A study of the *ompT* gene of *Escherichia coli* K-12

by

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I would like to dedicate this thesis to
my parents, my sister Alison and
my brother David but most
of all to Gary

Contents

Summary

The *ompT* gene of *E. coli* encodes a 40 kd outer membrane protein (OmpT) which, *in vitro*, exhibits proteolytic activity towards the ferric-enterochelin receptor protein. In this study the *ompT* gene was cloned from *E. coli* K-12 on a 4.3 kb *Eco*RI DNA fragment. Subcloning of this fragment, in conjunction with maxi-cell analysis, demonstrated that *ompT* was located on a 1.5 kb *Pst*I-*Sma*I DNA fragment.

DNA sequence analysis revealed that the *Pst*I-*Sma*I fragment contained an open reading frame (ORF) of 951 bp, the latter having a coding capacity of 35.6 kd. This deduced molecular weight was somewhat smaller than the molecular weight of 42 kd estimated by SDS-PAGE for pro-OmpT. Potential -10 and -35 promoter consensus sequences were identified upstream from the *ompT* coding region as was a putative ribosome binding site. A DNA sequence showing homology to a consensus sequence present in the putative promoter regions of iron-regulated genes, was also located upstream from the *ompT* coding region. With regard to the deduced amino acid sequence of OmpT, a potential signal sequence was found at the amino-terminal of the protein which could direct export from the cell cytoplasm. The protein was also examined for amino acid sequences displaying homology to other outer membrane proteins, but none were identified.

ompT-*phoA* gene fusions were constructed in order to assess the ability of the *ompT* transcription/translation initiation signals to drive the expression of cloned heterologous genes in *E. coli*. OmpT-PhoA fusion proteins were indeed produced and these were exported to the periplasm of the cell. Synthesis of the chimeric proteins was found to be 5-6 fold higher at 37°C than at 30°C which seemed to reflect the normal temperature-dependent production of the OmpT protein. Both *ompT*-*lacZ* operon and protein fusions were also created in an attempt to define the stage at which temperature affected the synthesis of OmpT. Studies indicated that this occurred at some post-transcriptional step. The effect of the *envZ* allele on the expression of *ompT* was also investigated. This mutation appeared to reduce the level of transcription of *ompT* as indicated by studies using the *ompT*-*phoA* and *ompT*-*lacZ* fusions.

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Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself, except for the use of PhoA antibodies in Western blotting, which was performed by D. R. Gill (Dept. of Biological Sciences, University of Warwick). All sources of information have been specifically acknowledged by means of reference.

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Abbreviations

Ap^r	ampicillin resistant
BCIG	5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
cAMP	cyclic adenosine 3',5'-monophosphate
cpm	counts per minute
d	daltons
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DFP	diisopropylfluorophosphate
EDTA	ethylenediaminetetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
Km^r	kanamycin resistant
kb	kilobase
kd	kilodaltons
IPTG	isopropyl-beta-D-thiogalactopyranoside
min	minute(s)
moi	multiplicity of infection
Mr	relative molecular mass

mRNA	messenger ribonucleic acid
OD	optical density at specified wave-length (nm)
ONPG	o-nitrophenyl beta-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming units
'phage	bacteriophage
PMSF	phenylmethylsulphonyl fluoride
psi	pounds/square inch
RNase	ribonuclease
rpm	revolutions per minute
sec	second(s)
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tc^r	tetracycline resistant
TLCK	tosyl-L-lysine chloromethyl ketone
TLPK	tosyl-L-phenylalanine chloro-methyl ketone
Tris	2-amino-2-hydroxy-methylpropane-1,3-diol
tRNA	transfer ribonucleic acid
UV	ultra-violet
v/v	concentration, volume by volume
w/v	concentration, weight by volume

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate

E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Restriction Endonucleases

Ba	BamHI
Ec	EcoRI
Pa	PstI
Rs	RsaI
Sa	SacI
Sm	SmaI
Hi	HindIII

Cl	*Cla*I
Dr	*Dra*I

Nucleotide Symbols

Letter	Base
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

All other abbreviations, symbols and units used in this study are defined in the Biochemical J. (1981) 193, 1-27.

Chapter One

Introduction

Preface

The present study is concerned with the *ompT* gene of *E. coli* which encodes the outer membrane protein OmpT. The following general introduction will outline the structure, composition and function of the outer membrane with particular emphasis being placed upon the functions, regulation, biosynthesis and assembly of the outer membrane proteins. Finally, the available literature concerning the OmpT protein and the *ompT* gene will be summarised and the aims of this work presented.

1.1 Introductory Comments

All cells are enclosed by a thin membrane, the cytoplasmic membrane, which retains the various molecules necessary for maintaining biological functions and which serves as a barrier between the outer environment and the cytoplasm of the cell. Most bacteria, except the *Mycoplasma*, contain an additional layer, external to the cytoplasmic membrane, which is referred to as the cell wall. Together, the membrane and the cell wall constitute the cell envelope. The cytoplasmic membrane is common to all bacteria and is very similar with respect to structure and function. The cell wall, however, varies in both composition and architecture depending upon the bacterial species and this is reflected by the Gram reaction. Gram-positive organisms (e.g., *Bacillus subtilis*) possess a thick, relatively homogeneous cell wall while that of Gram-negative bacteria (e.g., *Salmonella typhimurium*) is remarkably complex and consists of several layers (reviewed by Glauert and Thornley, 1969). The following discussion will be confined to the envelopes of Gram-negative organisms since the present work is concerned with a protein present in the cell wall of *Escherichia coli*.

1.2 The Cell Envelope of Gram-negative Bacteria

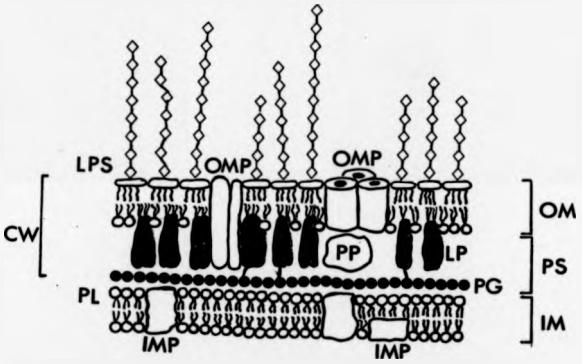
The cell envelope of a Gram-negative bacterium consists of three morphologically distinct layers; an inner cytoplasmic membrane, a rigid intermediate layer of peptidoglycan and an

outer membrane at the surface of the cell. This structure was initially demonstrated by the examination of fixed, sectioned cells of E. coli in the electron microscope (De Petris, 1967) and has since been confirmed by freeze-etching studies (Bayer and Remsen, 1970). Both the inner and outer membranes have a double-track appearance in the electron microscope and are separated from each other by the periplasmic space. The outer membrane and the peptidoglycan layer constitute the cell wall which is approximately 12 nm in width. A model for the cell envelope structure of Gram-negative bacteria is illustrated in Figure 1.2.

The cytoplasmic membrane consists mainly of protein and lipid (Miura and Mizushima, 1968) and is 7-8 nm thick. The so-called fluid-mosaic model describes this membrane as a phospholipid bilayer interspersed with protein molecules (Singer and Nicholson, 1972). It contains many energy-linked permeases which are responsible for the accumulation of nutrients into the cytoplasm by active transport. Enzymes of the electron transport system (Osborn et al., 1972a) and the ATPase of many bacteria (Salton, 1974) have also been located in the inner membrane. The membrane is also involved in the synthesis of cell wall polymers such as peptidoglycan (Salton, 1974), phospholipid (White et al., 1971) and lipopolysaccharide (Osborn et al., 1972b) and plays a role in the synthesis of proteins that are to be secreted from the cell (reviewed by Oliver, 1985). Evidence also exists

Figure 1.2 Diagrammatic representation of the Gram-negative bacterial cell envelope

The three main layers of the cell envelope are shown: outer membrane (OM), inner membrane (IM) and peptidoglycan (PG). Also shown are: the cell wall (CW), periplasmic space (PS), lipoprotein (LP), outer membrane protein (OMP), inner membrane protein (IMP), periplasmic protein (PP), lipopolysaccharide (LPS) and phospholipid (PL).



Redrawn from Lutenberg and van Alphen (1983).

that the membrane contains specific attachment sites for the chromosome suggesting a role in either the synthesis or segregation of replicating DNA strands (Dworsky and Schaechter, 1973).

The periplasm is located between the inner and outer membranes. It contains proteins which comprise approximately 4% of the total cell protein (Nossal and Heppel, 1966). Some perform a catabolic function (e.g., alkaline phosphatase) while others are involved in the degradation or modification of harmful substances (e.g., beta-lactamase). A further group of proteins, the binding proteins, have affinity for various nutrients and are essential components of many transport systems. The periplasm also contains oligosaccharides (van Golde et al., 1973) which comprise about 1% of the dry weight of the cell. They are a closely related family of highly branched molecules containing about nine residues of glucose as the sole sugar. These are thought to play a role in the osmoregulation of Gram-negative organisms (Kennedy, 1982).

The periplasm also contains the peptidoglycan (murein) layer which represents 1-10% of the Gram-negative cell wall (reviewed by Schleifer and Kandler, 1972). It consists of linear amino-sugar chains containing alternating residues of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Each strand contains 10-65 disaccharide residues and adjacent chains maybe covalently cross-linked to each other

by tetrapeptides which are attached to the N-acetylmuramic acid residues. The peptide linkages which hold neighbouring chains together, confer the structure of a molecular mesh upon the peptidoglycan which can form a continuous sac around the cell. The peptidoglycan layer, which is 2-3 nm wide, is regarded as being primarily responsible for maintaining cell shape and for the ability of the cell to withstand the very high internal osmotic pressure in dilute environments.

The outer membrane contains a complex mixture of protein, lipid, lipoprotein and lipopolysaccharide (Weidel et al., 1960). It appears to be attached to the peptidoglycan layer via protein molecules which have been identified by Braun and Rehn (1969) as lipoprotein. The outer membrane is discussed in more detail below.

1.3 The Outer Membrane

Many advances in the study of the outer membrane have come from the development of suitable procedures for the separation of this structure from the inner cytoplasmic membrane. The methods which have been devised are presented below.

1.3.1 Methods used to Prepare the Outer Membrane

1.3.1.1 Sucrose Density-gradient Centrifugation

Fractionation of the two membranes using this method is possible because the outer membrane has a higher density

than the cytoplasmic membrane due to the presence of lipopolysaccharide (Miura and Mizushima, 1968). Firstly cells are broken either mechanically with a French pressure cell (Schnaitman, 1970a), or by disrupting lysozyme-treated spheroplasts using either osmotic shock (Miura and Mizushima, 1968), or sonication (Osborn et al., 1972a). The resulting envelope fraction is isolated from other cell components by centrifugation before fractionation on sucrose gradients to give cytoplasmic and outer membranes.

1.3.1.2 Differential Membrane Solubility using Detergents

It has been reported that the treatment of isolated cell envelopes with certain detergents can selectively solubilise the cytoplasmic membrane while leaving the outer membrane intact. These include the non-ionic detergent Triton X-100 (Schnaitman, 1971) and sodium lauryl sarcosinate (Filip et al., 1973). Morphologically the outer membrane seems to remain intact after such treatment. However, chemical analysis has revealed that it contains only half of the lipopolysaccharide and one-third of the phospholipid normally found in the outer membrane though all constituent proteins are present (Schnaitman, 1971). Once the inner membrane has been solubilised, the outer membrane can then be isolated by centrifugation. This method has proved most useful in the preparation of proteins from the outer membrane.

1.3.1.3 Electrophoretic Methods

Owing to the presence of LPS, the outer membrane has a very large number of negatively-charged groups and can be separated from the less negatively-charged cytoplasmic membrane by the use of a preparative particle electrophoresis apparatus (White et al., 1972).

1.3.2 Composition of the Outer Membrane

The outer membrane is mainly composed of phospholipid, lipopolysaccharide (LPS) and protein (Schnaitman, 1970b; Miura and Mizushima, 1968, 1969). Also present in the Enterobacteriaceae is the enterobacterial common antigen (ECA). The protein content is higher than that of the cytoplasmic membrane and the protein species present are unique to and characteristic of the outer membrane. Phospholipid, LPS and ECA are discussed in more detail below largely with respect to E. coli and Salmonella typhimurium for which most data is available. The proteins of the outer membrane are described in sections 1.3.5 and 1.3.6. in relation to E. coli as an example of a Gram-negative species.

1.3.2.1 Phospholipid

The major phospholipid of the outer membrane of E. coli and Salmonella typhimurium is phosphatidylethanolamine. Also present are phosphatidylglycerol and cardiolipin (White et al., 1972). Phospholipid molecules are synthesised in the cytoplasmic membrane (White et al., 1972) and are

subsequently translocated to the outer one. Phospholipid molecules appear to move reversibly between the inner and outer membranes (Jones and Osborn, 1977a, 1977b). Translocation is rapid and is driven by the proton-motive force (Donohue-Rolfe and Schaechter, 1980). Newly synthesised phospholipid appears at multiple, random sites in the outer membrane though the exact mechanism of translocation has yet to be determined. Jones and Osborn (1977b) proposed that phospholipid transport occurs via zones of adhesion between the cytoplasmic and outer membranes (Bayer, 1968). Alternatively, the reversible translocation of phospholipid molecules could be catalysed by specific proteins. Such a method of phospholipid exchange between membranes is well established in animal cells (Wirtz, 1974) but the existence of appropriate exchange proteins has not been reported in bacteria.

1.3.2.2 Enterobacterial Common Antigen

The enterobacterial common antigen (ECA) is an acidic polymer containing N-acetyl-D-glucosamine, D-mannosaminuronic acid and 4-acetamido-4,6-dideoxy-D-galactose (Lugowski *et al.*, 1983). Its chemistry and biology have been extensively reviewed by Mayer and Schmidt (1979) and Makela and Mayer (1976). ECA appears to be linked to a phospholipid 'anchor' which presumably attaches the antigen to the outer membrane. In the so-called ECA-immunogenic strains, however, the ECA is associated with

lipopolysaccharide by an interaction with the R-core-lipid-A moiety (section 1.3.2.3). Using ferritin-labelled antibodies, Rinno et al. (1980) demonstrated the cell surface location of this antigen.

1.3.2.3 Lipopolysaccharide

Lipopolysaccharides (LPS) are ubiquitous among the Gram-negative bacteria. They are the major endotoxin of the cell and mediate a variety of pathophysiological effects in animals. LPS molecules are synthesised in the cytoplasmic membrane (Osborn et al., 1972b) and are extremely complex. They consist of a hydrophobic part, lipid-A, and a hydrophilic moiety comprising an oligosaccharide core (R-core) and a polysaccharide tail (O-antigen) which protrudes from the cell surface. The O-antigen may vary with respect to composition and structure even within a single genus and bacteria can produce either complete (S-type) or incomplete (R-type) LPS molecules. These are referred to as smooth and rough strains respectively.

The functions of LPS are varied. It can serve as the receptor for the adsorption of various bacteriophage (e.g., T7, T3, P1) although this function was probably not intended. LPS is required for the biological activity of some outer membrane proteins *in vitro* (Datta et al., 1977; Schindler and Rosenbusch, 1981) and the amount of certain proteins in the outer membrane is drastically reduced in strains producing defective Rc and Rd type LPS (Ames et al.,

1974; Koplow and Goldfine, 1974). These factors suggest LPS maybe important for the translocation of some proteins to the outer membrane and/or their correct assembly within it. Further details of LPS-protein interactions are presented in section 1.3.5.

Strains possessing defective LPS are more susceptible to the loss of periplasmic enzymes suggesting LPS molecules contribute to the stability of the outer membrane (Lindsay et al., 1973). LPS also plays an important role in protecting the cell from host defence factors (section 1.3.4.1) and deleterious hydrophobic compounds (section 1.3.4.3).

Newly synthesised LPS molecules appear at discrete sites on the surface of the outer membrane several hundred of which are present in a single cell (Muhlradt et al., 1973). Electron microscopy of thin sections has revealed that these areas are coincident with the sites of fusion between outer and inner membranes as reported by Bayer (1968). The appearance of LPS molecules at discrete sites is followed by the lateral diffusion of these areas resulting in a uniform distribution of LPS over the surface of the cell (Muhlradt et al., 1973). Translocation of LPS is an irreversible and unidirectional process (Osborn et al., 1972b) and it has been postulated that strong associations with proteins in the outer membrane are the cause of this.

1.3.3 Structure of the Outer Membrane

The outer membrane is approximately 7.5 nm thick and resembles the trilaminar structure of the cytoplasmic membrane in cross-section (Glauert and Thornley, 1969). A lipid bilayer forms the basic continuum of the membrane and this model is supported by X-ray diffraction data (Ueki *et al.*, 1970) and by freeze-fracturing which produces a characteristic cleavage in the centre of the membrane (van Gool and Nanninga, 1971; Smit *et al.*, 1975).

The nature of the lipid bilayer of the outer membrane has been most studied in *E. coli* and *S. typhimurium* and it appears quite asymmetric in structure. Studies with ferritin-labelled antibodies directed against the O-antigen show that LPS is located exclusively in the outer half of the membrane (Shands, 1965). Enzymatic modification of LPS in intact cells has also led to the same conclusion (Funahara and Nikaido, 1980). Furthermore, both chemical labelling and resistance to exogenous phospholipases have demonstrated that most phospholipid molecules are located in the inner leaflet of the outer membrane, at least in wild-type *Enterobacteriaceae* (Kamio and Nikaido, 1976; van Alphen *et al.*, 1977b). With respect to *S. typhimurium*, this rather extreme form of assymetry is also supported by the fact that the total amount of phospholipid in the outer membrane is hardly sufficient to cover one monolayer (Smit *et al.*, 1975). In addition, freeze-fracturing of the outer membrane

of both S. typhimurium (Smit et al., 1975) and E. coli (van Gool and Nanninga, 1971) has indicated that the inner layer consists of phospholipid and that the outer leaflet is filled with LPS and protein molecules though some of the latter span the lipid bilayer.

The model described supposes the complete absence of phospholipid bilayer regions in the outer membrane. However, a study by van Alphen et al. (1977b) suggests that a small amount of phospholipid bilayer structure is present in the outer membrane of E. coli but is well-shielded from both enzymatic attack and chemical labelling agents by proteins, LPS and divalent cations. The latter have strong affinity for LPS (Schindler and Osborn, 1979) and are thought to neutralise the electrostatic repulsion between neighbouring LPS molecules which would ordinarily result from their negative charge. This neutralisation allows the interaction of LPS molecules on the surface of the cell.

In contrast to the Enterobacteriaceae, there is a paucity of information regarding the outer membrane structure of other Gram-negative bacteria.

1.3.4 Functions of the Outer Membrane

The outer membrane performs many important tasks for the cell. These include nutrient transport, protection against deleterious hydrophobic compounds and host defence

mechanisms as well as maintenance of cell shape and these are discussed below.

1.3.4.1 Protection Against Host Defence Factors

Gram-negative bacteria are more resistant than Gram-positive species to a variety of host defence mechanisms due to the presence of the outer membrane (Donaldson et al., 1974; Patterson-Delafield et al., 1980). Protection is largely afforded by the LPS component of the outer membrane since strains producing defective LPS are far more sensitive to leukocyte proteins (Rest et al., 1977), lysozyme (Tamaki and Matsuhashi, 1974), complement activity (Guan and Scott, 1980) and phagocytosis (Medearis et al., 1968) than bacteria possessing complete LPS molecules. The structure of LPS can be altered quite readily, particularly with respect to the O-antigen which projects from the surface of the cell. Gram-negative pathogens can, therefore, avoid the action of antibodies which are produced by the animal host against a specific type of LPS. However, in the case of Neisseria gonorrhoeae, the ability of the cell to change its outer membrane protein profile is important in protecting the bacterium from host proteases (James and Swanson, 1978).

1.3.4.2 Maintenance of Cell Shape

Some lines of evidence suggest that the outer membrane helps to maintain cell-shape, once determined. For example, under certain conditions purified outer membranes retain the shape of the cells from which they have been isolated (Henning et

al., 1973) and the treatment of E. coli K-12 with lysozyme can result in degradation of the peptidoglycan layer without alteration of cell shape (Feingold et al., 1968). In addition, Birdsell and Cota-Robles (1967) have shown that disruption of both the murein layer and the outer membrane is necessary for the transformation of rods into spherical forms, a result strongly suggesting that the outer membrane contributes to the rigidity of the cell wall. A similar conclusion has been reached by Asbell and Eagon (1966) who showed that EDTA, which releases protein-LPS complexes from the outer membrane, can convert cells of Pseudomonas aeruginosa into osmotically sensitive forms. The latter, in the presence of EDTA-released protein-LPS complex and under non-metabolising conditions, can revert to osmotically resistant cells (Stinnett and Eagon, 1975).

1.3.4.3 Resistance to Deleterious Hydrophobic Compounds

Most biological membranes are known to allow the rapid, passive diffusion of lipophilic but not hydrophilic agents due to their phospholipid bilayer stucture. In contrast, the outer membrane of Gram-negative bacteria, enteric species in particular, has a low degree of permeability to hydrophobic molecules as shown by the strong resistance of these organisms to hydrophobic antibiotics (e.g., novobiocin), dyes (e.g., methylene blue) and detergents (e.g., SDS). Indeed, the difference in dye and detergent sensitivity is the basis for various selective media (e.g., deoxycholate

agar and eosin-methylene blue medium) for Gram-negative enterics such as E. coli and S. typhimurium.

The impermeable nature of the outer membrane to lipophilic compounds can be understood quite well on the basis of its asymmetric bilayer structure as presented in section 1.3.3. Either the lack of phospholipid bilayer domains (Smit et al., 1975) or the fact that any present are well-shielded (van Alphen et al., 1977b) are thought to be the major reasons for the low permeability of the outer membrane to external hydrophobic agents. LPS molecules may also contribute to the permeability barrier of the outer membrane since they are held together by both hydrophobic and ionic interactions. Lipophilic compounds may therefore have great difficulty penetrating between the associated hydrocarbon chains. The permeability of the outer membrane can be increased by a number of factors including mutations which alter the structure of LPS molecules and those which lead to a decrease in the amounts of certain outer membrane proteins. These factors are extensively reviewed Nikaido and Vaara (1985).

One can appreciate that membranes which exclude lipophilic compounds are important to wild-type enteric organisms like E. coli and S. typhimurium which inhabit environments where detergent bile salts and long-chain fatty acids are found in abundance. There is no a priori necessity, however, that all Gram-negative bacteria should produce hydrophobe-impermeable

outer membranes. In fact, some non-enteric Gram-negatives such as N. gonorrhoeae, a mucosal surface pathogen, are extremely sensitve to hydrophobic agents like erythromycin and crystal violet. These observations suggest that the outer membrane may contain areas of phospholipid bilayer which are exposed and therefore penetrable by lipophilic compounds. Alternatively, weaker LPS interactions on the surface of the cell may cause this increased sensitivity.

As mentioned, the outer membrane is freely permeable to hydrophilic agents such as nutrients (e.g., sugars, amino acids and inorganic salts) and antibiotics (e.g., neomycin, ampicillin and cycloserine). This property is correlated with the protein component of the outer membrane which is described in the next section.

1.3.5 Proteins of the Outer Membrane of E. coli

The identification of outer membrane proteins has been mainly dependent upon the use of SDS-PAGE (Laemmli, 1970). Initially, the outer membrane protein patterns obtained using this system differed drastically between laboratories partly because different gel systems were applied and partly because different solubilisation temperatures were used during preparation of protein samples for electrophoresis. Schnaitman (1970a), first reported that the E. coli outer membrane contained one major protein which accounted for 70% of the total outer membrane protein. Several laboratories have since demonstrated that Schnaitman's single protein

consists of up to four proteins with molecular weights between 33-40 kd. The nomenclature of these 'major outer membrane proteins' has been standardised by Reeves (1979) and is shown in Table 1.3 (a) along with previous designations. In addition to these proteins, many others have been identified and characterised and they will be described later in this section.

The proteins of the outer membrane perform a variety of functions for the cell. Some form either diffusion pores or specific transport systems which allow the uptake of nutrients by the energy-independent processes of passive and facilitated diffusion. Other proteins are involved in maintaining the structural integrity of the outer membrane while some have an enzymatic function. Although this section concentrates on *E. coli*, the outer membranes of other Gram-negative species contain similar proteins and details of these can be found in the review of Lutenberg and van Alphen (1983).

It is important to note that the protein profile of the outer membrane is dependent upon strain background, media composition (e.g., presence of specific nutrients, salt concentration), temperature, pH, lysogeny and various mutations (Schnaitman, 1974; Lutenberg *et al.*, 1976). These factors will be discussed in relation to each individual protein.

Table 1.3 (a) Nomenclature of the major outer membrane proteins of *E. coli* K-12

Previous designations	Uniform nomenclature
Protein a, 3b, 011	OmpT
Protein b, 1a, 09, Ia, TolF, A_1, 4	OmpF
Protein c, 1b, 08, Ib, 4, A_2	OmpC
Protein d, 3a, 010, II*, 7, B, TolG	OmpA

(Adapted from Lutenberg and van Alphen, 1983)

1.3.5.1 OmpC and OmpF

OmpC and OmpF are often referred to as porin proteins which reflects their function as general diffusion pores in the outer membrane. Initially, they were referred to as 'matrix protein' due to their tendency to associate with peptidoglycan when isolated from the cell (Rosenbusch, 1974). The two proteins are immunologically related, show regions of homology at the amino acid level (Nikaido and Wu, 1984) and have a high content of beta-structure (Rosenbusch, 1974). A comparison of their structural genes has also revealed extensive similarity with respect to nucleotide sequence, suggesting they have evolved from a common ancestral gene (Mizuno *et al.*, 1983a). Within the membrane, the relative amounts of the two porins can vary, but together they remain at a constant 100,000 copies per cell (Lutenberg *et al.*, 1976). OmpF and OmpC are found in the majority of *E.coli* strains but quantitative differences have been reported (Lutenberg *et al.*, 1976). Some features of the porin proteins are summarised in Table 1.3 (b).

The function of both proteins has been studied using reconstitution experiments. Such studies have demonstrated that the purified proteins are able to form aqueous pores in phospholipid-LPS liposomes through which various monosaccharides and amino acids can pass (Nakae, 1976; Nikaido and Rosenberg, 1983). *In vivo* work using porin-deficient mutants has also confirmed the pore function of

Table 1.3 (b) Characteristics of proteins OmpF and OmpC

Characteristic	OmpF	OmpC
Molecular weight (kd)	37,205	36,500
Receptor for 'phage or colicin	K20, TuIa, ColA, ColE2, ColE3, T2, TP1	Me1, TuIb, T4, PA-2, 434
Previous gene nomenclature	*tolF*. *colB*. *cos*. *cry*	*meoA*. *par*
Protein sequence	Chen *et al*. (1982)	Mizuno *et al*. (1983a)
Gene sequence	Inokuchi *et al*. (1982)	Mizuno *et al*. (1983a)

these proteins (Bavoil et al., 1977). Within the membrane, the pore consists of three protein monomers which interact to form a functional channel (Palva and Randall, 1978). Each channel is thought to have a triplet inlet converging to a single outlet on the periplamic side of the membrane (Dorset et al., 1984).

Diffusion of solutes through the channels is dependent upon a variety of factors including the size, electrical charge and hydrophobicity of the penetrating molecules. Small hydrophilic molecules (<600 d) can freely permeate the pores such that the uptake of monosaccharides and amino acids is not retarded by the outer membrane (Decad and Nikaido, 1976). Hydrophobicity impairs the penetration of solutes through OmpF and OmpC both in vitro and in vivo, so that small, hydrophobic molecules (>200 d) are excluded from the pores (Nikaido and Rosenberg, 1983; Nikaido et al., 1983). The presence of a negative charge on the solute also reduces the rate of diffusion through the porins (Nikaido and Rosenberg, 1983; Nikaido et al., 1983). Exclusion of hydrophobic and negatively charged molecules maybe of ecological advantage to E. coli whose natural habitat contains high concentrations of hydrophobic and anionic bile salts.

A number of observations have indicated that LPS is important in the assembly and/or the functioning of the porin proteins in the outer membrane. Bacterial strains

producing defective LPS possess decreased amounts of OmpF and OmpC in the outer membrane (Koplow and Goldfine, 1974; Lutenberg *et al.*, 1976) and both pore activity and 'phage receptor functions are dependent upon LPS *in vitro* (Datta *et al.*, 1977; Schindler and Rosenbusch, 1981).

The regulation of expression of the two porins has been widely investigated. Both culture conditions and a number of different genetic loci control the appearance of OmpC and OmpF in the membrane. Firstly, many studies have been concerned with the influence of osmolarity upon porin expression since cells grown in medium of a high osmotic strength express OmpC whereas OmpF synthesis is repressed under such conditions (van Alphen and Lutenberg, 1977). Genetic analysis of this regulation has shown that it is mediated through an unlinked locus, *ompB*, which encodes two proteins, OmpR and EnvZ. One model suggests that OmpR is a positive activator for porin expression and can exist in either a monomeric or a dimeric form. The monomer is required for activation of *ompF* and the dimer for expression of *ompC*. The model also suggests that the *envZ* gene product acts as an osmosensor in the cytoplasmic membrane and influences the monomer-dimer equilibrium of OmpR such that preferential synthesis of either OmpF or OmpC can occur, depending on the osmolarity of the medium (Hall and Silhavy, 1981a, 1981b). This regulation maintains a constant combined copy number of the two proteins. Mutations in the *osmZ* gene

affect the osmotic regulation of the porin genes. The synthesis of OmpC and OmpF is increased and reduced respectively, in *osmZ* mutants, as if the cell is responding to a higher osmolarity than that to which it is actually exposed (Higgins *et al.*, 1988). Mutations in *osmZ* lead to an increase in DNA supercoiling and the latter has been implicated in the expression of both *ompC* and *ompF* (Higgins *et al.*, 1988).

Two other genetic loci, *tolC* and *micF*, can influence the expression of the porin genes. Mutations in the *tolC* locus lead to a lack of OmpF in the outer membrane and this effect occurs at a post-transcriptional level (Morona and Reeves, 1982). In addition, *micF*, which is located upstream of *ompC*, reduces OmpF synthesis. Transcription of this gene produces a 173 base mRNA the 3' end of which is complementary to the 5' end of *ompF* mRNA. Stable hybrids can form between the two RNA molecules thereby inhibiting *ompF* mRNA translation (Mizuno *et al.*, 1984). The results of Misra and Reeves (1987) indicate that the primary effect of the *tolC* mutation is to increase the expression of *ompC* and *micF* such that the synthesis of OmpF is then prevented by *micF*. However, it is not understood where the *tolC* gene product acts at the molecular level.

OmpF synthesis is significantly reduced in strains grown on glucose and this can be overcome by addition of cAMP to the medium (Scott and Harwood, 1980). Cells deficient in either

adenylate cyclase (*cya*) or the cAMP activator protein (*crp*) also produced low levels of OmpF indicating that the cAMP system positively controls expression of the *ompF* gene (Scott and Harwood, 1980).

Temperature also influences the level of expression of *ompF* and *ompC* (Lutenberg *et al.*, 1976). At increased temperatures OmpF synthesis is reduced and OmpC raised to compensate. The temperature-dependent expression, however, is not exhibited by strains harbouring an *envY* mutation (Lundrigan and Earhart, 1984a). This locus encodes a 25 kd protein but how this can mediate the temperature-dependent fluctuation in porin proteins is not understood.

The pH of the culture greatly influences porin gene expression (Heyde and Portalier, 1987). OmpC synthesis is increased while OmpF production is reduced at low pH. This pH-dependent regulation acts at a transcriptional level on both *ompF* and *ompC* as well as at a post-transcriptional stage on *ompF* and is mediated, at least in part, by the EnvZ protein. Other proteins involved in the pH regulation of porin genes include TolA, TolB and ExcC (Heyde *et al.*, 1988).

A final factor which affects the synthesis of OmpF and OmpC is the bacteriophage PA-2. When *E. coli* is lysogenised by this 'phage, both porins are supplanted by a new, PA-2-

encoded protein, namely protein 2 (Pugsley and Schnaitman, 1978).

1.3.5.2 OmpA Protein

OmpA is present in about 100,000 copies per cell. It is a heat-modifiable protein in that its apparent molecular weight is increased from 28 kd to 35 kd if boiled in SDS-solution prior to polyacrylamide gel electrophoresis (Nakamura and Mizushima, 1976). This property is ascribed to the high beta-structure content of OmpA which tends to bind excessive amounts of SDS. On boiling, however, beta-structure can be reduced extending the molecule to an alpha-helical form which binds less detergent and so decreases the electrophoretic mobility of the protein (Nakamura and Mizushima, 1976; Heller, 1978). The complete amino acid sequence of OmpA has been determined by Chen *et al*. (1980) who also reported the true molecular weight of OmpA as 35 kd.

OmpA can serve as a receptor for various bacteriophage (e.g., K3 and TuII*) and for colicin L indicating that the protein is located at the surface of the cell. Mutants lacking OmpA are defective in F-pilus mediated conjugation (Skurray *et al*., 1974) where it seems the protein is needed to stabilise mating aggregates. The protein also plays a role in maintaining both the structural integrity of the outer membrane and the rod-shape of the cell. These functions were noted by Sonntag *et al*. (1978) who were

studying *ompA lpp* mutants. Such strains are spherical and require high concentrations of either magnesium or calcium ions for growth. Moreover, abundant blebbing of the outer membrane occurs and the peptidoglycan layer is no longer connected to the outer membrane in these mutant bacteria. OmpA-LPS interactions have been shown to protect the protein from denaturation and non-specific proteolytic degradation (Schweizer *et al.*, 1978). *In vitro*, the phage receptor activity of OmpA is dependent upon the presence of LPS (Schweizer *et al.*, 1978) and the purified protein will only inhibit conjugation when it is added together with LPS (van Alphen *et al.*, 1977a; Schweizer and Henning, 1977).

