CHARACTERIZATION OF MEMBRANE-BINDING BY FTSY, THE PROKARYOTE SRP RECEPTOR

By

JONATHAN SCOTT MILLMAN, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfilment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

MEMBRANE BINDING BY FTSY

DOCTOR OF PHILOSOPHY (2002) McMaster University
(Biochemistry) Hamilton, Ontario

TITLE: Characterization of membrane-binding by FtsY, the prokaryote SRP receptor

AUTHOR: Jonathan S. Millman, B. Sc. (McMaster University)

SUPERVISOR: Professor Dr. David W. Andrews

NUMBER OF PAGES: x, 242

ABSTRACT

The signal recognition particle pathway cotranslationally targets polytopic proteins to the inner membrane of *Escherichia coli*. FtsY is the receptor that is recognized by the ribosome-nascent chain-bound signal recognition particle in the bacterial targeting reaction. At the outset of this work a major unresolved issue, and current issue of some contention is the mechanism of FtsY assembly on the *E. coli* inner membrane. To clarify the nature of this process, this thesis describes the region of FtsY that binds the membrane and a site-specific cleavage event that defines this region upon membrane binding. The involvement of a specific lipid, phosphatidylethanolamine and an as-yet unidentified inner membrane protein in FtsY membrane targeting are also addressed. With this understanding of the mechanisms of FtsY membrane assembly in *E. coli*, additional investigations demonstrate divergent species-specific interactions with the membrane for FtsY homologues from *Bacillus subtilis* and *Streptomyces coelicolor*. Finally, the unique amino-terminal region was determined to be essential for the function of FtsY in *E. coli*.

ACKNOWLEDGEMENTS

For specific contributions I acknowledge Felicia Vulcu for Figure 3.4 and Hai-Yan Qi and Dr. Harris Bernstein for Figure 3.5.

I would like to thank my supervisor, Dr. David Andrews, for necessary guidance, and a willingness to allow me the freedom to pursue this area of investigation. I also thank my supervisory committee, Dr. Herb Schellhorn, Dr. Gerry Wright, Dr. Gerhard Gerber, and Dr. Turlough Finan, whose assistance in steering this project was invaluable.

I would also like to thank my friends and colleagues who helped me along the way: those who knew; those who thought they knew; and those who knew enough to know they did not know. In no particular order, they are: Iain, Brian, Jason Y, Jason N, Glenn, Alexandra, Josie, Simone, Scott, Martin, Helen, Amit, Aline, Kathleen, Stu, Scott, Alex, Kerri-Ann, Johnny T., Nawaid, Nandita, Steve, Paulina, Peter, Kyle, Matty, Ly, Min, Jeremy, Mina, Weijia, Bronwen, Jill, Shirley, Roy, Wen Hua, Rami, Mary, the boyz from bay 616 (Ace and Henk), and Grasshopper (Felicia).

I thank those who were close to me throughout this journey: Mum, Dad, Kate, Grandad and Chantel. Also in memory of Nana Pitman and Nana Millman, both of whom remain forever in my heart.

Finally, as this chapter of my life comes to a close and the next one begins, I thank Jordan and Karen. Jordan, you have brought great joy into my life and were responsible for the very last experimental step of this thesis. Karen, you have taught me so much about love, and that soul mates truly do exist.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP	alkaline phosphatase
ATP	adenosine 5'-triphosphate
CRMs	canine rough microsomes
CL	cardiolipin
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetate
EGTA	ethylene glycol-bis(ß-aminoethyl ether) N,N,N′,N′-tetraacetic acid
ER	endoplasmic reticulum
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
IM	inner membrane
INV	inner membrane inverted vesicle
IPTG	isopropyl-1-thio-β-D-galactopyranoside
kDa	kilodalton
LB	Luria-Bertini Broth
M	molar
mM	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
MW	molecular weight

PAGE	polyacrylamide gel electrophoresis
PA	phosphatidic acid
PBST	*Propionibacterium shermanii* transcarboxylase
PC	phosphatidylcholine.
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
RNC	ribosome-nascent chain
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SR	signal recognition particle receptor
SRα	α-subunit of the signal recognition particle receptor
SRβ	β-subunit of the signal recognition particle receptor
SRP	signal recognition particle
TM	transmembrane
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
WT	wild-type

CHAPTER I

Introduction

1.1 Overview:

All cells are encapsulated by a lipid-based membrane that provides a physical barrier between the interior of the cell and the external environment. In order for polar substances, including secretory and membrane proteins, to cross this barrier cells have developed various transport systems. Integration of proteins into membranes adds a further level of complexity as hydrophobic sequences of amino acids must be inserted into the bilayer without misfolding or aggregating.

The complexity and variety of protein targeting pathways employed by all cells is extensive. Bacteria provide a useful model system for the investigation of these process as they lack internal organelles and thus target proteins to a single membrane, and are amenable to cell-free analysis and genetic manipulations. Even though prokaryotes contain only one internal membrane they have developed at least three distinct pathways to direct proteins to this site: (1) The general secretory (Sec) pathway; (2) The twin arginine (Tat) pathway; (3) The signal recognition particle (SRP) pathway. The Sec pathway targets the vast majority of secretory proteins and some integral membrane proteins to the inner membrane (IM). The other pathways enable the targeting of a specific subset of proteins with unique translocation requirements. The Tat pathway is used to target and translocate proteins that cross the membrane in a fully folded state. Hydrophobic polytopic IM proteins that must be cotranslationally translocated across the IM utilize the SRP pathway.

This thesis will first review the mechanisms of protein targeting and integration

into the IM of a model bacterial species, *Escherichia coli*. After a general overview of this field the focus will shift to the SRP pathway of protein targeting. The two proteins that regulate cotranslational targeting through this pathway are Ffh and FtsY. The principal focus of this thesis is one essential step in the biogenesis of the SRP pathway: the mechanisms of membrane binding by FtsY.

1.2. The General Secretory Pathway:

Membrane targeting of proteins that are synthesized on a common pool of ribosomes that also synthesize cytoplasmic proteins requires that one class of proteins contain specific signals. In both prokaryotes and eukaryotes secretory and membrane proteins contain signal sequences located at their amino-termini. These consist of an amino-terminal positively charged region of 1-5 residues, a hydrophobic core of 7-15 residues, and a carboxyl-terminal span of 3-7 residues containing small aliphatic amino acids that may constitute a site of cleavage of the peptide chain (Izard and Kendall, 1994).

The vast majority of periplasmic and IM proteins in *E. coli* are targeted and translocated via the Sec pathway (reviewed in (Fekkes and Driessen, 1999)). It appears to be the 'default' targeting pathway for proteins not recognized by components of the Tat or SRP targeting pathways. The essential components of this system were identified in genetic screens for cells exhibiting severe protein export defects under non-permissive conditions (reviewed in (Wickner et al., 1991)).

Nascent membrane and secretory proteins that travel through the Sec pathway are bound in the cytoplasm by the chaperone SecB (Kumamoto, 1989). SecB directs the

nascent protein to the IM through an interaction with the peripheral membrane protein SecA. Targeted proteins are subsequently translocated into or across the IM though a single translocation complex, or "translocase" (Figure 1.1).

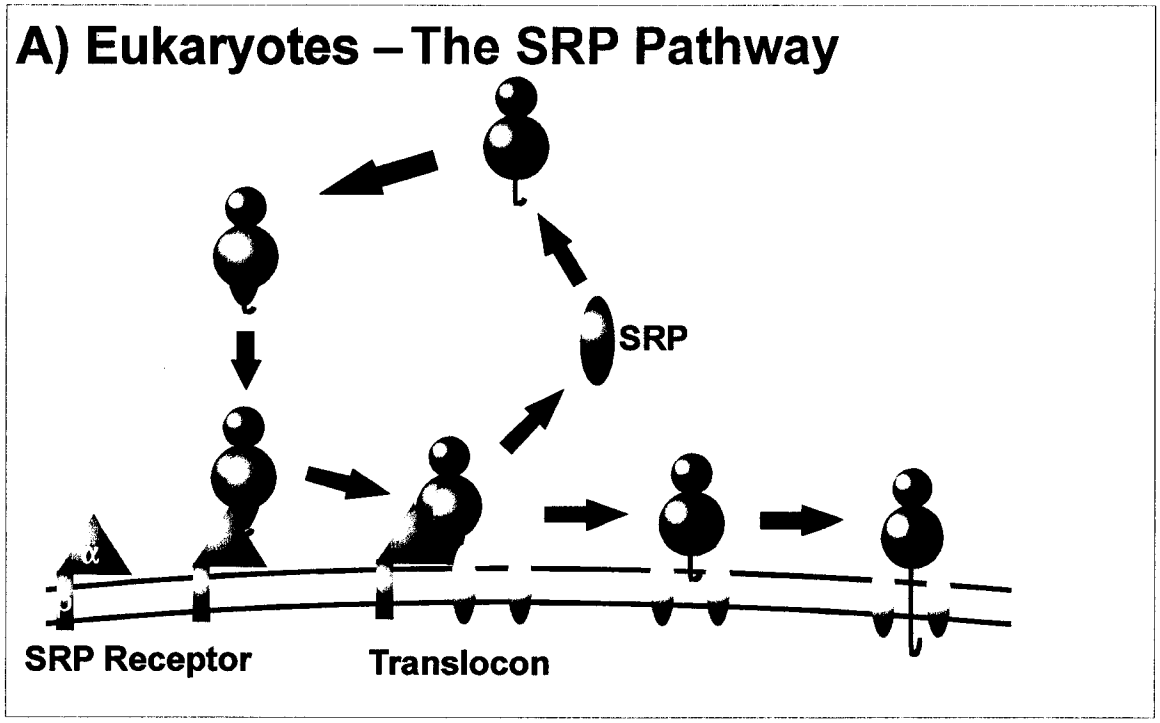
1.2.1. Protein Targeting in the Sec Pathway.

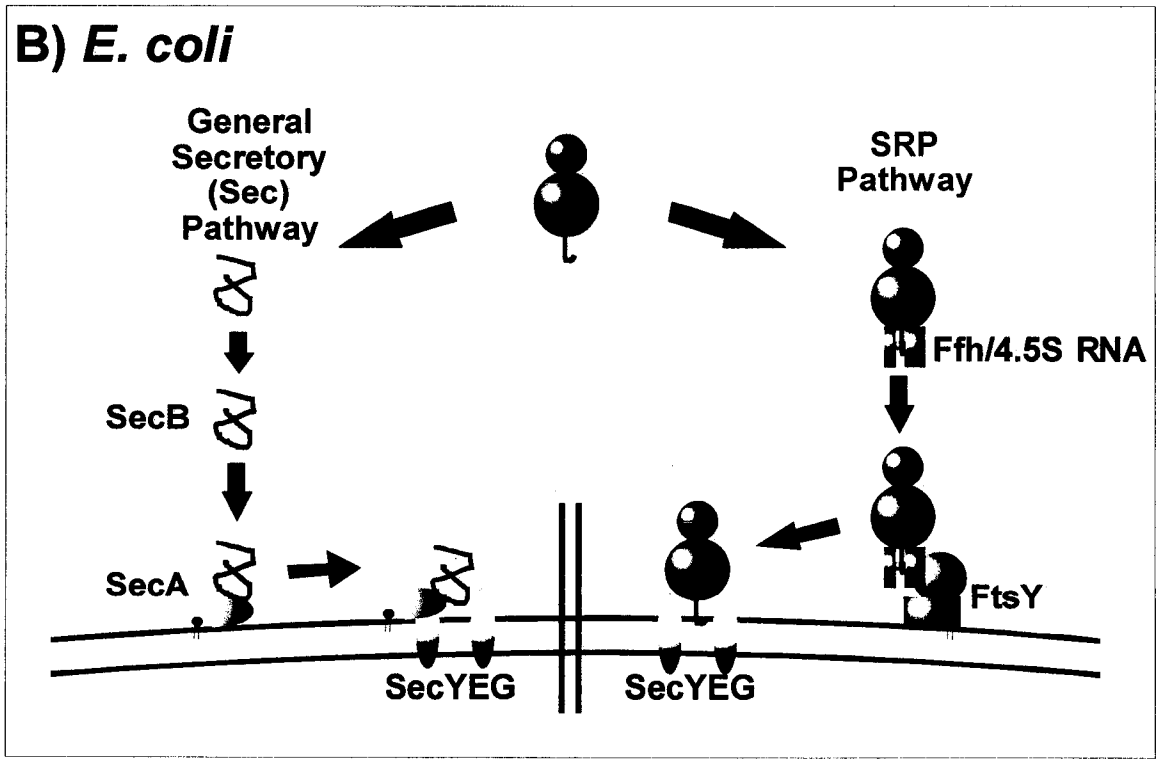
SecB is a highly acidic molecular chaperone of MW 17 kDa and is the first component of the Sec pathway to interact with newly synthesized secretory proteins (Kumamoto and Nault, 1989). SecB binds highly selectively to precursor secretory proteins either co- or post-translationally (Kumamoto and Francetic, 1993; Kumamoto, 1989). These interactions occur through the mature domains of precursor proteins, and not through direct interactions with signal sequences (Randall et al., 1998; Randall and Hardy, 1995). The presence of a signal sequence slows folding of the mature domain (Park et al., 1988), possibly allowing for discrimination between precursor and cytoplasmic proteins based on the rate at which they fold (Hardy and Randall, 1991). The binding motif recognized by SecB consists of approximately nine residues enriched in aromatic and basic residues (Fekkes et al., 1997). Based on the quaternary structure of SecB, binding of these motifs is proposed to occur in channels present on each side of the tetramer (Xu et al., 2000). SecB maintains precursor proteins in a conformation that is loosely folded and therefore competent for translocation (Lecker et al., 1990; Weiss et al., 1988). Although other chaperones are able to bind precursor secretory proteins, only SecB targets proteins to the translocase (Hartl et al., 1990).

Precursor proteins are targeted to the translocase via the peripheral membrane protein SecA, an ATPase that exists primarily as a homodimer of 102 kDa subunits

Figure 1.1. Common protein targeting pathways in Eukaryotes and in *E. coli*. *A*. In mammalian cells, membrane and secretory proteins are targeted to the endoplasmic reticulum cotranslationally via the signal recognition particle pathway. The targeting process begins with recognition of a hydrophobic signal sequence on a nascent secretory or integral membrane polypeptide by the 54 kDa protein of SRP (SRP54), resulting in binding of SRP to both the ribosome and nascent polypeptide and a concomitant slowing of translation elongation. The ribosome nascent chain complex is targeted to the endoplasmic reticulum (ER) membrane through an interaction with the α-subunit of the SRP receptor (SRα). This interaction leads to insertion of the nascent chain into the translocon; the aqueous pore through which proteins are translocated cotranslationally.

B. In *E. coli*, two pathways exist for targeting proteins to the inner membrane. In the General Secretory (Sec) Pathway, nascent secretory proteins are fully translated in the cytosol prior to targeting. These proteins are bound by SecB, which delivers the protein to the peripheral membrane protein SecA. Through successive cycles of SecA insertion and deinsertion into the membrane, the nascent protein is translocated through the SecYEG translocase pore into the periplasmic space. A second, cotranslational SRP targeting pathway exists for membrane assembly of polytopic inner membrane proteins. Ffh and 4.5S RNA form a SRP-like complex which interacts with nascent membrane proteins. This particle promotes cotranslational targeting of nascent chains via an interaction with FtsY, the SRα homologue. Proteins are ultimately cotranslationally translocated through the common SecYEG translocase.


A) Eukaryotes – The SRP Pathway
SRP
SRP Receptor
Translocon


B) E. coli
General Secretory (Sec) Pathway
SRP Pathway
Ffh/4.5S RNA
SecB
SecA
FtsY
SecYEG
SecYEG

(Cabelli et al., 1988; Akita et al., 1991). It is found both in the cytoplasm and on the membrane in approximately equal distribution (Cabelli et al., 1991). It binds the membrane through low affinity interactions with acidic phospholipids (Breukink et al., 1992; Hendrick and Wickner, 1991) and high affinity interactions between the carboxyl-terminal region of SecA and the core translocase component SecY (Manting et al., 1997; Matsumoto et al., 1997; Snyders et al., 1997).

The presence of a bound precursor protein increases the affinity of SecB toward SecA (Fekkes et al., 1998). Residues throughout SecB come together to form a negatively charged surface (Fekkes et al., 1998; Xu et al., 2000) that recognizes a 22 amino acid region that is enriched in basic residues at the carboxyl-terminus of SecA (Fekkes et al., 1997; Breukink et al., 1995). The interaction also requires a coordinated Zinc ion bound to SecA (Fekkes et al., 1999). The affinity of this interaction is greatly increased by the association of SecA with the translocase (Hartl et al., 1990; Fekkes et al., 1997), ensuring delivery of the preprotein to membrane-bound SecA.

The interaction between SecB and SecA transfers the preprotein to SecA (Fekkes et al., 1997; Fekkes et al., 1998), which binds directly to the signal sequence. Stimulation of ATP binding and hydrolysis by SecA (Miller et al., 2002; Kim et al., 2001a), releases SecB into the cytoplasm for further rounds of targeting (Fekkes et al., 1997).

1.2.2. Translocation across the IM: The translocase.

SecA plays a pivotal role in protein export, acting both as a membrane receptor for the preprotein and also as a component of the translocase complex of the IM. The other core components of this complex consist of the integral membrane proteins

SecYEG. Genes encoding SecY and SecE were identified in early screens for mutations that affected protein translocation (reviewed in (Wickner et al., 1991)). These proteins, along with SecA, are sufficient to reconstitute protein translocation across the IM (Brundage et al., 1990; Akimaru et al., 1991). SecG copurifies with SecYE and strongly stimulates translocation in a reconstituted system (Brundage et al., 1992; Nishiyama et al., 1993; Hanada et al., 1994).

Translocation of precursor proteins requires both ATP and the proton motive force (Geller et al., 1986; De Vrije et al., 1987; Yamane et al., 1987). ATP binding by SecA drives insertion of an approximately 2.5 kDa segment of the preprotein into the translocase pore (van der Wolk et al., 1997). ATP hydrolysis then releases the preprotein to the SecYEG complex where the preprotein is prevented from retrotranslocation by the presence of the proton-motive force (Schiebel et al., 1991). Through successive cycles of membrane insertion and deinsertion, the entire protein is ultimately translocated across the IM (van der Wolk et al., 1997).

The precise mechanism by which SecA drives translocation through the pore is unclear and somewhat controversial. Translocation begins with dissociation of the SecA dimer into monomers (Or et al., 2002). Cross-linking experiments have shown that the preprotein is associated with SecA throughout its membrane passage (Joly and Wickner, 1993). In the presence of SecYE, preprotein and ATP, a 30 kDa domain at the carboxyl-terminus of SecA is inserted into the inner membrane (Economou and Wickner, 1994; Price et al., 1996). This domain is inaccessible to proteases added from either face of the

membrane and becomes accessible only upon addition of detergent (Economou and Wickner, 1994; Kim et al., 1994). Furthermore, this proteolytic fragment is stabilized by non-hydrolysable ATP analogues (Economou et al., 1995). In the presence of SecYEG the 30 kDa domain is also protected from photoactive probes in the lipid bilayer (Eichler et al., 1997; van Voorst et al., 1998) suggesting SecA inserts into the translocase during preprotein translocation. A protease-protected amino-terminal 65 kDa domain containing the high affinity ATP binding site has also been observed upon trypsin digestion of SecA in the presence of vesicles and translocating protein (Eichler and Wickner, 1997). This has led to the suggestion that preprotein translocation is mediated by successive rounds of SecA insertion and deinsertion in the membrane. Alternate models whereby SecA simply undergoes conformational changes throughout the translocation process have also been proposed (see, for example (Manting and Driessen, 2000)) since neither the size of the SecYEG assembly nor the thickness of the lipid bilayer appear sufficient to accommodate large domains of SecA (Breyton et al., 2002).

The pore through which preproteins are translocated consists minimally of dimers of SecYEG (Bessonneau et al., 2002), which form a pore containing a 16x25 angstrom cavity (Breyton et al., 2002). In this complex, SecY spans the membrane 10 times, and has been demonstrated through cross-linking studies to be in close proximity to the translocating preprotein, shielding it from phospholipids (Joly and Wickner, 1993). Apart from SecA, it is the only component of the complex that has been demonstrated to interact directly with the preprotein. This, and its direct interactions with SecA suggest

that SecY is primarily responsible for formation of the translocation pore. SecE appears to stabilize SecY. In the absence of SecE, SecY is rapidly degraded by the protease FtsH *in vivo* (Kihara et al., 1995). SecG undergoes inversions of topology that are linked to the cycles of preprotein translocation, suggesting that SecG assists in the inversions of SecA (Nishiyama et al., 1996).

Although SecYEG along with SecA form a functional translocase, the accessory proteins SecD, SecF and YajC form a trimer that associates with the translocation apparatus (Duong and Wickner, 1997a). Depletion of SecD and SecF diminishes, but does not abolish protein translocation (Pogliano and Beckwith, 1994). The SecDFYajC heterotrimer appears to function by stabilizing the membrane-inserted conformation of SecA and preventing the reverse movement of translocation intermediates (Duong and Wickner, 1997b).

YidC is the most recently identified component of the translocase. It is essential for cell viability, and depletion of YidC interferes with insertion of a variety of membrane proteins (Samuelson et al., 2000). YidC was first identified as a membrane component required for membrane-integration of substrates that do not use the Sec translocase (Samuelson et al., 2000), a process previously believed to be spontaneous. In this capacity YidC may be involved in folding hydrophobic segments into a transmembrane conformation (Chen et al., 2002). YidC also cross-links to SecD and SecF (Nouwen and Driessen, 2002), suggesting it is tangentially associated with the translocase through the SecDFyajC complex.

YidC appears to be involved only in the integration of membrane proteins (Samuelson et al., 2001; Van der Does et al., 2001). A variety of cross-linking studies have demonstrated that when it is associated with the translocase YidC interacts with TM segments of nascent proteins late during translocation, even as additional TM segments are being translocated (Urbanus et al., 2001; Beck et al., 2001; Houben et al., 2002). This has led to the suggestion that YidC mediates partitioning of nascent proteins into the lipid bilayer.

1.3 The Twin Arginine (Tat) Pathway:

The Tat pathway is a distinct targeting and translocation pathway that transports proteins through membranes in a fully folded conformation (reviewed in (Robinson and Bolhuis, 2001)). Proteins that are targeted by this system contain signal peptides that resemble those of typical secretory proteins but contain an additional motif comprised of sequential arginine resides in the amino-terminal region of the signal (Chaddock et al., 1995). Certain variations on this motif may also direct proteins through this pathway (DeLisa et al., 2002). Most of the known and predicted substrates of the Tat pathway bind cofactors (Weiner et al., 1998; Santini et al., 1998). Although only 22 substrates have been identified, this pathway is of great importance, since most cofactors are only incorporated into proteins in the cytoplasm, necessitating a mechanism for translocating folded proteins across the membrane.

The Tat translocation apparatus contains at least three integral membrane proteins,

TatA, TatB, and TatC that are required for translocation of a Tat substrate, SufI, across INVs *in vitro* (Yahr and Wickner, 2001). These are encoded on a single operon, along with an additional component, TatD (Weiner et al., 1998). The amino acid sequences of each of the subunits resemble no other known protein translocase subunits.

Targeting mechanisms employed by this pathway are also unclear, although a potential binding protein for twin-arginine signals (DmsD) has been identified (Oresnik et al., 2001). Translocation of substrates through this apparatus requires an intact membrane potential, but does not require nucleotides (Yahr and Wickner, 2001). The mechanisms by which proteins are translocated are also unknown, but must be capable of translocating substrates as large as 5 nm in diameter across the membrane in a folded state without major losses in metabolites or ions.

1.4. The Signal Recognition Particle (SRP) Pathway:

The SRP pathway was first discovered in eukaryotes, where it targets signal sequence-containing proteins to the endoplasmic reticulum (ER) for cotranslational translocation through the translocon (Figure 1.1) (reviewed in (Keenan et al., 2001)). Targeting is initiated when SRP binds to the signal sequence of a nascent secretory protein as it emerges from the ribosome (Kurzchalia et al., 1986; Krieg et al., 1986). Translation is transiently arrested (Walter and Blobel, 1981) as the ribosome-nascent chain (RNC) complex is directed to the ER membrane through an interaction between SRP and the SRP receptor (SR) (Gilmore et al., 1982a; Meyer and Dobberstein, 1980).

The SR in eukaryotes is composed of two GTPase subunits; transmembrane SRβ anchors the peripherally bound SRα onto the membrane (Miller et al., 1995; Young et al., 1995). Ultimately, the RNC complex is transferred to the translocon, whose integral membrane components are analogous to those of their bacterial counterparts (reviewed in (Andrews and Johnson, 1996)).

Mammalian SRP exists as an extended ribonucleoprotein particle composed of six polypeptides of apparent MW 9 kDa, 14 kDa, 19 kDa, 54 kDa, 68kDa and 72 kDa assembled on a single 7S RNA (Walter and Blobel, 1980; Gilmore et al., 1982b; Walter and Blobel, 1982; Andrews et al., 1987). In most species the RNA is approximately 300 nucleotides long and forms eight helical segments. In this complex structure the 5' and 3' ends of the RNA are spatially proximal to each other and form multiple hydrogen bonds to form the 'Alu' domain while the central portion of the RNA folds into the 'S' domain (Gorodkin et al., 2001). The two smallest polypeptides, SRP9 and SRP14 form a homodimer that assembles on the Alu domain of the RNA (Strub et al., 1991; Janiak et al., 1992). This region of the particle is responsible for inducing elongation arrest of the nascent protein (Siegel and Walter, 1985). Arresting elongation increases translocation efficiency, likely because it increases the amount of time that the nascent chain remains in a translocation-competent state. (Mason et al., 2000). The 68 kDa and 72 kDa polypeptides also form a heterodimer (Scoulica et al., 1987). Alkylation of these components results in a particle that functions in elongation arrest but fails to interact with SR or promote translocation of nascent chains (Siegel and Walter, 1988). SRP19

appears to regulate biogenesis of the particle (Politz et al., 2000). It binds the S domain, causing localized changes in the helices of domain IV, and thereby allows SRP54 to assemble onto the particle (Diener and Wilson, 2000; Hainzl et al., 2002). The GTPase SRP54 is the main functional component of the particle. It is through SRP54 that SRP recognizes signal sequences and interacts with the ER-resident SR.

Regulation of targeting at the membrane is highly complex, and requires the coordinated GTPase activity of both SRP54 and SRα. Interactions of SRP54 with the ribosome and SRα with Sec61 (the primary component of the translocon) increase the affinity of SRP54 and SRα for GTP (Bacher et al., 1996). The concerted interaction of SRP54, SRα and two molecules of GTP produces a conformational change in both proteins that locks the molecules together and docks the RNC complex (Rapiejko and Gilmore, 1997). This docking allows transfer of the RNC to the translocon (Rapiejko and Gilmore, 1994). GTP hydrolysis by both SRP54 and SRα is required for release of SRP from SR and initiates another round of targeting (Connolly et al., 1991; Powers and Walter, 1995). Ribosomes may also play a role in regulating transfer of the ribosome or the nascent chain from SR to the translocon by stimulating the GTPase activity of SRβ (Bacher et al., 1999), which may in turn dissociate the SR complex (Legate et al., 2000).

1.4.1. Evidence for an SRP pathway in prokaryotes.

With the cloning SRP54 and SRα from mammalian cells, homologues of these proteins (referred to as Ffh and FtsY, respectively) were identified in the *E. coli* genome (Bernstein et al., 1989; Romisch et al., 1989) and demonstrated to be essential (Phillips

and Silhavy, 1992; Luirink et al., 1994). An essential RNA moiety (4.5S RNA) with high sequence identity to domain IV of 7S RNA was also identified in *E. coli* (Struck et al., 1988).

Since genetic screens for *E. coli* mutants with translocation defects failed to identify any homologues of the eukaryotic SRP pathway, the involvement of Ffh and FtsY in protein targeting was initially controversial (Bassford et al., 1991; Rapoport, 1991). The first evidence that these components may functionally resemble their eukaryotic counterparts came from studies showing that Ffh and 4.5S RNA assemble into a ribonucleoprotein particle similar to the eukaryotic SRP (Ribes et al., 1990; Poritz et al., 1990). Altering the expression of either component led to defects in targeting of a subset of proteins to the IM (Ribes et al., 1990; Poritz et al., 1990; Phillips and Silhavy, 1992; Jensen et al., 1994). Furthermore, 7S RNA and 4.5S RNA were found to be functionally interchangeable in certain assays (Ribes et al., 1990; Poritz et al., 1990; Brown, 1991). Identification of specific interactions between *E. coli* SRP and signal sequences of nascent secretory proteins provided evidence that this particle is truly involved in 'signal recognition' (Luirink et al., 1992; Bernstein et al., 1993). Interestingly, although Ffh was found to cross-link to the signal sequence of a eukaryotic secretory protein as part of a chimeric eukaryotic SRP, it could not promote translocation across microsomes (Bernstein et al., 1993).

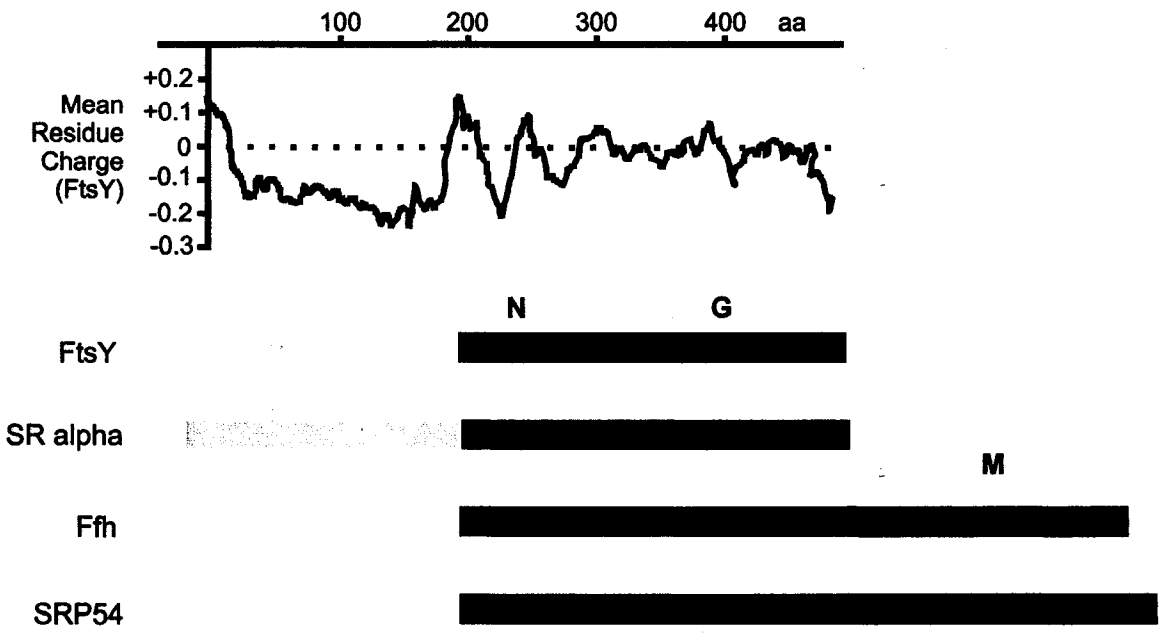
The role of FtsY in the *E. coli* SRP targeting system was confirmed when: (i) It was determined that *E. coli* SRP binds FtsY in a GTP-dependent manner (Miller et al.,

1994); (ii) This interaction resulted in GTP hydrolysis by both proteins (Miller et al., 1994); (iii) Depleting FtsY resulted in the accumulation of precursor membrane proteins in the cytoplasm (Luirink et al., 1994).

These, and further studies discussed below clearly indicated that a protein targeting pathway exists in *E. coli* that consists of components homologous to those of eukaryotic SRP. With the complete sequencing of the *E. coli* genome, however, no further homologues of the eukaryotic system have been identified. Thus the *E. coli* pathway is a model 'minimal' SRP system. A schematic describing the domain structures of the components of this system is presented in figure 1.2.

Ffh and FtsY share two common domains termed N and G that are also found in the eukaryotic homologues. The G regions are GTP-binding domains that constitute a specific sub-family of Ras-related low molecular weight GTPases (Bernstein et al., 1989; Romisch et al., 1989). The N regions are four helix bundles located amino-terminal to and making extensive contacts with the G domains (Murphy et al., 1997; Freymann et al., 1997). Ffh and SRP54 contain an additional carboxyl-terminal M domain. This methionine-rich region forms a crescent-shaped domain separated from the GTPase domain by a short linker (Czarnota et al., 1994). The M region is involved in binding both the signal sequence and SRP RNA (Romisch et al., 1990; Lutcke et al., 1992; Zopf et al., 1990). A highly negatively charged sequence has been identified at the amino-terminus of FtsY (amino acids 1-197) that is not found in the eukaryotic homologue SRα (Gill and Salmond, 1990). This amino-terminal 'A' region is involved in membrane

Figure 1.2. Charge Distribution, Domain Organization, and homologues of *E. coli* FtsY. *E. coli* FtsY has been conceptually divided into three regions: a highly negatively charged 'A' region at the amino-terminus (residues 1-197); a central 'N' region (residues 198-284); and a carboxyl-terminal 'G' region (residues 285-497). The N and G regions are also present in the FtsY homologue SRα and the related SRP-family GTPases Ffh and SRP54. Ffh and SRP54 contain an additional 'M' domain at their carboxyl-termini, whereas SRα contains an amino-terminal region that shares no homology with the 'A' region of FtsY. The residue charge diagram is adapted from *Gill and Salmond*, 1990.


100
200
300
400
aa
+0.2
+0.1
0
-0.1
-0.2
-0.3
Mean Residue Charge (FtsY)
N
G
M
FtsY
SR alpha
Ffh
SRP54

assembly (see below).

1.4.2. Role of GTPase activity in the SRP system.

Binding of *E. coli* SRP to FtsY is a prerequisite for release of the nascent chain at the translocon, resulting in GTP hydrolysis by both components (Miller et al., 1994) (Valent et al., 1998). A mutant FtsY with decreased GTPase activity has a reduced ability to interact with *E. coli* SRP and inhibits translocation of membrane proteins (Kusters et al., 1995). Together, these results demonstrate the importance of GTPase activity in regulating the SRP pathway. However, certain aspects of the GTPase cycle appear to be unique to this family.

Through the generation of a mutant FtsY in which the GTPase domain preferentially binds xanthosine 5'-triphosphate it was determined that FtsY and Ffh function as GTPase activating proteins for each other (Powers and Walter, 1995). The GTPase activity of FtsY can be stimulated by the NG region of Ffh, demonstrating that GAP activity resides in the NG domain of Ffh, and by extension is likely also in the NG region of FtsY (Macao et al., 1997). Furthermore, unlike most small GTPases, those in the SRP family have a very low binding affinity for GDP, which can be rapidly exchanged for GTP in the absence of an external guanine nucleotide exchange factor (Moser et al., 1997; Jagath et al., 1998). The interaction of *E. coli* SRP-bound RNCs with FtsY occurs independently of guanine nucleotides (Valent et al., 1998) and in the eukaryotic pathway both SRP54 and SRα can be in a nucleotide-free form prior to membrane-docking of the SRP-RNC complex (Rapiejko and Gilmore, 1997). The

nucleotide-free form of these GTPases is stabilized by a network of interactions between active site residues (Freymann et al., 1997). Several structural features contribute to the nucleotide occupancy of the GTPase domain. Amongst these, the interface between the N and G domains harbours many conserved residues that make extensive contacts (Murphy et al., 1997; Freymann et al., 1997). These are in close proximity to the nucleotide-binding fold, suggesting they may be involved in sensing or controlling the nucleotide occupancy of the G domain (Freymann et al., 1997). A unique 'closing loop', which is disordered in the absence of nucleotide, may act as a gate, promoting nucleotide exchange (Freymann et al., 1999).

The possibility that SRP and its receptor are in empty-site conformations upon membrane targeting of the SRP-RNC complex to the membrane initially seemed at odds with data demonstrating an increased affinity for GTP by SRP54 upon binding the ribosome and by SRα upon binding the translocon (Bacher et al., 1996). Both of these events can presumably occur prior to targeting of the nascent chain to the membrane. This apparent dichotomy can be explained by a 'concerted switch' model for SRP targeting, in which the steps that lead to docking of the ribosome nascent chain complex each result in increased affinity for GTP by one of the components but do not have to proceed in a specific order (Millman and Andrews, 1997). When the two GTP-bound molecules interact they lock together in the GTP-bound state and can only be separated by hydrolysis of GTP. This series of low-fidelity reactions imparts high fidelity to the targeting reaction (Millman and Andrews, 1997). This is a novel mechanism of GTPase

action that, due to the "locking" step, leads to unidirectional targeting of RNCs to the membrane (Figure 1.3).

1.4.3. The SRP system targets polytopic IM proteins cotranslationally.

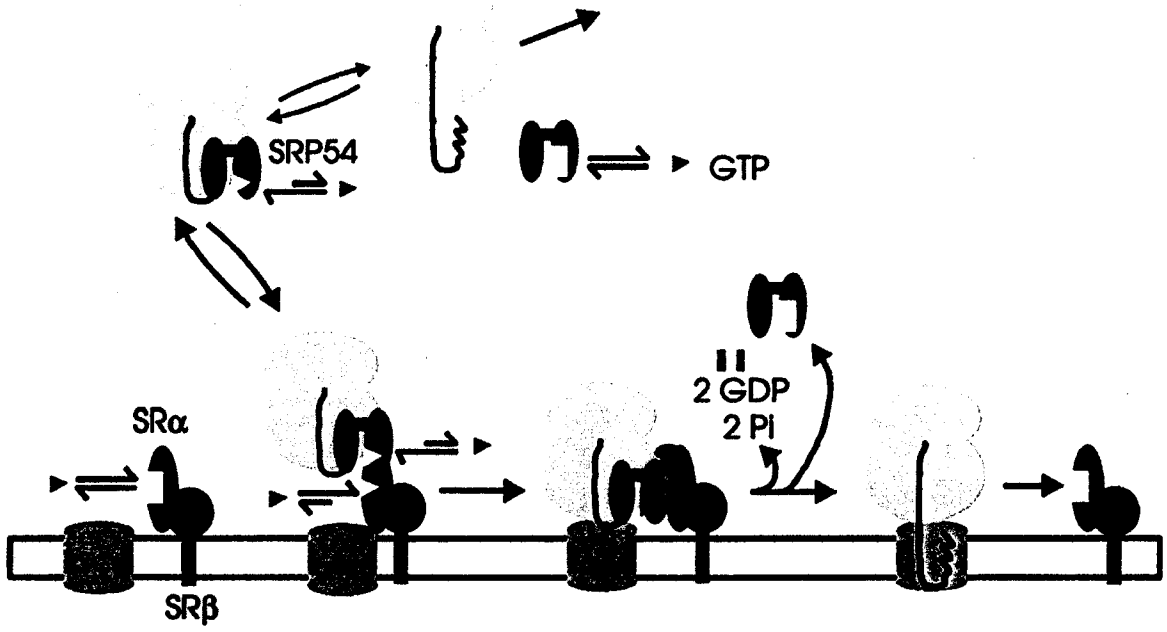
Initially it was not intuitively clear why *E. coli* and other prokaryotes maintained two parallel pathways for targeting proteins containing amino-terminal signal sequences to the IM. Investigations utilizing a small subset of membrane and secretory proteins demonstrated that predominantly polytopic inner membrane proteins displayed defects in membrane assembly upon depletion of SRP components. In contrast, depletion of the targeting components of the Sec pathway abolished translocation of secretory proteins, but had little effect on the assembly of many inner membrane proteins (de Gier et al., 1996; Seluanov and Bibi, 1997; Macfarlane and Muller, 1995). The assertion that the SRP pathway is involved in targeting of polytopic IM proteins was confirmed using an unbiased genome-wide screen for SRP-dependent substrates (Ulbrandt et al., 1997). Genes were isolated that conferred a synthetic lethality when overproduced in the presence of limiting Ffh, presumably by titrating available Ffh. Nine genes, all of which encoded IM proteins were identified in this screen. Each of these proteins displayed defects in membrane insertion upon depletion of SRP, whereas translocation of all tested secretory proteins was not affected by SRP depletion (Ulbrandt et al., 1997).

In *E. coli* the targeting signals of membrane proteins are generally more hydrophobic than those of secretory proteins. This apparently provides a mechanism for recognition of membrane proteins by SRP, which cross-links with greater efficiency to

Figure 1.3. A concerted switch model for SRP-mediated targeting. Stable targeting of the ribosome-nascent chain complex to the translocon requires the concerted interaction of SRα with SRP54 and two molecules of GTP. Binding of SRP54 to the ribosome increases the affinity of SRP54 for GTP but is not required for SRP54 binding to SRα. Similarly, binding of SRP54 to SRα increases the affinity of SRα for GTP. Simultaneous binding of SRP54, SRα, and two molecules of GTP results in a conformational change that prevents dissociation of the complex and transfers the ribosome nascent chain to a docking site on the endoplasmic reticulum. Subsequent hydrolysis of GTP dissociates SRα from SRP54 and restores the original conformations of the GTPases, which are primarily in the GTP-bound state due to the low affinity for GDP and the relatively high concentration of GTP in the cytoplasm.

GTP, green triangle; SRP54, red; SRP receptor, blue. The other polypeptides known to comprise SRP and the translocon are omitted for clarity.

Adapted from Millman and Andrews, 1997


SRP54
GTP
2 GDP
2 Pi
SRα
SRβ

more hydrophobic signal sequences (Valent et al., 1995; Valent et al., 1997). *In vivo*, substrates become more dependent on the SRP pathway when the hydrophobicity of their signals is increased (Lee and Bernstein, 2001; Kim et al., 2001b). Thus, the hydrophobicity of the targeting peptide is a signal for specific recognition by the SRP pathway. However, the targeting signal may not be the only factor affecting which pathway is utilized. Using a hybrid protein, the dependence on SRP was found to increase with increasing the size of the periplasmic domain (Newitt et al., 1999).

Although insertion of IM proteins is often impaired by depletion of *E. coli* SRP components, it is never completely blocked (Ulbrandt et al., 1997; Newitt et al., 1999). Conversely, depletion of Ffh reduces targeting of certain secretory proteins (Kim et al., 2001b). Thus, it appears that there may be some overlap in the ability of different substrates to use the SRP and Sec pathways with the general principle that the propensity to use the former increases with increasing hydrophobicity, number of transmembrane regions, and size of the periplasmic domain.

This raises the question of why SRP components are essential despite the ability of most IM proteins to use the Sec targeting pathway. Since most SRP substrates are polytopic IM proteins it is likely that, as in the eukaryotic pathway, SRP targets RNC complexes to the IM cotranslationally, thereby preventing protein aggregation and misfolding following synthesis in the cytoplasm. Cotranslational targeting has been demonstrated for one SRP substrate, MtlA (Neumann-Haefelin et al., 2000). Translationally-arrested nascent MtlA chains were found to associate with SecY on inner

membrane inverted vesicles (INVs) only when the polypeptide chains were associated with ribosomes, but not following release by puromycin (Neumann-Haefelin et al., 2000).

The mechanism of *E. coli* cell death upon SRP depletion was addressed recently by reducing the levels of SRP such that no growth defect was observed, but a heat shock response was initiated (Bernstein and Hyndman, 2001). When the heat shock response was suppressed then reduced SRP expression was lethal. The nature of the chaperones involved in the response suggested that the heat shock response protected the cells through increased degradation of mislocalized IMPs. This led to the conclusion that SRP has been maintained and is essential because efficient cotranslational IMP targeting is necessary to prevent a toxic accumulation of aggregated proteins in the cytoplasm (Bernstein and Hyndman, 2001).

1.4.4. SRP targets nascent proteins to the Sec Translocase and has a variable requirement for SecA.

Through cross-linking studies, it was found that the SRP pathway delivers nascent proteins to the same SecYEG translocase used by the general secretory pathway (Valent et al., 1998). A block in the translocase was found to prevent insertion of SRP substrates into INVs (Koch et al., 1999). The SRP pathway also appears to target proteins that do not utilize the Sec translocase for translocation. Several phage and artificial membrane proteins requiring SRP assemble in the IM upon severe SecE depletion (de Gier et al., 1998; Cristobal et al., 1999). Although insertion of these proteins was previously believed to be spontaneous there is growing evidence that 'Sec-independent' proteins

utilize YidC for membrane integration (see above, section 1.2.2).

A major area of focus and controversy in this field is whether SecA is involved in either the targeting or translocation of SRP substrates. Using purified components and *in vitro* assays for insertion of selected SRP substrates, targeting and stable integration into membranes required SRP components but not SecA (Koch et al., 1999; Scotti et al., 1999). Furthermore, *in vivo,* depletion of Ffh but not inhibition of SecA diminished LacY membrane integration (Macfarlane and Muller, 1995). In contrast, an SRP substrate cross-linked to SecA upon delivery to INVs (Valent et al., 1998). More compelling evidence for SecA involvement in targeting or translocation of SRP-dependent substrates was provided by the observation of a synthetic lethal effect with mild *secA* and *ffh* mutations and a block in membrane insertion of AcrB, a known SRP substrate, upon depletion of SecA *in vivo* (Qi and Bernstein, 1999). Mutations in *secA* also inhibit membrane assembly of MalF, a protein for which targeting is impaired by mutations in the gene encoding 4.5S RNA (Tian et al., 2000).

An appealing resolution for these apparently disparate results comes from studies in which targeting and translocation were separated (Neumann-Haefelin et al., 2000). SecA is not required for cotranslational targeting of a hybrid outer membrane protein containing the targeting signal from MtlA, an SRP substrate, but is essential for translocation of the substantial hydrophilic segment of this protein (Neumann-Haefelin et al., 2000). SRP and SecA therefore likely cooperate in a sequential, non-overlapping manner for targeting and translocation of SRP-dependent substrates with large

periplasmic domains.

Having clearly identified a role for SRP components in protein targeting in *E. coli*, the reasons they were not identified in initial screens for targeting defects should be considered. It is clear that since most screens looked for defects in protein secretion (rather than membrane integration) they would not have identified SRP components (see, for example (Lee et al., 1989)). Further, since there is some overlap between the GSP and SRP pathways, mutations which ablate or restore translocation of membrane proteins would only be observed in the rare circumstance that the Sec pathway could not at least partially substitute for the SRP pathway.

More recently, a very sensitive assay that can detect minor defects in assembly of a polytopic membrane protein has been employed to identify mutants in the SRP pathway (Tian and Beckwith, 2002). Using a hybrid protein consisting of an SRP-dependent substrate linked to β-galactosidase (which is inactive in the periplasm), colonies exhibiting a faint blue colouration on X-gal plates were selected as likely candidates for a partial defect in translocation of polytopic inner membrane proteins. This assay identified mutations in all components of the SRP pathway as well as mutants that reduced expression of SecA. No mutations in other components of the Sec pathway were reported (Tian and Beckwith, 2002).

1.4.5. Components of the SRP pathway may be involved in multiple cellular processes.

Prior to the observation that 4.5S RNA shares sequence identity with 7S RNA it was determined that depletion of 4.5S RNA in *E. coli* resulted in a dramatic global

decrease in protein synthesis (Brown and Fournier, 1984). It was subsequently found that expression of a mutated 4.5S RNA allele induces a heat shock response prior to cessation of protein synthesis (Poritz et al., 1990). The latter effect was therefore proposed to be a secondary effect of an accumulation of misfolded membrane proteins in the cytoplasm (Poritz et al., 1990). This, along with numerous studies cited herein demonstrating the role of the SRP complex in protein targeting, led to the assignment of targeting of IM proteins as the primary role for 4.5S RNA in *E. coli*.

A number of further investigations, however, provide strong evidence that 4.5S RNA plays a direct role in both translation and protein targeting. 4.5S RNA is present in a four-fold excess over Ffh *in vivo* (Jensen and Pedersen, 1994). A slight decrease in 4.5S RNA expression causes decreased translation of all proteins, whereas a further drop in 4.5S RNA expression is required for accumulation of precursor membrane proteins (Jensen et al., 1994). Several suppressor mutations in 16SrRNA and 23SrRNA, both involved in translation, reduce the cellular requirement for 4.5S RNA but not Ffh (Brunelli et al., 2002). 4.5S RNA cross-links to the ribosome in two places, one of which depends on the presence of Ffh and a nascent chain while the other is independent of Ffh and occurs on non-translating ribosomes (Rinke-Appel et al., 2002).

