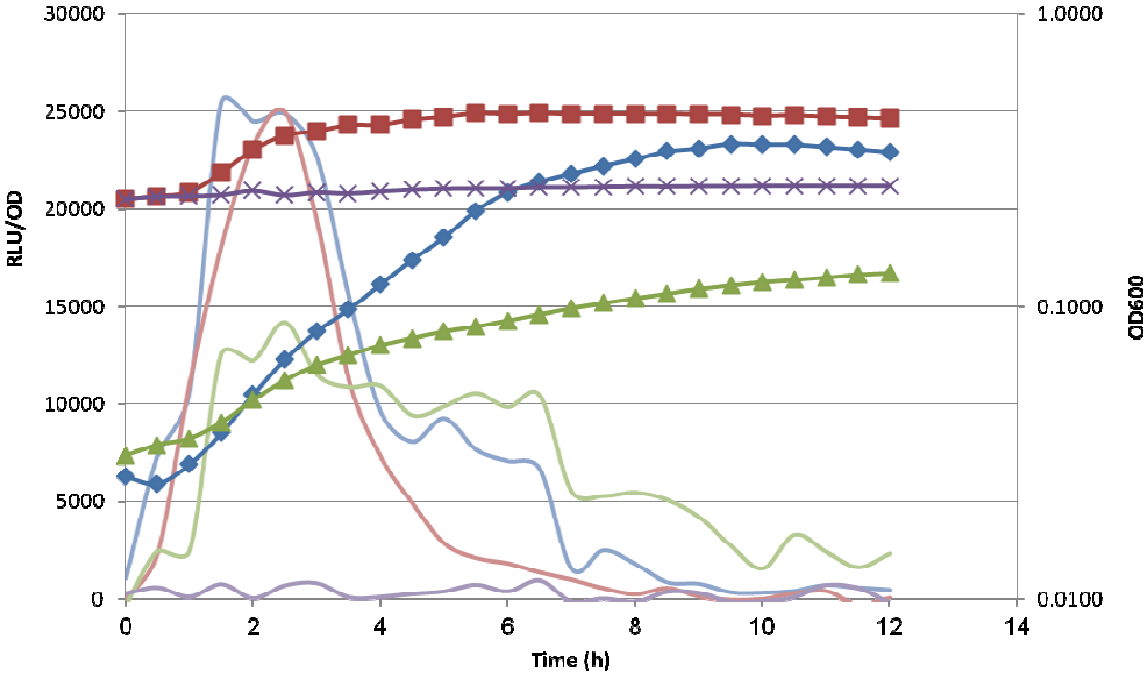



*L. monocytogenes* EGD transformed with pSB3008 orpLMO0516up were grown overnight in 10ml BHI broth at 37°C. The cultures were used to inoculate MCDB202 using two different inoculation levels ($OD_{600nm}$ =0.05 and 0.8). Samples (200µl) of each fresh culture were transferred into the wells of a microtitre plate and the plates incubated for 12 h in a Tecan plate luminometer at 37°C. The $OD_{600nm}$ readings and the bioluminescence levels were measured at 30 min intervals. Results are shown for *L. monocytogenes* (BS10) grown in BHI at 37 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* (pLMO0017up)at 37 °C with high ( ) and low inoculation( ).

From a comparison of the effect of inoculation size, it was seen that using a low inoculation level generally resulted in higher relative RLU levels than those achieved under the same conditions using a high level of inoculation. The peak level of light when using low inoculation in MCDB 202 (Blue line) was higher than that using a high inoculation (Orange line), and Peak of peak level of light when using low inoculation in BHI (Green line) was higher than that using a high inoculation in BHI (Purple line) in all four of these experiments. This indicates that the cell mass was linked to the level of gene expression recorded, and hence, would be affected by growth rate of cells. To understand more about the relationship between the expression of the two promoters and the cell growth, the light output was directly compared to growth phase by plotted both these parameters on one graph (Fig. 7.15-7.18).

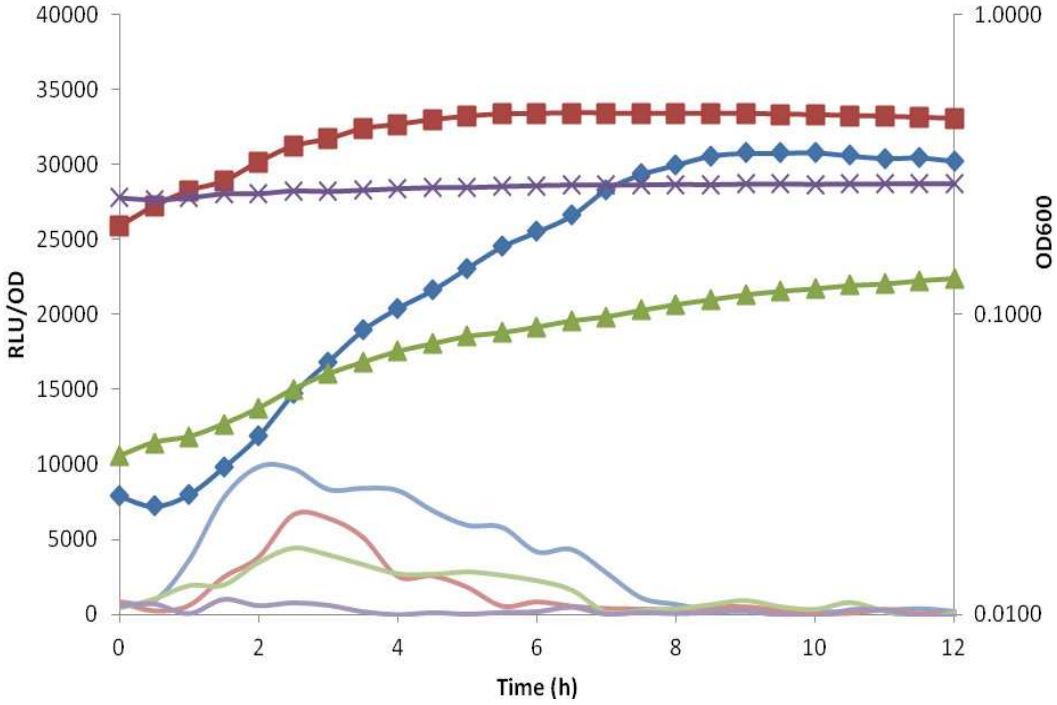
From the results of this analysis it is clear that there is a close linkage between the time of maximal expression of the promoters and the growth phase. Expression of both the *lmo0516* and *BS10* promoters is induced when the cells entered the exponential growth phase and expression levels then dropped when the cells started to enter into stationary phase, suggesting that the expression of the two promoters, in terms of expression pattern and time, is linked to grow phase rather than media condition.

**Figure 7.15: Growth and light output from *L.* monocytogenes (pSB3008) at 25°C**



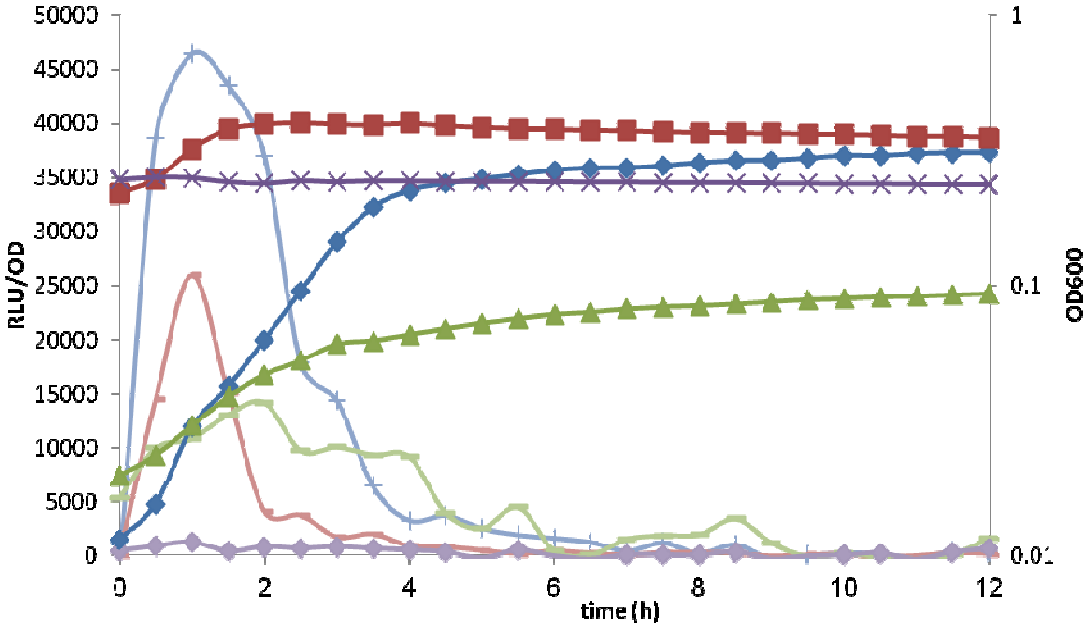

The graph shows a composite of the RLU/$OD_{600nm}$ data for *L. monocytogenes* (pSB3008) from Fig.s 7.15 and 7.16 and growth curves of each culture ($OD_{600nm}$). Cells were grown in either BHI or MCDB 202 broth at 25 °C with inoculation level of $OD_{600nm}$ 0.05 and 0.8. Growth data are shown for *L. monocytogenes* (BS10) grown in MCDB202 at 25 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation ( ). Results of RLU/$OD_{600nm}$ are shown for *L. monocytogenes* (BS10) grown in MCDB202 at 25 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation ( ).

**Figure 7.16: Growth and light output from *L.* monocytogenes (pLMO0516up) at 25°C**



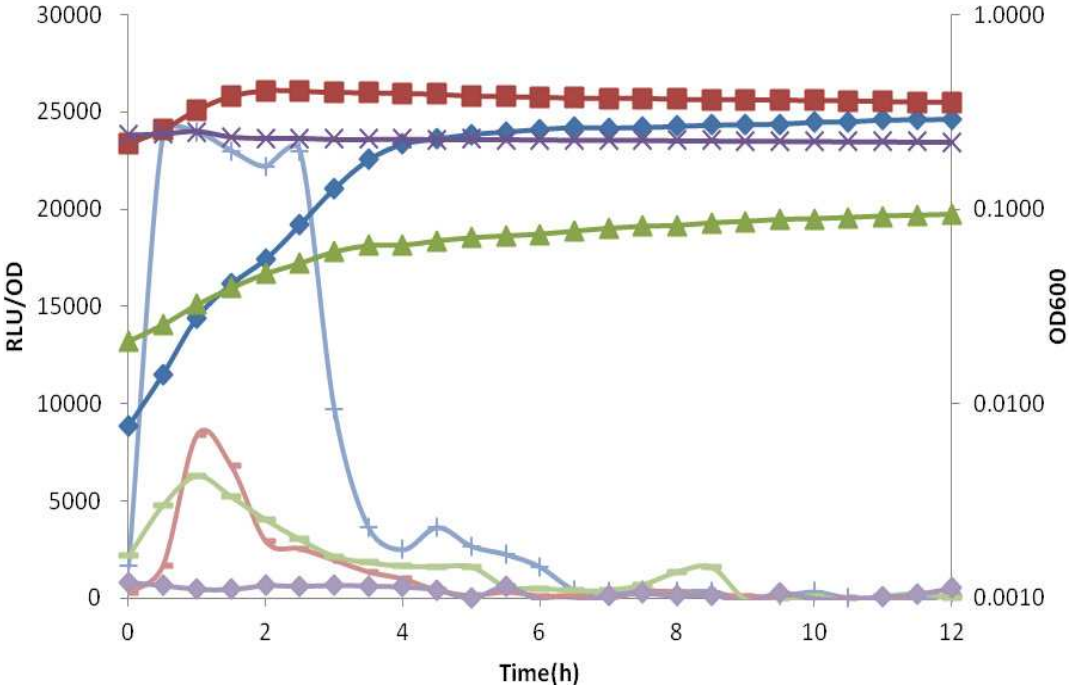

The graph shows a composite of the RLU/$OD_{600nm}$data for *L. monocytogenes* (pLMO0516up) from Fig.s 7.15 and 7.16 and growth curves of each culture ($OD_{600nm}$). Cells were grown in either BHI or MCDB 202 broth at 25 °C with inoculation level of $OD_{600nm}$ 0.05 and 0.8. Growth data are shown for *L. monocytogenes* (pLMO0516up) grown in MCDB202 at 25 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation( ). Results of RLU/$OD_{600nm}$ are shown for *L. monocytogenes* (BS10) grown in MCDB202 at 25 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation( ).

**Figure 7.17: Growth and light output from *L.* monocytogenes (pSB3008) at 37°C**




The graph shows a composite of the RLU/$OD_{600nm}$data for *L. Monocytogenes* (pSB3008) from Fig.s 7.17 and 7.18 and growth curves of each culture ($OD_{600nm}$). Cells were grown in either BHI or MCDB 202 broth at 37 °C with inoculation level of $OD_{600nm}$ 0.05 and 0.8.Growth data are shown for *L. monocytogenes* (BS10) grown in MCDB202 at37 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation( ). Results of RLU/$OD_{600nm}$ are shown for *L. monocytogenes* (BS10) grown in MCDB202 at37 °C with high ( ) and low ( )inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation( ).

**Figure 7.18: Growth and light output from *L.* monocytogenes (pLMO0516up) at 37°C**




The graph shows a composite of the RLU/$OD_{600nm}$data for *L. Monocytogenes* (pLMO0516up) from Fig.s 7.17 and 7.18 and growth curves of each culture ($OD_{600nm}$). Cells were grown in either BHI or MCDB 202 broth at 37 °C with inoculation level of $OD_{600nm}$ 0.05 and 0.8. Growth data are shown for *L. monocytogenes* (pLMO0516up) grown in MCDB202 at 37 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation ( ).Results of RLU/$OD_{600nm}$ are shown for *L. monocytogenes* (BS10) grown in MCDB202 at 37 °C with high ( ) and low ( ) inoculation and *L. monocytogenes* in BHI with high ( ) and low inoculation( ).

From this data the growth rates of the *Listeria* cells in each experiment were calculated using the growth rate equation:

$$\text{Specific Growth Rate} = \frac{(\log_{10}N - \log_{10}No) \times 2.303}{T - T_0}$$

No = $OD_{600nm}$ reading at $T_0$ in the early *Exponential* phase

N = $OD_{600nm}$ reading at T in the late *Exponential* phase

To = Time of first measurement in the early *Exponential* phase

T = Time of second measurement in the late *Exponential* phase

**Table 7.3: Growth rates of *L. monocytogenes strains grown* in either BHI or MCDB 202 broth**

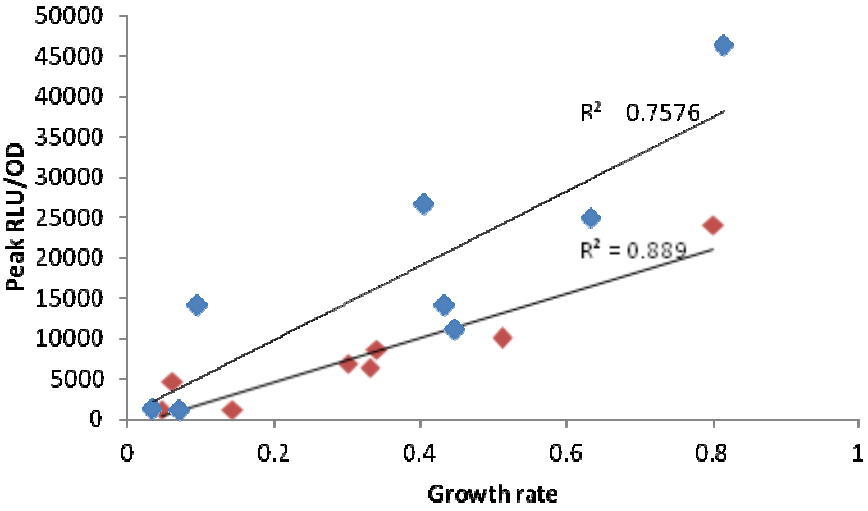
| **Promoter** | **Temperature** | **Inoculation Level[a]** | **Growth rate ($min^{-1}$)** | |
|---|---|---|---|---|
| | | | **MCDB202** | **BHI** |
| BS10 promoter | 25°C | Low | 0.0946 | 0.447 |
| | | High | 0.07028 | 0.634 |
| | 37°C | Low | 0.432 | 0.816 |
| | | High | 0.0335 | 0.405 |
| *lmo0516*promoter | 25°C | Low | 0.0478 | 0.4892 |
| | | High | 0.0325 | 0.2817 |
| | 37°C | Low | 0.312 | 0.772 |
| | | High | 0.128 | 0.3209 |

[a] low; initial $OD_{600nm}$ = 0.05 and high; initial $OD_{600nm}$ = 0.8

To investigate if there was any relationship between the expression of these two promoters and the growth rate, the cell growth rate of each cell samples (Table 7.4) was plotted against the corresponding peak value of expression as determined from graphs (7.16-7.19). From this analysis (Fig. 7.20) it is clear that there is a relationship between the bioluminescence output of the cells and the growth rate of the cells. The faster the growth rate, the higher the bioluminescent peak that was recorded. This was found to be true for both the *lmo0516* and BS10 promoters, and the correlation (Fig. 7.20) is better for *lmo0516* ($R^2$value = 0.889)than it is for BS10 ($R^2$ value =0.7576), which has previously been reported to be expressed in a growth-phase dependent manner (Li *et al.*, 1997). In addition this analysis confirms that the *lmo0516* promoter is weaker than the BS10 promoter since there is a lower amount of light produced. However it can be concluded that both promoters are growth rate and grow phase-dependent and therefore *lmo0516* is unlikely to be responsible for the induction of the *Listeria* EPS seen when the cells are grown in MCDB 202.

