Identification and Characterisation of an Old Yellow Enzyme (OYE) - NamA from *Listeria monocytogenes*

by

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Abstract

The food-borne pathogene *Listeria monocytogenes* has been considered a significant threat to human health worldwide. It mainly infects individuals suffering insuffecint immunity such as pregnant women. During pregnancy, *L. monocytogenes* is capable of causing a serious damage to the mother and the fetus. It can spread to different organs including the placenta via adaptation to interacellular lifestyle. To maintain pregnancy, the levels of the hormones progesterone and β-estradiol increase and reduction in hormone levels was proposed to be associated with fetal death and abortion. The objectives of this project therefore were to investigate the role of pregnancy hormones on the growth and virulence of *L. monocytogenes*, and to identify bacterial genes with possible roles in binding to pregnancy hormones. It was obsereved that the growth of *L. monocytogenes* in the presence of progesterone under anaerobic condition was affected by the action of the hormone and the effect was dose/time-dependent of exposure as increasing concentrations showed greater effect on the bacterial growth. Interestingly, bacterial growth was restored within 24 h of exposure to the hormone. In parallel, a Tn*917*-LTV3 insertion library was constructed and a number of mutants isolated that had reduced growth in the presence of β-estradiol were identified. However, reduction in growth was not microbiologically significant. Furthermore, bioinformatics analysis was performed to identify listerial genes with possible role in hormones degradation. It was observed that *L. monocytogenes* encodes for a protein that is possibly involved in steroid degradation; therefore, gene expression and a clear-deletion mutant were performed to test this hypothesis. This revealed no significant role of this protein in the growth restoration observed in the presence of progesterone. Also, the deleted gene was investigated of its ability to reduce NADPH in the presence of a possible substrate (progesterone, β-estradiol). This showed that this gene could possess an enzymatic activity toward pregnancy hormones. An attempt to purify this protein for further investigation was performed and protein expression in a soluble form was unsuccessful. The findings presented in this thesis represent an important view when considering the relation between pregnancy hormones and *L. monocytogenes*; however, further investigations of hormone-degrading proteins from *L. monocytogenes* are needed. This knowledge may form the basis of a therapy to protect pregnant individuals.

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Table of Contents

List of Figures

List of Tables

List of abbreviation

Abbrev.	Meaning	Abbrev.	Meaning
°C	Degree Celsius	LPS	lipopolysaccharide
µM	Micromolar	M	molar
amp	ampicillin	min	minute
ATP	adenosine triphosphate	ml	millilitre
BCFA	Branched Chain Fatty Acids	NADPH	Nicotinamide adenine dinucleotide phosphate
BHI	Brain Heart Infusion	NCBI	National Center for Biotechnology Information
BLAST	Basic local Alignment Search Tool	neo	neomycin
ble	bleomycin	NK	Natural killer cells
bp	base pair	NO	Nitric oxide
cat	chloramphenicol	OD	Optical density
CBP	Corticosteroid-Binding Protein	ORF	Open reading frame
CFU	Colony Forming Unit	OYE	Old yellow enzyme
CNS	Central Nervous System	PBP	Progesterone binding protein
DMSO	Dimethyl Sulfoxide	PBS	Phosphate buffer saline
DNA	Deoxyribonucleic acid	PCR	Polymerase chain reaction
DTT	Dithiothreitol	PDI	disulfide isomerase
EBP	Estrogen binding protein	pH	the negative log of hydrogen ion concentration
EDTA	Ethylenediaminetetraacetic acid	pM	Picomolar
erm	erythromycin	RNA	Ribonucleic acid
EVT	extravillous trophoblasts	RPM	Round per minute
h	hour	S	second
HCG	human chorionic gonadotropin	SDS-PAGE	Polyacrylamide gel electrophoresis
HSV	Herpes simplex virus	SYN	syncytiotrophoblast
IPTG	Isopropyl-β-D-thiogalactopyranoside	tet	tetracycline

kan	kanamycin	TLCC	Taylor Laboratory Culture Collection
kb	Kilo base	TLRs	Toll-like receptors
kDa	Kilo dalton	TNF	Tumor necrosis factor
LB	Luria-Bertani medium	TSB	Trypton soy broth
lin	lincomycin	v	volt
LLO	listeriolysin O	WT	Wild type
Log	Logarithmic phase	LHI	Hyperladder I

Chapter I

Introduction

1.1 Background

Listeria monocytogenes is a food-borne pathogen that can cause several clinical complications in humans including gastroenteritis, septicaemia, meningitis, abortion and foetal death in pregnant individuals (Farber *et al*., 1991). As the name indicates, the disease listeriosis has its name from *Listeria monocytogenes* that causes a high mortality rate, which can reach up to 30% of reported cases (Braun *et al*., 2000). *L. monocytogenes* mainly affects immunocompromised individuals such as the elderly, those undergoing immunotherapy and also pregnant individuals (Barbuddhe *et al*., 2008). It has been reported that as high as 60% of all listeriosis cases involve pregnant women with an age of less than 40 years old (Wing *et al*., 2002). Pregnancy factors are believed to play a crucial role in increasing the risk of listeriosis among pregnant individuals ~20-fold more than non-pregnant healthy adults, and this may be partially explained by the immunosuppression effects that permit fetoplacental-allograft tolerance (Sperandio *et al*., 2002). *L. monocytogenes* has adapted to survive in several environments from soil, water and food to mammalian intestine and cellular compartments making it a difficult pathogen to eradicate (Seveau *et al*., 2007).

Listeria infection usually begins with the ingestion of contaminated food and results in various symptoms that are similar in humans and animals (Figure 1.1). Thus, it has been recognized as a significant public health problem worldwide (Oevermann *et al*., 2010). Listeriosis can also lead to serious health complications including foetal death. In addition, pregnant individuals are advised not to eat certain types of food including soft cheese, unpasteurised milk products and ready to eat food since these types of food can harbour a very low number of *Listeria* microbes and they can still grow at very low temperature (refer to section 1.6 for details) which can cause huge problems in the food industry (Delgado *et al*., 2008). Aditionally, infections caused by *L. monocytogenes* have been reported in humans as well as a wide variety of animals, such as rodents, fish, birds, sheep and cattles (Gray *et al*., 1966).

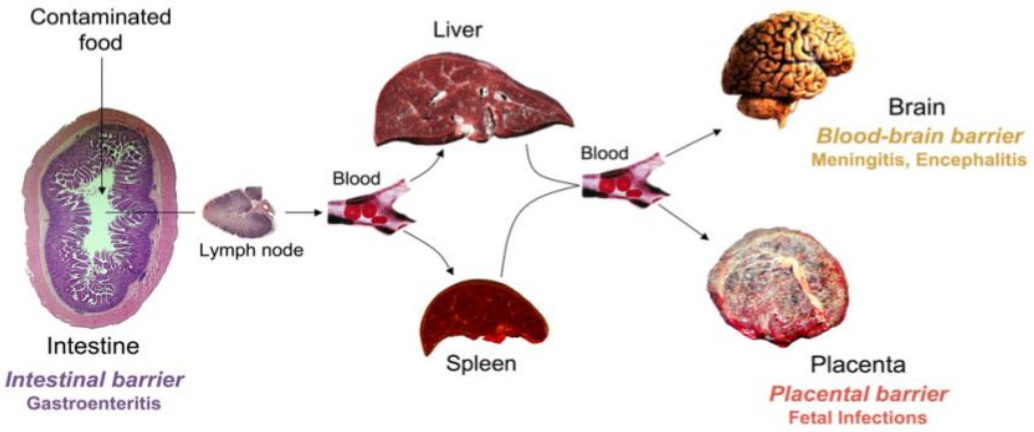


Figure 1.1 Routes for *L. monocytoegenes* transmission inside human body (from Lecuit 2007).

Fifteen *Listeria* species were identified; all have very close genetic similarity based on a phylogenetic analysis that used 16s rRNA and amino acid sequences of conserved loci (refer to figure 1.11 for illustration). Most species are recognised as non-pathogenic species including *L. marthii, L. innocua, L. gray, L. fleischmannii, L. aquatic, L. floridensis, L. rocourtiae, L. weihenstephanensis, L. cornellensis, L. grandensis* and *L. riparia* (den Bakker *et al*., 2014), whereas *L. seeligeri and L. welshimeri* have rarely been reported to have caused human infection. *L. ivanovii* has been recently reported to have caused human listeriosis (Guillet *et al*., 2010), leaving *L. monocytogenes* as the most infectious species that infects both animals and humans, especially of those who have less functional immune response.

Previous case review series showed that *L.monocytogenes* was the causative agent of approximately 10-20% of stillbirth or abortion in pregnant women in a study conducted on 722 listeriosis-reported cases, whereas 68% of the cases developed foetal infection. In addition, 62.8% of the infected-foetuses recovered while 12.7% suffered long-term sequelae and the rest of the infected neonates died (Mylonakis 2002).

1.2 Placenta and its role during pregnancy

The placenta is the organ that links the foetus to the maternal uterus during pregnancy and supports the normal growth of the foetus. It functions at maximum efficiency to keep the blood supply for the foetus separate from the maternal blood as well as many other essential functions (Gude *et al*., 2004). Food and oxygen pass into the placenta from the maternal blood supply where they are carried to the foetus via the umbilical cord. Waste products from the foetus are carried back along the umbilical cord to the mother bloodstream via the placenta. Moreover, the placenta helps to protect the foetus against microbial infections. However, several pathogens including bacteria, viruses and parasites are known to have the ability to cross the placental barrier and infect the foetus (Arce *et al*., 2009). This can result in very serious complications such as abortion, birth defects, brain damage, deafness and foetal death (Anderson 2007). In addition to the placental important functions, it is considered to be the main source of the hormone progesterone during middle and late stages of gestation where progesterone plays a crucial role in maintaining pregnancy.

In an experimental study that examined listerial infection in pregnant goats, it was demonstrated that listerial infection resulting in spontaneous abortion was accompanied by a drop in levels of progesterone (Engeland *et al*., 1997). Whether this reduction was a direct result of *Listeria* infection or bacterial-induced damaged tissues was not examined and warrants further investigation.

1.3 Placental infection

Placental infection is considered the major cause of maternal and fetal disease in humans, and is accounted for a major source of human morbidity and mortality (Beck *et al*., 2010). Pathogens trafficking from mother to foetus – vertical transmission- can occur at two sites of direct contact between maternal cells and the trophoblasts at the maternal-foetus interface in human placenta: Firstly, maternal blood surrounded specialised fetus syncytiotrophoblast (SYN) cells and secondly, the extravillous trophoblast in

the site of uterine implantation. Syncytiotrophoblast is known as the first line of defence against pathogens invasion and abortion mediated by inflammation may possibly be an advantage for the infected mother (Robbins *et al*., 2010).

Placental infection is considered the leading cause for an approximate number of 12.9 million cases of preterm delivery that occur annually worldwide (Beck *et al*., 2010). Many of these infants die before or shortly after delivery and others suffer from long-term neurological sequelae and health problems (Saigal *et al*., 2008). To date, the mechanism of how pathogens breach the placental barrier and colonise the placental cells is largely obscure. As an outcome of various intensive studies to elucidate the main routes for placental breaching, invasion of the cells can happen by ascending the genital tract, and most importantly through vertical transmission (Robbins *et al*., 2010). Although the overall immunity is compromised during pregnancy (Mor, 2010), relatively small number of pathogens causes foetal infection (Table1.1), and even for these invasive pathogens, colonising maternal cells cannot guarantee infection of the fetus (Robbins and Bakardjiev 2012).

The placenta is an organ that constitutes of tissues derived from both the mother and the fetus (Robbins *et al*., 2010). Its major tasks are to provide nutrients from maternal tissue to a growing embryo, gas exchange and protect the foetus from maternal immunity as well as infectious agents. In hemochorial placentas, fetal extraembryonic epithelial cells and/or trophoblasts highly invade the uterine and cause uterine endometrium (decidua) and maternal spinal arteries structure rearrangement (Robbins *et al*., 2010). Thus, creating an extensive network of foetal capillaries, which in turn form another interface where circulating maternal blood comes in direct contact with foetal cells for molecular exchange (Robbins *et al*., 2010). Moreover, almost all mammalian maternal-foetal interfaces exhibit a continuous layer of syncytiotrophoblast (SYN) that is bathed with circulating maternal blood (refere to figure 1.2 for placental structure). These multinucleated trophoblasts are suggested to facilitate protection of the placenta against invasive pathogens (Crocker *et al*., 2004). Several studies showed that SYN cells play a crucial role in resistance to infection by diverse pathogens including Cytomegalovirus (Fisher *et al*., 2000), herpes simplex virus (Koi *et al*., 2002) and the parasite *Toxoplasma*

gondii (Robbins *et al.,* 2012) and most importantly, resistance to colonisation by *Listeria monocytogenes* bacteria (Robbins *et al*., 2010). In general, microbes use different mechanisms to colonise host cells and several pathogens exhibit intracellular lifestyle to evade host immunity; for example, *L. monocytogenes* enters host epithelial cells and spreads from cell to cell intracellularly without exposing to the extracellular compartments (Lecuit *et al*., 2004). It binds host cells upon specific interactions between listerial invasive proteins and host-cell transmembrane proteins (E-cadherin) (refer to section 1.8.1 for detsils) that are expressed on the basolateral surface of mononuclear cells (Lecuit *et al*., 2004). Crucially though, expression of E-cadherin in the human placenta is controversial. One study conducted by Lecuit and colleagues (2004), suggested that extracellular invasion of the human placenta by *L. monocytogenes* occurs via interaction of listerial InIA with E-cadherin on the surface of SYN. However, other studies failed to prove that E-cadherin is expressed on these cells (Robbins *et al*., 2010). Importantly, most pathogens capable of breaching the intestinal barrier use receptors that are important components in host cell to cell junctions and by deliberately avoiding expression of such junction, the SYN may reduce multiple pathogens breach as well as excluding maternal leukocytes (Robbins *et al*., 2010).

The second layer of defence in the placenta is found where extravillous trophoblasts (EVT) invade the uterus. EVT cells function in a similar way to NK cells and macrophages to serve as a protection mechanism against pathogens. These cells have innate host defence and play important role to exclude invading pathogens (Mor, 2010). Also, trophoblasts express TLRs as well as antiviral factors, which induce apoptosis upon breaching of pathogens (Mor, 2010). Further evidence has shown that EVT innate defence highly restricted *L. monocytogenes* to escape the primary vacuole killing mechanism as compared to other epithelial cells (Robbins *et al*., 2010), as well as impaired growth of the parasite *Toxoplasma gondii* (Robbins *et al.,* 2012).

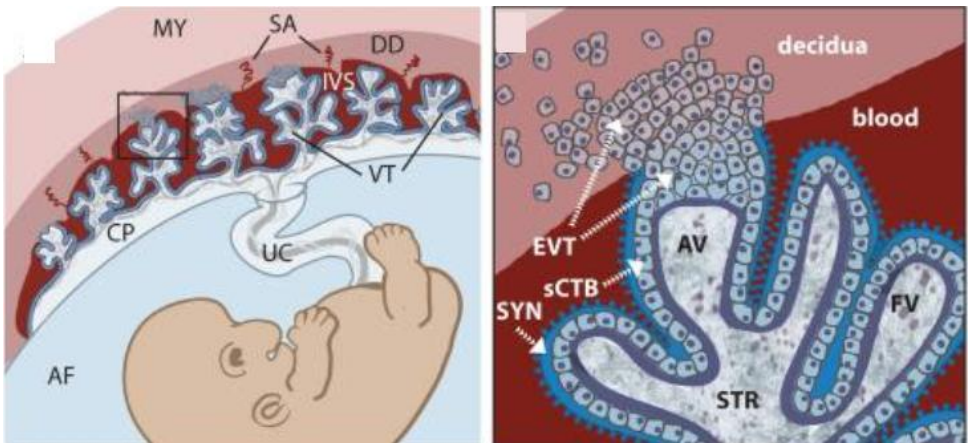


Figure 1.2 Structure of the placenta and fetus in uterus. MY: myometrium, SA: spiral arteries, DD: deciduas, IVS: interavillous space filled with maternal blood, VT: villous tree, CP: chronic plate, UC: umbilical cord, AF: amniotic fluid, AV: maternal blood surrounds the villous tree, FV: floating villi, SYN: syncytiotrophoblast, sCTB: subsyncytial cytotrophoblast, STR: stroma, EVT: extravillous cytotrophoblast. (Adopted from Robbins *et al*., 2010).

One possibility of how pathogens invade and colonise placental trophoblasts is by damaging and degrading of the SYN as shown by *Plasmodium falciparum*, the causative agent of malaria (Crocker *et al*., 2004). *P. falciparum* infects erythrocytes, thus resulting in accumulation of erythrocyte-membrane proteins that bind antigens in the intervillous space (Fried and Duffy, 1996). Another example can be seen with SYN detachment and apoptosis due to increased titers of *Trypanosoma cruzi* (Duaso *et al*., 2001). It was also proved that cell enzymatic damage results in an increased placental colonisation by *L. monocytogenes* and *T. gondii* (Robbins et al., 2010). In addition, experimental evidence shows that uterine-trophoblast interface is the most vulnerable site of pathogens entry in pregnant mice including *L. monocytogenes* (Le Monnier *et al*., 2007). In guinea pig, placental invasion and colonisation by *L. monocytogenes* can occur either by cell-to-cell spread or by direct invasion, and mutants incapable of spreading intracellularely are also incapable to trespass the placental barrier (Robbins et al., 2010).

Table 1.1 Several pathogens known of their capabilities to breach the placental barrier and cause diverse infection (Robbins *et al*., 2012a)

Pathogen	Type	Route of infection	Lifestyle
Brucella spp.	Bacteria	Ingestion	Intracellular
Coxiella burnetii	Bacteria	Inhalation	Intracellular
Listeria monocytogenes	Bacteria	Ingestion	Intracellular, Extracellular
Mycobacterium tuberculosis	Bacteria	Airborne	Intracellular
Treponema pallidum	Bacteria	Sexual	Extracellular
Leishmania spp.	Parasite	Vector	Intracellular, Extracellular
Plasmodium falciparum	Parasite	Vector	Intracellular
Toxoplasma gondii	Parasite	Ingestion	Intracellular
Trypanosoma spp.	Parasite	Vector	Intracellular, Extracellular
Cytomegalovirus	Virus	Droplet	Intracellular
Lymphocytic choriomeningitis virus	Virus	Ingestion, Inhalation	Intracellular
Parvovirus B19	Virus	Droplet	Intracellular
Rubella virus	Virus	Droplet	Intracellular
Varicella zoster virus	Virus	Airborne	Intracellular

Several pathogens are known to cross the placental barrier and infect the foetus in both humans and animals. All of the haematogenous placental pathogens have at least partially intracellular life style (Zeldovich *et al*., 2013). Most of bovine-diagnosed abortions are attributed to a variety of infectious pathogens including protozoa, viruses, fungus and bacteria, where 4.1% of the diagnosed abortions in cattle are linked to *Listeria* infection (Anderson, 2007). The following examples of microbes with the ability to invade human placenta share an intracellular life style within the infected-host cells.

1.3.1 Toxoplasma gondii

T. gondii is a parasitic protozoa belonging to the genus *Toxoplasma*. It is the cause of the disease toxoplasmosis that poses a high risk to pregnant individuals. Cats are the definitive host of the parasite that can be transferred to almost all warm-blooded animals including humans (Many *et al*., 2006). As for humans, the biggest concern is transmission of the parasite from an infected mother to the foetus. Previous studies showed that one third to one half of infants born to *T. gondii*- infected mothers were also infected (Dean *et al*., 2006). T. Gondi exhibits an intracellular life style within host-infected cells to escape exposure to host immunity, thus it has become an excellent model to examine intracellular parasitism (Sibley, 2003). During pregnancy, infection is usually asympotomatic however there is around 40% chance that the infants will become infected, which can result in stillbirth or abortion. Around 10% of infected infants are born with severe diseases (Many *et al*., 2006). In pregnant women, *T. gondii* crosses the placental barrier to gain access to core tissues by very sophisticated entry mechanism that differs from any other microorganism invasion. It actively penetrates target cells in a parasite myosin- and actin- dependent manner (Carruthers, 2002). Barragan and colleagues have also demonstrated that *T. gondii* has the ability to cross the placental barrier via active motility exploited by the parasite, which can result in dissemination within human tissues (Barragan *et al*., 2002).

1.3.2 Campylobacter jejuni

Members of the genus *Campylobacter* have been pointed to have association with spontaneous abortion, stillbirth and premature delivery in humans (Simor *et al*., 1986). *C. jejuni* is commonly found in untreated water and the intestinal tracts of animals. *C. jejuni* is the cause of the disease campylobacteriosis and causes watery diarrhea in infected persons accompanied by fever and headache (Dean *et al*., 2006). This pathogen thrives in environments with reduced oxygen and is easily inhibited by salt, acid, drying and eliminated by heat (Denton *et al*., 1992). In human intestine, *C. jejuni* penetrate the mucus and colonises the intestinal crypts in an efficient manner. Post colonisation, C*. jejuni* can cross the mucosal barrier and invade intestinal cells. It has evolved multiple mechanisms to gain access to host cells. The most efficient route is by migrating underneath cultured cells and invasion occurs from the basal cell side instead of the apical side. Once within the infected cell, it resides within a membrane-bound compartment called (CCV) - *Campylobacter* Containing Vacule and exhibits an intracellular life style (Bouwman *et al*., 2013). In addition, this microbe is able to infect the foetus in pregnant women by transmission through the placental unit and that results in foetal loss, preterm delivery or stillbirth. In previous study, *C. jejuni* was found in cow-placenta lesions and foetal tissues post abortion (Donkeersgoed *et al*., 1990). However, mechanisms underlying invasion by *C. jejuni* is poorly understood.

1.3.3 Salmonella enterica

Serovars of *S. enterica* cause serious infections worldwide. Although the common serotypes related to infection are Typhi, Enteritidis and Typhimurium, *S. Dublin* has been linked to the majority of reported abortion cases where bacterial infection is assumed to disseminate from the intestinal tract. Organ invasion by *Salmonella* can result in placental infection, leading to destruction of foetal vili that may lead to foetal loss without foetal infection (Anderson, 2007). In general, abortions caused by *Salmonella* and *Listeria* are often similar in that the infection can spread to the placenta via intracellular life

style, which lead to massive destruction of the foetal-liver cells, septicaemia and death (Anderson, 2007).

1.4 Influence of pregnancy hormones on immunity

Several pregnancy factors are thought to play an important role in compromising immunity during pregnancy. For example, the steroid hormone progesterone that is mainly produced by the corpus luteum and granulosa cells is considered a key factor to establish and maintain pregnancy. In addition to its pivotal role, several studies have shown that progesterone has an immune suppressive effect in mammals, which in turn, suppresses the production of the pro-inflammatory cytokines IL-1 and TNF-α (Su *et al*., 2009).

In general, when a microbe invades human cells, Toll-like receptors (TLRs) come into play where they recognise microbial molecules, and activate innate immune cells to secrete type1 interferon and pro-inflammatory cytokines for initial host defence. In 2009, Su and colleagues showed that the administration of progesterone that corresponded to peripheral blood levels seen during pregnancy resulted in immune suppressive effects, where IL-6 production was inhibited, along with NO secretions from macrophages. Therefore progesterone has a significant regulatory effect on maternal immunity during pregnancy, which results in suppression of pro-inflammatory cytokine production and TLR expression (Su *et al*., 2009). On the other hand, β-estradiol plays a key role by affecting epithelial cells of the reproductive tract including β-estradiol -enhanced thickening of the epithelium (smith *et al*., 2004). As consequence, pathogens are unable to access thickened cells (Kaushic *et al*., 2011). Hormone-enhanced changes can also play a pivotal role in reducing immune effectiveness against genital infections, because key cells in the defence line have been reduced as a result of hormonal fluctuation. For example, increased levels of progesterone during the luteal phase of the menstrual cycle leads to decreased levels of both IgG and IgA in cervical tissues (Kutteh *et al*., 1998). In addition, regulation of human immune responses by β-estradiol has been well studied. Changes in β-estradiol level can have either an anti-inflammatory or pro-inflammatory influence on

cytokine production and macrophage induction and migration (Zang *et al*., 2002). Low levels of β-estradiol induced antibody production in ovariectomized rats, while increased levels of the same hormone decreased inflammatory T cell migration to the infection site (Straub, 2007).

1.5 Production of pregnancy hormones and formation from cholesterol

The corpus luteum is one of the few endocrine glands that form from the remains of another organ. It is formed from granulosa cells, theca cells, capillaries and fibroblast. The wall of the follicle collapses into a folded structure, which is a characteristic of the corpus luteum. The main function of this gland is to produce progesterone. The formation, maintanence, regression and steroidogenesis of the corpus luteum are among the most significant and closely regulated events in mammalian reproduction. During pregnancy, the fate of the corpus luteum is affected by the ovarian, pituitary and placental regulators. The corpus luteum undergoes a process of regression, which leads to its disappearance from the ovary permitting a new cycle (Stocco *et al*., 2013).

Cholestrol is the main precursor of five main classes of steroid hormones including progesterone, glucocorticoids, mineralocorticoids, androgen and estrogen. The major site for synthesis of these classes of hormones are the corpus luteum; progesterone, ovaries; estrogen, testicals; androgen, and adrenal cortex for glucocorticoids and mineralocorticoids. The first stage in hormones synthesis from its precursor is the removal of 6-carbon unit from cholesterol side chain to form pregnenolone (figure 1.3). Cholesterol side chain is hydroxylated at C-20 and C-22, and the bond connecting these atoms is cleaved by the action of the enzyme desmolase. This step requires the oxidation of 3 NADPH and 3 O_2 molecules. The hormone corticotropin, whic is synthesised by the anterior pituitary gland, stimulate the conversion of cholesterol into pregnenolone, the precursor of all steroid hormones. The formation of progesterone from pregnelonone occurs in two steps: (i) the 3-hydroxyl group of pregnenolone is oxidised to 3-keto group, (ii) followed by isomerisation of the bond between C-4 and C-5 to a double bond.

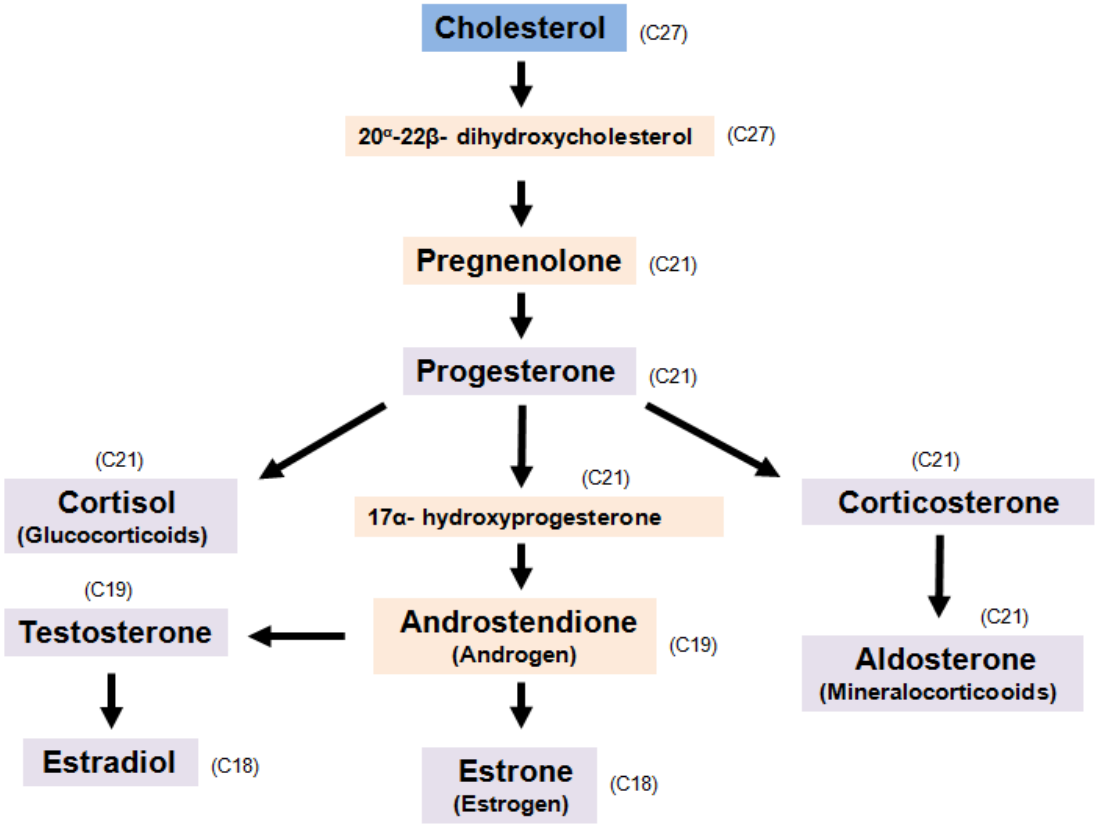


Figure 1.3 Pathways for the formation of steroid hormones from its precursor

Cortisol is synthesised from progesterone by hydroxylation at C-17, C-21 and C-11. Hydroxylation at C-17 must occur before C-21, wheras C-11 can be hydroxylated at any stage. Hydroxylation of progesterone at C-21 yields deoxycorticosterone that is hydroxylated at C-11 to form aldosterone post C-18 oxidation of the angular methyl group to an aldehyde. Progesterone plays an intermediate role to synthesise androgens and estrogen from pregnenolone. The synthesis starts with the hydroxylation of progesterone at C-17. The side chain that consists of C-20 and C-21 is then cleaved to form androstenedione, an androgen. The other androgen, testosterone is synthesised by the reduction of 17-keto group of androstenedione, which can be reduced by the action of 5α-reductase to yield dihydrotestosterone (DHT). Estrogen is formed from androgen by the loss of the C-19 angular methyl group and formation of an aromatic A-ring, by aromatase. Estrone, an

estrogen, is derived from androstenedione, while estradiol is derived from testosterone (Breg *et al*., 2002).

1.6 *Listeria monocytogenes* - organism and characteristics

L. monocytogenes was first discovered by E.G.D. Murray in 1926 and named after the British surgeon Joseph Lister. Before Harvey Pirie changed the genus name to *Listeria* in 1940, it was known as *Bacterium monocytogenes*. It was first isolated from infected guinea pigs and rabbits (Murray *et al*., 1926). Pirie and colleagues isolated a *Listeria*-similar organism from ill gerbil's liver in 1930 and named it *Listerella hepatolytica*. Because the name '*Listerella*' had been previously used for another group of moulds, scientists finally agreed the name *Listeria monocytogenes* (Pirie, 1940).

For many years, *Listeria* isolates were a laboratory rarity, and the incidence of the disease was an unresolved mystery. Nevertheless, the number of reported listeriosis cases started to increase between late 70s and beginning of 80s. In 1979, the first human listeriosis outbreak was reported, where the incidence was directly linked to contaminated food consumption. From that date onward, a series of human listeriosis outbreaks led the disease to be the leading cause of food-borne problems worldwide (Farber *et al*., 1991).

L. monocytogenes is a Gram positive rod-shaped facultative anaerobic bacterium which occurs naturally in soil, sewage, silage and stream water (Cairns *et al*., 2009). *Listeria* bacilli have been isolated from as many as 37 species of mammals and 17 species of birds (Gray and Killinger, 1996). The organisms are resistant to freezing, drying and heat with the ability to persist on food processing surfaces. *L. monocytogenes* are usually arranged in short chains and may appear coccoid on slide smears thus can easily be mistaken for *Streptococci. L. monocytogenes* is motile by flagella that are produced at room temperature but not at 37°C (Grundling *et al*., 2004). Instead, the bacteria exploit host-cell actin filaments to propel themselves intracellularly and between the cells. *L. monocytogenes* is an aerobic, facultative, intracellular parasite that is non-spore forming (Farber *et al*., 1991). Several diagnostic tests can be used to identify *Listeria* such as Gram stain, hydrolysis

of sodium hippurate and esculin, catalase, motility and alpha-hemolysis, all of which must be positive in addition to H_2S test that must be negative (Curiale *et al*., 1994). *L. monocytogenes* can utilize several carbon sources for energy including glucose-1-phosphate, glucose, fructose and mannose-6-phosphate, plus mediating the import of glucose-6-phosphate from the host-cell cytoplasm into the bacterium. Thereon, these carbon-derived energy sources are important for the rapid replication and bacterial survival within the infected cells (Farber *et al*., 1991).

One important characteristic of *L. monocytogenes* that is critical to its ability to cause human listeriosis is the capacity of the pathogen to divide and grow at wide range of temperature ranging from -0.4 to 45°C (Walker *et al*., 1990). *L. monocytogenes* is considered a psychrotolerant pathogen since its optimum growth temperature is ranging between 30 to 37°C, however, the microbe can still grow at temperatures less than 15°C (Walker *et al*., 1990). In 1984, Gray and colleagues were able to record the ability of *L. monocytogenes* to grow at low temperature for the first time. During that study, *Listeria* isolates from bovine brain suspension were able to grow at 4°C after storage for three months (Lee *et al*., 2013). The ability of *L. monocytogenes* to grow at refrigeration temperature is directly linked to the transmission of this food-borne infection. Many laboratory studies have shown that listerial growth is generally very slow when growing at 4°C and lower, with a doubling time of 12 to 50 h and lag phase of 60 to 477 h (Lee *et al*., 2013). However, Lag phase is considerably reduced as the temperature increases, thus increasing the risk of illness (Lee *et al*., 2013).

In order for *L. monocytogenes* to adapt and grow at refrigeration temperature, it has to overcome several problems that are directly linked to bacterial survival. As the temperature decreases the cell membrane fluidity also decreases, which results in impaired nutrient uptake by the bacteria. Other problems arise from reduced temperature include the ability of *L. monocytogenes* to transcribe DNA which is caused by increased DNA superhelical coiling, enzyme activity reduction, slow protein folding and proper function of ribosome (Bayles *et al*., 1996). The metabolic rate of listerial cells

also decreases as the temperature decreases; therefore, the cells respond to changes by altering the gene expression and changing their membrane composition (Lee *et al*., 2013). *L. monocytogenes* goes through different phases during adaptation to low temperature including initial cold shock, acclimation and cold adapted status. Synthesis of cold shock proteins (CSPs) occurs when reducing temperature and non-cold shock proteins are inhibited. CSPs in general represent a class of proteins, which are characterized by their expression patterns rather than a specific function. Some of the CSPs act as RNA chaperones and facilitate translation initiation. As cells become adapted to low temperature, the number of non-cold shock proteins increases again and CSPs decreases (Chan and Wiedmann, 2009). Reducing the temperature causes changes in lipid membrane fluidity and that significantly affect bacterial nutrient transport such as amino acids and carbohydrate; hence, reducing bacterial ability to grow (Bayles *et al*., 1996). One way that *L. monocytogenes* also uses in order to adapt to low temperature and changing its membrane fluidity is by alteration membrane-incorporated fatty acids (Walker *et al*., 1990). In *L. monocytogenes* grown at optimum temperature, 95% of the cell membrane constitute of branched-chain fatty acids (BCFA). While, during adaptation state, *L. monocytogenes* changes its membrane-incorporated fatty acids profiles so that the number of iso-form fatty acids is reduced while the number of anteiso-form is increased, that changes increase bacterial ability to survive unfavourable temperature (Carrasco *et al*., 2006).

1.7 Listeriosis and prevalence of infection

Listeria is found widely in nature, which is primarily foodborn in domestic animals and birds. Thus, it is considered a wide spread microbe with a characteristic of a common foodborne source. *L. monocytogenes* can be isolated from the stool of 1-10% of the population without apparent illness (Schlech *et al*., 1983). This microbe makes a high concern to food industry since it can survive and grow in harsh conditions such as wide pH range, high salt concentrations and temperatures between 0 and 45ºC (Hibi *et al*., 2006).

L. monocytogenes can contaminate a large variety of food, with variable incubation period (see table 1.2) ranging from 1 to 70 days (Hernandez-Milian

and Payeras-Cifre, 2014). The first reported outbreak of foodborn illness caused by *L. monocytogenes* occurred in Boston in 1979 in a hospital after 23 patients ingested contaminated vegetables prepared within the hospital. Further outbreak occurred in 1981 in Maritime Provinces, Canada due to consumption of contaminated coleslaw, and the isolate was identical to the ones isolated from the infected patients, which suggested a definitive proof of foodborn transmission (Schlech *et al*., 1983). Subsequent listeriosis outbreaks have been documented elsewhere (Farber and Peterkin, 1991).

Table 1.2 Incubation periods for *L. monocytogenes* in different clinical cases.

Reported cases	Incubation period (days)	
	Median	**range**
Pregnancy-associated cases	27.5	17-67
CNS-associated cases	9	1-14
Cases with bacteraemia	2	1-12
Febrile gastrointestinal cases	1	6h - 10

Food contaminated with *L. monocytogenes* may cause a serious health problem among elderly people and individuals with low immunity including those undergoing chemotherapy, HIV patients and pregnant women; the disease is not otherwise correlated with gender, ethnicity or dependence on geographical regions (Cairns *et al*., 2009). *L. monocytogenes* is ubiquitous and is considered to be the leading cause of food-borne death in the United States (Mead *et al*., 1999). Dairy products and ready to eat food have been implicated in outbreaks of listeriosis in Europe (Fretz *et al*., 2010). According to a raw milk-sampling survey, as low as 3-4% of the milk samples contained *Listeria* cells, however, the vast majority of listeriosis was linked to other dairy products (Kazmierczak *et al*., 2001). Sporadic episodes of *L. monocytogenes* in England and Wales showed a sharp increase between 2001-2004 when compared to previous reported cases of non-pregnancy associated listeriosis. Moreover, listeriosis epidemiology in the same regions has increased from 2.1

cases per million people in 1990-2000 to 3.6 cases per million in 2001-2009, which indicates increased occurrence (Mook *et al*., 2011). In addition, reported incidence of listerial infection across Europe ranged from 0 to 7.5 cases per million in 2002 (Goulet *et al*., 2008).

It was previously reported that the number of listeriosis incidence not associated with pregnancy increases among individuals as they become older, and this is correlated with reduction of host immunity. Indeed, the highest number of incidence was observed in the group aged between 70 and 90 years old (CDC, 2012). It was also reported that the number of global reported cases associated with listeiosis, including pregnancy-related cases, had increased between 2004 until 2012 (figure 1.4).

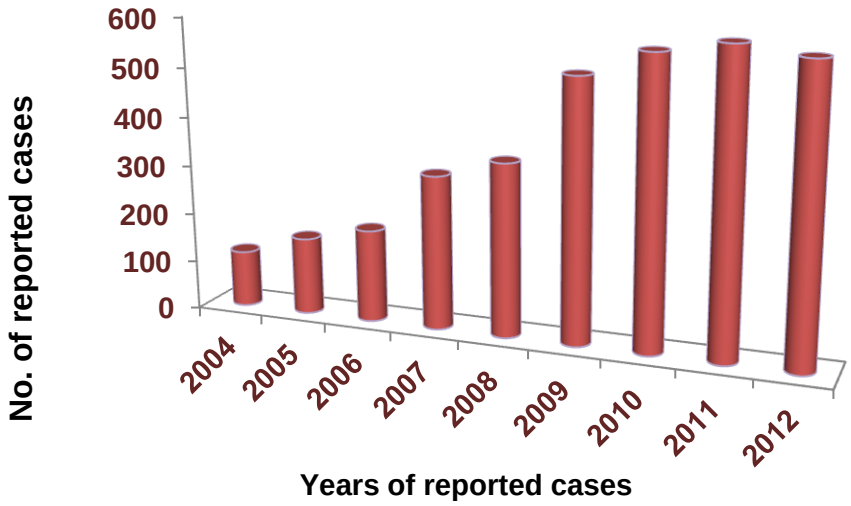


Figure 1.4 National listeriosis surveillance by year (CDC, 2012).

According to CDC (2012) analysis of 74 episodes of pregnancy-associated listeriosis, it was noted that Hispanic ethnicity was more commonly infected (34%) than in patients with invasive listeriosis not associated with pregnancy (11%). In addition, 21% of episodes of pregnancy-associated listeriosis led to foetal death; wheras 4% were reported to have led to death of live-borne infants (see table 1.3).

Table 1.3 Demographic and clinical characteristics of episodes of pregnancy-associated listeriosis cases.

Characteristics	n	%
Age		
Median (range)	28(21-42)	
Ethnicity		
Hispanic	21	34
Non-Hispanic	40	66
Race		
White	45	80
Black or African American	6	11
Asian	4	7
Multiracial	1	2
Source of most invasive isolates		
CSF from neonate	8	11
CSF from neonate and from mother	1	1.5
Blood from both mother and neonate	1	1.5
Blood from neonate	25	34
Blood from mother	32	43
Other product of conception	7	9
Hospitalised		
Mother (n=61)	42	69
Live-borne infants (n=40)	35	88
Pregnancy outcome		
Live birth, infant survived	27	38
Live birth, infant died	3	4
Live birth, unknown infant outcome	18	25
Foetal death	15	21
Still pregnant at time of reports	8	11

It has been reported that the annual incidence of *Listeria*-related infection ranges between 0.1 and 1 case per 100.000 populations. Although listeriosis is less common than other food-borne diseases, *L. monocytogenes* causes up to 19% and 17% of food-borne associated deaths in the USA and France; respectively. Currently, the number of listeriosis incidences has been increased in several countries in Europe, and the increase was suggested to occure due to an increase of populations with age over 60 years old or under 60 years old with predisposing conditions that cause reduced immunity (Goulet *et al*., 2013). It was previously reported that listeriosis incidence rate in

the USA was 0.27 cases per 100.000 populations between 2004 and 2009 (Silk *et al*., 2012), and the incidence had increased to 0.29 cases per 100.000 of populations, and in adults aged 65 years old the incidence increased 1.3 cases per 100.000 persons (CDC, 2013).

In a study conducted in China, listeriosis was reported 479 times between 1964 and 2010, and 82 of them were outbreak-related cases (Feng *et al*., 2013). In 2011, Europian countries provided data on the occurance of listeriosis and the average case reported was 0.31 per 100.000 populations (figure 1.5). The highest rate was noted in Denemark (0.88 cases/100.000 populations), and 57% of the patients were over 65 years old (EU summary report, 2013).

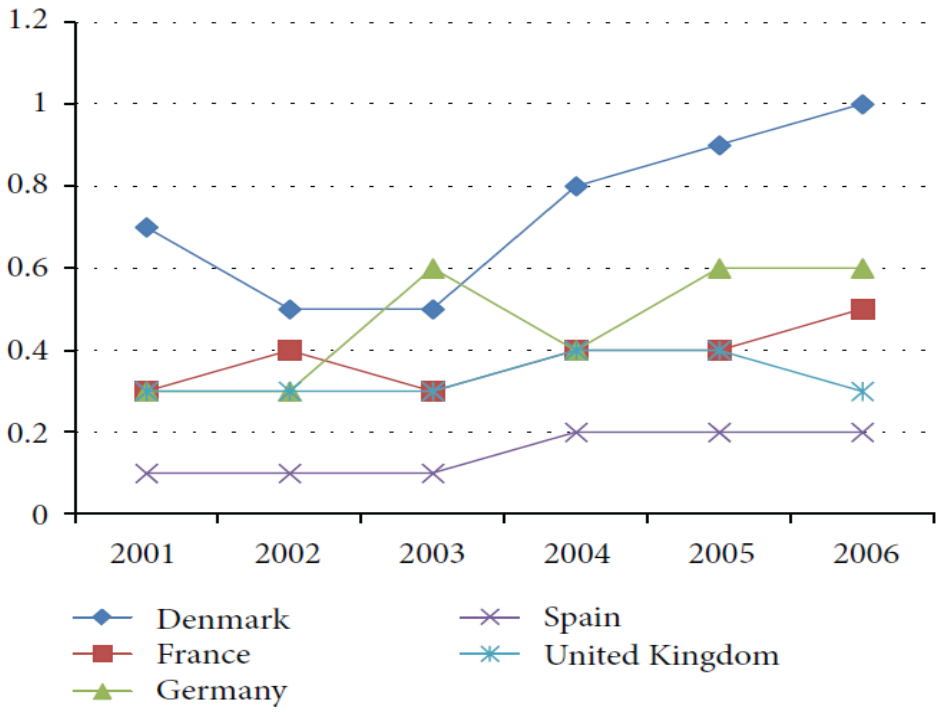


Figure 1.5 Incidence of listeriosis per 100.000 populations in five European countries from 2001 to 2006 (adopted from Hernandez-Milian *et al*., 2013).

In Spain, a study conducted to analyse data collected from listeriosis cases occurred between 2002 and 2013. It was observed that 41.6% of the patients were over 65 years old (Hernandez-Milian *et al*., 2012).

Listeriosis has been reported to be an infection of great concern to public health for two important reasons, (i) clinical severity with hospitailsation rate >90% and (ii) high fatality rate (up to 30% of reported cases- see table1.4) (Pontello *et al*., 2012).

Table 1.4 Listeriosis-reported cases with mortality percentage

Countries	Years	Death %
USA	2009-2011	17.6%
China	1964-2010	26%
Denmark	1994-2003	21%
Spain	2011	20-30%
Majorca	2002-2012	25%
Madrid	1986-2007	24.3%
Barcelona	2011	14%

A recent listeriosis-associated outbreak (August, 2014) was reported in Denemark. According to Danish State Serum Institute (SSI), a number of 15 people died as a consecuences of listerial infection, and 38 people had become ill. The outbreak was traced down to a popular deli meat called Rullepolse. Latest report from SSI showed that *Listeria* cells were recovered from fish; however, none of the hospitalised patients had contracted listeriosis from infected fish (Food Safety Tech, 2014). In 2012, a number of 147 persons became infected with *Listeria* in the USA, and the source of the outbreak was traced down to cantaloupes fruits from a farm in Colorado, USA. This outbreak was reported in different states across the country, which caused the number of *Listeria*-related deaths to increase to 33, among which was one pregnant woman who had a miscarriage (CDC, 2012).

In *Listeria* outbreak, the source of contaminated food had varied from vegetables products in the early 1980s to dairy products in the early 1990s (Farber and Peterkin, 1991). Currently, ready-to-eat meat contaminated with *L. monocytogenes* have been implicated with the transmission of the disease,

including a large outbreak in the United States due to consumption of contaminated hot dogs, which infected a large number of people and resulted in mortality of 21%. (Donelly, 2001). Studies involved in all major listeriosis outbreaks indicated that the main cause of the disease was *L. monocytogenes* serotype 4b. Other studies, however, showed that the serotype 4b may be relatively rare within raw food (Baek *et al*., 2000). In addition, serotype 4b was the strain responsible for the sudden increase of the reported cases of listeriosis between 1987-1989; however, increase awareness of the infection and withdraw of contaminated sources from the retails reduced the number of cases in subsequent years.

Another outbreak of listeriosis was documented in Italy in 1997 (table 1.5). The isolate was traced to consumption of listerial-contaminated tuna salad and maize, of which a number of 1566 infected individuals reported symptoms that included fever, stomachache, and headache. All reported cases, however, showed little evidence of serious risk and no fatality was ensued. Further investigation indicated that the serotype 4b was the main cause of the outbreak (Aureli *et al*. 2000). The manifestations of listeriosis include septicaemia, central nervous system complications (CNS) or placental/fetus infection in pregnant women. *Listeria* is involved in meningitis post targeting the CNS. In the United States, *Listeria* is considered the fifth most common cause of bacterial-derived meningitis. In addition, vertical transmission of *Listeria* in pregnant women may result in Bacteraemia and neonatal meningitis (Evans *et al*. 2004).

During pregnancy, women are particularly susceptible to bacterial infection including *Listeria*, which can cross the placental barrier and infect the foetus. The infection can be treated with antibiotics; however, the disease is fatal in about one-third of the cases (Schlech *et al*., 1983). Listerial isolates from patients can be reported to either the Public Health Laboratory System Communicable Disease Surveillance centre or in Scotland, the Scottish Centre for Infection and Environmental health. Presently, the establishment of a surveillance network in Europe has led increased awareness of *Listeria* infection and its complications. Nonetheless, epidemiological conclusion raised from reported-case investigations in European countries indicates that listeriosis incidence is on the increase (Gillespie *et al*., 2009).

Table 1.5 Examples of outbreak of human food-borne listeriosis (McLauchlin *et al*., 2004).

Country	Year	Food	Cases	Deaths
USA	1976	Raw salad	20	5
New Zealand	1980	Fish	22	7
Canada	1981	Coleslaw	41	18
USA	1983	Milk	49	14
USA	1985	Soft cheese	142	30
Switzerland	1983-1987	Soft cheese	122	34
UK	1987-1989	Pate	355	94
France	1993	Pork tongue	279	NA
France	1993	Pork rillettes	38	10
USA	1994	Milk	45	0
Sweden	1994-1995	Fish	9	2
France	1995	Soft cheese	17	4
Canada	1996	Crab meat	2	0
Italy	1997	Salad and tuna	1566	0
USA	1998-1999	Hot dog	50	>8
Finland	1998-1999	Butter	25	6
Finland	1999	Fish	5	NA
France	1999-2000	Pork rillettes	10	2
France	1999-2000	Pork tongue	32	10
USA	2000	Turkey meat	29	7
Switzerland	2005	Soft cheese	3	1

1.8 Pathogenesis of *L. monocytogenes*

L. monocytogenes has a high mortality in relation to food-borne infections, and its ability to grow at a wide range of temperature (Jemi and Stephan, 2006), as well as the ability to cross the important human barriers during the infection and exploit the host-cell machinery system (Figure 1.6) (Cossart *et al*., 2001). Bacterial internalization in both phagocytic and nonphagocytic cells is also considered a key element for *Listeria* pathogenesis and survival of the bacteria (Braun *et al*., 2000).

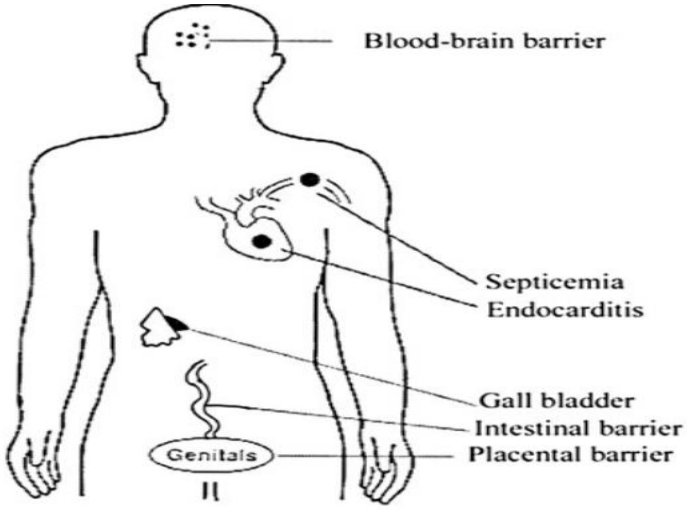


Figure 1.6 Important human barriers, (from Barbuddhe, 2008).

1.8.1 *L. monocytogenes* entry into host cells

L. monocytogenes enters host-cells by receptor-mediated endocytosis upon binding to the correct receptors displayed on relevant cell types including epithelial cells, endothelial cells, fibroblast, hepatocytes and macrophages. Entry of *L. monocytogenes* into host-cell is a dynamic process of which actin polymerization and membrane ruffling are required (Pizarro-Cerda *et al*., 2003).

At the cellular level, the pathogen adapts the process of pathogen-induced endocytosis to enter the cell after binding to displayed cell-surface receptors

that are recognized by bacterial invasive proteins. Adhesion of *L. monocytogenes* to host cells is a key step to establish an infection. Internalisation into host-cells is dependent on two listerial surface invasive proteins; namely Internalin A and B, which target the adhesion host cell-incorporated proteins; E-cadherin and the hepatocyte growth factor receptor Met; respectively (Seveau *et al*., 2007).

InIA interaction has a very high degree of specificity and replacement of amino acids in critical positions renders host cells resistant to *L. monocytogenes* infection (Lecuit *et al*., 2004). Indeed, InIA mutant strains showed significant loss of ability to cross the intestinal epithelial barrier in humans. Proteomic studies have also shown that all *Listeria* isolates from pregnant women diagnosed with listeriosis expressed the functional virulence protein, while only 65% of *Listeria* isolates from food sources expressed the protein (Lecuit *et al*., 2004).

These two invasive proteins are sufficient to mediate adhesion and internalisation into host cells. Internalin B receptor, Met, is ubiquitously expressed in a wide range of cell types, whereas InIA receptor is only expressed by a limited number of cell types, mostly of an epithelial origin. This indicates that InIA exhibits a more restricted cell tropism (Biern and Cossart, 2007).

Bacterial binding via InlA stimulates uptake by inducing cytoskeletal rearrangements in the host cell membrane, which interfere with the normal signalling pathway. Therefore the microbe will become contained and engulfed into the host-cell cytoplasm. Listerial internalisation upon E-cadherin binding is mediated by downstream cellular interactors involved in the formation of adherens junctions. These interactor molecules include α-catenin, β- catenin, actin and p120 catenin, which are recruited at the site of InlA-mediated bacterial entry (Lecuit *et al*., 2000).

Listerial InlA and InlB are part of a large protein family that share a common architecture, which includes a signal peptide at the N-terminal and a number of 22 amino acids leucine-rich repeat (LRR) that are involved in protein-protein interactions and receptor recognition (Reid *et al*., 2003). InlA is a protein that

consists of 800 amino acids and 15 LRR domains. Downstream of the LRR region, InlA harbours an inter-repeat region that has been shown to be crucial for the binding of the LRR domain to E-cadherin, followed by the C- terminal that is covalently bound to the bacterial cell surface (Lecuit *et al*., 1997; Marino *et al*., 1999). The distal N-terminus domain interacts with catenin that binds the intracytoplasmic domain of E-cadherin and promotes formation of the cell-cell junction (Seveau *et al*., 2007).

It was demonstrated that not only E-cadherin serves as a receptor for InlA, but also that upon ligand/receptor interaction, *Listeria* was able to exploit the downstream molecules involved in the formation of adherens junctions. This was apparent when the infection was impaired in cells expressing an E-cadherin variant that lack either the complete intracellular domain or only the β-catenin-binding domain, showing that the downstream interaction is crucial for cell invasion (Lecuit *et al*., 2000). Upon engagement of E-cadherin by InlA, the adherens junction machinery is activated inducing the recruitement of the junctional proteins α-catenin, p120 catenin, ARHGAP10 and myosin Vlla (Figure 1.7). InlA interaction with E-cadherin induces the caveolin-dependent clustering of E-cadherin and the activation of tyrosine kinase Src promotes E-cadherin phosphorylation, which triggers the recruitment of the ubiquitin-ligase Hakai and the ubiquitation of E- cadherin. This will induce the sorting of E-cadherin with clatherin-coated pits for bacterial internalisation. Alternatively, E-cadherin may persist within caveolin-rich domains and bacterial internalisation can occur through caveosomes (Bonazzi *et al*., 2009).