The structural gene encoding OmpA (*ompA, tolG, con, tut*) is located at 21.5 min on the *E. coli* chromosome and has been cloned by Henning *et al.* (1979). Attempts to clone *ompA* onto high copy number vectors have proven unsuccessful, presumably because high levels of OmpA are lethal to the cell (Bremer *et al.*, 1980). Sequencing of the gene has been reported by Movva *et al.* (1980a) and this has revealed that *ompA* mRNA can form extensive secondary structures which may contribute to the stability of *ompA* mRNA (Hirashima *et al.*, 1973). It has been shown that the amount of OmpA in the outer membrane remains constant throughout a wide range of bacterial doubling times (Lutenberg *et al.*, 1976). Such invariability requires a mechanism for adjusting the rate of biosynthesis of OmpA in direct proportion to the rate of

cell growth. The studies of Nilsson *et al*. (1984) indicate that the stability of *ompA* mRNA is growth-rate dependent and would therefore allow the cell to alter the rate of OmpA synthesis in response to the generation time of the cell. The stability of *ompA* mRNA appears to be controlled by endonucleolytic cleavage of the 5' non-coding region of the message (Melefors and von Gabain, 1988).

1.3.5.3 Lipoprotein

The Braun lipoprotein (7.2 kd) is the most abundant protein in the *E. coli* cell envelope present at $3x10^5$-$6x10^5$ copies per cell and representing 5-7% of the total cellular protein.

Approximately 30% of lipoprotein molecules are covalently linked, via the -amino group of the C-terminal lysine, to diaminopimelic acid residues of the underlying peptidoglycan layer (Braun and Rehn, 1969; Braun *et al*., 1976). The remaining lipoprotein is free in the outer membrane (Inouye *et al*., 1972). The N-terminal amino acid of lipoprotein, cysteine, is modified in a unique manner: its amino group is substituted with a fatty acid and its sulphydryl group is substituted with a diglyceride (Braun, 1975). These modifications are made in the cytoplasmic membrane. With respect to the bound form of lipoprotein, these fatty acid moieties interact with the outer membrane and so may serve to anchor this structure to the cell (Braun, 1975; Braun and Rehn, 1969). Lipoprotein is apparently not exposed at the

cell surface since, unlike other outer membrane proteins, it does not function as a receptor for bacteriophage. The protein is largely alpha-helical in structure (Braun et al.. 1976) and its entire amino acid sequence has been determined by Braun and Bosch (1972).

The isolation of lipoprotein-deficient mutants (lpp, lpo) indicates that the protein is not essential for survival of the cell. However, such strains do show a significant loss of outer membrane in the form of vesicles as well as increased sensitivty to EDTA and leakage of periplasmic enzymes. This suggests a role for lipoprotein in maintaining the structural integrity and stability of the outer membrane (Hirota et al., 1977; Suzuki et al., 1978).

Nakamura and Inouye (1979) have cloned and sequenced the lpp gene of E. coli which maps at 36.5 min. Attempts by Lee et al. (1981) to clone the gene into high copy number plasmids have proven unsuccessful, presumably due to the lethal overproduction of lipoprotein. The protein is constitutively produced and its mRNA is very stable with a half-life of 11.5 min (Hirashima et al., 1973).

1.3.5.4 The LamB Protein

This outer membrane protein plays a role in the uptake of maltose and maltodextrins (Szmelcman and Hofnung, 1975; Wandersman et al., 1979). It is the product of the lamB gene which is part of the maltose regulon located at 91 min on

the E. coli chromosome. Addition of maltose to the growth medium results in the expression of the structural genes of this regulon, including lamB (Raibaud et al., 1979; Silhavy et al., 1979). After such induction the LamB protein can be present at up to 100,000 copies per cell.

The protein forms transmembrane diffusion channels which allow the entry of maltose as well as amino acids (Nakae, 1979) and unrelated sugars (Luckey and Nikaido, 1980a). However, the pores become more discriminating for larger saccharides. For example, maltotriose can diffuse through the channel faster than other trisaccharides (Luckey and Nikaido, 1980a, 1980b). Maltose is able to enter the cell via OmpF and OmpC and it is thought, therefore, that the major role of LamB is in the transport of maltodextrins which are too large to penetrate the general diffusion pores.

The LamB protein has been purified by Luckey and Nikaido (1980a). It has a molecular weight of 47 kd, acts as a receptor for 'phages lambda, K10 and TP1 and is a trimer in its active form (Palva and Westerman, 1979). Randall (1975) noted that reduced amounts of LamB were present in LPS-deficient mutants and in vitro the protein has been shown to interact with LPS as well as lipoprotein and peptidoglycan (Yamada et al., 1981). These associations may be important

for the correct assembly and/or for the activity of LamB in the outer membrane, although this has not been demonstrated.

Amino acid homology with PhoE, OmpF and OmpC proteins has been reported (Nikaido and Wu, 1984). The *lamB* gene has been cloned and sequenced by Clement and Hofnung (1981). Synthesis of the protein is reduced in *perA* mutants (Wanner *et al*., 1979; Wandersman *et al*., 1980), increased at low temperatures (Lundrigan and Earhart, 1984a) and is favoured in media with an alkaline pH (Heyde and Portalier, 1987).

1.3.5.5 The PhoE Protein

The PhoE protein (Foulds and Chai, 1978), also referred to as protein E, Ic and e, is induced in wild-type cells by phosphate limitation (Overbeeke and Lutenberg, 1980) and, along with other proteins, helps the bacterium scavenge the last traces of phosphate or phosphate-containing components from the medium. The protein has been reported to form a more efficient pore for both inorganic and organic phosphate than porin OmpF (Korteland *et al*., 1982) and evidence suggests that PhoE has a recognition site for these solutes near the entrance of the pore (Overbeeke and Lutenberg, 1982).

The protein is encoded by the *phoE* gene which is located at 5.9 min on the *E. coli* chromosome and is part of the *pho* regulon (Tommassen and Lutenberg, 1980; Tommassen and Lutenberg, 1981) PhoE has a molecular weight of 36.8 kd and

can be present at up to 100,000 copies per cell though overproduction of the protein can be deleterious to the cell (Tommassen et al., 1982b). It has been purified (Chai and Foulds, 1979) and is able to act as a receptor for bacteriophages TC45, TC23 and K3 (Chai and Foulds, 1978, 1979).

Overbeeke et al. (1980) have shown that PhoE is immunologically related to OmpF and OmpC and recent work on the cloned genes ompF (Tommassen et al., 1982a) and phoE (Tommassen et al., 1982b) has demonstrated that their DNA's can be hybridised over the entire length of the genes (Tommassen et al., 1982a). The nucleotide sequence of the phoE gene has been established by Overbeeke et al. (1983) and a comparison of the predicted amino acid sequence of PhoE with that of OmpF (Chen et al., 1976) has revealed that 70% of the residues are identical (Overbeeke et al., 1983). Mizuno et al. (1983a) have also reported DNA homology between phoE and ompC as well as homology at the amino acid level of these two porins. In addition, Nikaido and Wu (1984) have determined amino acid homology with the LamB protein.

The synthesis of PhoE is adversely affected by mutations in the perA gene (Lundrigan and Earhart, 1981) and is also present in decreased amounts in strains producing defective LPS (Lutenberg et al., 1978).

1.3.5.6 The BtuB Protein

Vitamin B12 is too large to pass through the general pores of the outer membrane and therefore it requires a specific receptor protein for facilitating its uptake into the cell (White *et al.*, 1973). The receptor for this nutrient, the BtuB protein (60 kd), is specified by the *btuB* gene which is located at 89 min on the *E. coli* genetic map (Kadner and Liggins, 1973). Synthesis of the protein occurs during vitamin B12 limitation (Kadner, 1978) when BtuB can be present at 200-300 copies per cell. It is responsible for binding and translocating the vitamin to the periplasmic side of the outer membrane, the latter being dependent on the *tonB* gene product (Postle and Good, 1983). In addition to vitamin uptake, the protein functions as a receptor for E colicins, colicin A and bacteriophage BF23 (Cavard and Lazdunski, 1981; Di Masi *et al.*, 1973). Synthesis of BtuB is influenced by temperature, the protein being more prevalent at 27°C than at 37°C (Lundrigan and Earhart, 1984b).

1.3.5.7 Outer Membrane Proteins involved in Iron Transport

The solubility of ferric iron is extremely low and bacteria have developed systems to take up this essential nutrient from the environment by first complexing it with low molecular weight chelators known as siderophores (reviewed by Neilands, 1982). The iron-laden molecule is then taken up into the cell via specific receptors present in the outer membrane. Chelators used by *Enterobacteriaceae* include

aerobactin, enterochelin (enterobactin), citrate and ferrichrome. These iron-uptake systems are of particular importance to pathogenic bacteria since the iron present in animal hosts is generally complexed with proteins such as transferrin.

When E. coli is grown under conditions of low iron, it can synthesise certain siderophores and also produce a number of outer membrane proteins which are involved in the uptake of specific iron-chelator complexes. Together, the chelators and receptors constitute high affinity systems, these being dependent upon the tonB gene product (Postle and Good, 1983). The receptor proteins involved in the uptake of the chelated iron are summarised in Table 1.3 (c). Some of these proteins are found in reduced amounts in strains with a perA mutation (Lundrigan and Earhart, 1981).

1.3.5.8 Outer Membrane Proteins involved in Nucleoside Transport

Bacterial strains defective in the tsx locus, located at 9.2 min on the E. coli chromosomal map, are defective in the uptake of all nucleosides and deoxynucleosides except cytidine and deoxycytidine (Hantke, 1976; Krieger-Brauer and Braun, 1980). The tsx gene which has recently been cloned (Tommassen et al., 1982), encodes a 26 kd protein which forms pores in the outer membrane through which nucleosides

Table 1.3 (c) Outer membrane proteins involved in the uptake of chelated iron

Receptor protein	Mr (kd)	Structural gene	Receptor for 'phage/colicin	Proposed function
FecA*	80.5	fec (7 min)	-	Uptake of Fe(III)-citrate
FhuA (TonA)	78.0	fhuA (3.5 min)	T1, T5, 080, Colicin M	Uptake of Fe(III)-ferrichrome
FepA (Cbr, FeuB)	81.0	fepA (13 min)	ColB, ColD	Uptake of Fe(III)-enterochelin
Cir	74.0	cir (44 min)	ColI, ColV	Uptake of complexed Fe(III) ?
83K Protein	83.0	-	-	Uptake of complexed Fe(III) ?

* Presence of citrate required for expression. All other proteins are induced by Fe(III) limitation.

The Cir, FepA and 83K proteins are decreased in cells with a perA mutation and the synthesis of these proteins is co-ordinately regulated along with the production of enterobactin.

(Adapted from Lutenberg and van Alphen, 1983)

can diffuse (Krieger-Brauer and Braun, 1980). Synthesis of this protein is subject to catabolite repression (Manning and Reeves, 1978), and is therefore produced in very low amounts by cells grown in media containing glucose and by strains with cya and crp mutations (Krieger-Brauer and Braun, 1980). The protein is found in increased amounts in cytR and deoR mutants (Krieger-Brauer and Braun, 1980) since these loci encode repressor proteins which are involved in the control of expression of tsx and other nucleoside uptake systems. The Tsx protein acts as a receptor for the bacteriophage T6 and colicin K and is present in 10,000 copies per cell.

1.3.5.9 Other E. coli Outer Membrane Proteins

Other less well characterised proteins found in the outer membrane of E. coli are summarised in Table 1.3 (d). Some of these proteins are encoded by either bacteriophage or plasmids present in the cell.

1.3.6 Enzymes of the E. coli Outer Membrane

The outer membrane is poor in enzymatic activity compared to the cytoplasmic membrane. In E. coli the first enzyme to be detected in the outer membrane was phospholipase A (Scandella and Kornberg, 1971). Since, outer membrane preparations from this organism have been reported to carry out a variety of proteolytic cleavage reactions in vitro. These include descriptions of the cleavage of colicins Ia, A and A1 to inactive polypeptide fragments (Bowles and

Table 3 (d) Other outer membrane proteins of *E. coli*

Protein	Comments
NmpC	37 kd pore-forming protein. Synthesis subject to catabolite repression. The *nmpC* gene (12 min) is normally silent in wild-type cells.
TolC	52 kd protein. Shows some homology to OmpC, OmpF, PhoE and LamB. Mutations in the *tolC* gene (66 min) prevent expression of OmpF, NmpC and protein 2 at a post-transcriptional level.
Protein K	40 kd pore-forming protein. Found in encapsulated *E. coli* strains.
Protein III	17 kd protein. Found in diminished quantities in *E. coli* with defective LPS. Synthesis is negatively controlled by cAMP.
Protein G	15 kd protein. Implicated in the coupling of DNA replication and cell elongation.
LPS-binding protein	15 kd. Common antigen in the *Enterobacteriaceae*. Has high affinity towards LPS and is thought to anchor LPS to the outer membrane.
Protein 2	Encoded by the bacteriophage PA-2. Pore protein which supplants both OmpC and OmpF when *E. coli* is lysogenised by this 'phage. Synthesis is subject to catabolite repression by cAMP and is thermally regulated.
MRB Protein	Encoded by R factors. Function not understood.
TraT	The most studied outer membrane protein encoded by F factors. Prevents a bacterium already containing a F plasmid from acting as an acceptor cell during conjugation by blocking the stabilisation of mating aggregates.
FadL	Required for the uptake of long-chain fatty acids. 43 kd. Acts as a receptor for 'phage T2.

Konisky, 1980), the conversion of the precusor of alkaline phosphatase to the mature form (Inouye and Beckwith, 1977), the cleavage of plasminogen to plasmin (Leytus et al.. 1981), caseinolytic activity (Regnier and Thang, 1979), nitrate reductase solubilising activity (MacGregor et al., 1978), ferric enterobactin receptor activity (Hollifield et al.. 1978; Fiss et al., 1979) and the cleavage of bacteriophage M13 precoat protein to coat protein (Zwizinski et al., 1981). There is confusion in the literature as to whether these activities are the result of either separate or identical proteolytic enzymes. A number of proteases have been purified from the outer membrane and their activities tested in an attempt to clarify the situation; these are discussed below.

1.3.6.1 Protease IV and Protease V

Protease IV has been purified by Regnier (1981a, 1981b). It is an endoproteolytic enzyme with a Mr of 23,500 and is responsible for the caseinolytic activity associated with the outer membrane. It is sensitive to EDTA, p-aminobenzamidine, which inhibits trypsin-like proteases, as well as TLCK and TLPK which inactivate trypsin and chymotrypsin respectively. Pacaud (1982a, 1982b) reported the purification of two enzymes from membrane preparations of E. coli designated protease IV and protease V. Both are capable of degrading alkylated, high molecular weight cytoplasmic proteins in vitro. However, protease IV, as

described by Pacaud (1982b), is found in the cytoplasmic membrane and clearly differs from protease IV of Regnier (1981a, 1981b) having a molecular weight of 34 kd. Protease V is located in both the inner and outer membranes. It is inhibited by the serine protease inhibitors DFP and PMSF and by TLCK and TPCK .

1.3.6.2 Leader (Signal) Peptidase

This enzyme which converts M13 precoat protein into its mature form has been purified by Zwizinski and Wickner (1980). It is a 39 kd protein and is present in both the cytoplasmic and outer membranes. The enzyme also cleaves precursor forms of two periplasmic amino acid binding proteins and of the LamB protein (Zwizinski *et al.*, 1981).

1.3.6.3 Protease VI

Recent work has identified an enzyme in the outer membrane of *E. coli* termed protease VI (Palmer and St. John, 1987). It is a serine protease and has a molecular weight of 43 kd which is similar to that of the OmpT protein. The latter is responsible for modifying the ferric-enterobactin receptor protein in the outer membrane of *E. coli* (Hollifield *et al.*, 1978) and is discussed in more detail in section 1.4. Despite similarities in size, Palmer and St. John (1987) concluded that protease VI and protein OmpT were not identical enzymes on the basis of inhibitor studies. Protease VI is sensitive to PMSF whereas this inhibitor has

no effect upon OmpT. Protease VI is also inhibited by DFP, benzamidine, p-aminobenzamidine and TLCK.

1.3.6.4 Protease VII

This protease was recently discovered in E. coli producing human gamma interferon (Sugimura and Higashi, 1988). The enzyme cleaves the interferon specifically between basic amino acids (Lys-Arg and Arg-Arg). It is insensitive to PMSF, DFP, TLCK, EDTA and p-chloromercuribenzoate but is inhibited by benzamidine and by Zn^{2+} and Cu^{2+} ions.

1.3.7 Biosynthesis of Outer Membrane Proteins

Since the machinery for protein synthesis is located in the cell cytoplasm, proteins destined for the periplasm and the outer membrane must initially cross the inner membrane. This process has been studied in detail both genetically and biochemically and excellent reviews regarding this subject have been published (Oliver, 1985; Randall et al., 1987). This section will, therefore, focus upon how existing models which are used to describe protein translocation across the cytoplasmic membrane, can be applied to outer membrane proteins.

Firstly, the signal hypothesis of Blobel and Dobberstein (1975) proposes that exported proteins contain an amino-terminal extension of 20-40 amino acid residues, referred to as the signal sequence. This causes ribosomes engaged in the synthesis of exported proteins, to bind to the cytoplasmic

membrane. Once attached to the membrane, translation continues while the growing polypeptide chain is vectorially transferred through a proteinaceous pore. The signal sequence is eventually cleaved by a signal peptidase and the mature protein released. Such a mechanism is referred to as co-translational since protein synthesis and export are coupled.

Initially, this model was used to describe the export of proteins from eukaryotic cells. However, the fact that mammalian polypeptides with their own signal sequences can be exported from E. coli suggests a similar mechanism may operate in bacteria (Talmadge et al., 1980). Indeed, it is now well established that many outer membrane proteins are synthesised as pro-proteins with an amino-terminal peptide extension. A number of approaches have led to the identification of these precursor molecules including:

(a) The in vitro translation of mRNA for outer membrane proteins, for example, LamB (Randall et al., 1978) and lipoprotein (Hirashima et al., 1974).

(b) The accumulation of precursors through the uncoupling of protein synthesis and processing, examples including OmpT (Gayda et al., 1979b), OmpA and the porins (Sekizawa et al.. 1977).

(c) Structural gene and protein sequencing. Table 1.3 (c) shows the amino acid sequences of the peptide extensions found in precursors of outer membrane proteins as deduced by

Table 1.3 (e) Signal sequences of *E. coli* outer membrane proteins

Protein	Signal sequence	Reference
LP*	MKATKLVLGAVILGSTLLAG/C	Inouye *et al.* (1977)
LamB	MMITLRKLPLAVAVAAGVMSAQAMA/V	Hedgpeth *et al.* (1980)
OmpA	MKKTAIAIAVALAGFATVAQA/A	Movva *et al.* (1980b)
OmpF	MMKRNILAVIVPALLVAGTANAK/A	Mutoh *et al.* (1982)
OmpC	MKVKVLSLLVPALLVAGAAN/A	Mizuno *et al.* (1983b)
PhoE	MKKSTLALVVMGIVASASVQA/A	Overbeeke *et al.* (1983)
TolC	MKKLLPILIGLSLSGFSSLSQA/E	Hackett & Reeves (1983)

* Lipoprotein

(a) The amino acid following the dash (/) is the N-terminal amino acid of the mature protein.

(b) Hydrophobic amino acids (von Heijne, 1981) are underlined.

these procedures.

These amino acid extensions, or signal sequences, are cleaved by signal peptidases (Wolfe et al., 1982; Yamada et al., 1984) during or after export of the protein through the inner membrane. The signal sequence is important for the export of proteins since studies in which the leader peptide is totally or extensively deleted, prevent translocation across the cytoplasmic membrane (Emr et al., 1978). In some cases it appears that sequences in the mature protein are also important for translocation through the inner membrane. For example, it has been reported that the leader sequence and the first thirty-nine amino acids of the mature LamB protein are necessary to initiate export from the cytoplasm (Benson et al., 1984). In contrast, Freudl et al. (1987) showed that the export of OmpA occurred even when residues 1-229 of the mature protein were deleted. They concluded from this that sequence information within mature OmpA was unnecessary for translocation of the protein across the cytoplasmic membrane (Freudl et al., 1987; Freudl and Henning, 1988). The same conclusion was recently drawn from experiments on the export of PhoE (Bosch et al., 1986).

Studies have also indicated that outer membrane proteins are preferentially synthesised on polysomes which are bound to the cytoplasmic membrane (Randall and Hardy, 1977) and this again supports the signal hypothesis. However, the

synthesis of lipoprotein has been reported to occur on cytosolic ribosomes (Konings et al., 1975).

A second model, the loop model (Inouye and Halegoua, 1980) also assumes that protein synthesis and export are coupled. Here, the positively charged signal sequence binds to the inner membrane and the hydrophobic region then inserts into the lipid bilayer forming a loop-like structure. As polypeptide elongation continues, an increasingly larger portion of the loop is located on the periplasmic side of the inner membrane. Cleavage at the processing site releases the translocated protein while the signal peptide remains embedded in the cytoplasmic membrane. In fact, the presence of the cleaved signal peptide of lipoprotein in the inner membrane has been demonstrated (Hussain et al., 1982).

Although evidence exists for the coupling of synthesis and export of outer membrane proteins (Hall et al., 1983), both LamB and OmpA can be translocated through the cytoplasmic membrane after translation has been completed (Josefsson and Randall, 1981; Zimmerman and Wickner, 1983). A model, the membrane trigger hypothesis (Wickner, 1979), has been proposed to explain the post-translational export of some proteins. The role of the signal sequence in this particular postulate is to promote the folding of the newly made precursor into a conformation that is soluble and competent for export. Binding of the precursor protein to the cytoplasmic membrane triggers a conformational change

allowing the protein to spontaneously insert into and pass through the lipid bilayer. Cleavage of the signal peptide renders this an irreversible event. The model suggests that protein export does not require any complex secretory apparatus. However, genetic analysis has indicated that a number of proteins encoded by the sec genes (reviewed by Oliver, 1985), are needed for the export of many outer membrane proteins including OmpA, LamB, OmpF, lipoprotein (reviewed by Randall et al., 1987) and TonA (Baker et al., 1987). It seems, therefore, that some sort of export machinery is required for the translocation of outer membrane proteins across the inner membrane whether it be during or subsequent to protein synthesis.

In summary, it has not been conclusively established which, if any, of these models correctly describes the export of outer membrane proteins. Indeed, it maybe that a different mechanism operates depending upon the protein being exported.

1.3.8 Regulation of Outer Membrane Protein Synthesis

Many of the genes encoding outer membrane proteins are independently regulated and efficiently transcribed and translated, and in some cases they produce stable mRNA (Hirashima et al., 1973). Under conditions of maximum expression therefore, proteins such as LamB, the porins, OmpA and lipoprotein are among the most abundant in the cell. Since none of these proteins are enzymes whose

products can be sensed directly, it is unlikely that the cell can sense the levels of these proteins once they have been exported to the outer membrane. How does the cell therefore regulate the synthesis of outer membrane proteins to prevent their overproduction since the outer membrane has only a finite capacity for protein.

Datta *et al*. (1976) reported that the assembly of OmpA into the outer membrane was subject to feedback inhibition when overproduced in the cell but did not suggest how such a mechanism could operate. More recently Click *et al*. (1988) noted that the overproduction of OmpC resulted in almost complete inhibition of synthesis of both OmpA and LamB. This inhibition was mediated at the level of translation. The workers suggest that cells are able to monitor expression of exported proteins by sensing occupancy of some limiting component in the export machinery and use this in turn to regulate translation of these proteins. This translational regulation which may be coupled to export could provide a means for preventing the deleterious overproduction of outer membrane proteins.

1.3.9 Assembly of Proteins into the Outer Membrane

As previously discussed outer membrane proteins are synthesised in association with the cytoplasmic membrane, a subject which has received much attention. The subsequent translocation and assembly of proteins in to the outer membrane has been less well studied and an understanding of

how outer membrane proteins reach their final destination is far from complete. The following discussion serves to summarise some important features of outer membrane protein assembly along with the models which have been proposed to describe how the proteins reach their ultimate cellular location.

1.3.9.1 Kinetics of Assembly of Outer Membrane Proteins

The kinetics of insertion of newly made proteins into the outer membrane has been studied using the technique of pulse-labelling (Ito *et al.*, 1977; Lin and Wu, 1980). Such experiments have revealed that the integration of protein molecules into the outer membrane takes approximately three minutes although incorporation of OmpF and OmpC requires a longer period of time. The assembly of proteins into the outer membrane is somewhat faster than the assembly of proteins into the periplasm which takes approximately ten minutes (Ito *et al.*, 1977). Such data does not support the proposal that outer membrane proteins pass through the periplasm on route to their final destintion (Halegoua and Inouye, 1979). Lin and Wu (1980) also reported that the rate-determining step in the assembly of proteins into the outer membrane lies in the elongation of the nascent polypeptide chain.

1.3.9.2 Sites of Insertion

The sites of insertion of proteins into the outer membrane have been studied by a number of workers. Smit and Nikaido

(1978) reported that the 35 kd porin protein of S. typhimurium could be detected at numerous but discrete sites on the cell surface following its synthesis. Begg (1978) observed that OmpC and OmpF were inserted into the outer membrane of E. coli in a similar manner. As with LPS, these sites corresponded to areas of fusion between inner and outer membranes. It is thought that the sites of insertion move along the cell surface leaving behind newly incorporated protein. In this way proteins become located over the whole of the outer membrane (De Leij et al., 1979).

In contrast to the diffuse intercalation of porin proteins into the outer membrane, Begg and Donachie (1977) reported that newly synthesised T6 receptor protein was added exclusively at the poles of the cell. This was also noted for the OmpA protein (Begg and Donachie, 1984). The insertion of the 'phage lambda receptor (LamB) was examined by Ryter et al. (1975). Following induction of the protein with maltose, E. coli was infected with lambda and the location of 'phage particles on the cell surface was subsequently studied. These were seen mainly over the region of the division septum and it appeared that LamB was only synthesised during a short period in the cell-cycle just before cell division.

Similar cell-cycle-dependent synthesis (or insertion) has been reported for an iron-chelator receptor protein (Boyd and Holland, 1977) in E. coli. In contrast, synthesis and

insertion of the porin proteins occurs throughout the cycle (Churchward and Holland, 1976).

1.3.9.3 **Interaction with Lipopolysaccharide**

Although some evidence indicates that proteins continue to be incorporated into the outer membrane independently of LPS (Rick and Osborn, 1977), reports also show that strains producing defective LPS molecules contain reduced amounts of protein in their outer membranes (Ames *et al.*, 1974; Koplow and Goldfine, 1974). This suggests a certain structure of LPS may be required for either the translocation and/or the assembly of proteins into the outer membrane. It is known that the majority of *ompA*, *ompC* and *ompF* mutants, selected for resistance to 'phages and colicins, do not incorporate structurally altered proteins into the outer membrane (Osborn and Wu, 1980). Failure to assemble such proteins into their normal location may result from the inability of these molecules to interact properly with LPS. Whether or not a specific amino acid sequence is necessary for association with LPS has not been defined. The insertion of LPS and porin proteins at discrete sites in the outer membrane is at least consistent with the idea that some proteins may be translocated to their final location in association with LPS molecules. In the case of OmpA, evidence has been presented to indicate protein-LPS interactions play an essential role in the biosynthesis of this protein (Beher *et al.*, 1980)

1.3.9.4 Models for the Assembly of Proteins into the Outer Membrane

A number of models have been postulated for the assembly of proteins into the outer membrane. Firstly, it has been suggested that outer membrane proteins OmpA and lipoprotein appear in the periplasm before they are assembled into the outer membrane (Halegoua and Inouye, 1979). Such a mechanism has also been suggested for the assembly of TonA (Baker et al., 1986) and PhoE (Bosch et al., 1986). However, this model is energetically unfavourable unless the nascent proteins are coated with amphipathic molecules during transit through the periplasm. Also, the rate of assembly of bulk outer membrane proteins is significantly faster than periplasmic proteins which does not seem to support this particular model (Ito et al., 1977). It has been suggested by Pages et al. (1978), however, that periplasmic proteins first appear in the outer membrane as their precursor forms and are subsequently released into periplasm by proteolytic processing. This may account for their slow rate of appearance in the periplasmic space.

A model proposing the co-translational transfer of outer membrane proteins has also been described by De Leij et al. (1978). This suggests that nascent polypeptide chains are incorporated into the outer membrane while still being attached to ribosomes on the inner surface of the cytoplasmic membrane. Transfer of the protein to the outer

membrane is thought to occur via zones of adhesion and indeed newly synthesised porin proteins appear to be inserted at such sites (Smit and Nikaido, 1978; Begg, 1978). It can account for the rapid appearance of proteins in the outer membrane upon completion of protein synthesis (Lin and Wu, 1980; Ito et al., 1977).

A third model to explain the divergence of export of outer membrane proteins from that of periplasmic proteins has been proposed by Emr et al. (1980). It postulates the existence of a dissociation sequence within the mature protein that directs outer membrane proteins to their proper destination. The model proposes that soon after the dissociation sequence has been translated, transport of the nascent chain through the cytoplasmic membrane ceases. When translation is complete, the polypeptide is left spanning the membrane. The protein is subsequently translocated to the outer membrane by vesicles which bud off from the inner membrane and then fuse with the outer one. This method of translocating proteins to the outer membrane was first used to describe the export of LamB based on studies using LamB-LacZ hybrid proteins (Emr et al., 1980). However, it is doubtful that exocytosis via vesicle formation could be rapid enough to account for the kinetics of protein assembly into the outer membrane (Ito et al., 1977). In addition, this model is not compatible with the known orientation of lipoprotein and OmpA in the outer membrane (Braun, 1975; Schwaizer et

al.. 1978). Despite this, much research has been carried out to identify sequences which might direct the export and proper localisation of outer membrane proteins and this is discussed below.

Despite their necessity for protein export across the cytoplasmic membrane (Emr et al., 1978), the leader sequences of outer membrane and periplasmic proteins possess no obvious differences which might direct proteins to their respective compartments. It seems very likely, therefore, that information required for sorting proteins is contained within the mature polypeptide. Confirmation of this supposition was obtained using chimeric precursors comprising the signal sequence of a periplasmic protein (beta-lactamase) and the mature portion of a protein destined for the outer membrane (PhoE). Such a hybrid protein was directed to the outer membrane despite the presence of a signal sequence belonging to a periplasmic protein (Tommassen et al.. 1983). A number of approaches have been taken to identify information within the mature polypeptide which is needed to direct a protein to the outer membrane. These have included the use of gene fusion technology and deletion analysis.

Benson et al. (1984) created hybrid LamB-LacZ fusions in an attempt to determine which sequences were needed for the proper localisation of LamB. They concluded that amino acid residues located between positions 39 and 49 of the mature

protein were necessary to direct LamB to the outer membrane. Studies of LamB fused to the periplasmic protein alkaline phosphatase (PhoA), however, gave contradictory results (Hoffman and Wright, 1985). LamB-PhoA chimeras containing up to 115 amino acid residues of the mature LamB sequence were found in the periplasm rather than the outer membrane.

Similar studies have been undertaken to characterise sequences involved in directing the ferrichrome-iron (III) receptor (FhuA) to the outer membrane (Coulton et al., 1988). FhuA-PhoA chimeras containing 88 and 180 amino acid residues of mature FhuA were located in the periplasm and outer membrane respectively. This suggested a sequence between residues 88 and 180 was necessary to direct FhuA to the outer membrane and to form a stable association with it (Coulton et al. 1988).

Although hybrid proteins have been useful in analysing translocation to the outer membrane, the above data illustrate the possible contribution of LacZ and PhoA sequences to the results obtained. In addition, the use of cytosolic proteins (e.g., beta-galactosidase) in the formation of chimeras has led to conflicting data concerning the cellular location of hybrid proteins (Tommassen et al., 1985; Coulton et al., 1988).

Deletion analysis has also been used in an attempt to define specific amino acid sequences involved in directing proteins

to the outer membrane. For example, deletions were made in the gene encoding OmpA and the cellular location of the resulting truncated polypeptides examined (Henning et al., 1983; Freudl et al., 1985). All the OmpA deletion derivatives were transported across the inner membrane but some remained in the periplasm suggesting the absence of a possible sorting sequence within these particular proteins. Similarly, some internal deletions in PhoE caused the protein to be directed to the periplasm rather than to the outer membrane (Bosch et al., 1986). The study of LamB using this type of analysis has also revealed that certain amino acid sequences are required to direct the protein to the outer membrane (Benson and Silhavy, 1983).

Nikaido and Wu (1984) independently predicted that the amino acid sequence of LamB between residues 39 and 49 might be important for directing the protein to the outer membrane by comparing the amino acid sequences of LamB, PhoE, OmpF and OmpA. Statistically significant homology was found among these proteins and one of the most striking overlapped with residues 39-49 of LamB. Since sequences with some homology to this region were found in the other outer membrane proteins but none were found among proteins from the cytoplasm, periplasm or cytoplasmic membrane, it was suggested that this short sequence was used to identify proteins destined for the outer membrane (Nikaido and Wu, 1984). However, evidence that in OmpA the homologous

sequence (spanning residues 1-14) was not essential for localisation in the outer membrane, came from the studies of Freudl et al. (1985). They detected OmpA in the outer membrane using immunoelectron microscopy, despite the deletion of residues 4-45 from the mature protein. The outer membrane lipoprotein does not possess an area homologous to the sequence described by Nikaido and Wu (1984). Despite this, a short amino acid sequence has been implicated in directing lipoprotein to the outer membrane (Ghrayeb and Inouye, 1984; Yu et al., 1984).

In summary, conflicting information exists in the literature regarding the nature of a specific sequence used to direct proteins to the outer membrane. Indeed, it may be that proteins are localised in this stucture by virtue of their conformation as suggested by Freudl et al. (1985) for the OmpA protein. However, in addition to information that may reside within the protein itself, other factors such as interaction with LPS and in some cases a control that maybe cell-cycle dependent, play a role in the incorporation of proteins into the outer membrane.

