Mechanisms of protein translocation in _Escherichia coli_

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by

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Definitions of terminology used in this thesis:-

Export is the process of localisation of a protein to any extracytoplasmic destination,

Secretion involves complete export of a protein to the medium.

1 O.D. unit of cells is defined as 1ml of cells at an A_{450} = 0.1

Abbreviations

EDTA - diamineoethane tetra-acetic acid, disodium salt.

TCA - trichloroacetic acid

SDS PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

SN - supernatant

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CHAPTER ONE: Introduction

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I   Introduction

Gram-negative bacteria, such as Escherichia coli, are
characterised, as illustrated in Fig.1.1, by the presence of two surface
membranes enclosing the periplasm. This is an aqueous compartment which
contains the cell wall, a rigid, 3-dimensional peptidoglycan layer which
is largely responsible for giving the cell its shape. Fusion of the two
membranes has been proposed to occur at specific sites throughout the
cell surface to form zones of adhesion. Originally observed by Bayer
(1979) these zones are termed "Bayer" junctions and can be seen when
E.coli cells are plasmolysed in 20% sucrose, sectioned and examined under
the electron microscope. Structural details of these regions have
remained elusive however and their function, if any, is obscure. The
inner of the two membranes, the cytoplasmic membrane, contains a number
of proteins including many specific transport systems, ATP synthetase and
enzymes concerned with the synthesis of peptidoglycan and other surface
constituents. The outer membrane, which is unique to Gram-negative
bacteria, lacks such functions but plays a vital role in providing a
barrier between the cell and the environment (see reviews by Silhavy et
al., 1983; Nikaido and Vaara, 1985; Hancock, 1984). Finally, the
periplasm also contains a high concentration of proteins (Nossal and
Heppel, 1966) mostly concerned with the promotion of solute transport
through the inner membrane permeases.

I i   Structure and growth of the E.coli outer membrane

The outer membrane of Escherichia coli is a glycolipidphospho-
lipid bilayer which is composed of phospholipids, lipopolysaccharide and
in addition contains substantial amounts of protein. The glycolipid

<u>Figure 1.1</u>

Structure of the <u>E.coli</u> cell envelope (redrawn with modifications

from Lugtenberg & van Alphen, 1983).

In <u>E.coli</u> K12 strains the O antigen chains of LPS are absent

A - OmpA protein; BP - periplasmic binding protein; IM - inner membrane;

IMP - inner membrane protein; L - lipoprotein, Lip. A - lipid A; O Ag - <u>o</u>

antigen; OM - outer membrane; PG - peptidoglycan; PMP - periplasmic

protein; PMS - periplasmic space; PP - pore forming protein trimer e.g.

porins like OmpF.
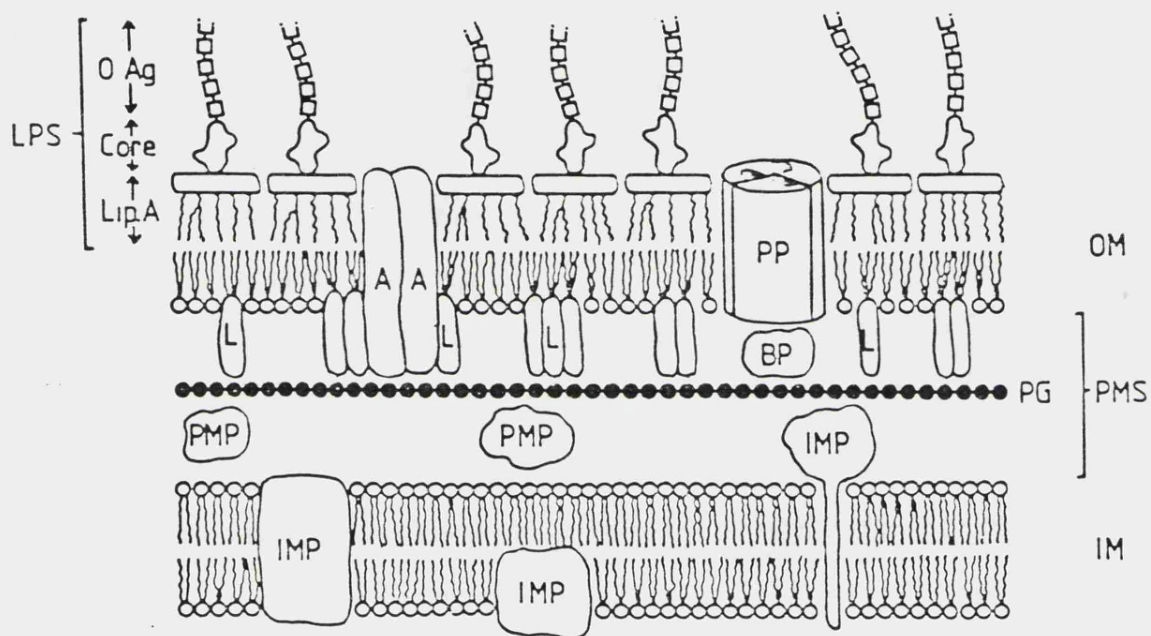
(lipid A) forms the outer leaflet of the membrane with the polysaccharide groups exposed on the outer surface. This results in an overall hydrophilicity of the cell surface, a feature which is thought to be important in evading phagocytosis and providing some resistance to complement (Nikaido and Vaara, 1985). Resistance to host defence systems is in fact one of the most important functions of the outer membrane as it creates an impermeable barrier to various harmful substances such as lysozyme, lysin, some antibiotics, detergents and even leukocytes (Donaldson et al., 1974).

Approximately half of the mass of the outer membrane of E.coli is composed of protein and interestingly the level of protein is tightly controlled. Therefore, although the surface area to mass ratio of cells growing at different rates varies considerably, the concentration of the major proteins per unit surface area remains constant. Indeed, kinetic studies have demonstrated that different polypeptides compete for assembly into the outer membrane (Boyd and Holland 1979). This important regulatory feature results in the ratio of ribosomal proteins, and at least the major surface porins, varying almost inversely with growth rate (Boyd, 1979). The basis of this control is unknown but may be achieved by limiting the concentration of some of the components of the protein translocatory machinery which will be discussed below.

The biogenesis of outer membrane and other envelope proteins is an extremely complex, dynamic process involving the generation of at least 25% of total cellular protein under some growth conditions (Boyd, 1979). Consequently, it has been calculated that membrane bound polysomes directing the synthesis of surface proteins in E.coli may occupy a substantial proportion of the inner surface of the cytoplasmic membrane throughout the cell cycle (De Leij et al., 1979). From such calculations and more importantly from direct analysis of the emergence of newly

synthesised proteins on the cell surface it now seems clear that protein

assembly occurs uniformly over the whole surface of E.coli and not at

localised zones of insertion (see Nanninga 1985).

Although a substantial amount of protein is found in the outer

membrane of E.coli, only about fifty different protein species can be

resolved by sodium dodecylsulphate polyacrylamide gel electrophoresis

(SDS-PAGE). Of these, only four or five polypeptides are extremely

abundant, being expressed at a level of at least 100,000 copies per cell

(Hall and Silhavy, 1981a). In contrast, the vitamin B12 receptor protein

BtuB is present in only a few hundred copies per cell (Sabet and

Schnaitman, 1973; Bassford and Kadner, 1977). Several other more minor

outer membrane proteins are regulated by growth conditions and are

consequently present at varying levels in the membrane. Many of these

outer membrane proteins have been shown to facilitate the entry of

nutrients and ions into the cell. In addition, the same proteins

frequently act as receptors for bacteriophage and colicins, highly

specific bacteriocidal proteins (Konisky, 1982; Hantke, 1976; Bradbeer et

al., 1976).

II i Outer membrane proteins

Table 1.1 summarises the properties of some outer membrane proteins

and gives a brief indication of their function. Two major outer membrane

proteins OmpA (Sonntag et al., 1978) and lipoprotein (Braun and Rehn,

1969) both appear to be associated with peptidoglycan (Endermann et al.,

1978; Palva, 1979) and contribute to the shape and structure of the

surface envelope. Lipoprotein is a small protein, with a molecular

weight of 7.2kD, which probably forms stable trimers (Choi et al., 1986).

Lipoprotein is tightly bound to the inner leaflet of the outer membrane

Table 1   Some outer membrane proteins of <u>E. coli</u>

Protein	Molecular weight	Gene	Function	Receptor for:
OmpA	35,159	<u>ompA</u>	conjugation, structural role	Phage TuII; colicins K and L
OmpF	37,205	<u>ompF</u>	general pores for nutrients with molecular weight < 600D	Phage Tula, T2, K20, TP1, T22, TP5; colicin A
OmpC	36,000	<u>ompC</u>		Phage Tulb, Mel, T4, λ434, SS1, TP2, TP5, TP6
PhoE*	36,800	<u>phoE</u>	phosphate uptake	Phage TC23, TC45
LamB*	47,000	<u>lamB</u>	maltose and malto-dextrin uptake	Phage λ, K10, TP1, TP5, 551
Lipoprotein	7,200	<u>lpo</u>	anchors outer membrane to peptidoglycan	
TonA (FhuA)	78,000	<u>fhuA</u>	ferric ferrichrome uptake	Phage T1, T5, Φ80; colicin M, Albomycin
Protein G	15,000		DNA replication?	
Phospholipase A	21,000			
Tsx	25,000	<u>tsx</u>	nucleoside transport	Phage T6; colicin K.
BtuB	60,000	<u>btuB</u>	vitamin B12 uptake	Phage BF23, colicin E

*Proteins which are amplified to high levels in the surface under particular nutritional conditions.

Figure 1.2

Hydrophobicity plots for three outer membrane proteins of E.coli.

Graphs were plotted according to the method of Kyte and Doolittle

(1982), scanning 11 amino acids at a time. Positive values are

hydrophobic, negative values are hydrophilic.

OMPA

OMPF

TONA.
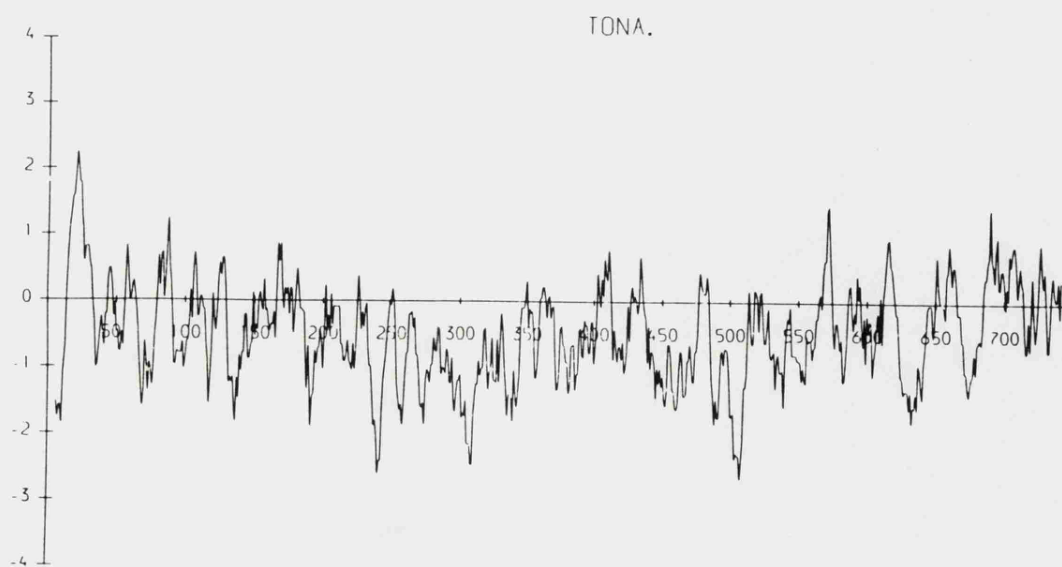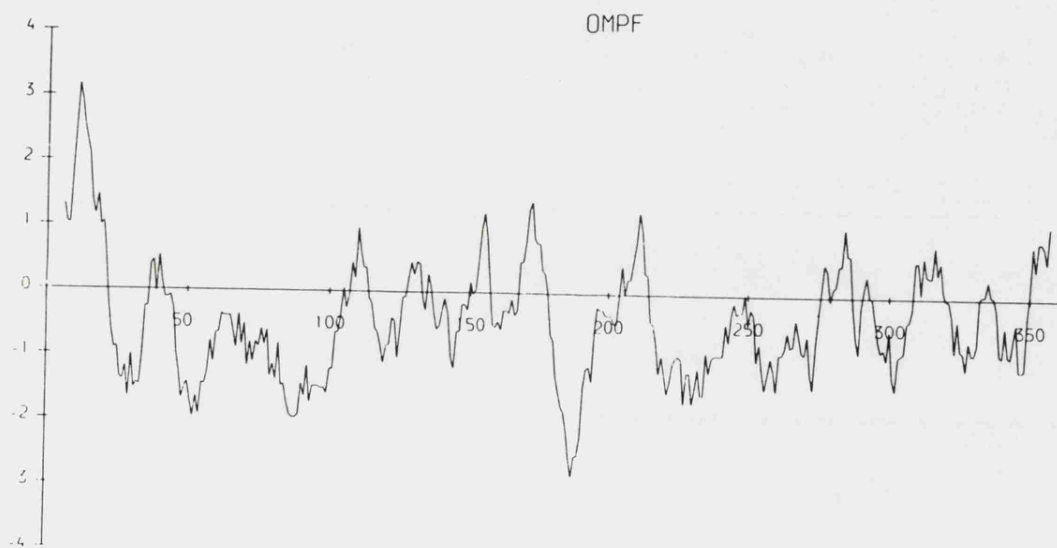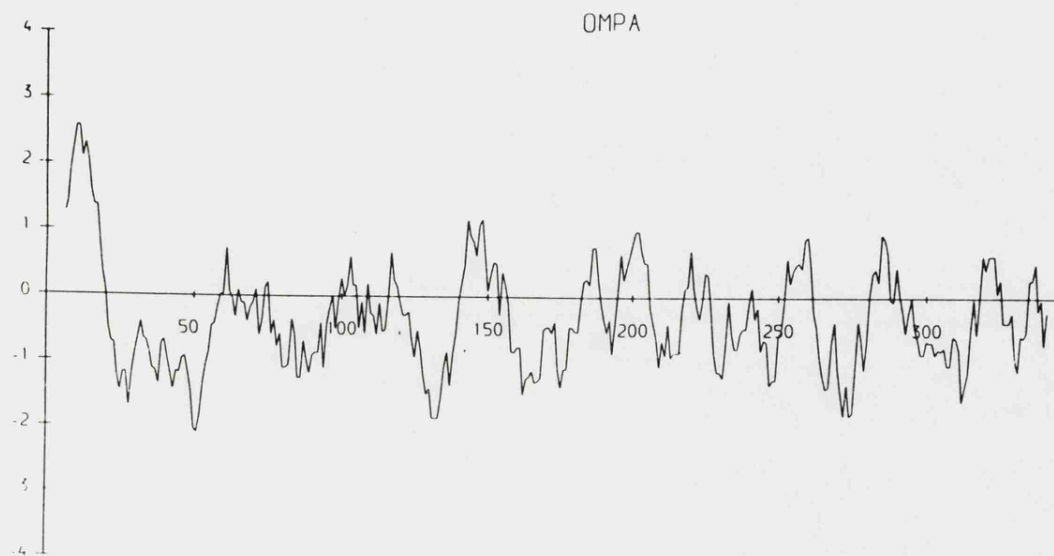
Figure 1.3

Structural model of the OmpA fragment 1-117, reproduced from Vogel and Jahnig (1986).

Rectangles represent membrane spanning β-strands with bold face lettering denoting the residues on the hydrophobic sides. Asterisks denote the beginning of β-turns, circles and diamonds indicate positively and negatively charged residues.

PERIPLASM

35 Å

0 (+)

3 (−)
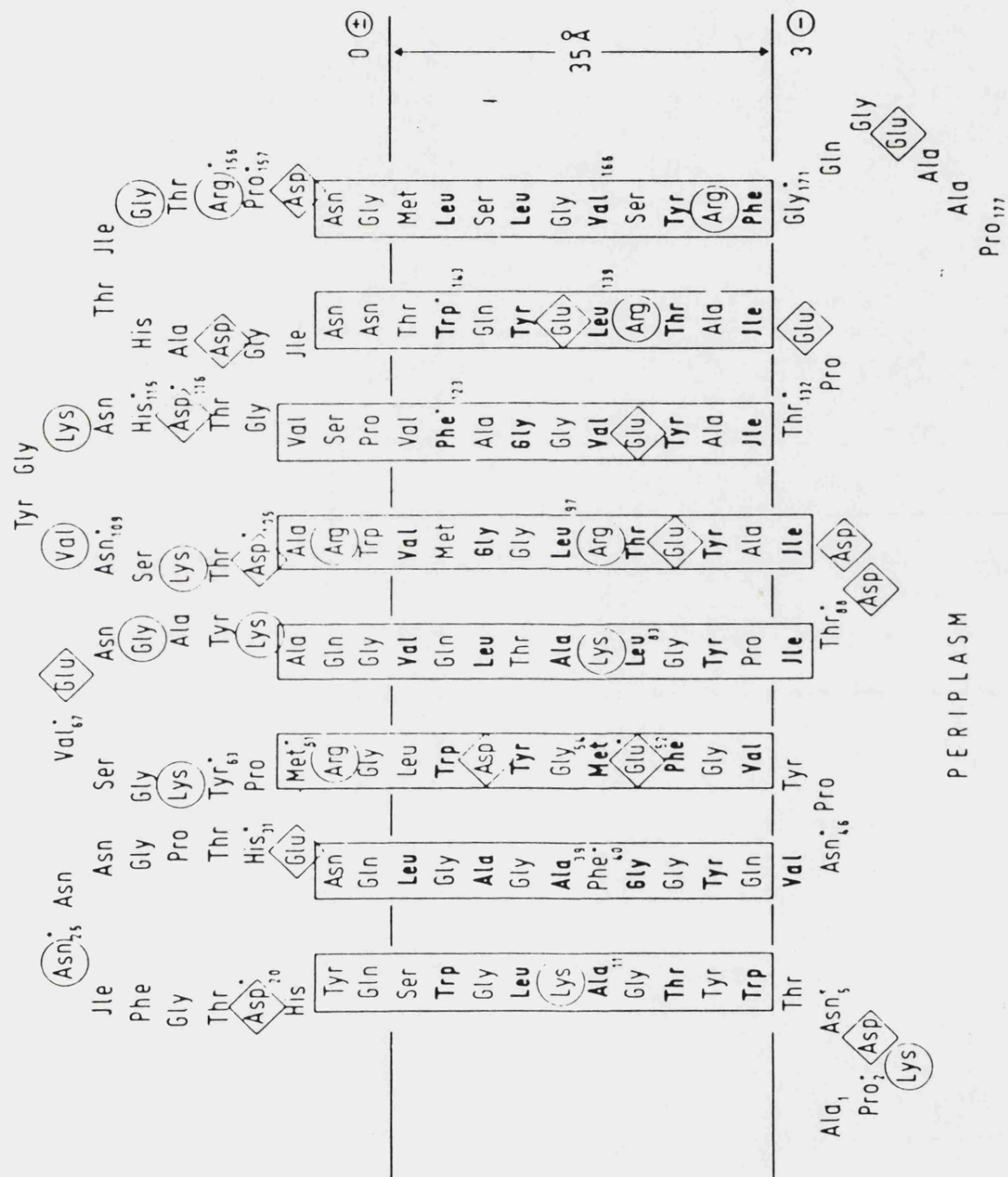
(Nikaido and Vaara 1985). This is achieved by post-translational

additions of a diglyceride moiety and an amide linked fatty acid at the

NH_2-terminus of the mature molecule which allows lipoprotein to interact

with the outer membrane. At the C-terminus many lipoprotein molecules

are covalently linked to the peptidoglycan through lysine residues

(Braun, 1975). OmpA is also a structural protein, lacking porin activity

(Nikaido and Vaara, 1985), although it appears to have an additional role

in conjugation mediated by the F (or fertility) plasmid and as a receptor

for phage TuII and also colicins K and L (Konisky, 1982). Unusually for

surface proteins, E.coli outer membrane proteins are not characterised by

large blocks of hydrophobic amino acid residues (Fig.1.2). This is

exemplified, as shown in Fig.1.3, by OmpA, which nevertheless has a large

N-terminal domain which probably crosses the outer membrane eight times

in an antiparallel β-sheet conformation, with four "loop" regions exposed

at the cell surface (Freudl et al., 1985). Other studies indicate that

residue 70 close to the NH_2-terminus is exposed at the cell surface (Cole

et al., 1982), whereas the C-terminal region from residue 177 to the end

is located in the periplasm (Chen et al., 1980).

Other common outer membrane proteins include OmpF and OmpC, porin

proteins which are very similar in structure and function. These

proteins form relatively non-specific pores or channels and allow small

water soluble molecules (less than 600 Daltons) to enter the cell

(Nikaido and Vaara, 1985; Luktenhaus, 1977). OmpF has been extensively

studied and the gene is now sequenced (Mutoh et al., 1982). Like OmpA,

OmpF appears to be high in β-sheet and low in α-helix structure and is

thought to form trimers in the membrane (Schindler and Rosenbusch, 1978;

Hawley and McClure 1983). There is believed to be a strong

evolutionary relationship between the non-specific porins OmpF and OmpC

and the PhoE porin, a protein which is produced in response to inorganic

phosphate limitation and facilitates the specific uptake of inorganic

phosphate and phosphate containing compounds (Korteland et al., 1982).

Overbeeke et al. (1983) compared the PhoE sequence with that of OmpF and

established that the proteins contained 63% identical amino acids with

homology increasing to 86% at the C-terminal end of the two proteins

(Fig.1.4).

No such relationship has been found to exist between these proteins

and LamB, the porin responsible for uptake of maltose and maltodextrins

(Luckey and Nikaido 1980). In addition to maltose uptake, LamB is also

the receptor for phage λh^+ and its host range derivative λhh^* (Hofnung et

al., 1976). A series of elegant experiments carried out by Hofnung and

his colleagues have made use of the phage sensitivity range to identify

LamB residues exposed on the surface of the cell which comprise the λ

recognition sites (Charbit et al. 1984). Strains resistant to phage λh^+

but sensitive to λhh^* were isolated and thus, cells with only slightly

altered, functional LamB, were obtained. By sequencing the DNA and

identifying the altered residues, it was therefore possible to pinpoint

amino acids which are exposed on the outer surface of the cell when LamB .

adopts its final conformation in the outer membrane. The insertion of

foreign epitopes which did not affect LamB synthesis and assembly,

confirmed the identity of some of these surface amino acids (Charbit et

al., 1986). Together with hydropathic plots, data on secondary

structure, the high incidence of β-sheet in LamB (Neuhaus, 1982) and the

interesting observation that monoclonal antibodies raised against cell

surface exposed proteins in E.coli interact with protein residues within

the last 70 amino acids of LamB

Figure 1.4

Dot matrix comparison of OmpF and PhoE.

The amino acid sequences of OmpF and PhoE were compared using the "Diagon" computer programme supplied by Staden. Sequential blocks of 11 amino acids were compared using a proportional algorithm which allows not only for identity, but also for similarity of amino acids within each block. A score of homology above that predicted by chance is indicated by a point on the matrix printer at a coordinate equivalent to the positions of the central amino acids in the blocks compared.

PhoE
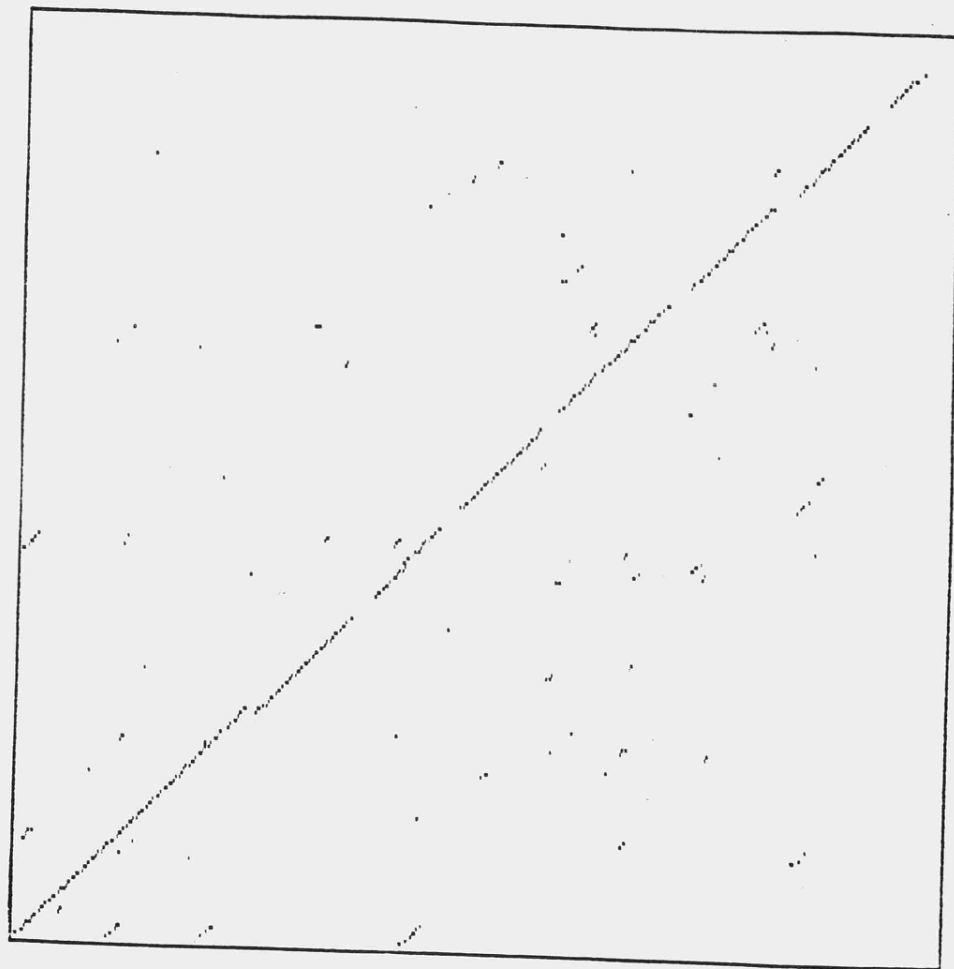
OmpF

(Gabay et al., 1983), a model of LamB structure in the membrane has been produced (Gehring et al., 1987). Thus, LamB is now thought to adopt a structure containing at least four loops, including the C-terminal 70 amino acids, exposed at the surface, each loop separated by a series of transmembrane stretches of polypeptide (Gehring et al., 1987). It seems therefore, that exposed loops and transmembrane β-sheets may be common to many outer membrane proteins.

Another abundant outer membrane protein is protein G, which has a molecular weight of 15kD and a supposed role in DNA replication and cell elongation (James, 1975) although this has not been confirmed. Several of the less common outer membrane proteins are involved in iron uptake and are expressed at high levels in response to low concentrations of ferric iron in the growth medium, (Braun et al., 1973, Hollifield and Nielands 1978). Proteins for the specific transport of other compounds such as vitamin B12 (Bradbeer et al., 1976) and nucleosides (Hantke 1976) have also been identified. However, only two enzymes have been reported to be associated with the outer membrane of E.coli, namely a serine protease which has the ability to cleave the cytoplasmic membrane protein nitrate reductase (MacGregor et al. 1979) and a phospholipase responsible for the modification of outer membrane phospholipids (Bell et al., 1971; Nishijima et al., 1977). Additionally, a temperature-dependent protein has been identified in the outer membrane (see e.g. Schnaitman, 1974). This protein has a molecular weight of 40kD and has been reported to have various functions including conversion of a ferric-enterochelin receptor from an 81kD to a 74kD protein (Fiss et al., 1979) or to regulate capsular polysaccharide synthesis (Gayda et al., 1979). The gene encoding this protein has subsequently been named ompT (Ruprecht et al., 1983) but no definite function has yet been assigned to this unusual

protein. Another heat inducible protein of 24kD, distinct from the
similar sized T6 receptor was also identified by Fairweather et al.
(1981). The synthesis of this protein was also stimulated by inhibitors
of DNA gyrase but its function remains unknown.

Of particular interest in this study is the outer membrane protein
TonA. TonA is essential for the uptake of ferric-ferrichrome (Braun et
al., 1973) but is a constitutive component of the outer membrane and does
not appear to be regulated by the amount of iron in the growth medium.
The TonA protein is encoded by the fhuA gene (Braun et al., 1983), which
has been sequenced, (Coulton et al., 1986) and has been shown to code for
an 80kD protein which is processed to form a mature protein of molecular
weight 78kD (Plastow, 1981). The study of high copy number clones of the
gene indicate that even when overproduced the protein retains its
function and is correctly assembled into the envelope (Menichi and Buu,
1986; Jackson et al., 1986).

II ii Regulation of outer membrane protein synthesis

The total amount of protein in the outer membrane is maintained at a
relatively constant level by a number of control mechanisms in E.coli.
Many proteins are controlled by nutritional conditions (see Table 1.1)
but others are controlled either at the level of translation or
transcription and expression may be increased by raising gene copy
number, or prevented by mutation (Case et al., 1986). The level of
major outer membrane proteins may thus be increased or decreased to
compensate for the alteration in environmental conditions (Schnaitman,
1974; Movva et al., 1978).

The relative amounts of porin protein in the outer membrane appear
to be particularly tightly controlled and seem to be interdependent. For

example, induction of iron binding protein reduces the level of OmpF by competition as expected (Boyd and Holland, 1979). In addition, growth in maltose induces LamB synthesis but reduces OmpC synthesis (Diedrich and Fralick, 1982) whereas a mutation in PhoE which causes constitutive synthesis of this protein, lowers OmpF and OmpC levels (Pugsley and Schnaitman, 1978). OmpF, OmpC and LamB all vary with growth temperature (Lundrigan and Earhart, 1984) and OmpF and OmpC vary reciprocally with osmolarity (van Alphen and Lugtenberg, 1977). No single regulatory system has been identified which controls these porins as a group however.

The reciprocal osmoregulation of ompF and ompC has been the subject of much recent research and appears to be controlled by three different mechanisms. Firstly, the regulatory ompB operon, consisting of envZ and ompR plays a major role in the control of porin synthesis (Hall and Silhavy, 1981a; Hall and Silhavy, 1981b). OmpR serves as a positive regulator that apparently binds upstream of ompF and ompC and triggers transcription (Dairi et al., 1985; Mizuno and Mizushima, 1986) whereas EnvZ located in the cell envelope may serve as an osmotic "sensor" in interaction with OmpR (Matsuyama et al., 1986). Secondly, antisense RNA may regulate OmpF/C synthesis at the translational level (Aiba et al., 1987). Control in this case is mediated by the micF gene which codes for RNA complementary to the 5' end of ompF RNA and therefore reduces translation (Mizuno et al., 1984). Gene dosage is important in the micF effect and the ratio of micF:ompF mRNA may determine the degree of inhibition. Finally, protein-coding regions are also thought to exert a "fine tuning" control on synthesis of OmpF and OmpC (Inokuchi et al., 1985). The physiological importance of osmoregulation is not known but it has been suggested that strict control may be required under certain conditions which occur in the natural environment (Aiba et al., 1987).

II iii <u>Cytoplasmic membrane and surface proteins</u>

The proteins of the cell surface and cytoplasmic membrane of <u>E.coli</u> are also very diverse in structure and function. For example some cytoplasmic membrane proteins are integral to the membrane whereas others are attached only by the C-terminus. Many cytoplasmic membrane proteins such as lactose permease (Ehring <u>et al</u>., 1980) and the Fo component of ATPase (Nielson <u>et al</u>., 1981) are synthesised without an NH_2-terminal signal sequence may integrate into the membrane by means of their overall hydrophobicity. However, within the group of inner membrane proteins known as penicillin-binding proteins (PBP's) there appear to be a number of proteins which require a signal sequence in order to assemble into the membrane. There are seven well characterised PBP's in <u>E.coli</u>, with molecular weights ranging from 40,000-90,000 daltons (Spratt, 1977) and all are involved in the synthesis of peptidoglycan (Spratt and Pardee, 1977). Although there is no evidence for a cleaved signal in either PBP1A or PBP1B (Broome-Smith <u>et al</u>., 1985), PBP5 and PBP6 are unusual among inner membrane proteins in that they are synthesised with an NH_2-terminal signal sequence (Broome-Smith <u>et al</u>., 1983; Pratt <u>et al</u>., 1981a). PBP5 is a carboxypeptidase and the active site is predicted to protrude into the periplasm to reach the substrate (Glauner <u>et al</u>., 1984). It is likely therefore, that a substantial proportion of PBP5 is translocated to the periplasmic face of the inner membrane with complete transfer prevented by sequences near the C-terminus (Pratt <u>et al</u>., 1986). Stretches of hydrophobic amino acids have been proposed to act as an anchor for other inner membrane proteins (Yost <u>et al</u>., 1983) but no visible anchor sequence is present in the amino acid sequence of PBP5. Studies of deletion mutants indicate however, that deletion of as few as 10 amino acids at the C-terminus results in the complete release of PBP5 into the periplasm (Pratt <u>et al</u>., 1986).

In addition to polypeptides that are translocated across or incorporated into the membrane many bacteria export proteins that are components of surface structures. Important surface proteins of E.coli are F pili, which are involved in conjugation (Manning and Achtman, 1979) and fimbriae, which allow specific adhesion to epithilial cells during infection (Jones and Rutter, 1972). Both structures are assembled from a pool of homogeneous subunits and final assembly involves the interaction of several gene products in addition to the structural components. Together these proteins create a firm anchor to components of the cell wall and allow transfer through the outer membrane. The assembly of these structures will be discussed in section VIIIi.

III i Protein translocation in eukaryotes

Before considering in detail mechanisms of protein translocation in bacteria, important findings from studies of higher organisms, which provided much of the early data will now be described.

In eukaryotes, two components have been isolated from mammalian endoplasmic reticulum (ER) which are thought to mediate the translocation of proteins across the ER. These include the signal recognition particle (SRP), a ribonucleoprotein particle (Walter and Blobel, 1980) and docking protein or SRP-receptor, which is an integral membrane component (Meyer et al., 1982; Gilmore et al., 1982). SRP was found to cause translational arrest of nascent secretory polypeptides in a wheat germ expression system by recognising and binding to the signal sequence, upon its emergence from the large ribosomal subunit (Walter and Blobel, 1981). Kurzchalia et al. (1986) demonstrated that SRP and the signal sequence interact when they used affinity labelling to show that

the signal sequence of a secretory protein could bind specifically to the 54kD protein component of SRP. The block imposed by SRP was apparently relieved by interaction between the arrested polypeptide chain and the membrane bound docking protein, presumably by the displacement of SRP from the ribosome (Gilmore and Blobel, 1983). The nascent chain subsequently integrates into the membrane in an unknown manner, although interaction between the signal sequence and the proposed receptor has been suggested to play a role in chain segregation across the membrane (Friedlander and Blobel, 1985). However, some aspects of the proposed overall mechanism have now been challenged. Thus, although the translation arrest-release cycle of SRP has been demonstrated with a number of proteins including, for example, vesicular stomatitus virus spike protein synthesised in a wheat germ translation system, at present this phenomenon cannot be demonstrated with reticulocyte or HeLa cell translation systems (Meyer, 1985). Moreover, even in the wheat germ system, Ainger and Meyer (1986) have recently shown that SRP can arrest translation at late times and that the translocation of large domains of at least some proteins can be uncoupled from synthesis.

Despite the identification of specific cellular components of the protein export machinery, it is important to emphasise that the precise mechanism of translocation of the polypeptide across the membrane, either through a proteinaceous pore (Blobel and Dobberstein, 1975), or through the bilayer itself remains a mystery.

III ii The prokaryotic secretion machinery

The presence of a similar NH_2-terminal signal sequence in both eukaryotes and prokaryotes, coupled to the fact that eukaryotic proteins such as preproinsulin (Talmadge et al., 1980) can be correctly processed

and translocated to the periplasm in E.coli, initiated a search for an analogous prokaryotic secretion apparatus.

One approach used to identify proteinaceous components of the export pathway present in E.coli was to isolate and characterise mutants with an altered cellular secretion machinery, resulting in pleiotropic effects on protein localisation. In principle, this could be achieved by selecting for cells which cannot export envelope proteins but retain precursors in the cytoplasm. However, such a direct approach offers no selection process for the putative mutants and can be extremely time consuming. Consequently, as discussed in later sections, a number of ingenious methods have been developed and used to identify several export proteins which appear to be involved in the secretion process.

III ii a NH$_2$-terminal signal sequence

Analysis of at least 32 different prokaryotic NH$_2$-terminal signal sequences has revealed the presence of three regions within the signal: (i) a postively charged amino-terminal region of variable length, (ii) a central hydrophobic region of between 7 and 15 amino acids which apparently folds to form an α-helix, (iii) an uncharged carboxy terminal region, including the cleavage site of leader peptidase which usually follows a glycine, alanine or serine residue (Perlman and Halvorson, 1983; von Heijne 1983). Once a protein or at least an NH$_2$-terminal domain has traversed the cytoplasmic membrane the signal sequence of the precursor is rapidly removed either by the action of leader peptidase, which is capable of cleaving the signal sequence of most exported proteins (Wolfe et al;, 1985), or by lipoprotein signal peptidase (Yamada et al., 1984). This latter protease is specific for lipoprotein and other precursors which have been modified either by glyceryl transferase or by O-acyl transferase (Hussain et al., 1982).

E.coli periplasmic and outer membrane protein signal sequences vary

in length from 20-30 amino acids and differ considerably in amino acid

sequence although they have the same overall structure and hydro-

phobicity, (von Heijne, 1983, Watson, 1984). For the analysis of the

function of signal sequences a number of methods have been used to

isolate mutants carrying defects in a given signal. These include in

vitro manipulations of DNA or direct selection for either loss of export

or second site alterations to correct frameshift mutations. Emr et al.

(1980) isolated mutations in the 25 amino acid signal of the outer

membrane protein LamB, many of which blocked export of LamB completely

and led to the accumulation of precursor in the cytoplasm. In the

majority of cases the mutations were found to alter residues in the

hydrophobic core region, either by the substitution of a charged amino

acid or by deletion of a critical subset of four amino acids contained

within the central region. Moreover, pseudoreversions of these mutations

were all found to re-establish a predicted α-helical structure, by the

replacement of proline/glycine by leucine/cysteine residues. This

indicated that the overall conformation of the signal sequence is one of

the most important features (Emr and Silhavy, 1982). In addition, signal

sequence mutations in LamB were reported to reduce the level of synthesis

of the precursor since no accumulation of precursor or degradation

products was observed in the cytoplasm (Hall et al., 1983). This led to

the suggestion that translation may be coupled to export and that signal

sequence mutations may also block translation release in bacteria (Hall

et al., 1983). Nevertheless, no direct evidence for such a coupling

exists.

III ii b <u>SecA</u>

A useful approach for identifying components of the secretion

machinery is based on the inabilty of a cell to export hybrid proteins

composed of the NH_2-terminal half of an exported protein fused to LacZ.

If translation of the hybrid is initiated, this can render the LacZ

portion of the molecule inactive and therefore it is possible to obtain

mutant cells which are completely unable to export such a hybrid protein

by selection for increased levels of β-galactosidase activity. One such

hybrid consisting of the NH_2-terminus of the periplasmic protein maltose

binding protein (MBP) fused to the cytoplasmic protein β- galactosidase

(Bassford <u>et al</u>., 1979) was found to be bound tightly to the cytoplasmic

membrane when synthesis was induced by maltose. In this presumably

partially translocated state, the hybrid retains only

minimal β-galactosidase activity perhaps due to the inability of the

bound molecules to tetramerise. However, any secretion defect, such as an

alteration in the MBP signal sequence or in the secretion machinery

itself, causing the hybrid to accumulate in the cytoplasm, leads to a

detectable increase in β-galactosidase activity. Thus, several

conditional lethal, temperature sensitive mutations in the secretion

machinery were identified using this system (see Fig.1.5). The first of

these was <u>secA</u>, which maps at 2.5min on the chromosome and lies close to

a large cluster of genes involved in envelope biosynthesis, (Oliver and

Beckwith, 1981; Oliver and Beckwith 1982a). Similarly, <u>secB</u> was

identified and maps at 81min close to <u>cysE</u> and <u>gpsE</u> (Kumamoto and

Beckwith 1983). A third gene, <u>secD</u>, maps at 10min and was isolated by

detecting an increase in β-galactosidase activity encoded by a fusion

between <u>phoA</u>, which codes for the periplasmic protein alkaline

phosphatase, and <u>lacZ</u> (Gardel <u>et al</u>., 1987); alleles at this locus

display cold sensitive defects in secretion.

Figure 1.5

Map of the <u>E.coli</u> chromosome showing location of genetic loci

discussed in the text.

<u>expA</u> - mutation causes a decrease in 6 periplasmic enzymes and some

modification of inner and outer membrane protein profiles; <u>lep</u> - signal

peptidase; <u>lpo</u> - lipoprotein; <u>malB</u> - maltose transport operon

(<u>malG,F,E,K</u> - transport, <u>lamB</u> - receptor for maltose); <u>ompA</u> - outer

membrane matrix protein, OmpA; <u>ompB</u> - regulatory locus comprising 2

genes, <u>ompR</u> & <u>envZ</u>, involved in regulation of expression of several

envelope proteins, including OmpF and OmpC.; <u>ompC</u> - encodes porin OmpC;

<u>ompF</u> - encodes porin OmpF; <u>phoE</u> - encodes inducible outer membrane

protein involved in phosphate uptake; <u>prlA</u>, <u>prlB</u>, <u>prlC</u> - mutations

causing suppression of certain signal sequence mutations; <u>secA</u>, <u>secB</u>,

<u>secD</u> - mutations causing generalised export defective phenotype, <u>ssyA</u>,

<u>secC</u> - mutations causing defect in protein synthesis; <u>tolC</u> - mutation

affecting expression of some outer membrane proteins; <u>tonA</u> - encodes the

ferrichrome receptor TonA; <u>ts215</u> - temperature sensitive mutation causing

slow processing of envelope proteins at the non-permissive temperature.

malG malF malE P malK lamB
malB
secA prlD
tonA
phoE
secD
100/0
10
90
ompF
ompA
expA
prlB
secB
80
20
ompR
envZ
ompB
ts215
[secY] prlA
30
prlC
secC
70
tolC
60
lpp
40
ssyA
lep
ompC
50
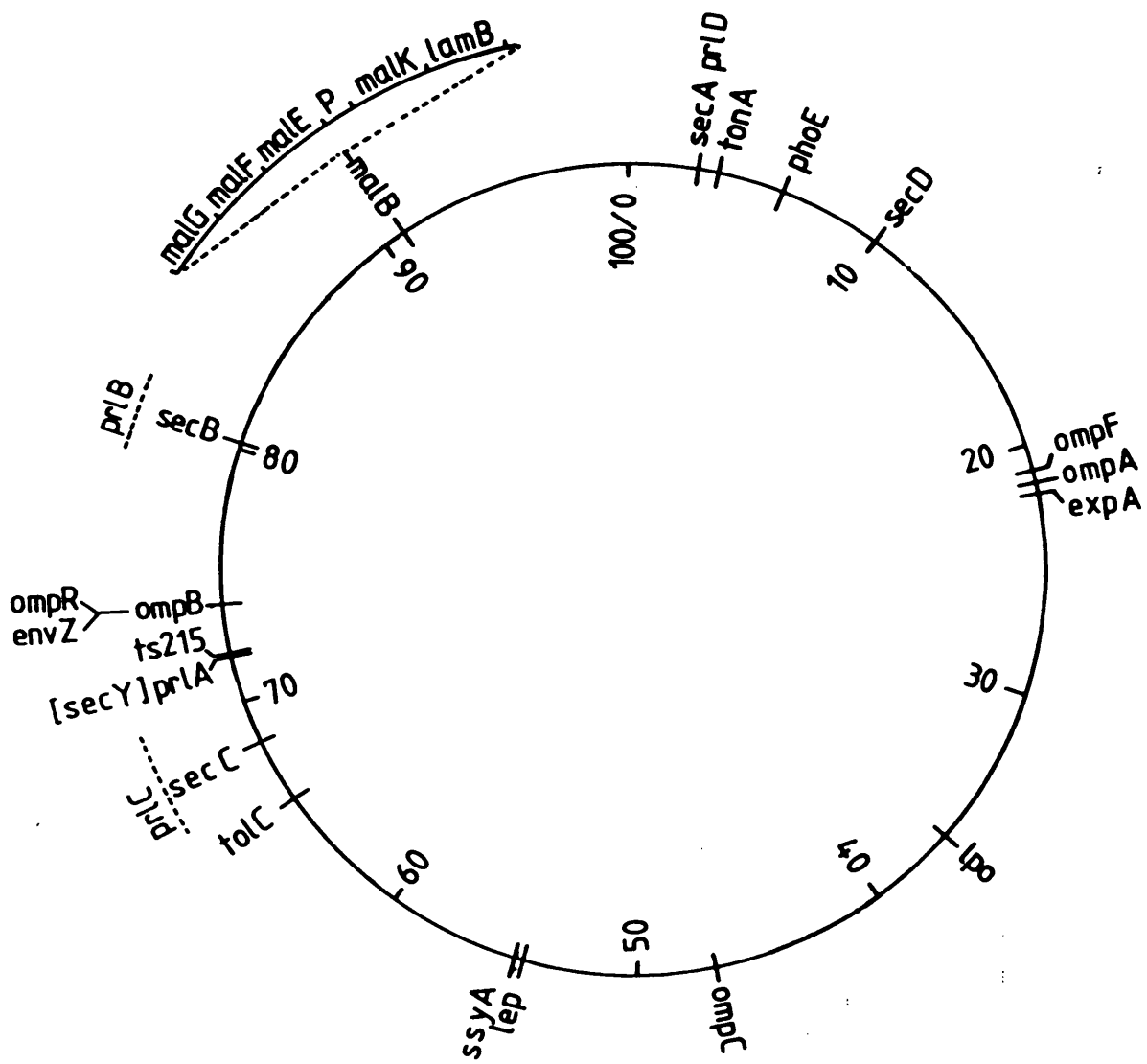
The secA^{ts} mutant showed reduced growth at 42°C and accumulated

precursors to several periplasmic and outer membrane proteins in the

cytoplasm under these restrictive growth conditions (Oliver and Beckwith,

1981). Moreover, other studies have shown that secA is apparently

required for the translocation and processing of fimbrial proteins (Dodd

et al., 1984) and specific inner membrane proteins (Jackson et al.,

1986). M13 coat protein is however, an exception and can assemble into

the inner membrane in the absence of secA (Wolfe et al., 1985).

Using antibody raised against a SecA-LacZ fusion protein, SecA

itself was identified as a peripheral cytoplasmic membrane protein with a

molecular weight of 92kD (Oliver and Beckwith, 1982a) and more recent

analysis carried out with purified antibody and isoelectric-focussing,

has revealed that SecA exists as several isoforms (Liebke, 1987). The

protein was calculated to be present in small amounts, perhaps as low a

500-1000 copies per cell based on an estimation of 10,000 - 20,000

copies of MBP per cell (Oliver and Beckwith, 1982b).

Synthesis of SecA was found to be derepressed 10-fold in a secA^{ts}

strain when secretion was blocked (Oliver and Beckwith, 1982b).

Therefore, in order to establish that overproduction of defective SecA

does not jam the membrane and so interfere with secretion, a secA (amber)

mutant strain containing a temperature sensitive suppressor was isolated.

Although very little SecA protein is produced in this strain, export of

protein from the cytoplasm was still inhibited, providing further

evidence that secA is specifically involved in the secretion process

(Oliver and Beckwith 1982b).

Although yet to be substantiated, further evidence to indicate that SecA is normally required for protein secretion was obtained when it was established that a protein isolated from Bacillus subtilis will cross react with SecA antibody. The B.subtilis protein was identified as part of the cells secretion machinery by its ability to associate with membrane fractions containing membrane bound polysomes and the difference in isolation procedure from that used to identify the E.coli SecA has been taken as strong circumstantial evidence that both proteins are involved in protein export (Oliver and Beckwith, 1982b)

Some studies also indicated that SecA might be required for the synthesis, as well as the secretion of envelope proteins, since no MBP or LamB was detected in pulse-labelling experiments using mutants lacking functional SecA (Oliver and Beckwith, 1982b; Strauch et al., 1986). It was therefore suggested that a translation-arrest system involving SecA, similar to that found in eukaryotes, might exist in prokaryotes. Furthermore, other studies also indicated that the block in MBP synthesis observed in secA strains could be reversed by signal sequence mutations. This suggested that translation inhibition was due to the recognition of the signal sequence by the secretion machinery (Kumamoto et al., 1984; Oliver and Liss, 1985b). However, the latter result was not confirmed in later studies and moreover recent evidence suggests that the reduction in synthesis of LamB, MBP and alkaline phosphatase is a secondary consequence of secA limitation and in fact no inhibition of synthesis was observed for OmpA, or lipoprotein (Liss and Oliver, 1986). Since the reduced synthesis of MBP was alleviated by cAMP, blocking SecA function may in fact cause cAMP levels to decline leading to catabolite repression of certain outer membrane protein genes (Strauch et al., 1986). OmpA and lipoprotein are both constitutively expressed proteins of

the E.coli cell envelope and should therefore be unaffected by catabolite repression. In contrast, MBP and LamB are both subject to transcriptional regulation (Chapon, 1982) and synthesis may be reduced if cAMP levels decline. In conclusion therefore SecA does not appear to be involved in coupling synthesis and secretion in E.coli.

Although these findings also cast some doubt on the theory that synthesis and secretion are tightly coupled in bacteria, another protein has been tentatively identified as causing translational control arrest in E.coli (Hengge-Aronis and Boos, 1986). These authors observed that when a truncated periplasmic glycerophosphodiesterase (GlpQ') is expressed, it is not translocated to the periplasm but becomes jammed in the inner membrane. A concomitant reduction in the synthesis of a number of periplasmic proteins including MBP and RBP was observed but no similar inhibition of synthesis of OmpA, OmpF/C or LamB occurred. The effect was shown to be independent of the level of cAMP and inhibition of synthesis was dominant over accumulation of precursor upon inactivation of secA. This led to the suggestion that GlpQ' blocks a protein which would normally act to catalyse restart of translation of a subset of periplasmic proteins, and thus may be involved in sorting. The putative protein has not however been identified and the position of the corresponding gene on the chromosome is not known.

Despite these various studies, in different secA mutants, a role for the SecA protein in secretion still remains to be directly demonstrated. In particular, no in vitro studies have been carried out using secA mutants. It is also important to note that no kinetic experiments have been attempted and in all the experiments described, cultures have been grown at the non-permissive temperature for several hours before analysis. Thus, it is difficult to determine whether it is primarily

secondary effects that are observed in these cultures. For example, secA mutations, by virtue of the method of isolation will probably be leaky and some residual export and assembly will occur even at the restrictive temperature. Also, it is possible that multiple pathways exist for export and assembly of envelope proteins and the true effect of SecA is may therefore be obscured.

III iii c SecY/PrlA

Emr et al. (1981) using an indirect approach for the isolation of secretion defective strains, identified mutants which were able to restore secretion of an envelope protein synthesised with a defective signal sequence. Many of the mutations were found to map in genes encoding ribosomal proteins. However, one particular mutation, which although located in the ribosomal protein operon, rplO, at 72 min on the E.coli chromosome, was distinct from the r-protein genes. This mutant, prlA, for protein localisation, was highly pleiotropic and suppressed mutations in the signal sequence of several exported proteins, including the periplasmic MBP and the outer membrane proteins OmpF and LamB (Emr and Bassford 1982, Michaelis et al., 1983). A number of prlA alleles have since been isolated and these suppress different signal sequence mutations to varying degrees. They do not however affect secretion of precursors synthesised with non-defective signal sequences. This suggests that an additional component of the secretion machinery may be required to mediate the interaction between the signal sequence and prlA gene product in order to promote secretion (Randall et al., 1987). Ito et al. (1983) isolated a similar mutation, termed secY, by localised mutagenesis around the 72 min region and subsequently screening for temperature sensitive mutants that accumulated precursor to MBP in

the cytoplasm. Further studies confirmed that this secY mutant was conditionally defective in the processing and localisation of many envelope proteins at 42°C (Ito, 1984; Shiba et al., 1984b). Few inner membrane proteins have yet been studies in secY mutants, but it appears that in addition to being secA independent, M13 coat protein is also secY independent (Wolfe et al., 1985). In contrast however, leader peptidase requires secY to assemble into the inner membrane (Wolfe et al., 1985).