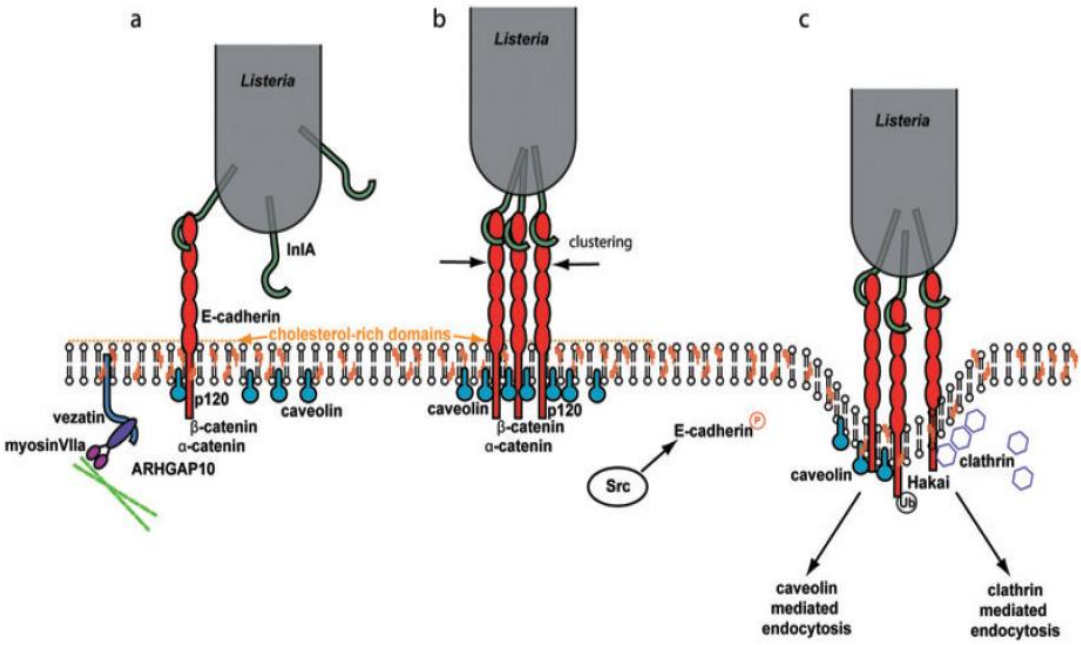


Figure 1.7 *L. monocytogenes* attachment to host cell mediating endocytosis and cytoskeletal rearrangement upon specific cell receptors binding and signalling changes (from Bonazzi *et al*., 2009).

Interestingly, the deletion of host-membrane juxtamembrane domain of E-cadherin, important for signalling cascade and binding to p120 catenin, was shown to have no apparent effect on listerial invasion.

In contrast, InlB, which mediates binding of InlA to host cell, is loosely attached to the bacterial cell surface through a series of domains (Cossart *et al*., 2001). InlB consists of leucine-rich repeats and inter-repeat regions in the N-terminus; these domains are sufficient to promote entry into the host cell. Previous studies have shown that InlA interaction with E-cadherin is dependent on a proline at position 16 (Figure 1.8). The presence of this amino acid allows the terminal loop of E-cadherin to be hydrophobic and uncharged, therefore strengthen the interaction with InlA (Bonazzi *et al*., 2009). In contrasts to humans and the guinea pig, species such as the mouse which express a different amino acid at this particular position are not permissive to orally acquired infection that depend on InlA interaction (Cossart *et al*., 2001).

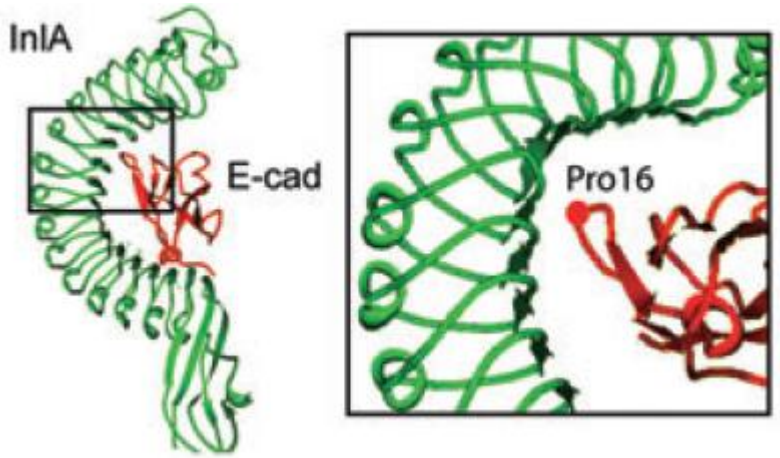


Figure 1.8 Species specificity of the InlA-E-cadherin interaction. Interaction depends on the presence of a prolin in position 16 of the N-terminal E-cadherin repeat (Bonazzi *et al*., 2009).

The human maternofetal barrier consists of the chorioallantoic placenta and the chorioamnion, two anatomically distinct components (Gude *et al*., 2004). The villous syncytiotrophoblast is a key component of the placental barrier as it comes into direct contact with intervillous space-circulating maternal blood. Lecuit (2004) and colleagues showed that E-cadherin is expressed in villous cytotrophoblasts and syncytiotrophoblasts in the maternofetal interface, which indicates that *L. monocytogenes* exploits the high degree specificity interaction between these proteins to cross the placental barrier. The syncytiotrophoblast layer is considered a specialised form of endothelium cells since it lies directly in the maternofetal interface and is in direct exposure to maternal blood, thereby *L. monocytogenes* directly adhere to the epithelium and colonise the trophoblast layer in an InlA-E-cadherin dependent manner, followed by the invasion of placental villi (Lecuit *et al*., 2004). In addition, InlB also plays an essential role in invading and colonisation of human placental tissues, since this protein has less degree of specificity and can bind to three types of host-cell surface proteins including c-Met, gC1q-R and glycosaminoglycans. InlB receptors are expressed in nearly all human cells (Barbuddhe *et al*., 2008) including the placenta. Thus invasion of the placenta requires co-ordinated action of both listerial invasion proteins (Disson *et al*., 2008).

1.8.2 Life cycle of *L. monocytogenes*

L. monocytogenes co-ordinately regulates a number of important virulence factors that enable it to undergo invasion and intracellular growth and the life cycle is depicted in figure 1.9. In brief, *L. monocytogenes* enters the host-cell upon binding of bacterial surface proteins to host-cell surface receptors that result in cytoskeletal rearrangement and membrane ruffling which subsequently leads to bacterial engulfment through endocytosis. Once inside the phagocytic vacule, *L. monocytogenes* virulence gene *hly* encoding listeriolysin O (LLO) is up-regulated, thus the lysing enzyme LLO is expressed along with other phospholipases including PlcA and PlcB. The virulence gene *hly* is pH-dependent and its optimum pH is 5.5 therefore LLO is expressed exclusively within the phagocytic compartment and not in the cytosol. LLO forms pores in the vacuole membrane, resulting in leakage of ions and phagocyte burst, thus allowing the microbe to escape the phagosome killing mechanism. In the neutral pH of the cytosol, *hly* is down-regulated in contrast to other virulence factors which are up-regulated. One such factor, ActA, polymerises host-cell actin filaments and allows *L. monocytogenes* to synthesise a propulsive 'comet tail' at one pole of the cell. The bacterium uses this to propel itself through the cytoplasm until it reaches the cell membrane and interacts with the membrane compartment and produce a new protrusion through cell signalling. This protrusion can be sensed and taken up by neighbouring cells through membrane-compartment rearrangements. *L. monocytogenes* becomes surrounded by double-membrane vacuole from the primary and secondary infected cell and LLO and phospholipases are once again up-regulated to lyse both vacuole membranes. The microbe escapes and moves freely in the secondary-infected cell where the lifecycle is repeated again. The approximate doubling time for *Listeria* cells inside the host-cell is 2-3h from escaping the vacuole (Pizarro-Cerda *et al*., 2004).

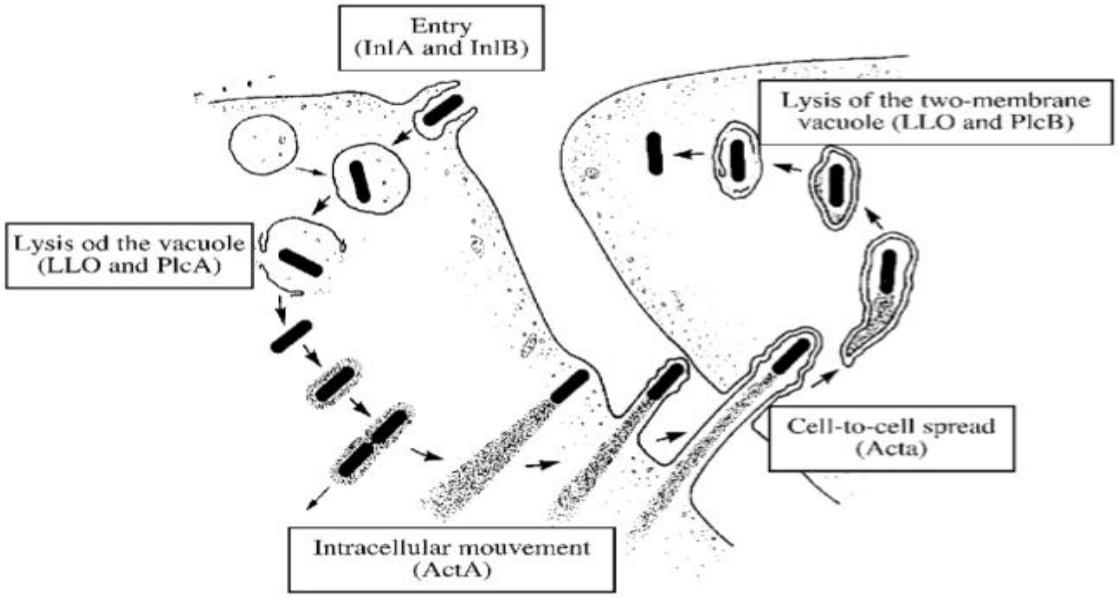


Figure 1.9 *L. monocytogenes* life cycle inside infected-host cell (from Pizarro-Cerda *et al*. 2003).

1.8.3 *Listeria* virulence factors and their contribution to bacterial survival

The pathogen *L. monocytogenes* possesses several virulence factors responsible for key steps required for intracellular parasitism namely *prfA, plcA, hly, mpl, actA* and *plcB*. Those listerial-pathogenicity dependent genes are linked to a 9-kb chromosomal segment reffered to as LIPL-1 (*Listeria* Pathogenicity Island-1). Physical and transcriptional organisation of the virulence cassette in some listerial species is depicted in figure 1.11. Gene-specific variations in LIPI-1 are directly linked to the pathogenicity of listerial species.

Other *L. monocytogenes* factors have been reported to have important roles in bacterial invasion. The surface protein p60 is considered as an invasion protein that plays an essential role in cell invasion. Previous studies have shown that strains affected in the production of p60 protein were defective in cell invasion (Bierne *et al*., 2007).

In *L. monocytogenes*, most of the genes involved in *Listeria* pathogenesis are under the control of the main transcriptional regulator PrfA. This regulator

binds as a dimer to a specific base pair sequence of dyad symmetry called the PrfA box, and positively regulates the expression of listerial virulence genes including the expression of *prfA* gene itself (Mauder *et al*., 2006). PrfA is a member of the cAMP receptor protein-fumarated and nitate reduction regulator (CRP/FNR) family of transcriptional regulators. The expression of *prfA* gene is dependent on three essential promoters P1, P2 and these are under the control of the house-keeping sigma factors; SigA and SigB respectively. The second promoter P2 can be also activated by SigA. In contrast, the third promoter is controlled by PrfA transcript itself; hence, allowing autoregulation of *prfA* gene transcription (Mauder *et al*., 2006). The critical virulence factor, PrfA, regulates the switch from flagellum-propelled bacterium in the extracellular compartment to intracellular pathogen.

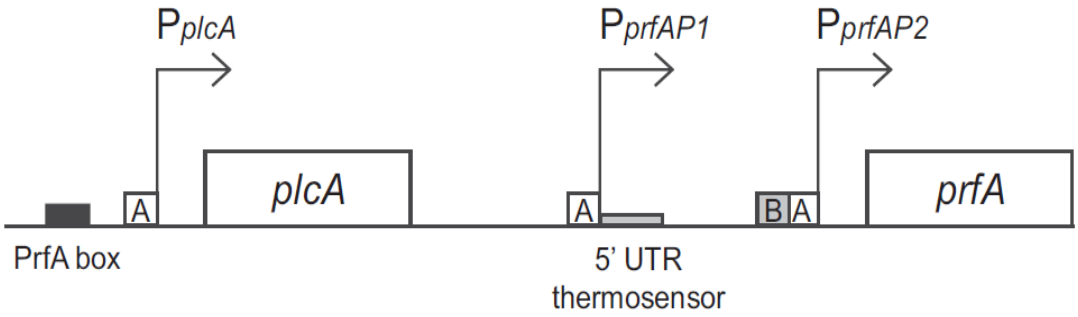


Figure 1.10 Schematic that shows the three promoters that induce *prfA* expression (from Lemon *et al*., 2010).

During the intacellular invasion, the PrfA-dependant transcription of virulence genes increases first in the phagocytic vacuole. After phagosytic escape into the host cytosol, a number of PrfA-dependent virulence genes are induced to a greater extent (Lemon *et al*., 2010). The production of PrfA is regulated at transcriptional, translational and protein activity levels. Moreover, the promoter of the upstream gene *plcA* (P*plcA*$_p$) contains a PrfA box and is positively aoutoregulated by PrfA itself. This aoutoregulation leads to a positive expression of the *plcA* and *prfA* genes. The resulting increased level of PrfA expression from P*plc*A is crucial for listerial virulence (Figure 1.10). P*prfA*$_{p1}$ is

σ^A-dependent of expression and its transcript contains a thermosensor in the 5' untranslated region (UTR), and is not efficintiley translated at 30ºC or below, but efficient at host body temperature. P*prfA*$_{p2}$ can be activated by both sigma factors A and B, and its transcript does not contain a translational thermosensor (Lemon *et al*., 2010). The activity of PrfA in the up-regulation of listerial virulence gene-cluster is crucial for the survival of the bacteria inside the host cell.

In addition, expression of InlA and InlB; key factors for cell invasion- (discussed in section 1.8.1), is regulated by both positive regulatory factor A (PrfA)-dependent and independent mechanisms (Lingnau *et al*., 1995). A sigmaB-dependent promoter was previously demonstrated to positively regulates and induce the expression of InlA; however, the presence of a putative sigmaB-dependent promoter upstream of InlB, suggests that contributions of sigmaB to *L. monocytogenes* invasion may not be solely limited to modulation of InlA expression (Kazmierczak *et al*., 2003). Identification of the sigmaB-dependent promoter that partially regulates the expression of PrfA, suggested that the contribution of sigmaB to listerial – virulence gene expression might be at least partially memdiated through PrfA. It was reported by Kim and colleagues (2005) that loss of sigmaB did not significantly reduce expression of Iap, ActA or ClpC, each of which have been associated to the invasion of *L. monocytogenes*. Invasion defects associated with loss of sigmaB-mediated transcription of the InlAB locus, with relatively minor indirect effect resulting from sigma-dependent expression of *prfA* (Kim *et al*., 2005).

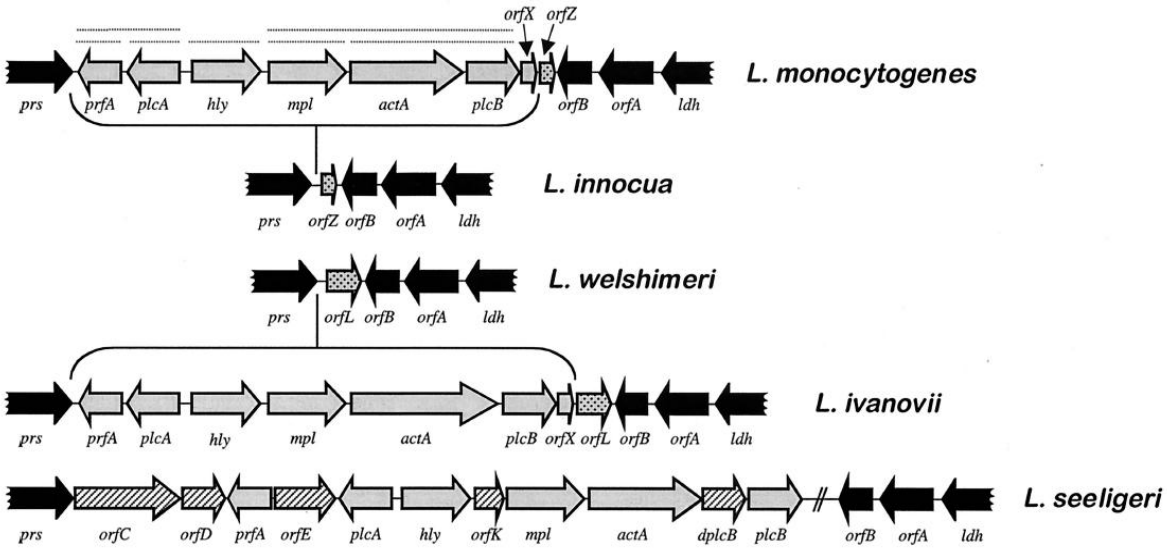


Figure 1.11 Physical organisation of the virulence-gene cluster in *L. monocytogenes* and other listerial species. Genes belonging to LIPI-1 are in grey and the flanking loci are in black. LIPI-1 is inserted in a region delimited by the *prs* and *ldh* genes, encoding for the housekeeping enzymes phosphoribosyl-pyrophosphate synthase and lactate dehydrogenase; respectively (adopted from Vazquez-Boland *et al*., 2001)

1.9 Listeriosis during pregnancy

Hormonal changes such as Human Chorionic Gonadotrophin HCG, progesterone and β-estradiol (oestrogen) are important to maintain pregnancy. For example, HCG is secreted in the early stages of pregnancy by the embryo, and by placental trophoblasts in late stages of gestation. It functions to maintain female hormones during pregnancy and prevents corpus luteum destruction, therefore maintaining progesterone secretions (Siiter *et al*., 1982). Moreover, developing placenta begins to release the hormone HCG that influences granulosa cells of corpus luteum to continue to secrete progesterone and β-estradiol in high levels, which in turn prevents the menstrual cycle to start and maintain pregnancy. At the end of the first two months of gestation, the placenta reduces the level of HCG secreted and further increase the amount of progesterone secreted (Siiter *et al*., 1982). However such changes may place pregnant individuals under increased risk

of infection as a result of hormone-mediated immune suppression as well as elevated levels of hormones (Mor, 2010).

Listeriosis during pregnancy has been correlated with several implications, which can occur as a consequence of infection. These complications include neonatal infection, premature delivery, still-birth, abortion and foetal death (Engeland *et al*., 1999). In addition, a sophisticated underlying mechanism exploited by *L. monocytogenes* is now documented to have a key role in microbe targeting to the maternofetal barrier. It targets and crosses the placental barrier depending on E-cadherin-InIA interaction at the villous trophoblast barrier level (Lecuit *et al*., 2004). Listeriosis is often asymptomatic for the mother and in some cases appears as a mild flu-like sickness, urinary problems or contractions of the uterus (Lecuit *et al*., 2004). If the growing foetus is infected, the disease is classified as either early or late onset. The average time for the start of the early onset is one and a half days, and this is more likely to happen in preterm infants where the disease is usually developed from maternal septicaemia (Schlech, 1996). In contrast, it can take a few days to several weeks for late onset infection, which may occur in infants who are delivered in term from infected-mother vaginal tract. In late onset of listeriosis, the mother usually presents no symptoms and the infant becomes infected due to passage through vaginal tract. As a consequence, the infected infant is more likely to develop neonatal meningitis syndrome within10 to 20 days post-parturition (Schlech, 1996). It is well known that *Listeria* infection is more severe in pregnant women due to sophisticated pathogen and vulnerable host characteristics. Indeed, non-pregnant healthy women suffer less complication from listeriosis (Delgado, 2008).

Despite its clinical importance, little is known about the underpinning molecular mechanisms involved in placental infection, and the role of pregnancy-associated factors in the development of listerial infection. Increased susceptibility of listeriosis during pregnancy can be partially explained by the unique immunological suppression in the mother, as the maternal immune system tolerates foreign allograft (Sperandio *et al*., 2002). In 1953, Peter Medawar investigated the possible mechanisms to clarify the paradox of the immunological condition during pregnancy (Medawar, 1953).

Since then, pregnancy has been regarded as a condition of cell-mediated immunological suppression (Wegmann *et al*., 1993). In fact, immunological suppression during pregnancy has been postulated as the reason for increased incidence of microbial infection; *L. monocytogenes* in particular (Mor, 2010). However, the exact state of the maternal immunity and its contribution to susceptibility to listerial infection remains unknown (Billington, 2003). It was evident by Bakardjiev and colleagues in a study conducted on guinea pig that only a single bacterium was sufficient to cause placental infection and the placenta can act as a reservoir for bacterial trafficking to other organs. Importantly though, it was found that the very low clearance rate of the bacteria from the placenta was the main reason of pregnant susceptibility to *Listeria* rather than immune suppression during pregnancy (Bakardjiev *et al*., 2006). Previous study showed that the number of *L. monocytogenes* in the placenta was 10^3-10^4- fold lower than other maternal infected organs including liver and spleen that showed higher penetration rate immediately after intravenous inoculation of the animal with the bacteria (Bakardjiev *et al*., 2005). Once the placenta has been seeded, however, it became the source of bacterial efflux and the number of bacteria increased by 10^7- fold as the foetus became infected over the three days course of infection, leading to a very slow clearance rate.

In general, expulsion of the infected placenta can be advantageous for the mother as a mechanism of defence against microbial infection. In the case of animals, the aborted placenta can cause cross contamination for other animals feeding in the same environment.

1.10 Hormone-pathogen interactions and its effects on bacterial growth

Generally, the exploration of the molecular cross-talk between pathogens and the host is a relatively unexplored area, however recent literature in this area demonstrates that this is an important area that warrants further investigation. Indeed, the issue of hormonal regulation and susceptibility to infections is a complex one and it is thought that female sex/pregnancy hormones play a role which results in females being more vulnerable to viral and bacterial infections

(Kaushic *et al*., 2011). During infection, microorganisms constantly monitor the surrounding environment, therefore regulating expression of virulence genes. For example, it has been shown that *Salmonella enterica* sv*.* Typhi interacts with host neuroendocrine stress hormones circulating in the host blood through *cpxAR*-dependent pathway, which lead to the release of bacterial haemolysin E thus augmenting bacterial virulence (Karavolos *et al*., 2011).

Experimental models of microbial infection with or without hormonal treatment have been studied to elucidate the role of sex/pregnancy hormones on the outcome of the infection (Leone *et al*., 2012). Some studies used endotoxin lipopolysaccharide (LPS) to determine the effects of the hormones on the infection in animals. LPS administration results in the production of sepsis that is driven by the overproduction of cytokines by macrophages, which recognise bacterial antigens and endotoxin through Toll-Like Receptors (TLRs) (Blackwell and Christman, 1996). It was observed that rats treated with β-estradiol had increased survival rate when compared to non-treated, and that can be explained by decreasing the oxidative stress following LPS challenge. This observation was reverted following removal of endogenous estrogen by ovariectomy, which caused increasing of mortality rate associated with LPS challenge (Merkel *et al*., 2001). Furthermore, sex hormones in males are accounted for the susceptibility to Mycobacterial infection that occurs more frequently in males than females (Neyrolles and Quintana-Murci, 2009). Experimental evidence showed that male mice infected with *M. marinum* had higher colonisation rate of the lung and spleen than female mice. Also, when female mice were injected with exogenous testosterone the susceptibility to infection increased (Yamamato *et al*., 1991). It has also been demonstrated that progesterone plays a role in attenuating the viability of *H. Pylori* (Hosoda *et al*., 2011). Thus, gerbil treated with 17-α-hydroxyprogesterone caproate, synthetic progesterone derivative, impaired the viability of *H. pylori*, while progesterone-treated gerbils resulted in less gastritis (Hosoda *et al*., 2011). Another example of the effects of hormones on bacterial infection can be seen during Q fever that is caused by *Coxiella burnetti.* Symptoms of the infection are reported more frequent in men than women. Furthermore, when mice were inoculated with *C. burnetti*, it was observed that the number of bacteria

was higher in the spleen of males than females, whereas exogenous treatment with β-estradiol caused a reduction of the colonisation rate (Leone *et al*., 2004).

In general, the effect of sex/pregnancy hormones on bacterial infection is dependent on the infection species and the levels of circulating hormones. For example, female mice infected with *Pseudomonas aeruginosa* were more susceptible to infection than males, and that can be determined by bacterial colonisation and inflammatory cytokines, which were higher in females than males (Guilbault *et al*., 2002). In line with this observation, it has been shown that exogenous administration of β-estradiol to male mice resulted in increased inflammation in lungs and elevated levels of IL-17 and IL-23 (Wang *et al*., 2010). As described above, sex/pregnancy hormones have differential role on the bacterial infection. Indeed, progesterone-treated female mice showed increased resistance to *Salmonella typhimurium* challenge, whereas β-estradiol treatment caused severe infection and increased susceptibility to the microbe (Kita *et al*., 1989). Moreover, pregnant mice infected with *S. typhimurium* showed higher colonisation rate of the spleen than non-pregnant (Pejcic-Karapetrovic *et al*., 2007).

Additionally, it was also documented that steroid hormones have predisposing effects on bacterial growth, metabolism and expression of virulence genes. For instance, the anaerobic bacteria *Prevotella intermedius* can uptake both progesterone and β-estradiol, which stimulate bacterial growth. Moreover, hormones can be substitute for vitamineK, a key growth factor for *P. Intermedius* (Kornman and Loesche, 1982). It was previously reported that sex/pregnancy hormones trigger susceptibility for several genital infections and even more so when coupled with the use of hormonal contraceptives that fluctuate hormone concentrations (Sonnex, 1998). Females are therefore considered to be more susceptible to a wide range of genital tract infections including Herpes Simplex Virus (HSV-2), HIV, Gonorrhoea, Candidiasis and Chlamydia (Brabin, 2002). Therefore, the higher the pregnancy hormones levels in human body, the more susceptible to microbial infection a woman would be, and this is true for the use of contraceptive pills except that the level of the hormone used is not as high as

the level observed during pregnancy. Previous studies reported that several pathogens possess extracellular receptors that tightly bind to circulating hormones in the blood stream and either increase or decrease susceptibility to infection (Powell *et al*., 1983).

Hormones produced by the ovaries have increasingly become recognised to have both direct and indirect effects on pathogens, where multiple indirect effects have been shown to be caused by effector molecules (Cunhan *et al*., 1985). It was previously reported that β-estradiol is able to influence gene transcription in several pathogens including Human Papillomavirus (Hall *et al*., 2011). Moreover, the parasitic microorganism *Trichomonas vaginalis* has been shown to possess β-estradiol receptors, which bind β-estradiol with high affinity, and stimulate transcription of genes in the microbe leading to increased pathogen infectivity (Sonnex, 1998). This shows that different microbes can be genetically affected by human hormones.

During pregnancy, the female hormonal system is altered from the norm as the level of certain hormones change in order to maintain pregnancy. Altered levels of progesterone and β-estradiol are also thought to correlate with increased occurrence and severity of infection (Hall *et al*., 2011). Increased levels of progesterone appear to augment infectivity of Herpes simplex virus HSV-2 (Sonnex, 1998), while high levels of β-estradiol enhance gonococcal infection in women more frequently during the menstrual cycle (sweet *et al*., 1986). Vaginal infection caused by *Candida albicans* is considered one of the most common gynaecological problems in the United States and Europe (Kinsman *et al*., 1986), where changes in hormone concentrations have predisposing effects on vaginal infection by *C. albicans* (Pung *et al*., 1984). Moreover, the incidence of candidiasis is more common in pregnant women than non-pregnant, which may be explained by hormonal changes that alter the immune system, along with modulation of virulence gene expression in the microbe. Indeed, the microbe *C. albicans* possesses a hormonal corticosteroid-binding protein (CBP) that can directly bind hormones, thus displacing them from their cognate host-cell receptors. This protein was found in all *Candida* species studied by Loose and colleagues (1983). However, other fungal species do not appear to posses CBP. In contrast,

Saccharomyces cerevisiae and *Paracoccidioides brasiliensis* display high affinity receptors for β-estradiol (Loose *et al*., 1983).

Chlamydia trachomatis is one of the most common sexually transmitted pathogens although infection caused by *C. trachomatis* is often asymptomatic. Crucially though, infection can lead to chronic disease in immunocompromised patients as well as patients experiencing hormonal changes (sweet *et al*., 1986). Furthermore, it was shown that *C. trachomatis* infection of human endometrial epithelial cells *in vitro* was higher when tissue explants that were used were collected during times of increased concentration of β-estradiol (Maslow *et al*., 1988). In detail, *C. trachomatis* gains entry to host infected-cell cytoplasm by utilisation of both β-estradiol - specific protein disulfide isomerase (PDI), and β-estradiol receptors that exist on the surface of HEC-1B cell-lines (Hall *et al*., 2011). *Chlamydia* is either attached directly to the ligand-binding domain on the β-estradiol receptor, or attach a region distal to the ligand-binding site and entry could be triggered when β-estradiol is present (Hall *et al*., 2011).

Another study demonstrated that the growth rate of the pathogenic fungus *Coccidioides immitis* was significantly simulated with the addition of progesterone and β-estradiol to the medium. This suggests that *C. immitis* grows faster in pregnant women where these hormones are circulating in significantly higher concentrations than non-pregnant women and hence pregnant women are at increased risk of infection (Powell *et al*., 1983). The increased risk to aquire coccidioidomycosis during pregnancy has been attributed to the immunosuppression and elevated levels of pregnancy hormones (i.e progesterone and β-estradiol) (Powell *et al*., 1983). Moreover, it was previously shown that *C. immitis* posseses hormonal-binding system that binds circulating progesterone, β-estradiol and androgen (Powell *et al*., 1984). In summary, changes of hormonal levels affect human cells and tissues, which alter the overall immunity that enhance bacterial growth (Figure 1.11). On the other hand, the metabolism, growth and gene expression of the bacteria can be influenced by steroid hormones, which can probably result in steroid degradation and/or overproduction of cytokines and inflammation.

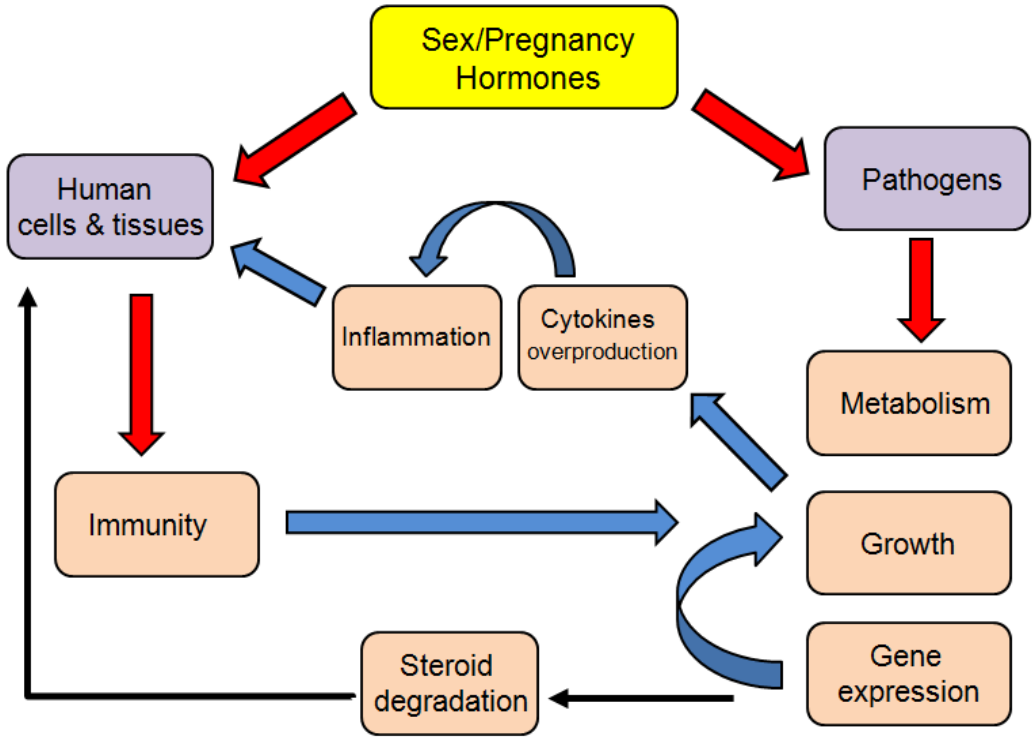


Figure 1.12 Schematic diagram summarises the possible effects of steroid hormones in human body during infection. Hormone-sensitive pathogens can be influenced by increased concentration of circulating hormones by several means depending on the hormone, concentration and species exposed. Hormone-driven bacterial gene expression can generally affect the growth of the microbe, and/or overexpress of virulence genes involved in steroid degradation, which might eventually affect the function of human cells and tissues. Growth of the microbes can also mediate cytokines production by specialised cells that result in inflammation, which in turn affecting the cells and tissues.

Hormones generally affect the outcome of the infection depending on the type of the hormone, infective species (depending on the capacity of binding to the hormone) and the hormone concentrations present; for example, high concentrations (100 µM) of progesterone can be bacteriocidal for *H. pylory*, whereas 50 µM of the same hormone only inhibits the growth (Hosoda *et al*., 2011). Indeed, pregnancy hormones have differential role toward host susceptibility to infection and this was evident in the study conducted by Emoto *et al.,* (1989). They observed that mice treated with estradiol were more susceptible to *Salmonella typhimurium* infection, whereas treatment with

progesterone reversed the result observed by increasing mice resistance to the infection. Moreover, increased concentration of pregnancy hormones was previously reported to influence the growth of several pathogens by alteration to the innate immune response, or more interestingly, by directly affecting the pathogen at the genetic level, which may lead to changes in gene expression (Drutz *et al*., 1981). Indeed, presence of steroid hormones including progesterone was previously demonstrated to influence the expression of steroid-degradation gene cluster in *Comamonas testosteroni* that uses specific enzymes (i.e. 5,3-ketosteroid isomerise, 3α-dehydrogenase and β-dehydrogenase) to convert several steroids to androsta-1,4-diene 3,7-dione. Theses enzymes facilitate the conversion of a hydroxyl group at C-17 position to make ketone and introducing a double bond at C-1 position of the A-ring, followed by hydroxylation at C-9 position of the B-ring. This leads to aromatization of the A –ring that is cleaved and 2 compounds are formed by hydrolysis (Horinouchi *et al*., 2012).

Another example of hormones influence on bacterial infection was seen when female mice inoculated with *L. monocytogenes* were treated with exogenous doses of β-estradiol. On day 3-7 after infection, the number of bacterial cells in liver and spleen was higher in treated mice than in control mice. This shows that administration of estradiol was associated with an increased mortality rate of the female mice, which suggests a vital role of the hormone in susceptibility to infection. In line with this observation, male mice treated with estradiol showed reduced level of resistance to infection, and increased level of lung inflammation and cytokines production were observed (Wang *et al*., 2010). Moreover, increased rate of microbial infection is directly influenced by elevated levels of steroid hormones. For example, it was previously reported that elevated levels of estradiol and progesterone in girls and testosterone in boys were positively correlated to the increased incidence of gum infection caused by *Prevotella intermediate* and *Prevotalla nigrescens* (Nakagawa *et al*., 1994). It was also reported that hormonal changes during pregnancy may lead to a 55-fold increase in levels of Bacteroides species in periodontal gum specimens when compared to non pregnant women (Kornman and Loesche, 1980). Furthermore, Steroid use causes physiological hormonal level to

increase, which in part mimics the increased hormone concentration during pregnancy. Individuals with such increase are at higher risk for listerial infection (Janakiraman, 2008). In contrast to the increase in bacterial growth, progesterone and estradiol have been shown to exert growth-inhibiting effects on a number of microorganisms. The antimicrobial action of steroids was mainly reported against a number of Gram-positive and Gram-negative bacteria and different species of fungus. Hosoda and colleagues (2011) demonstrated that progesterone, at a concentration of more than 50 μM, significantly reduced the growth of *Helicobacter pylori in vitro* through its bacteriocidal action, while a concentration of 100 μM completely inhibited the growth. In the case of estradiol, both concentrations of 50 and 100 μM showed similar inhibitory effect (CFU ml^{-1} > 10^4, more than control sample), which suggest that progesterone exhibit more potent bacteriocidal than estradiol. Different study conducted by Morse and Fitzgerald (1974), showed that *Neisseria gonorrhoeae* is highly sensitive to growth inhibition by the action of progesterone, which was shown to bind membrane-associated enzymes and reduce the activity of NADPH oxidase and L-lactate dehydrogenase. Interestingly, similar experiments conducted on fungus revealed that the bacteriostatic action of progesterone was abolished after an extended period of lag phase. For example, *Trichophyton mentagrophytes* was able to metabolise progesterone into different compounds with a reduced affinity to bind the fungal proteins involved in growth inhibition. Metabolising of progesterone resulted in restoring the growth (Clemons *et al*., 1989). Furthermore, a similar response was observed when different Gram-positive bacteria including *S. aureus* and *L. monocytogenes* were incubated with 20 μg ml^{-1} of progesterone. For *L. monocytogenes* the lag phase was prolonged and an inhibitory action of progesterone was observed in the first 8 hours of growth, whereas longer incubation enabled *L. monocytogenes* to restore the growth to a level comparable to that of the control (Yotis *et al*., 1966).

Taken together, these data demonstrate the role of sex/pregnancy hormones in the context of the overall tolerance and susceptibility to microbial infection. Also, there is a clear hormonal influence on the susceptibility and progress of bacterial infection in human and animal models of infection, which correspond

to the type and concentration of the hormone. Hence, intense investigations are needed to elucidate the role of steroid hormones and the mechanisms underpinning *L. monocytogenes* infection during pregnancy, effects of the hormones on growth and virulence gene expression.

1.11 Aims

There is evidence in the literature that several pathogens are influenced by different factors found in pregnant individuals. To date, little is known about the food-borne bacterium *L. monocytogenes* and how it is affected by pregnancy hormones, and so several questions need to be addressed. Based on other evidence described above it is possible that pregnancy hormones such as progesterone and β-estradiol, may lead to changes in bacterial virulence gene expression. In addition, it is possible that circulating pregnancy hormones may directly or indirectly influence *L. monocytogenes* growth, pathogenesis and survival in pregnant individuals.

The aims of this project therefore are to:

- Determine the effects of pregnancy hormones on the growth of *L. monocytogenes*.
- Generation of mutants exhibiting phenotype to pregnancy hormones using transposon-mediated mutagenesis
- Determine if *L. monocytogenes* possesses proteins with the ability to bind pregnancy hormones using bioinformatic approaches
- Characterise as yet unidentified genes in *L. monocytogenes* that may be involved in hormone modulation.

Together these approaches will aid the understanding of listerial infection during pregnancy and may reveal new insights that can be exploited to prevent infection.

Chapter II

Materials & Methods

2.1 Bacterial strains

L. monocytogenes EGD-e was the parental strain of *L. monocytogenes* used throughout this study and was obtained from the Taylor Laboratory Culture Collection (TLCC). *L. monocytogenes* DP-L910 (Camilli *et al*., 1990) containing Tn*917*-LTV3 was a kind gift from Prof D. Hodgson, University of Warwick, UK. *Escherichia coli* α-Select (Bioline, UK) was used for Tn*917*-LTV3 propagation and recovery of *L. monocytogenes* chromosomal DNA flanking the integrated transposon. Also, it was used for propagation of the MBΔpLSV101 plasmid for gene deletion. *Escherichia coli* XL1-Blue (Stratagene, UK) was used for cloning of the recombinant protein, NamA. *Escherichia coli* BL21 (DE3) (Bio-Rad, UK) was used for NamA protein expression (Table 2.1).

2.2 Bacterial growth media

L. monocytogenes was routinely grown in Brain Heart Infusion (BHI; Oxoid, UK) or Tryptic Soy Broth (TSB; Sigma-Aldrich, UK) at 37°C. For liquid culture, bacteria were normally grown with shaking at 200 rpm. For growth on solid medium, BHI or TSB agar was prepared by the addition of 1.5 (w/v) agar (Bacteriological, No. 3; Oxoid). Luria Bertani (LB) agar or broth was used for growth of *E. coli* and contained per litre, 10 g (1%) Tryptone, 5 g (0.5%) NaCl, 5 g (0.5%) yeast extract and 1.5% (w/v) agar where appropriate. M9 minimal media was also used to grow *E. coli* and contained per litre, 40 mM Na_2HOP_4 (w/v), 20 mM KH_2PO_4 (w/v), 9 mM NH_4Cl (w/v), 0.2% glucose (w/v), 1 mM $MgSO_4.7H_2O$, 3 µM Thiamine.HCl, 130 µM $CaCl_2$ and 8.5 mM NaCl. Media were prepared with distilled H_20 and sterilised by autoclaving at 121°C for 20 min.

2.3 Preparation of antibiotics

The following antibiotics were used for selection of Tn*917*-LTV3 in *L. monocytogenes* DP-L910 (final concentrations): tetracycline (tet; 12.5 µg ml^{-1}; prepared in 70% (w/v) ethanol), erythromycin (erm; 5 µg ml^{-1}; prepared in 70% (w/v), lincomycin (lin; 25 µg ml^{-1}; prepared in dH_2O). For selection of Tn*917*-LTV3 in *E. coli*, kanamycin (prepared in dH_2O) was used at 50 µg ml^{-1} (w/v). For selection of bacteria carrying pLSV101 plasmid, a final concentration of 5

µg ml^{-1} erm (w/v) was used for *L. monocytogenes* and 300 µg.ml^{-1} for *E. coli*. A final concentration of 100 µg.ml^{-1} ampicillin (prepared in dH_2O (w/v)) was used for selection of *E. coli* carrying pPAL7 plasmid. All antibiotics were filter sterilised before use and stored at -20°C, except for lincomycin, which was stored at 4ºC.

2.4 Defined minimal medium for growth of *L. monocytogenes*

HTM minimal media was prepared according to the method of Tsai *et al*., (2003). Briefly, media was prepared in distilled water by the sequential addition of each of the following components: MOPS pH 7.4; 100mM, KH_2PO_4; 4.82 mM, $MgSO_4$; 1.70 mM, Na_2HPO_4; 11.55 mM, Glucose; 55 mM, $(NH_4)_2SO_4$; 0.6 mg ml^{-1}. Agarose (Bioline) was added to a final concentration of 1.5% where appropriate. Media was sterilised by autoclaving at 121°C for 20 min. Cysteine, methionine, (each at a concentration of 0.1 mg ml^{-1}), thiamine (2.96 µM) and biotin (2.05 µM) were prepared separately in distilled water (few drops of NaOH [1 M] was added to dissolve methionine and biotin). Riboflavin (1.33 µM) and lipoic acid (24 pM) were prepared in DMSO. All vitamins, amino acids and lipoic acid were filter sterilised before addition to the media.

2.5 Exposure of *L. monocytogenes* to pregnancy hormones

L. monocytogenes was exposed to pregnancy hormones during growth in TSB or HTM, where appropriate. The medium contained different concentrations of progesterone or β-estradiol (≥99% purity), (Sigma-Aldrich, UK). Hormones were prepared in 100% ethanol and diluted to the appropriate concentration with the same. As a control, bacteria were grown in the same media as the testing samples except that 100% ethanol was added to the culture instead of hormones.

2.5.1 ..Bacterial growth monitored by optical density and viable counting

Bacterial growth was monitored by optical density, which was measured manually using 6315 UV/visible spectrophotometer (JENWAY, UK) for which, 1 ml of the bacterial culture was transferred into a disposable plastic

(Polystyrene) cuvette, and wavelength was set to 600 nm. The mean of three separate reading was recorded each time. Bacterial growth during hormone exposure was monitored by viable counting. In this case, bacteria were inoculated 1:50 from an overnight culture growing aerobically into TSB or HTM containing hormones, followed by growth at 37°C, 200 rpm for aerobic condition or anaerobically without shaking (Whitley DG500 Anaerobic Workstation; Whitley, UK). At an appropriate time point, an aliquot of bacteria was removed and immediately used for viable counting using the Miles and Misra spot dilution technique. Ten-fold serial dilutions were made using sterile PBS in 96-well plate and bacteria plated on TSA agar before incubation overnight at 37°C under either aerobic or anaerobic conditions. Experiments were carried out in triplicate on at least 3 independent occasions.

2.6 SDS-PAGE analysis of proteins

Bacterial proteins were analysed by SDS-PAGE using either a precast 4 -15% Tris-glycine gel (BioRad) or following manual preparation of a 12% gel. The gel apparatus was assembled according to the manufacturer's preparations.

2.6.1 Preparation of the resolving gel

A 12 % resolving gel was prepared by adding the following reagents in order: 4 ml H_20, 3.3 ml 30% acrylamide mix [29% (w/v) acrylamide: 1% (w/v) N,N'-methylenebiasacrylamide], 2.5 ml of 4X resolving-gel buffer (1.5 M Tris- HCl pH 8.8), 0.1 ml 10% SDS, 0.1 ml of 10% ammonium persulfate, 4 µl (0.4%) TEMED (N.N.N',N'- tetramethylethyelenediamene). These volumes were used to prepare 10 ml of the gel. The resolving gel was then applied to the space between the glass plates leaving enough space for the stacking gel to be prepared. Isopropanol was overlaid across the top of the gel, which was left to set at room temperature. Once it had set the isopropanol was removed by washing with water.

2.6.2 Preparation of the stacking gel

A 5 % stacking gel was prepared by mixing the following reagents in order: 1.4 ml H_20, 0.33 ml 30% acrylamide mix, 0.25 ml of the stacking-gel buffer (1M

Tris- HCl pH 6.8), 0.02 ml 10% SDS, 0.02 ml 10% ammonium persulfate and 2 µl (0.2%) of TEMED). This was poured on top of the stacking gel and the comb inserted. Once the stacking gel had set the comb was removed. The gel assemble was fitted into the electrophoresis tank and the tank filled with 1X SDS- page running buffer (25 mM tris-glycine, 250 mM glycine and 0.1% SDS).

2.6.3 Visualisation of proteins

Following electrophoresis, the gel was removed and stained using SimplyBlue SafeStain (Invitrogen) according to the manufacturer's instructions. Once stained, gels were photographed using a Chemidoc XRS+ system (BioRad).

2.7 Miniprep of plasmid DNA

Plasmid DNA was prepared using the QIAQuick Plasmid Miniprep Kit (Qiagen). *L. monocytogenes* was grown overnight at the appropriate temperature with shaking (200 rpm) in TSB containing selective antibiotic. Bacterial cells were pelleted by centrifugation at 15,000 rpm from 1.5 ml of overnight culture and cells were lysed by incubation of the cells at 37ºC for 30 min in 0.5 ml TE buffer (10mM Tris-Cl and 1mM EDTA, pH 8.0) containing lysozyme (2.5 mg ml^{-1}, prepared in dH_2O (w/v)), followed by another 30 min incubation after adding protinaseK (1 mg ml^{-1}, prepared in dH_2O (w/v)). Cell debris was pelleted by centrifugation at 15,000 rpm for 3 min and supernatant was transferred to the DNA-isolation column to prepare DNA according to the manufacturer's instructions. For preparation of plasmid DNA from *E. coli*, bacteria were grown overnight at the appropriate temperature with shaking (200 rpm) in LB containing selective antibiotic. A volume of 1 ml of the overnight culture was pelleted by centrifugation at 15,000 rpm, and DNA was prepared according to the manufacturer's instructions.

Table 2.1 Bacterial strains used in this study and their characteristics

Bacterial strain	Characteristics	Source
L. monocytogenes EGDe	Wild type- whole genome sequence: NCBI, NC_003210	TLCC
L. monocytogenes DP-L910	*L. monocytogenes* 10403S harboring Tn*917*-LTV3	Camili *et al*. 1990
L. monocytogenes MBΔEGDe	*L. monocytogenes* lacking NamA-encoding gene	This work
E.coli BL21(DE3)	*E. coli* B F- dcm ompT hsdSB(rB-, mB-) gal λ(DE3)	Bio-Rad
E.coli BL-PAL7	*E.coli* BL21(DE3) harboring plasmid pPAL7	This work
E.coli BL-NamA	*E.coli* BL21(DE3) harboring NamA construct on pPAL7	This work
E.coli α-select	F- *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Δ(*lac*ZYA-*arg*F)U169 Φ80*lac*ZΔM15 λ	Bioline
E.coli α-select (Tn*917*-LTV3)	*E.coli* α-select harbouring Tn*917* with recovered DNA from *L. monocytogenes*	This work
E. coli α-select (MBΔpLSV101)	*E.coli* α-select harbouring NamA on pLSV101 for NamA deletion	This work
E.coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*q*ZΔM15* Tn*10* (Tetr)]	Stratagene
E.coli XL1-Blue-PAL7	*E.coli* XL1-Blue harboring plasmid pPAL7	This work
E.coli NamA-PAL7	*E.coli* XL1-Blue harboring NamA construct on pPAL7	This work

Abbreviation include: TLCC, the Taylor Laboratory Culture Collection. pPAL7, plasmid pPAL7 is the expression vector which utilizes the T7lac promoter to strongly express a profinity eXact fusion protein in *E. coli* cells producing T7 RNA polymerase. The vector constitutively express the lacI repressor and confers ampicillin resistance to the host cell. *Tn917*-LTV3, transposon Tn917 carried on LTV3 plasmid vector. It was used to randomly interrupt *L. monocytogenes* genes.

2.8 Agarose gel electrophoresis

Agarose gels were prepared in 1x TAE (40 mM Tris-acetate, 1 mM EDTA). The gel solution was dissolved by heating in the microwave for 4 min and cooled to 55°C before the addition of SafeView DNA stain (NBS Biologicals, UK), (5 % SafeView DNA stain). An appropriate volume of DNA was mixed with 5X DNA loading buffer and applied to the wells of an agarose gel. Electrophoresis was carried out in 1X TAE at 100 V for 30 – 40 min. DNA was visualised using the Chemidoc XRS+ system (BioRad).

2.9 Transformation of *E. coli* with plasmid DNA

Chemically competent *E. coli* α-select and XL1-Blue were purchased from Bioline and Stratagen; respectively, and were used according to the manufacturer's instructions. Briefly, 3-5 µl of plasmid DNA was mixed with 50µl of *E. coli* cells and incubated on ice for 15 min. Cells were heat shocked at 42ºC for 90 s, followed by further incubation on ice for 2 min. A volume of 1ml of LB broth was added immediately to the mixture and further incubated for 1h at 37ºC (200 rpm). Cells were plated onto selective LB agar and incubated statically overnight at the appropriate temperature (30-37°C).

2.10 Preparation of chemically competent *E. coli*

E. coli was grown in 10 ml LB broth at 37ºC, overnight. Next day, cells were diluted 1:200 into fresh 100 ml LB broth containing 1.5 ml of 1M $MgCl_2$, and the cells were further incubated at 37ºC until the OD_{600} reached 0.4-0.6. The cells were harvested by centrifugation at 4000 rpm for 10 min (4ºC). the pellet was resuspended in 30 ml of ice cold-solution1 (600 µl of 1M $MgCl_2$, 3 ml of 1M $CaCl_2$, 12 ml of 50 mM MES, dH_2O up to 60 ml) and the mixture was incubated on ice for 20 min. Cells were harvested by 5 min centrifugation at 3000 rpm, 4ºC and the pellet was resuspended in a final volume of 6 ml ice cold-solution2 (120 µl of 1M $MgCl_2$, 600 µl of 1M $CaCl_2$, 2.4 ml of 50 mM MES, 3.6 ml of 50% glycerol and dH_2O up to 12 ml). Cells were stored at -80ºC in 100 µl aliquots.

2.11 Preparation of electrocompetent *L. monocytogenes* EGD-e

L. monocytogenes was prepared for electrotransformation according to the method of Park and Stewart (1990). Briefly, *L. monocytogenes* was grown overnight at 37ºC in TSB containing 0.5 M sucrose (TSB/S). A volume of 50 ml of the overnight culture was inoculated into 250ml of TSB/S and further incubated at 37ºC (200rpm) for 2 h or until OD 600nm reached 0.2. Penicillin G was added to a final concentration of 10 µg ml^{-1} (prepared in dH_2O (w/v)), and the culture incubated for further 2 h at 37°C. Cells were harvested at 4000 rpm for 10 min at 4ºC, and washed twice in an equal volume of 1mM HEPES and 0.5 M sucrose (HEPES/S). The resulting pellet was resuspended in $1/400^{th}$ volume of HEPES/S and placed on ice for immediate electrotransformation.

2.12 Electrotransformation of *L. monocytogenes* with plasmid DNA

An appropriate volume of plasmid DNA was added to *L. monocytogenes* electrocompetent cells and incubated on ice for 2 min. The mixture was transferred to a chilled electroporation cuvette and pulsed at 2.5 KV, 25µF and 200Ω using BioRad Gene Pulser (Xcell™ Microbial system). The cuvette was incubated on ice for 10 sec and 1ml TSB-sucrose was added to the cells. Cells were incubated at 30-37ºC (depending on the plasmid) for 3h followed by plating on selective TSB agar containing the appropriate antibiotic. Plates were incubated at 30-37°C for up to 48 h.

2.13 Tn*917*-mediated insertional mutagenesis

L. monocytogenes containing pLTV3 was cultured overnight in TSB containing erythromycin, lincomycin and tetracycline at 30°C with agitation (200 rpm). Next morning, the culture was back-diluted 1:800 into TSB containing erythromycin, and lincomycin only and grown at a temperature non-permissive for pLTV3 replication (43°C) overnight. Next morning, 1 ml of culture was removed and bacteria were serially diluted in sterile PBS and plated to obtain single colonies on TSB agar containing erythromycin and lincomycin only. Plates were incubated at the same non-permissive temperature for 2 days.

After 2 days, single colonies were picked up from the plate and replica patched onto TSB agar containing erm,linc, tet and erm, lin only (2 different agar plates) and those were incubated again at the same non-permissive temperature overnight. The number of tetracycline sensitive colonies and presumed recipients of the transposon were ascertained by comparison of bacteria that grew on medium containing either erm, lin, tet or erm, lin only. For storage of the transposon library, colonies that were sensitive to the presence of tetracycline was probagated in TSB in the presence of erm, lin and each culture was mixed thoroughly with an equal volume of 50% (v/v) glycerol and placed at -80°C in 1 ml aliquots.

2.14 Primary screening of transposon mutants

A high throughput primary screening to find transposon mutants sensitive to progesterone and β-estradiol was carried out by growing bacteria separately on TSB agar containing either 60 µg ml^{-1} progesterone or 60 µg ml^{-1} β-estradiol. In detail, mutant library was removed from -80°C freezer and thawed on ice. From this, a volume of 100 µl was mixed with 900 µl of steriled PBS and ten-fold serial dilutions were prepared for single colonies. Bacterial dilutions were spread on TSA agar containing erythromycin, linocmycin only and plates were incubated overnight at 37°C. Next day, single colonies were replica patched onto TSB agar containing erm, lin (control) only and erm, lin and 60 µg ml^{-1} of the hormone (either progesterone or β-estradiol). Plates were incubated anaerobically for 12 h at 37°C. Alternatively, the screening was carried out using liquid TSB in 96-well plate, of which 60 µg ml^{-1} of the hormone was added to the medium containing erm and lin, and plates were incubated in similar condition as above. For the control, the same procedure was followed with ethanol only (because hormone was dissolved in ethanol, so TSB received the same amount of ethanol only).The optical density of growth was measured using MRX revelation microplate reader (Dynex Technologies).

2.15 Extraction of *L. monocytogenes* genomic DNA

Genomic DNA was prepared from *L. monocytogenes* following overnight growth in TSB and antibiotics where appropriate. The Gentra PureGene Extraction Kit (Qiagen) was used according to the manufacturer's instructions to isolate genomic DNA from up to 3 ml of overnight culture. Alternatively, genomic DNA was prepared by phenol:chlorophorm extraction. Briefly, a volume of 1.5 ml of an overnight culture was pelleted by centrifugation at 15,000 rpm for 5 min, and pellet was washed by 0.1X SSC (1X SSC: 0.15 M NaCl, 0.015 M tri-sodium citrate; pH 7.0). The resulting pellet was resuspended in 100 µl of 0.01 M sodium phosphate buffer with 20% sucrose (w/v) and 2.5 $mg.ml^{-1}$ lysozyme and incubated for 1 h at 37°C. A volume of 900 µl of TE buffer (mM Tris-Cl, 1 mM EDTA pH 8.0) containing 1% (w/v) SDS and 1 $mg.ml^{-1}$ proteinase K was added to the mixture and further incubated at 37ºC for 45 min. A volume of 1 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the lysed cells and vortexd vigorously for 20 sec followed by centrifugation at maximum speed for 5 min. Resulting clear aqueous phase was transferred to a clean sterile propylene tube (top layer) to repeat the extraction using similar volume of phenol:chloroform:isoamyl alcohol. A 2-volume of ethanol and 0.1 volume of 3 M sodium acetate, (pH 5.2) were added to the clear aqueous phase. The mixture was inverted several times and spun at maximum speed for 5 min. The DNA was recovered by adding 50 µl of DNase-RNase free water to the bottom of the tube and incubated at 65ºC for 30 min.

