INVESTIGATION OF THE ASSEMBLY OF TonA PROTEIN INTO THE OUTER MEMBRANE OF ESCHERICHIA COLI

Thesis submitted for the degree of Doctor
of Philosophy at the University of Leicester
by

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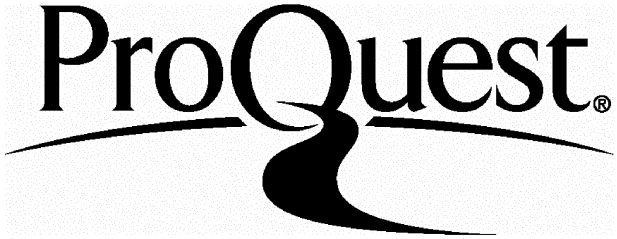


TO MY MOTHER AND FATHER

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor I. Barry Holland, for his advice and encouragement during the course of this project. Thanks are also due to all the members of the Genetics Department, especially those with whom I shared labs, for their assistance and for providing a happy working atmosphere (and for playing bridge!). Finally, I would like to thank Dr. Julie M. Pratt for her collaboration on the work described in Chapter 10 and for providing guidance and discussion throughout this project. I acknowledge the receipt of a research studentship from the Medical Research Council.

Definitions of terminology as I have used it here:

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

Secretion involves complete export of a protein to the medium (or intercellular space)

The prefix "pre" is used to indicate the presence of an intact signal sequence; "pro" is used to describe other protein precursors:

eg., proinsulin : insulin precursor

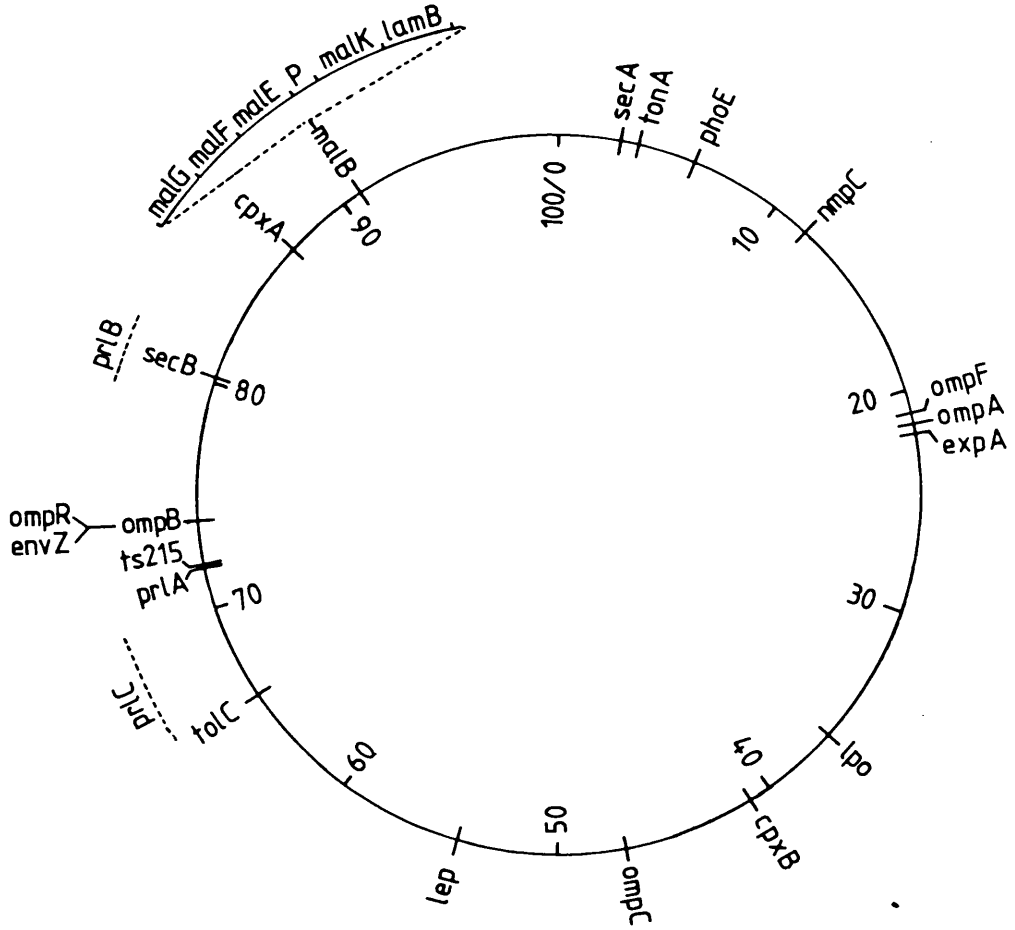
preproinsulin : insulin precursor carrying intact signal sequence

Abbreviations

CCCP	-	carbonylcyanide m-chlorophenylhydrazone
DNP	-	dinitrophenol
EDTA	-	diaminoethane tetra-acetic acid, disodium salt
LPS	-	lipopolysaccharide
SDS PAGE	-	sodium dodecyl sulphate polyacrylamide gel electrophoresis

Map of the Escherichia coli chromosome showing location of genetic loci discussed in this thesis

cpxA, cpxB	: mutations cause defective transcription and assembly of OmpF; inner membrane protein profile also altered
expA	: mutation causes decrease in 6 periplasmic enzymes and some modification of inner and outer membrane protein profiles
lep	: structural gene, Wickner's signal peptidase
lpo	: Braun's lipoprotein structural gene
malB - malG	: maltose transport
- malF	: inner membrane maltose transport protein
- malE	: periplasmic maltose binding protein
- malK	: maltose transport
- lamB	: structural gene, outer membrane LamB protein, receptor for maltose & maltodextrins
nmpC	: encodes a novel outer membrane protein, expressed in some ompF, ompC mutants
ompA	: encodes the outer membrane matrix protein
ompB	: regulatory locus comprising 2 genes, ompR & envZ, involved in regulation of expression of several envelope proteins, including OmpF & OmpC
ompC	: encodes outer membrane porin OmpC
ompF	: encodes outer membrane porin OmpF
phoE	: encodes inducible outer membrane protein PhoE, involved in phosphate uptake
prlA, prlB, prlC	: mutations causing supression of certain signal sequence mutations
secA, secB	: mutations causing generalised export defective phenotype
tolC	: mutation affecting expression of some outer membrane proteins
tonA	: encodes the ferrichrome receptor, TonA
ts215	: temperature sensitive mutation causing slow processing at the non permissive temperature


malG, malF, malE, P, malK, lamB
malB
cpxA
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tonA
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ompC
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lep
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tolC
prlC
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prlA
ts215
ompB
envZ
ompR
80
secB
prlB

CONTENTS

CHAPTER ONE

INTRODUCTION

I. STRUCTURE AND FUNCTION OF THE E. COLI CELL ENVELOPE

The cell envelope of Gram-negative bacteria, including Escherichia coli, is a complex structure, composed of inner (cytoplasmic) and outer membranes, separated by the periplasmic space, within which lies the peptidoglycan (murein) layer (Fig. 1.1). The structure of the envelope has been extensively reviewed (Nikaido & Nakae, 1979; Osborn & Wu, 1980; Hall & Silhavy, 1981a; Lugtenberg & Van Alphen, 1983) and, except where indicated, the following information on envelope structure has been taken from these reviews.

I.1 The inner membrane

The inner membrane, which possesses a typical bilayer structure, is functionally complex. Activities associated with the inner membrane include electron transport, oxidative phosphorylation, transport of nutrients into the cytoplasm and synthesis of phospholipids, peptidoglycan and lipopolysaccharide (LPS). In addition, some inner membrane proteins appear to be involved in cell division.

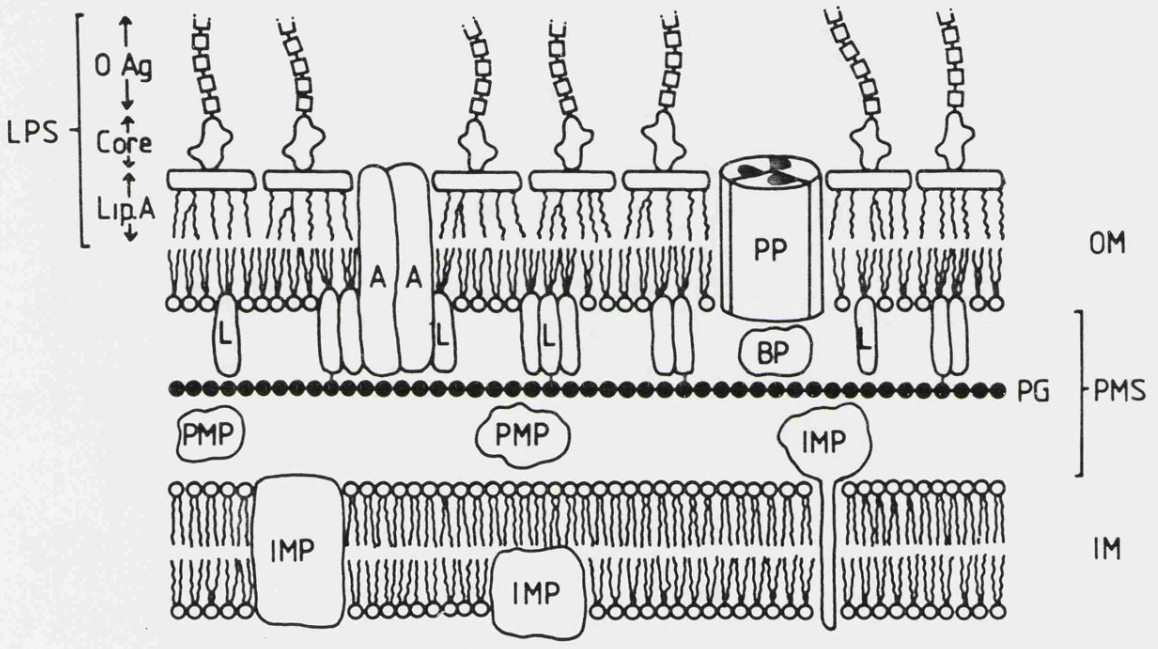
I.2 The periplasmic space

Periplasmic proteins comprise approximately 4% of the total cellular protein and are involved in (i) conversion of nutrients to forms which may be transported across the inner membrane (eg. phosphatases, proteases, nucleases); (ii) transport of nutrients (eg. maltose binding protein,

Fig. 1.1: Structure of the E. coli cell envelope (redrawn with modifications from Lugtenberg & van Alphen, 1983)

In E. coli 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 - O antigen
OM - outer membrane
PG - peptidoglycan
PMP - periplasmic protein
PMS - periplasmic space
PP - pore forming protein trimer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arabinose binding protein); and (iii) degradation and modification of harmful substances such as antibiotics and heavy metals (eg. β-lactamase).

I.3 The peptidoglycan

The peptidoglycan consists of a rigid monolayer of polysaccharide chains cross linked by peptide bridges. This layer is, at least in part, responsible for the maintenance of the characteristic rod shape of *E. coli*, and allows the cell to withstand the osmotic pressure of the cytoplasm (3.5 atm.).

I.4 The outer membrane

The natural environment of *E. coli* is the gut, where the bacterium is exposed to the degradative activity of proteases, lipases and glycosidases, and the detergent like activity of bile salts, fatty acids and glycerides. The outer membrane serves as a protective barrier against such substances - *E. coli* can grow in the presence of 5% SDS (a strong ionic detergent) whereas erythrocytes will lyse when exposed to as little as 0.001% SDS (Lugtenberg & Van Alphen, 1983); and, as will be discussed in Chapter 4, the intact *E. coli* cell is also highly protease resistant. However, the outer membrane must also allow permeation of nutrients, and, to achieve this end, possesses a variety of protein species involved in nutrient uptake, some with a wide specificity, and others which allow uptake of only one or a few related compounds. Many of these proteins are also used

as receptors by bacteriophages and colicins.

The structure of the outer membrane is unusual, in that there are two types of lipid molecule distributed asymmetrically, such that the outer leaflet of the bilayer consists mainly of LPS (Fig. 1.2), and the inner leaflet consists mainly of phospholipid. The phospholipid composition of the outer membrane is distinct from that of the inner membrane: the outer membrane contains mainly phosphatidylethanolamine, whereas the inner membrane contains considerably more phosphatidylglycerol and diphosphatidylglycerol (Lugtenberg & Van Alphen, 1983).

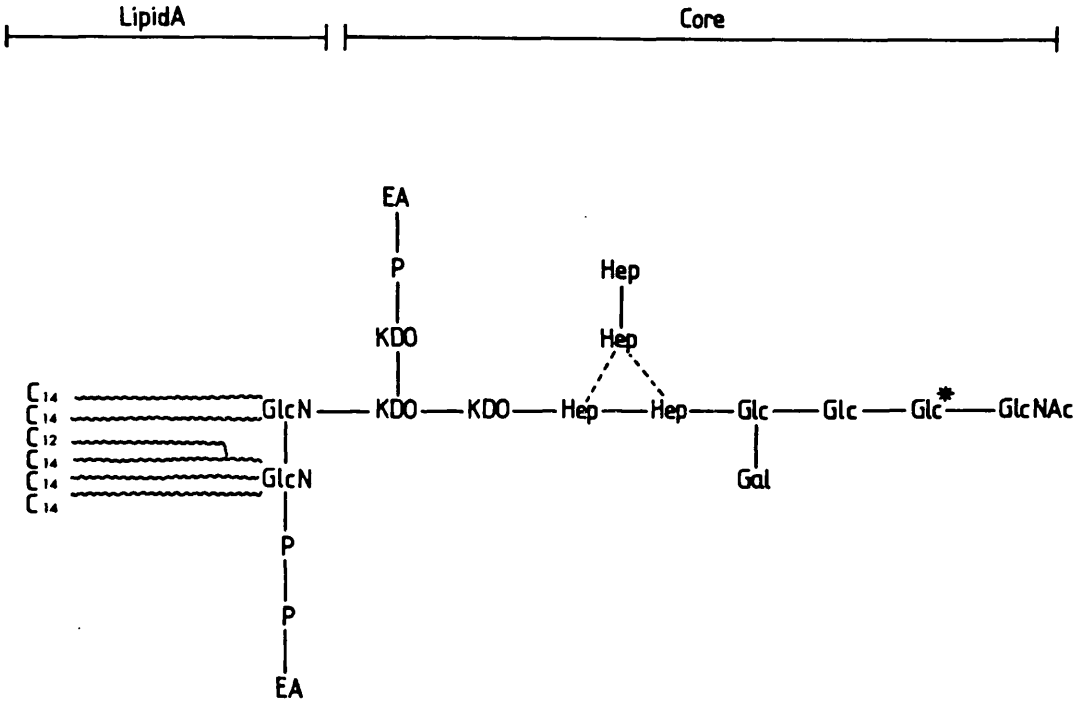
I.5 Fusion sites between inner and outer membranes

Electron microscopic studies by M. Bayer (reviewed in Bayer, 1979) raised the possibility that junctions exist between the inner and outer membranes; however, the molecular architecture of these "Bayer junctions" is not known. It has been proposed that these junctions represent the sites of export of outer membrane constituents from the cytoplasm and also serve as a junction through which phage nucleic acids are injected. However, it is questionable whether such sites represent permanent structures, or whether they are merely transient sites of biosynthesis and translocation of outer membrane constituents.

Fig. 1.2: Tentative structure of E. coli K12 LPS (data from Lugtenberg & van Alphen, 1983)

O antigen chains for other E. coli strains would be linked to the core via glc*

EA	- ethanolamine
Gal	- galactose
Glc	- glucose
GlcN	- glucosamine
GlcNAc	- N-acetyl-D-glucosamine
Hep	- L-glycero-D-mannoheptose
KDO	- 2-keto-3-deoxy-octulosonic acid
P	- phosphate


LipidA
Core
EA
P
KDO
Hep
Hep
C14
C14
C12
C14
C14
C14
GlcN
GlcN
KDO
KDO
Hep
Hep
Glc
Glc
Glc*
GlcNAc
Gal
P
P
EA

I.6 Separation of inner and outer membranes

Two methods are in general use for the separation of inner and outer membrane proteins for analysis. The first method, that of Osborn *et al* (1972), involves the production of spheroplasts by EDTA-lysozyme treatment, followed by osmotic or sonic lysis. The membranes are recovered from the lysate by ultracentrifugation and are separated into inner and outer membrane fractions on a sucrose equilibrium density gradient. The second method utilises the differential solubility of the proteins of the two membranes in non ionic detergents such as sarkosyl NL-97 (Filip *et al*, 1973). On resuspending the total envelope pellet in a solution of sarkosyl, the inner membrane proteins are solubilised, whilst the outer membrane proteins remain in a sarkosyl insoluble complex which can be pelleted by ultracentrifugation. This separation probably reflects a difference in structural organisation of the two membranes (eg. interactions of outer membrane proteins with LPS), and may not always be a true representation of the physical distribution of the proteins; improperly assembled proteins in the outer membrane may remain sarkosyl soluble. However, abnormal proteins often cannot be correctly localised by the use of Osborn gradients alone, and several techniques must be combined to determine the localisation of such proteins. Although outer membrane protein profiles produced by the two methods are largely identical, sarkosyl treatment does remove several minor proteins from the outer membrane as defined by Osborn gradients (Chopra & Shales, 1980).

Sarkosyl separation was used throughout the work

described here for the following reasons: (i) the sarkosyl separation is relatively rapid and allows simultaneous processing of large numbers of samples, and (ii) attempts to produce spheroplasts from labelled maxicells (in vivo plasmid expression system) failed; this was probably due to heterogeneity in cell size following irradiation.

II. OUTER MEMBRANE PROTEINS

The outer membrane proteins of E. coli serve two major functions: firstly the maintenance of the integrity of the outer membrane, and, secondly, nutrient uptake. The bulk of the outer membrane protein is composed of a few protein species expressed at high levels, with 10 - 20 minor proteins also present (see Table 1.1). Some of these minor proteins may, under certain conditions, be expressed at high levels (see below). The outer membrane proteins can be divided into three classes: lipoprotein, OmpA protein and the pore forming proteins.

II.1 Lipoprotein

Brauns lipoprotein (Braun & Rehn, 1969) is the most abundant of the outer membrane proteins, representing approximately 5% of the total cellular protein. The lipoprotein exists in two forms in the outer membrane: roughly 1/3 of the molecules are covalently bound to the peptidoglycan via their C-terminal lysine residues, whilst the remainder exist in a free form. The N-terminal cysteine residue of both forms is substituted with a diglyceride moiety and an amide linked fatty acid. Lipoprotein appears to function in stabilisation of the outer membrane. Mutants lacking lipoprotein have an increased sensitivity to EDTA, and an increased release of periplasmic proteins and outer membrane vesicles into the medium.

Table 1.1: Outer Membrane Proteins of E.coli

	Other names	M.W.	Function	Receptor for:
MAJOR PROTEINS				
OmpF	Ia, b, O-9 matrix protein	37,205	general pore for nutrients with M.W. 600	Phages TuIa, T2, K20, TP1, TP2, TP5,colicin A
OmpC	Ib, c, O-8	36,000	general pore for nutrients with M.W. 600	Phages TuIb, Mel, PA-2, T4, 434, SS1, TP2, TP5, TP6
OmpA	II*, 3a, d O-10, TolG	35,159	stabilisation of cell envelope	Phages TuII*,K3, colicin L, receptor in conjugation
Lipoprotein	IV	7,200	anchors OM to peptidoglycan	
INDUCIBLE MINOR PROTEINS				
LamB		47,392	maltose and maltodextrin uptake. Induced by maltose and maltodextrins	Phages λ, K10, TP1, TP5 SS1
PhoE	Ic, e	36,782	inorganic and organic phosphate uptake. Induced by phosphate limitation	Phages TC23, TC45
UNINDUCIBLE MINOR PROTEIN				
TonA	FhuA	78,000	ferric hydroxamate uptake	Phages T1, T5,ϕ80, Colicin M, Albomycin

II.2 OmpA protein

Together with Brauns lipoprotein, OmpA appears to be involved in the maintenance of the structural integrity of the outer membrane as well as in cell shape determination. A mutant lacking both OmpA and the lipoprotein requires high concentrations of Mg^{2+} or Ca^{2+} for growth and produces spherical cells in which the outer membrane is almost completely detached from the peptidoglycan (Sonntag et al, 1978). OmpA appears also to function in the stabilisation of mating pairs during conjugation.

II.3 Pore forming proteins

Due to their ability to remain bound to the peptidoglycan-lipoprotein complex even following incubation at 60°C in 2% SDS, the pore forming proteins have often been described as the "peptidoglycan associated proteins". However, this description is purely operational, and there is no evidence that these proteins are directly bound to the peptidoglycan.

The major pore forming proteins of E. coli K12, OmpF and OmpC, are immunologically related both to each other and to the inducible PhoE protein (see below). These two proteins form general diffusion pores for nutrients of molecular weights up to approximately 600. The biologically active form of the OmpF/C proteins appears to be a trimer, within which each monomer forms a channel, opening cooperatively with the other monomers of the trimer.

Other, minor pore forming proteins of the outer membrane have increased specificity for particular nutrients, for

example LamB, PhoE and TonA, which allow efficient uptake of maltodextrins, phosphate (both organic and inorganic) and Fe^{3+}-ferrichrome respectively. Some of the minor pore proteins may become major outer membrane proteins under certain conditions, for example LamB is induced by growth on maltose as the sole carbon source, and PhoE by phosphate limitation.

II.3.1 Iron transport systems:(reviewed in Konisky, 1979). Ferric iron has a very low solubility, and in order to obtain sufficient levels of this trace metal from the environment, bacteria have evolved systems which retrieve iron from insoluble polynuclear complexes, allowing absorption by the cells. Such iron uptake systems utilise low molecular weight iron chelators (termed siderophores or siderochromes), which are secreted into the medium and taken up again in complex with Fe^{3+}. Uptake of the siderophore-Fe^{3+} complex is mediated by specific outer membrane proteins. Each species of siderophore is recognised by a specific outer membrane receptor, for example the siderophores enterochelin and ferrichrome are recognised by the FepA and TonA proteins respectively. Several outer membrane proteins (Cir, FepA, TonA (FhuA), FecA and the 83kD protein) are involved in iron uptake. Although the other iron uptake proteins have been shown to be induced under conditions of Fe^{3+} limitation, TonA protein does not appear to be inducible by iron stress (Plastow et al, 1981).

II.4 LPS-outer membrane protein interaction

LPS appears to be important for the function of OmpA, OmpF and OmpC in phage receptor activity, promotion of conjugation (OmpA) and pore formation (OmpF and OmpC). Thus LPS added to purified OmpA, OmpF or OmpC (which have no *in vitro* activity) was found to restore the functions of these proteins (Datta *et al*, 1977; Schweizer & Henning, 1977). The lipid A moiety of LPS seems to be responsible for this effect since it is as efficient in reactivating purified OmpA protein as LPS itself (Schweizer *et al*, 1978). In addition, LPS appears to be important in the assembly of outer membrane proteins (see section IV.1).

II.5 Regulation of outer membrane protein synthesis

It has been observed that there is an overall limitation on the total amount of protein in the outer membrane. Boyd & Holland (1979) observed that the quantity of OmpF per unit surface area remained constant while the surface area:volume ratio varied widely with growth rate. Several groups have observed that when the expression of one of the major outer membrane proteins is increased by raising the gene copy number, or prevented by mutation, the levels of the other major proteins are decreased or increased to compensate for the alteration (Schnaitman, 1974; Movva *et al*, 1978; Henning *et al*, 1979). In addition, induction of the iron uptake proteins by iron stress results in a corresponding decrease in the expression of the OmpF protein whilst the total outer membrane protein remains constant (Boyd & Holland, 1979).

Since no "excess" outer membrane protein appears in other cell fractions under any of the above conditions, it is obvious that regulation is occurring, either at the level of synthesis, or via rapid degradation of superfluous protein. The limitation of outer membrane protein expression may depend on export site availability, or may be the result of a physical limit to the amount of protein which can be assembled into the outer membrane.

III.5.1 Regulation by OmpR and EnvZ

The relative levels of the OmpF and OmpC proteins are regulated by the action of two genes, envZ (tpo, perA) and ompR, which together form the OmpB locus. Hall & Silhavy (1981b) have proposed that the OmpR protein exists in a state of equilibrium between monomeric and multimeric forms, with the monomer switching on OmpF synthesis and the multimer switching on OmpC synthesis. The equilibrium is postulated to be controlled by the envZ product in response to osmotic changes in the medium. By this mechanism the total OmpF plus OmpC remains constant, but the relative levels of the two proteins fluctuate according to environmental conditions. Regulation by OmpR has been shown to act at the level of transcription in the following way (Hall & Silhavy, 1981b). The lacZ (β-galactosidase) gene was brought under the transcriptional control of ompF (or ompC) in an operon fusion, and under the translational control of ompF (or ompC) in a gene fusion. With both the operon and gene fusions, expression of β-galactosidase was found to mirror the level of expression of OmpF (or OmpC).

EnvZ protein also affects the expression of the maltose operons (Wandersman et al, 1980) and several periplasmic proteins (Wanner et al, 1979). Thus EnvZ may be part of a regulatory circuit controlling expression of many extracytoplasmic proteins. Hall & Silhavy (1981b) have proposed that the envZ gene product is essential for growth in E. coli, since repeated attempts to isolate insertion mutations in the gene have failed.

II.5.2 Post-transcriptional regulation of outer membrane protein synthesis

A mutation at the tolC locus (Morona & Reeves, 1982) prevents OmpF expression and has a similar effect on NmpC (a novel outer membrane protein first observed in strains lacking both OmpF and OmpC) and protein 2 (a phage PA-2 encoded protein). The tolC mutation has no effect on LamB or PhoE expression. From studies using an ompF-lacZ operon fusion, Morona & Reeves demonstrated that the block in OmpF expression occurs at a post-transcriptional level. This block is not absolute however, since OmpF expression still occurs in some media. Similarly, the coliphage PA-2 prevents expression of OmpF and OmpC, but has no effect on expression of β-galactosidase from ompF/C-lacZ operon fusions, thus the expression block appears to operate at a post-transcriptional level. In this case the host OmpF/C proteins are replaced by phage directed protein 2 (Hall & Silhavy, 1981a). Post-transcriptional regulation of outer membrane protein synthesis may occur, at least in part, via coupling of translation with translocation (see section III.6.3)

III. THE MECHANISM OF PROTEIN LOCALISATION

III.1 Introduction

As described in section I, there are four distinct compartments within the E. coli cell to which proteins may be localised: the cytoplasm, the inner membrane, the periplasm and the outer membrane; in addition some proteins appear to be secreted into the medium. Thus the cell must possess some mechanism by which newly synthesised proteins are directed to their correct destination. Eucaryotic cells face similar, but even more complex problems, since proteins must be localised to the cytosol, the endoplasmic reticulum, membranes and compartments within organelles (mitochondria, chloroplasts, lysosomes), the plasma membrane, or, finally, some proteins must be secreted. Since these proteins which must cross and/or integrate into membranes are often not strikingly different in total amino acid composition from cytoplasmic proteins, the question arises as to how polypeptides containing hydrophilic residues, and perhaps even whole hydrophilic domains, can enter into or pass through the hydrophobic barrier presented by the lipid bilayer.

III.1.1 Membrane bound ribosomes

Palade (1955, 1975) observed that eucaryotic cells possess both free and membrane bound ribosomes, with the proportion of bound ribosomes corresponding to the secretory activity of the cell. These ribosomes are bound to the outer surface of the endoplasmic reticulum (ER) via their 60s subunits (Sabatini et al, 1966), forming regions of "rough" ER (RER). Further investigation of membrane bound ribosomes revealed

functional differences between free and bound polysomes. Results from several laboratories suggested that membrane bound ribosomes were engaged in the synthesis of secreted proteins and proteins of the ER whereas free ribosomes synthesised cytoplasmic proteins (Siekevitz & Palade, 1960; Redman, 1968, 1969; Ganoza & Williams, 1969; Omura & Kuriyama, 1971).

With the finding of two functional classes of ribosome, the possibility of structural differences between the two classes was also examined. Several groups, using different eucaryotic sources of ribosomes, observed an extra protein in bound ribosomes that was not present in free ribosomes, and also a protein found in free but not bound ribosomes (Fridlender & Wettstein, 1970; Borgese et al, 1973; McConkey & Hauber, 1975). The additional proteins were found to be associated with the 60s subunit. Borgese et al (1973) reported that although the 40s subunits of membrane bound rat liver ribosomes exchange with the 40s subunits of free ribosomes in vitro, the 60s subunits remained membrane bound. A possible role for the additional protein from free 60s subunits was suggested by the finding of Blobel (1976) that a protease sensitive factor ("dissociation factor") from extracts of free ribosomes led to release of ribosomes from microsomal (vesicular ER) membranes in vitro. Thus it appeared that a system existed for in vivo exchange of ribosomes between the free and membrane bound forms, and that there were no fundamental differences between the two classes, merely associations with additional proteins

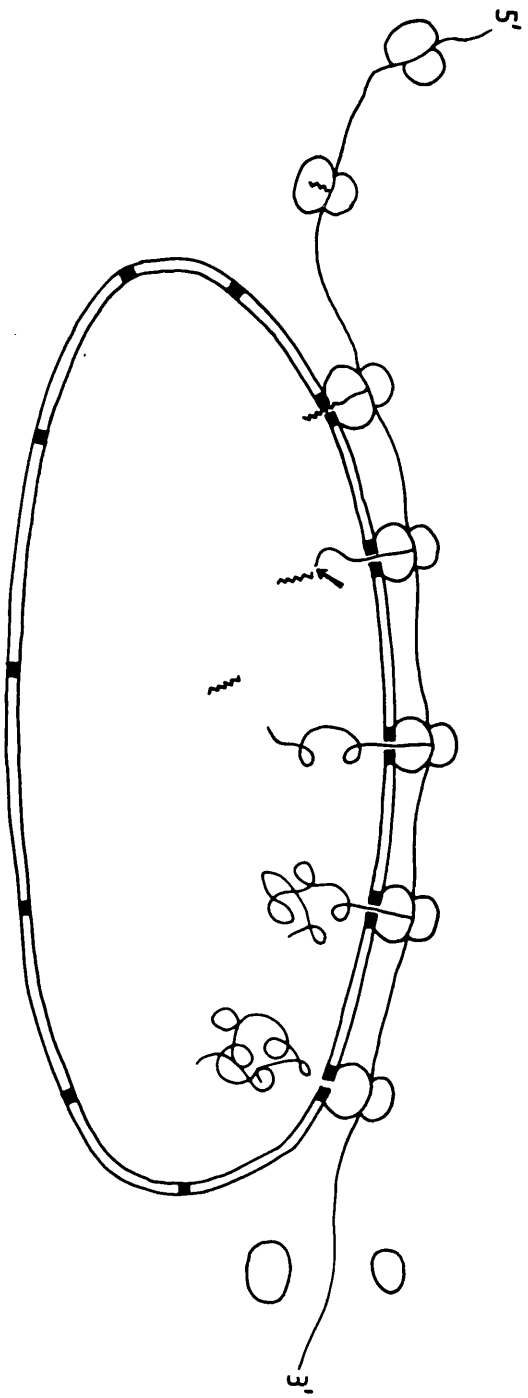
depending upon the current functional state of the ribosome. The extra proteins associated with 60s subunits of bound ribosomes might be explained by a ribosome binding protein being removed from the membrane together with the ribosome under the conditions used to release membrane bound ribosomes.

III.1.2 The Signal Hypothesis

Blobel & Sabatini (1971) proposed that either the mRNA or nascent polypeptide sequences for secreted proteins encode a signal which directs ribosomes to the ER. In fact, using *in vitro* translation systems, such polypeptide signals were subsequently demonstrated to be present at the N-terminus of several secreted proteins (Milstein *et al*, 1972; Blobel & Dobberstein, 1975). Moreover, addition of microsomal membranes to the *in vitro* system during translation was shown to promote cleavage of the "signal sequence" to form the mature protein (Cowan *et al*, 1973; Blobel & Dobberstein, 1975). On the basis of these studies, Blobel & Dobberstein (1975) presented a more detailed model for secretion, termed the Signal Hypothesis (Fig. 1.3). According to this hypothesis the mRNA encoding a secreted protein binds to free ribosomes in the cytoplasm where translation is initiated. Upon the emergence of the signal sequence from the ribosome, this polypeptide inserts into the membrane, where it interacts with receptor proteins resulting in the formation of a transmembrane channel. Vectorial transfer (Redman & Sabatini, 1966) of the nascent polypeptide then occurs through this channel, and the signal sequence, no longer

Fig. 1.3: The Signal Hypothesis (redrawn from Blobel & Dobberstein, 1975)

The signal sequence is indicated by a zig-zag line; following translation initiation in the cytoplasm, this sequence interacts with receptors in the (ER) membrane forming a proteinaceous pore through which the nascent protein is extruded cotranslationally. Processing of the signal sequence occurs during translocation.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required, can be proteolytically removed by a "signal peptidase". For integral membrane proteins, Blobel (1978) proposed that translocation is initiated in the same way, but is halted and the transmembrane channel dissociated, upon insertion of a "stop transfer" ("dissociation") sequence into the membrane channel.

III.1.3 Evolutionary conservation of the mechanism of protein localisation

Work on the mechanism of protein localisation has been carried out using many different organisms, both procaryotic and eucaryotic. From these studies it has emerged that the mechanism of protein localisation has been highly conserved during evolution. Thus signal peptides have been identified for many extracytoplasmic proteins from widely differing organisms, and these signal sequences are remarkably similar in overall structure (see section III.2.1). *In vitro* systems using components from heterologous sources are competent in protein localisation, for example, immunoglobulin synthesised from murine myeloma mRNA in a wheatgerm cell free system can be correctly processed by and sequestered into dog pancreas microsomes (Dobberstein & Blobel, 1977). In addition, ovalbumin (Fraser & Bruce, 1978) and preproinsulin (Talmadge *et al*, 1980a,b) genes cloned and expressed in *E. coli* give rise to periplasmic ovalbumin and proinsulin respectively. Yeast cells will synthesise and process bacterial pre-β-lactamase to form the mature, enzymatically active form (Roggenkamp *et al*, 1981). Thus protein translocation across the eucaryotic RER appears to occur by a similar process to

protein translocation across the E. coli inner membrane. Therefore, in the following sections, results obtained using both procaryotic and eucaryotic systems will be discussed in order to obtain an overall picture of the mechanism of protein localisation.

III.2 The signal sequence and processing

III.2.1 Signal sequence structure

Many signal peptides, both procaryotic and eucaryotic have now been identified and sequenced, and although varying considerably in length (15 - 30 amino acids) and in amino acid sequence, all share the following structural features (Austen, 1979; Inouye & Halegoua, 1980):

(i) The amino terminal section of the signal peptide is usually basic in character;

(ii) Regions of 11 - 25 residues in the central portion of the signal sequence are free of charge, with a highly hydrophobic core of 10 - 14 residues;

(iii) proline or glycine residues (usually 1 or 2) are present in the uncharged region;

(iv) the residue at the carboxyl side of the signal peptidase cleavage site is one with a short side chain, ie., gly, ala, ser or cys (Fig. 1.4).

Based on these common features of signal sequences, Inouye et al (1977) proposed a modified version of the Signal Hypothesis, the Loop Model, which emphasises the role of the polar residues in the initial membrane interaction. According to this model the basic N-terminal region of the signal

sequence remains bound to the cytoplasmic face of the membrane, which is negatively charged due to the presence of phosphoglycerides. The hydrophobic part of the signal peptide then inserts into the bilayer as a loop; the presence of the α-helix breakers proline and glycine would aid such loop formation. When the loop is exposed on the external face of the membrane, processing occurs and protein translocation continues in a vectorial fashion, leaving the signal peptide in the membrane (Fig. 1.4). This model predicts that a cleaved signal peptide should remain embedded in the membrane, as opposed to Blobel's original Signal Hypothesis, in which the signal sequence was entirely extruded across the membrane.

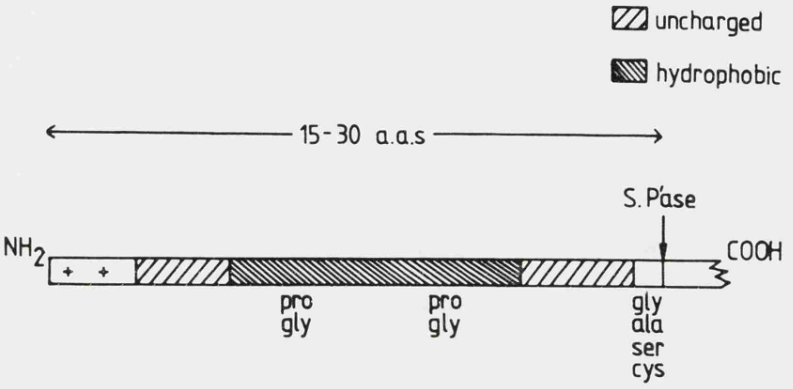
The importance of the basic residues at the start of the signal sequence was demonstrated by Inouye *et al* (1982), who, by site specific mutagenesis, isolated mutations in the hydrophilic region of the *E. coli* lipoprotein signal sequence, such that the wild type +2 overall charge was altered to +1, 0 or -1. These mutations reduced the quantity of lipoprotein appearing in the envelope to 70%, 40% and 7% respectively of the wild type amount. Furthermore, a mutation in the hydrophilic portion of the *E. coli* LamB signal sequence, altering the charge from +1 to 0, has a similar drastic effect on LamB expression (Hall *et al*, 1983). This mutant, *lam*B701-708, will be discussed further in section III.6.3.

Predictions of conformation for signal peptides reveal highly ordered secondary structures consisting of mainly

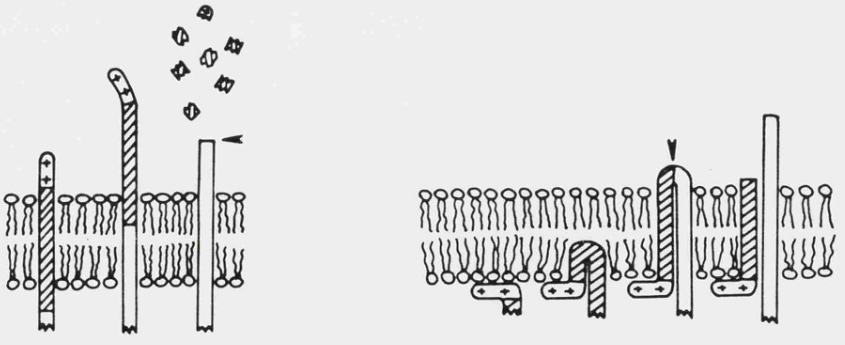
Fig. 1.4: Signal sequence structure, and the Linear and Loop Models for the signal sequence - membrane interaction

Top: Generalised signal sequence structure - a positively charged N-terminal section followed by an uncharged region with a highly hydrophobic core usually containing 1 or 2 α-helix breaking residues (pro & gly). At the carboxyl side of the signal peptidase (S. P'ase) cleavage site is an amino acid with a short side chain (gly, ala, ser or cys). The length of the signal sequence varies between 15 and 30 amino acids.

Bottom: Linear Model and Loop Model for the translocation of proteins across membranes (redrawn from Halegoua & Inouye, 1979b). Arrows indicate signal peptidase cleavage.



signal sequence structure



linear model

loop model

α-helix or β-sheet (Austen, 1979). A synthetic preproparathyroid hormone signal peptide (23 amino acid signal plus 7 amino acids of proPTH) analysed on the basis of circular dichroism spectra revealed a relatively high degree of secondary structure for such a short polypeptide (Rosenblatt et al, 1980). In aqueous buffer the analysis indicated 27% α-helix, 43% β-sheet and 30% random coil, and in a non polar solvent 46% α-helix and 54% random coil. In addition, revertants of an E. coli lamB mutant in which the central hydrophobic portion of the signal sequence is unable to assume a stable α-helical conformation were found to possess amino acid substitutions which may allow new areas of α-helicity to form (Emr & Silhavy, 1983). Thus aspects of the secondary structure rather than the amino acid sequence per se appear to be important for signal sequence function.

III.2.2 Protein localisation independently of signal sequences

A signal sequence is not an absolute requirement for an extracytoplasmic protein to reach its destination. For several integral membrane proteins of both eucaryotes and procaryotes it has been demonstrated that (i) there is no higher molecular weight precursor, and (ii) the N-terminal sequence does not resemble a signal sequence. Such proteins include the E. coli lactose permease (Ehring et al, 1980), the E. coli ATPase F_o c subunit (Nielsen et al, 1981) and bovine retinal opsin (Schechter et al, 1979). It is not known whether such proteins assemble into the membrane co- or post-translationally; it is possible that membrane integration in

these cases is a spontaneous event dependent on the overall hydrophobic character of the protein (see section III.4.3).

Some E. coli plasmids appear to encode independent transport functions which allow secretion of a particular plasmid encoded protein, for example α-haemolysin, which requires the action of at least two other plasmid encoded gene products in order to be secreted (Wagner et al, 1983). The N-terminal amino acid sequence for α-haemolysin as predicted from the DNA sequence does not resemble a signal peptide (M. Hartlein, pers. comm.), and thus the plasmid encoded transport system appears to operate via a different mechanism from the host signal sequence mediated system.

Another E. coli plasmid encoded protein, the F plasmid TraJ protein, localised in the outer membrane, has neither a cleaved signal peptide nor any sequence resembling a signal sequence at its N-terminus (Fowler et al, 1983). It is possible that F also encodes a specific protein export system, or that sequences elsewhere in the TraJ protein carry the information for assembly.

III.2.3 MalE-LacZ and LamB-LacZ hybrids

In order to observe the effect of signal sequences fused to the N-terminus of a normally cytoplasmic protein, Beckwith and his coworkers constructed hybrids between the malE or lamB (both maltose inducible) and lacZ genes of E. coli. Thus strains were isolated which synthesised hybrid proteins consisting of a nearly complete, enzymatically active β-galactosidase moiety fused to varying lengths of sequence from the N-terminus of the MalE (periplasmic) or LamB (outer

membrane) protein. Both MalE and LamB possess precursor forms with typical signal sequences, and thus the effect of these signals on localisation of β-galactosidase could be investigated.

(i) MalE-LacZ fusions: Three classes of MalE-LacZ hybrids were isolated, incorporating different lengths of the MalE N-terminus (Fig. 1.5) (Bassford et al, 1979; Silhavy et al, 1979). The first class of fusion was found to be cytoplasmically located, and probably did not carry an intact signal sequence. For the remaining four fusions tested, a significant quantity of the β-galactosidase activity separated with the envelope fraction. Of these envelope associated hybrids, the majority separated with the inner membrane on sucrose gradients. Class III fusions (72-47 and 179-3) caused the host strain to become extremely maltose sensitive, ie., induction of hybrid protein synthesis by growth on maltose was lethal. In addition, upon hybrid protein induction, several new proteins were observed, some of which have been identified as precursors of outer membrane and periplasmic proteins (Ito et al, 1981; Herrero et al, 1982). It was concluded that, although export of the hybrid protein was initiated by the action of the signal sequence, attempts to translocate the normally cytoplasmically located β-galactosidase moiety resulted in disruption of processing and/or translocation of many periplasmic and outer membrane proteins.

(ii) LamB-LacZ fusions: Again three classes of hybrids were produced (Fig. 1.6) (Silhavy et al, 1979): Class I fusions

Fusion class	Strain	Hybrid protein localisation (%) CY	PM	IM	OM	Approx. amount of malE DNA in hybrid gene
I	PB4-1	88	1	-	-	2/13
II	PB41-4	64	1	32	5	3/13
	PB62-32	32	1	65	5	4/13
III	PB72-47	24	1	73	5	8/13
	PB179-3	39	1	60	5	12/13

Figure 1.5: Properties of MalE-LacZ hybrid proteins. Localisation data based on quantification of β-galactosidase activity in each fraction. The malE gene has been divided into 13 units by deletion mapping; the values listed in the last column represent the number of segments present in the hybrid gene. (Silhavy et al, 1979).

Fusion class	Strain	Hybrid protein localisation (%) CY	PM	IM	OM	Approx. amount of lamB DNA in hybrid gene
I	61-4	90	<1	<10	<1	1/9
	52-4	85	<1	<15	<1	1/9
II	42-1	43	2	≃25	≃30	5/9
III	42-18	<10	-	<10	>80	6/9
	42-12	<10	-	<10	>80	6/9

Figure 1.6: Properties of LamB-LacZ hybrid proteins. Localisation data based on quantification of β-galactosidase activity in each fraction. The lamB gene has been divided into 9 units by deletion mapping; the values listed in the last column represent the number of segments present in the hybrid gene. (Silhavy et al, 1979).

contained very little LamB sequence, therefore probably not an intact signal sequence, and were cytoplasmically located; Class II fusions gave a maltose sensitive phenotype to the host similar to that caused by MalE-LacZ hybrids 72-47 and 179-3; Class III fusions, containing the most LamB sequence, were maltose resistant, and the hybrids were localised to the outer membrane by three different techniques: (a) class III fusion strains possess β-galactosidase activity outside the cytoplasmic membrane barrier since they can grow on lactose even in the absence of a lactose transport system; (b) on sucrose gradients the hybrids fractionate with the outer membrane, and (c) using immunofluorescence, β-galactosidase cross reacting material could be detected at the cell surface (Silhavy et al, 1979).

From these studies it was concluded that the signal sequence initiates translocation, but is not sufficient to ensure correct localisation. Thus E. coli is not able to translocate the β-galactosidase sequence across a membrane (except in one case - see section IV.1.2(iv) for further discussion of this fusion class) despite the fact that, in all cases where an intact signal sequence was present, translocation appeared to be initiated normally. Perhaps it is not surprising that the signal sequence itself does not contain the t al information for localisation. The overall similarity between signal sequences suggests that they may all function identically in initial binding to the membrane, and that regions of the mature protein sequence lead to correct choice of ultimate location. In the case of the

hybrids, it is possible that sequences within the ß-galactosidase moiety actually block the translocation process.

III.2.4 The signal peptidase

An E. coli signal peptidase (or "leader peptidase") responsible for processing of the M13 precoat protein has been purified (Zwizinski & Wickner, 1980) and a clone carrying the signal peptidase gene (lep) has been isolated (Date & Wickner, 1981). This signal peptidase has been reported to be equally distributed between the inner and outer membranes as isolated from Osborn gradients; such a dual localisation is unique among E. coli membrane proteins (Mandel & Wickner, 1979; Zwizinski et al, 1981). The signal peptidase which fractionates with the outer membrane may not be a true outer membrane protein; signal peptidase present in regions of the inner membrane where translocation of outer membrane proteins is taking place may fractionate with the outer membrane.

Sequencing of the signal peptidase gene revealed no signal sequence for this protein (Wickner, pers. comm.). This sequencing data also indicates that the signal peptidase gene is the second gene in an operon; the first gene remains unidentified. The signal peptidase activity is not specific to M13 coat protein; the same enzyme will also cleave the precursors of two E. coli periplasmic binding proteins and the outer membrane protein LamB (Zwizinski et al, 1981). Moreover, the signal peptidase appears to be essential for normal growth of E. coli, indicating an important role for

this enzyme in processing the envelope proteins (Silver & Wickner, 1983).

Signal peptidase activity has also been extracted from rough (but not smooth) ER (Jackson & Blobel, 1977), and a signal peptidase from rat liver microsomes has been partially purified (Mollay et al, 1982).

Intact signal peptides have been observed in both eucaryotic and procaryotic in vitro systems following processing, indicating that signal peptidases are endoproteases (Chang et al, 1978; Zwizinski & Wickner, 1980; Mollay et al, 1982; Hussain et al, 1982). As would be predicted from Inouye's Loop Model (Fig. 1.4), the cleaved signal peptide remains membrane bound, at least in the case of prelipoprotein (Hussain et al, 1982). Activities leading to degradation of the signal peptide have been observed in vitro. Zwizinski & Wickner (1980) reported a soluble activity, "leader peptide hydrolase", which degraded the signal peptide of M13 coat protein in E. coli. In contrast, Hussain et al (1982) observed a membrane bound "signal peptide peptidase", which degraded the cleaved signal peptide of the E. coli lipoprotein. These two activities may be an identical enzyme fractionated under different conditions, or may represent two distinct signal peptide degrading activities. Since no degradation of the signal peptide occurs when processing of prelipoprotein is inhibited by globomycin, which has no effect on signal peptide peptidase activity, the signal peptide peptidase can only degrade the signal peptide following cleavage by signal peptidase.

Substrate recognition by signal peptidase appears to involve residues on both sides of the cleavage site since an amino acid substitution at position 14 of the lipoprotein signal sequence (20 residues) almost completely prevents processing, while still allowing some translocation (Lin et al, 1978), and a mutation at residue 2 of mature M13 coat protein drastically decreases the rate of processing (Russel & Model, 1981). The signal peptidase does not rely on distance of the cleavage site from the N-terminus, since an E. coli signal peptidase will correctly cleave preproinsulin fused to 4, 12 or 25 amino acids from the N-terminus of pre-β-lactamase during translocation of the protein to the periplasm (Talmadge et al, 1980b). Preliminary analysis of an internal LamB deletion (amino acids 70 - 184) suggests that this deletion results in a loss of processing of the signal sequence (Benson & Silhavy, 1983). Thus the overall conformation of the preprotein may affect processing.

Eucaryotic signal peptidase(s) will correctly cleave procaryotic signal sequences and vice versa (see section III.1.3) and an antiserum raised against Wickner's E. coli signal peptidase cross reacts with a yeast protein (Wickner, pers. comm.).

III.2.5 Processing

By analysis of the N-termini of populations of nascent chains in exponentially growing E. coli, Joseffson & Randall (1981) demonstrated that processing occurs both co- and post-translationally. Some proteins may be processed entirely cotranslationally (eg, AmpC β-lactamase) and others entirely

post-translationally (eg, TEM ß-lactamase), but the majority of proteins analysed appeared to be processed in both modes (LamB, OmpA, alkaline phosphatase, MalE and arabinose binding protein). The timing of processing event appears to bear no relation to the ultimate location of each protein as can be seen with the two periplasmic ß-lactamases described above. In all cases studied by Josefsson & Randall, processing did not occur before the nascent chain had reached 80% of its final length; the need for attainment of this critical length may be due to (i) the requirement for correct folding of the N-terminus for recognition by the signal peptidase, or (ii) the inaccessibility of the nascent chain to the signal peptidase before the critical length has been reached.

There are several extracytoplasmic proteins which are not synthesised as higher molecular weight precursor forms, but their N-termini often possess many of the characteristics of signal sequences described in section III.2.1. Such proteins include cytochrome P450 of the ER (Bar-Nun *et al*, 1980) and the *E. coli* inner membrane ATPase F_o b subunit (Nielsen *et al*, 1981). Although ovalbumin, a eucaryotic secreted protein, is not synthesised as a higher molecular weight precursor, residues 25 - 45 of the protein bear some resemblance to a signal peptide. In addition, association of ovalbumin with microsomes in an *in vitro* translation system occurs when the nascent chains are only 50 - 60 residues in length (Meek *et al*, 1982). These association kinetics are comparable with those of secreted and membrane proteins which possess cleaved signal sequences. Thus, processing of signal

peptides may not normally be essential for export of some proteins which have therefore never evolved a signal peptidase cleavage site or have lost it.

