Characterization of the SecA2 Protein Export Pathway of Mycobacteria

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ABSTRACT

NATHAN WILLIAM RIGEL: Characterization of the SecA2 Protein Export Pathway of Mycobacteria
(Under the direction of Miriam Braunstein)

Nearly one-third of the world's population is infected with *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis. To establish and maintain infection, *M. tuberculosis* uses surface and secreted proteins to modulate the host immune response. There are several dedicated export machines that transport surface and secreted proteins from their site of synthesis in the cytoplasm across the bacterial cytoplasmic membrane. The bulk of protein export across the cytoplasmic membrane is carried out by the Sec pathway. Energy for Sec-dependent protein export is provided by the essential ATPase SecA. Recently, a small subset of Gram positive bacteria and mycobacteria were found to possess two SecA homologs, SecA1 and SecA2. In *M. tuberculosis* and the non-pathogenic model mycobacterium *M. smegmatis*, SecA1 is essential for general protein export and is the presumed "housekeeping" SecA, while SecA2 is an accessory SecA specific for a subset of exported proteins. In this work, we describe our initial attempts to characterize the mechanism behind SecA2-mediated protein export in mycobacteria. We began by comparing similarities and differences between SecA1 and SecA2. One striking difference we discovered was that SecA1 and SecA2 localize to different cellular compartments. By comparing a structural prediction of SecA2 to the published crystal structure of SecA1, we

identified putative structural differences between the two SecAs. Both SecA1 and SecA2 have predicted ATP binding sites. We showed that *M. tuberculosis* SecA1 and SecA2 bind and hydrolyze ATP in vitro. By constructing a *secA2* mutant that encodes a protein defective in ATP binding, we also demonstrated that ATP binding is required for normal SecA2 function in vivo. Upon subsequent analysis, we found that SecA2 mutants unable to bind ATP were dominant negative. We used this dominant negative phenotype as a tool to study SecA2 by performing a suppressor screen. We isolated intragenic suppressors and used them to identify structural subdomains of SecA2 that are important for function. While we have not yet identified the extragenic suppressors, we believe they represent a powerful tool for identifying SecA2-interacting proteins. Identifying SecA2-interacting proteins will be crucial to fully understand the accessory SecA2 protein export pathway of mycobacteria. Finally, we show for the first time in any bacterium with two SecA proteins that the canonical SecA1 protein is also required to export SecA2-dependent substrates. By developing a better understanding of the SecA2 pathway, we hope to increase our knowledge of mycobacterial physiology to enable the design of new anti-tuberculosis therapies.

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ABBREVIATIONS

2D	Two-dimensional
^{32}P	phosphorus
A	alanine
ABC	ATP binding cassette
ADP	adenosine diphosphate
AIDS	Acquired Immune Deficiency Syndrome
Asp	aspartic acid
ATP	adenosine triphosphate
BCG	bacillus Calmette-Guerin
BME	beta-mercapto ethanol
bp	base pair
C	cysteine
CFP-10	culture filtrate protein 10 kilodalton
CFU	colony forming units
CW	cell wall fraction
Cys	cysteine
D	aspartic acid
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
DSP	dithiobis(succinimidyl propionate)
E	glutamic acid

E.	*Escherichia*
ESAT-6	early secreted antigen target 6 kilodalton
ESX-1	ESAT-6 secretion system
F	phenylalanine
G	glycine
Glu	glutamic acid
H	hour
HA	hemagglutinin
His	histidine
HIS	hexahistidine tag
HIV	Human Immunodeficiency Virus
hyg	hygromycin resistance gene
I	isoleucine
Ile	isoleucine
K	lysine
kan	kanamycin resistance gene
kbp	kilobasepair
kDa	kilodalton
L	leucine
L.	*Listeria*
Leu	leucine
M.	*Mycobacterium*
MDR	Multi-drug resistant

MEM	membrane fraction
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
ng	nanogram
nm	nanometer
OD_{600}	optical density at 600 nanometers
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEL	pellet fraction
Prl	protein localization
R	arginine
S	serine
S.	*Streptococcus*
Suc	sucrose
T	threonine
Thr	threonine
SDS	sodium dodecyl sulfate
Sec	secretion
SOL	soluble fraction

SRP	Signal Recognition Particle
TB	tuberculosis
V	valine
WCL	whole cell lysate
XDR	extensively drug resistant
Y	tyrosine
β	beta
Δ	gene deletion
μl	microliter

Chapter 1

INTRODUCTION

Tuberculosis (TB) is caused by the acid-fast bacillus *Mycobacterium tuberculosis*. By current estimates, up to one-third of the world's population is infected with *M. tuberculosis* (71). In 2006, 1.7 million people died from TB while an additional 9.2 million new cases of TB were reported. TB is the leading cause of death from a bacterial disease (72). TB is also a leading cause of death of HIV positive individuals (71). As with other infectious diseases, the burden of TB is highest in developing countries, especially in Africa and Eastern Asia.

Spread of *M. tuberculosis* and development of TB disease. While public health strategies have been implemented in many countries to control the spread of TB, a new threat has surfaced with the rise of multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB) *M. tuberculosis* strains (45, 71). MDR-TB strains are resistant to the standard treatment with first-line antibiotics isoniazid and rifampicin. Treatment is still possible with less effective second-line antibiotics. However, if not treated properly, MDR-TB strains can acquire additional antibiotic resistance. This has led to the appearance of XDR-TB strains. These strains are resistant to the first-line antibiotics, any fluoroquinolone, and any of the second-line antibiotics. Depending on the drug resistance profile, some XDR-TB strains are untreatable.

As of February 2008, 45 countries, including the US, reported confirmed cases of XDR-TB (70). 40,000 new cases of XDR-TB are expected to emerge each year, underscoring the urgent need to devise new anti-TB treatments.

M. tuberculosis is spread person to person via aerosols that are expelled when a person with an active case of TB coughs or sneezes. *M. tuberculosis* is contained within these aerosols, which are then inhaled into the alveolar space of the lung. The bacteria are then phagocytosed by macrophages. *M. tuberculosis* is an intracellular pathogen, and as such, it has developed the means to survive inside host cells. The mechanism by which *M. tuberculosis* survives inside macrophages is complex and is a matter of active investigation (35, 55).

For individuals with a healthy immune system, primary infection with *M. tuberculosis* is usually controlled and TB disease does not immediately develop. During the onset of the cell-mediate immune response, *M. tuberculosis*-infected macrophages become surrounded by other immune cells (macrophages and T cells) and are effectively walled-off from the rest of the lung (37). This network of cells is termed a granuloma, one of the hallmarks of TB infection (20, 56). The metabolic state of *M. tuberculosis* inside a granuloma is not well understood. The most common description is that *M. tuberculosis* enters a dormant state, awaiting conditions that are more favorable for the bacterium to reactivate. In 10% of all individuals infected with *M. tuberculosis*, the bacilli reactivate later in life resulting in active infectious TB disease (29). The signals that trigger reactivation are not clear but do seem linked to the immune status of the infected individual. This has become particularly important with the advent of the HIV epidemic since TB is the leading cause of death among HIV patients (26).

Identification of the molecular mechanisms that *M. tuberculosis* uses to gain a foothold in the host is an active area of research. Protein export systems of *M. tuberculosis* are of particular interest given the importance of such systems to virulence in numerous and diverse bacterial pathogens (28, 40). Exported proteins are defined as any protein located beyond the cytoplasmic membrane and includes proteins attached to the bacterial cell surface or secreted into the extracellular environment. The extracytoplasmic location of exported proteins makes them ideally positioned to interact with host cells. Consequently, exported proteins and the requisite systems for exporting them often play important roles in bacterial pathogenesis.

The general Sec system of protein export. In bacteria, the bulk of protein export from the cytoplasm across the cytoplasmic membrane is carried out by the universally conserved general secretion (Sec) pathway (Figure 1.1) (22, 47). The Sec pathway is essential in all organisms tested. Much of the detailed characterization of the Sec pathway was performed using the model organisms *E. coli* and *B. subtilis* (22, 50).

The central component of the Sec apparatus is the membrane-bound channel called the Sec translocase. The translocase is composed of the integral membrane proteins SecY, SecE, and SecG (12). Together, these three proteins form a complex that spans the cytoplasmic membrane and serves as the channel through which pre-proteins are translocated in an unfolded state. Another key player in the Sec pathway is the multifunctional SecA ATPase (23). SecA can directly bind precursor pre-proteins destined for export through the SecYEG translocase (49). SecA can also interact with cytoplasmic chaperones, such as GroEL and SecB (8, 27). SecB recognizes a subset of pre-proteins, keeps them in an unfolded export-competent state, and binds directly to SecA to deliver them for export (33,

53). SecA also binds with low affinity to acidic phospholipids in the cytoplasmic membrane and with high affinity to SecYEG (34). SecA is an ATPase and the energy from ATP hydrolysis is required for Sec export (46). With each cycle of ATP binding and hydrolysis, SecA undergoes conformational changes that allow it to feed nascent pre-proteins through the SecYEG translocase, 20 amino acids at a time (58). Given the essential roles SecA plays in protein export, it is no surprise that in all cases tested SecA is essential for viability.

ATP binding and ATPase activity are essential for SecA function (46). SecA contains ATP-binding Walker Box motifs, a structural feature found in many ATPases (69). Mutations in the Walker Box motifs of *E. coli* SecA destroy the ability of SecA to bind ATP (46). Furthermore, mutations in the SecA Walker Box are nonfunctional as shown by the inability to complement temperature sensitive *secA* mutants. SecA Walker Box mutants are also nonfunctional for in vitro pre-protein translocation assays. Additionally, disruption of ATPase activity interferes with the ability of SecA to cycle between the cytoplasmic membrane and the cytoplasm (25). Normally, SecA is found evenly distributed between the cytoplasmic membrane and cytoplasm (14). However, a mutated Walker Box results in a SecA that is predominantly cell membrane-associated (46). In fact, treatment with carbonate is unable to dissociate Walker Box mutant SecA from the cytoplasmic membrane, indicating that this protein is permanently lodged in the membrane and unable to cycle upon ATP binding and hydrolysis. This fixed localization helps explain why a SecA Walker Box mutant is unable to complement temperature sensitive *secA* mutants.

The Sec pathway exports proteins that are synthesized as pre-proteins with an N-terminal sorting signal, called a signal sequence. Sec signal sequences are tripartite; they consist of a positively charged N domain, a hydrophobic H domain, and a polar C domain

that contains a cleavage site (22). During the process of export, a periplasmic signal peptidase, either LepB or LspA, cleaves the signal sequence to release the exported mature protein (66, 67, 77). LepB cleaves standard signal sequences while LspA specifically cleaves signal sequences of lipoproteins by recognizing a specific lipobox motif (L-A/S-G/A-C_{+1}).

The Sec pathway also participates in delivering integral membrane proteins to the cytoplasmic membrane (22, 76). This class of proteins is delivered to the SecYEG translocase cotranslationally with the aid of the *S*ignal *R*ecognition *P*article (SRP). SRP is a ribonucleoprotein composed of a 4.5S RNA and a GTPase Ffh (fifty-four homolog). As a newly synthesized pre-protein emerges from the ribosome, SRP recognizes the signal sequence or transmembrane domains of the nascent polypeptide chain. This complex then interacts with FtsY, which in turn interacts with both anionic phospholipids and the SecYEG translocase. Upon GTP hydrolysis, SRP and FtsY are released from the membrane, leaving the pre-protein associated with the SecYEG translocase.

The Sec pathway also plays a role in delivery of integral membrane protein into the cytoplasmic membrane (22, 76). SecA delivers some, but not all transmembrane proteins to the SecYEG translocase. Once inserted into the SecYEG pore, hydrophobic transmembrane domains are believed to exit the translocase through a lateral gate. The precise mechanism behind lateral exit and integration of transmembrane proteins into the cytoplasmic membrane is not fully understood. Furthermore, insertion of a subset of transmembrane proteins requires the membrane protein YidC in addition to the Sec pathway (16). YidC can associate with SecYEG and catalyze insertion of newly translocated membrane proteins. In addition,

YidC can function independently of the Sec pathway and directly insert transmembrane domains into the cytoplasmic membrane (76).

Specialized protein secretion systems have roles in bacterial pathogenesis. In addition to the universally conserved Sec pathway, many bacterial pathogens use specialized secretion systems to transport virulence factors (28). There are now six named specialized secretion systems in Gram negative bacteria (30). Some of these pathways export proteins in two steps. Such proteins first cross the cytoplasmic membrane through the Sec system and are then secreted across the Gram negative outer membrane by a separate export apparatus. Other pathways secrete proteins in one-step by a process that is completely independent of the Sec pathway. One of the best-characterized specialized secretion systems is the Type III secretion system (T3SS) found in bacteria including *Salmonella* and *Yersinia* (38). The T3SS spans the entire bacterial cell envelope (comprised of the cytoplasmic membrane and the cell wall) and forms a needle complex that extends beyond the bacterial cell surface. The T3SS directly injects proteins synthesized in the bacterial cytoplasm through the needle complex and into host cells. Once inside the host, these bacterial effector proteins can function in a wide variety of ways to mediate bacterial infection.

While there are a plethora of specialized secretion systems in Gram negative bacteria, only recently have specialized export systems been described in Gram positive bacteria. One such example is the ESX-1 system (1). In *M. tuberculosis*, the ESX-1 system is required for virulence (43). This system was first identified in mycobacteria as being required to export the small virulence factors CFP-10 and ESAT-6. Both of these proteins lack predicted Sec signal sequences, suggesting an alternate secretion pathway is required for their export. Subsequent analysis shows the genes adjacent to *esat-6* and *cfp-10* encode proteins that

constitute a specialized secretion system (11). Several Gram positive bacteria encode ESX secretion systems including *Staphylococcus aureus*, *Corynebacterium diphtheriae*, and *Listeria monocytogenes* (1, 62).

Another example of specialized protein secretion in Gram positive bacteria is the cytolysin-mediated translocation pathway. Examples of this pathway are found in *Streptococcus pyogenes* and *Listeria monocytogenes* (44, 52). In this system, a soluble cytolysin released from the bacterium forms a large oligomer within the membrane of a eukaryotic host cell. Once this pore is formed, other effector proteins exported from the bacterial cytoplasm can directly enter the host cell cytoplasm. In the *S. pyogenes* example, streptolysin O (SLO) forms a pore in membrane of human keratinocytes (44). Then, SPN (*S. pyogenes* NAD-glycohydrolase) translocates into the host cytoplasm. The combined activity of SLO and SPN is required for full cytotoxicity.

Accessory SecA2 protein export systems. It was long thought that all bacteria possess a single and essential SecA (24). Recently, mycobacteria and a small subset of Gram positive bacteria were found to encode two SecA homologs (5, 9, 10, 15, 18, 42, 54, 59). Included in this list are pathogens (*M. tuberculosis*, *Listeria monocytogenes*, *Streptococcus gordonii*, *Streptococcus parasanguinis*, *Staphylococcus aureus*, and *Bacillus anthracis*) and nonpathogens (*M. smegmatis*, *L. innocua*, and *Corynebacterium glutamicum*). In these bacteria, the SecA with highest homology to the essential SecA of *B. subtilis* is called SecA or SecA1 (Table 1.1). The other SecA is called SecA2. Bacteria with an accessory SecA2 can be divided into two groups: those that also possess an accessory SecY2 protein (SecA2/Y2) and those that do not (SecA2-only) (Figure 1.2).

The accessory SecA2/Y2 systems of *S. gordonii* and *S. parasanguinis*. The best-studied SecA2/SecY2 systems are those of *S. gordonii* and *S. parasanguinis*, two organisms found in the oral cavity (5, 18). The components and the mechanism of the accessory SecA2/SecY2 systems in these two bacteria are very similar. In both cases, the *secA2* and *secY2* genes are encoded in a locus that additionally possesses a suite of similarly arranged genes that include a SecA2-exported substrate and proteins involved in glycosylation and export of this substrate (Figure 1.3). In *S. gordonii*, the SecA2 system exports the serine-rich glycoprotein GspB (5). After crossing the cytoplasmic membrane, GspB becomes anchored to the cell wall where the protein is implicated in platelet binding. Platelet binding by *S. gordonii* is believed important to development of endocarditis (3). Similarly, the SecA2 system of *S. parasanguinis* exports a serine-rich glycoprotein called Fap1 to the cell wall (18). Fap1 forms fimbriae that are needed for attachment of *S. parasanguinis* to the surface of teeth and subsequent accumulation of dental plaque leading to periodontal disease (74). It seems highly likely that the proper export of these serine-rich glycoproteins by SecA2 is important to development of disease.

The accessory SecA2/SecY2 systems in *S. gordonii* and *S. parasanguinis* share many of the same components, including an accessory *secY2* homolog that encodes a protein with predicted transmembrane domains. In *S. gordonii*, Asp1-3 (Accessory secretory protein) are all required for GspB export (64). Asp2 is a predicted transmembrane protein. It is possible that Asp2 and SecY2 form a membrane-embedded translocase. The function of Asp1 and Asp3 is not clear as both proteins lack predicted transmembrane domains. The homologous proteins in *S. parasanguinis* are named Gap1-3 (Glycosylation associated protein). The function of these proteins appears to differ slightly from the Asp proteins. For example, in

the absence of Gap3, Fap1 is still exported but the pattern of glycosylation is different (51). The significance of the difference between Gap3 and Asp3 function is unclear. There are other differences between the SecA2/SecY2 systems of *S. gordonii* and *S. parasanguinis*. In *S. gordonii*, *asp4* and *asp5* encode proteins with homology to *B. subtilis* SecE (52% similarity) and SecG (55% similarity), respectively (65). Both proteins are required for GspB export. However, there are no obvious Asp4 or Asp5 homologs in *S. parasanguinis*. The significance of the slight differences between *S. gordonii* and *S. parasanguinis* is not clear.

Both GspB and Fap1 are heavily glycosylated proteins. In both the *S. gordonii* and *S. parasanguinis secA2/Y2* loci there are multiple genes encoding proteins with roles in glycosylation of the exported serine-rich proteins (13, 63, 73). Downstream of *secA2* are *gtf* genes required for glycosylation of GspB and Fap1. Although the genomic organization is slightly different, both *S. gordonii* and *S. parasanguinis secA2/Y2* loci also encode additional genes with homology to glycosyltransferase (*gly*) and nucleotide sugar synthetase (*nss*). Gly and Nss specify the types of carbohydrate linkages that are added to GspB. *S. parasanguinis* encodes two additional glycosylation factors, GalT1 and GalT2. GalT2 is required for full glycosylation of Fap1 (75).

There is evidence that GspB and Fap1 are glycosylated prior to export. While GspB export is blocked in *S. gordonii secA2* and *secY2* mutants, the GspB that is retained in the cytoplasm is still glycosylated (2). In *S. parasanguinis* cytoplasmic fractions, Fap1 is detected with both anti-peptide and anti-glycan antibodies, suggesting glycosylation occurs prior to export (18). This finding is unique because the known glycoproteins in *M. tuberculosis* and *Campylobacter jejuni* are glycosylated after export across the cytoplasmic

membrane (48, 68). It is possible that the accessory SecA2 system is important for exporting proteins with post-translational modifications.

However, data from both *S. gordonii* and *S. parasanguinis* indicates glycosylation is not required for SecA2-mediated export. Fap1 is still exported in a *S. parasanguinis gtf1* mutant although it lacks any detectable carbohydrate modifications, and unglycosylated GspB is still exported in *S. gordonii gtf* mutants (6, 73). Interestingly, glycosylation status partially explains why the GspB and Fap1 are not exported by the canonical Sec pathway. In *S. gordonii gtf* mutants, GspB is not glycosylated but is still exported, and this export is reduced in a *gtf secA2* double mutant. One demonstration of the effect of glycosylation comes from experiments showing more GspB is exported by a *gtf secA*2 double mutant than a *secA2* single mutant (6). This difference is attributed to export of unglycosylated GspB via the canonical Sec pathway. In support of this idea, treatment with azide, a known SecA inhibitor, diminishes the residual GspB export in the double mutant. Thus, it appears that glycosylation can block GspB export by the canonical Sec pathway. Perhaps, fully glycosylated GspB is too large for export through the SecYEG translocase. These findings also suggest that the accessory SecA2/Y2 systems are adapted to export proteins that are modified post-translationally.

In addition to glycosylation, the signal sequence is also important for directing GspB and Fap1 export (4, 6, 17). Both GspB and Fap1 have atypically long N-terminal signal sequences required for export. In *S. gordonii*, three key glycine residues (G3) are required for export of fully glycosylated GspB (4). The G3 residues serve a dual function by promoting export by SecA2 and by blocking export by the canonical Sec pathway. The Fap1

signal sequence does not have G3 residues, but there may be other features that specify export via SecA2.

SecA1 is the essential housekeeping SecA protein of mycobacteria. Genomic analysis shows that *M. tuberculosis* encodes all of the essential proteins of the Sec pathway, including the ATPase SecA1 and the translocase components SecY, SecE, and SecG (19). Notably, SecB is not encoded by any mycobacteria. The SecB chaperone is not required for export of all Sec-dependent proteins, even in *E. coli*. In mycobacteria, there is ample evidence that SecA1 functions as the housekeeping secretion factor, analogous to *E. coli* SecA. SecA1 is essential for growth in *M. tuberculosis* and *M. smegmatis*; *secA1* cannot be deleted from the *M. smegmatis* chromosome unless a copy of *secA1* is expressed from a plasmid (9, 57). Also in *M. smegmatis*, depletion of SecA1 leads to a loss of viability and prevents export of the MspA porin, which possesses a predicted Sec signal sequence (32). In contrast, the *secA2* gene is not essential as Δ*secA2* mutants have been constructed in *M. tuberculosis* and *M. smegmatis* (9, 10).

SecA2 is part of a specialized protein export system in mycobacteria. The accessory SecA2 system of mycobacteria appears unrelated to the accessory SecA2/SecY2 systems found in some Gram positive bacteria. However, the accessory *secA2* locus is highly conserved in *Mycobacterium* species. The genomic organization of the accessory SecA2 system in *M. tuberculosis* and *M. smegmatis* is significantly different from the SecA2/SecY2 systems found in Gram positive bacteria (54). One major difference is that there is no extra SecY2 in mycobacteria. Of the genes that surround *secA2* in mycobacteria, none is predicted to encode proteins involved in glycosylation. Furthermore, there are no homologs of the *asp*

or *gap* genes found in *S. gordonii* and *S. parasanguinis*, respectively. Additionally, there is no known SecA2 substrate encoded in the accessory *secA2* locus of mycobacteria.

On the other hand, the accessory SecA2 locus contains similar genes organized in the same way across all species of mycobacteria. Sequence alignments show that the proteins encoded by the genes in the *secA2* locus of *M. smegmatis* are >70% similar to the homologs encoded in the *M. tuberculosis secA2* locus. This degree of similarity is comparable for any protein conserved between these two organisms. Three genes downstream of *secA2* in both *M. smegmatis* and *M. tuberculosis* encode proteins with predicted transmembrane domains (Table 1.2), but the function of these proteins has not yet been determined. One possibility is that these proteins form a membrane-embedded translocase to export SecA2-dependent pre-proteins.

The gene immediately downstream of *secA2* encodes PgsA2, a phosphatidylglycerolphosphate homolog predicted to function in acidic phospholipid synthesis (21, 39). Since *E. coli* SecA interacts with acidic phospholipids, perhaps PgsA2 synthesizes phospholipids that specifically interact with SecA2. In *M. tuberculosis*, but not *M. smegmatis*, the gene immediately upstream of *secA2* encodes IlvG, a probable acetolactate synthase, a key enzyme for biosynthesis of branched chain amino acids. Upstream of *ilvG* is Rv1819c, a predicted ABC (*A*TP *b*inding *c*assette) transporter that has a homolog in *M. smegmatis* (Msmeg3655). ABC transporters are transmembrane proteins that use energy from ATP hydrolysis to transport molecules across the cytoplasmic membrane (7). PgsA2, IlvG, and Rv1819c/Msmeg3655 have not been studied in detail so their contribution to SecA2-dependent protein export remains unknown. The mechanism of SecA2-mediated protein export is probably very similar among different mycobacterial

species, including both pathogens and nonpathogens alike. However, genomic analysis does not support the idea of the mycobacterial SecA2 systems functioning similarly to the accessory SecA2/SecY2 systems in Gram positive bacteria.

Mycobacterial proteins dependent on SecA2 for export. In *M. smegmatis*, two proteins that require SecA2 for export to the cell wall were identified by 2D-PAGE. Msmeg1704 and Msmeg1712 (YtfQ) are encoded by genes in a putative operon, and possess N-terminal signal sequences (31). Unlike the SecA2-dependent proteins of *S. gordonii* and *S. parasanguinis*, both Msmeg1704 and Msmeg1712 are predicted lipoproteins and have homology to periplasmic sugar binding proteins. These proteins appear to be genuine lipoproteins, as both proteins are enriched by Triton X-114 extraction, a method used to isolate lipoproteins. Additionally, processing of these proteins is inhibited by treatment with globomycin, an inhibitor of the lipoprotein signal peptidase (31). It is worth noting that the effect of SecA2 on these lipoproteins is specific; other mycobacterial lipoproteins do not require SecA2 for their export. There are no homologs of Msmeg1704 or Msmeg1712 in *M. tuberculosis*. However, Msmeg1704 is not correctly processed when expressed in a *M. tuberculosis* Δ*secA2* mutant. This result indicates that the SecA2 systems are functionally conserved among mycobacterial species.

In *M. tuberculosis*, SecA2 substrates were discovered by comparing proteins secreted into the culture medium from cultures of wild-type and Δ*secA2* mutant bacteria (10). Three exported proteins are underrepresented in the Δ*secA2* mutant culture filtrate: SodA (superoxide dismutase), HspX (Acr, alpha-crystallin), and Rv0390 (protein of unknown function). Unlike the previously identified SecA2-dependent proteins, all three of these proteins lack N-terminal signal sequences. Of these three proteins, SodA was characterized

further. In the absence of SecA2, the amount of SodA exported into the culture medium is greatly reduced as shown by immunoblot and enzymatic activity assays (10, 36). Additionally, SodA accumulates in the cytoplasm of a Δ*secA2* mutant as expected of a true SecA2-dependent protein. KatG (catalase-peroxidase) is also exported into the culture medium by *M. tuberculosis*. Immunoblot assays show that KatG export depends on SecA2. Both SodA and KatG are antioxidant enzymes and could be important for protecting intracellular *M. tuberculosis* from the oxidative burst of macrophages. Taken together, the data obtained from studies of both *M. tuberculosis* and *M. smegmatis* consistently shows that SecA2 plays a role in exporting a select subset of proteins.

It is unclear why the SecA2-dependent proteins identified in *M. smegmatis* have signal sequences and the proteins identified in *M. tuberculosis* do not. One possibility is that proteins recognized by SecA2 include examples with and without signal sequences. Alternatively, SecA2 could recognize features of the mature protein, thereby eliminating the need for a signal sequence. It is also possible that role of SecA2 in exporting unconventional exported proteins like SodA is indirect. In this case, SecA2 exports proteins (not yet identified) that constitute a specialized secretion system that specifically recognizes proteins without signal sequences like SodA. An example of this indirect effect is found in the T3SS of *Yersinia pestis*. YscJ is a Sec-dependent lipoprotein that is part of the needle complex. Disruption of YscJ prevents export of Type III effector proteins, none of which has Sec-dependent signal sequences (60). To distinguish between these proposed models of SecA2 function, it is important to identify all the proteins that interact with SecA2 and require SecA2 for export out of the cytoplasm.