2.16 Purification of RNA from *L. monocytogenes*

L.monocytogenes was cultured in the presence of increasing concentrations of either progesterone or β-estradiol for 12h under anaerobic conditions at 37ºC. From this, total RNA was isolated from bacterial culture using either RNA isolation kit (Bioline; UK), or by Trizol-based isolation (Invitrogene; UK). In brief, approximately 2 ml of bacterial culture was mixed with 2 volumes of RNA protect (Qiagen; UK) and the mixture was incubated at room temperature for 5 min, followed by centrifugation at 13,500 rpm. Manual

instructions for each method were followed where appropriate. All Samples were run on 1.2% agarose gel for 30 min at 100 V.

2.16.1 Semi-quantative RT-PCR for gene expression

Concentrations of RNA samples were determined by using Nanovue spectrophotometer (GE Health care, UK), and the mean of 3 different reading was used to adjust the final concentration. All RNA samples were reversed transcribed to cDNA by using qScript^{TM} cDNA SuperMix (Quanta Biosciences; UK). A volume of 5 µl of each RNA sample (10 µg ml^{-1}) was added to 4 µl of qScript mix, and RNase/DNase free water was then added to a final volume of 20 µl. The reaction was carried out in the thermal cycler as following:

5 minutes	@ 25˚C	1 cycle
30 minutes	@ 42˚C	
5 minutes	@ 85˚C	

After completion of cDNA synthesis, 1/5th of the first strand cDNA (2 µl) was used to PCR amplify the target genes using specific primers (Table 2.2). The PCR cycle was set as in section 2.18. PCR products were analysed by agarose gel electrophoresis once cycling was complete.

2.17 Cloning and preparation of deletion-mutant in *L. monocytogenes* chromosomal DNA

Temperature sensitive plasmid DNA pLSV101 was used to create a deletion-mutant in *L. monocytogenes* according to the protocol of Joseph *et al*., (2006). In brief, two fragments of 604 bp and 426 bp were PCR amplified from up-stream (AB) and down-stream (CD) regions of the gene to be deleted and ligated via the introduced BglII restriction site. Following nested PCR using the oligonucleotides AD and the ligation mixture as a template, the resulted PCR product (1030 bp) was cloned into the similarly digested pLSV101 via BamHI and EcoRI restriction sites giving rise to MBΔpLSV101, which was electrotransformed into the previously prepared electrocompetent *L. monocytogenes* as described above in section 2.10 and 2.11. Cells harbouring MBΔpLSV101 were grown overnight in TSB supplemented with 5

µg ml^{-1} erm. This culture was used to streak TSA agar containing the same selective antibiotic and incubated overnight at a temperature non-permissive for the plasmid replication (43ºC), which trigger the first homologous crossover into the chromosomal DNA while not allowing the plasmid to replicate. Thus, only cells with the chromosomally integrated plasmid were able to grow. A single colony from the streaked plate was inoculated into TSB media without antibiotic and incubated at 30ºC overnight. From this, subsequent growth at 30ºC was prepared to ensure second crossover and excision of the integrated plasmid, hence the gene of interest. Genomic DNA was extracted and PCR was run to check success of the deletion using AD primers.

2.18 PCR cycles

PCR reactions were performed using My Taq HS mix (Bioline), which includes: Buffer, nucleotides and DNA polymerase. The annealing temperature and the length of the extension cycle were adjusted depending on the melting temperature of each primer used and the length of the PCR product. The reaction was set up to contain the following:

Per 50 µl reaction	µl
2x My Taq HS mix (Bioline)	25
Forward primer (10 µM) [0.5-1 µM]	0.5
Reverse primer (10 µM) [0.5-1 µM]	0.5
Template DNA [0.5-1 µg]	1 - 2
PCR grade water (Qiagen)	to 50 µl

PCR cycling was carried out under the following conditions:

5 min @ 95°C	1 cycle
30 s @ 95°C 30 s @ X°C 25 X min @ 72°C	cycles
10 min @ 72°C	1 cycle

PCR products were analysed by agarose gel electrophoresis as described in section 2.8 once cycling was complete.

2.19 Screening of clones

Single colonies were selected and inoculated into 10 ml LB supplemented with the appropriate antibiotics (100 µg ml^{-1} amp; used for pPAL7, and 5 µg ml^{-1} erm; used for pLSV101). The inocula were grown overnight at 30-37ºC, 200 rpm and 2 ml of the overnight culture were used for plasmid DNA isolation as previously described in section 2.7. Clones were verified by restriction endonuclease analysis using the isolated recombinant plasmid DNA to ascertain presence of the inserted gene. The restriction products were visualised on 0.5-1% agarose gels.

2.20 Restriction endonuclease digest of DNA fragments

Specific restriction endonuclease enzymes were used to digest DNA fragments or genomic DNA. The reaction mixture contained 10X digestion buffer, DNA to be digested, restriction enzyme(s) and dH_2O was added up to 20 µl. The reaction mixture was incubated in the PCR machine 2-16 h at 37ºC, to avoid condensation and to ensure that all reaction components are in the bottom of the tube.

2.21 Ligation

For cloning, PCR products were ligated 3:1 to the similarly digested plasmid vectors by using T4 DNA ligase. The reaction mixture contained DNA fragments, 10X ATP, ligation buffer and T4 DNA ligase. For the recovery of Tn*917* from *L. monocytogenes* genomic DNA, isolated genomic DNA was digested overnight at 37ºC using restriction enzymes internal to the transposon, and the digestion product was self ligated using the same components as above. Ligation reactions were prepared overnight at 4ºC or 1 hr at room temperature.

2.22 Overexpression of the recombinant proteins

Plasmid vector pPAL7 was ued to facilitate protein expression by the use of T7 lac promoter. This plasmid confers Profinity eXact tag located directly upstream of the cloned gene to facilitate co-expression and recognition of the tag for precise cleavage and purification. Amp-resistant gene, origin or replication, lacI gene, T7 lac promoter and terminator and MCS are shown on the map (figure 2.1). To overexpress recombinant proteins, the recombinant plasmid DNA was first transformed into chemically competent *E. coli* BL21 (DE3) cells and the transformants were selected on LB agar plates supplemented with 100 µg ml^{-1} amp. Vector DNA only was also transformed into *E. coli* BL21 (DE3) as a control.

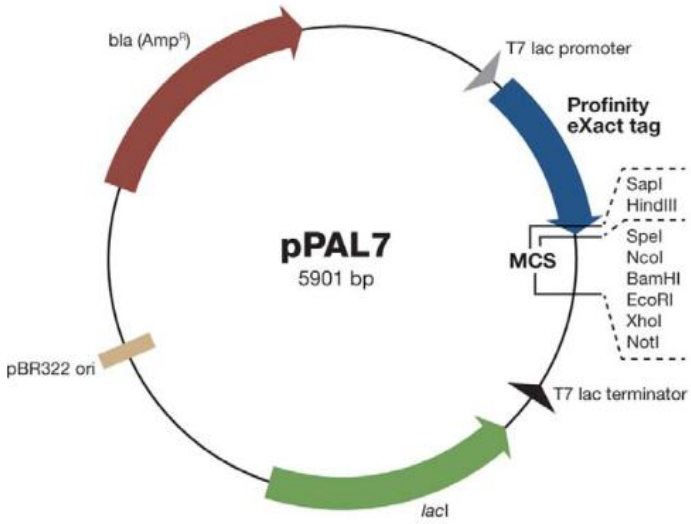


Figure 2.1 Map of the expression-plasmid vector used in this study**.**

An overnight culture of *E. coli* BL21 (DE3) harbouring recombinant plasmid or the vector only were inoculated into LB broth supplemented with 100 µg ml^{-1} amp. The cultures were grown at 37ºC with shaking (200 rpm) until the OD_{600} reached 0.4-0.6. An aliquot of 1 ml was removed from each culture and the remaining cells were induced with a final concentration of 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) (Bioline) and grown for 3-6 h or overnight. Samples were taken from each culture and the OD_{600} was measured to equalise the number of cells. From each, an appropriate volume was transferred to a fresh microcentrifuge

tube and cells were pelleted at 15,000 rpm for 5 mins at 4ºC. Cells were resuspended in 0.2 ml TE buffer containing 50 µl of 10X BugBuster (Merck4Biosciences, UK). The samples were vortexed vigorously for 20 sec followed by centrifugation at 15,000 rpm for 5 mins, at 4ºC. Supernatant was mixed with 2X SDS sample buffer (1M Tris; pH 6.8, 10% SDS, 50% glycerol, 1% bromophenol blue, 1M DTT, prepared in dH_2O) and loaded on SDS-PAGE gel as described in section 2.6.

2.23 Determination of recombinant protein solubility

The solubility of the recombinant protein was assessed by overexpression at 20-37ºC together with varying concentrations of IPTG (0.4-1 mM) in 10 ml LB broth supplemented with 100 µg ml^{-1} amp. When overexpressing at temperature lower than 25ºC, the induced cells were incubated overnight (~16 h), while a 3-6 h induction period was sufficient at 25ºC and above. After induction, 1 ml aliquots of induced cells were pelleted by centrifugation at 15,000 rpm for 5 mins, 4ºC. The supernatant was discarded and the pellet was resuspended in 0.2 ml TE buffer containing 1x BugBuster, followed by vortexing and further centrifugation at 15,000 rpm for 15 mins at 4ºC. The supernatant was transferred to a fresh tube (soluble fraction), whereas the cell debris were resuspended in 0.2 ml TE buffer (insoluble fraction). Aliquots (10 µl) of the soluble and insoluble fractions were analysed by SDS-PAGE as described in section 2.6.

2.24 Enzymatic activity assay

The activity of the enzymes exist in the bacterial lysate against progesterone and β-estradiol was measured by following the oxidation of β-NADPH to β-NADP at a wavelength of 340 nm. Firstly, both WT *L. monocytogenes* and a mutant lacking *namA* gene were grown overnight at 37ºC in TSB (200 rpm). From which, ten-fold serial dilutions were prepared and cfu ml^{-1} was determined to equalise the number of cells to be lysed. Approximately 1 ml of the overnight culture was spun for 5 min at 15,000 rpm and pellet was washed with 0.5 ml PBS. Cells were resuspended in 0.5 ml TE buffer and incubated for 30 min at 37ºC with lysozyme at a concentration of 3 mg ml^{-1}. A final

concentration of 1 mg ml^{-1} of protinaseK was also added to the mixture and further incubated for 30 min. Lysed cells were spun at 15,000 rpm for 5 min (4ºC), and supernatant was kept on ice and used immediately for the enzymatic activity. The reaction was initiated by adding bacterial lysate to the reaction mixture that contains β-NADPH (Sigma-Aldrich) at a concentration of 100-500 µM, substrate (100 µM of progesterone, β-estradiol or 2-cyclohexene-1-one as a positive control) in a total volume of 1 ml reaction buffer (potassium phosphate, pH7.0). The mixture was prepared in a quartz cuvette and the assay was performed at room temperature at 340 nm for 1 h.

2.25 Bioinformatics analyses

Sequences were obtained from the NCBI database and subjected to BLAST analyses using either the BLASTp or BLASTx algorithm (Benson *et al*., 2009) as appropriate. For multiple alignments of sequences, the ClustalW alignment tool (Larkin *et al*., 2007) and UGENE software were used.

2.26 Statistical analysis

Listeira monocytogenes growth differences in the presence and absence of pregnancy hormones and bacterial enzymatic assays were statistically analysed by the use of statistical software Minitab®17.1.0 (Lead Technologies, Inc), where one way ANOVA (Analysis of Variance) and paired t-test were performed.

Table 2.2 List of primers used in this study.

Name	Sequence (5'→3')	Details	Tm(°C)	Restrict. site
Tn*917*F	ACC TCC GAA GAT ACA CGC C	Specific for internal region of Tn*917-LTV3*	58.8	-
Tn*917*R	TTG TCG GCG AAT ATC GCG CTC	Specific for internal region of Tn*917-LTV3*	61.8	-
NamA-pal7F	GGA TGA **AAG CTT** TAA TGT CAA AAT TAT TTT CAG	Specific for cloning of NamA for expression	62	HindIII
NamA-pal7R	CTT **GGA TCC** TTA TTT CCA CGC GCG TG	Specific for cloning of NamA for expression	66	BamHI
*Lmo2470*A	TTA TTA CGT T**GG ATC C**AA GTT CTA ACC	Specific for upstream region of *NamA*-F	70	BamHI
*Lmo2470*B	GA**A GAT CT**A AAC TCT GAA CTT CCA GC	Specific for upstream region of *NamA*-R	74	BglII
*Lmo2472*C	GA**A GAT CT**C TAA TTC GCA TCC TTT TC	Specific for downstream region of *NamA*-F	72	BglII
*Lmo2472*D	CAC G**GA ATT C**TA GAG TAT TAT CGT AC	Specific for downstream region of *NamA*-F	72	EcoRI
Tn*917*-seq	CTC ACA ATA GAG AGA GAT GTC ACC G	Specific for sequencing of Tn*917*-recovered DNA	74	-
NamA-F	GAA TGG CAC TTG ATA ACC TGG	Specific for RNA gene expression-internal to *namA*	62	-
NamA-R	GCT ACT GAC TAT GCA CAT GG	Specific for RNA gene expression- internal to *namA*	60	-
Lmo2235-F	CAA ACG GAA CAA CGC AAC TTC G	Specific for RNA gene expression- internal to *lmo2235*	66	-
Lmo2235-R	GAA GTC CAT TCA AAC GTG CTG G	Specific for RNA gene expression- internal to *lmo2235*	66	-
Lmo0489-F	TGT GGG TCG TTT CAC AGA GC	Specific for RNA gene expression- internal to *lmo0489*	62	-
Lmo0489-R	CTC GTC TAG TTT TCC TGC TGC	Specific for RNA gene expression- internal to *lmo0489*	64	-

Chapter III

Influence of pregnancy hormones on the growth of *Listeria monocytogenes*

3.1 Introduction

The foodborne pathogen, *L. monocytogenes,* affects individuals with reduced cell-mediated immunity and causes devastating complications. The disease manifests primarily as abortion, septicaemia, or central nervous system (CNS) infections with an increased fatality rate among infected persons (Benshushan *et al*., 2002). During pregnancy, where levels of pregnancy hormones (i.e. progesterone and β-estradiol) are elevated (Rettew *et al*., 2010), pregnant women have increased susceptibility to infections such as malaria, measles, toxoplasmosis, leprosy (Hansen disease) and listeriosis (Jamieson *et al*., 2006). Alterations in pregnancy hormonal levels have been shown to directly contribute to host susceptibility to invading microbes by local immonosuppression and increased state of maternal immunocompetence (Devonshire *et al*., 2003).

In short, it is well documented that *L. monocytogenes* threaten the health condition of pregnant women (Benshushan *et al*., 2002); thus, it is thought that specific factors found in pregnant women may play an important role for the increased risk of listeriosis during pregnancy. The increased risk of listerial infection during pregnancy has shed light on several factors that might be directly/indirectly involved in the outcome of listeriosis, such as compromised immunity (Devonshire *et al*., 2003) and elevated levels of pregnancy hormones (Kaushic *et al*., 2011). As it can be seen, there is a clear evidence that pregnancy hormones have a role in the increased susceptibility and progress in bacterial infections in human and animal models of infection (Rettew *et al*., 2010). Since it is also known that pregnancy hormones influence the growth of several microbes (Table 3.1), which results in increased/decreased levels of microbial infection, it is anticipated that pregnancy hormones might also influence the growth of *L. monocytogenes*. Currently, it is not completely understood whether pregnancy hormones have a direct proliferative effect on *L. monocytogenes*. Hence, this study was undertaken to investigate the role of progesterone and β-estradiol on the growth of *L. monocytogenes in vitro* under both aerobic and anaerobic conditions.

Table 3.1 *In vitro* influence of steroid hormones on microbial growth

Type	Organism	Hormone	Effects	Reference
Fungus	*Candida albicans*	Estradiol	Increased fungal virulence	Madani *et al*. 1993
	Saccharomyces cerevisiae	Estradiol	Inhibited growth	Feldman *et al*. 1984
	Paracoccidioides brasiliensis	Estradiol	Blocked conversion to invasive form	Salazar *et al*. 1988
	Trichophyton mentagrophytes	Progesterone	Extended the lag phase	Clemons *et al*. 1989
	Trichophyton gypseum	Testesterone	Inhibited growth	Casas-Campillo *et al*. 1961
	Trichophyton puroureum	Testesterone	Inhibited growth	
Protozoa	*Trichomonas vaginalis*	Estradiol	Inhibited growth	Sugarman and Mummaw 1988
Gram positive bacteria	*Lactobacillus spp.*	Progesterone, estradiol	increased growth	Pelzer *et al*., 2012
	Bacillus thetaiotaomicron	Progesterone	Inhibited growth	Kornman and Loesche, 1981
	Nocardia asteroides	Ethyl estradiol	Inhibited growth	Casas-Campillo *et al*. 1961
	Staphylococcus spp.	Progesterone	Inhibited action of virulence factor	Yotis and Savov, 1969
	Staphylococcus spp	Androgen	Inhibited growth	Yotis And Fitzgerald, 1968
Gram negative bacteria	*Bacteroides melaninogenicus*	Progesterone, Estradiol	Increased growth, and can substitute vitamine k	Kornman and Loesche, 1982
	Bacteroides gingivali			
	Escherichia coli	Estradiol, Progesterone	Inhibited growth when efflux pumps-genes were knocked out	Elkins and Mullis, 2005
	Neisseria meningitidis	Progesterone	Inhibited growth	Miller and Morse, 1976
	Neisseria gonorrhoeae	Progesterone	Inhibited growth	
Acid fast Bacteria	*Mycobacterium tuberculosis*	Corticosterone	Inhibited growth	Casas-Campillo *et al*. 1961

3.2 Results

3.2.1 Influence of progesterone and β-estradiol on the growth of *L. monocytogenes* under aerobic conditions

To ascertain whether pregnancy hormones progesterone and β-estradiol have any growth proliferation effects on *L. monocytogenes*, experiments were conducted to assess the growth of *L. monocytogenes* aerobically during exposure to each of these hormones. A fresh bacterial culture was incubated overnight with shaking at 37ºC, and this was used to inoculate freshly prepared TSA medim with/without hormone. Bacterial cells were exposed to different concentrations of each hormone separately and growth curve was constructed for each based on the number of viable cells obtained at each time point. Each of the hormones was used at concentrations of 0, 20, 40 and 60 µg ml^{-1} (0, 63.6, 127.2 and 190.8 µmol/L, respectively) and the growth was monitored over a period of 12 h. The number of viable cells were determined every 3 h by performing a 10-fold serial dilution and a 10 µl-volume was spotted on TSA agar followed by static (aerobic) overnight incubation at 37ºC under aerobic conditions.

Growth of *L. monocytogenes* during exposure to pregnancy hormones under aerobic condition

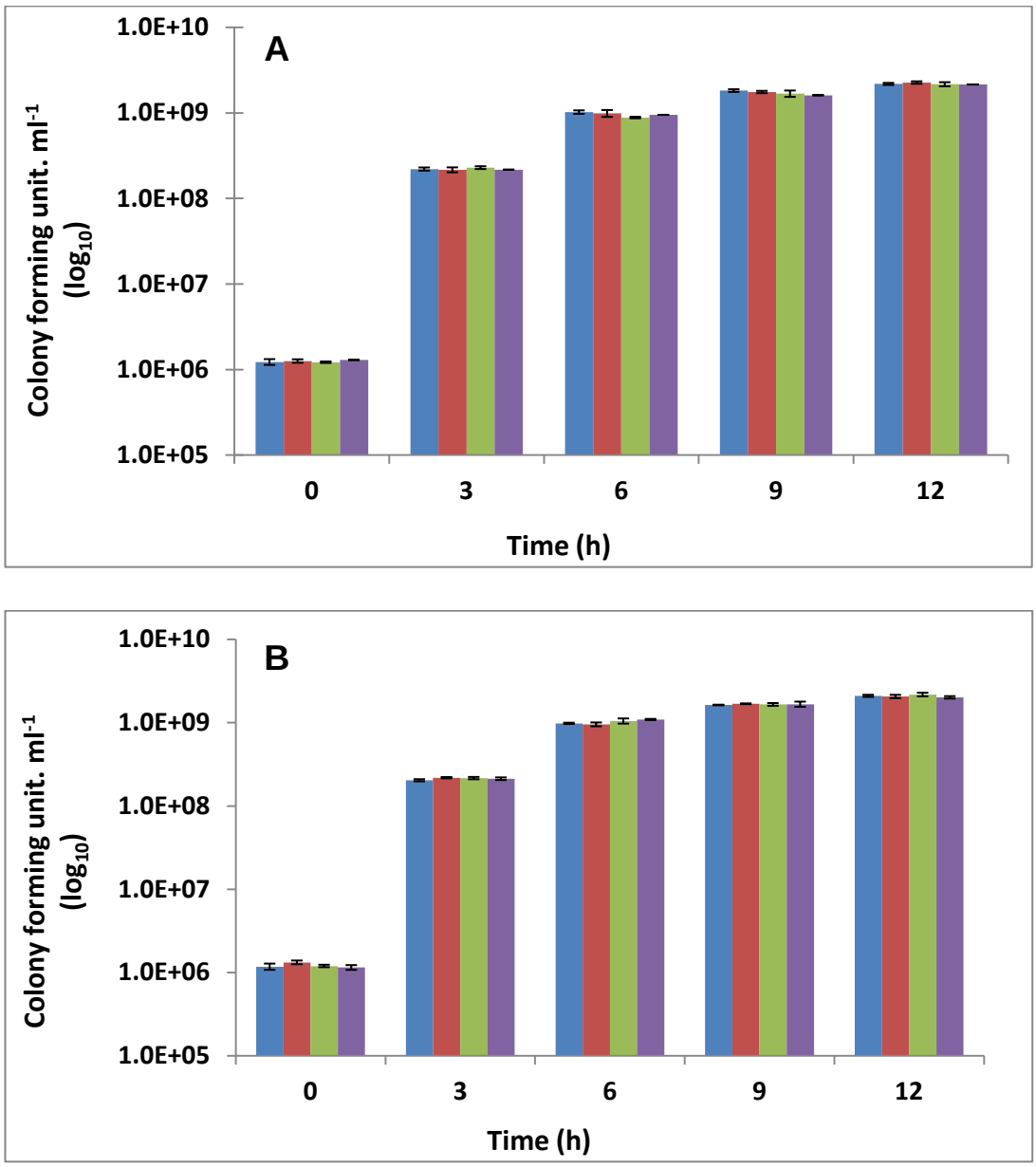


Figure 3.1 Growth curves of *L. monocytogenes* EGD-e during exposure to pregnancy hormones. Bacteria were incubated aerobically at 37°C, with shaking (200 rpm) in the presence of **(A)** progesterone and **(B)** β-estradiol at final concentrations of 0 μg ml^{-1}, 20 μg ml^{-1}, 40 μg ml^{-1} and 60 μg ml^{-1}. Bacterial growth was monitored for 12 h by Miles and Misra technique of counting the viable colony forming units, and the mean of three independent experiments was plotted on the graph (p-value for all 4 samples at 12 h=0.248 and 0.233; for graph A and B respectively). The number of viable cells is showing in a 10-based logharithmic scale. Error bars show the standard error of the mean, (n=9).

According to the data obtained, the growth of *L. monocytogenes* was not affected in the presence of either progesterone or β-estradiol under aerobic condition. From each treatment, viable count of the cells was comparable to the cell count of the control (Ethanol only). The same results were repeated when this experiment was performed 3 times in 3 different occasions. Statistical analysis indicates that no significant finding was observed in each treated group ($p>0.05$; CI= 95%) Therefore, it can be concluded that neither of the hormones has an effect on the growth of *L. monocytogenes* under the conditions of this experiment. Since *L. monocytogenes* is a facultative bacterium, similar experiments were conducted under anaerobic conditions (tissue-physiological level of oxygen *in vivo* is only 3%, Morrison *et al*., 2000) to investigate whether any of the hormones has an effect on *L. monocytogenes* when grown anaerobically. The work station (anaerobic cabinet) operates on one cylender of conventional anaerobic gas mixture of 10% hydrogen, 10% CO_2 and 80% N_2. Growth curves were monitored as described above.

3.2.2 Influence of progesterone on the growth of *L. monocytogenes* under anaerobic conditions

To assess the effect of progesterone on the growth of *L. monocytogenes* under anaerobic conditions, cells were incubated with different concentrations of progesterone. Firstly, only one bacterial culture was incubated overnight (~15h) anaerobically without the hormone. From this, a dilution of 1:200 was inoculated into each medium with/without progesterone, to give approximate inoculums of 1.0E+06 cfu ml^{-1}. Cultures were incubated statically (anaerobic) at 37ºC, and each culture was used to perform a 10-fold serial dilution that was spotted on TSA agar. The growth of *L. monocytogenes* was monitored by counting of viable colony forming units at the time points: 0, 6, 8, 10, 12 and 24 h. The time *Listeria* is taking to start replicating (lag phase) under anaerobic condition is between 3 and 6 h; hence, time points were selected based on that. The mean of 3 different expreriments was plotted on the graph.

Growth of *L. monocytogenes* during exposure to progesterone under anaerobic condition

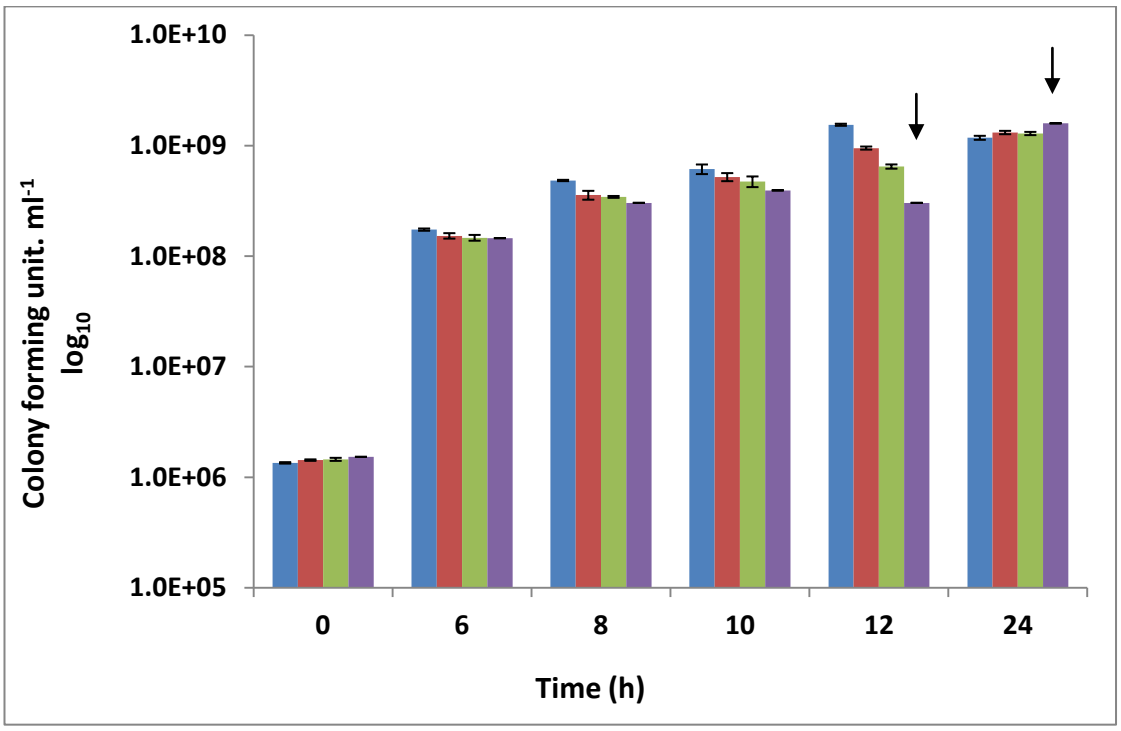


Figure 3.2 Growth curve of *L. monocytogenes* growing anaerobically during exposure to progesterone. *L. monocytogenes* EGD-e was incubated at 37ºC in the presence of progesterone at concentrations of: 0 μg ml^{-1}, 20 μg ml^{-1}, 40 μg ml^{-1} and 60 μg ml^{-1}. Growth was monitored by viable count of the cells over a period of 12 h plus the cell count at 24 h. Arrows show the highest effect (12 h) and the restoration observed (24 h). The mean of three independent experiments was plotted on the graph (p-value for samples at 12 h and 24 h =0.001 and 0.13; respectively). The number of viable cells is showing in a 10-based logharithmic scale. Error bars show the standard error of the mean, (n=9).

It was observed that bacterial growth was aignificantly affected by the presence of progesterone in a dose-dependent manner ($p<0.05$; CI=95%). Cells treated with increasing concentrations of progesterone showed a greater retardation in bacterial growth and this was most evident at the 12 h time point post incubation. The cell count at 12 h-point from cultures exposed to 20, 40 and 60 μg ml^{-1} progesterone showed 1.5-fold, 2.5-fold and 3.5-fold decrease; respectively, when compared to the control.

Data obtained, demonstrated that the bacterial cultures exposed to progesterone had been negatively influenced during the first 12 h of incubation in the presence of different concentrations of the progesterone, and this effect was dose-dependent. However, the negative effect of the hormone was reversed between 12 and 24 h post incubation as can be determined by the number of viable cells at 24 h point. In fact, progesterone-treated bacteria resumed a growth comparable to the control culture in the second half of the exposure.

In order to investigate whether pre-treatment with progesterone would change the rate of bacterial growth, cells previously exposed to different concentrations of progesterone were back diluted to fresh media containing the same concentrations of the hormone. Briefly, *L. monocytogenes* was grown anaerobically at 37ºC overnight. From this culture, all TSB media containing progesterone at final concentrations of 0, 20, 40, 60 µg ml^{-1} were inoculated with similar inoculums and cultures were incubated anaerobically at 37ºC for 24 h. Next day, similar inoculums from each treated culture was used to inoculate fresh TSB media containing the same final concentrations of progesterone as above, followed by further incubation at the same conditions. Bacteria were monitored only for 12 h since the dose-dependent effect was most evident at this time point. The viable count of the cells was determined as previously described.

Anaerobic pre-exposure of *L. monocytogenes* to progesterone

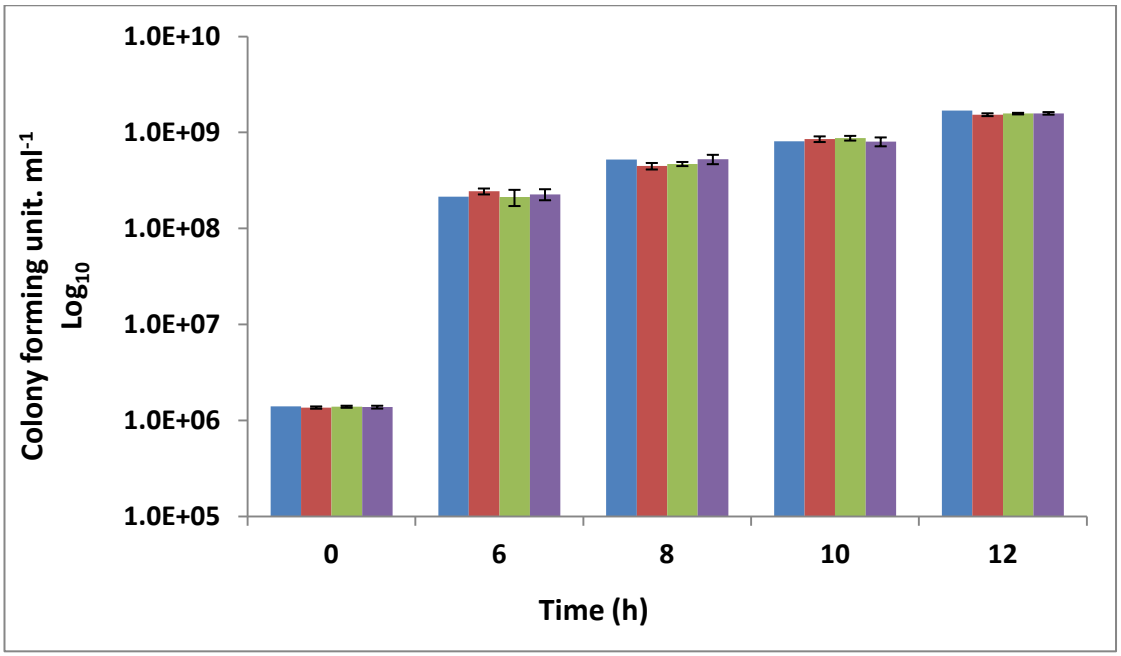


Figure 3.3 Growth curve of *L. monocytogenes* pre-exposed to progesterone. Listerial cells were incubated with different concentrations of progesterone for 24 h. TSB media contained progesterone at final concentrations of 0 μg ml^{-1}, 20 μg ml^{-1}, 40 μg ml^{-1} and 60 μg ml^{-1}. Post 24 h of anaerobic incubation at 37ºC, each culture was 1:200 back diluted into fresh medium supplemented with the same concentrations of progesterone. New cultures were further incubated at similar conditions. Bacterial growth was monitored by viable cfu count over a period of 12 h and the mean of three independent experiments was plotted on the graph (p-value for samples at 12h point = 0.09). Cells are plotted in log_{10} scale. Error bars show the standard error of the mean, (n=9).

It was observed that growth of *L. monocytogenes* was influenced by the presence of progesterone when bacteria were grown anaerobically at 37ºC as determined by the number of viable cells, and the effect was dose-dependent. The negative effect of progesterone on bacterial growth was not observed when cells were pre-exposed to progesterone. All treated cultures, including the control, showed similar growth during 12 h period of exposure. Pre-treatment with progesterone abolished the negative effect of progesterone that was observed when bacteria were exposed for the first time. Therefore, it is of interest to investigate weather pre-exposure of the cells to lower progesterone

concentration would result in a complete adaptation to higher concentration of the same hormone. Hence, *L. monocytogenes* was first exposed to progesterone at a final concentration of 20 µg ml^{-1} and the culture was incubated anaerobically at 37ºC for 24 h. From this, a fresh TSB media supplemented with progesterone at final concentrations of 20 and 60 µg ml^{-1} were inoculated and the cultures were incubated at similar conditions. Bacterial growth was monitored as previously described.

Adaptation of *L. monocytogenes* to progesterone at lower concentration

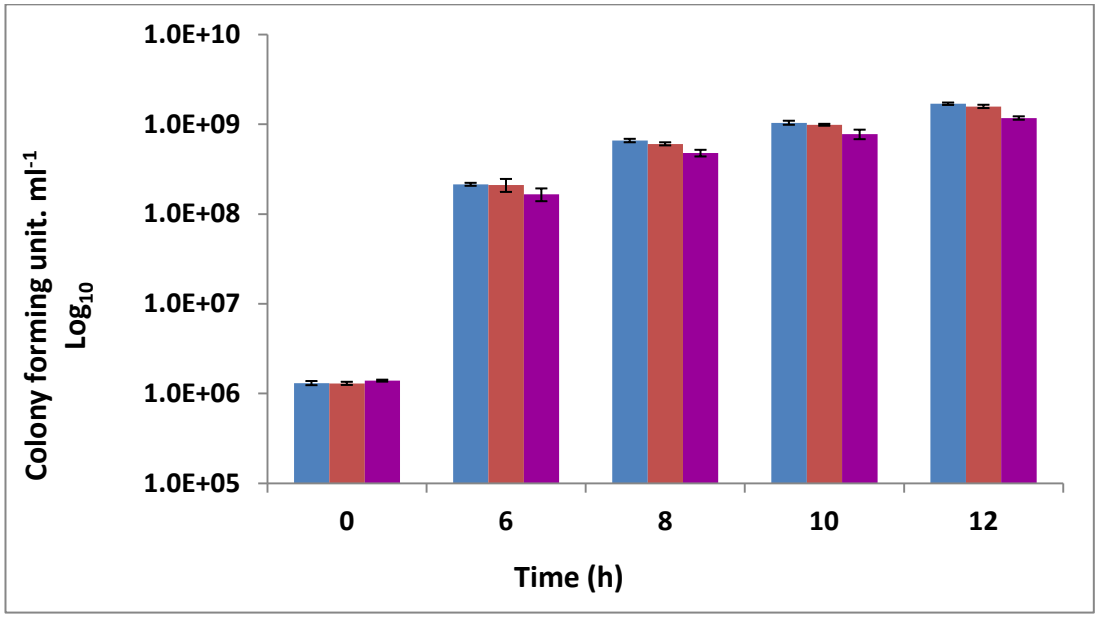


Figure 3.4 Growth curve of *L. monocytogenes* pre-exposed to progesterone. Listerial cells were pre-exposed to progesterone at a final concentration of 20 µg ml^{-1} and grown anaerobically at 37ºC for 24 h. From this, similar number of cells was used to inoculate fresh TSB supplemented with progesterone at concentrations of: 0 µg ml^{-1}, 20 µg ml^{-1} and 60 µg ml^{-1}. Bacterial growth was monitored by viable cfu count over a period of 12 h and the mean of three independent experiments was plotted on the graph (p-value for samples at 12 h =0.088). Cells are plotted in log_{10} scale. Error bars show the standard error of the mean, (n=9).

L. monocytogenes pre-exposed to 20 µg ml^{-1} showed similar growth to the control when cells were back diluted into fresh TSB supplemented with the same concentration of progesterone. Whereas cells back diluted to higher concentration (60 µg ml^{-1}) showed relatively unsignificant reduction in growth as determined by the number of viable colony forming units ($p>0.05$; Cl=95%). The number of cells exposed to progesterone at a concentration of 60 µg ml^{-1} was only decreased ~1.3-fold when compared to the control at 12 h point.

To further assess the negative effects of progesterone on the growth of *L. monocytogenes* and the adaptation observed in previous results (Figures 3.3 and 3.4). Progesterone at a final concentration of 60 µg ml^{-1} was added at different time points to the media. Briefly, bacteria were grown anaerobically at 37ºC overnight. From this, similar number of cells was used to inoculate TSB media and cells incubated at 37ºC for 24 h under anaerobic conditions. Each culture was supplemented with progesterone at different time point. Culture 1, received ethanol only as a control, culture 2 was supplemented with progesterone at 0 h, culture 3 was supplemented with progesterone at 12 h, and culture 4 was supplemented with progesterone at 0 and 12 h during incubation (double addition). The viable count of the cells was determined as previously described. The growth of *L. monocytogenes* was monitored during exposure to progesterone at a final concentration of 60 µg ml^{-1}. Ethanol only was equally added to each culture; hence, all cultures were identical during exposure except for the hormone.

Growth of *L. monocytogenes* in the presence of progesterone added during incubation

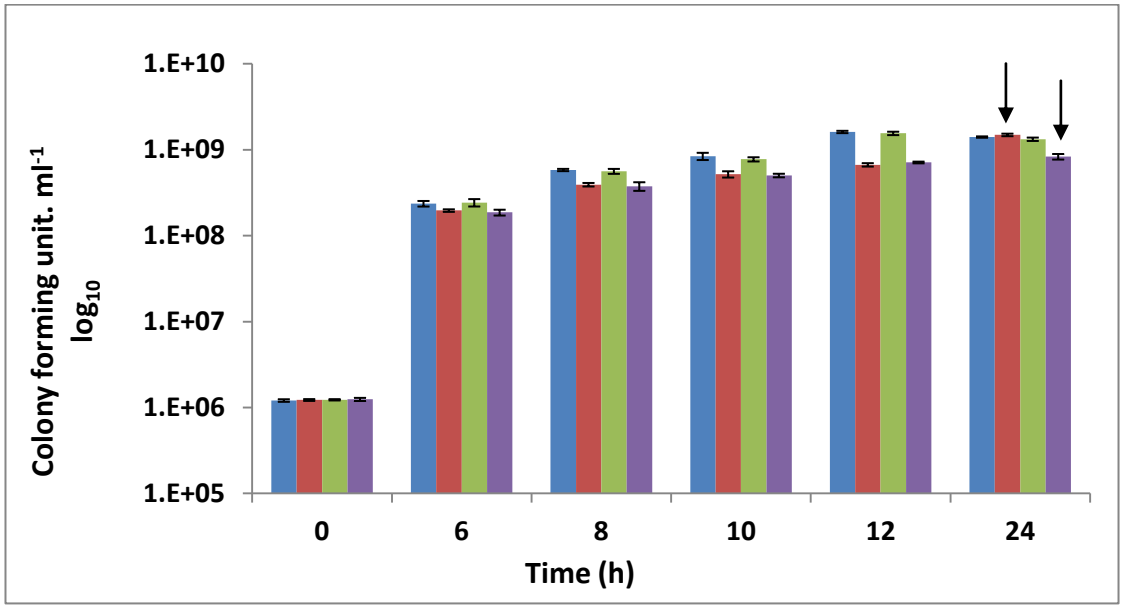


Figure 3.5 Growth curve of *L. monocytogenes* during exposure to progesterone added at the beginning and 12 h post-incubation. Bacterial cells were incubated in TSB media supplemented with progesterone at a final concentration of 60 µg ml^{-1}. Each culture received progesterone at different time as following: culture 1 ; control, culture 2 ; 0 h, culture 3 ; 12 h, culture 4 ; 0 and 12 h. Bacterial growth was monitored by viable cfu count over a period of 24 h and the mean of three independent experiments was plotted on the graph (p-value for samples at 12h and 24h =0.004 and 0.12; respectively). Arrows show the growth-restored cells; red, and the growth-slowed cells; purple. Cells are plotted in log_{10} scale. Error bars show the standard error of the mean, (n=9).

At 12 h time point, it was observed that cells received no progesterone in the first 12 h (culture 3) showed similar growth to the control, while cells received progesterone at 0 h time point (cultures 2 and 4) showed an approximately 3.5-fold reduction in the number of cells at 12 h post incubation. At 24 h time point, however, the least number of viable cells corresponds to culture 4 that received progesterone twice, while culture 2 and 3 showed little difference when compared to the control. From the data obtained, it is clear that progesterone negatively affects the growth of *L. monocytogenes* when cells are exposed to the hormone for the first time. Whereas cells pre-exposed to

the hormone grew similar to the control cells. It was also observed that adaptation to progesterone is time-dependent since adding of progesterone to cells already exposed to the hormone (culture 4), yet resulted in reduction in growth as determined by the number of cells at 24 h, ($p>0.05$; Cl=95%).

3.2.3 Influence of β-estradiol on the growth of *L. monocytogenes* under anaerobic conditions

To investigate the effect of β-estradiol on the growth of *L. monocytogenes*, cells were exposed to different concentrations of the hormone and growth was monitored during exposure by viable count of the cells. Briefly, bacterial cells were grown overnight at 37ºC. From this, a similar number of bacterial cells were used to inoculate TSB media supplemented with β-estradiol at final concentrations of: 0, 20, 40 and 60 µg ml^{-1} and cultures were incubated under anaerobic conditions for 12 h. Growth was monitored during exposure and the mean of 3 independent experiments was calculated and used to plot the graph.

Influence of β-estradiol on *L. monocytogenes* under anaerobic condition

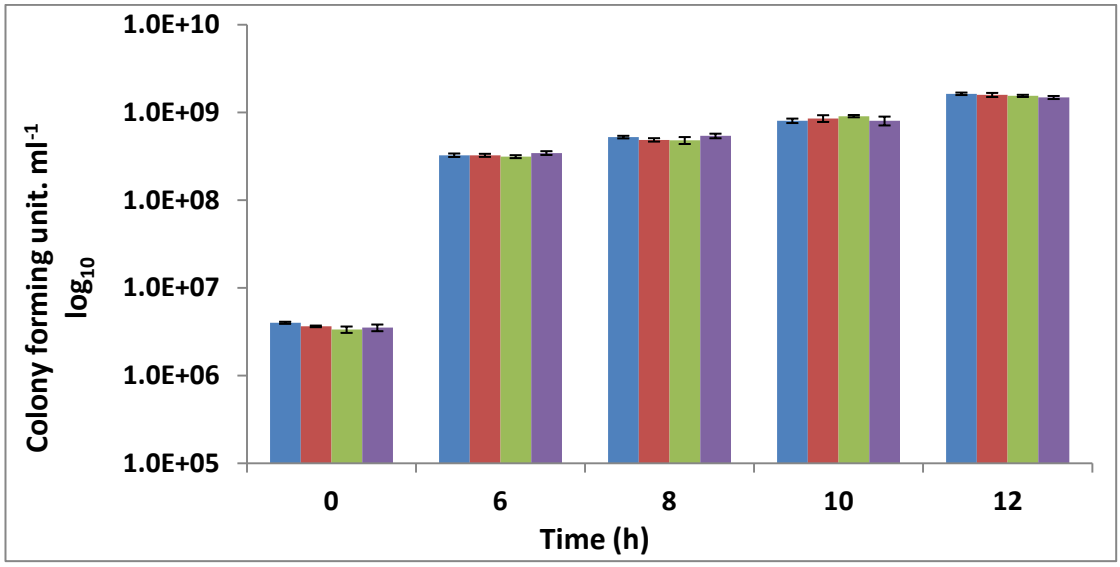


Figure 3.6 Growth curve of *L. monocytogenes* during exposure to β-estradiol. *L. monocytogenes* EGD-e was cultured anaerobically at 37°C in the presence of β-estradiol at different concentrations. 0 µg ml^{-1}, 20 0 µg ml^{-1}, 40 0 µg ml^{-1} and 60 0 µg ml^{-1}. Growth rate was monitored by viable count of the cells over a period of 12 h and the mean of three independent experiments was plotted on the graph (p-value at 12=0.155). Error bars show the standard error of the mean, (n=9).

From the data obtained, it was observed that *L. monocytogenes* grown under anaerobic conditions was not affected by the addition of β-estradiol as determine by the viable count of the cells ($p>0.05$; CI=95%). All cultures showed similar growth during the exposure period.

In order to investigate whether *L. monocytogenes* possess a mechanism involved in the phenotype observed with progesterone exposure through specific hormone-related genes, a transposon-related approach was conducted to identify genes with possible roles in hormones adaptation.

3.2.4 Generation of *L. monocytogenes* mutants using Tn*917*-LTV3

Generation of transposon-mediated mutagenesis in *L. monocytogenes* DP-L910 (contains Tn*917*-LTV3) was performed as described in chapter 2 section 13. The number of tetracycline sensitive colonies and presumed recipients of the transposon were ascertained by comparison of bacteria that grew on medium containing either erm, lin, tet or erm, lin only (Figure 3.7).

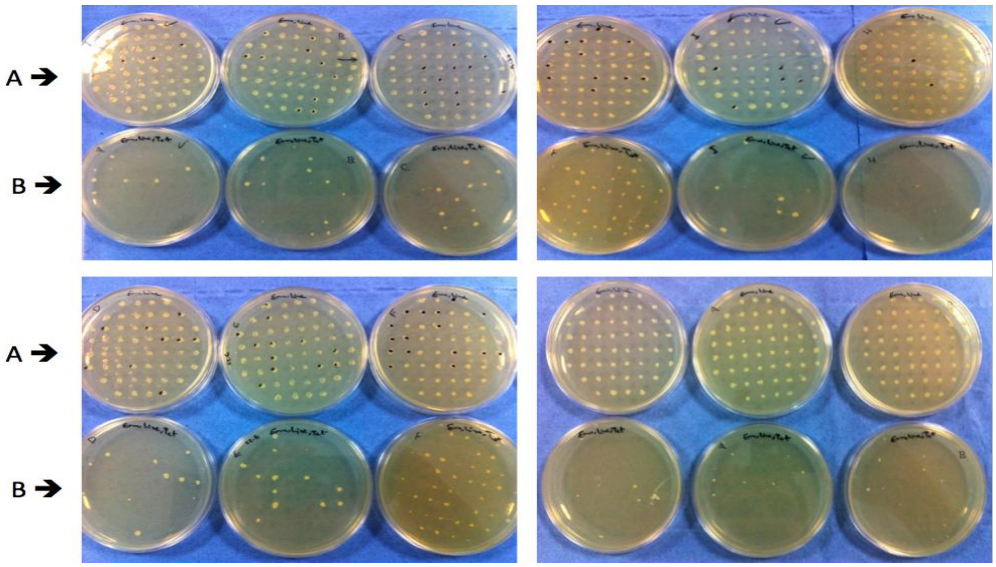


Figure 3.7 Transposon insertion mutants of *L. monocytogenes* DP-L910. Single colonies grown at the non-permissive temperature for plasmid replication were used to replica patch onto TSA agar supplemented with **(A)** erm, linc only **(B)** erm, linc and tet. Colonies were incubated at 43ºC overnight. Sensitive colonies to tet indicates presumed successful transposition into *L. monocytogenes* chromosomal DNA**.**

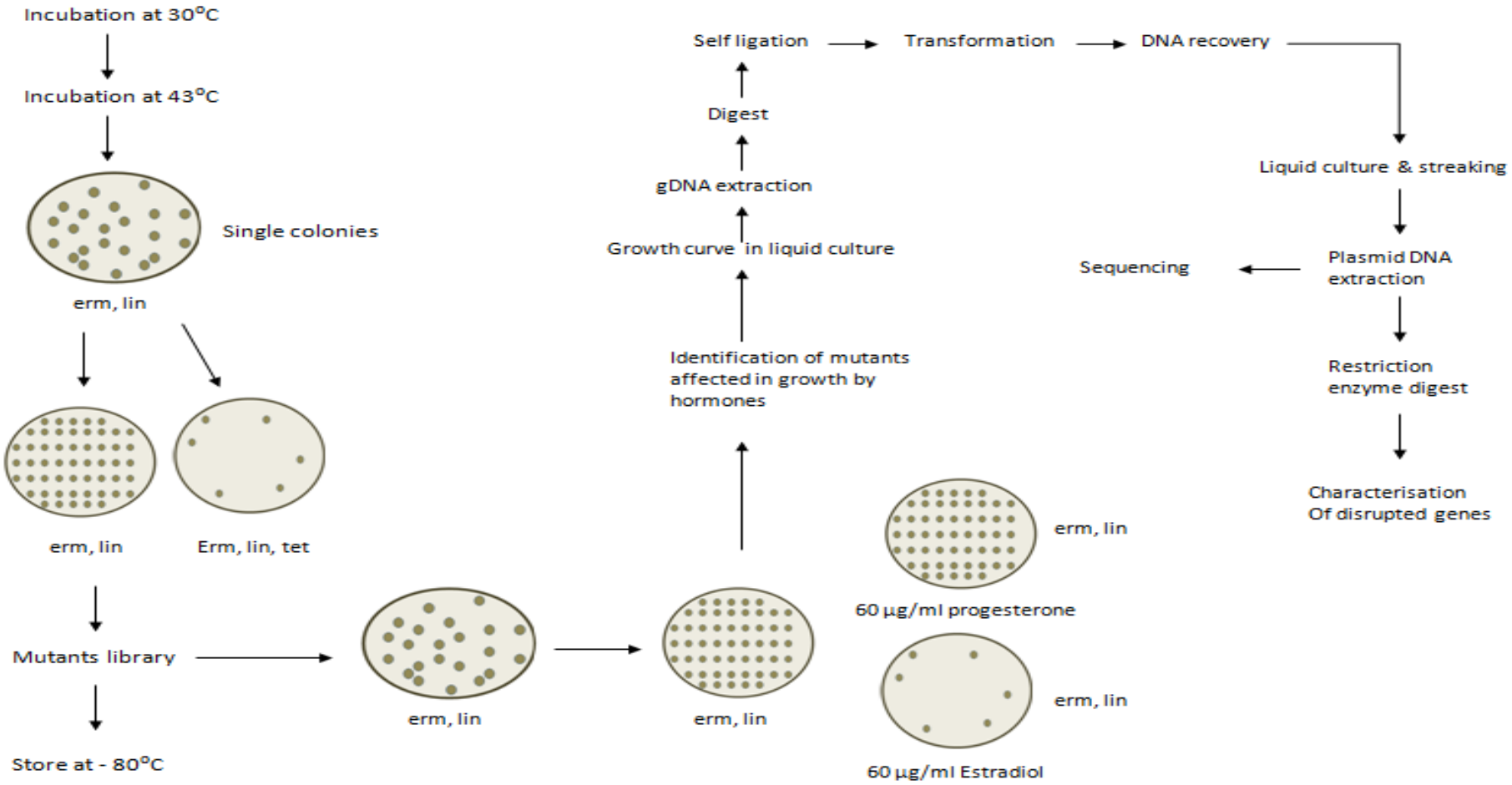


Figure 3.8 Schematic diagram illustrating the steps involved in the mutant generation and the identification of Tn*917*-interrupted genes.

The percentage of tet-sensitive colonies obtained was greater than 76% (480 of 624 colonies). To confirm that transposon insertion mutants were genuine, PCR was used to amplify a specific fragment internal to Tn*917*-LTV3. Genomic DNA was purified from 10 randomly chosen clones, which were erm, lin resistant but tet sensitive, indicating that they had lost the plasmid. The Tn*917*-LTV3 fragment was amplified by PCR using *L. monocytogenes* chromosomal DNA and pLTV3 plasmid as negative and positive controls, respectively (Figure 3.9).

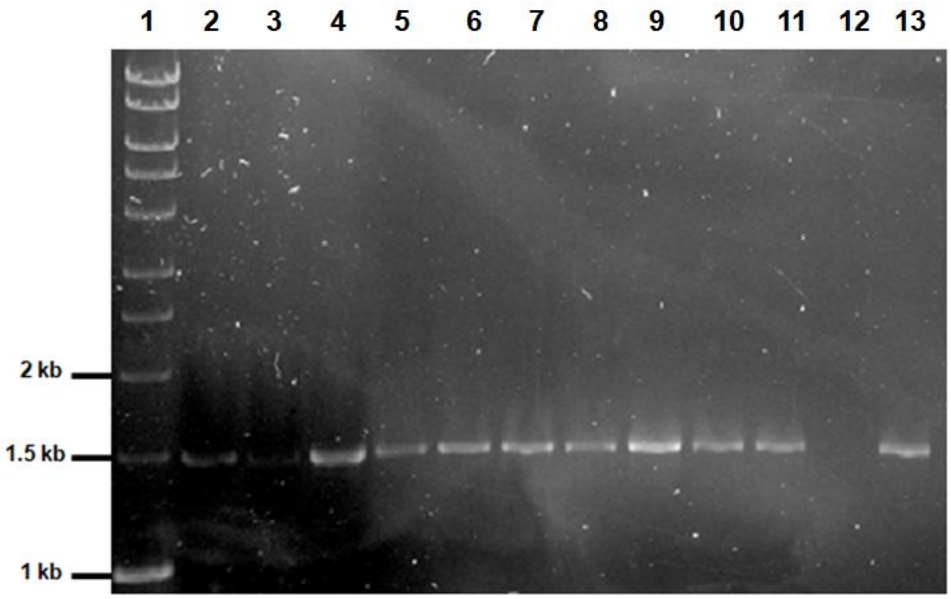


Figure 3.9 PCR Confirmation of Tn*917*-LTV3 insertion into *L. monocytogenes* chromosomal DNA. PCR was performed to amplify a Tn*917*-specific fragment from genomic DNA isolated from 10 randomly chosen mutants using specific primers internal to the transposon. Lane 1: molecular weight marker (HLI); lanes 2-11: PCR product isolated from genome DNA of mutants; lane 12: No PCR product from genomic DNA isolated from wild type *L. monocytogenes* (negative control); lane 13: PCR product from pLTV3 plasmid (positive control).

In each of the mutants selected, a 1.5 kb fragment was amplified which was identical to the fragment obtained from the plasmid pLTV3. This presumes that Tn*917*-LTV3 had inserted into the genome in each of the clones. Given that these clones were also tetracycline sensitive, we can assume that pLTV3

has been lost while the transposon has integrated successfully into the genome. Bacterial culture of which transposition has occurred was mixed with 50% glycerol and stored at - 80ºC in 1 ml aliquouts for later use. This hereby; reffered to as mutant libraries.

