Cloning, Expression and Purification of the Subunits of the Mannose PTS Permease of *Listeria monocytogenes* EGD

by

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PREFACE

The experimental work described in this dissertation for Master of Science in the discipline of Genetics, was carried out from February 2005 to March 2007, in the School of Genetics, Biochemistry, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg. The laboratory work was performed under the supervision of Dr Mervyn Beukes. The following dissertation was compiled under the supervision of Dr Gregory Watson.

These studies represent the original work by the author and have not otherwise been submitted in any form for any degree or diploma to any other University. Where use has been made of the work of others, it has been duly acknowledged in the text.

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Rizwana Mia (Ms)

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Dr G. Watson (supervisor)

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ABSTRACT

The disease listeriosis is caused by *Listeria monocytogenes.* This common food-borne disease has been responsible for about 0.1 to 10 cases per million inhabitants per year. However, this disease is serious with its high fatality rates of 20% - 30%, and 40% of all cases reported have been in pregnant women suffered from a foetal abortion. Recently the organism has acquired resistance to antibiotic treatment and the development of an alternative treatment is necessary. Class IIa bacteriocins such as leucocin A have been shown to be active against *L. monocytogenes*. However, the leucocin A receptor molecule responsible for growth inhibition within *L. monocytogenes* remains unclear. Various studies have implicated the mannose PTS permease (EII_t^{Man}) of *L. monocytogenes* as the putative receptor for class IIa bacteriocins. The results from studies reviewed indicate that the EII_t^{Man} of *L. monocytogenes* could be the chiral receptor needed for bacteriocin interaction at the surface of targeted cells. Specifically, the membrane associated IID^{Man} and IIC^{Man} subunits were implicated in direct interaction with class IIa bacteriocins. Our study focused on cloning, expression and purification of the subunits of the mannose PTS permease of *L. monocytogenes* EGD. Primers were designed to amplify the subunit genes of the *mptACD* operon. The *mptC*, *mptD* and *mptAB* genes which were then successfully cloned into pET28a expression vector and transformed into *E. coli* JM109(DE3) host strain. Recombinant plasmids were screened using colony PCR. Subsequently recombinant pET28-C, pET28-D and pET28-AB was once again transformed and expressed in the *E. coli* BL21(DE3) pLysS expression host strain. After an induction at 30°C for 5 hours, IIC^{Man} and IID^{Man} were found to be expressed in the cell membrane, whilst $IIAB^{Man}$ was expressed in the cytosol of the host expression strain. Membrane proteins His-IIC^{Man}, His-IID^{Man}, and cytosol associated His-$IIAB^{Man}$ were purified using Ni^{2+}-NTA affinity chromatography. Results for His-IIC^{Man} yielded a 28 kDa protein and a 55 kDa co-purified protein. Results for His-IID^{Man} yielded a 31 kDa protein and a 60 kDa co-purified protein. Results for His-$IIAB^{Man}$ yielded a 35 kDa protein and a 68 kDa co-purified protein. A western blot analysis revealed that all proteins purified carried an attached His-tag as detected by an anti-mouse peroxidase conjugate anti-His-tag antibody.

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LIST OF ABBREVIATIONS

$\Delta\psi$	Transmembrane potential
ΔpH	pH gradient
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280nm
AMP	Antimicrobial Peptide
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
cfu	Colony forming units
Cu^{+1}	Cuprous ion
Cu^{+2}	Cupric ions
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EII	Enzyme II of the bacterial phosphotransferase system
EII_t^{man}	Enzyme II of the mannose phosphotransferase permease
E. coli	*Escherichia coli*
Fwd	Forward
Glc	Glucose
H_20	Water
His	Histidine

HPK	Histidine Protein Kinase
HPr	Histidine Protein (phosphate carrier protein)
IF	Induction Factor
IP	Immunity Protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
L	length
l	litre
LB	Luria Bertani
L. gelidum	*Leuconostoc gelidum*
L. monocytogenes	*Listeria monocytogenes*
Lc. lactis	*Lactococcus lactis*
leuA	leucocin A
M	Molar
mM	millimolar
m v^{-1}	mass per volume
ml	millilitre
µl	microlitre
mg ml^{-1}	milligram per millilitre
µg ml^{-1}	microgram per millilitre
Man	mannose
MW	molecular weight
mpt	mannose phosphotransferase
Mtl	mannitol
nm	nanometre
ng $µl^{-1}$	nanogram per microliter
NaOH	Sodium Hydroxide
NCBI	National Centre for Bioinformatics Information

Ni^{2+}	Nickel Ion
NTA	Nitrilotriacetic Acid
OD_{600}	Optical Density at 600 nm
OGP	Octyl-β-D-glucopyranoside
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PMF	Proton Motive Force
PMSF	Phenylmethylsulphonyl fluoride
PCR	Polymerase Chain Reaction
%GC	Percentage Guanine:Cytosine
$RbCl_2$	Rubidium Chloride
Rev	Reverse
RO H_2O	Reverse osmosis water
RR	Response Regulator
RT	Room temperature
S2DHFR	Sodium Dihydropholate Reductase
ssDNA	single stranded DNA
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TE buffer	Tris-EDTA buffer
TFA	Trifluoroacetic acid
T*m*	Melting Temperature
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
TSC	Tris Sucrose Choline chloride buffer

v/v	volume per volume
w/v	weight per volume

CHAPTER 1

Literature Review

1.1. Introduction

Listeriosis caused by *Listeria monocytogenes,* is a common food-borne disease responsible for about 0.1 to 10 cases per million inhabitants per year. However, this disease is serious with its high fatality rates of 20% - 30%, and 40% of all cases reported have been in pregnant women suffered from a foetal abortion (WHO, 2008). The disease is clinically defined when the causal bacterial organism is isolated from blood, or cerebrospinal fluid (Charpenter & Courvalin, 1999). The manifestations of listeriosis include septicaemia, meningitis (or meningoencephalitis), encephalitis, and intrauterine or cervical infections in pregnant women which may cause spontaneous abortion in the second or third trimester of pregnancy or otherwise result in a stillbirth. The onset of the aforementioned disorders is usually preceded by influenza-like symptoms including persistent fever. It was reported that gastrointestinal symptoms such as nausea, vomiting, and diarrhoea may follow more serious forms of listeriosis or may be the only symptoms expressed. The onset time to serious forms of listeriosis usually ranges from a few days to three weeks. The onset time to gastrointestinal symptoms is usually greater than 12 hours. The bacterium is blood borne (septicaemic) and once it enters the host's monocytes, macrophages, or polymorphonuclear leukocytes, it can propagate within these host cells (Charpenter & Courvalin, 1999). The pathogenesis of *L. monocytogenes* centres on its ability to survive and multiply in phagocytic host cells. Its intracellular presence in phagocytic cells also permits access to the brain and probably transplacental migration to the foetus in pregnant women. *L. monocytogenes* is a remarkably well adapted bacterium which is able to grow in vegetable matter, yet invade human cells and undergo intracellular propagation (Charpenter & Courvalin, 1999).

Food-borne transmission constitutes the main route to acquisition of listeriosis. *Listeria* which is considered to be susceptible to almost all antibiotics, has been shown to acquire multi-drug resistance (Poyart-Salmeron *et al.,* 1990). This was perhaps due to the selective pressure exerted by over-prescription of drugs in clinical settings and their use as growth promoters for farm animals (Charpenter & Courvalin, 1999).

Bacteriocins are antimicrobial peptides produced by lactic acid bacteria. These peptides are known to inhibit closely related bacterial species. There are three major bacteriocin classes (Papagianni, 2003). However, much attention has been placed on the subset class IIa, which are small cationic heat-stable peptides, which exhibit inhibitory activity by affecting the bacterial cell membrane potential as well as the environmental pH of the cell (Hastings *et al.*, 1991; Klaenhammer, 1993; Dykes & Hastings, 1998). Class IIa bacteriocins are well known to successfully inhibit the growth of *L. monocytogenes* (Riley, 1998).

Due to the rise of antibiotic resistant strains and the severity of the disease listeriosis, class IIa bacteriocins such as leucocin A have been promoted to help control the growth of *L. monocytogenes* (Deegan *et al.*, 2006). The leucocin A receptor molecule responsible for growth inhibition within *L. monocytogenes* remains unclear. Various studies have implicated the mannose PTS permease of *L. monocytogenes* as the putative receptor for class IIa bacteriocins. However, no study has confirmed the theory using real time biomolecular interaction assays. We have undertaken the following study to set a precedent for future studies to determine the characteristics of each of the membrane bound subunits of the mannose PTS permease, and to answer the pertinent question: which subunit is the receptor molecule for class IIa bacteriocins? Therefore this study entails, cloning, expression and purification of the membrane bound subunits of *L. monocytogenes* EGD mannose PTS permease (EII_t^{Man}).

1.2. *Listeria monocytogenes*

L. monocytogenes was first described by Murray and co-workers, in 1926, as the causative agent of septicaemia in rabbits (*op. cit.* Harris *et al.*, 1989). The bacterium is a Gram positive rod-shaped organism about 0.5 µm in diameter and 1–2 µm in length. It is motile by means of a few peritrichious flagella when cultured at a temperature range of 20-25°C (Jawetz *et al.*, 1968; Rocourt & Bucherieser, 2007). Some studies suggest that 1-10% of humans may be intestinal carriers of *L. monocytogenes* (Charpenter & Courvalin, 1999).

The organism is found in no less than 37 mammalian species, both domestic and feral, as well as 17 species of birds and possibly some species of fish and shellfish. This microbe can be isolated from soil, silage, and other environmental sources. *L. monocytogenes* resists the lethal effects of freeze-drying and heat, which is remarkable for a bacterium that does not form spores (Charpenter & Courvalin, 1999).

The taxonomic classification of *L. monocytogenes* was initially unclear; hence it was included in the family *Corynebacteriaceae*, thereafter into the genera of uncertain affiliation. Studies that involved a taxonomic survey of *Listeria* and related bacteria, suggested the inclusion of *Listeria* in the family *lactobacillaceae* (Wilkins & Jones, 1977). Thereafter, the bacterium was classified in Bergey´s manual of Systematic Bacteriology under the family *Lactobacillaceae* (Harris *et al*., 1989). The taxonomic classification of *Listeria* at this time was based on numerical and chemotaxonomy (Harris *et al.*, 1989; Rocourt & Bucherieser, 2007). The advent of DNA/DNA hybridization, ribosomal RNA (rRNA) and DNA sequencing technologies, brought about an enhanced accuracy to the phylogenetic position of *Listeria* sp. Currently *Listeria* is grouped under the super kingdom Bacteria, phylum *Firmicutes*, class *Bacilli*, order *Bacillales* and family *Listeriaceae* (Glaser *et al*., 2001).

1.3. Antibiotic Resistance in *Listeria* species

The usual treatment of listeriosis involves the administration of β-lactam antibiotics such as ampicillin or penicillin G combined with an aminoglycoside such as gentamicin. An alternative to the administration of penicillin is tetracycline, whilst associative means of treatment include trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole. The most effective combination seems to be the trimethoprim which is synergized by using sulfamethoxazole (Biosivon *et al.*, 1990). However the remarkable adaptive capability of this bacterium was displayed when it was shown to confer resistance against most antibiotic combinations. The first of the *L. monocytogenes* strains to attain

antibiotic resistance to tetracycline was reported in 1988, with a minimum inhibitory concentration of 10 µg ml^{-1} tetracycline (Poyart-Salmeron *et al.*, 1990).

Antibiotic resistance is thought to be an acquired trait (Charpenter & Courvalin, 1999). The *L. monocytogenes* strain co-habituates in the digestive systems of humans and animals alongside diverse species of *Enterococcus* and *Streptococcus.* These bacterial species are known to harbour a large reservoir of conjugative plasmids and transposons, which have been implicated in the transfer of antibiotic resistant genes to *L. monocytogenes*, giving rise to antibiotic resistant *L. monocytogenes* (Charpenter & Courvalin, 1999). Table 1.1 gives an overview of the origin and nature of self-transferable plasmids or transposons responsible for the variety of antibiotic resistant genes that cause resistance in *Listerial* sp. The association between *Listerial, Enterococcal* and *Streptococcal* species is clearly evident as *Listeria* acquires resistance via self-transferable conjugative plasmids or transposons from these bacterial strains. Factors of concern are the circumstances which may lead to drug resistant *Listeria.* The administration of antibiotics in an indiscriminate manner by medical practitioners and its use in animal breeding programs led to the deterioration of the efficacy of these drugs (Poyart-Salmeron *et al.*, 1990). Selective pressure is exerted on these pathogenic organisms therefore resulting in broad spectrum antibiotic resistant strains. *Listeria´s* broad spectrum antibiotic resistance capability necessitates the need for alternative means in curbing potential infection and manifestation of *L. monocytogenes* during food processing and storage. Class IIa bacteriocins known as anti-*listerial* antimicrobial peptides have been shown to work well against *L. monocytogenes* and these antimicrobial peptides may be the answer to more effective treatment and prevention of listeriosis (Hastings *et al.*, 1991; Maftah *et al.*, 1993; Montville & Chen, 1998; Ennahar *et al.*, 2000; Hechard & Sahl, 2002; Chen & Hoover, 2003; Deegan *et al.*, 2006)..

Table 1.1. Conjugative plasmids and transposons, encoding antibiotic resistance genes responsible for antibiotic resistance in *L. monocytogenes*. The table was derived from a review study (Charpenter & Courvalin, 1999)**.**

Plasmids or Transposons	***Origin***	***Conferred Antibiotic Resistance to:****	**Mode of Transfer**	**Genes Transferred**
Plasmid pIP50	*Streptococcus agalactactiae*	*Cm microlides, lincosamides* and *streptogramins*	Conjugation. transfer, between *Listeria* and *Streptococcus*	Also able to replicate in *Listeria*. Therefore could transfer the *cat*221 gene (*Cm* acetyl transferase)
Plasmid pAMβ1	*Enterococcus faecalis*	*Em*	Conjugation. Self transferable by conjugation	*erm*AM (encodes an rRNA methylase)
Plasmid pRYC16	*Listeria*	*Em*	May mobilize a non-conjugative plasmid pDB1 of *Bacillus subtilis* between species of *Listeria*	Cryptic replicon plasmid compatible to pAMβ1 which transfers the Erythromycin resistant gene *erm*AM
Plasmid pIP811	*Enterococcus* and *Streptococcus*	*Cm, Em, Sm*	Self transferable by conjugation	Mediates transfer of *cat*221 gene (Cm acetyl transferase) *erm*AM (encodes an rRNA methylase) & *aad6* gene (encodes 6-N-aminoglycolase nucleotidyltransferase) respectively. Similar to pAMβ1
pIP813	*Enterococcus* and *Streptococcus*	*Tc*	Self transferable by conjugation	Harbours the *tet*L gene which encodes a protein involved in the active efflux of tetracycline from the bacterium.
pIP823	*Enterococcus faecalis* and *Staphylococcus aureus*	*Trimethoprim*	Self transferable by conjugation	*dfr*D gene encoding S2DHFR of a second class of high-level trimethoprim resistance. May also be found in *Bacillus subtilis*
Transposon Tn916	*Enterococcus faecalis*	*Tc, Mc*	Conjugation - Co-transfer of the *tet*M gene associated with *int-Tn* gene	*tet*M gene (directs synthesis of a cytoplasmic protein which protects the ribosome from inhibiting the antibiotic), and *int-Tn* gene (encodes the intergrase involved in the mobility of Tn *1545*-Tn*916* conjugative transposons)
Transposon Tn1545	*Streptococcus pneumoniae*	*Tc*	Conjugation.	Tn916-related. Encodes the *tet*M gene and *int-Tn* gene.

*Chloramphenicol (*Cm*), Erythromycin (*Em*), Streptomycin *(Sm)*, Tetracycline *(Tc)*, Minocycline (*Mc*).

1.4. Bacteriocins

Antimicrobial peptides such as bacteriocins are widely distributed in nature. They are produced by both eukaryotes and prokaryotes and form a diverse group, from the well-studied colicins of prokaryotes to the halocins of Archeabacteria. For centuries bacteriocins have played an important role in the preservation of food (Deegan *et al.*, 2006). The extensive range of these antimicrobial peptides have the potential to significantly impact the treatment and prevention of bacterial infections (Riley, 1998). Particular interest was focused on the antimicrobial activity of bacteriocins produced by lactic acid producing bacteria. Intensive investigations on these bacteriocins have elucidated the understanding of the structural, biosynthetic and innate activity of class IIa bacteriocins (Riley, 1998).

1.4.1. The ubiquitous nature of bacteriocins

Bacteriocins are produced by both Gram-positive and Gram-negative organisms. The most extensively studied bacteriocins from Gram-negative bacteria, were the colicins, from *Escherichia. coli* (Papagianni, 2003). This review focuses on the class IIa bacteriocins produced by lactic acid bacteria. The genera of lactic acid producing bacteria used in the food industry, to organically preserve food, include *Lactococcus, lactobacillus, Streptococcus thermophillus, Pediococcus, Leuconostoc, Carnobacterium* and *Enterococcus* (Diep & Nes, 2002). Numerous bacteriocins have been biochemically and genetically characterized from these organisms. Antimicrobial peptides of this nature have also been found for other non-food organisms of the Gram-positive genera, such as *Clostridium, Bacillus, Staphylococcus, Brevibacterium* and *Bifidobacterium* (Diep & Nes, 2002).

1.4.2. General mode of action

Bacteriocins are generally cationic, amphiphilic and most likely attack the susceptible bacterial cell by permeabilizing its membrane. It is assumed that their net positive charge facilitates interactions with the negatively charged bacterial phospholipid-containing membranes or acidic bacterial cell walls. Whilst it is also believed that their amphiphilic characteristics enable membrane permeabilization (Papagianni, 2003).

1.4.3. Classification and Characteristics of bacteriocins

Bacteriocins are classified into three major classes; class I, II and III. Table 1.2 highlights the sub-categories of each class. These peptides are roughly categorized into those that have a high content of a certain amino acid, most often being proline, those that contain disulfide bridges, and those harbouring an amphiphilic region within their molecule if they assume an α–helical structure (Papagianni, 2003).

Bacteriocins that are produced by Gram-negative organisms are of high molecular weight e.g. colicins from *E. coli*. These proteins are usually larger than 20 kDa. Bacteriocins produced by Gram-positive organisms such as lactic acid-producing bacteria have a smaller molecular weight many are under 20 kDa (Diep & Nes, 2002).

Table 1.2. Categories and sub-categories of each class of bacteriocin

Main Category	**The Predominant Amino Acids present**	**Sub-category**
Class I: Lantibiotics (modified bacteriocins)	Lanthionines	Type A: Elongated Molecules Type B: Globular Molecules
Class II: Non-modified, Heat Stable Bacteriocins	Proline and cysteine disulphide bridges	Class IIa: Pediocin-like/ anti-listerial Bacteriocins Class IIb: Two peptide Bacteriocins Other Bacteriocins
Class III: Large, Heat Labile Bacteriocins	Not well characterised	Larger than 30 kDa. But not well characterized

As mentioned before class IIa bacteriocins have been noted to inhibit growth of well known food pathogens, particularly *L. monocytogenes,* to the extent that this group of bacteriocins have been referred to as anti-listerial peptides. Bacteriocins have been used extensively in the control of listeriosis. Consequently this has led to the identification and description of a large number of anti-listerial bacteriocins (Riley, 1998).

Anti-listerial bacteriocins predominantly belong to the class IIa subgroup of the bacteriocin genre, classified on the basis of their primary structure, their highly similar amino acid sequence, distinctive N-terminal region and extreme anti-listerial activity (Klaenhammer, 1993; Riley, 1998). These bacteriocins remain the largest and most extensively studied subgroup of the class II bacteriocins (Ennahar *et al.*, 2000). They are commonly found to be cationic and amphiphilic, as mentioned previously (Section 1.4.2.). Most of them kill the bacterial organism by permeabilizing their cell membranes (Papagianni, 2003).

All class IIa bacteriocins have a YGNGVXC consensus sequence at the N-terminus (Ennahar *et al.,* 2000). The consensus motif has been shown to be part of a recognition sequence for a putative membrane-bound receptor (Ennahar *et al.*, 2000). The sequence similarity of various class IIa bacteriocins are highlighted in Figure 1.1. Peptides such as

leucocin A and mesentericin Y105 are almost identical to each other, differing by just two amino acids. Most other peptides in this group also share similar C-terminal sequence motifs.

Figure 1.1. Amino acid sequence alignments of class IIa bacteriocins highlighting the N-terminal YGNGV consensus motifs. Residues conserved in at least 10 of the stipulated sequences are shown in white boxes. Shaded boxes show the conserved C-terminal residues in at least two sequences. Amino acid residues shown in boldface font are positively charged; whilst those amino acids represented in lower case represent residues with an uncertain charge. The "X" represents unknown residues (Ennahar *et al.*, 2000).

1.4.4. The genetic organization of class IIa bacteriocins

Class IIa bacteriocins are either encoded on plasmids or chromosomes. These genes are involved in bacteriocin production, extracellular translocation, immunity of the producer organism and in some instances the regulation of bacteriocin synthesis (Ennahar *et al.*, 2000).

The general gene organization of class IIa bacteriocins was previously described (Ennahar *et al.* (2000). Conservation of class IIa bacteriocin gene arrangement is highlighted in Figure 1.2. The structural gene encodes a bacteriocin pre-peptide, which contains a leader sequence with two glycine residues at its C-terminus. The leader sequence may possibly serve as a recognition signal for a Sec-independent ABC transporter (Klaenhammer, 1993; Nes *et al.*, 1996). The ABC transporter in this context refers to an ATP-binding cassette. Class IIa bacteriocins contain at least two genes encoding proteins homologous to ABC-transporters and accessory proteins, which are required to drive the bacteriocin to the outside of the cell (Klaenhammer, 1993; Nes *et al.*, 1996). The bacteriocin structural gene precedes and is co-transcribed with an immunity protein. Some bacteriocin operons have additional open reading frames (ORF's) in the vicinity of the structural gene and in the same order forms a putative three component signal-transduction auto regulatory cassette. This cassette encodes an induction factor (IF), histidine protein kinase (HPK) and a response regulator (RR) (Ennahar *et al.*, 2000). The biosynthesis of bacteriocin production is explained in more detail in the next section (Section 1.4.6.).

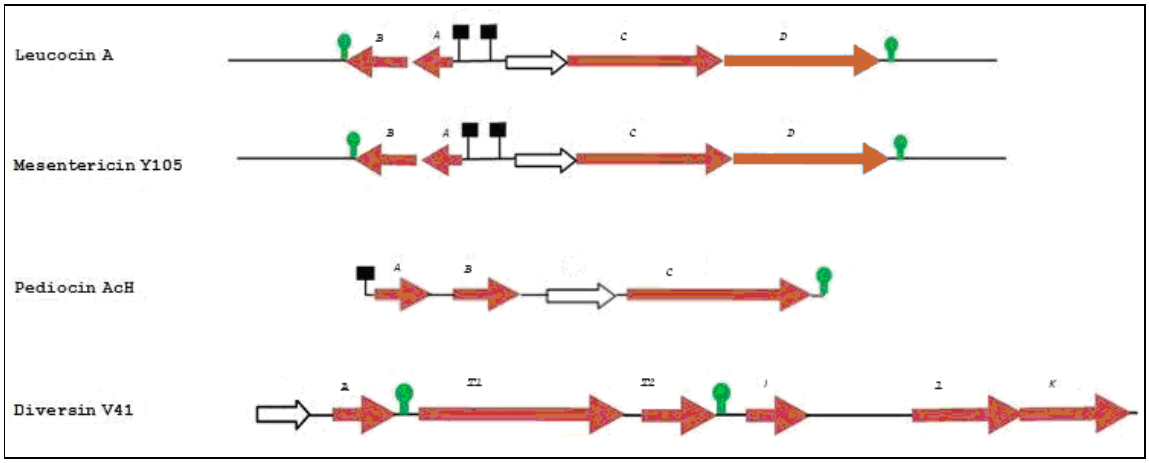


Figure 1.2. Organization of gene clusters involved in the immunity and production of class IIa bacteriocins. Shown are the open reading frames (ORF´s) encoding the three component regulated peptides namely; leucocin A, mesentericin Y105, pediocin AcH and the two component regulated Divericin V41. Promoters (black boxes); terminators (green lollipop-like symbols); A- *lcaA;* B- *lcaB* genes encoding leucocin A or bacteriocin, C- *lcaC* and D- *lcaD* encode the immunity peptide genes. The white arrow indicates the presence of the ORF (Ennahar *et al.*, 2000).

1.4.5. Biosynthesis of class IIa bacteriocins

The production of class IIa bacteriocins is referred to as a three component regulatory system involving the synthesis, processing, secretion of the bacteriocin and the host immunity protein, (see Figure 1.3) (Ennahar *et al.*, 2000). An exception to this system is divercin V41 of *Carnobacterium* V41 which has been noted to use a two-component signal transduction system (Metervier *et al.*, 1998).

The leader sequence on the class IIa bacteriocins serves as a recognition signal for both cleavage of the pre-peptide as well as transmembrane translocation of the mature peptide (Klaenhammer, 1993; Nes *et al.*, 1996; Riley, 1998; Ennahar *et al.*, 2000; Papagianni,

2003). The steps in the three component system as described in (Figure 1.3), would typically include:

- histidine protein kinase (HPK),
- response regulator (RR) and
- induction factor (IF).

IF´s are usually small heat-stable cationic and hydrophobic peptides that are first synthesized as pre-peptides with leader sequences of a double-glycine type (Nes *et al.*, 1996). An environmental stimulus triggers the pathway of biochemical reactions in the bacterial cell. The IF binds HPK. The HPK thereby phosphorylates the RR which in turn activates the bacteriocin operon therefore this enables the production of the immunity protein, pre-bacteriocin peptide and pre-induction factor. The pre-peptide form of the bacteriocin and IF, are biologically inactive. However, subsequent cleavage of the pre-peptide at a double-glycine proteolytic cleavage site removes the leader sequence from the bacteriocin peptide in tandem with the transmembrane translocation of the peptide to the periplasmic space of the cell (Havarstein *et al.*, 1995; Nes *et al.*, 1996; Ennahar *et al.*, 2000). The immunity protein plays an important role by binding to a transmembrane receptor of class IIa bacteriocins on the cytoplasmic part of the membrane receptor. Once the processed/active bacteriocin binds the receptor via the periplasmic binding site, the membrane permeabilizing ability of the bacteriocin is inhibited, leading to immunity of the host organism (Maftah *et al.*, 1993; Chen & Montville, 1995; Chen *et al.*, 1997a; Ennahar *et al.*, 2000). This immunity protein of class IIa bacteriocins are generally cationic and hydrophilic in nature, consisting of 88-114 amino acids. Apart from offering protection to the host, these immunity proteins offer partial immunity of the host strain against other class IIa bacteriocins (Ennahar *et al.*, 2000).