1.4 The OmpT Protein

OmpT is classed as a major outer membrane protein with an apparent Mr of 40,000 and is present at up to $4x10^4$ copies per cell. It is also referred to as protein a (Lutenberg et al., 1976) and protein 3b (Schnaitman et al., 1974). The level of OmpT in E. coli is strain dependent. For example,

in *E. coli* K-12 strains OmpT forms 7-12% of the total major outer membrane protein. In contrast, OmpT represents 36% of the total major outer membrane protein in strain K235 but very little is found in *E. coli* B (Lutenberg *et al*. 1976). Growth temperature also influences the amount of OmpT in the outer membrane. At 30°C it constitutes 1% of the total major outer membrane protein while at 37°C this level is increased to 5% (Lutenberg *et al*., 1976). Manning and Reeves (1977) also noted that OmpT was virtually absent from the outer membrane of cells cultured at 30°C but became a major outer membrane protein at 37°C.

Low iron concentrations also cause an increase in the amount of OmpT present in the outer membrane (Fiss *et al*., 1982) though the basis of this regulation has not been reported. Interestingly, the gene encoding OmpT lies close to the 13 min iron operon which is involved in the synthesis of enterochelin and proteins required for the uptake of the siderophore when complexed with iron (Elish *et al*., 1988). The amount of OmpT present in the outer membrane is also diminished in strains carrying mutations in the *perA* (*envZ*) locus (Lundrigan and Earhart, 1981; Rupprecht *et al*., 1983). but again the basis of this regulation has not been studied.

OmpT is synthesised as a 42 kd pro-protein (Gayda *et al*.. 1979b). The conversion of the precursor to its mature form is inhibited by procaine and classic trypsin inhibitors (e.g., TLCK). In keeping with these inhibitor studies, a

trypsin-sensitive site (after arginine or lysine) was predicted to be the point of cleavage for the conversion of pro-OmpT (42 kd) to OmpT (40 kd) yielding a 2 kd signal peptide (Gordon et al., 1984). However, Grodberg et al. (1988) reported that cleavage of pro-OmpT to give the mature protein occurred between an alanine and a serine residue which does not support the predictions of Gordon et al. (1984). Gayda et al. (1979b) reported that at 30°C the decrease of OmpT in the outer membrane was due to altered processing of the 42 kd pro-protein.

Mutants lacking protein a have been isolated (Earhart et al., 1979). Such strains have DNA deleted from the chromosome at approximately 12.5 min on the E. coli map.The structural gene encoding OmpT was shown to lie at 12.5 min by Rupprecht et al. (1983) who reported that when a protein a^- mutant was transformed with a plasmid containing the structural gene (ompT) for OmpT, the protein appeared in the outer membrane. This proved that the stuctural gene for OmpT was located at 12.5 min rather than a gene involved in regulating ompT expression.

The first indication of a function for the OmpT protein came from the studies of Hollifield et al. (1978). They reported a proteolytic activity in the outer membrane which converted the ferric enterobactin outer membrane protein receptor from an active 81 kd form to an inactive 74 kd (81^*) polypeptide by a trypsin-like cleavage. This activity was ascribed to

protein a by Fiss *et al*. (1979). These workers later demonstrated that a protein a$^-$ strain (UT4400) which originally lacked this proteolytic activity became capable of cleaving the 81 kd receptor when transformed with a plasmid carrying the *ompT* gene (Fiss *et al*., 1982). However, it is not known whether or not this processing activity occurs *in vivo*.

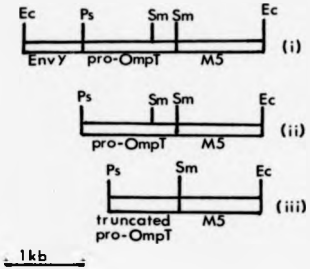
Recently a second proteolytic activity of OmpT has been identified by Grodberg and Dunn (1988). These workers noted that during the preparation of T7 RNA polymerase from *E. coli*. the enzyme was cleaved either between two lysine residues or between an arginine and a lysine residue. This was indicative of a trypsin-like cleavage and the protease responsible was found to be OmpT. The protease activity of OmpT is insensitive to DFP and PMSF (serine protease inhibitors), EDTA (metalloprotease inhibitor) and TPCK (chymotrypsin inhibitor) but is inhibited by benzamidine and *p*-aminobenzamidine which inactivate trypsin-like proteases (Fiss *et al*., 1979, 1982). In this respect it resembles protease VII recently described by Sugimura and Higashi (1988).

The gene encoding the OmpT protein was first cloned by a group attempting to clone the *capR* (*lon*) gene of *E. coli* (Berg *et al*., 1976). Strains containing a *capR* mutation overproduce capsular polysaccharide due to the derepression of enzymes involved in capsular polysaccharide synthesis.

They are sensitive to UV light and are unable to form colonies on complex medium. Berg *et al*. (1976) isolated a 3.1 kb *Eco*R1 fragment of DNA (Figure 1.4 a) which prevented both complex medium-induced killing and excessive capsular polysaccharide synthesis in a *lon* strain, but did not prevent UV sensitivity. The 3.1 kb fragment specified five proteins designated M1 (42 kd), M2 (40 kd), M4 (28 kd) and M5 (25 kd). The major polypeptides reproducibly observed were M1, M2 and M5 (Gayda and Markovitz, 1978). Protein M2 was found to be associated with the outer membrane while M5 was cytoplasmic. Gayda and Markovitz (1978) went on to show that M2 was in fact identical to OmpT and concluded that the gene encoding the protein was present on the 3.1 kb *Eco*RI fragment (Figure 1.4 a). M1 was found to be the precursor protein of OmpT while the M4 polypeptide has since been suggested to be the EnvY protein which is implicated in the thermal regulation of OmpF and OmpC (Lundrigan and Earhart, 1984a).

Further study demonstrated that suppression of the mucoid phenotype of *lon* strains was dependent upon the synthesis of both OmpT and the M5 polypeptide (Gayda *et al*., 1979a). Mutant plasmids unable to specify either of the proteins were no-longer able to suppress capsular polysaccharide synthesis. Gayda *et al*. suggested that protein a sterically interferes with the polysaccharide polymerases (Markovitz, 1977) at junctions between the inner and outer membranes

Figure 1.4 (a) Restriction maps of cloned DNA fragments specifying the OmpT protein and polypeptide M5



(i) 3.1 kb EcoRI fragment encoding pro-OmpT and M5

(ii) 2.3 kb PstI-EcoRI fragment encoding pro-OmpT and M5

(iii) SmaI deletion fragment encoding truncated pro-OmpT and M5

Adapted from Gordon *et al*. (1984).

while M5 represses the production of soluble enzymes involved in polysaccharide synthesis (Markovitz, 1977) although neither proposal has been substantiated.

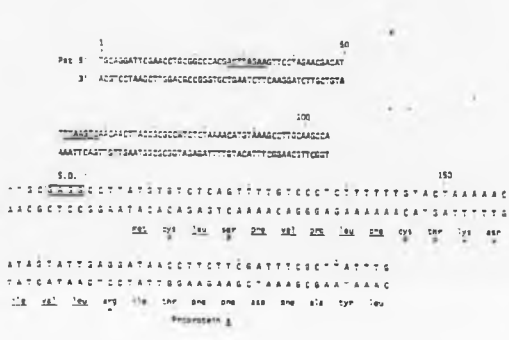
Gordon *et al*. (1984) mapped the coding regions of *ompT* and polypeptide M5 on the 3.1 kb *Eco*R1 fragment by subcloning and deletion analysis. They initially subcloned a 2.3 kb *Pst*I-*Eco*R1 fragment into pBR322 (Figure 1.4 a). This fragment encoded both proteins and was able to suppress the mucoid phenotype of a *lon* mutant. Subsequently, a mutant plasmid no-longer able to suppress capsular polysaccharide overproduction was isolated. This derivative was found to possess a DNA insert which prevented the synthesis of M5 and enabled the coding region of the M5 polypeptide to be defined (Figure 1.4 a).

Subsequently, the same workers deleted a 0.3 kb *Sma*I fragment from the 2.3 kb *Pst*I-*Eco*RI fragment in order to define the OmpT coding region (Figure 1.4 a). This deletion derivative was also unable to suppress the mucoid phenotype of a *lon* strain and no-longer encoded OmpT (40 kd) or its precursor protein (42 kd). Interestingly, the same deletion greatly diminished the proteolytic activity of OmpT against T7 RNA polymerase (Grodberg and Dunn, 1988) suggesting that the carboxy-terminus of OmpT could be involved either directly or indirectly in proteolysis. The *Sma*I deletion fragment encoded truncated polypeptides of 34 kd and 32 kd. Gordon *et al*. (1984) calculated that for the fragment to

encode a 34 kd protein, an open reading frame of 890 nucleotides would be required and so they proposed that the translational site of *ompT* would begin approximately 110 nucleotides downstream of the 5' *Pst*I site (Figure 1.4 a). They sequenced a total of 195 nucleotides starting from the *Pst*I site and located an ATG start codon (residues 118-120) followed by an open reading frame which continued to the end of the 195 nucleotides sequenced (Figure 1.4 b). A potential ribosome binding site was located and promoter consensus sequences at -10 and -35 identified. The deduced amino acid sequence predicted a putative signal sequence for pro-OmpT with a lysine at position 12 and an arginine at position 17 either of which would correlate with a trypsin-like cleavage site that would result in an approximate 2 kd reduction in the size of pro-OmpT to yield the mature protein (Figure 1.4 b). Secondary structure analysis predicted that processing would occur at the lysine residue so removing a 1389 dalton polypeptide consistent with the fact that the estimated Mr of pro-OmpT and OmpT are 42 kd and 40 kd respectively.

Gordon *et al*. also noted that the predicted promoter proximal mRNA of *ompT* could form a stable secondary structure that would sequester the Shine-Dalgarno sequence as well as the adjacent initiation AUG codon and could therefore lead to a decrease in translation (Figure 1.4 c). The secondary mRNA structure could be more stable at low temperatures (Gordon *et al*., 1984) which would correlate

Figure 1.4 (b) Nucleotide sequence of the promoter region and part of the ompT gene that specifies pro-OmpT



The deduced amino sequence is indicated below the nucleotide sequence. The Shine-Dalgarno (S.D.) sequence is enclosed in a box. The putative signal peptide is marked as follows: P = polar; + = basic; underlined = hydrophobic. The regions of RNA polymerase binding recognition sites (-10 and -35 homology regions) are underlined on the 5' strand.

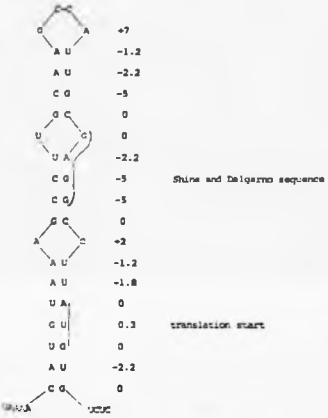
From Gordon et al. (1984).

with the observation that OmpT is synthesised in much reduced amounts at 30°C (Manning and Reeves, 1977). These workers also suggested that the secondary mRNA structure could be the site of action of the *perA* gene product. The latter could disrupt the stem-loop structure thereby allowing translation to occur.

1.5 Aims of the Project

The main interest in the *ompT* gene of *E. coli* stemmed from reports that the OmpT protein was synthesised in cells grown at 37°C but was absent from cells cultured at 30°C. Firstly, the regulation of OmpT synthesis with respect to temperature was to be analysed. Secondly, the usefulness of the *ompT* promoter and the OmpT signal sequence in directing the expression and export, respectively, of heterologous proteins from *E. coli* was to be studied. Here, it was of interest to see whether or not the naturally temperature-dependent synthesis of OmpT could be maintained in the production of other proteins. The initial aims of the present work were to clone and subsequently sequence the *ompT* gene and, in addition, the effects of various mutations on the expression of *ompT* were to be examined.

Figure 1.4 (c) Diagram to show the potential secondary structure of ompT mRNA.



The potential secondary structure of ompT mRNA around the Shine-Dalgarno sequence and translation start region is indicated. ΔG = -17.1 kcal.

From Gordon et al. (1984).

Chapter Two

Materials and Methods

2.1 Chemicals

Most chemicals used in this study were of analytical reagent grade and were obtained from either Fisons Laboratory Supplies, Loughborough, Leics., BDH Chemicals Ltd., Poole, Dorset, Sigma Chemical Company Ltd., Poole, Dorset or May and Baker Ltd., Dagenham, Essex. N,N'-methylene-bis-acrylamide, TEMED, ammonium persulphate and beta-mercaptoethanol were obtained from Bio-Rad Laboratories Ltd., Watford, Herts. Radioactive isotopes were supplied by Amersham International, Amersham, Bucks. Gelatin, nutrient broth and yeast extract came from Oxoid Ltd., Basingstoke, Hants., while Bacto-casamino acids, Bacto-tryptone and Bacto-agar were obtained from Difco Laboratories, East Molesey, Surrey. Ultra-pure phenol and ultra-pure urea came from Bethesda Research Laboratories, Paisley, Scotland.

2.2 Enzymes

Lysozyme and RNase A were obtained from the Sigma Chemical Company. All other enzymes, including restriction endonucleases, were supplied by Amersham International except for RsaI and Proteinase K which came from Boehringer-Mannheim, Lewes, East Sussex. The DNA polymerase I Klenow fragment was diluted with polymerase dilution buffer (100 mM KPO_4, pH7.5; 50% (w/v) glycerol) when required.

2.3 Media

The media used in this study are given below. All media and solutions listed were prepared in double-distilled water and

sterilised either by autoclaving at 121°C for 20 min or by filtration using Millipore GS disposable filters. Other buffers and solutions cited in the text were sterilised where appropriate. LB, MM, DD, TYE and 2xYT media were solidified with 2% (w/v) Bacto-agar where needed and DD was solidified with 0.6% (w/v) agar when required for preparing bacterial lawns. If necessary, MM was supplemented with amino acids at 20 $\mu g ml^{-1}$, vitamins at 1 $\mu g ml^{-1}$ and nucleotide bases at 40 $\mu g ml^{-1}$. These were prepared as 100x stock solutions in double-distilled water and filter sterilised.

Nutrient Broth (NB) Medium contained per litre:
13 g Oxoid nutrient broth.

Luria Bertani (LB) Medium contained per litre:
10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, pH7.4.

Stab Agar Medium was LB with 7 g per litre Bacto-agar.

Double Difco (DD) Medium contained per litre:
20 g Bacto-tryptone, 8 g NaCl. For bacteriophage lambda work 10 ml of 20% (w/v) maltose and 10 ml of 1 M $MgSO_4.7H_2O$ were added from sterile stock solutions. For bacteriophage P1 work 10 ml of 1 M $CaCl_2.2H_2O$ was added from a sterile stock.

2xYT Medium contained per litre:
10 g Bacto-tryptone, 10 g yeast extract, 5 g NaCl.

H-Top Agar contained per litre:
10 g Bacto-tryptone, 8 g NaCl, 8 g Bacto-agar.

TYE Medium contained per liter:
8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, pH7.4.

M9 Salts contained per litre:
6 g Na_2HPO_4 anhydrous, 3 g KH_2PO_4 anhydrous, 1 g NH_4Cl, 0.5 g NaCl, pH7.4.

M9 Minimal Medium (MM) was M9 salts with 10 ml of 10 mM $CaCl_2.2H_2O$, 10 ml of 0.1 mM $MgSO_4.7H_2O$ and 10 ml of 20% (w/v) glucose added, per litre, from sterile stocks.

Hershey Salts contained per liter:
3.0 g KCl, 5.4 g NaCl, 1.1 g NH_4Cl, 15 mg $CaCl_2.2H_2O$, 0.2 g $MgCl_2.6H_2O$, 0.2 mg $FeCl_3.6H_2O$, 87 mg KH_2PO_4 anhydrous, 12.1 g Tris base, pH7.4.

Hershey Medium was Hershey salts with 20 ml of 20% (w/v) glucose, 10 ml of 2% (w/v) proline and 1 ml of 0.1% (w/v) thiamine added, per litre, from sterile solutions.

K-Medium was MM with 50 ml of 20% (w/v) Bacto-casamino acids and 0.1 ml of 0.1% (w/v) thiamine added, per litre, from stock solutions.

Lambda 'Phage Buffer contained per litre:
7 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 2.5 g $MgSO_4.7H_2O$, 1 ml 1% (w/v) gelatin.

P1 Buffer contained per litre:

10 g Bacto-tryptone, 8 g NaCl and 10 ml of 1 M $CaCl_2.2H_2O$ which were added from a sterile stock.

Freezing Solution contained per litre:

126 g K_2HPO_4 anhydrous, 0.9 g sodium citrate, 0.18 g $MgSO_4.7H_2O$, 1.8 g $(NH_4)_2SO_4$, 3.6 g KH_2PO_4 anhydrous, 88 g glycerol.

2.4 Antibiotics

Antibiotics were prepared as 100x stocks, sterilised by filtration and used at the final concentrations shown in Table 2 (a) unless stated in the text. Tetracycline hydrochloride and chloramphenicol were dissolved in 50% (v/v) ethanol and stored at $-20^{o}C$. All other antibiotics were prepared in double-distilled water and stored at $4^{o}C$.

2.5 Bacterial Strains

The bacterial strains used in this study were *E. coli* K-12 derivatives and are given in Table 2 (b). Derivatives of strains carrying various plasmids were constructed by transformation and are defined in the text.

2.6 Bacteriophage

The bacteriophage used in this work are summarised in Table 2 (c). They were stored at $4^{o}C$ in the appropriate 'phage buffer over a few drops of chloroform. Bacteriophage M13mp8 (Messing and Vieira, 1982) was obtained from Bethesda

Table 2 (a) Antibiotic concentrations

Antibiotic	Abbreviation	Final concentration in medium (μg/ml)
Sodium ampicillin	Ap	100
Chloramphenicol	Cm	50
Kanamycin sulphate	Km	30
Streptomycin sulphate	Sm	100
Tetracycline hydrochloride	Tc	15
Cycloserine	Cy	200

Table 2 (b) Bacterial strains

Strain	Genotype	Source	Reference
CSH26	F⁻, ara, Δ(lac-pro), thi rpsL, Δ(recA-srl)F6, sup^{0}	D. Gill	Jones & Holland (1985)
CC118	araD139, Δ(ara-leu)7697, ΔlacX74, phoA 20, galE, galK, thi, rspE, rpoB, argE$_{am}$, recA1	J. Hinton	Manoil & Beckwith (1985)
LE392	F⁻, hsdR514, supE44, supF58, lacY1, galK2, galT22, metB1, trpR55, tonA, λ⁻	N. Crickmore	De Bruijn & Lupski (1984)
MCL31	HFrPO201Δ(gpt-lac)5, recA1, rpsEZ123, thi-1, supE44, TP3*, Δ(srl-recA)306::Tn10	J. Hinton	Lorence & Rupert (1983)
TG2	supE, Δ(lac-pro), thi, strA, recA, endA, sbcB15, hsdR4, F'-traD36, proA^{+} B^{+}, lacI^{q}, lacZΔM15	N. Minton	
C600	F⁻, thi-1, thr-1, leuB6, lacY1, tonA21, supE44, λ⁻	T. Gibbs	Appleyard, (1954)
JM83	ara, Δ(lac-pro), strA, thi, rpsL, Φ80, lacZΔM15	N. Minton	Vieira & Messing (1982)
RGC103	leu, pro, trp, thi, ade, canR	N. Mann	
MC4100	F⁻, araD139, rpsL, rbsR, relA1, Δ(argF-lac)U169, deoC1, ptsF25,	C. Guttierez	Guttierez et al. (1987)

Table 2 (b) Bacterial strains

Strain	Genotype	Source	Reference
MPH2	MC4100 Δ(brnQ-phoA-proC)	C. Gutierrez	Guttierez et al. (1987)
SG137	MC4100 ompR101, malA::Tn10.	C. Guttierez	Guttierez et al. (1987)
SG139	MC4100 envZ473. malA::Tn10	C. Guttierez	Guttierez et al. (1987)
CLG115	MC4100 envZ22. malA::Tn10	C. Guttierez	Guttierez et al. (1987)
EK23	CLG115 malA$^+$, Δ(srl-recA)306::Tn10	This study	
EK45	SG139 malA$^+$, Δ(srl-recA)306::Tn10	This study	
MC4100	MC4100 Δ(srl-recA) 306::Tn10	This study	
MPH21	MPH2 Δ(srl- recA) 306::Tn10	This study	
EK14	MPH2 malA::Tn10, envZ22	This study	
EK21	MPH2 malA::Tn10, envZ473	This study	
EK141	EK14 malA$^+$, envZ22, Δ(srl-recA)306::Tn10	This study	
EK211	EK21 malA$^+$, envZ473, Δ(srl-recA)306::Tn10	This study	

*TP3 = a transposition of lac to a region near the termination of transfer of HFr strain ED1032.

Table 2 (c) Bacteriophage

'Phage	Characteristics	Source	Reference
λTn*phoA*	Used for creating *phoA* gene fusions, Km^r	J. Hinton	Manoil & Beckwith (1985)
λ*plac*Mu3	Used for creating *lacZ* protein fusions *in vivo*	N. Crickmore	Bremer *et al*. (1984)
λ*plac*Mu51	Used for creating *lacZ* operon fusions *in vivo*	N. Crickmore	Bremer *et al*. (1985)
λ pMu507.3	Used as a 'helper 'phage' with the above two 'phages	N. Crickmore	Bremer *et al*. (1984)
$P1^{vir}$	General transducing 'phage of *E. coli*	T. Gibbs	
K20	OmpF specific 'phage	C. Gutierrez	Gutierrez *et al*. (1987)
hy2	OmpC specific 'phage	C. Gutierrez	Gutierrez *et al*. (1987)

Research Laboratories as double-stranded DNA and was stored at 4°C.

2.7 Plasmids

The plasmids used in this study are listed in Table 2 (d). They were kept at 4°C in TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH8.0).

2.8 Growth and Maintenance of Bacterial Strains

All *E. coli* strains were routinely grown from single colonies in LB, with antibiotics if necessary, at 37°C in a Gallenkamp orbital shaker (150 rpm).

When first obtained strains were checked for the presence of relevant antibiotic and auxotrophic markers by streaking onto appropriate media before storing on plates, stabs and in freezing solution as described below.

For maintenance, bacterial strains were streaked onto LB-agar plates, containing antibiotics where appropriate, and incubated overnight at 37°C. These plates were then stored at 4°C and strains subcultured every 1-2 months. Strains were also stored at room temperature on stab agar in 10 ml bijou bottles. Additionally, 10 ml bacterial cultures were grown overnight and then mixed with an equal volume of 2x freezing solution for long term storage at -20°C.

2.9 Preparation of Chromosomal DNA

A 500 ml overnight culture of the appropriate bacterial

Table 2 (d) Plasmids

Plasmid	Characteristics	Source	Reference
pBR325	Multicopy, Ap^r, Cm^r	N. Mann	Bolivar (1978)
pUC9	Multicopy, Ap^r	N. Minton	Vieira & Messing (1982)
pUC8	Multicopy, Ap^r	N. Minton	Vieira & Messing (1982)
pCH2	Contains a *bla-phoA* gene fusion, Tc^r	A. Wright	Hoffman & Wright (1985)
pAA182	*lacZ* operon fusion vector, multicopy, Ap^r	P. Jayaraman	Jayaraman *et al*. (1987)
pNM481	*lacZ* protein fusion vector, multicopy, Ap^r	N. Minton	Minton (1984)
pDIA15	*lacZ* operon fusion vector, low copy number, Km^r	H. de Reuse	De Reuse *et al*. (1986)
pDIA16	*lacZ* protein fusion vector, low copy number, Km^r	H. de Reuse	De Reuse *et al*. (1986)
pOMPT51	pUC9 with a 4.3 kb *Eco*RI insert	This study	
pOMPT36	pUC9 with a 4.3 kb *Eco*RI insert	This study	
pOMPT21	pUC9 with a 1.5 kb *Pst*I-*Sma*I insert	This study	
pOMPT30	pUC8 with a 1.5 kb *Pst*I-*Sma*I insert	This study	
pOMPT32	pUC9 with a 1.3 kb *Rsa*I-*Sma*I insert	This study	
pEH5	pAA182 with a 1 kb *Rsa*I-*Sma*I insert	This study	

Table 2 (d) Plasmids

Plasmid	Characteristics	Source	Reference
pEH10	pNM481 with a 1 kb *Rsa*I-*Sma*I insert	This study	
pDIA156	pDIA15 with a 1 kb *Eco*RI insert	This study	
pDIA168	pDIA16 with a 1kb *Eco*RI-*Bam*HI insert	This study	
pOMPT9	pOMPT30 carrying an *ompT*-*phoA* gene fusion	This study	
pOMPT10	As above	This study	
pOMPT6	As above	This study	
pOMPT2	As above	This study	
pOMPT5	As above	This study	
pOMPT7	As above	This study	
pOMPT12	pOMPT30 carrying an *ompT*-*lacZ* operon fusion	This study	
pOMPT14	pOMPT30 carrying an *ompT*-*lacZ* protein fusion	This study	
pBLA1	pBR325 carrying a *bla*-*lacZ* operon fusion	This study	
pBLA6	pBR325 carrying a *bla*-*lacZ* protein fusion	This study	

strain was harvested by centrifugation in a MSE 18 centrifuge (10,000 rpm, 10 min, 6 x 250 rotor). The pellet was resuspended in 10 ml of STE buffer (50 mM NaCl; 10 mM Tris-HCl; 1 mM EDTA; pH8.0) containing 10 $mgml^{-1}$ lysozyme. After incubation at 37°C for 15 min, 125 µl of proteinase K (20 $mgml^{-1}$) were added to the mixture followed by 3.25 ml of 10% (w/v) SDS. The mixture was placed at 37°C until clearing of the solution was observed. 4 ml of 5 M sodium perchlorate were then added and the mixture heated at 60°C for 15 min with occasional swirling. The lysate was subsequently transferred to a 50 ml polypropylene Oakridge tube, an equal volume of phenol-chloroform (section 2.12) added and the tube inverted gently to ensure thorough mixing of the contents. After centrifugation in a MSE 18 centrifuge (18,000 rpm, 30 min, 8 x 50 rotor), the upper aqueous layer was removed with a wide bore pipette and placed in a clean polypropylene tube. This phase was subsequently extracted twice, in the same manner, with an equal volume of chloroform-isoamylalcohol (24:1) to remove any traces of phenol. After the final chloroform-isoamylalcohol extraction, the upper aqueous phase containing DNA was precipitated by adding 0.02 volumes of 5 M sodium chloride and 2 volumes of 100% (v/v) ethanol and storing at -20°C overnight.

The precipitated DNA was collected by centrifugation in a MSE 18 centrifuge (18,000rpm, 15 min, 4°C, 8 x 50 rotor) and

washed in 70% (v/v) ethanol. Following vacuum desiccation for 30 min, the DNA was dissolved in 10 ml of TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH8.0) containing 100 $\mu g ml^{-1}$ of heat-treated RNase A and placed at 37°C for 30 min. The total volume was then made up to 30 ml with TE buffer (pH8.0) and 30 g of caesium chloride were added along with 3 ml of ethidium bromide (10 $mg ml^{-1}$). The mixture was placed in a Beckman 35 ml heat-sealable tube before centrifugation (45,000 rpm, 16 hours, 20°C) in a Beckman L8 Ultracentifuge using a VTi50 rotor. The DNA band was then visualised using long-wave UV light and removed through the side of the tube using a wide-bore needle and a 5 ml syringe. Ethidium bromide was removed from the DNA by extracting three times with isoamylalcohol, the upper layer being discarded each time. The DNA solution was then dialysed against 2 litres of TE buffer overnight and subsequently stored at 4°C in a sterile bijou.

2.10 Large-Scale Plasmid Preparation

The basic procedure was the alkaline lysis method of Maniatis et al. (1982). A 5 ml overnight culture was added to 500 ml of LB containing appropriate antibiotics for maintaining plasmid bearing strains. Following overnight incubation at 37°C in a Gallenkamp orbital shaker, cells were harvested (6000 rpm, 10 min, 4°C, 6 x 250 rotor) in a MSE 18 centrifuge. The cell pellet obtained was resuspended in 6ml of solution I (25 mM Tris-HCl; 10 mM EDTA; 50 mM

glucose; pH8.0) and transferred to a 50 ml polycarbonate Oakridge tube. After adding 13 ml of solution II (0.2 M NaOH; 1% (w/v) SDS) and gently agitating, the mixture was left on ice for 20-30 min to allow cell lysis. Chromosomal DNA and proteins were then precipitated by adding 13 ml of ice-cold solution III (60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml water; pH4.5). Following 30 min on ice, unlysed cells, denatured chromosomal DNA and protein were pelleted by centrifuging in a MSE 18 centrifuge (18,000 rpm, 20 min, 8 x 50 rotor). The supernatant was carefully decanted into a clean Oakridge tube, 0.64 volumes of isopropanol added and the mixture kept at -20°C for at least 1 hour to precipitate plasmid DNA. The latter was then recovered by centrifugation in a MSE 18 Centrifuge (18,000 rpm, 30 min, 4°C, 8 x 50 rotor) and the DNA pellet washed in 70% (v/v) ethanol. After drying under vacuum for 30 min, the plasmid DNA was dissolved in 3 ml of buffer (50 mM Tris-HCl; 1 mM EDTA; pH8.0) and then made to a total volume of 4.2 ml with the same solution. 4.7 g of caesium chloride and 0.5 ml of ethidium bromide (10 $mgml^{-1}$) were added to the plasmid solution and the mixture transferred to a 5 ml Beckman quick seal tube. The tubes were centrifuged in a Beckman L8 Ultracentrifuge using a VTi65.2 rotor (55,000 rpm, 16 hours, 20°C).

The plasmid DNA, visualised by long-wave UV light, was removed through the side of the tube with a 21-gauge needle

and a 1 ml syringe. The DNA was then extracted three times to remove ethidium bromide by mixing with an equal volume of isoamylalcohol followed by centrifugation in a MSE Microcentaur (1 min, high speed). The upper layer containing ethidium bromide was discarded each time. The DNA solution was dialysed against several changes of TE buffer and was then stored at 4°C. Plasmid DNA concentrations were determined by the spectrophotometric method of Maniatis et al. (1982) and were usually around 1 $mgml^{-1}$.

2.11 Small Scale Plasmid DNA Preparation

This was essentially the alkaline lysis method as described by Maniatis et al. (1982). A single bacterial colony was inoculated into 10 ml of LB containing selective antibiotics and grown overnight at 37°C with shaking. 1 ml of the culture was transferred to an Eppendorf tube, pelleted in a MSE Microcentaur (5 min, high speed) and the cells resuspended in 0.1 ml of solution I. 0.2 ml of solution II were added, mixed gently by inversion of the tube and then left on ice for 5 min. 0.15 ml of ice-cold solution III were then added and again the mixture was kept on ice for 5 min. Centrifugation in a MSE Microcentaur (4 min, high speed) was used to pellet the resulting precipitate and the supernatant was tranferred to a clean Eppendorf tube.

The supernatant was then extracted once with phenol-chloroform and once with chloroform-isoamylalcohol (section 2.12). The upper aqueous layer was retained and 0.64 volumes

of isopropanol added to precipitate plasmid DNA. Following incubation at -70°C for 20 min, plasmid DNA was recovered by centrifugation in a MSE Microcentaur (10 min, high speed), washed in 70% (v/v) ethanol and dried under vacuum for 5-10 min. The DNA pellet was resuspended in 50 µl of TE buffer and 10 µl samples were routinely used in restriction enzyme digests. RNase A was used at a final concentration of 100 $\mu g ml^{-1}$ to remove contaminating RNA when required.

2.12 Extraction and Precipitation of DNA Samples

Phenol-chloroform mix was prepared by dissolving 100 g of phenol and 100 mg of 8-hydroxyquinoline in 100 ml of chloroform and 4 ml of isoamylalcohol. This mixture was equilibrated by shaking twice with 0.2 volumes of 1 M Tris-HCl (pH8.0) and then once with 0.2 volumes of TE buffer. The mixture was then stored at 4°C under TE.

DNA samples to be extracted were mixed with an equal volume of phenol-chloroform until an emulsion formed. The two phases were separated by centrifugation in an MSE Microcentaur (1 min, high speed). The upper aqueous layer was removed taking care not to disturb the interface. This phase was then extracted with chloroform-isoamylalcohol (24:1) in the same manner. The DNA was precipitated from the aqueous phase by adding 0.1 volumes of 3 M sodium acetate (pH4.8) and 2.5 volumes of 100% ethanol followed by storage at -70°C for 20-30 min (or -20°C overnight). The DNA was recovered by centrifugation (10 min, high speed) in a MSE

Microcentaur. After discarding the supernatant, the DNA pellet was washed in 70% (v/v) ethanol and dried under vacuum for 10 min before resuspending in TE buffer and storing at 4°C.

2.13 Restriction Endonuclease Digestion

The required amount of DNA was restricted in a mixture containing 0.1 volumes of 10x restriction buffer, 1-5 units of enzyme/µg of DNA and sterile distilled water which was added to make a final volume of 20-50 µl. Restriction buffers were obtained from Amersham International. If these were not available, the high, medium and low salt buffers, as described by Maniatis et al. (1982), were prepared at 10x concentration and used accordingly. Digestions were carried out for at least 90 min at 37°C and for up to 4 hours when digesting chromosomal DNA. The reaction was then stopped by the addition of 0.5 M EDTA (pH7.5) to a final concentration of 10 mM.

If the restricted DNA was to be analysed by gel elecrophoresis, 0.2 volumes of sample buffer (10 mM EDTA, pH8.0; 10 mM Tris-HCl, pH8.0; 40% (w/v) sucrose; 0.25% (w/v) bromophenol blue) were added prior to loading into gel slots. If the DNA was to be digested with another enzyme, it was first extracted, precipitated and dissolved in TE buffer (section 2.12) before setting up a further restriction.

Vector DNA for use in cloning experiments was purified in the same way following digestion.

