INVESTIGATION INTO THE ROLE OF CHAPERONES IN THE SECRETION OF HAEMOLYSIN FROM *ESCHERICHIA COLI*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Jonathan Paul Whitehead.

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ABSTRACT **Jonathan P. Whitehead**

Investigation into the role of chaperones in the secretion of haemolysin from *Escherichia coli*

Haemolysin is a 107 kDa protein which is secreted independently of the general export pathway. It contains a C-terminal signal which directs its secretion through a trans-envelope translocator, comprised of the proteins HlyB, HlyD and TolC. It was reasoned that because haemolysin is exported post-translationally it may interact with molecular chaperones to maintain a "loosely folded" secretion-competent conformation.

Investigations carried out indicate that the general export chaperone SecB is not required for the efficient secretion of haemolysin. Preliminary studies using a *secB* null mutant, in which secretion was significantly reduced, suggested that SecB was essential for efficient secretion. However, further assays using a SecB sequestering approach and complementation of the *secB* null mutant indicated that SecB is not required either directly, to modulate haemolysin folding, or indirectly in the assembly of the membrane translocator. The reduced secretion by the *secB* null mutant is probably due to the pleiotropic effects of the mutation.

The SecB sequestering approach has also been reproduced in a T7 expression strain using a newly constructed T7 sequesterer. Following further characterisation, this T7 sequestering approach may be used in pulse-chase experiments without the need for immunoprecipitation, enabling the requirements of exported proteins for SecB to be determined in the absence of specific antisera.

Further investigations into the possible requirement of chaperones in the secretion of haemolysin have shown the presence of the fully functional chaperonin GroEL to be a strict requirement for efficient secretion of haemolysin. Using a temperature-sensitive *groEL* mutant, the level of haemolysin secretion was dramatically reduced at both the permissive and non-permissive temperatures. Secretion was restored by the introduction of plasmids carrying the *groEL* gene. In marked contrast, a similar requirement for the co-chaperonin GroES was not found using a temperature-sensitive *groES* mutant.

This is the first time that the secretion of a protein such as haemolysin, which contains a C-terminal secretion signal and is not secreted via a periplasmic intermediate, has been shown to require the activity of a chaperone.

CONTENTS

CHAPTER 1	Introduction	Page
1.1	Protein translocation - a fundamental process	1
1.2	Protein localisation	1
1.3	Cellular organisation in *Escherichia coli*	2
1.4	The *Escherichia coli* cell envelope	2
1.5	Targeting and translocation	3
1.6	Recognition and targeting - the signal peptide	3
1.7	Identification of components of the general export pathway (GEP)	4
1.8	SecA	5
1.9	Integral membrane Sec proteins	
	(i) SecY	7
	(ii) SecE	8
	(iii) SecD and SecF	8
	(iv) The translocase	9
1.10	Signal peptidases	10
1.11	Energy requirements for protein translocation	11
1.12	The periplasm	12
1.13	Outer membrane proteins (OMPs)	13
1.14	Translocation - a translocation-competent conformation	13
1.15	Role of molecular chaperones in translocation	14
1.16	The signal recognition particle like pathway	15
1.17	The general molecular chaperones	17
1.18	The GroEL/GroES chaperones	
	(i) Organisation	18
	(ii) General chaperone function	19
	(iii) Function in translocation	20
1.19	SecB	
	(i) Characterisation	21
	(ii) Chaperone function	22
	(iii) Recognition of precursors	23
	(iv) Targeting function	25
1.20	Flexibility of the chaperones	26

1.21 Secretion of haemolysin 26
(i) Organistion of the haemolysin operon 27
(ii) Structure of HlyA 27
(iii) Early stages of secretion - interactions with the inner membrane 29
(iv) Late stages of secretion - beyond the inner membrane 29
(v) Membrane proteins involved in secretion of HlyA
(a) HlyB 30
(b) HlyD 31
(c) TolC 32
1.22 Aims 32

CHAPTER 2 Materials and methods

2.1 Materials
(i) Sterilisation of solutions, media and materials 33
(ii) Nutrient medium 33
(iii) Nutrient agar 33
(iv) Luria-Bertani (LB) medium 33
(v) M9 minimal medium 33
(vi) M9 minimal agar 34
(vii) M63 minimal medium 34
(viii) Nutrient and M9 minimal blood agar 34
(ix) Minimal E medium 34
(xi) Antibiotics 34
2.2 Methods
(i) Growth curves 35
(ii) Pulse-chase immunoprecipitation 35
(iii) SDS polyacrylamide gel electrophoresis (SDS-PAGE) 36
(iv) Fluorography 36
(v) Staining gels 37
(vi) Western blotting 37
(vii) Densitometry 37
(viii) Analysis of haemolysin secretion 37
(ix) Short term secretion of haemolysin 38
(x) Stability of secreted haemolysin 38
(xi) Induction of cultures using IPTG or maltose 39
(xii) *In vitro* transcription-translation reactions 39

2.3 DNA manipulation methods
(i) Rapid $CaCl_2$ transformation procedure 39
(ii) Preparation and transformation of frozen competent cells 40
(iii) Synthesis of oligonucleotides 41
(iv) Elution of an oligonucleotide from its column after synthesis 41
(v) Agarose gel electrophoresis of DNA fragments 41
(vi) Purification of DNA using the Geneclean II® kit 42
(vii) Preparation of DNA by alkaline lysis 42
(viii) Purification of prepared DNA by NACS 43
(ix) Rapid preparation of plasmid DNA 44
(x) Rapid genomic DNA preparation 44
(xi) Restriction endonuclease analysis 45
(xii) Amplification of specific DNA sequences by PCR 45
(xiii) Treatment of PCR fragments for blunt-end cloning 46
(xiv) The removal of 5' phosphate groups from DNA 47
(xv) Ligation with T4 DNA ligase 48
2.4 Bacterial strains 49
2.5 Plasmids 50

CHAPTER 3 Investigation of haemolysin secretion in the *secB* null strain (CK1953)

3.1 Characterisation of the *secB* null strain (CK1953) 52
3.2 Secretion of haemolysin from the wild-type strain (MC4100) 53
3.3 Haemolysin secretion from the *secB* null strain
(i) Long term secretion and accumulation of HlyA 54
(ii) Short term secretion of HlyA 55
(iii) Stability of secreted haemolysin 55

CHAPTER 4 Investigation of haemolysin secretion in the "SecB sequestering" strains (HB1012pGL1 and HB1053pJW12)

4.1 The use of SecB sequesterers 57
4.2 The SecB sequestering strains (HB1012pGL1 and HB1053pJW12) 59

4.3 Characterisation of the SecB sequestering strains
(i) Effects of induction on the expression of the *malE* gene products 59
(ii) Effects of induction of the *malE* gene products on protein translocation 60
4.4 Haemolysin secretion in the SecB sequestering strains
(i) Haemolysin secretion following short term induction of the sequesterers 61
(ii) Haemolysin secretion following long term induction of the sequesterers 62
4.5 Introduction of SecB into the *secB* null strain (CK1953) 63

CHAPTER 5 Construction of a T7 sequesterer

5.1 The T7 RNA polymerase expression system 65
5.2 Basic strategy 65
5.3 Cloning of wild-type *malE* into pACYC184 (pJPW11) 66
5.4 Cloning of the wild-type *malE* gene under the control of the T7 promoter (pJPW12) 67
5.5 Cloning of a SecB sequestering *malE* gene under the control of the T7 promoter (pJPW15) 67
5.6 Characterisation of the T7 sequesterer
(i) Identification of the gene product 68
(ii) Effects of induction of the T7 sequesterer on protein translocation 68

CHAPTER 6 Investigation of haemolysin secretion in the *groEL44* temperature-sensitive strain (SF103)

6.1 The *groEL44* temperature-sensitive strain (SF103) 70
6.2 Characterisation of the *groEL44* temperature-sensitive strain
(i) Growth of the *groEL44* temperature-sensitive strain 71
(ii) Expression of the *groEL* and *groES* gene products 73
(iii) Protein translocation in the *groEL44* temperature-sensitive strain 74
6.3 Haemolysin secretion in the *groEL44* temperature-sensitive strain
(i) Secretion of functional haemolysin on blood agar 76
(ii) Haemolysin secretion in nutrient medium 77

CHAPTER 7 **Investigation of haemolysin secretion in the *groES619* temperature-sensitive strain (SF103)**

7.1 The *groES619* temperature-sensitive strain (CG2244) 80
7.2 Characterisation of the *groES619* temperature-sensitive strain
(i) Growth of the *groES619* temperature-sensitive strain 80
(ii) Expression of the *groEL* and *groES* gene products 81
(iii) Protein translocation in the *groES619* temperature-sensitive strain 82
7.3 Haemolysin secretion in the *groES619* temperature-sensitive strain
(i) Secretion of functional haemolysin on blood agar 84
(ii) Haemolysin secretion in nutrient medium 84

CHAPTER 8 **Discussion** 86

REFERENCES 93

ABBREVIATIONS

A	Absorbance
ABC	ATP binding cassette
BCIP	5-bromo-4-chloro-3 indolyl phosphate
bp	Base pair
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CIP	Calf intestinal phosphatase
d.d.	double distilled
DMSO	Dimethyl sulphoxide
ΔP	Protonmotive force
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
Ffh	Fifty four kDa homologue
Fts	Filamentation temperature sensitive
GEP	General export pathway
Hly	Haemolysin
HSP	Heat shock protein
IPTG	Isopropyl β-D-thiogalactoside
LPS	Lipopolysaccharide
m	Mature
MBP	Maltose binding protein
NBT	Nitro blue tetrazolium
OMP	Outer membrane protein
p	Precursor
PCR	Polymerase chain reaction
PMSF	Phenylmethyl-sulphonyl fluoride
PPO	2, 5-diphenyloxazole
prl	Protein localisation
RBP	Ribose binding protein
RTX	Repeat toxin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Secretory
SRP	Signal recognition particle
TCA	Trichloroacetic acid
Tris	Tris (hydroxy) methylamine
w.t.	Wild-type

CHAPTER 1

INTRODUCTION

1.1 Protein translocation - a fundamental process

"The basic life-endowing molecular processes had to exist prior to extensive evolutionary divergence. Thus, we should expect that these processes are governed by the same principles, and that even the molecular details will sometimes prove to be conserved throughout evolutionary history."

Saier and Jacobson, 1984

The translocation of proteins across biological membranes is a fundamental process occurring in all living cells. In eukaryotes it is essential for the organisation and maintenance of functional organelles, the insertion of membrane receptors and also for the secretion of substances including peptide hormones, digestive enzymes and immunoglobulins. In prokaryotes approximately 20% of the proteins synthesised are located partially or completely outside of the cytoplasm.

The majority of studies carried out in this thesis are concerned with a possible role for molecular chaperones in the secretion of haemolysin. The chaperones studied are involved in the general export pathway of *Escherichia coli* which is discussed and although the bulk of this discussion is restricted to the *E. coli* system, direct parallels can often be drawn with those systems engaged in eukaryotic protein translocation. A greater understanding of how proteins are exported and secreted in *E. coli* should provide further insights into protein translocation *per se*. In turn, this may shed light on a number of economically and medically important biological processes.

1.2 Protein localisation

Although the majority of proteins are synthesised within the cytoplasm, many are destined for localisation in non-cytoplasmic compartments or are secreted from the confines of the cell to the external *milieu*. In eukaryotes the presence of membrane-bound organelles into which specific proteins must be assembled results in a complex targeting problem. Prokaryotic cells do not contain such organelles and so present a simpler model for the study of translocation itself.

1.3 Cellular organisation in *Escherichia coli*

In the Gram-negative bacterium *Escherichia coli*, the majority of translocated proteins reach their final destination via the general export pathway (GEP), which includes their insertion into and translocation across the cytoplasmic membrane. In Gram-positive bacteria, fully translocated proteins are generally released into the extracellular *milieu*, whereas in Gram-negative bacteria they are released into the periplasm or are integrated into, or transported across, the outer membrane by one of several terminal branches of the GEP (Fig.1.1). For the purpose of this thesis proteins which remain within the confines of the cell, either completely or partially, will be termed exported proteins and those which reach the extracellular *milieu* secreted proteins.

1.4 The *Escherichia coli* cell envelope

The *E. coli* cell envelope consists of three major non-cytoplasmic compartments into which proteins are targeted and assembled (Fig. 1.1). The outer membrane consists of lipopolysaccharide in the outer leaflet; lipoprotein and phospholipids mainly in the inner leaflet, and proteins which generally span both leaflets of the bilayer. The outer membrane acts as a barrier to the external environment yet allows relatively selective entry of nutrients and ions through trimeric proteinaceous pores (Osborn and Wu, 1980). The constituent porin proteins are generally polytopic and quite hydrophilic compared to polytopic inner membrane proteins (Mizuno *et al.*, 1983). Generally the outer membrane has very little enzymatic activity with the majority of proteins acting structurally, such as lipoprotein and OmpA, or as porins, such as OmpF and OmpC.

The periplasmic compartment is relatively rich in proteins of diverse functions including nutrient transport, such as maltose and ribose binding proteins, nutrient metabolism, such as alkaline phosphatase, and in the breakdown of harmful substances, such as β-lactamases. The periplasm also contains the peptidoglycan cell wall which consists of a rigid network of polysaccharide chains crosslinked by peptide bridges and is important in maintaining cell shape.

The inner, cytoplasmic membrane encloses the cytoplasm and is relatively impermeable. It houses proteins which perform electron transport and oxidative phosphorylation, such as cytochrome oxidase and ATP synthetase, the import of

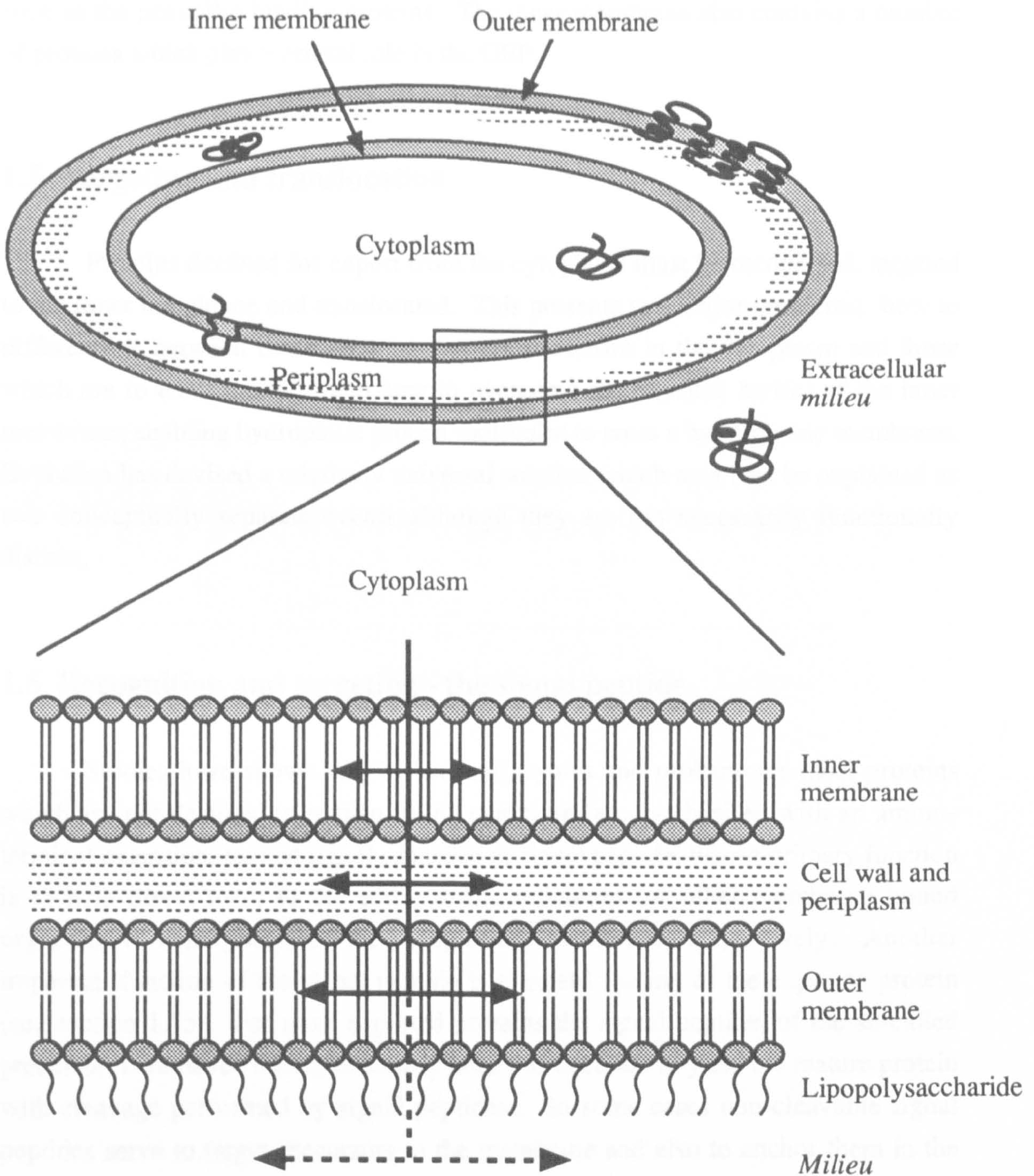

Fig. 1.1 Schematic representation of the *Escherichia coli* cell showing the general export pathway in Gram-negative bacteria. The basic pathway and its branches are indicated by arrows. Secreted (wholly extracellular) proteins follow the pathways indicated by both solid and dashed arrows; exported (i.e., extracytoplasmic but not extracellular) proteins follow only the pathways shown as solid arrows.

nutrients, such as lactose permease, and polysaccharide and peptidoglycan synthesis, such as the penicillin binding proteins. The inner membrane also contains a number of proteins which play a central role in the GEP.

1.5 Targeting and translocation

Proteins destined for export from the cytoplasm must be recognised, targeted to the inner membrane and translocated. This presents two major problems; how to differentiate between those proteins that are to remain in the cytoplasm and those which are to enter the GEP and how to overcome the physical barrier of the inner membrane, enabling hydrophilic protein molecules to cross a hydrophobic membrane. Evolution has devised a relatively universal solution which may best be explained as two conceptually separate events although they are not necessarily functionally distinct.

1.6 Recognition and targeting - the signal peptide

Studies have shown that in both eukaryotes and prokaryotes most proteins which are not destined to remain in the cytoplasm are synthesised with an amino-terminal extension, termed signal sequence or signal peptide, whose primary function is to help direct them to the endoplasmic reticulum (or other membrane bound organelle in eukaryotes) or the cytoplasmic membrane, respectively. Another important function of the signal peptide is to retard folding of the exported protein (see section 1.15). For most exported proteins the signal peptide, of the so-called precursor, is removed during or shortly after translocation to yield the mature protein with cleavage performed by signal peptidase. In some cases non-cleavable signal peptides serve to target precursors to the membrane and also to anchor them in the membrane (Lipp *et al.*, 1989).

Signal peptides have been characterised and found to lack any conserved amino acid sequence at the primary level, however detailed analysis of large numbers of signal peptides (von Heijne, 1983; 1985; 1986a; 1986b), have defined a number of characteristic features which are shared, to varying degrees, by all signal peptides (Fig.1.2). These features include:-

i) a total length of 15-35 residues (average of 21-23 residues).

ii) N domain: a net positively charged region spanning the first 2-10 residues, at the extreme N-terminus, which usually contains one or more Lys or Arg residues and is

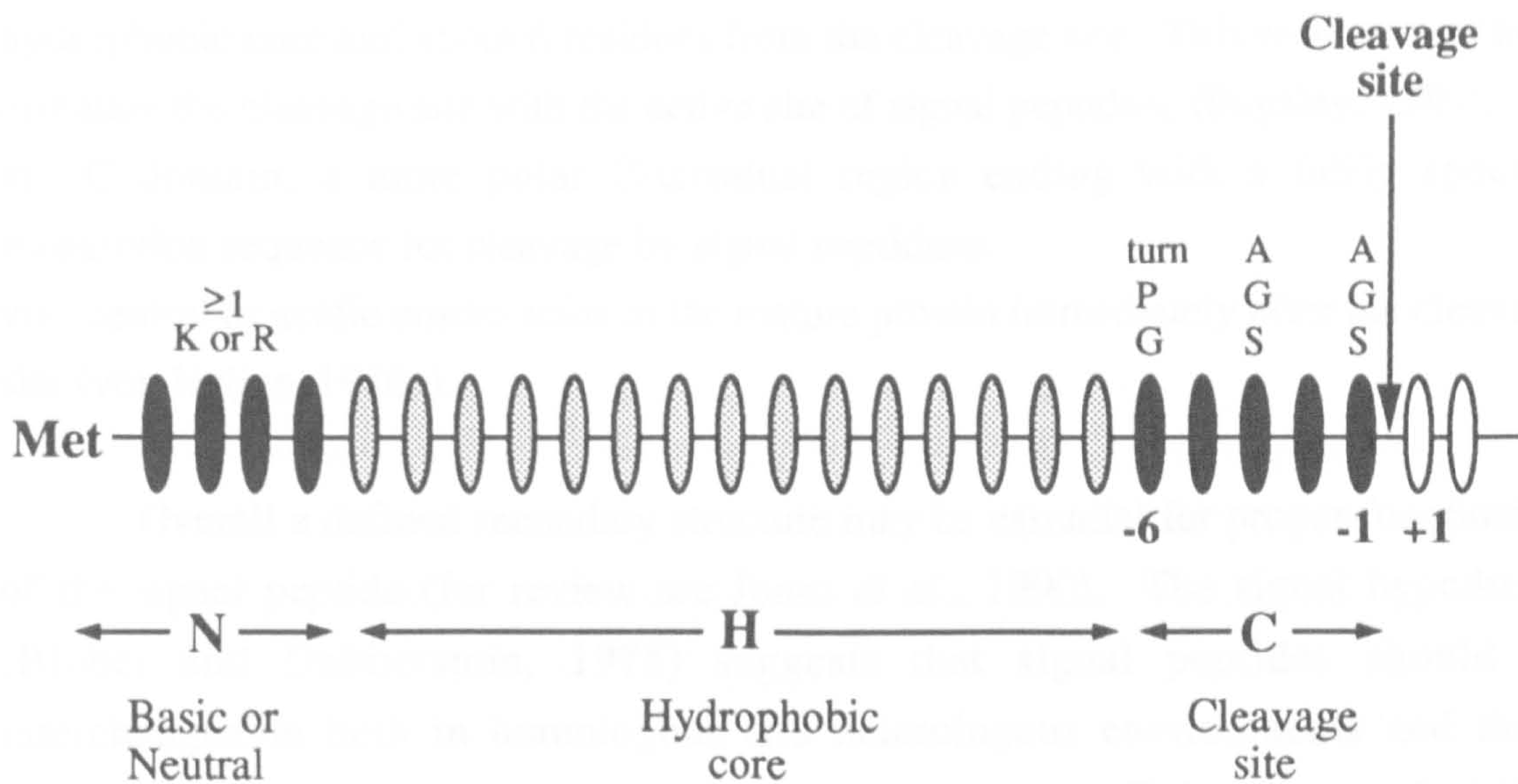

Fig. 1.2 Schematic representation of the principal features of a typical signal peptide found in *E. coli*. Solid ovals represent polar or charged residues and shaded ovals represent hydrophobic or apolar residues. Signal peptides have up to three domains ("N,""H," and "C"). The N domain (2 to 10 residues) is polar and carries a net positive charge. The H domain (> 8 residues) is composed of predominantly hydrophobic residues and alanine and lacks strongly polar or charged residues. The high leucine and alanine content causes signal peptides to adopt an α-helical configuration in apolar environments. The C domain is usually less hydrophobic and contains the signals that are recognised by signal peptidase i.e. small amino acids at positions -1 and -3 and a turn inducing residue at position -6 with respect to the cleavage site.

thought to be important for interaction with the protein SecA and the negatively charged head groups of the inner mebrane phospholipids (Akita *et al.*, 1990).
iii) H domain: a central core region of 7-16 residues containing uniquely hydrophobic or neutral residues and with a predicted strong tendency to form an α-helix in an apolar environment (Gierasch, 1989). This region may be critical in allowing the signal peptide to enter the bilayer.
iv) a turn-inducing amino acid (proline or glycine) immediately after the hydrophobic core and about 6 residues from the cleavage site. This residue may help orientate the cleavage site with the active site of signal peptidase (Pugsley, 1989).
v) C domain: a more polar C-terminal region ending with a fairly specific recognition sequence for cleavage by signal peptidase.
vi) neutral or acidic amino acids in the mature protein immediately after the cleavage site (von Heijne, 1986a).

Overall a defined secondary structure may be essential for proper functioning of the signal peptide (for review see Jones *et al.*, 1990). The signal hypothesis (Blobel and Dobberstein, 1975) suggests that signal peptides should be interchangeable both in homologous and heterologous environments and these predictions have been fulfilled in most, but not all, cases (Talmadge *et al.*, 1980; Ruohonen *et al.*, 1987; Lecker *et al.*, 1989a) suggesting a common mechanism of protein export. As a result, transport across the inner membrane of Gram-negative bacteria has been considered at least functionally equivalent to that across the eukaryotic endoplasmic reticulum membrane.

1.7 Identification of components of the general export pathway (GEP)

Proteins destined for the inner membrane, periplasmic space, outer membrane, or external *milieu* must initially be translocated, partially or completely, across the inner membrane and this is usually achieved by the GEP. Exceptions have been found, most notably the bacteriophage M13 coat protein, which assembles into the inner membrane in a manner independent of the GEP (Watts *et al.*, 1981; Saier *et al.*, 1989) however this belongs to an uncommon class of proteins of small size and unusual charge distribution (Zimmermann *et al.*, 1990). Several proteinaceous components of the GEP have been identified by a range of genetic approaches. One approach relies on the fact that certain precursor protein-β-galactosidase hybrids are toxic to cells because of jamming of the export machinery by the blocked translocation intermediate. Expression of these hybrid proteins confers a Lac^-

phenotype due to the inability of the normally cytoplasmic β-galactosidase moiety to tetramerise (Ito *et al.*, 1981; Lee *et al.*, 1989). Selection for Lac^+ phenotypes revealed a series of mutations with most occurring in the signal peptide coding region of the gene to which *lacZ* is fused. Indeed, when recombined genetically into the otherwise wild-type cognate gene, these signal peptide mutations also block export of the resulting protein (Emr *et al.*, 1978; Bassford and Beckwith, 1979; Emr *et al.*, 1980; Bedouelle *et al.*, 1980). However, mutations were also identified in the *sec* (*sec*retory) genes; *secA*, *secB* and *secD* (Oliver and Beckwith, 1981; 1982a; Kumamoto and Beckwith, 1983; 1985; Gardel *et al.*, 1987) which have since been shown to be important factors in the GEP. Further studies of the *secD* locus showed there were in fact two genes, named *secD* and *secF*, within a single operon (Gardel *et al.*, 1990).

Using a modified Lac selection where a *secA-lacZ* fusion was introduced into the cells, mutations in the genes *secA* and *secD* were again recovered, as well as cold-sensitive mutations in the *secY* gene (Schatz *et al.*, 1989). A new gene, *secE*, was also identified (Riggs *et al.*, 1988).

An alternative approach was used to identify the *prl* (protein localisation) genes. This involved the isolation of suppressor mutations which enhanced the export of proteins carrying defective signal peptides which in the absence of such suppressors resulted in decreased export and in some instances conferred a negative phenotype (Emr *et al.*, 1981). The *prlA*, *prlD*, and *prlG* suppressors were isolated using this approach (Ito *et al.*, 1983; Bankaitis and Bassford, Jr., 1985; Ryan and Bassford, Jr., 1985; Stader *et al.*, 1989) and further studies revealed these suppressors to be allelic forms of the *secY*, *secA* and *secE* genes respectively.

1.8 SecA

The *secA/prlD* gene is essential for viability and encodes the 102kDa protein SecA (Oliver and Beckwith, 1981; 1982a). SecA is thought to act as a homodimer (Akita *et al.*, 1991; Driessen, 1993) and is a strict requirement for translocation of proteins *in vivo* (Oliver and Beckwith, 1981; Wolfe *et al.*, 1985) and *in vitro* (Cabelli *et al.*, 1988; Watanabe and Blobel, 1993).

SecA is found in the cytoplasm and as an inner membrane protein, where it assumes both peripheral and integral states of association (Akita *et al.*, 1991; Cabelli *et al.*, 1991). Central to its function is the ability to bind and hydrolyse ATP (Lill *et*

al., 1989) with recent data suggesting two distinct ATP-binding domains per SecA protein (Mitchell and Oliver, 1993). This ATPase activity is stimulated by interaction with other components involved in translocation including; both the signal peptide and mature regions of exported proteins, acidic phospholipids, and SecY/SecE (Lill *et al.*, 1990). The interaction of SecA with precursors and SecY/SecE is supported by the isolation of *secA* mutants which can suppress signal peptide mutations (Bankaitis and Bassford, Jr., 1985) or temperature-sensitive *secY* mutations respectively (Fandl *et al.*, 1988). More recently, a direct association between SecA and a translocating precursor was shown by use of crosslinkers *in vitro* (Joly and Wickner, 1993).

SecA has also been shown to interact with SecB (see section 1.19iv) and data from McFarland *et al.*, (1993) indicates that a mutant SecA protein is able to partially compensate for the absence of SecB, suggesting an overlap in function of the two proteins at the level of targeting.

Studies of SecA using an *in vitro* system showed that a block in translocation caused by the removal of the protonmotive force could be overcome by the presence of an excess of SecA (Yamada *et al.*, 1989b). Further discussion of the role of SecA in the energetics of translocation is presented in section 1.11.

SecA appears to play a key role in translocation, possibly by acting as a membrane receptor for the precursor and also, in association with other proteins, promoting translocation. This is perhaps best illustrated by work of Brundage *et al.*, (1990) who using reconstituted vesicles containing only SecY/SecE/band1 complex and SecA effected translocation of pro-OmpA. It has been proposed that ATP binding and hydrolysis cause conformational changes in SecA which are thought to be important in its activities (Shinkai *et al.*, 1991), and that SecA undergoes successive cycles of precursor binding, ATP hydrolysis, and precursor release to SecY/SecE until translocation is complete (Schiebel *et al.*, 1991).

The expression of *secA* is regulated according to the protein export capability of the cell (Oliver and Beckwith, 1982b). Levels of SecA increase ten to twenty fold in cells carrying *sec* mutants (except *secB*), or when export is blocked by the addition of azide or synthesis of export defective β-galactosidase fusion proteins (Rollo and Oliver, 1988; Oliver *et al.*, 1990a; 1990b). The response is autoregulatory at the level of translation. Under normal export conditions SecA binds to a "secretion-responsive element" upstream from its translation start codon to repress translation (Dolan and Oliver, 1991; Schmidt *et al.*, 1991). SecA is the only component of the GEP that responds in this way, suggesting that overproduction of SecA is sufficient to

compensate for any temporary saturation or jamming of the GEP that might normally occur and again highlighting the pivotal role of SecA in the pathway.

1.9 Integral inner membrane Sec proteins

(i) SecY

The essential *secY/prlA* gene encodes the 49kDa integral membrane protein, SecY. Hydropathy plots and PhoA fusion analysis predict a structure of ten transmembrane domains with both the amino- and carboxy-termini in the cytoplasm (Akiyama and Ito, 1987). This organisation is similar to that of inner membrane proteins involved in solute transport such as LacY, lactose permease (Calamia and Manoil, 1990). The isolation of suppressor mutations in *secY* (Emr *et al.*, 1981; Emr and Bassford, Jr., 1982) coupled with its organisation in the membrane are consistent with the idea that SecY directly interacts with the signal peptide and also represents at least part of the apparatus that physically transfers proteins across the membrane, the so-called translocase (Ito, 1992; Brundage *et al.*, 1992). Further evidence for a direct interaction between the signal peptide and SecY was the discovery that *prlA* mutations cause defective translocation of staphylokinase and that mutants with alterations in the signal peptide can overcome this block (Iino and Sako, 1988; Sako and Iino, 1988). Also consistent with a role for SecY early in translocation is the fact that antibodies raised to the cytoplasmic C and N-termini of SecY inhibit translocation of pre-LamB *in vitro* (Watanabe and Blobel, 1989a).

Suppressors of signal peptide mutations have been isolated throughout the SecY protein (Ito, 1992), however recent work by Osborne and Silhavy (1993) indicates the presence of "hot spots" where suppressor mutations cluster in three distinct toplological domains, indicating functional importance. These suppressors are not allele-specific and the finding that a signal peptide is not a strict requirement for protein export in *prlA* mutants (Derman *et al.*, 1993) suggests that these suppressor mutations serve to broaden the specificity of the SecY protein to allow more efficient secretion of precursor proteins with altered or deleted signal peptides (Osborne and Silhavy, 1993).

Conditional lethal mutants have been obtained in the *secY* gene which result in the accumulation of precursors of exported proteins in the cytoplasm (Ito *et al.*, 1983; Shiba *et al.*, 1984; Riggs *et al.*, 1988). Vesicles made from a strain carrying the *secY24* temperature-sensitive mutation were inactivated for translocation *in vitro*

following incubation at the non-permissive temperature (Fandl and Tai, 1987) although activity could be restored by increasing the levels of SecA (Fandl *et al.*, 1988). This indicates that the SecY24 translocation defect is suppressed by SecA interacting, directly or indirectly, with SecY24 on the inner membrane which is consistent with data from Hartl *et al.*, (1990) which shows binding of SecA to the membrane at a high affinity receptor that includes SecY/SecE and also the finding that ATPase activity of SecA is stimulated by SecY (Lill *et al.*, 1990).

Watanabe and co-workers (Watanabe *et al.*, 1990; Watanabe and Blobel, 1993) found that reconstituted proteoliposomes containing less than 1% of wild-type levels of SecY were capable of efficient translocation and proposed that SecY is not an essential translocase component. This seems unlikely in view of the extensive evidence indicating direct SecY involvement in translocation (Watanabe and Blobel, 1989a; Bieker and Silhavy, 1990; Lill *et al.*, 1990; Nishiyama *et al.*, 1991; Ito, 1992) but a feasible explanation has not yet been put forward.

(ii) SecE

The essential *prlG/secE* gene encodes the 13.6kDa integral membrane protein, SecE, which spans the inner membrane three times with its amino-terminus in the cytoplasm (Schatz *et al.*, 1989; 1991). Conditional lethal cold-sensitive mutations have been isolated which cause decreased SecE synthesis and lead to precursor build up in the cytoplasm (Schatz *et al.*, 1989). Surprisingly, a small region of the protein containing only one of the three transmembrane domains is sufficient for viability and this essential transmembrane domain also contains two out of three *prlG* suppressor mutations (Schatz *et al.*, 1991; Nishiyama *et al.*, 1992).

(iii) SecD and SecF

SecD and SecF were identified by cold-sensitive mutations that cause the accumulation of precursors of exported proteins (Gardel *et al.*, 1987; 1990). The SecD and SecF proteins are not required for *in vitro* translocation systems (Brundage *et al.*, 1990; 1992) and no *prl* alleles have been found at the *secDF* locus (Schatz and Beckwith, 1990; Bieker-Brady and Silhavy, 1992) raising doubts about their importance in the GEP. However, recent experiments (Pogliano and Beckwith, 1993) showed that the cold-sensitive *secD* and *secF* mutants are phenotypically identical to cold-sensitive mutants in *secE* and *secY*. Also, Matsuyama *et al.*, (1993)

demonstrated that protein export in spheroplasts is inhibited after treatment with anti-SecD IgG, providing further support for the involvement of SecD and SecF *in vivo*. Most recently it has been shown that depletion of SecD and SecF from the cell significantly reduces protein translocation (Pogliano and Beckwith, 1994b). Also, overexpression of SecD and SecF results in a *prl* phenotype, with improved export of precursors with mutant signal sequences (Pogliano and Beckwith, 1994b).

Topology analysis of SecD and SecF (65kDa and 35kDa respectively) showed that both have large periplasmic domains situated between the first two of six transmembrane regions (Pogliano and Beckwith, 1994a). This topological organisation provides the basis for speculation that they are involved in a late step in the export process, such as folding or release of newly translocated proteins into the periplasm (Matsuyama *et al.*, 1993), recycling the export complex, or presenting newly translocated proteins to leader peptidase (Pogliano and Beckwith, 1994b). Genetic data also suggests that SecD and SecF form a complex (Pogliano and Beckwith, 1994b), however further studies are required to confirm these hypotheses.

(iv) The translocase

Both genetic and biochemical data suggest that SecY functions in a complex with SecE (Brundage *et al.*, 1990; 1992; Bieker and Silhavy, 1990; Bieker-Brady and Silhavy, 1992; Baba *et al.*, 1994) and perhaps one or more of several other integral inner membrane proteins including SecD and SecF (Bieker-Brady and Silhavy, 1992), band1 (Brundage *et al.*, 1990; 1992) and another protein, Ydr (Ito, 1992). *In vitro* reconstitution experiments have shown that a SecY/SecE/band1 complex is sufficient for SecA-dependent precursor protein translocation (Brundage *et al.*, 1990; 1992) although a strict requirement for band1 in the translocase has yet to be determined. Synthesis of large amounts of the Ydr protein has been shown to suppress export-negative effects of a *trans*-dominant mutation in SecY in a diploid strain that carries a wild-type *secY* allele (Ito, 1992; Shimoike *et al.*, 1992). This suppression is presumed to indicate that Ydr and SecY normally form a complex and that the product of the mutant *secY* allele titrates Ydr to prevent its interaction with wild-type SecY. Large amounts of Ydr saturate the defective SecY, allowing some functional SecY-Ydr complexes to form, however more information on the role of Ydr in the GEP is required. Overexpression of *secE* or *secD/F* also suppresses the *trans*-dominant effect of this *secY* allele (Ito, 1992), implying that the five proteins form a complex. More recently another protein which may be involved in the translocase has been identified (Nishiyama *et al.*, 1993). This protein, termed p12, stimulated protein

translocation when reconstituted into proteoliposomes together with SecE and SecY and appeared to be dependent on SecE for stability in a similar manner to SecY (Nishiyama *et al.*, 1993) (see below).

The interaction between SecY and SecE is widely accepted as a result of several lines of evidence. First, SecY overproduction is dependent on the simultaneous overproduction of SecE (Matsuyama *et al.*, 1990) which has been shown to be due to rapid association of newly synthesised SecY with SecE, preventing proteolysis of SecY (Taura *et al.*, 1993). Second, synthetic lethality is observed with specific *secY* and *secE* mutant alleles (Bieker and Silhavy, 1990; Osborne and Silhavy, 1993). Third, antibodies against SecY co-immunoprecipitate SecE, and band1 (Brundage *et al.*, 1990). Finally extensive studies using suppressor-directed inactivation (SDI) and Sec titration suggest that SecY is involved in a transient complex responsible for translocation, and this complex includes SecA, SecE, SecD/SecF and SecY itself (Fig.1.3) (Bieker and Silhavy, 1989; 1990; Bieker-Brady and Silhavy, 1992). Although the association of a transient complex is appealing (Bieker-Brady and Silhavy, 1992) this is at odds with data presented by Taura *et al.*, (1993) which suggests that the SecY/SecE complex is relatively stable and this is consistent with the isolation of the SecY/SecE/band1 complex (Brundage *et al.*, 1990; 1992). However SecY and SecE can be separated under relatively mild purification conditions and then recombined to form an active translocase (Matsuyama *et al.*, 1990), indicating that they are not necessarily tightly associated. Further characterisation of these interactions is required before the nature of the SecY/SecE complex is understood. Recently SecY, and also SecA, have been isolated in cross-linked complexes with partially translocated OmpA (Joly and Wickner, 1993) confirming close proximity to the translocating polypeptide.

1.10 Signal peptidases

Two types of signal peptidase have been identified in *E. coli*. The major signal peptidase (signal peptidase I or LepB) is an integral inner membrane protein, with its active site situated on the periplasmic face of the membrane. It is involved in the cleavage of the majority of exported proteins in *E. coli*. Lipoprotein signal peptidase (signal peptidase II or LspA) is a polytopic inner membrane protein which is structurally unrelated to LepB and cleaves lipoproteins which contain signal peptides that are different to those recognised by LepB (for review see Dev and Ray, 1990).

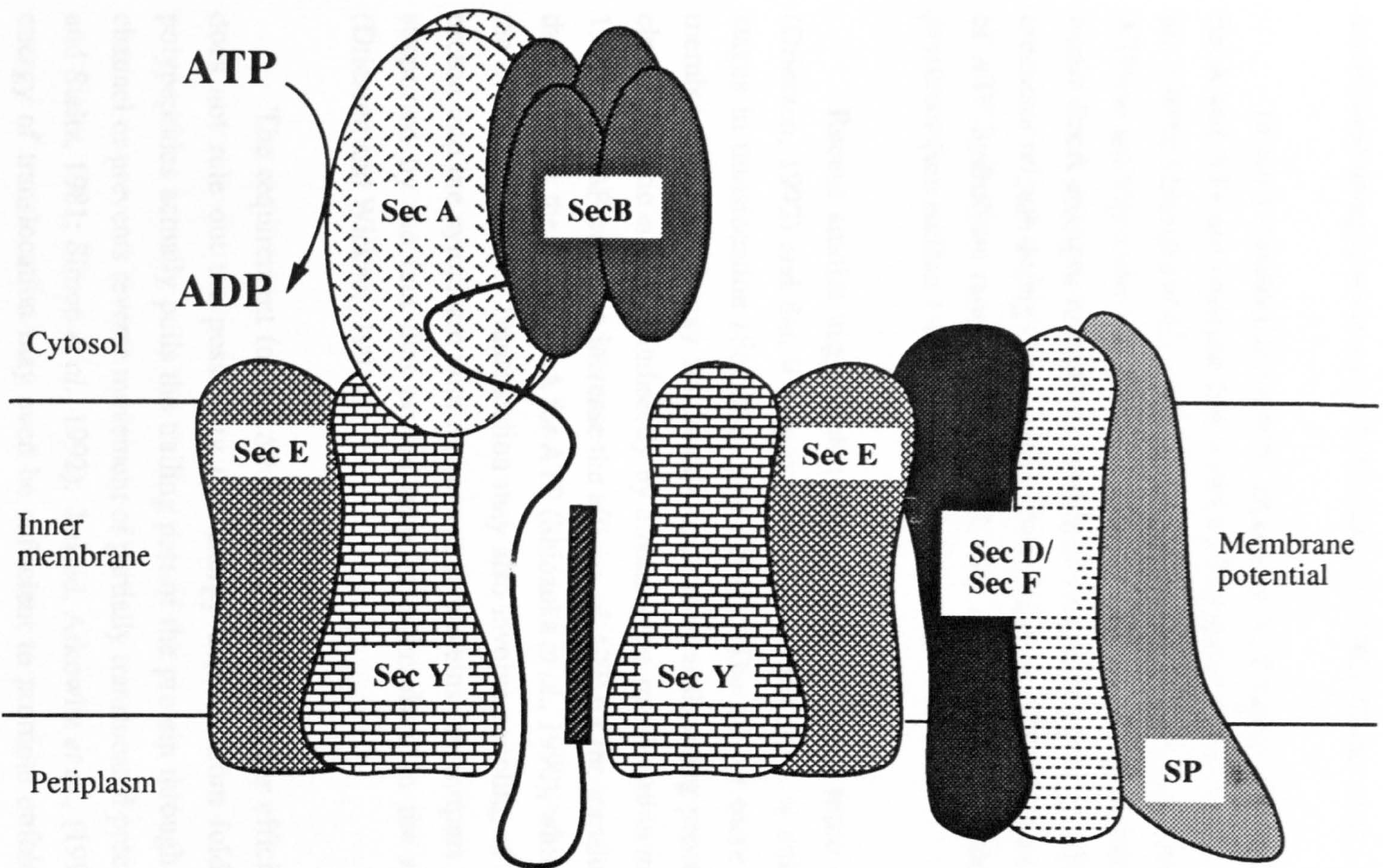

Fig. 1.3 Model showing possible organisation of the translocase in post-translational translocation featuring the chaperone SecB. The Sec proteins are shown in association with a partly translocated precursor. Signal peptidase (SP) is also shown. Band1 and Ydr are not shown. See text for details.

1.11 Energy requirements for protein translocation

The energy requirements for translocation are met by both ATP hydrolysis and the protonmotive force (ΔP). The requirement for ΔP has been demonstrated *in vivo* (Randall *et al.*, 1987) whereas the requirement for ATP hydrolysis by SecA was established using *in vitro* assays (Chen and Tai, 1985; Geller *et al.*, 1986).

In vitro translocation can be driven by ATP alone, although higher levels of SecA and ATP are required than when a ΔP is present (Tani *et al.*, 1989; Yamada *et al.*, 1989b; Shiozuka *et al.*, 1990). Translocase bound SecA was found to exhibit high ATPase activity even when translocation was blocked and a model was proposed where SecA engages in multiple rounds of precursor binding, ATP hydrolysis, and precursor release during translocation (Schiebel *et al.*, 1991). However the exact role of ATP hydrolysis remains unclear. It may induce release of SecB bound to the precursor (see section 1.19(iv)).

Recent studies suggest that the ΔP is the driving force for translocation (Driessen, 1992) and that it may operate alone when SecA is not bound or at late stages in translocation (Schiebel *et al.*, 1991). The ΔP (or more specifically, the membrane potential) may act directly upon the translocating precursor, exerting an electrophoretic effect, or indirectly by affecting the translocation machinery (Geller, 1991). The ΔP may also increase the affinity of ATP for the translocation apparatus, thus lowering the K_m of SecA for ATP (Shiozuka *et al.*, 1990), which would facilitate the cycling of SecA. Translocation may also involve coupling of the transport of a proton into the cytoplasm with movement of proteins (an antiport system), a theory supported by the fact that proton transfer is involved in the rate-limiting step (Driessen and Wickner, 1991).

The requirement for the ΔP and ATP hydrolysis for efficient translocation does not rule out the possibility that energy derived from folding of precursor polypeptides actually pulls the trailing part of the protein through the translocation channel or prevents reverse movement of partially translocated precursors (Engelman and Steitz, 1981; Simon *et al.*, 1992). Indeed, Arkowitz *et al.*, (1993) found that the energy of translocation may even be sufficient to promote unfolding of precursor domains. Furthermore, the interaction of imported proteins with a molecular chaperone in the mitochondrial matrix has been shown to drive import (Kang *et al.*, 1990; Manning-Krieg *et al.*, 1991).

1.12 The periplasm

Exported proteins adopt extensive tertiary and quaternary structure in the periplasm and proteins that are destined for insertion into the outer membrane or secretion across the outer membrane are usually close to their final conformation. Unlike the cytoplasm, the periplasm is not a reducing environment and as such, many periplasmic and extracellular proteins contain disulphide bonds. At least two enzymes are thought to be involved in the catalysis of disulphide bonds in translocated proteins, namely DsbA (Bardwell *et al.*, 1991; Kamitani *et al.*, 1992) and DsbB (Bardwell *et al.*, 1993; Missiakis *et al.*, 1993). Although the main activity of DsbA is catalysis of disulphide bond formation, like protein disulphide isomerase (PDI) found in the lumen of the endoplasmic reticulum, it can also catalyse disulphide bond exchange reactions (Bardwell *et al.*, 1991). The DsbB protein is an integral inner membrane protein (Bardwell *et al.*, 1993) and it has been suggested that the role of DsbB is to oxidise reduced forms of DsbA, allowing recycling of active DsbA (Bardwell *et al.*, 1993; Missiakis *et al.*, 1993). Mutations in the genes *dsbA* or *dsbB* in *E. coli*, or in related genes in other Gram-negative bacteria, result in a wide range of effects including: reduced formation of disulphide bonds in periplasmic proteins; reduced extracellular secretion (Pugsley, 1992); failure to assemble F pili in *E.coli* (Bardwell *et al.*, 1991) and failure to assemble or secrete heat-labile enterotoxin subunit B and cholera toxin (Peek and Taylor, 1992; Yu *et al.*, 1992). However DsbA and DsbB are not essential enzymes and the absence of either, or both, has no affect on translocation across the cytoplasmic membrane (Bardwell *et al.*, 1991).

The periplasm also contains a peptidylprolyl-*cis-trans* isomerase (rotamase) which catalyses the isomerisation of peptide bonds with proline in the second position (Liu and Walsh, 1990; Compton *et al.*, 1992).

It seems unlikely that enzymes with similar properties to the general molecular chaperones exist in the periplasm because significant levels of ATP are not thought to be present to support the activity of chaperones such as DnaK and GroEL which hydrolyse ATP during polypeptide release and folding (see section 1.15). However, molecular chaperones involved in pilus assembly are found in the periplasm and have several distinctive characteristics including shape, independence of ATP, and tightly folded ligands (Holmgren and Branden, 1989; Hultgren *et al.*, 1991; Bakker *et al.*, 1991). There are a number of candidate proteins which may act as periplasmic chaperones acting in the final stages of translocation and these have recently been reviewed by Wulfing and Pluckthun (1994).

1.13 Outer membrane proteins (OMPs)

The insertion of proteins into the outer membrane and the organisation of these proteins appear to be directly related. Hydropathy plots derived from sequences of the proteins concerned suggest the absence of regions of high overall hydrophobicity in the predicted transmembrane domains or in the entire polypeptide (Tommassen, 1988). This is in contrast to polytopic integral inner membrane proteins which contain hydrophobic α-helical membrane spanning sequences separated by hydrophilic outer membrane loops. However when such hydrophobic segments are inserted into outer membrane proteins (OMPs) they may act as stop transfer-membrane anchor signals and prevent polypeptide release from the inner membrane (MacIntyre *et al.*, 1988). The transmembrane domains of OMPs are composed of amphipathic β-sheets of alternating hydrophobic and hydrophilic residues as shown by X-ray diffraction and crystallization analyses of the OMPs, OmpF and PhoE (Cowan *et al.*, 1992) which also indicated that the transmembrane domains are not perpendicular to the plane of the membrane. It is thought that following translocation OMPs are released from the inner membrane, pass through the periplasm (Sen and Nikaido, 1990), and insert into the outer membrane, via a series of conformational changes (Jackson *et al.*, 1986; Fourel *et al.*, 1992) and by interactions with other outer membrane components. Studies suggest that LPS facilitates insertion into the outer membrane in some way (Ried *et al.*, 1990). Unlike polytopic inner membrane proteins slight alterations of the transmembrane domains of OMPs result in decreased assembly into the outer membrane although changes to extramembranous segments are relatively well tolerated (Klose *et al.*, 1988a; 1988b).

Although not widespread in Gram-negative bacteria such as *E. coli*, secretion across the outer membrane does occur and is accomplished by several alternative routes (for review see Holland *et al.*, 1990) which may be either dependent on or independent of the GEP for translocation across the inner membrane. One pathway which is independent of the GEP, that of haemolysin secretion, is described in section 1.21.

1.14 Translocation - a translocation-competent conformation

It is thought that translocated segments of exported proteins, which are generally devoid of regions of significant hydrophobicity, cross the bacterial cytoplasmic membrane via a specific channel formed, in *E. coli*, by one or more of

the integral membrane Sec proteins. A pre-requisite for this process is that the exported protein is maintained in a locally unfolded state or "translocation-competent conformation" (Randall and Hardy, 1986; Eilers *et al.*, 1987). Bacterial protein export occurs predominantly post-translationally (Josefsson and Randall, 1981a; 1981b; Randall, 1983) which allows a considerable length of precursor protein to be exposed in the cytoplasm and fold into more highly ordered structures prior to translocation. It is not yet clear how much tertiary structure can be tolerated by the translocation machinery (Tani *et al.*, 1990; Reed and Cronan, 1991; Lecker *et al.*, 1990) and to what extent folding is prevented or retarded prior to translocation. However, both *in vitro* (Chen and Douglas, 1987; Crooke and Wickner, 1987; Crooke *et al.*, 1988; Kusters *et al.*, 1989) and *in vivo* (Deshaies *et al.*, 1988; Weiss *et al.*, 1988; Kumamoto *et al.*, 1989) data suggest that extensive tertiary and especially quaternary structure makes precursor proteins translocation-incompetent. This provides strong evidence of a role for modulating factors, termed chaperones, to maintain precursor proteins in a translocation-competent conformation.

1.15 Role of molecular chaperones in translocation

Signal peptides have been shown to retard the folding of denatured precursors (Park *et al.*, 1988; Laminet and Pluckthun, 1989; Liu *et al.*, 1989) and such an effect *in vivo* would increase the time span during which precursors could interact with the translocation machinery at the membrane or bind to molecular chaperones to further retard their folding (Fig.1.4). Molecular chaperones prevent illicit inter- or intra-molecular interactions between polypeptides while allowing other, permissible interactions to occur. Bacteria have many cytoplasmic molecular chaperones and each is thought to bind to a number of proteins although strict binding-site specificities have not been recognised (Flynn *et al.*, 1991; Sherman and Goldberg, 1991; Landry *et al.*, 1992; Viitanen *et al.*, 1992). In addition to its role in modulating folding of cytoplasmic proteins the general chaperone, GroEL, has been shown to be capable of maintaining purified, denatured precursor proteins in a translocation-competent state when the denaturant is diluted out (Bochkareva *et al.*, 1988; Lecker *et al.*, 1989b) and this is thought to typify the activity of such chaperones. SecB has also been reported to prevent such denatured precursor proteins from losing translocation competence (Collier *et al.*, 1988; Weiss *et al.*, 1988; Kusters *et al.*, 1989; Lecker *et al.*, 1989b; Weiss and Bassford, Jr., 1990; Hardy and Randall, 1991). However, unlike GroEL, SecB is also thought to be involved in targeting the precursor to the translocase via its specific interaction with SecA (see section 1.19(iv); Hartl *et al.*, 1990). SecB has been shown to retard tertiary structure

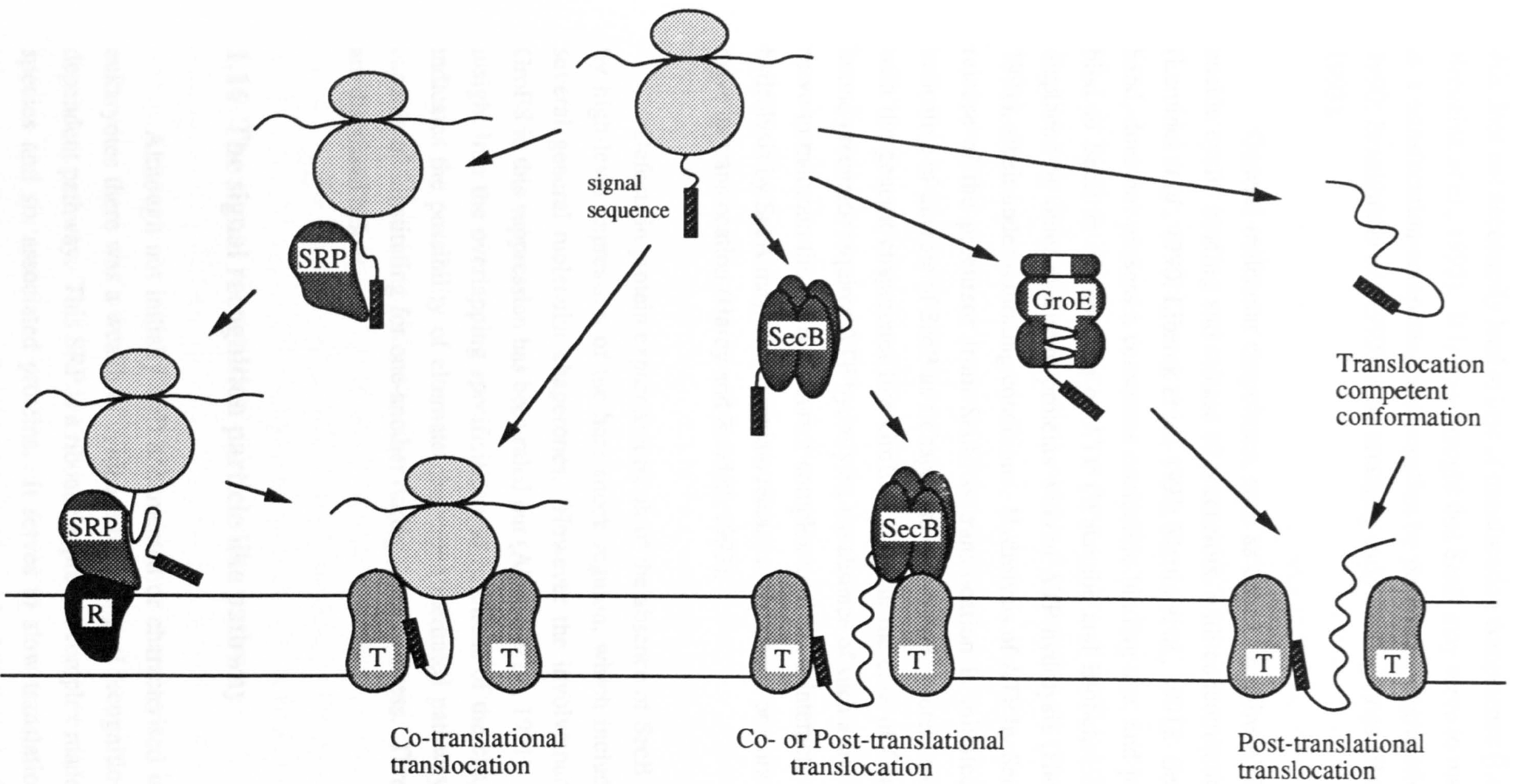

Fig. 1.4 Alternative pathways of translocation. Translocation may be co- or post-translational. A translocation competent conformation may be maintained by interactions of the signal recognition particle (SRP), SecB or GroE. T - represents the translocase incuding SecA, SecY, SecE, SecD and SecF. R - represents the SRP receptor. See text (sections 1.8-1.19) for details.

formation (Collier *et al.*, 1988; Liu *et al.*, 1989) but does not abolish it altogether as this does not necessarily lead to loss of translocation competence (Lecker *et al.*, 1990; Breukink *et al.*, 1992). It is now thought that SecB may serve to maintain precursors in a translocation-competent conformation by preventing aggregation (Lecker *et al.*, 1990; Breukink *et al.*, 1992) or by retarding early folding steps (Hardy and Randall, 1993).