3.2.5 Primary screening of mutant libraries for potential clones with response to pregnancy hormones

To simplify mutant-screening procedure, a number of 9000 (~ triple the number of listerial genes) colonies from the mutant libraries were screened on solid TSA agar supplemented with 60 µg ml^{-1} of either progesterone or β-estradiol, and cells were incubated anaerobically as described in chapter 2, section 14. Although growth of *L. monocytogenes* was not influenced by β-estradiol under both aerobic and anaerobic condition as shown in previous experiments, listerial cells were yet screened in the presence of β-estradiol since mutation in certain genes with the transposon could cause *L. monocytogenes* to become sensitive to the presence of the hormone. Single colonies were replica patched onto screening plates (control and hormone-supplemented agar), and incubated statically at 37ºC under anaerobic conditions (anaerobic work station). By visual comparison of clones on both plates (control and hormone-supplemented medium), it was observed that progesterone had no effect on bacterial growth as determined by the size of colonies grown on each plate. Colonies from the control plate were comparable in diameter to the cells grown in hormone-supplemented media, which suggests no effect of progesterone. In contrast to progesterone, a number of 13 different mutants were sensitive to the presence of β-estradiol at a concentration of 60 µg ml^{-1} and the size of each was largely reduced when compared to the size of the corresponding colonies grown without the hormone. All 13 potential mutants were propagated in 10 ml TSB media supplemented with erm and lin only, mixed with equal volume of 50% glycerol, and stored at -80ºC in 1 ml aliquots for later use.

3.2.6 Recovery of Tn*917*-integrated chromosomal DNA from *L. monocytogenes* and identification of interrupted genes

Potential mutants were cultured in 10 ml TSB media for genomic DNA isolation and further identification of listerial-integrated genes by restriction digest of each genomic DNA with the restriction enzyme *XbaI* (Figure 3.10). This enzyme cuts within the transposon downstream of ColE1 origin of replication once and at any of *XbaI*-specific site within the chromosomal gene adjacent to the transposon, which simplifies recovery by subsequent self ligation.

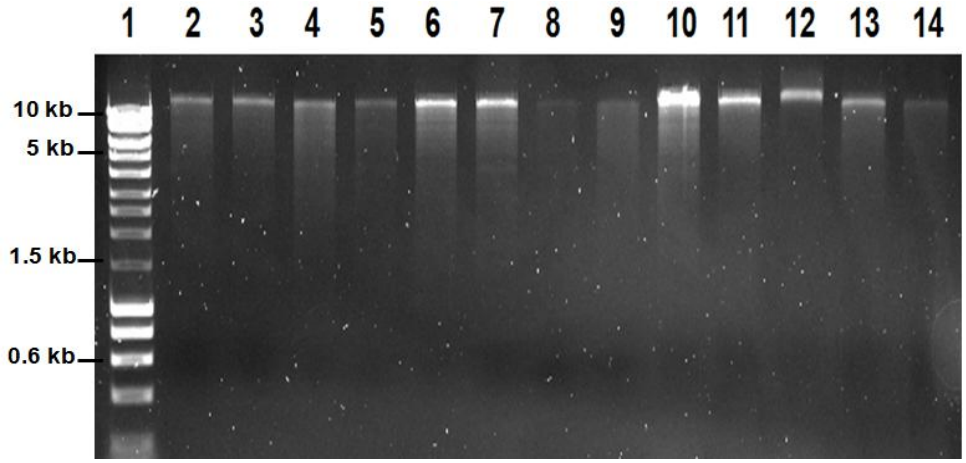


Figure 3.10 Restriction digest of *L. monocytogenes* genomic DNA containing the integrated Tn917. Genomic DNA from each of the 13 potential mutants was isolated and further digested with XbaI, which cuts within the transposon downstream of the ColE1 origin of replication. Lane 1: molecular weight marker; lanes 2-14: digested genomic DNA from potential mutants 1-13 in order.

Genomic DNA was added to the restriction digest reaction at a volume of 17 µl (37-71 µg μl^{-1}) and the reaction was set at 37ºC overnight in the PCR machine. The restriction enzyme was heat-inactivated at 65ºC for 20 min, and the reaction products were used for self ligation using T4 DNA ligase. Each of the ligation mixture was used to transform *E. coli* α-select competent cells for propagation and DNA isolation. Kanamycin at a final concentration of 50 µg ml^{-1} was used to select for clones with successful transformation of the Tn*917*-containing DNA, and isolated plasmids were further digested with *SalI* for

confirmation of transposon insertion and identification of the DNA size recovered from each mutant (Figure 3.11).

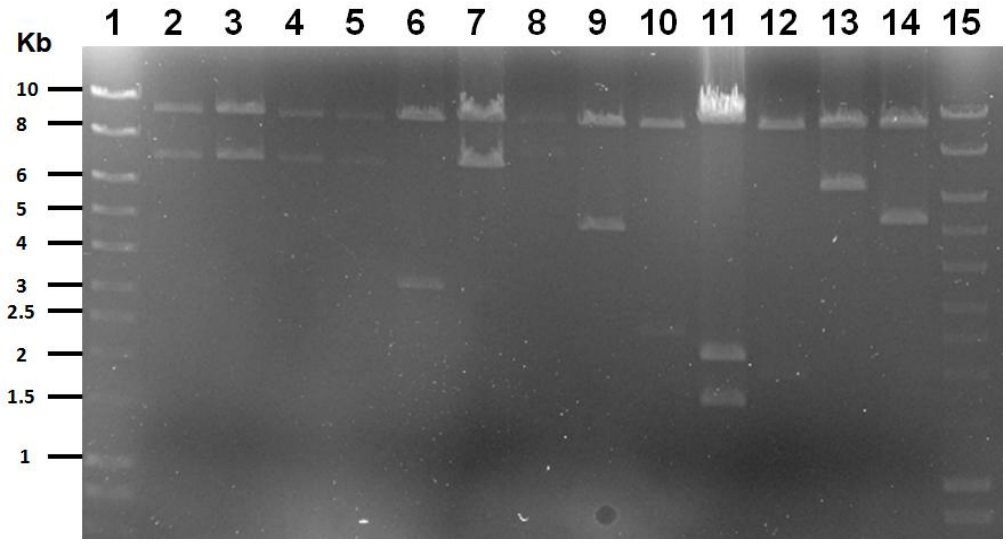


Figure 3.11 Restriction digest of Tn*917*-harbouring plasmid DNA. Isolation of plasmid DNA containing integrated Tn*917*-transposon from transformed *E. coli* was digested with SalI at 37ºC overnight. Products from restriction digest reactions were run on 0.5% agarose gel.

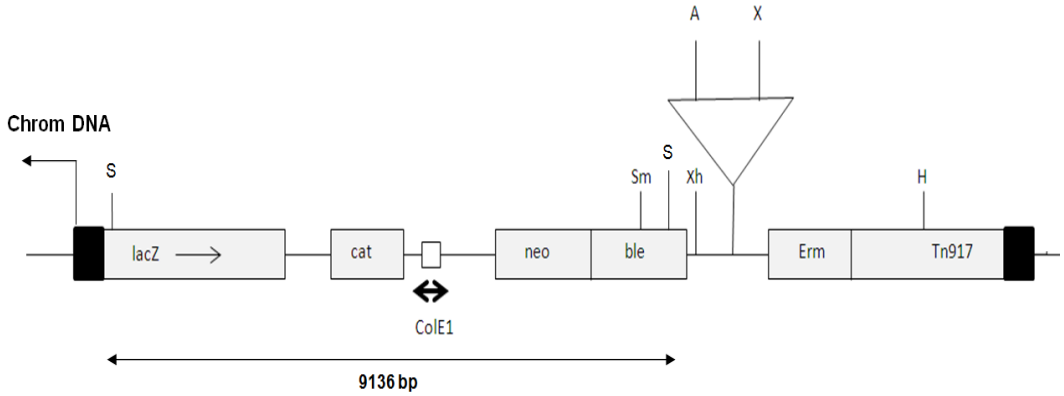


Figure 3.12 Hypothetical chromosomal insertions of Tn*917*-LTV3. In this case, a transcriptional fusion between the transposon-containing lacZ gene and a chromosomal DNA. Unique restriction sites in the transposon, which can be used to clone adjacent sequence flanking the left end of the transposon, are shown. Restriction endonuclease abbreviations: *HindIII* (H), *XbaI* (X), *Asp718* (A), *XhoI* (Xh) and *SmaI* (Sm), *SalI* (S). Antibiotic resistance genes: *erm*, erythromycin-lincomycin; *ble*, bleomycin; *neo*, neomycin-kanamycin; *cat*, chloramphinicol. Arrow at the proximal end of the transposon indicates the location of chromosomal DNA recovered. ColE1 box indicates the origin of replication.

DNA fragments post digestion, Lanes 1 and 15: molecular weight marker (HLI); Lanes 2-14: DNA fragments from mutants 1-13 in order. Large DNA fragment in lanes 2-14 (9136 bp) correspond to the predicted size of the digested transposon (Figure 3.11 – 3.12). Whereas other DNA fragments on the gel correspond to the recovered chromosomal DNA from *L. monocytogenes*. Lanes 2-5, 7 and 8: 9136 bp plus ~7000 bp; Lane 6: 9136 bp plus ~3100 bp; Lane 9: 9136 bp plus ~4500 bp; Lane 10: 9136 bp plus ~2100 bp; Lane 11: 9136 bp plus ~1900 bp and ~1500 bp; Lane 12: 9136 bp; Lane 13: 9136 bp plus ~6000 bp; Lane 14: 9136 bp plus ~5500 bp. Sequences recovered from all potential mutants were Blastx-searched against all protein sequences in the NCBI database; and all strains of *L. monocytogenes* proteins in particular. All 6 reading frames of the genes at the site of integration were analysed for the coding sequence and to identify the exact site of transposition (Figures 3.13 - 3.20).

Furthermore, DNA concentrations were measured (>150 µg μl^{-1}) and sent for sequencing using Tn*917*-seq primer (Table 2.2). The recovered sequences (ranging from 700-1100 bp) were analysed by matching with reference sequences in the database (National Centre for Biotechnology Information; NCBI), and interrupted genes were identified by sequence similarity to query sequence.

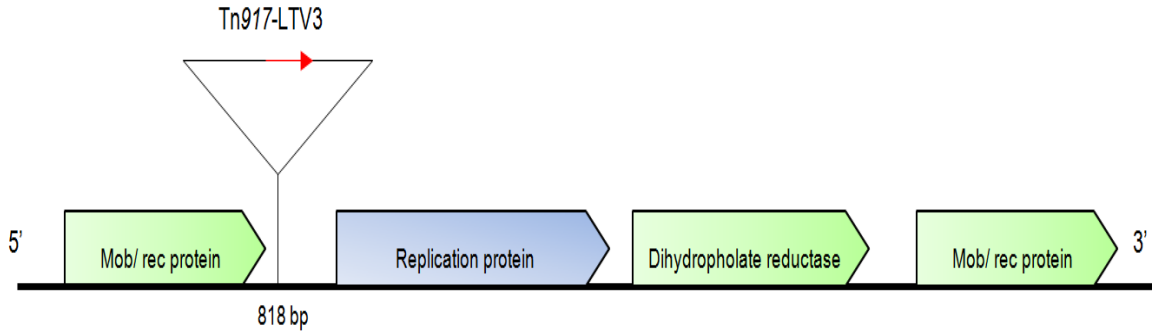


Figure 3.13 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutants 1-4, 6 and 7 showed that Tn*917* transposon was inserted in the protein replication operon. Multiple insertions into the exact site.This operon comprises of 4 genes; mobilisation/recombination protein, replication protein, dihydropholate reductase and mobilisation/recombination protein. Tn*917*-transposon was inserted in the plus strand at 818 bp upstream of the replication protein (blue arrow).

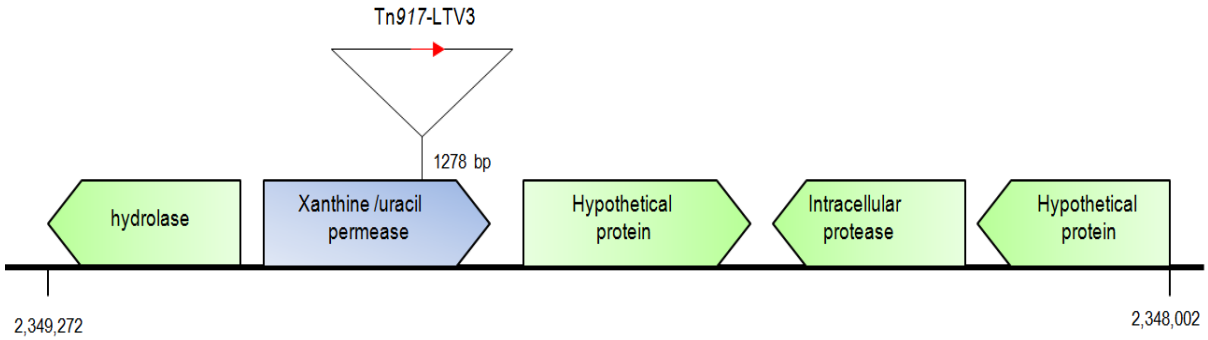


Figure 3.14 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 5 showed that Tn*917* transposon was inserted in the xanthine/uracil permease-coding gene (blue arrow). Tn*917*-transposon was inserted in the plus strand at 1278 bp downstream of the gene start codon.

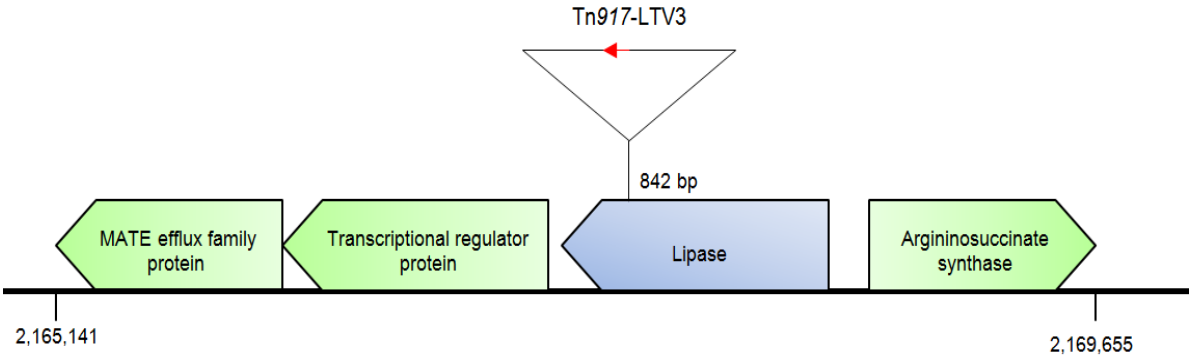


Figure 3.15 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 8 showed that Tn*917* transposon was inserted in the lipase-coding gene (blue arrow). Tn*917*-transposon was inserted in the minus strand at 842 bp upstream of the gene start codon.

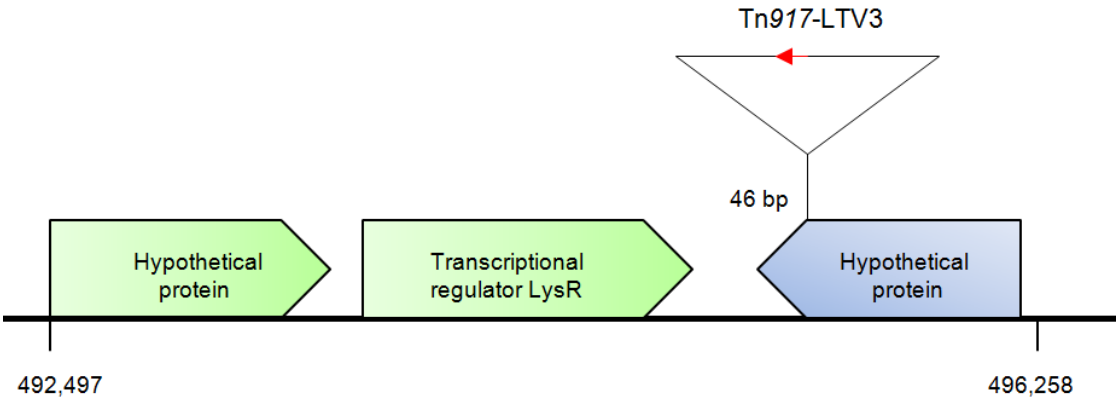


Figure 3.16 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 9 showed that Tn*917* transposon was inserted in a hypothetical protein-coding gene (blue arrow). Tn*917*-transposon was inserted in the minus strand at 46 bp upstream of the gene stop codon.

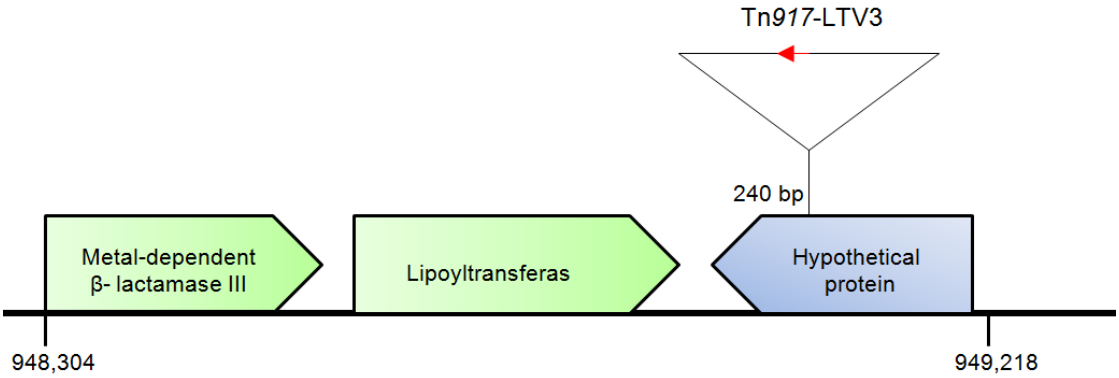


Figure 3.17 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 10 showed that Tn*917* transposon was inserted in a hypothetical protein-coding gene (blue arrow). Tn*917*-transposon was inserted in the minus strand at 240 bp downstream of the gene start codon.

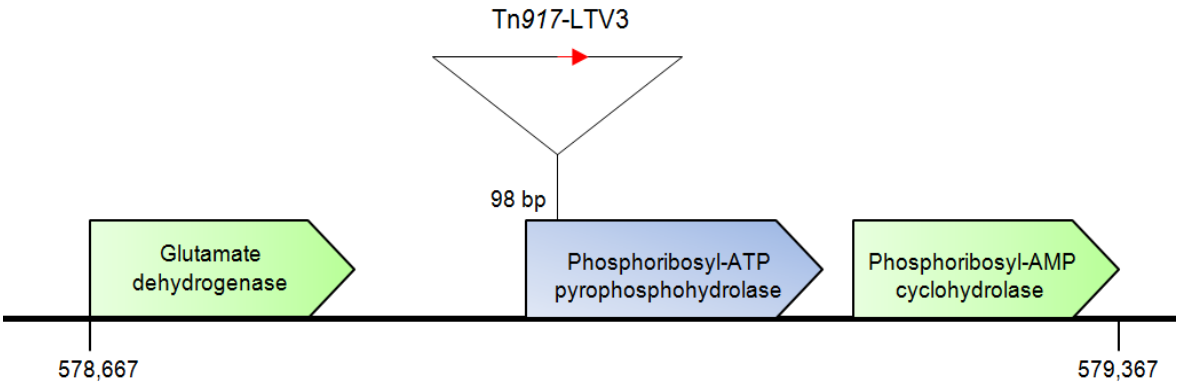


Figure 3.18 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 11 showed that Tn*917* transposon was inserted in a phosphoribosyl-ATP pyrophosphohydrolase-coding gene (blue arrow). Tn*917*-transposon was inserted in the plus strand at 98 bp downstream of the gene start codon.

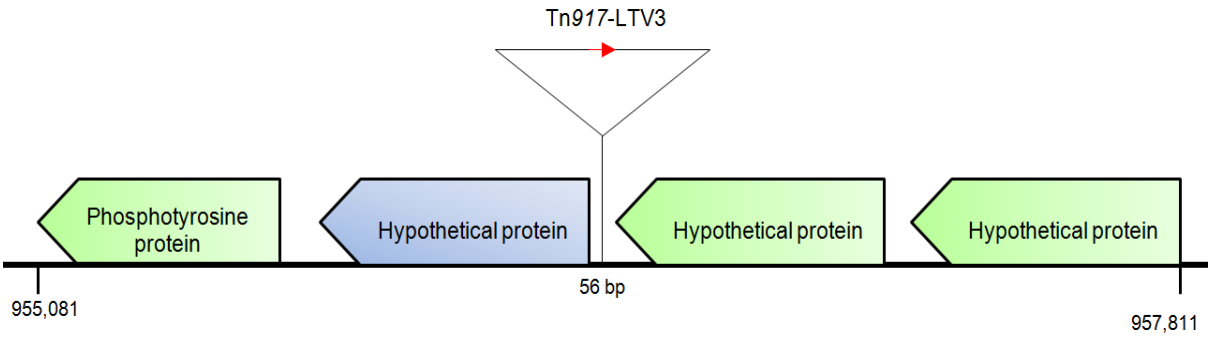


Figure 3.19 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 12 showed that Tn*917* transposon was inserted in the plus strand at 56 bp downstream of the start codon of a hypothetical protein-encoding gene (blue arrow).

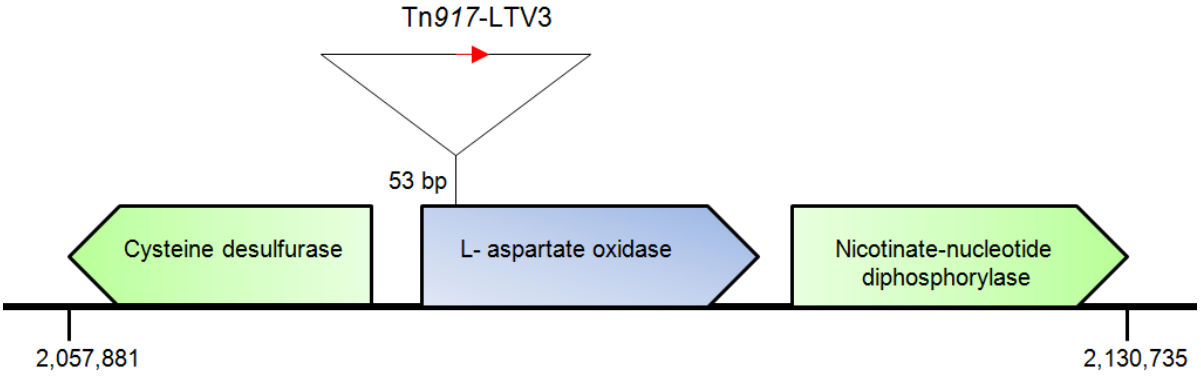


Figure 3.20 Physical map of *L. monocytogenes* interrupted gene. Analysis of sequences recovered from mutant 13 showed that Tn*917* transposon was inserted in the plus strand at 53 bp downstream of the start codon of a L-aspartate-oxidase-encoding gene (blue arrow).

Analysis of recovered sequences using blastx option in the NCBI database revealed a number of previously identified genes in *L. monocytogenes* (5 different genes) and 3 others hypothetical proteins with no similarity in the database to other organisms-encoded proteins. All query sequences showed a minimum of 98% similarity to sequences aligned from the database with a significantly small E-value (1E-115 to 1E-95) and total query coverage of 96-100%.

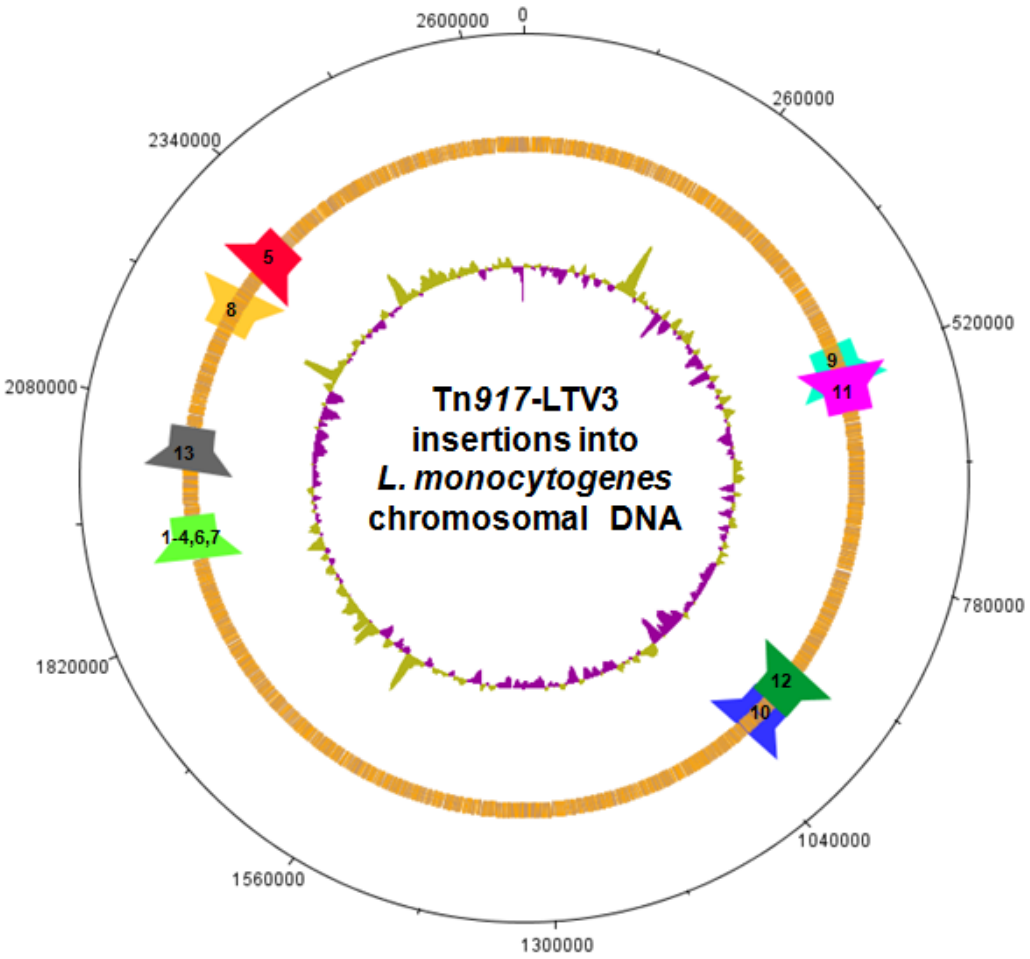


Figure 3.21 Physical map of Tn*917*- insertion sites in *L. monocytogenes* chromosomal DNA.

Transpson insertion pattern was distributed within the chromosome. It is unclear whether the transposon had integrated into sequence-favourable spots or a DNA secondary structure of the genome played a role in this phenotype (Garsin *et al*., 2004). Arrows indicate the sites of integration with 1-4, 5, 6, 7, 11, 12 and 13 in the plus strand, whereas 8, 9 and 10 in the minus strand. Light green arrow; 1-4, 6 and 7 represents mutants with integration at the replication protein operon, red arrow; number 5: xanthine/uracil permease-coding gene, yellow arrow; number 8: lipase-coding gene. Turquoise arrow; number 9: a hypothetical protein-coding gene, blue arrow; number 10: a hypothetical protein-coding gene, pink arrow; 11: a phosphoribosyl-ATP pyrophosphohydrolase-coding gene, dark green arrow; 12: a hypothetical protein-coding gene, grey arrow; 13: a L-aspartate-oxidase-encoding gene.

3.2.7 Secondary screening of Tn*917*-derived mutants

L. monocytogenes mutants with Tn*917*-chromosomal integration were further assessed to confirm their defect in growth in the presence of β-estradiol as previously observed in the primary screening. All mutants were grown in liquid cultures in the presence of β-estradiol at a final concentration of 60 µg ml^{-1} and growth was monitored for 24 h by viable count of the cells under anaerobic conditions. The number of viable cells was determined at 0, 6, 10 and 24 h intervals during exposure to the hormone. The mean of 3 independent experiments was used to construct the graphs.

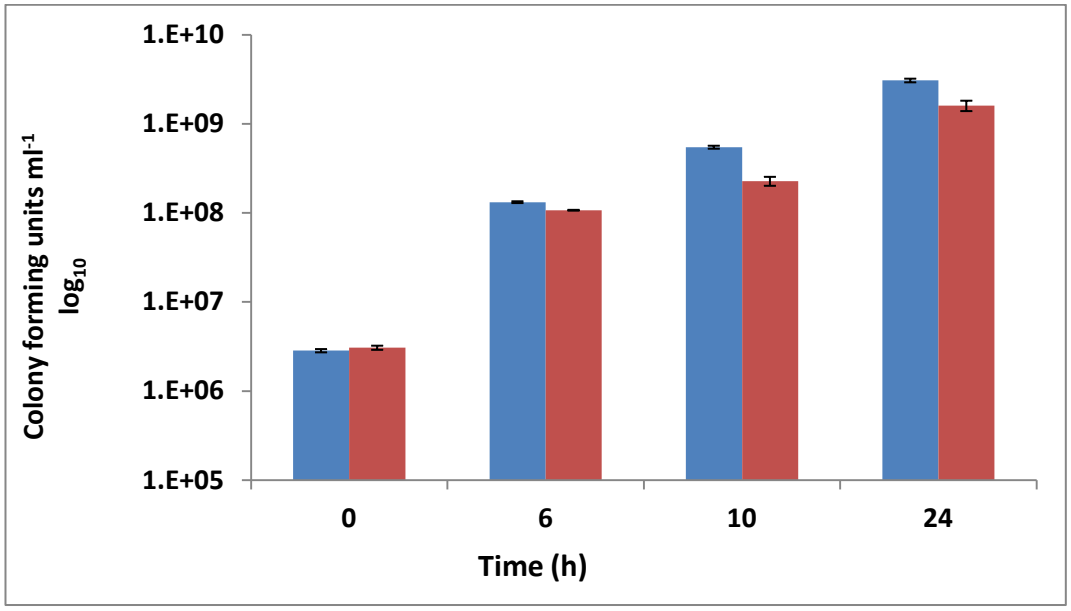


Figure 3.22 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutants with Tn*917*-integration in rep operon (M 1-4, 6 and 7) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h=0.04) Error bars show the standard error of the mean (n=9). This is only one set of representative data since all mutants are identical.

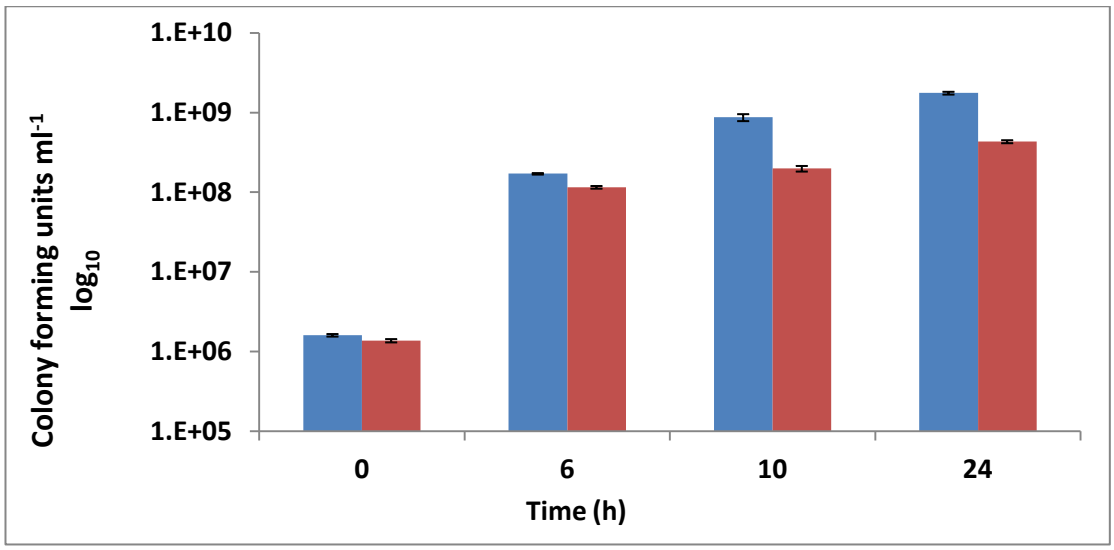


Figure 3.23 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in the gene coding for xanthine/uracil permease (M5) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.02). Error bars show the standard error of the mean (n=9).

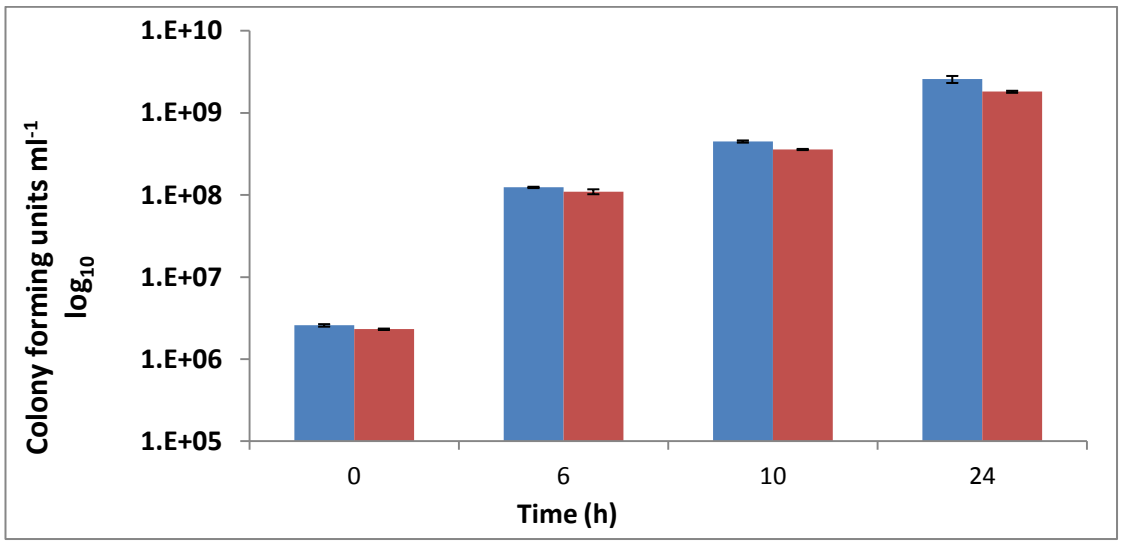


Figure 3.24 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in lipase-coding gene (M8) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.056). Error bars show the standard error of the mean (n=9).

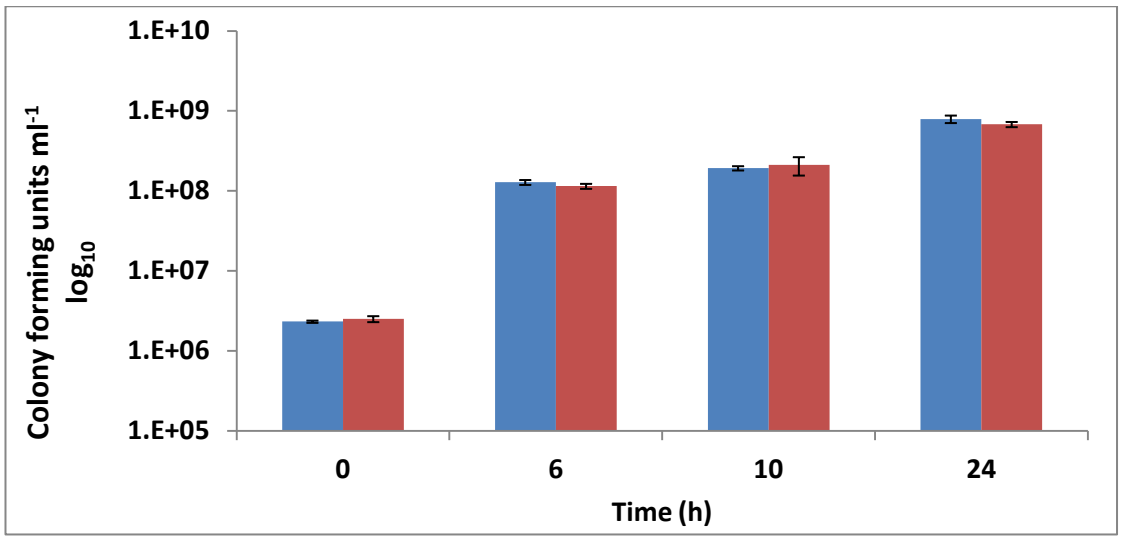


Figure 3.25 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in the gene coding for a hypothetical protein (M9) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.233). Error bars show the standard error of the mean (n=9).

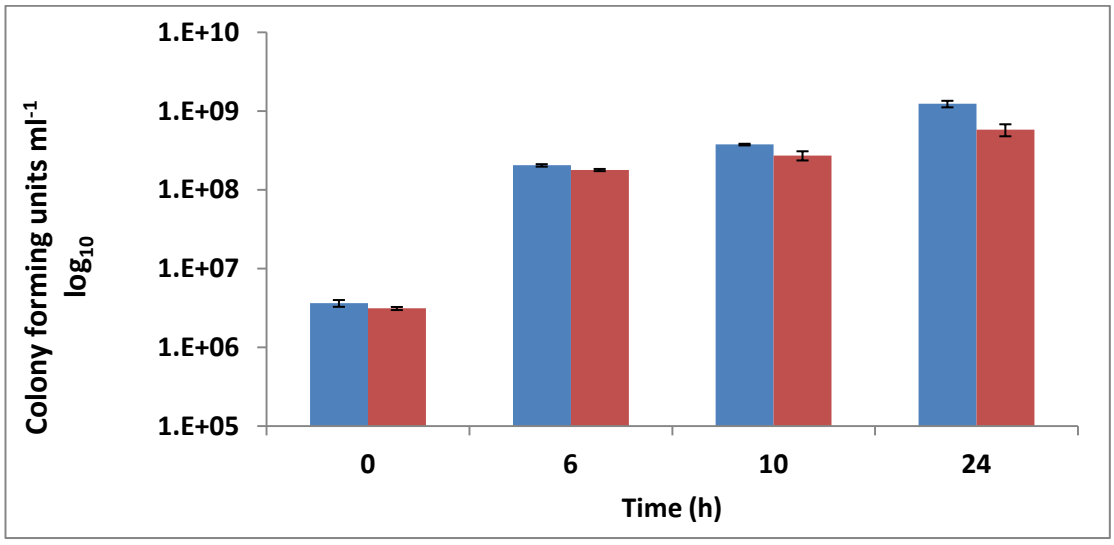


Figure 3.26 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in the gene coding for a hypothetical protein (M10) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.034). Error bars show the standard error of the mean (n=9).

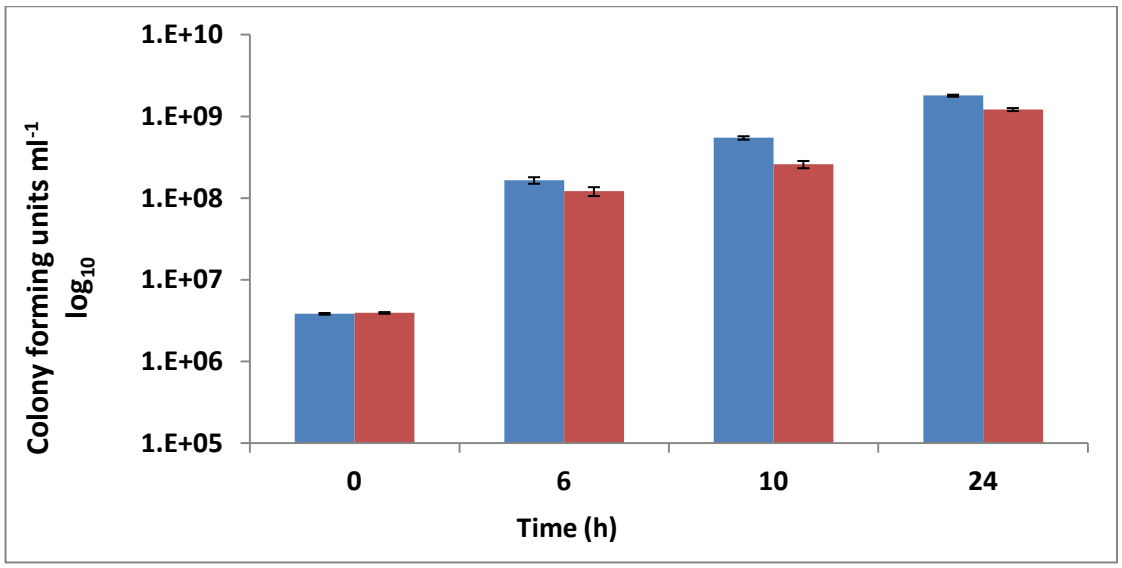


Figure 3.27 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in the gene coding for a phosphoribosyl-ATP pyrophosphohydrolase (M11) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.061). Error bars show the standard error of the mean (n=9).

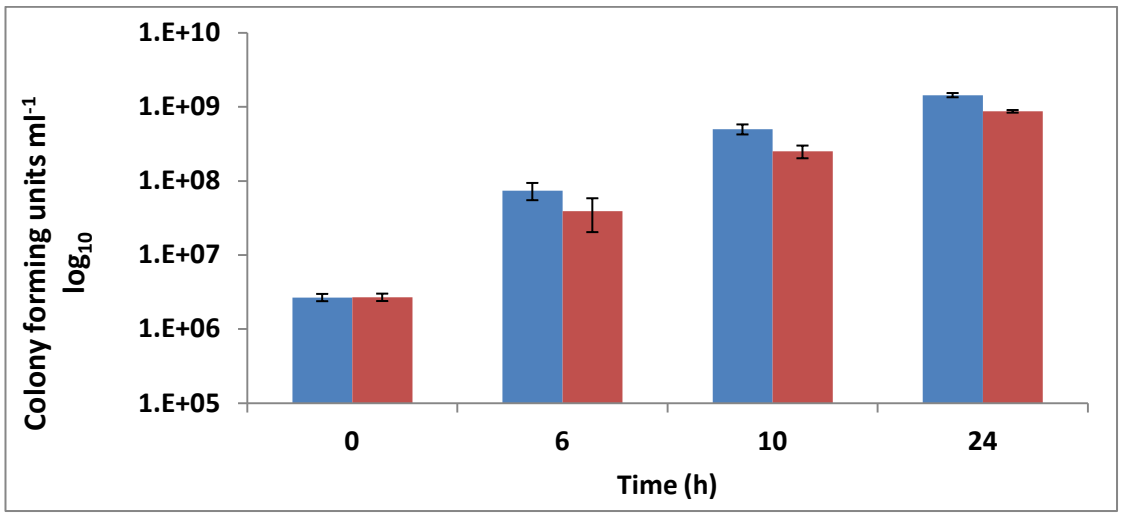


Figure 3.28 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in the gene coding for a hypothetical protein (M12) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.08). Error bars show the standard error of the mean (n=9).

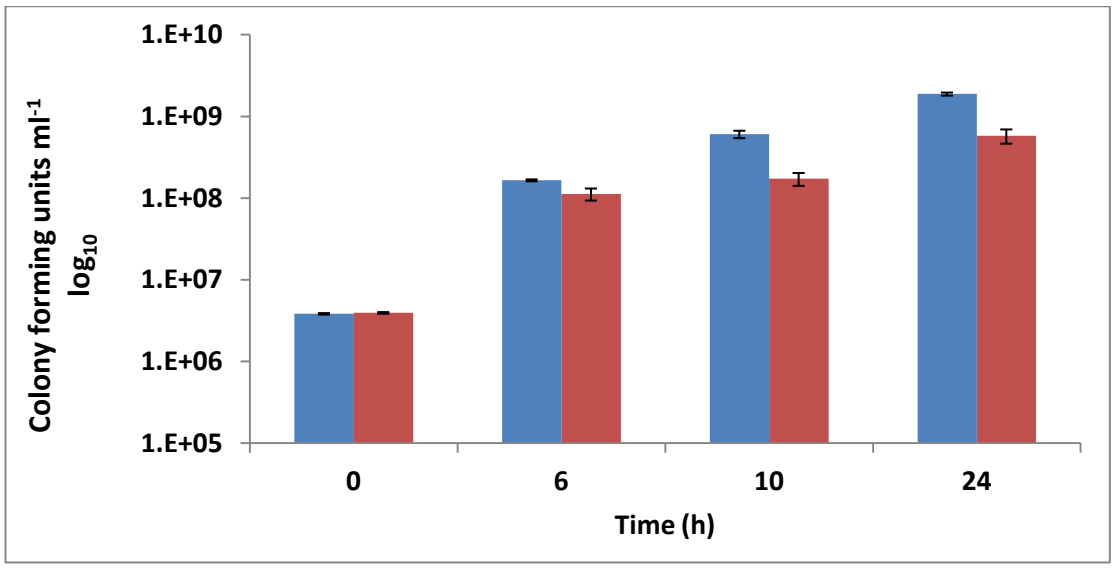


Figure 3.29 Growth curve of *L. monocytogenes* with Tn*917*-chromosomal integration. Growth of mutant with Tn*917*-integration in the gene coding for a L-aspartate oxidase (M13) was monitored for 24 h in the presence of β-estradiol. Control cells ; 0 µg ml^{-1} β-estadiol, Treated cells ; 60 µg ml^{-1} β-estadiol. The number of viable cells is showing in a log_{10} scale (p-value at 24 h =0.04). Error bars show the standard error of the mean (n=9).

L. monocytogenes Tn*917*-mediated mutants reduced in size when incubated on solid media in the presence of β-estradiol were further assessed in liquid cultures to ascertain their defect in growth when β-estradiol is present in the media. Although mutants were affected in growth by the addition of the hormone, reduction in growth was not microbiologically significant since plotting the graphs in logharitmic scale showed that values from the control and treated cells were not significantly different. Therefore, mutant libraries were further screened and assessed for their defect in growth in the presence of progesterone only since the growth of *L. monocytogenes* wild type was affected by the addition of this hormone. To simplify screening procedure, this was carried out in 96-well plates.

3.2.8 A high throughput screening of mutant libraries with progesterone in 96-wells plates

Additional screening of mutant libraries for clones with phenotype to progesterone was performed in liquid culture in 96-wells plate. In this case, a volume of 200 µl of TSB was supplemented with progesterone at a final concentration of 60 µg ml^{-1}, and mutants were incubated at 37ºC for 12 h under aerobic and anaerobic conditions. The optical density of bacterial growth was measured as previously described (Chapter 2, section 14). A number of 8800 clones were screened and the optical density from mutants grown with progesterone was compared to the optical density from the growth of the same mutants grown without the hormone (control, different plate). Results obtained indicate that there is no significant influence of progesterone on the growth of mutants under the conditions of this experiment. The O.D measurements from mutants were within the range of O.D measurements from the control cells when they were incubated in 200 µl total volume of TSB. For example, the O.D of bacterial cells grown as a control were in the range of 0.8 – 1.4, and cells grown with progesterone showed O.D withing this range.

3.2.9 Transposon-mediated random mutagenesis in *L. monocytogenes* chromosomal DNA

L. monocytogenes was investigated of its ability to encode for protein(s) that may play an essential role in the phenotype discussed earlier, which allow the bacteria to completely overcome the inhibitory effect of progesterone under aerobic conditions, or restoration of growth under anaerobic conditions post-exposure to progesterone. Random mutagenesis of *L. monocytogenes* chromosomal DNA was achieved by the use of transposon Tn*917*-LTV3 as previously described (Camilli *et al*., 1990). The approach of transposon-mediated insertional inactivation of *L. monocytogenes* was taken to identify clones with phenotype to pregnancy hormones that show growth inhibition/reduction when exposed to the hormones under aerobic/anaerobic conditions. Tn*917*-LTV3 comprises very important features, one of which is that it is carried by a highly temperature-sensitive derivative of vector pE194Ts, which allows the recovery of chromosomal insertions. Another

feature is that it contains a promoterless copy of the *E. coli lacZ* gene orientated such that insertions can derive *lacZ* transcriptional fusions. Tn*917*-LTV3 can replicate in *L. monocytogenes*, *Bacillus subtilis*, and *E. coli*. It comprises *E. coli* cloning vector that includes a gene selectable in *E. coli* immediately downstream from the *lacZ* coding sequence. It also contains ColE1 origin of replication and multiple cloning sites (MS) that facilitates the recovery in *E. coli* of chromosomal DNA adjacent to the insertion sites. Importantly, this transposon exhibits a very high transposition frequency that simplifies the generation of transposon-derived mutated libraries of *L. monocytogenes* (Figure 3.29). Plasmid LTV3 initiates its replication by the use of the temperature-sensitive origin of replication pE194Ts that loses its function at 42-43ºC. Tetracycline-resistance gene is carried by the plasmid, therefore loss of the plasmid renders the cells to become sensitive to tet, which indicates presumed successful transposition of the transposon into chromosomal DNA

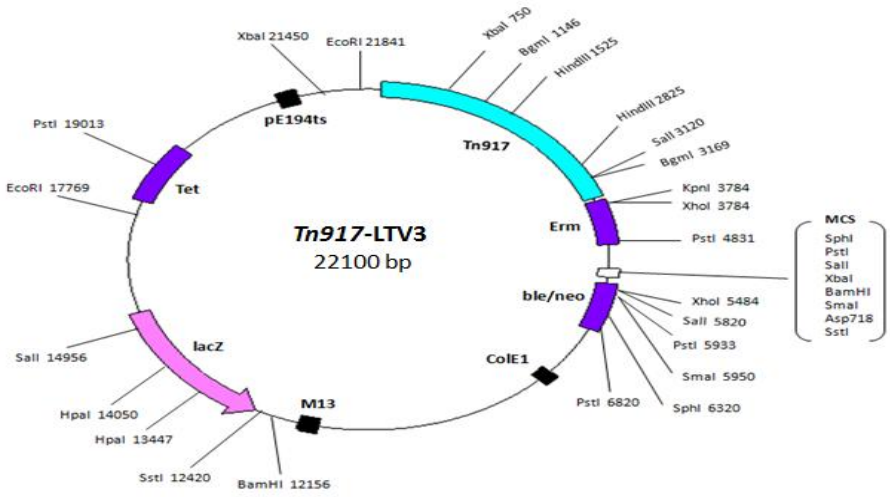


Figure 3.30 Restriction map of Tn*917*-LTV3. Plasmid vector contains of pE194Ts temperature-sensitive replicon, and ColE1-derived replicon. It is selectable by comprising of several antibiotic resistance genes including chloramphenicol acetyltransferase gene (*cat*), neomycin phosphotransferase II (*neo*), bleomycin (*ble*), tetracycline resistance gene (*tet*), and *Tn917* ribosomal methytransferase gene (*erm*). It also carries a Tn*917-lac* gene, which contains a promotorless lacZ gene from *E. coli* with translation initiation signals derived from *B. subtilis* gene *spoVG,* and M13mp19 polylinker. Restriction sites are indicated on the map, (Camilli *et al*., 1990)

Following generation of the mutants, a high throughput screening of the mutant libraries in the presence of progesterone showed no effect on bacterial growth as determined by the size of colonies (primary screening) and optical density (section 3.2.8). This could be explained by the possible presence of multiple genes in *L. monocytogenes* that are involved in hormone binding/degradation, which allow listerial cells to grow in the presence of progesterone without defect in growth. In contrast to progesterone, the hormone β-estradiol had negatively influenced a number of 13 mutants when cells were grown anaerobically on solid media (primary screening) and liquid culture (secondary screening). Each of the 13 mutants was cultured for genomic DNA isolation and subjected to restriction digest using the restriction enzyme *XbaI* that cuts once within the transposon downstream of the ColE1 origin of replication, which allows for the recovery of the adjacent DNA from *L. monocytogenes* chromosomal DNA, followed by self ligation and transformation into *E. coli* competent cells. Successful transformants harbouring the newly-constructed plasmids were selectable on Kanamycin supplemented medium.

Plasmid DNA harbouring the recovered sequence from *L. monocytogenes* chromosomal DNA were further subjected to endonuclease restriction digest using SalI for several reasons: (i) to confirm the existence of the transposon (ii) to show diversity of clones and (iii) to identify the approximate size of the DNA recovered from *L. monocytogenes.* Since *XbaI* was used to digest genomic DNA and further cloning, it cannot be used for this confirmation step as it will give a single large fragment only (comprising the transposon and the recovered DNA from *L. monocytogenes*). As shown in figure 3.11, mutants 1-4, 6 and 7 have a similar DNA sizes with the large fragment (9136 bp) corresponding to the transposon, whereas the smaller DNA fragment (~7000 bp) correspond to the sequence recovered from *L. monocytogenes*. This indicates that the transposon had frequent integration into the same position. The rest of the mutants showed different location of the transposon as indicated by the size of DNA fragments post the restriction digest with *SalI*, which indicates interruption of different genes from listerial chromosomal DNA.

Sequence analysis of the interrupted genes further confirms that the transposon had integrated into the exact site, which was found to be the replication protein operon in all of the 6 clones; M1, M2, M3, M4, M6 and M7, whereas integration was located differently in the other mutants. Each of these interrupted genes will be discussed in details in chapter 6.

In conclusion, the results obtained from the transposon mutagenesis were not of high importance to the the relation between pregnancy hormones and *L. monocytogenes* since the reduction in growth of each mutant was microbiologically insignificant when cells were screened in 10 ml liquid cultures, as the control cells and the mutant showed small differences when plotted in the logarithmic scale. For this reason, the bioinformatics approach was undertaken to further identify listerial proteins that are possibly involved in steroid degradation and the phenotype observed, as will be discussed in chapter 4.

3.3 Discussion

In order to understand the effect of pregnancy hormones on the growth of *L. monocytogenes*, cells were exposed to different concentrations of progesterone or β-estradiol, and growth was monitored during exposure. Concentrations of the hormones were chosen based on an earlier experiment conducted by Yotis and Stanke (1966). They examined the role of progesterone against 10 different microorganisms aerobically *in vitro*. *L. monocytogenes* was among the bacteria used in their study, which was exposed to progesterone at a final concentration of 20 µg ml^{-1}. The range of hormone concentrations used in Yotis study was also used in different studies. For example, the growth of the actinomycetes *Nocardia prasiliensis* was examined *in vitro* in response to steroid hormones (P_4 was used at [$4x10^{-6}$ M], (Hernandez *et al*., 1995). Another studis used P_4 at [0 - 40 µg ml^{-1}] or [10 µg ml^{-1}] to examine the inhibition dose for *N. gonorrhoeae* and *P. intermedia in vitro;* respectively (Jerse *et al*., 2003; Clark *et al*., 2006). In this study, increased concentrations of progesterone were also examined along with the concentration used earlier in Yotis study. The highest concentration of

progesterone used in the present study was 60 µg ml^{-1} since higher concentrations cannot be dispersed completely in the TSB media, as the hormone formed cotton-like particles in the media. Although both progesterone and β-estradiol exert an inhibitory action against the growth of several microorganisms (Table 3.1), no effect of the hormones was observed on the growth of *L. monocytogenes* under the conditions of these experiments when cells were grown aerobically at 37°C. The growth of aerobically grown cells in the presence of hormones was comparable to that of untreated bacteria as determined by the number of colony forming unit per millilitre of the culture. Monitoring bacterial growth was only possible by counting the viable cells and not by measuring the turbidity of the culture since supplementation with either progesterone or β-estradiol at higher concentrations (>40 µg ml^{-1}) changes the turbidity of the culture at 0 h and thereafter, leading to a false reading of the optical density. This suggests that the concentration of 60 µg ml^{-1} used in this study was less accurate as the hormone was coming out of the solution; hence the increased turbidity. This finding contradicts the result from Yotis study that showed the growth of *L. monocytogenes* was inhibited in the first 8 h of incubation when cells were exposed to progesterone at a concentration of 20 µg ml^{-1} under aerobic conditions, while in present study no difference was noted. Other study conducted by Hosoda and colleagues (2011) demonstrated the inhibitory action of a synthetic progesterone derivative namely 17α-hydroxyprogesterone caproate, but not the natural progesterone or its natural derivatives, on the growth of *H. pylori* under aerobic conditions. It was shown that 17α-hydroxyprogesterone caproate at a concentration of 100 µM completely inhibited the growth of *H. pylori* during exposure *in vitro*; whereas natural progesterone and its natural derivatives had no influence on the growth of the bacteria when grown for 24 h. Their findings suggest that the acylation at the carbon-17 position in the progesterone framework plays an important role in reinforcing the anti-*Helicobacter pylori* action of progesterone (Hosoda *et al*., 2011). The inhibitory concentration against *H. pylori* is equal to 31 µg ml^{-1}, which is within the range of the concentrations examined against *L. monocytogenes* in the present study where synthetic progesterone was used.