**Figure 7.20: Relationship between growth rate and promoter activity**

A




Graph shows the relationship between the growth rate of cells and promoter activity for *L.monocytogenes* EGD cells transformed with pLMO0516up or pSB3008, based on data presented in Figures 7.19-7.22 and Table 7.2. Peak RLU/$OD_{600nm}$ values are plotted against the growth rate of the culture. Red diamonds refers to data for *lmo0516* and blue diamonds to data for BS10 promoter. $R^2$ refers to the correlation coefficient determined using the least squares method.

## 7. 5 Discussion

It was seen from the results that the expression of the *lux* operon is highly affected by the nutrient content of the medium as well as the temperature and initial inoculation level. This makes it very hard to directly compare results between different experiments, and also with other published research. So the discussion of this chapter will mainly focus on the interpretation of the data presented here.

The BS10 promoter from *B. subtilis* is known to show growth-dependent expression in *L.monocytogenes*, suggesting a similarity in the regulation of these genes in these two different hosts. However, it was shown in previous work in the department that the expression intensity can varies among the different strains of *L. monocytogenes*, which may be linked to growth rate variation of different strains. However as only the expression of these reporters in *L. monocytogenes* EGD strains were studied here, and only as a reference to our tested reporter, variation in the expression level of BS10 is not our main concern.

The promoter of gene *lmo0017* produced no bioluminescence under the test conditions used. The structure of the clone was confirmed by DNA sequencing showing that the plasmid contained the expected insert. However, light production was seen when the plasmid was in *E. coli,* although evidence of promoter activity in a Gram-negative bacterium is not necessarily evidence that a functional promoter sequence exists for a Gram-positive bacterium. However the fact that some promoter activity was seen suggests that the promoter sequence present was not activated in *Listeria* under the test conditions used. However, due to limited of time, the reasons for this were not further investigated.

From the literature *lmo0017* gene is described as an "uncharacterised gene". There is only one particular paper identifying the gene as a CapA homologue (Desvaux *et al.*, 2010) but nothing else has been published about the gene. This suggests that there is no previous study on the gene *lmo0017* that have shown a particular phenotype. To further understand the role of this gene it may be useful to discover the condition that activates the gene. While the bioluminescence reporter gene provides a useful tool for screening many different growth conditions to try and identify when the gene is induced, there is no guarantee that a plasmid-based reporter will reflect the natural pattern of expression of the gene and therefore direct analysis of RNA levels by RT-PCR could be performed to confirm that the gene is expressed in *Listeria*.

As suggested in previous chapters, *lmo0516* seems to have an important function in *Listeria* cells. It was shown that a mutation in the gene causes impaired bile resistance of cells (Begley *et al.*, 2002). Expression of the gene was also shown to be highly up-regulated during cell invasion (Camejo *et al.*, 2009). Although it may not be linked to the extracellular polymeric substance formation, the data gathered so far suggests that the gene has an important function in the bacteria. Since the *capA* mutant could not be resuscitated from frozen storage, this may indicate that the cells are physiologically impaired in some way under cold stress. Fresh mutants would need to be constructed to allow further investigation of the biological role of this gene.

# Chapter 8

# General Discussion and Conclusions

## 8.1 Discussion

This PhD began following the discovery by a fellow PhD student of an extracellular polymeric substance that was produced when *L. monocytogenes* cells were grown in defined media. No specific descriptions of such capsule-like structure being produced by this organism could be found reported by other research groups in the recent decades, however, this was not the first time that it had been suggested that a capsule structure could be detected on the surface of *L. monocytogenes*. It was reported nearly 50 years ago in a paper in 1962 entitled "Demonstration of a Capsular Structure on *Listeria monocytogenes*"(Smith and Metzger, 1962). In this publication they showed an EM image of cells surrounding by a thin, blurry layer of capsule-like structure during incubation using trypticase soy broth with 10% rabbit serum and 5% glucose for 18 hours. However, due to the limits of the electron microscopy techniques used at the time, the image was not as clear as those that can be produced using current SEM technology. Hence it is not known if the structure seen in that research did represent the same structure detected by this group.

A year later, another group studying fine structure of *L. monocytogenes* did not detect any capsular structure (Edwards and Stevens, 1963). In this paper they commented on the findings of Smith and Metzger, saying that the capsule structure seen was due to the salt fixation during the treatment of the cells prior to EM imaging. However, it may be the difference in the growth condition used (in this publication 1% Difco tryptone was used) did not trigger the EPS formation. Unfortunately again the low quality of the EM images in this publication mean that it is difficult to provide any definitive analysis of their results.

In contrast to these early publications, the SEM analysis performed by my colleague clearly demonstrates the production of a layer of EPS present on the surface of cells grown in minimal media. As BHI or TSB (both nutrient rich media) are the most common media used to grow this organism, this suggests that a particular growth condition is required to induce production of the EPS layer by *L.monocytogenes*, and the specific condition that is required to produce this layer needs further identification.

There are many studies on biofilm formation that do suggest that *Listeria* does produce an extracellular matrix when it is grown on a surface. Some of the SEM images in these different publications showed a material that was very similar to that seen when EPS production was induced in our experiments when the cells were grown in defined media. However people seem to have believed that *Listeria* were incapable of producing capsular material, and they tend to have dismissed the possibility of these images indicating that EPS capsular structures were being formed.

However, it is believed that EPS is only produced during biofilm formation and is only produced when the cell attach to each other or to surfaces. In this case it seem that the EPS is made by planktonic cells, and therefore would have a different physiological role than that proposed for EPS that helps form the biofilm structure being the same substance by produced in different status. This could be indicated by the fact that the cells were seen to clump together to form clusters of cells and string-like structures when they were growing in this broth. It is not yet known if the EPS is produced prior to or after clumping occurs, to enhance cells attaching to each other. To do this a mutant that is unable to produce the EPS would be needed, and therefore this study would be advanced if any of the genes required for the biosynthesis of this molecule

could be identified. However, due to low number of reports on such Listeria EPS capsular structure and as a very new finding, knowledge on it is very limited

The EPS produced by *L. monocytogenes* was first suspected to be PGA, due to the present of a PGA biosynthesis gene in homologue and the fact that the material was stained by Giemsa stain in the same way as PGA. However, the bioinformatics analysis performed as part of this thesis, along with my colleague's chemical analysis of the polymer, has rejected this theory. From the work on the pattern of expression of the *capA* gene homologues it is unlikely that they are up-regulated under the conditions known to induce EPS production and the chemical analysis has shown that the EPS only contain small amount of amino acids, which is not the case for PGA. Instead, high levels of glycerol and phosphorous were detected, suggesting a possibility that the material is composed of phospholipids. However further analysis is needed to confirm this identification.

In a recently published study on*Lactococcus lactis showed* a novel cell wall polysaccharide pellicle has been identified on the surface of *L. lactis* when incubated in M17 medium supplemented with 0.5% glucose (Chapot-Chartier *et al.*, 2010). Structural TEM of the capsular substance produced results that were similar to those gained for the EPS in *Listeria*. They also studied phagocytosis assay as well as phage assay which correspond to the present work on EPS of *Listeria*. They have shown that the pellicles were shown to have enhanced phagocytosis ability and resistance to phages infection. Such protective function of the pellicles in *Lactococcus lactis*, were not seen in our Listeria EPS structure, as the EPS found in Listeria did not shown any enhancement invasion or in resistance to phages. They have also confirmed

the pellicle was composed of polysaccharides, but this is different to the chemical composition of the EPS of *Listeria*.

Since an $EPS^-$ mutant was not available, the main approach used in this research was a comparison of *Listeria* cell physiology and behaviour when the cells were grown under nutrient rich or defined media growth conditions. However, due to the great variation in nutrient availability and content, it is harder to design experiments that are directly comparable. In particular the different in cell mass, as well as cell survival strength and growth rate make it difficult to demonstrate that effects seen are not due to other changes in cell physiology. While recognising that it is not possible to eliminate these errors, attempts have been made here to try to diminish their effects.

While a lot of scientific methods use rich media in the laboratory to facilitate fast grow of cells to allow data to be generated rapidly, it is also important to bear in mind that in most real environmental situation nutrients available are often more minimal, and under these conditions the cells are found to survive and persist for years. Hence it may be useful to perform more experiments using a minimal nutrient state to give a better understanding of cell physiology that is likely to occur when the cells are growing in real environments.

One particular hypothesis that exists in the published literature that informed this study was the concept that AI-2 regulates biofilm formation in *Listeria*. However, the more research that was performed, the more questions that have has arisen about this theory, due to contradictions between different reports. First it was shown that AI-2-like molecules are produced in many different bacterial species, including *L. monocytogenes*. Moreover, many *luxS*

mutant bacteria which have reduced AI-2 levels have also been shown to have impaired biofilm formation, or other changes in cell physiology. So AI-2 was generally believed to be a quorum sensing signalling molecule, or even universal signalling molecule, among different bacteria. However, Holmes *et al.*(Holmes *et al.*, 2009) suggested that people may have overlooked the true function of AI-2. AI-2 has only directly been proved to be a signalling molecule in *Vibrio* species, shown by the induction of bioluminescence and the mechanism and metabolism of this *Vibrio* bioluminescence pathway has been identified. However, understanding of the role of AI-2 in other bacteria has relied on more indirect evidence, and has included some unconfirmed assumptions.

Some reports suggested that the change in cell physiology in *luxS* mutant may not be caused by the reduced AI-2 production. When we look at the metabolism of the AI-2 production of various bacteria, it is actually linked to an important metabolic pathway, called the activated methyl cycle (AMC). A mutation in *luxS*, of course may cause changes in AI-2 production level due to blocking of the biosynthetic pathway, but it will also cause a change in the full metabolism of the AMC. Research seems to have overlooked the importance of the biochemical role of AI-2, and ignored the fact that changes in cell physiology could be caused by the alternation of the metabolic cycle. AI-2, at least in the case of *L. monocytogenes*, seems to be a by-product of this metabolic pathway, which is exported out of the cells as waste and to date no direct evidence has shown a specific regulatory function of AI-2. This leads to a chicken and egg scenario. Is the fact that AI-2 is used as a cell-density related signalling molecule an evolutionary adaptation of the *Vibrios* that have capitalised on the fact that this molecule is produced by the cell, and that its concentration is proportional to rates of cell metabolism, and is then

naturally transported?

The presence of AI-2 among different species may indicate the activated methyl cycle is an ancient pathway that has been preserved along the evolution process, indicating the importance of the metabolic pathway. Cells with mutation in the cycle may not be able to survive due to natural selection, and were eliminated along the evolution process. This may explain why the AI-2 production was found to be universal in many bacterial species.

This idea is supported by the study of Rezzonico and Duffy (Rezzonico and Duffy, 2008). They have been working the genetics of AI-2 signalling and suggested that there is no evidence showing the presence of AI-2 receptors among different species, which is a critical component of a signalling system. In other words, a signalling system without a receptor is unable to give signalling function. A functional receptor was only identified in the *Vibrio* species. This is consistent with our results that addition of exogenous AI-2 has no particular effect on growth, biofilm formation or hydrophobicity of *Listeria*, suggesting a very minor function in AI-2.

One of the long term aims of understanding the role of AI-2 in bacterial gene expression is to develop the use of anti-quorum sensing agents, which could be used as a new generation food preservatives or to achieve other forms of microbial control. This idea is based on the theory that stopping the signalling between bacteria might prevent certain cell adaptations, such as spore forming or biofilm formation (Rasmussen and Givskov, 2006, March and Bentley, 2004, Choudhary and Schmidt-Dannert, 2010). However, the evidence to date suggests that AI-2 may not be a universal signalling molecule in bacteria, and hence the ideas of these anti-QS agents that

targets AI-2 in a range of genera may not be as useful as first suggested. The evidence is certainly beginning to point that was in the case of *L. monocytogenes*. However, it is important to note that there could be another signalling molecule used in quorum sensing, that does have a role in regulation of biofilm formation, infections etc, and therefore looking for QS-regulated gene systems, and for molecules that could block the induction of genes controlled by them, is still of interest.

The discovery of EPS production by *Listeria* is a new concept in the *Listeria* research field, but this may account for an important concern in food safety and the medical aspect as EPS were shown to be a virulence factors in many bacterial species and may also give protection to bacterial cells. Hence future work may focus on the role of *L. monocytogenes* EPS. The first aim is to identify the specific conditions that trigger its formation. This can be done by studying the nutrient content of the defined media used. Altering the media to observe the changes in EPS production may identify the specific nutrient limitation that is responsible for the up-regulation of the EPS production. A practical consequence of this would be to allow the mass production in EPS for further study and to improve experimental design to remove some of the effects of the very different culture conditions currently used as discussed above.

Further experiments to determine the function of the EPS may also be interesting, especially as my results indicate that EPS production by cells growing in the planktonic phase does not afford the same physical protection of the cells as is reported when cells grown in a biofilm. However there must be biological function of this EPS, as the production and secretion of EPS is a

high energy process and to commit to this, there must be a physiological pay back for the cells. Identifying the function could give us better understanding of the role of EPS in the wider sense, and also perhaps design ways to help control *Listeria* contamination of the food environment.

*Listeria* infection via the oral route presents bacteria with various challenging environmental conditions such as those found in the highly acidic stomach environment, or the blood environment where the cells are challenged by different components of the immune system, as well as the different physiological conditions that exist in different human organs. Rapid changes and adaptations in bacterial response to environment are critical to increase the chance of successful infection. Bacterial capsules have been reported to be a virulence factor for many bacterial species such as *Bacillus anthracis*, *Erwinia amylovora*, *Escherichia coli* as well as *Klebsiella pneumoniae* (Koehler*, 2002;* Bennett and Billing*, 1978,* Wu *et al.; 2008,* Goller and Seed*, 2010).* While there was no evidence that production of EPS made the *Listeria* cells more resistant to physical challenge, from the results gained here, the EPS capsule could also be one of the responses of *Listeria* to the host environment if the presence of this material protects the bacteria from engulfment by phagocytes. Hence the EPS capsule could also be part of the virulence responses of *Listeria* to the host. To investigate this further it would be necessary to either determine the virulence of an $EPS^-$ mutant, or to follow the pattern of expression of genes found to be necessary for EPS synthesis during growth *in vivo* and also during infection of a complete animal model, rather than just monitoring infection of tissue culture cells. This would be important as one of the CapA homologues, lmo0516 as shown to be upregulated during infections (Camejo *et al.*, 2009).

To further confirm whether or not the two *capA* homologues are linked to EPS produced in *Listeria*, mutagenesis of the two genes can be performed. If either of the two genes is linked to EPS formation, a mutant would be expected to show reduced EPS formation. As the interest of the two *capA* homologues in *Listeria* genome being an orphan gene of the PGA synthesis operon, it may be important to first identify and isolate the protein before further study and investigation in functioning, structure as well as localization of the protein in cells. Due to their similarity to the CapA proteins, there may be a chance that these proteins could be transporters for other secreted products. Making mutants can also be used to investigate the function of the two genes by studying changes in cell physiology compared to wild-type strains. Once the protein has been identified, study such as chemical analysis and structuring can be done to have better understanding of the protein as well as the genes.

**8.2 Conclusion**

It has been shown that *L. monocytogenes* cells grown in a defined minimal, MCDB202, showed enhanced production of EPS compared to cells grown in BHI. In this work it is shown that growth in MCDB202 causes an increase in surface hydrophobicity of the cells, presumably due to the presence of the EPS on the cells surface, but surprisingly this did not induce better attachment and biofilm formation, even on hydrophobic surfaces.

Listeria cells grown in MCDB202 were shown to be more sensitive to physical challenges including nisin or lysozyme or against phage infection. And cells grown in MCDB202 were found to be slightly less capable of infecting eukaryotic cells when this was measured using a cell invasion assay.

It was found that AI-2 production was relatively lower in *Listeria* cell grown in minimal media (MCDB202) than in cells growing in rich media (BHI) and supplementation experiments using synthetic AI-2 failed to find any relationship between AI-2 levels and the formation of biofilm.

Bioinformatic analysis has shown that the *Listeria* genes *lmo0516* and *lmo0017* are two conserved homologues of the *Bacillus* polyglutamate synthesis protein (*capA*). However, the use of reporter plasmids to study the pattern of expression of these genes suggest that they are unlikely to be genes contributing the induction of EPS biosynthesis seen when the *L. monocytogenes* cells were grown in MCDB media compared to BHI media.

This leaves us with the final mystery of exactly what is the primary role of this material in the life of *Listeria*? Perhaps solving this question will provide a new paradigm that will provide a wider understanding of the role of EPS in eubacterial physiology.

# Bibliography

Abee, T., Kovacs, . T., Kuipers, O. P. & Van Der Veen, S. **(2011)**. Biofilm formation and dispersal in gram-positive bacteria. *Current Opinion in Biotechnology,* 22**,** 172-179.

Allison, D. G. & Sutherland, I. W. **(1987)**. The role of exopolysaccharides in adhesion of freshwater bacteria. *Journal of General Microbiology,* 133**,** 1319-1327.

Alp, G. & Aslim, B. **(2010)**. Relationship between the resistance to bile salts and low ph with exopolysaccharide (eps) production of Bifidobacterium spp. Isolated from infants feces and breast milk. *Anaerobe,* 16**,** 101-105.

Andrew, G. M. **(2005)**. Formation of biofilms by *Listeria Monocytogenes* under various growth conditions. *Journal of Food Protection,* 68**,** 92-97.
Asad, S. & Opal, S. **(2008)**. Bench-to-bedside review: Quorum sensing and the role of cell-to-cell communication during invasive bacterial infection. *Critical Care,* 12**,** 236.

Auger, S., Krin, E., Aymerich, S. & Gohar,M. **(2006)**. Autoinducer 2 affects biofilm formation by *Bacillus cereus. Appl Environ Microbiol,* 72**,** 937-41.

Azeredo, J., Lazarova, V. & Oliveira, R. **(1999)**. Methods to extract the exopolymeric matrix from biofilms : A comparative study. *Elsevier Science,* 39**,** 243-250.

Bajaj, I. & Singhal, R. **(2011)**. Poly (glutamic acid) – An emerging iopolymer of commercial interest. *Bioresource Technology,* 102**,** 5551-5561.

Barnes, L.-M., Lo, M. F., Adams, M. R. & Chamberlain, A. H. L. **(1999)**. Effect of Milk Proteins on Adhesion of Bacteria to Stainless Steel Surfaces. *Applied and Environmental Microbiology,* 65**,** 4543-4548.