Similar to observations made in secA mutants, the synthesis of some but not all, envelope proteins studied appears to be affected in secY mutants. The synthesis of OmpA is not significantly altered (although translocation is inhibited) when the mutant is shifted to 42°C (Wolfe et al., 1985). In contrast, both the synthesis and translocation of MBP was reported to be rapidly reduced.

Further genetic analysis demonstrated that secY was identical to the prlA gene. The molecular weight and amino acid composition deduced from the DNA sequence of the cloned gene (Cerritti et al., 1983) indicated that SecY had a molecular weight of 49kD and clearly was not a ribosomal protein, although the gene was located at the end of the spc ribosomal gene cluster. Normally, expression of secY was translationally coupled to the upstream genes of the operon (Ito, 1984) but the cloned gene could be amplified by expressing from the lac promoter. Although over-expression of SecY was lethal for the cell, this method was used to identify the protein as an integral cytoplasmic membrane protein (Akiyama and Ito 1985). The hydropathic profile of the protein, deduced from DNA sequence data, has features similar to that of lactose permease (Foster et al., 1983) and contains several hydrophobic segments of 20-30 amino acids residues in length (Cerretti et al., 1983). This suggests that the protein may span the membrane several times (Akiyama and Ito, 1985).

SecY appears to be less stable than is usual for membrane proteins with a half life of 20-30 min (Ito, 1984). However, a small fraction of wild type, pulse-labelled SecY shows enhanced stability and this suggested that the stable fraction might represent protein involved in the hypothetical translocation complex.

No unequivocal role has been established for SecY but it seems that the protein may normally be involved in a closely coordinated interaction between nascent protein, ribosomal complexes and the cytoplasmic membrane. Evidence in support of this comes from the observation that ribosomes synthesising MBP carrying a defective signal sequence do not become membrane bound. In fact, increased synthesis of MBP on membrane bound polysomes can be observed in secY strains which also harbour prlA suppressor alleles (Rasmussen and Bassford, 1985). This suggests that the prlA (secY) gene product is involved in both recognition of the signal sequence and the process of binding the ribosome to the membrane. In this context, SecY may have a role similar to that of the eukaryotic ribophorins (Akiyama and Ito, 1985), integral membrane proteins specifically found in the rough endoplasmic reticulum (Kreibich et al., 1978), which are involved in vectorial translocation of exported proteins. Alternatively, in view of the highly amphiphilic nature of the SecY protein and the likelihood that it crosses the membrane several times, it is possible that the protein forms a proteinaceous pore which allows the passage of polypeptides through the membrane as originally postulated in the signal hypothesis (Blobel and Dobberstein, 1975).

III iii d SecB

The analysis of the secB gene, including the isolation of null

mutations (Oliver et al., 1982), indicated that this was a non-essential

gene which encoded a 12kD protein (Kumamoto and Beckwith, 1985). In

contrast to the more general effects observed in secA and secY strains

(Ito, 1984; Oliver and Beckwith, 1982), mutation in secB causes

precursor accumulation of only a subset of envelope proteins. The outer

membrane proteins OmpF, LamB and the periplasmic MBP were inhibited in

their secretion, but export of alkaline phosphatase and ribose binding

protein (RBP) was unaffected. This suggested that there might be

branches within the major export pathway.

The role of SecB in protein export is nevertheless not yet clear.

The close proximity of the secB gene to the rfa locus, which is involved

in the biosynthesis of lipopolysaccharide (Kumamoto and Beckwith, 1985),

may indicate that the assembly of some proteins and other surface

components share a common mechanism. Since secB is not apparently

essential for cell viability (Kumamoto and Beckwith, 1985) any major role

in surface assembly is however questionable.

III iii e Isolation of mutations which are suppressors of secA or secY

Isolation of suppressor mutations capable of reversing the export

defects in known secretion mutants is a useful means of identifying both

new export genes and also establishing which proteins might interact in

the secretion pathway. Disappointingly, most mutations isolated in this

way appear to be involved in protein synthesis rather than export per se.

For example, ssyA (a suppressor of secY), which maps separately from

other protein synthesis genes at 54.5min on the E.coli chromosome,

affects synthesis of not only envelope proteins but total protein

synthesis (Shiba et al., 1984b). This suggested that the altered ssyA

gene product may interact with ribosomes and so help to promote binding

to a defective SecY protein.

A second gene, secC, shows similar cold sensitive mutations to

ssyA. The mutant gene was identified by its ability to suppress defects

in secA^{ts} mutants (Ferro-Novick et al., 1984). The mutation maps at

68.5min and is allele specific, suggesting a specific interaction between

SecA and SecC. However, again synthesis rather than the export of

envelope proteins is inhibited in secC mutants although in this case the

effect is limited to envelope proteins.

In addition to mutations which cause a defect in protein synthesis,

chloramphenicol, an inhibitor of protein synthesis, also reverses the

effect of secA^{ts} mutants when added at low concentrations (Lee and

Beckwith, 1986). Consequently, these authors have suggested that any

reduction in protein synthesis may be able to compensate for the

secretion defect observed in secA mutants. Therefore, a defective

secretion machinery may perhaps function better if fewer proteins are

synthesised. Alternatively, if the rate of peptide bond formation is

reduced, nascent polypeptides may remain in conformationally competent

states for longer periods.

In addition to secC, a number of new prlA alleles were identified

as suppressors of secA defects (Brickman et al., 1984; Bankiatis and

Bassford, 1985). This strongly suggests that SecY and SecA are

components of the same pathway and interact to promote protein

translocation. Such an interaction seems increasingly likely in view of

the fact that changes in secA levels can suppress defects caused by

mutant prlA (Oliver and Liss, 1985a). However, isolation of other

suppressor alleles also identified a new locus, prlD, which was capable

of suppressing the effects of prlA mutations (Bankiatis and Bassford 1985). PrlD is a weak suppressor of MBP signal sequence mutations but has no effect on wild type protein export. The mutation is also allele-specific and is able to suppress only certain signal sequence mutations. The gene maps at 2.5min on the chromosome, distinct from secA. Although prlD⁻ alone has no detectable effect upon secretion some elegant genetic studies by Bankiatis and Bassford (1985) have indicated that it may play an important role in secretion. Using double mutants they found that certain combinations of prlA-prlD alleles resulted in normal growth, which implied that prlA and prlD interacted. On the other hand, other pairs of these alleles did not restore secretion and therefore interaction appeared to be allele specific. This and other data was interpreted as indicating that both prlA and prlD may act co-ordinately, through interaction with the signal sequence, to promote the efficient formation of nascent polypeptide complexes to be exported.

IV Kinetics of Synthesis and assembly of outer membrane proteins

The specificity of assembly into the outer membrane (as discussed below) appears to be determined by unique structural features of outer membrane proteins. It may be speculated therefore that this specificity may be expressed during translation, resulting in direct extrusion to the outer membrane, or it may be expressed post-translationally, in which case proteins might reside transiently either in the periplasm or in the inner membrane before entering the outer membrane. One approach to the problem of distinguishing between these possibilities is to study simultaneously the kinetics of both synthesis and assembly of outer membrane proteins.

A direct measurement of the rate of synthesis of an outer membrane protein can be made by analysis of the appearance, following pulse-labelling, of a particular protein in total cell lysates viewed by SDS-PAGE and autoradiography. Then, the time taken for the radioactivity in a given band to reach a constant level during a chase in non-radioactive medium, constitutes the total time required for synthesis of that protein. For an envelope protein, comparison of the time taken for a protein band to reach maximum radioactive intensity in both the total lysate and in the isolated outer membrane fraction gives a direct estimation of the time for assembly of that protein. Such an analysis was carried out by Boyd and Holland (1980), with the OmpF porin from E.coli B/r. First of all, these experiments demonstrated that the time required for peptide bond formation of an outer membrane protein was not obviously different from that of a cytoplasmic protein. Thus, if protein translocation across the membrane is co-translational, the rate of peptide bond formation is not appreciably affected. Similar results were obtained for another outer membrane protein, TonA and also for the periplasmic protein MBP (Jackson et al., 1986; Bassford, 1982). The kinetic experiments with OmpF, also demonstrated that processing was extremely rapid and significant levels of precursor were not detected. More importantly, the processed porin was never detected in a membrane free form since the newly synthesised protein became associated with the sedimentable cell envelope with the same kinetics as that required for its synthesis. Thus, no soluble, i.e. periplasmic intermediate was detected. The OmpF porin, subsequent to processing did however pass through a Sarkosyl-soluble pool in the envelope before attaining its final association with the Sarkosyl insoluble outer membrane fraction. Two possibilities could explain these findings. The intermediate is

truly in the cytoplasmic membrane or alternatively that OmpF, during

synthesis or very shortly afterwards is assembled into the outer

membrane, becoming Sarkosyl insoluble only after non-covalent

interactions with neighbouring membrane components are established. In

view of the fact that the Sarkosyl soluble intermediate was also the

processed form of OmpF and therefore lacking the hydrophobic signal which

might anchor the protein in the membrane, Boyd and Holland (1980)

preferred the latter alternative. Such Sarkosyl soluble outer membrane

forms of OmpF have been reported when synthesised in the presence of

phenethyl alcohol (Halegoua and Inouye, 1979a). In this case the protein

accumulated in the outer membrane as defined by density gradient

sedimentation, yet was solubilised by Sarkosyl.

In order to determine the export pathway for TonA Jackson et al.

(1986) created a series of truncated TonA polypeptides by Bal31 deletion.

These deleted polypeptides were unstable in whole cells but could be

labelled with ^{35}S-methionine in maxicells and envelopes subsequently

separated into Sarkosyl soluble and insoluble fractions. Consistent with

the accumulation of an inner membrane intermediate in the pathway of

translocation of the TonA polypeptide to the outer membrane, the

truncates always appeared to fractionate solely with the Sarkosyl soluble

fraction. In contrast however, kinetic experiments in which cells were

briefly pulsed with ^{35}S-methionine and then chased in the presence of

cold methionine, indicated that although precursor was never detected,

mature TonA was detected in an intermediate, envelope free form following

processing (Jackson et al., 1986). It therefore seems unlikely that any

Sarkosyl-soluble intermediates are present in the inner membrane and it

has been proposed (Jackson et al., 1986) that the apparent inner membrane

localisation is due to the inability of deleted forms of the protein to

complete folding and form Sarkosyl resistant complexes in the outer membrane. Therefore, as discussed further in section V ii, TonA may be assembled via a periplasmic intermediate whereas OmpF, LamB and OmpA (Crowlesmith et al., 1981) may assemble only via membrane bound intermediates.

It is important to note however that other data, which will be further discussed in section VII, are consistent with periplasmic intermediates for several outer membrane proteins. For example, kinetic studies with LamB have shown that there is a 30-50s delay as detected by antibody, in the appearance of newly synthesised, mature molecules at the cell surface (Vos Scheperkeuter and Witholt, 1984). This delay between synthesis and insertion could be due to a transient accumulation in the periplasm or, as anticipated by Hall and Silhavy (1981a) in the cytoplasmic membrane. This latter possibility was supported by recent in vitro studies by Watanabe et al. (1986). They established that LamB synthesised in the presence of membrane, added in the form of inverted vesicles prepared from E.coli cytoplasmic membranes, cannot be extracted from the vesicles at alkaline pH. Since when sodium hydroxide is added to increase the pH vesicles break open and translocated proteins are extracted, it appears that LamB, which sediments with the membrane fraction behaves as an integral membrane protein in this system.

V   Factors determining protein localisation to the envelope

All envelope proteins are synthesised on cytoplasmic ribosomes and since most E.coli proteins are uniquely found in one compartment, a mechanism must exist whereby nascent or newly synthesised proteins can be "sorted" and subsequently correctly localised. Rare exceptions to the

one cellular compartment rule involve envelope proteins reported to be found in both the inner and the outer membrane. Colicin lysis protein may be located in both inner and outer membrane (Pugsley, 1984) and recently certain penicillin-binding proteins (Rodriguez-Tebar et al., 1985) have all been claimed to be present in both membranes. However, fractionation techniques are not always reliable, particularly in the case of some inner membrane proteins which are also peptidoglycan associated. Thus, incomplete hydrolysis of peptidoglycan during, for example, spheroplast formation may result in significant amounts of such proteins being recovered with the gradient fractions corresponding to the outer membrane (Osborn et al., 1972). In fact, in the case of the major penicillin-binding proteins, PBP5 and PBP6, studies in other laboratories separating envelopes by Sarkosyl (Spratt, 1977), Osborn fractionation (Koyasu et al., 1980) and flotation (Jackson et al., 1985) demonstrated their exclusive localisation to the inner membrane.

V i   Role of the NH$_2$-terminal signal sequence

Virtually all outer membrane proteins in E.coli are synthesised with a classical, NH$_2$-terminal signal sequence which is removed by processing. Apparent exceptions are various F$^+$ (fertility plasmid) associated transfer proteins including TraJ (Achtman et al., 1979) which does not possess a signal sequence although recent evidence suggests that TraJ may not in fact be an outer membrane protein (Cuozzo and Silverman, 1986). More surprisingly, phospholipase A, which has an NH$_2$-terminal signal sequence is localised to the outer membrane without processing (de Geuss et al., 1984).

In addition to outer membrane proteins, periplasmic proteins and some inner membrane proteins (Pratt et al., 1981a) are also synthesised with an NH$_2$-terminal extension. Consequently, several studies have attempted to establish whether the signal sequence contains information

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required both for the initiation of export and for subsequent partitioning, or alternatively, that the mature polypeptide contains additional signals ensuring final localisation. One method used to investigate the extent of the information contained within the signal sequence was to exchange signal sequences from different classes of envelope protein. Tommassen and Lugtenberg (1984) constructed a gene fusion consisting of most of the gene for pre-β-lactamase, a periplasmic protein, fused to the gene for the mature form of the outer membrane protein PhoE and found that the resultant hybrid protein was correctly localised to the outer membrane. This indicated that the signal sequence of a periplasmic protein could efficiently direct export of PhoE across the cytoplasmic membrane, but that additional information for final localisation in the outer membrane must exist in the mature protein. Similarly, the signal sequence of the outer membrane protein OmpF was exchanged for that of DacA, an inner membrane penicillin-binding protein (PBP5) which also requires a signal sequence for assembly. The resulting DacA-OmpF hybrid protein was assembled into the outer membrane in a functionally active form (Jackson et al., 1985). In this particular case rather strikingly, the DacA signal is considerably less hydrophobic than that of OmpF yet functions as an effective signal.

In contrast to these rather clear cut findings, it is interesting that Sjostrom et al. (1987) have recently reported that signal sequences of inner membrane, periplasmic and outer membrane proteins can nevertheless be assigned to relatively discrete groups based upon a number of criteria including size and hydrophobicity. The precise significance of these findings is not clear but may indicate that the composition of signal sequences may be required to reflect the overall properties of a given class of molecules rather than specificity in

final localisation. Thus conformational requirements at early stages of export of periplasmic and outer membrane proteins may demand specific interactions between portions of the mature protein and the signal sequence in addition to the role of the latter in directing the protein to the membrane. Some evidence for interaction of the signal sequence with the mature region has been established by Lehnhardt et al. (1987). These authors used oligonucleotide-directed site-specific mutagenesis to shorten the hydrophobic region of the OmpA signal sequence and subsequently fused wild type and mutant signals to the mature regions of either Staph. aureus nuclease A or TEM β-lactamase. The ability of the signal peptide to direct processing of the hybrid produced was dependent not only upon the length of the signal but also the protein to which it was fused. Different deletions were also shown to retard or promote processing efficiency to different extents in different hybrids. This suggested either that the signal interacts with the mature region or that the mechanism of membrane binding and translocation varies for each protein.

V ii   Attempts to identify additional topogenic sequences

a   Gene fusions

Since the signal sequence alone is not sufficient for localisation to the outer membrane, attempts were made to identify internal localisation signals within the mature protein, for example, by the construction of gene fusions. Such hybrid proteins generally consist of a constant portion of a cytoplasmic protein such as β-galactosidase, fused to varying lengths of an exported protein. Subsequent localisation experiments then attempt to define the minimum portion of the envelope protein required to export β-galactosidase from the cytoplasm.

Unfortunately, the results must be interpreted with caution since recent
data indicate that standard cell fractionation techniques can result in
mis-localisation of such abnormal proteins. A problem which may be
exacerbated if the bacteria are export defective and are in an abnormal
physiological state (Tommassen, 1986).

Normal outer membrane proteins can be separated from inner membrane
proteins on the basis of their insolubility in detergents such as
Sarkosyl or Triton X100 (Filip et al., 1973). Alternatively, the inner
and outer membranes can be separated on the basis of density (Osborn et
al., 1972). However, with either method overproduction of given proteins
or the production of abnormal polypeptides, can lead to spurious
localisation to the outer membrane fraction due to aggregation of such
proteins. This pitfall can be avoided by applying the envelope sample in
a dense solution of sucrose at the bottom of a sucrose gradient, allowing
subsequent density separation through flotation (Hirst et al., 1984).
Alternatively, providing sufficiently high levels of an envelope protein
are present, it is possible to localise a protein by immune electron
microscopy and labelled colloidal gold particles (Tommassen et al.,
1985).

Initial gene fusion experiments carried out mainly by Benson and
Silhavy, concentrated on establishing any localisation signals contained
within the mature portion of the outer membrane protein LamB. Fragments
of increasing size from the NH_2-terminal region of LamB were fused to a
large functional C-terminal portion of β-galactosidase. The lamB-lacZ
fusion produced a protein which could be induced by maltose and yet still
retained some of the inherent enzymatic properties of the β-galactosidase
molecule. By monitoring the maltose sensitivity caused by different
hybrids and identifying the apparent cellular location of these molecules

specific regions within the mature LamB protein were suggested to be involved in protein export (Hall et al., 1982, Benson and Silhavy, 1983, Benson et al., 1984).

Hybrid LamB-LacZ proteins which contained a complete LamB signal sequence plus as many as 27 residues of mature LamB remained in the cytoplasm and cells were resistant to maltose. However, the export of slightly longer fusions containing 39 residues of mature LamB was initiated and hybrid protein, which became stuck in the cytoplasmic membrane, caused cells to become maltose sensitive. This data suggested that residues 27-39 of the mature protein in addition to the signal sequence, are required for translocation through the cytoplasmic membrane. On the other hand Benson et al. (1984) claimed that fusions which contained a complete signal sequence plus 49 amino acids of mature LamB are localised to the outer membrane with low efficiency. Consequently, it was suggested that residues 39-49 in some way interact with the cells secretion machinery in order to direct LamB to the outer membrane. Benson et al. (1984) also used immunofluorescence to localise fusions and the results obtained appeared to be consistent with previous data and to implicate a region at the NH$_2$-terminus of the mature LamB protein in localisation.

In similar experiments using ompA-lacZ fusions it was also suggested that a small NH$_2$-terminal segment of mature OmpA contains a signal necessary for final localisation to the outer membrane (Palva and Silhavy, 1984). In contrast however, in a slightly different system, where LamB was fused to the periplasmic protein alkaline phosphatase (PhoA), 170 residues of mature LamB were required to localise the LamB-PhoA hybrid to the outer membrane (Oliver, 1985).

As indicated above, gene fusion experiments should be interpreted with care. When Tommassen et al. (1985) constructed a PhoE-LacZ hybrid protein containing the NH$_2$-terminal 300 amino acid residues of PhoE, fused to β-galactosidase, it was shown by Sarkosyl and sucrose density gradient fractionation experiments to localise to the outer membrane. In contrast, by using immuno-cytochemical labelling on ultra-thin cryosections it was shown that the hybrid was in fact in the cytoplasm, re-emphasising the fact that conventional cell fractionation experiments are not reliable under these conditions. More importantly, this result also indicated that PhoE-LacZ hybrid proteins can never be transported out of the cytoplasm. In fact, in contrast to the studies of Benson and co-workers, immunochemical labelling techniques showed that LamB-LacZ hybrid proteins are also cytoplasmic, (Tommassen 1986), again indicating that β-galactosidase fusion proteins cannot be exported from the cytoplasm.

Data also implicating possible sorting signals for outer membrane proteins close to the NH$_2$-terminus was obtained from a comparison of the amino acid sequences of the three outer membrane proteins OmpA, OmpF and LamB (Nikaido and Wu, 1984). Statistically significant levels of homology in specific regions were found among these proteins, one of which overlapped, in the particular case of LamB, with regions 39-49. Sequences with similar homology were found in PhoE but none in a number of cytoplasmic, inner membrane or periplasmic proteins examined. Later evidence, discussed in the next section was subsequently also to refute this indication of specific sorting sequences.

V ii b <u>Protein deletions</u>

The limitations of the gene fusion technique has encouraged the development of alternative methods to identify localisation signals. For example, internal deletions can be created in outer membrane or periplasmic proteins and localisation of the corresponding polypeptides can then be examined. Recent experiments of this type carried out with OmpA (Freudl <u>et al</u>., 1985) and PhoE (Bosch <u>et al</u>., 1986) have cast considerable doubt on the existence of discrete localisation sequences in the mature form of outer membrane proteins. Henning and his co-workers created two deletions in the region of residues 4-84 of OmpA and in both cases, the proteins were shown by immunocytochemical labelling and electron microscopy to be localised to the outer membrane. In contrast, OmpA protein with deletions in amino acid residues 86-227 or 160-325 (see Fig. 1.3) was found only in the periplasm (Freudl <u>et al</u>., 1985). This indicated that no single discrete sorting signal appears to exist between residues 4 and 228 of mature OmpA. In particular, this result demonstrates that the proposed sorting signal identified by amino acid homology, which in the case of OmpA has been proposed to lie between residues 1-14 (Nikaido and Wu 1984) cannot be essential for routing the protein.

DNA technology also allows the ready production of truncated polypeptides, resulting from deletion of DNA encoding portions of the C-terminal end of a protein. This technique has been extensively used by Henning and his co-workers in an attempt to establish the importance of the C-terminus in either the initial crossing of the inner membrane or the final localisation of OmpA to the outer membrane. These authors observed that when the final 190-325 residues of the mature protein were removed, the protein was still exported and the NH_2-terminal region

localised to the outer membrane (Bremer et al., 1982; Henning et al., 1983). This was perhaps not surprising for OmpA since the C-terminal domain of this protein from residue 177 is normally present within the periplasm. In the case of TonA, however, studies in this laboratory have shown that even deletion of extremely short sections of the C-terminus appeared to block assembly into the outer membrane (Jackson et al., 1986). TonA truncates are extremely unstable and could only be identified and localised in maxicells. Nevertheless, it was demonstrated that the truncated polypeptides were all still processed and it was concluded that the C-terminal region was required for correct assembly into the outer membrane but not for translocation across the inner membrane. Similarly, C-terminal deletions of PhoE do not prevent processing and in this case periplasmic accumulation of truncates was demonstrated (Bosch et al., 1986). More importantly, overlapping internal deletions which cover the complete phoE sequence were revealed by immunological labelling on ultrathin cryosections to produce proteins which were transported to and accumulated in the periplasm (Bosch et al., 1986). The mature PhoE protein cannot therefore have discrete intragenic localisation signals and it is now doubtful if a common sorting signal exists. It seems more likely that the overall conformation of an outer membrane protein such as PhoE, OmpA and TonA, rather than a particular sequence of amino acids is important for assembly into the outer membrane. These data, together with the kinetic studies described above, are also more consistent with the hypothesis that many outer membrane proteins are initially translocated to the periplasm rather than directly extruded into the outer membrane through specialised sites in the inner membrane. Even in the case of OmpF, where no membrane free intermediate can be detected in vivo, other data support a periplasmic (perhaps

extremely short lived) intermediate. Thus, OmpF is exported efficiently to the medium when synthesised in spheroplasts, lacking an outer membrane (Metcalfe and Holland, 1980). Moreover, in contrast to the findings of Watanabe et al. (1986) with LamB, when OmpF and TonA are synthesised in vitro in the presence of inner membrane vesicles, the processed polypeptides can all be extracted from the vesicle lumen (J. Pratt and M Jackson, pers. comm,). The assembly of the LamB protein may therefore have features which differ from the assembly of other outer membrane proteins.

The signficance of the C-terminal region of the periplasmic protein TEM-β-lactamase has also been examined with respect to translocation across the inner membrane. TEM-β-lactamase exists in the periplasm as a monomer (Richmond, 1975) and it is important in conferring ampicillin resistance on cells. The protein is encoded by the bla gene and is synthesised as a high molecular weight precursor (Ambler and Scott, 1978) with an NH_2-terminal signal sequence 23 amino acids in length (Sutcliffe, 1978). In order to determine the importance of the C-terminus of this protein during translocation, Koshland and Botstein (1980) created a series of deletions in the β-lactamase secreted by the Gram negative bacterium Salmonella typhimurium. In initial experiments truncated β-lactamase failed to be released from the periplasm upon osmotic shock, which appeared to suggest that removal of as few as 21 amino acid residues from the carboxy-terminus were sufficient to inhibit transport across the cytoplasmic membrane although the truncated proteins were processed by removal of the NH_2-terminal signal sequence. Subsequently however, trypsin accessibility experiments using both intact and lysed spheroplasts prepared from cells synthesising prematurely

terminated β-lactamase, indicated that the truncated polypeptides are in reality exposed to the periplasm and it seems, that in intact spheroplasts, the truncates are weakly bound to the surface of the membrane. This weak interaction is disrupted when the cell undergoes osmotic lysis giving the protein an apparently cytoplasmic localisation. The absence of any large protected fragments of β-lactamase in the trypsin acessibility experiments indicates that the bulk of the protein is on the periplasmic side of the membrane and the carboxy-terminus is not required for translocation (Koshland and Botstein, 1982). A possible explanation for these conflicting results, is that the altered carboxy-terminus may reduce the efficiency of release of the translocated protein from the inner membrane and thus processed β-lactamase remains attached at the periplasmic surface of the inner membrane (Koshland and Botstein, 1982).

V iii <u>Protein localisation in eukaryotes</u>

Protein targeting in eukaryotes is a complex process, since unlike prokaryotes, eukaryotes contain a number of different membranous organelles. For example, a single eukaryotic cell may contain, in addition to the endoplasmic reticulum (ER), mitochondria, chloroplasts and peroxisomes. Transport to peroxisomes has been little studied and will not be discussed here (see e.g. Fujiki and Lazarow, 1985) but the biogenesis of chloroplasts and mitochondria has been extensively studied in recent years.

Although both organelles synthesise a proportion of their own proteins, as many as 90% of chloroplast and mitochondrial proteins, are encoded on nuclear chromosomes and imported after synthesis on

cytoplasmic ribosomes (Chua and Schmidt, 1979). Since both organelles comprise a number of discrete compartments this requires the transport of a wide variety of proteins to numerous specific locations, with up to three membrane bilayers present between the site of synthesis and function of a given protein.

A comparison of protein transport into chloroplasts and mitochondria reveals several common features (see e.g. Colman and Robinson, 1986) and although much of the following relates mainly to mitochondria, many of the basic principles are also applicable to chloroplasts and evidence suggested that presequences of chloroplasts and mitochondria may even be interchangeable (Hurt et al., 1986).

The majority of imported mitochondrial proteins are synthesised as larger precusors (Bohni et al., 1983) with a presquence which is cleaved by a signal peptidase located in the matrix (Maccecchini, 1979). The small number of proteins synthesised without NH_2-terminal extensions are largely proteins of the mitochondrial outer membrane (Freitag et al., 1982; Gasser and Schatz, 1983). Deletion analyses and gene fusion experiments have shown that the presequence is necessary for localisation and is sufficient to target passenger proteins to the expected suborganellar destination (Emr et al., 1986; van Loon et al., 1986; Ngugen et al., 1987).

The presequences of mitochondria have been carefully characterised and surprisingly seem to have little sequence homology. Presequences usually have a net positive charge and overall composition of predominantly polar residues interspersed with 1-3 hydrophobic amino acids (Roisie et al., 1986) to give the presequence an amphiphilic property. It seems therefore that aspects of secondary or tertiary structure are crucial for import function. Although no specific receptor

for the presequence has yet been identified, the observation that specific presequences can completely inhibit transport of proteins to the mitochondrion may be evidence for such receptors in the mitochondrial membrane (Gillespie et al., 1986).

Import of proteins to each mitochondrial compartment displays unique features but to a large extent import to the mitochondrial inner membrane and matrix appears to be analogous. Import is almost invariably dependent on the presence of a cleavable NH_2-terminal presequence and has a requirement for both ATP and an electrochemical gradient (Schleyer and Neupert, 1985; Eilers et al., 1987). The exact function of the ATP has not yet been established but it seems that mitochondrial proteins must at least partially unfold prior to translocation (Vassarrotti et al., 1987 and section VIii) and energy may be required for this unfolding (Chen and Douglas, 1987).

Studies on intermembrane space proteins such as cytochrome b_2 and cytochrome b_1 indicate that these proteins may be processed in two steps (Gasser et al., 1982). The first cleavage is carried out by the matrix-localised peptidase, indicating that precursors are at least partially imported into the matrix and a second processing step is thought to be mediated by a peptidase outside the matrix, although such a proteins has not yet been characterised (Reid et al., 1982).

The assembly of proteins into the outer membrane differs since there is no apparent energy requirement (Freitag et al., 1982; Hay et al., 1984) or cleavable presequence (Hay et al., 1984), although topogenic information may still be contained within the NH_2-terminal of the mature proteins (Hase et al., 1984). Import into mitochondria therefore appears to involve the following sequence of events; the NH_2-terminal presequence may initiate translocation and it is assumed that the

presequence is recognised by, and binds to receptors on the mitochondrial

surface (Hartl et al., 1986). Translocation across the membrane can then

proceed. During or after this process, which is dependent on an

electrochemical potential across the mitochondrial inner membrane (Gasser

et al., 1982) the presequence is removed by proteases present in the

matrix (Bohni et al., 1983) and/or intermembrane space (Hay et al., 1984)

and the protein is released into the correct mitochondrial compartment.

VI   Are outer membrane and periplasmic proteins normally assembled via a

      post-translational or co-translational mechanism?

Although a number of components of the secretion apparatus in both

prokaryotes and eukaryotes have now been identified, there has been

considerable controversy over whether protein export is co-translational

or post-translational. In the eukaryotic endoplasmic reticulum the early

evidence indicated that there is an obligatory coupling of the emergence

of a nascent polypeptide from the ribosome with extrusion across the

membrane. However, recent discoveries have shown that human glucose

transporter (GT) protein can be transported post-translationally into

microsomes in an SRP mediated manner (Mueckler and Lodish, 1986). In

addition, SRP can cause translational arrest of IgG and bovine prolactin

precursor after two thirds of the proteins have been synthesised (Ainger

and Meyer, 1986). Such findings have raised serious questions regarding

the nature of the early stages of protein export. Moreover, as discussed

below, strict coupling of the synthesis and export of periplasmic and

outer membrane proteins may not be essential in E.coli. Nevertheless,

this mode of translocation was favoured in the direct transfer model (von

Heijne and Blomberg, 1979) and the helical hairpin hypothesis (Engelman

and Steitz, 1981). The former proposed that the energy of elongation of the nascent chain derived through peptide bond formation is required to push the polypeptide through the bilayer. Consequently, translocation would then depend on a tight association between the ribosome and the membrane. In the latter hypothesis the distribution of polar and non-polar sequences in the polypeptide is important for insertion into the membrane. Halegoua and Inouye (1979b) also proposed a modified version of the signal hypothesis, the loop model, which similarly emphasises the role of polar residues in the initial membrane interaction. According to this model the basic NH_2-terminal region remains tightly bound to the cytoplasmic face of the membrane and the hydrophobic portion subsequently inserts into the bilayer as a loop, aided by the α-helix breakers proline and glycine. Processing can then occur and translocation continues in a vectorial fashion driven by elongation of the polypeptide through peptide bond formation. However, more recent evidence now appears to favour some aspects of the membrane trigger hypothesis for protein translocation in E.coli. This mechanism, proposed by Wickner (1979) in order to explain the clearly post-translational assembly of the small coat protein of bacteriophage M13, emphasises the existence of assembly information in the mature sequence and the importance of folding and hydrophobic interaction between the polypeptide and the hydrocarbon core of the membrane bilayer. Such a self assembly mechanism independent of translation, is nevertheless not necessarily post-translational and can be extended to include co-translational export by proposing that the conformation necessary to trigger insertion can be achieved before the protein is fully elongated (see section VI i).

VI i <u>Evidence for co-translational translocation</u>

The case for a co-translational mode of export was first postulated on the basis of the analogy drawn between the prokaryotic and eukaryotic secretion systems and the fact that many <u>E.coli</u> envelope proteins are synthesised on membrane bound ribosomes (Randall and Hardy, 1977). Moreover, direct evidence for co-translational translocation was obtained in 1977 by Davis and his co-workers for the periplasmic protein alkaline phosphatase. They showed that if spheroplasts were treated with a radioactive reagent which was unable to cross the membrane, a range of sizes of proteins were labelled. These proteins had incorporated radioactivity at different stages of synthesis and as a result displayed a broad molecular weight distribution (Smith <u>et al</u>., 1977). This indicated that part of the nascent chain was outside the inner membrane and accesssible to the external reagent and translocation must therefore be co-translational.

By analysis of the time of removal of the NH_2-terminal signal sequence from nascent chains in exponentially growing cultures of <u>E.coli</u>, Joseffson and Randall (1981) demonstrated that processing could occur either co- or post-translationally for a given protein. The time of processing did not however appear to correlate with the ultimate location of the polypeptide. For example, AmpC-β-lactamase was processed co-translationally whereas TEM-β-lactamase was processed entirely post-translationally, although both are periplasmic proteins. Many proteins however, such as OmpA, LamB and MBP appeared to be able to be processed either before or after completion. However, processing never occurred before the protein had reached 80% of its final length. These results were consistent with co-translational export at least under some conditions. However, these results also indicated that the nascent chain

may be inaccessible to certain components of the secretion machinery until a critical length has been reached or alternatively may reflect a requirement for a particular protein conformation for successful translocation.

VI ii Evidence for post-translational protein export

The first direct observations of a post-translational mode of translocation in bacteria came from studies involving M13 procoat (reviewed by Wickner, 1983). M13 procoat is the precursor of the phage coat protein and it contains a typical cleavable signal sequence although the mature protein remains transiently in the cytoplasmic membrane prior to assembly into M13 phage particles. In contrast to other E.coli envelope proteins the mechanism of procoat insertion appears to involve synthesis on membrane free polysomes, presumably due to the small size of the polypeptide. In fact, fully synthesised procoat should be released from ribosomes before an interaction of the signal sequence with any secretory apparatus can take place. The free polypeptide then appears to partition into the cytoplasmic membrane although this process is still dependent on membrane potential (Kuhn et al., 1986). M13 coat protein assembly, however, differs from that of other envelope proteins in being ATP, secA and secY independent (Wolfe et al., 1985). As a result of these special features it is not clear how far the M13 model can be applied to the export of periplasmic and outer membrane proteins. M13 procoat export may rather be analogous to export of the eukaryotic protein prepromelittin which contains only 70 residues (Suchanek et al., 1978) and unlike other larger eukaryotic proteins has been shown to interact with microsomes independently of SRP (Zimmermann and Mollay, 1986).

However, other examples of post-translational assembly have been demonstrated. Thus in S.typhimurium, a Gram negative bacterium closely related to E.coli, Koshland and Botstein (1982) observed, by means of pulse-labelling and trypsin protection experiments in vivo, that TEM-β-lactamase was rapidly processed and localised post-translationally to the periplasm. More surprisingly, Zimmerman and Wickner (1983) reported that in vitro, pre OmpA can be post-translationally processed by liposomes reconstituted with purified leader peptidase. Moreover, the processed form was then resistant to protease, demonstrating that translocation across the bilayer could occur spontaneously.

Randall (1983) also investigated the temporal translocation of polypeptides across the cytoplasmic membrane of E.coli on the basis of accessibility of nascent chains of periplasmic proteins to externally added proteinase K. After converting cells to spheroplasts, it was established that mature MBP and RBP were accessible to protease while nascent chains were only degraded subsequent to attaining their full molecular length and processing of the signal sequence. This data was interpreted to indicate that nascent chains of exported proteins remain on the cytoplasmic side of the membrane until the time of maturation. This observation supports the idea that entire domains of a polypeptide are translocated after synthesis since if extrusion were simultaneous with elongation, nascent chains would appear on the outside as soon as there were sufficient amino acids to span the bilayer.

A more extensive analysis of the kinetics of post-translational export in bacteria has been achieved in pulse-chase experiments in cells which are defective either in some component of the secretion apparatus or in the signal sequence of the exported protein (Wolfe et al., 1985; Ryan and Bassford 1985; Bacallao et al., 1986). Bacallao et al. (1986)

cloned wild type secY under lac transcriptional regulation so that

synthesis could be induced by the addition of IPTG. Functional SecY was

then introduced into a secY^{ts} strain growing at the restrictive

temperature. The processing of precursor accumulated under SecY

limitation was then examined at 42°C. Data obtained in these experiments

indicated that OmpA could translocate across the cytoplasmic membrane

post-translationally. MBP synthesised with a defective signal sequence

was slowly but inefficiently exported post-translationally when examined

during a chase period after first labelling with ^{35}S- methionine (Ryan

and Bassford, 1985). Nevertheless, in most of these experiments, some

precursor always apparently accumulated in a translocationally

incompetent form in the absence of SecY.

VI iii Polypeptide structure may determine translocational competence

Recent studies by Randall and Hardy (1986) on the periplasmic MBP

support the view that conformational changes are linked to MBP

translocation. Evidence for the importance of folding in protein export

has also been obtained on the basis of experiments carried out with

dihydrofolate reductase (DHFR) fused to a mitochondrial protein

presequence (Eilers and Schatz, 1986). Such a purified fusion protein

was imported into energised mitochondria. However, when a low molecular

weight antifolate agent capable of binding to DHFR was added to the

system import was then blocked. In this case therefore, rather than

never adopting a tertiary structure the results indicate that the protein

may be required to unfold in order to be transported across the

mitochondrial membrane. In turn, this implies that some component of the

putative export apparatus induces conformational changes if not extensive

unfolding in the protein during or before translocation.

An export mechanism involving either unfolding or prevention of folding, would eliminate any mechanistic differences between co- and post-translational translocation. Size and amino acid sequences may then be important determinants in the mode of translocation. A large protein, for example, may have several domains which fold independently and the secretion apparatus as suggested by Randall et al., (1987) may normally be capable of maintaining only the first domain in an unstructured form. If a polypeptide is synthesised at membrane export sites and translocated co-transationally, folding of C-terminal domains could be avoided. Finally, the stability of individual proteins may also dictate the degree to which the synthesis of polypeptides must be temporally coupled to transfer.

Further evidence that an interaction with the secretion machinery is not simply a function of the signal sequence comes from gene fusion experiments in which the signal sequences of secY independent M13 procoat and secY dependent OmpA have been exchanged (Kuhn et al., 1987). M13 coat protein with an OmpA signal sequence is still secY independent, whereas OmpA with an M13 signal requires a functional SecY protein. More importantly, both fusion proteins are exported with reduced efficiency, indicating that the mature region and signal sequence both contribute to the secretion process. The importance of the overall conformation of envelope protein precursors could also explain the relative heterogeneity of signal sequences and the variation in efficiency of export of different proteins.

VI iii Energy requirement for translocation

Although all exported proteins may interact with a complex secretion apparatus in order to facilitate secretion it seems likely that some form of energy must also be required for translocation across the

cytoplasmic membrane. The energy released through peptide bond formation, combined with tight binding of ribosomes to the membrane was originally proposed to promote the stepwise transfer of amino acids through the bilayer (von Heijne and Blomberg, 1979, Gilmore and Blobel, 1985). However, since synthesis and transport of envelope proteins are not necessarily tightly coupled, other mechanisms of providing energy must exist. Energy is indeed required in vivo for protein export since the addition of ionophores or uncouplers such as CCCP, DNP, valinomycin or ethanol to a growing culture, cause the cytoplasmic accumulation of precursors of exported proteins (Date et al., 1980; Enequist et al., 1981; Pages and Lazdunski 1982). In experiments in which the level of intracellular ATP was normal it was also shown that inhibition of export was not a result of secondary depletion of ATP (Bakker and Randall, 1984). Thus it was concluded that membrane potential was also required.

By the use of cell free translation systems (see section VII) in which synthesis can be coupled to export into inverted inner membrane vesicles it was possible to identify an ATP requirement in the process of translocation and to analyse the latter in more detail. Thus in vitro translocation of the outer membrane protein OmpA across the vesicular membrane was found to be absolutely dependent on ATP (Chen and Tai, 1985, Geller et al., 1986), but an additional requirement for a proton motive force was also demonstrated. Similarly, an OmpF-lipoprotein chimeric protein was shown to require both ATP and the proton motive force for translocation into vesicles (Yamane et al., 1987). As yet, however the mechanism whereby both ATP and proton motive force provide energy for translocation is not clear.

VII   Development of in vitro systems to study translocation of envelope proteins

Although a number of individual gene products involved in protein export in E.coli have now been identified, the exact function of the different components is not clear and it seems likely that in the future the exploitation of in vitro systems will be necessary in order to assign a role to each protein involved.   Such an in vitro system has been developed (Pratt, 1984) as a modification of the Zubay system (Zubay, 1973) and has been used extensively to study protein translocation.   The system consists of an S30, cell free extract of E.coli, capable of coupled transcription and translation of polypeptides directed by added DNA, supplemented with purified inverted inner membrane vesicles (Pratt et al., 1981a; Pratt, 1984).   Using this system efficient translocation and processing of both the inner membrane protein DacA and the outer membrane proteins, OmpF and TonA has been demonstrated (Pratt et al., 1981b; Pratt et al., 1986).   Processing efficiency can also be slightly enhanced by increased levels of leader peptidase (J. Pratt and M. Jackson, pers. comm.).   Importantly, in these in vitro experiments it was possible to demonstrate that the processed polypeptides could be recovered in re-isolated vesicles in a form inaccessible to protease demonstrating that translocation had also taken place.   In more recent studies the same systems have been used to demonstrate that translocation of another E.coli envelope protein, LamB, can be uncoupled from synthesis by subsequent addition of vesicles to the extracts (Muller and Blobel, 1984b).   Interestingly, the authors reported that in this case the system became saturated when only 25% of the molecules had been translocated.   Further addition of vesicles failed to increase processing.   This strongly suggested that other components were required

for efficient translocation providing evidence for a soluble component of the secretion machinery. The results also revealed that a small number of the translocated chains which were protease resistant also remained uncleaved. This indicated that translocation and processing are also not necessarily coupled events. This is consistent with the finding that some signal sequence mutations affect processing but not translocation (Silhavy et al., 1983). The signal sequence therefore, although important for translocation, (perhaps by recognising the secretion machinery or by inhibiting folding), need not be cleaved in order for successful translocation to take place. Uncleaved chains are probably anchored by the signal sequence to the membrane and the role of cleavage may be to release protein into the periplasm. Other studies, as indicated in the previous section, have enabled several groups to investigate the energy requirements for protein export in vitro systems (Chen and Tai, 1985; Geller et al., 1986; Yamane et al., 1987).

The use of in vitro systems has already allowed the identification of a soluble export factor by complementation of an export-deficient (salt washed), reconstituted cell-free system (Muller and Blobel 1984a). Under the conditions used, DNA-directed transcription-translation occurred only upon addition of both inverted vesicles and a 12S soluble export factor. Due to its high sedimentation coefficient, the latter was suggested to consist of a complex of molecules, but whether or not it is in any way related to the genetically defined components of the bacterial export pathway has not been established.

VIII   Secretion of proteins to the external medium

The previous sections have concentrated largely on the export

and assembly of envelope proteins, but in fact a small number of proteins

synthesised in Gram negative bacteria are able to cross both the inner

and outer membranes either to reach the external medium or to assemble on

the cell surface. The initial stages in export of these proteins appears

to be analogous to export of envelope proteins (Pugsley and Schwartz,

1985) but some additional mechanism must promote passage through the

outer membrane, which as described in section I, provides an effective

barrier against the release of the vast majority of proteins.

VIII i   Assembly of surface structures

Pili and fimbriae are filamentous appendages which protrude from

the surface of Gram negative bacteria. K88ab fimbriae are composed of

helically arranged identical 17kD subunits (Dodd et al., 1984) and once

assembled are totally reistant to disaggregation and denaturation by SDS

(Laemmli, 1970). Very little is known concerning the mechanism by which

fimbrial subunits are translocated across the outer membrane and

assembled but it appears that four or five gene products are required

(Dougan et al., 1983). The K88ab subunit is probably transported across

the cytoplasmic membrane co-translationally by means of a cleavable

signal sequence and once in the periplasm it may interact with two

proteins of molecular weight 17kD and 27kD respectively and possibly a

third of molecular weight 17.2kD (Mooi et al., 1982). These polypeptides

may be involved in modification of the subunit and assembly into fimbriae

during or after translocation through the outer membrane. Thus it

appears that fimbriae are assembled into the membrane surface in a two

step process (Mooi et al., 1984).

The F pilus of E.coli is also synthesised as homogeneous
subunits in the cytoplasm and translocated to the membrane surface but
again the mechanism of assembly remains obscure. The traA gene encodes
the F-pilin protein (Minkley et al., 1976; Kennedy et al., 1977) and
specifies a 14kD protein, prepilin, which is processed by TraQ to
produce a 7kD mature protein. As many as thirteen tra gene products are
required for assembly and it has been proposed (Ippen-Ihler, 1985) that
TraJ may span both inner and outer membranes and form a channel for
export of the pilus subunits. The reported localisation of TraJ, which
is also required for transcriptional activity of the tra operon, to the
outer membrane has recently been challenged (Cuozzo and Silverman, 1986)
and its role in an export mechanism is now uncertain.

VIII ii Secreted proteins in Gram negative bacteria

Probably in consequence of the additional permeability barrier,
provided by the outer membrane, the variety of proteins secreted in Gram
negative bacteria is small compared to the large numbers released from
Gram positive bacteria. However several secretion systems in Gram
negative bacteria have been extensively studied in recent years,
particularly in bacteria other than E.coli. Thus, pullulanase is
secreted by Klebsiella pnuemoniae/ aerogenes (Eisele et al., 1972),
Pseudomanas aeruginosa secretes toxin A (Lory et al., 1983) and cholera
toxin is secreted by Vibrio cholerae (Hirst et al., 1984). Due to the
commercial possibilities associated with proteins which are secreted into
the medium, the mechanism of release is now providing great interest,
however, as indicated below, the mechanism of translocation of these
proteins across the outer membrane has not yet been established.

Most proteins secreted by Gram negative bacteria are synthesised with an NH_2-terminal signal sequence and translocation across the inner membrane to the periplasm is mediated by this signal. The subsequent process of transfer across the outer membrane, however, appears to differ for each protein and may involve specific outer membrane proteins (Pugsley and Schwartz, 1985). For example, pullulanase accumulates on the cell surface during exponential growth with subsequent release into the medium after a considerable delay (Wohner and Wober, 1978) and some recent data even indicates that release may involve shedding of portions of the outer membrane into the medium (d'Enfert et al., 1987). Moreover, several genes appear to be involved in the secretion mechanism (M. Kornacker pers. comm.).

In contrast, it seems that the potent cholera enterotoxin synthesised by Vibrio cholerae, transiently enters the periplasm before crossing the outer membrane. Interestingly, E.coli synthesises a heat-labile enterotoxin (LT) which is strikingly similar in structure and function to cholera toxin (Hirst, 1986) but in contrast to cholera toxin, LT is only exported as far as the periplsmic space and cannot cross the outer membrane. Both LT and cholera toxin are comprised of 5 identical B subunits which bind to G_{H1} ganglioside receptors, and a single A subunit that catalyses ADP-ribosylation of adenylate cyclase. In E.coli, A and B subunits have signal sequences of 18 and 21 amino acids respectively and precursors are translocated through the cytoplasmic membrane and proteolytically processed before mature polypeptides are released into the periplasmic space. Mature monomers enter a pool of assembling subunits which gives rise to the holotoxin (AB_5) within the periplasmic space. In V.cholerae, the entire structure may then be secreted by a process which remains obscure. In E.coli the absence of

specific outer membrane export components, or the actual nature of the
E.coli outer membrane may prevent final translocation to the medium.

Exotoxin A, synthesised by Ps.aeruginosa, exhibits yet another
mechanism of release and appears to be co-translationally secreted
directly into the medium. It is suggested that this release is via
Bayer-like junctions, although this has not been confirmed and once more
the actual mechanism remains obscure (Lory et al., 1983).

One secretion system recently studied in detail is that of the
Gram negative bacterium Erwinia chrysanthemi. E.chrysanthemi, is of
particular interest since it is the causal agent of soft rot in a variety
of plants (Starr and Chatterjee, 1972). The bacterium is in fact capable
of producing several enzymes including pectinases, cellulases and
proteases, all of which depolymerise plant cell wall constituents.
Although several enzymes are released to the medium, extracellular
enzymic activity in these bacteria is specific and no β-lactamase
activity has been detected in the supernatant. It appears that export
components are required for transfer across the outer membrane and
secretion defective mutants which accumulate pectinase and cellulase
activity in the periplasmic fraction have been isolated (Andro et al.,
1984). These mutations have no effect on either synthesis or export to
the periplasm but the fact that export of cellulolytic and pectolytic
enzymes is inhibited by a single mutation has implicated a common step
for export of these enzymes to the external milieu (Thurn and Chatterjee,
1985). It has been proposed therefore that synthesis and translocation
across the inner membrane precede interaction with components which
direct translocation across the outer membrane and release into the
growth medium. When such Erwinia genes are expressed in E.coli the
enzymes are exported to the periplasm but not to the external medium,

suggesting that E.coli either has a significantly different outer

membrane or lacks genes which specify export components required for

transfer across the outer membrane (Thurn and Chatterjee, 1985).

In fact, there are several examples of toxins and enzymes which

are exported to the medium in Gram negative bacteria, but invariably

accumulate in the periplasm when expressed in E.coli. This indicates the

requirement for specific components in the normal host required for the

translocation of these proteins from the periplasm across the outer

membrane (Pugsley and Schwartz, 1985). One exception to this rule may

however be IgA1 proteases. IgA1 proteases are extracellular bacterial

enzymes that cleave the heavy polypeptide chain of human IgA1 at sites

within the hinge region (Kilian et al., 1979). These enzymes may be

determinants of virulence since they are elaborated from several human

pathogens such as Neisseria gonnorhoeae and also by clinical isolates of

Haemophilus influenzae (Bricker et al., 1986). IgA proteases have been

cloned and shown to be active in E.coli, but there are conflicting

reports as to whether of not the protein is exported beyond the

periplasm. Early evidence (Bricker et al., 1986) suggested that E.coli

could not release IgA proteases cloned from H.influenzae across the outer

membrane. More recently, in the case of an N.gonorrhoae enzyme, it has

been proposed that once the protease has reached the periplasm, an

amphiphilic C-terminal "helper" domain within the mature protease is able

to insert spontaneously into the outer membrane and form a pore for the

secretion of the protease domain (Pohlner et al., 1987) which is released

in a final autoproteolytic step. Moreover, this two step secretion

process is also reported to occur, but with reduced efficiency, when the

protease gene is expressed in E.coli (Halter et al., 1984) and may

represent a unique method of secretion in Gram negative bacteria.