Role of SecA2 in pathogenesis of *M. tuberculosis*. SecA2 is important for virulence of *M. tuberculosis* in both mouse and macrophage infection models. Mice infected with the *M. tuberculosis* Δ*secA2* mutant survive longer than mice infected with wild-type *M. tuberculosis* (10, 41). Specifically, the Δ*secA2* mutant has a growth defect during the early part of infection, prior to establishment of the cell-mediated immune response. Eventually, growth of the Δ*secA2* mutant plateaus and the bacteria persist for the duration of the infection. The bacterial burden of the Δ*secA2* mutant in lungs is lower than that of wild-type *M. tuberculosis*. During the early phase of infection when the Δ*secA2* mutant exhibits a defect in mice, *M. tuberculosis* is growing in macrophages. In macrophage cell culture, the Δ*secA2* mutant is defective for intracellular growth in comparison to wild-type *M. tuberculosis* (41). We hypothesize that the role of SecA2 is to promote export of proteins important to growth in macrophages. As mentioned above, the Δ*secA2* mutant is defective in exporting antioxidant enzymes. This export defect might partially account for the observed macrophage growth defect. However, the Δ*secA2* mutant also has a growth defect in macrophages that do not generate an oxidative burst (41). Therefore, SecA2 must export other proteins important for surviving inside host cells. Since macrophage infected with the Δ*secA2* mutant release more proinflammatory cytokines, one role of SecA2 may be to export proteins that modulate the host immune system in response to infection.

Interestingly, the Δ*secA2* mutant elicits better protective immunity in mice and guinea pigs than the vaccine strain *M. bovis* BCG (36). The enhanced protection was attributed to an increased $CD8^+$ T cell response. This result further reveals the importance of SecA2 for evading protective immune responses of the host.

Summary. It is clear that the mycobacterial accessory SecA2 system is responsible for exporting a select subset of proteins and it plays a role in the virulence of *M. tuberculosis*. Nevertheless, little is known about the mechanism of SecA2-dependent protein export in mycobacteria. A better understanding of the SecA2 protein export system has the potential to reveal new drug targets or vaccine strategies to combat infection with *M. tuberculosis*.

In this work, we undertook experiments to characterize the mechanism of SecA2-mediated protein export in mycobacteria. In the following chapters, we discuss biochemical and genetic analyses of the accessory SecA2 pathway of *M. tuberculosis* and *M. smegmatis*. In Chapter 2, we explore similarities and differences between the essential SecA1 protein and the accessory SecA2 protein. We demonstrate that SecA2 is an ATPase, and that ATP binding is required for normal SecA2 function in vivo (Chapter 3 and 4). Using a dominant negative *secA2* allele, we perform a suppressor screen in *M. smegmatis* and identify important functional domains of SecA2 (Chapter 3). We also uncover a role for SecA1 in exporting SecA2-dependent proteins (Chapter 5). We conclude with a discussion of our initial attempts undertaken to identify SecA2 interacting proteins (Chapter 6). Taken together, these studies represent an important first step for understanding the mechanism of SecA2-mediated protein export. As the SecA2 protein export system is found in several mycobacterial pathogens, we hope our studies will help in the search for new treatments for mycobacterial disease.

Table 1.1 Percent amino acid similarity to *B. subtilis* SecA

	SecA/SecA1	SecA2
B. subtilis	100	N/A
E. coli	65	N/A
S. gordonii	74	59
S. parasanguinis	73	60
S. aureus	78	58
M. tuberculosis	63	52
M. smegmatis	66	53
L. monocytogenes	81	65

Protein sequences were obtained from TIGR and NCBI and aligned using ClustalW.

Table 1.2 Homologous proteins encoded by the accessory SecA2 locus of mycobacteria

M. tuberculosis	*M. smegmatis*	% similarity	TM domain
Rv1819c	Msmeg3655	77	yes
IlvG	N/A	N/A	no
SecA2	SecA2	90	no
PgsA2	PgsA2	82	no
Rv1823	Msmeg3651	71	yes
Rv1824	Msmeg3650	94	yes
Rv1825	Msmeg3649	78	yes

Protein sequences were obtained from TIGR and NCBI.
Alignments were performed using ClustalW.
Transmembrane (TM) domains were predicted using TMHMM (61).

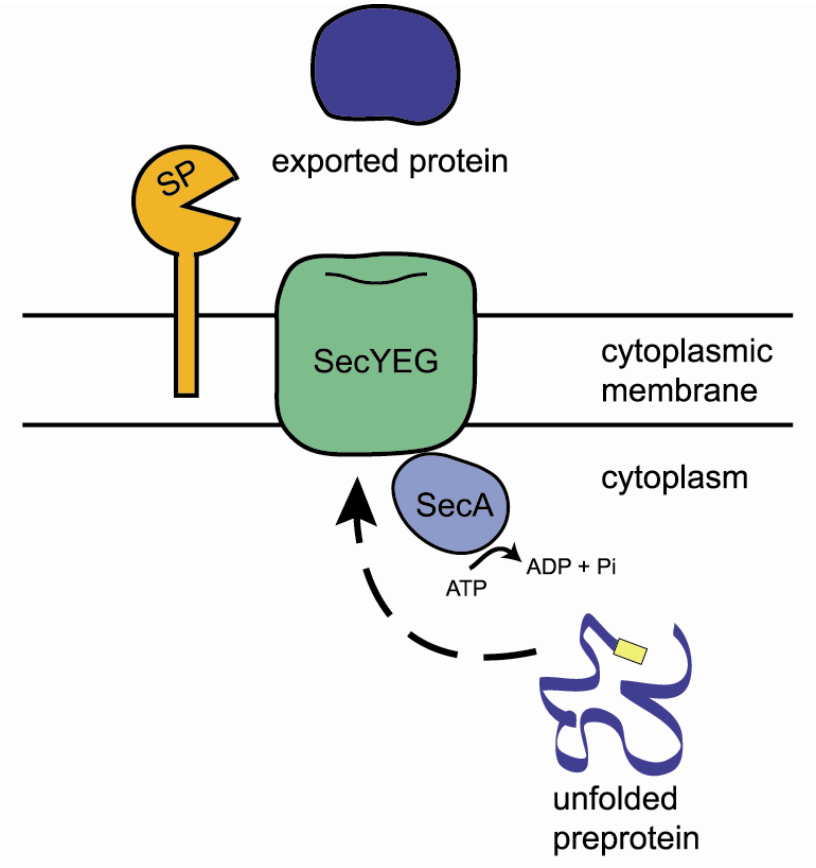

Figure 1.1. The general Sec pathway. The Sec pathway exports unfolded pre-proteins (dark blue ribbon) across the bacterial cytoplasmic membrane. Pre-proteins are targeted to the Sec pathway by an N-terminal signal sequence (yellow box), which is cleaved upon export by a signal peptidase (SP, orange) located in the periplasm. Through repeated cycles of ATP binding and hydrolysis, SecA (light blue) undergoes conformational changes and powers export of pre-proteins through the membrane-embedded SecYEG translocase (green).

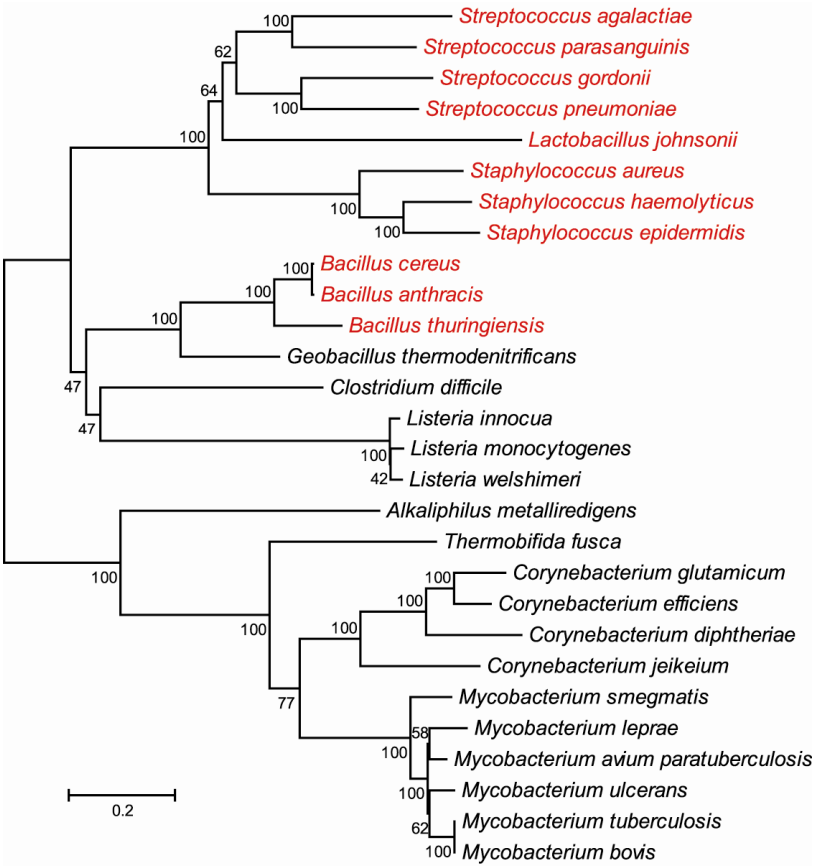

Figure 1.2. Evolutionary relationships of SecA2 proteins. The phylogenetic tree was generated in MEGA4 using the neighbor-joining method. The length of the branches reflects the number of amino acid changes between different SecA2s, as indicated by the bar. Bootstrap values are shown at the junctions. SecA2 sequences were obtained by searching all bacterial genome and protein databases available from NCBI and TIGR (as of April 2008) for the term SecA. For organisms with two SecA sequences, the one least like *B. subtilis* SecA was defined as SecA2 and used to build the tree. For simplicity, only one species of *Lactobacillus* with a SecA2 is included on the tree. Organisms in red possess two SecY homologs.

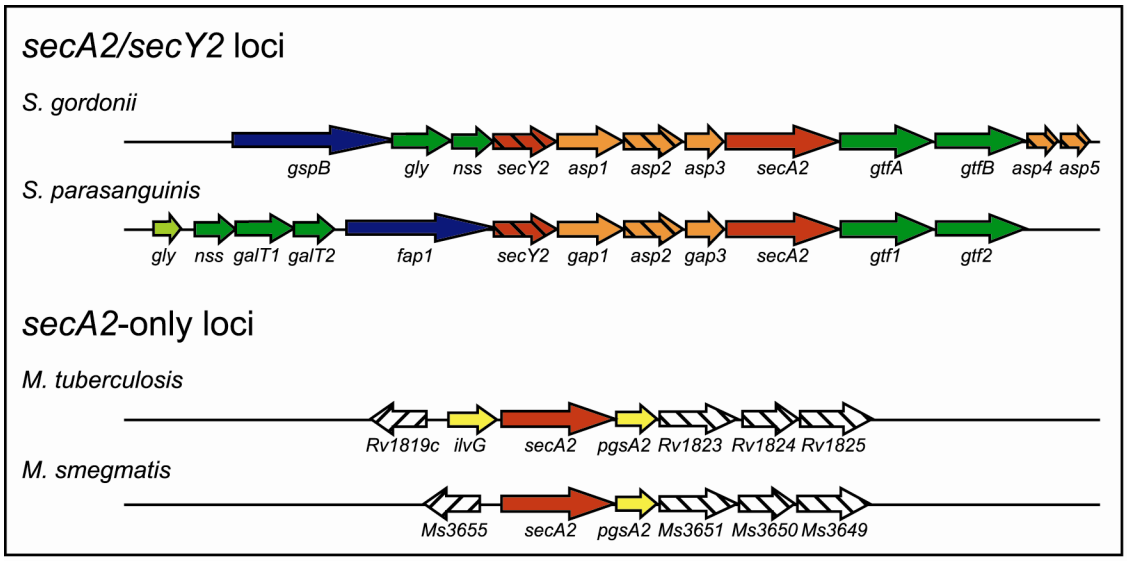

Figure 1.3. Organization of the genes encoding the accessory SecA2 system. Similar coloring indicates encoded proteins with homology or similar properties. Genes encoding SecA2-dependent substrates are in blue, glycosyltransferases in green, accessory Sec proteins in red, accessory SecA2-dependent secretion factors in orange, and biosynthetic enzymes in yellow. Any gene that does not fit in the above categories is white. Genes encoding proteins with predicted transmembrane domains are hatched.

References

1. **Abdallah, A. M., N. C. Gey van Pittius, P. A. Champion, J. Cox, J. Luirink, C. M. Vandenbroucke-Grauls, B. J. Appelmelk, and W. Bitter.** 2007. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol **5:**883-91.

2. **Bensing, B. A., B. W. Gibson, and P. M. Sullam.** 2004. The *Streptococcus gordonii* platelet binding protein GspB undergoes glycosylation independently of export. J Bacteriol **186:**638-45.

3. **Bensing, B. A., J. A. Lopez, and P. M. Sullam.** 2004. The *Streptococcus gordonii* surface proteins GspB and Hsa mediate binding to sialylated carbohydrate epitopes on the platelet membrane glycoprotein Ibalpha. Infect Immun **72:**6528-37.

4. **Bensing, B. A., I. R. Siboo, and P. M. Sullam.** 2007. Glycine residues in the hydrophobic core of the GspB signal sequence route export toward the accessory Sec pathway. J Bacteriol **189:**3846-54.

5. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol **44:**1081-94.

6. **Bensing, B. A., D. Takamatsu, and P. M. Sullam.** 2005. Determinants of the streptococcal surface glycoprotein GspB that facilitate export by the accessory Sec system. Mol Microbiol **58:**1468-81.

7. **Binet, R., S. Letoffe, J. M. Ghigo, P. Delepelaire, and C. Wandersman.** 1997. Protein secretion by gram-negative bacterial ABC exporters. Folia Microbiol **42:**179-83.

8. **Bochkareva, E. S., M. E. Solovieva, and A. S. Girshovich.** 1998. Targeting of GroEL to SecA on the cytoplasmic membrane of *Escherichia coli*. Proc Natl Acad Sci U S A **95:**478-83.

9. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J Bacteriol **183:**6979-90.

10. **Braunstein, M., B. Espinosa, J. Chan, J. T. Belisle, and W. R. J. Jacobs.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol **48:**453-64.

11. **Brodin, P., L. Majlessi, L. Marsollier, M. I. de Jonge, D. Bottai, C. Demangel, J. Hinds, O. Neyrolles, P. D. Butcher, C. Leclerc, S. T. Cole, and R. Brosch.** 2006. Dissection of ESAT-6 system 1 of *Mycobacterium tuberculosis* and impact on immunogenicity and virulence. Infect Immun **74:**88-98.

12. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. Driessen, and W. Wickner.** 1990. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649-57.

13. **Bu, S., Y. Li, M. Zhou, P. Azadin, M. Zeng, P. Fives-Taylor, and H. Wu.** 2008. Interaction between two putative glycosyltransferases is required for glycosylation of a serine-rich streptococcal adhesin. J Bacteriol **190:**1256-66.

14. **Cabelli, R. J., K. M. Dolan, L. P. Qian, and D. B. Oliver.** 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:**24420-7.

15. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch Microbiol **189:**605-10.

16. **Chen, M., K. Xie, F. Jiang, L. Yi, and R. E. Dalbey.** 2002. YidC, a newly defined evolutionarily conserved protein, mediates membrane protein assembly in bacteria. Biol Chem **383:**1565-72.

17. **Chen, Q., B. Sun, H. Wu, Z. Peng, and P. M. Fives-Taylor.** 2007. Differential roles of individual domains in selection of secretion route of a *Streptococcus parasanguinis* serine-rich adhesin, Fap1. J Bacteriol **189:**7610-7.

18. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol Microbiol **53:**843-56.

19. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.

20. **Cosma, C. L., D. R. Sherman, and L. Ramakrishnan.** 2003. The secret lives of the pathogenic mycobacteria. Annu Rev Microbiol **57:**641-76.

21. **de Vrije, T., R. L. de Swart, W. Dowhan, J. Tomassen, and B. de Kruijff.** 1988. Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes. Nature **334:**173-5.

22. **Driessen, A. J., and N. Nouwen.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem **77:**643-67.

23. **Economou, A.** 1998. Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. Mol Microbiol **27:**511-8.

24. **Economou, A.** 1999. Following the leader: bacterial protein export through the Sec pathway. Trends Microbiol **7:**315-20.

25. **Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner.** 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell **83:**1171-81.

26. **El-Sadr, W. M., and S. J. Tsiouris.** 2008. HIV-associated tuberculosis: diagnostic and treatment challenges. Semin Respir Crit Care Med **29:**525-31.

27. **Fekkes, P., C. van der Does, and A. J. Driessen.** 1997. The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. Embo J **16:**6105-13.

28. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev **61:**136-69.

29. **Flynn, J. L., and J. Chan.** 2001. Tuberculosis: latency and reactivation. Infect Immun **69:**4195-201.

30. **Gerlach, R. G., and M. Hensel.** 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int J Med Microbiol **297:**401-15.

31. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J Bacteriol **189:**5090-100.

32. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* using tetracycline repressors. J Bacteriol **189:**4614-23.

33. **Hartl, F. U., S. Lecker, E. Schiebel, J. P. Hendrick, and W. Wickner.** 1990. The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. Cell **63:**269-79.

34. **Hendrick, J. P., and W. Wickner.** 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:**24596-600.

35. **Hestvik, A. L., Z. Hmama, and Y. Av-Gay.** 2005. Mycobacterial manipulation of the host cell. FEMS Microbiol Rev **29:**1041-50.

36. **Hinchey, J., S. Lee, B. Y. Jeon, R. J. Basaraba, M. M. Venkataswamy, B. Chen, J. Chan, M. Braunstein, I. M. Orme, S. C. Derrick, S. L. Morris, W. R. Jacobs, Jr., and S. A. Porcelli.** 2007. Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. J Clin Invest **117:**2279-88.

37. **Houben, E. N., L. Nguyen, and J. Pieters.** 2006. Interaction of pathogenic mycobacteria with the host immune system. Curr Opin Microbiol **9:**76-85.

38. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev **62:**379-433.

39. **Jackson, M., D. C. Crick, and P. J. Brennan.** 2000. Phosphatidylinositol is an essential phospholipid of mycobacteria. J Biol Chem **275:**30092-9.

40. **Kurtz, S., and M. Braunstein.** 2005. Protein secretion and export in *Mycobacterium tuberculosis*, p. 71-138. *In* T. Parish (ed.), *Mycobacterium* molecular biology. Horizon bioscience, Norfolk, UK.

41. **Kurtz, S., K. P. McKinnon, M. S. Runge, J. P. Ting, and M. Braunstein.** 2006. The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. Infect Immun **74:**6855-64.

42. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol Microbiol **45:**1043-56.

43. **Lewis, K. N., R. Liao, K. M. Guinn, M. J. Hickey, S. Smith, M. A. Behr, and D. R. Sherman.** 2003. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. J Infect Dis **187:**117-23.

44. **Madden, J. C., N. Ruiz, and M. Caparon.** 2001. Cytolysin-mediated translocation (CMT): a functional equivalent of type III secretion in gram-positive bacteria. Cell **104:**143-52.

45. **Meya, D. B., and K. P. McAdam.** 2007. The TB pandemic: an old problem seeking new solutions. J Intern Med **261:**309-29.

46. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol Microbiol **10:**483-97.

47. **Murphy, C. K., and J. Beckwith.** 1996. Export of proteins to the cell envelope in *Escherichia coli*., p. 967-978. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*. ASM Press, Washington, D.C.

48. **Nita-Lazar, M., M. Wacker, B. Schegg, S. Amber, and M. Aebi.** 2005. The N-X-S/T consensus sequence is required but not sufficient for bacterial N-linked protein glycosylation. Glycobiology **15:**361-7.

49. **Papanikou, E., S. Karamanou, C. Baud, M. Frank, G. Sianidis, D. Keramisanou, C. G. Kalodimos, A. Kuhn, and A. Economou.** 2005. Identification of the preprotein binding domain of SecA. J Biol Chem **280:**43209-17.

50. **Papanikou, E., S. Karamanou, and A. Economou.** 2007. Bacterial protein secretion through the translocase nanomachine. Nat Rev Microbiol **5:**839-51.

51. **Peng, Z., H. Wu, T. Ruiz, Q. Chen, M. Zhou, B. Sun, and P. Fives-Taylor.** 2008. Role of *gap3* in Fap1 glycosylation, stability, in vitro adhesion, and fimbrial and biofilm formation of *Streptococcus parasanguinis*. Oral Microbiol Immunol **23:**70-8.

52. **Pizarro-Cerda, J., and P. Cossart.** 2006. Subversion of cellular functions by *Listeria monocytogenes*. J Pathol **208:**215-23.

53. **Randall, L. L., S. J. Hardy, T. B. Topping, V. F. Smith, J. E. Bruce, and R. D. Smith.** 1998. The interaction between the chaperone SecB and its ligands: evidence for multiple subsites for binding. Protein Sci **7:**2384-90.

54. **Rigel, N. W., and M. Braunstein.** 2008. A new twist on an old pathway--accessory Sec systems. Mol Microbiol **69:**291-302.

55. **Rohde, K., R. M. Yates, G. E. Purdy, and D. G. Russell.** 2007. *Mycobacterium tuberculosis* and the environment within the phagosome. Immunol Rev **219:**37-54.

56. **Russell, D. G.** 2007. Who puts the tubercle in tuberculosis? Nat Rev Microbiol **5:**39-47.

57. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

58. **Schiebel, E., A. J. Driessen, F. U. Hartl, and W. Wickner.** 1991. Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell **64:**927-39.

59. **Siboo, I. R., D. O. Chaffin, C. E. Rubens, and P. M. Sullam.** 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. J Bacteriol **190:**6188-96.

60. **Silva-Herzog, E., F. Ferracci, M. W. Jackson, S. S. Joseph, and G. V. Plano.** 2008. Membrane localization and topology of the *Yersinia pestis* YscJ lipoprotein. Microbiology **154:**593-607.

61. **Sonnhammer, E. L., G. von Heijne, and A. Krogh.** 1998. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol **6:**175-82.

62. **Sundaramoorthy, R., P. K. Fyfe, and W. N. Hunter.** 2008. Structure of *Staphylococcus aureus* EsxA suggests a contribution to virulence by action as a transport chaperone and/or adaptor protein. J Mol Biol **383:**603-14.

63. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2004. Four proteins encoded in the *gspB-secY2A2* operon of *Streptococcus gordonii* mediate the intracellular glycosylation of the platelet-binding protein GspB. J Bacteriol **186:**7100-11.

64. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2004. Genes in the accessory *sec* locus of *Streptococcus gordonii* have three functionally distinct effects on the expression of the platelet-binding protein GspB. Mol Microbiol **52:**189-203.

65. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2005. Two additional components of the accessory *sec* system mediating export of the *Streptococcus gordonii* platelet-binding protein GspB. J Bacteriol **187:**3878-83.

66. **Tokunaga, M., J. M. Loranger, and H. C. Wu.** 1984. A distinct signal peptidase for prolipoprotein in *Escherichia coli*. J Cell Biochem **24:**113-20.

67. **van Roosmalen, M. L., N. Geukens, J. D. Jongbloed, H. Tjalsma, J. Y. Dubois, S. Bron, J. M. van Dijl, and J. Anne.** 2004. Type I signal peptidases of Gram-positive bacteria. Biochim Biophys Acta **1694:**279-97.

68. **VanderVen, B. C., J. D. Harder, D. C. Crick, and J. T. Belisle.** 2005. Export-mediated assembly of mycobacterial glycoproteins parallels eukaryotic pathways. Science **309:**941-3.

69. **Walker, J. E., A. Eberle, N. J. Gay, M. J. Runswick, and M. Saraste.** 1982. Conservation of structure in proton-translocating ATPases of *Escherichia coli* and mitochondria. Biochem Soc Trans **10:**203-6.

70. **WorldHealthOrganization.** 2008. Global Tuberculosis Control 2008: Surveillance, Planning, Financing. World Health Organization, Geneva.

71. **WorldHealthOrganization** 2007, posting date. WHO Information tuberculosis fact sheet. [Online.]

72. **WorldHealthOrganization.** 2002. The World Health Report 2002: Reducing Risks, Promoting Healthy Life. World Health Organization, Geneva.

73. **Wu, H., S. Bu, P. Newell, Q. Chen, and P. Fives-Taylor.** 2007. Two gene determinants are differentially involved in the biogenesis of Fap1 precursors in *Streptococcus parasanguis*. J Bacteriol **189:**1390-8.

74. **Wu, H., K. P. Mintz, M. Ladha, and P. M. Fives-Taylor.** 1998. Isolation and characterization of Fap1, a fimbriae-associated adhesin of *Streptococcus parasanguis* FW213. Mol Microbiol **28:**487-500.

75. **Wu, H., M. Zeng, and P. Fives-Taylor.** 2007. The glycan moieties and the N-terminal polypeptide backbone of a fimbria-associated adhesin, Fap1, play distinct roles in the biofilm development of *Streptococcus parasanguinis*. Infect Immun **75:**2181-8.

76. **Xie, K., and R. E. Dalbey.** 2008. Inserting proteins into the bacterial cytoplasmic membrane using the Sec and YidC translocases. Nat Rev Microbiol **6:**234-44.

77. **Zwizinski, C., and W. Wickner.** 1980. Purification and characterization of leader (signal) peptidase from *Escherichia coli*. J Biol Chem **255:**7973-7.

Chapter 2

Comparative Analysis of the Properties of SecA1 and SecA2 of Mycobacteria

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The Sec-dependent translocation pathway is used to export most proteins across the cytoplasmic membrane. Recently, several bacteria, including the pathogen *Mycobacterium tuberculosis* and the non-pathogen *M. smegmatis*, were shown to possess two SecA homologs, SecA1 and SecA2. SecA1 is essential for general protein export. SecA2 is specific for a subset of exported proteins and is important for *M. tuberculosis* virulence. The mechanism for SecA2-dependent protein export via the accessory Sec system remains poorly defined. We also began to examine differences between SecA1 and SecA2. Using the experimentally determined crystal structure of SecA1 as a guide, we created a computer-generated homology model of the structure of SecA2, and identified two regions present in SecA1 that are absent in SecA2. We also used differential ultracentrifugation to create subcellular fractions of *M .smegmatis* and determine which compartments contain SecA1 and SecA2. SecA1 was found evenly distributed between cell envelope and cytoplasmic

fractions, while SecA2 was predominantly in the cytoplasmic fraction. Here, we used *M. smegmatis* as a model to characterize the accessory Sec system of mycobacteria. We redesigned a Δ*secA2* mutant of *M. smegmatis* for use in further genetic and biochemical analyses. This mutant had a larger in-frame, unmarked deletion and had the same phenotypes as the previously published Δ*secA2* mutant. These results are the first step in understanding the unique functions of the two SecA proteins of mycobacteria.

Introduction

In all bacteria, the general Sec pathway is used to export the bulk of proteins from the cytoplasm across the cytoplasmic membrane (15, 42). Given the substantial role the Sec pathway plays in exporting proteins to their proper location, it is not surprising that the Sec pathway is essential in all bacteria in which it has been tested.

