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Characterisation of the oligopeptide permease of Escherichia coli

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Characterisation of the Oligopeptide Permease of *Escherichia coli*

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Contents

Abbreviations

ϕ	Phi, backbone torsion angle about N-C^{α}
ψ	Psi, backbone torsion angle about C^{α}-C
AA	amino acid
ABC	ATP-binding cassete
ABTS	2,2`-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid
ADP	adenosine diphosphate
AIDS	Acquired immuno deficiency sindrome
AlaP	L-1-aminoethyl phosphonic acid
Ala_4P	Trialanyl-[1-aminoethylphosphonic acid]
alafosfalin	L-alanyl-L-1-aminoethyl phosphonic acid
APD	affinity peptide
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCA	bicinchoninic acid
Bpoc	2-(4-biphenyl)-isopropoxycarbonyl
BSA	Bovine serum albumine
NMR	nuclear magnetic resonance
CFCR	cystic fibrosis trnasmenbrane conductence regulator
CFS	competence and sporulation factor
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming units
Cm	chloramphenicol
CSP	competence-stimulating peptide
Da	Dalton
DAp	meso-diaminopimelic acid
DMF	dimethylformamide
DMS	dimethylsulfide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dpp(A)	Dipeptide-transport system (binding protein)

DTT	dithiothreitol
ECL	Western blotting detection reagents
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked inmuno sorbent assay
EtBr	ethidium bromide
Fc	fragment that crystallize
FCA	Complete Freund`s adjuvant
FCS	foetal calf serum
FIA	Freund`s complete adjuvant
Fmoc	9-fluorenylmethoxycarbonyl
FPLC	fast protein liquid chromatography
GBP	D-glucose/D-galactose binding protein
Glc	glucose
GlcNAc	N-acetyl glucosamine
HOBt	N-hydroxybenzotriazole
HPLC	High pressure liquid chromatography
Hpp	hexa-heptapeptide permease
HT	hypoxanthine-thymidine
IEF	isoelectric focussing
IMS	Industrial methylated spirit
IVM	*in vitro* mutagenesis
kb	kilo base
K_d	dissociation constant
KDO	3-deoxy-β-D-mannosyl-2-octulopyranosonic acid
LAOBP	lysine/arginine/ornithine binding protein
LB(medium)	Luria-Berthani
LIVBP	leucine/isoleucine/valine binding protein
lpp	lipoprotein gene
LPS	lipopolysaccharide
Lrp	leucine-responsive protein
LTA	lipoteichoic acid
MAb	monoclonal antibody
MDR	multidrug resistance p-glycoprotein
MIC	minimum inhibitory concentration
MOPS	3-[N-Morpholino] propane sulphonic acid
MBP	maltodextrin binding protein
MurNAc	N-acetyl muramic acid
NBDS	nucleotide-binding domains
NBT	nitro blue tetrazolin
OM	outer membrane
Omp	outer membrane protein
Opp(A)	oligopeptide permease (binding protein)

ORI	origin
Orn_3	triornithine
PEG	polyethylene glycol
PAGE	Polyacrylamide gel electrophoresis
BPD	binding protein-dependent
PBP	phosphate binding protein
PBS	phosphate buffer saline
pI	isoelectric point
PLG	prolyl-leucyl-glycine amide
PLS	proteliposoma
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
PPF	periplasmic protein fraction
PVC	polyvinyl chloride
RbsCDA	Ribose transporter
R_f	Relative mobility
RNA	ribonucleic acid
RPC	reverse phase chromatography
rpm	revolutions per minute
SBP	sulphate binding protein
ScrY	sucrose-specific channel forming proteins
SDS	sodium dodecyl sulphate
SFW	sterile filtered water
STET	(boiling method)
Tap	Dipeptide transport associated protein
t-Boc	butoxycarbonyl
TBS	Tris buffer solution
t-Bu	tert-butyl ether
Tc	tetracycline
TBE	Tris-borate
TFA	trifluoracetic acid
ThuBCD	iron-hydroxamate transporter
Tor	triornithine resistan
Tpp	tripeptide permease
Tris	tris (hydroxymethyl) aminomethane
Tsx	nucleotide-specific channel
UDP	uridyl diphosphate
Z	benzyloxycarbonyl

Summary

Chapter I

SUMMARY

The oligopeptide permease (Opp) in *E. coli* consists of five proteins, OppABCDF. The periplasmic oligopeptide binding protein (OppA) acts as the initial receptor for the uptake of oligopeptides, and interacts with the integral membrane components (possibly just OppB and OppC) which form a channel through which the peptide passes. The other two membrane associated components, OppD and OppF couple ATP hydrolysis to peptide translocation. Genetic and biochemical evidence that support a role for the Asp300 of *E. coli* OppA in the interaction with the membrane components are presented in this study.

Two *E. coli* mutants PA0522 and PA0523 with a point mutation in OppA [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA, respectively were used in this study. Both OppA mutant proteins have the ability to bind peptides. However, the oligopeptide permease system of these mutants is completely defective in peptide transport. It was concluded that the residue Asp-300 of *E. coli*, that is located at the surface of the oligopeptide binding protein (OppA), is intimately involved in the events that allow the translocation of the ligand from the OppA-ligand complex through the membrane associated components of the oligopeptide permease.

Compensative mutants able to recover the oligopeptide permease function for the OppA defect were isolated. The characterisation of these mutants and their oligopeptide binding protein, indicated that the recovery of the oligopeptide permease activity most likely arose from changes in the membrane components. These results provide supportive evidence of the role of Asp300 in the interaction with the membrane components.

Introduction

Chapter II

INTRODUCTION

AND

LITERATURE REVIEW

Peptides can serve as a sole carbon and nitrogen source for bacterial growth (Payne, 1980), and play an important role in the nutrition of most organisms including plants (Higgins and Payne, 1982) and animals (Matthews and Payne, 1975). Small peptides also serve many specific biological functions as hormones, toxins or antibiotics and synthetic peptides are increasingly being used in the design of novel antibiotics and chemotherapeutic agents (Higgins, 1987). Because peptide uptake systems have broad substrate specificity it has been possible to use peptides as carrier prodrugs. Thus, normally impermeable toxic moieties can be attached to a peptide and then be transported into the cell (Tyreman *et al.*, 1992).

2.1 Bacterial Cell Surface and its Influence on Substrate Transport

The transport systems involved in uptake of exogenous nutrients are located in the cytoplasmic membrane, which is protected by a rigid external structure called the cell wall. This structure that surrounds the cell, varies from species to species, but in all of them it is relatively porous so that it does not greatly restrict the flow of small molecules to or from the cytoplasmic membrane, although large molecules usually are unable to pass across it.

2.1.1 Gram-negative Bacteria

The cell envelope of Gram-negative bacteria consists of i) The outer membrane, which has been biochemically characterised, is composed mainly of lipopolysaccharides and phospholipids plus pore-forming proteins; ii) a thin layer of peptidoglycan that is linked to the outer membrane through a lipoprotein, and iii) between the outer membrane and the peptidoglycan layer the periplasmic space (Fig. 2.1).

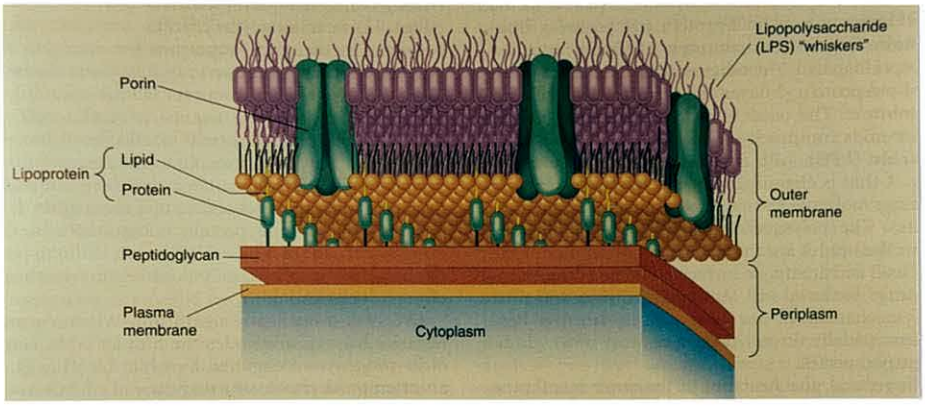


Figure 2.1

Schematic representation of the structure of cell wall of Gram-negative bacteria. Taken from McKane and Kandel (1996).

2.1.1.1 The Outer Membrane

Outer membrane of *E. coli* provides efficient protection from noxious agents in the environment, such as detergent, toxin and proteolytic enzymes. Its asymmetrical structure, consists of glycolipids in the outer layer and phospholipids in the inner layer, which provide an impermeable barrier to lipophilic as well hydrophilic molecules. Therefore, Gram-negative microorganisms require diffusion pathways for the translocation of nutrients and metabolites. The major components of the outer membrane are two types of lipids, lipopolysaccharides (LPS) and phospholipids; and a group of characteristic proteins (Table 2.1)

Table 2.1

Major Components of Outer Membrane

Components	Molecules/cell (10^5)	Fatty acid chain/cell (10^5)	Surface μ^2
LPS	34.6	242	4.9
Porins + OmpA	2		1.8
Lipoprotein	7	21	0.5
Phospholipids	87	174	4.1

Taken from Nikaido, 1996

Figure 2.2 shows a graphic representation of LPS. This consists in three portions: (i) the proximal, hydrophobic, lipid A, with an unusual structure in which a backbone in glucosamyl-β-(1→6) glucosamine is substituted with six or seven fatty acid residues, all of them saturated; (ii) the distal, hydrophilic, O-antigen polysaccharide region that protrudes into the medium, (iii) the core oligosaccharide region that connects the O-polysaccharide with

lipid A. The loss of O-antigen polysaccharide results in loss of virulence, suggesting its importance in escaping phagocytosis (reviewed by Nikaido, 1996).

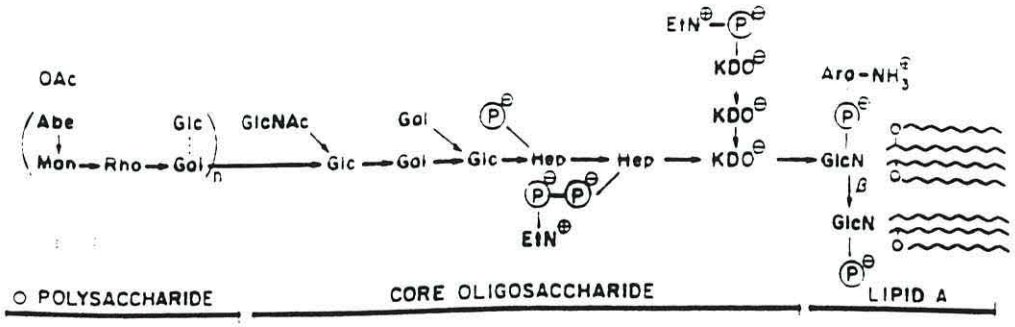


Figure 2.2

Structure of lipopolysaccharides from *S. Typhimurium*. Taken from Nikaido, 1996. Abbreviations used: Abe: abeguose; Man: mannose; Rha: L-rhamnose; Gal: galactose; OAc: O-acetyl; GlcNAc: N-acetyl-D-glucosamine; Hep: L-glycerol-D-mannoheptose; KDO: 2 keto-3-deoxy-D-mannooctulosonic acid; EtN: ehtanolamine; P: phosphate; GlcN: D-glucosamine; $AraNH_3$: 4-aminoarabinose. The wave lines represent fatty acid recidues.

Previous data also indicate that newly synthesised LPS, rather than preformed LPS, is necessary for proper assembly of outer membrane proteins (Reviewed by Dowhan, 1997).

The following classes of proteins are present in the outer membrane:

Murein Lipoprotein. This small protein (7.2 KD) is present in a large number of copies, 7 x 10^5 per cell. About one-third of the murein lipoprotein molecules are bound covalently to the peptidoglycan layer through the ϵ-group of its C-terminal lysine, thus fixing the outer membrane to the underlying peptidoglycan. Its role is still not very clear, mutants defective in the structural gene for this protein are fully viable (reviewed by Neidhardt *et al.*, 1990). However, mutants with deletion through the lipoprotein gene (*lpp*), produce unstable outer membranes, resulting in the release of outer membrane vesicle and periplasmic enzymes into the growth medium (Hirota *et al.*, 1977).

Porins. In Gram negative bacteria the impermeability of the outer membrane to nutrients is solved by special water filled channels formed by proteins known as porins. These permit the transmembrane diffusion of small hydrophilic compounds up to an Mr of about 600. Kinetic analysis of peptide uptake in whole cells of *E. coli* obtained by Alves and Payne (1980) showed that the permeability of these molecules across the outer membrane is through porins, confirming the early suggestion that the outer membrane acts as a molecular sieve for peptides with a size limit of about 650 Da. (Payne and Gilvarg, 1968b).

Porins are coded by *ompF*, *ompC* and *ompE* genes in *E coli* and the homologous *ompD* gene in *S. typhymurium*. These proteins are highly homologous and have similar solute exclusion sizes (Mr~600). Crystal structures of these porins reveal identical trimeric subunits, each subunit consisting of a 16-stranded anti-parallel β-barrel containing a pore. Each channel is constricted by a loop protruding into the β-barrel of the monomer about halfway through the membrane. This long loop inside the barrel contributes to a constriction of the channel where the charge distribution affects ion selectivity (Cowan *et al.*, 1992). The water filled channels exist in open and closed states, depending on the transmembrane potential, although this phenomenon is poorly understood. A recent study has been shown that the physical occlusion, due to a bulk movement of the constriction loop, was not the cause that led to a closed conformation (Phale *et al.*, 1997). The size of the channel, 1.1 and 1.2 nm diameter for OmpC and OmpF respectively, largely determines the exclusion limit of the outer membrane, and the penetration rate of solutes is inversely influenced by their size and hydrophobicity (Hancock, 1987; Nikaido, 1992). Most porins demonstrate little chemical selectivity, except for charge. OmpF and OmpC channels are weakly cations selective whereas PhoE is weakly anion selective. For example, Thanassi *et al.* (1995) showed that tetracycline cross the outer membrane via the porin OmpF as Mg^{2+} chelate, whereas in porin-deficient cells it crosses the outer membrane through its bilayer domain as uncharged tetracycline.

The total amount of porins present is relatively constant and is very large, making porins some of the more abundant protein in *E. coli* and *S. typhimurium*, in term of mass. OmpF, OmpC

and OmpD porins are produced using the usual culture medium, and the relative abundance of these proteins is under an efficient regulation by environment signals, while PhoE is produced only under conditions of phosphate starvation (Mizuno *et al.*, 1983)

Expression of OmpF and OmpC are regulated by the medium osmolarity. EnvZ a transmembrane histidine-kinase senses changes in the osmotic conditions of the growth environment and controls the phosphorylated state of the regulatory protein, OmpR. OmpR-phosphate regulate the expression of genes *ompF* and *ompC*. To date, the natural ligand that activates EnvZ, has not been identified and the role of the periplasmic domain of EnvZ is not clear, but it is not essential for sensing of osmolarity signals (Leonardo and Forst, 1996). OmpF is preferentially synthesized in a medium of low osmolarity, whereas OmpC is preferentially synthesized in a medium of high osmolarity (Mizuno and Mizushima, 1990), while PhoE expression is induced under conditions of phosphate starvation (Mizuno *et al.*, 1983). Recent studies using OmpR mutant lacking the potential phosphorylation site (Asp-55), suggest that OmpR is capable of functioning in a phosphorylation-independent manner under certain *in vivo* conditions (Kanamaru and Mizuno,1992).

Studies of permeability of outer membrane have shown that polyamines (putrescine, cadaverine, spermidine and spermine) inhibit chemotaxis and flux of β-lactam antibiotics through porins, suggesting that cadaverine might be a natural regulator of porin activity, and also suggesting that polyamines and related compounds could potentially serve as therapeutic agents specially targeted at the outer membrane (de la Vega and Delcour, 1995, 1996). Recently, Iyer and Delcour (1997) postulate a model for the possible molecular interaction between porins and polyamines. In this model the main mechanism for modulation of porin channels involve an alteration in the intrinsic rate constant for gating, leading to stabilise closed state, as consequence of the binding of the polyamines to an asymmetric site within the pore.

The expression of OmpF and OmpC porins of *E.coli* has been shown to be influenced by the leucine-responsive regulatory protein (Lrp). This protein has a positive effect on OmpC expression and a negative effect on OmpF expression (Ferrario *et al.*, 1995).

In vitro synthesis of outer membrane protein, PhoE has shown that it acts as a molecular chaperone in the assembly of outer membrane proteins (de Cock, H. and Tommassen, 1996).

OmpA protein. OmpA is one of the major proteins in the outer membrane in *E. coli* (Chen *et al.*, 1980). As well as OmpF and OmpC porins, OmpA is rich in β-sheet structure (Nakamura and Mizushima, 1976). OmpA is a monomer and consists of in two domains, its N-terminal domain is thought to cross the membrane 8 times in amphipathic β-strands (Klose *et al.*, 1988) and a water soluble, C-terminal domain (Vogel and Jhäing, 1986). This latter domain, which is digested by protease, is thought to reside in the periplasmic space (Vogel and Jhäing, 1986). Studies of mutants defectives in OmpA, suggest that this protein has a role in stabilising the structure of the outer membrane (reviewed by Nikaido and Vaara, 1985). OmpA protein of *E. coli* produces diffusion channels that allow the penetration of various solutes at very low rate compared with OmpF and OmpC (Sugawara and Nikaido, 1992), and has been suggested to be involved in fixing murein to the outer membrane (Leduc *et al.*, 1992).

Specific channels. The outer membrane possess proteins forming specific channels in *E. coli* and *S. typhimurium*. For example the LamB protein that allows the passage of maltose and maltodextrins across the outer membrane (Ferenci *et al.*, 1988); the Tsx protein involved in the specific diffusion of nucleotides (Bremer *et al.*, 1990) and the ScrY protein, which is encoded by a plasmid gene and produces sucrose specific channels (Schülein *et al.*, 1991).

High-Affinity Receptor in Outer Membrane. This class of proteins transport specific ligands across the outer membrane, for example B_{12}. These proteins bind the ligands much more tightly than specific channel proteins and the transport of these proteins requires the presence of TonB. TonB, a proline-rich protein, contains a highly rigid structure (Brewer *et al.*, 1990), which is anchored in the cytoplasmic membrane and extends through the periplasmic space and physically interacts with outer membrane receptors (Postle, 1993).

Proteins involved in direct import and export of proteins. Some proteins are translocated through the cytoplasmic membrane by a signal peptide-dependent step and are then transported out of the cell by a process that involves several specific secretion proteins located in the cell envelope. One of the best know is the TolC protein, which has been shown to be involved in the entry of some colicins, a class of bacteriocin, in *E. coli*, and the export of cytotoxins as hemolysin (Webster, 1990; Wandersman, 1990).

2.1.1.2 Murein Sacculus

The murein sacculus is a rigid structural component that is shape-determining for the bacterial cell wall (Weidel and Pelzer, 1964). This structure is a complex heteropolysaccharide, a class of peptidoglycan that contains roughly equal amounts of polysaccharide and peptides. The repeating unit, termed muropeptide in *E. coli*, is composed of N-acetyl glucosamine (GlcNAc), N-acetyl muramic acid (MurNAc: GlcNAc with D-lactic acid ether substituted at C-3), L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP), and D-alanine; all in equimolar amounts except for D-alanine, which may be present in slight excess. The sugars are linked together by β1→4 glycosidic bonds. Attached to the carboxyl group of each muramic acid by an amide linkage is a short peptide, L-alanyl-D-isoglutamyl-L-meso-diaminopimelyl- D -alanine (reviewed by Park, 1996). A molecule of lipoprotein (Braun

lipoprotein) is attached to about every tenth muropeptide, thus fixing the peptidoglycan layer with the outer membrane (Braun and Rhen, 1969).

2.1.1.3 Periplasmic Space

In Gram-negative bacteria, the compartment between the cytoplasmic membrane and the outer membrane, is called the periplasmic space. This region contains a high concentration of: binding proteins for the transport of amino acids, sugars, peptides, ions, vitamins; degradative enzymes (e.g., phosphatases and proteases); and detoxifying enzymes (e.g.β-lactamases) (Niedhardt *et al.*, 1990). Several processes that are vital to the growth and viability of the cell occur within this space.

Several techniques have been used to determine the volume of the periplasmic space, for example, electron microscopy studies have been suggested that the periplasm represents approximately 20-40 % of the cell volume. However, on the basis of the abundance of periplasmic proteins and their approximate atomic dimensions, it has been estimated, that at 10% of the cell volume, the concentration of periplasmic protein would form a highly viscous state (van Wielink and Duine, 1990). Based on cell envelope ultrastructure studies, Hobot *et al.* (1984) proposed a model for the organisation of the peptidoglycan within the periplasm, in which the peptidoglycan is in various states of polymerisation, and in the form of a gel. Thus, the peptidoglycan is more highly polymerised near the outer membrane and more loosely polymerised near the cytoplasmic membrane in form of a gel.

2.1.2 Gram-positive Bacteria

Gram-positive bacterial cell walls lack an outer membrane. Its cell envelope consists of (i) the cell wall that consists of many layers of peptidoglycan, forming a thick rigid structure. In addition to peptidoglycan, Gram-positive cell walls generally have another components,

teichoic acid, a polymer of ribitol or glycerol phosphate, which is linked to the peptidoglycan layer (Tortora *et al.*, 1995), and (ii) the cytoplasmic membrane, that contains characteristic lipid components as glycolipids and lipoteichoic acids, which is teichoic acid that spans the peptidoglycan layer (Fischer, 1988) (Fig. 2.3).

Lipoteichoic acid (LTA) plays a vital role in the growth and physiology in Gram-positive microorganism . In early study it was postulated that LTA modulates the autolysins (Fischer, 1988). Recently, Wecke *et al.* (1996), proposed that negatively charged lipoteichoic acid and/or wall teichoic acid serve *in vivo* to fix the cationic autolysins within the cell wall-membrane complex by electrostatic interaction. On the other hand, LTA has been shown to be involved in the binding of cations for enzyme function (Heptinstall *et al.*, 1970), and the electrochemical properties of the cell wall (Ou and Marquis, 1970). A recent study has suggested that LTA has a stabilisation effect on the membrane structure, via dynamic long-distance interactions of the hydrophilic poly(glycerolphosphate) chain with the phospholipids (Gutberlet *et al.*, 1997).

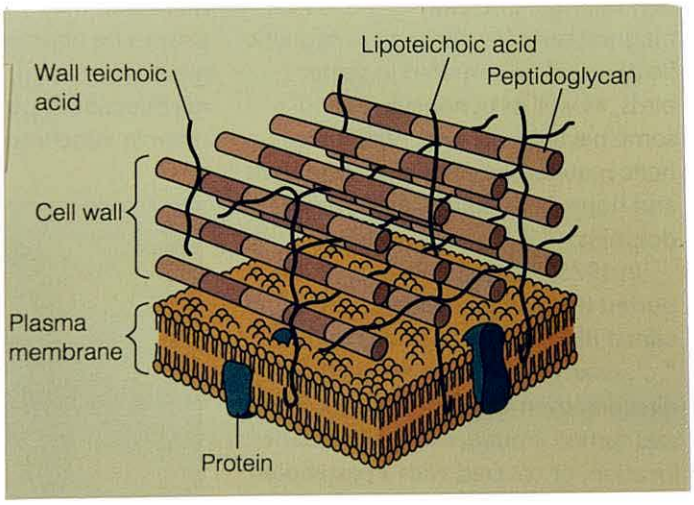


Figure: 2.3

Schematic representation of the structure of cell envelope of Gram-positive bacteria. Taken from Tortora *et al.* (1995)

2.2 Bacterial Transport Systems

The cytoplasmic membrane is impermeable to most water-soluble compounds, which therefore require specialised transport systems to cross it. In *E. coli* transport systems fall into three general classes: i) Phosphoenolpyruvate sugar phosphotransferase that phosphorylate specific mono-and disaccharides during transport; ii) transport systems that require for their function only membrane bound components that are energized by electrochemical ion gradient, and iii) the periplasmic binding protein-dependent (PBD) ABC transporters, using ATP to energize. These last transport systems are also referred to as osmotic shock-sensitive permease because they are inactivated during osmotic shock (Heppel, 1971).

2.3 Periplasmic Binding Protein-dependent Transport systems

Periplasmic binding protein-dependent transporters (PBD), form part of the largest family of active transport systems, for the uptake of sugars, amino acids, anions, peptides and other nutrients. These systems have been referred to as ABC transporters (Higgins, 1992), or traffic ATPases (Ames *et al.*, 1990). These ABC transporters comprise over 50 members, that are relatively specific for a given substrate. The majority are from prokaryotic species, although some have been reported in eukaryotic species.

The typical ABC transporter consists of four elements. Two of these domains are highly hydrophobic membrane-spanning proteins and form the pathway through which the substrate crosses the membrane. Two domains are peripherally located at the cytoplasmic face of the membrane, bind ATP, and couple ATP hydrolysis to the transport process. A fifth component, that consists of an extracellular ligand-specific binding protein, is present in all ABC bacterial transporter (reviewed by Higgins, 1992).This binding-protein is located in the periplasm of Gram-negative bacteria as soluble protein, while in Gram-positive bacteria it is a lipoprotein associated with the external face of the cytoplasmic membrane (Sutcliffe and Russell, 1995).

The individual components of an ABC transporter are frequently expressed as separated polypeptides, as for example Opp in *S. typhimurium* and spo0K in *S. subtillis* (Hiles et al., 1987; Perego *et al.*, 1991; respectively), while others are expressed as large multifunctional polypeptides. For example, the ATP-binding domains of *E. coli* ribose transport (RbsCDA) are fused in a large, single protein (Bell *et al.*, 1986). The two membrane-spanning elements in the iron-hydroxamate transporter (FhuBCD) of *E. coli* are fused into a single polypeptide (Coulton *et al.*, 1986). In eukaryotes two organisation of ABC transporters have been reported. The peptide transporter of the major histocompatibility complex (MHC), that consists of two polypeptides, RING 4 and RING 11, each of which comprise a hydrophobic domain at the N-terminus and an ATP-binding domain at the C-terminus (Trowslade *et al.*, 1990). The fusion of all four domains into a single polypeptide may occur, such as seen in the human multidrug resistance P-glycoprotein (MDR), amplification of which causes a multidrug resistance phenotype of cancer cells; and the cystic fibrosis transmembrane conductance regulator (CFTR) gene product, which is mutated in patients affected by cystic fibrosis (Chen *et al.*, 1986; Riordan *et al.*, 1989) (Fig. 2.4).

2.3.1 Structure of Binding Proteins and Proposed Mechanism for Active Transport by Periplasmic Binding Protein-dependent Transport Systems

Structure studies of binding proteins from several ABC transporters have revealed that, despite the lack of uniform size and sequence similarity, they show a similar tertiary structure overall (Quiocho, 1990, 1992; Quiocho and Ledvina, 1996). The common features between tertiary structure and atomic interaction of these proteins has been determined from X-ray crystal structure. The following characteristics were deduced in all binding proteins studied. i) All structures are ellipsoidal, consisting of two distinct globular domains separated by a deep cleft or groove; ii) the ligand-binding site is located deep in the cleft between the two domains; iii) a hinge-bending motion between the two domains modulates access to and from the binding site; iv) irrespective of the nature of the ligand, the specificity and affinities of the

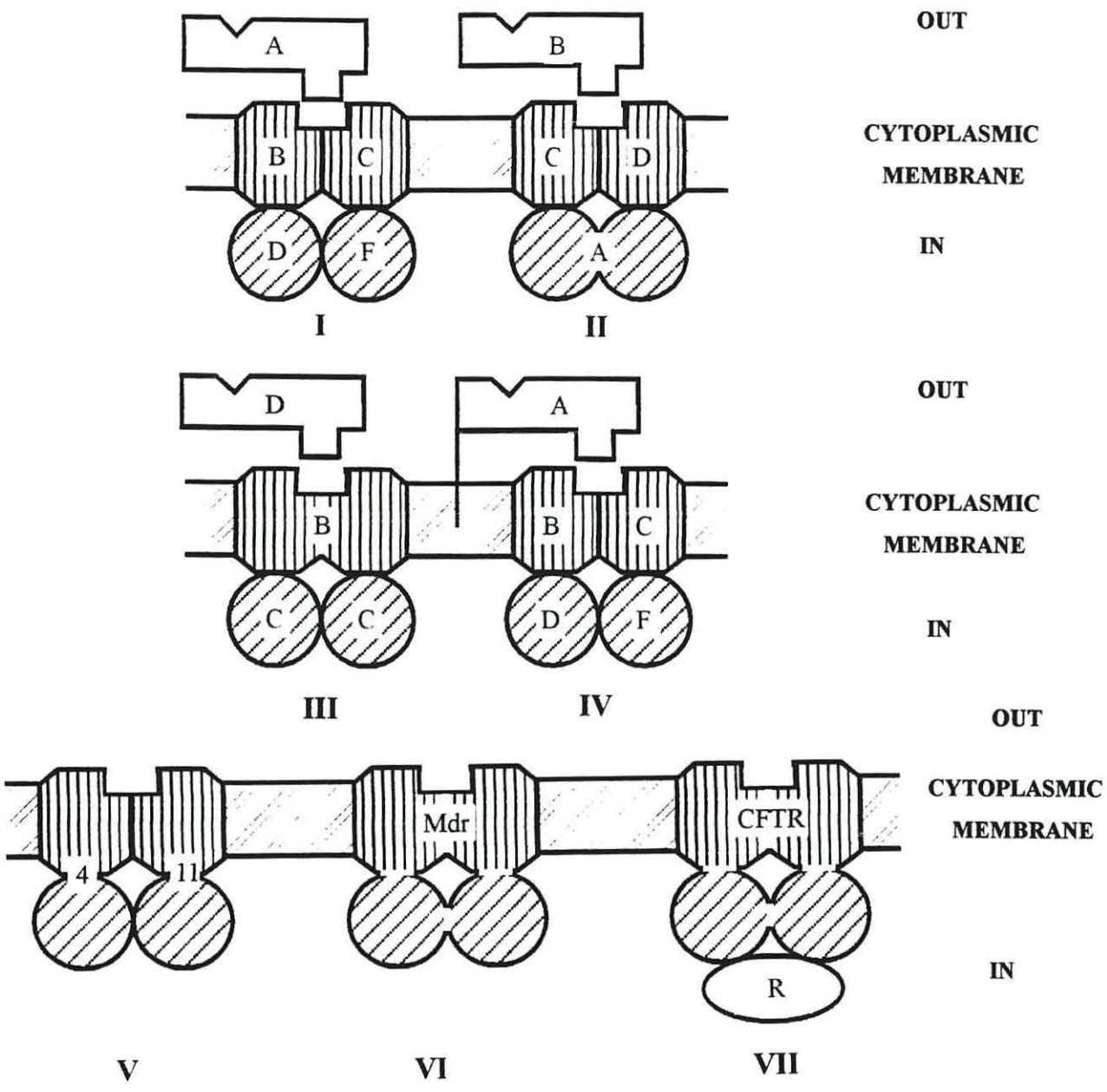


Figure 2.4

Structure organization of several ABC transporters. (I) oligopeptide permease (Opp) from *S. salmonella*; (II) ribose tramsporter (Rbs) from *E. coli*; (III) iron-hydroxamate from *E. coli*; (IV) peptide transporter (spoOK) from *B. subtilis*; (V) peptide transporter of MHC from man (Ring4-Ring11; (VI) multidrug resistance P-glycoprotein (Mdr) from eukariotic cells; and (VII) cystic fibrosis transmembrane conductance regulator (CFTR) from human cells.

binding sites are achieved through hydrogen-bonding interaction; v) binding of ligands induces a large protein conformational change; vi) three different structures have been observed among the binding protein: unliganded "open cleft", liganded "open cleft" and liganded "closed cleft".

On the basis of these observations, Quiocho has proposed the following mechanism: the unliganded form is the native state of binding protein, that binds the substrate preferentially to one domain of the open form, by virtue of the greater number of interactions (hydrogen bonds) than will be formed with the other domain; a hinge between the two domains then causes the other domain to participate in binding, producing the closed liganded form. This form interacts with membrane components, and conformational changes propagate throughout the entire system in synchrony with translocation, thus, initiating nutrient translocation or in case of chemotaxis flagella motion. The substrate is released from the binding protein and transferred to an active site or sites in the membrane components and finally translocated to the cytoplasm, completing a cycle (Fig. 2.5).

In contrast with the mechanism proposed by Quiocho above, Ames *et al.* (1996), based on results obtained by using chemical cross-linking method, showed that the liganded and unliganded forms of histidine-binding protein, HisJ, have equal affinity for the membrane-bound complex, HisQMP (HisQ and HisM are two hydrophobic subunit, and HisP consists in two identical copies of ATP-binding component). On the basis of these findings these workers have proposed a new model for the mechanism of action of periplasmic permease transport systems. In this model the receptor is associated with the membrane even in the absence of ligand. Upon ligand binding the receptor assumes the closed form , which induces fast ATP hydrolysis and lead to translocation (Fig. 2.6).

Both proposed models are based on conformational changes in both the periplasmic-binding protein and the membrane-bound components. The novel feature in the latter model is that the periplasmic binding protein does not leave the membrane bound-complex after translocation. On the other hand, this new model can explain better the mechanism for the

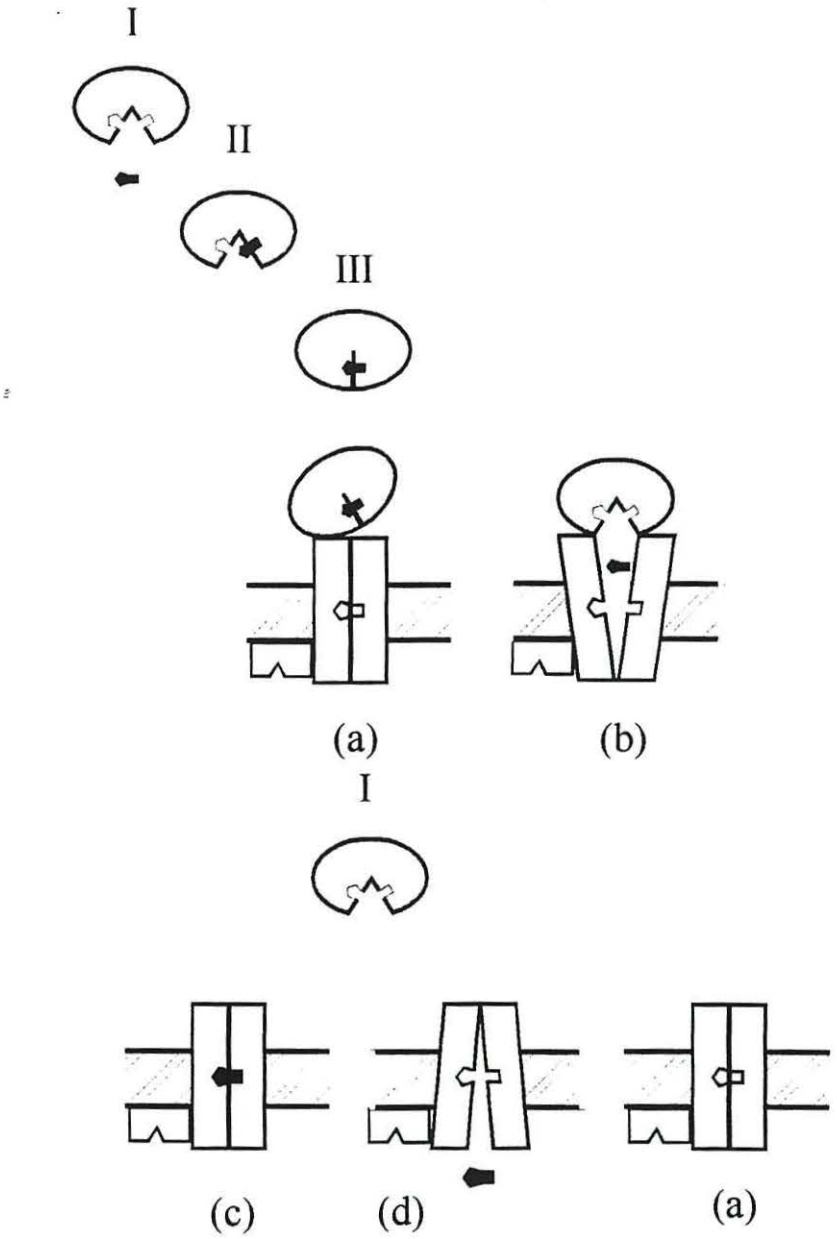


Figure 2.5

A model of mechanism for the binding protein-dependent transport system, proposed by Quiocho (1990). The different conformations of binding protein are referred to as (I) unliganded open cleft, (II) liganded open cleft, and (III) liganded closed cleft. The ligand-binding sites in the binding protein are asymmetric; one domain interacts with the substrate more extensively than the other domain. See text for further details.

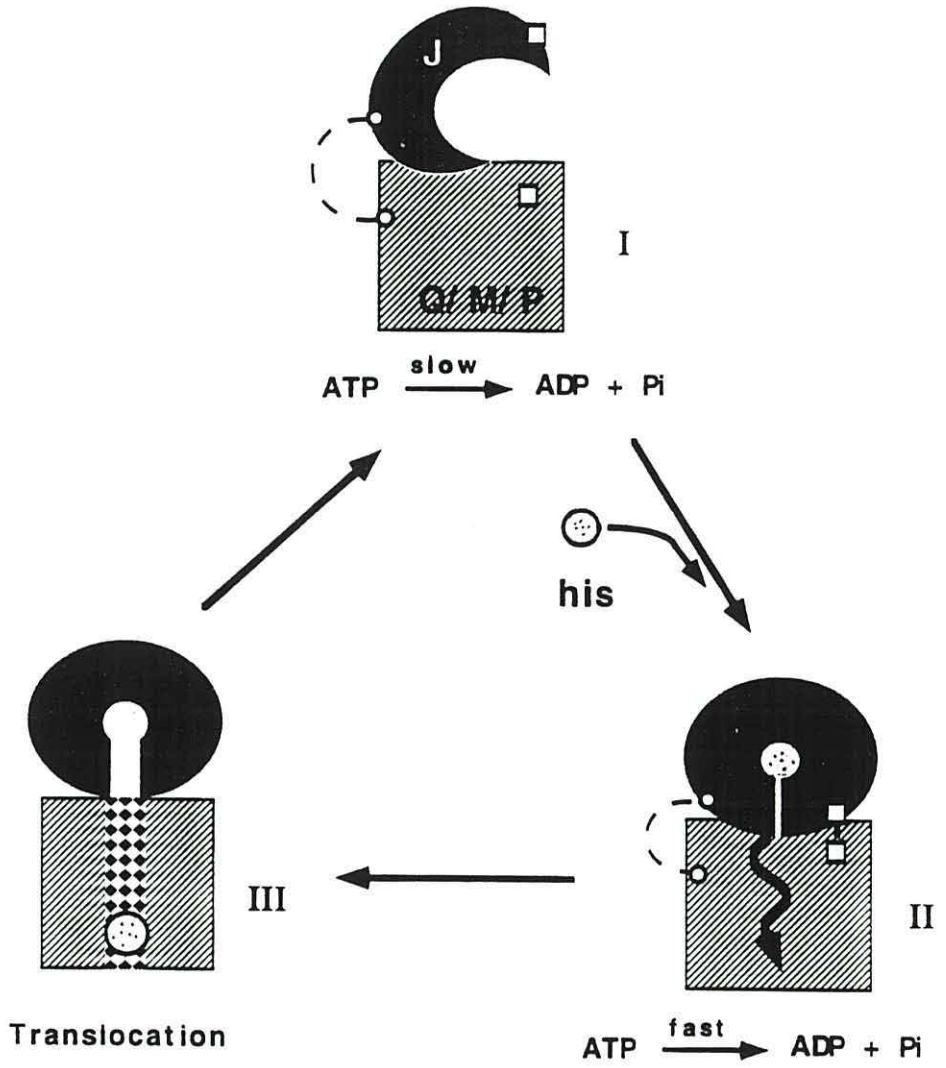


Figure 2.6

A new model for periplasmic binding protein-dependent transport proposed by Ames *et al.* (1996). The hatched box represents the membrane-bound HisQMP complex.Periplasmic binding protein (HisJ) is represented in solid black and is shown to undergo a conformational change upon binding of substrate (His) (a spotted circle) and during translocation; small blank squares and circles represent cross-linking sites for reagents.

active transport by permeases of Gram-positive bacteria, where the receptor or binding protein is a protein associated to the membrane as a lipoprotein.

Evidence for substrate-induced changes in OppA has been confirmed by isoelectric focusing (Tyreman, 1990; Tyreman *et al.*, 1992). Three species with different pI values have been observed. An unliganded form with a pI value of 6.20 and two liganded forms produced by addition of Ala_3 or LysAlaAla with pI values of 6.26 and 6.55 respectively. Analogous properties were found in DppA by the same workers.

Another interesting feature deduced from the crystallography studies of the binding protein, is the crucial role of hydrogen bonds in conferring substrate specificity. The two best examples of high specificity among these receptors are the sulphate binding protein (SBP) (Pflugrath and Quiocho, 1985) and the phosphate binding protein (PBP) (Lueke and Quiocho, 1990). Although phosphate and sulphate are structurally similar, at physiological pH, SBP and PBP exhibit no overlap in specificity. In PBP-phosphate complex, phosphate is completely dehydrated and sequestered in the protein cleft and bound by 12 hydrogen bonds. Eleven hydrogen bonds are formed with donor groups from the protein. The remaining hydrogen-bonding group, a carboxylate side-chain of Asp-56, serve as a charged hydrogen-bond acceptor at physiological pH. A binding of a fully ionized sulphate dianion is unsuccessful, by charge repulsion of the carboxylate that only recognises a proton on the phosphate (Lueke and Quiocho, 1990). The substrate of SBP is sulphate dianion, which is completely dehydrated and buried in the binding-site cleft. This sulphate is bound through seven hydrogen bonds donated entirely by uncharged polar protein groups (Pflugrath and Quiocho, 1985). The absence of a negatively charged residue in the binding site, as the carboxylate in PBP, account for its binding of fully ionised dianion ligand, including selenate and chromate, at physiological pH and its inability to bind phosphate (Jacobson and Quiocho, 1988).

In contrast to the high specificity showen by PBP and SBP, multiple related specificity is exhibited by lysine/arginine/ornithine binding protein (LAOBP), leucine/isoleucine/valine binding protein (LIVBP), and D-glucose/D-galactose binding protein (GBP). On the other

hand, the receptors that are designed to bind oligomeric substrates, as maltodextrin binding protein (MPB), dipeptide binding protein (DppA) and oligopeptide binding protein (OppA); are unable to recognise the monomeric unit (glucose or amino acid, respectively), however these exhibit a broad substrate specificity. In spite of, the diversities of ligands, hydrogen bonds are heavily involved in the binding of all of them. Details on specificity and atomic interaction between receptor and ligands of OppA, will be considered in section 2.5.3.1.

2.4 Peptide Permeases in Gram-negative Bacteria

Most of the knowledge of peptide transport in Gram-negative bacteria has arisen from studies carried out with *E. coli* and *S. salmonella*. Because the transport of peptides is severely reduced when bacteria are subjected to osmotic shock (Cowell, 1974) and appears to require a source of phosphate-bond energy, this system can be classified as a shock-sensitive transport system.

The existence of several peptide permeases is indicated from the results of competition studies showing minimal interactions between the substrates during uptake (Payne, 1980). Three genetically distinct permeases, with overlapping substrate specificities, serve to mediate the uptake of peptides in to the cytoplasm: i) the dipeptide permease (Dpp) which is relatively specific for dipeptides and also provides the chemoreceptor for peptide chemotaxis (Manson *et al.*, 1986); ii) the tripeptide permease (Tpp) with a restricted substrate specificity, showing greatest affinity for hydrophobic tripeptides (Gibson *et al.*, 1984); and iii) the oligopeptide permease (Opp) that provides the major pathway for the uptake of small peptides consisting of up to about five amino acid residues (Payne and Gilvarg, 1968a).

2.5 Oligopeptide Permease

The presence of the oligopeptide permease (Opp) was described through the study of a lysine auxotrophic strain of *E. coli*, in which growth can be satisfied by various lysine-containing oligopeptides. Mutants lacking Opp, can be selected by the use of triornithine, a toxic peptide transported through Opp. In this mutant, the transport of tripeptide and higher oligomers was largely lost, while dipeptide uptake was only slightly affected (Payne and Gilvarg, 1968b; Payne, 1968).

The oligopeptide transport system provides a major route for the uptake of many peptide antibiotics, both natural and synthetic (Ringrose, 1980). Studies carried out by Goodell and Higgins (1987), have suggested that the murein tripeptide produced during the degradation of the peptidoglycan from the murein sacculus, is transported into the cytoplasm via the oligopeptide permease, since *opp* negative strains do not incorporate externally added tripeptide into their murein sacculus. However, it has recently been demonstrated that Opp negative strains recycle murein-derived material efficiently (Park. 1993). A separate peptide uptake system, AmpG which is able to transport large muropeptides into the cell, has recently been suggested (Jacobs *et al.*, 1994).

2.5.1 Substrate Specificity of the Oligopeptide Permease

The substrate specificity of the oligopeptide transport system is unusually interesting in that, whilst it exhibits a stringent discrimination between free and peptide-bound amino acids, the number and nature of the amino acids that comprise the peptide is relatively unimportant. The specificity of the Opp has been defined predominantly through genetic experiments which illustrated that amino acid auxotropic strains of *E. coli* can use oligopeptides as a source of the required amino acid (Payne and Gilvarg, 1968a; Payne, 1968). Opp can transport oligopeptides up to hexapeptides, has a high affinity for substrates with a typical K_t value of 0.1-2.0 μM, and a typical V_{max} value of 2-20 nmol min^{-1} per mg protein (reviewed in Payne

and Smith, 1994). A detailed understanding of its specificity is important for the design of peptide antibiotics.

The Opp transport system has a requirement for the unsubstituted α-amino group (Gilvarg and Katchalski, 1965), however *E. coli* can utilize mono-α-N-substituted peptides in which the positive charge is retained (Payne *et al.*, 1971, 1974). Although a free terminal carboxyl group is not absolutely required for transport (Payne and Gilvarg 1968a), the Opp shows a reduced affinity for peptides in which the terminal carboxyl group has been modified (Payne and Bell, 1979). α-peptide linkages are required for peptide uptake in *E. coli* as well as in *S. typhimurium*, *Pseudomonas*, and *Streptococci.* Opp shows stereochemical specificity with preference for all L-residues but with some tolerance of D-residues within the chain (Payne, 1980). The nature of side chain residues influences individual kinetic parameters, but the system is characterised by its ability to handle an enormous range of natural and modified residues (Payne and Smith, 1994).

2.5.2 Oligopeptide Permease Components

Five proteins are required for the function of the Opp system. The genes encoding these proteins are cotranscribed as a single operon *oppABCDF* (Hiles *et al.*, 1987), which has been mapped near the *trp* locus at 35 min on *S. typhimurium* chromosome and 27 min on the *E coli* chromosome (Higgins *et al.*, 1983; Hogarth and Higgins 1983). The *opp* operon is located between *tonB* and *galU* in both *E coli* and *S. typhimurium* chromosome (Lenny and Margolin, 1980; Higgins *et al.*, 1983). The Opp share a typical organisation, comprising two integral membrane proteins which form a channel through which the solute passes, two components at the cytoplasmic surface of the membrane which couple ATP hydrolysis to solute translocation, and a periplasmic binding-protein, that acts as a receptor of substrate in the periplasmic space (Higgins *et al.*, 1982). All the proteins of the Opp system with the exception of the periplasmic OppA protein, are present at very low levels in the cell.

2.5.3 Oligopeptide Binding Protein

The *oppA* genes from *E. coli* and *S. typhimurium* encode polypeptides of 543 and 542 amino-acid residues, respectively (Kashiwagi *et al.*, 1990; Hiles and Higgins, 1986). The OppA protein is synthesised as a precursor, which is processed by removal of the signal peptide to generate a protein with a *Mr* value of approximately 58.8 k..The OppA is a soluble and abundant binding protein that binds substrate with high affinity, Km ~ 1μM (Payne and Bell, 1979).

2.5.3.1 OppA Crystal Structure and Interactions Between Receptor and Ligand

The OppA protein from *S. typhimurium* was crystallized for the first time by Tolley *et al.*, 1988, and structural data are recently arising from crystallographic analysis (Tame *et al.*, 1994, 1995; Sleigh, *et al.*, 1997).

Crystal structure of OppA, reveals high tertiary structure similarity with other periplasmic binding proteins. However, the most striking feature of the overall structure of this binding protein is the organisation of the polypeptide chain into three domains instead of two. In OppA the relative organisation of domain I and III with respect to each other and with respect to ligand, is similar to the arrangement of the two lobes of the other binding-proteins. The binding-protein structures have been classified into two groups depending on the nature of the hinge region (Yao *et al.*, 1994). The group I binding-proteins have three short connecting segments or hinges between the two domain. The transitions from domain to domain are from strand to helix for the first two cross-overs and strand for the third cross-over. The group II binding-proteins have differences in sheet topology, which is attributed to the nature of the cross-overs between domains. In these groups of proteins the first two interdomain connections are strand to strand and the third is helix to helix. OppA belongs to group II binding proteins.

The domain I of OppA consists of three separate segments of polypeptide chain, residues 1-44, 169-270 and 487-517, and contains a central seven-stranded β-sheet. The N-terminal polypeptide segment forms a random coil for 14 amino acid residues forming the central strand of sheet 1 which is followed by a further loop that includes residues 32-34 which form important contacts with the ligand. The second domain is made up of a contiguous segment of polypeptide from residues 45-168. It comprises a four-stranded β-sheet, one face of which is exposed to solvent, and the opposite face is buried by two α-helix and connecting segments in a well-ordered, highly hydrophobic core. The topology of domain III is similar to lobe 2 of other group binding proteins and contains a five-stranded mixed β-sheet. (Tame *et al.*, 1995).

As well as other periplasmic binding proteins, OppA contains two globular domains connected by a short segment of polypeptide chain, which acts as a hinge allowing relative domain movements to take place (Mao *et al.*, 1982). The structure of all liganded closed form, binding proteins show one or other lobe interacts more strongly than the other with the ligand. It has been suggested that this may improve the efficiency of ligand capture by providing a good binding in the open form of the protein (Oh *et al.*, 1993). The mechanism of binding has been linkened to that of a Venus flytrap (Quiocho, 1991). Venus flytrap model (Mao *et al.*, 1982) allows the participation of both domains in a binding mode accompanied by dehydration of ligands and expulsion of water molecules in the cleft, which contributes favourable entropy in protein-ligand formation. LAOBP (lysine/arginine/ornithine binding protein) and MBP (maltose- or maltodextrin binding portein) have been cryatallized in both closed (liganded) and open (unliganded) forms. Comparisons between these forms show that the lobes of the proteins move relatively to each other as rigid bodies, and the conformational change is brought about by changes in the ϕ and ψ angles of only a few residues in the strands connecting the two domains (Oh *et al.*, 1993; Spurlino *et al.*, 1991; Sharff *et al.*, 1992).

The crystal structure of OppA in complex with tripeptide and tetrapeptide ligand reveals that the ligand are completely sequestered from bulk solvent and enclosed in the protein interior

(Tame *et al.*, 1994, 1995). The peptide in OppA is bound in an extended conformation so that the charged ligand termini and its polar main chain hydrogen-bound groups, common to all peptides, are available to make interactions with the protein (Wilkinson, 1996). Tight peptide binding in due to main chain to main chain contacts, the ligand forming anti-parallel β-sheet-like interaction with an extended strand of sheet 3 (residues Gly-415 - Cys-417) on one side and parallel β-sheet-like interaction with a loop of domain I (residues Glu-32 - Val-34) on the other. The majority of the ligand backbone contacts are provided by domain III. In both tri- and tetrapeptide-OppA complex, a salt bridge is formed between the acidic side chain of Asp-419 on the protein and the α-amino group of the peptide ligand. This salt link explain the low affinity of OppA for peptides with acetylated α-amino group (Payne and Bell, 1979). From the structure of the OppA-tripeptide (OppA-KKK) and OppA-tetrapetide (OppA-KKKA) complexes, it is apparent that the protein has a series of positively charged side chain along the binding cavity, allowing that the C-terminus of peptide ligands of different lengths form ion pairs with different protein side chain. Two acetate ions found within the ligand-binding site in the OppA-KKK complex provide evidence that this is how longer peptide are accommodated. The first of these is bonded to His-371 and the second to Lys-307, whose side chain points into the protein. This second acetate ion presumably binds in the same place as the carboxyl group of a pentapeptide (Tame *et al.*, 1995).

Three side chain binding pockets have been identified in the structure of OppA-KKK complex (Tame *et al.*, 1994). These pockets that are able to accommodate side chains of peptide ligands, which can differ in size, shape, polarity and charge, by projecting into spacious hydrated cavities. Close to the ligand backbone the pockets are hydrophobic and there are not polar groups present that could compete for hydrogen bonds to these atoms. The side chains of Val-34 and the disulfide bridge between Cys-271 and Cys-417 lie against the main chain atoms of the second ligand residue and form part of the first and third side chain pockets. The first ligand side chain extends into the pocket and form hydrogen bonds with three water molecules. In the second side chain pocket, the apolar collar is formed by Trp-397, Trp-416, Leu-401, and the aliphatic portion of Glu-32. The pocket then widens and is flanked by

charged side chains indulging in ion pairs. Glu-32 and His-405 on one side and Glu-276 and Arg-404 on the other. The third lysine of trilysine extends into a cavity lined with asparagine amide group and tyrosine hydroxyl groups with the ligand's ϵ-amino group interacting with one of co-crystalized acetate ions. Few directed interaction are made between OppA and the ligand side chain, that could impose specificity.

Recently the crystal structure of OppA complexed with a dipeptide lysyllysine (OppA-KK) and the unliganded OppA, have been solved (Sleigh *et al.*, 1997). The overall structure of OppA-KK complex is identical to that of a series of OppA-peptide complex solved previously. The ligand main chain is very well ordered in the binding site. The α-amino group forms a salt bridge with the side chain carboxylate of Asp-419, as observed in complex with longer peptides. The interaction of ligand's α-amino groups, serve to anchor the peptides in the binding site, so the variation of length are accommodated by alternative handling of the carboxyl terminal residues. Unlike other OppA-peptide complexes, the α-carboxylate of the ligand dipeptide forms water-mediated interaction with the guanidinium groups of Arg-404 and Arg-413 rather than the direct salt bridge to Arg-413 and His-371 observed in tri- and tetrapeptide complexes, respectively (Tame *et al.*, 1994, 1995). The carbonyl and amino groups of the ligand's peptide bond are hydrogen bonded to the main chain amide and carbonyl groups of Cys-417 and Glu-32, respectively.

The affinity of OppA to bind lysine-containing peptides of different lengths using isothermal titration calorimetric was also measured by Sleigh and co-workers (1997). Their results showed that the dipeptide, KK, is bound with approximately 60-fold lower affinity than related tri- and tetrapeptide (KKK and KKKA, respectively), which were consistent with the results obtained by Guyer *et al.*, (1986) by using the equilibrium dialysis measurements. The following structure differences between the dipeptide and tripeptide complexes have been taken into account to explain the low affinity of OppA to bind dipeptides:(i) the α-carboxylate groups are handled in different ways, in a tripeptide complex the link of the α-COO^- group is mediated by directed ion pairing, while in dipeptide complex the α-carboxylate group form

charge-dipole interaction with water molecule (water-mediated ion pairing), (ii) the protein forms additional interactions with the main chain of the ligand in the tripeptide complex, including hydrogen bonding of the polar peptide linkage between residues 2 and 3, (iii) there are additional interactions with the side chain of residues 3 that include contacts to the aliphatic portion of the lysine 3 side chain and direct\water-mediated polar interaction with its charged ϵ-amino group, and (iv) additional ordered water molecules are buried in the dipeptide complex.

Comparison between the structure of liganded and unliganded OppA forms, has shown the following features: (i) both structures are related by a simple rigid body rotation of domain I and II with respect to domain III; (ii) the three-dimensional structure of individual domain are unaltered; (iii) the angle of opening unliganded OppA calculated by determination of the rotation required to superimpose the two lobes of the protein in unliganded form is 26°,which is a considerably smaller angle of opening than observed in other binding proteins for which the structure of the open, unliganded and closed, liganded forms have been determined (iv) the dramatic conformational change can be attributed to large changes in the ϕ and ψ angles of just a small number of residues located in two segments of the polypeptide chain linking the two domains, being the residues 270 and 485 responsible for the opening of the closed form; (v) A large number of water molecules are trapped in the protein upon ligand binding, which fill the volume not occupied by the smaller ligand and mediate interactions between polar ligand atoms and the protein (Sleigh *et al.*, 1997). Many of these water molecule are important in adapting the cavity in OppA to accommodate side chain of diverse structure (Tame *et al.*, 1996).

2.5.4 Membrane- associated Components

The membrane associated components, fall into two different classes, the highly hydrophobic proteins OppB and OppC thought to be responsible for peptide transport across the bilayer,

and the relatively hydrophilic, OppD and OppF proteins, which bind ATP and are believed to couple ATP hydrolysis to the transport process (Hiles *et al.*, 1987).

2.5.4.1 Integral Membrane Proteins

The OppB and OppC proteins share considerable sequence homology, have been shown to be membrane associated and are thought to function as a dimer (Higgins *et al.*, 1990). The amino acid sequence has been deduced from the nucleotide sequences of the corresponding genes. Comparison of these proteins with related integral membrane proteins from other permeases, reveals little sequence similarity, although all are highly hydrophobic and seem to be structurally related (Hiles *et al.*, 1987). Each of these two proteins consist of a core structure that spans the membrane six times, with the N-and-C termini both being located at the cytoplasmic face of the membrane (Higgins *et al.*, 1990; Pearce *et al.*, 1992). Short stretches of hydrophilic sequence, separating the core structure, appear to be conserved in many of these proteins (Fig. 2.7). This short sequence is probably exposed to the cytoplasmic face of the membrane and may interact with the peripherally located ATP-binding component (Dassa and Houfnun, 1985).

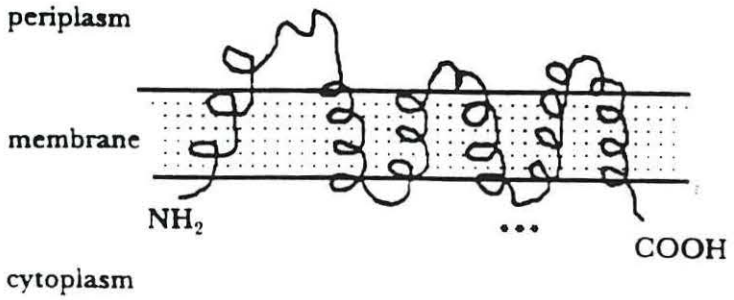


Figure 2.7

Diagram illustrating the structural model of an integral membrane protein determined for OppB; (***) indicates the location of the short hydrophilic sequence observed by Dassa and Hofnung, 1985. Taken from Higgins *et al.* (1990).

This sequence was later termed the EAA loop (Kerppola and Ames, 1992) when a similar segment was found in other inner membrane components from all proteins analysed. The high degree of conservation of the EAA loop (Saurin and Dassa, 1994) suggests an important common function of this sequence for all inner membrane components. However its exact function is not yet clear. It was suggested that it might interact directly with portions of the nucleotide binding-site of the membrane associated ATP-binding domain, or that it might be involved in signal transduction between the ATP hydrolysing subunit and the integral membrane components of the transport system (Saurin and Dassa, 1994).

The idea of the existence of specific substrate binding site(s) in the inner membrane components, arise from studies on the maltose transport system, where a *malE* mutant was able to transport maltose independently of any periplasmic protein (Shuman, 1982), and from isolation of mutations in the *hisM* gene (membrane component) of the histidine transport operon, that alter substrate specificity (Payne et al., 1985). Cheng and Ames (1997) carried out studies on histidine transport through the histidine permease of *E. coli*, by using reconstituted proteoliposomes. In this study the histidine permease was inhibited by high concentrations of internal histidine and ADP. The inhibition by histidine revealed the first evidence of the existence of a substrate binding-site in the membrane-bound complex of the histidine permease.

2.5.4.2 ATP-binding Domain

In Opp of both *E. coli* and *S. typhimurium*, the ATP- binding component comprises in two proteins OppD and OppF, which show extensive sequence homology (Higgins *et al.*, 1985). Unlike other characterised transport systems (Fig. 2.4), Opp requires two of these ATP-binding subunits, suggesting that such proteins function as a heterodimer, while in the other systems a single protein functions as homodimer (Higgins *et al.*, 1987). OppD and OppF, as well other ATP-binding components, are hydrophilic and contain no potential spanning

helices, they have been assumed to be peripherally associated with the cytoplasmic face of the inner membrane (Gallagher *et al.*, 1989).

OppD, HisP, MalK and the equivalent proteins from other transport systems, show extensive sequence homology, all possess a consensus ATP-binding site (Higgins *et al.*, 1985), and hydrolysis of ATP during transport has been demonstrated (Mimmack *et al.*, 1989). These proteins are examples of a large family of ATP-binding proteins that is associated with a number of biological processes in addition to transport (Higgins *et al.*, 1986, 1988). These include the HlyB protein involved in haemolysin secretion, the UvrA protein involved in DNA repair, the NodI protein that is required for nodulation in *Rhizobium*, the FtsE protein that is involved in cell division, and the P-glycoprotein from man. In addition to the nucleotide binding motifs, "Walker sites A and B" (Walker *et al.*, 1982), also called "linker peptide", there are several regions shared by these proteins that contain highly conserved amino acid residues. In contrast, a less well conserved sequence of approximately 100 amino acids, that connects the linker peptide has been postulated as a good candidate for involvement in the translocation. This sequence is relatively enriched in hydrophobic amino acids, and thought to fold in an α-helical structure, and therefore has been referred to as "helical domain" (Hyde *et al.*, 1990; Mimura *et al.*, 1991). In a recent study of the maltose ABC transporter, a putative helical domain in MalK (the ATPase component) has been reported. This domain encompasses 52 amino acid residues (89-140), and is crucial for a functional, high affinity interaction with MalF and MalG (membrane-integral proteins) (Wilken *et al.*, 1996).

2.5.5 Regulation of the *opp* Operon

Expression of Opp in *S. typhimurium* was shown to be constitutive (Jamieson and Higgins, 1984). OppA protein is expressed to a much greater extent than the distal OppBCDF proteins. The presence of an intercistronic region of 121 bp long between *oppA-oppB*, that can potentially form a stable stem-loop structure, has been suggested as possibly responsible

for such differential expression. This loop could function as a partial terminator of transcription or stabilizing upstream mRNA and therefore enhancing upstream expression (Hiles *et al.*, 1987).

In *E. coli*, the synthesis of a protein named polyamine-induced protein, was stimulated greatly by the addition of putrescine to growing cells of a polyamine-requiring mutant (Mitsui *et al.*, 1984). This protein was later identified as OppA (Kashiwagi *et al.*, 1990). Recently, it was found that the stimulation of the synthesis of OppA by polyamine, occurs mainly at the level of transcription, by measuring OppA synthesis and its mRNA level (Igarashi *et al.*, 1997).

Hiles and Higgins (1986), showed that OppA accumulates in stationary phase cells until it becomes a major component of the periplasm. Three possible reasons were postulated by these workers to explain this phenomenon (i) transcription of the operon increases (ii) the protein is more stable than any other protein periplasmic protein (iii) the *oppA* mRNA retains translatability longer than the RNA encoding other periplasmic proteins.

In *E. coli* (but not in *S. typhimurium*) the expression of the OppABCDF proteins, is increased by the presence of leucine in the growth medium (Andrew and Short, 1986). The leucine-responsive regulatory protein (Lrp) has been shown to regulate, either positively or negatively, the transcription of several *E. coli* genes in response to leucine (reviewed by Newman *et al.*, 1996). In *lpr* mutant of *E. coli*, the *oppABCDE* operon show a high constitutive expression, suggesting that it is repressed by Lrp, originally named OppI (Austin *et al.*, 1989).

A decrease in synthesis of OppA in *E. coli* was observed by phosphate limitation (Smith and Payne, 1990). This regulation is mediated by the PhoB/PhoR two components regulatory system know as the Pho regulon. PhoB, the activator protein, binds to a specific 'Pho box' sequence (10 bases upstream promoter region) facilitating binding of RNA polymerase and leading to enhanced transcription. Putative Pho boxes have been identified in *opp* operon,

which may allow PhoB to act as a repressor of transcription of these genes (Smith and Payne, 1990).

2.6 Dipeptide permease

Dpp is a peptide-transport system that handles dipeptides with high affinity (Kd values in the order of 0.1 μM) but with some overlapping substrate specificities with Opp and Tpp. The dipeptide system only transport peptides with a free or at least ionizable amino terminus (Gilvarg and Katchalsky, 1965; Payne, 1974) free carboxyl terminus (Payne and Gilvarg, 1968) and shows low specificity for the side chain of the peptide bound (Guyer *et al.*, 1985; Payne and Bell, 1979).

Dpp has been mapped to a single chromosomal locus between 77 min in *S. typhimurium* and 79.2 min in *E coli*.(Olson *et al.*, 1991; Abouhamad *et al.*, 1991). The *dpp* locus of *E. coli* which comprises an operon of five genes, *dppABCDE*, has been cloned (Abouhamad and Manson, 1994). Its organisation is the same as the oligopeptide permease (*opp*) operon of *S. typhimurium* and *spo0K* operon of *Bacillus subtilis*. The first gene in the operon, *dppA*, encodes a 57 kDa periplasmic dipeptide-binding protein (DppA). The membrane-associated components of Dpp, are similar to other periplasmic binding protein-depended transport system (Ames, 1986). The DppB and DppC proteins are highly hydrophobic and have a strong similarity to OppB and OppC, which are integral membrane proteins of Opp of *S. typhimurium*. The *dppD* and *dppE* reading frame overlap, as those of *oppD* and *oppF* from *S. typhimurium*. DppD and DppE are around 45% identical to OppD and OppF or Spo0KD and Spo0KE. DppD and DppE possess the consensus ATP-binding site present in a wide variety of periplasmic binding protein-depended transport systems (section 2.4.5).