The alternative function of 4.5S RNA appears to be regulating translation initiation, as its depletion results in an accumulation of ribosomes that can elongate chains but no longer initiate protein synthesis (Bourgaize and Fournier, 1987). A role in association or dissociation of elongation factor G (EF-G) has been proposed, as 4.5S

RNA competes with 23SrRNA for binding to EF-G (Nakamura et al., 1999).

1.5 Structure-Function relationships in the SRP pathway:

Protein targeting is mediated by the sequential interaction of the components of the SRP pathway. The main interactions involved are : (1) *E . coli* SRP with the RNC; (2) SRP with FtsY; (3) FtsY with the membrane; (4) Any of these components with the translocation apparatus (figure 1.1). Very little is known about the latter but some information has been gathered regarding each of the other interactions.

1.5.1. Interactions between E. coli SRP and the RNC.

Initial cross-linking studies indicated that the primary interactions between *E. coli* SRP and nascent signal peptides occur through the M domain of Ffh (Lutcke et al., 1992; Zopf et al., 1990). The crystal structure of the M domain indicates that the methionine-rich regions of the M domain form a hydrophobic groove (Keenan et al., 1998). These "methionine-bristles" are part of a highly flexible alpha helical region that may allow SRP to bind hydrophobic signal peptides that share little sequence similarity (Oh et al., 1996; Batey et al., 2000).

This conclusion has been challenged recently using a cross-linking approach that does not require an intervening spacer, and thus may be more precise (Cleverley and Gierasch, 2002). Signal peptides were shown to cross- link to the NG domain of Ffh but to only a very weak extent with the M-domain. Ffh was also shown to bind signal sequences even after removal of the M-domain (Cleverley and Gierasch, 2002). Thus, it

seems that the NG-domain also plays a major role in binding of the signal sequence. Modelling structures of hydrophobic peptide-binding proteins into a 10 angstrom resolution structure of SRP54 suggests that the methionine-rich clusters in the M domain line the inside of the cleft between the M and G domains (Czarnota et al., 1994), indicating that residues from both the M and NG domains could be involved in signal peptide binding.

Although the SRP54 holoenzyme is able to bind signal sequences, it requires SRP RNA to promote translocation of nascent chains (Hauser et al., 1995). Binding of SRP RNA to the ribosome stabilizes the interactions of Ffh with signal peptides (Zheng and Gierasch, 1997). Signal peptides can also bind directly to 4.5S RNA whereas unrelated peptides do not (Swain and Gierasch, 2001). The structure of the M domain bound to the minimal SRP RNA moiety shows that the RNA contributes one third of the proposed signal binding cleft, and is likely involved in signal recognition (Batey et al., 2000). Thus is appears that signal sequences are likely recognized by SRP through a combination of hydrophobic interactions and electrostatic contacts to both protein and RNA components.

1.5.2. Interactions between the SRP-RNC complex and FtsY.

As discussed in section 1.4.2., *E. coli* SRP binds tightly to FtsY in a GTP-dependent manner (Miller et al., 1994). 4.5S RNA has a role either directly or indirectly in this interaction, as it greatly facilitates both the assembly and disassembly of Ffh with FtsY (Peluso et al., 2000; Peluso et al., 2001). It also appears that the major contact

regions are in the NG domains of these proteins. Other regions are not necessary to stimulate binding to (Shepotinovskaya and Freymann, 2002) or GTPase activity of the reciprocal molecule (Macao et al., 1997). It has been suggested that the interaction may involve parts of the interface between N and G regions, as mutations at this interface on either molecule do not affect GTPase activity, but greatly reduce the interactions of *E. coli* SRP with FtsY (Lu et al., 2001).

1.5.3. E. coli FtsY interacts with the IM in an unusual manner.

Although most of the interactions that occur between SRP components appear to be highly conserved between eukaryotes and prokaryotes, it is clear that the mechanisms of membrane-assembly of the SRP receptors are divergent. The amino-terminal 140 amino acids of SRα, comprising two hydrophobic stretches of amino acids and a slightly basic region, are necessary and sufficient for binding directly to the transmembrane SRβ subunit (Young et al., 1995). Binding occurs independently of the SRP pathway, but cotranslationally during translation of SRα (Young and Andrews, 1996). It is assisted by a pause in translation immediately following the membrane binding domain (Young and Andrews, 1996).

No homologue of SRβ is encoded in the *E. coli* genome and the amino acid sequence of FtsY bears no resemblance to the amino-terminal region of SRα implicated in membrane assembly (Bernstein et al., 1989; Romisch et al., 1989). In place of the SRβ binding domain found in SRα, the amino-terminal 20 amino acids of the A region of FtsY are enriched in positively charged residues. The rest of the A region of *E. coli* FtsY

contains many acidic residues extending from amino acid 20 to the end of the A region at amino acid 197, bestowing a net negative charge of -47 on the A region (Zelazny et al., 1997).

Unlike most membrane proteins, FtsY is approximately equally distributed between the membrane and cytoplasm (Luirink et al., 1994). No clear explanation for this phenomenon has been put forth, although it is clear that binding to the membrane is required for the protein targeting function of FtsY (Valent et al., 1998).

The identity of the specific regions of FtsY that are involved in membrane assembly has been controversial. Deletion of even small regions of the amino-terminus of FtsY inhibit membrane assembly of FtsY and interfere with protein targeting (Chapter II) (Powers and Walter, 1997; Zelazny et al., 1997). Replacement of the A region with an unrelated transmembrane region allows cell growth and does not impart membrane-targeting defects under the assay conditions utilized (Zelazny et al., 1997). This was taken as evidence that the only role of the A region is binding the receptor to the membrane. However, as discussed further in Chapter IV, a growth defect can clearly be ascertained when the entire A region is replaced (Zelazny et al., 1997). Moreover, this defect was not apparent when a large portion of the A domain was present along with a transmembrane region (Zelazny et al., 1997). It has also been found that the A region and NG regions independently co-fractionate with membranes in pelleting assays (de Leeuw et al., 1997). Using an alternate assay described in Chapter II we found that the A and N regions together, but not the NG region, binds to membranes. It therefore appears that the

N region has overlapping roles in FtsY function. It is involved in regulating GTPase activity and binding FtsY to the membrane.(Gill and Salmond, 1990; Zelazny et al., 1997)

Both bilayer and non-bilayer phospholipids have been implicated in assembly and activity of several components of the secretory pathway in *E. coli*, including SecA (Lill et al., 1990; Hendrick and Wickner, 1991), and the SecYEG translocase (van der Does et al., 2000). The presence of a region in the carboxyl-terminal domain of FtsY that may be regulated by anionic phospholipids has been demonstrated (de Leeuw et al., 2000). It was also speculated that a second lipid-binding site exists in the amino-terminal region of FtsY (de Leeuw et al., 2000). We found that the region of FtsY responsible for membrane-binding binds liposomes containing the zwitterionic phospholipid phosphatidylethanolamine (PE) independent of a protein receptor (Chapter III). Surprisingly, in the absence of PE, SRP-dependent protein targeting remains functional, and FtsY binds to *E. coli* INVs via an interaction between the AN region and a trypsin-sensitive component of the membrane. This suggests that, similar to SecA of the general secretory pathway (Lill et al., 1990; Hendrick and Wickner, 1991), membrane assembly of FtsY involves interactions with both a specific lipid and a membrane protein.

1.6 FtsY proteins from different organisms contain highly divergent amino-terminal domains:

As a number of microbial genomes have been sequenced over the past several years, it has come as a surprise that the amino-terminal domains of FtsY proteins are

highly divergent amongst different bacteria. This variability suggests different modes of targeting of FtsY to the membrane.

The sequences can be broadly divided into four categories; 1) highly negatively charged regions with high homology to *E. coli* FtsY; 2) moderately charged regions with low homology to *E. coli* FtsY; 3) very short (<30 amino acid) regions with a small net positive charge; 4) regions containing a putative single-pass transmembrane region with no similarity to the A region of *E. coli* FtsY. Despite differences in the amino-terminal domains, all FtsY homologues share high amino acid identity in both the N and G regions.

As detailed in Chapter IV, representative proteins from each of these categories were selected for investigation. *H. influenzae* FtsY was selected as a relative of *E. coli* containing a much shorter, but still highly negatively charged A domain. *B. subtilis* FtsY is phylogenetically second most related to *E. coli* FtsY but completely lacks a recognizable A region. The region amino-terminal to the N domain extends for only 20 amino acids and bears a net charge of +3. It shares only 22% identity throughout this region with *E. coli* FtsY, and possesses a net negative charge of -18. *S. coelicolor* FtsY contains a putative type I signal anchor and has been postulated to insert into the membrane (Bibi et al., 2001). These proteins originate from species representing divergent orders, belonging to proteobacteria, bacillus, and actinobacteria, respectively.

We found that the A regions imparted differences in cellular localization for FtsY from different species. In *B. subtilis* FtsY was located primarily in the cytoplasm,

whereas the amino-terminus of FtsY from *S. coelicolor* integrates into the membrane. This prompted us to find that although endogenous FtsY is approximately evenly distributed between the cytoplasm and membrane in *E. coli*, this species can survive with an entirely integral membrane form of FtsY. However, the presence of the A region of *E. coli* FtsY was required for long-term survival of these cells, indicating that this region has an additional unknown function to that involved in membrane binding (Chapter IV).

The title of the report in which FtsY was first identified in *E. coli* described FtsY as "An Unusual Protein" (Gill and Salmond, 1990). Although the mechanisms of FtsY membrane assembly had not been determined when this report was published, the investigations presented in this thesis demonstrate that membrane assembly of *E. coli* FtsY is also unusual. In Chapter II, the regions within FtsY that are required for membrane binding are identified and shown to constitute a domain that can be cleaved from the protein upon membrane-binding. The ability of FtsY to bind both PE and a protein component of the membrane are demonstrated in Chapter III. Experiments presented in Chapter IV show that the manner of FtsY membrane assembly differs between organisms. Furthermore, an essential role for the unique amino-terminal region of *E. coli* FtsY other than in membrane binding of FtsY is also demonstrated.

CHAPTER II

A Site-specific, Membrane-dependent Cleavage Event Defines the Membrane Binding Domain of FtsY

adapted from

Millman, J.S. and Andrews, D.W.

Journal of Biological Chemistry, 1999, vol. 274, pp. 33227-33234

Preamble

The contents of this chapter were contributed entirely by the author of this thesis (Jonathan Scott Millman). It is adapted from the article published in the Journal of Biological chemistry to contain three additional figures (2.1, 2.9, and 2.10) which were originally presented at 'data not shown' due to space requirements. There is also a short addition to the discussion in this chapter regarding the nature of proteolytic cleavage of FtsY.

2.1. Summary

Targeting of many polytopic proteins to the inner membrane of prokaryotes occurs via an essential signal recognition particle-like pathway. Unlike the general secretory pathway, the proteins involved in this pathway and their activities appear in many respects to mirror closely those of their eukaryotic homologues. However, the *Escherichia coli* signal recognition particle receptor, FtsY, differs significantly at the amino-terminus from the eukaryote homologue α-subunit of the signal recognition particle receptor. In addition, there is no prokaryote homologue of the transmembrane β-subunit of the receptor. Therefore, FtsY must assemble on the membrane in a unique manner. Using assays designed to accurately discriminate membrane-bound proteins from aggregated material, we found that in contrast to a previous report, only amino acids 1-284 of FtsY are necessary and sufficient for membrane assembly. These amino acids together constitute a *bona fide* membrane binding domain that includes both the regions originally designated A and N based on sequence comparisons. Furthermore, we found that a membrane-bound factor mediates specific cleavage of some membrane-bound FtsY molecules between the N and G regions previously believed to be functionally linked to generate a novel membrane-bound isoform composed of only the AN domain.

2.2. Introduction

In mammalian cells, membrane and secretory proteins are targeted to the endoplasmic reticulum cotranslationally via the SRP pathway (reviewed in (Walter and

Johnson, 1994)). SRP is a cytoplasmic ribonucleoprotein particle composed of six polypeptides associated with an RNA scaffold. The targeting process begins with recognition of a hydrophobic signal sequence on a nascent secretory or integral membrane polypeptide by the 54-kDa protein of SRP (SRP54) resulting in the binding of SRP to both the ribosome and nascent polypeptide as well as a concomitant slowing of translation elongation. The ribosome nascent chain complex is targeted to translocation sites at the endoplasmic reticulum membrane through an interaction with the α-subunit of the SRP receptor (SRα) (Lauffer et al., 1985). This interaction leads to the insertion of the nascent chain into the translocon, the aqueous pore through which proteins are translocated cotranslationally (reviewed in (Andrews and Johnson, 1996)).

Homologues of the eukaryotic SRP pathway have been identified in many prokaryotes (Poritz et al., 1988; Struck et al., 1988; Bernstein et al., 1989; Romisch et al., 1989). In *Escherichia coli*, Ffh and 4.5 S RNA form an SRP-like complex that interacts with nascent secretory and membrane proteins (Valent et al., 1995; Luirink et al., 1992). This particle promotes cotranslational targeting of nascent chains via an interaction with FtsY, the SRα homologue (Powers and Walter, 1997). This pathway may be of particular importance for membrane assembly of hydrophobic inner membrane proteins (Ulbrandt et al., 1997; Macfarlane and Muller, 1995; Seluanov and Bibi, 1997; de Gier et al., 1996) . Although the targeting steps are distinct from those of the more ubiquitous Sec secretory pathway, at least some of the translocation apparatus is the same (Valent et al., 1995). An interesting divergence between the eukaryotic and prokaryotic SRP pathways is in the

membrane assembly of the receptors. In eukaryotes, SRβ, the transmembrane β-subunit of the SRP receptor, anchors SRα on the endoplasmic reticulum membrane through an interaction with the amino-terminal domain of SRα (Young et al., 1995). No homologue of SRβ has been identified in the *E. coli* genome sequence. Furthermore, the amino-terminal domains of FtsY and SRα are highly divergent (Bernstein et al., 1989; Romisch et al., 1989), suggesting that FtsY assembles on the membrane in a different manner. Because FtsY is believed to shuttle proteins to the membrane that are not efficiently inserted by the general secretory pathway and because FtsY is an essential gene in *E. coli*, it is likely that membrane assembly of FtsY is tightly regulated. A highly negatively charged region has been identified at the amino-terminus of FtsY (amino acids 1-197) that is not found in other eukaryotic or prokaryotic homologues (Gill and Salmond, 1990). In addition to this "A" region, central N (amino acids 198-284) and carboxyl-terminal G (amino acids 292-497) regions have been identified (Bernstein et al., 1989; Romisch et al., 1989). The G region is a GTP binding domain that together with the GTPase domains of SRP54, SRα, Ffh, and their homologues constitute a specific subfamily of Ras-related low molecular weight GTPases. The N region is found amino-terminal of the G region in all SRP family GTPases and has been assumed to have a role in GTPase activity (Montoya et al., 1997). The amino-terminal A region, by analogy with the membrane assembly domain of SRα, was expected to be involved in membrane assembly. Based on this supposition, the membrane assembly properties of the FtsY A region, NG region, and G region were each analyzed (de Leeuw et al., 1997).

Surprisingly, all three polypeptides fractionated with membranes after centrifugation, leading the authors to conclude that each independently binds to the *E. coli* inner membrane (de Leeuw et al., 1997).

Here we used a gel filtration chromatography-based assay to unambiguously distinguish membrane bound from aggregated FtsY. Using this assay, we determined that neither the A region nor the NG region were sufficient for membrane association, but the AN region of FtsY is both necessary and sufficient for membrane assembly. Furthermore, we found specific cleavage of some membrane-bound FtsY molecules generates a novel membrane-bound isoform of FtsY composed of only the A and N regions confirming that AN constitutes the membrane binding domain of FtsY.

2.3. Experimental Procedures

Materials and General Methods– General chemical reagents were obtained from Fisher, Sigma, or Life Technologies, Inc. SURE™ *E. coli* cells used for plasmid construction were purchased from Stratagene. Except where specified, restriction enzymes, other molecular biology enzymes, and reagents were from New England Biolabs or MBI Fermentas. RNA guard (an RNase inhibitor) was from Amersham Pharmacia Biotech.

Plasmids – Construction of plasmids, sequencing, and polymerase chain reactions were performed using standard methods (Sambrook et al., 1989). Deletion mutants and fusion proteins of FtsY are outlined in Fig. 2.3 and are described below.

FtsY was amplified from genomic DNA isolated from *E. coli* strain JM109 using the following oligonucleotides: 5'-CGCCCATGGCGAAAGAAA-3' and 5'-CAGTAGATGGGGATCCTGGAA-3'. To generate the FtsY expression plasmid pMAC897, the full-length coding sequence for FtsY was inserted behind tandem SP6 and tac promoters in the plasmid pSPtac using the restriction enzymes NcoI and BamHI. In addition to tandem SP6 and tac promoters, this plasmid contains a Shine-Dalgarno sequence to direct bacterial translation.

The entire FtsY coding sequence as well as the SP6 and tac promoter regions were excised from pMAC897 and inserted into the BglII site of the plasmid pSPMP366 (described previously (Janiak et al., 1994). The resulting plasmid, designated pMAC988, contains the coding region of FtsY followed by a sequence encoding the passive passenger protein gPa. In this plasmid, the endogenous termination codon of FtsY is present between the two coding regions. Plasmids encoding the various FtsY deletion mutants, FtsY fused to gPa, and FtsY deletions fused to gPa were generated using the technique described in (Hughes and Andrews, 1996) to delete the requisite regions from pMAC988.

Plasmid pMAC1000 encodes the polypeptide FtsYdSRY1 comprising amino acids 20 to the stop codon of FtsY. This polypeptide has a small positively charged region deleted from the amino-terminus of FtsY. Plasmid pMAC999 encodes the polypeptide FtsYdSRY2 comprising amino acids 59 to the stop codon of FtsY.

Plasmids pMAC997, pMAC1177, pMAC1178, pMAC1176, pMAC1424,

pMAC1423 pMac1422 and pMAC995 encode polypeptides F58-gPa, F96-gPa, F155-gPa, FtsYA-gPa, FtsYAN(Δ12)–gPa, FtsYAN(Δ8)-gPa, FtsYAN(Δ3)-gPa and FtsYAN-gPa consisting of the amino-terminal 58, 96, 155, 197, 272, 276, 281 and 284 amino acids of FtsY with the gPa domain at the carboxyl-terminus. Plasmid pMAC1310 encodes the polypeptide FtsYNG-gPa consisting of amino acids 198 to the final amino acid of FtsY with the gPa domain at the carboxyl-terminus.

Plasmids pMAC1252, pMAC1253, pMAC1485 and pMAC1486 encode polypeptides FtsYA, FtsYAN, FtsYAN(Δ12) and FtsYAN(Δ17), consisting of the amino-terminal 197, 284, 272 and 267 amino acids of FtsY, respectively, followed by Leu-Gln-Asp-Pro-Arg-stop codons.

Plasmid pMAC1062 encodes maltose binding protein with a premature stop codon behind a tac promoter to produce a carboxyl-terminally truncated protein of MW 40kDa.

Antibody Generation and Purification – Polyclonal antiserum against FtsY was raised in rabbits immunized with bacterially expressed fusion protein. Plasmid pMAC1042 encodes amino acids 41 to the stop codon of FtsY fused to the carboxyl-terminus of glutathione S-transferase in the vector pMAC241, a modification of pGEX2T (Amersham Pharmacia Biotech) with an enhanced polylinker. The fusion protein was purified using a glutathione-Sepharose column. Antibodies specific to FtsY were purified from serum as described (Bar-Peled and Raikhel, 1996).

Immunoprecipitations and Western Blots – Affinity purified anti-FtsY antibody and rabbit IgG were used for immunoprecipitation of FtsYAN-gPa. The former specifically recognizes epitopes in the AN region of this polypeptide derived from FtsY, whereas the latter is bound by gPa, which contains the IgG binding region of *Staphylococcus aureus* Protein A (Janiak et al., 1994). Following membrane targeting, fractions eluted from the CL-2B column were diluted with 1 ml of buffer A (100 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1% Triton X-100). Affinity purified IgG against FtsY (3 µl of 0.1 mg/ml) or 3 µl of buffer was added followed by a 2-h incubation at 4 °C. Protein A-agarose (Bio-Rad) was added to the fractions incubated with 3 µl of FtsY antibody. 3 µl of IgG-Sepharose were added to the other fractions. Following incubation for 2 h at 4 °C, the beads were washed 3 times with 1 ml of buffer A and then 2 times with 1 ml of buffer A without Triton X-100. To release the bound protein, the washed beads were incubated in 50 µl of SDS-PAGE loading buffer for 5 min at 80 °C, and 8 µl aliquots were analysed by SDS-PAGE.

To detect FtsY by immunoblotting, 5 µl of *E. coli* inner membrane inverted vesicles (INVs) containing approximately 15 µg of protein were solubilized in 50 mM Tris-Cl, pH 8.0, 1% SDS and then analysed by SDS-PAGE. Proteins were transferred to nitrocellulose using a semi-dry transfer apparatus (Hoeffer Instruments).

Cell-free Translation Systems – An S30 lysate was prepared from *E. coli* strain MRE600 (Cammack and Wade, 1965) as described previously (Muller and Blobel, 1984). To remove any remaining chromosomal DNA, micrococcal nuclease was added to a final

concentration of 25 units/ml of lysate along with 1 mM $CaCl_2$. The reaction was stopped after incubation for 30 min at room temperature by adding EGTA to a final concentration of 4 mM. A membrane-free S170 extract was obtained by centrifugation of 175 µl of S30/tube in the A-100/30 rotor of an Airfuge (Beckman) at 4 °C for 15 min at 28 p.s.i. (170,000 × g) and collecting the top 125 µl. Membrane-free ribosomes were isolated as described previously (Collier et al., 1988).

A typical 20-µl-coupled transcription and translation reaction contained 35 mM Tris acetate, pH 8.0; 190 mM potassium glutamate; 30 mM ammonium acetate; 2 mM DTT; 12 mM $Mg(OAc)_2$; 40 µM each of 19 amino acids (-methionine); 2 mM ATP; 0.5 mM each of CTP, UTP, and GTP; 20 mM phosphoenolpyruvate; 1 mM isopropyl-1-thio--D-galactopyranoside; 0.1 mg/ml *E. coli* tRNA; 35 mg/ml polyethylene glycol 8000; 20 µg/ml folinic acid; 12 µCi of L-[^{35}S]methionine; 1 µg of plasmid DNA; 6 µl of S170; and 0.2 µl of membrane-free ribosomes. Incubations were at 37 °C for 45 min.

Transcripts for cell-free translations in rabbit reticulocyte lysate were generated with SP6 polymerase as described previously (Gurevich et al., 1991). Translations performed in rabbit reticulocyte lysate and labeled with [^{35}S] methionine were described previously (Andrews, 1989). Radiolabeled translation products were separated by SDS-PAGE (Schagger and von Jagow, 1987), visualized by phosphorimaging using a Molecular Dynamics PhosphorImager 473, and quantified using the Imagequant software from Molecular Dynamics.

Inverted Vesicles -- FtsY-depelted INVs were prepared from strain N4156::pAra14-FtsY' in which FtsY expression is under control of the araB promoter. Cells were inoculated with saturated culture to OD_{550} of 0.02 and grown in 2YT media to OD_{550} of 0.6. FtsH-depleted INVs were prepared from strain AD315 in which FtsH expression is under control of the lac promoter. These cells were inoculated with saturated culture to OD_{550} of 0.04 and grown in M9 minimal media supplemented with a casein digest of amino acids to OD_{550} of 0.6. Crude inverted vesicles were prepared as described in (Muller and Blobel, 1984). 2-ml aliquots were then loaded on a sucrose step gradient consisting of 2.02 M (10 ml), 1.44 M (13 ml), and 0.77 M (13 ml) sucrose steps in 50 mM triethanolamine-acetate, pH 7.5, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Following centrifugation at 25,000 rpm for 18 h in the SW28 rotor in an Ultracentrifuge (Beckman), INVs were removed from the 0.77/1.44 M sucrose interface and diluted with 4 volumes of 50 mM triethanolamine-acetate, pH 7.5, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride. Vesicles were pelleted by centrifugation for 2 h at 150,000 × g_{av} and resuspended using a loose fitting Dounce homogenizer at 20-30 A_{280} units/cm path length in 50 mM triethanolamine-acetate, pH 7.5, 0.25 M sucrose, and 1 mM DTT.

Cell-free Translations and Membrane Targeting – In vitro translation reactions were terminated by chilling on ice and a post-ribosomal supernatant was prepared by centrifugation at 28 p.s.i. (170,000 × g) in the A-100/30 rotor of an Airfuge (Beckman) at 4 °C for 30 min. A 20-µl aliquot of the supernatant was incubated with 1 µl of INVs or buffer for 45 min at 37 °C.

To assay membrane binding by pelleting the mixture was overlaid on a 80- µl sucrose cushion (0.5M Sucrose, 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, 12 mM magnesium acetate). Following centrifugation in a TLA 100 rotor (Beckman) at 100,000 rpm for 15 min, the gradient was fractionated into 50-µl 'top' and 'middle' aliquots, and the pellet was solubilized in 10 mM Tris acetate, pH 8.0, 1% SDS at 65 °C for 10 min. 10-µl samples were analysed by SDS-PAGE.

Aggregation of FtsY translated in S170 extract was assessed by size-exclusion chromatography over Sepharose S200 resin. Following translation the reaction was layered on an S200 column 9 cm in height with a radius of 0.5 cm equilibrated with 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, 12 mM magnesium acetate. The void and total volumes were determined using blue dextran and 2,4-dinitrophenylalanine as indicators to be 1.5 ml and 7.2 ml respectively. 0.25-ml fractions from this column were analysed by SDS-PAGE and autoradiography.

To assay membrane binding by column chromatography, the mixture was loaded onto a 0.8-ml column of Sepharose CL-2B equilibrated with 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, 12 mM magnesium acetate in a 1-ml syringe. The column was eluted with the same buffer; fractions (2 drops each) were collected, and 8-µl samples were analysed by SDS-PAGE. The included and excluded volumes of CL-2B columns were calibrated as described

(Young et al., 1995).

To assay membrane binding by vesicle floatation the mixture was adjusted to 1.6 M sucrose final concentration, and 50 µl were overlaid with sucrose steps of 100 µl (1.25 M sucrose) and 50 µl (0.25 M sucrose). The steps also contained 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, 12 mM magnesium acetate. Following centrifugation in a TLA 100 rotor (Beckman) at 100,000 rpm for 90 min, the gradient was fractionated into 50-µl aliquots, and the pellet was solubilized in 10 mM Tris acetate, pH 8.0, 1% SDS at 65 °C for 10 min. 10-µl samples were analysed by SDS-PAGE.

To determine the elution and fractionation profiles of vesicles and non vesicle-associated proteins in both assays, purified vesicles and aliquots of membrane-free S170 lysate were subjected to assay as above and analysed by SDS-PAGE analysis. Using this approach the major proteins in both the vesicle membranes and cytosol could be followed unambiguously. Vesicles eluted in fractions 3 and 4 in the column chromatography assay and fractionated in both the second fraction from top and in the pellet in the floatation assay. Cytosol was found in fractions 5-12 in the column chromatography assay, in the bottom two 50-µl fractions, and the pellet in the floatation assay, as expected.

Mass spectroscopic analysis of FtsY58-gPa. FtsY58-gPa was purified on a 1-ml IgG-Sepharose column and eluted with 500 mM ammonium acetate (pH 3.4). Following dialysis in 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, further purification was obtained using a 1-ml diethylaminoethyl-Sepharose column and elution with 50 mM Tris-Cl, pH 7.5, 1

M NaCl. The sample was subjected to SDS-PAGE and blotted onto a Poly(vinylidene fluoride) support.

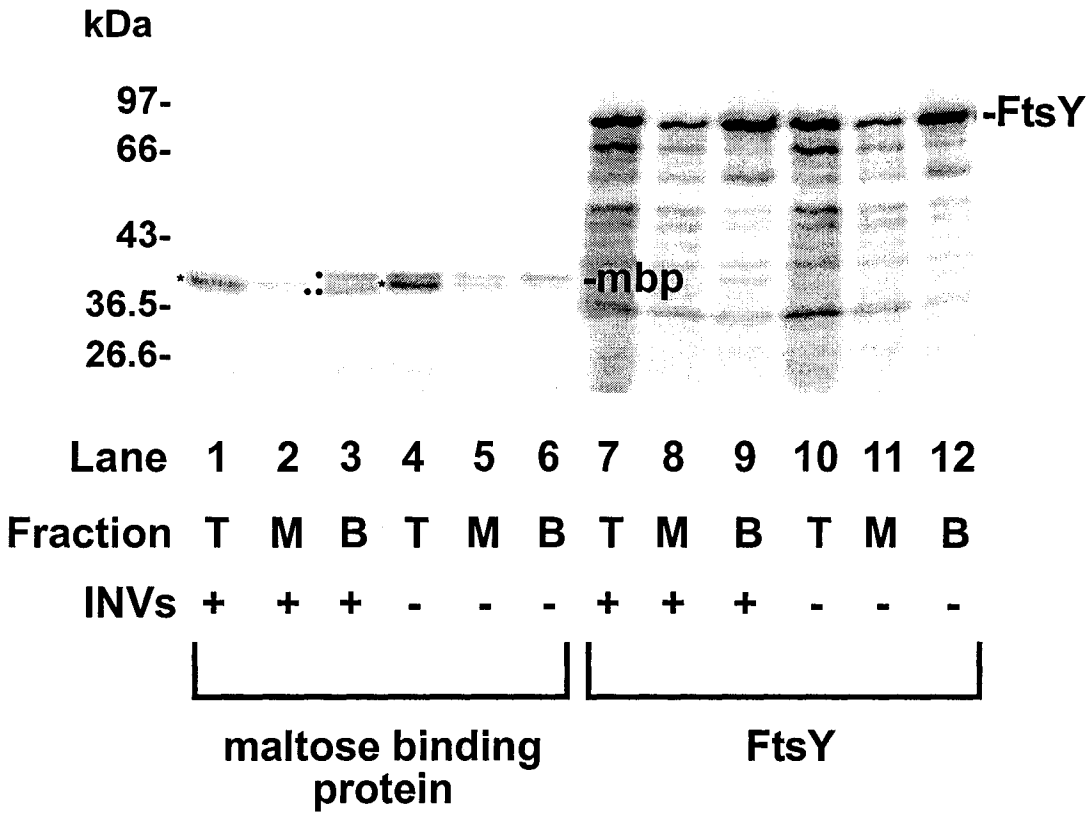
The protein sample was ionized from the solid support using Matrix Assisted Laser Desorption. In this process, a single laser pulse essentially instantaneously produces charged protein ions that are suitable for separation according to their mass to charge ratio (m/z) using Time-of-flight mass spectrometry. Separation of molecules by this technique is based on the principle that ions with differing m/z values have the same energy, but exhibit an inverse relationship between velocity and mass after acceleration out of the ion source. The velocity is determined by the length of time the molecule takes, under an applied voltage, to traverse a 'flight tube' of known distance. The multiple peaks obtained for proteins of differing ionization states were deconvoluted to derive the original molecular mass of the protein sample.

2.4. Results

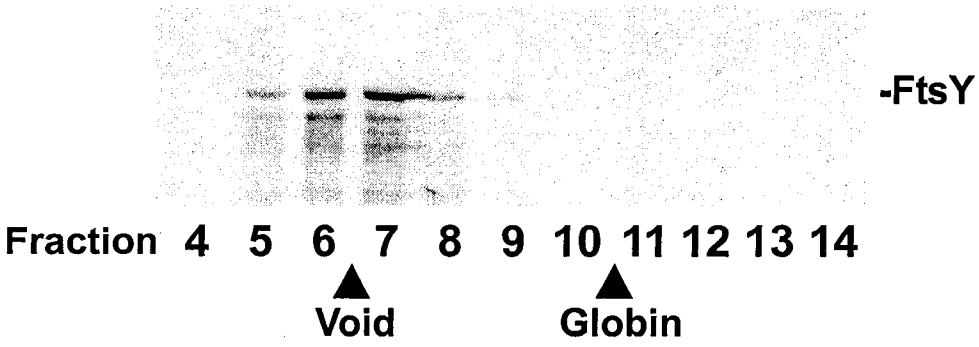
Membrane Binding of FtsY – Attempts to determine the membrane-binding domain of FtsY using a simple pelleting assay similar to that used in (Andrews et al, 1989) did not clearly distinguish membrane-bound FtsY from large aggregates. Using a truncated form of maltose binding protein (mbp-N) as a control, essentially all full-length and signal peptidase-cleaved mbp-N pellets in the presence of INVs (Fig. 2.1A, lane 3, dots), whereas in the absence of membranes most mbp-N remains in the supernatant fractions (Fig 2.1A, compare lane 4 with lane 6). An internal initiation product that lacks

Figure 2.1. FtsY Pellets in the Absence of Membranes. *A.* Truncated maltose binding protein (mbp-N – lanes 1-6) and FtsY (lanes 7-12) were synthesized in S170 extract and incubated with buffer (lanes 4-6, 10-12), or INVs (lanes 1-3, 7-9). Reactions were layered over 0.5 M sucrose containing 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, and 12 mM magnesium acetate. Following centrifugation, 50-µl fractions were taken from the top (T), and bottom (M) supernatant fractions and resolubillized pellet (B). The approximate migration positions of the expressed constructs are indicated at the sides of the panels. The position of full-length (one dot), leader peptidase cleaved (two dots) and an internal initiation product (asterix) of mbp-N are indicated. *B.* FtsY was synthesized in S170 extract and subjected to size-exclusion chromatography over a 7-ml Sepharose S-200 column. The peak elution fraction of blue dextran (void), and globin are indicated below the panel. The migration position of FtsY is indicated.

A


kDa
97-
66-
43-
36.5-
26.6-
-FtsY
-mbp
Lane 1 2 3 4 5 6 7 8 9 10 11 12
Fraction T M B T M B T M B T M B
INVs + + + - - - + + + - - -
maltose binding protein
FtsY

B


-FtsY
Fraction 4 5 6 7 8 9 10 11 12 13 14
Void
Globin

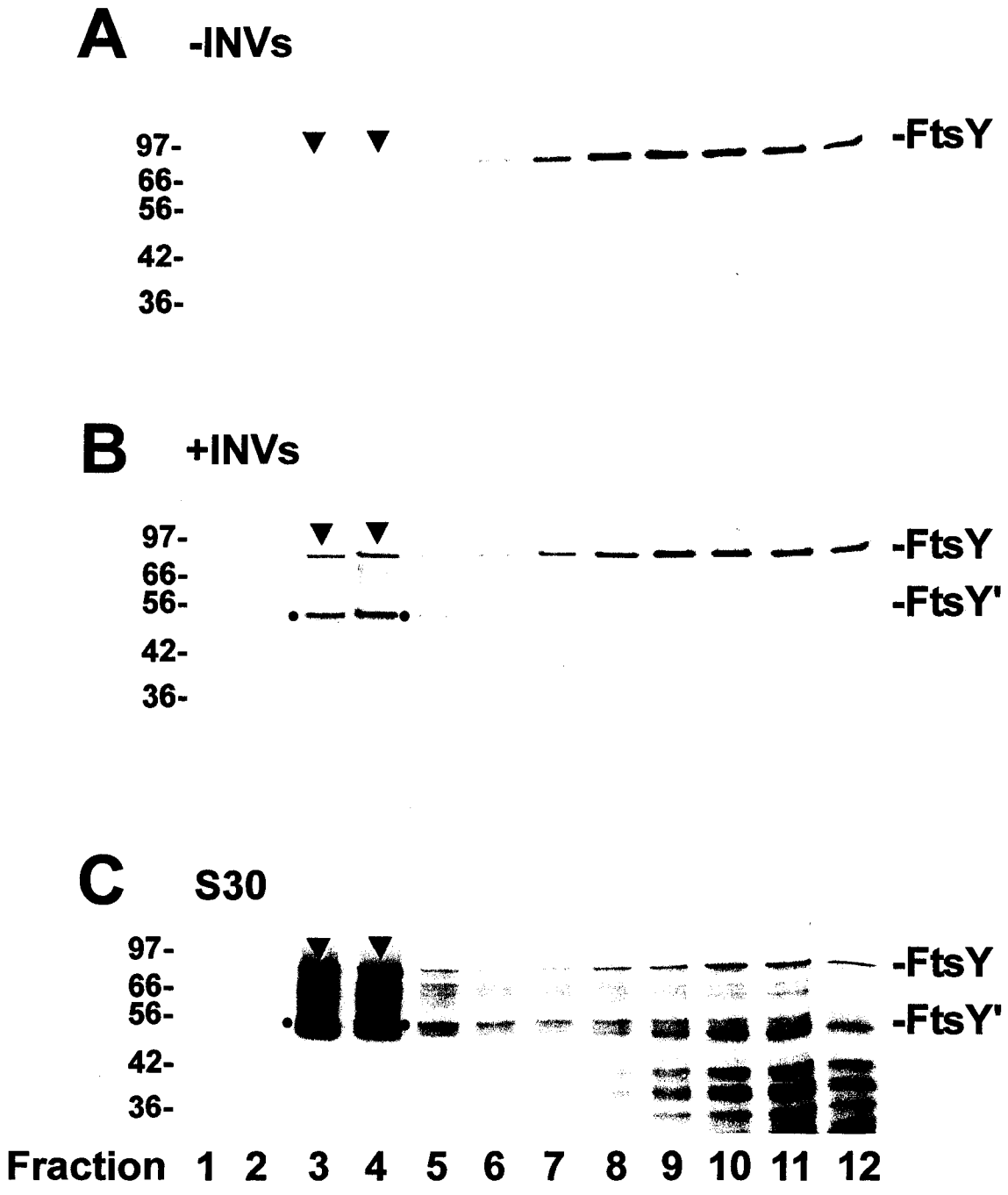
a signal sequence serves as a useful control, as it remains in the supernatant fractions both in the presence and absence of INVs (Fig. 2.1A, lanes 1 and 4 'asterisk')(Watanabe and Blobel, 1989).

In contrast, greater than 50% of FtsY pelleted both in the presence and absence of membranes (Fig. 2.1B, compare lanes 9 and 12). That this consisted largely or exclusively of aggregated protein was confirmed by Sepharose S200 size exclusion column chromatography, from which FtsY elutes exclusively in the void fractions (Fig.2.1B).

Therefore, to assay for stable membrane binding, translations of FtsY in S170 extract were incubated with or without INVs and then fractionated by gel exclusion chromatography using Sepharose CL-2B, a resin with a large enough exclusion limit to retain FtsY aggregates (Fig. 2.2). Vesicles and vesicle-associated proteins eluted in the excluded volume (fractions 3 and 4, marked with arrowheads in Fig. 2.2). The included volume (fractions 5-12) contains cytosolic proteins.

As expected in the absence of INVs, FtsY synthesized in S170 extract fractionated almost exclusively in the included volume of a CL-2B column (Fig. 2.2A, lanes 5-12). After incubation with INVs most of the full-length FtsY still fractionated in the included volume (Fig. 2.2B, lanes 5-12), likely representing FtsY molecules present as aggregates that do not assemble onto membranes. Nevertheless, a fraction of the full-length FtsY eluted in the excluded volume with membranes, in contrast to the incubations without INVs, demonstrating that some of the FtsY in S170 extracts binds to inverted vesicles

Figure 2.2. FtsY binding to membranes *in vitro*. FtsY was synthesized in S170 extract and incubated with buffer (A), INVs (B), or was synthesized in S30 extract (C). Membrane-bound molecules were separated from aggregates by chromatography on 0.8-ml Sepharose CL-2B columns equilibrated and eluted in buffer containing 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, and 12 mM magnesium acetate. Membranes eluted in the excluded volume (fractions 3 and 4), whereas the bulk of the *E. coli* lysate proteins eluted as a broad peak in the included volume (fractions 5-12). Full-length FtsY (arrowheads) and a 53-kDa species (dots) eluting with membranes in the excluded fractions are marked. The migration positions of molecular mass markers are indicated (in kDa) to the left of the panels.


A
-INVs
97-
66-
56-
42-
36-
-FtsY
B
+INVs
97-
66-
56-
42-
36-
-FtsY
-FtsY'
C
S30
97-
66-
56-
42-
36-
-FtsY
-FtsY'
Fraction 1 2 3 4 5 6 7 8 9 10 11 12

(Fig. 2.2B, lanes 3 and 4, arrowheads). Although the background is increased, it is clear that when translated in S30 lysate, which contains endogenous membranes, almost half of the full-length FtsY eluted in the excluded fractions (Fig. 2.2C). This increase in binding may be because of the high concentration of vesicles present in S30 lysate, as well as the elimination of the incubation time in the absence of membranes during which aggregation may occur.

In addition to the band corresponding to the previously described 92-kDa migration product of full-length FtsY (Luirink et al., 1994), a second band migrating as a 53-kDa species was observed when FtsY was incubated with membranes. This product eluted entirely in the excluded volume (Fig. 2.2B, lanes 3 and 4, dots). This band is not a result of membrane-dependent alterations in translation, such as premature termination or internal initiation, because membranes are added after translation was terminated and following removal of ribosomes from the extract. Although FtsY has a predicted mass of approximately 54 kDa, it migrates on SDS-PAGE with an apparent weight of 92 kDa (Luirink et al., 1994). The 53-kDa product could therefore represent full-length FtsY with an as yet unidentified modification removed or a proteolytically processed form of FtsY. As shown below, this band results from post-translational cleavage of FtsY between the N and G regions. After correcting for the number of methionine residues in each of these proteins, the cleaved product accounts for approximately 75% of the membrane-bound FtsY.

Regions of FtsY Required for Membrane Binding – It has previously been demonstrated that the GTPase domain located in the carboxyl-terminal two-fifths of FtsY interacts directly with the *E. coli* SRP particle (Miller et al., 1994; Powers and Walter, 1995). Therefore, it was predicted that the amino-terminal region would be involved in membrane binding. To determine which sequences in the amino-terminal region of FtsY were necessary for membrane binding, a set of plasmids was made (Fig. 2.3) encoding FtsY molecules with deletions of the amino-terminal 19 and 57 amino acids (SRY1 and SRY2). To identify FtsY sequences sufficient to bind INVs, test sequences were fused to gPa; a protein domain previously demonstrated to have no intrinsic targeting or membrane binding activity (Janiak et al., 1994). A series of plasmids were constructed encoding the amino-terminal 58, 96, 155, 197, and 284 amino acids of FtsY as well as amino acids 198-497 of FtsY fused to gPa. The latter three constructs have regions fused to gPa that correspond to the A region, AN regions, and NG regions of FtsY, respectively. Constructs encoding only the amino-terminal 197 or 284 amino acids of FtsY were also tested.

Deletion of amino acids 1-19 or 1-58 of FtsY resulted in polypeptides (FtsYdSRY1 and FtsYdSRY2, respectively) that did not bind INVs and therefore, fractionated identically by gel filtration chromatography in the presence and absence of INVs (Fig. 2.4A, compare lanes 1-10 with lanes 11-20). This demonstrates that the extreme amino-terminus of FtsY is necessary for membrane binding. To determine how much of the amino-terminal region of FtsY was sufficient for membrane assembly,

Figure 2.3. Mutants of FtsY. Diagram of the FtsY coding region (top bar) with domain designations listed above. Deletion mutants and fusions are diagrammed below with shaded bars indicating the regions expressed in each. The solid black bar indicates the gPa passenger protein domain. Amino acid positions of deletion points and fusions are indicated above the bars.

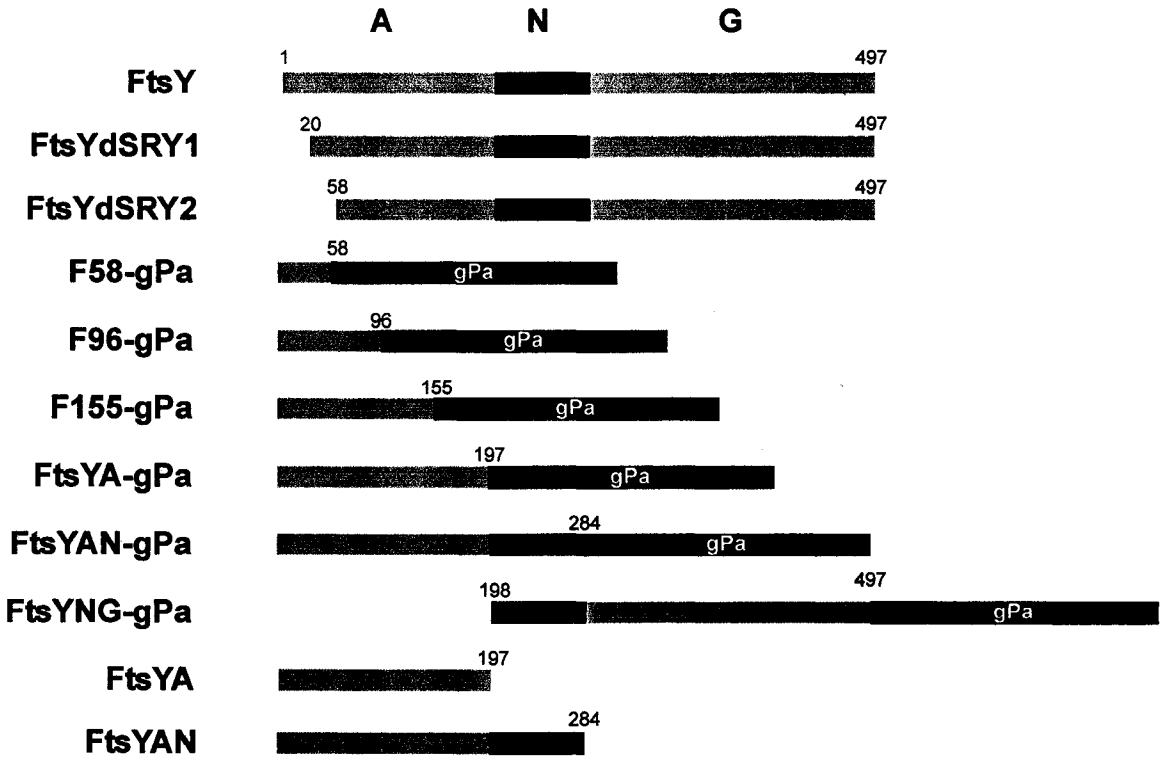
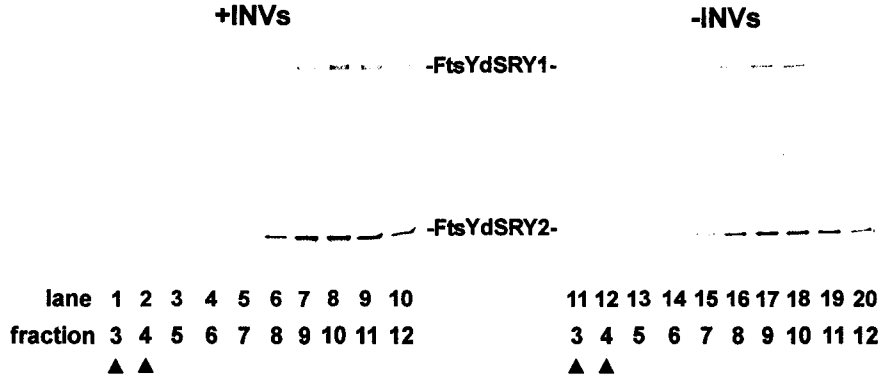
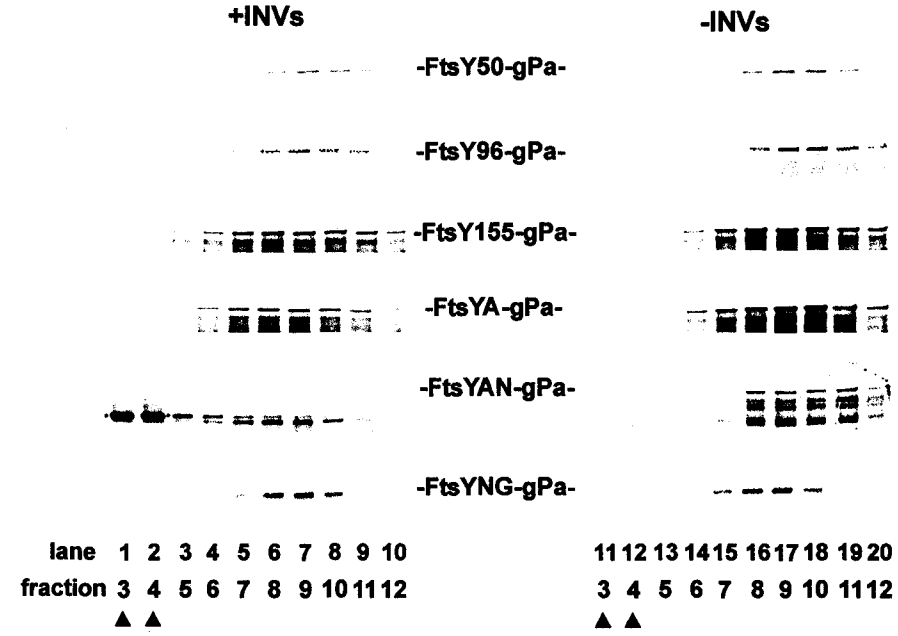

A
N
G
FtsY
1
497
FtsYdSRY1
20
497
FtsYdSRY2
58
497
F58-gPa
58
gPa
F96-gPa
96
gPa
F155-gPa
155
gPa
FtsYA-gPa
197
gPa
FtsYAN-gPa
284
gPa
FtsYNG-gPa
198
497
gPa
FtsYA
197
FtsYAN
284

Figure 2.4. Membrane binding of FtsY deletion mutants and fusion proteins. Deletion mutants and fusion proteins were synthesized in S170 lysate and incubated with membranes (lanes 1-10) or buffer (lanes 11-20), and membrane-bound molecules were separated from aggregates by chromatography on 0.8-ml Sepharose CL-2B columns as in Figure 2.2. Membranes and membrane-bound proteins eluted in the excluded volume (fractions 3 and 4, arrowheads), whereas cytosolic proteins eluted in the included volume (fractions 5-12). *A*, amino-terminal deletion mutants of FtsY. *B*, amino-terminal regions of FtsY fused to the passenger domain gPa. A 53-kDa putative cleavage product observed upon association of FtsYAN-gPa with membranes is indicated with dots. *C*, carboxyl-terminal deletion mutants consisting of the A domain or AN domains of FtsY. The migration positions of the expressed constructs are indicated at the sides of the panels.