Proteins destined for export via the Sec pathway are synthesized as pre-proteins with a characteristic N-terminal sorting signal, called a signal sequence. Signal sequences have a tripartite structure: a positively charged N domain, a hydrophobic H domain, and a polar C domain (15). The signal sequence is cleaved off by one of two periplasmic signal peptidases (LepB or LspA) during or immediately after transport across the cytoplasmic membrane (62, 64, 72). Only unfolded pre-proteins are compatible with the Sec pathway.

The Sec machinery is comprised of a Sec translocase embedded in the cytoplasmic membrane. The Sec translocase is composed of the integral membrane proteins SecY, SecE, and SecG (6, 16, 70). These proteins form a channel through which exported proteins may pass. The SecA ATPase is another protein required for export via the Sec pathway. As is the Sec pathway itself, SecA is essential in bacteria (17). Through repeated cycles of ATP

binding and hydrolysis, SecA ratchets pre-proteins through the SecYEG pore (19, 58). Subcellular localization experiments show that SecA is evenly distributed between the cell envelope (comprised of membrane and cell wall) and the cytoplasm (7, 18). This reflects the dynamic nature of SecA function in protein export. SecA interacts with lipids and the Sec translocase at the membrane (20, 28). It also interacts with chaperones, such as SecB, and newly synthesized pre-proteins in the cytoplasm (21, 22, 48). Thus, the presence of SecA in both membrane and cytosolic containing fractions is not surprising. In *E. coli*, mutations that destroy ATPase activity in SecA are unable to complement temperature sensitive *secA* mutants (41). This demonstrates ATPase activity is required for normal SecA function. Further, the SecA ATPase mutant proteins become stably associated with the cell envelope (18, 31, 41). These findings support the idea that ATP binding and hydrolysis is necessary for SecA to reversibly associate with the Sec translocase in its role of translocating a protein across the membrane.

Recently, some Gram positive bacteria and mycobacteria were discovered to possess two non-redundant SecA homologs (1, 3, 4, 8, 9, 38, 55, 60). All mycobacteria examined, including pathogens and nonpathogens, possess two SecA homologs. In bacteria with two SecAs, the SecA with higher sequence similarity to the canonical SecA of *E. coli* and *B. subtilis* is called SecA or SecA1. The other SecA homolog is called the accessory SecA or SecA2. SecA1 of *M. tuberculosis* is 63% similar to the well-characterized SecA of *B. subtilis*, and SecA1 of *M. smegmatis* is 66% similar to the canonical SecA protein. This sequence similarity is spread across the length of the protein, including the well-characterized Walker A and Walker B ATP binding motifs. Walker box motifs are characteristic of ATPases, and conserved among SecA proteins in all bacteria (15, 55, 69).

In both *M. smegmatis* and *M. tuberculosis secA1* cannot be deleted, indicating that *secA1* is an essential gene (3, 4). Additionally, depletion of SecA1 in *M. smegmatis* prevents the export of the Sec signal sequence-containing protein MspA (26). Taken together, these findings support the notion that in mycobacteria, SecA1 functions as the housekeeping export factor similar to SecA of *E. coli*.

SecA2 from *M. tuberculosis* and *M. smegmatis* are 52% and 53% similar to *B. subtilis* SecA, respectively. Notably, the ATP-binding Walker A and Walker B motifs are conserved in SecA2 (55). SecA2 is smaller than SecA1, partially due to a truncation at the C-terminus. The first characterization of any SecA2 protein was conducted in *M. smegmatis*, a fast-growing saprophytic organism often used as a model to study mycobacterial physiology (3).

In both *M. tuberculosis* and *M. smegmatis*, SecA2 is not essential as in-frame, unmarked Δ*secA2* deletion mutants have been constructed in both organisms (3, 4). In both species, a deletion in *secA2* is associated with several phenotypes. All of these phenotypes can be complemented by introduction of wild-type *secA2,* which shows the phenotypes of Δ*secA2* mutants are due to absence of SecA2. The Δ*secA2* mutant of *M. tuberculosis* is attenuated for virulence in murine and macrophage infection models, and is defective in secreting a small subset of proteins (4, 37). The *M. tuberculosis* Δ*secA2* mutant also has a colony morphology phenotype. When grown on minimal agar plates containing Tween 80 detergent, colonies of the Δ*secA2* mutant appear smooth and glossy compared to the dull and rough colonies of the wild-type parental strain (36). The Δ*secA2* mutant of *M. smegmatis* exhibits a growth defect on rich agar, is hypersensitive to azide and, like the *M. tuberculosis* Δ*secA2* mutant, is defective in exporting a small subset of proteins (3, 24). Together, these

phenotypic analyses show SecA2 is functional in mycobacteria with a role in exporting a specific subset of proteins.

Interestingly, overexpression of SecA1 does not compensate for a lack of SecA2. Conversely, overexpression of SecA2 does not allow construction of a Δ*secA1* deletion mutant (3). This shows that SecA1 and SecA2 are not functionally redundant and each has distinct roles in mycobacterial protein export.

In this chapter we discuss experiments designed to begin identifying properties that distinguish SecA1 and SecA2. They reveal similarities and differences that will serve as a guide for building and testing a model that describes how SecA1 and SecA2 function in protein export. Many of our experiments to characterize the mechanism of SecA2-mediated protein export in mycobacteria used the fast growing non-pathogen *M. smegmatis* as a model system. *M. smegmatis* is more convenient to manipulate experimentally than the slow-growing pathogen *M. tuberculosis* and it is often used to study basic biological processes of mycobacteria(54). To validate *M. smegmatis* as a legitimate experimental system for SecA2 export, we showed that expression of *M. smegmatis secA2* restores the ability of the *M. tuberculosis* Δ*secA2* mutant to grow in macrophages. Expression of *M. smegmatis* SecA2 also complements the colony morphology of a *M. tuberculosis* Δ*secA2* mutant. Furthermore, expression of *M. tuberculosis* SecA2 complements the rich agar growth defect and azide hypersensitivity of the *M. smegmatis* Δ*secA2* mutant. Taken together, the results of these cross-species complementation experiments show that SecA2 function is conserved between *M. smegmatis* and *M. tuberculosis*. These results validate using *M. smegmatis* as a model mycobacterium to study accessory SecA2 protein export.

The originally published *M. smegmatis* Δ*secA2* mutant ($mc^2$2522) is an in-frame, unmarked deletion of approximately one-third of the *secA2* gene (3). The deletion removes the predicted Walker B motif of the ATP binding site and it was therefore predicted to produce a nonfunctional protein. As stated above, several phenotypes are reported for $mc^2$2522, all of which are complemented by introduction of wild-type *secA2* into the mutant. This supports the notion that this mutant behaves like a null. However, because $mc^2$2522 should synthesize a truncated protein species, we still worried that the remaining two-thirds of SecA2 might complicate analysis of experiments using this Δ*secA2* mutant. To avoid any such problems, we constructed a new improved in-frame unmarked deletion mutant of *M. smegmatis secA2* where only three codons of the original open reading frame (ORF) remained. In this Chapter, we also describe our detailed analysis of this new *M. smegmatis* Δ*secA2* mutant (NR116) which is used in all subsequent work in this dissertation.

Our comparative analysis of SecA1 and SecA2 began by comparing the expression levels of each protein in *M. smegmatis* and *M. tuberculosis*. So far, this has not been performed for any organism possessing two SecA proteins. Using antibodies specific for SecA1 and SecA2, we performed quantitative immunoblots on whole-cell protein lysates from wild-type *M. smegmatis* and *M. tuberculosis*. In both cases, we found that the average number of moles of SecA2 and SecA1 per mg of protein was nearly equivalent. This indicates that the different functions of SecA1 and SecA2 are not due to a difference in expression level.

Next, we determined the subcellular localization pattern of SecA1 and SecA2 in *M. smegmatis*. In *E. coli*, SecA is evenly distributed between cell envelope and cytoplasmic fractions. We generated subcellular fractions of *M. smegmatis* and performed immunoblot

analysis to determine the location of SecA1 and SecA2. SecA1 was equally distributed between cell envelope and cytoplasmic fractions, further supporting the notion that SecA1 is the canonical SecA of mycobacteria. In contrast, SecA2 was found predominantly in the cytoplasmic fraction. These results reveal a distinguishing feature of SecA1 and SecA2, and it also provides a useful assay for future studies of SecA2 in mycobacteria.

Finally, we performed a comparison of the sequences and structures of SecA1 and SecA2. Sequence alignments show a high degree of similarity (~50%) between SecA1 and SecA2, even though SecA2 is 20 kDa smaller than SecA1. The crystal structure of *M. tuberculosis* SecA1 is solved (59), but the structure of *M. tuberculosis* SecA2 has not been determined. Using a computer-generated homology model of SecA2, built on to the crystal structure of SecA1, we identified potential structural differences between these two proteins. While strikingly similar across most of the structure, two regions present in SecA1 are notably absent in SecA2. We believe this model will be a useful tool in designing future experiments to characterize the accessory SecA2 protein of mycobacteria.

Materials and Methods

Bacterial strains and culture conditions. Middlebrook 7H9, 7H10, 7H11, and Mueller Hinton media (BD Biosciences) were used for growing *M. smegmatis*. For *M. smegmatis*, the growth medium was supplemented with 0.2% (w/v) glucose, 0.5% (w/v) glycerol, and 0.1% (w/v) Tween 80 (Fisher). For growing *M. tuberculosis*, 7H9 or 7H10 supplemented with 0.5% (w/v) glycerol and 1X ADS (0.5% (w/v) bovine serum albumin, 0.2% (w/v) dextrose, and 0.85% (w/v) NaCl) was used. When needed, kanamycin (Acros Chemicals) or hygromycin (Roche) was added to medium at 20 μg/ml or 50 μg/ml, respectively. LB was

used to grow *E. coli* cultures. When needed, kanamycin or hygromycin was added at 40 μg/ml or 150 μg/ml, respectively. The identity of all vectors used in this study was confirmed by DNA sequencing (UNC-CH automated DNA sequencing facility and Eton Biosciences).

Macrophage infections. Bone marrow macrophages were elicited from femurs of C57BL/6 mice, as described previously (37, 40), and 2.5 x 10^5 macrophages were seeded into wells of 8-well-chamber slides 24 h prior to infection. The *M. tuberculosis* strains were grown to mid-exponential phase, washed with phosphate-buffered saline containing 0.05% (w/v) Tween 80, diluted in tissue culture medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 1X nonessential amino acids [Gibco]) and added to the macrophage monolayer to achieve a multiplicity of infection of 1.0. Macrophage monolayers were infected with *M. tuberculosis* strains for 4 h at 37°C in 5% CO_2. On days 0, 1, and 5 post-infection, the contents of triplicate wells for each infection were washed to remove extracellular bacilli and then lysed with 0.05% (w/v) Tween 80. The resulting lysates were diluted and plated on Middlebrook 7H10 agar to enumerate intracellular bacteria during the course of infection.

Homology model of *M. tuberculosis* SecA2. To generate a structural model of SecA2, we entered the protein sequence of *M. tuberculosis* SecA2 into the 3D-PSSM server (33). This web tool compares the input sequence against a library of experimentally determined structures and scores compatibility between sequence and structure. The result was an alignment of SecA2 with *M. tuberculosis* SecA1 (PDB accession 1NKT (resolution 2.6 Å)) (59). Included in this alignment is a prediction of secondary structure for the input sequence (SecA2) along with the actual secondary structure for SecA1. Using this alignment, as well

as a raw sequence alignment generated using ClustalW, we determined where several insertions and deletions in SecA2 should be placed. This was achieved by comparing the sequence alignments versus the local environment of the SecA1 amino acids in the crystal structure. By examining the side chains of the amino acids that flank an insertion or deletion, we decided whether the position of a given insertion/deletion made structural sense. We then generated the homology model using Insight II (Accelrys, Inc.). To assess the validity of the model, we used the Profiles3D/verify function in Insight II to determine self-compatibility scores.

Subcellular fractionation. Cell envelope and soluble fractions were prepared by differential ultracentrifugation as described previously (24). Briefly, 100 ml cultures of *M. smegmatis* grown in Mueller Hinton were harvested by centrifugation at 3,000 x *g*. Cell pellets were resuspended in 4 ml of breaking buffer (PBS, 0.6 μg/ml each of DNase and RNase, and a cocktail of protease inhibitors (2 μg/ml each of aprotinin, E-64, leupeptin, and pepstatin A and 100 μg/ml Pefabloc SC) and then lysed by five passages in a French pressure cell. Unbroken cells were pelleted at 3,000 x *g* for 20 min to generate a clarified whole cell lysate, which was centrifuged at 100,000 x *g* for 2 h to separate the cell envelope (pellet) fraction from the soluble fraction (supernatant). The cell envelope fraction was washed once and then resuspended in PBS.

Immunoblot conditions. The anti-SecA2, anti-GroEL (HAT5/IT-64 from the World Health Organization collection), anti-MspA (gift from Michael Niederweis, (45)), and anti-HA (Covance) antibodies were used at 1:20,000 dilutions. The anti-SecA1 antibody was used at a 1:50,000 dilution. For most experiments, secondary antibodies conjugated to HRP (Biorad) were used along with ECL (Perkin Elmer) and film (Denville) to visualize blots. For

quantitative immunoblots, whole-cell lysates of exponential phase *M. tuberculosis* cultures grown in Middlebrook 7H9 medium were prepared following fixation in an equal volume of 10% (w/v) formalin for 1 h. Fixed cells were pelleted by centrifugation, resuspended in extraction buffer, and lysed by bead beating. For quantitative SecA1 and SecA2 Western blot analysis, 100 μg of formalin-fixed whole-cell lysates were electrophoresed on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel alongside known amounts of purified SecA1 or SecA2 protein (29). Proteins were transferred to nitrocellulose membranes and probed with rabbit polyclonal anti-SecA1 (1:50,000 dilution) or anti-SecA2 (1:20,000 dilution) antibody. The secondary antibody was goat anti-rabbit conjugated to alkaline phosphatase (1:20,000 dilution), and detection of the fluorescence from ECF (Amersham/GE Healthcare) was done with a PhosphorImager (Molecular Dynamics). The signal intensity values from six independently prepared whole-cell lysates were quantified by comparison with standard curve values to determine the number of moles of SecA1 and SecA2 per mg of cellular protein.

***secA2* suicide plasmid pNR6.** Sequences immediately upstream and downstream of *secA2* were PCR amplified from *M. smegmatis* genomic DNA using primers 5'-GTCGACCGACAGGTTCCAGCCGTAGAA-3' / 5'-GTCGACCACGGCGTCAGTTGTGCCTCG-3' and 5'-GTCGACTAGGCCCCAGCCATTAGGTTC-3' / 5'-TGATATCGAGCACCTCCCAGCCCCATTC-3', respectively. *Sal*I restriction sites were added to the ends of the upstream PCR product which was then cloned into the vector pCC1 using the Copy Control PCR cloning kit (Epicentre) to generate pNR3. The 901 bp downstream PCR product was engineered to contain a 5' *Sal*I site, and was cloned into the

vector pCR2.1 (Invitrogen) to generate pNR4. A 1087 bp *Sal*I fragment of pNR3 was ligated into *Sal*I-cut pNR4. The resulting plasmid, pNR5, contained a 2385 bp deletion of *secA2* leaving only three codons. A 1737 bp *Msc*I-*Eco*RV fragment containing the *secA2* deletion was cut from pNR5 and cloned into the *Eco*RV site of the counterselectable suicide plasmid pMP62, yielding vector pNR6.

Two step allelic exchange. The Δ*secA2* mutant strain NR116 was constructed by two step allelic exchange as described previously (2, 50). Wild-type *M. smegmatis* strain mc^2155 was electroporated with pNR6, and hygromycin-resistant transformants were selected on 7H10 plates. Transformants were screened by Southern blot to identify a single-crossover integration of pNR6 at the chromosomal *secA2* locus in strain SCO5. To resolve the single crossover strain, a saturated culture (7H9 0.2% glucose, 0.5% glycerol, 0.1% Tween 80, hygromycin 50 μg/ml) of SCO5 was diluted 1:100 into 7H9 without hygromycin and grown overnight at 37˚C. This culture was plated onto 7H10 supplemented with 4.5% (w/v) sucrose to select against the *sacB* marker encoded on the backbone of the suicide vector. Sucrose-resistant clones were screened for hygromycin sensitivity. Then sucrose-resistant, hygromycin-sensitive clones were screened by PCR and Southern blot for evidence of a second recombination event leading to chromosomal deletion of *secA2*. The resulting in-frame, unmarked Δ*secA2* deletion mutant derived from SCO5 was named NR116.

Southern blot analysis of SCO5 and NR116. Southern blot analysis was performed as previously described (56). Briefly, genomic DNA was isolated from *M. smegmatis* strains and digested with *Bam*HI. The probe used was a 1093 bp PCR product containing the sequence immediately upstream of *secA2*. The probe was labeled with [^{32}P]dCTP using the Ready-to-Go labeling kit (Amersham).

Azide assay. 200 µl of saturated (OD_{600nm} = 2.0) *M. smegmatis* culture was mixed with 3.5 ml of molten 7H9 top agar, and then poured onto a 7H10 bottom agar plate. After the top agar cooled, sterile 6 mm filter discs were placed onto the surface. 10 µl of 0.15 M sodium azide was then added to the disc. The plates were inverted and incubated for 2 days at 37˚C. After incubation, any resulting zone of inhibition on the plate was measured. Each strain was tested in triplicate, and untreated plates were included as a control.

Agar plate growth assays. All *M. smegmatis* strains were grown in 7H9 medium at 37˚C prior to plating. Serial dilutions of each strain were made in 7H9 and then plated onto the appropriate agar medium. All plates were incubated at 37˚C until colonies were visible.

Results

***M. smegmatis* is a valid model for studying the *M. tuberculosis* SecA2 system.** *M. tuberculosis* is difficult to manipulate experimentally because it is extremely slow growing and requires containment in a BSL3 laboratory. As a result, the fast growing nonpathogen *M. smegmatis* is often used as a model mycobacterium (54). Throughout this work, we take advantage of *M. smegmatis* as a convenient tool to study SecA2-mediated protein export in mycobacteria. It is already known that expression of *M. tuberculosis* SecA2 can complement the rich agar growth defect and azide hypersensitivity phenotypes of the *M. smegmatis* Δ*secA2* mutant (3). In order to validate using *M. smegmatis* as a model to study *M. tuberculosis* SecA2 export, we tested the ability of *M. smegmatis secA2* to complement two phenotypes of the *M. tuberculosis* Δ*secA2* mutant: a growth defect in macrophages and a smooth colony morphology when grown on plates containing Tween 80.

***M. smegmatis secA2* complements the macrophage growth defect of a *M. tuberculosis* Δ*secA2* mutant.** Unlike the wild-type *M. tuberculosis* H37Rv strain which can infect and grow inside macrophages over a period of several days, the Δ*secA2* mutant is defective in intracellular growth (37). A Δ*secA2* mutant of *M. tuberculosis* is attenuated for growth in macrophages and in mice (4, 37). Introduction of a wild-type copy of *M. tuberculosis secA2* at the *attB* locus complements the mutant phenotype. Here we tested if the *M. smegmatis secA2* gene can also complement the *M. tuberculosis* Δ*secA2* mutant phenotype. Murine bone marrow-derived macrophages were infected with wild-type *M. tuberculosis* (H37Rv), *ΔsecA2* mutant $mc^2$3112 (Δ*secA2*), the *ΔsecA2* mutant complemented with wild-type *M. tuberculosis secA2* (Δ*secA2/Mtb secA2*), or the *ΔsecA2* mutant expressing wild-type *M. smegmatis secA2* (Δ*secA2*/*Msm secA2*) (Figure 2.1A). As shown previously (37), H37Rv and the Δ*secA2* mutant complemented with *M. tuberculosis secA2* grew similarly in macrophages over a 5-day period of infection while the *ΔsecA2* mutant failed to grow in these macrophages. Introduction of *M. smegmatis secA2* in single copy at the chromosomal *attB* site in the mutant also fully restored the ability of the Δ*secA2* mutant to grow in macrophages. Western blot analysis confirmed that the Δ*secA2* mutant strains carrying either *M. tuberculosis* or *M. smegmatis secA2* expressed the corresponding SecA2 protein at similar levels (data not shown). These results indicated that *M. smegmatis* SecA2 was able to promote growth of *M. tuberculosis* in macrophages, just like *M. tuberculosis* SecA2.

Expression of *M. smegmatis secA2* complements the smooth colony morphology of the *M. tuberculosis* Δ*secA2* mutant. The colony morphology of the *M. tuberculosis* Δ*secA2* mutant has been described previously (36). In contrast to the dull, rough, irregularly

shaped colonies of wild-type *M. tuberculosis*, colonies of the Δ*secA2* mutant are round and smooth with a glossy appearance. This phenotype could indicate a difference in composition of the cell envelope between the two strains. Notably, this difference is only detected when the bacteria are grown on media containing Tween 80. When grown on 7H10 plates without Tween 80, wild-type and Δ*secA2* mutant colonies are indistinguishable. The rough colony morphology can be restored in the Δ*secA2* mutant by expression of a wild-type copy of *M. tuberculosis secA2*.

To further test the ability of *M. smegmatis secA2* to complement the *M. tuberculosis* Δ*secA2* mutant, we grew the bacterial strains described in Figure 2.1A on 7H10 plates supplemented with 0.05% Tween 80. After 3 weeks of growth, plates were removed from incubation and photographed. As shown in Figure 2.1B, the Δ*secA2* mutant is noticeably smoother than H37Rv. Expression of *M. tuberculosis secA2* is able to restore the rough colony morphology to the Δ*secA2* mutant. As expected, when *M. smegmatis secA2* is expressed in the Δ*secA2* mutant, the colonies appear rough (Figure 2.1B). This provides further indication that *M. smegmatis secA2* is able to function normally in *M. tuberculosis*. Together, these data showed that *M. smegmatis* SecA2 can substitute for *M. tuberculosis* SecA2 during growth in macrophages and under standard laboratory conditions. We believe our findings show functional conservation between *M. tuberculosis* SecA2 and *M. smegmatis* SecA2; this provides strong justification for using *M. smegmatis* as a model to understand how SecA2 functions in protein export.

Construction and characterization of a complete in-frame unmarked deletion of *M. smegmatis secA2*. Previously, a 1296 bp Δ*secA2* deletion mutant ($mc^2$2522) was constructed in *M. smegmatis* and used to assess the function of SecA2 (3). The deletion in

this mutant spans the ATP binding site of SecA2 (Figure 2.2A). Several in vitro phenotypes are reported for this mutant, including a growth defect on rich agar plates, hypersensitivity to sodium azide, and an export defect of the lipoproteins Msmeg1704 and Msmeg1712 (3, 24). Expression of a wild-type *secA2* allele in trans complements these phenotypes, showing that the phenotypes are attributable to the deletion in *secA2*.

Another phenotype observed in earlier studies is that while overexpression of SecA1 has no effect on wild-type *M. smegmati*s, overexpression of SecA1 in $mc^2$2522 exacerbates the rich agar growth defect of the Δ*secA2* mutant (3). This synthetic phenotype suggests a relationship between SecA1 and SecA2 or their respective export pathways. The Δ*secA2* deletion allele in $mc^2$2522 still encodes a protein of 366 amino acids, although no truncated SecA2 protein is observed by immunoblot (data not shown). In other bacteria, SecA is capable of forming dimers (12, 14, 31, 46). Thus, it is possible that the SecA proteins of mycobacteria also form dimers, perhaps even SecA1/SecA2 heterodimers. We considered the possibility that a truncated SecA2 protein might have dominant effects through interfering with SecA1-protein complexes and thereby produce the observed phenotype. To eliminate this potentially complicating factor, we constructed a new *M. smegmatis* Δ*secA2* mutant in which the *secA2* gene is fully deleted and only three codons remain. This strain, NR116, was then used for the remainder of this thesis work as an improved *M. smegmatis* Δ*sec*A2 mutant.

To construct the new Δ*secA2* mutant, we performed allelic exchange using a two-step counterselectable system (50). Wild-type *M. smegmatis* ($mc^2$155) was electroporated with the suicide plasmid pNR6 (Figure 2.2B). This plasmid lacks a mycobacterial origin of replication, and contains markers for hygromycin resistance (hyg^R) and sucrose sensitivity

(*sacB*). Hygromycin-resistant transformants were selected on 7H10 plates and then analyzed by Southern blot to confirm recombination of the deletion allele into the *secA2* chromosomal locus, yielding the single-crossover strain SCO5 (Figure 2.2D). Upon counterselection for sucrose resistance, the integrated suicide vector will undergo a second recombination event, either yielding a Δ*secA2* deletion mutant (Figure 2.2E) or regenerating the starting wild-type *secA2* allele (Figure 2.2C) without the intervening suicide vector backbone. Sucrose resistant colonies were screened for hygromycin sensitivity, which reports on loss of the vector backbone. Suc^R Hyg^S clones were analyzed by PCR and Southern blot until we identified NR116 as a Δ*secA2* deletion mutant (Figure 2.2F).

After constructing NR116, immunoblot analysis of whole cell lysates confirmed that SecA2 is no longer present in this new Δ*secA2* mutant. As expected, deletion of SecA2 in NR116 had no effect on expression of SecA1 (Figure 2.3A). NR116 was then tested for all the previously reported phenotypes of $mc^2$2522 (3, 24). When grown on rich Mueller Hinton agar, NR116 colonies were smaller than colonies of wild-type *M. smegmatis*. The phenotype of NR116 was indistinguishable from that of the original Δ*secA2* mutant $mc^2$2522 on rich agar (Figure 2.3B). As reported previously, neither *secA2* mutant exhibited a mutant phenotype when growing on minimal 7H10 agar media (3). We also tested NR116 for sensitivity to sodium azide. Sodium azide is known to target ATPases. In bacteria, the major target of azide is SecA (23). NR116 was more sensitive to azide than wild-type *M. smegmatis*; again the phenotypes of the Δ*secA2* mutants NR116 and $mc^2$2522 were indistinguishable from each other (Figure 2.3C). All these phenotypes associated with NR116 could be complemented by expressing a wild-type copy of *M. smegmatis secA2* on a plasmid vector.

We were particularly interested in retesting the exacerbated phenotype resulting from SecA1 overexpression in NR116. The results with NR116 were the same as shown previously with $mc^2$2522 (3); overexpression of SecA1 exacerbated the rich agar and azide hypersensitivity phenotypes of both *secA2* mutants. Since the same phenotype is observed when SecA1 is overexpressed in NR116, it eliminates the possibility that a truncated SecA2 protein in the original mutant $mc^2$2522 was responsible for the phenotype (Figure 2.4). This overexpression of SecA1 shows the inability of SecA1 to suppress *secA2* mutant phenotypes, and it also reveals a synthetic phenotype between high level SecA1 expression and absence of SecA2. We think this synthetic phenotype reflects a relationship between SecA2 and SecA1 or the general Sec Pathway.