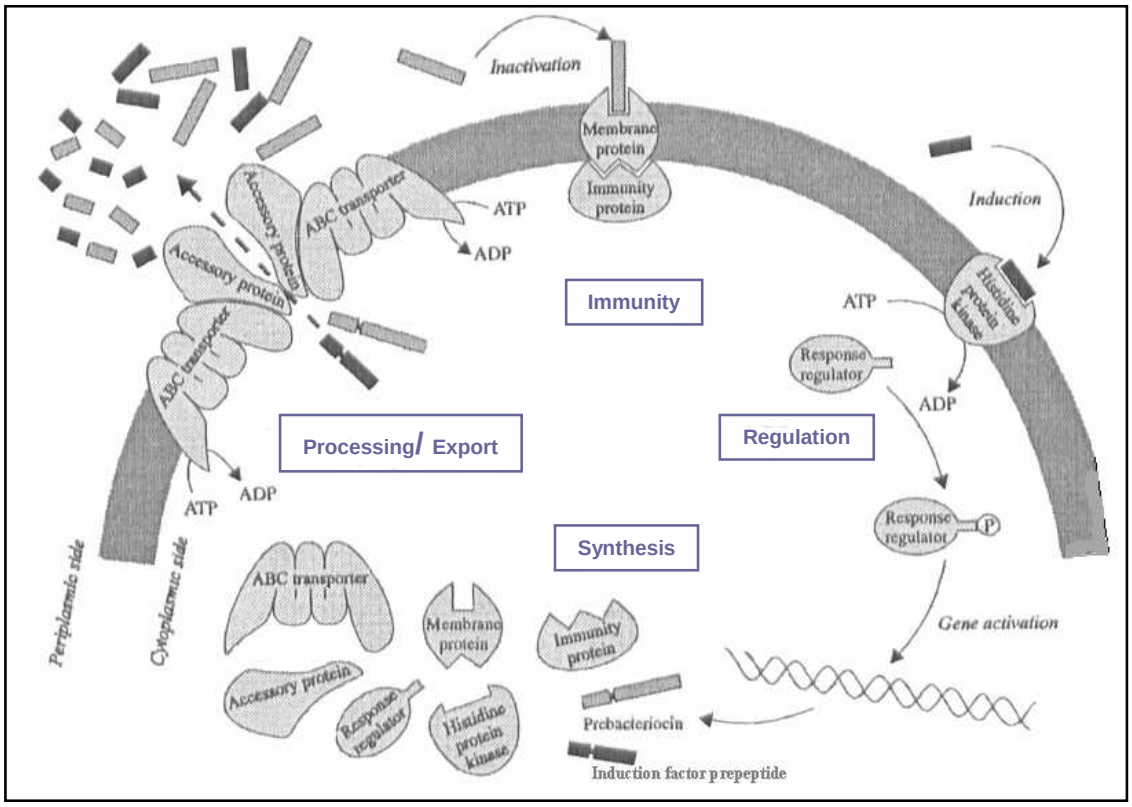


Figure 1.3. Schematic overview of the three component regulatory system for the production of class IIa bacteriocins within its host organism. The system involves the synthesis, processing, and excretion of the peptide and immunity of the host cell (Ennahar *et al.*, 2000).

1.4.6. The mode of action of bacteriocins

Similar to other bacteriocins produced by lactic acid bacteria, class IIa bacteriocins are bactericidal and act primarily by permeabilizing the membrane of vulnerable microorganisms, through the formation of poration complexes causing an imbalance in ionic concentration and leakage of inorganic phosphate (Klaenhammer, 1993). This has been clearly established for pediocin PA-1 (Chen & Montville, 1995), mesentericin Y105 (Maftah *et al.*, 1993), and bavericin MN (Kaiser & Montville, 1996). In the case of a susceptible organism such as *L. monocytogenes* a membrane disruption of this nature

causes steady dissipation of the proton motive force (PMF) which may lead to total dissipation of the pH gradient (ΔpH) and a partial dissipation of the transmembrane potential (Δψ) (Maftah *et al.*, 1993; Kaiser & Montville, 1996). The lethal activity of class IIa bacteriocins is mainly due to the dissipation of the PMF followed by an accelerated depletion of intracellular ATP and amino acids (Chen & Montville, 1995). However, no leakage of ATP has been observed in the mode of action of class IIa bacteriocins and the depletion may possibly be due to an increased consumption of ATP in order to maintain the PMF (Chen & Montville, 1995). One such bacteriocin, leucocin A, has been known to display activity against *L. monocytogenes* EGD.

1.4.7. The structure of class IIa bacteriocins

Structural models of class IIa bacteriocins in membrane mimicking environments were studied using nuclear magnetic resonance data, circular dichroism and computer simulation methods (Sailer *et al.*, 1993; Bhugaloo-Vial *et al.*, 1996; Kaiser & Montville, 1996; Chen *et al.*, 1997b; Fregeau Gallagher *et al.*, 1997). An important characteristic to note of class IIa bacteriocins is their cysteine content. Firstly it has been reported that they have at least two cysteines with disulfide bridges whilst their counterparts, the non-lanthionine containing bacteriocins, may include only one (lactococcin B, acidocin A) or no cysteine residues (lactococcin A, lactococcin M, lactococcin G and plantaricin A) (Riley, 1998). Protein sequence alignment studies of class IIa bacteriocins indicated that the two cysteine residues are present in conserved positions within the N-terminal region, and as a result the disulfide bridge which forms a four amino acid loop over these two residues is well conserved in all class IIa bacteriocins. The presence of disulfide bonds seems to be a crucial component in bacteriocin activity especially with those peptides having two disulfide bridges. Pediocin PA-1/AcH, enterocin A and divericin V41 are unique as they possess an extra disulfide bond involving a second pair of cysteine residues. This conformation gives class IIa bacteriocins an amphiphilic characteristic at the N-terminal region. The structure of the leucocin A peptide is highlighted in Figure 1.4. This antimicrobial peptide has an N-terminal β-sheet with two strands connected by cysteine

residues to form a β-hairpin at the position YGNGV, has been predicted for pediocin PA-1 and leucocin A. The C-terminal half of class IIa bacteriocins has been predicted to adopt an amphiphilic α-helix, which span similar regions in different molecules and is believed to be the transmembrane segment during pore formation in the affected cell (Fregeau Gallagher *et al.*, 1997). The secondary structure predicted for nine class IIa bacteriocins was previously investigated (Bennik *et al.*, 1998). The bacteriocins under scrutiny seem to have contained α-helical conformations spanning residues 15, 16-27 and 28, which are believed to form an oblique orientation and are mainly responsible for membrane rupture or perturbation (Fregeau Gallagher *et al.*, 1997; Bennik *et al.*, 1998).

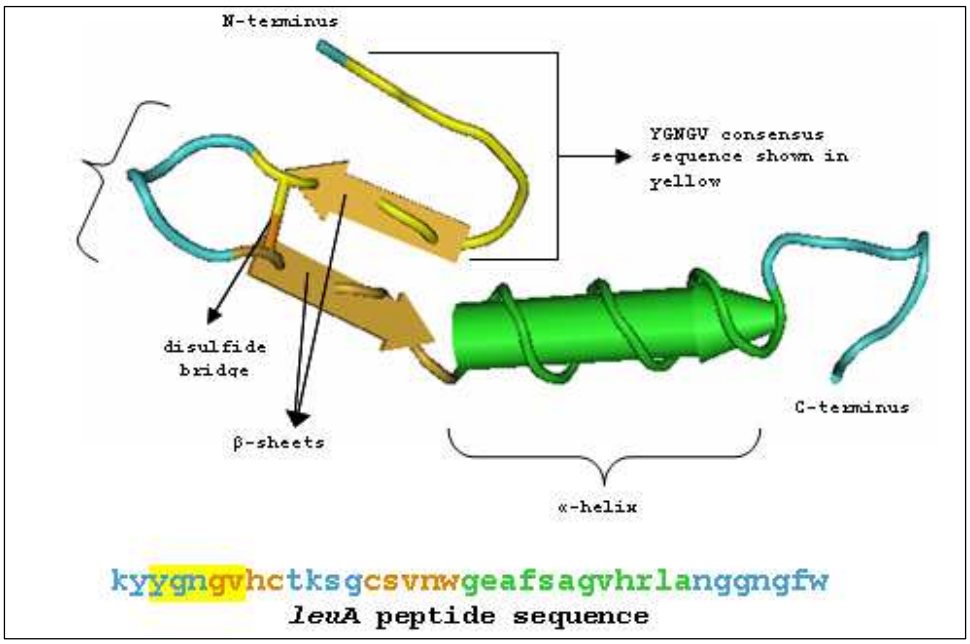


Figure 1.4. Three dimensional structure of the class IIa bacteriocin leucocin A. The N-terminus region, highlighted in yellow is the YGNGV consensus sequence followed by the six amino acid ring, bound by a disulfide bond (cysteine). β-sheets are displayed in brown toward the N-terminus and an α-helix toward the C-terminus in green The leucocin A amino acid sequence is shown as colour coded to the corresponding regions within the structure, and the consensus sequence also highlighted in yellow (Cn3D 4.1, NCBI software, USA) (Fregeau Gallagher *et al.*, 1997).

1.4.8. Leucocin A

In 1991, leucocin A produced by *Leuconostoc gelidum* UAL 187 was identified and characterized under the genera of the class IIa bacteriocins, (Hastings *et al.*, 1991; Hastings & Stiles, 1991). This anionic peptide of approximately 3.9 kDa is active against a wide range of lactic acid producing bacteria and well known food pathogens such as *L. monocytogenes* (Hastings *et al.*, 1991). However, its precise mechanism of interaction against *L. monocytogenes* has not been proven.

1.4.9. The putative receptor of class IIa bacteriocins

Over the years several postulated models of class IIa bacteriocin interaction with the target cell have been proposed. These include electrostatic binding to the membrane, which led to the suggestion of specific binding of the bacteriocin to a membrane associated component (Chen *et al.*, 1997a; Montville & Chen, 1998; Hechard & Sahl, 2002). Figure 1.5. highlights the postulated mechanism of interaction between class IIa bacteriocins and the susceptible bacterial membrane (Maftah *et al.*, 1993; Ennahar *et al.*, 2000).

Investigations into the molecular mode of bacteriocin interaction were performed utilizing leucocin A and its closely related counterpart mesentericin Y105. Firstly, the inactivation of the *rpoN* gene, encoding the sigma 54 (σ^{54}) subunit of RNA polymerase resulted in bacteriocin resistant *L. monocytogenes* and *E. faecalis* to mesentericin Y105 (Robichon *et al.*, 1997; Dalet *et al.*, 2000). These studies showed the σ^{54} subunit directs expression of the *mptACD* operon. The *mptACD* operon encodes a mannose permease phosphotransferase (PTS) system, (EII_t^{Man}). The EII_t^{Man} protein consists of three subunits $IIAB^{Man}$, IIC^{Man}, and IID^{Man}.

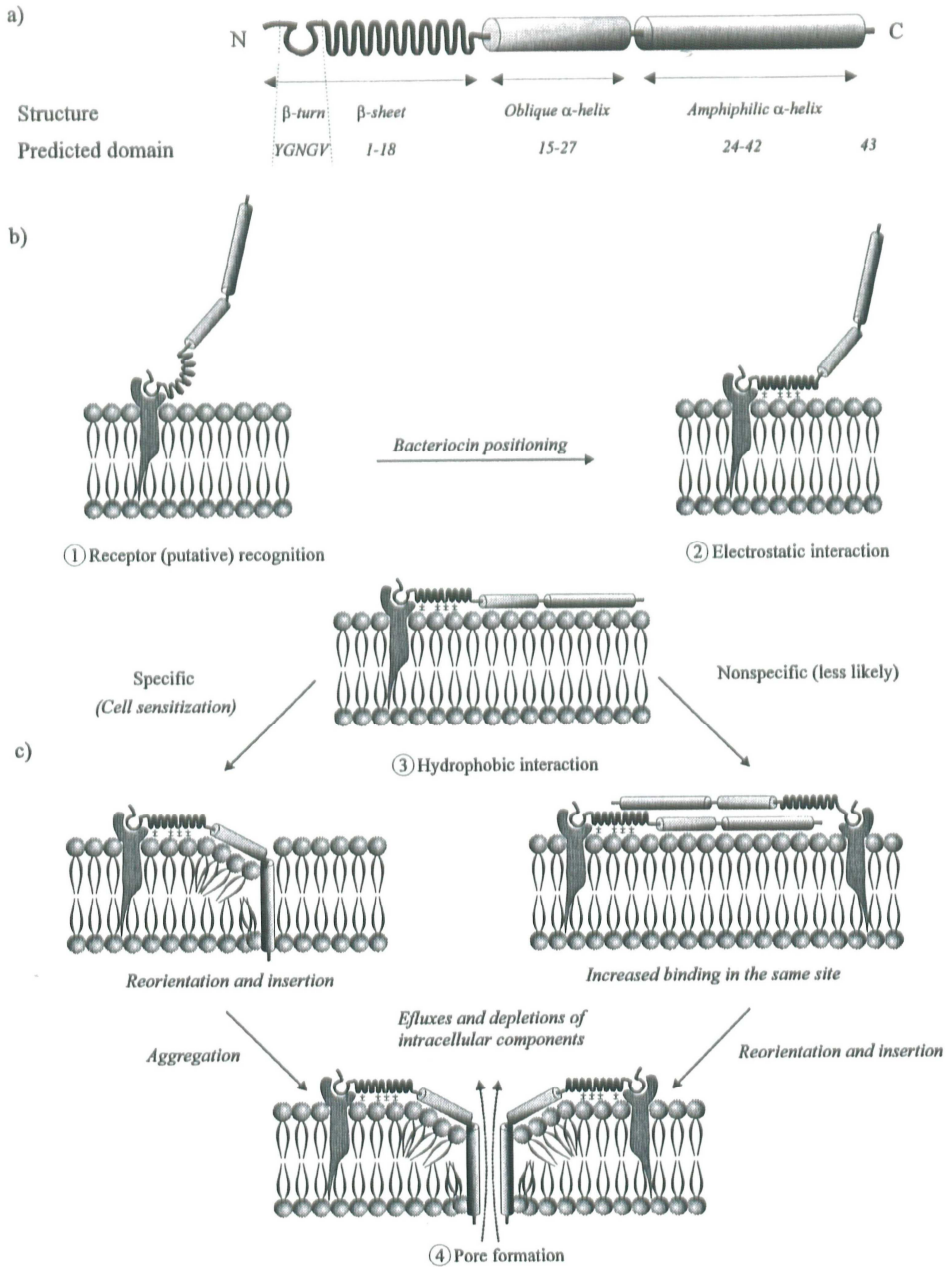


Figure 1.5. An illustration of a model class IIa bacteriocin and the predicted location of its domains with respect to interaction with the target cell membrane: (a) predicted bacteriocin structural domains; (b) possible interactions of each domain with the membrane surface; (c) bacteriocin insertion and formation of hydrophilic pores (Ennahar *et al.*, 2000).

Ramnath *et al.* (2000), were the initial group to describe the absence of a mannose PTS subunit in a *L. monocytogenes* strain conferring resistance to leucocin A. The protein described by this group was similar to the $IIAB^{Man}$ subunit of the EII_t^{Man} hence inferring, a similar if not an identical mechanism for resistance to both mesentericin Y105 and leucocin A (Ramnath *et al.*, 2001). Subsequently another study pointed out that leucocin A required an interaction with a chiral receptor at the surface of the target cell to be activated, as the D-enantiomer of leucocin A was inactive against 10 different strains who were all sensitive to the natural leucocin A (Yan *et al.*, 2000). Upon further investigation, the inactivation of the σ^{54}-dependent *mptACD* operon induced resistance of *L. monocytogenes* and *E. faecalis* to mesentericin Y105 (Dalet *et al.*, 2001; Hechard *et al.*, 2001). Furthermore, increasing the glucose or mannose concentration induced simultaneous expression of EII_t^{Man}, and sensitivity to mesentericin Y105 in *L. monocytogenes*, suggesting that the expression level of EII_t^{Man} and sensitivity to the bacteriocins were found to be associated (Dalet *et al.*, 2001). A deletion of the distal region of the *mptD* gene conferred leucocin A resistance in *L. monocytogenes* (Dalet *et al.*, 2001). Therefore, implicating the IID^{Man} subunit as an interactive molecule with class IIa bacteriocins (Dalet *et al.*, 2001; Hechard *et al.*, 2001). The *mptACD* operon of *E. faecalis* is 89% similar to *L. monocytogenes* (Hechard *et al.*, 2001). Another study showed a deletion in the proximal region of the *mptB* gene also led to bacteriocin resistance in *E. faecalis*. This study noted that the *mpt*ACD operon was not expressed due to a polar affect in gene transcription, therefore the indication that the $IIAB^{Man}$ subunit is involved in bacteriocin interaction, seems be incorrect. Subsequently, this was verified when a deletion of the *mptA* gene was shown to repress the expression of the *mptACD* operon, therefore suggesting that the $IIC^{Man} – IID^{Man}$ complex could function as a membrane bound target molecule for class IIa bacteriocins (Graversen *et al.*, 2002). However, studies performed on resistant *L. monocytogenes* strains show that resistant strains show alterations in their cell surface. These strains display a more positively charged cell wall, a cell membrane with a higher neutral charge and a more fluid cell membrane (Vadyvaloo *et al.*, 2004). Ramanth *et al.* (2004) investigated the role of the *mptACD* operon and derived plasmids containing various combinations of these three genes of the *mptACD* operon, namely pNM-ACD (utilized in this study), pNM-AC, pNM-AD, pNM-CD, pNM-C and pNM-D. These

constructs under the tight control of the nisin A promoter were transformed into a *Lc. lactis* strain deficient of the EII_t^{Man} protein. These constructs were induced using nisin A and exposed to class IIa bacteriocin leucocin A. The study showed that the phenotypic expression of IIC^{Man} rendered *Lc. lactis* sensitive to class IIa bacteriocin leucocin A (Ramnath *et al.*, 2004).

The results from studies reviewed indicate that the EII_t^{Man} of *L. monocytogenes* could be the chiral receptor (Figure 1.5) needed for bacteriocin interaction at the surface of targeted cells and the membrane associated IID^{Man} and IIC^{Man} subunits were implicated to be responsible for direct interaction with class IIa bacteriocins (Ramnath *et al.*, 2004).

Whilst the study by Vadyveloo *et al*, (2004) indicated the characteristics of bacterial cell surface alteration in class IIa resistant *L. monocytogenes* strains. The study concluded that strains with a high resistance to class IIa bacteriocins display the following characteristics:

(a) The cell wall had a more positive charge
(b) The cell membrane had a more neutral charge
(c) The cell membrane was more fluid
(d) There appeared to be a significant decrease in *mptA* expression

Therefore, the study, gave a strong indication that at least four cell surface changes contribute to class IIa bacteriocin resistance, and that each of these changes contribute to the degree of bacteriocin resistance (Vadyvaloo *et al.*, 2004). However, this study failed to acknowledge studies proving that the deletion in the *mptA* gene in *L. monocytogenes* resistant strains was due to the repression of the complete *mpt*ACD operon in these strains therefore the IIA^{Man} subunit may not be responsible for bacteriocin interaction.

1.5. The Mannose PTS Permease System

It is known that microorganisms are able to utilize many carbon sources to ensure their survival. They contain cell surface receptors which are able to monitor the environmental changes they may face. Thereby acquiring the ability to turn on or switch off the utilization

of a large number of carbon sources, sense concentration gradients of nutrients, and adapt to changes in osmotic strength as well as sense stress conditions such as availability of oxygen and limited nutrients. Many signals are sensed on the outside of the cell and are converted into a response inside the cell. This response may involve changes in protein synthesis and regulate enzyme activity, changes in behaviour (e.g. motility) or other processes. Many such sensory systems have been identified, with one common theme connecting most of these systems. Such as, the phosphorylation of the proteins involved in histidine, serine and aspartate residues (Postma *et al.*, 1993). One such system is the phosphoenolpyruvate (PEP) carbohydrate phosphotransferase system (PTS). The EII_t^{Man} is vital to most microorganisms (Postma *et al.*, 1993).This system is involved in both the transport and phosphorylation of a large number of carbohydrates in movement toward these carbon sources and the regulation of a number of other metabolic pathways (Postma *et al.*, 1993).

1.5.1. The role and organization of the PTS

The phosphoenolpyruvate-dependent carbohydrate transport system is responsible for the transport or uptake of a variety of carbohydrates such as hexoses and hexitols, from the periplasmic to the cytoplasmic space via facilitated diffusion, coupled with their phosphorylation. In addition, their function in phosphorylation of these very same carbohydrates takes place on the cytoplasmic side of the membrane (Robilllard & Broos, 1999). Therefore the PTS proteins accomplish both the translocation and phosphorylation of its substrates. The phosphoryl transfer potential of PEP is higher than that of ATP, enabling more efficient carbohydrate metabolism (Postma *et al.*, 1993). However, in non-PTS systems the accumulated carbohydrates of more than one ATP equivalent must be expended per monosaccharide unit for both transport and ATP-dependent phosphorylation to occur. This is the main reason the PTS system is found in obligate and facultative anaerobic bacteria, which synthesize ATP by substrate level phosphorylation (Postma *et al.*, 1993). The PTS proteins of the enteric bacteria *E. coli* and *Salmonella typhymurium*

have been intensively studied, whilst they have been partially characterized in a wide variety of other bacterial species (Postma *et al.*, 1993).

The following reaction constitutes the PTS-mediated translocation and phosphorylation of a given carbohydrate source:

Scheme I

P-enolpyruvate + (EI) $\rightleftharpoons$ P-EI + Pyruvate (1)

P-EI + HPr $\rightleftharpoons$ P-HPr + EI (2)

P-HPr + EIIA (domain or protein) $\rightleftharpoons$ P-EIIA + HPr (3)

P-EIIA + EIIB (domain or protein) $\rightleftharpoons$ P-EIIB + EIIA (4)

P-EIIB + carbohydrate$_{(out)}$ $\xrightarrow{\text{EIIC}}$ EIIB + carbohydrate-P$_{(in)}$ (5)

[P= phosphate; E1= enzyme I; HPr = Histidine protein (phosphate carrier protein)]

In most cases enzyme I (EI) and HPr phosphate carrier protein are soluble cytoplasmic proteins, which take part in the phosphorylation of all PTS carbohydrates in the organism. Therefore they have been referred to as the general PTS proteins, and the enzyme II´s (EII´s), are carbohydrate specific. These may consist of membrane bound proteins with subunit domains; a graphical description is shown in Figure 1.6.

Figure 1.6 shows the EI and HPr general proteins for all PTS´s, and the EII proteins, specific for mannitol (Mtl), glucose (Glc), and mannose (Man). Each of these EII proteins, contain two hydrophilic domains, IIA previously referred to as (EIII or III) consisting of the first phosphorylation site (P-His), and IIB consisting of second phosphorylation site (either a P-Cys or P-His residue). The membrane bound hydrophobic domains are split into two domains (IIC and IID). II^{Mtl}, II^{Glc}, and II^{Man} are specific for mannitol, glucose and

mannose, respectively. P~ indicates the phosphorylated forms of the hydrophilic polypeptide involved in the phosphorylation of the carbohydrate. The IIC^{Man} and IID^{Man} subunits are responsible for the translocation of a specific sugar from the outer cell membrane into the cytoplasm. The variation exemplified by the mannose PTS permease of *E. coli* show both the IIA and IIB domain fused into single hydrophilic proteins (Postma *et al.*, 1993; Robilllard & Broos, 1999).

The phosphotransfer reactions occur in a vectorial nature with respect to transport and phosphorylation for the mannitol, glucose and mannose phosphoenolpyruvate transferase systems of *E. coli*, shown in Figure 1.6 (Postma *et al.*, 1993; Robilllard & Broos, 1999). Commencing with P-enolpyruvate, the phosphate group is transferred via EI, HPr, IIA, IIB and it is speculated that the translocation of the carbon source occurs through the EIIC domain. This domain forms the integral portion of the translocation region and part of the substrate binding site of the IIC protein. Mannitol (EII^{Mtl}) and glucose (EII^{Glc}) have three domains (see Figure 1.6). In both cases the IIB and IIC domains are membrane bound and the IIA domain is soluble in the cytoplasm. The quaternary protein structure of the substrate-specific PTS of *E. coli* are found in almost all microorganisms.

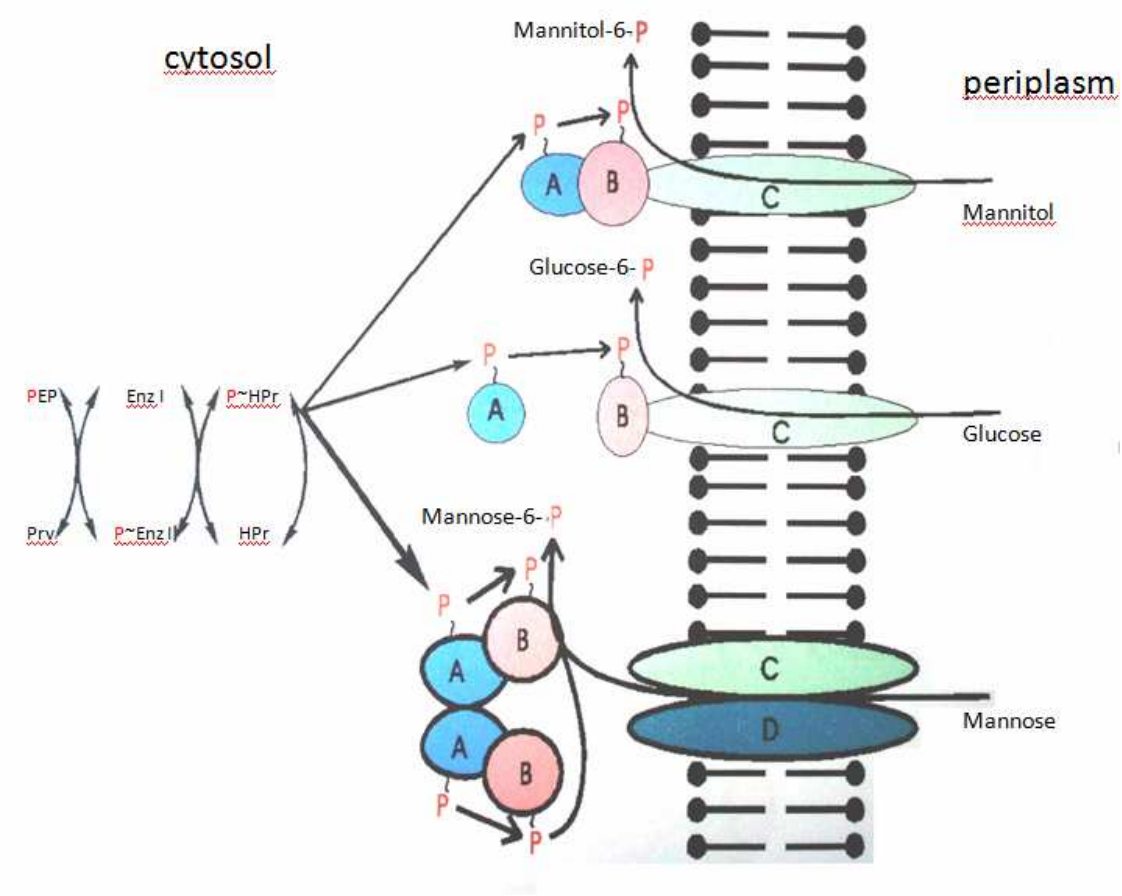


Figure 1.6. The organisation of the *E. coli* phosphotransferase system EII's, featuring the general HPr and Enzyme I (Enz I). Only three of the EII´s are shown, those specific to mannitol (Mtl), glucose (Glc) and mannose (Man). Each contains two hydrophilic domains, IIA containing the first phosphorylation site (P~His) and IIB containing the second P~Cys or P~His residue. The membrane bound hydrophobic domains include, one IIC domain in the case for mannitol and glucose and two in the case of mannose IIC and IID. The translocation of phosphate begins by transfer of the phosphate group from phosphoenolpyruvate (PEP) to EI, leaving behind pyruvate. This phosphate group is then transferred via HPr through to EII proteins (Postma, 1993; Robilllard & Broos, 1999).

1.5.2. Structure and function of the mannose PTS permease

Since the responsibility of EII_t^{Man} is that of vectorial translocation and phosphorylation of the transported solute, specifically mannose. The structure of the EII_t^{Man} protein is shown in Figure 1.7. It comprises of three subunits; the $IIAB^{Man}$ are fused together by two linkers with a molecular weight of 35 kDa. The IIC^{Man} and IID^{Man} units span the membrane several times and have the respective molecular weights of 28 kDa and 31 kDa and contain the substrate-binding site (Postma *et al.*, 1993; Huber & Erni, 1996).