As described earlier, the action of progesterone differs based on several factors including hormone derivatives, concentrations and the microbe tested.

In contrast to aerobic conditions, progesterone negatively influenced bacterial growth when cells were grown under anaerobic conditions (Figure 3.2, 3.4 and 3.5). The reduction in growth of *L. monocytogenes* under these conditions was dose-dependent where progesterone exerting higher inhibitory effects at increased concentrations. Hence, the highest inhibition of growth by progesterone was observed when cells were exposed to ~60 µg ml^{-1} at 12 h point ($p<0.05$; Cl=95%), and the viable count of the cells showed a 3.5-fold reduction when compared to the control. A possible explanation for this phenotype could be attributed to progesterone affecting listerial proteins that might be involved in energy generation within bacterial cells, subsequently reducing the overall energy and cell viability. Under anaerobic conditions, *L. monocytogenes* uses specific enzymes such as NADPH dehydrogenases and oxidoreductases for the production of energy that is achieved by electron transfer following oxidation of the NADPH cofactor and reduction of a terminal substrate (Lungu *et al*., 2010). Previous study demonstrated that steroid hormones including progesterone can form a complex with NADPH-dependent enzymes and inhibit their activities (Khan *et al*., 2005). Similar enzymes including NADPH-dependent dehydrogenases and oxidureductases from different bacteria and yeast were demonstrated to bind a variety of substrates including steroids (Madani *et al*., 1993, William and Bruce, 2002). While progesterone and other steroid hormones can act as potent inhibitors for this type of enzymes, some steroid hormones act as substrates (Buckman and Miller, 1998). Furthermore, enzymes involved in steroid binding are also involved in energy generation as a result of the electron transfer within molecules (Soory, 2000).

Interestingly, based on the observations from this work, *L. monocytogenes* was capable of resuming a positive growth in the second half of the exposure to a level similar to that of the control. This observation supports findings from previous studies that used *L. monocytogenes* (Yotis and Stanke, 1966), and *Trychophyton mentagrophytes* (Clemons *et al*., 1989). The finding from Yotis study showed bacterial evasion from the inhibitory action of progesterone after

8 h of incubation with the hormone, except that in the present study this observation occurred only when cells were grown under anaerobic conditions, but not aerobic, and the growth restoration time was between 12-24 h post-exposure to the hormone. Whereas Clemons and colleagues (1989) observed that *T. mentagrophytes* escaped the inhibitory action of progesterone by steroid metabolism and transformation into different compounds including 15-alpha-hydroxyprogesterone, 1-dehydroprogesterone and 11-alpha-hydroxyprogesterone. They also showed that hydroxylated metabolites of progesterone were less inhibitory to the growth of the organism. Thus, it was proposed that enzymatic conversion of progesterone was a mechanism of detoxification and evasion of the inhibitory effect of progesterone. Indeed, newly formed metabolites have decreased affinity for progesterone-binding protein (PBP) and, therefore, less potent as inhibitors of fungal growth. Based on Clemones findings, (although no significant similarity was found between PBP from *T. Mentagrophytes* and Listerial proteins) it is anticipated that metabolism of progesterone by *L. monocytogenes* into different metabolites could be a mechanism of escaping the inhibitory action of progesterone observed in the present study. Other possible explanations could be attributed to different 'inhibitory escape' mechanisms, one of which is that the presence of progesterone induces the expression of bacterial specific genes, which allows for utilisation of the carbon atoms within the backbone of the hormone for energy generation and proteins synthesis. For instance, microorganisms found in human mouth were previously demonstrated to synthesise enzymes needed for steroid metabolism. Indeed, presence of steroids enhances the expression of 5-α reductase activity in gingivia species, thus formation of 5-α dihydrotestosterone from androgen, which can influence protein synthetic activity in these pathogens (Markou *et al*., 2009). Another example of steroid metabolism is that periodontal microbes were shown to metabolise steroid hormones, which could play a role in nutritional requirements, energy generation and evasion of host defence mechanism by forming capsular proteins (Soory, 2000). The enzyme steroid 5-alpha reductase from *Treponema denticola* was also demonstrated to metabolise progesterone and other steroid hormones including testosterone and corticosterone (Clark and Soory, 2006). Interestingly, *Prevotella intermedia* was shown to substitute

estrogen and progesterone for vitamin K, which can act as a growth factor (Nakagawa *et al*., 1994).

In the present study, it was observed that *L. monocytogenes* treated with increasing concentrations of progesterone had been adapted to the presence of the hormone during the period of exposure by overcoming the inhibitory effect of progesterone and restoration of the growth to a level compared to the control was noted (Figure 3.2). Based on this phenotype, it was anticipated that listerial cells respond to the presence of the hormone by overexpression of enzymes that may have a role in the metabolism of progesterone, therefore, converting progesterone to less potent inhibitor metabolites. For this reason, it was proposed that pre-exposure of *L. monocytogenes* to progesterone will enable bacterial cells to grow comparable to the control cells by cellular adaptation. Hence*, L. monocytogenes* was incubated with different concentrations of progesterone, followed by back diluting each culture into a fresh media supplemented with the same concentration of progesterone as the previous culture and incubated at the same conditions (Figure 3.3). The results from this experiment support the proposed mechanism of bacterial adaptation to the hormone since all cultures treated with increasing concentrations of progesterone grew in a level comparable to the untreated cells after pre-exposure to the hormone. This observation can be explained as *L. monocytogenes* possess progesterone-inducible genes, which allow escaping from the inhibitory effect of progesterone by metabolising of progesterone and conversion to less potent metabolites. Previous study showed that *Comamonas testosteroni* was able to utilise C_{19} and C_{21} steroids, as well as a number of aromatic compounds as a sole carbon and energy sources via a complex metabolic pathway involving many steroid-inducible enzymes (Gong *et al*., 2012). These enzymes involve 3α-hydroxysteroid dehydrogenase/ carbonyl reductase (3α-HSD/CR) that is induced by steroids such as progesterone and testosterone (Oppermann and Maser, 1996). In order to further investigate the adaptation observed, similar experiments were performed to examine the particular role of the hormone concentration in cell adaptation. Therefore, bacterial cells were pre-exposed to 20 μg ml^{-1} of progesterone only and further incubated with the same and higher

concentrations as described in figure 3.4. Data obtained suggests a concentration-dependent adaptation to the hormone. In fact, bacterial cells treated with 20 µg ml^{-1} grew in a level similar to the control when cells were back diluted to the same concentration. Whereas back-diluting bacterial cells to a higher concentration of progesterone showed growth reduction of ~1.3-fold when compared to the control at 12 h time-point. Further adaptation experiments were performed to assess the role of the exposure time in the adaptation observed. To achieve this, progesterone at a concentration of 60 µg ml^{-1} only was used to supplement the media at different timing as described in figure 3.5. Results from this experiment indicates that cells treated with progesterone in the first 12 h were unable to restore the growth comparable to the control at 24 h post incubation because of the extra progesterone that was added at 12 h time-point (culture 4), while cells received progesterone once showed little difference when compared to the control at 24 h (culture 2 and 3).

The hormone β-estradiol was also examined in this study, because of the existence in high concentration during pregnancy, plus the potential of this hormone to influence the growth of several microorganisms (Table 3.1). For these reasons, it was proposed that β-estradiol has a proliferation effect on *L. monocytogenes.* To investigate this, *L. monocytogenes* was incubated with increased concentrations of β-estradiol as described in figure 3.6. As opposed to progesterone, the data obtained from this experiment suggests that this hormone has no influence on the growth of *L. monocytogenes* as can be determined by the viable count of the cell forming unit. Taken together, regardless of β-estradiol, it is suggested that the adaptation observed in all progesterone experiments is a dose-time-dependent of the exposure to progesterone. From the data obtained and the literatures described above, it can be hypothesised that *L. monocytogenes* encodes for enzymes that are progesterone-inducible of expression.

In order to test this hypothesis, two different approaches were taken to investigate whether *L. monocytogenes* possess genes that are influenced by the presence of pregnancy hormones, progesterone in particular. One of which is the generation of random mutagenesis in *L. monocytogenes*

chromosomal DNA by the use of Tn*917*-LTV3, and screen for bacterial phenotype that may be influenced by the presence of the hormone (discussed above). The second approach was achieved by the use of bioinformatics (chapter 4), where previously studied proteins from different microorganisms capable of hormone binding were used to identify listerial proteins with the potential to bind hormones.

Chapter IV
Bioinformatics analysis for the identification of hormone-binding proteins in *L. monocytogenes*

4.1 Introduction

The food-borne bacterium *L. monocytogenes* affects individuals with compromised immunity such as elderly, those receiving anti-cancer treatments, those who have received transplants, those with diseases that compromise their immunity, infants and pregnant women (Barbuddhe *et al*., 2008). During pregnancy, several factors are believed to increase severity of listeriosis in women and cause devastating outcome. One of which is the great increase of pregnancy hormones during this time, which was previously linked to an increased incidence of bacterial and fungal infections in women (Kaushic *et al*., 2011 and Pung *et al*., 1984). Several studies were previously conducted to find a relationship between hormones and the infectious agents, which take advantage of the suppressed body immunity and use of the circulating hormones. For example, bacteria *Prevotella intermedius* can uptake both progesterone and β-estradiol, which stimulate bacterial growth, therefore it is thought that specific proteins may exist that stimulate hormone influx (Kornman and Loesche, 1982). Also, *C. trachomatis* exhibits increased rate of infectivity during times of elevated level of β-estradiol by utilisation of the disulfide isomerase (PDI), which specifically binds human β-estradiol (Hall *et al*., 2011). Moreover, other microorganisms, such as *C. testosteroni* and *E. cloacae* were found to encode for enzymes that have enzymatic activity against a number of substrates including steroid hormones. Some of these steroids can inhibit the activity of the enzyme.

Madani *et al*., (1994) have previously demonstrated the presence of estrogen binding protein (EBP) in *Candida albicans* that exhibits high affinity and specificity for human steroid hormones. Moreover, it was also observed that the level of EBP expression was highest during the lag and early logarithmic phases of growth and declined near stationary phase, which could suggest a possible role in growth regulation and enhance pathogenesis during fungal infection (Madani *et al*., 1994). In line with this observation, Khan *et al*., (2005) demonstrated that progesterone can form a complex with PETN reductase from *Enterobacter cloacae* and inhibits its activity. This enzyme belongs to the old yellow enzyme (OYE) family, of which members can bind a variety of α/β unsaturated aldehydes, ketones and cyclic enones including steroid hormones. Some steroids act as substrates, whereas others are potent

inhibitors of proteins that belong to the OYE family (Khan *et al*., 2005). It was suggested that Tyr186 functions as the key proton donor during the reduction of the α/β unsaturated carbonyl compounds by PETN reductase (Khan *et al*., 2005). Other OYE members include xenobiotic reductase encoded by *Pseudomonas pudita* (Blehert *et al*., 1999) and YqjM from *Bacillus subtilis* (Fitzpatrick *et al*., 2003) have also been demonstrated to exhibit enzymatic activity against different substrates containing α/β unsaturated carbonyl compounds.

Taken together, it is evident from the literature that a number of microorganisms bind human hormones via specific proteins and use them for their advantage. Therefore, existence of such proteins in different microorganisms, and the growth restoration phenomenon observed earlier in chapter 3 led to the prediction that *L. monocytogenes* encodes for enzyme(s) that bind circulating pregnancy hormones. Also, listerial enzymes with a role in steroid hormones binding/degradation have been predicted. In order to investigate this prediction, a number of previously identified proteins from different microorganisms with the ability to bind/degrade steroid hormones were used to identify similar proteins in *L. monocytogens* via protein sequence alignment tools. Although the basic information about a protein comes from its sequence, it is difficult to infer anything about the function of the protein. However, the number of related sequences increases, so alignment of similar sequences can build a consensus for a protein family, or more accurately to identify conserved domains or highly conserved residues that may be essential for the function of the protein. For instance, the substrate binding site of an enzyme or the active site that confers the catalytic activity of the enzyme.

Although members of the OYE family bind a wide range of substrates, some have specificity to steroid hormones. This chapter discusses proteins that belong to the OYE family from *Candida albicans*, *Bacillus subtilis*, *Pseudomonas putida*, *Enterobacter cloacae*, *Escherishia coli* and Neisseria gonorrhoeae to identify similar proteins in *L. monocytogenes* via protein sequence alignment tools using NCBI and UniProt databases, ClustalW and UGENE bioinformatics softwares.

4.2 Results

4.2.1 Alignment of *C. albicans* EBP with *L. monocytogenes* proteins

Protein sequence of the Estrogen binding protein (EBP) from *C. albicans* was used to search for similar protein(s) in *L. monocytogenes* through the option Basic Local Alignment Search Tool program (BLASTp) in the NCBI database.

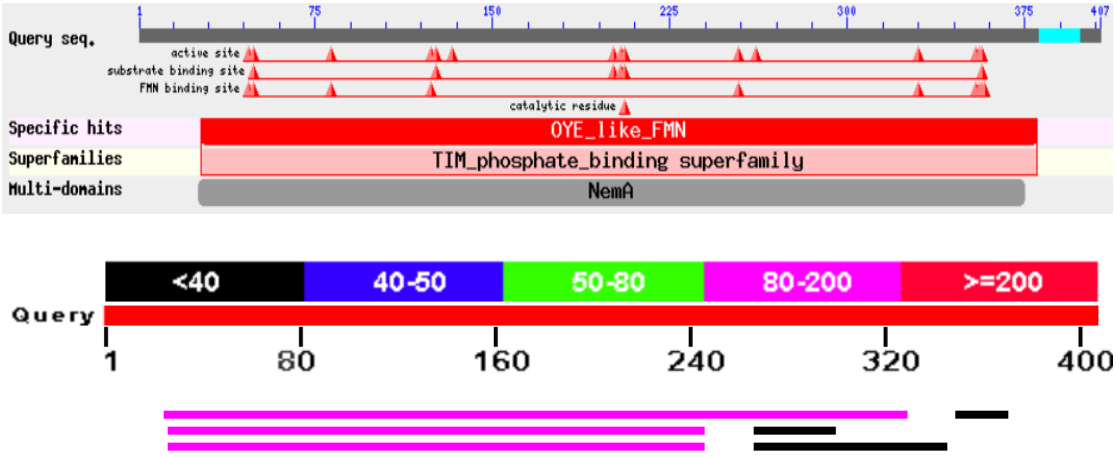


Figure 4.1 protein sequence alignment of EBP against *L.* monocytogenes proteins. A stretch of conserved domains have been detected, which belong to the family of Old Yellow Enzyme; OYE (top). A color key for alignment scores for proteins identity and the number of matched amino acids (bottom).

Table 4.1 Scores of sequences from *L. monocytogenes* proteins that show significant identity to EBP from *C. albicans*

Description	Max score	Total score	Query coverage	E- value	Maximum identity
NamA	119	119	74%	3E-31	31%
Lmo2235	105	105	54%	1E-25	34%
Lmo0489	96.7	96.7	54%	1E-22	33%

BLASTp search revealed significance similarity of the EBP from *C. albicans* to 3 different proteins found in *L. monocytogenes*. Each of these proteins showed more than 30% identity to the sequence from the query. Existence of NamA in listerial cells has been inferred from homology, while the other 2 proteins were only predicted (UniProt, Q8Y4H1, Q8Y541, Q8Y9N6).

A

Score	Expect	Method	Identities	Positives	Gaps
119 bits(299)	3e-31	Compositional matrix adjust.	97/308(31%)	153/308(49%)	32/308(10%)

```
Query  26   TKLFQPIKVGNNVLPQRIAYVPTTRFRA-SKDHIPSDLQLNYYNARSQYPGTLIITEATF  84
            +KLF   K+ +  L  RI   P   +   +KD I +D    +Y +R+     L+I EAT
Sbjct  2    SKLFSEYKLKDVTLKNRIVMSPMCMYSVENKDGIATDFHFAHYVSRAAGGTGLVILEATA  61

Query  85   ASERGGIDLHVPGIYNDAQAKSWKKINEAIHGNGSFSSVQLWYLGRVANAKDLKDSGLP-  143
              E G I     G++ND Q  + KK+   +H +G+ + +QL + GR A          LP
Sbjct  62   VQEVGRISEFDLGLWNDEQVPALKKLVGGLHYHGAKAGIQLAHAGRKAV--------LPG  113

Query  144  -LIAPSAVYWDENSEKLAKEAGNELRALTEEEIDHIVEVEYPNAAKHALEAGFDYVEIHG  202
             ++APSA+ +DE S+K  +         LT+E I  +V  ++  AA  A EAGFD +EIH
Sbjct  114  EIVAPSAIAFDEKSDKPVE--------LTKEAIKEVV-ADFKRAAYRAKEAGFDVIEIHA  164

Query  203  AHGYLLDQFLNLASNKRTDKYGCGSIENRARLLLRVVDKLIEVVGANRLALRLSPWASFQ  262
            AHGYL+ QFL+  +N+R D YG G   NR ++L  ++ K ++ V     + +R+S
Sbjct  165  AHGYLIHQFLSPITNRREDNYG-GPAGNRYKILSDII-KAVKEVWDGPIIVRVSATDYAH  222

Query  263  GMEIEGEEIHSYILQQLQQRADNGQQLAYISLVEPRVTGIYDVSLKDQQGRSNEFAYKIW  322
            G    G ++  +I      +AD       + L++    G+ +V+     G    FA +I
Sbjct  223  G----GLQLEDHIPFAKWMKADG------VELIDVSTGGLVNVAPPVFPGYQVPFADEIR  272

Query  323  KGNFIRAG  330
            +G  I  G
Sbjct  273  RGAGIATG  280
```

B

Score	Expect	Method	Identities	Positives	Gaps
105 bits(262)	1e-25	Compositional matrix adjust.	75/220(34%)	109/220(49%)	13/220(5%)

```
Query  28   LFQPIKVGNNVLPQRIAYVPTTRFRASKDHIPSDLQLNYYNARSQYPGTLIITEATFASE  87
            +F PI +G   +P R    P     A+ D   SD  L YY  R+     LI  EAT
Sbjct  6    MFSPIDIGPMKVPNRFVVSPMCNNYANTDGTLSDTSLAYYKERALGGFGLITFEATVVDV  65

Query  88   RGGIDLHVPGIYNDAQAKSWKKINEAIHGNGSFSSVQLWYLGRVANAKDLKDSGLPLIAP  147
            R     +   +Y+D Q  S+K++ +  H  G+  SVQL + G   N+   K SG PL
Sbjct  66   RAKGGANKACLYSDHQIASFKRVIDVCHDAGAKISVQLQHAGPEGNS---KVSGYPL---  119

Query  148  SAVYWDENSEKLAKEAGNELRALTEEEIDHIVEVEYPNAAKHALEAGFDYVEIHGAHGYL  207
                    S   +    N   A++ EE+  ++E+ Y  AA  A +AG D VE+H AHGYL
Sbjct  120  -----RAASAIASAAGRNTPEAISREELYELIEL-YGEAALRAKKAGADAVEVHCAHGYL  173

Query  208  LDQFLNLASNKRTDKYGCGSIENRARLLLRVVDKLIEVVG  247
            +  FL+  +NKR D++G G  ENR RL   +++ + + VG
Sbjct  174  VSSFLSARTNKRVDEFG-GCFENRMRLPRLIIESIRKRVG  212
```

C

Score	Expect	Method	Identities	Positives	Gaps
96.7 bits(239)	1e-22	Compositional matrix adjust.	75/224(33%)	115/224(51%)	19/224(8%)

```
Query  28   LFQPIKVGNNVLPQRIAYVPTTRFRASKDHIPSDLQLNYYNARSQYPGTLIITEATFA--  85
            +F+P+ V    +  R+   P     A  +    +  +NYY  R++    LI  E
Sbjct  7    IFEPLTVKRMTIKNRVIMPPMGTNLAGLNGEFLEEHMNYYEQRAKGGTGLITIENACVDF  66

Query  86   --SERGGIDLHVPGIYNDAQAKSWKKINEAIHGNGSFSSVQLWYLGRVANAKDLKDSGLP  143
                G   L    I ND     + K+ E +H +G+  S+Q+ + G  A+A   + +GL
Sbjct  67   PYGTNGTTQLR---IDNDQYIPGFYKLTERLHKHGTCVSIQINHAG--ASAYPARLNGLQ  121

Query  144  LIAPSAVYWDENSEKLAKEAGNELRALTEEEIDHIVEVEYPNAAKHALEAGFDYVEIHGA  203
             ++ S +         +K+ G   R LT EEI  IV  +Y +AA+ A +AGFD VEIHG
Sbjct  122  PVSASDIP--------SKKGGTVPRPLTVEEIYEIVN-KYGDAARRAQQAGFDAVEIHGG  172

Query  204  HGYLLDQFLNLASNKRTDKYGCGSIENRARLLLRVVDKLIEVVG  247
            H YLL QFL+   NKRTD++G G+ ENRAR++  +++K+   VG
Sbjct  173  HSYLLCQFLSPLYNKRTDEFG-GTPENRARIVKLILEKVRAEVG  215
```

Figure 4.2 Sequence alignments of EBP (query) against listerial proteins (subject). Alignment scores showed significance identity to **(A)** NamA 31%, **(B)** Lmo2235 34% and **(C)** Lmo0489 33%. Conserved amino acid residues stretched over the query sequence with NamA showing the highest coverage of 74%, and 54% for the other two proteins. Proteins consist of 407 aa (EBP), 338 aa (NamA), 641 aa (Lmo2235) and 664 aa (Lmo0489).

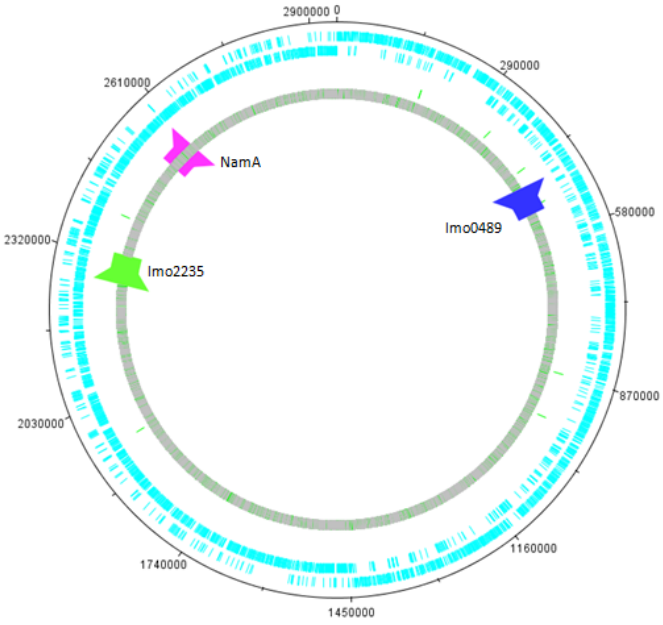


Figure 4.3 Map of *L. monocytogenes* chromosomal DNA and the locations of the genes encoding for putative OYE homologies, Lmo0489, Lmo 2235 and NamA-encoding gene (*Lmo2471*).

4.2.2 Alignment of *B. subtilis* YqjM with *L. monocytogenes* proteins

Protein sequence of the YqjM from *B. subtilis* was used to search for similar protein(s) in *L. monocytogenes* via BLASTp option in the NCBI database.

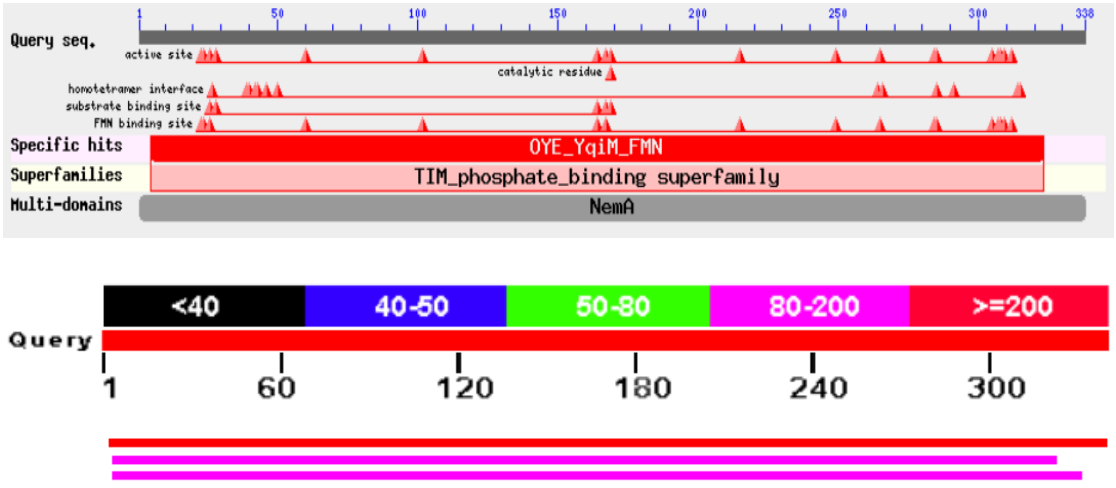


Figure 4.4 protein sequence alignment of YqjM against *L. monocytogenes* proteins. A stretch of conserved domains have been detected that belong to OYE (top). A color key alignment scores for proteins identity and the number of matched amino acids (bottom).

Table 4.2 Scores of sequences from *L. monocytogenes* proteins that show significant identity to YqjM

Description	Max score	Total score	Query coverage	E- value	Maximum identity
NamA	436	436	99%	1E-153	63%
Lmo0489	191	191	93%	1E-55	35%
Lmo2235	177	177	96%	1E-50	34%

BLASTp search revealed significance similarity of the YqjM from *B. subtilis* to 3 different proteins found in *L. monocytogenes*. The sequence from NamA showed the highest identity (63%) to YqjM, while the other 2 proteins showed less percentage of identity. The sequence from NamA covers 99% of the query sequence, while Lmo0489 and Lmo2235 cover 93% and 96%, respectively. YqjM consists of 338 aa, which is the same length as NamA.

A

Score	Expect	Method	Identities	Positives	Gaps
436 bits(1121)	1e-153	Compositional matrix adjust.	211/335(63%)	253/335(75%)	0/335(0%)

```
Query  4    KLFTPITIKDMTLKNRIVMSPMCMYSSHEKDGKLTPFHMAHYISRAIGQVGLIIVEASAV  63
            KLF+   +KD+TLKNRIVMSPMCMYS   KDG  T FH AHY+SRA G  GL+I+EA+AV
Sbjct  3    KLFSEYKLKDVTLKNRIVMSPMCMYSVENKDGIATDFHFAHYVSRAAGGTGLVILEATAV  62

Query  64   NPQGRITDQDLGIWSDEHIEGFAKLTEQVKEQGSKIGIQLAHAGRKAELEGDIFAPSAIA  123
              GRI++ DLG+W+DE +    KL   +   G+K GIQLAHAGRKA L G+I APSAIA
Sbjct  63   QEVGRISEFDLGLWNDEQVPALKKLVGGLHYHGAKAGIQLAHAGRKAVLPGEIVAPSAIA  122

Query  124  FDEQSATPVEMSAEKVKETVQEFKQAAARAKEAGFDVIEIHAAHGYLIHEFLSPLSNHRT  183
            FDE+S  PVE++ E +KE V +FK+AA RAKEAGFDVIEIHAAHGYLIH+FLSP++N R
Sbjct  123  FDEKSDKPVELTKEAIKEVVADFKRAAYRAKEAGFDVIEIHAAHGYLIHQFLSPITNRRE  182

Query  184  DEYGGSPENRYRFLREIIDEVKQVWDGPLFVRVSASDYTDKGLDIADHIGFAKWMKEQGV  243
            D YGG   NRY+ L +II  VK+VWDGP+ VRVSA+DY   GL + DHI FAKWMK  GV
Sbjct  183  DNYGGPAGNRYKILSDIIKAVKEVWDGPIIVRVSATDYAHGGLQLEDHIPFAKWMKADGV  242

Query  244  DLIDCSSGALVHADINVFPGYQVSFAEKIREQADMATGAVGMITDGSMAEEILQNGRADL  303
            +LID S+G LV+    VFPGYQV FA++IR  A +ATGA+G+IT G  AEEIL N RADL
Sbjct  243  ELIDVSTGGLVNVAPPVFPGYQVPFADEIRRGAGIATGALGLITRGEQAEEILCNERADL  302

Query  304  IFIGRELLRDPFFARTAAKQLNTEIPAPVQYERGW  338
            I +GRELLR+P+FA+ AAKQL   I AP QY R W
Sbjct  303  IIVGRELLRNPYFAKDAAKQLGETIEAPKQYSRAW  337
```

B

Score	Expect	Method	Identities	Positives	Gaps
191 bits(484)	1e-55	Compositional matrix adjust.	116/330(35%)	188/330(56%)	16/330(4%)

```
Query  5    LFTPITIKDMTLKNRIVMSPMCMYSSHEKDGKLTPFHMAHYISRAIGQVGLIIVEASAVN  64
            +F P+T+K MT+KNR++M PM    +   +G+    HM +Y  RA G  GLI +E + V+
Sbjct  7    IFEPLTVKRMTIKNRVIMPPMGTNLAG-LNGEFLEEHMNYYEQRAKGGTGLITIENACVD  65

Query  65   -PQGRITDQDLGIWSDEHIEGFAKLTEQVKEQGSKIGIQLAHAGRKA---ELEG-DIFAP  119
             P G      L I +D++I GF KLTE++ + G+ + IQ+ HAG  A    L G    +
Sbjct  66   FPYGTNGTTQLRIDNDQYIPGFYKLTERLHKHGTCVSIQINHAGASAYPARLNGLQPVSA  125

Query  120  SAIAFDEQSATPVEMSAEKVKETVQEFKQAAARAKEAGFDVIEIHAAHGYLIHEFLSPLS  179
            S I   +    P  ++ E++ E V ++  AA RA++AGFD +EIH  H YL+ +FLSPL
Sbjct  126  SDIPSKKGGTVPRPLTVEEIYEIVNKYGDAARRAQQAGFDAVEIHGGHSYLLCQFLSPLY  185

Query  180  NHRTDEYGGSPENRYRFLREIIDEVKQVWDGPLF---VRVSASDYTDKGLDIADHIGFAK  236
            N RTDE+GG+PENR R ++ I+++V+    GP F   +R SA ++T+ G  + D +
Sbjct  186  NKRTDEFGGTPENRARIVKLILEKVRAEV-GPFFPIVLRFSADEFTEGGNHLEDILELLD  244

Query  237  WMKEQGVDLIDCSSGA-----LVHADINVFPGYQVSFAEKIREQADMATGAVGMITDGSM  291
            + +E+  D++ S+        L    +N+  G++   A+ ++++ +  T   G I
Sbjct  245  YCQEEA-DILNVSAAINDNLYLQIDQMNLEDGWRSYLAKAVKDKFNKPTITSGNIRSPKA  303

Query  292  AEEILQNGRADLIFIGRELLRDPFFARTAA  321
            AE+IL  G ADL+ +GR L+ +P +    A
Sbjct  304  AEKILSEGYADLLAMGRGLIAEPNWVNKVA  333
```

C

Score	Expect	Method	Identities	Positives	Gaps
177 bits(448)	1e-50	Compositional matrix adjust.	114/337(34%)	185/337(54%)	12/337(3%)

```
Query  5    LFTPITIKDMTLKNRIVMSPMCMYSSHEKDGKLTPFHMAHYISRAIGQVGLIIVEASAVN  64
            +F+PI I  M + NR V+SPMC   ++  DG L+   +A+Y  RA+G  GLI  EA+ V+
Sbjct  6    MFSPIDIGPMKVPNRFVVSPMCNNYAN-TDGTLSDTSLAYYKERALGGFGLITFEATVVD  64

Query  65   PQGRITDQDLGIWSDEHIEGFAKLTEQVKEQGSKIGIQLAHAGRK--AELEG-DIFAPSA  121
             + +       ++SD  I  F ++ +  + G+KI +QL HAG +  +++ G  + A SA
Sbjct  65   VRAKGGANKACLYSDHQIASFKRVIDVCHDAGAKISVQLQHAGPEGNSKVSGYPLRAASA  124

Query  122  IAFDEQSATPVEMSAEKVKETVQEFKQAAARAKEAGFDVIEIHAAHGYLIHEFLSPLSNH  181
            IA      TP  +S E++ E ++ + +AA RAK+AG D +E+H AHGYL+  FLS  +N
Sbjct  125  IASAAGRNTPEAISREELYELIELYGEAALRAKKAGADAVEVHCAHGYLVSSFLSARTNK  184

Query  182  RTDEYGGSPENRYRFLREIIDEV-KQVWDG-PLFVRVSASDYTDKGLDIADHIGFAKWMK  239
            R DE+GG  ENR R  R II+ + K+V     +  R++++D  D GL + D    A +++
Sbjct  185  RVDEFGGCFENRMRLPRLIIESIRKRVGHSIAILCRINSTDGVDGGLSVQDSATVAAYLE  244

Query  240  EQGVDLIDCSSGALVH-----ADINVFPGYQVSFAEKIREQADMATGAVGMITDGSMAEE  294
            + G+D +  S    +      A   G+      +I+    +    VG  T+   AE
Sbjct  245  DCGLDGLHVSRSVHIRDEYMWAPTVLHAGFSSDLVTQIKRAVSIPVITVGRFTEPHYAEL  304

Query  295  ILQNGRADLIFIGRELLRDPFFA-RTAAKQLNTEIPA  330
            +++ GRADL+  GR+ L DP    + AA +L+  +P
Sbjct  305  MVREGRADLVAFGRQSLADPETPNKAAAGKLDELLPC  341
```

Figure 4.5 Sequence alignment of YqjM (query) against listerial proteins (subject). Alignment scores showed significance identity to **(A)** NamA 63%, **(B)** Lmo0489 35% and **(C)** Lmo2235 34%. Conserved amino acid residues stretched over the query sequence with NamA showing the highest coverage of 99%, while Lmo0489 and Lmo2235 showing 93% and 96%, respectively.

4.2.3 Alignment of *P. putida* xenobiotic reductase with *L. monocytogenes* proteins

Protein sequence of the xenobiotic reductase from *P. putida* was used to search for similar protein(s) in *L. monocytogenes* via BLASTp option in the NCBI database.

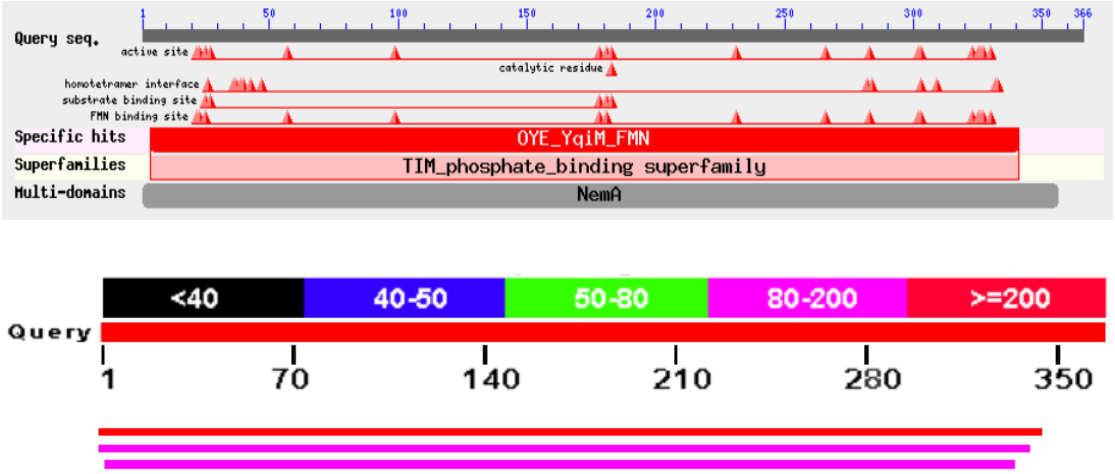


Figure 4.6 protein sequence alignment of xenobiotic reductase against *L. monocytogenes* proteins. A stretch of conserved domains have been detected that is related to the OYE (top). A color key alignment scores for proteins identity and the number of matched amino acids (bottom).

Table 4.3 Scores of sequences from *L. monocytogenes* proteins that show significance identity to xenobiotic reductase from *P. putida*.

Description	Max score	Total score	Query coverage	E- value	Maximum identity
NamA	217	217	93%	1E-67	40%
Lmo2235	141	141	92%	4E-38	31%
Lmo0489	138	138	90%	8E-37	29%

A

Score	Expect	Method	Identities	Positives	Gaps
217 bits(552)	1e-67	Compositional matrix adjust.	138/345(40%)	190/345(55%)	23/345(6%)

```
Query  1    MSALFEPFELKSHCLRNRIAVPPMCQYMA--NEGLVNEWHHIHYASIARGGAGLVIVEAT  58
            MS LF  ++LK   L+NRI + PMC Y     +G+  ++H  HY S A GG GLVI+EAT
Sbjct  1    MSKLFSEYKLKDVTLKNRIVMSPMCMYSVENKDGIATDFHFAHYVSRAAGGTGLVILEAT  60

Query  59   AVSPEGRITPSCAGLWSDKQGEAFIPIAQAIKAAGSVAGIQLAHAGRKASANRPWEGNGH  118
            AV   GRI+    GLW+D+Q  A   +   +   G+ AGIQLAHAGRKA    P E
Sbjct  61   AVQEVGRISEFDLGLWNDEQVPALKKLVGGLHYHGAKAGIQLAHAGRKAVL--PGE----  114

Query  119  IAEDDHRGWQTIAPSAIAFGAHLSKVPRAMSLSDIAQVREEFVMAARRALSAGFEWLELH  178
                    +APSAIAF     K P  ++   I +V  +F  AA RA  AGF+ +E+H
Sbjct  115  ----------IVAPSAIAFDEKSDK-PVELTKEAIKEVVADFKRAAYRAKEAGFDVIEIH  163

Query  179  FAHGYLGQSFLSAHSNRRSDGYGGNVENRRRFLIETMSAVREVWPEHLPLTVRLGVIEYD  238
             AHGYL   FLS  +NRR D YGG   NR + L + + AV+EVW    P+ VR+   +Y
Sbjct  164  AAHGYLIHQFLSPITNRREDNYGGPAGNRYKILSDIIKAVKEVWDG--PIIVRVSATDYA  221

Query  239  NQDEQTLLEAIALIRQLKNAGADLVNVSTGFNIPNTDTPWAPAFLAPIAERIRHEAGIPV  298
            +   Q L + I   + +K  G +L++VSTG  + N   P  P +  P A+ IR  AGI
Sbjct  222  HGGLQ-LEDHIPFAKWMKADGVELIDVSTG-GLVNVAPPVFPGYQVPFADEIRRGAGIAT  279

Query  299  AAAWGIESPALADRLVRDGQADLVMVGRAHLADPHWPYAAARTLG  343
             A   I     A+ ++ + +ADL++VGR  L +P++   AA+ LG
Sbjct  280  GALGLITRGEQAEEILCNERADLIIVGRELLRNPYFAKDAAKQLG  324
```

B

Score	Expect	Method	Identities	Positives	Gaps
141 bits(355)	4e-38	Compositional matrix adjust.	107/346(31%)	169/346(48%)	24/346(6%)

```
Query  1    MSALFEPFELKSHCLRNRIAVPPMCQYMAN-EGLVNEWHHIHYASIARGGAGLVIVEATA  59
             +++F P ++     + NR  V PMC    AN +G +++     +Y   A GG GL+  EAT
Sbjct  3    FNSMFSPIDIGPMKVPNRFVVSPMCNNYANTDGTLSDTSLAYYKERALGGFGLITFEATV  62

Query  60   VSPEGRITPSCAGLWSDKQGEAFIPIAQAIKAAGSVAGIQLAHAGRKASANRPWEGNGHI  119
            V    +   + A L+SD Q  +F  +       AG+   +QL HAG          EGN  +
Sbjct  63   VDVRAKGGANKACLYSDHQIASFKRVIDVCHDAGAKISVQLQHAG---------PEGNSKV  114

Query  120  AEDDHRGWQTIAPSAIAFGAHLSKVPRAMSLSDIAQVREEFVMAARRALSAGFEWLELHF  179
            +     G+  +  ++    A     P A+S  ++ ++ E +  AA RA  AG + +E+H
Sbjct  115  S-----GYP-LRAASAIASAAGRNTPEAISREELYELIELYGEAALRAKKAGADAVEVHC  168

Query  180  AHGYLGQSFLSAHSNRRSDGYGGNVENRRRFLIETMSAVREVWPEHLPLTVRLGVIEYDN  239
            AHGYL  SFLSA +N+R D +GG  ENR R     + ++R+     + +  R+     D
Sbjct  169  AHGYLVSSFLSARTNKRVDEFGGCFENRMRLPRLIIESIRKRVGHSIAILCRIN--STDG  226

Query  240  QDEQTLLEAIALIRQ-LKNAGADLVNVSTGFNIPNTDTPWAPA-----FLAPIAERIRHE  293
             D    ++  A +   L++ G D ++VS   +I + +  WAP       F + +  +I+
Sbjct  227  VDGGLSVQDSATVAAYLEDCGLDGLHVSRSVHIRD-EYMWAPTVLHAGFSSDLVTQIKRA  285

Query  294  AGIPVAAAWGIESPALADRLVRDGQADLVMVGRAHLADPHWPYAAA  339
              IPV       P  A+ +VR+G+ADLV  GR  LADP  P  AA
Sbjct  286  VSIPVITVGRFTEPHYAELMVREGRADLVAFGRQSLADPETPNKAA  331
```

C

Score	Expect	Method	Identities	Positives	Gaps
138 bits(347)	8e-37	Compositional matrix adjust.	100/343(29%)	163/343(47%)	31/343(9%)

```
Query  3    ALFEPFELKSHCLRNRIAVPPMCQYMAN-EGLVNEWHHIHYASIARGGAGLVIVEATAVS  61
            ++FEP  +K   ++NR+ +PPM   +A   G   E H  +Y   A+GG GL+ +E   V
Sbjct  6    SIFEPLTVKRMTIKNRVIMPPMGTNLAGLNGEFLEEHMNYYEQRAKGGTGLITIENACVD  65

Query  62   -PEGRITPSCAGLWSDKQGEAFIPIAQAIKAAGSVAGIQLAHAGRKASANRPWEGNGHIA  120
             P G    +   + +D+    F  + + +   G+   IQ+ HAG  A   R
Sbjct  66   FPYGTNGTTQLRIDNDQYIPGFYKLTERLHKHGTCVSIQINHAGASAYPAR---------  116

Query  121  EDDHRGWQTIAPSAIAFGAHLSKVPRAMSLSDIAQVREEFVMAARRALSAGFEWLELHFA  180
                G Q ++ S I        VPR +++ +I ++   ++  AARRA  AGF+ +E+H
Sbjct  117  ---LNGLQPVSASDIP-SKKGGTVPRPLTVEEIYEIVNKYGDAARRAQQAGFDAVEIHGG  172

Query  181  HGYLGQSFLSAHSNRRSDGYGGNVENRRRFLIETMSAVR-EVWPEHLPLTVRLGVIEYDN  239
            H YL   FLS   N+R+D +GG  ENR R +   +  VR EV P   P+ +R    E+
Sbjct  173  HSYLLCQFLSPLYNKRTDEFGGTPENRARIVKLILEKVRAEVGP-FFPIVLRFSADEFTE  231

Query  240  QDEQTLLEAIALIRQLKNAGADLVNVSTGFNIP--------NTDTPWAPAFLAPIAERIR  291
                LE I  +        AD++NVS   N           N +  W     + +A+ ++
Sbjct  232  GGNH--LEDILELLDYCQEEADILNVSAAINDNLYLQIDQMNLEDGWR----SYLAKAVK  285

Query  292  HEAGIPVAAAWGIESPALADRLVRDGQADLVMVGRAHLADPHW  334
             +   P   +  I SP  A++++ +G ADL+ +GR  +A+P+W
Sbjct  286  DKFNKPTITSGNIRSPKAAEKILSEGYADLLAMGRGLIAEPNW  328
```

Figure 4.7 Sequence alignment of xenobiotic reductase (query) against listerial proteins (subject). Alignment scores showed significance identity to **(A)** NamA 40%, **(B)** Lmo2235 31% and **(C)** Lmo0489 29%. Conserved amino acid residues stretched over the query sequence with NamA showing the highest coverage of 93%, while Lmo2235 and Lmo0489 showing 92% and 90%, respectively. Xenobiotic reductase consists of 366 aa.

4.2.4 Alignment of *E. cloacae* PETN reductase with *L. monocytogenes* proteins

Protein sequence of the PETN reductase from *E. cloacae* was used to search for similar protein(s) in *L. monocytogenes* via BLASTp option in the NCBI database.

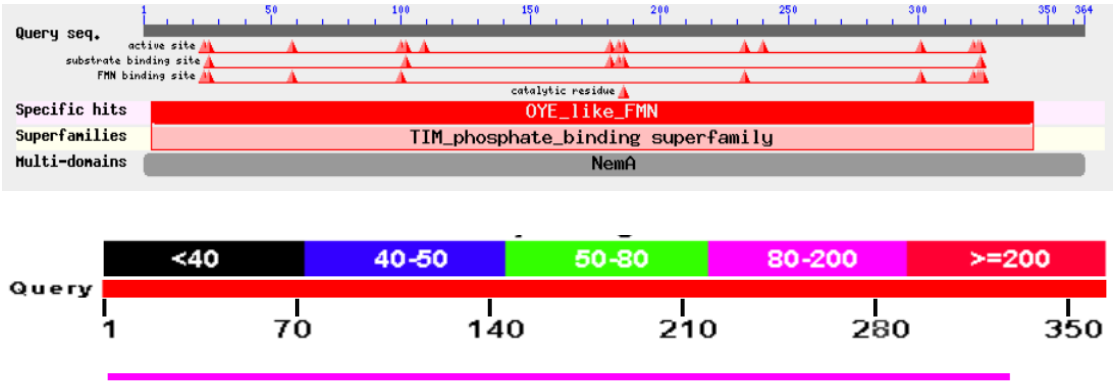


Figure 4.8 protein sequence alignment of PETN reductase against *L. monocytogenes* proteins. A stretch of conserved domains have been detected that belong to OYE (top). A color key alignment scores for proteins identity and the number of matched amino acids detected (bottom).

Table 4.4 Scores of sequences from *L. monocytogenes* proteins that show significance identity with PETN reductase from *E. cloacae*

Description	Max score	Total score	Query coverage	E- value	Maximum identity
NamA	142	142	89%	1E-39	32%

Score	Expect	Method	Identities	Positives	Gaps
142 bits(358)	1e-39	Compositional matrix adjust.	109/337(32%)	168/337(49%)	36/337(10%)

```
Query  4    KLFTPLKVGAVTAPNRVFMAPLTRLRSIEPGD-IPTPLMGEYYRQRAS--AGLIISEATQ  60
            KLF+  K+  VT  NR+ M+P+  + S+E  D I T     +Y  RA+   GL+I EAT
Sbjct  3    KLFSEYKLKDVTLKNRIVMSPMC-MYSVENKDGIATDFHFAHYVSRAAGGTGLVILEATA  61

Query  61   ISAQAKGYAGAPGLHSPEQIAAWKKITAGVHAEDGRIAVQLWHTGRISHSSIQPGGQAPV  120
            +    +      GL + EQ+ A KK+  G+H    +  +QL H GR    ++ PG
Sbjct  62   VQEVGRISEFDLGLWNDEQVPALKKLVGGLHYHGAKAGIQLAHAGR---KAVLPGEIVAP  118

Query  121  SASALNANTRTSLRDENGNAIRVDTTTPRALELDEIPGIVNDFRQAVANAREAGFDLVEL  180
            SA A          DE  +        P  L  + I  +V DF++A   A+EAGFD++E+
Sbjct  119  SAIAF---------DEKSD-------KPVELTKEAIKEVVADFKRAAYRAKEAGFDVIEI  162

Query  181  HSAHGYLLHQFLSPSSNQRTDQYGGSVENRARLVLEVVDAVCNEWSADRIGIRVSPIGTF  240
            H+AHGYL+HQFLSP +N+R D YGG   NR +++ +++ AV   W    I +RVS
Sbjct  163  HAAHGYLIHQFLSPITNRREDNYGGPAGNRYKILSDIIKAVKEVWDGPII-VRVSA----  217

Query  241  QNVDNGPNEEADALYLIEELAKRGIAYLHMSETDLAGGKP-----YSEAFRQKVRERFHG  295
             +  +G  +  D +   + +   G+  + +S   L    P     Y   F  ++R R  G
Sbjct  218  TDYAHGGLQLEDHIPFAKWMKADGVELIDVSTGGLVNVAPPVFPGYQVPFADEIR-RGAG  276

Query  296  VIIGA-GAYT-AEKAEDLIGKGLIDAVAFGRDYIANP  330
            +  GA G  T  E+AE+++     D +  GR+ + NP
Sbjct  277  IATGALGLITRGEQAEEILCNERADLIIVGRELLRNP  313
```

Figure 4.9 Sequence alignment of PETN reductase (query) against listerial proteins (subject). Alignment scores showed significance identity to NamA 32%. Conserved amino acids stretched over the query sequence with NamA showing a query coverage of 89%. PETN reductase consists of 364 aa.

4.2.5 Alignment of *E. coli* NemA with *L. monocytogenes* proteins

Protein sequence of the NemA from *E. coli* was used to search for similar protein(s) in *L. monocytogenes* via BLASTp option in the NCBI database.

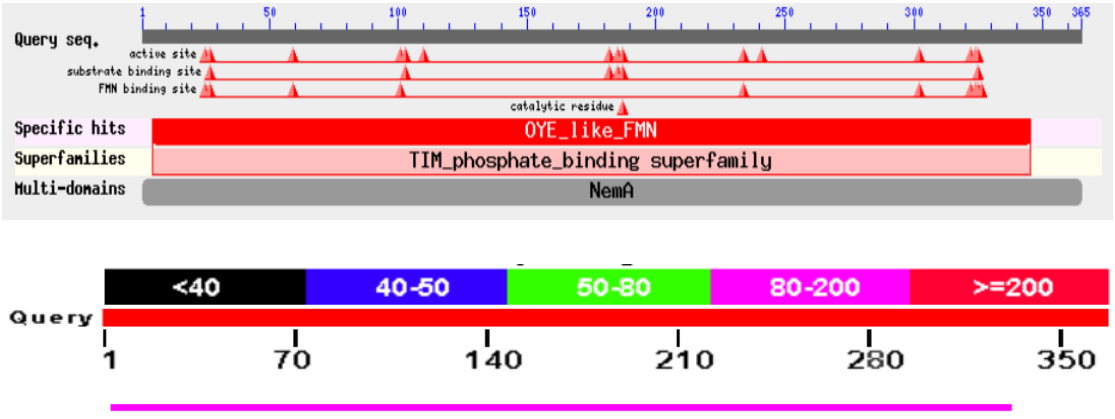
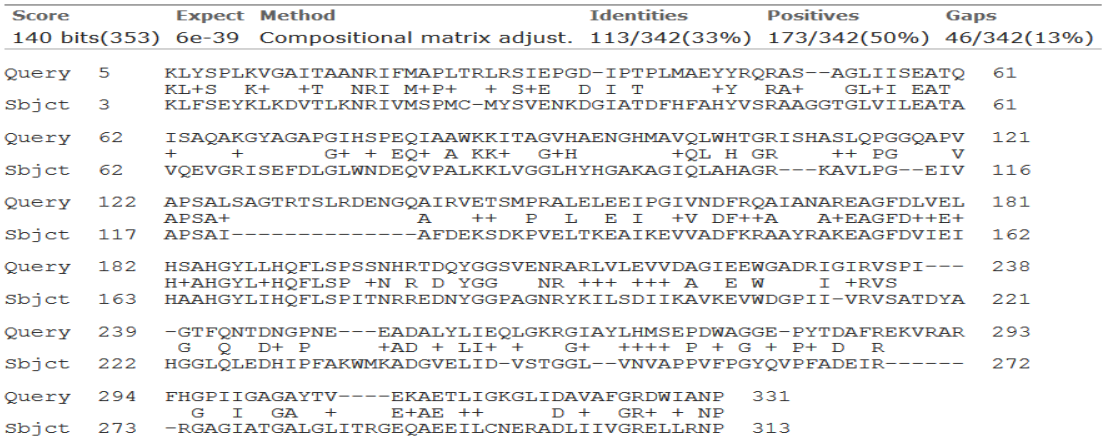


Figure 4.10 protein sequence alignment of PETN reductase against *L. monocytogenes* proteins. A stretch of conserved domains have been detected that belong to OYE (top). A color key alignment scores for proteins identity and the number of matched amino acids detected (bottom).

Table 4.5 Scores of sequences from *L. monocytogenes* proteins that show significance identity with NemA from *E. coli*

Description	Max score	Total score	Query coverage	E- value	Maximum identity
NamA	140	140	89%	6E-39	33%



Score	Expect	Method	Identities	Positives	Gaps
140 bits(353)	6e-39	Compositional matrix adjust.	113/342(33%)	173/342(50%)	46/342(13%)

```
Query  5    KLYSPLKVGAITAANRIFMAPLTRLRSIEPGD-IPTPLMAEYYRQRAS--AGLIISEATQ  61
            KL+S   K+   +T   NRI M+P+   + S+E   D I T      +Y   RA+    GL+I EAT
Sbjct  3    KLFSEYKLKDVTLKNRIVMSPMC-MYSVENKDGIATDFHFAHYVSRAAGGTGLVILEATA  61

Query  62   ISAQAKGYAGAPGIHSPEQIAAWKKITAGVHAENGHMAVQLWHTGRISHASLQPGGQAPV  121
            +    +        G+ + EQ+ A KK+   G+H          +QL H GR     ++ PG    V
Sbjct  62   VQEVGRISEFDLGLWNDEQVPALKKLVGGLHYHGAKAGIQLAHAGR---KAVLPG--EIV  116

Query  122  APSALSAGTRTSLRDENGQAIRVETSMPRALELEEIPGIVNDFRQAIANAREAGFDLVEL  181
            APSA+                A    ++   P   L   E I   +V DF++A    A+EAGFD++E+
Sbjct  117  APSAI--------------AFDEKSDKPVELTKEAIKEVVADFKRAAYRAKEAGFDVIEI  162

Query  182  HSAHGYLLHQFLSPSSNHRTDQYGGSVENRARLVLEVVDAGIEEWGADRIGIRVSPI---  238
            H+AHGYL+HQFLSP +N R D YGG   NR +++ +++ A   E W     I +RVS
Sbjct  163  HAAHGYLIHQFLSPITNRREDNYGGPAGNRYKILSDIIKAVKEVWDGPII-VRVSATDYA  221

Query  239  -GTFQNTDNGPNE---EADALYLIEQLGKRGIAYLHMSEPDWAGGE-PYTDAFREKVRAR  293
             G   Q   D+ P      +AD + LI+ +    G+   ++++ P + G + P+ D   R
Sbjct  222  HGGLQLEDHIPFAKWMKADGVELID-VSTGGL--VNVAPPVFPGYQVPFADEIR------  272

Query  294  FHGPIIGAGAYTV----EKAETLIGKGLIDAVAFGRDWIANP  331
               G   I   GA   +     E+AE ++      D +   GR+ + NP
Sbjct  273  -RGAGIATGALGLITRGEQAEEILCNERADLIIVGRELLRNP  313
```

Figure 4.11 Sequence alignment of NemA (query) against listerial proteins (subject). Alignment scores showed significance identity to NamA 33%, Conserved amino acids stretched over the query sequence with NamA showing a query coverage of 89%. NemA consists of 365 aa.