Only two classes of protein, colicins (see review by Pugsley, 1984), and haemolysin, are however secreted from E.coli into the environment and the mechanism of secretion of these two types of protein is very different.

The first class of proteins, colicins, are bacteriocidal factors and their synthesis and secretion is a complex process involving a number of different components and requiring derepression of several genes. Inactivation of the colicin operon repressor (e.g following DNA damage) allows the synthesis of large amounts of colicin and also the so called colicin lysis protein which has been localised to both inner and outer membranes (Cole et al., 1985; Oudega et al., 1984). This protein, unlike colicins themselves, is synthesised with a classical NH_2-terminal signal sequence. The subsequent release of colicins, which appear to accumulate in the cytoplasm, is absolutely dependent upon the lysis protein. The precise role of the lysis protein is unclear but activation of a cellular phospholipase in the outer membrane has been implicated in its action. This leads to increased permeability of the cell envelope and the release of several other proteins in addition to colicin (Baty et al., 1987; Pugsley and Schwartz, 1985). The secretion of colicins has been shown to be SecA dependent (Yamada et al., 1982) but this may be a secondary effect since it is likely that colicin lysis protein either requires SecA per se in order to reach the envelope or relies on the correct assembly of other SecA dependent protein components in the membrane. Thus colicin release appears to require several 'export' components and the release mechanism appears to be relatively non-specific. The second protein which is able to cross the outer membrane of E.coli, haemolysin, in contrast appears to involve release to the external growth medium by a highly specific and unique process and will be discussed in detail in the following sections.

VIII i   Secretion of haemolysin from E.coli.

The α haemolysin of E.coli causes lysis of red blood cells and
has been implicated in the pathogenicity of human and animal urinary
tract infections. Haemolytic determinants have been isolated from the
chromosome of such E.coli strains from humans (Welch et al., 1981) and
also as plasmid DNA from pathogens of animal origin (Smith, 1963).
Analysis of the DNA from different isolates has indicated that the
overall genetic organisation is extremely conserved throughout the
different systems (Mackman et al., 1986). Thus, the haemolytic
determinant has been established to be 7kb in size and to comprise four
contiguous genes (Hartlein et al., 1983; Mackman and Holland, 1984a).
Each of these genes has now been subcloned on to high copy number
plasmids and this has allowed the products to be identified. Thus the
3kb region encoding hlyC and hlyA, determines haemolytic activity and the
following 4kb region encoding hlyB and hlyD is essential for export
(Mackman et al., 1986; Muller et al., 1983). HlyA is the structural gene
and encodes a 107kD protein (Mackman and Holland, 1984b; Felmlee et al.,
1985b), but this protein has no haemolytic activity until activated by
the 20kD gene product of hlyC (Nicaud et al., 1985). The latter does not
however appear to be required for either synthesis or export of
haemolysin.

Identification of the hlyD and hlyB gene products proved
difficult due to the low level of expression of these proteins even after
subcloning. However, HlyD has been identified as a 53kD polypeptide
(Mackman et al., 1985a) whereas HlyB encodes two proteins, at least under
certain conditions; the major product in minicells is a 66kD polypeptide
but in vitro a 46kD protein predominates (Mackman et al., 1986; Felmlee
et al., 1985b). Localisation of the hlyB and hlyD gene products has

produced a variety of conflicting results and the proteins have variously been reported in both the inner and outer membranes (Hartlein et al., 1983; Wagner et al., 1983; Goebel et al., 1984; Mackman et al., 1985a). It has therefore been proposed that HlyB and HlyD form a specific secretion complex spanning both inner and outer membranes (Gray et al., 1986; Mackman et al., 1986).

VIII ii   Mechanism of export of Haemolysin

Over the past few years much research has gone into the mechanism whereby the various components of the haemolysin determinant interact to promote secretion. Although HlyC has been excluded from a role in the actual secretion process, the accumulation of the 107kD polypeptide in the cytoplasm in the absence of HlyB and HlyD supports a central role for these proteins in export.

Haemolysin is exported to the medium without proteolytic processing of the NH_2-terminus (Felmlee et al., 1985a) and DNA sequence data has confirmed that HlyB, HlyD and HlyA all lack a classical NH_2-terminal signal sequence (Felmlee et al., 1985b). In fact, it has been shown that it is the C-terminus of HlyA which carries specific information required for secretion and presumably interaction with the export components in the membrane (Nicaud et al., 1985; Gray et al., 1986). In particular, in the study by Nicaud et al. (1985) the results demonstrated that the C-terminal 23kD peptide of HlyA is even able to promote its own secretion. It is likely therefore that all the information required for secretion of the intact haemolysin is contained within this small C-terminal domain.

Early studies studies with the haemolysin determinant encoded by pANN202, carried out in minicells lacking export functions, suggested

that 70% of the haemolytic activity was in the periplasm (Hartlein et al., 1983). Moreover, significant levels of toxin were also reported in the periplasm of cells carrying the full haemolysin determinant. This led to the proposal that haemolysin, like may other secreted proteins, reached the outer membrane by means of a periplasmic intermediate. In contrast however, experiments in this laboratory have indicated that 90% of haemolytic activity encoded by Hly2001, is cytoplasmic in export defective strains (Gray et al., 1986) and insignificant levels of toxin activity were detected in the periplasm of strains expressing Hly2001 or pHly152. However, in these studies the periplasmic proteins were released by osmotic shock, and since it is known that certain large polypeptides cannot be shocked from the periplasm (Howard and Buckley, 1985) it is possible that a periplasmic pool of haemolysin would not be identified by this method. More definitive evidence is therefore required to confirm or deny the existence of a periplasmic intermediate of the 107kD protein en route to the external milieu.

The exact role of HlyB and HlyD still remains obscure although a variety of functions have been proposed. In particular, a key role for HlyB in the coupling of energy to secretion was suggested when a highly conserved sequence close to the C-terminus was found to correspond to an ATP binding site similar to that found in several bacterial transport proteins including the HisP protein (Higgins et al., 1986).

Currently however, although many of the individual components of the haemolysin secretion system have now been identified, the precise mechanism whereby the haemolysin toxin, crosses either or both membranes in E.coli and is released into the medium is still unclear. It is now important to establish the exact mode of transfer across the envelope and to determine the nature of the interactions between HlyA and other membrane components.

IX   Aims of this project

The growth of secA^{ts} or secY^{ts} mutants at the restrictive

temperature has been shown to inhibit the export of many envelope

proteins (Oliver and Beckwith, 1981). No detailed kinetic analysis has

however been carried out to investigate the immediate effect of

inactivation of these genes on export to the envelope. Also, although

evidence exists to indicate that mutations in secY suppress mutations in

secA (Brickman et al., 1984; Oliver and Liss, 1985a) it is still unclear

at which stage of the export pathway SecA and SecY may act and the exact

role of these proteins remains obscure.

In this project, kinetic experiments have been carried out to

determine the immediate effect, if any, of secA inactivation on protein

export. The export of the outer membrane protein TonA has also been used

as a model system in which to study the roles of SecA and SecY. In the

course of this study the kinetics of post-translational processing were

examined in an attempt to establish at which stage of export SecA and

SecY are required.

Also, in the hope of gaining some insight into the mechanism of

passage through the membrane of HlyA and OmpF, a chimeric protein

consisting of the C-terminal secretion signal of HlyA fused to the

majority of OmpF and a few residues of β-galactosidase was constructed.

Subsequent studies were concerned with analysis of the localisation of

this protein, both in the presence and absence of specific export

proteins HyB and HlyD.

Finally, the question of whether different proteins reach the

membrane via different pathways has also been addressed. Previous

evidence suggested that OmpF reaches the outer membrane without ever

existing in a membrane free form whereas TonA apparently passes through

the periplasm en route to the outer membrane (Boyd and Holland, 1979;

Jackson et al., 1986). In order to further analyse the method of

assembly of these proteins, export intermediates synthesised in

spheroplasts were localised. The role of export proteins HlyB and HlyD

in haemolysin secretion has also been examined in this system by

preparing spheroplasts from cells capable of synthesisng only HlyB or

HlyD.

CHAPTER TWO

Role of SecA and SecY in protein export

Introduction

As discussed in Chapter 1, several genes involved in secretion have

been identified in E.coli. As yet however, very little is understood

about the individual gene products and the way in which they interact to

promote export of proteins from the cytoplasm, across the inner membrane,

to either the external milieu or to the outer membrane. Currently the

best characterised of these proteins, which together very probably

comprise at least part of a cellular secretion machinery, are SecA and

SecY.

It has already been shown that loss of SecA function inhibits

processing of a number of envelope proteins, for example the periplasmic

proteins alkaline phosphatase (PhoA) and ribose binding protein (RBP).

In addition, precursors of the outer membrane proteins OmpF, LamB and

OmpA, accumulate in the cytoplasm when SecA is inactivated (Oliver and

Beckwith 1981; Wolfe et al., 1985). Although fewer inner membrane

proteins have been studied, it has also been demonstrated that the

processing of the inner membrane protein PBP5 (DacA) is secA dependent

(Jackson et al., 1985). However, not all proteins require SecA function

in order to reach their final destination, for example, the M13 coat

protein which is located in the inner membrane, is synthesised with an

NH_2-terminal signal sequence, but is completely unaffected by

inactivation of secA or secY (Oliver and Beckwith, 1981; Wolfe et al.,

1985). On the other hand, the finding that the translocation and

processing of proteins localised anywhere within the cell envelope can

apparently be _secA_ dependent, indicated that SecA function is probably

required for a secretory mechanism common to export of all types of

envelope protein, even including surface pili (Dodd _et al._, 1984).

Additionally however, the fact that M13 coat protein is synthesised as a

larger precursor and is matured by removal of the NH_2-terminal signal

sequence, demonstrates that there is no correlation between the presence

of a signal sequence and a requirement for _secA_. Moreover, these data

do not rule out the possibility that SecA may be involved in the export

of only a subset of envelope proteins and that alternative, _secA_

independent pathways may exist. In fact, Oliver and Beckwith (1981) have

reported that several unidentified periplasmic proteins are unaffected by

inactivation of _secA_ although this remains to be verified.

SecY inactivation also leads to the cytoplasmic accumulation of

several envelope protein precursors (Ito _et al._, 1983). SecY differs

from SecA in that it is an integral inner membrane protein but _secY_

mutations, like _secA_ mutations, have been shown to suppress mutations in

the signal sequence of exported proteins (Ryan and Bassford, 1985;

Schultz _et al._, 1984). These findings are consistent with an important

role in protein export for SecY although like SecA, the exact function of

SecY has not yet been established.

2.1 Kinetic analysis of protein incorporation into the inner and outer

     membranes in two secA mutants

Previous studies which have examined the effect of _secA_ inactivation

on protein export, have all analysed the localisation of membrane

proteins and accumulation of precursors several hours after a temperature

shift from 30°C to 42°C. This leaves open the possibility that the

effects observed are mostly secondary effects of _secA_ inactivation. It

was therefore important to examine the more immediate effects of loss of

SecA function , not only upon the export of outer membrane and

periplasmic proteins, but also on the export of inner membrane proteins.

In fact, few of the latter have so far been studied in secA defective

strains.

In order to determine the role of secA in protein export in E.coli,

two different secA^{ts} strains were grown in glucose minimal medium and

the effect of temperature shift, from 30°C to 42°C, on the rate of

incorporation of ^{35}S-methionine into total protein was investigated.

Cultures were initially grown at the permissive temperature and then

transferred to 42°C when they reached an A$_{450}$ = 0.1. Growth was

subsequently followed for a further 2h and at various intervals, 1ml

samples were pulse-labelled with ^{35}S-methionine. Fig.2.1 shows the mass

increase and rate of total protein synthesis in exponentially growing

cultures of the mutant (MM52) and the wild type parental strain (MC4100)

following a shift to the non-permissive temperature of 42°C. Both the

rate of incorporation of ^{35}S-methionine into total cellular TCA

precipitable protein and the increase in mass continued exponentially in

both the control and MM52 for at least 2h at 42°C indicating that growth

is apparently undisturbed over this period. The second secA strain,

MM66, behaved similarly (data not shown).

Having determined the effect of secA mutations upon total protein

synthesis, the experiment was then repeated, but in this case the

pulse-labelled cells were fractionated and isolated envelopes separated

into inner and outer membranes on the basis of Sarkosyl solubility.

Protein precipitates were washed with TCA to remove any traces of

unincorporated ^{35}S-methionine before the amount of radioactivity

incorporated into each fraction was determined using a scintillation

Figure 2.1

Effect of temperature shift on mass increase (A_{450}) and total
protein synthesis in a secA mutant.

100ml cultures of MM52 (secA^{ts}) and MC4100 were grown in M9 glucose
minimal medium and the temperature was raised from 30°C to 42°C at the
time indicated (⬆). 1ml samples were pulse-labelled with 15µCi
^{35}S-methionine for 5min and incorporation into TCA precipitable material
determined as described in Materials and Methods. Symbols: ● = cpm and ○
= A_{450}. ___ MM52 and _ _ _ MC4100.

no data?
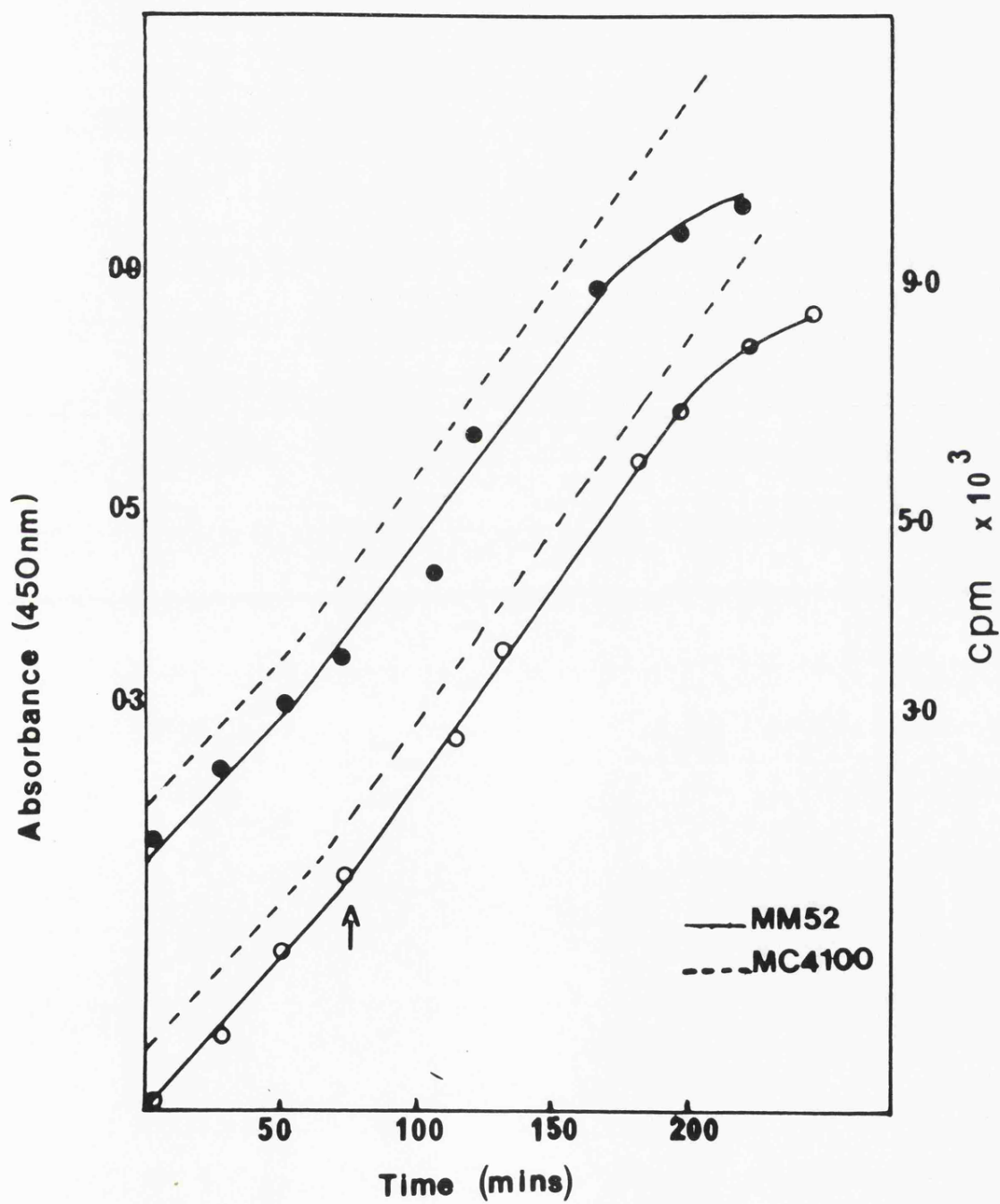
counter. In order to minimise errors caused by loss of material during

fractionation, an exponentially growing culture of the same strain was

labelled with ^{3}H-leucine at 30°C for several generations, and a constant

volume of these cells was added to each sample prior to sonication. The

need for quantitative recovery from sample to sample was then obviated

and radioactive incorporation could be recorded as a ratio of ^{35}S/^{3}H (see

Materials and Methods).

Fig.2.2 shows the effect of a temperature shift on the rate of

incorporation of acid precipitable counts into inner and outer membrane

protein relative to the total rate of protein synthesis. At 30°C all

strains showed a constant relative rate of incorporation of protein into

both inner and outer membranes. In the wild type strain, even after

transfer to 42°C, (Fig.2.2a), the relative rate of incorporation into

each membrane remained constant. However, in the secA^{ts} strain MM52

(Fig.2.2b), incorporation into both membranes was apparently inhibited

immediately after the temperature shift. This provides strong evidence

that SecA is indeed directly involved in protein export. Moreover, these

results suggested that inactivation of secA can affect the export of not

only periplasmic and outer membrane proteins but also many inner membrane

proteins.

In order to establish whether this effect was dependent on the

particular secA allele used, the experiment was repeated with MM66

(secA^{ts}), which contains a temperature sensitive amber mutation in the

secA gene. Again, upon transfer to 42°C, an immediate reduction of the

rate of incorporation of newly synthesised protein into both membranes

was observed (Fig.2.2c). Similarly, transformation of MM52 with plasmid

pLG513, encoding the outer membrane protein TonA (see section 2.2), again

showed a reduction in incorporation into both membranes (Fig.2.2d). This

Figure 2.2

Effect of temperature shift on the rate of synthesis of bulk inner membrane and outer membrane protein relative to total protein in secA$^+$ and secA$^-$ strains.

100ml cultures were grown at 30°C and the temperature was raised to 42°C at the time indicated ($\uparrow$) 1ml samples were pulse-labelled at intervals with 15μCi ^{35}S-methionine for 5min as described in Materials and Methods. The cells were lysed by sonication and the envelopes isolated and separated into inner and outer membranes as described in Materials and Methods. The relative rate of membrane protein synthesis was calculated as a percentage of total radioactive incorporation into whole cells. This figure shows: (a) MC4100 (b) MM52 secA^{ts} (c) MM66 secA amber (pLC513) and (d) MM52 (pLC513).

Symbols: O inner membrane, ● outer membrane.
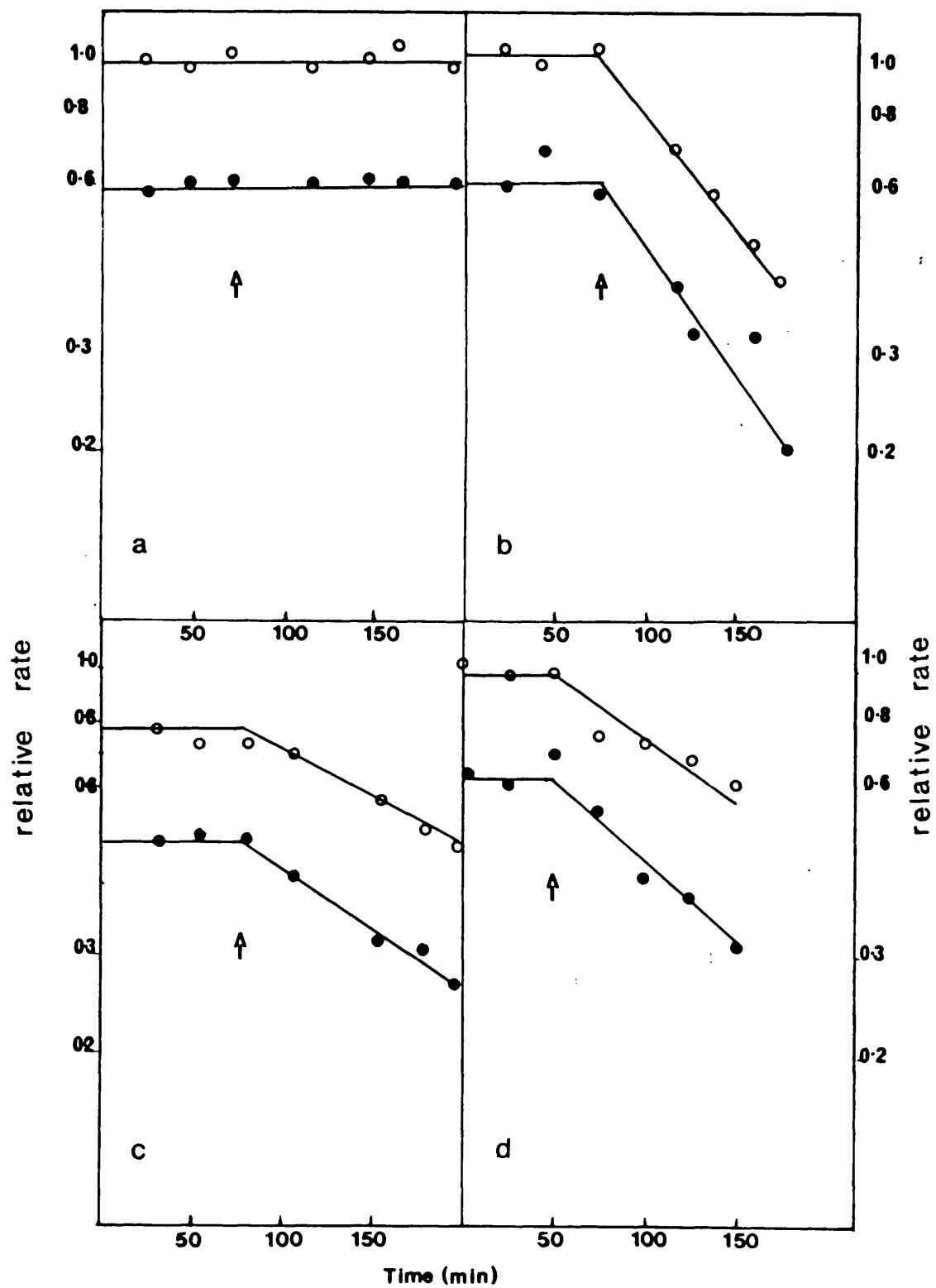
latter experiment was important in the light of evidence which indicated that preTonA, unlike other envelope protein precursors, fractionates with the outer membrane and might therefore contribute a significant level of radioactive incorporation to that fraction (see section 2.4).

It is important to note however, that although the reduction in the rate of incorporation of protein into the membranes occurred soon after the temperature shift in both secA mutants, incorporation did not fall to zero, but still continued at a level of approximately 30% of that observed at the permissive temperature, even after 2h at 42°C. Two alternatives could explain why assembly of newly synthesised protein into the membrane continues after 2h at the restrictive temperature. Either defective SecA may still operate, but inefficiently at this temperature, or, other SecA independent export pathways may exist in E.coli. Finally, it may be noted that, under the growth conditions used in these experiments, envelope proteins may only constitute about 10% of total protein (Boyd and Holland, 1979). Consequently, any inhibition of envelope protein synthesis after loss of secA function would not be reflected by an observable fall in the total cellular rate of protein synthesis.

2.3 Accumulation of preTonA in secA mutants

In an attempt to determine the actual role of SecA in protein export, the effect of secA inactivation on synthesis and export of a specific outer membrane protein was studied. The outer membrane protein TonA was chosen as a model system for this work. The TonA protein is encoded by the fhuA gene (Braun et al., 1973) and is an E.coli outer membrane protein which is essential for the uptake of ferric ferrichrome and also acts as a receptor for bacteriophage T1 (Konisky, 1979). The

gene has been sequenced (Coulton et al., 1986) and has been shown to code

for an 80kD protein with an NH_2-terminal signal sequence which is cleaved

presumably by leader peptidase, to form a mature protein of molecular

weight 78kD (Plastow et al., 1981). TonA is, however, not normally

produced at high levels in the cell and therefore the fhuA gene was

previously cloned into a high copy number plasmid based on pBR325, to

form pLC513 (Jackson, 1984; Jackson et al., 1986). Although still

transcribed from its own promoter, the level of TonA produced is vastly

increased and the protein now constitutes a major component of the outer

membrane. Fortunately, despite overproduction, it appears that TonA in

such strains is still correctly assembled into the envelope (Menichi and

Buu, 1986; Jackson et al., 1986) and it is therefore possible to study in

detail the export of this protein to the outer membrane.

It was of particular interest to study the mechanism of TonA

assembly, since there is some evidence which suggests that TonA is

assembled into the outer membrane via a periplasmic intermediate (Jackson

et al., 1986). Consequently, it was proposed that TonA crosses the inner

membrane concomitant with removal of the NH_2-terminal signal sequence,

once in the periplasm the protein may then adopt a conformation which

allows spontaneous partitioning into the outer membrane and final

maturation into a Sarkosyl insoluble form. In this respect TonA may

differ from OmpF, where there is some indication that the protein is

never in a membrane free form en route to the outer membrane (Boyd and

Holland, 1980).

Since OmpF is known to be secA/secY dependent (Oliver and Beckwith

1981; Ito et al., 1983), it was important to establish whether TonA also

required functional sec encoded proteins in order to reach the outer

membrane, or whether TonA reaches its final destination by a different,

SecA independent pathway. The first step in studying the affect of

secA/secY inactivation on export of TonA to the outer membrane was to

investigate whether TonA precursor accumulated in cells in the absence of

SecA. An exponentially growing culture of MM52 (secA^{ts}) containing

pLG513, encoding high levels of TonA, was transferred from 30°C to 42°C

and samples were pulse-labelled with ^{35}S-methionine at intervals. The

cells were then fractionated into cytoplasm and envelopes. Surprisingly,

analysis by SDS-PAGE and autoradiography revealed no preTonA in the

cytoplasm. In contrast, analysis of envelopes which had been separated

into inner and outer membrane fractions on the basis of Sarkosyl

solubility revealed that, preTonA was indeed accumulated under these

conditions. However, Fig.2.3a clearly shows that the 80kD protein

accumulated in the outer membrane fraction rather than the cytoplasm of

the secA mutant strain MM52 after shift to the non-permissive

temperature. This unexpected fractionation of preTonA with the outer

membrane will be reconsidered in a later section.

Fig.2.3 also shows that the ratio of apparent precursor to mature

TonA increased with time after the temperature shift, in agreement with

the kinetics of overall incorporation of protein into the envelope

following inactivation of secA shown in Fig.2.2b. This was confirmed by

densitometry as shown in Fig.2.3b which also indicates that significant

amounts of the mature form of TonA was recovered with the outer membrane

fraction even after 120min at the restrictive temperature. Thus some

processing, and therefore translocation, was obviously still taking place

after 2h at 42°C despite the inactivation of secA. In complete contrast

to the effect on TonA, labelling of the porins OmpF/C in the outer

membrane fraction was completely blocked shortly after the temperature

shift. Similarly the rate of assembly of mature OmpA into

<u>Figure 2.3</u>

Accumulation of preTonA in a <u>secA</u>^{ts} strain at 42^0C.

(A) Fractionation of preTonA with the Sarkosyl insoluble outer membrane fraction of strain MM52 (pLG513) at 42^0C. A 100ml culture of strain MM52 (pLG513) was grown in M9 glucose medium at 30^0C to an $A_{450} = 0.1$. The culture was then shifted to 42^0C and 1ml samples were taken at intervals and labelled with $15\mu Ci$ ^{35}S-methionine for 5min. Cells were then harvested and the isolated cell envelopes were separated into inner and outer membranes on the basis of Sarkosyl solubility and the outer membrane analysed by SDS-PAGE (11% acrylamide) and autoradiography. Membranes were loaded as identical cell absorbance equivalents. Lanes 1-4 show outer membrane fractions taken at 30, 60, 120 and 170min respectively after the temperature shift. The molecular weights of the standards are given in kilodaltons. In most cases the ovalbumin marker is marked as 43kD, the true molecular weight of this protein, although the marker actually runs with an apparent molecular weight of 46kD. OmpF and OmpC were not separated by the gel conditions used.

(B) Linear densitometer scan of the upper part of the gel shown in 2.3(A). Peak (a) corresponds to OmpF/C and disappears over the time course of the experiment, whereas peaks (b) and (c), which represent TonA and preTonA respectively, do not disappear but alter in their ratios with time after the temperature shift, as preTonA increases and mature TonA decreases.

A

pTonA →
TonA →

OmpF/C →
OmpA →

100—
69—

43—

30—

1 2 3 4

B

a   b   c   i

a   b   c   ii

a   b   c   iii
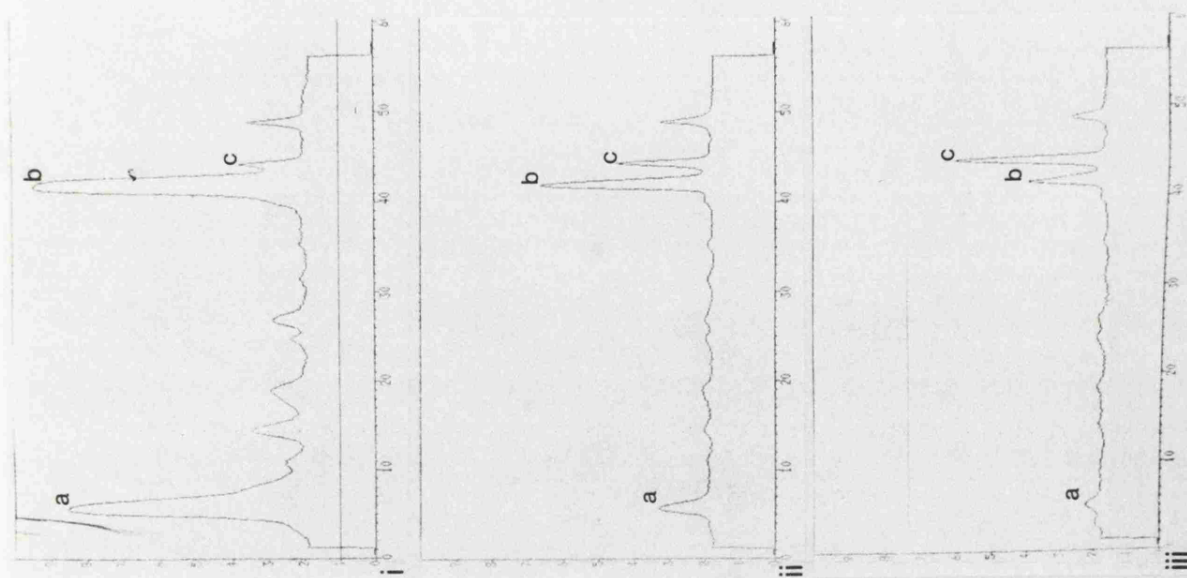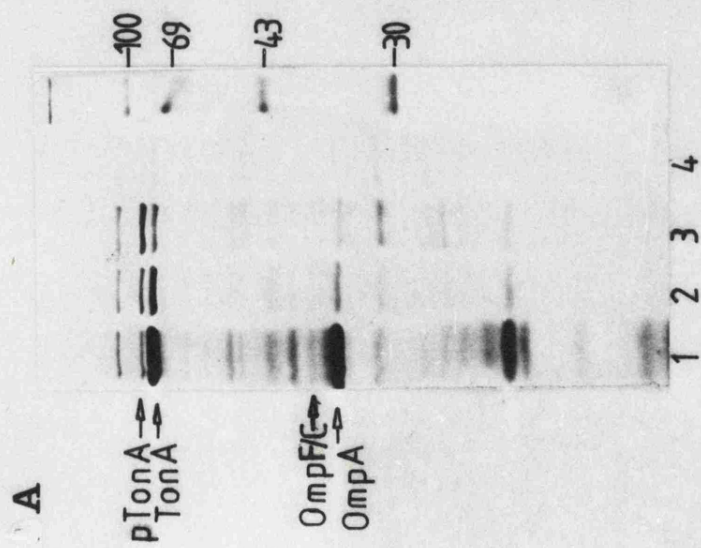
<u>Figure 2.4</u>

Immunoprecipitation of whole cell lysates of MC4100(pLG513) and MM52(pLG513) to identify preTonA and TonA.

Cultures were grown in M9 glucose minimal medium to an $A_{450} = 0.1$ and then transferred to $42^{o}C$. 0.5ml samples were then harvested at the times indicated, pulse-labelled with ^{35}S-methionine for 5min and precipitated with antisera raised against TonA. Lanes 1-3 show immunoprecipitates of MC4100(pLG513) at 0, 60 and 120min after the temperature shift. Lanes 4-6 show immunoprecipitates of MM52(pLG513) at 0, 60 and 120min after the temperature shift.
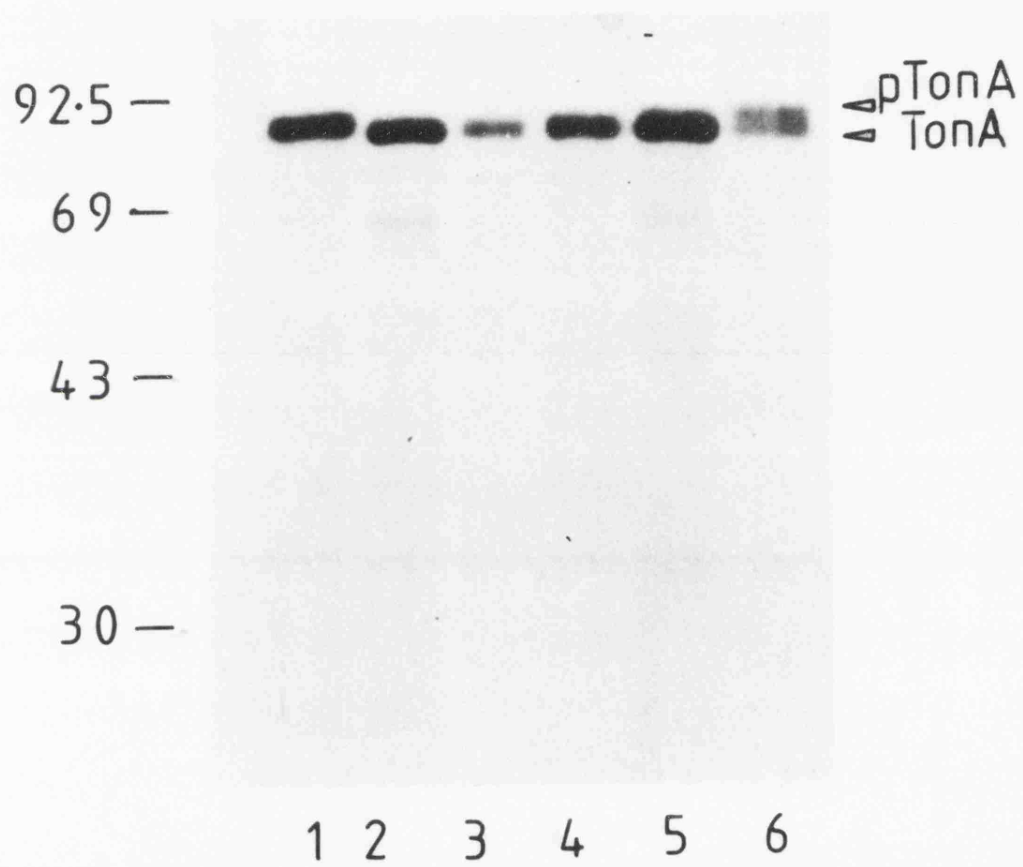
<u>Figure 2.5</u>

Analysis of the outer membrane of MM66(pLC513) shifted to 42°C.

A 100ml culture was grown to an $A_{450} = 0.1$ and shifted to 42°C. 1ml

samples were taken at intervals and pulse-labelled with 15μCi of

^{35}S-methionine for 5min. Cells were harvested and the isolated cell

envelopes were separated into inner and outer membranes on the basis of

Sarkosyl solubility and the outer membrane analysed by SDS-PAGE and

autoradiography. Membranes were loaded as identical A_{450} equivalents.

Lanes 1-4 show outer membrane fractions taken at 30, 60, 120 and 170min

after the temperature shift. C^{14} standards are indicated in kilodaltons.

pTon A
Ton A

OmpF/C →
OmpA →

92·5

69

43

30

1   2   3   4
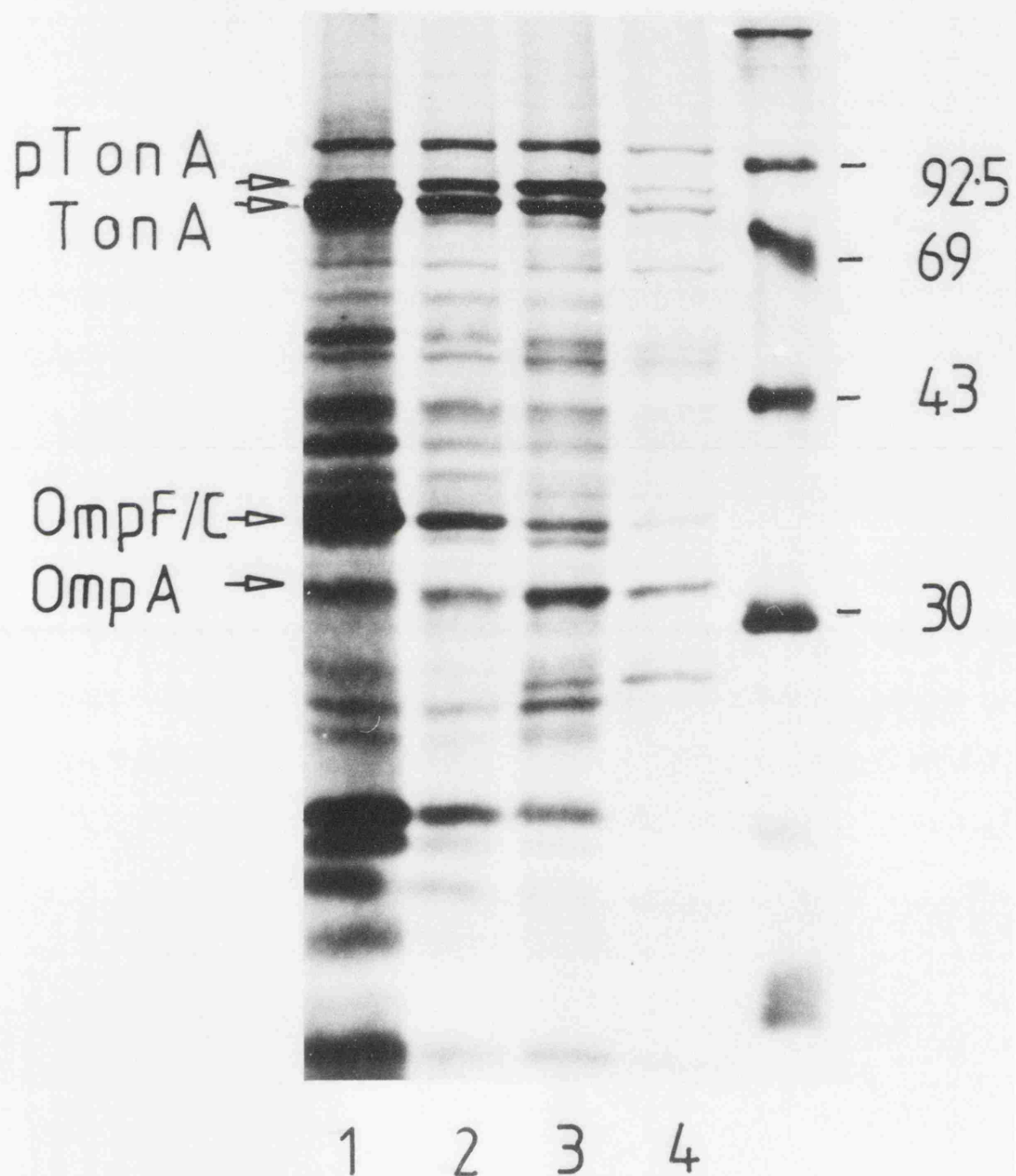
the outer membrane also decreased with time after the temperature shift, and only small amounts reached the outer membrane after 2h. This indicated that the processing and translocation of these proteins was either completely inhibited or the actual synthesis of these molecules was inhibited under these conditions. This point will be considered again in section 2.6.

In order to confirm that the 80kD protein localised to the outer membrane was indeed TonA, 0.5ml samples of an exponentially growing culture of MM52 were labelled with ^{35}S-methionine at both restrictive and permissive temperatures and total cell lysates immunoprecipitated with antiserum raised against TonA. When analysed by SDS-PAGE and autoradiography (Fig.2.4) both 80kD and 78kD bands could clearly be seen, indicating that these polypeptides do represent preTonA and mature TonA respectively. This experiment also illustrated that no signficant pool of precursor accumulated in the cytoplasm, since the amount of precursor in the total lysate was similar to the amount found in the outer membrane.

In order to investigate whether accumulation of preTonA in the outer membrane and the differing effects upon the other outer membrane proteins was allele specific, the experiment was repeated with the MM66 secA (amber) mutant isolated by Oliver and Beckwith (1982a). As Fig.2.5 shows, again preTonA fractionated with the outer membrane and mature TonA and OmpA continued to be synthesised and assembled whereas the synthesis/assembly of OmpF/C was rapidly inhibited. A further six strains carrying independently isolated secA alleles (see Table 8.1) gave similar results. Thus, the differing effects, accumulation of high levels of preTonA but not OmpA in the outer membrane and the rapid

disappearance of newly synthesised porin in the outer membrane were not

due to differential interactions between mutant forms of SecA and

individual membrane proteins.

2.3 Accumulation of preTonA in a secY mutant.

The effect of secY inactivation upon the assembly of TonA and other

outer membrane proteins was also examined in the secY^{ts} strain IQ85

(Shiba et al., 1984). This was investigated using the same conditions as

described above for the secA mutants and Fig.2.6 shows an SDS-PAGE

analysis of proteins fractionating with the outer membrane. Clearly,

processing of TonA was inhibited at 42°C and once again the precursor

associated with the outer membrane fraction. Moreover, virtually

identical results to those seen with secA strains were obtained for the

other outer membrane proteins. Thus, newly synthesised porins OmpF/C

disappeared from the outer membrane fraction in the secY^{ts} strain with

similar kinetics to those observed in MM52(secA^{ts}), whereas again

significant levels of mature TonA and small amounts of OmpA continued to

be assembled into the outer membrane even 2h after the temperature

shift. These results suggested that secA and secY are part of the same

export pathway for OmpA, TonA and OmpF/C, although the effects of

mutations in these genes on the net rate of synthesis and assembly of

different outer membrane proteins appears to vary widely (see section

2.6).

2.4 Localisation of pre TonA in secA^{ts} strains grown at the

    non-permissive temperature.

The observation that preTonA fractionated with the outer membrane in

the presence of inactive SecA or SecY was quite unexpected since in the

<u>Figure 2.6</u>

Fractionation of preTonA with the outer membrane in a <u>secY</u> mutant.

<u>E.coli</u> IQ85(pLG513) was grown in M9 glucose medium at 30°C to an

A_{450} = 0.1. The culture was then shifted to 42°C for 2h before labelling

with ^{35}S-methionine (15μCi per ml) for 5min at various times. Cell

envelopes were isolated and separated into inner and outer membranes on

the basis of Sarkosyl solubility. Proteins were analysed by SDS-PAGE

(11% acrylamide) and autoradiography. Lanes 1-4 show outer membrane

fractions 30, 60, 90 and 120min after the temperature shift.
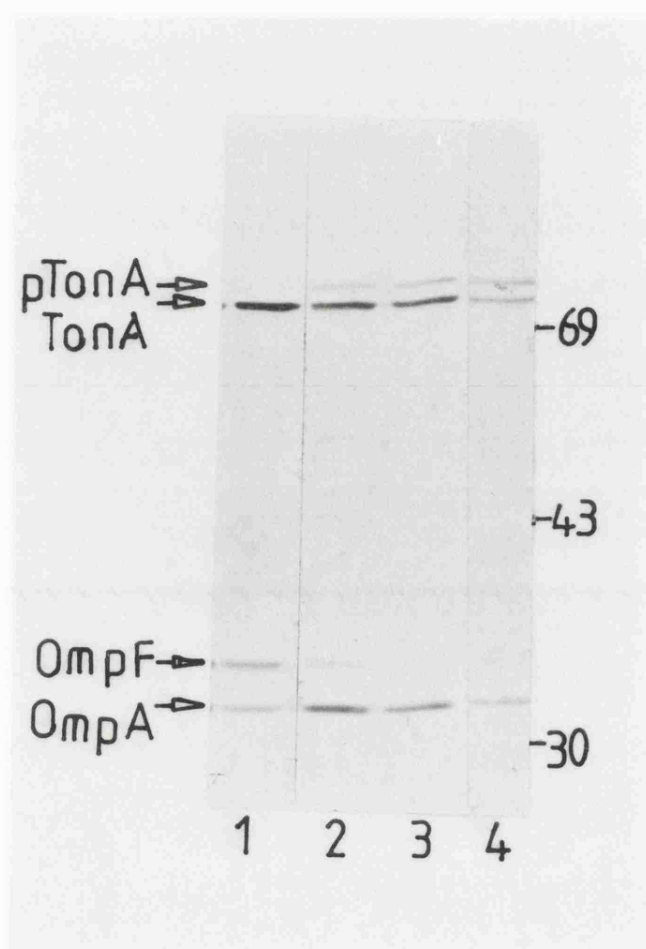
absence of proteolytic cleavage of the NH_2-terminal signal sequence most

models of outer membrane assembly would predict that precursor would

remain anchored in the inner membrane. The fact that SecA has been

identified as a cytoplasmic protein (see Chapter 1), is also at variance

with the presence of precursor in the outer membrane, since a role for

SecA in the initial stages of export has been envisaged. One possible

explanation for the results obtained was that preTonA was artefactually

associating with the outer membrane. In fact, several previous studies

have concluded that under certain conditions some modified envelope

proteins may associate with different cellular compartments depending on

the fractionation procedure employed (Halegoua and Inouye 1979b;

Tommassen et al., 1985; Tommassen, 1986).

    In order to determine the true location of the TonA precursor,

membranes were prepared by osmotic lysis of spheroplasts followeed by

separation on sucrose density gradients (Osborn et al.,1972). As

before, cells were grown in glucose minimal medium to an $A_{450} = 0.1$

before transfer to 42°C for 2h. Cells were then converted to

spheroplasts by addition of EDTA and lysozyme and lysed by vigourously

stirring in ice cold water (see Materials and Methods). The membrane

pellet was recovered by centrifugation and loaded on top of a 25-55%

sucrose step gradient and centrifuged to equilibrium. Inner and outer

membranes could then be separated on the basis of density. Fractions

were collected from the bottom of the gradient and the refractive index,

and hence the density of each sample was measured using a refractometer.

The determination of the distribution of labelled methionine revealed the

presence of four major peaks, corresponding to the four bands identified

by Osborn et al. (1972). Fig.2.7a shows these peaks and the

corresponding densities (in grams per cubic centimetre): H1 $\rho = 1.23$,

H2 $\rho = 1.20$, L2 $\rho = 1.15$ and

Figure 2.7

Fractionation of preTonA on an equilibrium sucrose gradient

(A) Cultures of (a) MC4100(pLG513) and (b) MM52(pLG513) were grown at 30°C to an A_{450} = 0.1 and then transferred to 42°C for 2h. 1ml samples were then pulse-labelled with ^{35}S-methionine for 5min and converted to spheroplasts by the addition of EDTA and lysozyme as described in Materials and Methods.

After osmotic lysis of the spheroplasts, membranes were separated by equilibrium sucrose density centrifugation and eleven separate fractions were collected. After TCA precipitation, 20µl of each fraction were added to 2ml of aqueous scintillation fluid and the number of counts per minute (cpm) were measured. The four peaks observed correspond to (H1) outer membrane, (H2) a mixture of inner and outer membranes, (L2) inner membrane contaminated with outer membrane and (L1) inner membrane.

(B) Localisation of preTonA, accumulating in MM52(pLG513) after 2h growth at 42°C, using sucrose gradient separation of envelopes. The samples, pulse-labelled and separated into inner and outer membranes as described above were analysed by SDS-PAGE (11% acrylamide). Lanes 2 and 3 represent the bulk of the outer membrane and lanes 9 and 10 the bulk of the inner membrane.
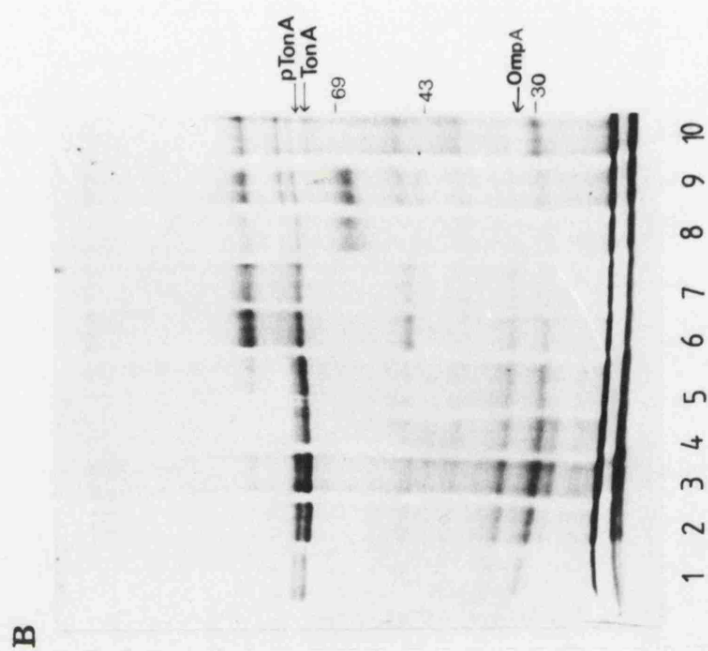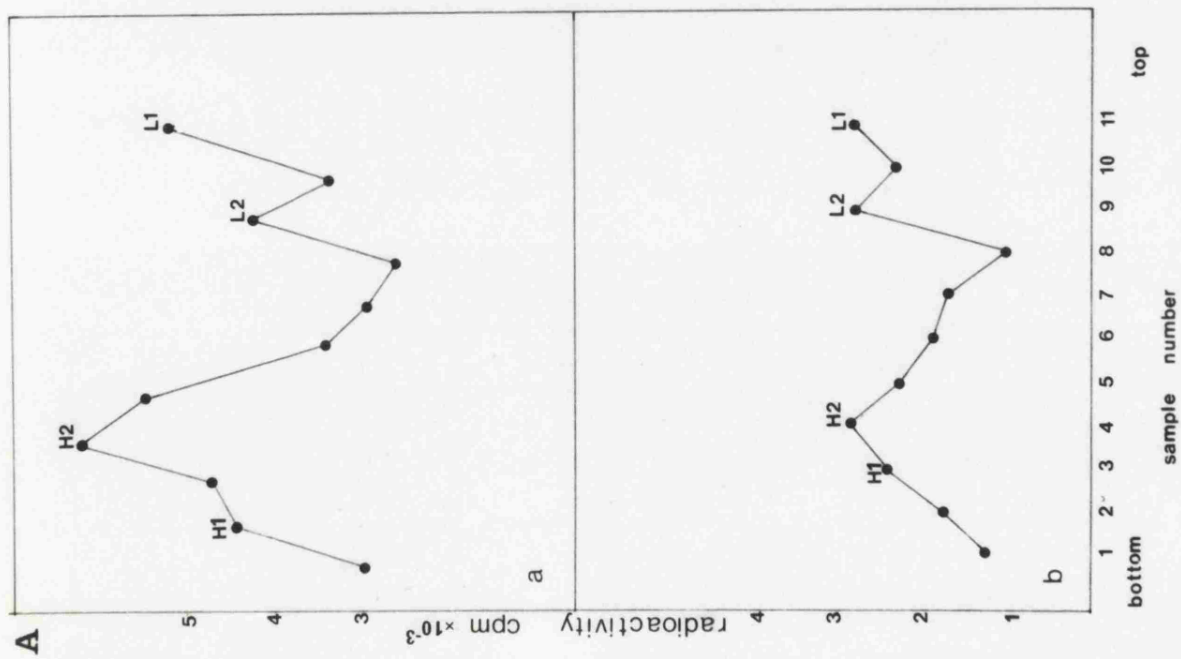
L1 ρ = 1.14. Fractions L1 and L2 have previously been established to consist of predominantly cytoplasmic membrane, with L2 reaching equilibrium banding at a higher density due to a greater contamination with outer membrane (Nicolaidis and Holland, 1978; A. Boyd PhD Thesis 1979). Similarly, H1 contains predominantly outer membrane and it appears that as expected, after 2h inactivation at 42°C there is less incorporation into this fraction in the secA[ts] (MM52) strain than in the wild type (MC4100) strain. Band H2 has previously been shown to contain a mixture of unseparated membranes (Holland and Darby, 1973). This separation of outer and inner membrane fractions was confirmed by SDS-PAGE and autoradiography. As shown in Fig.2.7b preTonA was still recovered with the outer membrane under these conditions.