New Δ*secA2* mutant phenotypes in *M. smegmatis*. During the course of these experiments, we attempted to narrow down the property or component of rich agar that accounts for the *M. smegmatis* Δ*secA2* mutant growth defect. We considered the possibility that the salt concentration in Mueller Hinton agar is higher than in 7H10 minimal agar and that the Δ*secA2* mutant might be more sensitive to media with a higher salt concentration. Mueller Hinton is a complex medium that includes digested casein and beef extract; thus, the exact chemical composition of this medium is not known. 7H10 is a defined medium; the identity and proportion of each chemical component is known. Given the colony phenotype displayed by the *M. tuberculosis* Δ*secA2* mutant and the growth defect exhibited by the *M. smegmatis* Δ*secA2* mutant on rich agar, we hypothesized that SecA2 might be important for assembling the protective cell envelope of the bacteria. One possible consequence of defective cell envelope assembly in the Δ*secA2* mutant could be sensitivity to high salt. To test this possibility, we supplemented standard 7H10 agar with 0.5 M NaCl. Normally, 7H10

does not contain any NaCl. Wild-type, the Δ*secA2* mutant NR116, and a complemented mutant strain were plated onto this high salt medium (Figure 2.5). Compared to standard 7H10 plates, all strains grew substantially slower on the salt-containing plates. In comparison to wild-type and the complemented strain, the Δ*secA2* mutant showed a growth defect on 7H10 salt plates. Normally, when grown on standard 7H10 plates there is no difference in growth between wild-type and Δ*secA2* mutant *M. smegmatis*. Thus, it seems the salt concentration of agar media has an impact on the rate of growth of the Δ*secA2* mutant.

7H11 is a commonly used mycobacterial growth medium. The composition of this medium is very similar to 7H10, with the notable addition of digested casein. Digest of casein is also a component of Mueller Hinton. *M. smegmatis* strains were plated onto Mueller Hinton, 7H11, or 7H10 supplemented with casein and incubated at 37˚C for 4 days. As shown in Figure 2.5, colonies of the Δ*secA2* mutant are slightly smaller than colonies of wild-type *M. smegmatis* when grown on media containing casein. Thus, casein digest is at least part of the explanation for why the Δ*secA2* mutant exhibits a growth defect on Mueller Hinton agar.

The Δ*secA2* mutant might be sensitive to a specific component of casein digest that slows its rate of growth on agar. Casein digest is rich in amino acids and other metabolic precursors, possibly including a high concentration of salt. Another possibility is that the rich agar growth defect of the *secA2* mutant is a consequence of growth rate. Wild-type *M. smegmatis* colonies appear one day earlier on rich media than on minimal media. When growing faster, a modest defect of the Δ*secA2* mutant may become more evident. If the Δ*secA2* mutant is compromised in cell envelope assembly or some other essential process, it

might take longer for colonies to form on rich agar. In any case, more work is required to determine the true cause of the rich agar growth defect.

SecA1 and SecA2 proteins are present in equivalent amounts in mycobacteria. The identification of bacteria that possess two SecA proteins is a relatively new discovery (55). Unlike SecA1, the SecA2 protein of mycobacteria has a limited role in exporting a select subset of proteins (4, 24). As a starting point to understanding the different roles of each SecA protein, we determined the relative amounts of SecA1 and SecA2 inside the bacterial cell. Quantitative immunoblot analysis was performed with whole-cell lysates prepared from the virulent *M. tuberculosis* strain H37Rv or wild-type *M. smegmatis* mc^2155, using antibodies specific to each SecA protein. These antibodies were raised against peptides specific for SecA1 or SecA2, and they recognize only the respective protein. By comparison to standard curves of purified *M. tuberculosis* SecA1 and SecA2 proteins (gift from Carol Teschke, University of Connecticut), quantitative immunoblots were used to determine the number of moles of SecA1 or SecA2 protein per mg of cellular protein. The results, from an evaluation of six independent whole-cell lysates, revealed nearly equivalent amounts of each protein (29, Chapter 4). In *M. tuberculosis*, the ratio of the average number of moles per mg of protein of SecA2 (2.07×10^{-11}) to that of SecA1 (2.25×10^{-11}) across these experiments was 0.93 +/- 0.12 (standard error). In *M. smegmatis*, the ratio was again similar (1.13 +/- 0.07 standard error). This indicates that differences between SecA1 and SecA2 are not due to different levels of expression, at least under standard laboratory conditions. Further, these data reveal similar results with *M. tuberculosis* and *M. smegmatis* SecA1 and SecA2 proteins.

SecA1 is equally distributed between cell envelope and soluble fractions, while SecA2 is predominantly cytoplasmic. Previous studies show SecA1 and SecA2 are not

functionally redundant; one SecA protein can not compensate for the absence of the other (3). However, little is known about properties that distinguish SecA1 and SecA2 function inside the cell. We generated cell envelope and soluble fractions of *M. smegmatis* and used SDS-PAGE and quantitative immunoblot analysis with anti-SecA1 and anti-SecA2 antibodies to determine the subcellular localization of the respective proteins. The cell envelope fraction contains cytoplasmic membrane and cell wall while a soluble fraction contains the cytosol. The purity of each fraction was assessed by immunoblots for the cell wall protein MspA (*Mycobacterium smegmatis* porin A) and the soluble cytoplasmic chaperone GroEL. As shown by quantitative anti-SecA1 immunoblots, SecA1 was found equally distributed between cell envelope (47 +/- 3%) and soluble (53 +/- 3%) fractions (Figure 2.6A and D). This result is like that reported for *E. coli* SecA, where the single essential SecA is evenly distributed between cell envelope and soluble fractions (7). This data is consistent with SecA1 being the "housekeeping" secretion factor of mycobacteria, analogous to SecA of *E. coli*.

In contrast to SecA1, SecA2 was predominantly localized to the soluble fraction (80 +/- 10%) instead of the cell envelope (20 +/- 10%) (Figure 2.6A and E). The same subcellular fractionation was also performed on the Δ*secA2* mutant NR116 (Figure 2.6B). As expected, no SecA2 is detected in that strain which demonstrates the anti-SecA2 antibody specificity. Furthermore, we also found that the absence of SecA2 had no impact on expression or localization of SecA1. In a Δ*secA2* mutant strain complemented with a wild-type copy of *M. smegmatis secA2*, localization of SecA1 and SecA2 was the same as in wild-type *M. smegmatis* (56 +/- 5% soluble vs. 44 +/- 5% cell envelope) (Figure 2.6C-E). This

indicates that the complementation vector used in our study produces SecA2 protein that localizes normally.

These results reveal an interesting difference between SecA1 and SecA2. It indicates that SecA1 behaves like the canonical SecA in being equally distributed between cell envelope and soluble fractions. In contrast, SecA2 is not equally distributed but, rather, enriched in the soluble fraction. This suggests significant differences in the role played by SecA2 versus SecA1 in directing protein export. Furthermore, by showing that the absence of SecA2 does not effect localization or expression of SecA1, it is clear that phenotypes of the Δ*secA2* mutant are not due to an effect on SecA1 activity.

Predicted structural differences between *M. tuberculosis* SecA1 and SecA2. Although the crystal structure of *M. tuberculosis* SecA1 has been solved (59), a crystal structure for SecA2 is not yet available. In the absence of an experimentally determined structure, we built a computer-generated model of SecA2 based on homology to SecA1 (Figure 2.7A). The model we constructed was a monomer of SecA2 built on a monomer of SecA1. To assess the validity of the model, we used the Profiles3D/verify function in Insight II to determine self-compatibility scores. This function checks if the structure assigned to a particular stretch of amino acids in the model is physically able to form. The score for the SecA2 model was 282.69; the expected score for a protein of this size is 330.86. For proteins this large, a score of 148.88 or less indicates an incorrect model. For comparison, we repeated the self-compatibility analysis for SecA1. The score was 360.31. For proteins of this size, the expected score is 376.61 and a score less than 169.47 is likely incorrect.

The crystal structure of *M. tuberculosis* SecA1 resembles the crystal structures of other SecA proteins (30, 47, 59, 65). From the genetic and biochemical analyses done with

E. coli and *B. subtilis*, SecA proteins can be divided into two large domains, the N-domain and the C- domain. Within these two domains, several structural subdomains that have been defined (15, 49, 67). As shown in Figure 2.7, the N-domain of SecA1, also known as the DEAD motor, consists of two subdomains called NBD1 (*n*ucleotide *b*inding *d*omain, dark blue) and IRA2 (*i*ntramolecular *r*egulator of *A*TPase activity, cyan). The highly conserved Walker A and Walker B motifs are found in the cleft formed between these two subdomains. ATP is bound within this cleft. When separated from the C-domain, the N-domain of SecA is still able to bind and hydrolyze ATP (32, 51).

The C-domain of SecA is divided into several substructures. Several α-helixes are collectively known as the α-helical scaffold domain (HSD). Included in this subdomain is the staple domain (SD, green) that joins the N-domain and C-domain together. In the HSD are also two α-helixes that form the regulatory region IRA1 (yellow). IRA1 modulates the ATPase activity of SecA (68). Also in the C-domain are the pre-protein crosslinking domain (PPXD, pink), the α-helical wing domain and the C-terminal linker (HWD and CTL, respectively; red). As expected from its name, SecA contacts pre-protein substrate via the PPXD (34, 43, 48). However, it is important to note this has only been experimentally demonstrated for a single pre-protein, pro-OmpA in *E. coli*. Activities that map to the CTL of *E. coli* include binding to phospholipids, binding to the chaperone protein SecB, and binding to a Zn^{2+} ion (5, 13, 21). However, the corresponding CTL region of *M. tuberculosis* SecA1 does not contain residues for a Zn^{2+} binding motif. The absence of the SecB/$Zn2^{+}$ binding site is not surprising since there is no SecB homolog in *M. tuberculosis* (11). The function of the HWD is not understood. Taken together, the structural subdomains of *M. tuberculosis* SecA1 are organized equivalently to *E. coli* and *B. subtilis* SecA.

Comparing the homology model of SecA2 to SecA1 reveals similarities and differences. The overall structures are highly similar. The nucleotide binding domains are nearly identical between the two proteins and SecA2 shares most of the subdomains in the SecA1 C-terminus and each domain is similarly structured. However, two regions are notably predicted as being absent in SecA2. The model predicts that SecA2 lacks the so-called Gram negative loop (orange) that is found within the IRA2 subdomain (25). Sequence alignments show that this region is present in all Gram negative SecA proteins but absent from Gram positive SecAs. This finding is interesting given that mycobacteria are neither Gram positive nor Gram negative. The function of this loop is not known. Part of the CTL/HWD is also missing in the SecA2 model. Loss of the Gram negative loop and part of the HWD/CTL account for the 20 kDa size difference between SecA1 and SecA2. The functional significance of the missing subdomains in SecA2 is not clear. Perhaps these subdomains are import for substrate recognition and thus sort pre-proteins to the appropriate SecA protein. Alternatively, these domains could be important for specifying interactions with different translocases. Using the homology model as a guide, detailed studies of each SecA2 subdomain can now be performed to characterize the mechanism of SecA2 function.

Discussion

In this Chapter, we sought to characterize SecA1 and SecA2 by comparing the similarities and differences between these two proteins. We started by asking basic questions about SecA1 and SecA2: Are they present in equal amounts? Are they localized in the same subcellular compartments? What accounts for the difference in size of each protein? We identified some similar features but we also uncovered differences. Also in this Chapter, we

performed experiments to justify the use of *M. smegmatis* as a model system, and developed new genetic and biochemical tools to use in our studies of the accessory SecA2 system of mycobacteria.

***M. smegmatis* is a valid model for studying the accessory SecA2 system of mycobacteria.** In two separate assays, we showed that *M. smegmatis* SecA2 can substitute for *M. tuberculosis* SecA2. The intracellular growth defect in macrophages and the colony morphology phenotype of the *M. tuberculosis* Δ*secA2* mutant were both complemented by *M. smegmatis secA2* expressed from a plasmid integrated into the chromosome in single-copy. These cross-species complementation experiments demonstrate *M. smegmatis* SecA2 can fulfill the role of SecA2 in *M. tuberculosis*. It supports our use of *M. smegmatis* as a model system to study SecA2-mediated protein export.

A new in frame, unmarked deletion mutant of *M. smegmatis secA2* has the same phenotypes as the original Δ*secA2* mutant. The initial *secA2* mutant of *M. smegmatis* ($mc^2$2522) is an in-frame deletion mutant. It was used to show SecA2 is not essential but is functional in *M. smegmatis* (3, 24). The deletion in *secA2* in $mc^2$2522 is not complete, but encompasses the ATP binding Walker B motif which is conserved in all SecA homologs. By deleting this critical region, it was predicted the resulting Δ*secA2* mutant would behave like a null mutant. Successful complementation experiments in $mc^2$2522 confirmed this prediction. However, much of the SecA2 coding sequence remains in $mc^2$2522. As subdomains of *E. coli* SecA are able to fully function alone, we worried that the truncated SecA2 species might interfere with SecA2-interacting protein complexes and complicate our subsequent genetic and biochemical analyses. For this reason, this thesis work started with the construction of an improved in-frame, unmarked full deletion of *M. smegmatis secA2* where only 3 codons

remained of the original ORF. This mutant, NR116, was tested in a battery of phenotypic assays and in all cases exhibited phenotypes identical to those of the original Δ*secA2* mutant. In Chapter 4, we further show that, like mc^{2}2522, NR116 is unable to export Msmeg1704 and Msmeg1712. Even though comparison of mc^{2}2522 and NR116 did not reveal any differences, we still chose to use NR116 in our subsequent studies.

During the course of our analysis of NR116, we also discovered new phenotypes. When grown on minimal agar supplemented with excess sodium chloride, NR116 colonies are smaller compared to wild-type *M. smegmatis*. The added osmotic pressure caused by high salt may be revealing a structural defect in the cell envelope of the Δ*secA2* mutant. One of the functions of the cell envelope is to act as a barrier to the external environment, and to provide structural integrity to the bacterial cell. If SecA2 is involved in establishing the proper cell envelope architecture, then the Δ*secA2* mutant may not be as effective at resisting stresses to the envelope like increased osmotic pressure. There are many examples of cell envelope proteins that are required for growth under high salt conditions (35, 39, 66). Interestingly, *Vibrio cholerae* Type II secretion mutants have outer membrane defects and thus are more sensitive to a variety of compounds including bile salts (61).

We also found that the Δ*secA2* mutant has a growth defect on 7H11 agar. One component found in 7H11 agar, but absent from 7H10 minimal agar, is digested casein. Casein is also a component of Mueller Hinton agar. Accordingly, supplementation of 7H10 agar with casein also resulted in a smaller colonies for Δ*secA2* mutant NR116. The Δ*secA2* mutant also has growth defect on LB agar (data not shown). We do not understand why growth on a nutrient source rich in amino acids or high in salt is detrimental to the Δ*secA2* mutant. One possibility is that some component of rich agar is toxic to Δ*secA2* mutant. In

this model, the Δ*secA2* mutant is unable to export a factor required to metabolize this toxic component. SecA2 might also export proteins that protect the cell from external stresses like increased osmotic pressure. Another possibility is that by increasing the growth rate of *M. smegmatis* on rich agar, a minor subtle phenotype becomes apparent. Without knowing the exact cause of the rich agar growth defect, it is difficult to distinguish between these possibilities. It is worth mentioning that the growth defect of the Δ*secA2* mutant in rich LB or Mueller Hinton is limited to agar media. Growth curves fail to demonstrate a phenotype for the Δ*secA2* mutant growing in any liquid media (data not shown). We do not understand the basis of the rich media growth defect, or why the rich media growth defect is observed only on solid media, not liquid media.

SecA1 and SecA2 are expressed at similar levels in mycobacteria. We began our comparative analysis of SecA1 and SecA2 by determining that the expression level of both proteins was the same. To the best of our knowledge, this has not been shown for any other organism with two SecA proteins. Importantly, this result rules out the possibility that the difference between SecA1 and SecA2 is due to the abundance of each protein. We also showed that the expression of each protein is the same in *M. tuberculosis* and *M. smegmatis*, supporting our use of *M. smegmatis* as a model for studying SecA2.

SecA1 and SecA2 localize differently in *M. smegmatis* subcellular fractions. Previous studies show that SecA1 and SecA2 are not interchangeable (3); overexpression of *secA1* does not compensate for the loss of *secA2*, and vice versa. Here, we showed that the subcellular localization of SecA1 and SecA2 differs in *M. smegmatis*. SecA1 partitioned equally between the cell envelope and soluble fractions, as is the case for the canonical SecA (7, 31), while SecA2 is predominantly in the soluble fraction. Interestingly, it was recently

shown that the localization of the two SecA homologs of *S. parasanguinis* also differs (10). In *S. parasanguinis*, the canonical SecA protein is also found equally distributed in cell envelope and soluble fractions. However, in this bacterium, SecA2 was predominantly associated with the cell envelope, which is opposite to our finding of SecA2 being predominantly localized to the cytosol in *M. smegmatis*. Nonetheless, both studies demonstrate a difference in how SecA1 and SecA2 behave in the cell. It also suggests that the SecA2 system of mycobacteria is functionally different from the SecA2/Y2 systems being studied in other bacteria.

The essential nature of SecA1 in mycobacteria suggests it is the housekeeping SecA, like SecA of *E. coli* (3, 57). In *E. coli*, SecA is shown to undergo repeated cycles of ATP binding and hydrolysis to drive export of pre-proteins through the SecYEG translocon (58, 63). In the process, SecA transiently associates with SecYEG and the cytoplasmic membrane while cycling back to the cytoplasm. In keeping with our proposal that SecA1 is the housekeeping SecA, we found SecA1 to be evenly distributed between cell envelope and soluble/cytosolic fractions. The localization profile of mycobacterial SecA2 is strikingly different and it suggests that SecA2 has a different mode of action than SecA1. Perhaps the primary function of mycobacterial SecA2 is in delivery of substrates to a translocase. In this regard, it may behave like a chaperone, such as SecB. SecB functions as a chaperone by binding to a subset of pre-proteins, keeping them unfolded, and targeting them to the translocase-associated SecA (27, 53). Like SecA2, only a subset of proteins is dependent on SecB for export. Also, all mycobacteria lack a SecB homolog (11). In this proposed model, SecA2 would function as a chaperone to recognize pre-proteins overlooked by SecA1, keep them competent for export, and then hand them off to a translocase. It is possible that SecA2

could deliver select substrates to SecA1 and the general Sec translocase or to a unique translocase that has not yet been discovered. While more experiments are needed to address this striking difference between SecA1 and SecA2, our findings provide a framework for future studies to determine the mechanistic differences between each SecA.

***M. tuberculosis* SecA2 lacks two subdomains found in the crystal structure of SecA1.** Overall, the SecA2 model closely resembles the SecA1 crystal structure (59). This is not surprising given the high amino acid sequence similarity (50%) shared by the two proteins. Both proteins are comprised of two large domains, the N-domain and the C-domain, typical of SecA proteins (15, 67). The N-domain of both SecA1 and SecA2 has an ATP binding pocket in the cleft formed by the NBD1 and IRA2 subdomains. Within this cleft are the well-described ATP-binding Walker box motifs (69). These motifs are found in ATPases, including SecA proteins (71). Our structural model is highly suggestive of SecA2 being a functional ATPase. In Chapter 4, we investigate the ATPase activity of both *M. tuberculosis* SecA1 and SecA2. The N-domain is linked to the C-domain through a long helix called the staple domain (SD) in both SecA1 and SecA2. With the exception of the two regions missing from SecA2, the C-domain of both proteins also exhibits a high level of similarity. Both proteins appear to possess the important regulatory subdomain called IRA1. In *E. coli*, IRA1 is important for controlling the overall ATPase activity of SecA (68). Both proteins also contain a domain with a proposed role in pre-preprotein recognition, PPXD. PPXD is exposed on the surface of SecA and thus could interact with many pre-proteins; however, such an interaction has only been experimentally proven for the model substrate pro-OmpA (34, 43, 48). It remains possible that other subdomains of SecA are involved in pre-protein binding, even possibly regions found in SecA1 but missing in SecA2. Since

SecA2 is important for exporting only a subset of proteins, it seems likely there will be some structural feature involved in routing pre-proteins to the appropriate SecA protein. Studies of the PPXD subdomain could provide insight into how substrate recognition occurs. With the identification of proteins that require SecA2 for their export, experiments will soon be feasible for determining where and how SecA2 interacts with pre-protein substrates.

The structural modeling identified two candidate regions that distinguish *M. tuberculosis* SecA1 and SecA2. Despite over 20 years of detailed biochemical and genetic analysis of *E. coli* SecA, the subdomains we found missing from SecA2 are among the least understood regions of this highly studied molecule. One of the regions missing from *M. tuberculosis* SecA2 corresponds to the Gram negative loop, a region found in all Gram negative SecAs but absent from all Gram positive SecAs (25). The function of this loop is completely unknown. Since mycobacteria are neither Gram positive nor Gram negative, it is interesting to discover *M. tuberculosis* SecA1 has the Gram negative loop and that SecA2 lacks it. However, the significance of this domain as a distinguishing property of each SecA remains to be resolved.

The other region missing from SecA2 is found in the helix wing domain/C-terminal linker (CTL). In *E. coli*, the CTL has several roles, including binding to anionic phospholipids and the cytoplasmic chaperone SecB (5). Additionally, the CTL also contains a Zn^{2+} binding motif that is required for SecB binding (13, 21). Despite these seemingly important roles, the CTL is dispensable for SecA function at least in *E. coli* (52). Notably, there is no SecB homolog in mycobacteria or any Gram positive bacteria (44).

Instead of interacting with chaperone proteins, the role of HWD/CTL in SecA1 might be to interact with pre-proteins or the membrane-embedded translocase. The domains of

SecA that are important for interacting with the SecYEG translocase are still poorly defined. Perhaps by deleting part of the HWD/CTL region, the specificity for pre-protein recognition is relaxed; in this case, pre-proteins normally rejected by SecA1 might now be recognized as competent for export by SecA2. This possibility could be directly tested by making the corresponding deletions in SecA1. Another possibility is that the missing regions are important for recognizing and binding to the appropriate translocase. Without these regions, SecA2 might be restricted to function as a cytoplasmic chaperone like SecB instead of associating with the canonical Sec translocase like SecA. Yet another possibility is that SecA2 uses the energy generated by ATP hydrolysis to power a unique membrane-embedded translocase. More experiments are needed to test these possibilities. Interestingly, sequence alignments of all known SecA2 proteins, including those from bacteria with an accessory SecY2, showed that SecA2 lacks part of the CTL due to a truncation at the C-terminus (data not shown).

In summary, the experiments presented in this Chapter uncovered similarities and differences in some of the basic properties of SecA1 and SecA2. Other experiments in this Chapter provide important justification for using *M. smegmatis* as a model for studying SecA2 export of mycobacteria and provide a foundation for further studies of the SecA proteins of mycobacteria presented throughout this work.

Acknowledgements

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Attributions

The experiments described here were performed by me with the following exceptions. Jessica R. McCann performed the macrophage infections. Henry S. Gibbons developed the subcellular fractionation technique for mycobacteria.

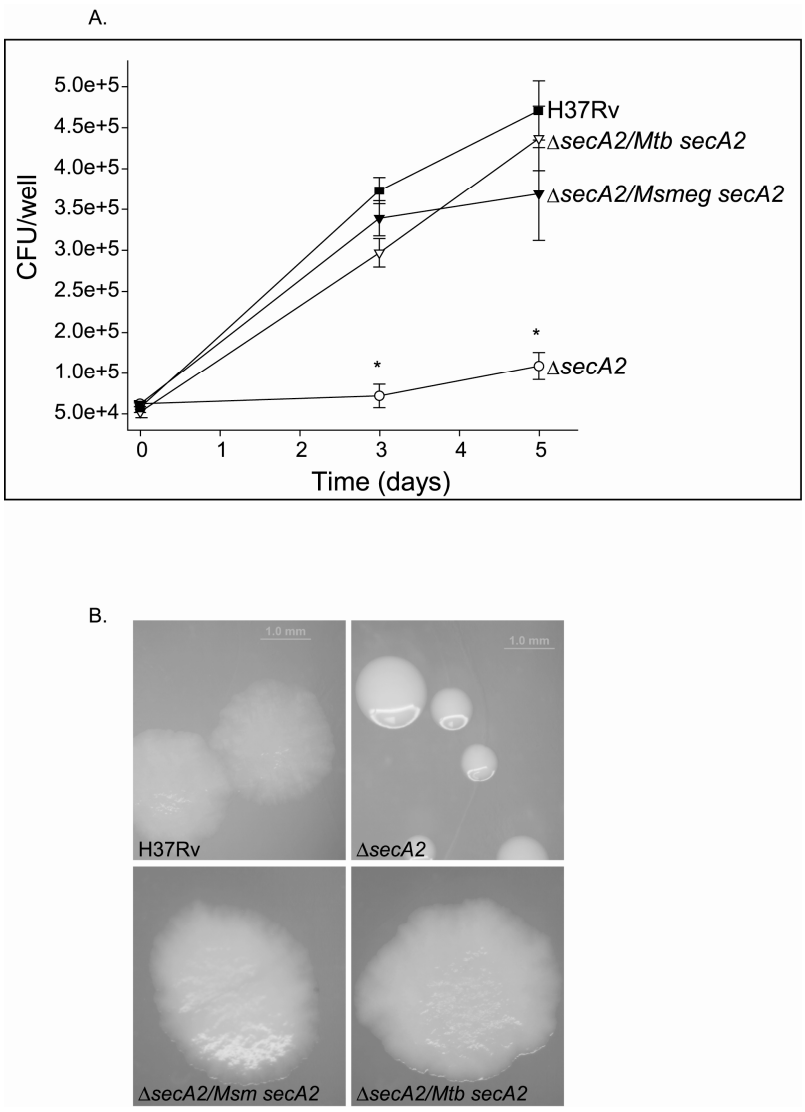

Figure 2.1. SecA2 from *M. tuberculosis* and *M. smegmatis* both restore the macrophage growth defect and altered colony morphology of a *M. tuberculosis* Δ*secA2* mutant. (A) Murine bone marrow-derived macrophages were infected at a MOI of 1.0 with the following strains: wild-type H37Rv (H37Rv), Δ*secA2* mutant (Δ*secA2*), Δ*secA2* mutant complemented with wild-type *secA2* from *M. tuberculosis* (Δ*secA2/Mtb secA2)*, and the Δ*secA2* mutant complemented with wild-type *secA2* from *M. smegmatis* (Δ*secA2/Msmeg secA2)*. CFU were determined by plating macrophage lysates at various times post-infection. The infection was performed with triplicate wells for each strain per time point. Error bars represent means +/- standard deviations for the triplicate wells. Data are representative of two independent experiments. *, (P<0.05) **(B)** Colony phenotype of *M. tuberculosis* strains. Wild-type (H37Rv), Δ*secA2* mutant (mc^{2}3112), Δ*secA2* mutant transformed with *M. tuberculosis secA2* (Δ*secA2/Mtb secA2*) and Δ*secA2* mutant transformed with *M. smegmatis secA2* (Δ*secA2/Msm secA2*) were plated onto 7H10 agar plates containing 0.05% Tween 80. Images of individual colonies were obtained after 3 weeks of growth at 37˚C. Scale bar = 1.0 mm.