4.2.6 Alignment of *N. Gonorrhoeae* NADH oxidase with *L. monocytogenes* proteins

Protein sequence of the NADH oxidase from ***N.*** *Gonorrhoeae* was used to search for similar protein(s) in *L. monocytogenes* via BLASTp option in the NCBI database.

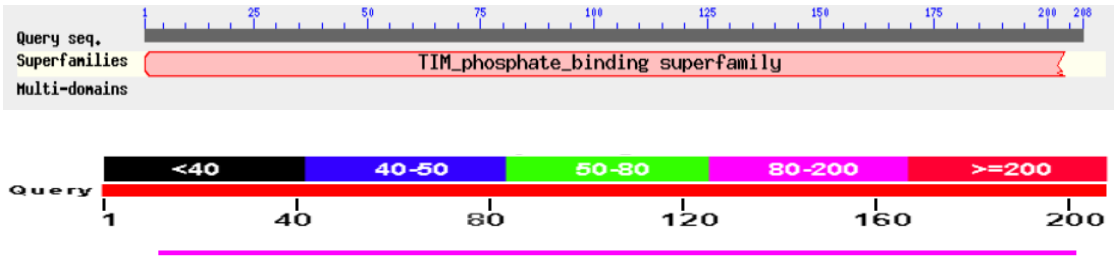


Figure 4.12 protein sequence alignment of NADH oxidase against *L. monocytogenes* proteins. Putative conserved domains have been detected (top), alignment scores for proteins identity and the number of matched amino acids detected (bottom).

Table 4.6 Scores of sequences from *L. monocytogenes* proteins that show significance identity with NADH oxidase from *N. Gonorrhoeae*

Description	Max score	Total score	Query coverage	E- value	Maximum identity
NamA	90.1	90.1	79%	2E-22	34%

Score	Expect	Method	Identities	Positives	Gaps
90.1 bits(222)	2e-22	Compositional matrix adjust.	56/166(34%)	91/166(54%)	5/166(3%)

```
Query  19   YGAWAEGGAGVLVTGNVMVAESGKGSINDVLISDDRALEMLKKWAKARTQNDTLLIMQIN  78
            Y + A GG G+++     V E G+ S  D+ + +D  +  LKK       +     +Q+
Sbjct  44   YVSRAAGGTGLVILEATAVQEVGRISEFDLGLWNDEQVPALKKLVGGLHYHGAKAGIQLA  103

Query  79   HAGKQSPAVVNKTPLAPSAVPLVGMNGFINPPRELSADEINGLIQQFVQTAKIAEQAGFS  138
            HAG++  AV+    +APSA+     +   + P EL+ + I  ++  F + A  A++AGF
Sbjct  104  HAGRK--AVLPGEIVAPSAI---AFDEKSDKPVELTKEAIKEVVADFKRAAYRAKEAGFD  158

Query  139  GVQIYAVHGYLISQFLSPHHNRRQDQWGGSLENRMRFLLETYTAIR  184
             ++I+A HGYLI QFLSP  NRR+D +GG   NR + L +   A++
Sbjct  159  VIEIHAAHGYLIHQFLSPITNRREDNYGGPAGNRYKILSDIIKAVK  204
```

Figure 4.13 Sequence alignment of NADH oxidase (query) against listerial proteins (subject). Alignment scores showed significance identity to NamA 34% from L. monocytogenes. Conserved amino acids stretched over the query sequence with NamA showing a query coverage of 79%. NADH oxidase consists of 208 aa.

Sequence identity of each protein aligned as a query was significantly similar to *L. monocytogenes* protein NamA with a minimum of 31% homology to EBP and maximum homology of 63% to YqjM. E-value from each alignment was significantly small which indicates greater chance of similarity and less probability of random background noise.

ClustalW software was also used to align multiple protein sequences derived from NCBI database of proteins belong to the OYE family. Each of these proteins was previously demonstrated to bind/degrade a variety of substrates including steroids.

Table 4.7 Multiple sequence alignment of proteins known to have a role in xenobiotic detoxification process. Length of each protein and identity scores (percentage) are shown. Red arrow indicates highest identity of YqjM to NamA.

SeqA	Name	Length	SeqB	Name	Length	Score
1	NamA	338	2	NADH	208	25.0
1	NamA	338	3	NemA	365	25.0
1	NamA	338	4	EBP	407	25.0
1	NamA	338	5	YqjM	338	62.0
1	NamA	338	6	PETN	364	26.0
1	NamA	338	7	Xenobiotic	366	40.0
2	NADH	208	3	NemA	365	28.0
2	NADH	208	4	EBP	407	26.0
2	NADH	208	5	YqjM	338	25.0
2	NADH	208	6	PETN	364	24.0
2	NADH	208	7	Xenobiotic	366	27.0
3	NemA	365	4	EBP	407	35.0
3	NemA	365	5	YqjM	338	28.0
3	NemA	365	6	PETN	364	87.0
3	NemA	365	7	Xenobiotic	366	26.0
4	EBP	407	5	YqjM	338	22.0
4	EBP	407	6	PETN	364	31.0
4	EBP	407	7	Xenobiotic	366	21.0
5	YqjM	338	6	PETN	364	30.0
5	YqjM	338	7	Xenobiotic	366	40.0
6	PETN	364	7	Xenobiotic	366	24.0

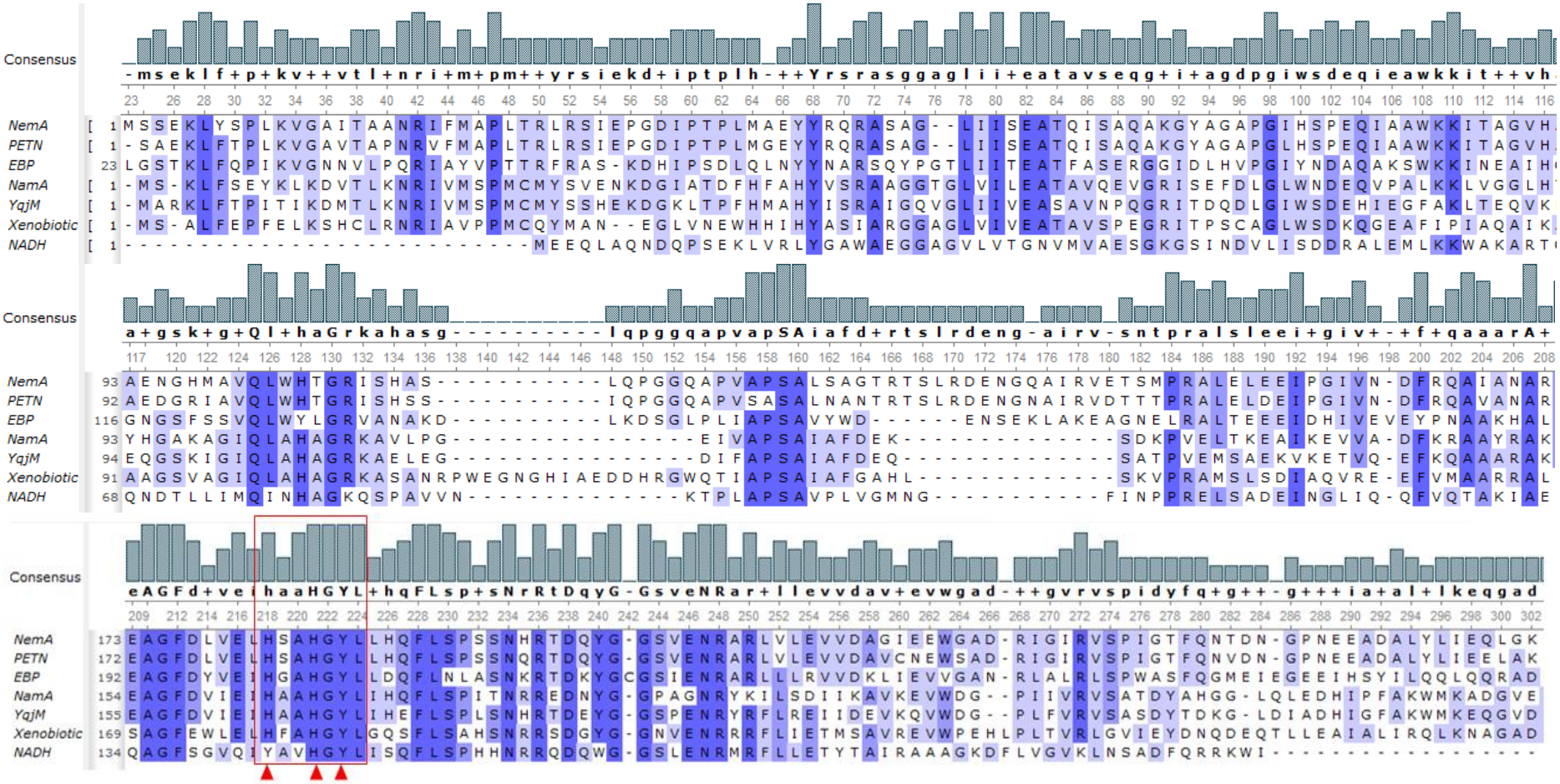


Figure 4.14 Multiple sequence alignments of members of the OYE family with NamA from *L. monocytogenes*. Conserved domains of the aligned sequences of proteins known to bind xenobiotic compounds and frequency of each amino acid (consensus) are shown. Protein sequences were taken from NCBI database. NemA; *Escherichia coli, PETN reductase; Enterobacter cloacae, EBP; Candida albicans,* NamA; *Listeria monocytogenes, YqjM; Bacillus subtilis,* Xenobiotic reductase; *Psedomonas putida, NADH oxidase; Neisseria gonorrhoeae.* Red box denotes conserved substrate binding site with red arrows showing amino acids (HxAHGYL) participate in binding and electron transfer to the substrate.

NamA from *L. monocytogenes* was shown to share sequence homology to a number of proteins belong to the OYE family. Although protein sequences differ in length and name, they share conserved amino acids where substrates bind.

Table 4.8 Characteristics of proteins belong to the family of OYE.

Organism	Name	Substrate Binding Site	Position	FMN Binding Site	Position	Length
L. monocytogenes	NamA	Y, H, H	27, 163, 166	R, R	214, 307	338
B. subtilis	YqjM	Y, H, H	28, 164, 167	R, R	215, 308	338
C. albicans	EBP	HGAH, Y, C	201-204, 206-225	Q, R,R	124, 254, 357	407
P. putida	Xenobiotic reductase	Unreviewed	N/A	A, Q, H, R, W	57,99,178, 231,358	363
E. cloacae	PETN reductase	Y, R, HSAH	69, 143, 182-185	A, Q, H, R, Y	59, 101, 182, 234, 352	365
E. coli	NemA	HSAH	182-185	Q, R, R	101, 234, 325	365
N. gonorrhoeae	NADH reductase	Unreviewed	N/A	N/A	N/A	208

Alignments of protein sequences belong to the family of OYE revealed conserved amino acid residues at the substrate binding site. Information derived from UniProt shows that the amino acid tyrosine (Y) was conserved in the substrate binding site of *L. monocytogenes* NamA, *B. subtilis* YqjM, *C. albicans* EBP and *E. cloacae* PETN reductase. Whereas histidine (H) was conserved in NamA, YqjM, EBP, PETN reductase and NemA from *E. coli*. Members of the OYE family are NADPH dependent of activity.

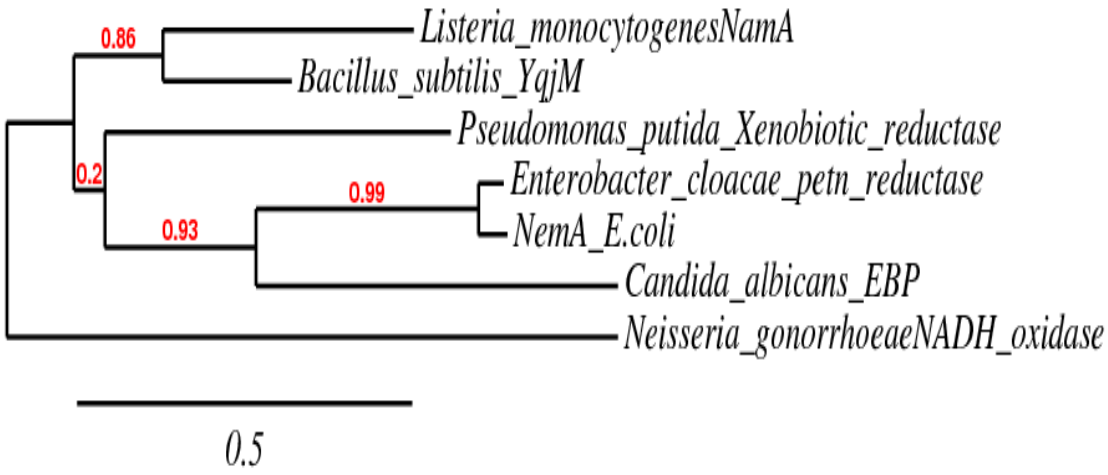


Figure 4.15 Phylogenetic tree showing minimum homology of amino acids substitution per site to the sequences aligned, with the highest homology to YqjM from *B. subtilis*. Diagram was generated using MABL software.

Further confirmation of sequence homologies were achieved by 3-dimentional comparison of protein structures. Each protein sequence was used to build a tertiary protein structure by the use of prediction software Phyre2. It builds up a structure for each protein based on amino acid sequence and similarity to other proteins in the database. Therefore, prediction is made upon fold of α-helices and β-sheets into compact molecule that becomes stable through specific tertiary interactions.

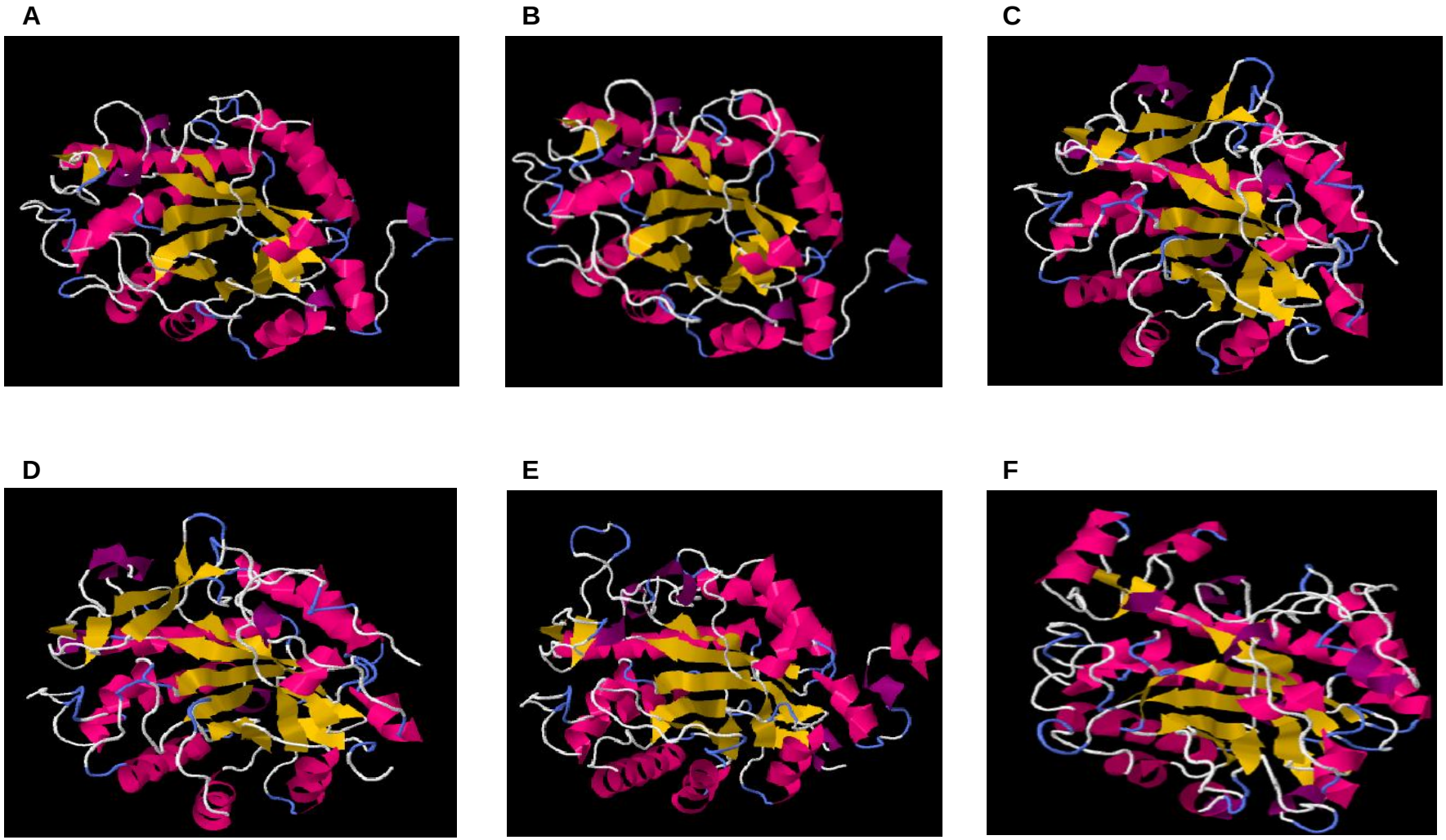


Figure 4.16 Comparison of the predicted tertiary structures of NADPH-dependent OYE. (A) *L. monocytogenes*: NamA, **(B)** *B. subtilis*:YqjM, **(C)** *E. cloacae*: PETN reductase, **(D)** *E. coli*: NemA, **(E)** *P. pudita*: xenobiotic reductase, **(F)** *C. albicans*: EBP. Tertiary structures were predicted using Phyre prediction software and the amino acid sequences of the proteins investigated were adapted from NCBI.

Distribution of α-helices and β-sheets from the tertiary protein structures were similar in all proteins, as α-helices lie at the outermost dimension, while β-sheets reside in the core of the molecule where substrates bind at the active binding domain.

4.3 Discussion

Bioinformatics and protein sequence alignment search was performed to identify *L. monocytogenes* proteins with the ability to bind steroid hormones. The search was conducted on the basis of existence of such proteins in different microorganisms. Therefore, sequences from these proteins were used for alignment with listerial proteins using the NCBI non-redundant protein database via BLASTp option. The results obtained showed that previously investigated proteins from different microorganisms with the ability to bind steroids and other unsaturated compounds share significant homologies to NamA, Lmo2235 and Lmo0489 proteins from *L. monocytogenes* with the highest sequence identity to YqjM from *B. subtilis* (63% identity, an E-value of 1e-153). All proteins used for the alignments were shown to be members of the OYE family since they possess a high degree of sequence identity with one another; thus were assigned similar functions. Based on the analysis presented in this study it is evident that NamA is qualified to be classified under the OYE family and propose similar function in the detoxification process of xenobiotic compounds.

In the last two decades, an increasing number of proteins have been isolated from different sources including Bacteria, yeast and plants, which share significant sequence identity with the first isolated OYE from yeast (Khan *et al*., 2005). The exact physiological role of these proteins is not fully understood; however, members of the OYE are capable of catalyzing a wide range of reactions, all having common step of the net addition of H_2 to the ligand (explained in details in chapter 5). Reduction of aromatic nitro compounds, denitrification of nitro-ester and nitro-aromatics, and the reduction of unsaturated carbon-carbon double bonds, including steroid hormones, are part of their reduction mechanism (Toogood *et al*., 2010). Furthermore, their

role in the degradation of explosives is a new insight of their physiological function (Gonzales-Perez *et al*., 2007). A number of these enzymes such as PETN reductase (*E. cloacae*), NemA (*E. coli*) and xenobiotic reductase (*P.putida*) have been isolated from bacteria found in explosive testing sites, which were later demonstrated to be involved in the degradation of organic nitrate esters (Khan *et al*., 2005 and Blehert *et al*., 1999). Therefore, it has been postulated that members of the OYE family are involved in the detoxification and degradation of xenobiotic compounds including steroid hormones.

Multiple alignments of OYE homologues with *L. monocytogenes* NamA showed that sequence identity is stretched over the sequence of the query, which revealed multiple conserved domains among aligned proteins. Substrate binding site among members of the OYE is conserved (Figure 4.14), and this includes NamA from *L. monocytogenes*. This site comprises histidine, alanine, glycine, tyrosine and leucine. They participate in ligand binding and reduction of the substrate by hydride and electron transfer via NADPH cofactor through the bound FMN. Moreover, prediction of the protein structures showed that all aligned proteins share similar conformation with 10 β-sheets in the core of the globule surrounded by 11 α-helices, which further confirms that these proteins are structurally and functionally similar. Indeed, proteins were shown to have sequence homologies to one another (Figure 4.15) with the strongest relation to YqjM from *B. subtilis* that was experimentally demonstrated to have a role in detoxification process (Kitzing *et al*., 2005).

In the present study, only NamA, and not the other two proteins (Lmo2235 and Lmo0489 – gene locations are depicted in figure 4.3), was experimentally demonstrated to be expressed in *L. monocytogenes* with/without the addition of hormones (refer to chapter 5 for details). In short, NamA from *L. monocytogenes* is hypothesised to bind pregnancy hormones, which in general influences the maintenance of healthy pregnancy. To investigate this hypothesis, NamA was subjected to further investigations in an attempt to understand the role of this enzyme toward pregnancy hormones. Therefore, gene expression, generation of deletion mutant, enzymatic activity and

purification of the protein were performed to determine its role in bacterial growth in the presence of hormones and the enzymatic activity of NamA toward progesterone and β-estradiol, which are explained in details in the next chapter.

Chapter V

Gene expression, Generation of deletion mutant and characterisation of NamA from *L. monocytogenes*

5.1 Introduction

The effect of pregnancy hormones on the growth and pathogenesis of *L. monocytogenes* during pregnancy was proposed to be related (Janakiraman, 2008). The ability of *L. monocytogenes* to infect pregnant women and cross the placental barrier led to the prediction that this bacterium takes advantage of the increased level of pregnancy hormones that are mainly produced from the placenta during mid and late stages of pregnancy. Therefore, it is anticipated that *L. monocytogenes* encodes for proteins allowing the pathogen to take use of the circulating hormones. To determine whether this prediction was correct, two approaches were taken to identify listerial proteins with the ability to bind pregnancy hormones. First approach used Tn*917*-LTV3-mediated mutagenesis to generate mutants with phenotype to pregnancy hormones; second approach was performed by the use of bioinformatics analysis of proteins capable of steroid binding from different microorganism and comparative analysis of the proteins involved. The results obtained from bioinformatics analysis showed that *L. monocytogenes* NamA protein shares significant identity to *C. albicans* EBP, *B. subtilis* YqjM, *E. cloacae* PETN reductase and many others that belong to the family of OYE, which were demonstrated to bind steroids (detailed in chapter 4). It was previously demonstrated that the expression of NADPH dehydrogenase (OYE) from *S. cerevisiae* was consistently up-regulated in response to progesterone (Banerjee *et al*., 2004). In line with this, *C. albicans* hsp90 was also shown to be up-regulated when cells were exposed to β-estradiol (Zhang *et al*., 2000). So, there is evidence that pregnancy hormones could play a role in microbial gene expression. For that reason, intense investigation of NamA including gene expression and deletion of NamA-encoding gene in *L. monocytogenes* were performed using semi-quantative RT-PCR and the temperature-sensitive *E. coli-Listeria* shuttle plasmid pLSV101(Joseph *et al*., 2006); respectively. The expression of NamA, bacterial growth of the NamA-deletion mutant and enzymatic activity were further investigated in the presence of pregnancy hormones progesterone and β-estradiol.

5.2 The family of Old Yellow Enzyme- OYE

Microbial degradation and detoxification of xenobiotic compounds has been an attractive field to researchers for many years. The search for microbial genes involved in degradation has revealed a number of proteins able to catalyse the reduction of unsaturated bonds through cofactor-dependent reactions. Sequence analysis and structural studies have indicated that these proteins belong to the family of the Old Yellow Enzyme (OYE). OYE was first isolated from brewer's bottom yeast in 1932 by Warburg and Christian during attempts to investigate the nature of the enzyme biological activity. The sugar glucose-6-phosphate (G6P) was oxidised by methylene blue in the presence of G6P-dehydrogenase. At the completion of the oxidation half reaction, Warburg identified another enzyme that permits completion of the respiratory chain reaction system by the use of molecular oxygen, which was later named the Old Yellow Enzyme (Figure 5.1). In 1953, Theorell purified another yellow enzyme from yeast that consists of a colourless apoprotein and a yellow dye, which both were found to be essential for the activity of the enzyme. Intensive research during this time showed that the yellow dye was biologically similar to riboflavin and later identified as riboflavin 5'-phosphate. Since then, the enzyme received considerable amount of investigation to elucidate the nature of the enzyme activity and the mechanism of function (Williams and Bruce, 2002).

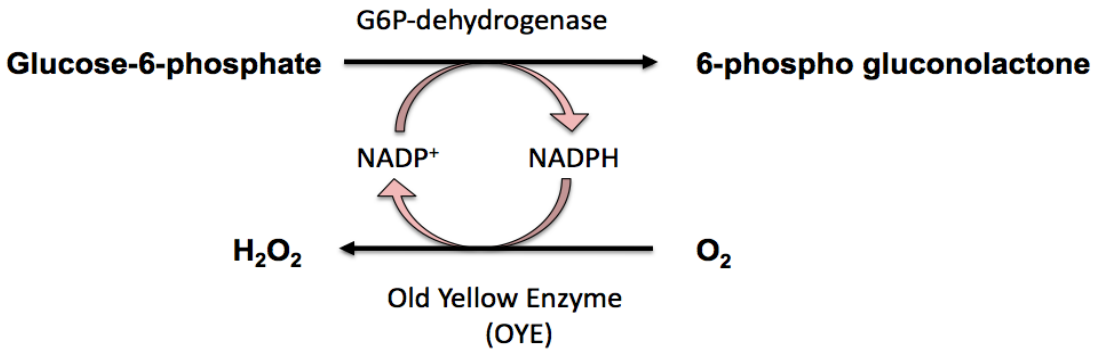
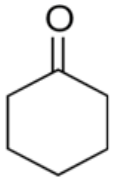
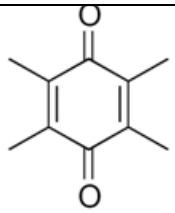
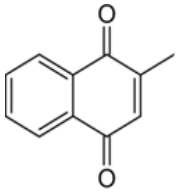
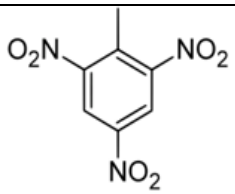
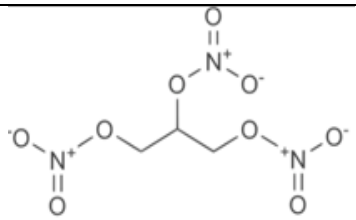
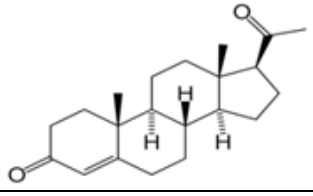
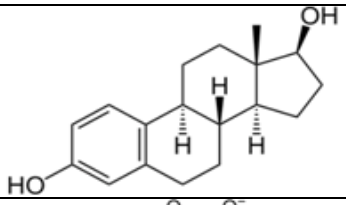
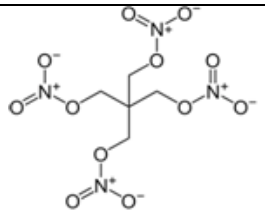
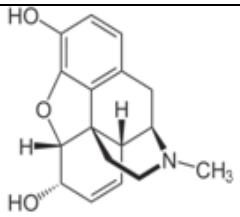
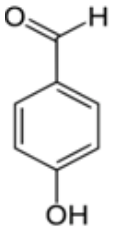
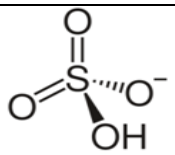
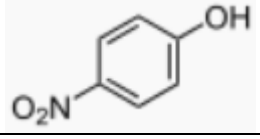


Figure 5.1 Schematic diagram shows the reaction system of Warburg and Christian.

The enzymatic cofactor Nicotinamide Adenine Dinucleotide Phosphate (NADPH) was found to be the physiological reductant of all OYE family members, where bound substrates become reduced upon NADPH oxidising. Members of the flavoprotein enzymes family catalyse the reduction of a wide variety of α/β unsaturated aldehydes, ketones and cyclic enones, plus reduce the nitro group, nitroester and nitroaromatic compounds (Table 5.1).

Table 5.1 Substrates capable of binding to proteins belong to the OYE family.

Name	**Chemical formula**	**Reference**
Cyclohexanone		William *et al*., 2002.
Duraquinone		William *et al*., 2002.
Menadione		William *et al*., 2002.
TNT (Trinitrotoluene)		Gonzalez-Perez *et al*., 2007
NG (Nitroglycerine)		Blehert *et al*., 1999
Progesterone		Khan *et al*., 2005

Estradiol		Madani *et al*., 1993.
PETN (Pentaerythritol tetranitrate)		Khan *et al*., 2005
Morphine		French and Bruce, 1994
pHBA (Hydroxybenzaldehyde)		Kitzing *et al*., 2005
Sulfate		Kitzing *et al*., 2005
pNP (*p*-nitrophenole)		Kitzing *et al*., 2005

Crystallization and X-ray studies revealed the structure of OYE. The enzyme consists of a single-domain protein of around 45 kDa, with α/β barrel fold. A small vitamin-derived molecule, flavin mononucleotide (FMN), was shown to bind the enzyme non-covalently and '*neither the enzyme alone nor FMN can catalyse the corresponding redox reaction, and only both together results in the active enzyme form*' (Theorell, 1955). The role of NADPH was established to serve as FMN reductant, and the flavin acts as an electron donor to reduce unsaturated substrates. The non-covalently bound FMN lies at the top of the barrel with the *si*-face accessible to the solvent, where it forms the bottom of the active site that permits stacking interaction between the FMN and the substrate. Moreover, oxygen atoms from the substrate contribute to hydrogen

bonding with amino acids that reside at the active binding domain of the enzyme (Figure 5.2).

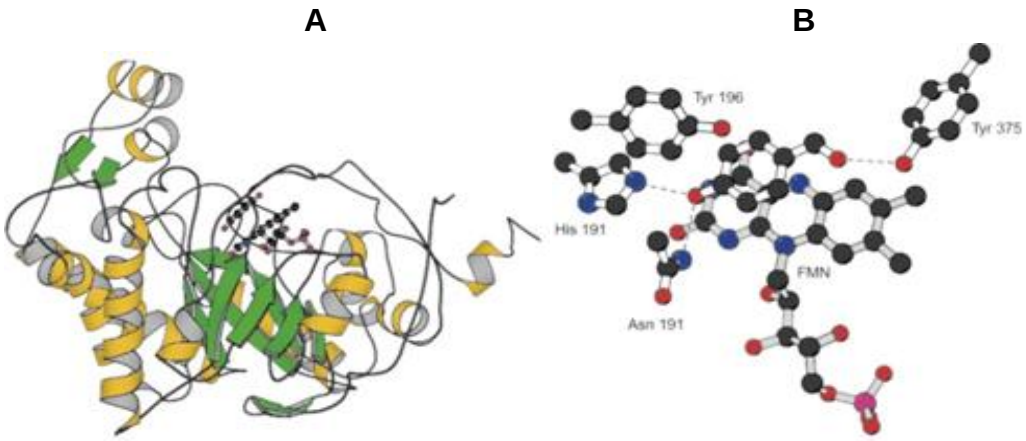


Figure 5.2 Structure of the OYE and substrate binding socket. **(A)** Three dimensional structure of the OYE represented in ribbon diagram. The enzyme is bound to *p*-hydroxybenzaldehyde through hydrogen bonding with amino acids from the active binding site. Substrate lies on top of the FMN. **(B)** Active binding domain of OYE in complex with *p*-hydroxybenzaldehyde. Dotted lines represent hydrogen bonding between oxygen atoms from the substrate and amino acids exist in the active site (adapted from William and Bruce, 2002).

In contrast to the OYE, the crystal structures of OYE members have revealed that these enzymes are mixture of homodimers and heterodimers with each monomer of ~45 kDa binding a molecule of flavin mononucleotide. Although the exact physiological function is still unknown, many ligands such as phenols and steroids have been identified, which indicates the possibility that these enzymes might be involved in steroid metabolism (Stott *et al*., 1993). Moreover, the crystal structure of OYE revealed several amino acid residues around the ligand binding site that affect catalysis and ligand binding (Brown *et al*., 1998). Phenolic compounds form parallel stacking interaction to the flavin by π-overlap with the isoalloxazine ring and oriented by hydrogen bonding of the phenolate oxygen with His-191, Asn-194 and Tyr-196. Construction of OYE mutant lacking these amino acids further confirmed the role of these residues in ligand binding and catalysis of the enzyme (Brown *et al*., 1998 and Kholi *et al*., 1998). It was previously demonstrated by Vas *et al*.,

(1995) that reduction of α/β- unsaturated carbolyl compounds occurs through stereospecific transfer of hydride from NADPH via the flavin to the carbon atom β to the carbonyl function and transfer of a proton to the α position, leading to trans-addition to the double bond that becomes saturated. Alignments of amino acid sequences of OYE homologues revealed high conservation in selected regions of the protein. For example, conserved amino acid residues involved in catalysis, flavin mononucleotide (FMN) and substrate binding (Adalbjornsson *et al*., 2010). Several structures of homologues proteins were solved, some exist as a monomer (PETN reductase), as a homo/heterodimer (OYE) or even as tetramer (YqjM) (Kahan *et al*., 2005, Fox and Karplus, 1994 and Kitzing *et al*., 2005). In all structures, FMN is bound on the top of the barrel and is attached to the structure by a network of hydrogen bonds. The active site contains a highly conserved motif (HxAHGYL) and mutational studies have confirmed their role in the catalytic cycle (Figure 5.3).

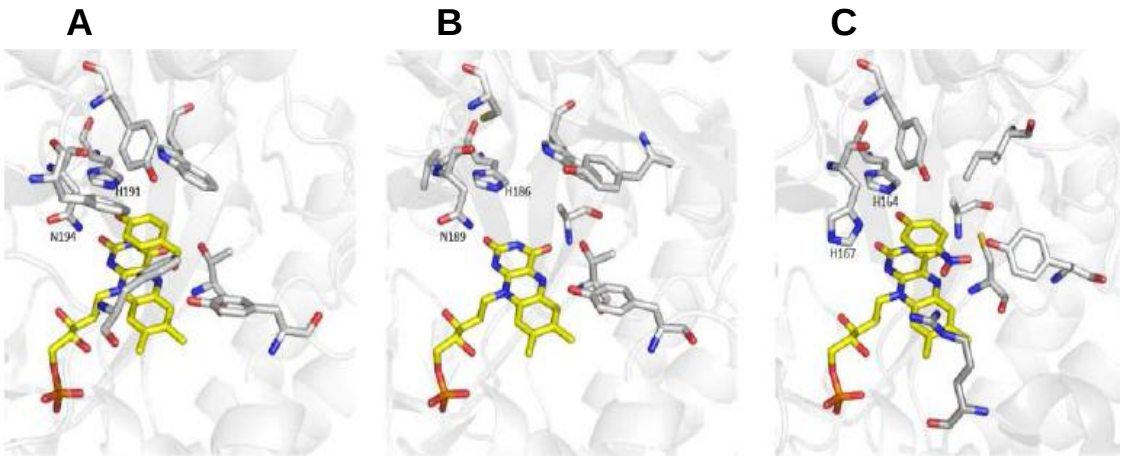


Figure 5.3 Comparison of the active sites from 3 different members of the OYE family. (A) OYE from yeast, **(B)** Morphine reductase (MR) from *P. putida* **(C)** YqjM from *B. subtilis.* All enzymes are bound to *p-hydroxybenzaldehyde* (white sticks). Residues within 5 Å around the bound substrate are shown (from Sabrina Kille, 2010).

The pair of His/Asn or His/His is involved in positioning and binding of the substrate, whereas tyrosine acts as a general acid by protonating the substrate after hydride transfer from the bound FMN molecule. The importance of the tyrosine residue is controversial since it plays a key role for

the catalytic activity of OYE (Vaz et al., 1995) but not for PETN reductase (Khan et al., 2005), while it is replaced by cysteine in the enzyme morphine reductase (MR), a member of the OYE family (French and Bruce, 1998). It can be seen from figure 5.3 that the phenolic ring of the ligand forms a stacking interaction with the enzyme-bound flavin. The ligand is anchored and positioned by the force of two hydrogen bonds between the His/His or His/Asn amino acid residues and the oxygen atom from the phenolic ring.

5.3 Results

5.3.1 Investigation of NamA Expression by semi-quatntative RT-PCR

Bioinformatic analysis of hormone binding proteins from *Listeria* and other microbes revealed that *L. monocytogenes* encodes for 3 similar proteins that share significant similarity with OYE members (Tables 4.1 and 4.2, chapter 4). To assess their expression with/without pregnancy hormones, specific primers were designed to amplify a small region internal to each of these genes by PCR using cDNA as a template. To achieve this, *L. monocytogenes* (exposed to increasing concentrations of either progesterone or β-estradiol) was used to extract total RNA as described in section 16, chapter 2. Before RNA extraction, the number of cells were equalised by measuring the O.D of each sample before centrifugation, and by counting cell forming units per millilitre of the sample. Each of the RNA samples was checked by PCR for DNA contamination using 16s-ribosomal RNA, followed by reverse transcription to cDNA and PCR amplification (section 16.1, chapter 2). The expression of *namA* only, and not the other two genes, (*lmo2235* and *lmo0489*) was detected by the use of semi-quantitive PCR. This protocol permits the preliminary detection of a transcript for specific-gene by the use of a set of primers internal to the gene to be amplified, without quantification of the PCR product.

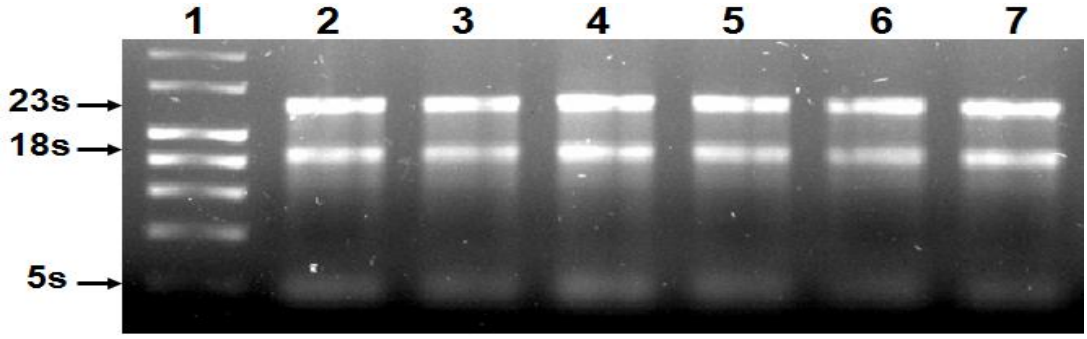


Figure 5.4 Total RNA isolated from *L. monocytogenes* cultured in the presence of either β-estradiol or progesterone. RNA isolated from cells exposed to; Lane 1: HL1, Lanes 2-4: cells exposed to β-estradiol at a final concentrations of 0, 20 and 60 µg ml^{-1}; E0, E20 and E60 respectively. Lanes 5-7: cells exposed to progesterone at a final concentrations of 0, 20 and 60 µg ml^{-1}; P0, P20 and P60 respectively. RNA bands show 23s, 16s and 5s RNA. RNA samples were run on 1.2% agarose gel for 30 min at 100v.

Table 5.2 Concentrations of RNA samples and purity ratio.

Sample	Concentration (µg ml^{-1})	A260/A280	A260/A230
E0	102	1.927	1.263
E20	110	1.939	0.0980
E60	110	2.095	0.914
P0	98	2.191	0.780
P20	90	2.145	0.690
P60	86	2.174	0.801

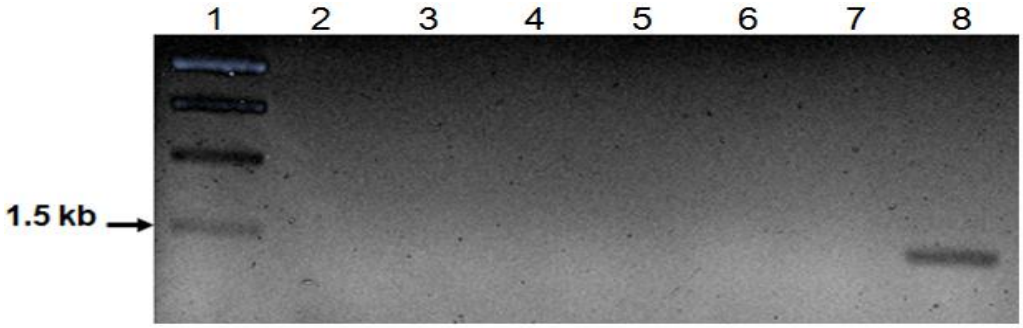


Figure 5.5 Check of DNA-contamination by PCR using 16s RNA. All RNA samples were contamination-free as indicated by PCR. Listerial genomic DNA was used as template for positive control. Lane1: HL1, Lanes: 2-7: no DNA bands (contamination free), lane 8: positive control.

Table 5.3 RNA samples and the volume used to adjust the final concentrations

Sample	Mean Conc. (µg ml^{-1})	Vol. To get 10 µg ml^{-1}	Vol. Of H_2O (µl)	Total Vol. (µl)	Final Conc. (µg ml^{-1})
E0	102	1	9	10	10
E20	110	1	9	10	10
E60	110	1	9	10	10
P0	98	1	9	10	10
P20	90	1.1	8.9	10	10
P60	86	1.2	8.8	10	10

Adjusment to the concentration of all RNA samples were performed using PCR grade water to insure equal amount of cDNA of all treated samples that will be used for gene expression along with the genomic DNA that was used as a positive control. Reverse transcription was carried out as described in section 16.1, chapter 2.

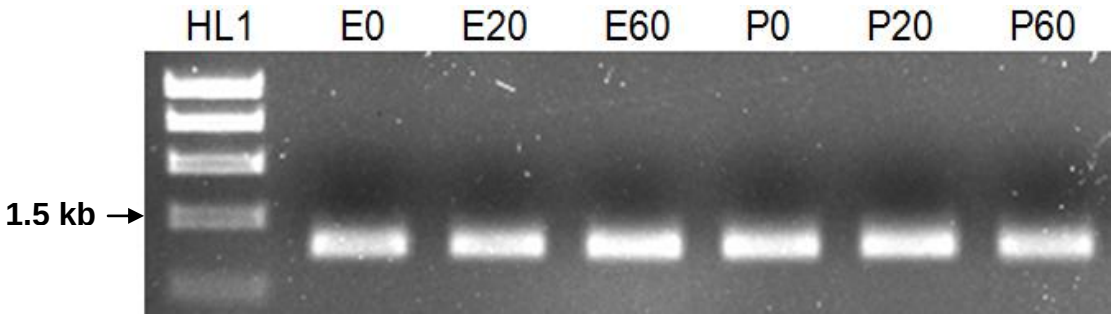


Figure 5.6 PCR product of 16sRNA using cDNA from all treated cultures to confirm success of reverse transcription reactions and equal intensity of each sample.

Post reverse-transcription reactions, cDNA from each sample were used as a tempelate for PCR reactions. Increased intensity of PCR-product bands suggests increased expression of the target gene and visa versa (no densitometry was involved; hence qualitative assessment only).

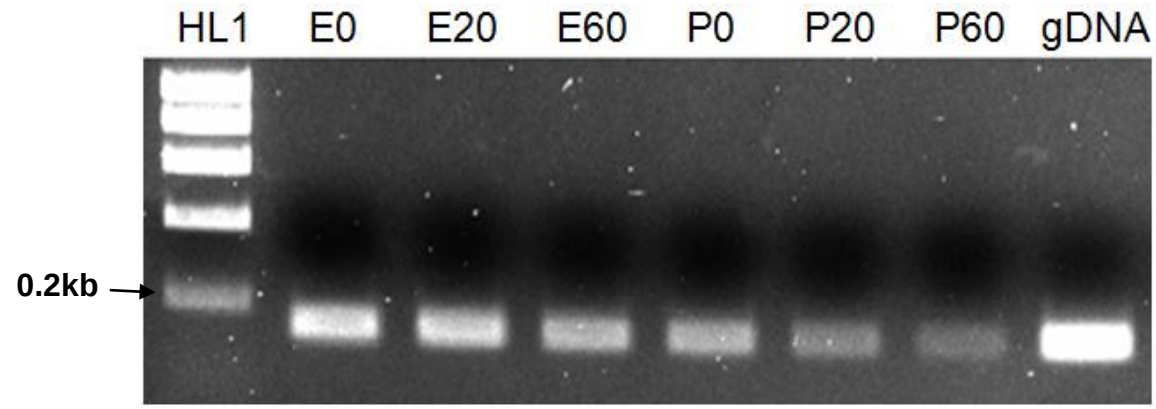


Figure 5.7 Semi-quantitative RT-PCR. Gene expression of NamA from *L. monocytogenes*. Specific primers were used to PCR amplify 153 bp from NamA-encoding gene (*lmo2471*). cDNA used for PCR amplification were derived from listerial cultures treated with: E0; 0, E20; 20 and E60; 60 µg ml^{-1} β-estradiol. P0; 0 , P20; 20, and P60; 60 µg ml^{-1} progesterone. Positive control used gDNA from *L. monocytogenes*. Similar results were obtained when this was performed 3 times in 3 different occasions.

Similar experiment was performed to assess the expression of *lmo2235* and *lmo0489* using the same cDNA used above and spesific designed primers for each gene. PCR was carried out as above.

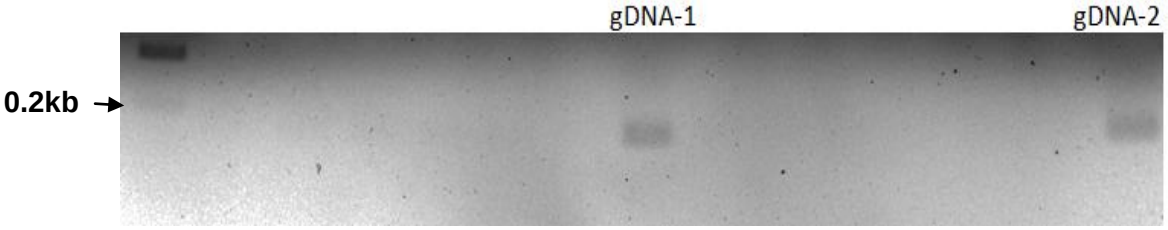


Figure 5.8 Semi-quantitative RT-PCR to assess the expression of *lmo2235* and *lmo0489*. Specific primers were used to PCR-amplify internal fragment of each gene. Positive controls used genomic DNA from *L. monocytogenes.*

Results show that only *namA* and not the other two genes were expressed in *Listeria*. The gene encoding for NamA was constitutively expressed in the presence and absence of both hormones (progesterone and β-estradiol); however, intensity of PCR bands were relatively decreased as the hormone concentration increases and this was more apparent when cells were exposed to progesterone (Figure 5.7).

5.3.2 Generation of *namA*-deletion mutant in *L. monocytogenes*

In order to investigate the role of NamA in the growth restoration phenomenon observed in chapter 3, and whether it has enzymatic activity toward pregnancy hormones, NamA-encoding gene (*Lmo2471*) was deleted and *namA*-deletion mutant strain was constructed. One hypothesis for the inability of the NamA mutant to grow in the presence of pregnancy hormones is therefore that the expression of NamA protein is required for resistance to the toxicity/growth retardation of pregnancy hormones.

The temperature-sensitive shuttle vector pLSV101 was used to delete NamA-encoding gene in *L. monocytogenes.* To accomplish this, two DNA fragments (Table 5.4) from *L. monocytogenes* chromosomal DNA were PCR-amplified and digested with the introduced *BglII* to facilitate ligation of both fragments together (detailed protocol in section 17, chapter 2).

Table 5.4 Characteristics of DNA fragments used for the deletion of NamA-encoding gene

Name	location	Size of PCR product	Restriction sites	Source of enzymes
Lmo2470-AB	Upstream	604 bp	*BamHI/BglII*	Roche,UK
Lmo2472-CD	Downstream	426 bp	*BglII/EcoRI*	

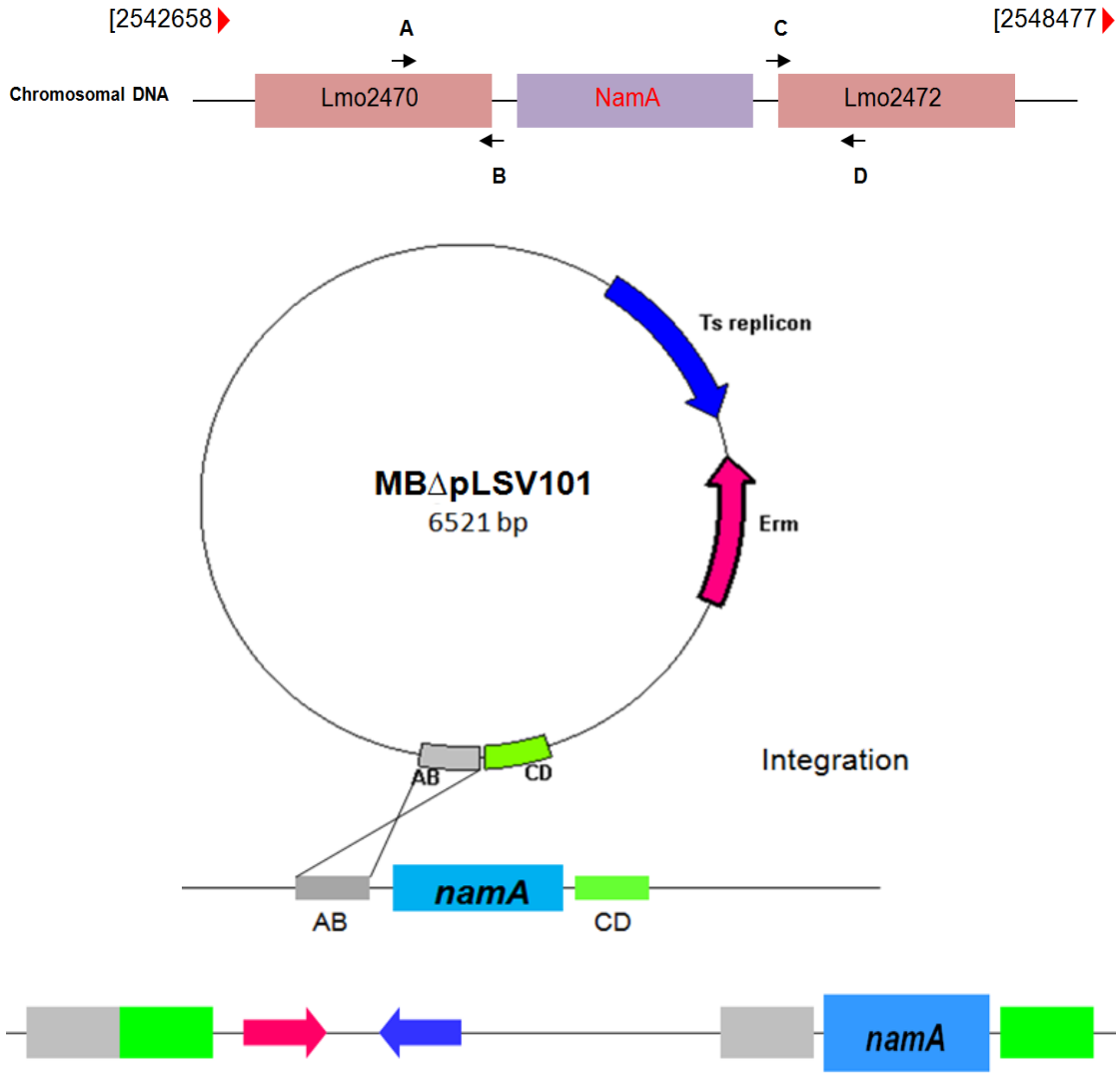


Figure 5.9 Schematic diagram of allelic exchange with MBΔpLSV101. **(A)** Fragments of *L. monocytogenes* chromosomal DNA used for cloning into shuttle vector pLSV101 were PCR-amplified using the AB and CD primer sets. **(B)** A single-crossover integration of the recombinant plasmid into *L. monocytogenes* chromosomal DNA. Integrated plasmid was excised by culturing the erm-resistant colonies at 30ºC without antibiotic and resulting erm-sensitive colonies were screened for by growing the cells in the presence of erm. Erm-sensitive cells were subjected to PCR amplification to confirm that gene deletion had taken place.

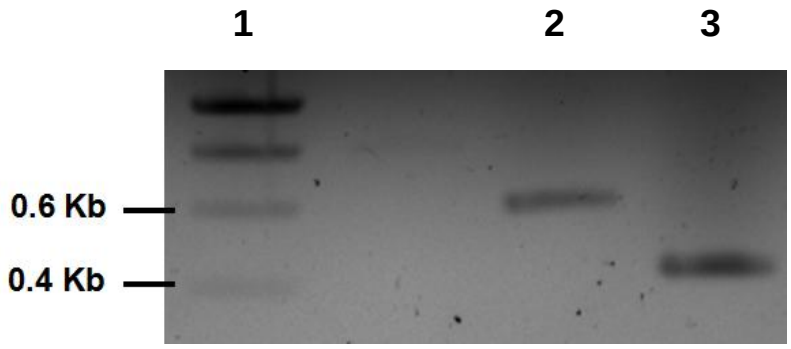


Figure 5.10 PCR amplification of DNA fragments from *L. monocytogenes* chromosomal DNA. Two fragments of 604 bp and 426 bp were PCR amplified from up-stream (AB, Lane 2) and down-stream (CD, Lane3) regions of the gene to be deleted. PCR products were run on 1% agarose gel for 40 min.

The remaining PCR products were purified using PCR purification kit (Qiagen) and digested with the restriction endonuclease *BglII* at 37ºC overnight. The restriction site of *BglII* (AGATCT) was introduced to B primer of the AB fragment and C primer of the CD fragment to facilitate fragments ligation followed by cloning into the multiple cloning site (MCS) on the shuttle vector. Post digestion with *BglII*, each of the fragments was gel extracted using gel extraction kit (Qiagen), since *BglII* enzyme cannot be heat-inactivated, and the DNA fragments were stitched together by T4 DNA ligase at 4ºC overnight at a ratio of 1:1. The ligation mixture was used as a template for PCR reaction to amplify AB/CD fragment using the outermost primers A and D.

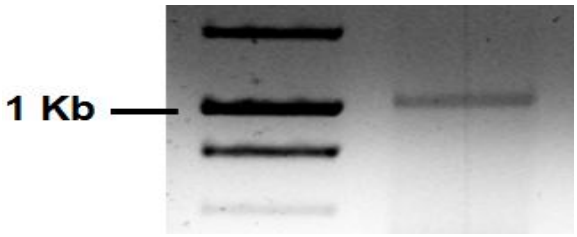


Figure 5.11 PCR amplification of AB/CD fragment from the ligation mixture. Previously PCR-amplified fragments of AB and CD were ligated together and PCR amplified using the outermost primers AD. PCR product was run on 1% agarose gel, which shows a DNA fragment of 1030 bp.

PCR product of AB/CD fragment was purified using PCR purification kit. Both of AB/CD PCR product and the plasmid vector pLSV101 were similarly digested at 37ºC overnight using *BamHI* and *EcoRI* (Table 5.4). Post digestion, restriction enzymes were heat-inactivated at 65ºC for 20 min followed by ligation at a ratio of 3:1 (Insert : vector) for construction of recombinant plasmid (MBΔpLSV101). A volume of 5 µl of the ligation mixture was used to transform *E. coli* α-select for propagation. Cells were incubated statically at 37ºC overnight and successful transformants were selected on LB agar supplemented with erm at a final concentration of 300 µg ml^{-1}. A number of 12 colonies were randomly chosen for propagation and isolation of the recombinant plasmid. Therefore, colonies were cultured overnight in LB broth supplemented with 300 µg ml^{-1} erm and plasmid was isolated as described in chapter 2, section 7.