The expression of *dpp*, was not affected by carbon source, presence of peptide in minimal medium, anaerobiosis or lack of activity of Opp and Tpp. Therefore its expression appear to be constitutive (Abouhamad *et al.*, 1991).

2.6.1 Dipeptide Binding Protein

The dipeptide binding protein (DppA) is located in the periplasm and function as the initial receptor for dipeptides. DppA and OppA proteins are 26.5% identical at the amino acid level (Olson *et al.*, 1991; Abouhamad *et al.*, 1991). The DppA also functions as the initial chemotaxis receptor for dipeptide, through its interaction with the product of the *tap* gene (Tap: taxis associated protein), a transmembrane protein which transmits the chemotactic signal into the cell (Manson *et al.*, 1986).

The crystal structure of DppA in complex with Glycyl-L-leucine has been determined at 3.2 Å resolution (Dunten and Mowbray, 1995) and the structure of its unliganded form has been determined and refined to a *R*-factor of 0.169 to 2 Å (Nickitenko *et al.*, 1995).

The overall structure of the DppA is similar to that of the *S. typhimurium* OppA. Domain I include residues 34-181, 183-260, and 479-507; domain II include residues 34-181; and domain III includes residues 267-478. The binding site is located between domain I and III, and is designed to recognise the backbone of the ligand, while providing space to accommodate a variety of side chains. This site is also designed to select ligands with charged termini, because both Asp-408 and Arg-355 prefer oppositely charged partners. The spacing of these groups favours binding of dipeptides as opposed to free amino acids. The ability of Dpp to accept some tripeptides, suggests that Arg-355 is free to reposition its side chain to form a favourable interaction with the carboxyl terminus of tripeptide ligands (Dunten and Mowbray, 1995).

Dunten and Mowbray (1995) described three domains in the structure of DppA-dipeptide complex, in contrast with the two domains (I and II) described by Nickitenko *et al.* (1995). These domains are connected by two hinge segments which form part of the base of the wide groove between the two domains. The relative orientation of the two domains gives the protein a pearlike shape. Domain I, which is composed of two integral subdomain (Ia and Ib), is folded from two separated polypeptide segments from the amino- and carboxyl terminal

end. The three domain I, II and III, in *S. typhimurium* OppA, are equivalent to subdomain Ia, subdomain Ib and domain II, in DppA, respectively.

In summary, DppA and OppA share with the other binding protein the critical feature for ligand binding, including the location for ligand site, the participation of both domains in peptide binding and the hinge-bending motion (Quiocho and Ledvina, 1996).

2.7 Tripeptide permease

Tpp system has been identified in *E. coli*. Studies using Opp and Dpp mutant strains, have shown that it can transport a wide range of di-and tripeptides (Alves and Payne, 1980) with a preference for those containing hydrophobic amino acids, particularly with N-terminal Val or Met residues (Payne, 1983).

Most toxic peptides that enter the cell through Tpp are also substrates for Opp, however, the synthetic peptide antibiotic alafosfalin (L-alanyl-L-aminoethyl phosphonic acid) is specifically transported by Tpp. Gibson and his coworkers (1984) isolated a *S. typhimurium* mutant deficient in Tpp, using alafosfalin. This mutation was mapped at two separate loci *tppA* to 74 min and *tppB* to 27 min. Locus *tppA* encodes a positive regulator of *tppB* (Jemieson and Higgins, 1984) and *tppB* presumably encode the structural component of Tpp.Tpp is induced by anaerobic growth and is positively regulated by the EnvZ/OmpR regulatory system (Gibson *et al.*, 1987).

2.8 ABC-Type Transporter Systems in Gram-positive Bacteria

Early work based on competition studies and peptide transport-deficient mutants (Nisbet and Payne, 1982) demonstrated the presence of two separate uptake systems for peptides in *Streptococcus faecalis*. One is a high-rate system used by dipeptides, and to a lesser extent

tripeptides; the other is a low-rate oligopeptide system. While a single peptide transport system which recognizes both di-and tripeptides was shown in *Staphylococcus aureus* (Perry, 1981). Peptide transport systems in Gram-positive bacteria are essential for the uptake of peptides as sources of nutrients, but they are also involved in determining other cellular functions and properties.

Several examples of operons encoding ABC-type transporters in Gram-positive bacteria have been reported, and are showed in Table 2.2. Some of these examples are considered below.

Table 2.2

ABC-type transporters in Gram-positive Bacteria

Substrate	Organism	Operon	Reference
Oligopeptides	*Bacillus subtillis*	*spo0K*	Perego *et al.* (1991); Rudner *et al.* (1991)
Dipeptides	*Bacillus subtillis*	*dciA*	Mathiopoulos *et al.* (1991)
Sugars	*S. mutans*	*msm*	Russell *et al.* (1992)
Oligopeptides	*Lactococcus*	*oppDFBCA*	Tynkkynen *et al.* (1993)
Maltose	*S. pneumoniae*	*mal*	Puyet and Espinosa (1993)
Oligopeptides	*S. pneumoniae*	*amiABCDEF*	Alloing *et al.* (1994)
Oligopeptides	*E. faecalis*	*oppABCDF*	Leornard *et al.* (1996)
Oligopeptides	*S. pyogenes*	*oppABCDF*	Podbielski *et al.* (1996)
Oligopeptides	*Streptomyces coelicor*	*bldK*	Nodwell *et al.* (1996)
Hexa-heptapeptides	*S. gordonii*	*Hpp*	Jenkinson *et al.* (1996)

The idea that substrate-binding lipoproteins in Gram-positive bacteria that lack a periplasm are functionally equivalent to the high affinity binding periplasmic proteins of Gram-negative bacteria was introduced by Gilson *et al.*(1988). These workers suggested that the proposed 'binding lipoprotein' in these microorganisms would be maintained in the proximity of the inner membrane by insertion of their N-terminal glyceride-cysteine into this membrane. This hypothesis has been supported by Sutcliffe *et al.* (1993) who showed that the *msmE* gene product is part of the multiple sugar transport in *S. mutans* is a lipoprotein.

2.8.1 Ami System of *Streptococcus pneumoniae*

Evidence that *S. pneumoniae*, possess a high affinity binding-protein dependent transport system, was presented by Gilson and co-workers (1988). These workers found two proteins, AmiA and MalX, that have extensive homology with periplasmic binding proteins, OppA and MalE involved in oligopeptide and maltose transport, respectively, in Gram-negative bacteria. Complete nucleotide sequences revealed the presence of six open reading frames of *amiABCDEF*, encoding six proteins, AmiABCDEF (Alloing *et al.*, 1990). The AmiA, C, D, E and F proteins exhibit homology with components of the oligopetide permease of *S. typhimurium* and *E. coli*. AmiE and AmiF are likely to be involved in coupling of energy, and are homologous to the peripheral membrane components OppD and OppF. AmiC and AmiD proteins are strongly hydrophobic and are more than 30% homologous to OppB and OppC respectively. AmiB protein has no counterpart in the *opp* operon and its function remains to be determined.

Two homologous genes, *aliA* and *aliB*, have been identified in *S. pneumoniae* (Alloing *et al.*, 1994). Both genes have been shown to encode putative lipoproteins highly homologous to AmiA. Ami system and AmiA-AliA-AliB family constitute the first example of a transport system reported, in which multiple substrate binding proteins, with largely overlapping specificities, interact with a single transmembrane complex. In this study it also was

demonstrated that the three proteins were metabolically labelled with [^{3}H]palmitic acid, providing strong support to the hypothesis that the binding protein in ABC-transporters of Gram-positive bacteria is a membrane-bound lipoprotein.

It was suggested that the peptide permease in *S. pneumoniae* could modulate transformation, possibly via a direct interaction between the extracellular pneumococcal activator and the oligopeptide binding protein (Pearce *et al.*, 1994). However, an unmodified heptadecapeptide pheromone, named CSP (competence-stimulating peptide) capable of eliciting competence for genetic transformation was recently identified and characterised (Håvarstein *et al.*, 1995).

This CSP pheromone required neither Ami nor any of the three highly homologous oligopeptide binding proteins (Alloing *et al.*, 1996).

In *pneumococci*, the reduction of adhesion of Opp mutants to human epithelial and endothelial cells, has been suggested to result from the loss OppA expression, which can act directly as an adhesin, or by loss of other adhesins due to regulation of adhesin expression by Opp (Cundell *et al.*, 1995).

2.8.2 Opp system and the PrgZ-Opp complex of *Enterococcus faecalis*

Conjugative transfer of the plasmids pCF10 by *E. faecalis* donor cells occurs in response to a heptapeptide, cCF10 (Leu-Val-Phe-Leu-Val-Thr-Val), that has been shown to be an active extracellular pheromone (Mori *et al.*, 1988). Recent genetic analyses have identified two plasmid-encoded genes, *prgZ* and *traC*, whose derived product are homologous to the peptide-binding protein (Ruhfel *et al.*, 1993). Experimental evidence suggest that these protein may bind the peptide pheromones and transport it into the cell through a chromosomally encoded oligopeptide permease (Fig. 2.8), thereby inducing intracellular aggregation (Leonard *et al.*, 1996).

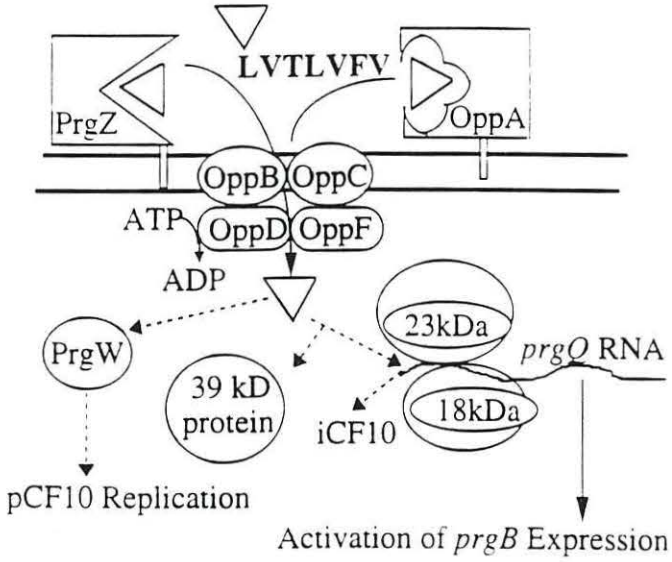


Figure 2.8

Model of cCF10 (LVTLVFV: the peptide pheromone) signalling during pCF10 conjugation, proposed for *E. faecalis*, by Leornard *et al.* (1996). Recipient-secreted pheromone (cCF10) interact with either the plasmid-encoded specific binding protein PrgZ or the lower affinity chromosomal binding protein OppA on the donor cell. cCF10 is then transported into the cell via chromosomally encoded oligopeptide permease system. Once inside the cell, the pheromone interacts with intracellular effector molecules, including possible displacing iCF10 from a ribonucleoprotein complex, leading to activation of prgB (aggregation substance) and the subsequent conjugative transfer of the plasmid to the recipients. Taken from Leonard *et al.* (1996).

2.8.3 Spo0K system and Dpp in *Bacillus subtilis*

Bacillus subtilis cells differentiate into spores resistant to heat, radiation and strong chemicals (Errington, 1993). In this microorganism, mutations at *spo0* loci are known to affect the initiation of sporulation. Studies in one of this loci, *spo0K*, demonstrated that the mutations were located within a five-gene operon encoding a binding protein-dependent oligopeptide

transport system (Perego *et al.*, 1991). The proteins encoded by *spo0K* operon are similar in sequence and organisation to the oligopeptide transport system of *S. typhimurium*. Spo0KA is the initial substrate binding protein, contains a signal sequence with a lipoprotein processing site, and is anchored to the membrane via an amino-terminal lipoyl moiety. OppB and OppC, encode two highly hydrophobic integral membrane proteins. OppD and OppF encode the ATP-binding component. However, unlike the Opp system of *S. typhimurium*, OppF, was not required for peptide transport or for sporulation (Perego *et al.*, 1991). These workers postulated that the accumulation of peptides that play a signalling role in the initiation of sporulation are transported by the oligopeptide transport system, which suggests a role in a sensory process rather than nutrient transport. The initiation of the sporulation may be triggered by the transport of signal peptides, such as degradation of products from peptidoglycan (Perego *et al.*, 1991; Rudner *et al.*, 1991).

Sporulation is a process that is characterised by substantial changes in patterns of gene expression. The initial gene expression required for development of spores is regulated by the Spo0A transcription factor. Spo0A is activated by phosphorylation, in response to a series of signals (for example, nutrient deprivation, cell cycle signals) and the levels of phosphorylation of this factor determines whether a cell will divide or sporulates (Burbulys *et al.*, 1991; Ireton *et al.*, 1993). Two pentapeptides, the competence- and sporulation factor (CFS) and the signal peptides encoded by the *phrA* gene that stimulate the sporulation, are examples of signalling molecules. Both CSF and PhrA peptides repress the activity of specific members of the Rap family of phosphatases. This repression stops the Raps proteins from dephosphorylating response-regulator molecules that themselves regulate genes involved in competence and sporulation (Perego and Hoch, 1996). These peptides are normally produced by secretion and processing of precursor molecules. The responses to these extracellular signalling molecules, later termed pheromones, CSF and PhrA, dependent on the *spo0K* (*opp*)-encoded oligopeptide permease (Solomon *et al.*, 1996; Perego and Hoch, 1996). Recently, Lazazzera and Solomon (1997) showed that both CSF and PhrA pheromones, are transported into cells by oligopeptide permease.

The dipeptide permease transport system represents another member of the ABC transporter described in *Bacillus subtilis*. The *dpp* operon originally *dciA* (Mathiopoulus and Sonenshein, 1989) encodes an early stationary phase-inducible dipeptide transport system (Mathiopoulus *et al.*, 1991). Expression of *dpp* is repressed during growth in media containing rapidly metabolisable sources of carbon and nitrogen or mixtures of amino acid, its expression is induced by all mechanisms known to turn on sporulation and is indirectly under Spo0A control (Slack *et al.*, 1991). A four gene operon (*codVWXY*) in which the promoter-distal codes for a product required for repression of the *dpp* operon has been reported. This repression take place during exponential growth phase in a nutrient-rich medium (Slack *et al.*, 1995).

Recently, a novel ABC transporter with a role in exoprotein production, sporulation and competence has been reported (Leskelä *et al.*, 1996). The *ecs* locus, a putative operon consists in three open reading frames, *ecsA*, *ecsB* and *ecsC*. The *ecsA* gene encode a putative polypeptide of 248 amino acids residues containing an ATP-binding site, and share about 30% sequence similarity with ATP-binding components of several ABC transporters. The *ecsB* gene was predicted to encode a hydrophobic protein with structural similarities to the integral membrane components of other ABC transporter. The structural prediction of *ecsC* gene product did not give an indication that EcsC is a second hydrophobic component of a putative ABC transporter.

2.8.4 Binding-Lipoprotein Dependent Oligopeptide Transport System in *Streptococcus gordonii*

A role for peptide permeases in bacteria adherence was first suggested for *S. gordonii*, an organism that together with *S. oralis* and *S. sanguis* are prominent components of human oral microbiota (Frandsen *et al.*, 1991). These *Streptococcal* species bind human salivary components including α-amylase, acidic proline-rich proteins, proline-rich glycoprotein,

mucin and salivary agglutinin. When these proteins are present in the acquired pellicle which coat oral surfaces, they act as receptors permitting the attachment for other *Streptococci* to the tooth surface and the formation of dental plaque.

A binding-lipoprotein-dependent oligopeptide transport system in *S. gordonii* has recently been reported (Jenkinson *et al.*, 1996). This Hpp (Hexa-heptapeptide permease) consists in three cytoplasmic membrane-bound lipoproteins that are the product of three genes (*hppA, hppG and hppH*). These proteins have apparent molecular mass of 76 to 78 kDa and are highly similar to the AmiA, AliA and AliB substrate binding protein components of the oligopeptide permease in *S. pneumoniae*.

The *hppA* gene was identified originally following insertional experiments. HppA$^-$ mutant cells of *S. gordonii* were reduced in their ability to be aggregated in human saliva or serum and were deficient in adherence to strain of Actinomyses naeslundii (Jenckinson and Easingwood, 1990). The Hpp system was essential to the binding and subsequently uptake of hexa- or heptapeptides. In addition to peptide transport and adherence, this system plays a role in the control of metabolic functions associated with the growth of streptococcal cells on complex nitrogen sources and with development of competence (Jenkinson *et al.*, 1996).

2.9 Antibacterial peptide analogues

The broad structural specificities of peptide permeases in microorganisms have provided an opportunity for development of a broad range of peptide-carrier prodrugs, such as derivatised amino acid residues or unnatural analogues, that might act as substrate. Figure 2.9 illustrates the process by which peptide transporters can be used to transport impermeant toxic moieties, which are inhibitors of an intracellular target, into the cell in the form of peptide carrier prodrugs via peptide permeases. Once inside the organism, it can be released enzymatically (most commonly by peptidase action). In this way, the toxic compound cannot undergo exodus and is actively accumulated (Payne and Smith, 1994; Payne, 1995).

Various terms have been proposed to describe peptide-mediated transport of impermeant moieties, these include 'illicit transport' (Ames *et al.*, 1973), 'Portage transport' (Gilvarg, 1981), 'Warhead delivery' (Ringrose, 1980). The word 'Smugglins' was suggested by Payne (1976) to define the peptide-carrier complex.

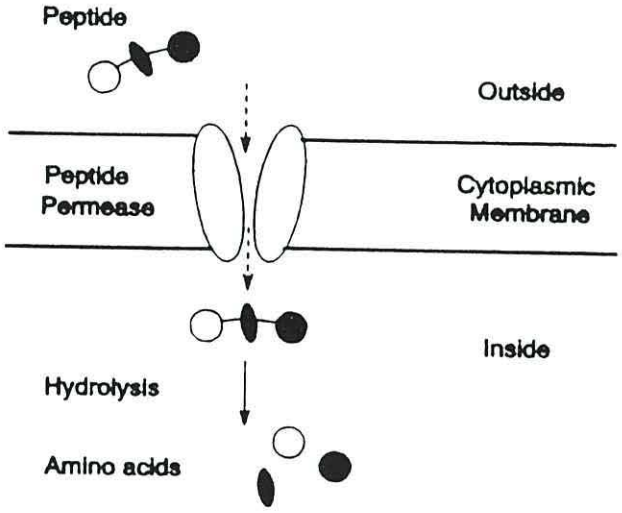


Figure 2.9

Exploitation of bacteria peptide permease by peptide carrier-prodrugs. The impermeant inhibitor is transporter into the cell as part of a peptide prodrug via the peptide permease. Within the cytoplasm, the inhibitor in released in free form, and being able to accumulate to high intracellular concentration, its activity against its target is increased. Taken from Payne, 1995.

2.9.1 Natural smugglins

There are many naturally occurring peptide smugglins active against bacteria whose structure and mechanism of actions have been extensive studied. Bacilysin is a dipeptide mimetic produced by *Bacillus subtilis* that was first identified by its ability to lysis growing culture of *Staphylococcus aureus* (Abraham *et al.*, 1946). Bacilysin is composed of an N-terminal L-alanine residue and C-terminal amino acid mimetic, L-β-(2-3-epoxycyclohexanono-4)-alanine know as anticapsin. Bacilysin is transported predominantly by dipeptide permease. Once inside the cell it is hydrolysed by intracellular peptidases to yield anticapsin, which acts as glutamine analogue to inhibit glucosamine synthetase and consequently peptidoglycan

biosynthesis. Thus, anticapsin itself is inactive but, in the form of peptide (bacilysin) it inhibits both Gram-negative and Gram-positive bacteria (Ringrose, 1980, 1983; Kenig and Abraham, 1976).

Phosphinothricyl-Ala-Ala (bialaphos) is a tripeptide phosphorus-containing amino acid analogue that has been well characterised. It is produced by *Streptomyces hydroscopicus* and is active against Gram-negative and Gram-positive bacteria (Diddens *et al.*, 1976, 1979). The toxic moiety, phophinothricin is a δ-phosphinate (L-2-amino-4 methylphosphino butiric acid) analogue of glutamic acid and consequently is a potent inhibitor of glutamine synthetase. Two tripeptides, closely related to phosphinothricyl-Ala-Ala are L-(N^5-phosphono)methionine-S-sulphoximiny-Ala-Ala and the synthetic analogue L-methionine-S-dioxydyl-Ala-Ala, both are potent inhibitors of glutamine synthetase, and as well as bialaphos are inactive against the isolated enzyme.

Examples of natural smugglins containing residues with β-lactam rings include the dipeptide Alanyl-3α-S-chloro-3-S-hydroxyl-2oxo-3-Azetidimylmethyl-S-alanine and the phytotoxin tabtoxin, both transported by di-and oligopeptide permeases. β-lactams interfere with the terminal stages of bacterial peptidoglycan polymerization and cross-linking by inhibiting specific enzyme and/or functional proteins located on the outer surface of the cytoplasmic membrane. The β-lactam group of antibiotics are the most widely used and probably the safest of all therapeutic agents (Ringrose, 1980, 1983).

2.9.2 Synthetic smugglins

As a consequence of the knowledge of natural peptides, various synthetic peptides analogues have been made to extend the "illicit transport" (Ames *et al.*, 1973) as a novel approach to designing chemotherapeutic agents.

Among the better studied antimicrobial synthetic peptides, the phosphonopeptides (a phosphonate group replacing the C-terminal carboxyl group) particularly alafosfalin has been

extensively studied. Alafosfalin is an analogue of L-alanyl-L-alanine with a phosphonic acid group instead of the C-terminal carboxyl group. It has a wide antibacterial spectrum, being more active against Gram-negative bacteria than Gram-positive bacteria (Allen *et al.*, 1978). The mode of action of Alafosfalin involves active transport by peptide permeases followed by rapid intracellular aminopeptidase cleavage to yield the L-1-aminoethyl-phosphonic acid warhead (AlaP), which is not able to leave the cell. Once inside the cell it inhibits alanine racemase and, thus, bacterial wall biosynthesis. This inhibition is reversible in Gram-negative bacteria and irreversible in Gram-positive bacteria (Atherton *et al.*, 1979a and b). *In vivo* studies have demonstrated that alafosfalin is active in animal infection, being absorbed reasonably well by the oral route (Allen *et al.*, 1978). The L-1-aminoethyl-phosphonic acid was incorporated into a range of smugglin prodrugs. This mimetic acted as an analogue of both L- and D-alanine and inhibit not only alanine racemase but also UDP-N-acetylmuramyl-L-alanine synthetase and D-Ala-D-Ala synthetase (reviewed by Payne, 1995).

Analogues of di-and tripeptides in which the peptide linkage (-CO-NH-) is replaced by -CO-NH-NH- (hydrazino analogue) or by -CO-NHO- (aminoxy analogue) have been shown to have antibacterial activity against *E. coli*, *Staphylococcus aureus* and *Salmonella dublin* (Morley *et al.*, 1983). Subsequent studies showed that aminoxy peptides are transported by all three peptide permeases of *E. coli* and that intracellular hydrolysis, to yield the free aminoxy amino acid, resulted in their toxicity (Payne *et al.*, 1984).

In addition to antibacterial compounds that act by inhibiting the cell wall biosynthesis, other synthetic antibacterial have been designed to inhibit the biosynthesis and assembly of the major lipopolysaccharide of the Gram-negative bacteria outer-membrane. The 2-deoxy analogue of β-KDO (3-deoxy-β-D-manosyl-2-octulopyranosonic acid) is a potent inhibitor of the CMP-KDO synthetase, but it fails to penetrate into bacteria. However, linked to a small peptide (e.g., Lys-Leu or Ala-Ala) it is actively transported through Opp to yield activity with a broad antibacterial spectrum (Hammond *et al.*, 1987; Goldman *et al.*, 1987; Smith and Payne, 1990).

2.10 General Objectives of this Study

The oligopeptide binding protein of *E. coli* and *S. typhimurium* has been extensively studied. These studies include genetic characterisation, kinetic parameters, substrate specificity, affinity for a broad range of substrate, tertiary structure and atomic structure. However, the interactions between OppA and the membrane components remain poorly understood. Thus the identification of the amino acid(s) of OppA involved in the interaction with the membrane components of oligopeptide permease, could be very important for understanding the molecular events for the translocation of the peptide across the membrane.

Previous studies carried on in this laboratory (Marshall, 1994), showed that a small fragment of OppA (residues 300-306), was involved in the function of oligopeptide permease. On the basis of these results experiments were performed to characterise the interactions of OppA with its ligands and the interactions between OppA and the membrane components.

To achieve this aim, the following objectives were proposed:

For the characterisation of *oppA*$^-$ mutant strains suitable for complementation with cloned OppA protein, monoclonal antibodies against *E. Coli* OppA, have to be re-cloned and characterised.

To investigate the role of Asp-300 of OppA protein, in the oligopeptide permease function, mutants able to express OppA containing a site-directed mutagenesis, have to be produced and characterised.

To test the ability of OppA mutant proteins to bind substrate, these proteins have to be produced and purified.

To produce strains carrying mutations affecting the membrane components, the strain PA0522 (containing [Asp^{300}→Ser]-OppA plasmid) able to express OppA but lack Opp function, has to be used to produce mutants able to compensate the loss of Opp function.

Peptides representing a specific region of OppA protein containing the Asp-300 residue has to be chemically synthesised, to be used in the production of antipeptide antibodies. Other synthetic peptides containing a different amino acid at the same position, also have to be produced.

For the screening of compensative mutants, anti-peptide antibodies against the synthetic peptides containing the Asp-300 or other residue at the same position of the OppA protein has to be produced.

These anti-peptide antibodies will be used in connection with bifunctional cross-linking reagent in future experiments to characterise the interaction of liganded-OppA with the oligopeptide permease protein in membrane vesicles, to explain its mechanism of action. On the other hand, the highly conserved amino acid sequence selected, makes these anti-peptide antibodies suitable to investigate similar systems in other species such as *Streptococci* and *Staphylococci*.

Chemicals and Methods

Chapter III

CHEMICALS

3.1 General Chemicals

4-chlohoro 1-naftol	Sigma Chemical Company Ltd., Poole, UK
α- mouse (anti-rabit)IgG	Sigma Chemical Company Ltd., Poole, UK
β-mercaptoethanol	Sigma Chemical Company Ltd., Poole, UK
ABTS	Sigma Chemical Company Ltd., Poole, UK
Acetic Acid (glacial)	General Laboratory supplies, Chester, UK
Acetonitrile	Rathburn Chemical Ltd., Walkerburn, UK
Acrylamide/Bisacrylamide (37.5:1)	Sigma Chemical Company Ltd., Poole, UK
Agar noble	Difco Laboratories, Detroit, USA
Agarose gel	(BDH) Merck Ltd., Liverpool, U.K
$AgNO_3$	Sigma Chemical Company Ltd., Poole, UK
Alkaline phosphatase	Sigma Chemical Company Ltd., Poole, UK
Bacto-agar	Difco Laboratories, Detroit, USA
Bacto-tryptol	Oxoid Unipath Ltd., Hampshire, UK
Bacto-yeast	Oxoid Unipath Ltd., Hampshire, UK
BCA	Pierce, Rockford, Illinois USA
BCIP	Sigma Chemical Company Ltd., Poole, UK
Brilliant Blue G	Sigma Chemical Company Ltd., Poole, UK
Bromophenol blue	Electra BDH Chemicals Ltd., UK
BSA (A grade)	Sigma Chemical Company Ltd., Poole, UK
$Ca_2Cl\ 2H_2O$	(BDH) Merck Ltd., Liverpool, U.K
CH_3COOK	(BDH) Merck Ltd., Liverpool, U.K
Chloramphenicol	Sigma Chemical Company Ltd., Poole, UK

Citrate-phosphate buffer with sodium perborate (capsules)	Sigma Chemical Company Ltd., Poole, UK
Citric acid	(BDH) Merck Ltd., Liverpool, U.K
CNBr	Sigma Chemical Company Ltd., Poole, UK
$CoCl_3$	(BDH) Merck Ltd., Liverpool, U.K
Comassie brilliant blue 250	(BDH) Merck Ltd., Liverpool, U.K
$CuSO_4$	(BDH) Merck Ltd., Liverpool, U.K
Dimethyl sulphoxide	(BDH) Merck Ltd., Liverpool, U.K
DMF	Sigma Chemical Company Ltd., Poole, UK
DMSO	(BDH) Merck Ltd., Liverpool, U.K
DTT	Sigma Chemical Company Ltd., Poole, UK
EDTA	Sigma Chemical Company Ltd., Poole, UK
Ethanol	Haymann, Withman (Essex), UK
Ethidiumbromide	(BDH) Merck Ltd., Liverpool, U.K
FCS	Gibco, nbl Northumberland, U.K
$FeCl_3$	Sigma Chemical Company Ltd., Poole, UK
$FeSO_4$ $7H_2O$	(BDH) Merck Ltd., Liverpool, U.K
Ficoll (type 400)	Pharmacia, Upsala, Sweden
Formaldehyde	(BDH) Merck Ltd., Liverpool, U.K
Formic acid	Sigma Chemical Company Ltd., Poole, UK
Glacial acetic acid	(BDH) Merck Ltd., Liverpool, U.K
Glucose	(BDH) Merck Ltd., Liverpool, U.K
Glutaraldehyde	Aldrich Chemical Company Ltd., UK
Glycerol	(BDH) Merck Ltd., Liverpool, U.K
Glycine	Sigma Chemical Company Ltd., Poole, UK
Goat anti mouse polyvalent immunoglobulin (IgG,IgA,IgM) peroxidase	Sigma Chemical Company Ltd., Poole, UK
Goat anti rabit IgG (whole molecule) labelled with Horseradish peroxidase type IV	Sigma Chemical Company Ltd., Poole, UK
HAT	Gibco, nbl, Northumberland, UK
H_2O_2	Sigma Chemical Company Ltd., Poole, UK
HCl	(BDH) Merck Ltd., Liverpool, U.K
Helium	Sigma Chemical Company Ltd., Poole, UK
High molecular weight satandard mixture	Sigma Chemical Company Ltd., Poole, UK
isoamyl alcohol	Gibco, nbl Northumberland, U.K
Isopropanol	Scientific Services, Tattenhall, UK
K_2HPO_4	(BDH) Merck Ltd., Liverpool, U.K
K hexaferricyamide	(BDH) Merck Ltd., Liverpool, U.K
KCl	Aldrich Chemical Company Ltd., UK
KH_2PO_4	(BDH) Merck Ltd., Liverpool, U.K
KOH	(BDH) Merck Ltd., Liverpool, U.K
Leucine	Sigma Chemical Company Ltd., Poole, UK

Liquid nitrogen	BOC Ltd, Guildford (Surrey), UK
Malonic acid	Sigma Chemical Company Ltd., Poole, UK
Marker proteins pI	Sigma pI standard
Methanol	Rathburn Chemical Ltd., Walkerburn, UK
$MgCl_2$	(BDH) Merck Ltd., Liverpool, U.K
$MgSO_4$	(BDH) Merck Ltd., Liverpool, U.K
Milk power (non-fat)	Marvel; Premier Brands U.K. Ltd.,Stafford, U.K.
$MnCl_2$ $4H_2O$	(BDH) Merck Ltd., Liverpool, U.K
MOPS	(BDH) Merck Ltd., Liverpool, U.K
Na_2CO_3	Fluka Chemie AG. Buchs., Switzerland
NaCOOH	(BDH) Merck Ltd., Liverpool, U.K
$NaHCO_3$	(BDH) Merck Ltd., Liverpool, U.K
NaOH	Scientific Services Ltd., Toteehnhall, UK
NH_4HCO_3	(BDH) Merck Ltd., Liverpool, U.K
$(NH_4)_2S_2O_8$	Sigma Chemical Company Ltd., Poole, UK
$(NH_4)_2SO_4$	Sigma Chemical Company Ltd., Poole, UK
NBT	Sigma Chemical Company Ltd., Poole, UK
Pyronine Y	Sigma Chemical Company Ltd., Poole, UK
RbCl	(BDH) Merck Ltd., Liverpool, U.K
Restriction enzyme *Eco*Rv	Promega Corporation, Madison, USA
Restriction enzyme *Hind*III	Promega Corporation, Madison, USA
Restriction enzyme *Puv*II	Promega Corporation, Madison, USA
Restriction enzyme *Sal*I	Promega Corporation, Madison, USA
RNasa A	Sigma Chemical Company Ltd., Poole, UK
SDS	Sigma Chemical Company Ltd., Poole, UK
Sodium citrate	(BDH) Merck Ltd., Liverpool, U.K
Sucrose	(BDH) Merck Ltd., Liverpool, U.K
TBS tween	Sigma Chemical Company Ltd., Poole, UK
TEMED	Sigma Chemical Company Ltd., Poole, UK
Thimerosal	Sigma Chemical Company Ltd., Poole, UK
Triornithine	Bachem Feinchemikalien AG, Budendorf
Trisodium citrate	(BDH) Merck Ltd., Liverpool, U.K
Triton X-100	Aldrich Chemical Company Ltd., UK
Trp	Difco, East Molesey, U.K.
Tween 20	Sigma Chemical Company Ltd., Poole, UK
Xylene cyanol FF	Aldrich Chemical Company Ltd., UK
Yeast extract	Oxoid Unipath Ltd., Hampshire, UK

3.2 Chemicals and Disposables for Cell Culture and Antibodies Production

Chemicals

CO_2	BOC, Ltd., Guilford (Surrey) U.K.
$CuSO_4$	Scientific Services, Tattenhall, U.K.
DMSO	(BDH) Merck Ltd., Lutterworth, U.K
Eosin	(BDH) Merck Ltd., Lutterworth, U.K.
FCS	Gibco, nbl, Northumberland, U.K.

Freund's complete adjuvant	Difco, East Molesey, U.K.
Freund's incomplete adjuvant	Difco, East Molesey, U.K.
HAT (50x)	Gibco BRL, Uxbridge, U.K.
Horse serum	Sigma Chemical Company Ltd., Poole, UK
N_2	BOC Ltd., Guildford (surrey), U.K.
NH_4Cl	Sigma Chemical Company Ltd., Poole, UK
Nystatine	Sigma Chemical Company Ltd., Poole, UK
Penicillin|Streptomycin (100x)	Gibco, nbl Northumberland, U.K
RPMI 1640 glutamax II	Gibco BRL, Uxbridge, U.K.
RPMI 1640 medium	Gibco BRL, Uxbridge, U.K.

Disposables

Container, 30 ml (universal flask)	Philip Harris, Manshester, U.K.
Cryovial, 1.2 ml (Nalgene)	Philip Harris, Manshester, U.K.
Needles, sterile (0.5 x 16 mm)	Becton Dickinson, Dublin, Ireland
Needle, sterile (0.6 x 25 mm)	Terumo, Europe N.V., Belgium
Pipette, disposable (10 ml)	Philip Harris, Manshester, U.K.
Polystyrene pipette, 1 ml	Philip Harris, Manshester, U.K.
Syringe, disposable (1.5 and 10 ml)	Becton Dickinson, Dublin, Ireland
Tisue culture cluster, 96 wells	Philip Harris, Manshester, U.K.
Tisue culture cluster, 24 wells	Philip Harris, Manshester, U.K.
Tisue culture flask, 25 cm^2	Philip Harris, Manshester, U.K.

3.3 Chemicals for the Solid Phase Peptide Synthesis

Diethyl ether	Prolabo, Manchester, UK
DIPCDI	Aldrich Chemical Company Ltd., UK
EDT (1,2-ethanedithiol)	Lancaster synthesys
Fmoc-Arg(Pmc)-OH	Calbiochem-Novabiochem Corp., UK
Fmoc-Lys(Boc)-OH	Calbiochem-Novabiochem Corp., UK
Fmoc-Asp(OtBu)-OH	Calbiochem-Novabiochem Corp., UK
Fmoc-Ser(tBu)-OH	Calbiochem-Novabiochem Corp., UK
Fmoc-Met-OH	Peboc Limited, Llangefni, Wales, UK
Fmoc-Leu-OH	Peboc Limited, Llangefni, Wales, UK
Fmoc-Val-OH	Peboc Limited, Llangefni, Wales, UK
Fmoc-Ala-OH	Peboc Limited, Llangefni, Wales, UK
Fmoc-Phe-OH	Peboc Limited, Llangefni, Wales, UK
Fmoc-Pro-OH	Peboc Limited, Llangefni, Wales, UK
Fmoc-Trp-OH	Peboc Limited, Llangefni, Wales, UK
Ninhydrin	Sigma Chemical Company Ltd., Poole, UK
Phenol	Sigma Chemical Company Ltd., Poole, UK
Potasium cyanide	Aldrich Chemical Company Ltd., UK
Pyridine	(BDH) Merck Ltd., Liverpool, U.K
TFA	Lancaster synthesys
Thioaniasole 991	Lancaster synthesys

Tis (Trisopropylsilane)	Lancaster synthesys
Wang resin	Calbiochem-Novabiochem Corp., UK

Chapter IV

GENERAL METHODS

4.1 Bacterial strains and plasmids

All *Escherichia coli* strains used during this study were derived from the wild type strain K12. *E. coli* derivative strains and plasmids are listed and described in Table 4.1.

4.2 Growth of bacterial cultures

Bacterial strains were grown initially by transferring cells from a frozen glycerol culture, with a sterile wooden stick to inoculate 5 ml of LB medium. This culture was grown overnight, with shaking at 37°C, subsequently it was streaked onto agar plate, by using a sterile wire loop, and grown overnight at 37°C. The growth of a bacterial culture was monitored using a Pharmacia LKB Ultrospec 4050 spectrophotometer (Pharmacia LKB Biotechnology Ltd., Bucks., UK). A graph of number of cells per ml of culture against A_{660}, as measure by this spectrophotometer, is shown in figure 4.1.

Table 4.1

Bacterial Strains and Plasmids

Strain or plasmid	Opp phenotype	Source
E. coli		
*M2034	Wild type	Morse and Guerlin (1972)
PA0309	OppA$^-$	This laboratory
PA0467	OppA$^-$	This laboratory
PA0470	OppA$^-$	This laboratory
PA0472	OppA$^-$	This laboratory
PA0479	OppA$^-$	This laboratory
PA0480	OppA$^-$	This laboratory
PA0481	OppA$^-$	This laboratory
PA0483	OppA$^-$	This laboratory
PA0484	OppA$^-$	This laboratory
PA0183	OppABCDF$^-$	This laboratory
PA0521	As PA0467,but complemented with pI5.1	This laboratory
PA0522	As PA0467, but complemented with pBAN2	This study
PA0523	As PA0467, but complemented with pBAN3	This study
PA0588	L and W revertant mutant	This study
PA0589	L and W revertant mutant	This study
PA0591	Compensative mutant	This study
PA0593	Compensative mutant	This study
PA0605	Compensative mutant	This study
CM01	PA0467 transformed with pBAN2-1	This study
CM02	PA0467 transformed with pBAN2-2	This study
CM03	PA0467 transformed with pBAN2-3	This study
CM04	PA0467 transformed with pBAN2-4	This study

Strain or plasmid	Opp phenotype	Source
CM05	PA0467 transformed with pBAN2-5	This study
CM06	Cured PA0591 transformed with pBAN2	This study
CM07	Cured PA0591 transformed with pBAN3	This study
CM08	Cured PA0591 transformed with pBAN2-1	This study
CM09	Cured PA0591 transformed with pPI5.1	This study
Plasmid		
pI5.1	Wild type OppA	Kashiwagi *et al.*,(1990)
pBAN2	OppA(^{300}Asp→Ser) mutant	This laboratory
pBAN3	OppA(^{300}Asp→Arg) mutant	This laboratory
pBAN2-1	Plasmid isolated from PA0588	This study
pBAN2-2	Plasmid isolated from PA0591	This study
pBAN2-3	Plasmid isolated from PA0589	This study
pBAN2-4	Plasmid isolated from PA0593	This study
pBAN2-5	Plasmid isolated from PA0605	This study

All mutant strains used in this study are derivatives of strain M2034, and were selected by its resistance to triornithine

* M2034 genotype: *trp*E 9851 *leu* 277 F' IN(rrnD-rrnE)

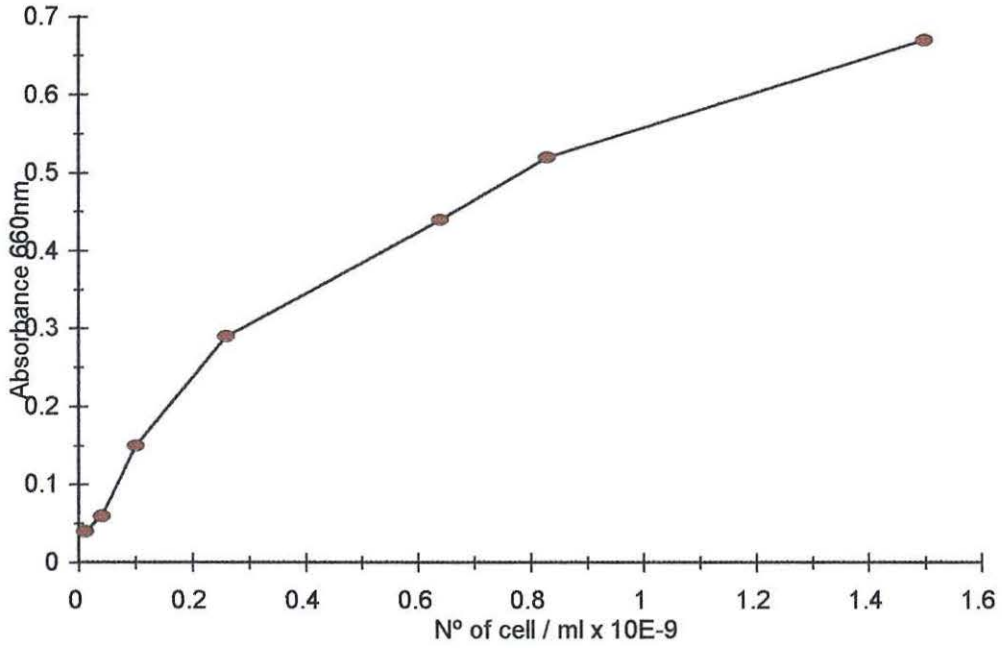


Figure 4.1:

Variation in absorbance at 660nm with number of *E. coli* cells. The variation in absorbance at 660nm with number of *E. coli* cells per ml of culture, as measured using a Pharmacia LKB Ultrospec 4050 spectrophotometer.

4.3 Storage of bacteria

Bacterial strains were maintained for short-term on minimal medium agar plates, containing all supplements needed, and antibiotic when required; wrapped in parafilm and stored at 4°C. Strains were subcultured every two months onto a fresh agar plate. For medium-term storage, strains were maintained on LB medium agar slopes at room temperature, antibiotic was added when required. These strains were used within 2 years. To preserve strains for long-term, glycerol cultures were prepared. Strains were grown in 5 ml LB medium, with shaking at 37°C, until late-logarithmic phase, and 0.6 ml of culture were mixed with the same volume of 80% sterile glycerol. This culture was frozen in liquid nitrogen and stored at -70°C.

4.4 Media

4.4.1 Minimal medium 'A'

Minimal medium 'A' (Davis and Mingioli, 1950) contained (per litre): 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$ and 0.5 g sodium citrate$\cdot 3H_2O$. The solution was made up at ten times concentration and sterilised by autoclaving. When required, this medium was diluted ten times in sterile distilled water, and 0.5% glucose and supplement(s) added as required. All supplements were sterilised by autoclaving except tryptophan, which was filter-sterilised using Oxoid cellulose acetate 0.45 µm filters.

4.4.2 Luria-Berthani medium

LB medium (Miller, 1972) contained 1% tryptone, 0.5% yeast extract and 1% NaCl. Some drops of 1 M NaOH were added to adjust the pH to 7.0

4.4.3 SOB medium

SOB medium contained 2% bacto-tryptone, 0.5% bacto-yeast, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$. The medium was made up without magnesium, pH was adjusted to 7.0 with 5 M NaOH. Just before use, 20 mM magnesium were added from a solution stock containing 1 M $MgCl_2$ and 1 M $MgSO_4$, sterilised by filtration.

4.4.4 SOC medium

SOC medium was prepared in the same way as SOB medium, except that 20 mM glucose was added from a sterile 1 M solution of glucose.

4.4.5 LM medium

LM medium contained 1% tryptone, 0.5% yeast extract, 10 mM NaCl and 10 mM $MgSO_4$.

4.4.6 Agar plates

Agar plates were prepared by adding 1.5% of bacto-agar to the appropriated medium, autoclaved and approximately 25 ml poured into Petri dishes.

4.4.7 Antibiotics

Chloramphenicol was prepared at 30 μg/ml in ethanol, when required.

4.5 Genetic techniques

4.5.1 Transformation of *E. coli*

E. coli strain PA0467 was transformed by using both a standard transformation method (Hanahan, 1983) and an electroporation method (Dower *et al*, 1988)

4.5.1.1 Standard transformation

Cells removed from streak on agar plate were picked off to inoculate 5 ml LB medium, and grown overnight at 37°C with shaking. 0.5 ml of this culture was used to inoculate 50 ml SOB medium, and grown at 37°C with shaking until the OD_{660} was 0.35-0.45 (in about 2-2.5 hours). 24 ml of cell culture was cooled on ice for 15 min, transferred to 50 ml polypropylene sterile centrifuge tubes, and harvested by centrifugation at 8,000 rpm, for 10 min at 4°C in a Beckman J2-21 centrifuge (Beckman Ltd., Cheshire, UK), using a

JA-20 rotor. The cells were resuspended by gentle swirling in 1/3 volume (8 ml) ice cold standard transformation buffer (TFB: 10 mM 2 N potassium morpholinoethane sulphonic acid-KOH pH 6.2, 100 mM RbCl, 45 mM $MnCl_2.4H_2O$, 10 mM $Ca_2Cl.2H_2O$, 3 mM $CoCl_3$), placed on ice for a further 15 min, and centrifuged as above. The supernatant was discarded and the cells resuspended in 2 ml of transformation buffer for frozen storage of competent cells (FSB: 10 mM CH_3COOK, 10 mM KCl, 45 mM $MnCl_2.4H_2O$, 10 mM $Ca_2Cl.2H_2O$, 3 mM $CoCl_3$ and 10% glycerol). Fresh DMSO (dimethyl sulphoxide) was added to 3.5%, swirled, and left on ice for 10 min. Dithiolthreitol (DTT) was added to 75 mM from a stock 2.25 M DTT containing 40 mM potassium acetate pH 6.0 (sterilised by filtration and stored at 20°C), and left on ice for 10 min. Another equal portion of DMSO was added and the cells were left on ice for a further 5 min. 200 µl aliquot of competent cells were transferred to sterile eppendorf tubes, frozen in liquid nitrogen and stored at -70°C. Competent cells were thawed, placed on ice for 10 min and the DNA (50-100 ng in 10 µl) was added. The mixture was swirled and incubated on ice for 30 min. The mixture was heat-pulse without agitation at 42°C for 2 min and placed on ice for at least 2 min. Then 800 µl of SOC medium at room temperature were added and the tubes incubated at 37°C, for 1 hour with agitation. A portion (10-100µl) of the culture was plated out onto LM plate, containing the appropriate antibiotic, and incubated overnight at 37°C. A negative control without DNA, was performed for each transformation experiment. Transformed cells were purified by streaking out for single colonies twice on the LM plates.

4.5.1.2 Electroporation

(a) Cell preparation

The *E.coli* strains were grown overnight in 5 ml LB medium at 37°C with shaking. 100 ml of LB medium was inoculated with 1 ml of the overnight cell culture, and grown with vigorous shaking at 37°C until the A_{660} was approximately 0.8 (2.5-3 hours). The cells were harvested by chilling the flask briefly on ice and centrifuging at 5,000 rpm for 10 min at 4°C in a Beckman J2-21 centrifuge, using a JA-14 rotor. The supernatant was

poured off and the pellet was resuspended in 50 ml of cold sterile filtered water (SFW), centrifuged as above, and resuspended in 25 ml SFW. The suspension was transferred to a JA-20 tube, and centrifuged at 6,000 rpm for 10 min at 4°C, resuspended and centrifuged as above. After the last washing the pellet was resuspended in 4 ml SFW. The suspension was split up in 4 eppendorf tubes and spun at 6,000 rpm in a microcentrifuge for 10 min at 4°C. Finally, the pellet of one of the 4 tubes was resuspended in 1 ml of cold 10% glycerol, the suspension was transferred to the next tube, using a cut-end blue tip, the pellet was resuspended and the same procedure was repeated with the last two tubes. Aliquots of 40 µl of the cell suspension were transferred to sterile eppendorf tubes, frozen in liquid nitrogen and stored at -70°C.

(b) Sterilisation of the electroporation cuvette

The cuvette was boiled in distilled water for 10 min, and left to cool. Each cuvette was washed with 1 ml of sterile de-ionised water, then washed with 1 ml 100% ethanol, and dried thoroughly in a flow cabinet.

(c) Transformation

The concentrated cells were gently thawed at room temperature, placed on ice and 1 µl of DNA in TE solution was added to give a final concentration of from 25 $ng.ml^{-1}$ to 2.5 $\mu g.ml^{-1}$. The suspension was mixed vigorously by flicking the tube, and left for 1 min on ice. The cells and DNA mixture were transferred to an ice cold electroporation cuvette and the suspension was shaken to the bottom of the cuvette. The cuvette was placed in the safety chamber, and the pulse was applied (2050 volts, for 5 msec.). After the pulse, the cells were immediately removed from the electrodes, mixed into 1 ml SOC medium and incubated at 37°C with shaking for 1 hour. Finally the cells were spread on LM medium containing the appropriate antibiotic and grown overnight at 37°C. Transformed cells were purified by streaking out for single colonies twice on the same LM plates.

4.5.2 Isolation and purification of plasmids from *E.coli*

Plasmid DNA was prepared by using three different methods. Alkaline lysis followed by Qiagen column purification, that produced a high quantity of pure DNA, suitable for transformation of strains with low transformation efficiency. Small-scale preparation of plasmid by alkaline lysis method, that produced DNA suitable for transformation. Minipreparation of plasmid by the boiling method (STET) that was used to isolate DNA rapidly for screening purposes.

4.5.2.1 Alkaline lysis isolation and Qiagen column purification of plasmid DNA

This method was performed by using a Kit from Qiagen Inc. (Chatsworth, CA, USA) following the manufacturer's instructions detailed below. The method is based on that described by Birnboim and Doli (1979), and Birnboim (1983).

(a) Alkaline lysis isolation

A single bacterial colony of *E. coli* strain containing the plasmid of interest, was transferred to 5 ml of LB medium containing the appropriate antibiotic and grown overnight at 37°C with vigorous shaking. Two Erlenmeyer flasks containing 100 ml of LB medium were inoculated with 1 ml of the overnight culture and grown overnight at 37°C with vigorous shaking. 150 ml of this culture ($O.D._{660}$ 1.5 ~ $1x10^9$ cells.ml^{-1}) were harvested by centrifugation in a sterile centrifuge bottle at 10,000 rpm for 10 min at 4°C, in a Beckman J2-21 centrifuge, using a JA-14 fixed-angle rotor. The bacterial pellet was resuspended in 4 ml buffer P1 (100µg.ml^{-1} RNAase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0), transferred to a fresh 50 ml centrifuge tube. 4 ml of buffer P2 (200 mM KOH, 1% SDS) were added, mixed thoroughly by inverting the tube 6 times and incubated at room temperature for 5 min. The mixture became viscous in this step. The lysate was neutralised by adding 4 ml of chilled buffer P3 (2.55 M potassium acetate pH 4.8). The

cloudy and viscous solution was mixed gently and thoroughly, immediately after addition of buffer P3 by inverting the tube 6 times. This suspension was centrifuged at 30,000 x g (18,000 rpm) at 4°C for 30 min, in the same centrifuge using a JA-20 fixed-angle rotor. The clear supernatant obtained was immediately pipetted out very carefully, in order to avoid contamination with the potassium dodecylsulphate precipitation layer obtained on the top of the supernatant. The supernatant was kept on ice.

(b) Purification on the Qiagen-tip

The QIAGEN-tip column was equilibrated with 3 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0). The cleared lysate supernatant (~13 ml) was applied onto the column, allowing it to enter the resin by gravity flow. The column was washed twice with 10 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0), and the DNA was eluted from the column by passing 5 ml of buffer QF (1.25 M NaCl, 50 mM MOPS, 15% ethanol, pH 8.2). DNA solution was stored at -20°C.

4.5.2.2 Minipreparation of plasmid DNA by the alkaline lysis method

This method is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

A single bacterial colony of *E. coli* strain containing the plasmid of interest, was transferred to 5 ml of LB medium containing the appropriate antibiotic and grown overnight at 37°C with vigorous shaking. The culture was harvested by centrifuging 1.5 ml of culture at 12,000 g for 30 seconds at 4°C in a microfuge, the medium was removed by aspiration and a further 1.5 ml of culture were transferred to the same tube and centrifuged as above. The bacterial pellet was resuspended in 100 µl of ice-cold Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) by vigorous vortexing. The bacterial lysis was done by adding 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS), and the content was mixed by inverting the tube rapidly 5

times. The reaction was neutralised by adding 150 µl of ice-cold Solution III (potassium acetate, 3 M with respect to potassium and 5 M with respect to acetate) and mixed gently by inverting the tube. The bacterial lysate was incubated on ice for 5 min, centrifuged at 12,000g for 5 min at 4°C in a microfuge, and the supernatant was transferred to a fresh tube. Both phenol extraction and the DNA precipitation were performed as described in section 4.5.3 and 4.5.4 respectively. Plasmid DNA was resuspended in 50-100 µl of TE buffer and stored at -20°C.

4.5.2.3 Minipreparation of plasmid DNA by boiling method (STET)

Holmes and Quigley (1981)

A bacterial strain containing the plasmid of interest was grown and harvested as described in section 4.5.2.2. The bacterial pellet was resuspended in 0.5 ml of STET buffer (8% sucrose, 5% Triton X 100, Tris 1 M pH 8.0, EDTA 50 mM) and 50 µl freshly prepared lysozyme (100mg.ml^{-1}) in STET buffer was added. The mixture was incubated in a boiling water bath for 50 seconds and centrifuge at 12,000g for 15 min at 4°C in a microfuge. The gelatinous pellet was removed by using a sterile wood. The plasmid DNA was precipitated by adding an equal volume of ice-cold isopropanol, left at -20°C for at least 10 min and centrifuged as above. The supernatant was decanted and the pellet washed with 0.5 ml ether, centrifuged as above and dried thoroughly under vacuum. The plasmid DNA was resuspended in 40 µl of TE buffer.

4.5.3 Phenol extraction

A mixture consisting of equal parts of water-equilibrated phenol and chloroform:isoamyl alcohol (24:1) was used to remove proteins from preparations of nucleic acids. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction.

An equal volume of phenol:chlorophorm:isoamyl alcohol (25:24:1 v/v/v) was added to the DNA preparation, mixed vigorously by flicking the tube, centrifuged at 2,000 rpm in a Beckman J2-21 centrifuge, using a JA-20 fixed-angle rotor, the upper aqueous was saved and a second volume of phenol:chlorophorm:isoamyl alcohol was added and centrifuged as above. To take off the residual phenol, 5 ml of water saturated ether was added, mixed by flicking the tube and centrifuged as above. The procedure was repeated twice.

4.5.4 Precipitation of the DNA

The double-stranded DNA was precipitated by adding 2 volumes of ethanol, mixed at room temperature and incubated at -20°C for 1 hour to allow DNA precipitation. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C, in a microfuge. The pellet was washed with 1 ml of cold 70% ethanol, air dried, and resuspended in 200 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

4.6 Antibacterial Susceptibility testing

The sensitivity of bacteria to toxic peptides such as triornithine (Orn_3) or Alanyl-di-alanyl-1-Aminoethyl phosphonic acid (Ala_4P) were determined by using two techniques.

4.6.1 Pour plate technique

Pour plate is a standard method (Island *et al.*, 1987) that is used to quantify an antibacterial agent, since the inoculum is dispersed uniformly in molten agar in a petri dish.

A single colony was picked off from a plate and grown in 5 ml LB medium for 6 hours at 37°C with rotary shaking. Suspensions of bacteria (3.3×10^7 CFU) were added to 3 ml melted agar (0.6% w/v) tubes that had been cooled at 42°C in hot plate. The bacteria and

agar medium were rapidly mixed by flicking, the suspension was poured onto minimal medium agar plates warmed at 37°C and allowed to set (~10 min). Three antibiotic susceptibility discs were applied to the surface of the overlay, using an Oxoid Disc Dispenser and different amounts of toxic peptide in the range 0.75 to 100 nmoles of Ala_4P or 0.8 to 2000 nmoles of Orn_3 were added to the disc. The plates were incubated overnight (14-15 hours) and the ability of a toxic peptide to inhibit bacterial growth was quantified by determining the size of the inhibition zone surrounding a disc containing the toxic peptide. Semi-logarithmic plots were made of toxic peptide concentration versus zone diameter, and the amount of inhibitor required to produce a zone of 25 mm diameter was calculated from the plot.

4.6.2 Radial streak technique

This technique was used for the simultaneous testing of the sensitivity of many strains to a single antibacterial agent.

Bacteria were removed from a streak on the agar plate using a fine, flame-sterilised platinum wire and streaked radially from the centre towards the outside of a minimal medium agar plate. A blank antibiotic susceptibility disc was placed at the centre of the plate and 10 µl of the toxic peptide were added. Plates were incubated overnight (14 hours) at 37°C. The strains resistant to the toxic peptide tested grew as blunt-ended streaks to the edge of the disc. Partial resistance was shown by tapered growth to the disc and sensitivity by a zone of growth inhibition. This test can only be semi-quantitative since it is difficult to control accurately the number of cells per streak.

4.7 Isolation of periplasmic proteins

4.7.1 Cold Osmotic Shock

This method was used to release periplasmic proteins from *E.coli* strains, and was performed using a modification of the procedure of Berger and Heppel (1972).

Bacteria grown in minimal medium were harvested by centrifugation 10,000 rpm for 10 min at 4°C in a Beckman J2-21 centrifuge, using a JA-14 fixed-angle rotor.

The procedure described below was followed for the cold osmotic shock of 800 ml portions of cell culture. Smaller scale preparations, for analytical purposes, were carried out on 20 ml of bacterial culture. In this case, the bacteria were harvested in the same way but using the JA-20 fixed-angle rotor or in a MSE bench centrifuge at 4,200 rpm for 10 min at room temperature. The volumes in parentheses are those used for these small scale preparation.

The bacterial pellet from 800 ml of culture (20 ml for small scale preparation) was resuspended in 20 ml (1 ml) of ice-cold 10 mM Tris-HCl pH 7.3, 30 mM NaCl, transferred to 50 ml centrifuge tube (1.5 ml eppendorf tube) and centrifuged as above using the JA-20 fixed-angle rotor. The supernatant was poured off, the pellet resuspended in 27 ml (1 ml) of the same buffer and centrifuged as above. The washed cells were resuspended in 13 ml (0.5 ml) of 33 mM Tris-HCl pH 7.3, followed by the addition of an equal volume of this buffer containing 40% sucrose and 2 mM EDTA. After mixing by swirling for 10 min at room temperature, the suspension was centrifuged at 12,000 rpm at 4°C. The pellet was immediately resuspended in 27 ml (1 ml) of ice-cold water, left on ice for 3 min, followed by the addition of 530 µl (20 µl) of 50 mM $MgCl_2$, gently mixed and kept on ice for a further 10 min, before the last centrifugation at 10,000 rpm at 4°C for 10 min (10,000 rpm at 4°C for 5 min). The supernatant which contained the periplasmic proteins was kept at 4°C. For large scale preparation, after centrifugation of the supernatant solution as above, this was filtered through a 0.45 µm cellulose acetate filter, dialysed against 5 mM NH_4HCO_3 and concentrated by lyophilization. For small scale preparation, the supernatant was transferred to a fresh tube and lyophilised without further treatment.

4.8 Purification of OppA and DppA

The OppA and DppA were purified following the original scheme described by Tyreman (1990) for *E. coli* K-12 strain M2034, that had been slightly modified (Fig.4.2).

4.8.1 Fast Protein Liquid Chromatography (FPLC)

This method offers high resolution, preparative capacity and rapid results allowing optimal conditions to maintain the biological activity and structural integrity of the molecules purified.

All solutions applied onto the FPLC columns were filtered by using a 0.45 μm cellulose acetate filter, and degassed for at least 5 min under vacuum.

4.8.1.1 Cation exchange chromatography

Freeze-dried osmotic shock fluid (section 4.7) was rehydrated in 5 ml 50 mM malonic acid-NaOH pH 4.8. Aliquot (1 ml) of this sample were loaded onto a Pharmacia Mono S HR 5/5 strong cation-exchange column (Pharmacia LKB Biotechnology, Bucks, UK) attached to a Pharmacia FPLC system, at a flow rate of 1.5 ml per min, that had been pre-equilibrated with the same buffer. Desorption of bound protein was achieved by increasing linear gradient of sodium chloride in 50 mM malonic acid-NaOH. pH 4.8 (0 to 1 M) maintaining the same flow rate. The absorbance of the eluent was continuously monitored at 280 nm. A typical profile is shown in figure.4.3. Peaks (II and III) eluting between 5-10% and 13-18% of sodium chloride were identified in SDS-PAGE as DppA and OppA, respectively. The fractions of each peak were dialysed against 5 mM NH_4HCO_3 (as described in section 4.8.2), lyophilised and stored at -20°C until required.

4.8.1.2 Anion exchange chromatography

Freeze-dried sample of OppA-containing fractions, was re-dissolved in 5 ml 20 mM Tris-HCl pH 7.8, and an aliquot (1 ml) of this sample were applied onto a Pharmacia Mono Q 5/5 anion-exchange column, at a flow rate of 1.5 ml per min, that has been pre-equilibrated with the same buffer. Desorption of bound protein was achieved by an increasing linear gradient of sodium chloride (0 to 1M) maintaining the same flow rate. The absorbance of the eluent was continuously monitored at 280 nm. The fractions of the eluted peak were pooled, dialysed against 5 mM NH_4HCO_3 (as described in section 4.8.2) and lyophilised and stored at -20°C until required.

4.8.1.3 Reverse phase chromatography

The purification procedure described above yields OppA and DppA that in part retain bound endogenous ligand. For some experiments, OppA was required free of ligands. Previous experiments (Tyreman, 1990) showed that reverse-phase chromatography of OppA and DppA removed bound peptide ligand. Purified OppA (100 µg dissolved in 100 µl of solvent A) was applied onto a Pharmacia Pro-RCP HR5/10 column. The solvent system used with this column consisted of an aqueous solution of 0.1% (v/v) trifluoroacetic acid (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). Before applying the sample, the column was equilibrated by washing with 15 ml of solvent B, followed by 7 ml linear gradient from 100% to 0% solvent B, and washed with solvent A until the baseline (absorbance 214 nm) remained stable. Freeze-dried OppA from Mono Q column was resuspended in solvent A. Samples (100-400 µg) of protein in a volume of 100 µl were loaded onto the column at a flow rate of 0.3 ml per min. Desorption of bound component was achieved by increasing linear gradient of solvent B. The absorbance of the eluent was continuously monitored at 214 nm. The fractions of the eluted peak were lyophilised and store at -4°C until required.

4.8.2 Dialysis of protein solutions

Protein solutions obtained in both types of ion exchange chromatography were dialysed against 5 liters of 5 mM NH_4HCO_3 overnight at 4°C. The dialysis tubing (Visking size 5-24/32", Medicell International. London, UK) was boiled for 5 min in 5% sodium carbonate (w/v), 50 mM EDTA. The tubing was rinsed with water and boiled in distilled water for a further 5 min. This tubing was stored in 25% ethanol and rinsed with distilled water before use. The dialysed solution was first pipetted as 1 ml aliquots in eppendorf tubes and centrifuged at 12,000 rpm for 5 min using a Micro-Centaur centrifuge, to remove precipitated proteins. The supernatant was used to load the FPLC column.

4.9 Protein estimation

Two methods with different sensitivities were used for the determination of the total protein content of a solution.

4.9.1 Micro-Biuret assay

Benedict's reagent consists of alkaline copper sulphate solution containing trisodium citrate and sodium carbonate. This method was used to quantify samples of about 0.1 mg of protein per ml of aqueous solution. A volume of 1 ml of samples was mixed with 750 µl of 1 M NaOH, followed by 50 µl of Benedict's reagent and incubated at room temperature for 15 min. Absorbance was read at 330 nm. The protein concentration of the samples tested were estimated from a BSA (Sigma chemical Co. Ltd., Dorset, UK) linear calibration curve.

4.9.2 Bicinchoninic acid (BCA)

Pierce BCA assay is a modification of the Lowry assay (Lowry *et al.* 1951) involving a dye binding step. The copper-protein complex which forms the basis of the Biuret and

the Lowry methods can be chelated by bicinchoninic acid to produce a very stable complex with a strong absorption maximum at 562 nm. The BCA method was carried out according to the manufacturer's instructions (Pierce, Rockford, USA). A volume of 10 µl of sample was mixed with 200 µl of BCA working reagent and incubated at 60°C in a hot block for 40 min. The samples were transferred to microtitre plates and the absorbance read at 540 nm with a microtitre plate reader (Titretek Twinreader, ICN Flow, High Wycombe, UK). The protein concentrations of the samples tested were estimated from a BSA linear calibration curve. The latter covered the range from 0.2 to 10 µg of BSA per total assay volume.

4.10 Electrophoresis

4.10.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Laemmli, 1970) is a method widely used for analysing protein mixtures qualitatively. In this study it was used for monitoring protein purification, screening mutants and to determine the relative molecular mass of protein. This method is based on the separation of proteins according to size, regardless of the original native charge on the molecule, since one molecule of SDS binds for every two amino acid residues, resulting in a structure negatively charged overall.