General molecular chaperones, such as GroEL, require ATP as a cofactor to enable cyclic binding and release of precursors, with concomitant ATP hydrolysis (Laminet *et al.*, 1990; Liberek *et al.*, 1991; Martin *et al.*, 1991). SecB, on the other hand, does not possess a consensus nucleotide binding site, and precursor proteins bind to SecB in the absence of ATP (Watanabe and Blobel, 1989b) and can be displaced by other precursor proteins without ATP hydolysis (Hardy and Randall, 1991), albeit under saturating conditions. Hydrolysis of ATP by SecA may stimulate release of the precursor from SecB as translocation is initiated. This may be indicative of the role of SecB as a chaperone dedicated to protein export as compared with the general chaperones like GroEL. If, as in the case of GroEL, release of a bound precursor requires ATP hydrolysis, the absence of such a site within SecB may serve to maintain the SecB-precursor complex until SecB interacts with SecA. ATP hydrolysis by SecA may then stimulate release of the bound precursor from SecB and drive its translocation (Hardy and Randall, 1993).

Defects in protein export as a result of the absence of SecB can be overcome by high-level expression of the heat shock regulon, which includes the genes for several general molecular chaperones. However the involvement of GroEL and GroES in this suppression has been ruled out (Altman *et al.*, 1991). This provides an insight into the overlapping specificities and functions of the chaperones and also indicates the possibility of alternate chaperone mediated pathways which may be capable of substituting for one-another during times of stress. Three such pathways are discussed below.

1.16 The signal recognition particle like pathway

Although not initially well documented or characterised in prokaryotes, in eukaryotes there was a wealth of evidence for a "signal recognition particle" (SRP) dependent pathway. This SRP is a ribonucleoprotein complex made up of a 7S RNA species and six associated proteins. It serves to slow translation of the nascent precursor and also to target the precursor to the endoplasmic reticulum via a specific

interaction with an integral membrane protein termed docking protein or SRP receptor (for reviews see Siegel and Walter, 1988; Rapoport, 1990).

Following the emergence of the signal sequence from the ribosome it is bound by SRP, through interaction with the 54kDa subunit (SRP54) (Walter and Blobel, 1980; Kurzchalia *et al.*, 1986), and this retards further elongation of the polypeptide so preventing uncoordinated folding of the nascent precursor (Walter and Blobel, 1980; Siegel and Walter, 1985; 1988). This nascent chain-ribosome-SRP complex is directed to the membrane by the specific SRP-SRP receptor interaction. GTP binding by the SRP receptor brings about release of the nascent chain-ribosome complex from SRP and subsequent insertion of the signal sequence into the membrane (Gilmore *et al.*, 1982; Meyer *et al.*, 1982). Hydrolysis of bound GTP by the SRP receptor facilitates release of SRP (Connolly *et al.*, 1991) completing the cycle.

In prokaryotes a number of factors have been identified which show both structural and functional homology with a number of the mammalian SRP components. A 4.5S RNA and a protein of M_r 48kDa, termed P48 or Ffh (fifty four homologue) protein, have been shown to be very similar to the 7S RNA and SRP54, respectively (Romisch *et al.*, 1989; Bernstein *et al.*, 1989) and are essential for cell viability (Brown and Fournier, 1984; Rapoport, 1991). A ribonucleoprotein complex made up of 4.5S RNA and Ffh has been identified in *E. coli* (Poritz *et al.*, 1990; Ribes *et al.*, 1990) and has been shown to interact specifically with the signal sequence of a nascent precursor protein (Luirink *et al.*, 1992). Early experiments showed only β-lactamase export to be retarded following depletion of 4.5S RNA (Poritz *et al.*, 1990; Ribes *et al.*, 1990), however a more recent study has demonstrated that depletion of the Ffh protein results in a general effect on protein export, with accumulation of the precursor forms of all exported proteins tested (Phillips and Silhavy 1992).

It is now thought that 4.5S RNA fulfils more than just a single role in *E. coli* with its mode of action determined by the availabilty of the Ffh protein. Studies suggest 4.5S RNA acts independently of Ffh in modulating the rate of translation (via a direct interaction with the ribosome) to allow proteins to fold properly (for review see Brown, 1991). The 4.5S RNA/Ffh complex is believed to have a more selective role, interacting directly with signal peptides of nascent precursors in a similar manner to SRP in eukaryotes (Luirink and Dobberstein, 1994). The *E. coli* SRP is thought to act as a chaperone specific for signal peptides in nascent precursors to maintain their translocation-competent conformation. This interaction may persist until SRP is contacted by another chaperone or a component of the translocation apparatus, such as SecA or SecY (Luirink and Dobberstein, 1994). A candidate for

an SRP receptor, the FtsY protein, has also been found in *E. coli* (Romisch *et al.*, 1989; Bernstein *et al.*, 1989) and recent data indicates that depletion of this protein results in the accumulation of β-lactamase, OmpF and RBP suggesting that FtsY is the functional *E. coli* homologue of the mammalian SRP receptor (Luirink *et al.*, 1994).

It has been suggested from these data that *E. coli* SRP may represent the first contact that a nascent precursor makes with a complex hierarchical set of chaperones involved in protein translocation in *E. coli* (Luirink and Dobberstein, 1994).

1.17 The general molecular chaperones

The involvement of molecular chaperones in *de novo* protein folding has been established for the stress proteins of the heat shock protein Hsp70 and Hsp60 families (Pelham, 1988; Fischer and Schmid, 1990; Gething and Sambrook, 1992). In prokaryotes the major heat shock proteins (HSPs) are coded by single genes which are expressed constitutively at all temperatures. Following a temperature shift or treatment with protein-damaging agents, such as ethanol, the rate of expression of these genes accelerates sharply. After a few minutes the rate of expression subsides, reaching a new steady state level, characteristic of a given temperature (Bukau, 1993). The function of many of these HSPs is to act as chaperones to ensure that polypeptide chains are correctly folded, processed, localised and in some cases complexed with other polypeptides to perform their biological function (for reviews see Craig *et al.*, 1993; Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993; Hartl *et al.*, 1994).

Members of the Hsp70 family are involved in diverse functions, including maintaining proteins in an unfolded state (Deshaies *et al.*, 1988; Chirico *et al.*, 1988; Zimmerman *et al.*, 1988; Phillips and Silhavy, 1990), dissociating protein aggregates, and facilitating renaturation (Pelham, 1988; Skowyra *et al.*, 1990). The *E. coli* Hsp70 homologue, DnaK, cooperates functionally with two additional heat shock proteins, DnaJ and GrpE (Georgopoulos *et al.*, 1990; Craig and Gross, 1991). Members of the Hsp60 family have been shown to mediate the folding of proteins to their native structure and facilitate oligomeric protein assembly (see section 1.18).

The Hsp70s and Hsp60s appear to interact with different structural elements of unfolded polypeptides. Members of the Hsp70 family interact with nascent chains and polypeptides in extended conformations (Beckman *et al.*, 1990; Palleros *et al.*, 1991) whereas the *E. coli* Hsp60, GroEL, may recognise secondary structure elements

(Landry and Gierasch, 1991) binding proteins as conformational intermediates formed early in folding (Martin *et al.*, 1991), although binding to exposed hydrophobic regions may be important in both (Hendrick and Hartl, 1993). Work carried out *in vitro* by Hartl and co-workers suggests that DnaK, DnaJ and GroEL interact sequentially with the folding polypeptide in a reaction coupled by GrpE which led them to propose a model where DnaK and DnaJ interact to prevent premature misfolding and aggregation, after which GroEL, in conjunction with GroES, mediates proper folding (Langer *et al.*, 1992a). This model is supported by data from mitochondrial studies where, following import from the cytoplasm, newly translocated polypeptides first interact with the DnaK-related Hsp70 in the matrix (Kang *et al.*, 1990; Scherer *et al.*, 1990; Manning-Krieg *et al.*, 1991) and are then passed on to Hsp60 (Cheng *et al.*, 1989; Ostermann *et al.*, 1989; Manning-Krieg *et al.*, 1991), the mitochondrial GroEL homologue to assist folding (Reading *et al.*, 1989).

It may be expected from this that exported proteins in *E. coli* would interact with DnaK and DnaJ prior to translocation and that this interaction would be sufficient for maintaining them in a loosely folded, translocation-competent conformation. Recently the export of alkaline phosphatase has been shown to be dependent on DnaK and DnaJ (Wild *et al.*, 1992), however the export of β-lactamase is dependent on GroEL and GroES for its efficient translocation (Kusukawa *et al.*, 1989). This allows interesting speculation as to the extent of tertiary structure which is both tolerated and necessary for translocation of a functional protein.

1.18 The GroEL/GroES chaperones

(i) Organisation

The *groEL* and *groES* genes are operonic (Tilly *et al.*, 1981), encode the proteins GroEL and GroES respectively, and have been shown to be indispensable for *E. coli* growth at all temperatures (Fayet *et al.*, 1989; Georgopoulos *et al.*, 1990). The GroEL and GroES proteins belong to a subset of the universal group of heat shock proteins, termed "chaperonins" (Ellis and van der Vies, 1991), which are also thought to be widely conserved and have been found in bacteria, mitochondria, chloroplasts, plastids (Hemmingsen *et al.*, 1988; McMullin and Hallberg, 1988; Lubben *et al.*, 1990) and more recently in the cytoplasm of thermophiles and eukaryotes (Hendrick and Hartl, 1993). These chaperonins form large oligomeric structures, with the 57kDa GroEL protein (Hemmingsen *et al.*, 1988) found as a decatetrameric homo-

oligomer, composed of two stacked heptameric rings (exhibiting a seven fold axis of symmetry) that form a central cavity (Hendrix, 1979; Hohn *et al.*, 1979). The GroEL chaperonin binds 1-2 molecules of unfolded protein in this cavity with one GroEL ring having the capacity for proteins of sizes up to 90kDa (Langer *et al.*, 1992b; Braig *et al.*, 1993), presumably in the conformation of a compact folding intermediate or molten globule (Martin *et al.*, 1991; van der Vies *et al.*, 1992). The 10kDa GroES protein may be thought of as a "co-chaperonin" acting as a regulatory factor that modifies the activity of GroEL. It forms a single heptameric ring of subunits (Hemmingsen *et al.*, 1988; Chandrasekhar *et al.*, 1986) which interacts with the GroEL oligomer in a 1:1 complex by binding to one end of the cylinder (Langer *et al.*, 1992b; Viitanen *et al.*, 1990).

(ii) General chaperone function

The exact role of GroES in the regulation of GroEL function is not yet clear, however recent work by Hartl and co-workers (Martin *et al.*, 1993a; 1993b) has considerably increased our understanding of how the two chaperonins function together. It was shown that GroES and the substrate protein have opposite effects on GroEL. GroES stabilises GroEL in the ADP bound state (Chandrasekhar *et al.*, 1986; Viitanen *et al.*, 1990; Martin *et al.*, 1991) inhibiting GroEL ATPase activity. Binding of unfolded polypeptide within the cavity of the GroEL cylinder triggers ADP and GroES release (Martin *et al.*, 1993a). Following ADP-ATP exchange (Martin *et al.*, 1991) GroES reassociates with GroEL and concomitant ATP hydrolysis discharges the bound protein for thermodynamically mediated folding. The principle of the reaction being that substrate and GroES go through mechanistically coupled cycles of binding and release until the folding protein has lost its affinity for GroEL.

The following model (Fig.1.5) was proposed to explain these and other findings:

i) the GroEL-GroES complex is the preferred state of the chaperonin proteins under physiological conditions (Jackson *et al.*, 1993). ADP is bound with high affinity to the seven subunits of the GroES-associated ring of GroEL and ADP binding in the opposite ring is less tight. Entry of unfolded protein at the GroES distal ring of GroEL triggers ADP dissociation from both GroEL rings and the release of GroES;

ii) in the absence of nucleotide, substrate protein is tightly bound and the affinity for GroES is significantly reduced;

iii) following entry of ATP, GroES rebinds at the alternative GroEL ring and the binding affinity for substrate is reduced;

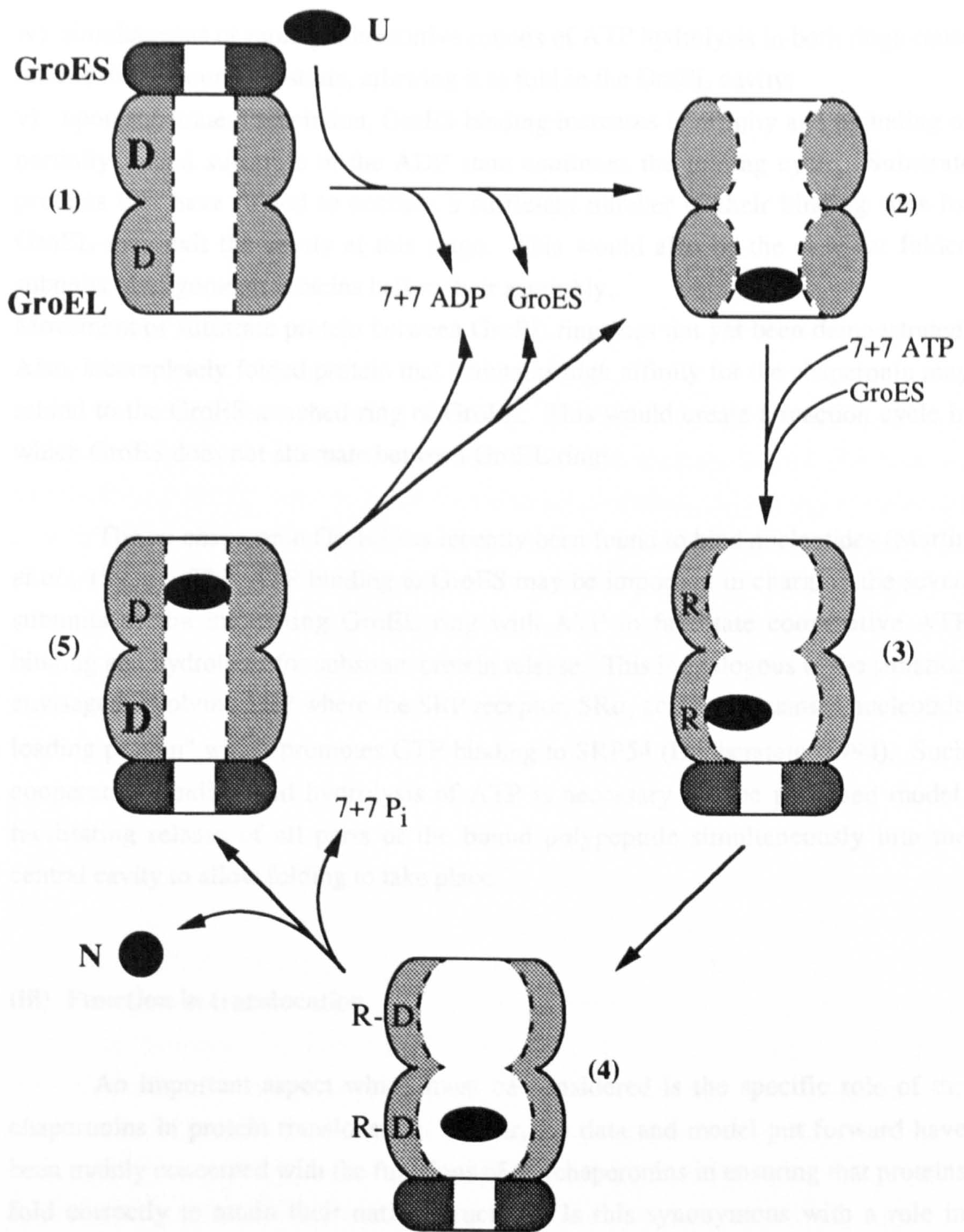

Fig. 1.5 Model for the reaction cycle of GroEL and GroES in chaperonin-assisted protein folding. **D**, the high affinity ADP state in the seven subunits of GroEL which are in direct contact with GroES; D, the relatively lower affinity of the subunits in the opposite GroEL ring which may hydrolyse ATP dependent on [K^+] and the concentration ratio of ATP/ADP; R, the subunits in a GroEL ring in the ATP-bound state; **U** and **N**, unfolded and native proteins, respectively. Initial binding and rebinding of substrate protein is only shown to the GroEL ring opposite GroES, however the principle of the reaction cycle is considered to be independent of the relative topology of substrate protein and GroES at GroEL. Between steps 5 and 2 GroEL is rotated by 180°, with the substrate protein remaining bound to the same ring. ATP binding and hydrolysis are co-operative events at the level of the 7-sub unit rings (taken from Martin *et al*, 1993a).

iv) simultaneous or rapidly consecutive rounds of ATP hydrolysis in both rings cause release of the bound substrate, allowing it to fold in the GroEL cavity;
v) upon substrate dissociation, GroES binding increases in affinity and rebinding of partially folded substrate in the ADP state continues the folding cycle. Substrate proteins that have folded to occlude a sufficient number of their binding sites for GroEL may exit the cavity at this stage. This would also be the case for folded subunits of oligomeric proteins before their assembly.
Movement of substrate protein between GroEL rings has not yet been demonstrated. Also, incompletely folded protein that maintains high affinity for the chaperonin may rebind to the GroES-attached ring of GroEL. This would create a reaction cycle in which GroES does not alternate between GroEL rings.

The co-chaperonin GroES has recently been found to bind nucleotides (Martin *et al.*, 1993b). This ATP binding to GroES may be important in charging the seven subunits of the interacting GroEL ring with ATP to facilitate cooperative ATP binding and hydrolysis for substrate protein release. This is analogous to the situation envisaged involving SRP where the SRP receptor, SRα, acts as a "guanine nucleotide loading protein" which promotes GTP binding to SRP54 (Dobberstein, 1994). Such cooperative binding and hydrolysis of ATP is necessary for the proposed model, facilitating release of all parts of the bound polypeptide simultaneously into the central cavity to allow folding to take place.

(iii) Function in translocation

An important aspect which must be considered is the specific role of the chaperonins in protein translocation. So far, the data and model put forward have been mainly concerned with the functions of the chaperonins in ensuring that proteins fold correctly to attain their native structure. Is this synonymous with a role in protein translocation via the maintenance of a translocation-competent conformation? Translocation of the periplasmic protein β-lactamase is known to be GroEL and GroES dependent (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989) which indicates that GroEL/ES are capable of maintaining a precursor in such a non-native conformation. A possible solution to this contrariety may be provided, at least in part, by the model above. If instead of a cyclic reaction, with GroES and substrate polypeptide no-longer flipping one another off the GroEL core, the substrate may bind and modify the GroEL/ES complex in some way so that the substrate remains bound to GroEL. This relatively stable complex (analogous to the formation of stable binary complexes observed by Lorimer *et al.*, 1993), may serve to maintain the

substrate in a translocation-competent conformation until the substrate interacts with another factor, possibly a Sec protein, which catalyses its release from GroEL and facilitates its translocation.

1.19 SecB

(i) Characterisation

The mutant selection procedure designed by Beckwith (Oliver and Beckwith, 1981; see section 1.7) enabled the isolation of the first mutants in *secB* (Kumamoto and Beckwith, 1983). Subsequent characterisation predicted the *secB* gene product to be a soluble protein, with high overall negative charge and estimated M_r, 16.6kDa (Kumamoto and Nault, 1989). The *secB* gene was found to be non-essential, although mutations within it resulted in a number of pleiotropic defects and viability only on minimal and not rich media (Kumamoto and Beckwith, 1985). Studies using a *secB* null mutant ($SecB^-$) indicated that only a subset of precursors were dependent upon SecB for their efficient translocation, as illustrated by an accumulation of precursors in the absence of SecB (Kumamoto and Beckwith, 1985). These "SecB-dependent" proteins included the periplasmic maltose binding protein (MBP) and also the outer membrane proteins OmpF, LamB, OmpA and PhoE (Kumamoto and Beckwith, 1985; Lecker *et al.*, 1989b). "SecB-independent" proteins included the periplasmic ribose binding protein (RBP) and alkaline phosphatase although it has since been proposed that the translocation of alkaline phosphatase is SecB dependent at reduced temperatures (Kusukawa *et al.*, 1989). Using an *in vitro* translocation system, SecB was independently identified as a component that enhanced the translocation of LamB and OmpA (Watanabe and Blobel, 1989b; 1989c; Kumamoto *et al.*, 1989; Hay, 1992). SecB protein has been purified (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989; Watanabe and Blobel, 1989c) and consistent with the predictions from sequence data is a cytoplasmic 16kDa protein of high negative charge that is thought to function as a tetramer (Watanabe and Blobel, 1989c). Complexes of SecB with precursors have been isolated from both *in vitro* (Lecker *et al.*, 1989b; Randall *et al.*, 1990; Weiss and Bassford, Jr., 1990) and *in vivo* sources (Kumamoto, 1989).

(ii) Chaperone function

Unlike the GroEL/ES chaperonins the function of SecB appears to be confined to a role in the GEP as it binds to the majority of precursor proteins but to only very few cytoplasmic proteins under extreme circumstances (Kumamoto and Beckwith, 1985; Kumamoto, 1989; MacIntyre *et al.*, 1991).

A role for SecB in the modulation of the folding of precursor proteins was proposed following initial evidence which indicated that SecB acts at an early stage in export, prior to the action of SecA or the translocation machinery at the inner membrane (Kumamoto and Beckwith, 1985). In the *secB* null mutant, co-translational processing of pre-MBP nascent chains was not observed (Kumamoto and Gannon, 1988), unlike in wild type cells (Joseffson and Randall, 1981a). However, limited post-translational translocation and processing was seen in the *secB* null mutant (Kumamoto and Gannon, 1988) as well as an increase in the amount of tightly folded intracellular pre-MBP. Randall and Hardy (1986) had previously demonstrated the importance of maintaining a relatively "loose" conformation which lacked stable tertiary structure for efficient translocation across the *E. coli* inner membrane. These findings are consistent with a role for SecB in influencing the conformation of pre-MBP. *In vitro* experiments (Collier *et al.*, 1988; Weiss *et al.*, 1988) confirmed this with the presence or absence of SecB dictating the pre-MBP conformation. Also, SecB has been shown to block the folding of denatured pre-MBP using purified proteins (Liu *et al.*, 1989; Hardy and Randall, 1991).

Complementary evidence of a role for SecB in the modulation of folding of precursor proteins came from *in vivo* studies in which functional SecB was "sequestered" from the cytoplasm by mutant precursors (see Chapter 4). It was first noted by Bankaitis *et al.*, (1984) that an export-defective LamB precursor, with two thirds of the hydrophobic core deleted from its signal peptide, was capable of entering the protein export pathway and competing with wild type precursors for SecB so interfering with general protein export in *E. coli.* Further investigation of this SecB sequestering effect revealed that mutant proteins which were most efficient in their sequestering activities often combined mutations in their mature region with mutations in the signal peptide. The mutation in the mature region served to slow the rate of folding, enabling SecB to interact with the mutant protein whilst the signal peptide mutation prevented further interaction of the mutant protein-SecB complex with the translocation apparatus (Collier *et al.*, 1988; Liu *et al.*, 1989). As expected, the interference effects which resulted were limited to those proteins which had previously been classified as SecB-dependent.

The previously classified SecB-independent RBP has recently been shown to be dependent on SecB under conditions in which export of RBP is less than optimal (Kim *et al.*, 1992). Addition of the uncoupler CCCP resulted in an accumulation of pre-RBP in the cytoplasm which could be improved by the addition of SecB (Kim *et al.*, 1992). It may be that any constraint imposed upon the translocation process could provide a situation in which a SecB effect can be observed.

To explain these and other results the model illustrated in Fig.1.6 was proposed by Kumamoto (1990). For fast and efficient translocation (co-translationally or immediately after translation) the nascent precursor must adopt a conformation that presents the signal peptide to the membrane in an appropriate manner. Alternative conformations may exist in which the signal peptide is less accessible to the membrane. For example, the signal peptide may interact with the mature sequence in order to retard folding. The equilibrium between the two forms (step 2) depends upon the properties of the mature sequence. However, binding of SecB shifts the equilibrium to favour the translocationally active conformation (step 1) and also prevents the competing reaction, that of folding into a tightly folded, export-incompetent conformation (step 3). The SecB-independent proteins may be those that have a high probability of spontaneously forming and maintaining the translocationally active conformation.

(iii) Recognition of precursors

Although only a subset of precursor proteins are SecB-dependent with others being SecB-independent this distinction is not clear cut. Most of the SecB-dependent proteins are exported, albeit more slowly, in *secB* null mutants and the SecB-independent export of other proteins, such as alkaline phosphatase is dependent on conditions (Kusukawa *et al.*, 1989). The features of proteins which render them SecB-dependent or independent have yet to be clearly established however certain characteristics have been identified as being important.

In vitro studies have shown that the ability of SecB to bind and block folding is dependent on the rate of folding of the protein in question (Liu *et al.*, 1989). In most cases the presence of the signal peptide, although not usually directly involved in this interaction, serves to decrease the rate of folding of the precursor protein and so increase the probability of SecB binding (Park *et al.*, 1988). Such a decrease in the rate of folding has also been achieved by introducing a mutation into the mature portion of the protein (Liu *et al.*, 1988), or conducting the experiments at lower

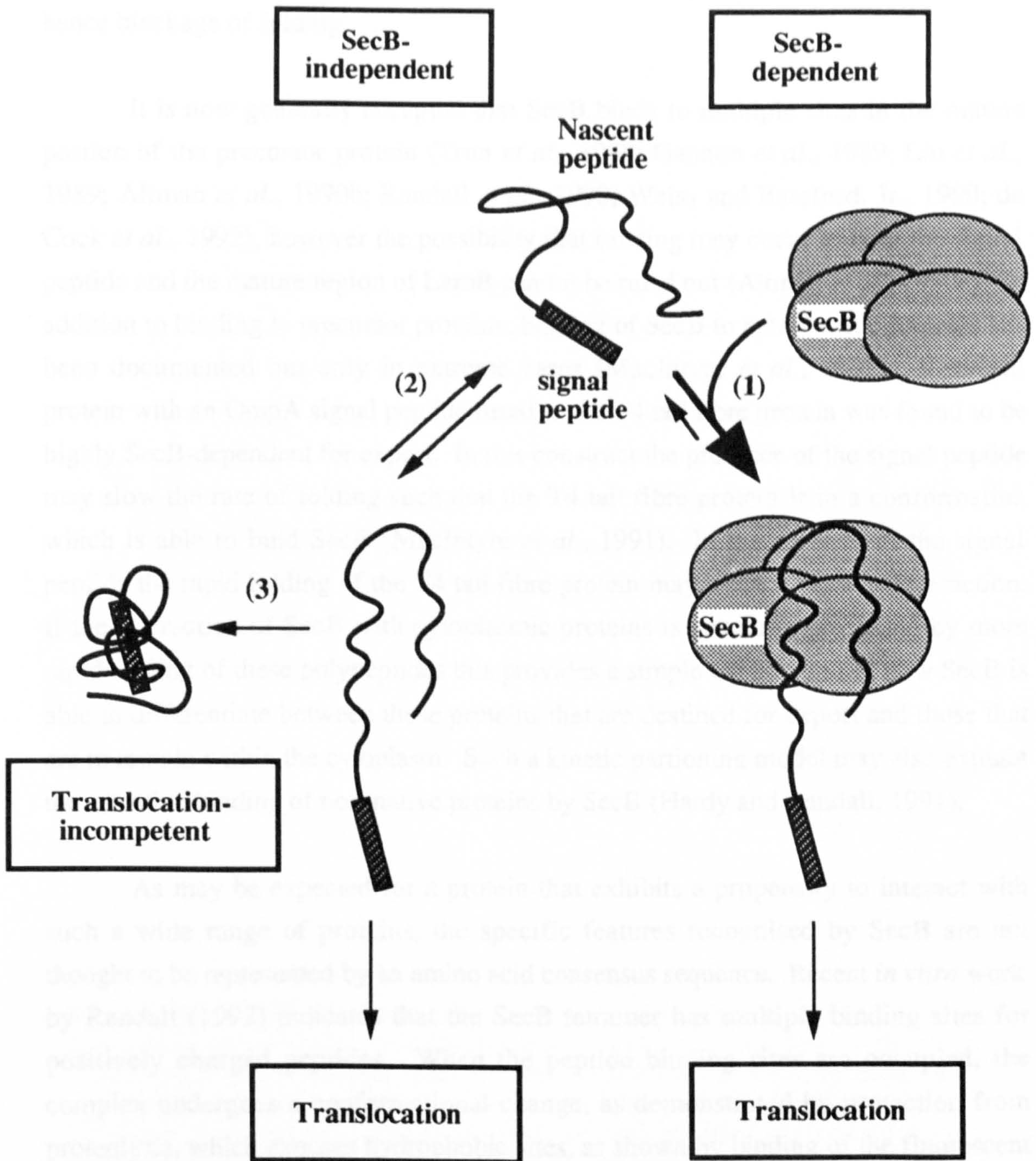

Fig. 1.6 Possible effects of SecB binding on the conformation of a nascent exported protein. The efficiency of translocation is determined by the accessibility of the signal peptide. Translocation-incompetent represents a conformation in which the mature sequence has formed a tightly folded structure or the signal peptide is inaccessible due to interaction with the mature sequence. Translocation represents conformations in which the signal peptide is highly accessible for interaction with the membrane-bound translocation apparatus. Binding of SecB maintains a translocationally active conformation, ensures the signal peptide is highly accessible and prevents transitions to other conformations (taken from Kumamoto, 1990).

temperatures (Liu *et al.*, 1989) with both cases resulting in increased SecB binding, hence blockage of folding.

It is now generally accepted that SecB binds to multiple sites in the mature portion of the precursor protein (Trun *et al.*, 1988; Gannon *et al.*, 1989; Liu *et al.*, 1989; Altman *et al.*, 1990b; Randall *et al.*, 1990; Weiss and Bassford, Jr., 1990; de Cock *et al.*, 1992), however the possibility that binding may occur to both the signal peptide and the mature region of LamB cannot be ruled out (Altman *et al.*, 1990a). In addition to binding to precursor proteins, binding of SecB to cytoplasmic proteins has been documented but only in extreme cases (MacIntyre *et al.*, 1991). A hybrid protein with an OmpA signal peptide fused to the T4 tail fibre protein was found to be highly SecB-dependent for export. In this construct the presence of the signal peptide may slow the rate of folding such that the T4 tail fibre protein is in a conformation which is able to bind SecB (MacIntyre *et al.*, 1991). In the absence of the signal peptide the rapid folding of the T4 tail fibre protein may prevent such an interaction. If the interaction of SecB with cytoplasmic proteins is generally precluded by more rapid folding of these polypeptides this provides a simple explanation of how SecB is able to differentiate between those proteins that are destined for export and those that are to remain within the cytoplasm. Such a kinetic partioning model may also explain the selective binding of non-native proteins by SecB (Hardy and Randall, 1991).

As may be expected for a protein that exhibits a propensity to interact with such a wide range of proteins, the specific features recognised by SecB are not thought to be represented by an amino acid consensus sequence. Recent *in vitro* work by Randall (1992) indicates that the SecB tetramer has multiple binding sites for positively charged peptides. When the peptide binding sites are occupied, the complex undergoes a conformational change, as demonstrated by protection from proteolysis, which exposes hydrophobic sites, as shown by binding of the fluorescent probe 1-anilino-naphthalene-8-sulphonate (ANS). These findings led to the proposal of a model to account for the interaction of precursors and SecB (Fig.1.7). At physiological ionic strength SecB has its hydrophobic site partly exposed, even in the absence of ligand. Saturation of the binding sites induces a conformational change that fully exposes the hydrophobic site, which is then available to bind hydrophobic regions of the polypeptide ligand. The presence of two different kinds of binding site allows the chaperone to have high selectivity for non-native proteins even though each site exhibits broad specificity. Also, exposure of the hydrophobic site after several of the peptide binding sites are occupied would prevent aggregation of SecB molecules with there being a high probability of filling it with the already bound polypeptide. This model is able to account for possible interactions between the

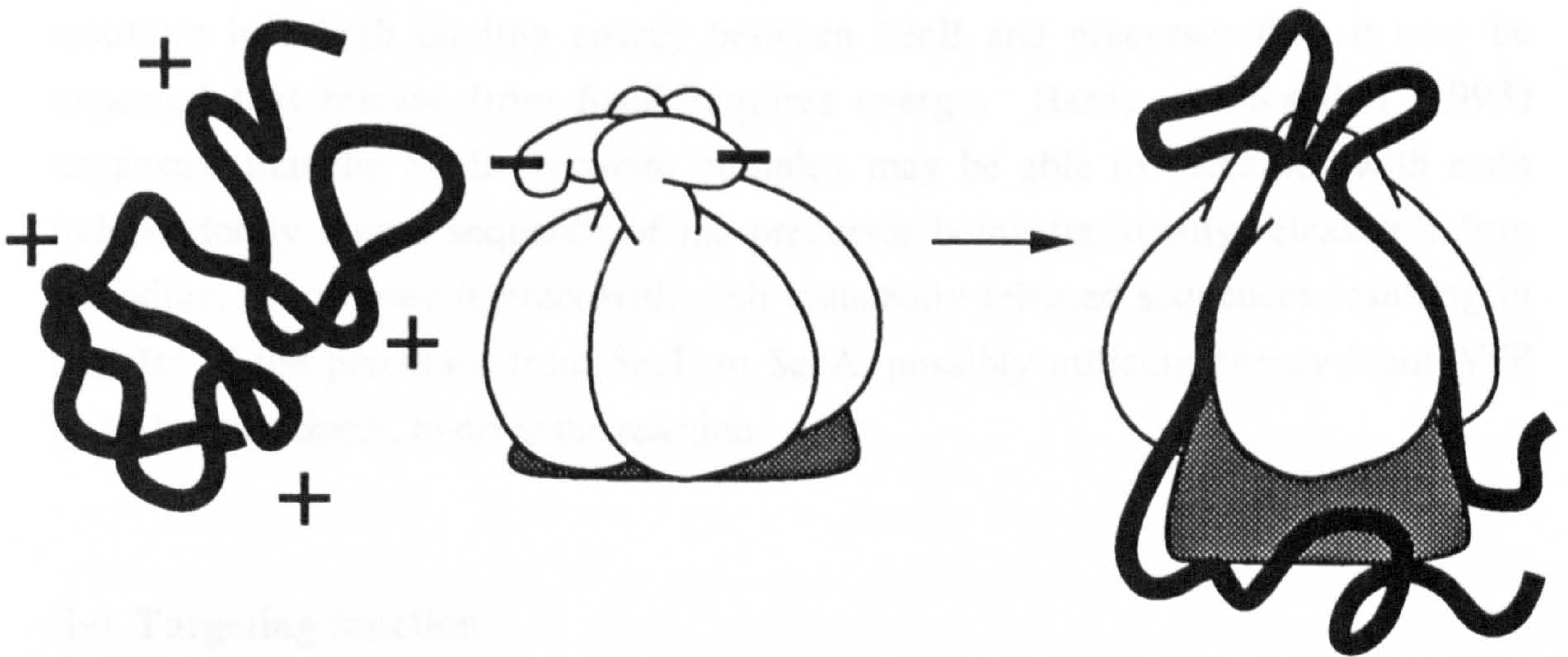

Fig. 1.7 Model of interaction of non-native polypeptide with SecB. A non-native polypeptide with several positive charges interacts with the negatively charged binding sites on the SecB tetramer to bring about a conformational change in SecB. The exposed hydrophobic binding site of the SecB tetramer (shaded) then binds to hydrophobic regions of the polypeptide resulting in the formation of a relatively stable complex (taken from Randall, 1992) .

signal peptide, which is positively charged, and SecB through one of the peptide binding sites but such a complex would not be stable unless further interactions occurred. It also explains why hybrid proteins, in which the signal sequences and mature regions derived from SecB-dependent and -independent proteins have been exchanged show SecB binding to those mature regions that are normally SecB-dependent irrespective of the nature of the signal peptide (Gannon *et al.*, 1989; Collier *et al.*, 1990). Such a model may also account for the stability of the SecB-precursor complex which is thought to be required for SecB to function efficiently as a chaperone in translocation (see section 1.15).

If the SecB-precursor complex is stable with the individual interactions resulting in a high binding energy between SecB and precursor then it may be expected that release from SecB requires energy. Hardy and Randall (1993) suggested that the SecB-precursor complex may be able to "breathe" with each independently bound sequence of the precursor being transiently released before rebinding. SecA may interact with such transiently released sequences resulting in transfer of the precursor from SecB to SecA, possibly utilising energy from ATP hydrolysis by SecA, to drive the reaction.

(iv) Targeting function

The model proposed by Kumamoto (1990) (see section 1.19(ii)) suggests that SecB acts to ensure signal peptide accessibility, so indirectly serving to target the precursor to the translocase. This is supported by the fact that the small proportion of MBP that is translocated in a *secB* null strain, is translocated slowly and post-translationally suggesting a poor targeting ability (Kumamoto and Gannon, 1988). Also, work by de Cock and Tommassen, (1992) suggests that SecB is mainly required for efficient targeting of pre-PhoE to the translocase. In an *in vitro* reconstituted system SecB has been shown to interact with SecA, the peripheral component of the translocase (Hartl *et al.*, 1990) both in the absence of precursor protein and with enhanced affinity in the presence of pro-OmpA. In addition, a variety of precursor proteins have been found to be complexed with SecB using SecB affinity chromatography (Kumamoto, 1989) and partial protease digests revealed that incomplete forms of these precursors were also associated. These data indicate a role for SecB not only in the modulation of folding of precursor proteins but also in targeting molecules, via a specific interaction with SecA (Hartl *et al.*, 1990), to the translocase and so stimulating co-translational translocation.

1.20 Flexibility of the chaperones

An insight into the flexibility of the chaperones was obtained recently when it was found that induction of the heat shock response can substitute for SecB function in *E. coli* (Altman *et al.*, 1991). Indeed, the accumulation of secretory protein precursors, caused either by mutations in *secB* or *secA* or by the overproduction of export-defective proteins, results in a two to five fold increase in the synthesis of heat shock proteins (Wild *et al.*, 1993). Somewhat surprisingly overproduction of GroEL/ES does not rescue the export defect caused when SecB is limiting or absent (Altman *et al.*, 1991). However, further studies indicated that simultaneous overproduction of DnaK and DnaJ is able to substitute for SecB in *secB* null strains, restoring the characteristic growth defect on rich media and the translocation kinetics of some SecB-dependent proteins (Wild *et al.*, 1992).

So far studies concerning the role of cytoplasmic chaperones have been limited to considering their involvement with proteins that are exported across the inner membrane only. It will be interesting to determine whether there is a similar role for chaperones in the processes involved in secreting proteins into the extracellular *milieu*. It seems logical to assume that proteins which are to be translocated across both the inner and outer membrane may require chaperoning to ensure that they remain in a secretion-competent conformation. The secretion of haemolysin provides a useful system for such studies and is described below.

1.21 Secretion of haemolysin

The secretion of polypeptides across both the inner and outer membranes of Gram-negative bacteria is relatively rare and is achieved by only a small number of proteins such as, IgA protease, aerolysin, cholera toxin and pullulanase and typically requires distinct outer membrane translocation mechanisms whose substrates are periplasmic intermediates generated by the GEP (Pohlner *et al.*, 1987; Wong and Buckley, 1989; Hirst and Holmgren, 1989; d'Enfert *et al.*, 1987). However *E. coli* haemolysin (HlyA) is secreted independently of the GEP (Gray *et al.*, 1989; Gentschev *et al.*, 1990) and belongs to the RTX (repeat toxin) family of proteins. These have several distinctive, yet characteristic features which include: a novel targeting signal located within the C-terminus of the toxins (Nicaud *et al.*, 1986; Gray *et al.*, 1986; Mackman *et al.*, 1987; Koronakis *et al.*, 1989; Hess *et al.*, 1990), the absence of any periplasmic intermediates in secretion (Gray *et al.*, 1986; Felmlee and

Welch, 1988; Koronakis *et al.*, 1989) and specific membrane proteins that export the toxins directly to the outside of the cell (Wagner *et al.*, 1983; Mackman *et al.*, 1985).

(i) Organisation of the haemolysin operon

The HlyA protein is encoded by the *hlyA* gene which is found on a contiguous stretch of DNA, termed the Hly determinant or Hly operon (Mackman and Holland, 1984b) as shown in Fig.1.8a which illustrates the organisation and transcription of the genes and molecular weights of their products. The four genes of the *hly* operon are co-transcribed from a promoter region proximal to *hlyC*, but expression of the structural (*hlyCA*) and export (*hlyBD*) determinants can be uncoupled by transcription termination at a *rho*-independent terminator located in the region between *hlyA* and *hlyB* (Welch and Pellet, 1988; Koronakis *et al.*, 1988b). Transcription termination between *hlyA* and *hlyB* can be alleviated by antitermination resulting in increased levels of *hlyCABD* transcripts leading to enhanced expression and subsequent secretion of HlyA (Nieto *et al.*, 1991; Vogel *et al.*, 1988).

HlyC is cytoplasmic (Nicaud *et al.*, 1985a) and plays no role in the secretion process but is involved in the activation of pro-HlyA (Issartel *et al.*, 1991). Secretion of HlyA is directed by the integral inner membrane proteins HlyB and HlyD (Wagner *et al.*, 1983; Mackman *et al.*, 1985) in close association with the *E. coli* outer membrane protein TolC (Wandersman and Delepelaire, 1990).

(ii) Structure of HlyA

The 107kDa protein, HlyA, does not possess an N-terminal signal peptide and does not undergo proteolytic processing (Felmlee *et al.*, 1985a; 1985b). As a member of the RTX group of proteins (Coote, 1992) it contains a series of glycine-rich nonapeptide repeat units near the C-terminal end which are required for efficient secretion and biological activity (Felmlee and Welch, 1988). Mature HlyA is secreted as an acylated form of the *hlyA* gene product pro-HlyA, following the covalent attachment of a fatty acid moiety in a cytoplasmic maturation mechanism directed by HlyC and dependent upon acyl carrier protein (Hardie *et al.*, 1991; Issartel *et al.*, 1991). Toxin maturation, by acylation, is not a requirement for HlyB/HlyD-dependent secretion of HlyA but is required to target the HlyA toxin to mammalian cell membranes, prior to forming cation-selective pores and disrupting the host cell (Bhakdi *et al.*, 1986).

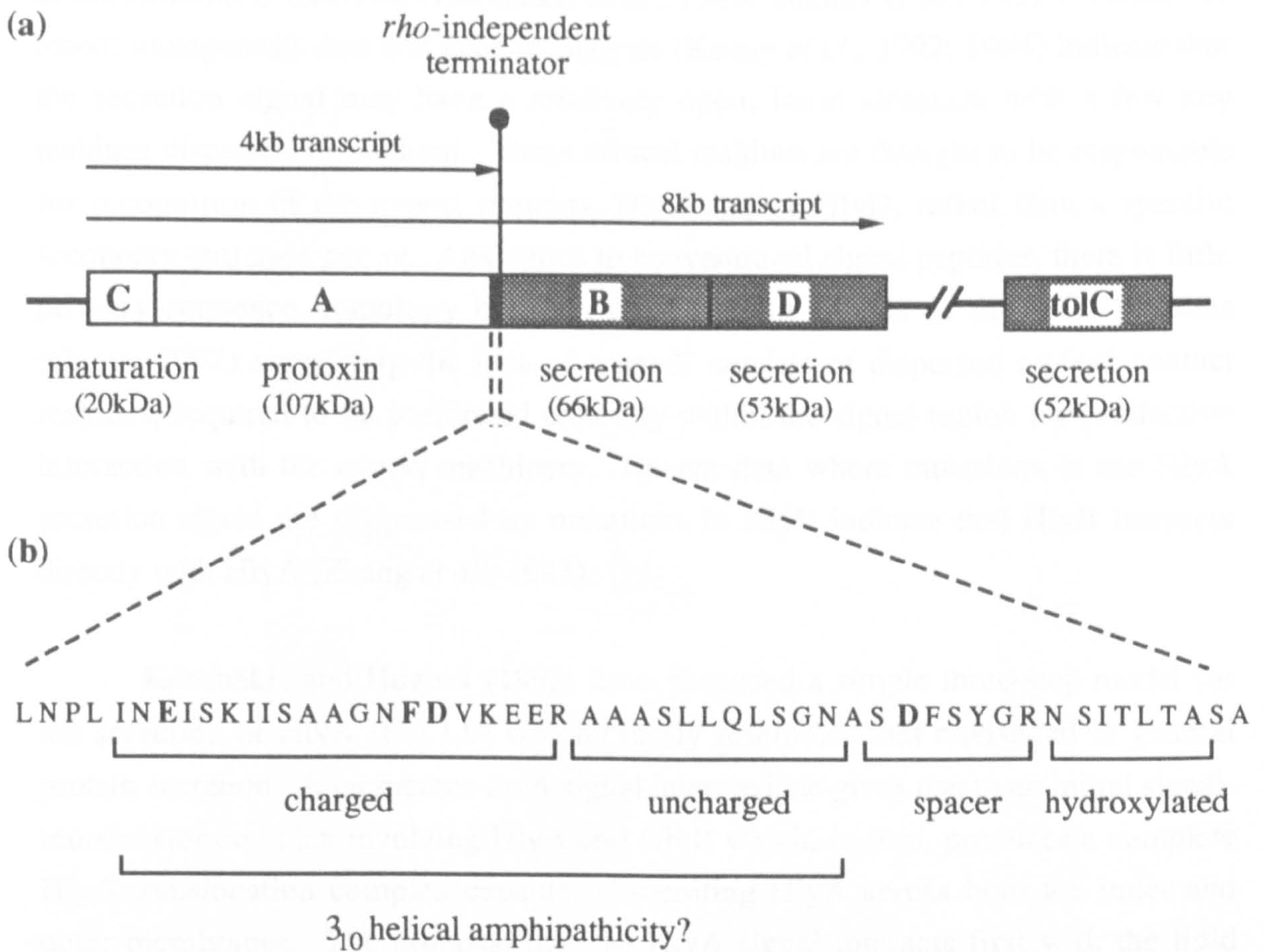

Fig. 1.8 (a) The haemolysin determinant which directs synthesis, maturation, and secretion of haemolysin. The *hlyA* gene encodes the inactive 1024 residue pro-HlyA which is converted to the mature toxin by acyl carrier protein-dependent fatty acylation directed by HlyC. HlyA is secreted across both membranes in a process dependent upon the inner membrane proteins HlyB, HlyD and also the *E. coli* outer membrane protein TolC. Expression of the operon generates two transcripts as a result of transcription termination and antitermination. (b) The HlyA C-terminal export signal (Felmlee *et al.*, 1985b) showing putative features and key residues (in bold). See text for details.

Post-translational targeting of HlyA to the inner membrane is directed by an unprocessed secretion signal situated within its C-terminal 46 amino acids (Kenny *et al.*, 1992). The sequence of this essential secretion signal suggests three domains (Fig.1.8b): an amphipathic charged helix, an uncharged region and a hydroxylated tail at the extreme C-terminus (Koronakis *et al.*, 1989; Stanley *et al.*, 1991). However, recent mutagenesis data and genetic analysis (Kenny *et al.*, 1992; 1994) indicate that the secretion signal may have a relatively open, loose structure with a few key residues dispersed throughout. These critical residues are thought to be responsible for recognition of the export proteins, HlyB and or HlyD, rather than a specific secondary structure *per se*. Analogous to conventional signal peptides, there is little primary sequence homology between the targeting signals of the RTX proteins (Coote, 1992) supporting the idea of a small number of dispersed critical contact residues, required to be positioned correctly within the signal region for productive interaction with the export machinery. Recent data where mutations in the HlyA secretion signal are suppressed by mutations in HlyB indicate that HlyB interacts directly with HlyA (Zhang *et al.*, 1993).

Koronakis and Hughes (1993) have proposed a simple three-step model for the secretion of HlyA (Fig.1.9) which closely resembles that envisaged in general protein secretion. A membrane lipid-signal intermediate gives rise to an initial signal-translocator complex involving HlyA and HlyB which, in-turn, produces a complete HlyA-translocation complex capable of secreting HlyA across both the inner and outer membranes. The proposal that the HlyA signal interacts first with the lipid bilayer and then the secretion proteins, is supported by investigations of targeting to the plasma membrane with fusions of the HlyA C-terminal signal to non-translocatable globular proteins (Oropeza-Wekerle *et al.*, 1990; V. Koronakis, unpublished results). The proposed initial interaction of HlyA with the lipid bilayer is also analogous to the suggested model for the export of lipophilic drugs by the mammalian multidrug resistance P-glycoprotein (Devaux, 1991; Higgins and Gottesman, 1992). The substrate is predicted to interact directly with membrane lipid prior to interaction with a substrate binding site on the transporter protein. This model also suggests that the transporter is not merely a hydrophilic hole which shields the substrate from the lipid bilayer but is able to open and close (Higgins and Gottesman, 1992). Such a model, which is able to "breathe" is appealing for the HlyA secretion system which must accomodate a protein of 1024 amino acids.

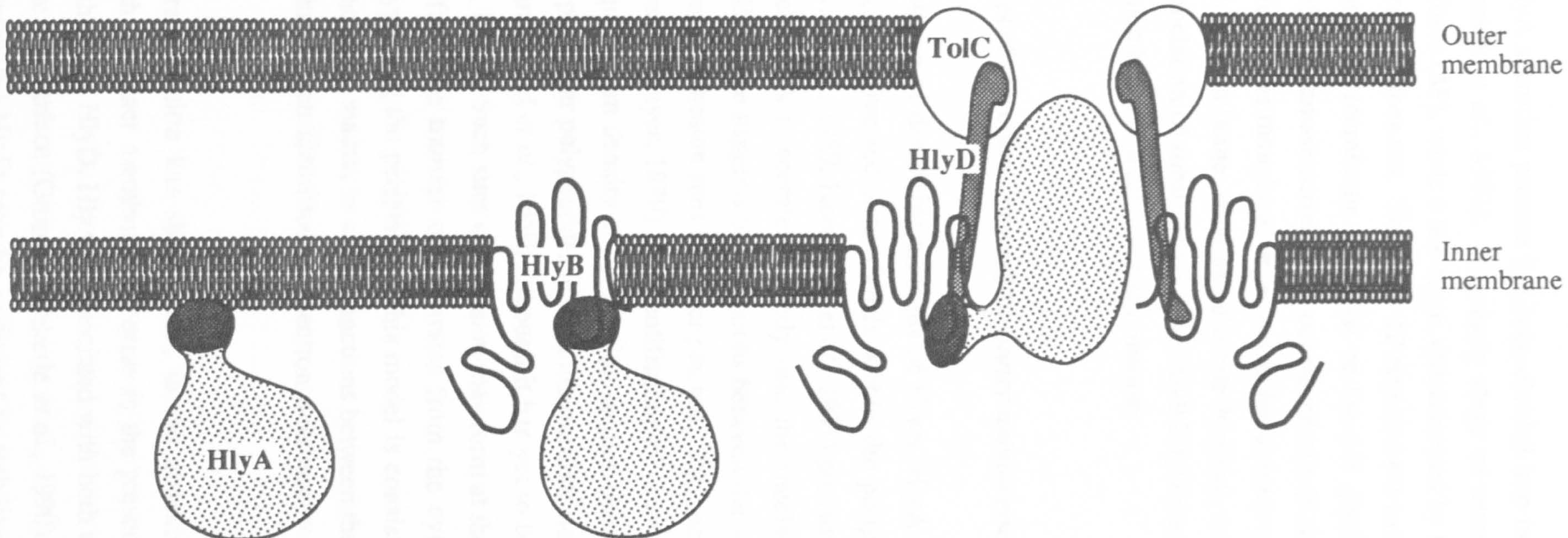

Fig. 1.9 Possible steps in the HlyB-dependent translocation of HlyA across the inner membrane and outer membrane during secretion without a periplasmic intermediate. The HlyA C-terminal export signal is represented by a dark stippled domain. The HlyA signal associates with the lipid bilayer and then interacts with HlyB. HlyA is then secreted across both membranes by the HlyB/HlyD/TolC complex.

(iii) Early stages of secretion - interactions with the inner membrane

The HlyA secretion process has been divided into two energetically distinct stages (Koronakis *et al.*, 1991). The early stage of secretion requires the total protonmotive force (ΔP), while a late stage, characterised by strong dependence upon pH and temperature, does not. The early ΔP requirement can be met by either of the ΔP components, the membrane potential or the pH gradient as is the case for translocation of β-lactamase across the inner membrane (Bakker and Randall, 1984). This ΔP requirement is thought to be essential for at least one or more of the early steps at the inner membrane, such as substrate binding, its subsequent release, or perhaps the association of substrate with the HlyB/HlyD complex which then inserts into the inner membrane in a ΔP dependent manner.