2.14 DNA Ligations

DNA samples to be ligated were first mixed together as follows. For subcloning DNA fragments, a 4:1 fragment to vector ratio was used with DNA concentrations <50 $\mu g ml^{-1}$. For construction of gene libraries and subcloning using 'blunt-end' restriction endonucleases, a ratio of fragment to vector of 6:1 was used and the DNA concentration was increased to 100 $\mu g ml^{-1}$. For vector re-circularisation the concentration was reduced to 10 $\mu g ml^{-1}$. The DNA mixture was then heated at 65°C for 5 min and incubated on ice for 60 min to allow DNA fragments to anneal slowly. After adding 0.1 volumes of 10x ligation buffer (4 mM ATP; 66 mM $MgCl_2$; 0.1 M dithiothreitol; 66 mM Tris-HCl, pH7.6) and T4 DNA ligase (0.1 units/μg of DNA), the mixture was made to 10-20 μl with sterile distilled water before incubation overnight at 15°C.

2.15 Agarose Gel Electrophoresis

Horizontal slab gels were prepared by boiling agarose (Sigma Medium Type EEO) in TBE electrophoresis buffer (0.089 M Tris-borate; 0.089 M boric acid; 0.002 M EDTA; pH8.3). Routinely, 0.6-0.8% (w/v) gels were used in either a large (25 cm x 15 cm x 1 cm) or a mini (10.5 cm x 8 cm x 1 cm) gel electrophoresis system. DNA samples were prepared by adding 0.2 volumes of loading buffer (section 2.13) before loading

into the gel slots. Electrophoresis was carried out, with the gels completely submerged in TBE buffer, at 125-150 volts or 50 volts overnight for large gels.

Gels were then stained in distilled water containing ethidium bromide (0.5 $\mu g ml^{-1}$) for 30 min and destained in distilled water for another 30 min. Gels were examined using a short-wave UV transilluminator and photographed when required using Polaroid Positive/Negative Land Pack Film Type 665.

HindIII-restricted bacteriophage lambda DNA (Bethesda Research Laboratories) was used to provide molecular weight markers giving fragments of 23.131, 9.418, 6.557, 4.361, 2.322, 2.028, 0.564 and 0.125 kb. The standards were used in gel electrophoresis according to the manufacturer's instructions. Restriction fragment sizes were determined with the DNASIZE programme (J. Hinton, pers. comm.) adapted from Schaffer and Sederoff (1981) and run on a BBC Model B Micro Computer.

2.16 Preparation of DNA Fragments from Agarose Gels

DNA fragments were prepared from agarose gels by electroelution into dialysis bags, the method being adapted from Maniatis *et al*. (1982). Following restriction with the appropriate enzyme(s), the DNA was electrophoresed on a 0.6% (w/v) agarose gel. After staining with ethidium bromide, DNA bands were visualised using long-wave UV light and the

desired fragment excised from the gel. The gel slice was then inserted into a small piece of dialysis tubing and covered with 0.2x TBE buffer. The tubing was secured at each end and then placed in a gel electrophoresis tank parallel to the electrodes and immersed in 0.2x TBE buffer. Electrophoresis was carried out at 200 volts for 2 hours.

To remove the DNA from the sides of the dialysis tubing, the current was reversed for a 10 min period. The buffer containing the DNA was then transferred from the tubing to a clean Eppendorf tube. The sample was subsequently extracted once with phenol-chloroform and once with chloroform-isoamylalcohol before precipitating the DNA (section 2.12) which was finally dissolved in 20-50 µl of TE buffer and stored at 4°C until required.

2.17 Transformation

The bacterial strain to be transformed was grown in 50 ml of LB at 37°C in a New Brunswick Gyrotory water bath shaker. The OD_{450} of the culture was monitored using a LKB Ultraspec 4050 until a reading of 0.6 was reached. Cells were then chilled on ice for 20 min, harvested by centrifugation in a MSE Chilspin (5000 rpm, 10 min) and the pellet resuspended in 20ml of ice-cold 0.1 M $MgCl_2$. The cells were immediately repelleted as before and resuspended in 2.5 ml of ice-cold 0.1 M $CaCl_2$ (Sigma grade I no. C-3881). Cells were left on ice for at least 2 hours before the addition of DNA for

transformation. If not for immediate use, competent cells were stored at 4°C for up to 2 days.

When required, a 0.1 ml aliquot of competent bacteria was mixed with the appropriate DNA sample and placed on ice for 30 min. The cells were then heat shocked (42°C, 2 min) and subsequently an equal volume of 2x LB was added to the sample. This mixture was incubated at 37°C for 1 hour with shaking, to allow expression of antibiotic resistance genes carried by the transforming plasmid DNA. 0.1 ml samples were then spread onto appropriate selective medium and the plates incubated at 37°C overnight.

2.18 Southern Transfer of DNA to Nylon Membranes

The basic procedure was that of Reed and Mann (1985). After electrophoresis of the DNA (section 2.15), the agarose gel was soaked in 2 volumes of 0.25 M HCl with gentle agitation for 5-10 min. Following a brief rinse with distilled water, the gel was placed on three sheets of Whatman 3MM paper saturated with 0.4 M NaOH. The bottom sheet was arranged such that the sides were immersed in 0.4 M NaOH which later served to transfer DNA from the gel to the nylon membrane.

Hybond-N nylon membrane (Amersham International) was cut to the size of the gel and then pre-wetted with distilled water. The membrane was placed on the surface of the gel and any air bubbles, which formed between the gel and the nylon, removed. Three sheets of Whatman 3MM paper, cut to the size

of the gel and soaked in 0.4 M NaOH, were placed on top of the membrane followed by several layers of absorbant paper. This was weighed down with a suitable object and left for 2 hours or more. During this time, DNA was transferred to the membrane as NaOH was drawn upwards from beneath the gel by capillary action. The membrane was then rinsed in 2x SSC (0.3 M NaCl; 0.03 M sodium citrate; pH7.0) and allowed to dry at room temperature. Membranes were stored between sheets of Whatman 3MM paper and wrapped in aluminium foil until required. Pre-wetting of nylon membranes prior to use in hybridisation experiments was not required.

2.19 Colony Hybridisation

The basic procedure was that of Grunstein and Hogness (1975). The bacterial colonies to be investigated by hybridisation were toothpicked onto two LB-agar plates which contained the necessary antibiotics for plasmid selection. One plate was incubated at 37°C overnight and then stored at 4°C and served as a 'master' plate. The second plate was overlayed with a sterile nitrocellulose filter (Schleicher and Schuell, ex. Anderman and Co. Ltd., Surrey), prior to streaking out colonies, for use in subsequent hybridisation experiments. This plate was incubated at 37°C for 6 hours. The filter was then transferred to a fresh LB-agar plate containing chloramphenicol (170 $\mu g ml^{-1}$) before further incubation at 37°C overnight. This step allows the amplification of plasmid copy number to take place.

Filters were then transferred to Whatman 3MM paper saturated with 0.5 M NaOH and left for 10 min in order to lyse the bacterial colonies. Subsequently, the filters were placed on paper saturated with 1 M Tris-HCl (pH7.5) for 10 min. This neutralisation step was repeated twice more. Finally, filters were transferred to paper saturated with 1.5 M NaCl and 0.5 M Tris-HCl (pH7.4), again for 10 min. The nitrocellulose filters were then immersed in 2x SSC containing proteinase K (1 $mgml^{-1}$) and left at room temperature for 60 min with occasional agitation. 2x SSC was then used to wash the filters thoroughly prior to drying at room temperature and baking under vacuum at 80°C for 2 hours. Filters were stored between sheets of Whatman 3MM paper and pre-wetted with 2x SSC before use in hybridisation experiments.

2.20 Labelling of Oligonucleotide DNA Probes

The 17 nucleotide *ompT* gene probe was a generous gift of N. P. Minton (Microbial Technology Laboratory, PHLS, CAMR, Porton Down, Wiltshire). The probe was stored at -20°C in oligonucleotide storage (10 mM Tris-HCl; 5 mM NaCl; 0.1 mM EDTA; pH7.2) buffer and was at a concentration of 1 $mgml^{-1}$.

When required, 100 ng of DNA probe were end-labelled using [gamma-^{32}P]dATP, which was provided in aqueous solution, and T4 polynucleotide kinase. An appropriate volume of oligonucleotide was mixed with 125 µCi of label (specific activity: 3000 Ci/mM), 2 µl of 10x kinase buffer (0.5 M

Tris-HCl, pH8.0; 0.1 M $MgCl_2$; 50 mM dithiothreitol; 1 mM spermidine hydrochloride; 1 mM EDTA, pH8.0), 10 units of T4 polynucleotide kinase and made to 20µl with distilled water. The mixture was left at 37°C for 1 hour and then the enzyme denatured by heating at 65°C for 15 min before making the reaction mixtures to 2 ml with distilled water. Unincorporated label was removed by passing the reaction mixture through a Pharmacia PD-10 disposable column containing Sephadex G-25M (Pharmacia Ltd., Milton Keynes, Bucks.) which had been pre-equilibrated with distilled water. The eluent was discarded and the labelled probe then eluted from the column with 3.5 ml of distilled water. The oligonucleotide was subsequently stored at -20°C until needed. Levels of $1x10^9$ cpm/µg of probe were routinely obtained using this method and in hybridisation experiments the labelled oligonucleotide was used at a concentration of 1 ng/ml of hybridisation fluid.

2.21 Hybridisations Using Oligonucleotide DNA Probes

Filters and membranes to be used in hybridisation experiments were firstly prehybridised in 6x SSC (0.9 M NaCl; 0.09 M sodium citrate; pH7.0), 10x Denhardts solution (0.2% (w/v) Ficoll; 0.2% (w/v) polyvinylpyrrolidone; 0.2% (w/v) bovine serum albumin (Pentax Fraction V), 0.1% (w/v) SDS and 100 $\mu g ml^{-1}$ denatured calf thymus DNA in a total volume of 100 ml. This solution was warmed at 55°C before prehybridisation which was carried out at the same

temperature for 2 hours. The prehybridisation fluid was then allowed to cool and labelled oligonucleotide probe was then added to a final concentration of 1 $ngml^{-1}$. Hybridisation was carried out at the desired temperature overnight. Filters were then washed in 6x SSC and 1% (w/v) SDS six times for 5 min each, rinsed in 6x SSC and dried at room temperature before exposure to Fuji X-ray film overnight at -70°C. The film was developed as described in section 2.25.

2.22 Gene Product Identification using the Maxi-Cell System

This procedure was an adaptation of the method proposed by Sancar *et al*. (1979) to label plasmid-encoded proteins *in vivo* with ^{35}S-methionine. Firstly, *E. coli* CSH26 was transformed with the plasmid under investigation. Following purification of the appropriate strain, maxi-cells were made.

The strain was grown in 10 ml of K-medium at 37°C to an OD_{600} of 0.5 and then placed on ice. The cells were subsequently UV-irradiated with a dosage of *ca* 50 Jm^{-2} in a Petri dish with constant agitation of the culture. Cells were then transferred to a sterile universal bottle and freshly prepared cycloserine was added to a final concentration of 200 μgml^{-1}. The culture was then incubated with shaking (150 rpm) for 14-16 hours at 37°C.

Cells were harvested by centrifugation in a MSE Chilspin (5000 rpm, 5 min), washed twice in an equal volume of Hershey salts and finally resuspended in 5 ml of Hershey medium. After incubation for a further 60 min, 30 μCi of ^{35}S-methionine were added and incubation continued for a further hour. Cells were then harvested in a MSE Chilspin (5000 rpm, 5 min) and washed twice in 10 mM Tris-HCl (pH 7.4). 50 μl of SDS-sample buffer were added to the cell pellet and samples boiled for 5 min to solubilise cell proteins. The samples were stored at -20°C if not immediately required for SDS-PAGE. Routinely, 20 μl aliquots were applied to gel slots and, after freezing, samples were reboiled for 3 min prior to electrophoresis.

2.23 SDS-Polyacrylamide Gel Electrophoresis of Proteins

Proteins were routinely analysed on 15% (w/v) linear, SDS-polyacrylamide gels measuring 22 cm x 15 cm x 2 mm. The buffers and acrylamide solutions used are listed in Tables 2 (e), (f) and (g). Both the separating gel and the stacking gel mixtures were degassed prior to the addition of TEMED and ammonium persulphate for polymerisation. Ammonium persulphate was always freshly prepared. The 70 ml separating gel was cast first and overlayed with butan-2-ol until set. After polymerisation, the surface of the gel was washed thoroughly to remove all traces of the butan-2-ol and a 10 ml stacking gel layered on top. Immediately after pouring, a 9-well Teflon gel comb was inserted into the

Table 2 (e) Stock solutions for SDS-PAGE

Stock solution	Composition
Lower gel buffer	3 M Tris-HCl, pH8.8.
Stacking gel buffer	0.5 M Tris-HCl, pH6.8.
40% acrylamide solution	40% (w/v) acrylamide, 0.5% (w/v) bis-acrylamide.**
Stacking gel acrylamide	10% (w/v) acrylamide, 0.5% (w/v) bis-acrylamide.
Electrophoresis buffer	0.025 M Tris-HCl, 0.192 M glycine, 0.1% (w/v) SDS.
SDS-sample buffer	0.0625 M Tris-HCl, pH6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol*, 0.001% (w/v) bromophenol blue.

*Correct volume always added fresh to each sample prior to boiling.

** N,N'-methylene-bis-acrylamide

Table 2 (f) Composition of polyacrylamide separating gels

Stock solution	Amount required for 15% (w/v) gel
Lower gel buffer	8.82 ml
40% acrylamide solution	26.25 ml
SDS, 10% (w/v)	0.70 ml
Water	34.05 ml
TEMED	168 µl
Ammonium persulphate, 10% (w/v)	16.8 µl

Table 2 (g) Composition of polyacrylamide stacking gel

Stock solution	Volume in stacking gel
Stacking gel buffer	2.4 ml
Stacking gel acrylamide	3.0 ml
Water	4.4 ml
SDS, 10% (w/v)	0.1 ml
Ammonium persulphate, 10% (w/v)	0.1 ml
TEMED	5.0 µl

stacking gel. The latter was then allowed to set for 30 min. Subsequently, the comb was removed and the gel transferred to a tank for loading. Electrophoresis was carried out using a discontinuous system (Laemmli, 1970) at a constant current of 20 mA overnight.

Molecular weight estimations were carried out by preparing a calibration curve (plotting $\log_{10}$ Mr against Rf value) using protein standards of known size. The standards used were obtained from Pharmacia Ltd. and consisted of phophorylase b (Mr 94,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), carbonic anhydrase (30,000), trypsin inhibitor protein (Mr 20,100), and alpha-lactalbumin (Mr 14,400). These were used according to the manufacturer's instructions.

2.24 Coomassie Blue Staining of Polyacrylamide Gels

Polyacrylamide gels were stained for at least 2 hours in 500 ml of a solution of 45% (v/v) methanol, 10% (v/v) glacial acetic acid and 0.2% (w/v) Page Blue 83. Destaining was carried out by soaking in the following solutions:

(i) 45% (v/v) methanol, 10% (v/v) glacial acetic acid for at least 1 hour.

(ii) 20% (v/v) propan-2-ol, 10% (v/v) glacial acetic acid for at least 1 hour.

(iii) 10% (v/v) propan-2-ol, 10% (v/v) glacial acetic acid to completion.

2.25 Fluorography and Autoradiography of Polyacrylamide Gels

Fluorography of polyacrylamide gels was carried out according to the method of Skinner and Griswold (1983). Following destaining, gels were immersed in dimethyl sulphoxide (DMSO) for 30 min and then soaked in 22.2% (w/v) 2,5-diphenyl oxazole (in DMSO) for 3 hours. The gels were then washed in distilled water and left to soak for 60 min. The gels were subsequently dried onto a sheet of Whatman 3MM paper using a Bio-Rad Model 1125B gel drier at 60°C for 3 hours. Gels were then exposed to Fuji X-ray film in a Harrier film cassette and left at -70°C for the required time.

If Amersham Beta-Max Hyperfilm (Amersham International) was used for autoradiography, the gels were not fluorographed prior to exposure but were dried at 80°C for 1 hour following destaining. The dried gels were then exposed to the X-ray film at room temperature, usually overnight.

The exposed film was subsequently developed using Kodak LX-24 developer (Kodak Ltd., Liverpool) for 5 min, rinsed in water and then fixed in Kodak FX-40 X-ray fixer (Kodak Ltd.) for either 5 min with ordinary film or for 15 min with Beta-Max film.

2.26 Isolation of Periplasmic Proteins

Periplasmic proteins were released in response to cold

osmotic shock treatment of cultures according to the method of Neu and Heppel (1965). Cultures were grown to an OD_{600} of 0.5 in 5 ml of LB at 37°C.

Bacterial cultures were then buffered by the addition of 0.5 ml of 0.5 M Tris-HCl (pH7.8) and, following a 10 min incubation at room temperature, the cells were pelleted by centrifugation in a MSE Chilspin (5000 rpm, 10 min). The cell pellet was resuspended in 0.8 ml of sucrose solution (30 mM Tris-HCl, pH7.8; 40% (w/v) sucrose; 2 mM EDTA) and, after a 10 min incubation at 30°C, the cells were re-pelleted by centrifugation in a MSE Microcentaur (1 min, high speed). The supernatant was carefully removed and the cells rapidly resuspended in 0.5 ml of ice-cold distilled water. The suspension was then left on ice for 10 min and the 'shocked' cells removed by centrifugationin a MSE Microcentaur (5 min, high speed). The resulting shock fluid was used directly in enzyme assays.

2.27 Isolation of Bacterial Cell Envelopes

Membrane fractions were purified from bacterial strains by the method of Ames (1974). If envelopes were to be prepared from 'shocked' cells, the latter were resuspended in 10 ml of HEPES buffer (pH7.4). If envelopes were to be prepared from maxi-cells, cultures were labelled and washed as in section 2.22 and then resuspended in 10 ml of HEPES buffer (pH7.4). Subsequently, the cellular membranes were disrupted using a French pressure cell set at 20,000 psi. Remaining

undisrupted cells were removed from the suspension by spinning in a MSE Chilspin (15 min, 10,000 rpm). Cell membranes were then pelleted by centrifugation of the supernatant fraction in 10 ml polycarbonate tubes using a MSE Super Speed 65 Centrifuge and a 10x10 Ti rotor (36,000 rpm, 1 hour, 4°C). The supernatant was removed and this represented the soluble cytoplasmic fraction of the cell. The pellet contained the cell envelope fraction and was resuspended in 0.5 ml of 10 mM Tris-HCl (pH7.4).

2.28 Separation of Outer and Inner Membranes

The membrane pellet, obtained as described in section 2.27, was retained in a 10 ml polycarbonate tube, resuspended in 0.2 ml of 0.5% (w/v) sodium lauryl sarcosinate (Sarcosyl) and incubated at room temperature for 30 min. The suspension was then centrifuged in a MSE Super Speed 65 Centrifuge using a 10x10 Ti rotor (1 hour, 35,000 rpm, 15°C). The supernatant represented the solubilised cytoplasmic membrane proteins and the pellet contained the Sarcosyl-insoluble outer membrane proteins. The pellet was washed by resuspending in 1 ml of 0.5% (w/v) Sarcosyl, incubating at room temperature for 30 min and centrifuging as before. The washed outer membrane pellet was finally resuspended in 100 µl of 10 mM sodium phosphate buffer (pH7.2) and 100 µl of SDS-sample buffer were added before boiling for 5 min and storing at -20°C until required for SDS-PAGE.

2.29 Preparation of High Titre Bacteriophage Lysates

High titre lysates of bacteriophage lambda derivatives, hy2 and K20 were prepared on the *E. coli* strain LE392. The strain was grown overnight in LB containing 0.2% (w/v) maltose and 10 mM $MgSO_4.7H_2O$. 0.2 ml of the culture were mixed with 10^{-5}-10^{-6} pfu of 'phage and left at room temperature for 10 min to allow the 'phage to adsorb to the cells. Following the addition of 3 ml of soft DD-agar, the mixture was poured onto a fresh, wet DD-agar plate. When set, plates were incubated at 37°C until confluent lysis was observed (by comparison with a 'phage-free control lawn). The top agar was transferred to a sterile universal and the plate washed with 3 ml of lambda buffer which was then pooled with the agar. 0.5 ml of chloroform were added and the mixture vortexed for 5 min to give a soft slurry. The agar was pelleted by centrifugation in a MSE Chilspin (5000 rpm, 20 min) and the supernatant containing the 'phage decanted into a sterile universal. This lysate was stored over a few drops of chloroform at 4°C.

High titre lysates of $P1^{vir}$ were prepared as described above except that strain C600 was used and grown overnight in LB with 10 mM $CaCl_2.2H_2O$ prior to 'phage infection. P1 buffer was used in place of lambda buffer.

Lysates were titred by spotting 10 µl samples of a range of dilutions of the lysate onto freshly poured lawns of either strain LE392 or C600 and incubating at 37°C overnight.

2.30 Pl Transductions

Transducing 'phage lysates of Pl^{vir} were prepared on the appropriate E. coli strain as described in section 2.29. The strain to be transduced was grown overnight in LB at 37°C. The cells were then harvested in a MSE Multex Centrifuge (5000 rpm, 10 min) and subsequently resuspended in 1 ml of Pl buffer. The culture was then infected with 0.1 ml of a transducing Pl^{vir} lysate (10^8 pfu/ml) and incubated at 37°C for 25 min. 10 ml of NB containing 0.05% (w/v) sodium citrate (w/v) were added to the culture and the cells were then pelleted by centrifugation in a MSE Multex (5000 rpm, 10 min). The cell pellet was resuspended in 10 ml of NB with 0.05% (w/v) sodium citrate and the culture was incubated with shaking at 37°C for 1 hour. The cells were then harvested by centrifugation in a MSE Multex (4000 rpm, 10 min) and finally resuspended in 0.8 ml of NB + 0.05% (w/v) sodium citrate. 0.1 ml aliquots of the culture were spread onto medium appropriate for the selection of the required transductants. The plates were incubated at 37°C overnight or until transductants appeared.

2.31 Enzyme Assays

2.31.1 Beta-galactosidse Assays

The method of Miller (1972) was used to assay beta-galactosidase. An aliquot (100-250 µl) of the sample to be assayed was mixed with Z buffer (60 mM Na_2HPO_4, anhydrous; 40 mM $NaH_2PO_4.2H_2O$; 10 mM KCl; 1 mM $MgSO_4.7H_2O$; 0.27% (v/v) beta-mercaptoethanol; pH7.0) to a volume of 0.5 ml. 0.1 ml of ONPG (4 $mgml^{-1}$ in Z buffer) was then added. The mixture was incubated at 37°C and the time of incubation monitored until a yellow colour appeared. The reaction was stopped by the addition of 0.25 ml of 1 M Na_2CO_3. The OD_{550} and the OD_{420} were measured and the units of beta-galactosidase calculated according to the formula:

$$\text{Units} = 1000 \times \frac{OD_{420} - (1.75 \times OD_{550})}{t \times v \times OD_{600}}$$

t = time of reaction (min), v = sample volume (ml)

2.31.2 Alkaline Phosphatase Assays

The method of Brickman and Beckwith (1975) was used to assay alkaline phosphatase. A 0.1 ml aliquot of the sample to be assayed was mixed with 0.9 ml of 1 M Tris-HCl (pH8.0) and the reaction started by the addition of 0.1 ml p-nitrophenyl phosphate disodium hexahydrate (4 $mgml^{-1}$ in distilled water). The mixture was incubated at 37°C and the time of incubation monitored until a yellow colour appeared. The reaction was stopped by the addition of 0.1 ml of 1 M KH_2PO_4. The OD_{550} and the OD_{420} were read. The units of

alkaline phosphatase were calculated by the formula in section 2.31.1.

2.31.3 Beta-lactamase

Beta-lactamase was assayed according to the method of O'Callaghan *et al*. (1982) in 1 ml microcuvettes. Assay mixtures contained 20-100 µl of sample made to 800 µl with 0.1 M potassium phosphate buffer (pH7.0). 20 µl of Nitrocephin (4 mg/ml in DMSO) were added and the absorbance at 500 nm at 30°C followed using a Pye Unicam SP1800 spectrophotometer. Enzyme activity was expressed as umoles (x10) of Nitrocephin hydrolysed per min per mg of protein.

2.31.4 NADH oxidase

This enzyme was measured using the method of Osborn *et al*. (1972a). Reaction mixtures contained 50 mM Tris-HCl (pH7.5), 0.12 mM NADH, 0.2 mM dithiothreitol and 100 µl of sample in a volume of 1 ml. The rate of decrease in the absorbance at 340 nm was monitored at 22°C as above. NADH oxidase activity was expressed as umoles of NADH oxidised per min per mg of protein.

2.31.5 Glucose-6-phosphate dehydrogenase

This enzyme was assayed according to Malamy and Horecker (1961). Assay mixtures contained 50 mM Tris-HCl (pH7.65), 10 mM $MgCl_2$, 1 µmole of glucose-6-phosphate, 0.4 µmoles of NADP and 100 µl of sample. NADP reduction was followed at 25°C at

340 nm as above. One unit of enzyme represented 1 µmole of NADP reduced per hour per mg of protein.

2.31.6 Protein Determinations

Protein determinations were performed using the Bio-Rad protein assay system (Bio-Rad Laboratories Ltd.) with bovine serum albumin standards.

2.32 DNA Sequencing

DNA was sequenced using the 'random shotgun' method as described by Bankier and Barrell (1983). This involved cloning sonicated DNA fragments into bacteriophage M13mp derivatives (Deininger, 1983), sequencing single-stranded DNA templates using the dideoxy-chain termination method (Sanger *et al.*, 1977) and separation of sequenced DNA on buffer gradient polyacrylamide gels (Biggin *et al.*, 1983).

2.32.1 Sonication of DNA

Sonication required that the DNA was in closed-circular form. Initially, therefore, the DNA to be sonicated was isolated on a fragment containing compatible sticky ends. This was done by restricting the DNA with an appropriate enzyme (section 2.13) electrophoresing it on an agarose gel (section 2.15) followed by electroelution into dialysis bags (section 2.16). After purification, the DNA fragment was self-ligated overnight at 15°C in a mixture containing 20-40 µg of DNA in a final volume of 100 µl. The DNA ligase was then inactivated (65°C, 10 min) and 60 µl of distilled

water added to the mixture. The DNA was subsequently sonicated twice for 8 sec using a MSE Ultrasonic Disintegrator (low frequency, amplitude 3), the DNA sample being spun in a MSE Microcentaur and cooled on ice between sonications. The ends of the sonicated DNA were then repaired by adding 20 µl of dNTP mix (dATP, dGTP, dCTP and dTTP all at 0.25 mM), 20 µl of 10x T4 DNA polymerase buffer (33 mM Tris-acetate, pH7.9; 66 mM potassium acetate, 10 mM magnesium acetate; 5 mM dithiothreitol; 0.1% (w/v) bovine serum albumin, Pentax Fraction V), 40 units of T4 DNA polymerase and 5 units of Klenow fragment of DNA polymerase 1. The mixture was incubated for 3-4 hours at 14°C. The sonicated DNA fragments were then separated on a 1.5% (w/v) agarose mini gel (section 2.15). Fragments between 500-1000 base pairs in size were purified by electroelution into dialysis bags (section 2.16) and, following extraction and precipitation, the DNA was dissolved in 50 µl of TE buffer.

2.32.2 Cloning of Sonicated DNA Fragments

The purified, sonicated DNA was cloned into the vector M13mp8 which had been restricted with the enzyme SmaI and purified as described in section 2.13. 1µl samples of the vector (20 ng/µl) were ligated to varying amounts of the purified DNA fragments in total volumes of 10-20 µl. The ligation mixtures were then used to transfect 100 µl samples of competent TG2 cells (section 2.17). Subsequent to heat-shock, the cells were added to 3 ml of H-Top agar containing

200 µl of exponential phase TG2, BCIG (20 µg/ml final concentration added from a stock solution made in dimethyl formamide) and IPTG (20 µg/ml final concentration added from a stock solution made in distilled water). Each mixture was poured over a 2xYT-agar plate and allowed to set before incubation overnight at 37°C. The plates were then examined for the presence of white plaques resulting from the growth of recombinant M13mp8 and blue plaques caused by re-ligated vector.

2.32.3 Preparation of Single-Stranded DNA Templates

Individual white plaques, containing recombinant M13 bacteriophage, were picked with sterile toothpicks and inoculated into 2 ml of 2xYT which contained 40 µl of an overnight culture of strain TG2. The samples, in sterile universal tubes, were then shaken at 37°C for 5-6 hours. 1.5 ml of the infected culture were then transferred to an Eppendorf tube and the cells pelleted in a MSE Microcentaur (5 min, high speed). 1 ml of the supernatant, containing the M13 'phage particles, was subsequently removed to a clean Eppendorf and 0.25 ml of 2.5 M NaCl + 20% (w/v) PEG 6000 added. The mixture was left at room temperature for 15 min to precipitate the 'phage which were then pelleted in a MSE Microcentaur (5 min, high speed). The supernatant was removed and the tubes re-centrifuged briefly to bring any remaining traces of PEG solution to the bottom of the tube. This was then removed by aspiration and the visible 'phage

pellet dissolved in 0.1 ml of 10 mM Tris-HCl/0.1 mM EDTA (pH8.0). 50 µl of phenol (Ultra-pure phenol equilibrated with 100x TE buffer, 99:1) were then added and the mixture vortexed for 2 min, left to stand for 5 min, vortexed again and finally centrifuged in a MSE Microcentaur (1 min, high speed) to separate the aqueous and phenol layers. The upper aqueous phase was transferred to a clean Eppendorf tube and traces of phenol were removed by mixing with diethyl ether. The upper layer was discarded and the bottom phase, containing the single-stranded M13 DNA, was precipitated by adding 0.1 volumes of 3 M sodium acetate (pH5.5) and 2 volumes of 100% ethanol. The mixture was frozen at -70°C for 20-30 min (or overnight at -20°C) and then the DNA pelleted in a MSE Microcentaur (5 min, high speed). The supernatant was discarded and the DNA washed in 70% (v/v) ethanol, vacuum dried for 10 min and dissolved in 25 µl of 10 mM Tris-HCl/0.1 mM EDTA (pH8.0) and stored at -20°C until required. The single-stranded DNA templates were usually checked on a 1% (w/v) agarose mini gel before sequencing.

2.32.4 DNA Sequencing Reactions

The DNA template to be sequenced was first annealed to the M13 17-base primer (Bethesda Research Laboratories). The annealing reaction was carried out in a 1.5 ml Eppendorf tube containing 5 µl of single-stranded template DNA (0.5-1 µg), 1 µl of 10x polymerase reaction buffer (70 mM Tris-HCl, pH7.5; 70 mM $MgCl_2$; 500 mM NaCl) and 2µl of primer DNA (4

ng) in a total volume of 10 µl. The tube was centrifuged briefly in a MSE Microcentaur to mix the contents and incubated at 80°C for 3 min to denature the DNA. The mixture was left to cool to room temperature to allow annealing of template and primer DNA and then the tube centrifuged as above to collect any condensation. 3 µl of [alpha-^{32}S]dATP (aqueous solution, 600 Ci/mmole, 9 µCi/ml), 1 µl of 0.1 mM dithiothreitol and 1 µl of DNA polymerase I Klenow fragment (1.5 units/µl) were added to the hybridised template and primer. 3 µl aliquots of the above mixture were then dispensed into four reaction tubes designated A, C, G and T. These were placed in ten hole centrifuge racks which enabled the tubes to be spun in an Eppendorf Model 5413 Centrifuge in order to mix the constituents of the sequencing reactions. The following were then added to the tubes:

	A	C	G	T
A°	1 µl	-	-	-
C°	-	1 µl	-	-
G°	-	-	1 µl	-
T°	-	-	-	1 µl
0.1 mM ddATP	1 µl	-	-	-
0.3 mM ddCTP	-	1 µl	-	-

0.5 mM ddGTP	-	-	1 µl	-
1.0 mM ddTTP	-	-	-	1 µl

The ddNTP solutions were prepared as 10 mM stocks and diluted in distilled water to the correct concentration for use. The N^{o} mixes were prepared as follows:

	A^{o}	C^{o}	G^{o}	T^{o}
0.5 mM dCTP	20 µl	1 µl	20 µl	20 µl
0.5 mM dGTP	20 µl	20 µl	1 µl	20 µl
0.5 mM dTTP	20 µl	20 µl	20 ul	1 µl
10x polymerase reaction buffer	20 µl	20 µl	20 ul	20 µl

The dNTP solutions were prepared as 10 mM stocks and diluted in distilled water for use.

The tubes were centrifuged to mix the contents and left at room temperature for 15 min to allow the sequencing reactions to proceed. 1 µl of 0.5 M dATP was then added to each tube and, after spinning briefly, the tubes were left at room temperature for a further 15 min. 5 µl of sequence dye (Table 2 h) were subsequently added and again the tubes centrifuged to mix the contents before boiling for 3 min to

denature the DNA. Samples of 2-3 µl were then applied to sequencing gels for electrophoresis as described below.

2.32.5 Sequence Gels

Samples of the sequence reactions were applied to 6% (w/v) buffer gradient polyacrylamide gels for electrophoresis. The solutions required for the preparation of these gels are listed in Table 2 (h). To prepare a sequence gel, 65 ml of solution I and 12 ml of solution II were measured into a beaker. 30 µl of 10% (w/v) ammonium persulphate and 70 µl of TEMED were added to solution I, while 60 µl of 10% (w/v) ammonium persulphate and 20 µl of TEMED were added to solution II. Each solution was thoroughly and rapidly mixed. 10 ml of solution I was drawn up into a 25 ml pipette followed by 10 ml of solution II. This mixture was cast between gel plates (20 cm x 50 cm) which were separated by Plastikard spacers of 0.3 mm and sealed with PVC tape. The back (sticky) plate was coated with a solution containing 7 µl of gamma-methacryloxy propyl-trimethoxysilane, 750 µl of 10% (v/v) glacial acetic acid and 24.25 ml of 100% ethanol. The front (repellent) plate was coated with 2% (v/v) dimethyldichlorosilane solution in trichloroethane. Any remaining solution I was used to fill the gel plates and then a Plastikard slot former (30 slots) placed in position. The gel was left to set for 30 min at a 45^{o} angle.