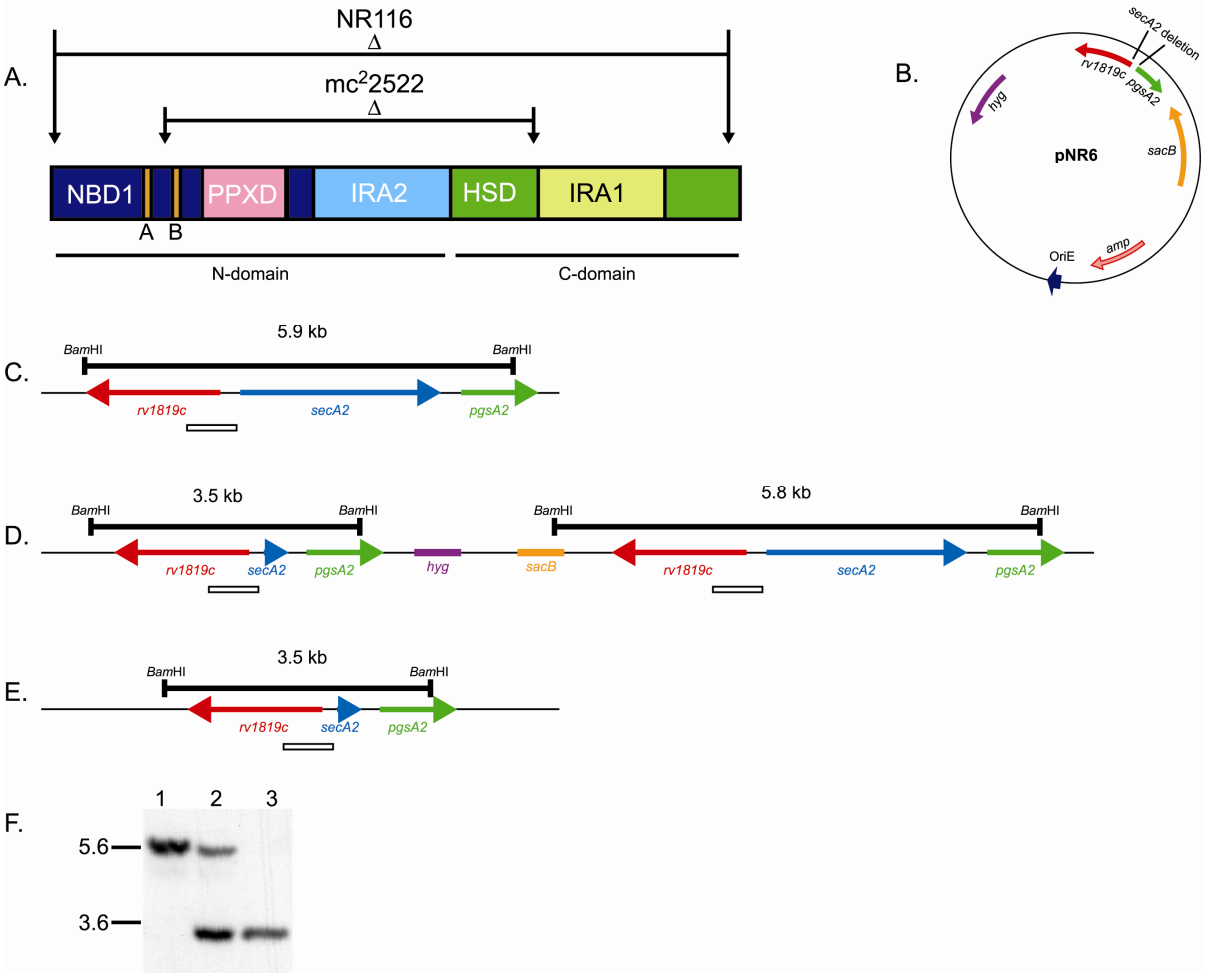

Figure 2.2. Construction of NR116, a new in-frame and unmarked *M. smegmatis* Δ*secA2* mutant. **(A)** Cartoon representation of the subdomains of SecA2. The deletion junction in each Δ*secA2* mutant is indicated by arrows. **(B)** Map of suicide plasmid pNR6 used to construct NR116. Diagram of *M. smegmatis secA2* locus in **(C)** wild-type strain mc^{2}155, **(D)** single crossover strain SCO5, and **(E)** Δ*secA2* mutant strain NR116. The bar below each line indicates site of probe hybridization. **(F)** Southern blot of genomic DNA digested with *Bam*HI. *Bam*HI restriction sites and predicted fragment sizes are indicated in A-C. Lanes 1: mc^{2}155, 2: SCO5, 3: NR116.

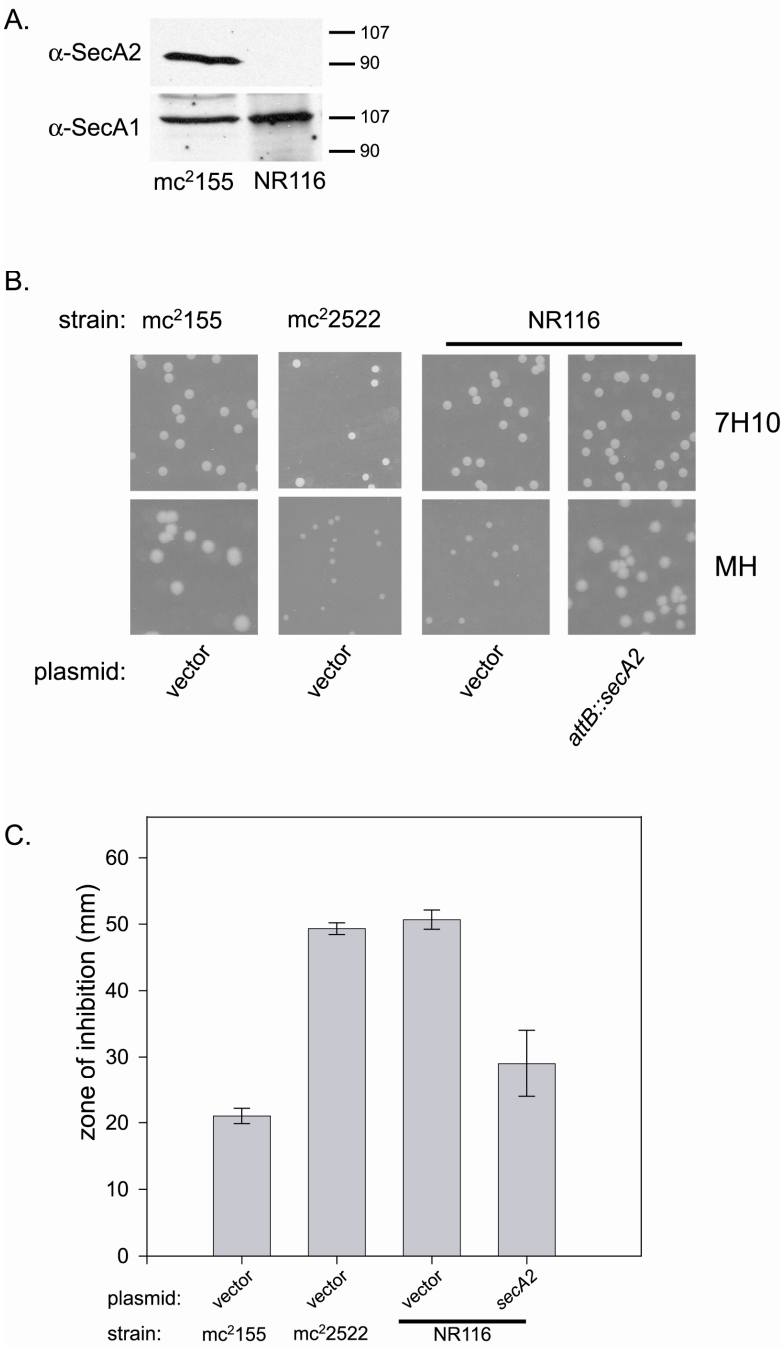

Figure 2.3. Comparison of phenotypes of two Δ*secA2* mutants: mc²2522 and NR116. **(A)** Whole cell lysates prepared from mc²155 and NR116 were analyzed by SDS-PAGE and immunoblot using anti-SecA1 and anti-SecA2 antibodies. **(B)** Colonies of *M. smegmatis* wild-type (mc²155), Δ*secA2* mutant (mc²2522 and NR116), and complemented Δ*secA2* mutant were grown on minimal (7H10, top) or rich (MH, bottom) agar at 37˚C. **(C)** Sensitivity to sodium azide was assessed by measuring inhibition of growth surrounding a filter disc impregnated with 10 µl of 0.15 M sodium azide. Each strain was assayed in triplicate. Error bars represent +/- standard error of the mean.

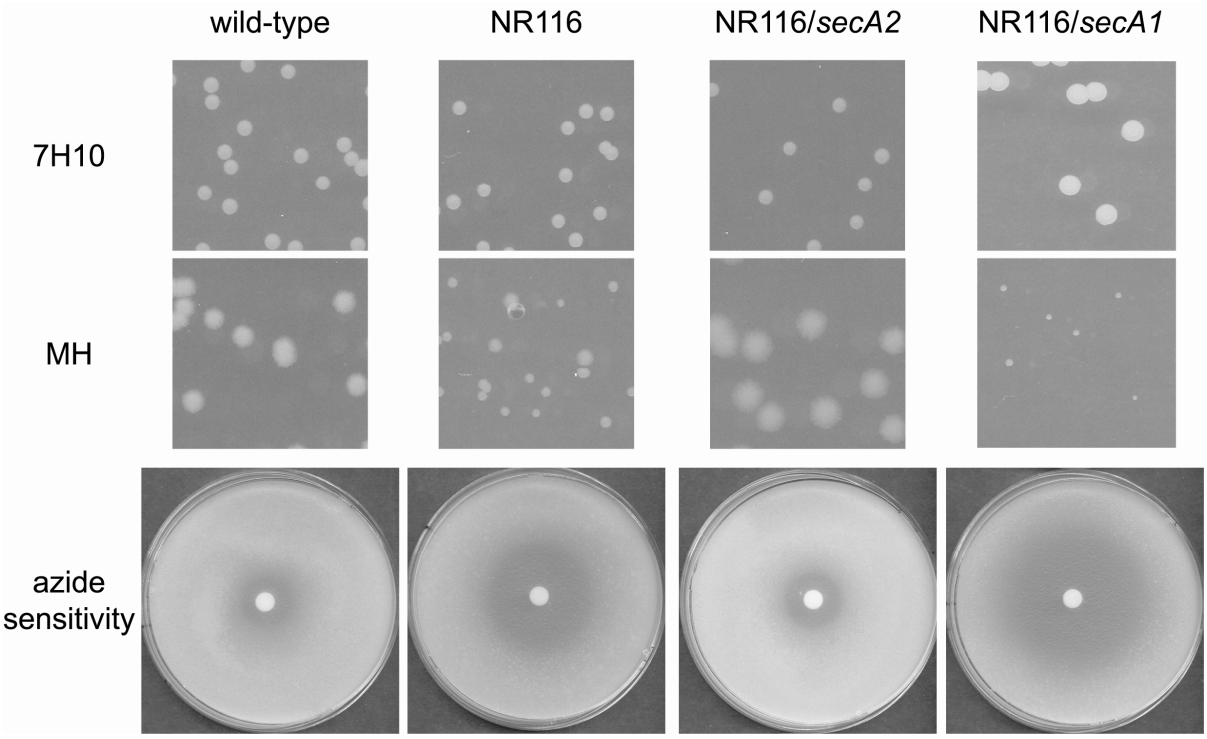

Figure 2.4. Overexpression of SecA1 fails to complement and exacerbates phenotypes associated with Δ*secA2* null mutant NR116. NR116 was electroporated with pYUB544, a multicopy plasmid that expresses *M. smegmatis* SecA1. The resulting strain was grown on minimal (7H10) or rich (MH) agar, and tested for sensitivity to sodium azide as described above. Wild-type, Δ*secA2* mutant NR116, and NR116 complemented with *M. smegmatis secA2* are shown for comparison.

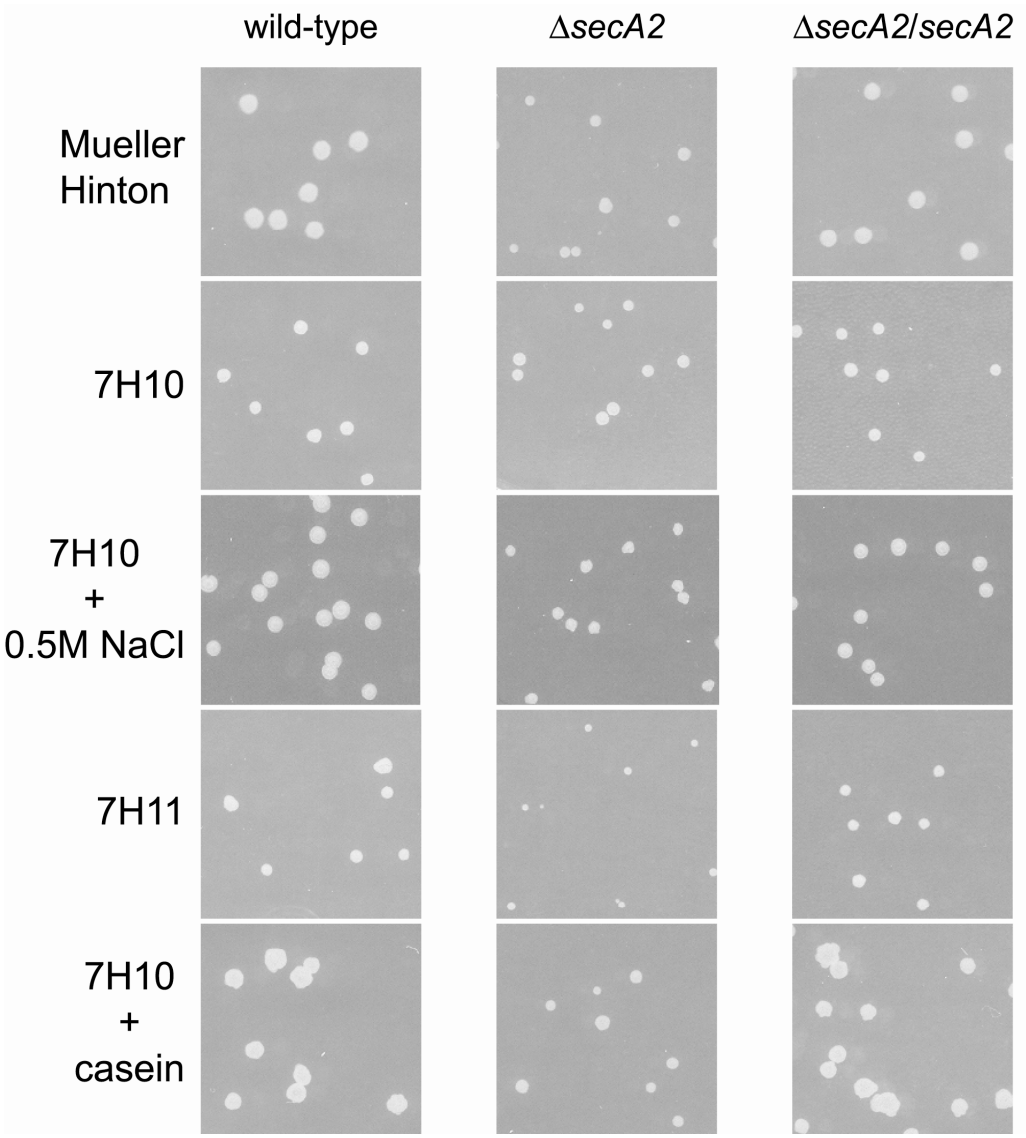

Figure 2.5. Identification of new Δ*secA2* mutant phenotypes. Serial dilutions of the wild-type, Δ*secA2* mutant (NR116), and complement mutant (Δ*secA2*/*secA2*) *M. smegmatis* strains were grown on 7H11 agar or 7H10 agar supplemented with casein or 0.5 M NaCl and incubated at 37˚C until visible colonies appeared. Strains were also grown on minimal (7H10) and rich (MH) agar plates as a control.

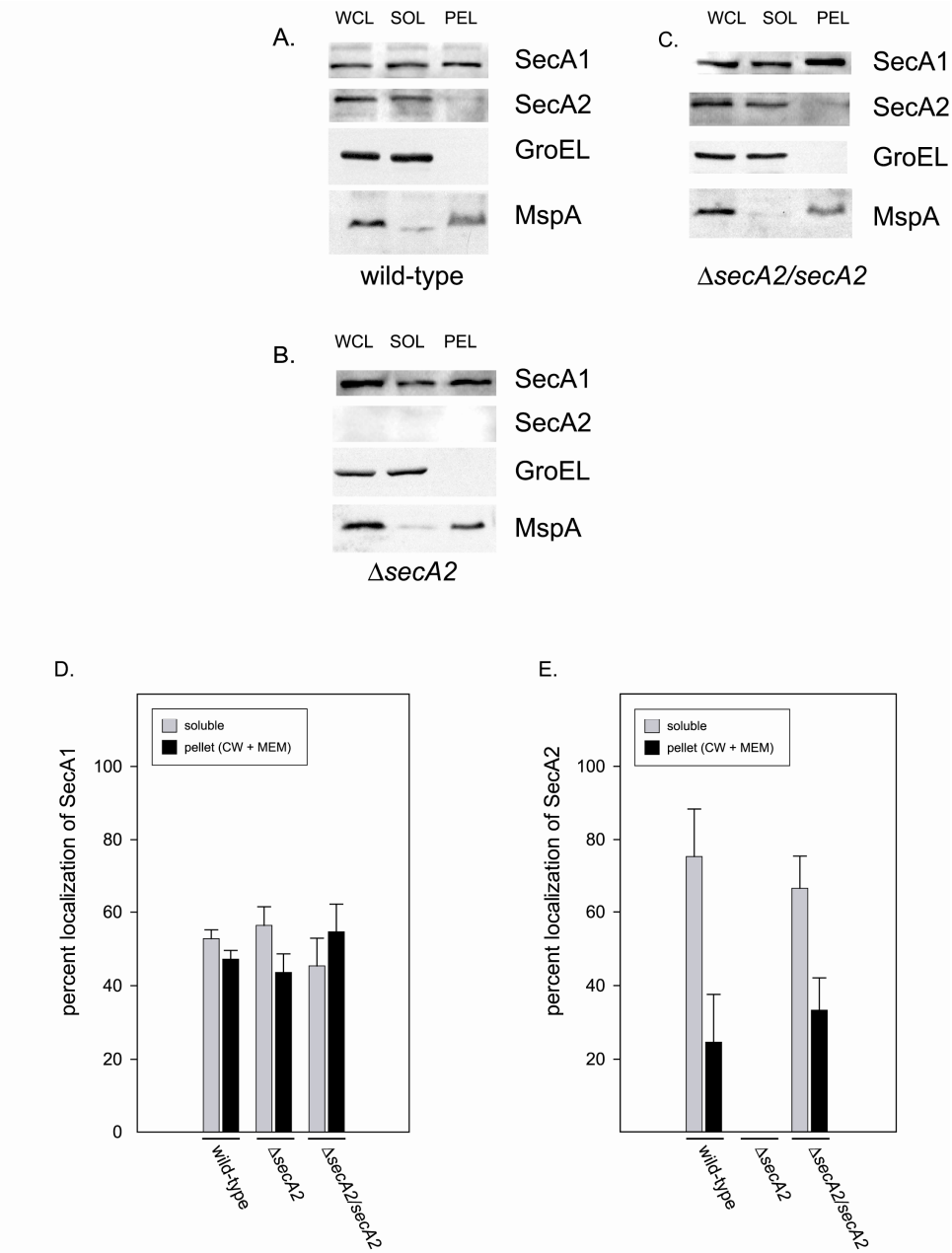

Figure 2.6. SecA1 is found equally distributed between cell envelope and soluble fractions, while SecA2 is found predominantly in the soluble fraction. Cultures of *M. smegmatis* were grown in Mueller Hinton until an approximate OD_{600nm} of 1.0. Whole-cell lysates were generated using a French pressure cell, and subcellular fractions were made by differential ultracentrifugation as described in the Methods. Protein derived from an equal number of cells was analyzed by quantitative immunoblot. Anti-SecA1 **(A)** and anti-SecA2 **(B)** antibodies were used at a 1:50,000 dilution and 1:20,000 dilution, respectively. Goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase (Amersham/GE Healthcare) were used according to the manufacturer. Immune complexes were developed using ECF and visualized using a phosphorimager (Molecular Devices). Data from quantitative anti-SecA1 **(C)** and anti-SecA2 **(D)** immunoblots are from three independent replicates. Error bars indicate +/- standard error of the mean.

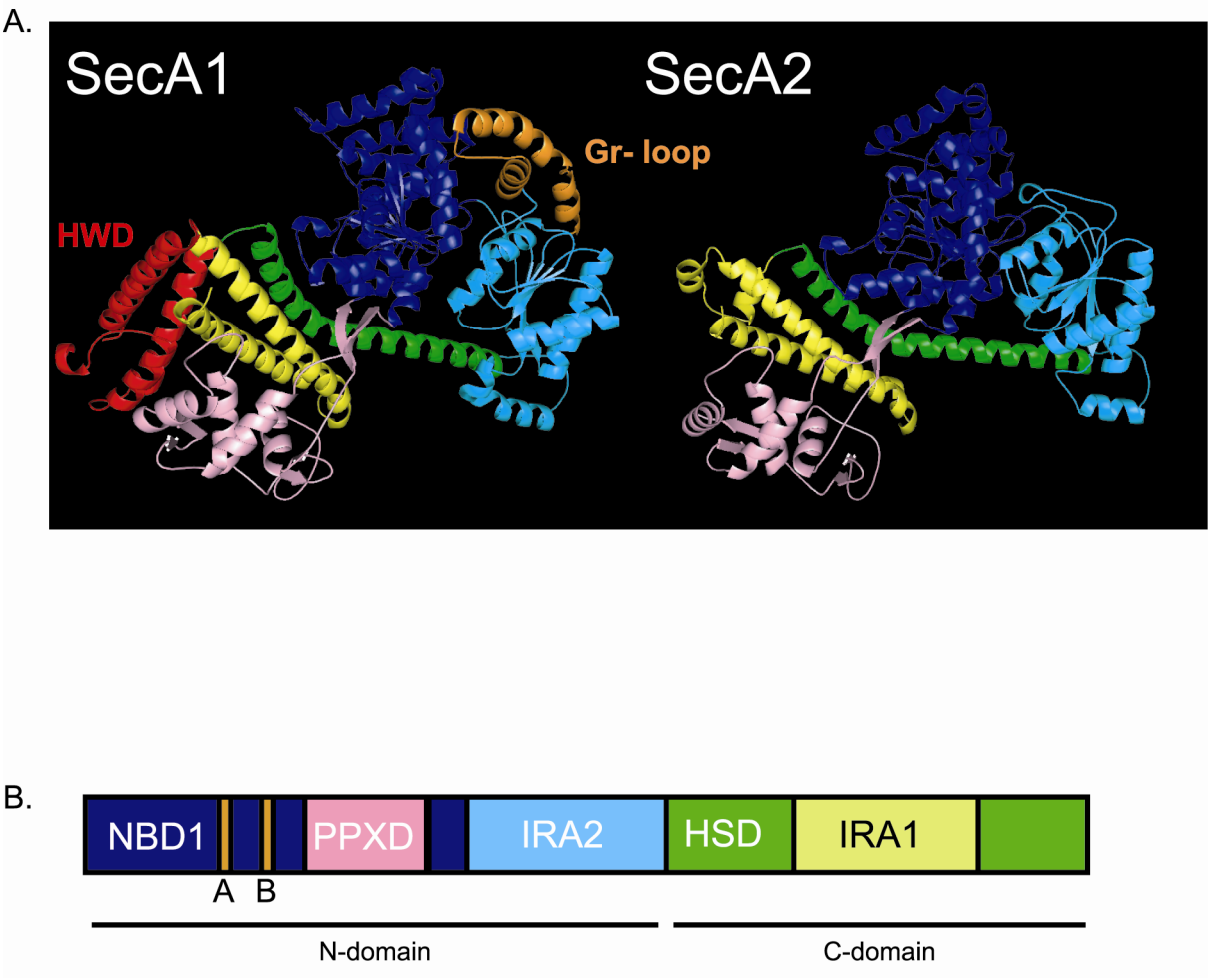

Figure 2.7. A computer-generated homology model of *M. tuberculosis* SecA2 is missing domains found in *M. tuberculosis* SecA1. **(A)** The crystal structure of SecA1 (PDB accession 1NKT) was used to generated the model structure of SecA2. The subdomains are named as in *E. coli* and *B. subtilis* SecA: NBD1 (*N*ucleotide *B*inding *D*omain, dark blue), IRA1 (*I*ntramolecular *R*egulator of *A*TPase, yellow), IRA2 (cyan), PPXD (*P*re*p*rotein *C*rosslinking *D*omain, pink), HSD (*H*elix *S*caffold *D*omain, green), and HWD/CTL (*H*elix *W*ing *D*omain/*C*-*T*erminal *L*inker, red). **(B)** A cartoon representation of the subdomains of SecA2.

References

1. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol **44:**1081-94.

2. **Braunstein, M., S. S. Bardarov, and W. R. J. Jacobs.** 2002. Genetic methods for deciphering virulence determinants of *Mycobacterium tuberculosis*, p. 67-99. *In* V. L. Clark and P. M. Bavoil (ed.), Methods in Enzymology, vol. 358. Academic Press, London.

3. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J Bacteriol **183:**6979-90.

4. **Braunstein, M., B. Espinosa, J. Chan, J. T. Belisle, and W. R. J. Jacobs.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol **48:**453-64.

5. **Breukink, E., N. Nouwen, A. van Raalte, S. Mizushima, J. Tommassen, and B. de Kruijff.** 1995. The C terminus of SecA is involved in both lipid binding and SecB binding. J Biol Chem **270:**7902-7.

6. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. Driessen, and W. Wickner.** 1990. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649-57.

7. **Cabelli, R. J., K. M. Dolan, L. P. Qian, and D. B. Oliver.** 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:**24420-7.

8. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch Microbiol **189:**605-10.

9. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol Microbiol **53:**843-56.

10. **Chen, Q., H. Wu, R. Kumar, Z. Peng, and P. M. Fives-Taylor.** 2006. SecA2 is distinct from SecA in immunogenic specificity, subcellular distribution and requirement for membrane anchoring in *Streptococcus parasanguis*. FEMS Microbiol Lett **264:**174-81.

11. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.

12. **de Keyzer, J., E. O. van der Sluis, R. E. Spelbrink, N. Nijstad, B. de Kruijff, N. Nouwen, C. van der Does, and A. J. Driessen.** 2005. Covalently dimerized SecA is functional in protein translocation. J Biol Chem **280:**35255-60.

13. **Dempsey, B. R., M. Wrona, J. M. Moulin, G. B. Gloor, F. Jalilehvand, G. Lajoie, G. S. Shaw, and B. H. Shilton.** 2004. Solution NMR structure and X-ray absorption analysis of the C-terminal zinc-binding domain of the SecA ATPase. Biochemistry **43:**9361-71.

14. **Ding, H., J. F. Hunt, I. Mukerji, and D. Oliver.** 2003. *Bacillus subtilis* SecA ATPase exists as an antiparallel dimer in solution. Biochemistry **42:**8729-38.

15. **Driessen, A. J., and N. Nouwen.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem **77:**643-67.

16. **Economou, A.** 1998. Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. Mol Microbiol **27:**511-8.

17. **Economou, A.** 1999. Following the leader: bacterial protein export through the Sec pathway. Trends Microbiol **7:**315-20.

18. **Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner.** 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell **83:**1171-81.

19. **Economou, A., and W. Wickner.** 1994. SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell **78:**835-43.

20. **Eichler, J., and W. Wickner.** 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:**5574-81.

21. **Fekkes, P., J. G. de Wit, A. Boorsma, R. H. Friesen, and A. J. Driessen.** 1999. Zinc stabilizes the SecB binding site of SecA. Biochemistry **38:**5111-6.

22. **Fekkes, P., C. van der Does, and A. J. Driessen.** 1997. The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. EMBO J **16:**6105-13.