(iv) Late stages of secretion - beyond the inner membrane

The absence of detectable amounts of HlyA, HlyA C-terminal peptides, or HlyA carrying defective secretion signals within the periplasm (Mackman *et al.*, 1987; Koronakis *et al.*, 1989; Koronakis *et al.*, 1991) provides strong evidence for a model in which HlyA is secreted directly into the medium with no periplasmic intermediate. Evidence suggests that junctions between the two membranes of *E. coli* exist in the form of adhesion sites or Bayer patches. This includes data from electron microscopy studies (Bayer, 1979), the identification of intermediate density envelope fractions in equilibrium density centrifugation studies (Bayer *et al.*, 1982) and the enrichment of particular polypeptides in such intermediate density fractions (Bayer *et al.*, 1987; Bourdineaud *et al.*, 1989) although it has yet to be widely acknowledged (Kellenberger, 1990). Such sites of adhesion may form at the sites of "proteinaceous bridges" and facilitate transfer of substrates from the cytoplasm to the external medium, so bypassing the periplasm. This model is consistent with protein import into the mitochondrial matrix, in which junctions between the two membranes during translocation have been identified by electron microscopy (Schleyer and Neupert, 1985).

Cell fractionation has shown that, in the absence of HlyB, HlyA still associates with the inner membrane, whereas in the presence of HlyB and the N-terminal two-thirds of HlyD, HlyA is associated with both membranes and partially exposed on the cell surface (Oropeza-Wekerle *et al.*, 1990). It was suggested from this that the truncated HlyD may be sufficient to stabilise a proteinaceous bridge spanning the two membranes but can not complete secretion. Studies of the

HlyB/HlyD/TolC homologues PrtD/E/F, which are involved in protease secretion in *Erwinia chrysanthemi*, suggest that the three proteins are unlikely to form a stable pore or adhesion site and a transient contact between inner and outer membranes is now thought to be more probable (Delepelaire and Wandersman, 1991).

A secretion intermediate in the late (ΔP-independent) stage of export has been shown to be inaccessible to protease in both whole cells or spheroplasts (Koronakis *et al.*, 1991) indicating that it is still situated within the inner membrane, or perhaps in a HlyB/D-induced or stabilised membrane contact site. Further translocation to the extracellular medium may be driven directly by HlyB ATPase activity, however from the persistence of late-stage secretion in the presence of high levels of ΔP-uncouplers it appears that this late transfer is energetically favourable, requiring no additional energy.

(v) Membrane proteins involved in secretion of HlyA

(a) HlyB

The integral inner membrane protein HlyB (66kDa) has a central role in the secretion of HlyA and belongs to the ABC (ATP-binding cassette) transporter family of proteins (Felmlee *et al.*, 1985a; Koronakis *et al.*, 1989; Blight and Holland, 1990; Koronakis *et al.*, 1991; Koronakis and Hughes, 1993) which are involved in secretion in both prokaryotes (for review see Fath and Kolter, 1993) and eukaryotes (for review see Higgins, 1992). They are characterised by an N-terminal membrane-binding domain and a large, highly conserved C-terminal domain containing a predicted ATP-binding site. They do not carry recognisable N-terminal signal sequences and their mechanism of membrane assembly is unresolved.

The topology of the N-terminal membrane-binding domain of HlyB has been tentatively assigned by generating fusion proteins of HlyB and the reporter protein β-lactamase (Wang *et al.*, 1991), and also analysis of HlyB-LacZ and HlyB-PhoA hybrids (Gentschev and Goebel, 1992). Both predicted eight membrane-spanning segments, although these were not identical and do not correspond entirely to the hydropathy predictions. The C-terminal domain has been shown to be hydrophilic and is located in the cytoplasm (Koronakis *et al.*, 1988a; Wang *et al.*, 1991; Gentschev and Goebel, 1992). This cytoplasmic domain bears closest homology to the ABC superfamily in a region of approximately 200 amino acids which contains characteristic features including: a glycine rich domain thought to be responsible for

nucleotide binding, another similar short glycine rich repeat domain and an aspartate residue which is thought to be essential for Mg^{2+} co-ordination (Mimura, *et al.*, 1991). In keeping with the requirements of most ABC transporters for two transmembrane domains and two ATP-binding domains, it is thought that the HlyB exporter may be a homodimer.

Other ABC transporters have been shown to bind ATP and it is thought that this, coupled with the ability to hydrolyse ATP is central to the activity of these transporters as membrane pumps. The purified sugar importer protein MalK has recently been shown to hydrolyse ATP *in vitro* (Walter *et al.*, 1992) and it has also been demonstrated that ATP hydrolysis occurs concomitantly with *in vivo* sugar uptake (Mimmack *et al.*, 1989). HlyB-directed ATP hydrolysis is believed to play a central role in the export of HlyA and amino acid substitutions in the ATP-binding region of HlyB decrease secretion (Koronakis *et al.*, 1988a). Recently Koronakis *et al.*, (1993) have shown that the C-terminal domain of HlyB does have ATPase activity although how this is coupled to HlyA transport is unknown.

(b) HlyD

The integral inner membrane protein HlyD (53kDa) has been shown, both by protease sensitivity and β-lactamase fusion topology mapping, to consist of a simple transmembrane domain and a large protease-sensitive periplasmic domain (Wang *et al.*, 1991; Gentschev and Goebel, 1992). HlyD is believed to play an important role in the formation and activation of the proteinaceous bridge which may straddle the inner and outer membranes, so bypassing the periplasm. This is supported by the presence of an additional highly conserved membrane protein in the case of the HlyB homologues, LktD and CyaD (Strathdee and Lo, 1989; Glaser *et al.*, 1988) which are involved in leukotoxin and adenylate cyclase secretion respectively. It is also consistent with recent data on ExbB, which shows homology to HlyD (M. Blight, personal communication), and has been shown to act, in combination with the transperiplasmic TonB protein, in transducing energy from the cytoplasmic membrane, to open channels in the outer membrane (Skare *et al.*, 1993).

(c) TolC

The minor outer membrane protein TolC (52kDa) has been shown to be essential for HlyA secretion (Wandersman and Delepelaire, 1990) and could perform an important late step in release of HlyA to the medium and/or its passage across the outer membrane. Consistent with this, TolC is also essential for the secretion of proteases in *Serratia marcescens* (Letoffe *et al.*, 1993).

1.22 Aims

HlyA secretion occurs post-translationally using a trans-envelope membrane translocator complex comprised of HlyB, HlyD and TolC. By analogy with requirements for export across the inner membrane, it was reasoned that secretion may require the nascent HlyA to remain in a "loosely folded" conformation before and during interaction with the secretion apparatus, and this may require the involvement of molecular chaperones.

It was intended to determine whether the chaperones SecB and GroEL/GroES, which have been previously identified as chaperones involved in protein translocation across the inner membrane, were involved in the secretion of HlyA.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Chemicals and materials were obtained as AnalaR® or equivalent grade from BDH. Other suppliers are indicated in the text. Chemicals and materials were stored and handled as recommended by the manufacturers, and to comply with COSHH regulations.

(i) Sterilisation of solutions, media and materials

Bacterial growth media and solutions were sterilised by autoclaving at 15 lbs/in^2 for 20 minutes. Antibiotic and vitamin solutions were sterilised by filtration. All glassware, micropipette tips and polypropylene microfuge tubes used in bacterial growth or DNA manipulation methods were autoclaved. Small volumes of double-distilled (d.d.) water were sterilised by autoclaving. All solutions used in DNA manipulation methods (section 2.3) were sterilised by autoclaving or filtration.

(ii) Nutrient medium

Nutrient medium contained 15g/l nutrient broth (Oxoid) in d.d. H_2O.

(iii) Nutrient agar

Bacto-agar (Difco) was added to nutrient medium to a final concentration of 1.5% (w/v). The medium was autoclaved, cooled to 55°C and poured into sterile Petri dishes.

(iv) Luria-Bertani (LB) medium

LB contained: 1% (w/v) NaCl; 1% (w/v) Bacto-tryptone (Difco); and 0.5% (w/v) Bacto-yeast extract (Difco). These components were dissolved in d.d. H_2O, the pH adjusted to 7.5 with 5M NaOH, and the solution made up to volume before autoclaving.

(v) M9 minimal medium

The defined M9 minimal salts contained: 6g/l Na_2HPO_4; 3g/l KH_2PO_4; 0.5g/l NaCl; 1g/l NH_4Cl. These components were dissolved in d.d. H_2O, and aliquots of 190ml were sterilised by autoclaving. Upon cooling the following previously

sterilised solutions were added to produce complete M9 minimal medium: 8ml of 20% (w/v) glucose or 8ml of 20% (w/v) glycerol; 0.2ml of 1M $MgSO_4$; 0.2ml of 1M $CaCl_2$ and 100µl of 0.1% (w/v) thiamine. Methionine assay medium (Difco) was added routinely to a final concentration of 0.25% (w/v).

(vi) M9 minimal agar

Bacto agar (Difco) was added to the M9 minimal salt solution to a final concentration of 1.5% (w/v) prior to autoclaving. Upon cooling to 55°C the solutions were added as described in (v) and the agar poured into sterile Petri dishes.

(vii) M63 minimal medium

The defined M63 minimal salts contained: 13.6g/l KH_2PO_4; 2g/l $(NH_4)_2SO_4$; 0.5mg/l $FeSO_4.7H_2O$. These components were dissolved in d.d. H_2O, the pH adjusted to 7.0 with KOH and aliquots of 190ml were sterilised by autoclaving. The solutions added in (v) were added to produce complete M63 minimal medium.

(viii) Nutrient and M9 minimal blood agar

As in (iii) and (vi) with defibrinated sheeps blood (Oxoid) added to a final concentration of 5% (v/v) before pouring.

(ix) Minimal E medium

The defined Minimal E salts medium described by Vogel and Bonner (1956) was made up as a 50 x stock composed of 10g/l $MgSO_4.7H_2O$; 100g/l citric acid; 500g/l K_2HPO_4 and 175g/l $NH_4NaHPO_4.4H_2O$. These components were dissolved in d.d. H_2O, and aliquots of 20ml were sterilised by autoclaving as required. 0.2% glucose (w/v) was sterilised by autoclaving. Upon cooling, sterile 50 x Minimal E salts were added to this to obtain a 1 x final concentration. Sterile thiamine and casamino acids (Difco) were added to final concentrations of 1µg/ml and 0.4mg/ml respectively.

(xi) Antibiotics

Stock solutions of the following antibiotics (Sigma) were made up and stored at 4°C for up to 2 weeks: Ampicillin (10mg/ml); Chloramphenicol (10mg/ml in 50% ethanol); Kanamycin (20mg/ml) and Tetracycline (10mg/ml in 50% ethanol). All antibiotics were used at a final concentration of 25µg/ml except Tetracycline which was used at 10µg/ml. Appropriate antibiotics were added to all media to ensure selective growth and maintenance of plasmids.

2.2 Methods

(i) Growth curves

(a) Overnight cultures were grown up in a shaking water bath in relevant media at the appropriate temperature. These were subcultured into fresh, prewarmed media to give an A_{600} of approximately 0.05. Growth was then monitored by reading the A_{600} value of 0.5ml samples of culture at 15 minute intervals using a CECIL CE303 spectrophotometer.

(b) As in (a) except following subculturing of the overnight culture and growth to an A_{600} of approximately 0.2, half the culture was transferred to a new flask and Isopropyl β-D-thiogalactoside (IPTG-Sigma) was added to a final concentration of 0.4mM. Growth was then monitored as in (a).

(c) As in (a) except following subculturing of the overnight culture and growth to an A_{600} of approximately 0.2, half the culture was transferred to a new flask which was incubated at 42°C. Growth was then monitored as in (a).

(ii) Pulse-chase immunoprecipitation

Cultures were propagated in M9 minimal medium except the *groE* mutants (and isogenic parents) which grew poorly and were cultured in nutrient medium, washed in minimal medium and resuspended in the same for radiolabelling. At the required A_{600} (usually 0.5) cultures were pulsed by the addition of ^{35}S-methionine (Amersham - 1000 Ci/mmole) to 30 µCi/ml. A sample of 0.5ml was taken after 30 seconds and prewarmed L-methionine added to a final concentration of 6.1mM to the remaining culture to initiate the chase. Further 0.5ml samples were taken at 15, 30, 60, and 120 seconds into the chase period. All samples were transferred to microfuge tubes on ice containing 0.15ml of 50% (w/v) trichloroacetic acid (TCA). TCA precipitates were pelleted by spinning for 2 minutes at 11000g and then washed in 0.5ml of 5% TCA (w/v). Pellets were resuspended in 50µl of 0.5% (w/v) SDS; 10mM Tris-acetate (pH 7.8) and incubated at 55°C for 10 minutes to solubilise proteins. A volume of 1ml of 1% (v/v) Triton X100; 10mM Tris-acetate (pH 7.8) was then added and the tubes spun for 5 minutes to pellet insoluble debris. 1-3µl of specific antisera was added to each supernatant and mixed in. Tubes were held on ice for at least 2 hours to permit antibody binding before addition of 100µl of *S. aureus* cell suspension prepared according to suppliers instructions (Sigma). Antibody adsorbtion to the cells was promoted by incubation at 37°C for 60 minutes, except in the case of β-lactamase immunoprecipitation reactions where incubation was carried out at 25°C. The cells were then pelleted by spinning the tubes for 20 seconds, and the pellet washed in 1ml of 0.15M NaCl; 0.1% (w/v) SDS; 5mM EDTA; 10mM Tris-

acetate (pH 7.6). The samples were finally washed in 1ml of 10mM Tris-acetate (pH 7.6). The pellet was resuspended in 30μl distilled H_2O and 30μl of 2 x Laemmli buffer (Laemmli, 1970) [0.125M Tris-HCl (pH 6.8); 4% (w/v) SDS; 20% (v/v) glycerol; 10% (v/v) β-mercaptoethanol; 0.002% (w/v) bromophenol blue] and the sample boiled for 5 minutes. The *S. aureus* cells were pelleted with a 20 second spin and the supernatant analysed by SDS-PAGE.

(iii) SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The procedure was based on that of Laemmli (1970) and was carried out using the Bio-rad PROTEAN® II xi slab cell kit or Bio-rad MINI-PROTEAN® II slab cell kit following the manufacturers instructions. Gels were of 0.75mm thickness and contained varying acrylamide concentrations depending on the samples to be analysed. Stock acrylamide (Severn Biotech Ltd.) containing 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide was used to make up the SDS-polyacrylamide gel mixtures with the final % acrylamide indicated:

For electrophoresis of	Stacking gel	Separating gel
HlyA	4%	8%
GroEL	7%	10%
GroES	7%	14%
Others	7%	12.5%
MBP	7%	12.5%*

* - the separating buffer used had a pH of 8.2. At this pH, the wild-type precursor, wild-type mature MBP and MBPΔ2-26,Y283D were clearly resolved (Liu *et al.*, 1989).

(iv) Fluorography

The procedure was based on that of Bonner and Laskey (1974). Gels were soaked in fixer [10% (v/v) acetic acid and 25% (v/v) isopropanol] for a minimum of 10 minutes. The fixer was discarded and the gel soaked in dimethyl sulphoxide (DMSO) for a minimum of 20 minutes. The DMSO was discarded and the gel was soaked in fresh DMSO for a minimum of 20 minutes. The DMSO was discarded and the gel was soaked in 22% (w/v) 2,5-Diphenyloxazole (PPO) in DMSO for at least 60 minutes. The PPO solution was placed back in its bottle for future use. The gel was washed in H_2O for at least 60 minutes and then dried. The gel was transferred onto a sheet of cling film, blotted dry and then transferred to a piece of Whatmann No.17

chromatography paper. The gel, on its paper backing was placed face up on the vacuum gel dryer (Bio-rad model 583) and covered with a fresh sheet of cling film. The gel was then dried for between 75 to 180 minutes at 60°C. The cling film was discarded and the gel placed against X-ray film (FUJI) in a cassette for autoradiography. The cassette was stored at -70°C for the required exposure time after which it was allowed to equillibrate at room temperature before the film was developed using an AGFA CURIX 60 machine. Repeated exposures were carried out until satisfactory results were obtained.

(v) Staining gels

Gels were stained by soaking in fixer containing 0.05% (w/v) Coomassie brilliant blue (Sigma) for at least 4 hours and then in a number of changes of destain [10% (v/v) acetic acid and 10% (v/v) isopropanol] to decrease background.

(vi) Western blotting

The procedure was based on that of Towbin *et al.*, (1979) and was carried out using the Bio-rad TRANS-BLOT® or Bio-rad MINI TRANS-BLOT® onto nitrocellulose (Schleicher and Schnell) or Polyscreen PVDF transfer membrane (Du Pont) following the manufacturers instructions. Goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-rad) was used routinely (1 in 3000 dilution) as the second antibody with 5-bromo-4-chloro-3-indolyl phosphate (BCIP-Sigma) and nitro blue tetrazolium (NBT-Sigma) colour development solutions. BCIP and NBT solutions were made up fresh prior to use. To 20ml carbonate buffer [0.1M $NaHCO_3$; 1.0mM $MgCl_2$; pH to 9.8 with 5M NaOH] 200µl of 30mg/ml NBT [in 70% (v/v) dimethylformamide] and 200µl of 15mg/ml BCIP [in 100% dimethylformamide] were added and poured onto the membrane. Incubation was carried out for up to 30 minutes for colour development. To stop colour development when the required sensitivity had been reached, the membrane was rinsed well in a number of changes of d.d. H_2O.

(vii) Densitometry

Quantification of bands on autoradiographs, stained gels and Western blots was carried out using a Shimadzu CS-9000 densitometer.

(viii) Analysis of haemolysin secretion

Nutrient medium was supplemented with $CaCl_2$ to a final concentration of 10mM to stabilise secreted haemolysin (Nicaud *et al.*, 1985(b)). When required, usually at an A_{600} of approximately 0.7, growth of cultures and secretion of haemolysin (HlyA) was terminated by plunging cultures into ice and adding "stop

cocktail" [5mM NaN_3; 100μg/ml spectinomycin] (1 in 25 dilution). To ensure that cell equivalents were analysed, the A_{600} values of the cell suspensions were measured.

The cell suspensions were transferred to centrifuge tubes and the cells harvested by spinning at 12000g for 10 minutes at 4°C. The supernatants ($^4/_5$ths) were transferred to fresh tubes containing 10μl of rifampicin (10mg/ml) to act as a marker to colour the fine precipitate. 50% TCA was added to a final concentration of 10% and the supernatants were incubated at 4°C overnight. The supernatant TCA precipitates were pelleted by spinning at 12000g for 10 minutes at 4°C. The pellets were then resuspended in 30μl of resuspension buffer [13ml bacterial buffer {0.3% (w/v) KH_2PO_4; 0.7% (w/v) NaH_2PO_4; 0.4% (w/v) NaCl; 0.01% (w/v) $MgSO_4.7H_2O$} (Stoker *et al.*, 1984); 5ml saturated Tris and 2ml 6mg/ml phenylmethyl-sulphonyl fluoride (PMSF-Sigma) in DMSO] and 30μl of 2 x Laemmli sample buffer. Samples were boiled for 5 minutes and analysed by SDS-PAGE and Coomassie blue staining or Western blotting.

From the cell pellet the remaining supernatant was carefully decanted off and the cells were washed in 1ml of bacterial buffer. The cells were re-pelleted by spinning at 12000g for 10 minutes and then resuspended in equal volumes of resuspension buffer and 2 x Laemmli sample buffer. Samples were boiled for 5 minutes and analysed by SDS-PAGE and Coomassie blue staining or Western blotting.

(ix) Short term secretion of haemolysin

During each experiment, all apparatus (centrifuge tubes, rotors) and media were prewarmed and maintained at the required temperature to avoid temperature shock. Following growth of cultures to an A_{600} of approximately 0.7, 25ml of each culture was transferred to centrifuge tubes and pelleted by spinning at 12000g for 10 minutes. The cells were washed once in the appropriate medium and then resuspended in 25ml of the same medium and transferred to a flask. The flask was incubated with shaking at the required temperature to permit secretion of HlyA. Following incubation for the required time secretion was terminated and the samples analysed as described in (viii).

(x) Stability of secreted haemolysin

Following growth of cultures to an A_{600} of approximately 0.7 "stop cocktail" (1 in 25 dilution) was added to prevent further secretion of HlyA. Incubation of the cultures was continued at the relevant temperature and samples were removed and placed on ice after 0, 10, 20 and 30 minutes. Samples were treated as described in

(viii) and the levels of HlyA present in the culture supernatants were determined by densitometry of samples analysed by Western blotting using HlyA antiserum.

(xi) Induction of cultures using IPTG or maltose

Following growth of cultures to the required A_{600}, IPTG (40mM stock) or 20% (w/v) maltose was added to a final concentration of 0.4mM or 0.2% respectively and incubation continued.

(xii) *In vitro* transcription-translation reactions

In vitro transcription-translation reactions were carried out using S30 extracts prepared from strain MC4100 containing plasmid pAR1219 (which encodes T7 RNA polymerase) using the method described by Nevin and Pratt (1991). The components (details of which are given in Pratt, 1984) of a typical reaction are listed below:

Low molecular weight mix	7.5µl
^{35}S-Methionine	2µl
S30 cell extract	5µl
MgAc (0.1M)	3.5µl
Rifampicin (10mg/ml DMSO)	2µl
DNA (2-2.5µg)	Yµl
TE	Xµl to give a final volume of 30µl

After mixing and incubation at 37°C for 45 minutes 15µl d.d. H_2O and 45µl of 2 x Laemmli sample buffer were added and the samples boiled for 5 minutes. Samples were analysed by SDS-PAGE and fluorography.

2.3 DNA manipulation methods

(i) Rapid $CaCl_2$ transformation procedure

Competent cells were produced by treatment of early logarithmic phase *E. coli* with cold $CaCl_2$ solution using a modification of the method of Cohen *et al.*, (1972). Cultures were propagated at the relevant temperature in appropriate media. At an A_{550} of 0.4 to 0.5 cultures were transferred to 15ml polypropylene falcon tubes and stood on ice for 20 minutes. Cells were then pelleted by spinning at 12000g for 5

minutes at 4°C. The cell pellets were washed in half the volume of ice-cold 0.1M $CaCl_2$ and then resuspended in 1/20th the volume of ice-cold 0.1M $CaCl_2$. The cells were then kept on ice for at least 2 hours to attain competence after which 100μl of competent cells were transferred to a fresh falcon tube containing 10-20ng of the DNA, usually in 5μl of TE [10mM Tris-HCl; 1mM EDTA; pH 7.5], and mixed gently by pipetting. The cells were held on ice for a further 40 minutes and then heat shocked by incubation at 42°C for 2 minutes to induce DNA uptake into the cells. The cells were left on ice for 2 minutes to recover and then 0.9ml SOC [made up as SOB containing: 2% (w/v) bacto-tryptone; 0.5% (w/v) bacto-yeast extract; 10mM NaCl and 2.5mM KCl which was autoclaved and then 1M $MgCl_2$ and 1M $MgSO_4$ were added to a final concentration of 10mM for each. Just prior to use glucose was added to a final concentration of 20mM (Hanahan, 1983)] was added and the cells incubated at the relevant temperature (usually 37°C, except for the *groE* temperature-sensitive strains which were incubated at 30°C for 90 minutes) for 60 minutes. The cells were then plated onto agar plates containing appropriate antibiotics for selective growth. As a control 100μl or competent cells were treated with 5μl of TE and processed as above.

(ii) Preparation and transformation of frozen competent cells

The method for preparing frozen highly competent *E. coli* cells and subsequent transformation was carried out as described by Inoue *et al.*, (1990). A 250ml volume of SOB in a 2 litre conical flask was inoculated with a streak of growth from a fresh overnight plate. The flask was incubated at room temperature (25°C) with vigorous shaking (200-250 rpm) until the A_{600} of the culture was approximately 0.6. The flask was placed on ice for 10 minutes and then the culture transferred to a centrifuge tube. The cells were then pelleted by spinning at 2500g for 10 minutes at 4°C. The cell pellet was resuspended in 80ml of ice-cold transformation buffer (TB) [which was made up as 10 x TB and diluted before use: 100mM HEPES; 550mM $MnCl_2$; 150mM $CaCl_2$ and 2.5M KCl; all components except $MnCl_2$ were dissolved in d.d. H_2O and the pH adjusted to 6.7 with KOH. The $MnCl_2$ was dissolved and the solution made up to volume. The solution was filter sterilised and stored at 4°C] and held in an ice bath for 10 minutes. The cells were then re-pelleted by spinning at 2500g for 10 minutes at 4°C. The cell pellet was then gently resuspended in 20ml of TB by pipetting. DMSO was added to 7% (1.51ml) and mixed in by gentle swirling. After incubating on ice for 10 minutes, the suspension was dispensed into 0.5ml aliquots in microfuge tubes (in the cold room). The tubes of cell suspension were rapidly chilled by dipping into liquid nitrogen and then stored at -70°C. Following thawing of competent cells at room temperature transformation was carried out as in (ii) except heat shock was carried out for only 30 seconds.

(iii) Synthesis of oligonucleotides

Oligonucleotides were synthesised using an APPLIED BIOSYSTEMS 381A oligonucleotide synthesiser on appropriate columns.

(iv) Elution of an oligonucleotide from its column after synthesis

To each column to be eluted a 1ml sterile plastic syringe was attached. In another 1ml syringe 0.8ml of .880 ammonia was taken up and this was attached to the other end of the column. Ensuring they were tight and whilst holding both syringes the column was filled with .880 ammonia by pushing the syringe containing ammonia and pulling the empty syringe, so that a volume of approximately 0.2ml was pushed through and drawn up into the empty syringe. The column filled with .880 ammonia was left for 20 minutes at an angle on the bench to aid the elution process. After 20 minutes the ammonia bathing the column was changed by pushing another 0.2ml through the column as before. The column was left for 20 minutes and the process repeated. Once the whole 0.8ml of .880 ammonia had been pushed through the column the solution was gently passed through the column to dislodge any remaining oligonucleotide. The .880 ammonia eluate, containing the released oligonucleotide, was transferred into a small glass vial. The sample was incubated at 55°C overnight taking care that the vial was tightly closed to prevent evaporation. The sample was allowed to cool before opening the vial. For each oligonucleotide, 4 microfuge tubes were labelled, and the oligonucleotide in ammonia was dispensed into the tubes as four aliquots of approximately 200μl. To each tube 20μl of 3M sodium acetate (pH 5) was then added and mixed in. 660μl of ethanol at -20°C was added and mixed by vortexing. The tubes were kept at -20°C for at least 30 minutes to precipitate the oligonucleotide. The tubes were spun in the microfuge for 20 minutes to pellet the precipitated oligonucleotide and the pellets were washed twice with 500μl of 80% (v/v) ethanol. The pellets were dried for 10 minutes in the vacuum dessicator and finally resuspended in 50μl of TE ready for use, or stored at -20°C as dry pellets.

The quantity and purity of the oligonucleotide was judged by reading the A_{260} and A_{280} of a 1 in 500 dilution of each oligonucleotide using a quartz cuvette. The oligonucleotide is acceptably pure if the ratio of A_{260}:A_{280} is greater than 1.7. An A_{260} of 1 means that there is an oligonucleotide concentration of approximately 33μg/ml.

(v) Agarose gel electrophoresis of DNA fragments

DNA prepared in this study was examined by submerged horizontal agarose gel electrophoresis. 0.8% (w/v) gels were cast using agarose (Appligene) in 1 x TAE buffer [40mM Tris-acetate; 2mM EDTA; pH 8.0]. Samples to be analysed were mixed with 0.2 volumes of 6 x sample buffer [15% Ficoll 400; 0.25% bromophenol

blue; 0.25% xylene cyanol FF] before loading and electrophoresis. Marker ladder (1kilobase DNA ladder, Gibco-BRL) was always loaded to allow size estimation and quantification of the DNA fragments on the gel. Following electrophoresis the gel was stained in aqueous ethidium bromide (1μg/ml) for 20 minutes, washed in water and viewed on a medium range UV transilluminator (A-VIOLET Products Ltd.).

(vi) Purification of DNA using the Geneclean II® kit (BIO 101 Inc.)

For the rapid purification of small DNA molecules, from an agarose gel slice, enzyme reaction mixture, or during a plasmid mini-preparation procedure, the Geneclean II® kit was used. The principle of Geneclean II® was described by Vogelstein and Gillespie (1979). In the presence of > 3M sodium iodide, DNA, but neither protein nor RNA, bind to a silica matrix (glassmilk). These contaminants can therefore be removed from a sample by washing the matrix before eluting the DNA into a low-salt buffer or water. The method for purification was carried out as described by the manufacturers.

(vii) Preparation of plasmid DNA by alkaline lysis

Plasmid DNA was required for use in restriction endonuclease analysis, for subcloning, as target sequence for PCR and *in vitro* transcription translation reactions and for transformation of competent *E. coli*. An alkaline lysis method based on that of Birnboim & Doly (1979) was used to prepare plasmid in sufficient quantity for all the above requirements. This procedure was combined with the use of NACS cartridges (Gibco-BRL) to purify prepared plasmid DNA.

A volume of 50ml of nutrient medium, containing the required antibiotic to select for the plasmid, was inoculated with the *E. coli* strain carrying the desired plasmid and incubated overnight at 37°C with shaking to produce a stationary phase culture. The culture was transferred to plastic Universal tubes and the cells pelleted by a 10 minute spin at 1500g in a bench centrifuge. Each cell pellet was resuspended in 0.5ml of ice-cold TEG buffer [25mM Tris-HCl; 10mM EDTA; 50mM glucose; pH 8.0] and transferred to separate microfuge tubes. The EDTA destabilised the bacterial outer membranes, while the glucose made the solution hypertonic to prevent premature cell lysis. The cells were immediately re-pelleted by a 30 second spin at 10000g in a microfuge. The pellets were then resuspended in 0.5ml of TEG buffer + 4mg/ml lysozyme (Sigma) and held on ice for 10 minutes to allow the lysozyme to enter the periplasm of the cells to degrade the peptidoglycan cell wall. To each tube, 0.5ml of freshly prepared NaOH/SDS solution [0.2M NaOH; 1% SDS] was added and mixed in by inversion. The tubes were held on ice for 5 minutes. The alkaline detergent solution caused immediate cell lysis and solubilisation of cellular components. The NaOH raised the pH to over 12.0 to denature chromosomal DNA

and high molecular weight RNA without affecting the supercoiled plasmid. A volume of 375µl of 3M sodium acetate (pH 5.0) was then added to the denatured cells. The tubes were inverted to mix, and held on ice for a further 10 minutes. The acetate neutralised the mixture to cause the denatured chromosomal DNA, high molecular weight RNA and protein-SDS complexes to aggregate and precipitate. The tubes were then spun for 15 minutes at 10000g to pellet insoluble cell debris. The supernatant containing the crude plasmid was dispensed into 0.5ml aliquots in fresh tubes and the plasmid precipitated with two volumes of ethanol at -20°C. The crude plasmid pellets were then resuspended in 300µl lots of TE buffer. Thus 12 pellets were resuspended in 4 lots of 300µl. To each 300µl of crude plasmid solution, 0.5 volumes of ice-cold 7.5M ammonium acetate were added. The tubes were then held on ice for 20 minutes to precipitate contaminating proteins and high molecular weight RNA. The tubes were spun for 10 minutes at 10000g to pellet the precipitated proteins. The supernatants were transferred to fresh microfuge tubes and two volumes of cold ethanol added to precipitate the plasmid. The plasmid was pelleted as before and the pellets dried in a vacuum dessicator for 10 minutes then resuspended in 0.5ml of 50mM NaCl in TE buffer for NACS.

(viii) Purification of prepared DNA by NACS

NACS is the product name for an ion-exchange resin sold by Gibco-BRL for nucleic acid purification. The principal of the system is that DNA binds to the resin in low salt conditions and is eluted by high salt. The protocol for the use of NACS cartridges was adapted from the instruction manual.

Contaminating RNA in the plasmid solution prepared by alkaline lysis was removed by adding 1µl of RNase T1 (100 Units) to the sample and incubating at 55°C for 15 minutes. During the RNase treatment, the NACS cartridge was prepared for use. Using a 10ml disposable plastic syringe, 2ml of 2M NaCl in TE was pushed through the cartridge to hydrate the resin. This was followed by 10ml of 0.2M NaCl in TE to equilibrate it. A sterile hypodermic needle was added to the other end of the prepared cartridge which was then clamped in a retort stand. After RNase treatment, 0.5ml of 375mM NaCl in TE buffer was added to the plasmid solution to adjust the salt concentration to 0.2M. The sample was then allowed to flow though the NACS cartridge by gravity. The eluate was re-passed across the column to ensure that the maximum amount of plasmid had bound. The NACS resin with bound DNA was then washed by gently pushing 5ml of 0.2M NaCl in TE through the cartridge using the syringe plunger. The plasmid DNA was then eluted from the NACS resin in two 400µl lots of 0.7M NaCl in TE. These eluates were allowed to flow through the cartridge by gravity and collected in separate microfuge tubes. The purified plasmid

DNA was precipitated with two volumes of cold ethanol and pelleted. The pellets were dried, dissolved in 50µl of TE buffer and stored at -20°C.

(ix) Rapid preparation of plasmid DNA

The STET plasmid mini-preparation method adapted from that of Holmes and Quigley (1981) was used to screen *E. coli* transformants for recombinants. Up to 50 transformants could be screened in a day using this method.

Transformant colonies were picked and streaked onto fresh selective agar. Following overnight incubation a toothpick streak of new growth was resuspended in 100 µl of STET buffer [8% (w/v) sucrose; 5% (v/v) Triton X100; 50mM EDTA 50mM Tris-HCl; pH 8.0] in a microfuge tube. The Triton X100 and EDTA destabilised the bacterial outer membrane while the sucrose made the solution hypertonic so that the cells were not prematurely lysed. 10 µl of a fresh 10mg/ml lysozyme solution was then added to each tube of cell suspension. The tubes were incubated at room temperature for 5 minutes, which allowed entry of the enzyme into the bacterial periplasm to degrade the peptidoglycan of the cell wall. To rupture the weakened cells and denature the proteins, chromosomal DNA and high molecular weight RNA, the tubes were placed in a boiling water bath for 1 minute. The lysed cell debris was pelleted by spinning the tubes for 15 minutes at 10000g. The supernatants containing released plasmid were transferred to fresh tubes. The crude plasmid DNA was then precipitated with an equal volume of isopropanol and pelleted. The pellets were washed with 80% (v/v) ethanol, dried for 10 minutes in the vacuum dessicator and resuspended in 15µl TE. The plasmid samples were examined directly by running 5µl on a 0.8% agarose gel or were digested with restriction endonucleases. To remove contaminating RNA, 1µl of RNase T1 (100 Units) was incorporated in the endonuclease master mix. When the number of transformants to be screened was small, the isopropanol precipitation was replaced by the Geneclean II® method which was quicker and gave a cleaner plasmid preparation.

(x) Rapid genomic DNA preparation

Genomic DNA was prepared by the method of Davis *et al.*, (1980). A toothpick streak of growth from a fresh overnight plate was resuspended in 0.5ml of genomic DNA analysis buffer [50mM Tris (pH 8.5); 50mM Na_2EDTA; 15% (w/v) sucrose and 1mg/ml lysozyme (added just prior to use)] into a microfuge tube. The tubes were left at room temperature for 10 minutes and 1µl diethyl oxydiformate was added. 10µl 10% (w/v) SDS was then added and the tubes inverted to mix. After incubation at 70°C for 5 minutes 50µl 5M KAc was added. The tubes were left on ice for at least 30 minutes and the precipitate sedimented by spinning in a microfuge at 10000g for 15 minutes. The supernatant was decanted into new tubes and 1ml

ethanol was added at room temperature. After spinning in a microfuge for 5 minutes the supernatant was discarded and the tubes inverted on a paper towel to drain. Care was taken to drain off all liquid (using a vacuum desiccator if necessary) but to not overdry. Precipitated DNA was dissolved in 50µl TE containing RNase T1 (10 Units).

(xi) Restriction endonuclease analysis

Restriction endonucleases were purchased from Gibco-BRL, Boehringer Mannheim, Pharmacia and Promega. A typical endonuclease digestion mixture included:

DNA solution	Wµl
10 x Reaction buffer	0.1 Vol.
Restriction endonuclease	Y Units
H_2O	Xµl to give the required total volume

The composition of the 10 x Reaction buffer depended on the particular restriction endonucleases to be used. Usually a buffer was supplied with each enzyme. The number of units of enzyme used was dependent on the mass of DNA to be digested. Usually 10 Units of enzyme were used to digest any quantity of $<$ 1µg. The digest mixtures were incubated for between 2 and 3 hours at the optimal temperature for each endonuclease (usually 37°C).

(xii) Amplification of specific DNA sequences by PCR

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985) was used to amplify the wild-type *malE* gene and plasmid borne *malE A14Q* gene. PCR is a method for amplifying specific DNA sequences bounded by two custom synthesised oligonucleotide primers, using a thermostable DNA polymerase in a temperature cycling procedure. As well as the primers of opposite senses, other components of a PCR reaction mixture included double stranded target DNA carrying the sequence to be amplified, all four dNTPs, the thermostable *Taq* DNA polymerase to catalyse DNA polymerisation, and a reaction buffer to give optimal conditions for polymerase activity.

The PCR temperature cycle had three consecutive incubation steps at high, low and intermediate temperatures. The first step at 94°C denatured the double stranded DNA template. The second at 50°C allowed the oligonucleotide primers to

anneal to complementary sequence on the template. And the third step at 72°C was the optimum for *Taq* polymerase activity to extend the primers along both template strands. This sequence of temperature changes was repeated to amplify the sequence bounded by the primers. After the first cycle, newly synthesised double stranded fragments acted as template for polymerisation. This meant that the amplification was exponential, so µg of fragment were produced from ng quantities of template.

The reaction mixtures for PCR were prepared in sterile microfuge tubes. The mixture composition used throughout this study was:

Plasmid template DNA (10ng)	Yµl
Primer 1 (2µM)	10µl
Primer 2 (2µM)	10µl
dNTPs (1mM)	5µl
10 x *Taq* DNA Pol. buffer	10µl
Taq DNA polymerase (1.25 Units)	0.25µl
H_2O	Xµl to give a total volume of 100µl per reaction

The composition of the 10 x *Taq* DNA polymerase buffer was 500mM KCl; 100mM Tris-HCl; 15mM $MgCl_2$; 0.1% (w/v) gelatin; pH 8.3.

After the mixtures had been assembled, a sterile mineral oil overlay (Sigma) was added to the tubes to prevent evaporation during the PCR. In this work the size of the fragment to be amplified was approximately 1200bp and the empirically determined reaction cycle parameters were: 60 seconds at 94°C, 30 seconds at 50°C and 90 seconds at 72°C. This cycle was performed using a HYBAID OMNIGENE thermal cycler, and was repeated 30 times. After the last extension step at 72°C, a further 10 minutes at this temperature were added to complete unfinished fragments. Once the reaction had been completed, the aqueous reaction mixture was taken from beneath the oil and transferred to a fresh tube.

(xiii) Treatment of PCR fragments for blunt-end cloning

In order to improve the efficiency of ligation and cloning of PCR products proteinase K digestion was performed to remove any bound *Taq* polymerase (Crowe *et al.*, 1991). A volume of the PCR mixture containing approximately 1µg of DNA was treated with proteinase K in the following reaction mixture:

PCR product (1μg DNA)	Yμl
Proteinase K (0.5mg/ml)	10μl
0.5% SDS in TE (w/v)	Xμl to give a total volume of 100μl per reaction

The mixture was incubated at 37°C for 30 minutes and then at 68°C for 10 minutes. The proteinase K treated DNA was then purified by genecleaning (vi). Following purification the DNA pellet was resuspended in 18μl distilled H_2O and the tube incubated at 70°C for 2 minutes to separate the ends of DNA duplex and allow access for the kinase to the 5' ends. The mixture was snap-cooled by placing the tube on ice and the following additions were made:

PNK/Klenow buffer + 10mM ATP	3μl
dNTPs (200μM)	3μl
DTT (50mM)	3μl
T4 polynucleotide kinase (10 Units)	1μl

The PNK/Klenow buffer was composed of 0.5M Tris-HCl (pH 7.6); 0.1M $MgCl_2$ and 6.05mg/ml ATP. The phosphorylation reaction was promoted by incubating the tube at 37°C for 30 minutes.

It was then necessary to treat the fragments with a DNA polymerase to remove 3' overhangs (usually a single A) on some fragments produced by terminal transferase activity of the *Taq* polymerase. After the kinase reaction 2μl (10 Units) of Klenow was added directly to the mixture and incubation continued at room temperature for 30 minutes. The reaction was stopped by adding 70μl TE and the PCR fragments were purified by genecleaning (vi). The purified fragment may then be used for ligation to the blunt-ended dephosphorylated vector.

(xiv) The removal of 5' phosphate groups from DNA

Calf intestinal phosphatase (CIP) was used to remove the 5' phosphates from linearised vector DNA molecules prior to cloning. This reduced vector recircularisation upon ligation, so that a greater proportion of transformant clones were recombinants.

Following linearisation of the vector DNA by digestion with the required restriction endonuclease(s), the pH of the reaction mixture was adjusted, if necessary, to 8.0. This was done by the addition of 10 x BRL REact 1 buffer to give double the volume of 1 x buffer, and the mixture made up to the required volume with sterile d.d. H_2O. CIP (1 Unit/μg DNA) was added and the mixture incubated. For vector molecules with recessed 3' hydroxyl termini or blunt-ended DNA, incubation was at 37°C for 30 minutes. For molecules with protruding single-stranded 3' termini, incubation was at 37°C for 30 minutes and then at 55°C for 30 minutes. The higher temperature step causes the ends of the DNA duplex to separate slightly, allowing access of the enzyme to the 5' phosphate.

(xv) Ligation with T4 DNA ligase

During the construction of recombinant DNA molecules, T4 DNA ligase was used to catalyse the joining of the ends of the vector and fragment DNAs. The ligation of DNA with cohesive termini and blunt-ended molecules was performed using reaction mixtures with the following composition:

Vector DNA (10-20ng)	Wμl
Fragment DNA (Yng)	Yμl
10 x T4 ligase buffer	1μl
DTT (10mM)	1μl
T4 Ligase (0.5-1 Units)	0.5-1μl
H_2O	Xμl to give a total volume of 10μl

The molar ratio of fragment to vector DNA was 3 to 5:1. 10 x T4 ligase buffer was composed of 700mM Tris-HCl; 70mM $MgCl_2$; 10mM ATP; pH 7.5. 0.5 Units of ligase were used for cohesive termini and 1 Unit for blunt-end ligations.

The ligation mixtures were incubated overnight at 14°C.

2.4 Bacterial strains

Strain	Genotype	
MC4100	*araD139* Δ(*lacIPOZYA*) *U169 rpsL relA1 thiA*	Casadaban, 1976
CK1953	*araD139* Δ(*lacIPOZYA*) *U169 rpsL relA1 thiA secB*::Tn5	Kumamoto and Beckwith, 1985
HB1012pGL1	*thi relA araD*139 *flbB pstF rpsL malE* Δ*312 lacI*^{q} / F'*lacI*^{q} Tn5 / pGL1	L. Randall personal comm.
HB1053pJW12	*thi relA araD*139 *flbB pstF rpsL lacI*^{q} / F'*lacI*^{q} Tn5 / pJW12	L. Randall personal comm.
DH5α	*sup44* Δ*lacU169* (φ*80 lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan, 1983
BL21(DE3)pRG1	*hsdS gal* (λcI^{ts}*857 ind1* $S_{am}7$ *nin5 lacUV5-T7gene1* / pRG1	Studier and Moffatt, 1986
TG1	Δ(*lac/proAB*) *supE thi hsd* Δ*5* / F' *traD proAB lacI*^{q} *lacZ* Δ*M15*	Gibson, 1984
SF103	Δ(*lac/proAB*) *supE thi hsd* Δ*5 zjd*::Tn10*groEL44* ^{ts}/ F' *traD proAB lacI*^{q} *acZ* Δ*M15*	P. Lund personal comm.
CG2245	*galE zjd*::Tn10 *groE*$^{+}$	Zeilstra-Ryalls, 1993
CG2244	*galE zjd*::Tn10 *groES619* ^{ts}*zje*::Kan^{r}	Zeilstra-Ryalls, 1993

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2.5 Plasmids

Plasmid	Antibiotic resistance	Relevant information	
pSF4000	Cm^r	11.7kbp *SalI* fragment carrying the haemolysin determinant from *E. coli* J96 in pACYC184	Welch *et al.*, 1983
pLG570	Amp^r	*Sau*3A fragment carrying the haemolysin determinant from *E. coli* LE2001 in pOU71	Mackman and Holland, 1984b
pDC2	Tet^r	Derived from pMB9 carrying the *secB* gene	Clark *et al.*, 1980
pCK11	Tet^r, Kan^r	Derived from pDC2 by transposon insertion in the *secB* gene (*secB*::Tn10)	Kumamoto and Beckwith, 1985
pACYC184	Cm^r, Tet^r	*E.coli* cloning vector	Chang and Cohen, 1978
pJPW11	Cm^r	1242bp blunt end fragment carrying the *malE* gene cloned into vector pACYC184	Section 5.3
pT7-5	Amp^r	Derived from pBR325 carrying the *T7* promoter	Tabor and Richardson, 1985
pJPW12	Amp^r	1229bp *SacI*-*Bam*HI fragment carrying the *malE* gene cloned into vector pT7-5	Section 5.4
pJPW15	Amp^r	Derived from pJPW12 carrying the gene for *malE A14Q,Y283D*	Section 5.5

pRG1	Cm^r	Derived from pACYC177 carrying the *lacI*^{q} gene	Griffin and Kolodner, 1990
pGL1	Amp^r	Derived from pBAR43 carrying the *lacUV5-malEΔ2-26,Y283D*	Liu *et al.*, 1989
pJW12	Amp^r	Carrying the *lacUV5-malE14-1*	Liu *et al.*, 1989
pOF39	Amp^r	Derived from pBR325 carrying the *groE* operon	Fayet *et al.*, 1986
pHO7	Amp^r	Derived from JF118EH carrying the *groEL* gene under *tac* promoter / *lacI*^{q} control (see Furste *et al.*, 1986)	P. Lund, personal communication
pHO8	Amp^r	Derived from pUC19 carrying the *groES* gene under its own and *lac* promoter control	P. Lund, personal communication
pBR325	Amp^r, Cm^r, Tet^r	Derived from pBR322 carrying the Cm^r gene	Bolivar, 1978
pAR1219	Amp^r	Carrying the *T7 RNA polymerase* gene under *lacUV5* control	Davanloo *et al.*, 1984

CHAPTER 3

INVESTIGATION OF HAEMOLYSIN SECRETION IN THE *secB* NULL STRAIN (CK1953)

HlyA is a large molecule which is secreted post-translationally using a trans-envelope membrane translocator complex comprised of the proteins HlyB, HlyD and TolC. It was reasoned that secretion may require the nascent HlyA to remain in a loosely folded conformation before interaction with the secretion machinery, and this might involve molecular chaperones such as SecB.

The involvement of SecB in the translocation of proteins across the inner membrane of *E. coli* has been well documented both *in vitro* and *in vivo* (see section 1.19; for review see, Kumamoto, 1990). The *secB* null strain CK1953 ($SecB^-$) was produced by transposon insertion (Kumamoto and Beckwith, 1985) and shows pleiotropic defects which include: an inability to grow on rich media (the basis of this selective growth defect is unknown, but has been consistently observed for many different *secB* null mutants; Kumamoto, 1990); a mucoid nature; and reduced translocation efficiency of a subset of *E. coli* exported proteins (Kumamoto and Beckwith, 1985).

3.1 Characterisation of the *secB* null strain (CK1953)

Preliminary experiments were carried out in order to confirm the identity of CK1953 as a *secB* null strain. Growth of this strain propagated in rich (nutrient broth and Luria broth) and minimal (M9 and M63) media was monitored (data not shown) and confirmed the viability of CK1953 in minimal media only (methods 2.2(i)). The growth rate in minimal media was almost comparable to that of the parent strain, MC4100, ($SecB^+$) (in agreement with Kumamoto and Beckwith, 1985).

To confirm that the *secB* null mutation reduced the translocation efficiency of OmpA, a protein which has been shown previously to be dependent on SecB for efficient translocation (Bankaitis and Bassford, Jr., 1984; Kumamoto *et al.*, 1989), a pulse-chase immunoprecipitation experiment was performed (methods 2.2(ii)). Following growth of cultures in minimal medium to an A_{600} of approximately 0.5 a pulse was initiated by the addition of ^{35}S-methionine. A sample of 0.5ml was taken after 30 seconds (which represents t=0) and L-methionine was added to the remaining

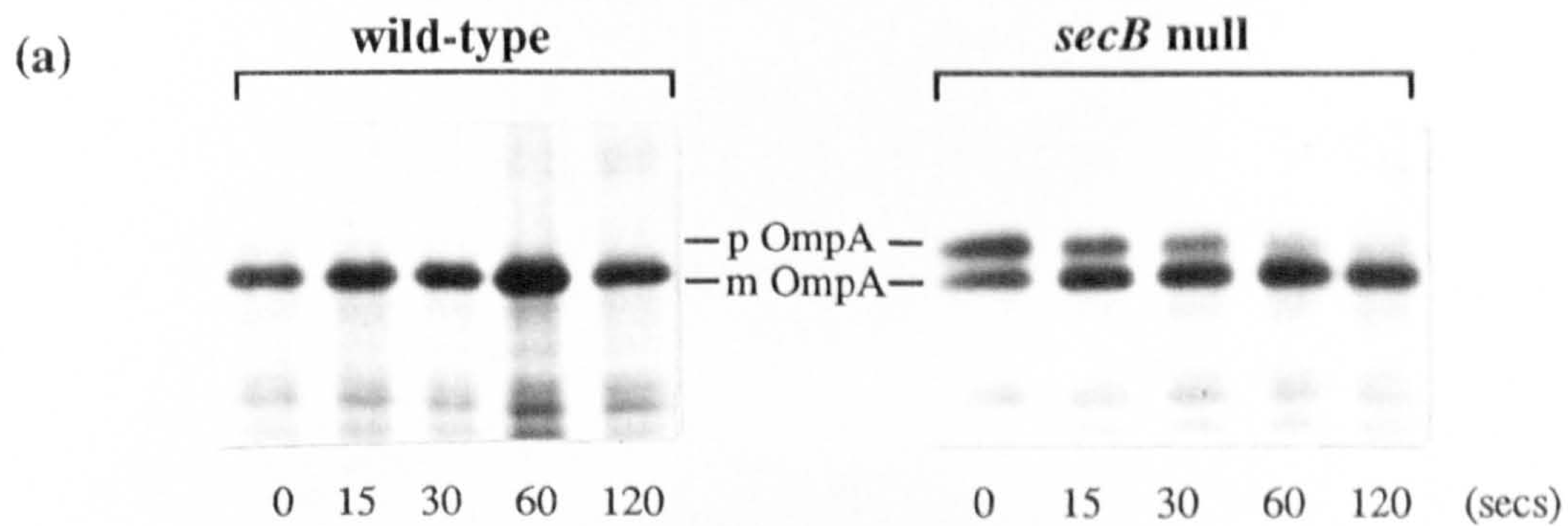
culture to initiate the chase. Further samples were taken at 15, 30, 60, and 120 seconds into the chase period. Immunoprecipitation was carried out overnight and samples were analysed by SDS-PAGE and fluorography.

Maturation of precursor to mature form by removal of the signal sequence is used routinely as a measure of the efficiency of protein translocation. This reaction may be visualised by a shift in mobility on SDS-PAGE of precursor following signal sequence processing to produce the mature protein. As shown in Fig.3.1(a) there is clearly a delay in signal sequence processing in the *secB* null mutant CK1953, compared with the wild-type strain MC4100, which reflects the dependence of OmpA on SecB for efficient export. Densitometric analysis produced quantitative data allowing the kinetics of translocation to be represented graphically (Fig.3.1(b)) and this illustrates how severely the rate of translocation of OmpA is decreased in the absence of SecB.

3.2 Secretion of haemolysin from the wild-type strain (MC4100)

Secretion of HlyA from the wild-type strain, MC4100, carrying the *E. coli* J96 haemolysin determinant on plasmid pSF4000 (Welch *et al.*, 1983) was assayed using two methods. The secretion of functional HlyA was identified by growth of cells on blood agar plates. Active HlyA secreted into the surrounding agar caused lysis of the red blood cells (Bhakdi *et al.*, 1986) resulting in the formation of halos around single colonies (Fig.3.2(b) and (c)). There is a significant decrease in the size of the halos produced by cells grown on minimal blood agar, compared with those produced by cells grown on nutrient blood agar (Fig.3.2 (b) and (c)). This agrees with the findings of Nicaud *et al.*, (1985b) who demonstrated that HlyA exhibits a 10 fold decrease in stability in minimal media compared with rich media (supplemented with calcium, see methods 2.2(viii)) at 37°C.