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

Bassler, B. L., Greenberg, E. P. & Stevens, A. M. **(1997)**. Cross-species inductionof luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol,* 179**,** 4043-5.

Beauregard, K. E., Lee, K.-D., Collier, R. J. & Swanson, J. A. **(1997)**. pH-dependent Perforation of Macrophage Phagosomes by Listeriolysin O from *Listeria monocytogenes*. *The Journal of Experimental Medicine,* 186**,** 1159-1163.

Begley, M., Gahan, C. G. M. & Hill, C. **(2002)**. Bile Stress Response in *Listeria monocytogenes* LO28: Adaptation, Cross-Protection, and Identification of Genetic Loci Involved in Bile Resistance. *Appl. Environ. Microbiol.,* 68**,** 6005-6012.

Begley, M., Gahan, C. G. M. & Hill, C. **(2005)**. The interaction between bacteria and bile. *FEMS Microbiology Reviews,* 29**,** 625-651.

Beloin, C., Michaelis, K., Lindner, K., Landini, P., Hacker, J., Ghigo, J.-M. & Dobrindt, U. **(2006)**. The Transcriptional Antiterminator RfaH Represses Biofilm Formation in *Escherichia coli*. *J. Bacteriol.,* 188**,** 1316-1331.

Bennett, R. A. & Billing, E. V. E. **(1978)**. Capsulation and virulence in *Erwinia amylovora*. *Annals of Applied Biology,* 89**,** 41-45.

Bereksi, N., Gavini, F., Benezech, T. & Faille, C. **(2002)**. Growth, morphology and surface properties of *Listeria monocytogenes* Scott A and LO28 under saline and acid environments. *Journal of Applied Microbiology,* 92**,** 556-565.

Bernheimer, H. P. & Tiraby, J.-G. **(1976)**. Inhibition of phage infection by *Pneumococcus* capsule. *Virology,* 73**,** 308-309.

Bierne, H., Sabet, C., Personnic, N. & Cossart, P. **(2007)**. Internalins: a complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*. *Microbes and Infection,* 9**,** 1156-1166.

Bishop, D. K. & Hinrichs, D. J. **(1987)**. Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. *The Journal of Immunology,* 139**,** 2005-9.

Bohne, J., Kestler, H., Uebele, C., Sokolovic, Z. & Goebel, W. **(1996)**. Differential regulation of the virulence genes of *Listeria monocytogenes* by the transcriptional activator prfA. *Molecular Microbiology,* 20**,** 1189-1198.

Bonazzi, M. & Cossart, P. **(2006)**. Bacterial entry into cells: A role for the endocytic machinery. *FEBS Letters,* 580**,** 2962-2967.

Boneca, I. G., Dussurget, O., Cabanes, D., Nahori, M.-A., Sousa, S., Lecuit, M., Psylinakis, E., Bouriotis, V., Hugot, J.-P., Giovannini, M., Coyle, A., Bertin, J., Namane, A., Rousselle, J.-C., Cayet, N., Prevost, M.-C., Balloy, V., Chignard, M., Philpott, D. J., Cossart, P. & Girardin, S. E. **(2007)**. A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system. *Proceedings of the National Academy of Sciences,* 104**,** 997-1002.

Borucki, M. K., Peppin, J. D., White, D., Loge, F. & Call, D. R. **(2003)**. Variation in Biofilm Formation among Strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.,* 69**,** 7336-7342.

Branda, S. S., Chu, F., Kearns, D. B., Losick, R. & Kolter, R. **(2006)**. A major protein component of the Bacillus subtilis biofilm matrix. *Molecular Microbiology,* 59**,** 1229-1238.

Bremer, P. J., Monk, I. & Osborne, C. M. **(2001)**. Survival of *Listeria monocytogenes* Attached to Stainless Steel Surfaces in the Presence or Absence of *Flavobacterium spp*. *Journal of Food Protection,* 64**,** 1369-1376.

Briandet, R., Meylheuc, T., Maher, C. & Bellon-Fontaine, M. N. **(1999)**. *Listeria monocytogenes* Scott A: Cell Surface Charge, Hydrophobicity, and Electron Donor and Acceptor Characteristics under Different Environmental Growth Conditions. *Appl. Environ. Microbiol.,* 65**,** 5328-5333.

Bruno, M. E., Kaiser, A. & Montville, T. J. **(1992)**. Depletion of proton motive force by nisin in *Listeria monocytogenes* cells. *Appl. Environ. Microbiol.,* 58**,** 2255-2259.

Burns, P., Vinderola, G., Reinheimer, J., Cuesta, I., De Los Reyes-Gavilan, C. G. & Ruas-Madiedo, P. **(2011)**. Technological characterization and survival of the exopolysaccharide-producing strain Lactobacillus delbrueckii subsp. lactis 193 and its bile-resistant derivative 193+ in simulated gastric and intestinal juices. *Journal of Dairy Research,* 78**,** 357-364.

Camejo, A., Buchrieser, C., Couve, E., Carvalho, F., Reis, O., Ferreira, P., Sousa, S., Cossart, P. & Cabanes, D. **(2009)**. *in vivo* transcriptional profiling of *Listeria monocytogenes* and mutagenesis identify new virulence factors involved in infection. *PLoS Pathog,* 5**,** e1000449.

Camilli, A., Tilney, L. G. & Portnoy, D. A. **(1993)**. Dual roles of plcA in *Listeria monocytogenes* pathogenesis. *Molecular Microbiology,* 8**,** 143-157.

Cammarota, M. C. & Sant'anna, G. L. **(1998)**. Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. *Biotechnology Letters,* 20**,** 1-4.

Campos, M. A., Vargas, M. A., Regueiro, V., Llompart, C. M., Alberti, S. & Bengoechea, J. A. **(2004)**. Capsule Polysaccharide Mediates Bacterial Resistance to Antimicrobial Peptides. *Infection and Immunity,* 72**,** 7107-7114.

Candela, T. & Fouet, A. **(2006)**. Poly-gamma-glutamate in bacteria. *Molecular Microbiology,* 60**,** 1091-1098.

Carpentier, B. & Cerf, O. **(2011)**. Review -- persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology,* 145**,** 1-8.

Carpentier, B. & Chassaing, D. **(2004)**. Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. *International Journal of Food Microbiology,* 97**,**111-122.

Challan Belval, S., Gal, L., Margiewes, S., Garmyn, D., Piveteau, P. & Guzzo, J. **(2006)**. Assessment of the Roles of LuxS, S-Ribosyl Homocysteine, and Autoinducer 2 in Cell Attachment during Biofilm Formation by L*isteria monocytogenes* EGD-e. *Appl. Environ. Microbiol.,* 72**,** 2644-2650.

Chapot-Chartier, M.-P., Vinogradov, E., Sadovskaya, I., Andre, G., Mistou, M.-Y., Trieu-Cuot, P., Furlan, S., Bidnenko, E., Courtin, P., Pechoux, C., Hols, P., Dufrene, Y. F. & Kulakauskas, S. **(2010)**. Cell Surface of *Lactococcus lactis* Is Covered by a Protective Polysaccharide Pellicle. *Journal of Biological Chemistry,* 285**,** 10464-10471.

Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M.-N. & Hebraud, M. **(2002)**. *Listeria monocytogenes* LO28: Surface Physicochemical Properties and Ability To Form Biofilms at Different Temperatures and Growth Phases. *Appl. Environ. Microbiol.,* 68**,** 728-737.

Cheigh, C.-I. & Pyun, Y.-R. **(2005)**. Nisin Biosynthesis and its Properties. *Biotechnology Letters,* 27**,** 1641-1648.

Chico-Calero, I., Suarez, M., Gonzalez-Zorn, B., Scortti, M., Slaghuis, J., Goebel, W., Consortium, T. E. L. G. & Vazquez-Boland, J. A. **(2002)**. Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in *Listeria*. *Proceedings of the National Academy of Sciences,* 99**,** 431-436.

Choudhary, S. & Schmidt-Dannert, C. **(2010)**. Applications of quorum sensing in biotechnology. *Applied Microbiology and Biotechnology,* 86**,** 1267-1279.

Chu, X., Lin, Y., Sun, Z., Huan, L. & Zhong, J. **(2010)**. Advances in the study of nisin resistance--a review.*Wei Sheng Wu Xue Bao,* 50**,** 1129-34.

Cossart, P. **(2000)**. Actin-based motility of pathogens: the Arp2/3 complex is a central player. *Cellular Microbiology,* 2**,** 195-205.

Cossart, P. **(2011)**. Illuminating the landscape of host–pathogen interactions with the bacterium *Listeria monocytogenes*. *Proceedings of the National Academy of Sciences,* 108**,** 19484-19491.

Cossart, P. & Lecuit, M. **(1998)**. Interactions of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. *EMBO J,* 17**,** 3797-3806.

Costerton, J. W., Irvin, R. T. & Cheng, K. J. **(1981)**. The Bacterial Glycocalyx in Nature and Disease. *Annual Review of Microbiology,* 35**,** 299-324.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. **(1995)**. Microbial Biofilms. *Annual Review of Microbiology,* 49**,** 711-745.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. **(1999)**. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science,* 284**,** 1318-1322.

Cotter, P. D., Draper, L. A., Lawton, E. M., Daly, K. M., Groeger, D. S., Casey, P. G., Ross, R. P. & Hill, C. **(2008)**. Listeriolysin S, a Novel Peptide haemolysin Associated with a Subset of Lineage I *Listeria monocytogenes*. *PLoS Pathog,* 4**,** e1000144.

Crandall, A. D. & Montville, T. J. **(1998)**. Nisin Resistance in *Listeria monocytogenes* ATCC 700302 Is a Complex Phenotype. *Appl. Environ. Microbiol.,* 64**,** 231-237.

Davies, E. A. & Adams, M. R. **(1994)**. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. *International Journal of Food Microbiology,* 21**,** 341-347.

De Keersmaecker, S. C. J., Varszegi, C., Van Boxel, N., Habel, L. W., Metzger, K., Daniels, R., Marchal, K., De Vos, D. & Vanderleyden, J. **(2005)**. Chemical synthesis of (s)-4,5-dihydroxy-2,3-pentanedione, a bacterial signal molecule precursor, and validation of its activity in *Salmonella typhimurium*. *Journal of Biological Chemistry,* 280**,** 19563-19568.

De Siqueira, R. S., Dodd, C. E. R. & Rees, C. E. D. **(2006)**. Evaluation of the natural virucidal activity of teas for use in the phage amplification assay. *International Journal of Food Microbiology,* 111**,** 259-262.

Delves-Broughton, J., Blackburn, P., Evans, R. J. & Hugenholtz, J. **(1996)**. Applications of the bacteriocin, nisin. *Antonie van Leeuwenhoek,* 69**,** 193-202.

Desvaux, M. L., Dumas, E., Chafsey, I., Chambon, C. & HeBraud, M. **(2010)**. Comprehensive appraisal of the extracellular proteins from monoderm bacterium: Theoretical and empirical exoproteomes of *Research,* 9**,** 5076-5092.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'orio, V., Vergara, A., Conter, M. & Ianieri, A. **(2008)**. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: Relationship with motility and cell surface hydrophobicity. *Journal of Applied Microbiology,* 104**,** 1552-1561.

Djordjevic, D., Wiedmann, M. & Mclandsborough, L. A. **(2002)**. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol,* 68**,** 2950-8.

Doherty, N., Holden, M. T. G., Qazi, S. N., Williams, P. & Winzer, K. **(2006)**. Functional analysis of LuxS in *Staphylococcus aureus* reveals a role in metabolism but not quorum sensing. *J. Bacteriol.,* 188**,** 2885-2897.

Dowd, G. C., Joyce, S. A., Hill, C. & Gahan, C. G. M. **(2011)**. Investigation of the mechanisms by which *Listeria monocytogenes* grows in porcine gallbladder bile. *Infect. Immun.,* 79**,** 369-379.

Drevets, D. A., Canono, B. P., Leenen, P. J. & Campbell, P. A. **(1994)**. Gentamicin kills intracellular *Listeria monocytogenes. Infection and Immunity,* 62**,** 2222-2228.

Dussurget, O., Cabanes, D., Dehoux, P., Lecuit, M., The European *Listeria* Genome, C., Buchrieser, C., Glaser, P. & Cossart, P. **(2002)**. *Listeria monocytogenes* bile salt hydrolase is a prfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. *Molecular Microbiology,* 45**,** 1095-1106.

Edwards, M. R. & Stevens, R. W. **(1963)**. FINE STRUCTURE OF *LISTERIA MONOCYTOGENES*. *J. Bacteriol.,* 86**,** 414-428.

Eisenberg, D. **(1984)**. Three-Dimensional Structure of Membrane and Surface Proteins. *Annual Review of Biochemistry,* 53**,** 595-623.

Eisenberg, D., Schwarz, E., Komaromy, M. & Wall, R. **(1984)**. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *Journal of Molecular Biology,* 179**,** 125-142.

Farber, J. M., Kozak, G. K. & Duquette, S. **(2011)**. Changing regulation: Canada's new thinking on Listeria. *Food Control,* 22**,** 1506-1509.

Farber, J. M. & Peterkin, P. I. **(1991)**. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Mol. Biol. Rev.,* 55**,** 476-511.

Flemming, H.-C., Neu, T. R. & Wozniak, D. J. **(2007)**. The EPS Matrix: The "House of Biofilm Cells". *J. Bacteriol.,* 189**,** 7945-7947.

Freeman, J. A., Lilley, B. N. & Bassler, B. L. **(2000)**. A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in Vibrio harveyi. *Molecular Microbiology,* 35**,** 139-149.

Fuqua, W. C. & Winans, S. C. **(1994)**. A LuxR-LuxI type regulatory system activates Agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J. Bacteriol.,* 176**,** 2796-2806.

Gaillard, J. L., Berche, P., Mounier, J., Richard, S. & Sansonetti, P. **(1987)**. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line caco-2. *Infect. Immun.,* 55**,** 2822-2829.

Gaillard, J. L., Berche, P., Frehel, C., Gouln, E. & Cossart, P. **(1991)**. Entry of L. Monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell,* 65**,** 1127-1141.

Gallardo-Moreno, A. M., Navarro-Perez, M. L., Vadillo-Rodriguez, V., Bruque, J. M. & Gonzalez-Martin, M. L. **(2011)**. Insights into bacterial contact angles: Difficulties in defining hydrophobicity and surface gibbs energy. *Colloids and Surfaces B: Biointerfaces,* 88**,** 373-380.

Gandhi, M. & Chikindas, M. L. **(2007)**. *Listeria*: A foodborne pathogen that knows how to survive. *International Journal of Food Microbiology,* 113**,** 1-15.

Geertsema-Doornbusch, G. I., Van Der Mei, H. C. & Busscher, H. J. **(1993)**. Microbial cell surface hydrophobicity the involvement of electrostatic interactions in microbial adhesion to hydrocarbons (math). *Journal of Microbiological Methods,* 18**,** 61-68.

Gellin, B. G., Broome, C. V., Bibb, W. F., Weaver, R. E., Gaventa, S., Mascola, L. & Group, L. S. **(1991)**. The epidemiology of listeriosis in the united states—1986. *American Journal of Epidemiology,* 133**,** 392-401.

Giotis, E. S., Blair, I. S. & Mcdowell, D. A. **(2007)**. Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *International Journal of Food Microbiology,* 120**,** 250-258.

Goldberg, M. B. **(2001)**. Actin-Based Motility of Intracellular Microbial Pathogens. *Microbiology and Molecular Biology Reviews,* 65**,** 595-626.

Goller, C. C. & Seed, P. C. **(2010)**. Revisiting the *Escherichia coli* polysaccharide capsule as a virulence factor during urinary tract infection: Contribution to intracellular biofilm development. *Virulence,* 1**,** 333-7.

Gouin, E., Welch, M. D. & Cossart, P. **(2005)**. Actin-based motility of intracellular pathogens. *Current Opinion in Microbiology,* 8**,** 35-45.

Goulet, V., Hedberg, C., Le Monnier, A. & De Valk, H. **(2008)**. Increasing incidence of listeriosis in france and other european countries. *Emerg Infect Dis,* 14**,** 734-40.

Graves, L. M., Helsel, L. O., Steigerwalt, A. G., Morey, R. E., Daneshvar, M. I., Roof, S. E., Orsi, R. H., Fortes, E. D., Milillo, S. R., Den Bakker, H. C., Wiedmann, M., Swaminathan, B. & Sauders, B. D. **(2010)**. Listeria marthii sp. Nov., isolated from the natural environment, finger lakes national forest. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 1280-1288.

Guenther, S., Huwyler, D., Richard, S. & Loessner, M. J. **(2009)**. Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Applied and Environmental Microbiology,* 75**,** 93-100.

Han, C. S., Xie, G., Challacombe, J. F., Altherr, M. R., Bhotika, S. S., Bruce, D., Campbell, C. S., Campbell, M. L., Chen, J., Chertkov, O., Cleland, C., Dimitrijevic, M., Doggett, N. A., Fawcett, J. J., Glavina, T., Goodwin, L. A., Hill, K. K., Hitchcock, P., Jackson, P. J., Keim, P., Kewalramani, A. R., Longmire, J., Lucas, S., Malfatti, S., Mcmurry, K., Meincke, L. J., Misra, M., Moseman, B. L., Mundt, M., Munk, A. C., Okinaka, R. T., Parson-Quintana, B., Reilly, L. P., Richardson, P., Robinson, D. L., Rubin, E., Saunders, E., Tapia, R., Tesmer, J. G., Thayer, N., Thompson, L. S., Tice, H., Ticknor, L. O., Wills, P. L., Brettin, T. S. & Gilna, P. **(2006)**. Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis*. *Journal of Bacteriology,* 188**,** 3382-3390.

Hanahan, D. **(1983)**. Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology,* 166**,** 557-580.

Harmsen, M., Lappann, M., Knochel, S. & Molin, S. **(2010)**. Role of extracellular DNA during biofilm formation by *Listeria monocytogenes. Appl. Environ. Microbiol.,* 76**,** 2271-2279.