23. **Fortin, Y., P. Phoenix, and G. R. Drapeau.** 1990. Mutations conferring resistance to azide in *Escherichia coli* occur primarily in the *secA* gene. J Bacteriol **172:**6607-10.

24. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J Bacteriol **189:**5090-100.

25. **Guina, T., D. Helfet-Hilliker, V. Ramamurthy, and D. Oliver.** 1998. Sequence and phylogenetic analysis of the *Borrelia burgdorferi secA* gene. Biochim Biophys Acta **1371:**24-30.

26. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* using tetracycline repressors. J Bacteriol **189:**4614-23.

27. **Hartl, F. U., S. Lecker, E. Schiebel, J. P. Hendrick, and W. Wickner.** 1990. The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* plasma membrane. Cell **63:**269-79.

28. **Hendrick, J. P., and W. Wickner.** 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:**24596-600.

29. **Hou, J. M., N. G. D'Lima, N. W. Rigel, H. S. Gibbons, J. R. McCann, M. Braunstein, and C. M. Teschke.** 2008. ATPase activity of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins and its importance for SecA2 function in macrophages. J Bacteriol **190:**4880-7.

30. **Hunt, J. F., S. Weinkauf, L. Henry, J. J. Fak, P. McNicholas, D. B. Oliver, and J. Deisenhofer.** 2002. Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. Science **297:**2018-26.

31. **Jilaveanu, L. B., C. R. Zito, and D. Oliver.** 2005. Dimeric SecA is essential for protein translocation. Proc Natl Acad Sci U S A **102:**7511-6.

32. **Karamanou, S., E. Vrontou, G. Sianidis, C. Baud, T. Roos, A. Kuhn, A. S. Politou, and A. Economou.** 1999. A molecular switch in SecA protein couples ATP hydrolysis to protein translocation. Mol Microbiol **34:**1133-45.

33. **Kelley, L. A., R. M. MacCallum, and M. J. Sternberg.** 2000. Enhanced genome annotation using structural profiles in the program 3D-PSSM. J Mol Biol **299:**499-520.

34. **Kimura, E., M. Akita, S. Matsuyama, and S. Mizushima.** 1991. Determination of a region in SecA that interacts with presecretory proteins in *Escherichia coli*. J Biol Chem **266:**6600-6.

35. **Kuroda, M., Y. Tanaka, R. Aoki, D. Shu, K. Tsumoto, and T. Ohta.** 2008. *Staphylococcus aureus* giant protein Ebh is involved in tolerance to transient hyperosmotic pressure. Biochem Biophys Res Commun **374:**237-41.

36. **Kurtz, S.** 2007. SecA2 of *M. tuberculosis* contributes to intracellular survival, immune modulation, and surface properties of the bacillus. Masters of Science. University of North Carolina at Chapel Hill, Chapel Hill.

37. **Kurtz, S., K. P. McKinnon, M. S. Runge, J. P. Ting, and M. Braunstein.** 2006. The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. Infect Immun **74:**6855-64.

38. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol Microbiol **45:**1043-56.

39. **Lithgow, J. K., E. Ingham, and S. J. Foster.** 2004. Role of the *hprT-ftsH* locus in *Staphylococcus aureus*. Microbiology **150:**373-81.

40. **McCann, J. R., J. A. McDonough, M. S. Pavelka, and M. Braunstein.** 2007. Beta-lactamase can function as a reporter of bacterial protein export during *Mycobacterium tuberculosis* infection of host cells. Microbiology **153:**3350-9.

41. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol Microbiol **10:**483-97.

42. **Murphy, C. K., and J. Beckwith.** 1996. Export of proteins to the cell envelope in *Escherichia coli*., p. 967-978. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*. ASM Press, Washington, D.C.

43. **Musial-Siwek, M., S. L. Rusch, and D. A. Kendall.** 2007. Selective photoaffinity labeling identifies the signal peptide binding domain on SecA. J Mol Biol **365:**637-48.

44. **Navarre, W. W., and O. Schneewind.** 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev **63:**174-229.

45. **Niederweis, M., S. Ehrt, C. Heinz, U. Klocker, S. Karosi, K. M. Swiderek, L. W. Riley, and R. Benz.** 1999. Cloning of the *mspA* gene encoding a porin from *Mycobacterium smegmatis*. Mol Microbiol **33:**933-45.

46. **Or, E., A. Navon, and T. Rapoport.** 2002. Dissociation of the dimeric SecA ATPase during protein translocation across the bacterial membrane. EMBO J **21:**4470-9.

47. **Papanikolau, Y., M. Papadovasilaki, R. B. Ravelli, A. A. McCarthy, S. Cusack, A. Economou, and K. Petratos.** 2007. Structure of dimeric SecA, the *Escherichia coli* preprotein translocase motor. J Mol Biol **366:**1545-57.

48. **Papanikou, E., S. Karamanou, C. Baud, M. Frank, G. Sianidis, D. Keramisanou, C. G. Kalodimos, A. Kuhn, and A. Economou.** 2005. Identification of the preprotein binding domain of SecA. J Biol Chem **280:**43209-17.

49. **Papanikou, E., S. Karamanou, and A. Economou.** 2007. Bacterial protein secretion through the translocase nanomachine. Nat Rev Microbiol **5:**839-51.

50. **Pavelka, M. S., Jr., and W. R. Jacobs, Jr.** 1999. Comparison of the construction of unmarked deletion mutations in *Mycobacterium smegmatis, Mycobacterium bovis* bacillus Calmette-Guerin, and *Mycobacterium tuberculosis* H37Rv by allelic exchange. J Bacteriol **181:**4780-9.

51. **Price, A., A. Economou, F. Duong, and W. Wickner.** 1996. Separable ATPase and membrane insertion domains of the SecA subunit of preprotein translocase. J Biol Chem **271:**31580-4.

52. **Rajapandi, T., and D. Oliver.** 1994. Carboxy-terminal region of *Escherichia coli* SecA ATPase is important to promote its protein translocation activity in vivo. Biochem Biophys Res Commun **200:**1477-83.

53. **Randall, L. L., S. J. Hardy, T. B. Topping, V. F. Smith, J. E. Bruce, and R. D. Smith.** 1998. The interaction between the chaperone SecB and its ligands: evidence for multiple subsites for binding. Protein Sci **7:**2384-90.

54. **Reyrat, J. M., and D. Kahn.** 2001. *Mycobacterium smegmatis*: an absurd model for tuberculosis? Trends Microbiol **9:**472-4.

55. **Rigel, N. W., and M. Braunstein.** 2008. A new twist on an old pathway--accessory Sec systems. Mol Microbiol **69:**291-302.

56. **Sambrook, J., and D. W. Russell.** 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

57. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

58. **Schiebel, E., A. J. Driessen, F. U. Hartl, and W. Wickner.** 1991. Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell **64:**927-39.

59. **Sharma, V., A. Arockiasamy, D. R. Ronning, C. G. Savva, A. Holzenburg, M. Braunstein, W. R. Jacobs, Jr., and J. C. Sacchettini.** 2003. Crystal structure of *Mycobacterium tuberculosis* SecA, a preprotein translocating ATPase. Proc Natl Acad Sci U S A **100:**2243-8.

60. **Siboo, I. R., D. O. Chaffin, C. E. Rubens, and P. M. Sullam.** 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. J Bacteriol **190:**6188-96.

61. **Sikora, A. E., S. R. Lybarger, and M. Sandkvist.** 2007. Compromised outer membrane integrity in *Vibrio cholerae* Type II secretion mutants. J Bacteriol **189:**8484-95.

62. **Tokunaga, M., J. M. Loranger, and H. C. Wu.** 1984. A distinct signal peptidase for prolipoprotein in *Escherichia coli*. J Cell Biochem **24:**113-20.

63. **van der Wolk, J. P., J. G. de Wit, and A. J. Driessen.** 1997. The catalytic cycle of the *Escherichia coli* SecA ATPase comprises two distinct preprotein translocation events. EMBO J **16:**7297-304.

64. **van Roosmalen, M. L., N. Geukens, J. D. Jongbloed, H. Tjalsma, J. Y. Dubois, S. Bron, J. M. van Dijl, and J. Anne.** 2004. Type I signal peptidases of Gram-positive bacteria. Biochim Biophys Acta **1694:**279-97.

65. **Vassylyev, D. G., H. Mori, M. N. Vassylyeva, T. Tsukazaki, Y. Kimura, T. H. Tahirov, and K. Ito.** 2006. Crystal structure of the translocation ATPase SecA from *Thermus thermophilus* reveals a parallel, head-to-head dimer. J Mol Biol **364:**248-58.

66. **Vijaranakul, U., A. Xiong, K. Lockwood, and R. K. Jayaswal.** 1998. Cloning and nucleotide sequencing of a *Staphylococcus aureus* gene encoding a branched-chain-amino-acid transporter. Appl Environ Microbiol **64:**763-7.

67. **Vrontou, E., and A. Economou.** 2004. Structure and function of SecA, the preprotein translocase nanomotor. Biochim Biophys Acta **1694:**67-80.

68. **Vrontou, E., S. Karamanou, C. Baud, G. Sianidis, and A. Economou.** 2004. Global co-ordination of protein translocation by the SecA IRA1 switch. J Biol Chem **279:**22490-7.

69. **Walker, J. E., A. Eberle, N. J. Gay, M. J. Runswick, and M. Saraste.** 1982. Conservation of structure in proton-translocating ATPases of *Escherichia coli* and mitochondria. Biochem Soc Trans **10:**203-6.

70. **Yahr, T. L., and W. T. Wickner.** 2000. Evaluating the oligomeric state of SecYEG in preprotein translocase. EMBO J **19:**4393-401.

71. **Ye, J., A. R. Osborne, M. Groll, and T. A. Rapoport.** 2004. RecA-like motor ATPases--lessons from structures. Biochim Biophys Acta **1659:**1-18.

72. **Zwizinski, C., and W. Wickner.** 1980. Purification and characterization of leader (signal) peptidase from *Escherichia coli*. J Biol Chem **255:**7973-7.

Chapter 3

Construction and Characterization of Walker Box Mutant Alleles of *secA2*

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The finding that two SecA homologs function in mycobacteria is a relatively recent discovery. SecA1 is essential, and is thought to function like the well-characterized SecA protein of *Escherichia coli*. SecA2 is not essential, but is required to export a subset of proteins. The mechanism of SecA2-dependent protein export remains poorly defined. Both SecA1 and SecA2 from *Mycobacterium tuberculosis* have been shown to possess ATPase activity, and this activity is required for normal function. Sequence alignments between *E. coli* SecA and mycobacterial SecA proteins show a high degree of similarity between the high-affinity ATP-binding Walker box motifs. Mutation of key residues in the Walker box of *E. coli secA* results in alleles that fail to complement temperature-sensitive *secA* mutants. We constructed Walker box mutant *secA2* alleles and tested the ability of these alleles to complement phenotypes of *M. tuberculosis* and *M. smegmatis* Δ*secA2* mutants. We show that *secA2 KR* fails to complement and exacerbates Δ*secA2* mutant phenotypes, and is a

dominant negative allele. SecA2 KR cannot support export of SecA2-dependent substrates and localized to a different subcellular fraction than wild-type SecA2. We isolated spontaneous intragenic and extragenic suppressors of *secA2 KR*, and subsequently identified important functional domains of SecA2. These results shed light on the mechanism of SecA2-mediated protein export.

Introduction

Protein export pathways are an important aspect of bacterial physiology. Exported proteins, those proteins transported beyond the cytosol to the cell envelope or released into the extracellular environment, are important for establishing cell structure, acquiring nutrients, and sensing changes in the environment. Exported proteins also have important roles in virulence because exported (secreted and surface-anchored) proteins are ideally positioned to interact with and modulate responses of host cells to invading bacterial pathogens (17). Because of the important role played by extracytoplasmic proteins in virulence, it is not surprising that the requisite export systems are equally important to virulence. Many bacterial pathogens use specialized systems to export virulence factors to the bacterial cell surface or release them into the extracellular environment (1, 19). Thus, identifying exported proteins and understanding their underlying transport systems could yield new targets for antimicrobial therapies.

In mycobacteria and a small subset of Gram positive bacteria, the accessory Sec pathway represents an example of a specialized protein export system (4, 8, 9, 11, 12, 33, 39, 44). The accessory Sec pathway is found in pathogens and non-pathogens alike. It has been linked to virulence in several cases including *Mycobacterium tuberculosis* and *Listeria*

monocytogenes (9, 23, 31, 32). The accessory Sec pathways studied so far are shown to export a select subset of proteins that include proven or predicted virulence factors (4, 9, 12, 20, 32). While the constituents of the accessory Sec pathway differs among bacterial species, one component they all share is the presence of an accessory SecA homolog called SecA2. SecA2 is homologous to the essential SecA (SecA1) that is part of the canonical essential Sec pathway found in all bacteria (13). In *E. coli*, SecA guides transit of unfolded pre-protein substrates through a channel in the cytoplasmic membrane comprised of the SecYEG proteins (22, 36). The power for this system is provided by the ATPase activity of SecA. SecA is essential, as is this ATPase activity (35). Mutants defective in SecA ATP binding/hydrolysis are unable to complement temperature sensitive *secA* mutants in *E. coli*. Both SecA1 and SecA2 from *M. tuberculosis* and *M. smegmatis* have predicted ATP-binding Walker box motifs (8, 24, 39). In Chapter 4, we show that *M. tuberculosis* SecA1 and SecA2 are able to bind and hydrolyze ATP *in vitro*. In this Chapter, we address the biological significance of SecA2 ATPase activity in *M. tuberculosis* and *M. smegmatis*.

SecA1 is essential in mycobacteria (8, 41). The *secA1* gene cannot be deleted unless *secA1* is present elsewhere on the chromosome or encoded on a plasmid. In contrast, *secA2* is dispensable for growth in both *M. tuberculosis* and *M. smegmatis*. Previous studies described construction and phenotypic characterization of Δ*secA2* mutants in both species (8, 9). The Δ*secA2* mutant of *M. tuberculosis* is attenuated in both mouse and macrophage models of infection (9, 31). In addition, when grown on agar plates that contain the detergent Tween-80, colonies of the Δ*secA2* mutant appear smooth and glossy compared to the rough and dull colonies of the wild-type strain H37Rv (30). The Δ*secA2* mutant of *M. smegmatis* exhibits several phenotypes, including growth defects on rich and high salt agar, and

hypersensitivity to sodium azide (Chapter 2) (8). The basis of these plate phenotypes is unknown. One possible explanation is that SecA2 is needed to export substrates that enable optimal growth under diverse conditions. *M. tuberculosis* and *M. smegmatis* Δ*secA2* mutants are both defective in exporting a small subset of proteins. Comparative 2D-gel analysis of exported proteins from both organisms led to the identification of a small number of proteins that depend on SecA2 for export (9, 20, 23). The phenotypes of mycobacterial Δ*secA2* mutants have proven useful for studying SecA2 function.

In order to study the importance of ATPase activity for SecA2 function, we tested the effect of amino acid substitutions in the ATP-binding Walker Box of SecA2. The Walker Box substitutions we engineered are in highly conserved positions and are predicted to prevent SecA2 from binding ATP. In Chapter 4, we confirm that SecA2 with a mutated Walker Box is unable to bind ATP. When tested in *M. tuberculosis*, we found that Walker box mutant *secA2* alleles fail to function as judged by the inability to complement the macrophage growth defect or colony morphology phenotype of the *M. tuberculosis* Δ*secA2* mutant. In *M. smegmatis*, we showed a Walker box mutation in SecA2 eliminates export of two SecA2-dependent lipoproteins, Msmeg1704 and Msmeg1712. This indicates that in *M. smegmatis* ATP binding is also necessary for normal SecA2 function. Furthermore, we discovered that in *M. smegmatis* the Walker box mutant *secA2* alleles not only fail to complement, but actually exacerbate plate phenotypes associated with the Δ*secA2* mutant.

The bulk of our studies focused on a particular Walker box mutant of *M. smegmatis* SecA2 K129R. We showed that this lysine to arginine amino acid substitution in the Walker Box of *M. smegmatis* SecA2 leads to dominant negative phenotypes. Dominant negative mutations combined with suppressor analysis can be a powerful way to identify proteins that

work together in biological pathways (2). The robust phenotypes associated with SecA2 K129R expression enabled us to isolate spontaneous suppressors of the dominant negative phenotypes. Both intragenic and extragenic suppressors were obtained. Analysis of the intragenic suppressors identified domains required for SecA2 function in vivo. Although the identity of the extragenic suppressors remains unknown, we are confident they will help to identify SecA2-interacting proteins. This work represents the first mutational analysis of any SecA2 and it provides an important first step in understanding the basic mechanism of SecA2-mediated protein export pathways in mycobacteria.

Materials and Methods

Bacterial strains and culture conditions. *M. smegmatis* was grown in Middlebrook 7H9 or on 7H10 (BD Biosciences) supplemented with 0.2% (w/v) glucose, 0.5% (w/v) glycerol, and 0.1% (w/v) Tween 80 (Fisher); or Mueller Hinton (BD Biosciences) supplemented with 0.1% (w/v) Tween 80. *M. tuberculosis* was grown in 7H9 or on 7H10 supplemented with 0.5% (w/v) glycerol and 1X ADS (0.5% (w/v) bovine serum albumin, 0.2% (w/v) dextrose, and 0.85% (w/v) NaCl). The antibiotics kanamycin (Acros Chemicals) or hygromycin (Roche) were added as needed at 20 μg/ml or 50 μg/ml, respectively. LB was used to grow *E. coli* cultures. When needed, kanamycin or hygromycin were added at 40 μg/ml or 150 μg/ml, respectively. All vectors used in this study were verified by DNA sequencing (UNC-CH automated DNA sequencing facility and Eton Biosciences). NR116 was used as the Δ*secA2* mutant of *M. smegmatis*, and $mc^2$3112 (9) was used as the Δ*secA2* mutant of *M. tuberculosis* for all experiments.

Construction of *secA2* Walker Box mutant plasmids pNR7, pNR8, pNR22, and pNR25. The DNA encoding the Walker boxes of *secA2* was mutated using the Quikchange Site Directed mutagenesis kit (Stratagene). The plasmid templates were pMB162 for *M. tuberculosis secA2*, pMB208 and pYA810 for *M. smegmatis secA2* (8, 20). pMB162 contains *M. tuberculosis secA2* under the control of the constitutive mycobacterial *hsp60* promoter and integrates in the *attB* site of the mycobacterial chromosome. *M. smegmatis secA2* is driven from the *hsp60* promoter in both pMB208 and pYA810; the difference between these vectors is that pMB208 is episomal while pYA810 integrates at the chromosomal *attB* site. The Walker A motif of *M. tuberculosis* SecA2 was mutated in plasmid pMB162 using the following primers: 5'-CGGTGAGGGCAGAACCCTTGCC-3' and 5'-CGGCAAGGGTTCTGCCCTCACC-3', and the Walker B motif was mutated using the primers: 5'-GACGTGGCTCTCATCAATGAAGCCGACTCCG-3' and 5'-CACGGAGTCGGCTTCATTGATGAGAGCCACG-3'. The resulting mutant plasmids were pNR7 and pNR8, respectively. To create the Walker A mutation in *M. smegmatis secA2*, primers 5'-CGGGTGAGGGCAGGACGCTGGC-3' and 5'-GCCAGCGTCCTGCCCTCACCCG-3' were used with pMB208 and pYA810 as the template to generate plasmids pNR22 and pNR25, respectively.

Construction of inducible *secA2 K129R* plasmid pNR54. Using pHSG86 as a template, we PCR amplified *M. smegmatis secA2* with primers 5'-AGGATCCATCCGGAGGAATCACTT-3' and 5'-AGGATCCCTAGTGGTGGTGGTGGTGGCGGAACACACCCGGCAGG-3'. The resulting PCR product was cloned into pCC1 to yield pNR52. Next, a 2.4 kb *Bam*HI fragment was cloned from pNR52 into similarly cut pMP715 (gift from Dr. Marty Pavelka,

University of Rochester) to give pNR53. pMP715 contains the TetO sequence upstream of the BamHI site, as well as the TetR repressor. TetR binds to the TetO sites and blocks gene expression (21). In the presence of anhydrotetracycline (Atc), TetR dissociates from TetO and allows gene expression. To mutate Walker box residue K129, pNR53 was subjected to site-directed mutagenesis as described. The resulting plasmid pNR54 carries *secA2 K129R* under control of the Atc inducible promoter. Induction of SecA2 K129R in *M. smegmatis* transformed with pNR54 was achieved by treating cultures with 300 ng/ml Atc for 21 hours at 37˚C.

Construction of suicide plasmid pNR23. Two-step allelic exchange was used to replace the Δ*secA2* deletion in the chromosome of strain NR116 with a full-length copy of *secA2* containing the *K129R* Walker A mutation (7, 38). First, pMB148 was digested with *Eco*RI to remove 1082 bp of extraneous vector sequence. pMB148 contains *M. smegmatis secA2* and additional upstream and downstream sequences. The remaining 7793 bp was then self-ligated to give a 7793 bp plasmid named pNR20. Site-directed mutagenesis was performed on pNR20 as described above, to generate pNR21. We used the same mutagenic primers on pNR20 that we used to make the *secA2* Walker A K129R mutation in pNR25. pNR21 contains full-length *secA2 K129R* driven from the native *secA2* promoter. Finally, a 5059 bp *Ase*I-*Eco*RV fragment from pNR21 was cloned into *Nde*I-*Eco*RV cut pMP62 to yield the counterselectable suicide vector pNR23.

Construction of *secA2 K129R* strain NR179. pNR23 was electroporated into Δ*secA2* mutant strain NR116, and hygromycin resistant transformants were selected on 7H10 plates. Strains where pNR23 integrated at the *secA2* locus by homologous recombination were analyzed by Southern blot to identify the single-crossover strain NR155. For the second

recombination event, NR155 was grown to saturation in 7H9 broth with 50 μg/ml hygromycin, diluted 1:100 in 7H9 without antibiotics, and grown overnight. Dilutions of the overnight culture were plated on 7H10 plates that contained 4.5% (w/v) sucrose to select for sucrose resistant colonies. Sucrose resistant, hygromycin sensitive clones were analyzed by colony PCR and Southern blot to identify strain NR179 as containing a full-length *secA2 K129R* allele at the chromosomal locus.

Southern blot analysis. Genomic DNA was prepared from *M. smegmatis* strains as described and digested with *Bam*HI (7). The probe was a 592 bp *Bam*HI-*Xma*I fragment from pMB156 that contained sequence homologous to the region immediately downstream of *M. smegmatis secA2*. A Ready-to-Go labeling kit (Amersham) was used to label the probe with [^{32}P]dCTP, and the resulting blot was developed by autoradiography.

Azide assay. 200 μl of saturated (OD_{600nm} = 2.0) *M. smegmatis* culture was mixed with 3.5 ml of molten 7H9 top agar, and then poured onto a 7H10 bottom agar plate. Sterile 6 mm filter discs were placed onto the surface of the cooled top agar. 10 μl of 0.15 M sodium azide was then added to the disc. The plates were inverted and incubated for 2 days at 37˚C, and resulting zones of inhibition were measured. Each strain was tested in triplicate, and untreated plates were included as a control.

Agar plate growth assays. All *M. smegmatis* strains were grown in 7H9 broth at 37˚C prior to plating except the Δ*secA2* mutant expressing pNR25. This strain was grown at 30˚C because of toxicity associated with expressing the *secA2 K129R* allele. Serial dilutions of each strain were made in 7H9 and then plated onto the appropriate agar medium. All *M. tuberculosis* strains were grown at 37˚C until saturation, diluted in 1X PBS with 0.05% (w/v) Tween 80, and plated onto 7H10. Plates were incubated at 37˚C until colonies were visible.

Export of Msmeg1704-HA and Msmeg1712-HA. To monitor export of Msmeg1704 and Msmeg1712, both proteins were tagged with the HA epitope at the C-terminus. pHSG51 and pHSG58 encode Msmeg1712-HA and Msmeg1704-HA under control of the constitutive *hsp60* promoter. Both of these plasmids are Kan^R and episomal. To generate Hyg^R derivatives of these plasmids, pHSG51 was cut with *Nhe*I and *Spe*I to generate a 4.2 kb fragment. This fragment was end-filled using the Klenow fragment (New England Biolabs), and ligated to the Hyg^R-containing 1.5 kb *Sma*I-*Eco*RV fragment from pYUB412 to generate pNR35. pNR36 (Msmeg1704-HA) was constructed by digesting pHSG58 with *Nhe*I and *Spe*I yielding a 5.4 kb fragment. The fragment was end-filled with Klenow and ligated to the Hyg^R-containing *Sma*I-*Eco*RV fragment from pYUB412. To monitor export of Msmeg1704-HA and Msmeg1712-HA, pNR35 and pNR36 were transformed into *M. smegmatis*. Strains were grown in Mueller Hinton broth until OD_{600nm} = 1.0, and then lysed in a French pressure cell. Lysates were fractionated by differential ultracentrifugation into cell wall (50,000 x *g* pellet), membrane (100,000 x *g* pellet), and soluble (100,000 x *g* supernatant) fractions (20). Protein derived from the same amount of starting cells for each fraction was analyzed by SDS-PAGE and western blots.

Immunoblot conditions. The anti-SecA2, anti-GroEL (HAT5, from the World Health Organization antibody collection), anti-MspA (gift from Dr. Michael Niederweis), and anti-HA (Covance) antibodies were used at 1:20,000 dilutions. The anti-SecA1 antibody was used at a 1:50,000 dilution. For most experiments, secondary antibodies conjugated to HRP (Biorad) were used along with ECL (Perkin Elmer) to visualize blots. For quantitative westerns, secondary antibodies conjugated to AP (Amersham/GE Healthcare) were used along with ECF. Fluorescence was quantified using a phosphorimager (Molecular Devices).

Results

ATP binding is required for SecA2 function in *M. tuberculosis* and *M. smegmatis*. Sequence alignments reveal the presence of conserved high-affinity ATP-binding Walker box motifs in SecA1 and SecA2 of mycobacteria (Figure 3.1A). In Chapter 4, we will demonstrate that *M. tuberculosis* SecA1 and SecA2 both possess ATPase activity *in vitro*. While the requirement for ATPase activity of *E. coli* and *Bacillus subtilis* SecA in its role in protein export is well established (34, 35), it is not known if the biological function of any accessory SecA2 proteins requires ATPase activity. To address this issue, we used a genetic approach. We constructed *secA2* Walker Box mutants predicted to prevent ATP binding (Figure 1A) and tested them for function by introducing the mutated alleles into Δ*secA2* mutants and assaying for complementation of Δ*secA2* mutant phenotypes. If ATPase activity is required for normal SecA2 function, then mutations that destroy ATPase activity should result in a *secA2* allele that does not complement Δ*secA2* mutant phenotypes. We chose to mutate key lysine and aspartic acid residues in the Walker box motif of SecA2. The side chains of these two amino acids function in nucleotide binding by interacting with the γ-phosphate of ATP (49). Indeed, mutations of the Walker Box lysine residue eliminates ATP binding of many diverse ATPases including *E. coli* SecA, the Type IV secretion protein DotB of *Legionella pneumophila* and type IV pilus protein PilQ of *E. coli* (35, 40, 43). In this Chapter, we describe experiments conducted in *M. tuberculosis* and in *M. smegmatis* in which *secA2* Walker box mutant alleles were tested for function.