In order to obtain more quantitative data the accumulation of secreted HlyA in culture supernatants was assayed (methods 2.2(viii)). Maximal secretion of HlyA has been shown to occur during late logarithmic to early stationary phase of cultures (Nicaud *et al.*, 1985b) and preliminary experiments confirmed this (data not shown). Accumulation of HlyA in culture supernatants was therefore assayed following growth of cultures to an A_{600} of approximately 0.7. Cells were harvested by centrifugation and culture supernatants were TCA-precipitated and analysed by SDS-PAGE and Coomassie blue staining or Western blotting using HlyA antiserum. MC4100 gave a largely protein-free culture supernatant (Mackman and Holland,



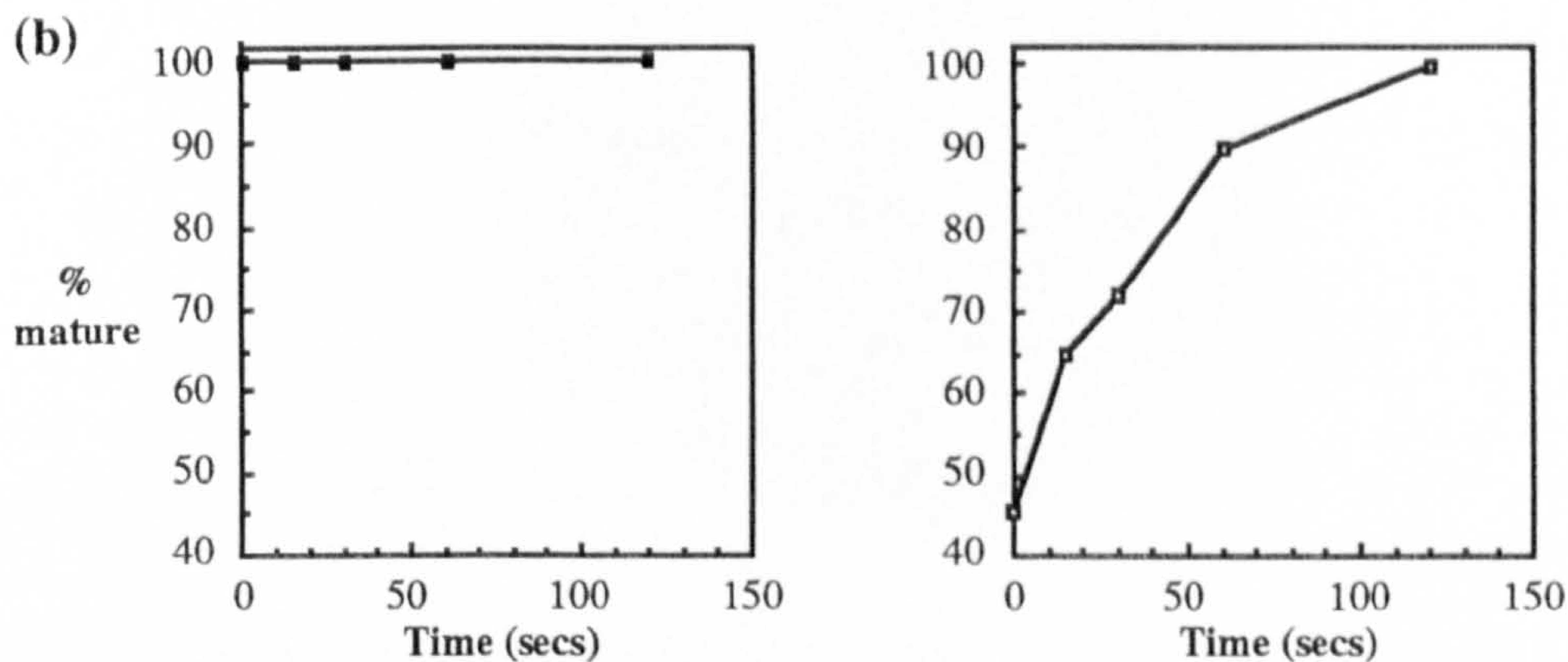
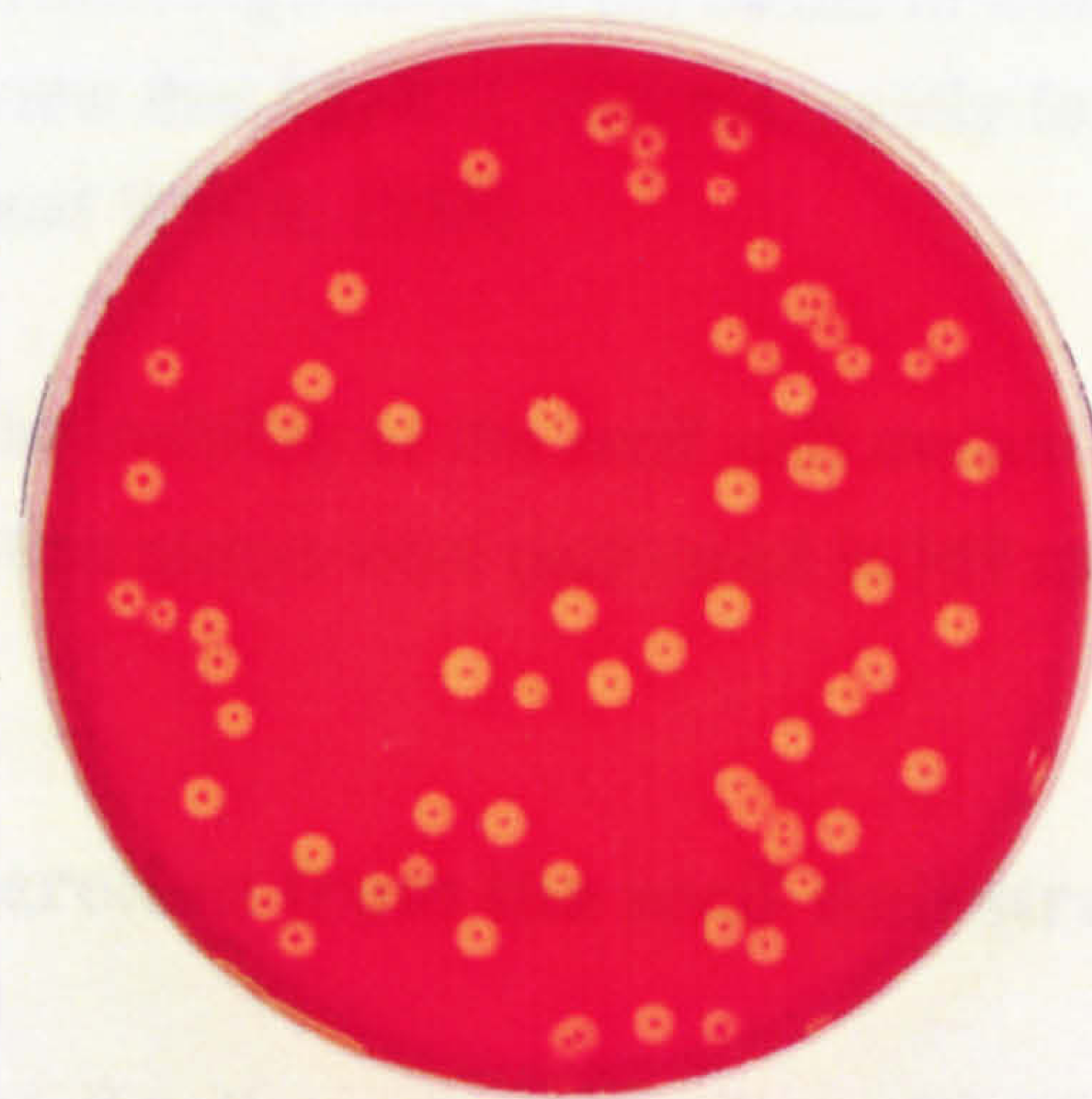


Fig. 3.1 Translocation of OmpA in the wild-type strain, MC4100, and *secB* null mutant, CK1953. Cultures were grown to early logarithmic phase in M9 minimal medium and pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with OmpA antiserum by SDS-PAGE and fluorography. The precursor and mature forms of OmpA are indicated. (b) Kinetics of OmpA translocation as determined by densitometry of the fluorographs in (a).

(a) wild-type

(b) wild-type pSF4000



(c) wild-type pSF4000

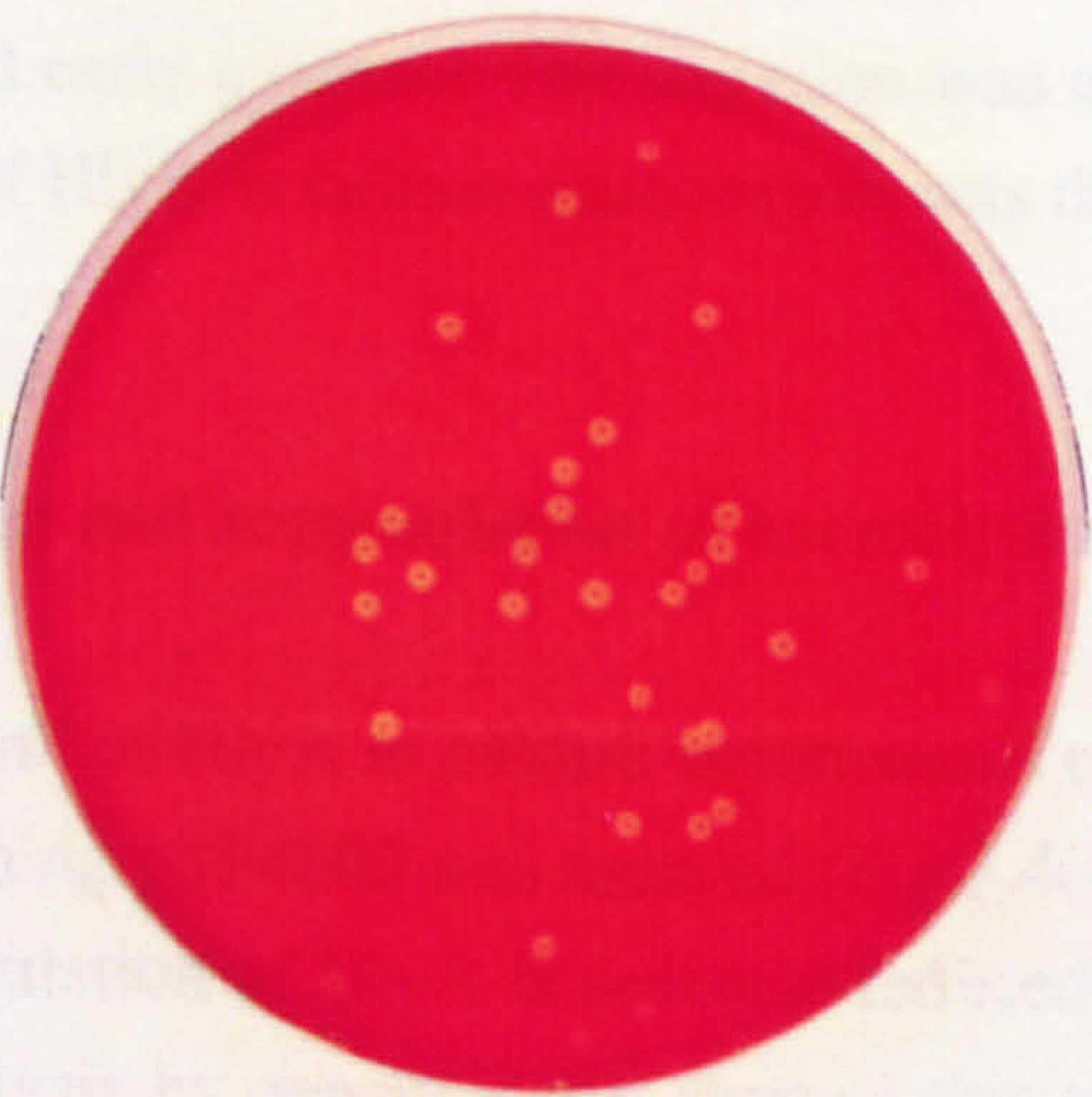


Fig. 3.2 Secretion of haemolysin from the wild-type (w.t.) strain, MC4100, on blood agar plates. Plates were incubated at 37°C for 16 hours. (a) No halos produced by w.t. grown on nutrient blood agar. (b) Large halos produced by w.t. pSF4000 grown on nutrient blood agar. (c) Reduced halos produced by w.t. pSF4000 grown on minimal blood agar.

1984a) in the presence or absence of pSF4000 as shown in the stained profile (Fig.3.3(a)). In both nutrient and minimal media the major band in the supernatants of cells containing pSF4000 was shown (by Western blot analysis using HlyA antiserum) to be HlyA (Fig.3.3(b)). Densitometry of the blot (data not shown) revealed a 10 fold decrease of HlyA accumulated in the supernatant of cultures grown in minimal media, compared with nutrient media, which is consistent with the decrease in size of the halos produced by cells grown on minimal blood agar, compared with those produced by cells grown on nutrient blood agar (Fig.3.2).

The cell pellets were also analysed by SDS-PAGE and Western blotting using HlyA antiserum (Fig.3.3(b)). However the levels of HlyA present in the cells remained relatively constant regardless of the media in which they were grown. This is consistent with the view that HlyA is secreted rapidly following synthesis (Nicaud *et al.*, 1985b; Felmlee and Welch, 1988).

As a result of the inability of the *secB* null mutant to grow in rich media all subsequent HlyA secretion assays were performed in M9 minimal medium.

3.3 Haemolysin secretion from the *secB* null strain

Growth of the *secB* null mutant, containing pSF4000, on minimal blood agar plates resulted in the production of very small halos around single colonies (data not shown). This provided early evidence that secretion was affected by the absence of SecB. Accumulation of HlyA in culture supernatants was then examined.

(i) Long term secretion and accumulation of haemolysin

The accumulation of HlyA in culture supernatants of the wild-type strain and *secB* null strain containing pSF4000 was assayed (Fig.3.4(a)) (methods 2.2(viii)). It is clear that the accumulation of HlyA is severely reduced in the supernatant of the *secB* null strain. Analysis by densitometry showed this to be a 6 fold decrease in HlyA accumulation (Fig.3.4(c)).

To confirm that this effect was not specific to HlyA encoded by the J96 determinant, the experiments were repeated using the *E. coli* LE2001 HlyA determinant carried on plasmid pLG570 (Mackman and Holland, 1984b). A similar reduction in HlyA accumulated in the supernatant of the *secB* null strain containing

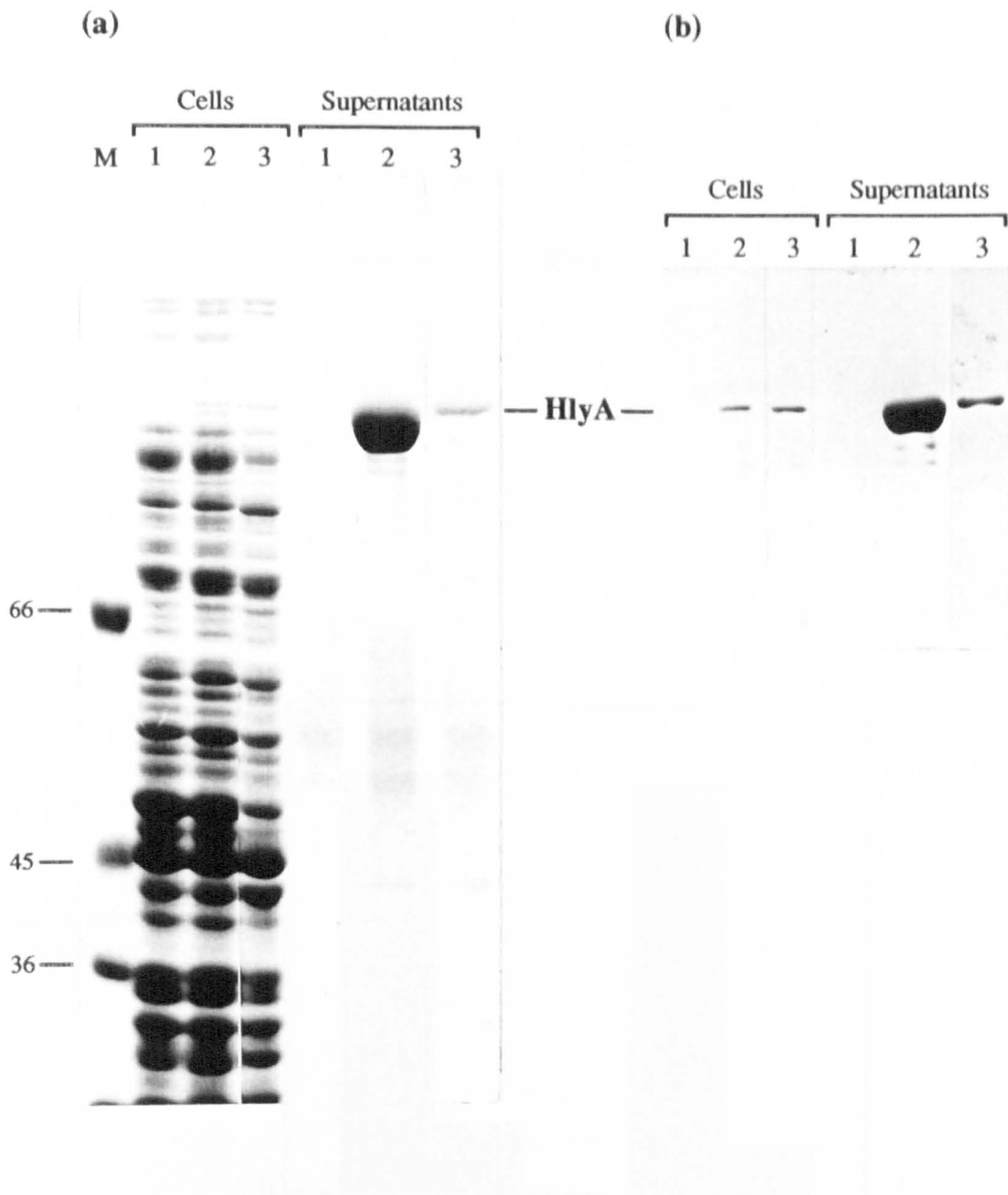


Fig. 3.3 Accumulation of haemolysin in culture supernatants of the wild-type (w.t.) strain, MC4100. Intracellular proteins and proteins accumulated in the supernatants of cultures grown in nutrient medium (1) w.t. and (2) w.t. pSF4000 or M9 minimal medium (3) w.t. pSF4000 were analysed by SDS-PAGE and (a) Coomassie blue staining or (b) Western blotting using HlyA antiserum (methods 2.2(viii)). The position of HlyA is indicated. M - SDS7 markers from Sigma. (Loading in A_{600} units: (a) cells 0.35: supernatants 7 (b) cells 0.1: supernatants 1.0)

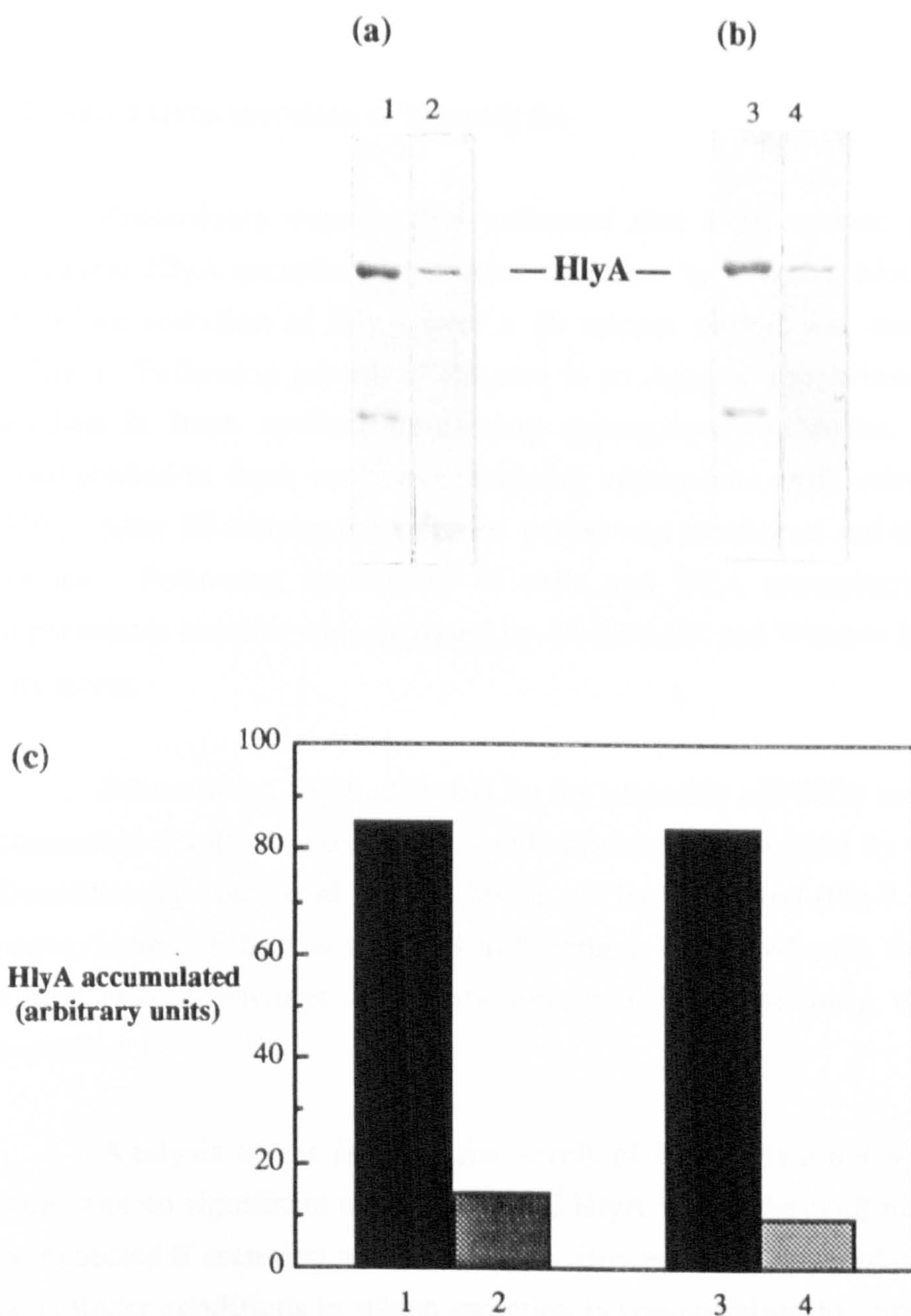


Fig. 3.4 Reduced accumulation of haemolysin from the *secB* null mutant, CK1953. Proteins accumulated in the supernatants of cultures (1) w.t. pSF4000, (2) *secB* null pSF4000, (3) w.t. pLG570, and (4) *secB* null pLG570 grown in M9 minimal medium were collected as described in materials and methods (2.2(viii)). (a) and (b) Equivalent samples (0.35 A_{600} units) were analysed by SDS-PAGE and Western blotting using HlyA antiserum. The position of HlyA is indicated. The lower molecular weight proteins in samples (1) and (3) are thought to be a previously recognised proteolytic breakdown product of HlyA (Nicaud *et al.*, 1985b). (c) Accumulation of HlyA as determined by densitometry of blots (a) and (b).

pLG570, compared with the wild-type strain containing pLG570, was seen (Fig.3.4(b) and (c)).

(ii) Short term secretion of haemolysin

Preliminary experiments indicated that a 30 minute period resulted in sufficient HlyA secretion for routine detection by Western blot (data not shown), therefore secretion of HlyA over a 30 minute period was determined (methods 2.2(ix)). Following growth of cultures to an A_{600} of approximately 0.7 cells were washed in fresh medium containing appropriate antibiotics. Cells were then resuspended in fresh medium containing appropriate antibiotics and incubated at 37°C. After 30 minutes the secretion period was terminated and cultures were placed on ice. Following harvesting of cells and TCA precipitation of the culture supernatants samples were analysed by SDS-PAGE and Western blotting using HlyA antiserum.

Secretion of HlyA, directed by the plasmids pSF4000 and pLG570 is again comparable, with slightly higher levels of secretion directed by pSF4000 (Fig.3.5). Densitometry confirmed that the levels of HlyA secreted (Fig.3.5(c)) were reduced approximately 6 fold in the *secB* null mutant, compared with the wild-type strain, which was consistent with data obtained from the long term accumulation experiments.

Analysis of the intracellular levels of HlyA (data not shown) showed that there was no significant accumulation of HlyA within the *secB* null cells which may be expected if secretion were decreased. However, the accumulation of HlyA within cells under conditions in which secretion is compromised has not been observed by other workers and has led to the proposal of a feedback mechanism (Wandersman and Letoffe, 1993) to prevent such an accumulation, although simple proteolysis of intracellular HlyA has not been ruled out. The absence of elevated levels of intracellular HlyA in the *secB* null mutant is consistent with the above.

(iii) Stability of secreted haemolysin

To ensure that the apparent reduction in secretion by the *secB* null strain was not the result of decreased stability of HlyA secreted by the mutant strain, compared to the wild-type strain, the half-life of secreted HlyA was determined (methods

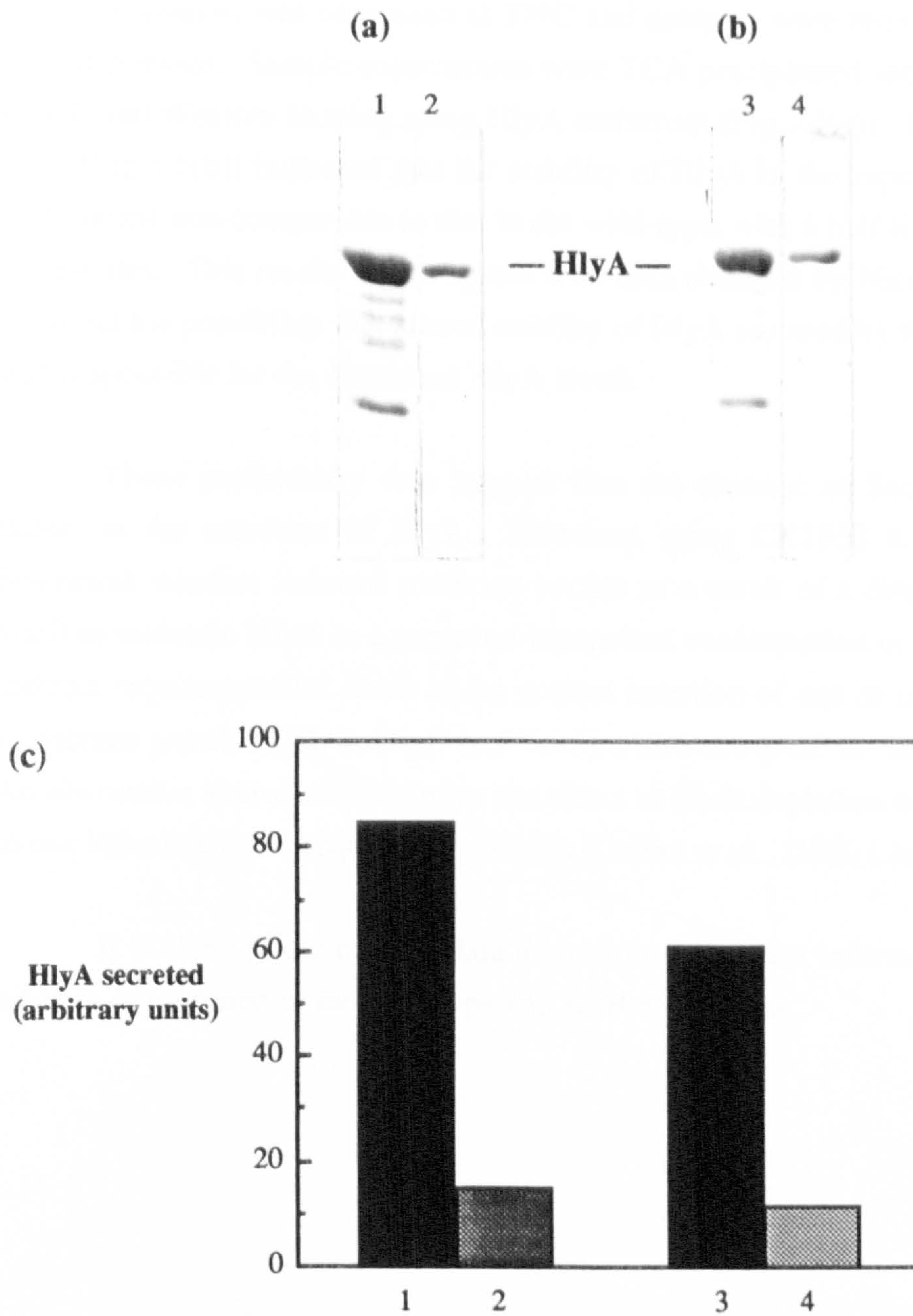
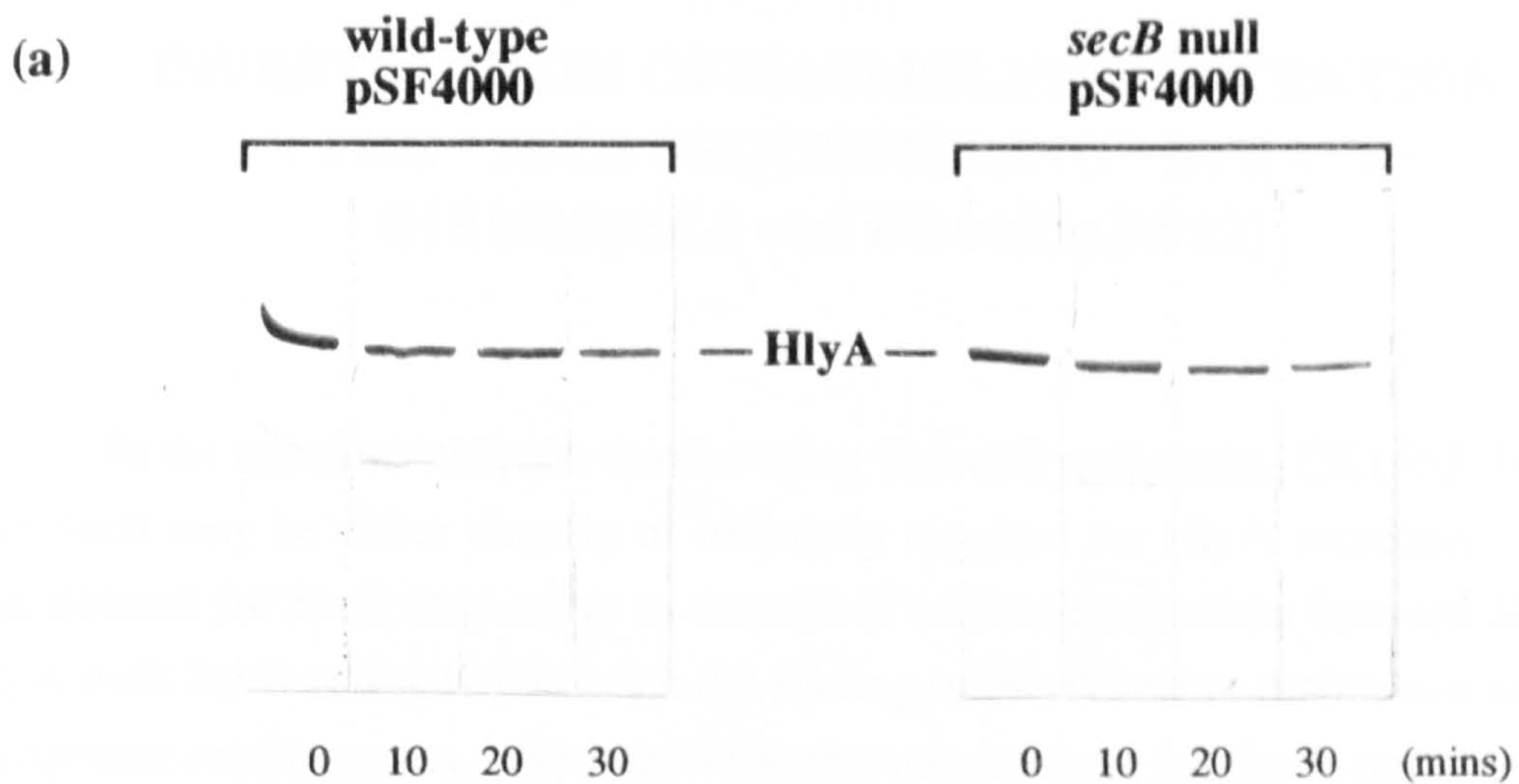


Fig. 3.5 Reduced secretion of haemolysin by the *secB* null mutant, CK1953, during a 30 minute period. Proteins accumulated over a 30 min period in the supernatants of cultures (1) w.t. pSF4000, (2) *secB* null pSF4000, (3) w.t. pLG570, and (4) *secB* null pLG570 grown in M9 minimal medium were collected as described in materials and methods (2.2(ix)). (a) and (b) Equivalent samples (1.4 A_{600} units) were analysed by SDS-PAGE and Western blotting using HlyA antiserum. The position of HlyA is indicated. (c) Secretion of HlyA as determined by densitometry of blots (a) and (b).

2.2(x)). Following growth of wild-type and *secB* null cultures containing pSF4000 to an A_{600} of approximately 0.7, growth and translation were terminated (designated t=0). Incubation was continued at 37°C and samples were removed after 0, 10, 20 and 30 minutes. Sample supernatants were TCA precipitated and analysed by SDS-PAGE and Western blotting using HlyA antiserum (Fig.3.6(a)). Densitometry of the blots (Fig.3.6(b)) indicated that the stability of HlyA in the supernatant of the *secB* null mutant was comparable to that in the wild-type, with a half-life of approximately 30 minutes. This result, which agrees with data obtained by Nicaud *et al.*, (1985b), ruled out the possibility that altered stability of HlyA secreted by the *secB* null mutant was responsible for the decreased HlyA levels.

These preliminary data suggest that the absence of SecB causes a severe defect in the secretion of HlyA. However, using CK1953 it is not possible to determine whether reduced secretion occurs as a result of a direct requirement for SecB to maintain HlyA in a secretion-competent conformation or perhaps reflects an indirect requirement for SecB in the correct insertion of one or more of the integral membrane proteins (HlyB, HlyD and TolC) which comprise the haemolysin exporter. An alternative approach to examine the effect of SecB depletion on HlyA secretion is to use inducible SecB sequestering strains (Collier *et al.*, 1988; Liu *et al.*, 1989).

It is also of note that the data do not provide direct information regarding the kinetics of secretion in the wild-type and *secB* null strains.



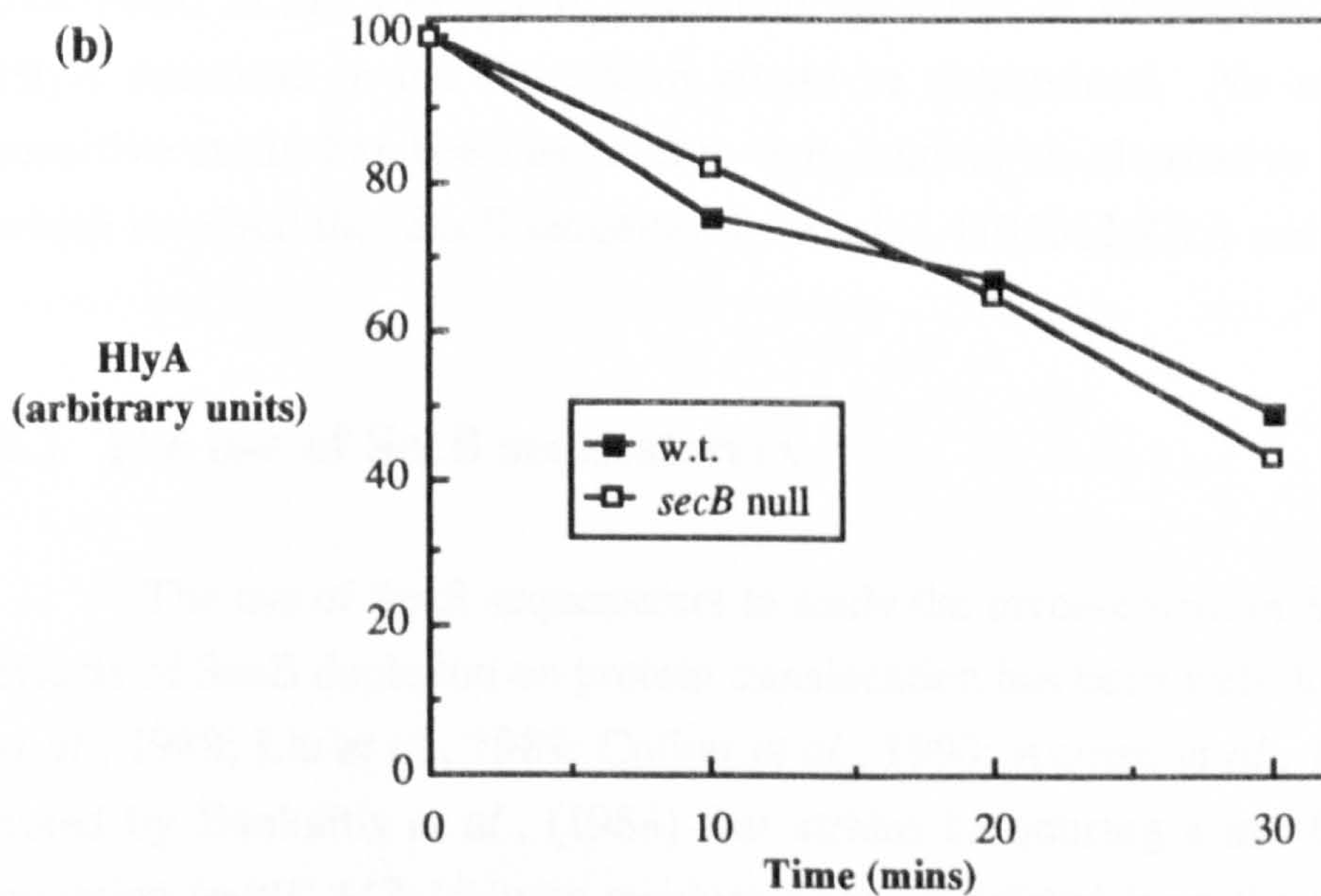


Fig. 3.6 Stability of haemolysin in culture supernatants. Growth of wild-type (w.t.) and *secB* null cultures containing pSF4000 in M9 minimal medium was terminated (t=0) and samples removed at the times indicated (methods 2.2(x)). (a) Proteins in the sample supernatants were analysed by SDS-PAGE and Western blotting using HlyA antiserum. The position of HlyA is indicated. (b) Stability of HlyA as determined by densitometry of blots in (a). Values for each culture are shown relative to that at t=0 which was given the arbitrary value of 100. (Loading in A_{600} units: wild-type 0.4: *secB* null 2.5)

CHAPTER 4

INVESTIGATION OF HAEMOLYSIN SECRETION IN THE "SECB SEQUESTERING" STRAINS (HB1012pGL1 and HB1053pJW12)

In the previous chapter, studies using the *secB* null strain, CK1953, indicated that SecB may be either directly or indirectly required for HlyA secretion. A direct requirement for SecB may occur as a result of a direct interaction between SecB and HlyA with SecB acting to moderate the folding of HlyA and so maintain a secretion-competent conformation. Alternatively, the requirement for SecB may be indirect with SecB necessary for the correct insertion of one or more of the accessory proteins involved in HlyA secretion (HlyB, HlyD and TolC). Finally, it may be that SecB is required in both the above processes. Attempts to demonstrate a direct requirement for SecB in HlyA secretion required that the proteins HlyB, HlyD and TolC should be assembled in the presence of SecB and the effect of subsequent SecB removal on HlyA secretion in the short term would be determined. No *secB* temperature-sensitive strain has been isolated to date, and so an alternative strategy was used which involved the "SecB sequestering" strains, HB1012pGL1 and HB1053pJW12.

4.1 The use of SecB sequesterers

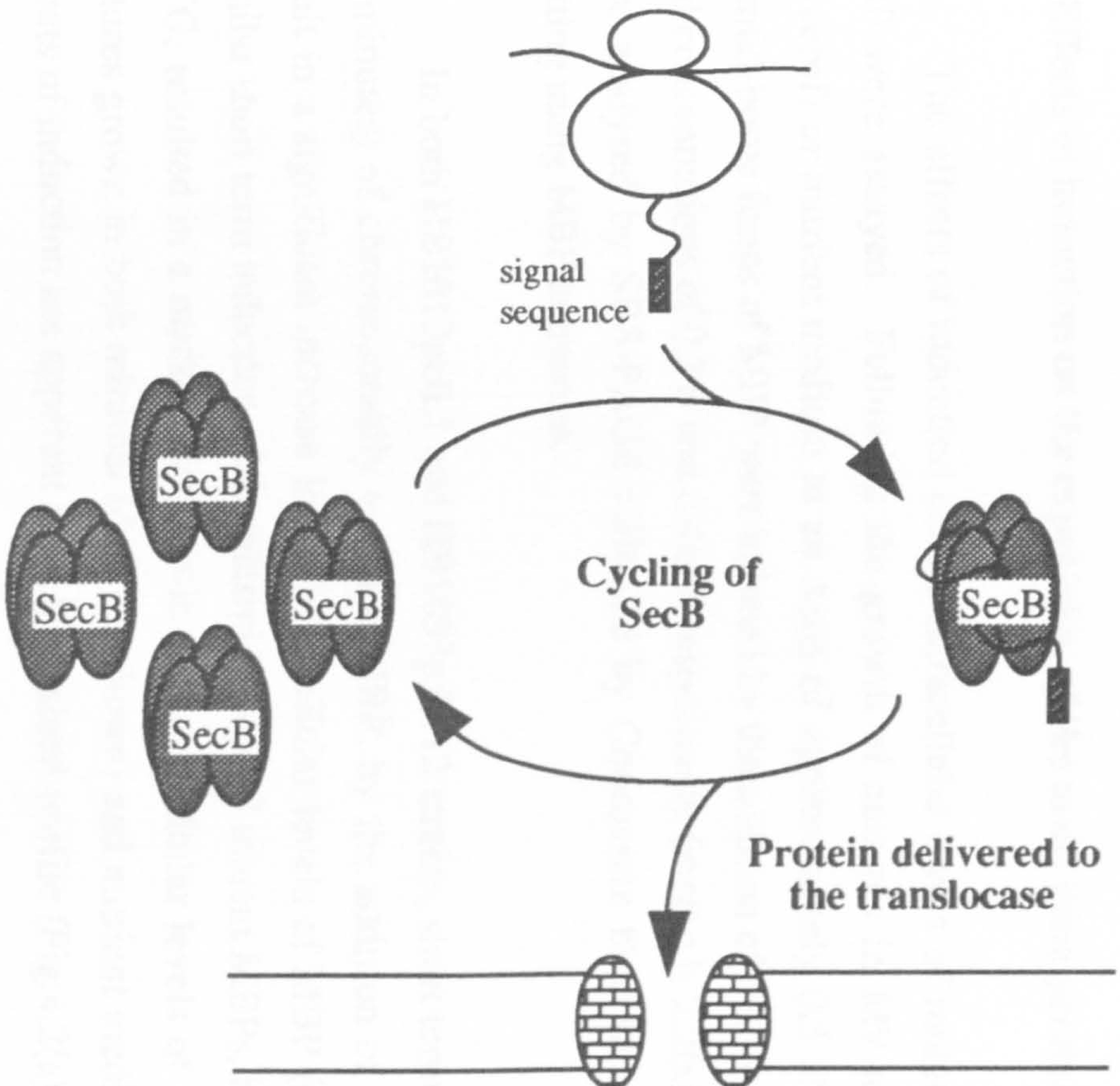
The use of SecB sequesterers to study the involvement of SecB and the direct effects of SecB depletion on protein translocation has been well documented (Collier *et al.*, 1988; Liu *et al.*, 1989; Collier *et al.*, 1990; Altman *et al.*, 1991). It was first noted by Bankaitis *et al.*, (1984) that strains harbouring a *malE* signal sequence mutation (*malE Δ12-18*, with residues 12 to 18 deleted from the hydrophobic core) and a *lamB* signal sequence mutation (*LamB S60*, with 12 residues deleted from the hydrophobic core which results in a block in LamB export) grew more slowly on maltose minimal medium than strains with the *malE* mutation alone. Further studies showed that synthesis of either of the mutant proteins interfered with the efficient translocation of several other wild-type proteins (Bassford and Bankaitis, 1984). This was the first documented case of an export-defective protein interfering with general protein export in *E. coli*. It was suggested that the export-defective precursors transiently enter the protein export pathway and compete with wild-type proteins for components of the cellular export machinery. Since then the ability of many different mutant protein molecules to interfere with normal protein export has been studied

(Collier *et al.*, 1988; Liu *et al.*, 1989). Collier *et al.*, (1988) noted that in general those proteins which were subject to interference by the mutant proteins were SecB-dependent and those which were unaffected were SecB-independent, indicating that interference was taking place at the level of SecB. Work by Liu *et al.*, (1989) characterised features of mutant proteins that are necessary and responsible for efficient interference with SecB. Most recently, such interference phenomena have been used to investigate the overlapping activities of SecB and the general chaperones including DnaK and GroEL (Altman *et al.*, 1991; Wild *et al.*, 1993).

From the above studies it has been concluded that interference requires that the interfering protein: exhibits a major export defect, is synthesised at a high rate and is actively synthesised at the time interference is being measured. It has been postulated that: export-defective proteins devoid of functional signal sequences retain some feature which is recognised by the export machinery (in this case SecB) and consequently they enter the export pathway while the export-defective protein is still a nascent polypeptide. The interference observed reflects a competition between export-defective proteins and other exported proteins for interaction with SecB. The inability of the export-defective protein to exit the export pathway via the normal route (translocation) may result in prolonged interaction with SecB. Availability of SecB may then become a limiting factor resulting in a delay in the export of some wild-type proteins. This interpretation of the data is represented in Fig.4.1.

By placing the interfering protein under the control of an inducible promoter the cytoplasmic pool of functional SecB may be "sequestered" following induction of the interfering, or "sequestering" protein. This facilitates rapid depletion of functional SecB at a specific stage of growth enabling conclusions to be drawn concerning the direct requirement for SecB in protein export.

It follows, that if the requirement of SecB for HlyA secretion is direct then after the removal of functional SecB, via induction of the sequesterers, an immediate decrease in the amount of HlyA secreted may be expected. If the requirement for SecB is indirect, then a delay between the removal of functional SecB and decreased HlyA secretion would be expected.



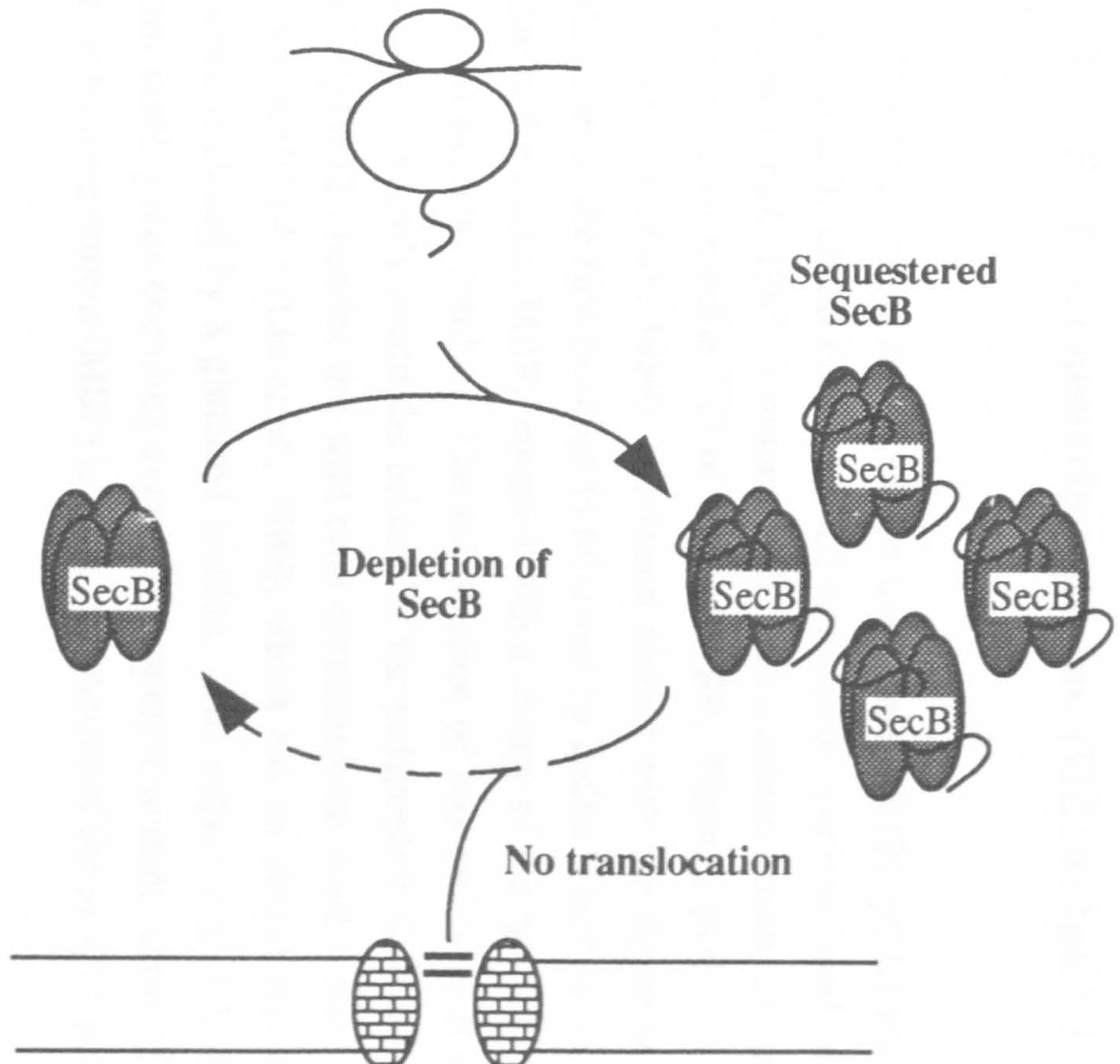


Fig. 4.1 Diagrammatic representation of SecB depletion using the SecB sequestering approach. (a) Cycling of functional SecB in wild-type cells. Interaction between the precursor and SecB is transient with SecB serving to maintain the precursor in a translocation competent conformation prior to interaction with the translocase and subsequent translocation. (b) Sequestering of SecB by a mutant precursor. The precursor is devoid of a functional signal sequence but is capable of interaction with SecB. Further movement along the GEP is prevented, by the absence of a functional signal sequence, leading to the sequestering of SecB.

4.2 The SecB sequestering strains (HB1012pGL1 and HB1053 pJW12)

The SecB sequestering strains used were HB1012pGL1 and HB1053pJW12 (see strains 2.4). Strain HB1012pGL1 encodes the chromosomal *malE Δ312* mutant (Rasmussen *et al.*, 1985); a mutant MBP with a deletion from residue 15 of the MBP signal peptide to residue 159 of mature MBP. Plasmid pGL1 encodes *malE Δ2-26,Y283D* (Liu *et al.*, 1989); a deletion mutant with the signal sequence removed (methionine in the first position is followed by lysine which is the first amino acid residue in the mature MBP), coupled with a change of the tyrosyl residue at position 283 to an aspartyl residue. The substitution of this amino acid residue has been shown to drastically retard the folding of the polypeptide (Liu *et al.*, 1988). Strain HB1053pJW12 encodes the wild-type chromosomal *malE* gene. Plasmid pJW12 encodes *malE 14-1* (Liu *et al.*, 1989); which has an alanyl residue in the signal sequence replaced by a glutamyl residue. The expression of both plasmid-borne mutant *malE* genes encoding export-incompetent mutant forms of the periplasmic maltose binding protein (MBP) is under the control of the *lacUV5* promoter.

4.3 Characterisation of the SecB sequestering strains

(i) Effects of induction on the expression of the *malE* gene products

The effects of induction on the intracellular levels of mutant and wild-type MBP were assayed. Following the growth of cultures in M9 minimal medium (glycerol) or nutrient medium to an A_{600} of approximately 0.5 chromosomal and plasmid-borne forms of MBP were induced by the addition of maltose and/or IPTG to final concentrations of 0.2% and 0.4mM respectively (methods 2.2(xi)). Cell samples were analysed by SDS-PAGE followed by Coomassie blue staining or Western blotting using MBP antiserum.

In both HB1012pGL1 and HB1053pJW12 strains, short term induction (up to 30 minutes) of chromosomally encoded MBP, by the addition of maltose, did not result in a significant increase in the intracellular levels of MBP (data not shown). Similar short term induction of the plasmid encoded mutant MBPs, by the addition of IPTG, resulted in a marked increase in the intracellular levels of mutant MBPs in cultures grown in both minimal (data not shown) and nutrient media (Fig.4.2). The effects of induction are apparent from the stained profile (Fig.4.2(a)). Expression of

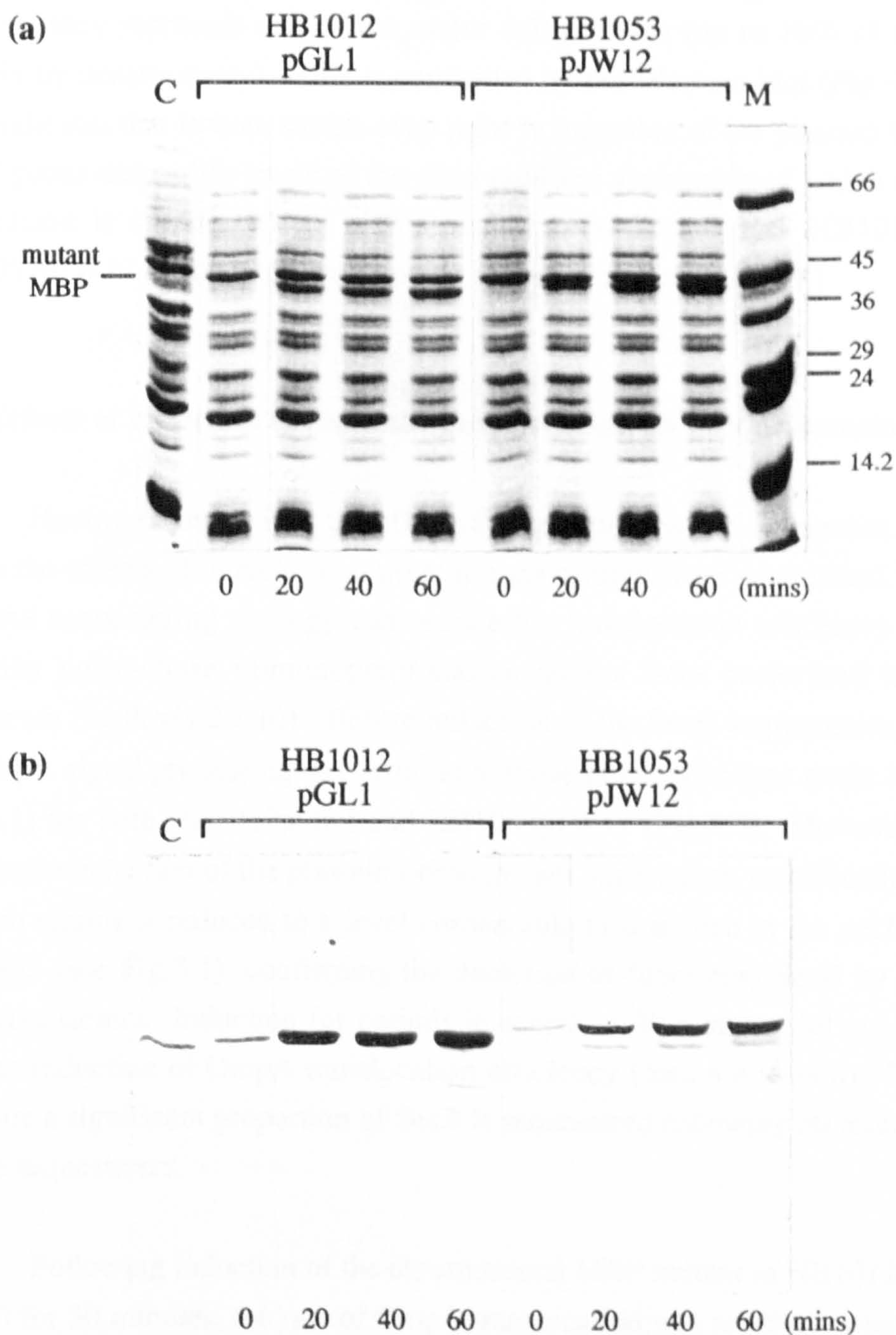


Fig. 4.2 IPTG-dependent induction of plasmid-borne mutant *malE* genes. Cultures were grown to mid logarithmic phase in nutrient medium and IPTG was added to a final concentration of 0.4mM. Samples were removed at the times indicated and cell equivalents (0.1 A_{600} units) were analysed by SDS-PAGE and (a) Coomassie blue staining or (b) Western blotting using MBP antiserum. M - SDS7 markers from Sigma. C - control, cell sample of MC4100 containing only chromosomal wild-type MBP.

the mutant MBPs is induced to such an extent that following induction for only 20 minutes they represent one of the major cell proteins (up to 10% of total cellular protein by densitometry). This is confirmed by the Western blot (Fig.4.2(b)) which also indicates that in both strains even prior to induction of the plasmid-borne mutant *malE* genes detectable levels of the gene products are expressed which suggests that expression is slightly leaky. (This occurs even though both HB1012pGL1 and HB1053pJW12 contain duplicate copies of the *lac* repressor, *lacI^q*.)

(ii) Effects of induction of the *malE* gene products on protein translocation

Having characterised the effects of induction on the expression of the *malE* genes the effects of induction on protein translocation were determined. To confirm that the sequestering strategy can reduce the translocation efficiency of exported proteins pulse-chase immunoprecipitation assays were performed using OmpA antiserum (methods 2.2(ii)). Before induction of the SecB sequesterers, the kinetics of OmpA signal processing were similar to those of the wild-type strain MC4100 (see Fig.3.1) for both HB1012pGL1 and HB1053pJW12 (Fig.4.3). However, following 30 minute induction of the plasmid-borne mutant *malE* genes, translocation efficiency in both strains is reduced to a level comparable to that seen in the *secB* null strain, CK1953 (see Fig.3.1), confirming the depletion of functional SecB by induction of the sequesterers. Induction for periods in excess of 30 minutes did not result in any further reduction of OmpA translocation efficiency (data not shown). These results indicate a significant proportion of SecB is sequestered following 30 minute induction of the sequesterers.

