Determining the Active Oligomeric State of SecA

by

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Chapter 1

Introduction
Gram-negative bacteria cell envelope structure

*Escherichia coli* and other Gram-negative bacteria have four main components of their cell envelope shown in Figure 1. The outermost and innermost components are both membranes. In between these are the periplasmic space and the peptidoglycan cell wall.

![Figure 1](image)

**Figure 1** Shows a simplified Gram-negative cell envelope containing an inner and outer membranes (yellow), a peptidoglycan cell wall (tan), and periplasmic space. The outer membrane is shown containing lipopolysaccharides (purple), and porins (blue), the inner membrane is shown containing other proteins (blue).

The outer membrane is a selectively permeable barrier made of a lipid bi-layer where the outer leaflet is composed of lipopolysaccharides shown to slow the diffusion of hydrophilic molecules (Nikaido, 2003). The outer membrane also contains channels and porins for aiding the diffusion of nutrients into the cell and wastes out (Nikaido, 1993), while protecting the cell against harmful environmental elements, often coming from host immune systems. In addition, the outer membrane contains adhesions and proteins implicated in drug resistance. The Gram-negative inner membrane, is a lipid bi-layer containing transporters for nutrients, wastes, proteins, lipopolysaccharides, and peptidoglycan. In addition to these transporters, the inner
membrane contains many proteins required for metabolism and bioenergetics such as the electron transport chain, and the proton ATPase. In between the inner and outer membranes is a matrix known as the periplasm, containing chaperones for assembling outer membrane beta barrel proteins (Sklar et al., 2007), as well as proteins that bind and aid in transport of sugars, amino acids, and vitamins. The periplasm also contains a changing mix of other cellular and environmental elements. The cell wall of Gram-negative bacteria is composed of peptidoglycan, and serves to give the cell structure and allow it to withstand environmental stresses such as osmotic pressure and low pH (Huang et al., 2008).

**Overview of protein translocation in eubacteria**

In eubacteria, RNA is translated into protein by ribosomes in the cytosol. However, proteins have functions in specific places throughout the cell, including the cytosol, inner membrane, periplasm, and outer membrane. In bacteria, as much as 30 percent (Driessen and Nouwen, 2008) of all proteins have functions that require them to be inserted into or translocated through the inner membrane. In order for the membranes and the periplasm to have the proteins necessary for cell growth, protection, and communication, there must be mechanisms of controlled transport across and into membranes that do not compromise the selective permeability of the membrane.

There are several translocation pathways in bacteria, some specific to the inner membrane and some specific to the outer membrane, each specializing in
transporting specific types of proteins under specific conditions. The main pathways of translocation are the following:

- The Sec-dependent pathway, or general secretion pathway, which translocates unfolded proteins (Randall and Hardy, 1986) into or across the plasma membrane.
- The Type I pathway, or ABC system, specializing in small toxins to be released from the cell (Rees et al., 2009).
- The Type II pathways, including the Twin arginine (Tat) system for transporting prefolded redox factors (Palmer et al., 2005).
- Type III, or contact dependent secretion, used to inject toxins directly into eukaryotic cells.

Once proteins have been translocated into the periplasm a new set of transporters transports them into or across the outer membrane, most notably the beta barrel assembly machinery (BAM) (Knowles et al., 2009).

**The Sec pathway**

The Sec pathway uses the energy from ATP and the proton motive force across the membrane to translocate preproteins across or into the plasma membrane (Geller et al., 1986). The Sec system is comprised of a group of several proteins, known as the Sec machinery. The main components of the Sec machinery are SecB, a chaperone; SecA, an ATPase translocation motor; and SecY, SecG, and SecE which make up a transmembrane channel. Also associated with these components is a group of less well characterized proteins, SecDF(YajC), and a signal peptidase.
All proteins destined to be translocated by the Sec system have a signal sequence at their amino terminus that is generally cleaved off as the protein is translocated. This signal sequence, while it has no precise consensus sequence, has a common pattern of about 1 to 5 positively charged residues at the amino terminal region (N domain), followed by a hydrophobic region of about 10 to 15 residues (H domain), followed by a hydrophilic region (C domain) (Fekkes and Driessen, 1999). The C domain is the cleavage site for the signal peptidase, and therefore has consistencies in residue type and placement (Driessen and Nouwen, 2008). A schematic diagram of a signal sequence is shown in Figure 2.

Figure 2 Shows a diagram of a typical signal sequence of a preprotein destined for translocation by SecA. Representations of the length and positioning of the positive N region, hydrophobic H region, and hydrophilic C region are shown. The signal peptidase cleavage site is represented by scissors. Figure adapted from (Fekkes and Driessen, 1999).

In cases of preproteins with more hydrophobic H domains in their signal sequences, or membrane proteins containing a transmembrane anchor sequence, such proteins are translocated co-translationally via recognition by a Signal Recognition Particle (SRP) during translation. The protein in this case is targeted to the SRP receptor, FtsY, which interacts directly with SecY (Angelini et al., 2005) and
bypasses both SecB and SecA. Trigger factor, a ribosome-bound protein, prevents proteins with less hydrophobic signal sequences from going through this pathway (Beck et al., 2000).

The SRP and SecB/SecA targeting pathways are visualized in Figure 3:

Figure 3 Shows the two routes a preprotein can take to get to the final step of translocation through SecYEG. A) Shows the chaperone SecB targeting the unfolded preprotein to the Sec translocon. B) Shows the Signal Recognition Particle binding to the protein which is then targeted to the Sec translocon by the SRP receptor, FtsY, bypassing SecA and SecB. C) Shows YidC, which can be used to completely bypass the Sec and SRP systems by directly inserting small proteins into the membrane.

Components of the Sec System

SecB

The majority of secretory proteins in Gram-negative bacteria are targeted to the Sec system by SecB (Watanabe and Blobel, 1989b), a homotetramer (Watanabe and Blobel, 1989a) chaperone shown in Figure 4.

Figure 4 Shows the homotetramer structure of the chaperone SecB, and its protein-binding channel. Figure adapted from (Xu et al., 2000).

SecB binds to the mature region of the preprotein (Randall et al., 1998) presumably by allowing the mature preprotein to wrap around it in the long grooves (Xu et al., 2000) shown in Figure 4, while leaving the signal sequence exposed. This wrapping allows the preprotein to reach the rest of the Sec machinery unfolded or loosely folded, which is the necessary state for translocation. In addition, SecB binds directly to SecA with highest affinity when SecA is bound to SecY and when SecB is bound to a preprotein (den Blaauwen et al., 1997) effectively targeting preproteins directly to the Sec machinery. This way, SecB acts not only to target the protein the
Sec machinery but to make sure it arrives in a translocatable state i.e., unfolded or loosely folded.

**SecYEG**

At the other end of the system is SecYEG. SecYEG is a protein conducting channel homologous to eukaryotic Sec61 (Flower, 2007) and phylogenetically conserved in all organisms (Stephenson, 2005). SecYEG can function in two ways: it can conduct proteins through the channel to the periplasm, or open laterally and insert proteins into the plasma membrane. It is thought that the hydrophobicity of the preprotein itself may influence this lateral opening (Xie et al., 2007).

SecYEG is composed of three parts, SecY, SecE, and SecG. The largest of these is SecY, a 49 kD protein containing 10 transmembrane segments (Akiyama and Ito, 1987). Tightly bound to SecY is SecE, a smaller protein with three transmembrane segments (only one of which is essential), thought to stabilize SecY (Kihara et al., 1995). Upon depletion of SecE, transport of proteins destined for the outer membrane was disrupted while transport of proteins into the inner membrane was increased indicating that SecE is involved with the lateral opening of SecY (Baars et al., 2008). SecG has 2 transmembrane segments, is less tightly bound to SecY and SecE, and is thought to undergo topology inversions that help couple SecA ATPase activity to the movement of proteins during translocation (Sugai et al., 2007) although this inversion activity has been disputed (van der Sluis et al., 2006).