The IIA^{Man} and IIB^{Man} subunits are hydrophilic. They sequentially transfer phosphoryl groups from the high-energy phosphoryl carrier protein phospho-HPr (the phosphorylated histidine-containing phospho carrier protein) to the substrate on the IIC^{Man} subunit. Phospho-HPr is regenerated by pyruvate in a reaction catalyzed by enzyme I. The phosphoproteins that have additional functions in chemotaxis and metabolic regulation (Huber & Erni, 1996). Although the mechanistic interactions between subunits are not clear, the interactions between domains play a major role in the mechanism of carbohydrate translocation by the membrane bound domains of the EII_t^{Man}. To highlight the operation of this phosphorylation cascade, Figure 1.7 show how the phosphate group is first transferred from HPr to His10 of IIA^{Man} domain and thereafter to His175 of IIB^{Man}, His86 and His 219 are also involved. IIB^{Man} interacts with the two membrane-spanning domains IIC^{Man} and IID^{Man} for the phosphoylation of the transported mannose molecule (Postma, 1993; Robilllard & Broos, 1999).

The membrane topology of the membrane bound subunits IIC^{Man} and IID^{Man} are clearly illustrated in Figure 1.8. Furthermore, this illustration, conveys an idea as to where the N and C termini regions reside. This information helped formulate cloning experiment strategies of these proteins

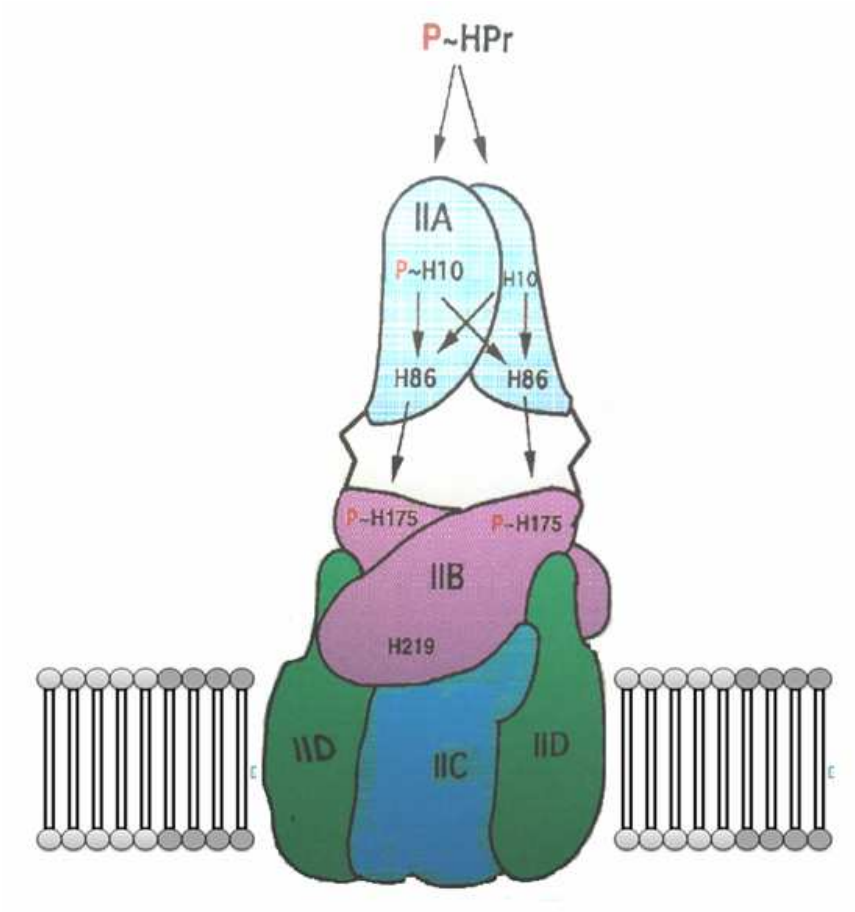


Figure 1.7. A graphical representation of the four domains of the mannose PTS permease of *E. coli*. The soluble $IIAB^{Man}$ consisting of a homodimer of two IIA^{Man} domains covalently linked to a IIB^{Man} domain. IIB^{Man} interacts with the two membrane spanning domains IIC^{Man} and IID^{Man} for the phosphorylated (Stolz *et al.*, 1993).

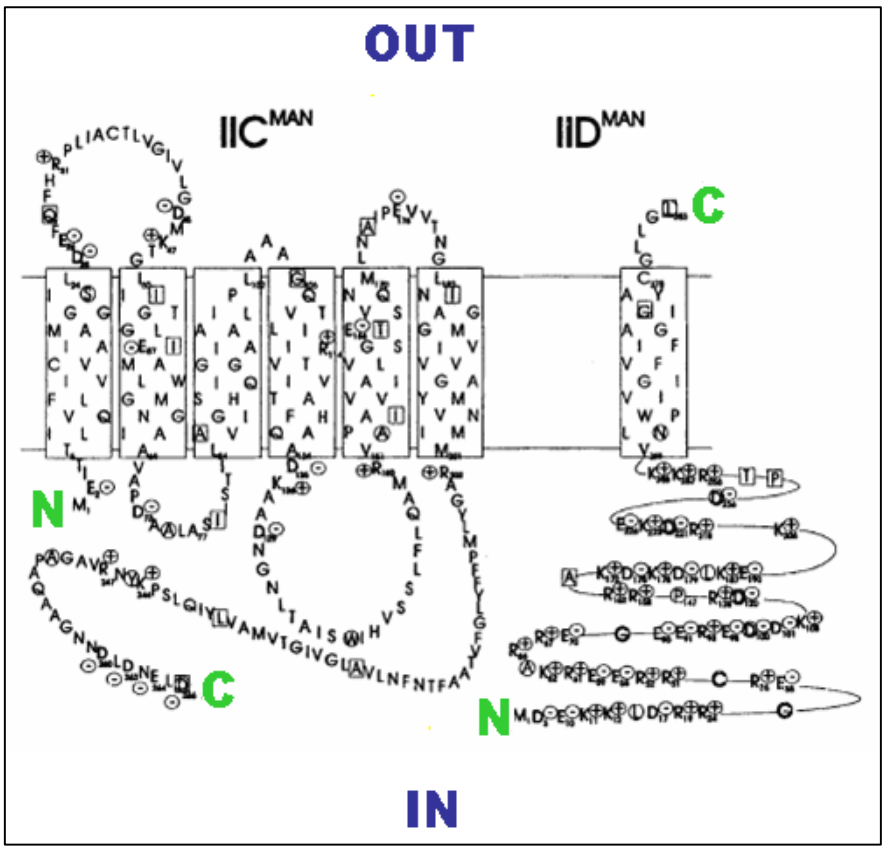


Figure 1.8. Transmembrane topology of the subunits of the EII_t^{Man}, IIC^{Man} and IID^{Man} of *E. coli.* Residues in membrane-spanning regions are arranged in α-helical arrays that are boxed. Charged residues are also indicated. Note the position of the N- and C- termini. The words "IN" refers to the cytosolic area within the cell and "OUT" referring to the periplasmic region. (Huber & Erni, 1996).

1.6. The Objectives of the Study

Various studies reviewed indicate that the EII_t^{Man} could be the chiral receptor for class IIa bacteriocin interaction at the surface of target cells and the membrane associated IID^{Man} and IIC^{Man} subunits are implicated in direct interaction with class IIa bacteriocins. The objective of the current study was to determine which subunit acts as a receptor molecule for class IIa bacteriocins. To this end we aim to amplify, clone, express and purify the individual subunits of the mannose PTS permease of *L. monocytogenes* EGD. The purpose of the study was to facilitate downstream Biacore molecular interaction studies with class IIa bacteriocins.

CHAPTER 2

Materials and Methods

Materials

2.1. Strains, And Plasmids

The bacterial strains, and plasmids, used in this study have been listed in Table 2.1.

Table 2.1. Strains and Plasmids used in this study

Strains or Plasmids	Characteristics	Reference/ Source
Leuconostoc gelidum UAL 187-22	*Lcn*$^{+}$, *Imm*$^{+}$, containing native plasmids pLG9.2 and pLG7.6	(Hastings *et al.*, 1991)
Leuconostoc gelidum UAL 187-13	*Lcn*$^{-}$, *Imm*$^{-}$ containing plasmid pLG9.2	(Hastings *et al.*, 1991)
Leuconostoc gelidum UAL 187-23	-	(Hastings *et al.*, 1991)
Leuconostoc gelidum Ta33a	*LeuB*	(Hastings *et al.*, 1991)
Listeria monocytogenes EGD	-	Accession: AL591824 (Hastings *et al.*, 1991)
Lactococcus lactis pNM -ACD	Plasmid pNM–ACD [insert *mptACD* cloned into pNZ-8020, *Cm*^{R}, *NisA*] and helper plasmid pNZ-9530 (*Em*^{R} , *NisPK)*	(Ramnath *et al.*, 2004)
Lactococcus lactis MG-CON	Plasmid MG-CON [pNZ-8020, (*Cm*^{R}, *NisA* & no insert] and helper plasmid pNZ-9530, (*Em*^{R}, *NisPK*)	(de Ruyter *et al.*, 1996; Ramnath *et al.*, 2004)
Escherichia coli JM109(DE3)	Plasmid pET28a (*Kan*^{R}*)*	Dr Alain Boulange
Escherichia coli JM109(DE3)	-	Promega, USA
Escherichia coli BL21(DE3) pLysS (competent cells)	-	Merck, South Africa

2.2. Media and Culture Conditions

All *E. coli* strains were cultured in Luria Bertani (LB) Broth and Luria Bertani (LB) agar as indicated in Table 2.2. However, *E. coli* JM109(DE3) cells were grown on minimal medium M9 including vitamin B1 (see Table 2.3.).

Table 2.2. Preparation of Luria Bertani (LB) Broth and LB Agar

Reagents	**Volume = 1 l**
Tryptone Powder (BDH Chemicals, Germany)	10 g
Yeast Extract Powder (Oxoid, Sigma-Aldrich, USA)	5 g
Sodium Chloride (BDH Chemicals, Germany)	10 g
To the above ingredients, 900 ml RO H_2O was added, the pH was adjusted to 7.5 with 1 M sodium hydroxide (NaOH),and the volume was adjusted to 1 l with RO H_2O.	
The media was dispensed into aliquots according to culturing requirements and autoclaved at 121°C for 15 minutes.	

Table 2.3. Preparation of Minimal Media M9 including vitamin B1

Reagents	**Volume = 200 ml**
$Na_2H\ PO_4$	1.2 g
KH_2PO_4	0.6 g
NaCl	0.1 g
NH_4Cl	0.2 g
Agar	3.0 g

To the above ingredients, approximately 200 ml of RO H_2O was added and the pH adjusted to 7.4 with 10 M NaOH. The mixture was autoclaved at 121°C for 15 minutes and cooled to 50ºC.

Thereafter, the following sterile solutions were added:

$MgSO_4$ [1 M]	400 µl
$CaCl_2$ [1 M]	20 µl
Glucose [20 % (w/v)] (filter sterilized)	2000 µl
Thiamine-KCl [1 M] (filter sterilized)	200 µl

Approximately 20 ml of medium was poured into 85 mm sterile Petri dishes, left to set at room temperature (RT) and stored at 4ºC.

Methods

2.3. Plasmid DNA Isolation of pNM-ACD and pNZ-8020 Plasmid

The isolation of the pNM-ACD and pNZ-8020 plasmid DNA from *Lc. lactis* a Gram positive organism, was performed using an alkaline lysis protocol as previously described (Holt *et al.*, 2001). MRS broth (10 ml) supplemented with chloramphenicol (10 μg ml^{-1}) and erythromycin (5 μg ml^{-1}), was used to prepare an overnight culture of *Lc. lactis* pNM-ACD, whilst non-supplemented MRS broth was used to prepare an overnight culture of *Lc. lactis* pNZ-8020 by single colony inoculation, respectively. The cultures were incubated overnight at 30ºC with vigorous shaking at 200 rpm. Table 2.4 highlights all the buffers that were utilized.

Table 2.4 Showing constituents of buffers used in plasmid isolation from Gram positive and Gram negative organisms (Sambrook & Russell, 2000; Holt *et al.*, 2001).

Name of Buffer	Constituents
GTE Solution	50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0
Lysozyme Solution*	50 mg ml^{-1} lysozyme dissolved in 25 mM Tris-HCl, pH 8.0. The solution was filter sterilized and stored at -20ºC
Lysis Solution	0.2 M NaOH dissolved in 1% SDS solution
Potassium Acetate Buffer	5 M potassium acetate, the pH was adjusted with glacial acetic acid and the solution stored at 4ºC.
TE Buffer	10 mM Tris-Cl, 10 mM EDTA, pH 7.5 and autoclaved at 121ºC for 15 minutes
Phenol : Chloroform Solution (50:50)*	15 ml phenol pH 7.4 mixed into 15 ml chloroform
Chloroform: Isoamyl Alcohol (24:1)*	24 ml chloroform mixed into 1 ml isoamyl alcohol

***These solutions were not used in the plasmid mini-prep from Gram negative organisms.

A 0.7 ml volume of the overnight cultures were removed and added to 0.3 ml 80% (v/v) glycerol for storage in 2 ml cryo-storage vials at -70ºC. The remaining cells were harvested by centrifugation at (6000 × *g*, 10 minutes, 4ºC) in a JA-20 rotor using a Beckman J2-21M centrifuge. The pellet was re-suspended in 100 µl GTE solution. Thereafter 40 µl lysozyme solution was added and the mixture was incubated at room temperature for 60 minutes. Subsequently, 200 µl lysis solution and 10 µl RNAse A (10 mg ml^{-1}) was added and the solution was mixed three times by inversion and incubated on ice for 5 minutes. The addition of lysozyme, lysis solution and RNAse A ensured complete cell lysis and degradation of RNA molecules. *Lc. lactis* are Gram-positive organisms having a thicker peptidoglycan cell wall structure, therefore this organism requires a stringent lysis procedure (Holt *et al.*, 2001). Chromosomal DNA and cellular debris were precipitated by addition of 150 µl cold potassium acetate buffer, pH 4.8. The mixture was mixed by inverting tubes for 10 seconds, incubated on ice for 5 minutes and centrifuged (15000 × *g*, 5 minutes, 4ºC) in a JA16 rotor in a Beckman J2-21M centrifuge. The supernatant was transferred to a new microfuge tube. In order to extract the plasmid DNA, an equal volume of phenol:chloroform (50:50) was added and the mixture centrifuged (13000 × *g*, 5 minutes, RT) in a microfuge. The upper phase was removed to a fresh tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged as described in the previous step and once again the upper phase extracted. Two volumes of 98.5% ethanol were added to precipitate the plasmid DNA. The precipitate was collected by centrifuging the mixture (13000 × *g*, 10 minutes, RT). The supernatant was discarded and the pellet washed with 500 µl 70% (v/v) ethanol to remove salt, and centrifuged (13000 × *g*, 10 minutes, RT) in a microfuge. The supernatant was discarded and the pellet was drained and left to dry in the laminar flow. Thereafter, the pellet was reconstituted in 20 µl TE buffer, pH 8.0. A 5 µl sample aliquot was used to verify the presence of plasmid DNA isolation by gel electrophoresis; the remaining 15 µl sample volume was stored at -20ºC.

2.4. Plasmid DNA Isolation of Cloning Vector pET28a

The expression vector pET28a is a mid-copy number plasmid, was purified from *E. coli* JM109(DE3) pET28a (Novagen, 2003). Mini-prep plasmid DNA isolations from *E. coli* JM109(DE3) pET28a, were performed using an alkaline lysis method and Table 2.4 highlights the constituents of the buffers utilized (Sambrook & Russell, 2000). However note that the lysozyme solution was not used for plasmid mini prep from Gram negative organisms. The method was performed as follows: LB medium (10 ml) containing kanamycin sulfate (30 μg ml^{-1}) was inoculated with a single colony of *E. coli* JM109(DE3) containing plasmid pET28a (Dr Alain Boulange, personal communication, 2005) and grown overnight at 37ºC with vigorous shaking. A 0.8 ml aliquot of the overnight culture was removed and added to 0.2 ml 80% (v/v) sterile glycerol for storage in 1.5 ml sterile cryo-storage vials.

The remaining cells were harvested by centrifugation at (12 000 × *g*, 10 minutes, 4ºC) in a JA-16 rotor using a Beckman J2-21M centrifuge. The pellet was re-suspended in 100 μl GTE solution, and left at room temperature. Thereafter 200 μl lysis solution and 2 μl RNAse A (10 mg ml^{-1}) was added to lyse cells and eliminate RNA molecules, respectively. The solution was mixed three times by inversion and incubated on ice for 5 minutes. Chromosomal DNA and cellular debris were precipitated by addition of 300 μl cold potassium acetate buffer, pH 4.8, The mixture was mixed by vortexing for 10 seconds, incubated on ice for 5 minutes and centrifuged (15000 × *g*, 5 minutes, 4ºC) in a JA16 rotor in a Beckman J2-21M centrifuge. The supernatant (800 μl) was transferred to a sterile microfuge tube. In order to extract the plasmid DNA, 600 μl isopropanol stored at 4°C was added to the supernatant and incubated at -20ºC for 30 minutes. The precipitated plasmid DNA formed a pellet by centrifugation (13000 × *g*, 5 minutes, RT). The supernatant was discarded and the pellet washed twice with 500 μl 70% (v/v) ethanol to remove salt, and centrifuged (13000 × *g*, 10 minutes, RT). The supernatant was discarded and the pellet was drained and left to dry in the laminar flow. Thereafter the pellet was reconstituted in 50 μl

TE buffer. A 5 µl aliquot was used to verify plasmid isolation on gel electrophoresis. The remaining 45 µl was stored at -20ºC.

For larger scale plasmid DNA preparations, the Nucleobond AX 100 Kit (Machery-Nagel, Germany) was used, as per manufacturer's instructions. The procedure as follows: LB broth (100 ml) containing kanamycin sulfate (30 µg ml^{-1}) was inoculated with a 1% inoculum (1 ml) from an overnight culture and grown at 37ºC with shaking for a period no longer than 12 -16 hours. Cells were harvested at (6000 × *g*, 15 minutes, 4ºC) using a J2-21M Beckman centrifuge. The supernatant was carefully discarded and the cell pellet was re-suspended in 8 ml buffer S1 [50 mM Tris-HCl, 10 mM EDTA, 100 µg ml^{-1} RNAse A, pH 8.0]. An 8 ml volume of buffer S2 [200 mM NaOH, 1% SDS] was added to the cell suspension, mixed thoroughly by gently inverting the tube 6-8 times, and incubated at room temperature for 5 minutes. Subsequently 8 ml of buffer S3 [2.8 M potassium acetate, pH 5.1] was added and the suspension mixed once again by gentle inversion until a homogeneous suspension containing an off-white flocculent was formed. The suspension was incubated on ice for 5 minutes. The suspension was then mixed and filtered through the folded filters supplied in the kit. During the filtration period the Nucleobond AX 100 cartridge was equilibrated with buffer N2 (2.5 ml) [100 mM Tris, 15% (v/v) ethanol, 900 mM KCl, 0.15% Triton X-100, adjusted to pH 6.3 with H_3PO_4]. Cartridges were allowed to empty by gravity flow. The filtered lysate was carefully removed and loaded onto the pre-equilibrated Nucleobond AX 100 cartridge. The cartridges were subsequently washed with buffer N3 (2 × 10 ml) [100 mM Tris, 15% (v/v) ethanol, 1.5 M KCl, adjusted to pH 6.3 with KH_2PO_4] and the flow-through was discarded. Plasmid DNA was eluted using 5 ml buffer N5 [100 mM Tris, 15% ethanol, 1 M KCl, adjusted to pH 8.5 with KH_2PO_4], precipitated by addition 3.6 ml room temperature isopropanol, and centrifuged immediately (15000 × *g*, 30 minutes, 4ºC). The plasmid DNA pellet was subsequently washed with 70% (v/v) ethanol (2 ml at RT), and centrifuged (15000 × *g*, 30 minutes, 4ºC). The supernatant was carefully removed with a micropipette, and the plasmid DNA pellet was briefly air-dried at room temperature and re-suspended in 50 µl TE buffer pH 8.0.

The purified plasmid DNA was analysed in 0.8% (w/v) agarose gels and quantified by UV/VIS spectrophotometry.

2.5. Quantification of Plasmid DNA by Spectrophotometry

Purified DNA was diluted 1:100 in sterile Milli-Q H_2O. The optical density at A_{280} and A_{260} were determined. DNA purity was indicated as a ratio between the readings at 260 nm and 280 nm. Pure DNA preparations of are expected as ratios from about 1.8. The DNA concentration (μg ml^{-1}) was calculated as follows:

Equation 2: $A_{260} \times$ dilution factor $\times 50 =$ **X** μg ml^{-1}

2.6. Analysis of Purified DNA by Agarose Gel Electrophoresis

Purified plasmid DNA was electrophoresed in a 0.8% (w/v), 1% (w/v) or 1.5% (w/v) horizontal agarose gels. Gels were prepared with electrophoresis grade agarose (Whitehead Scientific, South Africa) in 1 × TAE buffer [4.846 g Tris, 0.48 g anhydrous sodium acetate, 0.372 g Na_2EDTA_2, pH to 7.8 with glacial acetic acid and the volume adjusted to 1 l with RO H_2O] containing 0.5 μg ml^{-1} ethidium bromide (Promega, USA). Purified DNA samples were prepared by addition of 5 μl plasmid DNA, 5 μl RO H_2O and 2 μl loading buffer (Sigma-Aldrich, USA). Electrophoresis was performed at 8 V cm^{-1} (90 minutes). DNA bands present on gels were visualized and documented using a Versadoc Imager (Biorad, Germany).

2.7. Analysis of pNM-ACD Sequence Data and Primer Design

The strains of *Lc. lactis* containing plasmid constructs of the genes encoding the *mptACD* operon were obtained from a previous study Figure 2.1. (Ramanth *et al.* 2004). These constructs were previously sequenced, and the sequence data was used to formulate the primers utilized in this study. Primers were designed to amplify the individual subunit genes of the *mptACD* operon and to facilitate their incorporation into pET28a expression vector (Figure 2.2).

The genes encoding the individual subunits of the mannose PTS permease were analysed in the following way. The sequence data obtained (personal correspondence with Dr Yann Hechard) was entered onto an internet gene sequence database Genebank, to confirm its identity to a specific strain of *L. monocytogenes* (Benson *et al*., 2007). A complete alignment of the individual subunit genes of the *mptACD* operon gene sequence was entered into Clustal X 1.8 and each subunit gene sequence was located on the pNM-ACD vector. Protein sequence alignments were performed using Bioedit 7.0. The vector map of pNM-ACD (Figure 2.1) shows the *mpt* operon (*mptACD)* cloned into the multiple cloning site of a pNM-8020 plasmid vector. This data was used to determine the expected PCR product sizes.

Restriction sites *Nde*I and *Xho*I were incorporated at the 5´ end of the forward and reverse primers respectively. The use of these sites was determined by performing a search for DNA sequence restriction sites within the *mptACD* gene sequence, using web-based restriction site search software program, Webcutter 2.0 (Heiman, 1997). The gene sequence was copied and pasted to the program. The restriction site database detected all restriction sites present within the gene sequence. Restriction endonuclease sequences recognized by restriction enzymes *Nde*I and *Xho*I were found to be suitable, as they were not present in the *mptACD* gene sequence and were located within the multiple cloning site of the plasmid expression vector, (Figure 2.2). A software program, Primer Express 3.0 (Applied Biosystems, Switzerland), was responsible for generating the final primer sets. The primer sets were synthesized at the Molecular Biology Centre at the University of Cape Town using a Beckman 1000M Oligo synthesizer. Thereafter primers were chemically phosphorylated at the 5´ end.

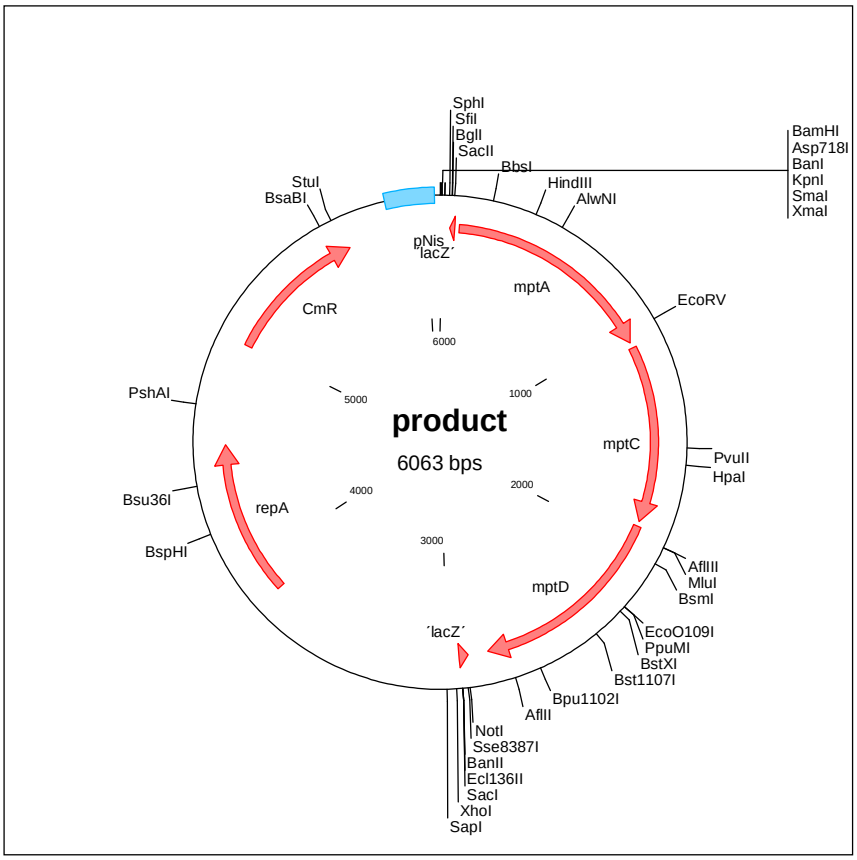


Figure 2.1. Plasmid vector map pNM-ACD, with the *mpt* operon cloned into the multiple cloning site of a pNZ-8020 food grade plasmid vector, transformed into *Lc. lactis* (Ramnath *et al.*, 2004).

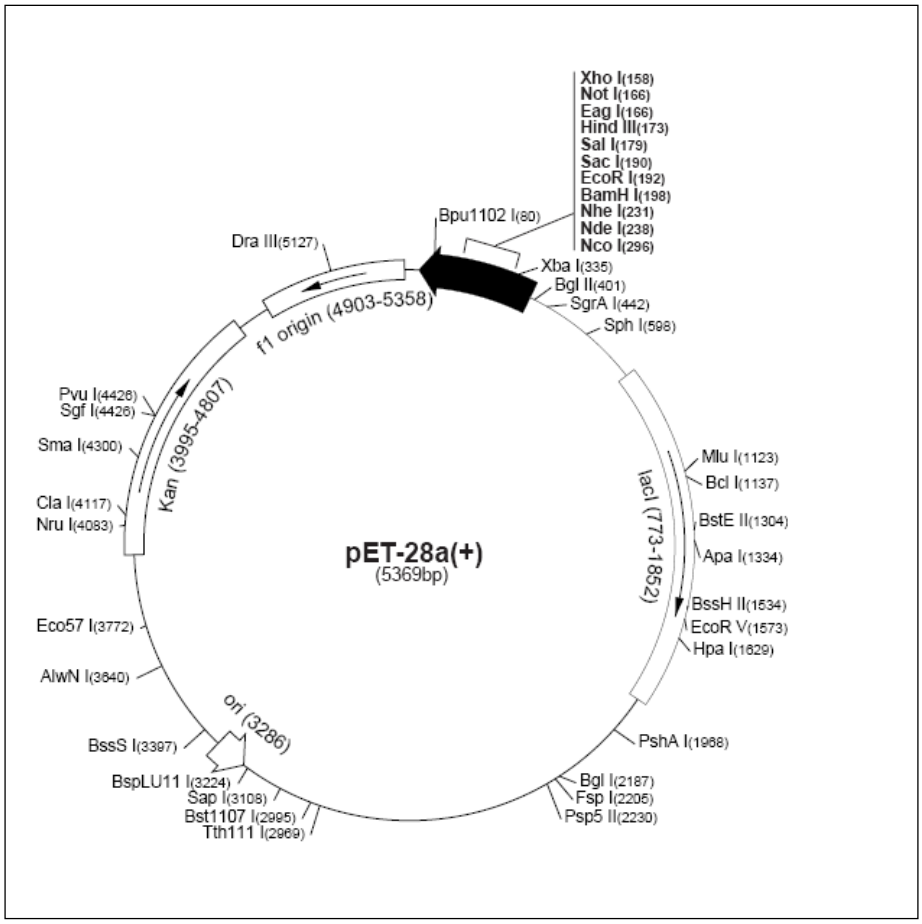


Figure 2.2. Map of the expression vector pET28a. This vector encodes an N-terminal His-tag/thrombin/T7-tag configuration (Novagen, 2003).