Following induction of the chromosomal MBP mutant in HB1012pGL1 (*malE Δ312*) for 30 minutes, the rate of OmpA translocation was relatively unaffected (data not shown). This is consistent with the finding that induction of chromosomal MBP for 30 minutes did not result in a significant increase in the levels of chromosomally expressed MBP (see section 4.3(i)). As expected the induction of both chromosomal and plasmid-borne mutant MBPs in HB1012pGL1 for 30 minutes produced a similar decrease in the rate of OmpA translocation (data not shown) as was seen following 30 minute induction of the plasmid-borne mutant alone (Fig.4.3). All subsequent experiments were carried out using induction of the plasmid-borne mutant MBPs (the sequesterers) alone.

To confirm that the sequestering effect was specific to SecB-dependent proteins, similar pulse-chase immunoprecipitation assays were performed using

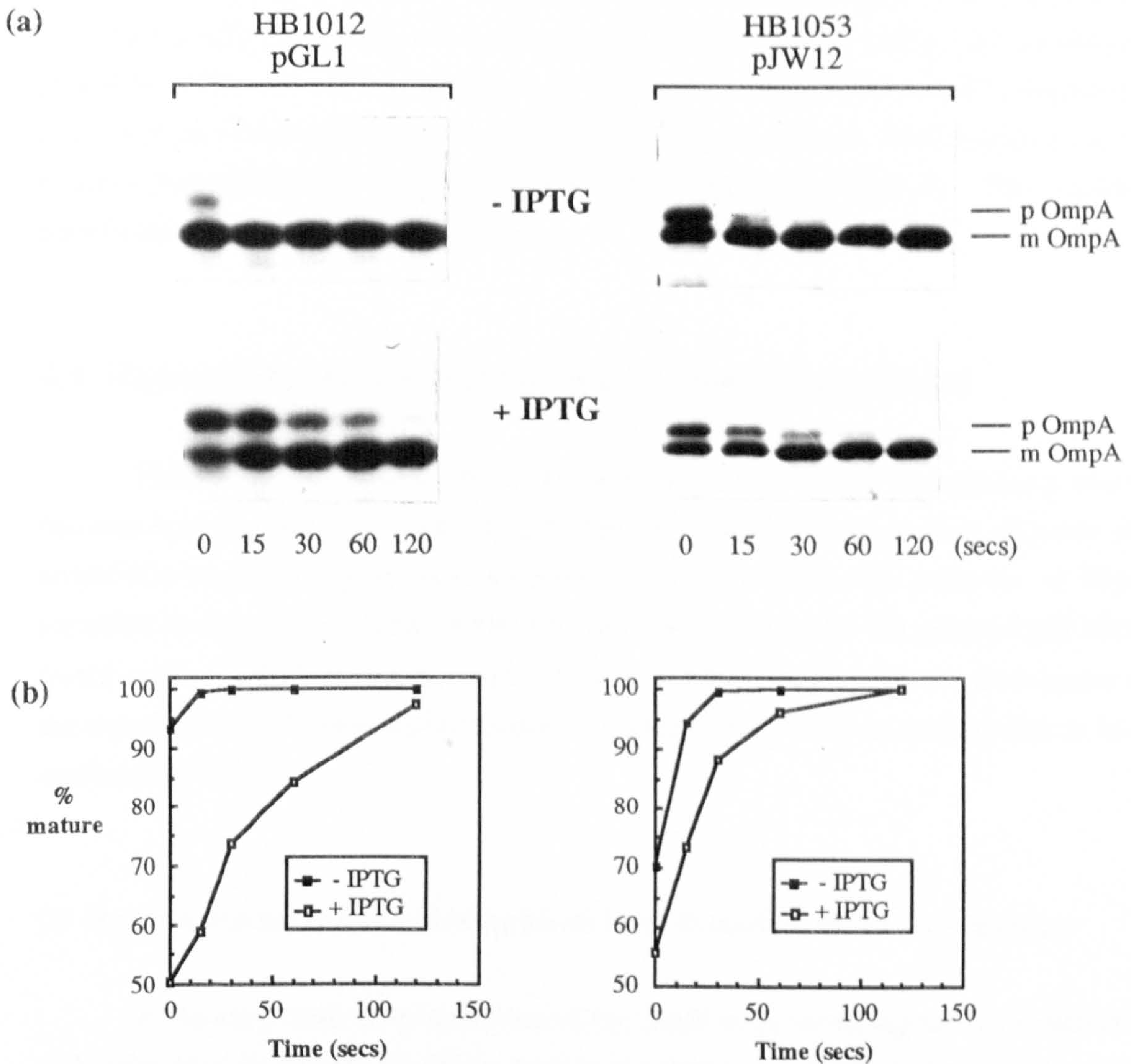


Fig.4.3 Reduced translocation efficiency of OmpA in the SecB sequestering strains following induction of the plasmid-borne *malE* genes. Strains HB1012pGL1 and HB1053pJW12 propagated in M9 minimal medium were grown to early logarithmic phase and the cultures split in two; one half had no additions (-IPTG) while the other had IPTG added to a final concentration of 0.4mM (+IPTG). Incubation was continued for 30 mins and then the cultures were pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with OmpA antiserum by SDS-PAGE and fluorography. The precursor and mature forms of OmpA are indicated. (b) Kinetics of OmpA translocation as determined by densitometry of fluorographs in (a).

antisera to ribose binding protein (RBP) and β-lactamase respectively. Following 30 minute induction of the plasmid-borne mutant *malE* gene in HB1012pGL1 the rate of translocation of RBP, a protein which has been shown previously to be independent of SecB for efficient translocation (Kumamoto and Beckwith, 1985), was unaffected (Fig.4.4). The rate of translocation of the SecB independent, GroEL-dependent protein, β-lactamase (Bochkareva *et al.*, 1988; Kusukawa *et al.*, 1989) appeared to be slightly increased following induction of the sequesterer (Fig.4.4). The possible significance of this is discussed below.

4.4 Haemolysin secretion in the SecB sequestering strains

Plasmid pLG570 could not be transformed into the SecB sequestering strains because it is incompatible with the plasmids pGL1 and pJW12 as they all carry the ampicillin resistance gene (see plasmids 2.5). Therefore the analysis of HlyA secretion in these strains was confined to secretion directed by the plasmid pSF4000. Initial studies indicated that the accumulation of HlyA in the culture supernatants of the uninduced SecB sequestering strains, containing pSF4000 was comparable in both strains (Fig.4.5).

(i) Haemolysin secretion following short term induction of the sequesterers

Following short term induction of the SecB sequesterering strains containing pSF4000 (for up to two doubling times) the secretion of HlyA over a 30 minute period was determined (methods 2.2(ix)). It is clear (Fig.4.6) that there is no significant effect on the secretion of HlyA with the levels accumulated from both strains remaining relatively constant. The reproducibility of these results was confirmed in repeated experiments. The levels of HlyA secreted by HB1012pGL1 containing pSF4000 were assayed either before or after 30 minute induction of the plasmid-borne sequesterer. The results were consistent with the above, (see Fig.4.7) with induction of the sequesterer for 30 minutes showing no significant effect on the levels of secretion of HlyA (data not shown).

These data appear to rule out the possibility of a direct interaction between SecB and HlyA itself although it may be inferred, in conjunction with the results from studies involving the *secB* null strain (see section 3.3), that SecB is required for the correct insertion of one or more of the accessory proteins involved in HlyA export (HlyB, HlyD and TolC).

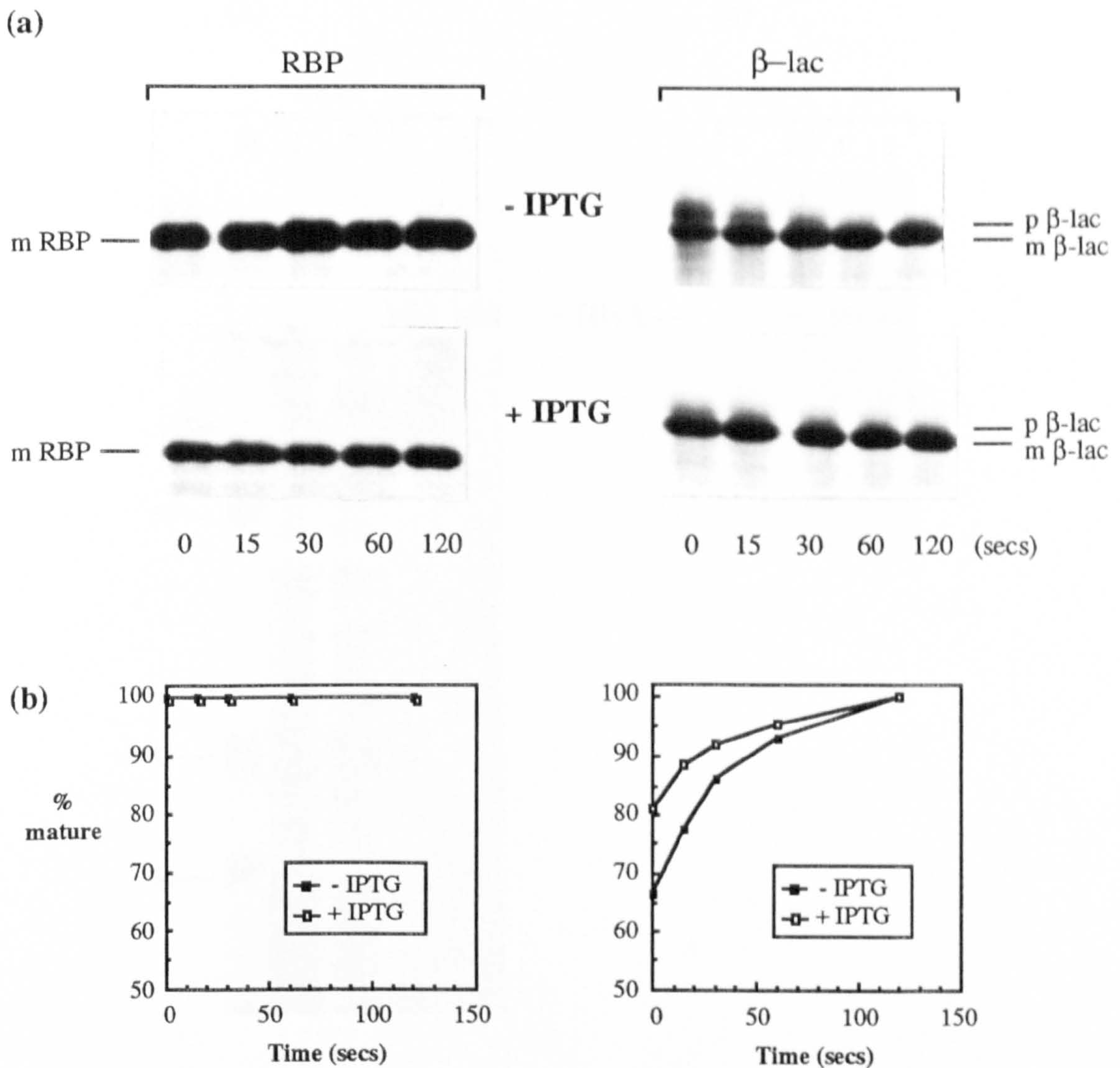


Fig. 4.4 The effect of induction of the plasmid-borne *malE* gene on RBP and β-lactamase translocation efficiency. Strain HB1012pGL1 propagated in M9 minimal medium was grown to early logarithmic phase and the culture split in two; one half had no additions (-IPTG) while the other had IPTG added to a final concentration of 0.4mM (+IPTG). Incubation was continued for 30 mins and then the cultures were pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with RBP antiserum or β-lactamase antiserum by SDS-PAGE and fluorography. The precursor and mature forms are indicated. (b) Kinetics of RBP and β-lactamase translocation as determined by densitometry of fluorographs in (a).

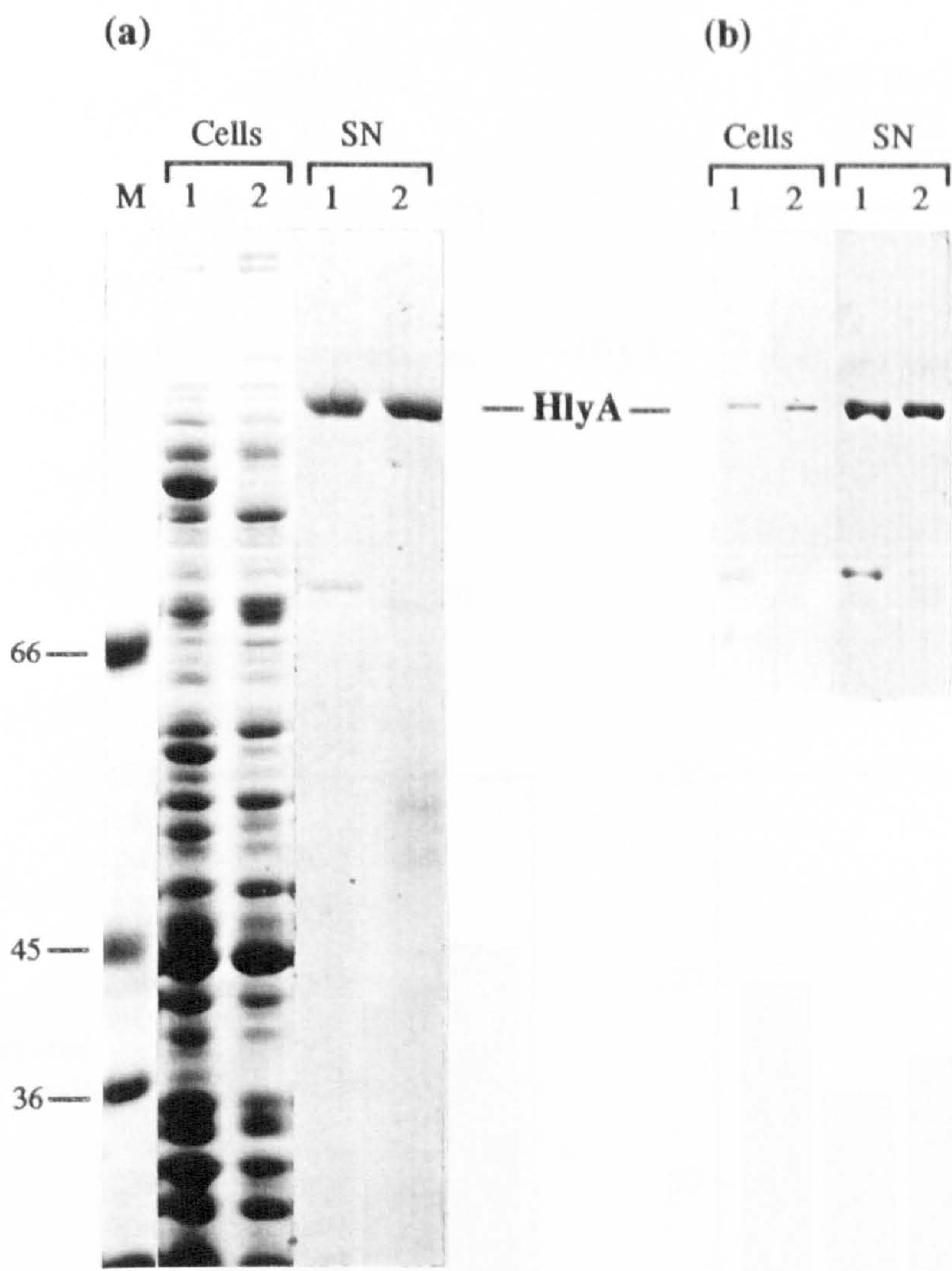


Fig. 4.5 Accumulation of haemolysin in culture supernatants of the SecB sequesterers. Intracellular proteins and proteins accumulated in the supernatants of (1) HB1012pGL1 and (2) HB1053pJW12 cultures containing pSF4000 grown in nutrient medium were analysed by SDS-PAGE and (a) Coomassie blue staining or (b) Western blotting using HlyA antiserum (methods 2.2(viii)). The position of HlyA is indicated. M - SDS7 markers from Sigma. SN - supernatants. (Loading in A_{600} units: (a) cells 0.35: SN 7: (b) cells 0.1: SN 1.0)

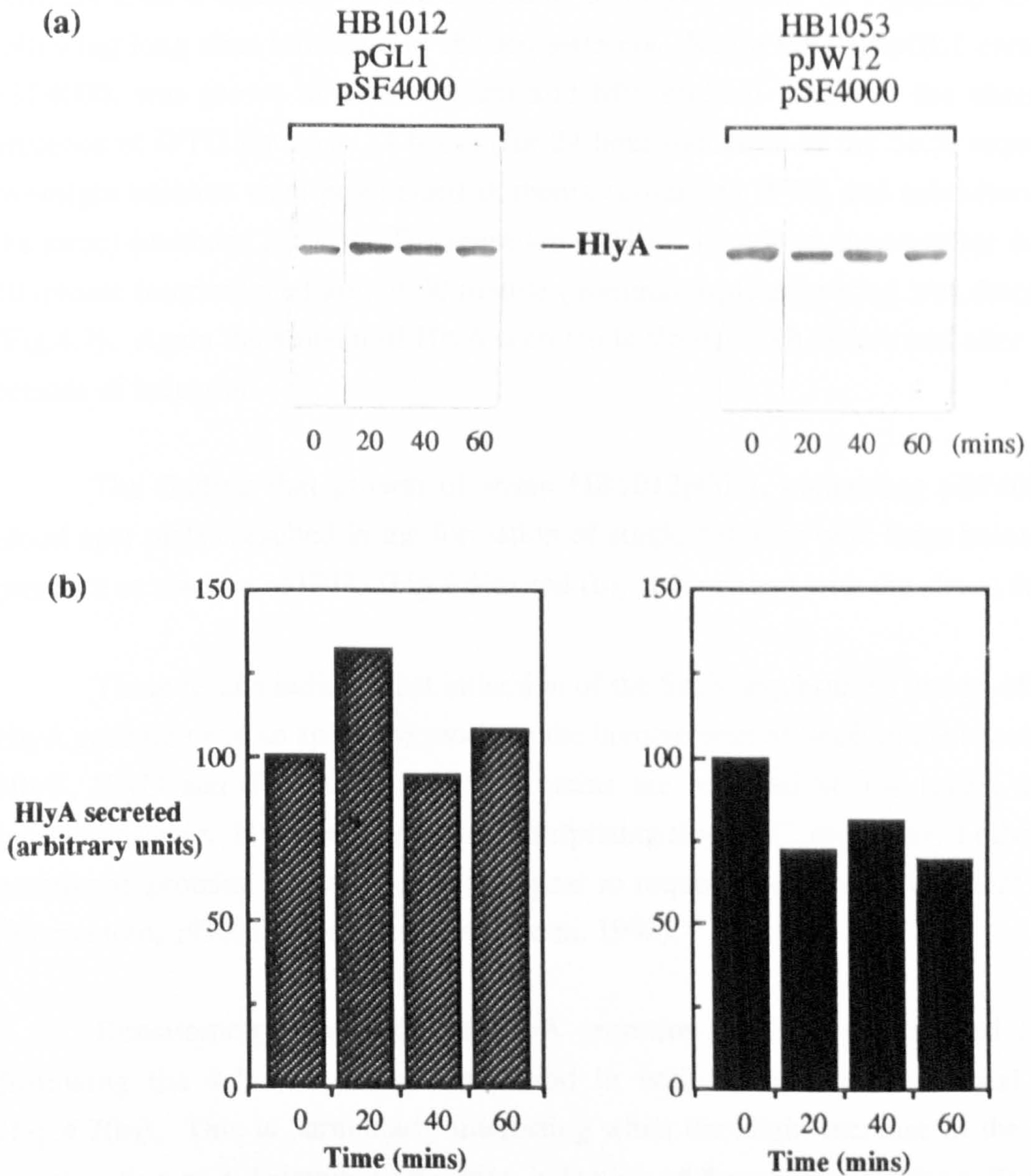


Fig. 4.6 Negligible effect on haemolysin secretion caused by short term induction of SecB sequesterers. (a) Proteins accumulated over a 30 minute period in nutrient growth medium from cultures of HB1012pGL1 and HB1053pJW12 containing pSF4000 were collected and equivalent samples (4.0 A_{600} units) analysed by SDS-PAGE and Western blotting using HlyA antiserum (methods 2.2(ix)). Separate cultures were non-induced (0), or induced with IPTG (0.4mM) for 20, 40 or 60 minutes before the accumulation assay as indicated. The position of HlyA is indicated. (b) HlyA secretion as determined by densitometry of blots in (a). Values are shown relative to that at time 0 for each strain, which was given the arbitrary value of 100.

(ii) Haemolysin secretion following long term induction of the sequesterers

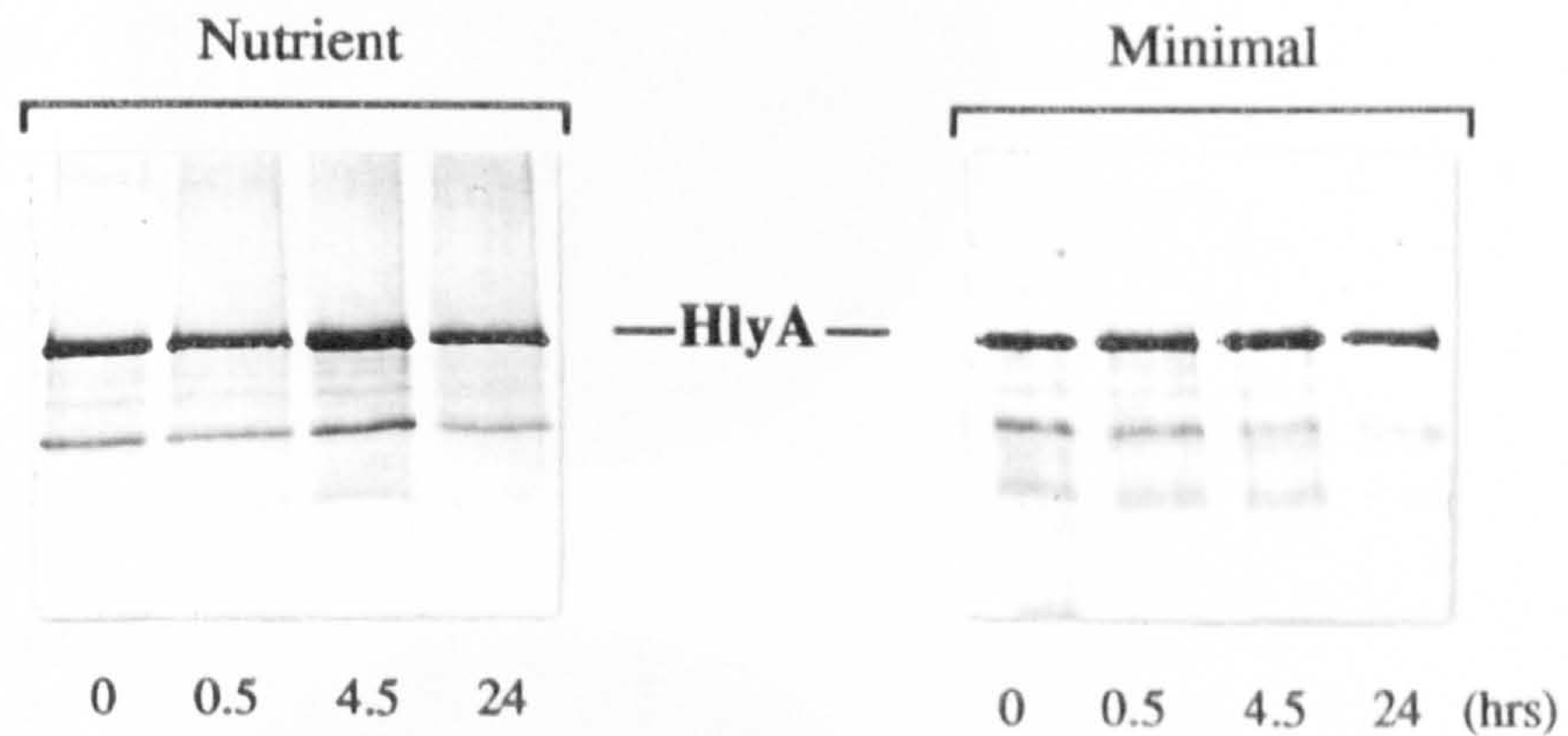
If SecB is required for the correct insertion of one or more of the accessory proteins then a decrease in the secretion of HlyA would be expected to occur following long term induction of the sequesterers. Strain HB1012pGL1 containing pSF4000, was grown in both nutrient and M9 minimal media in the absence or presence of IPTG for up to 24 hours (for 24 hour induction of the SecB sequesterer overnight cultures were propagated in media containing IPTG and subcultured into the same) (methods 2.2(xi)). The secretion of HlyA in culture supernatants during a 10 minute (nutrient medium) or 30 minute (minimal medium) period was determined (Fig.4.7). Again the amount of HlyA secreted is similar both before and after longer periods of induction.

The finding that growth of strain HB1012pGL1, containing pSF4000, on blood agar plates resulted in the formation of single colonies with large halos in the presence or absence of IPTG (Fig.4.8(a) and (b)) is consistent with the above data.

These results indicate that induction of the SecB sequesterers has no effect on HlyA secretion and so appear to preclude the involvement of SecB in the assembly of HlyB, HlyD and TolC unless these proteins are required at low levels and are unusually stable. However it does seem surprising that TolC, unlike most other outer membrane proteins studied, does not appear to require SecB for its efficient export (Kumamoto, 1990; de Cock and Tommassen, 1992).

Densitometry revealed that HlyA secretion is slightly increased (+30%) following the 4.5 hour induction period in both nutrient and minimal media (Fig.4.7(b)). This is particularly interesting when the slight increase in the rate of translocation of β–lactamase following induction of the sequesterer for a 30 minute period is considered (see Fig.4.4). It has been previously recognised that overproduction of export defective proteins can cause a 2 to 5 fold increase in the synthesis of heat shock proteins (Wild *et al.*, 1993). Indeed, analysis of cell samples by SDS-PAGE and Western blotting using GroEL antiserum showed slightly elevated levels of GroEL (up to 40 %) following induction of the sequesterer (data not shown). Although this is consistent with data from other groups (Wild *et al.*, 1993) work by Altman *et al.*, (1991) suggested that elevated levels of GroEL were not responsible for replacing SecB activity in *SecB* null strains. This does not rule out the possibility that the above observations may be indicative of a sequesterer induced stress response which is manifest after 30 minutes by a slight increase in the rate of β–lactamase translocation and after 4.5 hours by a slight increase in the secretion of HlyA.

(a)



(b)

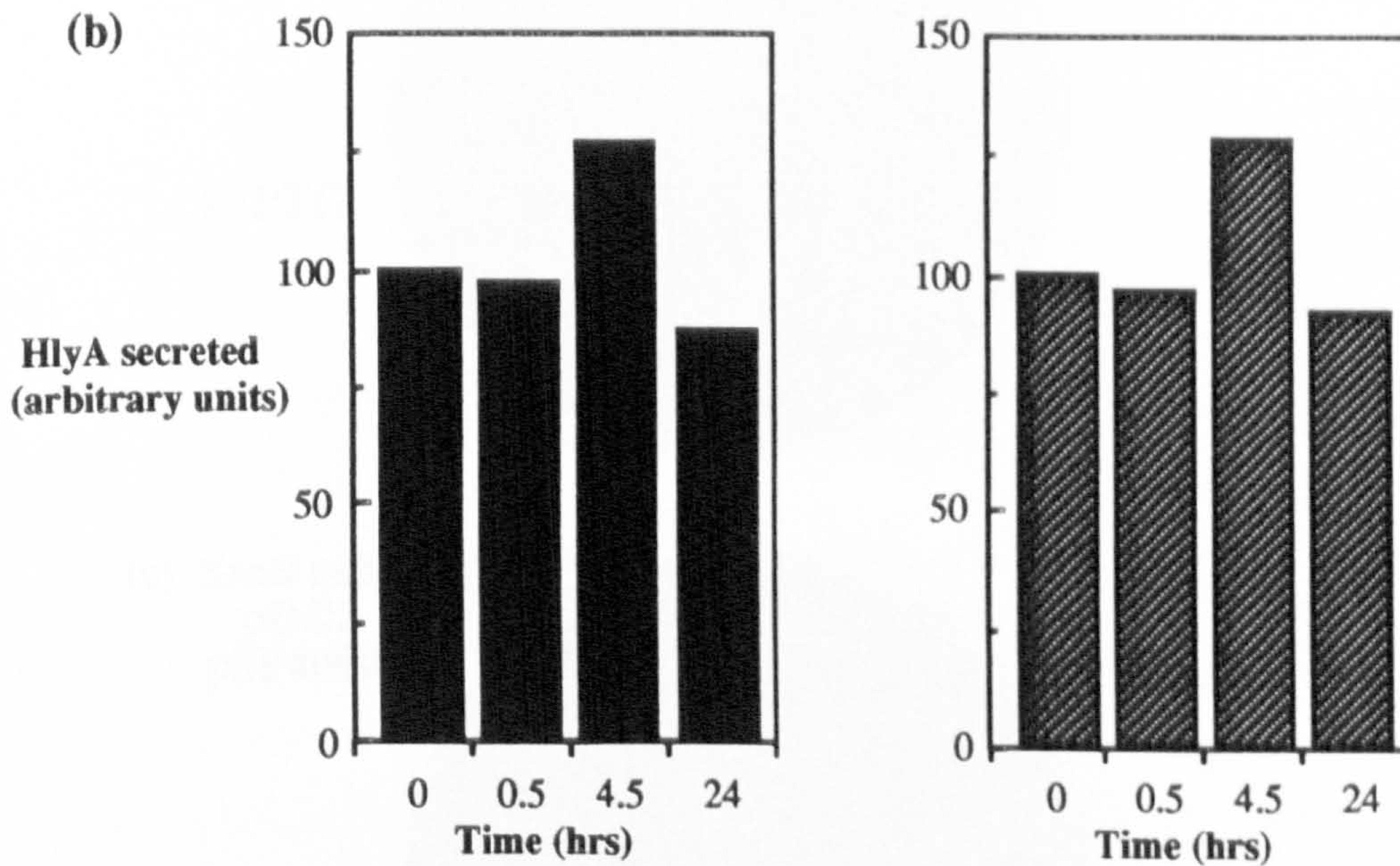
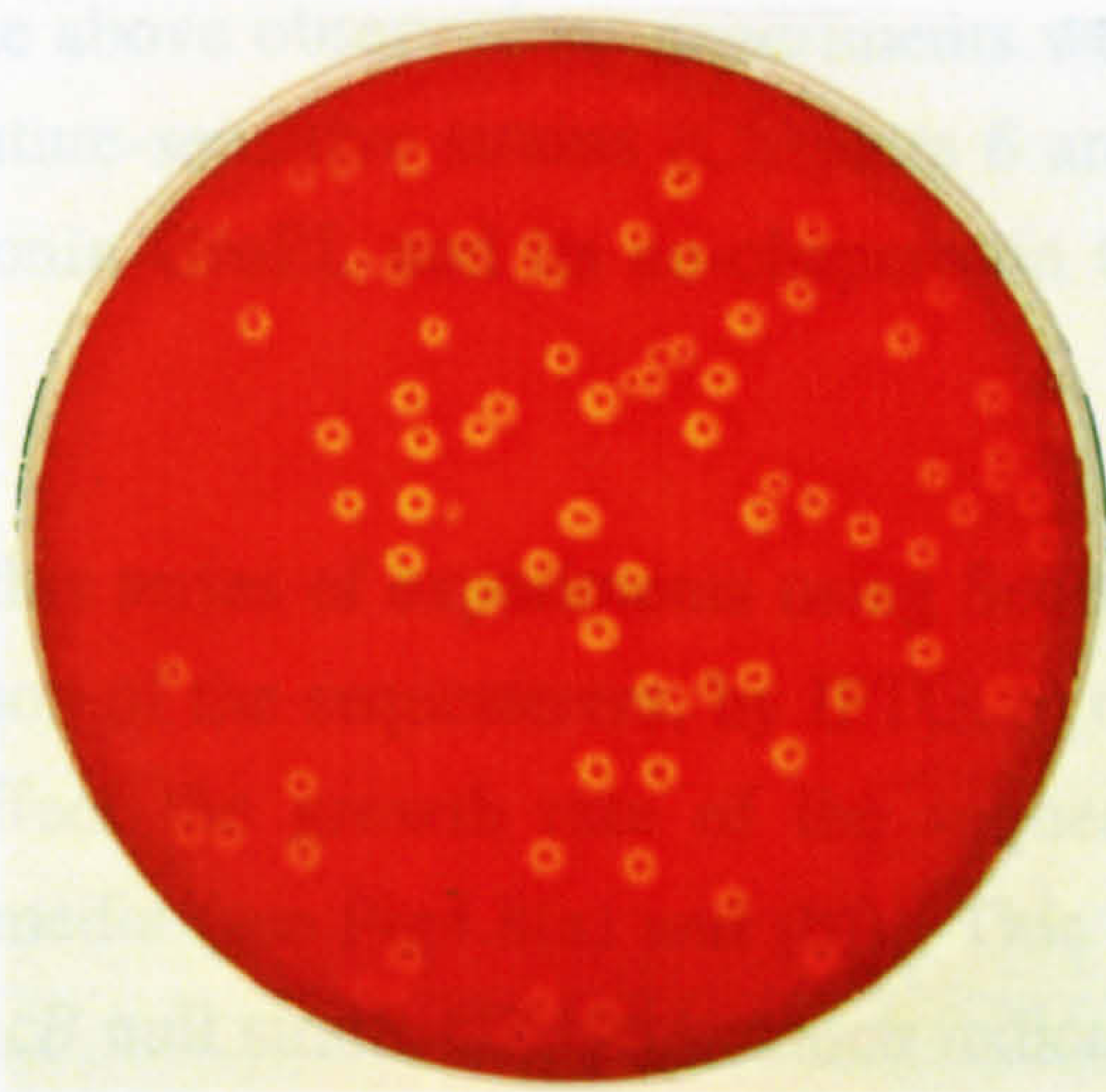


Fig. 4.7 Negligible effect on haemolysin secretion caused by long term induction of the SecB sequesterer HB1012pGL1. (a) Proteins accumulated over a 10 or 30 minute period in nutrient or minimal growth medium from cultures of HB1012pGL1 containing pSF4000 were collected and equivalent samples (2.0 A_{600} units for nutrient: 4.0 A_{600} units for minimal) analysed by SDS-PAGE and Western blotting using HlyA antiserum as described in materials and methods (2.2(ix)). Separate cultures were non-induced (0), or induced with IPTG (0.4mM) for 0.5, 4.5 or 24 hours before the accumulation assay as indicated. The position of HlyA is indicated. (b) HlyA secretion as determined by densitometry of blots in (a). Values are shown relative to that at time 0 for each medium, which was given the arbitrary value of 100.

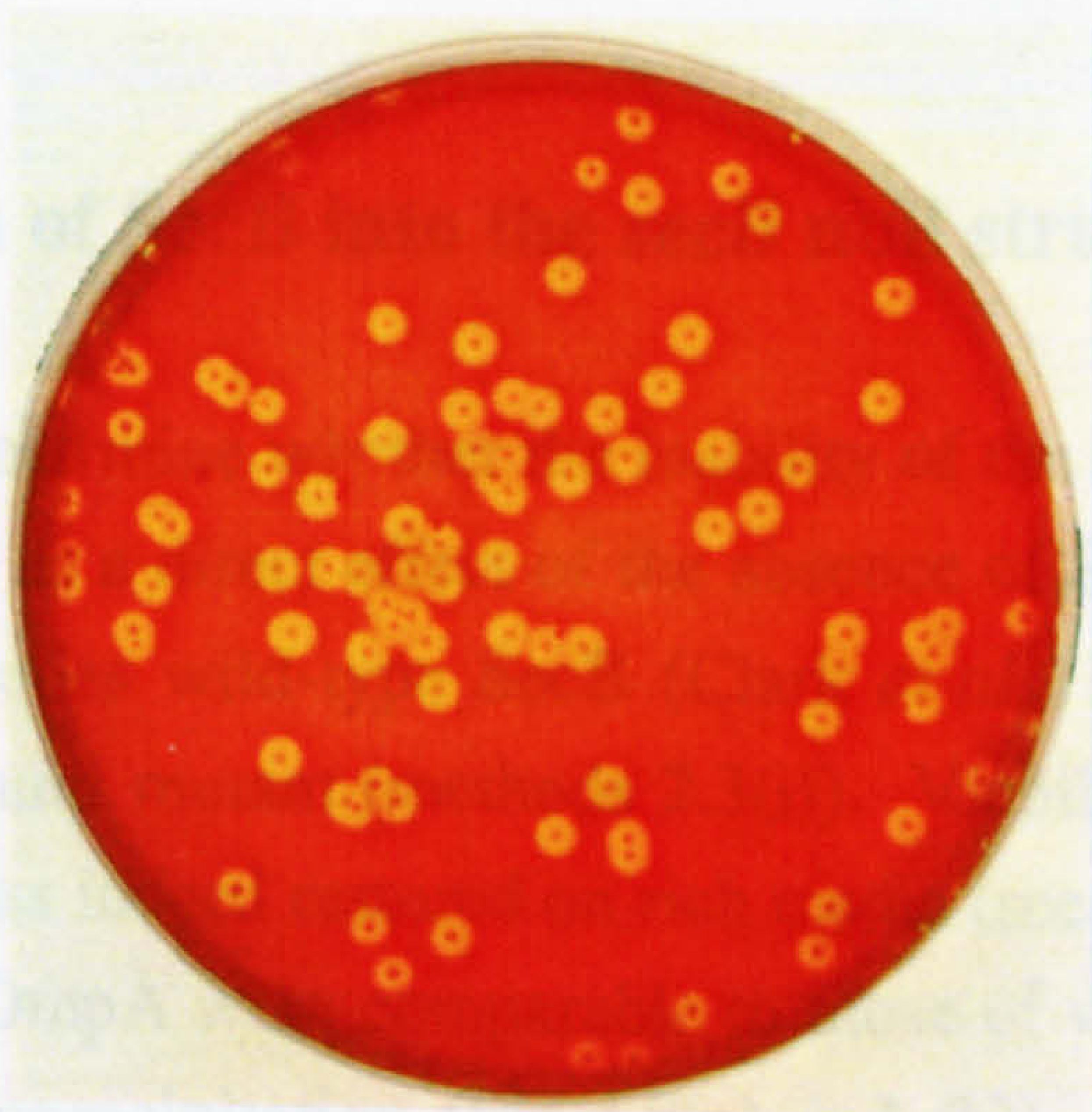
(a) HB1012
pGL1
pSF4000

- IPTG



(b) HB1012
pGL1
pSF4000

+ IPTG



(c) *SecB* null
pDC2
pSF4000

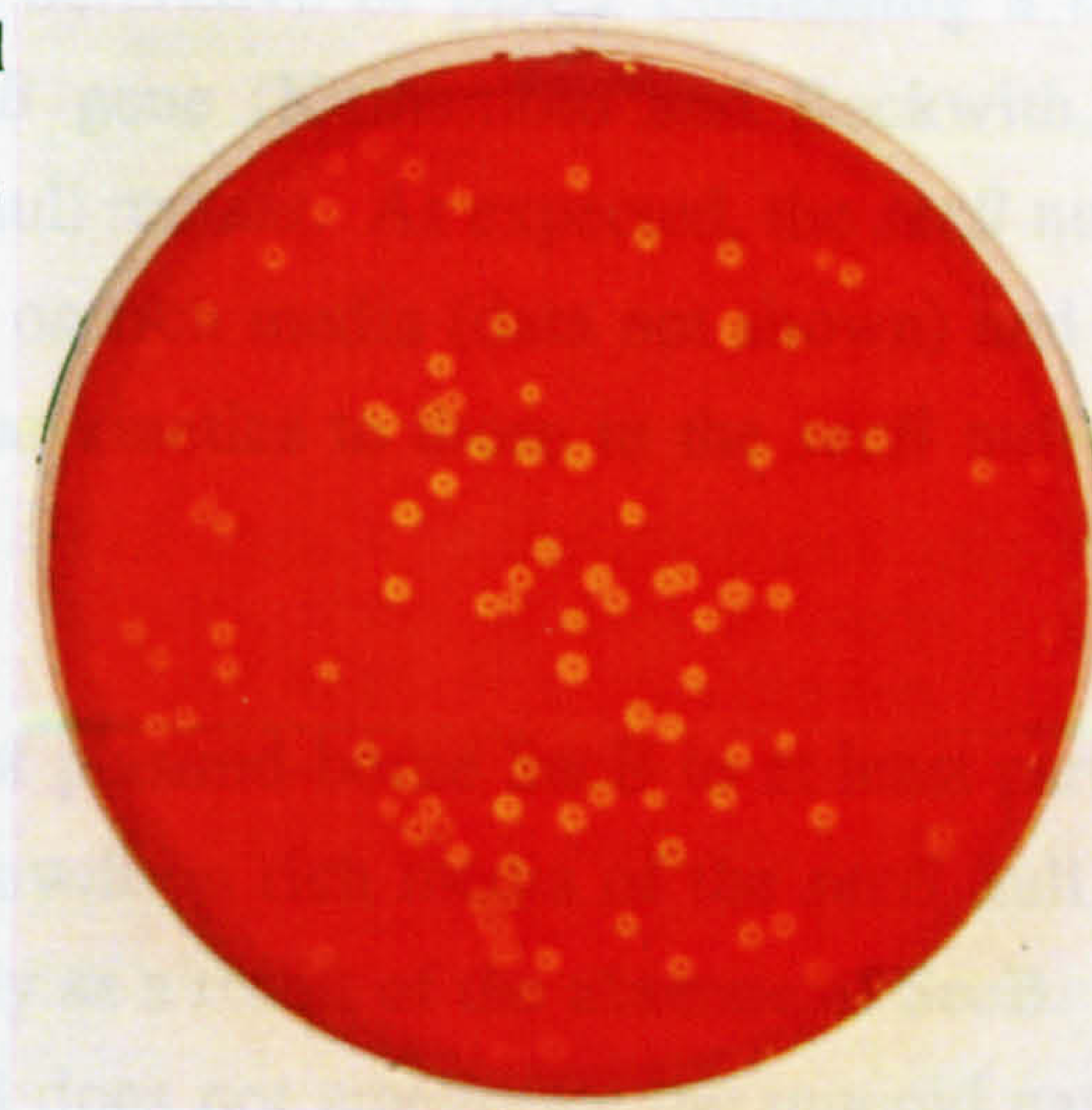


Fig. 4.8 Secretion of haemolysin on nutrient blood agar plates. (a) and (b) Halos produced by the SecB sequestering strain, HB1012pGL1 containing pSF4000, were unaffected by the presence of IPTG. (c) The *secB* null strain, CK1953, containing pDC2 and pSF4000 produced reduced halos on nutrient blood agar. Plates were incubated at 37°C for 16 hours.

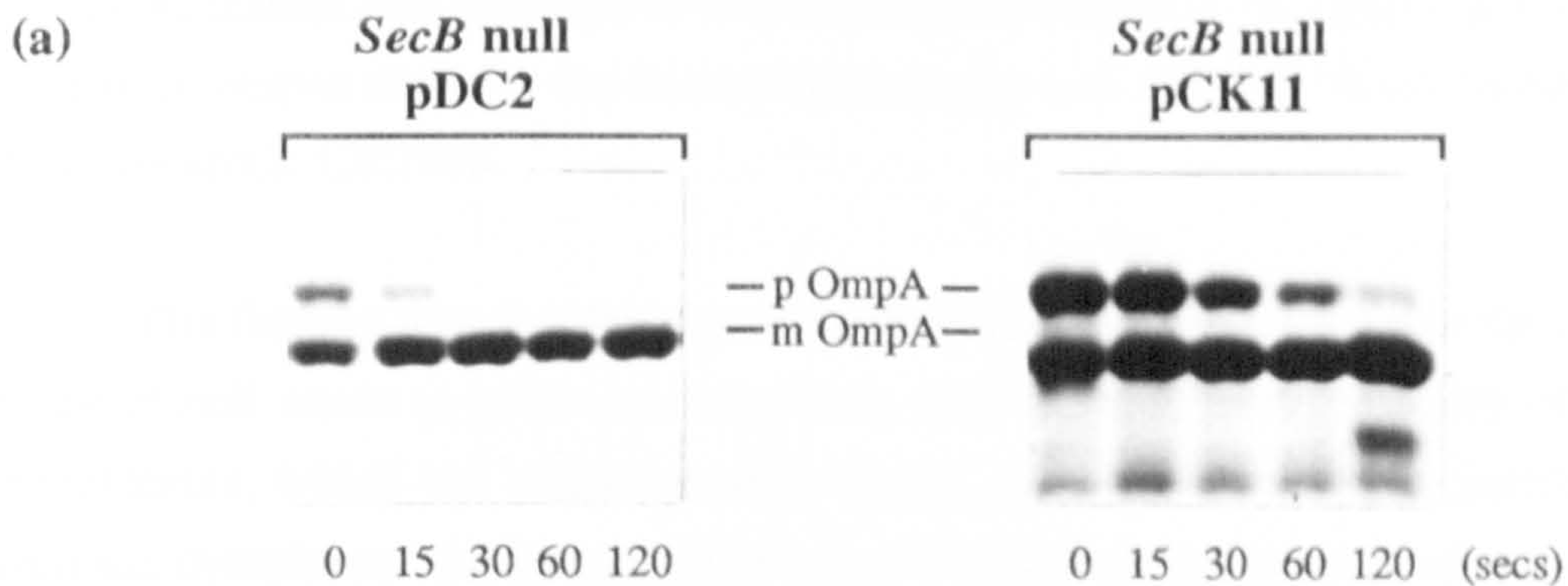
In view of the above observations experiments were carried out using *groEL* and *groES* temperature-sensitive strains (Chapters 6 and 7) in order to determine whether the chaperonin GroEL and its co-chaperonin GroES are involved in the secretion of HlyA.

Throughout this series of experiments using SecB sequestering strains it was apparent that induction of the sequesterers, by IPTG, to deplete functional SecB did not significantly affect the growth rate of the sequestering strains even when propagated on rich media (see Fig4.8(a) and (b)). This is in contrast with the data obtained from the *secB* null strain, CK1953, which indicated that cells were not able to grow on rich media in the absence of SecB (see section 3.1).

4.5 Introduction of SecB into the *secB* null strain (CK1953)

In order to determine whether the inability of the *secB* null strain to grow on rich media occurred as a direct effect of the absence of SecB, a plasmid pDC2, was obtained which encodes wild-type SecB (Clark *et al.*, 1980) and this was used to transform the *secB* null mutant (methods 2.3(i)). Viability of the *secB* null strain, containing pDC2, was indeed restored on rich media (see Fig.4.8(c)) and the kinetics of translocation of OmpA were comparable to those of wild-type cells as shown by pulse-chase immunoprecipitation (Fig.4.9(a) and (b)). As a control the plasmid pCK11, which is a derivative of pDC2 containing a transposon insertion which inactivates the *secB* gene (Kumamoto and Beckwith, 1985), was also used to transform the *secB* null mutant. As expected, the *secB* null strain containing pCK11 was unable to grow on rich media (data not shown) and the translocation of OmpA occurred with similar kinetics to those in the *secB* null strain without the plasmid (Fig.4.9(a) and (b)).

The ability of plasmid-borne SecB to at least partly restore the translocation defect and allow growth on rich media of the *secB* null strain, indicates that these defects occur directly as a result of the absence of SecB. In contrast, the presence of plasmid-borne SecB does not ameliorate the mucoid nature of CK1953 or improve the secretion of HlyA, as shown on blood agar (Fig.4.8(c) - see Fig.3.2(b) for a direct comparison). This suggests that the mucoid phenotype and reduced HlyA secretion may occur as a result of some other feature of CK1953 possibly caused by the strategy used to construct CK1953. Indeed, the above findings and the localisation of the *secB* gene close to the *rfa* locus (involved in lipopolysaccharide (LPS) core synthesis; Kumamoto and Beckwith, 1985) prompt the suggestion that LPS



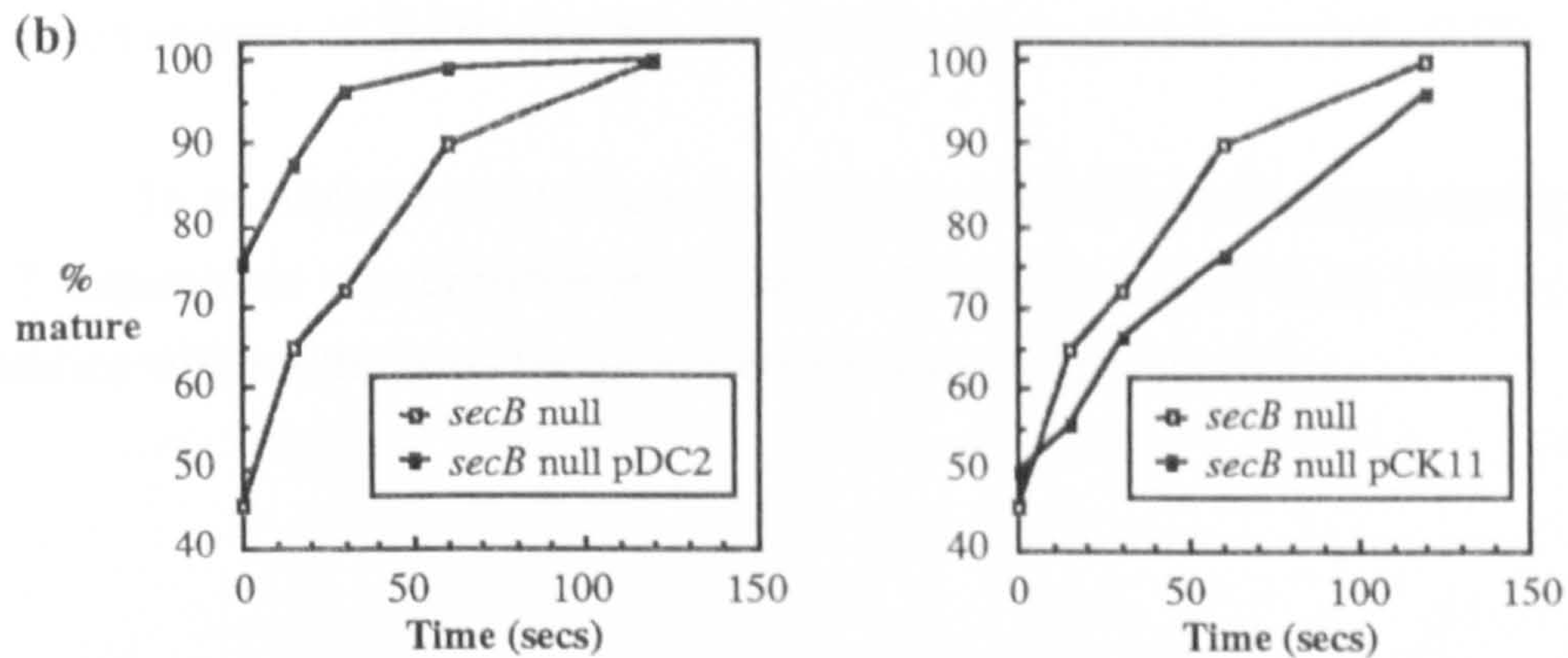


Fig. 4.9 Translocation of OmpA in the *secB* null mutant, CK1953, containing pDC2 or pCK11. Cultures were grown to early logarithmic phase in M9 minimal medium and pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with OmpA antiserum by SDS-PAGE and fluorography. The precursor and mature forms of OmpA are indicated. (b) Kinetics of OmpA translocation as determined by densitometry of fluorographs in (a). The data obtained for OmpA translocation in CK1953 (Fig.3.1) is also shown.

biosynthesis may have been disturbed in CK1953 (Parker *et al.*, 1992). Similarities between *secB* and *rfa* mutants were noted (sensitivity to phage U3) by Kumamoto and Beckwith, (1985) and led them to suggest a possible connection between components involved in the export of proteins and the export of polysaccharides. While this cannot be ruled out an alternative explanation, based upon the above, may be that the mutagenic transposon insertion is exerting a polar effect on *rfa* gene expression which is, in turn, responsible for the mucoid phenotype and reduced HlyA secretion of the *secB* null strain CK1953.

The finding that the absence of SecB is directly responsible for the inability of the *secB* null strain to grow on rich media also suggests that induction of the SecB sequesterers, which are viable on rich media, does not deplete all functional SecB from the cytoplasm. Although the effects of induction of the sequesterers resulted in translocation kinetics comparable to the *secB* null strain it is likely that small residual amounts of functional SecB remain in the cytoplasm following such induction. This limited amount of SecB may be sufficient to allow growth on rich media.

In an attempt to increase the efficiency of the SecB sequestering approach a T7 sequesterer was constructed (Chapter 5) which may also be used in pulse-chase studies without the need for subsequent immunoprecipitation.

CHAPTER 5

CONSTRUCTION OF A T7 SEQUESTERER

5.1 The T7 RNA polymerase expression system

The RNA polymerase and late gene promoters of bacteriophage T7 have been characterised and used to produce an efficient expression system both *in vivo* and *in vitro* (Studier *et al.*, 1990; Nevin and Pratt, 1991). The T7 RNA polymerase shows high activity, elongating chains about five times faster than does *E.coli* RNA polymerase (Chamberlin and Ring, 1973; Golomb and Chamberlin, 1974) and is highly specific for its own promoter, a 23bp sequence which is long enough to be unlikely to occur in any non-T7 DNA (Rosa, 1979; Panayotatos and Wells, 1979). Unlike the multisubunit RNA polymerases of bacteria and eukaryotes, T7 RNA polymerase is a monomeric enzyme of approximately 100kDa (Chamberlin and Ring, 1973; Moffatt *et al.*, 1984) which is unrelated to the *E.coli* polymerase.

E.coli RNA polymerase can be inhibited by the antibiotic rifampicin (Chamberlin *et al.*, 1970) which binds to the β-subunit of the holoenzyme. The T7 RNA polymerase however, is resistant to rifampicin (Chamberlin *et al.*, 1970). Therefore if rifampicin and T7 RNA polymerase are both present, the selective and high-level expression of a gene under the control of the T7 promoter can be achieved.

5.2 Basic strategy

Following characterisation of the SecB sequestering strains (Chapter 4), experiments were undertaken to determine whether it was possible to reproduce and enhance the sequestering effect using the T7 system. By placing the SecB sequesterer, and also the exported protein of interest (in the same or a compatible plasmid), under control of the T7 RNA polymerase, it should be possible to completely inhibit *E.coli* RNA polymerase (by addition of rifampicin) following induction of T7 RNA polymerase, such that only the sequesterer and protein of interest, under T7 expression, are synthesised. Pulse chase experiments could then be performed without the need for immunoprecipitation (assuming that the protein of interest shows different mobility to the sequesterer on SDS-PAGE) to determine the kinetics of translocation of the protein. Control experiments in which the effect of T7 expression of wild-type MBP upon export of the protein of interest would be carried

out in parallel. To facilitate such a strategy several DNA constructs were made and the preparation of these constructs is described below.

In order to construct the plasmids encoding wild-type and mutant MBP under the control of the T7 promoter it was decided to isolate *E. coli* genomic DNA and amplify the wild type *malE* gene by PCR using suitable primers (Fig.5.1(a)). Primers were designed such that the *malE* gene could be excised using the restriction enzymes *Sac*I and *Bam*HI respectively. The alteration of a single base in the *malE* 5' primer introduced a unique *Sac*I restriction site and alteration of a single base in the *malE* 3' primer introduced a unique *Bam*HI restriction site (Fig.5.1(a)).

Experience with cloning PCR fragments has led to it being the preferred strategy of the laboratory to blunt end clone PCR products into a suitable vector and then re-excise, using the engineered restriction sites, and transfer to the vector of choice, as this circumvents the problems associated with limited digestion of PCR products by restriction enzymes when restriction sites are situated at or near to the ends of the PCR products. The initial PCR product was cloned into pACYC184 and then transferred to the T7 expression vector, pT7-5.