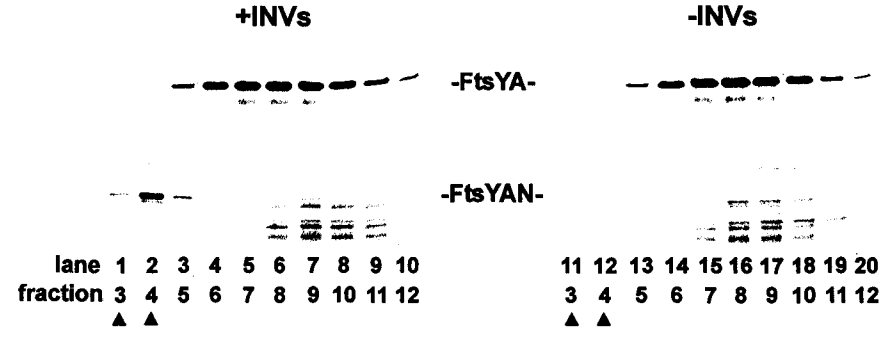
A) Amino-terminal deletions



B) Amino-terminal fusions



C) Carboxyl-terminal deletions



constructs containing amino-terminal segments of the A region of increasing size fused to gPa were fractionated in the presence or absence of membranes (Fig. 2.4B). Surprisingly, the elution patterns for all of these molecules are similar with and without INVs (Fig. 2.4B, compare lanes 1-10 with 11-20 for FtsY 58-gPa, FtsY 96-gPa, FtsY 155-gPa, and FtsYA-gPa). Therefore, fusions containing part or all of the A region of FtsY did not bind to INVs.

A single prominent band was obtained when a construct corresponding to the AN region fused to gPa (FtsYAN-gPa) was expressed *in vitro* and incubated with INVs. In contrast with the A region fusions, essentially this entire translation product fractionates with membranes in the excluded volume (Fig. 2.4B, lanes 1-2, dots). However, this band migrated at 53 kDa rather than the 92-kDa position observed for full-length FtsYAN-gPa in the absence of membranes (Fig. 2.4B, lanes 14-20). Unlike full-length FtsY where 75% of membrane-bound product was a 53-kDa species, in reactions containing FtsYAN-gPa essentially all of the membrane-bound protein migrated at 53 kDa.

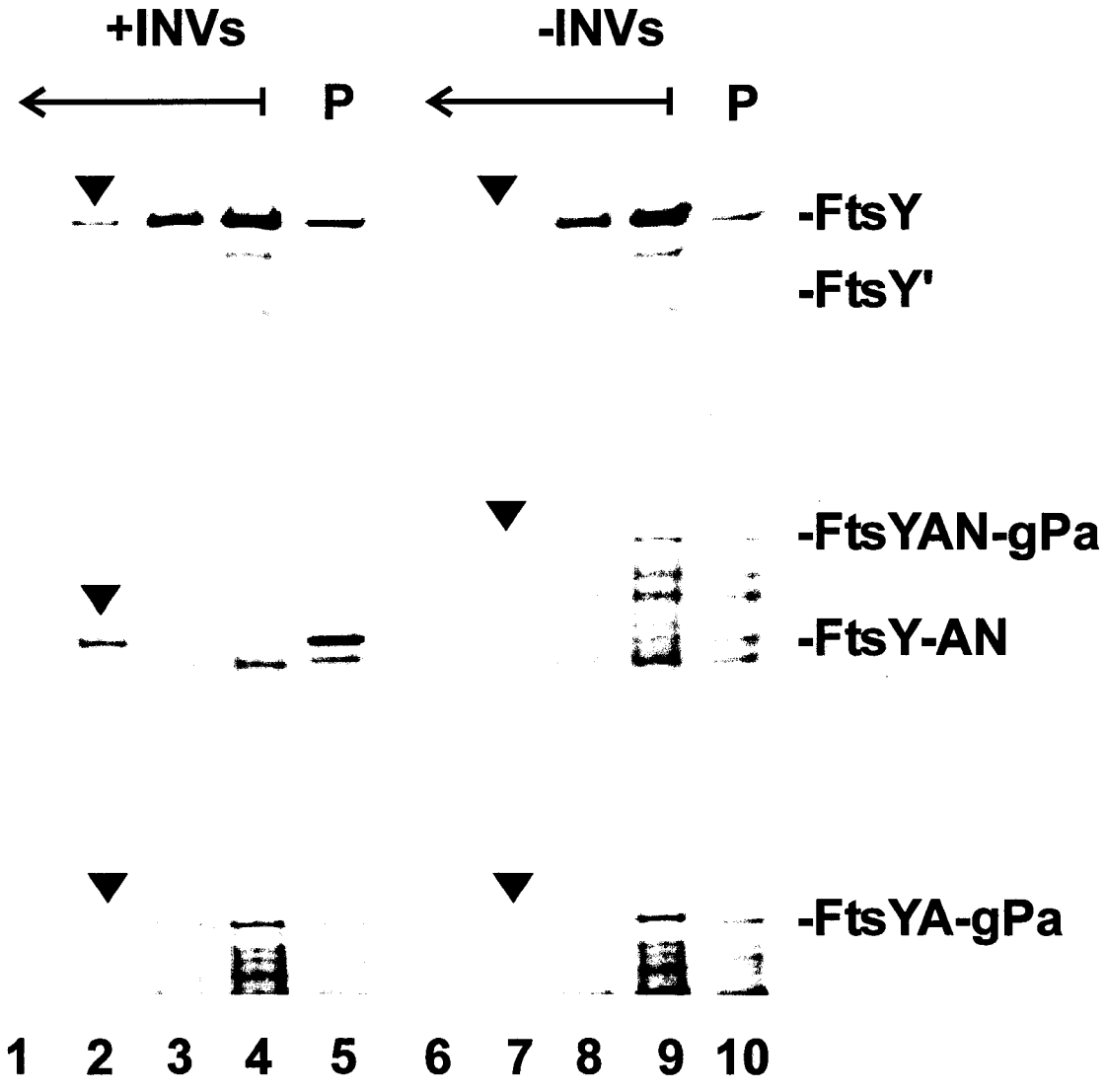
In the absence of membranes, the intensity of the 92-kDa band representing full-length product is greatly reduced (Fig. 2.4B, lanes 14-20). We attribute this to two factors. First, the product is dispersed over a larger number of fractions in the absence of membranes (at least 6 included fractions versus 2 excluded fractions). Second and more significantly, the FtsYAN-gPa product is apparently subject to nonspecific degradation if it is not targeted to membranes (see below, Fig. 2.7). Consistent with this interpretation, control experiments demonstrated that the amount of FtsYAN-gPa is maximal in S170

lysate immediately after transcription-translation and declines thereafter (data not shown). Unlike FtsYAN-gPa, the FtsYNG-gPa fusion is stable in the absence of INVs yet was unable to bind to membranes *in vitro* (Fig. 2.4B, compare lanes 1-10 with 11-20).

Because of the altered migration of the membrane-bound form of FtsYAN-gPa, we tested unfused versions of the A and AN sequences of FtsY individually for binding to INVs (Fig. 2.4C). Consistent with the behavior of the gPa fusions, the A region eluted in included fractions in the presence or absence of membranes (Fig. 2.4C, compare lanes 1-10 with 11-20 for FtsYA), demonstrating that the A region alone cannot bind to INVs. The AN region eluted in the excluded volume in the presence of membranes, demonstrating that this molecule bound efficiently to INVs (Fig. 2.4C, compare lanes 1-10 with 11-20 for FtsYAN). It is clear from this data that the AN domain, but not the A domain, of FtsY is both necessary and sufficient to direct membrane assembly. Moreover, consistent with the behaviour of FtsYAN-gPa, in the absence of INVs FtsYAN is degraded in lysate (Fig. 2.4C, lanes 11-20).

Vesicle lift assays were used to confirm these results. Control experiments demonstrated that sealed INVs migrate at the interface of the 0.25/1.25 M sucrose steps (Fig. 2.5, lanes 2 and 7) after centrifugation (data not shown). Some INVs were also found to pellet in this assay, presumably because they are leaky. Full-length FtsY and the putative cleavage product fractionated at the 0.25/1.25 M sucrose interface only when mixed with INVs (Fig. 2.5, top panel, compare lanes 2 and 7) confirming that they bound to membranes. Both full-length FtsY and the putative FtsY cleavage product were also

Figure 2.5. Floatation analysis for FtsY membrane binding. Full-length FtsY (top panel), FtsYAN-gPa (middle panel), and FtsYA-gPa (bottom panel) fusion proteins were synthesized in S170 lysate and incubated with membranes (lanes 1-5) or buffer (lanes 6-10). Reactions were adjusted to 1.6 M sucrose and overlaid with steps of 1.25 M sucrose and 0.25 M sucrose containing 35 mM Tris acetate, pH 8.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 1 mM DTT, and 12 mM magnesium acetate. Following centrifugation, 50-µl fractions were taken from the top of the gradient (lanes 1 and 6) to the bottom of the gradient (lanes 5 and 10). Membranes and associated proteins fractionate at the interface between the 1.25 and 0.25 M sucrose steps (arrowheads). The migration positions of the expressed constructs are indicated at the sides of the panels.


+INVs
-INVs
P
P
-FtsY
-FtsY'
-FtsYAN-gPa
-FtsY-AN
-FtsYA-gPa
1 2 3 4 5 6 7 8 9 10

observed to pellet in the presence of membranes, as expected for aggregates and molecules bound to leaky INVs (Fig. 2.5, top panel, compare lanes 5 and 10).

As above, FtsYAN-gPa was cleaved in the presence of membranes (Fig. 2.5, middle panel). This product fractionated at the 0.25/1.25 M sucrose interface only when membranes were added to the reaction (Fig. 2.5, middle panel, compare lanes 2 and 7). In contrast, FtsYA-gPa fractionates identically in the presence and absence of membranes, as expected for a protein that does not bind to membranes (Fig. 2.5, bottom panel).

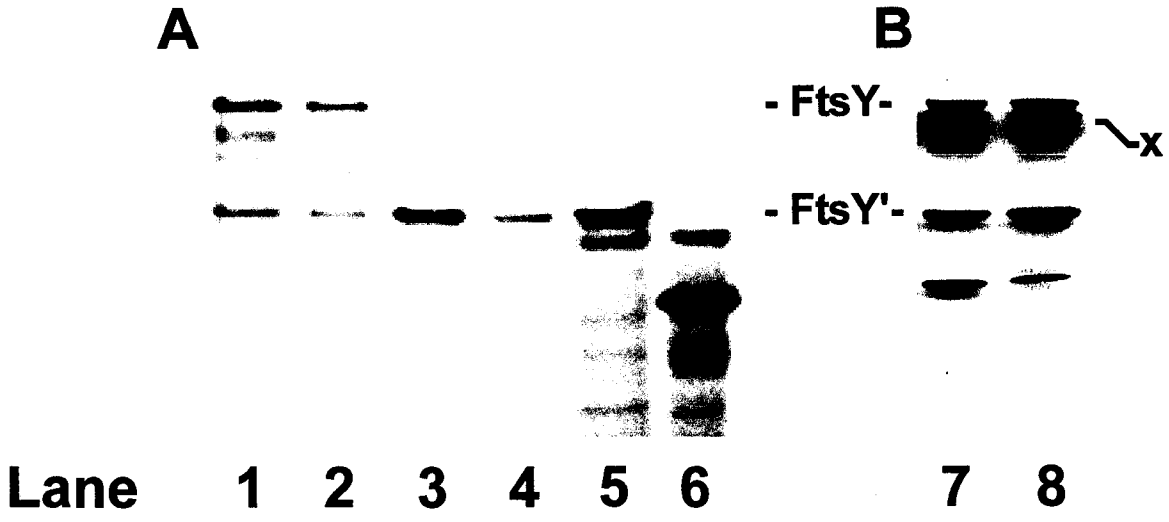
FtsY Is Cleaved upon Membrane Assembly – The lower molecular weight polypeptides bound to membranes following incubation of FtsY and FtsYAN-gPa translation products with INVs both migrate as 53-kDa species in SDS-PAGE. This is very close to the 54-kDa mass of FtsY expected from primary sequence data. Thus, it is possible that the band that migrates at approximately 92 kDa results from a modified form of FtsY, and the 53-kDa band is either not modified or may be generated from the 92-kDa species by the removal of some modifying group. Alternatively, the AN domain (calculated molecular mass 32 kDa) exhibits anomalous migration in SDS-PAGE and migrates at 53 kDa. To determine whether the amino-terminus of FtsY is modified or exhibits anomalous migration in SDS-PAGE, FtsY58-gPa, which has a calculated molecular weight of 39,527 but migrates with an apparent molecular mass of 48 kDa on SDS-PAGE, was expressed, purified from *E. coli*, and analysed by mass spectroscopy. The molecular weight measured for this molecule corresponded exactly to that expected

based on primary sequence analysis. Although this result does not rule out modifications in other regions of FtsY accounting for some of the unexpected apparent molecular weight, it suggests that the observed migrations of FtsY and AN in SDS-PAGE are anomalous because of physical properties inherent to the primary sequence of the polypeptide. Furthermore, FtsY and FtsYAN migrate as 92- and 53-kDa species, respectively, when synthesized in either *E. coli* S170 lysate or in reticulocyte lysate (see below). Because these lysates are unlikely to contain identical modification systems, this result further suggests that polypeptides containing the A region of FtsY migrate anomalously during SDS-PAGE.

To further assess the nature of the 53-kDa species that coelutes with membranes, the migration on SDS-PAGE of the products in the excluded fractions obtained from membrane binding assays for FtsY and FtsYAN-gPa were compared with those from translations of the A and AN regions of FtsY (Fig. 2.6). The 53-kDa bands observed for FtsY and FtsYAN-gPa corresponded exactly with each other, as well as with the migration of the AN polypeptide (Fig. 2.6, compare lanes 1-2 with 3-4 and lane 5). The simplest explanation for this data is that the 53-kDa band results from specific cleavage of FtsY and FtsYAN-gPa between the N and G regions.

To establish whether a membrane-bound species of FtsY with an apparent molecular mass of 53 kDa is present endogenously in *E. coli*, whole cells and INVs were separated by SDS-PAGE and analysed by immunoblotting with affinity purified antibodies to FtsY. The migration of the anti-FtsY reactive species was compared with

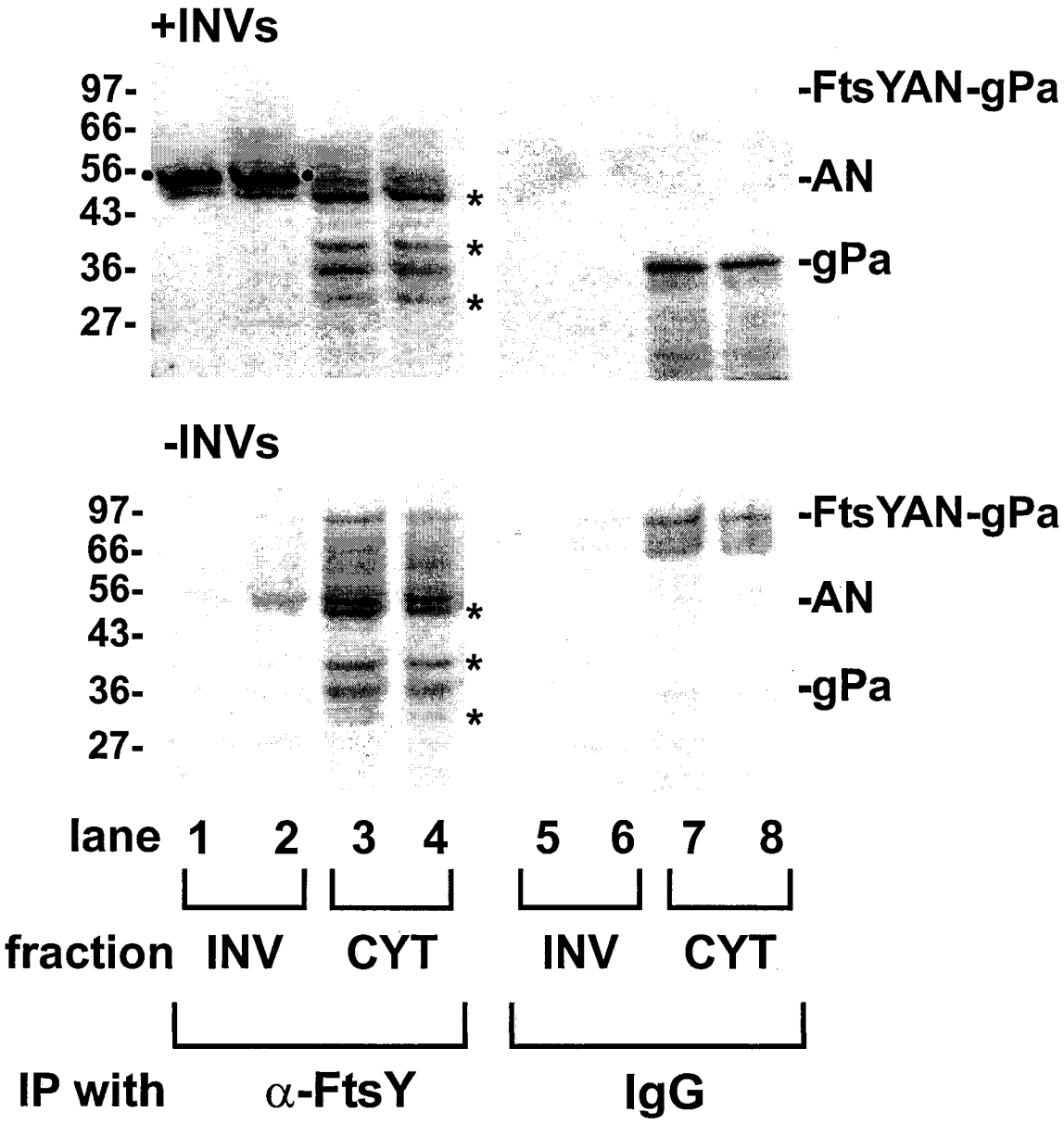
Figure 2.6. Identification of the 53-kDa membrane-bound product. *A*, two fractions corresponding to the excluded volumes from membrane binding assays for S170 synthesized FtsY (lanes 1-2), and FtsYAN-gPa (lanes 3-4) was analyzed by SDS-PAGE and phosphorimaging. FtsYAN (lane 5) and FtsYA (lane 6) synthesized in S170 lysate serve as size markers. *B*, *E. coli* cells at mid-log phase (lane 7) and inner membrane inverted vesicles (lane 8) were solubilized, and the component proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using antibodies generated against FtsY. The positions of full-length FtsY, the putative cleavage product observed associated with membranes (FtsY'), and a cross-reacting band (x) are indicated.


A
B
- FtsY-
- FtsY'-
x
Lane 1 2 3 4 5 6 7 8

the excluded fractions obtained in the presence of membranes from S170 translations of FtsY and FtsYAN (Fig. 2.6). Several prominent bands were obtained when INVs were analyzed by immunoblotting (Fig. 2.6, lanes 7 and 8). In addition to full-length FtsY, a 53-kDa band is detected that comigrates with the putative cleavage product of FtsY (Fig. 2.6, compare lanes 1-4 with lane 7), suggesting that both the 92- and 53-kDa isoforms of FtsY are present in *E. coli*. An uncharacterized band (indicated with an x) is also detected using these antibodies. A cross-reacting band of the same apparent molecular weight as that seen here has been observed previously (Luirink et al., 1994) using an independent FtsY antibody. We also observe a band with greater migration than the putative cleavage product of FtsY. The intensity of this band relative to both FtsY and the cleaved product is greatly decreased in purified vesicles versus whole cells (Fig. 2.6, compare lanes 7 and 8), suggesting that this product is predominantly cytosolic. The migration of this band does not correspond to the migration of the G domain of FtsY, thus the origin of this band is uncertain.

Further evidence that the 53-kDa bands correspond to membrane-dependent cleavage of FtsY between the AN and G sequences was obtained using differential immunoprecipitation of FtsYAN-gPa translation reactions after incubation with and without INVs (Fig. 2.7). FtsYAN-gPa synthesized in S170 extract was incubated in the presence (top panel) or absence (bottom panel) of INVs and then fractionated by Sepharose CL-2B gel exclusion chromatography. The translation products bound to INVs (excluded fractions 3 and 4) and in the cytosol (included fractions 9 and 10) were

Figure 2.7. FtsY is cleaved between AN and G domains upon membrane binding. The fusion protein FtsYAN-gPa was expressed in S170 lysate, incubated with membranes (top panel) or buffer (bottom panel), and analyzed for membrane binding as in Figure 2.2. Products in the excluded fractions (lanes 1-2 and 5-6) containing INVs and representative included fractions (lanes 3-4 and 7-8) containing cytosolic proteins (CYT) were identified by immunoprecipitation with affinity purified antibodies to FtsY (lanes 1-4) or with IgG-Sepharose (lanes 5-8). The migration positions of full-length FtsYAN-gPa, the AN domain of FtsY, and the gPa domain are indicated. Bands corresponding to FtsYAN are indicated by dots. The gPa domain contains the IgG binding domains of protein A and therefore binds to FtsY antibodies and to IgG-Sepharose. Asterisks indicate degradation products. The migration positions of molecular mass markers are indicated (in kDa) to the left of the panels. IP, immunoprecipitate.


+INVs
97-
66-
56-
43-
36-
27-
-FtsYAN-gPa
-AN
-gPa
-INVs
97-
66-
56-
43-
36-
27-
-FtsYAN-gPa
-AN
-gPa
lane 1 2 3 4 5 6 7 8
fraction INV CYT INV CYT
IP with α-FtsY IgG

identified by immunoprecipitation using either an anti-FtsY antibody (lanes 1-4) to bind the amino-terminus or IgG-Sepharose (lanes 5-8) to bind the gPa domain.

The anti-FtsY antibody efficiently precipitates the 53-kDa putative FtsYAN-gPa cleavage product from the vesicle containing excluded fractions (Fig. 2.7, lanes 1-2, dots). In contrast, this product is not precipitated with IgG-Sepharose (lanes 5-6). Because gPa contains four independent IgG binding domains, this result demonstrates that the 53-kDa putative cleavage product contains less than one-fourth of the gPa portion of FtsYAN-gPa.

Following incubation with INVs a band corresponding to the complete gPa domain is precipitated with IgG-Sepharose from the cytosolic fractions (Fig. 2.7, top panel, lanes 7-8). This product is not observed in the same fractions without added INVs (bottom panel, lanes 7-8). Thus, the 53-kDa membrane-associated band results from cleavage of FtsYAN-gPa immediately carboxyl-terminal of the AN region, and cleavage releases the gPa domain into the cytosol.

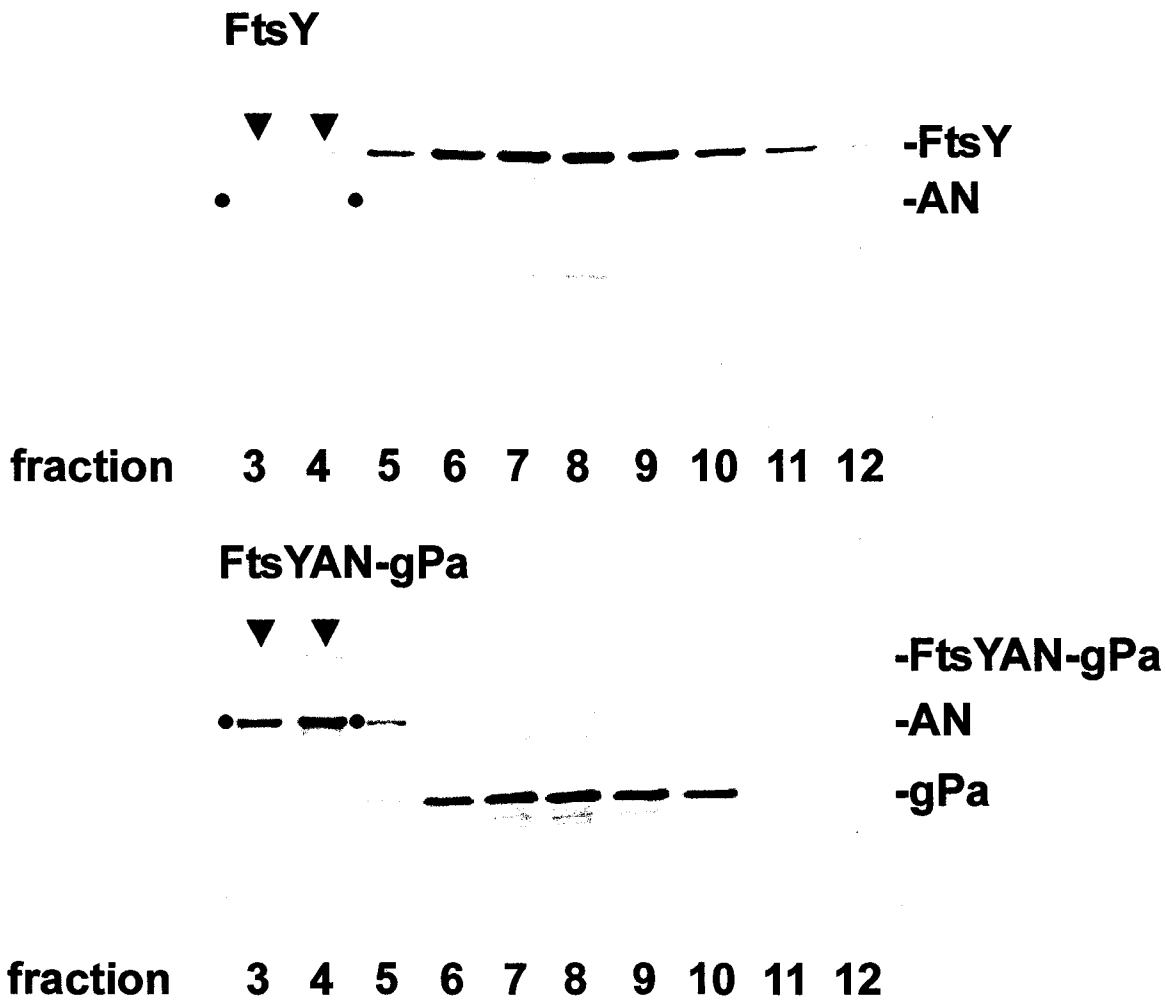
In the absence of INVs, specific cleavage of FtsYAN-gPa was not observed (Fig. 2.7, bottom panel). Furthermore, without INVs essentially no translation product precipitates from the excluded fractions with either anti-FtsY antibody or IgG-Sepharose, as expected (lanes 1-2 and 5-6). In the included fractions, several bands with greater migration than FtsYAN-gPa are immunoprecipitated with anti-FtsY antibody (lanes 3-4, asterisks). However, these bands are likely to result from relatively nonspecific degradation of FtsYAN. First, none of these bands correspond to the same size as the

specific cleavage product observed in the excluded fractions of A. Second, a specific band corresponding to gPa is not precipitated by IgG-Sepharose from the included fractions. Instead IgG-Sepharose primarily precipitates full-length FtsYAN-gPa from these fractions (bottom panel, lanes 7-8). Thus, specific cleavage of FtsYAN-gPa occurs only in the presence of membranes.

Taken together, these data suggest that full-length FtsY as well as FtsY fusion proteins containing both the A and N regions are competent for membrane binding and can be cleaved upon membrane binding. Even though only a small fraction of the total full-length FtsY molecules synthesized in *E. coli* lysate associated with INVs, the 53-kDa cleavage product, corresponding to the AN membrane binding domain, is efficiently retained on the membrane.

To obtain further evidence that membrane-dependent cleavage is because of a membrane-associated factor rather than a component of the *E. coli* cytosol, full-length FtsY and FtsYAN-gPa were translated in rabbit reticulocyte lysate, and INVs were then added. After incubation for 45 min at 37 °C the reactions were fractionated by Sepharose CL-2B gel exclusion chromatography as above. As was observed using S170-translated FtsY, most of the full-length FtsY synthesized in reticulocyte lysate did not bind to INVs and therefore fractionated in the included volume (Fig. 2.8, top panel, lanes 5-12). However, a small fraction of the full-length FtsY eluted in the excluded volume with INVs (Fig. 2.8, top panel, arrowheads). Significantly, these fractions also contained the 53-kDa product expected from cleavage of FtsY between the N and G regions (Fig. 2.8,

Figure 2.8. FtsY cleavage is membrane specific. Reticulocyte lysate translation reactions for FtsY (top panel) or FtsYAN-gPa (bottom panel) were incubated with INVs and analyzed for membrane binding as in Figure 2.2. INVs and membrane-bound proteins elute in the excluded volume (fractions 3 and 4). Cytosolic proteins eluted as a broad peak in the included volume (fractions 5-11). Arrowheads indicate bands corresponding to membrane-bound full-length FtsY and FtsYAN-gPa. Bands corresponding to the membrane-bound 53-kDa cleavage products of FtsY and FtsYAN-gPa are indicated by dots. The migration positions of full-length FtsYAN-gPa as well as the AN and gPa domains are indicated.


FtsY
-FtsY
-AN
fraction 3 4 5 6 7 8 9 10 11 12
FtsYAN-gPa
-FtsYAN-gPa
-AN
-gPa
fraction 3 4 5 6 7 8 9 10 11 12

top panel, lanes 3-4, dots).

Both membrane binding and cleavage are much clearer for FtsYAN-gPa synthesized in reticulocyte lysate (Fig. 2.8, bottom panel). In this reaction, essentially all of the full-length translation products fractionate with membranes in the excluded volume (Fig. 2.8, bottom panel, lanes 3-4, arrowheads). However, most of the molecules are cleaved, and the resulting 53-kDa band that comigrates with AN also elutes with INVs in the excluded volume (Fig. 2.8, bottom panel, lanes 3-4, dots). The gPa fusion domain that was cleaved from the AN portion behaves as a soluble protein and therefore elutes in the included fractions (Fig. 2.8, bottom panel, lanes 5-12) as expected. Thus, both FtsY and FtsYAN-gPa are cleaved carboxyl to the AN region only when INVs are added. The most likely explanation of this phenomenon is that cleavage is because of a proteolytic activity associated with INVs, although it remains possible that rabbit reticulocyte lysate contains a similar protease to one found in S170 lysate that performs cleavage only upon association of FtsY with the membrane. Furthermore, the AN domain remains tightly bound to the INVs after cleavage, demonstrating that the AN region of FtsY is a bona fide membrane binding domain.

In an attempt to determine the exact site in the polypeptide chain where cleavage occurs upon membrane-binding of FtsY, constructs were generated in which the carboxyl-terminal amino acids of the AN region were progressively deleted in carboxyl-terminal fusions to gPa. Following incubation with INVs and chromatography over a CL-2B column, products from constructs in which the carboxyl-terminal 3, 8, and 12

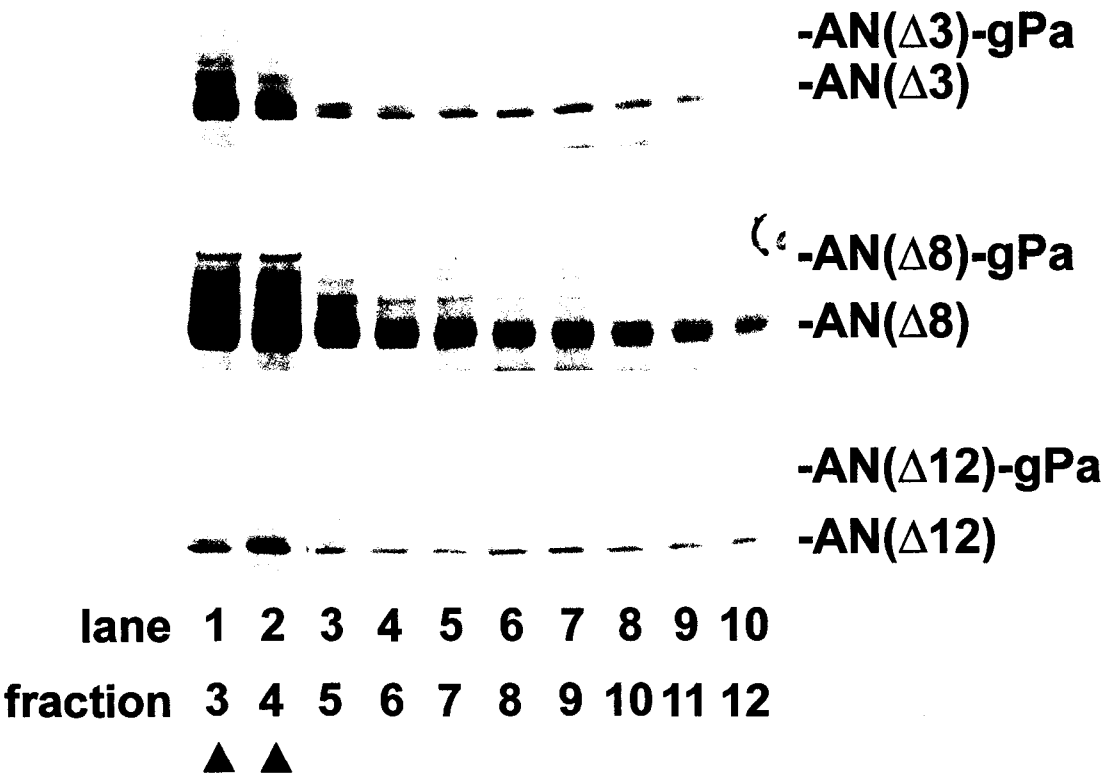
amino acids of the N region were deleted eluted primarily in the excluded fractions (Fig 2.9A, arrowheads). The migration of the predominant band in each case indicated that these fusion products were cleaved upon membrane-binding.

However, we considered that the membrane-binding might be a non-specific effect due to the presence of the carboxyl-terminal gPa and cleavage in these fusion proteins might actually occur in this alpha-helical region of the globin leader sequence. To test this hypothesis, the corresponding deletion mutants without gPa were generated and assayed for membrane-binding. A deletion mutant missing the carboxyl-terminal 12 amino acids from the N region elutes primarily in the excluded fractions following CL-2B column chromatography (Fig. 2.9B, top panel, lanes 1 and 2) confirming that at least for binding INVs these amino acids are dispensable. In contrast, a deletion mutant missing 17 amino acids elutes exclusively in the included fractions (Fig 2.9B, bottom panel, lanes 3-9), suggesting the presence of amino acids 267-272 is necessary for membrane binding. Since membrane-binding is a prerequisite for cleavage, we were unable to further investigate the site of cleavage in this manner.

A variety of factors (see below, discussion) led us to speculate that the integral membrane protein FtsH might be the protease that specifically cleaves FtsY. However, following incubation of full-length FtsY with INVs from FtsH-depleted *E. coli* the cleaved AN domain was observed in excluded fractions (Fig. 2.10, fractions 3 & 4, dots) following CL-2B size exclusion chromatography. This suggests that another protease can perform the observed site-specific cleavage of FtsY.

Figure 2.9. Membrane binding of FtsYAN deletion mutants and fusion proteins. Fusion proteins and deletion mutants were synthesized in S170 lysate and incubated with membranes. Membrane-bound molecules were separated from aggregates by chromatography on 0.8-ml Sepharose CL-2B columns as in Figure 2.2. Membranes and membrane-bound proteins eluted in the excluded volume (fractions 3 and 4, arrowheads), whereas cytosolic proteins eluted in the included volume (fractions 5-12). *A*, Deletions of the carboxyl-terminal regions of the AN domain of FtsY fused to the passenger domain gPa. *B*, carboxyl-terminal deletion mutants of the AN domain of FtsY. The migration positions of the expressed constructs and cleavage products are indicated at the sides of the panels.

A) Fusions to gPa



B) Deletions

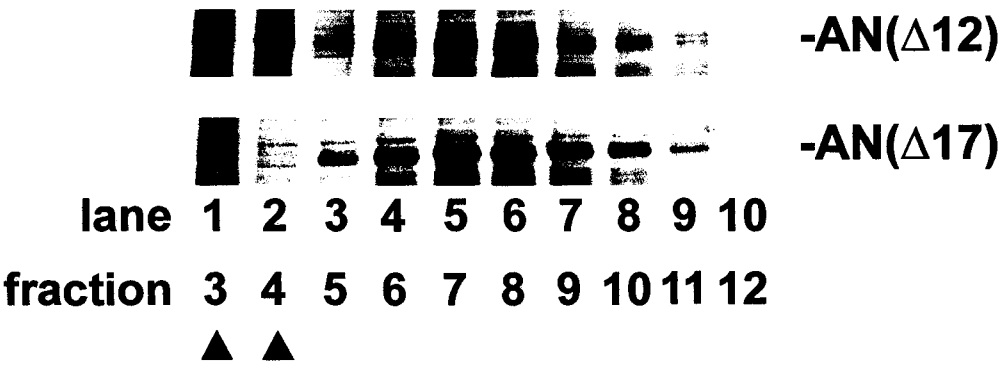
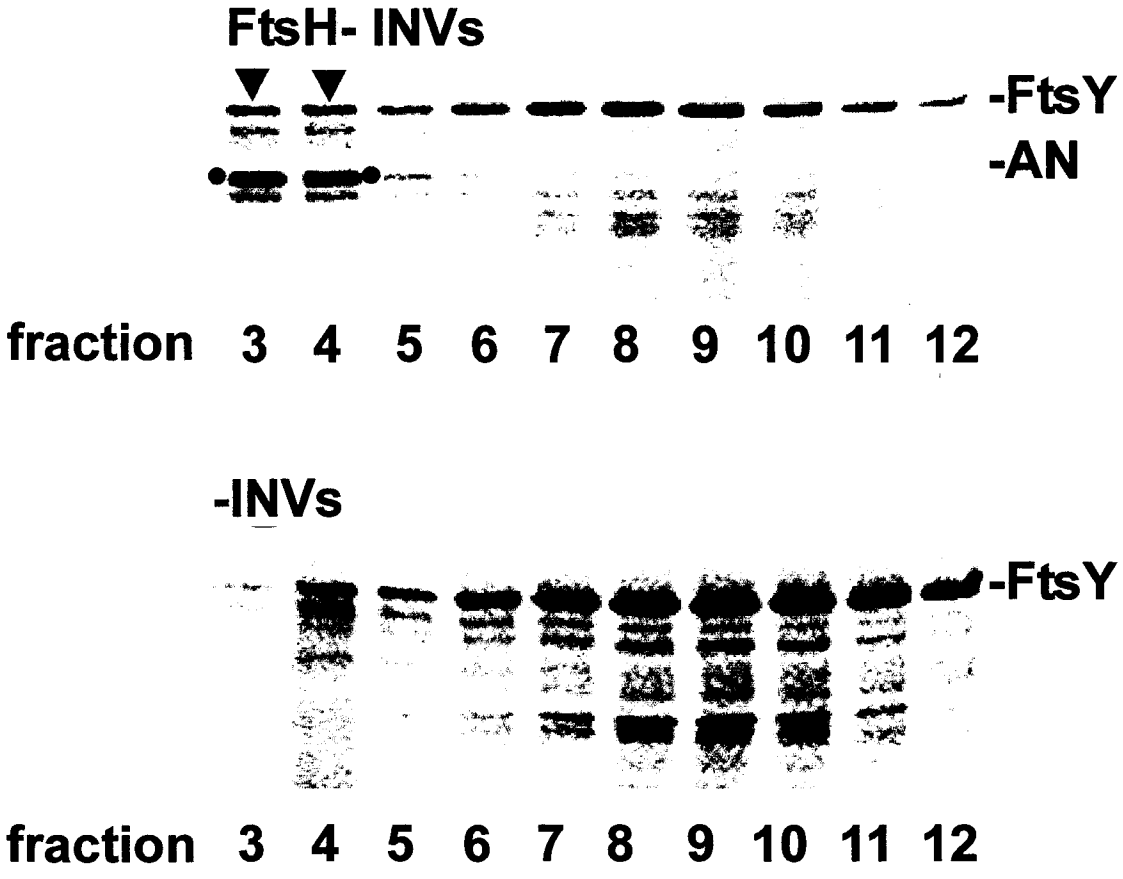


Figure 2.10. FtsY cleavage occurs upon binding FtsH-depleted INVs. FtsY was synthesized in S170 lysate, incubated with FtsH-depleted INVs (top panel) or buffer (bottom panel) and analyzed for membrane binding as in Fig. 2.2. INVs and membrane-bound proteins elute in the excluded volume (fractions 3 and 4). Cytosolic proteins eluted as a broad peak in the included volume (fractions 5-11). Arrowheads indicate bands corresponding to membrane-bound full-length FtsY. Bands corresponding to the membrane-bound 53-kDa cleavage products of FtsY are indicated by dots. The migration positions of full-length FtsYAN as well as the AN domains are indicated.


FtsH- INVs
-FtsY
-AN
fraction 3 4 5 6 7 8 9 10 11 12
-INVs
-FtsY
fraction 3 4 5 6 7 8 9 10 11 12

2.6 Discussion

We show here that although FtsY molecules synthesized in either *E. coli* or reticulocyte lysate aggregate, these aggregates can be clearly distinguished from membrane-bound molecules using gel filtration chromatography and by floatation in sucrose step gradients. Passing translation reactions incubated with INVs over Sepharose CL-2B (exclusion limit of 40,000 kDa) allow even relatively large protein aggregates to be retained in the included volume, whereas membranes and membrane-bound proteins elute in the excluded fractions (Young et al., 1995). Similarly, membrane-bound molecules that float in dense sucrose solutions can be distinguished unambiguously from pelleted aggregates. Moreover, because membranes undergo a very large dilution into buffer in either technique, proteins recovered with vesicles bound stably to membranes.

Although a large amount of aggregated full-length FtsY was found in the included fractions and in the pellet fractions of vesicle lift gradients, FtsY clearly cofractionated with membranes when translation reactions were incubated with INVs (Figs. 2.2 and 2.5). Furthermore, FtsY and FtsYAN-gPa also bound to membranes when synthesized in reticulocyte lysate suggesting that cytosolic *E. coli* proteins are not required for membrane binding (Fig. 2.8).

Identification of AN as the Membrane Binding Domain of FtsY –To determine which regions of FtsY mediate membrane binding, we constructed plasmids encoding deletion mutants and FtsY-gPa fusion proteins. Analysis of the deletion mutants and fusion proteins demonstrated that both the A and N regions of FtsY together constitute a

minimum region of FtsY that is both necessary and sufficient for membrane binding in either *E. coli* lysate (Fig. 2.4) or reticulocyte lysate (Fig. 2.8). That A and N together form a membrane binding domain in FtsY was surprising given the similarity of the N and G regions in FtsY to those in Ffh and SRP54. Indeed the homology of all SRP family GTPases has been interpreted as evidence that a gene duplication event led to the production of the cytoplasmic protein from the receptor or vice versa (Gribaldo and Cammarano, 1998). In SRP54, the N region appears to be involved in efficient signal sequence binding (Newitt and Bernstein, 1997). For this reason and because the N region is juxtaposed with the G region in both the membrane-bound receptor molecules and cytoplasmic homologues of SRP pathway GTPases, it has been assumed that the N region is involved in the GTPase domain function of FtsY. This is exemplified in previous structural and biochemical analyses of the N and G regions of FtsY as a single unit (Montoya et al., 1997; Zelazny et al., 1997). Although our data clearly indicate a role for the N region in FtsY membrane binding, it is not clear whether N is involved in the correct folding of the membrane binding domain or if it makes specific contacts with the putative FtsY receptor on the *E. coli* inner membrane. It is possible that in the cytoplasmic proteins the N domain evolved to function in signal binding, whereas in the *E. coli* receptor a role evolved for N in membrane receptor binding. The observation that the N region folds into a separate, four helix bundle in crystal structures of the NG regions of both FtsY and Ffh (Montoya et al., 1997; Freymann et al., 1997) is consistent with this hypothesis. Finally, our data do not rule out an additional function for the N

region in FtsY that may involve the G region.

Surprisingly, the FtsYAN-gPa fusion protein bound to INVs much more efficiently than did full-length FtsY. This enhanced efficiency versus wild type might be explained by the observation that unlike the G region, the gPa sequence contains a 23-amino acid linker region amino-terminal of the independently folded IgG binding domains of gPa (Janiak et al., 1994). This spacer may allow the AN and gPa domains to fold more efficiently in the fusion protein reducing aggregation and misfolding thereby leading to more efficient membrane binding. Consistent with this hypothesis, in S170 lysate FtsYAN binds to INVs better than FtsY does. This result also indicates that the low levels of FtsY membrane binding observed above (Fig. 2.2) are not because of a limiting number of binding sites for FtsY on the membrane, as both reactions contained similar quantities of INVs. Together with the demonstrations that in the absence of membranes FtsY forms large aggregates and that FtsY binds to the endogenous membranes in S30 lysate more efficiently than when INVs are added post-translationally, these results confirm that the low efficiency of FtsY membrane binding is because of misfolding and aggregation.

Membrane-dependent Specific Cleavage – Further support for a membrane binding function for the AN domain comes from the observation that a fraction of the FtsY molecules in *E. coli* (Fig. 2.6) and incubated with INVs *in vitro* (Figs. 2.2, 2.4, 2.5, 2.7, 2.8) are cleaved such that a 53-kDa product remains bound to membranes. That the 53-kDa cleavage product corresponds to the AN domain was demonstrated by differential

immunoprecipitation of the membrane-bound and cytosolic products that resulted from incubating the fusion protein FtsYAN-gPa with INVs (Fig. 2.7). Affinity purified antibodies generated against FtsY precipitated the AN region from membrane-bound fractions, whereas IgG-Sepharose bound to the gPa domain in fractions corresponding to cytosolic proteins.