Hardie, K. R. & Heurlier, K. **(2008)**. Establishing bacterial communities by 'word of mouth': Luxs and autoinducer 2 in biofilm development. *Nat Rev Microbiol,* 6**,** 635-43.

Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. **(2010)**. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents,* 35**,** 322-332.

Holmes, K., Tavender, T., Winzer, K., Wells, J. & Hardie, K. **(2009)**. AI-2 does not function as a quorum sensing molecule in *Campylobacter jejuni* during exponential growth in vitro. *BMC Microbiology,* 9**,** 214.

Hood, S. K. & Zottola, E. A. **(1997)**. Growth media and surface conditioning influence the adherence of *pseudomonas fragi*, *salmonella typhimurium*, and *listeria monocytogenes* cells to stainless steel. *Journal of Food Protection,* 60**,** 1034-1037.

Hughey, V. L. & Johnson, E. A. **(1987)**. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Appl. Environ. Microbiol.,* 53**,** 2165-2170.

Hyman, P. & Abedon, S. T. 2010. Chapter 7 - bacteriophage host range and bacterial resistance. *In:* Allen, I. L., Sima, S. & Geoffrey, M. G. (eds.) *Advances in applied microbiology.* Academic Press.

Ireton, K. **(2007)**. Entry of the bacterial pathogen *Listeria monocytogenes* into mammalian cells. *Cellular Microbiology,* 9**,** 1365-1375.

Isom, L. L., Khambatta, Z. S., Moluf, J. L., Akers, D. F. & Martin, S. E. **(1995)**. Filament Formation in *Listeria monocytogenes*. *Journal of Food Protection,* 58**,** 1031-1033.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M. & Cossart, P. **(2002)**. An RNA Thermosensor Controls Expression of Virulence Genes in *Listeria monocytogenes*. *Cell,* 110**,** 551-561.

Kalmokoff,M. L., Austin, J. W.,Wan, X. D., Sanders, G., Banerjee, S. & Farber, J. M. **(2001)**. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology,* 91**,** 725-734.

Kim, K. Y. & Frank, J. F. **(1995)**. Effect of Nutrients on Biofilm Formation by *Listeria monocytogenes* on Stainless Steel. *Journal of Food Protection,* 58**,** 24-28.

Kleemann, P., Domann, E., Chakraborty, T., Bernstein, I. & Lohoff, M. **(2009)**. Chronic prosthetic joint infection caused by *Listeria monocytogenes*. *Journal of Medical Microbiology,* 58**,** 138-141.

Koehler, T. M. **(2002)**. *Bacillus anthracis* genetics and virulence gene regulation. *Curr Top Microbiol Immunol,* 271**,** 143-64.

Kocianova, S., Vuong, C., Yao, Y., Voyich, J. M., Fischer, E. R., Deleo, F. R. & Otto, M. **(2005)**. Key role of poly-γ-dl-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. *The Journal of Clinical Investigation,* 115**,** 688-694.

Kocks, C., Hellio, R., Gounon, P., Ohayon, H. & P., C. **(1993)**. Polarized distribution of *Listeria monocytogenes* surface protein acta at the site of directional actin assembly. *Journal of Cell Science,* 105**,** 699-710.

Kreft, J. & Vazquez-Boland, J. A. **(2001)**. Regulation of virulence genes in *Listeria*. *International Journal of Medical Microbiology,* 291**,** 145-157.

Kyte, J. & Doolittle, R. F. **(1982)**. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology,* 157**,** 105-132.

Lang Halter, E., Neuhaus, K. & Scherer, S. **(2012)**. *Listeria weihenstephanensis sp. nov.*, isolated from the water plant Lemna trisulca of a German fresh water pond. *International Journal of Systematic and Evolutionary Microbiology*.

Leclercq, A., Clermont, D., Bizet, C., Grimont, P. A. D., Le Fleche-Mateos, A., Roche, S. M., Buchrieser, C., Cadet-Daniel, V., Le Monnier, A., Lecuit, M. & Allerberger, F. **(2010)**. *Listeria rocourtiae sp. nov*. *International Journal of Systematic and Evolutionary Microbiology,* 60**,** 2210-2214.

Lemon, K. P., Higgins, D. E. & Kolter, R. **(2007)**. Flagellar motility is critical for *listeria monocytogenes* biofilm formation. *J. Bacteriol.,* 189**,** 4418-4424.

Li, X., Lindahl, L., Sha, Y. & Zengel, J. M. **(1997)**. Analysis of the *Bacillus subtilis* s10 ribosomal protein gene cluster identifies two promoters that may be responsible for transcription of the entire 15-kilobase s10-spc-alpha cluster. *Journal of Bacteriology,* 179**,** 7046-54.

Lindsay, D. & Holy, A. V. **(2006)**. What food safety professionals should know about bacterial biofilms. *British Food Journal,* 108**,** 27-37.

Little, C. L., Amar, C. F. L., Awofisayo, A. & Grant, K. A. **(2012)**. Hospital-acquired listeriosis associated with sandwiches in the UK: A cause for concern. *Journal of Hospital Infection,* 82**,** 13-18.

Little, C. L., Sagoo, S. K., Gillespie, I. A., Grant, K. & Mclauchlin, J. **(2009)**. Prevalence and level of *Listeria monocytogenes* and other *listeria* species in selected retail ready-to-eat foods in the united kingdom. *Journal of Food Protection®,* 72**,** 1869-1877.

Liu, H. & Fang, H. H. P. **(2002)**. Extraction of extracellular polymeric substances (eps) of sludges. *Journal of Biotechnology,* 95**,** 249-256.

Loessner, M. J. **(1991)**. Improved procedure for bacteriophage typing of *Listeria* strains and evaluation of new phages. *Applied and Environmental Microbiology,* 57**,** 882-884.

Lombardia, E., Rovetto, A. J., Arabolaza, A. L. & Grau, R. R. **(2006)**. A luxs-dependent cell-to-cell language regulates social behavior and development in *bacillus subtilis. J. Bacteriol.,* 188**,** 4442-4452.

Lowery, C. A., Park, J., Kaufmann, G. F. & Janda, K. D. **(2008)**. An unexpected switch in the modulation of ai-2-based quorum sensing discovered through synthetic 4,5-dihydroxy-2,3-pentanedione analogues. *Journal of the American Chemical Society,* 130**,** 9200-9201.

Lund, N, J. M., Miettinen, M. K., Autio, T. J. & Korkeala, H. J. **(2000)**. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *Journal of Food Protection®,* 63**,** 1204-1207.

Lunden, J., Autio, T., Markkula, A., Hellstrom, S. & Korkeala, H. **(2003)**. Adaptive and cross-adaptive responses of persistent and non-persistent *Lsteria monocytogenes* strains to disinfectants. *International Journal of Food Microbiology,* 82**,** 265-272.

Lyon, W., Madden, J., Levin, J., Stein, J. & Caparon, M. **(2001)**. Mutation of LuxS affects growth and virulence factor expression in Streptococcus pyogenes. *Mol Microbiol,* 42**,** 145 - 157.

Mai, T. L. & Conner, D. E. **(2007)**. Effect of temperature and growth media on the attachment of *Listeria monocytogenes* to stainless steel. *International Journal of Food Microbiology,* 120**,** 282-286.

March, J. C. & Bentley, W. E. **(2004)**. Quorum sensing and bacterial cross-talk in biotechnology. *Current Opinion in Biotechnology,* 15**,** 495-502.

Maric, M., Arunachalam, B., Phan, U. T., Dong, C., Garrett, W. S., Cannon, K. S., Alfonso, C., Karlsson, L., Flavell, R. A. & Cresswell, P. **(2001)**. Defective Antigen Processing in GILT-Free Mice. *Science,* 294**,** 1361-1365.

Marvasi, M., Visscher, P. T. & Casillas Martinez, L. **(2010)**. Exopolymeric substances (EPS) from *Bacillus subtilis* : polymers and genes encoding their synthesis. *FEMS Microbiology Letters,* 313**,** 1-9.

Mclandsborough, L., Rodriguez, A., Perez-Conesa, D. & Weiss, J. **(2006)**. Biofilms: At the Interface between Biophysics and Microbiology. *Food Biophysics,* 1**,** 94-114.

Mcswain, B. S., Irvine, R. L., Hausner, M. & Wilderer, P. A. **(2005)**. Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge. *Appl. Environ. Microbiol.,* 71**,** 1051-1057.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R.-M. & Cossart, P. **(1996)**. E-Cadherin Is the Receptor for Internalin, a Surface Protein Required for Entry of *L. monocytogenes* into Epithelial Cells. *Cell,* 84**,** 923-932.

Merritt, J., Qi, F., Goodman, S. D., Anderson, M. H. & Shi, W. **(2003)**. Mutation of luxS Affects Biofilm Formation in Streptococcus mutans. *Infect. Immun.,* 71**,** 1972-1979.

Milenbachs, A. A., Brown, D. P., Moors, M. & Youngman, P. **(1997)**. Carbon-source regulation of virulence gene expression in *Listeria monocytogenes*. *Molecular Microbiology,* 23**,** 1075-1085.

Milohanic, E., Glaser, P., Coppee, J.-Y., Frangeul, L., Vega, Y., Vazquez-Boland, J. A., Kunst, F., Cossart, P. & Buchrieser, C. **(2003)**. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. *Molecular Microbiology,* 47**,** 1613-1625.

Mook, P., O'brien, S. J. & Gillespie, I. A. **(2011)**. Concurrent conditions and human listeriosis, England, 1999-2009. *Emerg Infect Dis,* 17**,** 38-43.
Moorman, M. A., Thelemann, C. A., Zhou, S., Pestka, J. J., Linz, J. E. & Ryser, E. T. **(2008)**. Altered hydrophobicity and membrane composition in stress-adapted *Listeria innocua*. *J Food Prot,* 71**,** 182-5.

Murray, E. G. D., Webb, R. A. & Swann, M. B. R. **(1926)**. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus Bacterium monocytogenes (n.sp.). *The Journal of Pathology and Bacteriology,* 29**,** 407-439.

Neu, T. R. & Marshall, K. C. **(1990)**. Bacterial polymers: Physicochemical aspects of their interactions at interfaces. *Journal of Biomaterials Applications,* 5**,** 107-133.

Ng, W.-L. & Bassler, B. L. **(2009)**. Bacterial quorum-sensing network architectures. *Annual Review of Genetics,* 43**,** 197-222.

Nielsen, P. H., Jahn, A. & Palmgren, R. **(1997)**. Conceptual model for production and composition of exopolymers in biofilms. *Water Science and Technology,* 36**,** 11-19.

Nwaiwu, O. **(2011)** The effect of growth conditions on the surface properties of Listeria monocytogenes; *PhD Thesis, University of Nottingham.*

Palmer, J., Flint, S. & Brooks, J. **(2007)**. Bacterial cell attachment, the beginning of a biofilm. *Journal of Industrial Microbiology & Biotechnology,* 34**,** 577-588.

Park, S. F. & Stewart, G. S. A. B. **(1990)**. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene,* 94**,** 129-132.

Park, S. F., Stewart, G. S. A. B. & Kroll, R. G. **(1992)**. The use of bacterial luciferase for monitoring the environmental regulation of expression of genes encoding virulence factors in *listeria monocytogenes*. *Journal of General Microbiology,* 138**,** 2619-2627.

Perehinec, T., Qazi, S., Gaddipati, S., Salisbury, V., Rees, C. & Hill, P. **(2007)**. Construction and evaluation of multisite recombinatorial (gateway) cloning vectors for gram-positive bacteria. *BMC Molecular Biology,* 8**,** 80.

Pestova, E. V., Havarstein, L. S. & Morrison, D. A. **(1996)**. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Molecular Microbiology,* 21**,** 853-862.

Peterson, S., Cline, R. T., Tettelin, H., Sharov, V. & Morrison, D. A. **(2000)**. Gene Expression Analysis of the *Streptococcus pneumonia* Competence Regulons by Use of DNA Microarrays. *Journal of Bacteriology,* 182**,** 6192-6202.

Pirie, J. H. **(1940)**. THE GENUS *LISTERELLA PIRIE*. *Science,* 91**,** 383.

Platt, R. M., Geesey, G. G., Davis, J. D. & White, D. C. **(1985)**. Isolation and partial chemical analysis of firmly bound exopolysaccharide from adherent cells of a freshwater sediment bacterium. *Canadian Journal of Microbiology,* 31**,** 675-680.

Poimenidou, S., Belessi, C. A., Giaouris, E. D., Gounadaki, A. S., Nychas, G.-J. E. & Skandamis, P. N. **(2009)**. *Listeria monocytogenes* attachment to and detachment from stainless steel surfaces in a simulated dairy processing environment. *Applied and Environmental Microbiology,* 75**,** 7182-7188.

Popowska, M., Kusio, M., Szymanska, P. & Markiewicz, Z. **(2009)**. Inactivation of the wall-associated de-n-acetylase (pgda) of *listeria monocytogenes* results in greater susceptibility of the cells to induced autolysis. *J Microbiol Biotechnol,* 19**,** 932-45.

Rasmussen, T. B. & Givskov, M. **(2006)**. Quorum-sensing inhibitors as anti-pathogenic drugs. *International Journal of Medical Microbiology,* 296**,** 149-161.

Rath, A. & Deber, C. M. **(2012)**. Protein Structure in Membrane Domains. *Annual Review of Biophysics,* 41**,** 135-155.

Rauch, M., Luo, Q., Muller-Altrock, S. & Goebel, W. **(2005)**. SigB-Dependent In Vitro Transcription of prfA and Some Newly Identified Genes of *Listeria monocytogenes* Whose Expression Is Affected by PrfA In Vivo. *Journal of Bacteriology,* 187**,** 800-804.

Renzoni, A., Cossart, P. & Dramsi, S. **(1999)**. PrfA, the transcriptional activator of virulence genes, is upregulated during interaction of *Listeria monocytogenes* with mammalian cells and in eukaryotic cell extracts. *Molecular Microbiology,* 34**,** 552-561.

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

Rickard, A., Palmer, R., Blehert, D., Campagna, S., Semmelhack, M., Egland, P., Bassler, B. & Kolenbrander, P. **(2006)**. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. *Mol Microbiol,* 60**,** 1446 - 1456.

Rieu, A., Briandet, R., Habimana, O., Garmyn, D., Guzzo, J. & Piveteau, P. **(2008)**. *Listeria monocytogenes* EGD-e Biofilms: No Mushrooms but a Network of Knitted Chains. *Appl. Environ. Microbiol.,* 74**,** 4491-4497.

Ripio, M. T., Dominguez-Bernal, G., Suarez, M., Brehm, K., Berche, P. & Vazquez-Boland, J. A. **(1996)**. Transcriptional activation of virulence genes in wild-type strains of *Listeria monocytogenes* in response to a change in the extracellular medium composition. *Research in Microbiology,* 147**,** 371-384.

Robbins, J. R., Skrzypczynska, K. M., Zeldovich, V. B., Kapidzic, M. & Bakardjiev, A. I. **(2010)**. Placental Syncytiotrophoblast Constitutes a Major Barrier to Vertical Transmission of *Listeria monocytogenes*. *PLoS Pathog,* 6**,** e1000732.

Roberts, I. S. **(1996)**. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annual Review of Microbiology,* 50**,** 285-315.

Rosenberg, M. **(2006)**. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. *FEMS Microbiology Letters,* 262**,** 129-134.

Rosenberg, M., Gutnick, D. & Rosenberg, E. **(1980)**. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiology Letters,* 9**,** 29-33.

Sahly, H., Podschun, R., Oelschlaeger, T. A., Greiwe, M., Parolis, H., Hasty, D., Kekow, J., Ullmann, U., Ofek, I. & Sela, S. **(2000)**. Capsule Impedes Adhesion to and Invasion of Epithelial Cells by *Klebsiella pneumoniae*. *Infection and Immunity,* 68**,** 6744-6749.

Sandasi, M., Leonard, C. M. & Viljoen, A. M. **(2008)**. The effect of five common essential oil components on *Listeria monocytogenes* biofilms. *Food Control,* 19**,** 1070-1075.

Schlech, W. F., Lavigne, P. M., Bortolussi, R. A., Allen, A. C., Haldane, E. V., Wort, A. J., Hightower, A. W., Johnson, S. E., King, S. H., Nicholls, E. S. & Broome, C. V. **(1983)**. Epidemic Listeriosis — Evidence for Transmission by Food. *New England Journal of Medicine,* 308**,** 203-206.

Schneider, R., Lockatell, C., Johnson, D. & Belas, R. **(2002)**. Detection and mutation of a luxS -encoded autoinducer in *Proteus mirabilis*. *Microbiology,* 148**,** 773 - 782.

Seeliger, H. P. R. & Jones, D. (eds.) 1986. *Genus Listeria. In Bergey's manual of systematic bacteriology* Williams & Wilkin Co.

Sela, S., Frank, S., Belausov, E. & Pinto, R. **(2006)**. A Mutation in the luxS Gene Influences *Listeria monocytogenes* Biofilm Formation. *Appl. Environ. Microbiol.,* 72**,** 5653-5658.

Serna, A. I. & Boedeker, E. C. **(2008)**. Pathogenesis and treatment of Shiga toxin-producing Escherichia coli infections. *Current Opinion in Gastroenterology,* 24**,** 38-47 10.1097/MOG.0b013e3282f2dfb8.

Seveau, S., Pizarro-Cerda, J. & Cossart, P. **(2007)**. Molecular mechanisms exploited by *listeria monocytogenes* during host cell invasion. *Microbes and Infection,* 9**,** 1167-1175.

Schnupf, P. & Portnoy, D. A. **(2007)**. Listeriolysin O: a phagosome-specific lysin. *Microbes and Infection,* 9**,** 1176-1187.

Schwede, T., Kopp, J., Guex, N. & Peitsch, M. C. **(2003)**. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research,* 31**,** 3381-3385.

Scortti, M., Monzo, H. J., Lacharme-Lora, L., Lewis, D. A. & Vazquez-Boland, J. A. **(2007)**. The PrfA virulence regulon. *Microbes and Infection,* 9**,** 1196-1207.