For proteins whose signal sequences are normally cleaved during assembly, processing may be required in many cases in order to achieve a functionally active protein conformation. Although preMalE has a maltose binding activity almost equivalent to the mature form (Ferenci & Randall, 1979), prealkaline phosphatase has a much reduced activity compared to the mature protein (Inouye & Beckwith, 1977). Conformational changes are known to take place following processing since the mature form of TEM β-lactamase is completely resistant to pronase or trypsin in whole cell lysates, whereas the precursor is completely degraded under the same conditions (Koshland & Botstein, 1982). In the case of the E. coli outer membrane lipoprotein, a mutation of the signal sequence, mlpA, which prevents processing also prevents modification at the N-terminal cysteine and binding to the peptidoglycan via the C-terminal lysine (Lin et al, 1980). Nevertheless, some translocation still appears to occur since 50% of this mutant prelipoprotein fractionates with the outer membrane on Osborn gradients.

III.3 Translocation

III.3.1 The mechanism of translocation

The signal hypothesis as proposed by Blobel & Dobberstein (1975) postulated the existence of specific integral membrane proteins which act to form a transmembrane pore through which

the nascent protein may pass. The lack of evidence for such channel forming proteins led to the formulation of models in which the nascent polypeptide is extruded directly through the bilayer (Engelman & Steitz, 1981; Von Heijne & Blomberg, 1979). Von Heijne & Blomberg proposed a "Direct Transfer Model", suggesting that following the initial insertion of the nascent signal sequence into the membrane the ribosome also becomes membrane bound. Thus the free energy of the ribosome membrane interaction can be harnessed to transfer the polypeptide chain through the membrane as it is being synthesised. Transfer will then continue provided that the energy required to insert each new residue into the membrane does not exceed the free energy of ribosome binding; if this energy limit is exceeded then the ribosome is released from the membrane, translocation stops and the polypeptide remains inserted in the membrane. Using these free energy considerations the correct topological orientation of a number of eucaryotic and procaryotic extracytoplasmic proteins can be predicted from the amino acid sequence (Von Heijne, 1980). In support of this model, two ribosome binding proteins, termed ribophorins, have been isolated from RER (Kreibich et al, 1978).

III.3.2 Stop transfer sequences

In translocation models based on the Signal Hypothesis the existence of a "stop transfer" ("stop translocation") sequence must be proposed in order to account for proteins which integrate into the membrane rather than crossing it completely (Blobel, 1978, 1980; Von Heijne, 1980). Such a

sequence might be visualised either as an extremely hydrophobic region which "sticks" in the membrane, or as a hydrophilic sequence which fails to insert into the membrane thus preventing further translocation. Clearly, a combination of both types of sequence might also act as an effective stop transfer sequence.

In the case of immunoglobulins, which occur in both secreted and membrane bound forms, it has been demonstrated that the bound form is produced by splicing an extra RNA sequence onto the immunoglobulin mRNA. The peptide sequence (M sequence) encoded by this extra RNA consists of a highly acidic stretch of 12 amino acids, of which 6 are glutamic acid, followed by 25 hydrophobic amino acids forming the membrane spanning region, and finally a cytoplasmic tail of 3 amino acids of which 2 are lysine (Rogers et al, 1980). Since the highly acidic part of the polypeptide chain actually appears to cross the membrane, it would seem that a hydrophilic sequence alone does not necessarily constitute a stop transfer sequence. Yost et al (1983) have shown that a hybrid protein consisting of β-lactamase as its N-terminus fused to globin behaves as a secretory protein. Thus, when analysed in an in vitro system, the protein was sequestered into added dog pancreas microsomes in a protease resistant form. When the above M sequence was inserted at the β-lactamase - globin junction, the hybrid protein became a transmembrane protein, with the β-lactamase moiety sequestered in the microsomes, and the globin moiety exposed to proteolytic digestion at the surface (Yost et al, 1983).

Thus the M sequence alone is able to function as a stop transfer sequence.

Influenza haemagglutinin is also anchored to the plasma membrane via its C-terminus, where there is a stretch of 24 hydrophobic amino acids, of which several are basic. Deletion of the 40 C-terminal amino acids results in complete secretion of the truncated protein (Gething & Sambrook, 1982; Sveda et al, 1982).

Similarly, bacteriophage f1 gene III protein, an E. coli inner membrane protein, appears to possess a stop transfer sequence since deletion of the C-terminal half of the protein results in a periplasmically located, truncated form of the protein (Boeke & Model, 1982). Like the haemagglutinin, the C-terminus of gene III protein contains a highly hydrophobic sequence.

The C-terminal 1/5 of the E. coli inner membrane protein penicillin binding protein 5 (PBP5) also appears to contain a stop transfer signal, since deletion of this region leads to a periplasmically located form of PBP5 (J. M. Pratt, pers. comm.). Interestingly, the C-terminus of PBP5 is not strikingly hydrophobic in nature. Thus the stop transfer signal operating in PBP5 may be different from those of immunoglobulin, haemagglutinin and the f1 gene III protein.

III.3.3 Cotranslational translocation

In the models discussed above, translocation of polypeptides across the membrane has been assumed to be cotranslational. Indeed, the majority of evidence accumulated to date points to a cotranslational mode of translocation for most groups of

extracytoplasmic proteins. The finding that, in both eucaryotes and procaryotes, membrane bound ribosomes synthesise extracytoplasmic proteins is, in itself, evidence that translocation is cotranslational. Rat liver membrane bound ribosomes have been shown to synthesise serum proteins, whereas the free ribosomes synthesise non-serum proteins (Ganoza & Williams, 1969; Redman, 1968, 1969). In E. coli, membrane bound ribosomes were found to be engaged in the synthesis of periplasmic (MalE, β-lactamase and arabinose binding protein) and outer membrane (LamB) proteins, whereas the free ribosome fraction were synthesising EFTU (Randall & Hardy, 1977, Randall et al, 1978; Baty et al, 1981).

The work of Smith et al (1977) demonstrated that the polypeptides synthesised by membrane bound ribosomes were actually crossing the membrane cotranslationally: the peptidyl tRNAs of membrane bound ribosomes were labelled in E. coli spheroplasts by an agent unable to penetrate the inner membrane, indicating that part of the nascent chains had crossed the membrane before synthesis was complete.

In vitro also, nascent eucaryotic and procaryotic extracytoplasmic proteins synthesised in cell free systems do not usually become correctly associated with added membrane vesicles unless these membranes are present during translation (Blobel & Dobberstein, 1975; Lingappa et al, 1978; Chang et al, 1979; Smith, 1980; Meek et al, 1982; J. M. Pratt, pers. comm.). Furthermore, there is now evidence that, for extracytoplasmic proteins in vivo, synthesis is tightly coupled to translocation such that translation is

halted if the signal sequence does not become membrane associated (see section III.6). However post-translational translocation appears to be characteristic of a distinct group of extracytoplasmic proteins, the mitochondrial and chloroplast proteins encoded by nuclear genes. In addition there is evidence to suggest that the M13 coat protein is assembled post-translationally in E. coli.

III.4 Post-translational assembly

III.4.1 Chloroplast and mitochondrial proteins

Both chloroplasts and mitochondria possess their own genetic information and protein synthesising machinery, which synthesise polypeptides destined for the matrix, the organelle membranes and, in mitochondria, the intermembrane space. The mechanism of localisation for these polypeptides appears to be similar to that occurring in bacteria and to that taking place across the RER. Thus, membrane bound ribosomes have been observed within chloroplasts and mitochondria (Ellis, 1979; Kuriyama & Luck, 1973); some of the non-matrix proteins are synthesised as higher molecular weight precursors (Sevarino & Poyton, 1980; Ellis & Barraclough, 1978); and both translocation and processing appear to be cotranslational (Sevarino & Poyton, 1980).

However, the majority of the proteins of chloroplasts and mitochondria are encoded by nuclear DNA (Schatz, 1979; Grossman et al, 1980). Many of these cytoplasmically synthesised proteins are initially synthesised as precursors with N-terminal extensions (Neupert & Schatz, 1981). The

proteins are assembled directly into the organelle, ie., there is no RER intermediate, and, in the case of mitochondria, receptor proteins on the organelle surface are required for protein import (R. Zimmerman, pers. comm.).

Some evidence has been obtained to suggest that, in cycloheximide treated cells, a subcellular fraction consisting of mitochondria-bound cytoplasmic ribosomes specifically engaged in mitochondrial protein synthesis can be isolated (Ades & Butow, 1980; Suissa & Schatz, 1982). However, since cycloheximide decreases the overall rate of protein synthesis, it is possible that the consequent increased availability of receptor sites on the mitochondrial surface allows lower affinity binding of polypeptides whose synthesis is not yet complete (Suissa & Schatz, 1982). When nuclear DNA encoded mitochondrial and chloroplast proteins are synthesised in vitro, they assemble efficiently and correctly into these organelles when added after translation is complete (Highfield & Ellis, 1978; Schatz, 1979; Chua & Schmidt, 1978; Matsuura et al, 1981; Gasser et al, 1982). Thus, although not completely ruled out by these studies, cotranslational membrane association and translocation does not appear to be obligatory for imported mitochondrial and chloroplast proteins, a situation significantly different from the apparent absolute requirement for cotranslational translocation across the RER.

The N-terminal extensions of cytoplasmically synthesised mitochondrial proteins vary considerably in size, and are frequently much longer than signal sequences (Neupert &

Schatz, 1981). For example, the precursor form of the ATPase proteolipid subunit of Neurospora crassa possesses a 66 amino acid N-terminal extension; moreover this sequence is primarily composed of basic and hydrophilic residues and in this characteristic also does not resemble a signal sequence (Viebrock et al, 1982). Consequently, for imported mitochondrial and chloroplast proteins, the N-terminal extension may not be acting in the same way as a signal sequence, and the mechanism of uptake of proteins into these organelles may be quite distinct from that generally occurring across bacterial and RER membranes.

III.4.2 Post-translational translocation in E. coli

Coliphage M13 coat protein has been extensively studied and appears to be an example of post-translational translocation in E. coli. An in vivo cytoplasmic intermediate in assembly has been demonstrated (Ito et al, 1979). Also, in vitro, the coat protein has been shown to be correctly assembled into and processed by membrane vesicles added post-translationally (Goodman et al, 1981), although the majority of precoat protein synthesised in vitro loses its ability to be incorporated into membranes within a few minutes of synthesis.

However, M13 coat protein is unusual in a number of ways: it is a very small protein, and consequently translation is probably complete before the signal sequence has emerged from the ribosome and is able to interact with membranes. In addition, although M13 coat protein resides transiently in the inner membrane, its ultimate location is in the non-

membraneous viral coat, and coat protein may therefore be structurally different from other proteins in order to achieve these two distinct localisations (Michaelis & Beckwith, 1982).

Analysing a quite different protein, Zimmerman & Wickner (1983) have reported that in vitro synthesised preOmpA can be post-translationally processed by liposomes reconstituted with purified signal peptidase. However, the OmpA processed in this way is not bound to the liposomes and thus in this system processing is not associated with translocation. Therefore this result cannot be taken as evidence for a post-translational mode of translocation for OmpA. Indeed, since recent results (to be discussed in section III.6.3) suggest that translation is tightly coupled to translocation for two other outer membrane proteins, LamB and lipoprotein, and the periplasmic protein MalE, a post-translational mode of assembly for OmpA does not seem likely.

Nevertheless, Wickner (1979) proposed a mechanism for post-translational translocation: the Membrane Trigger Hypothesis.

III.4.3 The Membrane Trigger Hypothesis

The Membrane Trigger Hypothesis (Wickner, 1979) proposes that membrane integration depends only on the structure of the protein to be integrated. A membrane (pre)protein existing in a soluble form in the cytoplasm is postulated to change to a more stable conformation on encountering the hydrophobic membrane environment, and will thus become membrane integrated; cleavage of the N-terminal extension alters the

folding pathway to render this integration irreversible. This hypothesis fits the M13 data, and may also be used as a model for post-translational uptake of proteins into chloroplasts and mitochondria. Cotranslational integration is not excluded by the Membrane Trigger Hypothesis, since the N-terminal region may undergo stable membrane integration while the C-terminus is still being synthesised.

Although the Membrane Trigger Hypothesis accounts for membrane integration, it is more difficult to envisage secretion by this mechanism except as a result of an inefficient diffusion process across the membrane. The Signal Hypothesis (Blobel, 1978; Inouye et al, 1977) is able to explain both secretion and stable membrane integration (by invoking stop transfer sequences). Nevertheless, membrane triggered folding may be involved in co- or post-translational integration for proteins which do not possess signal sequences and become integrated into the first bilayer they encounter (eg, the inner membrane proteins of E. coli and organelle synthesised inner membrane proteins of mitochondria). Finally, it is also possible that, following inital interaction of a nascent signal sequence with the membrane, subsequent translocation proceeds by transfer of completed domains of the polypeptide rather than by vectorial transfer (Randall, 1983).

III.5 Energy requirements for protein export

It has been demonstrated that energy is required for export and/or processing of many E. coli inner membrane, periplasmic and outer membrane proteins, since precursors of these proteins accumulate in the presence of uncouplers such as CCCP, DNP, valinomycin and ethanol (Date et al, 1980a; Enequist et al, 1981; Palva et al, 1981; Daniels et al, 1981; J. M. Pratt, pers. comm.). From studies using mutants in which the membrane potential can be dissipated without substantially altering cellular levels of ATP, Enequist et al (1981) demonstrated that membrane potential rather than ATP is required for protein export. Date et al (1980a) showed that the uncoupler CCCP had no direct effect on signal peptidase activity since in vitro processing of M13 precoat by the purified enzyme was not inhibited, although DNP did affect signal peptidase activity. The M13 precoat accumulated in the presence of CCCP was demonstrated to be trypsin resistant in cells in which the outer membrane had been rendered trypsin permeable (Date et al, 1980b). In this case it was concluded that translocation of coat protein across the inner membrane was blocked when the membrane potential was dissipated. Processing inhibition following dissipation of membrane potential may therefore be a result of the inability of the preprotein to achieve the correct conformation and/or localisation for signal peptidase activity.

III.6 The apparatus for protein localisation

III.6.1 Signal recognition particle and docking protein

Dog pancreas microsomes lose their ability to sequester newly synthesised secretory proteins following a high salt wash; upon readdition of the salt extract, this function is restored (Warren & Dobberstein, 1978). The component of the salt extract required for microsomal activity has been purified as an 11s ribonucleoprotein complex termed signal recognition particle (SRP), containing 6 polypeptides (Walter & Blobel, 1980) and a 7s RNA (Walter & Blobel, 1982). Surprisingly, the SRP was found to inhibit the *in vitro* translation of secretory (Walter *et al*, 1981) and transmembrane (Anderson *et al*, 1982) proteins until microsomes were made available to the system. The SRP was observed to bind to polysomes synthesising secretory proteins when the nascent polypeptide chains were approximately 70 residues long. At this point in translation about 30 amino acids should be exposed beyond the ribosome surface, and it has been suggested therefore that SRP interacts with both the signal sequence and the ribosome to prevent further translation until the appropriate membrane is encountered (Walter & Blobel, 1981).

Using the same *in vitro* system, a microsomal membrane component, "docking protein", was also identified, which relieved the SRP induced translation block (Meyer *et al*, 1982). Thus a model has been proposed (Meyer *et al*, 1982) in which SRP recognises and binds to the signal sequence as it emerges from the ribosome; translation is halted until the

SRP-ribosome complex interacts with the docking protein in the RER, when translation is resumed and translocation begins. Gilmore & Blobel (1983) have obtained evidence to suggest that, following release of the translation block, neither SRP nor the docking protein remain associated with the translation-translocation complex. This result indicates that SRP and docking protein may not be involved in translocation, only acting in the initial event which couples translation to translocation.

Anderson *et al* (1983) have shown that SRP is required for the integration of two proteins without cleaved signal sequences, rabbit sarcoplasmic reticulum calcium ATPase and bovine lens MP26 protein, into microsomal membranes *in vitro*. This result suggests that these two proteins possess SRP binding sequences, which may in fact be the equivalent of uncleaved signal sequences.

III.6.2 Export apparatus of E. coli

In contrast to the mammalian studies which rely on the use of *in vitro* systems, several genetic loci have been identified in *E. coli* which appear to be involved in protein localisation. The best characterised of these loci are *sec*A, *prl*A and *ts*215 (*sec*Y), which may be an allele of *prl*A.

*prl*A mutants (Emr *et al*, 1981; Emr & Bassford, 1982; Shultz *et al*, 1982) were originally selected as able to correctly localise a LamB protein with a 12 amino acid deletion (residues 10 - 21) in the signal sequence. These *prl*A mutants, although possessing no apparent growth defects, will suppress all known signal sequence mutations of MalE and

PhoE as well as those of LamB (except lamB701-708); see section III.6.3). However, the efficiency of suppression can vary from less than 5% for a 12 residue deletion in the LamB signal to nearly 100% for a point mutation. The degree of suppression also varies between different prlA alleles. The prlA locus maps to the promotor distal end of the spc operon, which encodes ribosomal proteins, although prlA itself does not encode any known ribosomal protein.

Two further loci, prlB and prlC, were identified by the same selection as prlA, ie., by their suppression of a lamB signal sequence mutation, although prlB and prlC are not as efficient in suppression as prlA (Emr et al, 1981, Emr & Bassford, 1982). However, only one prlB mutant has been isolated, the mutation suppresses only lamB signal sequence mutations, does not restore processing and has been found to map in the gene encoding the periplasmic ribose binding protein (Emr & Bassford, 1982; S. Benson, pers. comm.). Thus the significance of prlB is unclear; suppression of the lamB signal sequence mutations probably occurs via a specific bypass mechanism rather than through an alteration in the general export machinery of the cell. Two alleles of prlC have been isolated (Emr et al, 1981; S. Benson, pers. comm.); these mutations suppress signal sequence mutations of malE as well as of lamB. Processing of mutant signals does occur, but apparently at an abnormal site, so again, export may be occurring via an unusual route (Emr & Bassford, 1982).

A suppressor of malE signal sequence mutations, prlD, restores both processing and export, but has no effect on

lamB signal sequence mutations and does not suppress all malE signal sequence mutations (S. Benson, pers. comm.). A prlA, prlD double mutant exhibits a generalised export defective phenotype, accumulating precursors of several envelope proteins, suggesting important, interactive roles for the products of these genes.

The ts215 (secY) mutation maps to the same region of the E. coli chromosome as prlA, and leads to very slow processing of the MalE, OmpA and OmpF precursors, together with decreased levels of the ribosomal L15 protein at the non-permissive temperature (Ito, 1982). However, the mutation is not in the gene encoding L15, and is not a mutation of the lep gene (encoding Wickner's signal peptidase) (Ito et al, 1983).

secA mutants (Oliver & Beckwith, 1981) are also conditional lethals, selected for their inability to localise a MalE-LacZ hybrid to the inner membrane (ie., selected for maltose resistance). At the non permissive temperature, precursors of several periplasmic and outer membrane proteins accumulate in the cytoplasm, and these accumulated precursors do not chase into the mature form even though nascent preproteins are still being exported and processed at low levels. SecA appears to be a peripheral inner membrane protein although at low ionic strength it becomes partly soluble (Oliver & Beckwith, 1982). Interestingly, this protein undergoes a tenfold increase in synthesis when protein export is blocked by induction of the MalE-LacZ hybrid 72-47 (Oliver & Beckwith, 1982), indicating that

levels of SecA (and perhaps other components of the export machinery) can be adjusted to meet the export requirements of the cell.

Antiserum raised against SecA protein cross reacts with a Bacillus subtilis membrane protein associated with membrane bound polysomes (Oliver & Beckwith, 1982). In addition, the SecA antiserum immunoprecipitates an 11s ribonucleoprotein (RNP) complex from E. coli; this RNP contains a 6s RNA identical to that described by Brownlee (1971), which possesses regions of homology with the 7s RNA of the eucaryotic SRP (J. Beckwith, pers. comm.). Thus E. coli may possess an SRP equivalent, one component of which is the SecA protein. It is tempting to speculate that the PrlA protein may also be a component of the "E. coli SRP", perhaps involved directly in signal sequence recognition.

Mutations at the secB locus, selected for their inability to efficiently localise a MalE-LacZ hybrid to the inner membrane, are non-lethal and cause some defective export; a secA, secB double mutant possesses greater export defects than can be explained by the purely additive effects of the two mutations (Kumamoto & Beckwith, 1983).

Finally, genetic loci have been identified which may be involved in export, or, like the ompB and tolC loci (section II.5), merely in regulation of groups of exported proteins: expA (Dassa & Boquet, 1981) and cpxA and cpxB (McEwen et al, 1983).

The identification of several loci apparently involved in protein localisation in E. coli argues for a complex export

apparatus comparable to the SRP-docking protein system observed in eucaryotic *in vitro* systems (section III.6.1). The opportunity for mutational analysis and consequently *in vivo* studies of the functions of individual proteins perhaps makes *E. coli* a better system for a dissection of the export apparatus than the eucaryotic *in vitro* system.

III.6.3 Coupling of translation and translocation in E. coli

Since the SRP-docking protein apparatus presumably functions in the coupling of translation and translocation in eucaryotes, it might be reasonable to expect that a similar coupling occurs in procaryotes, since they appear to possess an SRP equivalent. There is some evidence for such a coupling of translation with translocation in *E. coli*: Firstly, when supression of a *secA*^{am} mutant is eliminated, less MalE protein is synthesised, suggesting that there is an *E. coli* activity which halts translation in the absence of export (Oliver & Beckwith, 1982). Secondly, a mutation of the hydrophilic amino acids of the LamB signal sequence (*lamB* 701-708) has been isolated, which drastically decreases the level of synthesis of wild type LamB protein, but when recombined into a cytoplasmically located LamB-LacZ fusion (ie., one containing very little LamB sequence), has no effect (Hall *et al*, 1983). Therefore the defect in *lamB*701-708 is not in transcription or translation initiation. Hall *et al* proposed the existence of a "stop translation" sequence early in the LamB protein, probably overlapping (or identical to) the signal sequence. This stop translation sequence must be partly absent in the LamB-LacZ hybrid. For wild type

LamB translation is presumably resumed when translocation is initiated, but the lamB701-708 mutation prevents translocation initiation and thus translation is never resumed. Finally, mutants of the hydrophilic region of the lipoprotein signal sequence lead to a decrease in synthesis by as much as 55% (Inouye et al, 1982). Thus again, inability to export a protein results in a fall in the level of synthesis.

Such a coupling of translation with translocation may explain the observation that, despite alterations in gene dosage or induction of new proteins, the quantity of protein in the E. coli outer membrane remains fairly constant, while no "excess" outer membrane proteins appear in other cell fractions. Although some control of outer membrane protein synthesis occurs at the transcriptional level, there is also evidence for control pathways operating at the level of translation (see section II.5). The postulated SRP-blocked outer membrane protein translation complexes may compete for assembly sites, possibly an equivalent of the eucaryotic docking protein. Other factors may be involved in the choice of which mRNAs to translate, and which nascent proteins in SRP-blocked translation complexes actually initiate translocation.

IV.ASSEMBLY OF E. coli OUTER MEMBRANE PROTEINS

IV.1 Introduction

The assembly of outer membrane proteins poses an additional problem for the E. coli cell. These proteins must either be translocated through the inner membrane without integrating into it, despite their ability to integrate into the outer membrane, or, alternatively, outer membrane proteins may be assembled via specialised sites which may not involve transmembrane passage.

An interesting possibility is that all outer membrane components (phospholipid, LPS and protein) are assembled at specialised sites in the inner membrane from where transfer to the outer membrane may occur via vesicles or regions of continuity between the two membranes. Phospholipid and LPS biosynthesis both occur in the inner membrane and therefore both these molecules must be translocated to the outer membrane (Bell et al, 1971; White et al, 1971; Osborn et al, 1972). Pages et al (1982) reported that LamB, OmpF and OmpC synthesis were inhibited when lipid synthesis was blocked, but OmpA and lipoprotein were unaffected. Heptose deficient LPS mutants of E. coli possess decreased amounts of OmpF (40% of the wild type level), OmpC (66% WT) and OmpA (64% WT), suggesting that LPS plays a role in assembly of outer membrane proteins (van Alphen et al, 1976). In addition, the level of OmpA expression has been shown to be correlated with its affinity for LPS (Beher et al, 1980). Thus it appears that outer membrane protein assembly is dependent to some extent on LPS and phospholipid, although it is uncertain

whether nascent outer membrane proteins interact with nascent LPS and phospholipid or with LPS and phospholipid already assembled into the outer membrane.

Some evidence exists that LPS may be transferred to the outer membrane via the Bayer junctions (section I.5) (Muhlradt et al, 1973) in Salmonella typhimurium, a Gram negative bacterium closely related to E. coli. The major outer membrane proteins of S. typhimurium have been reported to be preferentially inserted into the outer membrane through such zones of adhesion, randomly distributed over the cell surface (Smit & Nikaido, 1978). For E. coli, Vos-Scheperkeuter (1983) and N. Nanninga (pers. comm.) have used immune electron microscopy to obtain evidence that newly synthesised LamB and OmpF proteins are similarly inserted into the outer membrane at random sites. However, this result contradicts previous evidence by Ryter et al (1975) that newly synthesised LamB is inserted preferentially at the poles of the cell, this time using phage lambda binding to locate the LamB molecules on the cell surface. It is possible that λ binds more efficiently to receptor molecules at the poles of the cell, perhaps due to localised differences in outer membrane structure, whereas it might be expected that antibody binding would be a more accurate indication of the presence of LamB. Thus it appears that the major outer membrane proteins of S. typhimurium and LamB and OmpF in E. coli are inserted at random sites over the outer membrane surface.

IV.2 Inhibition of outer membrane protein assembly

Lipoprotein processing and assembly can be specifically inhibited by the action of the antibiotic globomycin. When cells are treated with globomycin, prelipoprotein accumulates in the inner membrane, which can be chased into mature lipoprotein in the outer membrane upon removal of the antibiotic (Hussain et al, 1980). This specific inhibition of lipoprotein processing and assembly is indicative of an assembly mechanism differing, at least in part, from that of other outer membrane proteins. Indeed, a second E. coli signal peptidase has been identified which is responsible for prelipoprotein processing (Yamagata et al, 1983).

General outer membrane protein assembly can be blocked in several ways. Firstly, as described in section III.5, energy is required in the form of a proton motive force across the inner membrane at some stage of translocation. Consequently, when this membrane potential is dissipated, precursors of outer membrane proteins accumulate. This energy requirement is shared in common with periplasmic proteins; in addition LPS assembly to the outer membrane requires both a membrane potential and ATP (Mulford & Osborn, 1983).

Secondly, toluene (Sekizawa et al, 1977; Inouye et al, 1977) or phenethyl alcohol (PEA) (Halegoua & Inouye, 1979; Pugsley et al, 1980) treatment of E. coli leads to the accumulation of precursors of lipoprotein, OmpA and OmpF. Interestingly, PEA, which drastically decreases membrane fluidity, has a differential effect upon the major outer membrane proteins of E. coli. OmpF processing is inhibited by

as little as 0.3% PEA, whereas inhibition of OmpA processing requires 0.5% PEA, at which concentration lipoprotein processing is still unaffected (Halegoua & Inouye, 1979). At 0.3% PEA, the accumulated preOmpF is membrane bound; on sucrose gradients this precursor fractionates with the outer membrane, but differs from normally assembled outer membrane proteins in being sarkosyl soluble. The preOmpF is trypsin sensitive in cells in which the outer membrane has been rendered permeable to the protease; OmpF is normally protease resistant under these conditions (Halegoua & Inouye, 1979). Following treatment with 0.3% PEA, lipoprotein and OmpA processing is not inhibited but these proteins can be released from cells by osmotic shock or by spheroplasting, ie., they fractionate with the periplasm. Thus PEA treatment appears to block assembly at a stage subsequent to translocation of the majority of the protein across the membrane. The effects of treatment with 0.3% PEA are reversible; the partly assembled outer membrane proteins can be fully assembled upon removal of the PEA, indicating that ongoing protein synthesis is not required for assembly (Halegoua & Inouye, 1979).

Outer membrane protein synthesis and/or assembly can also be blocked by decreasing membrane fluidity using fatty acid auxotrophs (Ito et al, 1977; DiRienzo & Inouye, 1979). Again, decreasing lipid fluidity has a differential effect on the major outer membrane proteins (DiRienzo & Inouye, 1979): OmpF synthesis is completely inhibited and preOmpA accumulates, whereas lipoprotein synthesis and assembly remain unaffected.

The accumulated preOmpA fractionates partly with the inner membrane in a sarkosyl soluble form and is more sensitive to trypsin than normally assembled OmpA when the outer membrane is permeabilised. Thus again the preOmpA is able to translocate, at least in part, across the inner membrane, but is unable to complete assembly into the outer membrane.

Finally, outer membrane protein processing and/or assembly is inhibited when certain hybrid proteins are induced, for example MalE-LacZ hybrid 72-47 (Ito et al, 1981; Herrero et al, 1982). Processing of periplasmic proteins is also inhibited, suggesting that some steps in assembly are common between outer membrane and periplasmic proteins (Ito et al, 1981).

Differential inhibition of outer membrane protein synthesis and assembly has been observed following several different treatments as described above. This may result from differences in assembly pathways, different affinities for other outer membrane components such as LPS, differences in ease of attaining structural configurations required during assembly, eg, at processing, and perhaps also differences in the sensitivities of regulatory circuits to disruption of outer membrane protein assembly. OmpF synthesis seems to be particularly sensitive to perturbations in assembly (Halegoua & Inouye, 1979; DiRienzo & Inouye, 1979; Pugsley et al, 1980). In the case of PEA, Halegoua & Inouye (1979) demonstrated inhibition of OmpF synthesis at the level of transcription.

IV.3 Kinetics of outer membrane protein assembly

Some studies of the kinetics of outer membrane protein assembly have indicated a considerable delay in synthesis and/or assembly of outer membrane proteins (Ito et al, 1977; Lin & Wu, 1980). There are two possible explanations for this delay: firstly that translation of outer membrane proteins is unusually slow, and, secondly, that there is a post-translational, rate limiting step in assembly of outer membrane proteins.

Boyd & Holland (1980) demonstrated that, in the case of OmpF, the translation time was close to that predicted and assembly of OmpF into the envelope fraction was cotranslational. However, within the envelope fraction the nascent OmpF protein appeared in a transient sarkosyl soluble pool prior to assembly into a sarkosyl insoluble complex. The transient sarkosyl soluble pool might represent either a true inner membrane intermediate or an incompletely assembled outer membrane intermediate. Complete assembly into the outer membrane probably involves the establishment of interactions with other outer membrane constituents (protein & LPS) which renders the protein sarkosyl insoluble.

Crowlesmith et al (1981) reported that preOmpA and preOmpF could be immunoprecipitated from the soluble fraction of a sonic lysate, or from the inner membrane (from sucrose gradients) of French pressed cells, again suggesting that there is a post-translational, intermediate stage before assembly into the outer membrane.

In contrast, DeLeij et al (1978) reported the detection

of nascent, incomplete polypeptides in the outer membrane fraction (from sucrose gradients) which could be chased into mature outer membrane proteins. These results indicated cotranslational assembly directly into the outer membrane.

The above sets of results are clearly contradictory as to whether post-translational assembly intermediates exist, and if so, whether they are of the mature or precursor form. With regard to experimental technique, the results of DeLeij et al (1978) and Lin & Wu (1980) were obtained from studies of bulk outer membrane protein, which is subject to greater inaccuracies than the study of individual proteins. The results described in section IV.2 demonstrate that the major outer membrane proteins are differentially affected by some treatments, and may therefore be assembled via differing pathways. Although the results of Boyd & Holland (1980) and Crowlesmith et al (1981) were based on studies of individual proteins, the assembly intermediates detected were different in one notable feature: Boyd & Holland detected a processed OmpF intermediate whereas Crowlesmith et al immuno-precipitated precursor forms of OmpA and OmpF from the inner membrane. The reults of DeLeij et al and Boyd & Holland are in agreement in so far as both indicate cotranslational assembly into the envelope, but the data of Boyd & Holland indicate an additional step prior to completion of assembly which is perhaps not detectable using sucrose gradients.

As described in section IV.2, outer membrane protein assembly can be perturbed by several treatments. In cases where the treatment can be reversed, for example with PEA and

ethanol, the blocked assembly intermediates can be correctly incorporated into the outer membrane following removal of the perturbant. These observations indicate that assembly is not constrained to a cotranslational mode, at least after the initial membrane interaction. Thus the mechanism of outer membrane protein assembly appears to be fairly flexible. Furthermore, as already discussed, Josefsson & Randall (1981) reported that outer membrane proteins were processed both co- and post-translationally in exponentially growing E. coli. It seems reasonable to suppose that the degree of cotranslational processing and also the longevity of any assembly intermediate might depend on the E. coli strain used and the growth conditions employed. If this is so, then perhaps it is not surprising that results obtained from different laboratories are not always in complete agreement.

IV.4 Requirement for processing

The requirement for processing in outer membrane protein assembly is difficult to assess. As described in section IV.2, several treatments lead to accumulation of outer membrane protein precursors, but it is difficult to determine in these cases whether a block in processing has led to translocation inhibition or vice versa, or whether both translocation and processing have been inhibited as primary effects of the treatment.

However, results with a lipoprotein signal sequence mutant, mlpA, suggest that processing is important for this protein (Lin et al, 1980). Only 4% of the lipoprotein in mlpA

strains is processed; this mature lipoprotein is of the bound form and fully modified (ie., diglyceride and fatty acids attached to the N-terminus). Of the remaining, unprocessed lipoprotein, 50% fractionates with the outer membrane on sucrose gradients. However, this prelipoprotein is of the free form and completely unmodified, indicating that removal of the signal sequence is essential for normal assembly.

In contrast to the above result, Benson & Silhavy (1983) have obtained evidence to suggest that a LamB deletion derivative lacking amino acids 70 - 186 is translocated to the outer membrane in an unprocessed form. Detection of the LamB derivative at the cell surface was achieved by immuno-fluorescence studies on whole cells using LamB antibody. However, it does remain a possibility that the protein was still anchored to the inner membrane via its signal sequence and/or not completely assembled into the outer membrane.

IV.5 Models for outer membrane protein assembly

The initial steps in assembly appear to be the same for most periplasmic and outer membrane proteins and perhaps some inner membrane proteins. As discussed in section III.2.1, signal sequences are similar in overall structure and appear to function only in initial recognition of a protein as extracytoplasmic. If this is so then the information directing proteins to the outer membrane must reside in another part of the protein molecule. There are several possibilities (Fig. 1.7):

(i) Outer membrane proteins may be translocated completely

Fig. 1.7: Models for assembly of outer membrane proteins in E. coli

(i) The protein is exported across the inner membrane to the periplasm, from where it spontaneously integrates into the outer membrane.

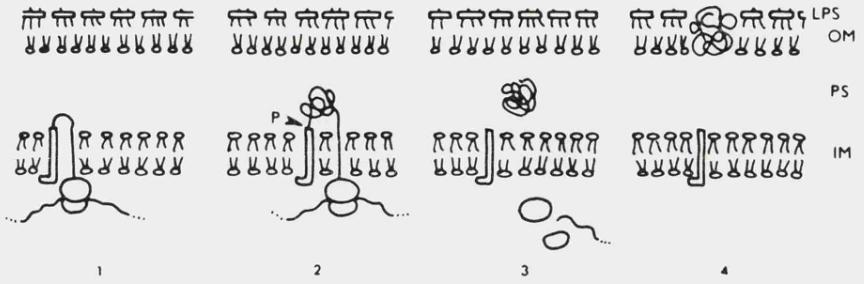
(ii) The protein is cotranslationally exported to the outer membrane, crossing both the inner membrane and periplasm.

(iii) Assembly occurs through specialised sites of fusion (Bayer Junctions) between the inner and outer membranes.

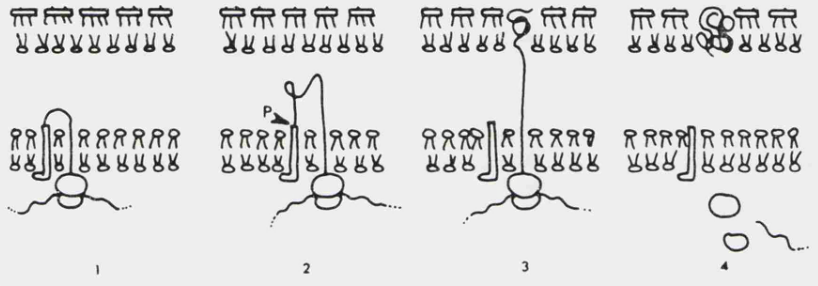
(iv) Assembly via a vesicular intermediate. The protein is translocated across the inner membrane until a stop translocation (dissociation) sequence is encountered (dark rectangle). The ribosome dissociates from the membrane and following completion of translation a vesicle buds from the inner membrane to fuse with the outer membrane. The N- and C-termini of the protein are marked; it is clear that, according to this model, protein domains (or lipid molecules) present on the cytoplasmic face of the inner membrane will eventually come to lie on the outer face of the outer membrane.

P - signal peptidase; PS - periplasmic space; IM & OM - inner and outer membranes. (i), (ii) & (iii) redrawn from Halegoua & Inouye (1979b); (iv) from Silhavy et al (1979).

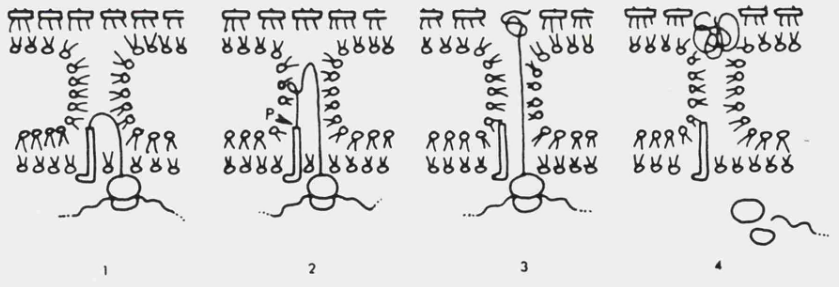
(i)



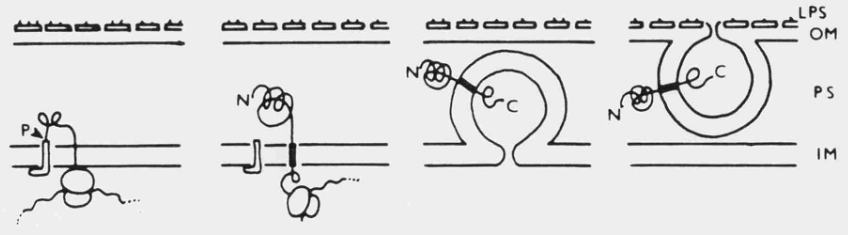
(ii)



(iii)



(iv)



across the inner membrane to the periplasm, from where they integrate spontaneously into the outer membrane. Integration into the outer rather than the inner membrane might rely on the unique structural features of the outer membrane, for example the presence of LPS, the different phospholipid content or specific protein-protein interactions. For such a mechanism, overall protein conformation would be important for integration into the outer membrane, with specific domains of the protein interacting with other outer membrane components.

(ii) Outer membrane proteins may be assembled cotranslationally directly into the outer membrane, crossing both the inner membrane and periplasm. In this case the informaton for initial outer membrane integration might be expected to lie in the mature N-terminal region of the nascent polypeptide.

(iii) Specialised sites may exist (the Bayer junctions) through which outer membrane proteins are cotranslationally assembled into the outer membrane. In this model, the signal sequence or sequences adjacent to it, must direct the translation complex to the appropriate sites. Of course, another possibility is that features of the nascent chains themselves induce formation of adhesion sites between the membranes, via which assembly occurs followed by membrane separation.

(iv) A fourth model for outer membrane protein assembly is based on the eucaryotic model for secretion via vesicles budding from the ER to fuse with the Golgi complex from where

further vesicles transport the proteins to the plasma membrane. Certain LamB-LacZ hybrids become localised to the E. coli outer membrane, with the β-galactosidase moiety at the cell surface (see section III.2.3). Since results with MalE-LacZ and smaller LamB-LacZ hybrids indicated that the attempted translocation of the β-galactosidase sequence across the inner membrane was lethal, Silhavy et al (1979) suggested a vesicular model of outer membrane protein assembly. According to this model, wild type LamB assembly is initiated in the usual way by signal sequence interaction with the export apparatus and translocation begins. However, translocation is stopped when a postulated "dissociation sequence" is encountered. The membrane integrated LamB protein then reaches the outer membrane via lateral diffusion to specialised regions of the inner membrane where vesicles are budded off to fuse with the outer membrane. It is apparent from Fig. 1.7 that the C-terminus of LamB need not physically cross a membrane by this mechanism, and thus the β-galactosidase moiety of a LamB-LacZ hybrid could reach the cell surface without translocation through a hydrophobic membrane barrier. The smaller LamB-LacZ hybrids which do not become localised to the outer membrane and result in cell death would presumably lack the proposed dissociation sequence.

Such a vesicular model of assembly might also be applied to phospholipid and LPS assembly. However, this model would predict that LPS should be initially assembled on the cytoplasmic face of the inner membrane bilayer (see Fig.

1.7), whereas Mulford & Osborn (1983) have obtained evidence for an LPS intermediate on the periplasmic face of the inner membrane in S. typhimurium.

V.AIMS OF THIS PROJECT

In order to elucidate the mechanism of assembly of outer membrane proteins it will be necessary to perform detailed studies of individual proteins, and compare the results obtained using different proteins. For this project the chosen protein was TonA (FhuA). This 78kD polypeptide is derived from an 80kD precursor (Plastow *et al*, 1981) and is normally a minor component of the outer membrane. TonA functions as a ferrichrome receptor, but unlike other outer membrane proteins involved in iron uptake, does not appear to be inducible by iron stress (Plastow *et al*, 1981). TonA also serves as a receptor for coliphages T1, T5 and 80, colicin M and the antibiotic albomycin, a ferrichrome analogue (Konisky, 1980). Thus T1 and T5 receptor activity provides a simple selection for the presence or absence of functional TonA protein. The *ton*A gene is carried by plasmid pLC19-19 from the Clarke & Carbon (1976) collection. This plasmid consists of a 12kb chromosomal fragment cloned into a colE1 derived vector. Thus by introducing this multicopy plasmid into *E. coli* strains, TonA protein synthesis is elevated such that it becomes a major outer membrane protein, an important factor for *in vivo* study. Furthermore, the availability of plasmid DNA encoding TonA allows (i) *in vitro* synthesis and assembly studies using a transcription-translation system and (ii) *in vitro* mutagenesis.

In this project I set out to investigate TonA assembly in the following ways: (i) the study of TonA assembly kinetics; (ii) the identification of the *ton*A DNA in order to carry out

subsequent *in vitro* mutagenesis; (iii) the study of the effects of C-terminal deletions on localisation; and (iv) the study of the effects of induction of the MalE-LacZ hybrid 72-47 on both TonA and the inner membrane penicillin binding proteins in an attempt to establish whether the hybrid does block specific sites of export for periplasmic and outer membrane proteins as suggested by Ito *et al* (1981).

CHAPTER TWO

SUBCLONING OF THE tonA GENE

2.1 Introduction

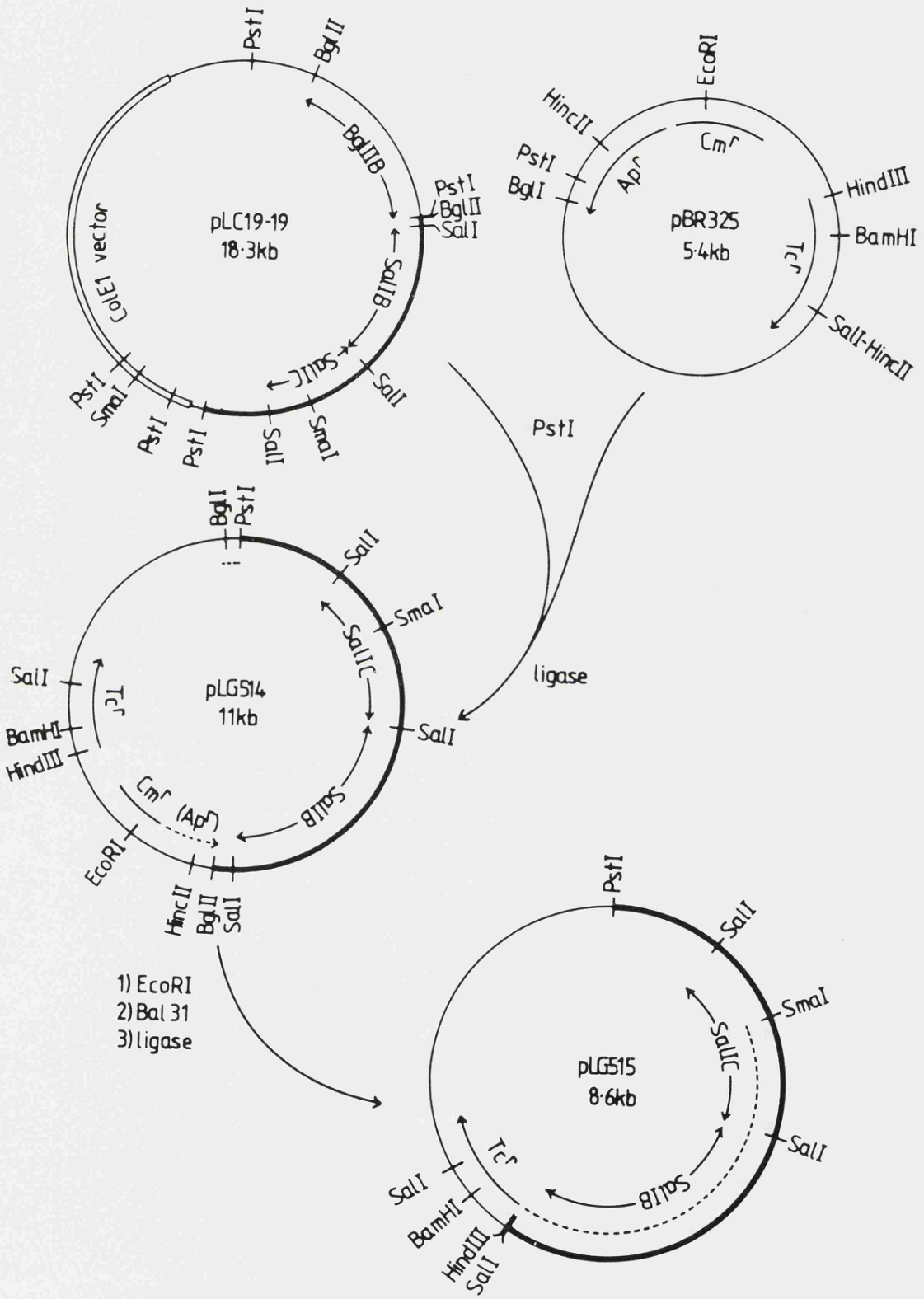
Plasmid pLC19-19 from the Clarke and Carbon (1976) collection was previously identified as carrying the E. coli tonA gene (Takeda et al, 1980). However, for the purposes of subsequent manipulation of the tonA gene this plasmid was difficult to work with for the following reasons. Firstly, the vector used in the construction of pLC19-19 was a colE1 derivative with no easily selectable markers, and therefore pLC19-19 was not easily transferable between different host strains. Secondly, the cloned DNA fragment carrying the tonA gene is 12kb in length, making pLC19-19 rather unwieldy for the analysis of the DNA encoding TonA protein and for the in vitro construction of tonA mutants. It was therefore decided to subclone a smaller fragment of pLC19-19 carrying the tonA locus.

Takeda et al (1980) constructed a restriction map of pLC19-19 (Fig. 2.1), and in addition, by deletion analysis, established that the SalIC fragment carries at least part of the tonA gene. Moreover, they showed that the BglIIB fragment could be deleted without loss of tonA. The tonA gene must therefore be located in the 6kb region between the BglII site and the colE1 vector. Thus it seemed likely that the complete tonA gene would be encoded within the 5.6kb PstI fragment. This fragment was therefore chosen for recloning into the PstI site in the Ap^r (bla) gene of pBR325 (Ap^r, Tc^r, Cm^r), a

Fig. 2.1: Construction of pLG514 and pLG515

DNA derived from the 5.6kb PstI fragment of pLC19-19 is indicated by heavy lines.

Dashed line indicates the localisation of the tonA gene within pLG515 as defined by the work described in this chapter


pLC19-19
18·3kb
ColE1 vector
PstI
BglII
BglIIB
SalI
SalIB
SalIC
SmaI
pBR325
5·4kb
EcoRI
HincII
BglI
Cmr
Apr
Tcr
HindIII
BamHI
SalI-HincII
ligase
pLG514
11kb
(Apr)
EcoRI
HincII
pLG515
8·6kb
1) EcoRI
2) Bal 31
3) ligase

high copy number vector (Fig. 2.1).

2.2 Recloning the 5.6kb PstI fragment of pLC19-19

The 5.6kb PstI fragment of pLC19-19 was purified from a preparative agarose gel, and ligated with a PstI digest of pBR325. A complete PstI digest of pLC19-19 DNA was also ligated into the same vector. Both ligation mixtures were used to transform E. coli strain Rec35-1 (recA, tonA) and transformants selected on nutrient agar containing tetracycline and chloramphenicol. Resultant colonies were screened for ampicillin sensitivity and phage T1 sensitivity. Two ampicillin sensitive, T1 sensitive clones were obtained: pLG513 from the ligation of purified 5.6kb PstI fragment, and pLG514 after ligation from the complete PstI digest of pLC19-19.

2.3 Characterisation of pLG513 and pLG514

Preliminary restriction analysis showed that pLG513 carried two 5.6kb PstI fragments of pLC19-19 (Fig. 2.2). pLG514 carried only one PstI fragment, with a deletion of one of the PstI sites expected from the construction (Fig. 2.1).

Plasmids pLC19-19, pBR325, pLG513 and pLG514 were used as templates in the Zubay in vitro transcription-translation system, and the protein products analysed by SDS PAGE. The results (Fig. 2.3) showed that both pLG513 and pLG514 coded for an 80kD protein identical in molecular weight to the pLC19-19 product previously shown to be the TonA precursor

Fig. 2.2: Restriction digests of pLG513, indicating the presence of two 5.6kb PstI fragments derived from pLC19-19

There are two SmaI and two BglII sites in pLG513; neither of these enzymes cut within pBR325, thus these sites must be derived from pLC19-19. The double SmaI x BglII digest yielded only one novel fragment size, 3.6kB, with the loss of the large BglII and large SmaI fragments (3.6kb is the expected length of the BglII-SmaI fragment in the 5.6kb PstI fragment of pLC19-19).

The restriction map of pLG513 is given opposite; clearly pLG513 was not formed by a simple vector - 5.6kb fragment ligation.