5.3 Cloning of wild-type *malE* into pACYC184 (pJPW11)

Genomic DNA was prepared from $malE^{+}$ (DH5α and MC4100) strains (Fig.5.1(b)) (methods 2.3(x)) and used as the template to produce relatively large quantities of the *malE* gene by PCR (methods 2.3(xii)) using the primers illustrated (Fig.5.1(a) and (c)). Approximately 1μg of the PCR fragment was treated with proteinase K and purified prior to treatment with polynucleotide kinase to add 5' phosphates and Klenow to produce blunt ends (methods 2.3(xiii)). The resultant DNA was re-purified (methods 2.3(vi)) and ligated (methods 2.3(xv)) into the *Eco*RV site of alkaline phosphatase treated (methods 2.3(xiv)) pACYC184. Following transformation into frozen competent DH5α cells (methods 2.3(ii)) and selection of transformants, by growth on nutrient plates with chloramphenicol, plasmid was isolated from 40 colonies (methods 2.3(ix)). Recombinant plasmids were identified by a decrease in mobility on 0.8% agarose gel compared with vector alone, and the correct fragment orientation for expression of the *malE* gene from the tetracycline promoter in pACYC184 was determined by *Nco*I digestion (Fig.5.2). Positive recombinants expressing MBP were then identified by Western blot analysis of cultures, grown in the presence of glucose to inhibit chromosomal *malE* expression, using MBP antiserum (data not shown). Following large scale preparation of the

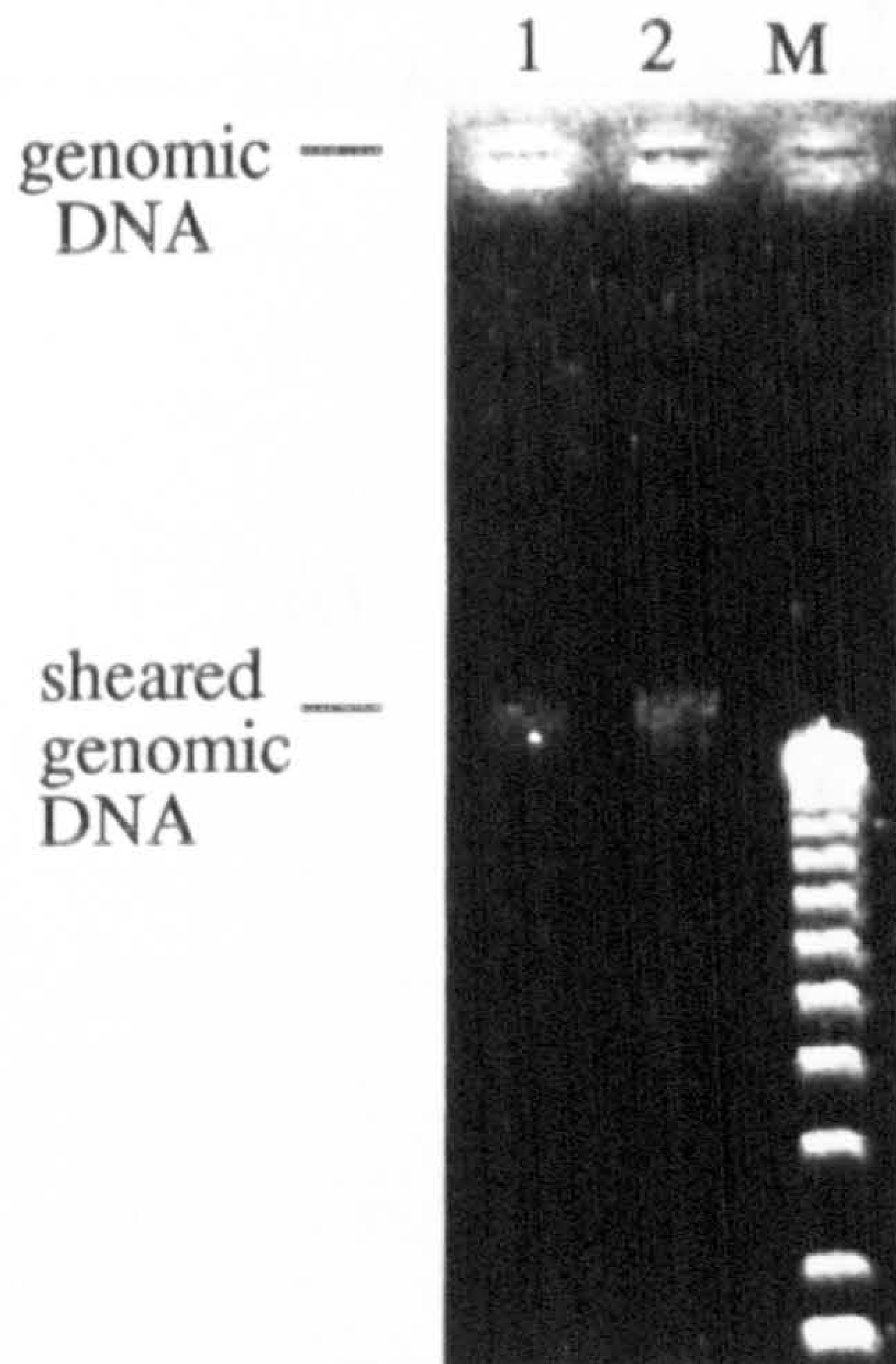
(a) ***malE* 5' primer**

5' - C GA G CT C TT CAC CAA CAA GGA CCA - 3'

***malE* 3' primer**

5' - C CG G AT CC G GCA TTT CAC AGC A - 3'

(b)



(c)

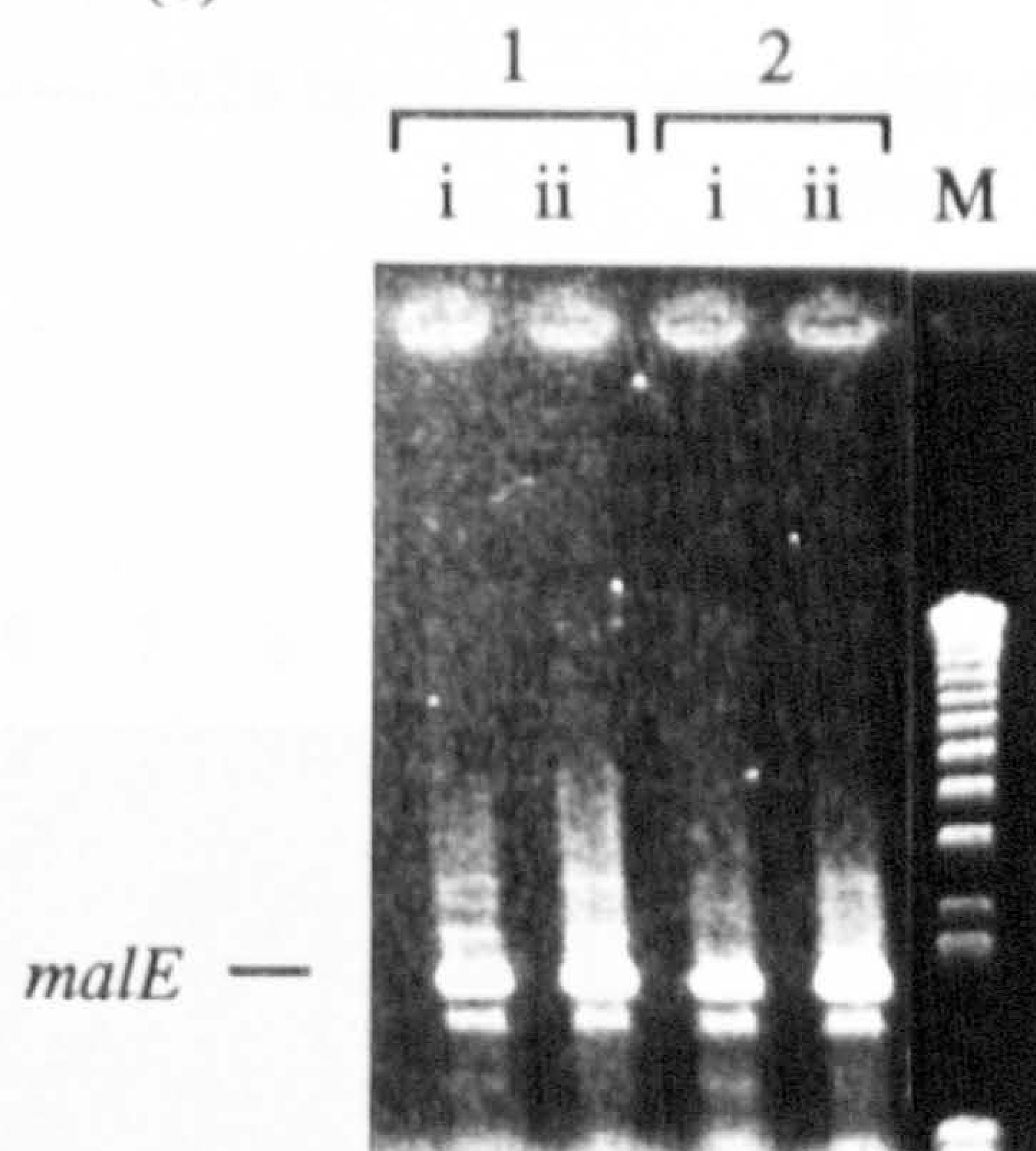
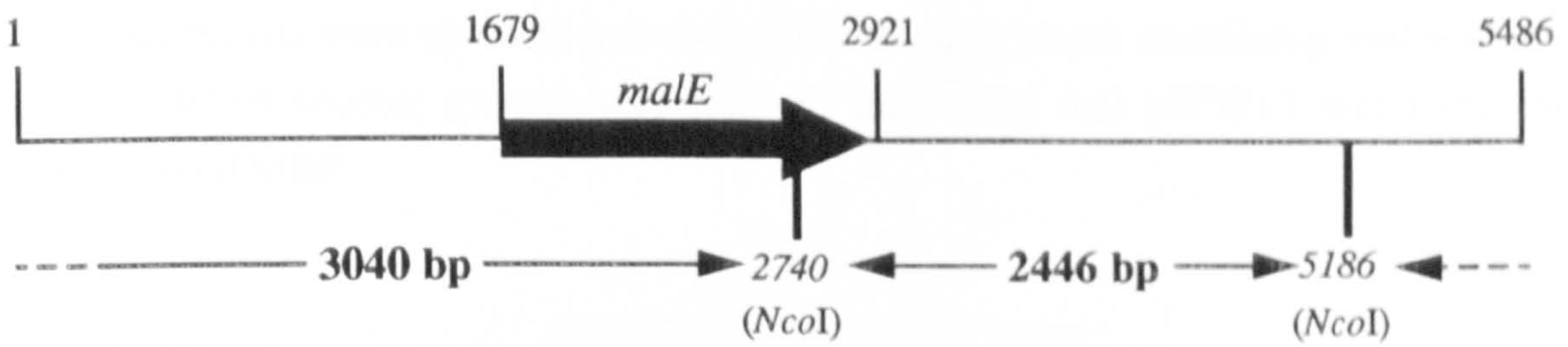
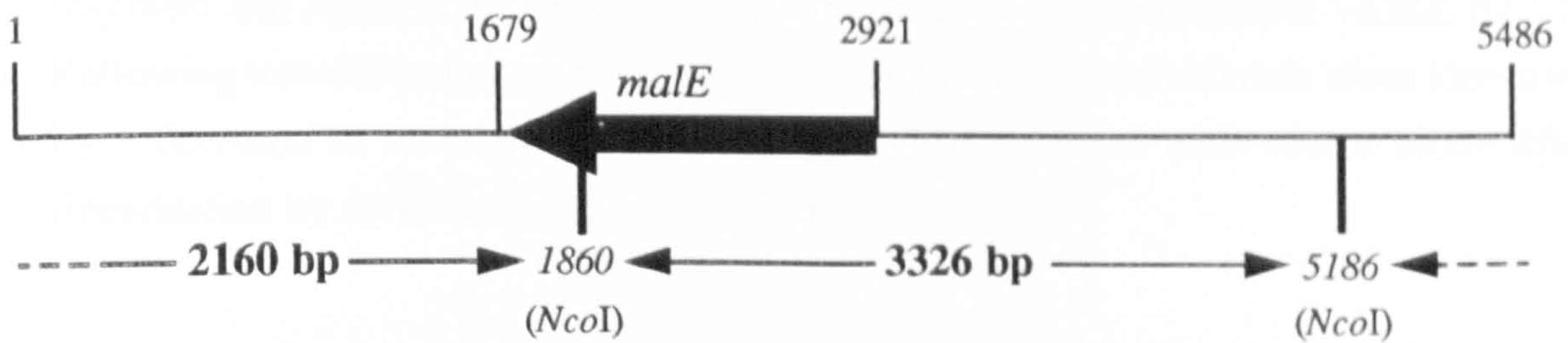


Fig. 5.1 Primers designed to PCR amplify the *malE* gene and analysis of genomic DNA preparations and PCR products. (a) Sequences of 5' primer with base alteration from A to T (shown in bold) to introduce a *Sac*I site (underlined) and 3' primer with base alteration from C to G (shown in bold) to introduce a *Bam*HI site (underlined). Analysis by electrophoresis on 0.8% agarose gels of; (b) Genomic DNA samples from (1) DH5α and (2) MC4100 strains prepared as described in materials and methods (2.3(x)). (c) PCR products from the genomic DNA samples in (b) using the primers in (a). PCR was carried out as described in materials and methods (2.3(xii)) using (i) 25mM Mg^{2+} PCR buffer (ii) 15mM Mg^{2+} PCR buffer. M - 1 kilobase ladder from Gibco-BRL.

(a) Correct orientation for expression of *malE*



(b) Incorrect orientation for expression of *malE*



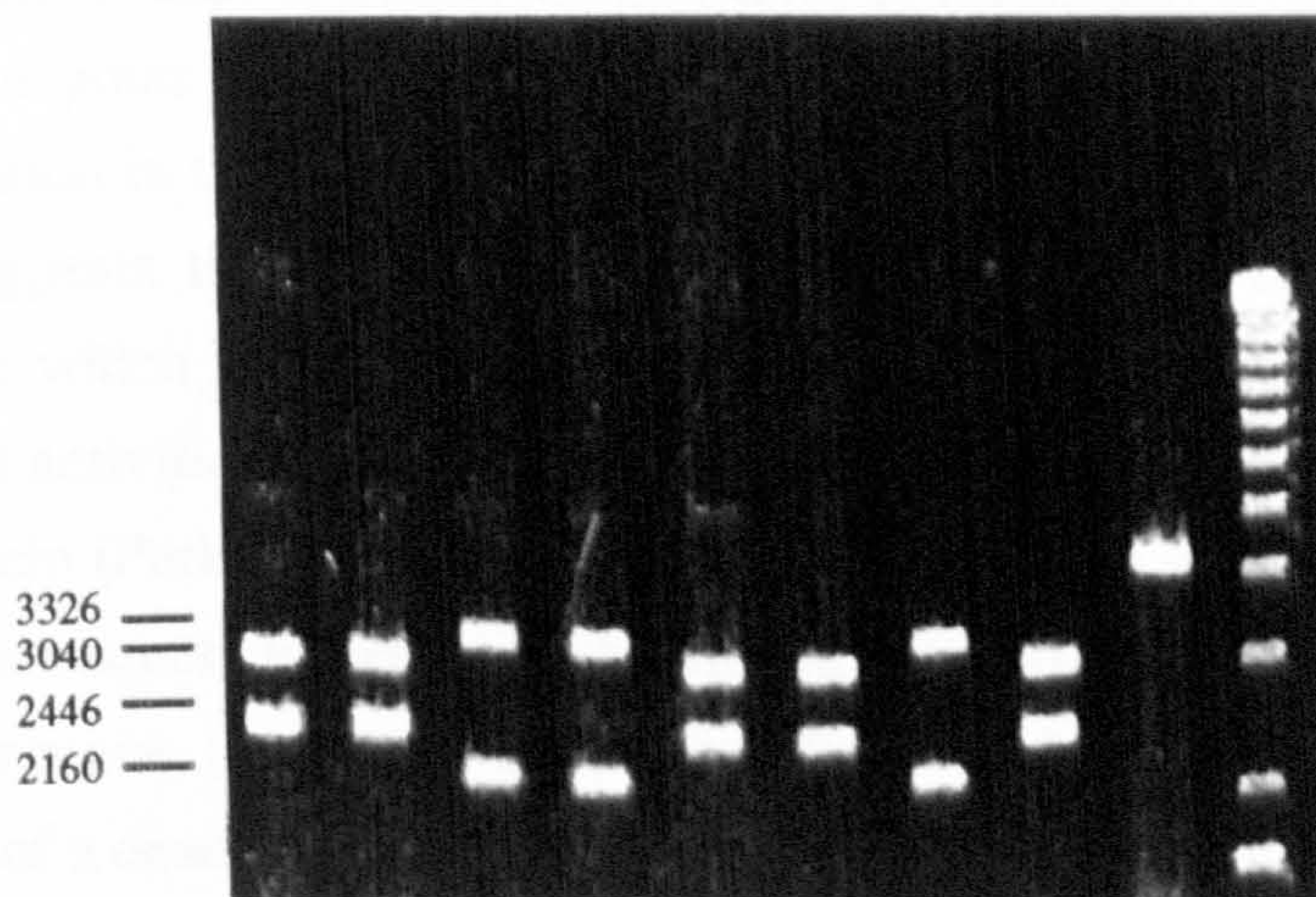


Fig. 5.2 Linear representation of the recombinant plasmid showing possible orientations of the *malE* gene and fragment sizes generated by *Nco*I digestion. All coordinates are relative to the G of the *Eco*RI site of pACYC184 at position 1; figures in bold - fragment sizes. Expression of *malE* in (a) is directed by the promoter of the tetracycline resistance gene of pACYC184, the function of which is abolished by insertion of the *malE* gene. (c) *Nco*I digests of recombinants. Samples 1, 2, 5, 6 and 8 - correct orientation for expression. Samples 3, 4 and 7 - incorrect orientation for expression. V - *Nco*I digest of vector pACYC184. M - 1 kilobase ladder from Gibco-BRL.

plasmid, named pJPW11, it was used to transform the strain HB1012 pGL1 (which is effectively *malE*⁻) (methods 2.3(vii) and (viii)). After growth on nutrient plates, transformants were streaked onto M9 minimal agar plates containing maltose as the sole carbon source; growth was restored indicating that pJPW11 was expressing functional MBP.

5.4 Cloning of the wild-type *malE* gene under the control of the T7 promoter (pJPW12)

The *malE* gene was excised from pJPW11 on a (1.2kbp) *Sac*I-*Bam*HI fragment and ligated into the *Sac*I-*Bam*HI digested, dephosphorylated vector, pT7-5. Following transformation and plasmid isolation positive recombinants were identified by a decrease in mobility on 0.8% agarose gel compared with vector alone after linearisation by *Hind*III digestion (Fig.5.3).

5.5 Cloning of a SecB sequestering *malE* gene under the control of the T7 promoter (pJPW15)

In an attempt to obtain a sequesterer of maximum efficiency it was decided to combine the point mutation in the MBP signal sequence, encoded by pJW12, with the point mutation in the mature region of MBP, encoded by pGL1. Work by Liu *et al.*, (1989) suggests that such a combination of mutations in MBP may result in a sequesterer which interacts with SecB with very high efficiency due to the combined antifolding activities of both the signal peptide and the mutation in the mature region of the protein (Park *et al.*, 1988; Liu *et al.*, 1988). The signal peptide point mutation prevents subsequent targeting of the mutant MBP to the translocation apparatus at the inner membrane (as is the case for MBP encoded by pJW12), resulting in the formation of a dead-end complex with SecB (see Fig.4.1).

The strategy used to construct the "T7 sequesterer", is shown in Fig.5.4. PCR was used to amplify the pJW12 encoded *malE* gene, using the 5' and 3' primers shown in Fig.5.1(a), so introducing a *Sac*I site 24 bases upstream of the start codon, to facilitate the isolation of a (459bp) *Sac*I-*Bgl*II fragment containing the DNA sequence encoding the MBP signal sequence mutation (Fig.5.4, step 1).

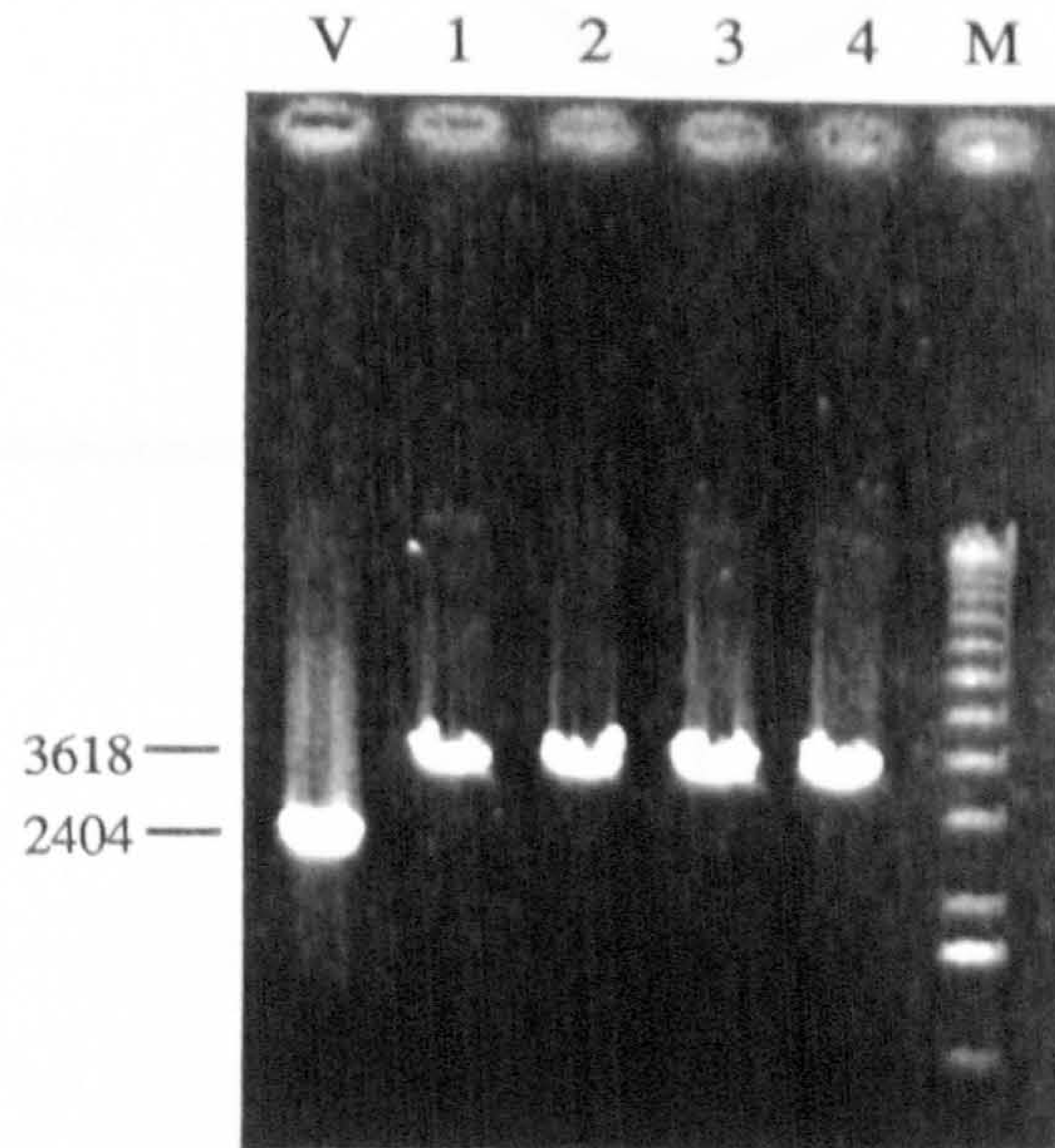


Fig. 5.3 Identification of successful pJPW12 recombinants by *Hind*III digestion. Following transformation and plasmid isolation of possible recombinants *Hind*III digestion was performed and samples analysed by electrophoresis on a 0.8% agarose gel. V - *Hind*III digest of vector pT7-5. M - 1 kilobase ladder from Gibco-BRL. Sizes of linearised recombinants and vector are indicated.

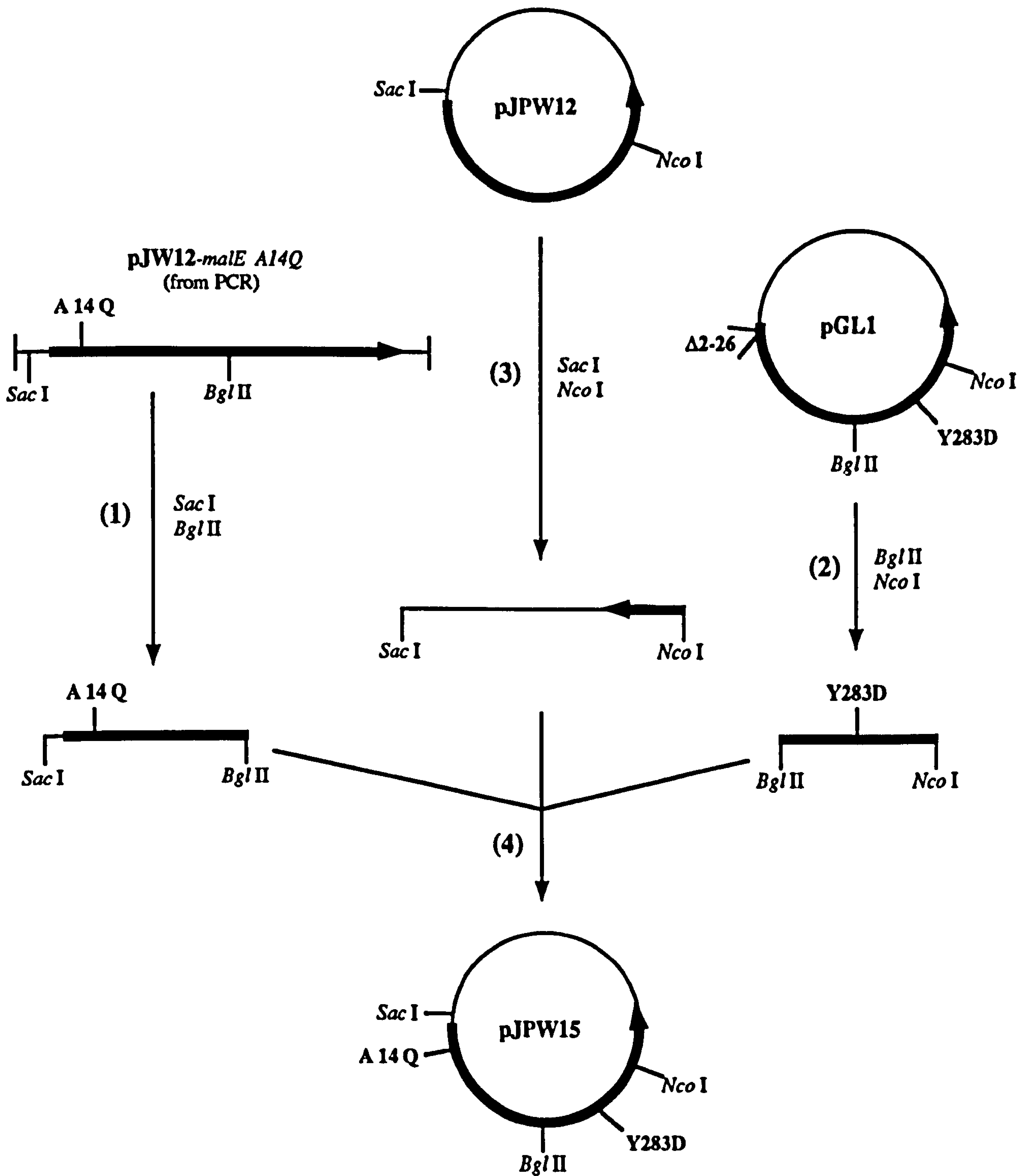


Fig. 5.4 Schematic diagram showing the strategy used to construct the T7 sequesterer (*malE A14Q, Y283D*). (1) Excision of a 459bp *Sac*I-*Bgl*II fragment from pJW12 PCR product. (2) Excision of a 602bp *Bgl*II-*Nco*I fragment from pGL1. (3) Excision of a 1061bp *Sac*I-*Nco*I fragment from pJPW12. (4) Three way ligation to produce pJPW15 (3618bp)

A (602bp) *Bgl*II-*Nco*I fragment containing the DNA sequence encoding the mutation in the mature domain of MBP was excised from pGL1 (Fig.5.4, step 2).

Following excision of the (1061bp) *Sac*I-*Nco*I fragment from pJPW12 (Fig.5.4, step 3) a three way ligation (Fig.5.4, step 4) was performed to create a double mutant *malE* gene and produce pJPW15. After transformation and plasmid isolation the identity of positive recombinants was confirmed by restriction analysis using *Bgl*II, *Nco*I and *Sac*I to confirm plasmid size and reconstruction of the restriction sites.

5.6 Characterisation of the T7 sequesterer

(i) Identification of the gene product

Expression from the T7 promoter was determined in T7 RNA polymerase dependent *in vitro* transcription-translation reactions performed in the presence of rifampicin (methods 2.2(xii)) (Fig.5.5).

Following transformation into the T7 expression strain BL21(DE3)pRG1 (see strains 2.4) the effects of T7 RNA polymerase induction on expression of the wild-type and mutant MBP proteins, encoded by pJPW12 and pJPW15 respectively, were assayed *in vivo* (Fig.5.6). It is clear from the stained profile that following induction of T7 RNA polymerase (methods 2.2(xi)) significant levels of the T7 sequesterer, encoded by pJPW15, accumulate after 20 minutes becoming more obvious after 30 minutes. A similar pattern of expression is seen for wild-type MBP, encoded by pJPW12, although it does not appear to be induced to the same levels for reasons which are not clear. Western blot analysis using MBP antiserum (Fig.5.6(b)) confirmed the induction of the two MBP forms.

(ii) Effects of induction of the T7 sequesterer on protein translocation

The effect of induction of the T7 sequesterer on the translocation of the SecB-dependent protein OmpA was then studied. Pulse-chase immunoprecipitation experiments were carried out (methods 2.2(ii)) in the presence of pJPW12 (wild-type MBP) or pJPW15 (T7 sequesterer) both before and after induction of T7 RNA polymerase respectively (methods 2.2(xi)). Wild-type MBP induction did not affect the translocation of OmpA which occurs with wild-type kinetics both before and after

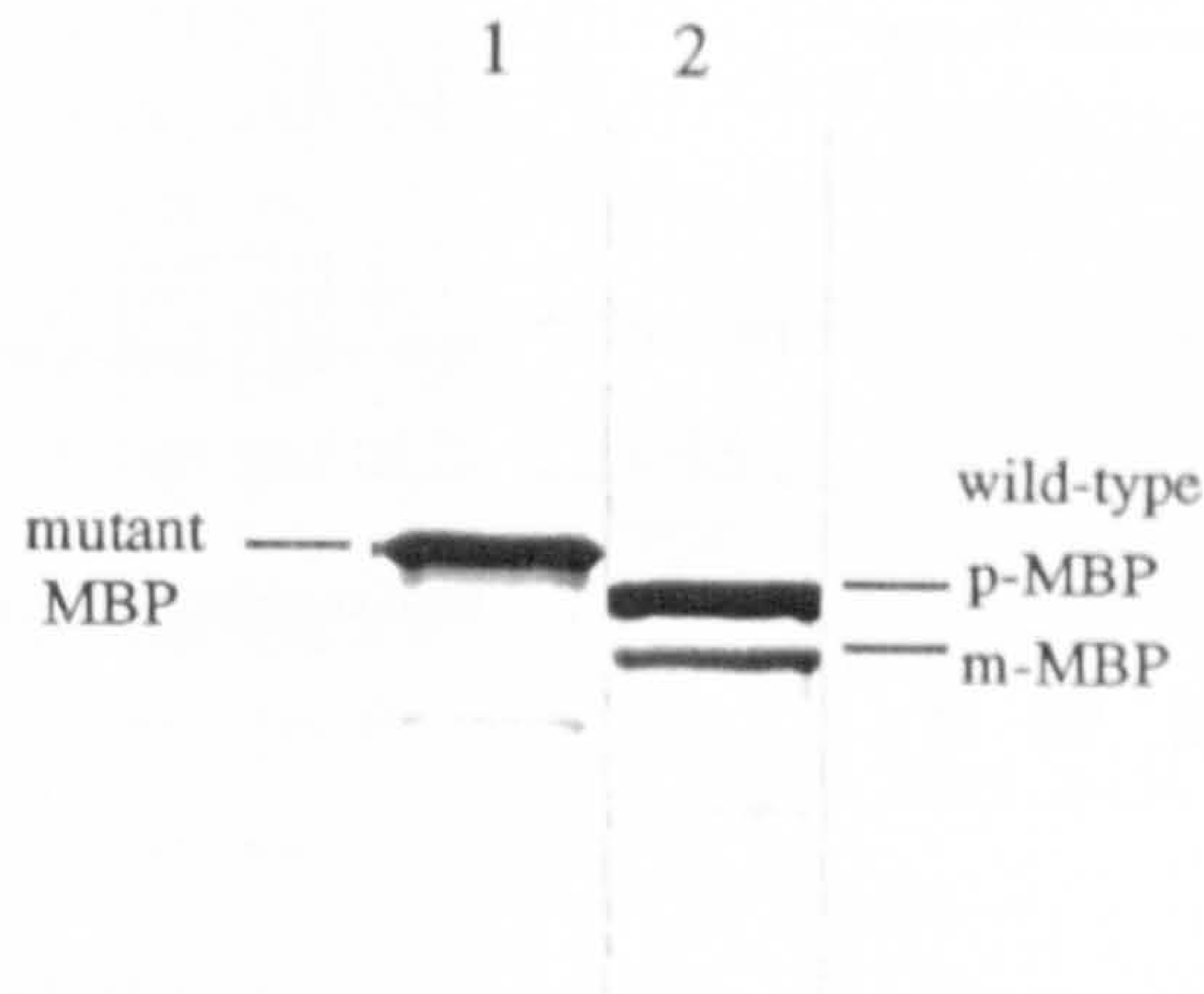


Fig. 5.5 *In vitro* expression of mutant and wild-type forms of MBP. *In vitro* transcription-translation reactions were carried out using (1) pJPW15 or (2) pJPW12 respectively, in the presence of rifampicin (at a final concentration of 0.6mg/ml), and analysed by SDS-PAGE and fluorography as described in materials and methods (2.2(xii)). The positions of the products are indicated. The presence of mature MBP (lane 2) is due to the presence of endogenous vesicles in the S30 extract.

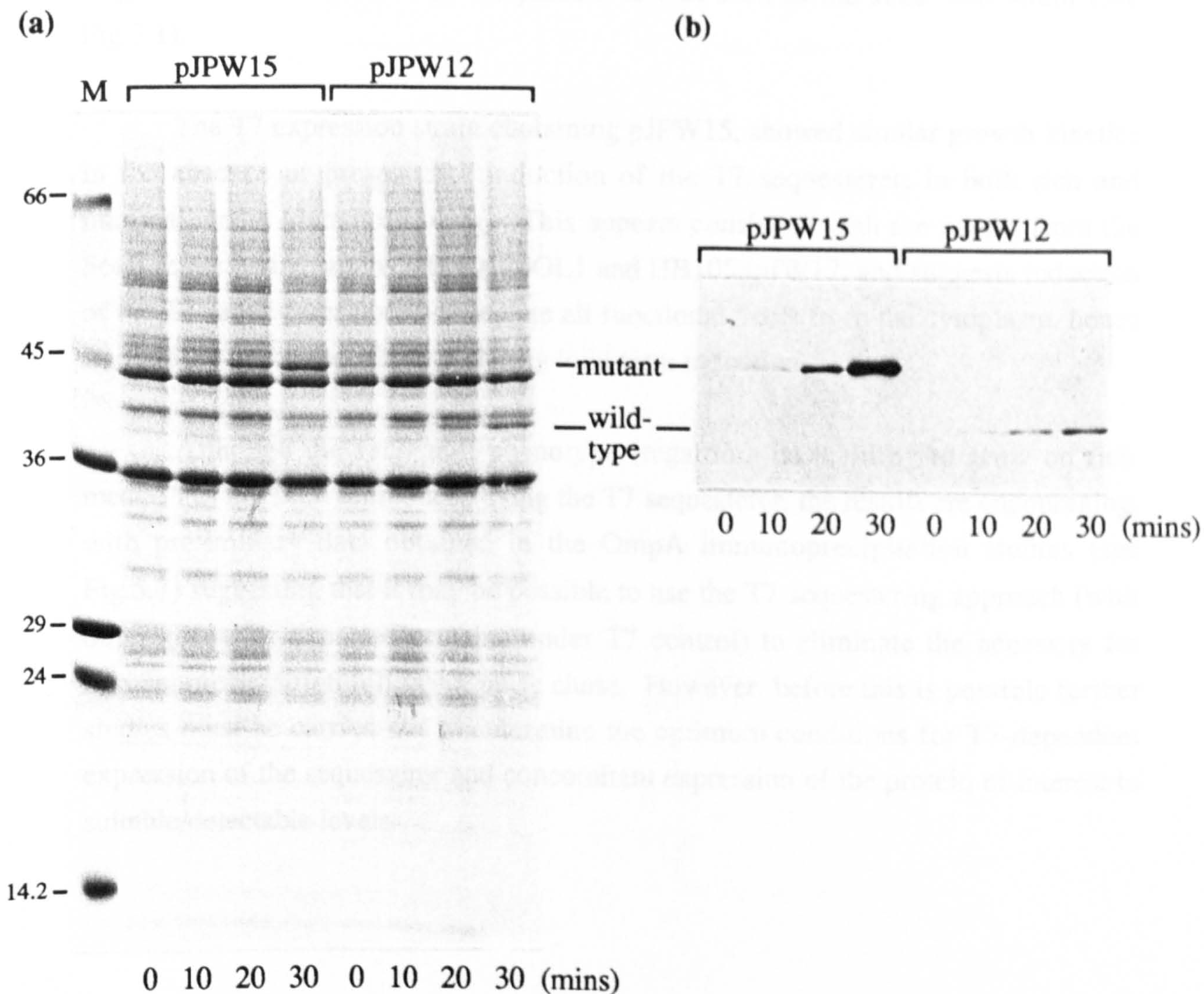


Fig. 5.6 Synthesis of mutant and wild-type MBPs encoded by pJPW15 and pJPW12 following T7 RNA polymerase induction. Cultures of the T7 expression strain BL21(DE3)pRG1 containing pJPW15 or pJPW12 were grown in nutrient medium and induced with IPTG (0.4mM) for the times indicated. Equivalent samples (0.1 A_{600} units in (a): 0.03 A_{600} units in(b)) were analysed by SDS-PAGE and (a) Coomassie blue staining or (b) Western blotting using MBP antiserum. The positions of mutant and wild-type MBP proteins are indicated. M - SDS7 markers from Sigma.

induction (Fig.5.7). Before induction of the T7 sequesterer, the kinetics of OmpA translocation are comparable to wild-type. However, following 30 minute induction of the T7 sequesterer the rate of translocation of OmpA is significantly decreased (Fig.5.7), to a level which is comparable to that seen in the *secB* null strain (see Fig.3.1).

The T7 expression strain containing pJPW15, showed similar growth kinetics in the absence or presence of induction of the T7 sequesterer, in both rich and minimal media (data not shown). This appears consistent with the results from the SecB sequestering strains, HB1012pGL1 and HB1053pJW12, and suggests induction of the T7 sequesterer does not deplete all functional SecB from the cytoplasm, hence its viability on rich media even during long term induction.

Although the *secB* null phenotype (regarding its inability to grow on rich media) has not been reproduced using the T7 sequesterer, the results are encouraging, with preliminary data obtained in the OmpA immunoprecipitation studies (see Fig.5.7) suggesting that it may be possible to use the T7 sequestering approach (with other genes for exported proteins under T7 control) to eliminate the necessity for immunoprecipitation following pulse chase. However, before this is possible further studies must be carried out to determine the optimum conditions for T7-dependent expression of the sequesterer and concomitant expression of the protein of interest to suitable/detectable levels.

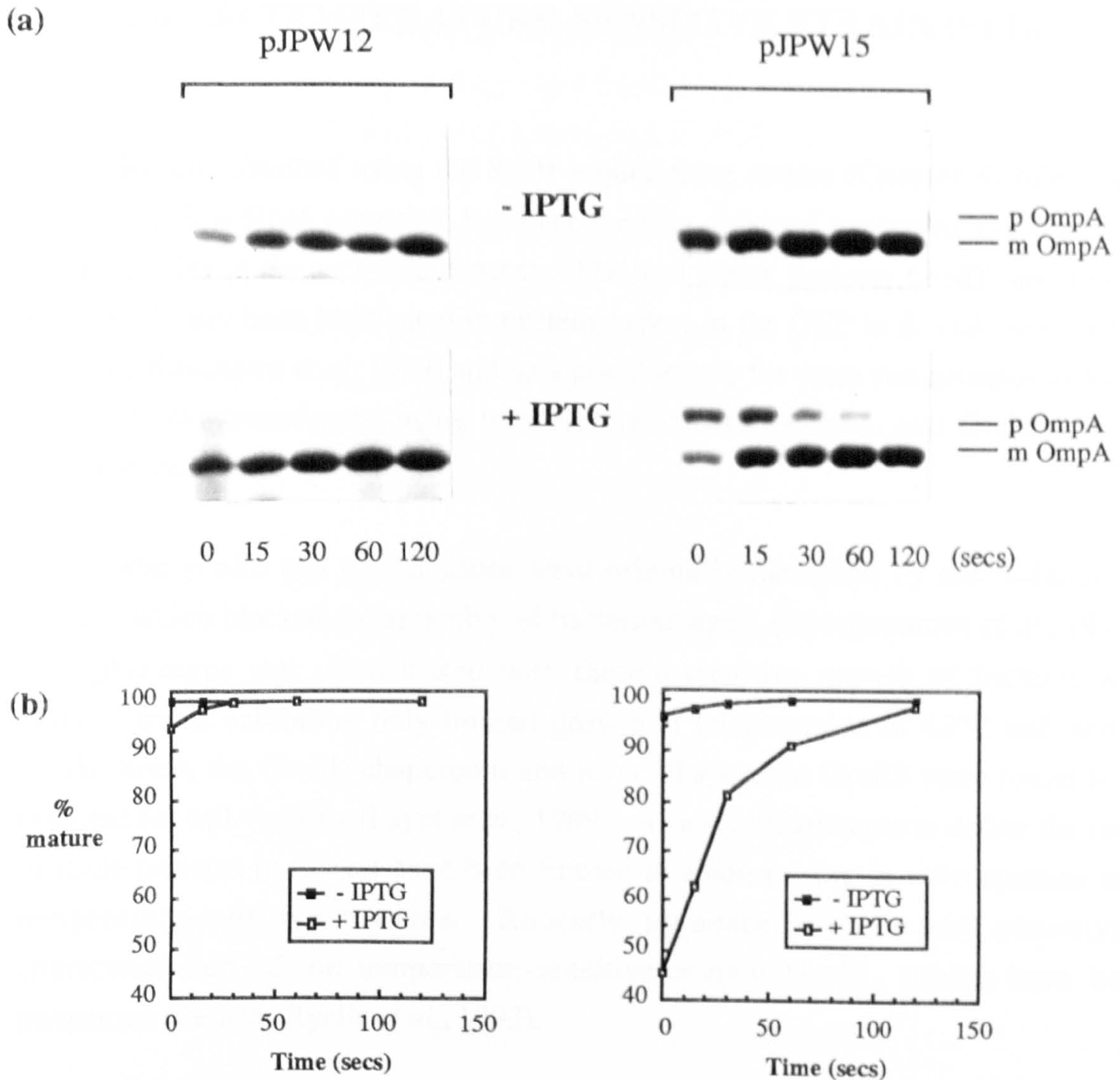


Fig. 5.7 Reduced efficiency of OmpA translocation following induction of the T7-sequesterer. Cultures of BL21DE3pRG1 containing pJPW12 or pJPW15 propagated in M9 minimal medium were grown to early logarithmic phase and the cultures split in two; one half had no additions (-IPTG) while the other was induced by addition of IPTG to 0.4mM (+IPTG). Incubation was continued for 30 mins and then the cultures were pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with OmpA antiserum by SDS-PAGE and fluorography. The precursor and mature forms of OmpA are indicated. (b) Kinetics of OmpA translocation as determined by densitometry of fluorographs in (a).

CHAPTER 6

INVESTIGATION OF HAEMOLYSIN SECRETION IN THE *groEL44* TEMPERATURE-SENSITIVE STRAIN (SF103)

Results obtained using the SecB sequestering strains (Chapter 4) ruled out a role for SecB in HlyA secretion, however the data obtained suggested a role for heat shock proteins in the secretion process. The heat shock proteins GroEL and GroES have previously been implicated in protein export in the GEP in *E. coli* (see section 1.18(iii); Kusukawa *et al.* 1989) and so a possible role for these two proteins in HlyA secretion was investigated using temperature-sensitive GroEL and GroES mutant strains respectively.

The *groEL* and *groES* genes were originally identified by the isolation of mutants which blocked the assembly of bacteriophage λ (Georgopoulos *et al.*, 1973). This phenotype was often linked with thermo-sensitive growth of bacteria with mutant strains exhibiting only limited growth at temperatures of 42°C and above. Unlike SecB, the GroEL chaperonin and its co-chaperonin GroES were found to be essential for cell viability (Fayet *et al.*, 1989). As a result, attempts to define the roles of these proteins in *E. coli* have been limited to studies using *in vitro* systems and temperature-sensitive mutants. Recently sequence analysis and phenotypic characterisation of the temperature-sensitive mutant GroEL strains have been performed (Zeilstra-Ryalls *et al.*, 1993).

This chapter documents studies which were carried out in order to determine whether the chaperonin GroEL is necessary for the efficient secretion of HlyA. In the following chapter (Chapter 7) experiments carried out to elucidate the potential involvement of the co-chaperonin GroES in HlyA secretion are discussed.

6.1 The *groEL44* temperature-sensitive strain (SF103)

The *groEL44* temperature-sensitive strain SF103 was produced by P1 transduction (see strains 2.4; personal communication, P. Lund) of the wild-type strain TG1 using the *groEL44* temperature-sensitive strain CG2241 (Zeilstra-Ryalls *et al.*, 1993). The *groEL44* allele contains a point mutation within the *groEL* coding sequence, at codon 191 (Glu to Gly), which is thought to be responsible for the temperature-sensitive phenotype (Zeilstra-Ryalls *et al.*, 1993).

6.2 Characterisation of the *groEL44* temperature-sensitive strain

The phenotype and characteristics of the *groEL44* mutant strains, with respect to growth and protein translocation in particular, have not been well documented to date and so preliminary experiments were carried out in order to identify and confirm the nature of strain SF103 as a *groEL* temperature-sensitive mutant.

It was hoped that the preliminary studies would provide interesting data, not only with respect to the *groEL44* mutant but also regarding the plasmids pHO7 (encoding GroEL) which was used in the GroEL studies and pHO8 (encoding GroES, which was used in Chapter 7), because these plasmids had not been used before in such experiments. In addition, experiments were carried out in order to obtain essential control data for subsequent HlyA secretion assays.

All experiments were performed in parallel using both the parent strain, TG1, and the *groEL44* mutant strain, SF103, and growth was carried out at the permissive temperature of 30°C unless otherwise stated.

(i) Growth of the *groEL44* temperature-sensitive strain

Transformations of the wild-type strain and *groEL44* mutant were carried out using the plasmids shown in Table 6.1 and transformants were selected by growth on nutrient agar plates containing appropriate antibiotics (methods 2.3(i)). Transformants were then plated onto fresh nutrient agar plates containing appropriate antibiotics and incubated at 30, 37 and 42°C for 24 hours respectively. The viability and growth of the parent and *groEL44* mutant strain, containing the various plasmids, at the different temperatures are summarised in Table 6.1.

It was clear (Table 6.1) that the *groEL44* mutant strain, SF103, was unable to grow at the non-permissive temperature unless a plasmid supplying wild-type GroEL was present. Plasmids pOF39 and pHO7 were both able to rescue the defect seen in SF103 at the non-permissive temperature. Plasmid pOF39 was derived from pBR325 containing a 2.2kb insert which encodes the *groEL* and *groES* genes expressed from their promoter (Fayet *et al.*, 1986). Plasmid pHO7 is derived from pJF118EH (Furste *et al.*, 1986) and encodes the *grcEL* gene under control of the *tac* promoter/*lac* repressor (*lacI^q*) (P. Lund, personal communication). The ability of plasmid pHO7 to rescue the temperature-sensitive growth defect of the *groEL44* mutant even in the

Wild-type	Growth		
Plasmids	30°C	37°C	42°C
-	++	+++++	++++
pBR325	++	+++++	++++
pSF4000	++	+++++	++++
pOF39	++	+++++	++++
pOF39pSF4000	++	+++++	++++
pH07	++	+++++	++++
pH07 (+IPTG)	++	+++++	++++
pH07pSF4000	++	+++++	++++
pH07pSF4000 (+IPTG)	++	+++++	++++

groEL44 mutant	Growth		
Plasmids	30°C	37°C	42°C
-	++	+++	n.c.
pBR325	++	+++	n.c.
pSF4000	++	+++	n.c.
pOF39	++	+++++	++++
pOF39pSF4000	++	+++++	++++
pH07	++	+++++	+++
pH07 (+IPTG)	++	+++++	+++
pH07pSF4000	++	+++++	+++
pH07pSF4000 (+IPTG)	++	+++++	+++

Table 6.1 Viability and growth of the wild-type and *groEL44* mutant strains at elevated temperatures. The wild-type strain, TG1, and *groEL44* mutant, SF103, containing the plasmids indicated were plated on fresh nutrient agar plates and incubated for 24 hours at 30, 37 and 42°C as indicated. Sizes of colonies produced are shown relative to those of TG1 grown at 37°C (+++++). n.c. - no colonies.

absence of inducer (IPTG) indicated that leaky expression was occurring, which has since been confirmed by P. Lund (personal communication).

Similar experiments were also performed in liquid media. Preliminary growth curves carried out in minimal media including M9, M63 and Minimal E medium indicated that growth was poor and inconsistent in minimal media (data not shown) (Horwich *et al.*, (1993) found that a more severe *groEL* mutant was incapable of any growth in minimal media). All subsequent growth was carried out in nutrient medium with relevant antibiotics. Following growth of cultures to early logarithmic phase (A_{600} of approximately 0.2) cultures were split and half was maintained at 30°C and half was transferred and incubated at 42°C. Growth of the cultures at 30°C and 42°C was monitored (methods 2.2(i)). The growth curves of cultures of particular interest are shown in Fig.6.1.

The growth rates of strains containing the haemolysin plasmid pSF4000 were comparable to those strains without pSF4000 indicating that the presence of pSF4000 did not adversely affect the strains (data not shown). Also, the growth rate of wild-type cells was unaffected by the presence of pOF39 or pHO7 (data not shown).

Following a shift from 30 to 42°C the *groEL44* mutant strain, containing pSF4000, continued to grow for approximately 60 minutes before a decrease in growth rate was seen, after which time the growth rate decreased until approximately 4 hours after the shift when growth ceased altogether (Fig.6.1(b)). However even growth of the wild-type strain for prolonged periods (over 2 hours) at 42°C resulted in a decrease in the rate of growth (Fig.6.1(a)). Complementation of the temperature-sensitive growth phenotype of the *groEL44* mutant by the GroEL plasmids was again apparent, with both pOF39 and pHO7 restoring growth to almost wild-type levels (Fig.6.1(c) and (d)). This was particularly surprising in strains containing pHO7 grown in the absence of (IPTG) inducer at 42°C, as the *groEL44* mutant has been shown to be weakly *trans*-dominant (Zeilstra-Ryalls *et al.*, 1993). Therefore, it may be expected that induction of mutant GroEL, following a shift to 42°C, would result in a "swamping out" of the uninduced plasmid-encoded wild-type GroEL by formation of mixed wild-type and mutant GroEL oligomers, as was suggested to occur by Zeilstra-Ryalls *et al.*, (1993). This would perhaps be expected to allow only limited complementation in the absence of IPTG. Strains containing pHO7 grown in the presence of (IPTG) inducer (added 30 minutes prior to temperature shift from 30 to 42°C) showed similar growth kinetics (data not shown) to those grown in the absence of inducer (Fig.6.1(d)).

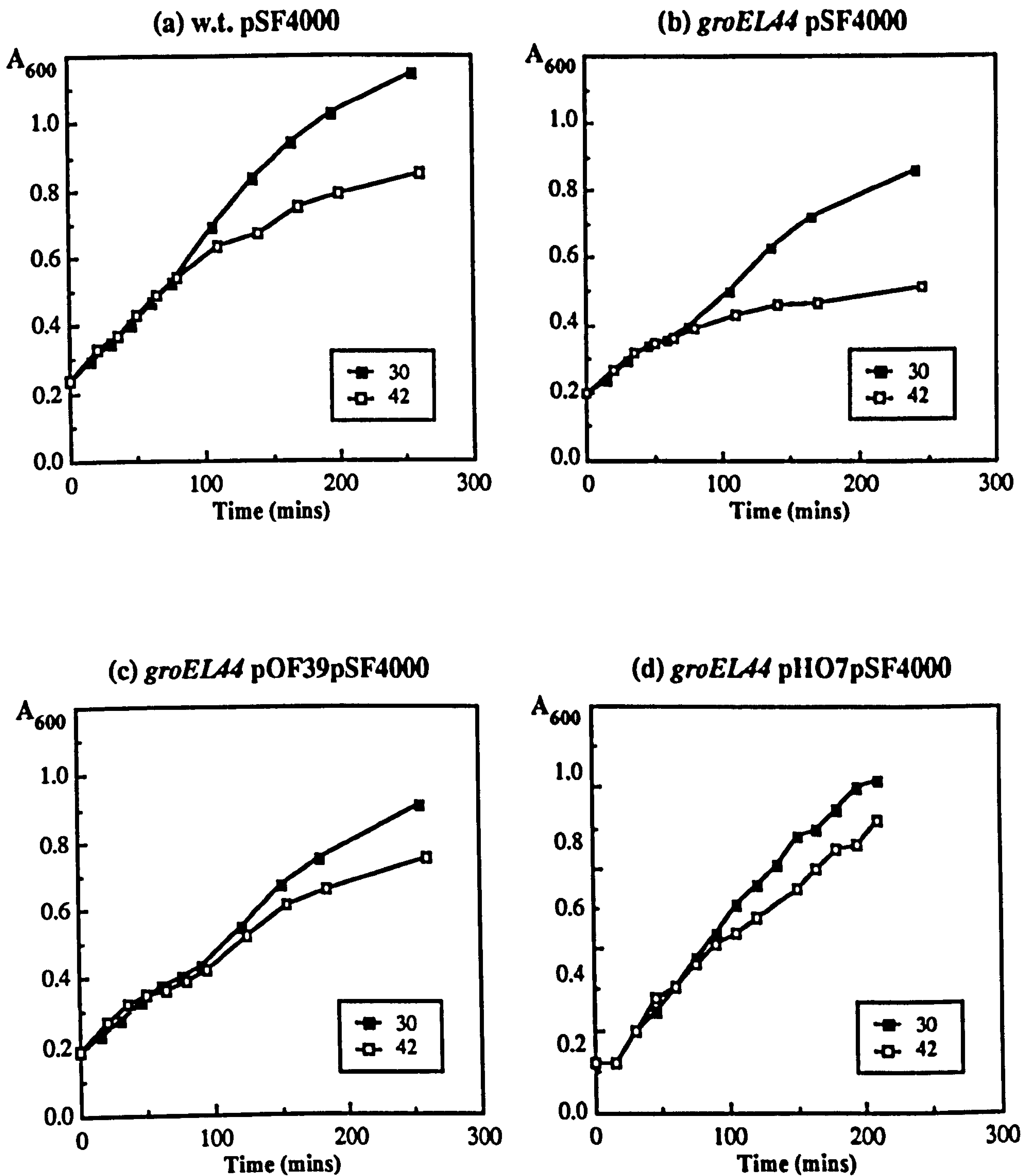


Fig. 6.1 Growth curves of wild-type (w.t.) and *groEL44* mutant cultures containing plasmids indicated. Cultures were propagated at 30°C in nutrient medium and grown to an A_{600} of approximately 0.2. Cultures were then split and half was maintained at 30°C and half was transferred to 42°C and growth was monitored (methods 2.2(i)).