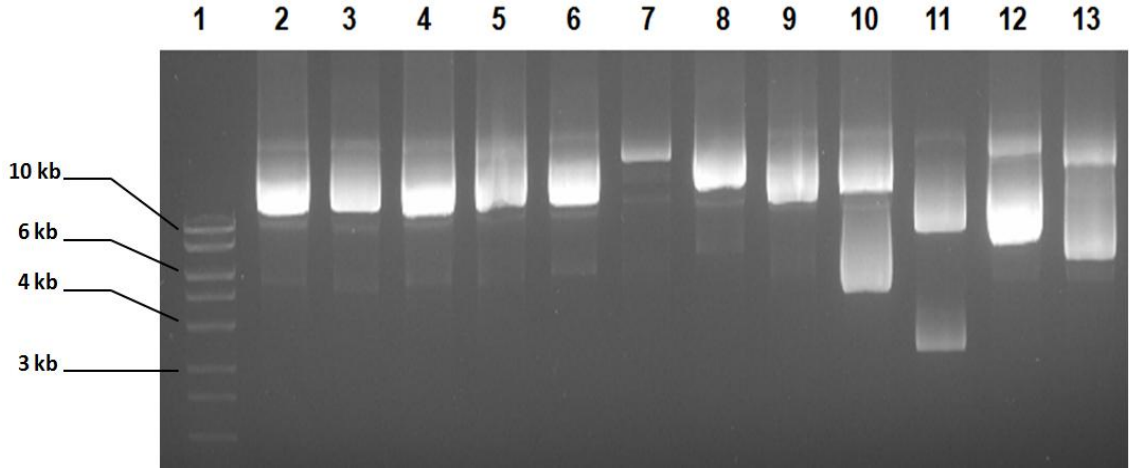


Figure 5.12 Isolation of recombinant plasmid DNA from *E. coli* α-select. A number of 12 random colonies of transformants were selected for isolation of recombinant plasmid MBΔpLSV101. Lane 1: molecular weight marker (HLI), Lanes 2-13: plasmid DNA isolated from 12 random colonies grown on LB agar supplemented with 300 µg ml^{-1} erm. DNA was run on 1% agarose gel.

Recombinant plasmid DNA from each of the 12 colonies was subjected to restriction digest using *HindIII*. This restriction endonuclease cuts twice within the plasmid vector pLSV101; which gives 2 fragments of 4170 bp and 1322 bp, and cuts once within the insert. Therefore, successful clones were

expected to show 3 bands of 4363 bp, 1322 bp and 837 bp (Figure 5.13). Restriction digest was carried out at 37ºC overnight.

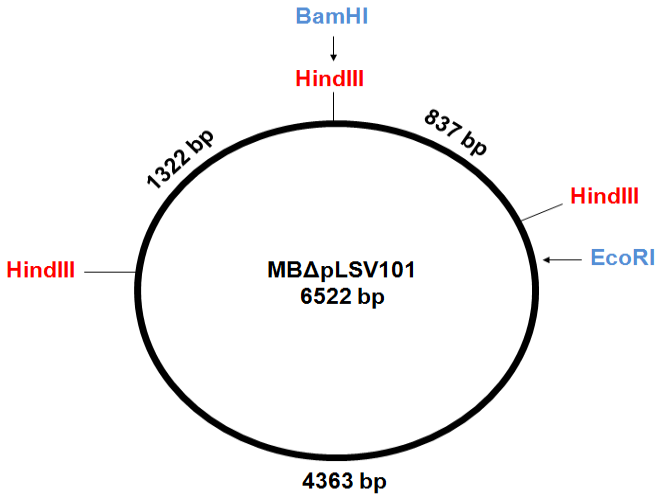


Figure 5.13 Restriction map of the constructed plasmid MBΔpLSV101 and selected restriction sites. The NamA-encoding gene was cloned into the shuttle vector pLSV101 via *BamHI* and *EcoRI* (Blue font). Restriction sites of *HindIII* (Red font) are shown on the map.

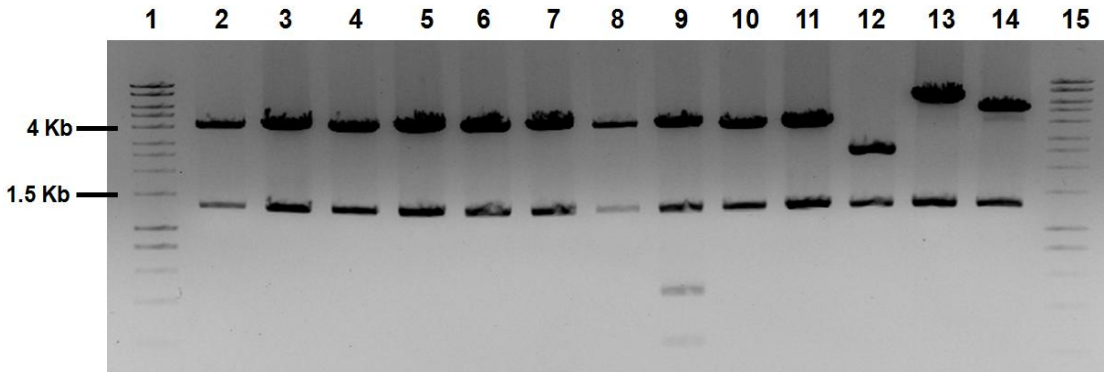


Figure 5.14 Restriction digest of the recombinant plasmid. Each of the isolated plasmid was digested overnight at 37ºC using *HindIII*, and DNA was run on 1% agarose gel. Lanes 1 and 15: molecular weight marker (HLI), Lane 2: vector only digested with *HindIII* (positive control), Lane 3-14: isolated plasmid DNA digested with HindIII.

DNA fragments in lanes 3-8 and 10-11match the sizes of DNA obtained from the vector only (Lane 2), which indicates unsuccessful cloning. However, DNA fragments in lane 9, 12, 13 and 14 vary in sizes. None of them correspond to the expected DNA fragments as depicted in figure 5.13. Therefore, more

colonies were picked for confirmation of successful cloning as described above.

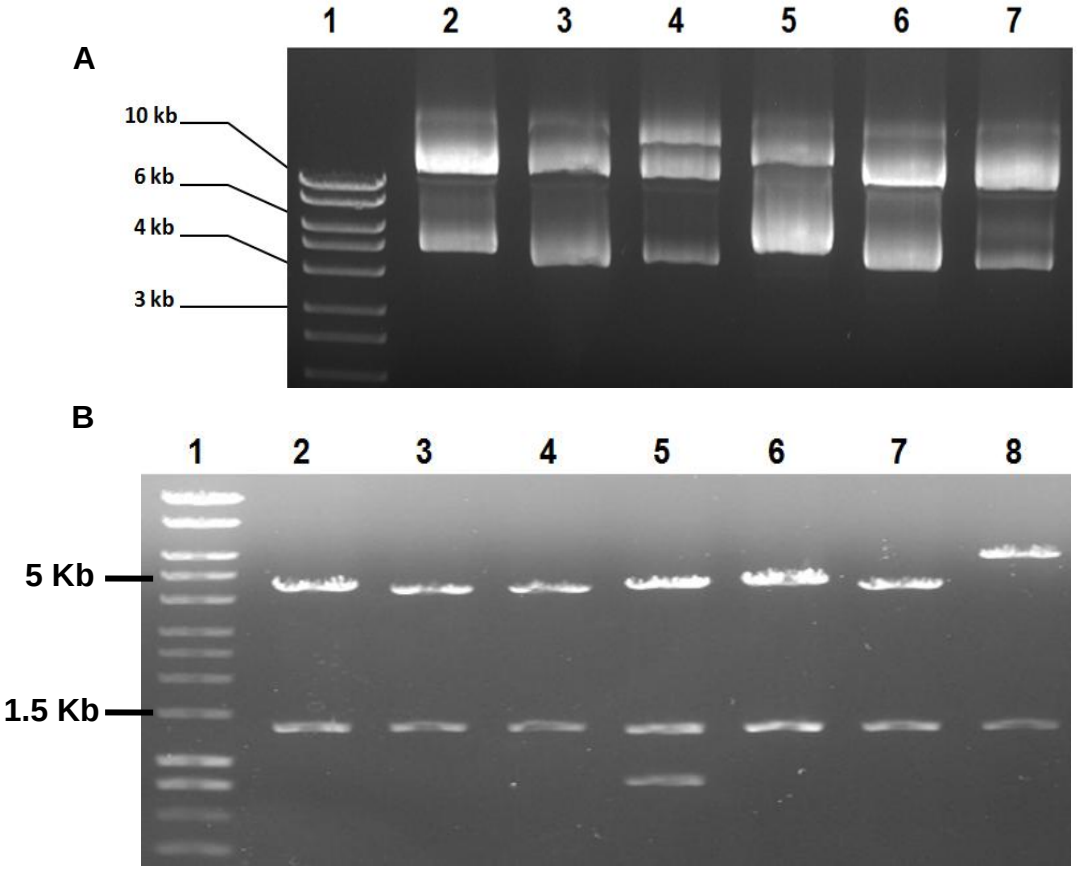


Figure 5.15 Isolation and digestion of recombinant plasmid. Another 6 randomly chosen colonies were selected to check for successful cloning. **(A)** Isolation of recombinant plasmids from 6 colonies grown on LB agar supplemented with 300 µg ml^{-1} erm. Lane 1: molecular weight marker (HLI), Lanes 2-7: recombinant plasmid DNA. **(B)** Restriction digests of the isolated recombinant plasmid using *HindIII*. Lane 1: molecular weight marker (HLI), Lane 2: pLSV101 vector (control), Lanes 3-8: recombinant plasmid digested with *HindIII*.

With the exception to the digest in lane 5 (clone 3), DNA fragments from the restriction digests indicate unsuccessful cloning since DNA fragments were the same size as the control (4170 bp and 1322 bp). However, digest from lane 5 represents 3 different fragments that correspond to 4363 bp, 1322 bp and 837 bp, which indicate possible successful clone. To further confirm this, recombinant plasmid (from lane 5) was used as a template to PCR-amplify

fragments of the insert, which were cloned from *L. monocytogenes* into the plasmid vector.

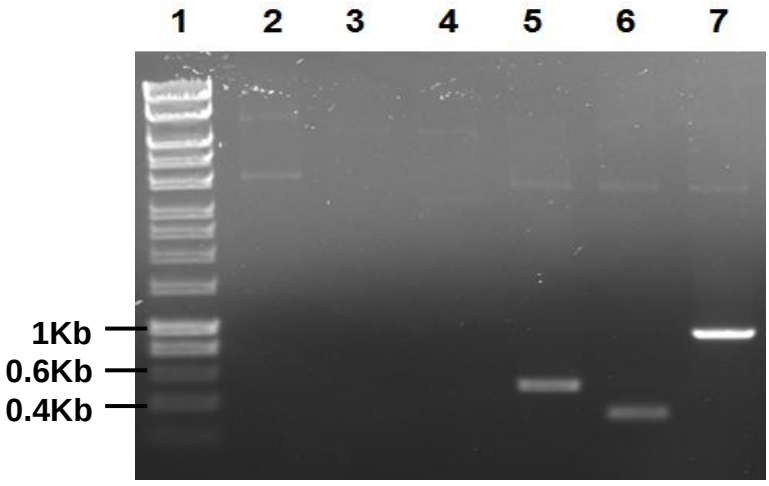


Figure 5.16 Confirmation of successful cloning by PCR amplification. Recombinant plasmid isolated from clone 3 was subjected to PCR amplification using the same primers used for cloning. Lane 1: molecular weight marker (HLI), Lane 2-4: no PCR product when using the vector only as a template, Lane 5: PCR product using the MBΔpLSV101as a template and AB primers (604 bp), Lane 6: PCR product using the MBΔpLSV101as a template and CD primers (426), Lane 7: PCR product using the MBΔpLSV101as a template and AD primers (1030 bp).

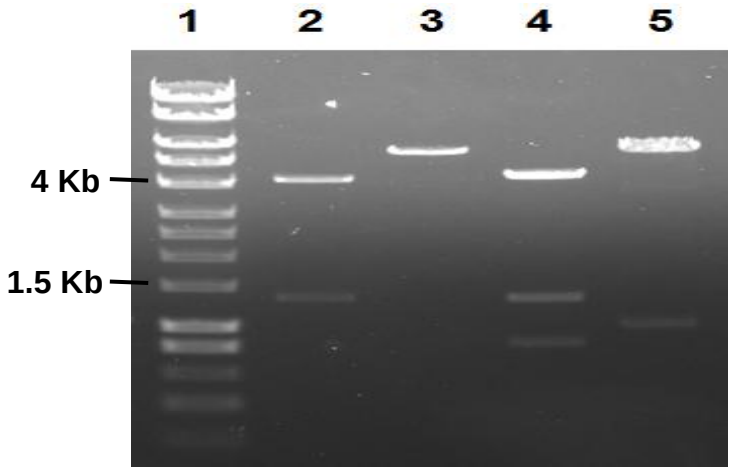


Figure 5.17 Confirmation of successful cloning by restriction digest. Successful cloning was further confirmed by restriction digest using: Lane 1: HLI, Lane 2: vector only digested with *HindIII*, Lane 3: vector only digested with *BamHI/EcoRI*, Lane 4: MBΔpLSV101 digested with *HindIII*, Lane 5: MBΔpLSV101 digested with*BamHI/EcoR*.

It is evident that clone 3 is the correct one, which harbours fragments derived from *L. monocytogenes* chromosomal DNA. As can be seen in figure 5.16, PCR products confirm that the inserted fragments were only in the recombinant plasmid, but not in the vector. Moreover, figure 5.17 further confirms that clone 3 harbours *L. monocytogenes*-derived fragments and gave DNA sizes corresponding to the correct bands expected. Plasmid vector digested with *HindIII* gave 4170 bp and 1322 bp, while digestion with *BamHI/EcoRI* gave DNA band correspond to the same size as the vector only since *BamHI* and *EcoRI* restriction sites are located in the multiple cloning sites, which are several nucleotides apart. Digestion of the recombinant plasmid with *HindIII* gave 3 different fragments of 4363 bp, 1322 bp and 837 bp, and the sum of these fragments matches the total size of the vector and the insert put together. Also, digestion of the recombinant plasmid with *BamHI/EcoRI* gave DNA sizes of 5491 bp and 1030 bp, which correspond to the size of the vector and insert, respectively.

Recombinant plasmid was electrotransformed into freshly prepared *L. monocytogenes* competent cells as described in chapter 2 section 12, and cells were incubated at 30ºC for 48 h on TSA agar containing 5 µg ml^{-1}. Successful transformation was confirmed by recombinant plasmid isolation from the transformants and further subjected to restriction digest.

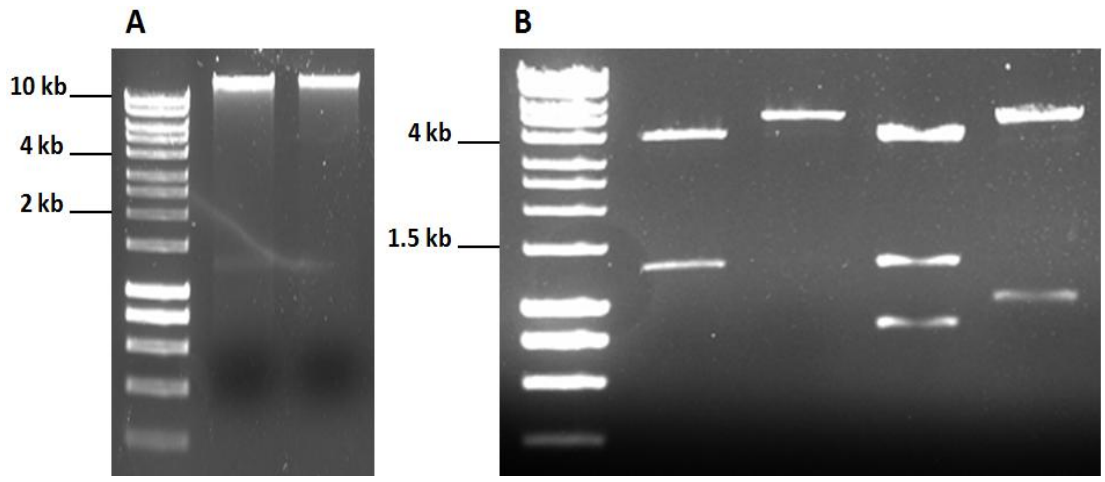


Figure 5.18 (A) Isolation of the recombinant plasmid DNA from *L. monocytogenes*. Lane 1: molecular weight marker (HLI), Lane 2-3: recombinant plasmid. **(B)** Restriction digests of the recombinant plasmid. Lane 1: HLI, Lane 2: vector only digested with *HindIII*, Lane 3: vector only digested with *BamHI/EcoRI*, Lane 4: MBΔpLSV101 digested with *HindIII*, Lane 5: MBΔpLSV101 digested with *BamHI/EcoRI*.

Recombinant plasmid (MBΔpLSV101) isolated from *L. monocytogenes* was digested with restriction enzymes *HindIII*, *BamHI* and *EcoRI* to confirm the correct plasmid. Bands sizes correspond to the sizes described in figure 5.17.

A single colony from of *L. monocytogenes* harbouring MBΔpLSV101 was streaked on TSA agar supplemented with 5 µg ml^{-1} erm and integration of the recombinant plasmid into the bacterial chromosome was selected for by incubating the plates at 43ºC overnight. Plasmid replication is not permitted at this temperature; therefore only cells with integrated plasmid will grow in the presence of 5 µg ml^{-1} erm. A single colony was cultured in 10 ml of TSB without antibiotic and incubated for 24 h with shaking (200 rpm) at 30ºC to facilitate second crossover and elimination of the integrated plasmid. These were subcultured 1:100 daily into fresh TSB without antibiotic at 30ºC for 3 cycles, then plated on TSA agar without antibiotic and incubated at 43ºC overnight. Colonies were replica plated with and without erm (Figure 5.19). Erm-sensitive colonies were selected and PCR was performed to determine whether successful allelic exchange had taken place. PCR amplification using AD primers was performed for all clones, which gives a PCR product of 2047 bp for the control and 1030 bp for the mutant.

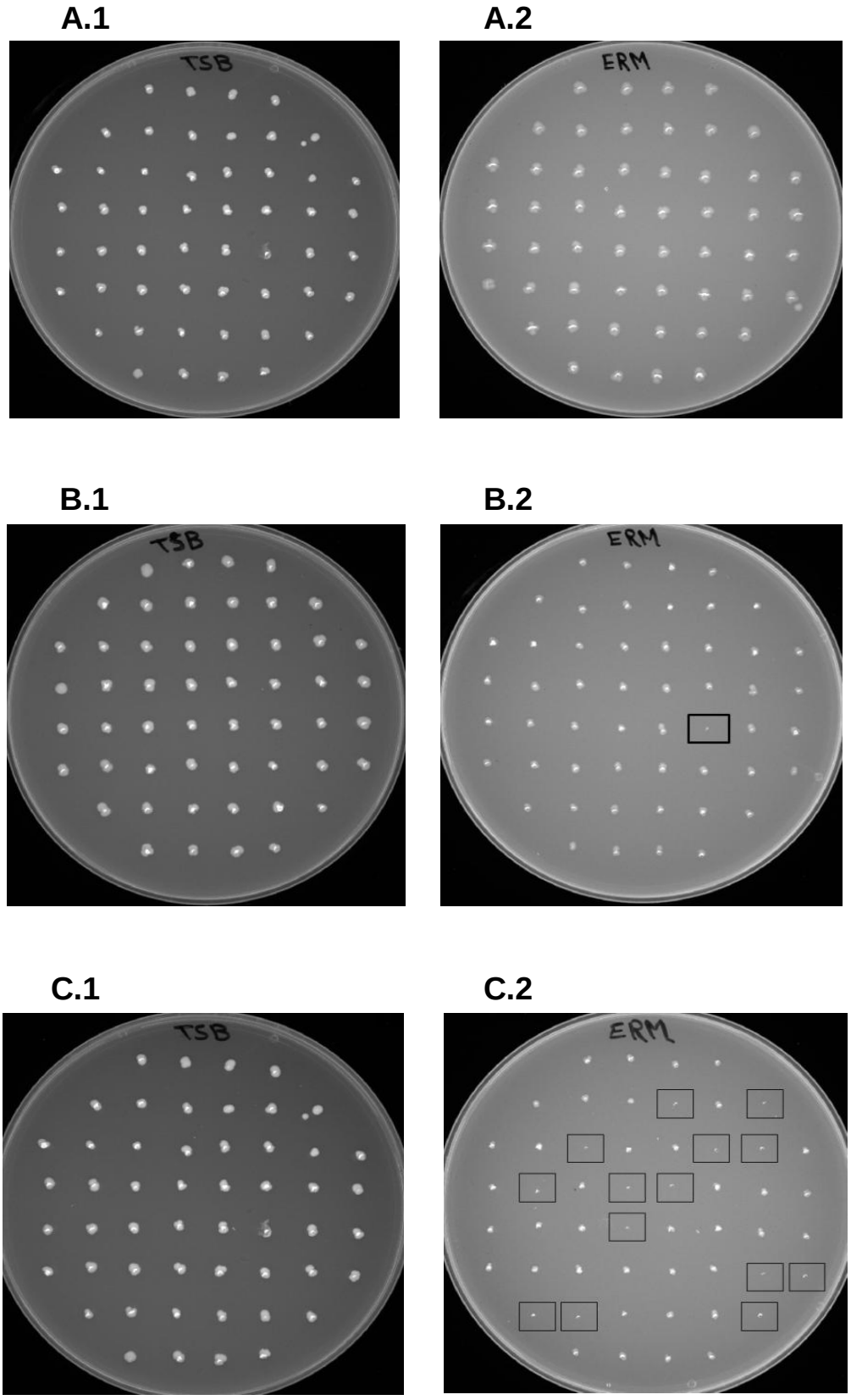


Figure 5.19 Screening for erm-sensitive colonies. Random single colonies grown at 43ºC were replica patched on TSA agar with and without erythromycin to identify erm-sensitive colonies. (A1-2) Cells prepared from the 1st cycle of 30ºC growing culture, (B1-2) Cells prepared from the 2nd cycle, (C1-2) Cells prepared from the 3rd cycle. Plates were incubated at 43ºC overnight.

Listerial cells with chromosomally integrated plasmid were first selected for by aerobically incubating *L. monocytogenes* harbouring the recombinant plasmid statically at 43ºC in the presence of 5 µg ml^{-1} erm. This temperature only allows cells with plasmid integration to grow, while cells with no integration will be sensitive to erm. The second crossover, to eliminate the integrated plasmid, was achieved by growing erm-resistant cells at 30ºC without antibiotic for several cycles. In the first cycle, none of the cells had lost the integrated plasmid, while subsequent culturing at the same conditions triggered the second allelic exchange and erm-sensitive clones were obtained. The chance of generating erm-sensitive clones from the 2nd cycle was 1.9%, whereas the chance had increased to 26.9% in the 3rd cycle.

Chromosomal DNA from erm-sensitive clones was isolated as described in chapter 2 section 15, and DNA was used for PCR amplification to confirm that deletion had taken place by using AD primers used earlier for the cloning.

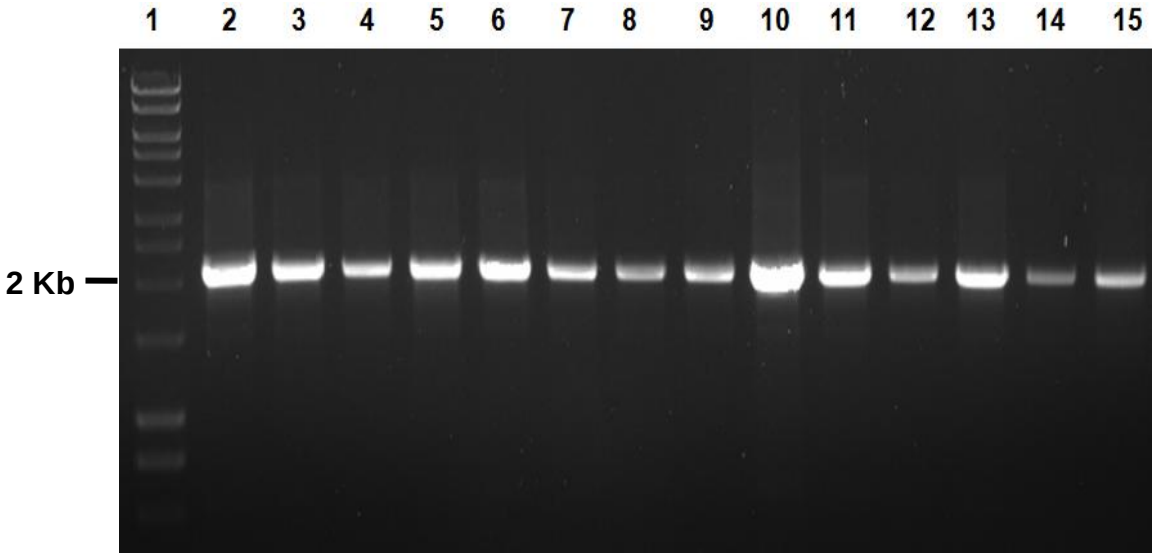


Figure 5.20 Screening for deletion of NamA-encoding gene by PCR. Erm-sensitive clones were screened for deletion mutant by amplifying NamA-encoding gene using AD primers. Lane 1: molecular weight marker (HLI), Lane 2: PCR product using genomic DNA from *L. monocytogenes* WT (positive control), Lane 3-15: PCR products using genomic DNA isolated from erm-sensitive colonies. PCR products were run on 1% agarose gel.

PCR amplification of *namA* gene from erm-sensitive clones showed that none of the colonies had lost the gene to be deleted. All PCR products were the same size as the positive control (2047 bp). Therefore, other erm-sensitive colonies were picked from the plate prepared from the 3rd cycle of subsequent incubation, and the PCR-amplification procedure was repeated as above.

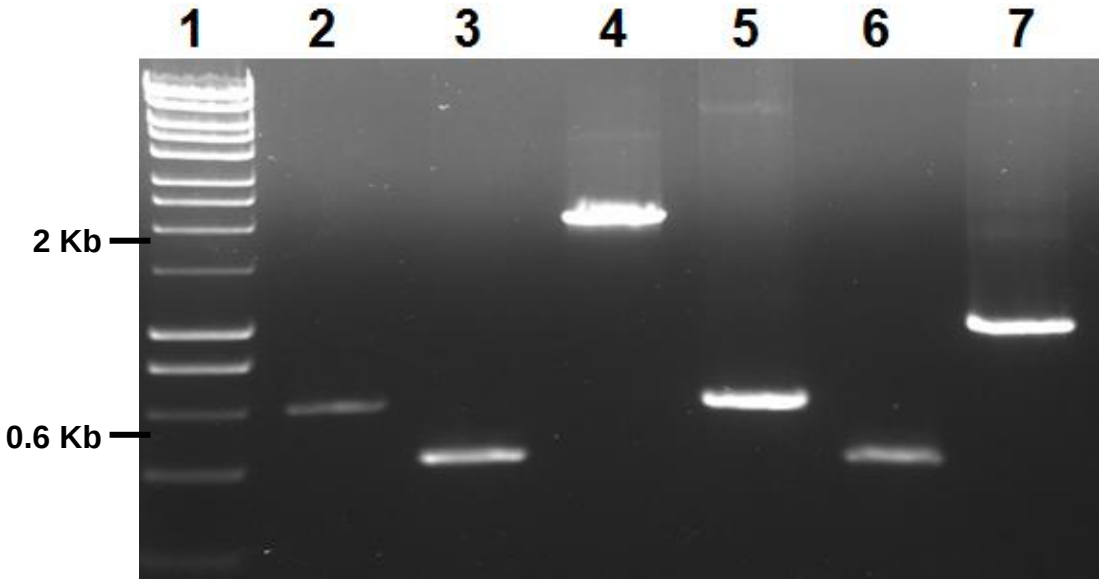


Figure 5.21 Confirmation of deletion mutant by PCR. Genomic DNA from an Erm-sensitive colony from the 3rd cycle was used to PCR amplify *namA*-gene using AD primers. Lane 1: molecular weight marker (HLI), Lane 2: AB fragment from WT genomic DNA (control 1), Lane 3: CD fragment from WT genomic DNA (control 2), Lane 4: AD fragment from WT genomic DNA (control 3), Lane 5: AB fragment from deletion-mutant genomic DNA, Lane 6: CD fragment from deletion-mutant genomic DNA, Lane 7: AD fragment from deletion-mutant genomic DNA.

It is evident from figure 5.21 that the gene encoding for NamA in *L. monocytogenes* was deleted as can be seen from the sizes of PCR products. The gene encoding for NamA is 1017 bp in size; therefore, DNA band in lane 7 (1030 bp) is 1017 bp smaller than DNA band in lane 4 (2047 bp). This confirms that mutant strain lacking NamA-encoding gene (*Lmo2471*) is now constructed. Having demonstrated the deletion of *Lmo247*1 from *L. monocytogenes* chromosomal DNA, the new strain hereby referred to as MBΔEGDe.

5.3.3 Characterisation of MBΔEGDe mutant strain

NamA protein from *L. monocytogenes* was proposed to play a key role in steroid hormones binding and degradation (chapter 4), which was pointed for the growth restoration phenomenon observed earlier (chapter 3). This proposal was based on the similarities of other proteins from different microorganisms that were previously demonstrated to be involved in steroid binding and degradation of xenobiotic compounds (detailed in chapter 4). For that reason, the gene coding for NamA was knocked out from *L. monocytogenes* chromosomal DNA to determine the inability of the mutant strain to grow in the presence of pregnancy hormones (progesterone and β-estradiol). Listerial mutant (MBΔEGDe) was incubated in the presence of progesterone and β-estradiol at a final concentration of 60 µg ml^{-1}; separately, and anaerobic bacterial growth was monitored for 24 h at 37ºC (as described in chapter 2, section 5.1). The mean of 3 independent experiments was used to construct the graphs.

Determination of MBΔEGDe phenotype in the presence of progesterone

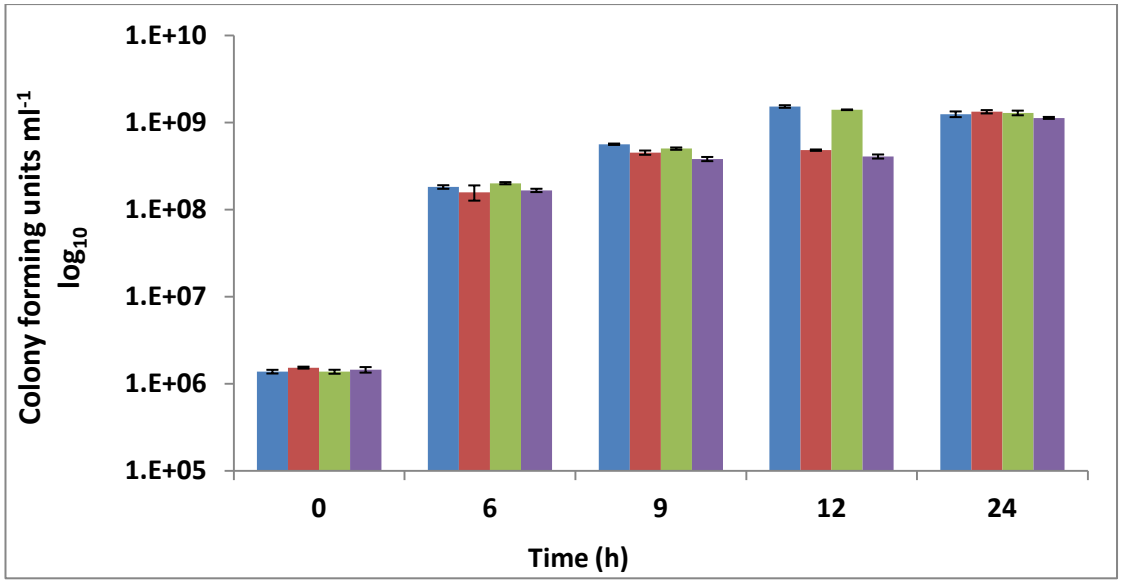


Figure 5.22 Growth of *L. monocytogenes* WT and mutant strain in the presence of progesterone. Growth of listerial cells was monitored in the presence of progesterone at a final concentration of 60 µg ml^{-1}. Bacterial growth was determined by viable count of the cells at 0, 6, 9, 12 and 24 h post incubation. WT without progesterone, WT with progesterone, mutant without progesterone, mutant with progesterone, (p-value for mutant samples at 24 h =0.033) Error bars show the standard error of the mean (n=9).

As can be seen from the growth of both wild type and the mutant, addition of progesterone to the media similarly affected the growth of both strains ($p>0.05$; Cl=95%; p-value was calculated based on the differences between WT and mutant strain with and without hormone at 12h and 24h, separately). WT and the mutant were reduced in growth in the first 12 h of incubation when progesterone was added, and that was most evident at 12 h post incubation (p-value=0.002). However, both strains restored normal growth comparable to the cells grown without progesterone as can be determine by the viable count of the cells at 24 h post incubation (p-value was calculated based on the differences between mutant strain with and without hormone at 24 h=0.033).

To determine whether the addition of β-estradiol to the media will have an effect on the growth of the mutant, bacterial cells were exposed to β-estradiol at a final concentration of 60 µg ml^{-1}, and growth was monitored under similar conditions as described above. The mean of 3 independent experiments was used to construct the graph.

Determination of MBΔEGDe phenotype in the presence of β-estradiol

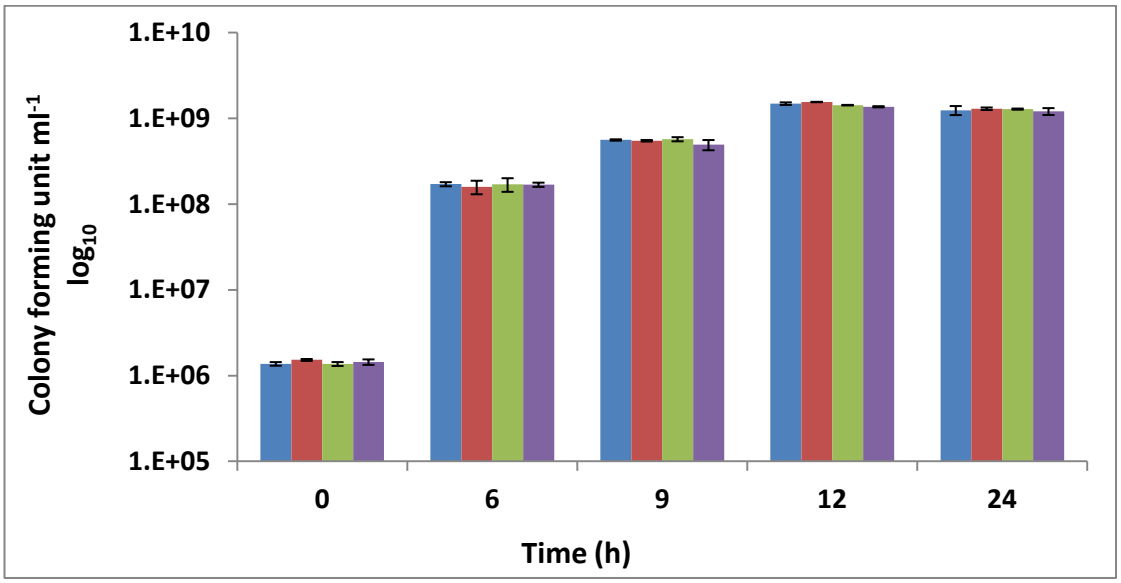


Figure 5.23 Growth of *L. monocytogenes* WT and the mutant strain in the presence of β-estradiol. Growth of listerial cells was monitored in the presence of β-estradiol at a final concentration of 60 µg ml^{-1}. Bacterial growth was determined by viable count of the cells at 0, 6, 9, 12 and 24 h post incubation. WT without β-estradiol, WT with β-estradiol, mutant without β-estradiol, mutant with β-estradiol. Cell numbers are presented in log_{10} scale (p-value for mutant samples at 24h =0.296). Error bars show the standard error of the mean (n=9).

Addition of β-estradiol to the media did not affect the growth of neither the WT nor the mutant as can be determined by the viable count of the cells ($p>0.05$; Cl=95%; p-value was calculated based on the differences between WT and mutant strain with and without hormone at 12h and 24h, separately). Indeed, both strains incubated with the hormone grew in a level comparable to the control in all time points measured.

To determine whether NamA possesses an enzymatic activity against pregnancy hormones, mutant strain was further characterised by enzymatic activity assay. Bacterial lysates from both strains (WT and mutant) were used to determine the oxidation rate of β-NADPH to β-NADP in the presence of either progesterone or β-estradiol as described in chapter 2 section 24. The reaction was initiated by adding bacterial lysate to the reaction buffer (potassium phosphate, pH 7.0) that contains the reaction components (substrate, β-NADPH) and the reaction was monitored by measuring the optical density at room temperature for 1 h at 340 nm. The mean of 3 independent experiments was used to construct the graph.

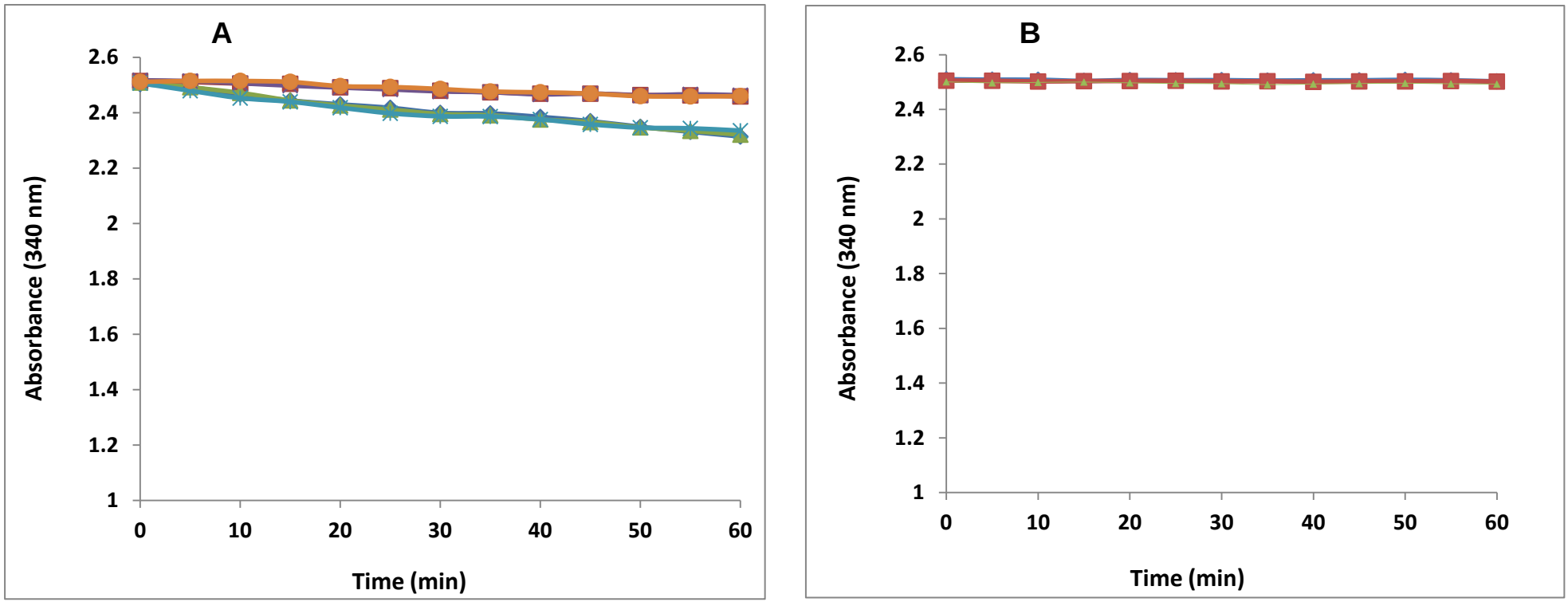


Figure 5.24 Enzymatic activity assay of *L. monocytogenes* WT and mutant strain in the presence of pregnancy hormones. Bacterial lysate from both strains were used to measure the enzymatic activity in the presence of 100 µM of progesterone or β-estradiol by following the oxidation of β-NADPH to β-NADP at 340 nm. **(A)** WT with progesterone (+ control), mutant with progesterone, WT with β-estradiol (+ control), mutant with β-estradiol, WT with 2-cyclohexene-1-one (+ control), and mutant with 2-cyclohexene-1-one. **(B)** Negative controls. Enzymatic reactions contained all the components except: hormone, bacterial lysate, 2-cyclohexene-1-one.The absorbance of β-NADPH was monitored for 60 min. Errors bars show the standard error of the mean, (n=3).

The results showed that enzymes in bacterial lysate isolated from the WT strain of *L. monocytogenes* could possess an enzymatic activity against both hormones (progesterone and β-estradiol) as well as 2-cyclohexene-1-one (positive control), and that was evident by the reduction in absorbance at 340 nm as β-NADPH became oxidised (p=0.061; Cl=95%; p-value was calculated based on the differences between WT with and without the hormone at 60 min). The initial absorbance showed a measurement of ~ 2.5 at 0 h and the measurement was reduced to ~2.31 at 1 h post incubation, which indicates that there was ~ 8% reduction after 1 h of initiating the reaction. Therefore, the oxidation of β-NADPH to β-NADP occurred at 0.133 % per min of the reaction. In contrast to the lysate from the WT strain, lysate isolated from the mutant strain showed reduced activity to both hormones and 2-cyclohexene-1-one. The absorbance of β-NADPH was only reduced by ~1.6 % after 1 h of initiating the reaction with the oxidation of 0.026 % per min of the reaction. On the other hand, reaction mixture lacking either bacterial lysate or the substrates (negative controls, figure B) showed no change in the initial absorbance of β-NADPH after 1 h of measuring the absorbance.

As can be seen from the results, bacterial lysate from the WT may harbour enzyme(s) that play a role in the oxidation of β-NADPH that becomes oxidised as a result of electron transfer, which subsequently reduces the enzyme-bound substrate. Comparison analysis of the enzymatic activity of both WT and the mutant strain showed that the oxidation occurred at a higher rate when used lysate from the WT (p=0.061; Cl=95%), whereas deletion of NamA-encoding gene reduced the oxidation rate by 7.9 % (p=0.25; Cl=95%; p-value was calculated based on the differences between the mutant strain with and without the hormone at 60 min). However, comparison of the oxidation achieved by the mutant (graph A) and the negative control from graph B, it can be seen that, although *namA* is deleted, absorbance of the β-NADPH was still reduced, although in a much slower rate than the one seen by the WT.

5.3.4 Purification of NamA protein from *L. monocytogenes*

In order to further investigate the *in vitro* activity of NamA, the expression vector pPAL7 (5.9 kb, Bio-Rad) (refer to the restriction map in section 2.22; chapter 2) was used for the expression of NamA-encoding gene. Expression of the cloned gene in the pPAL7 vector is under the control of the bacteriophage T7 RNA polymerase promoter. This promoter is not recognised by *E. coli* RNA polymerase, which aids a controlled level of expression of foreign genes. The products of which might otherwise be toxic to the host cells. The gene encoding for the T7 RNA polymerase is present in a bacteriophage λDE3 lysogen within the genome of *E. coli* host strain BL21 (DE3) and is regulated by the lacl repressor, also encoded by the host strain. Transcription of the T7 RNA polymerase gene is controlled by the lacP promoter and therefore expression is induced by the addition of the lactose analogue, isopropyl-β-D-thiogalacto-pyranoside (IPTG). The expression of the T7 RNA polymerase in turns initiates the expression of the target gene cloned in the plasmid. In the absence of T7 RNA polymerase or its inducers for expression, the host RNA polymerase does not recognise the T7 promoter in the cloning vector and therefore the background expression in minimal. In addition, the pPAL7 vector confers an ampicillin resistance gene for selection of *E. coli* cells harbouring the vector on LB medium supplemented with ampicillin and a resin-recognisable amino acid sequence (EEDKLFKAL) at the N-terminus, which facilitates cleavage of the fused-overexpressed protein.

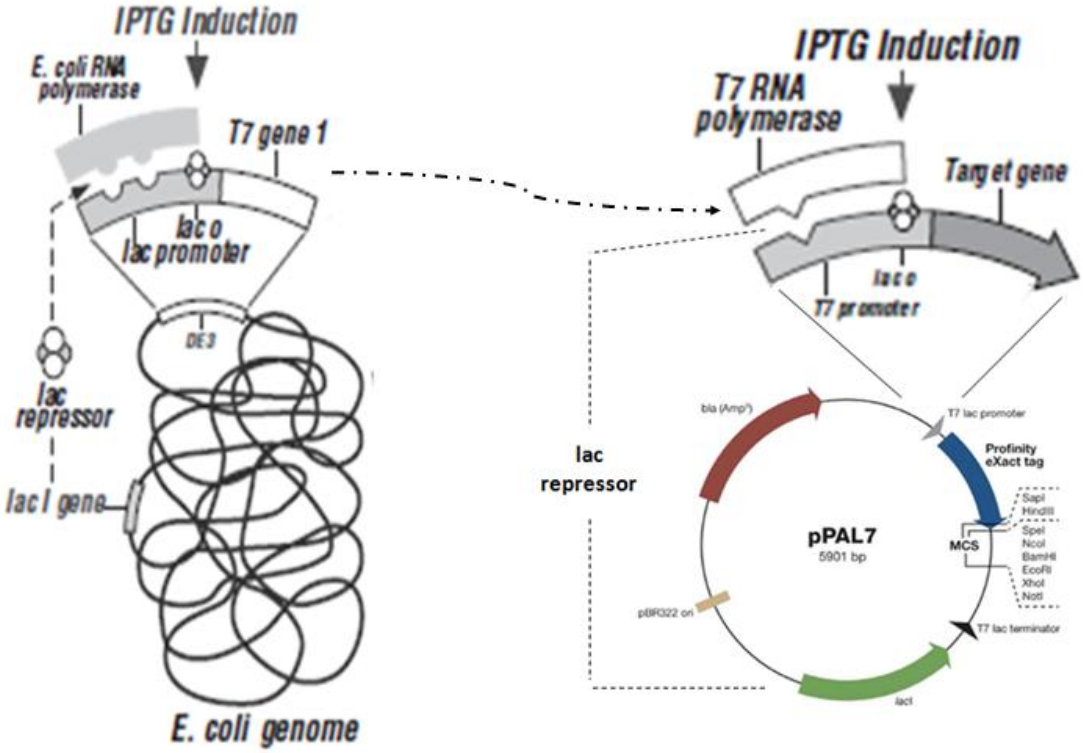


Figure 5.25 Expression system of pPAL7 vector. Transcription of the T7 RNA polymerase gene (T7 gene 1) by *E. coli* RNA polymerase is initiated from the promoter lac in the λ DE3 lysogen. The repressor (lacI) represses the transcription of the T7 RNA polymerase gene and by the addition of IPTG the expression is induced by the action of IPTG that binds to the lac repressor and allow for host RNA polymerase to bind and induce transcription of the T7 RNA polymerase of the lysogen. T7 promoter on the vector is induced of expression only by the action of the lysogen T7 RNA polymerase, which subsequently induce the expression of the fused-target gene.

5.3.5 Cloning of NamA-encoding gene into pPAL7 expression vector

L. monocytogenes NamA-encoding gene was amplified by PCR using primers NamA-pal7F and NamA-pal7R (listed in Table 2.2) and listerial chromosomal DNA as a template. These primers were designed to incorporate *HindIII* and *BamHI* restriction sites at the 5' and 3' ends of the amplicon, respectively. These two restriction sites are also present in the cloning vector pPAL7, but not in the gene encodes for NamA. A PCR product corresponding to the expected size of NamA-ORF (1017 bp) was obtained (Figure 5.26).

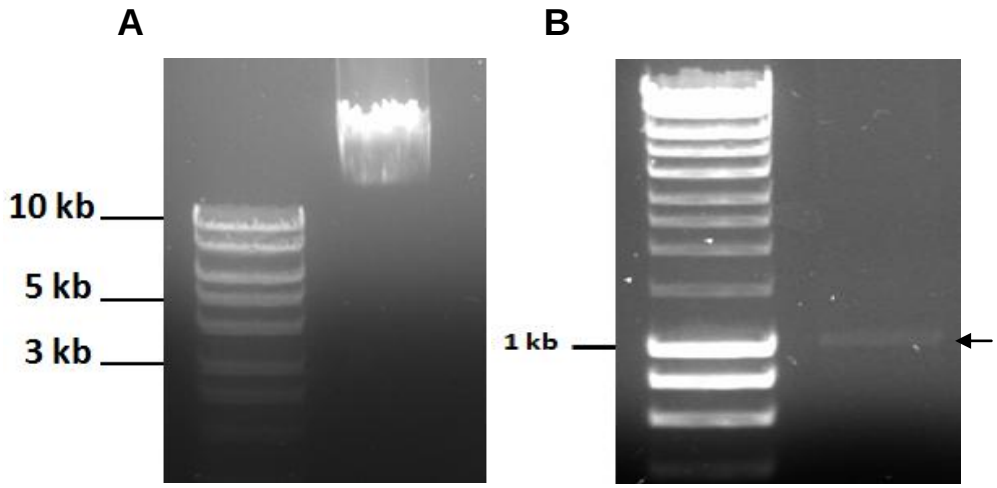


Figure 5.26 Amplification of NamA-encoding gene by PCR. *L. monocytogenes* chromosomal DNA was isolated **(A)**, and used as a template to PCR-amplify the gene encodes for NamA **(B)**. A DNA fragment of 1017 bp was obtained. DNA was run on 1% agarose gel.

Plasmid vector pPAL7 was transformed into *E. coli* XL1-Blue, and successful transformants were selected for by growing on LB agar supplemented with 100 µg ml^{-1} ampicillin. A single colony was picked and cultured in LB broth with ampicillin for propagation and plasmid isolation. The isolated plasmid was subjected to restriction digest using *EcoRV* that cuts twice within the vector and gives 2 DNA fragments of 4229 bp and 1672 bp. This step was performed to confirm the correct plasmid (Figure 5.27).

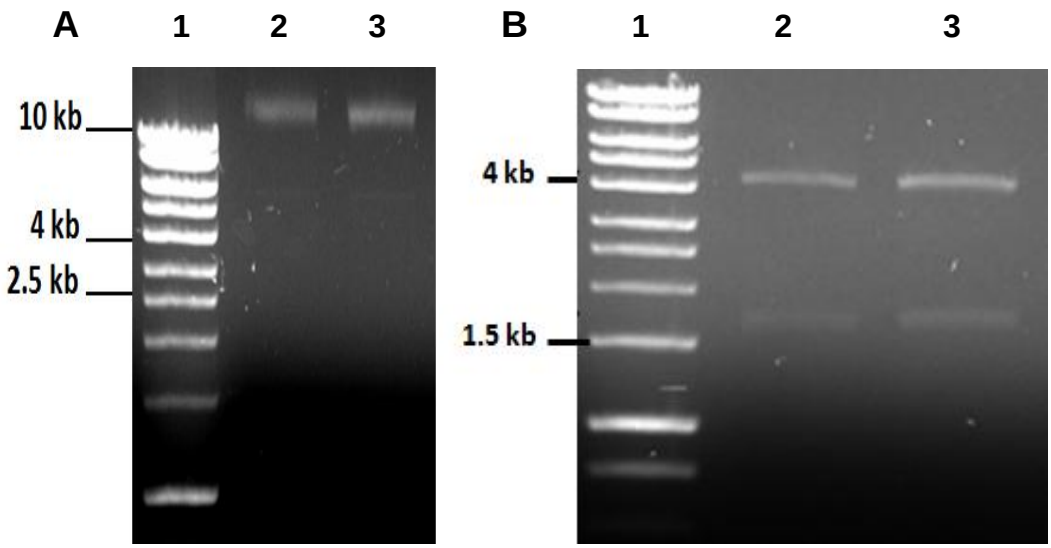


Figure 5.27 Confirmation of pPAL7 plasmid vector. **(A)** Isolation of plasmid vector from *E. coli* XL1-Blue transformants. Lane 1: HLI, Lane 2-3: plasmid vector. **(B)** Restriction digests of the plasmid vector using *EcoRV*. Lane 1: HLI, Lane 2-3: digested plasmid vector.

Following PCR amplification of the NamA-encoding gene (from figure 5.26), PCR-product of *namA*-gene was purified using PCR purification kit (Qiagen). Purified-PCR product and the pPAL7 expression vector were similarly digested with *HindIII* and *BamHI* restriction endonucleases to facilitate cloning. Post digestion, restriction enzymes were heat-inactivated for 20 min at 65ºC, and a ligation mixture was set to ligate the NamA-encoding gene (insert) into the vector at a ratio of 3:1; respectively. Ligation reaction was carried out at 4ºC overnight, and the ligation mixture was used to transform *E. coli* XL1-Blue for propagation of the constructed plasmid (*namA*-PAL7). Successful transformants were selected for by growing on LB agar supplemented with 100 µg ml^{-1} ampicillin at 37ºC overnight. A number of random colonies harbouring *namA*-PAL7 were picked and cultured in selective LB broth for plasmid isolation.

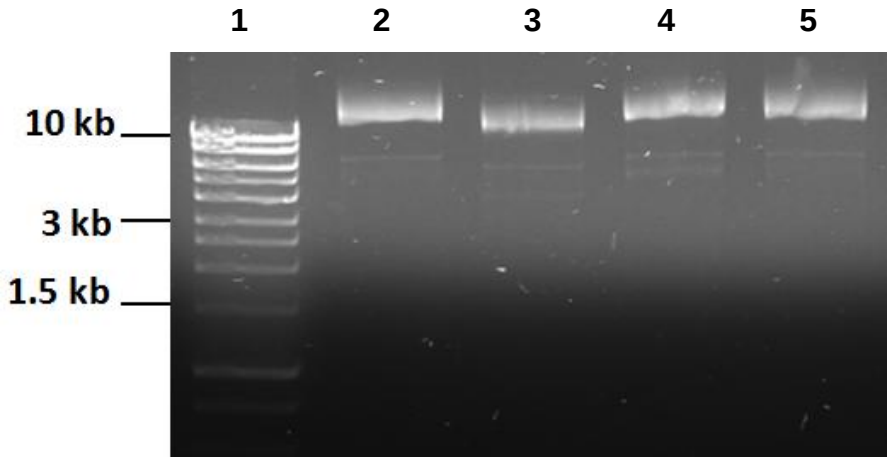


Figure 5.28 Isolation of *namA*-PAL7 recombinant plasmid from *E. coli* XL1-Blue. Lane 1: HLI, Lane 2-5: recombinant plasmid. DNA was run on 1% agarose gel.

In order to confirm successful cloning of the insert into plasmid vector, recombinant plasmid was further subjected to restriction digest using *HindIII* and *BamHI* (Figure 5.29), which gives 2 fragments of 5901 bp (vector) and 1017 bp (insert). Also restriction digest with *EcoRV* was performed for further confirmation (Figure 5.30), which gives 2 fragments of 4229 bp and 2689 bp. Digestion was set at 37ºC overnight.

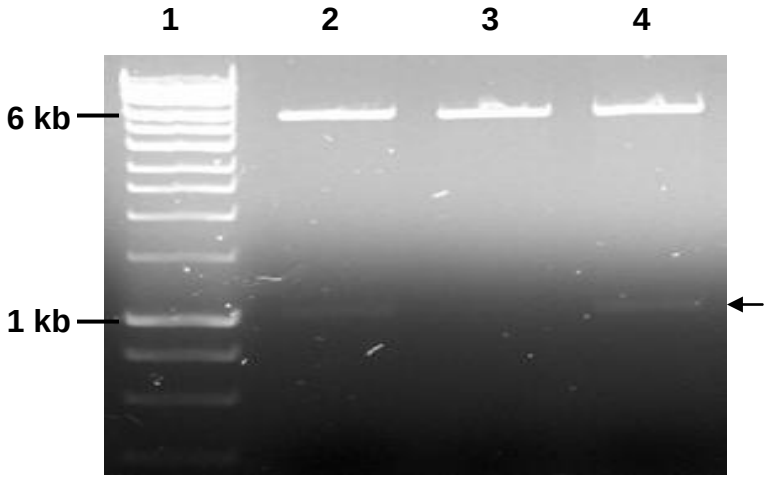


Figure 5.29 Agarose gel analysis of the recombinant plasmid restricted with endonuclease *HindIII* and *BamHI*. Successful construction of the recombinant plasmid was confirmed by restriction digest using *HindIII* and *BamHI*. DNA fragments correspond to the size of the vector only (5901 bp) and the insert (1017 bp). Lane 1: HLI, Lane 2-4: digested recombinant plasmid. DNA was run on 1% agarose gel.