The slot former was then removed, the gel assembled into a Raven vertical slab gel electrophoresis chamber and the

Table 2 (h) Reagents for sequencing gels

Solution	Components
40% (w/v) acrylamide	38 g acrylamide 2 g bis-acrylamide Dissolved in distilled water to a final volume of 100 ml Deionised by stirring with 2-4 g of Amberlite MB-1 for 60 min before filtration through Whatman 3MM paper. Stored at 4°C.
Solution II	25 ml 10x TBE 15 ml 40% (w/v) acrylamide 46 g ultra-pure urea 25 ml 40% (w/v) sucrose 5 mg bromophenol blue Made to 100 ml with distilled water, filtered and stored at 4°C. Degassed under vacuum before use.
Solution I	5 ml 10x TBE 15 ml 40% (w/v) acrylamide 46 g ultra-pure urea Made to 100 ml with distilled water, filtered and stored at 4°C. Degassed under vacuum before use.
Sequence dye	0.1 g xylene cyanol FF 0.1 g bromophenol blue 2 ml 0.5 M EDTA Made to 100 ml with deionised formamide. Formamide was deionised by stirring with 2 g of Amberlite MB-1 for 30 min. Filtered.

upper and lower reservoirs filled with 1x TBE buffer. The gel was pre-run for 30 min at 30 mA and the gel slots flushed with buffer to remove any urea and unpolymerised acrylamide. DNA sequence reactions were then loaded and electrophoresis carried out at 30 mA for 2-3 h.

After running, gels were fixed in 5% (v/v) methanol + 5% (v/v) acetic acid for 20 min and then transferred to Whatman 3MM paper for drying at 80°C for 15 min. The dried gel was exposed to Fuji X-ray film at room temperature and then developed as in section 2.25. The sequence data determined on both complementary strands were assembled into a complete sequence using the computer program described by Staden (1980).

Chapter Three

Cloning of the ompT gene of E. coli

3.1 Introduction

Before studies on the *ompT* gene could be carried out, it was necessary to clone the gene from *E. coli* K-12. Using published information (Gordon *et al.*, 1984), a DNA probe, specific for the *ompT* gene and 17 nucleotides in length, was constructed. This probe corresponded to residues 123 to 139 of the *ompT* gene sequence as reported by Gordon *et al.* (1984) and is shown below:

5' TCTCA GTTTT GTCCC TC 3'

The probe had a calculated melting temperature (Tm) of 50°C according to the formula: Tm (°C) = 4(G + C) + 2(A + T) where G, C, A and T are the numbers of the respective bases in the oligonucleotide (Wallace *et al.* 1979).

3.2 Results

3.2.1 Hybridisation of the ompT Probe to E. coli DNA

Chromosomal DNA from both *E. coli* JM83 and UT4400 was digested with the restriction endonuclease *Eco*RI. This enzyme was chosen since the *ompT* gene had been reported to lie within a 3.1 kb *Eco*RI restriction fragment of DNA (Gayda and Markovitz, 1978). The digested DNA was electrophoresed on a 0.8% (w/v) agarose gel before being transferred to Hybond-N nylon membrane by Southern blotting. A second membrane was prepared in exactly the same manner.

Both membranes were prehybridised at 55°C for 2 hours and then hybridised with ^{32}P-labelled *ompT* oligonucleotide

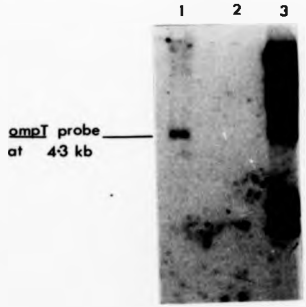
probe. Hybridisations were carried out overnight with one membrane at 40°C and the other at 45°C. These temperatures were chosen according to the guidelines of Suggs et al. (1981) who recommend a hybridisation temperature 5°C below the Tm of the oligonucleotide for perfectly matched sequences.

Following hybridisation, washing and drying, the membranes were autoradiographed for 24 hours. When developed, the exposed X-ray film showed that the probe had hybridised to JM83 DNA fragments of approximately 4.3 kb (Figure 3 a), hybridisation being strongest at 40°C. As expected, hybridisation to UT4400 DNA did not occur since the strain is deleted in a region of the chromosome containing the ompT gene (Earhart et al., 1979).

From the above results, it appeared that ompT was located on a DNA fragment 4.3 kb in size. EcoRI fragments of JM83 chromomsomal DNA 4-5 kb in length were therefore isolated in order to clone a fragment carrying the gene. EcoRI-restricted JM83 DNA was electrophoresed on a 0.8% (w/v) agarose gel and the appropriately sized fragments were prepared from the gel by electroelution. After purification, the DNA fragments were ligated into the EcoRI site of the multicopy plasmid pUC9 (Vieira and Messing, 1982) and the ligation mixtures were then used to transform E. coli JM83. Transformants were selected on the basis of ampicillin resistance and the selection plates also contained BCIG (20

Figure 3 (a) Autoradiogram of ^{32}P-labelled *ompT* oligonucleotide probe hybridised to *E. coli* chromomsomal DNA.

E. coli JM83 and UT4400 chromosomal DNA were restricted with *Eco*RI. electrophoresed on a 0.8% (w/v) agarose gel and then transferred to nylon membrane. The membrane was probed with ^{32}P-labelled *ompT* oligonucleotide. Track 1 = JM83 DNA; track 2 = UT4400 DNA; track 3 = ^{32}P-labelled lambda DNA restricted with *Hind*III



µg/ml final concentration added from a stock solution made in dimethyl formamide) and IPTG (20 µg/ml final concentration added from a stock made in distilled water). These allowed the differentiation of transformants containing religated pUC9 (blue colonies) from those carrying pUC9 with a DNA insert (white colonies).

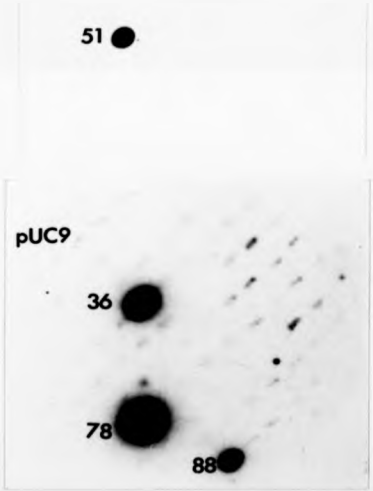
Six hundred white transformants were subsequently analysed by colony hybridisation using ^{32}P-labelled *ompT* oligonucleotide probe to detect any containing a recombinant plasmid carrying the *ompT* gene. Four of the transformants tested hybridised strongly with the probe (Figure 3 b). These were purified and designated strains 36, 51, 78 and 88. The recombinant plasmids they contained were referred to as pOMPT36, pOMPT51, pOMPT78 and pOMPT88 respectively.

3.2.2 Restriction Mapping of Positive Clones

The recombinant plasmids were prepared from their respective strains by caesium chloride density-gradient centrifugation. An initial restriction analysis of each plasmid with *Eco*RI indicated that all of them contained a DNA insert of 4.3 kb. Samples of each plasmid were then digested with a number of different enzymes in order to construct a preliminary restriction map of each 4.3 kb fragment by comparing the products of single and multiple digests (Maniatis *et al*., 1982). The enzymes used were *Eco*RI, *Sma*I and *Pst*I. The latter two endonucleases were chosen since both had previously been shown to cut the 3.1 kb *Eco*RI fragment on

Figure 3 (b) Autoradiogram of colony hybridisations with ^{32}P-labelled *ompT* oligonucleotide probe.

Four colonies hybridised strongly with the *ompT* probe and were designated strains 36, 51, 78 and 88. The recombinant plasmids carried by the strains were designated pOMPT36, pOMPT51, pOMPT78 and pOMPT88 respectively.



which the ompT gene was originally cloned (Gordon et al., 1984). The restriction maps of the 4.3 kb EcoRI fragments are illustrated in Figure 3 (c). These show that all the 4.3 kb fragments cloned were identical although pOMPT36 carried the fragment in the opposite orientation from the other three plasmids, with respect to the lacZ operator and promoter present on the pUC9 vector (Figure 3 c).

The map also shows the presence of two SmaI sites separated by 0.3 kb of DNA. A SmaI fragment of this size was also found on the original 3.1 kb EcoRI fragment of DNA containing the ompT gene (Gordon et al., 1984). However, two PstI sites were present on the fragment described in this work as compared to one PstI site located on the previously cloned fragment (Gordon et al., 1984). In addition, the DNA fragment cloned in this study was 1.2 kb larger than that reported by Gayda and Markovitz (1978) to carry the ompT gene.

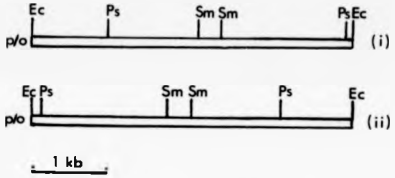
Since pOMPT78, pOMPT88 and pOMPT51 appeared to be identical on the basis of their restriction maps, pOMPT51 and pOMPT36 were selected for further analyses.

3.2.3 Hybridisation of the EcoRI DNA Insert to the ompT Probe

This experiment was carried out to ensure that the ompT DNA probe hybridised to the 4.3 kb EcoRI fragment and not to vector DNA sequences. The plasmids pOMPT36, pOMPT51 and pUC9

Figure 3 (c) Restriction maps of the 4.3 kb inserts in pOMPT36, pOMPT51, pOMPT78 and pOMPT88.

The map of the insert in pOMPT51, pOMPT78 and pOMPT88 is shown in (i) and the map of the insert in pOMPT36 is illustrated in (ii). The orientation of each insert, with respect to the lacZ promoter (p) and operator (o) of pUC9, is indicated.



were restricted with EcoRI, electrophoresed on a 0.8% (w/v) agarose gel and then transferred to Hybond-N nylon membrane. Subsequent hybridisation with ^{32}P-labelled ompT oligonucleotide probe showed that the probe hybridised to the cloned 4.3 kb DNA insert present in pOMPT36 and pOMPT51 but not to pUC9 DNA (Figure 3 d). This result demonstrates that DNA complementary to the probe was present on the cloned fragment rather than the vector.

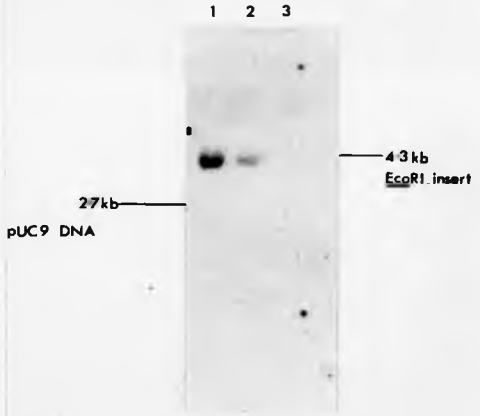
3.2.4 Labelling of the Proteins Encoded by pOMPT51 and pOMPT36

Previous studies had shown the OmpT protein to have an apparent Mr of 40,000. If the ompT gene was present on the cloned 4.3 kb EcoRI fragment, one would expect a protein of this size to be encoded by pOMPT51 and pOMPT36. The vector pUC9 is known to encode beta-lactamase (30 kd) and, therefore, both recombinant plasmids would be expected to encode this protein as well. In addition, one would expect the cloned fragment to encode proteins of 25 kd and 28 kd (Gayda and Markovitz, 1978) the former being the M5 polypeptide (Gayda et al., 1979a; Gordon et al., 1984). The 28 kd protein is possibly EnvY as reported by Lundrigan and Earhart (1984a).

To enable the identification of proteins encoded by the plasmids the maxi-cell technique of Sancar et al. (1982) was used. This method involves the use of strains which carry

Figure 3 (d) Autoradiogram of ^{32}P-labelled *ompT* oligonucleotide probe hybridised to the 4.3 kb inserts of pOMPT36 and pOMPT51.

Plasmids pUC9, pOMPT36 and pOMPT51 were restricted with *Eco*RI and electrophoresed on a 0.8% (w/v) agarose gel. The DNA was then transferred to a nylon membrane which was subsequently probed with ^{32}-labelled *ompT* oligonucleotide. Track 1 = pUC36; track 2 = pOMPT51; track 3 = pUC9.



mutations in the recA and uvrA genes. These mutations inactivate major DNA repair systems and prevent the cell from repairing DNA damaged by such agents as UV irradiation. The latter can be used to induce pyrimidine dimers in recA and uvrA mutants. These dimers inhibit transcription and result in the extensive degradation of chromosomal DNA by intracellular nucleases. Subsequent incubation of these UV-irradiated cells, therefore, results in very little protein synthesis. However, if such strains carry a multicopy plasmid, many plasmid molecules will survive the UV treatment due to their small size as compared with that of the bacterial chromosome. These plasmid molecules can therefore serve as templates for transcription during subsequent incubation and, the addition of a labelled amino acid to the incubated cells, leads to the preferential labelling of plasmid-encoded proteins.

The maxi-cell strain CSH26 was transformed with plasmids pUC9, pOMPT51 and pOMPT36. Transformants were selected on the basis of ampicillin resistance and the resulting strains designated CSH26 (pUC9), CSH26 (pOMPT36) and CSH26 (pOMPT51) respectively. The plasmid-encoded proteins were then labelled using the maxi-cell technique and electrophoresed on a 15% (w/v) SDS-polyacrylamide gel. Following fluorography and autoradiography of the gel, two labelled proteins of 30 kd and 40 kd were detected in the case of both pOMPT36 and pOMPT51 and one of 30 kd in the case of

pUC9 (Figure 3 e). The 30 kd protein was presumably beta-lactamase specified by the vector while the 40 kd protein was tentatively identified as the product of the *ompT* gene. A 42 kd protein which could represent pro-OmpT was not detected. Interestingly, a 25 kd protein which could represent the M5 polypeptide, the gene of which was reported to lie adjacent to *ompT* (Gordon *et al.*, 1984), was not labelled in these experiments. In addition, a 28 kd polypeptide which could represent EnvY was not apparent. This is possibly because the maxi-cells were labelled at 37°C, a temperature at which EnvY was not synthesised in the experiments of Lundrigan and Earhart (1984a).

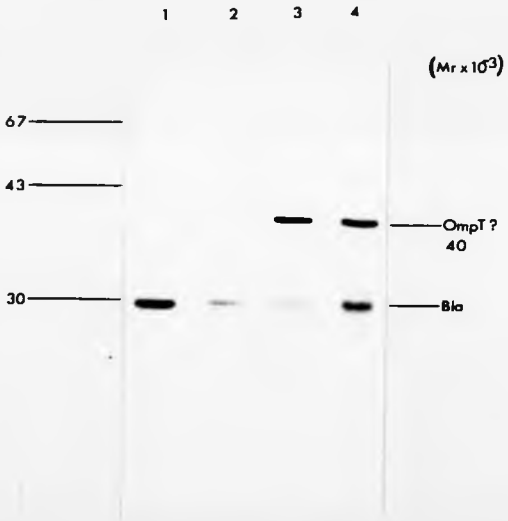
3.2.5 Do pOMPT36 and pOMPT51 Suppress Capsular Polysaccharide Synthesis ?

E. coli lon (*capR*) strains appear mucoid when grown on MM due to the overproduction of capsular polysaccharide. This phenotype can be suppressed by OmpT and M5 so that *lon* strains become non-mucoid (Berg *et al.*, 1976). The *lon* strain *E. coli* RGC103 was therefore transformed with plasmids pUC9, pOMPT51 and pOMPT36 to test this observation. The strain had been previously made *recA* by conjugation with *E. coli* MCL3I according to the method of Lorence and Rupert (1983).

Transformants were selected on the basis of ampicillin resistance on MM containing the required supplements for the

Figure 3 (e) Autoradiogram of ^{35}S-methionine labelled maxi-cell samples of pUC9, pOMPT36 and pOMPT51.

Samples were analysed by SDS-PAGE using a 15% (w/v) linear gel and subsequent fluorography. Track 1 = pUC9; track 2 = pUC9; track 3 = pOMPT36; track 4 = pOMPT51.



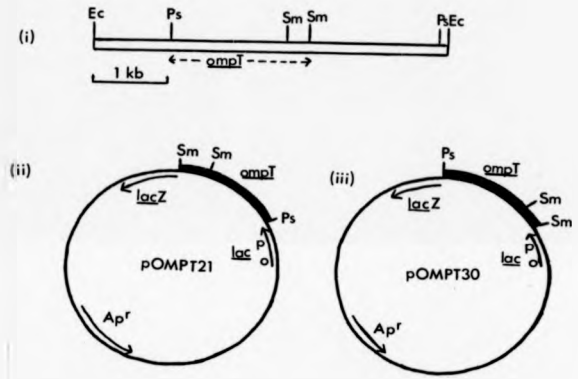
growth of RGC103. All transformants isolated retained a mucoid phenotype. This could be explained by either the fact that *ompT* and the gene encoding the M5 polypeptide had not been cloned or that they were not being expressed and were therefore unable to suppress excessive capsular polysaccharide synthesis. The former explanation was found not to be true as further analysis of the cloned 4.3 kb fragment showed that both genes had been isolated (Chapter 4). With regard to expression, the *ompT* gene appeared to be expressed in maxi-cells giving rise to a 40 kd protein but the 25 kd M5 polypeptide could not be detected in such experiments. This may explain the apparent failure of both pOMPT36 and pOMPT51 to suppress the mucoid phenotype exhibited by the *lon* strain *E. coli* RGC103.

3.2.6 Subcloning of the 4.3 kb EcoRI DNA Fragment

The 4.3 kb DNA fragment was subsequently subcloned to define the limits of the *ompT* gene. By comparing the restriction map of the 4.3 kb fragment (Figure 3 a) with that published by Gordon *et al* (1984), the *ompT* gene was probably located between the *Pst*I and *Sma*I sites as shown in Figure 3 (f). This 1.5 kb *Pst*I-*Sma*I fragment, carrying an internal *Sma*I site, was therefore cloned as described below.

Firstly the 4.3 kb *Eco*RI fragment was isolated by restricting pOMPT51 with *Eco*RI, electrophoresing the digested DNA on a 0.8% (w/v) agarose gel and purifying the required fragment by electroelution. This fragment was then

Figure 3 (f) Subcloning of the 4.3 kb EcoRI DNA fragment containing the ompT gene.



(i) Restriction map of the 4.3 kb EcoRI DNA fragment showing the probable location of the ompT gene.
(ii) The 1.5 kb PstI-SmaI fragment cloned into pUC9.
(iii) The 1.5 kb PstI-SmaI fragment cloned into pUC8.

restricted completely with PstI and partially with SmaI. Such digestion gave a mixture of PstI-SmaI fragments which were separated on a 0.6% (w/v) agarose gel. The 1.5 kb PstI-SmaI fragment was subsequently excised from the gel and isolated by electroelution. The purified fragment was ligated into PstI-SmaI restricted pUC8 and pUC9 (Vieira and Messing, 1982) and the ligation mixtures were then used to transform E. coli TG2. Transformants were selected on LB-agar containing Ap, BCIG and IPTG (section 3.2.1). A white colony from each transformation was purified and the resident recombinant plasmid checked for the presence of the 1.5 kb PstI-SmaI insert. pUC9 and pUC8 carrying this fragment were designated pOMPT21 and pOMPT30 repectively (Figure 3 f).

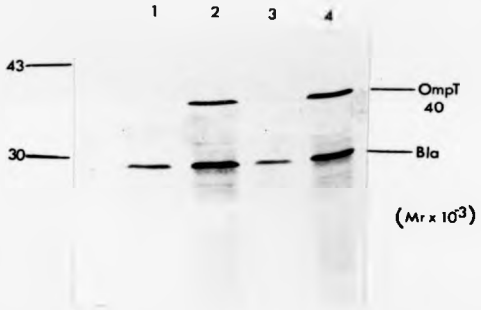
In order to determine if the 40 kd protein was encoded by either plasmid, E. coli CSH26 was transformed with each of them. Maxi-cell analysis of the resulting strains showed that both plasmids encoded a 40 kd protein (Figure 3 g) indicating that indeed the cloned gene was located on the 1.5 kb PstI-SmaI fragment. This result also indicated that a promoter which could drive expression of the cloned gene was present on the 1.5 kb fragment since the 40 kd protein was synthesised irrespective of the orientation of the insert with respect to the lacZ promoter present on the vector.

3.2.7 Cellular Location of the 40 kd Protein

As the OmpT protein is located in the outer membrane of E.

Figure 3 (g) Autoradiogram of ^{35}S-methionine labelled maxi-cell samples of pUC9, pOMPT21 and pOMPT30.

Samples were analysed by SDS-PAGE using a 15% (w/v) linear gel and subsequent fluorography. Track 1 = pUC9; track 2 = pOMPT21; track 3 = pUC8; track 4 = pOMPT30.



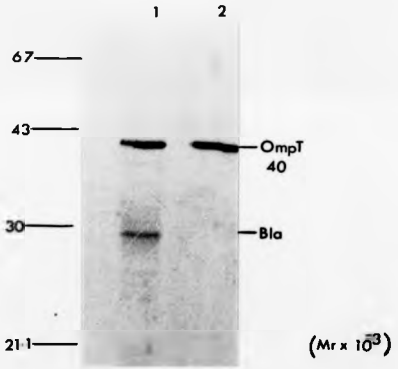
coli, fractionation of CSH26 (pOMPT21) was carried out in order to establish the cellular location of the 40 kd protein encoded by the plasmid. The maxi-cell technique was used first to label the 40 kd protein. Cell envelopes were then prepared from the labelled culture and the outer membranes separated from the inner membranes by virtue of their differential solubility in sodium lauryl sarcosinate (section 2.28). The isolated outer membrane fraction was electrophoresed on a 15% (w/v) SDS polyacrylamide gel which was then autoradiographed. The autoradiogram showed that the 40 kd protein was indeed located in the outer membrane of the cell (Figure 3 h).

3.3 Summary of Results

From the results obtained, it seems likely that the ompT gene has been cloned. Firstly, hybridisation with an oligonucleotide probe specific for ompT indicates that the gene is present on the cloned DNA fragment. Secondly, the cloned fragment encodes a 40 kd outer membrane protein, this being characteristic of the OmpT protein. Differences to previous reports concerning the cloning of ompT were, however, noted. In particular, the inability of the cloned fragment to suppress the mucoid phenotype of E. coli lon strains is contrary to previous work (Berg et al., 1976). This could be explained by the lack of synthesis of the M5 polypeptide which indeed could not be detected in maxi-cell experiments. A further difference concerns the size of the

Figure 3 (h) Autoradiogram of ^{35}S-methionine labelled maxi-cell samples of pOMPT21: Whole cell samples and outer membrane fractions.

Samples were analysed by SDS-PAGE using a 15% (w/v) linear gel and subsequent fluorography. Track 1 = pOMPT21, whole cell sample; track 2 = outer membrane-enriched sample.



DNA fragment on which the *ompT* gene was cloned. In this study a 4.3 kb *Eco*RI fragment carries the gene while Gayda and Markovitz (1978) originally isolated *ompT* on a 3.1 kb *Eco*RI fragment of DNA. This may reflect the fact that different *E. coli* strains were used.

Interestingly, cloning of the *ompT* gene into a multicopy plasmid appeared to have no detrimental effect upon cell growth. In contrast, the cloning of other outer membrane protein genes onto high copy number vectors, including *ompA* (Bremer *et al.*, 1980), *phoE* (Tommassen *et al.*, 1982b) and *lpp* (Lee *et al.*, 1981), has proved lethal to the cell due to the overproduction of the proteins which they encode.

To verify the cloning of the *ompT* gene and to define further restriction sites for subsequent manipulations, sequencing of the cloned DNA fragment was carried out and this is described in the next chapter.

Chapter Four

Sequencing of the cloned ompT gene

4.1 Introduction

Sequencing of the presumptive ompT gene was carried out to confirm its identity and to provide additional well defined restriction enzyme sites for subsequent manipulations. The DNA sequence can also be used to deduce the amino acid sequence of the protein. From this predictions concerning the secondary structure of the protein and its membrane topology can be made.

Studies in Chapter 3 have already demonstrated that the presumptive ompT gene is located on a 1.5 kb PstI-SmaI fragment of DNA (Figure 3 f). The preliminary restriction map of the 4.3 kb EcoRI DNA fragment indicates the presence of a second PstI site located 3.1 kb away from the first (Figure 3 f). Since this 3.1 kb PstI fragment has compatible sticky ends, it can easily be self-ligated for subsequent sonication and cloning into bacteriophage M13mp derivatives for DNA sequence analysis. The whole of the 3.1 kb fragment was therefore sequenced.

4.2 Results

4.2.1 Sequencing of the 3.1 kb PstI Fragment

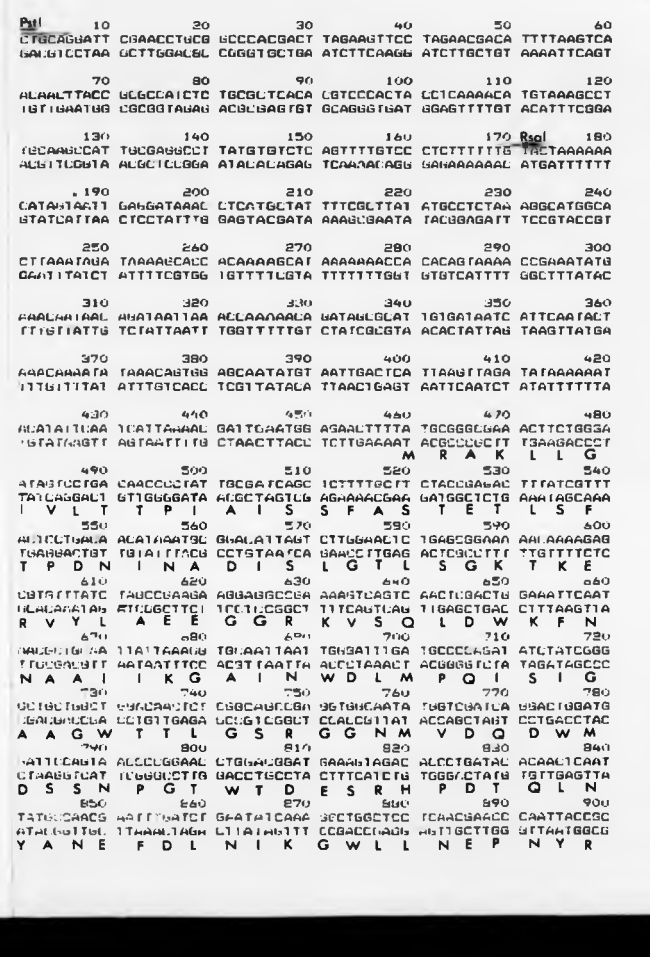
Plasmid pOMPT51 was restricted with PstI and electrophoresed on a 0.6% (w/v) agarose gel. The 3.1 kb fragment produced from this digest was isolated by electroelution, self-ligated and then sonicated (section 2.32.1). The sonicated DNA was electrophoresed on a 1.5% (w/v) mini-gel

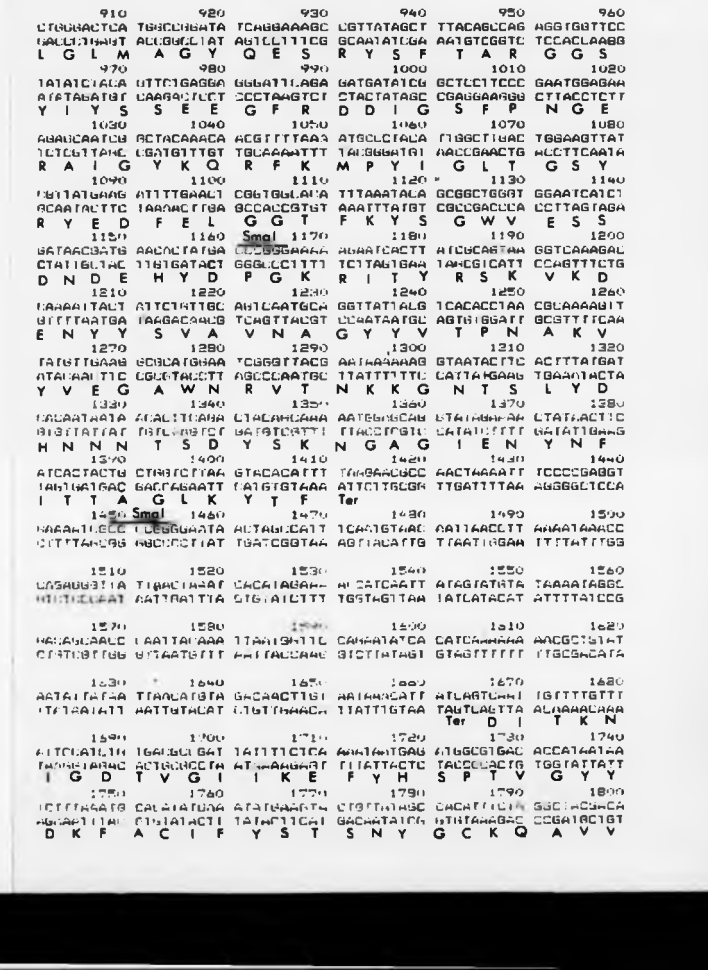
and fragments of approximately 0.5-1.0 kb were isolated and purified. These fragments were then cloned into the SmaI site of M13mp8 (Messing and Vieira, 1982) and the resulting ligation mixtures were used to transfect competent cells of TG2 (section 2.32.2). White plaques were later picked and used to produce single-stranded templates for sequencing by the dideoxy-chain termination method (section 2.32.3). The sequence data, determined on both complementary strands, were assembled into a complete sequence using the computer program described by Staden (1980). Once the complete nucleotide sequence of the 3.1 kb fragment had been compiled, the sequence was analysed by the program ANALSEQ (Staden, 1984) for potential open reading frames (ORF) which could encode the OmpT protein.

Analysis of the sequence revealed the presence of two significant open reading frames (Figure 4 a). The first was 951 bp (from nucleotide 460 to 1410) with a coding capacity of 35.6 kd. This ORF began with an ATG start codon (460 to 462) and was terminated by a TAA stop codon (1411 to 1413). The second ORF of 747 bp ran in the opposite direction (2411 to 1665) and could encode a protein of 28.8 kd. It began with an ATG codon (2411 to 2409) and ended with tandem termination codons TGA TAA (1664 to 1659). Notably, an ORF large enough to encode the pro-OmpT protein, which has an apparent Mr of 42,000, was not present on the DNA fragment.

Figure 4 (a) Nucleotide sequence of the 3.1 kb PstI fragment.

The nucleotide sequence is numbered from the 5' end of the PstI site. Two open reading frames are shown. One ORF runs from base 460 to 1410 (951 bp) and has a coding capacity of 35.6 kd (317 amino acid residues). The second ORF runs in the opposite direction from base 2411 to 1665 (747 bp) and has a coding capacity of 28.8 kd (249 amino acid residues). Restriction sites relevant to this study are indicated.