The exact position at which cleavage of the polypeptide chain occurs remains uncertain. Membrane binding and cleavage still occurs upon deletion of 12 amino acids at the carboxyl-terminus of the N region in a gPa fusion construct (Fig. 2.9). However, both the N region and the globin leader sequence of gPa form alpha-helical secondary structures and most proteases in prokaryotes do not recognize specific sequence motifs. Thus, the site of proteolytic cleavage in FtsY may have been deleted but was replaced with the alpha helical region of gPa. It was clear that deletion of 17 amino acids (but not 12) from the carboxyl-terminus of the N region prevented membrane-binding (Fig 2.9). Based upon the crystal structure of FtsY, this stretch of amino acids lies at the beginning of the fourth and final alpha-helical stretch of the N region (Montoya et al., 1997). Hence, although it is possible that these five amino acids play a specific role in membrane binding, it seems likely that removal of these five amino acids at this position causes a local or global disruption of the structure of the N region four-helix bundle, which in prevents membrane binding by the deletion mutant.

In the absence of membranes, the full-length FtsYAN-gPa protein behaves as a soluble cytosolic protein (Fig. 2.7). Although antibodies directed against FtsY also

immunoprecipitated a number of bands with greater migration than full-length FtsYAN-gPa from incubations without INVs, the pattern of bands obtained is indicative of relatively nonspecific cleavage and none of the bands obtained migrate at 53 kDa (Fig. 2.7). Moreover, degradation of cytosolic FtsYAN-gPa does not release intact gPa, as was observed for specific cleavage of FtsYAN-gPa in the presence of membranes. Indeed the major product precipitated with IgG-Sepharose from the cytosolic fractions was full-length FtsYAN-gPa. Thus, we conclude that when FtsYAN-gPa does not bind to membranes it is accessible to proteases that degrade the protein in a relatively nonspecific manner. The same phenomenon is observed when the AN domain alone is incubated in the presence of membranes (Fig. 2.4). In contrast, membrane-bound AN is protease resistant, suggesting that either the AN domain undergoes a conformational change upon membrane binding or it is stabilized by the association with another molecule on the membrane that masks potential cleavage sites for nonspecific proteases.

The above data demonstrate that upon binding to membranes a site-specific cleavage event occurs that defines the membrane-binding domain of FtsY. However, it does not resolve whether the protease is membrane-associated or free in the cytosol. Membrane-assembly of the AN region may result in a conformational change in the protein that exposes a previously inaccessible cleavage site to a soluble *E. coli* protease. To address this issue, FtsYAN-gPa was translated in reticulocyte lysate prior to incubation with membranes. Membrane assembly of the reticulocyte lysate translation products again resulted in specific cleavage of FtsYAN-gPa into membrane-bound AN

and free gPa domains (Fig. 2.8). In the absence of INVs there is much less nonspecific degradation of FtsYAN-gPa in reticulocyte lysate than in the S170 lysate, probably because the amount of nonspecific protease activity in reticulocyte lysate is less than in the *E. coli* system. Together, these results suggest that INV-dependent cleavage of FtsY is performed by a membrane-bound protease.

Although cleavage apparently occurs *in vivo* (Fig. 2.6), it is unclear at present whether cleavage is physiologically important or is simply a mechanism for dealing with excess FtsY on the membrane. One attractive but speculative possibility is that FtsY might function stoichiometrically rather than catalytically. In this scenario, FtsY could be cleaved subsequent to targeting as a mechanism for ensuring that targeting is unidirectional and to clear the binding site for reuse in future targeting reactions.

A candidate for the membrane-associated protease that cleaves FtsY remains uncertain. Several observations of the nature of the protease FtsH suggested it was an exceptional candidate: (1) Depletion of FtsH *in vivo* retards translocation of secreted proteins, but enhances translocation of normally anchored protein segments (Akiyama et al., 1994), a feature that would be expected if FtsH degrades a component of the SRP pathway; (2) FtsH is an inner membrane integral protein; (3) The cleavage specificity of FtsH shows preferences for positively charged and hydrophobic residues (Akiyama, 1999), which are highly enriched in the sequence 'LYGLLK' between the -17 and -12 positions of the N region at which our investigations into the site of cleavage suggest the protease may cleave. Hence, it was somewhat of a surprize that FtsY was cleaved upon

binding FtsH-depleted membranes (Fig 2.10). It is possible, however, that since only fmol quantities of FtsY are expressed in this assay the small amount of residual FtsH following depletion is sufficient for FtsY cleavage. Alternatively, FtsY may be cleaved by one or more of the numerous proteases in the cell.

Conclusions – We have clearly demonstrated that the AN region of FtsY is required for membrane binding. This suggests that the common practice of conceptually separating FtsY into the membrane binding A region and a GTPase region composed of both N and G sequences must be revisited. Because a structure for full-length FtsY has not been determined it was only possible to determine that AN is a bona fide protein domain by characterizing the biochemical properties of the molecule. Nevertheless, both the protease susceptibility and the membrane anchoring of both FtsY and FtsY fusion proteins strongly suggest that AN is the complete membrane binding domain of FtsY. In addition to revealing new information about the domain organization and functional properties of FtsY, identification of the membrane-binding domain is an essential first step in identifying the putative FtsY receptor. A possible physiological role for membrane-dependent cleavage of FtsY and the identity of the protease responsible for membrane-dependent cleavage can also now be elucidated.

CHAPTER III

FtsY Binds to the *Escherichia coli* Inner Membrane via Interactions with Phosphatidylethanolamine and Membrane Proteins

adapted from

Millman, J.S., Qi, H-Y, Vulcu, F., Bernstein, H.D. and Andrews, D.W.

Journal of Biological Chemistry, 2001, vol. 276, pp. 25982-25989.

Preamble

The contents of this chapter were contributed primarily by the author of this thesis (Jonathan Scott Millman). The experiments in Figure 3.4 were performed by Felicia Vulcu under the supervision of David Andrews and Jonathan Millman, and those in Figure 3.5 were performed entirely by Dr. Hai-Yan Qi in the lab of Dr. Harris Bernstein (National Institutes of Health, Bethesda MD). The text was written, in its entirety, by Jonathan Millman.

This chapter is adapted from the article published in the Journal of Biological chemistry with minor revisions to the text of the discussion that does not significantly alter its content.

3.1 Summary

Targeting of many polytopic proteins to the inner membrane of prokaryotes occurs via an essential signal recognition particle-like pathway. FtsY, the *Escherichia coli* homologue of the eukaryotic signal recognition particle receptor -subunit, binds to membranes via its amino-terminal AN domain. We demonstrate that FtsY assembles on membranes via interactions with phosphatidylethanolamine and with a trypsin-sensitive component. Both interactions are mediated by the AN domain of FtsY. In the absence of phosphatidylethanolamine, the trypsin-sensitive component is sufficient for binding and function of FtsY in the targeting of membrane proteins. We propose a two-step mechanism for the assembly of FtsY on the membrane similar to that of SecA on the *E. coli* inner membrane.

3.2 Introduction

In *Escherichia coli*, most integral membrane proteins are targeted to the inner membrane via SRP (Macfarlane and Muller, 1995; Ulbrandt et al., 1997; de Gier et al., 1996), a particle composed of Ffh and 4.5 S RNA (Luirink et al., 1992; Valent et al., 1995). SRP promotes co-translational targeting of nascent polypeptide chains via an interaction with FtsY, the membrane-associated SRP receptor (Valent et al., 1998; Powers and Walter, 1997). Although the targeting steps are distinct from those of the SecB secretory pathway (Koch et al., 1999; Scotti et al., 1999), both pathways converge at a common translocation pore in the membrane that comprises SecY, SecE, and SecG

(Valent et al., 1998; Qi and Bernstein, 1999). The components of the SRP pathway in *E. coli* closely resemble those of eukaryotes both in sequence (Poritz et al., 1988; Struck et al., 1988; Bernstein et al., 1989; Romisch et al., 1989) and in functional interactions (Valent et al., 1995; Luirink et al., 1992; Miller et al., 1994; Powers and Walter, 1995). An interesting divergence is observed in the mechanism of assembly of the SRP receptors onto membranes. In eukaryotes, the transmembrane β-subunit (SRβ) of the SRP receptor anchors the peripheral membrane α-subunit (SRα) on the ER membrane through an interaction between the GTP-binding domain of SRβ (Legate et al., 2000) and the amino-terminal domain of SRα (Young et al., 1995). No homologue of SRβ has been identified in the *E. coli* genome sequence. Although SRα and FtsY are homologous over two-thirds of their lengths, the amino-terminal domains are highly divergent (Bernstein et al., 1989; Romisch et al., 1989). Instead of the SRβ binding domain found in SRα, FtsY has a highly negatively charged A region at the amino-terminus (arbitrarily defined as amino acids 1-196) (Gill and Salmond, 1990) that, together with the central N region (amino acids 197-280), forms the minimum domain sufficient for assembly on the *E. coli* inner membrane (Chapter II). Mutations that abolish attachment of FtsY to the *E. coli* inner membrane interfere with protein targeting and cell viability (Luirink et al., 1994; Zelazny et al., 1997).

Both bilayer and non-bilayer phospholipids have been implicated in the assembly and activity of several components of the secretory pathway in *E. coli*, including SecA (Hendrick and Wickner, 1991; Lill et al., 1990) and the translocase (van der Does et al.,

2000). Recent investigations have demonstrated the presence of a region in the carboxyl-terminal region of FtsY that may be regulated by binding to anionic phospholipids (de Leeuw et al., 2000). It was also speculated that a second lipid-binding site exists in the amino-terminal region of FtsY (de Leeuw et al., 2000). Here we show that the previously defined membrane-binding domain of FtsY binds liposomes containing the zwitterionic phospholipid PE independent of a protein receptor. Surprisingly, in the absence of PE, SRP-dependent protein targeting remains functional, and the FtsY membrane assembly domain binds to *E. coli* inner membrane inverted vesicles (INVs) via an interaction with a trypsin-sensitive component. This suggests that, similar to SecA of the general secretory pathway (Hendrick and Wickner, 1991; Lill et al., 1990), membrane assembly of FtsY involves interactions with both a specific lipid and a membrane protein. Based on these results and previously published data, we propose that FtsY binding to the membrane occurs initially through phospholipid binding, followed by targeting to translocation sites via an interaction with a membrane protein.

3.3 Experimental Procedures

Strains, Plasmids, and Growth Conditions – Strains W3899, AD90, and AD93 (DeChavigny et al., 1991) were cultured in LB medium supplemented with 50 mM magnesium chloride. Plasmids pMAC988, pMAC1252, and pMAC1253, which encode full-length FtsY and the polypeptides FtsYA (amino acids 1-197 of FtsY) and FtsYAN (amino acids 1-284 of FtsY), respectively, have been described previously (Chapter II).

Plasmid pMAC141, encoding FtsYNG (amino acids 198-497 of FtsY), was created from plasmid pMAC1310 (Chapter II) using the technique described (Hughes and Andrews, 1996) to remove the portion of the construct encoding the fusion partner gPa. A derivative of plasmid pHDB3 containing the AcrB576-AP fusion cloned into the BamHI site (Ulbrandt et al., 1997) was first digested with SacI. To introduce a second HindIII site into the plasmid, the complementary oligonucleotides 5'-CATCGTAGAATCGGAAGCTTGCGTAGCT-3' and 5'-ACGCAAGCTTCCGATTCTACGATGAGCT-3' were then ligated to the linearized DNA. The resulting plasmid was digested with HindIII, and the fragment containing the AcrB576-AP fusion was ligated into the HindIII site of parc-FtsY(WT) or FtsY(G385A) (Ulbrandt et al., 1997) to make plasmids pJH10 and pJH11, respectively. A second HindIII site was introduced into plasmid pHP44 (Jander et al., 1996) by ligating the oligonucleotides listed above into the unique SacI site. The resulting plasmid was then digested with HindIII, and the fragment containing the AcrB576-PBST fusion was ligated into the HindIII site of parc-FtsY(WT) or FtsY(G385A) to make plasmids pJH12 and pJH13, respectively. In all of these plasmids, the *ftsY* and *acrB* genes are transcribed in opposite directions.

Membranes – INVs were isolated from *E. coli* strain W3899 or AD93 as described in Chapter II. To inactivate surface proteins on the INVs by proteolysis, INVs were adjusted to 10 mM calcium chloride (to stabilize the membranes) and treated with 50 μg/ml trypsin or 100 μg/ml proteinase K for 1 h at 24 °C. Following inhibition of

trypsin and other proteases with an inhibitor mixture (containing 20 μg/ml each chymostatin, antipain, leupeptin, and pepstatin and 40 μg/ml aprotinin) and 1 mM phenylmethylsulfonyl fluoride, vesicles were adjusted to 500 mM potassium acetate and collected by centrifugation over a 0.5 M sucrose cushion at 70,000 rpm (215,000 × g) in a Beckman TLA100.2 rotor. As an alternative means of inactivating surface proteins, separate aliquots of INVs were treated with 5 mM N-ethylmaleimide at 37 °C for 30 min at pH 7.5 and collected as described above. Phospholipid liposomes were generated using purified lipids (Avanti Polar Lipids, Inc.) in 50 mM Tris acetate (pH 7.5) by passing the suspension though a 0.1-μm filter 10 times using a mini-extruder (Avanti Polar Lipids, Inc.). Microsomes (CRMs) were isolated from canine pancreas as described (Young et al., 1995).

Cell-free Translations and Membrane Targeting – Plasmids were transcribed *in vitro* using SP6 polymerase, and then polypeptides were synthesized from unpurified transcription products and labeled with [^{35}S]methionine using a rabbit reticulocyte lysate translation system. Translation products (20 μl) were incubated for 45 min at 24 °C with 1 μl of canine pancreatic microsomes, inverted vesicles, or phospholipid liposomes. Where indicated, ethanolamine was added to a concentration of 200 mM and adjusted to pH 7.5 with acetic acid. To assess targeting to microsomes and inverted vesicles, the mixture was loaded onto a 0.8-ml column of Sepharose CL-2B equilibrated with 50 mM Tris acetate (pH 7.5) and 1 mM DTT in a 1-ml syringe. The column was eluted with the same buffer; fractions (two drops each) were collected; and 8-μl samples were analyzed

by SDS-PAGE. The included and excluded volumes of the Sepharose CL-2B columns were calibrated as described (Young et al., 1995).

Binding to liposomes was assayed by liposome flotation. The mixture was adjusted to a final sucrose concentration of 1.6 M, and 50 μl was overlaid with sucrose steps of 100 μl (1.25 M sucrose) and 50 μl (0.25 M sucrose) in 50 mM Tris acetate (pH 7.5) and 1 mM DTT. Following centrifugation in a Beckman TLA100 rotor at 100,000 rpm (435,000 × g) for 90 min, the gradient was divided into 40-μl fractions, and the pellet was solubilized in 40 μl of 10 mM Tris acetate (pH 7.5) and 1% SDS at 65 °C for 10 min. Equivalent aliquots of these fractions were separated by SDS-PAGE. After electrophoresis, radioactivity in the dried polyacrylamide gel was recorded using a PhosphorImager (Molecular Dynamics, Inc.).

Liposome Aggregation Assays – Myelin basic protein was a gift from Dr. George Harauz (MacMillan et al., 2000). FtsYAN-gPa and FtsYA-gPa are described in Chapter II and were purified on a 1-ml IgG-Sepharose column and eluted with 500 mM ammonium acetate (pH 3.4). Purified protein was dialyzed four times against a 1000-fold buffer excess consisting of 50 mM triethanolamine acetate and 50 mM NaCl (pH 7.5) and stored at a concentration of 1 mg/ml.

For light scattering measurements, at time 0, 10 μg of myelin basic protein, FtsYAN-gPa, FtsYA-gPa, or buffer alone was added to 1 ml of 0.01 mg/ml liposomes in a 1-cm path length cuvette using a remote sample injector and a cell equipped with a magnetic stirrer. Using a Photon Technology International Model C-44 fluorometer, light

of 550-nm wavelength was directed through the sample, and photons scattered at 90° to the sample were detected at 1-s intervals (collection time of 1 s/point) through a 1-nm slit using a photomultiplier and recorded using FeliXTM software (Photon Technology International). For graphical comparison, counts obtained using purified proteins at the indicated concentrations in buffer without lipids were subtracted from the values obtained in the presence of liposomes.

Conformational Change Assayed by Proteolytic Degradation – 40-µl fractions from flotation assays (described above) or equivalent amounts of reticulocyte lysate translations were incubated with trypsin at a final concentration of 0, 2, or 20 pg/µl for 30 min at 24 °C. Following this incubation period, the samples were immediately diluted in loading buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 0.01% bromphenol blue, 10% glycerol, and 0.5 M DTT), boiled for 5 min, and separated by SDS-PAGE. After electrophoresis, radioactivity in the dried polyacrylamide gel was recorded using the PhosphorImager.

Membrane Protein Insertion Assays -- To monitor membrane protein insertion, AD90 cells that were freshly cured of plasmid pDD72 as previously described (DeChavigny et al., 1991) and W3899 cells were both transformed with a plasmid from the pJH10-13 series. Cells containing plasmid pJH10 or pJH11 were grown overnight at 37 °C in MOPS minimal medium supplemented with all the amino acids except cysteine and methionine and 50 µg/ml ampicillin, washed, and added to fresh medium at A_{550} = 0.05. When the cultures reached A_{550} = 0.3, 2 mM isopropyl-D-thiogalactopyranoside

(IPTG) was added to cells containing pJH11. After an additional incubation of 40 min, cells were radiolabeled, converted to spheroplasts, and subjected to proteinase K digestion as described previously (Ulbrandt et al., 1997). AP-containing polypeptides were immunoprecipitated with a polyclonal antiserum (5 Prime 3 Prime, Inc.) and resolved by SDS-PAGE as previously described (Ulbrandt et al., 1997). Cells containing plasmid pJH12 or pJH13 were grown overnight at 37 °C in LB medium supplemented with 50 mM $MgCl_2$ and 100 μg/ml ampicillin, washed, and diluted. The cultures were divided in half when they reached $A_{550} = 0.1$, and 2 mM IPTG was added to one portion. Samples were removed from each culture after an additional 20-min incubation, and proteins were precipitated with cold 10% trichloroacetic acid. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and biotinylated AcrB-PBST fusion protein was detected as previously described (Jander et al., 1996).

3.4 Results

Membrane Binding of FtsY Does Not Require a Specific Protein Receptor – We have previously demonstrated, using a column chromatography-based assay, that the AN domain of FtsY (amino acids 1-284) specifically interacts with *E. coli* INVs, whereas the A region alone (amino acids 1-197) is unable to bind INVs (Chaper II). Similarly, deletion of as few as 19 amino acids from the amino-terminus abolishes binding (Chapter II). Thus, the AN domain is the minimum membrane-binding domain of FtsY.

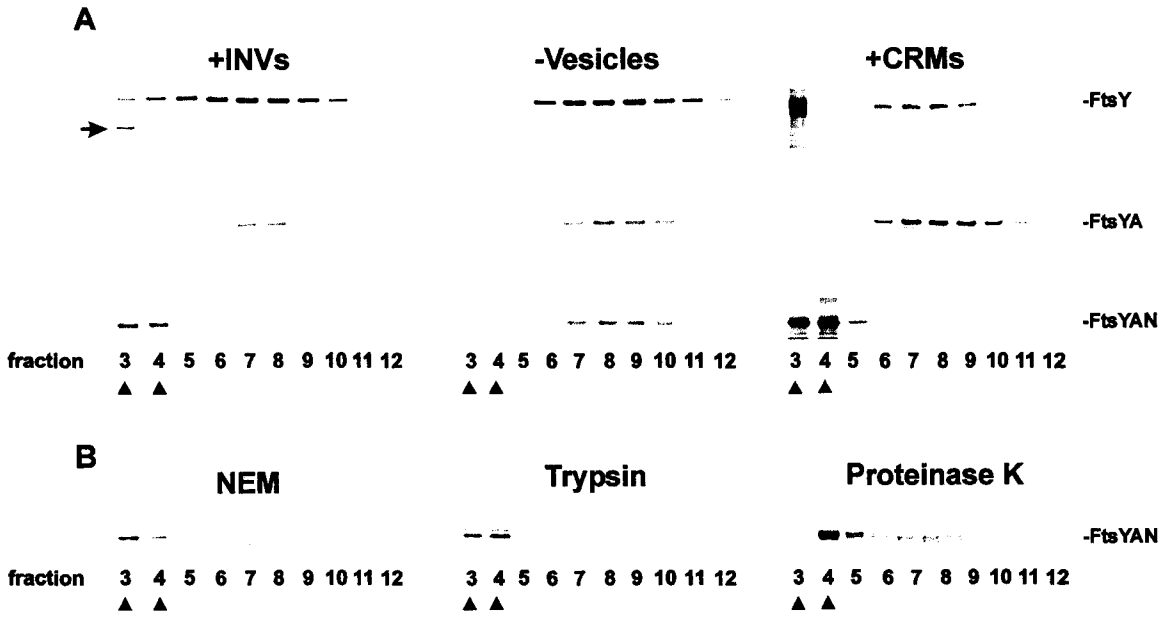
To further investigate membrane binding of FtsY, we compared binding of full-

length FtsY, FtsYAN, and, as a negative control, FtsYA synthesized in reticulocyte lysate to INVs and canine microsomes (Fig. 3.1). As expected, reticulocyte-translated FtsY and FtsYAN, but not FtsYA, bound INVs and therefore eluted in the excluded fractions when subjected to gel exclusion chromatography (Fig. 3.1A, fractions 3 and 4, arrowheads). As seen previously, a fraction of membrane-bound FtsY was cleaved between the N and G regions (Fig. 3.1A, arrow). Unexpectedly, full-length FtsY eluted in the excluded volume when incubated with canine microsomes (Fig. 3.1A, upper panel, +CRMs, fractions 3 and 4). Since the exclusion limit of this matrix is sufficient to retain cytosolic proteins and large aggregates of FtsY within the included volume, this indicates that FtsY can also bind to the eukaryotic endoplasmic reticulum membrane. As seen previously, a large fraction of the FtsY synthesized *in vitro* folded such that it would not bind to INVs, and although aggregated, it was recovered in the included fractions (Fig. 3.1A, upper panel, +INVs, fractions 5-12). Similarly, these molecules did not bind to microsomes (Fig. 3.1A, upper panel, +CRMs, fractions 5-12).

When FtsY bound microsomes, it was not cleaved (Fig. 3.1A, upper panel, +CRMs, fractions 3 and 4; the migration position of the cleavage product is indicated by the arrow). This confirms our prior suggestion that the proteolytic activity that cleaves FtsY resides on *E. coli* inner membranes (Chapter II).

Binding of FtsYA and FtsYAN to microsomes was assayed to determine if binding of FtsY requires the same minimum domain required for binding to the *E. coli* inner membrane. The A region of FtsY eluted entirely within the included volume

Figure 3.1. FtsY assembles on membranes from various sources. Reticulocyte lysate translation reactions of FtsY, FtsYA, and FtsYAN were incubated with INVs, CRMs, or buffer (Vesicles) (A) or with N-ethylmaleimide-treated INVs (NEM), trypsinized INVs (Trypsin), or proteinase K-treated INVs (Proteinase K) (B) as indicated. Membrane-bound molecules were separated from non-targeted molecules by chromatography on 0.8-ml Sepharose CL-2B columns equilibrated and eluted with buffer containing 50 mM Tris acetate (pH 7.5) and 1 mM DTT. Membranes eluted in the excluded volume (fractions 3 and 4, arrowheads), whereas non-targeted soluble cytosolic proteins and aggregates eluted as a broad peak in the included volume (fractions 5-12). The migration positions of expressed constructs are indicated on the right. The arrow on the left indicates the migration position of an FtsY cleavage product.


A
+INVs
-Vesicles
+CRMs
-FtsY
-FtsYA
-FtsYAN
fraction 3 4 5 6 7 8 9 10 11 12
B
NEM
Trypsin
Proteinase K
-FtsYAN
fraction 3 4 5 6 7 8 9 10 11 12

following Sepharose CL-2B chromatography (Fig. 3.1A, middle panel, +CRMs, fractions 5-12), demonstrating that it does not bind to microsomes. However, FtsYAN bound to microsomes as efficiently as it bound to INVs, confirming that the A and N regions together fold into a domain that binds to membranes (Fig. 3.1A, lower panels, compare fractions 3 and 4). The relatively high efficiency of binding of FtsYAN to vesicles *in vitro* compared with full-length FtsY is likely due to decreased aggregation when the G region is absent (Chapter II). Thus, the AN domain is sufficient for binding to either eukaryotic microsomes or *E. coli* inner membranes. We have previously demonstrated that the NG domain of FtsY does not bind INVs (Chapter II).

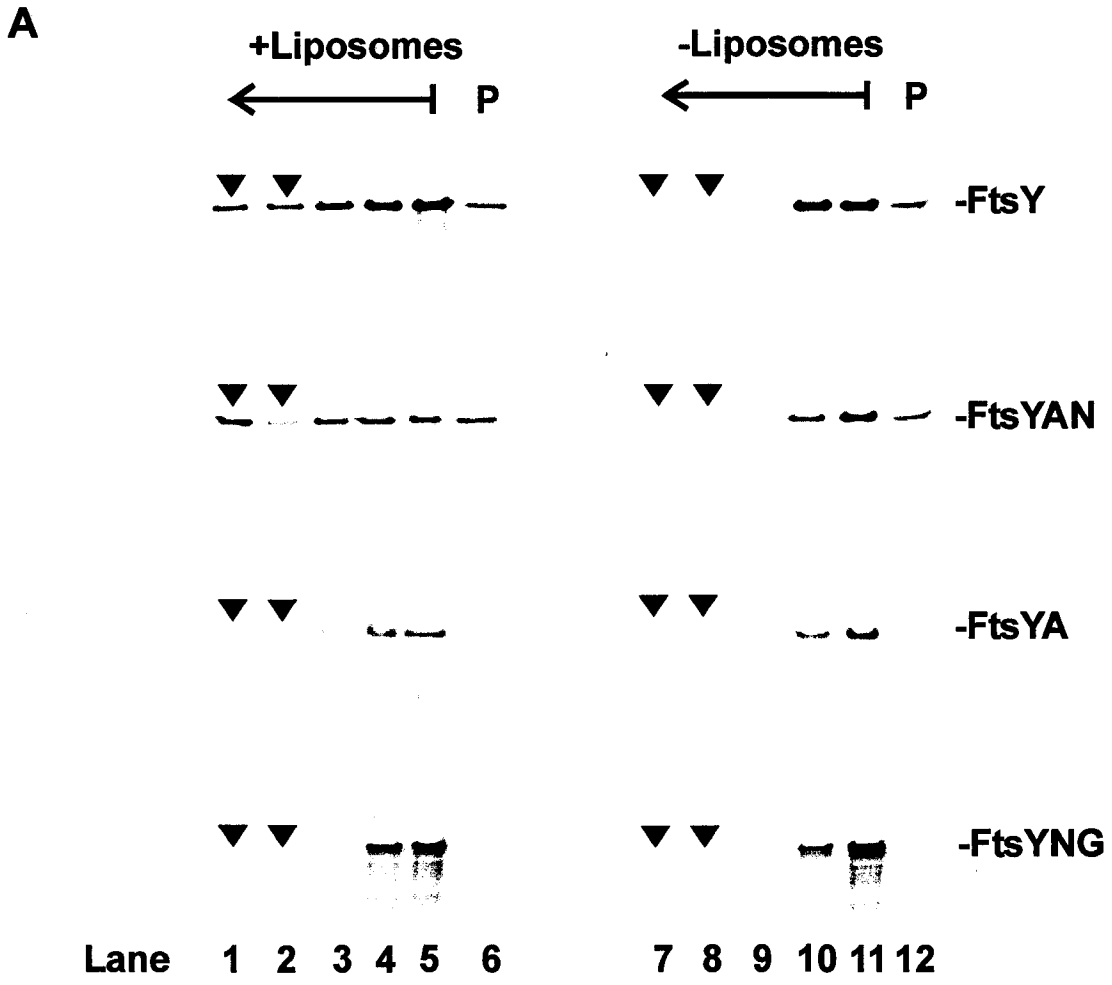
Binding of FtsY to eukaryotic microsomes via an interaction with a specific protein is unlikely, as there is no eukaryotic homolog for the FtsY membrane-binding domain. Therefore, it is possible that FtsY binding to INVs is also independent of a specific protein receptor. To determine whether a protein on the cytoplasmic face of the *E. coli* membrane is required for binding FtsY, proteins on the surface of the INVs were inactivated either by proteolysis or by alkylation of free sulfhydryl groups (cysteine) with N-ethylmaleimide (Fig. 3.1B). Alkylation of INVs with N-ethylmaleimide had no effect on binding of FtsYAN (Fig. 3.1B, fractions 3 and 4). Following incubation of FtsYAN with either trypsin- or proteinase K-treated vesicles, the vast majority of the protein continued to elute in the excluded volume following Sepharose CL-2B chromatography (Fig. 3.1B). Taken together, these results strongly suggest that FtsY binds to non-proteinaceous membrane component(s). Membrane phospholipids are therefore an

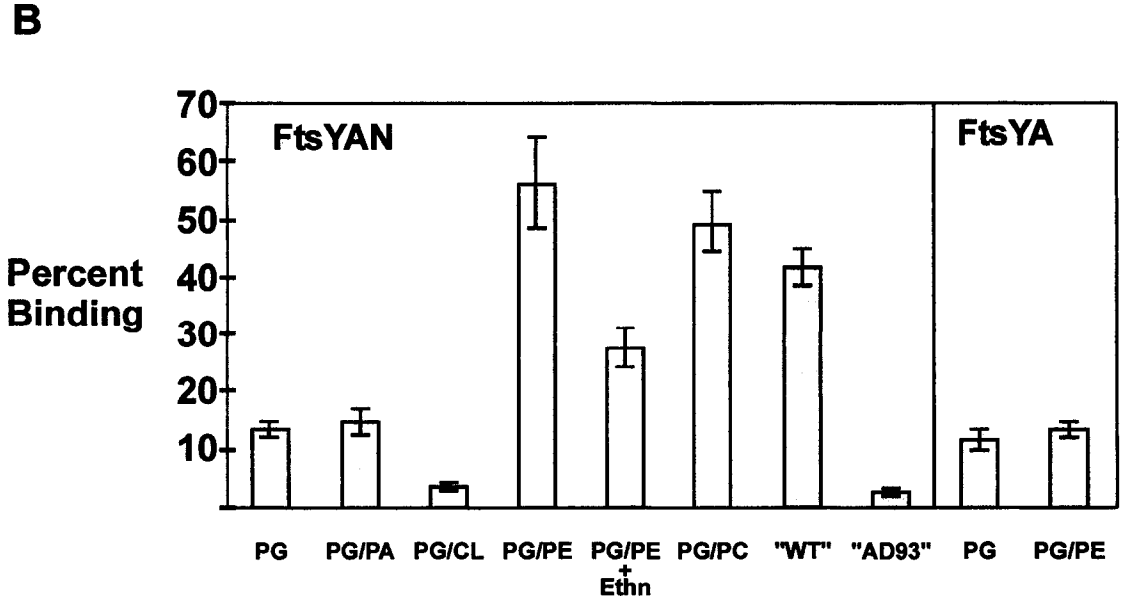
obvious potential binding site for FtsY.

The AN Domain of FtsY Binds to Phospholipid Liposomes via a Specific Interaction with PE – To investigate the possibility that FtsY binds to *E. coli* membranes through an interaction with phospholipids, a liposome flotation assay was employed (Fig. 3.2). The gradients in Fig. 3.2 were fractionated such that the floated vesicles were recovered in the 0.25 M sucrose step (lanes 1, 2, 7, and 8, arrowheads). In the absence of liposomes, full-length FtsY was found in dense fractions (Fig. 3.2A, upper panel, lanes 10-12). When incubated with liposomes, some of the full-length FtsY migrated into the 0.25 M sucrose step (Fig. 3.2A, compare lanes 1 and 2 with lanes 7 and 8), confirming that it bound to liposomes. Similarly, FtsYAN also fractionated at the 0.25 M sucrose step only when incubated with liposomes (Fig. 3.2A, compare lanes 1 and 2 with lanes 7 and 8). Compared with these molecules, only a small fraction of FtsYA migrated at the 0.25 M sucrose step in the presence of liposomes (Fig. 3.2A). Furthermore, under these conditions, the NG domain failed to migrate into the 0.25 M sucrose step in the presence of liposomes (Fig. 3.2A). Thus, the AN domain specifically mediates binding of FtsY to membranes via an interaction with one or more species of *E. coli* phospholipid. FtsY is not cleaved in this assay, even after binding to liposomes (Fig. 3.2A, upper panel), confirming that the protease responsible for specific cleavage of FtsY (Fig. 3.1) is not present in the reticulocyte lysate.

Using the flotation assay described above, lipid binding of the AN domain of FtsY was examined for lipids with different head groups (Fig. 3.2B). The inner membrane of

Figure 3.2. The FtsY membrane assembly domain binds phospholipid liposomes containing PE. A, FtsY, FtsYAN, FtsYA, and FtsYNG were synthesized in reticulocyte lysate and incubated with liposomes prepared from lipids extracted from *E. coli* using chloroform (lanes 1-6) or buffer (lanes 7-12). Reactions were adjusted to 1.6 M sucrose and overlaid with steps of 1.25 and 0.25 M sucrose in 50 mM Tris acetate (pH 7.5) and 1 mM DTT. Following centrifugation, 40-µl fractions were taken from the top of the gradient (decreasing density is indicated by the arrows). Pelleted material (P) was resuspended from the bottom of the gradient (lanes 6 and 12). Liposomes and associated proteins fractionated in the 0.25 M sucrose step (arrowheads). The migration positions of expressed constructs are indicated on the right. B, FtsYA and FtsYAN were synthesized in reticulocyte lysate and incubated with phospholipid liposomes consisting of PG, PA, CL, PE, and PC. When vesicles were made from two lipids, they were present in equimolar amounts. Vesicles with a composition similar to that of wild-type *E. coli* strain W3899 ("WT") contained 76% PE, 14% PG, and 10% CL. Vesicles similar to *E. coli* strain AD93 ("AD93") contained 50% PG, 46% CL, and 4% PA. In one reaction, the targeting buffer contained 200 mM ethanolamine (Ethn). Membrane binding was assayed as described in A, and radioactive counts corresponding to FtsYAN or FtsYA in each fraction were quantified using a PhosphorImager. Percent binding was determined to be the (radioactive counts in the top three fractions divided by the total radioactivity) × 100. Error bars indicate 1 S.D. from data from three independent experiments.


A
+Liposomes
P
-Liposomes
P
-FtsY
-FtsYAN
-FtsYA
-FtsYNG
Lane 1 2 3 4 5 6 7 8 9 10 11 12


B
Percent Binding
70
60
50
40
30
20
10
FtsYAN
FtsYA
PG
PG/PA
PG/CL
PG/PE
PG/PE + Ethn
PG/PC
"WT"
"AD93"
PG
PG/PE

E. coli contains four predominant phospholipids: PE, phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidic acid (PA). Although the ratios of these species vary between strains of *E. coli*, a typical wild-type strain (W3899) has a composition of 76% PE, 14% PG, 10% CL, and trace amounts of PA (Rietveld et al., 1993). Therefore, we investigated the binding of FtsYAN to liposomes prepared from different combinations of these lipids.

Since, in some instances, it was not possible to generate liposomes using a single lipid, we used PG as a "background" lipid in which to assess binding. PG was used for this purpose because, unlike PE, it was possible to generate liposomes from pure PG as well as other lipids in combination with PG. In each case, the liposomes were made from equal amounts of PG and the lipid being tested.

In flotation assays, ~12% of FtsYAN bound to liposomes generated from pure PG (Fig. 3.2B). This did not differ significantly from the amount of FtsYA (used as a non-membrane binding control) that bound to pure PG liposomes (Fig. 3.2B). A similar amount of FtsYA bound to liposomes composed of both PE and PG, whereas >50% of FtsYAN bound to liposomes containing PE (Fig. 3.2B). FtsYAN failed to bind above base-line levels to liposomes containing mixtures of either PA or CL with PG (Fig. 3.2B). Thus, it is likely that *in vivo* membrane assembly of FtsY involves binding to PE.

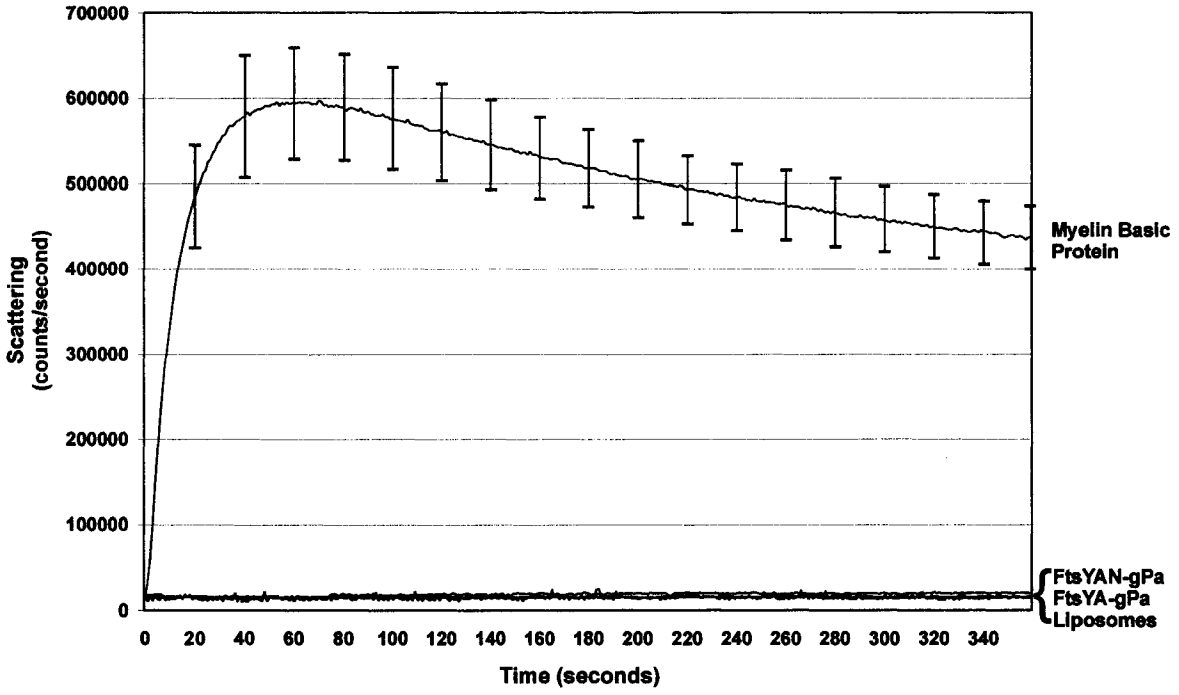
To determine if FtsY binding to liposomes containing PE depends on a direct interaction with the ethanolamine head group, we performed competition experiments by adding ethanolamine to the binding reaction. We found that, in flotation assays,

increasing concentrations of ethanolamine reduced the amount of FtsY that bound to liposomes. Ethanolamine at a concentration of 200 mM reduced FtsYAN binding to liposomes generated from either *E. coli* extracted lipids or PE/PG by 50% (Fig. 3.2B). Concentrations of ethanolamine >200 mM disrupted liposomes and thus could not be assessed for effects on binding. We conclude therefore that FtsY binds to membranes in part by an interaction with PE. However, PE is not a major component of canine pancreatic microsomes, and FtsY binds efficiently to these microsomes, suggesting that the head group specificity is not absolute. The head group of phosphatidylcholine (PC) differs from PE only in that it contains a tertiary amine, suggesting that FtsY may bind to microsomes via this lipid. In flotation assays, FtsY bound to liposomes containing PC as efficiently as to liposomes containing PE (Fig. 3.2B).

To ensure that binding of FtsYAN to membranes did not simply reflect liposome aggregation, light scattering measurements were taken for liposomes composed of 50% PE and 50% PG incubated with an equimolar amount of FtsYAN-gPa, FtsYA-gPa, or myelin basic protein (Fig. 3.3). FtsYAN-gPa has been shown to bind INVs (Chapter II) and liposomes (see Fig. 3.4). Fusion to gPa permits purification of the fusion proteins following expression in *E. coli* by virtue of the affinity of gPa for immunoglobulins.

As has been described previously (MacMillan et al., 2000), addition of myelin basic protein to liposomes leads to an immediate and extensive increase in light scattered due to aggregation of liposomes. The scattering observed upon addition of myelin basic protein plateaued and then decreased slowly due to the settling of aggregated liposomes

Figure 3.3. The membrane-binding domain of FtsY fails to elicit liposome aggregation. At time 0, myelin basic protein, FtsYAN-gPa, FtsYA-gPa, or buffer alone was injected into a cuvette containing liposomes composed of 50% PE and 50% PG. Light of 550-nm wavelength scattered at 90° to the sample was detected. and counts were corrected using the purified proteins in buffer. Error bars are indicated at every twentieth data point and represent 1 S.D. from data from four independent experiments for myelin basic protein. Error bars cannot be seen for other plots, but are <10% of the measured value for each data point.

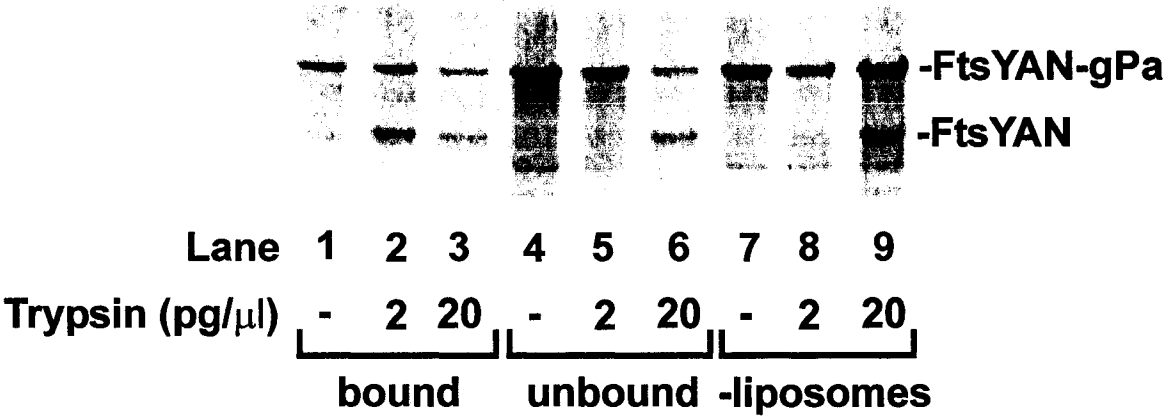

700000
600000
500000
400000
300000
200000
100000
0
Scattering (counts/second)
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
Time (seconds)
Myelin Basic Protein
FtsYAN-gPa
FtsYA-gPa
Liposomes

from solution (Fig. 3.3). Upon addition of either FtsYAN-gPa or the non-binding control FtsYA-gPa to identical liposomes, no difference in light scattering was observed compared with the liposomes alone (Fig. 3.3). This suggests that binding of FtsYAN to membranes cannot be accounted for by induced liposome aggregation.

FtsY Undergoes a Conformational Change upon Binding Liposomes – We have previously demonstrated that FtsY and FtsYAN-gPa are cleaved carboxyl-terminal to the N region upon membrane binding (Chapter II). Although the identity of the protease(s) responsible for this cleavage *in vivo* is unknown, we found that this site was sensitive to cleavage by trypsin at a concentration of 20 pg/μl even in the absence of membranes (Fig. 3.4, lane 9). However, when FtsYAN-gPa was bound to liposomes and isolated by flotation, the protein that cofractionated with liposomes in the top fraction of the sucrose gradient was cleaved carboxyl-terminal to the N region by trypsin at a concentration of 2 pg/μl (Fig. 3.4, lane 2). FtsYAN-gPa that was not associated with liposomes and hence eluted in the denser fractions still required a concentration of 20 pg/μl for cleavage (Fig. 3.4, lanes 5 and 6). Thus, it is apparent that a conformational change that makes the protease-sensitive site more accessible occurs when FtsYAN-gPa binds liposomes.

PE Depletion Does Not Block Assembly of Inner Membrane Proteins – The *E. coli* strains AD90 and AD93, which are unable to synthesize PE (DeChavigny et al., 1991), were used to further investigate the role of PE in membrane binding of FtsY. These strains can be grown in the presence of divalent cations, resulting in *E. coli* with an inner membrane composed of 46% PG, 50% CL, and 4% PA (Rietveld et al., 1993).

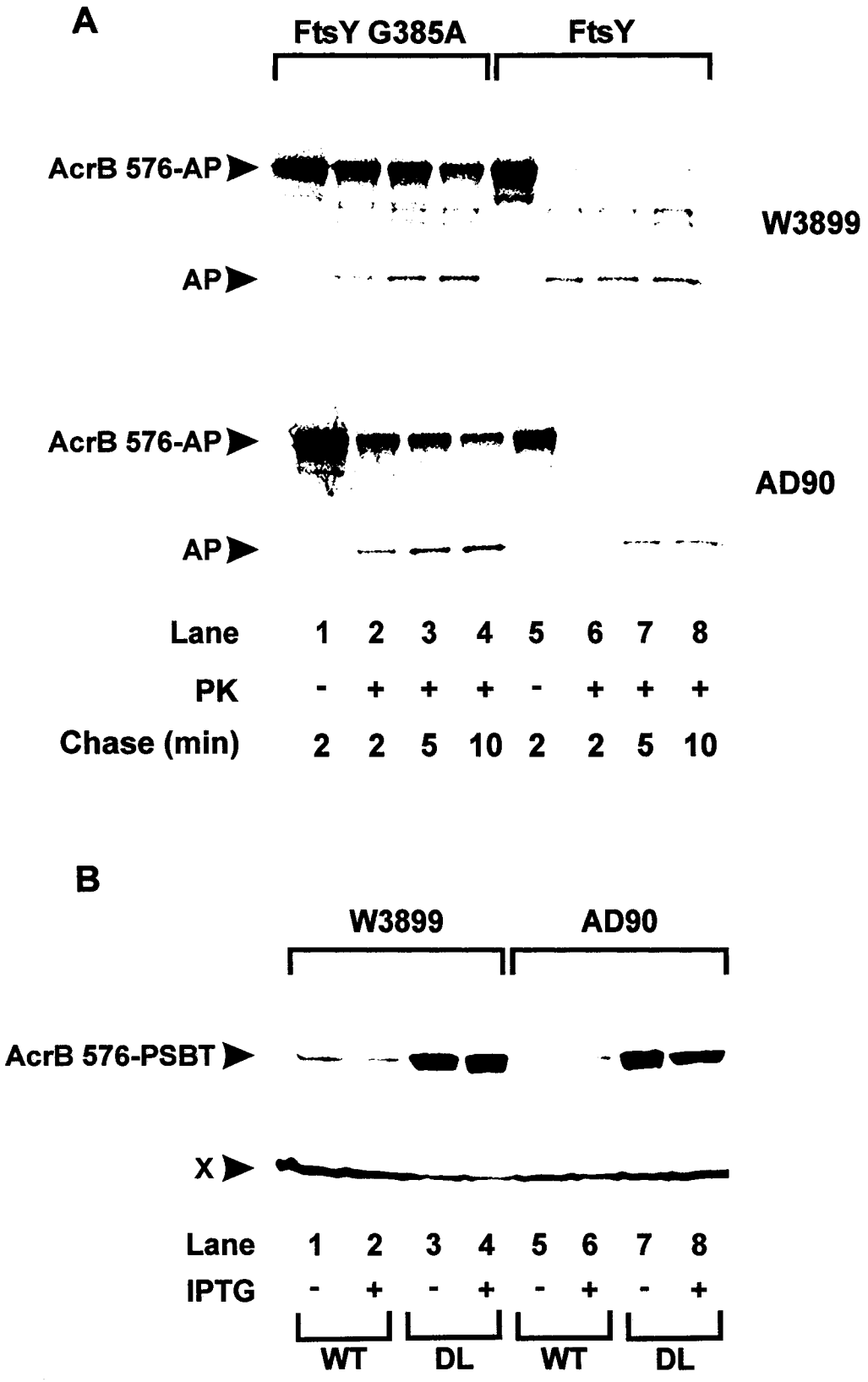
Figure 3.4. FtsY undergoes a conformational change upon binding liposomes. FtsYAN-gPa was synthesized in reticulocyte lysate and incubated with liposomes prepared from lipids extracted from *E. coli* (lanes 1-6). Flotation assays were performed on incubations containing liposomes as described in the legend to Figure 3.2. Following centrifugation, fractions from the top one-third (lanes 1-3, bound) or the bottom one-third of the sucrose gradient (lanes 4-6, unbound) and samples not incubated with liposomes (lanes 7-9, liposomes) were treated with trypsin at a final concentration of 0, 2, or 20 pg/μl. The migration positions of FtsYAN-gPa and the trypsin digest product FtsYAN are indicated on the right.