Shen, Y., Naujokas, M., Park, M. & Ireton, K. **(2000)**. InlB-Dependent Internalization of *Listeria* Is Mediated by the Met Receptor Tyrosine Kinase. *Cell,* 103**,** 501-510.

Shetty, A., Mclauchlin, J., Grant, K., O'brien, D., Howard, T. & Davies, E. M. **(2009)**. Outbreak of *Listeria monocytogenes* in an oncology unit associated with sandwiches consumed in hospital. *Journal of Hospital Infection,* 72**,** 332-336.

Sinde, E. & Carballo, J. **(2000)**. Attachment of *Salmonella spp.* and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiology,* 17**,** 439-447.

Sleator, R. D., Watson, D., Hill, C. & Gahan, C. G. M. **(2009)**. The interaction between *Listeria monocytogenes* and the host gastrointestinal tract. *Microbiology,* 155**,** 2463-2475.

Smith, C. W. & Metzger, J. F. **(1962)**. Demonstration of a Capsular Structure on *Listeria Monocytogenes*. *Pathologia et Microbiologia,* 25**,** 499-506

Smith, J. L., Fratamico, P. M. & Novak, J. S. **(2004)**. Quorum sensing: A primer for food microbiologists. *Journal of Food Protection,* 67**,** 1053-1070.

Smoot, L. M. & Pierson, M. D. **(1998)**. Effect of Environmental Stress on the Ability of *Listeria monocytogenes* Scott A To Attach to Food Contact Surfaces. *Journal of Food Protection,* 61**,** 1293-1298.

Stavru, F., Archambaud, C. & Cossart, P. **(2011)**. Cell biology and immunology of *Listeria monocytogenes* infections: novel insights. *Immunological Reviews,* 240**,** 160-184.

Stepanović, S., Ćirković, I., Ranin, L. & S Vabić-Vlahović, M. **(2004)**. Biofilm formation by *Salmonella* spp. and Listeria monocytogenes on plastic surface. *Letters in Applied Microbiology,* 38**,** 428-432.

Sternglanz, R., Dinardo, S., Voelkel, K. A., Nishimura, Y., Hirota, Y., Becherer, K., Zumstein, L. & Wang, J. C. **(1981)**. Mutations in the gene coding for *Escherichia coli* DNA topoisomerase I affect transcription and transposition. *Proceedings of the National Academy of Sciences,* 78**,** 2747-2751.

Stevens, K. A., Sheldon, B. W., Klapes, N. A. & Klaenhammer, T. R. **(1991)**. Nisin treatment for inactivation of *Salmonella* species and other gram-negative bacteria. *Appl. Environ. Microbiol.,* 57**,** 3613-3615.

Stewart, P. S. & William Costerton, J. **(2001)**. Antibiotic resistance of bacteria in biofilms. *The Lancet,* 358**,** 135-138.

Stoodley, P., Dodds, I., Boyle, J. D. & Lappin-Scott, H. M. **(1998)**. Influence of hydrodynamics and nutrients on biofilm structure. *Journal of Applied Microbiology,* 85**,** 19S-28S.

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,* 96**,** 1639-1644.

Takahashi, H., Suda, T., Tanaka, Y. & Kimura, B. **(2010)**. Cellular hydrophobicity of *Listeria monocytogenes* involves initial attachment and biofilm formation on the surface of polyvinyl chloride. *Letters in Applied Microbiology,* 50**,** 618-625.

Temple, M. & Nahata, M. **(2000)**. Treatment of listeriosis. *The Annals of Pharmacotherapy,* 34**,** 656-661.

Tilney, L. G. & Portnoy, D. A. **(1989)**. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *listeria monocytogenes*. *The Journal of Cell Biology,* 109**,** 1597-1608.

Toole, G. O., Kaplan, H. B. & Kolter, R. **(2000)**. Biofilm formation as microbial development. *Annual Review of Microbiology,* 54**,** 49-79

Tresse, O., Lebret, V., Benezech, T. & Faille, C. **(2006)**. Comparative evaluation of adhesion, surface properties, and surface protein composition of *Listeria monocytogenes* strains after cultivation at constant pH of 5 and 7. *Journal of Applied Microbiology,* 101**,** 53-62.

Tsuneda, S., Aikawa, H., Hayashi, H., Yuasa, A. & Hirata, A. **(2003)**. Extracellular polymeric substances responsible for bacterial adhesion onto solid surface. *FEMS Microbiology Letters,* 223**,** 287-292.

Turovskiy, Y. & Chikindas, M. L. **(2006)**. Autoinducer-2 bioassay is a qualitative, not quantitative method influenced by glucose. *Journal of Microbiological Methods,* 66**,** 497-503.

Urban, C. F., Lourido, S. & Zychlinsky, A. **(2006)**. How do microbes evade neutrophil killing? *Cellular Microbiology,* 8**,** 1687-1696.

Ute, S., Yuewei, H., Martin, W. & Kathryn, J. B. **(2005)**. Alternative Sigma Factor sigmaB Is Not Essential for *Listeria monocytogenes* Surface Attachment. *Journal of Food Protection,* 68**,** 311-317.

Van Der Veen, S. & Abee, T. **(2010)**. Dependence of continuous-flow biofilm formation by *Listeria monocytogenes* EGD-e on SOS response factor YneA. *Appl Environ Microbiol,* 76**,** 1992-5.

Vatanyoopaisarn, S., Nazli, A., Dodd, C. E., Rees, C. E. & Waites, W. M. **(2000)**. Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. *Appl Environ Microbiol,* 66**,** 860-3.

Vatanyoopaisarn, S. **(2000)** Formation of biofilms on stainless steel by Pseudomonas fluorescens and Listeria monocytogenes. *PhD Thesis, University of Nottingham.*

Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J. &

Kreft, J. **(2001)**. *Listeria* Pathogenesis and Molecular Virulence Determinants. *Clin. Microbiol. Rev.,* 14**,** 584-640.

Veiga, E. & Cossart, P. **(2005)**. *Listeria* hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. 7**,** 894-900.

Vilain, S., Pretorius, J. M., Theron, J. & Brozel, V. S. **(2009)**. DNA as an Adhesin: *Bacillus cereus* Requires Extracellular DNA To Form Biofilms. *Applied and Environmental Microbiology,* 75**,** 2861-2868.

Vilchez, R., Lemme, A., Thiel, V., Schulz, S., Sztajer, H. & Wagner-Dobler, I. **(2007)**. Analysing traces of autoinducer-2 requires standardization of the *Vibrio harveyi* bioassay. *Analytical and Bioanalytical Chemistry,* 387**,** 489-496.

Walker, S. J., Archer, P. & Banks, J. G. **(1990)**. Growth of *Listeria monocytogenes* at refrigeration temperatures. *Journal of Applied Microbiology,* 68**,** 157-162.

Watnick, P. & Kolter, R. **(2000)**. Biofilm, city of microbes. *J. Bacteriol.,* 182**,** 2675-2679.

Watson, S. P., Clements, M. O. & Foster, S. J. **(1998)**. Characterization of the Starvation-Survival Response of *Staphylococcus aureus*. *Journal of Bacteriology,* 180**,** 1750-1758.

Whisstock, J. C. & Lesk, A. M. **(2003)**. Prediction of protein function from protein sequence and structure. *Quarterly Reviews of Biophysics,* 36**,** 307-340.

Williams, P. **(2007)**. Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology,* 153**,** 3923 - 3928.

Wingender, J., Neu, H. R. & Flemming, H.-C. (eds.) 1999. *Microbial extracellular polymeric substances: Characterization, structure, and function*: Springer.

Wilson, J. W., Schurr, M. J., Leblanc, C. L., Ramamurthy, R., Buchanan, K. L. & Nickerson, C. A. **(2002)**. Mechanisms of bacterial pathogenicity. *Postgraduate Medical Journal,* 78**,** 216-224.

Wu, J. H., Wu, A. M., Tsai, C. G., Chang, X.-Y., Tsai, S.-F. & Wu, T.-S. **(2008)**. Contribution of Fucose-Containing Capsules in *Klebsiella pneumoniae* to Bacterial Virulence in Mice. *Experimental Biology and Medicine,* 233**,** 64-70.

Young, R. **(1992)**. Bacteriophage lysis: Mechanism and regulation. *Microbiol. Mol. Biol. Rev.,* 56**,** 430-481.

Zhang, Y. **(2008)**. Progress and challenges in protein structure prediction. *Current Opinion in Structural Biology,* 18**,** 342-348.

# Appendix

## Appendix I - Protein Sequence of lmo0017 and lmo0516 (Please refer to p.166)

### A) Hypothetical protein lmo0017 [Listeria monocytogenes EGD-e].

1 MKSRKKGIIL VLSVILIFSI GLLVNNLMTN NKDTAKPKKK TVAAVKKKKE TPPKPKEPFN
61 IDFTGDIMFD WDLRPVLAEK GMDYPFNNVR EELKSSDYTF VDLETAITTR TKKVPYQEFW
121 IKSDPSSLTA LKNAGVDMVN ISNNHILDYY EDGLLDTTAA LRANNLAYVG AGKNEDEAYQ
181 LKVADIKGNK VGFMSFCHFF PNTGWIADED TPGVTNGYDL NLVEEKIKEE RAKNKDIDYM
241 VVYFHWGVEK TNTPVDYQTQ YVKKLVDDNL VDAIVASHPH WLQGFEVYKD VPIAYSLGNF
301 LFPDYVSGHS AETGIYKLNF DQGKVTAHFD PGIISGNQIN MLEGSSKTAQ LNYLQSISPN
361 ATINSNGDIS AK

### B) Hypothetical protein lmo0516 [Listeria monocytogenes EGD-e].

1 MKKLTYVIIT GLVLVFIAGA FWITNSTNQS DQKATQTEPI KKISPANVKT ISSEAKKTLN
61 SLASSGADKA SISDLKQLIK ELKSYPTEKN DSGVYLQNLT ACLEAVKSYT TGKADEKTLG
121 KVYPAFLASE QKLSAIEKTN QYDWFYAAAA TNQQGLKEKG VVTLTMVGDN SFGTYPETPE
181 HLKFDNVFQK NNGNNTYVYK NCLPWFKSDD YTIINAESAF TNATKAENKM WRIKSDPAHV
241 AFLPASGVDA ANLANNHTMD YFQVGYDDTL KAFKENNIPV FNADAPLETT IKGMKTVLLG
301 YDCRMSQQSP AYLERIVKDV KKYKKEDTLV IVNMHWGVEY RETPTDYQTQ FGHAILDAGA
361 DIIMGSHPHR LESVEKYKDK YIVYSMGDFA FGADPTLLSR MTSMFQLRFT KEDNKIVLKD
421 ISIVPTYENS DGSTTENNYQ PLPVFGDDAK KIVDELNRIS KPIEGGVTEY TYFDPF

The protein sequence of A) lmo0017 and B) lmo0516 (Listeria monocytogenes EGD) was downloaded from NCBI online gene bank.

lmo0017: http://www.ncbi.nlm.nih.gov/protein/16802065

lmo0516: http://www.ncbi.nlm.nih.gov/protein/CAC98595.1

## Appendix II - Searching for the present of the conserved sequences in various (Please refer to page 171)

## A) NNH-$(X)_2$–DYY–$(X)_6$–DT and NNH-$(X)_2$–D–$(X)_4$–G–$(X)_2$-DT

**Sequences producing significant alignments:**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| YP_003598028.1 | putative capsule biosynthesis protein CapA [Bacillus megaterium DSM | 38.4 | 38.4 | 100% | 2e-06 | 80% |
| YP_003563259.1 | capsule biosynthesis protein CapA [Bacillus megaterium QM B1551] > | 38.4 | 38.4 | 100% | 2e-06 | 80% |
| YP_005280756.1 | capsule synthesis protein CapA [Gordonia polyisoprenivorans VH2] >g | 38.4 | 38.4 | 100% | 2e-06 | 80% |
| ZP_09269672.1 | hypothetical protein GOPIP_031_01230 [Gordonia polyisoprenivorans | 38.4 | 38.4 | 100% | 2e-06 | 80% |
| ZP_02621390.1 | encapsulation protein CapA [Clostridium botulinum C str. Eklund] >gb | 38.4 | 38.4 | 100% | 2e-06 | 73% |
| YP_005494579.1 | pgsA3 gene product [Bacillus megaterium WSH-002] >gb\|AEN89270. | 35.8 | 35.8 | 100% | 2e-05 | 80% |
| YP_003190897.1 | putative polyglutamate synthase CapA [Desulfotomaculum acetoxida | 33.7 | 33.7 | 100% | 8e-05 | 67% |
| ZP_01631694.1 | poly-gamma-glutamic synthesis PgsA protein-like protein [Nodularia s | 33.7 | 33.7 | 100% | 8e-05 | 67% |
| YP_001126007.1 | poly-gamma-glutamate biosynthesis protein [Geobacillus thermodenit | 32.9 | 32.9 | 100% | 1e-04 | 73% |
| ZP_01620698.1 | poly-gamma-glutamic synthesis PgsA protein-like protein [Lyngbya s | 32.9 | 32.9 | 100% | 1e-04 | 60% |
| YP_004982688.1 | capsule synthesis protein CapA [Geobacillus thermoleovorans CCB_US | 32.5 | 32.5 | 100% | 2e-04 | 73% |
| YP_147857.1 | poly-gamma-glutamate biosynthesis protein [Geobacillus kaustophilus | 32.5 | 32.5 | 100% | 2e-04 | 73% |
| YP_003253901.1 | capsule synthesis protein, CapA [Geobacillus sp. Y412MC61] >ref\|YP | 32.5 | 32.5 | 100% | 2e-04 | 73% |
| YP_003271379.1 | capsule synthesis protein, capA [Gordonia bronchialis DSM 43247] >g | 32.0 | 32.0 | 100% | 3e-04 | 73% |
| ZP_07388641.1 | ResB family protein [Paenibacillus curdlanolyticus YK9] >gb\|EFM0981 | 31.6 | 31.6 | 86% | 4e-04 | 56% |
| YP_002316008.1 | enzyme of poly-gamma-glutamate biosynthesis (capsule formation) [ | 30.8 | 30.8 | 100% | 7e-04 | 73% |
| ZP_08981253.1 | Capsule synthesis protein, CapA [Desulfosporosinus meridiei DSM 132 | 30.3 | 30.3 | 100% | 0.001 | 60% |
| ZP_09313157.1 | hypothetical protein FbacHQ_02315 [Flavobacteriaceae bacterium HQ | 30.3 | 30.3 | 66% | 0.001 | 90% |
| YP_001734719.1 | poly-gamma-glutamate biosynthesis protein [Synechococcus sp. PCC | 29.9 | 29.9 | 100% | 0.001 | 67% |
| YP_004310342.1 | capsule synthesis protein, CapA [Clostridium lentocellum DSM 5427] | 29.5 | 29.5 | 100% | 0.002 | 67% |
| YP_004112188.1 | HAD-superfamily hydrolase [Desulfurispirillum indicum S5] >gb\|ADU65 | 29.1 | 29.1 | 66% | 0.002 | 67% |
| ZP_08325924.1 | hypothetical protein HMPREF0491_00786 [Lachnospiraceae oral taxo | 29.1 | 29.1 | 46% | 0.002 | 100% |
| ZP_03756440.1 | hypothetical protein CLOSTASPAR_00424 [Clostridium asparagiforme | 29.1 | 29.1 | 46% | 0.002 | 100% |
| EIC95084.1 | bacterial capsule synthesis protein [Eubacterium saburreum F0468] | 29.1 | 29.1 | 46% | 0.002 | 100% |
| ZP_07905040.1 | protein of poly-gamma-glutamate biosynthesis (capsule formation) fa | 29.1 | 29.1 | 46% | 0.002 | 100% |
| YP_001530559.1 | Mur ligase middle domain-containing protein [Desulfococcus oleovora | 29.1 | 29.1 | 46% | 0.002 | 100% |
| YP_006181671.1 | capA2 gene product [Halobacillus halophilus DSM 2266] >emb\|CCG46 | 29.1 | 29.1 | 46% | 0.002 | 100% |
| YP_002802173.1 | MFS (major facilitator superfamily) transporter [Azotobacter vinelanc | 29.1 | 29.1 | 60% | 0.002 | 60% |
| YP_006188726.1 | hypothetical protein [Paenibacillus mucilaginosus K02] >gb\|AFH6096 | 29.1 | 29.1 | 46% | 0.002 | 100% |
| YP_005311934.1 | hypothetical protein PM3016_1879 [Paenibacillus mucilaginosus 3016 | 29.1 | 29.1 | 46% | 0.002 | 100% |
| YP_004640007.1 | hypothetical protein KNP414_01573 [Paenibacillus mucilaginosus KNP | 29.1 | 29.1 | 46% | 0.002 | 100% |
| EHJ20587.1 | hypothetical protein KIW_09030 [Pediococcus acidilactici MA18/5M] | 29.1 | 29.1 | 73% | 0.002 | 73% |
| ZP_07368099.1 | conserved hypothetical protein [Pediococcus acidilactici DSM 20284] | 29.1 | 29.1 | 73% | 0.002 | 73% |