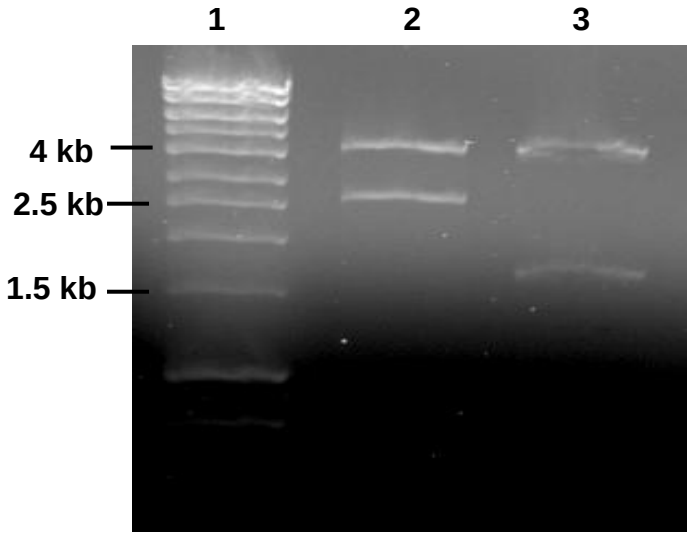


Figure 5.30 Agarose gel analysis of the recombinant plasmid restricted with endonuclease *EcoRV*. Analysis of the recombinant plasmid with different restriction enzyme further confirms the successful cloning. Lane 1: HLI, Lane 2: constructed plasmid restricted with *EcoRV*, Lane 3: parental plasmid restricted with *EcoRV* (control)

It is evident from the restriction analysis of the recombinant plasmid that NamA-encoding gene was successfully cloned into the expression vector as can be determined by the size of fragments obtained.

5.3.6 Overexpression of the *namA*-encoding gene

Recombinant plasmid (*namA*-PAL7) was then transformed into the expression strain *E. coli* BL21 (DE3) for expression of the target gene, and plated on ampicillin-supplemented agar. Single colonies were selected and recombinant plasmid DNA extracted for restriction analysis to confirm the presence of the clone *namA*-PAL7 (data not presented).

Having demonstrated the presence of *namA*-PAL7 within the expression strain, *E. coli* BL21 (DE3) harbouring the *namA*-PAL7, hereby referred to as *E. coli* BL-*namA*, was grown at 37ºC in LB broth supplemented with ampicillin. The overnight culture was subcultured 1:200 into fresh LB broth with ampicillin

and grown as described in chapter 2 section 22. The cultures were grown until an OD_{600} of ~ 0.5 and induced with IPTG to a final concentration of 1 mM. Prior to induction, 1 ml of cells were sampled (un-induced control cells) and sampling was also undertaken at 2 and 4 h post induction. It is important to note that *E. coli* BL21 (DE3), which harbours only the parent plasmid pPAL7 (referred to in this work as *E. coli* BL-PAL7) was used as a negative control and was subjected to the identical conditions to that of *E. coli* BL-*namA*. Post induction, the number of cells were equalised by measuring the optical density of each culture and the volume used for analysis was calculated accordingly. For example, after 3 h of IPTG induction the OD_{600} reached 2, therefore ~ 300-400 µl of the culture was centrifuged and the pellet was resuspended in 50 µl of 2x SDS loading buffer if the OD_{600} was 0.5, or 60 µl of the buffer if the OD_{600} was 0.6. Samples from non-induced and induced cultures were analysed by SDS-PAGE gel and the results obtained are presented in Figure 5.31.

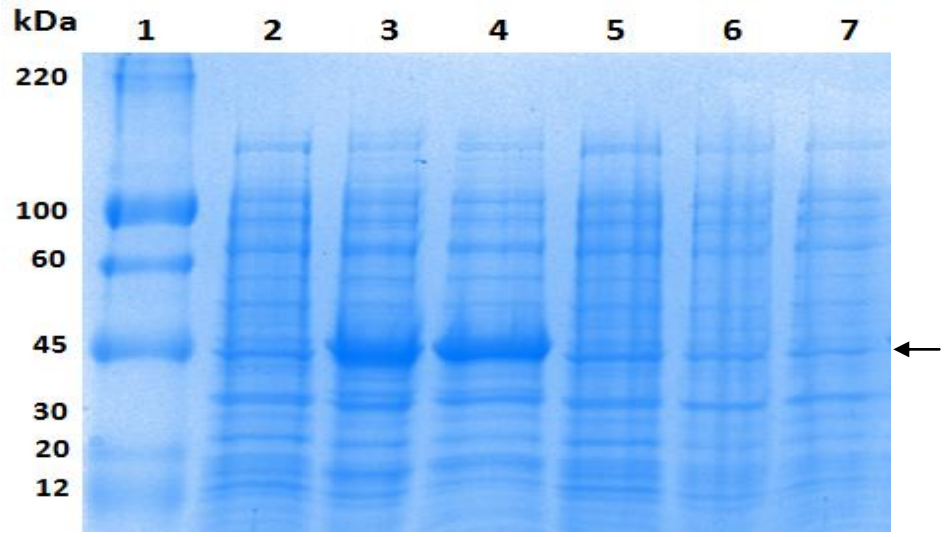


Figure 5.31 SDS-PAGE profile of *E. coli* BL-*namA* and E. coli BL-PAL7 cell extracts. Lane 1: ColorBurst marker protein (Sigma-Aldrich), Lane 2: cell extract of BL-*namA* sampled before induction with IPTG, Lane 3: cell extract of BL-*namA* sampled at 2 h post induction, Lane 4: cell extract of BL-*namA* sampled 4 h post induction, Lane 5-7: cell extracts of BL-PAL7 before induction, 2 and 4 h post induction, respectively. Arrows show the over-expressed protein (~45kDa). Analysis was performed on 12% SD-PAGE.

As demonstrated in Figure 5.31, a band with a molecular weight of approximately 45 kDa was present in *E. coli* BL-*namA* cells that have been induced with IPTG (lanes 3-4) but not in the control *E. coli* BL-PAL7 cells. (Lanes 6-7). The molecular weight calculated from the number of amino acid present in NamA protein (338 aa) is ~37kDa and the profinity eXact tag is ~ 8 kDa, which both give ~45 kDa when analysed on SDS-PAGE prior to the tag cleavage.

5.3.7 Determination of NamA-protein solubility

For the purpose of purification and assaying the *in vitro* activity of NamA protein, it was desirable for the overexpressed protein to be soluble in aqueous medium. The *E. coli* BL-*namA* cells were induced with 1 mM IPTG at 37ºC and harvested by centrifugation after 4 h of induction. The cells were disrupted by addition of 1X BugBuster solution (Merck4Biosciences) and the crude lysate was processed into soluble, inclusion bodies and cell debris fractions by centrifugation as described in Chapter 2 section 23, followed by analysis by SDS-PAGE. The majority of the induced recombinant protein NamA was observed in the inclusion bodies fraction and with some also present in the cell debris (Figure 5.32), indicating that the overexpressed protein NamA was in an insoluble form.

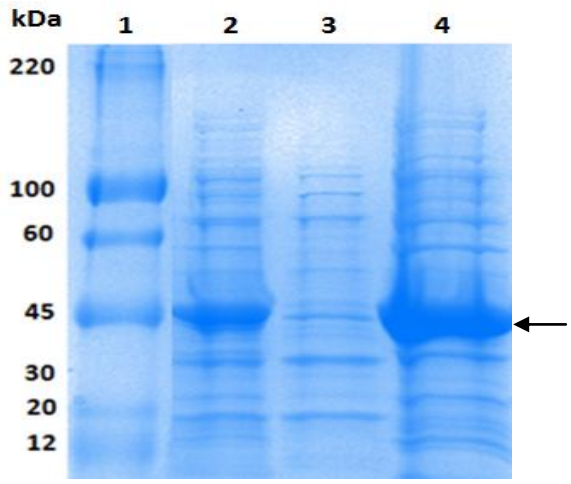


Figure 5.32 Determination of recombinant NamA solubility. Lane 1: represents proteins marker (ColorBurst), Lane 2: total cell extract, Lane 3: soluble fraction, Lane 4: inclusion bodies. Arrows show the over-expressed protein. Analysis was performed on 12% SD-PAGE.

As described above, the recombinant NamA was found to be in an insoluble form after it was induced at the optimum growth conditions for *E. coli*, the expression host. To enhance the solubility of the recombinant protein NamA, growth at lower temperatures, reduced concentration of IPTG for induction, different growth media, anaerobic growth and addition of glycoglycine were tested.

5.3.8 Optimisation of growth/induction factors to enhance NamA-protein solubility

To enhance the solubility of the recombinant protein NamA, growth temperature of 30, 25, 20ºC were tested with 1 mM IPTG for induction. BL-*namA* and BL-PAL7 were grown selectively in LB broth at 37ºC overnight. These cultures were diluted 1:200 into fresh LB broth supplemented with ampicillin at a final concentration of 100 µg ml^{-1} and cells were grown at 37ºC until the OD_{600} reached ~ 0.5. A volume of 1 ml sample was removed from each culture and cells were pelleted by centrifugation as described in chapter 2 section 23. The remaining culture was induced with IPTG at a final concentration of 1 mM and cells were further incubated at 30, 25ºC for 3-6 h or at 20ºC overnight (~ 16 h). The number of cells from both cultures was equalised as described above. The cells were centrifuged and the pellet was disrupted by the addition of 1x BugBuster as previously described.

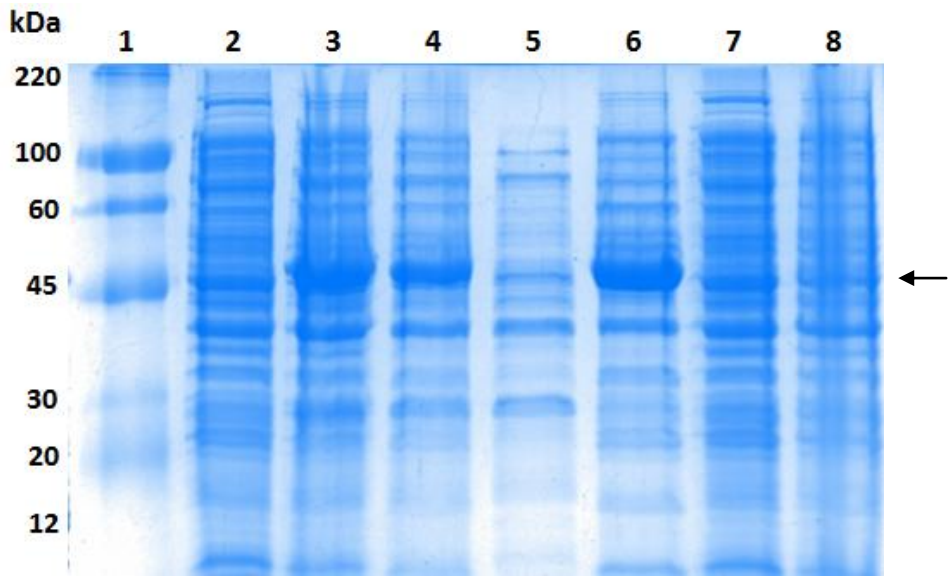


Figure 5.33 SDS-PAGE profile of the recombinant NamA expressed at 30ºC with 1 mM IPTG. Lane 1: protein marker (ColorBurst), Lane 2: pre-induced BL-*namA*, Lane 3: BL- *namA* 4 h post induction, Lane 4: BL- *namA* cell debris (total cells), Lane 5: BL- *namA* soluble fraction, Lane 6: BL- *namA* inclusion bodies, Lane 7-8: pre-induced and 4 h-induced BL-PAL7; respectively, (negative controls). Arrows show the over-expressed protein.

As can be seen from figure 5.33, NamA protein was induced at 30ºC for 4 h with 1 mM IPTG. Lanes 2 indicate that the recombinant protein was not expressed before adding IPTG and overexpression was achieved by incubating the cells for 4 h with IPTG. It is also noteworthy that the protein band corresponds to the expected size of NamA (~ 45 kDa) was not observed when BL-PAL7 was grown in the identical conditions as the induced BL-*namA*. The solubility of NamA protein was not achievable by reducing the growth temperature to 30ºC. Hence, the growth temperature was further reduced to 25 and 20ºC in an attempt to enhance the solubility of the protein. For the reason of testing the solubility of the protein, BL-PAL7 (negative control) was not included in the following analysis.

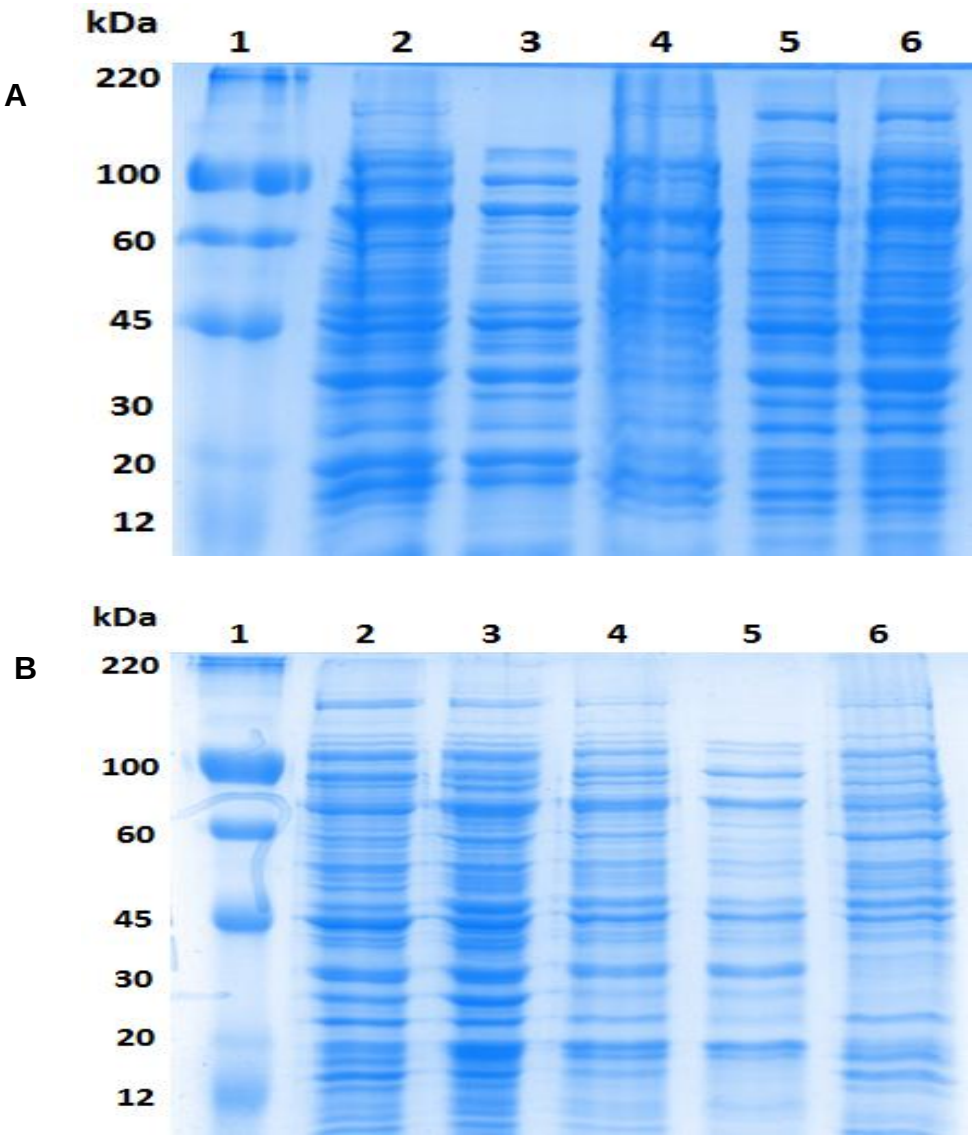


Figure 5.34 SDS-PAGE profile of the recombinant NamA expressed with 1 mM IPTG. Expression of the recombinant protein was performed at **(A)** 25ºC and **(B)** 20ºC. In both gels, Lane 1: protein marker (ColorBurst), Lane 2: pre-induced BL-*namA*, Lane 3: BL- *namA* 6 h post induction for **(A)** and ~ 16 h for **(B)**, Lane 4: BL-*namA* cell debris (total cells), Lane 5: BL- *namA* soluble fraction, Lane 6: BL- *namA* inclusion bodies.

Induction of the recombinant protein was not achievable at neither 25 nor 20ºC as can be seen from the pre-induced (lane 2, both gels) and induced cells (lane 3, both gels). The level of protein expression from lane 2 and 3 were comparable at 45 kDa (expected size of the recombinant protein). From the results obtained, it was noted that expression of NamA was minimal when cells where grown at a temperature below 30ºC, which will not allow for

expression-protein analysis. However, growth temperature of 30ºC and above permits high level of recombinant protein expression, but this protein is fractioned into the insoluble fraction.

Several attempts to enhance the solubility of the recombinant protein were also performed by decreasing the final concentration of IPTG to 0.4 mM. Therefore, cells were grown at 37 and 30ºC until reached an OD_{600} of 0.5, then induced with 0.4 mM IPTG as described above.

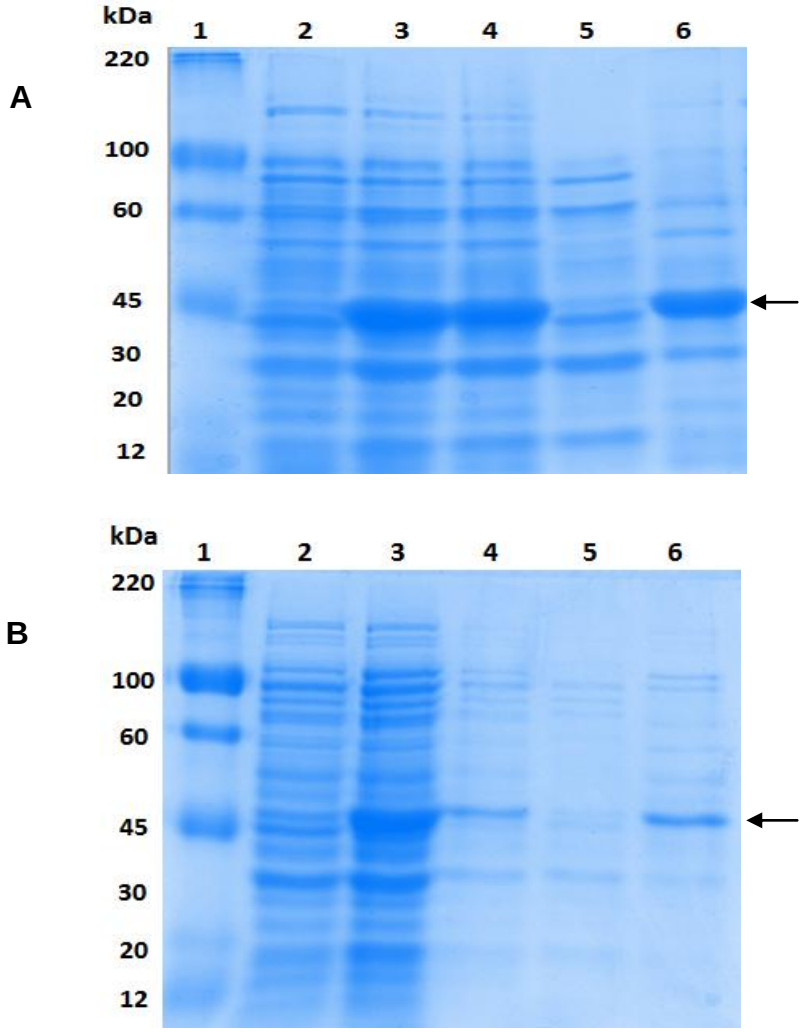


Figure 5.35 SDS-PAGE profile of the recombinant NamA expressed with 0.4 mM IPTG. Expression of the recombinant protein was performed at **(A)** 37ºC and **(B)** 30ºC. In both gels, Lane 1: protein marker (ColorBurst), Lane 2: pre-induced BL-*namA*, Lane 3: BL- *namA* 3 h post induction for (A) and ~ 4 h for (B), Lane 4: BL-*namA* cell debris (total cells), Lane 5: BL- *namA* soluble fraction, Lane 6: BL- *namA* inclusion bodies. Electrophoresis was run at 12% acrylamide gel. Arrows show the over-expressed protein.

Reduction of the final concentration of the IPTG used for induction was performed at 37 and 30ºC in an attempt to enhance the solubility of the recombinant protein NamA. As can be seen in figure 5.35 A-B, NamA was overexpressed with 0.4 mM IPTG (Lane 3, both gels). However, the recombinant protein remains in the insoluble form as it has fractioned into the inclusion bodies.

Further attempts to enhance the solubility of the recombinant protein were performed at 37ºC but in a different growth media, anaerobic conditions or by the addition of glycylglycine. Addition of glycylgycine at a concentration of 1 M to the growth media was previously demonstrated to increase the solubility of the expressed recombinant protein up to 170-fold (Gosh *et al*., 2004). Therefore, the expression strain BL-*namA* was first grown in LB broth supplemented with ampicillin at 37ºC overnight. From this, cells were diluted 1:200 into fresh M9 minimal media supplemented with ampicillin and incubated for 3 h with shaking (200 rpm) or until the OD_{600} reached ~0.5. A volume of 1 ml was sampled (pre-induced) and the remaining culture was induced with IPTG at a final concentration of 1 mM for further 6 h. Protein samples were prepared as previously described and run on 12% acrylamide gel.

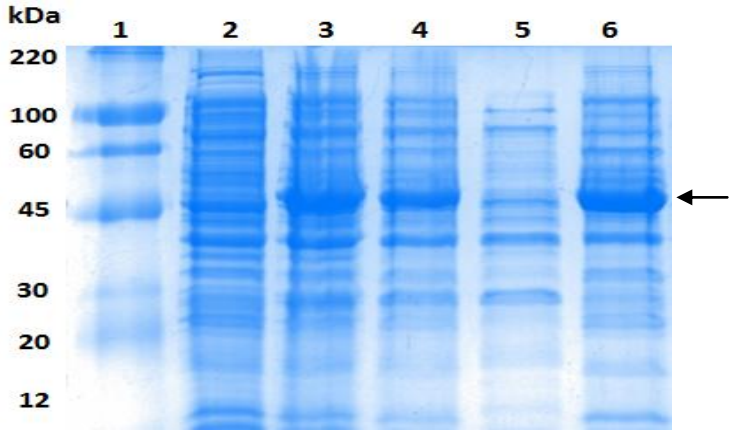


Figure 5.36 SDS-PAGE profile of the recombinant NamA expressed in M9 minimal medium with 1 mM IPTG. Expression of the recombinant protein was performed at 37ºC. Lane 1: protein marker (ColorBurst), Lane 2: pre-induced BL-*namA*, Lane 3: BL- *namA* 6 h post induction, Lane 4: BL- *namA* cell debris (total cells), Lane 5: BL- *namA* soluble fraction, Lane 6: BL- *namA* inclusion bodies. Electrophoresis was run at 12% acrylamide gel.

Culturing BL- *namA* in M9 minimal medium was performed to enhance the solubility of the recombinant protein. As can be seen in figure 5.36; once again, NamA was fractioned into the inclusion bodies indicating that the overexpressed protein was in an insoluble form (Lane 6).

Several further attempts were performed to enhance protein solubility including expression under anaerobic conditions and the addition of glycylglycine.

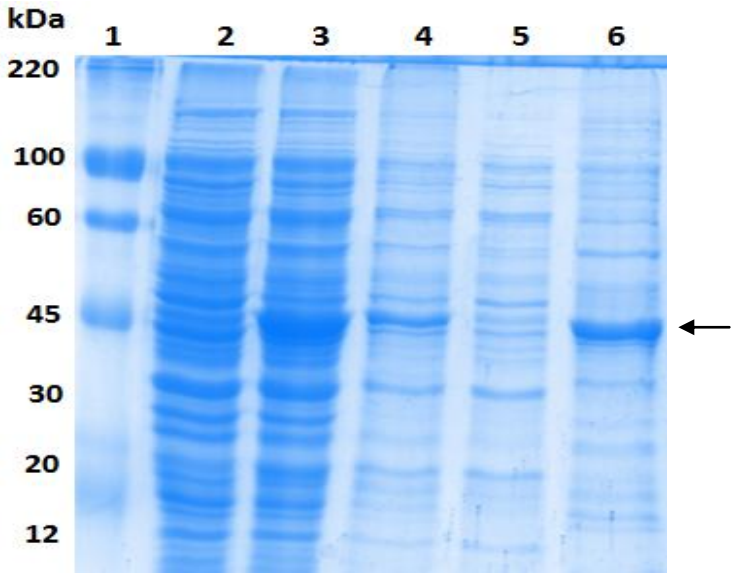


Figure 5.37 SDS-PAGE profile of the recombinant NamA expressed anaerobically in LB medium with 1 mM IPTG. Expression of the recombinant protein was performed at 37ºC. Lane 1: protein marker (ColorBurst), Lane 2: pre-induced BL- *namA*, Lane 3: BL- *namA* 5 h post induction, Lane 4: BL- *namA* cell debris (total cells), Lane 5: BL- *namA* soluble fraction, Lane 6: BL- *namA* inclusion bodies. Electrophoresis was run at 12% acrylamide gel.

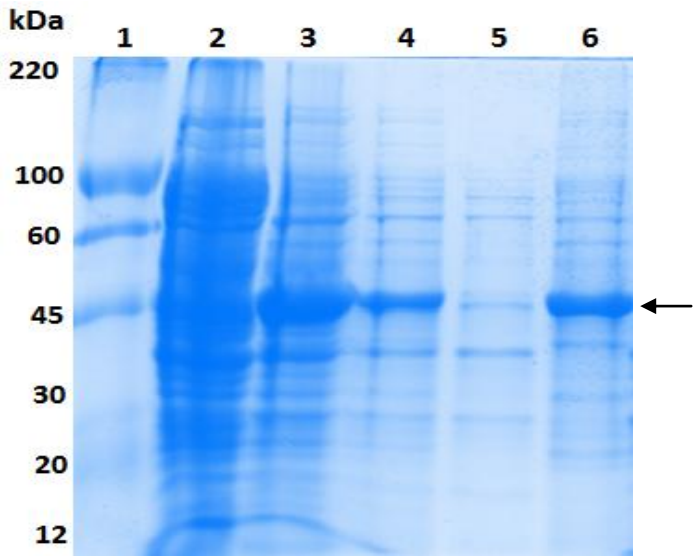


Figure 5.38 SDS-PAGE profile of the recombinant NamA expressed in LB medium with 1 mM IPTG and 0.5 M glycylglycine. Expression of the recombinant protein was performed at 37ºC. Lane 1: protein marker (ColorBurst), Lane 2: pre-induced BL- *namA*, Lane 3: BL- *namA* 5 h post induction, Lane 4: BL- *namA* cell debris (total cells), Lane 5: BL- *namA* soluble fraction, Lane 6: BL- *namA* inclusion bodies. Electrophoresis was run at 12% acrylamide gel.

Solubility of the recombinant protein NamA was not achievable in all conditions tested. In all growth conditions at 30 and 37ºC, the reduction of the IPTG concentration, expression in minimal medium, expression of anaerobically grown cells and finally the addition of 0.5 M glycylglycine did not enhance the solubility of the protein as can be seen from the gel pictures above. However, expression at low temperature (25, 20ºC) showed minimal recombinant protein expression of NamA, which was comparable to the protein levels from the pre-induced cells (control).

5.4 Discussion

The work presented in this chapter represents the first to demonstrate the gene expression and deletion of an OYE family member from *L. monocytogenes*, and subsequent characterisation of the deletion mutant strain and cloning of the gene for protein overexpression and purification. Semi-quantative RT-PCR was performed to qualtitively asses the expression of 3 different genes from *L. monocytogenes* in the presence and absence of pregnancy hormones. Each of these genes shares significant sequence homology to the hormone-binding proteins found in other microbes (discussed in chapter 4). For this reason, it was of interest to investigate the effect of pregnancy hormones on their expression and whether there is a link to the phenotype observed in chapter 3. As can be seen in figure 5.7, increasing concentrations of β-estradiol relatively down-regulated the expression of *namA*, while it was clear that high concentrations of progesterone largely reduced the expression of this gene. In the case of progesterone, addition of progesterone showed clear reduction of *namA* expression, which could be the reason of the growth retardation observed in chapter 3 (NamA was proposed to be involved in cell-energy generation). Since RNA samples were isolated 12h post exposure to the hormone, and the growth restoration phenomena was observed at 12-24h post exposure; it is unknown whether longer incubation would increase the expression of *namA* gene through different enzymatic oxidation of the hormone. Therfore, the result obtained here is inconclusive and requires further investigation. Although the addition of β-estradiol did not influence the growth of *L. monocytogenes* (chapter 3), addition of this hormone caused a small reduction in the expression of *namA* as can be seen from the bands intensity in the PCR products. This could be linked to the results obtained in chapter 3 where no apparent effect was observed in the growth of *L. monocytogenes*. Moreover, gene expression of both *lmo2235* and *lmo0489* genes were not detected in the presence and absence of both hormones when they were PCR-amplified by the use of the cDNA as tempelate (both genes were not transcribed into mRNA); hence, *namA* was the only gene to be further studied.

Utilisation of the shuttle vector pLSV101 facilitated the deletion of the target gene by homologous recombination process, which was achievable by the shift of the temperature of growing cells harbouring the constructed plasmid MBΔpLSV101 to one non-permissive for plasmid replication. Shuttle vector pLSV101 replicates by the rolling circle mechanism using a temperature-sensitive replicon pE194 that is non-permissive of replication at high temperature (Joseph *et al*., 2006). Plasmid MBΔpLSV101 confers two listerial-derived fragments located up-stream and down-stream of the target gene to be deleted from *L. monocytogenes* chromosomal DNA. In order for the first allelic crossover to take place, *L. monocytogenes* harbouring the recombinant plasmid (MBΔpLSV101) was incubated on TSA agar plate supplemented with erythromycin at a final concentration of 5 µg ml^{-1} at 43ºC overnight. This step allows for either up-stream or down-stream listerial fragments carried on the recombinant plasmid to crossover the identical fragment (region of homology) exists on the *L. monocytogenes* chromosomal DNA, which results in the integration of the entire plasmid into the chromosome. Therefore, plasmid-integrated cells were selected for by growing on selective media and cells without plasmid integration (harbouring the recombinant plasmid only) was not able to grow since the plasmid replication is dependent on the temperature-sensitive replicon. Erm-resistant colonies were selected and cultured in TSB broth and excision of the plasmid by the second crossover event was stimulated by decreasing the temperature to one permissive for plasmid replication in the absence of antibiotic selection. This triggered recombination and loss of the integrated plasmid. If the second crossover event took place at the region of homology used for integration the strain remains wild-type. However, if the recombination took place at the opposite region of homology the mutant allele is left in the chromosome. Excision at the same site as that used for integration should take place in theory at the same frequency as the heterologous site giving a mutation frequency of 50%. In practice, mutation frequency as low as 1% may occur (Bae and Schneewind, 2006). The allelic exchange recombination event for *namA* deletion is depicted in figure 5.9.

It was observed that the success of generation of erm-sensitive colonies (Figure 5.19) was negative when cells were grown at 30ºC for the first cycle, and the chance had increased by 1.9% and 26.9% in the second and third cycles, respectively. Growing the cells at low temperature in the absence of antibiotic triggered the excision of the plasmid that confers the erm-resistant gene, which resulted in increased number of cells sensitive to erythromycin. To confirm the deletion mutant, randomly-selected erm-sensitive colonies were subjected to PCR amplification using primers A and D. AD-PCR amplified fragment from the wild-type strain showed a band of 2047 bp, which includes NamA-encoding gene (1017 bp) and the up-stream/down-stream fragments used for cloning (604 and 426 bp, respectively). Hence, the deletion-mutant strain lacking NamA-encoding gene showed a 1017 bp-smaller band when PCR-amplified using AD primers.

To investigate the role of NamA protein in the growth of *L. monocytogenes* in the presence of pregnancy hormones, mutant strain was cultured anaerobically in liquid broth in the presence of 60 µg ml^{-1} of either progesterone or βestradiol. The results obtained in figure 5.22 and 5.23 showed that the growth of *L. monocytogenes* WT and mutant strains were similarly affected by the addition of the hormones (For both hormones $p>0.05$; Cl=95%), which suggests no role for NamA protein in the restoration phenomenon observed in chapter 3, or there may be multible genes of which functions resulted in the restoration phenomenon observed. Therefore, deletion of one gene did not largely affect the growth restoration of the mutant strain. Further investigations should be performed to elucidate the role of other genes in the chromosome that might be related to hormone binding/degradation. For example, the enzyme 3α-hydroxysteroid dehydrogenase/ carbonyl reductase (3α-HSD/CR) from *C. testosteroni* was previously demonstrated to play a pivotal role in steroid degradation, BLASTp search was perfomed to identify similar protein in *L. monocytogenes*. The results revealed that *L. monocytogenes* encodes for 3-ketoacyl-(acyl-carrier-protein) reductase that shares a significant identity of 37% (e-value 2e-27) to the query.

To further characterise NamA, enzymatic activity of bacterial lysate prepared from WT and mutant strain were used to measure the oxidation rate of β-NADPH to β-NADP at 340 nm. As a member of the OYE family, the enzyme NamA uses β-NADPH as a co-factor that participates in electron transfer to the substrate through the bound FMN molecule. This reaction results in the oxidation of β-NADPH that can be measured at 340 nm, but the oxidised form cannot be detected at this wavelength, which simplifies monitoring the activity of the enzyme (Khan *et al*., 2005). The oxidation rate of β-NADPH was monitored for 1 h at room temperature in the presence of 2-cyclohexene-1-one as a positive control, progesterone or β-estradiol. As can be seen in figure 5.24, the oxidation of the co-factor was minimal when the lysate from the mutant strain was used in the reaction (lacking NamA, $p>0.25$). However, the oxidation rate was relatively higher when using lysate prepared from the WT strain ($p=0.061$). The oxidation was observed when using 2-cyclohexene-1-one as well as progesterone and β-estradiol, which suggests that enzyme(s) exist in the lysate have different substrate specificity. Although the rate of oxidation observed by the mutant was minimal when compared to the WT, mutant oxidation of β-NADPH had a 1.6 % reduction after 1 h of incubation. This suggests that there may be other enzymes in the lysate that use β-NADPH as a co-factor, which led to this reduction. In general, the rate of oxidation from both lysates (WT and mutant lysates) were insignificantly different (cut-off for testing the null hypothesis is 0.05 with 95% confidence interval) and the small differences in absorbance are suggested to be (i) due to polar effect of the deleted gene, which may have affected the function of other enzymes within the lysate, (ii) the reduction of NamA activity is not related to the effect seen when the cells were grown with progesterone. On the other hand, the initial absorbance at the start of the assay was very high (O.D of 2.5), which may have led to an increased-measurement error since an O.D of 2 means 1% of light transmitted through the spectrophotometer. Therefore, reducing NADPH concentration to an O.D less than 1 will also reduce measurement error. Another explanation is that the reduction in NADPH absorbance was not due to genuine oxidation, rather the background noise from the spectrophotometer may have affected the measurement outcome. Problems encountered in this assay could be solved by using a pure

purified form of the enzyme, plus reducing measurement error by diluting the concentration of NADPH so that the initial absorbance is less than 1.

To further elucidate the importance of NamA, the gene encoding for NamA was cloned into pPAL7 expression vector for protein expression and purification. This gene was fused with EEDKLFKAL amino acids-encoding gene to facilitate recognition and precise cleavage by the use of the purification resin. A protein band corresponds to 45 kDa was shown to be extensively expressed when the bacterial cells were induced by the addition of IPTG at 37 and 30ºC. This band represents the NamA protein (~ 37 kDa) attached to a small amino acid fragment (~8 kDa) that is recognisable by the resin. For the purpose of purification, it was desirable for NamA to be in the soluble form to be biologically active. Therefore, overexpression at 37ºC was not the ideal temperature since the expressed protein aggregated as inclusion bodies, which indicates that NamA was in an insoluble form and improperly folded. Several attempts were taken to enhance protein solubility by reducing the growth temperature of the expression strain to 30, 25 and 20ºC. As can be seen from the results, expression at 30ºC yielded an insoluble protein, whereas minimal expression was observed at 25 and 20ºC. Other attempts to enhance the recombinant protein solubility was performed by decreasing the concentration of the inducer IPTG to 0.4 mM, growing the cells in minimal media, anaerobic growth or by the addition of glycylglycine to the media. All attempts resulted in the accumulation of the expressed protein in the inclusion bodies, which indicates non-biologically active form. It was previously demonstrated that the addition of 1M glycylglycine to the media resulted in a 170-fold increase in the soluble form (Gosh *et al*., 2004). In the present study, only 0.5 M was used since higher concentration was not dissolvable in any solvent including 100% ethanol or Dimethyl sulfoxide (DMSO).

Taken together, it was observed that deletion of NamA-encoding gene did not affect the growth of the mutant strain in the presence of the hormones tested. In addition, the prediction of NamA ability to have a significant role in the oxidation of pregnancy hormones was demonstrated to be invalid by the use of bacterial lysate prepared from the deletion-mutant strain; hence, further enzymatic studies are needed to investigate the pure form of NamA in the oxidation of steroid hormones, and to examine whether steroid hormones are potent inhibitor for the enzyme or the enzyme plays a role in the oxidation of these hormones. Furthermore, purification of NamA was not achievable by the use of pPAL7 for expression since the protein expressed aggregated in an insoluble form. Enhancement of protein solubility could be increased by the use of His-tag fusion plasmid or the use of expression plasmid that confers cytoplasmic-signal peptide.

Prosser and colleagues (2010) proved that purification of NemA protein (homologue of listerial NamA protein) in a soluble form was feasible by the use of His-tag fusion vector. Given the similarities in the structures of these proteins, and the fact that the protein in present study was produced as fusion protein in pPAL7 which includes a specific peptide tag, it is possible that this sequence was affecting the folding pathway leading to insolubility of the protein**.**

Chapter VI
Concluding discussion

Listeria monocytogenes is a food-borne pathogen well known worldwide for its ability to cause devastating complications during infection. This pathogen mainly affects individuals with suppressed immunity including elderly, HIV patients and pregnant women. In addition, infection caused by *L. monocytogenes* is considered the leading cause of deaths related to contaminated food in the United States (Mead *et al*., 1999). During pregnancy, infection caused by *L. monocytogenes* leads to serious health problems for the mother and the fetus, which may results in abortion or fetal death (Cairns *et al*., 2009). The mechanism of listerial infection during pregnancy and how this bacterium results in the loss of the fetus is not completely understood; however, several proposed mechanisms are believed to be involved in the outcome of the infection. First, *L. monocytogenes* invades the placenta and causes cells destruction [route 1] that results in reduction in progesterone levels or improper nutrients uptake [route 3], which both can lead to fetal death and abortion. Second, invasion by *L. monocytogenes* results in progesterone metabolism [route 2] through special listerial-encoded proteins that may lead to reduction in the levels of progesterone, consequently loss of the fetus (Figure 6.1).

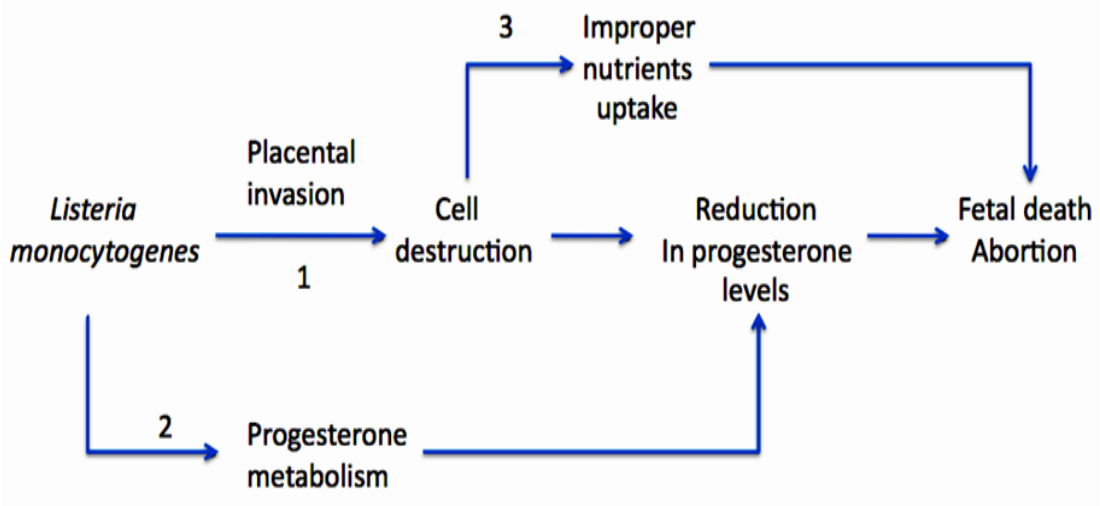


Figure 6.1 Proposed mechanisms of *L. monocytogenes* infection during pregnancy and the possible routes involved in the loss of the fetus.

In a study conducted on goats, it was previously demonstrated that reduction in progesterone levels was correlated to fetal death and abortion. Goats inoculated with *L. monocytogenes* in the first trimester showed clinical symptoms of the disease in connection to abortion, whereas incoculation in the second trimester resulted in more pronounced clinical symptoms few days after inoculation. Moreover, fetal death occurred either immediately or two days before abortion, and mummification was observed in the dead fetus that had been dead for two days before expulsion (Engeland *et al*., 1997). This observation suggests that *L. monocytogenes* causes reduction in progesterone levels in human and this could possibly be one of the reasons that results in abortion.

During pregnancy, the increased levels of hormones were demonstrated to have direct/indirect effect on microbial growth. Some of the microbes were shown to possess proteins with the ability to bind hormones. For example, *C. trachomatis* binds to human β-estradiol by utilisation of the fungus-encoded disulfide isomerase protein (Hall *et al*., 2011). Also, *C. albicans* was demonstrated to possess estrogen-binding protein (EBP) that tightly binds to β-estradiol (Madani *et al*., 1994). These and other observations described above led to the prediction that *L. monocytogenes* may also possess protein(s) that can bind to human hormones since the infection of this bacterium is significantly increased during pregnancy where the levels of pregnancy hormones are increased. In order to investigate this prediction, the effect of pregnancy hormones on the growth of *L. monocytogenes* was first studied *in vitro* by monitoring bacterial growth in the presence of different concentrations of either progesterone or β-estradiol. From the results obtained, it was observed that only progesterone but not β-estradiol caused significant growth retardation as it was determined by the viable cells count under anaerobic conditions. The reduction in growth was only observed in the first 12 h of growth and this was most evident when cells where exposed to a final concentration of 60 µg ml^{-1} of the hormone. Statistically, the null hypothesis (H_0) was set as no differences are detected between the treatment groups, and the alternative hypothesis was tested against H_0, and the cut-off for confidence interval was 95%.

The effect of the hormone was concentration-dpendent since increasing concentrations showed more reduction in growth. Interestingly, the growth of bacteria treated with progesterone showed a growth-restoration phenomenon in the second half of the incubation and this was observed at 24 h time-point. The cell counts from cells treated with increasing concentrations of progesterone were comparable to the cell count from untreated cells. The growth restoration event led to the prediction that *L. monocytogenes* encodes for proteins that were involved in overcoming the growth-slowing effect of progesterone, which allowed for normal growth as compared to the control cells in the second half of the incubation period. Therefore, further experiments were carried out to test whether pre-treatment of progesterone would abolish the retardation effect observed. As can be determined by the number of viable cells, bacteria pre-treated with increasing concentration of progesterone grew in a similar level to that of the control cells in the entire incubation period. It was also observed that the effect of progesterone was concentration/time-dependent since pre-treatment with low progesterone concentration did not fully recover the cells when they were back diluted to higher concentrations. Based on these observations, it was of interest to identify listerial genes that might be involved in the restoration event observed earlier and to identify genes with phenotype to pregnancy hormones. Therefore, generation of transposon-mediated mutants were performed using Tn*917*-LTV3, and mutants with phenotype to the hormones were screened in solid and liquid media. Mutants with reduced growth in the presence of β-estradiol were obsereved under anaerobic conditions and the genes interrupted were identified by sequencing and alignment to the NCBI database. A number of 8 different genes were shown to have been interrupted by the transposon integration. It was previously described by Charpentier *et al*., (1999) that *L. monocytogenes* possesses an operon that facilitates high resistance to the bacteriostatic antibiotic trimethoprim by synthesis of dihydropholate reductase. This operon comprises of 4 different genes, of which products are essential for conjugative mobilization among different species of Gram-positive bacteria via plasmid rolling circle (RC), which was suggested to have a key role in resistance to several antibiotics. In the present study, and based on sequence analysis of M1, it was observed that Tn*917*

was integrated at a site 818 bp upstream of the replication protein that facilitates initiation of plasmid replication. Integration of the transposon at this site may have affected the expression of rep protein, subsequently causing bacterial cells to become less resistance to toxic compounds on bacterial growth. On the other hand, analysis of the sequence recovered from M5 revealed that Tn*917* integration occurred at position 1278 bp from the start codon of the xanthine/uracil permease gene in *L. monocytogenes* chromosomal DNA. This gene encodes for a transmembrane transporter protein that consists of 427 amino acids. Xanthine/uracil permease participates in the uptake of xanthine and uracil for bacterial DNA synthesis, therefore interruption of the uptake process compromises DNA synthesis, which leads to leaky bacterial growth. Furthermore, it was previously suggested by Wurtzel *et al*. (2012) that xanthine/uracil permease in *L. monocytogenes* confers a regulatory mechanism of which inactivation results in simultaneous silencing of the opposing operon that encodes for an efflux pump of the multidrug and toxic compounds extrusion (MatE) protein. Members of the MatE-efflux family protein are responsible for excreting metabolic or xenobiotic organic compounds (Omote *et al*., 2006). It is therefore anticipated that reduction in growth of M5 in the presence of β-estradiol possibly occurs as a consequences of defects in the efflux system in addition to the leaky bacterial growth. Furthermore, listerial-encoded lipase is predicted to have an activity toward β-estradiol, of which the enzyme catalyses the hydrolysis of the hormone resulting in structurally different molecules. This prediction is based on the work reported by Lee *et al*., (1988), where they demonstrated that enzymes belong to the family of hormone-sensitive lipases were responsible for hydrolysis of steroid in bovine placenta and possible steroid hormone target tissues in general. Moreover, bacterial lipases were found to be closely related to mammalian hormone-sensitive lipases, of which significant homology of protein sequences was found. The evolutionary relationship of these proteins suggests possible similarity in functional properties (Hemila *et al*., 1994). In the present study, sequence analysis of the mutant disrupted in the lipase-encoding gene (M8) showed that the trasposon was integrated at 842 bp from the start codon. The mutant was reduced in growth when exposed to β-estradiol during anaerobic incubation.

Although bacterial growth was not largely affected, the number of viable cells showed a 0.6-fold reduction when compared to the control cells. This suggests that this gene does not play a vital role in bacterial resistance to the hormone.

Different transposon-mediated mutant (M9) was shown to be deficient in growth in the presence of β-estradiol. The transposon was inserted into a hypothetical protein of which product is predicted to be under the control of the steroid-inducible transcriptional regulator LysR. This prediction is derived from the work reported by Gong *et al*., (2012a). They have previously demonstrated that in the presence of steroid, the gene (*hsdA*) encoding for 3α-hydroxysteroid dehydrogenase/ carbonyl reductase (3α-HSD/CR) in *C. testosteroni*, a key enzyme in steroid degradation, is up-regulated upon steroid binding to the repressors, of which they bind to the promoter of the transcriptional regulator LysR-gene. Therfore, in the present study, it has been postulated that polar effect of the interrupted gene might have affected the expression of the transcrioptional regulator adjacent to the hypothetical protein, and this resulted in downregulation of a gene within the operon with a possible role in steroid degradation. For this reason, bioinformatics analysis of the genes adjacent to the hypothetical protein was performed to identify genes with possible roles in steroid degradation. The results showed that all hypothetical proteins within the operon have no significant similarity to other proteins in the NCBI database. Furthermore, bacterial genes encoding for L-aspartate oxidase was given a significant importance in the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) that is involved in the redox reactions of all living organisms. It catalyses the first step in the bacterial *de novo* biosynthesis of NAD^+ (Mattevi *et al*., 2011). In the present study, when L-aspartate oxidase gene in *L. monocytogenes* was interrupted by the trasnposon (M13), this showed that the mutant strain was deficient in growth when exposed to β-estradiol. This could possibly be the reason of a polar effect of the interrupted gene. However, analysis of adjacent genes in the same operon showed no significant findings. Therefore, it is currently unknown whether there is a relation between the hormone and the gene encoding for L-aspartate oxidase in *L. monocytogenes*. Likewise, other

interrupted genes in *L. monocytogenes* (M10, M11 and M12) were shown to be reduced in growth when exposed to the same hormone. Again, it is unknown whether the reduction of growth was a cause of a direct effect of the gene or the polarity of the gene was involved in the growth reduction observed. Complimentary cloning of the interrupted genes would have been an approach to answer this question, but this was not performed since the growth was not significantly affected in the presence of the hormone when mutants were screened in liquid media. For this reason, the mutant-library was further screened in the presence of progesterone for 12 h in 96-well plates under anaerobic conditions to identify genuine mutants. The period of incubation and the conditions used for this screening were selected based on the results observed in chapter 3. Unfortenatily, mutants were not affected by the presence of progesterone that was used at a final concentration of 60 µg ml^{-1}, and this was determined by measuring the optical density of all mutants alongside the controls. A possible explanation for this is that *L. monocytogenes* possesses multiple genes of which products function in combination to allow listerial resistance to the toxicity of progesterone; hence identifying a single gene with this role was not achievable.

Different approach was undertaken to identify listerial gene(s) with possible role in hormones binding and degradation using bioinformatics tools. Previously identified proteins with the ability to bind steroid hormones from different microorganisms were the basis of the alignment used to identify listerial proteins. The results obtained showed that previously investigated proteins from different microbes with the ability to bind steroids and other unsaturated compounds share significant homologies to NamA, Lmo2235 and Lmo0489 proteins from *L. monocytogenes* with the highest sequence identity to YqjM from *B. subtilis.* All proteins used for the alignments were shown to be members of the OYE family since they possess a high degree of sequence identity with one another; thus were assigned similar functions. Based on the analysis presented in this study it was evident that NamA is qualified to be classified under the OYE family and propose similar function in the detoxification process of xenobiotic compounds and a wide range of substrates including steroid hormones. Substrate binding site among

members of the OYE is conserved, and this includes NamA from *L. monocytogenes.* Intense investigations were carried out to determain the importance of NamA in listerial resistance to steroid hormones and whether it has an enzymatic activity toward either progesterone or β-estradiol. Therefore, *namA*-gene expression was performed using semi-quantative RT-PCR to preliminary investigate the expression of this gene in response to progesterone and β-estradiol. It was observed that *namA*-gene was down-regulated when cells were exposed to progesterone. This could be an explanation for the phenotype observed in chapter 3. Down-regulation of *namA* had possibly caused growth defect in the first half of the incubation, and progesterone degradation by other listerial-encoded enzyme(s) reduced progesterone concentration in the media; thus, restoration of normal gene expression and growth. As for the hormone β-estradiol, it was noted that *namA* expression was relatively down-regulated when cells where exposed to the hormone, and this could be linked to the unaffected bacterial growth when cells were exposed to this hormone. In order to quantitively assess the expression of *namA* gene; two approaches could be performed, (i) densitometry and (ii) RT-PCR. Each of which could deliver a quantitative measurement of the results obtained from gene expression experiments.

On the other hand, deletion mutant of the NamA-encoding gene was constructed and further characterised by testing the ability of the mutant strain to grow in the presence of the hormones in addition to measuring its enzymatic activity. Deletion of NamA from *L. monocytogenes* chromosomal DNA was achievable by the use of a temperature-sensitive vector that is dependent of expression on a replicon non-permissive to increased temperatures. Therefore, post the first allelic exchange and integration of the recombinant plasmid into listerial genomic DNA, the activity of the replicon is no longer necessary for the plasmid replication, since the integrated plasmid can use the host cell mechinary to replicate. The incorporated plasmid confers erm-resistance gene; hence, cells with successful recombination become resistant to erythromycin. To confirm deletion of the NamA-encoding gene, cells must become sensitive to erm, and this was achievable by growing the recombinant-plasmid harbouring cells for several consiquitive cycles without

antibiotic. It was observed that more cycles resulted in more chances for the cells to lose the integrated plasmid. From the data obtained, it was observed that the mutant strain was not significantly affected in growth when incubated in the presence of 60 µg ml^{-1} of either progesterone or β-estradiol under anaerobic conditions. Progesterone similarly affected the growth of both strains (WT and mutant) and the hypothesis of NamA role in listerial resistant to the hormone was demonstrated to be invalid. There must be other listerial genes that are involved in the restoration observed earlier with two possibilities to explain thier role for hormones binding; (i) hormones serve as substrates for the enzyme(s), which convert them to other compounds, or (ii) the hormones bind to the enzyme and therby modulate the oxidureductase activity.

In addition, bacterial lysates from both WT and mutant strain were used to measure the enzymatic activity of NamA by following the oxidation rate of β-NADPH to β-NADP at 340 nm. NamA and other members of the OYE family use β-NADPH as a cofactor for electron transfer to the substrate, which results in the oxidation of the cofactor and reduction of the substrate. Although the oxidation rate of β-NADPH was minimal, it was observed that in the presence of progesterone and β-estradiol the optical density of the cofactor was reduced during the period of incubation when WT lysate was used, but less reduction was observed when using the lysate prepared from the mutant strain. In conclusion, the rate of oxidation from both lysates (WT and mutant lysates) were insignificantly different, and the small differences in absorbance are suggested to be due to polar effect of the deleted gene, which may have affected the expression of other enzymes within the lysate. On the other hand, the initial absorbance at the start of the assay was very high, which may have led to an increased-measurement error since an O.D of 2 means 1% of light transmitted through the spectrophotometer. Therefore, reducing NADPH concentration to an O.D less than 1 will also reduce measurement error. Another explanation is that the reduction in NADPH absorbance was not due to genuine oxidation, rather the background noise from the spectrophotometer may have affected the measurement outcome. To further investigate NamA enzyme in its purified form, the gene coding for

NamA was cloned into the expression vector pPAL7 and the protein was overexpressed by the addition of IPTG. As described earlier, all the optimisation attempts to express the enzyme in a soluble form were not successful as the proteins aggregated in the inclusion bodies (insoluble) fraction, which indicates that this protein was not biologically active. Therefore, purification of the enzyme was not further performed.

In summary, the findings presented in this thesis represent a new insight in the relation between pregnancy hormones and *L. monocytogenes*. This pathogen is suggested to overcome the growth retardation/negative effect of the hormone by utilisation of the listeria-encoded enzyme(s) that may results in the reduction of hormonal levels in a human body. Furthermore, the identification of an OYE family member encoded by *L. monocytogenes* may contribute to the understanding of how this pathogen overcomes the toxicity encounterd inside the host and the possibility of degrading pregnancy hormones that are crucial to maintain pregnancy, which may lead to fetal death and abortion.

This knowledge may form the basis of a therapy to protect pregnant individuals against infections caused by *L. monocytogenes*.

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