-FtsYAN-gPa
-FtsYAN
Lane 1 2 3 4 5 6 7 8 9
Trypsin (pg/μl) - 2 20 - 2 20 - 2 20
bound
unbound
-liposomes

Binding of FtsYAN to phospholipid vesicles containing 46% PG, 50% CL, and 4% PA was no higher than that observed in the absence of membranes (Fig. 3.2B, "AD93"). Addition of magnesium ions at a concentration of 50 mM (similar to the concentration of Mg^{2+} in the growth medium used for PE-depleted strains) had no effect on binding of FtsY (data not shown). Thus, in AD90 or AD93 cells grown in Mg^{2+}, FtsY cannot bind to the inner membrane via an interaction with phospholipids. We therefore presumed that, in these strains, the assembly of inner membrane proteins that are targeted by SRP would be reduced or eliminated completely. One such protein is the multi-spanning transmembrane protein AcrB (Ulbrandt et al., 1997). To analyze the insertion of AcrB into the cytoplasmic membrane, we utilized an AP fusion to the amino-terminal 576 amino acids of AcrB (AcrB576-AP) that requires the SRP pathway for membrane insertion (Ulbrandt et al., 1997). Proper insertion of the fusion protein and transport of the AP domain into the cytoplasmic membrane were assessed by a protease protection assay (Traxler et al., 1992). When such fusion proteins were properly inserted into the inner membrane, the AP domain folded into a protease-resistant conformation that was cleaved from the remaining protein by the protease and that could be immunoprecipitated and observed by SDS-PAGE (Fig. 3.5A, AP).

As expected, in the WT strain expressing only wild-type FtsY, only bands corresponding to AP alone were immunoprecipitated following protease treatment of spheroplasts (Fig. 3.5A, upper panel, lanes 6-8). This indicates that insertion of the fusion protein into the cytoplasmic membrane in wild-type cells was essentially complete.

Figure 3.5. An inner membrane protein requiring the signal recognition particle is integrated in the cytoplasmic membrane of PE-deficient *E. coli*. *A*, wild-type W3899 cells (upper panel) and PE-deficient AD90 cells (lower panel) expressing AcrB576-AP as well as either FtsY (lanes 5-8) or dominant lethal FtsY(G385A) (lanes 1-4) were induced with 2 mM isopropyl--D-thiogalactopyranoside and then, after 40 min, labeled with radioactive cysteine and methionine and subjected to a chase of 2, 5, or 10 min as indicated. Insertion of AcrB576-AP was examined by treating spheroplasts with proteinase K (PK; lanes 2-4 and 6-8) or buffer alone (lanes 1 and 5) and immunoprecipitation with antibody to PhoA. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. The positions of full-length AcrB576-AP and cleaved AP are indicated. *B*, wild-type W3899 cells (lanes 1-4) and PE-deficient AD90 cells (lanes 5-8) expressing AcrB576-PBST as well as either FtsY (WT) or dominant lethal FtsY (DL) were grown at 37 °C in LB medium + 50 mM MgCl2 and induced with isopropyl--D-thiogalactopyranoside (IPTG) where indicated. Following precipitation of proteins with trichloroacetic acid, samples were subjected to Western blotting and probed with horseradish peroxidase-conjugated streptavidin. The positions of AcrB576-PBST streptavidin-biotin complexes and a background band of unknown identity (X) are indicated.


A
FtsY G385A
FtsY
AcrB 576-AP
W3899
AP
AcrB 576-AP
AD90
AP
Lane 1 2 3 4 5 6 7 8
PK - + + + - + + +
Chase (min) 2 2 5 10 2 2 5 10
B
W3899
AD90
AcrB 576-PSBT
X
Lane 1 2 3 4 5 6 7 8
IPTG - + - + - + - +
WT
DL
WT
DL

Consistent with previous results (Ulbrandt et al., 1997), overexpression of a dominant lethal allele of FtsY blocked membrane protein insertion, as demonstrated by the significant fraction of pulse-labeled AcrB576-AP that was protected from protease treatment and migrated as the full-length product (Fig. 3.5A, compare upper and lower panels, lanes 2-4). Surprisingly, the insertion of the fusion protein into the cytoplasmic membrane in cells lacking PE was apparently unaltered compared with WT cells since essentially all of the AP was also accessible to the protease in this strain (Fig. 3.5A, lower panel, lanes 6-8). Under these conditions, it is clear that although FtsY could not bind to the phospholipid component of the membrane, targeting of an SRP pathway-dependent substrate was not impaired.

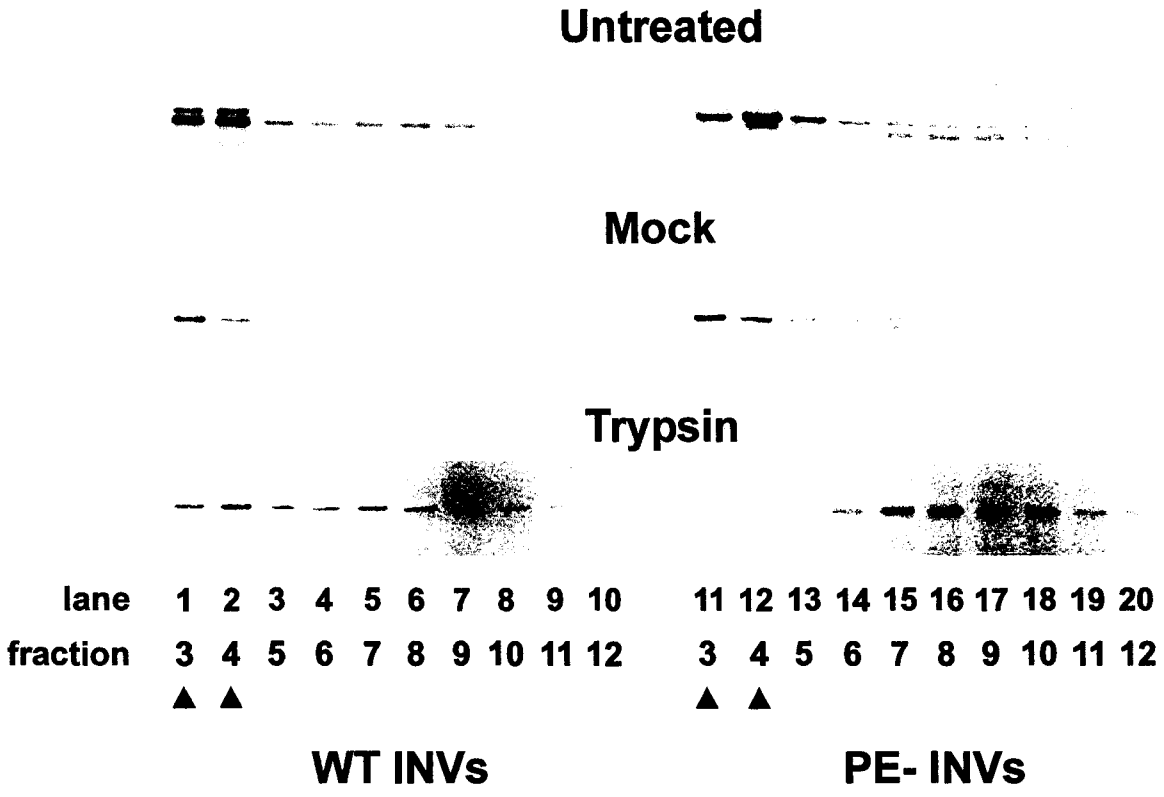
A defect in assembly of inner membrane proteins appears to be less prominent in cells growing in minimal medium (Ulbrandt et al., 1997). To address this concern, an alternate assay was used that employs conditions under which cell growth is more rapid (Jander et al., 1996). The 576-amino acid amino-terminal fragment of AcrB was expressed as a fusion to PBST. If the fusion fails to integrate into the cytoplasmic membrane, the 76-amino acid PBST is recognized and biotinylated by cytoplasmic *E. coli* biotin ligase. The biotinylated protein was detected with horseradish peroxidase-conjugated streptavidin on a Western blot (Fig. 3.5B). Expression of this protein in wild-type cells together with dominant lethal FtsY led to reduced integration of the fusion protein into the cytoplasmic membrane, as indicated by the recognition of biotinylated AcrB576-PBST with horseradish peroxidase-conjugated streptavidin (Fig. 3.5B, lanes 3

and 4). When wild-type FtsY was expressed, essentially no fusion product was recognized by horseradish peroxidase-conjugated streptavidin (Fig. 3.5B, lanes 1 and 2), showing that the fusion protein integrated appropriately into the cytoplasmic membrane. In cells deficient in PE, the protein integrated into cytoplasmic membranes of cells expressing wild-type FtsY (Fig. 3.5B, lanes 5 and 6), but not dominant lethal FtsY (lanes 7 and 8). Similar to the results with the AP fusion, depletion of PE failed to elicit a decrease in AcrB membrane assembly. It is therefore apparent that, despite the observation that the membrane assembly domain of FtsY binds PE, the presence of this phospholipid in the cytoplasmic membrane is not essential for the activity of the SRP pathway.

FtsY Membrane Assembly Involves Both PE and a Membrane-associated Protein – The absence of an apparent defect in assembly of SRP-dependent inner membrane proteins in strains devoid of PE suggested that FtsY might continue to bind to the cytoplasmic membrane in these cells. This hypothesis is supported by immunoblots of cytosol and INVs prepared from WT and AD93 cells that revealed an identical distribution of FtsY between the cytosol and membrane in both strains (data not shown). Therefore, in the presence of 50 mM Mg^{2+}, FtsY assembles on the inner membrane of AD93 cells that do not contain any PE.

Binding of FtsY to *E. coli* inner membranes lacking PE was confirmed *in vitro* using a column chromatography assay. FtsYAN binding to vesicles prepared from AD93 cells was the same as that to vesicles prepared from wild-type *E. coli* (Fig. 3.6, upper

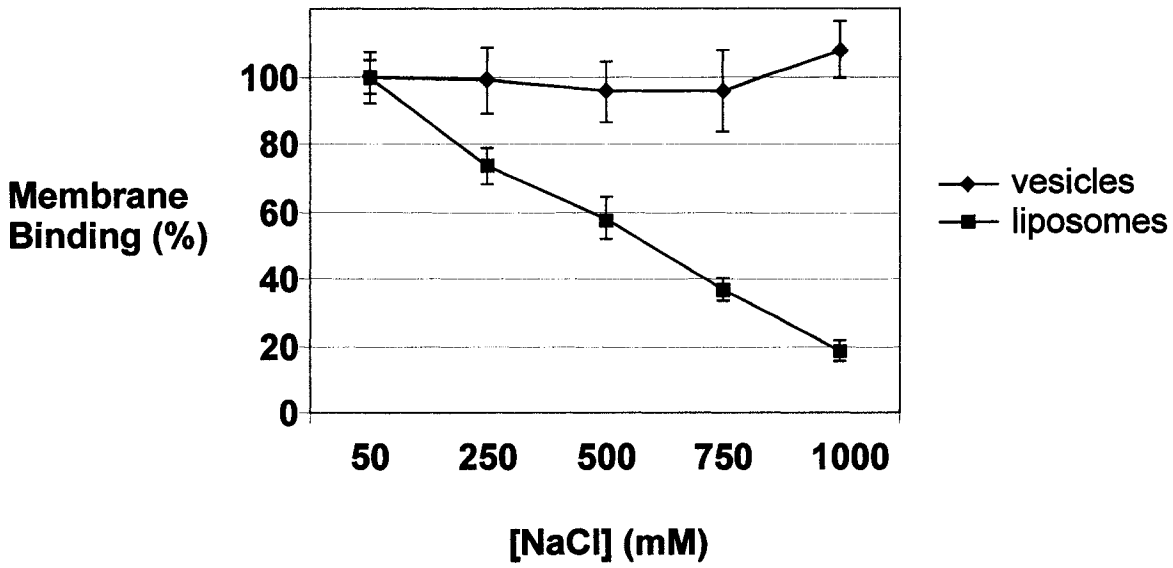
Figure 3.6. FtsY membrane assembly involves both PE and a protein moiety. Reticulocyte lysate translation reactions of FtsYAN were incubated with INVs generated from wild-type *E. coli* strain W3899 (lanes 1-10, WT INVs) or *E. coli* strain AD93 (lanes 11-20, PE-INVs), which were untreated (upper panels), treated with trypsin (lower panels), or mock-treated with buffer (middle panels). Membrane binding was assayed as described in the legend to Figure 3.1. INVs and membrane-bound proteins eluted in the excluded volume (fractions 3 and 4, arrowheads). Cytosolic proteins eluted as a broad peak in the included volume (fractions 5-11).


Untreated
Mock
Trypsin
lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
fraction 3 4 5 6 7 8 9 10 11 12 3 4 5 6 7 8 9 10 11 12
WT INVs
PE- INVs

panels, compare lanes 1 and 2 with lanes 11 and 12). Since the presence of divalent cations restores proper protein translocation across PE-depleted vesicles (Rietveld et al., 1995), we considered that magnesium or other cations present in *in vitro* translations may participate in FtsY binding to PE-depleted INVs. Therefore, in a separate control experiment, translation reactions were depleted of small molecules by passage over a Sephadex G-25 gel exclusion column, and the INVs were prepared in a buffer that did not contain Mg^{2+}. FtsYAN continued to bind INVs in the absence of Mg^{2+} (data not shown), suggesting that some component of INVs in addition to PE binds FtsY.

To examine the potential involvement of an *E. coli* inner membrane protein in binding FtsY to membranes, we assayed binding of FtsYAN to WT and AD93 vesicles treated with trypsin. FtsYAN bound to control (mock-treated) AD93 and WT vesicles (Fig. 3.6, middle panels, compare lanes 1 and 2 with lanes 11 and 12) and to trypsinized wild-type vesicles that contained PE (lower panels, lanes 1 and 2). However, when incubated with trypsin-treated vesicles prepared from AD93 cells, FtsYAN was essentially absent from the excluded fractions (Fig. 3.6, compare middle and lower panels, lanes 11 and 12). Since both PE depletion and protease degradation of INV proteins are required to eliminate binding to INVs, either lipid or a membrane-protein can bind FtsY to membranes. Other explanations such as loss of FtsY binding after trypsin treatment due to a proteolysis-induced physical change (e.g. curvature) in the membranes are unlikely since binding was lost only with INVs that did not contain PE.

Figure 3.7. FtsY binding to liposomes (but not INVs) is reduced as ionic strength increases. Reticulocyte lysate translation reactions of FtsYAN were incubated with liposomes prepared from lipids extracted from *E. coli* using chloroform (liposomes) or INVs from *E. coli* (vesicles) and then adjusted to NaCl concentrations of 100 mM to 1 M as indicated and isolated from the reaction by flotation. Proteins in the gradient fractions were separated by SDS-PAGE, and radioactive counts corresponding to FtsYAN in each fraction were quantified using a PhosphorImager. Percent binding was determined as (radioactive counts in the top two fractions divided by the total radioactivity) × 100. Error bars indicate 1 S.D. calculated from data from three independent experiments.


Membrane Binding (%)
100
80
60
40
20
0
50
250
500
750
1000
[NaCl] (mM)
vesicles
liposomes

The Avidity of FtsY Binding to Biological Membranes Is Greater than That to Artificial Liposomes – Binding of FtsY to both PE and a membrane protein suggests that FtsY binds to INVs in a manner qualitatively different than the manner in which it binds to liposomes. To test this, the avidity of FtsYAN for liposomes and INVs was compared by varying the ionic strength of the incubation buffer and then assaying membrane binding by flotation as described above. Binding of FtsYAN to INVs was unaffected by up to 1 M NaCl (Fig. 3.7, 'vesicles'). In contrast, binding to liposomes decreased with increasing salt concentration (Fig. 3.7, 'liposomes'). The amount of FtsYAN bound to liposomes in 1 M NaCl was 20% of that bound in 0.1 M salt (Fig. 3.7). Thus, at higher salt concentrations, FtsY binds to INVs much better than to liposomes. This difference in binding is likely due to the trypsin-sensitive FtsY-binding protein on INVs.

3.5 Discussion

The Membrane-binding Domain of FtsY Binds to Phospholipids – The ability of the membrane assembly domain of FtsY to bind proteolyzed or alkylated membranes provided the first clear evidence that membrane binding may involve a non-proteinaceous component. The concentration of trypsin used to treat these vesicles was 100 times greater than that previously found to be sufficient to prevent binding of SRα to canine microsomes (Andrews et al., 1989). This clearly shows that SRα and FtsY bind microsomes in a qualitatively different manner, the latter most likely through an interaction with a non-protein component of the membrane.

Our finding that FtsY and FtsYAN bind vesicles made from purified *E. coli* lipids confirmed that FtsY can bind membranes through an interaction with phospholipids. Concurrent with these experiments, the ability of FtsY to bind liposomes was reported independently (de Leeuw et al., 2000). Surprisingly, those authors detected binding of the NG domain of FtsY to liposomes. We found previously that removal of even the amino-terminal 19 amino acids from FtsY completely abolishes binding to INVs (Chapter II), and we detected only very inefficient binding of the NG domain to either INVs or liposomes under the relatively physiological conditions used here (Chapter II) (Fig. 3.2A). FtsY missing the amino-terminal 47 amino acids is also nonfunctional in translocation assays and does not bind microsomes (Powers and Walter, 1997). Thus, it seems likely that the AN domain is the major lipid-binding domain of FtsY.

Unlike the binding of FtsY to INVs (Chapter II), full-length FtsY was not cleaved between the AN and G domains when it bound to microsomes or liposomes (Fig. 3.2). This result, together with the demonstration that cleavage does not depend on the translation extract (Chapter II and Fig. 3.2), extends our previous experiments by demonstrating unequivocally that the protease that cleaves FtsY resides on the *E. coli* inner membrane. The cleavage site between the AN and G domains does become more sensitive to proteolytic cleavage upon binding liposomes (Fig. 3.4), which suggests that FtsY undergoes a conformational change upon binding to lipids. Although a role for this conformational change is unclear, it suggests that FtsY forms specific interactions with liposomes. Furthermore, since the N and G regions of FtsY are quite closely packed

together in protein crystals (Montoya et al., 1997), a conformational change upon membrane binding that increases the accessibility of the region between these domains lends greater credence to the observation that the A and N regions together are involved in membrane binding.

FtsY Binds Membranes through a Specific Interaction with Phosphatidylethanolamine – Of all the major *E. coli* lipids, FtsY binds only to liposomes containing PE (Fig. 3.2B). It also binds the closely related lipid PC (Fig. 3.2B). Since the A region of FtsY is highly negatively charged and the liposomes to which it binds have the greatest positively charged character of all those investigated, it was of some concern that the binding observed may reflect a simple ion-induced aggregation of liposomes, as has been previously observed (Duzgunes et al., 1981). This was ruled out by examining liposome aggregation by light scattering following addition of purified protein to liposomes (Fig. 3.3). Since addition of FtsYAN-gPa to liposomes resulted in no appreciable change in light scattering (Fig. 3.3), we conclude that the interaction of FtsYAN with liposomes does not result merely from aggregation of the liposomes.

It has previously been observed that full-length FtsY does cause liposome aggregation, leading to the suggestion that there were likely two different lipid-binding sites in FtsY (de Leeuw et al., 2000). Although two lipid-binding sites in FtsY remains a possibility, our deletion mutagenesis study suggests that only a single domain (AN) binds specifically enough to be physiologically relevant for membrane targeting and assembly. The two sets of results can be reconciled by the observation that FtsY aggregates in

solution. Any multimerization (including aggregation) of a protein containing a single lipid-binding domain would result in a protein expected to behave like a multivalent lipid-binding protein. Although we have not assayed oligomerization of FtsY rigorously, gel filtration chromatography revealed that most of FtsY synthesized *in vitro* forms aggregates (Chapter II). Consistent with multimerization, when FtsY purified from *E. coli* was analyzed by gel filtration chromatography, it eluted as a single peak with an apparent molecular mass of >200 kDa (Luirink et al., 1994).

It is also apparent that, although the AN domain is highly negatively charged (net charge of 48) (Gill and Salmond, 1990), binding to PE cannot be accounted for by a simple electrostatic interaction. The A region alone accounts for a net charge of 47, but does not bind PE/PG liposomes above background (Fig. 3.2). Thus, the inclusion of PE in liposomes does not enhance FtsYAN binding solely by reducing the electrostatic repulsion between the negatively charged AN domain and negatively charged phospholipid head groups. Rather, these data, together with our results demonstrating that ethanolamine competes for FtsY binding to PE-containing liposomes, suggest that the A and N regions fold into a domain containing a specific binding pocket that allows FtsY to bind PE.

Unlike the lipid interaction observed with the NG domain, which was speculated to involve both an electrostatic interaction and the insertion of the domain into the lipid bilayer (de Leeuw et al., 2000), we propose here that the interaction of the AN domain with the membrane is a true peripheral interaction involving a specific interaction with

the head group of PE (or PC) and a membrane protein. Our conclusion that binding is peripheral to the lipid core of the bilayer is consistent with our observation that FtsY is displaced from liposomes by 500 mM NaCl (Fig. 3.7), a salt concentration that is unlikely to extract a protein domain from a lipid bilayer.

FtsY Also Binds a Protein on the Cytoplasmic Membrane – Similar to depletion or disruption of FtsY function (Ulbrandt et al., 1997; Luirink et al., 1994), depletion of PE from wild-type *E. coli* leads to both cell division and protein translocation deficiencies (Ohta and Shibuya, 1977; Rietveld et al., 1995). The continued targeting of an SRP-dependent protein to the membrane in cells depleted of PE (Fig. 3.5) and our identification of a trypsin-sensitive binding site on INVs generated from these cells suggest that the defects seen in PE-depleted cells are not entirely due to the effect on the targeting of FtsY. However, it remains likely that the efficiency of FtsY-mediated targeting drops, contributing to the phenotype that eventually develops.

It is clear that the interaction of FtsY with PE is different from the interaction with the membrane protein. FtsY bound to the cytoplasmic membrane is not extracted with NaCl concentrations up to 1 M, whereas when bound to PE-containing liposomes, FtsY binding decreases with increasing NaCl concentrations (Fig. 3.7). This quantitative difference in binding shows that FtsY binds to the protein component of the membrane more tightly than to PE. Taken together with the abundance of PE in wild-type *E. coli* membranes (70%), it seems likely that FtsY would bind to *E. coli* membranes initially by an interaction with PE, and it may then be transferred to empty translocation sites via the

tighter interaction with a membrane-bound receptor. A precedent for this type of membrane binding exists with SecA, which requires an interaction with both acidic phospholipids and the SecY-SecE complex for high affinity binding to the *E. coli* inner membrane (Hendrick and Wickner, 1991).

The similarity to SecA suggests a strategic advantage of "dual specificity" binding. It is possible that membrane assembly occurs by a two-step process in which the first step (lipid binding) serves to reduce a three-dimensional problem to two dimensions. Initial binding of FtsY to the lipid membrane would be greatly facilitated by an interaction with PE, which constitutes >70% of membrane phospholipid. However, an abundant lipid is unlikely to provide sufficient targeting specificity for FtsY or an FtsY-Ffh-nascent protein complex. Thus, following initial binding, the restriction of FtsY to the two-dimensional surface of the membrane may increase the apparent affinity for the specific membrane receptor, possibly the translocation pore, by the restricted movement mechanism proposed in (Kholodenko et al., 2000). That no SRP-dependent translocation defect was observed in our assays when cells were depleted of PE is not surprising if this mechanism is presupposed. Under the conditions used in our assays, the initial lipid-binding step does not appear to be essential for targeting FtsY to the membrane, allowing the insertion into the cytoplasmic membrane of SRP-dependent proteins to proceed. However, under conditions of stress or rapid metabolism, the two-step mechanism may confer an advantage to cells and thus has been retained in both the SRP pathway for FtsY and the general secretory pathway for SecA.

The identification of a target membrane via binding to abundant lipids may provide sufficient initial targeting specificity in *E. coli* because there is only a single target membrane. In contrast, an initial lipid-binding step would impair targeting to the endoplasmic reticulum in eukaryotes because there are several noncontiguous membrane-delimited compartments that would compete for initial targeting. Initial binding to an incorrect membrane in the cell would then severely impair targeting to the correct membrane. This may explain why in eukaryotes membrane assembly of the SRP receptor requires a transmembrane β-subunit not found in prokaryotes.

CHAPTER IV

Different Amino-terminal Regions of FtsY confer Differences in Membrane Binding and Survival

4.1 Summary

A significant, yet unresolved issue in bacterial protein targeting is the mechanism of membrane assembly of FtsY, the prokaryotic equivalent of the SRP receptor. In *E. coli* FtsY is targeted to the membrane through interactions of its amino-terminal region with phospholipids and a membrane protein. However, the sequencing of coding regions for FtsY proteins from divergent microbial species and reports that the amino-terminus might be replaceable with a transmembrane targeting region (Zelzany et al., 1997; Bibi et al., 2001) have raised questions about the role of the amino-terminus of FtsY. We identified forms of FtsY in different organisms that are entirely cytoplasmic, entirely transmembrane, or distributed between these locations. By investigating the ability of FtsY proteins with divergent amino-termini to complement depletion of the endogenous FtsY in *E. coli* (a species with FtsY located in both cytoplasm and at the membrane) we found that expression of FtsY from *B. subtilis*, which is cytoplasmic, could not complement depletion of endogenous FtsY even when it was targeted to the membrane by the *E. coli* A domain. We also found that *E. coli* can tolerate a constitutively transmembrane form of FtsY but that the A region of *E. coli* FtsY is required for prolonged survival in liquid culture. The sequences of the A regions that are functional in *E. coli* are highly divergent in length and contain numerous disperse acidic residues.

4.2. Introduction

In mammalian cells, most protein targeting to the endoplasmic reticulum (ER) occurs through the signal recognition particle (SRP) pathway (reviewed in (Keenan et al., 2001)). SRP is a ribonucleoprotein complex composed of six polypeptides assembled on a scaffold RNA (Walter and Blobel, 1982). Signal sequences on nascent chains are recognized by SRP as they emerge from the ribosome (High and Dobberstein, 1991; Zopf et al., 1990; Newitt and Bernstein, 1997). The ribosome-nascent chain complex is then targeted to the ER membrane through an interaction with the SRP receptor complex, consisting of transmembrane SRβ and peripherally anchored SRα (Connolly and Gilmore, 1989; Young et al., 1995). Ultimately, nascent proteins are delivered to the translocon and cotranslationally translocated across the endoplasmic reticulum (ER) membrane (Rapoport et al., 1996).

Escherichia coli contain SRP composed of Ffh and 4.5S RNA (Valent et al., 1995; Luirink et al., 1992), which targets polytopic proteins to the inner membrane (de Gier et al., 1996; Ulbrandt et al., 1997; Valent et al., 1998; Koch et al., 1999). SRP recognizes transmembrane segments cotranslationally and targets nascent polypeptide chains through an interaction with FtsY, the homologue of the mammalian SRP receptor (Newitt et al., 1999; Beck et al., 2000; Powers and Walter, 1997; Valent et al., 1998). Nascent proteins are translocated across the same translocation pore used to translocate most secreted proteins (Valent et al., 1998; Qi and Bernstein, 1999) with the additional component YidC playing a fundamental role in translocation of nascent membrane

proteins (Samuelson et al., 2000; Scotti et al., 2000). Ffh, 4.5S RNA and FtsY are all essential for *E. coli* viability (Phillips and Silhavy, 1992; Bourgaize and Fournier, 1987; Luirink et al., 1994). Depletion of either protein component leads to defects in both protein targeting and cell division (Phillips and Silhavy, 1992; Luirink et al., 1994).

Whereas most of the components of the SRP pathway in *E. coli* closely resemble those of eukaryotes both in sequence (Poritz et al., 1988; Struck et al., 1988; Bernstein et al., 1989; Romisch et al., 1989) and functional interactions (Luirink et al., 1992; Bacher et al., 1999; Powers and Walter, 1995; Miller et al., 1994), FtsY displays significant differences both in sequence and membrane assembly compared to the eukaryotic SRP receptor. In eukaryotes, a transmembrane β-subunit of the SRP receptor anchors the peripheral membrane α-subunit on the ER membrane through an interaction between the GTP-binding domain of SRβ (Legate et al., 2000) and the amino-terminal domain of SRα (Young et al., 1995). Although in principle this system could permit regulated release of SRα from SRβ (and therefore the ER membrane) (Legate and Andrews, 2001), SRα has only been found tightly bound to the ER membrane (Tajima et al., 1986; Rubins et al., 1990). SRα and FtsY are homologous over two-thirds of their lengths. However, the amino-terminal domains are highly divergent (Bernstein et al., 1989; Romisch et al., 1989). In place of the SRβ-binding domain found in SRα, *E. coli* FtsY has a highly negatively charged "A" region at the amino-terminus (arbitrarily defined as amino acids 1-196) (Gill and Salmond, 1990). This region, together with the central "N" region (amino acids 197-280), forms the minimum domain sufficient for assembly on the *E. coli*

inner membrane (Chapter II). Unlike SRα, in *E. coli* FtsY is found equally distributed between the cytoplasm and inner membrane (Luirink et al., 1994). FtsY membrane-attachment involves interactions both with lipid and protein components (Chapter III),(de Leeuw et al., 2000). Mutations in the A region of FtsY that abolish binding to the *E. coli* inner membrane interfere with protein targeting and cell viability (Powers and Walter, 1997; Zelzany et al., 1997), suggesting either that FtsY must be able to bind to the *E. coli* inner membrane to function or that the A region provides some other essential function in *E. coli.*

Sequence analysis of FtsY proteins from a variety of microbes shows that the A region is highly variable (Gorodkin et al., 2001). This variability suggests different modes of targeting FtsY to the membrane are employed by these organisms. Amongst the amino-terminal regions identified, members of the bacterial order Actinomycetales contain a putative transmembrane region at the amino-terminus (Bibi et al., 2001). This observation, together with a report in which the A region of *E. coli* FtsY could be replaced with an unrelated transmembrane sequence, led to the conclusion that the A region functions only in FtsY membrane binding (Zelazny et al., 1997; Bibi et al., 2001). It also suggests that the cytoplasmic form of FtsY found in *E. coli* is not essential for cell viability.

In this study we investigated the cellular location of FtsY proteins from *Bacillus subtilis*, *E. coli*, and *Streptomyces coelicolor.* Fractionation indicates that in *B. subtilis* FtsY is located entirely in the cytoplasm, *S. coelicolor* FtsY is targeted to the membrane,

and *E. coli* FtsY is present in both locations. Growth analysis of *E. coli* in liquid culture indicated that the closely related homologue to *E. coli* FtsY from *Haemophilus influenzae* complements depletion of endogenous FtsY, *B. subtilis* FtsY fails to complement FtsY-depletion even when targeted to the membrane by the *E. coli* A region, and that a fusion protein containing the *S. coelicolor* TM region amino-terminal to the *E. coli* NG region provides partial complementation of endogenous FtsY depletion. Using another, unrelated single-pass transmembrane sequence fused to the *E. coli* NG region, we found that, in contrast to a previous report (Zelazny et al., 1997), a constitutively transmembrane form of FtsY must contain a least part of the A region to allow for extended survival upon depletion of wild-type FtsY. Furthermore, the AN and NG regions must be expressed as a single polypeptide.

4.3 Experimental Procedures

Phylogenetic Analysis -- We used the internet-accessible tool phyloBLAST (Brinkman et al., 2001) (http://www.pathogenomics.bc.ca/phyloBLAST/) to compare sequences related to *E. coli* FtsY. Full-length *E. coli* FtsY was compared to sequences in the SWISSPROT database using WU-BLAST2 (Altschul et al., 1990). Twenty-six unique sequences representing microbial FtsY proteins were selected for further analysis. The program CLUSTALW (Thompson et al., 1994) was used with default settings to align individual protein data sets. A phylogenetic tree was constructed using the PHYLIP 3.5c programs and the neighbour-joining method (Saitou and Nei, 1987).

General procedure and reagents – Genomic DNA from *Bacillus subtilis* was a kind gift from Dr. Eric Brown (McMaster University); from *H. influenzae* a gift from Dr. Gerry Wright (McMaster University); and *S. coelicolor* from Dr. Justin Nodwell (McMaster University). Strain N4156::pAra14-FtsY' was a kind gift from Dr. Joen Luirink (Biocentrum Amsterdam, The Netherlands)(Luirink et al., 1994). General chemical reagents were obtained from Fisher, Sigma, or Life Technologies Inc. DH5α *E. coli* cells used for plasmid construction were purchased from Invitrogen. Restriction enzymes and other molecular biology enzymes were from New England Biolabs or MBI Fermentas.

Plasmid Construction – Construction of plasmids, sequencing, and PCR reactions were performed using standard methods (Sambrook et al., 1989). Plasmid pMAC897 and pMAC1253, which encode full-length FtsY and the polypeptide FtsYAN-gPa (amino acids 1-284 of FtsY) have been described previously (Chapter II). Plasmid pMAC873 contains the FtsY coding sequence directly behind the UTK untranslated leader and start codon of pSPUTK (Falcone and Andrews, 1991). Plasmid pGBM2 (Manen et al., 1997) was obtained from the American Type Culture Collection.

Genes encoding FtsY from *B. subtilis*, *H. influenzae* and *S. coelicolor* were amplified from their respective genomic DNA using the following oligonucleotides: 5' GCAGGTACCAGAGCGTGCGCTTACTGCTA 3', and 5' ATAGGATCCGGCCAGCTGCTGGTTACTAT 3' (*B. subtilis*), 5' ATAGGTACCCCTGCAATAATGCGAACCTC 3', and 5'

ATAGGATCCGCTGTGTTGCGCCATGATAG 3'(*H. influenzae*) and 5' ATAGGTACCGATGCGGCCTCCTCCATCGT 3' and 5' ATCGGATCCCGTCTCCCGATCAGCAAC 3' (*S. coelicolor*). The products were cloned into plasmid pSPUTK using the restriction enzymes KpnI and BamHI to generate plasmid pMAC1512, pMAC1513 and pMAC1514, respectively . The full-length coding sequences from each of these plasmid were amplified using the common primer 5' CGTTAGAACGCGGCTACAAT 3' and one of the primers 5' CGAGGAAAGAGAAGACAACATGAGCTTTTTT 3' (pMAC1512), 5' GACGGTCTCGCATGAACGACATTTTTATCGGATTA 3' (pMAC1513) and 5' TCAGGTCTCCCATGACGGGGGTCACGCATA 3' (pMAC1514). To generate expression plasmid the amplified products were inserted behind the tandem SP6 and *tac* promoters in the plasmid pMAC897 using the restriction enzymes NcoI and BamHI (pMAC897) and BpiI (amplified *B. subtilis* coding sequence) or Eco31I (the other amplified sequences) and BamHI to generate plasmid pMAC1542, pMAC1543 and pMAC1545, respectively.

To generate a transmembrane fusion to FtsY the coding sequence of a canine-mouse hybrid SRβ isolated from plasmid pMAC455 (Young et al., 1995) digested with BamHI and EcoRI was inserted into pMAC873 digested with BglI and EcoRI to generate plasmid pMAC1097. From this plasmid coding regions for both SRβ and FtsY were cloned into pBluescript (Stratagene) using HindIII and EcoRI. To generate a gene encoding a fusion of amino acids 1-85 of SRβ to amino acids 44-497 of FtsY, a PCR-

based deletion strategy was used as described in (Hughes and Andrews, 1996) with primers 5' GTGCATGCCTGTGAAGGCCTC 3' and 5' GTGCATGCCAGTTAACAACCT 3'. The coding sequence was cloned into plasmid pMAC897 digested with NcoI and BamHI to produce the expression vector pMAC1100. Plasmid pMAC1586, encoding a fusion of amino acids 1-85 of SRβ to amino acids 198-497 (NG region) of FtsY was generated as described in (Hughes and Andrews, 1996) using pMAC1100 as a template with the primers 5' GTGCATGCCAGTTAACAACCTG 3' and 5' TTCGCATGCCTGAAACGCAGCCTGTT 3'.

A plasmid encoding a fusion of amino acids 1-197 (A region) from *E. coli* FtsY to amino acids 20–329 (NG region) of *B. subtilis* FtsY was generated by first amplifying the region encoding the *B. subtilis* NG domain from plasmid pMAC1512 with the primers 5' CGTTAGAACGCGGCTACAAT 3' and 5' GCCGCATGCTAAAGTTTAAGGATGGCCTTGAA 3'. This product was cloned into plasmid pMAC1176 using SphI and BamHI. To generate an in-frame fusion this product was digested with SphI, subjected to 3' end removal with DNA polymerase I (Klenow fragment) and re-ligated.

A fusion of the transmembrane region (amino acids 1-113) from *S. coelicolor* FtsY to *E. coli* FtsY amino acids 198-497 was generated by amplifying plasmid pMAC1545 with the primers 5' CAGGCATGCGACCAGTCGGCCGGCGGTGGG 3' and 5' AACCGACTCTGACGGCAGTT 3'. The amplified region was cloned into plasmid pMAC1100 using SphI and HindIII.

The coding regions generated above were cloned into the plasmid pGBM2, which contains the pSC101 replicon for survival in strain N4156::pAra14-FtsY', with Bsp1407I and BamHI. The plasmid and corresponding coding regions are listed in Table 4.1.

Growth of cells – E. coli N4156::pAra14-FtsY' cells transformed with expression vectors were grown overnight at 37°C in Luria-Bertani medium (LB) supplemented with 0.2% arabinose, ampicillin (50 μg/ml) and streptomycin (15 μg/ml). To produce the growth curves in Figures 4.4C and 4.6B, overnight cultures were washed twice with LB and diluted to a cell density (OD_{550}) of 0.1 in fresh LB containing 0.02 mM IPTG with or without arabinose (0.2%), as indicated. After the initial 1.5 hours cell density was measured every hour for the construction of growth curves. Cells were diluted 1:100 following 3.5 hours of growth.

To obtain growth curves in which cells were maintained in an exponential growth state (Fig. 4.6C), overnight cultures were washed twice with LB and diluted to a cell density (OD_{550}) of 0.005 into fresh LB containing 0.02 mM IPTG with or without arabinose (0.2%). For the duration of the measurement of growth, prior to reaching a cell density of 0.4 cultures were diluted 1:50 into prewarmed LB containing 0.02 mM IPTG with or without arabinose (0.2%). Growth measurements were obtained at twenty-four hours and beyond by continuing to dilute cells into the same media supplemented with ampicillin (50 μg/ml) and streptomycin (15 μg/ml) to prevent plasmid loss. Twenty-four hours following their initial inoculation (and prior to reaching a cell density of 0.4) cultures were diluted to a cell density of 0.05. Cell density was measured every hour for

the construction of growth curves.

Antibodies, Expression, Cellular Fractionation and Immunoblotting – Antibodies directed against the epitope CGVNGVGKTTTIGKL from the G region of FtsY were raised in rabbits using a keyhole limpet haemocyanin-coupled peptide with this sequence. Affinity purified antibody raised against amino acids 41-497 of *E. coli* FtsY was a gift from Dr. Harris Bernstein (NIH, Bethesda MD).

To detect constructs expressed in *E. coli* N4156::pAra14-FtsY', overnight cultures were diluted to a cell density (OD_{550}) of 0.02 in LB containing 0.02 mM IPTG and arabinose. Following four hours of growth, cells were washed twice in LB and diluted into fresh LB containing 0.02 mM IPTG. Following 1.5 hours growth, approximately $2x10^8$ cells were pelleted by centrifugation at 17 000 x g for 30 seconds and resuspended in 60 µl of 20 mM Tris-Cl, pH 7.6. An equal volume of 2X SDS-loading buffer (100 mM Tris-CL, pH 7.6, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 250 mM DTT) was added followed by incubation at 95°C for 10 minutes. Samples were subjected to centrifugation at 17 000 x g for 2 minutes and 10 µl aliquots were analyzed by SDS-PAGE and immunoblotting.

For fractionation experiments *E. coli* N4156::pAra14-FtsY' expressing the indicated constructs were grown in 100 ml of LB containing 0.02 mM IPTG to a cell density (OD_{550}) of 0.6. Cells were pelleted at 3000 x g for 15 minutes, washed once with deionized filtered H_2O, and resuspended in 3 ml buffer A (0.25 M sucrose, 50 mM Triethanolamine-acetate, pH 7.5, 1 mM EDTA, 1 mM DTT, 2 mM

phenylmethylsulfonylfluoride). Cells were passed twice through a French pressure cell at 8000 psi. Debris was removed by centrifugation at 18 000 x g for 15 minutes. Membranes were pelleted by centrifugation at 100 000 x g for one hour and resuspended in 1/10th the original buffer volume. Vesicles (30 µl) were adjusted to 0.1 M sodium carbonate, pH 11.5 with three volumes of 0.133 M sodium carbonate or to 50 mM Triethanolamine-acetate, pH 7.5, 50 mM NaCl and incubated on ice for 30 minutes. Membranes were pelleted through an 80 µl 0.5 M sucrose cushion containing 0.1 M sodium carbonate or 50 mM Triethanolamine-acetate, pH 7.5, 50 mM NaCl by centrifugation at 100 000 x g for 30 minutes and resuspended in 200 µl of 50 mM Tris-Cl, pH 8.0, 1% Triton X-100.

B. subtilis strain LS087 (Briehl et al., 1989) was grown to an OD_{600} of 0.8 in 100 ml LB broth, washed twice and resuspended in 3 ml buffer B (0.25 M sucrose, 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 1mM phenylmethylsulfonylfluoride). Following passage twice through a French pressure cell at 8000 psi, debris was removed by centrifugation at 30 000 x g for 10 minutes. Membranes were pelleted at 100 000 x g for 1 hour and resuspended in 0.75 ml buffer B.

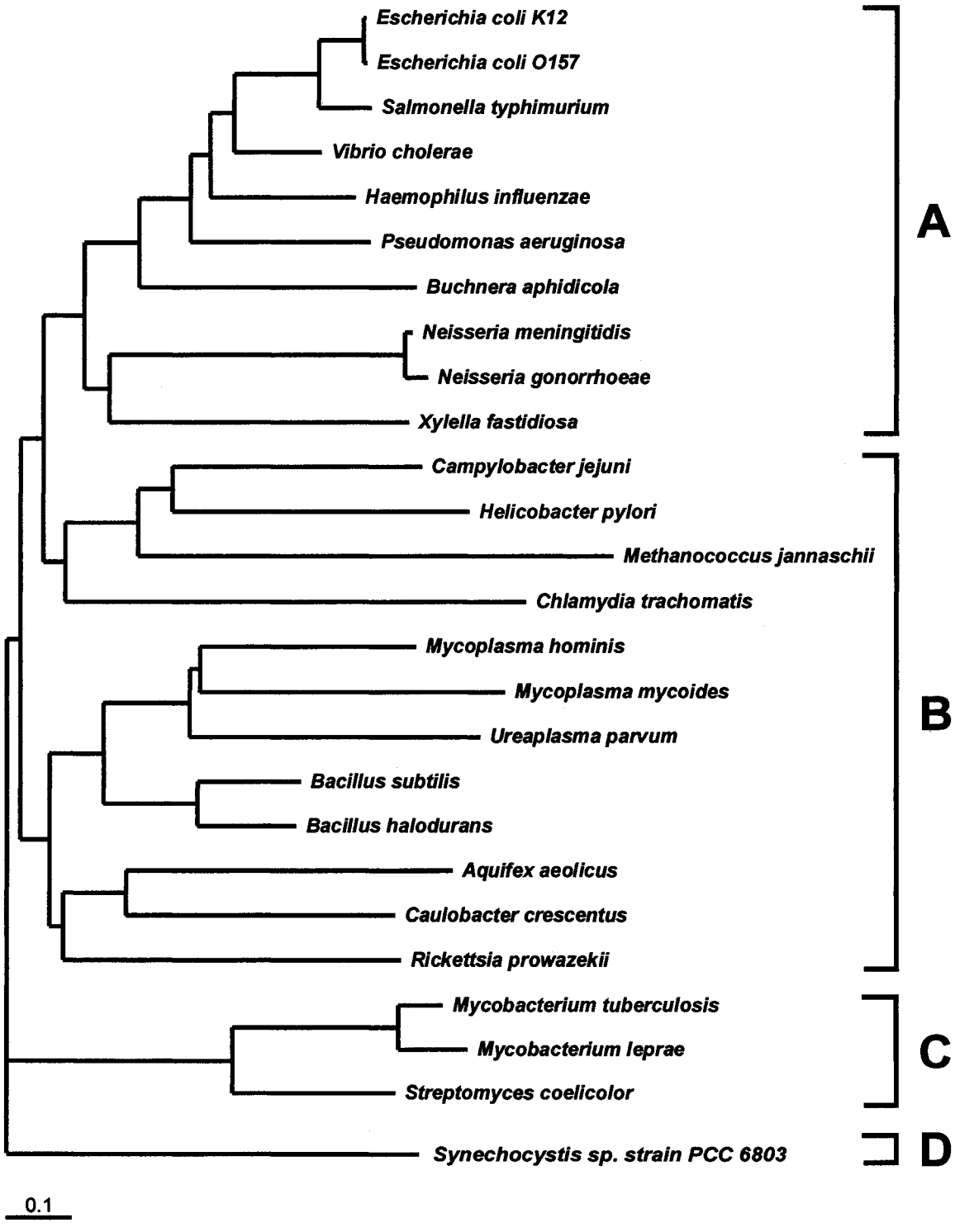
Volumes corresponding to an equivalent number of cells (approximately $5x10^8$) from each fraction were precipitated with trichloroacetic acid, resuspended in sample buffer, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose using a semi-dry transfer apparatus (Hoeffer Instruments). The nitrocellulose membrane was probed with affinity purified IgG raised against *E. coli* FtsY (expression in *E. coli)* or

anti-FtsY epitope serum (*B. subtilis* sample) diluted 1:20 000 in 10 mM KPO_4, pH 7.4, 0.1% Triton X-100, 560 mM NaCl, 0.02% SDS, 1% BSA. Secondary anti-rabbit IgG linked to horseradish peroxidase was used to detect the primary antibodies on the washed blot. Bound antibody was visualized by soaking in substrate solution (Western Lightning™, PerkinElmer Life Sciences, Inc.) and exposure to film for approximately 20 seconds.