These results did not however exclude the possibility that preTonA was co-sedimenting with the outer membrane as a protein aggregate originating from the cytoplasm. Such aggregates can be separated from membrane fractions by introducing the sample at the bottom of a 30-55% sucrose step gradient (Hirst et al., 1984). During centrifugation any membrane associated proteins will float up the gradient with the membrane to its equilibrium density. However, aggregated protein not associated with membrane would remain at the bottom of the gradient since sedimentation of proteins is negligible in solutions of such density and viscosity. Membranes isolated from both MC4100 (pLG513) and MM52 (pLG513) grown at 42°C for 120min were separated using this technique. Bovine serum albumin (BSA) was used as a soluble protein control in a parallel gradient and was found to remain entirely in the bottom fraction (data not shown). Fractions were collected starting at the top of the gradient and after separation by SDS-PAGE, proteins were identified by staining in Coomassie blue. Fig 2.8 shows that mature TonA floated up

Figure 2.8

Analysis of membrane proteins in MM52 (pLG513) grown at 42°C using a flotation gradient.

Membranes were prepared from a culture of MM52(pLG513) grown at 42°C for 2h and then separated from aggregates by means of a flotation gradient as described in Materials and Methods. Proteins were analysed by SDS-PAGE followed by staining in Coomassie blue. The figure shows the pellet (lane 8) and bottom 7 fractions (out of a total of 11) of the gradient. A strong band running across the gel is lysozyme which was added to facilitate TCA precipitation through sucrose. The absence of lysozyme from the pellet (lane 1) has resulted in this track appearing narrower than those further up the gradient.
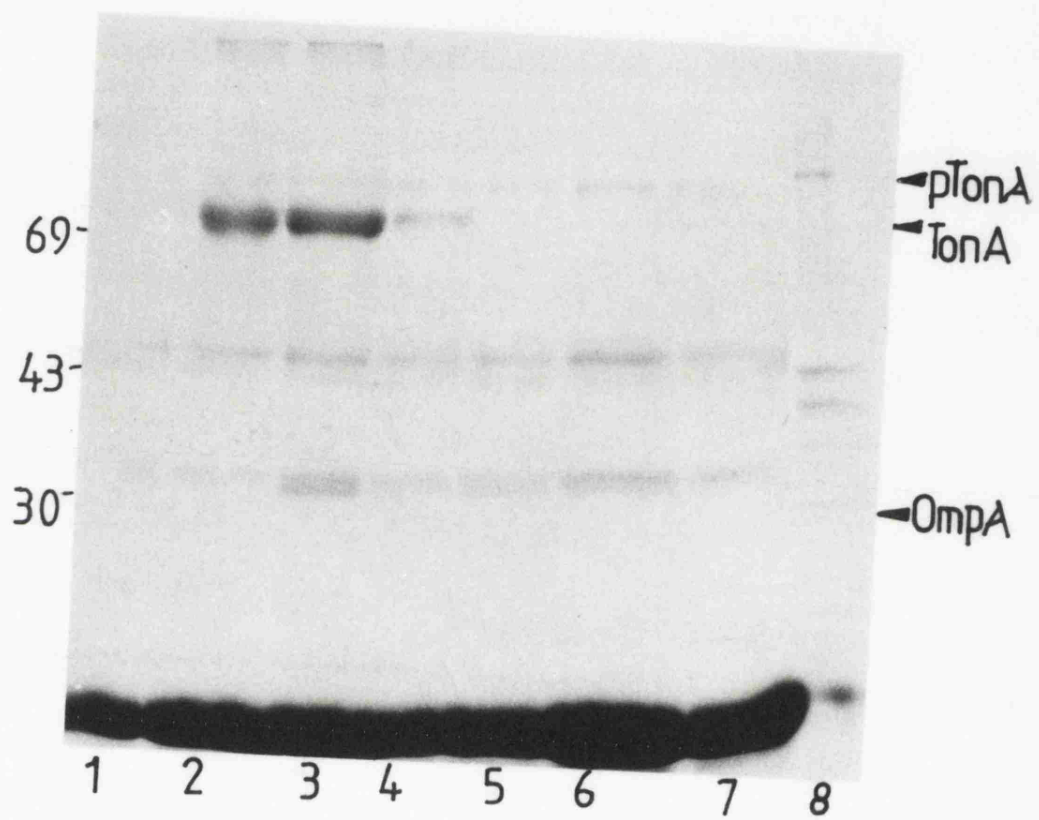
the gradient with the membrane associated protein, whereas preTonA did

not move from the position at which it was applied at the bottom of the

gradient. An identical result was obtained when the procedure was

carried out in the $sec\underline{Y}^{ts}$ strain, IQ85 (data not shown). It can

therefore be concluded that preTonA fractionating with the outer membrane

is not a membrane associated protein but may in fact be in the form of

protein aggregates, which may, form during breakage and fractionation of

the cells.

2.5 Protease sensitivity of pre TonA

PreTonA also appears to associate with the outer membrane in the

form of a protein aggregate in the presence of a malE-lacZ fusion in

strain PB72-49. In this strain the accumulation of large amounts of

hybrid protein, MalE-LacZ, inside the cell inhibits processing of many

envelope proteins and leads to accummulation of their precursors (Ito et

al., 1981; Herrero et al., 1982;). The preTonA which is found in the

outer membrane under these conditions has been shown to be completely

degraded by pronase E (M. Jackson PhD Thesis 1984) whereas mature TonA,

like most correctly assembled outer membrane proteins (Reithmeier and

Bragg, 1977) was largely resistant to pronase E . Therefore it can be

concluded that preTonA molecules probably accumulate in the cytoplasm

when assembly is inhibited either by induction of the malE-lacZ gene

fusion or by inactivation of secA/secY, and that aggregation of these

preTonA molecules results in co-sedimentation with the outer membrane

fraction. This fortuitous occurrence therefore provides a convenient

system in which to monitor simultaneously upon cell fractionation the

relative levels of preTonA and mature TonA in the outer membrane under

different conditions.

2.6 Effect of secA inactivation on the synthesis of outer membrane proteins.

Before any further experiments were carried out on the role of SecA and SecY in export of TonA and other proteins to the outer membrane, the effect of secA inactivation on the synthesis of TonA was investigated. This was considered to be important since SecA has been implicated in translation-arrest of exported proteins (Kumamoto et al., 1984). It was therefore essential to carry out as far as possible a quantitative analysis of the synthesis of both pre and mature TonA following the inactivation of secA.

Wild type (MC4100) and secA^ts (MM52) strains were grown for 2h at $42^{o}C$ before they were pulse-labelled with ^{35}S-methionine and cell extracts were immunoprecipitated with antiserum against either TonA, OmpF or OmpA. Subsequent analysis of the samples by SDS-PAGE followed by autoradiography is shown in Fig.2.9. Bands corresponding to both precusor and mature proteins were cut from dried gels and the amount of radioactivity in each band measured in a scintillation counter. It was then possible to compare the number of radioactive counts incorporated into mature protein in the wild type strain at $42^{o}C$, with the combined total number of counts incorporated into precursor and mature protein in the same number of A_{450} units in the secA^ts strain. The values obtained indicated that in strain MM52 the combined rate of incorporation of ^{35}S-methionine into TonA mature and precursor forms was at least 70% of that found in MC4100 under the same conditions. Similar results were found for OmpA, indicating that secA inactivation inhibited the processing of TonA and OmpA with little effect on their synthesis. In contrast however, the synthesis of either mature or unprocessed OmpF/C was reduced to undetectable levels in the MM52 mutant strain after 2h at $42^{o}C$.

Figure 2.9

Effect of temperature shift on synthesis of outer membrane proteins in secA strain MM52.

Strains MC4100(pLC513) and MM52(pLC513) were grown at 42°C for 2h after the temperature shift and a volume of culture equal to 0.3 A_{450} OD units was pulse-labelled with ^{35}S-methionine (15μCi per 0.5ml). Total cell lysates were then immunoprecipitated against either OmpA, TonA or OmpF antibody. This figure shows lane 1, MC4100(pLC513) and lane 2, MM52(pLC513) immunoprecipitated against OmpA; lane 3, MC4100(pLC513) and lane 4 MM52(pLC513) immunoprecipitated against TonA; lane 5, MC4100(pLC513) and lane 6, MM52(pLC513) immunoprecipitated against OmpF. The dye front was run off the gel in order to separate preOmpA and mature OmpA.
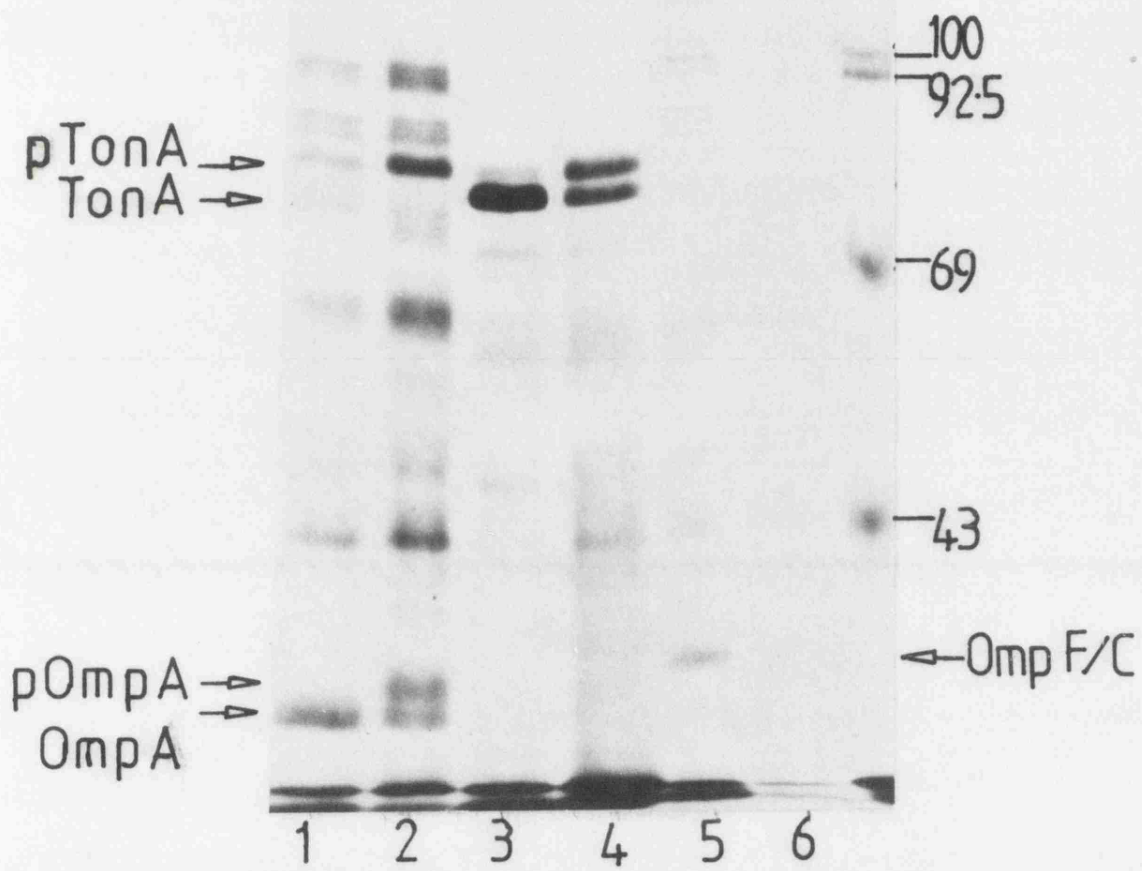
Discussion

Although it has already been established that many E.coli outer membrane and periplasmic proteins are affected by loss of SecA function, (Oliver and Beckwith, 1981), it has proved less easy to study the effect of secA inactivation on proteins which are normally localised to the inner membrane. One reason for this is that inner membrane proteins generally lack a cleavable NH_2-terminal signal sequence and consequently it is not possible to monitor the accumulation of precursor as a convenient index of blocked assembly. In fact two inner membrane proteins which have been studied in the absence of functional SecA, namely M13 coat protein and PBP5 (DacA), are unusual in that they are both synthesised with NH_2-terminal cleavable signal sequences. These two proteins differ however, in that PBP5 is SecA dependent (Jackson et al., 1985) whereas M13 coat protein does not appear to require SecA in order to reach the inner membrane (Wolfe et al., 1985).

Previous studies also failed to include kinetic experiments, and generally an analysis of envelope protein assembly was carried out at least 2h after the temperature shift. Since mutant cells are at this stage considerably abnormal, secondary effects have complicated interpretation of the data. Therefore, in this study a series of kinetic experiments were been carried out in order to investigate the affect of secA inactivation on the export of proteins to both the outer and the inner membrane. The results, shown in Fig. 2.2 indicated that functional SecA was required for the assembly of many proteins to both the outer and inner membranes. Moreover, the effect of secA inactivation upon assembly of envelope proteins was apparently immediate, strengthening the conclusion that SecA does have a key role in protein export. It can be

seen however, that export to the membranes is not completely abolished, and in fact, in the secA^{ts} strain (MM52) even after 2h at the restrictive temperature the rate of incorporation of newly synthesised protein into both membranes was about 30% of that at 30°C. The amount of protein still reaching the envelope might reflect the existence of alternative, SecA independent pathways. However, the secA mutation may also be leaky, a possible affect of the original method of isolation of the secA mutant (see Chapter 1).

In addition to the analysis of export of bulk protein to the inner and outer membranes under SecA limitation, a more detailed investigation was carried out on export of the outer membrane protein TonA. It has been suggested (Jackson et al., 1986) that export of TonA to the outer membrane involves cleavage of the NH$_2$-terminal signal sequence during the initial stage of translocation across the inner membrane to the periplasm. Subsequently, the processed periplasmic intermediate adopts a conformation which results in spontaneous insertion into the outer membrane. However, if this does indeed represent the pathway of assembly of TonA to the outer membrane, large amounts of preTonA would not be expected to fractionate with the outer membrane. Data obtained from cell frationation in the absence of functional SecA therefore appeared inconsistent with this model.

By applying envelopes prepared by the lysis of spheroplasts to the bottom of a 30-55% sucrose step gradient, it was possible to separate preTonA, which remained in a pellet at the bottom of the gradient, from membraneous material which floated up the gradient. This indicated that the association of preTonA with the outer membrane fraction during membrane separation on the basis of Sarkosyl solubility or by the

position after sedimentation in an equilibrium sucrose gradient was simply due to the formation of high molecular weight aggregates which fortuitously associate with the outer membrane fraction. This interpretation is further substantiated by the fact that although, as expected, mature TonA assembled in the outer membrane is protease resistant (Reithmeier and Bragg, 1977), outer membrane associated preTonA is protease sensitive (M. Jackson Ph.D Thesis 1984). The localisation of TonA observed after cell fractionation in MM52 (secA^{ts}) at the restrictive temperature does not therefore contradict previously proposed models of TonA export. It is also possible that a similar formation of high molecular weight aggregates may explain the previous observation that unprocessed LamB can be associated with the outer membrane fraction (Clement et al., 1982).

Previous studies indicated that secA mutations resulted in a reduction in the level of translation of porins and MBP at the non-permissive temperature (Oliver and Beckwith, 1981; Oliver and Beckwith, 1982a). Therefore, the affect of secA inactivation on the synthesis, rather than the export of TonA, was also investigated in this study. In order to quantitate the effect of loss of SecA function on outer membrane protein synthesis, whole cell lysates were immuno-precipitated with antisera raised to either TonA, OmpA or OmpF and the amount of radioactive incorporation at the restrictive temperatures was calculated. The data obtained indicated that although processing and export of TonA was rapidly inhibited at 42°C, synthesis of TonA continued at a level reasonably comparable with that observed in the wild type strain MC4100 grown under the same conditions. Similar results were obtained for OmpA which suggests that although SecA is required for

translocation of TonA and OmpA across the inner membrane, it does not

have a role in coupling synthesis to export of these proteins. In

agreement with this observation, a recent study by Strauch et al. (1986)

has provided evidence that the inhibition of envelope protein synthesis

of MBP and LamB seen as a result of loss of SecA function, may in fact be

a secondary affect causd by catabolite repression accompanying a

reduction in the level of cAMP. This work although carried out in strain

MM66 (secA[am]) on the envelope proteins MBP, alkaline phosphatase and

LamB, may also be applicable to strain MM52. Since the possibility that

different secA alleles affect different proteins to different extents has

now also been ruled out in this study, an alternative explanation for

lack of OmpF/C precursor accumulation could be extreme instability of

these envelope protein precursors when accumulating in the cytoplasm.

This could then result in an apparent inhibition of protein synthesis.

If envelope protein precursors are complexed with SecA, an allelic affect

on stability might be expected, but once again this was not detected.

Nevertheless, inherent instability of cytoplasmic porin precursors cannot

be ruled out. The observation that the addition of casamino acids to the

growth medium results in continued porin synthesis (see Chapter 3), is

also a strong indication that inhibition of envelope protein synthesis is

a secondary affect and it therefore now seems unlikely that SecA causes

translational arrest in E.coli.

The observed cytoplasmic accumulation of envelope precursors upon

secA and secY inactivation, could of course also be due to a secondary

effect resulting from a shortage of leader peptidase. The assembly of

this protein into the envelope has, during the course of this study been

established to be SecA and SecY dependent (Wolfe et al., 1985). However,

it is likely that if a shortage of leader peptidase caused precursor

accumulation, precursor would accumulate in the inner membrane. In addition, the kinetic data, which shows an apparently immediate decrease in incorporation of newly synthesised protein to the outer membrane, also favours a primary rather than a secondary affect on protein export. Moreover, in a subsequent section, evidence will be presented which shows that efficient processing of preTonA accumulated in a secY mutant after 2h at 42°C, can take place upon return to the permissive temperature, even in the absence of further protein synthesis, demonstrating that functional leader peptidase is still present and not limiting.

CHAPTER THREE

Post-translational translocation of TonA to the outer membrane of E.coli

Introduction

The defective export of protein to the periplasm and outer membrane

seen in the absence of functional sec encoded proteins results in the

cytoplasmic accumulation of many envelope protein precursors (Oliver and

Beckwith 1981, Ito, 1983, Kumamoto et al., 1984). Since maturation of

precursor to mature protein and cleavage of the NH_2-terminal signal

sequence is thought to occur at the cytoplasmic face of the inner

membrane, it therefore appears that the proteins tentatively identified

as components of the cellular secretion machinery probably operate at an

early stage in protein translocation (Oliver and Beckwith, 1981; Shiba et

al., 1984). Coupled with the fact that certain prl or sec mutations are

able to suppress mutations associated with other sec alleles (Brickman et

al., 1984; Oliver and Liss, 1985), this has led to the suggestion that

proteins encoded by these genes form a cytoplasmic complex and secretory

machinery, analogous to eukaryotic SRP and docking protein, and essential

for catalysis of the early stages in protein translocation.

In this study in order to determine whether SecA and SecY are

components of such a pathway and act sequentially to facilitate protein

export, an analysis of the extent if any, of maturation of preTonA (i.e.

post-translational translocation) to mature TonA was carried out in both

MM52(secA^{ts}) and IQ85(secY^{ts}) mutant strains.

3.1 Processing of preTonA in a secA mutant

In order to examine the post-translational processing of preTonA which had accumulated in the absence of SecA, an exponentially growing culture of strain MM52(pLG513) was transferred from 30°C to 42°C and growth continued at this temperature for a further 2h. A 10ml sample was then pulse-labelled with ^{35}S-methionine for 30s at 42°C before the sample was returned to 30°C and chased in the presence of excess cold methionine to prevent further radioactive incorporation. Samples were removed at intervals during the chase and fractionated into inner and outer membranes on the basis of Sarkosyl solubility. Proteins were subsequently analysed by SDS-PAGE and autoradiography.

Fig.3.1a shows the outer membrane fractions prepared at intervals during a short chase. The amount of preTonA, accumulating in the cytoplasm, but fractionating with the outer membrane (as shown in Chapter 2) appears to remain fairly constant or to decline only slowly, throughout the chase. In fact, even over a longer chase period of 30min, the preTonA was calculated to have a half life of 7min (Fig 3.2 and Materials and Methods), eight-fold greater than the expected value of approximately 50s for TonA assembly in a wild type strain. It is not clear whether this slow reduction of precursor is due to proteolysis, an effect which might be exacerbated by the induction of proteases at high temperature (Phillips et al., 1984) or to authentic, but inefficient processing. Further analysis of the outer membrane fractions prepared after a short chase at 30°C, also revealed other complexities. Thus, although precursor from the cytoplasm had apparently been processed with greatly reduced efficiency, there was a significant increase in the level of mature TonA fractionating with the outer membrane (Fig.3.1). Some increase in TonA production would be expected in such a short chase as a

<u>Figure 3.1</u>

Processing of TonA in a <u>secA</u>^{ts} strain during a short chase.

A 100ml culture of strain MM52(pLG513) was grown in M9 glucose supplemented with 0.5% casamino acids for 2h at $42^{\circ}C$ before a 10ml sample was labelled with 150μCi ^{35}S-methionine for 30s. The culture was then returned to $30^{\circ}C$ and chased with excess cold methionine as described in Materials and Methods. The membranes were separated on the basis of Sarkosyl solubility. Proteins were analysed by SDS-PAGE (11% acrylamide) and autoradiography). Molecular weights of standards are indicated in kilodaltons.

(A) Lanes 1-4 outer membrane fractions after 0, 30, 60 and 80s of chase respectively.

(B) Lanes 1-4 inner membrane fractions after 0, 30, 60 and 80s of chase respectively.
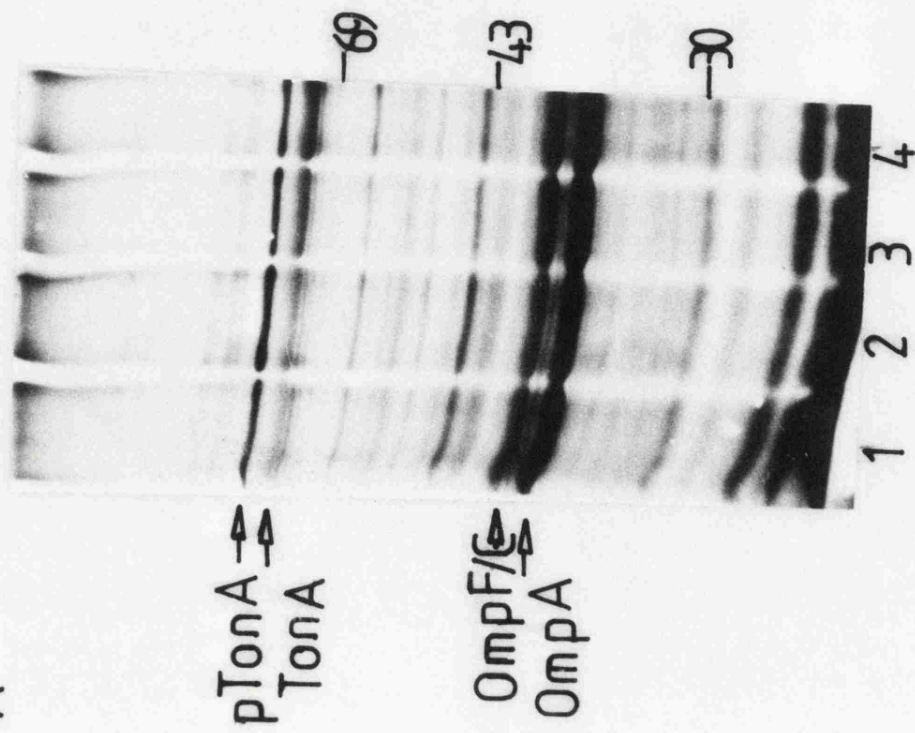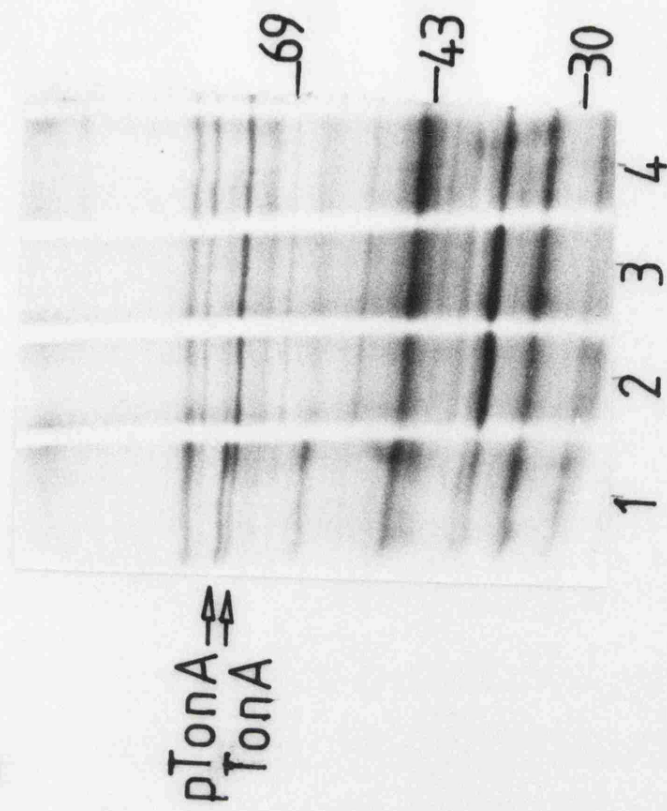
Figure 3.2

Processing during a long chase of preTonA accumulating in a secA mutant.

(A)   Graph to show decrease in the amount of radioactivity in the
80kD, preTonA band with time. The amount of radioactivity present in
preTonA at each time point was determined by cutting out the bands in (B)
and counting in a scintillation counter (see Materials and Methods). The
amount of radioactivity in the band of interest was plotted as a
percentage of the total number of counts in the whole track. This
eliminated errors which might occur as a result of slightly different
amounts of material loaded in each track, e.g. track 2 contains more
material compared to track 1.

(B)   A 100ml culture of MM52(pLG513) was grown for 2h at $42^{o}C$ in
glucose minimal medium supplemented with 0.5% casamino acids before a
10ml sample was pulse-labelled with ^{35}S-methionine for 30s. Excess cold
methionine was added to prevent further radioactive incorporation and
samples were taken at intervals and separated into cytoplasm and
envelopes by centrifugation. Lanes 1-5, envelope fractions 0, 5, 10, 15,
and 20min into the chase. Molecular weights are given in kilodaltons.

B

92·5—

69—

43—

30—

pTonA
TonA

OmpA

1 2 3 4 5

A

radioactivity pTonA/total

4

2

1

5   10   15   20   Time min
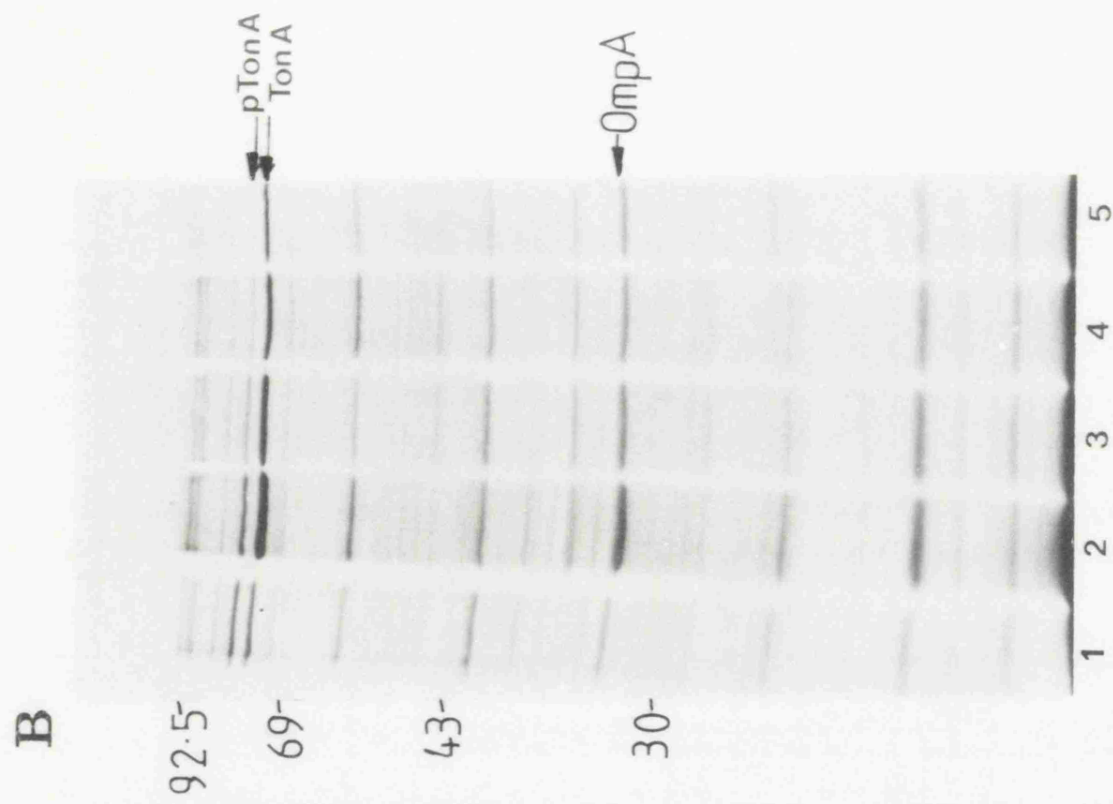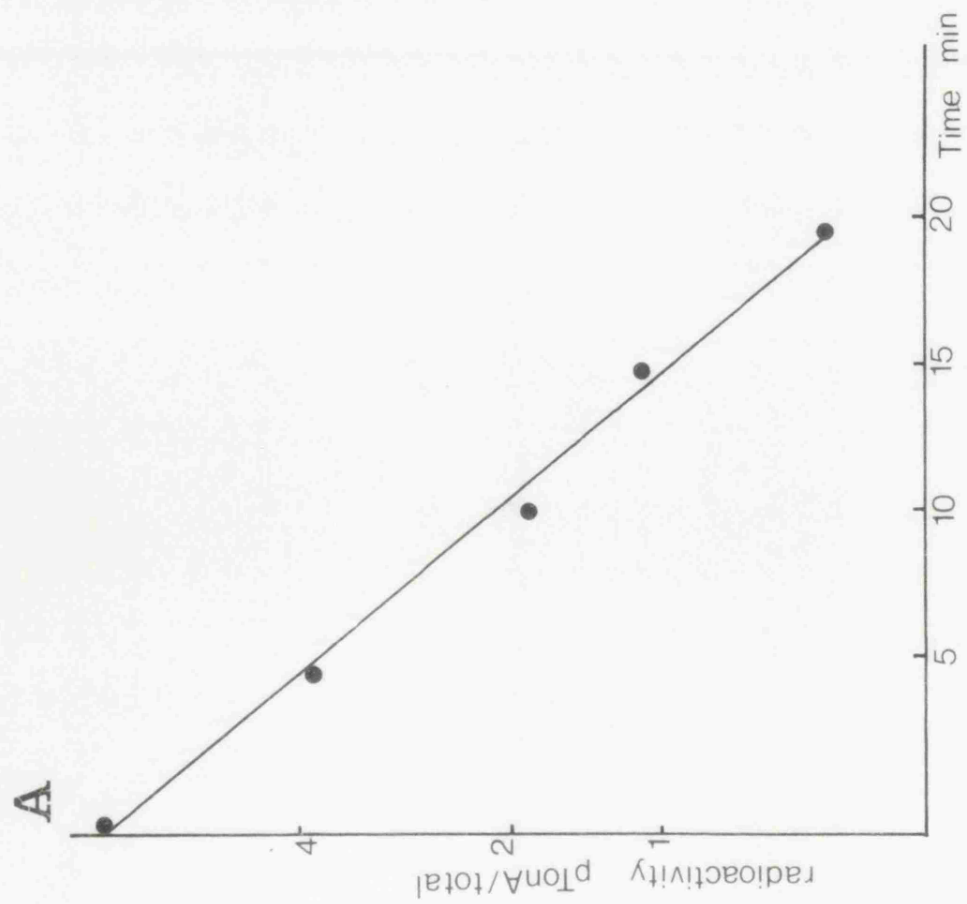
result of the completion of nascent chains initiated during the short pulse. These chains will be completed with a $t_{1/2}$ of 50s (Jackson et al., 1986). Nevertheless, one would have expected a corresponding decrease in the level of precursor unless other sub-populations of preTonA, not fractionating with the outer membrane, give rise to mature TonA.

Indeed, Fig.3.1b shows that a small amount of preTonA which sediments with the inner membrane disappeared completely 80s into the chase. Therefore, it seems that some of the observed increase in mature TonA fractionating with the outer membrane may be due to processing of a small inner membrane pool of precursor.

The results obtained in this analysis indicate that the majority of the large pool of precursor which accumulates in the cytoplasm under SecA limitation is not in a translocationally competent form and may in fact have adopted a conformation which precludes processing and correct assembly into the outer membrane. In contrast, the small inner membrane associated pool of precursor, appears to remain translocationally competent and rapid processing followed by translocation to the outer membrane occurs upon return to 30°C.

It can also be seen in Fig.3.1a, that in this experiment, in contrast to those described in Chapter 2, the porins OmpF/C are synthesised even after 2h at 42°C. The reasons for this are not clear, although one possibility is that addition of casamino acids to the growth medium results in physiological conditions which eliminate the secondary effects of secA inactivation. Supplementation of the growth medium with casamino acids has previously been proposed to increase the ratio of preMBP to mature MBP when added to maltose minimal medium (Ito et al., 1983). Under the conditions used in this experiment however, the only

effect appears to be that OmpF/C synthesis continues despite inactivation of secA. The basis of this phenomenon has not been further pursued, but it is a further indication that inhibition of porin synthesis is indeed only a secondary effect of secA inactivation as suggested by Strauch et al. (1986).

3.3 Processing of preTonA in a secY mutant

In order to compare these observations in MM52(secA^{ts}) with the fate of preTonA which accumulates in a secY mutant, a similar experiment was carried out in strain IQ85(secY^{ts}). A culture of IQ85(pLC513) was grown in glucose minimal medium supplemented with casamino acids, pulse-labelled with ^{35}S-methionine for 30s and chased at 30°C as before. An analysis of the inner and outer membrane fractions prepared on the basis of Sarkosyl solubility is shown Fig.3.3. In complete contrast to the inefficient processing observed in MM52, Fig.3.3 shows that preTonA accumulating at the restrictive temperature in IQ85 is rapidly processed with a maximum half life (calculated as indicated in Materials and Methods and Fig.3.2a) of 90s. A simultaneous increase in the ratio of mature TonA reflects this disappearance and is indicative of post-translational processing of the precursor although some of this increase in mature TonA will again represent completion of synthesis of nascent chains initiated during the pulse.

Finally, it is interesting that small amounts of a polypeptide running at the expected position of preOmpA (between porin and preOmpA) rapidly disappears during the chase in the secY mutant in a similar manner to the disappearnce of TonA (Fig.3.3)

Figure 3.3

Processing of TonA in a <u>secY</u>^{ts} strain during a short chase.

A 100ml culture of strain IQ85(pLG513) was grown in glucose minimal medium supplemented with 0.5% Casamino acids for 2h at 42°C. 10ml samples were labelled for 30s with 150μCi ^{35}S-methionine as in the previous figure, and transferred to 30°C. The culture was then chased in the presence of excess cold methionine. The membranes were separated on the basis of Sarkosyl solubility. Proteins were analysed by SDS-PAGE (11% acrylamide) and autoradiography. Lanes 1-4, outer membrane fraction after 0, 30, 60 and 80s of chase respectively.

Figure 3.4

Processing of preTonA in a secA mutant in the absence of protein

synthesis.

A culture of MM52(pLG515) was grown in glucose minimal medium at the

restrictive temperature for 2h before a 10ml sample was pulse-labelled

with ^{35}S-methionine for 30s and transferred to 30°C. The culture was

then chased in the presence of excess cold methionine and chloramphenicol

(final concentration 250mg/ml). The membranes were separated on the

basis of Sarkosyl solubility and proteins were analysed by SDS-PAGE.

Lanes 1-4, outer membrane fractions 0, 5, 10 and 30min into the chase

respectively
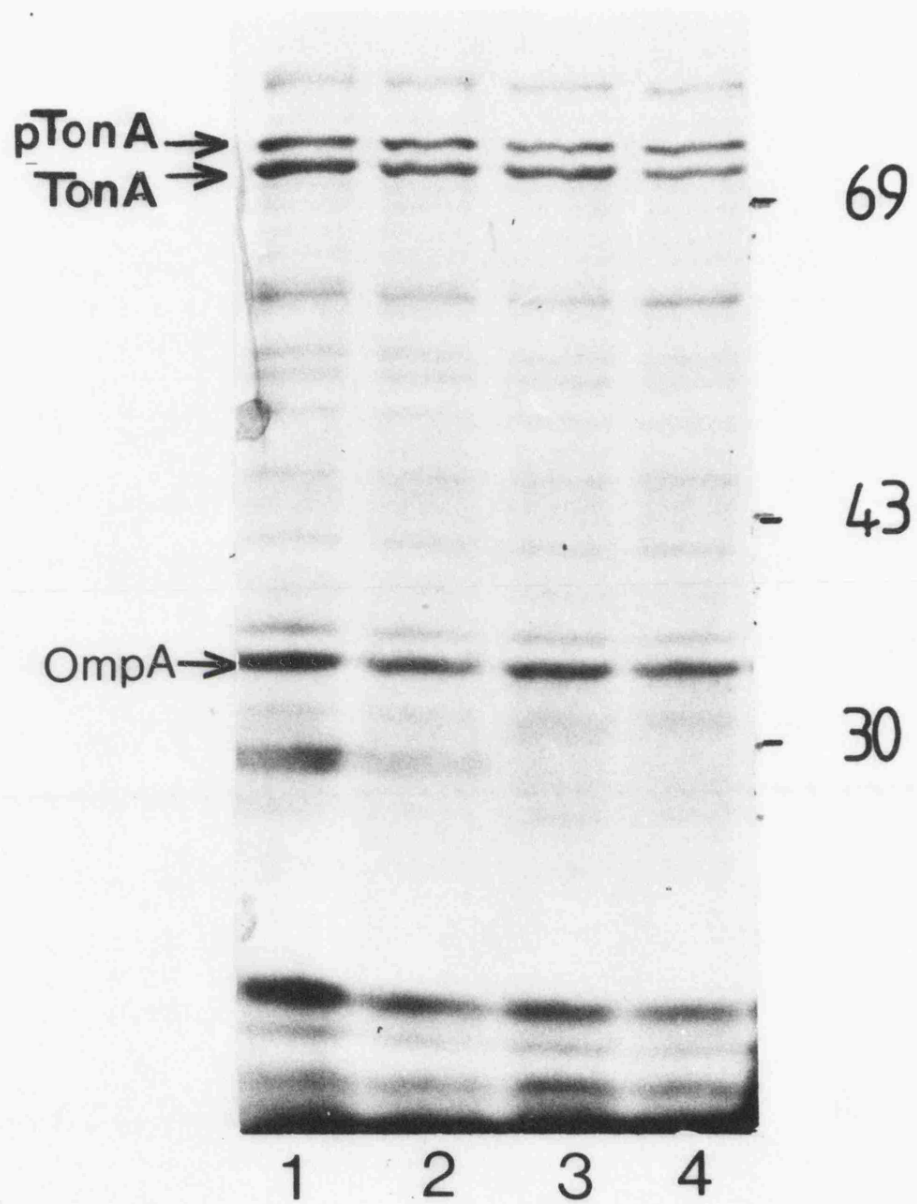
3.4 Processing of preTonA accumulating in secA and secY mutants in the absence of protein synthesis.

In order to examine further the nature of any post-translational processing of preTonA, the pulse-chase experiments were repeated in the absence of protein synthesis. If protein synthesis is inhibited, TonA molecules initiated during the pulse will not be completed during the chase and degradation of precursor by proteases should also be reduced, if induced synthesis of proteases at $42^{\circ}C$ is involved. Thus, plasmid pLG515, a derivative of pLG513 was transformed into strain MM52. This plasmid, although still carrying the tetracycline resistance marker, has a deletion in the chloramphenicol resistance gene (Jackson et al., 1986). It is therefore possible to inhibit protein synthesis during the chase by addition of chloramphenicol, a bacteriostatic agent capable of inhibiting peptide bond formation. Fig.3.4 shows that the presence of chloramphenicol completely inhibits any detectable processing of the accumulated precursor. This is consistent with inhibition of protease synthesis capable of promoting non-specific cleavage of preTonA or with the synthesis of active molecules of SecA which might otherwise be produced during the chase. However, in other experiments, an increase in the level of the mature band was observed between the first two samples consistent with the rapid processing of a small sub-population of preTonA seen above (data not shown).

In order to carry out a similar pulse-chase experiment in IQ85 (secY^{ts}) in the absence of protein synthesis, it was necessary to add kanamycin to the chase mixture. This was due to the fact that it was impossible to construct a strain including pLG515 by selection since strain IQ85 itself codes for tetracycline resistance, the selectable marker on pLG515. Kanamycin is however also an inhibitor of protein

synthesis and can bind to bacterial ribosomes causing misreading of mRNA. Fig.3.5, shows that processing of preTonA in the secY mutant carrying pLG513 still occurs extremely rapidly in the absence of protein synthesis. This is accompanied by an apparently concomitant increase of the mature form during the chase. In this experiment, in the absence of protein synthesis the increase in mature TonA can wholly be ascribed to maturation since any completion of nascent polypeptides initiated during the chase will be blocked. It can therefore be concluded that processing and presumably translocation in this case, is not dependent upon new protein synthesis at the permissive temperature. Consequently, the results indicate that processing of preTonA is promoted through rapid renaturation of the mutant form of SecY allowing rapid resumption of protein translocation. In contrast, in the case of the secA mutant either SecA does not renature and/or preTonA remains translocationally incompetent under these conditions. The results in this section, do however, demonstrate quite clearly that preTonA remains in a translocationally competent form despite the inactivation of SecY.

3.5 Reconstitution of SecA function

The results obtained in the previous section indicated either that in the absence of active SecA, preTonA rapidly becomes translocationally incompetent, or alternatively that SecA function is only restored extemely slowly upon return to permissive temperature. In order to determine the rapidity with which SecA function is regained, a culture of MM52(pLG513) was grown in glucose minimal medium for 2h at the restrictive temperature. The culture was then returned to 30°C and 1ml samples were pulse-labelled with ^{35}S-methionine at intervals. Fractionation into inner and outer membranes on the basis of Sarkosyl solubility was followed by SDS-PAGE analysis.
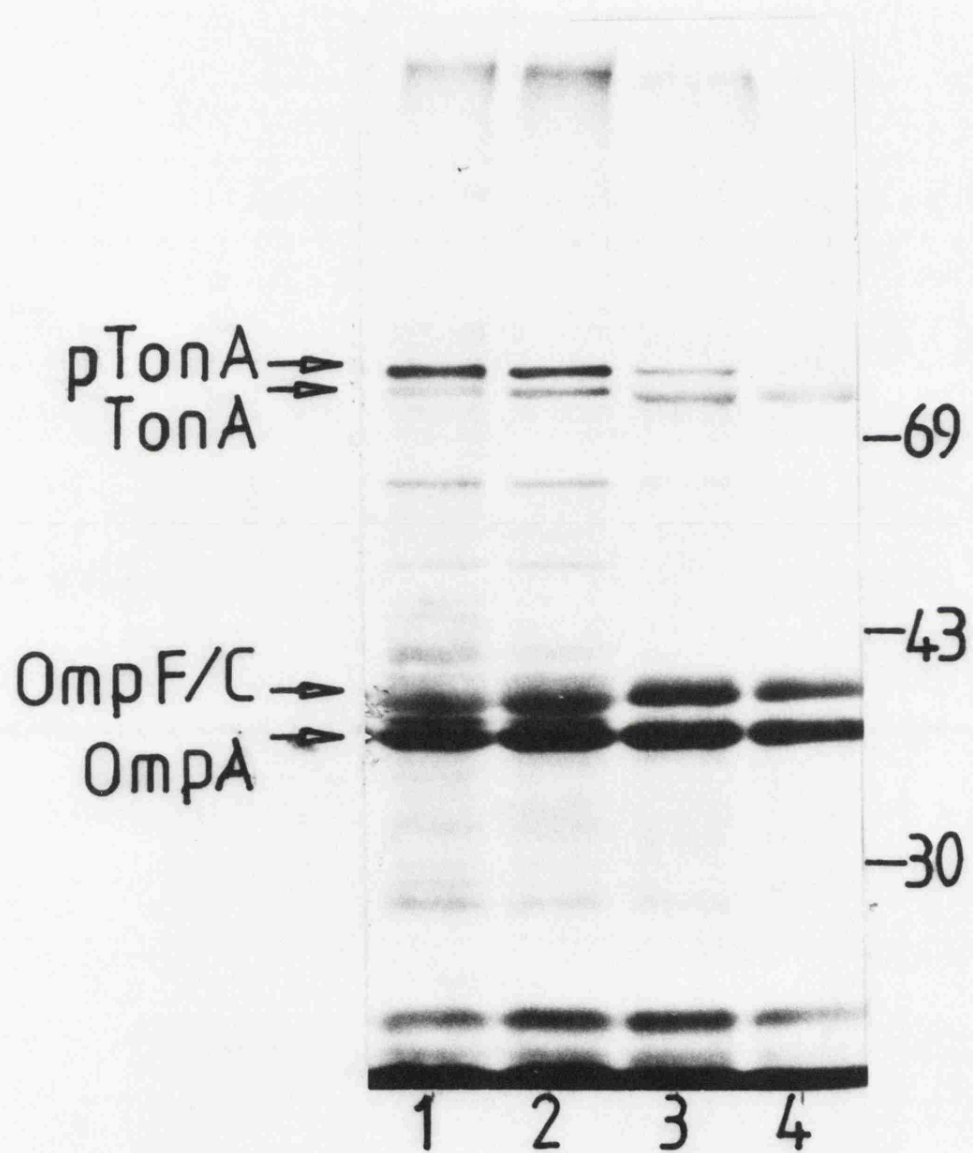
Figure 3.5

Procesing of preTonA in a secY mutant in the absence of protein synthesis.

A culture of IQ85(pLC513) was grown in glucose minimal medium at the restrictive temperature for 2h and then a 10ml sample was labelled for 30s with ^{35}S-methionine as above and chased in the presence of excess cold methionine and kanamycin (final concentration 250mg/ml). Lanes 1-5 outer membrane fractions after 0, 30, 60, 80 and 120s of chase.
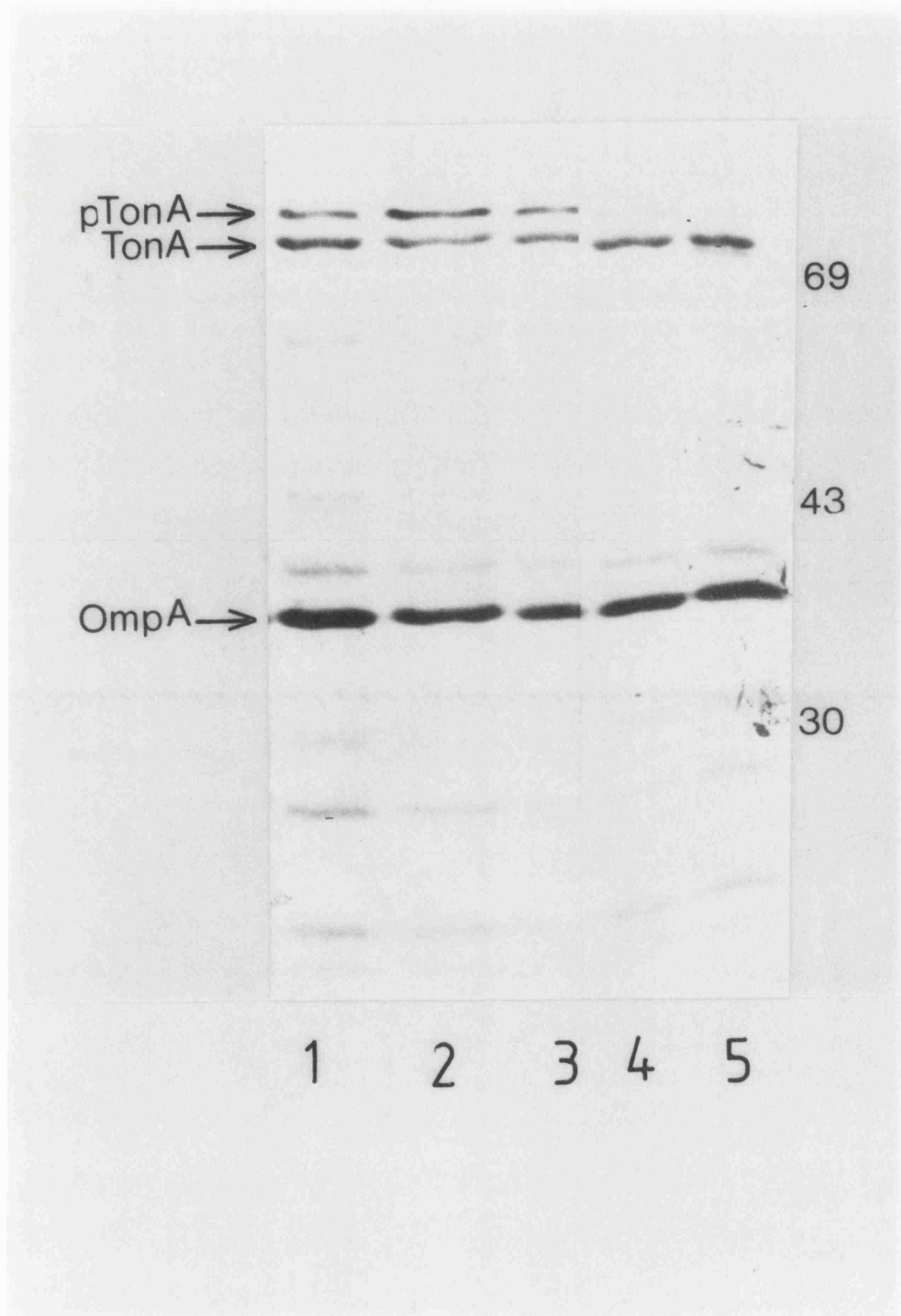
Figure 3.6

Synthesis of TonA upon return to 30°C.