2.8. Amplification of *mptAB, mptC* and *mptD* Genes

Primers were designed for the amplification of each subunit gene of the mannose PTS permease *mptAB, mptC, mptD* as described in the Results (Chapter 3, Section 3.2). Primers were purchased from the Molecular Biology Unit (University of Cape Town, South Africa) with a known 5´ chemical phosphorylation, optical density (OD ml^{-1}), primer length and melting temperature. The primer solutions were received at an approximate 2 M concentration and a 5 µM working stock solution of each primer was prepared in TE buffer pH 8.0.

The preparation for each PCR reaction was performed as stipulated in Table 2.5. and the PCR of the *mptAB, mptC* and *mptD* genes were performed on a GeneAmp 9700 (Applied Biosystems, South Africa). PCR was performed in 100 µl volumes to ensure sufficient amount of PCR product was obtained per reaction. The following protocol, for primer pairs *mptAB* and *mptD* was performed: 94°C for 5 minutes; 30 cycles (94°C for 60 seconds; 58°C for 60 seconds; 72°C for 90 seconds); 72°C for 7 minutes; and then held at 4°C. Whilst for primer pair *mptC* the same cyclic procession as stipulated for *mptAB* and *mptD* was followed. However, the annealing temperature was changed from 58°C to 55°C. The reaction samples were analysed on a 1% (w/v) agarose gel at 9 V cm^{-1} (90 minutes).

Table 2.5. The preparation of PCR reactions for the synthesis of each subunit of the mannose PTS Permease

Reagents	**Required concentration per reaction**	**Test Reaction (Plasmid pNM-ACD) [µl]**	**Control (No Enzyme) [µl]**	**Control (No Template) [µl]**	**Control (No Insert: plasmid MG-CON) [µl]**
10 × PCR Buffer(with $MgCl_2$) (Roche)	1 ×	10	10	10	10
10 mM dNTP´s (Roche)	0.2 mM	2	2	2	2
Forward Primer [5 µM]	0.3 µM	6	6	6	6
Reverse Primer [5 µM]	0.3 µM	6	6	6	6
Sterile Milli-Q H_2O	-	66.6	67.6	74.6	66.6
dsDNA Template Plasmid pNM-ACD	50 ng	8	8	-	-
dsDNA Template Plasmid MG-CON	50 ng	-	8	-	8
Enzyme Taq Polymerase [5 U/ µl] (Roche)	0.05 U μl^{-1}	1	-	1	1
TOTAL REACTION VOLUME		100	100	100	100

2.9. Purification of PCR Products

In preparation of restriction enzyme digestion of PCR products the performance of the restriction enzyme is dependent on the purity of the DNA sample. High salt concentration may inhibit the activity of restriction enzymes (Roche-Applied-Science, 2002; Roche-Applied-Science, 2004). Hence, it was necessary to purify PCR products.

Purification of all PCR products was performed using the Nucleospin Extract II Kit (Machery–Nagel). To adjust DNA binding conditions one volume (100 µl) of PCR reaction was mixed with two volumes (200 µl) of buffer NT. A Nucleospin Extract II column was placed into a 2 ml collecting tube and the sample mixture was loaded, in order to allow DNA binding onto the column. The column and collecting tube was then centrifuged (11000 × *g*, 1 minute, RT). The flow through was discarded and the column placed back into the collecting tube. The silica membrane was subsequently washed by addition of 600 µl buffer NT3. The column and collecting tube was once again centrifuged (11000 × *g*, 1 minute, RT) and the flow through was discarded. Placing the column back into the collecting tube and centrifuging (11000 × *g*, 2 minutes, RT) allowed for removal of buffer NT3. The flow through was once again discarded making sure it did not come into contact with the spin column upon removal. Residual ethanol from buffer NT3 might inhibit subsequent reactions. Therefore in addition to centrifugation, total removal of ethanol was achieved by incubation of the column at 70ºC for 2-5 minutes. For elution of the DNA fragments, the spin column was placed in a sterile (RNAse/DNAse free) microfuge tube, 50 µl of pre-warmed (70ºC) elution buffer NE was added to the spin column and incubated at room temperature for 1 minute. Thereafter the column and microfuge tube was centrifuged (11000 × g, 1 minute, RT) for complete elution of PCR products.

2.10. Cloning of PCR Products *mptAB*, *mptC* and *mptD* into Plasmid pET28a Expression Vector

In order to express the individual subunits that make-up the mannose PTS permease (EII_t^{Man}), amplified gene sequences encoding the subunits of the EII_t^{Man} were cloned into expression vector pET28a at the *Nde*I and *Xho*I sites within the multiple cloning site (MCS) of the vector

2.10.1. Restriction enzyme digest of plasmid vector pET28a and PCR products

All restriction enzymes used in this study were purchased from Roche Applied Science, Germany. Restriction digestion using enzymes *Nde*I and *Xho*I were performed on expression plasmid vector pET28a and PCR products *mptAB*, *mptC* and *mptD*. The protocol used was described by Sambrook and Russell (2000).

*Nde*I and *Xho*I (Roche, Germany) are restriction endonucleases recognise the respective gene sequences CA/TATG and C/TCGAG and they both have 100% efficiency in the presence of SuRE/Cut buffer H [0.5 M Tris-HCl, 1 M NaCl, 100 mM $MgCl_2$, 10 mM dithioerythritol, pH 7.5] (Roche, Germany). Restriction digest reactions were set up as described in Table 2.6, with three pET28a controls. Each reaction was carried out at 37ºC with reactions 1-3 incubated for a period of 3 hours and reactions 4-7 incubated for a period of 5 hours. All reactions were stopped by addition of 2 µl electrophoresis grade DNA sample loading buffer [0.25% bromophenol blue; 0.25% xylene cyanol; added to a 30% glycerol solution] and immediately loaded onto a 1% (w/v) agarose gel and electrophoresed (9 V cm^{-1}). A Versadoc Imaging system the gel was first documented and digested DNA was gel purified.

Table 2.6. Restriction endonuclease digest reactions**.** Sample preparations of the amplified genes *mptAB*, *mptC*, *mptD*, plasmid vector pET28a and control reactions for restriction endonuclease digests with *Nde*I and *Xho*I were performed as stipulated.

Reagents	**Reactions**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	***mptAB* (*Nde*I /*Xho*I) [µl]**	***mptC* (*Nde*I /*Xho*I) [µl]**	***mptD* (*Nde*I /*Xho*I) [µl]**	**pET28a (*Nde*I/ *Xho*I) [µl]**	**Control pET28a uncut [µl]**	**Control pET28a (*Nde*I) [µl]**	**Control pET28a (*Xho*I) [µl]**
Intact DNA	15	15	15	15	15	15	15
10 × Sure/Cut Buffer H (Roche)	2	2	2	2	2	2	2
Enzyme *Nde*I (10 U μl^{-1}) (Roche)	1	1	1	1	-	1	1
Enzyme *Xho*I (10 U μl^{-1}) (Roche)	1	1	1	1	-	1	1
Sterile Milli-Q H_2O	1	1	1	1	3	1	1
Total Volume	20	20	20	20	20	20	20

2.10.2. Gel extraction of restricted plasmid DNA and PCR product´s from a 1% (w/v) agarose gel

DNA extraction from an agarose gel matrix was carried out using a Nucleospin Extract II kit (Machery-Nagel, Germany).The 1% (w/v) agarose gel used to analyse the restriction digest was documented and thereafter a hand held long wave UV lamp was utilized to illuminate DNA. The DNA bands of interest were carefully excised using a clean scalpel, the weight of the gel slice was determined and the slice transferred into a clean tube. For each 100 mg of agarose gel 200 µl buffer NT and the sample was incubated at 50ºC for a total time of 5-10 minutes until the gel slices were dissolved. However, to aid the dissolution of the gel slice the sample was vortexed every 2-3 minutes until the sample was completely dissolved. A Nucleospin Extract II column was placed into a 2 ml collecting tube and the sample mixture was loaded, in order to allow DNA binding onto the column. The column and collecting tube were then centrifuged (11000 × *g*, 1 minute, RT). The flow-through was discarded and the column placed back into the collecting tube. The silica membrane was subsequently washed by addition of 600 µl buffer NT3. The column and collecting tube was once again centrifuged (11000 × *g*, 1 minute, RT) and the flow-through was discarded. Placing the column back into the collecting tube and centrifuged at (11000 × *g*, 2 minutes, RT), this allowed for the quantitative removal of buffer NT3. The flow-through was once again discarded making sure it did not come into contact with the spin column upon removal. Residual ethanol from buffer NT3 might inhibit subsequent reactions. Therefore in addition to centrifugation total removal of ethanol was achieved by incubation of the column at 70ºC for 2-5 minutes. For elution of the DNA fragments the spin column was placed in a sterile (RNAse/DNAse free) microfuge tube, 50 µl of pre-warmed (70ºC) elution buffer NE was added to the spin column and incubated at room temperature for 1 minute. Thereafter the column and microfuge tube was centrifuged (11000 × *g*, 1 minute, RT) for complete elution of the DNA fragments.

2.10.3. Ligation of PCR products into pET28a

Following restriction digest, PCR products *mptAB*, *mptC*, and *mptD* were ligated into pET28a expression vector (restricted with *Nde*I *and Xho*I) as stipulated in Table 2.7. Thereafter, $RbCl_2$ competent *E. coli* JM109(DE3) cells were transformed (see section 2.11) with the ligated pET28a.

Table 2.7. Preparation of samples for ligation of the genes *mptAB*, *mptC*, and *mptD* into expression vector pET28a

Reagents	**mptAB [µl]**	**mptC [µl]**	**mptD [µl]**	**pET28a (*Nde*I) [µl]**	**pET28a uncut [µl]**
Insert	10	10	10	-	-
Vector pET28a **(*Nde*I/*Xho*I)**	2	2	2	2	2
Sterile RO H_2O	4	4	4	14	18
The above reagents were added to sterile microfuge tubes. Controls: pET28a (*Nde*I) with no insert DNA; and unrestricted pET28a were also prepared. The reaction was heated at 65ºC for 5 minutes, and cooled on ice. The following buffers and enzymes were added.					
10 × ligation Buffer	2	2	2	2	-
T_4 DNA ligase (1×10^3 U ml^{-1})	2	2	2	2	-
Total Volume	20	20	20	20	20
Each reaction was subjected to an incubate at 16 ºC overnight and the reaction was stopped by heating at 65ºC for 5 min.					

2.11. *E. coli* JM109(DE3) competent cell preparation using rubidium chloride ($RbCl_2$)

For most strains of *E. coli* $RbCl_2$ competent cells are known to give better transformation efficiencies than $CaCl_2$ procedure (Hanahan, 1985). Competent cells prepared by the $RbCl_2$ method, remain stable for 1 year when stored at -70°C. The following procedure is a modification of a previous procedure (Hanahan, 1985). Table 2.8 illustrates the constituents and preparation of buffers used in this protocol.

Table 2.8. Preparation of reagents used in $RbCl_2$ Competent Cell Preparation of *E. coli* JM109(DE3) (Hanahan, 1985)

Name of Buffer	Constituents
TFB1 buffer*	30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM $RbCl_2$, and 15% (w/v) glycerol. Adjusted to pH 5.8 with 1 M acetic acid. The solution was filter sterilized (0.22 μm) and stored at 4ºC
TFB2 buffer	10 mM Mops, 75 mM $CaCl_2$, 10 mM $RbCl_2$, and 15% (w/v) glycerol. The solution was adjusted to pH 6.5 with 1 M KOH. The solution was filter sterilized (0.22 μm) and stored at 4ºC

*Caution when adjusting the pH of TFB1 buffer, make sure the pH does not drop lower than 5.8, because a black precipitate would form.

A single bacterial colony of *E. coli* JM109(DE3) initially grown on minimal medium M9/ B1 (Table 2.3) was inoculated into 25 ml LB medium and incubated at 37ºC and shaking at 225 rpm overnight. The overnight culture was sub-cultured at a 1:100 dilution by inoculating 2.5 ml into 250 ml of LB media supplemented with 20 mM $MgSO_4$. Cells were grown in a 1 l flask until an OD_{600} of 0.4-0.6 was reached. Cells were harvested in 250 ml aliquots by centrifugation (4500 × *g*, 5 minutes, 4ºC) and the resultant pellet from the 250 ml culture was gently re-suspended in 0.4 × original volume (100 ml) ice-cold TFB1

buffer. The re-suspended pellets were combined into one bottle. For the remaining steps, the cells were maintained on ice, and all pipettes, pipette tips, flasks and tubes, were kept chilled (either at -20 ºC or on ice). The re-suspended cells were further incubated on ice for 5 minutes. Cells were subsequently harvested by centrifuging (4500 × *g*, 4 ºC, 5 minutes). The pellet was re-suspended in 0.4 × previous volume (40 ml) ice-cold TFB2 buffer. Lastly the cells were incubated on ice for 15 minutes, then dispensed into pre-chilled (-20ºC) cryo-storage-vials at 100 µl aliquots, on ice. A dry ice/isopropanol bath was prepared by chilling the isopropanol overnight at -20ºC, and placing the dry ice into the isopropanol. To quick freeze cells, each of the cryo-storage vials containing the competent *E. coli* JM109(DE3) cells was dipped into the bath for 15 seconds and then stored at -70ºC.

2.12. Transformation of *E. coli* JM109(DE3)

Transformation efficiency of competent $RbCl_2$ *E. coli* JM109(DE3) was determined using purified pET28a plasmid DNA. An aliquot of 100 µl competent cells was thawed on ice. Plasmid DNA pET28a (0.1 ng) was added to 10 µl competent cells in a sterile microfuge tube, gently mixed by swirling the pipette and incubating on ice for 30 minutes.

Transformation test reactions were performed using ligation reactions (see Chapter 2, Section 2.13). Approximately 10 ng (≈ 10 µl) of the desired plasmid DNA (control plasmid pET28a, pET28-AB, pET28-C and pET28-D) were each added to 100 µl aliquot of competent *E. coli* JM109(DE3) cells (thawed on ice), in sterile 1.5 ml microfuge tubes and incubated on ice for 30 minutes. Each tube was heat shocked in a 42ºC water bath for 60 seconds and immediately placed on ice to cool for 2 minutes. SOC media (Novagen, Germany) (900 µl) was added to the microfuge tubes containing transformed cells and incubated in a 37ºC shaking water-bath (152 rpm) for 45 minutes. Subsequently, 200 µl aliquots of the incubated culture were plated onto LB agar plates supplemented with kanamycin sulfate (30 µg ml^{-1}). The plates were left to dry in an upright position at 37ºC for 20 minutes, thereafter these plates were inverted and incubated overnight at the same temperature for approximately 16 hours.

2.13. Screening for Recombinants using Colony PCR

Transformants, were selected on kanamycin supplemented media and colony PCR was used to select colonies which appeared on selection media. (Sambrook & Russell, 2000; Novagen, 2003). However, some colonies may not contain recombinant vectors and therefore colony forming units (cfu) had to be tested for the presence of the inserted gene.

The controls used for colony PCR, were colonies *Lc. lactis* pNM-ACD containing the *mptACD* template; colonies *Lc. lactis* pNZ-8020 containing no template and *E. coli* pET28a were used.

The master mix was prepared according to Table 2.9. Test reactions (Table 2.10) were prepared,: freshly prepared LB agar plates (3 per screen) supplemented with kanamycin sulfate were labelled as follows: each plate was divided with a grid of 25 blocks drawn in and numbered from 1-25, 26-50, and 51-55 respectively. These plates were to serve as a reference plate for colonies under going PCR. PCR tubes were also labelled 1-55, C_1, C_2, C_3 and C_4 respectively. The PCR master mix solution (see Table 2.9) was dispensed into each vial and placed on ice, thereafter the enzyme *Taq* polymerase was added and where necessary the substituted volumes of sterile Milli-Q H_2O. Reaction vials were kept on ice and outside the PCR workstation, each test colony was aseptically picked using a sterile toothpick and touched onto a numbered square of the new agar plate and then lastly shaken into a PCR vial bearing the same number, and promptly placed back on ice. Once all reactions set-up in this fashion was complete, reactions for each primer pair were carried out according to PCR protocol as previously described in PCR of *mptAB*, *mptC* and *mptD*. The newly prepared agar plates were incubated overnight at 37 ºC.

Table 2.9. Preparation of master mix for colony PCR of 55 transformed *E. coli* JM109(DE3)

Reagents	**Required concentration per vial**	**Required volume for single test reaction [µl]**	**Master mix for 60 reactions [µl]**
10× PCR Buffer (without $MgCl_2$) (Roche)	1 ×	2.5	150
*25 mM $MgCl_2$			
For *mptD*	3 mM	3	180
For *mptAB* & *mptC*	2 mM	2	120
10 mM dNTP's (Roche)	0.2 mM	0.5	30
Forward Primer [5 µM stock solution]	0.3 µM	1.5	90
Reverse Primer [5 µM stock solution]	0.3 µM	1.5	90
**Sterile Milli-Q H_2O	-	15.75	945
For *mptD*		16.75	1005
For *mptAB* & *mptC*			
Enzyme *Taq* polymerase [5 U μl^{-1}] (Roche)	0.05 U μl^{-1}	0.25	-
TOTAL VOLUME		25	1485

* The $MgCl_2$ concentration varies for reactions containing primers *mptD*.

** Due to the varying concentrations of $MgCl_2$, the amount of H_2O also varies for the specified reactions containing primers *mptD*.

Table 2.10. Sample preparation for colony PCR of transformed *E. coli* JM109(DE3).

Reagents	**Required concentration per vial**	**Test Reaction 1-55 (colony JM109(DE3) - pET28a-X)** [µl]	**Control (C_1 -No Enzyme)** [µl]	**Control (C_2 -No colony)** [µl]	**Control (C_3 -colony *Lc. lactis* pNM-ACD)** [µl]	**Control (C_4 -colony *Lc. lactis* MG-CON)** [µl]
Master mix		24.75	24.75	24.75	24.75	24.75
Sterile Milli-Q H_2O		-	0.25	-	-	-
Enzyme Taq Polymerase [5 U/µl] (Roche)	1 U μl^{-1}	0.25	-	0.25	0.25	0.25
Test cfu lactic acid bacteria 1-55	50 ng	1 colony	-	-	-	-
Lc. lactis pNM-ACD	50 ng	-	1 colony	-	1 colony	-
Lc. lactis pNZ-8020	50 ng	-	-	-	-	1 colony
TOTAL REACTION VOLUME		25	25	25	25	25

Analysis of PCR reactions were carried out on 1% (w/v) agarose gels (9 V cm^{-1}). Samples were prepared by addition of gel loading buffer (2 µl) (Promega, USA) and run along side Molecular Weight Maker III (Roche, Germany). The results were documented and clones showing the amplified gene insert were selected. These clones were grown further by transferring and streaking the reference colonies onto fresh agar plates supplemented with kanamycin sulphate (30 µg ml^{-1}), following an overnight incubation at 37ºC.

Selected clones were once again grown overnight at 37ºC in 10 ml LB broth and used for preparation of glycerol stocks (0.1 ml 80% (v/v) sterile glycerol added to 0.9 ml overnight culture) and mini-prep plasmid DNA isolation using the alkaline lysis method (see Section 2.4). As previously described, purified plasmid DNA constructs were analyzed on a 0.8% (w/v) agarose gel and quantified by determining absorbance readings at 260/280 nm on a Beckman UV/VIS spectrophotometer.

2.14. Transformation of Recombinant DNA into Expression Strain *E. coli* BL21(DE3) pLysS

The appropriate number of vials containing competent cell were removed from the -70ºC freezer and placed on ice. In order to determine the transformation efficiency of these commercially competent cells, 1 µl (0.2 ng) of the supplied test plasmid was added to a vial of 20 µl competent cells. Using the pipette tip the mixture was gently stirred and immediately placed on ice. Purified recombinant pET28a –X (2 µl) was added to 20 µl competent cells and immediately placed on ice. Tubes were incubated on ice for 5 minutes, and heat-shocked in a preheated water bath (42ºC, for 30 seconds). Immediately thereafter the tubes were incubated on ice for 2 minutes. Room temperature SOC media (250 µl) was added to each cell containing vial (Novagen, Germany). Vials were incubated in a 37ºC water bath, shaking at 250 rpm for 30 minutes. Thereafter 50 µl of the test transformation were plated using the spread plate technique. Plates were left to dry for 20 minutes, inverted and incubated overnight at 37ºC. Once again transformed colonies were analysed for the presence of the insert using colony PCR (see section 2.13) and analysed on a 1%

(w/v) agarose gel (9 V cm^{-1}). Clones yielding a positive result for the insert were tested for expression of the target gene.

2.15. Expression of the $IIAB^{Man}$, IIC^{Man} and IID^{Man} Polypeptides of EII_t^{Man}

E. coli BL21(DE3) pLysS cells were transformed with recombinant plasmid pET28a and used for the expression of His-tagged subunits His-$IIAB^{Man}$, His-IID^{Man} and His-IIC^{Man} of the mannose PTS permease ($EIIt^{Man}$).

Each strain of *E. coli* BL21(DE3) pLysS containing pET28-AB, pET28-C, or pET28-D was grown in LB broth supplemented with kanamycin sulfate (30 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}), to select for the recombinant plasmid.

Overnight broth cultures (10 ml LB broth supplemented with chloramphenicol (34 μg ml^{-1}) and kanamycin sulfate (30 μg ml^{-1}) were prepared by single colony inoculation, and subsequently inoculated into 100 ml LB broth supplemented containing chloramphenicol (34 μg ml^{-1}) and kanamycin sulfate (30 μg ml^{-1}). The cultures were grown to an OD_{600} of 0.5, an aliquot (1 ml) was retained for sodium dodeocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Cultures were induced with 0.4 mM isopropyl-β -D-thiogalactopyranoside (IPTG), for 3 hours at 37ºC, and once again induced for 5 hours at 30°C. Thereafter shake flasks were cooled on ice. A 1 ml aliquot from each culture was taken for SDS-PAGE analysis. The remaining culture was discarded, by autoclaving at 121ºC for 15 minutes.

The dispensed sample volumes were each microfuged and the bacterial pellet was re-suspended in 50 μl phosphate buffer saline, pH 8.0. SDS-PAGE samples were prepared using 50 μl sample and 50 μl SDS sample buffer (Biorad, Germany). The soluble cytosolic and insoluble membrane fractions were prepared as stipulated previously (Von Jagow *et al.*, 2003). A 12% (w/v) SDS-PAGE gel was used to analyse expression of each subunit.

2.16. Analysis of Protein Samples on SDS-PAGE Gels

2.16.1. SDS-PAGE gel preparation

Protein samples were analysed by SDS-PAGE, on a 10% (w/v) SDS-PAGE gel, using a Hoefer SE 250 Mighty Small II vertical gel system as previously described (Sambrook & Russell, 2000). SDS-PAGE gels were prepared as shown on Table 2.10. The stipulated volumes were used to prepare two gels.

Table 2.11. Reagents used to prepare SDS-PAGE gels

REAGENTS	Running gel (10%)	Stacking gel (4%)
Acrylogel (37.5:1 bis-acrylomide ratio) (Merck, Germany)	3.15 ml	650 µl
RO H_2O	5.40 ml	3.0 ml
0.5 M Tris (Roche,Switzerland)	-	1.25 ml
3 M Tris (Roche, Switzerland)	1.25 ml	-
50% (v/v) Glycerol (Merck, Germany)	200 µl	-
20% (w/v) SDS (Sigma-Aldrich, USA)	50 µl	25 µl
TEMED (Promega, USA)	15 µl	5 µl
10% (w/v) Ammonium persulfate (BDH chemicals Ltd, UK)	36 µl	25 µl

For reducing SDS-PAGE, samples were combined with an equal volume of reducing buffer [125 mM Tris-HCl, 4% SDS, 20% (v/v) glycerol, 0.2 mM dithiothreitol (DTT) (Boehringer Mannheim, South Africa), 0.02% (w/v) bromophenol blue, pH 6.8] and incubated as follows: samples from expression studies were either incubated in a boiling water bath for 5 minutes or at 85°C for 3 minutes, prior to loading onto the gel. Whilst samples analysed from purification studies were heated at 45ºC for 30 minutes before loading onto the gel. Volumes between 15-30 µl of the treated samples were loaded into

the appropriate wells of the stacking gel. All samples were run alongside a ready to use protein standard (Biorad, Germany). Electrophoresis was performed at 18 mA per gel, until the bromophenol blue tracker dye reached the bottom of the gel. Proteins were visualized by Coomassie Blue staining.

2.16.2. Coomassie blue staining of SDS-PAGE gel

Following SDS-PAGE, gels were placed in a standard Coomassie staining solution [0.025% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol, 7% (v/v) acetic acid] for 4 hours to overnight. Staining solution we replaced with de-staining solution [7% (v/v) acetic acid, 5% (v/v) methanol], until the gel background was clear. Gel images were documented using a Versadoc 5000 gel image system (Biorad, Germany).

2.17.Culturing and Over expression of Target Genes in *E. coli* BL21(DE3) pLysS Clones, pET28-AB, pET28-C, and pET28-D

A single colony of the transformed *E. coli* BL21(DE3) pLysS was inoculated into 20 ml LB broth supplemented with 30 μg ml^{-1} kanamycin sulfate and 34 μg ml^{-1} chloramphenicol. The culture was incubated at 37°C in an orbital shaking water-bath at 230 rpm. The culture was grown until an OD_{600} was between 0.4 -0.6.

The 20 ml inoculum was transferred into a 2 l Erlenmeyer flask containing 800 ml LB broth and grown at 37°C in a shaking water-bath at 230 rpm. When cells reached mid-exponential phase (OD_{600} = 0.5, ≈ 3 hours), overproduction of the His-IID^{Man} and His-IIC^{Man} was induced by the addition of 0.4 mM isopropyl-β -D-thiogalactopyranoside (IPTG) and incubated at 30°C for 5 hours. At this stage (OD_{600} = 1.0) the cells were rapidly cooled in an ice-bath and harvested by centrifugation (8000 × *g*, 10 minutes, 4°C). The cell pellet was weighted and washed with an approximate volume of 5 ml g^{-1} TSC buffer (20 mM Tris, 250 mM sucrose, 140 mM choline chloride), and centrifuged at 8000 rpm, 10

minutes, 4°C. The pellet was drained, the wet weight of each pellet was determined and recorded, and thereafter pellets were stored overnight at -20°C.

2.18. Preparation of Cytosol and Membrane Fractions from *E. coli* BL21(DE3) pLysS Clones

The wet weight of the pellet as determined in Section 2.17, was used to re-suspend cells in 5 ml g^{-1} TSC Buffer. The frozen cell pellets were thawed by a single freeze-thaw cycle at -70°C in TSC buffer. A successful rupture of the cells was indicated by the colour of the suspension, which turned from milky white to brownish.