## B) $(X)_2$-H-(X)-P-(X)-V-$(X)_4$-E-(X)-Y and G-(X)-HPH-$(X)_5$-Y

**Sequences producing significant alignments:**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| ZP_02212747.1 | hypothetical protein CLOBAR_02366 [Clostridium bartlettii DSM 1679 | 32.0 | 32.0 | 100% | 2e-04 | 77% |
| YP_723575.1 | putative branched-chain amino acid aminotransferase [Trichodesmiu | 29.5 | 29.5 | 84% | 0.001 | 73% |
| YP_004440225.1 | capsule synthesis protein CapA [Treponema brennaborense DSM 121 | 29.1 | 29.1 | 100% | 0.002 | 69% |
| YP_001734204.1 | putative GTPase [Synechococcus sp. PCC 7002] >gb\|ACA98948.1\| | 29.1 | 29.1 | 84% | 0.002 | 71% |
| YP_001002846.1 | exodeoxyribonuclease V subunit alpha [Halorhodospira halophila SL1] | 29.1 | 29.1 | 61% | 0.002 | 100% |
| YP_003798630.1 | hypothetical protein NIDE3009 [Candidatus Nitrospira defluvii] >emb\| | 29.1 | 29.1 | 61% | 0.002 | 100% |
| ZP_03681775.1 | hypothetical protein CATMIT_00396 [Catenibacterium mitsuokai DSM | 28.6 | 28.6 | 100% | 0.002 | 69% |
| NP_781225.1 | encapsulation protein capA [Clostridium tetani E88] >gb\|AAO35162.1 | 28.2 | 28.2 | 84% | 0.003 | 73% |
| ZP_02621390.1 | encapsulation protein CapA [Clostridium botulinum C str. Eklund] >gb | 28.2 | 28.2 | 84% | 0.003 | 73% |
| YP_001099887.1 | regulatory protein PII [Herminiimonas arsenicoxydans] >emb\|CAL617 | 27.8 | 27.8 | 76% | 0.004 | 80% |
| ZP_01995862.1 | hypothetical protein DORLON_01857 [Dorea longicatena DSM 13814] | 27.8 | 27.8 | 84% | 0.005 | 73% |
| ZP_08849797.1 | hypothetical protein HMPREF9457_01506 [Dorea formicigenerans 4_6 | 27.8 | 27.8 | 84% | 0.005 | 73% |
| ZP_02235401.1 | hypothetical protein DORFOR_02287 [Dorea formicigenerans ATCC 27 | 27.8 | 27.8 | 84% | 0.005 | 73% |
| ZP_10403944.1 | Protein of unknown function, DUF481 [Thiovulum sp. ES] >gb\|EJF07 | 27.4 | 27.4 | 92% | 0.006 | 75% |
| ZP_04753410.1 | hypothetical protein AM305_09136 [Actinobacillus minor NM305] >gb | 27.4 | 27.4 | 69% | 0.006 | 78% |
| ZP_10065416.1 | hypothetical protein DOK_12586 [gamma proteobacterium BDW918] | 26.9 | 26.9 | 76% | 0.008 | 80% |
| ZP_09550326.1 | Capsule synthesis protein, CapA [Caldithrix abyssi DSM 13497] >gb\|E | 26.9 | 26.9 | 84% | 0.008 | 73% |
| YP_004471613.1 | capsule synthesis protein CapA [Thermoanaerobacterium xylanolyticu | 26.9 | 26.9 | 100% | 0.008 | 62% |
| YP_006390662.1 | capsule synthesis protein CapA [Thermoanaerobacterium saccharoly | 26.9 | 26.9 | 100% | 0.008 | 62% |
| YP_003852786.1 | capsule synthesis protein, CapA [Thermoanaerobacterium thermosac | 26.9 | 26.9 | 100% | 0.008 | 62% |
| ZP_03777926.1 | hypothetical protein CLOHYLEM_04980 [Clostridium hylemonae DSM | 26.9 | 26.9 | 84% | 0.008 | 73% |
| YP_001307381.1 | encapsulation protein CapA [Clostridium beijerinckii NCIMB 8052] >gb | 26.9 | 26.9 | 100% | 0.008 | 69% |
| ZP_10363844.1 | sigma54 specific transcriptional regulator, Fis family [Ralstonia sp. PE | 26.9 | 26.9 | 61% | 0.008 | 88% |
| EHT06500.1 | hypothetical protein HMPREF9690_03759 [Klebsiella oxytoca 10-524 | 26.9 | 26.9 | 69% | 0.009 | 78% |
| ZP_08739798.1 | Metal-dependent phosphohydrolase [Vibrio tubiashii ATCC 19109] >g | 26.5 | 26.5 | 61% | 0.010 | 88% |
| ZP_09462497.1 | ArsR family transcriptional regulator [Acetivibrio cellulolyticus CD2] | 26.5 | 26.5 | 53% | 0.010 | 100% |
| EIF02578.1 | metal-dependent phosphohydrolase [Vibrio tubiashii NCIMB 1337 = A | 26.5 | 26.5 | 61% | 0.011 | 88% |
| ZP_06163129.1 | HAD hydrolase, IIB family [Actinomyces sp. oral taxon 848 str. F033 | 26.5 | 26.5 | 76% | 0.011 | 80% |
| YP_004981872.1 | FMN adenylyltransferase / riboflavin kinase [Geobacillus thermoleovo | 26.5 | 26.5 | 53% | 0.011 | 100% |
| YP_003671833.1 | riboflavin biosynthesis protein RibF [Geobacillus sp. C56-T3] >gb\|AD | 26.5 | 26.5 | 53% | 0.011 | 100% |
| YP_003253140.1 | bifunctional riboflavin kinase/FMN adenylyltransferase [Geobacillus sp. | 26.5 | 26.5 | 53% | 0.011 | 100% |

## C) YSLGNF-(X)-F and YS-(X)-G-(X)-F-(X)-F

**Sequences producing significant alignments:**

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident |
|---|---|---|---|---|---|---|
| ZP_03754453.1 | hypothetical protein ROSEINA2194_02878 [Roseburia inulinivorans DS | 29.5 | 29.5 | 100% | 8e-04 | 100% |
| ZP_05092087.1 | hypothetical protein CDSM653_473 [Carboxydibrachium pacificum DS | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08321144.1 | bacterial capsule synthesis protein [Paraprevotella xylaniphila YIT 11 | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| EIY65519.1 | hypothetical protein HMPREF1069_01927 [Bacteroides ovatus CL02T | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08596189.1 | hypothetical protein HMPREF1017_03297 [Bacteroides ovatus 3_8_4 | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_07042073.1 | putative capsule biosynthesis protein [Bacteroides sp. 3_1_23] >gb\| | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_09935600.1 | hypothetical protein BSGG_0987 [Bacteroides sp. D2] >gb\|EFS30287 | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_05416437.1 | CapA domain protein [Bacteroides finegoldii DSM 17565] >gb\|EEX443 | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_02068468.1 | hypothetical protein BACOVA_05484 [Bacteroides ovatus ATCC 8483 | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08449260.1 | bacterial capsule synthesis protein [Capnocytophaga sp. oral taxon | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_005436840.1 | hypothetical protein RGE_20000 [Rubrivivax gelatinosus IL144] >db\| | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_09060610.1 | hypothetical protein HMPREF9469_03647 [Clostridium citroniae WAL- | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08403320.1 | putative poly-gamma-glutamate biosynthesis enzyme [Rubrivivax ber | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_284418.1 | poly-gamma-glutamate biosynthesis enzyme [Dechloromonas aromat | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08503216.1 | Putative poly-gamma-glutamate biosynthesis enzyme [Methyloversa | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08275700.1 | putative poly-gamma-glutamate biosynthesis enzyme [Oxalobacterac | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_006412262.1 | putative enzyme of poly-gamma-glutamate biosynthesis (capsule for | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_293341.1 | putative poly-gamma-glutamate biosynthesis enzyme [Ralstonia eutr | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_003241368.1 | capsule synthesis protein CapA [Paenibacillus sp. Y412MC10] >gb\|AC | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_001875220.1 | putative poly-gamma-glutamate biosynthesis protein [Elusimicrobium | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08278331.1 | bacterial capsule synthesis protein [Paenibacillus sp. HGF5] >gb\|EGG | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_003009665.1 | hypothetical protein [Paenibacillus sp. JDR-2] >gb\|ACS99578.1\| con | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_006433923.1 | putative enzyme of poly-gamma-glutamate biosynthesis (capsule for | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_08470535.1 | hypothetical protein HMPREF9456_02130 [Dysgonomonas mossii DSM | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_09003347.1 | Capsule synthesis protein, CapA [Paenibacillus lactis 154] >gb\|EHB6: | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_003852786.1 | capsule synthesis protein, CapA [Thermoanaerobacterium thermosac | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_003701615.1 | capsule synthesis protein, CapA [Syntrophothermus lipocalidus DSM | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_003190897.1 | putative polyglutamate synthase CapA [Desulfotomaculum acetoxida | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| ZP_07898623.1 | Capsule synthesis protein, CapA [Paenibacillus vortex V453] >gb\|EFL | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| NP_624217.1 | putative enzyme of poly-gamma-glutamate biosynthesis (capsule for | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_958922.1 | poly-gamma-glutamate biosynthesis protein [Marinobacter aquaeolei | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_006233433.1 | hypothetical protein MY9_3645 [Bacillus sp. JS] >gb\|AFI30177.1\| hy | 29.5 | 29.5 | 100% | 9e-04 | 100% |
| YP_004879149.1 | capsule biosynthesis protein CapA [Bacillus subtilis subsp. spizizenii T | 29.5 | 29.5 | 100% | 9e-04 | 100% |

## Appendix III - Searching of other cap protein homologue in Listeria genome (Please refer to p.172)

### A) CapB Protein

1 MKNIKIVRIL KHDEAIRIEH RISELYSDEF GVVYAGNHLI FNWYQRLYLS RNILISKKSK

61 SRKGLIQMIF IIGICTVFLI IYGIWEQRCH QKRLNSIPIR VNINGIRGKS TVTRLITGVV

121 QEAKYKTVGK TTGTSARMIY WFTDEEQPIK RRKEGPNIGE QRRVVKEAAD LEAEALICEC

181 MAVQPDYQII FQNKMIQANV GVIVNVLEDH MDVMGPTLDE VAEAFTATIP YNGHLVTIES

241 EYLDYFKEVA EERNTKVIVA DNSRISEEFL RKFDYMVFPD NASLALAVAE ALGIDEETAF

301 RGMLNAHPDP GAMRITRFAD QSKPAFFVNG FAANDPSSTL RIWERVDDFG YSNLAPIVIM

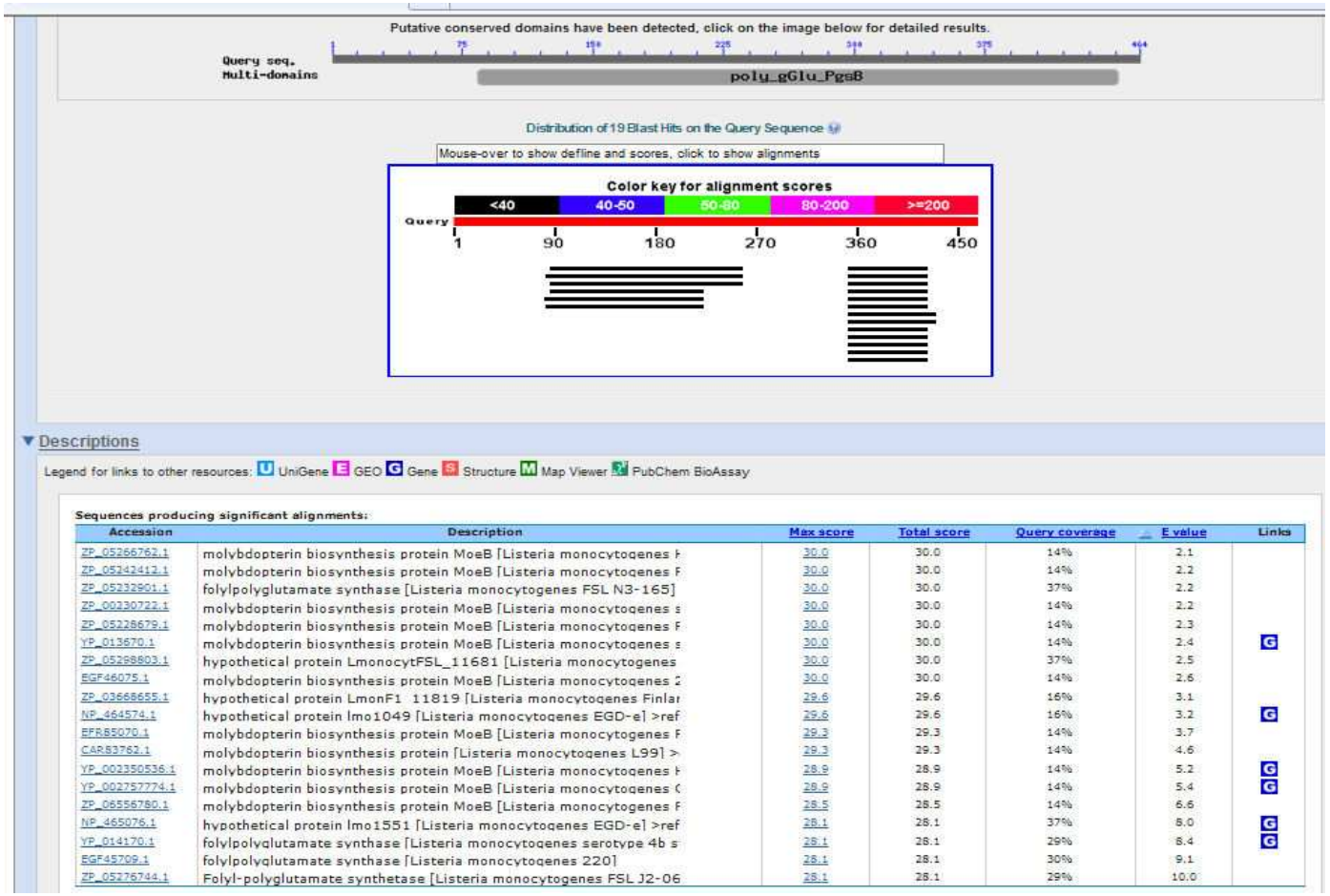
361 NCRPDRVDRT EQFARDVLPY IKAEIVIAIG ETTAPITSAF EKGDIPTQEY WNLEGWSTSE

421 IMSRMRPYLK NRIVYGVGNI HGAAEPLIDM IMEEQIGKKQ AKVI

ATGAAAAACATAAAAATTGTAAGAATATTGAAACATGATGAGGCAATACGCATTGAACATAGGATTTCAG
AATTATACTCAGATGAATTCGGTGTTGTATATGCAGGGAACCACCTAATTTTTAATTGGTATCAACGACT
CTACTTAAGTCGAAATATCTTAATAAGCAAGAAATCGAAAAGCAGGAAGGGATTAATACAGATGATCTTC
ATAATAGGTATATGTACAGTGTTTTTGATTATTTATGGTATATGGGAACAACGTTGCCATCAGAAAAGGC
TCAATTCTATCCCAATTCGAGTAAACATAAATGGAATTCGAGGTAAATCTACCGTTACAAGACTAATTAC
AGGTGTTGTACAAGAAGCGAAATATAAGACTGTAGGGAAAACAACTGGTACATCTGCGCGAATGATATAT
TGGTTTACTGACGAGGAGCAACCGATTAAGCGCCGTAAAGAAGGTCCTAATATCGGTGAGCAACGCAGGG
TAGTTAAAGAGGCTGCTGATTTAGAAGCAGAAGCACTTATTTGTGAATGTATGGCAGTTCAACCCGATTA
TCAAATTATCTTCCAAAATAAAATGATTCAAGCAAATGTTGGAGTGATTGTAAATGTTTTAGAAGATCAT
ATGGATGTTATGGGACCTACACTTGACGAAGTAGCTGAAGCTTTCACTGCTACCATTCCATATAATGGAC
ATTTAGTCACTATTGAAAGTGAATACTTGGATTACTTTAAAGAGGTTGCAGAAGAGAGAAATACAAAAGT
GATTGTTGCGGATAATTCTAGAATTTCAGAAGAATTCTTACGAAAATTTGATTACATGGTCTTCCCAGAT
AATGCATCGCTTGCTTTAGCGGTAGCAGAGGCTCTTGGGATTGATGAGGAAACAGCATTCCGTGGTATGT
TGAATGCTCATCCGGATCCAGGAGCAATGAGAATTACACGTTTTGCTGACCAATCTAAGCCTGCGTTCTT
CGTAAATGGTTTTGCAGCGAATGATCCCTCATCAACATTACGTATTTGGGAACGTGTGGATGATTTTGGA
TATAGTAATCTAGCTCCAATTGTAATTATGAATTGCCGCCCTGACCGCGTTGATCGTACTGAGCAGTTTG
CTAGGGATGTTTTGCCATATATTAAAGCGGAAATAGTTATTGCGATTGGAGAAACGACTGCACCTATTAC
AAGTGCTTTTGAAAAAGGAGATATTCCAACGCAAGAGTATTGGAACTTAGAAGGCTGGTCAACAAGTGAA
ATTATGTCTCGTATGCGTCCATATTTAAAAAATCGGATTGTATATGGAGTGGGTAATATTCATGGTGCAG
CTGAGCCATTAATCGATATGATTATGGAAGAACAAATTGGCAAAAAGCAAGCAAAAGTGATTTAA

The DNA and protein sequence of *Bacillus anthracis* CapB (str. A0248) was downloaded from NCBI online gene bank.