A culture of MM52(pLG513) was grown in glucose minimal medium for 2h
at 42°C. A 1ml sample was then pulse-labelled with ^{35}S-methionine for
30s immediately before the culture was transferred to 30°C. Additional,
1ml samples were subsequently pulse-labelled at intervals at 30°C and
fractionated into inner and outer membranes on the basis of Sarkosyl
solubility. Samples were analysed by SDS-PAGE followed by
autoradiography. Lanes 1-4, outer membrane fractions 0, 2, 8 and 10min
after transfer to 30°C.
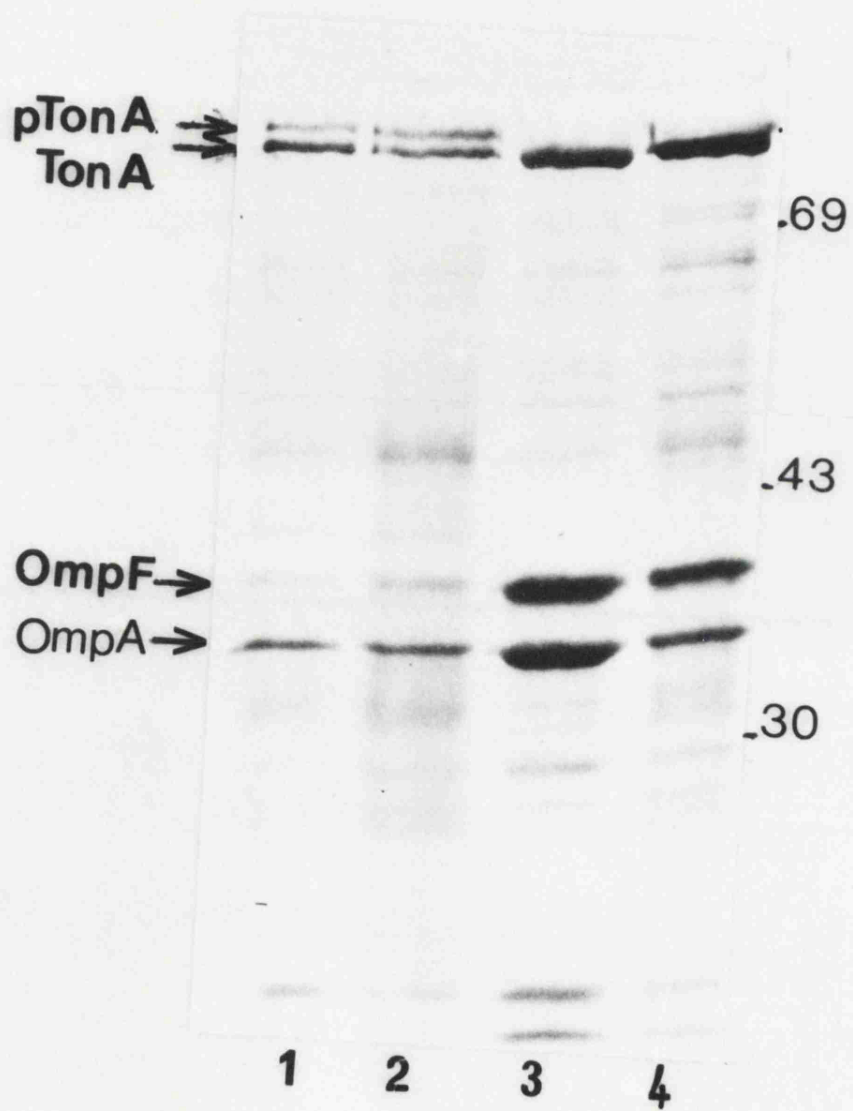
Fig.3.6 shows that upon return to 30°C, SecA rapidly appeared to regain the ability to catalyse secretion. Thus, after a maximum of 8min, all newly synthesised TonA protein was observed in the correctly processed form, similarly, the ability to synthesise porins was also restored rapidly. Therefore, the failure to observe efficient post-translational processing of preTonA labelled at 42°C in the experiments described in the previous section, is not primarily due to the inability to synthesise new active forms of SecA upon return to permissive temperature. Rather the results are more consistent with the accumulation of preTonA in a translocationally incompetent form in the absence of SecA.

Discussion

These studies on the kinetics of processing of preTonA in either secA^{ts} or secY^{ts} strains upon return to the permissive temperature, have revealed important differences between the two mutations. The bulk of preTonA accumulating in the secA^{ts} strain and sedimenting with the outer membrane fraction, was apparently processed extremely inefficiently to the mature form during a chase at 30°C. In fact in the presence of chloramphenicol during the chase, processing of the precursor was apparently completely inhibited. This may indicate that "processing" in the absence of chloramphenicol is occurring via inducible increases in protease levels rather than via alternative translocation pathways or even via residual SecA activity. This was supported by the apparent instability of preTonA at 42°C which disappeared without a concomitant increase in mature TonA in the outer membrane (data not shown).

Interestingly, small but significant amounts of preTonA detected in the inner membrane after pulse-labelling, at 42°C, appear to chase

rapidly at the permissive temperature. This may reflect the processing
of a pool of preTonA trapped in the inner membrane, for example in
association with components of the secretory pathway beyo'nd SecA.

The observation that accumulated preTonA only inefficiently crosses
the inner membrane post-translationally in a secA mutant, agrees with the
observed kinetics of processing of preMBP in the same strain. Oliver and
Beckwith (1981) established that in MM52(secA^{ts}), after a short pulse at
the restrictive temperature, preMBP, which accumulated in the cytoplasm,
although stable for up to 20min, could not be processed to the mature
form, at either the permissive or restrictive temperature.

This reduced efficiency of preTonA or preMBP to chase into the
mature forms even upon restoration of SecA function suggests that the
conformation adopted by precursor accumulating in the cytoplasm in the
absence of active SecA, greatly inhibits subsequent post-translational
translocation across the inner membrane. There may indeed be a number of
explanations for the slow processing of preTonA under these post-
translational conditions. These might include non specific protease
digestion of precursor independently of translocation, or residual levels
of protein assembly which can occur in a secA^{ts} strain even after 2h at
42°C. Such translocation may be a result either of low levels of SecA
activity or of the presence of an alternative inefficient secretion
pathway normally masked by the presence of efficient secA dependent
secretion.

The kinetics of restoration of SecA at 30°C are much more rapid than
the kinetics of processing of preTonA accumulated at 42°C. This
therefore indicates that the failure to process precursor efficiently is
not due to the inability to resynthesise SecA rapidly. Rather the data
are more consistent with the formation of defective SecA/preTonA

complexes at 42°C having a greatly reduced capability of continuing along the translocation pathway. This would be most readily explained if, in the absence of SecA, preTonA folds into a form incompatible with translocation.

PreTonA which accumulates in a secY mutant appears to differ from precursor in a secA mutant since it apparently maintains a translocationally competent form. In fact, preTonA which accumulates in the outer membrane is rapidly processed to mature TonA upon return to 30°C. This post-translational processing seems to occur regardless of whether or not new SecY protein is synthesised. This result indicates that rapid renaturation of the protein encoded by the IQ85 secY allele, can take place.

Previous reports have also suggested that envelope protein precursors accumulating in secY[ts] strains can subsequently be chased efficiently into the mature form (Ito et al., 1983). However, during a chase carried out at 42°C, Shiba et al. (1984) observed that although all precursors examined were converted to the mature form, different proteins seemed to chase into the mature form at different rates. For example, preMBP showed slow conversion whereas preOmpA was completely converted into OmpA 5min into the chase. This post-translational export of OmpA was attributed to either residual SecY activity at 42°C or the emergence of an alternative, less efficient, but SecY independent pathway which is used by different proteins with different degrees of efficiency. In these experiments however, the chase was carried out at 42°C and as a result, spurious processing may occur due to the synthesis of heat shock inducible proteases (Phillips et al., 1984).

In support of the findings in this study, Wickner, Ito and their colleagues also concluded that the role of SecY in protein export is not

limited to nascent polypeptide chains. These authors observed that the

processing of full length preOmpA which had accumulated at 42°C in a secY

mutant is accelerated by inducing synthesis of plasmid encoded wild type

SecY. They therefore suggested that under certain circumstances, SecY is

capable of catalysing post-translational translocation and that SecY

function is therefore not intimately related to ribosome function or

chain elongation (Bacallao et al., 1986).

As a result of this and other studies on secA and secY mutants it is

therefore possible to propose that SecA may function as part of a complex

which acts to maintain or to promote the production of envelope

precursors in a translocationally competent form. A function which may

be achieved by binding to the signal sequence and also perhaps to parts

of the mature protein to prevent folding of the nascent chain.

Alternatively, SecA may promote conformational changes in the nascent

polypeptide which allow partial unfolding and subsequent passage across

the bilayer. In either case, it is possible to speculate that once

precursors like preTonA have been delivered to translocation sites in the

inner membrane, which may contain for example SecY, SecA function is then

no longer required.

A method of protein secretion may therefore be envisaged which

relies upon the binding of SecA to nascent polypeptides in order to

prevent precursor folding into a translocationally incompetent form.

Such a SecA/precursor complex could possibly remain translocationally

competent for long periods until subsequent interaction with membrane

bound SecY allows translocation across the inner membrane. SecY itself,

which from the pulse chase kinetic studies appears to act at a later step

than SecA, could conceivably play a number of different roles. Thus SecY

protein with its location in the inner membrane may bind nascent

secretory complexes in order to link energy transducing processes to translocation itself. Alternatively, SecY may bring the nascent secretory complex into close juxtaposition with the membrane "translocator" or even form part of a polypeptide transport channel itself.

CHAPTER FOUR

Effect of secA and secY inactivation on haemolysin export

Introduction

Haemolysin is one of the few proteins secreted by E.coli into the
external growth medium, and to reach the external environment, the large
107kD polypeptide must cross both inner and outer membranes. Since the
latter normally provides a barrier to release of proteins from the cell,
some mechanism must therefore have evolved whereby haemolysin can cross
the outer membrane in the absence of cell lysis.

Haemolysin is unusual, not only in the fact that it is released into
the environment, but also, although it crosses both the periplasm and
outer membrane, it is synthesised without a classical NH_2-terminal signal
sequence (Felmlee et al., 1985a). In addition, as discussed in Chapter 1,
the secretion of haemolysin is absolutely dependent on the presence of two
proteins, synthesised from contiguous genes (Mackman et al., 1985a).
These genes have been mapped and the products identified (see Chapter 1).

The observation that secretion of HlyA has an absolute requirement
for HlyB and HlyD proteins, has led to the proposal that these two
proteins might form a specific secretion complex which spans the inner and
outer membranes and is able to bind to haemolysin and promote direct
translocation to the medium (Mackman et al., 1986). Whether translocation
via this pathway is also secA/secY dependent had not however been
established. It was therefore of interest to ascertain whether, in
addition to a requirement for two novel proteins, HlyB and HlyD, export of
haemolysin to the external medium is also dependent on SecA and SecY.

4.1 Haemolytic activity in MM52(secA^{ts})

Preliminary experiments carried out in an attempt to investigate the affect of secA inactivation on haemolysin secretion, were designed to compare the level of cellular and released haemolytic activity in the temperature sensitive secA mutant, MM52 at 30°C and at 42°C. Therefore plasmid pANN018, a clone of a chromosomal haemolysin determinant provided by W. Goebel (N. Mackman, Ph.D thesis, 1984) which contains the whole haemolytic determinant, was transformed into MM52. This plasmid produces only low levels of haemolytic activity but was used in preference to pLG570, since although pLG570 directs the synthesis of higher levels of haemolysin, it is a temperature sensitive "runaway" plasmid and therefore promotes the synthesis of increased levels of haemolysin at 42°C (Mackman and Holland, 1984a). This would make comparisons of haemolytic activity at 30°C and 42°C difficult to interpret.

Cells were grown in nutrient broth supplemented with calcium chloride which has been shown to increase the amount of haemolytic activity, probably through stabilisation of the toxin (Mackman and Holland, 1984b). At an A_{450} = 0.4 the culture was transferred from 30°C to 42°C and 1ml samples were taken at intervals and centrifuged (5min, 14,000rpm) to separate supernatant from cells. 200μl of each supernatant sample was then assayed for the ability to lyse red blood cells (see Materials and Methods). Haemolytic activity for each supernatant sample was plotted against increase in absorbance of the culture, thus, although growth was more rapid at 42°C the values obtained at different temperatures were directly comparable. Intracellular haemolytic activity was also monitored but was negligible in both cases.

As the results in Fig.4.1a indicate, haemolytic activity continues to accumulate apparently undisturbed in the secA mutant at 42°C, although

Figure 4.1

Accumulation of external haemolytic activity during the growth of strain MM52(pANNO18).

(A) 100ml cultures were grown in nutrient broth supplemented with 10mM calcium chloride, at 30°C and 42°C respectively. At intervals, 1ml samples were taken, from each culture, centrifuged to separate the growth medium from the cells and 200μl of supernatant were assayed for external haemolytic activity which was measured as described in Materials and
Methods. The haemolytic activity was plotted against A_{450} so that the peaks for the different cultures were directly comparable.

Symbols: ___ haemolytic activity in MM52(pANNO18) at 30°C,

- - - haemolytic activity in MM52(pANNO18) at 42°C.

(B) Stability of haemolytic activity in the medium at 0°C, 30°C and 42°C respectively.

A culture of MM52(pANNO18) was grown in nutrient broth in the presence of 10mM calcium chloride to an A_{450} = 0.8. 10ml of cells were harvested by centrifugation and supernatant samples were placed at each of three different temperatures. 200μl samples were assayed for haemolytic activity as described in Materials and Methods.

Symbols: ● ice, O 30°C, ■ 42°C.
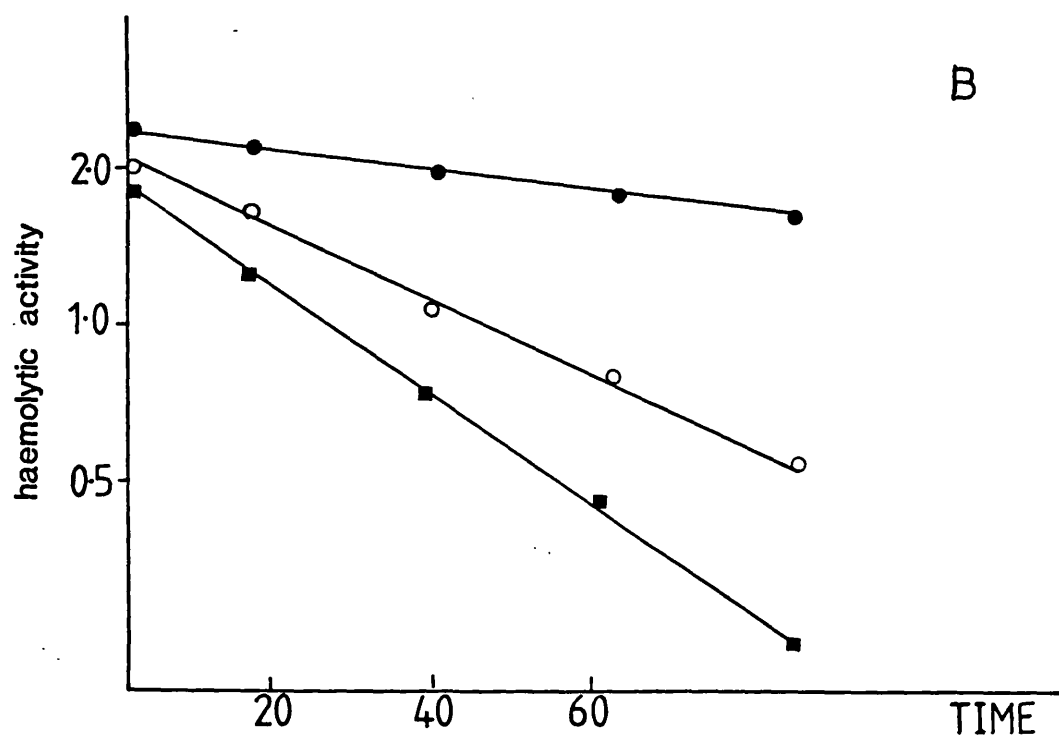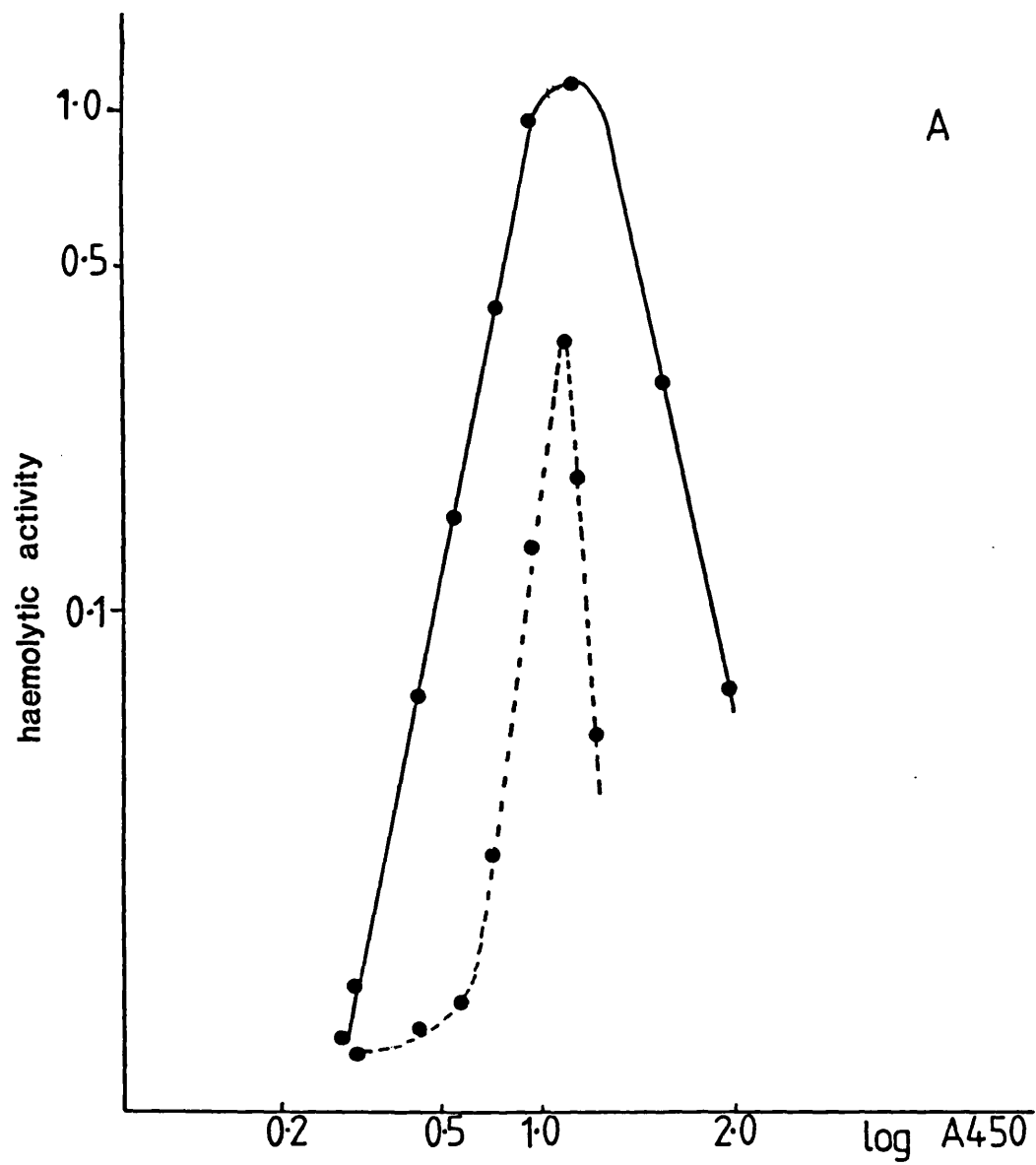
at $42^\circ C$ maximum haemolytic activity was lower than at $30^\circ C$. It has already been established that the stability of haemolysin is sensitive to many factors, including increase in temperature (Nicaud et al., 1985), therefore the decrease in activity observed at $42^\circ C$ (Fig.4.1a) could be due to greater instability and therefore a shorter half life of haemolysin. In order to quantitate this, the stability of haemolysin produced from pANN018 in strain MM52 was calculated. A 100ml culture of strain MM52(pANN018) was grown in nutrient broth plus calcium chloride to an A_{450} = 0.8, the cells were harvested by centrifugation (14,000 rpm; 10min) and the supernatant was incubated at various temperatures. Thus, 10ml of supernatant was placed either on ice, at $30^\circ C$ or at $42^\circ C$ and $200\mu l$ samples were assayed for haemolytic activity at 10min intervals. Fig.4.1b shows that, at $0^\circ C$ haemolysin is reasonably stable over long periods but the half life at $30^\circ C$ is 44min and at $42^\circ C$ the half life is 18min. The results obtained therefore appeared to indicate that in a $secA^{ts}$ mutant external haemolytic activity is diminished only as a result of the greater instability of haemolysin at the restrictive temperature and that export of haemolysin to the external medium is not grossly affected by defective SecA.

4.2 Pulse-labelling of the 107kD polypeptide in a $secA^{ts}$ mutant

Although the results in the previous section indicated that secA inactivation had little effect on the production of extracellular haemolysin, this assay is not particularly sensitive, especially, when residual secA activity may be quite significant even after 2h at $42^\circ C$ (see e.g Fig.2.3). Consequently, an alternative test of haemolysin production was sought.

In order to produce lysis of red blood cells, a chromosomally encoded

haemolysin requires the presence of not only hlyB and hlyD but also a

third gene, hlyC, the product of which is responsible for converting

inactive haemolysin to active haemolysin (Nicaud et al., 1985). These

various components of the haemolysin determinant have now been subcloned

and hlyA is fully expressed in the absence of hlyC (Mackman et al., 1985).

Moreover, the haemolysin produced, although inactive, can still be

exported from the cytoplasm in the presence of HlyB and HlyD (Nicaud et

al., 1985). Additionally, haemolysin which has not been activated by HlyC

has been found to be relatively more stable with a half life of 42min at

37°C compared to 25min for active haemolysin (Nicaud et al., 1985). These

developments therefore allowed haemolysin in this study to be labelled

with ^{35}S-methionine at both 30°C and 42°C. The amount of haemolysin

produced in MM52(secA^{ts}) after the temperature shift to 42°C was therefore

compared with the level of production in MC4100 under the same conditions.

MM52 (secA^{ts}) and MC4100, the wild type parental strain, were

transformed with plasmids pLG583 and pLG575. The former plasmid expresses

HlyA from a promoter within the tet gene in the low copy number plasmid

pLG339 and the latter expresses export proteins HlyB and HlyD from a

promoter within the tet gene in pACYC184 (Mackman et al., 1985).

Initially, strain MM52(pLG583, pLG575) was grown in glucose minimal medium

at 30°C, until, at an A$_{450}$ = 0.4, the culture was transferred to 42°C.

1ml samples were taken at intervals and pulse-labelled with ^{35}S-methionine

for 5min. Supernatant and cells were separated by centrifugation and

protein in the supernatant was precipitated by the addition of TCA. The

protein profiles were then analysed by SDS-PAGE and autoradiography. The

time course of secretion shown in Fig.4.2a indicates

Figure 4.2

Effect of secA inactivation on haemolysin secretion

(A) A 100ml culture of MM52(pLG575,pLG583) was grown in glucose minimal medium at 30°C to an $A_{450} = 0.4$. The culture was then transferred to 42°C and 1ml samples were labelled at intervals with 15 μCi of ^{35}S-methionine. Cells were separated from the growth medium by centrifugation and any protein in the supernatant was precipitated by the addition of TCA. Fractions were analysed by SDS-PAGE (11% acrylamide) and autoradiography. In order to identify the position of the 107kD protein on the gel, and also to show the expected degree of cytoplasmic contamination in a sample prepared from cells which did not contain the haemolytic determinant, samples from SE5000(pLG570) and SE5000 were also prepared and loaded on the gel.

Lane 1, SE5000(pLG570)supernatant; lane 2, SE5000 supernatant; lane 3 molecular weight standards; lanes 4 to 7 supernatant fraction from a culture of MM52(pLG575,pLG583) after 0, 60, 90 and 120min respectively at 42°C. Supernatant from exactly 0.2 A_{450} units of cells was loaded in lanes 4 to 7.

(B) 100ml cultures of MM52(pLG575,pLG583) and MC4100(pLG575,pLG583) were grown in glucose minimal medium at 30°C to an $A_{450} = 0.4$. Cultures were then transferred to 42°C and after 2h, 1ml samples of both wild type and mutant were pulse-labelled with ^{35}S-methionine for 5min. Samples were analysed by SDS-PAGE (11% acrylamide) and autoradiography. Lane1, MM52 (pLG575,pLG583) cells; lane 2, MC4100(pLG575,pLG583) cells; lane 3, MM52(pLG575,pLG583) supernatant; lane 4, MC4100(pLG575,pLG583) supernatant. Samples were loaded as identical A_{450} cell equivalents.

A

92.5 -
69 -
43 -
30 -

1 2 3 4 5 6 7

◀107

B

200 -
92.5 -
69 -
46 -
30 -
14 -

1 2 3 4

◀107
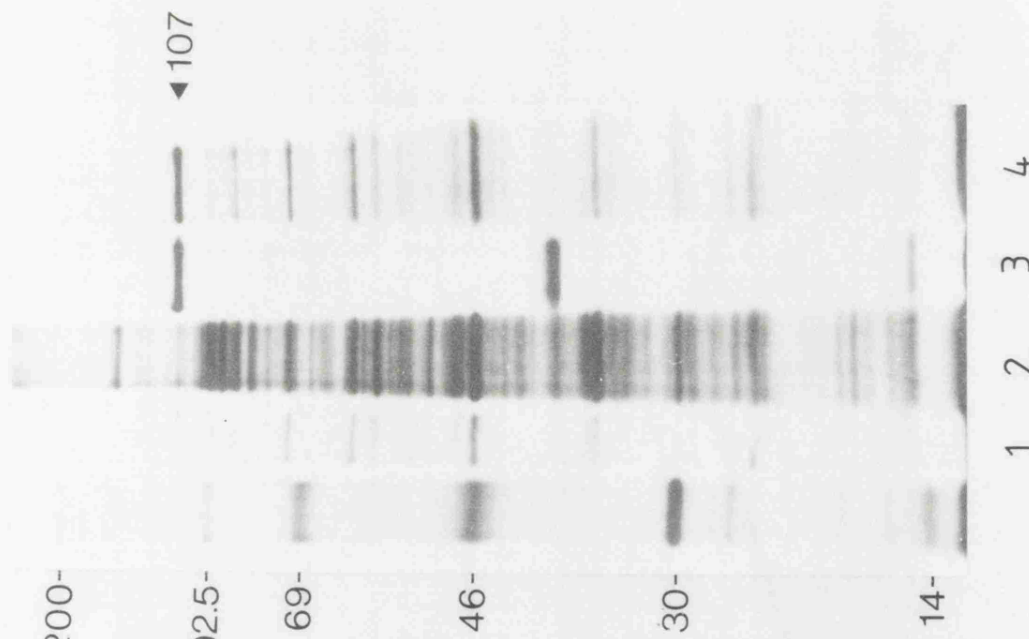
that there is no apparent loss of ability to secrete haemolysin in strain MM52(secA^{ts}) even after 2h at 42°C. This is corroborated in Fig.4.2b, which shows a comparison of cell and supernatant fractions from MM52(pLG583,pLG575) with the wild type parental strain, MC4100 (pLG583,pLG575) grown in parallel in glucose minimal medium at 42°C. After 2h at 42°C, 1ml samples of both cultures were labelled with 15μCi ^{35}S-methionine for 5min at 42°C. Cells were harvested and any protein in the supernatant was precipitated by the addition of TCA before analysis by SDS-PAGE followed by autoradiography. Fig.4.2b shows protein from cells and supernatant from the two strains loaded at exact A$_{450}$ equivalents. There was no evidence to indicate that the synthesis and secretion of haemolysin is inhibited in the mutant. The effect of the secA mutation in MM52 is nevertheless evident by the observation that less radioactivity was incorporated into the cells in this strain than in MC4100, presumably due to defects in the membrane structure which have inhibitory effects on bulk proteins synthesis after 2h.

In some, but not all, experiments a second protein was observed to be released into the medium together with HlyA (see Fig4.2b, lane 3). This protein migrated with a molecular weight of 34kD and comparison with a normal outer membrane profile indicated that the band probably represented OmpA. Residual levels of this protein reach the outer membrane even in the absence of functional SecA but the fact that OmpA was not always found in the growth medium after secA inactivation indicates that there is not consistently a pool of this protein in the supernatant. It is likely that the protein is unable to assemble correctly when the membrane is abnormal and is therefore released into the medium. The presence of this pool of OmpA in the supernatant may simply depend on the exact state of the membrane in any given experiment.

4.3   Effect of secY inactivation on the secretion of haemolysin

Since SecA and SecY have been proposed to be components of the same

export pathway (Brickman et al., 1984), it seemed likely that the export

of haemolysin from the cytoplasm to the external medium which appears to

be largely independent of SecA function would also be independent of SecY

function.   To investigate this, E.coli strains IQ85(secY^{ts}) and IQ86, the

wild type parental strain, were transformed with plasmid pLG583, encoding

HlyA, and pLG575, encoding export functions HlyB and HlyD, and

transformants were selected by the appropriate antibiotic resistance.

100ml cultures were subsequently grown in glucose minimal medium at 30°C

and at an A$_{450}$ of approximately 0.4, cultures were transferred to 42°C.

As shown in Fig.4.3, although both strains grow at 30°C with a doubling

time of 55min, upon transfer to 42°C, increase in absorbance in the mutant

strain immediately slowed and there was a concomitant reduction in protein

synthesis, indicated by the failure of cells to incorporate significant

levels of ^{35}S-methionine soon after transfer to 42°C (data not shown).

In order to investigate whether this effect was a direct result of

abortive secretion of haemolysin upon secY inactivation at high

temperature, strains synthesising either export functions HlyB and HlyD,

or HlyA alone were constructed and the increase in optical density after

the temperature shift was monitored at A$_{450}$.   Fig.4.3 shows the effect of

temperature shift on mass increase of these strains.   It seems that

individually, neither export functions nor the haemolysin toxin itself

have the same deleterious effect on cell growth after transfer to 42°C,

and in fact the growth rate actually increases for a short time before

growth is inhibited.   This may indicate that when cell growth is inhibited

immediately it is not a specific effect caused by interaction of defective

Figure 4.3

Growth of IQ85(secY^{ts}) synthesising various components of the haemolysin determinant.

100ml cultures of IQ85(pLG575,pLG583), IQ85(pLG583), IQ85(pLG575) and IQ86(pLG575,pLG583) were grown at 30°C in glucose minimal medium supplemented with casamino acids and at an A$_{450}$ of approximately 0.4 the cultures were transferred to 42°C (↑). Mass increase was followed by monitoring A$_{450}$ at intervals throughout the growth period and the increase in absorbance was plotted against time.

Symbols: ——●——IQ86, ——○——IQ85(pLG583), ⁻⁻●⁻⁻IQ85(pLG575), ⁻⁻○⁻⁻IQ85(pLG575,pLG583).
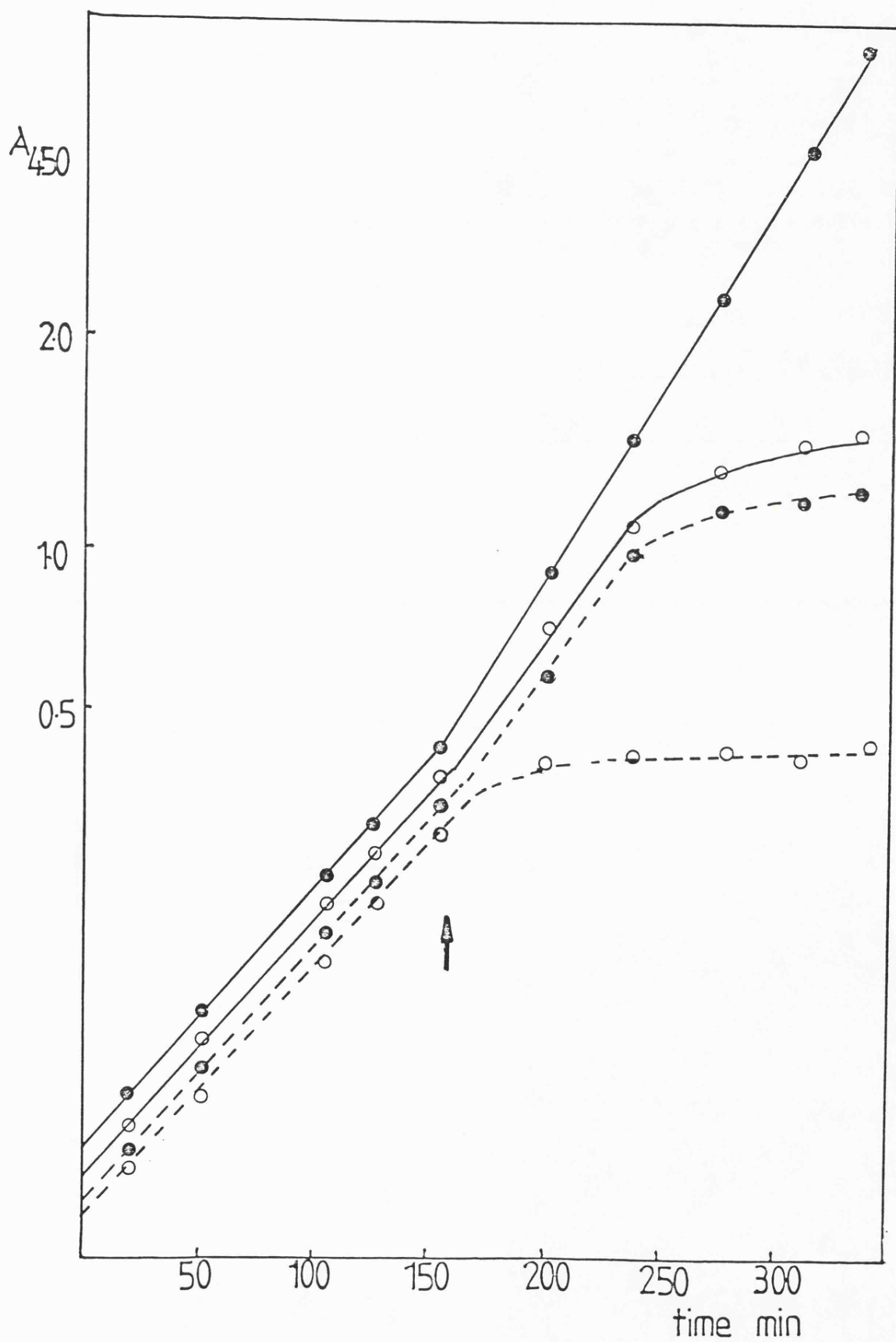
Figure 4.4

Intracellular accumulation of haemolysin in IQ85 at 30°C.

100ml of IQ85(pLG575,pLG583) and IQ86(pLG575,pLG583) were grown in nutrient broth plus calcium chloride at 30°C and when the cultures reached an A_{450} = 0.8, 20ml samples were taken, cells were harvested by centrifugation and any protein in the supernatant was precipitated by the addition of TCA. The protein profiles of the different fractions were analysed by SDS-PAGE (11% acrylamide).

(A) Coomassie blue stained profile of, lanes 1 and 2, IQ86 (pLG575, pLG583) cells and supernatant and lanes 3 and 4 IQ85 (pLG575,pLG583) supernatant and cells. Cells equivalent to 0.5 A_{450} units of cells and protein from the supernatant equivalent to 5.0 A_{450} units of cells were loaded in each lane.

(B) Western blot of cell and supernatant samples from IQ85(pLG575, pLG583) were using polyclonal antibody raised against HlyA. Lane 1, supernatant and lane 2, cells. Exactly equivalent amounts of cells and supernatant were loaded in each lane.
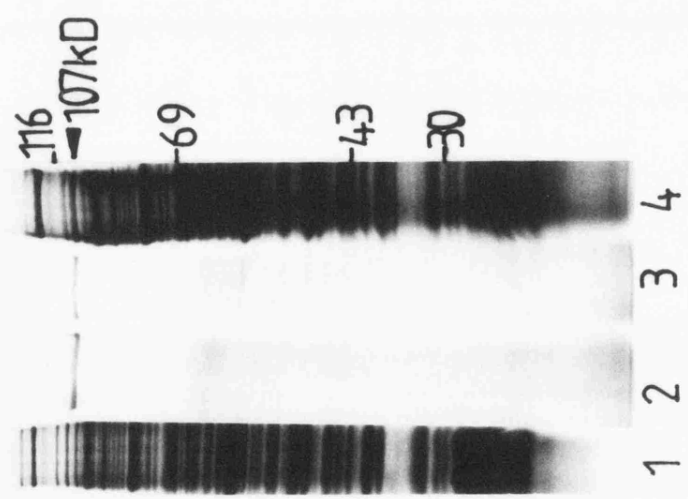
SecY with either export components or HlyA itself, and rather suggests that if an interaction between the 107kD protein and SecY is important, it may occur at a stage of export when protein actually translocates across the membrane. However, it may be concluded that whatever the reason, it seems that rapid inhibition of growth precludes any direct test of the effect of secY on the synthesis and secretion of the 107kD protein in stain IQ85.

4.4. Intracellular accumulation of haemolysin at the permissive temperature in a secY^{ts} mutant.

Since it has been observed that the secY mutation is leaky and envelope protein precursors may accumulate at low levels even at 30°C in the secY^{ts} strain IQ85 (see e.g.Ito, 1986), it is possible that mutant SecY may lead to inhibition of haemolysin export even at 30°C. If this is the case, low levels of haemolysin may accumulate intracellularly at the permissive temperature. In order to establish whether an effect on haemolysin export could be observed at 30°C, secY^{ts} cells carrying appropriate haemolysin clones were grown in nutrient broth plus calcium chloride at the permissive temperature to an $A_{450} = 0.8$. Supernatant and cells were separated by centrifugation (14,00rpm, 10min) and any protein in the supernatant was precipitated by the addition of TCA. Samples analysed by SDS-PAGE and stained with Coomassie blue (Fig4.4a) showed that although the 107kD polypeptide appears to be efficiently secreted at 30°C, a band running at the same position in the total cell lysate of IQ85 but absent in IQ86 may represent low levels of intracellular haemolysin at 30°C. In order to confirm this, exactly equivalent amounts of cells and supernatant were analysed by a Western blot using polyclonal antibody raised to HlyA. Fig4.4b shows that

surprisingly, high levels of haemolysin are retained within the cell at 30°C, almost equivalent in fact, to the amount secreted. In the wild type strain, no intracellular accumulation is observed at either 30°C or 42°C (data not shown).

It is still possible that the accumulation of the 107kD polypeptide at 30°C is also a secondary effect caused by minor alterations in the membrane even at 30°C. This seems unlikely however and it seems more probable that either HlyB and HlyD or HlyA, directly require SecY for correct assembly or export to the extracellular growth medium respectively.

Discussion

The experiments described here indicate that the actual mechanism of export of haemolysin from the cytoplasm to the external medium is apparently independent of SecA function. Assessment of a possible role for secY was complicated by the fact that growth of secY cells carrying the haemolysin determinant was rapidly inhibited at 42°C. Since this effect was most marked when HlyA, HlyB and HlyD were all synthesised by the cells it seems that this growth defect may be due to abortive translocation of haemolysin involving interaction with a defective SecY protein. This hypothesis was supported by the observation that haemolysin appears to accumulate intracellularly even at 30°C in the secY[ts] mutant. It may be postulated therefore, that although independent of SecA function, either haemolysin itself normally requires SecY in order to pass through the membrane or SecY is in some way vital for the correct assembly of HlyB and HlyD. It must be noted however, that the decrease in growth observed in the secY strain may simply be the result of a non-specific effect caused by the high plasmid load.

It has already been established that haemolysin export is absolutely
dependent on the presence of HlyB and HlyD (see e.g. Mackman et al.,
1986). These two proteins, although located at least in part in the inner
membrane (Mackman et al., 1986) and synthesised without a signal sequence
(Felmlee et al., 1985a) may require either or both SecA and SecY in order
to assemble correctly into the membrane. In the case of the secA mutant,
relative stability and low turnover of functional HlyB and HlyD might
allow haemolysin export to continue, even if SecA is required for assembly
of the export components to the membrane. SecY, is an integral membrane
protein, and even if not directly required for assembly of HlyB and HlyD,
defective protein may sufficiently disrupt the membrane to prevent HlyB
and HlyD from mediating the export of haemolysin.

Currently, high expression systems in normal cells which allow
synthesis of HlyB or HlyD in sufficient quantity to visualise labelled
material in whole cell membranes pulse-labelled with ^{35}S-methionine, have
not been obtained. Therefore, it is not possible to assess directly the
effect of secA or secY inactivation on assembly of these proteins into the
membrane. It is likely however, that the construction of plasmids
expressing high levels of HlyB and HlyD in the near future should allow
for a direct analysis of the effect of secA/secY inactivation on assembly
into the envelope.

As indicated in Chapter 2, the function of SecY is not known but the
fact that HlyA may be secY dependent is consistent either with a role as a
ribosome receptor or perhaps in coupling energy transfer to protein
export. It is also possible, that if haemolysin is exported via a
periplasmic intermediate, (but see Chapters 1 and 6), SecY might mediate
translocation to the periplasm, whereas HlyB and HlyD allow transport
through the outer membrane.

CHAPTER FIVE

Secretion of a chimeric protein into the medium using the C-terminal

secretion signal of haemolysin

Introduction

Previous studies have indicated that removal of the last 27 amino

acids from the C-terminus of haemolysin abolishes secretion from the

cytoplasm to the external medium (Gray et al., 1986). It therefore

appears that this C-terminal region may contain a topogenic sequence

which can interact with the export components HlyB and HlyD in order to

promote secretion. In fact, the 23kD fragment encoded by the C-terminus

of hlyA has been subcloned to form plasmid pLG609 (Nicaud et al., 1986).

In the presence of pLG575, encoding the Hly export functions, the protein

encoded by pLG609 can be secreted into the medium even in the absence of

the intact haemolysin (Nicaud et al., 1986). This strongly suggests that

a specific region close to the C-terminus contains all the information

necessary for secretion.

Since haemolysin export differs in many ways from conventional

protein export, and moreover the presence of a topogenic sequence so

close to the C-terminus is unique, the possibility of using the

C-terminus to promote release of a membrane protein which is unable to

utilise its normal export pathway was investigated. In particular it was

anticipated that a hybrid molecule, consisting of the core of an envelope

protein fused to the C-terminus of haemolysin, even if not secreted,

should provide valuable information as to the actual route followed by

different proteins as they leave the cytoplasm. For this reason a

chimeric molecule was constructed which consisted of the C-terminus of

haemolysin fused to the core of OmpF, an outer membrane protein whose

export is normally dependent on a classical NH_2-terminal signal sequence

and is mediated by SecA. In all probability therefore, OmpF, normally

crosses the inner membrane and periplasm using a completely different

mechanism from that used by haemolysin.

5.1   Construction of a lacZ-ompF-hlyA chimeric molecule

The C-terminus of hlyA was fused to the core of ompF in a two stage

process (Fig.5.1). The first step involved the formation of a lacZ-ompF

hybrid in the high copy number vector pUC12. In order to do this, the

central portion of ompF was cut out on a 0.9kb BglII - HincII fragment,

from plasmid pLG361, which encodes the entire ompF gene (Jackson et al.,

1985). This fragment contains the core of OmpF but lacks the signal

sequence and also DNA encoding both the first 11 amino acids of the

NH_2-terminus and the final 30 amino acids at the C-terminus of the mature

molecule. Removal of the DNA encoding the NH_2-terminal signal sequence

was an advantage, since competitive translocation across the inner

membrane via the usual OmpF export pathway is thus avoided.

The purified ompF fragment was ligated with a BamHI - HincII digest

of pUC12 and the ligation mix was subsequently transformed into E.coli

strain JM101. Transformants were selected on minimal agar containing

ampicillin, IPTG and Xgal and white colonies which contained inserts were

characterised by digestion with restriction enzymes (Fig.5.2).   One

transformant, pLG631, was identified as containing an in frame fusion of

ompF to the NH_2-terminus of ρ-galactosidase. The resultant lacZ-ompF

gene was predicted to encode a hybrid protein consisting of the first

ten residues of β-galactosidase attached to the central 300 residues of

OmpF.

Figure 5.1

Construction of a lacZ-ompF-hlyA gene fusion.

The lacZ-ompF-hlyA gene fusion was created in two steps. First, a lacZ-ompF hybrid gene was made in pUC12. The core of the ompF gene, lacking DNA sequences encoding the first 11 amino acids of the NH$_2$-terminus and the final 30 amino acids of the C-terminus of mature OmpF, was removed from pLG361 (Jackson et al., 1985) on a 0.9kb BglII - HincII fragment. This was inserted downstream of the lac promoter of pUC12 which had been digested with BamH1 and HincII. The intermediate plasmid, pLG631, was predicted to encode a chimeric protein containing at its NH$_2$-terminus the first 10 amino acids of a β-galactosidase moiety fused to the central 300 amino acids of OmpF. Secondly, pLG609 was digested with EcoRI and filled in using Klenow to make blunt ends, before being further digested with HindIII to generate a 1.6kb fragment. This contained the 3' end of hlyA and was inserted into pLG631, which had been digested with HincII and HindIII. The final construct, pLG632 was selected by resistance to ampicillin and its composition was confirmed by restriction enzyme analysis.

SsI?
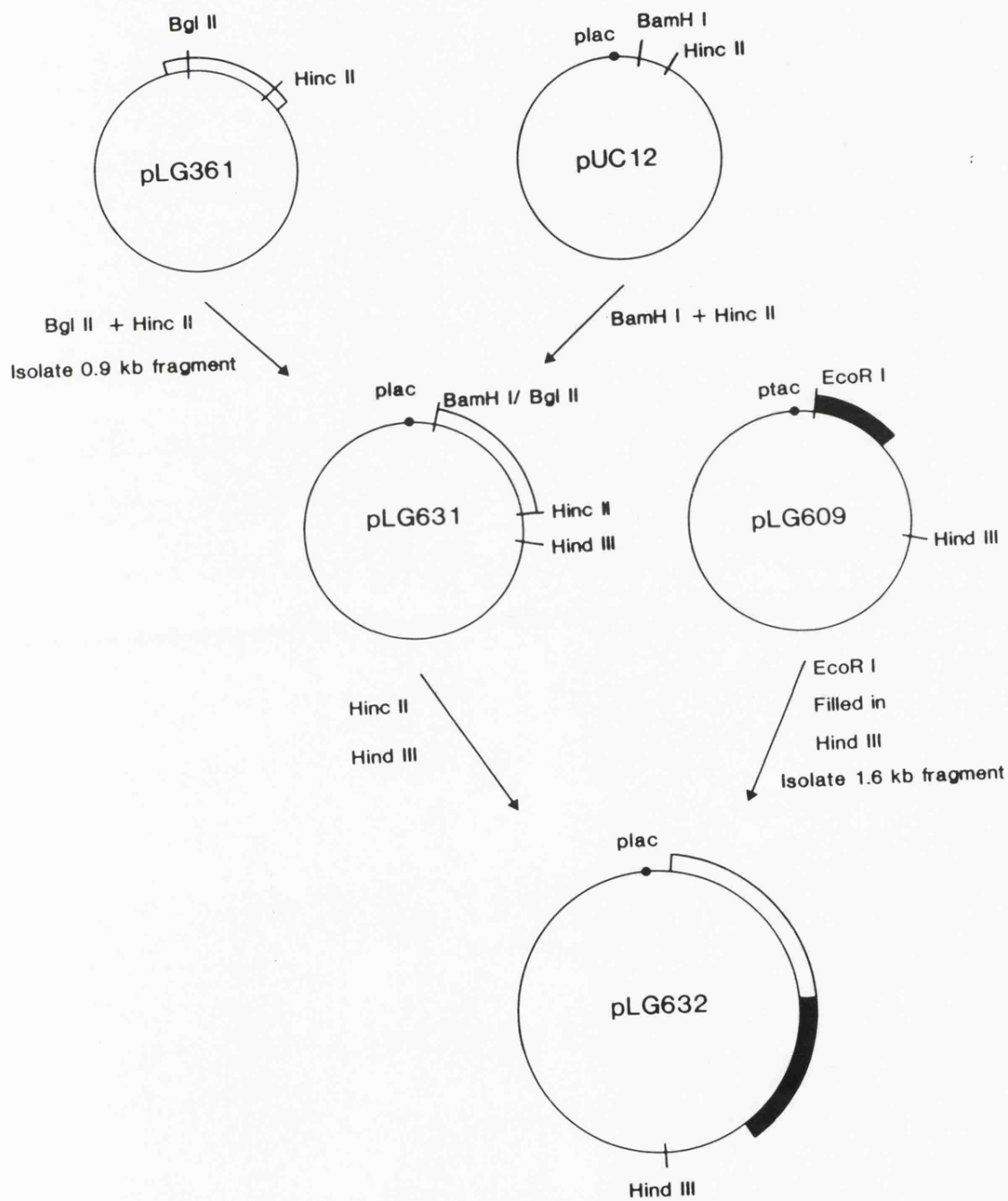
Figure 5.2

    Restriction digests, indentifying insertion of a 0.9kb fragment

    derived from pLG361 into pUC12.

(A) The restriction enzymes HindIII and SstI both cut at opposite ends

within the multi-cloning site of the vector pUC12. Since neither of

these enzymes cut within the 0.9kb fragment of pLG361, it is possible to

remove the 0.9kb fragment inserted into pUC12 by double digestion with

these enzymes. A double HindIII x SstI digest should yield one fragment

of size 0.9kb and one of size 2.68kb.

    Tracks 1-11 show HindIII x SstI double digests of DNA prepared from

colonies identified as containing inserts from plating on IPTG and Xgal.

Track 1? shows a similar digest of pUC12 and track 12 contains λ HindIII

size markers. Track 5 appears to contain the predicted 0.9kb and 2.68kb

fragments.

(B) Further characterisation of clone 5 (pLG631 in Fig. 5.1) indicated

that this was indeed the required recombinant. Track 1, uncut clone 5;

track 2, clone 5 digested with HindIII and SstI; track 3, HincII cut

clone 5; track 4, HindIII cut clone 5 and track 5, λ HindIII standards,

with the 1.96 and 0.59 markers indicated.

Figure 5.3

   Restriction digests, identifying the lacZ-ompF-hlyA chimeric gene.

   Ligation of the 1.6kb fragment from pLG609 into pLG631 is predicted

to recreate a unique HindIII site within the recombinant plasmid. It

should therefore be possible to linearise the new construct by digestion

with HindIII.

(A) Tracks 1-8 show HindIII digests of DNA prepared from CSH26

transformed with the pLG631/1.6kb fragment ligation mix. Recombinants

2-5 are approximately 5.3kb in size and were therefore predicted to

contain the 1.6kb insert. Track 9 contains λ HindIII markers.