Because SecYEG spans the membrane, it is responsible for the gating of the channel it forms to prevent leakage between periplasm and cytosol. A domain of
SecY has been identified that acts as a plug in the channel when no translocation is occurring. Figure 5 shows SecYEG structure and a schematic of the proposed plug.

Figure 5 Shows a representation on SecY, flanked by SecE and SecG, changing conformation from A) plugged to B) unplugged. C) Shows a cartoon schematic of this movement. Figure adapted from (Flower, 2007).

This plug domain not only prevents leakage but it has been shown to regulate the rate of translocation (Maillard et al., 2007) and to discern the specificity of signal sequences (Li et al., 2007).

**SecA**

SecA is a 102 kDa ATPase which serves as the bridge between SecB and SecYEG by binding directly to both of them. The role of the SecA protein is to take the preproteins from SecB or in some cases directly from the cytosol, and by coupling
motion to its ATPase activity, move the preprotein into the SecYEG channel. A
ribbon diagram of SecA is shown in Figure 6A.

Figure 6  A) Shows a ribbon model of SecA, B) shows a schematic of the protein’s
domains (In this figure, nucleotide binding fold 2 is labeled IRA2). Figures adapted
from (Papanikolau et al., 2007).
SecA can be divided into the following domains shown and labeled in Figure 6B: A noticeable part of SecA is the long central helix which is part of the helical scaffold domain (HSD), necessary for the ATPase activity of SecA to be coupled to the movement of translocation (Gold et al., 2007). SecA contains two nucleotide binding folds (NBFI, NBFII), which bind ATP and ADP. Together the two nucleotide binding folds and the helical scaffold make up the ATP driven motor which is comparable to the DEAD motors of helicases (Sharma et al., 2003) and provides the movement required for translocation. Preproteins are bound by SecA through the preprotein binding domain (PPXD). In addition to these basic domains, SecA also contains a helical wing domain (HWD) and at the end, the carboxy-terminal domain (CTD) The carboxy-terminal region has been shown to inhibit signal peptide binding, though this is alleviated in the presence of SecB (Gelis et al., 2007).

Regulation of SecA activity

The translocation activity of SecA is regulated in several ways to ensure the right activity with the right protein at the right time. When SecA is bound to SecYEG, it is activated (Karamanou et al., 2007), and binds tightly to the signal sequence of a preprotein bound to SecB. SecA ATPase activity is also increased by the binding of SecA to SecYEG, and when ATP is associated with SecA in the nucleotide binding domain, initiation of translocation through SecYEG can occur. These interactions effectively regulate SecA activity to situations when translocation can occur (Natale et al., 2004). SecA ATPase activity can also be upregulated by the
binding of signal peptide (Driessen and Nouwen, 2008) or downregulated by the binding of magnesium in the nucleotide binding domain (Gold et al., 2007).

It was also shown that the conformation and oligomeric state of SecA can vary with the presence of nucleotides. In vitro, in the absence of nucleotides, dimeric SecA was found. ADP and non-hydrolyzable ATP was shown to shift SecA into the monomeric form. ATP-bound SecA was shown to be in a more “expanded” state than ADP-bound SecA (Bu et al., 2003).

**SecDF(YajC)**

The full function of SecDF(YajC), a membrane protein complex associated with SecYEG, is unclear as of now. It has been proposed that it regulates insertions and deinsertions of SecA into the membrane (Duong and Wickner, 1997). Alternately, SecDF(YajC) may influence the formation of the SecYEG channel since it was shown that upon depletion of SecDF(YajC), membrane bound SecG was also depleted (Kato et al., 2003). It has also been noted that cells deficient in SecDF(YajC) are cold sensitive for growth and protein secretion (Pogliano and Beckwith, 1994).

**Oligomeric state of SecA**

SecA was first shown to exist as a dimer using simple size exclusion chromatography (Akita et al., 1991). Questions still existed however as to whether this was the active state of SecA or simply a storage state, and which of the possible dimers SecA existed in. SecA from various types of bacteria has been crystallized
and analyzed producing a range of dimeric possibilities. *Bacillus subtilis* SecA was first shown using FRET to exist in solution as an antiparallel dimer shown in Figure 7 (Ding et al., 2003).

![Figure 7](image)

**Figure 7** Shows a proposed FRET supported dimer of *B. subtilis* SecA (Ding et al., 2003).

Next, an antiparallel dimer of *Mycobacterium tuberculosis* SecA was proposed (Sharma et al., 2003) shown in Figure 8. In 2006, *Bacillus subtilis* SecA was crystallized as a slightly shifted anti parallel dimer (Zimmer et al., 2006) shown in Figure 9, and *Thermus thermophilus* SecA was crystallized into a parallel dimer (Vassylyev et al., 2006) shown in Figure 10. Most recently, an *Escherichia coli* SecA head-to-head dimer has been crystallized (Papanikolau et al., 2007), shown in Figure 11.
**Figure 8** Shows a crystallized anti-parallel dimer of *M.tuberculosis* SecA with one protomer colored green.

**Figure 9** Shows a crystallized dimer of *B.subtilis* SecA with one protomer colored green.
Figure 10 Shows a crystallized dimer of *T.thermophilus* SecA with one protomer colored green.

Figure 11 Shows a crystallized head to head dimer of *Escherichia coli* SecA with one protomer colored green.

Since crystal structures do not necessarily report on the active state of a protein, the question of the active oligomeric state of SecA has been approached in many other ways. Originally, SecA was shown by Fluorescence Resonance Energy
Transfer (FRET) to translocate as a dimer (Driessen, 1993), in addition, SecA was originally thought to exist entirely as a dimer in solution, but both of these ideas have been questioned. SecA was shown by light scattering to in fact be in a dynamic equilibrium between its monomeric and dimeric state (Woodbury et al., 2002), and many other studies have been done to address the translocation state of SecA.

By using EDAC crosslinking, SecA was shown dissociate into monomers in the presence of synthetic signal peptides and to bind SecYEG as a monomer (Or et al., 2002). Considering the importance of SecYEG and signal peptide binding in translocation, this appeared to be strong evidence for SecA functioning as a monomer during translocation. SecA was also shown by FRET to shift its monomer-dimer equilibrium towards the monomer in the presence of acidic phospholipids (Benach et al., 2003). Because SecA functions bound to the membrane, this was also taken as evidence for SecA functioning as a monomer. However, in this same study (Benach et al., 2003), the influence of signal peptides on SecA was looked at again with a more sensitive method and signal peptides were found to promote oligomerization of SecA while lipids induced monomerization. Other studies have also detected the presence of SecYEG-bound dimers (Jilaveanu et al., 2005).

Studies of translocation and ATPase activity have been carried out on several SecA mutants. One of the first showed that a SecA derivative strongly biased towards the monomeric form (SecA/95-6Ala) was still slightly active (4.5%) in vitro for translocation of proOmpA (Or et al., 2002).

Later, in vitro studies were done using a version of SecA missing the first 11 amino acids and the last 65, shown to exist mostly in monomeric form called
SecAΔ11/95. In addition to SecAΔ11/95 another SecA derivative, SecAΔ11, missing only the first 11 amino acids was studied. SecAΔ11 is shown in Figure 12.

![Figure 12](image.png)

**Figure 12** Shows an anti-parallel SecA dimer from *B.subtilis*. The residues missing in SecAΔ11 are shown in white. These are thought to be important dimer contact sites, supported by the fact that when deleted, SecA is 99% monomeric (Or et al., 2004).