Resolving gels (large or mini) were composed of 12.5% or 8% (w/v) acrylamide (acrylamide-bisacrylamide = 37.5:1) in 0.38 M Tris-HCl pH 8.3. Stacking gel was composed of 3 % (w/v) acrylamide (acrylamide-bisacrylamide = 37.5:1) in 0.12 M Tris-HCl pH 6.8. Both resolving and stacking gels contained 0.1 % (w/v) SDS, persulphate and TEMED. Wells were produced in the stacking gel by allowing it to polymerise in the presence of a 1 mm thick comb. The running buffer contained 0.2 M glycine, 0.025 M Tris and 0.1% SDS.

Proteins were prepared by mixing 5 µl of protein solution containing 5 µg of total crude protein or 0.5 µg of pure protein, with 20 µl of 5 x sample buffer (0.4 M Tris-HCl pH

6.8, 0.01 % (w/v) SDS, 8 % (v/v) glycerol, 2.5 % (w/v) bromophenol blue, and 5 % β mercaptoethanol). The samples were boiled in a water bath for 3 min, and applied to the gel (3-10 µl per well).

A high molecular weight standard mixture (Sigma SDS-6H) was used as markers. Proteins and their molecular weights are as follows: rabbit muscle Myoglobin (20.5 K); *E.coli* β galactosidase (116K); rabbit muscle Phosphorylase b (97.4K); bovine serum albumin (66K); egg albumin (45K), and bovine erythrocytes Carbonic Anhydrase (29K). These proteins were treated in the same way as protein samples.

Large gels (water-cooled) were run in a home-made electrophoresis tank at constant current of 15 mA until the bromophenol blue dye reached the resolving gel, then the current was turned on at 35 mA until the dye had migrated to the botton of the gel.

Small gels were run at constant current of 25 mA for the stacking gel and 50 mA for the resolving gel on a Mini-PROTEAN II electrophoresis cell (Bio-Rad Laboratories ltd). When this gel was used for blotting, 1 µl of pyronine Y (in 80% glycerol) was added in all wells to mark the position of the slots on the blot.

4.10.2 Isoelectric focussing (IEF)

IEF is a technique that separates proteins according to their different isoelectric point, and was used in this study to characterise proteins that had undergone a mutation, and to study the changes in pI consequent upon ligand binding to OppA.

This method was performed in a Pharmacia Phast system (Pharmacia LKB Biotechnology, Bucks, UK) on pI 5-8 IEF phast gels, from the same source.

Freeze-dried, pure OppA samples were dissolved in distilled water (12 µg/µl) and incubated for one hour at 37°C, before their uptake into 8 x 0.5 µl phast gel sample applicator. When exogenous ligand was added to the protein, 5 µl of an aqueous solution of peptide was incubated with 1 µl of OppA (12 µg/µl). Sigma pI standard calibration

markers in the range 5.1 to 7.2 were similarly applied. These markers and their pI values are as follows: β Lactoglobulin pI 5.1, Carbonic Anhydrase II pI 5.9, Carbonic Anhydrase I pI 6.6, and Myoglobin pI 6.8 and 7.2 (for acidic and basic forms respectively).

The gels were prefocused for 8 min at 2 mA, 3.5 W. Electrophoresis was carried out at 2000v, 25 mA for 20 min at 15°C. Proteins were fixed with 20% trichloroacetic acid, washed in distilled water and stained with Coomassie blue as described in section 4.14.1. pI was calculated from the linear calibration plot of $\log_{10}$ of marker proteins pI versus their mobility.

4.10.3 Gel agarose electrophoresis

Gel agarose electrophoresis can separate DNA molecules according to their size; they migrate towards the positive pole because of their negative charge. The composition of the gel determines the size of DNA molecule that can be separated. 0.6% (w/v) of agarose that has an efficient range of separation of linear DNA molecules of 1-21 Kb, was used to separate digested plasmid DNA.

Agarose gel was prepared by mixing 0.6% (w/v) agarose in 0.5 x TBE buffer (0.045 M Tris-borate, 1 mM EDTA) in an Erlenmeyer flask with loose-fitting cap. The mixture was heated until all agarose grains dissolved. The solution was cooled to 60°C, and ethidium bromide (from a stock aqueous solution of 5 mg/ml) was added to a final concentration of 0.5 μg/ml. The solution was mixed thoroughly. A home-made mold was sealed with autoclave tape and a comb was positioned 0.5 mm above the plate before pouring the warm agarose solution into the mold. When the gel was completely set (30-45 min at room temperature) the comb and autoclave tape were carefully removed and the gel was mounted in a home-made electrophoresis tank containing just enough 0.5 x TBE buffer to cover the gel.

DNA samples were digested with restriction endonucleases following the commercial

instructions, and 4 µl of 6 x gel-loading buffer II (aqueous solution of 0.25% (w/v) bromophenol blue, 0.25% xylene cyanol FF (w/v) 15% (w/v) Ficoll (Type 400; Pharmacia) were added and mixed. Molecular weight markers were diluted in TE, and mixed with the same gel-loading buffer. The submerged gel was loaded using a micropipette. The electrophoresis was run at 1 V/cm until the bromophenol blue and xylene cyanol FF had migrated the appropriate distance through the gel. The gel was examined by ultraviolet light and photographed. The sizes of the DNA fragments were estimated from a calibration curve constructed with the $\log_{10}$ of the size (Kb) of the *HindIII*-fragment versus the distance they have migrated (cm).

4.11 Western blotting

Western blot technique was used to detect on a cellulose membrane immobilised proteins, which had been previously separated on SDS-PAGE. This technique can detect 10 pg of protein with horseradish peroxidase or alkaline phosphatase labelling.

Before starting the procedure, the nitrocellulose membrane (0.45 µm), filter papers (whatman 3MM), absorbent paper and fibre pads, were cut large enough to cover the gel and pre-wetted in transfer buffer (24.4 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) ethanol) for 5 min.

A piece of plastic (mylar mask) with a window slightly smaller than the membrane was placed over the anode of the TE 70 Semi-Phor Semi Dry Tranfer Unit (Hoefer Scientific Instrument, San Francisco, USA), which was covered with two wetted fibre pads and one wetted filter paper. The polyacrylamide gel-membrane sandwich was assembled by placing the gel (with the left top on the right) on a wetted absorbent paper and covered with the nitrocellulose paper. Air bubbles trapped between the gel and the membrane were removed by rolling a small test tube over the sandwich. the assembled gel was transferred to the transfer chamber in the inverse position, the absorbent paper was removed and a second layer of two wetted filter papers and two wetted fibre pads was placed. The transfer chamber was closed and transfer was run at 100 mA (constant current) for 1 hour at room temperature. Proteins were detected immunochemically as described in section

4.12, and the electroblot containing the molecular weight markers were silver stained as described in section 4.13.

4.12 Immunostaining of proteins on Western blot

This method was carried out using a modification of the method of Blake *et al.* (1984). All steps were carried out with gentle agitation at room temperature.

After removing the electroblot from the transfer chamber, the nonspecific binding sites were blocked by incubating the membrane in an aqueous solution of 3% nonfat milk in TBS-Tween 20 (20 mM Tris-HCl pH 7.3, 0.9% NaCl, 0.05% Tween 20) for one hour, and washed with TBS-Tween 20 for 5 min with 3 changes. The antigens were incubated with the first antibody diluted 500-fold in TBS-Tween 20, for two hours, and washed as above. The antigen-antibody complex was detected by adding the second antibody, anti-mouse (or anti-rabbit) IgG peroxidase conjugate diluted 2000-fold in the same buffer, incubated for two hours and washed as above. The bands were visualized by addition of freshly made substrate (5 mg 4-chloro-1-naftol, 1 ml (v/v) ethanol, 5 ml TBS at 37°C, 2.5 µl H_2O_2) and incubated until the bands had developed to a sufficient intensity. The reaction was stopped by washing the membrane with distilled water for 30 min with 3 changes. The blot was placed on absorbent paper, air-dried and stored in the dark.

4.13 Silver staining of proteins on Western blots

This method stains total proteins on a membrane and was used to monitor the efficiency of transfer and for identifying immunochemical detected bands.

The blot was washed in distilled water and incubated in freshly prepared colloidal silver reagent (aqueous solution of 0.2 g trisodium citrate and 0.08 g $FeSO_4.7H_2O$ to which 0.1 ml 20% (w/v) $AgNO_3$ solution was added under vigorous agitation) until the bands reached the desired intensity. The reaction was stopped by washing with distilled water. To remove surface staining, the gel was briefly rinsed with Farmer's reducer solution as

described in section 4.14.2

4.14 Staining and de-staining PAGE gel

Before staining, the stacking gel was removed from the resolving gel. All steps were carried out with gentle agitation at room temperature.

4.14.1 Coomassie blue staining

This procedure is a modification of the coomassie blue staining method described by Merril (1990), and detects as little as 0.1 µg of protein in a single band.

The resolving gel was simultaneously fixed and stained for at least 2 hours with coomassie blue stain (aqueous solution containing 0.2% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) glacial acetic acid, 50% (v/v) ethanol). The gel was de-stained by incubation with an aqueous solution containing 20% (v/v) ethanol and 10% (v/v) glacial acetic acid, until the background staining had been removed.

4.14.2 Silver staining

A modification of the silver staining method described by Heukeshoven and Dernick (1988) was used, which can detect as little as 2 ng of protein in a single band.

After removing the stacking gel, the resolving gel was fixed in an aqueous solution of 30% (v/v) ethanol, 10% (v/v) glacial acetic acid for 30 min, washed in distilled water and incubated in an aqueous solution of 0.01% sodium thiosulphate, 30% (v/v) ethanol, 0.4 M sodium acetate pH 6.0 buffer, for 1 hour. The gel was washed with distilled water for 20 min, with 3 changes, and incubated for 30 min with silver reagent (50 µl of 37% formaldehyde per 200 ml 0.2% silver nitrate). A brief water wash was carried out before placing the gel with the developing reagent (100 µl of 37% formaldehyde per 200 ml 2.5% (w/v) sodium carbonate until it reached the desired intensity of the bands. The

staining reaction was stopped by placing the gel in an aqueous solution of 1% glacial acetic acid. Subsequently, the gel was water washed for 10 min, with 3 changes. To remove surface staining the gel was briefly rinsed with Farmer's reducer solution (0.3% w/v sodium thiosulphate, 0.15% (w/v) potassium hexaferricyamide, 0.05% (w/v) sodium carbonate) water washed and left in water to remove traces of Farmer's.

4.15 Drying gels

Stained polyacrylamide gels were air-dried by placing the gel between two sheets of pre-wetted cellophane in a gel drying frame. The gels were dried in a SE1200-Easy Breeze TM air gel dryer (Hoefer Scientific Instruments, San Francisco,USA).

Chapter V

SOLID-PHASE PEPTIDE SYNTHESIS

The fundamental concept of solid-phase peptide synthesis (SPPS), introduced by Merrifield in 1963, is based on the sequential addition of N-α-protected amino acids residues to an insoluble polymeric support. The resulting peptide is bound covalently through its C-terminus to the linker of the support. The product remains attached to the resin throughout all the synthesis steps and is separated from soluble reagents and solvent by simple filtration and washing.

5.1 The Solid Support

The solid support is the unique feature which distinguishes solid-phase synthesis from all other techniques. The support must contain reactive sites at which the peptide chain can be attached, and later removed, and yet it must be stable to the physical and chemistry condition of the synthesis (Atherton and Sheppart, 1989). The solid support used in this procedure is a p-Benzyloxybenzyl alcohol, Wang resin [4-(Hydroxymethyl) phenoxymethyl-copoly (styrene-1% divinylbenzene) resin], figure 5.1

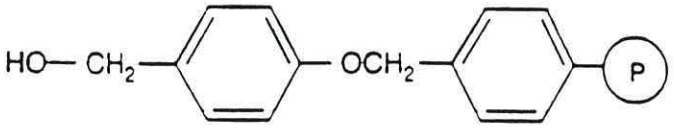


Figure 5.1:

p-Benzyloxybenzyl Alcohol Resin (Wang Resin)

This resin consists of beads (200 - 400 mesh) made from a copolymer of polystyrene crosslinked with 1% of divinylbenzene (DVB).The peptide C-terminal amino acid residue, N-α-protected with Fmoc group, is attached to the polymer by nucleophilic displacement (Fig. 5.2). A typical degree of substitution is 0.2-0.7 mmoles of amino acid per gram of resin.

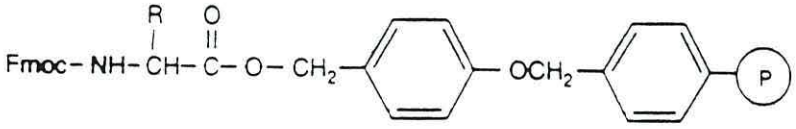


Figure 5.2:

Fmoc-amino acid on Wang Resin

Polystyrene cross-linked with 1% DVB is the support of choice for the large majority of applications. Molecular events take place in the interior of a resin bead in the same manner as they do in homogeneous solution, e.g. amine components attached to this polymer and free in solution have shown the same rate of reaction and similar degrees of freedom

of rotation. Furthermore, the solvating effect of the resin on the synthesised peptide may be very considerable: a given peptide may be perfectly solvated when it is attached to the resin whereas the same peptide, in the same solvent, may precipitate when it is cleaved from the polymer (Van Regenmortel, *et al.*, 1988).

5.2 Protecting Groups

All the functional groups of the amino acids, except those that will be actually involved in the formation of the peptide bond, must be protected. This includes amino and carboxylic acid groups, as well as any other reactive groups (such as nucleophilic alcohols or thiols, if those are present). The protecting groups used must be chosen so that they can be removed without rupturing the newly made peptide bond(s). Figure 5.3 shows the protecting groups and cleavage strategy for Fmoc chemistry.

5.2.1 α amino Protection

The protection of amino groups is not straightforward, because the best way to render the nitrogen non-nucleophilic is to protect it as amide. The only way to remove the amide-protecting group without simultaneously cleaving any peptide bond is to use C-alkoxyamide, which can be cleaved under milder conditions than normal amide.

Alkoxycarbonyl derivatives that are commonly used include: Benzyloxycarbonyl (Z), tButoxycarbonyl (Boc), 2-(4Biphenyl)-isopropoxycarbonyl (Bpoc), 9-Fluorenylmethoxycarbonyl (Fmoc). The use of a particular protecting group is associated with the methodology used.

Fmoc protection was chosen as the alkoxycarbonylamino derivative, which is very stable

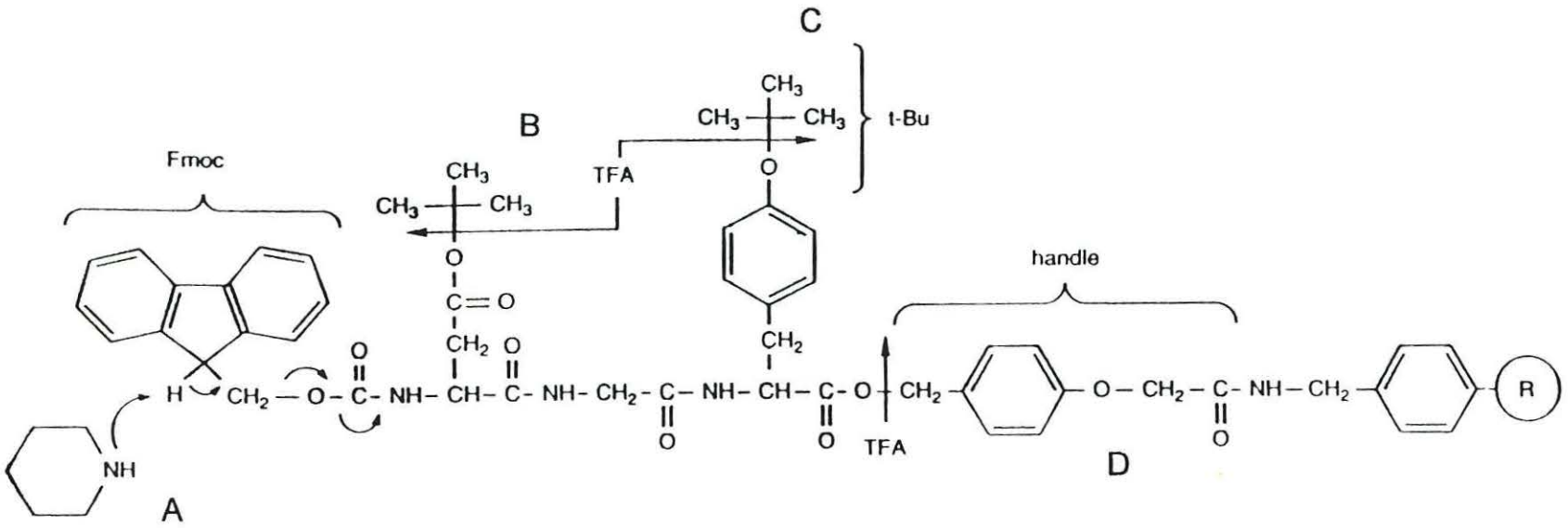


Figure 5.3

General protecting group and cleavage strategy for Fmoc chemistry, showing the amino acid deprotection with piperidine and the cleavage with TFA. Key: **A**: Piperidine; **B**: Asp residue showing the side-chain (carboxyl group) protected with tert-Butyl ether (tBu); **C**: Tyr residue showing the side-chain (hydroxyl group) protected with tert-Butyl ether (tBu). **D**: Solid support.

Table 5.1

Protected amino acids used in the peptide synthesis

Fmoc protected amino acid	Protecting group	Comments
Fmoc- Arg (Pmc)-OH	2,2,5,7,8,-Pentamethyl-chroman 6 sulfonyl	Pcm blocks the ω nitrogen, is extremely stable and was removed with TFA/Thioanisole
Fmoc-Lys(Boc)-OH	t-Butoxycarbonyl	Boc is a alkyl chloroformate. Basic and nucleophilic reagent share no affect at all on the Boc group.
Fmoc-Asp(OtBu)-OH	Benzyl/t-Butyl	The most common side reaction of Asp residues is cyclisation to form succinimide and subsequentlly re-opening of the ring to form β aspartyl peptide. This reaction is overcome by the use of Asp (OtBu)
Fmoc-Ser(tBu)-OH	tert-Butyl ether	The Butyl ether function is resistent to nucleophilic and basic attack. The tBu group is cleaved with TFA
Fmoc-Met-OH		
Fmoc-Leu-OH		
Fmoc-Val-OH		
Fmoc-Ala-OH		
Fmoc-Phe-OH		
Fmoc-Pro-OH		
Fmoc-Trp-OH		Fmoc-Trp was used without a protecting group for the indole nucleus, because in the tetrapeptide synthesis, scavengers were included in the deprotection solvent
Fmoc-Gly-OH		

to acidic reagents, is cleaved swiftly under certain basic conditions. Routinely this was achieved with 20% Piperidine in DMF.

5.2.2 Protection of Side Chain Functions

Tert-butyl derivatives are used for the protection of some side chain functions: tert-butyl esters for Asp and Glu; tert-butyl ethers for Ser, Thr and Tyr; tert-butoxycarbonyl for Lys and His.

The N-α-Fmoc protected amino acid used in the peptide synthesis (table 5.1) are listed in table 5.1.

5.3 Steps of the Synthesis

Solid-phase peptide synthesis was performed on a Biosearch 9500AT peptide synthesiser, using a Biosearch SAM II reagent delivery system and reactor module, controlled in an on-line mode by microcomputer running Biosearch Peptide Workstation.

The steps of the synthesis are summarised in figure 5.4. All the peptides synthesised and their amino acid sequences are listed in table 5.2.

Table 5.2
Amino acid sequence of the peptides synthesised using solid-phase methodology

Synthetic Peptide name	Amino acid sequence
PWLA	Pro-Trp-Leu-Ala
APD^{300}	Leu-Gly-Met-Asp- Arg- Asp-Ile-Ile-Val
APR^{300}	Leu-Gly-Met-Asp- Arg- Arg-Ile-Ile-Val
APS^{300}	Leu-Gly-Met-Asp- Arg- Ser-Ile-Ile-Val

GENERAL SCHEME OF SOLID PHASE PEPTIDE SYNTHESIS

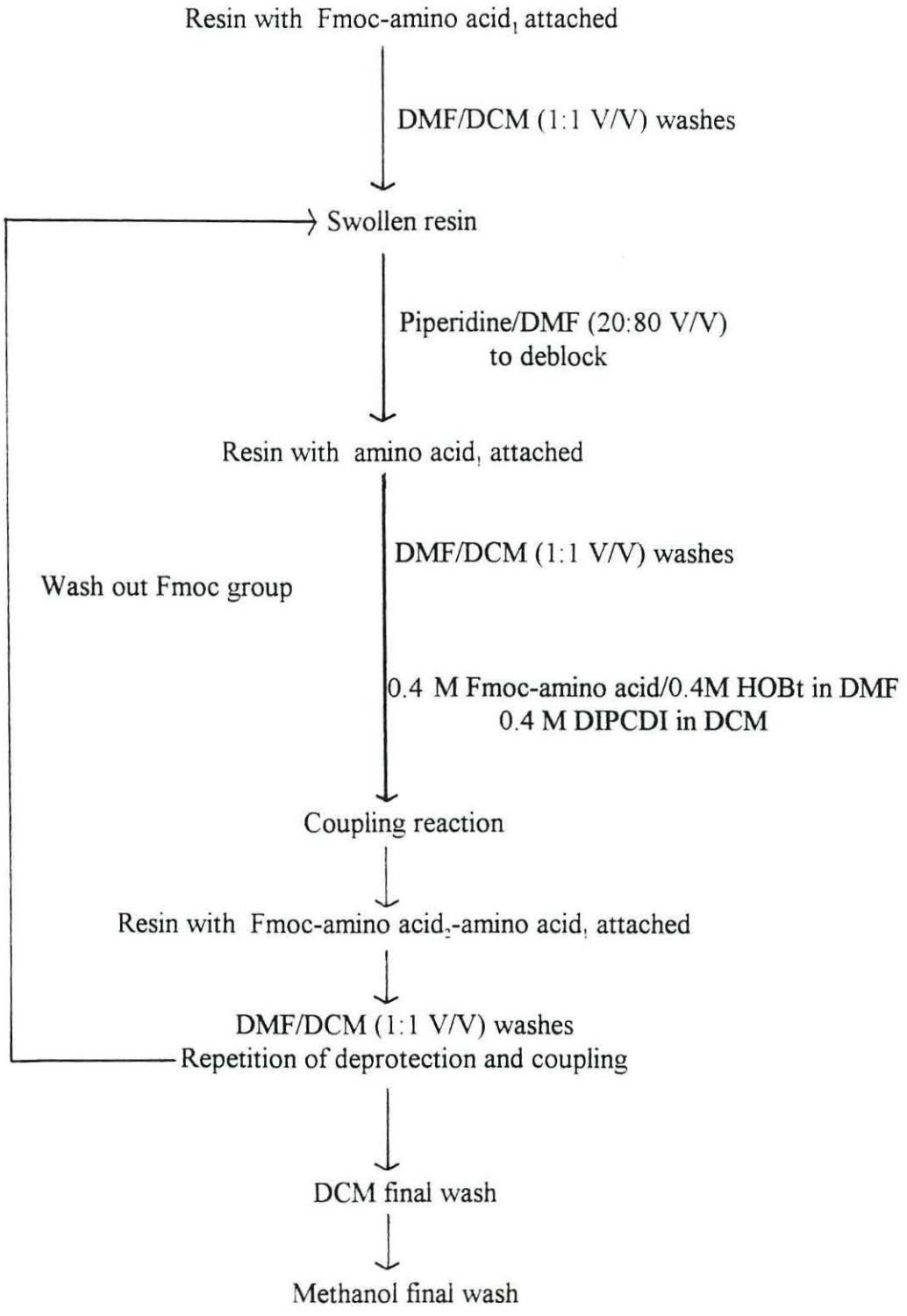


Figure 5.4 :
Flow chart of the reaction scheme used for the peptide synthesis

5.3.1 Solvation of the Resin

The Fmoc-Ala or Fmoc-Val Wang resin was swollen by solvation with three changes of a 1:1 (v:v) DMF/DCM, with agitation for 30 seconds by a stream of nitrogen.

Solid-phase synthesis requires that the dispersing medium be a good solvating agent for both the polymer support and the protected peptide. It must also constitute a good reaction medium for the various chemical steps involved in the synthesis.

5.3.2 Deprotection

The base-labile Fmoc group is cleaved swiftly under certain basic conditions. Deprotection was carried out by washing the swollen Fmoc-AA-resin or Fmoc-peptide-resin with two washes of 20% (v/v) piperidine in DMF. The first wash was for 3 min, the second for 6 min, followed by five 30 second washes of 1:1 (v:v) DMF/DCM, to remove any free Fmoc. The deprotection takes only a matter of seconds at room temperature. The mechanism of cleavage is via the stabilized dibenzocyclopentadienide anion, giving a free amino group, and the dibenzofulvene produced react with piperidine giving a co-product, Figure 5.5.

5.3.3 Coupling Reaction

The DIPCDI was used as coupling reagent. The primary activating event is addition of the carboxyl group to the carbodiimide functionality to give, a potent acylating agent, *O*-acylisourea. The carbodiimide -N=C=N-, contains a central carbon atom that is very electron deficient, and is therefore attacked rapidly by nucleophile. The carbonyl group of the carboxylic acid derivative is now quite reactive, and the addition of an amine results in the formation of an amide bond (Fig. 5.6). Since the urea is only sparingly soluble in most solvents, its separation from the desired product is very straightforward in solution synthesis.

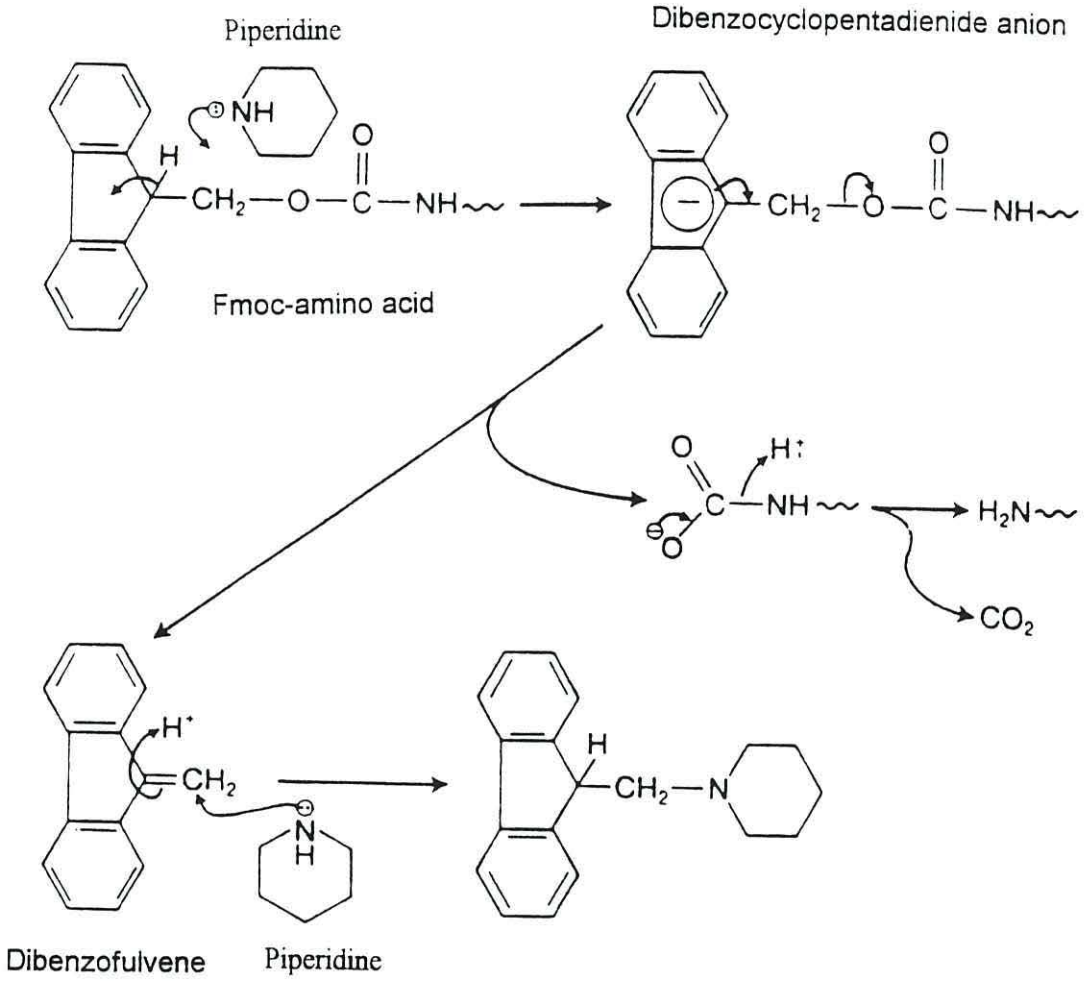
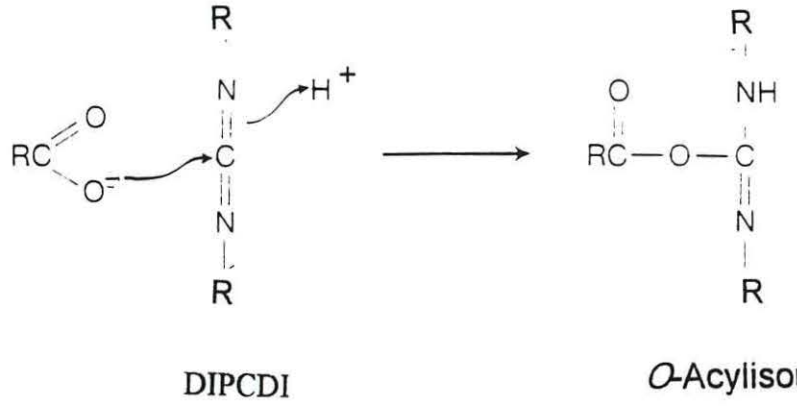


Figure 5.5

Fmoc amino acid cleavage in basic conditions (20% Piperidine in DMF)

The coupling reaction was achieved by simultaneous addition of equimolecular amounts of the following solutions: 0.4M Fmoc amino acid and (0.4M)1-hydroxy-benzotriazole (HOBt) in DMF, and (0.4M) diisopropylcarbodiimide (DIPCDI), in DCM, to the vessel containing the deprotected AA-resin. The mixture was agitated, with a nitrogen stream, for 1 hour at room temperature. The resulting resin-bound peptide was washed with 1:1 (v/v) DMF/DCM as above, and the synthetic cycle repeated until the last Fmoc amino acid was incorporated.



DIPCDI *O*-Acylisourea

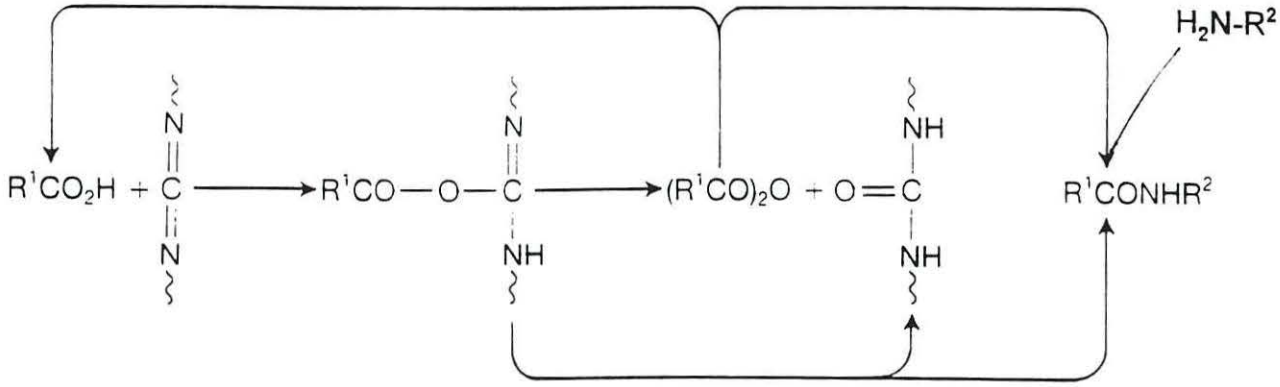
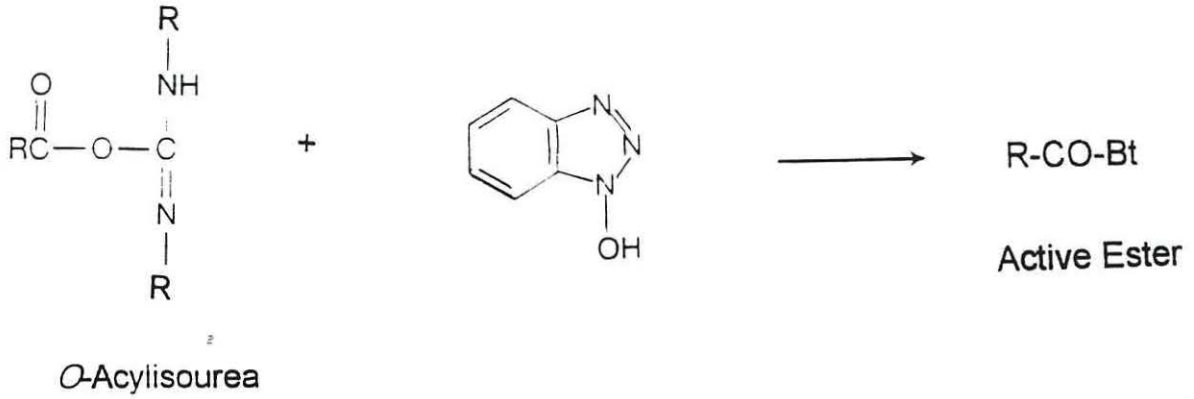


Figure 5.6:

Peptide bond formation

The intermediates shown in figure 5.6, are highly reactive. Side reactions such as racemisation and the collapse of the *O*-acylisourea, to give the less reactive *N*-acylurea, can take place. These problems are reduced by performing the coupling in the presence of a suitable α-nucleophile like HOBt, which is able to react very rapidly with the *O*-acylisourea before side-reaction can intervene (Fig.5.7). The rate-accelerating effect of HOBt is almost certainly due to formation of the intermediate benzotriazolyl ester.



O-Acylisourea

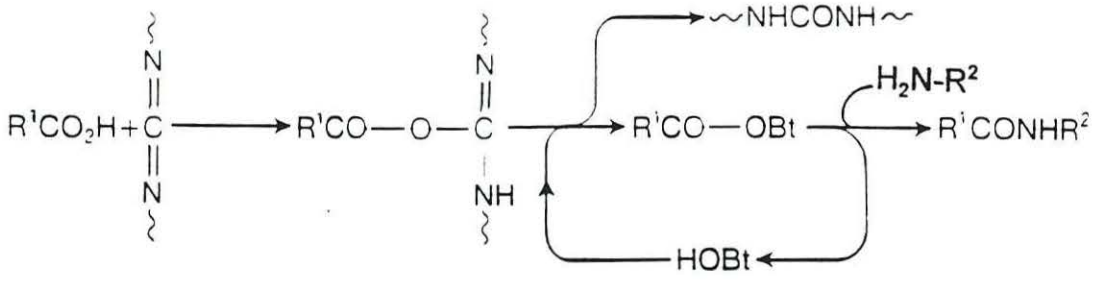


Figure 5.7: Activation and coupling of amino acid derivative

After the last Fmoc amino acid was incorporated, final deprotection was completed and the resin washed as above (section 5.3.2) to remove the free Fmoc group. At the end of the synthesis five 30 seconds washes with methanol were completed to shrink the resin. The resin was removed from the vessel and dried overnight under vacuum.

5.3.4 Monitoring of the Coupling Reaction

To monitor the coupling reaction a simple ninhydrin spot test (Kaiser, 1970) was used. A small sample of resin was removed after the DCM/DMF wash that follows the

coupling reaction, transferred to a small glass tube and washed several times with ethanol. Two drops of the following solution were added i) ninhydrin solution (5% (w/v) of ninhydrin in ethanol), ii) 80% (v/v) liquified phenol in ethanol, iii) 1mM aqueous solution of potassium cyanide in pyridine; mixed and heated for 5 min at 129°C. A straw-yellow colour with no colouration of the beads, indicated complete acylation. A strong dark blue-purple colour is obtained in presence of free amino acid groups.

5.3.5 Cleavage of the Peptide from the Resin

The first (C-terminal) amino acid is linked to the support through a benzyl ester-type linkage, and a strong acidic reagent, such as trifluoroacetic acid (TFA), is required for its cleavage. Because all peptides synthesised (Table 5.2) contained residues of arginine or tryptophan, electrophilic scavengers were added to the reaction mixture to reduce attack of nitronium ions, *t*-butyl or benzyl cations on sensitive amino acids. The cleavage mixture used consisted of: 81.5% (v/v) TFA, 5% (v/v) bi-distilled Phenol, 5% distilled water, 2.5% (v/v) ethanedithiol (EDT), 5% (v/v) thioanisole, 1% (v/v) triisopropylsilane.

The dried resin was placed in a flask and 10 ml of cleavage mixture was added and left at room temperature for 3 hours with gently swirling. The resin was filtered under gentle vacuum and washed with 5 ml of cleavage mixture. The filtrate was reduced almost to dryness by rotatory evaporation under vacuum. The peptide was precipitated by adding 10 volumes of cold diethyl ether to the flask and after setting on ice most of the diethyl ether was taken off by aspiration. The precipitated peptide was dissolved in water and lyophilised. All peptides were synthesised with a yeild of 85 to 70 %.

5.4 Characterisation of Peptides Synthesised

All peptides synthesised were analysed by high-performance liquid chromatography (HPLC). The PWLA was also identified by nuclear magnetic resonance (NMR) spectroscopy. Large peptides were identified by mass spectrometry.

5.4.1 PWLA

5.4.1.1 Reverse-phase HPLC of PWLA

Chromatographic separation by HPLC is the result of interactions between sample molecules and both the stationary and mobile phases. Sample components are selectively retained by the stationary phase and eluted by changes in the mobile phase. Reverse-phase chromatography was used to routinely analyse the peptides synthetised.

An aliquot of 5 nmoles peptide in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, at a flow rate of 1 ml/min. The column was pre-equilibrated with an aqueous solution of 0.1% (v/v) TFA. A linear gradient (0-100%) of (HPLC grade) acetonitrile containing 0.1% TFA, was used to elute the sample from the column. The absorbance of the eluent was monitored at 214nm. Figure 5.8 shows the elution profile of the synthesised product. Two peaks were observed, a major peak at 40 min and second minor peak at 42 min.

5.4.1.2 ^{1}HNMR of PWLA

A ^{1}HNMR spectra of PWLA is shown in figure 5.9 and its interpretation is given in Table 5.3. The functional groups of the side chains from the four amino acids that make up the tetrapeptide were identified in the spectra. The scavengers (ethanedithiol, thianisole, triisopropylsilane and phenol) used in the cleavage of the peptide from the resin, were not identified in the spectra.

5.4.1.3 ^{13}CNMR of PWLA

A ^{13}CNMR spectra of PWLA is shown in figure 5.10 and its interpretation is given in Table 5.4. Functionality was determined by chemical shift, the theoretical shifts given in figure 5.11 A being taken as reference. The three methyl peaks expected from Leu and Ala, were observed in the region 20 to 24 ppm. Six peaks, expected for the β-CH_2 and

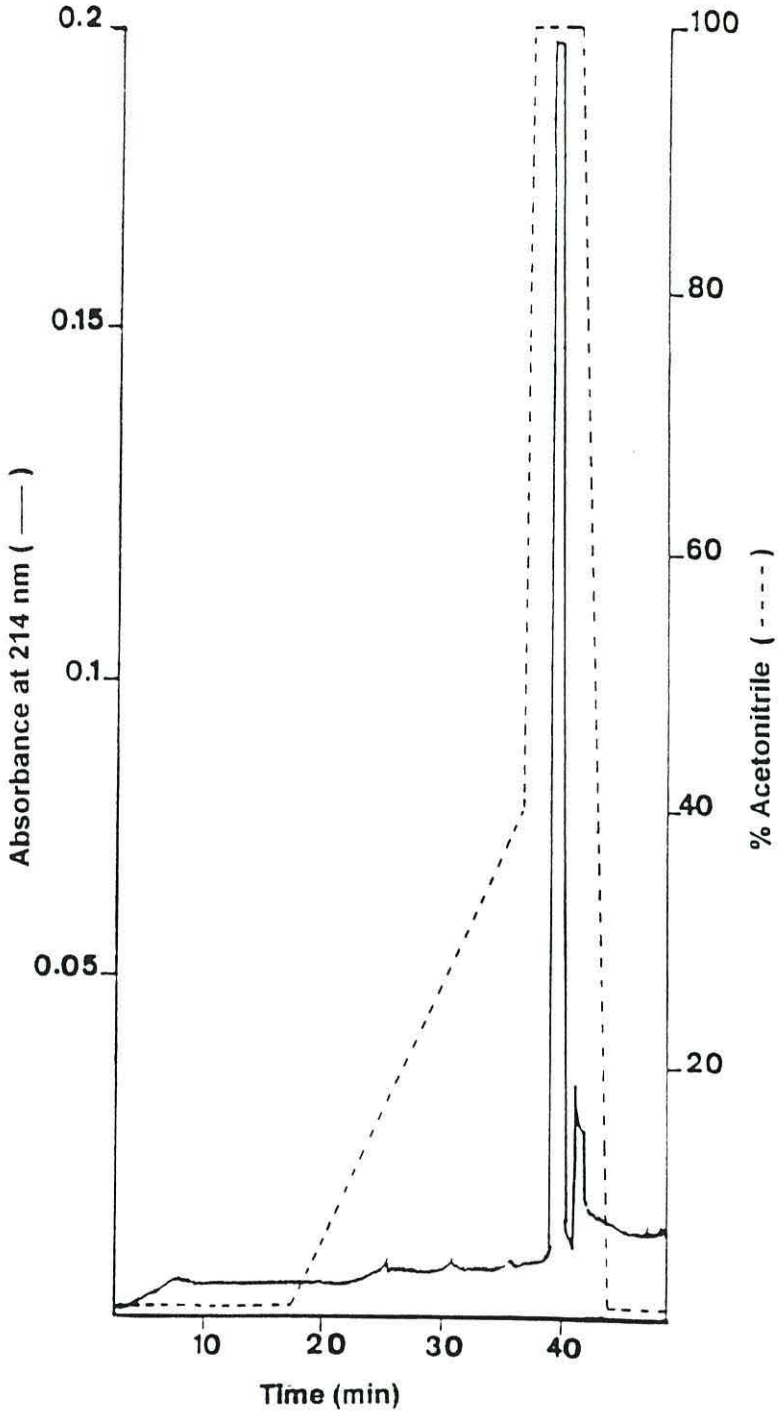


Figure 5.8

Reverse-phase HPLC of chemically synthesised PWLA tetrapeptide. The peptide was cleaved from the resin by TFA in the presence of phenol, ethanedithiol, thioanisole and triisopropylsilane, precipitated and washed with diethyl ether and lyophilised. 5 nmoles of crude peptide dissolved in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, at a flow rate of 1 ml/min. The column was pre-equilibrated with an aqueous solution of 0.1% (v/v) TFA, and a linear gradient (0-100%) acetonitrile (HPLC grade) containing 0.1% TFA, was used to elute the sample from the column. The absorbance of the eluent was monitored at 214nm

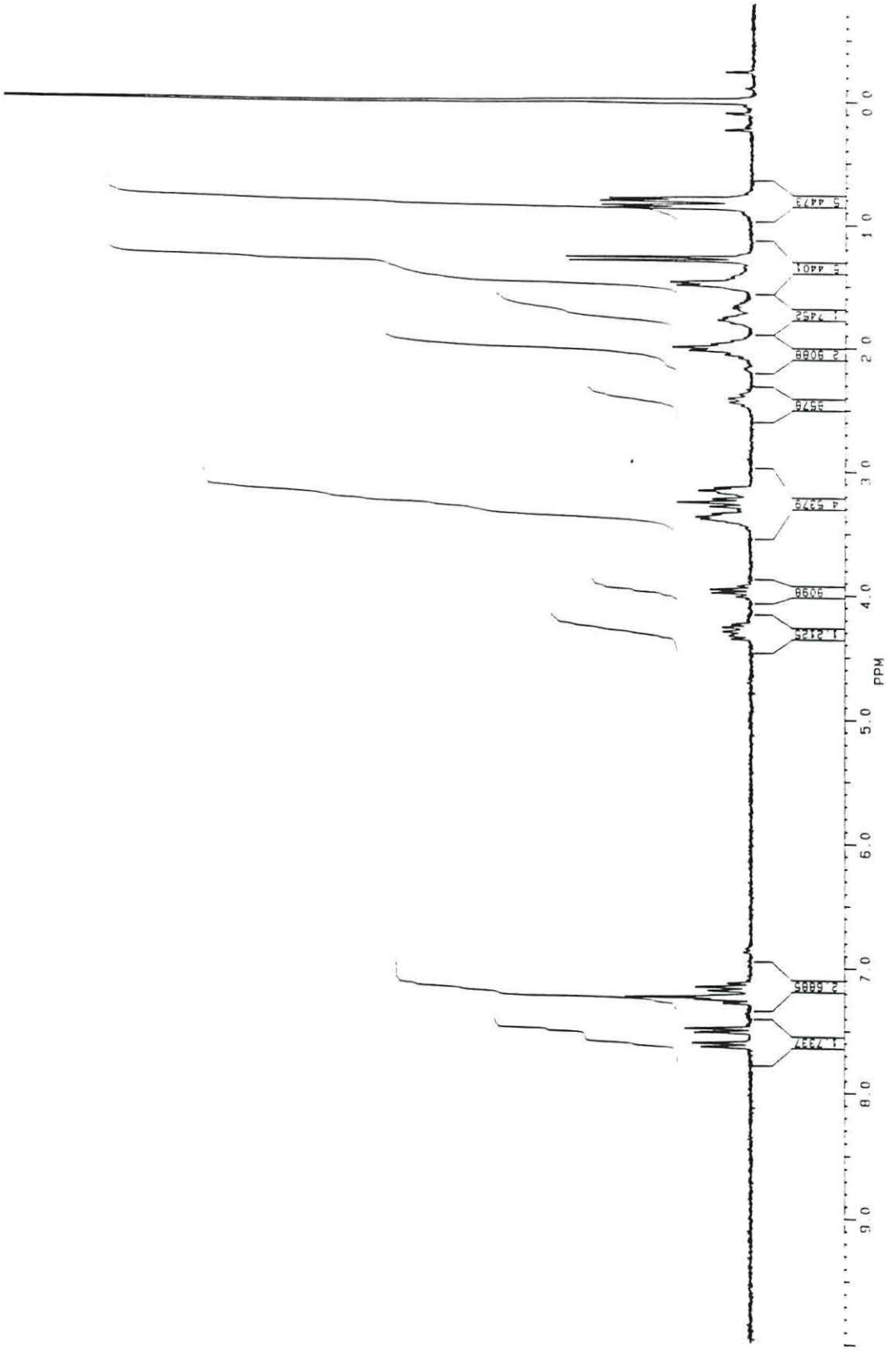


Figure 5.9

^{1}HNMR spectrum of chemically synthesised PWLA tetrapeptide

Table 5.3

^{1}H-NMR interpretation of PWLA

Chemical shift δ (ppm)	Multiplicity	Integral (cm)	Number of hydrogens	Functionality
0.89	Quaternary	10.3	6	δ-CH_3 Leucine
1.3	Doublet	5.0	2	β-CH_3 Alanine
1.5	Doublet	4.6	2	γ-CH Leucine
1.75	Multiple	3.2	1	γ-CH_2 Proline
2.05	Multiple	5.1	2	β-CH_2 Proline
2.35	Quaternary	1.5	None	
3.25	Multiple	8.7	5	δ-CH_2 Proline
4.0	Quaternary	1.5	None	α-CH Alanine
4.3	Multiple	2.1	None	β-CH_2 Tryptophan
7.15 - 7.2	Multiple	5.2	2	ζ^3-C, η^2-CTryptophan (Indole C-5, C-6)
7.54 - 7.6	Quaternary	3.3	2	ϵ^3C, ζ^2C Tryptophan (Indole C-4, C-7)

both γ- and δ-CH_2 from Pro, were observed in the region 25 to 47 ppm. Four peaks expected for α-CH of each amino acid, were observed in the region 53 to 62 ppm. The eight aromatic peaks expected for indole-tryptophan, were observed in the region 111 to 130 ppm. Two carbonyl peaks were observed in the region 172 to 175 ppm instead of an expected four, the third and four peaks could be the two small peaks observed in the region 180 to 190 ppm. Four unspecific peaks were observed, which could be due to impurity and could account for the minor peak observed in the HPLC. The scavengers (ethanedithiol, thianisole, triisopropylsilane and phenol) used in the cleavage of the peptide from the resin, were not identified in the spectra. Theoretical shifts from the scavenger are shown in Figure 11 B.

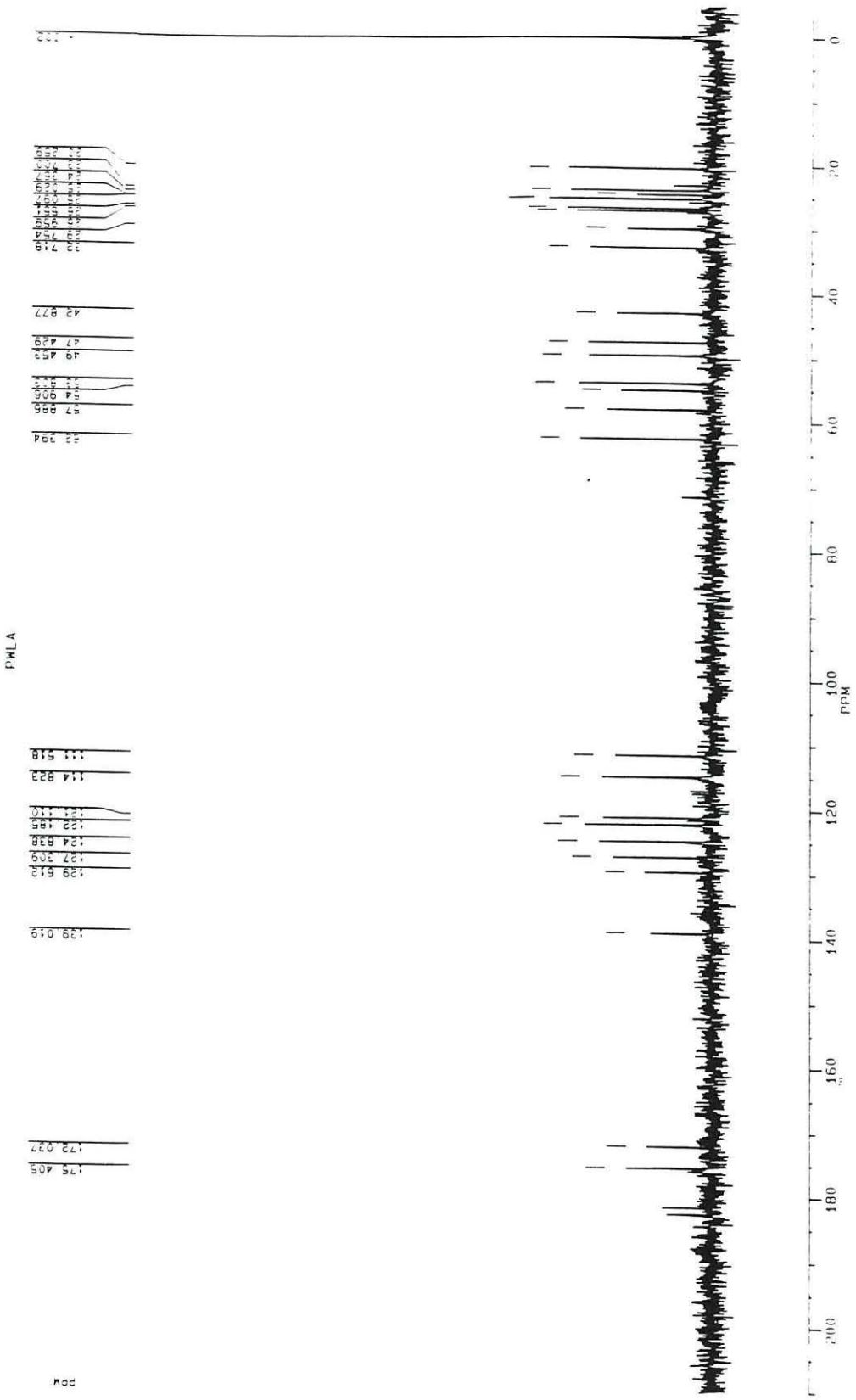


Figure 5.10

^{13}CNMR spectrum of chemically synthesised PWLA tetrapeptide

Table 5.4

^{13}C-NMR interpretation of PWLA

Chemical shift δ (ppm) taken from spectrum	Functionality
20.26	δ^1-CH_3 Leucine
23.70	δ^2-CH_3 Leucine
24.37	β-CH_3 Alanine
25.03	γ-CH_2 Proline
25.10	Unspecific
26.55	β-CH_2 Proline
26.96	Unspecific
29.75	Unspecific
32.72	β-CH_2 Tryptophan
42.88	β-CH_2 Leucine
47.43	δ-CH_2 Proline
49.45	Unspecific
53.80	α-CH Alanine
54.91	α-CH Tryptophan
57.87	α-CH Leucine
62.40	α-CH Proline
111.52	ζ^2 -C Tryptophan
114.82	γ -C Tryptophan
121.11	η^2 -C Tryptophan
122.19	ϵ^3 -C Tryptophan
124.84	ζ^3 -C Tryptophan
127.31	δ^1 -C Tryptophan
129.61	δ^2 -C Tryptophan
139.20	ϵ^2 -C Tryptophan
172 - 175	Carbonyl

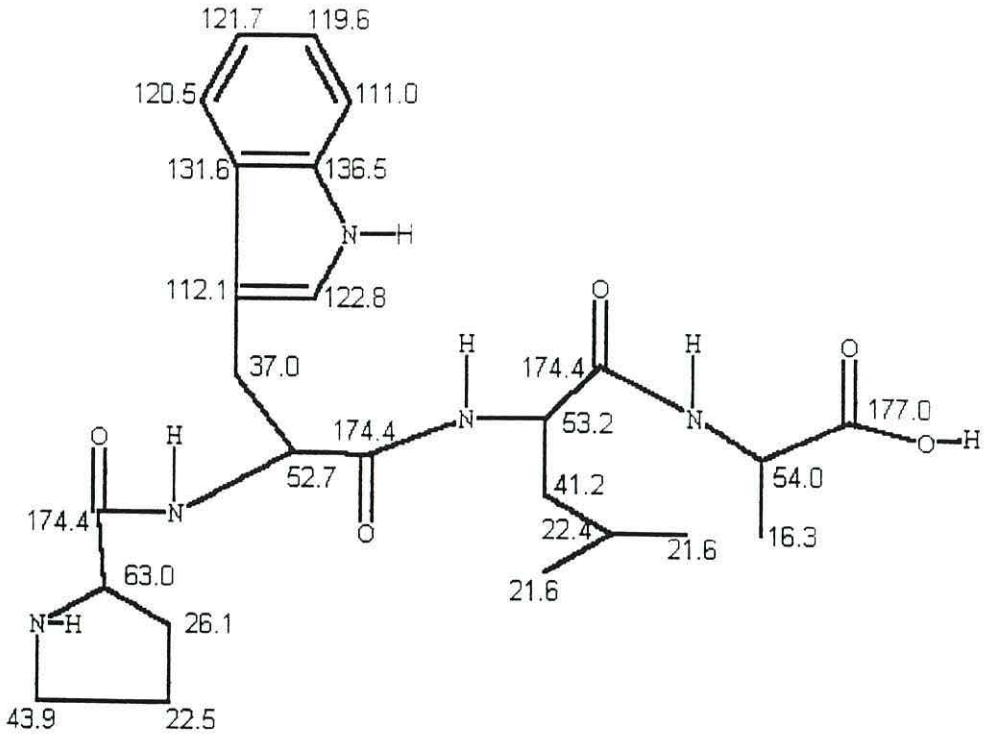


Figure 5.11(A)

PWLA structure showing the theoretical ^{13}C chemical shift.

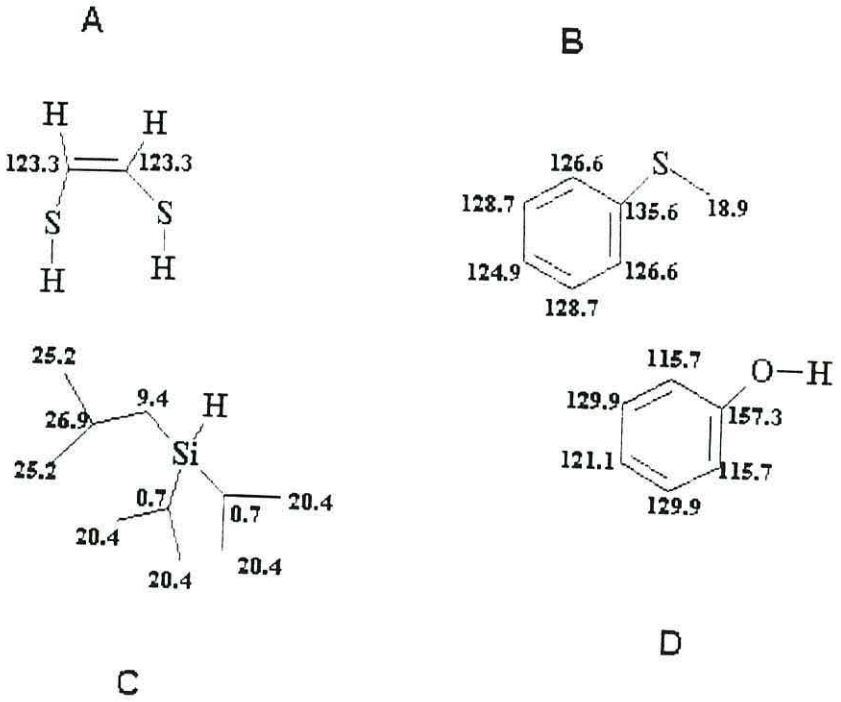


Figure 5.11 (B)

Scavenger structure showing the theoretical chemistry ^{13}C. (A) ethanedithiol (B) thioanisole (C) triisopropylsilane (D) phenol.

5.4.2 APD^{300}

An aliquot of 5 nmoles of APD^{300} in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, as described in section 5.4.1.1. Figure 5.12 shows the elution profile of the synthesised product. A single peak eluting at 35 min was observed.

Electrospray Mass spectrum is shown in fig 5.13 A. The expected ion Mr 1031 was present in high abundance. A peak was also observed at 1253.8 Da/e, which could be due to impurities. However, the single peak observed by HPLC, suggest the presence of low amount of impurity in the crude preparation.

5.4.3 APR^{300}

An aliquot of 20 nmoles of APR^{300} in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, as described in section 5.4.1.1. Two peaks were observed in the HPLC elution profile of the synthesised product (Fig. 5.14). One peak with high absorbance at 25 min, and another peak with low absorbance at 35 min.

The Electrospray Mass spectrum shows the expected compound at 1074 Da/e (Fig. 5.15 A), although peaks in range 1116 - 1541 Da/e, were showed in the spectrum, which possibly represent impurities in the crude preparation (Fig. 5.15 B).

5.4.4 APS^{300}

An aliquot of 20 nmoles of APS^{300} in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, as described in section 5.4.1.1. Figure 5.16 shows a single peak in the HPLC elution profile of the synthesised product.

The expected ion Mr 1003, is observed at the expanded view of the region 970 to 1100 in the electrospray mass spectrum shown in the figure 5.17 B. Also impurities were observed in the range 1021 - 1091 Da/e.

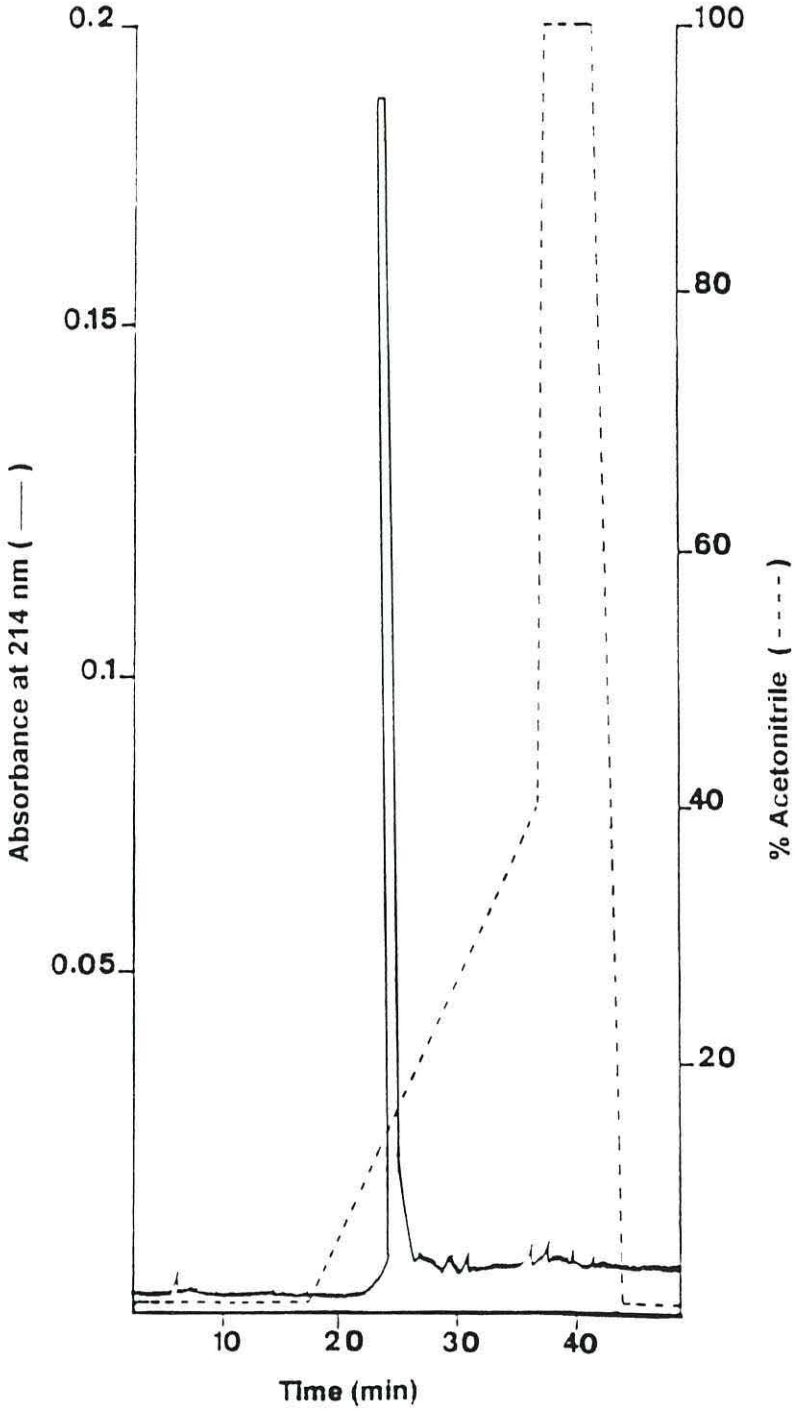


Figure 5.12

Reverse-phase HPLC of chemically synthesised APD^{300} nonapeptide. The peptide was cleaved from the resin by TFA in the presence of phenol, ethanedithiol, thioanisole and triisopropylsilane, precipitated and washed with diethyl ether and lyophilised. 5 nmoles of crude peptide dissolved in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, at a flow rate of 1 ml/min. The column was pre-equilibrated with an aqueous solution of 0.1% (v/v) TFA, and a linear gradient (0-100%) acetonitrile (HPLC grade) containing 0.1% TFA, was used to elute the sample from the column. The absorbance of the eluent was monitored at 214nm.