(B) Clone 2 and clone 8 were selected for further analysis by digestion

with SstI and HindIII. Track 1, clone 2 digested with HindIII and SstI;

track 2, clone 2 digested with HindIII; track 3, clone 8 digested with

HindIII and SstI; track 4, clone 8 digested with HindIII; track 5, pLG631

digested with HindIII and SstI; track 6, pLG631 digested with HindIII and

track 7 λ HindIII markers.   Clone 8, as predicted, appears to be a

recircularisation of pLG631, but clone 2 contains the 1.6kb from pLG609

and is therefore the required recombinant.
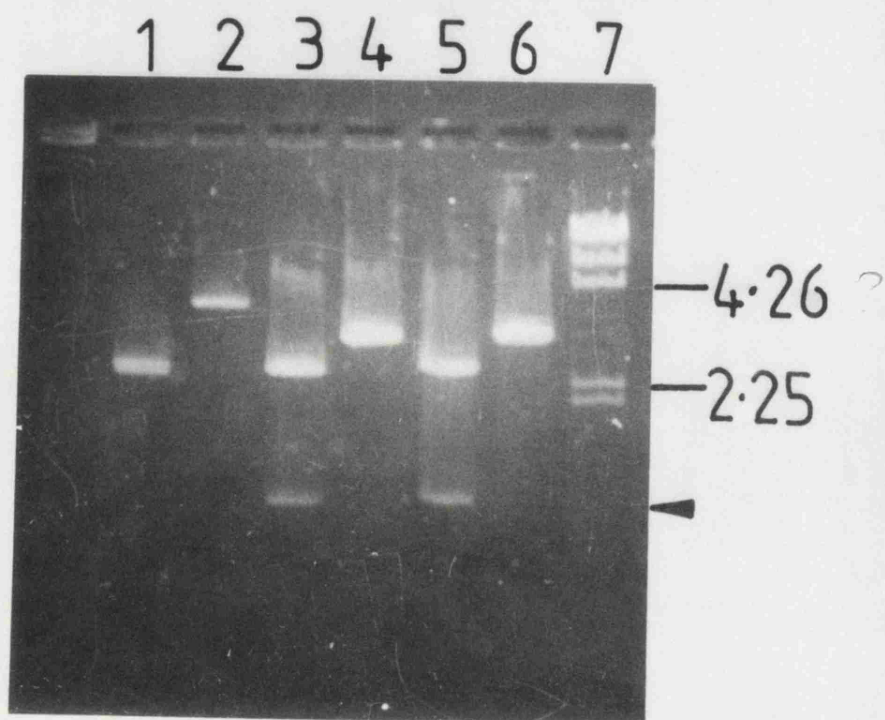
Table 5.1

Assay of differential release of β-lactamase from JM101(pLG575,pLG632) after induction with IPTG

| Time after induction (hours) | Enzyme Activity (units/ml) | | |
	SN Fraction	Cell pellet	% released
0	0.85	40	2.1
1.5	1.25	42	2.9
2.0	1.25	39	3.2

A 100ml culture of JM101(pLG575,pLG632) was grown in Luria broth containing the appropriate antibiotics until at an A_{450} = 0.2, IPTG was added to derepress the lac promoter. 0, 1.5, and 2h post-induction 1ml samples were taken and cell and supernatant (SN) fractions were separated by centrifugation. Cells were washed in Buffer B (see table 8.2), resuspended and sonicated as described in Materials and Methods. Aliquots of cells and supernatant equal to 0.2 and 0.4 A_{450} units were added to the nitrocefin assay buffer (see Materials and Methods) and the final volume was increased to 3.5ml. Hydrolysis monitored at 482nm in a Unicam spectrophotometer.

The second stage in the production of the lacZ-ompF-hlyA chimeric gene involved insertion of the C-terminus of hlyA into pLG631 at the C-terminus of ompF (see Fig.5.1). Plasmid pLG609 was therefore digested with EcoRI and filled in with Klenow to make blunt ends before further digestion with HindIII to generate a 1.6kb fragment. This fragment was purified from a low melting point agarose gel and was subsequently ligated into a HindIII - HincII, digest of pLG631 which had been treated with alkaline phosphatase. Recombinants were of necessity identified by restriction analysis. However, 35% of the transformants appeared to contain the 1.6kb insert when screened for the anticipated regeneration of a unique HindIII site and the production of two fragments of size 2.5kb and 2.8kb upon digestion with SstI and HindIII (see Fig.5.3).

A plasmid constructed in this way, pLG632, should therefore contain the 3' end of hlyA in frame with the lacZ-ompF hybrid to create a lacZ-ompF-hlyA gene. This gene, is predicted to encode a 56kD chimeric protein which consists of 10 amino acids of β-galactosidase, 300 residues of OmpF and the final 218 amino acids of HlyA. Transcription is under the control of the lac promoter and translation initiation is controlled by the start signals of β-galactosidase.

5.2 Secretion of a 56kD chimeric protein into the medium

The fact that inactive haemolysin and the 23kD C-terminal region of hlyA are both specifically secreted from the E.coli cytoplasm, but only in the presence of export functions hlyD and hlyB, indicated that export of the chimeric protein would probably also only occur in the presence of these two proteins. However, in order to ascertain whether this was indeed the case, pLG632 was transformed into strain JM101 (F'lacI^q), both in the presence and absence of hlyB and hlyD encoded on pLG575 (Mackman et al., 1985a).

Cells were grown in Luria broth plus the appropriate antibiotics and at an A_{450} = 0.2 the lac promoter was derepressed by the addition of IPTG. Cells and supernatant from both JM101(pLG632) and JM101(pLG632,pLG575) were harvested at intervals and protein in the supernatant fraction was precipitated by the addition of TCA. Proteins in each sample were separated by SDS-PAGE and visualised by staining with Coomassie brilliant blue.

Fig.5.4 shows a stained profile of a time course of accumulation of the 56kD chimeric protein after induction of cells containing plasmids encoding both the chimeric protein and export functions. There is no obvious cellular accumulation but the chimeric protein is the major band in the supernatant and increases with time after induction. Although there is no visible associated cell lysis, the precise level of non-specific release of intracellular material was monitored by assaying for the presence of β-lactamase in the supernatant. This periplasmic enzyme is encoded by plasmid pLG632, and although low levels of release into the medium might be expected, a large increase in the amount of activity associated with the supernatant would be indicative of cell lysis.

The β-lactam activity was measured by monitoring the change in absorbance of a cephalosporin, nitrocefin, when cleavage of the amide bond takes place (O'Callaghan et al., 1972). Aliquots of supernatant and sonicated cells were assayed for β-lactamase activity as described in Materials and Methods, both before induction and 90min and 120min post-induction and the percentage release of β-lactamase was calculated at each time point. Table 5.1 indicates that over the course of the experiment, release of β-lactamase from the periplasm increases from 2.1% to only 3.2% of total cellular activity detected in cell sonicates.

Figure 5.4

Time course of the secretion of the 56kD chimeric protein.

A culture of JM101 (pLG632,pLG575) was grown in L-broth containing ampicillin (100μg/ml) and chloramphenicol (25μg/ml), before IPTG was added at A_{450} = 0.4 to derepress the lac promoter. Cell and culture supernatant samples were taken at the time of induction, and 1, 2 and 3h post-induction. 0.5 A_{450} units of cells and protein from the supernatant, which had been precipitated with TCA, equal to 5.0 A_{450} units of cells were loaded on the gel. Proteins were analysed by SDS-PAGE (11% acrylamide) and were visualised by staining with Coomassie brilliant blue. Molecular weights are given in kilodaltons.

Cells, lane 1, t=0h; lane 2, t=1h; lane 3, t=2h; lane 4 t=3h.

Supernatant, lane 5, t=0h; lane 6, t=1h; lane 7, t=2h; lane 8, t=3h.

-92.5

-69

-43

-30

-14.3

1 2 3 4 5 6 7 8

cells   supernatant
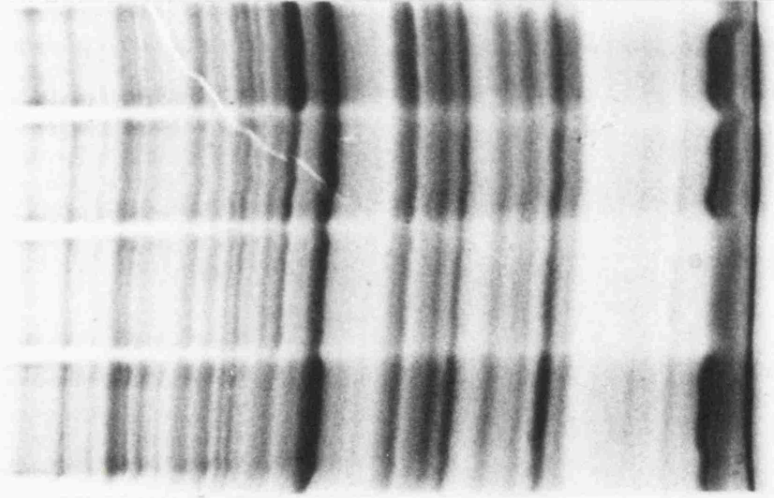
Figure 5.5

Identification of the 56kD chimeric protein in cells and medium.

Cultures of JM101 (pLG632) and JM101 (pLG632,pLG575) were grown in

L-broth, containing the appropriate antibiotics, before IPTG was added at

A_{450} = 0.4 to derepress the lac promoter upstream of the lacZ-ompF-hlyA

hybrid gene. Cell and culture supernatant samples (SN), were taken at

the time of induction and 2h post induction. 0.5 A_{450} units of cells and

supernatant were loaded. Proteins were analysed by SDS-PAGE (11%

acrylamide) and were visualised using polyclonal antibody to HlyA.

JM101 (pLG632) ; lane 1, t=0h (cells); lane 2 t=0h (SN); lane 3, t=2h

(cells); lane 4, t=2h (SN).

JM101 (pLG632,pLG575) : lane 5, t=0h (cells); lane 6, t=0h (SN); lane 7,

t=2h (cells); lane 8, t=2h (SN).
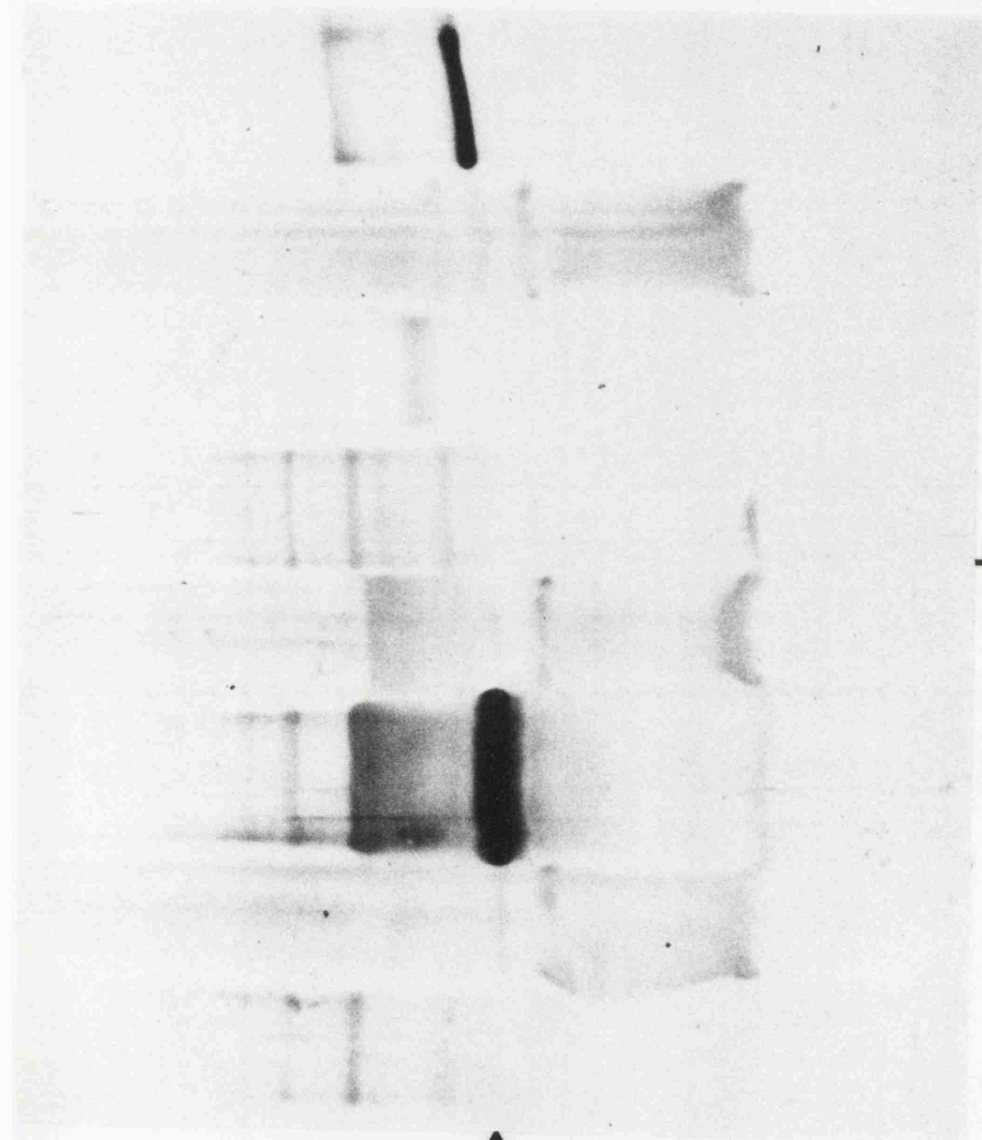
1   2   3   4 | 5   6   7   8

– export   + export

Figure 5.6

Nature of the chimeric molecule released into the medium.

(A) 100ml culture of JM101 (pLG632,pLG575) was grown in L-broth,

containing appropriate antibiotics and at an A_{450} = 0.2, IPTG was added

to derepress the lac promoter. Cell and supernatant samples were

prepared before induction and 1h and 2h post induction as described in

Materials and Methods. Protein in the supernatant was precipitated by

addition of TCA and resuspended in 0.5ml 50mM Tris/Cl pH 7.8 for

treatment with protease from S.griseus (as described in Materials and

Methods). Following proteolysis, samples were separated by SDS-PAGE and

visualised using polyclonal antibody to OmpF. Lane 1, supernatant pre

induction; lanes 2 and 3 supernatant sample 1h and 2h post induction

incubated with protease; lanes 4 and 5, supernatant samples 1 and 2h post

induction; lane 6 cells 2h post induction, lane 7, pre induced cells.

(B) 100ml culture of JM101 (pLG632,pLG575) was grown in L-broth and

synthesis of the 56kD protein induced as described above. 2h post

induction 50ml of supernatant was centrifuged (40k, 4h, 50Ti) to remove

vesicles. Three separate regions were identified, a pellet, a turbid

zone above the pellet and the supernatant. The protein in each fraction

was identified by Western blot analysis using polyclonal antibody to

OmpF. Lane 1, pellet; lane 2, turbid zone, lane 3, supernatant.

A

1 | 2 3 | 4 5 | 6 7

B

1 2 3
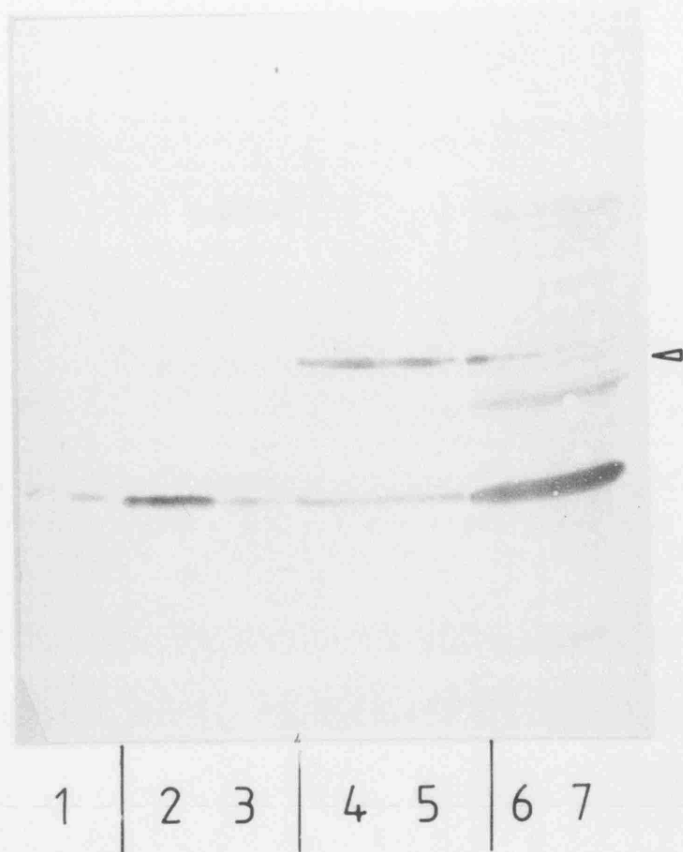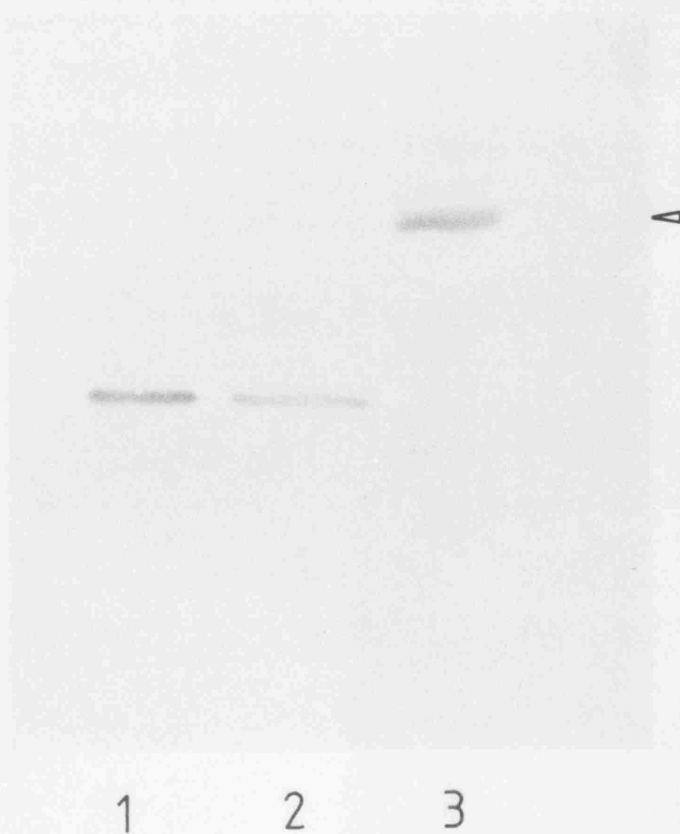
Similarly, when the level of malate dehydrogenase (MDH) a cytoplasmic

enzyme, was compared in supernatant and cells in an identical experiment,

negligible levels of activity were observed in the culture medium (R.

Haig personal communication). It can therefore be concluded that the

increased level of the 56kD protein in the supernatant is not due to

non-specific lysis but rather specific release of the 56kD protein into

the medium from the bacteria.

In contrast to the results obtained in the presence of HlyB and

HlyD, in the absence of export functions there was no evidence of

chimeric protein in the medium in Coomassie blue stained acrylamide gels

(data not shown). However, a Western blot analysis using polyclonal

antibody to HlyA confirmed the identity of the chimeric protein released

from the cells and additionally revealed that in the absence of HlyB and

HlyD, the 56kD protein does remain cell associated (Fig.5.5). There was

no evidence of cell associated chimeric protein when HlyB and HlyD export

functions were present. These results clearly demonstrated that the 56kd

protein was specifically recognised by the haemolysin export pathway and

efficiently secreted into the medium.

5.3 Nature of the released chimeric protein

Western blot analysis using polyclonal antibody to OmpF indicated

that in addition to the 56kD polypeptide, OmpF itself is present in the

growth medium (Fig.5.6). This is not surprising in view of the fact that

it has previously been observed that even under normal growth conditions

small amounts of OmpF are released into the medium in a vesicle

associated form (Mug-Opstelten and Witholt 1978). It was therefore

important to test whether the 56kD chimeric protein was truly secreted or

was released in the form of vesicles. In order to investigate this, the

culture supernatant, containing both mature OmpF and chimeric protein was treated with protease obtained from Streptomyces griseus. Mature OmpF, which has been integrated into the membrane will of course be protease resistant (Jackson et al.,1985) however, if the 56kD hybrid protein, rather than being integrated into the membrane, is in a membrane free form, it should be protease sensitive. As Fig.5.6a shows, the 56kD hybrid protein was completely degraded by protease whereas the mature OmpF embedded in the vesicle membrane was completely resistant.

This result does not however exclude the possibilty that the chimeric molecule is inserted into a vesicle membrane in a protease sensitive conformation. Therefore, vesicles in the culture supernatant were sedimented by centrifugation (40rpm, 4h, 50Ti rotor). Fig.5.6b shows that all the OmpF protein was in the pellet under these conditions, whereas the chimeric protein remained in the supernatant. It was therefore concluded that the secretion of the chimeric protein was specific and did not involve release of limited portions of the outer membrane.

5.4   Nature of the functional secretion signal

The observation that the haemolysin system is able to recognise and specifically release a protein which contains 300 residues of OmpF and the final 218 residues of HlyA is a strong indication that all the information which is necessary to initiate and carry out secretion of intact haemolysin is contained in the C-terminus of the molecule. The C-terminal 200 amino acids of HlyA were therefore carefully examined for regions which might form a structural resemblance to a classical

NH$_2$-terminal signal sequence. The only possible feature of note however, appeared to be a region between residues 166 and 180 which is predicted to form an α-helix (see Fig.5.7 and Gray et al., 1986).

This could, for example, represent an integral constituent of the signal itself or could have a role in presenting the C-terminal secretion signal (further downstream) as a domain distinct from the rest of the haemolysin protein. In order to investigate whether the α-helix itself was sufficient to promote export, a synthetic oligonucleotide encoding 22 amino acids (residues 164-185) and containing the predicted α-helix was obtained and ligated into the C-terminus of the lacZ-ompF fusion in pLG631 as illustrated in Fig.5.8.

The oligonucleotide was 74 base pairs in length and had HincII and HindIII ends. The vector, pLG631, was therefore prepared by digestion with HindIII and HincII followed by purification of the large 3.8kb fragment from a low melting point agarose gel, to remove the small 13 base pair fragment created by the double digestion. This was an important step since competition from this very small fragment during ligation could not be disregarded on account of the size of the oligonucleotide to be inserted. The prepared oligonucleotide (see Materials and Methods) was ligated into the vector, transformed into CSH26 and selected for resistance to ampcillin. Transformants were patched on to ampicillin plates and colonies screened for the presence of a unique BglII site present in the oligonucleotide. Sixteen of a possible 24 colonies were linearised upon digestion with BglII and must therefore contain the oligonucleotide.

The plasmid created, pLG635, which is predicted to encode a 35kD polypeptide was transformed into JM101 both in the presence and absence of pLG575 (which encodes HlyB and HlyD). Cells were then grown in Luria broth and IPTG in order to induce transcription, which, as before, was

Figure 5.7

Hydropathy plot of the last 82 amino acids of HlyA. The unique
BglII restriction site is indicated together with regions of proposed α-
helical secondary structure as predicted by Chou and Fasman (1978). The
unshaded boxes represent regions of weak α-helix and the shaded boxes
represent regions of strong α-helix.
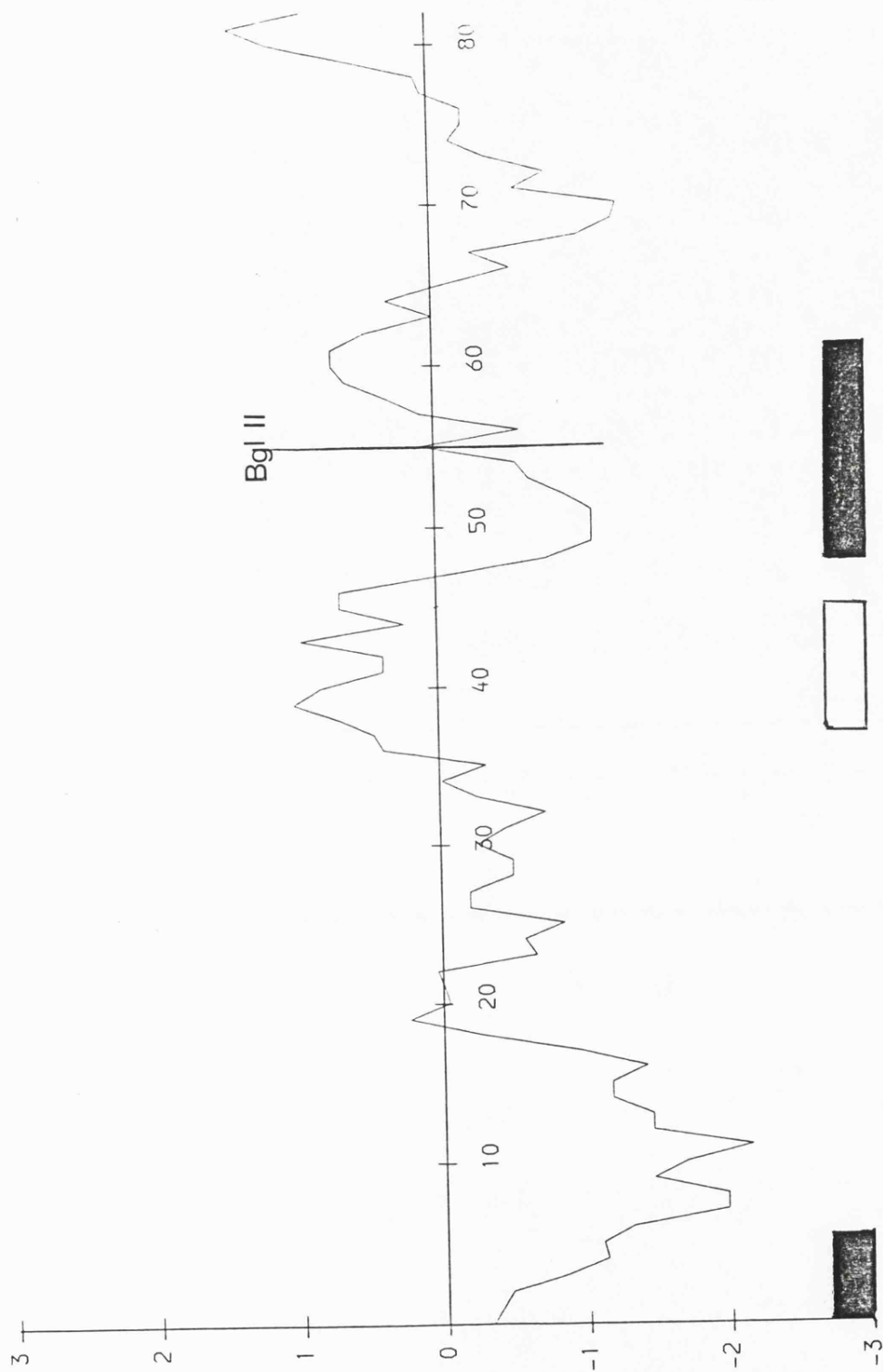
Figure 5.8

Construction of a lacZ-ompF-oligonucleotide gene fusion.

The lacZ-ompF-oligonucleotide fusion was created by ligating the prepared oligonucleotide (see Materials and Methods) with the 3.8kb fragment isolated from a HindIII-HincII digest of pLG631. Transformants were isolated by their resistance to ampicillin and pLG635 was identified by the presence of a unique BglII site which had originated from the presence of the oligonucleotide.

Oligonucleotide
74 bp

HindIII   HincII

plac

Bam HI / Bgl II

HincII
HindIII

pLG631

HindIII + HincII

Isolate 3·8 kb fragment

plac

HincII
BglII
HindIII
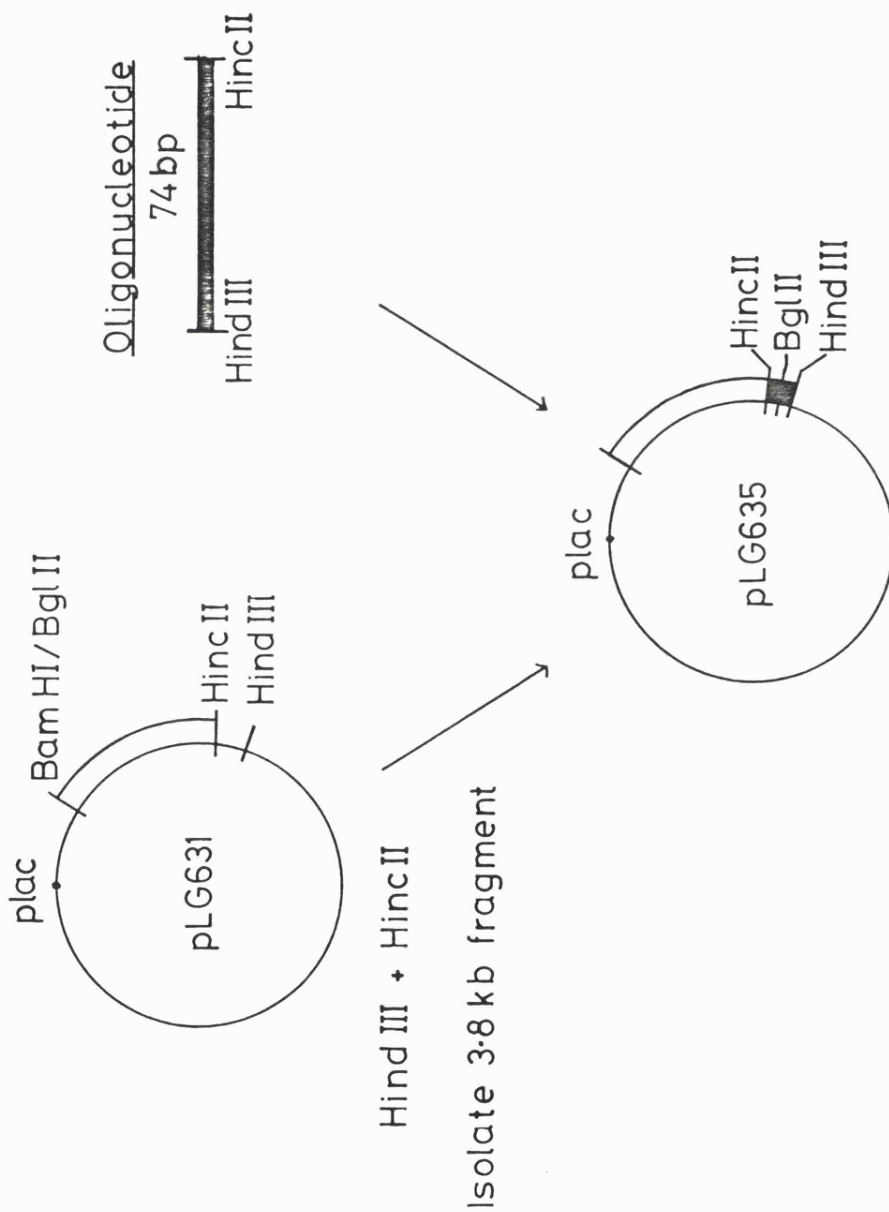
pLG635

<u>Figue 5.9</u>

The LacZ-OmpF-Oligonucleotide chimeric protein is not secreted into

the medium by the haemolysin export system.

<u>E.coli</u> strain JM101 containing pLG575 (encoding HlyB and HlyD) and

pLG635 (encoding the <u>lacZ-ompF</u>-oligonucleotide chimeric gene) was grown

in Luria broth containing ampicillin (100μg/ml) and chloramphenicol

(25μg/ml) before IPTG was added as described in Materials and Methods to

derepress the <u>lac</u> promoter. Samples were taken at the time of induction

and 1, 2, and 3 hours post-induction. Cell samples were prepared by

harvesting the cells and resuspending in SDS-sample buffer. Culture

supernatant samples were prepared by removing cells by centrifugation and

adding TCA to precipitate protein. Samples prepared from 0.5 A_{450} units

of cells or supernatant from 5.0 A_{450} units of cells were analysed by

SDS-PAGE (11% acrylamide) and visualised by staining with Coomassie

brilliant blue. Lane 1, cell sample pre-induction; lanes 2-4 cell

samples, 1h, 2h and 3h post-induction. Lane 5, supernatant sample

pre-induction; lanes 6-8, supernatant samples 1h, 2h and 3h post-

induction. Molecular weights are given in kilodaltons. In track 4 a

band of molecular weight approximately 35kD can be seen accumlating in

the cells.

Figure 5.10

Intracellular accumulation of the LacZ-OmpF-Oligonucleotide chimeric protein.

E.coli JM101 and JM101 containing pLG635 either in the presence or absence of pLG575 encoding export functions, was grown in Luria broth supplemented with the appropriate antibiotics. IPTG was added at an A_{450} = 0.2 to derepress the lac promoter and samples were taken at the time of induction and 3 and 5 hours post-induction. Cells were harvested and resuspended in SDS-sample buffer analysed by SDS-PAGE (11% acrylamide) and visualised by staining with Coomassie brilliant blue. Cells equivalent to 0.5 A_{450} units were loaded per sample. Lanes 1-3 show at the time of induction, lane 1, JM101; lane 2, JM101(pLG635); lane 3, JM101(pLG575,pLG635). Lanes 4-6 show cells from the same culture 3h after induction and lanes 7-9 cell samples 5h after induction. Molecular weights are given in kilodaltons.
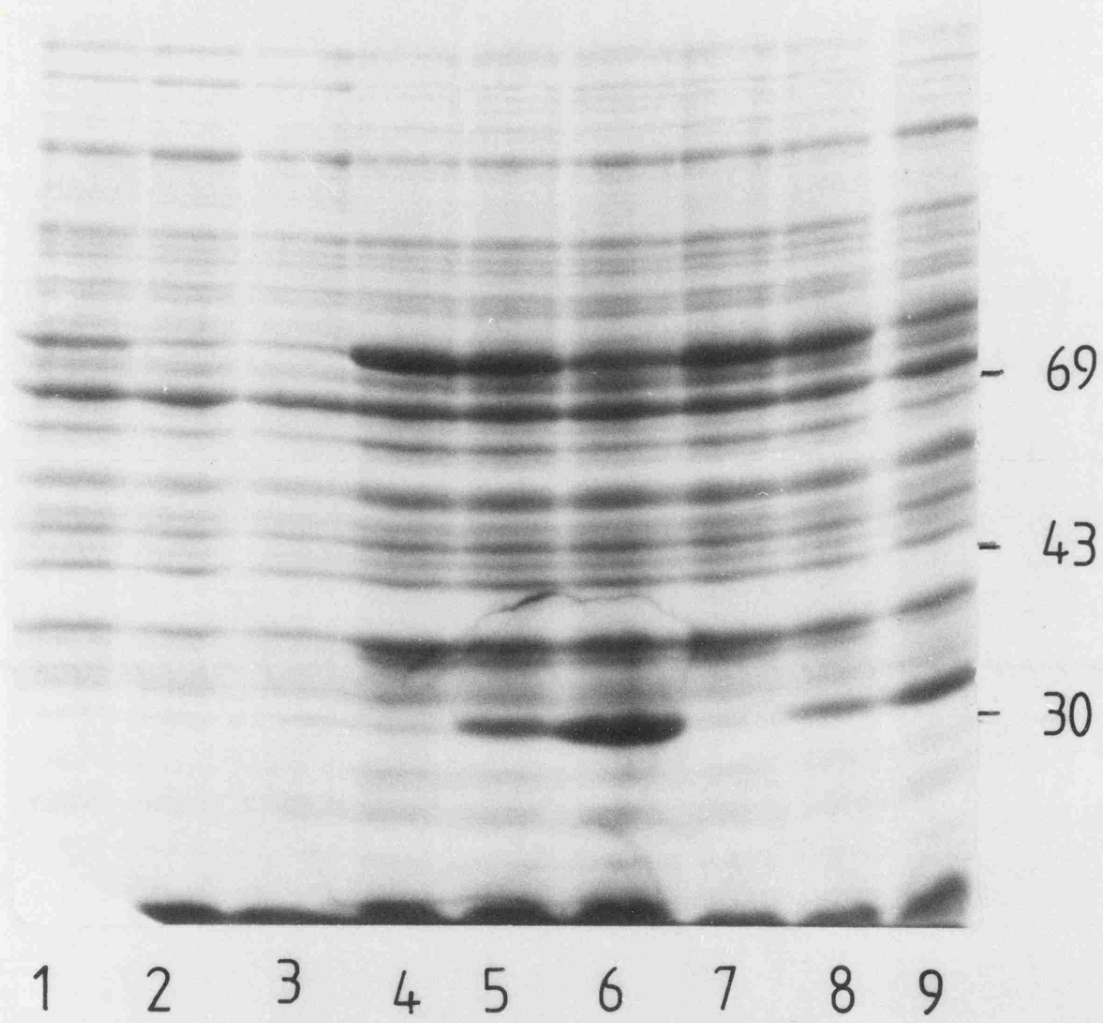
under the control of the lac promoter. Samples were taken at intervals and any protein in the supernatant fraction obtained from the culture was precipitated by addition of TCA and analysed by SDS-PAGE, followed by staining with Coomassie blue. Fig.5.9 shows the cell and supernatant fractions obtained from JM101(pLG575,pLG635). There is no evidence of any specific protein secreted from the cells and it appears that this chimeric protein remains cell associated. Indeed, 2h after induction, a protein of molecular weight approximately 35kD accumulated in the cells and this may represent the chimeric protein. Interestingly, in cells which lacked the HlyB/HlyD export functions substantially higher levels of the 35kD protein accumulated intracellularly (Fig.5.10). Thus, if one function of HlyB and HlyD is to maintain haemolysin in an unfolded (and potentially protease sensitive) form prior to export less protein carrying an incomplete signal might be expected to accumulate when these proteins were present. Unfortunately, since the chimeric protein is of a similar size to normal OmpF, attempts to confirm the identity by Western blot, using antibody raised to OmpF, failed, since induced protein, if present, was obscured by the porins OmpC and OmpF, which have a virtually identical molecular weight. Also insufficient epitopes were present in the portion of HlyA present to allow cross reaction with polyclonal antibody raised against HlyA.

These findings indicated that although unable to direct secretion, the α-helix from the C-terminal region might nevertheless be able to interact with the secretion machinery in some way. Future experiments might therefore be directed towards attempts to demonstrate such an interaction, for example, by cellular localisation of the 35kD polypeptide.

Discussion

Current knowledge of the mechanism of secretion of haemolysin from the cytoplasm to the external medium indicates that the secretion process represents a unique mechanism for crossing the inner and outer membranes of E.coli. This secretion process must necessarily be post-translational since the critical export signal is located at the C-terminus of the intact molecule. It has been proposed that this C-terminal region can interact with the export proteins HlyB and HlyD and initiate translocation across the membrane (Nicaud et al., 1985).

Although recognition of the export components must be specific it now appears that the haemolysin export pathway is also able to recognise and release into the medium a 56kD chimeric protein containing 300 residues of OmpF and 218 residues of HlyA. Localisation studies on this chimeric molecule are particularly informative, since although the route taken by the chimeric protein to the outer membrane is unknown, it seems that there is no opportunity for the OmpF portion of the molecule to insert into the outer membrane. This would certainly be consistent with direct extrusion of HlyA to the medium by a mechanism which bypasses the periplasm, possibly similar to the pathway used by many outer membrane proteins. However, it is possible that the protein may have folded in such a way so that the OmpF portion of the molecule was precluded from any interaction with the outer membrane. In addition, when interpreting the results, it must be remembered that both the first 11 and last 30 amino acids of the mature OmpF were removed during construction of the chimera. The first 11 amino acids are known to be non-essential for assembly to the outer membrane (Jackson et al., 1985) but it is possible that OmpF would be unable to assemble into the outer membrane in the absence of the final 30 amino acids.

Current models of protein export envisage translocation of unfolded molecules. Assuming that haemolysin export also involves an unfolded intermediate the results are consistent with translocation of the chimeric molecule through the inner and outer membranes within a continuous transenvelope "pore", perhaps composed of HlyB and HlyD. The OmpF portion would thus be prevented from interacting with the outer membrane during translocation. The possibiity must not be ignored however, that the haemolysin export system and the C-terminus of the mature haemolysin molecule allows access of the chimeric molecule only to the periplasm. The chimeric molecule may then insert spontaneously into the outer membrane due to inherent properties of the 300 amino acid residues which are derived from mature OmpF molecule. Subsequently, the inability to form trimers and the water soluble nature of the chimera might result in release from the membrane to the external medium. However, the observation that the C-terminus of haemolysin is also able to direct the specific release of prochymosin, a eukaryotic cytoplasmic protein with no affinity for the E.coli outer membrane (B. Kenny pers. comm.), argues against this mode of secretion for chimeric molecules which contain the C-terminus of haemolysin.

However, it has been proposed (Gray et al., 1986) that several amino acids, in particular those following the α-helix close to the C-terminus of HlyA, may be important in forming part of a "signal" structure which is recognised by the secretion machinery. Consequently, further investigations concerning the nature of the secretion signal are currently being carried out in this laboratory by adding a larger oligonucleotide, encoding the final 37 amino acids of the intact haemolysin molecule, to the lacZ -ompF gene fusion. This portion of the

molecule may in addition to the α-helix, include amino acids down stream of the helix essential for recognition of the export machinery. Clearly in this study it was shown that the C-terminal α-helical region of HlyA alone was insufficient to promote secretion of the OmpF chimera.

CHAPTER SIX

Localisation of Exported proteins synthesised by spheroplasts

Introduction

As described in Chapter 1, the structure of a Gram-negative bacterium is completely dependent on the presence of a rigid peptidoglycan layer. Thus, if the peptidoglycan is slowly digested by treatment with EDTA and lysozyme, the outer membrane peels away from the cell to leave the cytoplasm surrounded by only the inner membrane. The outer membrane remains largely intact and is apparently still attached at one or more points on the cell surface (Birdsell and Cota-Robles, 1967). Under these conditions, the cells, devoid of peptidoglycan, no longer have any inherent structure, and must be maintained in osmotically buffered minimal medium to prevent lysis. In this state, it has been established that both protein synthesis and also translocation to the inner membrane and periplasm may continue (Metcalfe and Holland, 1980). In contrast, the outer membrane is in a physiologically abnormal state and therefore it is presumably impossible for outer membrane proteins to assemble correctly. As a result, proteins normally localised to the outer membrane might be expected to accumulate in the cytoplasm or inner membrane, or alternatively be released into the periplasm, which in spheroplasts is synonomous with the external medium.

By determining the localisation of outer membrane proteins synthesised in spheroplasts some insight into the pathway of export of outer membrane proteins might be gained. For example, if translocation cannot be initiated in the absence of a normal outer membrane, precursor might accumulate in the cytoplasm. Alternatively, in the absence of an intact envelope, translocation may be initiated but not completed and

outer membrane proteins may become jammed in the inner membrane. Finally, if transfer to the outer membrane is normally a two step process and protein exists transiently as a soluble periplasmic intermediate, outer membrane proteins may be released from spheroplasts into the medium. Release of protein into the supernatant is not unequivocal evidence of a periplasmic intermediate however, since a localisation external to spheroplasts may also be observed if separation of the outer and inner membranes has disrupted an export pore or channel which normally bridges the two membranes. In this case, protein might conceivably be released into the supernatant although normally the protein would not exist in a membrane free form.

The export pathway of secreted proteins may also be analysed in spheroplasts. In particular, the important question of whether haemolysin crosses the inner and outer membranes at specialised zones of adhesion and thus bypasses the periplasm, or alternatively passes through the periplasm before subsequently crossing the outer membrane may be addressed using this system.

Secretion of the majority of proteins from Gram negative bacteria appears to be analogous to the assembly of fimbriae on the surface of E.coli (Mooi et al., 1982) occuring via a two step process involving initial translocation to the periplasm followed by transfer across the outer membrane (see Chapter 1 and Pugsley and Schwartz, 1985). In contrast however, E.coli seems to lack essential outer membrane components which allow proteins to cross this permeability barrier. Thus, proteins which are secreted from other Gram negative bacteria accumulate in the periplasm when expressed in E.coli (Hirst et al., 1984; Pugsley and Schwartz, 1985). Haemolysin, the exception in E.coli, is of course specifically released into the growth medium but whether or not a

periplasmic intermediate exists is the subject of some controversy, complicated by uncertainty as to the exact location of the specific export proteins HlyB and HlyD.

Early reports based on separation of minicell membranes by sucrose density equilibrium centrifugation indicated that HlyB and HlyD were predominantly outer membrane proteins (Hartlein et al., 1983), However, serious technical problems are associated with the formation of spheroplasts from minicells. This, coupled with the fact that Hartlein et al. (1983) did not show that classical inner and outer membrane proteins synthesised in the same cells fractionate at different positions on the gradient, means that it is difficult to evaluate these results. In contrast, localisation studies using Sarkosyl treatment of envelopes isolated from sonicated minicells and maxicells expressing HlyB and HlyD from the export subclone pLG575, suggested that the majority of both HlyB and HlyD fractionate with the inner membrane, although in the same study, small, but clearly significant amounts of HlyD were detected in the Sarkosyl insoluble fraction (Mackman et al., 1985b). The Hly proteins B and D have therefore been proposed to form a specific secretion complex spanning the inner and outer membranes (Mackman et al., 1986). As discussed in Chapter 1, however, the basis of separation of E.coli envelope proteins by Sarkosyl solubility is not understood and these experiments may also be subject to artefacts. Therefore, the behaviour or HlyB and HlyD in this detergent cannot be assumed.

Another method used to assess whether haemolysin is exported via the periplasm, was to determine the localisation of haemolytic activity in export defective mutants. Thus, using either strains containing mutations within the haemolytic determinant, or strains containing plasmids encoding only part of the export system, Hartlein et al. (1983)

reported that the majority of haemolytic activity produced from cells

which lacked export functions was periplasmic. A similar result was

obtained in HlyB⁻ cells, whereas in HlyD⁻ cells, haemolytic activity was

apparently associated with the outer membrane. In addition mutations at

the C-terminus of HlyA encoded by pANN202-3124 containing a 60 base pair

deletion at the 3' end of hlyA, or pANN202-3122 containing Tn5 insertions

at the C-terminus, were reported to result in a cytoplasmic localisation

of HlyA. Finally, Goebel and co-workers (Springer and Goebel, 1980)

reported that even in the presence of the whole haemolysin determinant,

up to 50% of the toxin was normally found in the periplasm and outer

membrane. These results led to the suggestion that sequences within the

intact haemolysin were capable of promoting transport across the inner

membrane whereas HlyB and HlyD mediated transport across the outer

membrane.

In contrast, in this laboratory, complementation tests using

subclones carrying one or both export genes from Hly2001, indicated that

if either export function was absent at least 90% of the activity was

cytoplasmic (Gray et al., 1986). Moreover, in contrast to these

(Springer and Goebel, 1980; Hartlein et al., 1983) significant levels of

periplasmic haemolytic activity were never detected with wild type

Hly2001 determinants. These results suggested that no periplasmic

intermediate exists in the case of Hly2001 and are therefore consistent

with a translocation mechanism whereby HlyA crosses both membranes

simultaneously. In fact, other studies in this laboratory (L. Gray Ph.D

Thesis, 1987, Gray et al., 1986) have suggested that the extremely low

levels of haemolytic activity produced by pANN202 plasmids may lead to

spurious localisation using osmotic shock treatments. Nevertheless,

the osmotic shock data obtained in this laboratory must

also be interpreted with some caution. This method, developed by Nossal and Heppel (1966) relies upon increased permeability of the outer membrane and as a result large polypeptides in particular might not be released from the periplasm. For example, even aerolysin, which has a molecular weight of 54kD and is known to exist transiently in the periplasm of Aeromonas hydrophila during normal export (Howard and Buckley, 1983), cannot be shocked from the periplasm of export defective mutants (Howard and Buckley, 1985). It was therefore important to test for the presence of possible periplasmic haemolysin intermediates by examining the periplasmic fraction prepared after the formation of spheroplasts by treatment with EDTA and lysozyme rather than by osmotic shock.

6.1 Localisation of outer membrane proteins synthesised in spheroplasts

Strain MC4100(pLG513), synthesising high levels of OmpA, OmpF/C and TonA was grown in glucose minimal medium to an $A_{450} = 0.5$ and spheroplasts were prepared as described in Materials and Methods. Once conversion to spheroplasts was at a level of 90%-95% (as monitored by phase contrast microscopy), the intact spheroplasts were separated from the supernatant by low speed centrifugation. The spheroplasts were carefully resuspended in glucose minimal medium which had been osmotically buffered by the addition of 0.3M sucrose. Spheroplasts were pulse-labelled with ^{35}S-methionine for 10min and the pulse was terminated by the addition of excess cold methionine. Labelled spheroplasts were harvested, osmotically lysed and inner and outer membranes were separated from the isolated envelopes on the basis of Sarkosyl solubility. Any protein in the supernatant of the spheroplast suspension was precipitated by the addition of TCA and the protein

profiles for each fraction were analysed by SDS-PAGE followed by autoradiography.

Fig.6.1 shows that the outer membrane fraction contained no labelled material indicating that assembly of these proteins was completely blocked. Nevertheless, the cytoplasmic, "periplasmic" and inner membrane fractions contained ^{35}S-methionine labelled proteins and the "periplasm" appeared to comprise a discrete subset of proteins distinct from either the inner membrane or cytoplasm. Although the degree of lysis was not monitored in these particular experiments by assaying for the cytoplasmic enzyme malate dehydrogenase, there is no evidence of either Ef-Tu (molecular weight 42kD) or the β or β' subunits of RNA polymerase (molecular weight 150kD and 155kD) released from the cytoplasm into the "periplasmic" fraction. This appears to rule out non-specific cell lysis and the proteins observed in the "periplasm" are probably either normal periplasmic proteins or translocation intermediates which have been unable to reach the outer membrane.

Since there were a large number of labelled proteins in each cellular fraction, it was impossible to unequivocally determine the localisation of individual membrane proteins of interest. Therefore, cytoplasmic, "periplasmic" and inner membrane fractions were immuno-precipitated with antibody raised against TonA, OmpA or OmpF respectively. The latter antiserum also cross reacts with OmpC and it is important to note that the majority of porin synthesised in spheroplasts is probably OmpC, since under conditions of high osmolarity, OmpF synthesis is repressed and OmpC synthesis is induced (van Alphen and Lugtenberg, 1977; Kawaji et al., 1979).

Fig.6.1b,c reveals that all of the OmpF/C protein and the majority of the OmpA protein synthesised in spheroplasts was released into the

Figure 6.1

Localisation of outer membrane proteins synthesised in spheroplasts.

A 100ml culture of MC4100(pLG513) was grown in M9 glucose minimal medium to an A_{450} = 0.5. 10ml of culture was then harvested, washed, resuspended in Tris-sucrose and converted to spheroplasts as described in Materials and Methods. Spheroplasts were resuspended in glucose minimal medium osmotically buffered with 0.3M sucrose, pulse-labelled for 10min with ^{35}S-methionine and chased with excess cold methionine. The intact spheroplasts were again harvested and protein in the supernatant was precipitated by the addition of TCA. Spheroplasts were osmotically lysed and membranes were separated on the basis of Sarkosyl solubility.

(A) Samples equivalent to 0.5ml of culture were analysed by SDS-PAGE (11% acrylamide) and autoradiography. Lane 1, inner membrane; lane 2, periplasm; lane 3 cytoplasm; lane 4, outer membrane.

(B) Immunoprecipitation against OmpF antibody. Fractions prepared from 5ml of culture were immunoprecipitated with polyclonal antibody raised against OmpF and analysed by SDS-PAGE (11% acrylamide) followed by autoradiography. Lane1, cytoplasm; lane 2, periplasm, lane 3, inner membane. Molecular weight standards are indicated.