B - BamHI

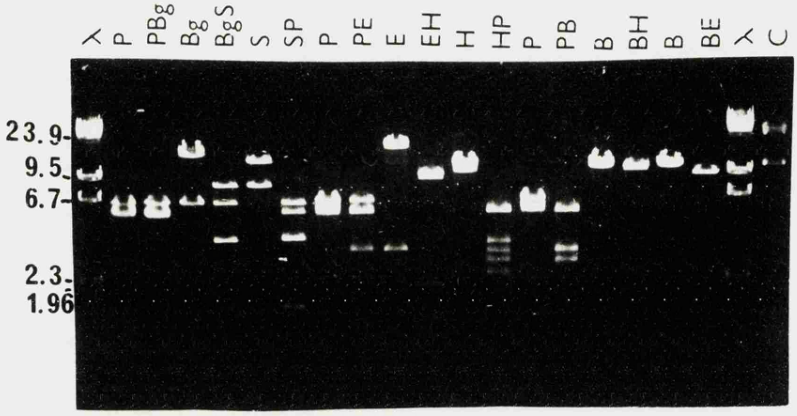
Bg - BglII

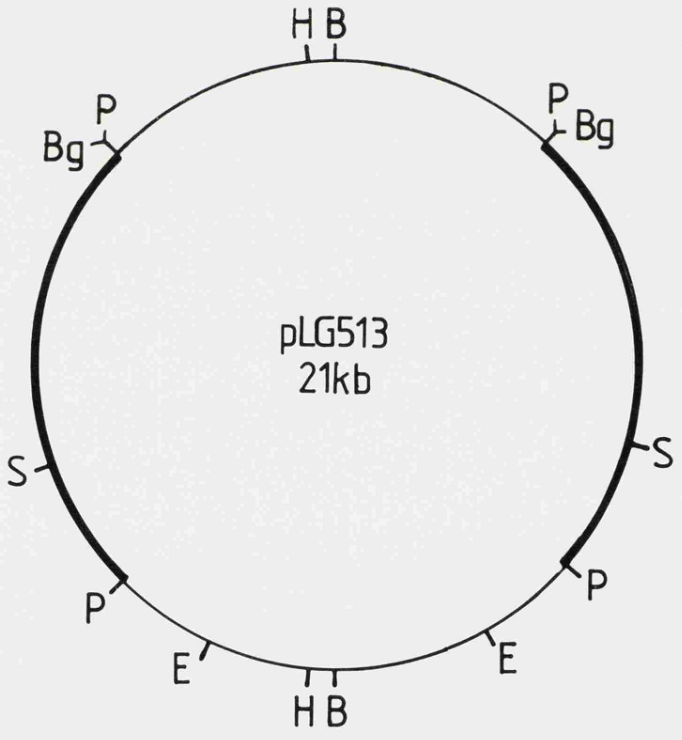
E - EcoRI

H - HindIII

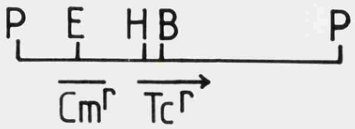
P - PstI

S - SmaI





5·6kb PstI frag.



pBR325 x PstI

Fig. 2.3: Proteins encoded by pBR325, pLC19-19, pLG513, pLG514, pLG515 and pLG516

^{35}S -methionine labelled proteins synthesised in the Zubay transcription-translation system from pBR325, pLC19-19, pLG513, pLG514 and pLG515 templates:
LE316 extract used; 2.5µg of each DNA was used and equal volumes of each reaction mix subsequently loaded on an 11% acrylamide I gel, and the gel autoradiographed.

Expression in minicells of pLG514, pLG515 and pLG516:
Equal amounts of ^{35}S -methionine labelled minicells were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

Positions of ^{14}C labelled standard proteins are marked to the left of each gel
pTonA = preTonA; β-lac = β-lactamase; CAT = chloramphenicol acetyl transferase

kD
100
92.5
69
46
30
14.3
pBR325
pLC19-19
pLG513
pLG514
pLG515
no DNA
pTonA
65k
40k
β-lac
30k
CAT
kD
100
92.5
69
46
30
pLG514
pLG515
pLG516
pTonA
TonA
65k
40k
30k
CAT

(Plastow et al, 1981). Together with the fact that pLG513 and pLG514 conferred T1 sensitivity on a tonA$^-$ host, these results indicated that both plasmids carried an intact tonA gene. The novel 65kD polypeptide encoded by pLG514 (Fig. 2.3) is probably a readthrough product from the bla promotor into the cloned fragment, since this protein was lost when the bla promotor was deleted (see section 2.6).

2.4 Recloning the SalI fragments of pLG514

By deletion analysis, Takeda et al (1980) established that the 1.67kb SalIC fragment of pLC19-19 encoded at least part of the tonA gene. Subcloning the SalI fragments of pLG514 would identify any other SalI fragments encoding parts of the tonA gene. A SalI digest of pLG514 was therefore ligated into pBR325 cut with SalI, and the ligation mixture was used to transform GI65 (tonA), selecting for chloramphenicol and ampicillin resistance. A single clone was isolated which conferred T1 sensitivity on the host strain; when analysed this clone was found to contain both the SalIB and SalIC fragments of pLG514 (equivalent to the SalIB and C fragments of pLC19-19 (Fig. 2.1)). The SalIC fragment alone recloned into pBR325 did not confer T1 sensitivity on the host. Thus the tonA gene must span the two SalI fragments.

2.5 Deletion of Cm^r from pLG514

The major protein product of pLG513 and pLG514 in both the in vitro transcription-translation system, and in minicells, is chloramphenicol acetyltransferase (CAT), the product of

the Cm^r gene. TonA protein forms a relatively minor band, often detected only with difficulty and requiring long exposures of the gels. Under the conditions of these expression systems, it seemed possible that highly efficient transcription and translation of the Cm^r gene might be engaging a large proportion of the protein synthesising capacity of the system, and therefore a larger proportion of the ^{35}S -methionine label.

In order to test this, the Cm^r gene was deleted from pLG514 in the following way (Fig. 2.1): pLG514 DNA was cut with EcoRI which possesses a single target site within the Cm^r gene of the plasmid. The linearised DNA was treated with Bal31 exonuclease, removing samples at various times during the incubation in order to produce a range of DNA deletions. The samples were pooled before recircularisation of the DNA, which was used to transform CSH26ΔF6tonA. Transformants were selected on nutrient agar containing tetracycline, and the resultant colonies screened for chloramphenicol sensitivity and T1 sensitivity.

Mini-Birnboim plasmid preparations were carried out for 11 of the Cm^s, $T1^s$ clones, the DNA was run on agarose gels, and the two clones yielding the smallest plasmids, pLG515 and pLG516, were analysed further.

2.6 Characterisation of pLG515 and pLG516

pLG516 was found to behave identically with pLG515 in all respects. The restriction map of pLG515 is shown in Fig. 2.1,

where it can be seen that a 2.4kb region of pLG514 has been deleted, including the whole of the Cm^r gene and the bla promotor. A comparison of the plasmid encoded proteins from pLG514 and pLG515 in the minicell and Zubay systems is shown in Fig. 2.3. Since equal amounts of minicells or Zubay reaction mixture were loaded in each gel slot, it is apparent that the labelling of plasmid encoded TonA protein is much more efficient when the Cm^r gene has been deleted. In addition, two protein products (∿30kD & ∿40kD) which are visible amongst the Zubay products of pLG514 but not apparent amongst the minicell products, can be seen clearly amongst the products programmed by pLG515 in both the minicell and the Zubay systems. Thus, labelling of several proteins is enhanced when the Cm^r gene is absent. The 65kD novel polypeptide encoded by pLG514 (section 2.3) is not coded by pLG515 or pLG516 (Fig. 2.3). Thus it was concluded that this protein was probably a product of readthrough from the bla promotor, which was deleted in the construction of pLG515 and pLG516. The ladder of polypeptides visible below the TonA band in Zubays and minicells of pLG515 and pLG516 are probably incomplete or aborted translation products of tonA.

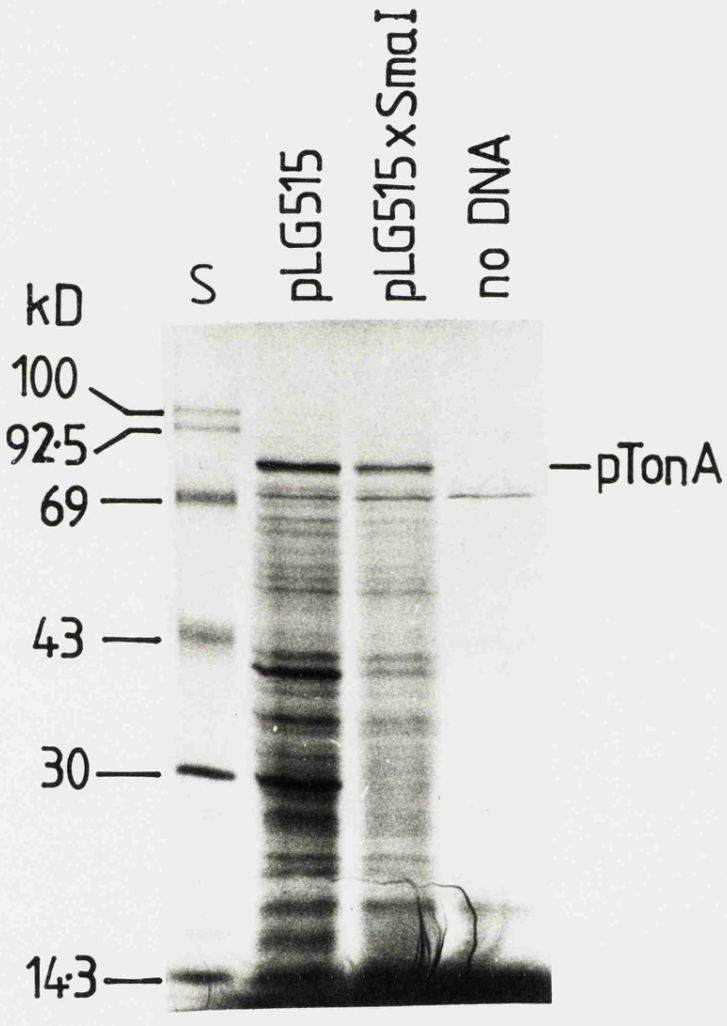
2.7 Use of linear DNA templates in the Zubay system to further localise the site of the tonA gene

Pratt et al (1981a) demonstrated that restriction endonuclease fragments of DNA could be used to programme the Zubay system and thereby assign specific polypeptide products to the DNA fragments encoding them. This technique was applied to pLG515

Fig. 2.4: Products of *in vitro* expression of pLG515 and pLG515 linearised with SmaI

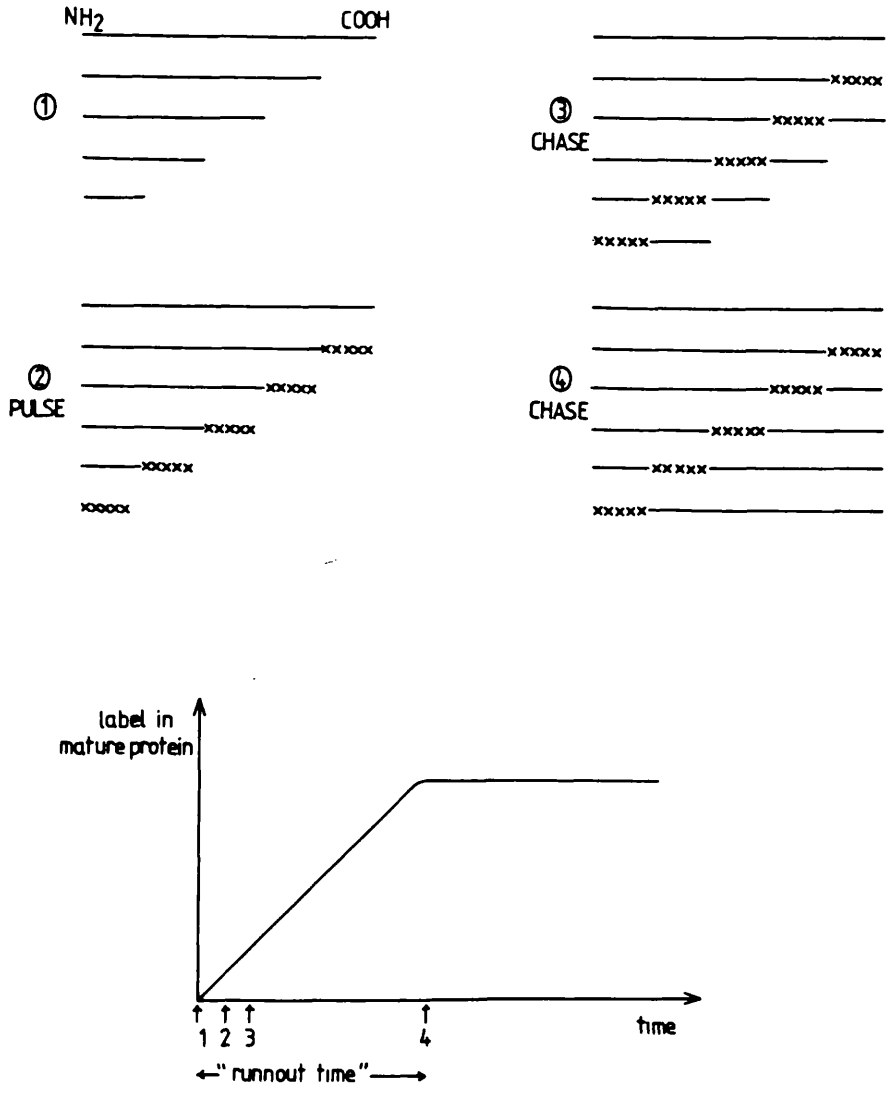
^{35}S -methionine labelled proteins synthesised in the Zubay transcription-translation system from 2.5μg of pLG515 and SmaI digested pLG515. N138 (*recB*^{ts}) extract used; equal volumes of each reaction mix loaded on an 11% acrylamide I gel.

S - ^{14}C -labelled protein standards


kD
S
pLG515
pLG515 x SmaI
no DNA
100
92·5
69
43
30
14·3
pTonA

in order to establish whether the tonA gene spans the SmaI site in the SalIC fragment (Fig. 2.1). As Fig. 2.4 shows, pLG515 linearised by restriction with SmaI is still able to programme the synthesis of the 80kD TonA precursor in the Zubay system. Thus it was deduced that the tonA gene does not span the SmaI site. This result located the tonA gene to the 3.5kb region of pLG515 indicated in Fig. 2.1.

Fig. 3.1: Theoretical basis for kinetic experiments



CHAPTER THREE

ASSEMBLY KINETICS OF TonA PROTEIN

3.1 Introduction

As discussed in Chapter 1 (section IV.3), the evidence which exists concerning the kinetics of outer membrane protein assembly is, in part, contradictory. However, some of these studies indicated the existence of post-translational assembly intermediates for outer membrane proteins. It is of course entirely possible that different outer membrane proteins may follow any one of several distinct assembly pathways, or that the same protein may be assembled via an alternative pathway under different growth conditions.

As part of the study of the assembly of TonA protein, the kinetics of assembly of TonA were investigated using the procedure of Boyd & Holland (1980). The theoretical basis of this method is outlined below.

3.2 Basis of run-out experiments

At any given time point during exponential growth there will be a series of incomplete translation products existing for each protein ranging from newly initiated chains to nearly complete chains (Fig. 3.1(1)). If the cells are exposed to a short pulse with a radioactive amino acid, label will be incorporated into all the growing chains, including some newly initiated chains (Fig. 3.1(2)). During the subsequent chase period the labelled polypeptide chains will gradually be completed (Fig. 3.1(3)). Thus it can be seen that

incorporation into the mature protein will increase. When polypeptides whose synthesis was initiated during the pulse are completed, the level of incorporation of label into mature protein will plateau (Fig. 3.1(4) & (5)). The time at which the plateau is reached is therefore equivalent to the translation time of the protein. For a membrane protein, if any time elapses between synthesis and assembly into the membrane then the run-out time (time taken to reach a plateau of radioactivity) for that protein in the membrane fraction will be equal to the sum of the translation time (measured by runnout in the whole cell lysate) and the assembly time.

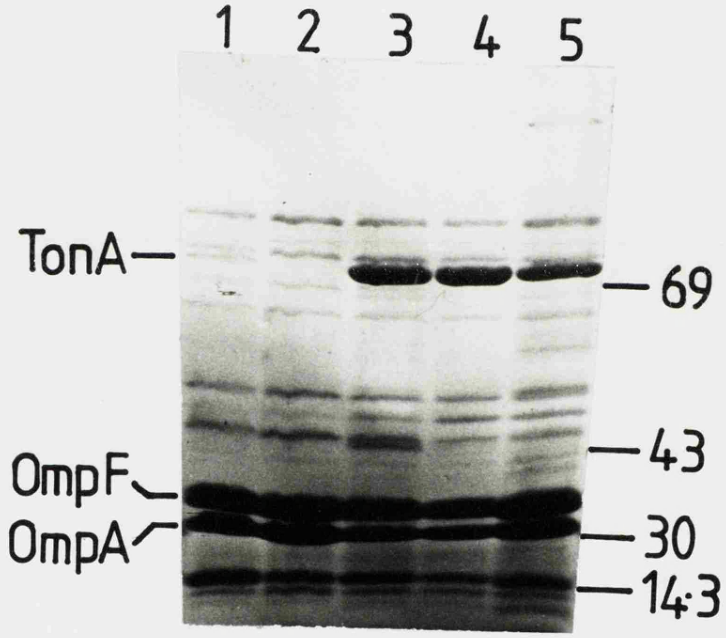
3.3 Overproduction of TonA protein in a host strain carrying pLG513

The host strain used for the experiments described in this chapter was CSH26ΔF6*tonA*, isolated as a spontaneous T5 resistant mutant of CSH26ΔF6 (carrying a *recA* deletion). Outer membrane preparations of CSH26ΔF6, CSH26ΔF6*tonA*, CSH26ΔF6*tonA*/pLG513 and JA200/pLC19-19 (Fig. 3.2) showed that the *tonA* mutant (track (ii)) had lost a 78kD outer membrane protein, ie., TonA. This protein formed a minor band in CSH26ΔF6 (track (i)) and was vastly overproduced in CSH26ΔF6*tonA*/pLG513 (tracks (iii) & (iv)) and in JA200/pLC19-19 (track (v)). It is also interesting to note that when TonA protein was overproduced, the production of OmpA and OmpF/C proteins decreased (compare tracks (i) & (ii) with (iii) & (iv)). This is indicative of a controlling mechanism at some level in the synthesis and assembly of outer membrane

Fig. 3.2: Outer membrane preparations analysed on 8.5% acrylamide II gels of:

1. CSH26ΔF6
2. CSH26ΔF6tonA
3. CSH26ΔF6tonA/pLG513
4. "
5. JA200/pLC19-19

The positions of molecular weight markers are shown on the right.


1
2
3
4
5
TonA
OmpF
OmpA
69
43
30
14·3

proteins, which limits the total quantity of protein reaching the outer membrane (see Chapter 1, section I.5).

3.4 Quantification of TonA specific material in the 78kD band of whole cell lysates

Exponentially growing cultures of CSH26Δ F6*tonA* and CSH26Δ F6*tonA*/pLG513 were pulse labelled with ^{3}H -alanine, unlabelled carrier cells of CSH26Δ F6*tonA*/pLG513 were added to each, and sonic lysates and outer membrane fractions were prepared from both samples. The proteins were separated by SDS PAGE and specific bands, including the 78kD TonA band, were cut from the gels for determination of radioactivity. The results (Table 3.1) show that approximately 80% of the material in the 78kD band of whole cell lysates of CSH26Δ F6*tonA*/pLG513 is pLG513 specified material. Therefore, assuming that pLG513 does not encode any other 78kD polypeptides, 80% of the material in the 78kD band of the lysate was TonA. Consequently, it should be possible to follow the kinetics of TonA synthesis in whole cell lysates of CSH26Δ F6*tonA*/pLG513.

3.5 Translation and assembly time of TonA protein

Exponentially growing cells of CSH26Δ F6*tonA*/pLG513 at $30^{o}C$ were prelabelled with ^{3}H -leucine as an internal standard, and then pulsed for 30 sec with ^{35}S -methionine. The pulse was terminated by addition of excess cold methionine and samples taken during the chase were fractionated to produce whole cell lysate, total membrane and outer membrane

Fraction	Band	Radioactivity in band as % of total radioactivity in sample	
		CSH26ΔF6*tonA*	CSH26ΔF6*tonA*/pLG513
	TonA	0.75	35
OM	OmpF/C	42	20
	OmpA	36	18
	78kD(TonA)	1	5
LY	EFTU	8.8	8.5
	70kD	3.8	3.8
	85kD	4.3	4.2

Table 3.1: Determination of TonA specific protein in the 78kD band of whole cell lysates of CSH26ΔF6*tonA*/pLG513 by comparison with the parent strain, CSH26ΔF6*tonA*, which produces no visible TonA protein.

2ml of exponentially growing cells at A_{450} 0.2 in M9 minimal medium at 37°C were labelled for 5 min with 20μCi of ^{3}H - alanine and then chased with excess cold alanine for 2 min. 8ml (25 OD units) of cold carrier cells of CSH26ΔF6*tonA*/pLG513 were added to each sample, and total lysate and outer membrane fractions prepared. The samples were run on 8.5% acrylamide II gels and the gels stained and dried. Individual bands were cut from the gels for determination of radioactivity. The remainder of each track was used to determine total radioactivity.

OM - outer membrane; LY - total cell lysate

fractions. These samples were electrophoresed on 8.5% polyacrylamide gels to give good resolution of proteins of high molecular weight. The relevant protein bands (including the mature TonA band) were cut from the gels and the ratio of ^{3}H to ^{35}S was determined.

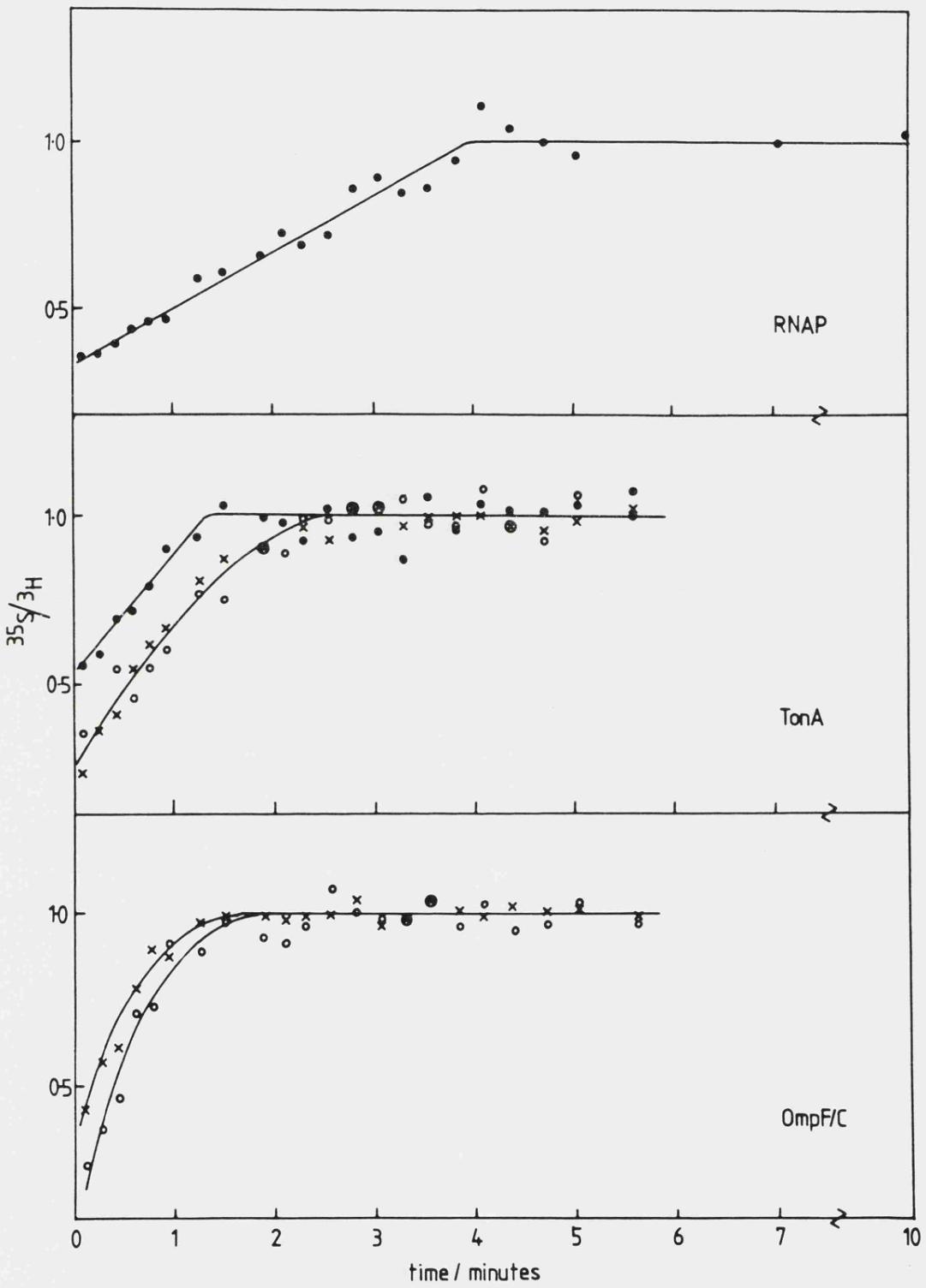
Using an average molecular weight of 115 for amino acid residues in a protein and 152.5kD for the RNA polymerase doublet (150kD and 155kD), the RNA polymerase subunits were estimated to possess approximately 1326 residues. The run-out time of 245s for RNA polymerase therefore gave a translation rate of 5.4aa/s. The 78kD TonA protein was calculated to possess approximately 678 residues. Therefore, if translated at the same rate as RNA polymerase, TonA would be expected to have a run-out time of 125 sec in whole cell lysates. In fact, from the results in Fig. 3.3, the run-out time for TonA was calculated as 100 sec. This apparently faster translation time than expected can be explained by the loss of the N-terminal methionine during processing of the signal sequence; the run-out time would therefore represent the translation time from the first methionine in the mature protein to the C-terminus. A cytoplasmic protein such as RNA polymerase would be far more likely to have retained an intact N-terminus. However, an underestimation of translation time will not affect the main point of the experiment: the comparison of run-out times for TonA between different cell fractions.

From Fig. 3.3 it can be seen that there was a lag of approximately 60 sec between run-out time for TonA in the

Fig. 3.3: Assembly kinetics for TonA protein

A 30ml exponential culture of CSH26ΔF6tonA/pLG513 in M9 minimal medium at 30°C and A_{450} 0.08 was prelabelled with 250μCi of ^{3}H -leucine for 60 min as an internal standard. Cold leucine was added to a final concentration of 100μg/ml and the cells grown for a further 15 min. The cells were then pulsed with 250μCi of ^{35}S -methionine for 30 sec; the pulse was terminated by the addition of excess cold methionine (time = 0). 1ml samples were taken during the chase into 1ml of crushed ice made from 0.5M sodium azide in 10mM sodium phosphate buffer pH7.2. Carrier cells (25 OD units) were added to the samples which were then fractionated into total lysate, total membrane and outer membrane fractions. The samples were run on 8.5% acrylamide II gels, and the ratio of ^{3}H to ^{35}S measured for individual protein bands cut from the stained, dried gels.

● : whole cell lysate; x : total membrane; o : outer membrane


1·0
0·5
RNAP
1·0
0·5
TonA
1·0
0·5
OmpF/C
$^{35}S/^{3}H$
0
1
2
3
4
5
6
7
10
time / minutes

lysate, and the run-out time measured in both the total envelope and outer membrane fractions. Since the OmpF/C proteins does not produce a clearly identifiable band in E. coli K12 lysates, it was not possible to measure the translation time for these proteins in this system. However, it is apparent from Fig. 3.3 that, in contrast to the TonA data, there was a 10 - 15 sec delay between the appearance of OmpF/C in the sarkosyl soluble fraction and its appearance in the sarkosyl insoluble fraction of the envelope. This result was therefore qualitatively similar to that obtained by Boyd & Holland (1980) with E. coli B/r, in which system a transient sarkosyl soluble pool of OmpF porin in the envelope fraction was observed.

3.6 Discussion

In contrast to previous studies with OmpF porin (Boyd & Holland, 1980), the results in section 3.5 revealed no detectable sarkosyl soluble, membrane bound intermediate in TonA assembly. During the 60 sec which elapsed before association with the envelope fraction, the mature TonA protein must therefore have been: (i) still in the cytoplasm; (ii) loosely associated with the envelope, becoming dissociated during the fractionation procedure; or (iii) present in the periplasmic space. It seems unlikely that a processed assembly intermediate would accumulate in the cytoplasm, since the signal sequence would then have no apparent function. Crowlesmith et al (1981) reported that both preOmpA and preOmpF protein could be detected as

assembly intermediates which fractionated with the soluble fraction when the cells were sonicated and with the inner membrane when the cells were French pressed. This indicated an assembly intermediate loosely bound to the membrane. Since cells were sonicated as a first step in fractionation in the TonA kinetic experiment described here, it is possible that a similar dissociation of an intermediate from the membranes was occurring. Alternatively the TonA assembly intermediate might be of the free periplasmic form proposed by Halegoua & Inouye (1980) (Fig. 1.7), or of the periplasmic vesicular form proposed by Silhavy et al (1979) (Fig. 1.7).

Boyd & Holland (1980) reported that there was no detectable delay before insertion of mature OmpF into the cell envelope, but that the protein remained sarkosyl soluble for 25 sec prior to its incorporation into a sarkosyl insoluble form. This result indicated either a true inner membrane intermediate in assembly or a sarkosyl soluble outer membrane intermediate. Although it was not possible to analyse OmpF/C run-out in addition to TonA run-out in K12 lysates, OmpF/C did appear in the sarkosyl soluble fraction approximately 15 sec before incorporation into the sarkosyl insoluble fraction (Fig. 3.3), indicating that this aspect of assembly is similar in both K12 and B/r strains. Such a sarkosyl soluble mature OmpF intermediate cannot be equated with the unprocessed, inner membrane intermediate detected by Crowlesmith et al (1981). However, the two forms may represent different stages in the same assembly pathway or slightly divergent pathways. Joseffson & Randall (1981) have

provided evidence that a given protein may be processed both co- and post-translationally as part of its normal synthetic pathway, therefore differences detected in time of processing may only reflect experimental conditions.

The TonA intermediate detected here is of the mature form; no precursor was seen even in short pulses, suggesting that processing is either cotranslational or occurs rapidly after translation is complete. The TonA intermediate may represent a more loosely membrane associated equivalent of the sarkosyl soluble OmpF intermediate detected by Boyd & Holland (1980) or it may represent an intermediate stage in an assembly pathway distinct from that of OmpF.

In order to be able to study more accurately OmpF assembly kinetics in CSH26ΔF6tonA, it would be necessary to transform this strain with the multicopy plasmid clone of ompF which is now available. Thus the kinetics of assembly of OmpF and TonA could be compared under the same experimental conditions. To establish whether an intermediate form of TonA was being lost from the envelope fraction following sonication it would be interesting to vary the lysis conditions, perhaps using osmotic lysis. Although fractionation by the Osborn technique of a large number of samples would be technically difficult, it would be interesting to take samples at early time points to process by the Osborn method, to compare assembly intermediate localisation by this method and by the sonication-sarkosyl method. Similarly, it would be interesting to look for possible periplasmic intermediates in TonA assembly by

isolating periplasmic fractions from the samples by osmotic shock or spheroplast formation.

To conclude, the results obtained here indicate that translation of TonA procedes at approximately the same rate as that of RNA polymerase. The 60 sec delay between completion of TonA synthesis and its apparent insertion into the envelope must be due to the occurrence of a post-translational (and, in this case, post-processing) assembly intermediate. Once recoverable in an envelope associated form there was no detectable delay before integration into the sarkosyl insoluble complex.

CHAPTER FOUR

PROTEASE TREATMENT OF TonA IN INTACT CELLS AND ISOLATED ENVELOPES

4.1 Introduction

E. coli outer membrane proteins are necessarily accessible to the environment as receptors for nutrients, colicins and bacteriophages, whilst they must also be resistant to attack by proteases present in the gut. The OmpF/C proteins are completely resistant to proteolysis, even in isolated envelope fractions, and can only be degraded following solubilisation in SDS (Reithmeier & Bragg, 1977). Although the OmpA protein is not affected by pronase E treatment of intact cells (Reithmeier & Bragg, 1977), both pronase E and trypsin treatment of isolated envelope fractions yield membrane bound OmpA fragments, of 20kD and 25kD respectively (Inouye & Yee, 1972). Holland & Tuckett (1972) observed the production of a similar 25kD OmpA fragment following trypsin treatment of intact cells in phosphate buffer. However, subsequent studies by Halegoua & Inouye (1979) indicated that the E. coli outer membrane is permeable to trypsin in the absence of magnesium. Halegoua & Inouye found that in the presence of 10mM Mg^{2+} OmpA protein remains trypsin resistant in intact cells, indicating that the outer membrane is impermeable to trypsin under these conditions.

The pattern of protease resistance of TonA might be a useful tool in determining the location of mutant TonA polypeptides and intermediates in TonA assembly. Therefore

intact cells of PB72-47/pLG513 and envelope fractions isolated from these cells were treated with various proteases to determine the effect on TonA.

The proteases chosen were: trypsin, which cleaves peptide bonds on the carboxyl side of lysine and arginine; chymotrypsin, which preferentially cleaves at the carboxyl side of aromatic and large hydrophobic residues; proteinase K and subtilisin, which are non-specific serine proteases, although proteinase K preferentially cleaves at the carboxyl side of aromatic and hydrophobic residues; and pronase E, which is a partially purified mixture of proteases, including aminopeptidases, carboxypeptidases, and neutral and alkaline peptidases, and has no specificity.

4.2 Results of protease treatment

(i) Intact cells: Protease treatment of intact cells was carried out in Tris/Cl buffer and therefore in the absence of Mg^{2+}, under which conditions the outer membrane is permeable to trypsin (Halegoua & Inouye, 1979).

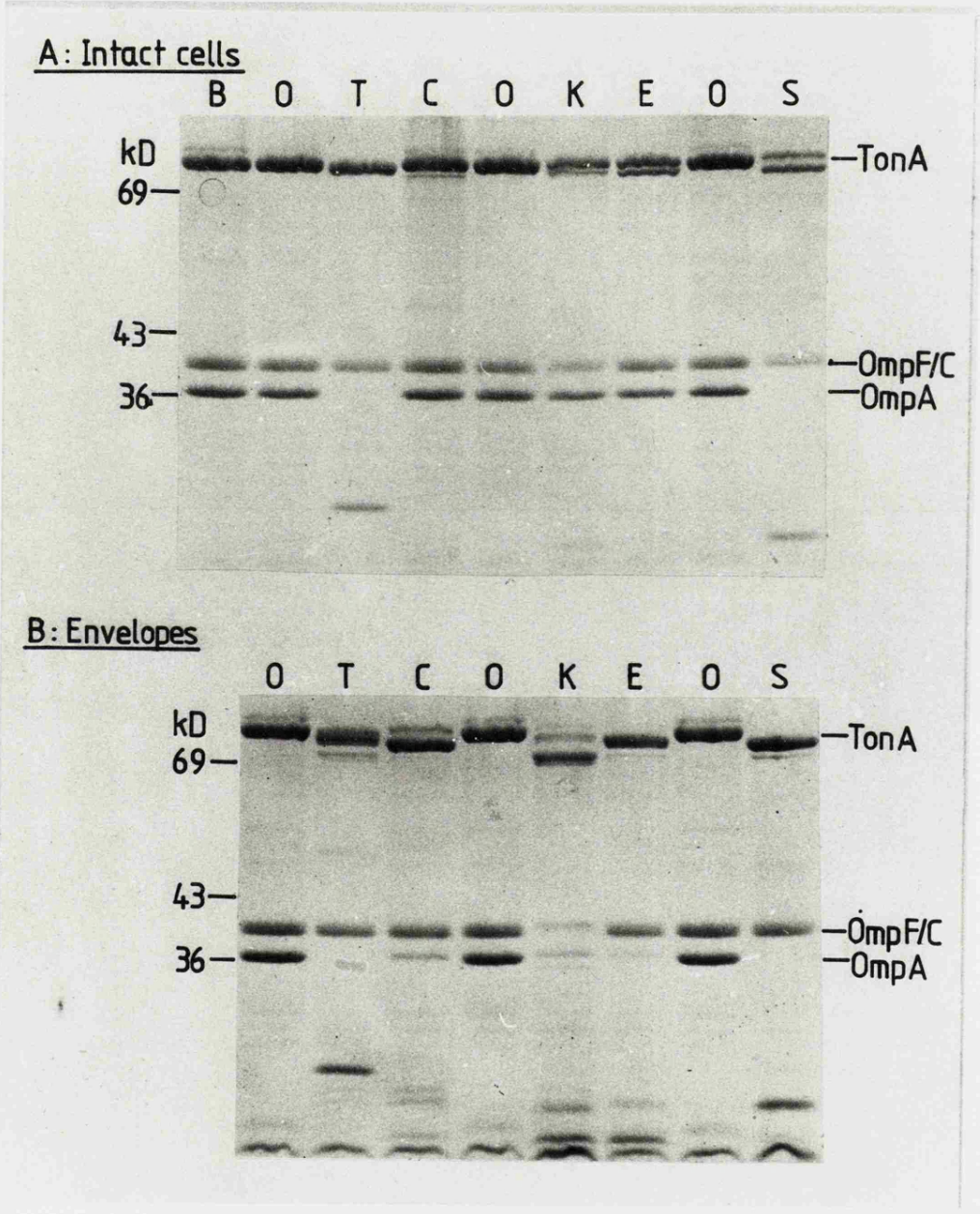
As expected, the OmpF/C proteins were resistant to digestion by pronase E and trypsin, and in addition were not visibly affected by chymotrypsin, proteinase K and subtilisin (Fig. 4.1a). OmpA was resistant to digestion by pronase E, proteinase K and chymotrypsin in intact cells, but was partly degraded by both trypsin and subtilisin, leaving membrane bound fragments of 25kD and 20kD respectively.

Although TonA appeared to be at least partly accessible

Fig. 4.1: Protease sensitivity of outer membrane proteins in whole cells and isolated envelopes

800 OD units of exponential PB72-47/pLG513 grown in Luria broth at 37°C were harvested and resuspended in 25ml of 50mM Tris/Cl pH7.8. Half of the cells were used to determine the sensitivity of intact cells to proteases; envelopes were isolated from the remainder for proteolytic treatment of membranes. Outer membrane proteins were isolated from all samples and analysed by SDS PAGE (11% acrylamide I) and Coomassie blue staining.

O, B	control samples
T	trypsin
C	chymotrypsin
K	proteinase K
S	subtilisin
E	pronase E


A: Intact cells
B O T C O K E O S
kD
69
43
36
TonA
OmpF/C
OmpA
B: Envelopes
O T C O K E O S
kD
69
43
36
TonA
OmpF/C
OmpA

to all five proteases, only small regions (totalling about 2 - 3kD) at the N- and/or C-terminus were degraded, leaving the majority of the protein molecule intact and membrane bound. Trypsin appeared to cleave all the TonA molecules present, but the remaining four proteases affected only a fraction of the TonA molecules.

(ii) Isolated envelopes: Proteolytic treatment of isolated envelopes under the same conditions (i.e., in Tris/Cl buffer) showed OmpF/C to be resistant to trypsin, chymotrypsin, subtilisin and pronase E (Fig. 4.1b). A large proportion of the OmpF/C present was, however, susceptible to attack by proteinase K. All of the OmpA present was degraded by trypsin and subtilisin, producing membrane bound fragments of 25kD and 20kD respectively. A small proportion of the OmpA molecules were unaffected by proteinase K, chymotrypsin and pronase E, but the majority were degraded to membrane bound fragments of approximately 20kD.

TonA protein was sensitive to all the proteases, being degraded to membrane bound fragments of 70 - 75kD, indicating that very little of TonA was protease accessible, even in isolated envelopes.

4.3 Discussion

Since proteinase K and pronase E, which are both relatively non-specific proteases, did not appear to cleave OmpA in intact cells, it seems probable that the sensitivity of OmpA to trypsin and subtilisin under the same conditions was due to the permeability of the outer membrane to these proteases

in the absence of Mg^{2+} (Halegoua & Inouye, 1979). TonA in intact cells was partially degraded by all five proteases, even those which did not appear to penetrate the outer membrane, although not all the TonA present was cleaved by chymotrypsin, proteinase K, pronase E and subtilisin. This could be due to only a proportion of the TonA molecules being accessible on the cell surface. Menichi & Buu (1983) demonstrated that of the total phage T5 receptors detectable using phage inactivation by solubilised outer membrane proteins, only 25% were accessible to T5 phage in whole cells. When the same strain was transformed with pLG513, the number of T5 receptors in solubilised outer membranes increased 40 fold, but only 3% of these receptors were accessible to T5 phage in intact cells. Thus TonA protein may exist in different states in the outer membrane, perhaps some of the receptor sites being masked by other proteins (Menichi & Buu, 1983). Alternatively, the proteolysis reaction may not have gone to completion, and with a longer incubation time, or additional protease, all of the TonA molecules might have been cleaved by the proteases. However, in view of the fact that in isolated envelopes the same protease concentration led to cleavage of all the TonA molecules present (Fig. 4.1b), this explanation seems unlikely.

In isolated envelopes the OmpF/C proteins were found to be proteinase K sensitive, but unaffected by pronase E and subtilisin. This result may indicate that proteinase K was able to penetrate into the membrane bilayer, perhaps via the

pores postulated to be formed by OmpF/C, and could therefore cleave these proteins. All of the OmpA molecules in isolated envelopes were accessible to trypsin and subtilisin, but some OmpA molecules were unaffected by chymotrypsin, proteinase K and pronase E. Again, this could be due to masking of some of the protein molecules, or due to incomplete proteolysis.

The TonA protein in isolated envelopes was accessible to partial proteolysis by all the proteases, but the majority of each protein molecule was resistant to degradation. Regions close to the N- and/or C-terminus of the TonA molecule must be the protease sensitive parts of the protein, since the majority of TonA remains intact following proteolysis. This high level of resistance of TonA protein to proteolytic degradation in envelopes can be used as an indication of correct assembly of the TonA molecule.

The protease resistance of TonA and OmpF/C may be attributable to these proteins being buried in the bilayer with only small regions exposed on either face of the membrane. Alternatively, substantial portions of these outer membrane proteins may be accessible to the proteases, but not susceptible to proteolytic digestion due to a protease resistant conformation. Such a conformational effect has been observed with TEM β-lactamase: the precursor form of β-lactamase is protease sensitive, but, following processing, the mature β-lactamase molecule assumes a protease resistant conformation (Koshland & Botstein, 1982).

CHAPTER FIVE

EFFECT OF MalE-LacZ HYBRID INDUCTION ON PROCESSING AND ASSEMBLY OF PENICILLIN BINDING PROTEINS AND TonA

5.1 Introduction

As described in Chapter 1 (section III.2.3), induction of hybrid proteins composed of the N-terminal portion of MalE or LamB fused to ß-galactosidase is, in several cases, lethal to the cell. Hybrid protein induction leads to accumulation of precursors of several outer membrane and periplasmic proteins, and the inhibition of cell division (Silhavy et al, 1979; Ito et al, 1981; Herrero et al, 1982). Ito et al suggested that the hybrid proteins were blocking an early stage in secretion common to both periplasmic and outer membrane proteins, perhaps involving specific export sites. However, no investigation of the effect of the hybrids on inner membrane protein assembly was carried out. Therefore, as part of a study of the effects of induction of the MalE-LacZ hybrid 72-47 on envelope protein assembly, the effect of the hybrid on a group of inner membrane proteins, the penicillin binding proteins (PBPs) was investigated initially. The PBPs are a set of inner membrane proteins easily identifiable by their interaction with ^{14}C -benzyl-penicillin (Spratt, 1975). Using the in vitro Zubay transcription-translation system together with SDS PAGE analysis of the products, Pratt et al (1981) identified larger molecular weight precursor forms of PBP5 and PBP6, which were processed to the mature size in the presence of

inverted inner membrane vesicles. Similarly, a higher molecular weight form of PBP3 has been identified in an *in vitro* system, suggesting that PBP3 is also synthesised with a signal sequence (Nakamura *et al*, 1983). Thus the effect of the MalE-LacZ hybrid protein on processing and assembly of inner membrane proteins could be investigated.

In addition, in order to study the effects of hybrid protein induction on TonA assembly, pLG513 was transformed into PB72-47, carrying the *malE-lacZ* hybrid gene 72-47 (Fig. 1.5). It was hoped that blocking assembly of TonA in this way might reveal intermediates in the assembly process which are not normally detectable.

5.2 Effect of the MalE-LacZ hybrid on the PBPs

Maltose was added to cultures of PB72-47, carrying the *malE-lacZ* hybrid gene 72-47, and to cultures of the parent strain MC4100, in order to induce transcription from the *malB* operons, including the hybrid gene. After two mass doublings, the cells were harvested and fractionated. Cytoplasmic and envelope fractions were incubated with ^{14}C -benzylpenicillin to label the PBPs and the samples were analysed by SDS PAGE. No significant benzylpenicillin binding was observed with the cytoplasmic or outer membrane fractions. The inner membrane fractions are shown in Fig. 5.1. PBPs 1a, 1b, 2 and 3 appear to be incorporated normally into the inner membrane in the presence of hybrid protein. A greatly reduced amount of PBP4 was detected in the inner membrane of maltose induced PB72-47. A slight decrease in the levels of PBP5 and

Fig. 5.1: Accumulation of putative prePBP6 (PBP6*) following induction of the MalE-LacZ hybrid protein

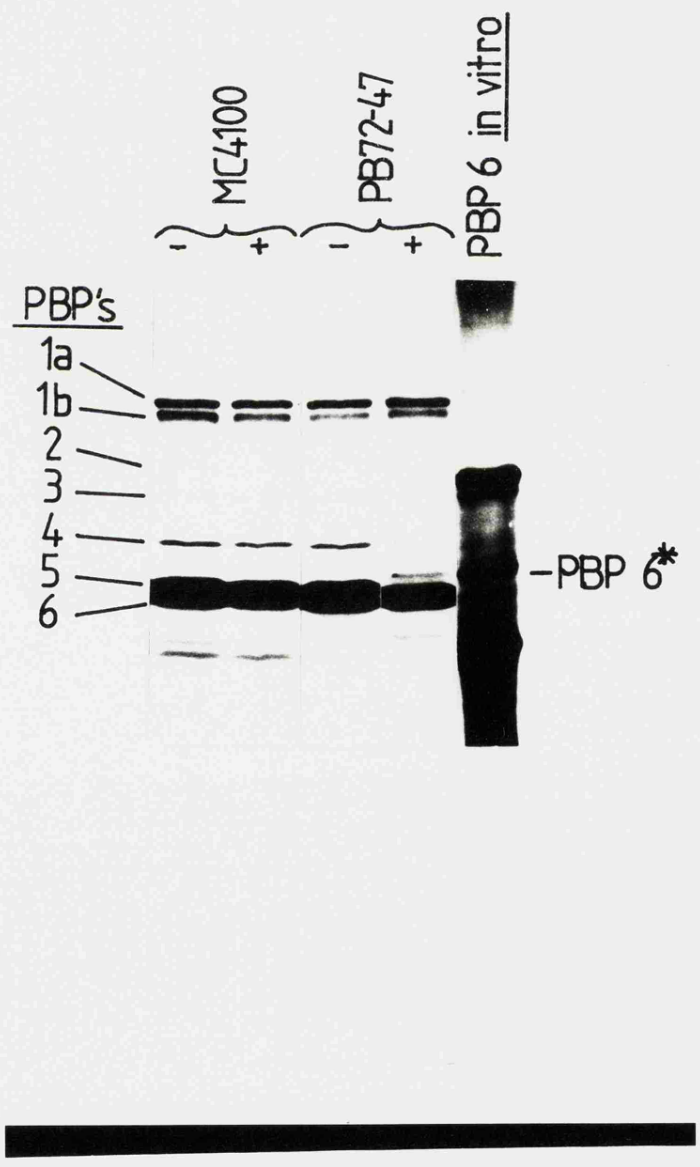
Strains MC4100 and its derivative PB72-47 were inoculated into 600ml of M9 minimal medium with glycerol (0.4%) instead of glucose as sole carbon source, to A_{450} 0.03. The cultures were grown at 30°C to A_{450} 0.05, and 300ml of each culture transferred to a prewarmed flask containing 6ml of filter sterilised maltose. Each culture was incubated at 30°C until 2½ mass doublings had taken place; the cells were then harvested and fractionated into total membrane and cytoplasmic samples for ^{14}C -benzylpenicillin labelling. The membranes were then further fractionated into inner and outer membrane samples. The samples were analysed by SDS PAGE and fluorography.

The outer membrane and cytoplasmic fractions showed no significant ^{14}C -benzylpenicillin binding. Inner membrane samples are shown opposite.

- glycerol grown cultures

+ maltose induced cultures

PBP6* synthesised in vitro: 2.5µg of pLG310 DNA was used to programme the Zubay transcription-translation system, using the MRE600 extract and a ^{35}S -methionine label.


MC4100
PB72-47
PBP 6 in vitro
- + - +
PBP's
1a
1b
2
3
4
5
6
-PBP 6*

PBP6 was also observed, together with the appearance of two new bands on the gel at the positions expected for the precursor forms of these two proteins (the putative PBP5 precursor band is only visible on the original autoradiogram). Although PBP3 also appears to possess a cleavable signal sequence (Nakamura et al, 1983), no prePBP3 could be detected here. The ^{14}C -benzylpenicillin labelled bands appearing below PBP6 in Fig. 5.1 are difficult to characterise; one of these bands may be "PBP7", which is seen in PBP profiles of some strains under certain conditions (Spratt, 1977).

The conclusions which can be drawn from these results are limited since it is not known to what extent benzylpenicillin will bind to PBPs which are (i) not membrane integrated; (ii) incorrectly integrated into the membrane or (iii) unprocessed. However, there was a significant effect on the amount of PBP4 available for benzylpenicillin binding in the inner membrane; this could reflect either a decreased synthesis of PBP4 or a cytoplasmic or abnormal membrane bound form of the protein unable to bind benzylpenicillin. Processing of PBP5 and PBP6 appeared to be inhibited; the degree of inhibition was difficult to determine since, as indicated above, unprocessed PBPs 5 and 6 may not bind benzylpenicillin.

In the case of PBP5, these ambiguities were resolved using a recombinant λ phage encoding PBP5. As described in the next section, this allowed the relative amounts of mature and unprocessed forms to be determined directly without

recourse to ^{14}C -benzylpenicillin labelling.

5.3 Expression of PBP5 from cloned DNA in the presence of hybrid MalE-LacZ protein

λpBS10 (Spratt et al, 1980) carries an E. coli chromosomal fragment encompassing the genes leuS, pbpA (encoding PBP2), rodA and dacA (encoding PBP5) and a phage immunity region derived from λimm434. MC4100 and PB72-47 were transformed with the recombinant plasmid pGY101 (Levine et al, 1979), which carries the gene encoding λimm434 repressor in a mutant form enhancing repressor synthesis (λimm434T). MC4100/pGY101 and PB72-47/pGY101 were grown in glycerol or induced for malB operon expression by maltose addition. After two mass doublings the cultures were heavily irradiated to prevent host gene expression and infected with λpBS10 in order to observe the effect of the hybrid MalE-LacZ protein on PBP5 processing. This expression system has the following advantages over the procedure used in section 5.2:

(i) Since only proteins from the cloned genes carried by λpBS10 were expressed (phage specific genes were repressed by λimm434 repressor encoded by pGY101), ^{35}S -methionine could be used as a label, thus eliminating the uncertainties involved with benzylpenicillin binding;

(ii) The PBP5 labelled in this system was all newly synthesised, whereas using benzylpenicillin, all PBP5 synthesised both before and after hybrid protein induction was labelled.