Using site-directed mutagenesis, we constructed two mutant alleles of *M. tuberculosis secA2* with mutations in the sequence encoding the conserved Walker A or Walker B high-affinity ATP binding motifs. In plasmid pNR7, the lysine codon at residue 115 in the Walker

A motif was replaced with an arginine codon (SecA2 K115R). In plasmid pNR8, the aspartic acid codon in the Walker B motif was changed to asparagine codon at residue 216 (SecA2 D216N). We also constructed plasmids pNR22 and pNR25, which carry *M. smegmatis secA2* with a Walker A lysine to arginine substitution at amino acid 129 (SecA2 K129R), which correspond to K115 of *M. tuberculosis* SecA2. In *E. coli*, the corresponding KR and DN Walker box mutations result in *secA* alleles that are unable to complement a temperature-sensitive *secA* mutant (35). The various expression constructs were transformed into the *M. tuberculosis* wild-type (H37Rv) and Δ*secA2* mutant ($mc^2$3112) and the *M. smegmatis* wild-type ($mc^2$155) and Δ*secA2* mutant (NR116). For each construct, SecA2 expression was driven from a constitutive *hsp60* promoter. Based on the rationale described above, if ATPase activity is important for SecA2 function, then *secA2* Walker box mutant alleles will fail to complement Δ*secA2* mutant phenotypes. Indeed, we found that none of the mutant alleles compensated for the absence of *secA2*. These results indicated that ATPase activity is critical for normal SecA2 function. Much to our surprise, in some assays these alleles not only failed to complement, but actually exacerbated the Δ*secA2* mutant phenotypes and behaved as dominant negative mutations.

Phenotypes of *secA2* Walker Box mutant alleles expressed in *M. tuberculosis*.

***M. tuberculosis* SecA2 K115R does not restore the ability of a *M. tuberculosis* Δ*secA2* mutant to grow in macrophages.** A Δ*secA2* mutant of *M. tuberculosis* is attenuated for growth in macrophages and in mice (9, 31). To test the idea that SecA2 functions as an ATPase in mycobacteria, we asked whether the intracellular phenotype of the Δ*secA2* mutant of *M. tuberculosis* could be complemented by a *M. tuberculosis secA2* allele that encodes a lysine to arginine (K115R) substitution at position 115 of the Walker box motif. This *secA2*

K115R allele was electroporated into the Δ*secA2* mutant mc^{2}3112 and integrated in single copy at the chromosomal *attB* site. Introduction of a wild-type copy of *secA2* at the *attB* locus was previously shown to complement the Δ*secA2* mutant phenotype (31). Murine bone marrow-derived macrophages were infected with wild-type *M. tuberculosis* (H37Rv), *ΔsecA2* mutant mc^{2}3112 (Δ*secA2*), the *ΔsecA2* mutant complemented with wild-type *M. tuberculosis secA2* (Δ*secA2*/*secA2*), or the *ΔsecA2* mutant expressing the *secA2 K115R* allele (Δ*secA2*/*secA2* KR) (Figure 3.1B). As shown previously, H37Rv and the *secA2* complemented strain grew similarly in macrophages over a 5-day period of infection while the *ΔsecA2* mutant failed to grow in these macrophages (31). Introduction of *secA2 K115R* did not restore the ability of the Δ*secA2* mutant to grow intracellularly; the Δ*secA2* mutant expressing SecA2 K115R failed to grow in macrophages. Western blot analysis confirmed that the Δ*secA2* mutant strains carrying wild-type *secA2* or *secA2 K115R* expressed SecA2 protein at similar levels (Figure 3.1C) ruling out the possibility that the lack of growth in macrophages was due to a problem with expression of *secA2 K115R*. The intracellular growth defect of the *secA2 K115R* expressing strain was also not a result of a general growth defect. The *secA2 K115R* strain had a mean generation time that was 1.07 +/- 0.06 times that of the wild-type strain when the two strains were grown in parallel in 7H9 liquid media. These results indicated that substitution of the conserved lysine in the Walker A motif renders SecA2 inactive in its biological role of promoting *M. tuberculosis* growth in macrophages.

Expression of *M. tuberculosis secA2 K115R* does not complement the smooth colony phenotype of a Δ*secA2* mutant. In contrast to the dull, rough, irregularly shaped colonies of wild-type *M. tuberculosis*, colonies of the Δ*secA2* mutant of *M. tuberculosis* are

round and smooth with a glossy appearance (30). This phenotype could indicate a difference in composition of the cell envelope between the two strains. Notably, this difference is only detected when the bacteria are grown on media containing Tween 80. When grown on 7H10 plates without Tween 80, wild-type and Δ*secA2* mutant colonies are indistinguishable (data not shown). The rough colony morphology can be restored in the Δ*secA2* mutant by expression of a wild-type copy of *secA2* (30). To further test the Walker Box mutant *secA2* alleles, we grew the same set of *M. tuberculosis* strains tested in macrophages on 7H10 plates supplemented with 0.05% Tween 80. After 3 weeks of growth, plates were removed from incubation and photographed. As shown in Figure 3.2A, colonies of the Δ*secA2* mutant appeared smooth in comparison to H37Rv. Expression of wild-type *secA2* restored the rough colony morphology to the Δ*secA2* mutant. In contrast, when the *secA2 K115R* mutant allele is expressed in the Δ*secA2* mutant, the colonies remained smooth (Figure 3.2B). This provided further indication that Walker Box mutant *secA2* alleles are unable to function normally. It is worth mentioning that expression of the *secA2 K115R* allele in H37Rv changed the rough appearance of these colonies to a smooth phenotype similar to that of the Δ*secA2* mutant. This is consistent with *secA2 K115R* being a dominant negative allele. Further demonstration of *secA2* Walker box alleles being dominant negative is presented later in this Chapter.

Phenotypes of *secA2* Walker box mutant alleles expressed in *M. smegmatis*.

SecA2 K129R expressed from the native *secA2* chromosomal locus causes Δ*secA2* mutant phenotypes with increased severity. Two-step allelic exchange was used to create an *M. smegmatis* strain that expressed *secA2 K129R* at the native *secA2* locus (7, 38). An advantage to this approach is that SecA2 K129R expression will be controlled by

the native SecA2 promoter, not the constitutive *hsp60* promoter used on mycobacterial plasmid vectors. Suicide vector pNR23 was constructed as described in Methods. This construct encodes a full-length copy of *M. smegmatis secA2* with a single point mutation to change the codon for lysine 129 in the Walker Box to arginine. pNR23 was electroporated into Δ*secA2* mutant (Figure 3.3A), and the resulting transformants were screened for a single crossover event; the desired strain was subsequently named NR155 (Figure 3.3B). Upon counterselection, we performed Southern blot analysis to identify a strain carrying *secA2 K129R* at the chromosomal locus and named this strain NR179 (Figure 3.3C and D). Expression of SecA2 K129R in NR179 was confirmed by immunoblot (Figure 3.3E). Strain NR179 was then tested for Δ*secA2* mutant phenotypes. When compared to wild-type *M. smegmatis* strain, NR179 showed a growth defect on rich MH agar (Figure 3.3F). NR179 was also hypersensitive to azide treatment (data not shown). These results demonstrated that ATP binding is important for the normal function of SecA2 in *M. smegmatis*. Interestingly, the Mueller Hinton rich agar growth defect of NR179 was more severe than that of a Δ*secA2* mutant (compare colony size of Δ*secA2* to NR179). Thus, when SecA2 K129R is the only SecA2 expressed in a cell, a more severe growth defect is observed on rich agar plates than if no SecA2 is present. The azide hypersensitivity displayed by the Δ*secA2* mutant was also more severe in NR179 (data not shown). As described below, increased severity of Δ*secA2* mutant phenotypes was a recurring theme with Walker Box *secA2* mutants.

Expression of *M. tuberculosis* Walker Box mutant *secA2 K115R* or *D216N* alleles do not complement and they exacerbate the phenotypes of a *M. smegmatis* Δ*secA2* mutant. To continue our evaluation of the importance of ATP binding for SecA2 function, we tested the ability of *M. tuberculosis secA2* Walker Box mutant alleles to function in *M.*

smegmatis. Wild-type SecA2 from *M. tuberculosis* was previously shown to function in *M. smegmatis* by the ability to complement the rich agar growth defect and azide hypersensitivity phenotypes associated with a *M. smegmatis* Δ*secA2* mutant (8). *M. smegmatis* wild-type, Δ*secA2* mutant (Δ*secA2*), Δ*secA2* mutant complemented with wild-type *M. tuberculosis secA2* (Δ*secA2*/*secA2*), and Δ*secA2* mutant complemented with *M. tuberculosis secA2 K115R* (Δ*secA2*/*secA2 K115R*) or *secA2 D216N* (Δ/*secA2 D216N*) cultures were grown in 7H9 broth at 37˚C and a series of dilutions were plated onto Mueller Hinton rich agar. The plates were incubated at 37˚C for 3-4 days. In comparison to wild-type *M. smegmatis* colonies, Δ*secA2* mutant colonies were smaller on rich agar (Figure 3.4A). The Δ*secA2* mutant carrying pMB162, a plasmid that encodes wild-type *M. tuberculosis* SecA2, grew like wild-type. However, plasmids derived from pMB162 that express *M. tuberculosis secA2 K115R* (pNR7) or *D216N* (pNR8) mutant alleles were unable to restore growth of the Δ*secA2* mutant to wild-type levels. The *secA2 K115R* and *D216N* alleles not only failed to complement, but they exacerbated the growth defect of the Δ*secA2* mutant on rich agar plates.

We also assayed the Walker Box mutant *secA2* alleles for the ability to complement the azide hypersensitivity phenotype (Figure 3.4B). Saturated cultures of *M. smegmatis* were mixed with molten top agar and spread onto 7H10 bottom agar plates. Sterile filter discs were placed in the middle of the top agar overlay and then impregnated with 10 µl of 0.15 M sodium azide. The plates were inverted and incubated for two days until zones of clearing around the filter discs were measured. As previously reported, the Δ*secA2* mutant was more sensitive to azide than wild-type *M. smegmatis*, and this increased sensitivity was reversed by expression of a wild-type copy of *M. tuberculosis secA2* (8). As was seen with the growth

phenotype on rich agar, expression of the Walker Box mutant *secA2* alleles in the Δ*secA2* mutant not only failed to complement the azide sensitivity phenotype but resulted in a more severe phenotype than the parental Δ*secA2* mutant strain.

Expression of *M. smegmatis secA2 K129R* fails to support export of SecA2-dependent proteins in *M. smegmatis*. We also tested the ability of SecA2 Walker Box mutants to function in *M. smegmatis*. We started by testing the ability of *M. smegmatis* SecA2 K129R to export two SecA2-dependent lipoproteins, Msmeg1704 and Msmeg1712. Plasmid pNR25, expressing *M. smegmatis secA2 K129R*, was generated by performing site-directed mutagenesis on wild-type *secA2* carried on pYA810. Wild-type and Δ*secA2* mutant *M. smegmatis* strains were transformed with pNR25, and the transformants were tested for Δ*secA2* mutant phenotypes, starting with export of SecA2-dependent proteins.

The *M. smegmatis* Δ*secA2* mutant is defective in exporting Msmeg1704 and Msmeg1712 lipoproteins from the cytoplasm to the cell wall (20). To directly test if ATPase activity is important for SecA2 function in promoting protein export, we tested if Walker box mutant *secA2* alleles could support export of these two SecA2-dependent proteins of *M. smegmatis*. To monitor export, each protein was tagged with the HA-epitope at the C-terminus. The tagged proteins were then expressed in wild-type (WT) *M. smegmatis*, Δ*secA2* mutant (Δ), *ΔsecA2* mutant complemented with wild-type *secA2* (Δ/WT), or the *ΔsecA2* mutant expressing the *secA2 K129R* allele (Δ/KR) mutant and grown in Mueller Hinton broth at 37˚C. Expression of SecA2 and SecA2 K129R in the complemented strains was confirmed by western blot (data not shown). Cells were lysed in a French pressure cell and then fractionated into cell wall (CW), membrane (MEM), and soluble (SOL) fractions by differential ultracentrifugation. Protein derived from an equivalent number of starting cells

was then analyzed for each fraction by SDS-PAGE and western blot with anti-HA antibodies (Figure 3.5). As expected from published studies, the Δ*secA2* mutant failed to export Msmeg1704-HA and Msmeg1712-HA to the cell wall fraction (20). Moreover, both proteins accumulated in the soluble fraction of the Δ*secA2* mutant which is consistent with an export defect of the proteins following synthesis in the cytoplasm. Expression of wild-type *secA2* in the Δ*secA2* mutant complemented the export defect of Msmeg1704-HA and Msmeg1712-HA to the cell wall, indicating the export defect is due to the absence of *secA2*. When *secA2 K129R* was expressed in a Δ*secA2* mutant, Msmeg1704-HA and Msmeg1712-HA failed to reach the cell wall, and accumulated in the soluble fraction. Purity of each fraction was confirmed by immunoblots for the cell wall protein MspA and the cytoplasmic chaperone GroEL (data not shown). These results indicated that SecA2 K129R is unable to function in exporting these two known *M. smegmatis* SecA2-dependent proteins.

***M. smegmatis secA2 K129R* fails to complement and exacerbates the rich agar growth defect and azide hypersensitivity phenotypes associated with a *M. smegmatis* Δ*secA2* mutant.** As previously reported, colonies of the *M. smegmatis* Δ*secA2* mutant have a growth defect on rich agar plates like Mueller Hinton when compared to wild-type bacteria (8). This growth phenotype is restricted to plate media; no growth defect is observed when the Δ*secA2* mutant is grown in rich broth. The rich agar growth defect is complemented by wild-type *secA2* from *M. smegmatis*, demonstrating that this phenotype is due to deletion of *secA2*. We tested if SecA2 K129R expressed from the native *secA2* chromosomal locus was functional by using the rich agar and azide assays. By expressing SecA2 K129R from the native SecA2 promoter, we could avoid any artifacts associated with expression of SecA2 K129R from the constitutive *hsp60* promoter as is the case with pNR25. As expected from

the earlier studies, *M. smegmatis secA2 K129R* failed to complement and exacerbated the rich agar growth defect (Figure 3.6A) and azide hypersensitivity (Figure 3.6B) of the Δ*secA2* mutant.

We made an additional observation with this allele. When *secA2 K129R* was expressed from plasmid pNR25 in wild-type *M. smegmatis*, Δ*secA2* mutant phenotypes were observed. This strain (wild-type/*secA2 K129R*) exhibited a rich agar growth defect and azide sensitivity phenotype that is comparable to that seen with the Δ*secA2* mutant. Since this strain carries wild-type *secA2* at the chromosomal locus and *secA2 K129R* on a plasmid, this result suggested that *M. smegmatis secA2 K129R* is a dominant negative allele.

***M. smegmatis secA2 K129R* is a dominant negative allele.** Dominant negative mutations produce a gene product that can interfere with the function of the normal gene product in merodiploids; the mutant phenotype is observed in the presence of a wild-type allele (2). The net result for the cell is a loss of activity of the wild-type gene product. Dominant negative mutations usually occur when a mutant protein can still interact with the same proteins as the wild-type protein, but the mutation blocks its function such that only non-productive protein complexes form (40, 43).

Dominant negative alleles are typically dosage dependent (2, 10, 46). If expression of a dominant negative allele is increased, a stronger phenotype arises. Therefore, we tested the effect of increasing expression of *secA2 K129R* in a wild-type strain. Plasmid pNR25 is a single copy vector; it integrates at the *attB* site of the *M. smegmatis* chromosome. To increase expression of *secA2 K129R,* we constructed a multi-copy episomal plasmid carrying *secA2 K129R*. This plasmid, pNR22, was electroporated into wild-type and Δ*secA2 M. smegmatis*. Despite repeated attempts, we were unable to obtain transformants in either

strain background (data not shown). Control plasmids were successfully electroporated into *M. smegmatis* in parallel. Although a negative result, the failure to obtain transformants suggested that high level *secA2 K129R* expression is toxic, which is consistent with there being a dosage dependent effect and the *secA2 K129R* Walker box allele being dominant negative.

To better show the dosage-dependent dominant negative phenotype, we used an inducible promoter system recently shown to function in mycobacteria (14, 21). By inducing expression of *secA2 K129R* after transformation into mycobacteria, we hoped to be able to first construct the strains and then test the effects of high-level expression in a controlled fashion. The inducible system we used is based on the Tet operator (21). We cloned *secA2 K129R* downstream of two TetO sites on plasmid pMP715 thus generating pNR54. Also encoded on pNR54 is the repressor protein TetR. TetR binds to the TetO sites and blocks expression of *secA2 K129R*. However, in the presence of the inducer molecule anhydrotetracycline (Atc), TetR dissociates from the TetO sites allowing expression of *secA2 K129R* (Tet ON). The level of *secA2 K129R* expression can be controlled by adjusting the concentration of Atc inducer.

Wild-type and Δ*secA2* mutant *M. smegmatis* strains were electroporated with pNR54, and the resulting transformants were grown in 7H9 in the absence of Atc at 37˚C. Each strain was used to inoculate Mueller Hinton broth +/- Atc induction and growth was monitored by measuring optical density. After 21 hours of induction, serial dilutions of each strain were plated onto Mueller Hinton plates +/- Atc supplementation (500 ng/ml) and incubated at 37˚C for 3-4 days. As the concentration of inducer increased in the growth media, a corresponding increase in SecA2 K129R expression was observed by SecA2

immunoblots (data not shown). High level expression of SecA2 K129R in both wild-type and Δ*secA2* mutant strain backgrounds led to reduced growth in rich liquid media as revealed by growth curves (Figure 3.7A and C). This is the first time we observed a phenotype in liquid media related to SecA2. As expression of SecA2 K129R increased, the rich agar growth defect of both wild-type and Δ*secA2 M. smegmatis* also became more severe (Figure 3.7B and D). As a control, we also determined that overexpression of wild-type SecA2 does not exacerbate the rich agar growth defect of wild-type of Δ*secA2* mutant *M. smegmatis* (data not shown). These results confirmed that the rich agar growth defect associated with expression of SecA2 K129R is dosage dependent, and are consistent with *secA2 K129R* being a true dominant negative allele.

A model to explain the exacerbated and dominant negative phenotypes caused by Walker box mutant *secA2* alleles. In the previous sections, we described a collection of experiments in *M. tuberculosis* and *M. smegmatis* in which *secA2* Walker box mutants were tested. The results are consistent across the various studies. First, they clearly show that the Walker box is required for normal SecA2 function. Second, they show that failure to express any *secA2* is better than expressing a Walker box mutant *secA2* allele. Said another way, the phenotype associated with a Walker Box mutant *secA2* allele is worse than a Δ*secA2* deletion allele. Third, they show *secA2* Walker box mutants behave as dominant negative alleles. A model to explain the *secA2* Walker box mutant phenotype is as follows. The Walker box mutations render SecA2 catalytically "dead". However, this "dead" protein may still interact with its normal binding partners, but due to the inability to hydrolyze ATP, remains in a locked complex. In this way, the "dead" SecA2 Walker box molecules could titrate up interacting proteins. If the SecA2-interacting proteins tied up in non-functional complexes

have additional and possibly essential functions in the cell, this would lead to exacerbated phenotypes like those we observed. With this model in mind, as the levels of SecA2 K129R increase, the observed phenotypes would become more severe due to the increased production of non-functional complexes. The translocase complex that functions with SecA2 to promote export of a subset of proteins remains to be identified. One strong possibility is that SecA2 works with the general Sec translocase. With this possibility in mind, it is possible that the interacting proteins tied up by SecA2 K129R are components of the essential canonical Sec pathway (Figure 3.8). The SecA2 Walker box mutants could bind irreversibly to the SecYEG translocase, thus compromising export of proteins in addition to Msmeg1704 and Msmeg1712.

The subcellular distribution of the dominant negative SecA2 K129R differs from the cytosolic distribution of wild-type SecA2. As presented in Chapter 2, subcellular fractionation showed that *M. smegmatis* SecA2 is primarily localized to the cytoplasm of *M. smegmatis*, unlike *M. smegmatis* SecA1 and *E. coli* SecA (Figure 3.9A) (35). As a way to understand phenotypes associated with the *secA2 K129R* allele, we tested the localization pattern of SecA2 K129R. In fact, we found that SecA2 K129R is not enriched in the cytosolic/soluble fraction like wild-type SecA2 but, rather, is predominantly associated with the cell envelope that is comprised of cell membrane and cell wall (Figure 3.9D and E).

This shift in localization suggested that the SecA2 K129R protein is trapped at the membrane (Figure 3.8). This striking shift in subcellular localization is consistent with the model that SecA2 normally interacts transiently with a membrane-embedded channel to deliver proteins for export from the cytoplasm and that the Walker Box substitution in SecA2 K129R causes the protein to become permanently associated with the membrane channel.

Permanent association of SecA2 K129R, due to a defect in ATPase activity, could compromise the efficiency of the translocase channel. If this channel were essential, like the SecYEG translocase, the expected result would be impaired growth in a dosage-dependent manner, not unlike the phenotypes associated with the *secA2 K129R* allele.

We also examined the localization of SecA1 in the presence of SecA2 K129R. Of all the proteins in the canonical Sec pathway, we considered SecA1 a prime candidate for interacting with SecA2. Although it remains controversial whether SecA functions as a dimer during the process of protein export, in other bacteria SecA is shown to form dimers. Thus, it seemed possible that SecA1 would form mixed dimers with SecA2. If so, then SecA1 dimerization with SecA2 K129R would be expected to also shift SecA1 primarily to the cell envelope fraction. In wild-type *M. smegmatis*, SecA1 is equally abundant in cytoplasmic and cell envelope fractions. Examination of subcellular fractions of the Δ*secA2*/*secA2 K129R* strain revealed that localization of SecA1 is unaffected by the presence of SecA2 K129R (Figure 3.9D and E). This result does not preclude the interaction of SecA2 with the canonical Sec pathway. It does however suggest that the point of interaction is not SecA1.

Isolation of spontaneous suppressors of the exacerbated rich agar growth defect caused by Walker Box mutant alleles. Suppressor mutations can be useful in identifying functional domains of a protein (intragenic suppressors) or additional gene products involved in a biological process (extragenic suppressors) (2, 5, 16, 25, 28). During initial experiments with the Δ*secA2 M. smegmatis* strains expressing *M. tuberculosis secA2 K115R*, *M. tuberculosis secA2 D216N*, or *M. smegmatis secA2 K129R*, spontaneous suppressors of the severe rich agar growth defect were observed (Figure 3.10A). We therefore sought to

characterize suppressors of the dominant negative phenotype as a means of identifying SecA2 functional domains and SecA2-interacting proteins.

Intragenic and extragenic suppressors of *secA2 K115R*. As a starting point, we collected suppressors of the exacerbated rich agar growth defect caused by *M. tuberculosis secA2 K115R* (pNR7) expression in the *M. smegmatis* Δ*secA2* mutant. Suppressors were collected from independently grown cultures of the *M. smegmatis* Δ*secA2* mutant carrying pNR7; this plasmid integrates in single copy into the *attB* site of the bacterial chromosome. Serial dilutions of each culture were plated onto Mueller Hinton agar to collect suppressors, and on 7H10 agar to obtain total CFU counts. Using this method, we determined that the spontaneous suppressors arose at a relatively high frequency of 10^{-4}. All the suppressors we collected grew better than the starting strain (Δ*secA2*/*secA2 K115R*) on Mueller Hinton agar. They did not, however, grow like wild-type *M. smegmatis* on rich agar. Instead, they resembled the Δ*secA2* mutant in exhibiting a minor growth defect on Mueller Hinton agar. As a first step to characterize the suppressors, we tested each for normal SecA2 expression by anti-SecA2 western blot. Of the 11 suppressors we isolated, 9 still expressed full-length SecA2 at normal levels (data not shown).

To determine whether these suppressors were intragenic or extragenic, we prepared genomic DNA from each of the SecA2-expressing suppressors. We then PCR amplified *secA2 K115R* integrated at the chromosomal *attB* site and sequenced the entire gene. One of the suppressor mutations mapped to the *secA2 K115R* allele. One of these intragenic suppressors contained an in-frame, unmarked 8 amino acid deletion of residues 681-688 in the IRA1 subdomain (13, 48). Each of the subdomains of SecA2, including IRA1, will be discussed in more detail below.

The remaining suppressors did not have mutations in *secA2 K115R*; this implies the suppressor mutation is elsewhere in the genome. To confirm that the suppressor mutation is unlinked to the *secA2 K129R* allele, we used a technique to reintroduce a fresh Walker Box mutant *secA2 K129R* allele into the *attB* site (37). In these suppressors, SecA2 K115R is encoded on a plasmid that is integrated into the chromosome based. By electroporating a second copy of this integrating plasmid, the original resident vector (encoding SecA2 K115R) is excised while the incoming vector integrates in the chromosome. This technique allows replacement of the resident *secA2 K115R* allele with a fresh copy of *secA2 K115R*. In all cases we tested, the final resulting strain still exhibited the suppressor phenotype, confirming that the suppressor is indeed extragenic (data not shown). Together, these results demonstrated the feasibility of performing a suppressor screen in *M. smegmatis* to identify intragenic and extragenic suppressors of Walker Box *secA2* mutant alleles.

Comprehensive screen to identify suppressors of the Δ*secA2* mutant rich agar growth defect exacerbated by expression of *secA2 K129R*. To expand our search for suppressors, we isolated suppressors of the exacerbated rich agar growth defect using the *M. smegmatis* Δ*secA2* mutant expressing *M. smegmatis secA2 K129R* from the chromosomal *attB* locus. By choosing this starting strain, we hoped to avoid any possible complications that could arise from mixing bacterial systems as might occur by expressing *M. tuberculosis secA2 K115R* in a *M. smegmatis* Δ*secA2* mutant. Suppressors were isolated by plating serial dilutions of independently grown cultures onto Mueller Hinton agar plates. As above, we also plated dilutions onto 7H10 agar to obtain total CFU counts and calculate how frequently suppressors arose. Again, the frequency of recovering spontaneous suppressors was 10^{-4}. Interestingly, we noticed some of the suppressors had a smaller (S) colony size than other

larger colonies (B). However, in further analysis, both S and B colony types displayed similar sized colonies on rich agar plates. Like the suppressors of *M. tuberculosis secA2 K115R*, the suppressors of *M. smegmatis secA2 K129R* did not exhibit the severe growth defect of the starting strain, and the suppressors again resembled Δ*secA2* null mutants when grown on rich agar plates and when tested for sensitivity to sodium azide.

In total, we collected 55 suppressors, 34 of which expressed full-length SecA2 at normal levels as determined by Western blot (data not shown). As described above, we PCR amplified *secA2* from genomic DNA isolated from each suppressor strain, and sequenced the entire *secA2 K129R* allele in the *attB* locus to determine if the suppressor were intragenic or extragenic. We thus identified 9 suppressors with intragenic mutations (within *secA2 K129R*), and 23 with no mutation in *secA2 K129R*, which presumably represent extragenic suppressors. In the following sections, we describe the identity and analysis of the intragenic suppressors. We have yet to identify the extragenic suppressors, but we outline a potential strategy for doing so.

Intragenic suppressors of SecA2 K129R map to different subdomains of SecA2. The sequenced intragenic suppressor mutations are shown in Table 1. These mutations fall into four subdomains of SecA2: NBD1, IRA1, IRA2, and PPXD (13, 47) (Table 1). With the exception of the IRA2 suppressor, we identified at least two suppressor mutations within any given subdomain. We mapped the location of each intragenic suppressor mutation onto the structural prediction model of SecA2. When viewed as a space-filled image, the NBD1, IRA1, and PPXD mutations all appear to be surface exposed (Figure 3.10B and Figure 3.11). Since these suppressor mutations are restricted to the surface exposed regions of SecA2 K129R, it is possible that the mechanism of suppression is similar. These surface exposed

regions could be important for protein-protein interactions. We discuss the possibility that the intragenic suppressors disrupt protein interactions in more detail below.