Samples were then treated with the following reagents, which were added to give a final concentration of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 2-mercaptoethanol, 2.5 mM $MgCl_2$ and 2 μg ml^{-1} DNAase I, 10 μg ml^{-1} RNAse A and subsequently incubated for 15 minutes at room temperature. The DNAse rapidly digests DNA, therefore reducing the viscosity of the cellular extract. The resulting suspension was then centrifuged in a Beckman centrifuge model J2-21M using a fixed angle rotor JA14, at 12000 × *g* for 5 minutes at 4°C to eliminate unbroken cells.

The supernatant was transferred to polyallomer ultracentrifuge tubes and ultra-centrifuged in a Beckman Coulter preparative ultracentrifuge using a swing bucket rotor SW40, at 230 000 × *g*, for 60 minutes at 4°C. The supernatant contained the soluble cytosolic fraction and the resulting pellet contained the membrane fraction.

The supernatant containing the $IIAB^{Man}$ subunit was quantified using a Bradford assay and purified via metal chelate affinity chromatography. The membrane pellet was re-suspended in TSC buffer (10 ml) using a 10 ml syringe with a blunt needle (no 18), ultracentrifuged at 230 000 × *g* for 60 minutes at 4°C and re-suspended in TSC buffer at a final membrane protein concentration of 10 mg ml^{-1}. Samples were flash frozen in liquid nitrogen and

stored at -70°C. The second ultracentrifugation simply a wash step to eliminate any mercaptoethanol from the membrane fraction.

2.19. Determination of Protein Concentration using the Bradford Assay

Determination of protein concentration was achieved using the Bradford assay (Bradford, 1976; Read & Northcote, 1981). Standard protein concentrations were made from a 1 mg ml^{-1} stock solution of bovine serum albumin (BSA), at the following concentrations 10 µg ml^{-1}, 20 µg ml^{-1}, 30 µg ml^{-1}, 40 µg ml^{-1} and 50 µg ml^{-1} in TSC buffer. These standards were performed in triplicate and tested with 100 µl sample diluted in 900 µl Bradford reagent [50 mg Serva blue G, 50 ml 88% (v/v) phosphoric acid, 23.5 ml 99.5% (v/v) ethanol stirred for 1 hour and made up to 500 ml with Milli-Q H_2O. the mixture once again stirred for 30 minutes and filtered with Whatman No 1 filter paper], and 100 µl TSC added to 900 µl Bradford reagent served as the blank. The OD_{595} was determined on a Beckman DU640 Spectrophotometer. The readings for each standard were averaged and a scatter curve plot was generated using Microsoft Office Excel 2003 (Microsoft Corporation, USA). The membrane fraction samples were also tested using 100 µl membrane fraction added to 900 µl Bradford reagent. The spectrophotometry readings of membrane fraction samples were used to determine the protein concentration in the membrane fractions, by extrapolating it from the graph.

2.20. Affinity Purification of His-tagged, EII_t^{Man} Subunits on the Ni^{2+}-NTA matrix

2.20.1. Solubilisation of bacterial cell membrane

The 9 ml membrane fraction was mixed with 18 ml solubilisation buffer [100 mM MOPS/KOH pH 7.0, 20% (v/v) glycerol, 1% (w/v) octyl-β-D-glucopyranoside (OGP).

Solutions were mixed freshly before use, pH checked and the solution filtered (0.22 μm)] and incubated for one hour at 4°C with gentle stirring. The solution was ultra-centrifuged (180000 × *g*, 60 minutes, 4°C) in a Beckman Coulter preparative ultracentrifuge. The supernatant containing the His-tagged subunits of EII_t^{Man}, whilst the pellet contained the bacterial membrane.

2.20.2. Metal chelate affinity chromatography (Ni^{2+} -NTA)

Loading of the Ni^{2+}-NTA resin was most efficiently done in batch. An aliquot of 5 ml Ni^{2+}-NTA agarose resin (Qiagen-Hilden, Germany) was pre-treated by washing twice with 20 l Milli-Q H_2O, and centrifuged at 1000 × *g* for 15 minutes, 4ºC. Thereafter, the resin was equilibrated by re-suspending the resin pellet in aliquots of 20 ml binding buffer (pH 7.9), centrifuged (1000 × *g*, 15 minutes, 4 ºC) and the pH of the supernatant tested. This process was repeated until the pH of the supernatant was at pH 7.9. The solubilised protein fraction was mixed with the resin and incubated at 4°C for one hour with gentle rotation (25 rpm, on ice). The resin was then transferred into a 10 ml column (Biorad, Germany) and allowed to settle until tightly packed and the flow-through collected under gravity flow into 2 ml aliquots. The column was washed with 120 ml binding buffer, followed by 80 ml washing buffer, these and all following chromatographic steps were performed with a flow rate of 2 ml $minute^{-1}$ by natural gravity flow. Elution was conducted with 20 ml elution buffer and fractions of 2 ml were collected and their protein concentration determined (A_{280}). It was imperative that the entire procedure had to have been carried out at 4°C as the His-tag tends to aggregate at room temperature and at very acidic pH 4-5.

The His-tagged proteins were usually found in those fractions having the highest A_{280} reading and were confirmed by SDS-PAGE gels and western blot analysis. The protein containing fractions were transferred to dialysis tubes (12-15 kDa molecular weight cut off), and dialyzed twice (1 hour and then overnight) in 150 ml dialysis buffer [25 mM K-Acetate (pH 5.0); 100 mM KCl; 5 mM $MgCl_2$; 0.03% (w/v) OGP], at 4°C.

2.21. Western Blot Analysis

This procedure was performed to confirm the presence of the His-tagged protein subunits of the mannose PTS permease. SDS-PAGE was carried out on duplicate gels. One gel was stained with Coomassie Blue staining procedure to the total protein profile while the other was used for blotting. The gels contained a Biorad precision plus molecular weight protein standard marker (Biorad, Germany), and a His-tagged MBP protein (Mr Derek Van Rooyen, personal communication, 2007) as a positive control. Proteins separated by SDS-PAGE, were transferred onto nitrocellulose membrane

SDS-PAGE was carried out on duplicate gels. One gel was stained with Coomasie Blue staining procedure to visualize the protein profile, whilst the second gel was utilized for protein transfer onto a nitrocellulose membrane. Proteins transferred onto nitrocellulose membrane using a mini-protean transblot cell (Biorad, Germany). A sandwich consisting of two filter pads (Biorad, Germany), the SDS-PAGE gel and the nitrocellulose membrane, were immersed in a mini-protean transblot cell (Biorad, Germany) filled with ice-cold Towbin transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3] (Towbin *et al.*, 1979). Transfer was performed for 90 minutes at 90 V. In order to assess the efficiency of protein transfer, the gel and nitrocellulose membrane were carefully removed from the sandwich and stained with Coomassie and Ponceau S (Sigma-Aldrich, USA), respectively. The lane containing the Biorad molecular weight marker (Biorad, Germany) now visually apparent on the nitrocellulose membrane due to the Ponceau S staining was carefully cut out and left to air dry between two sheets of Whatman 3mm filter paper. The remaining nitrocellulose membrane was de-stained by adding a few drops of 1 M NaOH to Milli-Q H_2O, in which the membrane was immersed until all traces of the Ponceau S stain had been removed. The membrane was placed between two sheets of Whatman 3mm filter paper and allowed to air dry for 1.5 hours.

2.22. Detection of His-tagged Proteins

Anti-His tag antibodies (Roche, Germany) were used for the identification of the His-tagged subunits of the EII_t^{Man} proteins. The nitrocellulose membrane containing the blotted proteins, was blocked for one hour with 5% (w/v) no-fat milk powder in phosphate buffered saline (PBS) [10 mM phosphate, 150 mM sodium chloride, pH 7.2]. Thereafter the membrane was washed and incubated in PBS for 5 minutes. The wash procedure was repeated three times. The membrane was subsequently incubated for two hours with Anti-His_6 peroxidase-conjugate mouse monoclonal antibody (Roche, Germany) in 0.5% (w/v) bovine serum albumin in phosphate buffered saline (BSA-PBS). The membrane was incubated in the antibody solution for 2 hours. Subsequently, the membrane was once again washed thrice in PBS as described above. BM Blue peroxidase (POD) precipitating, chromogenic substrate (5 ml) (Roche, Germany) was added and protein bands became visible. The membrane was removed from the substrate solution, washed in Milli-Q H_2O and dried between Whatman 3 mm filter paper. An image of the blot was captured using the Versadoc 5000 (Biorad, Germany) imager and subsequently analysed using Quant One software (Biorad, Germany). Results and discussions for experiments carried out are outlined in chapters 3 and 4.

CHAPTER 3

Cloning the *mptAB*, *mptC*, and *mptD* genes of *L. monocytogenes* EGD

3.1. Introduction

The mannose PTS permease (EII_t^{Man}) of *L. monocytogenes* has been implicated as the putative receptor of class IIa bacteriocins (Ramnath *et al.*, 2004). The *mptACD* operon encodes the $IIAB^{Man}$, IIC^{Man} and IID^{Man} membrane-bound subunits that make up the complete EII_t^{Man} protein. The focus of our study was to clone the individual genes *mptAB, mptC, mptD* encoding each of the subunits.

Plasmid pNM-ACD, is a derivative of pNM-8020. It contains the mannose PTS operon (Figure 3.1). The sequence data and construct that was transformed into *Lc. lactis* was kindly provided by Dr Yann Hechard (personal communication, 2004).

3.2. Analysis of pNM-ACD Sequence Data

Utilizing the sequence data of pNM-ACD, we developed primers specific to genes encoding the individual subunits of the mannose PTS permease.

The nucleotide sequence supplied by Dr Yann Hechard (personal communication, 2004) was used to perform a $BLAST^N$ search. This revealed that the *mptACD* operon, cloned into the pNM-8020 vector was 100% identical to *L. monocytogenes* EGD accession number AL591982**.** The mannose PTS permease encoded by this plasmid may be seen in Figure 2.1 (Chapter 2, Section 2.7). The *mptAB*, *mptC* and *mptD*, genes were found at positions 87-1052 bp, 1076-1882 bp and 1905-2815 bp relative to the multiple cloning site of pNM-ACD. Their gene sequence lengths were 965 bp, 806 bp and 910 bp, respectively. This data was used in primer design for subsequent PCR, described in Chapter 2 (Section 2.8). Protein sequence alignments were performed using Bioedit 7.0., in order to facilitate the alignment of the *mptACD* protein sequence to the protein sequence of *L. monocytogenes* EGD and it was confirmed to be 100% identical to *L. monocytogenes* EGD (data not shown).

The pET28a expression vector was seen as the most suitable expression vector to clone and express the PCR amplified *mptAB*, *mptC* and *mptD* genes. As seen in Figure 3.1 the pET28a multiple cloning site (MCS) encodes an N-terminal His-tag which was used to express the target gene as a His-tagged protein. The presence of a specific thrombin protease cleavage site could facilitate the removal of the N-terminal His-tag (Novagen, 2003).

A restriction recognition site search was performed to determine which restriction sites were present within the *mptACD* gene. Figure 3.2 highlights the presence of those restriction enzyme recognition sites found in the MCS of pET28a and therefore could not be used to clone into the expression vector. Restriction endonuclease enzymes *Nde*I and *Xho*I recognition gene sequences found in the MCS of pET28a (Figure 3.1), were not present within the *mptACD* gene sequences. Therefore *Nde*I and *Xho*I sequences were incorporated onto the 5′ end of the forward and reverse primers, respectively. These restriction enzymes (Roche, Germany) were used because they generate sticky ends. This ensures unidirectional cloning, within the pET28a expression vector (Sambrook & Russell, 2000).

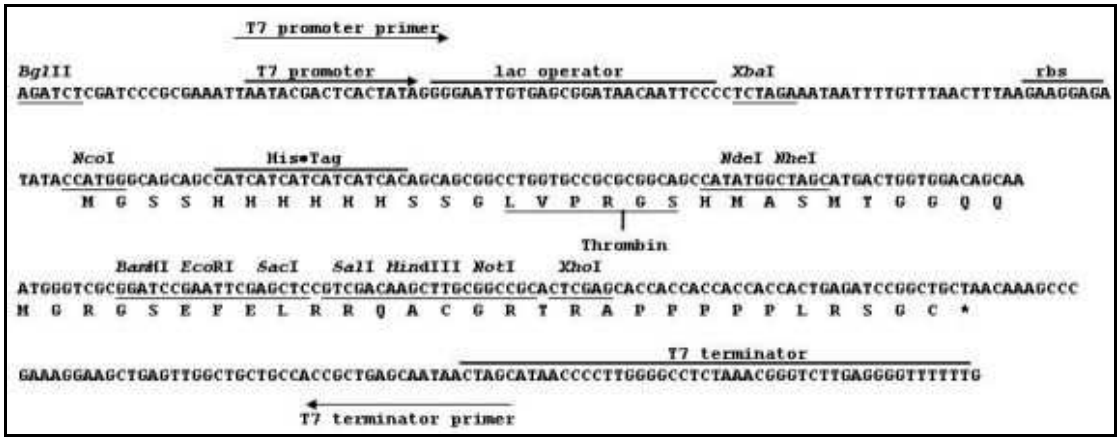


Figure 3.1. The Multiple Cloning Site (MCS) of pET28a expression vector. The MCS contains an N-terminal His-tag, Thrombin protease cleavage site and the appropriate *Nde*I and *Xho*I restriction sites (Novagen, 2003).

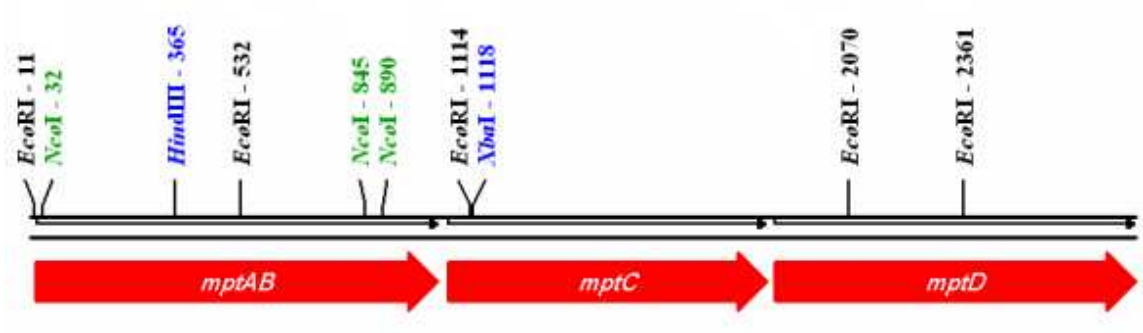


Figure 3.2. A graphic presentation of the *mptACD* operon, showing the restriction sites from MCS of pET28a present within the operon sequence. Restriction sites *Bam*HI, *Bgl*II, *Nde*I, *Nhe*I, *Not*I, *Sac*I, SalI, and *Xho*I were not found within the *mptACD* operon. The figure was generated by pDraw32 1.0.106 (Acaclone software, USA).

3.3. Primer Design

The restriction enzyme data analysis was used to inform the design of primers for PCR amplification. Restriction endonuclease recognition sequences *Nde*I and *Xho*I identified in the previous Section (3.1), were incorporated on the 5´ terminus of each primer. These sites were designed so that individual genes could be directionally cloned into the pET28a vector, such that the open reading frame was fused to the N-terminal His-tag.

The initial primer sets, specific to the target genes, with their 5´ sequence modification were then subjected to analysis on a primer design software program, Primer Express 3.0 (Applied Biosystems, South Africa). The program was used to generate the final primer sets. Primer express assessed primer pairs according to the following characteristics: the primer length (L), the melting temperature (T_m), and the GC content (%GC) of each primer sequence. In addition, the probability of possible primer dimer, and loop formation were also visually illustrated by the primer design program.

Primer dimer formation was minimized by deleting base pairs on the 3′-end, at the same time random base pairs were added to each primer at the 5′-end to accommodate for efficient binding of the restriction enzymes to its recognition sequence. These steps were performed on each primer sequence. The objective was to decrease the probability of primer dimer and loop formation, whilst ensuring that a high but comparable T_m, %GC and length, between primer pairs was maintained. The final primer pairs that resulted from this analysis are shown in Table 3.1. A Blast analysis of the final primer pairs (see Table 3.1), were performed to verify primer specificity to *L. monocytogenes* EGD *mpt*ACD. Figure 3.3 shows the position at which each primer sequence annealed to the subunit genes of the *mptACD* operon.

Table 3.1. The final primer pairs that were designed to amplify *mpt*AB, *mpt*C and *mpt*D genes

Primer name	**Primer sequence** [1]	**L**	**%GC**	**T_m** [2]
mptAB Forward	5′-*AATCATATG*ATGGTAGGAATTATCCTCG-3′	28	39.3	51.9
mptAB Reverse	5′-*ATGAGCTC*TTATTGTGTCTTTAATTCG-3′	27	33.3	44.3
mptC Forward	5′-*AATCATATG*ATGTCTGTCATATCAATAATTTTAG-3′	34	24.2	50.8
mptC Reverse	5′-*ATGAGCTC*TTAATAGTCGTTTAATATATCGC-3′	31	35.5	49.5
mptD Forward	5′-*AATCATATG*GGCAGAAAAAATCG-3′	25	33.3	40.7
mptD Reverse	5′-*GCGAGCTC*TTACAGAAGCCCGATTAAGTGAC-3′	31	56	58.3

[1] Primer sequence in italics include, base pairs in blue, represent the added sequences to facilitate restriction digests; those in green, represent the restriction site sequence *Nde*I and *Xho*I on the forward and reverse primers respectively.

[2.] T_m calculated according to the number of base pairs specific to the template sequence (*mptACD*), i.e. for *mptAB* the sequence length of 19 bp recognises the *mptACD* sequence.

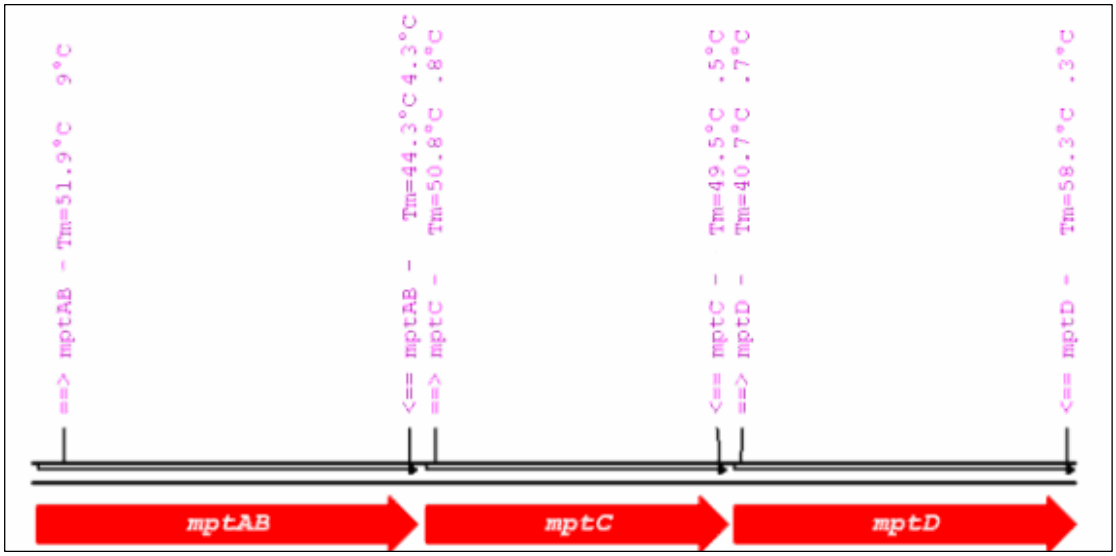


Figure 3.3. A graphic presentation of the *mptACD* operon from *Lc. lactis*, highlighting the priming sites of the *mptAB*, *mptC* and *mptD* primers. The symbols "^" represents the position at which the forward primer binds, and "v" represents the position at which the reverse primer binds. This figure was generated by pDraw32 1.0.106 (Acyclone software,USA).

3.4. Isolation of Plasmid pNM-ACD from *Lc. lactis*

The aim was to purify plasmid DNA pNM-ACD from *Lc. lactis*, to serve as a template for subsequent PCR. The preparation of plasmid DNA from *Lc. lactis*, incorporated the addition of lysozyme to assist in cell lysis and the usual alkaline lysis method was used as modified by Holt *et al*. (2001). However, the protocol by Holt *et al*. (2001) did not incorporate a step for treatment of the sample with RNAse. During our attempts at plasmid DNA isolation, the presence of RNA in the final sample was noticed. Therefore to eradicate the presence of RNA, the addition of RNAse A (10 mg ml^{-1}) (Roche, Germany) was incorporated during the initial lysis step.

Figure 3.4, indicates that plasmid DNA was successfully isolated from *Lc. lactis*. The distinct banding patterns represent the different conformations of plasmid DNA. Agarose gel electrophoresis gave an indication of the level of purity of the plasmid DNA sample. No RNA was present in the final plasmid DNA sample. Plasmid pNM-8020 containing no *mptACD* insert was also isolated to serve as a control in downstream experimentation. Results from a UV spectrophotometry analysis indicated that plasmid pNM-ACD and pNM-8020 were isolated, with a concentration of 39.5 ng μl^{-1} and 38.1 ng μl^{-1}, respectively.

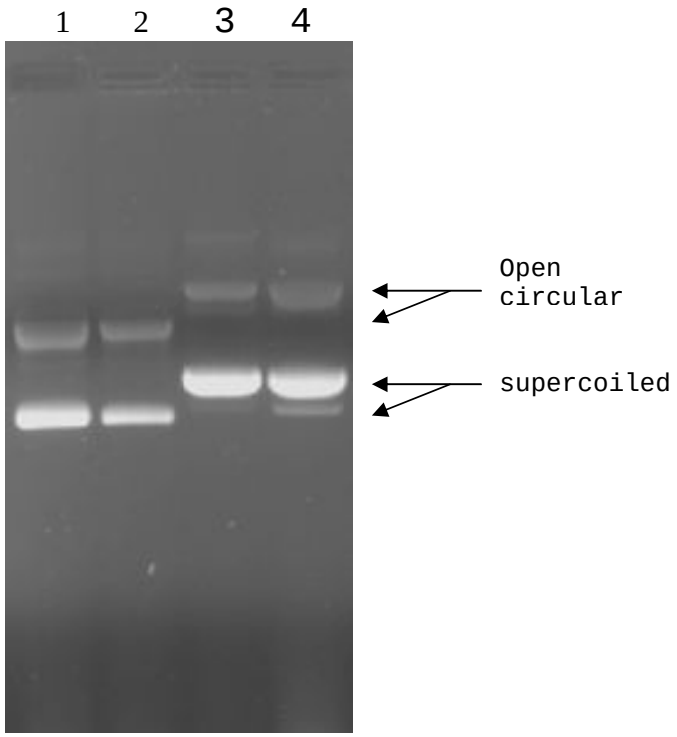


Figure 3.4. A 0.8% (w/v) agarose gel of plasmid DNA pNM-8020 and pNM-ACD isolated from *Lc. lactis*. A total of 8 ng plasmid DNA was resolved in 0.8% (w/v) TAE agarose gel. Lanes 1 and 2: plasmid pNM-8020; lanes 3 and 4: plasmid pNM-ACD.

3.5. Mini prep and plasmid isolation of pET28a expression vector from *E. coli* JM109(DE3)

E. coli JM109(DE3) pET28a strain was grown on LB medium containing 30 µg ml^{-1} kanamycin sulfate. A mini-prep, plasmid DNA isolation was performed to purify plasmid DNA which was analysed by agarose gel electrophoresis. The experiment confirmed the presence of plasmid DNA. To isolate plasmid DNA at a much higher concentration and enhanced purity, the Nucleobond AX 100 plasmid DNA isolation kit (Machery-Nagel) was used. Figure 3.5 highlights that plasmid DNA pET28a was successfully isolated. Results from a UV spectrophotometry analysis confirmed the sample purity and determined that the concentration of plasmid pET28a was 5.7 µg $µl^{1}$.

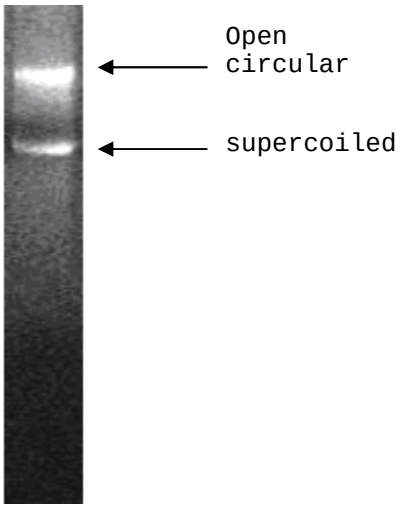


Figure 3.5. A 0.8% (w/v) agarose gel of plasmid DNA pET28a isolated from *E. coli* JM109(DE3). A total of 4 ng plasmid DNA was resolved in 0.8% (w/v) TAE agarose gel. The banding pattern shows the two possible conformations of Plasmid DNA. Plasmid pET28a was prepared from *E. coli* JM109(DE3) using a Nucleobond AX100 plasmid DNA isolation kit (Machery-Nagel, USA).

3.6. Amplification of *mptAB, mptC* and *mptD* Genes from pNM-ACD

The PCR technique was used to amplify genes of *mptACD*. PCR reactions were setup utilizing primer pairs outlined in Table 3.1. In order to generate the correct DNA fragment, primer specificity which is reliant on the correct annealing temperature and the appropriate titration of divalent ions such as Mg^{2+}, was taken into consideration.

Initial PCR of *mptC* yielded a product at an annealing temperature of 55°C and a 1.5 mM $MgCl_2$ concentration. The result shown in Figure 3.6, lane 2, highlights a 830 bp *mptC* PCR product resolved in a 0.8% (w/v) agarose gel.

The conditions utilized to amplify *mptC* were repeated in order to amplify *mptAB* and *mptD*. Primer pairs *mptAB* and *mptD*, at an annealing temperature of 55°C and 1.5 mM $MgCl_2$, amplified multiple PCR products per reaction, (data not shown), indicating that these conditions did not work. Thereafter the annealing temperature was varied from 55°C to 60°C for both *mptAB* and *mptD* primer pairs. Single PCR products of 930 bp, *mptD*, and 968 bp, *mptAB*, were obtained at an annealing temperature of 58°C (Figure 3.7, lane 2 and Figure 3.8 lane 2).

Performing PCR reaction with 1.5 mM $MgCl_2$ concentration did not prove sufficient. It was observed that the intensity of bands on agarose gel electrophoresis of 8 ng PCR products *mptAB* and *mptD* were faint. Hence the $MgCl_2$ concentration was varied from 1.5 mM to 2.5 mM. The adjustments to the $MgCl_2$ concentration in PCR reactions showed a proportional increase in the concentration of PCR product, this was observed as an increase in the band intensity of the 8 ng PCR product upon resolution in 0.8% (w/v) agarose gels. Equal concentration of PCR products were resolved on 0.8% (w/v) agarose gels. At 1.5 mM $MgCl_2$ concentration, faint single PCR products were observed for both *mptAB* and *mptD* whilst darker bands were observed at a 2 mM concentration. No distinct band intensity differences were observed at concentrations of 2 mM and 2.5 mM $MgCl_2$, even though equal volumes of sample were loaded on agarose gel (data not shown).

Figures 3.6 to 3.8, lanes 3 to 5 represent the appropriate control reactions and as expected there was no evidence of DNA amplification in these reaction tubes. Particular attention must be drawn to lane 3 of each Figures (3.6 to 3.8), represents the result of the negative control reaction. The reaction contained pNM-8020 (the vector without the *mptACD* insert) as the template DNA strand with primer pair's, *mptAB*, *mptC* or *mptD* per reaction tube. The control reactions were performed to confirm that neither of the primer pairs had a binding affinity to the pNM-8020 vector DNA.