Fig. 5.2 shows the proteins synthesised in the presence

Fig. 5.2: Accumulation of prePBP5 (PBP5*) following λpBS10 infection of UV irradiated cells induced for MalE-LacZ hybrid synthesis

Tracks 1 - 3: ^{35}S -methionine labelled products of λpBS10 infection of UV irradiated cells of

1: MC4100/pGY101, maltose induced for 2 generations

2: PB72-47/pGY101, glycerol grown

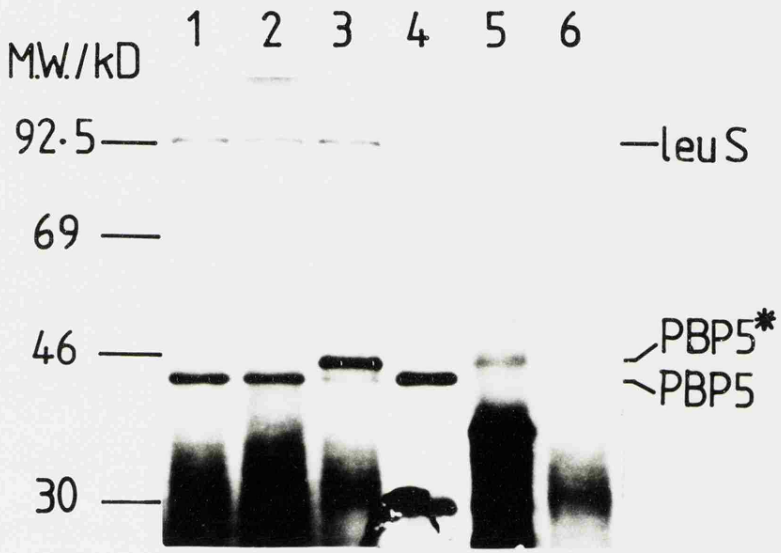
3: PB72-47/pGY101, maltose induced for 2 generations

Track 4: ^{35}S -methionine labelled products of minicells prepared from DS410/pBS42 (plasmid encoding PBP5)

Track 5: ^{35}S -methionine labelled products of *in vitro* expression of λpBS10 in the Zubay transcription-translation system (MRE600 extract)

Track 6: Control for tracks 1 - 3, irradiated cells of PB72-47/pGY101 maltose induced, incubated with 100μl lambda buffer and labelled as samples 1 - 3

Samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography.


M.W./kD
1
2
3
4
5
6
92·5
69
46
30
leuS
PBP5*
PBP5

and absence of hybrid MalE-LacZ protein in UV irradiated cells infected with pBS10. It is apparent that in maltose induced MC4100/pGY101 (track 1) and in glycerol grown PB72-47/pGY101 (track 2) the major product of λpBS10 infection was mature PBP5, comigrating with the PBP5 synthesised in minicells (track 4). In contrast, in maltose induced cultures of PB72-47/pGY101 (track 3), where the MalE-LacZ hybrid was present, relatively little mature PBP5 was produced. However, a new protein appeared which comigrated with the PBP5 precursor (PBP5*) synthesised in the Zubay in vitro system (track 5). This result showed conclusively that PBP5 processing was almost completely inhibited in cells expressing the MalE-LacZ hybrid. Although PBP2 is also encoded by λpBS10, this protein was not expressed at sufficient levels in the UV irradiation-λinfection system for a similar analysis.

5.4 Kinetics of inhibition of PBP5 processing following maltose induction of PB72-47/pGY101

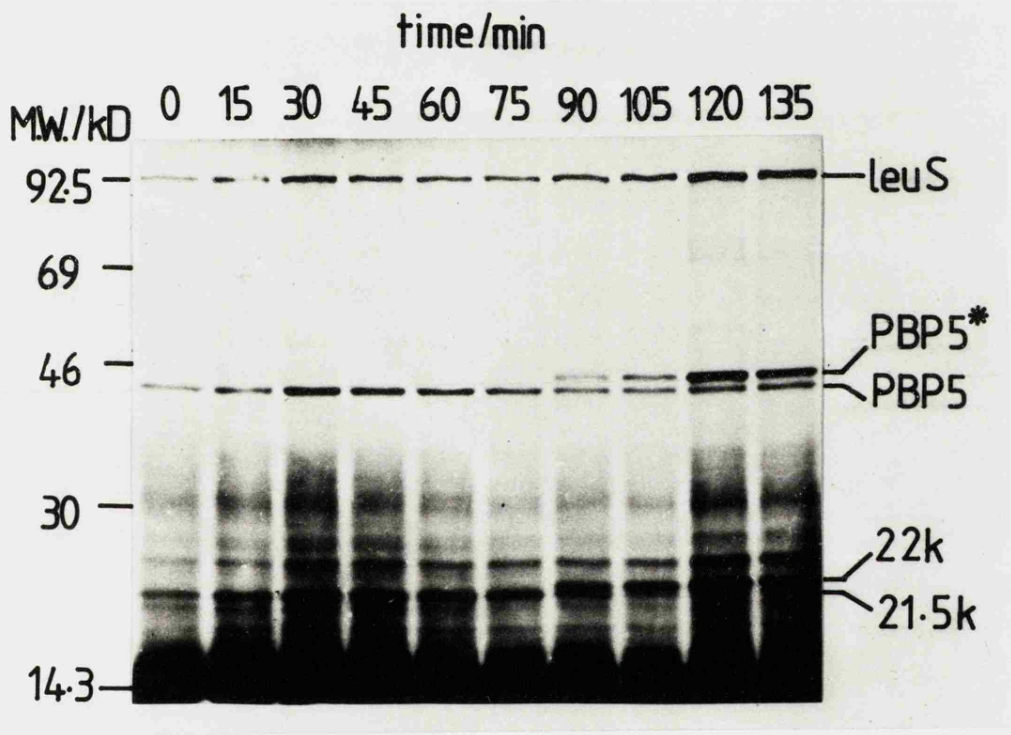
If the observed inhibition of PBP5 processing was a direct effect of the MalE-LacZ hybrid protein blocking some aspect of the secretory apparatus, it might be expected that the kinetics of this processing inhibition would follow closely the kinetics of synthesis of the hybrid protein.

A culture of PB72-47/pGY101 was induced with maltose, and samples taken at intervals during the induction for subsequent UV irradiation and λpBS10 infection. The results (Fig. 5.3) showed the PBP5 precursor to be initially

Fig. 5.3: Kinetics of inhibition of PBP5 processing on maltose induction of PB72-47/pGY101

Maltose (0.4%) was added at time 0 to an exponential culture (A_{450} 0.1) of glycerol grown PB72-47/pGY101. At intervals samples were taken to examine the proteins synthesised following λpBS10 infection of the UV irradiated cells as before. These samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

2ml samples were taken from the culture at the same time intervals for determination of kinetics of induction of hybrid protein by pulse labelling (see Fig. 5.4).

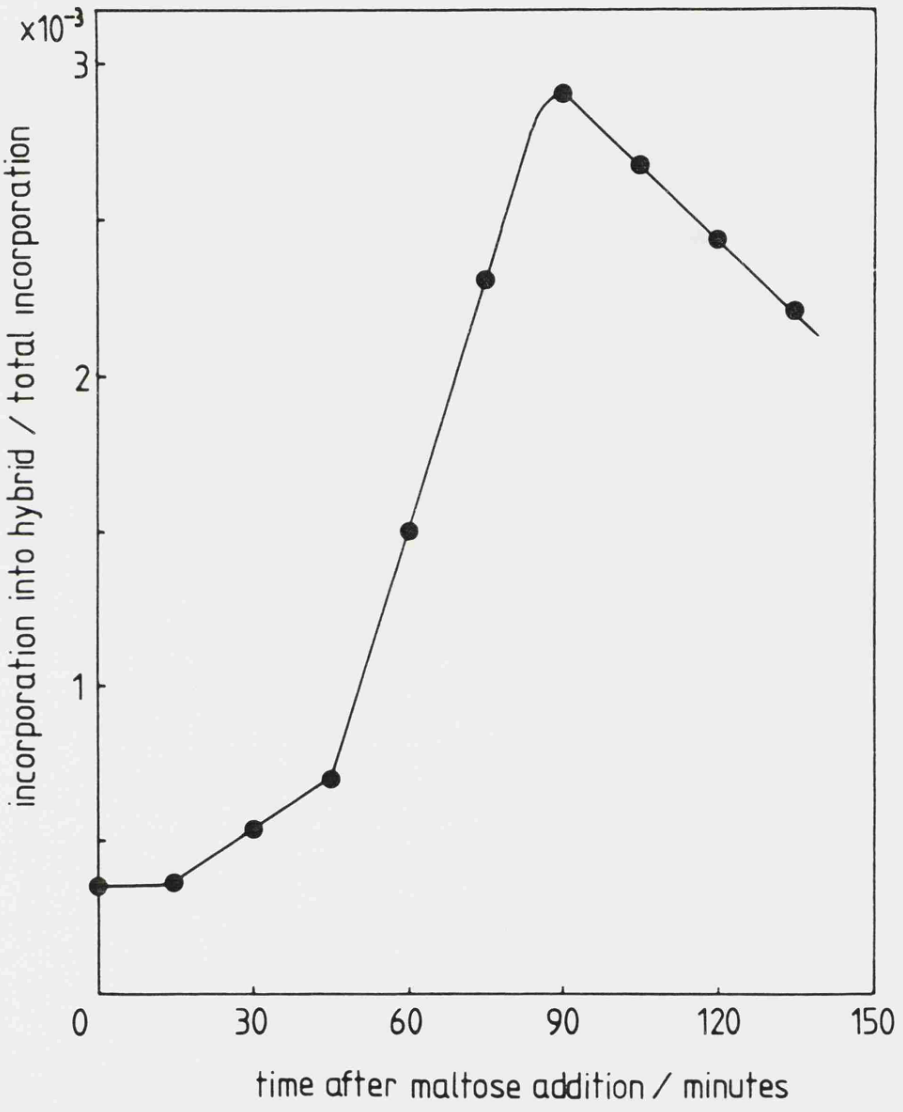

time/min
0 15 30 45 60 75 90 105 120 135
MW./kD
92·5
69
46
30
14·3
leuS
PBP5*
PBP5
22k
21·5k

detectable at 75 min after addition of maltose (not visible in this photograph) with progressively more PBP5 remaining unprocessed at later times. Interestingly, a 21.5kD protein, encoded by λpBS10 and previously localised to the inner membrane (Spratt et al, 1980; N. Stoker, pers. comm.), also apparently accumulated in an precursor form in maltose induced PB72-47/pGY101 (Fig. 5.3). Not visible in Fig. 5.3, but apparent on the original autoradiogram was a similar accumulation of a possible precursor form of a 58kD protein. This protein has also been shown to be localised in the inner membrane (Spratt et al, 1980; N. Stoker, pers. comm.). Both the 21.5kD precursor and the 58kD precursor accumulated with the same kinetics as the PBP5 precursor.

The kinetics of synthesis of the hybrid MalE-LacZ protein were followed by pulse labelling with ^{35}S -methionine of samples taken during the maltose induction of PB72-47/pGY101. The cellular proteins were separated by SDS PAGE, the hybrid protein band cut from the gel and ^{35}S incorporation measured relative to the total incorporation in each sample. Maximal induction of hybrid protein was found to occur at 90 min after addition of maltose, correlating well with the observed inhibition of processing (Fig. 5.4). The lag in induction of the MalE-LacZ hybrid protein, and also of the lamB gene product (Herrero et al, 1982) was probably due to glucose contamination of the maltose. The cells would preferentially use glucose as a carbon source, and only switch to maltose catabolism when the glucose supply was exhausted.

Fig. 5.4: Kinetics of induction of the MalE-LacZ hybrid during the experiment described in Fig. 5.3

2ml samples were taken from the maltose induced culture (see legend to Fig. 5.3) at various times following maltose addition. Each sample was pulse labelled with 10μCi of ^{35}S - methionine for 2 min, then 2ml of ice cold 600μg/ml chloramphenicol, 2% methionine added and the samples chilled on ice. The proteins were separated on 8.5% acrylamide II gels, the bands corresponding to the hybrid protein were cut from the stained, dried gels and the radioactivity determined; the remainder of each track was used to determine total radioactive incorporation.


x10⁻³
3
2
1
incorporation into hybrid / total incorporation
0
30
60
90
120
150
time after maltose addition / minutes

5.5 Effect of MalE-LacZ hybrid on TonA assembly

In order to discover whether TonA assembly into the outer membrane was affected by induction of the hybrid MalE-LacZ protein, pLG513 was transformed into PB72-47. A glycerol grown culture of PB72-47/pLG513 was maltose induced, and after two mass doublings the cells were labelled for 10 min with ^{35}S -methionine. After fractionation into cytoplasm, inner membranes (sarkosyl soluble) and outer membranes (sarkosyl insoluble), the samples were analysed by SDS PAGE and autoradiography. The MalE-LacZ hybrid protein could be seen clearly in the sarkosyl soluble membrane fraction of maltose induced cells, but smaller amounts were also visible in the sarkosyl insoluble membrane fraction (Fig. 5.5). The level of labelled OmpA in the outer membrane of induced cells was slightly less than that in uninduced cells. The effect of the hybrid on OmpF/C proteins was more pronounced, with only a relatively small amount of these proteins still being assembled into the outer membrane. However, TonA protein was labelled at the same levels in the outer membranes of both induced and uninduced cells, and a considerable amount of a protein 2 - 3kD larger than TonA, presumably preTonA, was also apparent in the outer membrane of the maltose induced culture of PB72-47/pLG513. This appearance of preTonA in the sarkosyl insoluble fraction was unexpected, since preOmpF/C, when detected at all by these techniques, accumulated in the sarkosyl soluble fraction under similar conditions (Herrero et al, 1982). A protein migrating slightly slower than mature LamB in the outer membrane fractions of maltose induced cells

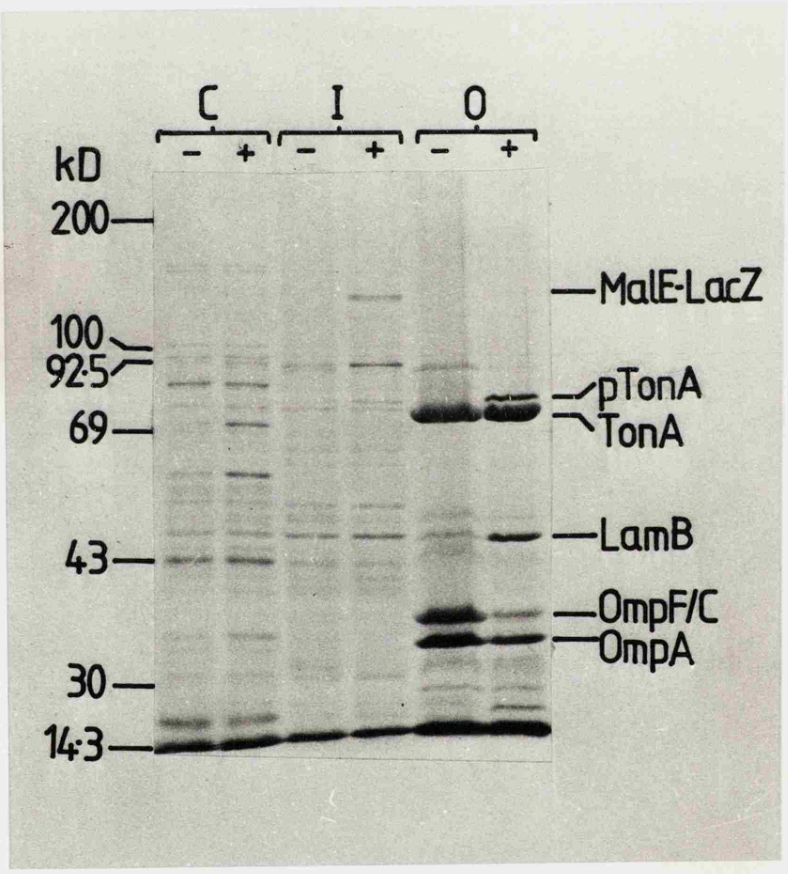
Fig. 5.5: Inhibition of TonA processing by MalE-LacZ hybrid induction

A 300ml culture of PB72-47/pLG513 was grown in glycerol minimal medium to A_{450} 0.1 at 30°C, then half the culture transferred to a prewarmed flask to which maltose (0.4%) was added. After two generations 10μCi of ^{35}S -methionine was added and the cultures incubated for a further 10 min. Excess cold methionine was added, and cytoplasmic, inner membrane and outer membrane fractions prepared. The samples were analysed by SDS PAGE (8.5% acrylamide II) and autoradiography.

Positions of ^{14}C -labelled molecular weight markers shown at left.

C - cytoplasm; I - inner membrane; O - outer membrane

\- glycerol grown cells; + maltose induced cells


C
I
O
- + - + - +
kD
200
100
92.5
69
43
30
14.3
MalE-LacZ
pTonA
TonA
LamB
OmpF/C
OmpA

probably represents the LamB precursor.

5.6 Pronase E treatment of TonA assembled in the presence of hybrid MalE-LacZ protein

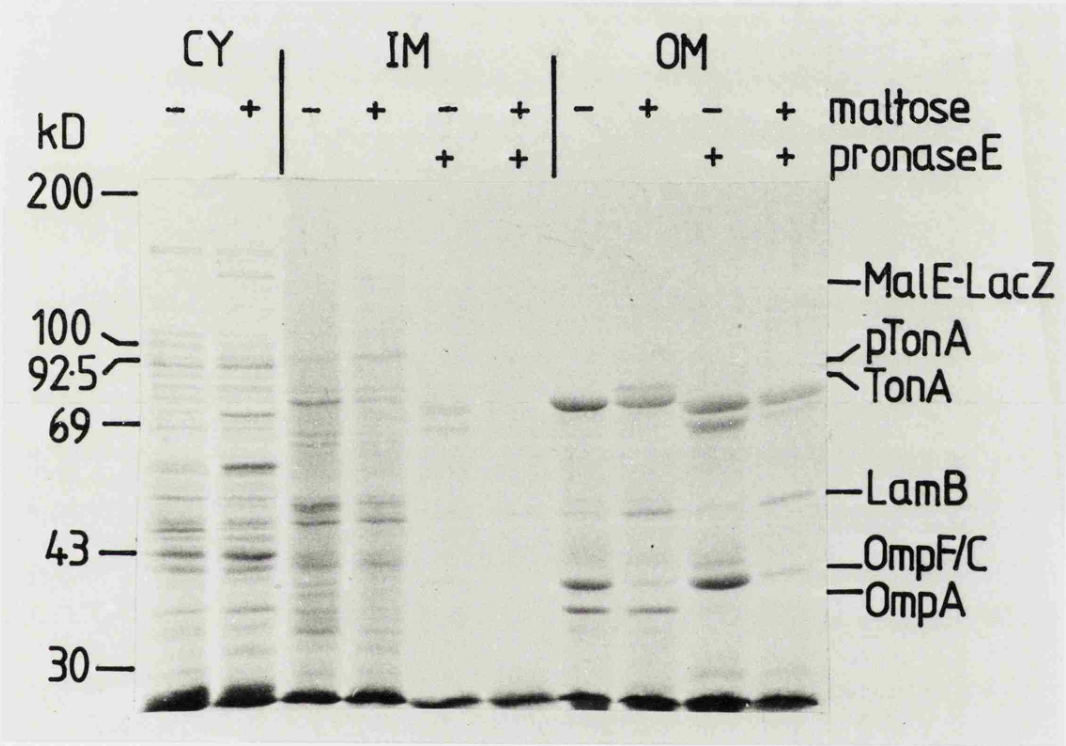
TonA protein is normally relatively inaccessible to proteases added to envelope fractions (see section 4.2). If TonA protein synthesised in the presence of the MalE-LacZ hybrid is assembled correctly into the outer membrane it would be expected to possess the same properties of protease resistance as TonA protein assembled under normal conditions. Therefore, glycerol grown and maltose induced cultures of PB72-47/pLG513 were labelled for 10 min with ^{35}S -methionine as before, and the isolated envelopes treated with pronase E prior to separation into sarkosyl soluble and insoluble fractions (Fig. 5.6). The results clearly demonstrated that TonA protein processed and assembled into the outer membrane in the presence of the hybrid protein was as resistant to protease treatment as TonA assembled under normal conditions, although the larger of the two major membrane bound cleavage products appeared to predominate in the former case. The fate of the preTonA after protease treatment was more uncertain. The precursor band disappeared following pronase treatment but no unique cleavage products appeared, indicating either cleavage to the same size as the mature protein pronase product, or, alternatively, complete degradation. Similarly to TonA, the LamB, OmpF/C and OmpA proteins all showed the same pattern of protease sensitivity whether they were assembled in the presence or absence of the hybrid protein.

Fig. 5.6: Pronase E sensitivity of outer membrane proteins assembled in the presence of the MalE-LacZ hybrid

A 400ml culture of PB72-47/pLG513 was grown in glycerol minimal medium at 30°C to A_{450} 0.05, then half the culture was transferred to a prewarmed flask containing maltose (0.4%). After 2 generations, 10µCi of ^{35}S -methionine was added to each culture, incubation continued for 10 min, then excess cold methionine added and the cultures cooled on ice. Cytoplasmic and total membrane fractions were isolated and the washed membrane pellets resuspended in 0.5ml 50mM Tris/Cl pH7.8 for pronase E treatment. Following proteolysis, inner and outer membrane proteins were separated by sarkosyl treatment. The samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

The positions of ^{14}C -labelled molecular weight markers are shown to left of gel

CY - cytoplasm; IM - inner membrane; OM - outer membrane


CY
IM
OM
kD
maltose
pronaseE
200
100
92.5
69
43
30
MalE-LacZ
pTonA
TonA
LamB
OmpF/C
OmpA

5.7 Chase of sarkosyl insoluble preTonA into mature TonA

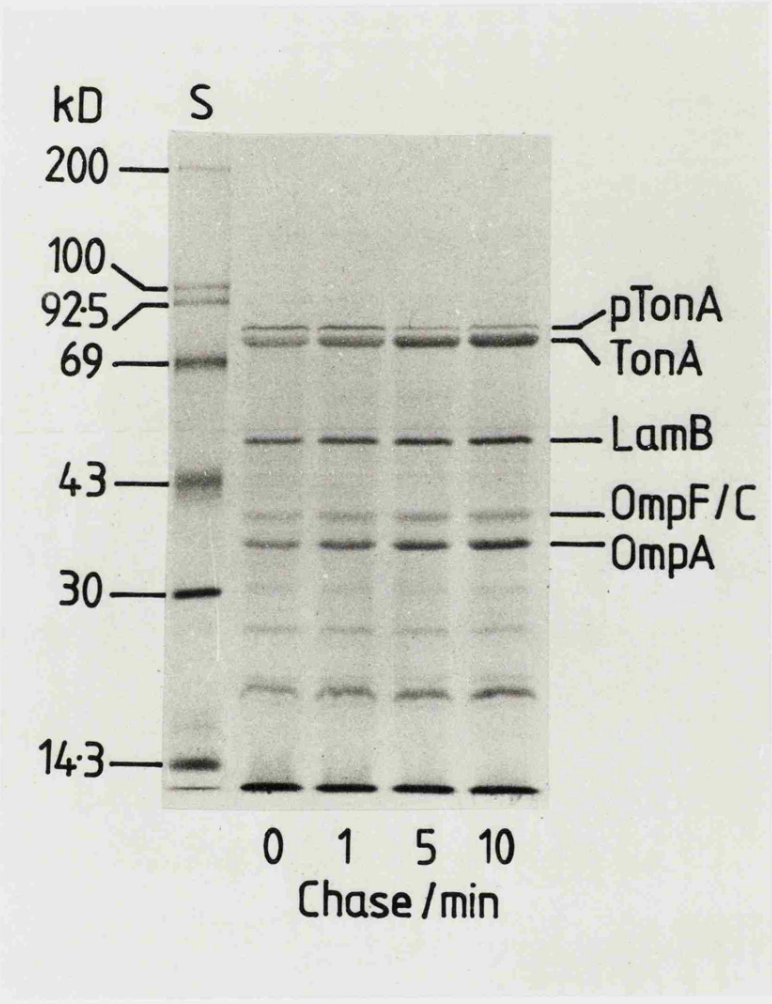
In order to ascertain more clearly the protease sensitivity of the preTonA accumulated in the sarkosyl insoluble envelope fraction in the presence of MalE-LacZ hybrid, it was necessary to increase the ratio of preTonA to mature TonA in the labelled fraction. A pulse chase experiment was therefore carried out to determine whether a higher ratio of preTonA to mature TonA could be obtained using a shorter labelling period.

A culture of maltose induced PB72-47/pLG513 was labelled with ^{35}S -methionine for 60 sec, excess cold methionine added to terminate the pulse, and samples taken during the subsequent chase. The sarkosyl insoluble envelope material was prepared from these samples and analysed by SDS PAGE (Fig. 5.7). The total amount of labelled preTonA plus mature TonA was found to increase during the chase period; this was to be expected since, even under conditions of normal growth, TonA synthesis and assembly takes approximately 3 min to complete at 30^{o}C (Fig. 3.3). However, it was apparent that at early times during the chase the ratio of preTonA to mature TonA was indeed higher than that observed after 10 min of chase (Fig. 5.7). These results also demonstrated that although preTonA became sarkosyl insoluble under the conditions of this experiment, the signal sequence was still accessible to signal peptidase.

Fig. 5.7: Outer membrane fractions isolated from samples taken during the chase period following a 1 min pulse with ^{35}S -methionine of maltose induced PB72-47/pLG513

An 800ml culture of PB72-47/pLG513 was maltose induced for 2½ generations as previously. The cells were pulse labelled for 1 min with 50µCi of ^{35}S -methionine, then excess cold methionine added (time 0) and 200ml samples transferred to GS3 bottles containing 200ml ice cold bacterial buffer at times 0, 1 min, 5 min and 10 min. Outer membrane fractions were isolated and analysed by SDS PAGE (11% acrylamide I) and autoradiography.

S - ^{14}C -labelled marker proteins


kD
S
200
100
92·5
69
43
30
14·3
pTonA
TonA
LamB
OmpF/C
OmpA
0 1 5 10
Chase/min

5.8 Pronase E sensitivity of preTonA accumulated in the sarkosyl insoluble fraction during a short pulse labelling

Having established that the ratio of preTonA to mature TonA could be increased by using a shorter labelling period, the pronase sensitivity of the accumulated preTonA was investigated.

A culture of maltose induced PB72-47/pLG513 was labelled with ^{35}S -methionine for 60 sec, fractionated into cytoplasm and envelopes, and the envelope fraction treated with pronase E. Following fractionation of the envelopes into sarkosyl soluble and insoluble components, the samples were analysed by SDS PAGE and autoradiography (Fig. 5.8).

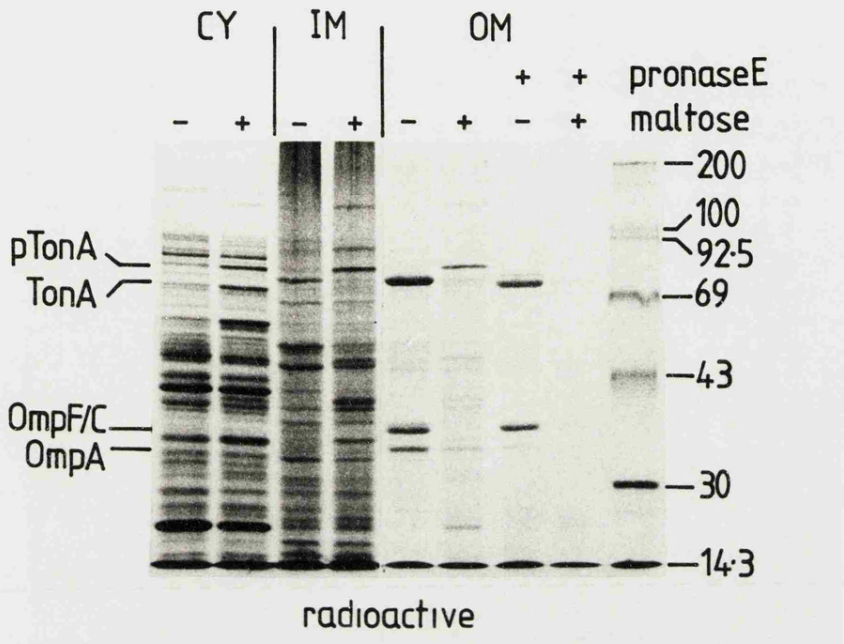
Using this technique the preTonA was found to be completely pronase E sensitive - no protease resistant membrane bound fragment was discernable in either membrane fraction. This result clearly indicated that in the absence of processing, the consequent abnormal localisation and/or folding of the protein rendered it extremely sensitive to added protease.

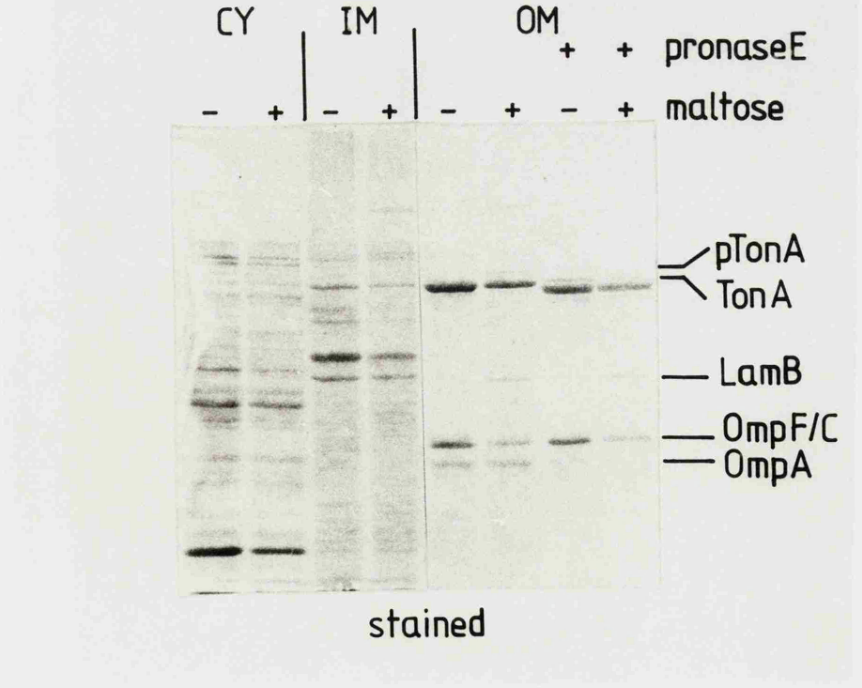
Also apparent from Fig. 5.8 is a striking difference between the radioactive gel profiles of membrane fractions from glycerol grown and maltose induced cultures. In addition to the appearance of labelled TonA predominantly in an unprocessed form in maltose induced cells, new bands which might correspond to the precursor forms of LamB, OmpF/C and OmpA were also visible in both the sarkosyl soluble and sarkosyl insoluble membrane fractions from induced cells. In each case the putative precursor was degraded by pronase E,

Fig. 5.8: Pronase E sensitivity of preTonA accumulated in the sarkosyl insoluble fraction during a short pulse labelling

A 400ml culture of PB72-47/pLG513 in glycerol minimal medium was grown to A_{450} 0.05, and half the culture maltose induced as previously. After 2 generations each culture was pulse labelled with 25μCi of ^{35}S -methionine for 60 sec then immediately poured into 50ml of ice cold bacterial buffer containing 0.5M sodium azide. Cells were fractionated and the membranes pronase E treated as described in the legend to Fig. 5.6. The samples were analysed by SDS PAGE (11% acrylamide I) and (i) autoradiography and (ii) Coomassie blue staining.

CY - cytoplasm; IM - inner membrane; OM - outer membrane


CY
IM
OM
pronaseE
maltose
pTonA
TonA
OmpF/C
OmpA
200
100
92·5
69
43
30
14·3
radioactive


CY
IM
OM
pronaseE
maltose
pTonA
TonA
LamB
OmpF/C
OmpA
stained

whereas the mature forms of LamB amd OmpF/C in the sarkosyl insoluble fraction were pronase E resistant.

5.9 Discussion

Previous results (Silhavy et al, 1979; Ito et al, 1981) have shown that the MalE-LacZ hybrid protein blocks the processing and assembly of outer membrane and periplasmic proteins; the results described in this chapter demonstrate that the hybrid also blocks processing of several inner membrane proteins, including PBP5 and PBP6. This result suggests that, rather than blocking a specific export system for outer membrane and periplasmic proteins, the MalE-LacZ hybrid may be inhibiting some part of the assembly/translocation pathway common to inner membrane, periplasmic and outer membrane proteins. Alternatively, the hybrid may be causing a generalised disruption of the organisation of the cell envelope.

It is conceivable that many signal peptidase molecules are sequestered into abortive processing complexes with the hybrid protein; however, increasing signal peptidase synthesis using the cloned signal peptidase gene does not alleviate the maltose sensitivity of PB72-47 (C. Watts, pers. comm.). The observation that processing is merely slowed down and not completely blocked in maltose induced cultures of PB72-47 is a further indication that signal peptidase inactivation may not be the primary effect of hybrid protein induction. Thus from pulse-chase experiments reported by Ito et al (1981), it was apparent that accumulated precursors were gradually processed to the mature form, although a small

proportion of the molecules remained unprocessed. This is true of TonA, which, in the presence of MalE-LacZ hybrid, accumulated in a precursor form that was slowly processed to the mature form (Fig. 5.7). Again, it appeared that a proportion of preTonA molecules were never processed, or processed extremely slowly, since substantial amounts of precursor were visible even in stained profiles (Fig. 5.8). It is possible that the refractory nature of this class of preTonA molecules was due to the attainment of an abberrant conformation or location which did not allow processing.

The fact that processing still occurs, albeit at a reduced rate, in maltose induced PB72-47, may explain the finding that ^{14}C -benzylpenicillin labelling only facilitated the detection of a small accumulation of prePBP5 and prePBP6 (Fig. 5.1), whereas, in the UV infection system, where only newly synthesised PBP5 was labelled, an almost complete inhibition of PBP5 processing was observed (Fig. 5.2).

The finding that preTonA was apparently able to associate into the sarkosyl insoluble fraction was unexpected. Halegoua & Inouye (1979a) demonstrated that preOmpF/C accumulated in the presence of phenethyl alcohol, although fractionating with the outer membranes on sucrose gradients, was sarkosyl soluble and completely degraded following trypsin treatment of whole cells with permeabilised outer membranes. On the basis of these results, Halegoua & Inouye (1979b) proposed that the accumulated preOmpF/C was anchored to the inner membrane by its N-terminal signal sequence, and associated

with the outer membrane fraction via ionic interactions (Fig. 5.9). Similarly, the results of DiRienzo & Inouye (1979) suggested that the preOmpA which accumulated under conditions of decreased membrane fluidity had, at least in part, crossed the inner membrane but remained sarkosyl soluble.

One explanation for the sarkosyl insolubility of preTonA is that this protein is able to associate into a sarkosyl resistant complex independently of the outer membrane, for example by forming protein aggregates in the inner membrane, cytoplasm or periplasm. Protein aggregates in the soluble fraction might be of sufficient mass to fractionate with the envelope during ultracentrifugation of cell lysates. An analogous situation has been described by S. J. S. Hardy (pers. comm.), who demonstrated that bacterial heat labile enterotoxin subunit B, a periplasmically located protein, may under some conditions (for example following osmotic lysis in Tris/Cl-EDTA buffer) fractionate with the envelope due to protein aggregation.

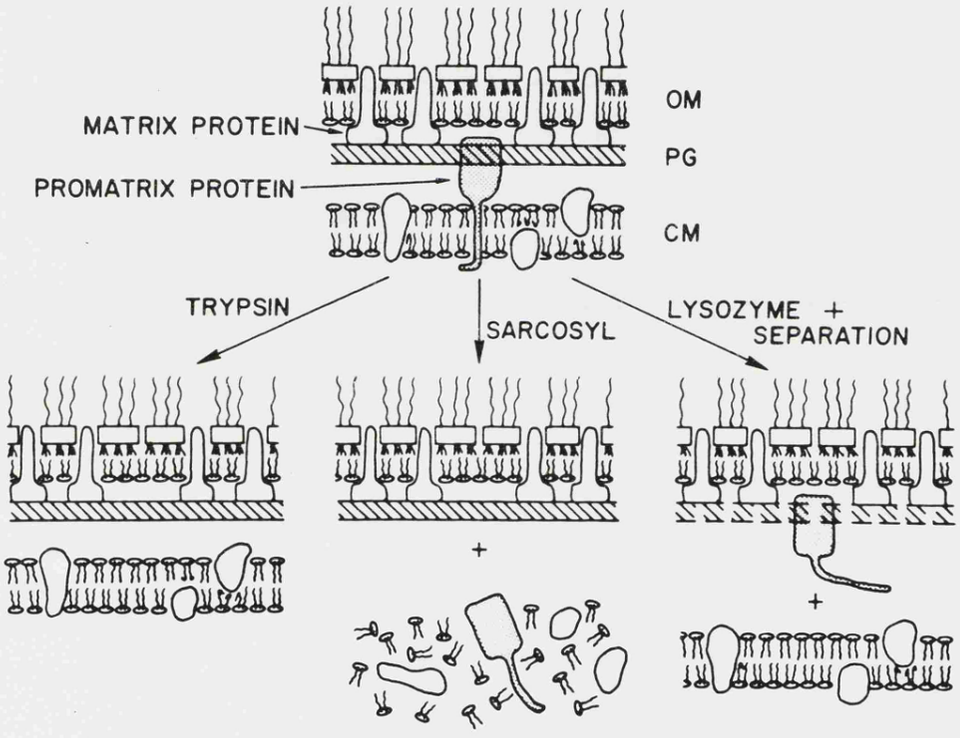
Alternatively, the preTonA accumulating in the presence of the MalE-LacZ hybrid protein might be anchored to the inner membrane by its signal sequence, with the bulk of the protein extending across the periplasmic space to the outer membrane as proposed by Halegoua & Inouye (1979b) for preOmpF/C. However, in the case of preTonA, perhaps the conformational restraints imposed by the persistence of the signal peptide are insufficient to prevent association into a sarkosyl insoluble complex involving interaction with outer membrane constituents. Whatever the nature of the sarkosyl

Fig. 5.9: Possible localisation in the envelope of preOmpA (prematrix protein) produced in phenethyl alcohol treated cells (Halegoua & Inouye, 1979b)

CM - inner membrane

PG - peptidoglycan

OM - outer membrane


MATRIX PROTEIN
PROMATRIX PROTEIN
OM
PG
CM
TRYPSIN
SARCOSYL
LYSOZYME +
SEPARATION
+
+

insoluble form of preTonA, the molecule was not correctly assembled into the outer membrane, as indicated by its sensitivity to pronase E. However, the fact that most of the TonA assembled in the presence of the hybrid protein ultimately became correctly integrated into the outer membrane, suggests that the location and conformation of the preTonA molecule was not completely non-physiological, and that by slowing down processing and/or translocation an intermediate in TonA assembly had been detected.

Ito et al (1981) reported that preOmpA and preOmpF/C which accumulated in the presence of the MalE-LacZ hybrid fractionated with the envelope when the cells were lysed in 200mM KCl, but with the soluble fraction following lysis in the absence of KCl. Thus Ito et al concluded that these precursors were only weakly associated with the envelope. The envelope associated preOmpF/C was further shown by Herrero et al (1982) to fractionate with the sarkosyl soluble envelope material, in contrast to the results obtained here for preTonA. Novel protein bands which might correspond to preLamB, preOmpF/C and preOmpA are detectable in the sarkosyl insoluble envelope fraction in Fig. 5.8. However, the majority of these precursors were recovered from the sarkosyl soluble envelope fraction, in agreement with the results of Herrero et al for preOmpF/C.

Some preTonA was recovered in the cytoplasmic and sarkosyl soluble envelope fractions (see Figs. 5.6 and 5.8). This may be indicative of weak association of preTonA with the envelope fraction as reported by Ito et al (1981) for

OmpA and OmpF. Alternatively, the cytoplasmic and sarkosyl soluble preTonA may represent genuine assembly intermediates. However, the assembly process occurring in the presence of the MalE-LacZ hybrid does not necessarily resemble the true *in vivo* pathway.

In order to more accurately localise the precursors accumulated in the presence of the MalE-LacZ hybrid, it would be necessary to carry out protease treatments of cells in which the outer membrane had been rendered protease permeable. If the precursors have partially or completely crossed the inner membrane they should be sensitive to such proteolytic treatment. Cytoplasmic or periplasmic protein aggregates could be separated from genuine envelope associated material by introducing the envelope sample at the bottom of a sucrose gradient. During subsequent centrifugation the membranes would float away from any protein aggregates, which remain at the bottom of the gradient (S. J. S. Hardy, pers. comm.). Such an experiment would determine whether the preTonA accumulated in the presence of the MalE-LacZ hybrid was in fact membrane associated, or merely present in the cell lysate in an aggregative form.

CHAPTER SIX

Tn1000 MUTAGENESIS OF THE tonA GENE

6.1 Introduction

In order to carry out *in vitro* mutagenesis of the *tonA* gene it was necessary to know the precise location and orientation of the *tonA* gene within the SalI B and C fragments of pLG515 (Fig. 2.1). Mutagenesis by Tn1000 (formerly known as $\gamma\delta$)(Guyer, 1978) represents a relatively rapid method for defining regions of plasmid DNA encoding particular genes. Tn1000 is carried by the conjugative plasmid F. Plasmid pBR325 is non-mobilisable, ie., even in the presence of an F plasmid, cannot be independently transferred to a recipient. However, as part of a transposition complex with the Tn1000 element of F, pBR325 may be transferred to the recipient cell. The transposition complex is resolved in the recipient, producing a pBR325 derivative bearing a random Tn1000 insertion (Fig. 6.1). By restriction analysis of Tn1000 insertions inactivating particular plasmid encoded functions, regions of the DNA required for each function may be defined. It was decided to apply this technique of Tn1000 mutagenesis to pLG515, a pBR325 derivative. In addition, it was hoped that analysis of the truncated TonA' polypeptide products resulting from insertion of Tn1000 into *tonA* might reveal the direction of transcription of the gene.

Fig. 6.1: Postulated mechanism of Tn1000 mutagenesis

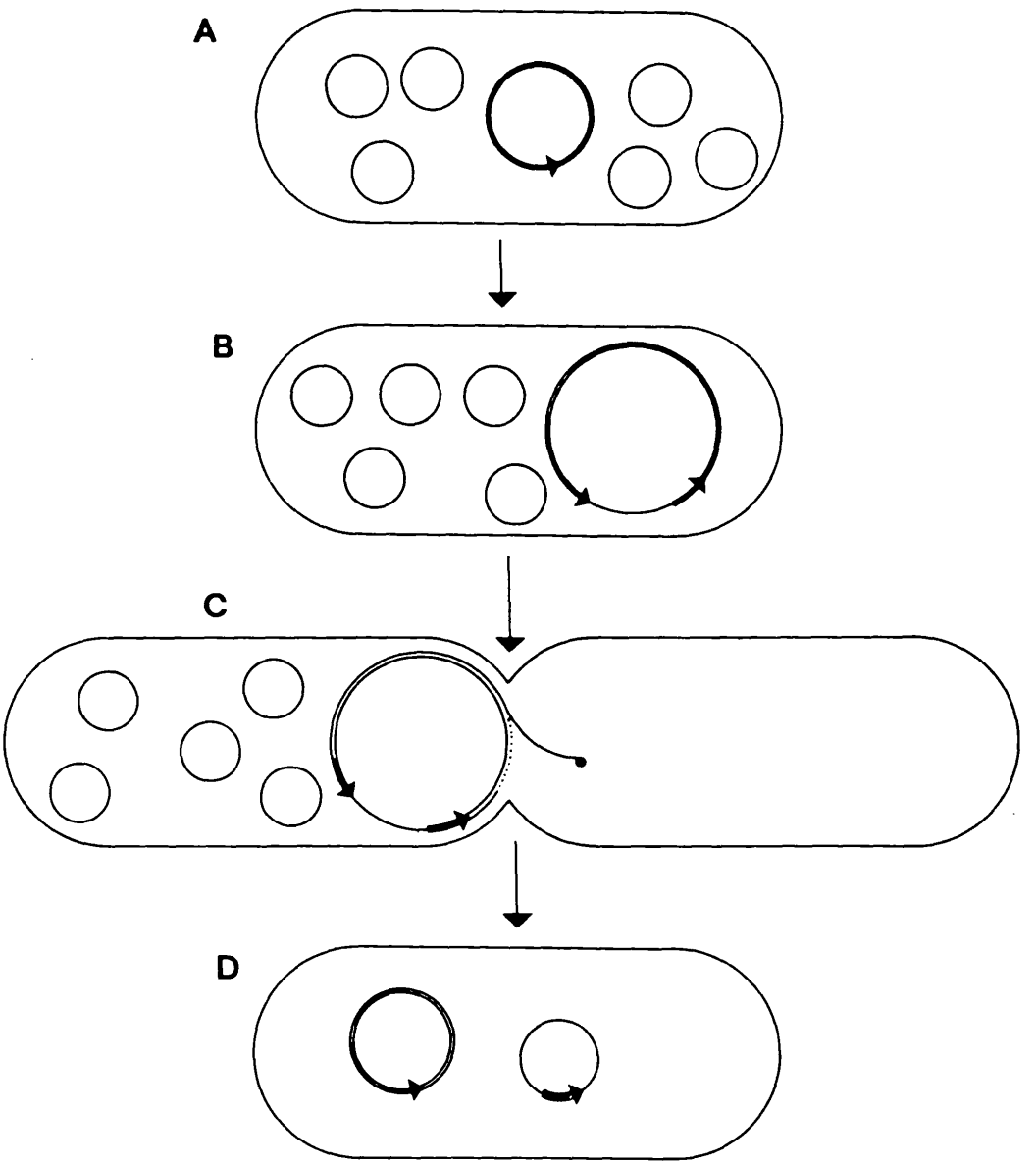
A: The non-mobilisable plasmid to be mutagenised is introduced into a strain harbouring an F factor carrying Tn1000.

B: Transposition by Tn1000 into a copy of the non-mobilisable plasmid results in formation of a cointegrate plasmid.

C: The cointegrate plasmid is transferred to a recipient during conjugation.

D: Resolution of the cointegrate in the recipient cell, forming the F plasmid in its original state, and the non-mobilisable plasmid carrying a copy of Tn1000.

(Diagram from an original by N. G. Stoker)


A
B
C
D

6.2 .2 Tn1000 mutagenesis of pLG515

In n order to facilitate the analysis of the protein products enco:ncoded by the pLG515::Tn1000 plasmids, the minicell prod:roducing strain DS410 (Sm^r) was chosen as a recipient. In orde:rder to allow detection of active plasmid encoded tonA gene:enes, a tonA$^-$ derivative of DS410 was isolated. pLG515 was tran:ransformed into RB308 (Sm^s), which carries an F^+ conjugative plas:lasmid. The resultant strain, RB308/pLG515, was mated with DS41S410tonA, and the mating mixture plated on nutrient agar plat:lates, selecting for resistance to: (i) streptomycin; (ii)ii) tetracycline; (iii) streptomycin and tetracycline; and (iv)iv) streptomycin, tetracycline and bacteriophage T5.

' Tc^r, Sm^r transconjugants were obtained at a frequency of 1.3 x.3 x 10^{-4} per donor cell, and $T5^r$, Tc^r, Sm^r transconjugants at at a frequency of 4.9 x 10^{-5} per donor cell. These $T5^r$, Tc^r, Sm^r m^r clones were subsequently used to map the tonA gene. The trans:ransconjugants selected on streptomycin and tetracycline were:ere screened for T5 sensitivity, and those clones which were T5 5 sensitive were also mapped. In addition the trans:ransconjugants were scored for sensitivity to phage T1. All clon:lones were found to have the same sensitivity to T1 as to T5. 5.