Studies showed the ATPase activity of SecAΔ11/95 as moderately equivalent to wild type SecA though the differences in ATPase activity were heightened in the presence of SecY. In this same study, SecAΔ11/95 was shown to have a lower but comparable translocation activity in vitro, and to complement in vivo about 85%. Protein levels in the in vivo study of WT SecA and SecAΔ11/95
were shown through immunoblotting to be about equal (Or et al., 2004). SecA was
also shown to be non-functional when cross linked in its dimeric state (Or and
Rapoport, 2007).

In seeming opposition to these SecA derivative studies, it was found that
SecA cross-linked in its dimeric state was functional in vitro in ATPase assays and
for in vitro protein translocation (Jilaveanu and Oliver, 2006). It was also shown that
SecAΔ11 missing the first 11 amino acids (shown above in Figure 12) was poorly
functional for ATPase activity and nonfunctional for in vitro protein translocation.
These defects were exacerbated under salt conditions that favor monomerization
(Jilaveanu et al., 2005). It was also pointed out that the low level of translocation seen
in vitro with SecA/95-6Ala (Or et al., 2004) could be due to a low level of
dimer (Jilaveanu et al., 2005).

A study using a technique called membrane trapping exposed the existence of
membrane bound SecA dimers by using a strain that co-expressed two forms of
SecA: a membrane stuck mutant form of SecA along with his-tagged wild type.
When these cells were screened, his-tagged SecA was found to be almost entirely
associated with the membrane along with the membrane-stuck SecA mutant protein,
consistent with the existence of SecA membrane-bound dimer (Jilaveanu et al., 2005).

A somewhat inconclusive study was done with genetically linked SecA
dimers, showing that that SecA dimers covalently linked head to tail in a genetic
fashion are fully functional in vivo. This result supports the hypothesis that SecA
does not need to fully dissociate into monomers in order for translocation to occur.
However, it does not rule out that a “tethered” monomer may be acting in the cell in this case (Wang et al., 2008).
The current study

Since these previous studies produced seemingly contradictory results and indicated complexity in the monomer-dimer associations and dissociations of SecA during translocation, the present study attempts an in vivo approach to investigating this question of the active oligomeric state of SecA by allowing cells to naturally select for suppressors of the SecAΔ11 defect, then using biochemical techniques to analyze the suppressing protein. The goal of this study is therefore to isolate suppressors of the SecAΔ11 defect and analyze the method of suppression in order to gain insight on the physiological dimer and its role in the cell. It is hypothesized that the SecAΔ11 defect will be suppressed if the monomer dimer equilibrium can be shifted back towards the dimer, thus proving the importance of the SecA dimer. This can in theory be done in the several ways, outlined in Table 1:

<table>
<thead>
<tr>
<th>Type of suppressor</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Increased affinity in the dimer interface</td>
<td>Allow SecAΔ11 monomers to dimerize</td>
</tr>
<tr>
<td>2) Allosteric changes in protein that shift dimer interface</td>
<td>Allow SecAΔ11 monomers to dimerize</td>
</tr>
<tr>
<td>3) Overexpression of SecAΔ11</td>
<td>Use law of mass action to increase the amount of dimerized SecAΔ11</td>
</tr>
<tr>
<td>4) Changes that bypass the role of the dimer</td>
<td>The physiological role of the dimer will be accounted for by altering SecAΔ11 in such a way that it can perform the necessary function without dimerization</td>
</tr>
</tbody>
</table>

Class 1 suppressors can address the hypothesis directly. Since it is hypothesized that class 1 mutations may be found along the dimer interface and therefore increase the SecA monomer’s affinity for the other subunit, these mutations
may be used gain insight on the real physiological dimer. If these mutations are found and the monomer-dimer equilibrium is shifted towards the dimer, this will support the importance of the SecA dimer.

If class 2 mutations are found that shift the monomer dimer equilibrium towards the dimer, without aligning with a d known dimer interface, this will still support the importance of the SecA dimer.

It was recently shown in this lab that SecAΔ11/95 is overproduced about two to three fold in vivo compared to the SecAΔ11 protein (possibly due to the loss of the unstructured C-terminus which may be a target for proteolytic destruction), and when this overproduction is reduced using strains containing pLysS which down regulates SecAΔ11/95 overexpression, the cells are no longer viable. It was therefore hypothesized that the SecAΔ11/95 activity in vivo can be accounted for by overexpression which by the law of mass action would shift the monomer-dimer equilibrium towards the dimer. Class 3 mutations can address this. If strains are found that suppress the SecAΔ11 defect only by overproducing the protein, the SecAΔ11/95 activity in vivo can be explained.

In addition, class 4 suppressors while not strictly under the hypothesis, can be used in the future to help elucidate the role of the SecA dimer as well as the complexity of the monomer-dimer cycling. This can be done by investigating the effect the changes the class 4 mutants have on activities such as signal peptide binding and ATP hydrolysis, and should not be overlooked.
Chapter 2

Materials and Methods
Background strains

An *Escherichia coli* strain containing a chromosomal secA amber allele and a temperature sensitive amber suppressor, BL21.19 [secA13(Am) supF(Ts) trp(Am) zch::Tn10 recA::CAT clpA::KAN (lambdaDE3)], was used as a host for plasmids containing alleles of secA. 30º C and 42º C were used as the permissive and restrictive temperatures, respectively, where at 30º C the chromosomal secA and the plasmid secA are both expressed, while at 42º C only the plasmid secA is expressed.

Stratagene’s XL1-Blue supercompetent *E.coli* genotyped as recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZΔM15 Tn10 (Tetr)] (listed genes are mutant) was used in transformations

Stratagene’s XL1-gold supercompetent *E.coli* genotyped as TetrΔ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F’ proAB lacIqZ ΔM15 Tn10 (Tetr) Amy Camr] was also used.

Plasmid

A derivative of the Novgen pET29b plasmid was used, pT7secAhis, containing a T7 promoter as well as a lac operator and repressor (*lacO*, *lacI* respectively), and a his-tagged secA gene.

Stratagene’s pUC18 plasmid was used as a control in PCR and transformations.
SecA mutants and characterization

Four alleles of secA were used in this study on the described plasmid:

- pT7secAhis, wild type SecA, efficiency of plating: 0.91
- pT7secAΔ11his, SecA missing residues 2-11, efficiency of plating 5x10^{-5}
- pT7secAΔ8his, SecA missing residues 2-8, efficiency of plating 3x10^{-5}
- pT7secAΔ11/95, SecA missing residues 2-11 and the last 65 residues at the carboxy terminus, efficiency of plating 0.93

Plating efficiency is defined as the ratio of number of colonies when grown at 42^o C (only plasmid SecA expressed) to number of colonies when grown at 30^o C (plasmid and chromosomal SecA both expressed) and was determined previously (Das et al., 2008).

Selection of spontaneous SecAΔ11 suppressor mutants

BL21.19 (pT7secAΔ11his) was grown overnight at 30^o C in LB (Sigma) media containing 100 µg/mL ampicillin (amp). This culture was then diluted 1/40 in plain LB, and 100 µL of the diluted cells were plated on LBamp plates and incubated for 24 hours at 42^o C. Colonies that grew were then purified by streaking twice at 42^o C. To ensure that the colonies were suppressing the secAΔ11 defect by an alteration in the plasmid, plasmid DNA was isolated from each potential suppressor using the Qiagen mini-prep kit, and retransformed into calcium chloride competent BL21.19. Transformants were selected on LBamp plates grown for 24 hours at 30^o C, suppressors were then struck out twice at 42^o C and 30^o C to compare growth and purify them.
Characterization of SecAΔ11 suppressor mutants

SecA level characterization

The purified plasmid-linked suppressors were then screened for SecA overproduction using Western blot. To screen for overproduction, BL21.19(pT7secAhis), BL21.19(pT7secAΔ11his), BL21.19(pT7secAΔ11/95), and the isolated suppressors were grown overnight at 30º C in LBamp, then subcultured 1 to 50 and grown at 42º C until mid log phase (OD$_{600}$ approx .8). Cell number was then normalized, and cells were harvested by centrifugation. Cells were then resuspended in SDS loading buffer and boiled for five minutes. Before loading, boiled samples were compared for protein concentration using the BioRad Bradford assay, and overall protein amount was normalized and loaded onto SDS 7.5% polyacrylamide gels. After running, protein was transferred with BioRad Transblot using 15 volts for 20 minutes. SecA was then probed with anti SecA sera and visualized with SuperSignal West Pico, and Syngene gel box.