(ii) Expression of the *groEL* and *groES* gene products

The effects of temperature shift on expression of both chromosomal and plasmid-borne *groEL* and *groES* genes was examined. Cultures, containing relevant plasmids, were grown in nutrient medium containing relevant antibiotics at 30°C. At appropriate times (determined from growth curves) samples were removed from the cultures and incubated at 42°C for 30 and 60 minutes respectively. Growth of all cultures was terminated at an A_{600} of approximately 0.7 and cells were harvested by centrifugation. Cell samples were analysed by SDS-PAGE and stained using Coomassie blue or Western blotted using GroEL or GroES antisera respectively (Fig.6.2).

The results for the wild-type strain (Fig.6.2) were identical to those results obtained from the *groEL44* mutant (data not shown) indicating that the *groEL44* mutant shows levels of GroEL and GroES expression comparable to the wild-type strain both before and after heat shock. Following a shift to 42°C for 30 and 60 minutes the levels of GroEL are increased as shown in the stained profile (Fig.6.2(a)) and confirmed by the Western blot (Fig.6.2(b)). Consistent with previous findings (Bukau, 1993) the levels of GroEL were increased approximately 2 fold following a shift to 42°C (from densitometry, data not shown). It is apparent from the stained profile that the presence of plasmid pOF39 results in a large increase in the levels of GroEL, even at 30°C. The *groEL* gene (and *groES* gene) in pOF39 is under the control of its normal chromosomal (σ^{32} controlled) promoter and therefore incubation at 42°C results in the increased expression of GroEL becoming even more pronounced. Cells containing plasmid pHO7 exhibit similar levels of GroEL expression (in the absence of IPTG induction) to those cells containing only a single chromosomal copy of GroEL. This indicates that although the presence of pHO7 in the *groEL44* mutant was able to restore growth at the non-permissive temperature without induction (Table 6.1 and Fig.6.1(d)), only low levels of expression must be required for such complementation.

Although the induction of GroES was not obvious from the stained profile (Fig.6.2(a)) Western blotting using GroES antiserum revealed that GroES induction had occurred in all cultures following a shift to 42°C (Fig.6.2(c)). As with GroEL, the levels of GroES were markedly increased in cells containing pOF39 particularly at 42°C. As expected the presence of plasmid pHO7, which encodes only GroEL, had no effect on the levels of GroES.

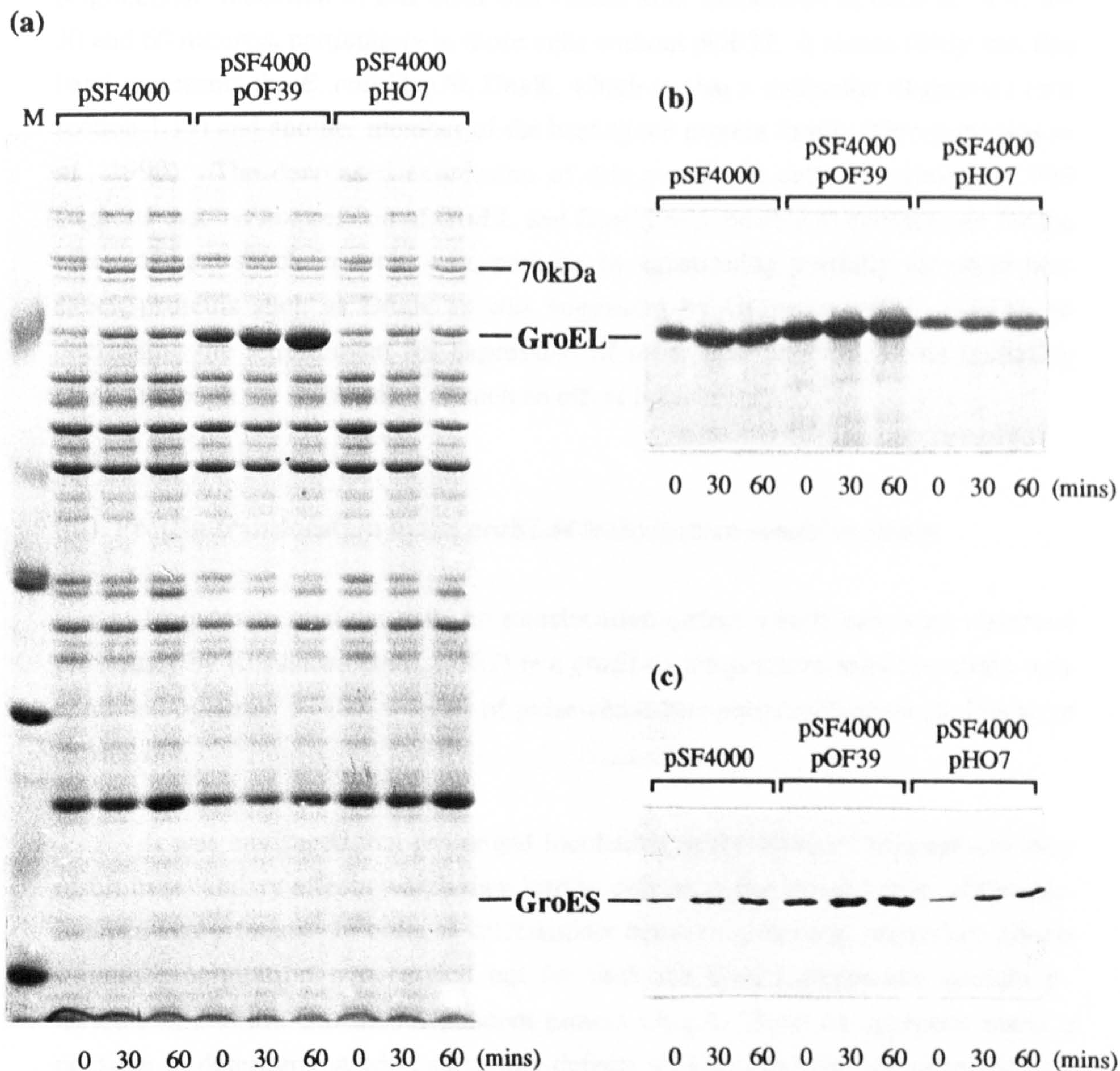


Fig. 6.2 Effects of temperature shift on expression of chromosomal and plasmid-borne *groEL* and *groES* genes. Wild-type, TG1, cultures containing the plasmids indicated were grown in nutrient medium and incubated at 30°C (0), or shifted to 42°C for 30 or 60 mins. Equivalent samples (0.1 A_{600} units in (a): 0.03 A_{600} units in (b) and (c)) were analysed by SDS-PAGE and (a) Coomassie blue staining or Western blotting using (b) GroEL antiserum or (c) GroES antiserum. The positions of GroEL and GroES are indicated. M-SDS7 markers from Sigma.

Also of interest was a band which migrated at a M_r of approximately 70kDa (Fig.6.2(a)). Induction of this band was visible after incubation of cells at 42°C for 30 and 60 minutes, particularly in those cells without pOF39. It seems likely that this band represents the *E. coli* Hsp70, DnaK, which is also a molecular chaperone (see section 1.17) and another member of the heat shock protein family (Georgopoulos *et al.*, 1990). The decreased expression of this protein in cells containing pOF39 suggests that overexpression of GroEL and GroES may be able to compensate for the effect of heat shock in some way, perhaps by substituting partially for other heat shock proteins such as DnaK as was suggested by Gragerov *et al.*, (1992), so decreasing the requirement for expression of other heat shock proteins including DnaK although the mechanism of such an effect is not clear.

(iii) Protein translocation in the *groEL44* temperature-sensitive strain

In order to confirm that the translocation defect which had been observed previously by Kusukawa *et al.*, (1989) in a *groEL44* temperature-sensitive strain was exhibited by strain SF103, a series of pulse-chase immunoprecipitation studies were carried out.

It was envisaged that prolonged incubation at the elevated temperature may result in secondary effects which may lead to defects in the translocation of GroEL-independent proteins. In order to differentiate between direct and secondary effects immunoprecipitation was carried out for both the GroEL-dependent protein β-lactamase, and the GroEL-independent protein OmpA. Such an approach made it possible to determine at what stage any defects seen in translocation occurred as a direct result of the *groEL44* allele.

As a result of the inconsistent growth of cultures in minimal media, strains, containing pBR325 (which encodes β-lactamase), were initially propagated in nutrient medium containing relevant antibiotics at 30°C. At appropriate times (determined from growth curves) samples were removed from the cultures and incubated at 42°C for 30, 60 and 120 minutes respectively. Cultures were then harvested (at an A_{600} of approximately 0.7) by centrifugation taking care to maintain them at the relevant temperature. Cells were washed in fresh, prewarmed M9 minimal medium and were then re-harvested. Following resuspension in fresh, prewarmed M9 minimal medium pulse-chase experiments were carried out in the usual manner (methods 2.2(ii)). Samples were split and subsequent immunoprecipitation was performed using antisera to β-lactamase and OmpA

respectively. Samples were processed and then analysed by SDS-PAGE and fluorography.

The β-lactamase immunoprecipitation results are shown in Fig.6.3. In the wild-type strain translocation of β-lactamase occurs efficiently at both 30°C and after a shift to 42°C for 60 minutes. Interestingly, there appears to be a decrease in the rate of translocation of β-lactamase following incubation at 42°C for 30 minutes. The reason for this is not clear. Also apparent from the densitometry is a slight decrease in efficiency of β-lactamase translocation in the wild-type strain after incubation at 42°C for 120 minutes, compared with translocation after 60 minutes at 42°C (discussed below).

In the *groEL44* mutant the most obvious feature is the presence of significant levels of pre-β-lactamase, compared with wild-type, which suggests that the translocation efficiency of β-lactamase is severely decreased in the *groEL44* mutant strain. However densitometry indicated (Fig.6.3(b)) that the translocation of β-lactamase occurs with only slightly reduced efficiency in the *groEL44* mutant compared with the wild-type strain at 30°C. Following incubation at 42°C for 30, 60 and 120 minutes the rate of β-lactamase translocation is significantly decreased in the *groEL44* mutant as expected.

The OmpA immunoprecipitation results are shown in Fig.6.4. It is clear that the translocation of OmpA occurs with virtually wild-type kinetics in the *groEL44* mutant even after incubation at 42°C for a period of 120 minutes. Densitometry revealed that the rate of translocation of OmpA was significantly increased in both the wild-type and *groEL44* mutant following a shift from 30°C to 42°C (Fig.6.4(b)) which is in contrast with the results obtained for β-lactamase (Fig.6.3(b)). However, after incubation at 42°C for 120 minutes a slight decrease in the rate of translocation of OmpA was seen in both the wild-type strain and *groEL44* mutant, although the decrease was more marked in the *groEL44* mutant (Fig.6.4(b)). This was consistent with the findings that a decrease in the growth rate and a slight decrease in the efficiency of β-lactamase translocation were seen in the wild-type strain after incubation at 42°C for 120 minutes.

It was concluded from the above data that the direct effects of the *groEL44* mutation are seen following incubation of the temperature-sensitive mutant at 42°C for periods of up to 60 minutes. After incubation at 42°C for 60 minutes the wild-type strain exhibits efficient translocation of both β-lactamase and OmpA, unlike the *groEL44* mutant which shows a decrease in the efficiency of β-lactamase

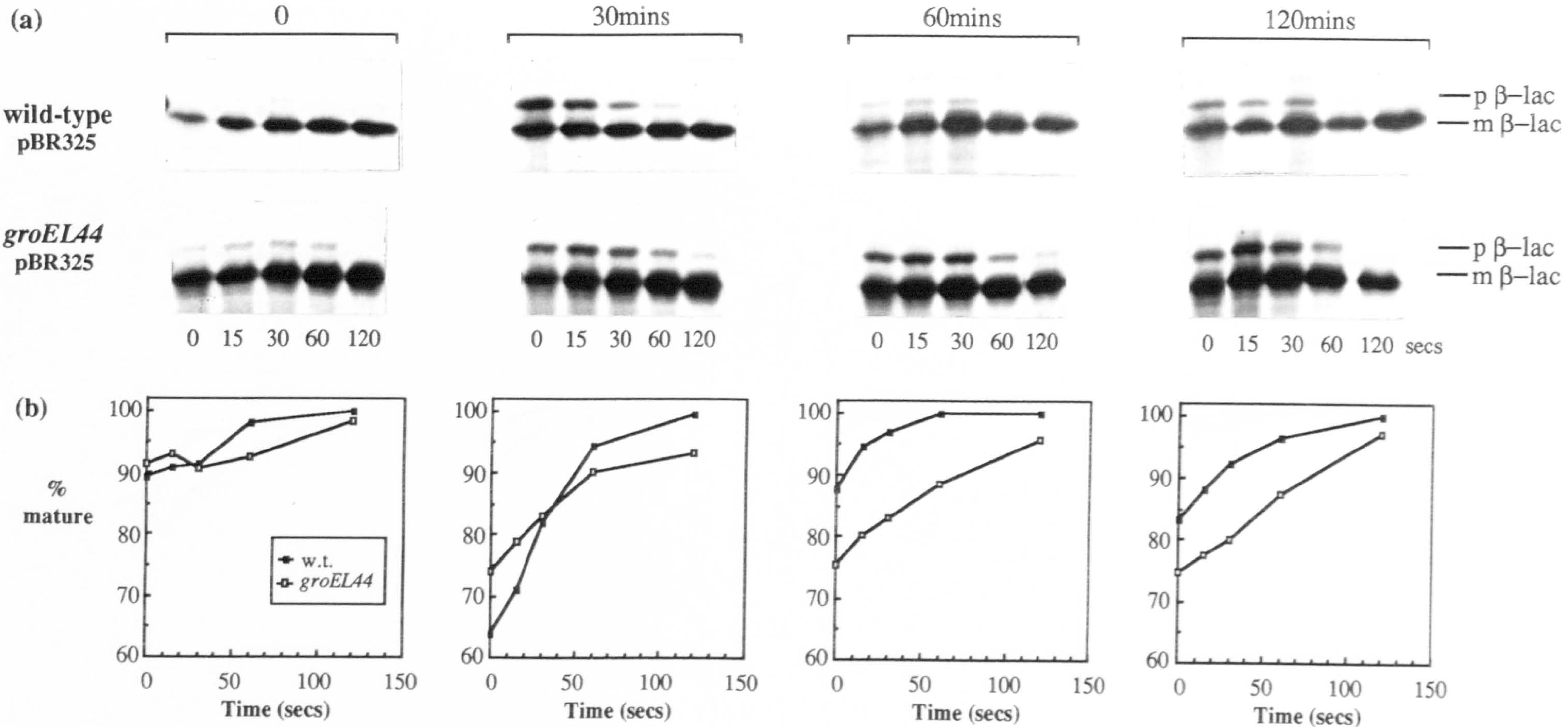


Fig. 6.3 The effect of depletion of functional GroEL on β-lactamase translocation efficiency. Separate cultures of the wild-type (w.t.) and *groEL44* strains, containing pBR325, were incubated at 30°C (0), or shifted to 42°C for 30, 60 or 120 mins before pulse chase assays as indicated. Each was pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with β-lactamase antiserum by SDS-PAGE and fluorography. The precursor and mature forms of β-lactamase are indicated (b) Kinetics of β-lactamase translocation as determined by densitometry of fluorographs in (a).

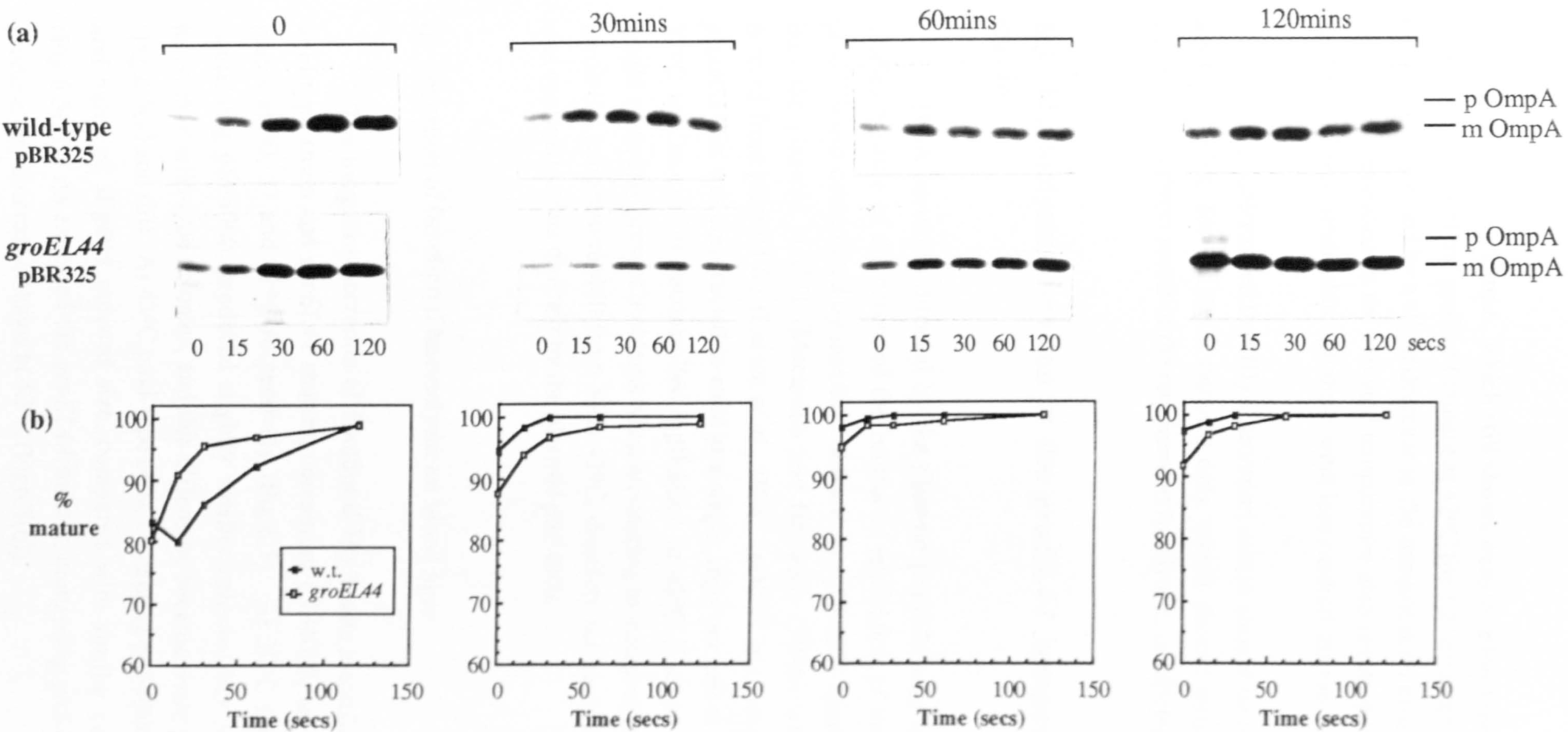


Fig. 6.4 The effect of depletion of functional GroEL on OmpA translocation efficiency. Separate cultures of the wild-type (w.t.) and *groEL44* strains, containing pBR325, were incubated at 30°C (0), or shifted to 42°C for 30, 60 or 120 mins before pulse chase assays as indicated. Each was pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with OmpA antiserum by SDS-PAGE and fluorography. The precursor and mature forms of OmpA are indicated (b) Kinetics of OmpA translocation as determined by densitometry of fluorographs in (a).

translocation but not OmpA, which still shows translocation kinetics similar to wild-type. Incubation of the *groEL44* mutant at 42°C for 120 minutes results in secondary effects, as illustrated by a slight decrease in the translocation kinetics of OmpA. Such prolonged incubation at the elevated temperature also resulted in deleterious effects on the wild-type strain although these were less marked than in the *groEL44* mutant.

It was determined that HlyA secretion assays should be carried out following a shift to 42°C for up to 60 minutes only which should ensure that any effects occurred as a direct result of the requirement for GroEL in efficient HlyA secretion.

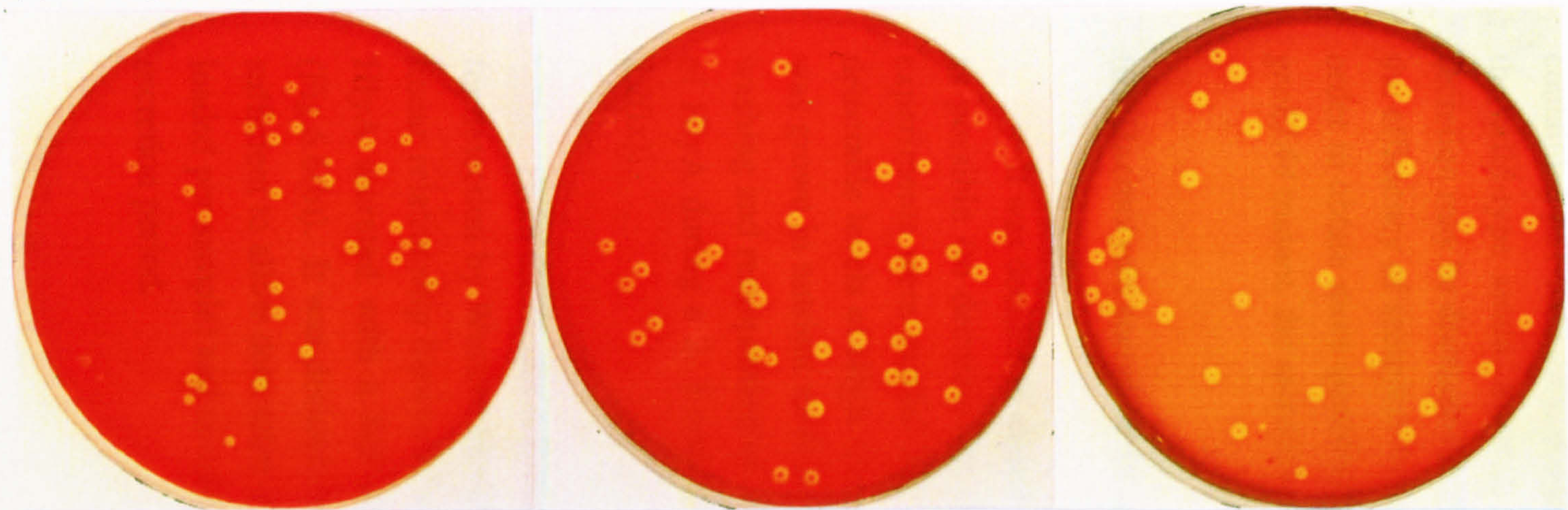
6.3 Haemolysin secretion in the *groEL44* temperature-sensitive strain

HlyA secretion directed by the plasmid pLG570 was not assayed in these studies because of the nature of the control of replication of the plasmid. Plasmid pLG570 was constructed by insertion of the *E. coli* LE2001 haemolysin determinant into the plasmid pOU71 (Mackman and Holland, 1984b) which was originally derived from plasmid R1 (Larsen *et al.*, 1984). pOU71 is a temperature amplifiable plasmid that was constructed to exist at a single copy per cell at temperatures below 37°C and to exhibit uncontrolled replication at 42°C (Larsen *et al.*, 1984). This would obviously present problems when attempting to interpret results in experiments involving temperature shift from 30 to 42°C, therefore the analysis of HlyA secretion was confined to that directed by the plasmid pSF4000.

(i) Secretion of functional haemolysin on blood agar

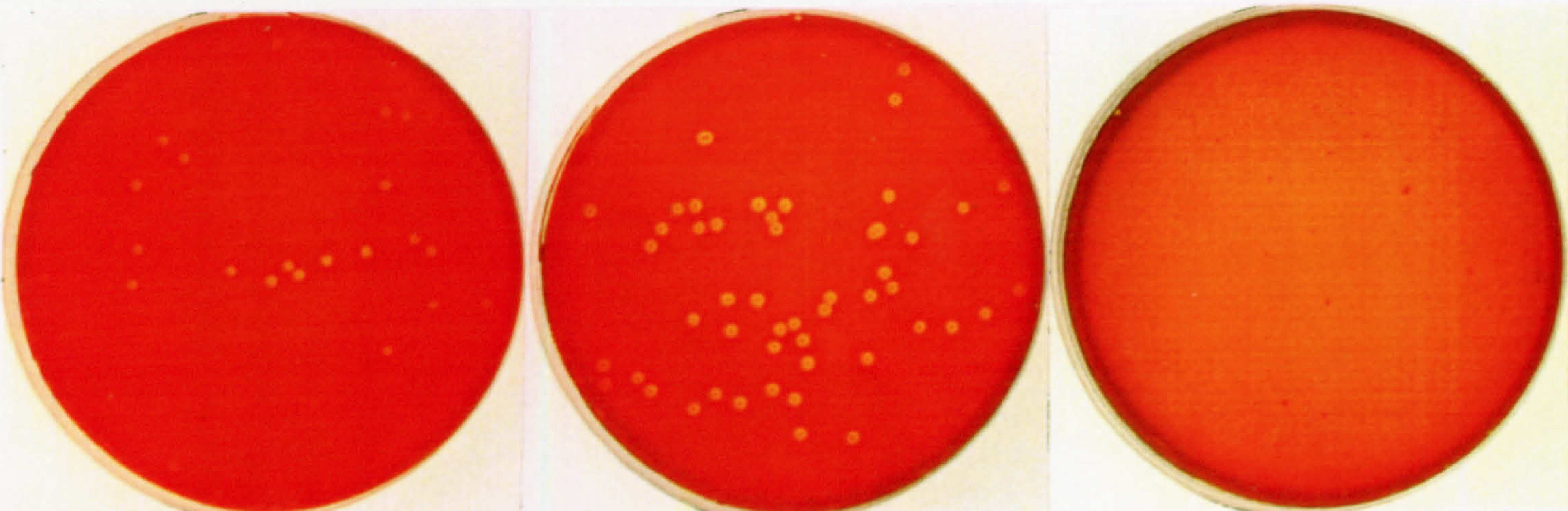
The long term secretion of functional HlyA was identified by growth of the wild-type strain and *groEL44* mutant, containing pSF4000, on nutrient blood agar plates at 30, 37 and 42°C respectively (Fig.6.5). At 30°C the *groEL44* mutant, containing pSF4000, produced slightly smaller colonies but considerably smaller halos than wild-type colonies, and this difference became more pronounced at 37°C (Fig.6.5(a) and (b)). At 42°C wild-type cells, containing pSF4000, produced colonies and halos of slightly reduced size, compared with similar cells grown at 37°C (Fig.6.5(a)). As expected the *groEL44* mutant, containing pSF4000, was unable to produce any colonies or halos at 42°C (Fig6.5(b)).

(a) w.t. pSF4000



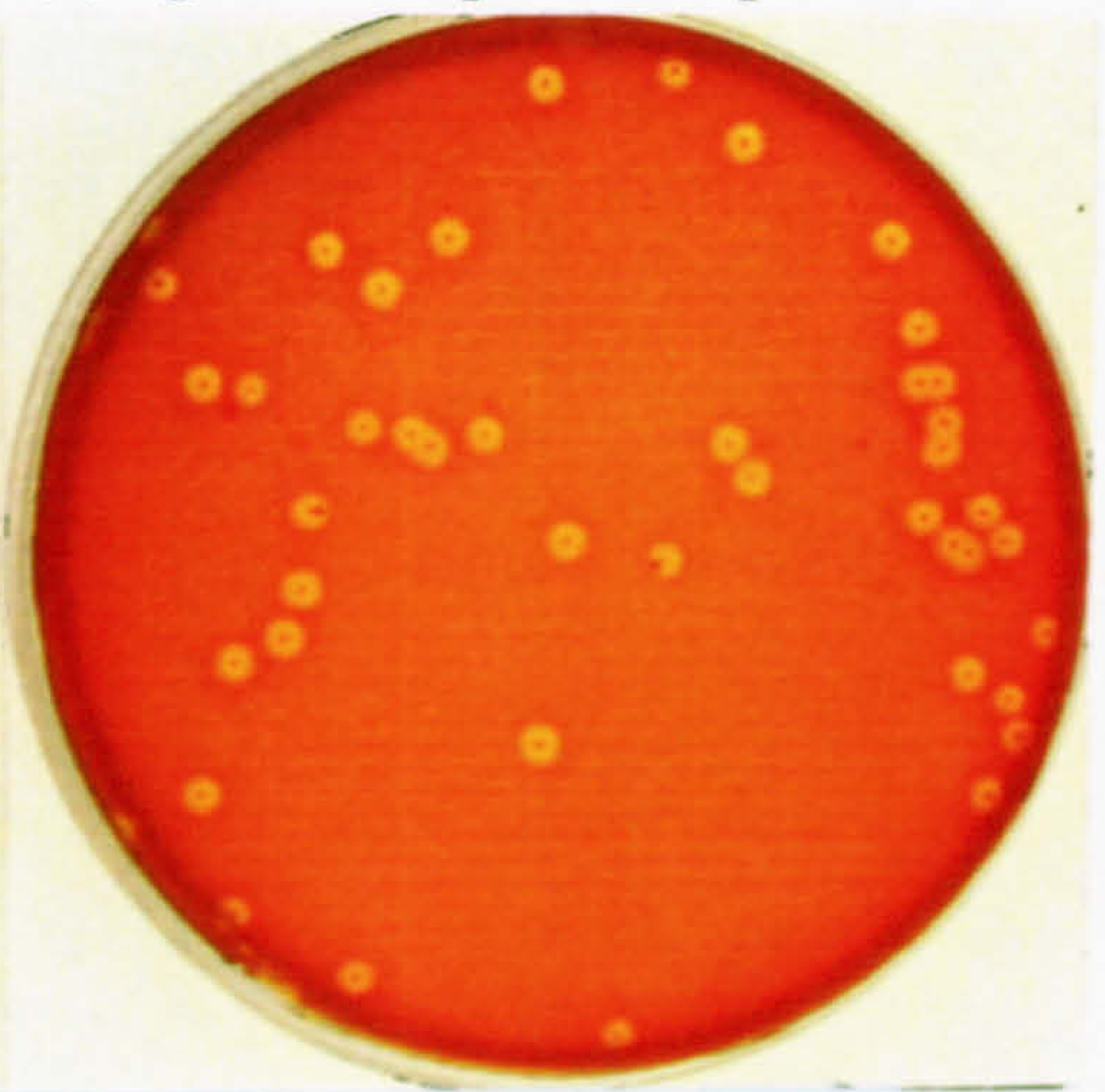
30 37 42

(b) *groEL44* pSF4000



30 37 42

(c) *groEL44* pSF4000pOF39



42

Fig. 6.5 Secretion of haemolysin from the wild-type (w.t.) strain and *groEL44* mutant on nutrient blood agar plates. Plates were incubated for 16 hours at 30, 37 and 42°C as indicated.

At all temperatures, growth of the *groEL44* mutant containing pSF4000 and either pOF39 or pHO7, resulted in increased colony and halo size compared with the *groEL44* mutant containing pSF4000 alone. Complementation of the *groEL44* mutant was particularly striking at 42°C (Fig.6.5(c)). Inclusion of the GroEL encoding plasmids in the wild-type strain, containing pSF4000, had no significant effects on colony or halo size (data not shown) and growth was consistent with results obtained in Table 6.1.

These results indicate that secretion of functional HlyA occurs in both the wild-type strain and *groEL44* mutant, containing pSF4000. The *groEL44* mutant, containing pSF4000 only, produced consistently smaller halos than those produced by the wild-type strain, even at the permissive temperature, suggesting that HlyA secretion may be decreased in the *groEL44* mutant. As it was not possible to estimate the efficiency of secretion from the *groEL44* mutant at 42°C from the above, and in order to obtain more quantitative data regarding the rate of secretion of HlyA in the *groEL44* mutant, the secretion of HlyA into culture supernatants was determined.

(ii) Haemolysin secretion in nutrient medium

It was decided to carry out the secretion assays with as short a secretion period as possible (to decrease the likelihood of secondary effects) and so preliminary experiments were carried out to determine the shortest secretion period in which detectable levels of HlyA were secreted. The wild-type strain, containing pSF4000, was grown in nutrient medium at 30°C to an A_{600} of approximately 0.7. Following washing and resuspension of cells in fresh prewarmed nutrient medium the secretion of HlyA during a 2, 5 and 10 minute period was determined (Fig.6.6) (methods 2.2(ix)).

From the results it is clear that sufficient secretion occurs in a 2 minute period for detection by Western blotting (Fig.6.6(a)). Analysis by densitometry indicated a linear relationship between the duration of the secretion period and the amount of HlyA secreted with a half-life for HlyA secretion of approximately 1 minute (Fig.6.6(b)). It was decided from this that a secretion period of 1 minute should be sufficient to allow detectable levels of HlyA secretion (if double the A_{600} units were loaded per sample) and so subsequent experiments were carried out with a 1 minute secretion period.

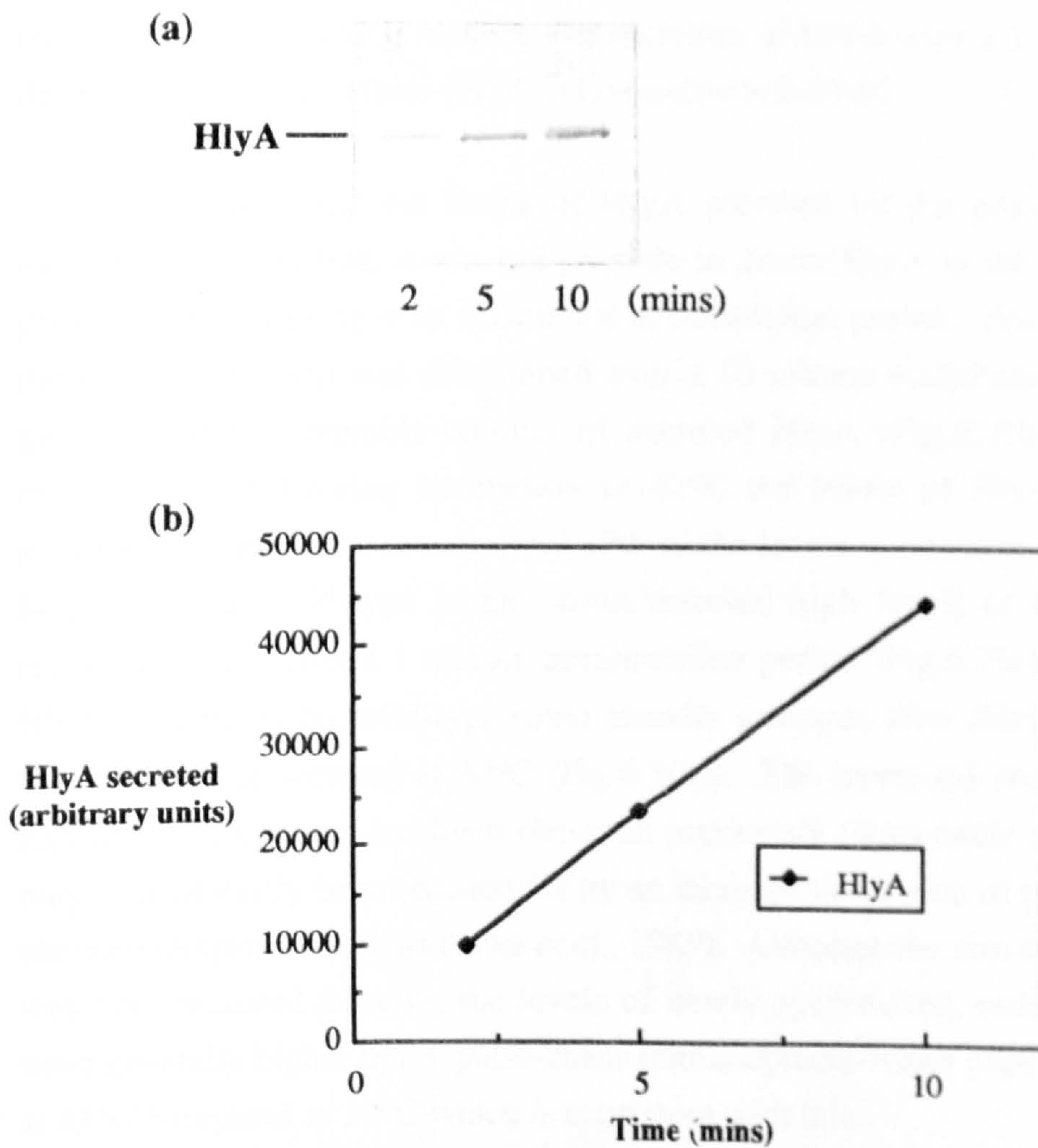


Fig. 6.6 Relationship between the secretion of HlyA and the duration of the secretion period. (a) Proteins secreted over a 2, 5 and 10 minute period in nutrient medium from the wild-type strain, TG1, containing pSF4000 were collected and equivalent samples (2.0 A_{600} units) analysed by SDS-PAGE and Western blotting using HlyA antiserum (methods 2.2(xi)). The position of HlyA is indicated. (b) Secretion of HlyA from the wild-type strain containing pSF4000 as determined by densitometry of blot in (a).

Wild-type and *groEL44* mutant cultures, containing pSF4000, were grown in nutrient medium at 30°C. At the appropriate times (determined from growth curves, such that all samples were harvested at an A_{600} of approximately 0.7) samples were transferred and incubated at 42°C. Following washing and resuspension of cells in fresh prewarmed nutrient medium the secretion of HlyA over a 1 minute period was determined for each culture (Fig.6.7(a)) (methods 2.2(ix)).

It is clear that the levels of HlyA secreted by the *groEL44* mutant are extremely low. In fact, it was not possible to detect HlyA in the supernatant of the *groEL44* mutant using only a 1 minute accumulation period. Secretion of HlyA by the *groEL44* mutant was determined over a 10 minute accumulation period which gave a readily detectable amount of secreted HlyA (Fig.6.7(b)). Densitometry revealed that following incubation at 42°C the levels of HlyA secreted by the *groEL44* mutant decrease to around 55% of the levels secreted at 30°C (Fig.6.8(b)). In contrast the wild-type parent strain secreted high levels of HlyA which were readily detected after a 1 minute accumulation period (Fig.6.7(a)). The amount of HlyA secreted by the wild-type strain actually increases after shifting to 42°C, by up to 200% of that secreted at 30°C (Fig.6.8(a)). The increased secretion of HlyA at elevated temperatures has been observed previously (Koronakis *et al.*, 1991). This may at least partly be accounted for by an increase in the rate of protein synthesis at elevated temperatures (Kusukawa *et al.*, 1989). Although the rate of protein synthesis was not measured directly, the levels of newly synthesised, radiolabelled proteins were generally higher in the pulse-chase immunoprecipitation experiments performed at 42°C compared to 30°C which is consistent with this.

The levels of HlyA secreted in the wild-type strain and the *groEL44* mutant are directly compared in Fig.6.8(b). Whilst it must be remembered that secretion was measured over a 1 minute and 10 minute period respectively the significance of the effect of raised temperature on the levels of secretion becomes apparent. This serves to confirm the requirement of functional GroEL for efficient secretion of HlyA.

Identical experiments were performed using strains containing the GroEL encoding plasmids as well as pSF4000. The results obtained confirmed that depletion of functional GroEL was the cause of the reduced HlyA secretion by the *groEL44* mutant (Fig.6.7(a)). In the presence of either complementing plasmid, pOF39 or pHO7, the secretion of HlyA was significantly restored in the *groEL44* mutant (Fig.6.8(a)). Levels of secretion were still below those for the wild-type strain which was possibly due to the formation of partially functional mixed wild-type and mutant GroEL oligomers (see section 6.3(i)). In the complemented *groEL44* mutant,

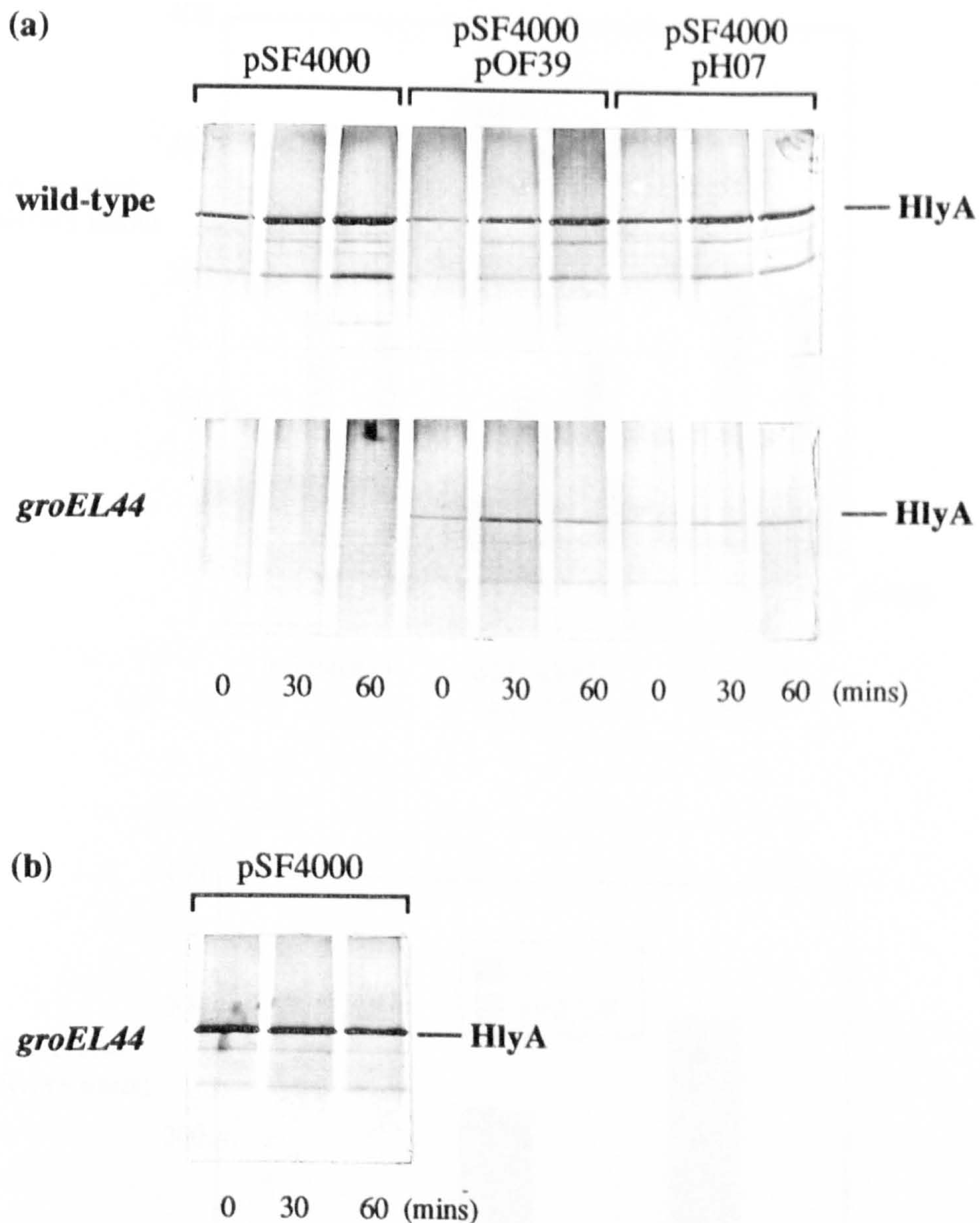
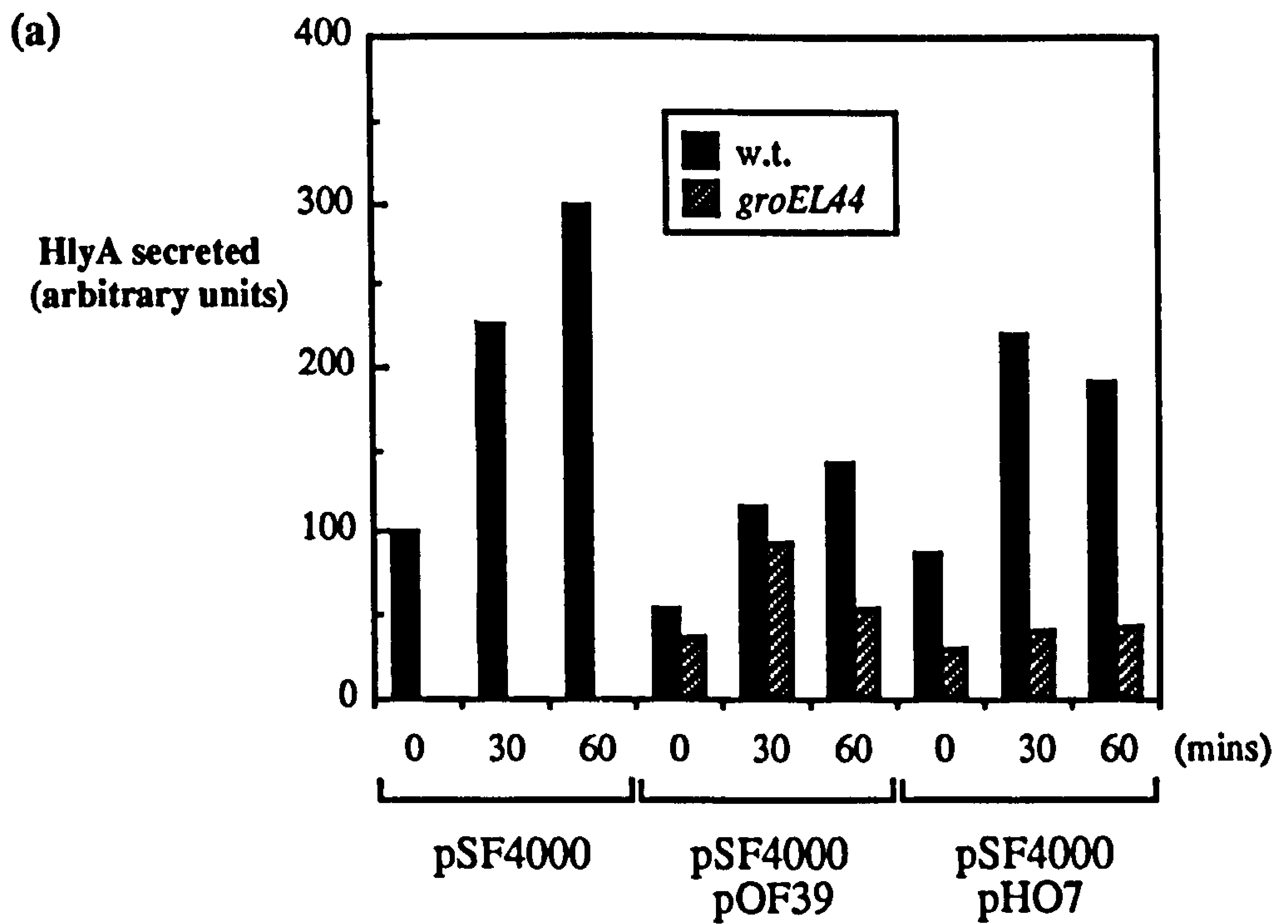


Fig. 6.7 Reduced secretion of haemolysin caused by the depletion of functional GroEL and restoration of secretion by complementing plasmids. (a) Proteins accumulated over a 1 min period in nutrient growth medium from cultures of wild-type and *groEL44* mutant strains containing the plasmids indicated were collected and analysed by SDS-PAGE and Western blotting using HlyA antiserum (methods 2.2(ix)). (b) To confirm secretion by the *groEL44* mutant, HlyA accumulated over a 10 min period by the *groEL44* mutant strain containing pSF4000 is also shown. Separate cultures were grown at 30°C (0), or shifted to 42°C for 30 or 60 mins before secretion assays as indicated. The position of HlyA is indicated. (Loading: 4.0 A_{600} units in (a) and (b)).



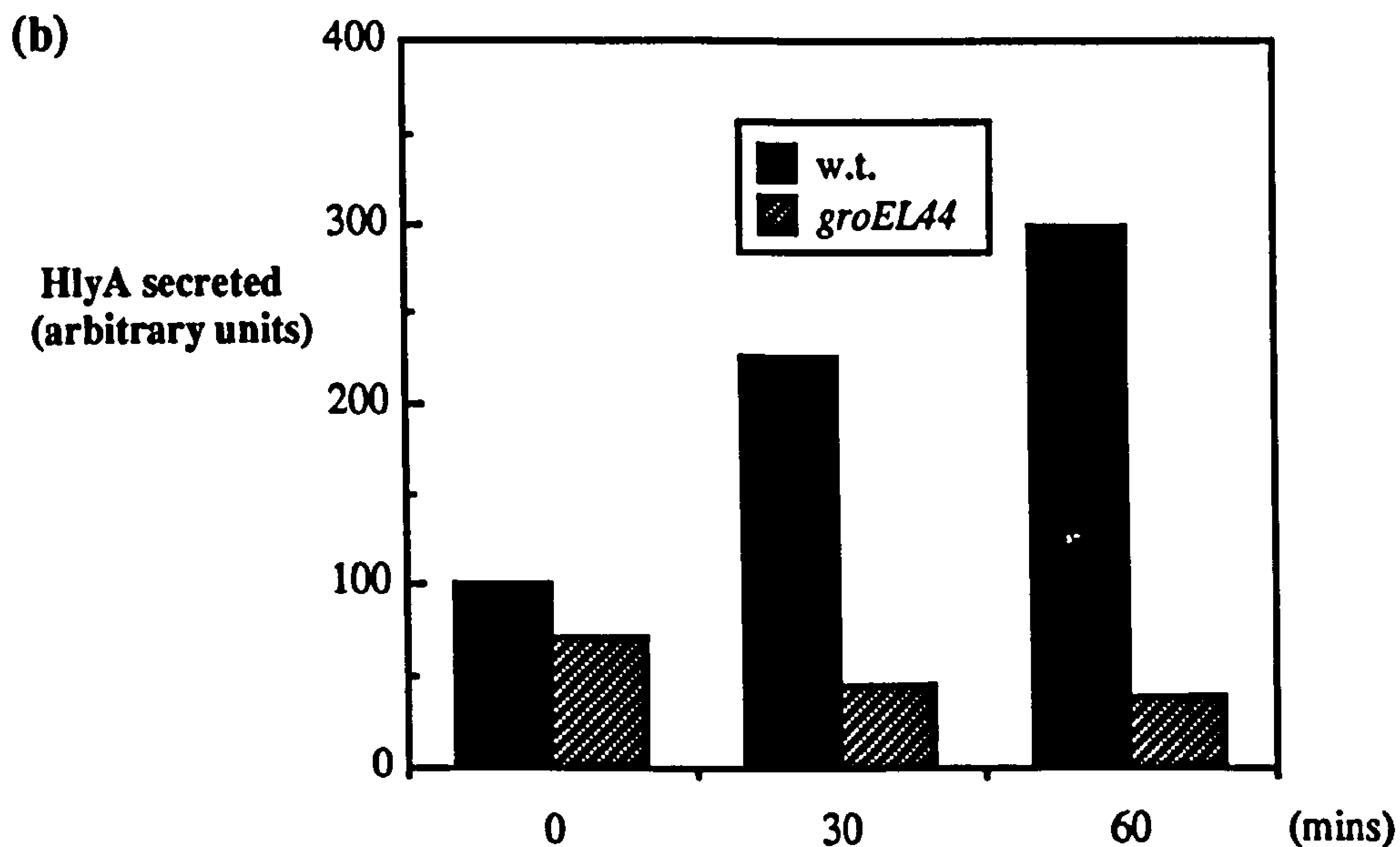


Fig. 6.8 Haemolysin secretion as determined by densitometry of blots in Fig.6.7. (a) HlyA accumulated over a 1 min period in nutrient growth medium from cultures of wild-type (w.t.) and *groEL44* mutant strains containing the plasmids indicated. (b) HlyA accumulated over a 1 min and 10 min period in the wild-type and *groEL44* mutant strains containing pSF4000 respectively. All values are shown relative to that of w.t. pSF4000 at 30°C which was given the arbitrary value of 100. Separate cultures were grown at 30°C (0), or shifted to 42°C for 30 or 60 mins before secretion assays as indicated.

secretion increases with incubation at the elevated temperature as previously desribed for the wild-type strain. In the wild-type strain the presence of the plasmids encoding GroEL causes a reduction in secretion efficiency which suggests that HlyA secretion may be reduced if an optimal level of GroEL is exceeded.

In order to determine whether the decreased secretion of HlyA from the *groEL44* mutant resulted in an accumulation of intracellular HlyA the intracellular levels of HlyA were determined by Western blotting (data not shown). Consistent with previous findings in which HlyA secretion is compromised (see section 3.3(ii); Wandersman and Letoffe, 1993), the intracellular levels of HlyA were similar in both wild-type and mutant strains at the permissive and non-permissive temperatures respectively.

From these results it may be concluded that the chaperonin GroEL is directly required for the efficient secretion of HlyA, as shown by the decrease in secretion from the *groEL44* mutant following incubation at both 30°C and 42°C. The finding that HlyA secretion is reduced so dramatically in the *groEL44* mutant, even at the permissive temperature, suggests that HlyA may have a strict requirement for fully functional GroEL.

The data presented rule out the possibility that pleiotropic effects of the mutation in GroEL are responsible for the decreased secretion from the *groEL44* mutant, as no such pleiotropic effects are observed at the permissive temperature. However, whilst it remains unlikely, the possibility that the *groEL44* mutation has a direct effect on the synthesis of HlyA cannot be entirely ruled out.

CHAPTER 7

INVESTIGATION OF HAEMOLYSIN SECRETION IN THE *groES619* TEMPERATURE-SENSITIVE STRAIN (CG2244)

In the previous chapter studies using the *groEL44* mutant SF103, indicated that GroEL was required for the efficient secretion of HlyA. In attempts to determine whether the co-chaperonin GroES is also required for the efficient secretion of HlyA similar experiments were performed using the *groES619* temperature-sensitive strain, CG2244 (Landry *et al.*, 1993).

7.1 The *groES619* temperature-sensitive strain (CG2244)

The *groES619* temperature-sensitive strain CG2244 contains a point mutation within the *groES* coding sequence, at codon 24 (Gly to Asp) which is reponsible for the temperature-sensitive phenotype (Landry *et al.*, 1993).

7.2 Characterisation of the *groES619* temperature-sensitive strain

As with the *groEL44* mutant strains the phenotype and characteristics of the *groES619* mutant strain have not been well documented to date and so preliminary experiments were performed to confirm the nature of strain CG2244 as a *groES* temperature-sensitive mutant.

All experiments were carried out in parallel using both the wild-type parent strain, CG2245, and the *groES619* mutant strain, CG2244, and growth was carried out at the permissive temperature of 30°C unless otherwise stated.