6.3 F.3 Restriction analysis of the Tn1000 insertions

(i) S) Strategy: Fig. 6.2 shows the restriction map of Tn1000 and id the two possible orientations of the transposon within pLG51LG515. Mini-Birnboim plasmid preparations were used to prepprepare DNA for restriction analysis. The positions of Tn1000

Fig. 6.2a: Mapping Tn1000 insertions inactivating tonA within pLG515

Transpositions in orientation A:
EcoRI cuts the smaller SmaI fragment into fragments of 0.8kb, 1.1kb and fragment X. Fragment X is sized, and X - 0.9kb (the distance of the end from the nearest EcoRI site) is the distance of the Tn1000 insertion from the SmaI site of pLG515

Transpositions in orientation B:
The smaller SmaI fragment Y is sized and Y - 2.5kb (the distance between the Tn1000 SmaI site and the end) is the distance of the Tn1000 insertion from the SmaI site of pLG515

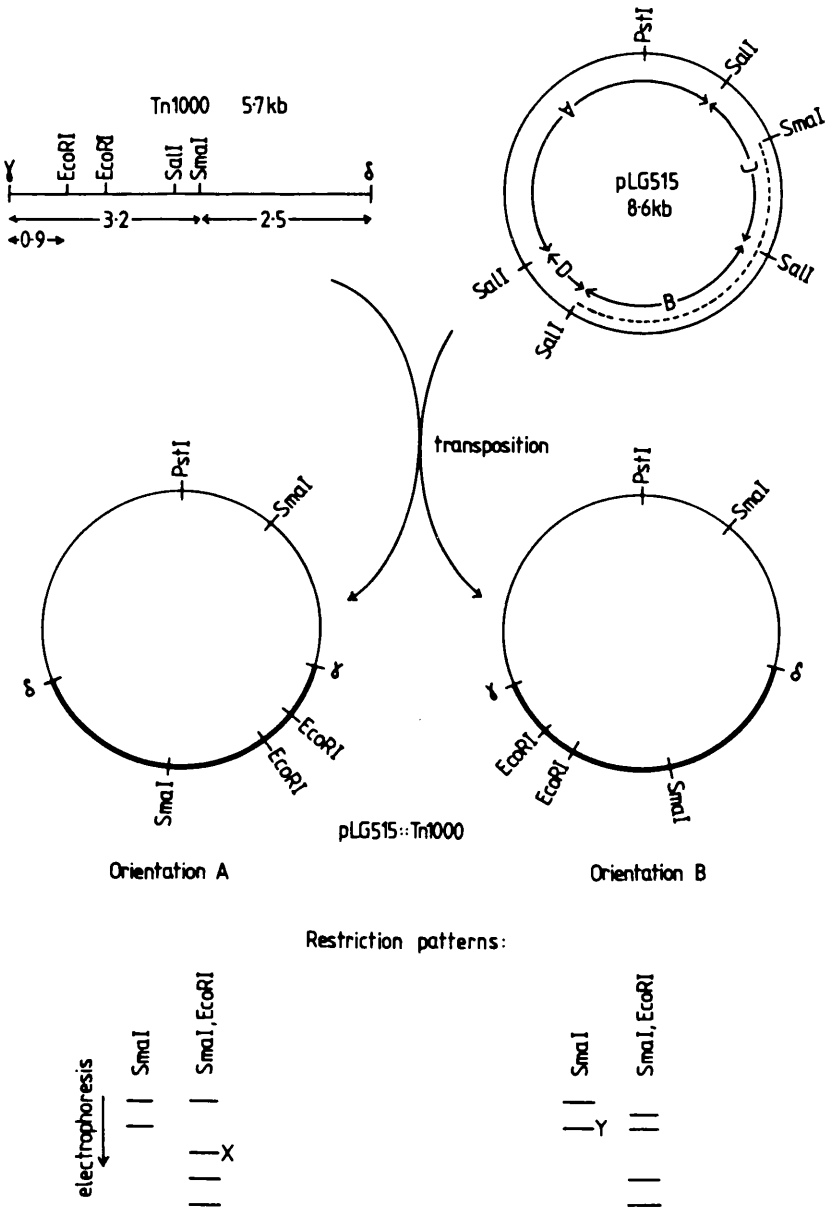

Tn1000 5·7kb
EcoRI
EcoRI
SalI
SmaI
γ
δ
0·9
3·2
2·5
pLG515
8·6kb
PstI
SalI
SmaI
A
C
D
B
transposition
pLG515::Tn1000
Orientation A
Orientation B
Restriction patterns:
electrophoresis
SmaI
SmaI, EcoRI
X
Y

Fig. 6.2b: Restriction digests of pLG515::Tn1000 clones

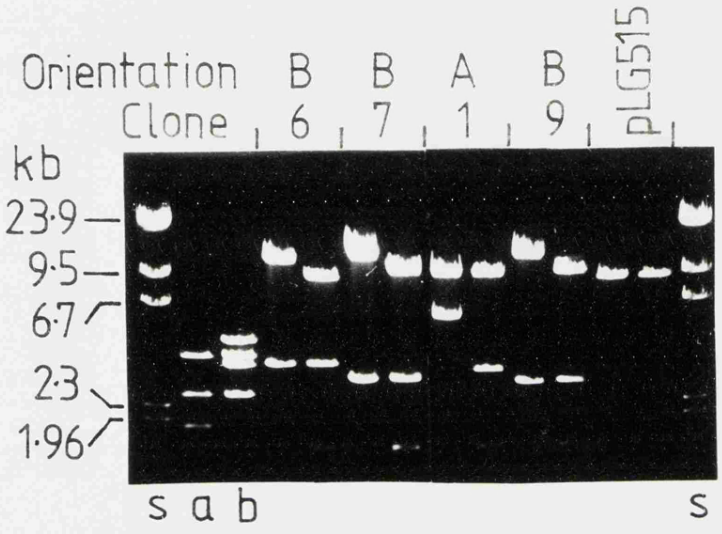
s - λ x HindIII standards

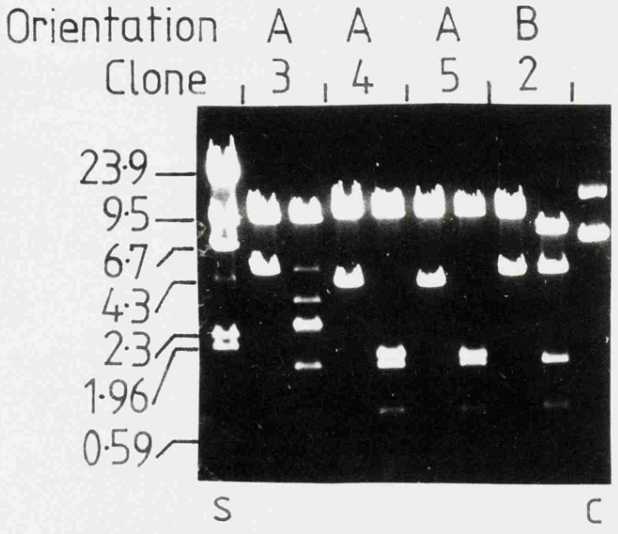
a - pLG515 x SalI

b - clone 9 x SalI

c - clone 9, unrestricted

In each case, first track is the SmaI digest, second track is the SmaI x EcoRI double digest.


Orientation B B A B
Clone 6 7 1 9 pLG515
kb
23·9
9·5
6·7
2·3
1·96
s a b s

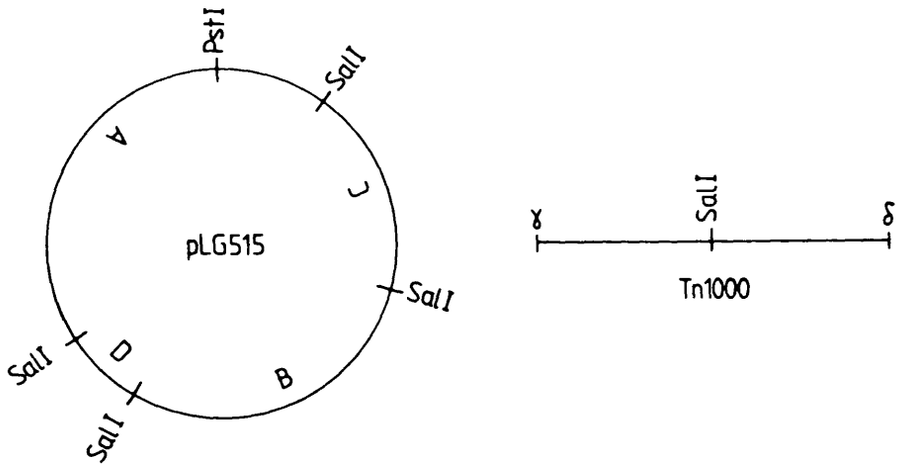

Orientation A A A B
Clone 3 4 5 2
23·9
9·5
6·7
4·3
2·3
1·96
0·59
s c

insertions which inactivated tonA were mapped using SmaI and SmaI x EcoRI double digests. A SmaI digest yields two fragments; by sizing the smaller fragment and subtracting either 2.5kb or 3.2kb depending on the orientation of the transposon, the distance of the insertion from the SmaI site was determined. The orientation of Tn1000 was determined using the SmaI x EcoRI double digest (Fig. 6.2).

To map insertions which did not inactivate tonA, a SalI digest was first performed to locate the transposon to one of the four SalI fragments (Fig. 6.3). Insertions mapping in the SalI A and D fragments were not analysed further. Insertions into SalI B were mapped as for insertions inactivating the tonA gene. Insertions into the SalI C fragment were mapped by sizing the two novel SalI fragments and combining this data with that from the SmaI and SmaI x EcoRI digests.

(ii) Results: Ten insertions which inactivated tonA mapped as shown in Fig. 6.4. DNA was prepared from 35 clones still possessing an active tonA gene; of these, 21 yielded plasmids of the size expected for pLG515 carrying a Tn1000 insertion, and 14 yielded either no plasmid DNA or plasmids of the wrong size. These 14 clones were discarded. SalI digests of the remaining 21 clones showed 16 insertions to map in SalI A, 3 in SalI B, and 2 in SalI C. These latter 5 insertions were mapped more accurately as shown in Fig. 6.4. The insertions inactivating the tonA gene defined a 2kb region, therefore providing a minimum estimate of the region of DNA encoding TonA. Since the TonA precursor is an 80kD protein, a tonA

Fig. 6.3a: Assignment of Tn1000 insertions not inactivating tonA to one of the four SalI fragments of pLG515



SalI digests of:-

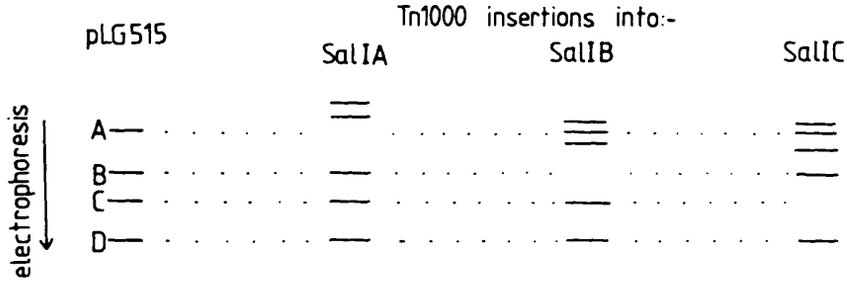


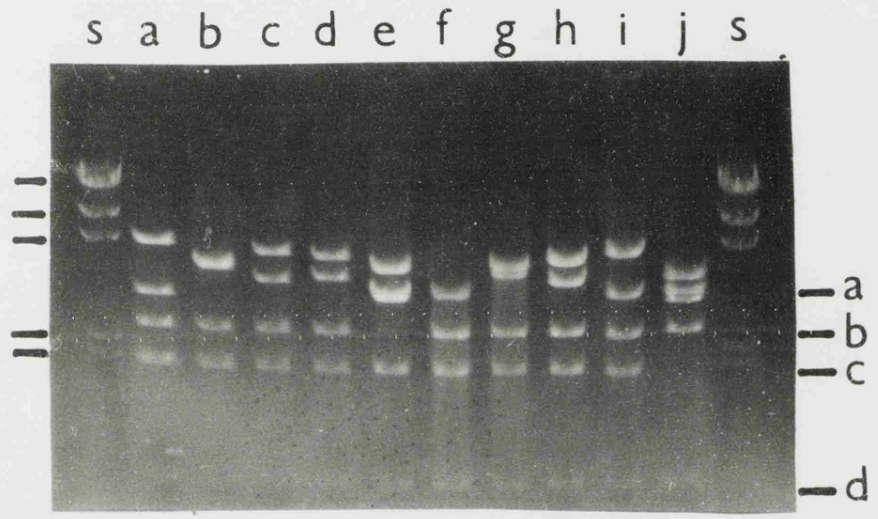
Fig. 6.3b: SalI digests of pLG515::Tn1000 clones

s - λ x HindIII standards

f - pLG515 x SalI

j - pLG515::Tn1000-9 x SalI

other tracks represent pLG515::Tn1000 clones not used in subsequent analysis


s a b c d e f g h i j s
a
b
c
d

gene of approximately 2kb would be expected. Thus it appears that insertions over the whole length of tonA inactivate the gene, and therefore it can be concluded that the whole of the TonA protein is important in assembly or function, at least with respect to T1 and T5 adsorption.

6.4 Analysis of proteins encoded by pLG515::Tn1000 plasmids

The polypeptides encoded by the pLG515::Tn1000 plasmids were analysed both in vitro, using the Zubay transcription-translation system, and in vivo, using minicells, and later, maxicells. Maxicells were eventually chosen as an in vivo expression system rather than minicells due to difficulties experienced in isolating usable minicells from these strains. The pLG515::Tn1000 plasmids were therefore transformed into a tonA$^-$ derivative of CSH26ΔF6, a strain suitable for use in the maxicell system. The products of in vivo and in vitro expression are shown in Fig. 6.5.

Both in vivo and in vitro, a family of apparently truncated TonA' polypeptides were synthesised from the plasmids carrying a Tn1000 insertion inactivating the tonA gene. By comparison of the map positions of Tn1000 insertions with the size of the corresponding TonA' polypeptides, the direction of transcription of the tonA gene was deduced (Fig. 6.4).

The doublet bands produced in maxicells by several of the pLG515::Tn1000 plasmids, including pLG515::Tn1000-9 (encoding wild type TonA protein), probably represented precursor and mature forms of the same polypeptide. This was confirmed by

Fig. 6.4: Restriction map of pLG515, showing positions of 14 Tn1000 insertions (arrows)

Insertions inactivating the tonA gene (including 1-8) define the minimum coding sequence of the gene; direction of transcription is deduced from sizing the truncated polypeptides produced (see section 6.4). Sequences derived from pLC19-19 are indicated by a heavy line.

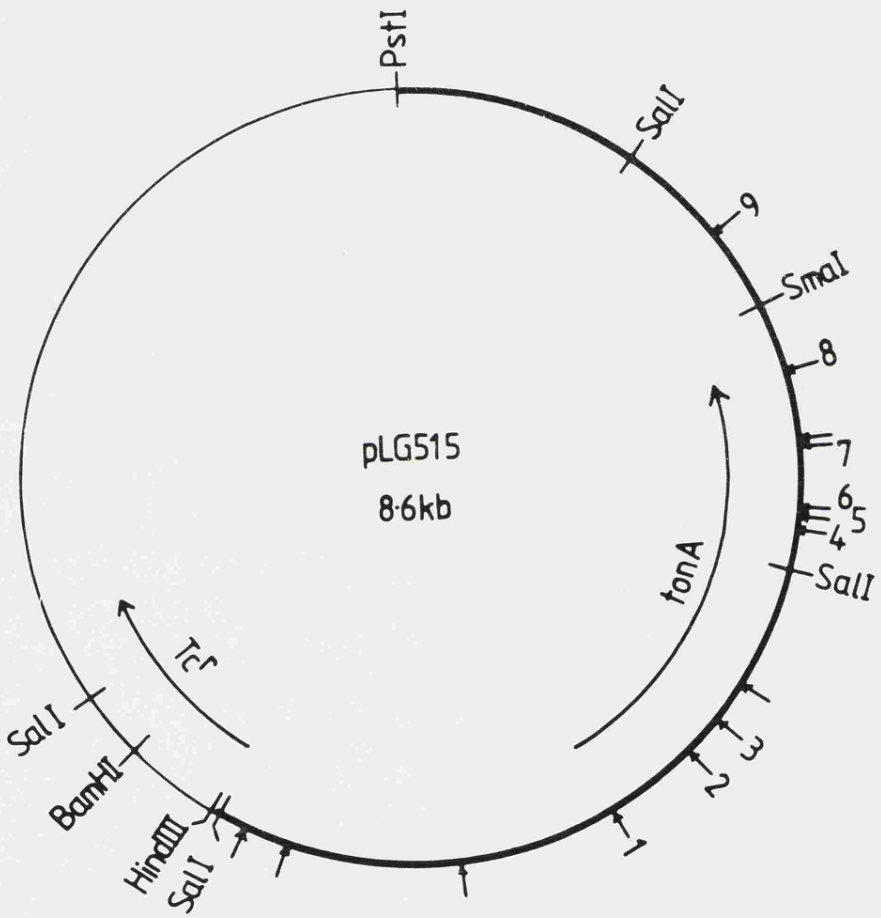

PstI
SalI
9
SmaI
8
7
6
5
4
SalI
tonA
1
2
3
Tc
SalI
BamHI
HindIII
SalI
pLG515
8·6kb

Fig. 6.5: Products of in vitro and in vivo expression of pLG515::Tn1000 plasmids

^{35}S -labelled products of (i) transcription-translation incubations using 2.5µg pLG515::Tn1000 DNA with MRE600 extract, and (ii) maxicells carrying pLG515::Tn1000 plasmids, run on 11% acrylamide I gels and autoradiographed.

Positions of marker proteins shown at left of each gel; position of in vitro TonA' products indicated at right of Zubay gel.

C - control tracks, no plasmid

pTonA - preTonA

pLG515::Tn1000-9 encodes wild type TonA

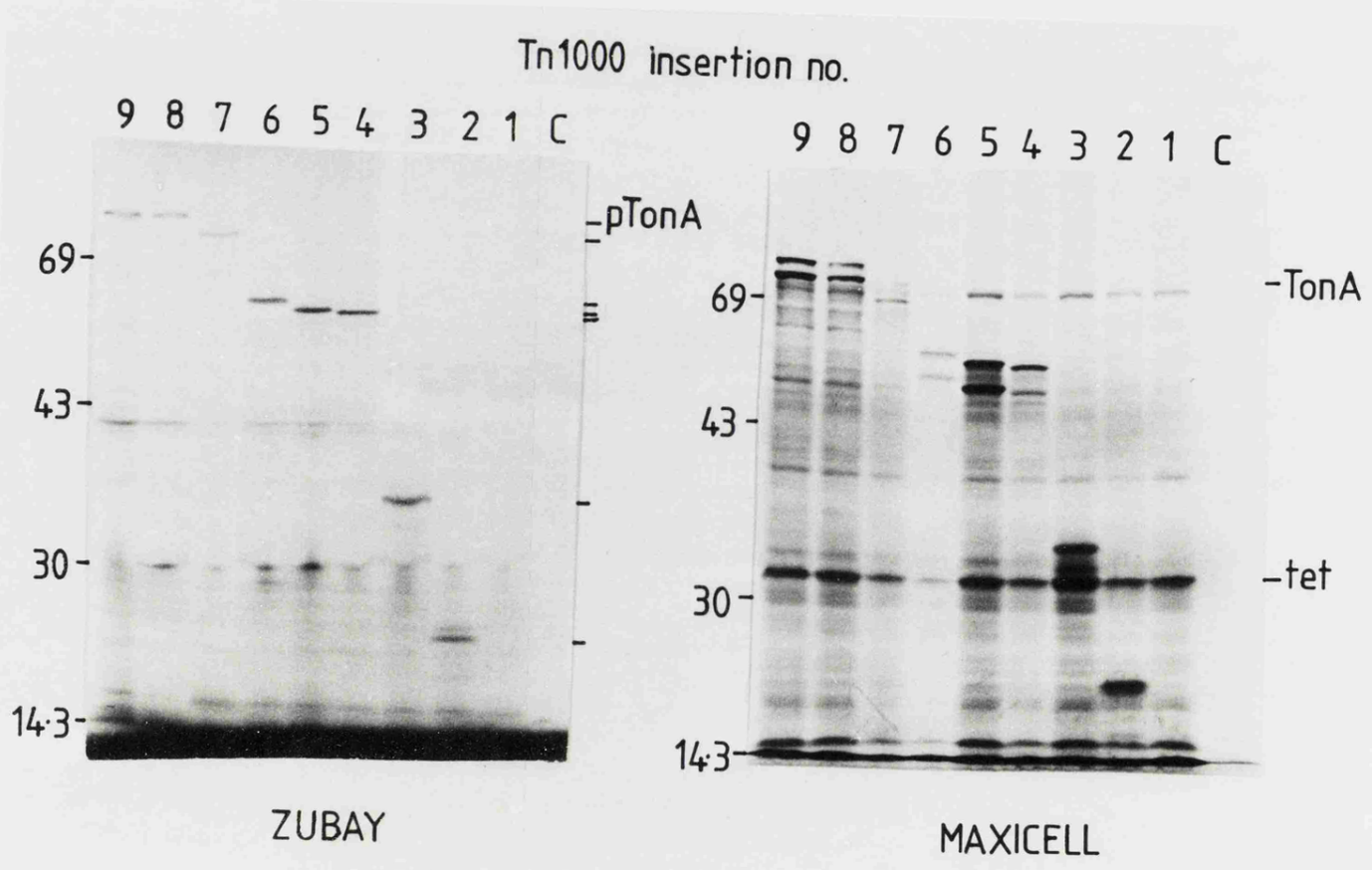

Tn1000 insertion no.
9 8 7 6 5 4 3 2 1 C
9 8 7 6 5 4 3 2 1 C
pTonA
TonA
tet
69
43
30
14·3
69
43
30
14·3
ZUBAY
MAXICELL

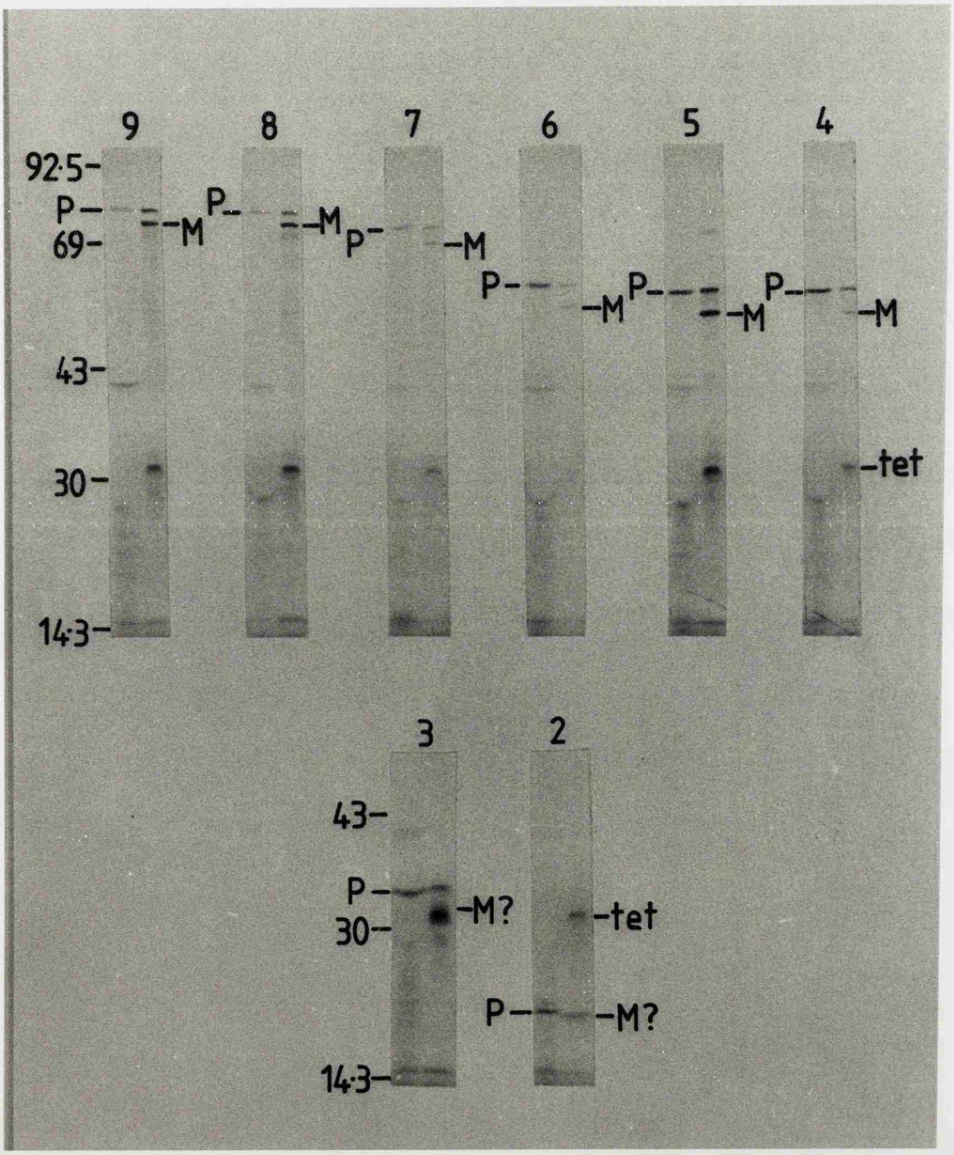
Fig. 6.6: Comparison of *in vitro* and *in vivo* products synthesised from pLG515::Tn1000 plasmids

Zubay and maxicell samples from Fig. 6.5 were rerun to give a direct comparison between the *in vitro* and *in vivo* products from each plasmid. In each case first track is the Zubay sample, second track is the maxicell sample.

Position of M.W. standards is shown to left of gels

P - precursor; M - mature form

For pLG515::Tn1000-3 a band just above tet^r may be a processed form (visible more clearly in Fig. 6.5). For pLG515::Tn1000-2 the maxicell product does appear slightly smaller and may represent a processed form.


9
8
7
6
5
4
92·5-
P-
69-
-M
P-
-M
P-
-M
P-
-M
P-
-M
P-
-M
43-
30-
-tet
14·3-
3
2
43-
P-
30-
-M?
-tet
P-
-M?
14·3-

running the *in vitro* and *in vivo* products side by side on SDS polyacrylamide gels (Fig. 6.6). The *in vitro* synthesised precursor forms of wild type TonA and of TonA' polypeptides 8 and 7 comigrated with the larger of the two *in vivo* synthesised proteins; thus these polypeptides do indeed appear to display precursor and processed forms.

6.5 Discussion

Using the Tn1000 mutagenesis data, both the position and direction of transcription of *tonA* were mapped. In addition, the truncated TonA' polypeptides produced could be used to investigate the requirement for an intact C-terminus in TonA assembly (see Chapter 7).

Strikingly, pLG515::Tn1000-8 encoded a polypeptide virtually identical in molecular weight (by SDS PAGE analysis) to the wild type TonA protein encoded by pLG515::Tn1000-9, implying that Tn1000 insertion 8 is very close to the C-terminal coding region of the gene, and yet pLG515::Tn1000-8 did not confer T1 or T5 sensitivity on the host strain.

This result might be explained in several ways:

(i) The C-terminus of TonA is important for phage receptor activity. Thus a truncated TonA polypeptide might assemble correctly into the outer membrane, yet still be functionally inactive;

(ii) The C-terminus might be of critical importance in determining the correct localisation of TonA protein.

Specific sequences present at or near the C-terminus might be important in this respect;

(iii) The TonA' polypeptides may possess Tn1000 encoded amino acid sequences at their C-termini which prevent normal function and/or assembly. Such an effect on assembly has been observed with β-galactosidase fusions to MalE or LamB (Silhavy et al, 1979);

(iv) Finally, the TonA' polypeptides may be degraded very rapidly in exponentially growing cells, either before, or during, the assembly process. Some of these possibilities are investigated further in Chapter 7.

As discussed in Chapter 1, processing does not normally occur until synthesis of periplasmic and membrane proteins is at least 80% complete. Assuming translocation to be cotranslational, a large proportion of the molecule must therefore have been translocated in cases where processing occurs. Similarly, treatment of cells with phenethyl alcohol or dissipation of the membrane potential leads to inhibition of both translocation and processing (section 1.IV.2), indicating the dependence of the latter on at least the early stages of assembly. On the basis of this argument it appears probable that translocation of the larger TonA' polypeptides is at least initiated normally, bringing the polypeptides into contact with a signal peptidase. Experiments undertaken in order to localise the TonA' polypeptides within the cell are described in Chapter 7.

CHAPTER SEVEN

INVESTIGATION OF TRUNCATED TonA POLYPEPTIDES PRODUCED BY Tn1000 MUTAGENESIS

7.1 Introduction

As described in Chapter 6, a family of truncated TonA polypeptides were isolated using Tn1000 mutagenesis of the tonA gene. It was hoped that further study of these truncated forms of TonA might reveal the necessity for an intact C-terminus in TonA assembly.

All the isolated Tn1000 insertions into tonA led to inactivation of the gene. Four possible explanations for this effect are as follows:

(i) The C-terminus is important in phage receptor activity;

(ii) Assembly of TonA requires an intact C-terminus;

(iii) Tn1000 encoded sequences at the C-terminus interfere with assembly;

(iv) Truncated TonA polypeptides are degraded before they can be assembled.

By the experiments described in this chapter it was hoped to distinguish between these possibilities.

7.2 Stability of truncated TonA polypeptides in maxicells and evidence for processing

It was observed in several different maxicell experiments that labelling of the truncated TonA polypeptides tended to be inefficient when compared to the quantity of labelled wild type TonA present. This effect could be due to instability of

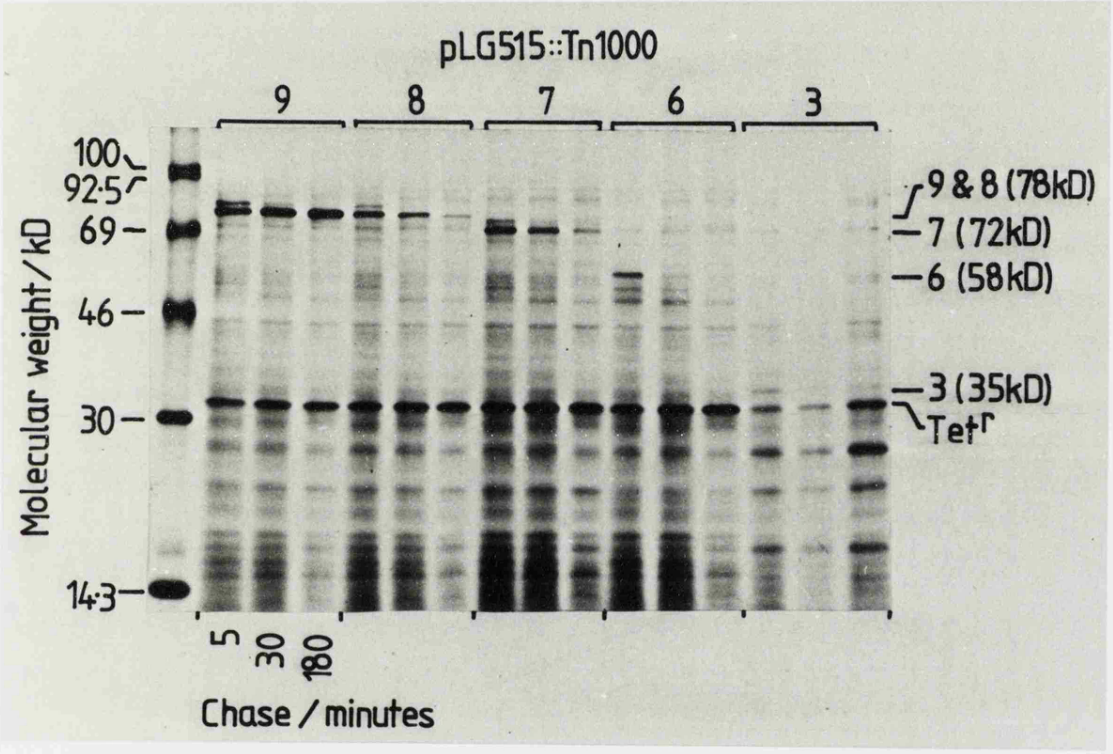
the truncated polypeptides. Such instability might be confirmed by a pulse-chase experiment. In addition, a pulse-chase would show whether, as discussed in Chapter 6, a precursor-product relationship exists between the two apparent forms of several of the TonA' polypeptides observed after SDS PAGE (Figs. 6.5 & 6.6).

Maxicells were prepared from CSH26ΔF6*tonA* carrying various pLG515::Tn1000 plasmids, labelled for 15 min with ^{35}S -methionine, and then chased with cold methionine. Samples taken during the chase were analysed by SDS PAGE (Fig. 7.1). The wild type TonA protein encoded by pLG515::Tn1000-9 was completely stable during the 180 min chase period; moreover, processing of the precursor to the mature form was observed during the chase. However, it was clear that the TonA' polypeptides encoded by pLG515::Tn1000-8, 7 and 6 were all unstable, and mostly degraded during the chase. Nevertheless, it is apparent that the upper band of the doublet synthesised by maxicells of pLG515::Tn1000-8 and 7 disappears at a similar rate to that shown by the precursor form of wild type TonA in the same system. Thus it can be concluded from these experiments that the doublet bands produced in maxicells do indeed represent precursor and mature forms of the same protein.

Fig. 7.1: Pulse-chase of maxicells synthesising TonA' polypeptides truncated by Tn1000 insertion

600µl of maxicells were incubated with 50µCi of ^{35}S - methionine for 15 min at 30°C, then 50µl of 44mg/ml cold methionine was added (time 0) and 200µl samples taken at times 5, 30 and 180 min during the chase. Samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

pLG515::Tn1000-9 encodes wild type TonA protein


pLG515::Tn1000
9
8
7
6
3
100
92·5
69
46
30
14·3
Molecular weight / kD
9 & 8 (78kD)
7 (72kD)
6 (58kD)
3 (35kD)
Tet^r
5
30
180
Chase / minutes

7.3 Amino acid sequences encoded by Tn1000 ends

The observed instability of the TonA' polypeptides did not appear severe enough to account for the T1 and T5 resistance of the mutant clones. An alternative explanation of the inability of even the larger TonA' polypeptides to act as phage receptors is that a substantial portion of the sequence of these polypeptides is encoded by Tn1000 rather than by the tonA gene. The first 50 base pairs at either end of Tn1000 have been sequenced (Reed et al, 1979) and thus the amino acid sequences which might be fused to the tonA encoded sequences can be predicted (Fig. 7.2). In 2 of the 6 reading frames, the third codon encountered is a "stop" codon, and thus only 2 amino acids should be added to the polypeptide. In one case, translation should terminate after 11 amino acids have been added to the tonA encoded sequences, but for the remaining 3 reading frames there are no stop codons within the sequenced region. A considerable length of abnormal polypeptide sequence may be added to the TonA specific sequences in these cases.

7.4 Comparison of TonA' polypeptides encoded by pLG515::Tn1000-8 and 9 by partial proteolysis

As indicated in the previous section, a considerable proportion of the C-termini of the TonA' polypeptides may be encoded by Tn1000 DNA in some cases. A comparison of the partial proteolysis products of wild type TonA with those of the truncated polypeptides should reveal any large differences in sequence.

γ end

```
                -10             -20             -30             -40             -50
  G G G G T T T G A G G G C C A A T G G A A C G A A A A C G T A C G T T T A T G G T A T A A C T T A T

frame A . gly . val . STOP
      B   ? . gly . leu . arg . ala . asn . gly . thr . lys . thr . tyr . val . tyr . gly . ile . thr . tyr ......
      C ? . gly . phe . glu . gly . gln . trp . asn . glu . asn . val . arg . leu . trp . tyr . asn . leu ......
```

δ end

```
                -10             -20             -30             -40             -50
  G G G G T T T G A G G G C C A A T G G A A C G A A A A C G T A C G T T A A G G A G A T A A T T C G T

frame A . gly . val . STOP
      B   ? . gly . leu . arg . ala . asn . gly . thr . lys . thr . tyr . val . lys . glu . ile . lys . arg ......
      C ? . gly . phe . glu . gly . gln . trp . asn . glu . asn . val . arg . STOP
```

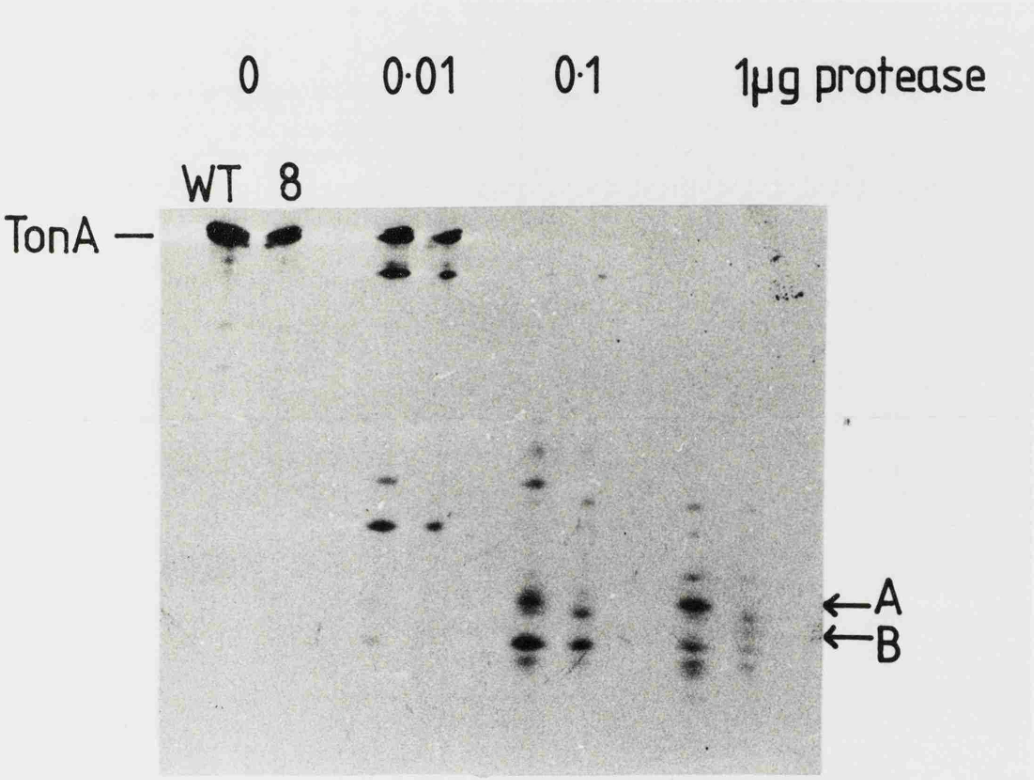
Fig. 7.2: DNA sequences encoded by Tn1000 ends (Reed et al, 1979) and possible translation products in all three frames.

Fig. 7.3: Comparison of TonA derived polypeptides encoded by pLG515::Tn1000-8 and 9(WT) by partial proteolysis

The ^{35}S -methionine labelled 78kD products from maxicells of CSH26 F6tonA/pLG515::Tn1000-8 and 9 were excised from gels and subjected to partial proteolysis with Staphylococcus aureus V8 protease (0, 0.01, 0.1 and 1µg). The products of proteolysis were separated on a 15% acrylamide I gel and detected by fluorography.

A : Fragment unique to digest of wild type TonA

B : Fragment unique to digest of TonA'-8


0
0·01
0·1
1µg protease
WT
8
TonA
A
B

Maxicells of CSH26 ΔF6*tonA* harbouring pLG515::Tn1000-8 and 9 were labelled with ^{35}S -methionine, the products subjected to SDS PAGE and the 78kD band cut out prior to partial proteolysis with *Staphylococcal* V8 protease. Analysis of the resulting peptides by SDS PAGE (Fig. 7.3) showed that the 78kD polypeptide encoded by pLG515::Tn1000-8 was indeed mainly composed of peptides in common with wild type TonA, although one fragment was missing and another novel peptide was present (arrowed). This result provided confirmation that the 78kD polypeptide encoded by pLG515::Tn1000-8 is indeed a derivative of the TonA protein, and maximally possesses only a small region of Tn1000 encoded sequence. Peptide fragment A, absent in the digest of TonA' polypeptide 8 may represent the wild type C-terminal fragment, and the novel peptide B present in the digest of polypeptide 8 may represent the new, partly Tn1000 encoded C-terminus.

7.5 Localisation of truncated TonA polypeptides in maxicells

Since processing of the larger TonA' polypeptides seemed to occur in maxicells with the same efficiency as processing of wild type TonA, it appeared that at least the initial stages of assembly were occurring for these truncated polypeptides. Consequently, attempts were made to determine the precise cellular localisation of these truncated forms of TonA. Although, as shown in Fig. 7.1, these proteins are relatively unstable, they do persist long enough in maxicells to allow their localisation to be determined. Maxicells were chosen

for these initial studies on localisation because of the low background labelling of chromosomally encoded proteins.

Fig. 7.4 shows the distribution in various maxicell fractions of the truncated TonA polypeptides. Also included in Fig. 7.4 are soluble and envelope fractions of maxicells harbouring the plasmid vectors pACYC184 and pBR325 as controls in order to confirm that the normal system of fractionation was operable in maxicells. Thus the cytoplasmic protein, CAT, encoded by both of these plasmids was found mainly in the soluble fraction, as expected, although there was some contamination of the envelope fraction; ß-lactamase, a periplasmic enzyme, was also found in the soluble fraction, which here includes both periplasm and cytoplasm. The 32kD tetracycline resistance protein, an inner membrane protein, fractionated with the envelope, subfractionating with the sarkosyl soluble material.

Fig. 7.4 shows that the wild type TonA protein encoded by pLG515::Tn1000-9 fractionated, as anticipated, mainly with the sarkosyl insoluble (outer membrane) fraction of the envelope. Some of the TonA protein fractionated with the cytoplasmic/periplasmic and sarkosyl soluble fractions; this may be due, in part, to overloading of the outer membrane with TonA since the cells were continuously synthesising this protein without corresponding growth of the outer membrane. It is interesting to note that in the cytoplasmic/ periplasmic fraction the ratio of precursor to mature protein was reversed from that found in all the other fractions, perhaps indicating an unprocessed assembly intermediate in

Fig. 7.4: Fractionation of maxicells prepared from strain carrying various pLG515::Tn1000 plasmids

For each fractionation, 10ml of cells at A_{450} 0.5 were irradiated and labelled as described in 11.13. Fractionation was carried out as described in 11.6 with the following modifications: 10mM $MgCl_2$ was present in the sodium phosphate buffer and envelopes were pelleted from the sonicates for 60 min at 58krpm. Samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

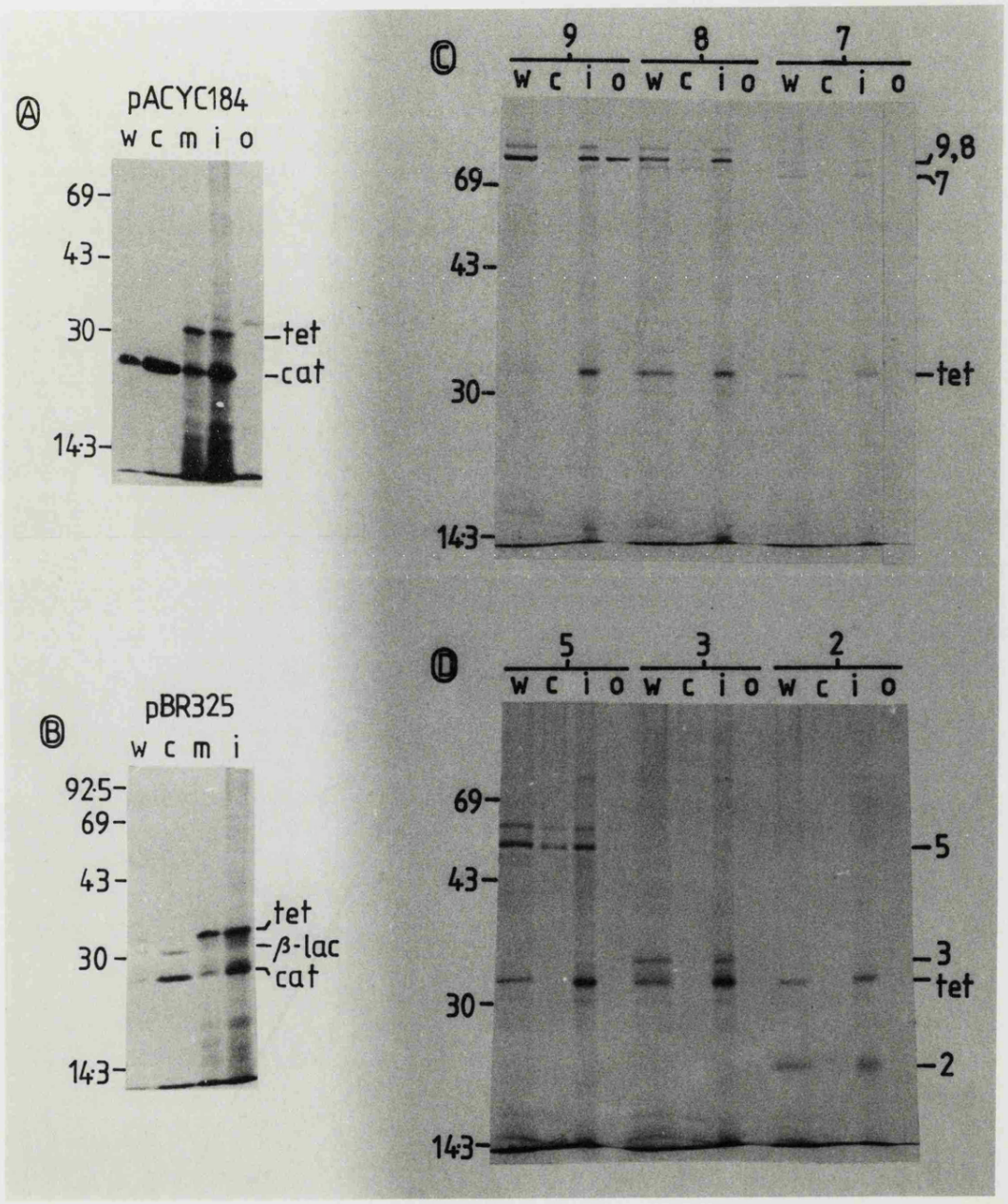
A & B : control fractionations of maxicells prepared from CSH26ΔF6tonA carrying pACYC184 and pBR325

C & D : fractionations of maxicells prepared from CSH26ΔF6 tonA carrying pLG515::Tn1000 clones 9, 8, 7, 5, 3 and 2

Positions of M.W. standards shown to the left of each panel

Positions of the TonA' polypeptides are indicated by the clone numbers to the right of panels

- tet - tetracycline resistance protein (im)
- β-lac - β-lactamase (periplasmic)
- cat - chloramphenicol acetyl transferase (cyto)
- w - whole cell
- c - cytoplasm plus periplasm
- m - total membrane
- i - inner membrane
- o - outer membrane


A
pACYC184
w c m i o
69-
43-
30-
143-
-tet
-cat
B
pBR325
w c m i
92·5-
69-
43-
30-
143-
tet
-β-lac
cat
C
9
8
7
w c i o w c i o w c i o
69-
43-
30-
143-
9,8
7
-tet
D
5
3
2
w c i o w c i o w c i o
69-
43-
30-
143-
-5
-3
-tet
-2

this system. In complete contrast to wild type TonA, the TonA' polypeptides appeared to fractionate mainly with the sarkosyl soluble envelope fraction, showing quite clearly that the TonA' polypeptides behaved differently from wild type TonA. This indicates either:

(i) A true inner membrane localisation, with the truncated forms, although processed, unable to complete transfer to the outer membrane;

(ii) That the truncated polypeptides are present in the outer membrane, but unable to associate into the sarkosyl insoluble complex due to the absence of the C-terminus; or

(iii) That the truncated polypeptides are spanning both membranes.

7.6 Fractionation of pulse labelled exponentially growing cells carrying pLG515::Tn1000 plasmids

Exponentially growing cells are a more truly *in vivo* system than maxicells; for instance precursor forms of envelope and periplasmic proteins are difficult to detect in growing cells, whilst, as can be seen from Fig. 7.1, precursors persist for several minutes in maxicells. The TonA' polypeptides fractionated with the envelope of maxicells, therefore, assuming that they behave similarly in growing cells, it should be possible to detect the truncated polypeptides. On the other hand, if the truncated polypeptides fractionated with the cytoplasm of growing cells they would be difficult to detect amongst the other cellular proteins.

Exponentially growing cells at 30°C of MC4100tonA carrying different pLG515::Tn1000 plasmids were pulse labelled for 1 min with ^{35}S -methionine, followed by a 1 min chase. The cells were then fractionated into total lysate, cytoplasm, periplasm, total envelope, inner membrane (sarkosyl soluble) and outer membrane (sarkosyl insoluble) and the fractions analysed by SDS PAGE. As can be seen from Fig. 7.5, although wild type TonA was clearly visible in the total lysate, envelope and outer membrane fractions, the TonA' polypeptides were difficult to detect. Very small amounts of protein in the outer membrane fractions which probably correspond to the TonA' polypeptides 7 and 8 were observed. A protein of the expected molecular weight of TonA'-7 was also apparent in total envelope and inner membrane fractions. However, except perhaps in the case of TonA'-7, the detectable levels of the truncated polypeptides are severalfold less than the amount of wild type TonA protein.

It is apparent from these results that the TonA' polypeptides behave radically differently from wild type TonA protein. The truncated polypeptides may be undetectable in lysates and inner membrane fractions due to a combination of lower levels of TonA' polypeptides and masking by other proteins. Such low levels of TonA' polypeptides relative to wild type TonA protein could be attributable to a far greater instability of the truncated proteins in growing cells compared with maxicells. Alternatively, the TonA' polypeptides may not be synthesised in growing cells at the

Fig. 7.5: Fractionation of pulse labelled exponentially growing cells carrying pLG515::Tn1000 plasmids

For each strain 5ml of exponential cells at A_{450} 0.2 in M9 minimal medium at 30°C were pulsed for 60 sec with 30μCi of ^{35}S -methionine, then excess cold methionine added and the cells incubated for a further 60 sec followed by the addition of approximately 3ml of crushed ice made from 0.5M sodium azide in 10mM sodium phosphate buffer pH7.2. 100 OD units of cold carrier cells were added and each sample fractionated into total lysate, cytoplasmic, periplasmic (osmotic shockate), total membrane, inner membrane and outer membrane samples. The samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography)

s - ^{14}C standard protein mix

l - whole cell lysate

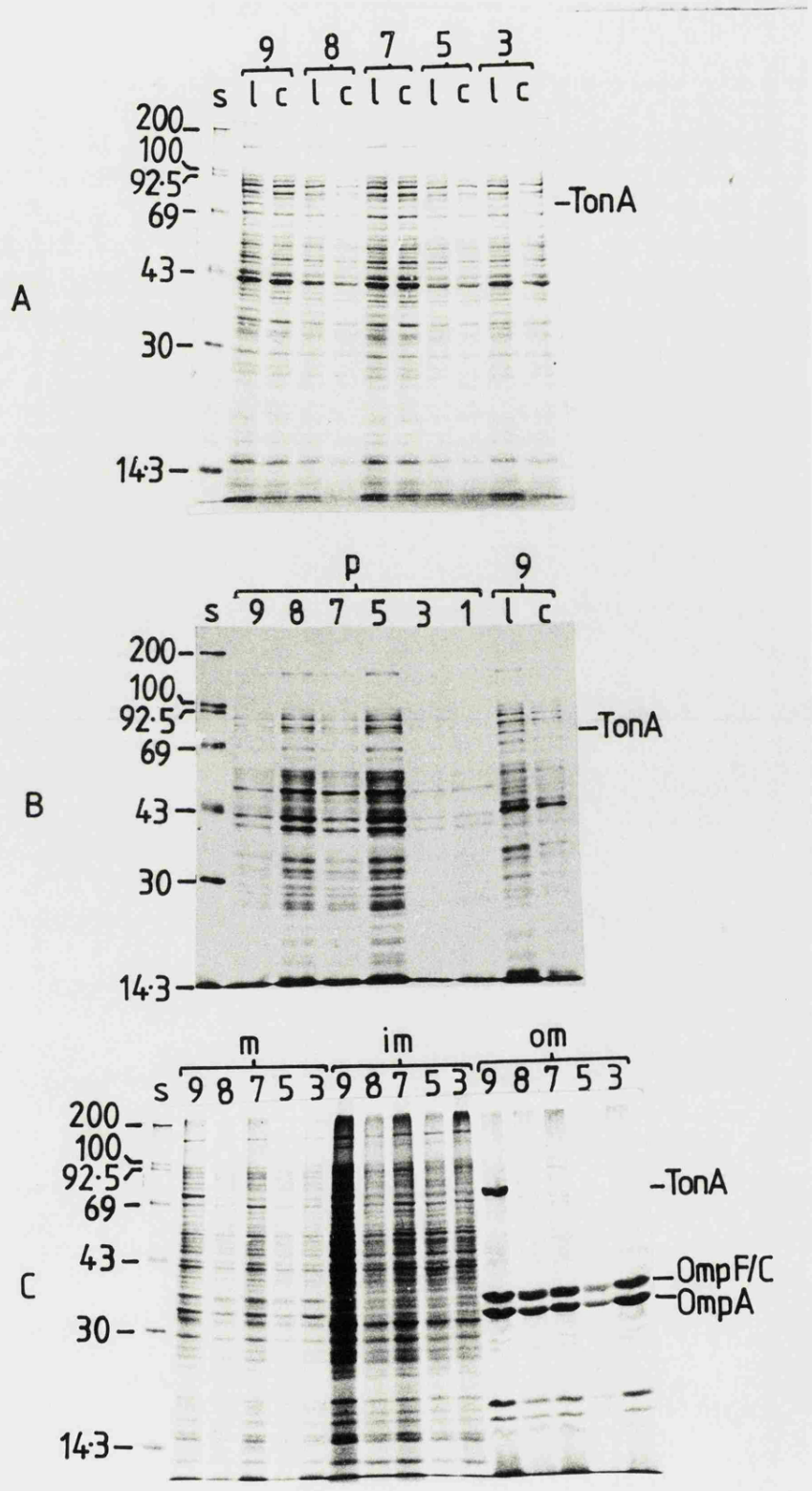
c - cytoplasm plus periplasm

p - periplasm (osmotic shockate)

m - total membrane

im - inner membrane

om - outer membrane


A
9 8 7 5 3
s l c l c l c l c l c
200
100
92·5
69
43
30
14·3
-TonA
B
p 9
s 9 8 7 5 3 1 l c
200
100
92·5
69
43
30
14·3
-TonA
C
m im om
s 9 8 7 5 3 9 8 7 5 3 9 8 7 5 3
200
100
92·5
69
43
30
14·3
-TonA
-OmpF/C
-OmpA

same rate as wild type TonA.