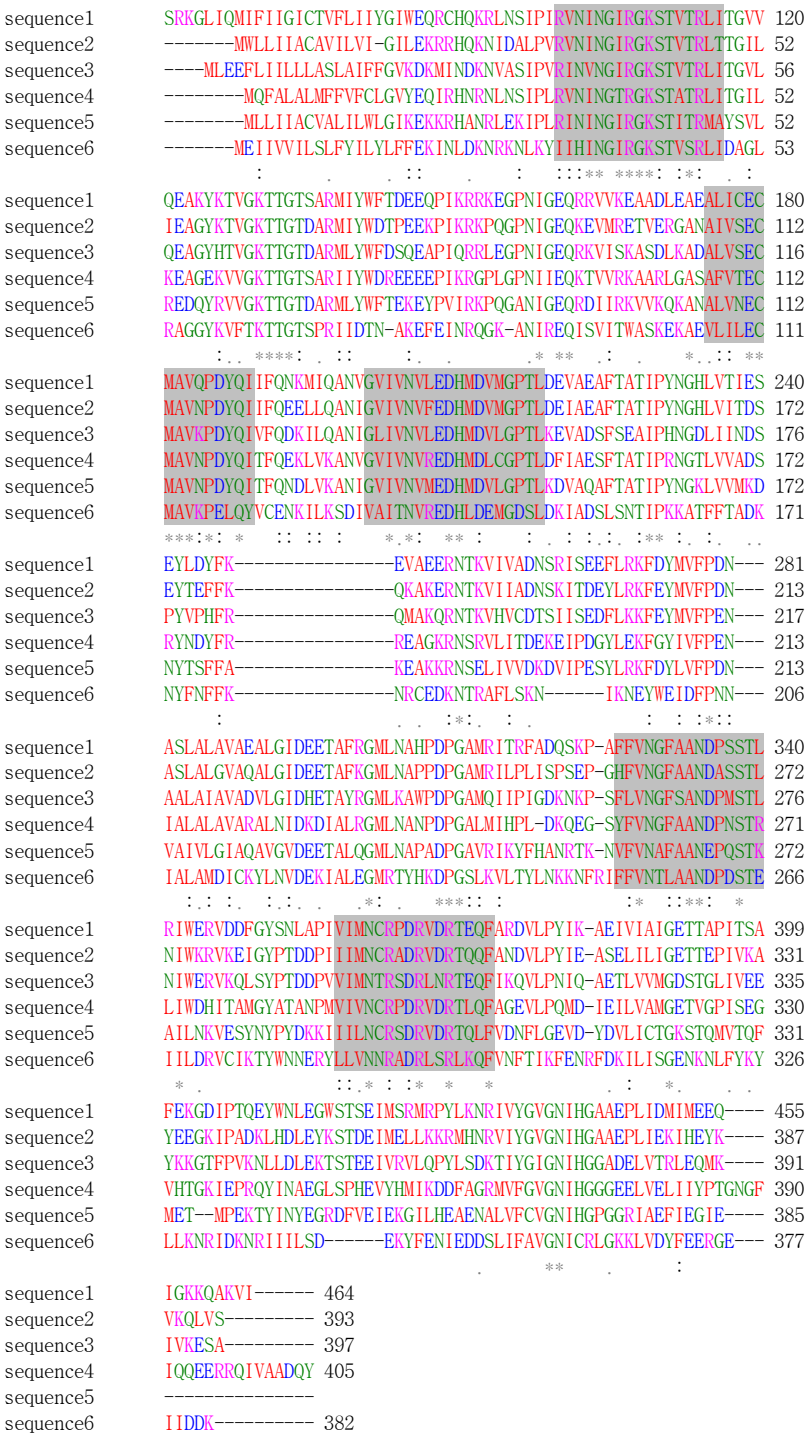
http://www.ncbi.nlm.nih.gov/gene/7847607




**BLAST results of *B. anthracis* CapB in *L.monocytogenes* EGD genome**

BLASTp results of *B. anthracis* CapB in *L. monocytogenes* EGD genome using NCBI website BLAST software. Blast results only gave very low score hits indicating that no similar proteins were present in *Listeria*.

## Multiple sequence alignment of various capB proteins




Alignment were generated using ClustalW2software with capB Proteins from *1)Bacillus anthracis 2)Bacillus subtilis 3)Oceanobacillus iheyensis 4) Staphylococcus epidermidis 5) Fusobacterium nucleatum 6) Desulfitobacterium hafniense*. Conserved sequences (in grey boxes) were Blast in Listeria genome for possible homologues.

## B) CapC Protein

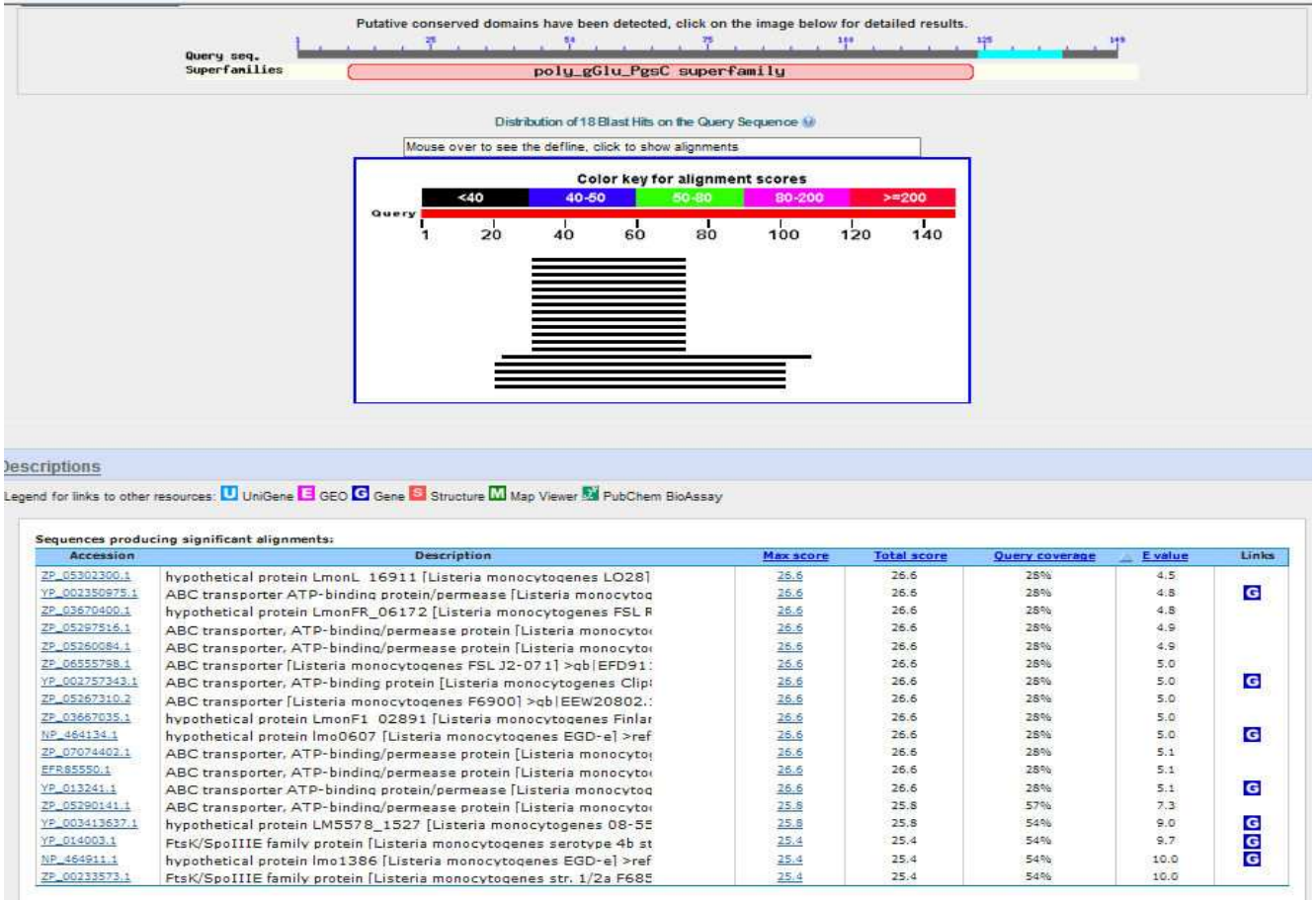
### *Bacillus anthracis* CapC protein and DNA sequences

```
  1 MFGSDLYIAL VLGVTLSLIF TERTGILPAG LVVPGYLALV FNQPVFMLVV LFISILTYVI
 61 VTYGVSRFMI LYGRRKFAAT LITGICLKLL FDYCYPVMPF EIFEFRGIGV IVPGLIANTI
121 QRQGLPLTIG TTILLSGATF AIMNIYYLF
```

```
ATGTTTGGATCAGATTTATATATTGCATTAGTATTAGGAGTTACACTGAGCCTTATTTTTACAGAAAGAA
CAGGTATTTTACCTGCAGGTTTAGTTGTACCTGGTTATTTAGCACTCGTTTTTAATCAGCCCGTATTTAT
GTTGGTTGTTTTATTTATCAGTATTTTAACATATGTAATCGTTACGTATGGTGTTTCAAGATTCATGATT
TTATATGGCCGTAGAAAATTTGCGGCAACGCTAATTACAGGTATTTGTTTAAAACTTTTATTTGATTATT
GTTATCCTGTTATGCCATTTGAGATTTTTGAATTCCGTGGTATTGGAGTTATTGTTCCAGGATTAATTGC
AAATACAATTCAAAGACAAGGGTTACCATTAACAATTGGAACTACAATTTTGTTAAGTGGTGCAACATTT
GCAATCATGAATATTTATTACTTATTTTAA
```

The DNA and protein sequence of *Bacillus anthracis* CapC (str. A0248) was downloaded from NCBI online gene bank.

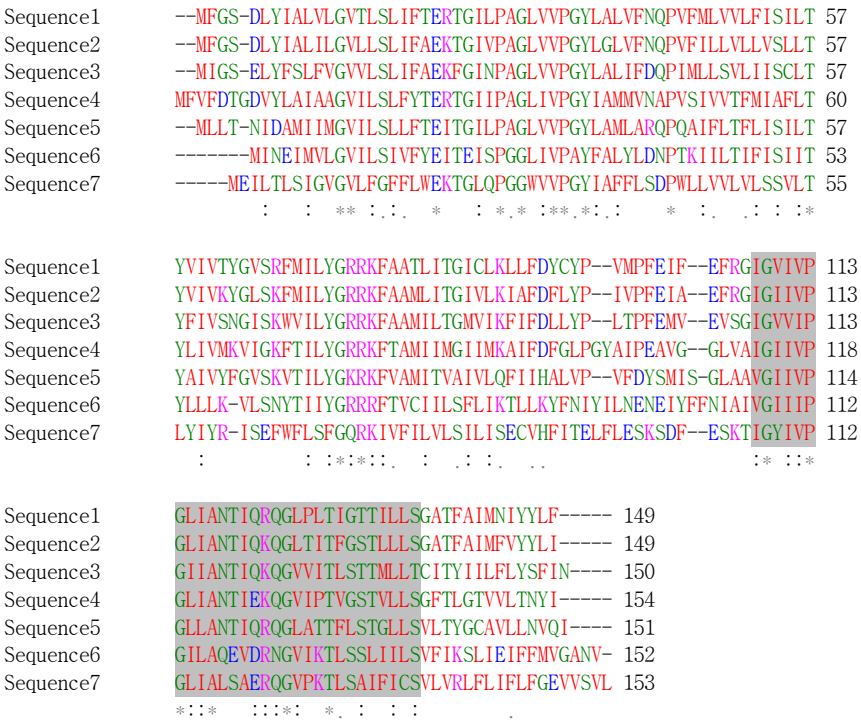
http://www.ncbi.nlm.nih.gov/gene/7847528




**BLAST results of *B. anthracis* CapC in *L.monocytogenes* EGD genome**

BLASTp results of *B. anthracis* CapC in *L. monocytogenes* EGD genome using NCBI website BLAST software. Blast results only gave very low score hits indicating that no similar proteins were present in *Listeria*.

**Multiple alignment of capC protein from various bacteria**




Alignment were generated using ClustalW2 software with CapC proteins from *1)Bacillus anthracis 2)Bacillus subtilis 3)Staphylococcus epidermidis 4)Oceanobacillus iheyensis 5)Desulfitobacterium hafniense 6)Fusobacterium nucleatum 7)Leptospira interrogans*. Conserved sequences (in grey boxes) were Blast in Listeria genome for possible homologues.

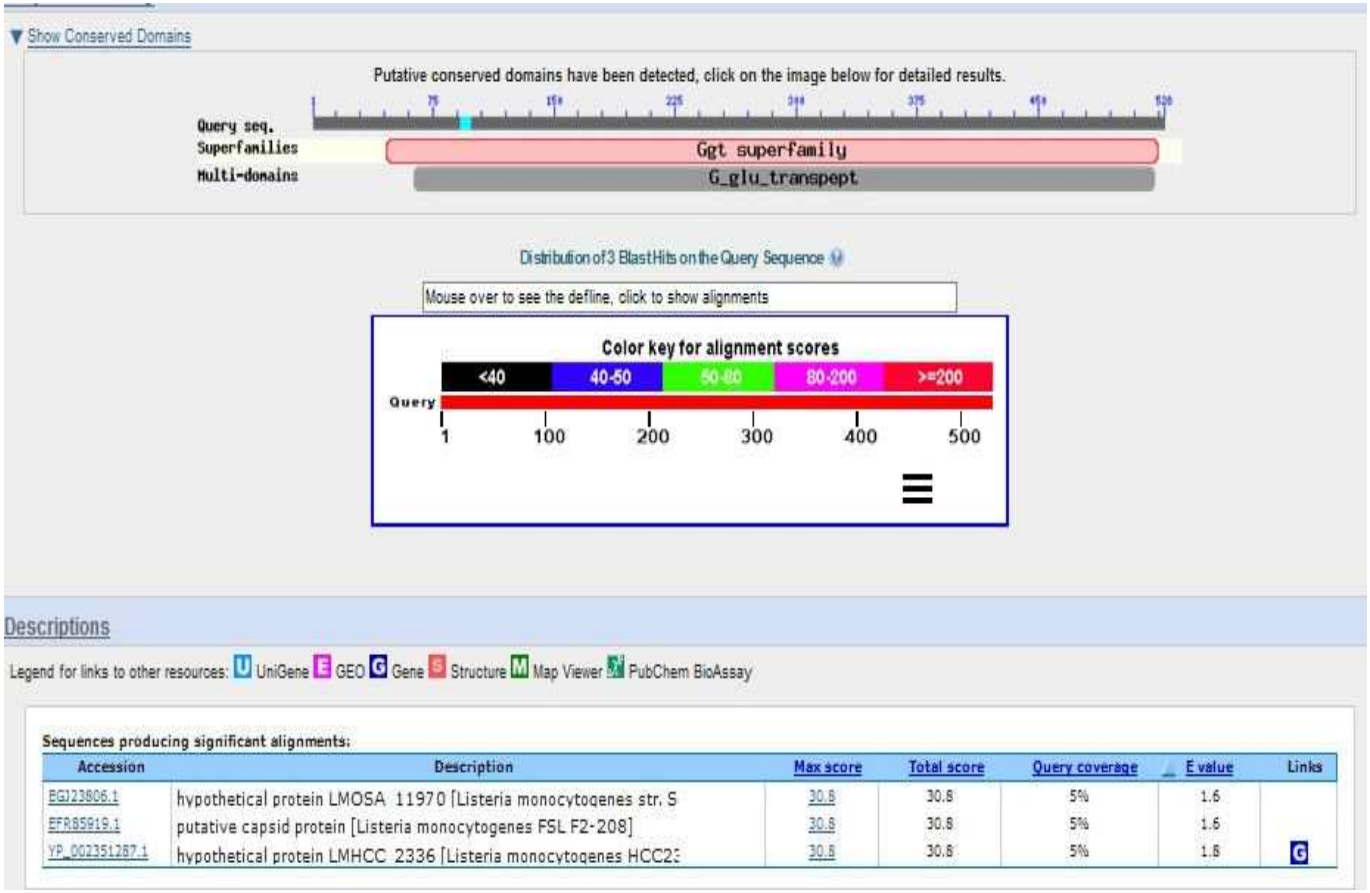
## C) CapD protein

```
  1 MNSFKWGKKI ILFCLIVSLM GGIGVSCSFN KIKDSVKQKI DSMGDKGTYG VSASHPLAVE
 61 EGMKVLKNGG SAVDAAIVVS YVLGVVELHA SGIGGGGGML IISKDKETFI DYRETTPYFT
121 GNQKPHIGVP GFVAGMEYIH DNYGSLPMGE LLQPAINYAE KGFKVDDSLT MRLDLAKPRI
181 YSDKLSIFYP NGEPIETGET LIQTDLARTL KKIQKEGAKG FYEGGVARAI SKTAKISLED
241 IKGYKVEVRK PVKGNYMGYD VYTAPPPFSG VTLLQMLKLA EKKEVYKDVD HTATYMSKME
301 EISRIAYQDR KKNLGDPNYV NMDPNKMVSD KYISTMKNEN GDALSEAEHE STTHFVIIDR
361 DGTVVSSTNT LSNFFGTGKY TAGFFLNNQL QNFGSEGFNS YEPGKRSRTF MAPTVLKKDG
421 ETIGIGSPGG NRIPQILTPI LDKYTHGKGS LQDIINEYRF TFEKNTAYTE IQLSSEVKNE
481 LSRKGLNVKK KVSPAFFGGV QALIKDERDN VITGAGDGRR NGTWKSNK
```

```
TTGAATTCCTTTAAATGGGGAAAGAAGATAATTCTTTTCTGTTTGATAGTCAGCTTAATGGGGGGTATCG
GGGTATCCTGTTCTTTCAATAAAATAAAAGACAGTGTTAAGCAAAAAATTGATAGTATGGGTGATAAAGG
AACTTATGGAGTGAGTGCCTCTCACCCCCTTGCGGTTGAGGAAGGTATGAAAGTATTAAAGAACGGTGGA
AGTGCAGTAGATGCAGCGATTGTGGTCTCATATGTTTTAGGCGTTGTAGAACTGCATGCCTCAGGAATAG
GTGGGGGCGGTGGAATGCTCATTATATCTAAAGATAAAGAAACCTTTATTGATTATCGTGAAACAACTCC
GTACTTTACAGGAAACCAAAAGCCACATATTGGAGTACCCGGATTTGTGGCTGGAATGGAGTATATTCAT
GATAATTATGGTTCATTACCGATGGGTGAGTTATTACAACCAGCCATTAATTATGCGGAAAAAGGGTTCA
AGGTAGATGATTCCTTAACAATGCGATTAGACCTTGCGAAGCCACGTATTTATTCTGATAAGCTAAGTAT
CTTCTATCCGAATGGTGAACCTATTGAAACTGGAGAAACACTTATCCAGACAGATTTAGCGAGAACCTTA
AAGAAGATTCAAAAAGAAGGGGCTAAAGGCTTTTATGAAGGAGGAGTCGCTAGGGCAATCAGTAAAACTG
CAAAAATATCGTTAGAAGATATAAAAGGATATAAAGTAGAGGTACGTAAACCAGTAAAAGGTAACTACAT
GGGATATGATGTTTATACCGCTCCACCACCTTTTTCAGGAGTTACTTTATTACAAATGTTGAAATTAGCT
GAAAAGAAAGAAGTATATAAAGATGTAGATCATACGGCAACTTATATGTCTAAAATGGAAGAGATTTCAA
GGATTGCCTATCAAGATAGAAAGAAAAACCTAGGGGATCCAAATTACGTTAATATGGATCCAAATAAAAT
GGTGAGTGACAAATATATATCAACAATGAAGAATGAGAATGGTGATGCGCTTTCGGAAGCAGAGCATGAA
AGCACAACGCATTTTGTTATCATTGATAGAGATGGAACGGTTGTCTCTTCAACTAATACACTAAGCAATT
TCTTTGGAACAGGAAAGTACACAGCAGGGTTCTTCTTAAATAATCAATTGCAGAACTTTGGAAGTGAGGG
ATTTAATAGTTATGAACCTGGTAAACGTTCACGAACGTTTATGGCCCCCACTGTATTAAAGAAAGATGGG
GAAACGATCGGCATTGGGTCACCAGGTGGTAACCGTATTCCGCAAATTTTAACCCCAATATTGGATAAAT
ATACGCATGGTAAGGGTAGCTTGCAAGACATTATCAATGAATACCGTTTTACTTTTGAAAAAAATACAGC
GTATACAGAGATTCAGCTAAGTTCAGAAGTGAAAAATGAGTTATCTAGAAAAGGATTGAACGTAAAGAAG
AAAGTATCCCCTGCCTTTTTTGGTGGGGTACAGGCCTTAATTAAAGACGAGAGAGATAATGTTATCACCG
GCGCTGGAGATGGCAGAAGAAATGGAACTTGGAAATCAAATAAATAG
```

The DNA and protein sequence of *Bacillus anthracis* CapD (str. A0248) was downloaded from NCBI online gene bank.

http://www.ncbi.nlm.nih.gov/gene/7847571




**BLAST results of *B.anthracis* CapB in *L.monocytogenes* EGD genome**

BLASTp results of *B. anthracis* CapD in *L. monocytogenes* EGD genome using NCBI website BLAST software. Blast results only gave very low score hits indicating that no similar proteins were present in *Listeria*.