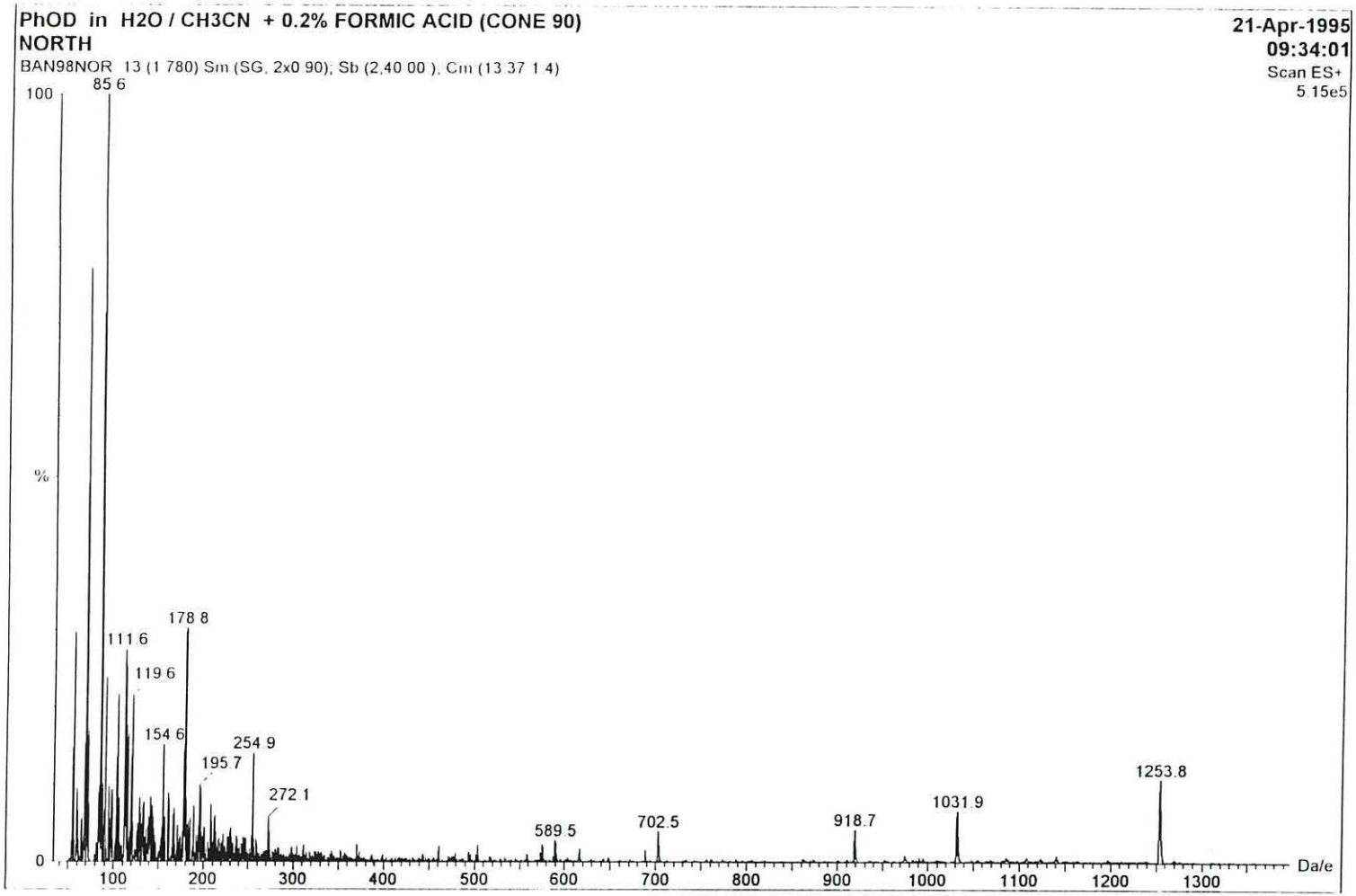


Figure 5.13 (A)

Electrospray-mass spectrum of chemically synthesised APD[300] nonapeptide.

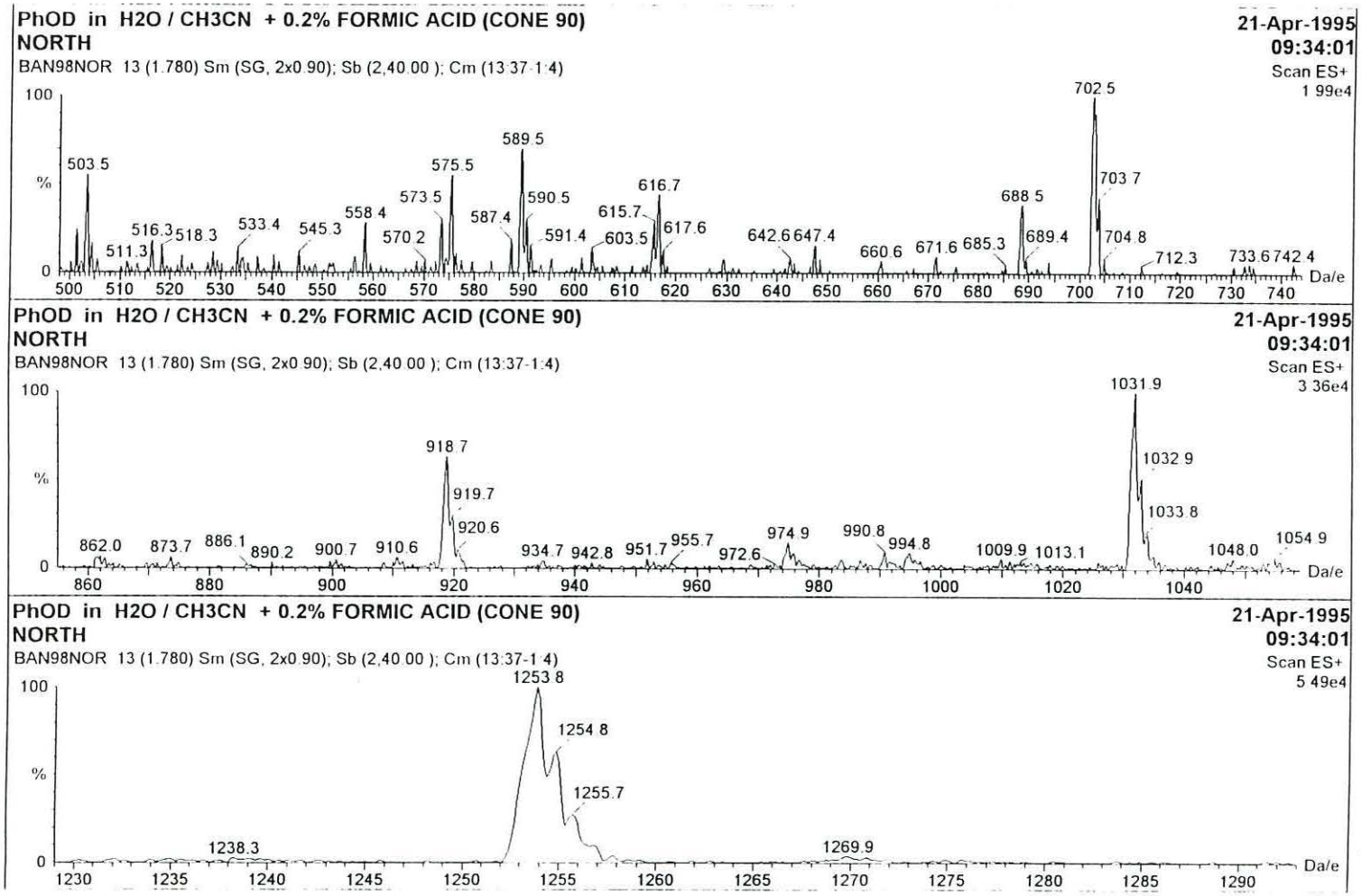


Figure 5.13 (B)

An expansion of region 500 to 1290 Da/e from the Electrospray-Mass Spectrum of chemically synthesised APD[300] nonapeptide.

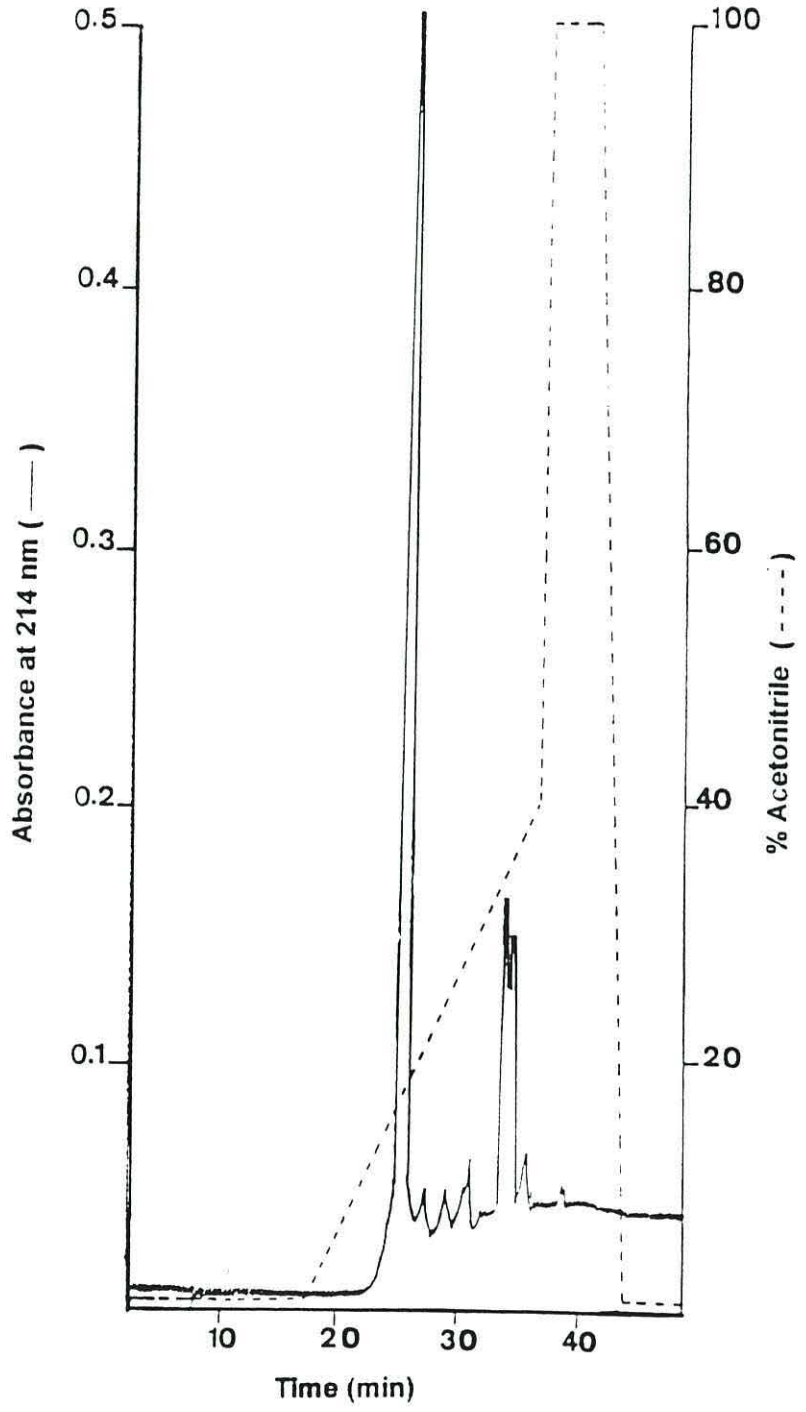


Figure 5.14

Reverse-phase HPLC of chemically synthesised APR^{300} nonapeptide. The peptide was cleaved from the resin by TFA in the presence of phenol, ethanedithiol, thioanisole and triisopropylsilane, precipitated and washed with diethyl ether and lyophilised. 20 nmoles of crude peptide dissolved in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, at a flow rate of 1 ml/min. The column was pre-equilibrated with an aqueous solution of 0.1% (v/v) TFA, and a linear gradient (0-100%) acetonitrile (HPLC grade) containing 0.1% TFA, was used to elute the sample from the column. The absorbance of the eluent was monitored at 214nm.

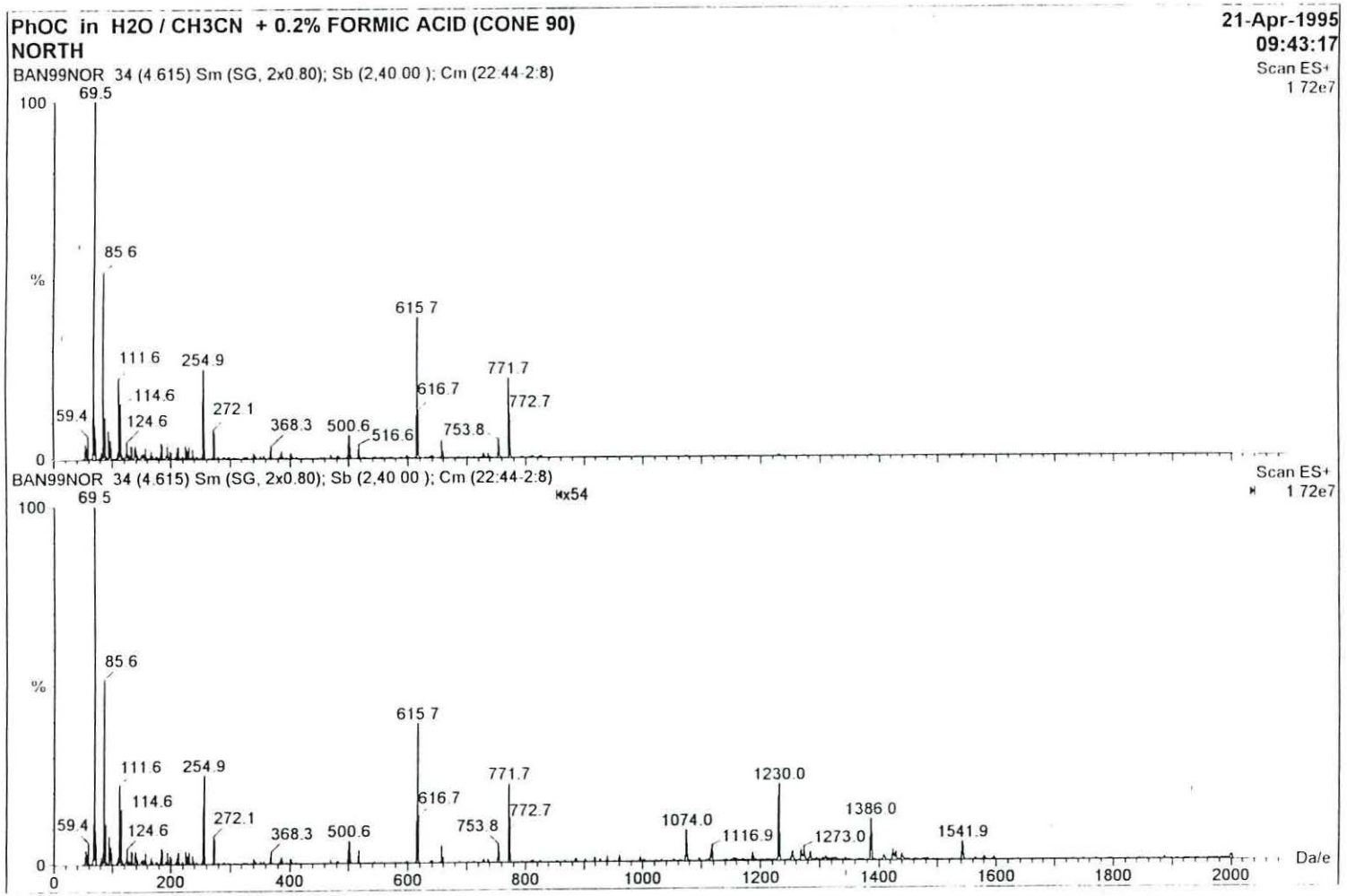


Figure 5.15 (A)

Electrospray-mass spectrum of chemically synthesised APR^{300} nonapeptide.

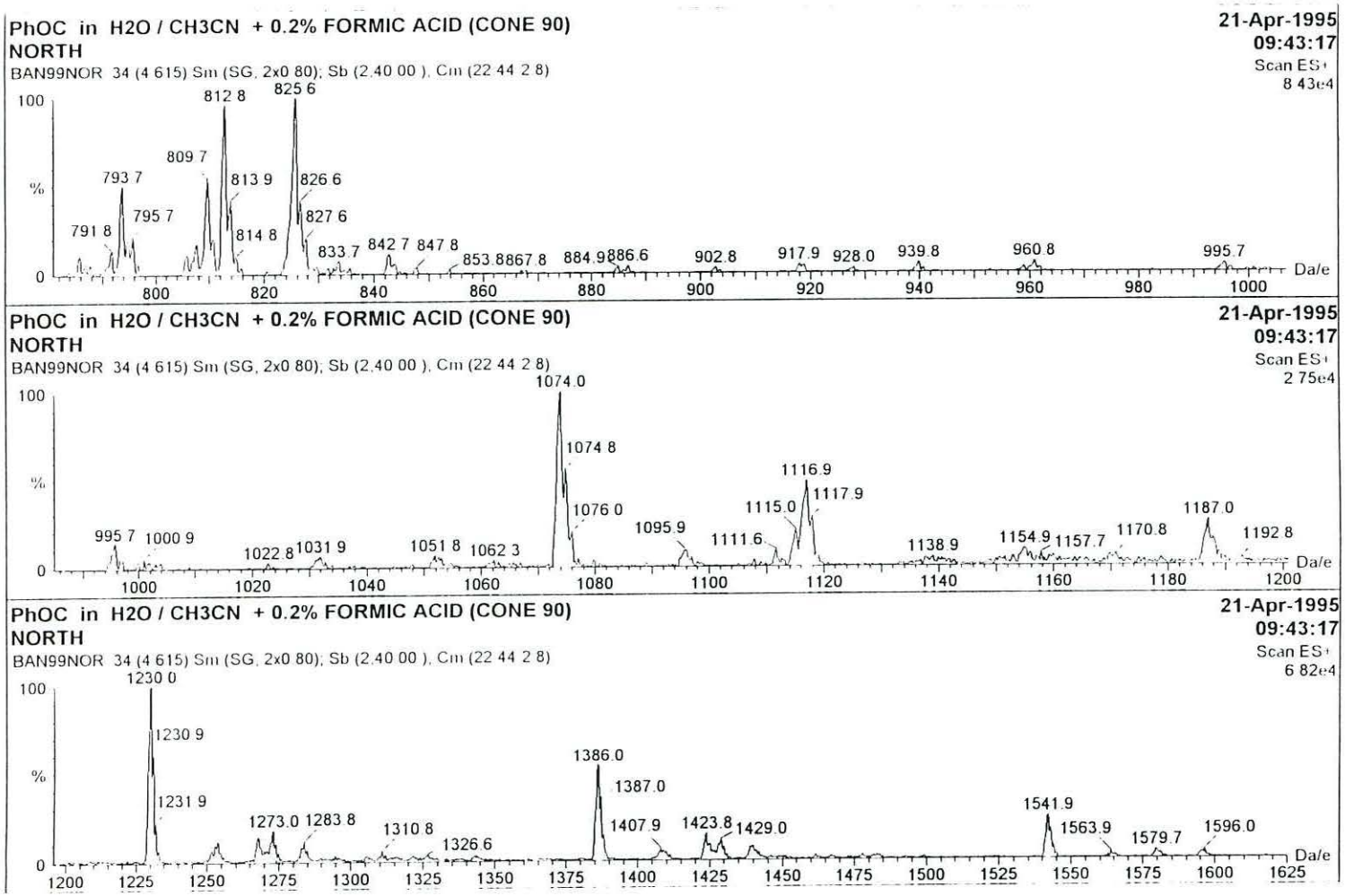


Figure 5.15 (B)

An expansion of region 800 to 1621 Da/e from the Electrospray-Mass Spectrum of chemically synthesised APR[300] nonapeptide.

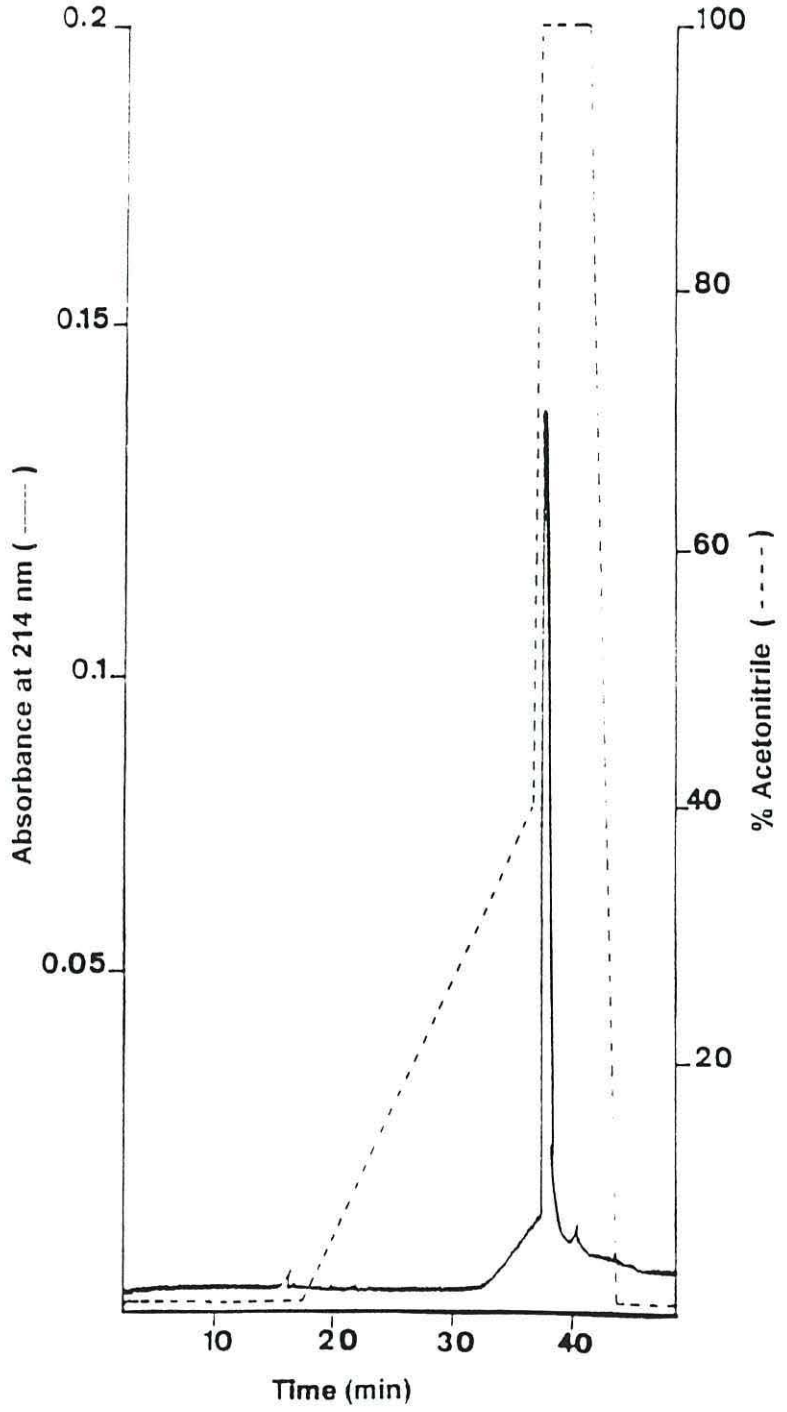


Figure 5.16

Reverse-phase HPLC of chemically synthesised APS^{300} nonapeptide. The peptide was cleaved from the resin by TFA in the presence of phenol, ethanedithiol, thioanisole and triisopropylsilane, precipitated and washed with diethyl ether and lyophilised. 20 nmoles of crude peptide dissolved in 100µl aqueous solution of 0.1% (v/v) TFA, was applied to a Vydac C18 column, at a flow rate of 1 ml/min. The column was pre-equilibrated with an aqueous solution of 0.1% (v/v) TFA, and a linear gradient (0-100%) acetonitrile (HPLC grade) containing 0.1% TFA, was used to elute the sample from the column. The absorbance of the eluent was monitored at 214nm.

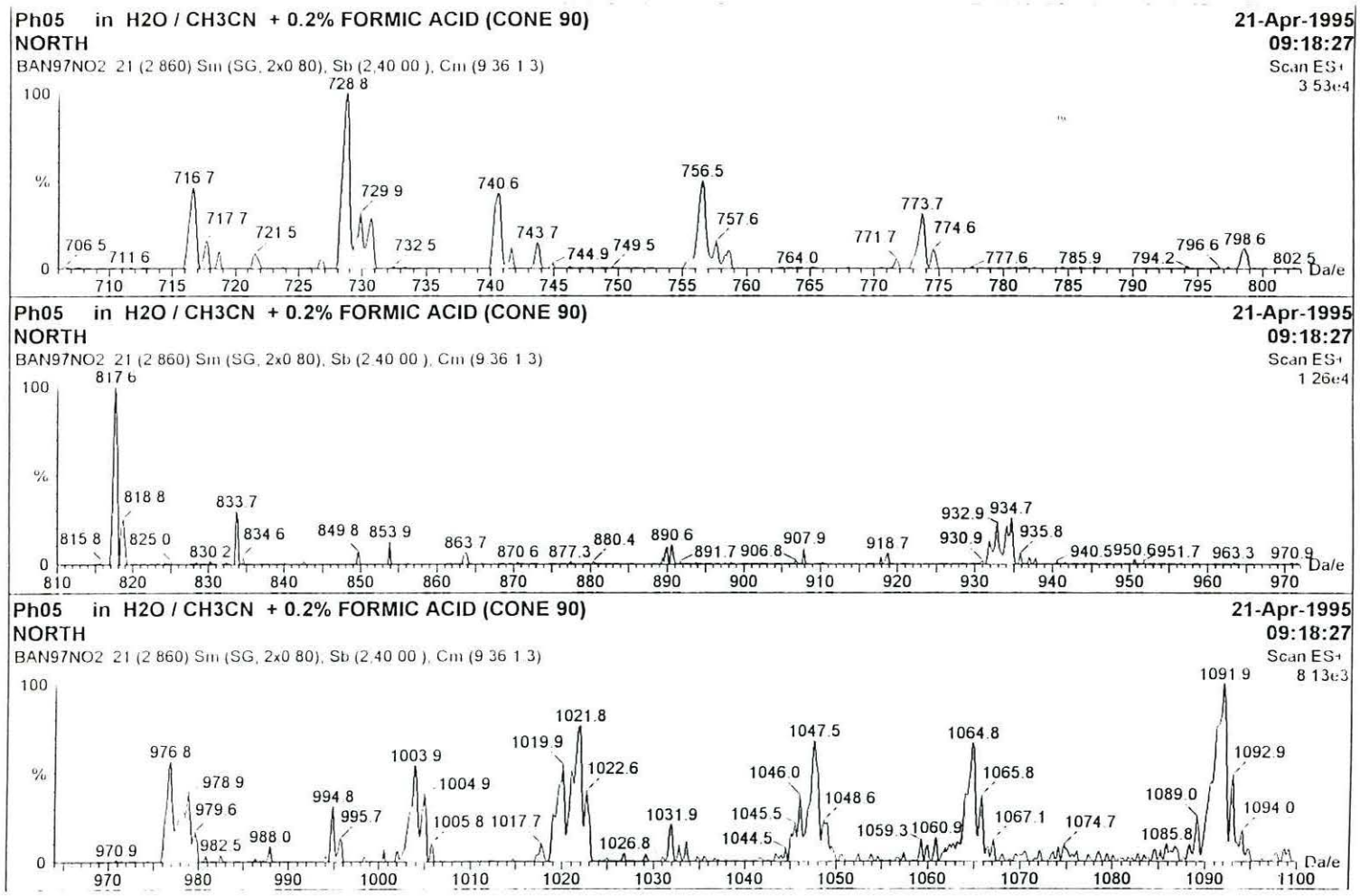


Figure 5.17 (A)

Electrospray-mass spectrum of chemically synthesised APS^{300} nonapeptide

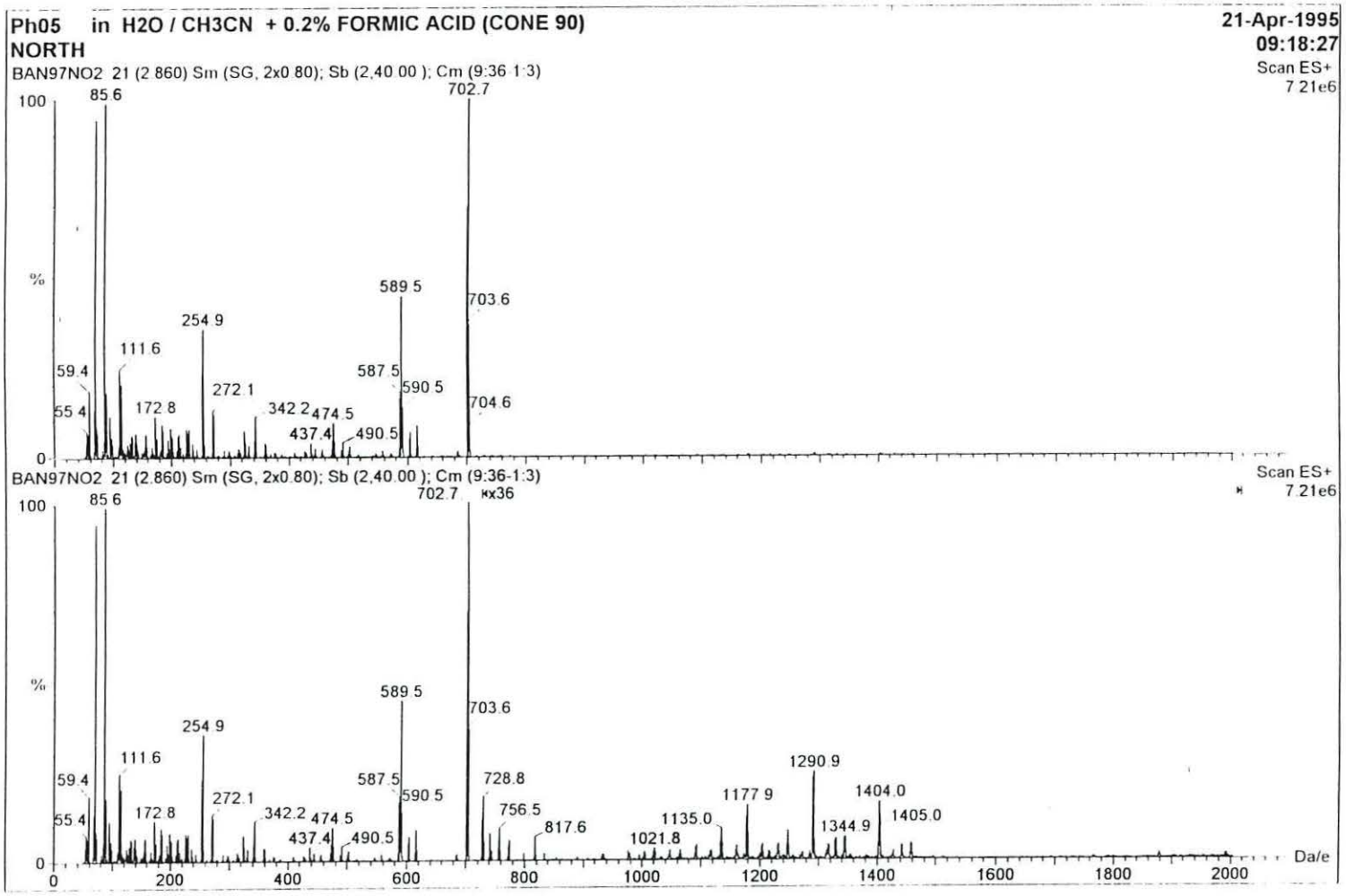


Figure 5.17 (B)

An expansion of region 700 to 1100 Da/e from the Electrospray-Mass Spectrum of chemically synthesised APS[300] nonapeptide

Results and Discussion

Chapter VI

PRODUCTION AND CHARACTERISATION OF ANTI-PEPTIDE ANTIBODIES

6.1 Introduction

The use of anti-peptide antibodies is highly recommended when a cloned DNA sequence is known and site-directed antibodies are needed. These anti-peptide antibodies, which have cross-reactivity with the complete protein, have been designated antibodies of predetermined specificity, since they react with a single antigenic region of the protein (Lerner, 1984). The fact that anti-peptide antibodies have the property of binding to a specific region of the protein antigen make them similar to monoclonal antibodies; however, they differ from monoclonal antibodies in that they are less specific for the native conformation of the protein and usually bind to the protein with a lower affinity constant. Most synthetic peptides are good immunogens, and there is usually no difficulty to produce antibodies against them by classical immunisation procedures (Niman *et al.*, 1983).

Anti-peptide antibodies are useful not only for detecting the protein product of a gene

(Lerner *et al.*, 1981), but also have been used successfully to detect conformational changes in proteins (White and Wilson, 1987) and to follow the protein maturation during processing leading to virus formation, as the maturation of the glycosylated envelope polyprotein Pr80 of a murine retrovirus (Green *et al.*, 1981). Peptides have been used to elicit neutralising antibodies to human immunodeficiency disease virus (Javaherain *et al.*, 1989), and malaria (Patarroyo *et al.*, 1988).

The aim of this study was to produce anti-peptide antibodies using chemically synthesised peptides representing putative ligand binding site of OppA from *E. coli* (Marshall, 1994). These antibodies could be used for the screening of OppA with a point mutation in this specific region of the protein, and in future experiments to characterise the interaction of liganded-OppA with the oligopeptide permease protein in membrane vesicles, to explain its mechanism of action. On the other hand, the highly conserved amino acid sequence selected, makes these anti-peptide antibodies suitable to investigate similar systems in other species such as *Streptococci* and *Staphylococci*.

6.2 Production of Anti-peptide Antibodies

6.2.1 Introduction

A synthetic 9-amino acid peptide corresponding in amino acid sequence to a region in Domain III of the *E. coli* OppA, designated APD^{300} (Table 6.1), was chemically synthetised, conjugated to a protein carrier, and used as antigen to produce anti-peptide antibodies. Two other synthetic peptides designated as APR^{300} and APS^{300}, that represent the same region in OppA with a site-directed mutagenesis, were also used to produce anti-peptide antibodies (Table 6.1). The proteins that contain these two latter peptides, have been referred as [Asp^{300}→Arg]-OppA and [Asp^{300}→Ser]-OppA, respectively.

Table 6.1

Peptide Name	Amino acid sequence
APD^{300}	$^{+}NH_3$-Leu-Gly-Met-Asp-Arg-**Asp**-Ile-Ile-Val-COO^-
APS^{300}	$^{+}NH_3$-Leu-Gly-Met-Asp-Arg-**Ser**-Ile-Ile-Val-COO^-
APD^{300}	$^{+}NH_3$-Leu-Gly-Met-Asp-Arg-**Arg**-Ile-Ile-Val-COO^-

The major disadvantage with anti-peptide antibodies is that they may not recognise the native protein, however some criteria can be taken into account to predict a good response of these antibodies. The peptides selected for this proposal contain an internal hydrophilic region, which is required to predict the possible surface location at the native protein. On the other hand, small peptides such as those listed in Table 6.1, have fewer problems for coupling to carrier molecules than longer peptides, and their use ensures that the site-specific character is retained.

6.2.2 Methods

6.2.2.1 Coupling peptide to carrier Protein by Using Glutaraldehyde

The carrier protein used for coupling the peptides prior to immunisation, was bovine serum albumin (BSA). This carrier was chosen for its availability of reactive sites, size, solubility, commercial availability and cost.

A single-step coupling procedure was performed, using a 1:1 (mol/mol) ratio between the synthetic peptide and available Lys in the carrier protein to couple 5 mg of BSA with peptide. A coupling molar ratio of 1:1 was used to take into account the total Lys available for coupling (30 residues per molecule of BSA) in the carrier protein. Peptide (2.5mg) dissolved in 1 ml of distilled water and BSA (5mg) dissolved in PBS, were

stirred together, in the fume hood, and 1 ml of freshly prepared glutaraldehyde aqueous solution (21 mM) was added dropwise, with stirring over a period of 10 min at room temperature. The mixture was incubated for one hour with stirring at room temperature, then dialysed against PBS for 24 hours at 4°C with two changes of buffer. The peptide/BSA conjugate was split in 1ml aliquots and stored, at -20°C, as 1 mg/ml solution. This concentration was determinated solely on the concentration of the peptide used.

6.2.2.2 Immunisation

Three New Zealand White rabbits, female, 10-12 weeks old, were immunised with 500µg of each individual conjugate peptide emulsified in complete Freud's adjuvant (CFA) (1:1 v/v) by multiple subcutaneous injections. Booster immunisation was carried out two weeks past the first injection using the same conditions and doses of antigen, but emulsified in incomplete Freud's adjuvant (IFA). Three BKTO Mice, female, 8-10 weeks, were immunised for each antigen using the same protocol described for rabbit, but the doses of immunogen were 100µg of conjugate peptide, and a second booster immunisation was given two weeks past the first booster.

To prepare the immunogen (water-in-oil emulsion), the complete or incomplete adjuvant was added to a tube, and while mixing in a vortex, an equal volume of the antigen solution was added and mixed vigorously until a thick emulsion was developed. CFA contains two components, one is a mineral oil designed to form a deposit protecting the antigen from rapid catabolism. The second component is heat-killed *Mycobacterium tuberculosis*, which stimulates the immune response nonspecifically. IFA only contains the first mineral oil component.

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6.2.2.3 Sampling blood and Serum Preparation

Samples of blood were taken each 2 weeks, starting the second week after the first booster to monitor the antibody response in each animal. A first blood sample was taken just before immunisation, to be used as a negative control in future tests. This sample was referred to as pre-immune serum. Small amounts of samples were collected until the desired antibodies were detected.

Test bleeds on rabbits was done from the ear vein, using a needle connected to a syringe through a plastic catheter. About 5 ml of blood was collected at any one time.

Blood samples from mice were collected from the tail vein. Using a sterile scalpel, the underside of the tail was cut and drops of blood were collected in an eppendorf tube (about 100 µl at one time).

After collection, the blood samples were allowed to clot by incubation at 37°C for 1 hour, then transferred to 4°C and incubated overnight to allow the clot to contract. The serum was then removed from the clot and any remaining insoluble material removed by centrifugation at 10,000g for 10 min. at room temperature. Serum was stored aliquoted in plastic tubes at -20°C.

6.2.2.4 Antibody Titre test (ELISA)

The antibody titre and the antigenic activity of synthetic peptides were measured by using Enzyme Linked immunosorbent assay (ELISA), following a modification of the method described by Engvall and Perlmann, 1972.

The assay consisted of the followed steps.

(a) Depending upon the antigen tested, two kinds of microtitre plate were used. Falcon plates (polyvinyl) were used when the antigen absorbed was the whole protein, or MaxiSorp plates when the soluble peptide was used to coat the plate.

Microtitre wells were coated with antigen by incubation overnight at 4°C with 100 µl of 20 µM peptide solution (2µg/well) or 0.018 µM of whole protein solution (0.1µg/well) in coating buffer. Carbonate buffer at pH 9.6 (15 mM Na_2CO_3; 35 mM $NaHCO_3$; 0.02% Thimerosal) was used as coating buffer

(b) Un-adsorbed antigen was aspirated and wells were repeatedly washed (minimum 3 times) with phosphate buffered saline, pH 4.0, containing 0.05% Tween 20 (PBS-Tween).

(c) The antiserum to be analysed was diluted in PBS-Tween and 100µl incubated with the adsorbed antigen, for 2 hours at 37°C. Serial dilution (1:2) in PBS-Tween starting from 1:50 for anti-peptide antibodies and 1:100 for anti-whole protein antiserum, were used.

(d) Un-bound antibody was washed off as described in (b).

(e) A suitable, enzyme-labelled anti-immunoglobulin diluted 1:2000 in PBS-Tween was added to the antigen-antibody complex, and incubated for 2 hours at 37°C. To detect antibodies raised in both rabbits and mice antisera, the secondary antibody used was goat anti-rabbit IgG (whole molecule) labelled with Horseradish peroxidase Type IV and goat anti-mouse polyvalent immunoglobulin (IgG, IgA, IgM) labelled with Horseradish peroxidase Type IV, respectively.

(f) Un-bound secondary antibody was washed off as described in (b).

(g) A volume (100µl) of freshly prepared chromogen substrate was added per well and incubated at room temperature until the colour developed sufficiently (20-30 min). The subtrate system used was 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS). The substrate was prepared by diluting 0.3 ml of 0.02% ABTS in 9.7 ml 0.1 M citrate buffer pH 4.0 containing 0.015% H_2O_2.

(h) Optical density was read at 405 nm in a microtitre plate reader (Titertek Twinreader ICN Flow Laboratories.

6.2.3 Results and Discussion

The titre of anti-peptide antibodies and their specificity was determined by ELISA, which is a solid-phase immunoassay used to quantify the amount of antibodies present in an antisera raised against a peptide which are capable of reacting with an immobilised peptide or the protein containing the peptide.

6.2.3.1 Determination of optimal conditions for ELISA

Optimal conditions such as antigen concentration for coating plate, blocking solution, antisera dilution, and anti-immunoglobulin enzyme conjugate dilution were predetermined empirically. The absorption of synthetic peptides to the wells of microtitre plates, was improved by using polystyrene plate (Maxi-Sorp™ Nalge Nunc Inter. Ltd) which is recommended for coating with peptides. The use of this plate avoided the necessity of conjugating the peptide to an alternative carrier protein. Although different proteins are used as carriers to make the peptide conjugate to inoculate and peptide conjugate to coat the wells, the presence of antibodies directed to the carrier protein used as inoculum, could show cross-reactivity with the peptide/carrier conjugate used to coat the wells, which for instance, could contribute to the overall reaction leading to an overestimation of the level of peptide antibodies present in the antiserum (Van Regenmortel *et al.*, 1988).

The maximum attachment of antigen to the solid-support plastic is reached with carbonate buffer at pH 9.6, however free binding sites could still be present on the wells, which can cause non-specific binding of the antibodies, leading to an overestimation of the level of specific antibodies tested. Blocking is commonly achieved with a range of protein or detergent solution. The protein most commonly used to block is BSA, but because this protein was used as carrier, it was necessary to seek a different blocking solution. A 5% (w\v) nonfat dry milk in PBS Tween was tested as blocking solution, but a high background was observed. Repeated washing with PBS-Tween, however was enough to minimise the nonspecific binding, as shown in the control incubations where the wells

contained no antigen.

6.2.3.2 Determination of titre and specificity of anti-peptide antibodies from rabbit's antisera

The results of ELISA titre test of the sera collected in the final bleeding of rabbits immunised with APD/BSA conjugate, APR/BSA conjugate and APS/BSA conjugate, are showed in figures 6.1 A, B and C, respectively. The minimal detectable response above the negative control was observed at 1:3000, 1:6000 and 1:500 dilution of anti-APD, anti-APR and anti-APS, respectively. The development of antipeptide antibodies titre in these antisera is showed in figure 6.2. All rabbit antisera showed high titre of antibodies against the peptide used as antigen. Anti-APD and Anti-APR antisera, showed high titre from the 3rd week until 29th and 50th week after booster, respectively. While anti-APS antiserum showed high titre from the 9th until the 45th week after the booster.

In order to test the specificity of these anti-peptide antibodies, the ability of each anti-peptide antiserum (anti-APD, anti-APR and anti-APS) to react with APD, APR and APS, which differ in only one amino acid at the same position (Table 6.1), was also determined (Fig. 6.3 A, B and C). The three anti-peptide antibodies, showed cross-reactivity against the peptides tested, for instance, anti-APD antiserum was able to react with APR and APS (Fig. 6.3 C and D), but with lower affinity as compared with the reaction observed for APD (Fig. 6.3 A). However, in each case greatest specificity was shown for the cognate antigen, and in the case of anti-APR the relative affinities were markedly different.

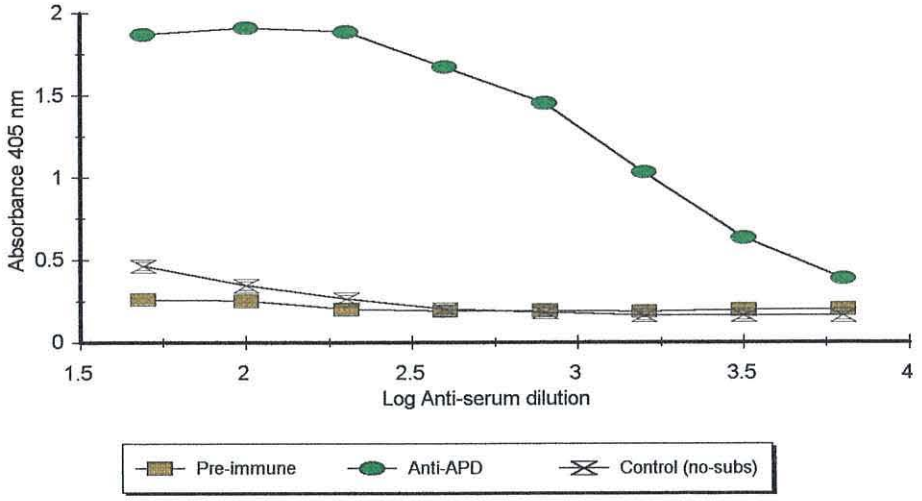


Figure 6.1 A

Anti-APD antibody titre from rabbit antiserum by ELISA. Microtitre plate was coated with APD ((2μg\200μl). The antiserum tested was taken 50 weeks after booster (final bleeding). Minimal detectable response above negative control, was at 1:3000 dilution.

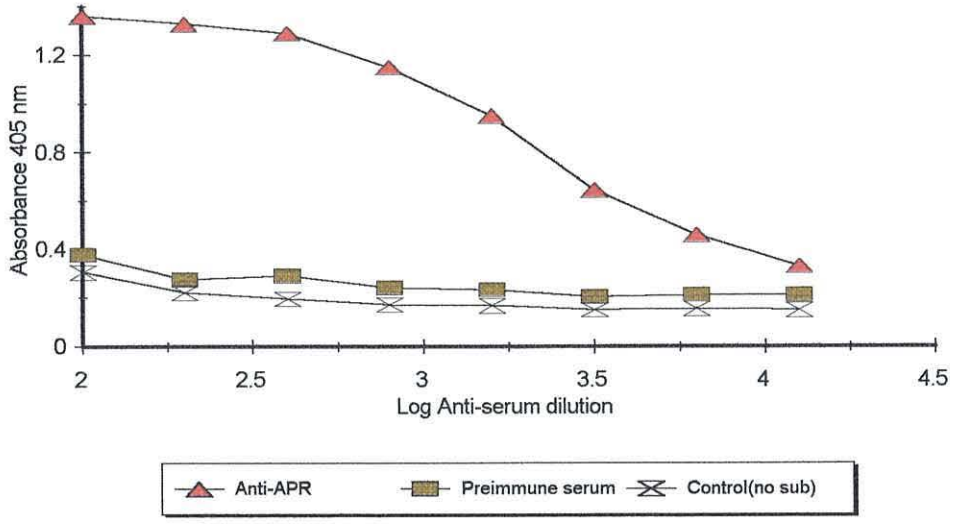


Figure 6.1 B

Anti-APR antibody titre from rabbit antiserum by ELISA. Microtitre plate was coated with APR (2μg\200μl). The antiserum tested was taken 29 weeks after booster (final bleeding). Minimal detectable response above negative control, was at 1:6000 dilution.

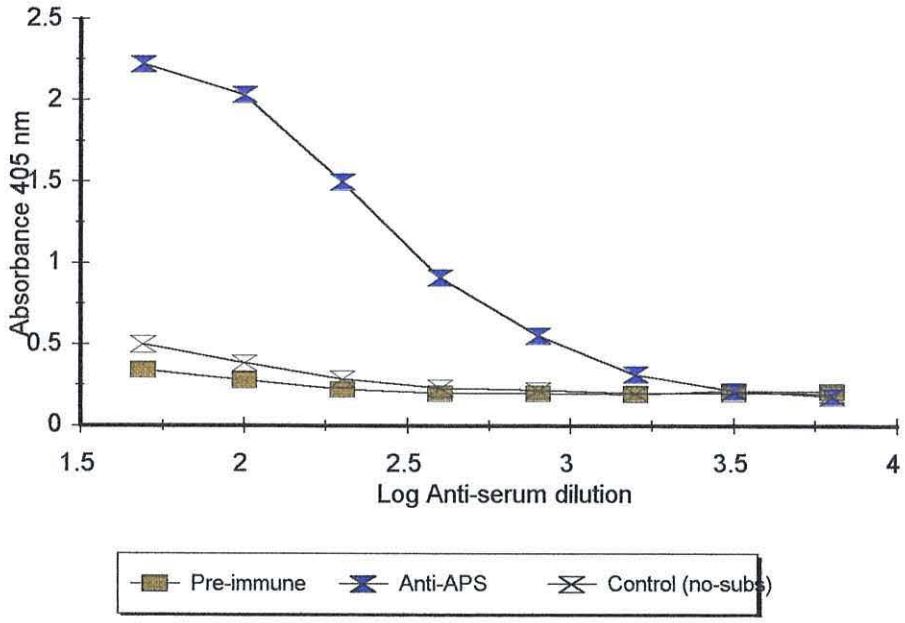


Figure 6.1 C

Anti-APS antibody titre from rabbit antiserum measured by ELISA. Microtitre plate was coated with APS (2µg\200µl). The antiserum tested was taken 45 weeks after booster (final bleeding). Minimal detectable response above negative control, was at 1:500 dilution.

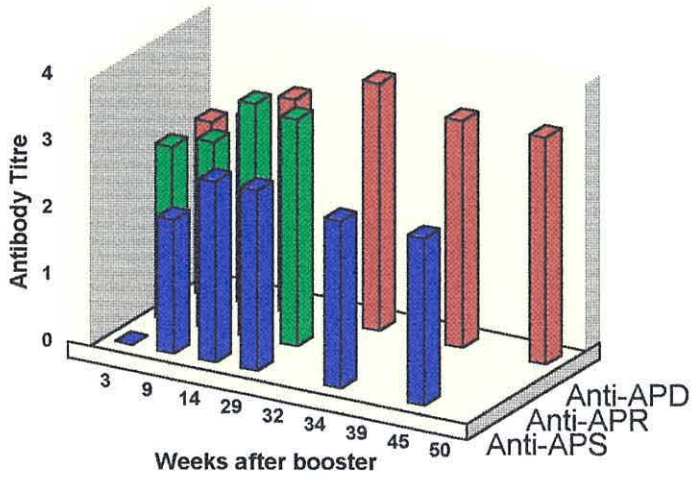


Figure 6.2

Development of anti-peptide antibodies titre in rabbit antisera. Antibody titre was expressed as the log of the half -maximal binding above negative control.

6.2.3.3 Determination of titre and the specificity of anti-peptide antibodies from mice antisera

Three mice were immunised with the same antigen APD/BSA conjugate, APR/BSA conjugate and APS/BSA conjugate, and the development of an immune response was monitored every 2 or 4 weeks after booster in each animal. ELISA titre test of the sera collected in the final bleeding are shown in Figures 6.4 A, B and C. Antisera from mice immunised with APD/BSA conjugate and APS/BSA conjugate were able to recognise APD and APS, respectively, and the minimal detectable response above the negative control was observed at 1:800 and 1:400 antiserum dilution, respectively (Fig. 6.4 A and C). Both anti-APD and anti-APS antisera showed high titre from the 2nd week until the 28th week after the second booster, while no activity was shown in mouse's serum immunised with APR/BSA conjugate (Fig. 6.5). The use of several animal for immunisation with the same antigen is recommended, because even genetically identical animals receiving the same immunisation schedule may develop different immune responses However, none of the three mice immunised with APR showed any anti-APR activity (Fig. 6.4 B and 6.5).

The ability of mouse anti-APD to bind APS and APR was tested. Figure 6.6 shows that anti-APD was specific to bind APD . Cross-reactivity of anti-APD with APR and APS was not shown.

6.3 Cross-reactivity of anti-peptide antibodies with the whole protein

6.3.1 Introduction

In order to test the ability of anti-APD, anti-APR and anti-APS antibodies to recognise WT-OppA, Ser^{300}-OppA and Arg^{300}-OppA, respectively, the purified protein or the mixture of proteins containing the whole protein, were separated on SDS-PAGE, immobilised by western-blotting and immunostained with anti-peptide antibodies from rabbits.

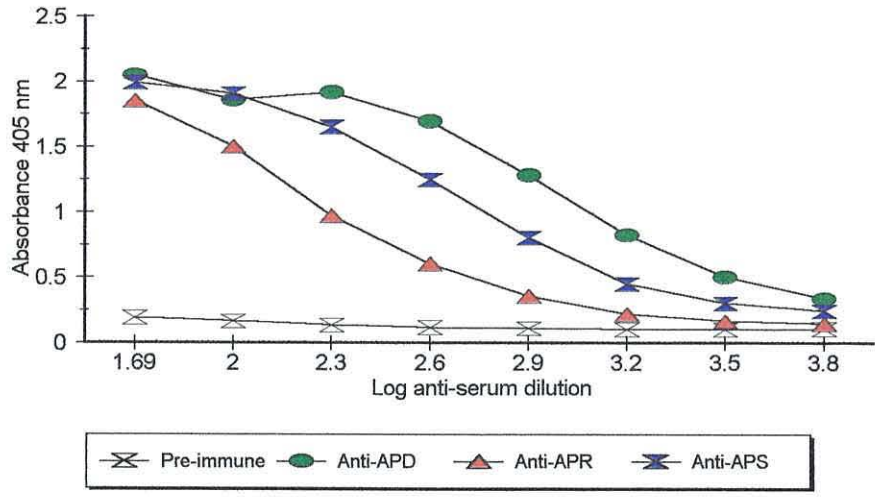


Figure 6.3 A

Ability of anti-APD, anti-APR and anti-APS to bind APD. Microtitre plate was coated with APD (2μg\200μl).

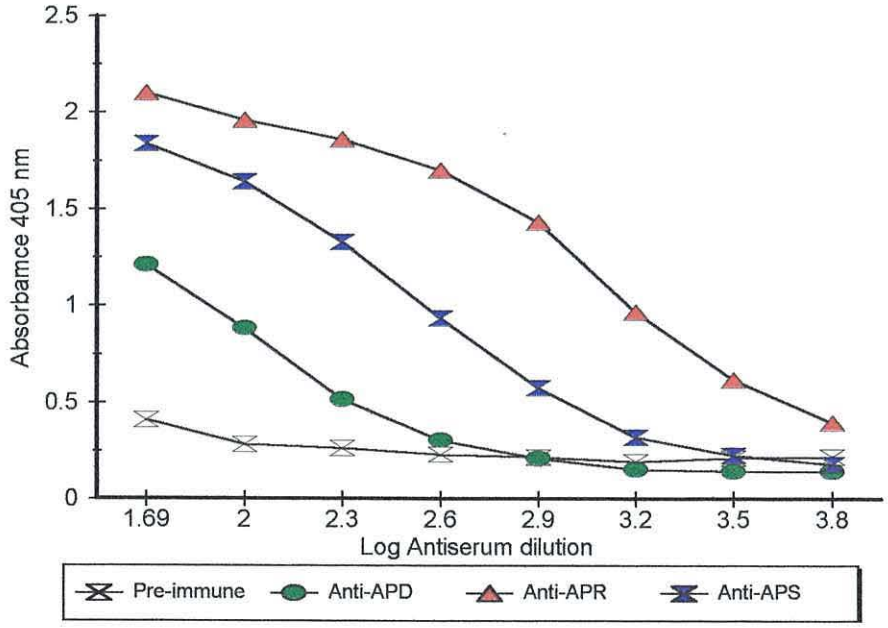


Figure 6.3 B

Ability of anti-APD, anti-APR and anti-APS to bind APR. Microtitre plate was coated with APR (2μg\200μl).

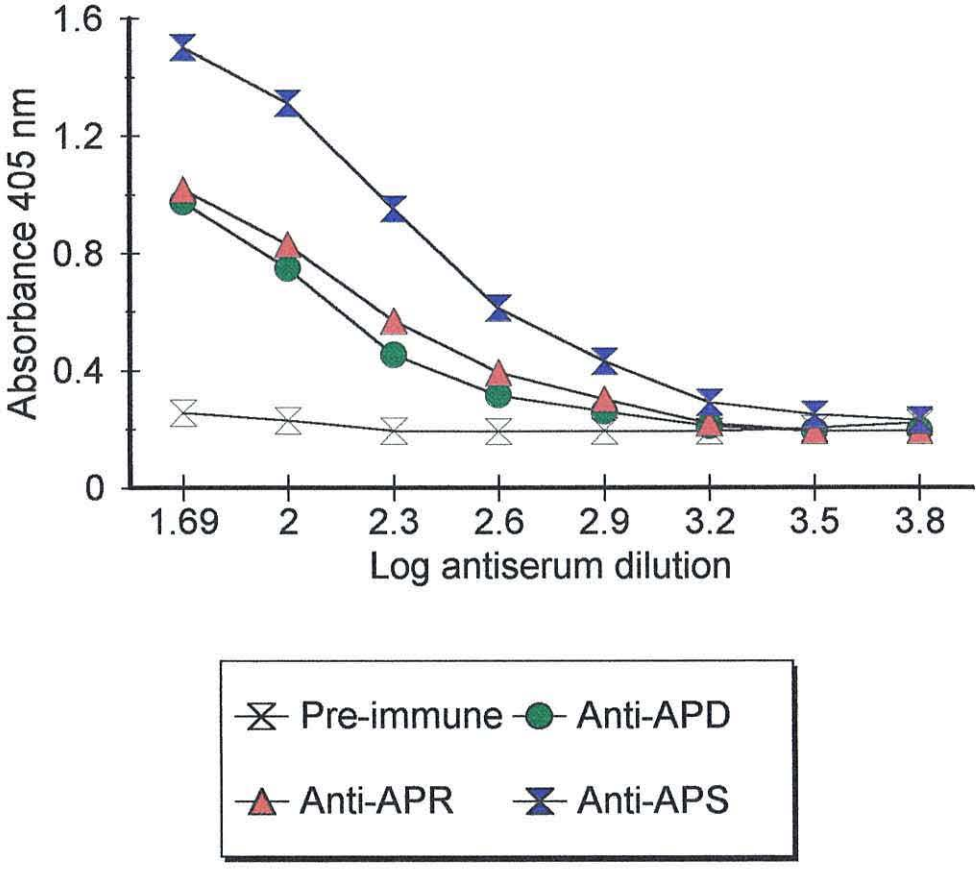


Figure 6.3 C

Ability of anti-APD, anti-APR and anti-APS to bind APS. Microplate was coated with APS (2µg\200µl).

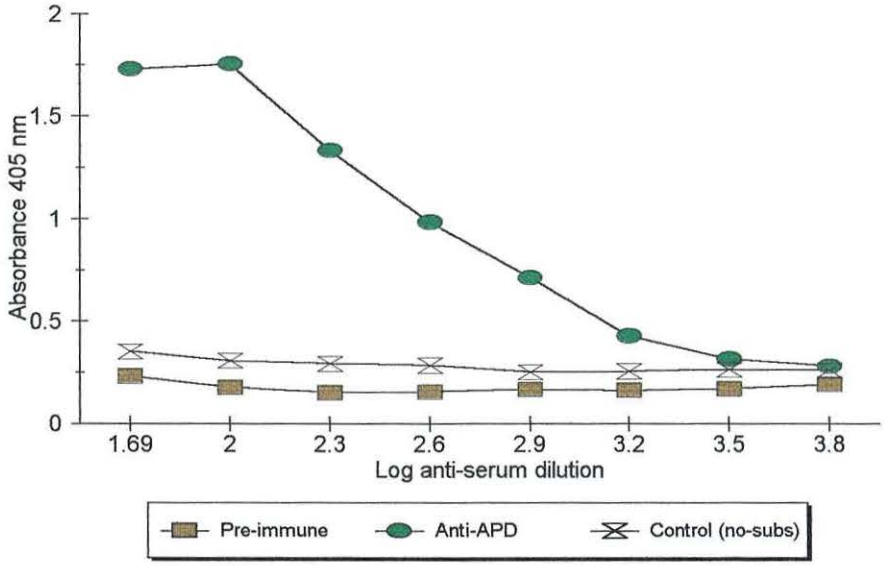


Figure 6.4 A

Anti-APD antibody titre of mouse antiserum by ELISA. Microtitre plate was coated with APD (2μg\200μl). The antiserum tested was taken 38 weeks after booster (final bleeding). Minimal detectable response above negative control, was at 1:800 dilution.

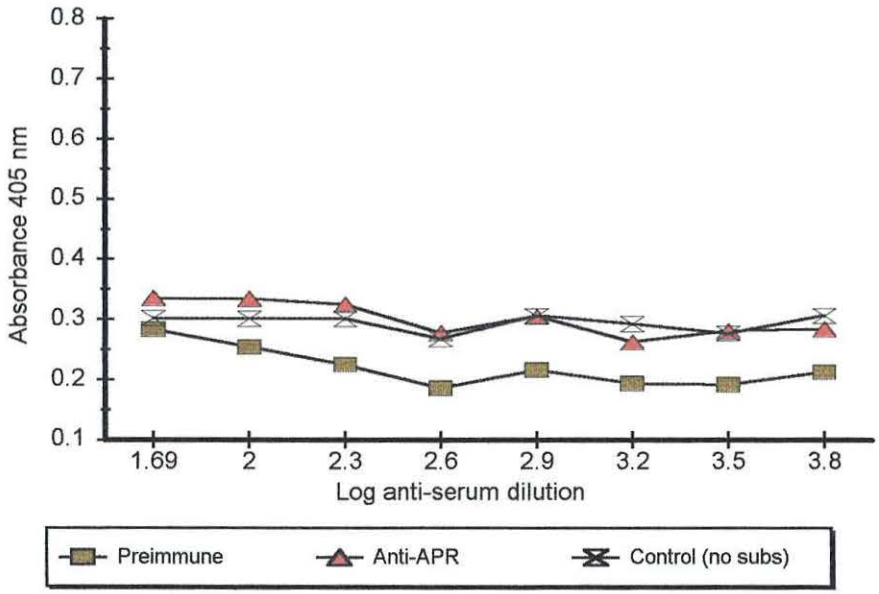


Figure 6.4 B

Anti-APR antibody titre of mouse's antiserum by ELISA. Microtitre plate was coated with APR (2μg\200μl). The antiserum tested was taken 38 weeks after booster (final bleeding).

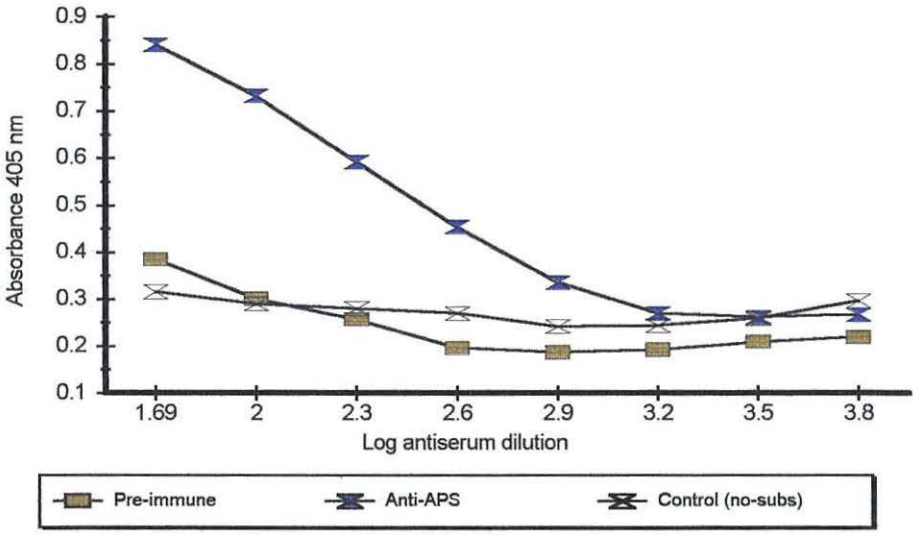


Figure 6.4 C

Anti-APS antibody titre from mouse antiserum by ELISA. Microtitre plate was coated with APS (2μg\200μl). The antiserum tested was taken 38 weeks after booster (final bleeding). Minimal detectable response above negative control, was at 1:400 dilution.

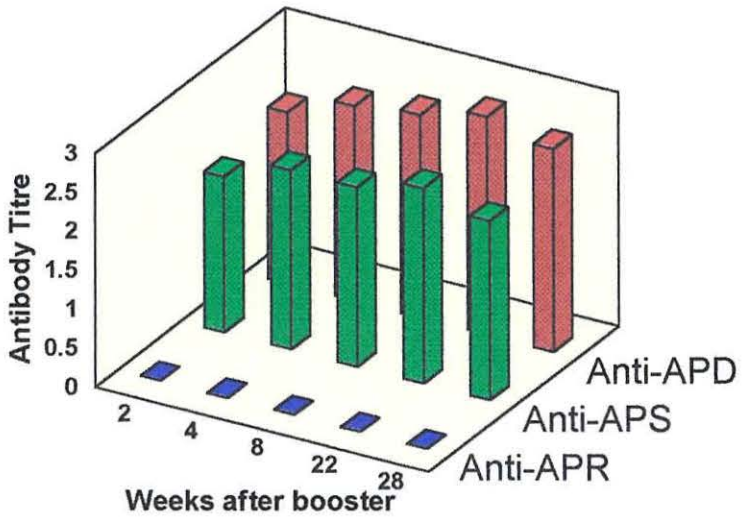


Figure 6.5

Development of anti-peptide antibodies titre in mice antisera. Antibodies titre was expressed as the log of the half-maximal binding above negative control.

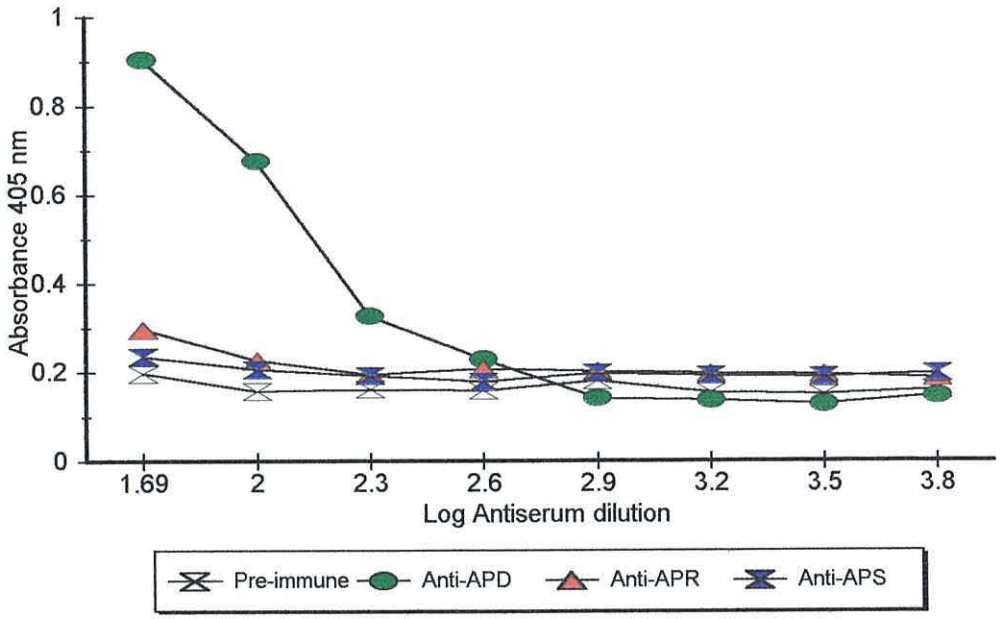


Figure 6.6

Ability of anti-APD, anti-APR and anti-APS, from mouse antiserum to bind APD. Microtitre plate was coated with APD (2µg\200µl).