(C) Immunoprecipitation with OmpA antibody. Fractions prepared fom 5ml of culture were immunopreciitated against antibody raised against OmpA and analysed by SDS-PAGE followed by autoradiography. Lane 1, cytoplasm; lane 2, periplasm,lane 3, inner membrane.
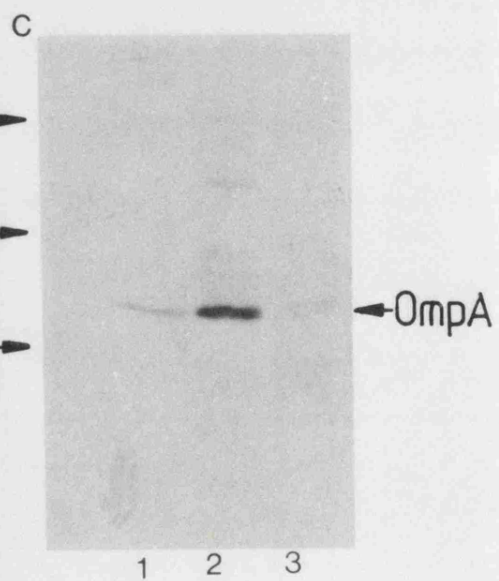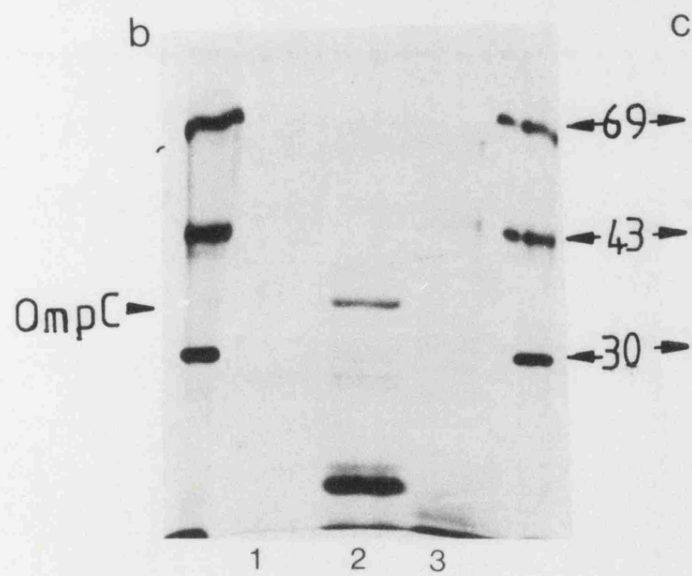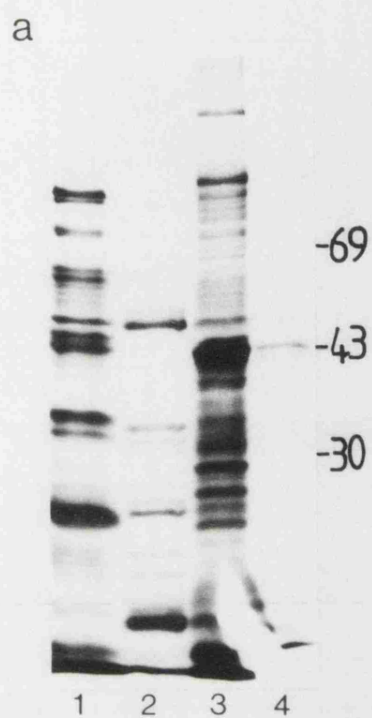
Figure 6.2

Effect of high osmolarity on the synthesis of TonA

100ml cultures of MC4100 and MC4100(pLG513) were grown in minimal

medium for 2 generations either in the presence of absence of 0.3M

sucrose. Cell samples equivalent to 0.5 A_{450} units were loaded on an 11%

acrylamide SDS-PAGE gel and visualised by staining with Coomassie

brilliant blue. Lane 1,, MC4100; lane 2, MC4100 in 0.3M sucrose; lane 3,

MC4100(pLG513); lane 4, MC4100(pLG513) in 0.3M sucrose. Molecular

weights are given in kilodaltons.
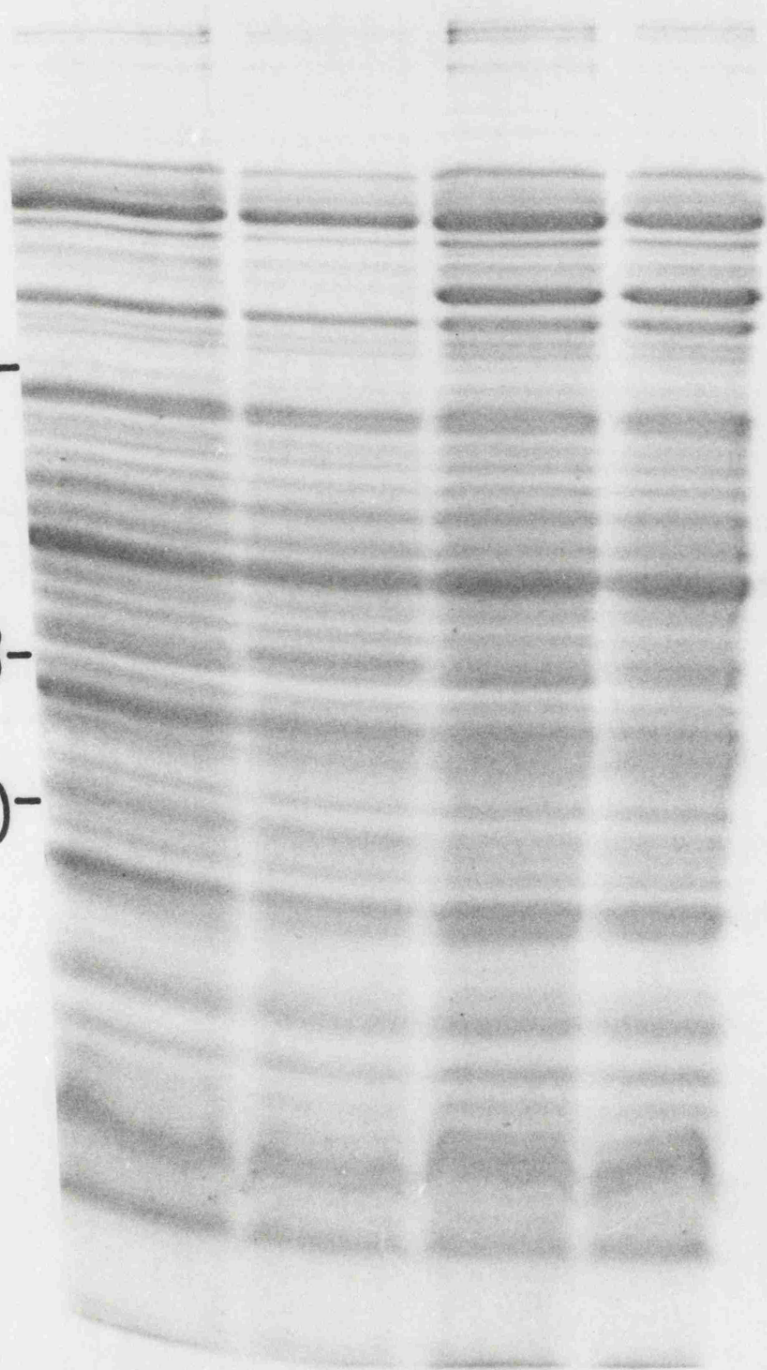
supernatant fraction (periplasm). The ability of these proteins to reach

the periplasm in the absence of an intact outer membrane indicated that

the presence of neither the outer membrane nor any normal points of

contact between inner and outer membranes are essential for the

initiation of translocation or transfer across the inner membrane.

Moreover, the results were also consistent with a periplasmic

intermediate in the assembly of porins and OmpA.

Surprisingly, there was no evidence of TonA or preTonA in any of the

three different fractions immunoprecipitated with TonA antiserum. The

presence of pLG513, encoding TonA, was confirmed by preparing whole cell

stained profiles and identifying the TonA polypeptide by SDS-PAGE and

staining with Coomassie blue, therefore it seemed possible that TonA

synthesis might also be subject to osmoregulation. In order to

investigate this possibility, E.coli strain MC4100(pLG513) was grown in

0.3M sucrose, cells were harvested and the protein profiles analysed by

SDS-PAGE and staining with Coomassie blue. The results shown in Fig.6.2,

indicated that the amount of TonA synthesised was in fact independent of

the osmolarity of the medium and therefore high osmolarity does not

appear to inhibit synthesis of TonA. Although, it is not clear therefore

why TonA synthesis is blocked in spheroplasts, this fact precluded any

comparison of the localisation of OmpF/C and TonA synthesised in the

absence of the outer membrane. Similarly, it was impossible to deduce

anything about possible periplasmic intermediates in TonA assembly.

6.2 Localisation of haemolysin in the absence of export functions

In the light of the contradictory reports concerning possible

periplasmic intermediates in haemolysin export detected in the absence of

export functions (Hartlein et al., 1983; Gray et al., 1986), it was

considered important to establish whether in the absence of both HlyB and

HlyD, HlyA is released into the supernatant when the peptidoglycan is

digested by the action of EDTA and lysozyme. A culture of SE5000(pLG583)

expressing the 107kD protein from a promoter within the tet gene was

grown in Luria broth to an $A_{450} = 0.5$, the cells were harvested and the

periplasmic fraction was separated by treatment with EDTA and lysozyme

as described in Materials and Methods. Cytoplasm and membranes were

separated by osmotic lysis of the cells and all fractions, prepared from

exactly equivalent A_{450} units were analysed by SDS-PAGE and visualised by

a Western blot analysis with antibody raised against HlyA. Fig.6.3 shows

that all the haemolysin synthesised was intracellular and no periplasmic

pool was detected. This result therefore clearly supports the previous

findings by Gray et al. (1986) that no periplasmic intermediate

accumulates in the absence of export functions. Similarly, no

periplasmic pool of haemolysin was obtained when the periplasmic fraction

was prepared by lysozyme treatment of cells expressing the 107kD protein

in the presence of export functions (data not shown). However, as a final

note of caution these results do not rule out the possibility that the

107kD protein accumulating in the periplasm is rapidly degraded.

6.3 Localisation of haemolysin synthesised in spheroplasts

Localisation of haemolysin synthesised in spheroplasts in the

presence of only one of the specific export functions might be expected

to indicate the sequential or co-ordinated activity of HlyB and HlyD in

the secretion of HlyA. Therefore, in order to analyse independently the

effect of HlyB and HlyD on haemolysin export, strains were constructed

which contained pLG583, encoding hlyA (Mackman et al., 1985) and various

combinations of export functions. The strains were grown in glucose

minimal medium to an A_{450} = 0.5, converted to spheroplasts, pulse-

labelled with ^{35}S-methionine and fractionated into periplasm, cytoplasm

and membranes as described above. Each fraction was then analysed by

SDS-PAGE and autoradiography.

When spheroplasts were prepared from cells containing both export

functions HlyB and HlyD, variable localisation of haemolysin was observed

and haemolysin accumulated either in the periplasm or in association with

the inner membrane (data not shown). In the absence of either export

function no intracellular or extracellular accumulation of haemolysin was

observed and obviously levels of synthesis were very low or degradation

rapid (Fig.6.4c). The variable results obtained in the presence of both

export proteins may indicate that a membrane channel or export pore is

required for HlyA export and that this "pore" is not always disrupted

upon formation of spheroplasts. If the pore remains intact, protein is,

in effect, transported to the external medium and not the periplasm,

whereas if the pore is disrupted, the 107kD protein may not be released

from the membrane. Thus, the presence of the 107kD polypeptide in the

supernatant may represent true secretion through an intact pore and not

transfer to the periplasm.

In an attempt to dissect the translocation system further,

spheroplasts expressing one or other haemolysin export protein were

analysed. Unfortunately, very little haemolysin is synthesised in

spheroplasts if one component of the export system is missing

(Fig.6.4a,b). This is not unexpected, since haemolytic activity in

export defective mutants was observed to be only 5% of levels in wild

type cells (Mackman et al., 1986). Under these conditions transcription

from the hly promoter is apparently reduced by a similar amount (D.

Figure 6.3

Localisation of haemolysin synthesised in the absence of export functions.

100ml culture of SE5000(pLG583) was grown in minimal medium to an $A_{450} = 0.5$. 10ml of cells were then harvested and converted to spheroplasts as described in Materials and Methods. After separation of the periplasm by low speed centrifugation (6,500rpm, 2min) the spheroplasts were lysed osmotically and cytoplasm and membranes separated by centrifugation (35,000rpm, 40min). Proteins were separated by SDS-PAGE and visualised by Western blot analysis using polyclonal antibody raised against HlyA. Lane 1, 107kD standard, lane 2, membranes, lane 3, periplasm, lane 4 cytoplasm.

←107kD

1 2 3 4
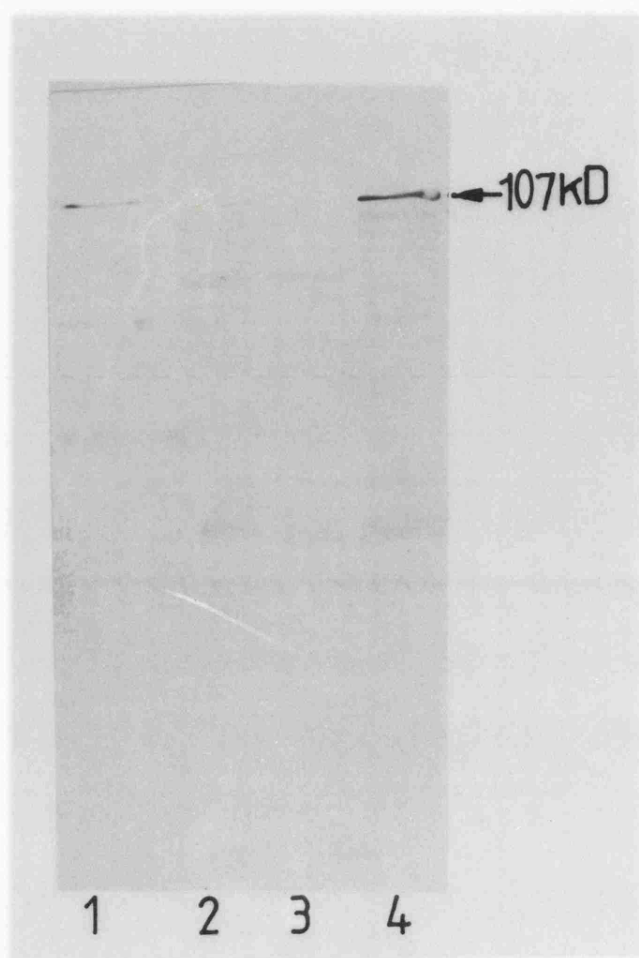
Figure 6.4

Localisation of haemolysin in export defective spheroplasts.

100ml culture of SE5000(pLG583,pLG579) and SE5000(pLG583,pLG594) were grown in M9 glucose minimal medium to an A_{450} = 0.5. 5ml of each culture were harvested and resuspended in 1ml Tris sucrose. Spheroplasts were prepared as described in Materials and Methods and pulse-labelled with ^{35}S-methionine for 10min. The various fractions were then analysed by SDS-PAGE followed by autoradiography.

(A) SDS-PAGE analysis of fractions obtained from spheroplasts of SE5000(pLG583,pLG579). Lane 1, cytoplasm; lane 2, periplasm, lane 3, inner membrane. Longer exposure of the periplasmic fraction did not yield any evidence of the 107kD polypeptide in the periplasm.

(B) SDS-PAGE analysis of fractions obtained from spheroplasts of SE5000 (pLG583,pLG594). Lane 1, cytoplasm, lane 2, periplasm, lane 3, inner membrane. The intensity of the faint band observed in lane 2 could be increased by longer exposure but this resulted in the other bands becoming too dark to distinguish.

(C) SDS-PAGE analysis of fractions obtained from spheroplasts of SE5000(pLG513). Lane 1, cytoplasm; lane 2, periplasm; lane 3, membranes.
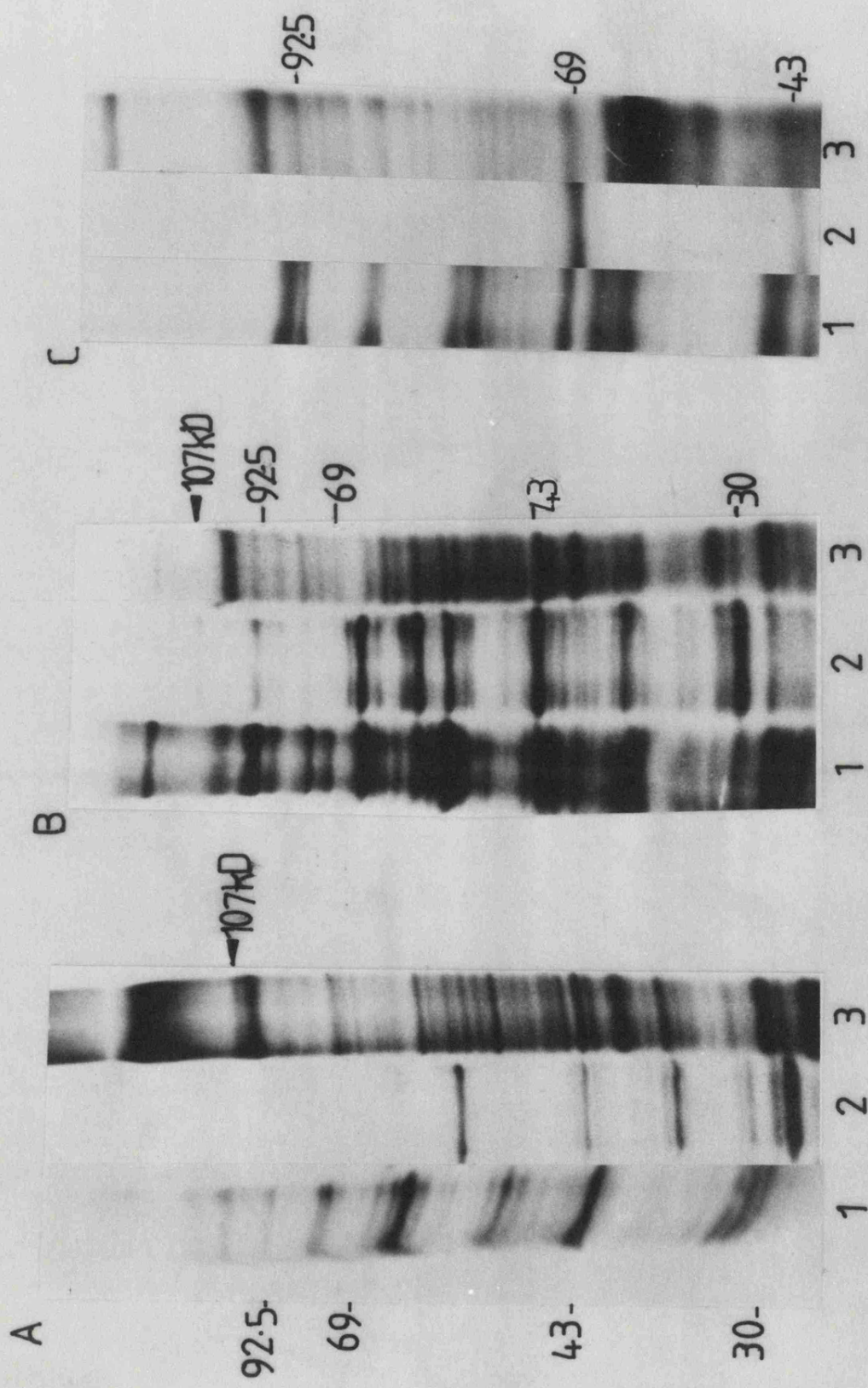
Donnelly and I.B. Holland, unpublished data). Since feedback control

should only occur at a level equivalent to that observed in HlyB⁻/HlyD⁻

cells it also seems likely that in the presence of one export function,

export may be initiated but not completed and the 107kD protein may thus

be more sensitive to degradation by proteases and not dectable in any

cellular fraction (see also section 6.6). Also, a further reduction in

the level of synthesis of haemolysin is apparently caused by high osmotic

pressure, although this effect is very slight (data not shown) and the

mechanism of control is not known. It must be noted however, that with

plasmid pLG583 the majority of transcription is not from a haemolysin

promoter but from a promoter within the tet gene (Mackman et al., 1985)

and therefore any regulation involving feedback control must be exerted

at the level of translation.

Fig.6.4 shows the inner membrane, periplasm and cytoplasm prepared

from spheroplasts after a 10min pulse. From the protein profiles it can

be seen that the final destination of haemolysin synthesised in

spheroplasts, apparently varies depending on the export functions

present. Thus, in HlyD⁻ spheroplasts (Fig.6.4a) haemolysin appears to

remain bound to the inner membrane (lane 3) whereas in HlyB⁻

spheroplasts, the haemolysin was present primarily in the supernatant

(periplasm). This band appears very weak in the figure but can be seen

more clearly on the original autoradiograph. It may be important to note

however, that in spheroplasts formed from HlyB⁻ cells it was impossible

to prepare the periplasm from spheroplasts without some associated cell

lysis. The samples of HlyB⁻ and HlyD⁻ periplasms shown in Fig.6.4, were

in fact prepared simultaneously, and yet in HlyB⁻ cells there is a degree

of lysis which is not seen in HlyD⁻ cells. This may reflect a

requirement for the presence of HlyB in order to stabilise the membrane

and prevent lysis, or it may be caused by the presence of HlyD alone

resulting in a fragile membrane and easy disruption of spheroplasts.

6.4 Trypsin accessibility of membrane associated haemolysin

Since cell-fractionation experiments are subject to ambiguities and artefacts is is possible that protein which is synthesised in spheroplasts appears to be membrane bound, for example the 107kD haemolysin in Fig 6.4a synthesised in hlyB$^+$ cells could be associated with either the periplasmic or cytoplasmic face of the inner membrane. In order to resolve these possibilities the localisation of the 107kD protein was probed by trypsin accessibility experiments (Halegoua and Inouye, 1979b).

The rationale for such experiments is simple, if the haemolysin has been translocated to the periplasmic face of the inner membrane then it should be digested by protease added to the labelled spheroplasts. However, if the protein is integrated into the membrane or present on the cytoplasmic face it should be protected fom the degradative action of protease. The protease chosen for these experiments, was trypsin, on the basis of the observation that computer analysis revealed a large number of trypsin sensitive sites and the purified toxin is very sensitive to trypsin (data not shown).

Cells containing pLG583 and pLG579, were converted to spheroplasts, resuspended and subdivided into 200μl aliquots. Each aliquot was then labelled with ^{35}S-methionine for 2min and different aliquots were treated with increasing amounts of trypsin as described in Materials and Methods. Trypsin digestion was stopped after 10min by the addition of antitrypsin and each sample was analysed by SDS-PAGE followed by autoradiography. The results of the trypsin accessibility experiment are shown in Fig.6.5. In addition to the inner membrane fraction, a supernatant sample prepared from an exponential culture of cells capable of synthesising both export functions and the 107kD polypeptide was also

treated with trypsin. This provided a control to check that trypsin was

active under the experimental conditions used (see Materials and

Methods). Fig.6.5 shows that the 107kD protein accumulating in the

membrane fraction in hlyB$^+$/hlyD$^-$ cells was not digested by trypsin. In

contrast, the secreted 107kD protein was completely digested even by

50µg/ml trypsin. This result indicates that the protein is not attached

to the external surface of the spheroplast but is either on the

cytoplasmic face or integral to the membrane.

One way of confirming the identity of the haemolysin present in

various fractions throughout these experiments would be to immuno-

precipitate each fraction with polyclonal antibody to HlyA. However, it

was considered that the very low level of synthesis of the 107kD protein

when export was disrupted would probably be insufficient for detection by

immunoprecipitation. For example, Fig.6.1b shows that after

immunoprecipitation of the periplasmic fraction with antibody raised

against OmpA or OmpF, ten times more material had to be loaded to obtain

a band of equal intensity to that observed in Fig.6.1a. As an

alternative to the 107kD polypeptide in these experiments it was

therefore decided to repeat the spheroplast experiments using either the

OmpF chimeric molecule (see Chapter 5) or the 23kD C-terminal polypeptide

of haemolysin, both of which are normally expressed and accumulate inside

cells at much higher levels in the absence of export functions.

6.5 Localisation of the lacZ-ompF-hlyA chimeric protein and 23kD protein

in spheroplasts

Although it has already been established that haemolysin itself does

not accumulate at high levels in the absence of export functions (Gray et

al., 1986; Mackman et al., 1986), both the lacZ-ompF-hlyA chimeric

Figure 6.5

Trypsin sensitivity of membrane associated haemolysin synthesised in

spheroplasts..

Spheroplasts of SE5000(pLG583,pLG579) were labelled with ^{35}S-

methionine as in fig.6.4. the spheroplasts were then treated with

increasing concentrations of trypsin as described in Materials and

Methods. Each mixture was incubated on ice for 30min and the reaction

was stopped by the addition of antitrypsin. In order to ensure that the

reaction was working and show that haemolysin is sensitive to the

degradative action of trypsin, a supernatant sample from cells secreting

the 107kD protein was also treated with trypsin and run on the same gel.

The samples were analysed by SDS-PAGE (11% acrylamide) and

autoradiography. Lanes 1-3, inner membrane samples treated with; lane 1,

10μl 0.1M Tris/sucrose pH 8.0; lane 2, 50μg/ml trypsin, 100μg/ml trypsin;

lanes 4-6, supernatant samples treated with 10μl 0.1M Tris/sucrose pH

8.0; lane 5, 50μg/ml trypsin; lane 6, 100μg/ml trypsin. Molecular weight

standards are shown at the right.
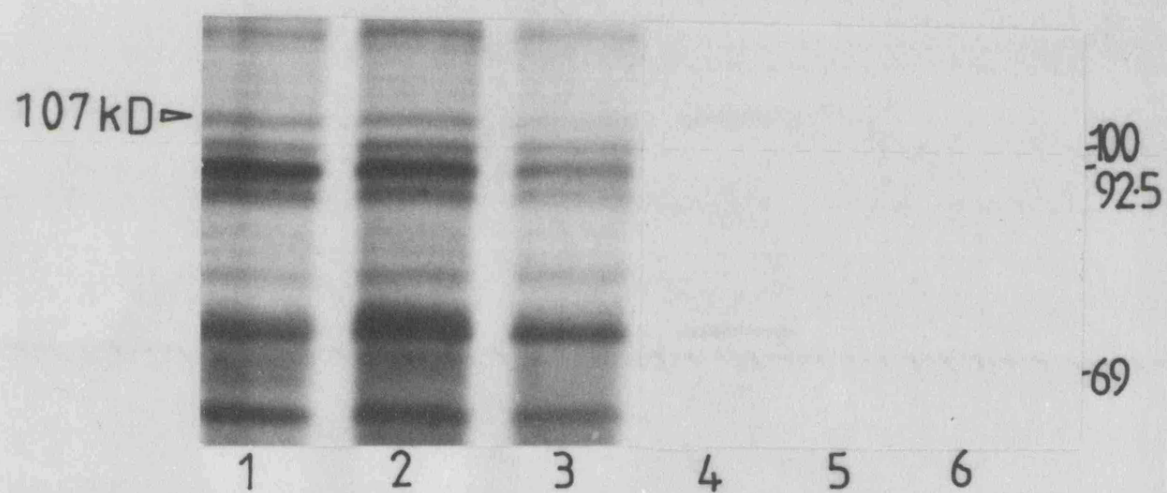
Figure 6.6

Localisation of the 23kD C terminal polypeptide of HlyA accumulating

in cells lacking export functions.

(A) 100ml cultures of SE5000(pLG609,pLG575), SE5000(pLG609,pLG579),

SE5000(pLG609,pLG594) and SE5000(pLG609) were grown in luria broth

supplemented with the appropriate antibiotics and at an A_{450} = 0.2, IPTG

was added to derepress the tac promoter. 3h post-induction 20ml of each

culture was harvested and any protein in the supernatant was precipitated

by the addition of TCA. Cells equivalent to 0.5 A_{450} units were then

analysed by SDS-PAGE and visualised by antibody raised against HlyA.

Lanes 1-4 are cell samples; lane 1, SE5000(pLG609); lane 2

SE5000(pLG609,pLG579); lane 3, SE5000(pLG609,pLG594), lane 4

SE5000(pLG609,pLG575); lane 5 supernatant from SE5000(pLG609,pLG575).

(B) 100ml of SE5000pLG609,pLG575) were grown in luria broth and at an

A_{450} = 0.2, IPTG was added to derepress the tac promoter. 3h

post-induction, 6ml of culture was harvested and cells were converted to

spheroplasts as described in Materials and Methods. Each fraction was

analysed by SDS-PAGE (11% acrylamide) and staining with Coomassie blue.

Lane 1, cytoplasm; lane 2, periplasm; lane 3, membranes, lane 4

supernatant and lane 5 molecular weight markers in kilodaltons.
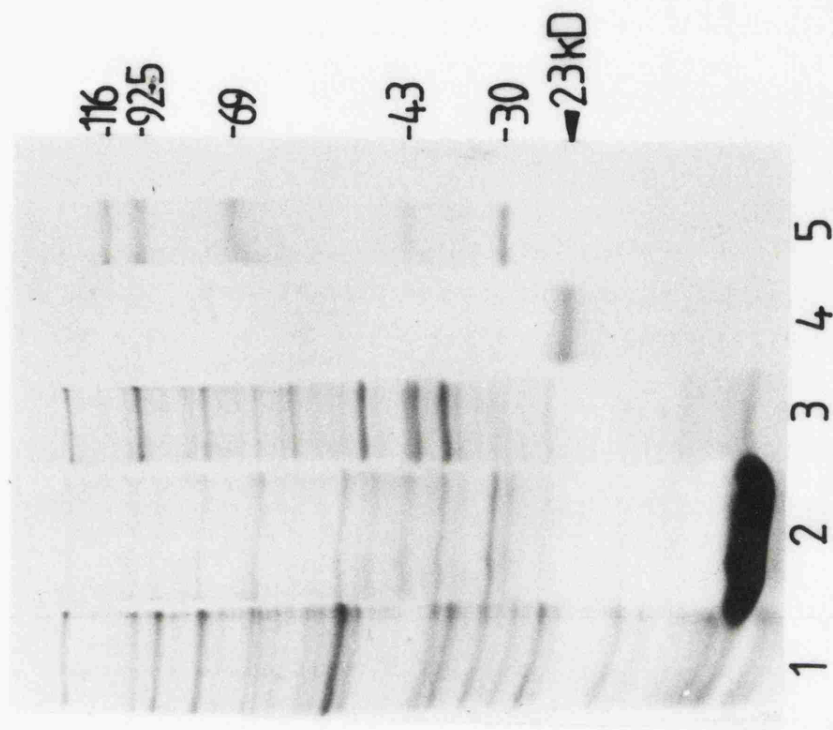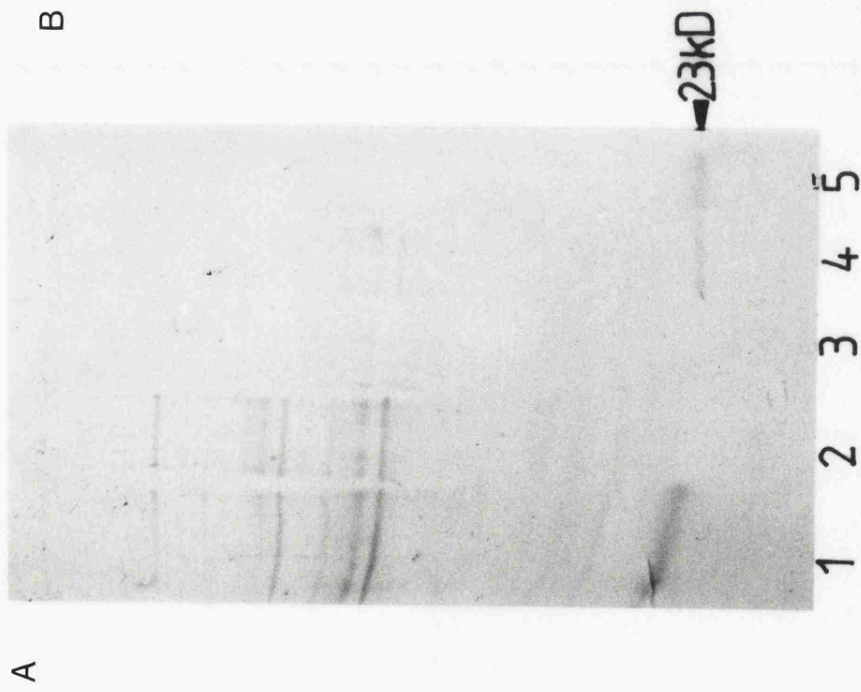
protein (Chapter 5) and the 23kD C-terminal fragment of haemolysin

(Nicaud et al., 1985) do accumulate intracellularly to significant

levels. The localisation of either of these proteins in spheroplasts

lacking one or more export proteins was therefore expected to be quite

feasible using antibodies.

Initially, SE5000(pLG632), encoding the lacZ-ompF-hlyA chimeric

protein was transformed together with either pLG579, pLG594 or, as a

control, pLG575. However, although a large number of SE5000

(pLG632,pLG575) transformants were obtained, no transformants of cells

which contained pLG632 and either HlyB(pLG579) or HlyD(pLG594) alone

formed colonies. This failure could not be attributed to the lack of

competence of cells or the quality of the DNA, since both were

satisfactory in other transformations carried out under idential

conditions. It must therefore be concluded that in these constructions

the synthesis of the chimeric protein combined with the presence of

either one of the export components is lethal to the cells.

Construction of strains expressing the 23kD protein (pLG609)

carrying one or other of export gene presented no problem. Cultures of

SE5000(pLG609,pLG579) and SE5000 (pLG609,pLG594) were therefore grown in

Luria broth with the appropriate antibiotics and at an A_{450} = 0.2, IPTG

was added to derepress the tac promoter. After 3h to allow maximum

induction, cells were harvested and proteins in cell and supernatant

(medium) samples were analysed by SDS-PAGE and visualised by either

Coomassie blue stain or by Western blot analysis. Stained profiles, as

expected, revealed no secreted polypeptide if either export component was

absent (data not shown) but the 23kD protein was exported when HlyB and

HlyD were present (a small amount of intracellular 23kD polypeptide can

however be observed in Fig.6.6, lane 4). Although there is no indication

that the 23kD protein accumulates intracellularly when export functions are present, spheroplasts were prepared from SE5000(pLG609,pLG575) and separate fractions examined by Western blot analysis using antibody against HlyA. Fig.6.6b (lane 2) clearly shows that there is no 23kD in the periplasm under these conditions. A similar experiment with cells which lacked export functions showed that the 23kD protein was entirely intracellular in the absence of HlyB and HlyD. Thus, the Western blot analysis in Fig.6.6a shows the accumulation of the 23kD protein in cells in the absence of both export functions and also confirms that the protein is exported to the growth medium in the presence of both HlyB and HlyD. However surprisingly, when only one export component was present, no 23kD protein could be detected by antibody in any cellular fraction (Fig.6.6, lanes 2 and 3) or in the culture medium (data not shown).

In view of the unexpected result that the 23kD protein was not apparently produced under these conditions it was important to carry out further control experiments to ensure that the gene encoding the 23kD protein was intact. Analysis of the DNA from these cultures indicated that pLG609 was indeed still present and there was no evidence of mini-plasmids indicating deletion of the haemolysin gene fragment (data not shown). Therefore, attempts were made to reconstitute secretion by transforming the missing export components into cells lacking either hlyB or hlyD. Subsequent experiments showed however that although it was possible to transform an additional plasmid into these cells, after several generations resistance to ampicillin, the selective marker, was lost, indicating loss of pLG609 encoding the 23kD protein. It was therefore not possible to look for restoration of secretion by this method. As a final attempt to check that SE5000 strains were still at least capable of synthesising the 23kD protein, DNA was prepared from

SE5000(pLG579,pLG609) and re-transformed, together with pLG575, into

SE5000. Transformants were selected only for resistance to ampicillin

encoded by plasmid pLG609 and chloramphenicol encoded by plasmid pLG575

in the hope that the additional complication of selecting pLG579 which is

resistant to tetracycline would be avoided. When these constructs were

examined efficient secretion of the 23kD protein was observed upon

induction of the tac promoter with IPTG (data not shown). This result

confirmed the integrity of the 23kD gene present in the constructs and

therefore the results obtained above indicated that any 23kD protein

synthesised in the presence of only one export function was probably

extremely unstable.

Discussion

The experiments described in this chapter indicate that the OmpC and

OmpA proteins synthesised in spheroplasts are exported across the

cytoplasmic membrane into the medium. This is consistent with a

mechanism of export whereby these outer membrane proteins are normally

fully translocated to the periplasm with subsequent assembly into the

outer membrane. The results are less consistent with translocation via

specific structures connecting inner and outer membranes as these would

be expected to be disrupted by spheroplasting. It has also been proposed

that outer membrane proteins may pass directly from the inner membrane to

the outer membrane by means of a continuous polypeptide chain which

stretches across the periplasm and assembles into the outer membrane

concomitant with the final stages of synthesis in the cytoplasm. If such

a mechanism is involved in the assembly of OmpC and OmpA, the results

obtained here indicate that translocation is not dependent upon coupled

assembly into the outer membrane.

Unfortunately TonA does not appear to be synthesised at all in spheroplasts and therefore the experiments revealed nothing of the assembly mechanism for this protein. The reasons for the absence of TonA synthesis are unclear and the possibility remains that TonA synthesis is tightly coupled to some aspect of transport which is disrupted in spheroplasts. Alternatively, processed TonA may be extremely unstable if it is not inserted into the membrane. Indeed this is known to be the case in mutant forms of TonA, when very small deletions of the the C-terminus are introduced resulting in inhibition of assembly into the outer membrane (M. Jackson Ph.D thesis, 1984).

Analysis of export of haemolysin synthesised in spheroplasts has proved more complicated. The apparent translocation of the 107kD protein to the supernatant (periplasm) in $HlyB^-/HlyD^+$ cells and its inner membrane localisation in $HlyD^-/HlyB^+$ cells is consistent with a role for HlyD as the actual translocation pore in the membrane, with HlyB, anchored in the inner membrane, required perhaps to provide energy for final extrusion through the outer membrane. A number of alternative interpretations are however possible. For example, release in $HlyD^+$ cells may be primarily due to lysis caused by fragility of the inner membrane. Also perhaps, HlyB in spheroplasts forms a translocation complex in the membrane and in the absence of HlyD, the protein becomes stuck in this complex during translocation. It is also possible that patches of outer membrane remain attached at certain places on the surface of spheroplasts depending on the particular preparation in any given experiment. If these zones of outer membrane correspond to haemolysin export sites, an intact export structure could remain in which case export to the supernatant is in fact true secretion. This could explain why, if HlyB and HlyD are both present in the membrane of

spheroplasts, protein is sometimes in the supernatant and sometimes in the membrane. Notwithstanding this result, the lack of quantitative translocation of the 107kD protein synthesised in spheroplasts to the medium (periplasm), in the absence of either HlyB or HlyD is further evidence against a periplasmic intermediate in normal export.

Previously a number of roles have been proposed for the two export components of the haemolysin export system. On the basis of minicell fractionation and evidence of periplasmic haemolytic activity in both wild type and in export defective cells, Hartlein et al., (1983) proposed that haemolysin (encoded by pANN202) is transported first to the periplasm and then subsequently interacts with HlyB and HlyD allowing the 107kD polypeptide to cross the outer membrane. In this model HlyB mediates translocation whereas HlyD, which was identified as an outer membrane protein by Hartlein et al. (1983), releases haemolysin into the medium from the surface of the outer membrane. In contrast to these data, studies in this laboratory with Hly2001 indicated that HlyB and HlyD were probably located in the inner membrane (Mackman et al., 1986). Moreover, these authors failed to detect any haemolytic activity in the osmotic shockate prepared from either export defective or wild type cells (Gray et al., 1986). However, it is known that osmotic shock treatment does not necessarily release all periplasmic proteins (Howard and Buckley, 1985) and the possibility of the presence of a pool of periplasmic haemolysin could not be excluded. In this study, this point was examined further by isolating the periplasm from wild type or export defective cells by treatment with EDTA and lysozyme. In agreement with the studies of Gray et al. (1986) the 107kD protein was detected in the cytoplasm and not in the periplasm. Therefore the results indicate that translocation of haemolysin across the inner membrane is normally

dependent upon the export functions HlyB and HlyD. The results do not however rule out the possibility that haemolysin is normally translocated via the periplasm but any attempts to disrupt this process leads to rapid degradation of any periplasmic HlyA. This latter alternative is less likely in view of the fact that the 23kD protein which is stable and can accumulate intracellularly in the cytoplasm, nevertheless is also never detected in the periplasm.

In an attempt to establish the individual roles of HlyB and HlyD, spheroplasts were prepared from cells which were either HlyB⁻ or HlyD⁻ and the localisation of haemolysin synthesised during a 10min pulse with ^{35}S-methionine was examined. Unfortunately however, the results have proved difficult to interpret, partly due to the low levels of the 107kD protein synthesised in the absence of export functions.

From the results in Fig.6.3, it seems that in cells expressing only HlyB, the 107kD protein becomes associated with the inner membrane and cannot be released into the supernatant. It is not possible however to assess whether this is because HlyD normally forms part of the export pore or whether the 107kD protein, presumably complexed with HlyB, cannot be released in the absence of HlyD. Nevertheless, this observation differs from previous proposals which suggested that HlyB is required to release the toxin from the outer membrane (Hartlein et al., 1983). On the other hand in some experiments, the 107kD polypeptide was apparently released into the spheroplast supernatant in spheroplasts expressing only HlyD. This result would indicate that HlyD is in fact sufficient to translocate the 107kD polypeptide across the inner membrane. The results must however be interpreted with some caution, particularly since in every experiment using HlyD⁺, HlyB⁻ cells it proved impossible to prepare the spheroplast supernatant fraction (periplasm) without some

concomitant spheroplast lysis. This could be due to fraglity of the membrane of HlyD$^+$ cells or could be the result of attempted translocation of the 107kD polypeptide in the absence of HlyB. It is also possible, that HlyB and HlyD together form a transenvelope pore which is not fullly disrupted upon spheroplast formation. HlyD may constitute the central pore, with HlyB in some way responsible for pore specificity by forming a specific recognition site or "gate", which prevents non-specific passage of proteins from the cytoplasm. Moreover, if the inner membrane portion of the pore remains intact in spheroplasts the 107kD protein may be released into the "periplasm" although no periplasmic intermediate would exist in vivo. Irrespective of the precise organisation of HlyB and HlyD in the export system, the results obtained in this study and the primarily inner membrane localisation of both HlyB and HLyD (Mackman et al., 1986) argues for a role for both HlyB and HlyD export components in translocation across the inner membrane and is not compatible with an exclusively outer membrane role for either HlyB or HlyD as envisaged by Hartlein et al. (1983). The data are more consistent with either export through a single transenvelope pore composed of a complex of HlyB and HlyD, or a two step sequential export process mediated by the two export proteins respectively, in a manner which is normally tightly coupled.

An interesting model for the export of haemolysin may also be proposed on the basis of a recent suggestion as to the mode of export of surface or secreted proteins by Singer et al. (1987a). These authors propose that protein translocation may proceed through aqueous filled pores created by an aggregate of non-identical subunits which together form a transmembrane aqueous channel down the central axis of the aggregate. It is proposed that a part of the protein to be translocated

binds to a subunit of the export pore exposed on the cytoplasmic surface. Alteration of the secondary structure would then act to open the pore and allow translocation. Energy required to separate the subunits of the pore and promote translocation was proposed by the authors either to be supplied by some external source or by binding of export proteins to one of the subunits. Translocation could then proceed domain by domain, each portion of protein leaving the channel open for succeeding portions. The data obtained in the spheroplast system may point to HlyB and HlyD functioning concomitantly as such a hetero-multimeric pore. HlyD may form the central pore and HlyB may function to provide entry specificity and to open and close the pore and/or promote translocation through the hydrolysis of ATP. Thus, in the absence of HlyB, HlyD might remain open under certain conditions, for example in spheroplasts, and allow passage of protein.

It is interesting to speculate upon the intriguing observation that in the absence of one or other export protein, no 23kD polypeptide could be detected inside cells, which is in complete contrast to the result obtaned when both HlyB and HlyD were absent. This failure to observe intracellular 23kD protein in the presence of only one export function might be due to either HlyB/D normally acting to maintain the 23kD protein in an unfolded state or one or other export function promoting unfolding of the polypeptide. In either situation an abortive translocation intermediate in an unfolded form might be more sensitive to degradation by proteases and therefore fail to be detected by means of a Western blot analysis. Such a hypothesis could be investigated by, for example, the isolation of temperature sensitive mutants of either HlyB or HlyD and an analysis of the fate of pulse-labelled HlyA at the non-permissive temperature. Finally, it is interesting to note that the failure to detect the 23kD protein when either export function was

missing and the failure to export the 107kD protein from the cytoplasm in spheroplasts which are HlyB⁻ or HlyD⁻ argues in favour of both export functions being accessible to cytoplasmic proteins. Similarly, these results suggest that neither HlyB or HlyD are localised to the outer membrane.

All these suggestions are however tentative, particularly since some of the spheroplast data were variable and difficult to assess due to low level synthesis of HlyA. Considerably more data are therefore required in order to determine the precise localisation of HlyB and HlyD in the envelope, the organisation of the two proteins with respect to each other and consequently their precise roles in secretion of haemolysin across the cell envelope.

CHAPTER SEVEN

Conclusion

The translocation of nascent or newly synthesised polypeptides across biological membranes appears to be a complex process, involving a series of components which together comprise the export machinery. In E.coli, proteins destined for both the periplasm and outer membrane appear to share a common pathway for at least the initial stages of export and data obtained in this thesis (Chapter 2) and elsewhere (Rothman and Lodish, 1977, Singer et al., 1987a) suggest similar mechanisms for the assembly of some inner membrane proteins. In contrast, although secretion may share some features with envelope protein assembly, at least two separate mechanisms exist in E.coli for secretion of proteins to the extracellular medium. Thus, although some secreted proteins may be transferred to the periplasm via the normal envelope protein export pathway and subsequently translocated through the outer membrane, haemolysin, at least, is apparently secreted via a completely novel pathway and even the early stages appear to differ from envelope protein export (see e.g. Mackman et al., 1986 and Chapter 4).

It is now an undisputed fact that the presence of an NH_2-terminal signal sequence is important in initiating translocation of proteins across membranes. However it appears that some parts of the mature protein and a complex cellular secretion machinery are also probably essential for efficient transfer across the bilayer. Thus the possibility that extrusion is coupled to elongation as assumed in many of the early models of protein export (Blobel and Dobberstein, 1975; von Heijne and Blomberg, 1979) and the role of specific export components has been investigated. The importance of the conformation of a protein

during translocation and the route taken by proteins which are finally assembled in the outer membrane are also considered. In addition, some of the differences between envelope protein assembly and the secretion of haemolysin have been analysed.

In agreement with proposals that export must be co-translational (Blobel and Dobberstein, 1975), an analysis of the post-translational processing of preTonA accumulated in cells in which secA was inactivated, indicated that precursor accumulated in the absence of SecA was largely translocationally incompetent (Chapter 3). In contrast however, in the absence of SecY the majority of the preTonA remained in a translocationally competent form and could cross the membrane upon restoration of functional SecY. This result is consistent with a role at a late step in translocation for SecY and demonstrated that this protein can function efficiently in post-translational export.

A similar conclusion for the role of SecY was reached by Bacallao et al. (1986) using a completely different experimental approach. These authors prepared inverted vesicles from secY$^+$ and secY^{ts} strains grown at the restrictive temperature and analysed the post-translational processing of the outer membrane protein OmpA in vitro. In secY$^+$ vesicles, preOmpA was converted to OmpA and was inaccessible to digestion with protease, whereas secY^{ts} vesicles were unable to promote the translocation and therefore processing of preOmpA. However, when synthesis of functional SecY was induced, processing of the preOmpA molecules rapidly commenced and it was therefore concluded that SecY function is not limited to an interaction with the nascent polypeptide. Unfortunately however, the authors did not analyse the efficiency of co-translational export in secY deficient vesicles, a process which is more likely to occur in vivo. Nevertheless, it is also clear from these

results that in vitro systems should facilitate a functional assay for a more detailed analysis of SecY and other sec encoded proteins.

Notwithstanding the SecA data presented here, recent data both in this and other studies, appears to indicate that bacterial protein export can, in principle, involve translocation after the completion of synthesis. It may also be concluded that cytoplasmic proteins such as SecA do not appear to function in any step involving an essential translational arrest of nascent polypeptides prior to export. Evidence indicates however that SecA protein does have a direct role in protein translocation and may in fact function as part of a cytoplasmic secretory complex of proteins, consisting not only of SecA but possibly also SecB, SecD and PrlD, all of which are known to interact with each other and with the signal sequence (Bankiatis and Bassford, 1985; Brickman et al., 1984; Chapter 1). A role for such a complex may be envisaged in the light of recent data which suggests that the conformation and tertiary structure of a protein is one of the most important features of an envelope protein if it is to remain in a tranlocationally competent form. These studies have indicated that at least some mitochondrial and prokaryotic proteins must maintain or adopt an unfolded structure (Eilers and Schatz 1986; Randall and Hardy, 1985; Chapter1) in order to pass through the bilayer. A cytoplasmic complex could therefore bind to nascent chains to prevent the growing chain from folding and adopting a tertiary structure which may inhibit subsequent translocation (Randall and Hardy, 1986).

Recent analysis of intragenic suppressors of MBP signal sequence mutations has indicated that other factors in addition to a cellular secretion machinery and the signal sequence itself may be involved in determining the translocation competence of a given protein (Cover et

al., 1987). Many of these intragenic suppressors are located within the signal sequence itself and alter the overall hydrophobicity of the MBP signal sequence (Bankiatis et al., 1984). In addition, least one suppressor was found to map within the mature portion of the protein indicating that direct interaction between the signal sequence and mature protein was involved in translocation. However, in view of the fact that MBP may only be competent for export in the absence of tertiary structure (Randall and Hardy, 1985), it has also been proposed that export might be restored if a change in the mature sequence simply retards folding. Thus, if the signal sequence normally interacts with a component of the export machinery which prevents the protein from folding, a mutation in the signal sequence might hinder the interaction. Any subsequent mutation which retards folding would however increase the time during which the protein was competent for transloction and processing of MBP might be observed.

If proteins fold before binding to the soluble complex they may be irreversibly destined to remain in the cytoplasm and can no longer enter the secretion pathway. This would explain the export incompetent pool observed by several groups under certain conditions (e.g. Ryan and Bassford, 1985; Wolfe et al., 1985) and also the translocationally incompetent preTonA observed in secA^{ts} cells at the restrictive temperature (Chapter 3). Individual proteins within the putative cytoplasmic secretory complex may have a variety of functions but it is likely that at least one of the components is able to interact with a second complex of proteins, located in the cytoplasmic membrane. SecY and leader peptidase probably comprise at least part of this membraneous complex, acting, possibly together with other as yet unidentified components, to remove the signal sequence, release the soluble complex

and most importantly to participate in transfer through the bilayer.

This latter process may involve further unfolding of the polypeptide

coupled to the translocation process.

Although as indicated above, knowledge of the early stages of the

secretory mechanism in bacteria has increased substantially in recent

years, this has not revealed anything of the subsequent pathway taken by

proteins which assemble into the outer membrane. Several different

mechanisms have been proposed for this process (see Chapter 1 and

Fig.7.1) and it is possible that alternative mechanisms exist for the

translocation of different outer membrane proteins. Thus, previous data

described in Chapter 1, implicated distinct final assembly pathways for

OmpF and TonA with only the latter proceeding via a periplasmic

intermediate. However, preliminary experiments carried out in

collaboration with Dr Julie Pratt have suggested that both TonA and OmpF

synthesised in vitro, in a cell free system supplemented with inverted

vesicles prepared from E.coli inner membranes, could be extracted from

the lumen rather than the membrane of the vesicle under controlled

conditions. This suggests that both of these proteins had been processed

and fully translocated across the cytoplasmic membrane. These data have

recently been confirmed (J. Pratt pers. comm.) and are in agreement with

the data presented in Chapter 6 involving spheroplasts, which suggest

that OmpF is normally translocated to the periplasm prior to assembly in

the outer membrane. In contrast however, it seems that LamB, synthesised

in the same in vitro system in the presence of inverted vesicles, is

processed but behaves as though still tightly bound to the membrane (J.