4.4 Results

To examine the relatedness of FtsY proteins from a variety of microbial genomes to *E. coli* FtsY a phyloBLAST analysis was performed (Brinkman et al., 2001). Sequences were selected for analysis by comparison of the *E. coli* FtsY protein sequence to the SwissProt/TREMBL database using WU-BLAST2 (Altschul et al., 1990). The 26 sequences selected as representative of diverse microbial species were used to perform a phylogenetic analysis using the neighbour-joining method of PHYLIP (Brinkman et al., 2001) (Fig. 4.1). Analysis of the sequences of FtsY proteins clustering at different positions on this phylogenetic tree suggested that the amino-terminal regions were highly divergent, and permits grouping based on sequence similarities. Group A (Fig. 4.1), including *E. coli* FtsY, contain amino-terminal regions that are highly negatively charged. Group B contains proteins that are phylogenetically second most related to *E. coli* FtsY but completely lack a recognizable A region. The region amino-terminal to the N domain is short (between 10 and 25 amino acids) and bears a net positive charge. This virtual

Figure 4.1. Phylogenetic Classification of FtsY proteins from Prokaryotes. Protein sequences from 26 homologues of *E. coli* FtsY from the indicated species were identified in the SWISSPROT database using WU-BLAST2. These sequences were aligned using CLUSTALW, and the phylogeny tree was generated using the neighbour-joining method (Saitou and Nei, 1987). The scale bar indicates 0.10 changes per amino acid position


Escherichia coli K12
Escherichia coli O157
Salmonella typhimurium
Vibrio cholerae
Haemophilus influenzae
Pseudomonas aeruginosa
Buchnera aphidicola
Neisseria meningitidis
Neisseria gonorrhoeae
Xylella fastidiosa
Campylobacter jejuni
Helicobacter pylori
Methanococcus jannaschii
Chlamydia trachomatis
Mycoplasma hominis
Mycoplasma mycoides
Ureaplasma parvum
Bacillus subtilis
Bacillus halodurans
Aquifex aeolicus
Caulobacter crescentus
Rickettsia prowazekii
Mycobacterium tuberculosis
Mycobacterium leprae
Streptomyces coelicolor
Synechocystis sp. strain PCC 6803
A
B
C
D
0.1

absence of an A region was observed in 12 of 26 sequences examined including FtsY proteins from *Rickettsia, Caulobacter* and *Mycoplasma* species. Group C consists of FtsY proteins that contain a putative type I signal anchor within 15 residues of the amino-terminus; a single acidic residue followed by a stretch of 21 aliphatic hydrophobic residues then four basic residues. Of the sequences analyzed, FtsY from the blue-green algae *Synechocystis sp. PCC 6803* alone makes up group D. The amino-terminal region of this protein is of similar length (199 amino acids) to the A region of *E. coli* FtsY, but with no regions of significant sequence similarity and a net negative charge of -18 compared with -47 in FtsY from *E. coli*.

We have previously identified the AN domain as the region responsible for membrane-binding of *E. coli* FtsY (Chapter II). The diversity of sequences at the amino-termini of FtsY proteins from different species suggests that at least three of these classes of proteins have profoundly differing mechanisms and ability to bind membranes. FtsY from *E. coli*, in group A, is located both in the cytoplasm and at the membrane (Luirink et al., 1994). We hypothesized that group B proteins, which lack an A region, would be entirely cytoplasmic. Based on the 'positive-inside rule', proteins in group C are postulated to insert into the membrane with the bulk of the protein on the cytoplasmic face.

To test this hypothesis, we attempted to fractionate representative organisms from each of these three groups, *E. coli* (Group A), *B. subtilis* (Group B) and *S. coelicolor* (Group C), into membrane and cytosol fractions and detect endogenous FtsY proteins

using immunoblotting. Unfortunately, we were unable to unambiguously detect *S. coelicolor* FtsY, either in its native organism or when expressed in *E. coli* (data not shown). Instead, in order to determine the localization of FtsY containing the putative TM region, a hybrid protein consisting of the amino-terminal 113 amino acids of *S. coelicolor* FtsY to the NG domain of *E. coli* FtsY was expressed in *E. coli* (*Str*TM-NG, table 4.1 and figure 4.2). As has been observed previously (Luirink et al., 1994), *E. coli* FtsY is distributed between the membrane and cytosol fractions (Fig 4.3). As predicted, we found that in *B. subtilis* FtsY is located entirely in the cytosol, and that *Str*TM-NG is located almost exclusively at the membrane when expressed in *E. coli* (Fig 4.3). Thus, the amino-terminal region from *S. coelicolor* does function to direct proteins to the inner membrane.

Analysis of cell growth in liquid culture indicates differing capacities to complement depletion of FtsY in E. coli– Using *E. coli* strain N4156::pAra14-FtsY', in which endogenous FtsY is under control of the araB promoter, cells can be depleted of FtsY by transfer into arabinose-free media, resulting in filamentation and rapid cell death (Luirink et al., 1994). We used this strain to test whether viability could be restored through the expression of proteins from groups A through C from our phylogenetic classification, each corresponding to differing subcellular localization. (Fig. 4.4A). Although in *E. coli* N4156::pAra14-FtsY' the chromosomal copy of the *ftsy* gene is expressed from an arabinose promoter (araB), in the context of this strain it will be referred to as 'endogenous' FtsY.

Table 4.1. Expression constructs utilized in this study. The plasmid designation, and details of the constructs used in this study are listed.

Plasmid	Construct	Description
pMAC1581	FtsY (*E. coli)*	WT FtsY from *E. coli*
pMAC1582	FtsY (*H. influenzae)*	WT FtsY from *H. influenzae*
pMAC1604	FtsY (*B. subtilis)*	WT FtsY from *B. subtilis*
pMAC1607	FtsYA(*coli*)-NG(*subtilis*)	Amino acids 1-197 from *E. coli* FtsY fused to amino acids 21-329 from *B. subtilis* FtsY
pMAC1618	*Str*TM-NG	Amino acids 1-113 from *S. coelicolor* SRβ fused to amino acids 199-497 from *E. coli* FtsY
pMAC1584	βTM-ANG	Amino acids 1-86 from *C. familiaris* SRβ fused to amino acids 44-497 from *E. coli* FtsY
pMAC1585	βTM-NG	Amino acids 1-86 from *C. familiaris* SRβ fused to amino acids 199-497 from *E. coli* FtsY
pMAC1583	FtsYAN	Amino acids 1-284 from *E. coli* FtsY
pMAC1617	FtsYAN and βTM-NG	Co-expression of both constructs in a single plasmid
pGBM2	Vector	pGBM2 vector containing no insert

Figure 4.2. Domain structure of constructs utilized in this study. Domain diagrams of *A.* the FtsY proteins and *B.* the fusion constructs used in this study are presented. Domain designations are listed in the *E. coli* coding region.. The percent sequence identity in the domains of each homologue compared to *E. coli* FtsY is listed. The sequence of the putative transmembrane sequence located 10 amino acids from the amino terminus of *S. coelicolor* FtsY is expanded below the corresponding domain structure. The checkerboard pattern indicates the putative transmembrane sequence of *S. coelicolor* FtsY or canine SRβ.

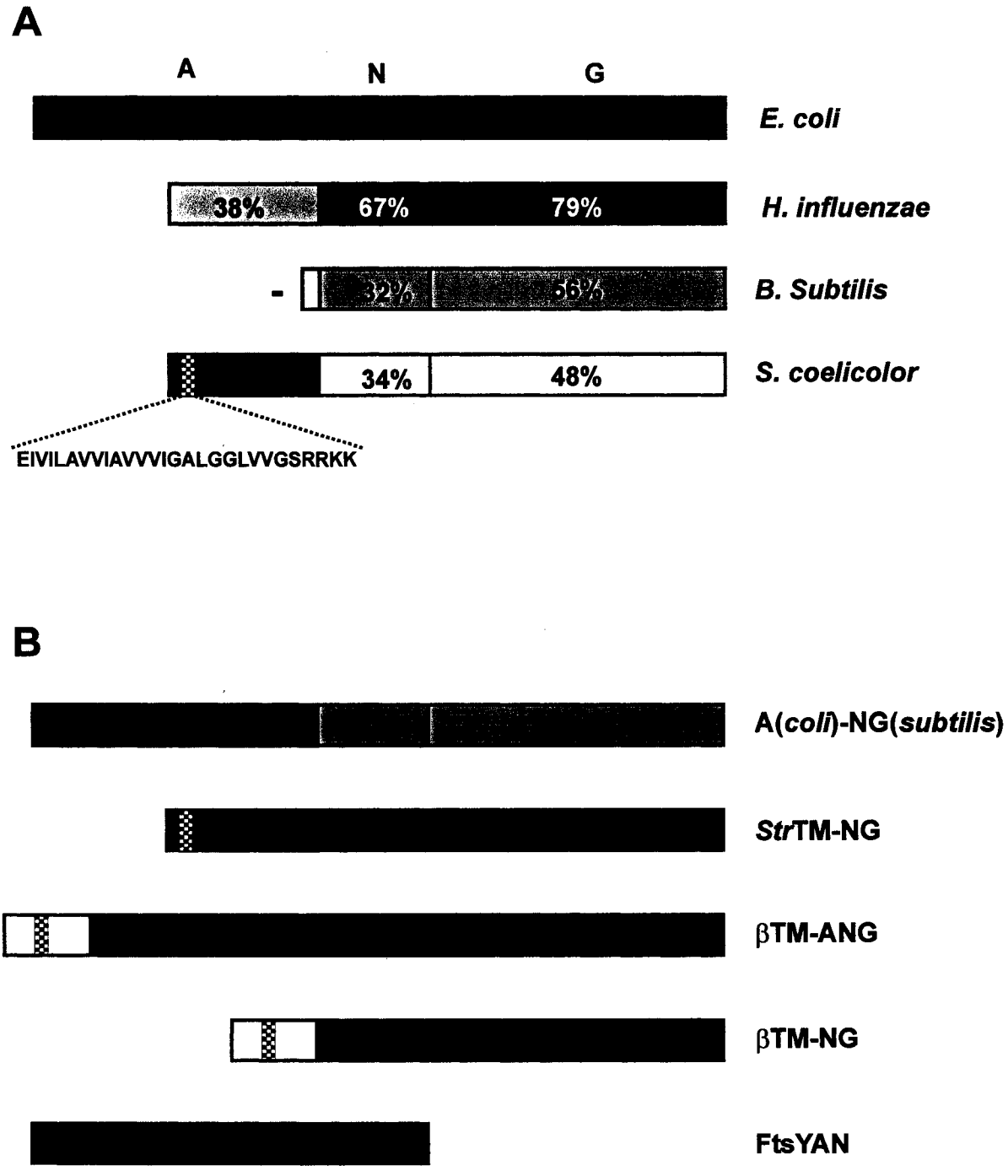

A
A
N
G
E. coli
38%
67%
79%
H. influenzae
32%
56%
B. Subtilis
34%
48%
S. coelicolor
EIVILAVVIAVVVIGALGGLVVGSRRKK
B
A(coli)-NG(subtilis)
StrTM-NG
βTM-ANG
βTM-NG
FtsYAN

Figure 4.3. Cellular localization differs between FtsY proteins from different prokaryotes. *E. coli* Strain N4156::pAra14-FtsY' (top panel), the same strain expressing *Str*TM-NG (middle panel) and *B. subtilis* strain LS087 (bottom panel) were grown to a cell density (OD_{550}) of 0.6 and whole cells (lane 1) were fractionated into cytosol (lane 2) and membrane (lane 3) fractions. Equivalent cell volumes were analyzed by SDS-PAGE, and immunoblots were probed with anti-FtsY antibodies.

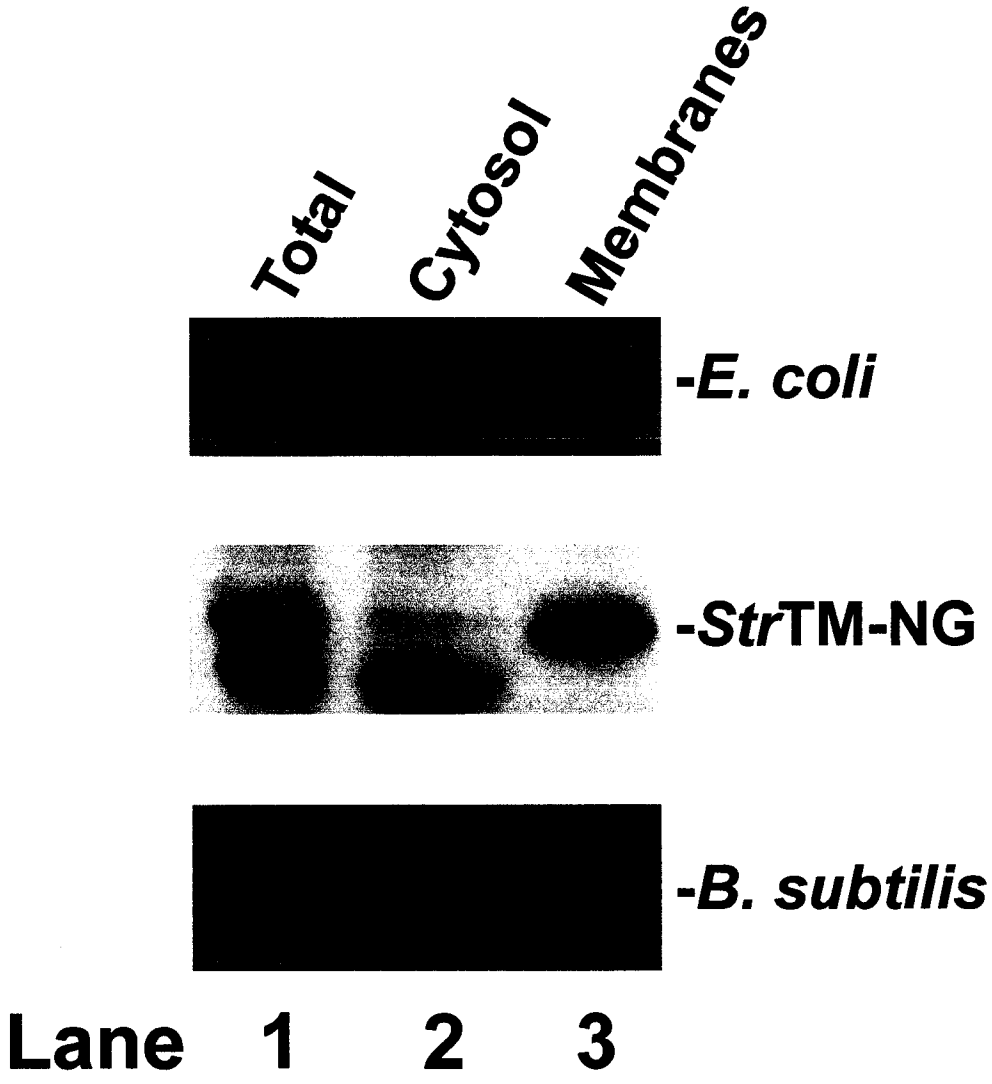
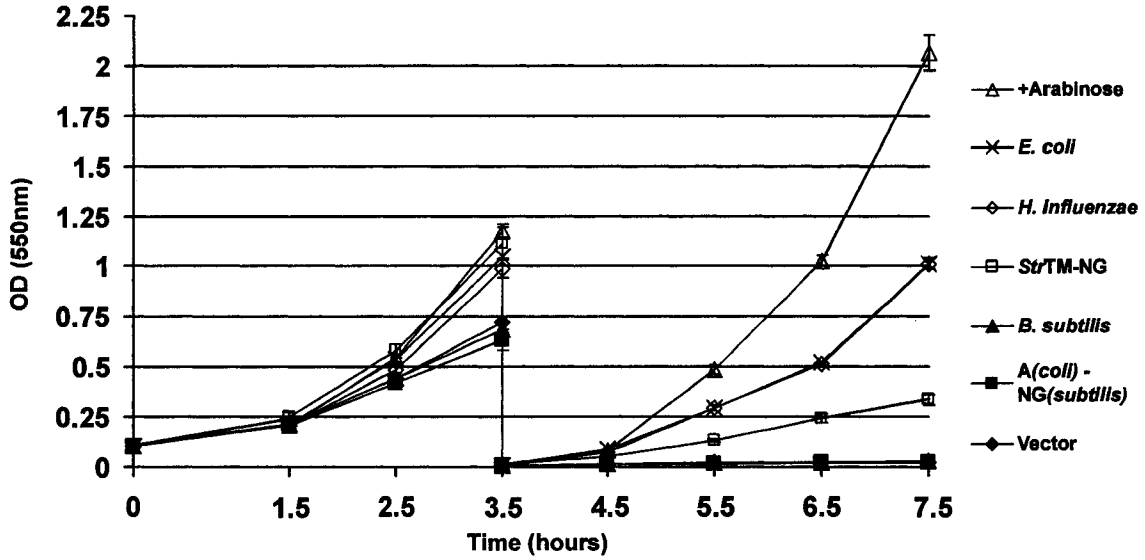
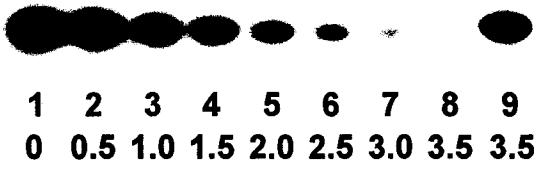

Total
Cytosol
Membranes
-E. coli
-StrTM-NG
-B. subtilis
Lane 1 2 3

Figure 4.4. Complementation of FtsY-depleted *E. coli*. *A. E. coli* strain N4156::pAra14-FtsY' transformed with the indicated constructs were grown overnight in LB containing ampicillin, streptomycin, and 0.2% arabinose. Cells were washed twice with LB, and diluted in LB supplemented with 0.02 mM IPTG. 3.5 hours following the initial dilution cultures were diluted 1:100 in the same media. Error bars represent 1 standard deviation obtained from three independent cultures. *B. E. coli* strain N4156::pAra14-FtsY' transformed with empty vector (lanes 1-8) or vector expressing FtsY (lane 9) were grown overnight in LB containing ampicillin, streptomycin, and 0.2% arabinose. Cells were washed twice with LB, and diluted in LB supplemented with 0.02 mM IPTG (all lanes) and arabinose (lane 1). At the indicated times equivalent number of cells were analyzed by SDS-PAGE and immunoblotting with anti-FtsY antiserum. *C. E. coli* strain N4156::pAra14-FtsY' transformed with empty vector (lane 1) and constructs expressing *H. influenzae* FtsY (lane 2), *B. subtilis* FtsY (lane 3), A(coli)-NG (*subtilis*) (lane 4), and *Str*TM-NG (lane 5) were grown overnight in LB containing ampicillin, streptomycin, and 0.2% arabinose. Cells were diluted and grown for 4 additional hours in the same medium containing 0.02mM IPTG. They were then washed twice with LB, and diluted in LB supplemented with 0.02 mM IPTG. Following 1.5 hours of further growth an equivalent number of cells were analyzed by SDS-PAGE and immunoblotting with anti-FtsY antiserum. The migration positions of bands corresponding to expressed proteins are indicated with arrowheads.

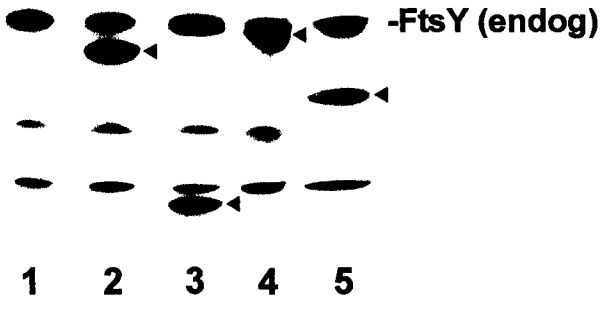
A


2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
OD (550nm)
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
Time (hours)
+Arabinose
E. coli
H. influenzae
StrTM-NG
B. subtilis
A(coli) - NG(subtilis)
Vector

B


Lane 1 2 3 4 5 6 7 8 9
Hours (-ara) 0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 3.5

C


-FtsY (endog)
Lane 1 2 3 4 5

In the first 1.5 to 2.5 hours of growth, cells transfected with any of the constructs, including an empty vector used as a control, grew at similar rates (Fig. 4.4A). A similar phenomenon has been observed previously following depletion of arabinose from growth media (Luirink et al., 1994; Zelazny et al., 1997). Endogenous FtsY levels drop off substantially during this period (Fig 4.4B) until they reach an apparently critical concentration that is no longer sufficient to sustain wild type growth levels 2.5 to 3.5 hours after dilution into arabinose-free media.

Following the initial 3.5 hours of growth, cultures were seeded into fresh media at a dilution of 1:100 to maintain a logarithmic growth state. We have repeated all growth experiments with an initial seeding at a further 1:100 dilution and observed growth rates at time points beyond 3.5 hours that are the same as observed with multiple dilutions (data not shown). However, the higher dilution does not permit accurate measurements at times less than three hours.

Complementation with plasmid-borne *E. coli* FtsY resulted in growth rates approximately 0.85 times those obtained upon expression of endogenous FtsY (Fig. 4.4A). We observed these reduced growth rates whether expressing FtsY from the tac or trc promoters in two different plasmid backbones, pGMB2 (Manen et al., 1997) and pJH15 (Lu et al., 2001) (data not shown). The level of FtsY expression was similar when expressed from either the endogenous gene in the presence of arabinose or from the plasmid-encoded gene through induction by IPTG (Fig 4.4B, compare lanes 1 and 9). In cells transformed with vector, growth in arabinose-containing media was not altered by

the presence of the plasmid-selective antibiotic. Since cells could not survive without a plasmid under selective conditions this indicates that the rate of plasmid loss was below the sensitivity of our growth analysis and thus can not account for the observed differences in growth when FtsY is expressed from the chromosome versus a plasmid. Thus it appears that expression of plasmid-borne FtsY in this strain affects cells in a slightly deleterious manner compared to expression of the chromosomal FtsY.

We also tested whether expression of FtsY from *H. influenzae*, a related species with a significantly shorter A region than that of *E. coli* FtsY, was sufficient for survival. When the FtsY homologue from *H. influenzae* was expressed, upon depletion of endogenous FtsY cells grow at a rate that is indistinguishable from that observed upon expression of plasmid-borne *E. coli* FtsY (Fig 4.4A). Thus, *E. coli* tolerate an FtsY with a divergent but acidic A region.

Using immunoblotting with antibodies raised against a significant portion of *E. coli* FtsY (amino acids 41-497), expression was detected from the construct encoding *H. influenzae* FtsY, as well as the other constructs for which growth was examined in Fig 4.4A was detected (Fig 4.4C). With the exception of plasmid-borne *E. coli* FtsY (Fig 4.4B), the intensity of the bands on the immunoblot corresponding to each construct did not match the level of endogenous FtsY in the presence of arabinose. This was not unexpected, however, since the constructs expressed in this investigation consisted of FtsY proteins with sequences that differ from *E. coli* FtsY, to which the antibody was generated, especially in the A domains. Another antibody raised against an epitope

common to the G region of most FtsY proteins produced too much background in *E. coli* lysates to be useful for these investigations. Nevertheless, sufficient sequence similarity was present to observe expression of each construct in *E. coli* (Fig 4.4C). Furthermore, the expression level of each construct was comparable to or greater than the level of endogenous FtsY expression 1.5 hours following dilution into arabinose-free medium (Fig 4.4C). At this time point, the expression of FtsY is sufficient to promote a rate of growth that is indistinguishable from that observed in the presence of arabinose (Fig 4.4A). Additionally, *H. influenzae* FtsY expressed at the detected levels complemented depletion of endogenous FtsY as well as *E. coli* FtsY. Therefore, the level of expression observed from each construct should be sufficient to promote survival if the encoded protein is fully functional in *E. coli*.

We next determined whether FtsY from *B. subtilis*, in which the protein is located primarily in the cytosol, could complement depletion of endogenous FtsY in *E. coli*. Expression of the FtsY homologue from *B. subtilis* resulted in growth rates comparable to vector alone. Following the initial 3.5 hours of growth and dilution into fresh media cells failed to exhibit any further growth (Fig 4.4A). This indicates that *B. subtilis* FtsY is unable to complement depletion of FtsY in *E. coli*.

The most apparent difference between FtsY from *B. subtilis* and *E. coli* is the absence of an A region in *B. subtilis* FtsY (Fig 4.2). To test whether the failure of FtsY from *B. subtilis* to permit cell growth could be accounted for entirely by this difference we generated a construct that expressed the A region from *E. coli* FtsY fused to the NG

region from *B. subtilis* FtsY (A(*coli*)-NG(*subtilis*)). This construct also failed to complement FtsY depletion, resulting in growth indistinguishable from cells transformed with vector alone (Fig. 4.4A). Using a column chromatography assay described previously (Chapter II), we found that A(*coli*)-NG(*subtilis*) bound INVs as well as *E. coli* FtsY (data not shown). Thus, differences exist between the NG regions of *B. subtilis* FtsY and *E. coli* FtsY that prevent these domains from being functionally interchangeable in *E. coli*.

As stated above, expression of a construct encoding FtsY from *S. coelicolor* could not be detected in strain N4156::pAra14-FtsY'. Furthermore, the failure of A(*coli*)-NG(*subtilis*) to complement FtsY depletion in *E. coli* suggested that proteins containing NG regions from more distantly related FtsY proteins would not be functional in *E. coli*. Thus, in order to test whether the putative TM region from *S. coelicolor* FtsY could functionally replace the A region of *E. coli* FtsY we measured the growth of cells expressing a construct encoding the amino-terminal region of *S. coelicolor* FtsY (amino acids 1-113) fused to the NG region from *E. coli* FtsY (amino acids 198-497). Expression of this construct following depletion of FtsY resulted in continued growth beyond the time at which growth ceased with vector alone, but at a lower rate than when cells expressed FtsY proteins from *E. coli* or *H. influenzae* (Fig 4.4A, '*Str*TM-NG').

E. coli FtsY fused to a transmembrane sequence is functional only when part of the A region is present – We were surprised that expression of *Str*TM-NG resulted in greatly reduced growth rates in comparison to expression of *E. coli* FtsY. It has

previously been reported that when transmembrane segments from LacY are fused to the NG region of FtsY the protein is fully functional (Zelazny et al., 1997). Our results suggest that the conclusion of this previous study, that the only function of the A region in *E. coli* FtsY is targeting to the membrane, may be incorrect.

Although we found *Str*TM-NG is targeted to the membrane, in order to show it is in fact integrated into the membrane we performed carbonate extractions (Fig 4.5). As has been observed previously, *E. coli* FtsY is approximately equally distributed between the membrane and cytosol (Fig. 4.5, top panel)(Luirink et al., 1994). However, when membranes were treated with sodium carbonate (pH 11.5) and subjected to high speed centrifugation FtsY was found entirely in the supernatant, confirming that it is a peripheral membrane protein (Fig. 4.5, top panel).

We tested membrane integration of *Str*TM-NG four hours following dilution into arabinose-free media when endogenous FtsY levels are below the critical threshold for growth. Since integration of membrane proteins in *E. coli* is dependent on FtsY (Ulbrandt et al., 1997), it was possible that if the transmembrane constructs were not functional in SRP-dependent protein targeting, a synthetic defect might be observed preventing membrane integration of these constructs. No such defect was apparent, as *Str*TM-NG was observed entirely in *E. coli* membranes following fractionation (Fig 4.5, second panel) and this protein was found in the pellet fraction following carbonate treatment and high speed centrifugation (Fig. 4.5, second panel), indicating that it is integrated into the membrane.

Figure 4.5. Membrane integration of FtsY fusion proteins. *E. coli* Strain N4156::pAra14-FtsY' expressing the indicated constructs were grown in the presence (top panel) or absence (bottom three panels) or arabinose to a cell density (OD_{550}) of 0.6 and fractionated into cytosol (C) and membrane (M) fractions. Membrane fractions were treated with 0.1 M carbonate or Tris-NaCl buffer and subjected to centrifugation at 100 000 x g resulting in supernatant (S) and pellet (P) fractions. Following SDS-PAGE blots were probed with anti-FtsY antibodies.

Since *Str*TM-NG is integrated into the membrane in *E. coli* we considered that the partial restoration of growth observed upon expression of this construct in cells depleted of endogenous FtsY might be the result of an unexpected membrane toplogy of *Str*TM-NG. To address this concern we generated a second construct (βTM-NG) in which the unrelated but well characterized single-pass transmembrane sequence from canine SRβ (amino acids 1-85) was fused amino-terminal to the NG region (amino acids 198-497) of *E. coli* FtsY. SRβ spans the membrane only once, is not related to any microbial protein, but contains an optimal type I signal anchor for integration into prokaryotic membranes (von Heijne, 1989). This construct was expressed at levels equal to or greater than endogenous FtsY 1.5 hours following depletion of arabinose (Fig 4.6B, lane 3). It localizes entirely in the membrane fraction of *E. coli* and is resistant to extraction with carbonate (Fig 4.5, bottom panel), indicating that it is integrated into the *E. coli* inner membrane

As with *Str*A-NG, expression of βTM-NG following depletion of endogenous FtsY resulted in growth beyond the time at which growth ceased with vector alone, but at lower rates than when full-length *E. coli* FtsY was expressed (Fig 4.6A). The observation of sub-optimal growth in two separate transmembrane fusions to the NG region of *E. coli* FtsY (*Str*TM-NG and βTM-NG) led us to re-analyze the initial report that the NG region constitutes a fully-functional FtsY when fused to an unrelated TM domain (Zelazny et al., 1997). This analysis (see below, discussion) led us to consider that the A region may be required for the function of FtsY even when it is targeted to the membrane through an

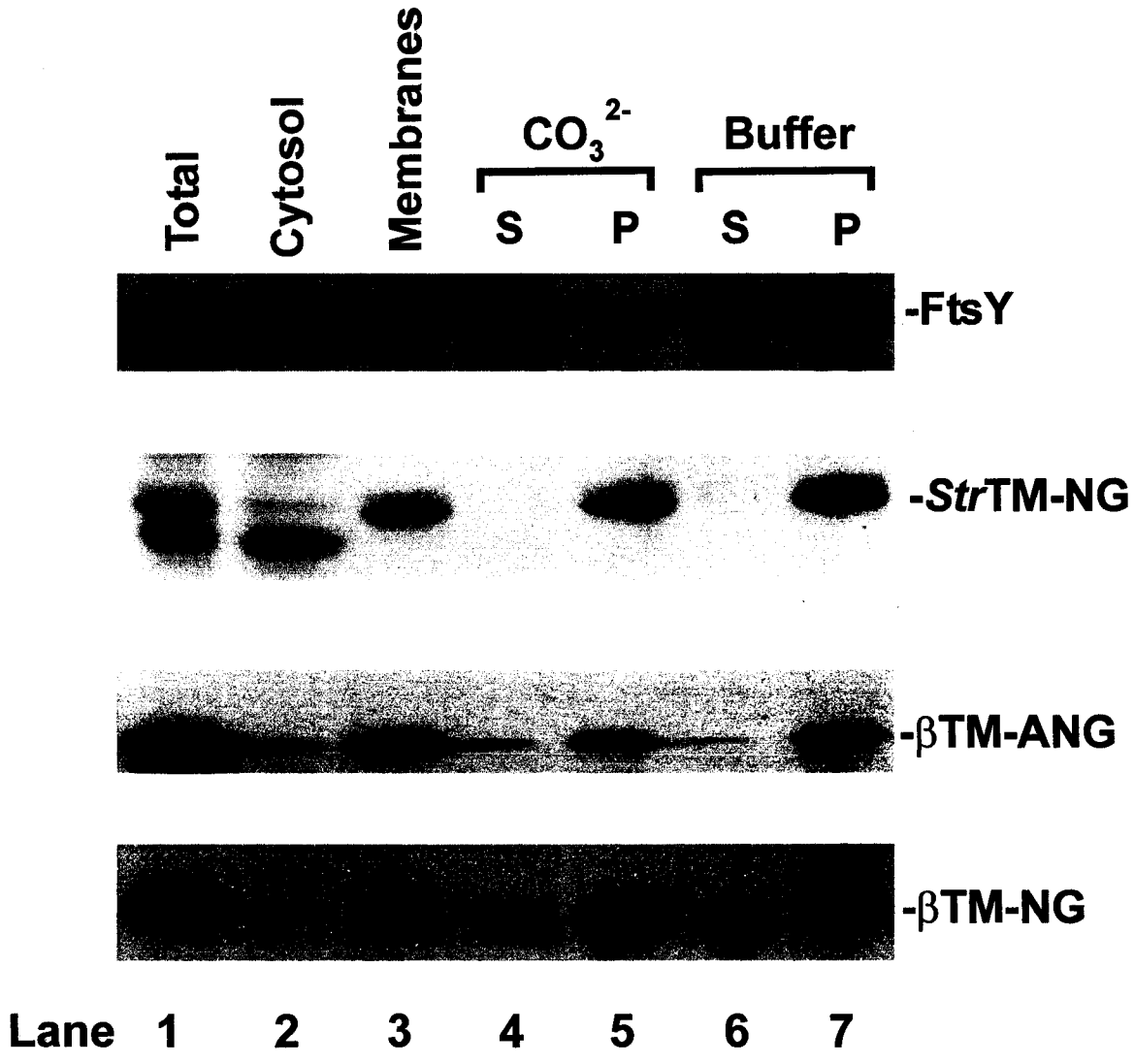
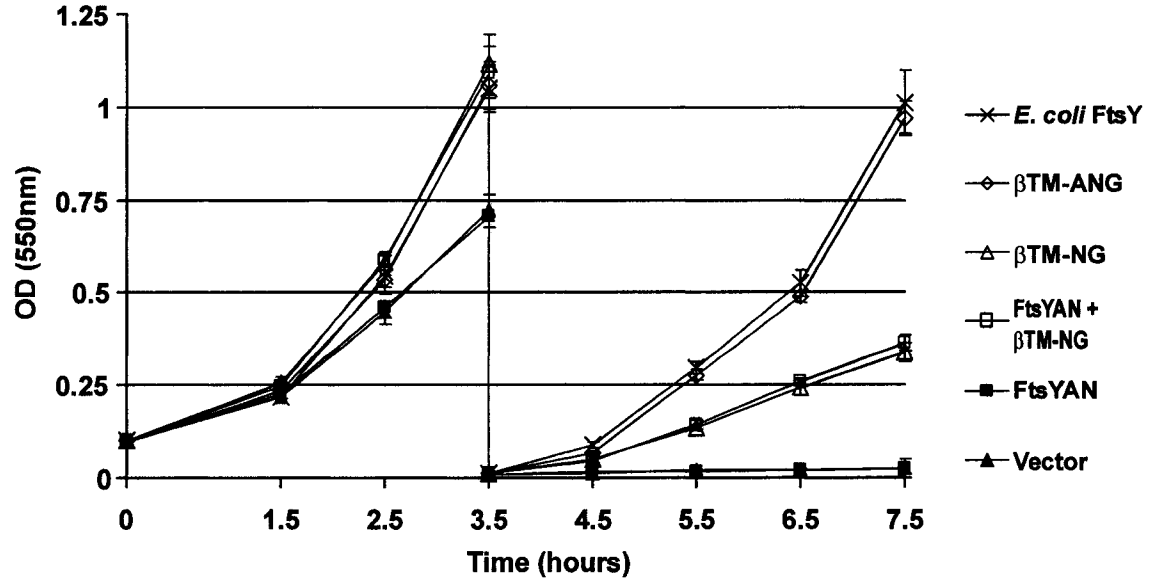
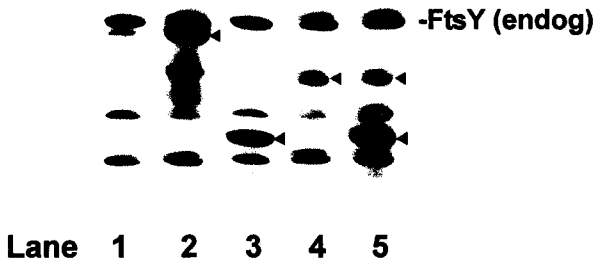

Total
Cytosol
Membranes
CO_3^{2-}
Buffer
S
P
S
P
-FtsY
-StrTM-NG
-βTM-ANG
-βTM-NG
Lane 1 2 3 4 5 6 7

Figure 4.6. Complementation of FtsY-depleted *E. coli* by FtsY deletions and fusion proteins. *A. E. coli* strain N4156::pAra14-FtsY' transformed with the indicated constructs were grown overnight in LB containing ampicillin, streptomycin, and 0.2% arabinose. Cells were washed twice with LB, and diluted in LB supplemented with 0.02 mM IPTG. 2.5 hours following the initial dilution cultures were diluted 1:100 in the same media. Error bars represent 1 standard deviation obtained from three independent cultures. *B. E. coli* strain N4156::pAra14-FtsY' transformed with empty vector (lane 1) and constructs expressing TM-ANG (lane 2), TM-NG (lane 3), FtsYAN (lane 4) and both FtsYAN and TM-NG (lane 5) were grown overnight in LB containing ampicillin, streptomycin, and 0.2% arabinose. Cells were diluted and grown for 4 additional hours in the same medium containing 0.02mM IPTG. They were then washed twice with LB, and diluted in LB supplemented with 0.02 mM IPTG. Following 1.5 hours growth equivalent number of cells were analyzed by SDS-PAGE and immunoblotting with anti-FtsY antiserum. The migration positions of bands corresponding to expressed proteins are indicated with arrowheads. *C. E. coli* strain N4156::pAra14-FtsY' transformed with the indicated constructs were grown overnight in LB containing ampicillin, streptomycin, and 0.2% arabinose. Cells were washed twice with LB, and diluted in LB supplemented with 0.02 mM IPTG. Cultures were maintained in conditions of exponential growth by repeated dilutions into prewarmed media. Cell density measurements are presented from 2.5 to 8.5 hours and from 24 to 28 hours following dilution into arabinose-free medium. Data points were obtained by multiplying the cell density at each hour by the dilution factor and dividing this value by the initial cell density at 2.5 hours (first 7 points) or 24 hours (last 5 points) following transfer into arabinose-free media. These values are expressed as a logarithmic function versus time.

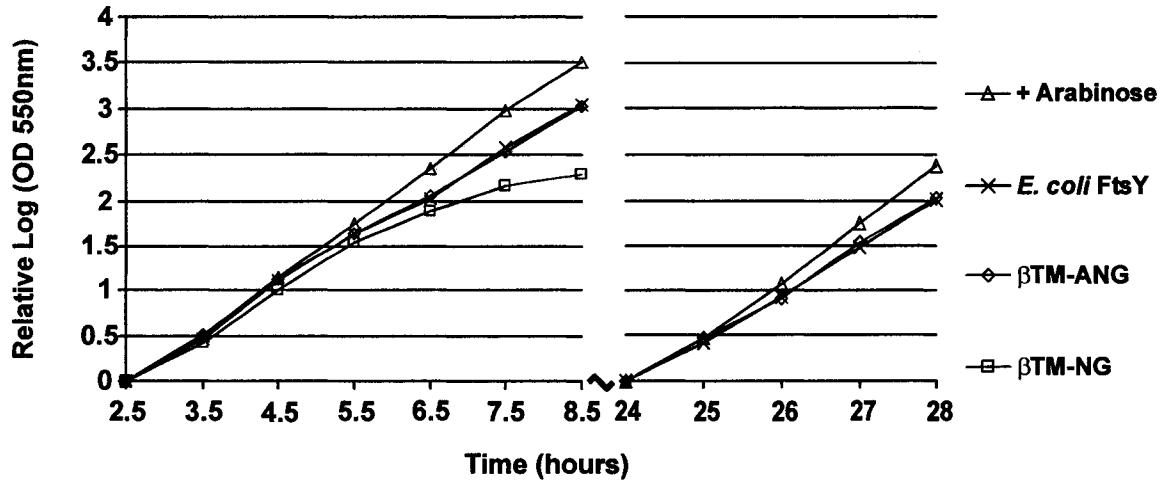
A


1.25
1
0.75
0.5
0.25
0
OD (550nm)
0
1.5
2.5
3.5
4.5
5.5
6.5
7.5
Time (hours)
E. coli FtsY
βTM-ANG
βTM-NG
FtsYAN + βTM-NG
FtsYAN
Vector

B


-FtsY (endog)
Lane 1 2 3 4 5

C


4
3.5
3
2.5
2
1.5
1
0.5
0
Relative Log (OD 550nm)
2.5
3.5
4.5
5.5
6.5
7.5
8.5
24
25
26
27
28
Time (hours)
+ Arabinose
E. coli FtsY
βTM-ANG
βTM-NG

independent transmembrane domain.

We generated a construct encoding the TM region from SRβ fused to most of the A and NG regions from *E. coli* FtsY (amino acids 41-497, βTM-ANG). This protein is expressed at levels equal to or greater than endogenous FtsY 1.5 hours following depletion of arabinose (Fig 4.6B, lane 2). βTM-ANG fractionated with membranes and is found in the pellet fraction upon centrifugation following incubation in carbonate (Fig 4.5, third panel), indicating that is integrated into the membrane. Depletion of FtsY from cells expressing TM-ANG resulted in growth rates indistinguishable from those observed for cells expressing plasmid-borne *E. coli* FtsY (Fig.4.6A). Thus, only when a substantial portion of the A region is present can a constitutively transmembrane form of FtsY fully complement depletion of endogenous FtsY.

The growth of cultures expressing TM-NG appeared to be stagnating 7.5 hours following depletion of arabinose although at this cell density (OD_{550} ~ 0.3) cells are expected to be in conditions conducive to exponential growth. We investigated whether cells expressing TM-NG were experiencing a growth defect by performing a growth analysis of strain N4156::pAra14-FtsY' expressing plasmid-borne FtsY, βTM-ANG, and βTM-NG under conditions where cultures were constantly maintained in conditions conducive to exponential growth (Fig 4.6C). In each case, cells were continuously diluted 1:50 into fresh, prewarmed media prior to reaching a cell density (OD_{550}) of 0.4. The data points following the initial 2.5 hours of endogenous FtsY depletion were obtained by multiplying the cell density at each hour by the dilution factor and dividing

this value by the initial cell density 2.5 hours following transfer into arabinose-free media. By expressing this as a logarithmic function versus time a linear curve is indicative of exponential growth.

Growth measurements of cells expressing plasmid-borne FtsY and βTM-ANG produced essentially linear curves on a logarithmic plot for the duration of this analysis (Fig 4.6C). In contrast, cells expressing βTM-NG grew exponentially for the first three hours of this analysis, albeit at a lower rate than the cells expressing FtsY and βTM-ANG (Fig. 4.6C). In the three following hours the growth curve of cells expressing βTM-NG became non-linear. Furthermore, when subjected to further dilutions beyond 9.5 hours no further growth was detected for cells expressing βTM-NG, whereas cells expressing FtsY and βTM-ANG continue to grow indefinitely.

To determine whether cells expressing plasmid-borne FtsY and βTM-ANG continue to grow at the same, decreased rate in comparison to those expressing FtsY from the chromosomal araB promoter we maintained cultures in exponential growth conditions through further dilutions into LB (containing streptomycin to maintain plasmid in all cells) until 24 hours following transfer to arabinose-free medium. Cell density measurements were performed on these cultures under exponential growth conditions (Fig. 4.6C). Growth of cells expressing plasmid-encoded FtsY and βTM-ANG was indistinguishable at 2.5 hours and 24 hours following inoculation into arabinose-free media. Thus, expression of FtsY from a plasmid in strain N4156::pAra14-FtsY' in the absence of arabinose results in growth slower than cells expressing FtsY from the

arabinose promoter. Thus this difference in growth rates is maintained for at least twenty eight hours of growth. Taken together, the results in Figs 4.4A, 4.6A and 4.6C suggest that in *E. coli* the A region of FtsY has a role in FtsY function other than membrane binding.

Since we found an acidic A region was required for full complementation of FtsY depletion in *E. coli* but that βTM-NG supported growth for at least seven hours following FtsY depletion, we tested whether the amino-terminal region and carboxyl-terminal regions of FtsY could function independently. Previous examination of the separability of domains in FtsY examined the expression of A and NG as separate polypeptides (Herskovits et al., 2001). However, we have previously demonstrated that the A and N regions must be expressed together in order to bind membranes (Chapter II). For this reason we expressed AN together with βTM-NG in strain N4156::pAra14-FtsY'. As expected, expression of the AN region alone failed to complement FtsY depletion (Fig. 4.6A). Growth of cells expressing AN and βTM-NG was the same as in cells expressing TM-NG alone (Fig. 4.6A). Thus, full complementation requires that the A and NG regions are physically linked.

4.5 Discussion

Prokaryotic species exist in which FtsY is primarily cytoplasmic, membrane-bound or in mixed populations. The N and G regions are highly homologous between FtsY and its mammalian homologue, SRα (Bernstein et al., 1989; Romisch et al., 1989).

Furthermore, they share a high degree of sequence similarity to the N and G regions of the cytoplasmic SRP GTPases; Ffh in prokaryotes and SRP54 in eukaryotes (Bernstein et al., 1989; Romisch et al., 1989). However, the amino-terminal A region of *E. coli* FtsY is unique, bearing no resemblance to the amino-terminal domain of SRα or any other translocation GTPase. As a number of microbial genomes have been sequenced over the past several years, it has come as a surprise that the amino-terminal domains of FtsY proteins are also highly divergent amongst prokaryotic species. They can be broadly divided into four categories (Fig 4.1); Group A: highly negatively charged regions that resemble the A region of *E. coli* FtsY; Group B: short (<35 amino acid) regions with a net positive charge; Group C: regions containing a putative single-pass transmembrane region; Group D: identified only in the blue-green algae *Synechocystis sp. PCC6803* thus far, consisting of a moderately negatively charged (net charge of -18) region with barely detectable (22%) similarity to the A region from *E. coli* FtsY.

Previous investigations have demonstrated that the amino-terminal region of *E. coli* FtsY is involved in membrane binding (Chapters II and III)(Powers and Walter, 1997). The differences in the sequences of amino-terminal regions of FtsY between species suggest that either the A region has a limited membrane-binding function that can be provided by a variety of types of sequences, or that the A regions, where present, have species-specific functions. The report that *E. coli* depleted of endogenous FtsY survived when the A region of *E. coli* FtsY was replaced with a multi-spanning transmembrane domain from LacY (Zelazny et al., 1997) suggests that the only function of the A domain

is binding FtsY to the membrane. Extrapolation of this result to other prokaryotes predicts that the FtsY proteins from group B organisms are targeted to the membrane very differently than the FtsY proteins from groups A and C.

We confirmed that *E. coli* FtsY exists in a mixed population between the membrane and cytoplasm (Fig 4.3), and that it is only peripherally associated with the membrane (Fig 4.5) Our predictions regarding the cellular localization of proteins from groups B and C were confirmed using representative FtsY proteins from each group. *B. subtilis* FtsY, which contains a 20 amino acid positively charged amino-terminal region, does not target to the membrane during vegetative growth (Fig. 4.3). At the other end of the spectrum the amino-terminal region from *S. coelicolor* constitutively integrates FtsY into the membrane (Figs. 4.3 and 4.5). Although we were unable to follow endogenous FtsY upon fractionation of *S. coelicolor*, the characteristics of TM segments across species are conserved, as are the latter steps in the translocation process (Wallin and von Heijne, 1998). Therefore, our observation that the *S. coelicolor* amino-terminal region functions as a transmembrane signal anchor in *E. coli* suggests a similar function in *S. coelicolor*. It therefore appears that different prokaryotic species have developed FtsY proteins with divergent amino-terminal regions, resulting in differing subcellular localization.

The NG domain of B. subtilis FtsY is not functionally interchangeable with the NG domain from E. coli FtsY– We found that although *B. subtilis* survive with an FtsY protein that is entirely cytoplasmic, expression of this protein in *E. coli* fails to

complement depletion of endogenous FtsY (Fig 4.4). This was not surprising, since this protein contains only a short, positively charged region amino-terminal to the NG regions. Expression of the *E. coli* FtsY NG region alone fails to support growth of *E. coli* upon depletion of full-length FtsY (Zelazny et al., 1997).

We were surprised, however, that when the *B. subtilis* NG domain was targeted to the *E. coli* inner membrane by the membrane-binding domain of *E. coli* FtsY, complementation of FtsY depletion was not observed (Fig 4.4). It seems unlikely that this is due to defects in the GTPase activity of *B. subtilis* NG, since both species contains the four consensus GTPase elements of the GTPase superfamily (Althoff et al., 1994). Comparing *B. subtilis* FtsY to the NG regions of FtsY proteins from *E. coli* and *H. influenzae*, both of which complement FtsY depletion in *E. coli* (Fig 4.4), the greatest differences are found in the N region (Fig. 4.2). This region in *B. subtilis* FtsY shares only 32% amino acid identity with *E. coli* FtsY, compared to the 67% identity shared between *E. coli* and *H. influenzae* FtsY. The N region makes extensive contacts with the GTPase domain that may be important for interactions with Ffh (Lu et al., 2001; Shepotinovskaya and Freymann, 2002) and signal sequences (Cleverley and Gierasch, 2002). In this regard, one significant amino acid substitution occurs at position 36 of *B. subtilis* FtsY, in which a lysine residue replaces a leucine that contacts multiple conserved residues from both the N and G region (Montoya et al., 1997). This may cause a significant conformational change in this region that could affect the activity of the NG region.