6.3.2 Methods

Proteins were separated on SDS-polyacrylamide gel electrophoresis as described in section 4.10.1

Western blotting was carried out as described in section 4.11, but the conditions were optimised to use anti-peptide antibodies as first antibody. To obtain the highest signal with minimum background, a solution of 1% gelatine in PBS-Tween was used as blocking agent.

Immunodetection was carried out by enhanced chemiluminescence (ECL) according to the manufacturer's instructions. The follows steps were carried out:

(i) The blot was washed with TBS-Tween for 5 min with 3 changes. (ii) An equal volume of detection reagent solution 1 and 2, were mixed to give sufficient volume to cover the membrane (0.125 ml /cm^2 membrane). The washed blot was drained of excess buffer and placed in a fresh container. The detection reagent was added directly to the blot, on the surface carrying the protein, mixed with gently shaking and incubated at room temperature for 1 min. Excess detection reagent was drained off the blot by holding it vertically and touching the edge against tissue paper. The drained blot was then wrapped in plastic film, and placed in a film cassette protein side up. In the dark, a sheet of autoradiography film was placed on the top of the blot, the cassette closed, and exposed for between 15 and 60 seconds. The photographic film was then removed and developed immediately.

6.3.3 Results and discussion

The reactivity of anti-peptide antibodies against the whole protein was first analysed on ELISA, figure 6.7 A, B and C shows the results obtained. In spite of the fact that optimal conditions for ELISA were used to test the cross-reaction of the antipeptide antibodies with the proteins containing the peptide, no measurable response was observed. For

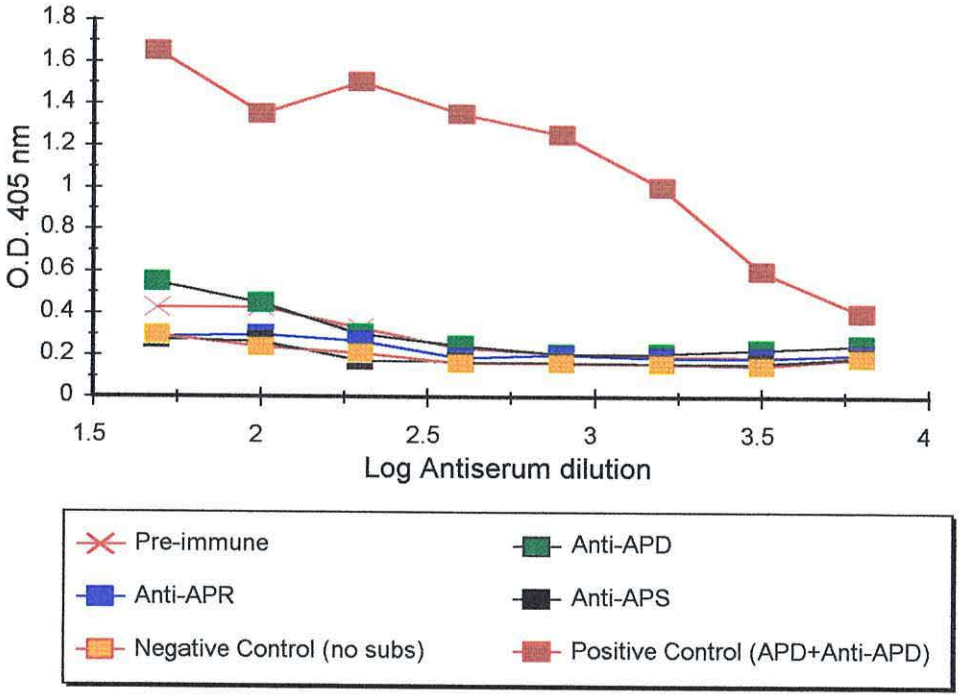


Figure 6.7 A:

Ability of anti-APD, anti-APR and anti-APS from rabbits to recognise wild type-OppA by using ELISA. Microtitre plate was coated with wild type-OppA (0.1μg/200μl) or APD (2μg/200μl). The antigen APD was included as positive control. All anti-serum tested were taken 3 weeks after booster.

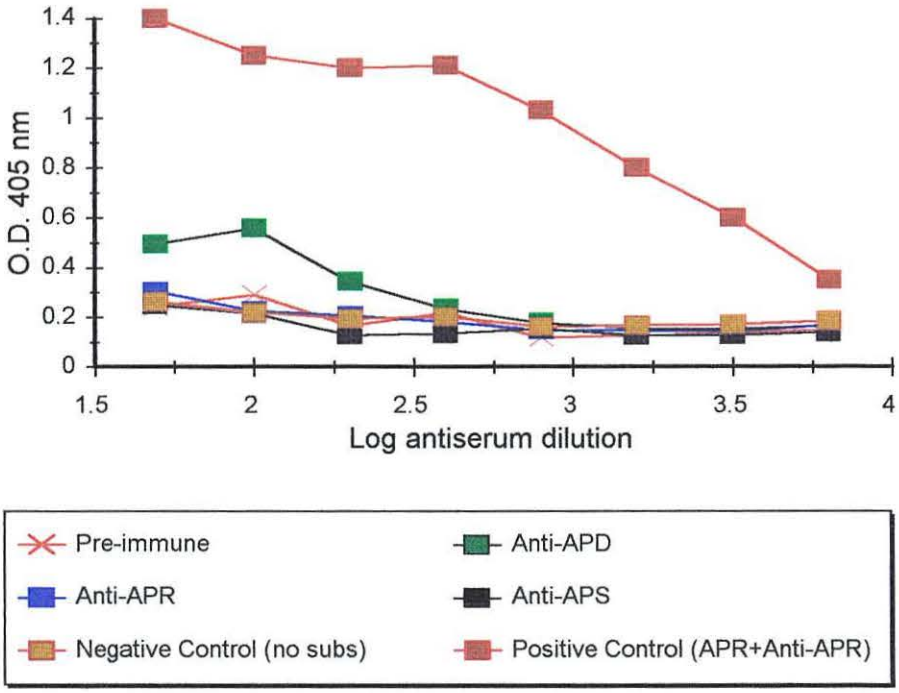


Figure 6.7 B:

Ability of anti-APD, anti-APR and anti-APS from rabbits to recognise Arg^{300}-OppA by using ELISA. Microtitre plate was coated with Arg^{300}-OppA (0.1µg/200µl) or APR (2µg/200µl). The antigen APR was included as positive control. All anti-serum tested were taken 3 weeks after booster.

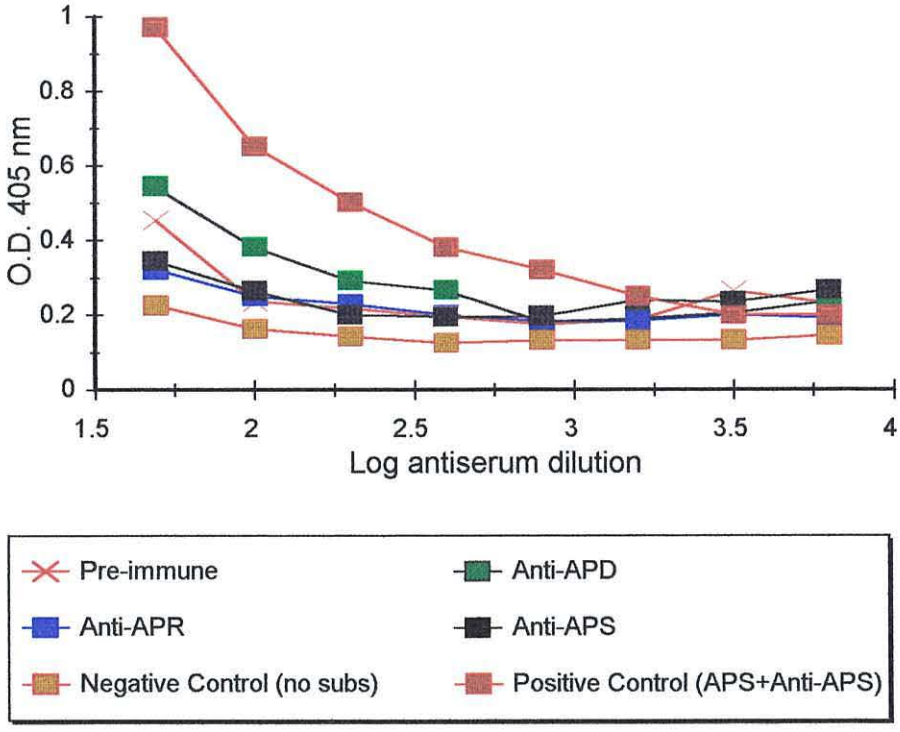


Figure 6.7 C:

Ability of anti-APD, anti-APR and anti-APS from rabbits to recognise Ser^{300}-OppA by using ELISA. Microtitre plate was coated with Ser^{300}-OppA (0.1µg/200µl) or APS (2µg/200µl). The antigen APS was included as positive control. All anti-serum tested were taken 3 weeks after booster.

example the antigen concentration used was 0.1μg of OppA, which had the highest absorbance when monoclonal antibody raised against OppA is used as first antibody. Also the antiserum dilution used produced a good response when APD, APR or APS were used for coating the plate (positive control). The lack of a positive response could be due to a failure of the antipeptide antibodies to recognise the whole protein, however other factors such as how the protein is adsorbed onto the plate could be involved. For example the antigenic peptide may be displayed differently or be less accessible on the plate when it is in the whole protein as compared to free or conjugated peptide as a coating.

The reactivity of these anti-peptide antibodies was then tested by using western blot. Both anti-APD and anti-APS antibodies were unable to recognise WT-OppA and Ser ^{300}-OppA, respectively, on western blotting. However, when anti-APR was tested against the purified Arg^{300}-OppA or the periplasmic protein fraction from *E. coli* PA0523 (containing [Arg^{300}-OppA]-plasmid), a thick band was observed at the OppA position (Fig. 6.8).

All rabbit's antisera contained a high titre of antibodies against the homologous peptide (Fig. 6.2), but only one peptide (APR) gave antiserum which reacted with the whole protein Arg^{300}-OppA mutant. The three peptides used as antigen, with the exception of one amino acid are homologous with reference to protein location, hydrophilicity and size, and would presumably have the same ability to produce an antiserum capable to react with the whole protein. However only APR showed this cross-reaction. A possible explanation could be due to the lack of a single, well-defined conformation in these peptides (APD and APS). In other words, these peptides were able to stimulate the production of antibodies, but only one elicited antibodies able to recognise the peptide in the conformation adopted in the whole protein. Consistent with this is the evidence that regions of high flexibility, for example the N- and C-termini of protein, will obviously cross-react better than regions of rigid structure (Crupton, 1986).

The anti-APD, anti-APR and anti-APS antibodies, were produced with the idea to be used in the screening of spontaneous compensative mutants produced in this study (section 9). However this was not possible, due to the cross-reaction observed among these antibodies

and the three peptides (APD, APR and APS) and the lack of reaction between both anti-APD and anti-APS rabbit's antisera and the whole protein. However the anti-APR antibody from rabbit could be a useful tool to study the interaction of liganded-OppA with the oligopeptide permease protein in membrane vesicles, to explain its mechanism of action and to investigate similar systems in other species such as *Streptococci* and *Staphylococci*.

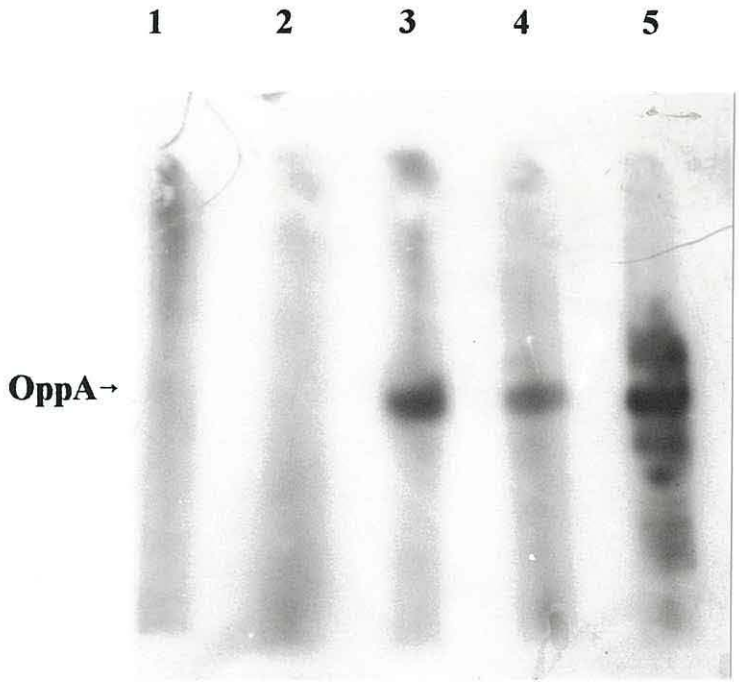


Figure 6.8

Western blot of 8% polyacrylamide-SDS gel showing the cross-reaction of anti-peptide antibodies against the whole protein containing the peptide. All anti-peptide antibodies are from rabbits and were diluted 1:25 in PBS-tween before use. Anti-OppA polyclonal antibody diluted 1:500 in PBS-Tween was added as control. Lane 1: 3µg of WT-OppA immunostained with anti-APD; lane 2: 3µg of Ser^{300}-OppA immunostained with anti-APS; lane 3: 3µg of Arg^{300}-OppA immunostained with anti-APR; lane 4: 15µl (30µg of total protein/µl) of periplasmic protein fraction from PA0523 (containing the Arg^{300}-OppA plasmid); lane 5: 1 µg of purified WT-OppA immunostained with anti-OppA polyclonal antibody.

Chapter VII

PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES

7.1 Introduction

Serum from animals immunised with an immunogenic substance, contains in addition to specific antibodies against this particular antigen, a mixed population of antibodies which usually creates a variety of problems in immunochemical techniques. Even when the antigen is purified the serum antibodies are heterogeneous because the multiple epitopes on the antigen induce the proliferation of a variety of B-cell clones.

The preparation of homologous antibodies with a defined specificity was achieved with the development of the technology for hybridoma production by Köhler and Milstein (1975). This technique consists of fusion of a normal B-cell (lymphocytes that mature in the bone marrow and are precursors of antibodies-secreting plasma cells) with a myeloma cell (a cancerous plasma cells), to generate a hybrid with growth properties of a myeloma cell but secreting the antibodies product of the B-cell. These hybrid cells, or hybridomas, can be maintained for an indefinite time and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by these hybridomas are known as

monoclonal antibodies.

The usefulness of monoclonal antibodies stems from three characteristics: their specificity for binding, their homogeneity, and their ability to be produced in unlimited quantities.

Hybridomas secreting monoclonal antibodies specific for OppA, have been prepared in our laboratory by Schuster (1995). To go further into the characterisation of these monoclonal antibodies, two cell lines, one secreting αDppA Mab and another secreting αOppA MAb, were thawed, expanded and recloned. The aim of this study was to characterise monoclonal antibodies against *E. Coli* OppA, to be used in the study of the oligopeptide permease.

7.2 Methods

7.2.1 Culture conditions

RPMI supplemented with 5 or 10% FCS (foetal calf serum) and 1x HT (13.6 mg/L hypoxanthine-3.88 mg/L thymidine), was used as growth medium; RPMI Glutamax II with 1 mM sodium pyruvate and 1x streptomycin, as propagation medium; RPMI without supplements, as wash medium; and 90% (v/v) FCS, 10%(v/v) DMSO, as freezing solution.

Cell cultures were grown in a GA2SN CO_2 incubator (LEEC Ltd., Nottingham, U.K.) With 5% (v/v) CO_2 at 100% humidity maintained by placing sterile water containing $CuSO_4$ at the bottom of the incubator at 36.5°C. Cell growth was monitored by simple observation under an inverse microscope (Wilovert, Will, Wetzlar, FRG). All media were pre-warmed to 37°C, in a water bath, before use.

7.2.2 Viability test and cell count

Before counting the viable cells, a volume of cell suspension was washed to remove FCS, which interferes with vital dyes, and resuspended in the same volume or diluted when required. The percentage of viable cells was determined by mixing a volume of cell suspension with a volume of 0.3 % (w/v) eosin in 0.9 % (w/v) NaCl and observed under the microscope. Vital dyes are excluded from living cells, but stain dead cells resulting in their dark red staining. When an exact cell count was needed, the number was determined by using a hemocytometer (improved Neubauer counting chamber).

7.2.3 Hybridoma Cloning

Hybridomas cells were recloned by limiting dilution. A sample of the cells to be cloned were resuspended in RPMI supplemented with 10% FCS and 1x HT and counted. An aliquot of cell suspension was removed and diluted in the same growth medium at 1 x 10^3 cell/ml. Using a multipipetter 100 µl of growing medium was added to each well of a 96-well plate. The first row of the plate was filled with 100 µl of cell suspension giving a final volume of 200 µl per well. One in two serial dilution was carried out across the plate, starting with a average of 50 cells per well until a average of 0.5 cell per well. Cells were mixed by gently pipetting up and down three times. Each well was topped up with more growth medium. Cells were fed at day seven and then each three days with two drops of growing medium. After two weeks the supernatant were assayed, by ELISA, for specific antibodies production, and the positive wells originated from a single clone were selected for propagation. Aliquots of these clones were also stored in liquid nitrogen as described in section 7.2.4.

7.2.4 Propagation of selected clones

Once the useful clones were identified, isolated and a sample stored, propagation was

performed. Cells were transferred from the culture in the 96-well plate to a 24-well plate containing 0.5 ml RPMI supplemented with 10% FCS and 1x HT medium. After the 24-well culture became dense, it was transferred into a 25 cm^2 tissue culture flask containing 5 ml growth medium. When the cell concentration reached saturation (5 x 10^5 to 10^6 cell per ml), subculturing were carried out by aseptically removing most of supernatant, resuspending the cells in the remain medium and splitting the cell suspension in two or more tissue culture flasks. The correct time for passaging the cells was determined by observing them under the microscope.

Feeding of cells was carried out by removing the old medium and then addition of fresh growth medium. These supernatants were screened for specific antibody production and stored at 4°C. For final collection of tissue culture supernatants, each individual culture was allowed to grow until the cell density was so great that cell death occurred. This allows the collection of higher-titre supernatant without affecting the quality of antibodies.

7.2.5 Storage of cells

7.2.5.1 Freezing cells for liquid nitrogen storage

Healthy cells, growing in exponential phase, were aseptically transferred to a sterile centrifuge tube and spun down at 400g for 5 min. The supernatant was removed and cells were resuspended in freezing solution (see section 7.2.1) to a final concentration of 5 x 10^6 to 5 x 10^7 cells/ml. Aliquots (0.5 ml) of cell suspension were transferred to a cryo-vial (Cryovial Nunc) and frozen by lowering the temperature uniformly by 1°C per min. to -30°C and then at -5°C per min. until -80°C, using a cell freezer R204 (Palmer products Ltd., Sumbury-on-Thames, U.K.). For long term storage, vials were transferred to a liquid nitrogen tank (-185°C).

7.2.5.2 Recovering cells from liquid nitrogen storage

The vial was removed from the liquid nitrogen storage and transferred to a 37°C water bath to thaw. In order to minimise the exposure time of the cells to DMSO, which is toxic to the cell at this temperature, cells were immediately washed just before the cells had completely thawed. The outside of the vial was sterilised with 70 % ethanol, and cell suspension was carefully transferred to a sterile centrifuge tube containing 10 ml of wash medium at room temperature, using a sterile Pasteur pipette. Cells were pelleted by centrifugation at 400g for 5 min. The supernatant was discarded and cells were resuspended in 10 ml growing medium (10 % FCS), seeded into a 24-well plate, inspected after settling and incubated at 36.5°C in a CO_2 incubator.

7.2.6 Purification of Monoclonal Antibodies

Two methods were tested to concentrate and purify antibodies contained in the hybridoma culture supernatant, lyophilization and precipitation by ammonium sulphate.

7.2.6.1 Lyophilization

A volume (100 ml) of sample was dialyzed overnight against 5 litres 10 mM NH_4HCO_3 at 4°C. This was lyophilized and then rehydrated by the addition of 3 ml of phosphate buffer saline (PBS), containing 0.01% of thimerosal and stored at -20°C.

7.2.6.2 Precipitation by ammonium sulphate

Four concentrations of ammonium sulphate were tested. The volume of saturated ammonium sulphate solution required to reach a final concentration of 40, 50, 55 and

60% was slowly added to the hybridoma culture supernatant, and left stirring overnight at 4°C. The mixture was then centrifuged at 16K rpm for 20 min at 4°C, and each pellet resuspended in 1 ml of PBS. Both pellet and supernatant were dialyzed overnight against PBS at 4°C, then 0.01% thimerosal was added and the samples stored at -20°C.

7.2.7. Determination of monoclonal titre

The titre of monoclonal antibodies (MAbs) were tested by ELISA. Optimal conditions such as antigen concentration for coating plate, blocking solution, antisera dilution, and anti-immunoglobulin enzyme conjugate dilution used in this assay were those predetermined by Schuster (1995), slightly modified. Purified OppA antigen diluted in coating buffer (50 mM $NaCO_3/NaHCO_3$ pH 9.6) was absorbed onto multiwell plates by incubating 100µl of 1µg/ml per well overnight at 4°C. The unbound sites were blocked by incubation of 100µl of 5 mg BSA/ml PBS/0.05% tween 20 per well for one hour at 37°C. The MAb to be tested was added as a two fold serial dilution across the plate, and incubated for one hour at 37°C. The antigen-antibody complex was detected by addition of second antibody (anti-Mouse IgG-Peroxidase conjugate) diluted 2000-fold and left for one hour at 37°C. After each incubation the solutions were aspirated and the wells washed three times with PBS/0.05% tween 20. Freshly prepared substrate (ABTS/H_2O_2/citric acid pH4) was added and incubated at room temperature until the colour developed sufficiently. The absorbance was measured in a microtitre plate spectrophotometer at 405 nm.

The protein concentration in each sample was determined by micro-Biuret assay as described in section 4.9.1, and the antibody activity was expressed as OD/total volume, and the specific activity as OD/mg protein.

7.2.8 Immunodots:

Optimal conditions were determined empirically by testing different concentrations of purified antigens and different dilutions of antibodies.

One μg of antigen was adsorbed directly to a nitrocellulose sheet and left for one hour to allow the protein to bind to the membrane. The nonspecific binding sites were blocked by placing the membrane in a solution of 3% nonfat milk in TBS/tween for one hour with shaking at room temperature. The antigens were incubated with the first antibody diluted 500-fold. The antigen-antibody complex was detected by adding the second antibody (anti-mouse IgG peroxidase conjugate) diluted 2000-fold. The bands were visualized by addition of freshly prepared substrate. This was prepared by mixing 5mg of 4-chloro-1-naphthol dissolved in 1 ml methanol, then mixed with 5 ml of pre-warm TBS, at 37°C, containing 2.5μl of H_2O_2 was added to the mixture.

7.2.9 Gel electrophoresis and immunoblots

SDS polyacrylamide gel electrophoresis was carried out on 12.5% gel (acrylamide/bisacrylamide ratio 37:1), as described in section 4.10.1, and western blotting was carried out as described in section 4.11. Proteins were detected immunologically as described in section 4.12, and the electroblot containing the molecular weight markers was silver stained as described in section 4.13.

7.3 Results and Disscusion

7.3.1 Concentration and Purification of Monoclonal Antibodies

Monoclonal antibodies produced *in vitro* are highly diluted and contain contaminant

proteins from foetal calf serum. Two methods were tested for concentration and purification of monoclonal antibodies, lyophilization and precipitation by ammonium sulphate. The supernatant collected from the hybridoma clone VI/22/2 (Schuster, 1995) was selected to test both methods.

Results showed that high monoclonal antibodies activity is recovered after lyophilization, and the ammonium sulphate method allowed a partial purification leading to a higher specific activity (Table 7.1).

Most of the immunoglobulins precipitate at 50% of saturated ammonium sulphate, but not all of them have the same behaviour, and for this reason experiments using different concentrations of saturated ammonium sulphate were carried out. A saturation of 55% proved to be the best concentration to obtain a higher specific activity without significant lost of monoclonal antibodies (Table 7.2). To support these results the fractions obtained during the purification process with 55% ammonium sulphate, were analysed by SDS-PAGE (Fig. 7.1). The lower concentration of albumin, which is the most abundant protein in the serum, was observed in the pellet fraction (Fig. 7.1, lane 4), also both heavy and light chains from the antibodies were detected in the same fraction. No antibodies were detected in the crude preparation due to their low concentration (Fig. 7.1, lane 2).

7.3.2 Cloning

Two hybridoma cell lines (II/15/2) secreting anti-DppA and (VI/22/2) secreting anti-OppA isolated by Schuster (1995), were subjected to further cloning. Four cell clones secreting anti-DppA were obtained from (II/15/2), and two cell clones secreting anti-OppA MAb were obtained from VI/22/2 cell line. These clones were named: DppA F5, DppA D9, DppA F3, DppA F4, OppA E3 and OppA C2, respectively (Table 7.3); and the antibodies secreted by them: αDppA F5, αDppA D9, αDppA F3, αDppA F4, αOppA E3 and αOppA C2, respectively.

Table 7.1

Comparison of Two methods for the Concentration and Purification of MAb secreted by VI/22/2 hybridoma cell line

Samples	OD/ml	% Activity recovered	Protein mg/ml	% Protein Recovered	OD/mg protein
Initial	9920/ml		2.56mg/ml		3887
50% $(NH_4)_2SO_4$ Pellet	67916 /ml	65%	6.29mg/ml	42%	10795
50% $(NH_4)_2SO_4$ Supernatant	1020/ml	20%	0.88mg/ml	68%	1176
Initial	10000/ml		2.25mg/ml		4444
Lyophilized	40000/ml	83%	60mg/ml	80%	6667

The results are the average from two ELISA assay

Table 7.2

Comparison of different percentages of Saturation of Ammonium Sulphate to Concentrate and Purify Monoclonal Antibody Secreted by VI/22/2 hybridoma cell line by precipitation

Samples	% Activity recovered	OD/ml	Protein mg/ml	% Protein recovered	OD/mg protein
Initial		10000/ ml	3.5mg/ml		3048
45% $(NH_4)_2SO_4$ Pellet	8.5%	4480/ml	4.75mg/ml	18%	943
45% $(NH_4)_2SO_4$ supernatant	70%	24666/ml	0.85mg/ml	53%	2638
50% $(NH_4)_2SO_4$ Pellet	43.2%	25444/ml	6mg/ml	21%	4241
50% $(NH_4)_2SO_4$ supernatant	20%	560/ml	0.9mg/ml	65%	623
55% $(NH_4)_2SO_4$ Pellet	99.8%	105000/ml	5.76mg/ml	23%	8677
55% $(NH_4)_2SO_4$ supernatant	6.3%	156/ml	0.77mg/ml	63%	201
60% $(NH_4)_2SO_4$ Pellet	90.5	50526/ml	7.5mg/ml	27%	6739
60% $(NH_4)_2SO_4$ supernatant	1.08%	25/ml	0.5mg/ml	44%	50

Table 7.3

Concentration and Partial Purification of Monoclonal Antibodies Secreted by Several Hybridoma Cell Lines

Hybridoma [1]	Hybridoma [2]	Specificity of MAb secreting	Activity (OD/ml)	MAb Dilution to give half-maximal binding
II/15/2		Anti-DppA	15600	1:12800
	DppAF5	"	18800	1:18000
	DppAD9	"	17060	1:15100
	DppAF3	"	60800	1:51200
	DppAF4	"	51200	1:30400
VI/22/2		Anti-OppA	86600	1:43000
	OppAE3	"	102400	1:51200
	OppAC2	"	172000	1:86000

(1) Hybridoma cell lines isolated by Schuster (1995). (2) Hybidoma cell lines isolated in this study

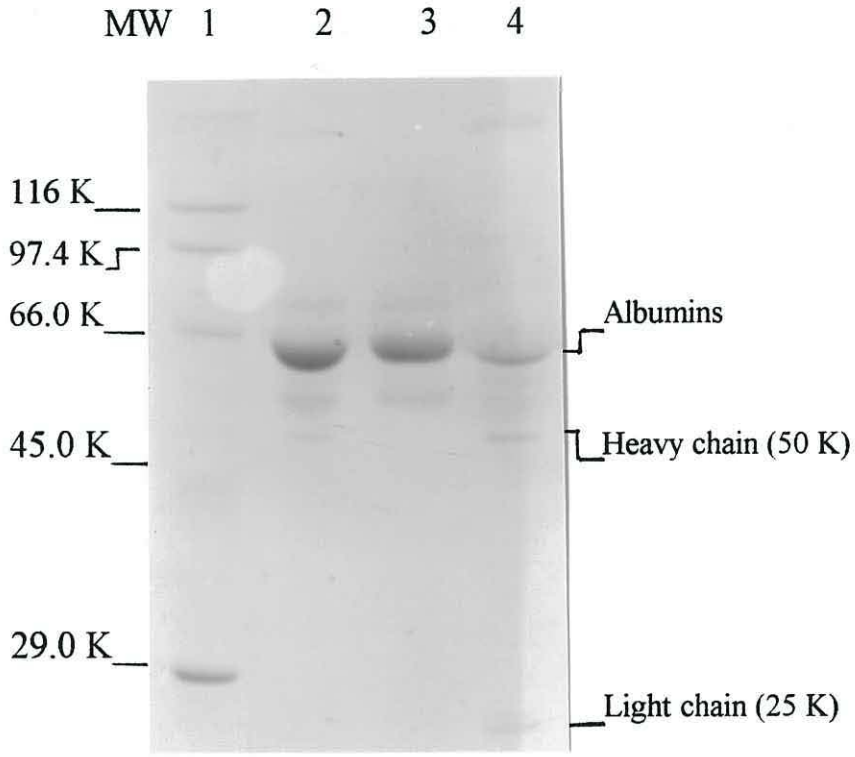


Figure 7.1:

Coomassie blue stained gel showing the different fractions obtained during MAb concentration and purification by 55% of ammonium sulphate precipitation. Lane 1: molecular weight marker. Lane 2: crude preparation of VI/22/2 MAb (5µg of proteins). Lane 3: 55% ammonium sulphate supernatant (5µg of proteins). Lane 4: 55% ammonium sulphate pellet (5µg of proteins).

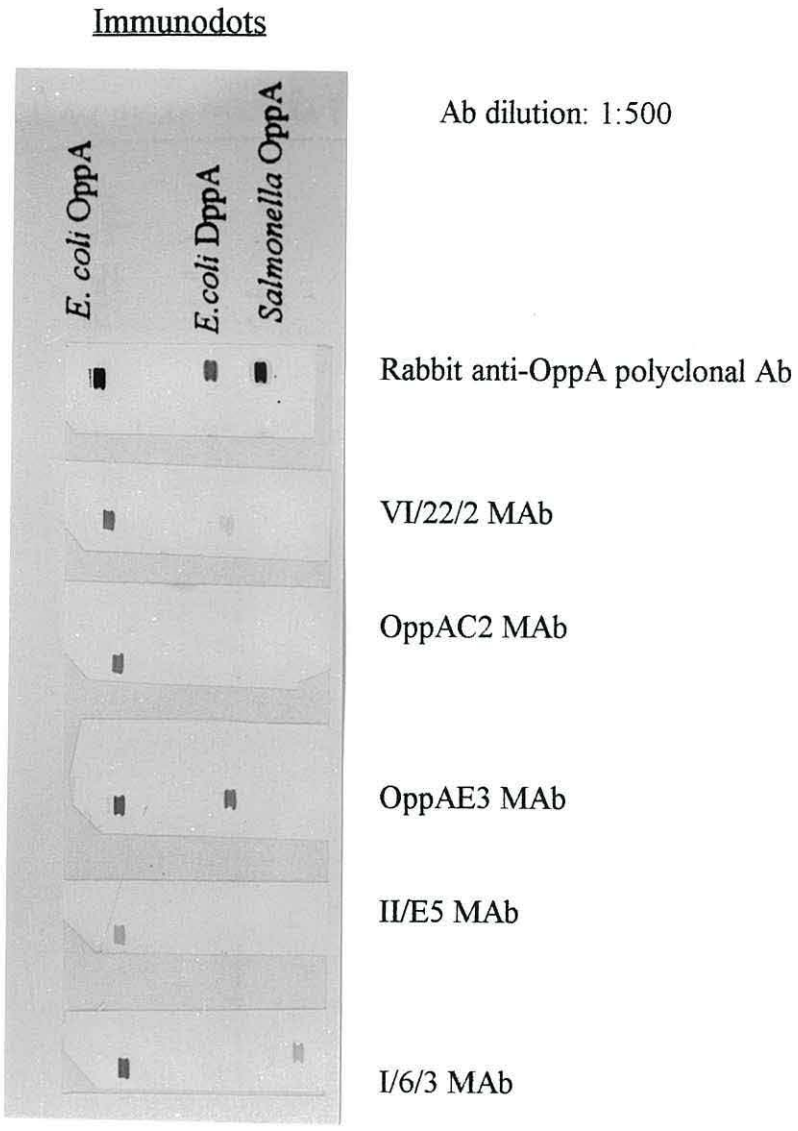


Figure 7.2:

Immunodots showing the immunoreaction of *E. coli* OppA, *E. coli* DppA and *S. tiphymurium* OppA with several anti-OppA raised against *E. coli* OppA. Antisera were used at 1:500 dilution.

After the concentration and partial purification by precipitation with 55% ammonium sulphate the titre of the monoclonal antibodies was determinated in the cell culture supernatant from all cell lines, and the antibody activity expressed as the dilution required to give half-maximal binding detected by ELISA .

7.3.3 Characterisation of Monoclonal antibodies

In order to test the specificity of several monoclonal antibodies raised against OppA from *E. coli*, the ability of these antibodies to recognise other peptide-binding proteins was analysed by immunodot.

Purified OppA and DppA from *E. coli*, and purified OppA from *S. typhimurium*, were used as antigens. Polyclonal antibodies which show cross-reaction with all the antigens tested (Schuster, 1995) were used as positive control, and II/E5 MAb raised against DppA was used as negative control. Also an anti-OppA I/6/3 MAb (Schuster, 1995) was included in this study.

The anti-OppA I/6/3 MAb showed cross-reaction with OppA from *S. typhimurium*, and both VI/22/2 MAb and anti-OppAE3 MAb showed cross-reaction with DppA from *E. coli*. No-cross-reaction was detected either with anti-OppAC2 MAb or anti-DppA II/E5 MAb (Fig. 7.2). Both anti-OppAC2 and αOppAE3 hybridoma cells were isolated from VI/22/2 hybridoma cell line, however different specificities were observed when both monoclonal antibodies were tested using purified DppA from *E. coli*. These results indicated the presence of more than one clone secreting anti-OppA antibodies against different epitopes in the OppA, in the original cell line.

On the other hand, the cross-reaction observed in the monoclonal antibodies above indicated homology between the periplasmic binding proteins studied. The amino acid sequence of OppA from *E. coli* shares 93.4% and 48.7% of similarity with OppA from *S. typhimurium* and DppA from *E. coli*, respectively. These results testify to an immunological relation among the three proteins studied.

Surprisingly, and in contrast to this study, results obtained with anti-OppA polyclonal antibodies raised against OppA from *S. typhimurium* did not show immunological relation between OppA and other periplasmic binding proteins (including DppA) of the same species (Hiles and Higgins, 1986).

These monoclonal antibodies were used to characterise *E. coli* Opp$^-$ mutants (section 8.2) and to detect the expression of OppA in cured compensative mutants (section 9.6.4.2.3).

Chapter VIII

A POINT MUTATION IN OppA THAT ABOLISHES THE OLIGOPEPTIDE PERMEASE FUNCTION IN *E. COLI*

8.1 Introduction

Photoaffinity reagents have been used to probe many diverse biological systems (Bayley and Knowles, 1977). A dipeptide photoaffinity label, alanyl-4-azido-2-nitro-phenylalanine (Ala-Phe[N_3NO_2]) synthesised by Hardy and Payne (1991) as ^{14}C Ala-Phe[N_3NO_2], has been used as an initial attempt to identify residue(s) that may be involved at or near the substrate binding site of OppA (Smith, 1992; Marshall, 1994). The peptide photoaffinity label in which the aromatic ring of phenylalanine residue has been modified with a photoreactive moiety, can be recognised by the receptor as a reversible inhibitor. This label Ala-Phe[N_3NO_2] upon activation by UV light, becomes irreversibly bound to the protein. Cleavage of the photoaffinity labelled-protein with proteases yields labelled peptide fragments suitable for sequencing. Marshall (1994), employing this technique, identified a sequence at residues 300-305, Asp-Ile-Ile-Val-Asn-Lys, as a putative binding site in *E. coli* OppA.

This assumption was supported by the fact that this sequence was well conserved in both *E. coli* and *S. typhimurium* OppA and *E. coli* DppA; the presence of an acidic residue (Asp300), that should satisfy the requirement for a protonated N-terminal amino group of a peptide for it to be bound effectively by both OppA and DppA (Payne, 1971, 1974; Guyer *et al.*, 1986); and the conserved Ile-Ile region that might be involved in hydrophobic interaction, as reported by Morley *et al.* (1983).

The aim of this study was the production and the characterisation of a mutant OppA containing an alteration in the residue Asp300, that either removed the negative charge (Asp^{300}→Ser) or changed the negative charge for a positive charge (Asp^{300}→Arg).

8.2 Characterisation of *E. coli* Opp⁻ mutants

8.2.1 Introduction:

In the search of a bacterial strain suitable to express cloned *oppA*, previous workers isolated a group of oligopeptide transport-deficient (Opp^-) mutants from *E. coli* M2034. These Opp^- mutants were selected with triornithine (Orn_3), a toxic peptide inhibitor of protein synthesis (Gilvarg and Levin, 1972) which is transported specifically by oligopeptide permease (Barak and Gilvarg, 1974), and designated TOR mutant.

The genetic defect in these mutants might be in any of the different genes that are essential for the normal activity of the system, or near the *opp* locus. Figure 9.1 shows the linkage map of *E. coli* K12, the M2034 parent strain (Berlyn *et al*, 1996). In this respect a first screening to detect mutants with a defect in the *oppA* gene was carried out in this laboratory. TOR mutants were analysed by SDS gel, and those mutants that apparently failed to synthesise the binding protein, were also characterised concerning OppA expression by using rabbit anti-OppA antiserum (Schuster, 1995) and with respect to the ability of cloned *oppA* to complement for oligopeptide permease function (Marshall, 1994). Based on the results obtained in these studies, the Opp^- mutant designated PA0309, was selected as host for cloned *oppA*.

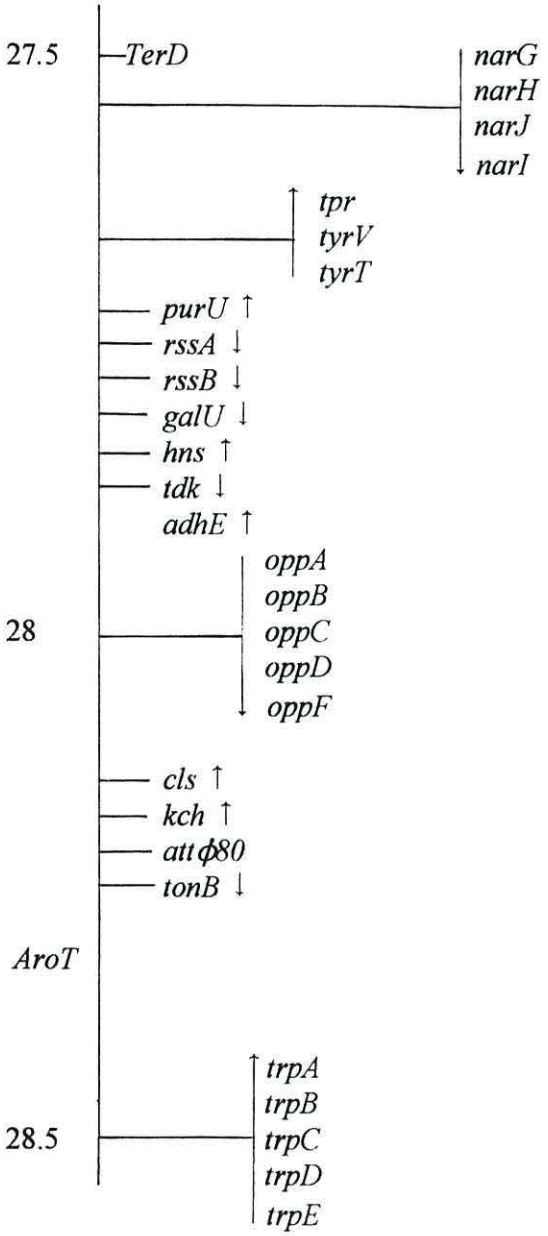


Figure 9.1:

Linear drawing of circular linkage map of E. coli K-12 between 27.5 and 28.5 minutes. Arrows shows direction of transcription. *TerD*: DNA replication fork inhibition; *nar*:nitrate reductase; *tpr*: protamine like protein; *tyrV*: tyrosine; *tyrT*: tyrosine; *purU*: purine; *rssA*: regulator of σ^s; *rssB*:regulator of σ^s; *galU*: glucose-1-phosphate uridyltransferase; *hns*: histone-like protein (HN-S); *tdk*: thymidine kinase; *adhE*: alcohol/acetaldheyde deshydrogenase; *opp*: oligopeptide permease operon ;*cls*: cardiolipin synthetase; *kch*: K^+ channel; *attϕ80*: integration site for phago ϕ80; *tonB*: uptake of chelated iron and cyanobalinin, sensitivity to phago T1, ϕ80 and colicins; *AroT*: transport of aromatic amino acids, alanine and gycine; *trp*: tryptophan synthesis.(Taken from Berlyn *et al.* 1996).

8.2.2 Detection of OppA in Periplasmic Protein Fraction of *E coli* Strain PA0309

8.2.2.1 Introduction

Strain PA0309, which was complementable with pPI5.1 (plasmid containing wild type OppA) and apparently failed to synthesise OppA, was chosen as host to check the expression of various mutated OppA. During these studies, problems arose in the use of PA0309 that led to the idea that perhaps recombination might be occurring between chromosomal and plasmid *opp* genes (Marshall, 1994).

In order to investigate the problems associated with the use of PA0309 as a host for complementation and transformation, further studies were carried out.

8.2.2.2 Methods

PA0309 stored in glycerol at -70°C, was grown in 5 ml LB medium, overnight with shaking at 37°C, and streaked onto minimal medium agar plate, supplemented as required.

Periplasmic protein fraction was obtained by cold osmotic shock (small scale preparation) as described in section 4.7.1. Proteins were separated by SDS-PAGE (see section 4.10.1) and coomassie blue or silver stained as described in sections 4.14.1 and 4.14.2, respectively.

Western blot was carried out as described in section 4.11, and the immunodetection was carried out as described in section 4.12 by using anti-OppA monoclonal antibody as first antibody.

8.2.2.3 Results and Discussion

The periplasmic proteins fraction from PA0309, was analysed by SDS-PAGE. The periplasmic protein fraction from M2034 (wild type) was included as a control, and both purified OppA and DppA as markers. Analysis of the Coomassie stained periplasmic proteins from PA0309 showed two thin bands at the position of OppA (Fig. 8.2, lanes 6 and 8), although other proteins might also migrate to this position.

In order to test better the OppA expression in PA0309, immunoblotting analysis was performed, using as controls periplasmic protein fraction from M2034 (wild type) and PA0183 (*oppABCDF* $^-$), respectively and PA0309 vesicle. Two different anti-OppA monoclonal antibodies were used as immunostains. The C2 MAb which specifically reacts with *E.coli* OppA and the E3 MAb which cross-reacts with *E. coli* DppA (see section 7). When E3 MAb was used as a probe, two low intensity bands were observed in the PA0309 periplasmic protein fraction, one of them at the OppA position and another lower-molecular weight at DppA position (Fig. 8.3 lane 4). These results suggest that strain PA0309 is able to express a small amount of OppA, possibly due to a mutation in the promoter region, and that it is not a deletion mutation in the *oppA* gene.

8.2.3 Test of Periplasmic Proteins of Several *E. coli* Opp$^-$ mutants

8.2.3.1 Introduction

Based on the above results, it became necessary to identify an alternative strain to PA0309 that could be used as a successful host for transformation and complementation studies. From amongst many Opp$^-$ strains some had been tentatively characterised as *oppA*$^-$; these were now re-examined.

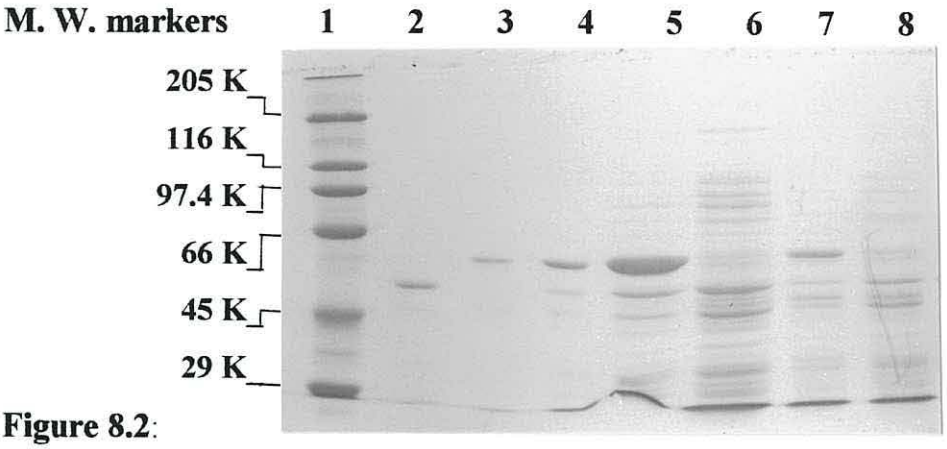


Figure 8.2:

Coomassie blue-stained gel showing the periplasmic proteins fraction (PPF) from *E. coli* PA0309. Lane 1: M.W. markers, lane 2: 0.5 µg of purified DppA, lane 3: 0.5 µg of purified OppA, lanes 4, 5 and 7: 5, 25 and 10 µg of PPF from M2034 (W.T), respectively; lanes 6 and 8: 25 and 10 µg of PPF from PA0309, respectively.

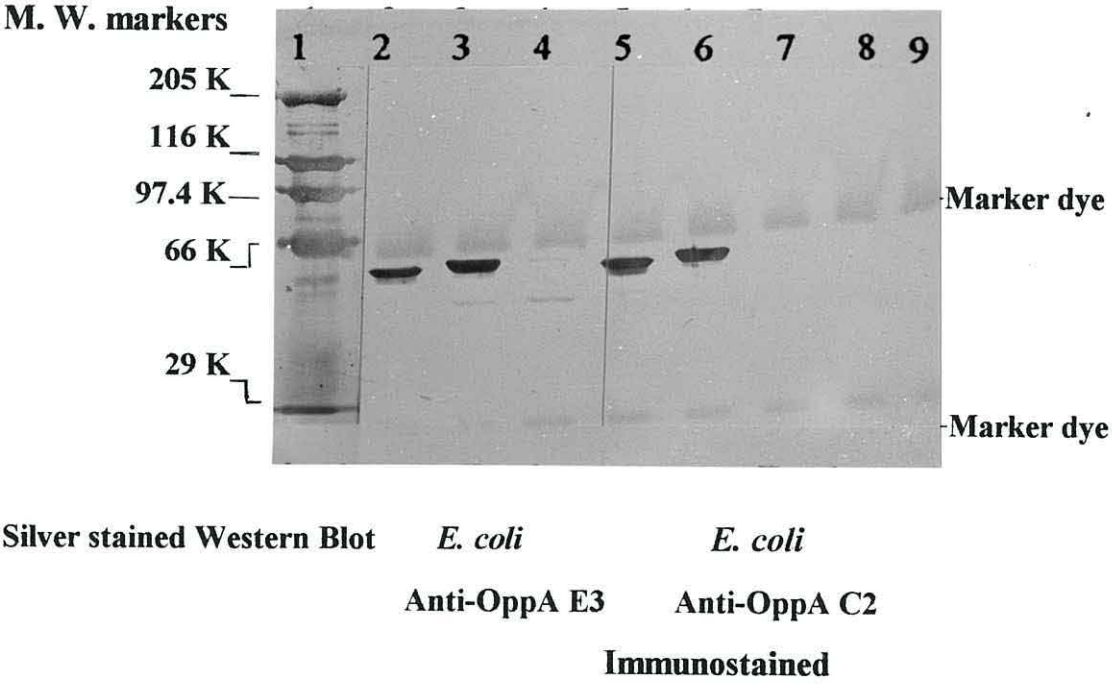


Figure 8.3:

Western blot of 8% polyacrylamide-SDS gel showing immunoreaction of OppA from several *E. coli* strains. Lane 1: molecular weight markers; lanes 2 and 5: 0.5 µg of purified OppA; lanes 3 and 6: 5 µg of periplasmic protein fraction (PPF) from M2034; lanes 4 and 7: 5 µg of PPF from PA0309; lane 8: 5 µg PA0309 vesicle; lane 9: 5 µg of PPF from PA0183.

8.2.3.2 Methods

TOR mutants stored in glycerol at -70°C were grown in 5 ml LB medium, overnight with shaking at 37°C, and streaked onto minimal medium agar plate, supplemented as required.

Periplasmic protein fractions were obtained by cold osmotic shock (small scale preparation) as described in section 4.7.1. Proteins were separated by SDS-PAGE (see section 4.10.1) and silver stained as described in sections 4.14.2.

Western blot was done as described in section 4.11, and the immunodetection was carried out as described in section 4.12 by using anti-OppA E3 monoclonal antibody (see section 7 and 8) as first antibody.

8.2.3.3 Results and Discussion

Periplasmic proteins of the following Opp$^-$ mutants PA0467, PA0470, PA0472, PA0479, PA0480, PA0481, PA0483, and PA0484 were fractionated in SDS-PAGE. Periplasmic protein fraction of M2034 (wild type) were included as control, and both purified *E coli* OppA and DppA as markers. Analysis of the silver stained gel showed similarity between the bands observed in PA0309 and the bands observed in all strains tested in this experiment (Fig. 8.4 A and B, respectively). However when these strains were analysed by immunoblotting using the E3 anti-OppA monoclonal antibody as first antibody, no bands were observed at the position of OppA, while a low molecular weight at the DppA position cross-reacted with E3 anti-OppA monoclonal antibody in all Opp$^-$ mutants studied (Fig. 8.5).

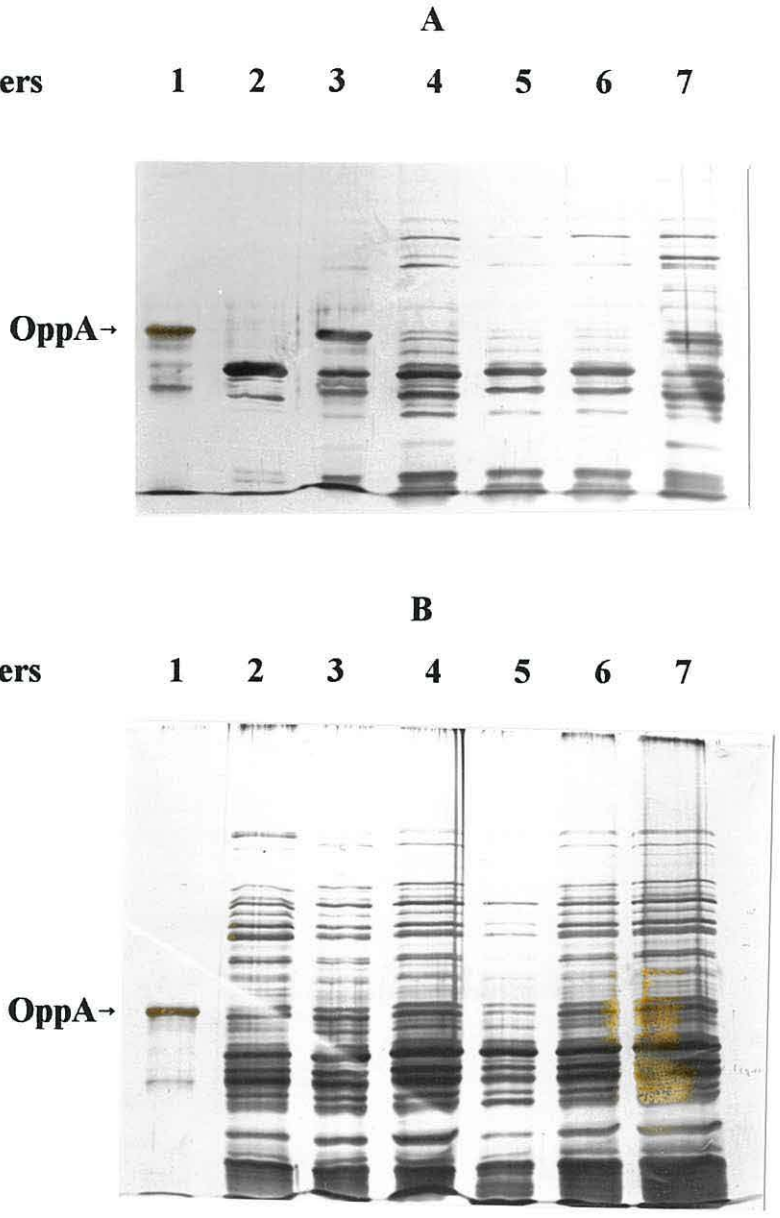


Figure 8.4:

Silver stained-gel showing the periplasmic protein fraction (PPF) from several *E. coli* Opp⁻ mutants.

Gel A) Lane 1: 0.5 µg of purified OppA; lane 2: 0.5 µg of purified DppA; lane 3: 5 µg of PPF from M2034 (WT); lane 4-7: 5 µg of PPF from PA0467, PA0470, PA0472 and PA0479 Opp⁻ mutants, respectively.

Gel B) Lane 1: 0.5 µg of purified OppA; lane 2, 3, 5 and 6: 5 µg of PPF from PA0480, PA0481, PA0483 and PA0484; lane 4 and 7: 10 µg of PPF from PA0481 and PA0484.

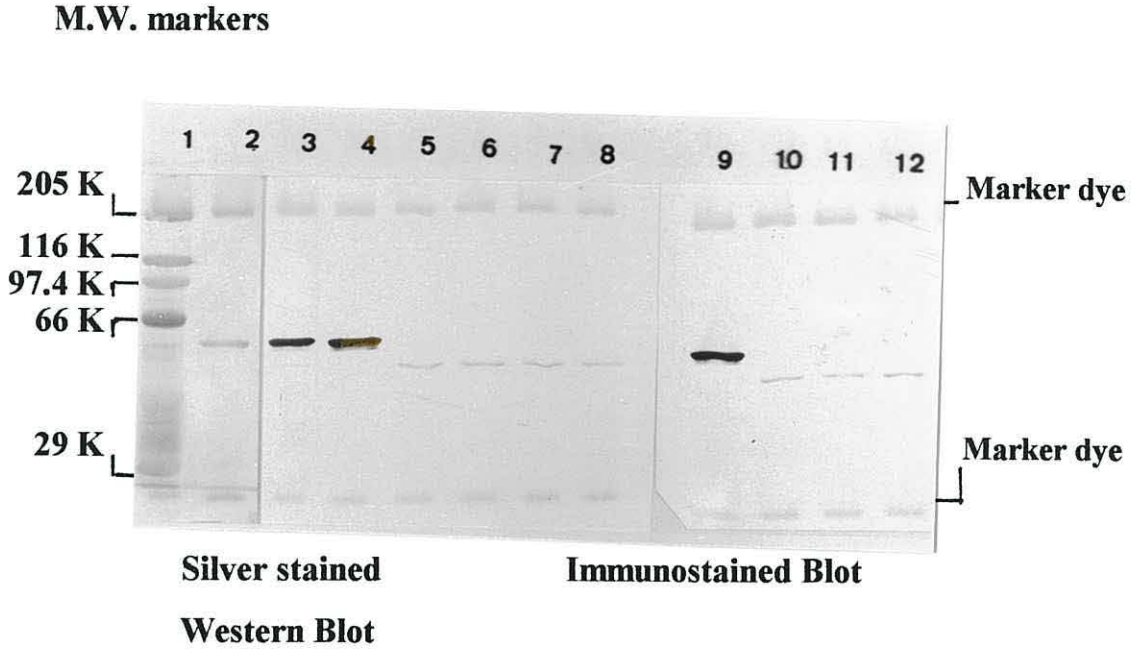


Figure 8.5

Immunoblot of 8% polyacrylamide gel showing immunoreaction of periplasmic protein fraction (PPF) from several *E. coli* Opp⁻ mutants, using anti-OppA monoclonal antibody. Lane 1: silver-stained western blot of molecular weight markers, lane 2: silver-stained western blot of OppA, lane 3: 5µg of PPF from M2034 (wild type), lanes 4 and 9: 0.5 µg purified OppA, lanes 5-8 and 10-12: 5 µg of PPF from PA0481, PA0483, PA0484, PA0467, PA0470 and PA0472, respectively.

8.3 Characterisation of PA0521

8.3.1 Introduction

Based on the results obtained in section 8.2.3.3, the strain PA0467 was selected for complementation and transformation studies. PA0467 was complemented in this laboratory with pPI5.1, which contains the 7.81 kb DNA including wild type *OppA* (Fig. 8.6) to give the strain PA0521. To establish the phenotype of PA0521, the expression of cloned *oppA* was analysed on SDS-PAGE, and the biological function of the oligopeptide permease was established by testing the sensitivity to toxic peptides.

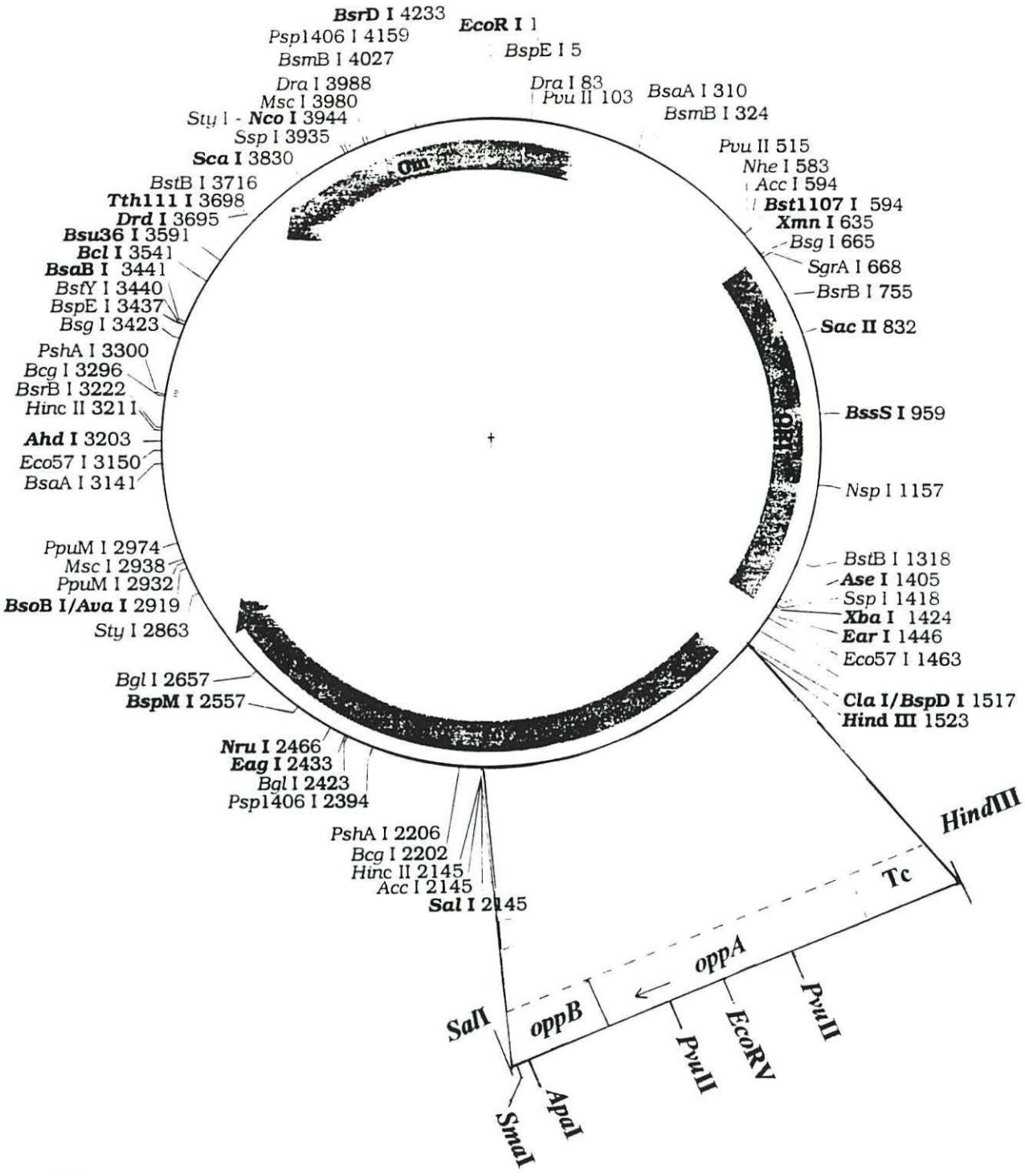


Figure 8.6

Circular map of pACY184 (Chang and Cohen, 1978; Rose, 1988) showing the 3.8 kb insertion containing the *oppA* gene, to give pPI5.1 (as described by Kashiwagi et al., 1990). The map shows the location of sites for enzyme that cleave the molecule once or twice, unique site are shown in **bold** type. The relative positions of antibiotic resistance gene and the origin of replication, are represented as **Cm** (chloramphenicol), **Tc** (tetracycline) and **ORI** origin), respectively. Restriction map of the insert are also shown. The direction of transcription is indicated with arrows.

8.3.2 Methods

Bacteria were grown as described in section 4.2

Periplasmic protein fraction were obtained by cold osmotic shock (small scale preparation) as described in section 4.7.1. Proteins were separated by SDS-PAGE (see section 4.10.1) and coomassie blue stained as described in sections 4.14.1.

Pour plate assays were carried out as described in section 4.6.1

8.3.3 Results and Discussion

On SDS-PAGE gel the presence of a tight band (Fig. 8.7) migrating at the same position as OppA expressed by M2034 (wild type strain), showing the ability of PA0521 to reconstitute the expression of OppA.

To determine the biological function of the oligopeptide permeases, with cloned *OppA*, the ability of two toxic peptides to inhibit the growth of PA0521, were tested. Ala_4P, which is an inhibitor of D-alanine racemase, and Orn_3, which is an inhibitor of protein synthesis, and specifically are transported by the oligopeptide permease in *E.coli* (Allen *et al.*, 1978; Payne and Gilvarg, 1968) were used as antibiotics (Fig. 8.8). The ability of a toxic peptide to inhibit bacterial growth was quantified by determining the diameter of the inhibition zone surrounding a disk containing the toxic peptide. PA0521 was sensitive to both toxic peptides tested, being 100 nmoles of Orn_3 and 13 nmoles of Ala_4P the amount of toxic peptides to give 25 mm zones of inhibition (Fig. 8.9 A and B respectively). Similar results were obtained with M2034 (wild type strain). PA0467 (*oppA*$^-$) was completely resistant to Ala_4P and Orn_3 (Fig. 8.8 C).

These results indicate that PA0467(*oppA*$^-$) regains the wild type phenotype, by complementation with cloned OppA, showing full restoration of oligopeptide permease function. On the other hand, these results confirm that PA0467 that has been shown to

be defective in OppA production, is a suitable host for transformation with plasmid containing mutated OppA.

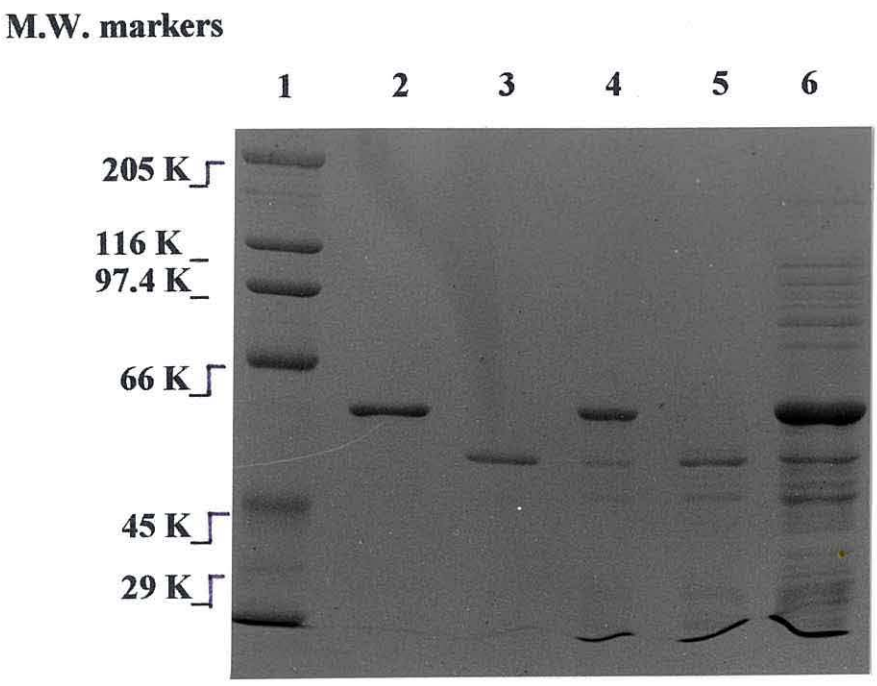


Figure 8.7

Coomassie blue stained gel showing the periplasmic protein fraction (PPF) from PA0521 (PA0467 *oppA*⁻ strain, transformed with pPI5.1, that contain the wild type OppA). Lane 1: molecular weight markers, lane 2: 0.5µg OppA, lane 3: 0.5µg DppA, lane 4: 5µg of PPF from M2034 (wild type strain), lane 5: 5µg of PPF from PA0467 (*oppA*⁻), lane 6: 25µg of PPF from PA0521.