Pratt, pers. comm.). It is likely therefore that as previously suggested

by Watanabe et al., (1985) the primary site for integration of LamB

Figure 7.1

Models indicating possible sorting mechanisms for E.coli outer-

membrane proteins (upper bilayer). Reproduced from Jackson et al 1986.

In A, polypeptides pass directly through the inner membrane at sites

of adhesion with the outer membrane; cleavage of the signal sequence

releases the nascent protein from the inner membrane and allows complete

folding into the outer membrane. This model presumes the presence of

discrete N-terminal signal sequences in the mature polypeptide that

ensure insertion into the adhesion site and/or recognition of factors

unique to the outer membrane. In B, the protein is first extruded to the

periplasm, facilitated by cleavage of the signal; the polypeptide then

partitions into the outer membrane, triggered by a specific topographic

sequence or by the overall structure of the polypeptide. In C, an

extreme case of A, the protein, initially inserted into the inner

membrane, diffuses to sites of biogenesis of the outer membrane that are

recognised through specific topographic sequences. The protein is then

translocated to the outer membrane in the form of a vesicle, which buds

from the inner membrane. The vesiscle may not exist in free form as

drawn but may simply coalesce with the outer membrane as it is forming in

the inner membrane. Importantly, in model C, if for some reason cleavage

of the signal sequence is blocked, assembly to the outer membrane should

not be blocked. Finally, all these models assume that the initial stages

of assembly involve the interaction of the N-terminal signal with a sec

dependent export pathway.

Possible sorting mechanisms for localisation of *E. coli* outer membrane proteins

A

B

C
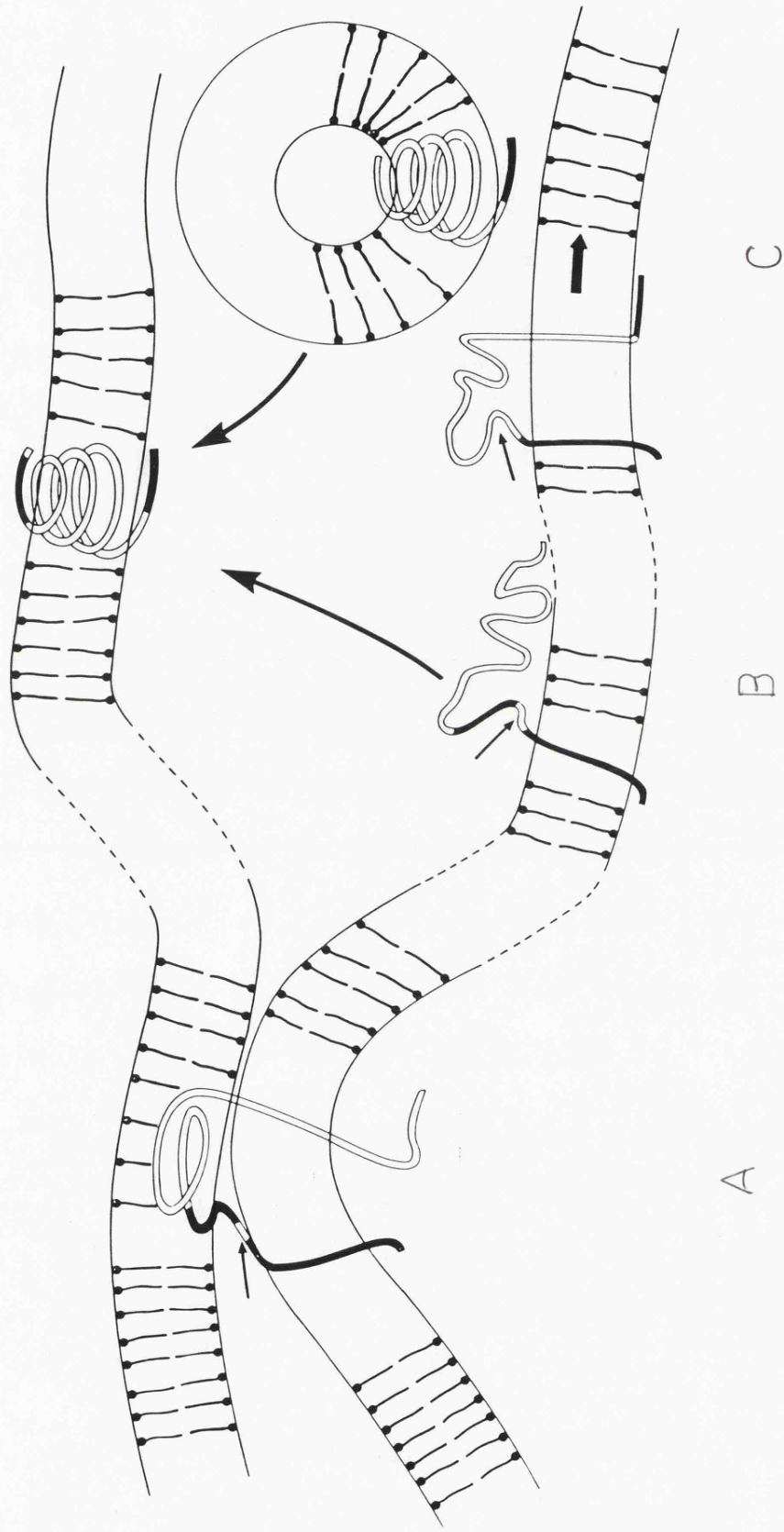
protein prior to subsequent sorting to the outer membrane is the cytoplasmic membrane. The possibility cannot be ignored however that particular properties of LamB result in a spurious localisation in these in vitro studies which depend upon differential solubility in urea or high pH. In conclusion, it seems that both TonA (Jackson et al., 1986) and PhoE (Bosch et al., 1986) are assembled into the outer membrane via a periplasmic intermediate which spontaneously folds into the outer membrane. In addition, although it was previously suggested that OmpF is extruded directly to the outer membrane (see Chapter 1), the spheroplast data presented in Chapter 6 and in vitro experiments carried out by Dr. J. Pratt now indicate that OmpF may follow the same assembly pathway as TonA and PhoE. Presumably the half life of any membrane free intermediate of OmpF is very short, explaining the failure to detect a periplasmic form. In contrast, LamB may be sorted from the inner membrane and may possibly follow a different route to the outer membrane.

Although some basic principles of protein translocation may be the same for many exported proteins, the observation in the kinetic studies described in Chapter 2 that even after 2h at the restrictive temperature assembly to the membrane of secA^{ts} mutants still occurs at a level of about 30% of that seen in the wild type, raised the possibility that more than one export pathway may exist in E.coli. Nevertheless, it must be noted that as a result of the method of isolation (see Chapter 1) these mutants could obviously be leaky, which could account for this residual activity. In addition, as discussed already, evidence indicates that haemolysin may cross the bilayer using a pathway distinct from that utilised by the majority of envelope proteins. It appears likely however, that at least the initial stages in export of most envelope proteins is the same, although this does not exclude later branches

within the main pathway. The existence of a divergence at least at one stage is exemplified by lipoprotein signal peptidase and leader peptidase which both act to remove the signal sequence from a different subset of proteins. In addition, the observation that the secB mutation has pleiotropic effects on export but does not affect all proteins, is another indication that branches occur within the main export pathway. Of particular interest in this respect are the two periplasmic proteins MBP and RBP (ribose binding protein) since although the former is secB dependent, the latter does not require secB in order to reach the periplasm. Similarly, the outer membrane lipoprotein does not appear to require SecB for assembly (Hayashi and Wu, 1985). Some of these differences are described in Table 7.1.

Returning to the central question of protein translocation the basic similarities in export of different proteins will now be discussed. Although there are obvious differences among the systems, it seems likely that the underlying principles in the molecular mechanisms of protein translocation across the ER and the bacterial cytoplasmic membrane and into a variety of organelles may well be similar. On this assumption Singer et al. (1987a) have proposed an interesting model for protein translocation. This model takes into account a set of stringent requirements which have been proposed to be essential for translocation, in particular, that translocation occurs by transfer of successive segments of an unfolded polypeptide chain and that the ionic groups remain in contact with water throughout the transfer process. The mechanism stipulates that each translocation event is mediated by one of a set of special integral proteins in the membrane, termed translocator proteins (TPs) and it has been proposed that each TP is an aggregate consisting of n homologous but not identical transmembrane subunits that

Table 7.1

LOCALISATION	PROTEIN	GENE	MOLECULAR WEIGHT	SIGNAL SEQUENCE	SECA	SECY	SECB	PROCESSING by lep or lpp
INNER MEMBRANE	PBP5	dacA	42,000	+	+			lep
INNER MEMBRANE	leader peptidase	lep	32,000	-	+	+		
INNER MEMBRANE	lactose permease	lacY	31,000	-				
PERIPLASM	MBP	malE	39,000	+	+	+	+	lep
PERIPLASM	alkaline phosphatase	phoA	49,700	+	+	+	-	lep
OUTER MEMBRANE	lipoprotein	lpo	7,200	+	+	+	-	lpp
OUTER MEMBRANE	OmpA	ompA	35,000	+	+	+	-	lep
OUTER MEMBRANE	OmpF	ompF	37,000	+	+	+	+	lep
OUTER MEMBRANE	TonA	tonA	78,000	+	+	+		lep
SECRETED	Haemolysin	hlyA	107,000	-	-	?		-

Molecular weights are given in daltons.

form a membrane-spaning, water-filled channel down the central axis of the aggregate. The special feature ascribed to these integral membrane proteins is that at one of the n non-identical interfaces between neighbouring subunits within the membrane, the two subunits are only weakly bound to each other. This interface can then provide the passageway for the translocation of a hydrophobic polypeptide. Successive segments of the polypepteide can form subdomains of secondary structure in the interface and each subdomain can be translocated in an energy dependent process. Assembly of other proteins into the inner membrane is likely to use the same mechanism and the only additional feature required would be the thermodynamic discrimination by the TP of for example, long hydrophobic sequences which might constitute a stop-transfer sequence (Singer et al., 1987b).

One advantage of this model is that the entire translocation process may in principle be either co- or post-translational. For post-translational translocation, unfolding of the chain would be necessary. An SRP complex or a complex containing SecA might therefore be required either to unfold proteins or maintain partially or fully synthesised chains in an unfolded state (Singer et al., 1987a). This model also explains many of the features observed in the export of haemolysin. Perhaps HlyB and HlyD form a TP-like complex which spans both inner and outer membranes and acts to allow specific export of haemolysin (Chapter 6) in a manner similar to that proposed by Singer et al. (1987) to explain translocation of envelope proteins across the cytoplasmic membrane. With respect to haemolysin export the situation is additionally complicated since HlyA appears to require both HlyB and HlyD and possibly also SecY to cross the membranes but is apparently independent of SecA. Thus, if SecA is indeed required to maintain

exported proteins in a translocationally competent form the observation
that haemolysin export is SecA independent may be a reflection of the
ability of either HlyB or HlyD to substitiute for SecA in haemolysin
export. Rather than acting to prevent folding however, haemolysin export
components may actively unfold the haemolysin chain whose signal sequence
does not apparently appear until the last 30-50 amino acids of the
C-terminus are synthesised (Nicaud et al., 1985; Mackman et al., 1986).
Consequently, a two step export process can be proposed whereby the
haemolysin molecule is first unfolded and subsequent translocation is
mediated by a "TP-complex" which may comprise HlyB, HlyD and SecY
together with other membrane components. Currently it is not however
possible to envisage the exact function of individual export components
in this system. The possibility exists however, that haemolysin
secretion involves the same fundamental process as envelope proteins to
traverse the bilayer but translocation is initiated and then mediated by
entirely unique export components ensuring that only the haemolysin
molecules are secreted.

These observations therefore, together with diferences in
translocation observed for LamB and other outer membrane proteins
described above, suggest that although a central pathway may exist for
the export of many proteins, this pathway may be adapted or modified to a
form more suited to the requirements of individual proteins. The
haemolysin export pathway may even be an extreme example of an export
pathway which has adapted to the special process of traversing both
membranes. Thus, haemolysin, although secA independent may require secY
and perhaps other components of the envelope protein export pathway to
reach the external milieu.

Over the past few years, our knowledge of the prokaryotic secretion machinery has increased considerably although it is still not clear to what extent the prokaryotic and eukaryotic systems represent an essentially conserved, common mechanism and to what extent earlier perceived differences are important. Thus, translation-arrest during eukaryotic secretion may not be as universal in eukaryotes as at first assumed and its apparent absence from prokaryotes therefore may not be significant. The next few years however, are likely to result in the elucidation of the exact function of the various components of the prokaryotic secretion apparatus as in vitro translation systems become more sophisticated and functional assays are developed. This will then allow a more direct comparison with the eukaryotic SRP and docking protein. Finally, although it is likely that the fundamental essentials of protein translocation through membranes may be highly conserved, it is still possible that a variety of systems have evolved in order to present polypeptides in the appropriate conformation at the site of export and each system may reflect the requirements of different types of proteins including the various classes of E.coli envelope proteins.

CHAPTER EIGHT

Materials and Methods

1. Bacterial Strains

The bacterial strains used in this study are listed in table 8.1.
Strains in current use were maintained on nutrient or M9 minimal agar
plates at 4°C. For long term storage, frozen cultures of each strain
were prepared in nutrient broth containing 30% v/v glycerol and stored at
-80°C.

2. Media and Buffers

All media and buffers used are described in Table 8.2. Plasmid
bearing strains were grown in, and maintained on, freshly prepared media
containing the appropriate antibiotic(s) as shown in table 8.2. For
isolation of clones containing inserts in pUC12, IPTG was added to a
final concentration of 0.5mM and Xgal to a final concentration of
50mg/ml, from a stock prepared in dimethylformamide.

3. Growth of E.coli in liquid culture

Liquid cultures were grown in a New Brunswick Gyrotory shaker and
cell density was monitored by measuring absorbance at 450nm using a
Gilford Microsample Spectrophotometer 300N.

4. Induction of expression regulated by the lac/tac promoter

Cultures containing required genes under control of either the lac
or the tac promoter were induced to express these genes in liquid culture
by addition of 0.5mM IPTG. Stationary phase cultures were diluted to an

Table 8.1

Bacterial strains

Strain	Genotype	Source
MC4100	araD139, Δ(lacIPOZYA) U169 thiA, rpsL, relA	P. Bassford
MM52	MC4100 secA^{ts}	J. Beckwith
MM66	MC4100 secA^{am} Tn10	J. Beckwith
MQ3		
MQ4	F$^-$ lac, U169, araD,	
MQ6	relA, mat, rpsL	J. Beckwith
MQ11	secA^{ts}	
8.29		
IQ85	MC4100 Tn5 secY^{ts}	J. Beckwith
IQ86	MC4100 Tn5	J. Beckwith
CSH26	F6, Δ(lac-pro), ara, thi, Δ(recA-srl), rpsL	
JM101	supE, thi, Δ(lac-proAB), F',traD36, proAB, lacI^{q}ZΔM13 .	
SE5000	rpsL, ara139, Δ(lacIPOZYA), U169, recA57	

Plasmids

pUC12	Ap^R, lacZ	Norrander et al., (1983)
pLG575	Cam^R hlyB,D,A	Mackman et al., (1984)
pLG579	Tet^R hlyB	Mackman et al., (1985)
pLG583	Kan^R hlyA	"
pLG594	Cam^R hlyD	"
pLG609	Ap^R	Nicaud et al., (1985)
pLG513	Tet^R, Cam^R fhuA	Jackson et al., (1986)
pLG515	Tet^R, fhuA	"
pLG361	Tet^R, ompF	Jackson et al., (1985)
pLG339	Tet^R, Kan^R	Stoker et al., (1979)
pLG631	ApR	This study
pLG632	Ap^R	"
pLG635	Ap^R	"

Table 8.2 Media and Buffers

Media

Nutrient Broth	Oxoid No 2	2.5% w/v
Luria Broth	Tryptone	1.0% w/v
	Yeast extract	0.5% w/v
	NaCl	0.5% w/v
Minimal medium	Na_2HPO_4	0.6% w/v
	KH_2PO_4	0.3% w/v
	NH_4Cl	0.1% w/v
	Glucose (autoclaved separately)	0.4% w/v
	$MgSO_4$ "	10mM
	$CaCl_2$ "	1mM
	As required:-	
	Thiamine (filter sterilised)	10μg/ml
	Casamino acids	0.5% w/v

Nutrient/Luria/Minimal agar: media as above, solidified with 1.45% agar.

Antibiotics

Antibiotics	Ampicillin	100μg/ml
	Chloramphenicol	25μg/ml
	Kanamycin	20μg/ml
	Tetracycline	10μg/ml

Ampicillin and Kanamycin were filter sterilised, stocks of tetracycline and chloramphenicol were made in 50% ethanol.

Buffers

Bacterial buffer	Na_2HPO_4	0.7% w/v
	NaCl	0.4% w/v
	KH_2PO_4	0.1% w/v
	$MgSO_4.7H_2O$	0.01% w/v

A_{450} = 0.01 and IPTG was added when the cultures reached an A_{450} of

approximately 0.1. Aliquots of the culture were taken at various times

and assayed for the presence of induced proteins.

5.   Recovery of E.coli cell fractions

(a)   Whole cell lysates

Cells were harvested from growing cultures (Sorvall SS34 rotor,

10,000rpm, 5min, 4°C) and washed in ice cold 10mM sodium phosphate buffer

pH 7.2. The washed pellet was resuspended in 50μl of phosphate buffer and

transferred to an Eppendorf tube before 50μl of SDS sample buffer was

added (see Table 8.3). In order to complete cell lysis and solubilise

protein, samples were immediately boiled for 5-10min before vortexing to

shear DNA. Cell lysates were stored at -20°C and always re-boiled before

electrophoresis.

(b)   Culture media

Cells were harvested from culture media using a Sorvall SS34 rotor

(8,000rpm, 10min, 4°C), supernatants were then transferred to a fresh

tube and recentrifuged using an SS34 rotor (14,000rpm, 10min, 4°C) to

remove all cell debris. The protein in the supernatant was precipitated

by the addition of trichloracetic acid (TCA) to a final concentration of

10% and harvested (Sorvall HB4 rotor, 8,000rpm 10min, 4°C). The pellet

was resuspended in saturated Tris and SDS sample buffer.

(c)   Preparation of bacterial cell envelopes from sonic lysates

The basic procedure was that described by Churchward and Holland

(1976) except $MgSO_4$ was omitted from the buffer. Labelled cells were

transferred to 25ml beakers and in many experiments unlabelled carrier

cells were added to each sample. Carrier cells were taken from an exponential phase culture grown to an A_{450} = 0.5 in the same medium as that used in the experiment.

The combined sample (final volume adjusted to 7ml by addition of ice-cold 10mM sodium phosphate buffer pH 7.2) was sonicated for three 30s intervals, with 30s cooling periods, using the 3/4 inch end diameter probe in a 150 Watt MSE ultrasonic disintegrator. This and all subsequent operations were carried out at 4°C.

Sonicated samples were transferred to centrifuge tubes and centrifuged using a Sorvall SM24 rotor (7,000rpm, 5min) to remove unlysed cells. The cleared lysates were transferred to another centrifuge tube and re-centrifuged using a Beckman 50Ti rotor (35,000rpm, 40min). The supernatant represented the cytoplasm and periplasm and was precipitated with TCA (final concentration 10%) while the envelope pellet was drained, resuspended in 1ml sodium phosphate buffer pH 7.2 and washed by re-centrifugation (50Ti, 35,000rpm, 4°C). The washed pellet was again drained and fractionated into inner and outer membranes.

(d) Separation of outer and cytoplasmic membranes by the use of

     Sarkosyl

The method used was based on that of Filip et al. (1973). Washed envelope pellets were resuspended in 100-200μl of 0.5% w/v Sarkosyl NL97, and incubated for 30min at room temperature. The outer membrane, which is insoluble in this detergent, was recovered by centrifugation in a Beckman ultracentrifuge using a 50Ti rotor (35,000rpm, 90min). The supernatant representing the cytoplasmic membrane was transferred to Eppendorf tubes. The centrifuge tube containing the membrane pellet was

carefully drained and the pellet resuspended by freeze-thawing and vortexing. Both membrane fractions were subsequently prepared for SDS-PAGE by addition of sample buffer and boiling for 5min. Samples were stored at -20°C and re-boiled before electrophoresis.

(e) Preparation of cell envelopes by osmotic lysis of spheroplasts

The basic method was that of Osborn et al. (1972) and was performed at 4°C. Cells were harvested by centrifugation in a Sorvall GSA rotor (7,000rpm, 10min) and resuspended in 6ml ice-cold 10mM Tris-HCl pH 7.8 containing 0.75M sucrose. Egg white lysozyme was added from a stock prepared in 10mM sodium phosphate buffer pH 7.2 to a final concentraion of 100µg/ml, and the suspension incubated for 3min on ice, with continuous slow stirring. This was followed by very slow dropwise addition of 6.4ml 1.5mM EDTA pH 7.5. The samples were incubated on ice with constant stirring for 10-15min until conversion to spheroplasts was greater than 95% (as monitored by phase contrast microscopy). The spheroplasts were lysed by addition of 2/3 volume ice-cold distilled water and vigorous stirring for 10min. The lysate was transferred to centrifuge tubes, vortexed to shear DNA and cell envelopes were isolated by centrifugation as above.

(f) Separation of outer and cytoplasmic membranes on sucrose gradients

The washed envelope pellet was resuspended in 1ml of 25% w/w sucrose containing 5mM EDTA pH 7.5. Gradients were prepared by layering successive 2.1ml vol of 50%, 45%, 40%, 35%, 30% w/w sucrose over a cushion of 1.5ml of 55% w/w sucrose. All these solutions contained 5mM EDTA, pH 7.5. The resuspended envelope material was layered on to the

gradient and then centrifuged in a Beckman ultracentrifuge using the SW40 rotor (35,000rpm, 14h).

Fractions were collected from the bottom of the gradient by puncturing the cellulose nitrate centrifuge tube with a fine needle. For determination of radioactivity in each fraction 200μl samples were taken and counted in aqueous scintillation fluid (see Table 8.4). For isolation of membrane material, fractions from the various peaks of radioactivity were taken and precipitated wih TCA. The refractive index of samples was measured using a refractometer (Bellingham and Stanley Ltd., London). Fractions were then analysed by SDS-PAGE and autoradiography.

(g) Flotation of membranes away from non-membrane associated protein on sucrose gradients

The procedure was basically as described by Hirst et al (1984). The washed envelope pellet was resuspended in 1ml of 60% w/w sucrose containing 5mM EDTA pH 7.5. A step gradient consisting of 2.1ml of 55%, 50%, 45%, 40%, 35% and 30% w/w sucrose in 5mM EDTA was carefully layered on top of the 60% w/w sucrose cushion containing the evelope fraction. The gradients were then centrifuged for 48h at 38,000rpm in a Beckman SW40 rotor. The tube was marked into 11 equal fractions and samples were carefully removed from the top of the gradient with a Pasteur pipette.

6. SDS polyacrylamide gel electrophoresis

(a) Preparation and running of gels

The basic system was that of Laemmli (1970). The constitution of various buffers and solutions used is given in table 8.3. Only one acrylamide solution was used in any one gel, acrylamide I was used for

Table 8.3   SDS-PAGE solutions, buffers and gel recipes

Gel Composition

		11%
Separating Gel:	Buffer A	13.5ml
	Acrylamide (I or II)	6.8ml
	H_2O	6.0ml
	Ammonum persulphate (APS)	
	freshly made, 10mg/ml	1.0ml
	N,N,N,N',N'-tetramethyl	
	ethylenediamine (TEMED)	75µl
Stacking Gel	Buffer B	10.0ml
	Acrylamide (I or II)	3.3ml
	H_2O	6.7ml
	APS	0.5ml
	TEMED	40µl

TEMED was always added immediately before the gel was poured.

Buffer A	Tris/Cl pH 8.8	0.75M
	SDS	0.2% w/v

Buffer B	Tris/Cl pH 6.8	0.25M
	SDS	0.2% w/v

Acrylamide I	Acrylamide	44% w/v
	N, N'-methylene-bis-	
	acrylamide (bis)	0.8% w/v

Acrylamide II	Acrylamide	44% w/v
	Bis	0.3% w/v

| Electrophoresis | Trisma base | 0.125M |
buffer	Glycine	0.192M
	SDS	0.1% w/v

Sample buffer	Tris/Cl pH 6.8	0.125M
	Glycerol	20% v/v
	β-mercaptoethanol	10% v/v
	SDS	4% w/v
	Bromophenol blue	0.05% w/v

1/3 volume of this sample buffer was added to each sample before boiling
and electrophoresis.

Destain	Isopropanol	25% w/v
	Acetic Acid	10% v/v

Stain	Coomassie	
	brilliant blue	0.05% w/v
	in destain	

resolution of proteins such as TonA since this achieved better

separation of high molecular weight bands (Hancock et al., 1976).

Better overall resolution was however obtained by use of acrylamide II

and this was therefore used routinely.   Gels were 1mm thick and were

composed of a 7% stacking gel and an 11% separating gel.   Freshly

prepared ammonium persulphate solution was always used.

Electrophoresis was carried out on a Biorad slab gel system at a

constant current of 25mA per gel, until the tracking dye was

approximately 5mm from the end of the gel (unless otherwise stated in

the text).

(b)   Molecular weight   markers

For radioactive molecular weight markers, a ^{14}C-methylated mix was

used.   It contained myosin (200kD), phosphorylase B (100kD and 92.5kD),

bovine serum albumin (69kD), ovalbumin (43kD), carbonic anhydrase (30kD)

and lysozyme (14.3kD).   1ηCi of this mixture together with sample buffer

was loaded per slot.

For stainable molecular weight markers, 20μl of a solution

containing 50ηg of each required protein as indicated in the text, was

loaded per slot.

(c)   Autoradiography and Fluorography

Radioactive gels were dehydrated by two successive washes in

dimethylsulphoxide, followed by impregnation with 2,5-diphenyloxalzole

(PPO) exactly as described by Bonner and Laskey (1974).   The gels were

then dried on to a sheet of Whatman No 17 Chromatography paper in a

Bio-Rad gel drying unit.   The dried gels were then placed in a Kodak RPR

X-Omat X-ray film cassette and exposed at -80°C.

(d) Staining gels in Coomassie blue

Gels were stained for 2-3h in 300ml 10% acetic acid, 25% v/v isopropanol, 0.05%w/v Coomassie brilliant blue. Diffusion destaining was carried out by shaking the gel in 300ml 10% v/v acetic acid, 25% v/v isopropanol for two three hour periods.

7. Direct measurement of radioactivity in individual gel bands

The method used was that of Ames (1974). Radioactive gels were fluorographed and dried as above and then exposed and developed. If it was not possible to prestain the gel, the exact position of the X-ray film on the gel was carefully noted and pins were then stuck through the film in order to mark the position of the band of interest on the fluorograph. Bands were carefully cut out using small scissors and placed in plastic scintillation vials, with care being taken to ensure each slice was thoroughly soaked. After 10min, 2ml of scintillation fluid was added, (Table 8.4). The vials were stoppered, vortexed and incubated in a 37°C constant temperature room overnight in the dark before cooling, vortexing and counting in a Minnaxi Scintillation Counter.

This method was used to determine the relative radioactive content of different proteins in the same sample, or the reduction in radioactivity in one protein band in consecutive samples.

8. DNA manipulation, General Techniques

(a) Handling of DNA

When handling DNA, disposable gloves were worn, all buffers, reagents, glass and plastic ware was sterilised by autoclaving and all glassware coming into contact with DNA was siliconised.
Many of the techniques used are modified versions of those described in Maniatis et al. (1982).

Table 8.4 Scintillation fluid

Non-aqueous scintillation fluid	PPO	5g
	dimethyl POPOP	0.3g
	toluene	1000ml
NCS scintillation fluid	NCS solubiliser	83.5ml
	PPO	4g
	dimethyl POPOP	60.5mg
	toluene	1000ml

dimethyl POPOP - 1,4,di-2 (4methyl-5-phenoxazolyl) benzene

(b) Phenol Extraction

DNA solutions were mixed with an equal volume of phenol, chloroform, isoamyl alcohol, 8 hydroxyquinoline (100:100:4:0.4; w:v:v:w) saturated with 10mM Tris-Cl, pH 7.5. The upper aqueous phase containing the DNA was removed and the phenol layer re-extracted with an equal volume of 10mM Tris-HCl, pH 7.5. The phenol was AR grade and was not re-distilled.

(c) Ethanol precipitation

DNA was precipitated from solution by the addition of 0.1 volume of 2M sodium acetate, pH 6.5 and 2.5 volumes of ethanol. The mixture was chilled for 20min at -80°C before precipitated DNA was pelleted either using a Sorvall HB4 rotor (13,000rpm, 15min, 4°C) or an Eppendorf microfuge for 10min at maximum speed. The supernatant was then discarded, the pellet washed in 70% ethanol and recentrifuged. DNA was dried under vacuum and redissolved as appropriate for further manipulation.

9.   Preparation of plasmid DNA

For solutions and buffers used see Table 8.5

(a) Rapid plasmid preparation for screening

1.5ml of an overnight culture of bacteria grown in nutrient broth plus the appropriate antibiotic(s) was transferred to an Eppendorf and centrifuged for 3min in an Eppendorf microfuge. Cells were resuspended in 100µl of TEG buffer and incubated at room temperature for 10min. 200µl of alkaline SDS solution was added and the contents of the tube mixed by several sharp inversions, followed by 5min incubation on ice. 150µl of potassium acetate was then added, the solution was gently mixed

and left for 5min on ice. The chromosomal DNA was pelleted by
centrifugation for 5min at $4^{\circ}C$ and the supernatant removed using a
Gilson P200 pipette. Plasmid DNA was recovered by phenol extraction and
ethanol precipitation. The pellet was washed in 70% ethanol, dried
under vacuum and resuspended in 50μl TE buffer. RNA was removed by
incubation with Ribonuclease A (20μg/ml) for 30min at $37^{\circ}C$.

(b) Large scale plasmid preparation

To prepare DNA from an amplifiable plasmid, a 400ml culture in
nutrient broth containing appropriate antibiotics was grown to late log
phase at $37^{\circ}C$. Chloramphenicol (170μg/ml) was added and the culture was
shaken overnight. Cells were harvested and washed in fresh nutrient
broth before resuspending in 3ml 10% sucrose in 50mM Tris/Cl pH8.0 and
0.5ml lysozyme (10mg/ml), Ribonuclease A (300μg/ml) was also added. The
mixture was incubated at room temperature for 5min. 4ml 2% TritonX-100
in 50mM Tris pH 8.0 were added to the tube and the tube was inverted
several times until lysis was complete. The lysate was cleared by
centrifugation using a Sorvall SS34 rotor (5,000rpm, 10min, $4^{\circ}C$) and the
supernatant decanted into a Sorvall GSA bottle. Two-thirds volume of
polyethylene glycol (PEG)/NaCl was added and the mixture was left at $4^{\circ}C$
overnight. The precipitate was collected by centrifugation using a
Sorvall GSA rotor (5,000rpm, 10min, $4^{\circ}C$), drained and resuspended in
exactly 1.1ml TES buffer. The lysate was transferred to a Beckman VTi65
self sealing tube and underlaid with 4ml CsCl-EtBr solution. The
refractive index of the CsCl-EtBr solution was adjusted to 1.293 by
addition of further CsCl or TE buffer. The tube was then centrifuged to
equilibrium in a Beckman VTi65 rotor (55,000rpm, 3h, $15^{\circ}C$) with slow
acceleration. The plasmid band was recovered by insertion of a 0.8mm

needle and transferred to a siliconised glass tube before the ethidium

bromide was removed by several extractions with an equal volume of

sodium chloride saturated propan-2-ol. The sodium chloride was

subsequently removed by dialysis against TE buffer and the plasmid

solution was precipitated with ethanol.

10. Agarose Gel Electrophoresis

Horizontal slab agarose gels (0.5-1% w/v) were prepared using

Seakem analytical agarose in TAE buffer. Ethidium bromide was added to

both gel and running buffer to a concentration of 0.5μg/ml. All samples

were mixed with 1/6 volume agarose gel sample buffer before loading (see

table 8.6). Electrophoresis was carried out with the gel completely

submerged in electrophoresis buffer at 100V, until the dye front had

migrated through 3/4 of the gel. DNA was visualised by trans-

illumination with long wave (260nm) UV light. λ DNA restricted with

HindIII was used to supply the molecular weight markers and gave sizes

of 23kb, 9.4kb, 6.5kb, 4.4kb, 2.3kb, 2.0kb, 0.5kb, and 0.1kb

respectively.

11. Restriction endonuclease digestion

All manipulations were carried out wearing disposable gloves, using

sterile buffers and plastic ware. 0.5μg of plasmid DNA was used per

restriction digest; and 0.5μl of 6mg/ml Ribonuclease A (previously

boiled for 5min to destroy any DNase activity) was added to the

restriction mixture to remove any excess RNA. 1/10 of the final volume

of 10 times concentrated restriction buffer was added. BRL React

Buffers were used as recommended by the manufacturers. In all cases the

final reaction volume was at least 10 times the volume of enzyme added. Digestions were carried out for 1h at 37°C.

12. Isolation of DNA fragments from agarose gels

After digestion with the appropriate restriction enzymes, DNA was electrophoresed in a 1.0% low melting point agarose gel. Bands were visualised under long wave U.V. light and the required band excised from the gel. 2-3 volumes of H_2O were added to the gel slice and the mixture was heated at 70°C until the agarose melted. After rapid chilling on ice, phenol was added, the mixture was vortexed and centrifuged (2,500rpm, 5min). The supernatant was removed and phenol extracted until the interface was clear before DNA was precipitated by the addition of ethanol.

13. Ligation

Restricted plasmid vector DNA and fragments for ligation were mixed in a 1:3 molar proportion. Total DNA concentration was usually between 5-50ng/ml in a 10-20µl total volume. 1/10 volume of 10 times concentrated ligation buffer (Table 8.7) and 1µl T4 DNA ligase were added and the mixture incubated overnight at 15°C. Blunt ended ligations were carried out at a DNA concentration of 100µg/ml, with 10 times the normal ligase concentration. Ligation was carried out at 15°C overnight for both blunt ended ligations and for fragments with overlapping complementary ends. T4 DNA ligase was obtained from New England Biolabs.

Table 8.6 Agarose gel electrophoresis

Electrophoresis buffer	Tris/acetate pH 8.0	267mM
	EDTA	1mM
Sample buffer	Tris/Cl pH6.8	125mM
	Glycerol	20% w/v
	EDTA	5mM
	Bromophenol blue	0.05%

Table 8.7 Restriction and ligation of DNA

Ligation buffer x10

1M Tris/Cl pH7.4	660mM
1M MgCl$_2$	100mM
10mM ATP	1mM
DTT	25mM
BRL nuclease free BSA	1% w/v

Klenow buffer x10

Tris/Cl pH 7.8	500mM
MgCl$_2$	50mM
β-mercaptoethanol	100mM

Alkaline phosphatase (CIP) buffer x10

Tris/Cl pH 9.0	500mM
MgCl$_2$	10mM
ZnCl$_2$	1mM
spermidine	10mM

Linker kinase buffer x10

Tris/Cl pH	100mM
MgCl$_2$	100mM
DTT	50mM

DTT - dithiothreitol

14. **Filling in single strand projected ends**

Aliquots of DNA were incubated with 1 unit of E.coli polymerase I

Klenow fragment (Boehringer), 1/10 volume of Klenow buffer (Table 8.8)

and 0.25mM of each dNTP for 30min at 37°C. DNA was recovered by phenol

extraction and ethanol precipitation and resuspended in TE buffer.

15. **Alkaline phosphatase treatment of linear, plasmid vector DNA**

To minimise recircularisation of plasmid DNA, 5' phosphates were

removed from both ends of linear DNA by treatment with calf intestinal

phosphatase (Ullrich et al., 1977). Digested vector DNA was phenol

extracted and precipitated with ethanol before the DNA was resuspended

in a minimum volume of 10mM Tris/Cl pH 8.0. 1/10 volume of 10x

concentrated CIP buffer (see table 8.7) and 1μl of calf intestinal

phosphatase was added. The mixture was incubated at 37°C for 30 min and

the reaction stopped by phenol extraction.

16. **Preparation of Oligonucleotide DNA**

The short 74bp oligonucleotide used to create the construct

described in Chapter 5, was provided as 4 short single stranded

complementary HincII - BglII and BglII - HincII strands of DNA. In order

to prepare the four synthetic oligonucleotides for ligation, the 3'- 5'

HincII - BglII and the 5'- 3' BglII - HincII oligonucleotides were

phosphorylated by the addition of 1/10 volume of linker kinase buffer

(Table 8.8), 10mM ATP and 1.5μl Klenow. The mixture was incubated at

37°C for 30min to allow phophorylation to take place and boiled to

inactivate the kinase. The oligonucleotides were stored at -20°C until

used. Before ligation, non-phosphorylated oligo- nucleotides were added

to equimolar amounts of the phosphorylated

oligonucleotides and all four were boiled for 5min and allowed to cool

to room temperature. The oligonucleotides were then ligated together

and the unfractionated mixture was ligated with vector DNA.

17. Transformation of bacteria with plasmid DNA

(a) Preparation of Competent Cells

Stationary phase cultures of E.coli were diluted in nutrient broth

to an A_{450} of less than 0.01 and grown rapidly with good aeration to

mid-exponential phase (A_{450} = 0.3-0.5). Cultures were immediately

placed on ice before 10ml of these cells were harvested by

centrifugation for 3min at 4,000rpm in an M.S.E. chillspin. Cell pellets

were resuspended in 5ml of ice cold 100mM $MgCl_2$, reharvested and

resuspended in 5ml of ice cold 100mM $CaCl_2$. The cells were kept on ice

for 20min before centrifuging as before and resuspending in 600µl of ice

cold 100mM $CaCl_2$. Cells were then kept at $4^{\circ}C$ for a minimum of 1h

before use, and were used within two days.

(b) Transformation

200µl of the prepared competent cells were incubated on ice for 1h

with 10 - 500ng of plasmid DNA. After a 2min heat shock at $42^{\circ}C$ the

bacteria were added to 2ml of prewarmed nutrient broth in a 25ml flask

without shaking. Undiluted culture and culture diluted to 10^{-1} and 10^{-2}

were spread on nutrient agar containing the necessary antibiotics to

select for plasmid transformed cells.

18. Pulse-labelling of exponential cultures

In many experiments protein synthesis over a specified short period of the growth cycle was measured by pulse-labelling. Samples of culture were tranferred either to an Eppendorf tube or prewarmed 25ml flask containing a cocktail of ^{35}S-methionine or ^{3}H-leucine and unlabelled methionine/leucine as stated in the text. After a fixed period, ice cold unlabelled methionine (30mg/ml) or leucine (30mg/ml) was added to a final concentration of 1.0% and also, where indicated, chloramphenicol (final concentration 250μg/ml). Samples were stored on ice until required.

19. Use of an internal standard of ^{3}H-leucine-labelled cells.

In many experiments described, incorporation of radioactive amino acids into cell protein fractions and individual gel bands was measured. In order to obviate the need for reproducible quantitative recovery from sample to sample , a 30ml exponentally growing culture (A_{450} = 0.1) was labelled by addition of 100-200μCi of ^{3}H-leucine (53Ci/mmol). After two generations of growth, unlabelled leucine was added (final concentration 20μg/ml) ad the cells chilled.

A constant volume of ^{3}H-leucine-labelled culture, was added to samples labelled with ^{35}S-methionine immediately prior to preparation of cell envelopes. The ratio of ^{35}S/^{3}H radioactivities in any protein fraction or gel and derived from these samples was thus a measure of the relative amounts of ^{35}S radioactivity in the whole of the original samples.

Double-labelled samples were processed for determination of radioactivity by the normal procedure described elsewhere. Counting was performed on the ^{14}C/^{3}H setting of a Minnaxi Liquid Scintillation Spectrophotometer. Samples labelled with each of the isotopes alone

were always processed in parallel with experimental samples for measurement of cross-channel spilllover. The spillover from the ^3H channel to the ^{14}C channel was always less than 1% and was disregarded, spillover in the opposite direction was 40-100% and ^3H counts per minute were always corrected for this spillover before calculation of the isotope ratios. The ratio of ^3H radioactivity/ ^{14}C radioactivity in samples was usually greater than 10:1, so that the spillover correction was less than 10% of total ^3H radioactivity.

20. Pulse-chase analysis of assembly of protein into the outer

membrane

A 10ml sample was taken from a 100ml culture growing in M9-glucose minimal medium and labelled by addition of 180μCi of ^{35}S-methionine (50Ci/mmol). After a specified time (see text) cold methionine was added to a final concentration of 1% and 1ml samples were removed at given intervals to ice-cold M9 minimal medium containing chloramphenicol to a final concentration of (250mg/ml) or kanamycin (250mg/ml) as indicated in the text was then added.

21. Radioactive incorporation into protein

Synthesis of protein in bacterial cultures was measured as the incorporation of ^{35}S-methionine into TCA precipitable material. TCA precipitates were collected on to 27mm membrane filters (Sartorius, 0.45μm pore size) by suction, and washed extensively with several 5-10ml volumes of ice-cold 5% TCA. Filters were dried under an infra-red lamp and tranferred to plastic scintillation vials. To each vial 0.5-1.0ml of non-aqueous scintillation fluid was added (see Table 8.4). The plastic vials were stoppered and placed in standard Packard glass scintillation vials. The samples were then counted in a Minnaxi Liquid Scintillation Spectrophotometer.

22. Preparation and pulse-labelling of spheroplasts

The method used was adapted from the basic method of Osborn et al.

(1972). 5ml of an exponentially growing culture (A_{450} = 0.1) were

harvested, washed in Buffer B (see table 8.2) and resuspended in 1ml

0.3M sucrose, 0.12M Tris pH 7.6. 66μl of 2mg/ml egg white lysozyme in

the same sucrose-Tris buffer and 20μl of 0.25M EDTA pH 8.0 were added

and the mixture was incubated on ice for approximately 15min until

conversion to spheroplasts was at a level of about 90% (monitored by

phase contrast microscopy).

The spheroplasts were harvested at low speed in an Eppendorf

microfuge (6,500rpm, 3min). The supernatant represented the periplasm

and the pellet contained whole spheroplasts. Spheroplasts were

carefully resuspended in 1ml of minimal glucose medium containing 0.3M

sucrose. The cells were incubated at 37°C and pulse-labelled with 20μCi

of ^{35}S-methionine for 10min. The pulse was terminated by placing the

flask on ice and chasing with excess cold methionine. Spheroplasts were

harvested by centrifugation (6,500rpm, 3min) and any protein in the

supernatant was precipitated by addition of TCA. The spheroplasts were

lysed by addition of 7ml of ice cold 10mM sodium phosphate buffer pH 7.2

and membranes were separated on the basis of Sarkosyl solublity.

23. Protease treatment of cell fractions

(a) Trypsin Accessibility of membrane proteins

This procedure was followed in order to determine whether material

which fractionated with the inner membrane after conversion to

spheroplasts was truely integrated into the membrane or whether it was

merely associated with the surface of the inner membrane.

Cells were grown, converted to spheroplasts, pulse-labelled with ^{35}S-methionine and chased with cold methionine as described above. 20s after the initiation of the chase, the cells were chilled in an ice-water bath, harvested and resuspended in 1ml of Tris-sucrose. Four 100μl aliquots of the suspension were taken for digestion with trypsin. To the first tube was added 10μl of 0.1M Tris/sucrose (pH 8.0), to the second 10μl of 2mg/ml trypsin, to the third 10μl of 100μg/ml trypsin and to the fourth 10μl of 50μg/ml trypsin (all trypsin solutions were made up in 0.1M Tris/sucrose pH 8.0). After 10min incubation on ice, the digests were transferred to 50μl of pre-warmed SDS sample buffer which contained 10μl of 2mg/ml antiptrypsin. Samples were boiled for 5min and analysied by SDS-PAGE and autradiography.

(b)   Protease treatment of culture supernatant samples

Protease from Streptomyces griseus (Sigma) was used. 10μl of a protease stock (10mg/ml) was added to 50μl of supernatant. The mixture was incubated at room temperature for 30min before the reactin was stopped by the addition of 1μl of PMSF (6mg/ml) and SDS-sample buffer.

24. Immunological reactions

(a) Preparation of the cells of Staphylococcus   aureus.

For all buffers used see Table 8.8

Staphylococus aureus cells were prepared as described by Kessler (1975). Before use, 1ml of these cells was harvested in an Eppendorf microfuge and washed twice in 1ml RIPA followed by washing in 1ml NET buffer to which had been added 1mg/ml BSA (Table8.8). This saturated the binding sites of the S. aureus cells. The cells were then resuspended in 0.9ml RIPA before use.

(b)   Immunoprecipitation

Strains were grown in glucose minimal medium and 0.5ml samples were labelled with 15µCi of ^{35}S-methionine for 5min as described in the text. The labelled sample was sedimented in and Eppendorf microfuge (5min, 13,00rpm, ^{4}°C) and washed in 0.5ml of ice-cold 10mM Tris pH 8.0. After re-centrifugation the sample was resuspended in 1% SDS, 10mM Tris, 1mM EDTA pH 8.0 and boiled for 2min. At this stage samples were added to 0.65ml of 2% Triton X-100, 50mM Tris, 150mM NaCl, 1mM EDTA pH 8.0. The mixture was vortexed and debris was removed by centrifugation in an Eppendorf microfuge for 5min. 2µl of antiserum raised agains' either OmpF, OmpA or TonA was added and the samples were incubated overnight at ^{4}°C. The following day, 10µl of 10% v/v suspension of bacterial adsorbent (see above) were added and samples were incubated for 30min on ice. The cells were then washed thoroughly three times in RIPA buffer, harvested and solubilised in 30µl urea, SDS, β-mercaptoethanol and boiled for 3min before addition of 30µl SDS sample buffer. The cells were again harvested (3min, 13,000rpm), the pellet was discarded and the supernatant was analysed by SDS-PAGE and autoradiaography.

(c)   Western blotting

Electrophoretic transfer of proteins from acrylamide gels onto nitrocellulose and subsequent immunological detection of proteins was caried out using polyclonal antibody and horseraddish peroxidase conjugated to goat anti-rabbit IgG (Nordic Immunological) . as described by Towbin et al. (1979) except that the substrate, o-diamisadine, was replaced by 0.5mg/ml 3,4,3',4'-tetra-aminobiphenyl hydrochloride.

25. Measuring levels of Haemolytic Activity

The method used was based on the procedure developed by Noegel et al. (1979). External haemolytic activity was measured by taking the supernatant from cultures grown in nutrient broth and 10mM $CaCl_2$. The supernatant was separated from cells by centrifuging 1ml samples in an Eppendorf microfuge for 10min. Haemolytic activity was measured by the amount of haemoglobin released from an incubation mixture containing 3% defibrinated sheep erythrocytes, 10mM KPO_4, 155mM NaCl and 10mM $CaCl_2$, pH 7.5. The reaction tubes were incubated at 37°C for 30min and any remaining intact erythrocytes were removed from the mixture by centrifugation for 2min in an Eppendorf microfuge. The amount of haemolysin released was determined by measuring the absorbance of the supernatant at 540nm (Waalwijk et al., 1983). The reaction was linear for 30min and was proportional to the amount of culture supernatant added below a haemolytic concentration of 10units/ml (Mackman et al., 1984a).

26. Assay for β-lactamase activity

In order to monitor the degree of lysis of cells during preparation of supernatant samples, the level of β-lactamase released into the supernatant was assayed with increasing time after induction. β-lactamase activity was assayed by measuring the hydrolysis of nitrocefin, a commeriallly available chromogenic β-lactam. A 100ml culture of JM101(pLG575,pLG632) was grown in Luria broth supplemented with the appropriate antibiotics and at an A_{450} = 0.2, IPTG was added to derepress the lac promoter. At intervals after induction 1ml samples were harvested by centrifugation for 5min in an Eppendorf microfuge at maximum speed. Cells were washed in Buffer B (see Table 8.2) and then resuspended in 1ml 50mM sodium phosphate buffer, pH7.0. The cells were

sonicated for three 30s intervals in an M.S.E. ultrasonic disintegrator and cell and supernatant samples equivalent to 0.2 and 0.4 A_{450} units were assayed for β-lactamase activity. The volume of each sample to be assayed was made up to 1ml with 50mM sodium phosphate buffer pH 7.0 and 2.5ml of assay buffer (50mM $NaPO_4$ containing 10μg/ml nitrocefin) were added. At various times after induction, supernatant and cell samples equivalent to 0.2 and 0.4 A_{450} O.D. units were assayed and the activity was taken as the mean of the two values. Each assay mixture was made up to a total volume of 1ml by the addition of 50mM sodium phosphate, pH 7.0 and 0.01mg of nitrocefin were added per ml. Hydrolysis was monitored by change in absorbance at 482nm using a Unicam spectrophotometer.

Table 8.10   Sources of chemicals

Radiochemicals were obtained from Amersham International; all other chemicals were obtained from Fisons (AR grade) except:-

    Acetic acid: Fisons SLR
    Acrylamide: Eastman Kodak
    Alkaline phosphatase: Sigma
    Casamino acid: Sigma
    Ammonium persulphate: Biorad
    Ampicillin: Sigma
    Chloramphenicol: Sigma
    Coomassie brilliant blue: Gurr's
    8-hydroxyquinoline: AnalaR
    Goat anti rabbit IgG: Nordic Immunological
    Klenow: Boehringer
    Isoamylalcohol: Sigma
    lysozyme: Sigma
    Kanamycin: Sigma
    β-mercaptoethanol: Sigma
    NCS tissue solubiliser: Amersham
    Nirocefin: Oxoid
    N.N'-methylene-bis-acrylamide: Eastman Kodak
    Restriction endonucleases: BRL
    Streptomyces griseus protease: Sigma
    Trypsin, Antitrypsin: Sigma
    Sodium dodecyl sulphate: Biorad
    T4 ligase: Biolabs
    Tetracycline: Sigma
    N,N,N',N'-tetramethylethylenediamine: Eastman Kodak
    Trisma base: Sigma
    Tryptone: Oxoid
    Thiamine HCl: Sigma

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Abstract

Mechanisms of protein translocation in E.coli.

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A wide variety of proteins which are synthesised in the cytoplasm of
E.coli are subsequently directed either to non-cytoplasmic compartments or
transported to the extracellular medium. Proteins which are exported from
the cytoplasm are thought to interact with a complex cellular machinery
and a number of mutations affecting this secretion machinery have been
isolated.
        In this study, the export of the outer membrane protein TonA was used
as a model system to examine the effect on protein translocation of two
temperature sensitive secretion mutants, secA and secY. Initial analysis
of the effect of secA^{ts} mutations on bulk envelope protein synthesis
confirmed the key role of SecA in protein transport, including many
proteins assembled into the inner membrane. Analysis of the rate of
processing of preTonA, pulse-labelled at the restrictive temperature and
chased at the permissive temperature revealed differences between SecA and
SecY mutants. In particular these data indicate that SecA and SecY may
interact sequentially to promote protein export and that SecA may be
required to maintain preTonA in a translocationally competent form prior
to interaction with SecY.
        In order to investigate the nature of a specific "export" signal
within a protein to be exported, the possibility of using the novel
secretion signal at the C-terminus of E.coli haemolysin to direct chimeric
protein into the medium was also investigated. The C-terminal signal was
successfully fused to a hybrid protein containing a few residues
of β-galactosidase and the majority of E.coli outer membrane protein OmpF
lacking its own NH$_2$-terminal signal sequence. The chimeric protein is
specifically translocated across the inner and outer membranes and is
released into the medium. Consistent with a transport system which
bypasses the periplasm, other studies indicated that haemolysin transport
is secA independent but may involve secY.
        Finally, the localisation of haemolysin and several outer membrane
proteins synthesised in spheroplasts was also examined in the hope of
gaining some further insight into the route taken by proteins which reach
the outer membrane or the external medium.