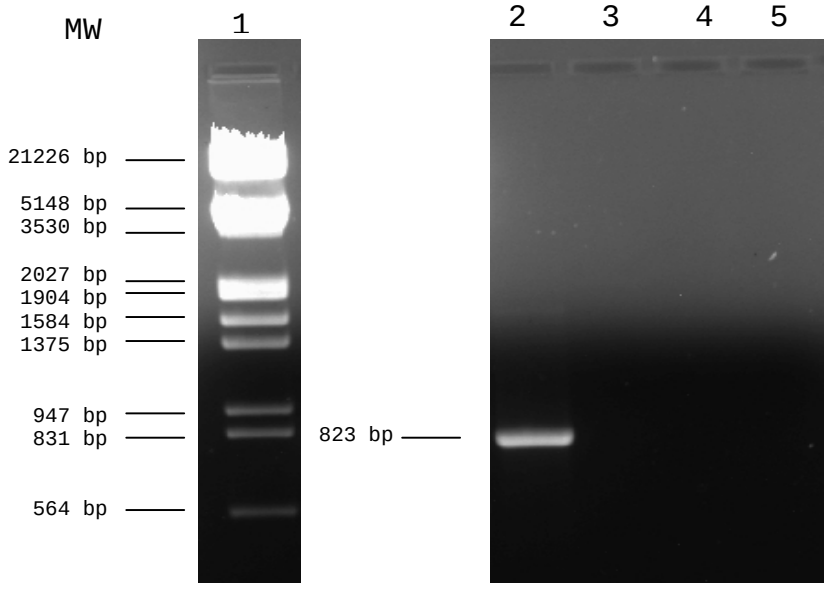


Figure 3.6. A 1.5 % (w/v) agarose gel highlighting a 823 bp *mptC* PCR gene product from plasmid DNA template, pNM-ACD. The PCR product was amplified at an annealing temperature of 55°C and 1.5 mM $MgCl_2$. Lane 1: Molecular weight marker III (Roche, Germany); lane 2: PCR product *mptC* fragment (830 bp) (Quant One, Biorad, Germany); lane 3: negative control, plasmid pNM-8020; lane 4: negative control, no *Taq* polymerase in the reaction; and lane 5: negative control, no template pNM-ACD in the reaction. The DNA profile was analysed using Quant One image analysis software program (Biorad, Germany)

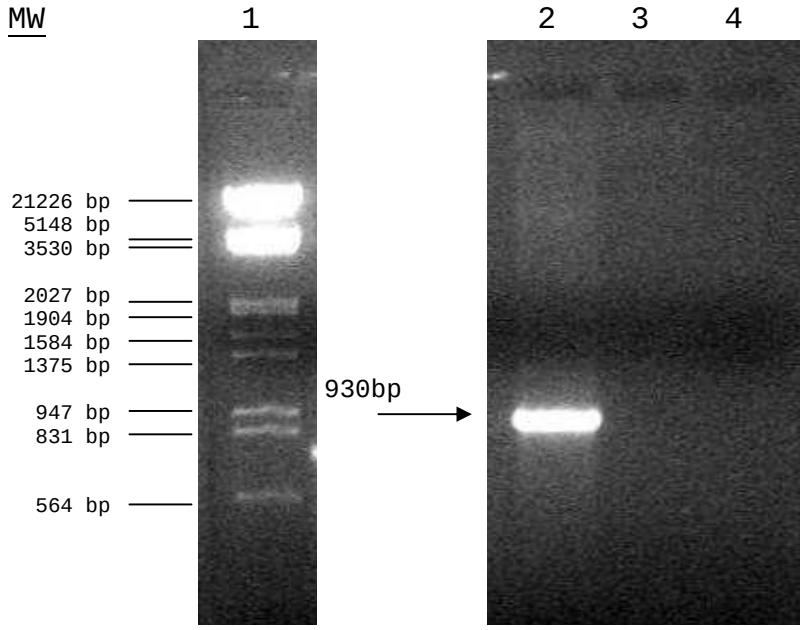


Figure 3.7. A 1.5% (w/v) agarose gel highlighting a 927 bp *mpt*D PCR gene product from plasmid DNA template, pNM-ACD. The PCR product was amplified at an annealing temperature of 58°C and 2 mM $MgCl_2$. Lane 1: Molecular weight marker III (Roche, Germany); lane 2: PCR product *mpt*D fragment (930 bp) (Quant One, Biorad, Germany); lane 3: negative control, plasmid pNM-ACD; lane 4: negative control, no template pNM-ACD in the reaction. The DNA profile was analysed using Quant One image analysis software program (Biorad, Germany)

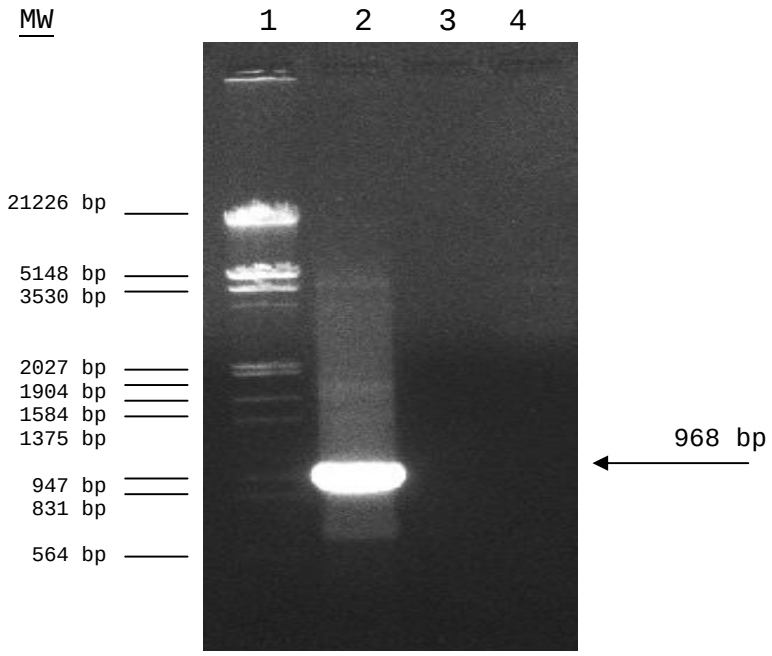


Figure 3.8. A 1.5 % (w/v) agarose gel highlighting a 972 bp *mpt*AB PCR gene product from plasmid DNA template, pNM-ACD. The PCR product was amplified at an annealing temperature of 58°C and 2 mM $MgCl_2$. Lane 1: Molecular weight marker III (Roche, Germany); lane 2: PCR product *mpt*AB fragment (968bp) (Quant One, Biorad, Germany); lane 3: negative control, plasmid pNM-8020; lane 4: negative control, no *Taq* polymerase in the reaction; and lane 5: negative control, no template pNM-ACD in the reaction. The DNA profile was analysed using Quant One image analysis software program (Biorad, Germany)

3.7. Cloning of *mptAB, mptC* and *mptD* Genes into pET28a

The purified plasmids and PCR products *mptAB*, *mptC* and *mptD* were purified from the above mentioned gels (seen in Figures 3.5 – 3.9), via gel elution, using a Nucleospin Extract II kit (Machery-Nagel, Germany) and resuspended in TE buffer (Chapter 2, Section 2.10.2). PCR products were double digested with *Nde*I and *Xho*I and analysed on agarose gel electrophoresis, using Quant One image analysis software. The correct product size was enough to confirm that PCR fragments were intact at its correct size and spurious digestion did not occur.

Purified pET28a plasmid vector was restricted using *Nde*I and *Xho*I endonucleases and analysed via agarose gel electrophoresis. Figure 3.9, lanes 2 and 3 illustrate that both *Nde*I and *Xho*I are functional. The linearised supercoiled pET28a plasmid, gave a plasmid size of 5400 bp, as analysed on Quant One (Biorad, Germany) gel analysis program. The digest was run for 4 hours so as to prevent partial digestion of DNA. Lane 5 confirmed that the reaction conditions promoted specific nuclease activity as pGEX-3X (GE Healthcare, USA), which does not contain either *Nde*I nor *Xho*I restriction sites remained uncut. The double digested pET28a expression plasmid, seen in lane 4, was gel purified to avoid the presence of non-hydrolysed plasmid pET28a.

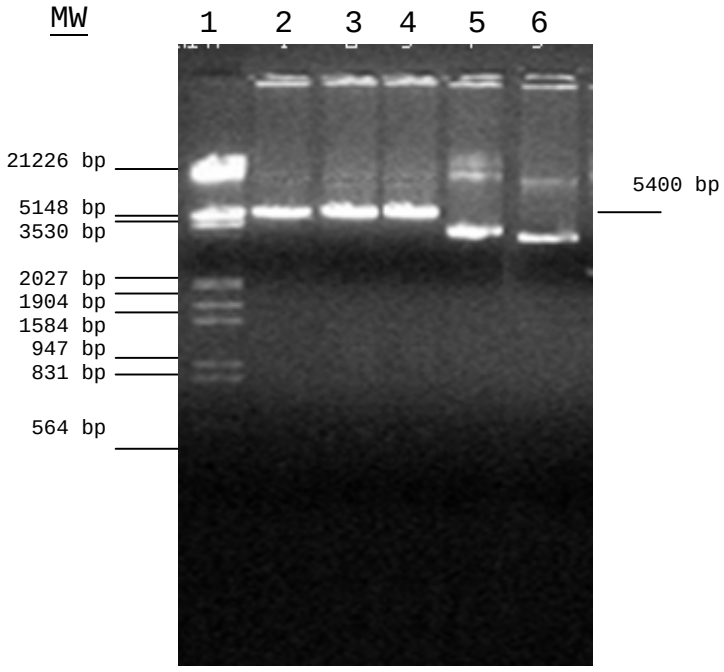


Figure 3.9. A 1.5% (w/v) agarose gel highlighting a restriction digest of pET28a plasmid DNA. Lane 1: Molecular weight marker III (Roche, Germany); lane 2: pET28a -restricted with *Nde*I; lane 3: pET28a -restricted with *Xho*I; lane 4: pET28a- restricted with both *Nde*I and *Xho*I; lane 5: Uncut plasmid pET28a; and lane 6: negative control, plasmid pGEX-3X restricted with *Nde*I and *Xho*I. DNA was analysed using Quant One image analysis software program (Biorad, Germany)

Restricted PCR products *mptAB, mptC* and *mptD* were ligated individually into the pET28a plasmid expression vector. The pET28a cloning system lacks the blue-white colony selection mechanism and therefore it was imperative that transformation and screening processes was optimal. The putative pET28-AB, pET28-C and pET28-D constructs were transformed into *E. coli* JM109(DE3) rubidium chloride ($RbCl_2$) competent cells. This particular strain of *E. coli*, has an array of significant genes to facilitate cloning. However, the F´ episome with a *recA* gene present helps maintain

plasmids within the cell (Goeddel, 1991). Furthermore, *E. coli* BL21(DE3) pLysS, the expression host for the newly developed pET28a recombinant vectors, were observed to display very low transformation efficiency when transforming a ligation mix as opposed to transforming a plasmid free from salt-containing buffers (Hanahan, 1985). Hence, $RbCl_2$ *E.* coli JM109(DE3) competent cells were prepared as they have high transformation efficiency. The purification of ligated plasmids did not prove feasible as this would result in loss of DNA concentration. Therefore, it seemed most feasible to transform the ligation reactions into *E.* coli JM109(DE3) competent cells.

Screening for recombinant pET28a and verification of insert DNA was performed using colony PCR and insert specific primers. Figure 3.11 illustrates results from a colony PCR to screen for the presence of the *mpt*C insert. Control reactions showed that the *mptC* primer pair was specific to the *mpt*C gene yielding an 830 bp fragment for colonies containing the *mpt*C insert.

Similar screening procedures were followed to screen for the presence of *mptD* and *mptAB* recombinant pET28a, with the identical control reactions used as stipulated for colony PCR screening of *mptC*. Results for the screen for the *mptD* insert showed that the *mptD* primer pair was specific to the *mptD* gene yielding a 930 bp PCR product for colonies containing the *mptD* insert (Figure 3.12). Whilst, results for the screen of *mptAB* insert showed that the *mptAB* primer pair was specific to the *mptAB* gene yielding a 968 bp fragment from colonies containing the *mptAB* insert (Figure 3.13).

During the screening process for the *mptC* insert, we noticed a distinct difference between transformants and non-transformants. Transformants generated a very translucent colony, whilst non-transformants were observed to have a very opaque white colony phenotype. This observation was verified during subsequent colony PCR reactions. During the screening for *mptD* and *mptAB* inserts, the translucent colonies selected, gave a 98% and 100% positive result, for the respective inserts.

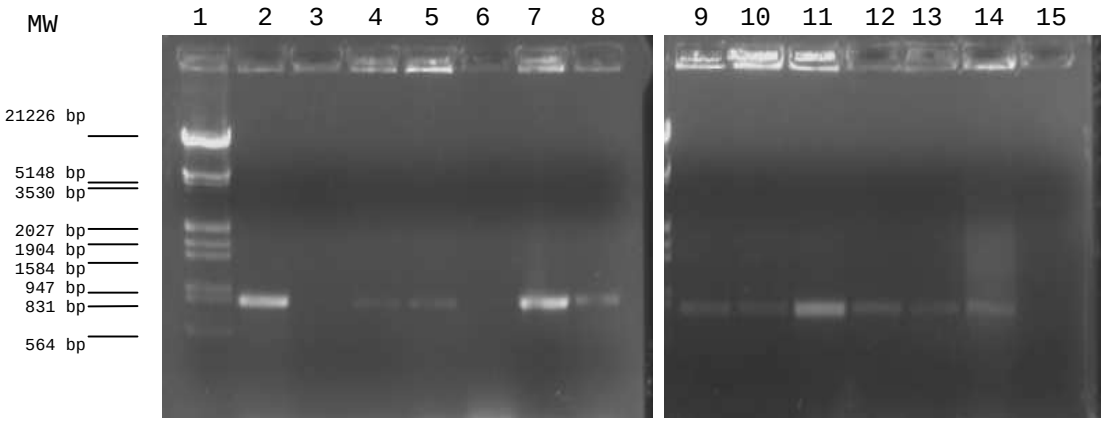


Figure 3.10. Agarose gels 1.5 % (w/v) highlighting results from colony PCR screen for the *mptC* insert in *E. coli* JM109(DE3) pET28-C. Lane 1: Molecular weight marker III (Roche, Germany); lane 2: positive control - *Lc. lactis* pNM-ACD colony (803 bp *mptC* PCR product); lane 3: negative control - *Lc. lactis* pNZ-8020 colony (no PCR product); lane 4-14: *E. coli* JM109(DE3) pET28-C test colonies numbered 1-10 (830 bp *mpt*C PCR product); and lane 15: negative control colony-*E. coli* JM109(DE3) pET28a with no insert (no PCR product). The DNA profile was analysed using Quant One image analysis software program (Biorad, Germany).

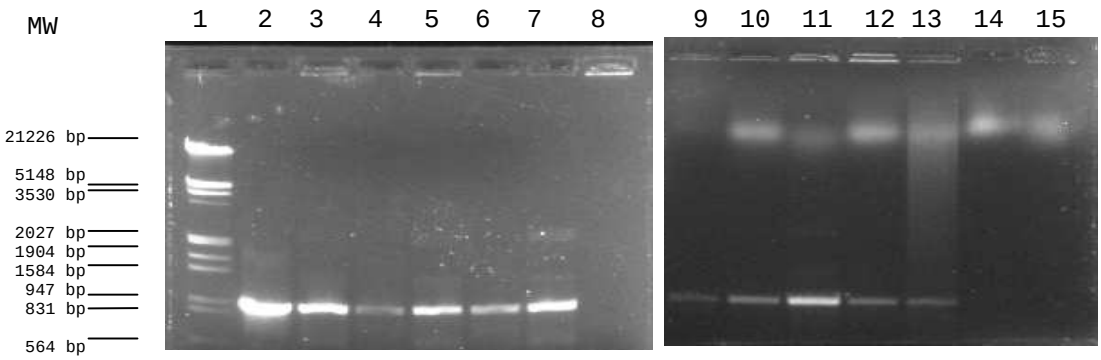


Figure 3.11. Agarose gels 1.5 % (w/v) highlighting results from colony PCR screen for the *mptD* insert in *E. coli* JM109(DE3) pET28-D. Lane 1: Molecular weight marker III (Roche, Germany); lane 2: positive control colony-*Lc. lactis* pNM-ACD (930 bp *mptD* PCR product); lane 3- 13 : *E. coli* JM109(DE3) pET28-D test colonies numbered 1-10 (930 bp *mptD* PCR product); lane 14: negative control colony- *Lc. lactis* pNZ 8020 (no PCR product); and lane 15: negative control colony *E. coli* JM109(DE3) pET28a with no insert (no PCR product). DNA was analysed using Quant One image analysis software program (Biorad, Germany).

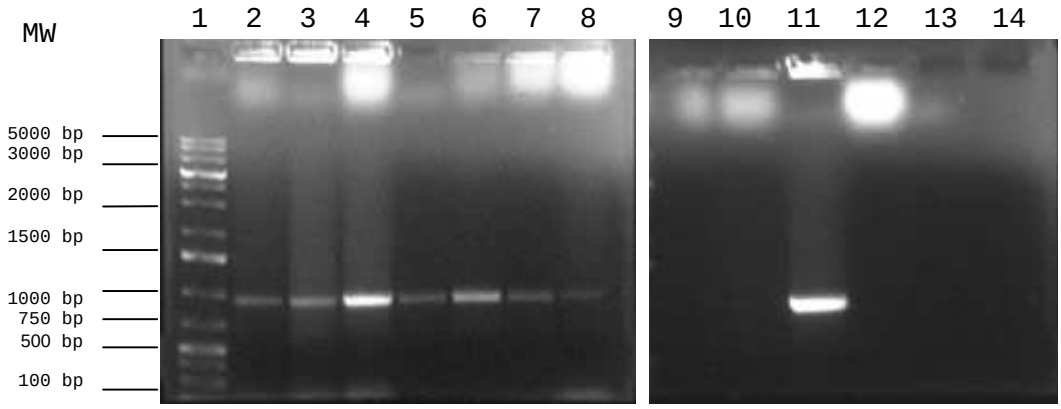


Figure 3.12. Agarose gels 1.5 % (w/v) highlighting results from colony PCR screen for the *mptAB* insert DNA in *E. coli* JM109(DE3) pET28-AB.. Lane 1: O´ Gene Ruler molecular weight marker (Fermentas, South Africa); lanes 2- 10: *E. coli* JM109(DE3) pET28-AB test colonies numbered 1-9 (968 bp *mptAB* PCR product); lane 11: positive control colony - *Lc. lactis* pNM-ACD (968 bp *mptAB* PCR product); lane 12: negative control colony-*Lc. lactis* pNZ-8020 (no PCR product);and lane 13: negative control colony -*E. coli* JM109(DE3) pET28a with no insert (no PCR product). Lane 14: Empty. The DNA profile was analysed using Quant One image analysis software program (Biorad, Germany)

The recombinant plasmids from *E. coli* JM109(DE3), were subjected to a plasmid isolation procedure. These recombinant plasmids were once again restricted with *Nde*I and *Xho*I, analysed by 1.5% (w/v) agarose gel electrophoresis to confirm the presence of the inserted gene in pET28a. Results (not shown) confirmed the presence of the *mptC*, *mptD* and *mptAB* inserts, within their respective pET28a plasmids. These recombinant plasmids were then transformed into an expression strain *E. coli* BL21(DE3) pLysS. Colony PCR was performed once more, to ensure the correct plasmid was present in the host cell. We therefore infer that *mptC*, *mptD* and *mptAB* were successfully cloned into pET28a and transformed into *E. coli* BL21(DE3) pLysS the host expression strain.

3.8. Summary

A pNM-ACD plasmid, from a previous study was used as a template for the amplification and cloning of the genes encoding the individual subunits of the mannose PTS permease. Primers were designed to amplify the subunit genes of the *mptACD* operon of *L. monocytogenes*. These primers incorporated restriction enzymes *Nde*I and *Xho*I on the 5´ end of the forward and reverse primers, respectively. The subunit genes of the *mptACD* operon were successfully amplified. Restriction digests of PCR products and plasmid DNA was performed and analysed by 1.5% (w/v) agarose gel electrophoresis. Following restriction digests, gel elution of both pET28a plasmid and PCR amplified insert DNA was performed to facilitate subsequent cloning. The *mptC*, *mptD*, and *mptAB* genes were individually cloned into a pET28a expression vector, and transformed into *E. coli* JM109(DE3). Recombinant pET28a was screened for the presence of each insert, *mptC, mptD, and mptAB*, using colony PCR and insert specific primers. During the screening process for *mpt* inserts, we noticed a distinct difference between transformants and non-transformants. Transformants generated a translucent colony, whilst non-transformants were observed to have an opaque white colony phenotype. Recombinant plasmid DNA from *E.* coli JM109(DE3) was once again isolated, subsequently restricted, and analysed on 1.5% (w/v) agarose gel, to confirm the presence of insert DNA. Thereafter these recombinant plasmids pET28-C, pET28-D and pET28-AB were individually transformed into *E. coli* BL21(DE3) pLysS host expression strain, to express the target gene.

CHAPTER 4

Expression and Purification of the Subunits of the Mannose PTS Permease

4.1. Introduction

As mentioned previously in order to express and purify the subunits of the EII_t^{Man}, *E. coli* BL21(DE3) pLysS strain was transformed with recombinant plasmids pET28-C, pET28-D and pET28-AB respectively. This particular strain of *E.* coli is deficient of the *lon* protease and the *ompT* outer-membrane protease genes. The absence of these proteases, ensure a reduction in proteolytic activity within the host expression strain. Furthermore the pLysS plasmid secretes low levels of the enzyme lysozyme. It is both useful in preventing expression of the target gene in the non-induced cells and helps in complete cell rupture, during cell lysis (Goeddel, 1991; Novagen, 2003).

4.2. Analysis of Protein Expression of the Subunits of the EII_t^{Man}

Overproduction of the His-tagged, subunits of $EIIt^{Man}$ (His-IIC^{Man}, His-IID^{Man} and His-$IIAB^{Man}$), was achieved by inducing protein production with IPTG during mid-exponential growth phase (OD_{600} = 0.6). Induction of each subunit was monitored at a varied time between 3 to 5 hours and the temperature was varied from 37°C to 30°C. The induced and non-induced cell protein content of broth cultures was analysed in 10% (w/v) SDS-PAGE gels for differences in protein banding pattern between the total cell protein of the induced sample, non-induced sample and the different cell fractions. Analysis on total cell protein from ruptured cells served as the control. The cell culture supernatant and the cytosolic fraction, which was further split into the soluble and membrane fractions, were prepared. These fractions were analysed in order to determine the location of expressed protein (Von Jagow *et al.*, 2003).

The induction of the IIC^{Man} subunit was initially performed at 37°C for 3 hours. It was clear that a protein of the expected size was synthesized. However, the level of synthesise was relatively low compared to the other proteins present in the cell extract (Figure 4.1). Figure 4.1, lane 4 highlighted the over-expression of a 24 kDa protein in the cell membrane fraction. The non-induced fraction in lane 2 showed an absence of a 24 kDa

protein band. Therefore the appearance of this band was attributed to the expression of the IIC^{Man} protein.

The induction temperature was decreased from 37°C to 30°C and the induction time was increased from 3 hours up to 5 hours, in an attempt to decrease the amount of cellular protein expression relative to the expression of the target membrane protein. Figure 4.2, lane 4 show clearly that an induction at 30°C for 5 hours relatively increased the level of IIC^{Man} (24 kDa) expression as indicated by the intensity of the banding pattern. Therefore, relative expression of the IIC^{Man} was greatly improved by induction at 30°C for 5 hours (Figure 4.2).

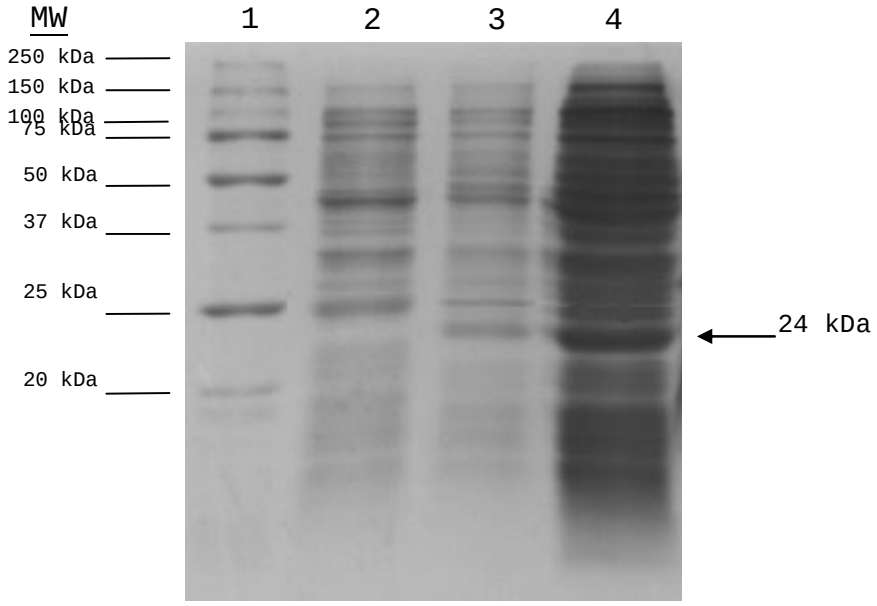


Figure 4.1. A 10% (w/v) SDS-PAGE gel showing expression of His-tagged IIC^{Man} in *E. coli* BL21(DE3) pLysS pET28-C at 37°C. Lane 1: Precision plus protein standard (Biorad, Germany); lane 2: non-induced control, total cell protein; lane 3: induction control, total cell protein; lane 4: crude membrane fraction. The arrow indicates expression of the His-tagged IIC^{Man} at approximately 24 kDa. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

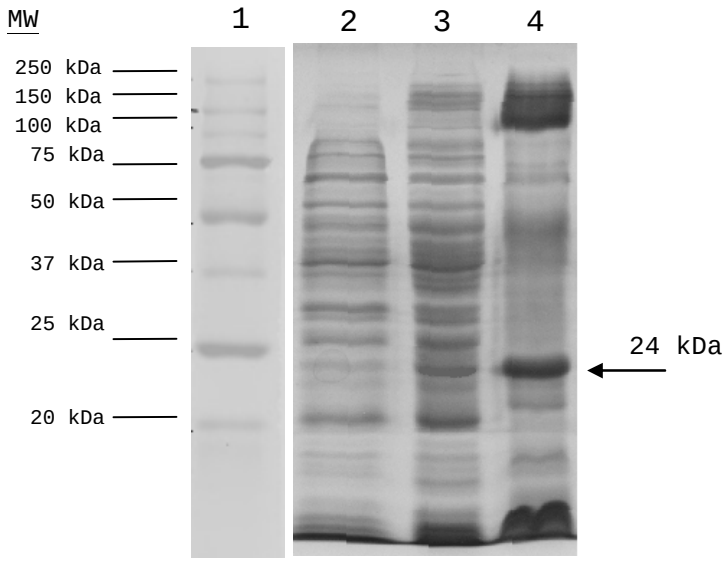


Figure 4.2. A 10% (w/v) SDS-PAGE gel showing expression of His-tagged IIC^{Man} in *E. coli* BL21(DE3) pLysS pET28-C at 30°C. Lane 1: Precision plus protein standard (Biorad, Germany); lane 2: non-induced control, total cell protein; lane 3: induction control, total cell protein; lane 4: crude membrane fraction. The arrow indicates expression of the His-tagged IIC^{Man} at approximately 24 kDa. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

Similar studies were performed to induce the IID^{Man} subunit. An initial induction of IID^{Man} at 37°C for 3 hours showed, that a protein of the expected size was synthesized. However, the level of synthesise was relatively low compared to the other proteins present in the cell extract (Figure 4.3). Figure 4.3, lane 4 of this figure shows the over-expression of protein in the cell membrane fraction, with a molecular weight of 27 kDa. The non-induced fraction (Figure 4.3., lane 2) showed an absence of a 27 kDa protein band. Therefore the appearance of this band was attributed to the expression of the IID^{Man} protein.