Sequence analysis

secA mutants were sequenced by the University of Pennsylvania DNA sequencing facility using 7 overlapping primers spanning the secA gene and promoter region.
Biochemical characterization

To examine the effects of SecA overproduction on the monomer-dimer equilibrium, the monomer-dimer dissociation constants of SecA and its derivatives was found. To do this, SecA was purified by Novagen his-bind resin as described by the manufacturer or Sigma SP sepharose as described previously by Ding et al 2003. SecAhis, SecAΔ11his, SecAΔ11/95 and SecAΔ8his were sent to the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale. Equilibrium dissociation constants were found by using size-exclusion chromatography and static light scattering.

Size exclusion chromatography was done through a Superose 6 10/30 HR column (GE Healthcare). This was connected to a liquid chromatography system (Alliance 2965, Waters Corp) with auto sampler. The size exclusion column eluate was monitored with a photodiode array UV/vis detector (996 PDA; Waters Corp), a differential refractometer (OPTI-Lab or OPTI-rEx, Wyatt Corp) and a static multi angle laser light scattering detector. (DAWN-EOS; Wyatt Corp). Data collection was done with the aid of Millennium Software (Waters Corp) and ASTRA software (Wyatt Corp).

The preceding procedure was done at two different salt concentrations (100 and 300 mM KCl) since it was previously shown that salt concentration influences the SecA monomer-dimer equilibrium (Woodbury et al., 2002).
PCR mutagenesis of pT7secAΔ11

In order to address more directly the effect of the pT7secAΔ11 defect, secA was mutagenized directly using Stratgene’s Genemorph II EZ Clone Domain Mutagenesis Kit using the following protocol. The kit contains error prone polymerases shown to give equal mutation rates at all bases. Mutation rate can be controlled both by amount of input DNA and number of cycles of PCR carried out.

Primer design and PCR

Primers were designed to mutagenize and amplify secAΔ11 from pT7secAΔ11his:

Forward: 5’ GATATACATATGAGTCGTAACGATC 3’ Tm 50.4º C
Reverse: 5’ TTGTTAGCAGCCGGATCTCAG 3’ Tm 57.0º C

These primers were used in two PCR reactions using the following set-ups to create two conditions of differing mutation frequencies:

Low mutational frequency (1-3 mutations per secA gene)

5µL 10x mutazyme buffer
19.4 µL 85.9 ng/µL pT7secAΔ11his DNA for approx 1000 ng of target secAΔ11
1 µL of forward and reverse primers at 125 ng/µL
1 µL of 200 µM dNTP
1 µL mutazyme polymerase
dH2O to total volume of 50 µL

High mutational frequency (10 mutations per secA gene), performed same as above but using 10.5 µL of template plasmid for approx 500 ng of target secAΔ11.

PCR was run with the following parameters in an Applied Biosciences Veriti Temperature Cycler:
Product was gel purified on 1% agarose (Shelton scientific) gel using QIAquik Gel Extraction Kit according to manufacturer’s protocol. This product of mutagenized secAΔ11 was then used as a primer to replicate the pT7secAΔ11his plasmid containing unmutated secAΔ11 as follows:

Each reaction contained:

- 25 µL 2x EZclone enzyme mix
- 2 µL 50ng template pET29 with pT7secAΔ11his
- 500 ng megaprimer purified in last step
- 3 µL EZclone solution
d H2O to final volume of 50 µL

PCR was run with the following parameters:

- 1 min 95º C
- 25 cycles of the following:
  - 50 sec 95º C
  - 50 sec 60º C
  - 10 minutes (2 min/kb of plasmid) 68º C

The product was digested with Dpn I for two hours.

**Selection of suppressor mutants**

The Dpn I-digested product above was transformed into XL1-Gold supercompetent cells from Stratagene following the manufacturer’s protocol to create a library of mutagenized pT7secAΔ11. Transformants were plated on LBamp plates and incubated at 37º C for 24 hours. The plates were then flooded, cells were
harvested by centrifugation, and DNA was extracted using Wizard Plus SV Miniprep kits according to manufacturer’s protocol.

This DNA was then transformed into BL21.19 electrocompetent cells using the parameters outlined in the BTX Electro Cell Manipulator 600 manual. The cells were grown at 30º C with shaking for one hour, then serial dilutions in LB were plated on LBamp plates and incubated overnight at 30º C or 42º C. Six colonies were then picked from two plates grown at 42º C (B1-6 and C1-6) for a total of 12 candidate suppressors, and purified twice by streaking at 42º C. Candidates C1-C3 died during purification.

**SecA Overproduction screen**

Surviving candidate suppressors (named B1-B6 and C4-C6) were grown overnight in 30º C cultures, then Western blotted using the protein level normalization protocol outlined above (See “SecA level characterization”).

SecA levels of some mutants were ambiguous so T7 promoter/lacO region of the remaining candidate suppressors not obviously overproducing SecA was sequenced by the University of Pennsylvania DNA sequencing facility. No mutations were found in this region.

The plasmids of all candidate suppressors were isolated and transformed into new BL21.19 cells to check for plasmid linkage by their growth phenotype at 30º C and 42º C.
**secA mutation characterization**

**Sequencing**

The *secA* gene in the remaining candidate suppressors (B2-B6 and C4-C5) were sequenced at the University of Pennsylvania DNA sequencing facility. Mutations were visualized on the SecA X-ray structure using WebLabViewer Lite or DS Viewer Pro.

**Recreation of secA mutations in pT7secAΔ11his**

The *secA* mutations contained in the strains, B2, B4, C4 (found to be the same as B2, so this mutation is addressed as “B2” in this section), and C5 (two mutations were found in C5, named C5.1 and C5.2) were recreated using Stratagene’s QuikChange kit.

Primers were designed using Stratagene’s QuikChange primer design website as follows in Table 2. Only the forward primers are shown:

**Table 2**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Change</th>
<th>Melting Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td>5’ GATGCGTATTTTTTGGCTTCCGAGATCCGGCATG 3’</td>
<td>C to A</td>
<td>65.3º C</td>
</tr>
<tr>
<td>B4</td>
<td>5’ CGCGACCAATATGGCGATCGTGGAGATATTG 3’</td>
<td>G to A</td>
<td>64.9º C</td>
</tr>
<tr>
<td>C5.1</td>
<td>5’ CGCGCACCACATCATGGTGATGCACCACGTAAC 3’</td>
<td>C to G</td>
<td>66.7º C</td>
</tr>
<tr>
<td>C5.2</td>
<td>5’ CGTGCACGGTCTGTAACATGCTGCTCTGGAATACAAGCAGTGCCA 3’</td>
<td>A to G</td>
<td>65.6º C</td>
</tr>
</tbody>
</table>
Four PCRs and a blue/white PCR control were set up as follows using Pfu polymerase Ultra Hotstart 2x Mastermix from Stratagene:

- 25 µL PCR mix
- 1 µL 100 ng template (pT7secAΔ11his or control pUC18 plasmid)
- 1 µL each primer at 125 ng/µL
- dH₂O to total volume 50 µL

PCR was run with the following parameters:

- 30 sec 95º C
- 16 cycles of the following:
  - 30 sec 95º C
  - 1 min 57.5º C
  - 16 min 72º C

PCR product was digested with Dpn I for 1 hour at 37º C, and transformed into XL1-Blue super competent cells following the manufacturer’s protocol. 100 µL of cells were plated on LBamp plates which were incubated for 20 hours at 37º C to select for cells containing plasmid. Two colonies from each plate were then purified twice, and colonies were grown overnight at 37º C in LBamp media. Plasmid DNA was extracted from each and retransformed into BL21.19 electrocompetent cells. Transformants were selected on LBamp plates by incubation for 20 hours at 30º C. Colonies from these plates were then purified twice at 30º C. Growth at 30º C and 42º C was compared to growth of both BL21.19(pT7secAΔ11his) and the original suppressors. As a more quantitative measure of suppression, the recreated mutants were grown to log phase (OD600 0.6 - 0.8) in LBamp media and plating efficiencies were done by serially diluting the culture and plating on LBamp plates at 30º C and 42º C. Plating efficiency was defined as the ratio of colonies at 42º C to the number of colonies at 30º C.
Reversion of secA mutations

To further address the role of the mutations found within secA in strains B2, B4, C4 and C5, these mutations were reverted back to wild type secA bases by QuikChange mutagenesis. Plasmid extracted from the suppressors was used as template DNA. Primers used are shown in Table 3 and were identical to those used to create the mutation as described earlier, except the changed base was changed back to wild type:

Table 3

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Change</th>
<th>Melting Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2 and C4</td>
<td>5’ GATGCGGTATTTTTTGCTTCCGA CGAGTATCCGGCATG 3’</td>
<td>A (mut) to C (WT)</td>
<td>66.3º C</td>
</tr>
<tr>
<td>B4</td>
<td>5’ CGCGACCAATATGGCGGATTTCGTGGAGATATTG 3’</td>
<td>A (mut) to G (WT)</td>
<td>66.3º C</td>
</tr>
<tr>
<td>C5.1*</td>
<td>5’ CCGGCCAACATCATGCTGATGCACCACGTAAC 3’</td>
<td>G (mut) to C (WT)</td>
<td>66.9º C</td>
</tr>
<tr>
<td>C5.2</td>
<td>5’ CGTGCCTTTCTTGGTAAAAAATA ACAAGCAGTGCCA 3’</td>
<td>G (mut) to A (WT)</td>
<td>64.8º C</td>
</tr>
</tbody>
</table>

*This reaction has not been done yet

PCR parameters were identical to those previously described (See “Recreation of secA mutations”). Selection and purification protocol is the same as described above (see “Recreation of secA mutations”).
Creation of multiple secA mutations

To investigate the role of the secA mutations further, the mutations were combined using the templates and primers shown in Table 4:

Table 4

<table>
<thead>
<tr>
<th>Template plasmid (1 µL)</th>
<th>Primers (1 µL at 125 ng/µL)</th>
<th>Resulting plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recreated pT7SecAΔ11*B2</td>
<td>B4 forward and reverse</td>
<td>pT7SecAΔ11<em>B2</em>B4</td>
</tr>
<tr>
<td>Recreated pT7SecAΔ11*B2</td>
<td>C5.1 forward and reverse</td>
<td>pT7SecAΔ11<em>B2</em>C5.1</td>
</tr>
<tr>
<td>Recreated pT7SecAΔ11*B4</td>
<td>C5.1 forward and reverse</td>
<td>pT7SecAΔ11<em>B4</em>C5.1</td>
</tr>
<tr>
<td>Recreated pT7SecAΔ11*C5.2</td>
<td>C5.1 forward and reverse</td>
<td>pT7SecAΔ11<em>C5.1</em>C5.2</td>
</tr>
</tbody>
</table>

PCR was run with the concentrations and parameters described above (see “Recreation of secA mutations”) to amplify the entire plasmid. Selection and purification was done as described above (see “Recreation of secA mutations”).

Cold sensitivity testing

Plasmid from the reverted B2 was transformed into XL1-Blue cells along with plasmid containing pT7secAΔ11his as a control, following the XL1-Blue manufacturer’s protocol (except SOC was used in place of NZY+ broth) and plated at 30º C and 42º C. Plates were incubated for 24 hours to test for loss of ampicillin resistance at 30º C.
Chapter 3

SecAΔ11 mutants that suppress by protein overproduction

Adapted from Reexamining the Role of the Amino Terminus of SecA in Promoting its Dimerization and Functional State (Das et al., 2008).
Results

There have been conflicting reports on the active oligomeric state of SecA based on the varying functionality of different monomer-biased SecA derivatives. SecAΔ11/95, an altered form of SecA missing residues 2-11 and the last 65 residues, was shown to be monomer-biased and functional in vivo (Or et al., 2004). It was shown in this lab however that another monomer-biased mutant, SecAΔ11 missing only residues 2-11 was not active in vivo (Jilaveanu et al., 2005). It was also previously shown in this lab that SecAΔ11/95 is overproduced in vivo (Das et al., 2008). To investigate whether there are inherent activity differences between these two monomer-biased proteins (e.g. the presence of the carboxy terminus of SecAΔ11/95 which could potentially act to somewhat block signal peptide binding (Gelis et al., 2007) or to target SecA for proteolytic destruction) or if production level itself is enough to account for the differing growth properties of the two strains, mutants that suppress the SecAΔ11 defect were isolated and investigated.

By plating cells containing pT7 SecAΔ11 at high concentrations and incubating at the restrictive temperature, spontaneous suppressor mutants were found and purified. About fifty percent of these were shown to be plasmid linked, and these had plating efficiencies of around 1.

To investigate overproduction of SecA, the isolated, plasmid-linked suppressors were analyzed on Western blot by running an equal amount of protein from each strain. Nearly all suppressors were had some level of overproduction shown in Figure 13.
Figure 13 Shows a preliminary Western blot screen of the plasmid linked suppressor mutants, showing BL21.19 containing WT secA on the left, and a series of mutants overproducing SecA in the remaining lanes.

To investigate the cause of this overproduction, plasmids from nine of the overproducing mutants were sequenced. Four of the nine sequenced suppressor mutants had mutations in the lacO region shown in Figure 14 that controls the expression of secA.

Figure 14 Shows the pET29b vector with the secA gene under control of the lac system where the lac repressor (lacI) can bind to the lack operator (lacO) just upstream of secA and prevent expression of secA.

Mutations in this area have been previously shown to cause serious defects in the binding of the lac repressor, between 4 and 6 percent of wild type binding (Barkley and Bourgeois, 1980), resulting in this case in pT7secAΔ11 being nearly constitutively on, causing overproduction of SecAΔ11. The lacO region and the relevant mutations are shown in Figure 15A. Protein levels of a lacO mutant are
shown in Figure 15B compared with expression levels of pT7secA, pT7secAΔ11, and pT7secAΔ11/95.

**Figure 15.** A) Shows the three mutated bases in the lacO region of the plasmid, the numbers refer to the percent repressor binding when each base is mutated. B) Shows the different levels of secA expression with pOmpA as a control for overall protein loaded. The gel shows pT7secAhis and pT7secAΔ11his with comparable amounts of SecA protein while pT7secAΔ11/95 shows a higher amount, and the lacO mutant (original mutant, second from right; recreated mutant, far right) shows very high SecA levels.