PstI
RsaI


910 920 930 940 950 960
L G L M A G Y Q E S R Y S F T A R G G S
970 980 990 1000 1010 1020
Y I Y S S E E G F R D D I G S F P N G E
1030 1040 1050 1060 1070 1080
R A I G Y K Q R F K M P Y I G L T G S Y
1090 1100 1110 1120 1130 1140
R Y E D F E L G G T F K Y S G W V E S S
1150 1160 SmaI 1170 1180 1190 1200
D N D E H Y D P G K R I T Y R S K V K D
1210 1220 1230 1240 1250 1260
E N Y Y S V A V N A G Y Y V T P N A K V
1270 1280 1290 1300 1310 1320
Y V E G A W N R V T N K K G N T S L Y D
1330 1340 1350 1360 1370 1380
H N N N T S D Y S K N G A G I E N Y N F
1390 1400 1410 1420 1430 1440
I T T A G L K Y T F Ter
1450 SmaI 1460 1470 1480 1490 1500
1510 1520 1530 1540 1550 1560
1570 1580 1590 1600 1610 1620
1630 1640 1650 1660 1670 1680
Ter D I T K N
1690 1700 1710 1720 1730 1740
I G D T V G I I K E F Y H S P T V G Y Y
1750 1760 1770 1780 1790 1800
D K F A C I F Y S T S N Y G C K Q A V V

```
      1810        1820        1830        1840        1850        1860
TTGATAAAAT AAGAGTTTGA ABTTATGAGT TTTTTTGCAT ACCTCATCCT AGTATCTCTC
AACTATCTTA TTCTCAAACT TCAATAACTA AAAAAACGTA TGAGTAGGAG TCATAGAGAG
  N   I   S   Y   S   R   S   T   I   L   K   K   A   Y   R   M   R   T   D   R
      1870        1880        1890        1900        1910        1920
AATATTTCAA TAAATGACGT TCCTTCATCC CTTAATCTTT TTTTTATTAA ACTTTCACTC
TTATAAAGTC ATTTACTGCA AGGAAGTAGA GAATTAGAAA AAAATAATT TGAAAGTGAG
  L   I   E   T   F   S   T   G   E   D   R   L   R   K   K   I   L   S   E   S
      1930        1940        1950        1960        1970        1980
GTATAAATCA ATTCCTCAAT ATCTTTTAAA TGCCATTGCC GCTCAATAAT AAAACTGATT
CATATTTAGT TAAGGCGTTA TAGAAAATTT ACGGTAACGG CGAGTTATAA TTTTGACTAA
  T   Y   I   L   E   A   I   D   K   L   H   W   Q   R   E   I   N   F   S   I
      1990        2000        2010        2020        2030        2040
ATTCCAATAA TTTTACAGGT AAATGTATTT ATATTTGTTA GTATAAATGA ATTTACACTT
TAAGGTCATT AAAATGTCCA TTTACATAAA TATAAACAAT CATATTTACT TAAATGTGAA
  I   G   T   I   K   C   T   F   T   N   I   N   T   L   I   F   S   N   V   S
      2050        2060        2070        2080        2090        2100
TCGCGTTTTT TGAACATGAC AAGTAAGGAT ATACATAGTC TTTCTTTTAA CCAAAGGAAG
AGCGCAAAAA ACTTGTACCG TTCATTCCTA TATGTATCAG AAAGAAAATT GGTTTCCTTC
  E   R   K   K   F   M   A   L   L   S   I   C   L   R   E   K   L   W   L   S
      2110        2120        2130        2140        2150        2160
TGTGAGTCTG CTATTTTAAT CCCTTCAAAC AGATAAAAAA CAAGCGATAA TGGAGGTTCC
ACACTCAGAC GATAAAATTA GGGAAGTTTG TCTCTTTTTT GTTCGCTATT ACCTCCAAGG
  H   S   D   A   I   K   I   G   E   F   L   S   F   V   L   S   L   P   E
      2170        2180        2190        2200        2210        2220
TCTTCAACAA TATAATCCAT CTTATCAAAA GTAAATTTGC CAGGCAGCTC ATTATTCACG
AGAAGTTGTT ATATTAGGTA GAATAGTTCT CATTTAAACG GTCCGTCGAG TAATAAGTGC
  E   E   A   I   Y   G   N   K   D   L   T   F   K   G   P   L   E   N   N   V
      2230        2240        2250        2260        2270        2280
TCGATAAAAA AGAATAAACA TGTTTCTCTA TCTATATCAA CAATCTTAG TTTAGAGGGG
AGCTATTTTT TCTTATTTGT ACAAAGGATT AGATATAGTT GTTAAGAATC AAATCTCCCC
  D   I   F   F   S   L   C   T   K   K   D   I   D   V   I   R   L   K   S   P
      2290        2300        2310        2320        2330        2340
CATACTAGTA ACTCTCTTCT AATCTTGTCG CTTACAATAA ACAATGAATT TTTTTGAAC
GTATGATCAT TGAGAGAAGA TTAGAACAGC GAATGTTATT TGTTACTTAA AAAAAACTTG
  C   V   P   L   E   R   I   K   D   S   V   I   F   L   S   N   K   F
      2350        2360        2370        2380        2390        2400
AAAAATAACTC TCCTGTTTAT AATTAAATCA AATGATTGAC AGATGAAAAC TACGGAGCAA
TTTTTATTGAG AGGACAAATA TTAATTTAGT TTACTAACTG TCTACTTTTG ATGCCTCGTT
  S   I   V   R   N   I   I   L   D   F   S   Q   C   I   F   V   S   C
      2410        2420        2430        2440        2450        2460
ACATAAATCA TCTTTGCACT AICATAAAAT TAAAAACAAGT TGATAGTAGT CAATACAACA
TGTATTTAGT AGAACGTGGA TAGTATTTTA ATTTTGTTCA ACTATCATCA GTTATGTTGT
  V   Y   D   M
      2470        2480        2490        2500        2510        2520
ACCAGTTAAA TACACAATCA TAATCAACAT GATGTGCAIT TATATTTTTA TACACAAAAT
TGGTCAATTT ATGTGTTAGT ATTAGTCCTA CTACACGTAA ATATAAAAAT ATGTGTTTTA

      2530        2540        2550        2560        2570        2580
TATAGTTTGC AAATTTTAAT AAATTTCATT TAAGATTAAA TTATTATATG TATGTTGTTT
ATATCAAACG TTTAAAATTA TTTAAAGTAA ATTCTAATTT AATAATATAC ATACAACAAA

      2590        2600        2610        2620        2630        2640
TTTATTCTAA CTTTATTTAA AGTTAIATTT TTCAACGCTT ACTATGCTTT TTATTAACAT
AAATAAGATT GAATAAATTT TCAATGTAAA AAGTTGCGAA TGATACGAAA AATAATTGTA

      2650        2660
AAACTCACTA CAACCCACCT GAAACC
TTTGAGTGAT GTTGCGTGGA CTTTGG
```

The 5' terminus of the sequence determined here was compared to the partial *ompT* gene sequence as reported by Gordon *et al*. (1984). Differences between the two were apparent (Figure 4 b). From the 5' *Pst*I site, the sequence found in this study is identical to that published by Gordon *et al*. (1984) up to nucleotide residue 81. A twenty-three base pair DNA insert between nucleotide residues 81 and 82 is then found in the sequence determined in this work (Figure 4 b). The two DNA sequences are then the same upto and including residue 179 (155 of the published sequence). At position 180 (156 of published sequence), however, an A residue is inserted in the sequence presented here which alters the reading frame of the DNA. The deduced amino acid sequences (from nucleotide 142 of the sequence determined here or 118 of the published sequence) are, therefore, identical for the first twelve residues. After this, however, the amino acid sequence deduced in this work is different from that published and ends with a TGA termination codon at nucleotides 190-192 (Figure 4 b). This stop codon is followed by a sequence of 244 base pairs, which includes eight stop codons, before the beginning of the 951 bp ORF at nucleotide 460 (Figure 4 b). Other differences between the published sequence (Gordon *et al*., 1984) and that determined in this study are indicated in Figure 4 (b). The results obtained suggested that the ORF designated by Gordon *et al*. (1984) to encode the amino-terminus of OmpT, may not be correct and the question to be answered was whether or not

Figure 4 (b) Comparison of the 5' terminus of the DNA sequence reported by Gordon *et al*. (1984) to encode OmpT with that found in this study.

(i) Sequence of the 5' terminus of the DNA encoding OmpT as determined in this study.
(ii) Sequence of the 5' terminus of the DNA encoding OmpT as determined by Gordon *et al*. (1984).

The nucleotide residues are numbered from the 5' *Pst*I site and relevant restriction sites indicated. The -10 and -35 consensus sequences, the Shine-Dalgarno sequence (SD) and the ATG translational start codon, as designated by Gordon *et al*. (1984) for *ompT*. are shown in (ii). The amino acid sequence proposed by Gordon *et al*. (1984) for the amino-terminus of pro-OmpT is also shown in (ii). The two nucleotide sequences are aligned and differences between them are boxed in (i) and (ii) as are differences between the deduced amino acid sequences. The translational stop codons occurring between the end of the first ORF found in this study and the 951 bp ORF are underlined in (i).

```
(i)  CTGCAGGATTCGAACCTGCGGCCCACGACTTAGAAGTTCCTAGAACGACATTTT
(ii) CTGCAGGATTCGAACCTGCGGCCCACGACTTAGAAGTTCCTAGAACGACATTTT
     PstI                      -35

                                        81
(i)  AAGTCAACAACTTACCGCGCCATCTCTGCGCTCACACGTCCCACTACCTCAAAA
     -10                        81
(ii) AAGTCAACAACTTACCGCGCCATCTCT----------------------AAAA

                                        142
(i)  CATGTAAAGCCTTGCAAGCCATTGCGAGGCCTTATGTGTCTCAGTTTTGTCCCTC
                                          M  C  L  S  F  V  P  L
                                  SD      118
(ii) CATGTAAAGCCTTGCAAGCCATTGCGAGGCCTTATGTGTCTCAGTTTTGTCCCTC
                                          M  C  L  S  F  V  P  L

     RsaI                 179
(i)  TTTTTTGTACTAAAAAACATAGTAATTGAGGATAAACCTCATGCTATTTTCGCTT
       F  C  T  K  K  H  S  N  -
     RsaI                 155
(ii) TTTTTTGTACTAAAAA-CATAGTA-TTGAGGATAA-CCTTCTTTCGATTT-CGCTT
       F  C  T  K  N  I  V  L  R  I  T  F  F  D  F  A  Y

(i)  ATTTGCCTCTAAAGGCATGGCACTTAAATAGATAAAAGCACCACAAAAGCATAAA
(ii) ATTTG----------------------------------------------------
       L   end of published sequence

(i)
AAAACCACACAGTAAAACCGAAATATGAAACAATAACAGATAATTAAACCAAAAACAG

ATAGCGCATTGTGATAATCATTCAATACTAAACAAAATATAAACAGTGGAGCAATATGT

AATTGACTCATTAAGTTAGATATAAAAAATACATATTCAATCATTAAAACGATTGAATGG
                                        460
AGAACTTTTATGCGGGCGAAACTTCTGGGAATAGTCCTGACAACCCCTATTGCGATCAGC
          M  R  A  K  L  L  G  I  V  L  T  T  P  I  A  I  S

TCTTTTGCTTCTACCGAG

S  F  A  S  T  E
```

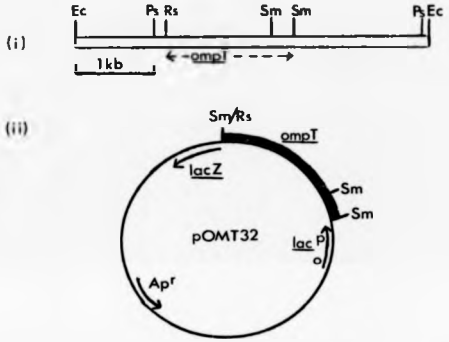
the 42 kd pro-OmpT protein could be encoded by the 951 bp ORF.

4.2.2 **Does the 951 base pair ORF encode the OmpT Protein ?**

To answer this question subcloning of the 1.5 kb PstI-SmaI fragment, known to encode ompT, was carried out. Examination of the sequenced DNA revealed the presence of a RsaI site located towards the end of the ORF reported by Gordon et al. (1984) and this is shown in Figure 4 (a). It was therefore decided to isolate a restriction fragment of DNA beginning at this RsaI site and terminating at the second downstream SmaI site. This would eliminate the promoter sequences, the Shine-Dalgarno sequence, the ATG start codon and DNA encoding most of the signal peptide of OmpT as designated by Gordon et al. (1984).

pOMPT21 was partially digested with both RsaI and SmaI giving a mixture of restriction fragments which were ligated into the SmaI site of pUC9. Following transformation of TG2, white transformants were screened for the presence of a recombinant plasmid carrying the appropriate RsaI-SmaI 1.3 kb insert. Such a plasmid was isolated and designated pOMPT32 (Figure 4 c). E. coli CSH26 was transformed with pOMPT32 and the resulting strain was used to analyse the proteins encoded by the recombinant plasmid by the maxi-cell technique. As shown in Figure 4 (d), pOMPT32 still encoded a protein with a Mr of 40,000 and fractionation of maxi-cells demonstrated that the protein was located in the outer

Figure 4 (c) Subcloning of the 1.5 kb PstI-SmaI DNA fragment which carries the ompT gene.

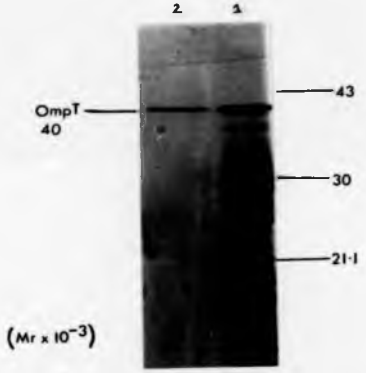


(i) The location of the ompT gene on the 1.5 kb PstI-SmaI DNA fragment is indicated.

(ii) pOMPT32 = pUC9 with the 1.3 kb RsaI-SmaI DNA fragment cloned into the SmaI site (Sm/Rs = SmaI-RsaI junction).

Figure 4 (d) Autoradiogram of ^{35}S-methionine labelled maxi-cell samples of pOMPT32: Whole cell sample and outer membrane fraction.

Samples were analysed by SDS-PAGE using a 15% (w/v) linear gel. Track 1 = pOMPT32, whole cell sample; track 2 = outer membrane-enriched sample.



membrane (Figure 4 d). Clearly the RsaI-SmaI 1.3 kb fragment could encode a protein with an apparent Mr of 42,000 (pro-OmpT) despite the fact that it carried an ORF (951 bp) with a potential coding capacity of only 35.6 kd. It was concluded, therefore, that the 951 bp ORF encoded the OmpT protein. The ompT promoter and the ompT transcription and translation initiation signals were assumed to be located on the RsaI-SmaI 1.3 kb DNA fragment as well since the protein was synthesised in maxi-cells when the fragment was cloned in the opposite direction to the lacZ promoter present on the vector (Figure 4 c). This is not in agreement with the published information concerning ompT (Gordon et al., 1984).

This study is not unique in the finding that an outer membrane protein has an apparent molecular weight, on polyacrylamide gels, larger than that predicted from its deduced amino acid composition. Indeed, sequencing of phoE revealed that it could encode a protein of 38.8 kd which is lower than the Mr of 40,000 estimated from SDS-PAGE of the purified PhoE protein (Overbeeke et al., 1983).

4.2.3 Analysis of the Nucleotide Sequence of the ompT Gene

The nucleotide sequence preceding the start-point of the 951 bp ORF (nucleotide 460) was analysed for sequences that could be involved in the interaction with RNA polymerase and which are known to be rather well-conserved in the promoters of E. coli genes (Rosenberg and Court, 1979). These conserved sequences are centred at nucleotides -10 and at -

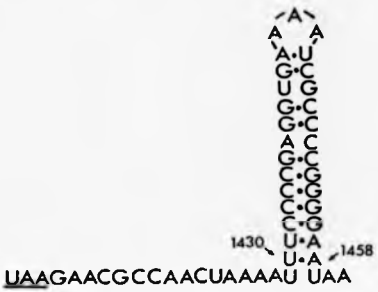
35 prior to the transcriptional start point of the gene. The -10 consensus sequence is referred to as the Pribnow box (Pribnow, 1975) and has the sequence TATAAT where the underlined nucleotides are those most stringently conserved (Rosenberg and Court, 1979). The -35 consensus sequence is involved in the recognition of the sigma factor of RNA polymerase holoenzyme and has the sequence TTGACA where the underlined nucleotides are highly conserved (Rosenberg and Court, 1979). These two consensus regions are usually separated by 16-18 nucleotides (Hawley and McClure, 1983). From the RsaI site (169 to 174), a number of sequences with varying degrees of homology to the Pribnow box were located. These are shown in Figure 4 (e). Potential -35 sequences are also indicated. The -35 region located between nucleotides 393-398 shows good homology to the consensus sequence but both -10 sequences lying downstream of this (421-426 and 419-424) are rather poorly conserved.

Considering the possible termination of transcription, a stable RNA hairpin structure can be formed corresponding with nucleotides 1430 to 1458 (Figure 4 f) with a ΔG value of -16.8 kcal/mol (25^{o}C) calculated according to the methods of Tinoco et al. (1973) and Borer et al. (1974). The stability of this G+C-rich stem and loop structure indicates that this sequence may play an important role in the termination of transcription (Pribnow, 1979; Platt, 1981). However, this RNA hairpin structure is not followed by a

Figure 4 (e) Analysis of the 5' terminus of the ompT gene

Possible -35 and -10 consensus sequences are indicated. A potential Shine-Dalgarno sequence (SD) is boxed upstream from the designated ATG translational start codon for ompT which is underlined. The potential OmpT signal peptide is indicated as are relevant restriction sites.

Figure 4 (f) Possible stem-loop structure characteristic of the prokaryotic transcription termination site found in the 3' end non-coding region of ompT



The G-C rich stem-loop structure at the 3' end of ompT mRNA is shown. The translational termination codon is underlined. Numbers of the corresponding nucleotides in the DNA sequence (Figure 4 a) are indicated. A ΔG value of -16.8 kcal/mol was calculated for the stem-loop structure.

poly(U) sequence which is a well known feature of rho-independent terminators (Pribnow, 1979; Platt, 1981). Transcription termination of the *ompT* gene may therefore be dependent upon the rho factor.

The nucleotide sequence GGAG (449 to 452), preceding the nucleotide sequence encoding the pro-OmpT protein (Figure 4 e), is complementary to the 3' end of the 16S rRNA (Shine and Dalgarno, 1974) and has been found in all analysed prokaryotic sequences at a distance of four to nine nucleotides preceding the start of translation (Gold *et al.*, 1981). In the case of *ompT*, this ribosome binding site is located seven nucleotides upstream from the translational ATG start codon (460 to 462).

Gordon *et al.* (1984) reported the existence of potential secondary structure in *ompT* mRNA around the proposed Shine-Dalgarno sequence and translation start region (Fig. 1.4 c) This was suggested to decrease the efficiency of translation of *ompT* mRNA at 30°C and therefore lead to a decrease in the amount of the protein synthesised at this temperature. However, from the results presented here, the Shine-Dalgarno sequence and translation start codon designated by Gordon *et al.* (1983) do not appear to be correct though indeed secondary structure around the translation start site can influence the efficiency of translation (Gold *et al.*, 1981). The nucleotide sequence encompassing the Shine-Dalgarno sequence and ATG start codon designated in this study, was

found not to have the potential to form an extensive, stable secondary structure which could sequester the ribosome binding site and translation initiation codon such that translation of *ompT* mRNA could be reduced at 30°C.

The [A-T] content of the *ompT* coding region (residues 460-1413) is 57%. This figure is higher than the average [A-T] content of the *E. coli* chromosome which is 49% (Orvskov, 1978). The DNA sequence preceding the *ompT* coding region and encompassing the potential *ompT* promoter region (residues 169-428), also has a high [A-T] content of 73%. The abundance of [A-T] base pairs in the promoter region of *E. coli* is considered to facilitate the unwinding of the duplex DNA helical structure, such that initiation of transcription can take place (Mizuno *et al.*, 1983a).

The codon usage of *ompT* is summarised in Table 4 (a). It appears from this, that the *ompT* gene shows no preference for the major isoaccepting tRNA species unlike many other outer membrane proteins. Indeed, the genes encoding lipoprotein and OmpA (Movva *et al.*, 1980a) and OmpC, OmpF and PhoE (Mizuno *et al.*, 1983a) all selectively use major isoaccepting species of tRNAs (Ikemura, 1981). This may reflect the fact that *ompT* encodes an outer membrane protein which is less abundant than the previously mentioned proteins, in terms of copy number per cell. Of the codons used in the *ompT* coding sequence, 9.8% are rare codons (ATA, ACG, TCG, CCT, CCC, CAA, AAT, AGG) as defined by Konigsberg

Table 4 (a) The distibution of codons in the ompT reading frame

TTT Phe	8	TCT Ser	6	TAT Tyr	14	TGT Cys	0
TTC Phe	4*	TCC Ser	3	TAC Tyr	9*	TGC Cys	0
TTA Leu	1	TCA Ser	3	TAA End	1	TGA End	0
TTG Leu	2	TCG Ser	1	TAG End	0	TGG Trp	8
CTT Leu	4	CCT Pro	4	CAT His	0	CGT Arg	4*
CTC Leu	7	CCC Pro	4	CAC His	3	CGC Arg	2*
CTA Leu	1	CCA Pro	0*	CAA Gln	4	CGA Arg	2
CTG Leu	5*	CCG Pro	2*	CAG Gln	3*	CGG Arg	2
ATT Ile	6	ACT Thr	10*	AAT Asn	16	AGT Ser	7
ATC Ile	8*	ACC Thr	3*	AAC Asn	10*	AGC Ser	7
ATA Ile	4	ACA Thr	8	AAA Lys	16*	AGA Arg	5
ATG Met	6	ACG Thr	2	AAG Lys	2	AGG Arg	0
GTT Val	5*	GCT Ala	5*	GAT Asp	12	GGT Gly	9*
GTC Val	6	GCC Ala	4	GAC Asp	7	GGC Gly	11*
GTA Val	0*	GCA Ala	8*	GAA Glu	13*	GGA Gly	12
GTG Val	1*	GCG Ala	3*	GAG Glu	4	GGG Gly	1

* Major isoaccepting species of tRNA (Ikemura, 1981).

and Godson (1983). The latter workers defined a total of twenty-three codons in E. coli, including the eight rare codons, that are used infrequently. In the ompT reading frame these twenty-three codons appear at a frequency of 30.2%. Konigsberg and Godson (1983) noted that the infrequently used codons comprised 24-29% of the total number of codons used in regulatory genes (e.g., lacI, dnaG). In contrast, they were found at a level of only 12% in genes encoding non-regulatory proteins (e.g., trpE, ompA). It has been suggested that the cell uses rare codons to differentiate two classes of genes, regulatory and non-regulatory, and that rare codon usage may be part of a general mechanism for modulating protein synthesis (Konigsberg and Godson, 1983). Whether the use of rare codons in the ompT gene reflects either of these suppositions is unknown.

4.2.4 Analysis of the Deduced Amino Acid Sequence of OmpT

The deduced amino acid composition of the OmpT protein is summarised in Table 4 (b). The protein contains a high percentage of hydrophobic amino acids which may reflect the fact that it is normally found in a lipid-rich environment.

Examination of the deduced amino acid (Figure 4 e) sequence revealed the presence of a potential signal peptide of twenty amino acids (encoded by nucleotides 460 to 519). This peptide has many characteristics in common with other signal sequences (Oliver, 1985). Its amino terminus is basic with

Table 4 (b) Amino acid composition of OmpT

Ala	20 (6.3)	Leu	20 (6.3)
Arg	15 (4.7)	Lys	18 (5.7)
Asn	26 (8.2)	Met	6 (1.9)
Asp	19 (6.0)	Phe	12 (3.8)
Cys	0 (0.0)	Pro	10 (3.1)
Gln	7 (2.2)	Ser	27 (8.5)
Glu	17 (5.3)	Thr	23 (7.2)
Gly	33 (10.4)	Trp	8 (2.5)
His	3 (0.9)	Tyr	23 (7.2)
Ile	18 (5.7)	Val	12 (3.8)
End	1 (0.3)		

Acidic	(Asp + Glu)	36 (11.3)
Basic	(Arg + Lys)	33 (10.4)
Aromatic	(Phe + Trp + Tyr)	43 (13.5)
Hydrophobic	(Aromatic + Ile + Leu + Met + Val)	99 (31.1)

Figures in parentheses represent the number of residues as a percentage of the total.

an arginine (-19) and a lysine (-17) residue. This is followed by a stretch of predominantly hydrophobic amino acids (-16 to -2). The residue at the cleavage site is alanine (-1). In addition to these three basic features, the potential OmpT signal peptide also contains a glycine (-14) and a proline (-8) residue. These two amino acids are found within the hydrophobic core of many signal peptides (Oliver, 1985). A serine and/or a threonine residue are often present towards the distal end of the hydrophobic core of many leader sequences. Indeed two serine residues are located in such a position in the potential OmpT signal sequence. The first twenty amino acids therefore constituted a characteristic leader peptide which could be cleaved during the export of pro-OmpT through the cytoplasmic membrane of the cell. Recently this has been confirmed by the work of Grodberg *et al*. (1988) who isolated the mature OmpT protein from the outer membrane of *E. coli*. They showed that the amino-terminal residue of the protein was a serine in keeping with the data presented here. In addition, these workers confirmed the nucleotide sequence of the *ompT* gene as found in this study.

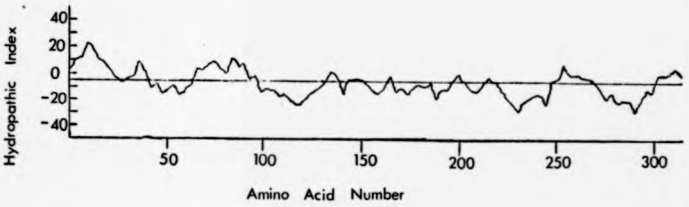
The overall hydropathy along the amino acid sequence of the OmpT protein was evaluated by the method of Kyte and Doolittle (1982) using the computer program of Staden (1984). The average hydropathy of segments containing eleven amino acid residues was calculated progressively from the

amino-terminus of the protein with values being plotted at the mid-point of each segment. Hydrophobic regions gave positive scores; hydrophilic segments gave negative scores. Figure 4 (g) displays the hydropathy profile for the OmpT protein and indicates that it is not appreciably hydrophobic in overall character. Alternating regions of hydrophobic and hydrophilic character are indicated throughout the protein. The most hydrophobic segments of the protein are the signal sequence, one domain close to the amino-terminus (residues 63-97) and two other domains located towards the carboxy-terminus of the protein (residues 250-270 and 302-317). Such hydrophobic segments could potentially span the outer membrane if they assume beta-sheet structures, but not if they assume alpha-helical structures (Yu and Hong, 1986). Indeed, the hydrophobic segment containing residues 250-270, can assume a beta-sheet structure (Figure 4 h). This potential protein secondary structure was determined by the alogorithm method of Garnier et al. (1978) using the Beckman MicroGenie Sequence Analysis computer program. However, such predictions are theoretical and a better understanding of the secondary structure of OmpT and its organisation within the outer membrane would be gained from a study of the purified protein.

4.2.5 DNA Sequence Homologies

The Genetic Sequence Data Bank (GenBank) of published nucleic acid sequences was searched using the Beckman

Figure 4 (g) Hydropathy profile of the OmpT protein



The hydropathy index was evaluated according to the parameters presented by Kyte and Doolittle (1982), using a span setting of 11 amino acid residues. Hydrophobic regions extend above the centre line.

Figure 4 (h) Secondary structure predictions for the OmpT protein.

```
M R A K L L G I V L T T P I A I S S F A S T E T L S F T P D
A A A A A B B B B B B B B B B                 T   T       T

N I N A D I S L G T L S G K T K E R V Y L A E E G G R K V S
      B B B B B B                   A B B A A A A A A T A A B

N L D W K F N N A A I I K G A I N W D L M P Q I S I G A A G
B B T T A A A A A A A A B B B   T T         B B B B       T

W T T L G S R G G N M V D N D W M D S S N P G T W T D E S R
B B B B             B B B B B B B   T                 T T T T

H P D T Q L N Y A N E F D L N I K G W L L N E P N Y R L G L
  T T T           A A A A A A T T T           T T T T B B B

M A G Y Q E S R Y S F T A R G G S Y I Y S S E E G F R D D I
B B   B B   T T T B B B T T T T       B B     T T T T T T T

G S F P N G E R A I G Y K N R F K M P Y I G L T G S Y R Y E
T             T A A A A A T T T B B B B B B               B B

D F E L G G T F K Y S G W V E S S D N D E H Y D P G K R I T
B B A A T T       T   T             T T T T T T   T T T B B B

Y R S K V K D Q N Y Y S V A V N A G Y Y V T P N A K V Y V E
B B B T T T T T B B B B B B B B B B B B B B B B T B B B B B B

G A W N R V T N K K G N T S L Y D H N N N T S D Y S K N G A
A A A A A A T   T T T   B B B B T T T T T T T T T T T T

G I E N Y N F I T T A G L K Y T F END
  B B B T B B B B B           B B
```

The secondary structure predictions were made according to Garnier et al. (1978). A = alpha helix; B = beta-sheet; T = turn; blank = random coil.

MicroGenie Sequence Analysis computer program, to find any global similarities between *ompT* and nucleotide sequences present in the data base. These efforts failed to demonstrate any overall similarities between the *ompT* gene and nucleic acid sequences contained in GenBank.

It has been reported that upstream regions of iron-regulated genes such as *fhuA*, *cir*, *fepA* and *iucA* show a certain degree of homology: an apparent 19 base pair dyad consensus sequence 5'-GATAATGATAATCATTATC-3' (De Lorenzo *et al*., 1987). This sequence has been proposed as the site of binding of the Fur protein which represses the expression of iron-regulated genes when the nutrient is available (Schaffer *et al*., 1985). Since the OmpT protein was reported to be synthesised in greater amounts in cells growing in iron-limited medium (Fiss *et al*., 1982), a similar sequence was searched for in the upstream region of the *ompT* gene. A possible consensus sequence was located beginning at nucleotide residue 339 and ending at residue 356 (Figure 4 e) and this is shown in Figure 4 (i) along with the proposed consensus sequence and the actual sequences found in the upstream regions of the aforementioned iron-regulated genes. It must be stressed, however, that only in the case of the *iucA* gene has experimental characterisation of the promoter region been carried out (De Lorenzo *et al*., 1987) and therefore the sequence comparisons should be taken with caution.

Figure 4 (i) Comparison of sequences from promoter regions of iron-regulated genes

Promoter	Sequence
iucA primary Fur binding site	TGATAATGAGAATCATTATTG
iucA secondary Fur binding site	CATAATTGTTATTATTTTAC
fhuA	TCTTTATAATAATCATTCTCG
fepA	TATTATGATAACTATTTGCA
cir	TGGATTGATAATGATTATC
ompT	CATTGTGATAATCATTCAA
Consensus	GATAATGATAATCATTATC

The most conserved nucleotides are indicated with vertical lines and the sequences are written 5' to 3'. The consensus is proposed to bind the Fur repressor protein in the promoter region of the iron-regulated genes (De Lorenzo *et al.*, (1987).

4.2.6 Protein Sequence Homologies

General searches for similarities with proteins recorded in the National Biomedical Research Foundation (NBRF) protein data bank were performed using the Beckman MicroGenie Sequence Analysis computer program. These failed to find any global similarities between OmpT and proteins in the data base. The same program was used to align and find any local regions of homology between OmpT and other *E. coli* outer membrane proteins (OmpF, PhoE and TolC) in the data bank. Previously, Nikaido and Wu (1984) had examined amino acid homology between LamB, TolC, OmpF, OmpC, OmpA and PhoE. This revealed several regions of local homology that were statistically significant. In particular, one region of ten amino acid residues, located towards the amino-terminus of each protein, showed homology between all six outer membrane proteins. This was suggested to play a part in either directing outer membrane proteins to their ultimate destination or in their interaction with LPS molecules. However, computer assisted searches failed to locate such a sequence in the OmpT protein and no other significant homologies were found.

Reports have suggested that the OmpT protein has trypsin-like proteolytic activity (Fiss *et al.*, 1979; Grodberg and Dunn, 1988) and indeed it is inhibited by benzamidine and *p*-benzamidine which inhibit trypsin-like proteases. OmpT was therefore examined for the presence of an amino acid

sequence known to occur in the active site of mammalian and bacterial trypsin, these being GSDGGP and GDSGG respectively (Chin et al., 1988). The only amino acid sequence with any homology to this consensus was GSRGG encoded by nucleotide residues 742-759 of the DNA sequence presented in Figure 4 (a).

4.3 Summary of Results

In summary, this chapter has described the sequencing of the ompT gene which was found to be encoded by a 951 base pair ORF. The deduced amino acid sequence of the OmpT protein failed to show any homology to other outer membrane proteins. Upstream of the ompT coding region, a potential consensus sequence, which is present in the putative promoters of iron-regulated genes, was found. The significance of this has yet to be examined in other iron-regulated genes as well as in ompT.

Finally, a second ORF of 747 bp was located on the 3.1 kb PstI fragment with a potential coding capacity of 28.8 kd. However, a protein of this size was never labelled in maxi-cell experiments. One possibility is that the ORF encodes the so-called M5 polypeptide (25 kd) previously reported by Gordon et al. (1984).

Chapter Five

Construction of phoA-ompT gene fusions

5.1 Introduction

5.1.1 Aim of this work

Achieving a high-level expression of foreign proteins in *E. coli* using various expression vectors has become a much studied area of recombinant DNA technology. One of the aims of this work was to assess the possibility of using the *ompT* promoter and the *ompT* transcription and translation initiation signals to drive the expression of cloned heterologous genes in *E. coli*, ultimately those encoding eukaryotic proteins (e.g., human growth hormone). In addition, the ability of the OmpT signal sequence to direct heterologous polypeptides out of the cell cytoplasm was to be studied.

When producing foreign proteins in *E. coli* the advantages of exporting the protein of interest to the periplasm are several. Firstly, the amino-terminal amino acid of the secreted product can be identical to that of the natural product as a result of cleavage of the leader peptide. Secondly, proteolytic activities are considered to be much lower in the periplasmic space than in the cytoplasm thus stabilising the secreted gene product. Finally, many cloned gene products, particularly when synthesised in very large amounts, may be toxic to the cell in the cytoplasmic compartment (Ghrayeb *et al.*, 1984). A number of expression vectors which also direct the secretion of foreign proteins have been constructed. These have made use of *E. coli* signal

sequences from either periplasmic (Villa-Komaroff *et al.*, 1978) or outer membrane proteins (Takahara *et al.*, 1985).

As well as directing proteins from the cell cytoplasm, it is also advantageous in large-scale fermentations to be able to limit the expression of the cloned gene to the end of the growth phase of the culture. This has been achieved by the use of metabolically controlled promoters such as those of the *lac* (Ghrayeb *et al.*, 1984) and *trp* operons (Mizukami *et al.*, 1986). Temperature-controlled systems have also been utilised, particularly the powerful promoters obtained from the bacteriophage lambda, P_L and P_R, both controllable by the temperature-sensitive lambda repressor encoded by the gene cI_{857}. At temperatures above 37°C, the repressor protein is denatured and allows expression of cloned genes from the P_L and P_R promoters. Thus, cultures can be grown below 37°C to a high cell density before the temperature is increased to induce synthesis of the cloned gene product (Schauder *et al.*, 1987). The natural temperature-dependent synthesis of OmpT was a feature which could possibly be exploited to control the production of foreign proteins from an expression-secretion vector based on the *ompT* gene.

5.1.2 Approach

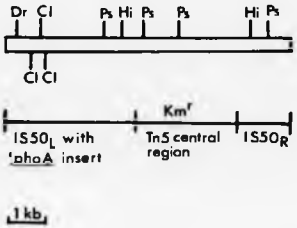
To assess the usefulness of the *ompT* gene in driving expression and export of cloned gene products, gene fusion technology was exploited. This involved the creation of hybrid genes between the cloned *ompT* gene and the gene

encoding alkaline phosphatase, *phoA*. Fusions were constructed such that expression of a chimeric gene resulted in the synthesis of a hybrid protein in which the amino-terminus consisted of OmpT while the carboxy-terminus comprised the PhoA protein. Since alkaline phosphatase is an easily assayable enzyme, the ability of the OmpT signal sequence to direct export of a heterologous protein could be examined by determining the location of the OmpT-PhoA chimera. In addition, the assay of alkaline phosphatase would enable the monitoring of gene expression at different temperatures to determine if the natural temperature-dependent synthesis of the OmpT protein was retained in the production of the fusion proteins.