The induction temperature was decreased from 37°C to 30°C and the induction time was increased from 3 hours up to 5 hours, in an attempt to decrease the amount of cellular

protein expression relative to the expression of the target membrane protein. Figure 4.4, lane 4 show clearly that an induction at 30°C for 5 hours, relatively increased the level of IID^{Man} (27 kDa) as indicated by the intensity of the banding pattern. Therefore, relative expression of the IID^{Man} was greatly improved by induction at 30°C for 5 hours (Figure 4.4).

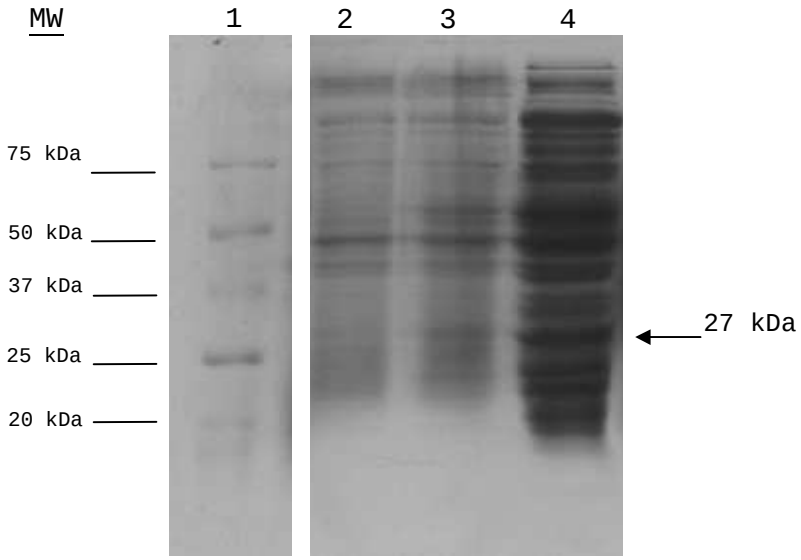


Figure 4.3. A 10% (w/v) SDS-PAGE gel highlighting the expression of His-tagged IID^{Man} in *E. coli* BL21(DE3) pLysS pET28-D at 37°C. Lane 1: Precision plus protein standard (Biorad, Germany); lane 2: non-induced control, total cell protein; lane 3: induction control, total cell protein; lane 4: crude membrane fraction. The arrow indicates expression of the His- tagged IID^{Man} at approximately 27 kDa. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

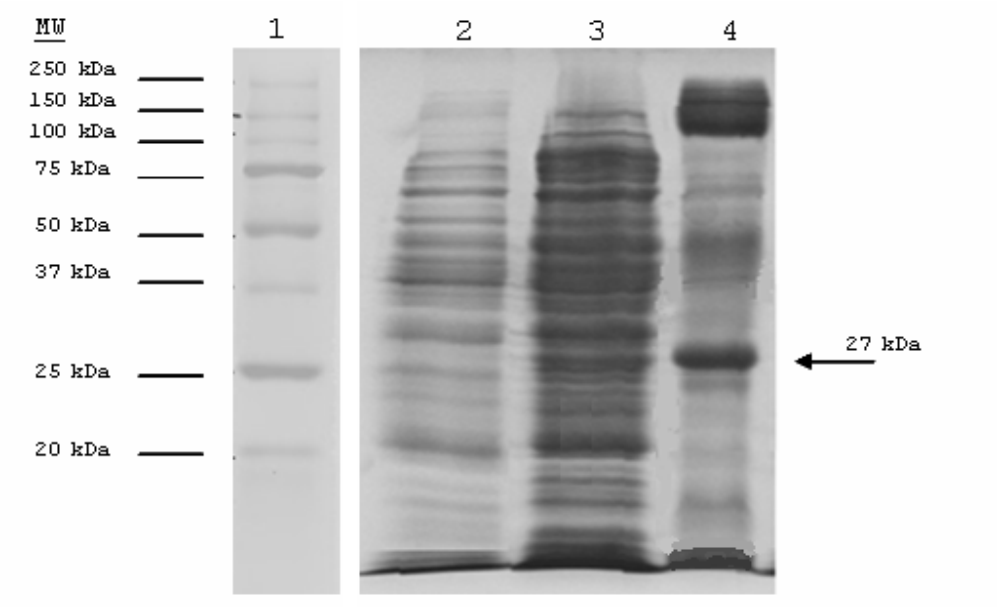


Figure 4.4. A 10% (w/v) SDS-PAGE gel highlighting the expression of His-tagged IID^{Man} in *E. coli* BL21(DE3) pLysS pET28-D at 30°C. Lane 1:; Precision plus protein standard (Biorad, Germany); lane 2: non-induced control, total cell protein; lane 3: induction control, total cell protein; lane 4: crude membrane fraction. The arrow indicates expression of the His- tagged IID^{Man} at approximately 27 kDa. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

Initially induction of $IIAB^{Man}$ protein at 37°C for 3 hours was performed. It was clear that a 38 kDa protein of the expected size was synthesized. Figure 4.5, lanes 3 and 4, showed that over-expression of the $IIAB^{Man}$ protein in the cytosolic and membrane fractions had occurred. This is because the $IIAB^{Man}$ subunit is membrane associated and not an integral membrane protein. Therefore, it is not surprising that the $IIAB^{Man}$ was expressed in both the cytosolic and membrane fractions.

However, after induction at 30°C for 5 hours, over-expression of the 37.6 kDa $IIAB^{Man}$ in the soluble cytosolic fraction was relatively higher than that found in the membrane fraction (Figure 4.6, lanes 4 and 5). Therefore the lowered temperature effect on protein expression of $IIAB^{Man}$ greatly enhanced the solubility of the protein.

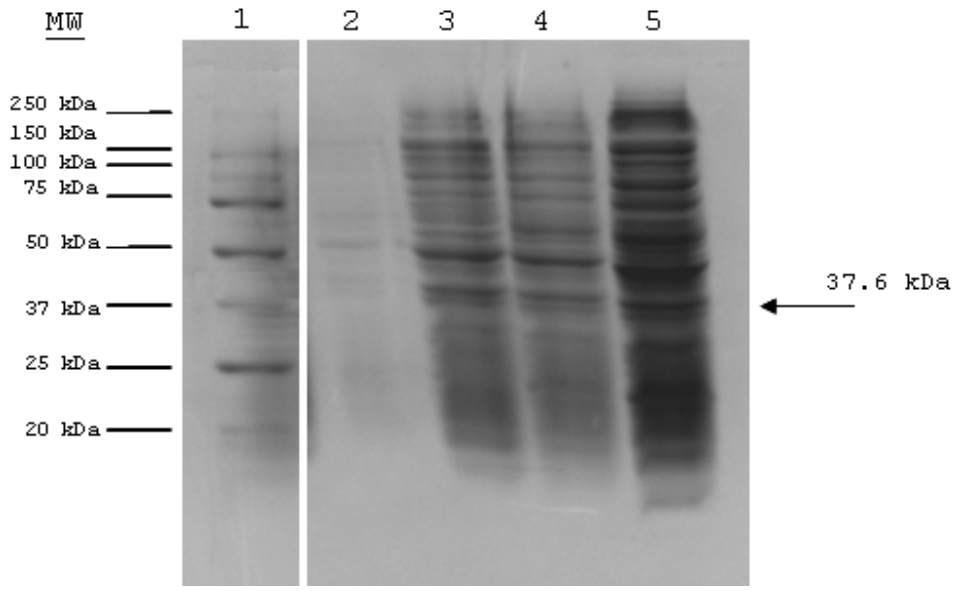


Figure 4.5. A 10% (w/v) SDS-PAGE gel highlighting the expression of His-tagged $IIAB^{Man}$ in *E. coli* BL21(DE3) pLysS pET28-AB at 37°C. Lane 1: Precision plus protein standard (Biorad, Germany); lane 2: non-induced control, total cell protein; lane 3: induction control, total cell protein; lane 4: cytosolic protein fraction; Lane 5: crude membrane fraction. The arrow indicates expression of the His- tagged $IIAB^{Man}$ in lane 3, 4 and 5 at 37.6 kDa in both the cytosolic and crude membrane fractions. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

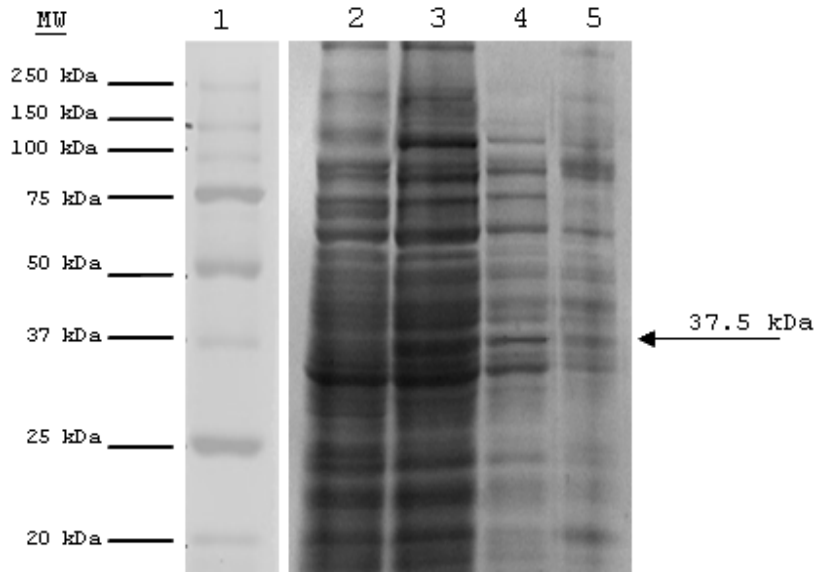


Figure 4.6. A 10% (w/v) SDS-PAGE gel highlighting the expression of His-tagged $IIAB^{Man}$ in *E. coli* BL21(DE3) pLysS pET28-AB at 30°C. Lane 1: Precision plus protein standard (Biorad, Germany); lane 2: non-induced control, total cell protein; lane 3: induction control, total cell protein; lane 4: cytosolic protein fraction; Lane 5: crude membrane fraction. The arrow indicates expression of the His- tagged $IIAB^{Man}$ in lane 3, and 4 at 37.5 kDa in the cytosolic protein fraction. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

4.3. Purification of the Individual Subunits of the Mannose PTS Permease of *L. monocytogenes* EGD

The helper plasmid pLysS found in *E. coli* BL21(DE3) pLysS encodes for the enzyme T7 lysozyme. The release of lysozyme played a dual role in protein purification. It was responsible for the decrease of basal protein expression and aided in the lysis of newly transformed *E. coli* BL21(DE3) pLysS host strain. A single freeze thaw cycle was sufficient to disrupt the cells integrity and ensure efficient cell lysis. This method ensured

that proteins released, were not subjected to mechanical denaturing, which is usually associated with more stringent lysis procedures such as sonication (Novagen, 2003).

4.3.1. Preparation of insoluble membrane and soluble cytosolic cell fractions

A total volume of 3 l cell culture of *E. coli* BL21(DE3) pLysS pET28-C, *E. coli* BL21(DE3) pLysS pET28-D and *E. coli* BL21(DE3) pLysS pET28-AB were used to prepare membrane and cytosolic fractions of each expression strain. The fractions were ultra-centrifuged, pooled and analysed on SDS-PAGE. The total protein concentration was determined, to indicate the level of protein expression in the cell fractions.

A Bradford assay was used to determine protein concentration (Bradford, 1976; Read & Northcote, 1981). Standard protein concentrations were made from a 1 mg ml^{-1} stock solution of bovine serum albumin (BSA) and a standard concentration curve was generated (Figure 4.7). This standard concentration graph was used to determine the unknown protein concentration in the sample (Figure 4.7).

The membrane protein content within isolated membranes was determined, and re-suspended in TSC buffer at a final membrane protein concentration of 10 µg $µl^{-1}$ as previously described (Von Jagow *et al.*, 2003). All membrane fractions were flash-frozen in a dry-ice isopropanol bath and stored at -70°C. This was performed in order to maintain protein integrity within the membrane fraction. A volume of 3 l gave a total yield of 17.2 mg and 15.8 mg protein from membrane fractions for *E. coli* BL21(DE3) pLysS pET28-C and *E. coli* BL21(DE3) pLysS pET28-D, respectively.

The cytosolic fraction containing the $IIAB^{Man}$ subunit was collected after ultra-centrifugation (see Chapter 2, Section 2.18). The membrane fraction or insoluble fraction was discarded, the supernatant immediately pooled and quantified. The total cytosol protein content was shown to be 38 mg from a 3 l volume of *E. coli* BL21(DE3) pLysS pET28-AB culture.

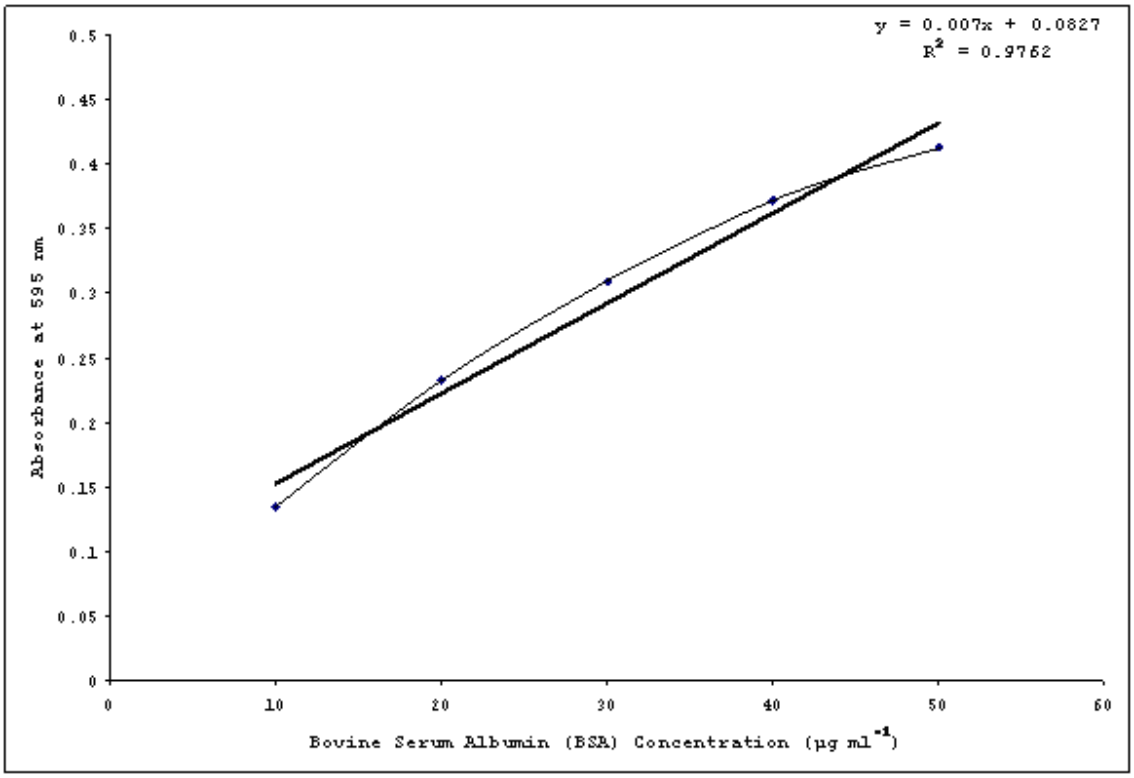


Figure 4.7. A Bradford assay standard concentration curve (BSA), to estimate the protein concentration of membrane, cytosolic and purified protein fractions.

4.3.2 Affinity Purification of His-Tagged $IIAB^{Man}$ IIC^{Man} and IID^{Man}

Membrane protein purification requires detergents to solubilise the cellular membrane. Most detergents are amphiphilic molecules that form micelles in water. They enable protein solubilisation by binding to the hydrophobic portion of the proteins on one side and interact with the aqueous phase on the other side. Properties and uses of detergents maybe classified according to their hydrophobic and hydrophilic portions. The most critical characteristic of a detergent would be the charge and the critical micelle concentration (CMC) value. The CMC value would therefore inform, what the minimal concentration of free detergent would be required to keep membrane proteins in solution (Von Jagow *et al.*, 2003). A sugar detergent which has a hydrophobic portion linked by a glycosidic bond to a sugar moiety was found to be most compatible in order to solublize the membrane fractions. A neutrally charged sugar detergent called octyl-β-D-glucopyranoside (OGP) was used for membrane solubilization. This sugar detergent with an appropriate ionic strength was found to be most suitable for extraction of the EII_t^{Man} integral membrane proteins, to ensure that an active form of the integral membrane protein was to be isolated (Von Jagow *et al.*, 2003).

Imidazole is a component of the His-tag structure. The imidazole ring within the 6 × His-tag structure binds to the nickel ions (Ni^{2+}) immobilized by nitrilotriacetic acid (NTA) groups on the matrix. Therefore, the purification procedure was performed over an imidazole gradient. Loading of the Ni^{2+}-NTA resin was most efficiently done in batch to ensure efficient binding of His-tagged proteins to the Ni^{2+}-NTA resin. Furthermore, 5 mM imidazole was added to the lysate, to decrease non-specific binding of cellular proteins to Ni^{2+}-NTA resin (Von Jagow *et al.*, 2003). It was imperative that the purification procedure was carried out at 4°C as the His-tag tends to aggregate at room temperature (Qiagen, 2002).

The purification was monitored by collecting 2 ml fractions and their protein concentration determined at an absorbance of 280 nm (A_{280}). The absorbance readings were plotted, in order to generate chromatographs for each purification procedure (Figures 4.8, 4.10 and

4.12). These chromatographs gave an indication at which point His-tagged proteins were eluted.

Therafter, fractions with a 280 nm absorbance greater than 1.2 were analysed on SDS-PAGE gels (see Figures 4.9, 4.11 and 4.13). Each gel profile shows the pooled fractions of the cell lysate, flow through and wash procedures, respectively (see Figures 4.9 (a), 4.11 (a) and 4.13 (a)).

Chromatograph profiles generated from each purification procedure indicated that proteins were eluted between fractions 20 to 25. This was confirmed by SDS-PAGE gel analysis of these fractions (see Figures 4.9 (b), 4.11(b) and 4.13(b)). The , IIC^{Man}, IID^{Man} and $IIAB^{Man}$ proteins resolved on SDS-PAGE at molecular weights, 28 kDa, 31 kDa and 38 kDa respectively. Despite stringent wash steps during the procedure all elution's showed the presence of a co-eluted protein, observed at molecular weights 68 kDa, 60 kDa and 69 kDa respectively.

The eluted fractions were dialysed, to decrease the imidazole and detergent concentration. Subsequently the eluants were quantified using a Bradford Assay, to give an estimate of the protein content present after purification. Preparations of IIC^{Man}, IID^{Man}, and $IIAB^{Man}$ subunits contained 274 µg ml^{-1}, 256 µg ml^{-1} and 380 µg ml^{-1}, respectively. We proceeded with western blot analysis to see if these proteins were in fact His-tagged proteins, corresponding to the appropriate molecular weight of the subunits of EII_t^{Man}.

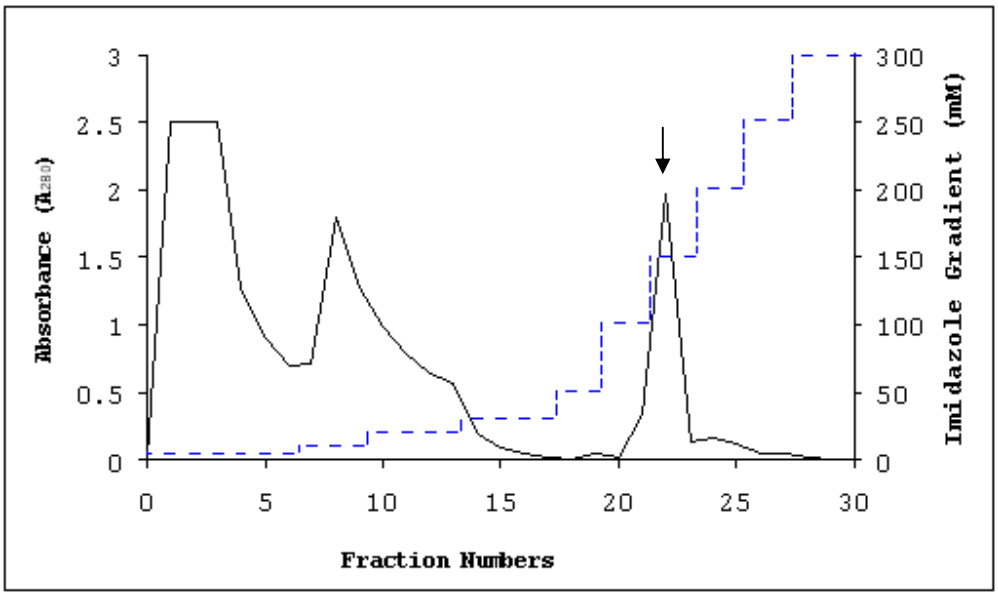
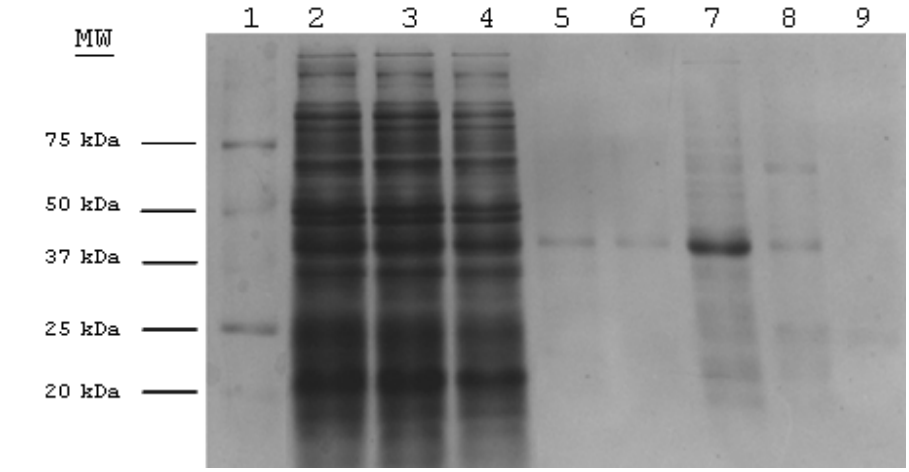
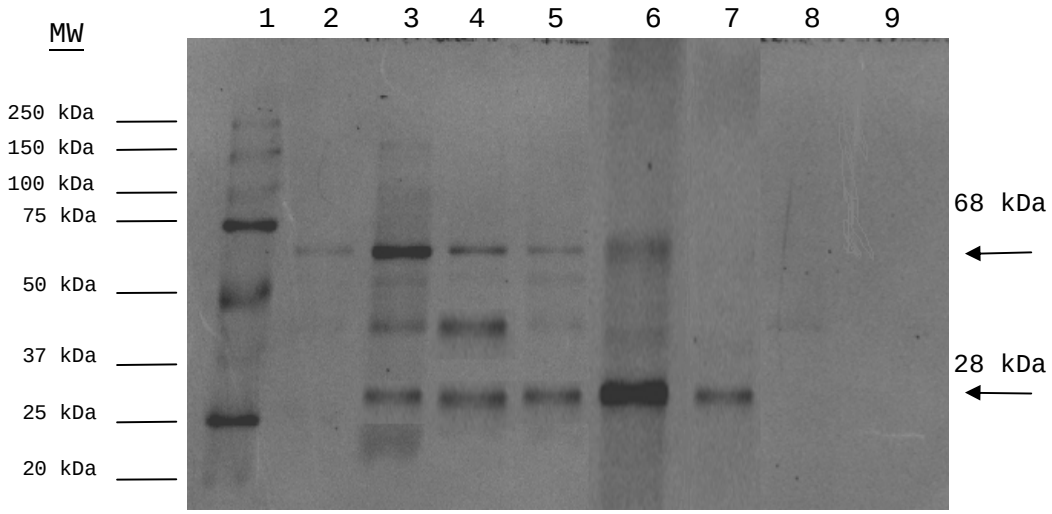


Figure 4.8. Chromatograph outlining the Ni^{2+}-NTA purification of His-tagged IIC^{Man} subunit of the mannose PTS permease. Fractions 1-7: flow-through with 5 mM imidazole; fractions 8-18: wash steps at various imidazole concentrations; fractions 20 – 25: elutions. The arrow depicts fractions containing the eluted IIC^{Man} subunit. The procedure was performed on an imidazole step gradient (depicted by the broken line), ranging from 5 mM to 300 mM.