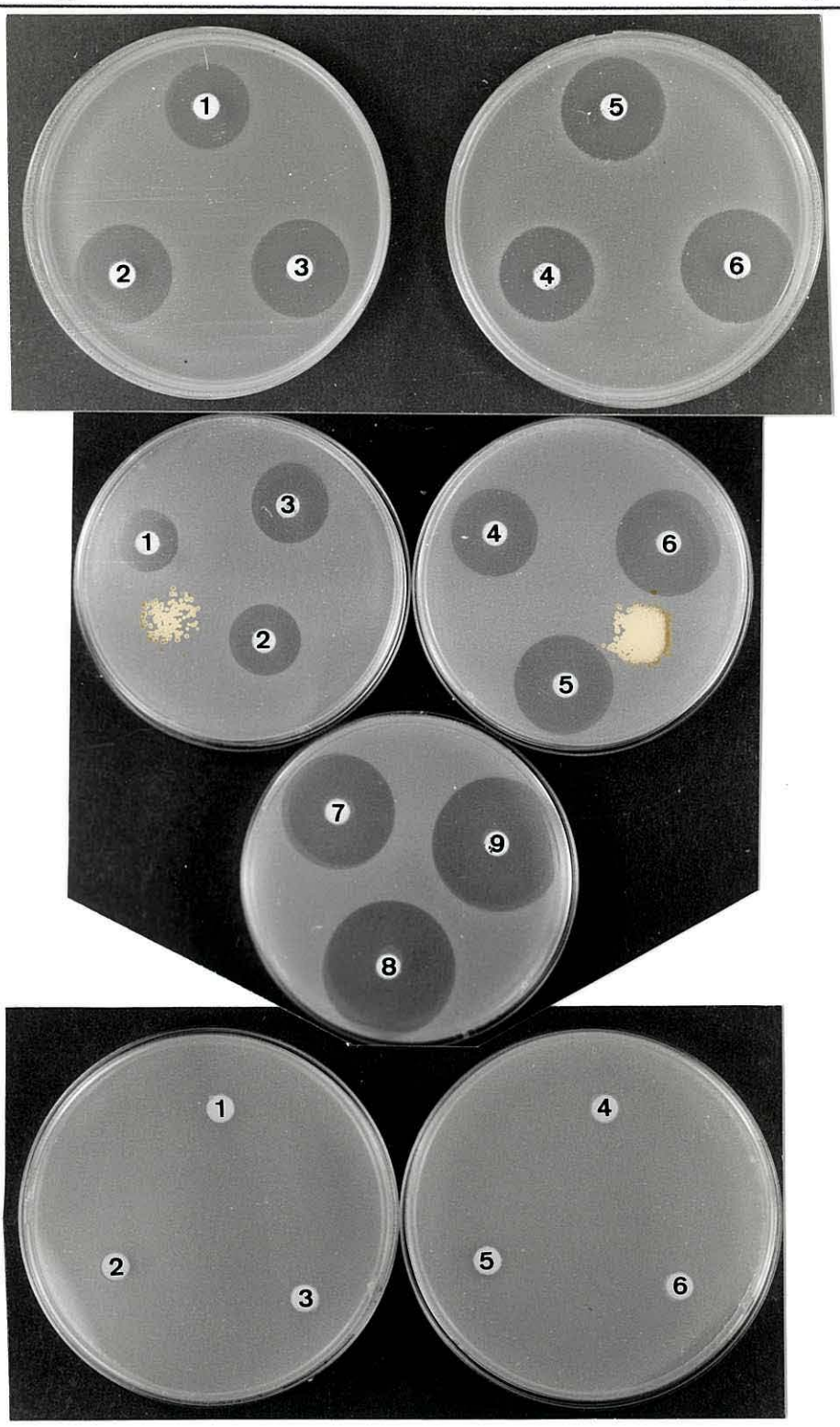


Figure 8.8:

Sensitivity of *E. coli* PA0521 (containing the wild type plasmid) and PA0467 (*oppA*$^-$) to toxic peptide. **A)** PA0521, 1-6 represent 3, 6, 12, 25, 50 and 100 nmoles of Ala_4 P, respectively. **B)** PA0521, 1-9 represent 8, 16, 30, 60, 125, 250, 500, 1000 and 2000 nmoles of Orn_3, respectively. **C)** PA0467 1-3 represent 25, 50 and 100 nmoles of Ala_4 P, respectively; 4-6 represent 500, 1000 and 2000 nmoles of Orn_3, respectively.

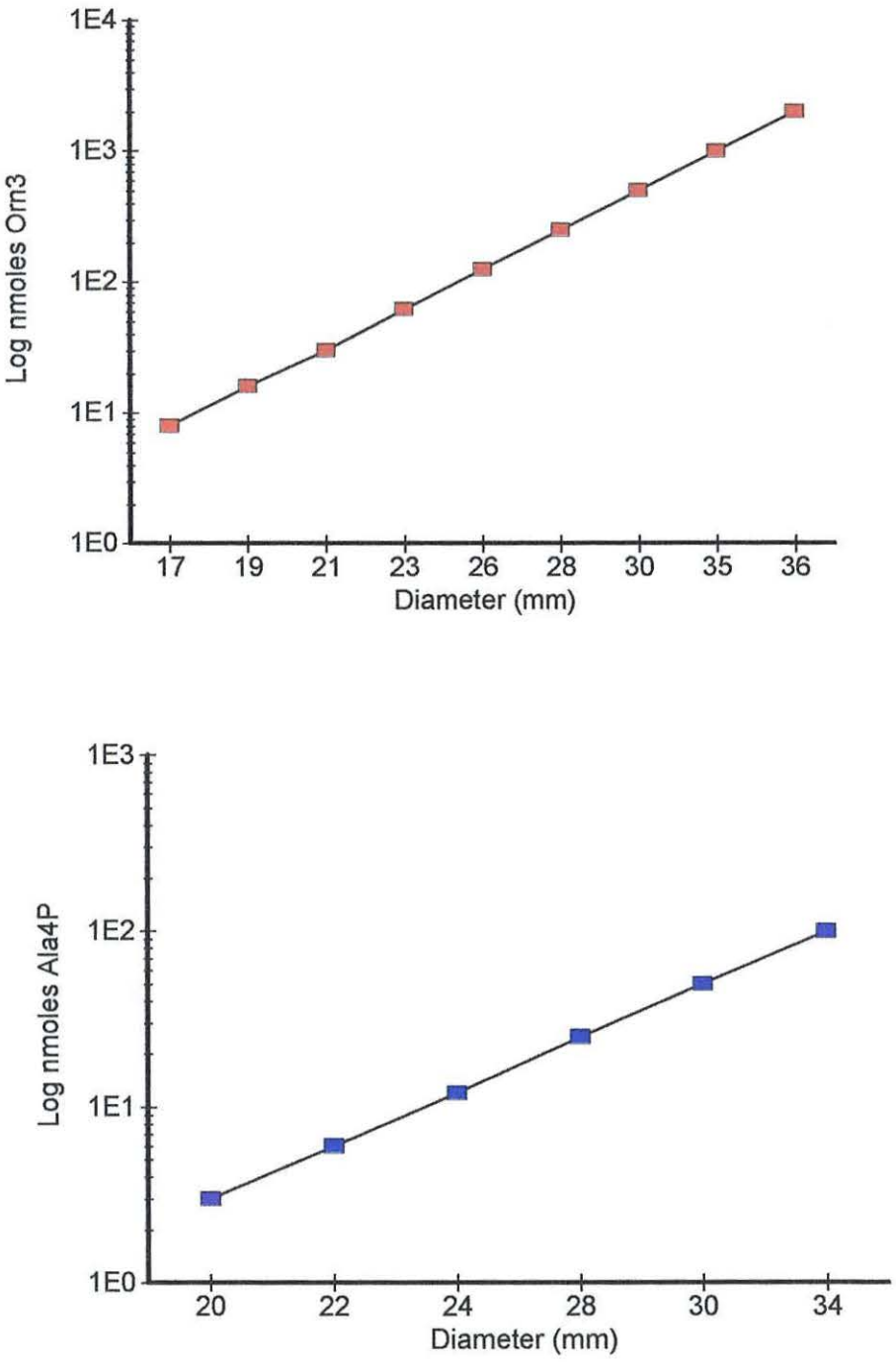


Figure 8.9

Graphic representation of the toxic peptides sensitivity in E. *Coli* PA0521. The amount of toxic peptide to give 25 mm inhibition zone, were 100 nmoles and 13 nmoles of Orn_3 and Ala_4P, respectively.

8.4 Production of Strains Carrying A Point Mutation in OppA

8.4.1 Introduction

Based on the putative peptide-binding region 300-306 (Asp-Ile-Ile-Val-Asn-Lys) for *E. coli* OppA, identified earlier by Marshall (1994), two strains with a point mutation in the residue Aspartyl-300, were produced and characterised.

The residue Asp300 was chosen to assess the relative contribution of its negatively charged group to satisfy the requirement for a protonated N-terminal amino group of a peptide. This residue was mutagenised to Serine ($Asp^{300}\rightarrow$Ser) and to Arginine ($Asp^{300}\rightarrow$Arg).

8.4.2 Methods

Both pBAN2 (containing the $Asp^{300}\rightarrow$Ser OppA) and pBAN3 (containing the $Asp^{300}\rightarrow$Arg OppA) plasmids were constructed in this laboratory by Gillian Payne. A point mutation was generated by site-directed mutagenesis, using the Sculptor *in vitro* mutagenesis (IVM) system RPM 1526 (Amersham International plc, Bucks., U.K.) according to manufacturer's instructions, as previously described (Marshall, 1994).

Plasmid DNA used for transformation, was purified by alkaline lysis followed by Qiagen column purification, as described in section 4.5.2.1. Minipreparation of plasmid DNA by alkaline lysis or boiling method, as described in section 4.5.2.2 and 4.5.2.3, respectively, was used for screening.

PA0467 was transformed with pBAN2 by using a standard transformation method and with pBAN3 by using an electroporation method, as described in section 4.5.1.1 and 4.5.1.2, respectively.

Bacterial strains were grown as described in section 4.2.

Periplasmic protein fraction (PPF) were prepared by cold osmotic shock as described in section 4.7.1, and SDS-PAGE was performed as described in section 4.10.1.

8.4.3 Results and Discussion

Two mutants with a point mutation in OppA were produced. The first was obtained by transformation of PA0467 *(oppA⁻)* with pBAN2, in which the residue Aspartyl-300 of OppA was changed to Serine (Asp^{300}→Ser). The resulting transformant was designated PA0522. The second, was obtained by transforming PA0467 with pBAN3, in which the residue Aspartyl 300 of OppA was changed to Arginine (Asp^{300}→Arg), and designated PA0523.

Transformant cells were selected on agar plates containing chloramphenicol (34μg/ml). Two colonies of each transformant strain, were picked up and re-isolated, for further screening.

The periplasmic protein fraction from PA0522 and PA0523 was analysed on SDS-PAGE. Figure 8.10 A and B shows the ability of both PA0522 and PA0523 strains, respectively, to reconstitute the expression of mutated OppA.

8.5 Characterisation of PA0522 and PA0523

8.5.1 Introduction

To characterise the oligopeptide permease function in both PA0522 (containing [Asp^{300}→Ser]-OppA plasmid) and PA0523 (containing [Asp^{300}→Arg]-OppA plasmid) mutants, two different assays were performed, the ability of both Ala_4P and Orn_3 toxic peptides to inhibit their growth and the ability of these mutants (Leu and Trp auxotrophs) to grow in minimal medium containing prolyl-leucyl-glycine amide, as sole source of leucine.

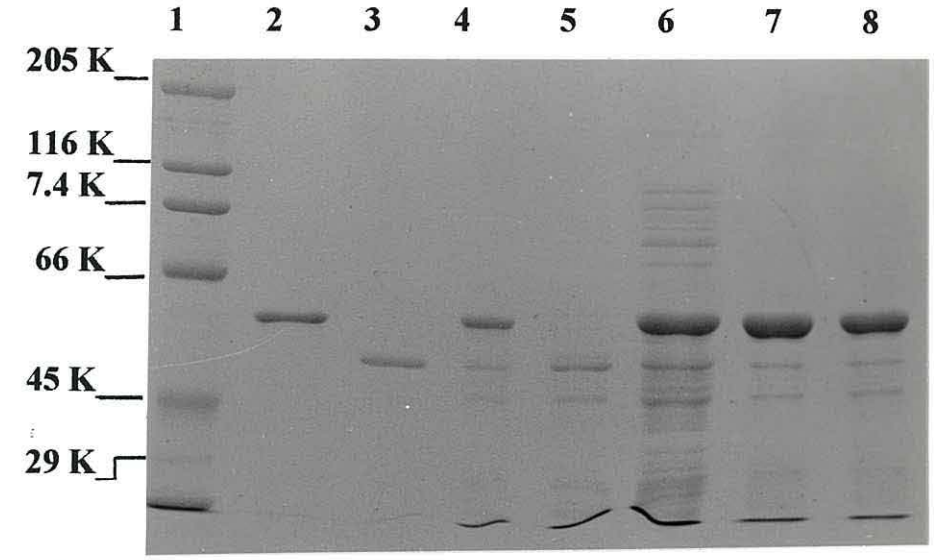


Figure 8.10 A
Coomassie blue stained gel showing the periplasmic protein fraction (PPF) from PA0467 (*oppA*$^-$) transformed with pBAN2 (containing the Asp ^{300}→Ser OppA). Lane 1: molecular weight markers, lane 2: 0.5µg purified OppA, lane 3: 0.5µg purified DppA, lane 4: 5µg of PPF from M2034 (wild type), lane 5: 5µg of PPF from PA0467 (*oppA*$^-$), lane 6: 25µg of PPF from PA0521, lanes 7 and 8: 5µg of PPF from two cell clones of PA0467 transformed with pBAN2.

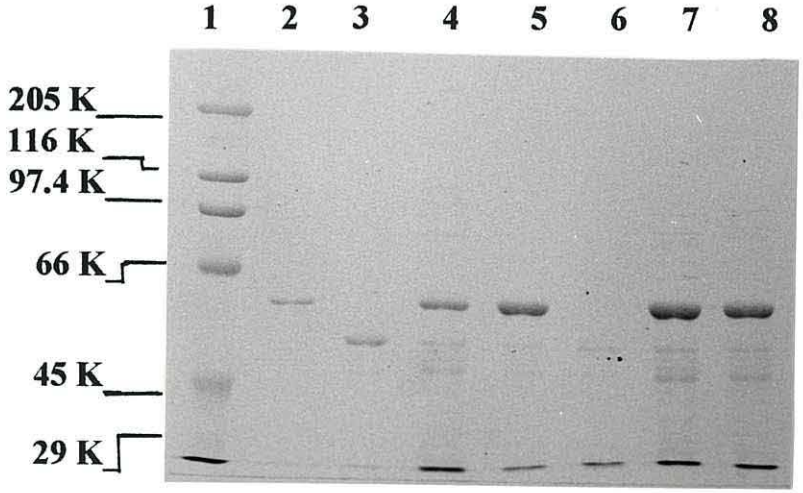


Figure 8.10 B
Coomassie blue stained gel showing the periplasmic protein fraction (PPF) from PA0467 (*oppA*$^-$) transformed with pBAN3 (containing the Asp ^{300}→Arg OppA). Lane 1: molecular weight markers, lane 2: 0.5µg purified OppA, lane 3: 0.5µg purified DppA, lane 4: 5µg of PPF from PA0521 (containing the wild type plasmid), lane 5: 5µg of PPF from PA0522 (containing the OppA) lane 6: 5µg of PPF from PA0467 (*oppA*$^-$), lanes 7 and 8: 5µg of PPF from two cell clones of PA0467 transformed with pBAN3.

8.5.2 Methods

Bacteria were cultured in a microtitre plate, to allow the growth of bacteria in a small volume of medium. A volume of 230µl of minimal medium containing 0.5% (w/v) Glc, 0.2 mM Trp and chloramphenicol (34µg/ml), was supplemented with 0.5mM prolyl-leucyl-glycine amide (PLG). Minimal medium (Glc,Trp and cm), without additional supplement or supplemented with 0.5 mM leucine, were included as negative and positive controls. These media were inoculated with 20µl of washed cells (2.5 x 10^7 cells). Bacterial strains were grown at 37°C with shaking and the growth was monitored by measuring the absorbance at 630nm, each hour using a microtitre plate reader (Titertek Twinreader ICN Flow Laboratories, U.K.).

Sensitivity to toxic peptides was tested by using a pour plate technique as described in section 4.6.1.

8.5.3 Results and Discussion

In order to investigate the biological activity of the oligopetide permease, the capacity of both PA0522 (containing [Asp^{300}→Ser]-OppA plasmid) and PA0523 (containing [Asp^{300}→Arg]-OppA plasmid) Leu and Trp auxotrophs, to grow in minimal medium supplemented with prolyl-leucyl-glycine amide was examined. PA0521 (containing the wild type OppA plasmid) was included as control. Figure 8.11 shows the growth curve of all strains analysed. PA0522 and PA0523 were unable to grow in medium where leucine was added as peptide. Only PA0521 was able to grow in minimal medium containing PLG as sole source of leucine.

When the sensitivity to toxic peptides was tested, both PA0522 and PA0523 mutants, were completely resistant to both Ala_4P and Orn_3, while PA0521 was sensitive. Typical pour plates are shown in figure 8.12. These results together with the growth curve, confirm the loss of the biological function of the oligopeptide permease in both PA0522 and PA0523 strains, that express OppA carrying a point mutation.

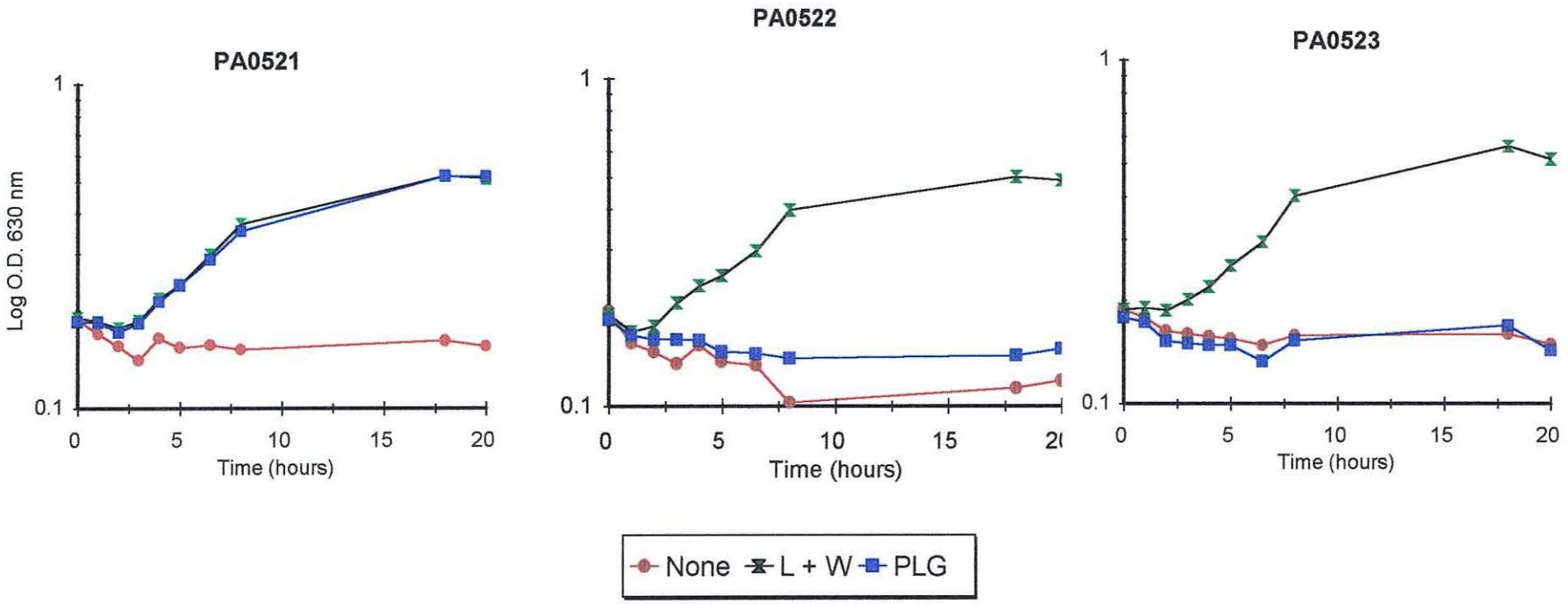


Figure 8.11

Growth curve of the following *E. coli* Leu and Trp auxotrophs: PA0521 (containing the wild type OppA plasmid), PA0522 (containing the [Asp ^{300}→Ser]-OppA plasmid) and PA0523 (containing the [Asp ^{300}→Arg]-OppA plasmid), using the peptide prolyl-leucyl-glycine amide as sole source of leucine. The assay was performed in a microtitre plate, and each well contained: 230 µl of minimal medium, in presence of chloramphenicol (34µg/ml) supplemented as follows: i) 0.5% Glc, 0.2 mM Trp (referred as none) ii) 0.5% Glc, 0.2 mM Trp and 0.5mM Leu (referred as L+W) iii) 0.5% Glc and 0.5 mM of prolyl-leucyl-glycine-amide (referred as PLG). Each well was inoculated with 2.5 x 10^7 cells, and grown with shaking at 37°C. The growth was determined by measuring the absorbance at 630 nm, each hour.

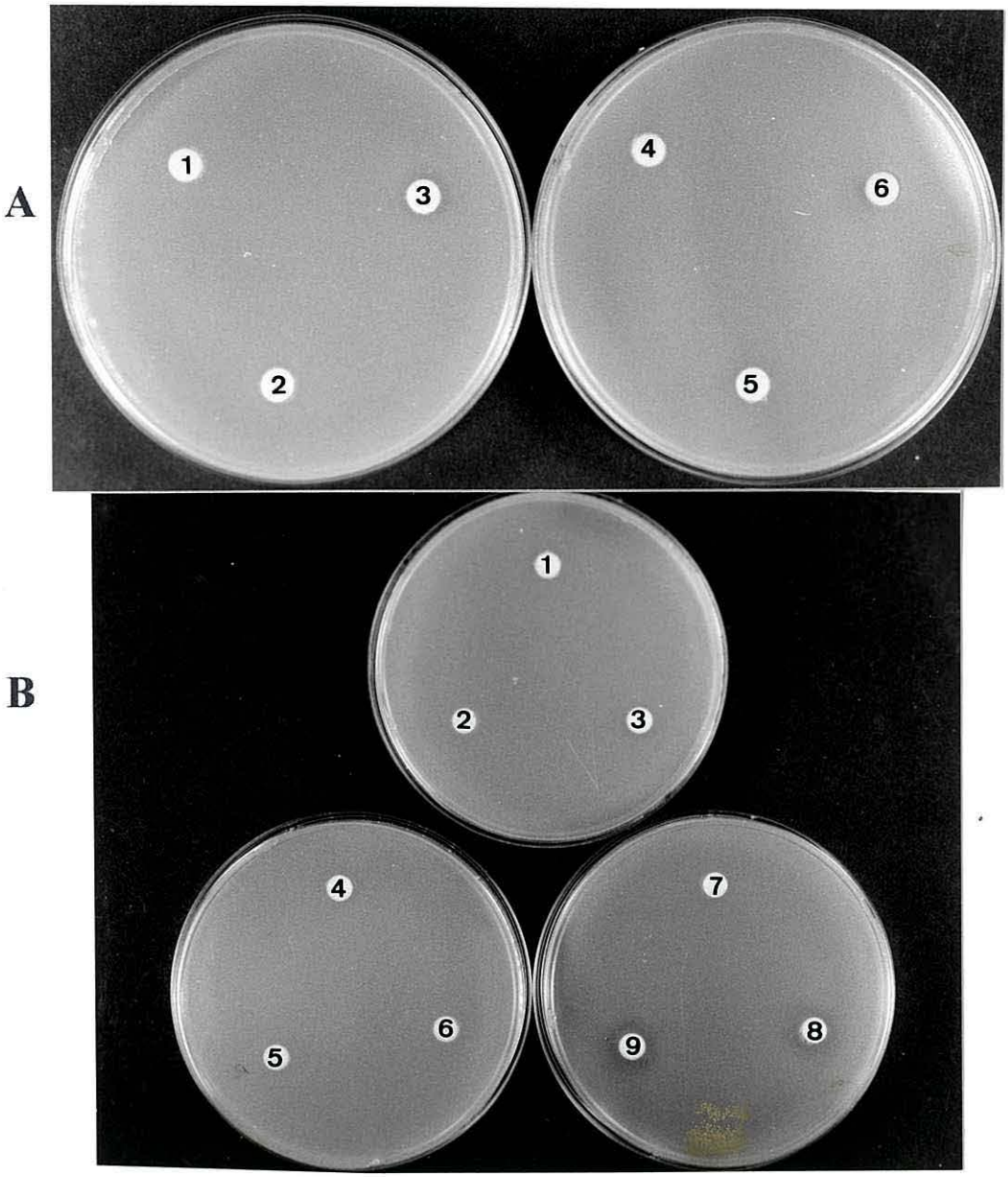


Figure 8.12:

Typical pour plate obtained by testing the sensitivity of *E. coli* PA0522 (containing [Asp^{300}→Ser]-OppA plasmid) and *E. coli* PA0523 (containing [Asp^{300}→Arg]-OppA plasmid) to toxic peptide. **A)** PA0522, 1-6 represent 60, 125, 250, 500, 1000 and 2000 nmoles of Orn_3, respectively. **B)** PA0523, 1-9 represent 8, 16, 30, 60, 125, 250, 500, 1000 and 2000 nmoles of Orn_3, respectively.

Point mutations affecting the function of several members of the ATP-binding cassette (ABC) family of transporter, have been described. A single mutation in TAP (transporter associated with antigen processing), resulted in an alteration of the peptide transport specificity (Armandola *et al.*, 1996); a spontaneous mutation in *mdr*1 (P-glycoprotein) in which the amino acid 185 is substituted, resulted in altered affinity of P-glycoprotein for drugs transport (Choi *et al.*, 1988); a point mutation in one of the nucleotide-binding domains (NBDs) of the cystic fibrosis transmembrane conductance regulator (CFCR), alters the ability to translocate chloride ion (Anderson and Welsh, 1992). Other mutations affecting the function of ABC transporters are nucleotide substitution in the binding protein gene, leading to the generation of premature stop codons and loss of expression, as has been reported for MDR1 (Gottesman and Pastan, 1993) and CFTR (Welsh and Smith, 1993). However, a point mutation which does not affect the gene expression, but is able to stop the complete function of an ABC transporter, has not yet been reported.

8.6 Purification of [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA Proteins

8.6.1 Introduction

In order to study the molecular characteristics of mutated OppA, both [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA proteins, were purified from periplasmic protein fraction of PA0522 and PA0523, respectively.

8.6.2 Method

Mutated OppA proteins were purified by following the purification scheme shows in Figure 4.2 (see section 4.8).

Bacterial strains were grown as described in section 4.2

Periplasmic protein fraction was isolated by cold osmotic shock as described in section 4.7.1, using 800 ml portions of cell culture.

The protein mixture was separated by Fast Protein Liquid Chromatography, as described in section 4.8.1.1 and 4.8.1.2. In order to obtain unliganded form of mutated OppA, the purified protein was applied onto reverse phase chromatography as described in section 4.8.1.3.

8.6.3 Results and discussion

Both mutated OppA as well wild type OppA were purified by the procedure outline in Figure 4.2 (see section 4.8). The filtered and dialysed cold osmotic fluid (the periplasmic protein fraction or PPF) was concentrated 10-fold by freeze-drying. This PPF was fractionated on cation exchange chromatography. A typical elution profile obtained for all OppA (wild type and mutants) is shown in Figure 8.13. Peaks (II and III) eluting between 5 to 10 % and 13 to 18% of sodium chloride were identified by SDS-PAGE as DppA and OppA, respectively. Peak I corresponds to unbound proteins. The fractions of peak III (containing OppA) were pooled and subjected to anion exchange chromatography as shown in Figure 8.14. Two peaks with poor resolution eluting between 6 and 12% of sodium chloride were obtained, and identified as OppA by SDS-PAGE. These peaks corresponded to a mixture of the liganded and unliganded forms as was demonstrated by reverse phase chromatography that removes bound peptide (Tyreman, 1990). Proteins that have a high affinity for their ligand, such as OppA and other binding proteins, are co-purified with bound ligand. A phenomenon referred to as the retention effect (Silhavy *et al.*, 1975).

Starting with 1.6 litres of culture of PA0522 and PA0523, the total amount of purified protein, was 17.31 and 16.16 mg, of [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA respectively, which represent 22 and 23 % of the total periplasmic protein. These mutant strains produced 2.9 to 4.6-fold of the amount of OppA typically reported in wild type strain.

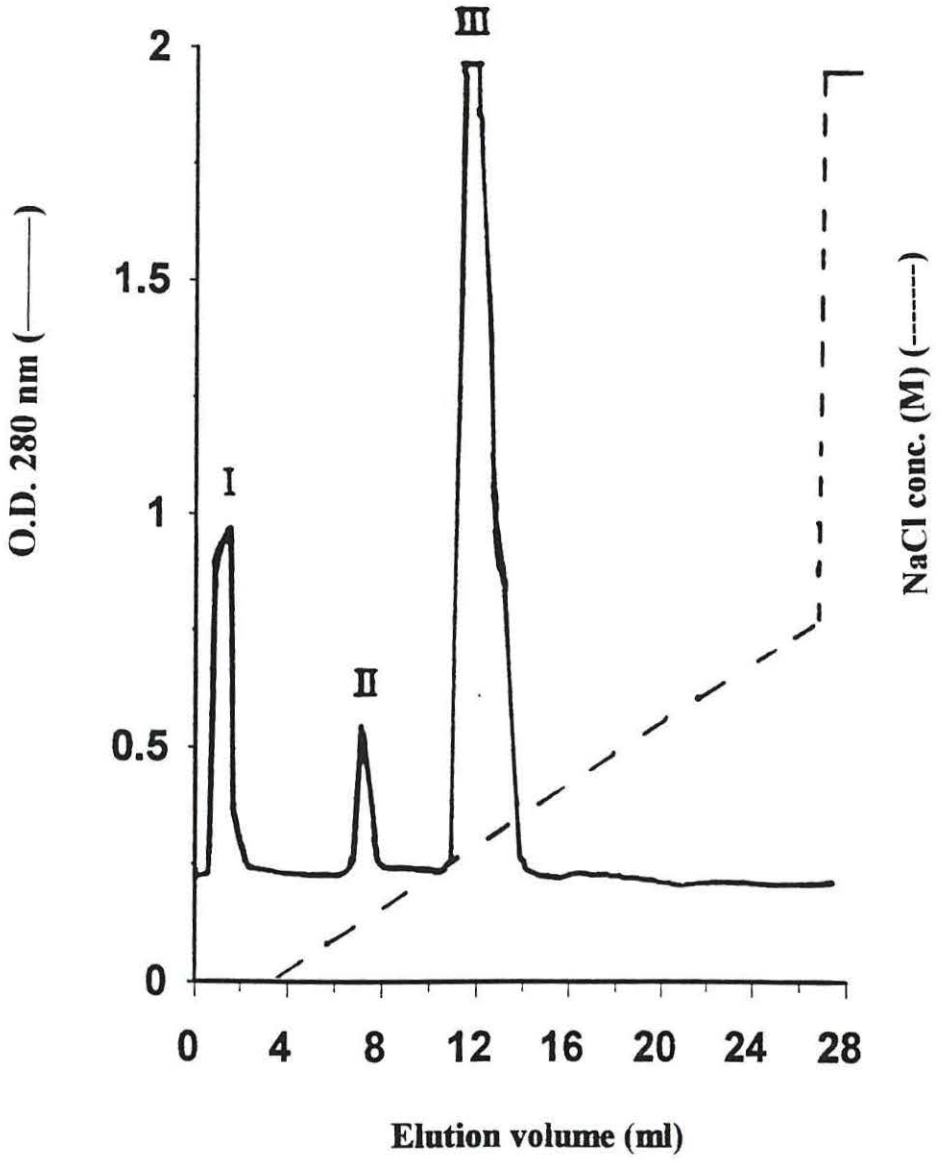


Figure 8.13

Cation-exchange chromatography of the concentrated periplasmic protein fraction

The column (FPLC MonoS HR5/5) was eluted by increasing linear gradient of sodium chloride in 50 mM malonic acid-NaOH (-----) as described in section 4.8.1.1. Protein was determined by measuring absorbance at 280 nm (——). Samples from each peak containing protein were subjected to SDS-polyacrylamide gel electrophoresis. Peak I, unbound components; peak II, DppA; peak III, OppA.

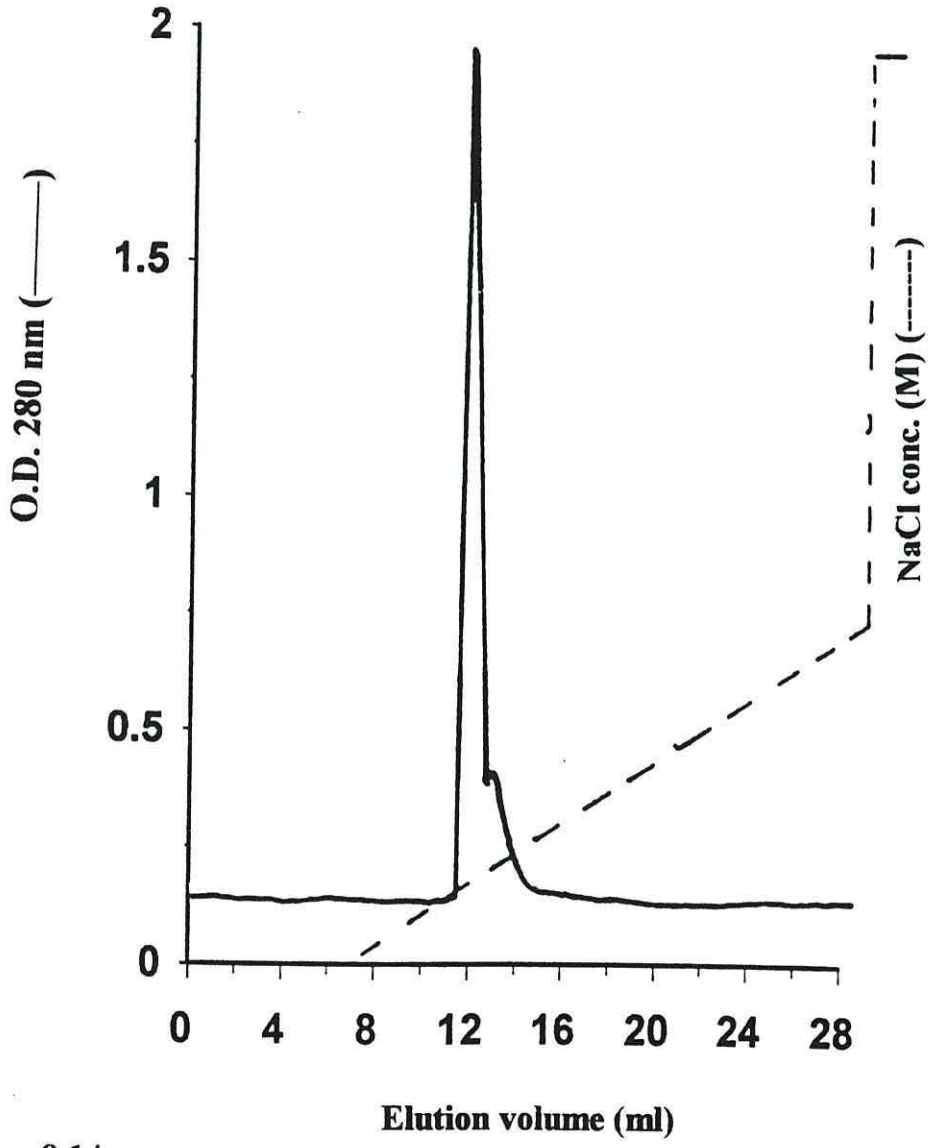


Figure 8.14

Anion exchange chromatography of the oligopeptide-binding protein.

Fraction of MonoS column containing OppA (peak III) were pooled, concentred and dissolved in 20 mM Tris pH 7.8. Aliquots of this sample were applied onta a column QHR5/5, and eluted by increasing linear gradient of sodium chloride in 20 mM Tris-HCl pH 7.8 (- - - -) as described in section 4.8.1.2. Proteins were determined by measurement absorbance at 280 nm (———). Samples from peak containing protein was subjected to SDS-polyacrylamide gel electrophoresis, migrating a as single band at the OppA position.

Binding proteins free of ligand (unliganded form) of both mutated as well wild type OppA, were obtained by applying purified OppA onto an FPLC Pro-RPC column. A single peak eluting at 40 % acetonitrile was obtained (Fig. 8.15).

The purification obtained for both OppA mutants was similar to that obtained with OppA wild type.

8.7 Isoelectric point change associated with OppA wild type and mutants upon peptide binding.

8.7.1 Introduction

In order to characterise mutated OppA, the pI of these binding proteins at two different points in the purification (ion exchange chromatography and reversed phase chromatography), were analysed. The changes in pI consequent upon ligand binding to OppA were studied by incubation with exogenous substrate.

8.7.2. Methods

The affinity and pI change of OppA upon ligand binding of mutated-OppA and wild type-OppA were studied by analysing the pI of these proteins complexed with Ala-Ala Ala (Ala_3) and Lys-Ala-Ala (Lys-Ala_2), at 312, 62, 12, 2.5, and 0.1 μM.

Isoelectric focussing (IEF) was carried out as described in section 4.10.2 and pI was calculated from the linear calibration plot of $\log_{10}$ of marker proteins pI versus their mobility.

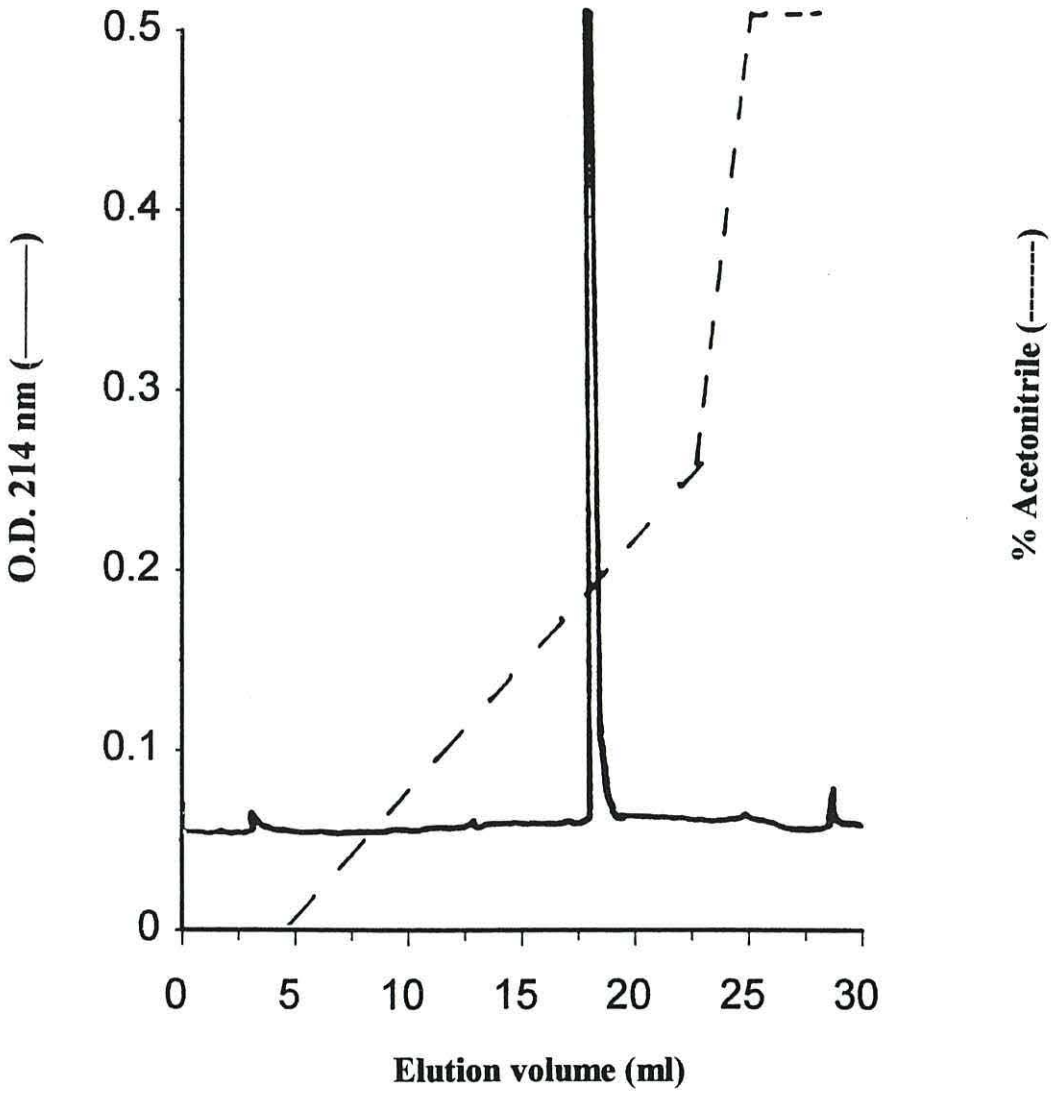


Figure 8.15

Reverse-phase chromatography of purified oligopeptide-binding protein by both ion and cation-exchange chromatography.

Aliquots of purified OppA were applied onto a column PRO-RCP HR 5/10. Desorption of bound components was achieved by increasing linear gradient of acetonitrile in 0.1% (v/v) trifluoracetic acid (-----) as described in section 4.8.1.3. Protein was determined by measuring absorbance at 280 nm (___). Samples from peak containing protein was subjected to isoelectric focusing electrophoresis, migrating as a single band.

8.7.3 Results and Discussion

IEF analysis of [Asp^{300}→Ser]-OppA, purified on ion-exchange column, revealed four main components. Which on the narrow range phast gel (pH 5-8) had pI in the range 6.54 to 6.8, while [Asp^{300}→Arg]-OppA revealed three main components which had pI in the range 6.96 to 7.12, as compared with OppA wild type that revealed three main components, which had pI in the range 6.2 to 6.8 (Fig. 8.16 lane 2 in B, C and A gels, respectively).

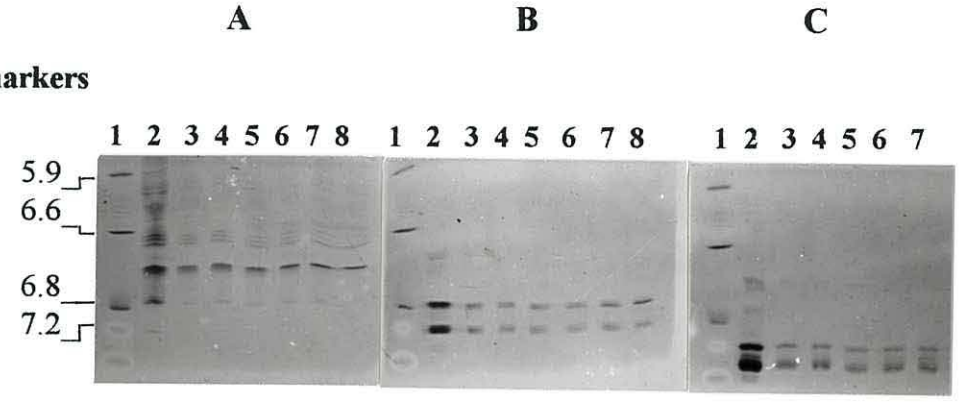


Figure 8.16

Isoelectric focusing analysis of the peptide binding ability of wild type, [Asp^{300}→Ser] and [Asp^{300}→Arg]-OppA proteins. All proteins were purified by ion-exchange chromatography and incubated with substrate as described in section 4.10.2. Gel **A**: lane 1, pI markers; lane 2:wild-type-OppA protein purified by ion-exchange chromatography; lanes 3-8 wild type-OppA incubated with 312, 62, 12, 25, 0.5 and 0.1 μM of Ala_3, respectively. Gel **B**: lane 1, pI markers; lane 2: [Asp^{300}→Ser]-OppA protein purified by ion-exchange chromatography; lanes 3-8 [Asp^{300}→Ser]-OppA incubated with 312, 62, 12, 25, 0.5 and 0.1 μM of Ala_3, respectively. Gel **C**: lane 1, pI markers; lane 2, [Asp^{300}→Arg]-OppA protein purified by ion-exchange chromatography; lanes 3-7 [Asp^{300}→Arg]-OppA incubated with 312, 62, 12, 25 and 0.5 μM of Ala_3, respectively.

The ability to bind substrate was analysed in all three OppA by incubating them with Ala_3.When [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA were incubated with different concentration of Ala_3 (312, 62, 12, 2.5, and 0.1 μM), a pI shift was observed with progressive loss of the three components of pI 6.28, 6.75 and 6.8 of [Asp^{300}→Ser]-OppA, and progressive loss of the two components 7.03 and 7.12 of [Asp^{300}→Arg]-OppA, with a concomitant increase in the abundance of the one of pI 6.54 and pI 6.96 of [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA, respectively (Fig. 8.16 B and C). The same phenomenon was observed for OppA wild type but, the concentration of Ala_3 to yield a single band was 12.5μM as compared with the mutants which needed a higher concentration (312μM) to produce the same result (Fig. 8.16 A).

IEF experiments employing the single protein component obtained from reverse phase chromatography (OppA unliganded form) were carried out. IEF gels showed a single band with pI 6.54, 7.0 and 6.2, for [Asp^{300}→Ser]-OppA, [Asp^{300}→Arg]-OppA and wild type OppA, respectively (Fig. 8.17).

When these RPC-purified proteins were incubated with a high concentration of Ala_3, a shift pI to 6.48, 6.9 and 6.26 was observed for [Asp^{300}→Ser]-OppA, [Asp^{300}→Arg]-OppA and wild type OppA, respectively (Fig. 8.17 A). While when these proteins were incubated with Lys-Ala_2, a pI shift to 6.75, 7.1 and 6.43 was observed for [Asp^{300}→Ser]-OppA, [Asp^{300}→Arg]-OppA and wild type OppA, respectively (Fig. 8.17 B).

The fact that the pI shift (0.34 or 0.80 pI units) as a consequence of substitution of the Asp300 residue to serine or arginine, was bigger than the theoretically predicted pI shift (0.13 and 0.27 pI units, respectively), suggests that the OppA mutation to serine or to arginine possible induced a slight conformational change of the protein, which does not abolish its ability to bind substrate, although a decrease in the affinity to bind Ala_3 and Lys-Ala_2 was observed.

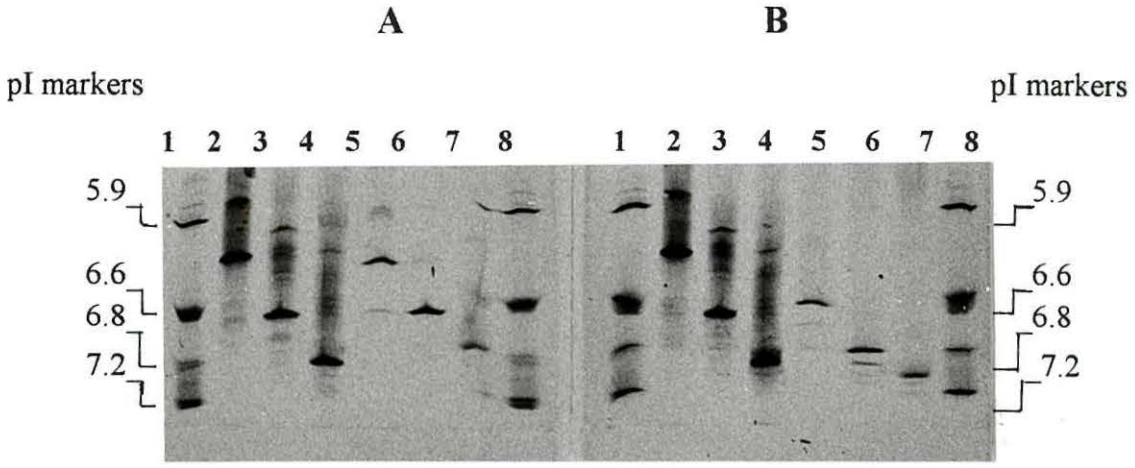


Figure 8.17

Isoelectric Focusing analysis of the peptide binding ability of wild type, [Asp^{300}→Ser] and [Asp^{300}→Arg]-OppA proteins, in their unliganded form. Free-ligand proteins were obtained by reversed-phase chromatography, as described in section 4.8.1.3. Gel A: Lanes 1 and 8, pI markers; lanes 2-4: 12μg of wild type, [Asp^{300}→Ser] and [Asp^{300}→Arg]-OppA protein, respectively; lanes 5-7: wild type, [Asp^{300}→Ser] and [Asp^{300}→Arg]-OppA, incubated with Ala_3. Gel B: Lanes 1 and 8, pI markers; lanes 2-4: 12μg of wild type, [Asp^{300}→Ser] and [Asp^{300}→Arg]-OppA protein, respectively; lanes 5-7: wild type, [Asp^{300}→Ser] and [Asp^{300}→Arg]-OppA, incubated with Lys-Ala_2.

8.8 General Discussion

Many periplasmic binding proteins have been characterised structurally, some with and some without ligand. These include arabinose-binding protein (Quiocho and Vyas, 1994), galactose-glucose binding protein (Zou *et al.*, 1993, Vyas *et al.*, 1994), ribose-binding protein (Mowbray and Cole, 1992), sulphate-binding protein (Pflugrath and Quiocho, 1998), leucine and leucine-isoleucine-valine-binding protein (Sack *et al.*, 1989a and 1989b), and histidine-binding protein (Oh *et al.*, 1994, Yao *et al.*, 1994). All these protein show common features in their tertiary structure and atomic interaction between proteins and ligands, despite observed differences in size, sequences and specificity. They are ellipsoidal, constituting of two globular domain, the polypeptide crossing between domain

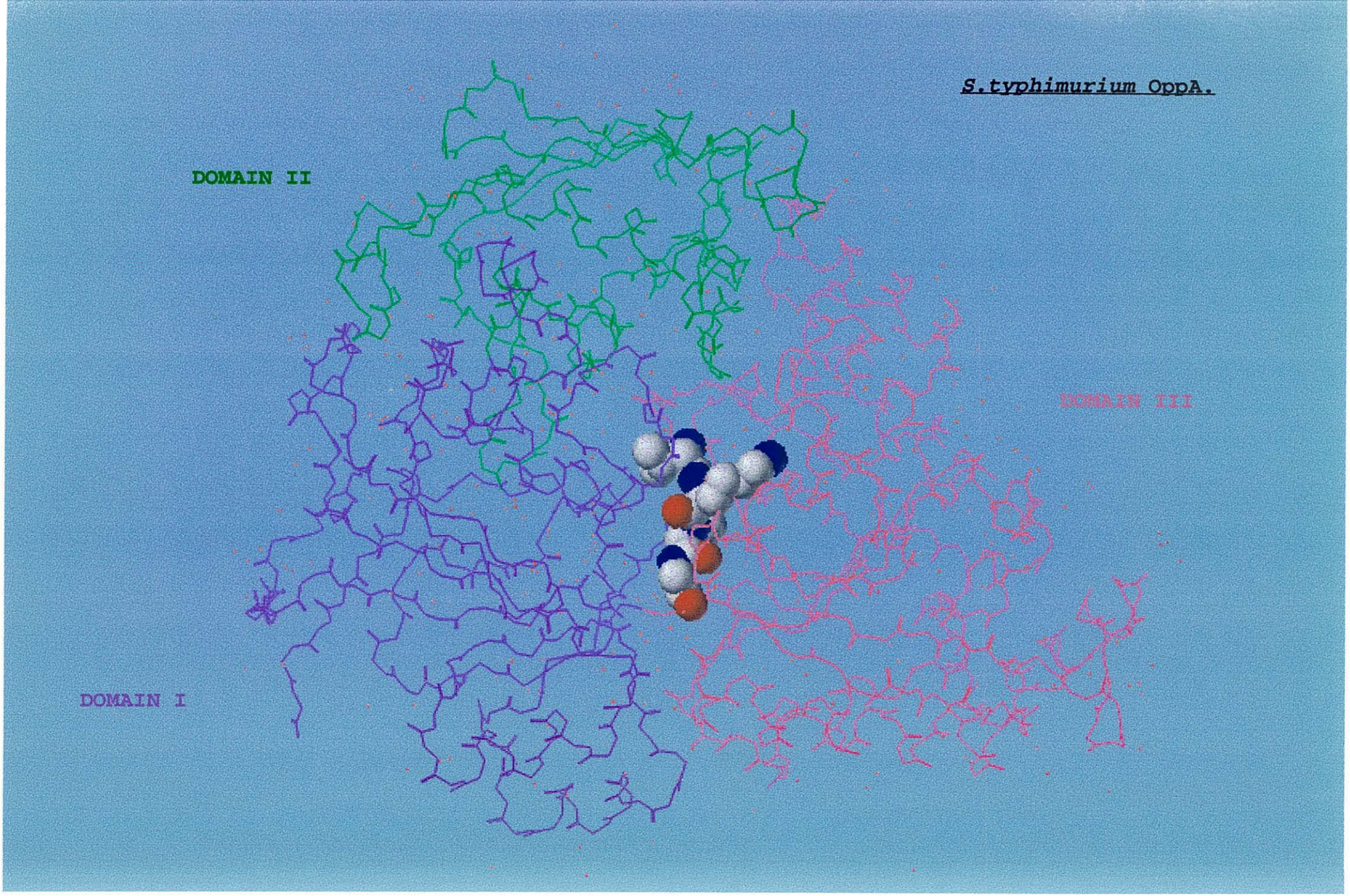
two and three time to form a hinge which opens and closes on ligand binding and released. Binding of ligand to the open form induce a large conformational change in the protein, producing the closed liganded form that interacts with membrane components to released the substrate (Quiocho, 1990, 1992).

OppA from *S. typhimurium* that shows 94.5% of similarity with OppA from *E. coli* (Kashiwagi *et al.*, 1990), and is presumably very similar structurally, has been crystallized (Tolley *et al.*, 1988) and its structure has been solved crystalographically (Tames *et al.*, 1994; Tames *et al.*, 1995). The structure of *S. typhimurium* OppA reveals a three-domains organization. Two of the domains (I and III) present surfaces that enclose the ligand and are linked by two segments that allow them open and close (Fig. 8.18). The crystal structure of OppA from *S. typhimurium* complexed with the peptide KKK and KKKA have been refined to 1.4 and 2.1Å, respectively (Tames *et al.*, 1995). This later study revealed that tight peptide binding is due to main-chain contacts, the majority of them provided by domain III, the ligand forming antiparallel β-sheet-like interaction with an extended strand of sheet 3 (residues Gly415-Cys417) on one side and parallel β-sheet-like interaction with a loop of domain I (residues Glu32-Val34) on the other. The charges at the N and C termini of the peptide are countered in the complex by oppositely charged side chains, Asp419 and Arg413 (Fig. 8.19).

In order to establish the spatial relationship between the photoaffinity peptide and the ligand (VKPG), the distance between the N-terminus of the ligand and the residue Asp300 of the photoaffinity peptide, was calculated by using the X-ray diffraction data from the Brookhaven Protein Databank and the SYBYL software package by Barry Grail in this laboratory. The photoaffinity peptide lies in domain III exposed at surface (Fig. 8.20), and the distance between the negative charge of Asp300 and the N-terminus of the ligand is 28.06 Å, that is incompatible with any atomic interaction (Fig. 8.21). This figure also shows the putative electrostatic interaction between Asp419 and the N-terminus of the ligand proposed by Tame *et al.* (1994). These data together with the pI shift observed in both mutated OppA, confirm that Asp300 has no direct role in the final binding of the ligand in its 'ligand binding site' (section 8.6). However, the fact that the substitution of Asp300 for Ser or Arg abolishes the function of oligopeptide permease, implies that the

Figure 8.18

Crystal structure of the OppA-VKPG complex from *Salmonella typhimurium*, showing the three domain organization. Domain I, II and III are shown in *purple*, *green* and *pink*, respectively. Ligand (Val-Lys-Pro-Gly) is shown in spacefill.


S.typhimurium OppA.
DOMAIN II
DOMAIN III
DOMAIN I

residue is involved in another function, for example in the interaction with the membrane complex.

Based on the mechanism proposed by Quiocho (1992), these combined results, for example: i) the lack of biological function shown by PA0522 (containing [Asp^{300}→Ser]-OppA plasmid) and PA0523 (containing [Asp^{300}→Arg]-OppA plasmid), ii) the difference in pI observed between the wild type OppA and both mutated OppA, which might indicate a conformational change, and iii) the spatial relationship of Asp300 with the N-terminus of the ligand, are compatible with the idea that residue Asp300 could be involved in the interactions with the other domains, being unable to produce an effective closed liganded form and thus failing to interact successfully with the membrane components (*oppBCDF*) that is required for transport.

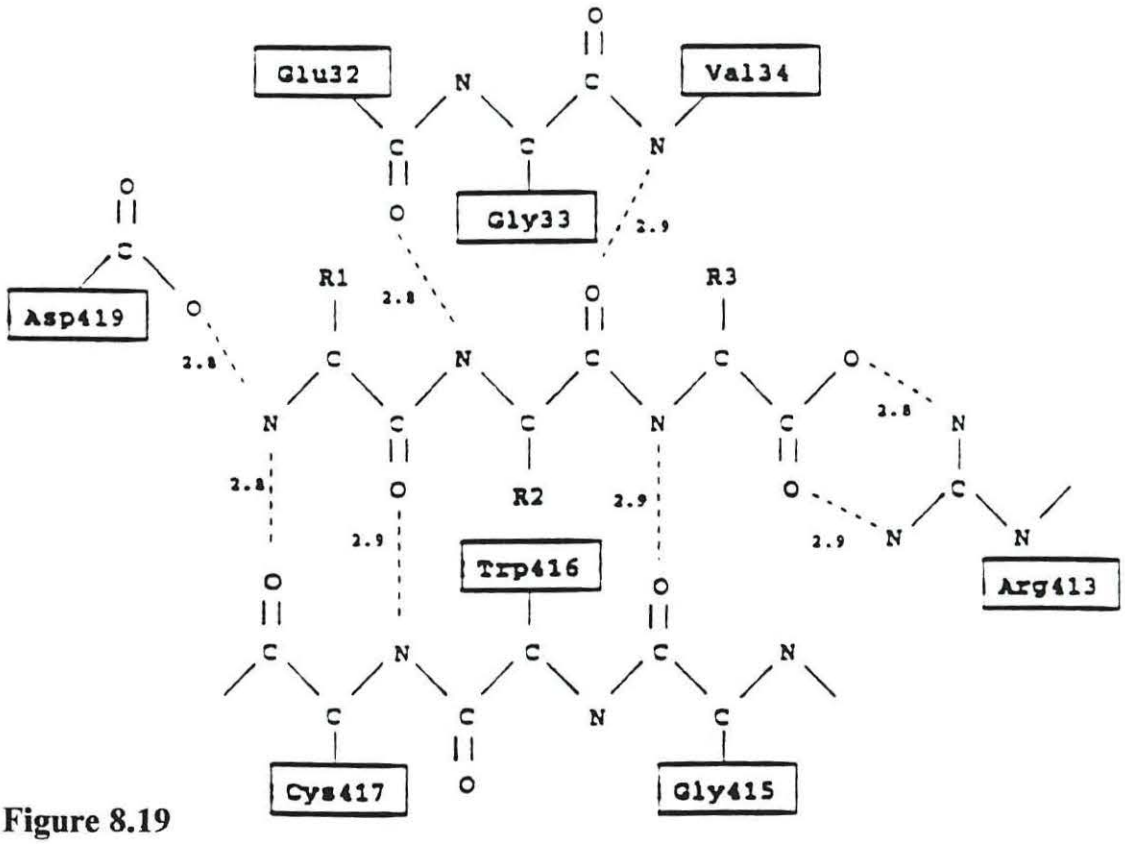


Figure 8.19

Schematic diagram illustrating the interaction made by main chain of the trilysine ligand with OppA. Protein residues are labelled. Hydrogen bound and electrostatic interaction are indicated by the dotted line. R1, R2, and R3 indicate the ligand side chain. Taken from Tame et al., 1995.

Figure 8.20

Crystal structure of the OppA-KVPG complex from *Salmonella typhimurium*, showing the Photoaffinity peptide (*yellow*). Domain I, II and III are shown in *purple*, *green* and *pink*, respectively. Ligand (Val-Lys-Pro-Gly) is shown in spacefill. Photoaffinity peptide is shown in ball-and-stick

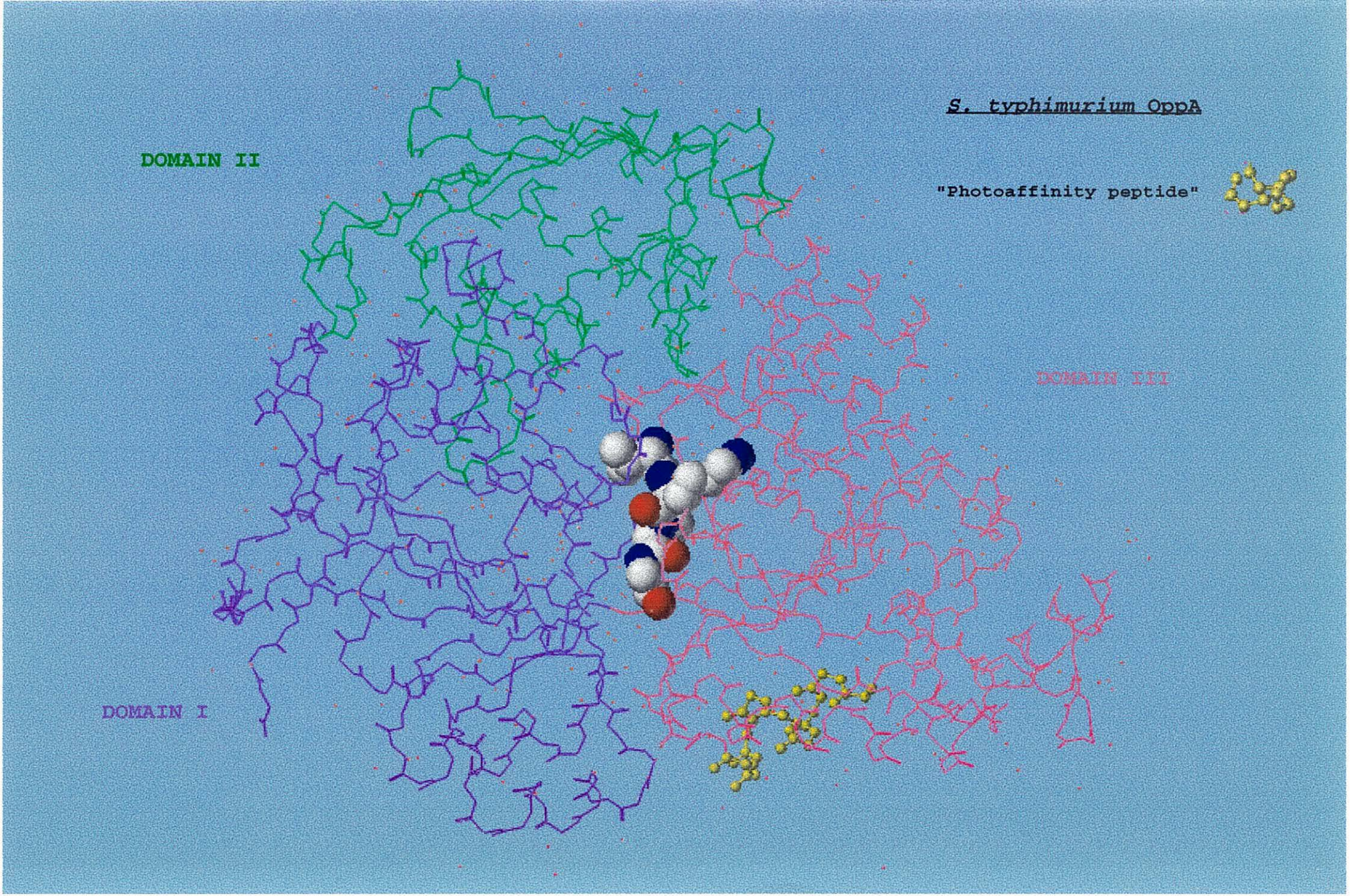
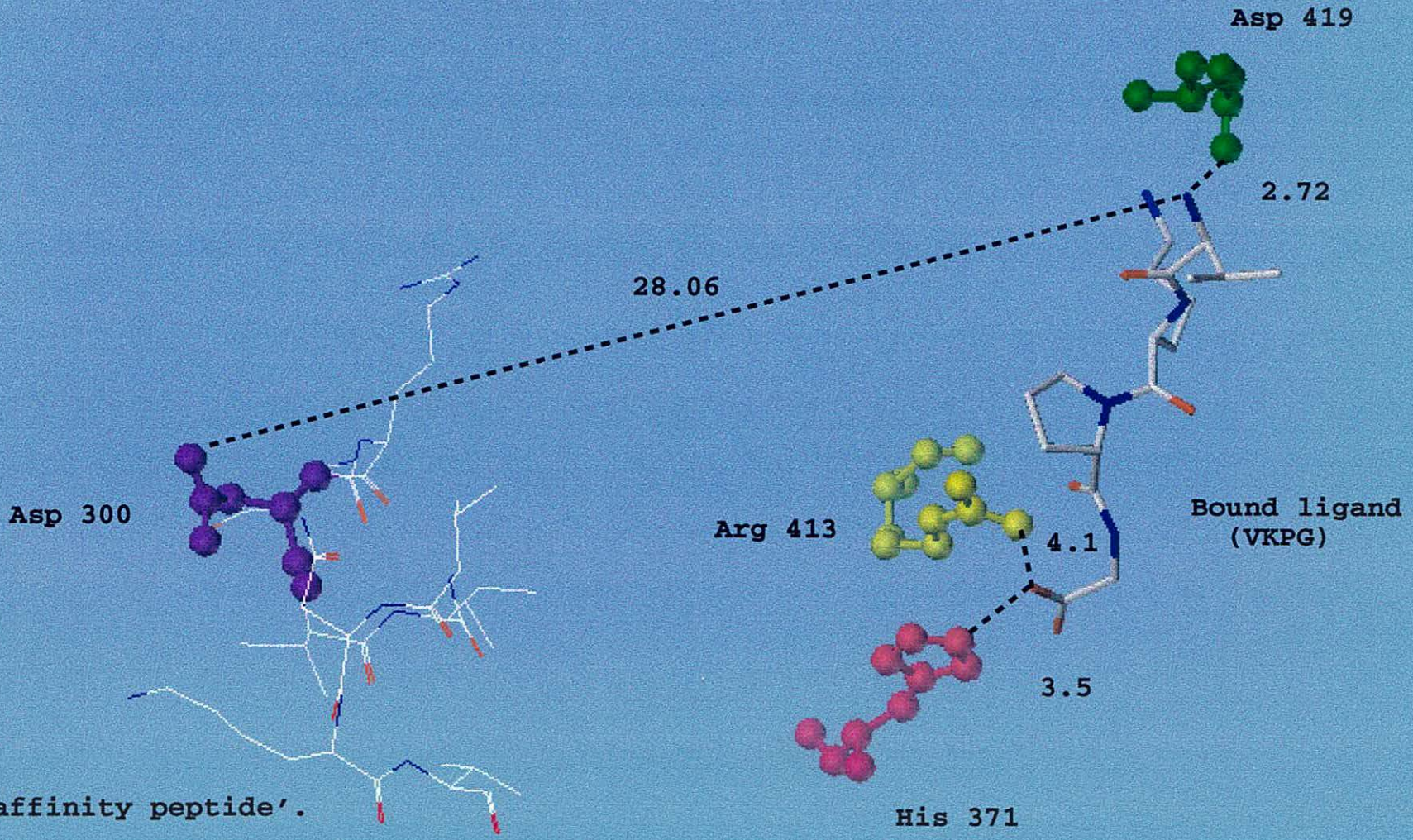

S. typhimurium OppA
DOMAIN II
"Photoaffinity peptide"
DOMAIN III
DOMAIN I

Figure 8.21

Schematic drawing showing the spatial relationship between the photoaffinity peptide and the bound ligand of *Salmonella typhimurium*. Ligand (Val-Lys-Pro-Gly) is shown in thick lines. Residues Asp419, Arg413 and His317 involved in ligand-binding are shown in green, yellow and pink ball-and-stick, respectively. Photoaffinity peptide is shown in thin lines and the residue Asp300 in *purple* spacefill.