The mutations in the lacO region were then engineered into new pT7secAΔ11 background to check for sufficiency of this mutation, and comparable suppression was seen (plating efficiency of .64) as well as comparable SecA levels shown in Figure 15B (compare lanes 4 and 5). To check for necessity of these mutations, the
mutations were reverted, and the growth monitored. Growth of these reverted mutants was then comparable to growth of BL21.19 containing pT7secAΔ11. This showed that these mutations in the lacO region are necessary and sufficient to cause overproduction of SecAΔ11, and that over production of SecAΔ11 is enough to cause suppression of the SecAΔ11 defect.

Since the results showed that overproduction itself is indeed enough to suppress the SecAΔ11 defect, we wanted to determine if this overproduction affects the monomer-dimer equilibrium, thus providing an explanation why the lacO mutants, and SecAΔ11/95 while monomer biased can still support the growth of cells when overproduced. To do this, static light scattering was used. Light scattering determined the average size of the molecules coming out of a column, which then could be interpreted as the fraction of SecA dimer. This analysis was performed on purified SecA, SecAΔ8, SecAΔ11, and SecAΔ11/95 to determine the dissociation constants (see Figure 16).

Figure 16 Shows monomer-dimer dissociation curves for SecA. The curves show SecAΔ11 and SecAΔ11/95 having identical dimer association constants (shifted only slightly because SecAΔ11/95 is 5 kDa smaller than SecA). Curves show that at the higher concentrations likely achieved in the cell by overproduction (Das et al., 2008), there is indeed more dimer present. A) Refers to 100mM KCl while B) refers to 300mM KCl.
Static light scattering measurements gave rise to the following equilibrium constants at two different salt concentrations given in Table 5:

<table>
<thead>
<tr>
<th>Protein</th>
<th>100mM KCl</th>
<th>300mM KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>SecAHis</td>
<td>&lt;1x10^-9 M</td>
<td>2.2x10^-6 ( +/- 0.2x10^-6 ) M</td>
</tr>
<tr>
<td>SecAΔ8his</td>
<td>7x10^-8 +/- 1x10^-8 M</td>
<td>2.41x10^-5 +/- 0.05x10^-5 M</td>
</tr>
<tr>
<td>SecAΔ11his</td>
<td>3.5x10^-5 +/- 0.2x10^-6 M</td>
<td>&gt;2.3x10^-4 M</td>
</tr>
<tr>
<td>SecAΔ11/95</td>
<td>3.8x10^-5 +/- 0.09x10^-6 M</td>
<td>&gt;2.3x10^-4 M</td>
</tr>
</tbody>
</table>

The concentrations at which dimerization of SecAΔ11 is seen may well be physiologically attainable. A typical E.coli has between 2,500 and 5,000 SecA molecules (Matsuyama et al., 1992), throughout a cylindrical cell with average dimensions of 1.8 μm long with a diameter of .3 μm. This, along with the 5 to 15 times increase in SecA production, as well as macromolecule crowding (Konopka et al., 2006), could produce high enough concentrations (200 μM) of SecAΔ11 to form dimers in vivo.
Discussion

The decreasing plating efficiencies of BL21.19 (pT7secAhis), BL21.19 (pT7secAΔ8his), and BL21.19 (pT7secAΔ11his), can be correlated to the increasing monomer-dimer dissociation constant. BL21.19 (pT7secAΔ11/95) appears to stand out as a viable mutant of monomer-biased SecA since the dissociation constant of SecAΔ11/95 is comparable to that of SecAΔ11his yet BL21.19 (pT7secAΔ11) is inviable. However, when in vivo levels of SecAΔ11/95 were examined, they were shown to be two to three times higher than SecAΔ11his. Collectively the results indicate that SecAΔ11his and SecAΔ11/95 proteins have the same properties in vivo but that the overproduction of SecA previously observed in BL21.19 (pT7secAΔ11/95) may be sufficient to suppress the lethal dimerization defect observed in BL21.19(pT7secAΔ11). This, when taken with the knowledge that SecA is in a dynamic equilibrium between monomer and dimer, suggests that the observed overproduction of SecAΔ11/95 repopulates the dimer by the law of mass action.

This hypothesis is supported by the series of mutants found in this study that suppress the dimerization defect. These mutants were able to suppress the secAΔ11 defect by significantly overproducing SecAΔ11his. The results of the static light scattering confirm that at higher concentrations, like those produced by the overproducing lacO mutants, and to a lesser extent by BL21.19 (pT7secAΔ11/95), SecAΔ11his does form dimers. This level of dimer may then be enough to meet the physiological requirement of the cell.
Chapter 4

Intragenic secAΔII suppressor mutants
Results

Because all the spontaneously produced mutants described in the previous chapter overproduced SecA, a more direct approach to isolate intragenic secA suppressor mutants was taken to investigate the question of the oligomeric requirements for SecA activity. In this study, secA was directly mutagenized by error prone PCR in an attempt to isolate strains that suppressed the SecAΔ11 defect by an alteration in the SecA protein itself.

Primers were designed to amplify SecAΔ11, and PCR was run with mutagenic polymerases, with concentrations of reagents set to obtain 1-3 mutations per secA gene. A second PCR was run with concentrations set to obtain about 10 mutations per secA gene. These fragments were then inserted back into the pET29 plasmid and a library of mutated secAΔ11 was created. This library was used to transform electro-competent cells which were then grown at the restrictive temperature to select for mutations that supported suppression.

Suppressors from the cells transformed with plasmid from the PCR reaction with the lower mutation rate arose at a frequency about 2 logs higher (a rate of 3x10⁻³) than the spontaneous mutation rate. No suppressors were found using plasmid from the PCR reaction with the higher mutation rate, presumably due to the presence of mutations that rendered SecA nonfunctional. 12 suppressors (named B1-B6 and C1-C6) were then purified by streaking and checked for plasmid linkage. During purification, three strains (C1-C3) died out. All of the remaining nine were found to be plasmid linked by extracting plasmid, retransforming, and assessing
growth. The comparable growth at the permissive and restrictive temperatures of the nine plasmid linked suppressors is shown in Figure 17.

**Figure 17** Shows the growth of all the mutants found to not be clearly overproducing SecA. The top plate was grown at 42° C, the bottom plate at 30° C. Clockwise from top right of both plates are: Δ11, B2, B3, B4, B5, B6, C4, C5. The plates show comparable growth at the restrictive and permissive temperatures on all except Δ11 which as expected, failed to grow at the restrictive temperature.
The nine plasmid linked suppressors were then Western blotted to screen for overproduction. The Western blot only clearly ruled out two candidate suppressors, B1 and C6 due to clear overproduction. The other lanes were equivocal and could not be ruled in or out with certainty due to possible loading inconsistencies. See Figure 18.

![Western blot image](image)

**Figure 18** Shows SecA levels from all the mutants as well as from strains containing wild type SecA, SecAΔ11, and SecAΔ11/95. B1 and C6 are clearly overproducing, while the rest are somewhat questionable.

Mutants B2-B6, C4, and C5, were sequenced to investigate possible alterations in the SecA protein. They were also checked for mutations in the *lacO* region because of the results of the previous chapter. No mutations were found in the *lacO* region of any of these mutants. Four suppressors, B2, B4, C4, and C5, were found to have mutations in the *secA* gene itself while the remaining three B3, B5, B6, either had “synonymous” mutations (base change did not change the amino acid) or no mutations. B2 and C4 were found to have the same mutation in *secA*. 
The following crystal structures (Figures 19A-19D and 20A-B) show the identified secA mutations placed on the x-ray structure (Papanikolau et al., 2007) of the E.coli SecA monomer.