7.7 Discussion

Evidence is beginning to accumulate concerning the requirement for an intact C-terminus in correct localisation of proteins to the periplasm and membranes of E. coli. For the periplasmic proteins β-lactamase and MalE, it has been demonstrated that the C-terminus is not required for translocation across the inner membrane, but, in the case of β-lactamase, is required for the release of this protein into the periplasm (Koshland & Botstein, 1980, 1982; Ito & Beckwith, 1981)

A substantial portion of the C-terminus of OmpA may be deleted without any apparent effect on the function or localisation of the product: a partially deleted ompA' gene encoding 193 of the 325 residues of mature OmpA protein (33kD) was found to produce a 24kD outer membrane protein which still expressed all known functions of OmpA (Bremer et al, 1982a). However, this 24kD polypeptide was not present in the outer membrane at the same levels as wild type OmpA. Interestingly, the products of ompA' genes encoding more than 193 amino acids were all degraded to the same 24kD polypeptide, suggesting the presence of an independent protein domain possessing all the information required for outer membrane integration (Movva et al, 1980; Bremer et al, 1982b). OmpA' polypeptides containing only 133 or 45 residues were found to be rapidly degraded (Bremer et al, 1982b).

Similar indications of instability were obtained from

studies of a 20kD amber fragment of the F plasmid encoded TraT protein (25kD). This polypeptide was shown to be incorporated into the outer membrane in a sarkosyl insoluble form, although the polypeptide fragment could not be detected in envelopes from exponentially growing cells (Achtman et al, 1979). Nevertheless, it may be concluded that for OmpA and TraT the C-terminus does not appear to be essential per se in localisation.

In the case of OmpA, the 148 residues cleaved from the C-terminus by pronase treatment of membrane fractions are dispensible with respect to all known functions of the protein, and may be unassociated with the membrane, perhaps forming a separate functional domain in the periplasm (Bremer et al, 1982b). OmpA may be unusual among the outer membrane proteins in this respect. The C-terminal regions of both OmpA and TraT do appear to be necessary for stability and may also affect the efficiency of translocation or assembly.

Following mutagenesis of a plasmid borne phoE gene by Tn5 insertion, truncated polypeptide products were observed in minicells but not in exponentially growing cells (Overbeeke et al, 1983). Tommassen et al (1982) reported that no polypeptide products of Tn5 mutagenised ompF genes were detectable in minicells or exponentially growing cells. These results, and those obtained using OmpA and TraT, suggest that truncated outer membrane proteins are either rapidly degraded or not synthesised at wild type levels.

Hirashima et al (1973) demonstrated that outer membrane protein mRNA molecules had unusually long half lives (5.5

min) when compared to cytoplasmic protein mRNAs. It is therefore possible that the altered 3' terminus of mRNA molecules encoding truncated outer membrane proteins decrease mRNA stability. Both OmpA and lipoprotein mRNAs have a high potential for secondary structure formation in all regions except those on either side of the initiation codon, and it has been proposed that mRNA stability is due to this secondary structure (Pirtle *et al*, 1980; Nakamura *et al*, 1980; Movva *et al*, 1980). In order to eliminate the possibility that unstable mRNA molecules were responsible for the absence of truncated outer membrane polypeptide products, Overbeeke *et al* (1983) mutated the *pho*E gene by insertion of a tetranucleotide at codon 286 (PhoE has 330 residues). The change of reading frame leads to premature chain termination, but the mRNA secondary structure should not be significantly altered. Since no product was detectable in minicells or in growing cells for this mutant gene, it is apparent that mRNA instability is not a major cause of lack of expression of truncated outer membrane proteins.

The C-terminus of LamB does not appear to be important for localisation, since the protein products of hybrid *lam*B-*lac*Z genes containing only 6 of the 9 deletion units defining *lam*B, are assembled into the outer membrane (see section 1.III.2.3(iii)). Similarly, the polypeptide product of a *pho*E-*lac*Z hybrid gene containing the majority of the *pho*E sequence is assembled into the outer membrane. However, a *pho*E' gene produced by Tn5 insertion at the same point as the junction between the fused genes produces no apparent product

in growing cells (J. Tommassen, pers. comm.). This result indicates that the C-terminus of PhoE is not essential for assembly, but that a truncated PhoE protein, containing an abnormal C-terminus, is rapidly degraded. The resistance of the PhoE-LacZ hybrid to degradation can be attributed to possession of a normal C-terminus, albeit from another protein.

The results described in this chapter show TonA' polypeptides to be relatively unstable in maxicells, and largely undetectable in growing cells; results similar to those described for TraT and PhoE fragments. The early stages of translocation and assembly into the envelope of the TonA' polypeptides appeared to occur in maxicells, where the larger polypeptides were apparently processed normally. However, although the TonA' fragments became envelope associated, they remained sarkosyl soluble. For TonA protein therefore, the C-terminus is required at least for association into a sarkosyl insoluble form and for stability of the protein. It is possible that, like the OmpA' and TraT' fragments, the TonA' polypeptides are located in the outer membrane but in a sarkosyl soluble form. Sequences at the C-terminus of TonA may be essential for interactions with LPS and/or proteins in order to form a sarkosyl insoluble complex. Alternatively, the TonA' fragments may reach an intermediate stage of assembly and be prevented from completing transfer to the outer membrane by their lack of specific sequences at the C-terminus, or by failure to attain an overall conformation required for the final stages of assembly. Such a

conformational effect may be the factor preventing release of truncated ß-lactamase polypeptides into the periplasm (Koshland & Botstein, 1982). In addition, it remains a possibility that the inability of the truncated TonA molecules to associate into the sarkosyl insoluble material was due to the presence of unusual, Tn1000 encoded sequences at their C-termini (see Fig. 7.2).

Further study is required to unambiguously define the location of the TonA' polypeptides. Attempts were made to fractionate the inner and outer membranes by the Osborn technique, but this failed due to the presence of a large proportion of filamented cells in the irradiated maxicell culture which would not form spheroplasts. Protease treatment as described in Chapter 4 might also be applied to further localise the truncated TonA polypeptides.

CHAPTER EIGHT

ISOLATION OF tonA C-TERMINAL DELETIONS USING BAL31 EXONUCLEASE

8.1 Introduction

As discussed in the previous chapter, although Tn1000 mutagenesis provided a good method of mapping the tonA gene and determining the direction of transcription, studies of the truncated products resulting from Tn1000 insertions into the tonA gene were limited by the uncertainty of how much of the polypeptide was coded by tonA DNA, and how much resulted from readthrough into Tn1000.

By linearising pLG515 at the unique SmaI site and digesting with Bal31 exonuclease it would be possible to delete various lengths of the promotor distal end of the tonA gene. Ligating such fragments to DNA sequences known to contain stop codons would produce truncated genes without the problem of readthrough. The plasmid pFURK (Fig. 8.1) carries a spectinomycin resistance gene flanked by sequences containing stop codons in all three frames (Fig. 8.2). Restriction of pFURK with SmaI produces a blunt ended fragment such that, reading inwards from either end, stop codons are encountered in all three frames after 4, 5 and 6 amino acids respectively (Fig. 8.2). Thus, by ligating this pFURK "stop" fragment to a mixture of pLG515 x SmaI x Bal31 fragments of various lengths, it should be possible to create a family of pLG515 deletion derivatives carrying different lengths of the tonA gene. The truncated polypeptides encoded

Fig. 8.1: construction of tonA' genes using Bal31 exonuclease and the pFURK "stop" fragment

DNA derived from pLC19-19 is indicated by heavy lines; ▷ indicates the oligo-stop codon sequence of the pFURK "stop" fragment.

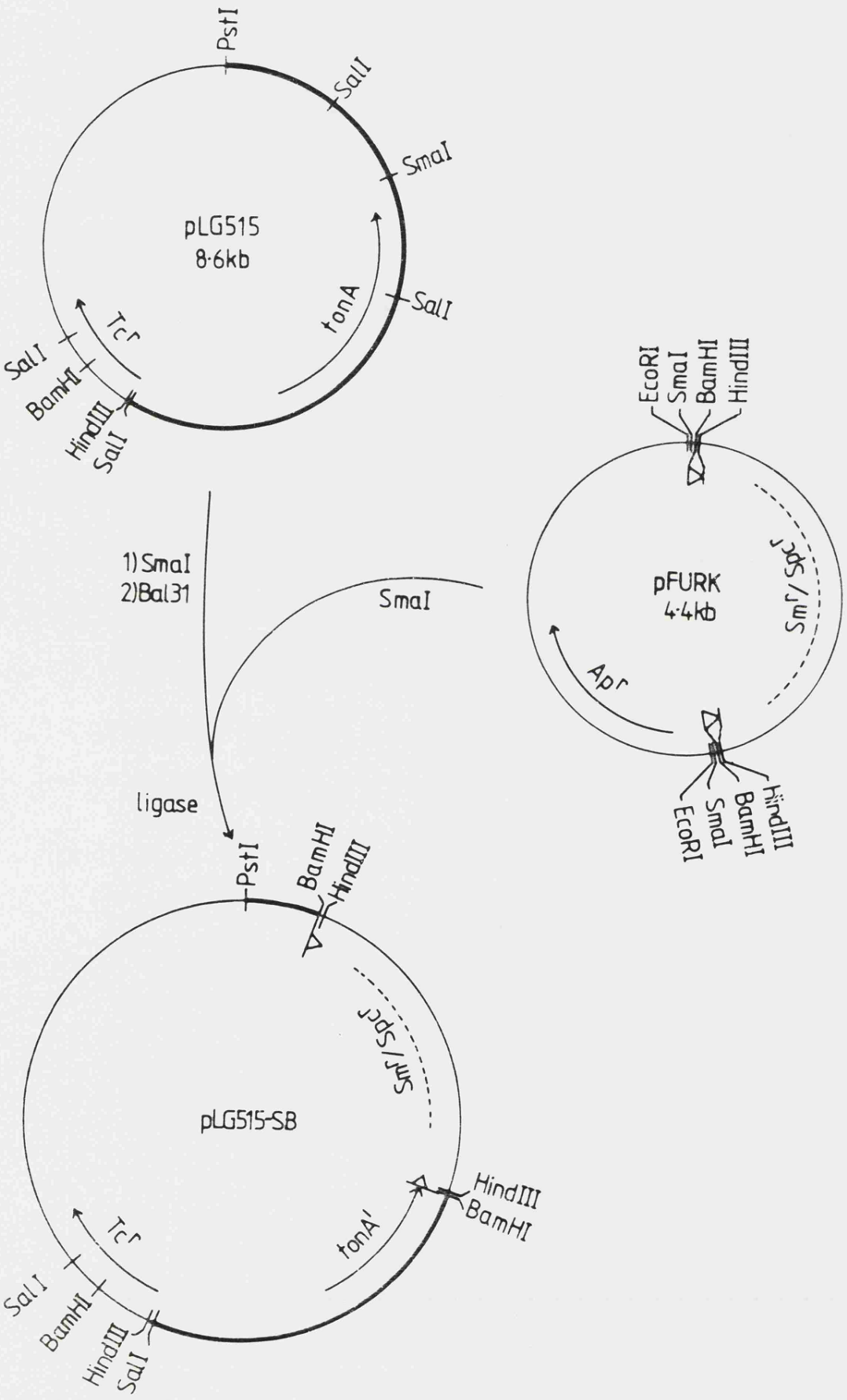

PstI
SalI
SmaI
SalI
pLG515
8·6kb
tonA
Tc r
SalI
BamHI
HindIII
SalI
EcoRI
SmaI
BamHI
HindIII
pFURK
4·4kb
Sm r/Spc r
Ap r
EcoRI
SmaI
BamHI
HindIII
1) SmaI
2) Bal31
SmaI
ligase
PstI
BamHI
HindIII
Sm r/Spc r
pLG515-SB
HindIII
BamHI
tonA'
Tc r
SalI
BamHI
HindIII
SalI

Fig. 8.2: Construction of pLG515-SB plasmids showing nucleotide sequence of the oligo-stop DNA and the BamHI (B) and HindIII (H) sites used in the mapping

The HindIII - BamHI fragment carrying tonA derived DNA was identified by comparison of a BamHI single digest with a BamHI x HindIII double digest; both BamHI fragments were sized in order to determine the extent of the Bal31 deletion.

Translation of oligo-stop:

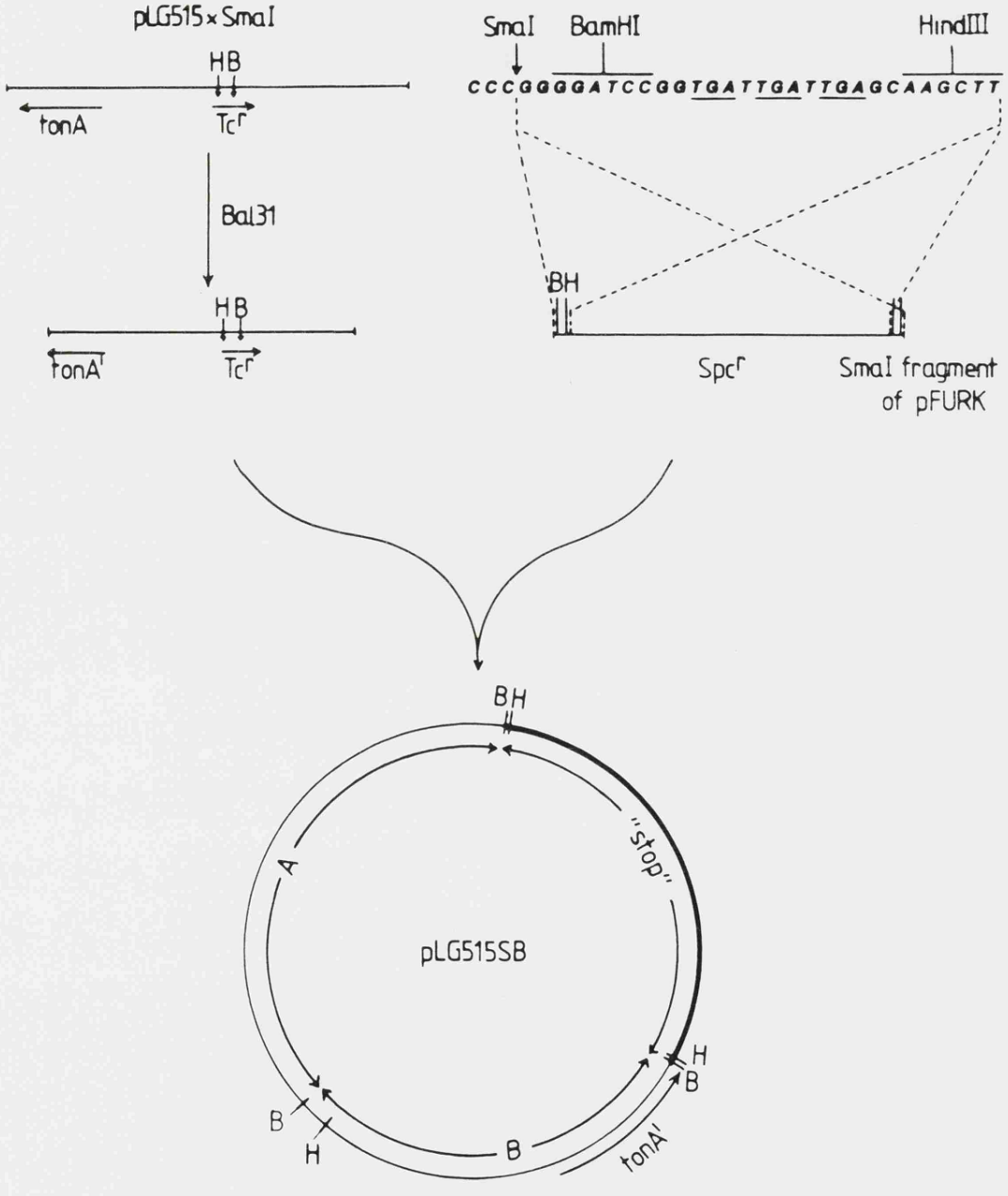
SmaI BamHI HindIII

G G G G A T C C G G T G A T T G A T T G A G C A A G C T T

gly . tyr . pro . val . ile . asp . STOP

? . gly . ser . gly . asp . STOP

? . gly . ile . arg . STOP


pLG515 × SmaI
H B
tonA
Tc^r
Bal31
H B
tonA'
Tc^r
SmaI
BamHI
HindIII
C C C G G G G A T C C G G T G A T T G A T T G A G C A A G C T T
B H
Spc^r
SmaI fragment
of pFURK
B H
"stop"
A
pLG515SB
H
B
B
H
B
tonA'

by these genes would possess mainly tonA specific amino acids with very little foreign sequence at the C-terminus.

Analysis of such truncated polypeptides might confirm the conclusion from the previous chapter that the C-terminal sequences of the tonA gene are extremely important for correct localisation to the outer membrane.

8.2 Isolation of tonA deletion derivatives using Bal31 exonuclease

SmaI digested pLG515 was incubated with Bal31 exonuclease and samples taken at 1.5 min intervals over 40 min, during which time a maximum of 2kb was digested from each end of the linear fragment. The samples were pooled and ligated with SmaI digested pFURK (Fig. 8.1). The ligation mix was used to transform CSH26ΔF6tonA and transformants were selected on nutrient agar containing streptomycin, tetracycline and spectinomycin. These transformants were screened for ampicillin sensitivity and scored for T1 resistance. All 28 ampicillin sensitive clones obtained (pLG515-SB101 - 128) were T1 resistant, ie., the plasmids had lost part or all of the tonA gene. In order to obtain clones which still carried a complete, or nearly complete, tonA gene, the Bal31 digestion of pLG515 x SmaI was repeated, halving the amount of Bal31 enzyme used and taking samples at 1 min intervals over 10 min. Ligation, transformation and screening of transformants were carried out as before. Of 23 Ap^S clones isolated 20 were T1 sensitive (pLG515-SB201 - 220) and 3 were T1 resistant (pLG515-SB221 - 223)

8.3 Mapping of the deletions

As shown in Fig. 8.2, BamHI will cut the pLG515-SB plasmids into 3 fragments: a pFURK "stop" fragment, and two fragments, derived from pLG515, which can be distinguished in a double digest with BamHI and HindIII, where HindIII will cut the BamHI B fragment. In addition, the BamHI digest shows the stop codon sequence to be intact (see Fig. 8.2). The extent of the Bal31 deletions of pLG515 for eight of the clones is shown in Fig. 8.3.

8.4 Proteins encoded by pLG515-SB plasmids in vitro

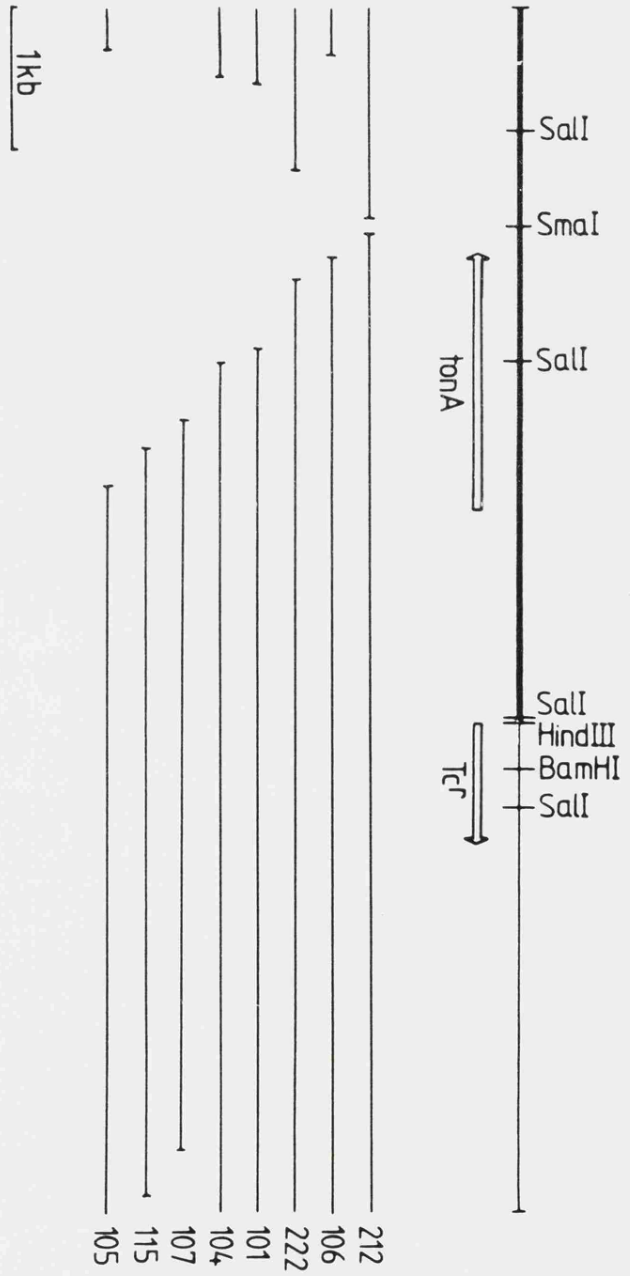
Plasmid DNA isolated from the clones shown in Fig. 8.3 was used to programme the Zubay transcription-translation system; the proteins synthesised are shown in Fig. 8.4. For clones 212 (*tonA*$^{+}$), 101, 104, 107, 115 and 105, the sizes of the polypeptide products corresponded well with the restriction mapping data (Table 8.1). However, clone 106, from which a TonA' product of nearly 78kD was expected, programmed the synthesis of two smaller products, one of 37kD and one of 14kD. Among the translation products of 222 DNA was a TonA' polypeptide of the expected size (≈72kD), but, in addition, an extra product of 35kD was synthesised.

8.5 Further analysis of clones 106 and 222

The plasmid DNA isolated from clones 106 and 222 may have consisted of a heterologous population, which arose from independent point mutation events following initial transformation. Such mutations would not alter the

Fig. 8.3: Diagrammatic representation of pLG515 derived sequences deleted in the pLG515-SB plasmids

The map of pLG515, linearised at the unique PstI site, is shown at the top of the figure. Below are 8 pLG515-SB plasmids showing the pLG515 derived DNA remaining in these constructs. The diagram does not show the pFURK "stop" fragment, which is present in all the pLG515-SB plasmids at the deletion site. Plasmid pLG515-SB212 confers T5 sensitivity on the host strain; all other derivatives of pLG515 are *tonA*$^-$.


1kb
105
115
107
104
101
222
106
212
SalI
SmaI
SalI
tonA
SalI
HindIII
BamHI
SalI
Tcr

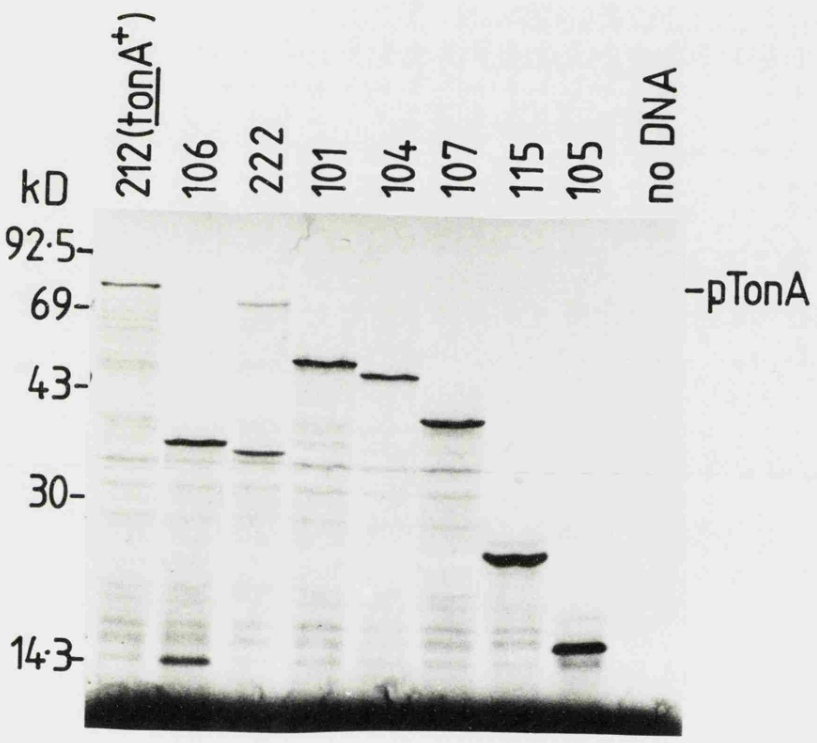
Clone	Size of BamHIB fragment (kb)	Size of TonA' polypeptide (kD)
212	3.85	80
222/2	3.5	72
101	3.02	53
104	2.92	50
107	2.5	40
115	2.3	21.5
105	2.01	16

Table 8.1: Comparison of truncated polypeptides synthesised from pLG515-SB plasmids *in vitro* with the extent of Bal31 deletion in each case.

Fig. 8.4: ^{35}S -methionine labelled polypeptide products from *in vitro* expression of the pLG515-SB plasmids

Transcription-translation reactions were carried out using the LE316 extract; polypeptide products were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

Positions of ^{14}C -labelled standards indicated at left


212(tonA+)
106
222
101
104
107
115
105
no DNA
kD
92·5-
69-
43-
30-
14·3-
-pTonA

BamHI and HindIII restriction patterns and would explain the presence of two different polypeptide products instead of the single TonA' product expected from each clone. To test this, DNA from clones 106 and 222 was transformed into MC4100 in order to isolate secondary clones derived from cells bearing individual plasmids. Plasmid DNA was prepared from three of the secondary 222 clones and four of the secondary 106 clones. This DNA was used to programme the Zubay system. From Fig. 8.5 it is apparent that whilst the plasmid DNA first isolated from clone 222 encoded two products, of 72kD and 35kD, clones 222/1 and 222/2 encoded only the 72kD product, and clone 222/3 encoded only the 35kD product. All four of the secondary 106 clones encoded a 37kD product, but the 14kD polypeptide, if present, was not sufficiently resolved. Thus it appears that the plasmid DNA isolated from the original 222 clone was heterogenous, encoding two different TonA' products, although the restriction maps of these plasmids were identical with respect to BamHI and HindIII. The possibility remains that the 106 clone likewise carried a heterogenous plasmid population; further tests would be required to establish this.

8.6 Products of pLG515-SB plasmids synthesised in maxicells

Plasmids pLG515-SB101, 104, 105, 107, 115, 212 and 222/2 were transformed into SE5000, a *recA*$^-$ host suitable for use in the maxicell expression system. The protein profiles obtained from expression of these plasmids in the maxicell system are

Fig. 8.5: Products of *in vitro* expression of plasmids from clones 222 and 106, and plasmids from secondary clones derived by transformation of the first plasmid preparation

Transcription - translation reactions were carried out using MRE600 extract, polypeptide products were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

Positions of ^{14}C -labelled marker proteins shown at right; positions of 72kD, 35kD and 37kD TonA' polypeptides shown to left of appropriate panel.

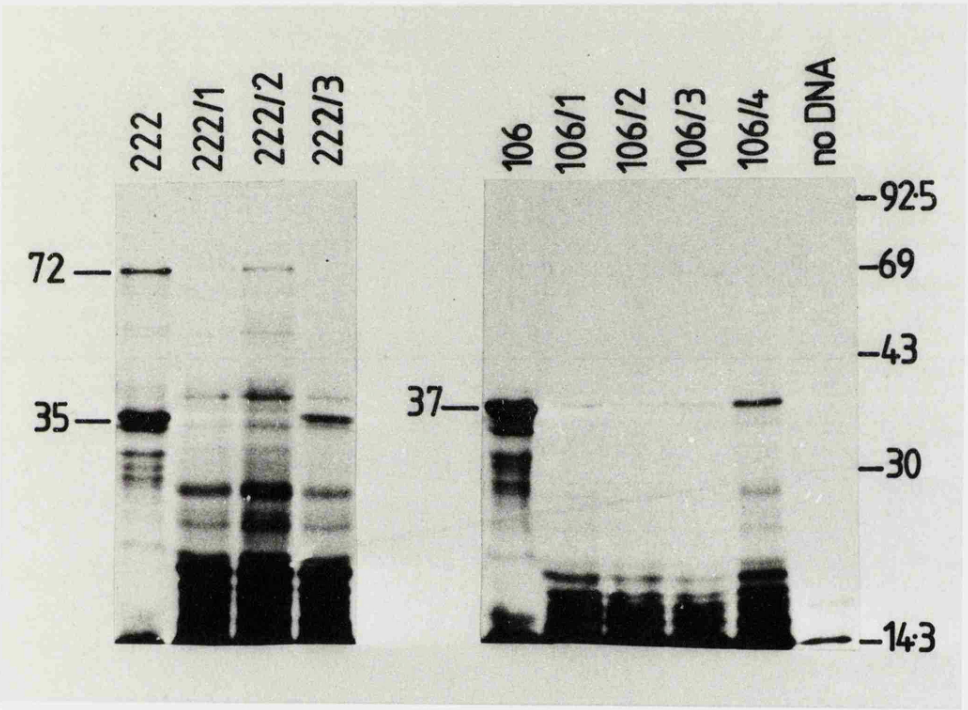

222
222/1
222/2
222/3
106
106/1
106/2
106/3
106/4
no DNA
72
35
37
92·5
69
43
30
14·3

Fig. 8.6: Products of in vivo expression in maxicells of pLG515-SB plasmids

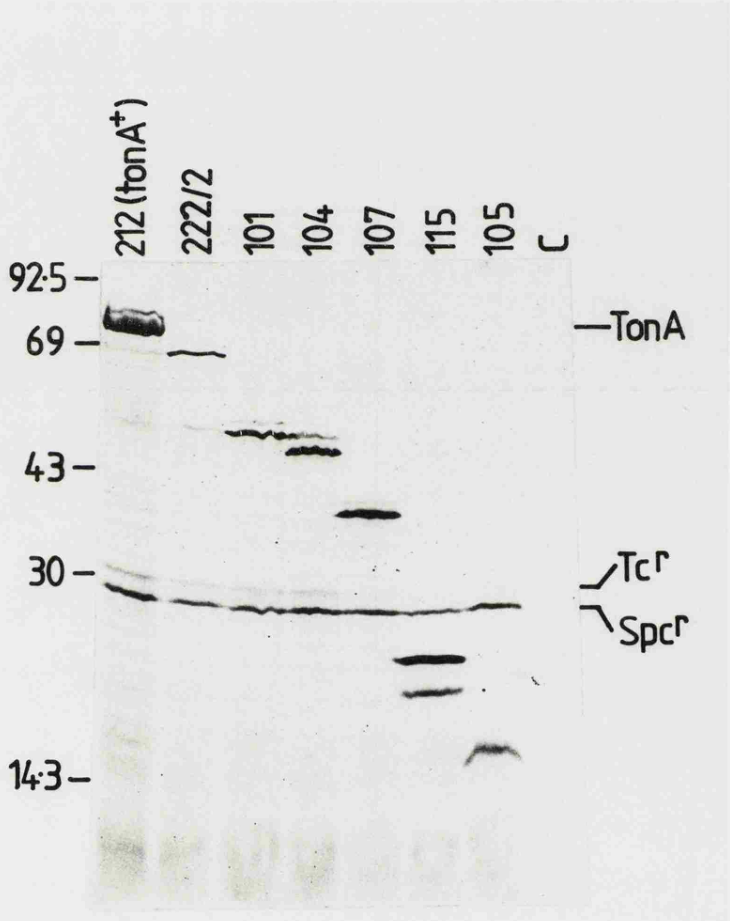
^{35}S -methionine labelled products of maxicells prepared from SE5000 carrying the pLG515-SB plasmids indicated were run on 11% acrylamide I gels and analysed by autoradiography

Positions of the ^{14}C -labelled marker proteins shown at left of gel

C - control track, SE5000 without plasmid

Tc^r - tetracycline resistance protein

Sp^r - spectinomycin resistance protein ?


212 (tonA+)
222/2
101
104
107
115
105
C
92·5
69
43
30
14·3
TonA
Tcr
Spcr

shown in Fig. 8.6, where the expected family of TonA' polypeptides can be seen. In the cases of clones 212 (wild type TonA), 222/2, 101, 104 and 107 an additional protein, 2 - 3kD larger than the major TonA/TonA' product, was observed. These higher molecular weight polypeptides presumably represent the precursor forms of wild type TonA and its truncated derivatives.

8.7 Localisation of the truncated TonA polypeptides in maxicells

The results obtained in Chapter 7 indicated that TonA' polypeptides derived from Tn1000 insertion into the tonA gene were localised in the sarkosyl soluble envelope fraction. It was possible that the localisation of these polypeptides was affected by Tn1000 encoded amino acids present at the C-terminus. Therefore a similar determination of localisation was carried out for the TonA' polypeptides created by Bal31 deletions, which should possess very few additional amino acids at their C-termini owing to the presence of the pFURK "stop" fragment.

Fig. 8.7 shows the gel profiles obtained upon fractionation of maxicells harbouring various pLG515-SB plasmids. The results were very similar to those obtained when maxicells carrying pLG515::Tn1000 plasmids were fractionated. Wild type TonA protein (encoded by pLG515-SB212) fractionated with the envelope fraction, partitioning approximately equally between the sarkosyl soluble and sarkosyl insoluble fractions, whereas the larger TonA'

Fig. 8.7: Fractionation of maxicells carrying plasmids pLG515-SB212, 222/2 and 104

^{35}S -methionine labelled maxicells of SE5000 carrying the above plasmids were prepared and fractionated as described in the legend to Fig. 7.4. Samples were analysed by SDS PAGE (11% acrylamide I) and autoradiography.

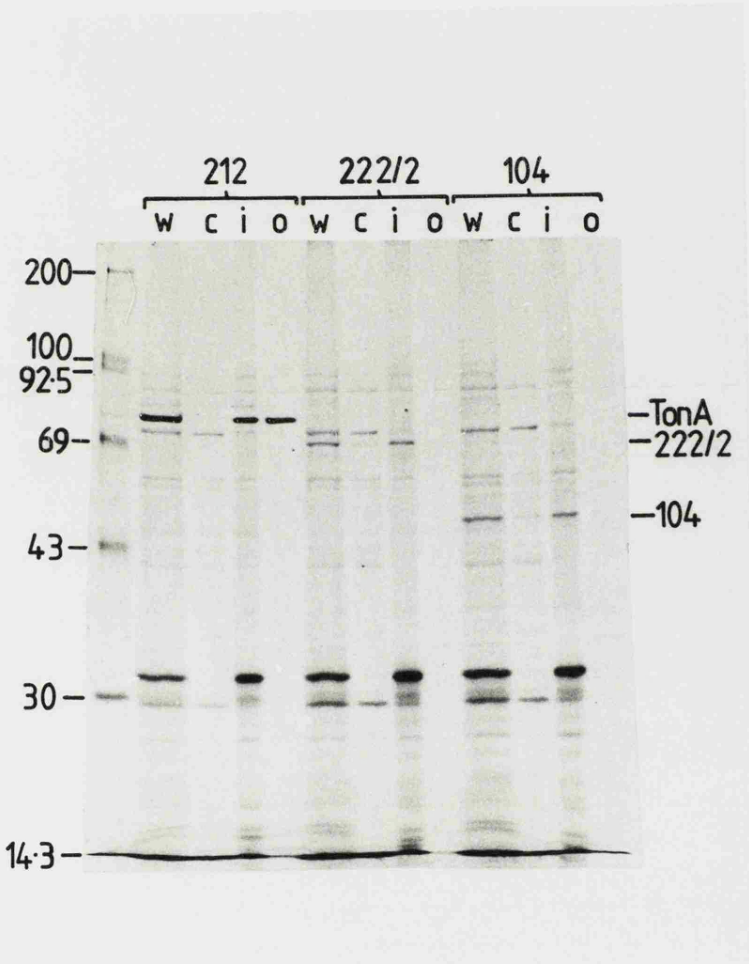
Positions of ^{14}C -labelled marker proteins indicated at left, positions of TonA' polypeptides indicated at right.

w - whole cell lysate

c - cytoplasm plus periplasm

i - inner membrane

o - outer membrane


212
222/2
104
w c i o w c i o w c i o
200
100
92·5
69
43
30
14·3
TonA
222/2
104

polypeptides (222/2 and 104) fractionated mainly with the sarkosyl soluble fraction with the remainder of the protein present in the soluble (cytoplasm plus periplasm) fraction (Maxicells carrying pLG515-SB107 and 105 were also fractionated in this experiment, but labelling of the TonA' polypeptides encoded by these plasmids was insufficient).

8.8 Discussion

Clones pLG515-SB101, 104, 107, 115 and 105 encode TonA' polypeptides with molecular weights ranging from 50kD to 14kD. No more than 6 amino acids (Fig. 8.6) at the C-termini of these polpeptides can be encoded by sequences from the "stop" fragment. Thus the cellular localisation of these polypeptides should reflect only the information present in the tonA encoded sequences.

Clones 106 and 222 clearly behaved differently from the other clones analysed. The DNA obtained from clone 106 did not encode a product of the molecular weight predicted from the restriction mapping data, encoding instead two novel products of 37kD and 14kD. Plasmid DNA isolated from clone 222 encoded a product of the expected molecular weight (72kD) but encoded an additional polypeptide of 35kD. For clone 222 it was demonstrated that a heterogenous plasmid population was present, in which individual plasmids encoded either the 72kD or the 35kD product, yet no such plasmid heterogeneity was indicated by the restriction mapping data.

A hypothesis to account for this anomalous behaviour of clones 106 and 222 is as follows: if the synthesis of TonA' molecules lacking only a short region at the C-terminus is in some way detrimental to cell growth, then a strong selective pressure would exist for mutations to arise which prevented the synthesis of those particular polypeptides. Thus clones 106 and 222 might have originated by transformation of the host by a single plasmid carrying a detrimental tonA' gene. Within the subsequent plasmid population mutations would arise and be selected for, which alleviated the detrimental effect, for example by nonsense mutations, frameshifts or deletions, creating a tonA' gene encoding a shorter polypeptide. In the case of nonsense or frameshift mutations the BamHI and HindIII restriction patterns would not be altered, and thus heterogenous plasmid populations could arise which possessed identical restriction maps but encoded two (or more) different TonA' products. Gradually, plasmids still encoding detrimental genes would be selected out of the population.

Two further observations were made which support this hypothesis. Following inoculation from plates into Luria broth, cultures of 222 grew extremely slowly, requiring 24 hours incubation before any growth was visible (normally a stationary phase culture is obtained following overnight incubation). However, the growth rate then appeared to accelerate. This growth pattern fits a model in which growth is slow until advantageous mutations have become fixed in the population, after which the growth rate of cells carrying

such mutations increases. All three of the secondary 222 clones possessed normal growth patterns; thus the synthesis of the 72kD and 35kD proteins was not deleterious.

Restriction mapping of clones 220 and 221 (which were *tonA*$^-$, possessing small Bal31 deletions) indicated that one of the expected BamHI sites had been deleted at some stage from both of these plasmids. No such deletions were observed in any other clones analysed, therefore it is possible that these deletions had arisen subsequent to primary transformation to alleviate the effects of deleterious *tonA'* genes.

The above explanation of the behaviour of clones 106 and 222 assumes that deleting small regions at the C-terminus of TonA is deleterious, whereas larger deletions have no apparent effect on growth. B. Bouges-Bocquet (pers. comm.) has recently obtained direct evidence that the synthesis of LamB' proteins with deletions of the 10 C-terminal amino acids is lethal, consistent with the hypothesis proposed here for TonA. The reason for this lethality is not clear. Bouges-Bocquet found no evidence for inhibition of processing or translocation of other extracytoplasmic proteins following induction of synthesis of lethal LamB derivatives. The truncated LamB polypeptides fractionated with the outer membrane on Osborn gradients, although it is possible that they are incorrectly assembled and interfere with outer membrane structure and/or assembly. In order to carry out a similar study to confirm that the synthesis of the smaller C-

terminal deletions of TonA is lethal, it will be necessary to construct an inducible system either by substituting an inducible promotor for the tonA promotor, or by recloning the tonA gene onto a temperature sensitive runaway vector.

Fractionation of maxicells synthesising the truncated TonA' polypeptides derived from Bal31 deletion of the tonA gene gave results essentially identical to those obtained using Tn1000 to truncate the gene. Thus TonA' polypeptides are able to associate with the envelope fraction in a sarkosyl soluble form. The inability of these truncated polypeptides to complete assembly and become sarkosyl insoluble must be due to the absence of an intact C-terminus since the presence of the pFURK "stop" fragment ensures that no more than 6 amino acids are added to the tonA encoded sequences. Either specific sequences at the C-terminus are required or the C-terminus is important for the protein to achieve the necessary conformation for the stages of assembly subsequent to initial membrane interaction. Randall (1983) has suggested that translocation of outer membrane proteins across the inner membrane occurs by whole domains of the protein being translocated at a time rather than by vectorial transfer. Protein conformation would probably be important for such a mechanism. On the other hand, it is possible that overall protein conformation is important for the final stages of assembly into a sarkosyl insoluble complex in the outer membrane. Equally, it may be that specific, C-terminal amino acid sequences of TonA are required for interaction with LPS and other outer membrane

constituents in the formation of the sarkosyl insoluble complex. Of course, the possibilities presented above are not mutually exclusive, and the C-terminus of TonA may be important at several stages of assembly.

CHAPTER NINE

DISCUSSION

The assembly of E. coli outer membrane proteins is evidently a complex process, requiring several steps. The signal sequence plays an important role in early stages of export of outer membrane proteins, but since the signal sequences of outer membrane proteins do not appear strikingly different from those of inner membrane and periplasmic proteins, the determination of ultimate localisation does not seem to rely on features of the signal sequence. Tommassen et al (1983) have constructed a hybrid protein consisting of the signal sequence and amino terminus of the periplasmic β-lactamase protein fused to the mature outer membrane PhoE protein. The PhoE sequences of this hybrid protein are localised to the outer membrane despite the replacement of the normal PhoE signal sequence with that of β-lactamase. Similarly, recent results from our laboratory have demonstrated that the substitution of the OmpF (outer membrane protein) signal sequence with that of the inner membrane protein PBP5 does not prevent correct localisation and assembly of the OmpF sequences into the outer membrane (M. E. Jackson, J. M. Pratt, N. G. Stoker & I. B. Holland, manuscript in preparation). Several other observations support the hypothesis that early stages of translocation and assembly are identical for outer membrane, periplasmic and some inner membrane proteins: (i) secA^{ts} mutants accumulate precursors to both periplasmic and outer membrane proteins at the non-

permissive temperature (section 1.III.6.2); (ii) prlA mutations supress signal sequence mutations of both periplasmic and outer membrane proteins (section 1.III.6.2); (iii) Wickner's signal peptidase, which processes the M13 precoat protein, an inner membrane protein, will also process the signal sequences of outer membrane and periplasmic proteins (section 1.III.2.4); and (iv) as described in Chapter 5, induction of the MalE-LacZ hybrid 72-47 inhibits processing of inner membrane, periplasmic and outer membrane proteins. These results can be rationalised if we assume that the signal sequence carries only the information required for the initial membrane interaction. Therefore sequences within the mature protein would be expected to contain the additional information conferring an ultimate localisation on outer membrane proteins. A great deal of work has been carried out in order to locate such sequences for several different outer membrane proteins by the study of mutant proteins carrying internal and C-terminal deletions. However, no such sequences have been unambiguously identified.

There are in fact indications that more than one sequence is required for efficient localisation of outer membrane proteins. For example, in the case of LamB, although the signal sequence plus 49 amino acids is sufficient to localise 15 - 20% of the molecules of a LamB-LacZ hybrid to the outer membrane, the efficiency of localisation is increased to 85% when the signal sequence plus 240 or more amino acids is present in the hybrid (S. Benson, pers. comm.). This may reflect a number of different requirements for final

localisation. As discussed in Chapter 1 (section IV.1), there is a large body of evidence to suggest that interaction with LPS is one important factor in the assembly of at least some outer membrane proteins. In cells synthesising mutant forms of LPS the quantities of the major outer membrane proteins are markedly reduced. Thus it seems reasonable to assume that mutant outer membrane proteins lacking specific sequences or domains which interact with LPS and other outer membrane constituents might be inefficiently assembled. If interaction with LPS occurs at an early stage of assembly, while translation is incomplete, it is also possible that outer membrane protein synthesis itself may be halted if particular associations with LPS are not achieved. For TonA, sequences interacting with LPS or other outer membrane constituents may lie, in part, close to or at the C-terminus. This would explain the apparent failure of the truncated TonA' polypeptides investigated in Chapters 7 and 8 to assemble correctly into the outer membrane, thus remaining sarkosyl soluble. Taking this speculation one step further, perhaps the apparent detrimental effect of small C-terminal deletions of TonA (see Chapter 8, Discussion) and the lethality of similar deletions of LamB (B. Bouges-Boquet, pers. comm.) is due to incorrect or partial association with LPS and/or proteins which result in disruption of the overall membrane structure or its biogenesis.

Recent evidence suggests that outer membrane proteins assume conformations within the membrane which involve a large degree of folding. By a combination of techniques it

has been established that the LamB molecule loops back and forth across the membrane several times (M. Schwartz, pers. comm.). Such techniques include (i) the mapping of mutations preventing phage interaction; (ii) the mapping of monoclonal antibody binding sites with respect to (a) the membrane, (b) functional sites and (c) the polypeptide chain; and (iii) protease accessibility following prolonged incubation with subtilisin and trypsin. Studies of hydropathy plots according to the method of Kyte & Doolittle (1982) have shown that, unlike inner membrane proteins which, in general, possess hydrophobic domains, mature outer membrane proteins have an overall hydrophilic character when only the primary structure is considered (N. G. Stoker & J. M. Pratt, pers. comm.) (Fig. 9.1). Thus folding within the membrane and protein-protein interactions involving the hydrophilic domains are probably required to ensure that the surfaces of the protein in contact with the lipid bilayer are hydrophobic in character.

Given that complex folding within the bilayer is characteristic of outer membrane proteins, it follows that that amino acids from several regions of the primary sequence may be important in establishing associations with other outer membrane constituents. In addition, attainment of a precise overall protein conformation may be necessary in order to align these sequences correctly on the protein surface.

Completion of association of newly synthesised protein with the outer membrane to form a stable structure appears to be a relatively slow stage of assembly which occurs post-

Fig. 9.1: Hydrophobicity plots for cytoplasmic, inner membrane, periplasmic and outer membrane proteins of E. coli

Graphs were plotted according to the method of Kyte & Doolittle (1982), scanning the protein 19 amino acids at a time. Thus the first point represents the hydropathic index averaged for amino acids 1 - 19, the second point the average hydropathic index for amino acids 2 - 20 and so on. Positive values are hydrophobic, negative values are hydrophilic. Regions of the proteins with hydropathy indices above the dashed line are likely to be sequences interacting with membranes. The signal sequences of M13 coat protein, β-lactamase, lipoprotein, OmpA, OmpF and LamB are clearly highly hydrophobic (in all cases the sequence used is that of the initial translation product).

CAT - cytoplasmic chloramphenicol acetyl transferase

BLA - periplasmic β-lactamase (precursor)

M13CP - inner membrane M13 coat protein (precursor)

LacY - inner membrane lactose permease

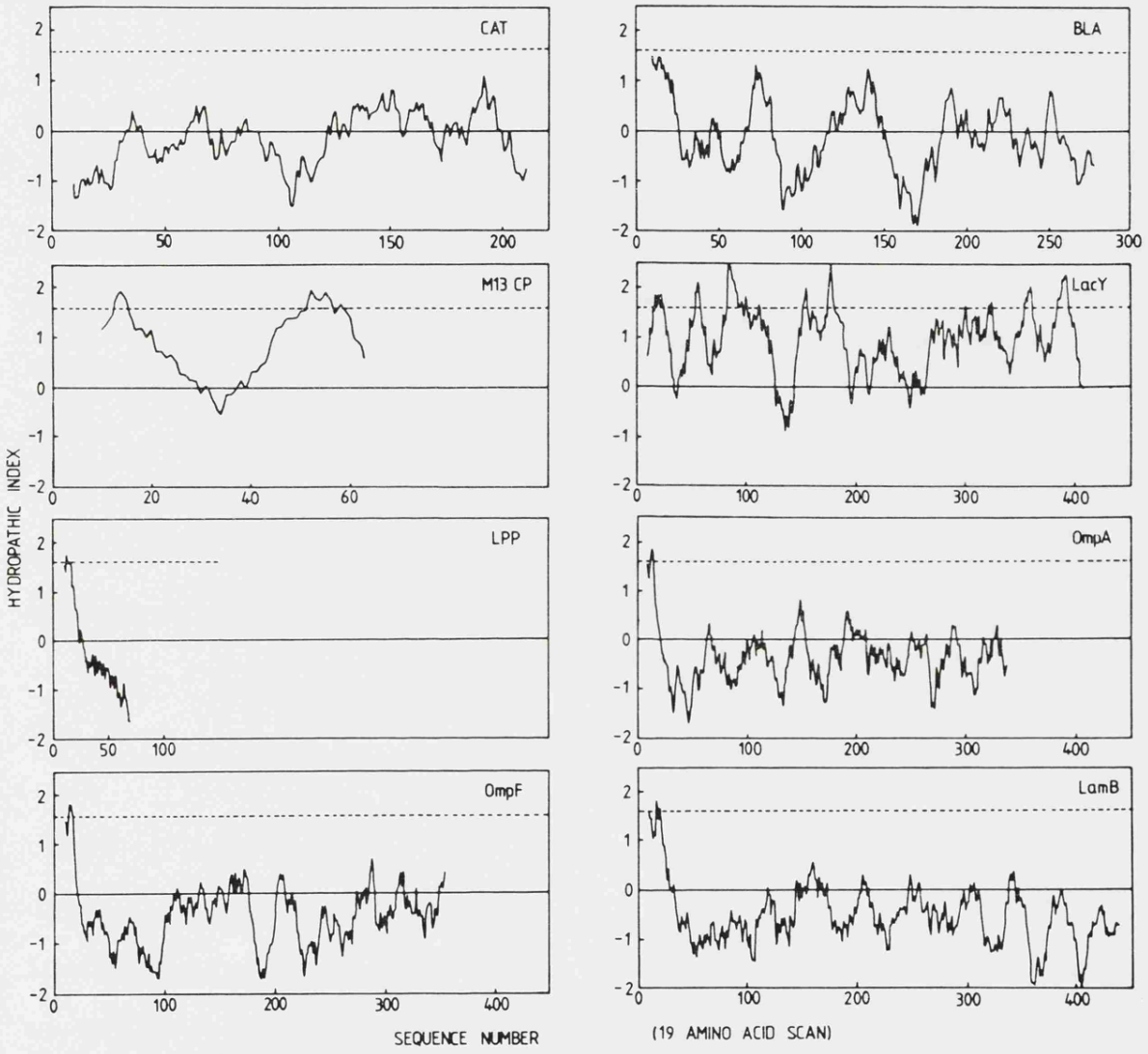
LPP - outer membrane lipoprotein

OmpA - outer membrane OmpA (precursor)

OmpF - outer membrane OmpF (precursor)

LamB - outer membrane LamB (precursor)

(From plots prepared by N. G. Stoker)


CAT
BLA
M13 CP
LacY
LPP
OmpA
OmpF
LamB
HYDROPATHIC INDEX
SEQUENCE NUMBER
(19 AMINO ACID SCAN)

translationally. By the use of antibodies raised against native monomers and trimers of LamB protein, vos Scheperkeuter (1983) has recently obtained evidence to suggest that (i) there is a delay (46 sec at 37^{o}C) before antigenic determinants detectable in whole cell lysates become exposed at the cell surface and (ii) association of the monomers into stable trimers takes 3 - 5 min. This result supports the conclusions of Boyd & Holland (1980) for OmpF, Crowlesmith et al (1981) for both OmpA and OmpF, and also the kinetic study carried out here for TonA that assembly of outer membrane proteins is not an entirely cotranslational process. However, the precise nature and location of the intermediate(s) have not been unambiguously defined.