(i) Growth of the *groES619* temperature-sensitive strain

Transformations of the wild-type strain and *groES619* mutant were carried out using the plasmids shown in table 7.1 (methods 2.3(i)) and transformants were selected by growth on nutrient agar plates containing appropriate antibiotics. Transformants were plated on fresh nutrient agar plates containing appropriate

Wild-type	**Growth**		
Plasmids	**30°C**	**37°C**	**42°C**
-	++	+++++	+++++
pBR325	++	+++++	+++++
pSF4000	++	+++++	+++++
pOF39	++	+++++	+++++
pOF39pSF4000	++	+++++	+++++
pH08	++	+++++	+++++
pH08 (+IPTG)	++	+++++	+++++
pH08pSF4000	++	+++++	+++++
pH08pSF4000 (+IPTG)	++	+++++	+++++

***groES619* mutant**	**Growth**		
Plasmids	**30°C**	**37°C**	**42°C**
-	++	+++	n.c.
pBR325	++	+++	n.c.
pSF4000	++	+++	n.c.
pOF39	++	+++++	+++++
pOF39pSF4000	++	+++++	+++++
pH08	++	++++	++++
pH08 (+IPTG)	++	++++	++++
pH08pSF4000	++	++++	++++
pH08pSF4000 (+IPTG)	++	++++	++++

Table 7.1 Viability and growth of the wild-type and *groES619* mutant strains at elevated temperatures. The wild-type strain, CG2245, and *groES619* mutant, CG2244, containing the plasmids indicated were plated on fresh nutrient plates and incubated for 24 hours at 30, 37 and 42°C as indicated. Sizes of colonies produced are shown relative to those of CG2245 grown at 37°C (+++++). n.c. - no colonies.

antibiotics and incubated at 30, 37 and 42°C for 24 hours. The viability and growth of the wild-type and *groES619* mutant, containing the various plasmids, at the different temperatures are summarised in Table 7.1.

The wild-type strain, CG2245, grew well at the elevated temperatures (37 and 42°C) as expected, with growth at 42°C comparable to that at 37°C. This suggests improved tolerance to prolonged incubation at 42°C compared with the wild-type strain, TG1, described in the previous chapter (see Table 6.1). The presence of pOF39 or pHO8 (*groES* under the control of both its own promoter and the *lac* promoter in pUC19, P. Lund, personal communication), in the wild-type strain had no detectable effect on growth.

The *groES619* mutant was unable to grow at the non-permissive temperature (42°C) and also exhibited only limited growth, compared with wild-type, at 37°C suggesting severe temperature-sensitivity. Plasmid pOF39 was able to restore growth comparable to wild-type levels at the elevated temperatures. Plasmid pHO8 was able to restore growth of the *groES619* mutant at 42°C in the presence or absence of IPTG as expected, although to a slightly lesser extent than pOF39.

Growth curves were carried out in nutrient medium (methods 2.2(i)) and the growth curves of particular interest are shown in Fig. 7.1.

Consistent with results obtained from growth on nutrient agar plates, the *groES619* mutant, containing pSF4000, showed severe temperature sensitivity following a shift from 30°C to 42°C, compared with the wild-type strain (Fig. 7.1(a) and (b)). After approximately 75 minutes at the non-permissive temperature growth of the *groES619* mutant apparently ceased. Complementation of the temperature-sensitive growth phenotype by the GroES plasmids was seen with pOF39 restoring the growth rate to that of the wild-type (Fig.7.1(c)). Plasmid pHO8 exhibited more limited recovery of the temperature-sensitive growth phenotype both in the absence (Fig.7.1(d)) or presence (data not shown) of IPTG induction, consistent with the results from growth on nutrient agar (Table 7.1).

(ii) Expression of the *groEL* and *groES* gene products

The effects of temperature shift on expression of both chromosomal and plasmid-borne *groEL* and *groES* genes was examined. Following growth of cultures in nutrient medium at 30°C samples were shifted to 42°C and incubated for 30 and 60

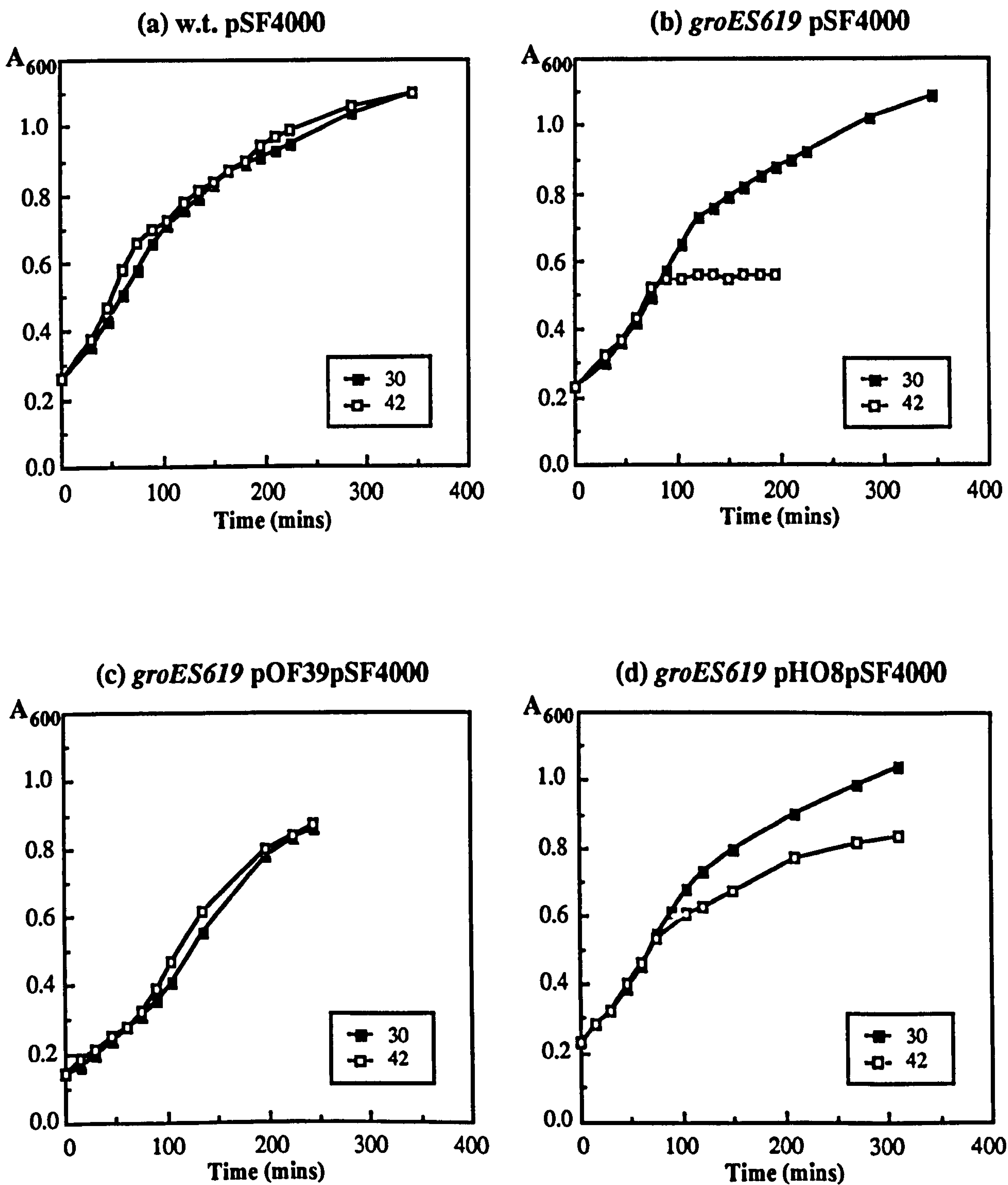


Fig. 7.1 Growth curves of wild-type (w.t.) and *groES619* mutant cultures containing plasmids indicated. Cultures were propagated at 30°C in nutrient medium and grown to an A_{600} of approximately 0.2. Cultures were then split and half was maintained at 30°C and half was transferred to 42°C and growth was monitored (methods 2.2(i)).

minutes respectively. The results from the wild-type strain (data not shown) and the *groES619* mutant (Fig.7.2) were identical and comparable with those results obtained in the *groEL44* mutant studies (see Fig.6.2).

Induction of GroEL was again particularly apparent in cells containing pOF39 following a shift to 42°C for 30 and 60 minutes respectively. As expected the presence of pHO8 (which encodes only GroES) had no effect on the levels of GroEL which were comparable to those in cells without pHO8.

Induction of GroES was again not visible from the stained profile however Western blot analysis using GroES antiserum indicated that GroES induction had occurred following a shift to 42°C, especially in cells containing pOF39 (Fig.7.2(c)). A similar increase in the levels of GroES produced in cells containing pHO8 was expected, however this was not seen (Fig.7.2(c)). The *groES* gene in pHO8 is under the control of its normal (σ^{32} controlled) promoter (as in pOF39), however the presence or absence of pHO8 did not appear to affect the levels of GroES at either 30 or 42°C. Other workers (P. Lund, personal communication) have found that expression of either GroES or GroEL directed from their normal promoter is variable when encoded on plasmids. The reason for this is not clear, however it may explain the apparent lack of induction of pHO8 encoded GroES at the elevated temperature. It may be that expression (in the absence of IPTG) is occurring to a limited extent as a result of leaky transcription from the *lac* promoter, also present on pHO8, and that this limited expression is sufficient for viability at the elevated temperature. This would be directly analogous to the leaky expression seen from the *tac* promoter of pHO7 in the absence of IPTG (see section 6.2(ii)).

As was observed in the *groEL44* mutant studies (see section 6.2(ii)) induction of a band of around 70kDa, which is likely to be DnaK, was apparent following incubation at 42°C, particularly in cells without pOF39.

(iii) Protein translocation in the *groES619* temperature-sensitive strain

In order to differentiate between possible direct and indirect effects occurring as a result of the *groES619* mutation, identical pulse-chase immunoprecipitation studies to those performed in section 6.2(iii) were carried out. Immunoprecipitation was again performed using antisera to the GroES-dependent protein, β-lactamase, and the GroES-independent protein, OmpA, respectively.

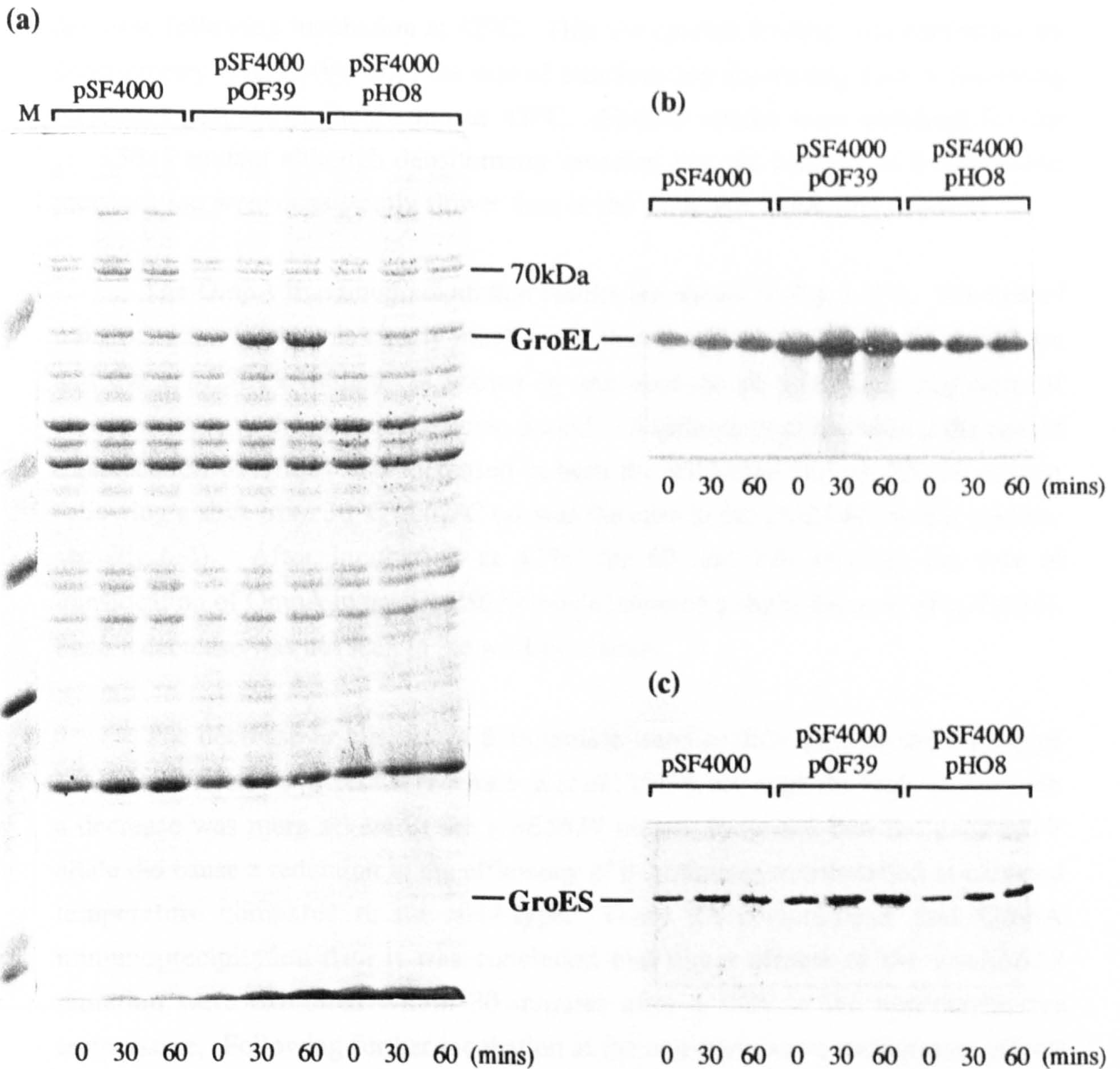


Fig. 7.2 Effects of temperature shift on expression of chromosomal and plasmid-borne *groEL* and *groES* genes. Cultures of the *groES619* mutant, CG2244, containing the plasmids indicated were grown in nutrient medium and incubated at 30°C (0), or shifted to 42°C for 30 or 60 mins. Equivalent samples (0.1 A_{600} units in (a): 0.03 A_{600} units in (b) and (c)) were analysed by SDS-PAGE and (a) Coomassie blue staining or Western blotting using (b) GroEL antiserum or (c) GroES antiserum. The positions of GroEL and GroES are indicated. M-SDS7 markers from Sigma.

Results from the β-lactamase immunoprecipitation assays are shown in Fig.7.3. In the wild-type strain the rate of translocation of β-lactamase appeared to decrease following incubation at 42°C. This unexpected finding was confirmed by densitometry (Fig.7.3(b)) with the rate of translocation decreasing further following increased periods of incubation at 42°C. Similar results were obtained for the *groES619* mutant although densitometry revealed that the kinetics of β-lactamase translocation were consistently slower than in the wild-type strain (Fig.7.3(b)).

The OmpA immunoprecipitation results are shown in Fig.7.4(a). The rate of translocation of OmpA is clearly much faster than β-lactamase in both the wild-type and the *groES619* mutant, as shown by the absence of significant amounts of precursor even at the start of the chase period. Densitometry revealed that the rate of translocation of OmpA was increased in both the wild-type and *groES619* mutant following a shift from 30°C to 42°C (as was the case in the *groEL44* mutant studies, see Fig.6.4). After incubation at 42°C for 60 and 120 minutes the rate of translocation of OmpA in the *groES619* mutant showed a slight decrease (Fig.7.4(b)). Such a decrease was not seen in the wild-type strain.

The decrease in kinetics of β-lactamase translocation seen in the wild-type strain at 42°C was unexpected (Kusukawa *et al.*, 1989), although the finding that such a decrease was more severe in the *groES619* mutant indicated that the *groES619* allele did cause a reduction in the efficiency of β-lactamase translocation at elevated temperature compared to the wild-type. From the β-lactamase and OmpA immunoprecipitation data it was concluded that direct effects of the *groES619* mutation were exhibited within 30 minutes after a shift to the non-permissive temperature. Following further incubation at the non-permissive temperature effects of a secondary nature began to occur, as illustrated by the slight decrease in the efficiency of OmpA translocation following incubation of the *groES619* mutant at 42°C for 60 minutes. However, such secondary effects were judged to be of a very minor nature and so the effects on HlyA secretion were assessed following a shift to 42°C for up to 60 minutes, consistent with the experiments performed in section 6.3(iii).

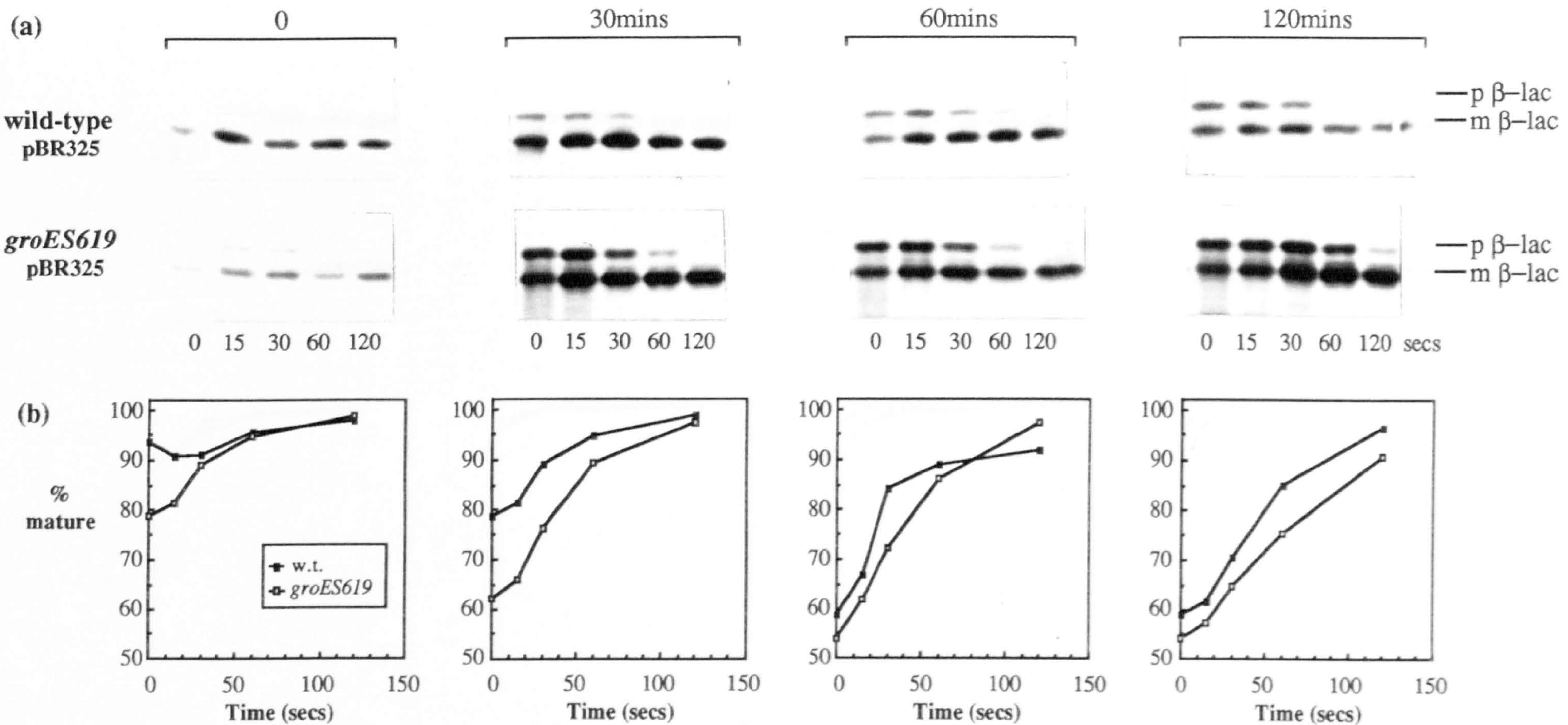


Fig. 7.3 The effect of depletion of functional GroES on β-lactamase translocation efficiency. Separate cultures of the wild-type (w.t.) and *groES619* strains, containing pBR325, were incubated at 30°C (0), or shifted to 42°C for 30, 60 or 120 mins before pulse chase assays as indicated. Each was pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with β-lactamase antiserum by SDS-PAGE and fluorography. The precursor and mature forms of β-lactamase are indicated. (b) Kinetics of β-lactamase translocation as determined by densitometry of fluorographs in (a).

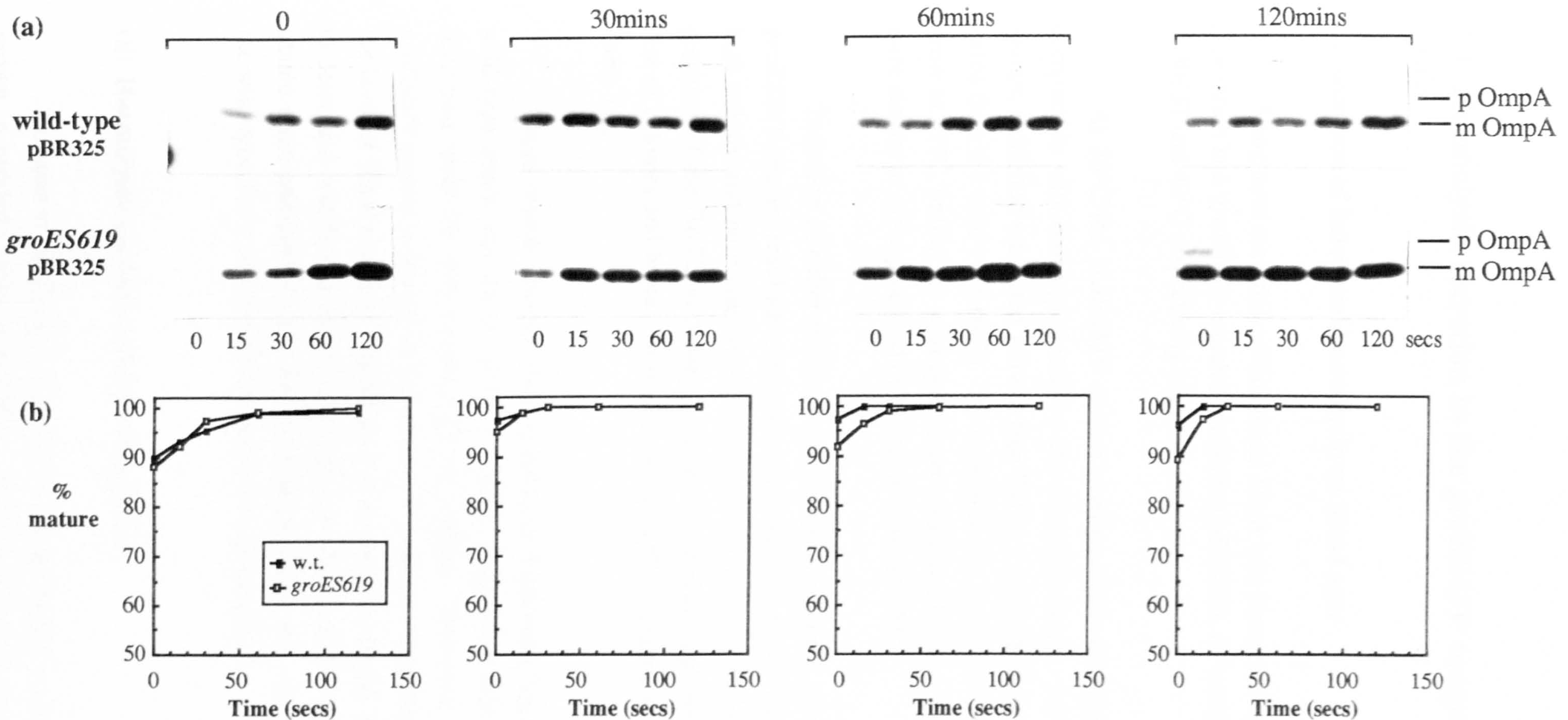


Fig. 7.4 The effect of depletion of functional GroES on OmpA translocation efficiency. Separate cultures of the wild-type (w.t.) and *groES619* strains, containing pBR325, were incubated at 30°C (0), or shifted to 42°C for 30, 60 or 120 mins before pulse chase assays as indicated. Each was pulsed with ^{35}S-Methionine for 30 sec and then chased, for the indicated times, with unlabelled L-Methionine as described in materials and methods (2.2(ii)). (a) Analysis of samples immunoprecipitated with OmpA antiserum by SDS-PAGE and fluorography. The precursor and mature forms of OmpA are indicated. (b) Kinetics of OmpA translocation as determined by densitometry of fluorographs in (a).

7.3 Haemolysin secretion in the *groES619* temperature-sensitive strain

(i) Secretion of functional haemolysin on blood agar

Long term secretion of functional HlyA was identified by growth of the wild-type strain and *groES619* mutant, containing pSF4000, on nutrient blood agar plates at 30, 37 and 42°C respectively (data not shown).

At 30°C the *groES619* mutant produced colonies and halos which were comparable with those produced by the wild-type strain. At 37°C the *groES619* mutant exhibited restricted growth (see Table 7.1) producing smaller colonies and halos than the wild-type strain. As expected, the *groES619* mutant was unable to grow at 42°C, unlike the wild-type strain which produced colonies and halos which were similar to those produced by the same strain at 37°C.

Inclusion of either of the complementing plasmids, pOF39 or pHO8, in the *groES619* mutant resulted in recovery of growth at the elevated temperatures and both colony and halo size were comparable to those of the wild-type strain. The presence of the GroES encoding plasmids in the wild-type strain did not affect the size of colonies and halos produced at 30 or 42°C consistent with results obtained in Table 7.1.

These results indicate that secretion of functional HlyA occurs in both the wild-type strain and the *groES619* mutant, suggesting that the levels of HlyA secretion from the two strains may be similar. However, the inability of the *groES619* mutant to produce colonies at 42°C made it impossible to estimate whether the level of HlyA secretion was reduced at the non-permissive temperature. In order to determine whether secretion was reduced in the *groES619* mutant at 42°C and to obtain more quantitative data, the secretion of HlyA into culture supernatants from the wild-type strain and *groES619* mutant were determined.

(ii) Haemolysin secretion in nutrient medium

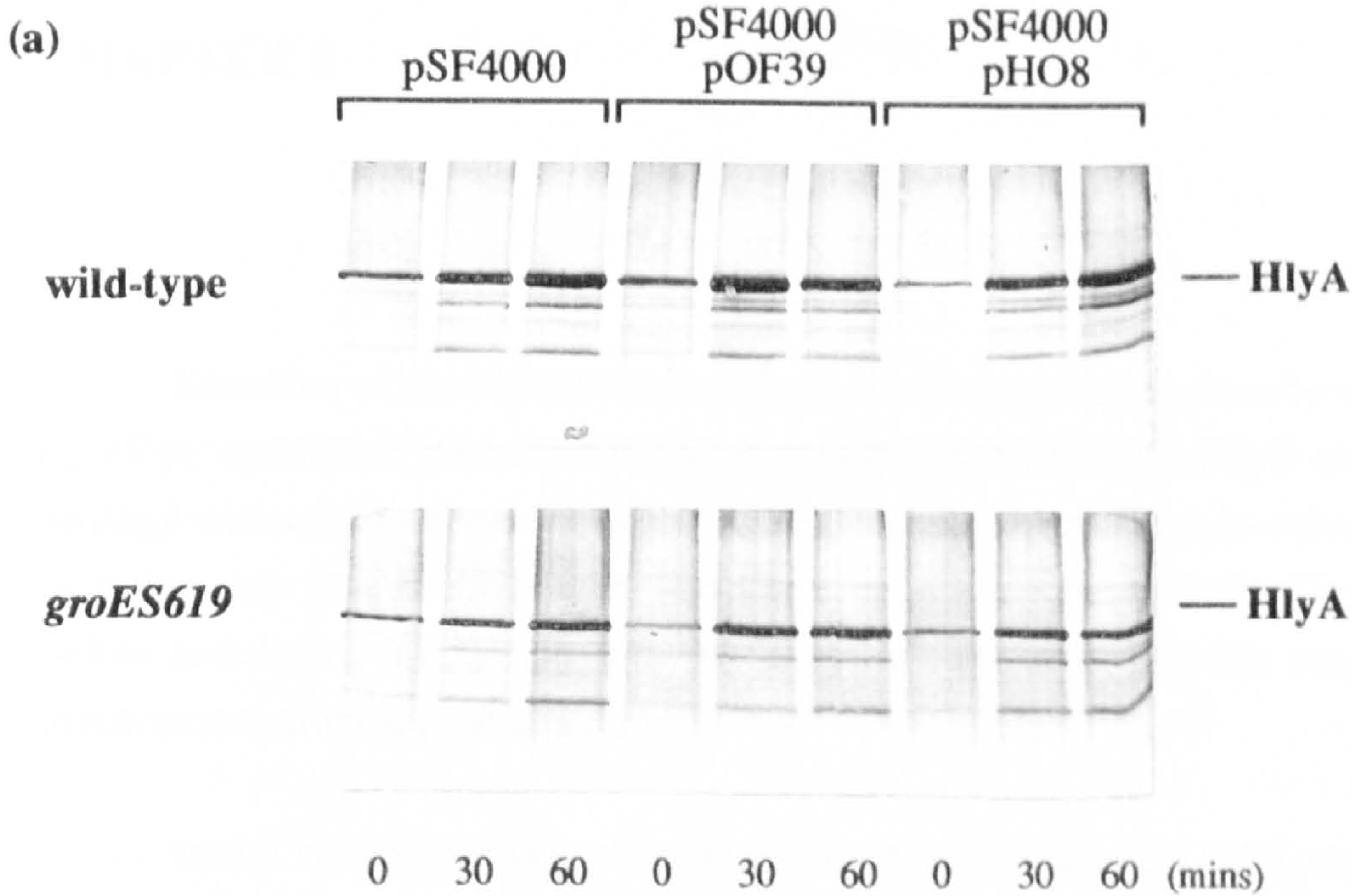
Cultures of wild-type and *groES619* mutant strains, containing pSF4000, were grown in nutrient medium at 30°C. At the appropriate times (determined from growth curves) samples were removed and grown at 42°C for up to 60 minutes.

Following washing and resuspension of cells in fresh prewarmed nutrient medium the secretion of HlyA over a 1 minute period was determined for each culture (Fig.7.5) (methods 2.2(ix)).

In contrast to the GroEL data (section 6.3(ii)) the absence of fully functional GroES appears to have no adverse effect on HlyA secretion. Although slightly reduced, the levels of HlyA which accumulate in the growth medium of the *groES619* mutant are similar to those in the growth medium of the parent strain at 30 and 42°C respectively. Secretion is increased in both the wild-type strain and *groES619* mutant following incubation at the elevated temperature, as was seen for the wild-type strain in the GroEL experiments (section 6.3(ii)). The inclusion of the complementing plasmids, pOF39 or pHO8, encoding GroES has little noticeable effect on HlyA secretion in either the wild-type strain or *groES619* mutant.

These data suggest that wild-type GroES activity is not required for the efficient secretion of HlyA. In order to state conclusively that the GroES co-chaperonin does not participate in HlyA secretion studies using different *groES* mutants may be performed, however the finding that all the *groES* temperature-sensitive mutants isolated (Landry *et al.*, 1993) contain mutations within the same domain suggests that similar results would be obtained.

The finding that GroES is not involved in HlyA secretion also rules out the possibility that filamentation, which was observed in both the *groEL44* and *groES619* mutants (using phase contrast microscopy - data not shown) consistent with previous observations (Georgopoulos and Eisen, 1974), may be responsible for the reduced secretion of HlyA in the *groEL44* mutant.



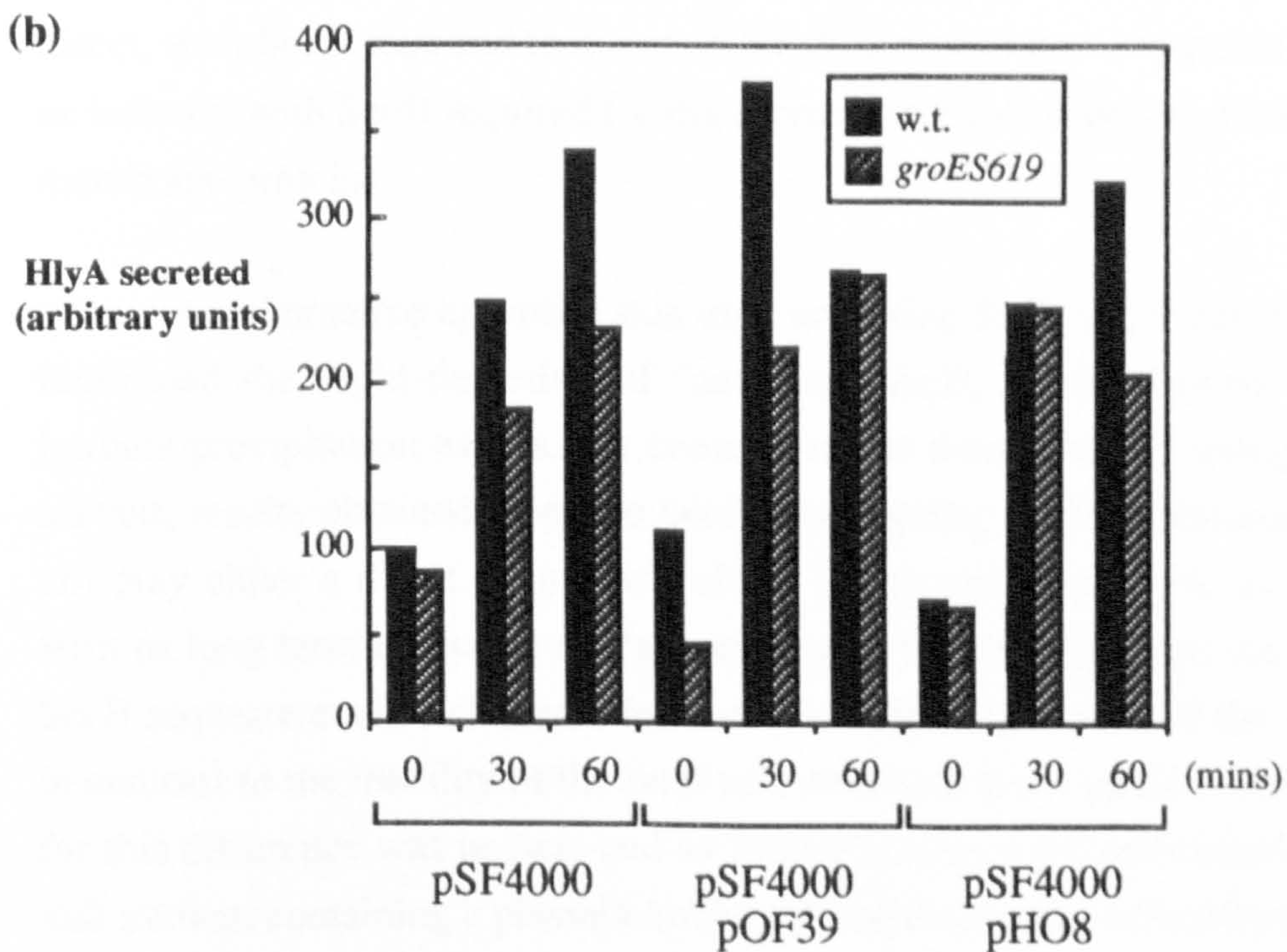


Fig. 7.5 Negligible effect on secretion of haemolysin caused by the depletion of functional GroES. (a) Proteins accumulated over a 1 min period in nutrient growth medium from cultures of wild-type (w.t.) and *groES619* mutant strains containing the plasmids indicated were collected and analysed by SDS-PAGE and Western blotting using HlyA antiserum as described in materials and methods (2.2(ix)). Separate cultures were grown at 30°C (0), or shifted to 42°C for 30 or 60 mins before secretion assays as indicated. The position of HlyA is indicated. (b) HlyA secretion as determined by densitometry of blots in (a). All values are shown relative to that of w.t. pSF4000 at 30°C which was given the arbitrary value of 100. (Loading: 4.0 A_{600} units)

CHAPTER 8

DISCUSSION

Secretion of the 107kDa toxin, HlyA, occurs post-translationally using a trans-envelope membrane translocator complex comprised of HlyB, HlyD and TolC. By analogy with requirements for export across the inner membrane, it was reasoned that secretion may require the nascent HlyA to remain in a "loosely folded" conformation before and during interaction with the secretion apparatus, and this may require the involvement of molecular chaperones.

Initial studies performed using the *secB* null strain, CK1953, suggested that the secretion of HlyA was strongly dependent on the chaperone SecB, with secretion severely reduced in the *secB* null background compared to wild-type. However, using this approach it was not possible to determine whether the dependence on SecB was direct, with SecB required to maintain HlyA in a secretion-competent conformation, or indirect, with SecB required for the correct insertion of one or more of the integral membrane proteins.

An alternative approach was used involving SecB sequestering strains, which facilitated the rapid depletion of functional SecB, as determined by pulse-chase immunoprecipitation assays. In contrast to the data obtained using the *secB* null mutant, results obtained from the SecB sequestering strains indicated that SecB did not play either a direct or indirect role in the secretion of HlyA, as shown in short term or long term induction experiments respectively. However, the viability of the SecB sequesterers in rich media following long term induction of the sequesterer was in contrast to the inability of the *secB* null mutant to grow on rich media. The reason for this difference was unclear and so further studies were performed using the *secB* null mutant, containing a plasmid-borne wild-type copy of *secB*, effectively making it wild-type with respect to SecB. As expected, the translocation kinetics of the SecB-dependent exported protein OmpA were restored to almost wild-type, and the viability on rich media was also restored. This indicated that the absence of SecB in the *secB* null mutant was directly responsible for these phenotypes. In contrast, the mucoid nature and the reduced secretion of HlyA by the *secB* null mutant were not recovered by the presence of wild-type SecB, suggesting that these phenotypes did not occur as a direct result of the absence of functional SecB but perhaps as an indirect effect of the strategy used to construct the *secB* null mutant. The localisation of the *secB* gene close to the *rfa* locus (Kumamoto and Beckwith, 1985), which is

involved in LPS biosynthesis (Parker *et al.*, 1992), and the above findings prompt the suggestion that the mutagenic transposon insertion may exert a polar effect on *rfa* gene expression. The involvement of LPS in HlyA secretion has been studied, with data suggesting that altered LPS biosynthesis results in decreased secretion of HlyA (Wandersman and Letoffe, 1993) and also decreased activity in secreted HlyA (Stanley *et al.*, 1993). It therefore seems likely that the decreased secretion seen in the *secB* null mutant is in fact due to altered LPS biosynthesis and not the absence of SecB. It would be interesting to determine whether the limited amounts of HlyA secreted by the *secB* null mutant show decreased activity, consistent with the results of Stanley *et al.*, (1993).

The inability of the SecB sequesterers to reproduce the characteristic selective viability of the *secB* null mutant on only minimal media suggested that the SecB sequesterers did not remove all functional SecB from the cytoplasm. This is even though induction of the sequesterers resulted in translocation kinetics comparable to those in the *secB* null mutant.

The T7 RNA polymerase expression system is used routinely for selective high level expression of proteins under control of the T7 RNA polymerase and so a "T7 sequesterer" was constructed in an attempt to increase the efficiency of the SecB sequestering approach. By constructing a plasmid with the sequesterer under the control of T7 RNA polymerase it was shown that the SecB sequestering approach could be reproduced in a T7 expression strain, with induction of the T7 sequesterer resulting in translocation kinetics comparable to those in the *secB* null mutant.

Interestingly, long term induction of the T7 sequesterer did not result in selective viability on only minimal media, as seen in the *secB* null mutant. This may be due to insufficient sequesterer expression to completely deplete cytoplasmic SecB but it is more likely to be as a result of the action of SecB itself, and may represent a drawback with any SecB sequestering approach. If, as is envisaged, SecB is directly involved in targeting of the precursor to the translocase (Hartl *et al.*, 1990) then sequesterers, in a complex with SecB, may still be directed to the translocase by the activity of SecB. Even though the sequesterer may be incapable of subsequent translocation it may be released from SecB as a result of interaction with the translocase, namely SecA. Release of the sequesterer from SecB would allow SecB to re-enter the translocation cycle. Thus, irrespective of how efficient the sequesterer is in forming the initial complex with SecB it may only be bound transiently. High level synthesis of sequesterers for prolonged periods may also produce secondary effects which probably result from the accumulation of export-incompetent

polypeptides at the translocase (Liu *et al.*, 1989). With this in mind it is interesting that long term induction of the sequesterers does not result in cell death on both rich and minimal media.

Even so, following further characterisation, the T7 sequestering approach may be used in pulse-chase experiments without the necessity for immunoprecipitation, enabling the requirements of exported proteins for SecB to be determined in the absence of specific antisera.

The finding that TolC does not appear to require SecB for its translocation is surprising (Kumamoto, 1990; de Cock and Tommassen, 1992). However, it remains possible that the low levels of functional SecB, which are thought to remain even following long term induction of the sequesterers, may be sufficient for export of TolC to the outer membrane and so facilitate HlyA secretion. Although it may be argued that the limited functional SecB, remaining after induction of the sequesterers, may be sufficient to maintain HlyA in a secretion-competent conformation via a direct interaction with HlyA, this appears highly unlikely, particularly as it has been shown that SecB has targeting activity (Hartl *et al.*, 1990) and that the Sec-dependent translocation pathway and the Hly secretion pathway compete when proteins contain both a HlyA secretion signal and a typical N-terminal signal peptide (Gentschev *et al.*, 1990).

Although it was concluded that the secretion of HlyA is SecB-independent, the data suggested a possible role for the heat shock proteins. Following induction of the SecB sequesterers for several hours, which resulted in induction of the heat shock protein GroEL in agreement with previous observations (Wild *et al.*, 1993), the secretion of HlyA was slightly increased.

Preliminary experiments were carried out which confirmed the translocation defect of β–lactamase in the *groEL44* and *groES619* mutant strains, as was seen by Kusukawa *et al.*, (1989) following a shift to the non-permissive temperature. The data of Kusukawa *et al.*, (1989), omits mention of the relative rates of β–lactamase translocation in the wild-type and mutant strains at the permissive temperature (30°C) and only includes data obtained following a shift to the non-permissive temperature (42°C) for 60 minutes. The corresponding results presented here are entirely consistent with their data, although the additional data shown here make it more difficult to come to such obvious conclusions about the requirements of functional GroEL and GroES for efficient β–lactamase translocation *in vivo*. However, the results obtained in the OmpA studies, which were carried out simultaneously with the

β–lactamase studies, were sufficiently consistent to conclude that the indirect effects of the mutations in *groEL* and *groES* were seen only after incubation at the elevated temperature for periods of 60 minutes or more. Experiments were then performed to determine whether GroEL and GroES were directly required for efficient HlyA secretion.

Studies using the *groEL44* temperature-sensitive mutant indicated that functional GroEL was a strict requirement for the efficient secretion of HlyA. The dramatically reduced secretion of HlyA even at the permissive temperature is consistent with the findings that temperature-sensitive and cold-sensitive mutants exhibit some defects even at the permissive temperatures (Zeilstra-Ryalls *et al.*, 1993; Landry *et al.*, 1993; Pogliano *et al.*, 1993) and suggests that HlyA may have a strict requirement for functional GroEL. Indeed, the translocation kinetics of β–lactamase were slightly slower in the *groEL44* mutant, than wild-type strain, at the permissive temperature. These results may be indicative of proteins exhibiting different levels of requirement for GroEL, which is discussed below.

In contrast to the wild-type strain, and previous data indicating the temperature-dependence of HlyA secretion (Koronakis *et al.*, 1991), secretion of HlyA by the *groEL44* mutant decreased following a shift to the non-permissive temperature. The presence of the complementing plasmids, particularly pOF39 and to a lesser extent pHO7, was able to at least partially restore secretion of HlyA in the *groEL44* mutant. The presence of either of the complementing plasmids in the wild-type strain TG1 resulted in decreased HlyA secretion, however a similar reduction was not seen in the wild-type strain CG2245 containing pOF39 or pHO8. The reason for this difference is not clear although it may be that for efficient HlyA secretion to occur optimum levels of GroEL may be required and exceeding these levels may have an adverse effect on secretion.

It is not clear how the formation of mixed wild-type and mutant GroEL oligomers (Zeilstra-Ryalls *et al.*, 1993) affects the activity of the GroEL tetradecamer. The formation of such mixed wild-type and mutant tetradecamers is probably responsible for the limited recovery of secretion seen in the *groEL44* mutant containing the complementing plasmids. It may be envisaged, that for maximum efficiency each of the GroEL monomers must be wild-type. Incorporation of increasing numbers of mutant monomers into the GroEL tetradecamer may result in a corresponding decrease in the efficiency of its activity. It may be that smaller proteins, such as the 11kDa protein barnase (Gray and Fersht, 1993), are able to tolerate the inclusion of mutant monomers in the tetradecamer without severe

deleterious effects. However a larger protein such as β-lactamase, which may be expected to interact more extensively with several of the GroEL monomers, may be less tolerant of inclusion of such mutant monomers. Such a model suggests that the larger a protein, the stricter the requirement for fully functional GroEL and the poorer the tolerance for inclusion of mutant monomers in the tetradecamer. Therefore it may be inferred, that the severely decreased secretion of HlyA even at the permissive temperature reflects a strict requirement for a fully functional GroEL tetradecamer for efficient secretion. This is amplified at the non-permissive temperature where the levels of HlyA secreted are even further reduced. In order to confirm this model experiments using the truncated C-terminal, 23kDa HlyA polypeptide (Nicaud *et al.*, 1986) could be performed to determine whether this polypeptide exhibits a decreased requirement for fully functional GroEL.

The ability of pOF39 to restore secretion in the *groEL44* mutant to higher levels than pHO7 may be due to the large increase in the intracellular levels of GroEL observed in the presence of pOF39. Such an increase in wild-type GroEL monomers makes the formation of fully functional tetradecamers more probable. The plasmid pHO7 produces only low levels of GroEL (in the absence of IPTG) and these would be less able to "swamp out" the mutant GroEL monomers, particularly at elevated temperatures where synthesis of the mutant, but not wild-type, monomers would be increased. It would be interesting to determine whether induction of pHO7 encoded GroEL (by the addition of IPTG) would result in a further increase in the levels of HlyA secreted as might be predicted.

Results using the *groES619* temperature-sensitive strain indicate that fully functional GroES, the GroEL co-chaperonin is not required for the secretion of HlyA. It would seem unlikely that the use of other *groES* mutants would give different results as recent data from Landry *et al.*, (1993) have shown that the mutation in *groES619* occurs in a "mobile loop" on the GroES protein. This mobile loop has been shown to be important in the correct interaction between GroEL and GroES, with the mutation in *groES619* altering this interaction. It is thought that this altered interaction is responsible for the temperature-sensitive phenotype of the *groES619* mutant strain. Consistent with this, all eight of the *groES* mutants identified in the initial screen (Georgopoulos *et al.*, 1973) have mutations within this mobile loop (Landry *et al.*, 1993).

The finding that GroES is not involved in HlyA secretion ruled out the possibility that filamentation, which was observed in both the *groEL44* and *groES619* mutants consistent with previous observations (Georgopoulos and Eisen, 1974), may

be responsible for the reduced secretion of HlyA in the *groEL44* mutant. It also makes it seem unlikely that reduced secretion from the *groEL44* mutant occurs as a result of some specific effect on the biosynthesis of HlyA.

There are a few precedents for GroEL acting independently of GroES in protein folding assays conducted *in vitro* (Lorimer *et al.*, 1993). However, GroES is present *in vivo* and essential for cell viability (Fayet *et al.*, 1989), so how might GroEL act in HlyA secretion without the activity of its co-chaperonin? A single precedent exists for the substitution of GroES activity by another protein. During phage T4 head assembly, structural protein Gp23 oligomerises in a process which is thought to require GroEL but not GroES (Tilly and Georgopoulos, 1982; Keppel *et al.*, 1990). It has been shown recently that the phage T4 protein, Gp31 is capable of interacting with GroEL and this complex is involved in T4 phage head assembly, (van der Vies *et al.*, 1994). Functional replacement of GroES by Gp31 was also shown with growth of *groES* mutants at the non-permissive temperature (van der Vies *et al.*, 1994). This functional replacement of GroES by Gp31 occurs even though GroES and Gp31 show no significant homology at the amino acid level (including the apparent absence of a consensus ATP binding site in Gp31) or in secondary structures (Keppel *et al.*, 1990). This suggests that the ATP binding site which is present on GroES (Martin *et al.*, 1993b) is not essential for function, although it may be required for optimum efficiency.

It is an intriguing possibility that HlyB might participate in the release of HlyA from the GroEL complex at the entrance to the membrane translocator complex. Available data suggest a requirement for ATP binding or hydrolysis at an early stage of secretion, prior to or during the association of HlyA with the inner membrane (Koronakis *et al.*, 1991). The nucleotide binding site of HlyB found in the cytoplasmic portion of the molecule exhibits ATPase activity (Koronakis *et al.*, 1993) and is thought to provide the energy for the early stages of HlyA secretion (Koronakis *et al.*, 1991; 1993).

A hypothetical model of the early stages in HlyA secretion is presented in Fig.8.1. It can be envisaged that the nascent HlyA emerges from the ribosome and initially binds to DnaK-DnaJ to prevent folding into a secretion incompetent state. This reaction has been proposed as a common first step in the folding pathway of proteins in *E. coli* (Langer *et al.*, 1992; Hartl *et al.*, 1994). The unfolded HlyA is released by DnaK after the intervention of GrpE and is then transferred to GroEL. Recent data suggest that GroEL binds unfolded proteins within the cavity of the tetradecameric double ring structure (Langer *et al.*, 1992; Braig *et al.*, 1993). It has

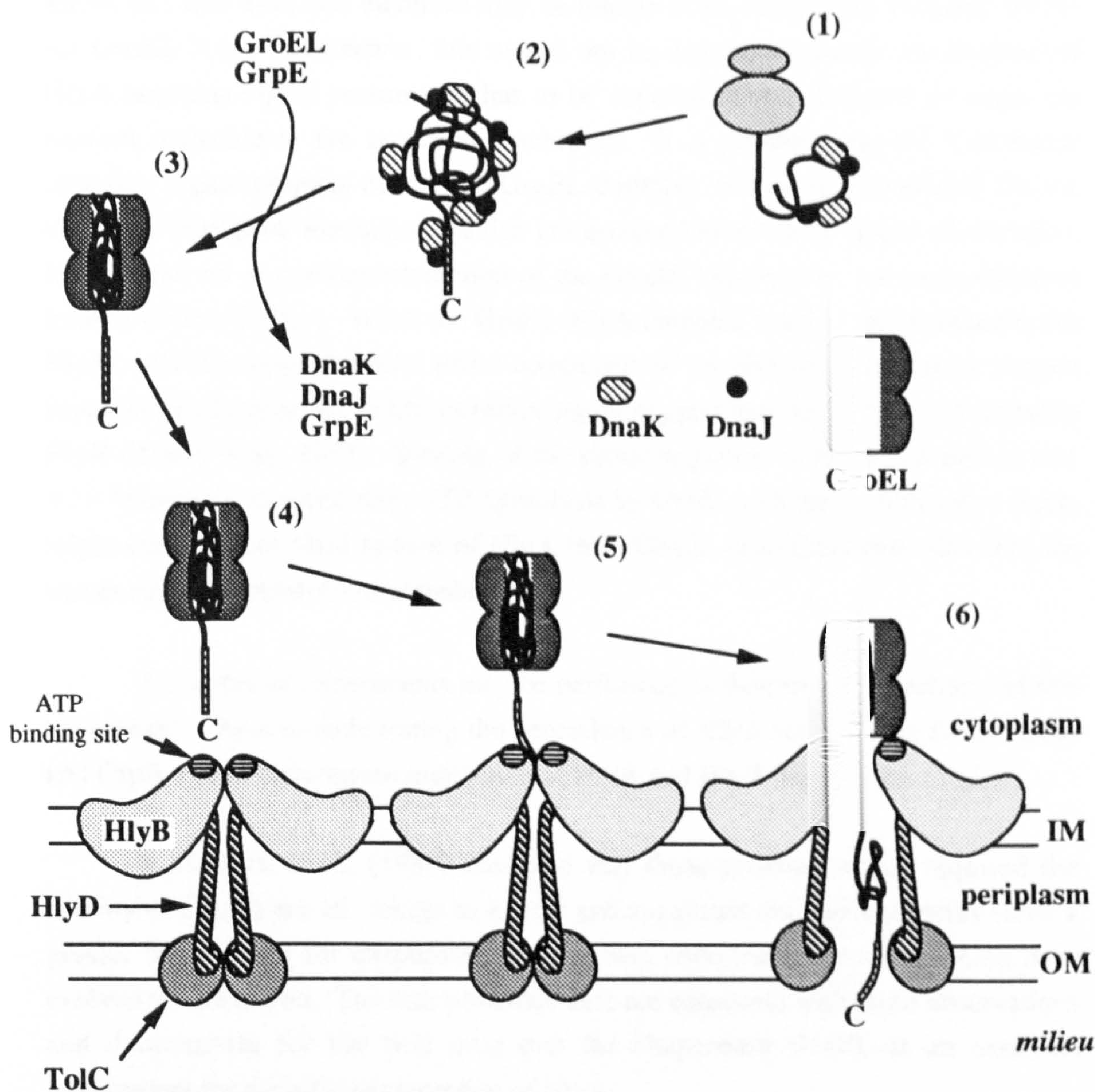


Fig. 8.1 Schematic diagram representing a hypothetical model of the early stages in haemolysin secretion showing the proposed involvement of GroEL. As HlyA emerges from the ribosome it interacts with DnaK and DnaJ to prevent folding (**1**). The completed nascent HlyA is held in an extended form by DnaK/DnaJ (**2**). HlyA is transferred to GroEL after the intervention of GrpE and is held in an intermediate state with secondary structure and some loose tertiary structure. The C-terminal secretion signal projects from the GroEL complex to prevent interaction with GroES (**3**). The GroEL/HlyA complex approaches the inner membrane and cytoplasmic domain of HlyB (**4**). The HlyA secretion signal interacts with HlyB, possibly via initial interaction with phospholipid head groups (**5**). The C-terminus of HlyA enters the translocator and rounds of ATP hydrolysis cause controlled release of HlyA into the translocator (**6**).

been estimated that within the GroEL cavity there is sufficient space to accomodate a single unfolded polypeptide of up to 90kDa (Braig *et al.*, 1993). HlyA is slightly above this size limit and therefore may be unable to be completely enclosed within the GroEL "cage". However, this would not be desirable because the C-terminal HlyA targeting signal presumably has to be exposed after translation to target the nascent molecule to the secretion machinery. It is possible that the C-terminal targeting signal projects out of the GroEL complex so that it is available for the necessary molecular interactions which are involved in the early stages of secretion. It may also act to prevent interaction of the GroES heptamer to prevent premature folding of bound HlyA. When the GroEL-HlyA complex reaches the membrane, the HlyA secretion signal interacts with a component of the translocator. Recent data on suppression of mutations in the secretion signal suggest that this receptor is probably HlyB (Zhang *et al.*, 1993). Binding of the signal sequence to HlyB and subsequent ATP hydrolysis, coupled with ATP hydrolysis by GroEL and the protonmotive force, might cause a controlled release of HlyA from GroEL and direct extrusion into the transmembrane translocator complex.

A number of experiments may be performed to determine the validity of this hypothesis. These include testing the dependence of HlyA secretion on DnaK, DnaJ and GrpE, and examining the interaction of HlyA and HlyB with GroEL *in vitro*.

Kusukawa *et al.*, (1989) observed that those proteins which required the activity of GroEL are all foreign to *E. coli* and suggested that these proteins show a greater requirement for chaperone function than endogenous proteins which have evolved within *E. coli*. The data presented here are consistent with these observations and demonstrate for the first time that the chaperonin GroEL is an essential requirement for the efficient secretion of HlyA.

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