To create such *phoA* fusions to the cloned *ompT* gene, the transposon Tn*phoA* was used (Manoil and Beckwith, 1985). This is a derivative of Tn5 and carries a *phoA* gene which has no promoter or signal sequence (Figure 5 a). When the transposon inserts into a gene in the correct orientation and reading frame, alkaline phosphatase becomes fused to the amino-terminus of the protein encoded by that gene. Alkaline phosphatase only becomes active, however, when it is fused to a protein that is exported from the cytoplasm. The transposon can be introduced into *E. coli* on a defective lambda transducing phage (Gutierrez *et al.*, 1987) and strains bearing fusions to exported proteins selected on medium which allows the detection of alkaline phosphatase

Figure 5 (a) Restriction map of transposon Tn*phoA*

Redrawn from Manoil and Beckwith (1985). All abbreviations for restriction enzymes have been listed earlier.



activity. Fusions can be made to genes located on the chromosome or to those cloned on plasmid vectors.

This system has been used successfully in the study of the regulation of gene expression (Gutierrez et al., 1986), in the analysis of protein export (Coulton et al., 1988; Hinton and Salmond, 1987) and in the examination of the topology of membrane proteins (Manoil and Beckwith, 1986).

5.2 Results

5.2.1 Isolation of ompT-phoA Gene Fusions

E. coli CC118 was transformed with plasmid pOMPT30 monomer. One Ap^r transformant was purified and the resulting strain was grown overnight in LB with Ap, 20% (w/v) maltose and 10 mM $MgCl_2$. 1 ml samples of the culture were then infected with lambda TnphoA (moi = 1). The mixtures were incubated at 30°C for 15 min to allow 'phage adsorption and infection. The infected cultures were then diluted 1:10 with fresh LB and incubated at 30°C for 4-6 hours with aeration. 200 µl samples were plated onto LB-agar containing Ap to select for pOMPT30, Km to select for the transposon (300 µg/ml) and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (40 µg/ml final concentration added from a stock solution made in dimethyl formamide) to detect strains with alkaline phosphatase activity. The latter appear blue on such medium while those strains in which TnphoA has not fused to an exported protein remain white. The high concentration of Km

used to isolate fusion strains, preferentially selects those in which the fusion is contained on a multicopy plasmid.

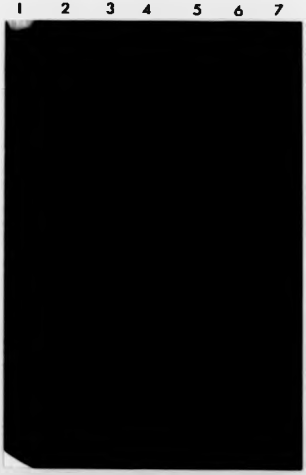
After incubation for 2 days at 37°C, the plates were examined. Km^r and Ap^r colonies arose at a frequency of 10^{-6} per pfu of lambda TnphoA. Blue fusion strains of various colour intensities appeared at a frequency of 2.8% and these colonies were purified. The plasmid DNA was prepared from each strain and was then used to transform E. coli CC118. Transformants were selected on the medium described above. One blue colony from each transformation was purified since these were assumed to contain a plasmid carrying a phoA gene fusion. Six fusion-bearing plasmids were then analysed by restriction mapping to determine the site of the TnphoA insertion. These were designated pOMPT2, pOMPT5, pOMPT6, pOMPT7, pOMPT9 and pOMPT10.

5.2.2 Defining the Site of the phoA Fusion

Each plasmid was digested with the enzyme PstI. This restriction endonuclease cuts the transposon TnphoA at four sites (Figure 5 a) and pOMPT30 at one site. Thus, digestion of the pOMPT30::TnphoA fusion plasmids with PstI gave rise to five fragments (Figure 5 b). The two largest fragments were assumed to contain the fusion joints between the plasmid and the transposon while the smaller fragments represented the internal PstI fragments from TnphoA (Figure 5 a). To ascertain which fragment contained the phoA fusion, the two largest PstI fragments from each plasmid were

Figure 5 (b) Agarose gel electrophoresis of the Tn*phoA*-insertion plasmids pOMPT10, pOMPT5, pOMPT2, pOMPT6, pOMPT9 and pOMPT7.

Samples were run on a 0.8% (w/v) agarose gel. Track 1 = lambda DNA digested with *Hin*dIII; tracks 2-7 = pOMPT2, pOMPT6, pOMPT9, pOMPT5, pOMPT10 and pOMPT7 respectively, digested with *Pst*I. In each case the smaller of the two largest *Pst*I fragments was later found to carry the *phoA* fusion.

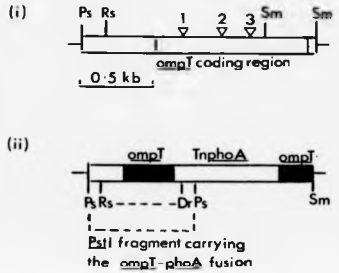


prepared by electroelution and ligated into the PstI site of pBR325 (Bolivar, 1978). The ligation mixtures were used to transform E. coli CC118 and transformants were selected on LB-agar containing Cm and BCIP. In each case, only recombinant plasmids carrying the smaller of the two PstI fragments gave rise to colonies with alkaline phosphatase activity and were, therefore, assumed to contain the phoA fusions. Each PstI fragment which carried the fusion, was then restriction mapped to determine the site of insertion of TnphoA using single and multiple restriction endonuclease digestions as described by Maniatis et al. (1982). The enzymes used were ClaI. DraI. RsaI and EcoRI. In each case, the restriction map indicated that the transposon had inserted into the cloned ompT gene and the position of TnphoA in the different fusion plasmids is shown in Figure 5 (c). Fusion plasmids pOMPT10 and pOMPT5 appeared to be very similar as did pOMPT2, pOMPT6 and pOMPT9, on the basis of this restriction mapping.

To define the exact site of fusion between ompT and the transposon, the RsaI-DraI DNA fragment (Figure 5 c), which contained the fusion joint, was sequenced as described in section 2.31 and the results are shown in Figure 5 (d). These indicate that indeed pOMPT9, pOMPT2 and pOMPT6 are indentical as are pOMPT10 and pOMPT5.

The high frequency at which certain fusions were isolated suggest that insertion of the transposon into the ompT gene

Figure 5 (c) Diagram to indicate the relative positions of insertion of Tn*phoA* in the cloned *ompT* gene.



(i) Position 1 (153) = pOMPT2, pOMPT6 and pOMPT9; position 2 (420) = pOMPT5 and pOMPT10; position 3 (590) = pOMPT7. The figures in parentheses represent the last bp of *ompT* present at the fusion site. Fusion joints between *ompT* and *phoA* were estimated from restriction mapping of the *PstI* fragment carrying the *phoA* fusion as represented in (ii).

Figure 5 (d) Sequence analysis of the insertion sites of TnphoA into ompT.

Plasmid	Sequence
pOMPT2	ATGCGGGCG----------AGCGGAAAAA^{133}A'CTGAC--
pOMPT6	M R A -38 amino acids---S G K T D--AP
pOMPT9	
pOMPT5	ATGCGGGCG----------CTGAATATC^{409}A'CTGAC--
pOMPT10	M R A -133 amino acids--L N I T D--AP
pOMPT7	ATGCGGGCG----------ATCGGCTAC^{577}A'CTGAC--
	M R A -188 amino acids--I G Y T D--AP

Sequences show the beginning of ompT and the insertion site of TnphoA in ompT (indicated by the vertical line). The last bp of ompT is underlined and numbered. The amino acid which is found at the OmpT-PhoA junction is underlined. AP: Alkaline phosphatase.

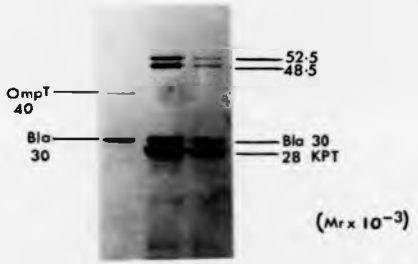
may not be random, although TnphoA has been reported to show a low sequence specificity of insertion (Manoil and Beckwith, 1986). Alternatively, the topological organisation of some fusion proteins might be lethal to the cell and therefore are not selected. No fusions were isolated which mapped in the last 122 amino acids of OmpT. This may have been due to the low alkaline phosphatase activity of such fusions as determined by their colour on medium containing BCIP in the initial selection. Indeed, colonies carrying pOMPT7, which contained the largest fusion, appeared a very pale blue on indicator medium.

5.2.3 Detection of Fusion Proteins

Initially, the maxi-cell labelling technique was used in an attempt to detect OmpT-PhoA fusion proteins. Each time the method was used, a 30 kd protein was detected, presumably beta-lactamase encoded by the vector, along with a 28 kd protein which was assumed to be kanamycin phosphotransferase, the latter being encoded by TnphoA. Two other proteins of 48.5 kd and 52.5 kd were also labelled in maxi-cell experiments and these were possibly encoded by the transposon (Rossetti et al., 1984; Rothstein and Reznikoff, 1981). As expected OmpT was no-longer labelled in such experiments but potential fusion proteins could not be detected either (Figure 5 e), indicating they could be unstable and possibly degraded before the labelling period was completed. Indeed, Bla-PhoA hybrid proteins have been

Figure 5 (a) Autoradiogram of ^{35}S-methionine labelled maxi-cell samples of pOMPT30, pOMPT9 and pOMPT10

Samples were analysed by SDS-PAGE using a 15% (w/v) linear gel. Track 1 = pOMPT30; track 2 = pOMPT9; track 3 = pOMPT10.



reported to be unstable and rapidly degraded to fragments of 47 kd, the size of mature alkaline phosphatase (Manoil and Beckwith, 1985) as have App-PhoA chimeras (Boquet et al., 1987). Labelling times were reduced (5 min) in an attempt to detect OmpT-PhoA hybrids but this was not successful.

Some fusion proteins could be detected, however, by probing Western blots of proteins extracted from fusion strains, with antibody specific for alkaline phosphatase (D. Gill, pers. comm.). This method identified potential OmpT-PhoA hybrids in extracts from three of the six strains analysed (Figure 5 f). These hybrids were encoded by pOMPT2, pOMPT6 and pOMPT9 which carried the smallest fusions. The size of the chimeric proteins which could be detected was in agreement with the size predicted from the point of insertion of the transposon assuming that the signal sequence was correctly cleaved from the hybrid protein when exported from the cell (Table 5 a). In addition to the largest protein band which was assumed to represent the OmpT-PhoA fusion, bands of lower molecular weight were also detected (Figure 5 f). These could possibly arise from proteolysis of the fusion protein. Such degradation has been noticed for Bla-PhoA chimeras by Hoffman and Wright (1985) who detected multiple protein bands below the position of the hybrid protein on Western blots.

5.2.4 Stability of the Fusion Plasmids

The stability of the fusion plasmids was examined after

Figure 5 (f) Identification of OmpT-PhoA fusion proteins

Cell extracts of strains carrying fusion plasmids were made and proteins of these extracts separated by electrophoresis on an 11% (w/v) polyacrylamide gel. The proteins were then blotted on to nitrocellulose filters and the OmpT-PhoA fusion proteins were detected with purified alkaline phosphatase antibodies (D. Gill, pers. commun.). Track 1 = CC118 (pOMPT30); track 2 = CC118 (pOMPT2); track 3 = CC118 (pOMPT5); track 4 = CC118 (pOMPT6); track 5 = CC118 (pOMPT7); track 6 = CC118 (pOMPT9); track 7 = CC118 (pOMPT10).

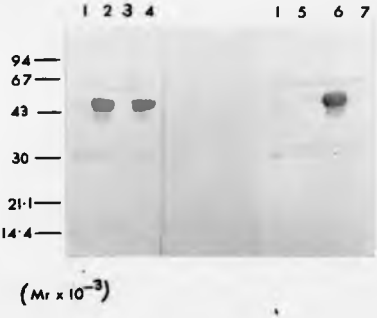


Table 5 (a) Size of OmpT-PhoA fusion proteins

Fusion plasmid	Size of hybrid protein (d)	
	Predicted*	Observed**
pOMPT2	49,750	49,500
pOMPT5	60,200	nd
pOMPT6	49,750	49,500
pOMPT7	66,250	nd
pOMPT9	49,750	49,500
pOMPT10	60,200	nd

* Calculated from the position of the Tn*phoA* insertion determined by DNA sequence analysis (Figure 5 d) and assuming processing of the signal peptide from OmpT-PhoA hybrid proteins (Mr of alkaline phosphatase = 47,000; average molecular weight of one amino acid residue = 110; Boquet *et al.*, 1985).

** Determined by SDS-PAGE (Figure 5 f).

nd = not detected.

growth of the fusion strains for 25 generations. The appropriate strain was grown overnight in LB containing Km and Ap. A 1:100 dilution of the culture was then made and 25 µl of this inoculated into 25 ml of fresh LB with Ap to maintain the plasmid. The culture was incubated at 37°C for 12 h. Dilutions of the culture were then made (10^{-6}-10^{-8}) and 0.1 ml aliquots of each dilution spread onto LB-agar with Ap and BCIP (40 µg/ml). After incubation overnight at 37°C, the plates were examined for any loss of alkaline phosphatase activity. White colonies were obtained at a high frequency with some of the clones (Table 5 b).

Of the strains which had lost alkaline phosphatase activity, the vast majority (>98%) were also no-longer resistant to Km. This indicated that either all or part of the Tn5 central region containing the Km^r gene (Figure 5 a) had been deleted from the transposon as well as the phoA fusion. In addition, approximately 50% of the colonies which had retained alkaline phosphatase activity were also sensitive to Km. HindIII restriction of the fusion plasmids carried by these strains indicated that the plasmids had lost the internal HindIII from the transposon (Figure 5 a) and therefore the gene encoding Km^r. The stabilty of the latter HindIII deletion plasmids was tested as described above. The alkaline phosphatase activity of these deletion derivatives was not lost as readily as in the parental fusion plasmid and white colonies were obtained at a much reduced frequency

Table 5 (b) Instability of alkaline phosphatase activity in CC118 strains carrying *ompT*-*phoA* gene fusions.

Fusion Plasmid	Strains carrying *ompT*-*phoA* plasmids		Strains with *ompT*-*phoA* (Δ*Hin*dIII) plasmids	
	blue colonies (%)	white colonies (%)	blue colonies (%)	white colonies (%)
OMPT2	74	26	93	7
OMPT6	54	46	88	12
OMPT9	97	3	100	0
OMPT10	100	0	-	-
OMPT5	100	0	-	-
OMPT7	100	0	-	-

(Table 5 b). Such instability has been reported previously for plasmids carrying pulA-phoA fusions (D'Enfert and Pugsley, 1987) and hlyA-phoA fusions (Erb et al., 1987). In each case the plasmids could be stabilised when most of the Tn5 part of TnphoA was removed as was found here.

5.2.5 Cellular Location of the OmpT-PhoA Fusion Proteins

Fractionation of the cells containing the ompT-phoA fusion plasmids was carried out in order to ascertain the location of the OmpT-PhoA chimeras. The CC118 fusion strain was grown in LB (10 ml with Ap and Km) at 37°C to an OD_{600} of 0.5 and then left on ice for 20 min before a final reading at OD_{600} was taken. A 5 ml sample of the culture was removed at this point and the cells disrupted in a French pressure cell (20,000 psi). Any unlysed cells were removed from the extract by centrifugation in a MSE Chilspin (10,000 rpm, 4°C, 10 min). Samples of the resulting cell extract were assayed for alkaline phosphatase (section 2.31.2) and represented the activity of the enzyme in the whole cell. The remaining 5 ml of culture was fractionated into periplasmic, cytosolic and membrane fractions.

Periplasmic proteins were isolated from the culture as described in section 2.26. Membrane and cytosolic fractions were then prepared from the 'shocked' cells as in section 2.27. Each fraction was then assayed for alkaline phosphatase activity (section 2.31.2). The purity of the periplasmic, cytosolic and membrane fractions was assessed

using beta-lactamase, glucose-6-phosphate dehydrogenase and NADH-oxidase, respectively.

The results (Table 5 c) indicated that most of the alkaline phosphatase activity of strains carrying the fusion proteins was found in the periplasmic fraction and was released by cold osmotic shock under conditions which left cytoplasmic proteins cell associated (as determined by the activity of glucose-6-phosphate dehydrogenase). This periplasmic location of alkaline phosphatase activity implies that the signal peptide which promotes OmpT export from the cytoplasm can also function to promote the export of a hybrid protein. The periplasmic location of the alkaline phosphatase activity also indicated that the fusion proteins did not become stably associated with the outer membrane, this being the normal cellular location of the OmpT protein. This may be due to a lack of sequences within the hybrid protein which normally allow OmpT to become associated with the outer membrane. Alternatively, the chimeric protein may not adopt the correct conformation needed to become integrated into the outer membrane. Larger amounts of alkaline phosphatase activity were associated with the membrane fraction in strains carrying pOMPT5, pOMPT7 and pOMPT10 compared to those carrying pOMPT2, pOMPT6 and pOMPT9. However, it was not determined whether this activity was associated with the cytoplasmic membrane or the outer membrane.

Table 5 (c) Sub-cellular fractionation of CC118 strains carrying *ompT*-*phoA* gene fusions

Fusion	Whole cell*	Periplasm**	Cytoplasm**	Membrane**
OMPT2	881	76.1	6.1	17.9
OMPT6	822	81.7	6.0	12.3
OMPT9	830	79.7	5.1	15.3
OMPT5	45	62.2	8.9	28.9
OMPT10	51	66.7	3.9	29.4
OMPT7	23	52.2	8.7	39.1

Fractionation was carried out on three separate occasions. Each time samples were assayed in triplicate from two separate cultures.

*Alkaline phosphatase activities are expressed as units/OD_{600}/ml. The activities are corrected for background from assays performed on CC118 (pOMPT30) which gave <2 units of alkaline phosphatase.

**Percentage of total alkaline phosphatase activity present in the fraction.

Over 85% of the total NADH-oxidase activity (section 2.31.4) was recovered in the membrane fraction.

Greater than 79% of beta-lactamase activity (section 2.31.3), encoded by pUC8, was associated with the periplasmic fraction.

Over 75% of glucose-6-phosphate dehydrogenase activity (section 2.31.5) was found in the cytosolic fraction.

5.2.6 Alkaline Phosphatase Activity of Fusion Strains

To determine if the synthesis of the OmpT-PhoA fusions was temperature-dependent, each fusion strain was cultured at both 30°C and 37°C to an OD_{600} of 0.5. The cultures were then treated as above (section 5.2.5) before being assayed for alkaline phosphatase activity (section 2.31.2). A strain harbouring a plasmid containing a bla-phoA gene fusion was also included in the experiments as a control and, in addition, the beta-lactamase activity of each strain was measured (section 2.31.3). Temperature would not be expected to affect the synthesis of this enzyme (Lundrigan and Earhart, 1984a). The results are presented in Table 5 (d).

These indicated that the level of synthesis of the OmpT-PhoA chimeras was dependent upon the growth temperature. The level of alkaline phosphatase activity was 5-6 fold higher in cells grown at 37°C compared to those cells cultured at 30°C. This increase does seem to reflect the pattern of OmpT production in E. coli as reported by Lutenberg et al. (1976). The absolute levels of alkaline phosphatase activity were dependent upon the point of TnphoA in the ompT gene. The closer the fusion to the amino-terminus of OmpT the higher the activity of alkaline phosphatase. This has been noted for other phoA fusions including those made to the appA (Boquet et al., 1987) and bla genes (Hoffman and Wright, 1985). This lower activity may be caused by the

Table 5 (d) Effect of temperature on the synthesis of OmpT-PhoA fusion proteins

Fusion plasmid	Alkaline phosphatase activity		Ratio 37:30
	30°C	37°C	
OMPT2	173	881	5.1
OMPT6	171	822	4.8
OMPT9	157	830	5.3
OMPT5	8	45	5.6
OMPT10	11	51	4.6
OMPT7	4	23	5.8
pCH2	910	1050	1.2

The assays were repeated on at least three separate occasions. Each time two cultures were grown for each strain and the alkaline phosphatase activity was assessed in triplicate on both cultures. The figures are corrected for background alkaline phosphatase activity from assays performed on CC118 (pOMPT30) which gave units of <2.

Alkaline phosphatase activity is expressed as units/OD_{600}/ml.

The beta-lactamase activity was assayed as in section 2.30.3. At 30°C and 37° the enzyme activity measured 12 units/ml of original culture and 13 units/ml of original culture, respectively. Ratio of activity, 37°:30°C = 1.1.

inefficient dimerisation of hybrid monomers in the larger fusion proteins.

Although the production of the OmpT-PhoA fusion proteins was increased at higher growth temperatures, this could reflect an increase in the overall rate of protein synthesis at 37°C. However, the synthesis of beta-lactamase and of a Bla-PhoA hybrid protein, increased only slightly at the higher temperature. This indicated that the higher levels of OmpT-PhoA protein production at 37°C was not accounted for solely by an increase in the overall rate of protein synthesis.

It was also possible that the copy number of the plasmid carrying the ompT-phoA increased at 37°C giving an increased gene dosage and, therefore, a higher production of OmpT-PhoA. However, the copy number of pUC8 is generally higher when cells are grown at lower temperatures due to decreased rates of growth (N. Minton, pers. commun.). One might also expect an increase in the synthesis of beta-lactamase if plasmid copy number was raised at 37°C, but this was not the case.

A further reason for the increase in alkaline phosphatase activity at 37°C could be that the hybrid proteins are more stable than at 30°C. This was not tested in the present study.

Finally, it is possible that the synthesis of the OmpT-PhoA fusion proteins reflects the normal pattern of OmpT

production which is reported in the literature (Lutenberg *et al*., 1976); in cells grown at 30oC OmpT is hardly detected compared to those cultured at 37°C. Considerable amounts of alkaline phosphatase activity were detected in some of the fusion strains (those carrying pOMPT9, pOMPT6 and pOMPT2) even when grown at 30°C. It may be that this reflects the normal expression of the *ompT* gene on the *E. coli* chromosome and that this effect is amplified when *ompT* is present on a high copy number plasmid. Indeed, the proteolytic activity of the OmpT protein has been detected at 30°C (Grodberg and Dunn, 1988) and Gayda *et al*. (1979b) reported that synthesis of OmpT did occur at 30°C but that the protein was rapidly degraded. Alternatively, it may be that the synthesis of OmpT is normally reduced at 30°C due to the presence of a repressor protein which inhibits some stage of OmpT production. When the *ompT* gene is cloned onto a high copy number vector the repressor may not be present in sufficient quantities to control OmpT synthesis. Ideally, to use the *ompT*-*phoA* fusions to study gene expression, they need to be present at low copy number to reflect more accurately the expression of *ompT* on the *E. coli* chromosome.

5.3 Conclusion

The work carried out to assess the ability of *ompT* to direct the synthesis of a heterologous protein, indicated that although *ompT* could perform this function, production of the cloned gene product still occurred at 30°C. In other

expression vectors the production of the cloned gene product is normally totally inhibited by either low temperatures or other metabolic controls until the cell density has reached a sufficiently high level. The OmpT signal sequence was able to direct the export of the PhoA protein to the periplasm of the cell and could be used in conjunction with a more tightly regulated promoter to promote the export of heterologous proteins in E. coli.

Chapter Six

Construction of ompT-lacZ operon and protein fusions

6.1 Introduction

6.1.1 Aim

Previous studies have shown that OmpT is found in very low amounts in cells grown at 30°C compared with those cultured at 37°C (Lutenberg *et al*., 1976; Manning and Reeves, 1977). Gayda *et al*. (1979b) suggested that this was due to altered processing of pro-OmpT at the lower temperature. A different control mechanism was proposed by Gordon *et al*. (1984) who postulated that the Shine-Dalgarno sequence and the AUG initiation codon, in the *ompT* message, were sequestered by a stable stem-loop structure resulting in a decrease in the synthesis of OmpT at 30°C. However, this seems unlikely considering the results of Chapter 4. The main aim of this work was to determine whether the temperature-dependent synthesis of the OmpT protein was regulated at the level of transcription, translation or at some post-translational stage. This involved the use of *lacZ* gene fusions.

6.1.2 Approach: The Use of lacZ Fusions

Gene fusion technology using *lacZ* has been widely employed in the analysis of basic biological problems, particularly in the study of the regulation of gene expression (Silhavy and Beckwith, 1985). Fusions are usually created such that the gene of interest controls the expression of *lacZ*. By assaying the activity of beta-galactosidase, therefore, the expression of the hybrid gene can be monitored which in turn reflects the activity of the exogenous promoter. A variety

of methods have been developed which allow one to fuse *lacZ* to the gene under investigation. These include *in vivo* systems which utilise the transposition functions of bacteriophage Mu to move the *lac* genes into the chromosome of the cell (Casabadan and Cohen, 1979). These functions have also been exploited in the construction of hybrid lambda p*lac*Mu 'phages which have allowed *lacZ* fusions to genes cloned onto high-copy number plasmids to be made (Bremer *et al*., 1984, 1985). In addition to this random, *in vivo* approach, many plasmid vectors have been developed for the creation of *lacZ* gene fusions. These allow one to construct defined fusions between the gene of interest and *lacZ* and they may be either single or multicopy vectors (Simons *et al*., 1987).

One of the attractive features of using the *lacZ* fusion system is the possibility of constructing both operon and protein fusions to the gene under study. In an operon fusion, the translational signals of the *lacZ* gene are intact and the expression of the *lac* genes is dependent only on the transcriptional signals of the gene to which it is attached. In protein fusions, however, both the transcriptional and translational signals of the *lacZ* gene have been replaced by those of the gene to which it is fused and a hybrid protein is produced as a result. If a regulatory mechanism is operating at a transcriptional level, then the expression of *lacZ* gene in both operon and

protein fusions will be affected. However, if regulation is occuring at the initiation of translation, only the protein fusion will be affected. This approach has been used to demonstrate that a gene coding for a ribosomal protein was regulated by autogenous control at the initiation of translation (Miura et al., 1981). In the case of the synthesis of gluconate-6-phosphate dehydrogenase, post-transcriptional regulation was shown by the use of protein and operon fusions (Baker and Wolfe, 1983). Recently, it has been shown that translation of a particular mRNA can be inhibited by pairing with a complementary RNA sequence. These include the translation of ompF mRNA (Mizuno et al., 1984) and the mRNA of Tn10 transposase (Simons and Kleckner, 1983). In both these cases, lac fusions were useful for demonstrating that regulation occurred at the level of translation. In view of this, both ompT-lacZ operon and protein fusions were created in order to determine if the temperature-dependent synthesis of OmpT was controlled at a transcriptional or at a post-transcriptional level. Both in vivo and in vitro approaches were taken to construct the fusions.

6.2 Results

6.2.1 Creation of ompT-lacZ Fusions: An In Vivo Approach

The hybrid bacteriophages lambda placMu3 and lambda placMu51 (Bremer et al., 1984, 1985) were used to produce protein and operon fusions, respectively, to the cloned ompT gene. High

titre, mixed lysates of lambda pMu507.3 and either placMu3 or placMu51 were prepared on the strain E. coli CSH26 (pOMPT30). The helper 'phage lambda pMu507.3 provides a functional Mu B gene whose product, although not essential for 'phage Mu transpositon, is necessary for Mu replication and stimulates the frequency of Mu transposition by 10-100 fold (Bremer et al., 1984). E. coli CSH26 (pOMPT30) was grown overnight in LB with 20% (w/v) maltose, 10 mM $MgCl_2$ and Ap. 1 ml samples of the culture were then infected with 0.1 ml of lambda pMu507.3 (10^8-10^9 pfu/ml) and 0.1 ml of either lambda placMu3 or lambda placMu51 (both at 10^8-10^9 pfu/ml). The infected cultures were left at 30°C for 30 min to allow 'phage adsorption. 5 ml of fresh LB were added to each infected culture and the cells centrifuged in a MSE Multex (5000 rpm, 5 min) to remove any unadsorbed 'phages. In each case, the supernatant was discarded and the cell pellet resuspended in a further 5 ml of LB, centrifuged again in a MSE Multex (5000 rpm, 5 min) and finally resuspended in 1 ml of LB. Dilutions of each infected culture were then made (10^{-1}-10^{-7}) in LB and 0.1 ml aliquots of each dilution mixed with 3 ml of soft DD agar. The mixtures were poured over DD-agar plates which were subsequently incubated at 37°C overnight. Mixed lysates were then prepared, from plates showing confluent lysis, as described in section 2.29.

To identify insertions of the lambda placMu 'phages into pOMPT30, specialised transducing 'phages carrying the entire plasmid were identified by their ability to transduce Ap^r. The mixed lysates prepared above were used to transduce E. coli CSH26 to Ap^r and to a Lac^+ phenotype. The strain was grown overnight in LB with 20% (w/v) maltose and 10 mM $MgCl_2$. 0.5 ml samples of the culture were infected with 50 µl aliquots of various dilutions (10^{-1}-10^{-7}) of the mixed lysates and incubated at 30°C for 30 min. 0.1 ml samples of each infected culture were then spread onto LB-agar containing Ap (125 µg/ml) and BCIG (20 µg/ml) and the plates incubated overnight at 37°C.

Subsequent examination of the plates revealed that Ap^r transductants were isolated at a frequency of 2×10^{-5}/pfu in the case of lambda placMu3 and at a frequency of 1×10^{-5}/pfu with lambda placMu51. Of the Ap^r strains, 4.3% and 2.3% were Lac^+ for lambda placMu51 and lambda placMu3, respectively. Protein fusions may have been isolated at a lower frequency than operon fusions because the former need to create an in-phase fusion to give rise to beta-galactosidase activity.

The Lac^+ strains were purified and the plasmid prepared from each. This was used to re-transform E. coli CSH26 in order to ensure that the lacZ fusion was contained on the plasmid. The plasmids were then analysed by restriction mapping using single and multiple restriction endonuclease digestions

(Maniatis *et al.*, 1982) to ascertain the position of insertion of the *lacZ* fusion. From ten promoter fusions analysed two mapped within the *ompT* gene and from twelve protein fusions examined only one mapped within *ompT*. Figure 6 (a) indicates the position of these fusions in the cloned *ompT* gene.

6.2.2 Creation of ompT-lacZ Fusions: An In Vitro Approach

Protein and operon fusions between the *ompT* gene and *lacZ* were also created by cloning defined fragments of *ompT* into plasmid vectors. The vectors used are listed below:

(i) pAA182. A multicopy vector used to create operon fusions.

(ii) pNM481. A multicopy plasmid used to make protein fusions.

(iii) pDIA15. A low copy number plasmid for creating operon fusions.

(iv) pDIA16. A low copy number vector for making protein fusions.

The construction of each *ompT-lacZ* fusion using the above vectors is described below.

6.2.2.1 Fusions in pAA182

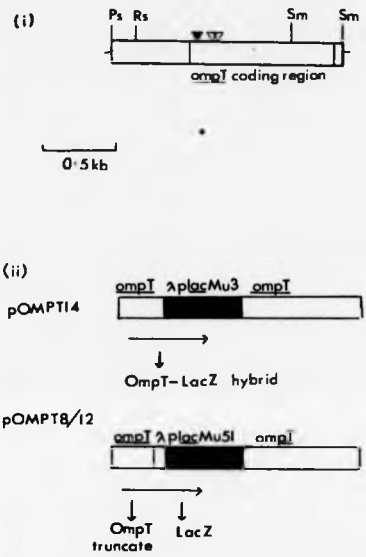
For creation of an operon fusion in pAA182 (Jayaraman *et al.*, 1987), the 1 kb *Rsa*I-*Sma*I DNA fragment from the *ompT* gene (Figure 6 b) was prepared from pOMPT21 by digesting with the restriction endonucleases *Rsa*I and *Sma*I. Following electrophoresis on a 0.8% (w/v) agarose gel, the appropriate

Figure 6 (a) Positions of lambda placMu3 and lambda placMu51 into the ompT gene carried by pOMPT30

(i) The position of fusion between ompT and lambda placMu3 (▲) and ompT and lambda placMu51 (△) are indicated. These were ascertained by restriction mapping of the fusion plasmids with the restriction endonucleases PstI. BamHI. SacI. HindIII. SmaI and EcoRI used in single and multiple digestions (Maniatis et al., 1982). The two operon fusions made by lambda placMu51 insertion were designated pOMPT8 and pOMPT12 and mapped at base pair 138 and 175 of the ompT coding sequence, respectively. The protein fusion made by lambda placMu3 was estimated to map at bp 45 of the ompT coding region and the fusion plasmid was designated pOMPT14.

(ii) The proteins synthesised from these fusions are shown. In the case of the protein fusion (pOMPT14), an OmpT-LacZ hybrid is produced while the operon fusions (pOMPT12 and pOMPT8) give rise to native LacZ.

A bla-lacZ operon fusion and a bla-lacZ protein fusion were obtained in pBR325 using the appropriate 'phage and were designated pBLA1 and pBLA6 respectively. These were isolated as for the fusions in pOMPT30 but insertions into the plasmid were selected on medium containing Cm.


(i)
Ps Rs
Sm Sm
ompT coding region
0·5kb
(ii)
ompT λplacMu3 ompT
pOMPT14
OmpT–LacZ hybrid
pOMPT8/12
ompT λplacMu51 ompT
OmpT truncate
LacZ

fragment was isolated by electroelution. The fragment was then ligated into the SmaI site of pAA182. The ligation mixture was used to transform the lac-deleted strain E. coli CSH26. Transformants were selected on LB-agar containing Ap and BCIG (20 μg/ml) to select the plasmid and to detect beta-galactosidase activity, respectively. Lac^+ colonies arising from the transformation were purified and checked for presence of the correct insert in the appropriate orientation for expression of the lac genes. One plasmid carrying the 1 kb RsaI-SmaI insert was designated pEH5 and this is shown in Figure 6 (b).