On the assumption that sequences from several regions of the primary sequence may be required for complete association with outer membrane components, it is possible to speculate that deletion of one such sequence from the protein, if not drastically altering conformation, may not significantly influence the ultimate location of the protein, only the stability of the association and the efficiency with which an outer membrane location is attained. Thus the finding of J. Tommassen (pers. comm) that internal deletions over the C-terminal 2/3 of PhoE did not prevent processing or association into the Triton insoluble fraction (equivalent to the sarkosyl insoluble fraction) does not necessarily mean that sequences in the deleted regions are not important in normal, functional assembly of PhoE. In the case of LamB, sequences from two or more parts of the molecule appear to be

required for efficient assembly of this protein (Benson & Silhavy, 1983; S. Benson, pers. comm.).

The results described in Chapters 7 and 8 indicate that for TonA sequences close to the C-terminus are essential at some stage of assembly, either directly or via a conformational effect. Other regions of the mature molecule may contain additional assembly information.

Although for TonA the C-terminus appears to affect assembly in some way, the question remains as to whether specific sequences, perhaps close to the mature N-terminus, direct nascent outer membrane proteins to a particular assembly pathway, resulting in transfer to the outer membrane, or alternatively, whether subsequent to the initial interaction with and translocation across the inner membrane facilitated by the signal sequence, translocation to the outer membrane occurs via successive interactions of the completed protein with components of the outer membrane. The observation that preTonA was able to associate into a sarkosyl insoluble complex although remaining protease sensitive (see Chapter 5) suggests that this molecule can establish some interactions with other outer membrane components although remaining partially assembled due, presumably, to constraints imposed by the persistence of the signal sequence. Thus it is possible that the apparently essential sequence(s) at the TonA C-terminus are able to commence assembly into the outer membrane, despite the fact that the molecule may be anchored to the inner membrane by its signal sequence.

The route by which proteins are translocated to the outer membrane remains uncertain. The models proposed (reviewed in Chapter 1 (section IV.5) include (i) direct co- and/or post-translational translocation across the inner membrane and periplasm into the outer membrane; (ii) translocation through regions of close contact between inner and outer membranes and (iii) transfer via budding of vesicles from the inner membrane which subsequently fuse with the outer membrane. From the level of outer membrane protein synthesis, it would be expected that periplasmic vesicles, if they exist as assembly intermediates, would be common structures in the envelope. However, such vesicles have not been seen in electron micrographs of freeze fractured E. coli (Witholt, pers. comm.). Although the kinetics of TonA assembly indicated the possible occurrence of a periplasmic intermediate, either of the free or vesicular form, it remains possible that the "soluble" intermediate was in fact membrane associated in the intact cell, only becoming soluble following sonication.

When the envelopes from French pressed cells are fractionated by the Osborn technique the mixed membrane fraction is enriched for nascent outer membrane proteins compared to the inner and outer membrane fractions (DeLeij et al, 1979). This result indicates that assembly of outer membrane proteins occurs via transient or permanent regions of fusion between the two membranes, or that specialised regions of the inner and/or outer membrane exist through which outer membrane proteins are assembled. However, there

is as yet no evidence concerning the structural organisation or permanence of such sites.

Future study of TonA assembly

The identification of sequences important in assembly of TonA would be greatly facilitated by DNA sequencing, which would allow specific, *in vitro* mutagenesis of the *tonA* gene. The isolation of internal deletions would allow the identification of any sequences required for assembly additional to those apparently present at the C-terminus. Of course, as discussed above, deletion of just one of several important sequences may not alter the outer membrane location of the deletion derivative, merely the efficiency of the assembly process, thus the kinetics of assembly into the outer membrane of such TonA derivatives would have to be compared with those of wild type TonA. Protein hybrids between various sequences from the TonA molecule and, for example, an inner membrane protein or periplasmic protein could be constructed by gene manipulation and the localisation of such hybrids studied. A study of the C-terminal sequences of TonA, perhaps by site specific mutagenesis, might prove particularly interesting in view of their apparent importance in assembly. By placing the *tonA* gene under the control of an inducible promotor it would also be possible to study the putative lethal or detrimental TonA' polypeptides which lack only a short sequence at the C-terminus.

The production of an antiserum to TonA would greatly

facilitate the localisation in exponential cells of unstable TonA derivatives. If monoclonal antibodies to TonA were isolated, this would also allow an analysis of the folding of TonA within the membrane similar to that carried out for LamB by M. Schwartz. In addition, the availability of monoclonal antibodies to the TonA C-terminus would permit easy identification of in-frame internal deletions of the molecule.

Finally, the kinetics of assembly of TonA could be studied in greater detail; in particular it would be important to localise more rigorously the intermediate(s) in TonA assembly, perhaps by a combination of several fractionation techniques and studies of protease sensitivity in whole cells, and cells in which the outer membrane has been permeabilised.

CHAPTER TEN

ENHANCED POLYPEPTIDE SYNTHESIS PROGRAMMED BY LINEAR DNA FRAGMENTS IN CELL-FREE EXTRACTS LACKING EXONUCLEASE V

10.1 Introduction

In order to select a restriction fragment of pLC19-19 (carrying tonA, see Chapter 2) for recloning into pBR325 (or a similar multicopy vector), an attempt was made to use linear fragments of pLC19-19 as templates in the Zubay transcription-translation system. Pratt et al (1981a) reported that linear DNA fragments could be used successfully in the in vitro system, and it was hoped that in this way the shortest possible restriction fragment of pLC19-19 still encoding TonA could be identified. However, the efficiency of linear fragments of pLC19-19 as templates in the transcription-translation system was found to be very low. Yang et al (1980) demonstrated that the recBC exonuclease V was responsible for the degradation of linear DNA fragments by S30 cell free extracts of E. coli, and that restriction fragments were stable in S30 extracts prepared from a recB mutant. However, the use of such an extract for protein synthesis was severely limited due to the high background resulting from endogenous DNA which apparently remains intact in the recB extract (Yang et al, 1980; and our observations). Thus, during the preparation of extract from $recB^+$ strains, endogenous DNA is normally degraded during an 80 min preincubation stage, presumably by the action of the recBC exonuclease V. In an attempt to overcome the high backgrounds

associated with the absence of exonuclease V, we decided to prepare an extract from an E. coli strain (N138) carrying a temperature sensitive recB allele (Tomizawa & Hirofumi, 1972). We anticipated that, during extract preparation at the permissive temperature, the exonuclease V would be active, whilst in transcription-translation assays carried out at the non-permissive temperature, linear DNA would be stable.

10.2 Results

(a) Background incorporation in N138 and MRE600 extracts

S30 extracts were prepared from N138 (recB^{ts}, grown at 30^{o}C) and from MRE600 (grown at 37^{o}C) and the incorporation of ^{35}S -methionine above background was determined for both extracts using a supercoiled plasmid template (Table 10.1). Using the MRE600 extract, incorporation with a pBR325 template was 74 times the background, whilst using the N138 extract, incorporation was 30 times above background. The background level of incorporation obtained with the N138 extract therefore appeared sufficiently low for the extract to be usable in the transcription-translation system.

(b) Comparative exonucleolytic activity of MRE600 and N138 S30 extracts

The level of exonucleolytic activity in the S30 extracts was determined by carrying out incubations with either linear or supercoiled DNA as template. Samples were removed at intervals for analysis of the remaining DNA by agarose gel electrophoresis. As shown in Fig. 10.1, with the MRE600 extract the restriction fragments were almost completely

strain	cpm	
	+DNA	-DNA
MRE600	100 396	1 346
N138	75 698	2 544

Table 10.1: Background incorporation in N138 & MRE600 extracts

Transcription-translation incubations were set up using either MRE600 or N138 S30 extracts, with 3μg of pBR325 as template or without DNA as a control. After 30 min incubation a 2μl sample was removed and ^{35}S -methionine incorporated into protein estimated by trichloroacetic acid precipitation and scintillation counting. The figures presented are the average from several experiments.

Fig. 10.1: Stability of linear DNA templates in MRE600 and N138 extracts

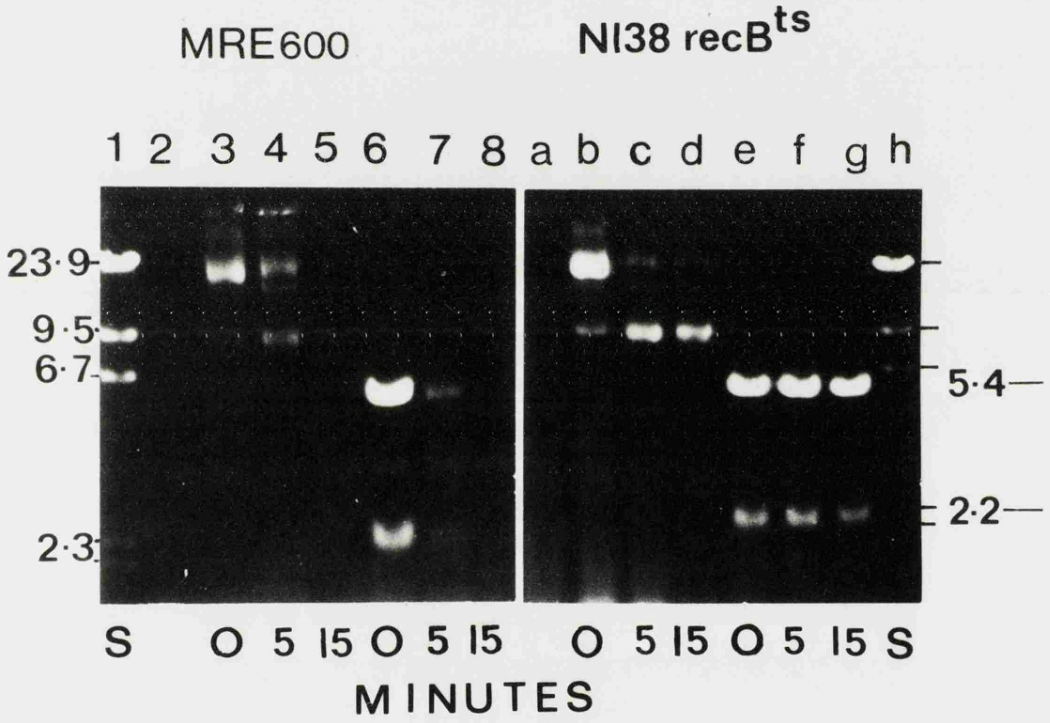
MRE600 and N138 (*recB*^{ts}) S30 extracts were incubated with 2.5µg of either pLG282 or pLG282 cut with EcoRI. At intervals 5µl aliquots were removed for analysis on a 0.6% agarose gel stained with ethidium bromide. Samples (0.5 - 1µg DNA) were removed at the times indicated. Further samples removed at 60 and 120 min were identical in each case.

Tracks 1 & h : molecular weight standards

Tracks 2 & a : extract incubated without DNA

Tracks 3,4,5,b,c,d : supercoiled pLG282 template

Tracks 6,7,8,e,f,g : pLG282 EcoRI fragments


MRE600
NI38 recB^ts
1 2 3 4 5 6 7 8 a b c d e f g h
23·9
9·5
6·7
2·3
5·4
2·2
S O 5 15 O 5 15 O 5 15 O 5 15 S
MINUTES

degraded within 5 min, whilst with the N138 extract no DNA degradation could be detected even after 120 min incubation.

(c) Kinetics of the transcription-translation reaction

The kinetics of the transcription-translation reaction using the MRE600 and N138 extracts with linear DNA templates were determined by removing samples at intervals for measurement of ^{35}S -methionine incorporation (Fig. 10.2). The results show that protein synthesis using the N138 extract could be sustained for up to 120 min, significantly longer than the corresponding MRE600 reaction.

(d) Effect of DNA concentration on protein synthesis

Standard transcription-translation assays were carried out using a range of concentrations of linear DNA template. The DNA used was the 5.4 kb plasmid pLG282 linearised with PstI which cuts within the ß-lactamase gene. The genes for chloramphenicol acetyl transferase (CAT, 24.8kD) and tetracycline resistance are also present on pLG282. As shown in Fig. 10.3, as little as 375ng of linear template was sufficient to promote the synthesis of readily detectable amounts of ^{35}S -methionine labelled CAT with the N138 extract compared to more than 5µg with an MRE600 extract. Longer exposure of the gel in Fig. 10.3 detected CAT programmed by 50ng of DNA with the N138 extract, and by 3-5µg of DNA with the MRE600 extract.

(e) Decay of exonucleolytic activity with age

Pratt et al (1981a) reported the successful use of restriction fragments as DNA templates in the Zubay transcription-translation system. In contrast, the results

Fig. 10.2: Protein synthesis programmed by a linear DNA template

10μg of pBR325 cut with HindIII was used as a template to programme protein synthesis with either MRE600 or N138 as the source of S30; ^{35}S -methionine incorporation into protein was measured by trichloroacetic acid precipitation and scintillation counting of 2μl samples removed at intervals from the incubation.

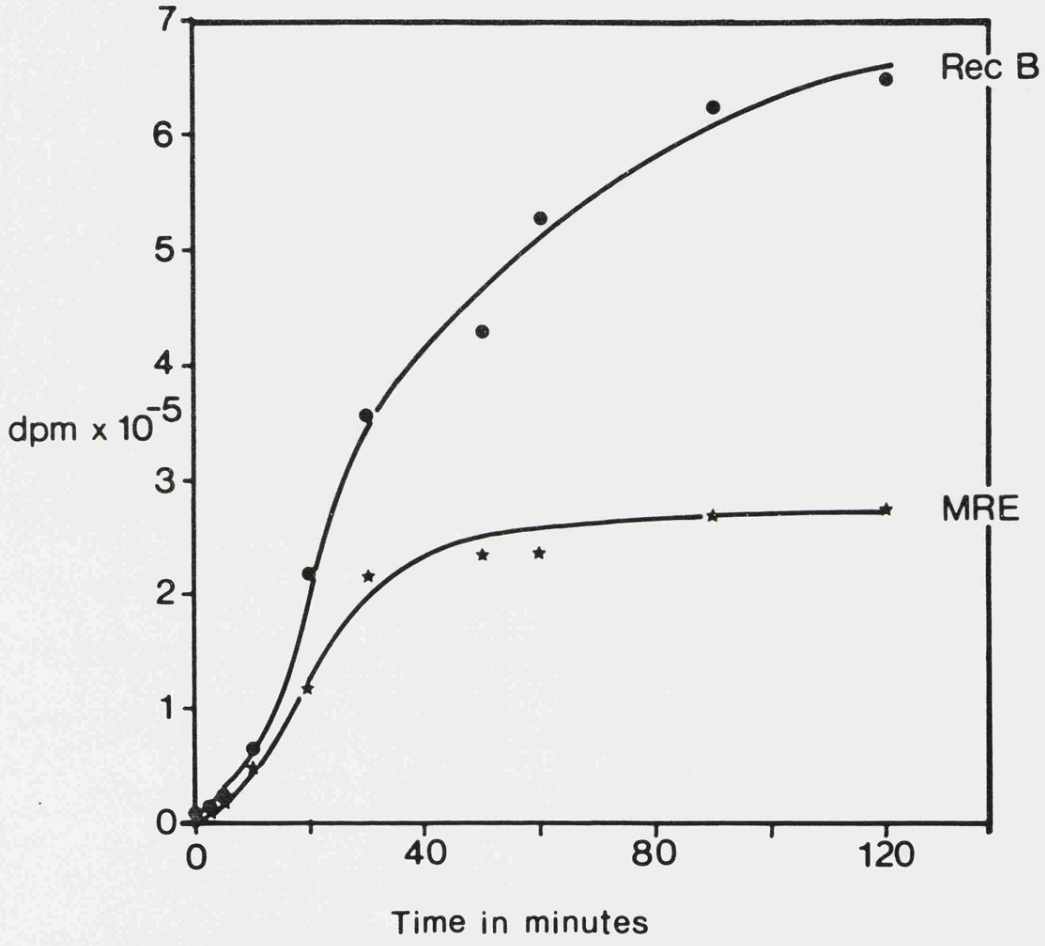
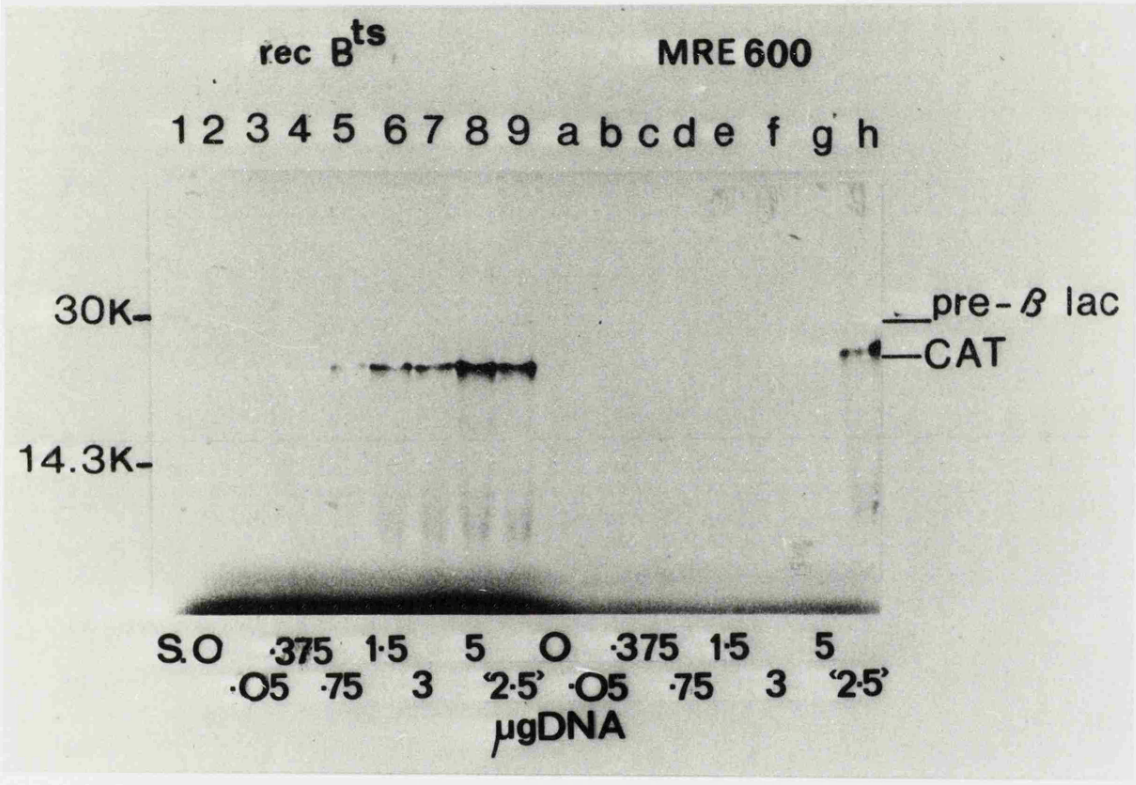

7
6
5
4
3
2
1
0
dpm x 10^-5
Rec B
MRE
0
40
80
120
Time in minutes

Fig. 10.3: Effect of DNA concentration on protein synthesis by MRE600 and N138 extracts

pBR325 (encoding CAT and β-lactamase) was cut at the unique PstI site (within the β-lactamase gene) and various amounts of the linear fragments were used as templates in transcription-translation incubations with either MRE600 or N138 S30 extract. Samples were analysed by SDS PAGE (15% acrylamide I) and autoradiography.

Track 1 : molecular weight standards


rec B ts
MRE 600
1 2 3 4 5 6 7 8 9 a b c d e f g h
30K-
14.3K-
pre-β lac
CAT
S.O ·375 1·5 5 O ·375 1·5 5
·O5 ·75 3 '2·5' ·O5 ·75 3 '2·5'
μgDNA

described here indicated that linear DNA was rapidly degraded and inefficient as a template when an MRE600 extract was used. Similar results indicating instability of linear DNA templates have been obtained using another E. coli K12 strain (J. Merrick, pers. comm.). Since the extracts used in the latter two cases were freshly prepared, whereas the extract used by Pratt et al (1981) had been stored in liquid nitrogen for some time, the stability of a linear DNA template was measured in fresh and old extracts. From Fig. 10.4 it is apparent that exonuclease activity in extracts of MRE600 and GI65 which were 1 - 2 years old was as low as that in fresh extracts of N138. Thus exonuclease V activity does appear to decay during storage of extracts in liquid nitrogen.

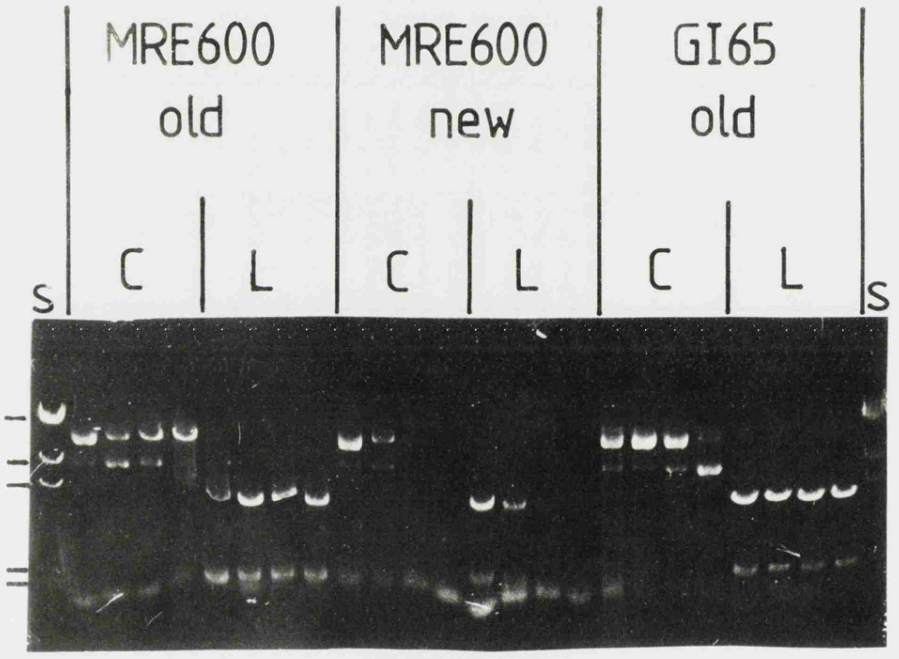
10.3 Conclusion

The use of E. coli strain N138 (recB^{ts}) as a source of S30 extract greatly improves the efficiency of linear DNA as a template in the in vitro transcription-translation system. The high background incorporation normally obtained when the S30 extract is made from a recB^{-} mutant appears to be eliminated by the use of the recB^{ts} mutant. Exonuclease activity in extracts of MRE600 and E. coli K12 strains appears to decay with age, thus linear DNA templates can be successfully used with recB^{+} extracts when these extracts are a year or more old.

Fig. 10.4: Stability of linear DNA templates in fresh and old S30 extracts

Transcription-translation incubations were set up with 2.5µg of either pLG282 (C) or pLG282 cut with EcoRI (L) as template, using old MRE600 S30 extract (stored approximately 1.5 years in liquid nitrogen), fresh MRE600 extract or old GI65 S30 extract (stored approximately 2 years in liquid nitrogen). At times 0, 5, 15 and 40 min 5µl aliquots were removed for analysis on a 0.6% agarose gel stained with ethidium bromide.

S - x HindIII standards : 23.9kb
9.5kb
6.7kb
2.3kb
1.96kb


MRE600
old
MRE600
new
GI65
old
C
L
C
L
C
L
S
S

CHAPTER ELEVEN

MATERIALS AND METHODS

11.1 Bacterial strains and bacteriophage

The bacterial strains and bacteriophage used in this study are listed, with their genotypes, in Table 11.1. Cultures were maintained on nutrient or M9 minimal agar plates at 4°C when in current use, and frozen at -20°C in nutrient broth containing 20% glycerol for long term storage.

11.2 Media and buffers

All media and buffers used are described in Table 11.2. For thymine requiring mutants all media were supplemented with 80μg/ml thymine. Plasmid bearing strains were grown in and maintained on freshly prepared media containing the appropriate antibiotic(s) as shown in Table 11.2.

11.3 Growth of bacteria in liquid culture

Liquid cultures were grown in a New Brunswick Gyrotary shaker at temperatures indicated in the text, and mass increase followed by measuring absorbance at 450nm or 600nm using a Gilford Microsample spectrophotometer 300N.

11.4 Preparation of bacteriophage

a)T1 and T5: The phage were diluted in lambda buffer to 1-2x10^6 plaque forming units (pfu)/ml, and 0.1ml of this dilution added to 0.2ml of a stationary phase culture of MC4100 in nutrient broth. After 10 minutes at 37°C to allow

Table 11.1: Bacteria and bacteriophage

Bacterial strains

MC4100	*ara*D139, Δ(*lac*IPOZYA)U169, *thi*A, *rel*A1, *rps*L	P.Bassford
PB72-47	MC4100/λp72-47, lysogen for λ phage carrying *mal*E-*lac*Z fusion 72-47	P.Bassford
GI65	*thr*, *leu*, *thi*, *lac*, *ton*A, *sup*E	lab stock
Rec35-1	*thr*, *leu*, *his*, *ile*, *arg*, *thi*, *pyr* *thy*A, *lac*, *tsx*, *ton*A, *str*, *rec*A35	I.B.Holland
CSH26ΔF6	Δ(*lac*,*pro*), *ara*, *thi*, Δ(*rec*A,*srl*), *rps*L	P.Oliver
CSH26ΔF6 *ton*A	spontaneous $T5^r$ derivative of CSH26ΔF6	this study
SE5000	MC4100*rec*A56 : *ara*D139, *rel*A1, Δ(*arg*F,*lac*)U169, *rps*L150, *flb*-301, *deo*C1, *pts*F, *rec*A56	C.Higgins
DS410	sup^o, *lac*Y, *min*A, *min*B, *rps*L, *ara*, *mal*A, *azi*	J.Reeve
DS410*ton*A	spontaneous $T5^r$ derivative of DS410	this study
N138	*met*, *leu*, *his*, *arg*, *gal*, $rec B^{ts}$, *lac*, *rps*L	G.Churchward
RB308	F^+, *deo*C, *lac*Y, *thy*A	G.Boulnois
MRE600	$RNase^-$	Porton Down
LE316	*met*B, *arg*E, *ilv*, *tna*, *sup*E, *gyr*B	E.Orr
JA200 /pLC19-19	Host: F^+, *trp*E5, *thr*, *leu*, *lac*Y, *rec*A (F^+ from *E.coli* Ymel)	B.Spratt
159 /pGY101	Host: *gal*, *ara*, *uvr*A, sup^o, *str* Plasmid: Km, λ*imm*434T	N.Stoker
DS410 /pBS42	Plasmid: Km, PBP5	B.Spratt
DS410 /pLG310	Plasmid: Ap, PBP6	N.Stoker

Table 11.1 cont.

Bacteriophage strains	
T1 and T5	G.Plastow
λpBS10	B.Spratt

Table 11.2: Media and Buffers

Media

Nutrient broth	Oxoid No. 2 Nutrient Broth	2.5% w/v
Luria broth	Tryptone	1.0% w/v
	Yeast extract	0.5% w/v
	NaCl	0.5% w/v
M9 Mininal medium	Na_2HPO_4	0.6% w/v
	KH_2PO_4	0.3% w/v
	NH_4Cl	0.1% w/v
	NaCl	0.05% w/v
	Glucose (autoclaved separately)	0.4% w/v
	$MgSO_4$ "	10mM
	$CaCl_2$ "	1mM
Plus, as required:		
	Thiamine (filter sterilised)	10μg/ml
	Amino acids	40μg/ml

Nutrient/Luria/minimal agar: media as above, solidified with 1.45% agar
BTL agar : 1% BBL trypticase, 0.5% NaCl, 0.7% agar
Soft nutrient agar : nutrient broth solidified with 0.5% agar

Antibiotics	Ampicillin (Ap)	25μg/ml
	Streptomycin (Sm)	100μg/ml
	Tetracycline (Tc)	10μg/ml
	Chloramphenicol (Cm)	25μg/ml
	Spectinomycin (Sp)	100μg/ml

Ap and Sm filter sterilised, stocks of Tc and Cm 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
Lambda buffer	Tris/Cl pH7.2	6mM
	$MgSO_4$	10mM
	Gelatin	0.5% w/v

phage adsorption, 4ml of soft nutrient agar (at about 45°C) was added and the mixture poured onto a luria agar plate. Several plates were prepared in this way, and incubated overnight at 37°C. 3ml of lambda buffer was then added to each plate, and the top layers scraped off together with the buffer, into a McCartney bottle. The mixture was shaken vigorously with 5 drops of chloroform and allowed to stand for 10 minutes before spinning out the cell debris in an MSE bench centrifuge at full speed for 20 minutes. The supernatant was collected and stored over chloroform.

Phage titres were assayed by mixing 0.1ml of various phage dilutions with 0.1ml of a stationary phase culture of MC4100, adsorbing for 10 minutes at 37°C, adding 4ml of soft nutrient agar, and pouring onto Luria agar plates.

b)Lambda: λ phage were prepared and assayed essentially as for T1 and T5 phage, except that the bacteria were grown up in Luria broth supplemented with 0.4% maltose to induce the receptor, BTL agar was used in place of soft nutrient agar, and the Luria agar plates contained 10mM $MgSO_4$ or $MgCl_2$.

11.5 Isolation of tonA mutants

0.1ml of 10^0, 10^{-1} and 10^{-2} dilutions of a stationary phase culture of the appropriate bacteria were spread over nutrient agar plates which had previously been spread with 0.1ml of T5 phage (approximately 10^{10}pfu/ml). The plates were incubated at 37°C overnight; resultant colonies were purified on nutrient agar plates and retested by cross streaking against T5 phage.

11.6 Cell fractionation

The basic procedure was that described by Churchward and Holland (1976), except that $MgSO_4$ was ommitted from the buffer. 120ml of exponentially growing cells at A_{450} 0.5 were harvested and resuspended in 10ml ice cold 10mM sodium phoshate buffer, pH7.2, transferred to a 25ml beaker, and sonicated for 3 x 30 sec with 30 sec cooling on ice, at 6μ amplitude, using the 3/4 inch end diameter probe in a 150 Watt MSE ultrasonic disintegrator. The sonicated samples were centrifuged in a Sorvall SM24 rotor for 5 min at 7krpm to remove unlysed cells. If required, 0.5ml of the supernatant was removed at this stage as a total cell sample, before pelleting the membranes in a Beckman 50Ti rotor for 30 min at 30krpm and 5°C. 0.5ml of the supernatant was retained as the cytoplasmic fraction, the rest was discarded. The membrane pellet was resuspended in 1ml of 10mM sodium phosphate buffer, pH7.2 and repelleted in the 50Ti rotor for 30 min at 30krpm and 5°C.

The washed membrane pellet was resuspended in 200μl of 0.5% w/v sarkosyl NL97, and 50μl removed for a total envelope fraction, if required. The remainder was incubated at room temperature for 30 min before centrifuging in the 50Ti rotor for 1 hour at 35krpm and 15°C. The supernatant from this centrifugation represented the solubilised inner membrane proteins; the pellet, containing the sarkosyl insoluble outer membrane proteins, was washed by resuspension in a further 1ml of 0.5% sarkosyl, incubating for 30 min at room temperature, and centrifuging for 1 hour at 35krpm as before.

The pellet was then resuspended in 150µl of 10mM sodium phosphate buffer, pH 7.2. 1/3 volume of acrylamide gel sample buffer was added to each fraction in an eppendorf tube, and the samples boiled for 5 min and frozen at -20°C. Resuspension of membrane pellets at each stage was found to be easier if the pellets were frozen in the resuspension solution and resuspended as they thawed.

When loading the samples thus prepared onto acrylamide gels, sample volumes used were as follows: 100µl of total cell, cytoplasmic and inner membrane samples, and 10 - 20µl of outer membrane samples per gel slot.

11.7 SDS polyacrylamide gel electrophoresis

a) Preparation and running of gels: The procedure was based on that of Laemmli (1970), using a Biorad 220 slab gel apparatus without cooling. The buffers, solutions and gel recipes used are given in Table 11.3. Gels were usually 1mm thick, and composed of either a 7% acrylamide stacking gel with an 11% or 15% acrylamide separating gel or a 5% acrylamide stacking gel with an 8.5% acrylamide separating gel, with 1cm of effective stacking distance between the sample wells and the surface of the separating gel. All samples were boiled for 5 min before loading and electrophoresis carried out at 25 mAmps/gel, until the dye front was within 5mm of the bottom of the gel. Gels were then either fixed by shaking in 200 - 300ml of destain for at least 30 min, or shaken in 200 - 300ml of stain overnight. Stained gels were destained by diffusion in several changes

Table 11.3: SDS PAGE solutions, buffers and gel recipes

Gel composition

		8.5%	11%
Separating gel:	Buffer A	13.5	13.5ml
	Acrylamide (I or II)	5.3	6.8ml
	H_2O	7.5	6.0ml
	Ammonium persulphate (APS) freshly made, 10mg/ml	1.0	1.0ml
	N,N,N',N'-tetramethyl ethylenediamine (TEMED)	75	75µl
		5%	7%
Stacking gel:	Buffer B	10.0	10.0ml
	Acrylamide (I or II)	2.4	3.3ml
	H_2O	7.6	6.7ml
	APS	0.5	0.5ml
	TEMED	40	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 buffer	Trisma base	0.125
	Glycine	0.192M
	SDS	0.1% w/v
Sample buffer	Tris/Cl pH 6.8	0.125M
	Glycerol	20% v/v
	-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% v/v
	Acetic acid	10% v/v
Stain	Coomassie brilliant blue	0.05% w/v in destain

Table 11.3(a): Acrylamide gel recipe for PBP separation

All buffers made up as in Table 11.3, except that Sigma SDS (95% pure) was used instead of Biorad SDS.

Acrylamide III	Acrylamide	30% w/v
	Bis	0.4% w/v

Gel composition

Separating gel (11%):	Buffer A	13.5ml
	Acrylamide III	10ml
	Water	2.8ml
	APS	0.95ml
	TEMED	75µl
Stacking gel (7%):	Buffer B	10ml
	Acrylamide III	4.8ml
	Water	5.2ml
	APS	0.5ml
	TEMED	40µl

of destain, shaking throughout.

b) Molecular weight markers: For stainable molecular weight markers 20µl of a solution containing sample buffer and 50ng/µl of each required protein was loaded per slot.

For radioactive molecular weight markers, a ^{14}C - methylated protein mixture was used, which contained myosin (200kD), phosphorylase B (100kD and 92.5kD), bovine serum albumin (69kD), carbonic anhydrase (30kD) and lysozyme (14.3kD). 5nCi of this mixture, with sample buffer added, was loaded per gel slot, or, if the gel was to be fluorographed, 1nCi per slot.

c) Autoradiography and fluorography: For autoradiography, fixed gels were dried onto a sheet of Whatman No. 17 chromatography paper using a Biorad slab gel drier model 1125. The dried gels were placed in a cassette with a sheet of Kodak XR P5 X-ray film for exposure, and the films developed using Kodak DX-80 developer, a 1% acetic acid wash, and Kodak FX-40 fixer.

Fluorography was carried out using the method of Bonner and Lasky (1974): fixed gels were dehydrated by two 30 min washes in 300ml dimethylsulphoxide (DMSO), after which the gels were soaked for 90 min in 200ml 22% PPO in DMSO and then rehydrated by soaking in water for 60 min. The gels were then dried and autoradiographed as above, except that cassettes were stored at -80^{o}C during exposure.

d) Estimation of radioactivity in individual gel bands: The method of Ames (1974) was employed: bands of interest were cut from stained, dried gels and placed in scintillation

vials, together with the backing paper. The slices were covered with 50µl of distilled water and rehydrated for 10 min at room temperature, after which 5ml of NCS scintillation fluid (Table 11.7) was added to each vial. The vials were then stoppered and vortexed. After overnight incubation at 37°C, the samples were cooled and revortexed before counting in a Packard TriCarb 3255 scintillation counter.

11.8 Preparation of plasmid DNA

When handling DNA, disposable gloves were worn, all buffers, reagents, glass and plastic ware used were sterilised by autoclaving, and all glassware coming into contact with the DNA was siliconised.

a)Rapid plasmid preparation for screening: 1.5ml of an overnight culture in Luria broth plus the appropriate antibiotic(s) was harvested into an eppendorf tube (3 min in Eppendorf microfuge). The cells were resuspended by vortexing in 100µl of a freshly made solution of 4mg/ml lysozyme in TEG buffer (Table 11.4). The tube was incubated at room temperature for 5 min. 200µl of of alkaline SDS solution was added and the contents of the tube mixed by several sharp inversions, followed by a further 5 min incubation on ice. 150µl of potassium acetate (Table 11.4) was then added and the tube vortexed gently for 10 sec. The chromosomal clot was pelleted by centrifugation for 5 min at 4°C and the supernatant removed into a fresh eppendorf tube using a Gilson P200 pipette. An equal volume of phenol mix was added to the supernatant, the tube was vortexed, and then

Table 11.4: Plasmid DNA preparation

TEG buffer	Tris/Cl pH 8.0	25mM
	EDTA	10mM
	Glucose	50mM
Alkaline SDS	NaOH	200mM
	SDS	1% w/v
Potassium acetate	5M KAc	60ml
	Glacial acetic acid	11.5ml
	H_2O	28.5ml
Phenol mix	Phenol	500g
	Chloroform	500ml
leave to dissolve, then add:		
	8-hydroxyquinoline	0.5g
	Isoamylalcohol	20ml
store under 1cm layer of TE buffer at 4°C		
TE buffer	Tris/Cl pH 7.5	10mM
	EDTA	1mM
Caesium chloride-isopropanol	Saturated CsCl solution	10ml
	Isopropanol	250ml
add 10ml amounts of H_2O and mix well until an aqueous phase persists, and all the CsCl is in solution		

centrifuged for 2 min. The aqueous layer was carefully removed, taking care not to disturb the interface. The nucleic acids were then precipitated from the aqueous layer by the addition of 1/10 volume of 3M sodium acetate, pH 4.8 and 2.5 volumes of ethanol, vortexing briefly and leaving at room temperature for 2 min. The precipitate was collected by centrifuging for 5 minutes, and the pellet well drained. 1ml of 70% ethanol was added to the pellet, the tube vortexed briefly and recentrifuged. The supernatant was removed, the pellet dried briefly under vacuum, and resuspended in 50µl TE. For restriction digests 10µl of DNA was used; contaminating RNA was degraded by addition of RNase.

b)Large scale preparation : This was basically a scaled up version of the rapid preparation. 500ml of Luria broth was innoculated from an overnight culture to A_{450} 0.05 - 0.1, and the cells grown at 37°C to A_{450} 0.9, when 85mg chloramphenicol (or 150mg spectinomycin for Cm resistant strains) was added to amplify the plasmid. After shaking overnight at 37°C, the cells were harvested in the Sorvall GS3 rotor for 10 min at 5krpm. The cells were resuspended in 8ml of 4mg/ml lysozyme in TEG buffer, and subsequent operations carried out as for the rapid preparation, scaling up the volumes, until just prior to the phenol extraction stage. At this point, the nucleic acids were precipitated by the addition of 2.5 volumes of ethanol, incubated at -20°C for 1 hour, pelleted (GSA rotor, 5krpm, 10 min, -5°C) and resuspended in 7.7ml of TE buffer. 1.2ml of 5mg/ml ethidium bromide and 9g caesium chloride were then added and the CsCl

dissolved. The refractive index of the mixture was adjusted to 1.393 by addition of further CsCl or TE buffer. The mixture was then transferred to a Beckman quickseal tube and, if necessary, liquid paraffin was added to fill the tube. After centrifugation in the Beckman 50Ti or 75Ti rotor for 40 - 48 hours at 38krpm and 15^{o}C the plasmid band, below the usually faint chromosomal band, was visualised with long wave ultraviolet light (although sometimes this was not necessary) and collected by insertion of a syringe through the tube wall. The plasmid DNA solution was then extracted 3 times with an equal volume of CsCl saturated isopropanol to remove ethidium bromide, the CsCl was removed by dialysis against TE buffer for 1 hour, then 1/4 TE buffer for 2 hours, and finally against 2 changes of 1/10 TE buffer for 2 hours each. If necessary the plasmid solution was concentrated by ethanol precipitation, adding 1/10 volume of sodium acetate and 2.5 volumes of absolute ethanol, leaving at -20^{o}C overnight, spinning down the nucleic acids, and resuspending in 1/10 TE buffer. Plasmid yield was usually about 0.5 - 1mg.

11.9 Agarose gel electrophoresis

Horizontal agarose slab gels (0.5 - 0.7%) were prepared by boiling agarose in electrophoresis buffer (Table 11.5) to dissolve, and adding ethidium bromide to 0.5µg/ml before pouring. All samples were mixed with 1/3 volume of agarose gel sample buffer before loading. Electrophoresis was carried out with the gel completely submerged in electrophoresis buffer, at 100 volts, until the dye front had migrated

Table 11.5: 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 11.6: Restriction and ligation of DNA

Restriction buffers (x5)

Enzyme	Tris/Cl	pH	$MgCl_2$	NaCl	KCl	DTT	βSH	EDTA
EcoRI	500	7.4	50	250	-	2.5	-	-
BamH1	30	7.4	30	250	-	-	30	-
HindIII	50	7.4	50	250	-	2.5	-	-
PstI/BglII	30	7.4	30	-	-	2.5	-	-
SalI	40	7.6	30	750	-	-	-	1
SmaI	75	8.0	30	-	75	-	-	-

figures represent mM concentrations

Ligation buffer (x10)

1M Tris/Cl pH 7.4	660µl
1M $MgCl_2$	100µl
10mM ATP	100µl
DTT	15mg
BRL nuclease free BSA	10µl
H_2O	90µl

Bal31 exonuclease buffer (x5)

Tris/Cl pH 8.0	100mM
$MgCl_2$	60mM
$NaCl^2$	3M
$CaCl_2$	60mM
$EDTA^2$	5mM

DTT - dithiothreitol; βSH - β-mercaptoethanol

through 3/4 of the gel. DNA was visualised by transillumination with short wave (260nm) ultra violet light. λ DNA cut with HindIII restriction endonuclease was used to give a set of molecular weight markers.

11.10 Restriction endonuclease digestion

All manipulations were carried out wearing gloves, using sterile buffers and plasticware. 0.5μg of plasmid DNA was used per restriction digest; if the DNA was heavily contaminated with RNA, 0.5μl of 6mg/ml RNase (previously boiled for 5 min to destroy any DNase activity) was added to the restriction mixture. Final reaction volume was usually 10μl, thus, 2μl of x5 restriction buffer (Table 11.6) was added, but this volume was increased if more DNA was to be restricted (eg. for a ligation); in all cases total reaction volume was at least 10 times the volume of enzyme added. Digestions were carried out for 1 hour at 37^{o}C and if necessary (for ligation) enzymes were then inactivated by a further 10 minute incubation at 65^{o}C.

11.11 Ligation of DNA fragments

DNA to be ligated was first phenol extracted and ethanol precipitated (see section 11.8a). After resuspension of the precipitate in TE buffer, the DNA fragments to be ligated were mixed, heated to 65^{o}C for 5 minutes to break apart the "sticky ends" and then left on ice for 60 minutes to allow reannealing. 1/10 volume of x10 ligation buffer (Table 11.6) and T4 DNA ligase were then added and the mixture incubated

overnight at 16°C. 5 - 20µg of DNA was usually used per ligation, and 0.5µg samples taken before addition of ligase, and after overnight incubation, to be run on a gel to check the ligation reaction.

For cloning of a restriction fragment into a vector, a 4:1 ratio of fragment to vector was used, at a DNA concentration of about 50µg/ml. For recircularisations a lower DNA concentration of about 10µg/ml was used. Blunt ended ligations were carried out at a DNA concentration of 100µg/ml, with 10 times the normal ligase concentration.

11.12 Transformation of bacteria with plasmid DNA

A culture of the bacteria to be transformed was grown in luria broth from A_{600} 0.01 - 0.05 to A_{600} 0.4. 10ml of these cells were harvested into a sterile capped Sorvall SS34 tube (5 minutes, 10krpm) and resuspended in 5ml of sterile $MgCl_2$. Immediately the cells were repelleted and resuspended in 5ml of sterile 100mM $CaCl_2$. After incubation on ice for 20 minutes, the bacteria were harvested and resuspended in 0.5ml of 100mM $CaCl_2$. 200µl of these cells were added to the DNA in an eppendorf tube and the mixture incubated on ice for 60 min. After a 5 min heat shock at 42°C, the bacteria were diluted into 2ml prewarmed nutrient broth and grown for two hours at 37°C. 0.1ml aliquots of 10^0, 10^{-1} and 10^{-2} dilutions were then spread over nutrient agar plates containing antibiotics to select for plasmid transformed cells. Dilutions of 10^{-5} and 10^{-6} were also plated on nutrient agar to assay viable cells. Plates were usually incubated

overnight at 37°C; occasionally a further days incubation was required to produce easily visible colonies. Transformants were restreaked and purified on nutrient agar containing the appropriate antibiotics.

With untreated, supercoiled plasmid DNA, 1μg was used per transformation; the whole of a ligation mix was used in a transformation.

11.13 The maxicell system

The strain used for this *in vivo* expression system was CSH26ΔF6*ton*A, which carries a *rec*A deletion and is therefore highly sensitive to ultraviolet irradiation. The method used here is based on that of Sancar *et al* (1979), who found that low UV doses cause extensive degradation of chromosomal DNA in *rec*A strains, while most plasmid DNA copies remain intact due to a lower chance of receiving a UV hit. Plasmid encoded proteins can therefore be identified by labelling the proteins synthesised after chromosomal degradation has occurred.

Plasmid bearing strains of CSH26ΔF6*ton*A were grown in M9 minimal medium containing the appropriate antibiotics, from A_{450} 0.05 to A_{450} 0.5. 2.5ml of each strain was transferred to a small (2" diameter) sterile petri dish, and irradiated, while shaking, with 40 ergs, given in 10 sec, from a Hanovia bactericidal lamp. A control culture of plasmid free CSH26ΔF6*ton*A was also irradiated. 2ml of each culture was then transfered to a foil covered 6" x 5/8" test tube, and the tubes shaken at 37°C for 60 min. Ampicillin was added to

each tube to a final concentration of 25µg/ml, to kill surviving cells, before shaking the tubes overnight at 37°C.

1ml of irradiated cells of each strain were harvested into sterile eppendorf tubes, and the bacteria resuspended in 0.5ml fresh, prewarmed minimal medium containing 25µg/ml ampicillin. The tubes were incubated at 37°C for 60 min then 2µl of ^{35}S -methionine was added to each to label the plasmid encoded proteins. After 60 min incubation at 37°C (where truncated polypeptide products were being studied, this incubation was reduced to 10 min), 20µl of 44mg/ml methionine was added to each tube, the bacteria pelleted in an eppendorf centrifuge (5 min) and resuspended in 60µl of bacterial buffer. 20µl of SDS PAGE sample buffer was added to each sample before boiling and analysing on acrylamide gels.

11.14 Minicells

The minicell producing strain DS410tonA was used; the minicells were purified from a 1 litre overnight culture grown in Luria broth containing appropriate antibiotics. The cells were harvested for 10 min at 8krpm, resuspended in 10ml of M9 minimal medium, and layered onto 2 sucrose gradients in polycarbonate Sorvall tubes (20% sucrose in M9 minimal medium, previously frozen in the tubes and allowed to thaw overnight at 4°C) which were centrifuged at 5krpm for 20 min in the HB4 rotor. The minicells, banding in the central region of the gradients, were removed with a pasteur pipette and pelleted in the SS34 rotor for 10 min at 13krpm. The pellet was resuspended in 2.5 ml of M9 minimal medium,

layered onto a single sucrose gradient and centrifuged as before. Again, the minicell band was removed, the cells pelleted, and resuspended in 1ml of M9 minimal medium before layering onto a sucrose gradient and centrifuging for 20 min at 5krpm. The top half of the minicell band was removed, pelleted, and resuspended in 0.5ml of M9 minimal medium. 10µl of the minicells were plated out for a viable count, and the cells were also checked under a phase contrast microscope.

To label plasmid encoded proteins, 100µl of minicells were transferred to an eppendorf tube, 400µl of minimal medium added, and the minicells pelleted in an eppendorf microfuge for 5 min. After resuspending in 100µl of M9 minimal medium the minicells were incubated at 37°C for 60 min, then 25µCi of ^{35}S -methionine was added and incubation continued at 37°C for 30 min. 20µl of 44mg/ml methionine was then added and the cells incubated for a further 15 min at 37°C. The minicells were then pelleted in the microfuge and resuspended in 60µl of SDS PAGE sample buffer.

11.15 λ infection of UV irradiated cells

4.5 A_{450} units (8 - 15ml) of cells growing exponentially in the media indicated in the text were transferred to a large glass petri dish and heavily irradiated (12,000 ergs mm^{-2}) on a platform shaker under a Hanovia bactericidal lamp. 3 A_{450} units of cells were transferred to a McCartney bottle and harvested in a bench centrifuge (full speed, 10 min). The cell pellet was resuspended in 2ml prewarmed M9 minimal medium containing 10mM $MgCl_2$. For each sample, 100µl of cells

were transferred to an eppendorf, and 100µl of λ phage (titre 5 x 10^8 to give a m. o. i. of 10, dialysed to remove any methionine). The tubes were incubated at 37°C for 10 min to allow phage adsorption, then 200µl of prewarmed medium was added and the tubes incubated at 37°C for a further 20 min. 25µCi of ^{35}S -methionine was then added and the tubes incubated for 10 min at 37°C followed by the addition of 200µl of 8mg/ml unlabelled methionine and chilling on ice. The cells were pelleted (3 min) and the supernatent removed using a Gilson P1000 pipette. The pellet was resuspended in 50µl of bacterial buffer, 20µl of SDS PAGE sample buffer added and the samples boiled for 3 min.