Figure 19 A) Shows the mutation (original residue shown in ball and stick form) found in both B2 and C4, Asp601Glu, in the nucleotide binding fold II domain on the monomer. B) Shows the mutation (original residue shown in ball and stick form) found in B4, Gly508Ser, in the nucleotide binding fold domain on the monomer. C) Shows mutation 1 found in C5 (called C5.1) Leu306Val in the PPXD, shown on the residue before and after the uncrystallized region of the monomer. D) Shows mutation C5.1 (Leu306Val in the PPXD) and 2 (Called C5.2, Lys892Arg in the carboxy terminal domain) on the monomer turned 90 degrees.
Figure 20 Shows A) all four mutations (original residues or closest crystallized residues shown in ball and stick form) on the same SecA molecule, and B) the molecule turned 90 degrees.
While not lining up in a dimer interface (see Appendix for all mutations shown on the x-ray dimer structures), all the mutations were in regions implicated in regulation of SecA activity or dimerization i.e. the preprotein binding domain, the nucleotide binding domain and the carboxy terminal domain. The possible roles of the mutations in these domains will be address further in the discussion.

In order to investigate these mutations further, the necessity and sufficiency had to be determined, to rule out the possibility of the mutations being coincidental non-deleterious mutations. To this end, mutations were re-engineered into unmutagenized pT7SecAΔ11 plasmids, as well as reverted in their original background plasmid by QuikChange mutagenesis.

The recreated mutants grew only slightly better than cells containing SecAΔ11. Plating efficiencies are shown in Table 6

<table>
<thead>
<tr>
<th>Strain (in BL21.19)</th>
<th>Efficiency of plating</th>
</tr>
</thead>
<tbody>
<tr>
<td>SecAΔ11</td>
<td>5.8x10^{-6}</td>
</tr>
<tr>
<td>Recreated B2</td>
<td>8.2x10^{-6}</td>
</tr>
<tr>
<td>Recreated C4</td>
<td>8.2x10^{-6}</td>
</tr>
<tr>
<td>Recreated B4</td>
<td>1.3x10^{-5}</td>
</tr>
<tr>
<td>Recreated C5.1*</td>
<td>2.14x10^{-5}</td>
</tr>
<tr>
<td>Recreated C5.2*</td>
<td>1.02x10^{-5}</td>
</tr>
</tbody>
</table>

*recreation not yet confirmed by sequencing

While this data shows that the mutations in secA are not sufficient for suppression, it does not rule out that the mutations may be necessary. To address this, mutations were each reverted in their original background plasmid. The reverted mutants showed unpredictable suppression levels shown in Table 7
As the above tables show, the mutations are not “typical” suppressor mutations that suppress when remade and do not suppress when reverted. However, neither do they seem to be purely coincidental. When B2 (Asp601Glu in the nucleotide binding domain) was reverted, it grew slowly and with ill-defined colonies at the restrictive temperature, with no observable growth at the permissive temperature. When C4 containing the same Asp601Glu mutation was reverted, the cells failed to grow entirely at the restrictive temperature while growing healthily at the permissive temperature, as one might expect from a “typical suppressor” This strongly indicates two different secondary mutations elsewhere on the plasmid of B2 and C4.

To investigate whether the secondary plasmid mutation in B2 caused the plasmid itself to become cold sensitive and therefore causing the cells to lose ampicillin resistance at 30° C, non-TS DH5a cells were transformed with the B2 reverted plasmid and grown at 30° C and 42° C. DH5a cells containing the reverted B2 plasmid grew comparably to DH5a cells containing pT7SecAΔ11. This showed that the poor growth of Bl21.19 cells containing the reverted the B2 plasmid at 30° C cannot be attributed to a cold sensitive mutation in the plasmid containing ampicillin.
resistance. This seems to implicate the presence of a secondary mutation in secA, though this was not found upon sequencing.

When B4 (Gly508Ser in the nucleotide binding domain) was reverted, it grew very slowly at the restrictive temperature, 42º C, and not at all at the permissive temperature 30º C. The B4 reverted plasmid was not yet tested for cold-sensitivity. When C5.2 (Lys892Arg) was reverted it grew similarly at both temperatures indicating that this mutation may be irrelevant. C5.1 (Leu306Val) has not yet been reverted though it suppressed the best when recreated.

This data is summarized in Table 8 (on next page).
<table>
<thead>
<tr>
<th>Mutant</th>
<th>C6</th>
<th>Comparable</th>
<th>C5</th>
<th>Comparable</th>
<th>C4</th>
<th>Comparable</th>
<th>B6</th>
<th>comparable</th>
<th>B5</th>
<th>comparable</th>
<th>B4</th>
<th>comparable</th>
<th>B3</th>
<th>comparable</th>
<th>B2</th>
<th>comparable</th>
<th>BI</th>
<th>comparable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype when reverted</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
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<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td></td>
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</tr>
<tr>
<td>Lys892Arg</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
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</tr>
<tr>
<td>Leu306Val</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
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<td>No</td>
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<tr>
<td>Asp601Glu</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
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<tr>
<td>Gly508Ser</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
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<td>Not tested</td>
<td>No</td>
<td>Not tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation in lacO region?</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td>L396Val</td>
<td>Not tested</td>
<td></td>
</tr>
<tr>
<td>Over-producing?</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
<td>Yes</td>
<td>Not tested</td>
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Because the recreated mutations only increased suppression to a degree of less than one log, in order to further investigate the potential roles of the mutations, plasmids with combinations of mutations were created. In addition to the two mutations found in C5 being combined on one plasmid to recreate the “complete” C5, the mutations from B2 and B4 were combined, the mutations from B2 and C5.1 were combined and the mutations from B4 and C5.1 were combined (all combinations confirmed by sequencing). Initially plating showed no apparent increase in suppression, but because the increase may be as little as one log, more sensitive methods are necessary to determine suppression levels. Preliminary plating efficiencies (data not shown) indicate that SecAΔ11 containing the combined B2 and C5.1 alterations show increased suppression of the SecAΔ11 defect.
Discussion

When cells were transformed with plasmids containing PCR-mutagenized pT7secAΔ11his, the plating efficiency was about two logs higher than the regular plating efficiency (~mutation rate) of BL21.19(pT7secAΔ11his). This indicates that the mutations created in secA are contributing to suppression of the SecAΔ11 defect. The PCR with the higher mutation rate created deleterious mutations, as indicated by growth at the permissive temperature and no growth at the restrictive temperature.

However, while the suppressors arose at a higher frequency, the very weak suppression in the recreated secA mutants indicates that the mutations in secA are not sufficient to suppress the defect. This is most likely because the weak suppression they offered allowed secondary spontaneous mutations elsewhere in the plasmid to form in such a way that neither the secA mutation or the secondary plasmid mutation was strong enough to suppress on its own, as indicated by the poor or slow growth seen in most strains when the mutations were reverted thus far, and the very weak suppression exhibited by the recreated strains. However, the total lack of growth at the restrictive temperature when C4 was reverted shows that this mutation is vital. The importance of the C4 mutation is also supported by its appearance twice in such a small pool of 12 mutants.

Proposed mechanisms of suppression

B2 and C4

The mutation in B2 and C4 (Asp601Glu) which was shown to be necessary for C4 to survive at the restrictive temperature, is a change from an aspartic acid to a
glutamic acid. These two amino acids are almost identical in properties of general polarity and pH, but glutamic acid has a longer R group. If the reversion experiments in B2 and C4 had not caused such unexpected and drastic results, the weak suppression shown in the recreated mutants might indicate that this mutation was an artifact of artificially mutagenizing the plasmid, an almost-silent mutation that is not too deleterious for functioning of the protein due to similarities in characteristics. However, since the reversion of C4 showed this mutation to be essential, it is possible that the extra length gained by the change from aspartic acid to glutamic acid is useful in making more or stronger dimer contacts. Since this protein itself has not been analyzed yet, it cannot be determined if this protein has altered characteristics in monomer-dimer equilibrium.