Spatial relationship between the 'photoaffinity peptide' & the bound ligand of *S. typhimurium* OppA.



(Dimensions in angstroms.)

Chapter IX

PRODUCTION AND CHARACTERISATION OF SPONTANEOUS COMPENSATIVE MUTANTS

9.1 Introduction

The oligopeptide permease of *E. coli* is a periplasmic binding protein-dependent system, which consists of five proteins. A soluble protein OppA, which provides the primary receptor for transport (Payne and Bell, 1979). Two highly hydrophobic proteins OppB and OppC, that are thought to be responsible for peptide transport across the bilayer (Hiles *et al.*, 1987). Two relatively hydrophobic proteins peripherally located on the cytoplasmic face of the membrane (Gallagher *et al.*, 1989), that are believed to couple ATP hydrolysis to the transport process (Higgins, 1990).

OppA has been extensively characterised: the membrane proteins have been characterised at the genetic level, but the proteins themselves, and their interaction with the binding protein, remain poorly understood. There are several reason for this. (i) Their low abundance, approximately 50-100 molecules per cell. (ii) The fact that they are

hydrophobic and hence their purification is difficult. (iii) Their only activity is to transport peptides across an intact membrane, a function that is lost as soon as the cell is disrupted. (iv) As is commonly found, they are deleterious to cell growth when overproduced (Higgins *et al.*, 1990).

The aim of this work was to produce strains carrying mutations affecting the membrane components, able to compensate the loss of oligopeptide permease function showed by the strain PA0522 (containing [$Asp^{300}\rightarrow$Ser]-OppA plasmid). Thus, the characterisation of these mutants could be very important for understanding the molecular events between OppA and the membrane proteins for the translocation of the peptide across the membrane.

9.2 Selection of Spontaneous Mutants Able to Grow with PWLA

9.2.1 Introduction

The strain *E. coli* PA0522 (*Leu*$^-$, *Trp*$^-$, pBAN2 [$Asp^{300}\rightarrow$Ser]-OppA) was selected to produce a mutant able to compensate the site-directed mutation in *oppA* gene. This mutant overexpresses [$Asp^{300}\rightarrow$Ser]-OppA, but has no biological function of the oligopeptide permease (see section 8.5 and 8.6, respectively). PA0522 was obtained as described in Figure 9.1, (see Table 9.1 for genetic markers details). The strain M2034, used to produce *oppA*$^-$ strains has a *trpE*9851 ochre mutation in the first gene of the *trp* operon, and an amber mutation in one of the genes involved in the biosynthesis of leucine, which confer auxotrophy for tryptophan and leucine (Morse and Guertin, 1972). PA0467 has a mutation in the *oppA* gene, that abolishes the OppA expression, and consequently the oligopeptide permease function. PA0522 was produced by transformation of PA0467 (*oppA*$^-$) with pBAN2, a plasmid carrying the *oppA* gene with a site-directed mutagenesis ($Asp^{300}\rightarrow$Ser).

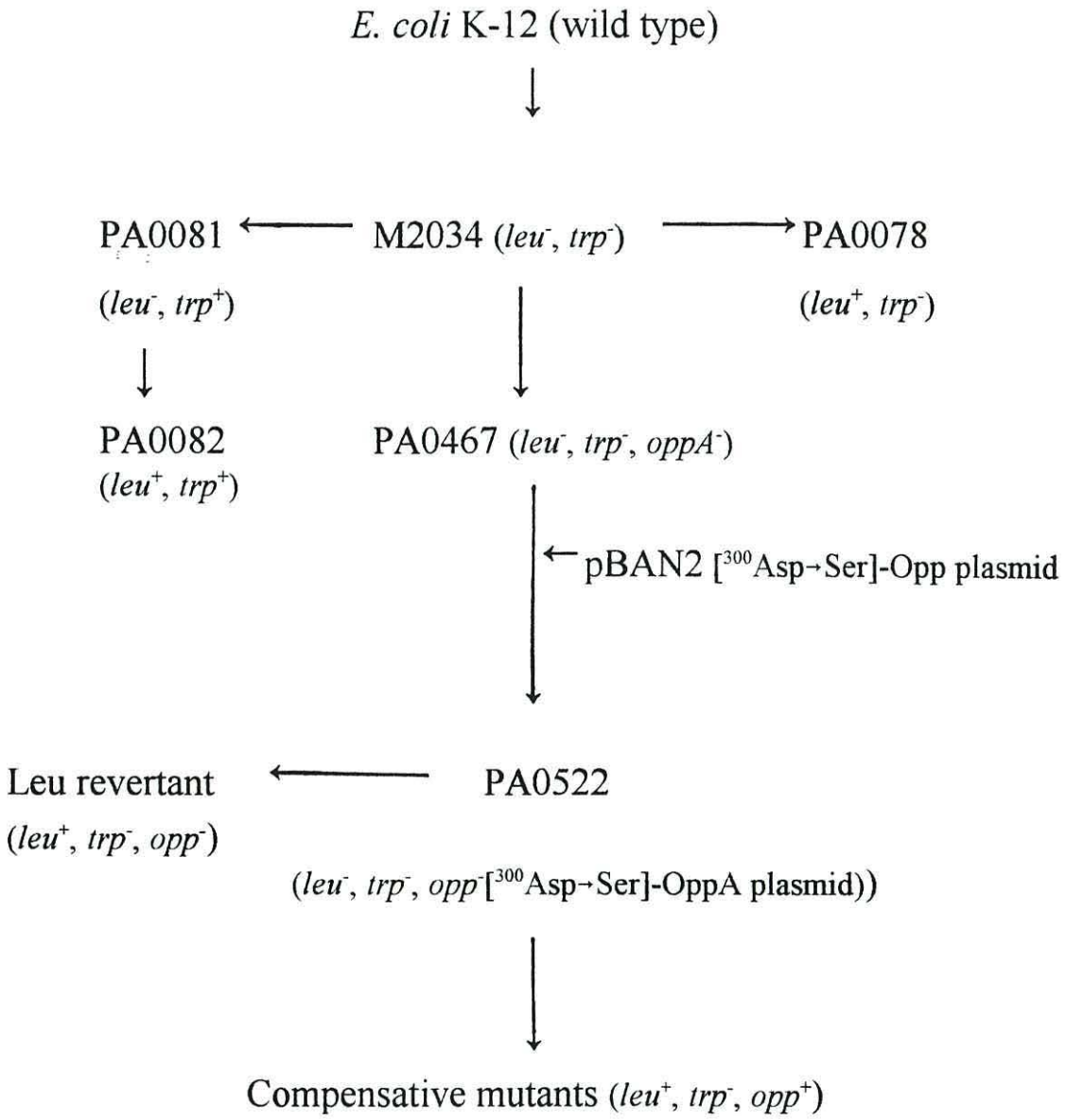


Figure 9.1

Flow chart showing the *E. coli* K-12 derivatives used to produce compensative mutants and control strains. Genetic markers are described in Table 9.1

Table 9.1

Genetic markers used in *E. coli* referred in Figure 9.1

Symbol	Description	Effect
trp$^-$	Ochre mutation in the first gene of the *trp* operon	Requires tryptophan for growth on minimal medium
leu$^-$	Amber mutation in one of the genes of the leucine synthesis	Requires leucine for growth on minimal medium
leu$^+$	Reversion of auxotrophic mutation for leucine	Abolition of leucine requirement for growth in minimal medium
oppA$^-$	Mutation in the *oppA* gene	Block the synthesis of OppA and consequently abolish the function of the oligopeptide permease
pBAN2	Plasmid containing the *oppA* gene with a site-directed mutagenesis, that changes the residue 300-Asp for Ser ([Asp $^{300}\rightarrow$Ser]-OppA)	Overproduction of [Asp$^{300}\rightarrow$Ser]-OppA mutant, with no biological function of oligopeptide permease
Opp$^+$	Reversion of the *oppA* mutation by restoration of the original base sequence or by some other mutation in the *opp* operon	Restoration of the oligopeptide permease function

The tetrapeptide Pro-Trp-Leu-Ala (PWLA) used to select the compensative mutants, was designated taking into account the following parameters: i) complete specificity for oligopeptide permease, and ii) requirement of leucine and tryptophan by the parent strain (PA0522) to grow in minimal medium.

9.2.2 Methods

PWLA was synthesised as described in section 5.

A single colony of *E. coli* PA0522 was grown in 5 ml minimal medium supplemented with Glc, Trp, Leu and chloramphenicol (34µg/ml), overnight, at 37°C with shaking. Twenty tubes containing 5 ml minimal medium supplemented as above, were inoculated with 10^2 cells from the overnight culture and grown under the same conditions. Twenty minimal medium agar plates supplemented with Glc and chloramphenicol and with top agar containing 0.5 mM PWLA, as the only source of Leu and Trp, were spread with 200 µl (5×10^8 cells) of each overnight culture, to obtain single colonies. The plates were incubated at 37°C for two days.

9.2.3 Results and Discussion

A mutation frequency of 4×10^{-8} was observed. An average of two colonies per plate was picked and re-isolated by streaking for single colonies on a minimal medium agar plate containing Glc, chloramphenicol (34µg/ml) and 0.5 mM PWLA in top agar. The re-isolated colonies were then selected for auxotrophic phenotype (*leu*$^-$ and *trp*$^-$) and PWLA transport.

9.3 First Screening to Detect Auxotrophic Phenotype in Mutants Selected with PWLA

9.3.1 Introduction

In order to verify the phenotype of the mutant selected with PWLA, experiments were designated to test the ability of these mutants, to grow in absence of either leucine or tryptophan, or both amino acids.

9.3.2 Methods

Individual colonies streaked onto PWLA were tested by transferring a small portion of bacteria with a sterile wooden stick to agar plates containing chloramphenicol (34μg/ml) and supplemented as follows: i) only Glc, ii) Glc and Leu iii) Glc and Trp iv) Glc and PWLA, and v) Glc, Leu and Trp. Plates were incubated overnight at 37°C. PA0521 (containing WT-OppA plasmid) and PA0522 (containing [Asp^{300}→Ser]-OppA plasmid), were included as positive and negative controls, respectively.

9.3.3 Results and Discussion

Table 9.2 summarises the results obtained in the growth test of mutants selected with PWLA. Normal growth was detected in all strains patched on plate containing PWLA and on plate containing both Leu and Trp amino acids (positive controls). In plates containing only Glc or Glc and one of the amino acid tested (Leu or Trp), two types of growth were observed, normal growth and poor growth as compared with the positive controls (Table 9.2). Growth without one or both amino acids, suggested the occurrence of reversion for one or both auxotrophic phenotypes, while poor growth, in the same conditions could be the result of nutrient traces in the medium and/or, essential nutrients leaking from dead cells, which could provide enough nutrients to allow some growth.

Table 9.2

Phenotype Screening of Mutant Selected with PWLA

	Minimal Medium, supplemented as follows:				
Mutants selected with PWLA	**Glucose**	**Glucose Leucine**	**Glucose Tryptophan**	**Glucose PWLA**	**Glucose Leu + Trp**
1	+	+	+	+	+
2	±	±	+	+	+
3	+	+	+	+	+
4	±	±	+	+	+
5	±	±	+	+	+
6	+	+	+	+	+
7	±	±	+	+	+
8	±	±	+	+	+
9	±	±	+	+	+
10	+	+	+	+	+
11	±	±	+	+	+
12	±	±	+	+	+
13	+	+	+	+	+
14	+	+	+	+	+
15	±	±	±	+	+
16	±	±	±	+	+
17	±	±	±	+	+
18	+	+	+	+	+
19	±	±	±	+	+
20	±	±	±	+	+
21	+	+	+	+	+
22	±	±	±	+	+

Mutants selected with PWLA	Glucose	Glucose Leucine	Glucose Tryptophan	Glucose PWLA	Glucose Leu + Trp
23	±	±	±	+	+
24	+	+	+	+	+
25	±	±	±	+	+
26	±	±	±	+	+
27	±	±	±	+	+
28	+	+	+	+	+
29	±	±	±	+	+
30	±	±	+	+	+
31	+	+	+	+	+
32	+	+	±	+	+
33	+	+	±	+	+
34	+	+	±	+	+
35	+	+	+	+	+
36	+	+	+	+	+

Bacterial were streaked in minimal medium agar plate supplemented with Glc, Leu and Trp, in presence of chloramphenicol (34μg/ml), and grown for 24 hours, at 37°C. Bacterial strains were then patched on minimal medium agar plate supplemented as indicated above, and grown overnight at 37°C. Symbols: (±): poor growth, (+): normal growth.

9.4 Second Screening to Detect Auxotrophic Phenotype in Mutants Selected with PWLA

9.4.1 Introduction

Based on the results obtained in section 9.3.3, 16 from 20 strains, that showed poor growth in both minimal medium agar plate, supplemented only with Glc and Glc and Leu, respectively (Table. 9.2), were selected for a second screening to detect auxotrophic reversion. This test was performed by using a more sensitive method, in order to minimise the presence of traces of amino acids in the growth medium. Two strains that showed a normal growth (referred in Table 9.2 as 3 and 6, respectively) were included as controls.

9.4.2 Methods

The 16 mutant strains selected as described in section 9.4.1, were designated (in order) as PA0590 to PA0605, also the two strains that showed the reversion of both Leu and Trp, were selected as control for future experiments. these strains were designated as PA0588 and PA0589.

Above-mentioned strains, including other controls (M2034, PA0522, PA0078, PA0081 and PA0082) were grown overnight in 5 ml minimal medium supplemented with Glc, Leu and Trp, with shaking at 37°C. Cells were harvested by centrifugation and washed with two volumes of minimal medium without supplements. Testing was performed by spotting 3µl of washed cells (3 x 10^6 cells) on Noble agar plates supplemented as follow: i) Only Glc, ii) Glc and Leu iii) Glc and Trp iv) Glc and PWLA, and v) Glc, Leu and Trp. Plates were incubated 24 hours at 37°C.

Table 9.3
Phenotype Screening of Mutants Selected with PWLA, by testing the growth in Minimal Medium, Noble Agar plate supplemented as follows:

Mutants selected with PWLA	Glucose	Glucose Leucine	Glucose Tryptophan	Glucose PWLA	Glucose Leu + Trp
PA0590	–	–	+	+	+
PA0591	–	–	+	+	+
PA0592	–	–	+	+	+
PA0593	–	–	+	+	+
PA0594	–	–	+	+	+
PA0595	–	–	+	+	+
PA0596	–	–	+	+	+
PA0597	+	+	+	+	+
PA0598	–	–	+	+	+
PA0599	–	–	+	+	+
PA0600	–	–	+	+	+
PA0601	–	–	+	+	+
PA0602	–	–	+	+	+
PA0603	–	–	+	+	+
PA0604	–	–	+	+	+
PA0605	–	–	+	+	+
Controls					
PA0588	+	+	+	+	+
PA0589	+	+	+	+	+
M2034 (*leu*⁻, *trp*⁻)	–	–	–	±	+
PA0522 (*leu*⁻, *trp*⁻, [pBAN2])	–	–	–	–	+
PA0078 (*leu*⁺, *trp*⁻)	–	–	+	+	+
PA0081 (*leu*⁻, *trp*⁺)	–	+	–	±	+
PA0082 (*leu*⁺, *trp*⁺)	+	+	+	+	+

Bacterial strains were grown overnight in 5 ml minimal medium supplemented with Glc, Leu and Trp, in presence of chloramphenicol (30μg/ml) when required, with shaking at 37°C. Portion of 3 μl of washed cells were spotted onto the plates and grown overnight. Symbols:(–)no growth, (±)poor growth, (+)normal growth, pBAN2([Asp ^{300}→Ser]-OppA).

9.4.3 Results and Discussion

In this method, the presence of small amounts of specific nutrients in the growth medium was minimised by using Noble agar and washed cells. Table 9.3 summarises the results obtained in this growth test. All mutants selected with PWLA, showed normal growth in both plates supplemented with Glc and PWLA, or supplemented with Glc, Leu and Trp (Fig. 9.2 D and E, positive controls).

Mutants selected in PWLA, spotted on tryptophan-containing plate (Fig. 9.2 A), showed the same intensity of growth as the positive controls, while strains with *leu*⁻ genotype (M2034, PA0522 and PA0081) were unable to grow in the same conditions, suggesting the reversion of the requirement of leucine for growth in minimal medium in all mutants selected with PWLA.

Analyses of plates where tryptophan or PWLA was absent, (Fig. 9.2 B and C), showed that only PA0588, PA0589 and PA0597 (Fig. 9.2, B and C, square 1,2 and 10), were able to grow. These results show that the mutants selected with PWLA, except the strain PA0597, retained the Trp⁻ phenotype, and confirm the auxotrophic reversion in both, PA0588 and PA0589 strains, observed in previous experiments (Table 9.2).

The results obtained in this screening indicated that all strains tested gained Leu^+ phenotype. In *E. coli*, a typical gene has a mutation rate between 2×10^{-6} and 2×10^{-8} per gene per generation (Witkin, 1994). Considering these data, the frequency for obtaining a double mutation in *E. coli* per generation, could be between 1×10^{-12} and 1×10^{-16}, which could be almost impossible to find on a single agar plate inoculated with approximately 5×10^8 cells.

The double mutation observed in these strains, could be interpreted in the following way. The growth of strain PA0522 (*leu*⁻, *trp*⁻, pBAN2 - [$Asp^{300} \rightarrow Ser$]) on minimal medium plate containing Glc and 0.5 mM PWLA in top agar, as only source of Leu and Trp, could occur by a mutation in the oligopeptide permease, regained a phenotype resembling wild type strain. This mutation allows PWLA transport, which satisfies the requirements for tryptophan, but is insufficient to cover the requirement for leucine, which is much higher [the concentration of leucine in *E. coli* is 428μmol/g of dried cells, whereas the concentration of tryptophan is 54μmol/g of dried cells, (Neidhardt and Umbager, 1996)].

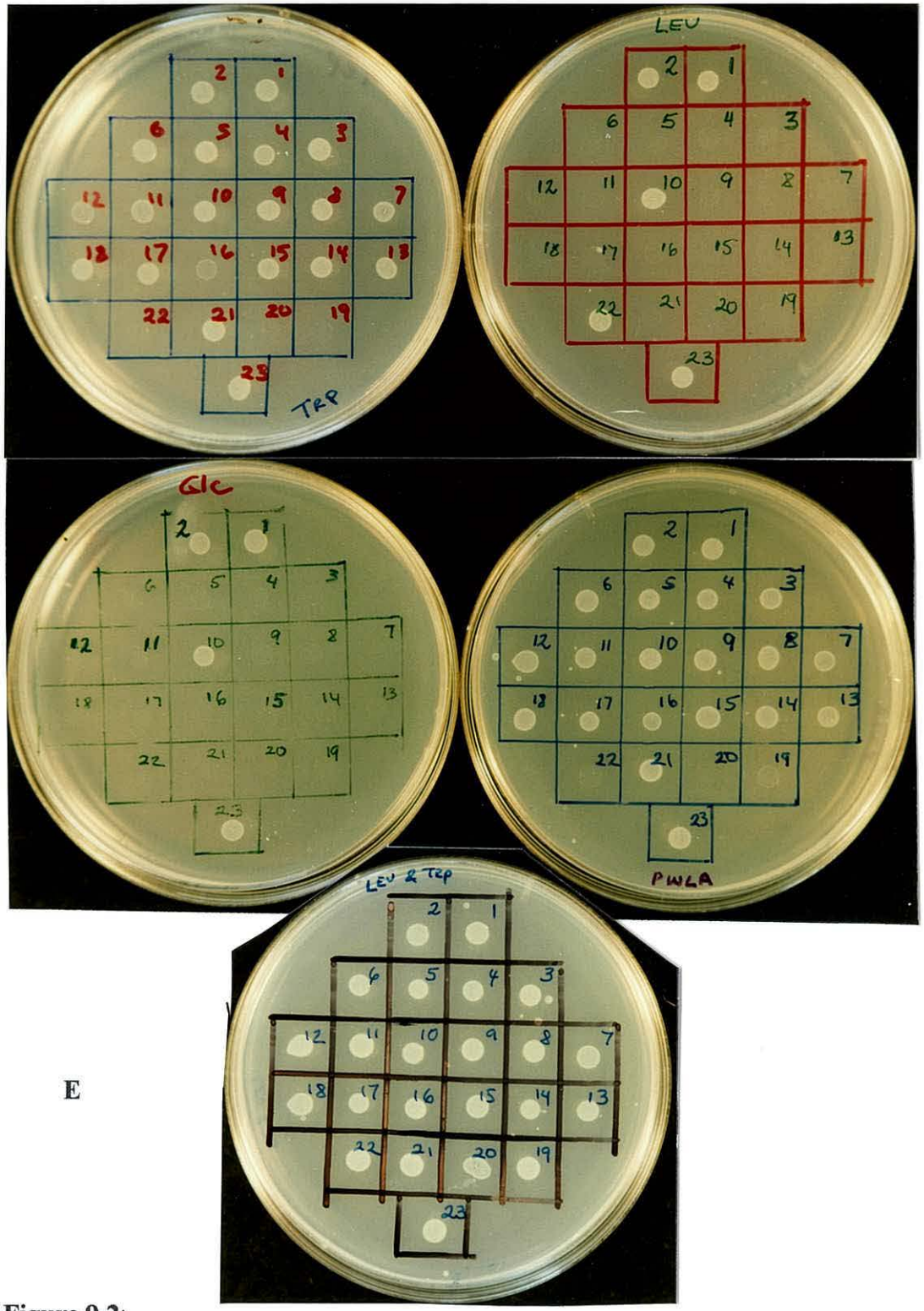


Figure 9.2:
Screening for requirement of Leu and Trp for grow, in mutants selected with PWLA. Minimal medium Noble agar plates containing Glc and supplemented as indicated above, were spotted with approximately 3 x 10^6 washed cells, and grown overnight at 37°C. (1-2):PA0588 and PA0589, included as controls; (3-18): PA0590 to PA0605 (mutants selected with PWLA); (19): M2034 (*leu*$^-$, *trp*$^-$); (20): PA0522 (*leu*$^-$, *trp*$^-$, pBAN2); (21): PA0078 (*leu*$^+$, *trp*$^-$); (22): PA0081 *leu*$^-$ *trp*$^+$); (23) PA0082 (*leu*$^+$, *trp*$^+$).

Consequently, only slow leu-limited growth could be possible. This situation allows circumstances for enrichment of fast-growing Leu^+ revertants to arise. This when the mutants were streaked for single colonies on PWLA (section 9.2.3) and individual colonies were grown as streaks for phenotype testing (section 9.3.2) the opportunity for faster-growing, Leu^+ revertants to accumulate in the culture arose.

This assumption was supported by the fact that the strain M2034 (*leu*$^-$, *trp*$^-$) as well PA0081 (*leu*$^-$, *trp*$^+$), showed a very poor growth in minimal medium supplemented only with Glc and PWLA (Fig. 9.2, D square 19 and 22), while the same strains showed a good growth in Glc-minimal medium plate supplemented with both PWLA and leucine (Fig. 9.3). A further, related feature is that leucine is involved in the action of Leucine-responsive regulatory protein or Lrp, a global regulator of gene expression in *E. coli*, and specifically increases the expression of OppA (Newman *et al.*, 1996; Newman and Lin, 1995); the possible influence of this on the effect seen here if difficult to predict.

The group of mutants that gained Opp^+ phenotype, was referred as compensative mutants, and selected for further screening.

9.5 Production and Characterisation of Leucine Revertant Mutants

9.5.1 Introduction

As shown in section 9.4.3, all compensative mutants regained *Leu*$^-$phenotype, therefore control experiments were designated to confirm that a leucine reversion alone in PA0522 strain (*Leu*$^-$, *Trp*$^-$, pBAN2), could not enable the strain to grow with PWLA as sole tryptophan source.

9.5.2 Methods

Leucine revertant mutants, were selected in minimal medium agar plate containing chloramphenicol (34µg/ml), and supplemented with Glc and 0.2 mM tryptophan, by using the methodology described in section 9.2.2, and re-isolated by streaking for single colonies in the same selection medium.

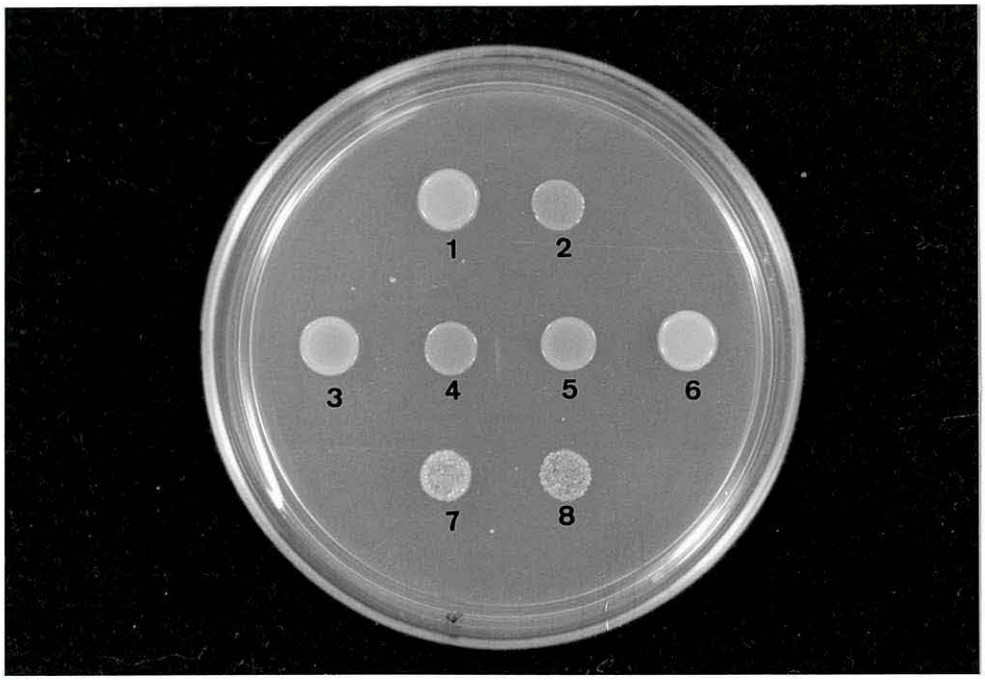


Figure 9.3:

Spot-patch to test the ability of several *E. coli* strains to grow with PWLA in presence of leucine. Bacteria grown overnight in minimal medium supplemented as required, were washed and spotted as described in section 9.4.2. (1): M2034 (*leu*$^-$, *trp*$^-$);(2): PA0521 (*leu*$^-$, *trp*$^-$,pPI5.1); (3): PA0078 (*leu*$^+$, *trp*$^-$); (4): PA0081 (*leu*$^-$, *trp*$^+$); (5): PA0082 (*leu*$^+$, *trp*$^+$); (6): PA0588 (*leu*$^+$, *trp*$^+$); (7 and 8): PA0596 and PA0597, respectively (mutants selected with PWLA).

The phenotype of these mutants was tested by spotting cell suspension, on minimal medium Noble agar plates, supplemented with Glc and i) Trp, ii) PWLA and iii) Trp and Leu; using the same methodology and controls described in section 9.4.2.

The sensitivity to toxic peptides Ala_4P and Orn_3, was measured by using both radial streak and pour plate techniques, as described in sections 4.6.2 and 4.6.1, respectively. The amount of Ala_4P applied onto the antibiotic disc were 0.2µmol and 12.5nmol for radial streak and pour plate, respectively, and the amount of Orn_3 applied onto the disc was 1µmol and 0.5µmol for both radial streak and pour plate, techniques.

9.5.3 Results and Discussion

The frequency of auxotrophic reversion for Leucine was 8 x 10^{-9}. An average of two colonies per plate was picked and re-isolated by streaking for single colonies on a minimal medium agar plate containing Glc, chloramphenicol (34μg/ml) and 0.2 mM tryptophan. The re-isolated colonies were characterised by testing their ability to transport peptides. All Leu revertant strains showed normal growth in plates where tryptophan or both Leu and Trp were added (Fig. 9.4). Analyses of these revertant strains spotted on plate containing PWLA, showed some slight growth at the edge of the drops and some single colonies inside the circumference of the drops.

Experiments were designated in order to verify the activity of the oligopeptide permease in these mutants. The sensitivity to toxic peptides Ala_4P and Orn_3, which are transported specifically by the oligopeptide permease in *E.coli* (Allen *et al.*, 1978; Payne and Gilvarg, 1968), were tested.

Table 9.4 shows the results obtained with both antibiotics tested by radial streak. The 19 Leu revertant strains tested, were resistant to both Ala_4P and Orn_3 toxic peptides. Because this test is semi-quantitative since it is difficult to control the number of cells per streak, four Leu revertant strains were randomly selected to test the same toxic peptides by using pour plate technique. The latter method is used to quantify antibacterial agents. No inhibition growth was detected in any of the Leu revertant strains tested with both toxic peptides. Typical radial streak plate and pour plate obtained in this test, are shown in Figure 9.5 A and B, respectively.

Because all the Leu revertant mutants were resistant to both toxic peptides tested, the growth at the edge of the drops observed in plate containing PWLA, could be due to the presence of new mutants.

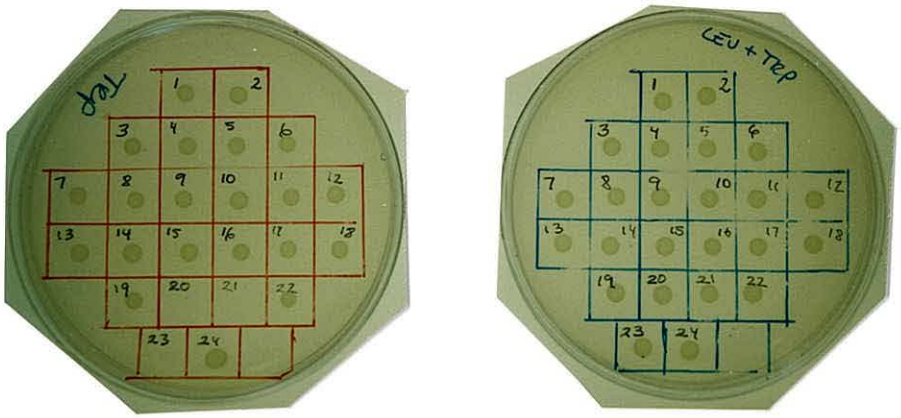


Figure 9.4:

Spot-patch to test the ability of Leu revertant strains to grow in: (**A**) Minimal medium Noble agar plates containing glucose and supplemented with Trp, and (**B**) Minimal medium Noble agar plates containing glucose and supplemented with Trp and Leu. (1-19): mutants selected with Trp, (20): M2034 (*leu*$^-$,*trp*$^-$), (21): PA522 (*leu*$^-$, *trp*$^-$,pBAN2), (22): PA0078 (*leu*$^+$, *trp*$^-$), (23): PA0081 (*leu*$^-$, *trp*$^+$), (24): PA0082 (*leu*$^+$, *trp*$^+$).

Table 9.4

Sensitivity of Several *E. coli* Leucine Revertant Mutants to Toxic Peptides, Analysed by Using Radial Streak Technique

Leu-revertant Mutants	Orn_3 (1 µmol)	Ala_4P (0.2 µmol)
I	R	R
II	R	R
III	R	R
IV	R	R
V	R	R
VI	R	R
VII	R	R
VIII	R	R
IX	R	R
X	R	R
XI	R	R
XII	R	R
XIII	R	R
XIV	R	R
XV	R	R
XVI	R	R
XVII	R	R
XVIII	R	R
XIX	R	R
Controls		
PA0467	R	R
M2034	S	S
PA0522	R	R
PA0523	R	R
PA0591	S	S
PA0593	S	S

PA0467, *oppA*$^-$; M2034, wild type; PA0522, containing (^{300}Asp→Ser) plasmid; PA0523, containing (Asp^{300}→Arg) plasmid; PA0591 and PA0593, compensative mutants (Opp$^+$, *trp*$^-$, *leu*$^+$).

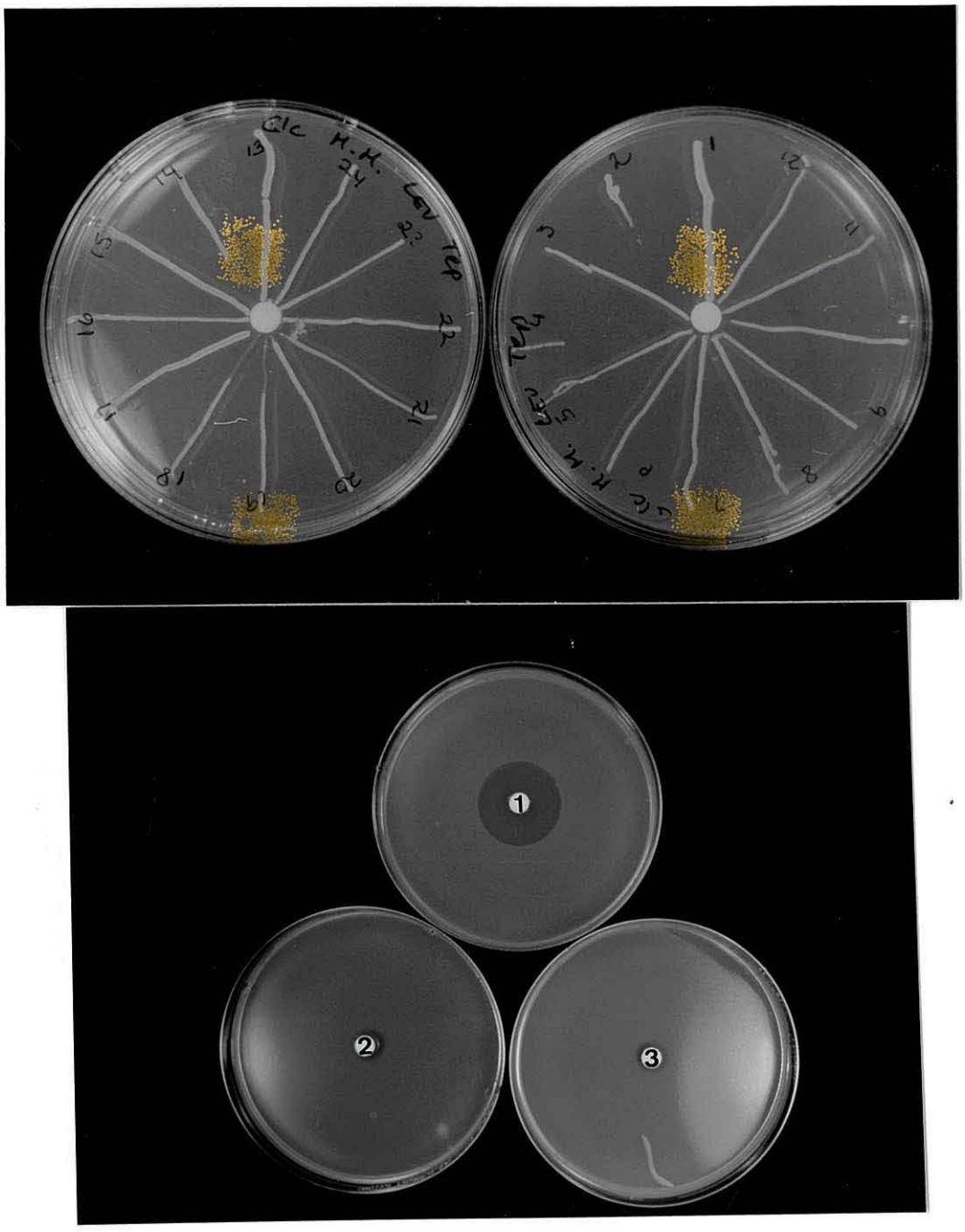


Figure 9.5:
Typical inhibition plates obtained by testing the sensitivity of Leu revertant mutants to toxic peptides. (**A**) radial streak: (1&13) PA0467, (2&14) M2034 (wild-type), (3&15) PA0522 [Asp^{300}-Ser]-OppA mutant, (4) PA0591 (Compensative mutant), (5-12) Leu revertant mutant strains, (16) PA0522, (17) PA0523, (18&19) PA0591 & PA0592 (Compensative mutant), (20-24) Leu revertant mutant strains and (**B**): pour plate: (1) PA0521, (2&3) Leu revertant mutant strains.

9.6 Characterisation of Compensative Mutants

9.6.1 Response of the Compensative Mutants to Toxic Peptides

9.6.1.1 Introduction

To study the reversion of the oligopeptide permease activity, in the compensative mutants, sensitivity to toxic peptides was examined. Ala_4P, which is an inhibitor of D-alanine racemase, and Orn_3, which is an inhibitor of protein synthesis, were used as antibiotics. Both are transported specifically by the oligopeptide permease in *E.coli* (Allen *et al.*, 1978; Payne and Gilvarg, 1968).

9.6.1.2 Method

Sensitivity to antibiotics was tested using the pour plate method described in section 4.6.1. The ability of an antibiotic to inhibit bacterial growth was quantified by determining the diameter of the growth zone surrounding the antibiotic containing disc.

9.6.1.3 Results and Discussion

Figure 9.6 shows typical pour plates obtained from the compensative mutants, using 3, 6 and 12 nmole of Ala_4P and 125, 250 and 500 nmole of Orn_3. Table 9.5 shows the inhibition zones (mm) determined for each concentration of antibiotic used.

All compensative mutants were inhibited by both antibiotics tested. Strains were divided into two groups: strains that showed inhibition zones approximately equal or smaller than the zones found for the wild type strain (PA0521), were classified under Group I; and strains that showed inhibition zones bigger than those found for the wild type strain (PA0521), were classified under Group II (Table 9.5).

The diffuse inhibition zone, observed when Orn_3 was tested in the compensative mutants (Fig. 9.6 A and B) as compared with wild type strain (Fig. 9.6 C) could be interpreted as a low level of restoration of the oligopeptide permease function, which may allow growth on PWLA and uptake of Ala_4P, which are normally good substrates, but not allow uptake of Orn_3, which is normally a poor substrate (Tyreman, 1990), at a rate sufficient to produce the same inhibition showed in the wild type strain.

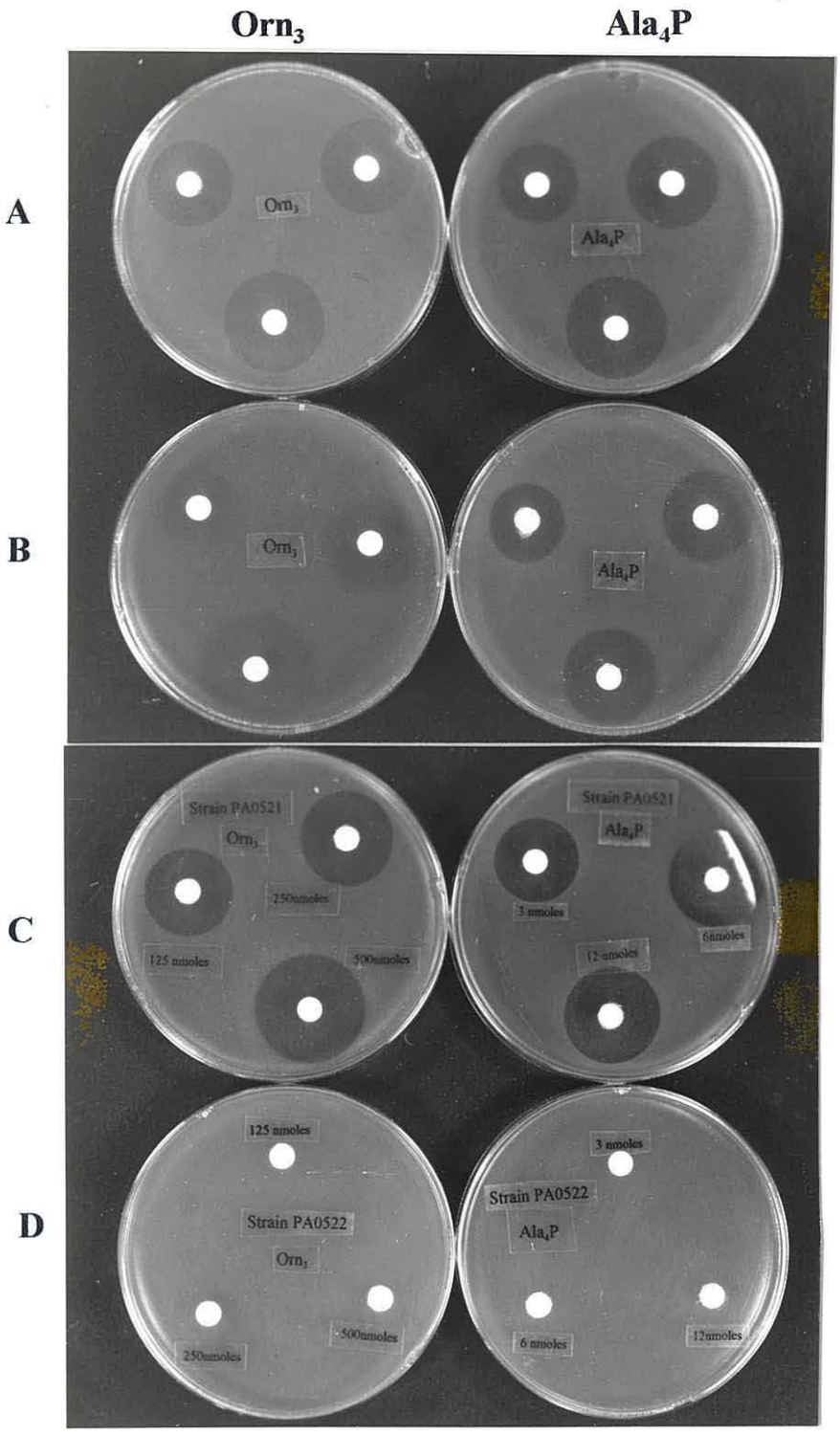


Figure 9.6:

Inhibition plate assay testing the sensitivity to toxic peptides, of the following *E. coli* strains: (**A**): PA0591(compensative mutant, from Group I), (**B**) PA0605 (compensative mutants, from Group II), (**C**): PA0521 (containing pPI5.1 [WT-OppA plasmid]), (**D**): PA0522 containing the [Asp $^{300}\rightarrow$Ser]-OppA plasmid. Discs 1-3: 125, 250 and 500 nmoles of Orn_3, respectively. Discs 4-6: 3, 6 and 12 nmoles of Ala_4P, respectively.

Table 9.5

Characterisation of compensative mutants according to their sensitivities to antibiotics, by using pour plate technique

	mm of inhibition zones						
	nmoles of Orn_3			nmoles of Ala_4P			
	125	**250**	**500**	**3**	**6**	**12**	
Strain							**Group**
PA0590	17	22	27	ND	ND	ND	I
PA0591	9	20	24	19	22	24	I
PA0592	18	22	29	19	21	25	I
PA0593	19	22	26	20	22	25	I
PA0594	16	21	31	21	24	26	I
PA0595	15	25	29	20	22	24	I
PA0596	15	24	30	21	23	25	I
PA0598	18	25	31	21	22	27	I
PA0599	15	24	30	21	24	29	I
PA0600	27	31	34	22	25	36	II
PA0601	27	29	32	21	23	26	II
PA0602	28	30	33	22	24	25	II
PA0603	28	30	32	21	24	27	II
PA0604	26	28	32	22	29	32	II
PA0605	31	36	41	25	27	29	II
PA0521	22±0.5	25 ±	28±0.	22±1.	25.5±0.	27±1.1	Control
PA0522	R	R	R	R	R	R	Control

Pour plate was performed as described in section 4.6.1. PA0590 to PA605: compensative mutants; PA0521, containing wild type OppA plasmid; PA0522, is the parent strain (containing [$Asp^{300} \rightarrow Ser$]-OppA plasmid).Abbreviation used :ND, test not determined.

Both groups I and II showed antibiotic sensitivities that suggests a mutation in the oligopeptide permease that compensates the loss of biological activity showed by the parent strain PA0522. However, their phenotypes are not strictly wild type (PA0521). Group I generally show lower antibiotic sensitivity, particularly to Orn_3, whereas group II generally show increased antibiotic sensitivity. It is not clear whether this effect may arise from changes in OppA or a membrane protein(s) of the oligopeptide permease system.

9.6.2 Expression of OppA

9.6.2.1 Introduction

OppA is one of the most abundant periplasmic proteins, representing 5-8% of the total (Higgins and Hardie, 1983; Guyer *et al.*, 1985; Tyreman *et al.*, 1992). Both *E.coli* PA0522 (containing the [Asp^{300}→Ser] OppA plasmid) and PA0523 (containing the [Asp^{300}→Arg] OppA plasmid) overexpress [Asp^{300}→Ser] OppA and [Asp^{300}→Arg] OppA, respectively (see section 8.6). In order to investigate the expression in the compensative mutants, a crude periplasmic protein fraction of the strains selected in section 9.1.3 was prepared and analysed by SDS-PAGE.

9.6.2.2 Methods

Bacterial strains were grown in 3 ml LB medium as described in section 4.2.

Cold osmotic shock was carried out as described in section 4.7.1.

Periplasmic protein fractions were analysed by SDS-PAGE, which was performed as described in section 4.9.1.

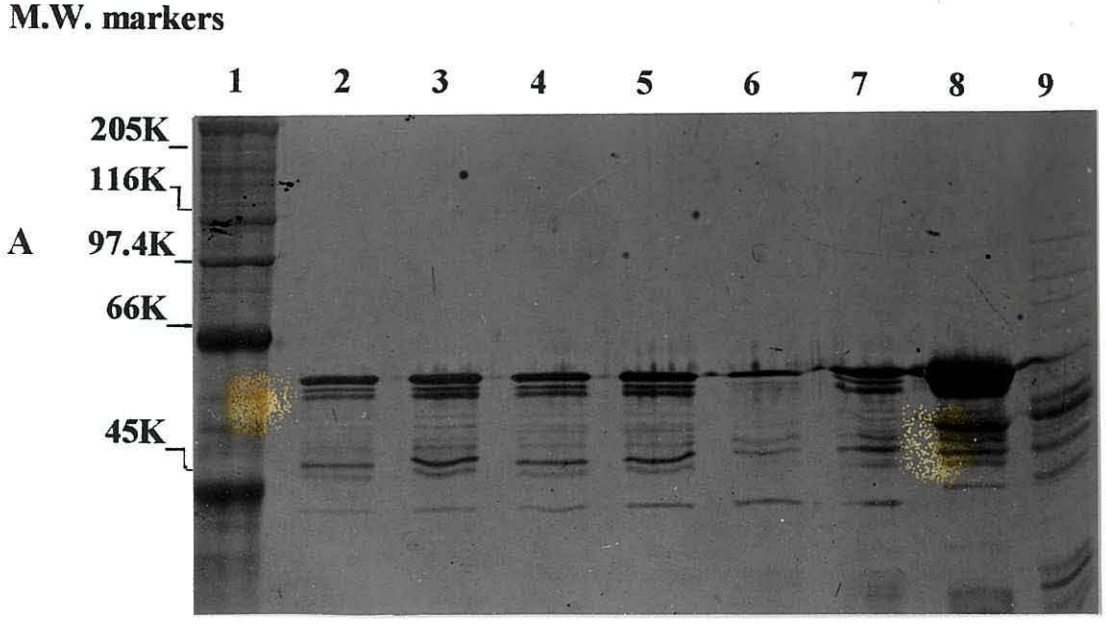
9.6.2.3 Results and Discussion

The individual components of the crude periplasmic protein fraction, from the 15 compensative mutants, were resolved by SDS-PAGE (Fig.9.7). The following strains were included as controls: PA0521, containing the wild type OppA plasmid (lane 7 in Gels C and D, respectively); PA0467 the parent *OppA*$^-$ strain (lane 9 in Gels A and B, respectively); PA0522 the parent strain containing the [Asp $^{300}\rightarrow$Ser]-OppA plasmid (lane 8 Gels C and D, respectively); PA0597 (Gel B, lane 3), PA0588, and PA0589 (lanes 3 and 4 in Gel C), that are Leu and Trp revertant mutants; WT-OppA isolated by ion-exchange chromatography (lane 8 in Gels A and B, respectively), and [Asp $^{300}\rightarrow$Ser]-OppA isolated by ion-exchange chromatography (lane 9 in Gels C and D, respectively). All compensative mutant strains showed expression of OppA.

9.6.3 Characterisation of Compensative Mutants According to their Isoelectric Point

9.6.3.1 Introduction

In previous studies Tyreman (1990), using an isoelectric focusing assay, showed that both peptide binding proteins, OppA and DppA, from *E. coli* underwent characteristic pI changes upon peptide binding. In section 8.7, evidence that indicates changes in the pI of a protein can be taken as an indication of conformational change, was presented. In order to investigate if OppA from compensative mutants showed a pI characteristic of the wild type, the Asp→Ser mutant and/or underwent a characteristic conformational change in its structure, pI analysis of OppA from these mutants was carried out.



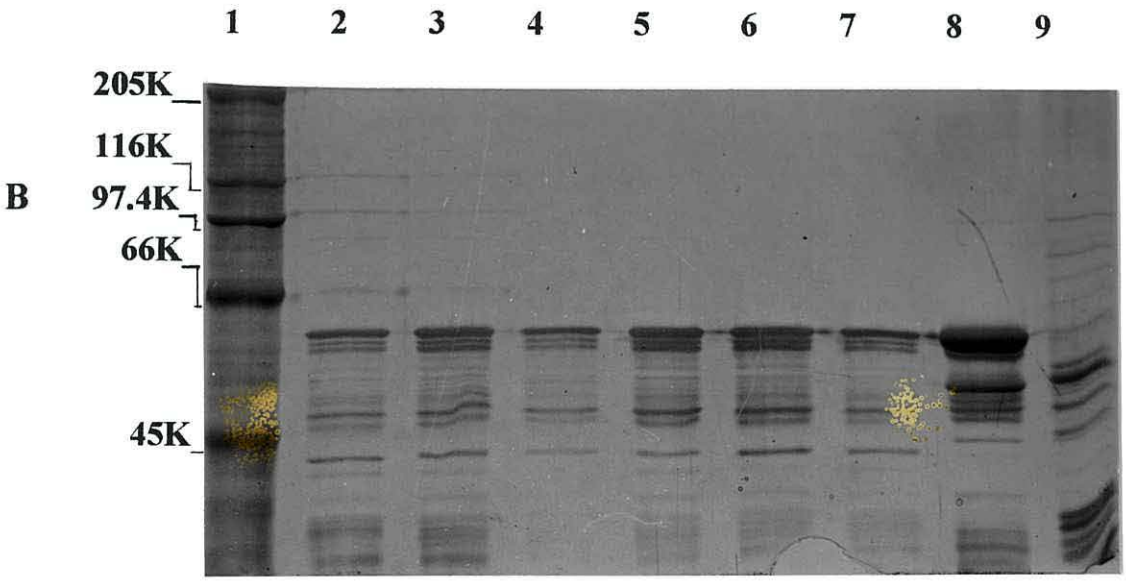
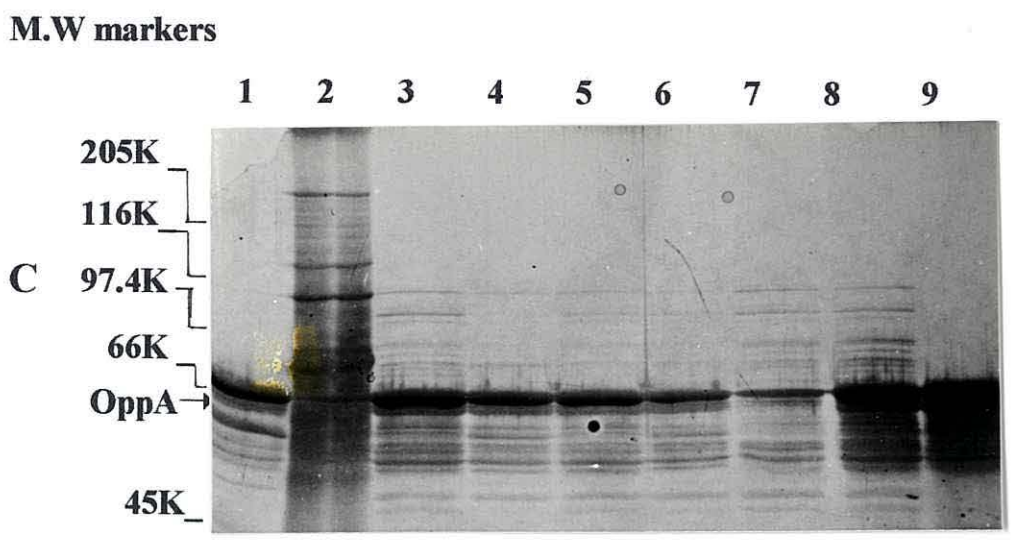


Figure 9.7 A and B:

Coomassie blue-stained SDS gel, showing the periplasmic protein fraction from the compensative mutants. Gel **A**: lane 1: M.W. markers; lanes 2- 7: 2.0, 2.25, 2.5, 2.25, 2.5 and 2.0µg of P.P.F from PA0590, PA0591, PA0592, PA0593, PA0594, and PA0595 compensative mutants, respectively; lanes 8: 5µg of WT-OppA; lane 9: 5µg of P.P.F. PA0467 (*oppA*$^-$). Gel **B**: lane 1: M.W. markers; lanes 2- 5: 1.75,1.75, 2.4 and 2.25µg of P.P.F from PA0596, PA0597, PA0598, and PA0599 compensative mutant, respectively; lane 6: 2.5µg of P.P.F. from PA0521 (containing the WT-OppA plasmid); lanes 7: 2.5µg of P.P.F. from PA0522 (containing the [Asp^{300}→Ser] OppA plasmid); lanes 8: 5µg of WT-OppA; lane 9: 5µg of P.P.F. PA0467 (*oppA*$^-$).



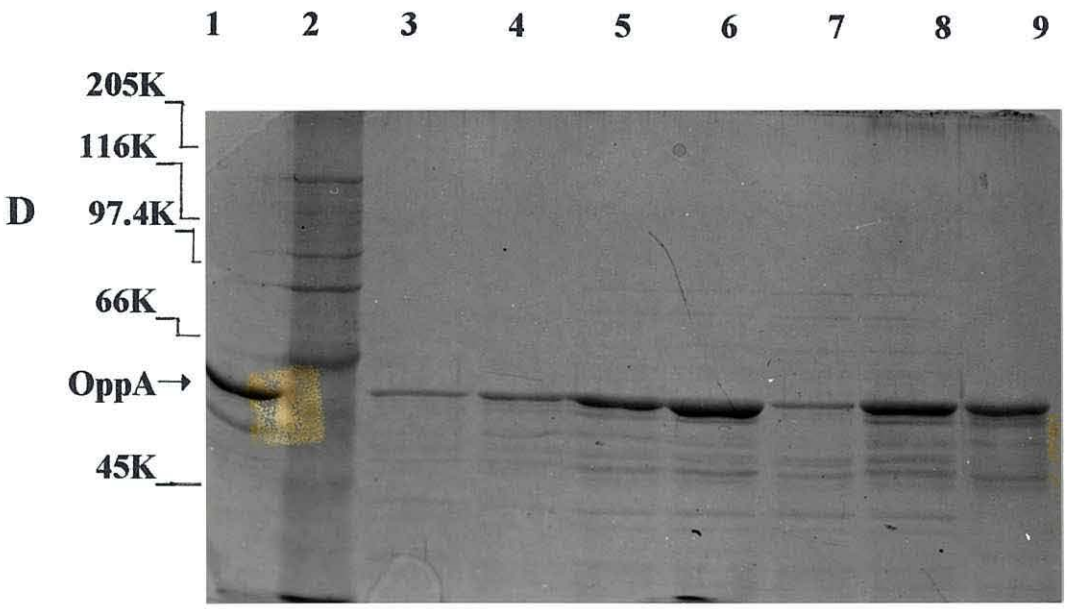


Figure 9.7 C and D:

Coomassie blue-stained SDS gel, showing the periplasmic protein fraction (P.P.F.) from the compensative mutants. Gel **C**: lane 1: 3µg WT-OppA; lane 2: M.W. markers; lanes 3 and 4: 5µg of P.P.F. from PA0588 and PA0589 Leu and Trp revertant mutants, respectively; Lanes 5 and 6: PA0600 and PA0601 compensative mutants, respectively; lanes 7 and 8: 5µg of P.P.F. from PA0521(containing the WT-OppA plasmid) and PA0522 (containing the [Asp^{300}→Ser] OppA plasmid), respectively; lane 9: 5µg of [Asp^{300}→Ser] OppA. Gel **D**: lane 1: 3µg OppA-WT; lane 2: M.W. markers; lanes 3 - 6: 5µg of P.P.F. from PA0602, PA0603, PA0604 and PA0605 compensative mutants, respectively; lanes 7 and 8: 5µg of P.P.F. from PA0521(containing the WT-OppA plasmid) and PA0522 (containing the [Asp^{300}→Ser] OppA plasmid), respectively; lane 9: 5µg of [Asp^{300}→Ser] OppA.

9.6.3.2 Methods

All strains studied were grown in both LB and minimal media, as described in section 4.2, and 20 ml of bacterial culture were used for isolation of periplasmic protein by using cold osmotic shock (see section 4.7.1). The pI of OppA was determined using isoelectric focusing and calculated from a linear calibration plot as described in section 4.9.2. The theoretical pI was calculated by using the Isoelectric Point Program from CGC package on Seqnet Daresbury Laboratory.

9.6.3.3 Results and Discussion

A typical IEF gel of the periplasmic protein fraction is shown in Figure 9.8. Table 9.6 shows the pI from all groups of the compensative mutants grown in LB medium.

OppA from group I showed a single band with pI values in the range 6.5 to 6.67; when the periplasmic protein fraction was incubated with $LysAla_2$ a single band was observed with pI value in the range 6.8 to 6.88, showing a shift in the range 0.15 to 0.3 pI units.

Different results were observed between the individual strains of group II. Four strains (PA0601, PA0602, PA0603, and PA0604) revealed a single band with pI value in the range 6.56 to 6.61. When the periplasmic protein fraction was incubated with $LysAla_2$ a single band with pI value in the range 6.82 to 6.88 was observed. The strain PA0600, showed a single band with pI value of 6.9, but did not show a significative pI shift when the preparation was incubated with $LysAla_2$. The strain PA0605, showed two bands with pI value of 6.84 and 6.88. Incubation with $LysAla_2$ caused an increase in the abundance of the pI 6.88 band. The former results were similar to those found in group I.

A periplasmic protein fraction from PA0521 (containing the wild type OppA plasmid) and PA0522 (containing the [Asp^{300}→Ser] OppA plasmid), were used as controls. IEF analysis of both WT-OppA and [Asp^{300}→Ser] OppA showed a single band with pI of 6.12 and 7.01, respectively. When both protein preparations were analysed after incubation with $LysAla_2$, a shift of 0.34 pI units was observed in the WT-OppA whereas no shift was observed in the [Asp^{300}→Ser] OppA (Table 9.6).

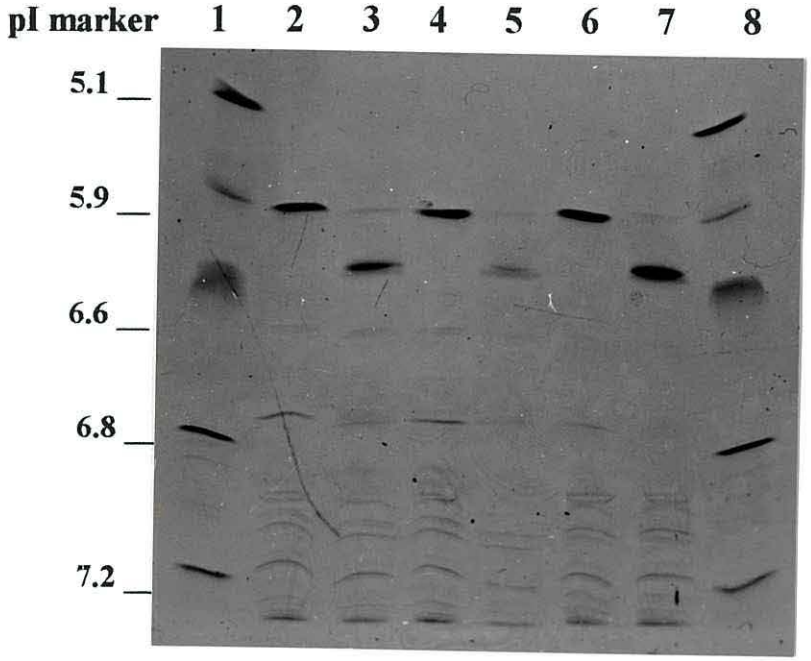


Figure 9.8:

IEF analysis of periplasmic protein fractions of compensative mutants and the ability of OppA to bind peptide. Lane 1 and 8: pI markers; lanes 2, 4 and 6, 12µg of P.P.F. from PA0604, PA0591, and PA0592 (compensative mutant), respectively; lanes 3, 5 and 7, 6µg of P.P.F.from PA0604, PA0591, and PA0592 (compensative mutant), respectively incubated with 0.01moles of $LysAla_2$.

Table 9.6

Characterisation of compensative mutants according to their OppA pI

Strain	Group	OppA	OppA-$LysAla_2$	pI shift
PA0590	I	6.5	6.8	0.3
PA0591	I	6.61	6.88	0.27
PA0592	I	6.61	6.88	0.27
PA0593	I	*6.64	*6.86	0.22
PA0594	I	6.67	6.82	0.15
PA0595	I	6.6	6.84	0.24
PA0596	I	6.6	6.84	0.24
PA0598	I	6.53	6.82	0.29
PA0599	I	6.53	6.82	0.29
PA0600	II	6.9	6.87	0.03
PA0601	II	6.56	6.82	0.26
PA0602	II	6.56	6.82	0.26
PA0603	II	6.56	6.82	0.26
PA0604	II	6.61	6.88	0.27
PA0605	II	*6.84-6.88	6.88	0.04
PA0521	Control	6.12	6.46	0.34
PA0522	Control	*7.01	*7.01	0
WT-OppA	RPC	6.2	6.43	0.23
[Asp^{300}→Ser]Opp	RPC	6.54	7.02	0.46

Bacterial strains, were grown in LB medium as described in section 4.2. 12 µg of periplasmic protein preparation, were used to load the IEF gel, also 5 or 10 mM $LysAla_2$ were incubated with the preparation to produce the liganded form of OppA. IEF was done as described in section 4.10.2 and pI was determined as described in the same section. *Average of two or three experiments; PA590 to PA605, compensative mutants; PA0521, containing WT OppA plasmid; PA0522, containing [Asp^{300}→Ser] OppA plasmid; OppA purified by using Reversed Phase Chromatography (RPC).

However, IEF analysis of both WT- and [Asp^{300}→Ser] OppA proteins purified by ion-exchange chromatography (Table 9.7), showed more than one band. Three main components for WT-OppA (pI in the range 6.2 to 6.8) and four main components for [Asp^{300}→Ser] OppA (pI in the range 6.54 to 6.8) corresponding to a mixture of liganded and unliganded forms. In addition to these results, IEF experiments were carried out using the single protein components purified by reverse phase chromatography. Both WT-OppA and [Asp^{300}→Ser] OppA proteins showed a single band with pI values of 6.2 and 6.54 respectively. A shift to pI 6.43 and 7.02 was observed for both proteins in the presence of the substrate $LysAla_2$ (Table 9.7).