6.2.2.2 Fusions in pDIA15

In order to make an operon fusion in pDIA15 (De Reuse et al., 1986), the 1 kb RsaI-SmaI fragment from the ompT gene (Figure 6 b) was prepared from pOMPT21 as described above The fragment was initially cloned into the SmaI site of pMTL24 (N. Minton pers. commun.; Chambers et al., 1988).This enabled the insert to be re-isolated on an EcoR1 fragment of DNA which could then be ligated into the EcoRI site of pDIA15. Following ligation of the EcoRI fragment into pDIA15, the ligation mixture was used to transform strain E. coli CSH26. Transformants were selected on LB-agar containing Km (25 μg/ml) to select for the plasmid and BCIG (20 μg/ml) to indicate beta-galactosidase activity. Again Lac^+ colonies were purified and checked for the presence of the correct insert. One plasmid containing the insert was

Figure 6 (b) Construction of *ompT*-*lacZ* operon fusions using pAA182 and pDIA15

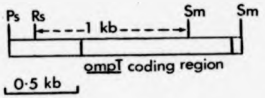
(i) 1.5 kb *Pst*I-*Sma*I fragment showing the position of the 1 kb *Rsa*I-*Sma*I fragment used to construct *ompT*-*lacZ* operon fusions.

(ii) EH5 = pAA182 with the 1 kb *Rsa*I-*Sma*I fragment cloned into the *Sma*I site (Rs/Sm = *Rsa*I-*Sma*I junction).

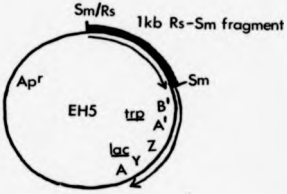
(iii) pDIA156 = pDIA15 containing the 1 kb *Rsa*I-*Sma*I fragment. The latter was firstly cloned into the *Sma*I site of pMTL24 in order to re-isolate the fragment with *Eco*RI sticky ends.

The arrows indicate the direction of transcription.

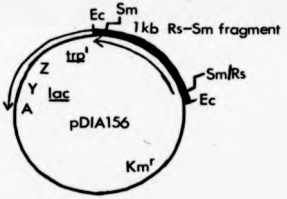
(i)


Ps
Rs
1 kb
Sm
Sm
ompT coding region
0·5 kb

(ii)


Sm/Rs
1kb Rs–Sm fragment
Ap^r
Sm
EH5
trp
B'
A'
lac
Z
Y
A

(iii)


Ec
Sm
1kb Rs–Sm fragment
trp'
Z
Y
A
lac
Sm/Rs
Ec
pDIA156
Km^r

designated pDIA156 (Figure 6 b) and was used throughout this study.

6.2.2.3 Fusions in pNM481

OmpT-LacZ protein fusions were made by cloning the 1 kb RsaI-SmaI fragment (Figure 6 c) of ompT into the SmaI site of pNM481 (Minton, 1984). If the fragment is cloned in the correct orientation with respect to lacZ, an in-phase fusion would be formed between ompT and the lacZ gene. After ligation of the RsaI-SmaI fragment into the SmaI site of pNM481, recombinant plasmids containing a lacZ fusion were isolated by transforming E. coli CSH26 with the ligation mixtures and selecting transformants on LB-agar with Ap and BCIG (20 µg/ml). Lac^+ colonies were purified and the fusion plasmids checked for the presence of the 1 kb RsaI-SmaI insert. One such plasmid was designated pEH10 and is shown in Figure 6 (c).

6.2.2.4 Fusions in pDIA16

OmpT-LacZ protein fusions were constructed by again using the 1 kb RsaI-SmaI fragment from the ompT gene (Figure 6 c) cloned into pDIA16. Initially, the fragment was cloned into the SmaI site of pMTL20 (N. Minton, pers. commun.; Chambers et al., 1988) which allowed the fragment to be re-isolated on a piece of DNA with EcoRI and BamHI sticky ends. The latter fragment was then cloned into the BamHI-EcoRI site of pDIA16 to create an in-phase fusion between ompT and lacZ (Figure 6 c). The ligation mixtures were used to transform

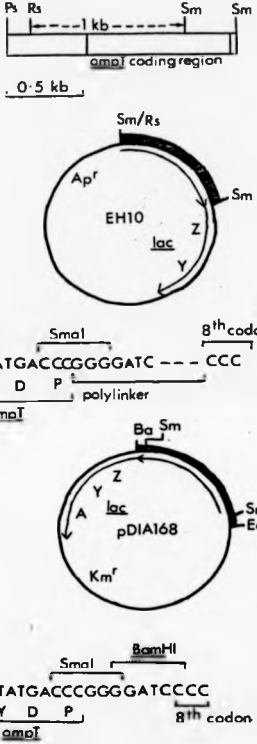
Figure 6 (c) Construction of *ompT*-*lacZ* protein fusions using pNM481 and pDIA16

(i) 1.5 kb *Pst*I-*Sma*I fragment showing the position of the 1 kb *Rsa*I-*Sma*I fragment used to construct *ompT*-*lacZ* protein fusions

(ii) EH10 = pNM481 with the 1 kb *Rsa*I-*Sma*I fragment cloned into the *Sma*I site to create an in-phase *ompT*-*lacZ* fusion (Rs/Sm = *Rsa*I-*Sma*I junction)

(iii) pDIA168 = pDIA16 containing the 1 kb *Rsa*I-*Sma*I fragment. The latter was initially cloned into the *Sma*I site of pMTL20 in order to re-isolate the fragment on a piece of DNA with a *Bam*HI and an *Eco*RI sticky end. This enabled the fragment to be cloned into the *Bam*HI-*Eco*RI site of pDIA16 to create an in-phase *ompT*-*lacZ* fusion.

The arrows indicate the direction of transcription.


Ps Rs
1 kb
Sm
Sm
amaT coding region
0·5 kb
Sm/Rs
Ap^r
Sm
EH10
Z
lac
Y
SmaI
8th codon of lacZ
TATGACCCGGGGATC --- CCC
Y D P
polylinker
amaT
Ba Sm
Z
Y
A
lac
pDIA168
Sm/Rs
Ec
Km^r
BamHI
SmaI
TATGACCCGGGGATCCCC
Y D P
amaT
8th codon of lacZ

E. coli CSH26 and transformants were selected on LB-agar containing Km (25 μg/ml) and BCIG (20 μg/ml). Lac^+ colonies were purified and checked for the presence of the correct insert of DNA. One such plasmid was designated pDIA168 (Figure 6 c).

6.2.3 Beta-galactosidase Assays of the Fusion Strains

Each fusion strain was grown to an OD_{600} of 0.5 at either 37°C or 30°C in LB containing the appropriate antibiotics for the selection of the fusion plasmid. The cultures were then placed on ice for 20 min before the OD_{600} was finally checked. A cell extract of each culture was then made using a French pressure cell set at 20,000 psi. Unlysed cells were removed from the extracts by centrifugation in a MSE Chilspin (10 min, 10,000 rpm, 4°C) and then samples of each extract used in the beta-galactosidase assay as described in section 2.31.1. Beta-galactosidase activities were calculated and expressed as units/OD_{600}/ml. All assays were performed in triplicate on separate cultures and on at least two separate occasions to ensure consistency in the assay. The results are presented in Table 6 (a) and Table 6 (b) for the *ompT-lacZ* operon and protein fusions respectively.

6.3 Discussion

6.3.1 Summary of Results

The strains carrying *ompT-lacZ* protein fusions displayed lower beta-galatosidase activities than those carrying *ompT-*

Table 6 (a) Beta-galactosidase assays of *ompT-lacZ* operon fusions measured at 37°C and 30°C

Operon Fusion	Beta-galactosidase Activity 37°C	30°C	37°C:30°C
pOMPT30	4.0	3.8	1.1
pOMPT12	2044	2025	1.0
pAA182	21	20	1.2
pEH5	400	389	1.0
pDIA15	1.4	1.2	1.2
pDIA156	104	98	1.1
pBLA1	2123	2113	1.0

Beta-galactosidase activity = units/OD_{600}/ml.

pBLA1 = pBR325::λ*plac*Mu51.

pOMPT12 = pOMPT30::λ*plac*Mu51.

Where possible, the beta-lactamase activity of each strain carrying the *ompT-lacZ* fusion plasmids was also assayed. The ratio of activity of beta-lactamase activity at 37°C:30°C was found to be approximately 1 in each case.

Table 6 (b) Beta-galactosidase assays of *ompT-lacZ* protein fusions measured at 37°C and 30°C

Protein Fusion	Beta-galactosidase Activity 37°C	30°C	37°C:30°C
pOMPT30	4.0	3.8	1.1
pOMPT14	298	61	4.9
pNM481	1.9	1.3	1.5
pEH10	236	46	5.1
pDIA16	2.0	1.8	1.1
pDIA168	70	12	5.8
pBLA6	389	372	1.0

Beta-galactosidase activity = units/OD_{600}/ml.

pOMPT14 = pOMPT30::λ*plac*Mu3

pBLA6 = pBR325::λ*plac*Mu3

Where possible, the beta-lactamase activity of each strain carrying the *ompT-lacZ* fusion plasmids was also assayed. The ratio of activity of beta-lactamase activity at 37°C:30°C was found to be approximately 1 in each case.

lacZ operon fusions. This may be due to inefficient tetramerisation of the hybrid monomers in the chimeric OmpT-LacZ proteins. The difference in the relative values of beta-galactosidase activity in strains containing *ompT-lacZ* operon fusions is probably an effect caused by the copy number of the vector on which the fusion is carried. This may also be true in the case of the *ompT-lacZ* protein fusions. In addition, it is also possible that the site of fusion between *lacZ* and the *ompT* gene affects the relative activity of beta-galactosidase. Indeed, those fusions occurring close to the amino-terminus of the OmpT protein (e.g., pOMPT14) result in a higher beta-galactosidase activity than those located towards the carboxy-terminus (e.g., EH10). This could again reflect inefficient tetramerisation of OmpT-LacZ monomers in the larger fusion proteins.

The results indicated that the level of beta-galactosidase activity did not vary significantly with growth temperature in any of the strains carrying *ompT-lacZ* operon fusions. This suggested that the temperature-dependent synthesis of the OmpT protein was mediated by a post-transcriptional mechanism. Indeed, the yield of beta-galactosidase from the *ompT-lacZ* protein fusions did reflect this in that the activity of the enzyme in cells grown at 37°C was approximately five times that found in cells cultured at 30°C. The increase in the production of the OmpT-LacZ

chimeras at 37°C appeared to reflect the normal pattern of synthesis of the OmpT protein and this effect was apparent in both the low and high copy number vectors used. The low copy number vectors were used to avoid possible complications due to gene dosage effects when multicopy plasmids are employed to study gene expression. At 30°C, OmpT-LacZ fusions were still synthesised even from the low copy number plasmid pDIA168. This indicated that OmpT is probably synthesised at low temperatures albeit at a much reduced level. In contrast to the effects of temperature upon the production of OmpT-LacZ fusions, the levels of beta-galactosidase produced from a bla-lacZ appeared not to be dependent upon this parameter. In addition, the levels of beta-lactamase expressed from some of the vectors used, were not increased at 37°C relative to 30°C.

6.3.2 What could cause the increase of OmpT synthesis at 37°C?

If the growth temperature-dependent synthesis of OmpT is indeed mediated by a post-transcriptional mechanism, what control models could cause this effect?

It is possible that at 30°C stable secondary structures form within ompT mRNA which serve to decrease translation at low temperatures. However, examination of the ompT DNA sequence (Figure 4 a) encompassing the ribosome-binding site (449-452) and the ATG start codon (460-462), did not reveal

sequences which could form stable stem-loop structures in *ompT* mRNA and so reduce translation at 30°C. In addition, there did not appear to be any sequences upstream of the *ompT*-*lacZ* fusion joint in pOMPT14 which could potentially form stable secondary structures in *ompT* mRNA, yet expression of OmpT-LacZ hybrids from this plasmid was temperature-dependent.

It is also possible that the synthesis of OmpT is dependent upon the growth-rate of the culture which is itself affected by temperature. Other genes whose expression is growth-rate dependent include the *rrnE* operon (Miura *et al.*, 1981) and the *gnd* (6-phosphogluconate dehydrogenase) gene (Baker and Wolfe, 1983). In both these cases gene expression is regulated at a post-transcriptional stage as revealed by the analysis of *lacZ* fusions. At higher growth rates, the half-life of the *ompT* mRNA could be increased leading to a greater yield of OmpT. However, factors leading to the stabilisation of *ompT* mRNA at 37°C would have to act at the 5' end of the message since the OmpT-LacZ fusions are also produced in higher amounts at this temperature.

A further possibility is that translation of *ompT* mRNA is inhibited at low temperatures by a putative translational repressor but no evidence for this has been reported.

To define the mechanism responsible for controlling the temperature-dependent production of the OmpT protein, it

would be advantageous to isolate mutants which no-longer display this regulatory pattern but none were isolated in the present study.

Chapter Seven

Effect of envZ on the expression of ompT

7.1 Introduction

The *ompB* region of the *E. coli* chromosome includes two genes, *ompR* and *envZ*, which are responsible for the osmolarity-sensitive biosynthetic regulation of the outer membrane porin proteins OmpF and OmpC (Hall and Silhavy, 1981a, 1981b). A number of missense mutations in the *envZ* locus (e.g. *envZ473*, *perA*, *tpo*) have been isolated by several laboratories. Strains bearing these mutant alleles are phenotypically $OmpF^-$, $OmpC^+$ and are also depressed for the synthesis of several exported proteins. These include MalE, LamB, PhoE, PhoA, the *cpr* protease, OmpT and several iron-regulated proteins (Wanner *et al.*, 1979; Wandersman *et al.*, 1980; Lundrigan and Earhart, 1981; Cavard *et al.*, 1982). Garrett *et al.* (1983) reported that this effect was the result of decreased transcription of the respective gene in the case of PhoA, LamB and MalE. In contrast, *envZ* amber mutations (e.g., *envZ22*) had no noticeable pleiotropic effects on the expression of *phoA*, *lamB* and *malE* (Garrett *et al.*, 1983). Such results suggested that the pleiotropic effect of the *envZ473* mutation was due to an aberrant EnvZ protein rather than reflecting a normal function for the protein. However, it has been demonstrated that EnvZ, along with the OmpR protein, is required for the normal expression of the *tppB* gene of *S. typhimurium* which encodes the tripeptide permease (Gibson *et al.*, 1987). In this case OmpR and EnvZ do act as pleiotropic regulatory proteins. Recent work has shown that the pleiotropic effects of the *envZ473*

mutation are mediated through the OmpR protein (Slauch et al., 1988). OmpR is thought to affect gene expression through the alpha subunit of RNA polymerase (Slauch et al., 1988) since envZ473 mutations can be suppressed by mutations which map in the gene (rpoA) encoding the alpha subunit of this enzyme (Garrett and Silhavy, 1987).

As mentioned above, the envZ473 allele reduces the synthesis of the OmpT protein (Lundrigan and Earhart, 1981). It was the aim of this work to establish at which level (e.g., transcription, translation) the expression of ompT was affected by mutations in envZ. This was assessed by using the ompT-phoA and ompT-lacZ fusions created in Chapters 5 and 6 respectively. Initially this required the construction of appropriate envZ mutants in which the fusion plasmids could be stably maintained and which provided either a lacZ or a phoA background as required.

7.2 Results

7.2.1 Construction of E. coli Strains EK23 and EK45

E. coli SG139 (lacZ, envZ473, malA::Tn10) and CLG115 (lacZ, envZ22, malA::Tn10) needed to be recA in order to stably maintain the ompT-lacZ fusion plasmids constructed in Chapter 6. The method used in this study to obtain recA derivatives (Lorence and Rupert, 1983) required that the strains be sensitive to tetracycline. Initially, therefore, malA$^+$ derivatives of both strains were isolated to remove

the malA::Tn10 allele. This was achieved by preparing a transducing P1^{vir} lysate on E. coli C600 and using the resulting lysate to transduce both SG139 and CLG115 to malA^{+} (section 2.30). Transductants were selected on MM containing 20% (w/v) maltose and amino acids needed to support the growth of both strains. Mal^{+} colonies arose on the selective medium at a frequency of $1x10^{-6}$ for SG139 and $2x10^{-6}$ for CLG115 per pfu of P1^{vir} and these colonies were purified. Strains were subsequently screened for sensitivity to tetracycline which would indicate loss of Tn10 and their sensitivity pattern to bacteriophage K20 (OmpF specific) and hy2 (OmpC specific) was tested (Gutierrez et al., 1987). These 'phage enable one to check if the envZ allele has been retained in the malA^{+} transductants by comparing their sensitivity/resistance pattern with that of the parental strains SG139 and CLG115. This is tested by cross-streaking the strains against the 'phage on TYE medium and incubating the plates at 37^{o}C overnight (C. Gutierrez, pers. commun.).

The results of the cross-streaking experiments are summarised in Table 7 (a) and these show that strains designated EK23 and EK45 are derivatives of CLG115 and SG139 respectively, which have lost Tn10, are malA^{+} and possess the appropriate envZ allele. Strains EK23 and EK45 were, therefore, made recA using the method of Lorence and Rupert (1983) before being transformed with plasmids carrying ompT-lacZ fusions (Chapter 6) as listed in Table 7 (c). In

Table 7 (a) Isolation of strains EK23 and EK45.

Strain	Relevant Genotype	Resistance to	
		hy2	K20
CLG115	*lacZ*, *malA*::Tn*10*, *envZ22*	+	+/-
SG139	*lacZ*, *malA*::Tn*10*, *envZ473*	-	+
SG137	*lacZ*, *malA*::Tn*10*, *ompR101*	+	+
MC4100	*lacZ*. *malA*$^+$, *ompR*$^+$, *envZ*$^+$. *recA*	-	-
EK23	*lacZ*. *malA*$^+$. *envZ22*. *recA*	+	-
EK45	*lacZ*. *malA*$^+$. *envZ473*. *recA*	-	+

- = sensitive; + = resistant

addition, strain MC4100 was made recA using the same method. This would serve as a strain with an envZ^{+} background.

7.2.2 Construction of Strains EK141 and EK211

In order to assess the effect of envZ upon the expression of ompT-phoA fusions, envZ22 and envZ473 derivatives of strain MPH2 (phoA) were constructed by P1 transduction (C. Guttierez, pers. commun.). Transposon Tn10 in the malA gene of SG139 and CLG115 provides a selectable marker which is ca 50% linked by P1 transduction to the ompB locus (envZ and ompR; Garrett et al., 1983). Transducing 'phage lysates were therefore prepared by growing P1^{vir} on strains SG139 and CLG115 (section 2.30). The lysates were used to transduce MPH2 to Tc^{r}, transductants being selected on LB-agar containing Tc (section 2.30). Colonies arose on this medium at a frequency of $1x10^{-5}$ per pfu of P1^{vir} and these were purified and then screened for the presence of malA on MM with and without maltose (20% (w/v)). All Tc^{r} strains tested were unable to grow on MM without maltose and were therefore assumed to contain the malA::Tn10 allele. Cross-streaking against 'phage K20 and hy2 was then used to screen malA derivatives for the presence of either envZ22 or envZ473 (section 7.2.1). The results of the cross-streaking experiments indicated that the envZ22 and envZ473 alleles were co-transduced with malA::Tn10 at frequencies of 41% and 50% respectively. The results are summarised in Table 7 (b) and indicate that strains EK14 and EK21 are derivatives of

Table 7 (b) Isolation of strains EK141 and EK211

Strain	Relevant Genotype	Resistance to	
		hy2	K20
CLG115	lacZ, malA::Tn10, envZ22	+	+/-
SG139	lacZ, malA::Tn10, envZ473	-	+
SG137	lacZ, malA::Tn10, ompR101	+	+
MPH2	phoA, malA$^+$, ompR$^+$, envZ$^+$, recA	-	-
EK14	phoA, malA::Tn10, envZ22	+	+/-
EK21	phoA, malA::Tn10, envZ473	-	+
EK141	phoA, malA$^+$, envZ22, recA	+	-
EK211	phoA, malA$^+$, envZ473, recA	-	+

- = sensitive; + = resistant

MPH2 which are malA::Tn10 and envZ22 and envZ473 respectively.

The transposon Tn10 was removed from strains EK14 and EK21 as described in section 7.2.1 so that each strain could be made recA. A malA$^+$ derivative retaining envZ22 after transduction was designated EK141 while one retaining envZ473 was designated EK211. Both strains were then made recA by the method of Lorence and Rupert (1983) before transformation with the ompT-phoA fusion plasmids isolated in Chapter 5. Strain MPH2 was also made recA so that expression of the ompT-phoA could be measured in an envZ$^+$ background.

7.2.3 Effect of envZ on ompT-lacZ expression

Strains EK23, EK45 and MC4100 were transformed with the ompT-lacZ low-copy number fusion plasmids, pDIA156 and pDIA168, previously isolated in Chapter 6. Transformants were selected on LB-agar containing Km (25 $\mu g ml^{-1}$) and BCIG (20 $\mu g ml^{-1}$) to detect beta-galactosidase activity. The envZ strains were much paler in colour than MC4100 which contained the envZ$^+$ allele, indicating a lower production of the enzyme from the fusion plasmid. The parental plasmids pDIA15 and pDIA16, used to create the fusions, were also used to transform the three strains for use in control experiments. Once the necessary strains had been selected and purified (Table 7 c), they were assayed for beta-galactosidase exactly as described in section 6.2.3 at both

30°C and 37°C. The results of the assays are presented in Table 7 (c).

7.2.4 Effect of envZ on ompT-phoA expression

Strains EK141 and EK211 were transformed with the ompT-phoA plasmid pOMPT9 isolated in Chapter 5. Transformants were selected on LB-agar containing BCIP (20 $\mu g ml^{-1}$), Km (300 µg/ml) and Ap. One transformant for each strain was purified. In addition, EK141 and EK211 were transformed with pOMPT30 for use in control experiments. Strain MPH2 was also transformed with pOMPT30 and pOMPT9. These derivatives would allow a comparison between ompT-phoA expression in an envZ$^+$ strain with that in an envZ strain to be made. After isolation of the necessary strains, each was assayed for alkaline phosphatase activity as described previously in section 5.2.6 at 30°C and 37°C. The results are summarised in Table 7 (d).

7.3 Discussion

The results indicated that only the envZ473 mutation affected the expression of both ompT-phoA and ompT-lacZ fusions. This mutation reduced the alkaline phosphatase activity of strains carrying pOMPT9 by approximately nine fold compared to the parental envZ$^+$ strain. This decrease occurred even though the ompT-phoA fusions were cloned onto a high copy number plasmid. The decrease in activity was apparent whether the cells were cultured at 30°C or 37°C.

Table 7 (c) Beta-galactosidase activity of strains containing *ompT-lacZ* fusion plasmids

Strain		Plasmid	Beta-galactosidase	
			30°C	37°C
EK23	(*envZ22*)	pDIA15	1.3	1.6
EK23	(*envZ22*)	pDIA156	95	101
EK45	(*envZ473*)	pDIA15	1.4	1.6
EK45	(*envZ473*)	pDIA156	9.3	10.5
MC4100	(*envZ*$^+$)	pDIA15	1.3	1.7
MC4100	(*envZ*$^+$)	pDIA156	90	102
EK23	(*envZ22*)	pDIA16	1.8	2.0
EK23	(*envZ22*)	pDIA168	13	72
EK45	(*envZ473*)	pDIA16	1.3	1.5
EK45	(*envZ473*)	pDIA168	1.8	9
MC4100	(*envZ*$^+$)	pDIA16	1.3	1.6
MC4100	(*envZ*$^+$)	pDIA168	9.5	53

Beta-galactosidase activity = units/OD_{600}/ml.

Table 7 (d) Alkaline phosphatase activity of strains containing *ompT-phoA* fusion plasmid pOMPT9

Strain	Plasmid	Alkaline phosphatase	
		30°C	37°C
EK141 (*envZ22*)	pOMPT30	2	2
EK141 (*envZ22*)	pOMPT9	152	827
EK211 (*envZ473*)	pOMPT30	2	3
EK211 (*envZ473*)	pOMPT9	18	97
MPH2 (*envZ*$^+$)	pOMPT30	3	2
MPH2 (*envZ*$^+$)	pOMPT9	146	836

Alkaline phosphatase activity = units/OD_{600}/ml.

Such a result indicated that the EnvZ protein did not control the temperature-dependent synthesis of OmpT since in *envZ* mutants the expression of *ompT*-*phoA* fusions was still affected by temperature.

The *envZ473* also reduced the beta-galactosidase activity of strains carrying either *ompT*-*lacZ* operon or *ompT*-*lacZ* protein fusions by up to nine fold. This indicated that the mutation had its effect at the transcriptional level. Such a result is in agreement with Garrett *et al*. (1983) who reported that the effect of the *envZ473* mutation on *phoA*, *malE* and *lamB* was also at the transcriptional level. However, the result is contrary to the suggestion by Gordon *et al*. (1984) that the product of the *perA* gene acted at a post-transcriptional stage by activating the translation of *ompT* mRNA. The *envZ473* mutation did not affect the temperature-dependent synthesis of beta-galactosidase in strains carrying *ompT*-*lacZ* protein fusions. An increased beta-galactosidase activity was still apparent in strains grown at 37^{o}C compared to those cultured at 30^{o}C.

It would have been interesting to investigate the effect of *rpoA* mutations on *envZ473* with regard to *ompT* expression and to see if the effect of *envZ473* was mediated by the OmpR protein.

Chapter Eight

General Discussion

8.1 A Summary of the Major Results

The major aims of this project were to clone and sequence the *ompT* gene of *E. coli* and analyse the effect of temperature upon its expression. In addition, it was of interest to determine if the *ompT* promoter and OmpT signal sequence could direct the expression and export of heterologous proteins in *E. coli* and, in doing so, retain the natural temperature-dependent synthesis of the OmpT protein. The effect of *envZ* on the expression of *ompT* was also to be examined. The main results gained from this work in relation to the factors mentioned, are summarised below.

(i) The *ompT* gene was successfully cloned from *E. coli* using an oligonuleotide probe specific for *ompT* (Chapter 3). Differences were apparent from previous reports concerning the cloning of *ompT*. In particular, the 4.3 kb *Eco*RI fragment on which the gene was cloned, failed to suppress the over production of capsular polysaccharide in an *E. coli lon* (*capR*) strain (section 3.2.5). Suppression of this phenotype had previously been used to identify recombinant plasmids carrying *ompT* (Berg *et al.*, 1976). The 4.3 kb *Eco*RI fragment was subcloned and the *ompT* gene found to lie within a 1.5 kb *Pst*I-*Sma*I fragment of DNA which apparently included the *ompT* promoter (section 3.2.6).

(ii) Sequencing of the *ompT* gene (Chapter 4) gave rise to some interesting results that were contrary to a previous publication which reported the DNA sequence of the *ompT* promoter and part of the *ompT* gene encoding the OmpT signal peptide (Gordon *et al.*, 1984). Studies carried out here indicated that when these published sequences were deleted from the 1.5 kb *Pst*I-*Sma*I fragment carrying *ompT*, the OmpT protein was still expressed in maxi-cells, apparently from its own promoter (section 4.2.2).

The 1.5 kb *Pst*I-*Sma*I DNA fragment carried an ORF of 951 bp (section 4.2.2). The latter has the capacity to encode a 35.6 kd protein (section 4.2.1). However, published information described OmpT as a 40 kd protein which was initially synthesised as a pro-protein with a 2 kd signal peptide. Indeed, the product of the *ompT* gene cloned in this study was 40 kd as determined by SDS-PAGE (section 3.2.6). Despite this, the OmpT protein was apparently encoded by the 951 bp ORF (section 4.2.2). Anomalous molecular weights estimated from SDS-polyacrylamide gels have been reported before. In the case of PhoE, for example, the Mr estimated by SDS-PAGE is 40,000 while that predicted from its deduced amino acid sequence is 38.8 kd (Overbeeke *et al.*, 1983).

Analysis of the DNA sequence of the cloned *ompT* gene allowed the identification of potential promoter consensus sequences, a Shine-Dalgarno sequence and a possible transcriptional terminator. Of particular interest was the homology found in the promoter region of *ompT* with that of the promoter regions of genes expressed during growth in iron-limited medium. OmpT has been reported to be synthesised in greater amounts when *E. coli* is grown under such conditions (Fiss *et al*., 1982) but the cause of this has not been reported.

Examination of the deduced amino acid sequence of the OmpT protein revealed the presence of a potential 2.2 kd signal peptide which has since been reported to be the actual signal sequence by Grodberg *et al*. (1988). Homologies between the protein sequence of OmpT and the sequences of other outer membrane proteins were not found.

(iii) The transposon Tn*phoA* was used to create *ompT-phoA* fusions. Synthesis of the resulting hybrid proteins was dependent upon temperature (section 5.2.6) this refecting the normal pattern of OmpT production. Five to six times more alkaline phosphatase activity was detected in cells grown at 37^{o}C compared to those cultured at 30^{o}C. Although synthesis of the OmpT-PhoA chimeras was still detected at the lower temperature,

this may be due to the *ompT-phoA* fusion being carried on a multicopy vector. Alternatively, it may reflect the normal pattern of expression of *ompT*, the latter still being expressed at 30°C albeit at a lower level. The OmpT signal sequence was able to direct the export of a heterologous protein, PhoA, to the periplasm of the cell (section 5.2.5). Since the expression of *ompT* did not appear to be tightly regulated by temperature, at least when cloned on a high copy number plasmid, use of the gene in the construction of an expression-secretion vector was not considered further.

(iv) The construction of *ompT-lacZ* operon and protein fusions indicated that the temperature-dependent synthesis of OmpT was the result of some post-transcriptional mechanism (Chapter 6).

(v) Mutations in the *envZ* gene lowered the expression of *ompT* at the level of transcription as determined by studies on both *ompT-phoA* and *ompT-lacZ* fusions (Chapter 7).

8.2 Further Work

The information concerning the OmpT protein is severely limited. In particular, no *in vivo* function for the protein has been described. All the proteolytic activities so far assigned to the protein have been discovered *in vitro* and have not been demonstrated to occur within the cell. This is

an obvious area for further study. It would also be of interest to examine the export of the protein. For example, are there amino acid sequences within OmpT required to direct the protein through the inner membrane and then to the outer membrane ? In addition, are the *sec* genes necessary for the export of OmpT as they are needed for the export of other outer membrane proteins (Randall *et al*., 1987)? Analysis of the topology of OmpT within the outer membrane could also be investigated using a variety of techniques including the binding of monoclonal antibodies and 'phages to OmpT, OmpT mutants and possibly chimeric proteins consisting of OmpT and other outer membrane proteins.

With respect to the *ompT* gene, the promoter and transcriptional start point need to be accurately defined probably by the technique of S1 mapping.

The regulation of expression of the *ompT* gene is another interesting area which can be examined in more detail than was possible in this study. Although the regulation of *ompT* expression appeared to occur at some post-transcriptional level (Chapter 6), the mechanism of this control was not determined. The isolation of mutant strains which no longer exhibit temperature-dependent synthesis of the OmpT protein may aid in discovering the regulatory mechanism involved in reducing OmpT production at 30^{o}C.

The effect of iron limiting conditions on the expression of *ompT* is also a subject for investigation. A consensus sequence has been suggested to be common to the promoter region of genes which are expressed during iron limitation. This is postulated to be the binding site of the Fur protein which, in conjunction with Fe^{2+}, represses the expression of iron-regulated genes (e.g., *fepA*, *fhuA*, *cir*) when this nutrient is available (De Lorenzo *et al*., 1987). OmpT has been reported to be synthesised in increased amounts in iron-limited medium (Fiss *et al*., 1982) and indeed the *ompT* gene has a sequence in its postulated promoter region that is homologous to proposed binding site of Fur (section 4.2.5). Of course accurate mapping of the *ompT* promoter has to be carried out before any real significance can be attached to this consensus.

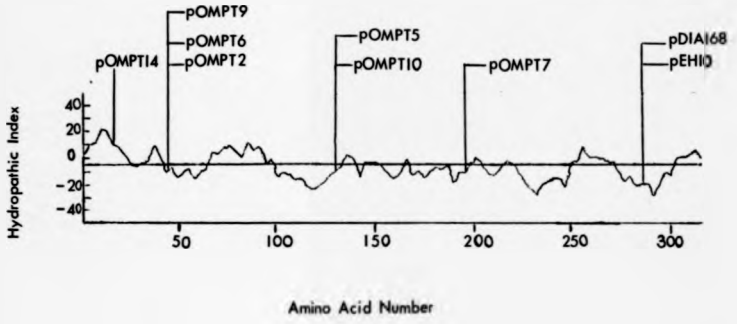
Further investigations into the effect of the EnvZ protein on the expression of *ompT* could also be carried out, particularly to see if the decrease in transcription caused by the *envZ473* mutation is mediated by the OmpR protein. Also of interest is the observation that in *E. coli* strains carrying a *gyrB* mutation the amount of OmpT found in the outer membrane is substantially increased (N. Mann, pers. commun.).

In the case of several ribosomal proteins it has been shown that regulation of gene expression occurs at a post-transcriptional level by binding of the ribosomal protein to

the 5' end of its own mRNA thereby preventing translation. Since the expression of *ompT* has been shown in this work to be controlled post-transcriptionally, it is tempting to speculate that some similar mechanism of autogenous control may be occurring for *ompT* expression and this could provide an interesting basis for future research.

Appendix I Hydropathy profile of the OmpT protein indicating the sites of fusion between the OmpT protein and PhoA, and OmpT and LacZ

The hydropathy index was evaluated according to the parameters presented by Kyte and Doolittle (1982), using a span setting of 11 amino acid residues. Hydrophobic regions extend above the centre line. The site of fusion between OmpT and PhoA is indicated as found in pOMPT2, pOMPT6, pOMPT9, pOMPT10, pOMPT7 and pOMPT5. The position of fusion between OmpT and LacZ is shown as found in pOMPT14, pDIA68 and pEH10.


pOMPT9
pOMPT6
pOMPT2
pOMPTI4
pOMPT5
pOMPTI0
pOMPT7
pDIAI68
pEHI0
Hydropathic Index
40
20
0
-20
-40
50
100
150
200
250
300
Amino Acid Number

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