SecA is an ATPase, and it is noted that the presence of ATP and ADP have varying effects on dimerization, activity, and conformation of SecA. Most notably ATP has been shown to promote monomerization of SecA (Shin et al., 2006), and to shift SecA to a closed conformation (Bu et al., 2003). Since the mutation found in B2 and C4 is in the nucleotide binding domain, it is plausible that it may be suppressing by inducing the opposite of some of the conformational changes that a bound ATP molecule causes. It is also possible that the mutation may be causing a slight conformational change that would normally be caused by dimerization that then allows the nucleotides to regain their full effect on SecA, that may be lost when SecA is monomer biased.

More research is necessary to determine which if any of these proposed models of suppression are occurring in the cell. Upon further investigation however,
this mutation may shed light on the physiological role and importance of the dimer, as well as where dimerization is necessary in the complex cycling of SecA.

**B4**

B4 (Gly508Ser) has changed a glycine to a serine which is a radical change from a small nonpolar residue to a larger more polar residue. Because the mutation found in B4 is a bit deeper in the SecA molecule than the other mutations (see Figure 19) it is possible that this mutation could drastically shift the surrounding regions giving rise to an altered surface structure. B4, also being in the nucleotide binding domain like B2 and C4, may be suppressing in ways similar to those described above, potentially altering the ATP and ADP binding and through this, SecA dimerization and activity, or it may make more dimer contact sites by shifting the exposed face. B4 will have to be studied more to understand why it fails to grow at the permissive temperature before the role of this mutation in the suppression of the SecAΔ11 can be understood.

**C5**

In suppressor C5, two mutations were found. C5.1 (Leu306Val in the PPXD) is again a change between similar residues. Leucine and valine vary by only one carbon, valine being one carbon smaller, and this residue (L306) is conserved as either leucine or valine in all SecA proteins (Gelis et al., 2007). This residue is one of 13 residues that exhibit a significant shift on NMR when signal peptide is present. When this residue was mutated to a polar residue in a previous study, signal peptide
binding was significantly reduced as was translocation (Gelis et al., 2007). It is plausible then that the leucine to valine change may be suppressing the SecAΔ11 defect by mimicking changes associated with preprotein binding which normally would shift SecA to a more open conformation, promote dimerization (Shin et al., 2006), and upregulate SecA activity (Driessen and Nouwen, 2008). The reverse situation is also plausible; the mutation may mimic changes associated with dimerization which then may promote preprotein binding, upregulating SecA activity.

The mutation found in C5.1 had the highest suppression rate when recreated (though still quite low), indicating that it may be essential. In addition, in preliminary screens of the combined mutants, the combined B2/C5.1 had the highest plating efficiency. The essential nature of C5.1 may be confirmed when the reversion of C5.1 is complete. If it is confirmed as essential, the positioning of this mutation shows that the binding of preprotein may be an essential function of the dimer.

In addition to this, it was shown that the C terminus can block the signal peptide from binding, and therefore downregulate translocation. If mutation C5.1 does in fact serve to upregulate SecA activity by increasing preprotein binding, it makes sense then that C5 would have two mutations, one in the PPXD and one in the C terminus, as they may act together to increase signal peptide binding, and therefore upregulating SecA activity. However, this may not be the case since the reversion of C5.2 shows that C5 can grow well without this mutation. This mutation in the CTD is also a more extreme change as lysine and arginine vary structurally. This region of SecA, being unstructured however, may simply be able to tolerate more drastic alterations that do not act to change SecA activity.
**Proposed dimers**

As shown in Figure 20, all the mutations found in this study align on one face of the monomer. This arrangement could indicate a dimer face. These mutations may directly stabilize the dimer, or they may be alterations in the monomer in areas where dimer contact would otherwise function to alter SecA activity. Based on the positioning of the mutations, one parallel and one anti parallel dimer shown in Figure 21 may be proposed, neither of which directly coincide with a previously crystallized dimer.

However, because of some of the expected results during the recreation and reversion experiments, more investigation must be done before the role of the mutations can be really understood and these dimers can be realistically supported. The fact that these mutations do not correlate with any previously crystallized dimers indicates that the “active” dimer may be quite different from the dimers existing in solution.
Figure 21 Shows two proposed dimers based on the mutations found. A) shows a parallel dimer, and B) shows an anti parallel dimer.
**Future Directions**

This project, particularly the PCR-created mutants, opens many interesting doors to future research. In the future, the *secA* mutations found can be created in pT7SecAΔ8 plasmid since it was shown to have a slightly higher affinity for the dimer. The mutations then might be able to suppress without the aid of a secondary plasmid mutation.

In hopes of finding more *secA* mutations, the PCR mutagenesis may be performed again using already mutated plasmid from one of the four mutants. This may increase the chances of finding a second beneficial mutation within *secA*.

To get a more definitive answer as to whether these mutations are shifting the monomer-dimer equilibrium, purified SecA protein from the mutants identified in this study can be analyzed by light scattering as described in chapter 3 and their dissociation constant can be found. If a slightly lower dissociation constant is found, this would give insight on the cause of the partial suppression observed.

Mutations that do not shift the equilibrium dissociation constant can be used to analyze the physiological role of the dimer by analyzing what activity of SecA has been enhanced by the mutation that has allowed the SecAΔ11 dimerization defect to be suppressed without repopulating the dimer. Signal peptide binding can be investigated in the case of mutant C5, as well as ATP-ADP binding in the case of B2 and C4.

Because reverted B2 and B4 seem to exhibit the phenotype of lethal overproduction at 30º C, to understand the unexpected results of the reversion experiments, plasmid levels could be investigated directly by normalizing cell number before extracting plasmid then measuring plasmid amount, rather than
measuring protein level through Western blot. If overproduction of plasmid is seen, this may partially explain the lethality of reverted B2 and B4 at 30º C when chromosomal secA is also expressed.

In addition, in the future more mutants can be screened to give a better sample size. Considering four mutations (three different mutations one identical) in secA were found from a pool of seven non-overproducing suppressors, it is reasonable to assume that screening more suppressors will yield more secA mutants. Mutations that do result in suppression or partial suppression when re-engineered into new pT7secAΔ11 can be purified and the dimer dissociation constant can be found. The mutants can then be analyzed to possibly construct the physiological dimer.

**Conclusion**

Overproduction of SecAΔ11his protein is sufficient to suppress the secAΔ11 defect, and at higher concentrations of protein, SecAΔ11his forms dimers, supporting the hypothesis that SecA requires the dimer to function.

The mutations in secAΔ11 reported in this study are not sufficient to suppress the dimerization defect in SecAΔ11his, but they may play a role in stabilizing the dimer or increasing SecA activity.
6. Appendix

Mutations shown on x-ray structures of SecA dimers

Key to all structures:
- **B2** Asp601Glu
- **B4** Gly508Ser
- **C5.1** Leu306Val
- **C5.2** Lys892Arg
Figures A-E Show the four mutation found in B2, B4, C4 and C5 on the x-ray structure of SecA dimers from A) *M. tuberculosis* (Sharma et al., 2003) B) *E. coli* (Papanikolau et al., 2007) C) *B. subtilis* (Ding et al., 2003). D) *T. thermophilus* (Vassylyev et al., 2006) E) *B. subtilis* (Zimmer et al., 2006) Corresponding residues were found using the alignment from (Vassylyev et al., 2006).
7. References

Orientations of proteins in membranes database. http://opm.phar.umich.edu/


Watanabe, M., and Blobel, G. (1989a). Cytosolic factor purified from Escherichia coli is necessary and sufficient for the export of a preprotein and is a homotetramer of SecB. Proc Natl Acad Sci U S A 86, 2728-2732.