Table 9.7

Isoelectric Point Analysis of Both WT-OppA and [Asp^{300}→Ser] OppA at Different Steps of Their Purification

Protein	Sample preparation	N° of bands	pI		
			pI range	OppA+ Lys-Ala_2	Shift
WT-OppA	PPF	1	6.12	6.46	+0.34
[Asp^{300}→Ser] OppA	PPF	1	7.01	7.01	0
WT-OppA	IEC		6.2 to 6.8		
[Asp^{300}→Ser] OppA	IEC	1	6.54 to 6.8		
WT-OppA	RPC	1	6.2	6.43	+0.23
[Asp^{300}→Ser] OppA	RPC	1	6.54	7.02	+0.46

Abbreviation used: IEC, ion-exchange chromatography; RPC, reverse phase chromatography; PPF, periplasmic protein fraction.

The results shown in Table 9.6, suggest that the predominant form of OppA in the crude periplasmic protein fraction, is the OppA liganded form. This could be explained by the large amount of peptides that may be present in the culture medium used. On the other hand, these pI values were different to those obtained from the periplasmic protein fraction for both controls, WT-OppA and [Asp^{300}→Ser] OppA.

In view of the above results and the complexity of PPF samples, a representative of each group was selected for further OppA pI analysis. In order to avoid a large amount of peptides in the crude preparation, all the strains were grown in minimal medium, that does not contain proteins or peptides. The pI determined with both growth conditions are summarised in Table 9.8. The results obtained for both controls PA0521(containing the WT-plasmid) and PA0522 (containing the [Asp^{300}→Ser] OppA plasmid), grown in minimal medium, matched those obtained with both OppA proteins purified by ion-exchange chromatography. The pI obtained from all the compensative mutant strains studied, after their incubation with $LysAla_2$ were very similar. Their pI values matched up with those obtained from the parent strain (PA0522). To confirm these results the periplasmic protein fractions were incubated with Ala_3, and similar results were observed (Table 9.8).

In previous experiments, it was suggested that the OppA mutation Asp^{300}→Ser caused a conformational change in the protein, and as a result the loss of its biological function, without losing the ability to bind substrates (see section 8.7). These suggestions stem from the fact that the pI shift (0.34 pI units), as a consequence of the substitution of Asp^{300}→Ser, was bigger than the theoretical pI shift expected, 0.13 pI units (Table 9.9). The same fact was observed in the liganded form of both WT-OppA and [Asp^{300}→Ser] OppA. It was not possible to compare between the pI determined from the purified OppA and the pI of the compensative mutants, because the unliganded form of the OppA was not easily detected in the crude periplasmic protein preparation. However, when these preparations were incubated with $LysAla_2$, the pI observed were similar to the pI obtained from the [Asp^{300}→Ser] OppA incubated with the same substrate (Table 9.8).

These results indicate that the recovery of the biological function of the Opp system observed in both Groups I and II, was apparently not caused by a change in their OppA.

Table 9.8

Summary of the OppA pI value obtained in three of the compensative mutants grown in different conditions

		OppA pI				
		Grown in LB medium		Grown in Minimal medium		
Strain (Sample)	Group	OppA	OppA-Lys Ala_2	OppA	OppA-Lys Ala_2	OppA -Ala_3
PA0591 (PPF)	I	6.61	6.88	6.6	6.86	6.6
PA0593 (PPF)	I	6.64	6.85	6.7	6.87	6.68
PA0605 (PPF)	II	6.84 - 6.88	6.88	6.63 - 6.73	6.87	6.63
PA0521 (PPF)	Control	6.12	6.46	6.25 - 6.61	6.56	6.68
PA0522 (PPF)	Control	7.01	7.01	6.69 - 6.83	7.0	6.78
WT-OppA (RPC purified)	Control	6.2	6.43			6.26
[Asp^{300}→Ser]-OppA (RPC purified)	Control	6.54	7.02			6.48

12 µg of total periplasmic proteins fraction (PPF) from strains grown in LB medium or minimal medium, were used to load the IEF gels, also 5 mM LysAla_2 or Ala_3 were preincubated with the preparation to produce the liganded form of OppA. IEF was done as described in section 4.10.2 and pI was determined as described in the same section. PA0521, containing WT-OppA plasmid; PA0522, containing [Asp^{300}→Ser]-OppA plasmid. PA0591, PA0593 and PA0605 are compensative mutants.

Table 9.9

Comparation between the theoretical pI of *E. coli* OppA and the pI determined by using IEF of purified OppA from both *E. coli* PA0521 (containing the wild type OppA) and PA0522 (containing the [Asp^{300}→Ser]-OppA plasmid).

Protein	Theoretical OppA pI		pI determined in RPC- purified OppA	
	pI	Shift (pI units)	pI	Shift (pI units)
WT-OppA	6.13		6.20	
WT-OppA-LysAla$_2$	6.27	+0.14	6.43	+0.23
WT-OppA-Ala$_3$	6.13	0	6.26	+0.06
[Asp^{300}→Ser] OppA	6.26		6.54	
[Asp^{300}→Ser] OppA-LysAla$_2$	6.40	+0.14	7.02	+0.48
[Asp^{300}→Ser] OppA-Ala$_3$	6.26	0	6.48	-0.06

The pI of OppA was determined by using IEF and calculated from linear calibration plot as described in section 4.10.2. The theoretical pI of both unliganded and liganded OppA forms, were calculated by using the Isoelectric Point Program from GCG package on Seqnet Daresbury Laboratory.

9.6.4 Characterisation of the OppA Gene

9.6.4.1 Studies of the Plasmid Profile

9.6.4.1.1 Introduction

In order to verify the presence of recombinant plasmid containing OppA, plasmid DNA from compensative mutants was extracted and analysed by comparing the restriction pattern with that of the original recombinant strain.

9.6.4.1.2 Methods

Plasmid DNA was purified by using the alkaline lysis method described in section 4.5.2.2, digested with restriction endonuclease enzymes, and separated on agarose gel electrophoresis as described in section 4.9.3.

9.6.4.1.3 Results and Discussion

Figure 9.9 shows the restriction analysis of plasmid isolated from three compensative mutants (lanes 3, 5, and 6). The following plasmid DNA were included as controls, pBAN2 ([Asp^{300}→Ser] OppA plasmid); pPI5.1 (WT-OppA plasmid); and plasmid isolated from PA0588 and PA0589 (Leu and Trp revertant mutants). All plasmid DNA were digested with *Sal* I and *Hind* III. The agarose gel shows two bands with size of 4.2 and 3.8 Kb, respectively. These DNA sizes equal the size of the vector (pACYC184) and the DNA insert, respectively.

The restriction pattern obtained after digesting the same sample, with *Eco*RV is shown in Figure 9.10. A single band with a size of 8.0 Kb was shown in all plasmids isolated from compensative mutant (lanes 3, 5, and 6), PA0588 (lane 2), PA0589 (Lane 4) and pBAN2 plasmid (lane 7), whereas the wild type plasmid showed two bands of 5.4 and 2.2 Kb (lane 8). The plasmid pPI5.1, which contains the wild type gene, has two *Eco*RV restriction sites. One in the vector and another within the region of the *oppA* gene targeted for the site directed mutagenesis. The plasmid pBAN2 has only a single restriction site for *Eco*RV in the vector, because the cleavage site for *Eco*RV within the *oppA* gene was destroyed by changes of a single base pair (A.T pair was exchanged with a G.C pair), without modifying the amino acid specified. With regard to plasmid size, amount and restriction sites for EcoRV and both *Sal* I and *Hind* III, no differences were observed between the plasmids isolated from the compensative mutants and the pBAN2 plasmid from the parent strain.

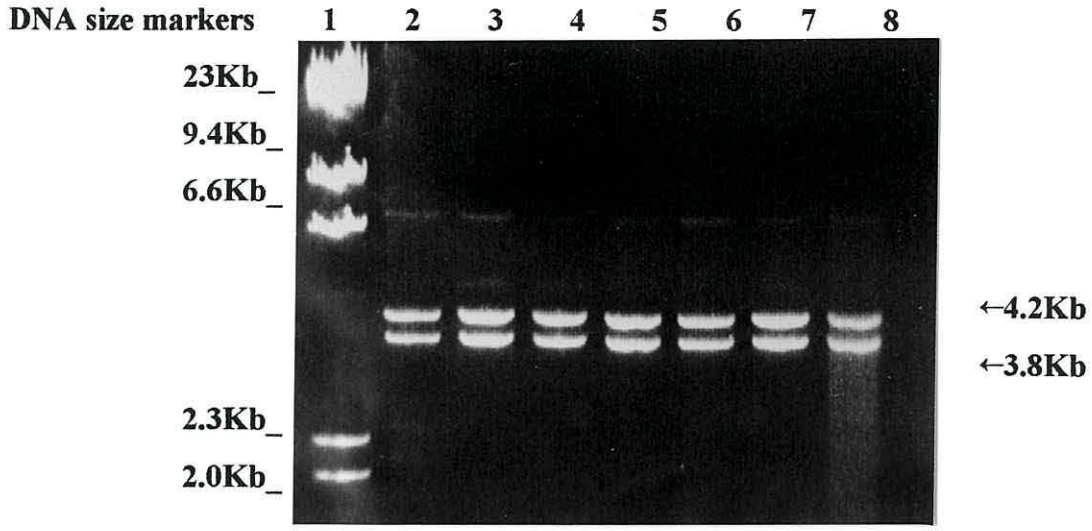


Figure 9.9:

Plasmid DNA from the compensative mutant strains digested with both restriction enzymes *Sal* I and *Hind*III. Lane 1: DNA size markers; lanes 3, 5, and 6: plasmid DNA from PA0591, PA0593 and PA0605 compensative mutants respectively; lanes 2 and 4: PA0588 and PA0589 (Leu and Trp revertant mutants); lane 7: pBAN2 ([Asp^{300}→Ser]OppA plasmid); lane 8: pPI5.1 (WT-OppA plasmid).

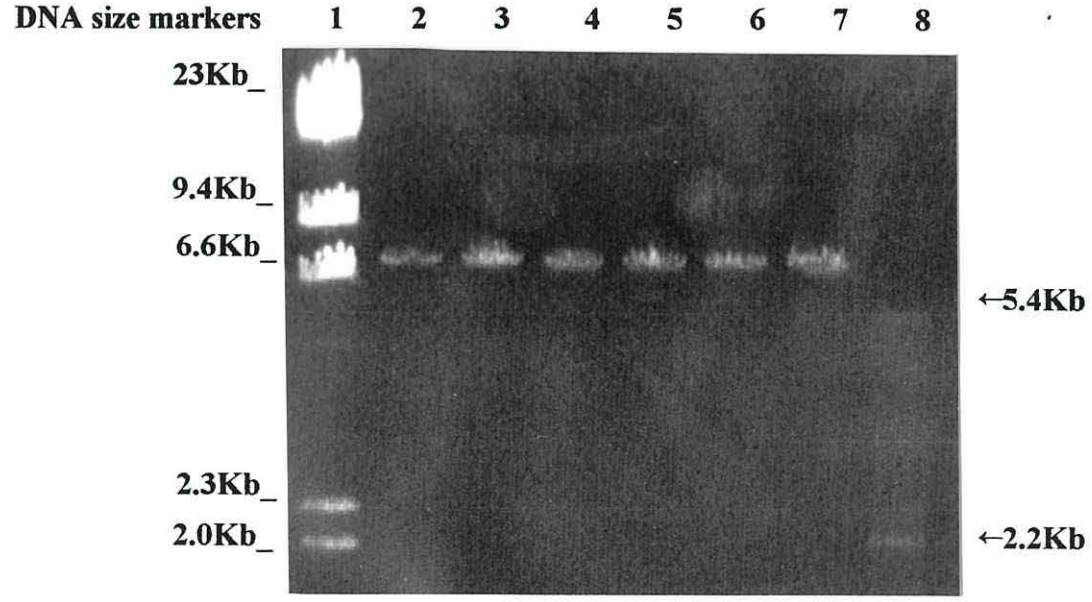


Figure 9.10:

Plasmid DNA from the compensative mutant strains digested with *Eco*RV restriction enzyme. Lane 1: DNA size markers; lanes 3, 5, and 6: plasmid DNA from PA0591, PA0593 and PA0605 compensative mutants respectively; lanes 2 and 4: PA0588 and PA0589 (Leu and Trp revertant mutants); lane 7: pBAN2 ([Asp^{300}→Ser]OppA plasmid); lane 8: pPI5.1 (WT-OppA plasmid).

9.6.4.2 Transformation Studies

9.6.4.2.1 Introduction

In order to investigate whether the mutation in compensative mutants is in the OppA or in one of the membrane components of the oligopeptide permease, two types of transformation studies were performed: i) transformation of *oppA*$^-$ strain by using plasmid DNA extracted from the compensative mutants, and ii) transformation of a cured compensative mutant with plasmid DNA containing either WT-OppA gene or plasmid DNA containing OppA gene with site directed mutations.

9.6.4.2.2 Methods

Plasmid DNA, used for transformation was purified by using the alkaline lysis method described in section 4.5.2.2. Plasmid DNA used for screening purposes was prepared by using the boiling method, as described in section 4.5.2.3.

Curing of plasmid was carried out by inoculation of 10 ml minimal medium supplemented as required without chloramphenicol, with 0.01 ml of an overnight culture, and incubated for 16 hours at 37°C with shaking. Part (0.01 ml) of this culture was then used as inoculum for 10 ml of the same medium and incubated for another 16 hours at 37°C with shaking. Cells were spread for single colonies on minimal plate, and screened for presence of the plasmid by patching the colonies onto plates with and without chloramphenicol. Colonies that had lost the trait were analysed for their plasmid content to confirm loss of the plasmid.

Standard transformation was carried out as described in section 4.5.1.1.

Cold osmotic shock was done as described in section 4.7.1.

Western blot was performed as described in section 4.11, and immunostained by using the E3 anti-OppA monoclonal antibody as first antibody.

9.6.4.2.3 Results and Discussion

a) Transformation of PA0467 with the Plasmids Isolated from Compensative mutant

Results obtained from the pI analysis of OppA from the compensative mutants, indicated that the recovery of the biological function of the Opp system, was not apparently caused by a change in their OppA. In order to verify this result, PA0467, the *oppA*$^-$ strain, was transformed with plasmids isolated from compensative mutant strains. Plasmids isolated from PA0591, PA0593 and PA0605, compensative mutants were designated pBAN2-1, pBAN2-2 and pBAN2-3, respectively. PA0467 transformed with pBAN2-1, pBAN2-2 and pBAN2-3, were designated CM01, CM02 and CM03, respectively. Plasmids isolated from both PA0588 and PA0589 (Leu and Trp, revertant mutants), were designated pBAN2-4 and pBAN2-5, respectively. PA0467 transformed with pBAN2-4 and pBAN2-5, were designated CM04 and CM05, respectively. The last two transformant strains were included as controls. An agarose gel profile of the plasmids extracted from the transformants is given in figure 9.11.

In order to verify the expression of OppA, a periplasmic protein fraction was prepared and analysed by SDS-PAGE. Figure 9.12 shows the presence of OppA in all transformed strains.

The biological function of the oligopeptide system was tested by patching the transformant strains on minimal medium plates containing 50 mM of PWLA in top agar. All transformant strains were unable to grow in the presence of PWLA as the only source of Leu and Trp. PA0591, PA0593, and PA0605 (compensative mutants, containing the pBAN2-1, pBAN2-2, and pBAN2-3 plasmids, respectively); PA588, and PA0589 (Leu and Trp revertant mutant, containing the pBAN2-4 and pBAN2-5, respectively); PA0522

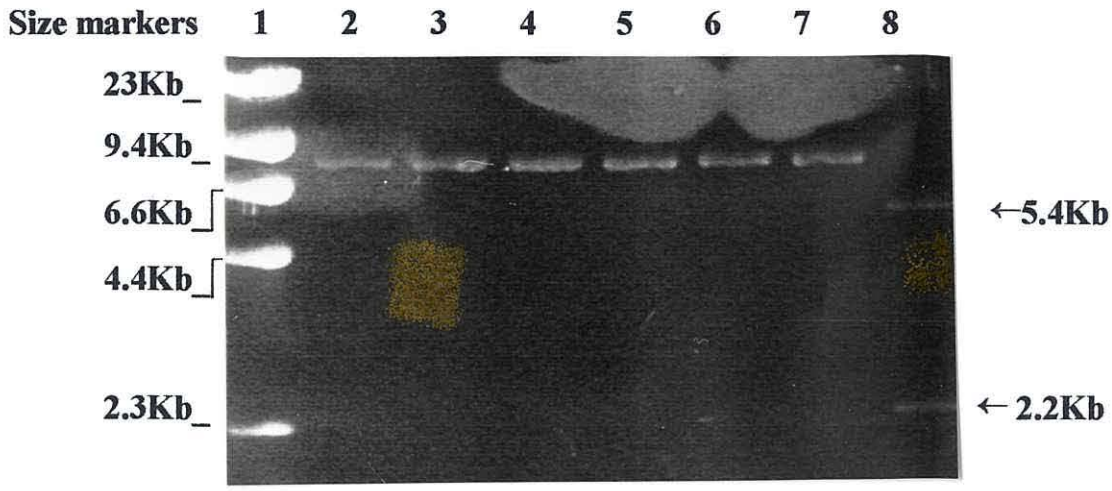


Figure 9.11

Agarose gel of plasmid DNA, extracted from *E. coli* PA0467 (*oppA*$^-$) transformed with plasmid isolated from, PA0591 (containig pBAN2-1), PA0593 (containig pBAN2-2) and PA0605 (containig pBAN2-3), compensative mutant; and PA0588 (containig pBAN2-4) and PA0589 (containig pBAN2-5), Leu-Trp revertant mutants. Lane 1: size DNA markers; Lanes 2 -6: pBAN2-1, pBAN2-2, pBAN2-3, pBAN2-4 and pBAN2-5, respectively; lane 7: pBAN2 [Asp^{300}→Ser] OppA plasmid; lane 8: pPI5.1 (WT-OppA plasmid). All plasmid were digested with *Eco*RV.

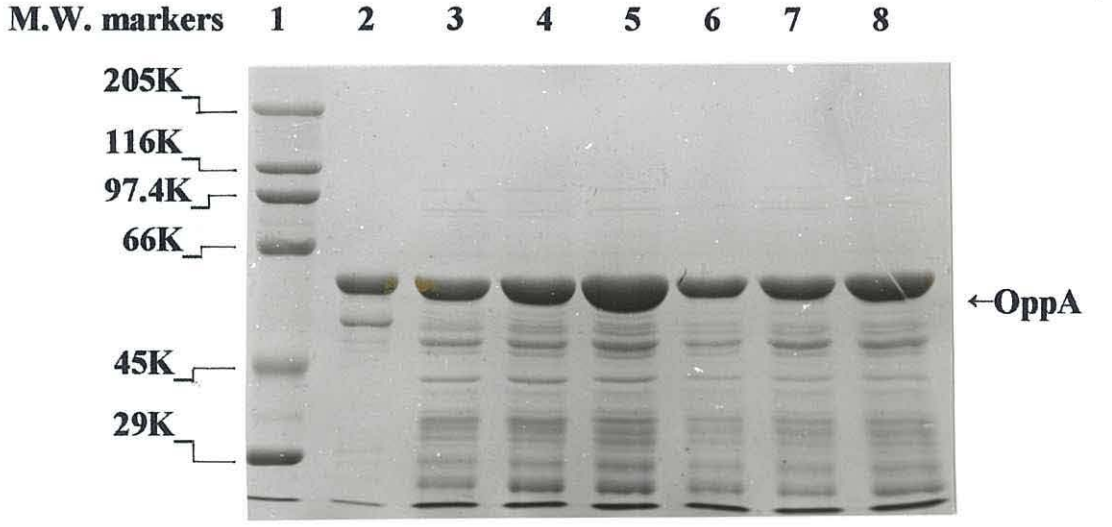


Figure 9.12

Coomassie blue-stained SDS gel showing the periplasmic protein fraction (P.P.F.) of *E. coli* CM01, CM02, CM03, CM04, CM05 and PA0522. Lane 1: M.W. marker; lanes 2: 5µg of WT-OppA; lanes 3 - 8: 3.5, 4.6, 4.5, 4.0, 3.7 and 2.6µg of P.P.F. from *E. coli* CM01(containing pBAN2-1), CM02 (containing pBAN2-2), CM03 (containing pBAN2-3), CM04 (containing pBAN2-4), CM05 (containing pBAN2-5).and PA0522 (containing pBAN2 [Asp^{300}→Ser]).

(containing the [Asp^{300}→Ser] OppA plasmid) and PA0467 (*oppA*$^{-}$) were included as controls. The first two groups of control strains (compensative mutants, and Leu and Trp revertant mutants) were able to grow in presence of PWLA, whereas both PA0522 and PA0467 strains did not show growth.

The sensitivity to both toxic peptides Ala_4P and Orn_3, was also tested in CM01 (PA0467 containing pBAN2-1), CM02 (PA0467 containing pBAN2-2) and CM03 (PA0467 containing pBAN2-3), by using pour plate. All strains tested, were resistant to both toxic peptides.

b) Curing Plasmid in Compensative Mutant Strains

Cured strains were selected by patching on minimal medium plates with and without chloramphenicol. The elimination of plasmid was verified by analysis of plasmid DNA preparations by agarose gel electrophoresis, which showed the absence of plasmid in the cured strains (Fig. 9.13, lanes 8-12). Expression of OppA in the periplasmic protein fraction was analysed by both SDS-PAGE (Fig. 9.14, lanes 3-7) and western blotting (Fig. 9.15, lanes 2-6). No OppA was detected in the cured strains. Purified OppA and the periplasmic protein fraction from PA0522, showed high intensity bands at the OppA position (Fig. 9.15, lanes 7 and 8).

c) Transformation of Cured PA0591 Strain

The cured PA0591 compensative mutant strain, was designated CPA0591, and selected for further screening. CPA0591 was transformed with the following plasmids: pBAN2 ([Asp^{300}→Ser]-OppA plasmid), pBAN3 ([Asp^{300}→Arg]-OppA plasmid), pBAN2-1 (OppA plasmid extracted from PA0591) and pPI5.1 (WT-OppA plasmid). The transformants were designated CM06 (CPA0591 transformed with pBAN2), CM07 (CPA0591 transformed with pBAN3), CM08 (CPA0591 transformed with pBAN2-1), and CM09 (CPA0591

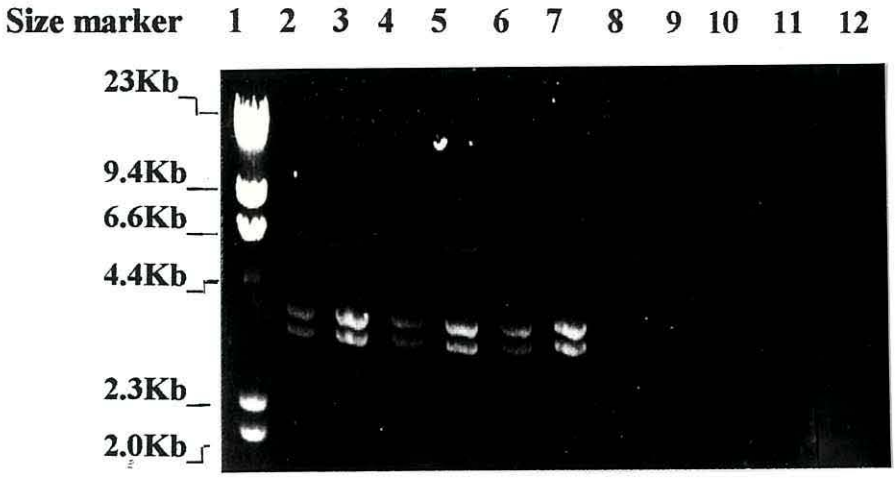


Figure 9.13:

Agarose gel showing plasmid DNA digested with both *Sal*I and *Hind*III. Lane 1: DNA size markers; lanes 2, 4 and 6: pBAN2-1 from PA0591, pBAN2-2 from PA0593 and pBAN2-3 from PA0605, compensative mutants respectively; Lanes: 3 and 5: pBAN2-4 from PA0588 and pBAN2-5 from PA0589, Leu-Trp revertant mutants; lane 7: pBAN2 containing [$Asp^{300} \rightarrow Ser$] OppA plasmid) from PA0522; lanes 8 - 12: Plasmid DNA preparation from CPA0588, CPA0591, CPA0589, CPA0593, CPA0605 (cured strains), respectively.

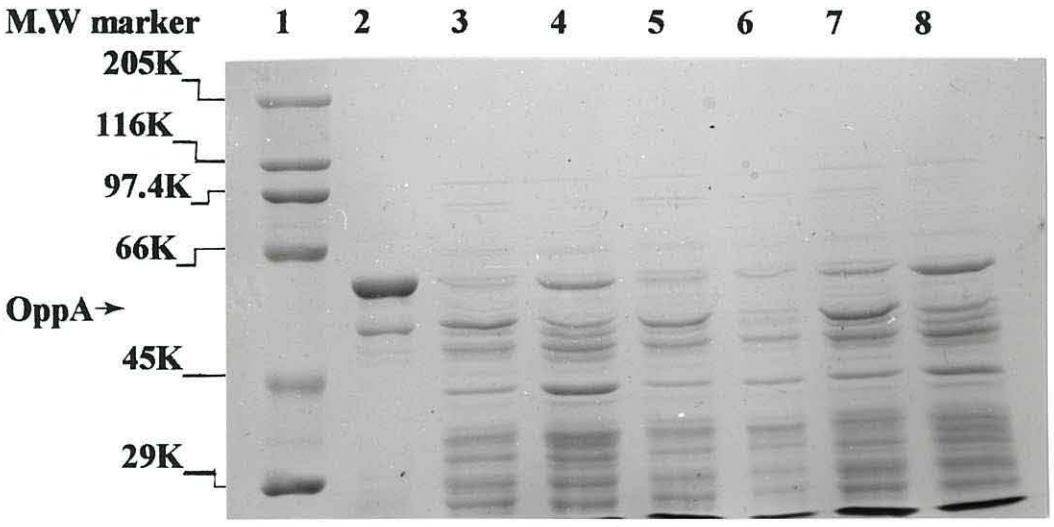


Figure 9.14:

Coomassie blue-stained SDS gel of the periplasmic protein fraction (P.P.F.) From cured strains. Lane 1: M.W. markers; lane 2: 5µg of WT-OppA; lanes 3- 7: 3.8, 5.2, 2.6, 3.0 and 5.0 µg of P.P.F. from CPA0588, CPA0591, CPA0589, CPA0593, CPA0605 (cured strains), respectively. Lane 8: 4.7µg of P.P.F. from PA0467 (*oppA*$^-$).

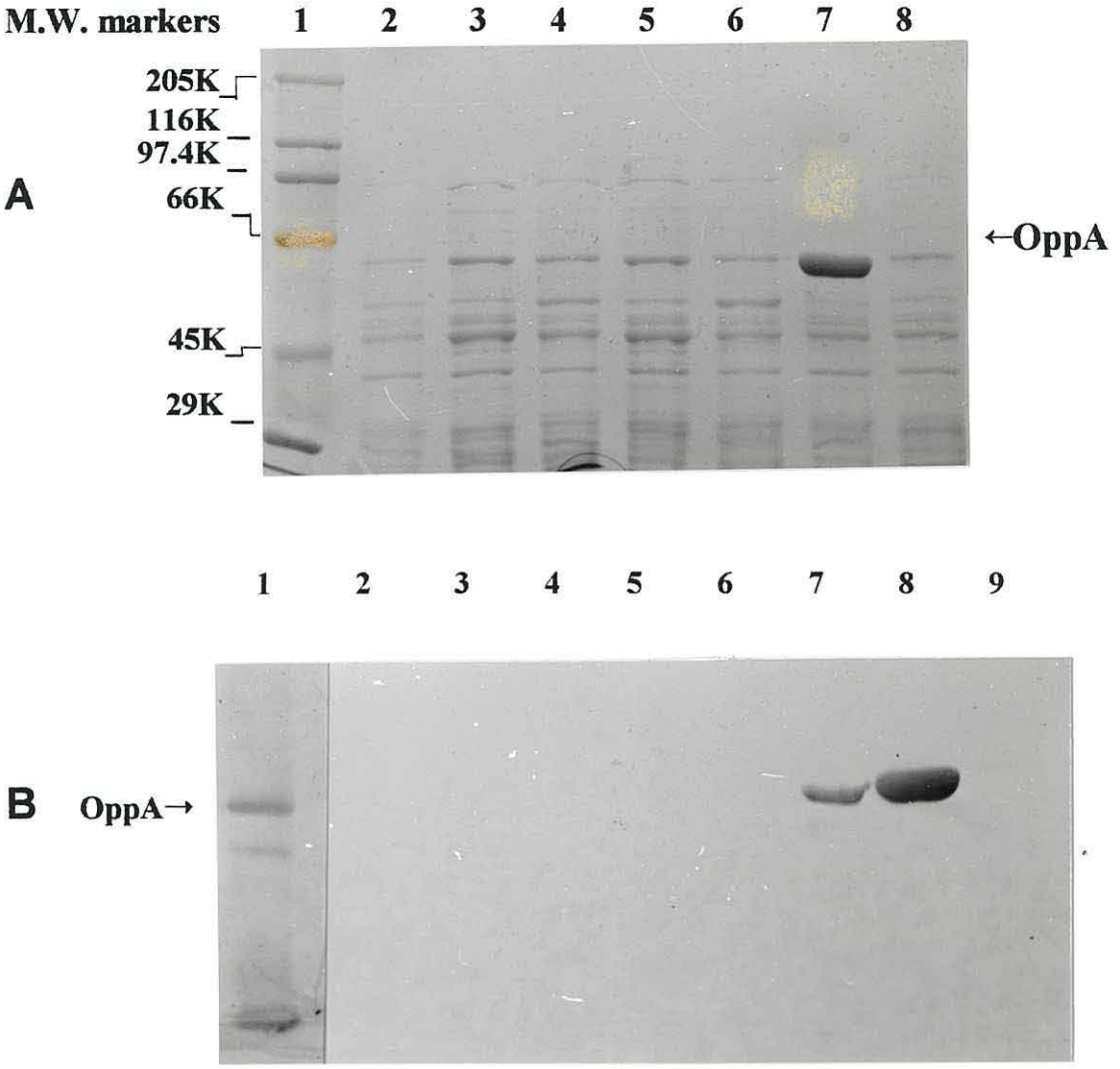


Figure 9.15:

A: Coomassie blue-stained SDS gel showing the cured compensative (*oppA*$^-$) mutant. Lane 1: M.W. markers, lanes: 3, 5 and 6 CPA0591, CPA0593 and CPA0605, cured compensative mutants respectively; Lanes: 2 and 4: CPA0588 and CPA0589, cured Leu-Trp revertant mutants, respectively; lane 7: PA0522 (containing the [Asp^{300}→Ser] OppA plasmid); lane 8: PA0467 (*oppA*$^-$). All samples contained 8 µg of periplasmic protein fractions (P.P.F).

B: Immunoblot showing immunoreaction of P.P.F from the cured strains. Lane 1 silver-stained OppA; lanes: 3, 5 and 6 CPA0591, CPA0593 and CPA0605, cured compensative mutants respectively; Lanes: 2 and 4: CPA0588 and CPA0589, cured Leu-Trp revertant mutants, respectively; lane 7: 5µg purified OppA from PA0521 (containing the WT-OppA plasmid); lanes 8 and 9: 20µg of P.P.F. from PA0522 (containing the [Asp^{300}→Ser] OppA plasmid) and PA0467 (*oppA*$^-$), respectively.

transformed with pPI5.1). Presence of plasmid in the transformed strains above-referred was verified by analysis of plasmid preparations by agarose gel electrophoresis (Fig. 9.16). The expression of OppA in both CM06 (CPA0591 transformed with pBAN2) and CM07 (CPA0591 transformed with pBAN3), were also assessed by SDS-PAGE (Fig. 9.17).

The sensitivity to both toxic peptides Ala_4P and Orn_3 was tested in all transformed strains, by using pour plate technique. The results obtained are shown in Table 9.10. CM07 (CPA0591 transformed with pBAN3), and CM08 (CPA0591 transformed with pBAN2-1) were sensitive to both toxic peptides tested, however, CM06 (CPA0591 transformed with pBAN2) was sensitive to Ala_4P, but surprisingly resistant to Orn_3. These results suggest that the recovery of the oligopeptide permease function in PA0591, could be due to a change in the membrane components, which allow a good interaction not only with [Asp^{300}→Ser]-OppA but also with [Asp^{300}→Arg]-OppA. The lack of sensitivity of CM06 (CPA0591 transformed with pBAN2) to Orn_3 is difficult to explain but could be due to the low level of restoration of the oligopeptide permease function in this strain, as was observed in PA0591 (see section 9.6.1.3, Fig. 9.7 A and Table 9.5). On the other hand, the resistance to both Ala_4P and Orn_3, observed in CM09 (CPA0591 containing pPI5.1, WT-plasmid), suggest that the cured PA0591, has not the same phenotype as PA0467 (*oppA*$^-$), which is complementable with pPI5.1 (WT-plasmid), producing a wild type phenotype. In other words, these results are compatible with a change in the membrane components.

Table 9.10

Sensitivity of Several *E. coli* transformed strains to Toxic Peptides

Strain	Orn_3	Ala_4P
CM06 (CPA0591 containing pBAN2)	R	S
CM07 (CPA0591 containing pBAN3)	S	S
CM08 (CPA0591 containing pBAN2-1)	S	S
CM09 (CPA0591 containing pPI5.1)	R	R

Sensitivity to antibiotic was tested by using pour plate technique, as described in section 4.6.1. The amount of Ala_4P and Orn_3 applied onto the antibiotic disc was 12 nmol of Ala_4P and 0.5µmol of Orn_3.

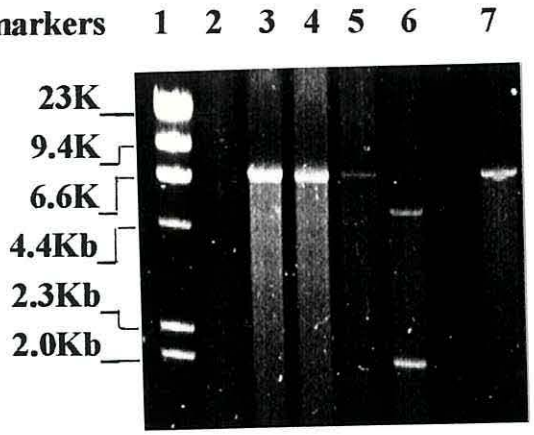


Figure 9.16:

Agarose gel of plasmid DNA, isolated from several *E. coli* mutant strains. Lane 1: DNA size markers; lanes 2 - 5: **pBAN2** ([Asp^{300}→Ser] OppA plasmid), extracted from CM06; **pBAN3** ([Asp^{300}→Arg] OppA plasmid), extracted from CM07; **pBAN2-1**, extracted from CM08 (compensative mutant) and **pPI5.1** (WT-OppA plasmid),extracted from CM09, respectively; lane 6: plasmid DNA preparation from cured PA0591 (compensative mutant); lane 7: **pBAN2-1**, from CM01 (PA0467 (*oppA*$^{-}$) transformed with pBAN2-1). All plasmid DNA were digested with *Eco*RV.

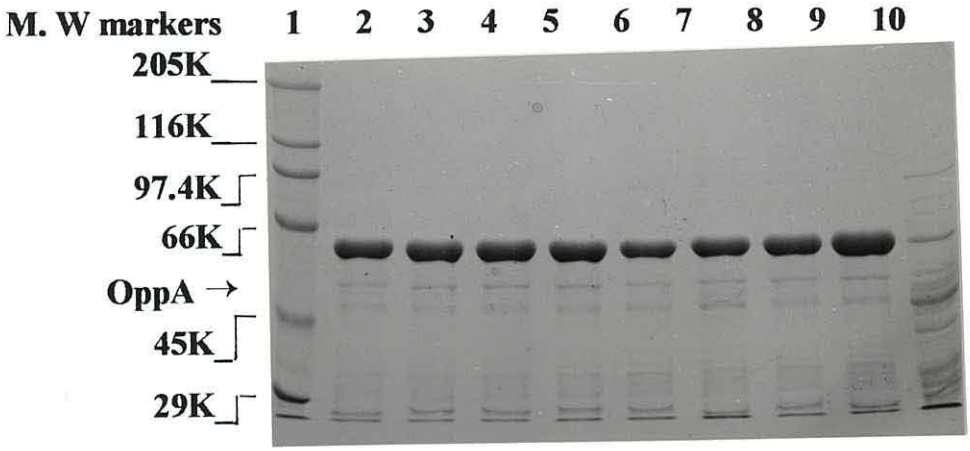


Figure 9.17:

Coomassie blue-staining gel showing the periplasmic protein fraction (P.P.F.) from cured CPA0591 compensative mutant transformed with pBAN2 (containing the [Asp^{300}→Ser] OppA plasmid) and pBAN3 (containing the [Asp^{300}→Arg] OppA plasmid), respectively. Lane 1: M.W. markers; lanes 2 - 5: four clones of cured PA0591 compensative mutant transformed with pBAN2; lanes 6 - 9: four clones of cured PA0591 compensative mutant transformed with pBAN3; lane 10: cured PA0591 compensative mutant. All samples were loaded with 10μg of total P.P.F.

9.6.5 Peptide Transport

9.6.5.1 Introduction

In order to investigate the biological function of the oligopeptide permease in the compensative mutants, peptide transport was studied in PA0591 compensative mutant and some derivative strains.

9.6.5.2 Methods

Peptide transport was performed by using a fluorescamine assay (Nisbet and Payne, 1979). Unprotonated forms of primary amino group react with fluorescamine to give a fluorescent derivative (excitation maximum, 390 nm; emission maximum, 485 nm). The fluorescamine is unstable in aqueous solution, for this reason the reagent must be prepared in acetone. This method can be used specifically to measure peptide, in the presence of amino acids, if a pH near to 6 is used.

9.6.5.2.1 Growth of Bacteria

Cells were grown overnight in minimal medium, supplemented as required at 37°C with shaking. Fresh medium 20ml was inoculated with 1 ml of overnight culture, and grown at 37°C with shaking for approximately 3 hours. Exponential-phase cells were harvested by filtration on a cellulose acetate filter (45mm, 0.45μm), washed with two volumes of 50mM potassium phosphate buffer, pH 7.0, containing 0.2% w/v glucose, and resuspended in the same buffer at 3.3×10^8 cells ml^{-1}.

9.6.5.2.2 Peptide Uptake Studies

Cells were incubated in 50mM potassium phosphate buffer, pH 7.0, containing 0.2% w/v glucose at 37°C with mixing, after 10 min peptide was added to a final concentration of 66μM for trialanine and 33μM for tetralanine. A sample (0.5ml) from the incubation medium was removed periodically and filtered immediately by passing the suspension through a 1 cm, glass fibre filter to remove microorganisms. The filtered incubation medium (0.5ml) was added to 2ml 0.1M disodium tetraborate/HCl buffer pH 6.2, and 0.5ml of fresh fluorescamine solution (0.15mg ml^{-1} in acetone) was added while vortex mixing. After 2 min the fluorescence of the solution was measured in a Perkin-Elmer fluorimeter (excitation 390nm; emission 485nm).

Peptide uptake was calculated by measuring the rate at which substrate was removed from the medium. The relation between the amount of tri-or tetraalanine and its fluorescence was shown to be linear in the range used (Fig. 9.18).

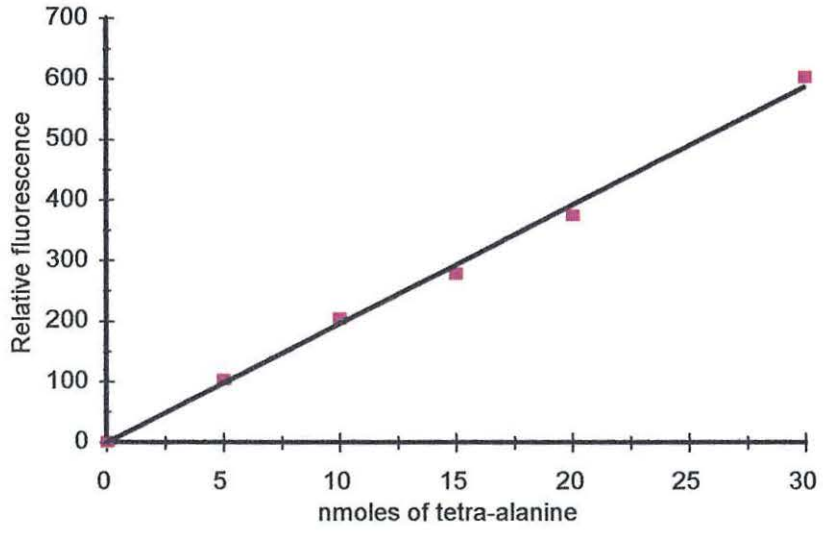


Figure 9.18

Calibration curve of tetraalanine versus fluorescence using fluorescamine

9.6.5.3 Results and Discussion

Guyer *et al* (1986) in studies of binding specificity, showed that both trialanine and tetraalanine are good substrates for *E. coli* OppA. Figure 9.19 shows two of the plots obtained when the transport of tetraalanine was assayed for both strains M2034 (WT strain) and PA0591 (compensative mutant), respectively; and Table 9.11 shows the rate (nmoles of peptide transported/min/mg/dry weight) of all the strains studied. Trialanine was transported by all the strains but at different rates. Both the PA0591 (compensative mutant), and CM07 (CPA0591 transformed with pBAN3 [Asp $^{300}\rightarrow$Arg] OppA plasmid) were able to transport trialanine at the same rate as the wild type M2034. The rate of transport of the same peptide observed in the following strains: cured PA0591 (compensative mutant), PA0521 (containing the WT-OppA plasmid), PA0522 (containing the [Asp$^{300}\rightarrow$Ser] OppA plasmid), PA0467 (*oppA*$^-$) and CM06 (CPA0591 transformed with pBAN2 [Asp$^{300}\rightarrow$Ser] OppA plasmid) was lower than the rate observed in the wild type M2034.

Andrews and Short (1985) showed evidence for the existence in *E. coli* of at least two transport systems which recognize tripeptides. The distinction between these systems has been defined not only genetically but also on the basis of transport substrates preferred by each system in *E. coli*, most tripeptides can be transported through both the tri- and the oligopeptide permease (Higgins *et al.*, 1983). The low rate of transport of trialanine found in both strains PA0467 (*oppA*$^-$) and PA0522 (containing [Asp$^{300}\rightarrow$Ser] OppA plasmid), confirm that trialanine is transported only through the tripeptide permease in these strains. The high transport rate observed in PA0591 (compensative mutant) and CM07 (CPA0591 transformed with pBAN3 [Asp$^{300}\rightarrow$Arg] OppA plasmid), suggest the function of both tri- and oligopeptide permease in these strains.

When the transport of tetraalanine was tested, only both wild type strains (M2034 and PA0521), PA0591 (compensative mutant), CM07 (CPA0591 transformed with pBAN3 [Asp$^{300}\rightarrow$Arg] OppA plasmid), and CM08 (CPA0591 transformed with pBAN2-1) were able to transport the tetrapeptide. Transport of tetraalanine was not detected in the strain

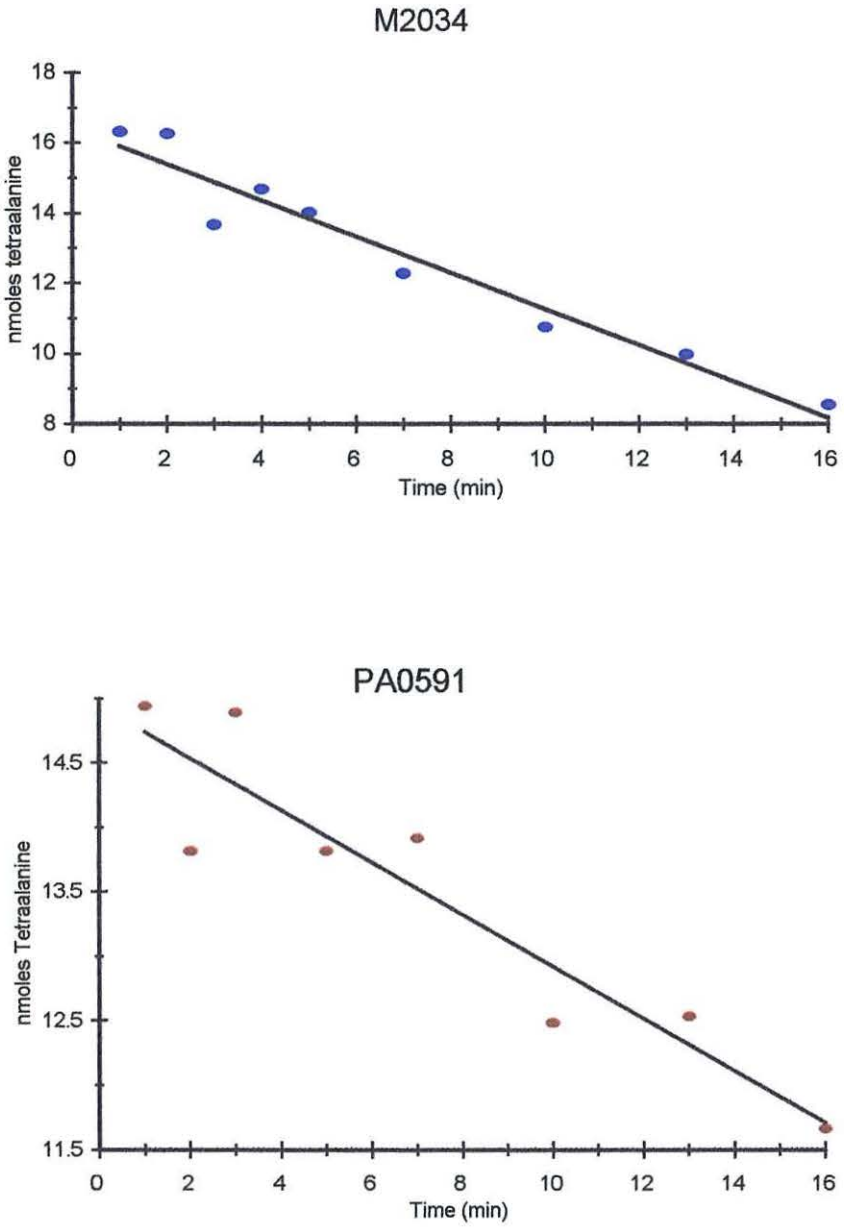


Figure 9.19:

Uptake of tetraalanine by strains M2034 (wild type) and PA0591 (compensative mutant). Cells were harvested by filtration, washed and resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2% w/v glucose, and then resuspended at 3.3 x 10^8 cells ml^{-1}. Tetraalanine was added to a concentration of 33μM. Samples were removed periodically and assayed by using fluorescamine. The line was fitted by linear regression, and the rates for M2034 and PA0591 were 6.87 and 2.55 nmoles/min/mg dry weight, respectively.

ORIGINAL

Table 9.11

Transport of Peptides by Several *E.coli* Strains

	nmoles peptide/min/mg dry weight (±SD)		
	Tri-L-alanine	**Tetra-L-alanine**	
Strain	**66µM**	**33µM**	**10µM**
M2034	19.09 ± 2.37	6.87 ± 0.62	
PA0521	12.98 ± 3.6	3.82 ± 0.72	
	9.2 ± 3.96	4.51 ± 1.33	
PA0522	10.75 ± 3.3	0.23 ± 0.89	
	14.79 ± 3.7		
PA0523	ND	1.02 ± 0.9	0.29 ± 0.32
PA0467	12.44 ± 2.05	0.38 ± 1.46	
PA0591	20.17 ± 1.6	2.55 ± 0.53	
CPA0591	20.08 ± 8.3	1.22 ± 0.9	
CM06 (CPA591+pBAN2)	12.13 ± 7.14	0.07 ± 1.21	0.18 ± 0.49
CM07 (CPA0591+pBAN3)	19.70 ± 6.2	3.11 ± 0.92	
		2.83 ± 1.17	
CM08 (CPA0591+pBAN2-1)	ND	5.6 ± 2.25	

The transport of both trialanine and tetraalanine were measured by using the fluorescamine assay, as described in 2.5.1.2. **M2034**, wild type strain; **PA0521**, containing WT OppA plasmid; **PA0522**, containing [Asp ^{300}→Ser] OppA plasmid; **PA0523**, [Asp^{300}→Arg] OppA plasmid; **PA0467**, *oppA*$^{-}$; **PA0591**, compensative mutant; **CPA0591**, cured compensative mutant; **CM06**, CPA0591 transformed with pBAN2 [Asp^{300}→Ser] OppA plasmid; **CM07**, CPA0591 transformed with pBAN3 [Asp^{300}→Arg] OppA plasmid; **CM08**, CPA0591 transformed with pBAN2-1(plasmid extracted from PA0591). Abbreviation used: SD, standard deviation; ND, test not determined.

CM06 (CPA0591 containing pBAN2 [Asp^{300}→Ser] OppA plasmid). The strains PA0467 (*oppA*$^-$), PA0522 (containing pBAN2) and PA0523 (containing pBAN3) included as control, were unable to transport tetraalanine. These results indicate a functioning oligopeptide permease system in both CM07 and CM08 strains. The difference in transport rate between the two wild type M2034 and PA0521, could be attributed to the low amount of OppA expressed by PA0521 (Fig. 9.7, lane 7).

Previous results obtained in this study (see section 8.7.3) showed that [Asp^{300}→Arg] OppA expressed by PA0523, is able to bind substrate but may fail to interact with the membrane components for successful transport. The fact that the cured PA0591 (compensative mutant) transformed with pBAN2-1(plasmid extracted from PA0591) or with pBAN3 ([Asp^{300}→Arg] OppA plasmid), was able to transport tetraalanine through the oligopeptide permease, is compatible with the idea that the PA0591 (compensative mutant) underwent a change (a forward-mutation) in the membrane components that compensates for the OppA defect. The results with the pBAN2 ([Asp^{300}→Ser] OppA plasmid) transformant, which might have been expected to behave similarly, is not readily explicable.

9.6.6 Growth Curve

9.6.6.1 Introduction

In order to study the biological function of the peptide transport system of the compensative mutant and some derivative strains, growth curves were performed. PWLA, the selection peptide, was used as sole source of leucine and tryptophan.

9.6.6.2 Methods

All strains were grown overnight in 10 ml minimal medium supplemented with Glc, Trp and Leu, in the presence of chloramphenicol (34μg/ml) when required, at 37°C, with shaking. Cells were washed with 0.9% sterile NaCl and 1 x 10^6 cells were used to inoculate 5 ml of minimal medium supplemented with Glc and 50 mM PWLA, and chloramphenicol when required. Two controls were included, a positive control, using minimal medium supplemented with Glc, Trp, Leu and chloramphenicol if required, and a negative control using the same medium supplemented only with Glc. The optical density was monitored each hour during 9 hours, and the doubling time was determined from a semi-logarithmic plot of the exponential-phase.

9.6.6.3 Results and Discussion

Figure 9.20 A and B shows the growth curves using minimal medium supplemented with Glc and PWLA, or supplemented with Glc, Trp and Leu, respectively; and Table 9.12 shows the doubling time of the strains studied.

Table 9.12

Doubling Time (minutes)

Strain	Minimal medium Glc + Leu + Trp	Minimal medium Glc + PWLA
CM06	90	120
CM07	90	53
CM08	90	50
PA0521	90	60
PA0523	ND	NG

Doubling time of several *E. coli* strains grown in minimal medium supplemented with Glc, Trp and Leu or with Glc and PWLA. CM06, is CPA0591 transformed with pBAN2 ([Asp^{300}→Ser] OppA plasmid); CM07, is CPA0591 transformed with pBAN3 ([Asp^{300}→Arg] OppA plasmid); CM08, CPA0591 transformed with pBAN2-1 (plasmid extracted from PA0591); PA0521, containing the WT-OppA plasmid; PA0523, containing [Asp^{300}→Arg] OppA plasmid Abbreviation used: ND, not determined; NG, no growth was observed.

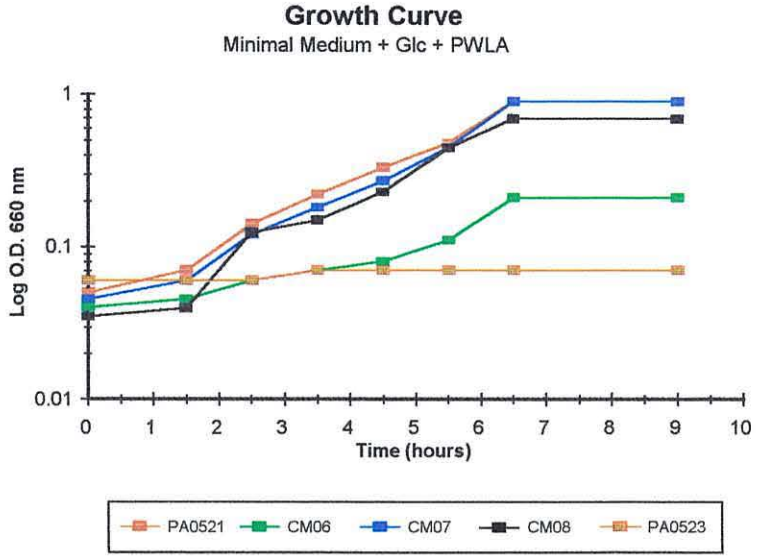


Figure 9.20 A

Growth curve of some *E. coli* PA0591 derivatives, grown in minimal medium supplemented with Glc and PWLA. PA0591, compensative mutant; PA0521 (containing the wild type plasmid); CM06, CM07 and CM08, are cured PA0591 transformed with pBAN2 ([Asp^{300}→Ser] OppA plasmid), pBAN3 ([Asp^{300}→Arg] OppA plasmid) and pBAN2-1 (plasmid isolated from PA0591), respectively; PA0523 (containing ([Asp^{300}→Arg] OppA plasmid).

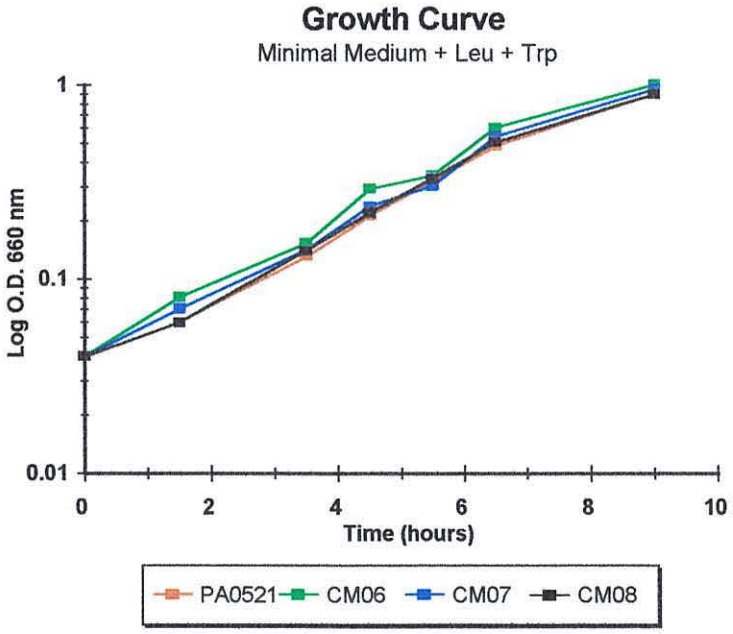


Figure 9.20 B

Growth curve of some *E. coli* PA0591 derivative, grown in minimal medium supplemented with Glc Leucine and Tryptophan. PA0591, compensative mutant; PA0521 (containing the wild type plasmid); CM06, CM07 and CM08, are cured PA0591 transformed with pBAN2 ([Asp^{300}→Ser] OppA plasmid), pBAN3 ([Asp^{300}→Arg] OppA plasmid) and pBAN2-1 (plasmid isolated from PA0591), respectively.

When the culture medium was supplemented with both amino acids Leu and Trp, all strains grew with the same doubling time. PA0521 (wild type); CM07, CPA0591 containing pBAN3 [$Asp^{300}\rightarrow$Arg] OppA plasmid; and CM08, CPA0591 containing pBAN2-1, extracted from PA0591, were able to grow well in presence of PWLA. In contrast, with CM06 that showed poor growth in presence of PWLA.

In order to confirm the lack of function in PA0523 that contain the pBAN3 [$Asp^{300}\rightarrow$Arg] OppA plasmid, this strain was included as control, and no growth was observed in presence of PWLA. These results endorse the results obtained with the antibiotic test and in the peptide transport assay. The "anomalous" behaviour of CM06 is at least consistent, i.e. poor growth on PWLA, negligible uptake of Ala_4 and resistance to Orn_3.

9.7 Concluding Remarks

The characteristics of the strains studied in this chapter are given in Table 9.13. The sensitivity to toxic peptides (Orn_3 and Ala_4P), the transport of tetraalanine and the growth using PWLA, observed in PA0591 (compensative mutant), all support the idea that the biological function of the oligopeptide permease system was recovered in this strain.

The lack of function in the oligopeptide permease system of PA0467 (*oppA*$^-$) transformed with the plasmid from the compensative mutants, suggest that the recovery of the oligopeptide permease system is due to a change in the membrane components.

The pI study of OppA from the compensative mutants, and the cleavage studies with *Eco*RV, apparently showed that this protein did not undergo a reversion to wild type OppA.

The lack of sensitivity to Orn_3 coupled with sensitivity to Ala_4P, the undetected tetraalanine transport, and the high doubling time when growing in minimal medium supplemented with Glc and PWLA, observed in CM06 (CPA0591 transformed with pBAN2 [$Asp^{300}\rightarrow$Ser] OppA plasmid), could be explained as a low level of restoration of the oligopeptide function, although would repay further examination.

Further work is needed to characterise the compensative mutants and to provide more details about the changes in membrane proteins that restore the biological function of the oligopeptide permease in these mutants.

Table 9.13
Summary of the Characteristics of the Compensative Mutants and their Derivative Strains

	Nutritional Requirements		Antibiotics Sensitivity					Peptide Transport	
	M.M Glc PWLA	M.MGlc	Orn_3	Ala_4P	Chloram-phenicol	OppA expression	Presence of Plasmid	Ala_3	Ala_4
Strain									
PA0588 (L and W revertant mutant)	+	+	ND	ND	R	+	+	ND	ND
PA0591 (Compensative mutant)	+	-	S	S	R	+	+	+	+
PA0589 (L and W revertant mutant)	+	+	ND	ND	R	+	+	ND	ND
PA0593 (Compensative mutant)	+	-	S	S	R	+	+	ND	ND
PA0605 Compensative mutant)	+	-	S	S	R	+	+	ND	ND
Cured PA0588	+	+	ND	ND	S	-	-	ND	ND
Cured PA0591	-	-	R	R	S	-	-	+	-
Cured PA0589	+	+	ND	ND	S	-	-	ND	ND
Cured PA0593	-	-	R	R	S	-	-	ND	ND

Continued Table: 10.13

Strain	M.M Glc PWLA	M.M + Glc	Orn_3	Ala_4P	Chloram-phenicol	OppA expression	Presence of Plasmid	Ala_3	Ala_4
Cured PA0605	-	-	R	R	S	-	-	ND	ND
CM01 (PA0467 transformed with pBAN2-1)	-	-	R	R	R	+	+	ND	ND
CM02 (PA0467 transformed with pBAN2-2)	-	-	R	R	R	+	+	ND	ND
CM03 (PA0467 transformed with pBAN2-3)	-	-	R	R	R	+	+	ND	ND
CM04 (PA0467 transformed with pBAN2-4)	-	-	ND	ND	R	+	+	ND	ND
CM05 (PA0467 transformed with pBAN2-5)	-	-	ND	ND	R	+	+	ND	ND
CM06 (Cured PA0591 Compensative mutant transformed with pBAN2)	±	-	R	S	R	+	+	+	-
CM07 (Cured PA0591 Compensative mutant transformed with pBAN3)	+	-	S	S	R	+	+	+	+

Continued Table: 10.13

Strain	M.M Glc PWLA	M.M + Glc	Orn_3	Ala_4P	Chloram-phenicol	OppA expression	Presence of Plasmid	Ala_3	Ala_4
CM08 (Cured PA0591 Compensative mutant transformed with pBAN2-1)	+	-	S	S	R	+	+	ND	+
CM09 (Cured PA0591 Compensative mutant transformed with pPI5.1)	-	-	R	R	R	+	+	ND	ND
PA0467 (*oppA*$^-$)	-	-	R	R	S	-	-	+	-
PA0521 (PA0467 complemented with pPI5.1)	+	-	S	S	R	+	+	+	+
PA0522 (PA0467+pBAN2)	-	-	R	R	R	+	+	+	-

Abbreviations used: R: resistant, S: sensitive, ND: test not determined, M.M. minimal medium. PA0467 is *oppA*$^-$; PA0521 is PA0467 transformed with pPI5.1 (WT OppA plasmid); PA0522 is PA0467 transformed with pBAN2 [Asp^{300}→Ser] OppA plasmid; pBAN3 [Asp^{300}→Arg] OppA plasmid; pBAN2-1, pBAN2-2, pBAN2-3, pBAN2-4 and pBAN2-5 are plasmids isolated from PA0588, PA0591, PA0589, PA0593 and PA0605 compensative mutants, respectively.

Chapter X

GENERAL DISCUSSION

In order to improve the tools available in this laboratory to investigate the oligopeptide permease in E. *coli* as well in other species, hybridoma cell lines secreting anti-OppA monoclonal antibodies were subjected to further cloning and characterisation. From the VI\22\2 cell line isolated by Schuster (1995) two cell lines (OppAE3 and OppAC2) were obtained and the monoclonal antibodies were concentrated, partially purified and characterised . Both VI\22\2 and OppAE3 monoclonal antibodies, showed cross-reaction with *E. coli* DppA, while OppAC2 showed no cross-reaction with either DppA or *S. typhimurium* OppA. This result is a strong indication that the two monoclonal antibodies recognise distinct epitopes in the OppA. OppAE3 and OppAC2 monoclonal antibodies were sufficiently sensitive to detect a very small amount of OppA expressed in a TOR mutant (PA0309), that had not been detected in previous experiments by using polyclonal antibodies raised against OppA.

Two *E.coli* OppA mutants PA0522 and PA0523, that express a point mutation in OppA, [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA, respectively were produced in this laboratory. Both OppA mutant proteins can bind peptides with relatively less affinity

compared with wild type-OppA. In contrast, the oligopeptide permease system of these mutants is completely defective in peptide transport. The hypothesis that the residue Asp-300 of *E. coli* OppA is intimately involved in the events that allow the translocation of the ligand from the OppA-ligand complex through the membrane associated components of the oligopeptide permease, is proposed in this study. The surface location of Asp-300 and its negatively charged carboxylate group, at physiological pH, make this residue a good candidate to be involved in protein-protein interaction. On the other hand, the OppA amino acid residues that interact with different ligands (di-,tri- and tetrapeptides), have been identified from crystal structure studies of OppA complexed with ligand (Tame *et al.*, 1994; 1995; Sleigh *et al.*, 1997), and Asp-300 is not directly involved in these interactions. Taking these results into consideration, Asp-300, together with other contiguous amino acid residues, might be directly involved in the interaction with the exterior surface(s) of one or both membrane components.

The structure of periplasmic binding proteins and their mechanism of ligand binding are all similar, which implies they may have a common mode of interaction with the membrane components of their respective system. In most of the binding proteins of the ABC-type transporters, the ligand initially interacts with one of the two domains in the open form, the second domain closes over the ligand, and interactions between the ligand and the second domain then serve to stabilise the closed form of the protein. Therefore, another possible explanation for the complete loss of biological function in both PA0522 and PA0523 mutants is that Asp-300 may be involved in the stabilisation of the liganded closed form.This form of the protein has an external surface quite distinct from that of the open form, and it is the form that is recognised by the membrane components.

Mutants able to recover the oligopeptide permease function for the OppA defect were isolated from PA0522. The characterisation of these compensative mutant strains and their oligopeptide binding protein, indicated that the recovery of the oligopeptide permease activity arose from a change in the membrane components, thus providing strong evidence of the role of Asp300 in the interaction with the membrane components.

Biochemical evidences for an interaction between the periplasmic binding protein, HisJ

(analogous to OppA) and one of the membrane components, Q protein (analogous to OppC) has been reported in the histidine permease of *S. pyphimurium*. (Prossnitz *et al.*, 1989). In this study, a mutant HisJ protein (Arg-176→Cys) which binds histidine, but does not support transport, showed a reduced ability to interact with Q protein, as compared with the wild type, implying that this mutation resulted in a defect in the interaction with the membrane complex.

Anti-peptide antibodies against a specific region in OppA containing the residue Asp-300 (Ser-300 or Arg-300) were produced. Unfortunately these antibodies could not be used for the screening of compensative mutants, first due to the cross-reaction observed between the three antisera and the three homologous peptides. Only one peptide (APR) gave antisera which reacted with the whole protein, when these anti-peptide antibodies were tested by using Western-blotting. It can be speculated that when a protein antigen is adsorbed on a solid support, this may fail to react with an anti-peptide antibody by virtue of the antigens protein being in an orientation that sterically precludes the interaction. The fact that only one anti-peptide antibody was able to recognise the adsorbed protein, is difficult to explain, because of the similarity among the three proteins, wild type-OppA, [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA, used as antigens. Further characterisation of these anti-peptide antibodies is required, for example interaction of the anti-peptide antibodies with the native protein in solution by using immunoprecipitation.

The use of the anti-peptide antibodies produced here in a study with membrane vesicles or the newly developed proteoliposome (LPS) reconstitution method (Cheng and Ames, 1997) and cross-linking reagents, will be very useful to study the ability of [Asp^{300}→Ser]-OppA and [Asp^{300}→Arg]-OppA, to interact with the membrane components, and to identify the region in OppA and in the membrane components involved in the interaction. Overall this should lead to a better understanding of the translocation mechanism of the ligand through the membrane complex of the oligopeptide permease.

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