Taken together, the observations that in *B. subtilis* FtsY is cytoplasmic and that its NG domain is not functionally interchangeable with the NG domain from *E. coli* FtsY suggest that in *B. subtilis* the mechanism of protein targeting differs from that in *E. coli*. Although *B. subtilis* contain a homologue of Ffh, the protein that binds FtsY in *E. coli*, it is not clear that in this organism FtsY is involved in protein targeting. No homologue of SecB is present in *B. subtilis*, and Ffh binds directly to SecA (Bunai et al., 1999), an event that has not been observed in *E. coli*. Furthermore, although in *E. coli* Ffh and FtsY are primarily responsible for targeting integral membrane proteins (Ulbrandt et al., 1997; Valent et al., 1998), over 80% of secreted proteins depend on both SecA and Ffh in *B. subtilis* (Hirose et al., 2000). Since the NG domain of *B. subtilis* FtsY is not functional in *E. coli* when fused to the A region and it is difficult to reconcile an entirely cytoplasmic SRP receptor with membrane-targeting of nascent proteins in *B. subtilis*, it may be that Ffh and SecA constitute the protein targeting pathway in *B. subtilis*, and that the cytoplasmic FtsY is involved in a separate function.

Constitutively transmembrane FtsY requires the A region for extended survival of E. coli – A putatively transmembrane form of FtsY, comprised of four transmembrane domains from LacY fused to the NG domain of FtsY has been demonstrated to rescue *E. coli* cells from death and protein-targeting defects when FtsY was depleted (Zelazny et al., 1997). This led the authors to conclude that the NG domain is "fully functional", and that the A region "is required only for the targeting of the C-terminal NG domain of FtsY to the membrane." (Zelazny et al., 1997). Given the unique nature of the A region of *E.*

coli FtsY, its extremely negatively charged character, and the observation that over 50% of FtsY is cytoplasmic in *E. coli*, we were surprised by this observation.

However, our demonstration of a naturally-occurring transmembrane region in FtsY from *S. coelicolor* (Figs 4.3 and 4.5) suggested that prokaryotes from group C (Fig 4.1) survive with FtsY that lacks an A region and is localized entirely at the membrane. We determined that a fusion of the transmembrane region from *S. coelicolor* FtsY to the *E. coli* NG region supported growth in FtsY-depleted *E. coli* to a greater extent than a vector control (Fig 4.4) even though *Str*TM-NG apparently quantitatively integrated into the membrane (Fig. 4.5). Although this result confirmed that a transmembrane form of FtsY was at least partially functional, the observation that cells expressing *Str*TM-NG eventually die led us to question the extent to which complementation has been achieved by other transmembrane FtsY fusion proteins.

Upon careful appraisal of the report by Zelzany *et al.* (1997) it is clear that the colonies observed upon complementation with a transmembrane fusion protein containing the *E. coli* NG region alone were smaller than equivalent cultures expressing wild type FtsY (Zelazny et al., 1997). The growth curves reported in (Zelazny et al., 1997) also leave some question as to whether the NG region is "fully functional" when fused to an unrelated TM domain. Cells expressing a multi-spanning TM region fused to most of A and NG from *E. coli* FtsY (pCLN4-Δ92) approached saturation (OD_{600}~1) approximately 7 hours following depletion of arabinose. In contrast, at this time cultures expressing the multi-spanning TM region fused only to NG (pCLN4-Δ198) exhibited an

optical density (OD_{600}) of less than 0.5 and failed to approach saturation even 9 hours following depletion of arabinose (Zelazny et al., 1997). Nevertheless, the authors reported that the A region was dispensable and could be replaced with a transmembrane region.

In order to re-examine the importance of the A region in more detail we generated growth curves for an extended period (Fig 4.6C). Also, rather than utilizing the multi-spanning transmembrane segments employed previously (Zelazny et al., 1997), we attempted to emulate what is observed in *S. coelicolor* and used a single-pass TM region at the extreme amino-terminus of the protein (Fig 4.2).

When all but 43 amino acids of the A region of *E. coli* FtsY were present along with NG in the transmembrane protein, growth was slightly slower than when chromosomal FtsY was expressed from the arabinose promoter, but indistinguishable from complementation with plasmid-borne *E. coli* FtsY (Figs 4.6A and C, "βTM-ANG"). These cells survive for at least 28 hours following depletion of arabinose, and continue to grow at a constant rate (Fig 4.6C). This confirmed that an entirely transmembrane form of FtsY could function as well as wild type FtsY in *E. coli* when all but the amino-terminal 43 amino acids of *E. coli* FtsY are present. This is consistent with the previous report in which cells depleted of endogenous FtsY but expressing a fusion to all but the amino-terminal 92 amino acids of FtsY (containing a large portion of the A region) grew at rates marginally slower than when endogenous FtsY was expressed from the arabinose promoter, and produced colonies of comparable size (Zelazny et al., 1997).

However, when the A region of *E. coli* FtsY was absent from the fusion protein, cells survived for only approximately six generations longer than a vector control following removal of arabinose from the growth media (Fig 4.6A and C, βTM-NG). These cells also grew much more slowly than those expressing WT FtsY or βTM-ANG and eventually exhibited limited proliferative potential approximately 9.5 hours following arabinose depletion. Our analysis of growth for an extended period demonstrates that the conclusions derived from the previous study (Zelazny et al., 1997) do not accurately describe the role of the A region.

Together, these results suggest that *E. coli* FtsY can be fully functional when it is entirely integrated into the membrane if the carboxyl-terminal 154 amino acids (our investigation) or 105 amino acids (Zelazny et al., 1997) of the A region are present. Furthermore, since a strain expressing TM-NG survives for an extended period compared to a vector control, it appears that constitutive localization of the NG domain to the membrane allows *E. coli* to survive better than in the complete absence of FtsY, or when most of the A region is present but targeting to the membrane is blocked. The observation that *E. coli* grow indefinately with a constitutively transmembrane FtsY but require a significant portion of the A region suggests an additional function for the A region aside from membrane binding.

It was not clear whether the additional role for the A region requires that it be physically linked to the NG region. A previous attempt to determine this found that cells continue to grow when the NG region is proteolytically cleaved from the A region

following targeting to the membrane (Herskovits et al., 2001). However, growth was only assessed for five hours following initiation of cleavage, during which time a slight defect was observed (Herskovits et al., 2001). *E. coli* continue to grow for longer than five hours when expressing βTM-NG, and since longer-term growth was not assessed, it can not be ruled out that following cleavage of the A region in the previous study cells would have been ultimately inviable. Furthermore, we have previously found that the full AN region is required for membrane binding, and that proteolytic cleavage occurs between the N and G regions in a portion of membrane-bound FtsY (Chapter II). Therefore, to accurately determine whether the functions of the amino-terminal and carboxyl-terminal regions of FtsY could be separated at the membrane we measured cell growth upon expression of FtsYAN and βTM-NG in the same cells (Fig 4.6A). Since these cells grew at the same rate as those expressing βTM-NG alone, it appears that the additional function of the A region is only observed when it is physically linked to the GTPase domain of FtsY.

Diverse acidic regions can perform the role of the A region of FtsY in E. coli – Of the genomes sequenced to date, the FtsY protein encoded by *Salmonella typhimurium* is the most closely related to that of *E. coli*. Since these proteins are 83% identical, we considered it of greater interest to analyze FtsY from a close relative that had more distinct differences in sequence. *H. influenzae* FtsY is 53% identical to *E. coli* FtsY, with 38% identity in the A region (Fig. 4.2). Although it is one of the most closely related proteins identified thus far, the A region is surprisingly different in that it is

approximately half the length of the A region from *E. coli* FtsY. Despite this difference, *H. influenzae* FtsY complements depletion of endogenous FtsY with growth rates essentially identical to wild type *E. coli* FtsY (Fig. 4.4). Furthermore, FtsY from the less-related organism *Neissseria gonorrhoeae* (also from Group A, Fig. 4.1) is at least partially functional in *E. coli,* as it restores protein targeting in *E. coli* depleted of endogenous FtsY, and can replace *E. coli* FtsY in an *in vitro* translocation assay (Arvidson et al., 1999).

The primary feature common to the A regions of FtsY in these species is the very high ratio of acidic amino acids, ranging from .214 acidic residues/position in *N. gonorrhoeae* to .294 acidic residues in *E. coli*. Other amino acids are poorly conserved in this region. Furthermore, no clear periodicity of the acidic residues is apparent, but they are essentially evenly dispersed throughout the sequence. The presence of these acidic residues and interspersed short aliphatic residues leads to a predicted alpha-helical secondary structure for most of the A region using the method described in (Pollastri et al., 2002).

The most striking difference between the A regions is the variability of length. That the length of the A region does not appear to be critical to its function is supported by complementation with TM fusions that contain portions of this domain. In our investigations, cells expressing a TM fusion missing the 44 amino-terminal amino acids grew as well as those expressing full-length FtsY (Fig. 4.6A and C). Furthermore, our analysis of (Zelazny et al., 1997) suggests that the TM fusion missing the 92 amino-

terminal amino acids of FtsY also fully complements growth (see above).

The diversity in length, predicted secondary structure, and presence of numerous disperse acidic residues in the A regions of Group A FtsY proteins is reminiscent of eukaryotic tropomyosin. The tropomyosins are a highly conserved family of actin binding proteins found in most eukaryotic cells. They are two-chained parallel coiled-coil alpha-helical proteins that bind cooperatively in the long pitch grooves of helical actin filaments (reviewed in (Stewart, 2001)). Although longer in higher eukaryotes, tropomyosin homologues in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are of similar length (161 to 199 amino acids) to the A regions of group A FtsY proteins, and have a similar acidic residue ratio (0.21 to 0.29 per amino acid position). These similarities suggest that in *E. coli* and related organisms the A regions of FtsY might function as dimerization motifs, and may interact with filaments within the cell.

Also common to all FtsY proteins except those containing a transmembrane region is a basic region of approximately 10 to 20 amino acids at extreme amino-terminus. We have previously shown that in *E. coli* FtsY this region is necessary, but not sufficient for membrane binding (Chapter II). Our observation that expression of βTM-ANG, in which the positively charged region is missing, is sufficient for growth indistinguishable from cells expressing *E. coli* FtsY (Fig. 4.6) indicates that the region at the extreme amino-terminus of FtsY is not required for the additional function of the A region beyond membrane-binding.

The basis for the necessity of the A region in *E. coli* and related FtsY proteins is

currently unclear. We and others have demonstrated that the A region is required for normal membrane assembly of FtsY (Chapter II)(Powers and Walter, 1997). We have also shown that FtsY targeting to the membrane occurs through interactions of the A and N regions with both a lipid component and an as-yet unidentified protein (Chapter III). We speculate that the presence of a TM region may substitute for the lipid-binding properties of the A region in initial targeting of the receptor to the membrane. However, it is likely that extended survival of *E. coli* requires that the AN region interacts with another membrane protein counterpart, which may only be possible when at least part of the A region is present. Significantly, we have shown that binding to the membrane protein is sufficient to maintain viability when lipid binding is abolished (Chapter III).

We can not exclude the possibility that cells are not viable for an extended period when expressing βTM-NG because of a secondary effect due in part to misregulation of FtsX and FtsE. Since *ftsY* exists on an operon with *ftsE and ftsX*, it is possible that expression of these genes is reduced in the absence of arabinose. This effect is not lethal, however, as cells complemented with FtsY or TM-ANG survive indefinitely. Thus it appears more likely that if deregulation of FtsX and FtsE has an effect it accounts for reduced growth rates observed when FtsY depletion is complemented with wild type FtsY (Figures 4.4 and 4.6). However, it remains a formal possibility that the limited proliferative potential of cells expressing βTM-NG may be a synthetic lethal effect due to both the absence of the A region and disregulation of FtsX and FtsE. This scenario would suggest either a role for FtsX and FtsE in protein targeting, or for the A region of FtsY in

a second pathway that involves the other proteins located in the same operon. Regardless of whether there is a synthetic lethal effect with FtsX and E or alternatively that the A region is in some way necessary for the function of FtsY in the SRP pathway of *E. coli*, it is clear that the A region has at least one function beyond targeting FtsY to the inner membrane of bacteria. The challenge before us is to discover the roles of the various A regions in different organisms.

CHAPTER V

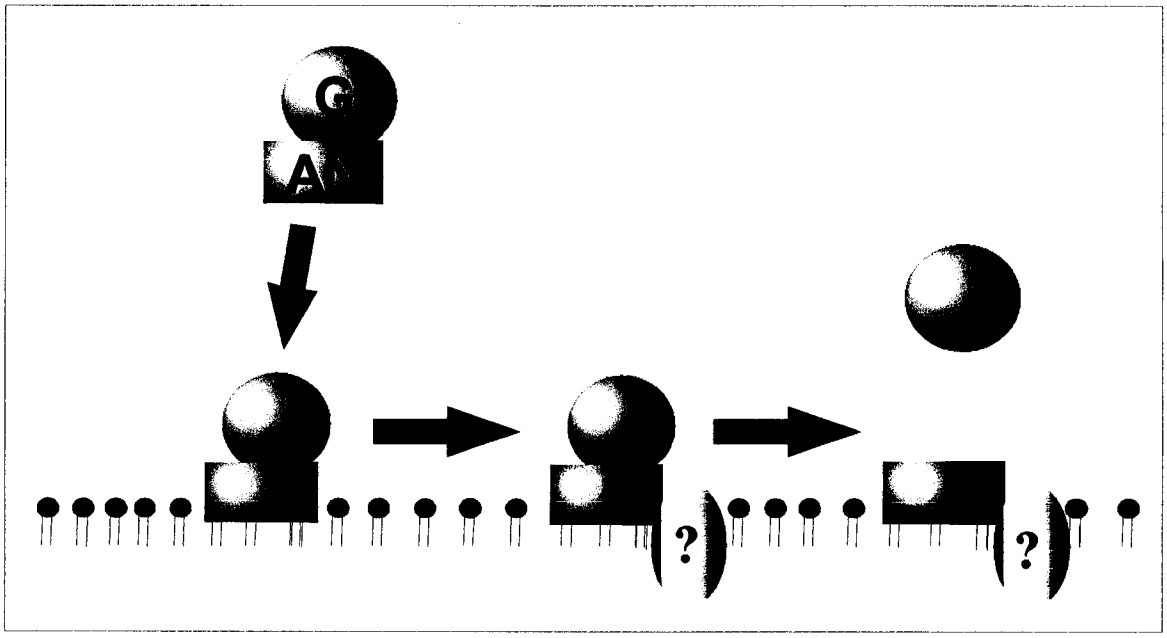
Conclusions

5.1. A simple model for binding of *E. coli* FtsY to the membrane

The nature of FtsY binding to the *E. coli* inner membrane has been a matter of some confusion over the past half-decade (de Leeuw et al., 1997; Zelazny et al., 1997; de Leeuw et al., 2000). The data presented in this thesis provides a comprehensive analysis of this process. We found that *E. coli* FtsY binds the inner membrane via an amino-terminal domain consisting of the A region, which contains a short positively charged stretch at its extreme amino-terminus followed by a highly negatively charged region of 177 amino acids, and the N region (Chapter II). This entire region is defined by a specific proteolytic event between the AN and G regions that cleaves a large portion of membrane-bound FtsY (Chapter II). The mechanism by which FtsY binds the membrane involves a specific interaction with the phospholipid PE and an as-yet unidentified membrane protein (Chapter III). This basic model for FtsY membrane binding in *E. coli* is depicted in Fig. 5.1.

Additional complexities in this process in *E. coli* and across species were also identified (Chapter IV). The A region, responsible in part for membrane binding, is highly divergent between bacterial species. Different microbial species exist in which FtsY is entirely cytoplasmic, entirely transmembrane, or mixed between the cytoplasmic and membrane populations (Chapter IV). Furthermore, although FtsY from *E. coli* can exist localized entirely at the inner membrane, we determined that its A region is required for an additional function that is necessary for extended survival (Chapter IV).

Figure 5.1. Simple Model for Membrane-binding by *E. coli* FtsY. The AN region of FtsY binds the membrane initially through an interaction with the abundant phospholipid PE (red). Once at the membrane the AN region binds to an unknown membrane protein (blue) and FtsY is cleaved between the AN and G regions by a membrane-bound protease.


G
A
?
?

5.2 The region of FtsY involved in membrane binding

We found that the entire AN region of FtsY was both necessary and sufficient for membrane-binding in *E. coli* (Chapter II). In contrast to a previous report (de Leeuw et al., 1997), neither the A region alone, nor the NG region were found to bind membranes in our investigations. One of the primary differences that may account for this discrepancy is our utilization of column chromatography and floatation assays for membrane-binding. FtsY aggregates *in vitro* and a significant proportion pellets in the absence of membranes (Chapter II). Since the assertion that the A and NG regions bound membranes independently was based primarily upon simple pelleting assays (de Leeuw et al., 1997), we speculate that aggregated protein found in the pellet following centrifugation may have been mistakenly identified as membrane-bound.

In a later study, lipid-binding activity was ascribed to the NG region, and used as further evidence that this region can independently interact with the membrane (de Leeuw et al., 2000). Two essential differences between this report and the data presented in Chapter III may account for the apparently conflicting observations. Firstly, in (de Leeuw et al., 2000) the ability of NG to bind liposomes was demonstrated only in the presence of purified full-length FtsY. Since FtsY either aggregates or forms multimers when purified (Luirink et al., 1994), it seems likely that FtsYNG was observed to cofractionate with liposomes because it was bound to full-length FtsY, which we have demonstrated binds liposomes (Chapter III). Furthermore, the apo forms of FtsY and FtsYNG were used in these investigations, and liposome binding was performed in the absence of nucleotide

(de Leeuw et al., 2000). In our assays for membrane- and liposome-binding, both GTP and GDP were abundant (Chapter II, III). Thus, it is possible that in its conformation in the absence of nucleotide FtsYNG displays lipid-binding activity, whereas when bound to nucleotide as in our investigations this activity is diminished or absent. In this manner binding to membrane lipids may regulate GTPase activity or vice-versa.

A later report by some members of the same group acknowledged that, as we found in Chapter IV, the NG domain must be linked to a membrane-targeting region to function in the cell (Herskovits et al., 2001). However, once targeted to the membrane, the A region could be proteolytically removed *in vivo* and cells survived for at least 5 hours longer than cells not expressing FtsY (Herskovits et al., 2001). However, extended viability of these cells was not tested, and the data presented indicates growth is slightly slower when the A region is removed than when the full-length FtsY is expressed. The rate of growth may be similar to our observations when the NG domain alone was targeted to the membrane by a TM domain (Chapter IV). If this were to be verified, it would suggest that when the NG domain is released from the A region at the membrane, cells experience the same growth defect as when the A region is absent in transmembrane fusions to FtsYNG.

The abovementioned report (Herskovits et al., 2001) does raise the possibility that the NG region may interact with the membrane under some conditions. However, the NG region is clearly not responsible for targeting the full-length protein to the membrane. Other studies have supported our identification of the amino-terminus of FtsY as the *bona*

fide membrane-binding domain. Removal of as little as 47 amino acids from the amino-terminus of FtsY prevents membrane-binding *in vitro* (Powers and Walter, 1997), and removal of 92 amino acids prevents integration of an integral membrane protein and complementation of FtsY depletion *in vivo* (Zelazny et al., 1997). Furthermore, in the latter study, as well as in Chapter IV of this thesis, replacing only part of the A region of FtsY with an unrelated transmembrane region reconstituted membrane-binding and function. Thus, it is clear that when part or all of the A region is present NG does not function as the membrane-assembly domain. Any lipid-binding activity the NG region may have must play some other, as-yet unidentified, role in FtsY function.

Several regions with distinct characteristics make up the minimal membrane-binding domain of FtsY. The core of this region is the highly negatively charged sequence between amino acid residues 20-197 of the A region. Our determination that *E. coli* FtsY binds a phospholipid with a zwitterionic head group (PE) may partially explain the role of this region, although the entire AN domain is required for binding phospholipid vesicles (Chapter III), refuting the possibility that this is a simple bulk charge-charge attraction. The analysis of this region from FtsY proteins of multiple related species, at least one of which complements endogenous FtsY depletion *in vivo*, suggested that the main requirement in this region may be a high concentration of disperse acidic amino acid residues (Chapter IV).

The sequence of amino acids enriched in basic residues at the extreme amino-terminus of FtsY (amino acids 1-20) is also required for membrane-binding by FtsY

(Chapter II). However, this region is not sufficient for membrane-binding (Chapter II). A short, positively charged region at the extreme amino-terminus is the only characteristic that is common to the amino-terminal regions of all prokaryotic FtsY homologues except those containing predicted TM sequences at this position (Gorodkin et al., 2001). Whether the positively charged sequence makes specific contacts with the membrane or is important for the overall folding of the membrane-binding domain of *E. coli* FtsY will probably be resolved only when a molecular structure of the entire FtsY molecule is obtained.

The necessity of the N region for FtsY membrane-binding was unexpected. Homologuous N regions are present in the cytoplasmic Ffh and SRP54 proteins (Bernstein et al., 1989; Romisch et al., 1989), as well as SRα, in which regions outside the N domain are responsible for membrane-binding (Young et al., 1995). The N region makes extensive contacts with the GTPase domain in FtsY (Montoya et al., 1997), and in Ffh and SRP54 binds to the signal sequence (Newitt and Bernstein, 1997; Cleverley and Gierasch, 2002).

One intriguing possibility is that the N domain may serve as a conduit of information between the GTPase domain and the membrane-binding region. The N region undergoes a conformational change upon the interaction of FtsY with Ffh (Shepotinovskaya and Freymann, 2002). We have also observed a conformational change in the N region upon binding to PE (Chapter III). Furthermore, we observe close to 100% membrane-binding when the G region is absent from FtsY, whereas the membrane-

binding efficiency of the uncomplexed full-length molecule is greatly reduced (Chapter II). Thus, membrane-binding of FtsY may be regulated by additional factors that interact with the G domain, such as binding to SRP-RNC complexes, and this regulation may be transmitted through conformational changes in the N region. To examine this possibility the efficiency of FtsY membrane-binding could be compared in the presence and absence of *E. coli* SRP-RNC complexes and GTP. A structure of the entire FtsY protein, especially bound to SRP, would also provide invaluable insight in this area.

5.3 Cleavage between the AN and G domains

Following membrane-assembly, FtsY is efficiently cleaved between the AN and G regions *in vitro* (Chapter II). This cleavage is also observed *in vivo*, but its extent and functional significance are unclear. A number of possible roles for cleavage can be envisaged, several of which are considered below.

Possibly the most simple explanation for cleavage of FtsY is protein turnover. Excess FtsY that is bound to the membrane through an interaction with PE, but is not associated with the other protein components with which it functions in protein targeting could lead to unproductive targeting reactions by SRP-RNC complexes. This would likely have an effect similar to other disruptions in the SRP pathway, causing a build-up of mis-targeted inner membrane proteins, and an eventual toxic accumulation of aggregated proteins in the cytoplasm (Bernstein and Hyndman, 2001). Cleavage in our investigations occured with FtsY targeted to the membrane as a single entity, not in a

complex with SRP. We also observed more efficient cleavage when gPa replaced the G domain (Chapter II). While this may be a consequence of greater accessibility at the cleavage site, this protein may instead be recognized and degraded because it is not a genuine FtsY protein. It would not be surprising, therefore, if, like SecY of the translocase (Kihara et al., 1995), FtsY is specifically targeted for degradation when it is not part of a functional translocation complex.

Alternatively, cleavage of FtsY following membrane-binding may serve to promote unidirectional targeting of the RNC complex to the translocase. In eukaryotes, this is accomplished by a series of steps that increases the binding affinity of the SRP GTPases (section 1.4.2). In *E. coli*, if cleavage is coupled to the targeting and translocation of nascent polypeptides then the conformational change that occurs in the N domain following the interaction with the SRP-RNC complex (Shepotinovskaya and Freymann, 2002) and membrane-binding (Chapter III) may provide access to a protease. Through coupling of FtsY-cleavage with membrane targeting of the RNC, targeting of the nascent chain to the IM would be unidirectional. Although this would require the turnover of a single FtsY molecule for each cycle of the targeting reaction, it would provide the important function of ensuring that, once targeted, nascent proteins remain at the membrane.

A further potential role for cleavage at the membrane may be exposure of a hidden epitope following membrane-binding. Analogous to zymogens, which require proteolytic processing to become active (Khan and James, 1998), a functional region in the AN

domain might be masked by the G domain until membrane-binding and subsequent cleavage occurs. If this region is dedicated to a process that involves FtsY at the membrane, it might be important that this region not be exposed in the significant cytoplasmic population of the protein, to prevent sequestration by FtsY of a component that is necessary at the membrane. Our observation that expression of the AN region in cells is not detrimental to growth (data not shown) partially refutes this suggestion. However, we have not defined the exact site of cleavage in the N region so our construct encoding FtsYAN may differ in a fundamentally important way from the cleaved product.

The identity of the protease that cleaves FtsY upon membrane binding is also still unknown. A number of criteria suggested that the integral membrane protease FtsH was a likely candidate, but depletion of this protease from INVs failed to elicit a reduction in cleavage (Chapter II). However, the low residual levels of FtsH that probably remained in these INVs may have been sufficient for cleavage of the small quantity of *in vitro*-translated FtsY bound to the membrane. A more conclusive analysis might be obtained through testing the ability of purified FtsH reconstituted in liposomes to cleave bound FtsY.

In follow-up investigations to determine the identity of the protease that cleaves FtsY, Felicia Vulcu observed FtsY membrane-binding and cleavage in INVs derived from strains in which the genes encoding the proteases YaeL, Lon, and ClpP had been deleted (data not shown). An exhaustive study of all known proteases in *E. coli* was beyond the scope of this investigation. Adding further complexity to such investigations, our

determination that cleavage by trypsin between the N and G regions is enhanced upon membrane binding (Chapter III) suggests this region may be accessible to multiple proteases.

To examine the role of FtsY cleavage in *E. coli* it will be essential to determine the exact cleavage site. Although generating deletions in this region was not successful in identifying this position (Chapter II), substitution of individual amino acids towards the carboxyl-terminus of the N domain might prove more successful. Alternatively, purification and mass spectroscopic analysis of a cleaved product either from whole cells or following targeting purified FtsY or FtsYAN-gPa to INVs would indicate the position of cleavage. Following this identification, the generation of a non-cleavable form of FtsY and the analysis of phenotypic properties of cells expressing this mutant would be valuable in elucidating the functional role of FtsY cleavage.

5.4 Why does FtsY bind PE?

Like its peripheral membrane counterpart SecA in the general secretory pathway (Breukink et al., 1992; Hendrick and Wickner, 1991), we found that FtsY binds to the membrane in part through an interaction with a phospholipid (Chapter III). SecA binds to acidic phospholipids whereas FtsY specifically binds the zwitterionic phospholipid PE (Chapter III).

Since SRP-dependent protein targeting was not impaired in the absence of PE (Chapter III), binding of FtsY to PE does not appear to be essential for SRP-dependent

protein targeting. This might be explained by the two-step membrane assembly process described in Chapter III, in which PE-binding initially restricts FtsY to the two-dimensional surface of the membrane and thereby may increase the apparent affinity for a specific proteinaceous membrane receptor. Although this could provide an evolutionary advantage under conditions of stress, PE binding would not be essential for FtsY function under most circumstances.

Alternatively, PE binding may regulate the function of FtsY. We found that FtsY undergoes a conformational change in the N region when FtsY binds PE (Chapter III). Due to the extensive contacts between the N and G regions (Montoya et al., 1997), lipid binding may act as a signal that regulates the activity of FtsY. Binding of SecA to acidic phospholipids regulates its ATPase activity (Lill et al., 1990), and may serve as an indicator that SecA is membrane bound. Binding to PE may similarly regulate FtsY, effecting either binding to the SRP-RNC complex, or the GTPase activity of FtsY. This hypothesis could be addressed by determining both the GTPase activity and ability of purified FtsY to bind SRP-RNC complexes in the presence and absence of phospholipid liposomes.

5.5 The identity of the membrane-bound receptor for FtsY.

At present, the identity of the protease-sensitive membrane receptor for FtsY is unknown (Chapter III). Furthermore, a mechanism must exist for the targeting of the RNC-SRP complex from the receptor to the translocation apparatus. It is possible that a

single protein may fulfill both roles. FtsY may bind an adaptor protein that links the SRP pathway to the translocase, or may bind directly to a component of the translocase.

The recently identified translocase-associated protein YidC is one candidate for an FtsY 'receptor'. Like FtsY, YidC is required for assembly of membrane proteins that use the SecYEG translocase as well as those that integrate into the IM in a 'Sec-independent' manner (Nouwen and Driessen, 2002; Samuelson et al., 2000), but is not required for translocation of secretory proteins via the general secretory pathway (van der Laan et al., 2001). By delivering nascent proteins directly from FtsY to YidC, the presence of the latter, required for IM protein integration, would be ensured in these translocation complexes.

Core components of the translocase are also candidates for the FtsY receptor. In Gram negative bacteria SecE contains three transmembrane domains instead of the single transmembrane domain in all other bacterial species in which the *secE* gene has been sequenced (Schatz et al., 1991). Although this connection is entirely circumstantial, the observation of the unique A region of FtsY and the extra TM domains of SecE in Gram negative bacteria suggests SecE should be considered as a candidate FtsY receptor.

A proteomic approach to identify proteins that interact with FtsY *in vivo* has recently been embarked upon by Felicia Vulcu (Andrews Lab). Using the 'tandem affinity purification' approach pioneered to identify protein complexes (Rigaut et al., 1999), Felicia has identified a number of potential candidates for the FtsY receptor (F. Vulcu, personal communication). Although connections with protein targeting are not

readily apparent for most of the copurifying proteins, SecA was identified as one target. However, since SecA is dispensable for the translocation of some SRP-dependent substrates (Koch et al., 1999; Scotti et al., 1999; Macfarlane and Muller, 1995) it would be surprising if this were the FtsY 'receptor'. Once candidates have been identified using this or another screen, further characterization will be required to verify that these proteins function as receptors for FtsY. Coimmunoprecipitations and Far Western analysis with FtsY would support an assertion that a candidate protein binds FtsY. Ultimately, putative receptors will have to be reconstituted in liposomes devoid of PE to authenticate them as FtsY receptors. Genetic studies would nicely complement such investigations. For example, depletion of the receptor should reduce SRP-dependent targeting and potentially alter cellular morphology in the same manner as FtsY depletion (Luirink et al., 1994).

5.6 SecA and FtsY display similarities in membrane binding.

One outcome of our investigation and those of other investigators is the identification of numerous similarities between *E. coli* FtsY and its Sec pathway counterpart, SecA. In addition to the obvious similarity that both proteins serve as membrane 'receptors' for the corresponding cytoplasmic component of the targeting reaction, they also share common features in their mechanisms of membrane-assembly: (1) FtsY and SecA are distributed between the membrane and cytosol in *E. coli* (Chapter IV)(Luirink et al., 1994; Cabelli et al., 1991) (2) Both bind a lipid component of the

membrane with low affinity and a membrane protein with high affinity (Chapter III)(Hendrick and Wickner, 1991); (3) Binding lipid results in conformational change in the protein (Chapter III)(Lill et al., 1990); (4) Both FtsY and SecA may contain two phospholipid binding sites, as each is able to cause aggregation of phospholipid vesicles (de Leeuw et al., 2000; Breukink et al., 1993). These similarities in parallel, but non-homologous pathways suggest that in *E. coli* these features are advantageous for membrane-targeting of nascent presecretory and membrane proteins.

The extent of these similarities suggests other potential parallels should be explored, such as whether, analogous to SecA, FtsY binds directly to the translocase and assists in translocation of nascent inner membrane proteins. Furthermore, SecA binds the nascent chain directly (Fekkes et al., 1997; Fekkes et al., 1998), and a recent report suggests that the NG domain of Ffh directly contacts signal sequences (Cleverley and Gierasch, 2002). Thus, it seems likely that the homologous NG domain of FtsY may interact with nascent IM proteins. SecA also performs an additional function by binding its encoding mRNA and down regulating its own expression (Dolan and Oliver, 1991). The possibility that FtsY has an additional function beyond its role in protein targeting is discussed in section 5.8.

5.7 FtsY A regions and cellular localization differ between prokaryotic species.

One of the great surprises of our investigations was the divergence between species in the amino acid sequence of the FtsY protein A regions. Furthermore, bacterial

species were identified in which FtsY was predominantly cytoplasmic, entirely transmembrane, or mixed between both locations (Chapter IV). It is clear from our investigations that the A region is important for more than just directing FtsY to the membrane, as in *E. coli* the A region is required for extended survival even when the protein is targeted to the membrane through an independent domain (Chapter IV).

The divergence in A region sequence and membrane localization suggests either that different bacteria have evolved separate specific requirements for SRP-dependent protein targeting, or that FtsY may have an additional or different function in some cells. Differences between species of prokaryotes in the A region and membrane localization may have arisen due to differences in membrane architecture or protein composition. It is conceivable, for instance, that in certain organisms FtsY is directed to the membrane only at times when a large number of polytopic proteins must be integrated. Alternatively, FtsY might function in a common pathway such as protein targeting for which the NG region is required, and alternate pathways that utilize the divergent A regions and cellular localization to accommodate species-specific requirements. As discussed in Chapter IV, it is equally likely that in species which contain only a short stretch of amino acids amino-terminal to the NG region (e.g. *B. subtilis*), the cytoplasmic FtsY may not function in protein targeting at all.

5.8 Is FtsY involved in cell division?

I present here circumstantial evidence suggesting that FtsY may be involved in cell division, a pathway in which its potential function has been largely overlooked. The *ftsY* gene was initially identified in a screen for temperature sensitive filamentation mutants as part an operon with two other genes, *ftsE* and *ftsX* (Gill and Salmond, 1987). Mutations in *ftsE* and *ftsX* that block cell division have not been reported to result in generalized defects in protein targeting or translocation (de Leeuw et al., 1999; Ukai et al., 1998).

Furthermore, although sequences homologous to all the other domains of the *E. coli* SRP pathway components can be identified in their eukaryotic counterparts, FtsY contains a unique amino-terminal domain that bears little resemblance to other protein sequences. We have demonstrated that for a fraction of membrane-targeted FtsY the amino-terminal domain is cleaved from the GTPase domain (Chapter II). Since the G domain is essential for the function of FtsY in SRP-dependent protein targeting (Chapter IV)(Miller et al., 1994), cleavage may define a domain which has a separate function at the membrane. We also determined that in *E. coli* the A region of FtsY is required for an unknown function in addition to its essential role in attachment of FtsY to the membrane (Chapter IV). Furthermore, at least one SRP-dependent substrate, LacY, appears to target to the membrane *in vivo* when the A region of FtsY is replaced with a TM region (Zelazny et al., 1997). Thus, the essential requirement for the A region may be in another cellular process.

Binding to two separate components on the membrane, PE and a protein (Chapter III) may also indicate a dual-role for FtsY. PE-binding by FtsY is dispensable for SRP-dependent protein targeting (Chapter III). In the absence of PE, *E. coli* proteins essential for early steps in cytokinesis localize to division sites, but no constriction occurs at these sites (Mileykovskaya et al., 1998). This suggests that an as-yet unidentified component essential for cell division is mislocalized or non-functional in the absence of PE. Together with the other supporting circumstantial evidence it is tempting to speculate that FtsY is the PE-binding protein required for cell division.

Further support for a cell-division role for the A domain of *E. coli* FtsY comes from the identification of DivIVA, a protein found only in Gram positive bacteria (Edwards and Errington, 1997). Although not sufficiently identical in sequence to FtsYA to be identified at the threshold of a BLAST sequence homology search, DivIVA is similar in length (169 amino acids) and contains numerous, disperse acidic residues (Fig 5.2). DivIVA is targeted to nascent division sites in *B. subtilis* (Edwards et al., 2000) and over expression of this protein or mutations in its encoding gene result in cell division defects (Cha and Stewart, 1997). Since FtsY proteins in Gram positive bacteria lack sequences homologous to the A region of *E. coli* FtsY, it is plausible that in these organisms DivIVA performs the same functions as does the A region of FtsY in *E. coli*.

If FtsYA is involved in cell division its activity may resemble that of the eukaryotic tropomyosin proteins. Like DivIVA and the A region of FtsY, tropomyosins contains numerous disperse acidic amino acids residues throughout their sequence; TPM1 from *Saccharomyces cerevisiae* contains 58 acidic residues out of 199 amino acids (Fig.

Figure 5.2. FtsYA, TPM1, and DivIVA Contain Numerous Disperse Negative Charges But Share Little Sequence Homology. *A.* The amino acid sequences of the A regions of *E. coli* FtsY (amino acids 1-197) and *N. gonorrhoeae* FtsY (amino acids 1-119), TPM1 from *Saccharomyces cerevisiae* and DivIVA from *B. subtilis* are presented. Acidic residues aspartate and glutamate are coloured red. The net negative charge per amino acid position is indicated. *B.* The above sequences were subjected to multiple alignment using the CLUSTALW method (Thompson et al, 1994). The Network Protein Sequence Analysis tool (Combet et al, 2000) was used to present the sequence alignment with identical residues indicated below the alignment with an asterix (*), strongly similar residues indicated with a colon (:), and weakly similar residues indicated with a period (.).

A

FtsYA (*E. coli*):
MAKEKKRGFFSWLGFGQKEQTPEKETEVQNEQPVVEEIVQAQEPVKASEQAVEEQPQAHTEAEAE
TFAADVVEVTEQVAESEKAQPEAEVVAQPEPVVEETPEPVAIEREELPLPEDVNAEAVSPEEWQA
EAETVEIVEAAEEEAAKEEITDEELETALAAEAAEEAVMVVPPAEEEQPVEEIAQEQEKPTKEGF
FA
(.294 acidic residues/position)

FtsYA (*N. gonorrhoeae*):
MFSFFRRKKKQETPALEEAQVQETAAKVESEVAQIVGNIKEDVESLAESVKGRAESAVETVSGAV
EQVKETVAEMPSEAGEAAERVESAKEAVAETVGEAVGQVQEAVATTEEHKLGWA
(.210 acidic residues/position)

TPM1:
MDKIREKLSNLKLEAESWQEKYEELKEKNKDLEQENVEKENQIKSLTVKNQQLEDEIEKLEAGLS
DSKQTEQDNVEKENQIKSLTVKNHQLEEEIEKLEAELAESKQLSEDSHHLQSNNDNFSKKNQQLE
EDLEESDTKLKETTEKLRESDLKADQLERRVAALEEQREEWERKNEELTVKYEDAKKELDEIAAS
LENL
(.291 acidic residues/position)

DivIVA:
MPLTPNDIHNKTFTKSFRGYDEDEVNEFLAQVRKDYEIVLRKKTELEAKVNELDERIGHFANIEE
TLNKSILVAQEAAEDVKRNSQKEAKLIVREAEKNADRIINESLSKSRKIAMEIEELKKQSKVFRT
RFQMLIEAQLDLLKNDDWDHLLEYEVDAVFEEKE
(.226 acidic residues/position)

B

```
FtsYA(coli)MAKEKKRGFFSWLGFGQKEQTPEKETEVQNEQPVVEEIVQAQEPVKASEQAVEEQPQAHT
FtsYA (gon)--------MFSFFRRKKKQETP----------------------ALEEAQVQETAAKV
TPM1       --MDKIREKLSNLKLEAESWQEKYEELKEKNKDLEQENVE-----KENQIKSLTVKNQQL
DivIVA     -------MPLTPNDIHNKTFTKSFR--------------------GYDEDEVNEFLAQV
                   ::      :                                :         :

FtsYA(coli)EAEAETFAADVVEVTEQVAESEKAQPEAEVVAQPEPVVEETPEPVAIEREELPLPEDVNA
FtsYA (gon)ESEVAQIVGNIKEDVESLAESVKGRAESAVET-----VSGAVEQVKETVAEMPSEAGEAA
TPM1       EDEIEKLEAGLSDSKQTEQDNVEKENQIKSLTVKNHQLEEEIEKLEAELAESKQLSEDSH
DivIVA     RKDYEIVLRKKTELEAKVNELDERIGHFANIEET---LNKSILVAQEAAEDVKRNSQKEA
           . :   .     :      : .   .          :.           :

FtsYA(coli)EAVSPEE--WQAEAETVEIVEAAEEEAAKEEITDEELETALAAEAAEEAVMVVPPAEEEQ
FtsYA (gon)ERVES------AKEAVAETVGEAVGQVQEAVATTEEHKLGWA-----------------
TPM1       HLQSNNDNFSKKNQQLEEDLEESDTKLKETTEKLRESDLKADQLERRVAALEEQREEWER
DivIVA     KLIVR-----EAEKNADRIINESLSKSRKIAMEIEELKKQSKVFRTRFQMLIEAQLDLLK
           .           .    . : :  .  :     .* .

FtsYA(coli)PVEEIAQEQEKPTKEGFFA-------
FtsYA (gon)-------------------------
TPM1       KNEELTVKYEDAKKELDEIAASLENL
DivIVA     NDDWDHLLEYEVDAVFEEKE------
```

5.2). It binds to and stabilizes actin filaments by inhibiting the dissociation of subunits at the end of the filament (reviewed in (Cooper, 2002)). Although bacteria were previously believed to lack actin filaments, it has recently been demonstrated that the prokaryotic protein MreB forms actin-like strands that assemble in the same manner as actin (van den Ent et al., 2001). Thus, one way in which FtsYA might be involved in cell division is through interactions with protofilaments like MreB that produce the requisite changes in morphology that enable cellular division. An inability to stabilize cellular protofilaments would also explain why *E. coli* cells depleted of FtsY undergo gross changes in cellular architecture (Luirink et al., 1994).

An alternative, or perhaps additional manner by which *E. coli* FtsY may function in cell division is suggested by the properties of FtsY from *N. gonorrhoeae.* This protein, which contains a similar A region, binds DNA *in vitro* (Arvidson and So, 1995). It has been postulated that this binding occurs not through sequence-specific interactions, but rather through recognition of secondary or tertiary structure formed by the DNA (Arvidson and So, 1995). Conceivably, FtsY proteins containing A regions that resemble that found in *E. coli* may function in cell division by interacting with the chromosome at the membrane. Perhaps not coincidentally, *B. subtilis* DivIVA interacts with the chromosome segregation machinery to help position the origin of replication at the cell pole in preparation for polar division (Thomaides et al., 2001).

A role for the A region of FtsY in cell division would provide a satisfactory answer as to why this region is so divergent between prokaryotic species, and may exist as a separate protein in others. That is, because the process of cell division is so diverse.

E. coli and other proteobacteria are rod-shaped, have two membranes and divide symmetrically in a constriction process (reviewed in (Nanninga, 2001)). *Bacillus* species, whilst rod-shaped, contain only a single membrane, divide asymmetrically using an ingrowing septum and can form spores. *S. coelicolor* has a complex life cycle that includes the growth of aerial hyphae, and cyanobacterial cell division occurs in response to circadian cycles. Although the great divergence in the A region and consequent FtsY subcellular localization may be surprising for a protein that functions in a well-conserved pathway such as SRP-dependent protein targeting, it may not be so for a protein that may function in cell division, a pathway with diverse requirements between species.

Various approaches might be taken to determine whether FtsY has a role in cell division. Observing FtsY colocalized with the cell division apparatus using fluorescence microscopy with immunolabelled cells or GFP tagged FtsY would suggest a role in this process. Also, if, as predicted, the A region of FtsY is integral to this function then βTM-NG may be localized differently than βTM-ANG in the cell. Cells depleted of FtsY or expressing βTM-NG may exhibit spiral structures similar to those observed upon depletion of PE (Mileykovskaya et al., 1998). Furthermore, *B. subtilis* might provide a natural system in which to determine whether the A region of FtsY can function in cell division. If the A region of FtsY is functionally analogous to the DivIVA protein then expression of FtsYA or FtsYAN in a DivIVA deletion strain may alleviate cell division defects.

5.9 Concluding remarks

Increasingly, scientists are being charged to not only provide an understanding of the fundamental systems that govern natural systems, but to do so in the context of enhancing the general well-being of humankind. During the course of our investigations it has been determined that the SRP pathway in prokaryotes is responsible for membrane-targeting of polytopic IM proteins, especially those with extensive hydrophilic regions (Newitt et al., 1999). One class of proteins that fit these criteria are those responsible for multi-drug efflux (Van Bambeke et al., 2000). Membrane-integration of at least one such transporter, AcrB, is SRP-dependent in *E. coli* (Ulbrandt et al., 1997). Since multi-drug efflux may be responsible for up to 70% of clinical isolates of drug-resistant prokaryotic pathogens (Zhong and Shortridge, 2000), determining ways by which this pathway can be blocked might be of great therapeutic value.

Our identification of the A region as an essential part of *E. coli* FtsY responsible for membrane-binding and another, as-yet unidentified, role could provide a starting point for studies that lead to the development of a therapy to reduce antibiotic drug resistance in pathogens. Through the development of drugs that target the membrane-binding properties of the A region, which is unique to prokaryotes, or developing drugs that target the as-yet unidentified role of the A region beyond membrane-binding, such treatments may arise. Furthermore, since A regions are highly divergent between prokaryotic species it may be possible to design highly specific therapies against certain classes of pathogens.

Although the investigations detailed in this thesis were performed due to a natural

curiosity and a desire for greater understanding of the basic principles that govern a single system in prokaryotes, they may unexpectedly provide the basis for the advancement of medicine and greater well-being in our society. It is precisely because we often can not predict the beneficial outcomes of such research that basic scientific inquiry should be encouraged and enabled in all fields of investigation

REFERENCES

Akimaru,J., Matsuyama,S., Tokuda,H., and Mizushima,S. (1991). Reconstitution of a protein translocation system containing purified SecY, SecE, and SecA from *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A *88*, 6545-6549.

Akita,M., Shinkai,A., Matsuyama,S., and Mizushima,S. (1991). SecA, an essential component of the secretory machinery of *Escherichia coli*, exists as homodimer. Biochem. Biophys. Res. Commun. *174*, 211-216.

Akiyama,Y. (1999). Self-processing of FtsH and its implication for the cleavage specificity of this protease. Biochemistry *38*, 11693-11699.

Akiyama,Y., Ogura,T., and Ito,K. (1994). Involvement of FtsH in protein assembly into and through the membrane. I. Mutations that reduce retention efficiency of a cytoplasmic reporter. J. Biol. Chem. *269*, 5218-5224.

Althoff,S., Selinger,D., and Wise,J.A. (1994). Molecular evolution of SRP cycle components: functional implications. Nucleic Acids Res. *22*, 1933-1947.

Altschul,S.F., Gish,W., Miller,W., Myers,E.W., and Lipman,D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Andrews,D. (1989). Examining protein translocation in cell-free systems and microinjected Xenopus oocytes. Biotechniques *7*, 960-967.

Andrews,D.W. and Johnson,A.E. (1996). The translocon: more than a hole in the ER membrane? Trends. Biochem. Sci. *21*, 365-369.

Andrews,D.W., Lauffer,L., Walter,P., and Lingappa,V.R. (1989). Evidence for a two-step mechanism involved in assembly of functional signal recognition particle receptor. J. Cell Biol. *108*, 797-810.

Andrews D.W., Walter P., and Ottensmeyer, F.P. (1987). Evidence for an extended 7SL RNA structure in the signal recognition particle. EMBO J. *6*, 3471-3477

Arvidson,C.G., Powers,T., Walter,P., and So,M. (1999). *Neisseria gonorrhoeae* PilA is an FtsY homolog. Journal of Bacteriology *181*, 731-739.

Arvidson,C.G. and So,M. (1995). Interaction of the *Neisseria gonorrhoeae* PilA protein with the pilE promoter involves multiple sites on the DNA. J. Bacteriol. *177*, 2497-2504.

Bacher,G., Lutcke,H., Jungnickel,B., Rapoport,T.A., and Dobberstein,B. (1996). Regulation by the ribosome of the GTPase of the signal-recognition particle during protein targeting. Nature *381*, 248-251.

Bacher,G., Pool,M., and Dobberstein,B. (1999). The ribosome regulates the GTPase of the beta-subunit of the signal recognition particle receptor. J. Cell Biol. *146*, 723-730.

Bar-Peled,M. and Raikhel,N.V. (1996). A method for isolation and purification of specific antibodies to a protein fused to the GST. Anal. Biochem. *241*, 140-142.