(A)



(B)

Figure 4.9. SDS-PAGE gels 10% (w/v), analysis of protein fractions from Ni^{2+}-NTA purification of His-tagged IIC^{Man} subunit. Gel **(A)** lane 1: Precision plus protein standard (Biorad, Germany); lane 2: crude membrane cell lysate; lane 3: flow through pooled fractions 1-7 from Ni^{2+}-NTA chromatography; lane 4-9: wash steps 1-6 (fractions 8-18) with a varying concentration of imidazole ranging from 5 mM to 30 mM. Gel **(B)** Fractions were eluted over an imidazole concentration gradient. Lane 1: Precision plus protein standard (Biorad, Germany); Lanes 2-9: elution of His-IIC^{Man} (fractions 20-26) with the imidazole concentration varying from 60 mM to 300 mM. The arrows show a 68 kDa protein and a 28 kDa His-IIC^{Man} protein. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

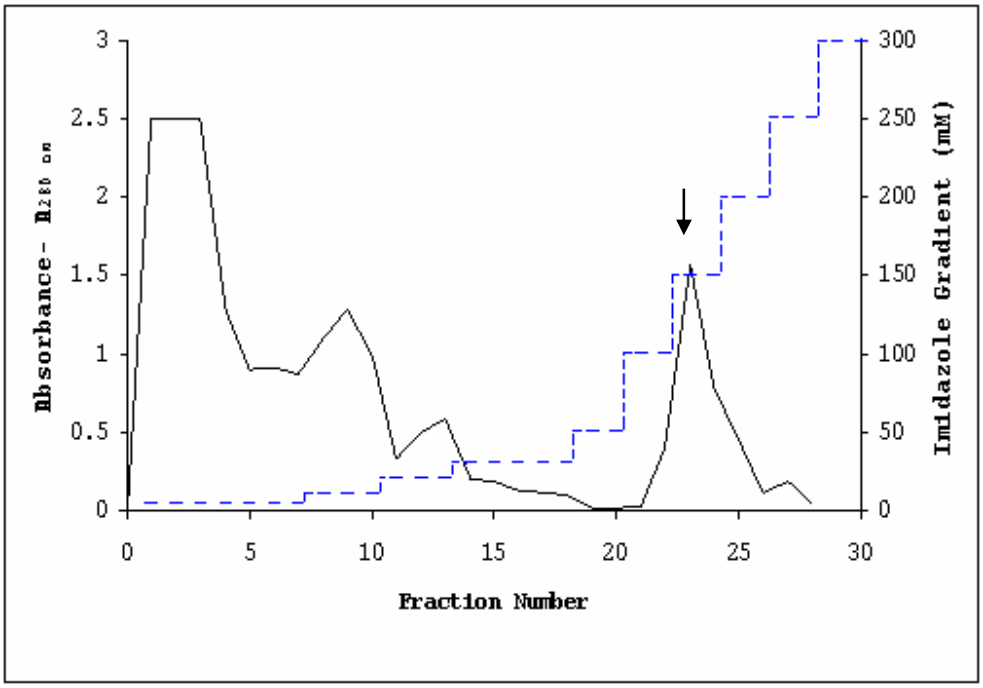
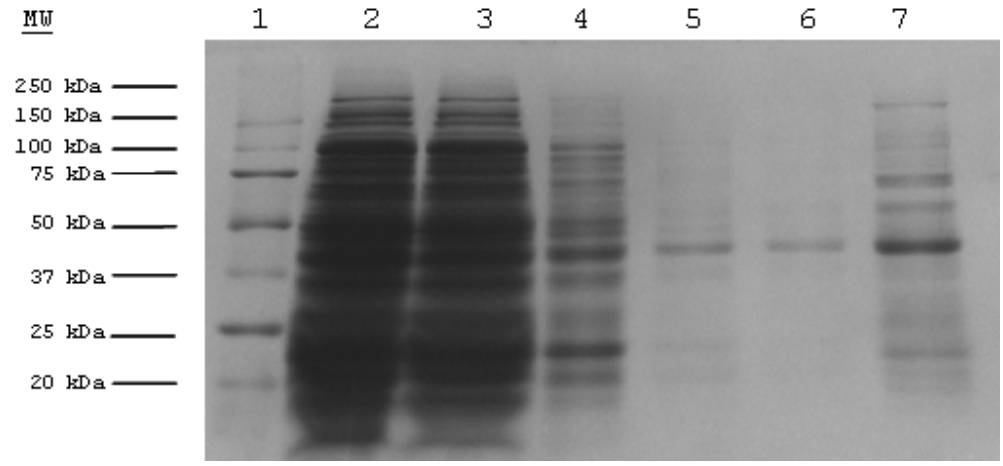
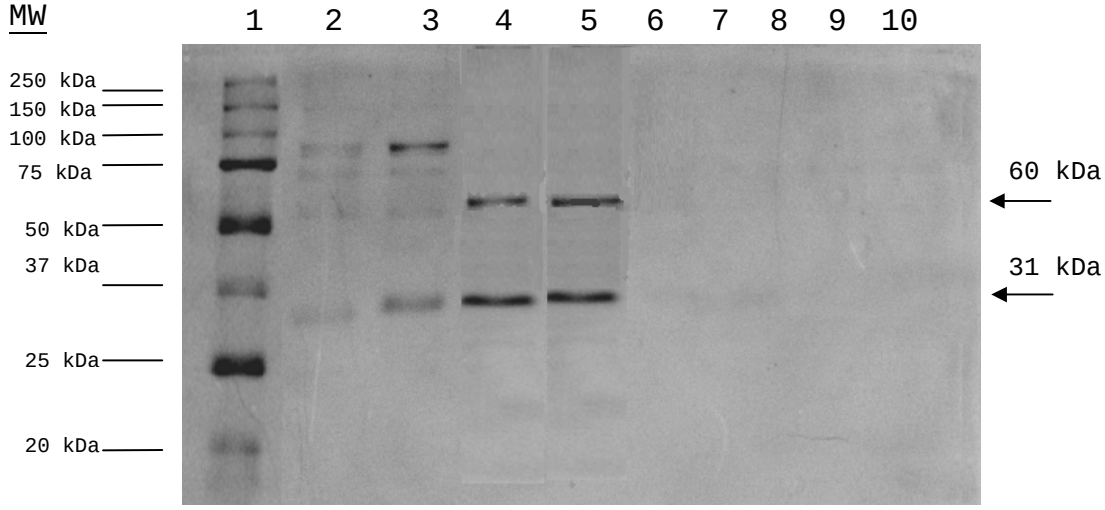


Figure 4.10. Chromatograph outlining the Ni^{2+}-NTA purification of His-tagged IID^{Man} subunit of the mannose PTS permease. Fractions 1-7: flow-through with 5 mM imidazole; fractions 8-18: wash steps; fractions 20 – 25: elutions- the arrow depicting the major optical density peak, contains the eluted IID^{Man} subunit. The procedure was performed on an imidazole step gradient (depicted by the broken line), ranging from 5 mM to 300 mM.



(A)



(B)

Figure 4.11. SDS-PAGE gels 10% (w/v), analysis of protein fractions from Ni^{2+}-NTA purification of His-tagged IID^{Man} subunit. Gel **(A)** lane 1: Precision plus protein standard (Biorad, Germany); lane 2: crude membrane cell lysate; lane 3: flow through pooled fractions 1-12 from Ni^{2+}-NTA chromatography; lane 4-9: wash steps 3-6 (fractions 8-18) with a varying concentration of imidazole ranging from 20 mM to 35 mM. Gel **(B)** Fractions were eluted over an imidazole concentration gradient. Lane 1: Precision plus protein standard (Biorad, Germany); Lanes 2-8: elution of His-IID^{Man} (fractions 20-26) with the imidazole concentration varying from 60 mM to 300 mM. Lanes 9-10: empty. The arrows indicate a 60 kDa protein, and the 31 kDa IID^{Man} protein. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

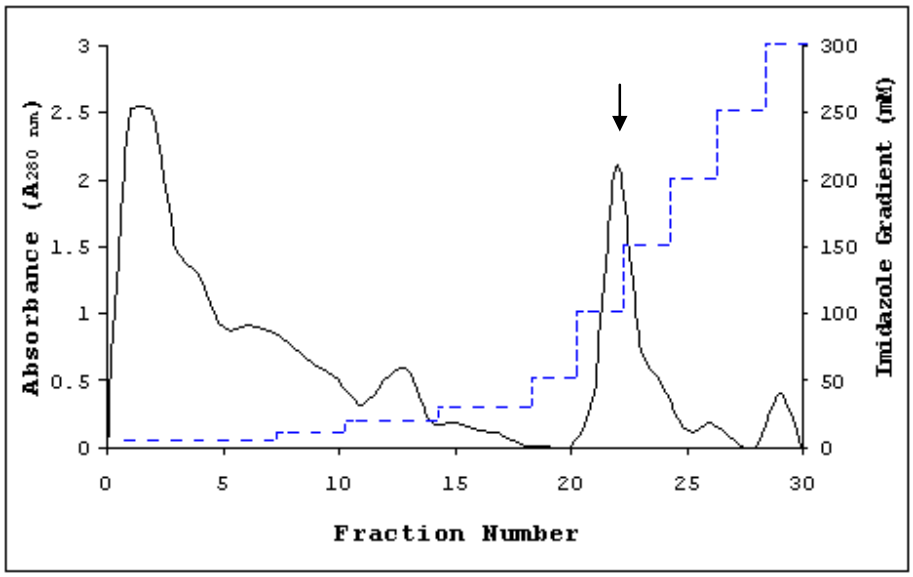
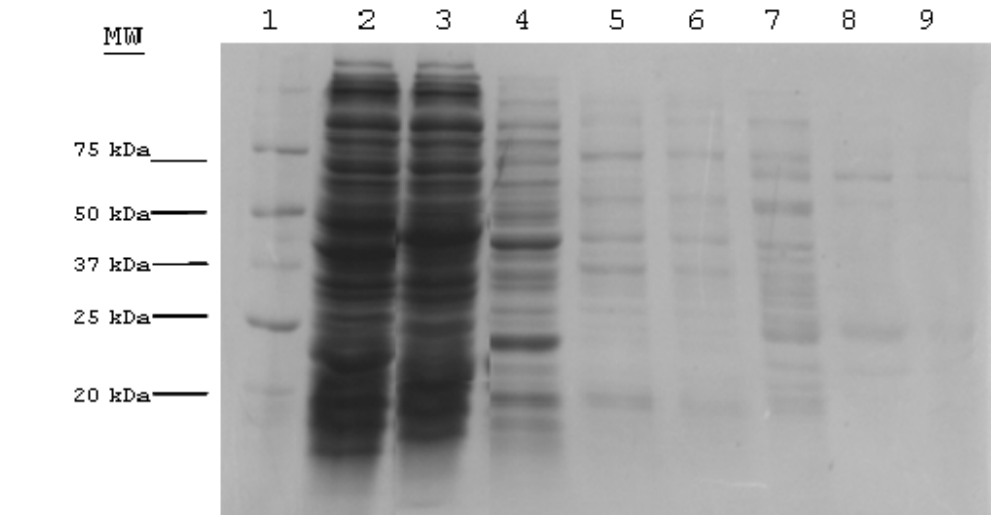
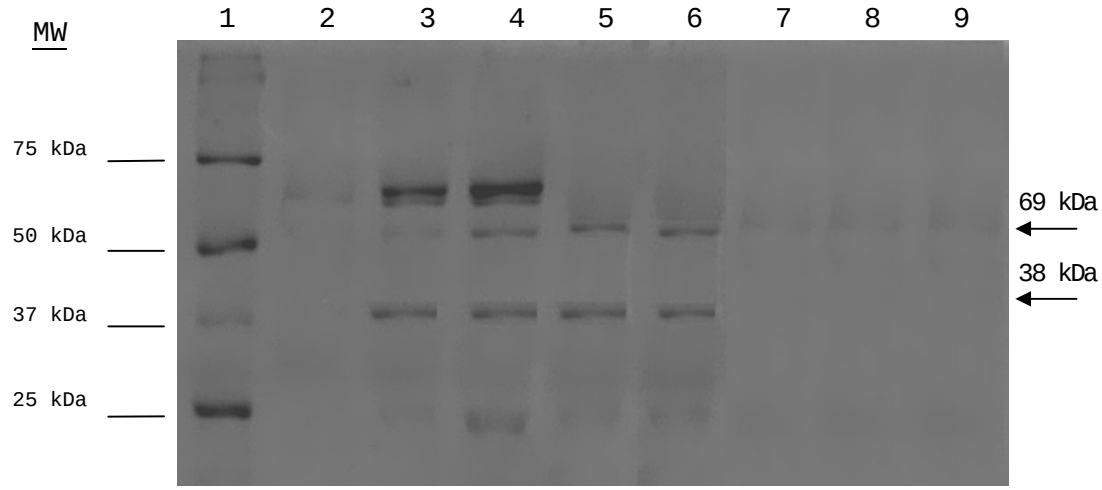


Figure 4.12. Chromatograph outlining the Ni^{2+}-NTA purification of His-tagged $IIAB^{Man}$ subunit of the mannose PTS permease. Fractions 1-7: flow-through containing 5 mM imidazole; fractions 8-18: wash steps; fractions 20 – 25: elutions- the arrow depicts the major optical density peak, containing the eluted $IIAB^{Man}$ subunit. The procedure was performed on an imidazole step gradient ranging from 5 mM to 300 mM (depicted by the broken line).



(A)



(B)

Figure 4.13. SDS-PAGE gels 10% (w/v), analysis of protein fractions from Ni^{2+}-NTA purification of His-tagged $IIAB^{Man}$. Gel **(A)** Lane 1: Precision plus protein standard (Biorad, Germany); lane 2: crude cytosolic fractions; lane 3: flow through pooled fractions 1-12 from Ni^{2+}-NTA chromatography; lanes 4-9: wash steps 1-6 (fractions 8-18) with a varying concentration of imidazole ranging from 10 mM to 35 mM. Gel **(B)**. Lane 1: Precision plus protein standard (Biorad, Germany); Lanes 2-9: elution of His-$IIAB^{Man}$ (fractions 20-26) with the imidazole concentration varying from 60 mM to 300 mM. The arrows (A) show what maybe referred to as the 69 kDa protein, whilst arrow (B) to shows the expected 38 kDa His-$IIAB^{Man}$ protein. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

4.4. Western Blot of Proteins Purified from Ni^{2+} -NTA Purification of Metal Chelate Chromatography

The aim of the following experiment was to confirm the purification of the His-tagged subunits of the mannose PTS permease

SDS-PAGE was carried out on duplicate 12% (w/v) SDS-PAGE gels. One gel was stained with Coomassie Blue staining procedure to visualize the protein profile (Figure 4.14), whilst the second gel was utilized for protein transfer onto a nitrocellulose membrane. Detection of His-tagged proteins was determined using anti-His-tag antibodies (Roche, Germany). BM Blue peroxidase (POD) (Roche, Germany) precipitating, chromogenic substrate was used to aid in visually observing detected His-tagged protein.

Western blot results, shown in Figure 4.15 confirm the presence of His-tagged fusion proteins. Gel quantitation using Quant One image analysis software (Biorad, Germany), indicated that a total of seven proteins were detected. The detection of the positive control (His-tagged MTI-MMP ectodomain protein) indicated that the experimental conditions were appropriate to detect His-tag proteins.

The IIC^{Man} preparation resulted in the presence of two His-tagged proteins at molecular weight 28 kDa and 55 kDa (see Figure 4.15, Lane 6). IID^{Man} preparations resulted in the presence of His-tagged proteins of molecular weights 31 kDa and 60 kDa (see Figure 4.15, lane 7). $IIAB^{Man}$ preparations showed a presence of a 38 kDa and 68 kDa proteins (see Figure 4.15, lane 5). Proteins with molecular weights of 28 kDa, 31 kDa and 35 kDa, correspond to the IIC^{Man}, IID^{Man}, and $IIAB^{Man}$ respectively. It was concluded that all three subunits of the mannose PTS permease were in fact expressed and purified as they were determined to have the correct molecular weight. However, it was interesting to observe that these three preparations also contained co-eluted proteins, with attached His-tags, evident by western blot analysis (Figure 4.15, lanes 5,6 and 7). The co-eluted proteins seem to resolve in 12% (w/v) SDS-PAGE gels at almost twice the molecular weight of their corresponding proteins' in (Figure 4.15 lanes 5, 6 and 7).

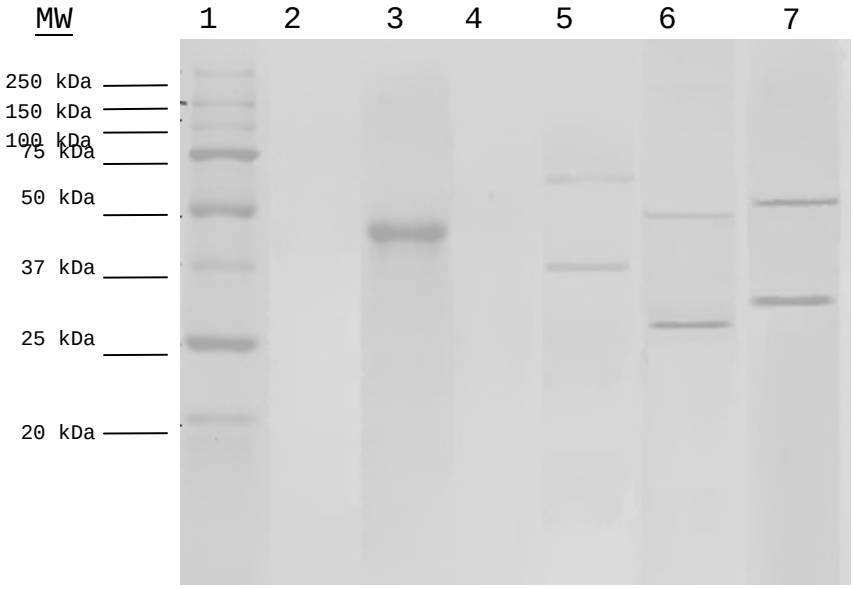


Figure 4.14. An SDS-PAGE 12% (w/v) reference gel, with the protein profile of purified subunits of the mannose PTS permease. Lane 1: molecular weight marker Precision plus protein standards (Biorad, Germany). Lane 3: 6 × His-tagged MTI-MMP ectodomain, (44 kDa), positive control (Mr D. Van Rooyen, personal communication, 2006). Lane 5: eluant containing, 69 kDa co-purified and 35 kDa His-IIAB^{Man} proteins. Lane 6: eluant, containing, 55 kDa co-purified and 28 kDa His-IIC^{Man} proteins. Lane 7: eluant containing, 60 kDa co-purified and 31 kDa His-IID^{Man} proteins. Lanes (2) and (4) were left empty. This gel served as a reference gel for Western blot analysis. The protein profile was analysed using Quant One image analysis software program (Biorad, Germany).

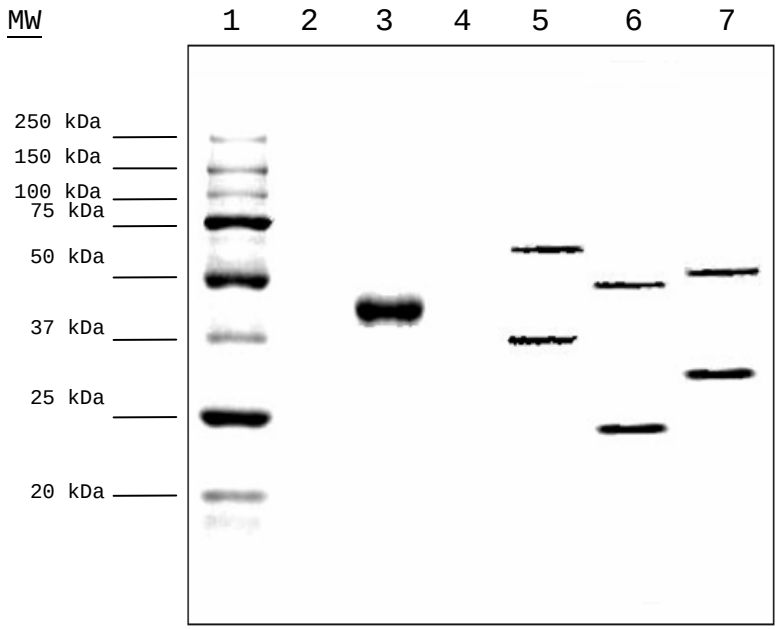


Figure 4.15. A Western Blot profile, of purified His-tag proteins on a nitrocellulose membrane, detected with anti-His-tag peroxidase conjugate mouse monoclonal antibodies. Lane 1: the transferred molecular weight marker stained with Ponceau S. Lane 3: shows the detected positive control. 6 × His-tagged MTI-MMP ectodomain, (44 kDa). Lane 5: detected 35 kDa His-IIAB^{Man} and co-purified 69 kDa proteins. Lane 6: detected 28 kDa His-IIC^{Man} and co-purified 55 kDa proteins. Lane 7: detected 31 kDa His-IID^{Man} and co-purified 60 kDa proteins.

4.5 Summary

Expression studies were performed with the temperature varying from 37°C to 30°C and a time variation from 3 hours to 5 hours. Results showed that the subunits of the EII_t^{Man} were expressed at an optimal temperature of 30°C for 5 hours.

These studies indicated that at 37°C and 30°C the IIC^{Man} and IID^{Man} subunits were expressed in the membrane fraction of the *E. coli* host strain. However, at 30°C, these proteins displayed an increased level of expression relative to the expression of other cellular proteins, within the host strain.

Expression of the $IIAB^{Man}$ at 37°C occurred in both the soluble cytosolic and insoluble membrane protein. However at 30°C expression of the membrane associated subunit was significantly greater in the soluble cytosolic fraction as opposed to the membrane fraction.

A Bradford assay was used to determine the total protein content present in induced cultures. A volume of 3 l broth culture gave a total yield of 17.2 mg and 15.8 mg total protein in the membrane fractions of IIC^{Man} and IID^{Man} respectively. The total cytosol protein content was shown to be 38 mg from a 3 l volume of culture for preparation of $IIAB^{Man}$. A freeze thaw method was followed to disrupt cells as per the manufacturer's instructions. However, the relative protein yield was low. Therefore other means of cell disruption should have been used.

Individual subunits of the EII_t^{Man} were purified by metal chelate chromatography. All subunits, IIC^{Man}, IID^{Man} and $IIAB^{Man}$ eluted at an imidazole concentration of approximately 150 mM. However, each preparation contained the protein corresponding to the correct molecular weight as well as a co-eluted protein. These preparations of IIC^{Man}, IID^{Man} and $IIAB^{Man}$ were subsequently dialysed, analysed on SDS-PAGE and quantified using the Bradford assay. An estimated protein concentration for IIC^{Man}, IID^{Man} and $IIAB^{Man}$ preparations were 274 µg ml^{-1}, 256 µg ml^{-1} and 380 µg ml^{-1}, respectively.

The Bradford assay, is a dye binding method, using Coomassie blue G-250, to determine protein concentration. However, the assay may become biased by the presence of a high concentration of detergent in the test sample (Dennison, 2003; Von Jagow *et al.*, 2003). Therefore it was imperative that the eluted preparations were first dialysed before quantifying the protein content in each preparation. The reason being is that dialysis enabled buffer exchange, to decrease the imidazole and detergent content within samples

Preparations were then analysed on a western blot. The analysis confirmed the presence of His-tagged proteins, His-IIC^{Man}, His-IID^{Man} and His-$IIAB^{Man}$ with molecular weights of 28 kDa, 31 kDa and 35 kDa, respectively. It was surprising that the co-purified proteins also bound the anti-His-tag peroxidase conjugate mouse monoclonal antibody. Therefore, this suggests that the co-purified proteins also contain a His-tag. We may infer that the subunits of EII_t^{Man}, were partially purified due to the presence of the co-purified proteins. A pure sample was not obtained. The association of these His-tagged co-purified proteins were not known.

CHAPTER 5

Final Conclusion and Future Directions

5.1. Amplification and Cloning the *mptC, mptD,* and *mptAB* Genes of *L. monocytogenes* EGD

The initial step in the project was to amplify each of the subunit genes of the mannose PTS permease. This was successfully achieved. However, an error was made during primer design; an extra ATG codon was incorporated in the forward primers. This ATG codon may well have added an extra methionine residue at the N-terminus of each target protein. This error was only noticed during the preparation of this manuscript and ideally should be corrected in any future work. However, it seems unlikely this error caused any significant affect on downstream experimentation.

The individual subunit genes of the mannose PTS permease were successfully cloned into the pET28a expression vector. The presence of the cloned insert was confirmed by colony PCR. During screening for the presence of recombinant pET28a, transformants were observed to have a distinct phenotype: colonies which appeared to have a translucent phenotype were shown to contain the recombinant pET28a, as opposed to non-recombinant pET28a found in colonies with an opaque white phenotype.

Recombinant pET28a clones (pET28-C, pET28-D, and pET28-AB) were sent for sequencing to verify if any mutations were introduced. However, poor quality data was returned. Due to the additional cost implications for a second sequencing procedure, this part of the project remained incomplete. *Taq* polymerase was used for PCR amplification. *Taq* polymerase has a relatively high mutation rate. The possibility of introducing mutations could have been minimized by using a polymerase with proof-reading ability (e.g. *Pfu* polymerase). However, as a matter of course, all newly developed recombinant products should have been sequenced.

5.2. Expression and Purification of the IIC^{Man}, IID^{Man} and $IIAB^{Man}$ Polypeptides in *E. coli* BL21(DE3) pLysS Clones

Over expression of membrane proteins is problematic because it may interfere with the membrane potential, upon insertion into the host cell membrane. This may prove toxic to the cell, therefore it was important to minimize background expression of target genes. *E.*coli BL21(DE3) pLysS was used as the host expression strain. This strain encodes for T7 lysozyme which lowers the background expression of target genes, and allowed for expression of membrane proteins.

Each construct was transformed into *E. coli* BL21(DE3) pLysS. Initially the His-IIC^{Man} (24 kDa), His-IID^{Man} (27 kDa) and His-$IIAB^{Man}$ (38 kDa) were successfully expressed at 37°C for 3 hours. Expression of His-IIC^{Man} and His-IID^{Man} was localized in the membrane fraction, whilst the His-$IIAB^{Man}$, was localized in both the soluble cytosol and membrane fractions. In an attempt to maximize protein expression, an induction at 30°C for 5 hours showed a relative increase in the expression level of all proteins. Under these conditions the His-IIC^{Man} and His-IID^{Man} remained in the membrane fractions, whilst the expression level of the $IIAB^{Man}$ subunit was observed to be relatively higher in the soluble cytosolic fraction as opposed to the membrane fraction. The $IIAB^{Man}$ is a dimeric structure which consists of the IIA soluble cytosolic domain and the IIB domain which enables association with the integral membrane subunits (Robilllard & Broos, 1999). It is possible that the lowered temperature may have affected the conformation of the dimer structure of this subunit such that relatively more of this protein appears in the cytosolic faction.

Crude membrane-bound proteins analysed in 10% (w/v) SDS-PAGE gels seem to resolve at a lower molecular weights than expected. In crude extracts, after expression of the IIC^{Man} and IID^{Man} proteins, their apparent molecular weights were 24 kDa and 27 kDa, respectively. However, after purification the IIC^{Man} and IID^{Man} had apparent molecular weights of 28 kDa and 31 kDa, respectively. A previous study exploring the purification of the subunit proteins of mannose PTS permease of *E. coli* showed that migration of membrane proteins on SDS-PAGE, tend to resolve at a slightly lower molecular weights

than the expected protein size, and this was attributed to hydrophobic nature of membrane proteins (Erni *et al.*, 1989) Perhaps the presence of high concentrations of hydrophobic proteins in crude membrane fractions in someway modifies the migration of target proteins in SDS polyacrylamide gels.

In each instance purification of the individual $EIIt^{Man}$ subunits yielded two proteins; His-IIC^{Man} (28 kDa) and co-eluted 55 kDa protein; His-IID^{Man} (31 kDa) and a co-eluted 60 kDa protein; and His-$IIAB^{Man}$ (35 kDa) and a co-eluted 68 kDa protein. The nature of the co-purified proteins is unknown. It was assumed that these proteins are possibly His-tagged as it certainly had a strong binding affinity to the Ni-NTA resin. Western blot analysis showed that these co-purified proteins cross reacted with the anti-His-tag peroxidase conjugate mouse monoclonal antibodies. Therefore Western blot results suggest that these proteins may have an attached His-tag.

In these experiments samples were prepared as described by Von Jagow *et al*. (2003). This method requires that samples are pre-treated by heat incubation at 45°C and subsequently run on SDS-PAGE gels. Careful observation of the eluted and co-purified proteins showed that these proteins seem to have resolved in 10% (w/v) SDS-PAGE at twice the molecular weight of its corresponding eluant. This implies that these co-purified proteins could very well be the protein dimer version of its corresponding eluant. Hence analysis of purified proteins could be repeated using an SDS-PAGE sample preparation procedure which incorporates boiling samples before electrophoresis, this should be sufficient to disrupt the dimers formed (Dennison, 2003).

However, should this postulation be incorrect, then it would be necessary to further purify these proteins. Dimers maybe identified using methods such as isoelectric focusing or peptide finger printing. These methods have previously been performed for membrane protein isolation (Erni & Zanolari, 1985; Erni *et al.*, 1987; Erni *et al.*, 1989; Huber & Erni, 1996; Robilllard & Broos, 1999).

5.3. Future Direction

During the early stages of this study leucocin A, a class IIa bacteriocin was purified as previously described (results not shown) (Uteng *et al.*, 2002). The intended use of the class IIa bacteriocins and the purified subunits of the EII_t^{Man} were to facilitate subsequent protein interaction studies using Biacore, real time biomolecular interaction assay.

Therefore, once a purified form of the individual subunits is obtained, these His-tagged subunits of the EII_t^{Man}, could be individually attached to a nickel ion Biacore chip. The class IIa bacteriocin could be passed through the flow channel in order to determine which subunit of the mannose PTS permease is the receptor molecule for class IIa bacteriocins.

The increasing occurrence of antibiotic resistance pathogens means that alternative strategies of their control need to be developed. Therefore, leucocin A and an understanding of its mechanisms of interaction with the target cell maybe the proposed solution. The above mentioned study is fundamental in forming a basis to establish an understanding if the mannose PTS permease is the receptor molecule of class IIa bacteriocins. The advent of multi-drug resistant pathogens implies that there is there is a need for antimicrobial peptides as an alternative treatment against harmful bacteria such as *L. monocytogenes*.

Chapter 6

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