11.16 Zubay in vitro transcription-translation system

The E. coli extracts were prepared as described by Pratt et al (1981a) using strains MRE600 (RNase$^-$) or LE316. For preparation of extract from N138 (recB^{ts}) the following modifications were made: the cells were grown at 30°C rather than 37°C and the extracts were preincubated at 30°C for 160 min to ensure complete degradation of endogenous DNA before storage in liquid nitrogen. Transcription-translation incubations were carried out at 37°C and contained:

7.5µl	low molecular weight mix
2.0µl	^{35}S -methionine (25µCi)
3.5µl	0.1M magnesium acetate
5.0µl	S30 extract (strain indicated in text)
2 - 5µg	DNA in 10mM Tris/Cl 1mM EDTA pH 7 +10mM Tris/acetate pH 7 to 30µl

After 30 min incubation, 5µl of prewarmed 44mg/ml unlabelled methionine was added and the incubation continued for a further 5 min. The protein products were analysed by SDS PAGE and autoradiography.

11.17 Osmotic shock (for periplasmic fraction)

The method used was that of Nossal & Heppel (1966). 50ml of exponentially growing cells (A_{450} 0.5) were harvested (8k, 5 min, SS34) and washed twice in 10mM Tris/Cl 30mM NaCl pH7.1. The cell pellet was then resuspended in 3 ml of 30mM Tris/Cl pH 7.1 and 0.5 ml taken as the whole cell sample. 2.5 ml of 40% sucrose in 30mM Tris/Cl pH7.1 was added to the remainder of the cells, followed by 6µl of 100mM EDTA, stirring rapidly. The tube was shaken at 25°C for 10 min. The cells were then harvested (6k, 10 min, SS34) and the pellet well drained before rapid resuspension in 5 ml of ice cold 0.5mM $MgCl_2$. The tube was shaken in an ice bath for 10 min and the cells harvested (6k, 10 min, SS34). The supernatent (periplasm) was decanted into a SM24 tube and centrifuged (8k, 10 min) to remove any remaining cells. 1ml of 50% TCA was added to the cleared supernatent, the tube left on ice for at least 30 min, the precipitate harvested (5k, 5 min, SM24), well drained and resuspended in 200µl of bacterial buffer to which was added 70µl SDS PAGE sample buffer plus sufficient saturated Tris solution to restore blue colour to the sample buffer. The cell pellet was resuspended in 200µl bacterial buffer and 70µl SDS PAGE buffer added.

When smaller initial quantities of cells were used (eg,

Table 11.7: Scintillation fluid

Non-aqueous scintillation fluid	PPO	5g
	dimethylPOPOP	0.3g
	toluene	1000ml
NCS scintillation fluid	NCS solubiliser	83.5ml
	PPO	4g
	dimethylPOPOP	60.5mg
	toluene	1000ml

Table 11.8: 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
Amino acids : Sigma
Ammonium persulphate : Biorad
Ampicillin : Sigma
Bal31 exonuclease : Biolabs
Chloramphenicol : Sigma
Coomassie brilliant blue : Gurr's
Gelatin : Difco
8-hydroxyquinoline : AnalaR
Isoamylalcohol : Sigma
Isopropanol : Fisons SLR
Lysozyme : Sigma
ß-mercaptoethanol : Sigma
NCS Tissue Solubiliser : Amersham
N,N'-methylene-bis-acrylamide : Eastman Kodak
Restriction endonucleases : BRL
Sodium dodecyl sulphate : Biorad
Spectinomycin (Trobicin) : Upjohn
Streptomycin : Glaxo
T4 ligase : Biolabs
Tetracycline : Sigma
N,N,N',N'-tetramethylethylenediamine : Eastman Kodak
Trisma base : Sigma
Tryptone : Oxoid
Thiamine HCl : Sigma
Yeast extract : Oxoid

^{35}S-methionine was used at a specific activity of approximately 1300 Curies/mmol, and a radioactive concentration of approximately 15 mCi/ml.

for maxicells), the volumes of reagents used were scaled down accordingly

11.18 ^{14}C -benzylpenicillin labelling of penicillin binding proteins

(a) Membrane fraction

The method used was that of Spratt (1977). A washed total membrane pellet was isolated from the cells as described in section 11.6. The pellet was resuspended in 100µl of 50mM sodium phosphate, pH7.2, 10µl of ^{14}C -benzylpenicillin was added, and the mixture incubated for 10 min at 30°C. 10µl of 20% sarkosyl was then added and incubation continued at room temperature for 15 min. 5µl of 120mg/ml cold benzylpenicillin was added and the outer membranes pelleted by centrifugation (35krpm, 60 min, 50Ti). The supernatent represented the inner membrane fraction.

(b) Cytoplasmic fraction

50µl of ^{14}C -benzylpenicillin was added to 1ml of the cytoplasmic fraction and the sample incubated at 30°C for 10 min, followed by the addition of 50µl of 20% sarkosyl, a 15 min incubation at room temperature and, finally, the addition of 5µl of 120mg/ml cold benzylpenicillin.

Samples prepared in this way were electrophoresed on 11% acrylamide III gels, using the recipes in table 11.3(a).

11.19 Partial proteolysis with Staphylococcus aureus V8 protease

Partial proteolysis was carried out according to the method of Cleveland et al, 1977). ^{35}S -methionine labelled maxicells were prepared for electrophoresis and each sample run in four slots on an 8.5% acrylamide II gel of 1mm thickness. The gel was stained for 90 min, then immediately placed over a light box and the TonA band (or equivalent region of the gel for CSH26ΔF6tonA/pLG515::Tn1000-8) cut out. The slices were soaked for 30 min in 10ml of 0.125M Tris/Cl, 0.1% SDS, 1mM EDTA pH6.8 (TSE), then frozen at -20°C overnight. After thawing, the slices were loaded into the slots of a 15% acrylamide I gel, 1.5mm thick with a 4cm stacking gel. The slices were overlaid with 10µl of 20% glycerol in TSE followed by 10µl of 10% glycerol in TSE containing protease and bromophenol blue. The samples were electrophoresed 2cm into the stacking gel, the current switched off for 30 min to allow proteolysis, and then electrophoresis continued normally followed by fluorography.

11.20 Proteolysis of whole cells and isolated envelopes

(a) Treatment of intact cells

10µl of a 10mg/ml solution of protease (as indicated in the text) was added to 60 OD units of cells in 2ml 50mM Tris/Cl pH 7.8. The samples were incubated at 37°C for 30 min followed by the addition of 100µl of 0.1M PMSF in ethanol (final concentration 5mM) and a further 15 min incubation at 37°C. 5ml of 10mM sodium phosphate pH7.2 was then added and

the cells harvested (10krpm, 5 min, SM24). The cell pellet was resuspended in 7ml of sodium phosphate buffer containing 1mM PMSF and the cells fractionated.

(b) Treatment of isolated envelopes

Envelopes were prepared from 60 OD units of cells and resuspended in 1ml of 50mM Tris/Cl pH7.8. 10µl of 10mg/ml protease was added and the samples incubated at 37°C for 30 min. 50µl of 0.1M PMSF was added and the samples incubated at 37°C for 15 min. 4ml of 10mM sodium phosphate buffer pH7.2 was then added, the envelopes pelleted and resuspended in 0.5% sarkosyl containing 1mM PMSF for separation of inner and outer membrane proteins.

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Investigation of the assembly of TonA protein into the outer membrane of Escherichia coli

Maria E. Jackson

ABSTRACT

The majority of outer membrane, periplasmic and some inner membrane proteins of Escherichia coli are synthesised with signal sequences which initiate the translocation process. It has been suggested that other polypeptide sequences within the mature protein carry additional information which determines the final localisation of the product.

The aim of this project was to investigate the assembly into the outer membrane of the E. coli ferrichrome receptor protein, TonA. The tonA gene was subcloned onto pBR325 in order to maximise expression of this normally minor outer membrane protein.

A study of the kinetics of assembly of TonA in a strain harbouring a multicopy plasmid carrying tonA revealed the occurrence of a processed assembly intermediate which separated with the soluble (cytoplasmic plus periplasmic) fraction of sonicated cells.

The position and direction of transcription of tonA was deduced by Tn1000 mutagenesis followed by analysis of the resultant truncated TonA' polypeptides synthesised in vitro and in maxicells. All the TonA' polypeptides thus produced, even those with apparently small C-terminal deletions, fractionated with the sarkosyl soluble envelope material in maxicells (wild type TonA is sarkosyl insoluble), suggesting an important role for the C-terminus in assembly. A similar result was obtained when the tonA gene was truncated using an "oligo-stop translation" sequence. This eliminated the possibility that complete assembly of the TonA' polypeptides truncated by Tn1000 insertion was prevented by Tn1000 encoded sequences at their C-termini.

Synthesis of the hybrid MalE-LacZ protein, 72-47, was demonstrated to inhibit the processing of TonA and several inner membrane proteins. Since this hybrid was already known to block the assembly and processing of periplasmic and outer membrane proteins, this result suggests that all three classes of exported protein share common steps in their assembly.

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Enhanced polypeptide synthesis programmed by linear DNA fragments in cell-free extracts lacking exonuclease V

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Preparation of an in vitro coupled transcription-translation system from *E. coli* strains lacking exonuclease V has greatly improved the system for use with added linear DNA fragments. In fact, in extracts of these mutants linear fragments are stable for several hours. However the cell extracts show a high level of endogenous background. To avoid this complication extracts were prepared at 30°C from a mutant carrying a temperature-sensitive exonuclease V. Polypeptides coded by a specific DNA region, e.g., delineated by restriction endonuclease sites, can now be easily identified.

Exonuclease V *Zubay* E. coli rec*B* *DNA* *Polypeptide*

1. INTRODUCTION

We have described the use of a modified Zubay [1] in vitro system to direct the synthesis of polypeptides from various DNA templates. In those studies [2] we were able to demonstrate that supercoiling of the DNA template was not essential for efficient polypeptide synthesis. Consequently we were able to use successfully linear DNA restriction enzyme fragments in this system. Nevertheless we noted that substantially higher concentrations of linear templates were required to achieve results comparable to those obtained with the corresponding supercoiled or covalently closed DNA molecules. We conclude that this was probably due to exonuclease digestion of the DNA. We therefore investigated the role of the *rec*B,C exonuclease V, in the loss of template activity. In mutants completely lacking the enzyme high backgrounds were obtained due to large amounts of endogenous DNA fragments, active as templates. In this paper, we describe the capacity of cell-free extracts, derived from a mutant carrying a temperature-sensitive *rec*B allele, to support polypeptide synthesis programmed by DNA. We found that at 37°C linear templates were stable for at least 120 min in this extract, backgrounds are low and the efficiency of polypeptide synthesis approached that of covalently closed (relaxed) templates. Thus as little as 50 ng of a linear DNA fragment was sufficient to programme the synthesis of a specific polypeptide.

2. MATERIALS AND METHODS

Strain *E. coli* N138, *rec*B^{ts} was kindly provided by Dr G.G. Churchward, University of Geneva; MRE600 *rec*B^{+} was described in [2]. The plasmids used were pBR325 (*Ap,Cm,Tc*) 5.4 kb and its derivative pLG282 which carries a 2.2 kb *Eco*RI fragment from the *ColI* plasmid coding for *api*.

The preparation of all DNA templates including restriction endonuclease generated fragments and the standard procedure for the preparation of cell-free extracts (S30) for in vitro synthesis of polypeptides programmed by DNA have been described in [2]. For the extract prepared from strain N138 the cells were grown at 30°C rather than at 37°C and the extracts were preincubated at 30°C for 160 min to ensure complete digestion of endogenous DNA before storage in liquid nitrogen. All incubations were carried out at 37°C and contained 7.5 μl LMM (low molecular weight mix), 2 μl [^{35}S]methionine (or 2 μl unlabelled methionine),

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Table 1

Strain	cpm	
	+ DNA	– DNA
MRE600	100396	1346
N138 (*recB*^{ts})	75698	2544

Incubations were performed with 3 μg of pBR325 as added template and either MRE600 or N138 (*recB*^{ts}) as source of S30. A 2 μl sample was removed after 30 min incubation and the [^{35}S]methionine incorporated into protein estimated by trichloroacetic acid precipitation. The figures presented are the average from several experiments

3.5 μl of 0.1 M magnesium acetate, 5 μl of S30 extract, DNA in 10 mM Tris, 1 mM EDTA (pH 7.0) and 10 mM Tris/acetate buffer (pH 7.0) to a final volume of 30 μl [2].

3. RESULTS AND DISCUSSION

Initial experiments with a *rec*B mutant showed that S30 extracts completely lacking exonuclease V gave extremely high background incorporation in the absence of DNA templates, rendering such a system quite inoperable. This was presumably due to substantial amounts of endogenous DNA which is normally degraded by exonuclease V during preparation of the extracts. To overcome this difficulty we decided to use an alternative strain car-

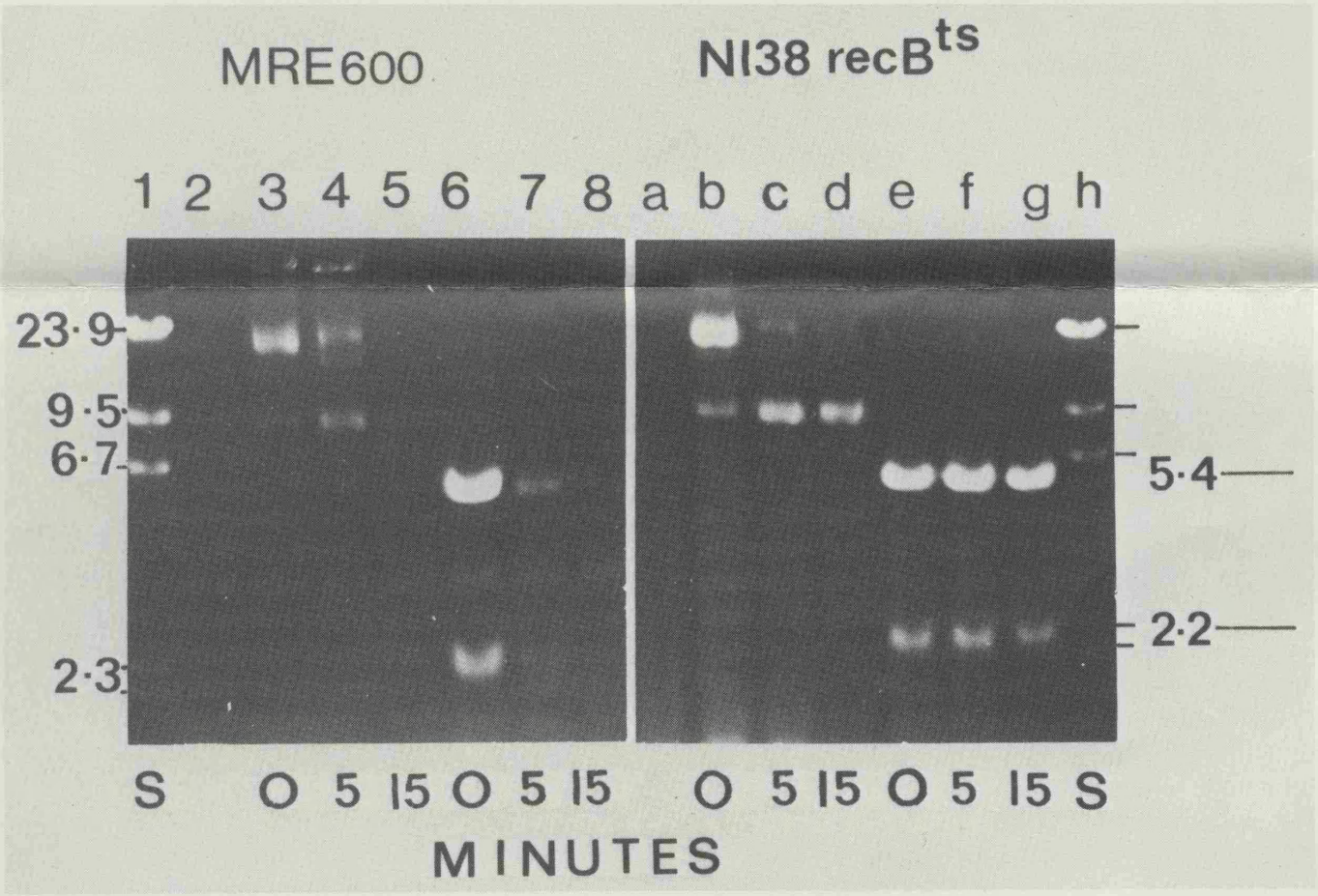

Fig.1. Stability of linear DNA templates in a *recB*^{ts} extract. S30 extracts were prepared from MRE600 (*recB*$^{+}$) and N138 (*recB*^{ts}) and incubated with 2.5 μg of either pLG282 or pLG282 cut with *Eco*RI. 5 μl aliquots were removed at intervals and analysed on a 0.6% agarose gel stained with ethidium bromide. (Tracks 1 and h) molecular mass standards (kb); (2 and a) no DNA; (3,4,5,b,c and d) 2.5 μg of pLG282; (6,7,8,e,f and g) 2.5 μg *Eco*RI fragments. Samples (0.5–1 μg DNA) were removed at the times indicated. Further samples removed at 60 and 120 min were identical to the 15 min sample in each case.

rying a temperature-sensitive mutation in *rec*B [3]. Extracts were prepared at 30°C as described in section 2 and preincubated at 30°C to reduce background incorporation. As shown in table 1 a low level of incorporation in the absence of added DNA comparable to that of the MRE extract, could be achieved by this strategy.

Extracts prepared from both *E. coli* MRE600 and *E. coli* N138 (*rec*B^{ts}) were incubated at 37°C together with either supercoiled pLG282, or the *Eco*RI restriction fragments (5.4 kb and 2.2 kb) of pLG282 as DNA templates. Samples were removed at intervals and analysed by agarose gel electrophoresis to determine the stability of the added DNA. As shown in fig.1 using the MRE600 extract the restriction fragments were almost completely degraded within 5 min, whilst in the N138 extract no DNA degradation could be detected even after 120 min of incubation. These results indicate that under these conditions exonuclease V is the major degradative activity in wild-type extracts but is largely absent in N138 extracts at 37°C. When this extract was incubated at 37°C in the presence of an added linear DNA template (fig.2) stimulation of

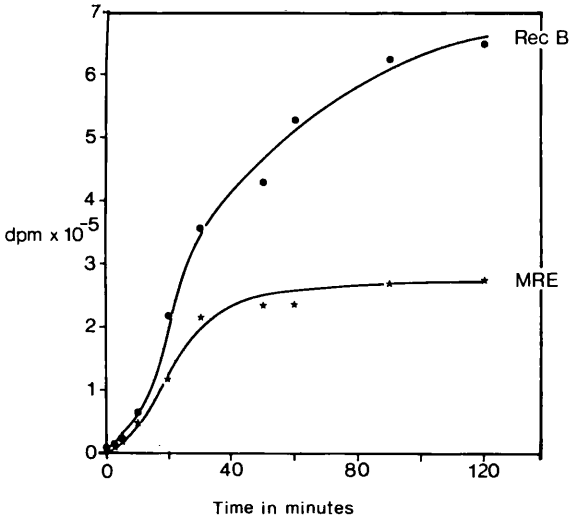

Fig.2. Protein synthesis programmed by a linear template. 10 μg of pBR325 cut with *Hin*dIII was used as a template to programme protein synthesis with either MRE600 or N138 as the source of S30; [^{35}S]methionine was included to label the synthesised peptides and 2 μl samples were removed at intervals and the radioactivity incorporated into protein determined by trichloroacetic acid precipitation.

protein
~~DNA~~ synthesis was obtained in a reaction which could be sustained for up to 120 min and significantly longer than that obtained with extracts from *E. coli* MRE600 containing exonuclease V.

As a further test of the ability of N138 extracts to support the synthesis of polypeptides programmed by linear DNA templates, standard 30 min reactions were carried out over a range of DNA concentrations. The template used was the 5.4 kb plasmid pBR325, linearised with *Pst*I which cuts within the β-lactamase gene. This plasmid codes for a 24.8 kDa protein, chloramphenicol acetyl transferase (CAT). As shown in fig.3 as little as 375 ng of template was sufficient to promote readily detectable amounts of [^{35}S]methionine-labelled CAT with the *rec*B^{ts} extract compared to more than 5 μg with extracts prepared from the MRE600 strain. In fact, longer exposures of the gel in fig.3 detected CAT programmed by 50 ng of DNA.

In our initial studies [2] with linear DNA templates we reported that extracts of MRE600 did support the synthesis of a number of polypeptides, although with a reduced efficiency compared to covalently closed circular molecules. In the present study we found, as indicated in fig.2, that in contrast, linear molecules were rapidly degraded and were quite inefficient as templates. Similar results, using the basic system described in [2], have also been obtained with another *E. coli* K12 strain (J. Merrick, personal communication). The basis for this apparent discrepancy has now been satisfactorily resolved with the finding that freshly prepared extracts of strain MRE600 and K12 strains contain high levels of an exonuclease activity, presumably exonuclease V which decays upon storage of extracts. Thus, extracts kept, for example, for 1 year in liquid nitrogen have quite low levels of exonuclease activity.

In conclusion, we now find a substantial improvement in the efficiency of linear DNA templates in this cell-free extract system, reflecting the increased stability of the template when extracts were prepared from a *rec*B^{ts} mutant. Furthermore, provided the preparative procedures and the preincubation period indicated in section 2 are followed, the high backgrounds, which normally accompany the complete absence of exonuclease V (see also [4]) can be successfully avoided.

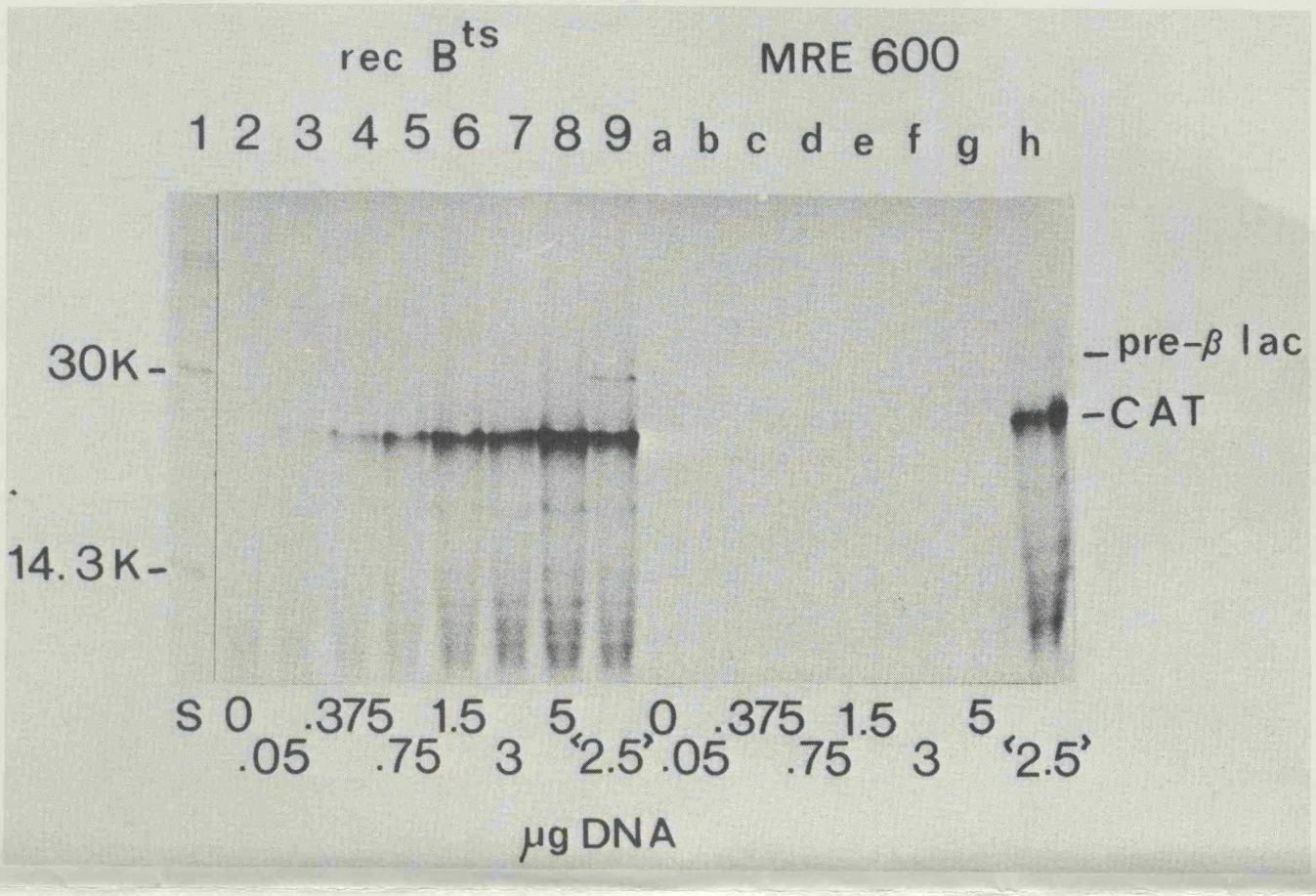

Fig.3. Effect of DNA concentration on polypeptide synthesis. pBR325 coding for major products, CAT and pre-β-lactamase was cut at the single *Pst*I site and the linear fragments incubated in the presence of [^{35}S]methionine with N138 (*rec*B^{ts}) S30 extract (tracks 1–9), or MRE600 (*rec*B^{+}) S30 (tracks a–h). After 30 min incubation samples were removed and analysed by SDS–PAGE (15% acrylamide) and autoradiography. The amount of template DNA used in each incubation is indicated; tracks 9 and h programmed by 2.5 μg supercoiled pBR325. Molecular mass standards, carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). Note that longer exposures of samples programmed by 3–5 μg linear DNA in the MRE600 extract revealed a band at the CAT position. K, kDa.

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Insertion of a MalE β-Galactosidase Fusion Protein into the Envelope of *Escherichia coli* Disrupts Biogenesis of Outer Membrane Proteins and Processing of Inner Membrane Proteins

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The synthesis of a membrane-bound MalE β-galactosidase hybrid protein, when induced by growth of *Escherichia coli* on maltose, leads to inhibition of cell division and eventually a reduced rate of mass increase. In addition, the relative rate of synthesis of outer membrane proteins, but not that of inner membrane proteins, was reduced by about 50%. Kinetic experiments demonstrated that this reduction coincided with the period of maximum synthesis of the hybrid protein (and another maltose-inducible protein, LamB). The accumulation of this abnormal protein in the envelope therefore appeared specifically to inhibit the synthesis, the assembly of outer membrane proteins, or both, indicating that the hybrid protein blocks some export site or causes the sequestration of some limiting factor(s) involved in the export process. Since the MalE protein is normally located in the periplasm, the results also suggest that the synthesis of periplasmic and outer membrane proteins may involve some steps in common. The reduced rate of synthesis of outer membrane proteins was also accompanied by the accumulation in the envelope of at least one outer membrane protein and at least two inner membrane proteins as higher-molecular-weight forms, indicating that processing (removal of the N-terminal signal sequence) was also disrupted by the presence of the hybrid protein. These results may indicate that the assembly of these membrane proteins is blocked at a relatively late step rather than at the level of primary recognition of some site by the signal sequence. In addition, the results suggest that some step common to the biogenesis of quite different kinds of envelope protein is blocked by the presence of the hybrid protein.

Several periplasmic and outer membrane proteins in *Escherichia coli* are synthesized as preproteins which are processed by removal of the N-terminal signal sequence to form the mature polypeptide (5, 14, 17, 27). The signal peptidase(s) involved in processing some or all of these proteins has not been specifically identified, but some evidence suggests that it is located in the inner membrane (8, 21, 25). Unprocessed intermediates if detected in vivo are normally short lived (10, 19). Furthermore, kinetic studies have indicated that the synthesis and assembly of many outer membrane proteins are tightly coupled (11), and in particular we have demonstrated that growing chains of the 36,500-molecular-weight matrix protein (26) in *E. coli* B/r are inserted directly into the envelope (9). The studies are consistent with the loop model used by Halegoua and Inouye (14) for protein secretion in which, for example, certain proteins are extruded directly into the outer membrane, accompanied by processing within the inner membrane.

The total polypeptide content per unit surface area of the *E. coli* and *Salmonella typhimurium* outer membrane is strictly regulated (1, 6), and different polypeptides appear to compete with each other for correct processing and assembly into the outer membrane (6). Smit and Nikaido (29) have presented electron microscope evidence for the emergence of protein molecules at about 200 discrete locations on the surface of *S. typhimurium*. These could represent nascent polypeptides being assembled at any one particular time and need not necessarily indicate the existence of specific structural sites for protein export.

Strong evidence for the role of the N-terminal signal sequences in the localization of membrane proteins in *E. coli* has been obtained by Bassford

et al. (3, 4), Emr et al. (12), and Silhavy et al. (28) by the construction of hybrid genes coding for proteins containing the N-terminal region of membrane or periplasmic proteins fused to an active β-galactosidase molecule. However, fusion proteins involving the N-terminus of the periplasmic, MalE protein, do not apparently cause β-galactosidase to be transported to the periplasm but result in its association with the cytoplasmic membrane when synthesis of the hybrid is induced by the addition of maltose (3). Bassford and Beckwith (3) also observed that strain PB72-47, producing the MalE β-galactosidase hybrid protein, grew as filaments in liquid culture and failed to form colonies after the addition of maltose. From these results, it was concluded that the large-molecular-weight fusion protein blocked the export sites to the periplasm, causing disruption of normal growth. In addition, while this paper was in preparation, Ito et al. (18) reported that precursors of both periplasmic and outer membrane proteins accumulated in strain PB72-47 after the addition of maltose, indicating that processing but not synthesis of at least some exported proteins was also blocked.

Previous studies in this laboratory (6, 9, 16) indicate that a high level of outer membrane protein synthesis is an essential requirement for division. We therefore decided to investigate in detail the rate of division and the synthesis of outer membrane proteins during the development of maltose sensitivity in a strain carrying a MalE β-galactosidase hybrid gene. Surprisingly, in the course of these studies, we also observed that processing not only of outer but also of inner membrane proteins was inhibited.

MATERIALS AND METHODS

Bacterial strains. Strain PB72-47 (Δ*lac-169 araD139 rpsL relA thiA* λp72-47) carrying the *malE-lacZ* fusion and its wild-type parent MC4100 and MC4100 (λpRI-1), containing a mutation in the fusion gene rendering the hybrid protein cytoplasmic, have all been described previously (3). For UV-λ infection experiments, MC4100 and PB72-47 were transformed with pGY101 carrying λ *imm*^{434} and Kan^r. λBS10 carrying *dacA* (penicillin-binding protein 5 [PBP5]) and pGY101 (20) were obtained from B. G. Spratt and N. Stoker. Bacteria were grown in 0.4% (wt/vol) glycerol-M9 minimal medium at 30°C supplemented with 0.4% (wt/vol) maltose where indicated.

Cell number. Bacterial cell numbers were measured in a model B Coulter counter as previously described (6).

Isolation of membranes. Bacterial envelopes were isolated from sonicated cells and separated into inner and outer membranes by treatment with Sarkosyl NL97 as previously described (6). Unless stated otherwise, inner membranes are defined as the Sarkosyl-soluble fraction and outer membranes are defined as the Sarkosyl-insoluble fraction. In some experiments, envelopes were also isolated from spheroplasts after osmotic lysis and inner and outer membranes separated by centrifugation to equilibrium on sucrose density gradients as described by Osborn et al. (23).

Measurement of rates of synthesis of membrane proteins. Samples (1 ml) of cultures were pulse-labeled with [^{35}S]methionine (15 μCi; 50 μCi/μg) for 5 min; incorporation was terminated by the addition of excess methionine and chloramphenicol (250 μg/ml) and incorporation into separated inner and outer membranes determined as described previously (6). To minimize errors in measurements of incorporation into different cell fractions and into individual proteins separated by polyacrylamide gel electrophoresis, a standard sample of cells previously grown for several generations in the presence of [^{3}H]leucine (50 Ci/mmol) was added to [^{35}S]methionine-labeled cells as an internal standard before isolation of envelopes (6). The rates of synthesis of membrane fractions and individual proteins are therefore expressed as the ratio of ^{35}S to ^{3}H. In addition, synthesis is expressed as a relative rate, i.e., the rate of synthesis of membrane proteins relative to total protein synthesis measured in the same pulse.

SDS-PAGE. Envelope samples or total cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 11% gels as previously described (6). The radioactivity in individual gel bands was measured as described by Ames (2).

Infection of heavily irradiated cells with λ. Infection of heavily UV-irradiated bacterial hosts PB72-47(pGY101) or MC4100(pGY101) with the transducing phage λpBS10 to label the *dacA* gene product with [^{35}S]methionine was carried out as described previously (24). To identify the PBP5 precursor, cultures of PB72-47 were grown in the presence of 0.4% maltose before irradiation and infection.

Penicillin-binding protein assay. Cell envelopes were incubated with [^{14}C]benzylpenicillin to specifically label penicillin-binding proteins in the inner membrane as described by Spratt (30).

Isotopes. Radioactive isotopes were obtained from the Radiochemical Centre, Amersham, U.K.

Molecular weight standards. Proteins used as molecular weight standards were phosphorylase A (94,000) transferrin (74,000), bovine serum albumin (68,000), lactic dehydrogenase (36,000), chymotrypsinogen (25,000), and lysozyme (14,300).

RESULTS

Effect of maltose on the growth and division of a strain synthesizing the fusion protein. Strain PB72-47, carrying the *malE-lacZ* fusion gene, and its wild-type parent were grown to exponential phase in minimal glycerol medium, and then maltose was added. The appearance of the fusion protein was readily detected in total cell lysates as described by Bassford and Beckwith (3). The rate of mass increase in the mutant compared with the wild type is reduced particularly between 2 and 3 h after maltose addition (Fig. 1). The rate of division was also reduced in the mutant, with the mass per cell increasing almost threefold over 4 h as the cells filamented.

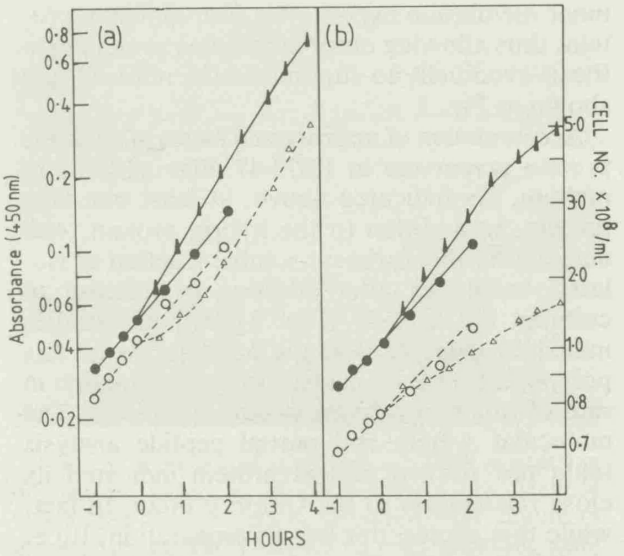

FIG. 1. Effect of addition of maltose on mass increase (absorbance at 450 nm) and cell number (Coulter counts) in strain MC4100 (a) and PB72-47 (b). Cultures were grown in M9-glycerol medium at 30°C, and maltose was added at time zero. Solid curve, mass; broken curve, cell number. Symbols: ▲ and △, maltose added; ● and ○, no maltose added.

The mass per cell in the wild type also increased to nearly double after the addition of maltose (Fig. 1), but this quickly adjusted to a new steady-state level. MC4100(λpRI-1), a maltose-resistant revertant of PB72-47, in which a mutation affecting the MalE signal sequence causes the fusion protein to accumulate in the cytoplasm (3), was also analyzed and was found to behave similarly to the wild-type parental strain (data not shown). Although clearly cells do filament after maltose induction of PB72-47 as observed by Bassford and Beckwith (3), division

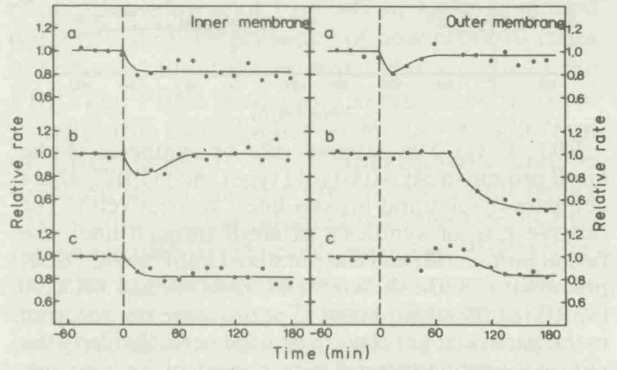

FIG. 2. Effect of maltose addition on the relative rate of synthesis of bulk inner membrane and outer membrane protein in MC4100 (a), PB72-47 (b), and MC4100 (λpRI) (c). Maltose was added at time zero to cultures growing exponentially in M9-glycerol medium at 30°C, and samples were pulse-labeled at intervals with [^{35}S]methionine as in Materials and Methods.

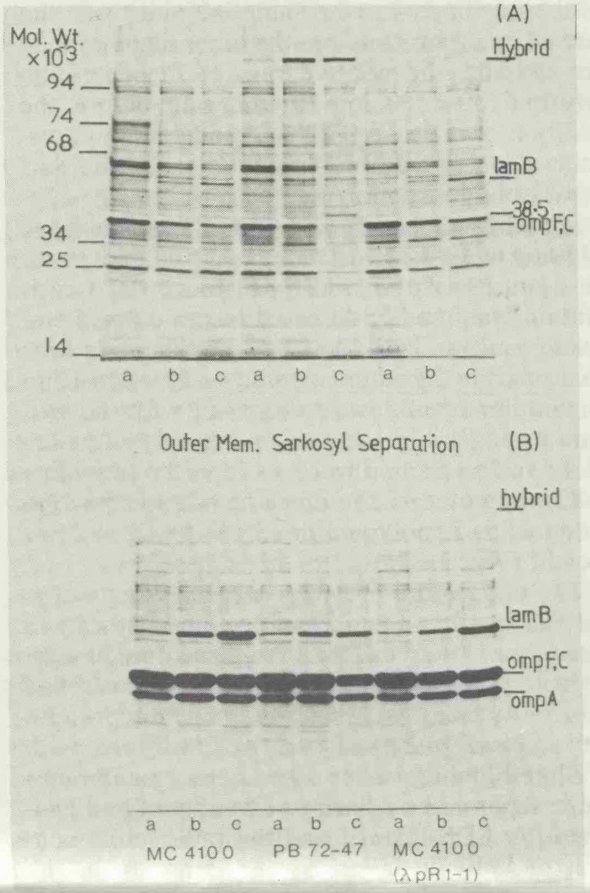

FIG. 3. Coomassie blue-stained gel profiles after SDS-PAGE analysis of inner membrane (A) and outer membrane (B) from cultures growing in M9-glycerol medium at 30°C. Samples were taken at time zero (a), 90 min (b), and 180 min (c) after the addition of maltose.

is by no means completely blocked, continuing at about 50% of the normal rate. Figure 2 shows the effect of the addition of maltose on the rate of synthesis and assembly of inner and outer membrane proteins, expressed relative to the total rate of protein synthesis, in strain PB72-47, its wild-type parent, and the maltose-resistant revertant MC4100(λpRI-1). Inner and outer membrane fractions were obtained by Sarkosyl treatment of envelopes from sonicated cells. In the wild-type strain, the relative rate of outer membrane protein synthesis was essentially unaffected by maltose. In contrast, after about 75 min, the rate of outer membrane protein synthesis in strain PB72-47 fell rapidly to about 50% of the initial rate, indicating that synthesis of the hybrid protein was blocking the sites of synthesis, assembly, or both, of outer membrane proteins. There was also a slight reduction of outer membrane protein synthesis in the revertant strain consistent with the presence of small amounts of fusion protein in the membrane of this strain (Fig. 3). Finally, although as in the

wild type there was the same initial drop in the rate of incorporation into the inner membrane in the mutant, the relative rate of incorporation returned after 60 min to the rate before the addition of maltose. This may indicate the presence of outer membrane precursors in the Sarkosyl-soluble fraction (see below).

Synthesis of LamB and fusion proteins after addition of maltose. After the addition of maltose to a culture of the wild-type strain, the LamB protein was readily detected in the outer membrane, and at least one new polypeptide was observed in the inner membrane fraction (Fig. 3). Similar results were observed with the mutant strain, but in addition, substantial amounts of the fusion protein were, as reported previously (3), detected in the inner membrane fraction along with a polypeptide of 38.5K molecular weight (Fig. 3).

The data in the previous section showed that the fall in outer membrane protein synthesis in the mutant strain did not take place until at least 60 min after the addition of maltose. To relate this to the rate of synthesis of the fusion protein, cells were pulse-labeled at intervals with [^{35}S]methionine, inner and outer membranes were separated by Sarkosyl treatment and analyzed by SDS-PAGE, and the radioactive content of individual bands was determined. The results obtained (expressed relative to the rate of total protein synthesis determined in each pulse) are shown in Fig. 4b. Two major features should be noted; the addition of maltose was followed by a lag of 30 to 45 min before synthesis of the hybrid protein and the LamB protein was detected (in the inner and outer membrane fractions, respectively), and the maximal rates of synthesis at 60 to 75 min coincided exactly with the overall fall in outer membrane protein synthesis shown in Fig. 2. These results clearly suggest that as maximal rates of synthesis of the fusion protein are reached, a substantial portion of sites for the synthesis and assembly of outer membrane proteins are blocked by the fusion protein. These results can be seen in better perspective when compared with the kinetics of LamB synthesis in the wild-type strain shown in Fig. 4a. First, the initial kinetics are essentially the same, demonstrating that the early lag in synthesis is not due to the presence of the hybrid protein. Second, the maximal rate of synthesis again peaked after about 75 min and then gradually declined to a new steady-state level approximately 40% of the maximum rate. This presumably reflects the gradual adjustment, involving several factors, to a new steady-state level of transcription from the maltose operons. The gradual decline in the synthesis of the fusion protein can be attributed to similar regulation of the *malE* promoter. This in turn should result in the appearance of some inner membrane export sites free of fusion protein, thus allowing outer membrane protein synthesis eventually to continue at the reduced rate shown in Fig. 2

Accumulation of unprocessed forms of envelope protein precursors in PB72-47 after addition of maltose. As indicated above, at least one new protein, in addition to the hybrid protein, was detected in the Sarkosyl-soluble fraction of isolated envelopes after addition of maltose to cultures of PB72-47 (Fig. 3). Kinetic studies indicated (Fig. 4) that the appearance of this polypeptide also coincided with the reduction in rate of outer membrane protein synthesis. The molecular weight and partial peptide analysis (data not shown) of this protein indicated its close relatedness to the OmpF protein. In fact, while this manuscript was in preparation, Ito et al. (18) reported that several outer membrane proteins, including OmpF and periplasmic protein precursor forms, accumulated in PB72-47 after addition of maltose. These results indicated that

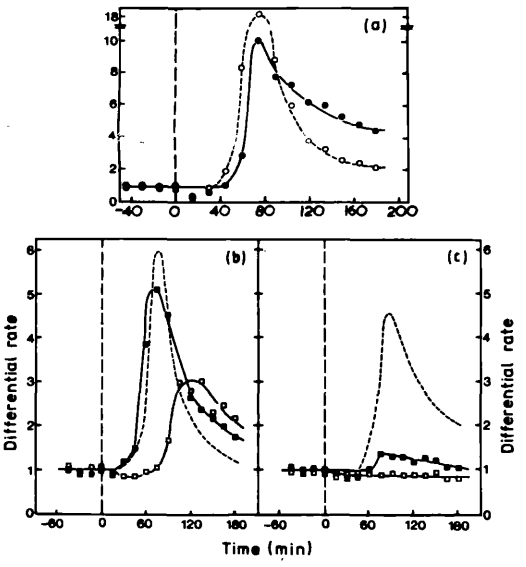

FIG. 4. (a) The relative rate of synthesis of the *lamB* protein in MC4100 (wild type) and in PB72-47 are compared (solid and broken lines, respectively). The relative rate of synthesis of *lamB* (broken line), the fusion protein (■) and the putative OmpF and C 38.5K precursor (□) are shown for PB72-47 (b) and MC4100 (λpRI) (c). The OmpF and C porins were not resolved in the particular gel conditions used here. Similarly the putative precursor band may consist of both porins. Maltose was added at time zero, and the [^{35}S]methionine/[^{3}H]leucine values (see Materials and Methods) are normalized to a value 1 for glycerol grown cells. For the analysis of *lamB*, bands were cut out from gel samples of outer membranes, whereas bands were cut out from gels of inner membrane samples for fusion protein and the porin precursor.

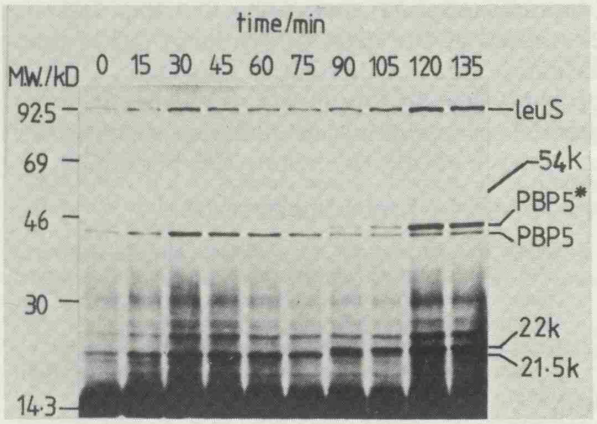

FIG. 5. Kinetics of inhibition of PBP5 processing on maltose induction of PB72-47(pGY101). Maltose was added at time zero to an exponential culture of glycerol grown PB72-47(pGY101). At intervals, samples were removed to examine the proteins synthesized after λpBS10 infection of the UV-irradiated cells, using a [^{35}S]methionine label. The products of the labeled samples were then separated by SDS-PAGE (11% gel). The position of molecular weight standards is indicated on the left, and the polypeptides coded by the transducing phage are indicated on the right. Other details are described in the text.

the fusion protein not only blocked sites of export for outer membrane and periplasmic proteins but also blocked access to the signal peptidase(s). To determine whether this effect was specific to outer membrane and periplasmic proteins, we also investigated the effect of induction of the fusion protein on the processing of inner membrane proteins. We have recently shown (25) that two inner membrane polypeptides, PBP5 and PBP6, are synthesized as larger-molecular-weight precursors which can be processed in vitro by the addition of inner membrane vesicles. Labeling of cells with [^{14}C]benzylpenicillin after growth of PB72-47 in maltose indeed demonstrated the presence of new proteins (data not shown) identical in molecular weight to the PBP5 and PBP6 precursors synthesized in vitro. Finally, this same higher-molecular-weight form of PBP5 labeled with [^{35}S]methionine can be detected when maltose grown cells (after heavy UV irradiation) are infected with the λ-transducing phage pBS10 (31) carrying *dacA* (PBP5) (Fig. 5). The appearance of the precursor could be detected as early as 75 min after addition of maltose, again coincident with the maximal rate of synthesis of the hybrid protein. Further inspection of the gel in Fig. 5 indicates the presence of an additional protein, apparently accumulating as a higher-molecular-weight precursor in the presence of maltose. This protein, approximate molecular weight 22,000, is identical to a protein coded by phage λpBS10 previously identified by Spratt et al. (31; N. Stoker, personal communication) and predominantly localized in the inner membrane.

DISCUSSION

The results described above indicate that although the MalE protein is normally localized in the periplasm, attempted export of this protein fused to β-galactosidase blocked at least some of the sites of synthesis and assembly of outer membrane proteins. In addition, these results indicate that the syntheses of both periplasmic proteins, e.g., the MalE protein, and outer membrane proteins are also normally subject to the availability of the same limiting factor(s). However, our study does not necessarily provide evidence for specific structural sites concerned with outer membrane biosynthesis. We have argued previously (6) that the capacity of *E. coli* cells to synthesize outer membrane proteins is normally saturated, and therefore the presence of the fusion protein in the inner membrane may simply reduce the availability of some limiting factor essential for translocation, synthesis, or both, of outer membrane proteins.

Although outer membrane proteins in *E. coli* are synthesized at a constant rate which doubles at a discrete time in the cell cycle (6, 9; A. Boyd, Ph.D. thesis, University of Leicester, U.K., 1979), the limiting factor might be due either to an abrupt doubling in the number of export sites or equally to a doubling in the rate of expansion for the underlying peptidoglycan (6). If such a bilinear mode of growth of the cell surface is an essential element in division control (15), differential inhibition of outer membrane protein synthesis might be expected to be correlated with inhibition of cell division. We have recently reported such a correlation (16) under conditions in which polynucleotide gyrase is inactivated. In some contrast, in this study, whereas the synthesis and assembly of outer membrane proteins were markedly reduced, the rate of cell division, although also reduced, still continued at about 50% of the rate before the addition of maltose. In this case, either we underestimated the amount of protein assembled into the outer membrane or high levels were not required for the division process. Although the membrane fractionation procedure may have been perturbed when high levels of the hybrid protein were present (see below), the slight inflation of the inner membrane fraction in the strain PB72-47 compared with the wild type (Fig. 2) was not sufficient to account for the apparent deficit of outer membrane protein. An alternative explanation is that the rate of peptidoglycan synthesis might be the primary determinant in bilinear surface growth, in which case the rate of outer membrane pro-

tein assembly might not be a major limiting factor as observed here.

Under conditions in which outer membrane protein synthesis was reduced, evidence was obtained for the accumulation of a precursor form of at least one of the major porins (22) in the envelope of strain PB72-47. While this manuscript was in preparation, Ito et al. (18), using a quite different approach, identified several outer membrane and periplasmic proteins in precursor form in strain PB72-47 after the addition of maltose. These results indicate that the presence of the fusion protein blocks some translocation system in the envelope or access to the signal peptidase, presumably located in the inner membrane. The processing of at least two inner membrane polypeptides, PBP5 and PBP6, is also inhibited, suggesting that this inhibition is due to a general disruption of the organization of inner membrane polypeptides or the sequestering of many signal peptidase molecules into abortive processing complexes, rather than the blockage of a specific export system common to outer membrane and periplasmic proteins. We have not ruled out the possibility that some envelope proteins accumulate in the cytoplasm and are therefore not processed, but clearly unprocessed polypeptides can be recovered with the envelope fraction. For PBP5 and PBP6, we could not detect any proteins capable of binding [^{14}C]benzylpenicillin in the cytoplasmic fraction after growth on maltose.

In an attempt to localize the fusion polypeptide in the envelope, we observed that the protein fractionated either with the outer or the inner membrane, depending upon the method (sucrose gradient or Sarkosyl) of membrane separation used (18; unpublished data). This apparent ambiguity may reflect the association of unprocessed periplasmic and other membrane proteins in vivo with both membranes, as found previously by Halegoua and Inouye (13). Nevertheless, until the precise localization of the fusion protein and in particular any unprocessed outer membrane proteins is established, it is perhaps premature to conclude whether processing is being blocked at an early or a late step in the normal translocation and final maturation of envelope proteins.

Finally, the kinetics of induction of the LamB and MalE proteins in both wild-type and mutant strains were quite unexpected. Although the cultures responded immediately in terms of the increased growth rate upon the addition of maltose, indicating that maltose was being metabolized, the synthesis of these proteins was not detected for at least 30 min. Identical kinetics were obtained in two other wild-type strains. Moreover, the appearance of the hybrid protein in total cell lysates followed the same kinetics (unpublished data), indicating that this delay is expressed at the level of synthesis rather than assembly into the envelope. This effect is most probably due to low levels of glucose in commercial preparations of maltose, despite our exhaustive efforts to exclude this possibility.

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