Bassford,P., Beckwith,J, Ito,K., Kumamoto,C., Mizushima,S., Oliver,D., Randall,L., Silhavy,T., Tai,P.C., and Wickner,B. (1991). The primary pathway of protein export in *E. coli*. Cell *65*, 367-368

Batey,R.T., Rambo,R.P., Lucast,L., Rha,B., and Doudna,J.A. (2000). Crystal structure of the ribonucleoprotein core of the signal recognition particle. Science *287*, 1232-1239.

Beck,K., Eisner,G., Trescher,D., Dalbey,R.E., Brunner,J., and Muller,M. (2001). YidC, an assembly site for polytopic *Escherichia coli* membrane proteins located in immediate proximity to the SecYE translocon and lipids. EMBO Rep. *2*, 709-714.

Beck,K., Wu,L.F., Brunner,J., and Muller,M. (2000). Discrimination between SRP- and SecA/SecB-dependent substrates involves selective recognition of nascent chains by SRP and trigger factor. EMBO J. *19*, 134-143.

Bernstein,H.D. and Hyndman,J.B. (2001). Physiological basis for conservation of the signal recognition particle targeting pathway in *Escherichia coli*. J. Bacteriol. *183*, 2187-2197.

Bernstein,H.D., Poritz,M.A., Strub,K., Hoben,P.J., Brenner,S., and Walter,P. (1989). Model for signal sequence recognition from amino-acid sequence of 54K subunit of signal recognition particle. Nature *340*, 482-486.

Bernstein,H.D., Zopf,D., Freymann,D.M., and Walter,P. (1993). Functional substitution of the signal recognition particle 54-kDa subunit by its *Escherichia coli* homolog. Proc.

Natl. Acad. Sci. U. S. A *90*, 5229-5233.

Bessonneau,P., Besson,V., Collinson,I., and Duong,F. (2002). The SecYEG preprotein translocation channel is a conformationally dynamic and dimeric structure. EMBO J. *21*, 995-1003.

Bibi,E., Herskovits,A.A., Bochkareva,E.S., and Zelazny,A. (2001). Putative integral membrane SRP receptors. Trends in Biochemical Sciences *26*, 15-16.

Bourgaize,D.B. and Fournier,M.J. (1987). Initiation of translation is impaired in *E. coli* cells deficient in 4.5S RNA. Nature *325*, 281-284.

Breukink,E., Demel,R.A., Korte-Kool,G., and de Kruijff,B. (1992). SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study. Biochemistry *31*, 1119-1124.

Breukink,E., Keller,R.C., and de Kruijff,B. (1993). Nucleotide and negatively charged lipid-dependent vesicle aggregation caused by SecA. Evidence that SecA contains two lipid-binding sites. FEBS Lett. *331*, 19-24.

Breukink,E., Nouwen,N., van Raalte,A., Mizushima,S., Tommassen,J., and de Kruijff,B. (1995). The C terminus of SecA is involved in both lipid binding and SecB binding. J. Biol. Chem. *270*, 7902-7907.

Breyton,C., Haase,W., Rapoport,T.A., Kuhlbrandt,W., and Collinson,I. (2002). Three-

dimensional structure of the bacterial protein-translocation complex SecYEG. Nature *418*, 662-665.

Briehl,M., Pooley,H., and Karamata,D. (1989). Mutants of *Bacillus subtilis* 168 thermosensitive for growth and wall teichoic acid synthesis. Journal of General Microbiology *135*, 1325-1334.

Brinkman,F.S., Wan,I., Hancock,R.E., Rose,A.M., and Jones,S.J. (2001). PhyloBLAST: facilitating phylogenetic analysis of BLAST results. Bioinformatics. *17*, 385-387.

Brown,S. (1991). Genes for 7S RNAs can replace the gene for 4.5S RNA in growth of *Escherichia coli*. J. Bacteriol. *173*, 1835-1837.

Brown,S. and Fournier,M.J. (1984). The 4.5 S RNA gene of *Escherichia coli* is essential for cell growth. J. Mol. Biol. *178*, 533-550.

Brundage,L., Fimmel,C.J., Mizushima,S., and Wickner,W. (1992). SecY, SecE, and band 1 form the membrane-embedded domain of *Escherichia coli* preprotein translocase. J. Biol. Chem. *267*, 4166-4170.

Brundage,L., Hendrick,J.P., Schiebel,E., Driessen,A.J., and Wickner,W. (1990). The purified E. coli integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell *62*, 649-657.

Brunelli,C.A., O'Connor,M., and Dahlberg,A.E. (2002). Decreased requirement for 4.5S

RNA in 16S and 23S rRNA mutants of *Escherichia coli*. FEBS Lett. *514*, 44-48.

Bunai,K, Yamada,K, Hayashi,K., Nakamura,K., and Yamane,K. (1999). Enhancing effect of *Bacillus subtilis* Ffh, a homologue of the SRP54 subunit of the mammalian signal recognition particle, on the binding of SecA to precursors of secretory proteins in vitro. J. Biochem. (Tokyo) *125*, 151-159.

Cabelli,R.J., Chen,L., Tai,P.C., and Oliver,D.B. (1988). SecA protein is required for secretory protein translocation into *E. coli* membrane vesicles. Cell *55*, 683-692.

Cabelli,R.J., Dolan,K.M., Qian,L.P., and Oliver,D.B. (1991). Characterization of membrane-associated and soluble states of SecA protein from wild-type and SecA51(TS) mutant strains of *Escherichia coli*. J. Biol. Chem. *266*, 24420-24427.

Cammack,K.A. and Wade,H.E. (1965). The sedimentation behaviour of ribonuclease-active and -inactive ribosomes from bacteria. Biochem. J. *96*, 671-680.

Cha,J.H. and Stewart,G.C. (1997). The divIVA minicell locus of *Bacillus subtilis*. J. Bacteriol. *179*, 1671-1683.

Chaddock,A.M., Mant,A., Karnauchov,I., Brink,S., Herrmann,R.G., Klosgen,R.B., and Robinson,C. (1995). A new type of signal peptide: central role of a twin-arginine motif in transfer signals for the delta pH-dependent thylakoidal protein translocase. EMBO J. *14*, 2715-2722.

Chen,M., Samuelson,J.C., Jiang,F., Muller,M., Kuhn,A., and Dalbey,R.E. (2002). Direct interaction of YidC with the Sec-independent Pf3 coat protein during its membrane protein insertion. J. Biol. Chem. *277*, 7670-7675.

Cleverley,R.M. and Gierasch,L.M. (2002). Mapping the signal sequence-binding site on SRP reveals a significant role for the NG-domain. J. Biol. Chem. *277*, 46763-46768.

Collier,D.N., Bankaitis,V.A., Weiss,J.B., and Bassford,P.J.J. (1988). The antifolding activity of SecB promotes the export of the E. coli maltose-binding protein. Cell *53*, 273-283.

Combet C., Blanchet C., Geourjon C. and Deléage G. (2000). NPS@: network protein sequence analysis. Trends Biochem. Sci. *25*, 147-50

Connolly,T. and Gilmore,R. (1989). The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. Cell *57*, 599-610.

Connolly,T., Rapiejko,P.J., and Gilmore,R. (1991). Requirement of GTP hydrolysis for dissociation of the signal recognition particle from its receptor. Science *252*, 1171-1173.

Cooper,J.A. (2002). Actin dynamics: tropomyosin provides stability. Curr. Biol. *12*, R523-R525.

Cristobal,S., Scotti,P., Luirink,J., von Heijne,G., and de Gier,J.W. (1999). The signal

recognition particle-targeting pathway does not necessarily deliver proteins to the sec-translocase in *Escherichia coli*. J. Biol. Chem. *274*, 20068-20070.

Czarnota,G.J., Andrews,D.W., Farrow,N.A., and Ottensmeyer,F.P. (1994). A structure for the signal sequence binding protein SRP54: 3D reconstruction from STEM images of single molecules. J. Struct. Biol. *113*, 35-46.

de Gier,J.W., Mansournia,P., Valent,Q.A., Phillips,G.J., Luirink,J., and von Heijne,G. (1996). Assembly of a cytoplasmic membrane protein in *Escherichia coli* is dependent on the signal recognition particle. FEBS Lett. *399*, 307-309.

de Gier,J.W., Scotti,P.A., Saaf,A., Valent,Q.A., Kuhn,A., Luirink,J., and von Heijne,G. (1998). Differential use of the signal recognition particle translocase targeting pathway for inner membrane protein assembly in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. *95*, 14646-14651.

de Leeuw,E., Graham,B., Phillips,G.J., Hagen-Jongman,C.M., Oudega,B., and Luirink,J. (1999). Molecular characterization of *Escherichia coli* FtsE and FtsX. Mol. Microbiol. *31*, 983-993.

de Leeuw,E., Poland,D., Mol,O., Sinning,I., ten Hagen-Jongman,C.M., Oudega,B., and Luirink,J. (1997). Membrane association of FtsY, the E. coli SRP receptor. FEBS Lett. *416*, 225-229.

de Leeuw,E., te Kaat,K., Moser,C., Menestrina,G., Demel,R., de Kruijff,B., Oudega,B.,

Luirink,J., and Sinning,I. (2000). Anionic phospholipids are involved in membrane association of FtsY and stimulate its GTPase activity. EMBO J. *19*, 531-541.

De Vrije,T., Tommassen,J., and de Kruijff,B. (1987). Optimal posttranslational translocation of the precursor of PhoE protein across *Escherichia coli* membrane vesicles requires both ATP and the protonmotive force. Biochim. Biophys. Acta *900*, 63-72.

DeChavigny,A., Heacock,P.N., and Dowhan,W. (1991). Sequence and inactivation of the pss gene of *Escherichia coli*. Phosphatidylethanolamine may not be essential for cell viability. J. Biol. Chem. *266*, 5323-5332.

DeLisa,M.P., Samuelson,P., Palmer,T., and Georgiou,G. (2002). Genetic analysis of the twin arginine translocator secretion pathway in bacteria. J. Biol. Chem. *277*, 29825-29831.

Diener,J.L. and Wilson,C. (2000). Role of SRP19 in assembly of the *Archaeoglobus fulgidus* signal recognition particle. Biochemistry *39*, 12862-12874.

Dolan,K.M. and Oliver,D.B. (1991). Characterization of *Escherichia coli* SecA protein binding to a site on its mRNA involved in autoregulation. J. Biol. Chem. *266*, 23329-23333.

Duong,F. and Wickner,W. (1997a). Distinct catalytic roles of the SecYE, SecG and SecDFyajC subunits of preprotein translocase holoenzyme. EMBO J. *16*, 2756-2768.

Duong,F. and Wickner,W. (1997b). The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. EMBO J. *16*, 4871-4879.

Duzgunes,N., Wilschut,J., Fraley,R., and Papahadjopoulos,D. (1981). Studies on the mechanism of membrane fusion. Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. Biochim. Biophys. Acta *642*, 182-195.

Economou,A., Pogliano,J.A., Beckwith,J., Oliver,D.B., and Wickner,W. (1995). SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell *83* , 1171-1181.

Economou,A. and Wickner,W. (1994). SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell *78*, 835-843.

Edwards,D.H. and Errington,J. (1997). The Bacillus subtilis DivIVA protein targets to the division septum and controls the site specificity of cell division. Mol. Microbiol. *24*, 905-915.

Edwards,D.H., Thomaides,H.B., and Errington,J. (2000). Promiscuous targeting of Bacillus subtilis cell division protein DivIVA to division sites in *Escherichia coli* and fission yeast. EMBO J. *19*, 2719-2727.

Eichler,J., Brunner,J., and Wickner,W. (1997). The protease-protected 30 kDa domain of

SecA is largely inaccessible to the membrane lipid phase. EMBO J. *16*, 2188-2196.

Eichler,J. and Wickner,W. (1997). Both an N-terminal 65-kDa domain and a C-terminal 30-kDa domain of SecA cycle into the membrane at SecYEG during translocation. Proc. Natl. Acad. Sci. U. S. A *94*, 5574-5581.

Falcone,D. and Andrews,D.W. (1991). Both the 5' untranslated region and the sequences surrounding the start site contribute to efficient initiation of translation in vitro. Mol. Cell Biol. *11*, 2656-2664.

Fekkes,P., de Wit,J.G., Boorsma,A., Friesen,R.H., and Driessen,A.J. (1999). Zinc stabilizes the SecB binding site of SecA. Biochemistry *38*, 5111-5116.

Fekkes,P., de Wit,J.G., van der Wolk,J.P., Kimsey,H.H., Kumamoto,C.A., and Driessen,A.J. (1998). Preprotein transfer to the *Escherichia coli* translocase requires the co-operative binding of SecB and the signal sequence to SecA. Mol. Microbiol. *29*, 1179-1190.

Fekkes,P. and Driessen,A.J. (1999). Protein targeting to the bacterial cytoplasmic membrane. Microbiol. Mol. Biol. Rev. *63*, 161-173.

Fekkes,P., van der Does,C., and Driessen,A.J. (1997). The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. EMBO J. *16*, 6105-6113.

Freymann,D.M., Keenan,R.J., Stroud,R.M., and Walter,P. (1997). Structure of the conserved GTPase domain of the signal recognition particle. Nature *385*, 361-364.

Freymann,D.M., Keenan,R.J., Stroud,R.M., and Walter,P. (1999). Functional changes in the structure of the SRP GTPase on binding GDP and Mg^{2+}GDP. Nat. Struct. Biol. *6*, 793-801.

Geller,B.L., Movva,N.R., and Wickner,W. (1986). Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into *Escherichia coli* inner membrane vesicles. Proc. Natl. Acad. Sci. U. S. A *83*, 4219-4222.

Gill DR, Salmond GP. (1987). The *Escherichia coli* cell division proteins FtsY, FtsE and FtsX are inner membrane-associated. Mol. Gen. Genet. *210*, 504-8

Gill,D.R. and Salmond,G.P. (1990). The identification of the *Escherichia coli* ftsY gene product: an unusual protein. Mol. Microbiol. *4*, 575-583.

Gilmore,R., Blobel,G., and Walter,P. (1982a). Protein translocation across the endoplasmic reticulum. I. Detection in the microsomal membrane of a receptor for the signal recognition particle. J. Cell Biol. *95*, 463-469.

Gilmore,R., Walter,P., and Blobel,G. (1982b). Protein translocation across the endoplasmic reticulum. II. Isolation and characterization of the signal recognition particle receptor. J. Cell Biol. *95*, 470-477.

Gorodkin,J., Knudsen,B., Zwieb,C., and Samuelsson,T. (2001). SRPDB (Signal Recognition Particle Database). Nucleic Acids Res. *29*, 169-170.

Gribaldo,S. and Cammarano,P. (1998). The root of the universal tree of life inferred from anciently duplicated genes encoding components of the protein-targeting machinery. J. Mol. Evol. *47*, 508-516.

Gurevich,V.V., Pokrovskaya,I.D., Obukhova,T.A., and Zozulya,S.A. (1991). Preparative in vitro mRNA synthesis using SP6 and T7 RNA polymerases. Anal. Biochem. *195*, 207-213.

Hainzl,T., Huang,S., and Sauer-Eriksson,A.E. (2002). Structure of the SRP19 RNA complex and implications for signal recognition particle assembly. Nature *417*, 767-771.

Hanada,M., Nishiyama,K.I., Mizushima,S., and Tokuda,H. (1994). Reconstitution of an efficient protein translocation machinery comprising SecA and the three membrane proteins, SecY, SecE, and SecG (p12). J. Biol. Chem. *269*, 23625-23631.

Hardy,S.J. and Randall,L.L. (1991). A kinetic partitioning model of selective binding of nonnative proteins by the bacterial chaperone SecB. Science *251*, 439-443.

Hartl,F.U., Lecker,S., Schiebel,E., Hendrick,J.P., and Wickner,W. (1990). The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. Cell *63*, 269-279.

Hauser,S., Bacher,G., Dobberstein,B., and Lutcke,H. (1995). A complex of the signal sequence binding protein and the SRP RNA promotes translocation of nascent proteins. EMBO J. *14*, 5485-5493.

Hendrick,J.P. and Wickner,W. (1991). SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. J. Biol. Chem. *266*, 24596-24600.

Herskovits,A.A., Seluanov,A., Rajsbaum,R., Hagen-Jongman,C.M., Henrichs,R., Bochkareva,E.S., Phillips,G.J., Probst,F.J., Nakae,T., Ehrmann,M., Luirink,J., and Bibi,E. (2001). Evidence for coupling of membrane targeting and function of the signal recognition particle (SRP) receptor FtsY. EMBO Reports *2*, 1040-1046.

High,S. and Dobberstein,B. (1991). The signal sequence interacts with the methionine-rich domain of the 54-kD protein of signal recognition particle. J. Cell Biol. *113*, 229-233.

Hirose,I., Sano,K., Shioda,I., Kumano,M., Nakamura,K., and Yamane,K. (2000). Proteome analysis of *Bacillus subtilis* extracellular proteins: a two-dimensional protein electrophoretic study. Microbiology *146 (Pt 1)*, 65-75.

Houben,E.N., Urbanus,M.L., Van der Laan,M., Hagen-Jongman,C.M., Driessen,A.J., Brunner,J., Oudega,B., and Luirink,J. (2002). YidC and SecY mediate membrane insertion of a type I transmembrane domain. J. Biol. Chem.

Hughes,M.J. and Andrews,D.W. (1996). Creation of deletion, insertion and substitution mutations using a single pair of primers and PCR. Biotechniques *20*, 192-196.

Izard,J.W. and Kendall,D.A. (1994). Signal peptides: exquisitely designed transport promoters. Mol. Microbiol. *13*, 765-773.

Jagath,J.R., Rodnina,M.V., Lentzen,G., and Wintermeyer,W. (1998). Interaction of guanine nucleotides with the signal recognition particle from *Escherichia coli*. Biochemistry *37*, 15408-15413.

Jander,G., Cronan,J.E.J., and Beckwith,J. (1996). Biotinylation in vivo as a sensitive indicator of protein secretion and membrane protein insertion. J. Bacteriol. *178*, 3049-3058.

Janiak,F., Glover,J.R., Leber,B., Rachubinski,R.A., and Andrews,D.W. (1994). Targeting of passenger protein domains to multiple intracellular membranes. Biochem. J. *300*, 191-199.

Janiak,F., Walter,P., and Johnson,A.E. (1992). Fluorescence-detected assembly of the signal recognition particle: binding of the two SRP protein heterodimers to SRP RNA is noncooperative. Biochemistry *31*, 5830-5840.

Jensen,C.G., Brown,S., and Pedersen,S. (1994). Effect of 4.5S RNA depletion on *Escherichia coli* protein synthesis and secretion. J. Bacteriol. *176*, 2502-2506.

Jensen,C.G. and Pedersen,S. (1994). Concentrations of 4.5S RNA and Ffh protein in *Escherichia coli*: the stability of Ffh protein is dependent on the concentration of 4.5S RNA. J. Bacteriol. *176*, 7148-7154.

Joly,J.C. and Wickner,W. (1993). The SecA and SecY subunits of translocase are the nearest neighbors of a translocating preprotein, shielding it from phospholipids. EMBO J. *12*, 255-263.

Keenan,R.J., Freymann,D.M., Stroud,R.M., and Walter,P. (2001). The signal recognition particle. Annu. Rev. Biochem. *70*, 755-775.

Keenan,R.J., Freymann,D.M., Walter,P., and Stroud,R.M. (1998). Crystal structure of the signal sequence binding subunit of the signal recognition particle. Cell *94*, 181-191.

Khan,A.R. and James,M.N. (1998). Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci. *7*, 815-836.

Kholodenko,B.N., Hoek,J.B., and Westerhoff,H.V. (2000) Why cytoplasmic signalling proteins should be recruited to cell membranes. Trends Cell Biol. *10*, 173-178.

Kihara,A., Akiyama,Y., and Ito,K. (1995). FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. Proc. Natl. Acad. Sci. U. S. A *92*, 4532-4536.

Kim,J., Miller,A., Wang,L.G., Muller,J.P., and Kendall,D.A. (2001a). Evidence that SecB

enhances the activity of SecA. Biochemistry *40*, 3674-3680.

Kim,J., Rusch,S., Luirink,J., and Kendall,D.A. (2001b). Is Ffh required for export of secretory proteins? FEBS Lett. *505*, 245-248.

Kim,Y.J., Rajapandi,T., and Oliver,D. (1994). SecA protein is exposed to the periplasmic surface of the E. coli inner membrane in its active state. Cell *78*, 845-853.

Koch,H.G., Hengelage,T., Neumann-Haefelin,C., Macfarlane,J., Hoffschulte,H.K., Schimz,K.L., Mechler,B., and Muller,M. (1999). *In vitro* studies with purified components reveal signal recognition particle (SRP) and SecA/SecB as constituents of two independent protein-targeting pathways of *Escherichia coli*. Mol. Biol. Cell *10*, 2163-2173.

Krieg,U.C., Walter,P., and Johnson,A.E. (1986). Photocrosslinking of the signal sequence of nascent preprolactin to the 54-kilodalton polypeptide of the signal recognition particle. Proc. Natl. Acad. Sci. U. S. A *83*, 8604-8608.

Kumamoto,C.A. (1989). *Escherichia coli* SecB protein associates with exported protein precursors in vivo. Proc. Natl. Acad. Sci. U. S. A *86*, 5320-5324.

Kumamoto,C.A. and Francetic,O. (1993). Highly selective binding of nascent polypeptides by an *Escherichia coli* chaperone protein in vivo. J. Bacteriol. *175*, 2184-2188.

Kumamoto,C.A. and Nault,A.K. (1989). Characterization of the *Escherichia coli* protein-export gene secB. Gene *75*, 167-175.

Kurzchalia,T.V., Wiedmann,M., Girshovich,A.S., Bochkareva,E.S., Bielka,H., and Rapoport,T.A. (1986). The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. Nature *320*, 634-636.

Kusters,R., Lentzen,G., Eppens,E., Vangeel,A., Vanderweijden,C.C., Wintermeyer,W., and Luirink,J. (1995). The Functioning of the SRP Receptor FtsY in Protein-Targeting in *Escherichia coli* Correlated with Its Ability to Bind and Hydrolyze GTP. FEBS Lett. *372*, 253-258.

Lauffer,L., Garcia,P.D., Harkins,R.N., Coussens,L., Ullrich,A., and Walter,P. (1985). Topology of signal recognition particle receptor in endoplasmic reticulum membrane. Nature *318*, 334-338.

Lecker,S.H., Driessen,A.J., and Wickner,W. (1990). ProOmpA contains secondary and tertiary structure prior to translocation and is shielded from aggregation by association with SecB protein. EMBO J. *9*, 2309-2314.

Lee,C., Li,P., Inouye,H., Brickman,E.R., and Beckwith,J. (1989). Genetic studies on the inability of beta-galactosidase to be translocated across the *Escherichia coli* cytoplasmic membrane. J. Bacteriol. *171*, 4609-4616.

Lee,H.C. and Bernstein,H.D. (2001). The targeting pathway of *Escherichia coli*

presecretory and integral membrane proteins is specified by the hydrophobicity of the targeting signal. Proc. Natl. Acad. Sci. U. S. A *98*, 3471-3476.

Legate,K.R. and Andrews,D.W. (2001). Assembly strategies and GTPase regulation of the eukaryotic and Escherichia coil translocons. Biochem. Cell Biol. *79*, 593-601.

Legate,K.R., Falcone,D., and Andrews,D.W. (2000). Nucleotide-dependent binding of the GTPase domain of the signal recognition particle receptor beta-subunit to the alpha-subunit. J. Biol. Chem. *275*, 27439-27446.

Lill,R., Dowhan,W., and Wickner,W. (1990). The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell *60*, 271-280.

Lu,Y., Qi,H.Y., Hyndman,J.B., Ulbrandt,N.D., Teplyakov,A., Tomasevic,N., and Bernstein,H.D. (2001). Evidence for a novel GTPase priming step in the SRP protein targeting pathway. EMBO J. *20*, 6724-6734.

Luirink,J., High,S., Wood,H., Giner,A., Tollervey,D., and Dobberstein,B. (1992). Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex. Nature *359*, 741-743.

Luirink,J., ten Hagen-Jongman,C.M., van der Weijden,C.C., Oudega,B., High,S., Dobberstein,B., and Kusters,R. (1994). An alternative protein targeting pathway in *Escherichia coli*: studies on the role of FtsY. EMBO J. *13*, 2289-2296.

Lutcke,H., High,S., Romisch,K., Ashford,A.J., and Dobberstein,B. (1992). The methionine-rich domain of the 54 kDa subunit of signal recognition particle is sufficient for the interaction with signal sequences. EMBO J. *11*, 1543-1551.

Macao,B., Luirink,J., and Samuelsson,T. (1997). Ffh and FtsY in a *Mycoplasma mycoides* signal-recognition particle pathway: SRP RNA and M domain of Ffh are not required for stimulation of GTPase activity *in vitro*. Molecular Microbiology *24*, 523-534.

Macfarlane,J. and Muller,M. (1995). The functional integration of a polytopic membrane protein of *Escherichia coli* is dependent on the bacterial signal-recognition particle. Eur. J. Biochem. *233*, 766-771.

Mac Millan SV, Ishiyama N, White GF, Palaniyar N, Hallett FR, Harauz G. (2000) Myelin basic protein component C1 in increasing concentrations can elicit fusion, aggregation, and fragmentation of myelin-like membranes. Eur. J. Cell Biol. *79*, 327-35

Manen,D., Pougeon,M., Damay,P., and Geiselmann,J. (1997). A sensitive reporter gene system using bacterial luciferase based on a series of plasmid cloning vectors compatible with derivatives of pBR322. Gene *186*, 197-200.

Manting,E.H. and Driessen,A.J. (2000). *Escherichia coli* translocase: the unravelling of a molecular machine. Mol. Microbiol. *37* , 226-238.

Manting,E.H., van der Does,C., and Driessen,A.J. (1997). In vivo cross-linking of the

SecA and SecY subunits of the *Escherichia coli* preprotein translocase. J. Bacteriol. *179*, 5699-5704.

Mason,N., Ciufo,L.F., and Brown,J.D. (2000). Elongation arrest is a physiologically important function of signal recognition particle. EMBO J. *19*, 4164-4174.

Matsumoto,G., Yoshihisa,T., and Ito,K. (1997). SecY and SecA interact to allow SecA insertion and protein translocation across the *Escherichia coli* plasma membrane. EMBO J. *16*, 6384-6393.

Meyer,D.I. and Dobberstein,B. (1980). Identification and characterization of a membrane component essential for the translocation of nascent proteins across the membrane of the endoplasmic reticulum. J. Cell Biol. *87*, 503-508.

Mileykovskaya,E., Sun,Q., Margolin,W., and Dowhan,W. (1998). Localization and function of early cell division proteins in filamentous *Escherichia coli* cells lacking phosphatidylethanolamine. J. Bacteriol. *180*, 4252-4257.

Miller,A., Wang,L.G., and Kendall,D.A. (2002). SecB modulates the nucleotide-bound state of SecA and stimulates ATPase. Biochemistry *41*, 5325-5332.

Miller,J.D., Bernstein,H.D., and Walter,P. (1994). Interaction of E. coli Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor. Nature *367*, 657-659.

Miller,J.D., Tajima,S., Lauffer,L., and Walter,P. (1995). The beta subunit of the signal recognition particle receptor is a transmembrane GTPase that anchors the alpha subunit, a peripheral membrane GTPase, to the endoplasmic reticulum membrane. J. Cell Biol. *128*, 273-282.

Millman,J.S. and Andrews,D.W. (1997). Switching the model: a concerted mechanism for GTPases in protein targeting. Cell *89*, 673-676.

Montoya,G., Svensson,C., Luirink,J., and Sinning,I. (1997). Crystal structure of the NG domain from the signal-recognition particle receptor FtsY. Nature *385*, 365-368.

Moser,C., Mol,O., Goody,R.S., and Sinning,I. (1997). The signal recognition particle receptor of *Escherichia coli* (FtsY) has a nucleotide exchange factor built into the GTPase domain. Proc. Natl. Acad. Sci. U. S. A *94*, 11339-11344.

Muller,M. and Blobel,G. (1984). In vitro translocation of bacterial proteins across the plasma membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. *81*, 7421-7425.

Murphy,E.C., Zheng,T., and Nicchitta,C.V. (1997). Identification of a novel stage of ribosome/nascent chain association with the endoplasmic reticulum membrane. J. Cell Biol. *136*, 1213-1226.

Nakamura,K., Fujii,Y., Shibata,T., and Yamane,K. (1999). Depletion of *Escherichia coli* 4.5S RNA leads to an increase in the amount of protein elongation factor EF-G associated with ribosomes. Eur. J. Biochem. *259*, 543-550.

Nanninga,N. (2001). Cytokinesis in prokaryotes and eukaryotes: common principles and different solutions. Microbiol. Mol. Biol. Rev. *65*, 319-333.

Neumann-Haefelin,C., Schafer,U., Muller,M., Koch,H.G., Yahr,T.L., and Wickner,W.T. (2000). SRP-dependent co-translational targeting and SecA-dependent translocation analyzed as individual steps in the export of a bacterial protein. EMBO J. *19*, 6419-6426.

Newitt,J.A. and Bernstein,H.D. (1997). The N-domain of the signal recognition particle 54-kDa subunit promotes efficient signal sequence binding. Eur. J. Biochem. *245*, 720-729.

Newitt,J.A., Ulbrandt,N.D., and Bernstein,H.D. (1999). The structure of multiple polypeptide domains determines the signal recognition particle targeting requirement of *Escherichia coli* inner membrane proteins. J. Bacteriol. *181*, 4561-4567.

Nishiyama,K., Mizushima,S., and Tokuda,H. (1993). A novel membrane protein involved in protein translocation across the cytoplasmic membrane of *Escherichia coli*. EMBO J. *12*, 3409-3415.

Nishiyama,K., Suzuki,T., and Tokuda,H. (1996). Inversion of the membrane topology of SecG coupled with SecA-dependent preprotein translocation. Cell *85*, 71-81.

Nouwen,N. and Driessen,A.J. (2002). SecDFyajC forms a heterotetrameric complex with YidC. Mol. Microbiol. *44*, 1397-1405.

Oh,D.B., Yi,G.S., Chi,S.W., and Kim,H. (1996). Structure of a methionine-rich segment of *Escherichia coli* Ffh protein. FEBS Lett. *395*, 160-164.

Ohta,A. and Shibuya,I. (1977). Membrane phospholipid synthesis and phenotypic correlation of an *Escherichia coli* pss mutant. J. Bacteriol. *132*, 434-443.

Or,E., Navon,A., and Rapoport,T. (2002). Dissociation of the dimeric SecA ATPase during protein translocation across the bacterial membrane. EMBO J. *21*, 4470-4479.

Oresnik,I.J., Ladner,C.L., and Turner,R.J. (2001). Identification of a twin-arginine leader-binding protein. Mol. Microbiol. *40* , 323-331.

Ota,K., Sakaguchi,M., von Heijne,G., Hamasaki,N., and Mihara,K. (1998). Forced transmembrane orientation of hydrophilic polypeptide segments in multispanning membrane proteins. Mol. Cell *2*, 495-503.

Park,S., Liu,G., Topping,T.B., Cover,W.H., and Randall,L.L. (1988). Modulation of folding pathways of exported proteins by the leader sequence. Science *239*, 1033-1035.

Peluso,P., Herschlag,D., Nock,S., Freymann,D.M., Johnson,A.E., and Walter,P. (2000). Role of 4.5S RNA in assembly of the bacterial signal recognition particle with its receptor. Science *288*, 1640-1643.

Peluso,P., Shan,S.O., Nock,S., Herschlag,D., and Walter,P. (2001). Role of SRP RNA in the GTPase cycles of Ffh and FtsY. Biochemistry *40*, 15224-15233.

Phillips,G.J. and Silhavy,T.J. (1992). The *E. coli* ffh gene is necessary for viability and efficient protein export. Nature *359* , 744-746.

Pogliano,J.A. and Beckwith,J. (1994). SecD and SecF facilitate protein export in *Escherichia coli*. EMBO J. *13*, 554-561.

Politz,J.C., Yarovoi,S., Kilroy,S.M., Gowda,K., Zwieb,C., and Pederson,T. (2000). Signal recognition particle components in the nucleolus. Proc. Natl. Acad. Sci. U. S. A *97*, 55-60.

Pollastri,G., Przybylski,D., Rost,B., and Baldi,P. (2002). Improving the prediction of protein secondary structure in three and eight classes using recurrent neural networks and profiles. Proteins *47*, 228-235.

Poritz,M.A., Bernstein,H.D., Strub,K., Zopf,D., Wilhelm,H., and Walter,P. (1990). An E. coli ribonucleoprotein containing 4.5S RNA resembles mammalian signal recognition particle. Science *250*, 1111-1117.

Poritz,M.A., Strub,K., and Walter,P. (1988). Human SRP RNA and *E. coli* 4.5S RNA contain a highly homologous structural domain. Cell *55*, 4-6.

Powers,T. and Walter,P. (1995). Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. Science *269*, 1422-1424.

Powers,T. and Walter,P. (1997). Co-translational protein targeting catalyzed by the

Escherichia coli signal recognition particle and its receptor. EMBO J. *16*, 4880-4886.

Price,A., Economou,A., Duong,F., and Wickner,W. (1996). Separable ATPase and membrane insertion domains of the SecA subunit of preprotein translocase. J. Biol. Chem. *271*, 31580-31584.

Qi,H.Y. and Bernstein,H.D. (1999). SecA is required for the insertion of inner membrane proteins targeted by the *Escherichia coli* signal recognition particle. J. Biol. Chem. *274*, 8993-8997.

Randall,L.L. and Hardy,S.J. (1995). High selectivity with low specificity: how SecB has solved the paradox of chaperone binding. Trends Biochem. Sci. *20*, 65-69.

Randall,L.L., Topping,T.B., Suciu,D., and Hardy,S.J. (1998). Calorimetric analyses of the interaction between SecB and its ligands. Protein Sci. *7*, 1195-1200.

Rapiejko,P.J. and Gilmore,R. (1994). Signal sequence recognition and targeting of ribosomes to the endoplasmic reticulum by the signal recognition particle do not require GTP. Mol. Biol. Cell *5*, 887-897.

Rapiejko,P.J. and Gilmore,R. (1997). Empty site forms of the SRP54 and SR alpha GTPases mediate targeting of ribosome-nascent chain complexes to the endoplasmic reticulum. Cell *89*, 703-713.

Rapoport,T.A. (1991). Protein translocation: A bacterium catches up. Nature *349*, 107-

108

Rapoport,T.A., Jungnickel,B., and Kutay,U. (1996). Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. Annu. Rev. Biochem. *65*, 271-303.

Ribes,V., Romisch,K., Giner,A., Dobberstein,B., and Tollervey,D. (1990). *E. coli* 4.5S RNA is part of a ribonucleoprotein particle that has properties related to signal recognition particle. Cell *63*, 591-600.

Rietveld,A.G., Killian,J.A., Dowhan,W., and de Kruijff,B. (1993). Polymorphic regulation of membrane phospholipid composition in *Escherichia coli*. J. Biol. Chem. *268*, 12427-12433.

Rietveld,A.G., Koorengevel,M.C., and de Kruijff,B. (1995). Non-bilayer lipids are required for efficient protein transport across the plasma membrane of *Escherichia coli*. EMBO J. *14*, 5506-5513.

Rigaut,G., Shevchenko,A., Rutz,B., Wilm,M., Mann,M., and Seraphin,B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. *17*, 1030-1032.

Rinke-Appel,J., Osswald,M., von Knoblauch,K., Mueller,F., Brimacombe,R., Sergiev,P., Avdeeva,O., Bogdanov,A., and Dontsova,O. (2002). Crosslinking of 4.5S RNA to the *Escherichia coli* ribosome in the presence or absence of the protein Ffh. RNA *8*, 612-625.

Robinson,C. and Bolhuis,A. (2001). Protein targeting by the twin-arginine translocation pathway. Nat. Rev. Mol. Cell Biol. *2*, 350-356.

Romisch,K., Webb,J., Herz,J., Prehn,S., Frank,R., Vingron,M., and Dobberstein,B. (1989). Homology of 54K protein of signal-recognition particle, docking protein and two *E. coli* proteins with putative GTP-binding domains. Nature *340*, 478-482.

Romisch,K., Webb,J., Lingelbach,K., Gausepohl,H., and Dobberstein,B. (1990). The 54-kD protein of signal recognition particle contains a methionine-rich RNA binding domain. J. Cell Biol. *111*, 1793-1802.

Rubins,J.B., Benditt,J.O., Dickey,B.F., and Riedel,N. (1990). GTP-binding proteins in rat liver nuclear envelopes. Proc. Natl. Acad. Sci. U. S. A *87*, 7080-7084.

Saitou,N. and Nei,M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. *4*, 406-425.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989). Molecular Cloning - A laboratory Manual. (Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press).

Samuelson,J.C., Chen,M., Jiang,F., Moller,I., Wiedmann,M., Kuhn,A., Phillips,G.J., and Dalbey,R.E. (2000). YidC mediates membrane protein insertion in bacteria. Nature *406*, 637-641.

Samuelson,J.C., Jiang,F., Yi,L., Chen,M., de Gier,J.W., Kuhn,A., and Dalbey,R.E.

(2001). Function of YidC for the insertion of M13 procoat protein in *Escherichia coli*: translocation of mutants that show differences in their membrane potential dependence and Sec requirement. J. Biol. Chem. *276*, 34847-34852.

Santini,C.L., Ize,B., Chanal,A., Muller,M., Giordano,G., and Wu,L.F. (1998). A novel sec-independent periplasmic protein translocation pathway in *Escherichia coli*. EMBO J. *17*, 101-112.

Schagger,H. and von Jagow,G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. *166*, 368-379.

Schatz,P.J., Bieker,K.L., Ottemann,K.M., Silhavy,T.J., and Beckwith,J. (1991). One of three transmembrane stretches is sufficient for the functioning of the SecE protein, a membrane component of the *E. coli* secretion machinery. EMBO J. *10*, 1749-1757.

Schiebel,E., Driessen,A.J., Hartl,F.U., and Wickner,W. (1991). Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell *64*, 927-939.

Scotti,P.A., Urbanus,M.L., Brunner,J., de Gier,J.W., von Heijne,G., van der Does,C., Driessen,A.J., Oudega,B., and Luirink,J. (2000). YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. EMBO J. *19*, 542-549.

Scotti,P.A., Valent,Q.A., Manting,E.H., Urbanus,M.L., Driessen,A.J., Oudega,B., and

Luirink,J. (1999). SecA is not required for signal recognition particle-mediated targeting and initial membrane insertion of a nascent inner membrane protein. J. Biol. Chem. *274*, 29883-29888.

Scoulica,E., Krause,E., Meese,K., and Dobberstein,B. (1987). Disassembly and domain structure of the proteins in the signal-recognition particle. Eur. J. Biochem. *163*, 519-528.

Seluanov,A. and Bibi,E. (1997). FtsY, the prokaryotic signal recognition particle receptor homologue, is essential for biogenesis of membrane proteins. J. Biol. Chem. *272*, 2053-2055.

Shepotinovskaya,I.V. and Freymann,D.M. (2002). Conformational change of the N-domain on formation of the complex between the GTPase domains of *Thermus aquaticus* Ffh and FtsY. Biochim. Biophys. Acta *1597*, 107-114.

Siegel,V. and Walter,P. (1985). Elongation arrest is not a prerequisite for secretory protein translocation across the microsomal membrane. J. Cell Biol. *100*, 1913-1921.

Siegel,V. and Walter,P. (1988). Each of the activities of signal recognition particle (SRP) is contained within a distinct domain: analysis of biochemical mutants of SRP. Cell *52*, 39-49.

Snyders,S., Ramamurthy,V., and Oliver,D. (1997). Identification of a region of interaction between *Escherichia coli* SecA and SecY proteins. J. Biol. Chem. *272*, 11302-11306.

Stewart,M. (2001). Structural basis for bending tropomyosin around actin in muscle thin filaments. Proc. Natl. Acad. Sci. U. S. A *98*, 8165-8166.

Strub,K., Moss,J., and Walter,P. (1991). Binding sites of the 9- and 14-kilodalton heterodimeric protein subunit of the signal recognition particle (SRP) are contained exclusively in the Alu domain of SRP RNA and contain a sequence motif that is conserved in evolution. Mol. Cell Biol. *11*, 3949-3959.

Struck,J.C., Toschka,H.Y., Specht,T., and Erdmann,V.A. (1988). Common structural features between eukaryotic 7SL RNAs, eubacterial 4.5S RNA and scRNA and archaebacterial 7S RNA. Nucleic. Acids. Res. *16*, 7740.

Swain,J.F. and Gierasch,L.M. (2001). Signal peptides bind and aggregate RNA. An alternative explanation for GTPase inhibition in the signal recognition particle. J. Biol. Chem. *276*, 12222-12227.

Tajima,S., Lauffer,L., Rath,V.L., and Walter,P. (1986). The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. J. Cell Biol. *103*, 1167-1178.

Thomaides,H.B., Freeman,M., El Karoui,M., and Errington,J. (2001). Division site selection protein DivIVA of *Bacillus subtilis* has a second distinct function in chromosome segregation during sporulation. Genes Dev. *15*, 1662-1673.

Thompson,J.D., Higgins,D.G., and Gibson,T.J. (1994). CLUSTAL W: improving the

sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673-4680.

Tian,H. and Beckwith,J. (2002). Genetic screen yields mutations in genes encoding all known components of the *Escherichia coli* signal recognition particle pathway. J. Bacteriol. *184*, 111-118.

Tian,H., Boyd,D., and Beckwith,J. (2000). A mutant hunt for defects in membrane protein assembly yields mutations affecting the bacterial signal recognition particle and Sec machinery. Proc. Natl. Acad. Sci. U. S. A. *97*, 4730-4735.

Traxler,B., Lee,C., Boyd,D., and Beckwith,J. (1992). The dynamics of assembly of a cytoplasmic membrane protein in *Escherichia coli*. J. Biol. Chem. *267*, 5339-5345.

Ukai,H., Matsuzawa,H., Ito,K., Yamada,M., and Nishimura,A. (1998). ftsE(Ts) affects translocation of K+-pump proteins into the cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. *180*, 3663-3670.

Ulbrandt,N.D., Newitt,J.A., and Bernstein,H.D. (1997). The *E. coli* signal recognition particle is required for the insertion of a subset of inner membrane proteins. Cell *88*, 187-196.

Urbanus,M.L., Scotti,P.A., Froderberg,L., Saaf,A., de Gier,J.W., Brunner,J., Samuelson,J.C., Dalbey,R.E., Oudega,B., and Luirink,J. (2001). Sec-dependent

membrane protein insertion: sequential interaction of nascent FtsQ with SecY and YidC. EMBO Rep. *2*, 524-529.

Valent,Q.A., de Gier,J.W., von Heijne,G., Kendall,D.A., Hagen-Jongman,C.M., Oudega,B., and Luirink,J. (1997). Nascent membrane and presecretory proteins synthesized in *Escherichia coli* associate with signal recognition particle and trigger factor. Mol. Microbiol. *25*, 53-64.

Valent,Q.A., Kendall,D.A., High,S., Kusters,R., Oudega,B., and Luirink,J. (1995). Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. EMBO J. *14*, 5494-5505.

Valent,Q.A., Scotti,P.A., High,S., de Gier,J.W., von Heijne,G., Lentzen,G., Wintermeyer,W., Oudega,B., and Luirink,J. (1998). The *Escherichia coli* SRP and SecB targeting pathways converge at the translocon. EMBO J. *17*, 2504-2512.

Van Bambeke,F., Balzi,E., and Tulkens,P.M. (2000). Antibiotic efflux pumps. Biochem. Pharmacol. *60*, 457-470.

van den Ent,F., Amos,L.A., and Lowe,J. (2001). Prokaryotic origin of the actin cytoskeleton. Nature *413*, 39-44.

van der Wolk,J.P., de Wit,J.G., and Driessen,A.J. (1997). The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events. EMBO J. *16*, 7297-7304.

van der,Does.C., Swaving,J., van Klompenburg,W., and Driessen,A.J. (2000). Non-bilayer lipids stimulate the activity of the reconstituted bacterial protein translocase. J. Biol. Chem. *275*, 2472-2478.

Van Der Laan,M., Houben,E.N., Nouwen,N., Luirink,J., and Driessen,A.J. (2001). Reconstitution of Sec-dependent membrane protein insertion: nascent FtsQ interacts with YidC in a SecYEG-dependent manner. EMBO Rep. *2*, 519-523.

van Voorst,F., van der Does,C., Brunner,J., Driessen,A.J., and de Kruijff,B. (1998). Translocase-bound SecA is largely shielded from the phospholipid acyl chains. Biochemistry *37*, 12261-12268.

von Heijne,G. (1989). Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. Nature *341*, 456-458.

Wallin,E. and von Heijne,G. (1998). Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. Protein Sci. *7*, 1029-1038.

Walter,P. and Blobel,G. (1980). Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A *77*, 7112-7116.

Walter,P. and Blobel,G. (1981). Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. J. Cell

Biol. *91*, 557-561.

Walter,P. and Blobel,G. (1982). Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature *299*, 691-698.

Walter,P. and Johnson,A.E. (1994). Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. Annu. Rev. Cell Biol. *10*, 87-119.

Watanabe M and Blobel G. (1989). Binding of a soluble factor of *Escherichia coli* to preproteins does not require ATP and appears to be the first step in protein export. Proc Natl Acad Sci U S A *86*, 2248-52

Weiner,J.H., Bilous,P.T., Shaw,G.M., Lubitz,S.P., Frost,L., Thomas,G.H., Cole,J.A., and Turner,R.J. (1998). A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. Cell *93*, 93-101.

Weiss,J.B., Ray,P.H., and Bassford,P.J., Jr. (1988). Purified secB protein of *Escherichia coli* retards folding and promotes membrane translocation of the maltose-binding protein in vitro. Proc. Natl. Acad. Sci. U. S. A *85*, 8978-8982.

Wickner,W., Driessen,A.J., and Hartl,F.U. (1991). The enzymology of protein translocation across the *Escherichia coli* plasma membrane. Annu. Rev. Biochem. *60*, 101-124.

Xu,Z., Knafels,J.D., and Yoshino,K. (2000). Crystal structure of the bacterial protein

export chaperone secB. Nat. Struct. Biol. *7*, 1172-1177.

Yahr,T.L. and Wickner,W.T. (2001). Functional reconstitution of bacterial Tat translocation in vitro. EMBO J. *20*, 2472-2479.

Yamane,K., Ichihara,S., and Mizushima,S. (1987). *In vitro* translocation of protein across *Escherichia coli* membrane vesicles requires both the proton motive force and ATP. J. Biol. Chem. *262*, 2358-2362.

Young,J.C. and Andrews,D.W. (1996). The signal recognition particle receptor alpha subunit assembles co- translationally on the endoplasmic reticulum membrane during an mRNA- encoded translation pause in vitro. EMBO J. *15*, 172-181.

Young,J.C., Ursini,J., Legate,K.R., Miller,J.D., Walter,P., and Andrews,D.W. (1995). An amino-terminal domain containing hydrophobic and hydrophilic sequences binds the signal recognition particle receptor alpha subunit to the beta subunit on the endoplasmic reticulum membrane. J. Biol. Chem. *270*, 15650-15657.

Zelazny,A., Seluanov,A., Cooper,A., and Bibi,E. (1997). The NG domain of the prokaryotic signal recognition particle receptor, FtsY, is fully functional when fused to an unrelated integral membrane polypeptide. Proc. Natl. Acad. Sci. U. S. A. *94*, 6025-6029.

Zheng,N. and Gierasch,L.M. (1997). Domain interactions in *E. coli* SRP: stabilization of M domain by RNA is required for effective signal sequence modulation of NG domain. Mol. Cell *1*, 79-87.

Zhong,P. and Shortridge,V.D. (2000). The role of efflux in macrolide resistance. Drug Resist. Updat. *3*, 325-329.

Zopf,D., Bernstein,H.D., Johnson,A.E., and Walter,P. (1990). The methionine-rich domain of the 54 kd protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence. EMBO J. *9*, 4511-4517.