**Multiply alignment of capD from various bacteria**


Alignment were generated using ClustalW2 software with CapD proteins from *1) Bacillus anthracis 2) Oceanobacillus iheyensis 3) Bacillus subtilis 4) Leptospira interrogans5) Desulfitobacterium hafniense 6)Staphylococcus epidermidis.* Conserved sequences (in grey boxes) were Blast in Listeria genome for possible homologues.

**D) Cap E protein**

***Bacillus anthracis* polyglutamate capsule biosynthesis protein CapE**

1 MVKKVFGWIM PILIVGLLLV TMGTFKRSET LTTDEQKKIS DYLQANP

ATGGTTAAAAAAGTTTTTGGATGGATTATGCCGATTTTAATTGTAGGTTTATTACTTGTAACAATGGGGA
CCTTTAAACGTTCGGAAACATTAACGACTGATGAGCAGAAGAAGATTAGTGATTATCTACAGGCTAACC
CCTAA

The DNA and protein sequence of *Bacillus anthracis* CapE (str. A0248) was downloaded from NCBI online gene bank.

http://www.ncbi.nlm.nih.gov/gene/7847606

**BLAST results of *B. anthracis* CapB in *L.monocytogenes* EGD genome**


BLASTp results of *B. anthracis* CapE in *L. monocytogenes* EGD genome using NCBI website BLAST software. Blast results show no similar proteins were present in *Listeria*.

**Multiply alignment of capE proteins from various bacteria**


Alignment were generated using ClustalW2 software with CapE proteins from *1) Bacillus anthracis 2) Bacillus thuringiensis 3) Bacillus cereus 4) Bacillus licheniformis.*

**Appendix IV - Blasting of *Bacillus cereus* CapA against Listeria homologues (Please refer to p.173)**

A)

```
 Score =  363 bits (933),  Expect = 3e-105, Method: Compositional matrix adjust.
 Identities = 184/372 (49%), Positives = 239/372 (64%), Gaps = 7/372 (2%)

Query  1    MKTLLKRFLLI--AFCITPIVVLINHSFTS-KAKDKPDFQNKSSKTASTSEKKIENPEIT  57
            MK+  K  +L+     I  I +L+N+  T+ K   KP  +  ++
Sbjct  1    MKSRKKGIILVLSVILIFSIGLLVNNLMTNNKDTAKPKKKTVAAVKKKKETPPKPKEPFN  60

Query  58   LTFSGDTMFDWQLRPVIEKNGADYPFQHVKEEITKADISFVNLESAFTTREKKAPGQLFW  117
            + F+GD MFDW LRPV+ + G DYPF +V+EE+  +D +FV+LE+A TTR KK P Q FW
Sbjct  61   IDFTGDIMFDWDLRPVLAEKGMDYPFNNVREELKSSDYTFVDLETAITTRTKKVPYQEFW  120

Query  118  IKSDPSTLQAIKNTGYDIVNIGNNHTLDYGQDGLLDTISHVEKLKFPYIGAGKNAKDAYT  177
            IKSDPS+L A+KN G D+VNI NNH LDY +DGLLDT + +      Y+GAGKN  +AY
Sbjct  121  IKSDPSSLTALKNAGVDMVNISNNHILDYYEDGLLDTTAALRANNLAYVGAGKNEDEAYQ  180

Query  178  AREMTVKGKKFKFLSFVRFMPDTNWVAGDNKPGVANGYDLNLVTKTIKEQK---KDADYL  234
             +   +KG K  F+SF  F P+T W+A ++ PGV NGYDLNLV + IKE++   KD DY+
Sbjct  181  LKVADIKGNKVGFMSFCHFFPNTGWIADEDTPGVTNGYDLNLVEEKIKEERAKNKDIDYM  240

Query  235  IVYMHWGVEKSNRPVEYQKQYVPKMVEAG-ADAIVGSHPHWLQGFEYYNKVPIAYSLGNF  293
            +VY HWGVEK+N PV+YQ QYV K+V+    DAIV SHPHWLQGFE Y  VPIAYSLGNF
Sbjct  241  VVYFHWGVEKTNTPVDYQTQYVKKLVDDNLVDAIVASHPHWLQGFEVYKDVPIAYSLGNF  300

Query  294  LFPSYVNGKSAETGVLTLTFKGKDVQMSFNPYIIRNNQVSPVNEEEKRKALQYLQTISTD  353
            LFP YV+G SAETG+  L F    V   F+P II  NQ++ +    K   L YLQ+IS +
Sbjct  301  LFPDYVSGHSAETGIYKLNFDQGKVTAHFDPGIISGNQINMLEGSSKTAQLNYLQSISPN  360

Query  354  VDIDDTGNIKNK  365
              I+  G+I  K
Sbjct  361  ATINSNGDISAK  372
```

B)

```
 Score =  125 bits (315),  Expect = 2e-33, Method: Compositional matrix adjust
 Identities = 96/305 (31%), Positives = 146/305 (48%), Gaps = 36/305 (12%)

Query  44   ASTSEKKI-ENPEITLTFSGDTMF--------DWQLRPVIEKNGAD--YPFQHVKEEITK  92
            A+T+++ + E   +TLT  GD  F          +   V +KN  +  Y +++
Sbjct  149  AATNQQGLKEKGVVTLTMVGDNSFGTYPETPEHLKFDNVFQKNNGNNTYVYKNCLPWFKS  208

Query  93   ADISFVNLESAFTTREKKAPGQLFWIKSDPSTLQAIKNTGYDIVNIGNNHTLDYGQDGLL  152
             D + +N ESAFT    KA  +++ IKSDP+ +  +  +G D  N+ NNHT+DY Q G
Sbjct  209  DDYTIINAESAFT-NATKAENKMWRIKSDPAHVAFLPASGVDAANLANNHTMDYFQVGYD  267

Query  153  DTISHVEKLKFPYIGAGKNAKDAYTAREMTVKGKKFKFLSFVRFMPDTNWVAGDNKPGVA  212
            DT+   ++   P   A     DA    E T+KG K   L +   M           P
Sbjct  268  DTLKAFKENNIPVFNA-----DA--PLETTIKGMKTVLLGYDCRM-------SQQSPAY-  312

Query  213  NGYDLNLVTKTIKEQKKDADYLIVYMHWGVEKSNRPVEYQKQYVPKMVEAGADAIVGSHP  272
                L  + K +K+ KK+   +IV MHWGVE    P +YQ Q+   +++AGAD I+GSHP
Sbjct  313  ----LERIVKDVKKYKKEDTLVIVNMHWGVEYRETPTDYQTQFGHAILDAGADIIMGSHP  368

Query  273  HWLQGFEYYNKVPIAYSLGNFLFPSYVNGKSAETGVLTLTFKGKDVQMSFNPYIIRNNQV  332
            H L+  E Y    I YS+G+F F +     S  T +  L F  +D     N  ++++  +
Sbjct  369  HRLESVEKYKDKYIVYSMGDFAFGADPTLLSRMTSMFQLRFTKED-----NKIVLKDISI  423

Query  333  SPVNE  337
              P  E
Sbjct  424  VPTYE  428
```

Aligned Blastp result of *B. cereus* CapA against A) lmo0017 and B) lmo0516 in *Listeria* EGD from the NCBI online Blast software.
(http://blast.ncbi.nlm.nih.gov/Blast.cgi)

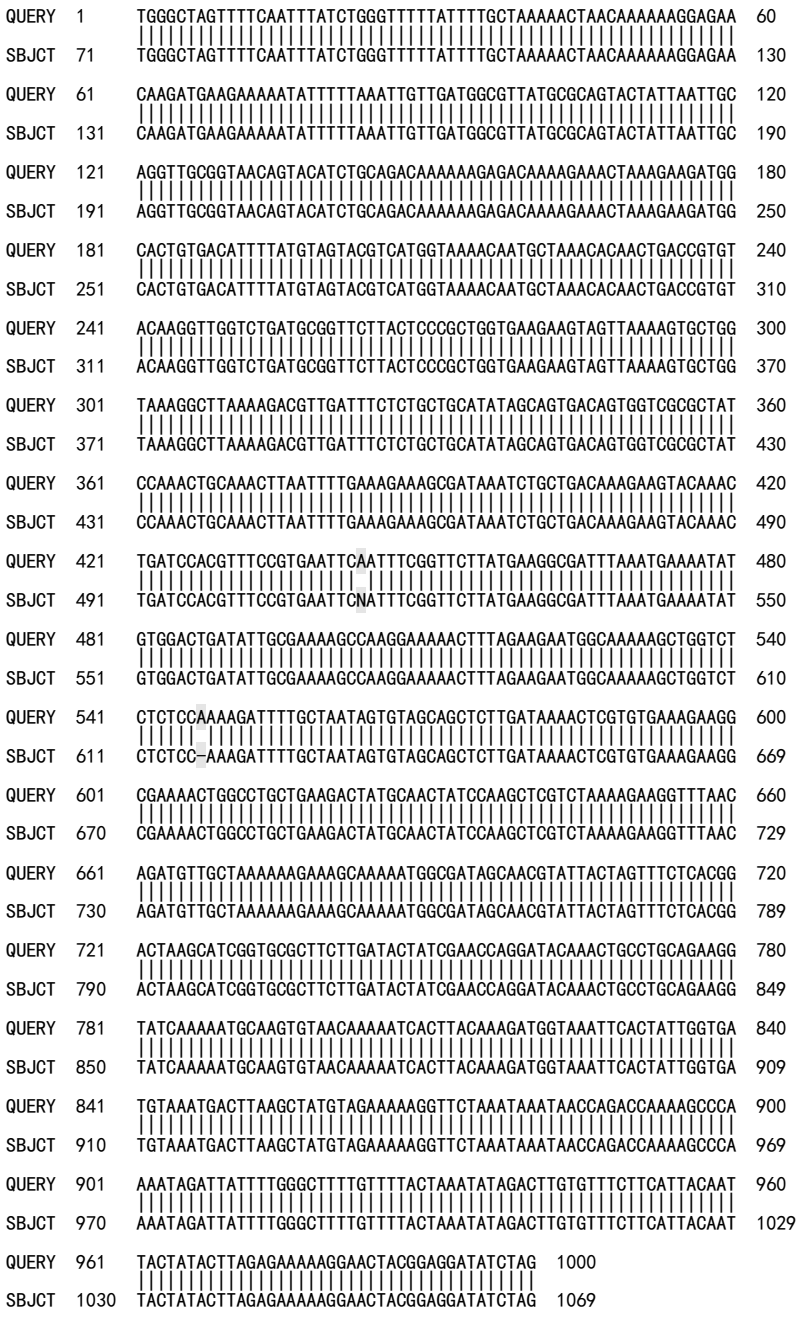
## Appendix V - Plasmid extracts sequencing results (Please refer to p.210)

## A) lmo0017 and pLMO0017up

```
QUERY  1     CGATAGACTTCCAGACATCTTTTGGATTACAAGTCATTGGATATGTGTTACCCGCTGCAA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  78    CGATAGACTTCCAGACATCTTTTGGATTACAAGTCATTGGATATGTGTTACCCGCTGCAA  137

QUERY  61    GCATACAACCGATTTGATTTTCAGGATTGATTTCATGACCAAGTTTAGTTGCAAGTGCTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  138   GCATACAACCGATTTGATTTTCAGGATTGATTTCATGACCAAGTTTAGTTGCAAGTGCTG  197

QUERY  121   ATGCAACTAATTGATGATGAGCAGCTTGATATTTAACTTCCTCTGGATTATCCTCTTTAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  198   ATGCAACTAATTGATGATGAGCAGCTTGATATTTAACTTCCTCTGGATTATCCTCTTTAG  257

QUERY  181   TAACGTCTAAACCTCCACCAATGTATGGAAGATGAAGAATCATGTTAATTTCATTAAATG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  258   TAACGTCTAAACCTCCACCAATGTATGGAAGATGAAGAATCATGTTAATTTCATTAAATG  317

QUERY  241   TCATCCAATATTTTACTTTATCTTTATAACGCGTGAAAATTGCTTCACAGAAATTCAAGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  318   TCATCCAATATTTTACTTTATCTTTATAACGCGTGAAAATTGCTTCACAGAAATTCAAGT  377

QUERY  301   AAAAGTCGATGCATTTACGATTTTTCCAACCACCATATTTTTTGAAAACTTCTAGCGGAG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  378   AAAAGTCGATGCATTTACGATTTTTCCAACCACCATATTTTTTGAAAACTTCTAGCGGAG  437

QUERY  361   TATCAAAATGGTTGATTGTAACAACTGGTTCGATACCATATTTGTGACATTCATCAAAAA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  438   TATCAAAATGGTTGATTGTAACAACTGGTTCGATACCATATTTGTGACATTCATCAAAAA  497

QUERY  421   CTGCATCATAAAATGCTAAACCTTTTTCATTTGGTGTCGTTTCATCACCATTCGGGAAAA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  498   CTGCATCATAAAATGCTAAACCTTTTTCATTTGGTGTCGTTTCATCACCATTCGGGAAAA  557

QUERY  481   TACGTGGCCAACTGATGGACATACGGAAACATTTGAATCCCATTTCAGCCATTAATTTAA  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  558   TACGTGGCCAACTGATGGACATACGGAAACATTTGAATCCCATTTCAGCCATTAATTTAA  617

QUERY  541   TGTCTTCTTTATAACGATGATAAAAATCAATTGATTCGTGACTTGGGTAAAAACCATAAT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  618   TGTCTTCTTTATAACGATGATAAAAATCAATTGATTCGTGACTTGGGTAAAAACCATAAT  677

QUERY  601   CTGTAGCAAGCGCTTTCGATGGATTGAATAAAGCTTCCCAACGTCCGTCCTCTACTGTTG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  678   CTGTAGCAAGCGCTTTCGATGGATTGAATAAAGCTTCCCAACGTCCGTCCTCTACTGTTG  737

QUERY  661   GAAGGATATCAACAAGTGAAAGTCCTTTACCGTCTTCAAGATAAGCTCCTTCACATTGGT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  738   GAAGGATATCAACAAGTGAAAGTCCTTTACCGTCTTCAAGATAAGCTCCTTCACATTGGT  797

QUERY  721   TGGCAGCAACTGCTCCGCCCCATAAAAAGTCTTTAGGAAATTTTGATTCTGTCATTTTTG  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  798   TGGCAGCAACTGCTCCGCCCCATAAAAAGTCTTTAGGAAATTTTGATTCTGTCATTTTTG  857

QUERY  781   TATATCTCCTCTCACGAATATAATTTACTTTTCCACTATAGAATATGTAATGCATTACAT  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  858   TATATCTCCTCTCACGAATATAATTTACTTTTCCACTATAGAATATGTAATGCATTACAT  917

QUERY  841   AGCAAGCAATAATTTCAATTTTTTTAAATTCGTCTTCCATTTTATGATTTTAAAGTTCAC  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  918   AGCAAGCAATAATTTCAATTTTTTTAAATTCGTCTTCCATTTTATGATTTTAAAGTTCAC  977

QUERY  901   AATTTAGCCATTAAAATCCATAAAAAAGTCTATTTTCGATAAAAACCTTTTTCTCCATAC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
SBJCT  978   AATTTAGCCATTAAAATCCATAAAAAAGTCTATTTTCGATAAAAACCTTTTTCTCCATAC  1037

QUERY  961   TGATAGAATTAGATAACTTTTTAATTTAGGAGGAGAAAAA  1000
             |||||||||||||||||||||||||||||||||||||||||
SBJCT  1038  TGATAGAATTAGATAACTTTTTAATTTAGGAGGAGAAAAA  1077
```

Aligned Blastp result of pLMO0017up Plasmid extract sequencing output against original lmo0017 in *Listeria* EGD strain from the NCBI online Blast software. (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

## B) lmo0516 and pLMO0516up




Aligned Blastp result of pLMO0516up Plasmid extract sequencing output against original lmo0516 in *Listeria* EGD strain from the NCBI online Blast software. (http://blast.ncbi.nlm.nih.gov/Blast.cgi)