To confirm that the suppressor phenotype was due to the sequenced mutation in *secA2 K129R* and not to a second site mutation, we reconstructed a representative suppressor of each subdomain. Genomic DNA was prepared from suppressor mutants and used as templates to PCR amplify the *secA2 K129R* suppressor allele. The resulting PCR products were subcloned into pCR2.1 and sequenced to verify that the *secA*2 gene still contained the *K129R* mutation, as well as the intragenic suppressor mutation. The *secA2 K129R* suppressor allele was then subcloned into pMV306, the same vector originally used to integrate *secA2 K129R* into the *attB* site of the chromosome. The resulting vectors were electroporated into Δ*secA2* mutant *M. smegmatis*, and transformants were tested for rich agar growth defects and sensitivity to sodium azide. For all intragenic suppressors tested, the suppressed phenotype was again seen when reintroduced into the Δ*secA2* mutant strain. All reconstructed suppressor mutants again exhibited a phenotype equivalent to a Δ*secA2* mutant background as opposed to the exacerbated phenotype of the Δ*secA2* mutant expressing *secA2 K129R* (Figure 3.12). This confirmed that the sequenced intragenic mutations are sufficient to account for suppression of the *secA2 K129R* phenotype.

Three intragenic suppressors hit the same amino acids in NBD1 (nucleotide binding domain). NBD1 and IRA2 together form the ATP-binding N-domain (13, 47). In two independent isolates, we identified a deletion of residues 182-185 (STAD) and in another suppressor, a duplication of residues 182-185 (STADSTAD) was found. NBD1 is 261 amino acids in length and it includes the Walker Boxes required for ATP binding. However, it seems highly unlikely these mutants have any impact on ATP binding in SecA2 K129R. The

NBD1 and IRA2 suppressor mutations are not found in the Walker Box motifs, and since the K129R substitution is intact in these suppressors as confirmed by DNA sequencing, we expect the intragenic suppressors remain defective for ATP binding.

Another intragenic suppressor has an amino acid substitution mutation in the IRA2 subdomain where threonine 459 is replaced with isoleucine (T459I). As with the NBD1 mutations, this IRA2 mutation is also far from the ATP binding site of SecA2 and seems unlikely to have any effect on ATP binding. On one helix of the IRA1 subdomain, we found two independent 8 amino acid in-frame deletions of residues 732-739 or 734-741 of *M. smegmatis* SecA2 K129R. With *M. tuberculosis* SecA2 K115R, we found an 8 amino acid in-frame deletion on the other helix composed of residues 681-688. IRA1 forms two helices joined by a loop, and is important for regulating SecA ATPase activity (48). Recently, a crystal structure of a SecY-SecA complex revealed a role for IRA1 in contacting the opening of the SecY pore as well as pre-proteins (15, 50). Finally, we identified two independent mutations in pre-protein binding domain PPXD: a substitution of aspartic acid 326 to histidine (D326H), and an insertion of a glutamic acid residue at position 364.

How do these intragenic suppressors function? Our model to explain the exacerbated dominant negative phenotypes associated with SecA2 K129R is that disrupting the Walker Box motif renders SecA2 catalytically "dead" (Figure 3.13). However, this "dead" protein can still interact with its normal binding partners, including some factors that are part of an essential process. By titrating essential factors in non-functional complexes, the "dead" SecA2 K129R molecules could produce the exacerbated and dosage-dependent phenotypes we observed. We hypothesize that surface exposed intragenic suppressor mutations might disrupt protein interaction domains. In this case, SecA2 K129R is still "dead" because the

Walker Box is still mutated, but SecA2 K129R is no longer able to form non-functional complexes. Such suppressors would effectively lack functional SecA2 protein. The resulting phenotype of the suppressor then should resemble that of a Δ*secA2* null mutant; indeed, all of the intragenic suppressors we isolated phenotypically resemble a Δ*secA2* mutant. Mutations that decrease the structural stability of SecA2 K129R could also lead to the suppressor phenotype. However, all of our intragenic suppressors expressed SecA2 K129R normally with no evidence of protein degradation, which makes this explanation seem unlikely (data not shown). One other possibility is that the suppressor mutations restore ATP binding to SecA2 K129R. However, since the K129R substitution is maintained in each suppressor, and none of the suppressor mutations map in the immediate vicinity of the Walker box, this mechanism of suppression also seems an unlikely explanation.

Localization of SecA1 and SecA2 differs in the intragenic suppressors. Using our subcellular fractionation scheme, we determined the localization of SecA1 and SecA2 in the intragenic suppressor strains. We determined previously that SecA1 is equally distributed between soluble and cell envelope fractions, wild-type SecA2 is predominantly cytoplasmic, and SecA2 K129R is found predominantly in the cell envelope fraction. Earlier, we proposed that the altered localization of SecA2 K129R is linked to the dominant negative phenotype. Based on the model we described above, if the intragenic suppressors disrupt interaction of SecA2 K129R with a membrane-associated protein, we predicted localization of SecA2 would change in the suppressor strains. This turns out to be the case. Figure 3.14A shows that representative intragenic SecA2 K129R suppressors in IRA1, IRA2, and PPXD changed the localization of SecA2 K129R from primarily being membrane-associated to being enriched in the soluble fraction. The localization change is not as dramatic in the

NBD1 suppressor, but there is still a significant shift to the soluble fraction. The fact that suppressors of the *secA2 K129R* phenotype result in restoration of the normal SecA2 localization profile provides strong support for our proposal that the entrapment of SecA2 K129R at the membrane is critical to the dominant negative phenotype. It is also consistent with our proposal that the intragenic suppressors we collected alleviate the growth defect of *secA2 K129R* allele by eliminating protein interactions with a membrane-associated complex.

We also looked at the localization of SecA1 in the intragenic suppressor strains (Figure 3.14B). In the IRA1 suppressor, SecA1 distribution was unaltered. It remains equally divided between cell envelope and cytoplasm. However, in the IRA2 and NBD1 suppressors, the localization of SecA1 skewed toward the envelope fraction. In the PPXD suppressor, the localization of SecA1 was skewed toward the soluble fraction. There is no obvious explanation for the different effects the individual suppressors have on SecA1 localization; however, this result was consistently reproduced when fractions were made from independent experiments.

Intragenic suppressor mutations reveal residues important for SecA2 function. The residues mutated in our intragenic suppressors are not reported in any of the prior extensive mutational analyses of SecA proteins in other bacteria (5, 6, 16, 25, 26, 28, 48). However, ours is the first study directed at finding suppressors of a Walker Box mutant of any *secA*. It is also the first suppressor analysis of any *secA2* mutant. We propose that the intragenic suppressor mutations we identified were able to suppress the phenotype associated with SecA2 K129R because they prevent SecA2 K129R from interacting with an essential SecA2-interacting protein complex. If true, then these suppressor mutations would also be expected to disrupt wild-type SecA2 function even in the absence of the K129R substitution.

To test this prediction, we used site-directed mutagenesis to restore the Walker Box of three representative SecA2 K129R intragenic suppressors (IRA1 Δ734-741, IRA2 T459I, and NBD1 STADSTAD) back to the wild-type lysine residue. The result was three new *secA2* mutant alleles, all of which have an intact Walker box capable of binding ATP. The resulting plasmids were then electroporated into wild-type and Δ*secA2* mutant *M. smegmatis*. Each mutant was then tested for Δ*secA2* mutant phenotypes. All three of these mutant alleles failed to complement the azide hypersensitivity (Figure 3.15A) or the rich agar growth defect (Figure 3.15B and C) of a Δ*secA2* mutant. Expression of these allele in wild-type *M. smegmatis* had no effect. Each protein was expressed normally as determined by anti-SecA2 immunoblot (data not shown). From these results, we conclude that the intragenic suppressor mutations generate recessive null alleles of *secA2*. These results support the idea that intragenic suppressors of *secA2 K129R* alleviate the exacerbated plate phenotypes by rendering SecA2 K129R nonfunctional, probably by disrupting interactions between SecA2 K129R and factors involved in the SecA2 export pathway.

Efforts to identify extragenic suppressors of *secA2 K129R*. After sequencing *secA2 K129R*, 23 suspected extragenic suppressors of the rich agar growth defect remained. Cloning the suppressor by standard molecular biology protocols has proved technically challenging and, to date, we have not identified the genetic locus of these extragenic suppressors. Because we consider it a possibility that SecA2 interacts with components of the essential Sec pathway, we considered *secA1* and *secY* attractive candidates for being the site of extragenic suppressor mutations. However, direct sequencing showed no mutations mapped to these two genes in the extragenic suppressors (data not shown).

Currently, we are constructing genomic libraries to clone the suppressor by complementation. The library needed depends on the nature of the suppressor mutation. The first scenario is if the suppressor is recessive. In this case, a merodiploid that contains the suppressor mutant allele and the parental wild-type allele will exhibit the severe rich agar growth defect of the starting Δ*secA2*/*secA2 K129R* strain. A library made from wild-type genomic DNA will be electroporated into the suppressor strain. The resulting transformants will be plated onto 7H10 minimal agar plates. Next, we will screen the transformants for the severe growth defect on Mueller Hinton rich agar by patching. Patches that grow poorly on rich agar should contain a vector carrying the wild-type parental suppressor allele.

If the suppressor is dominant, however, the library needs to be constructed from each suppressor and transformed into the starting strain where the suppressors were isolated: the *M. smegmatis* Δ*secA2* mutant expressing *secA2 K129R*. In this case, the resulting transformants will be screened by patching to identify clones without the severe rich agar growth defect. Said another way, vectors that carry a dominant suppressor allele will alleviate the rich agar growth defect associated with *secA2 K129R*. Not knowing whether the suppressors are dominant or recessive is a complicating factor. Another problem is the rate at which we initially obtained the suppressors. The observed frequency of the spontaneous rich agar suppressors is 10^{-4}, which precludes the use of libraries with small DNA inserts. Consequently, we want to use cosmid libraries in this project. In addition, we wanted to use a single-copy cosmid library to avoid artifacts associated with multicopy suppression. Since the *attB* site is occupied by *secA2 K129R* in all our suppressors, we could not use the existing integrating *M. smegmatis* genomic libraries. Recently, we obtained a new cosmid vector that integrates elsewhere in the mycobacterial chromosome to use in construction of new *M.*

smegmatis libraries for this project (gift from Dr. William Jacobs, Albert Einstein College of Medicine).

Localization of SecA1 and SecA2 differs in the extragenic suppressors. As with the intragenic suppressors, we determined the localization profile of SecA1 and SecA2 in two candidate extragenic suppressors. We chose two suppressors, 7S and 20B, which were isolated from independently grown cultures as having small and large colonies, respectively. Like all of the isolated suppressors, 7S and 20B suppress the rich agar growth defect to the level of a Δ*secA2* mutant. As observed with the intragenic suppressors, we found SecA2 K129R to be released from the envelope fraction in extragenic suppressors 7S and 20B (Figure 3.16A). This finding supports the idea that the extragenic suppressors alleviate the toxic phenotype of SecA2 K129R by eliminating the interaction between SecA2 K129R and the membrane-associated complex. One way this could occur is if the 7S and 20B suppressors have mutations in components of the membrane-associated complex that prevent interaction with SecA2. For this reason, we believe the extragenic suppressors have great potential to identify proteins that work with SecA2 in export.

We also examined the localization of SecA1 in 7S and 20B. In 7S, SecA1 was slightly more abundant in the envelope fraction; in wild-type *M. smegmatis*, SecA1 is evenly split between the envelope and the soluble fraction. Strikingly, in 20B, SecA1 was found predominantly in the soluble fraction (Figure 3.16B). The drastic change in localization pattern of SecA1 in 20B would be consistent with a suppressor mutation that maps to *secA1*. However, direct sequencing of *secA1* in 20B showed this is not the case. It is possible that the suppressor mutation is in another component of the Sec pathway and that this mutation alters the subcellular localization of SecA1.

Msmeg1712-HA is not exported to the cell wall in extragenic suppressor 7B and 20S. In an effort to link our extragenic suppressors to export of SecA2-dependent protein export, we examined the ability of two extragenic suppressors to export the Msmeg1712. As shown previously, Msmeg1712 is exported to the cell wall of *M. smegmatis* in a SecA2-dependent manner (20). We transformed 7S and 20B with pNR35 which expresses Msmeg1712 tagged at the C-terminus with the HA epitope. The resulting transformants were grown in Mueller Hinton broth and the cultures were fractionated as described above. As shown in Figure 3.17, Msmeg1712-HA is exported to the cell wall only when wild-type SecA2 is present. When SecA2 K129R is present, Msmeg1712-HA fails to reach the cell wall and accumulates in the cytoplasm. In suppressor 7S and 20B, Msmeg1712-HA is similarly retained in the cytoplasm. This indicates that the extragenic suppressor mutations in these two strains do not allow Msmeg1712-HA to bypass the requirement of SecA2 for export. It also confirms our other data indicating that the extragenic suppressors behave like Δ*secA2* null mutations.

Discussion

In this study, we showed that the Walker Box motif is required for normal SecA2 function. We exhaustively tested this by using different assays in both *M. tuberculosis* and *M. smegmatis*, and our results all lead to the same conclusion – SecA2 must bind ATP to function normally. We also showed that mutations in the SecA2 Walker box region are dominant negative and change the normal subcellular localization of SecA2 from the cytoplasm to the membrane. Dominant negative alleles can be extremely useful in identifying proteins that work together in a biological pathway. Using a dominant negative *secA2 K129R* allele, we were able to isolate spontaneous suppressor mutations. We believe

our collection of intragenic and extragenic suppressors have great potential to uncover functional domains of SecA2 and identify proteins that work with SecA2 in protein export.

SecA2 must bind ATP to function *in vivo*. Our other studies showed that SecA1 and SecA2 of *M. tuberculosis* are functional ATPases in vitro (Chapter 4) (24). Here we tested the biological significance of ATP binding by evaluating mutants with an amino acid substitution in the ATP binding site of SecA2. Mutations in the Walker A (K108R) and Walker B (D209N) motifs of *E. coli* SecA destroy ATP binding and render SecA nonfunctional in a variety of in vitro and in vivo assays (27, 35). In *E. coli* SecA, these Walker Box mutations also alter the subcellular localization of SecA by preventing its dissociation from the membrane as a consequence of failing to cycle between ATP binding and hydrolysis. Here, we constructed the corresponding Walker Box mutations in *M. tuberculosis* (K115R and D216N) and *M. smegmatis* (K129R) SecA2 and tested each for function in vivo. This is the first study of ATPase activity in any accessory SecA2 protein.

The K115R Walker Box substitution prevented SecA2 from carrying out its function in promoting growth of *M. tuberculosis* in macrophages. The same K115R mutation also failed to complement the smooth colony morphology phenotype of the Δ*secA2* mutant of *M. tuberculosis* (30). These two results indicated that normal SecA2 function in *M. tuberculosis* requires ATP binding. While the explanation for the intracellular growth defect and smooth colony morphology of the Δ*secA2* mutant is not yet known, we believe both phenotypes result from the inability of the Δ*secA2* mutant to export proteins that rely on the accessory SecA2 system. In regards to the altered colony morphology of the Δ*secA2* mutant, often such changes are due to alterations to the cell surface. This could arise from the Δ*secA2*

mutant failing to export cell wall biosynthetic enzymes. Interestingly, a Δ*secA2* mutant of *Listeria monocytogenes* also has altered colony morphology (33).

We also directly tested the ability of SecA2 K129R to support protein export in *M. smegmatis*. Previous studies in our laboratory identified Msmeg1704 and Msmeg1712 as *M. smegmatis* lipoproteins that require SecA2 for export from the cytoplasm to the cell wall (20). We found SecA2 K129R was incapable of exporting these two SecA2-dependent lipoproteins. This result directly demonstrates the requirement for the ATPase activity of SecA2 in promoting export of its select substrates.

Walker Box mutant *secA2* alleles are dominant negative. Early on we were surprised to discover Walker Box mutant alleles were not only non-functional, as shown by the failure to complement Δ*secA2* mutant phenotypes, but in several assays the phenotypes associated with the Walker Box *secA2* alleles were actually worse than that of a Δ*secA2* null mutant. This type of result is common for dominant negative mutations (10, 40, 43, 46). In other experiments, we noticed expression of mutant Walker Box *secA2* alleles in wild-type mycobacteria resulted in dominant negative Δ*secA2* mutant phenotypes. Expression of Walker Box mutant *secA2* alleles (*secA2 K115R*, *secA2 D215N*, and *secA2 K129R*) in wild-type *M. tuberculosis* resulted in smooth colonies like those exhibited by the Δ*secA2* mutant. Expression of *M. smegmatis* SecA2 K129R in wild-type *M. smegmatis* also resulted in dominant negative phenotypes as assessed by rich agar plating and azide sensitivity. Often, dominant negative mutations exhibit dosage dependent effects (2). By using multicopy and inducible *secA2 K129R* alleles expressed in wild-type *M. smegmatis*, we observed that severity of the phenotypes associated with SecA2 K129R was indeed dosage dependent.

Together our data convincingly show substitutions in the Walker Box of SecA2 to be dominant negative.

There is a precedent for Walker Box mutations being dominant negative. As mentioned above, the *E. coli* SecA K108R mutant was instrumental in demonstrating the importance of ATPase activity for the canonical SecA (35). SecA K108R inhibits proOmpA export in vitro in the presence of wild-type SecA. While not specifically mentioned, this phenotype is consistent with a dominant negative. The DotB ATPase is part of a Type IV secretion system of *Legionella pneumophila* that is required for growth of this pathogen in host cells (43). A DotB K162Q Walker Box mutant fails to support bacterial growth of a Δ*dotB* mutant inside host cells. Furthermore, a *dotB*/*dotB K162Q* merodiploid fails to grow intracellularly. DotB K162Q forms mixed multimers with wild-type DotB, effectively disrupting the Type IV secretion system. Finally, Walker box mutants of the PilQ subunit of *E. coli* type IV pili are also dominant negative (40). In the presence of wild-type PilQ, PilQ K238Q inhibits pilus formation. These diverse studies demonstrate the ability of Walker Box mutations to poison the processes with which they are involved.

Figure 3.13 shows a model to explain the phenotypes associated with SecA2 K129R. In a wild-type cell, SecA2 interacts with a membrane-embedded translocase to promote its export of a select subset of proteins. Because expression of SecA2 K129R causes phenotypes more severe than when there is no SecA2 present and because high level expression of SecA2 K129R prevents growth, we propose that SecA2 K129R interferes with an essential process. For this reason, we consider components of the essential Sec pathway to be attractive candidates for SecA2-interactors tied up by SecA2 K129R. The primary function of SecA2 may be to deliver proteins to the translocase. Such a role would help

explain the largely cytosolic location of wild-type SecA2, in comparison to SecA1. Alternatively, the role of SecA2 may be to provide energy to drive a select subset of proteins through the membrane-embedded translocase. In either case, when the Walker Box of SecA2 is mutated, SecA2 K129R is no longer able to bind and hydrolyze ATP like wild-type SecA2 (24). Accordingly, SecA2 K129R would be unable to undergo conformational changes associated with ATP hydrolysis. As a result, SecA2 K129R could become irreversibly locked to a component of the SecYEG translocon. This proposal is consistent with our observation of altered SecA2 K129R localization. Since the canonical Sec pathway is essential, locking SecA2 K129R to the translocase would effectively poison the cell by disrupting export of other proteins via the general Sec pathway.

The azide hypersensitivity phenotype further supports the possibility of an interaction between SecA2 and the essential Sec pathway (8). Azide is an inhibitor of ATPases, and in mycobacteria, SecA1 is the major target of azide (18). The azide hypersensitivity associated with the *M. smegmatis* Δ*secA2* mutant could reflect a role for SecA2 in assisting SecA1 to export essential proteins. Furthermore, inhibiting SecA1 by azide treatment coupled with expression of SecA2 K129R would then greatly impair the essential Sec pathway. In fact, the exacerbated azide hypersensitivity observed in a *secA2 K129R* strain supports this model.

In the model above, we propose the toxicity associated with expression of SecA2 K129R, is due to formation of non-functional complexes between SecA2 and components of the essential Sec translocase. However, it is also possible that SecA2 interacts with an unidentified membrane-embedded translocase to export pre-proteins out of the cytoplasm. Based on the exacerbated phenotypes associated with SecA2 K129R, we believe that this

mystery translocase would have to either export essential factors or itself be essential for some other reason.

Spontaneous suppressors of *secA2 129R* arise frequently. Isolating suppressors of secretion defects in *E. coli* led to the discovery of several components of the essential Sec pathway (6, 16, 25). We hypothesized a similar approach would be useful for identifying components of the accessory SecA2 pathway in *M. smegmatis*. Although numerous suppressors of various protein export defects in *E. coli* have been studied in the past, suppressors of Walker Box mutant *secA* alleles have never before been collected. Such a screen would be possible because the *E. coli* SecA K108R Walker box mutant displays a dominant negative phenotype (35). Adding SecA K108R inhibits translocation of proOmpA in vitro in the presence of wild-type SecA.

Intragenic suppressors. We identified seven different intragenic suppressors of *M. smegmatis secA2 K129R* and one intragenic suppressor of *M. tuberculosis secA2 K115R*. All of the intragenic suppressors behaved like Δ*secA2* null mutations in several different assays. With the model we proposed (Figure 13), it follows that a suppressor mutation that prevents binding between SecA2 K129R and a SecA2-interacting protein would be expected to behave like a null mutations. Indeed, when the intragenic suppressors were retested in the context of a corrected (wild-type) Walker box, they behaved like nulls. We grouped the possible mechanisms of suppression into three broad categories: 1) disrupting protein interaction domains, 2) destabilizing the structural integrity of SecA2 K129R, and 3) restoring ATPase activity. Although more experiments are required to determine the mechanistic basis of suppression, we think the most likely explanation is that the intragenic suppressor mutations disrupt protein interaction domains, thereby preventing SecA2 K129R

from interfering with an essential membrane-associated complex. All suppressors expressed equal levels of SecA2 K129R protein as the starting mutant strain and there was no evidence of protein degradation (data not shown). Moreover, since each suppressor still contains the K129R substitution, and none of the suppressors map directly to the Walker Box amino acids, we find it unlikely ATP binding and ATPase activity could be restored in the intragenic suppressors.

As stated earlier, the exact mutations we identified as intragenic suppressors have not previously been hit in any of the prior extensive mutational analyses conducted on *E. coli* SecA (16, 25, 28, 48). The resulting amino acid substitutions do map to structurally and functionally defined domains of *E. coli* SecA (13, 47). However, it is important to emphasize that all these domains are large regions that remain to be fully characterized and might possess functions in addition to those currently known. Furthermore, there could be novel functions associated with these domains in accessory SecA2 proteins. Therefore, caution is required when attempting to interpret the significance of the intragenic suppressors of SecA2 K129R uncovered in our studies.

We mapped each suppressor mutation onto a structural homology model of SecA2. Ideally, we hope to identify suppressor mutations in protein interaction domains of SecA2. Unfortunately, the domains of SecA2 that are most relevant to our model of how the intragenic suppressors might function (i.e. those domains that participate in or stabilize SecYEG interactions) are only just starting to be defined even for *E. coli* SecA.

The intragenic suppressor mutations we identified have not been picked up in any of the prior extensive studies of *E. coli* SecA. Most of the intragenic suppressor mutations we identified were surface exposed and thus positioned at sites that could possibly participate in

interactions with other proteins. Two intragenic mutations mapped to the PPXD subdomain, which in *E. coli* is important for interaction with pre-protein substrates (36). Two substructures within PPXD, Stem and Bulb, are involved in binding the signal sequence and the mature domain of a pre-protein substrate (36). However, it is important to emphasize that this has only been shown for one native SecA substrate, proOmpA. The D326H mutation we discovered in SecA2 is just 3 amino acids upstream of two residues important for normal *E. coli* SecA function: Y326 and I327 (Figure 3.11) (28, 29). Site-directed mutagenesis studies have shown that Y326 is critical for *E. coli* SecA to interact with pre-protein substrates, but not for interactions with SecYEG or the membrane. The affinity of SecA I327S for pre-proteins was also predicted to be reduced.

Elsewhere in PPXD, we obtained an insertion mutation at residue 364 of SecA2. This mutation is near the *E. coli secA* suppressor allele *prlD5* (A373V). The *prlD5* mutant was isolated as a suppressor that rescued export of a mutant maltose-binding protein with a defective signal sequence (16). The *prlD5* suppressor mutation reveals a role for the PPXD domain of SecA in pre-protein interaction. Interestingly, NMR structural studies showed that residue A373 is found on the surface of the Stem substructure (36). We have not tested the affinity of our PPXD mutants for pre-proteins, but such an experiment could help us understand the function of the PPXD domain in SecA2.

One explanation for how the PPXD suppressors alleviate the phenotypes associated with SecA2 K129R is that the mutations in PPXD prevent interactions between SecA2 K129R and the membrane-embedded translocase. A recent crystal structure of a SecA-SecY complex from *Thermotoga maritima* shows that PPXD makes substantial contacts with SecY (50). In fact, the two IRA1 helices are inserted into the entrance of the SecY channel.

Crosslinking studies show that IRA1 can also interact with pre-proteins and feed them into the translocase (15). This suggests a plausible mechanism for how mutations in IRA1 can suppress the SecA2 K129R mutant. Since IRA1 is important for SecA-SecY interactions, mutations in IRA1 could prevent occupation of the SecY channel by SecA2 K129R. Mutations in IRA1 could also prevent SecA2 K129R from binding and sequestering essential pre-proteins substrates. It will be important to identify the translocase channel used by SecA2 to distinguish between these possible models. Furthermore, some mutations in SecA PPXD prevent stable interactions with SecY.

In *E. coli* SecA, the IRA1 subdomain is important for coupling ATPase activity to the process of protein translocation (48). Directed mutagenesis studies of the SecA IRA1 domain have helped to dissect the regulatory functions of this subdomain (Figure 3.11). One of these mutants, F811A, is found in the region where we found a deletion in SecA2 IRA1; in *E. coli*, *secA F811A* was able to complement a temperature sensitive *secA* mutant, although not as efficiently as wild-type *secA* and when tested for *in vitro* translocation SecA F811A protein exhibits a protein export defect.

Another explanation for how the *secA2 K129R* suppressors in PPXD could function is that one role of SecA2 is to assist SecA1 to export a subset of proteins needed for normal growth on rich agar. In the absence of SecA2, export of such proteins is still possible, but much less efficient. The result would be the rich agar growth defect observed with the Δ*secA2* mutant. Perhaps SecA2 K129R irreversibly binds to these pre-proteins and delivers them to the translocase, but by failing to release them as a result of the ATPase defect, prevents their export altogether. This would lead to the exacerbated rich agar growth defect that is characteristic of the Δ*secA2* mutant expressing *secA2 K129R*. If mutations in the

PPXD of SecA2 relax interactions with pre-proteins, perhaps the pre-proteins can now be exported as in a Δ*secA2* mutant. Regardless of the mechanism, the phenotype of a SecA2 K129R suppressor in PPXD is equivalent to a Δ*secA2* mutant.

Some of the SecA2 K129R suppressor mutations are in close proximity to characterized SecA mutations. For example, temperature-sensitive mutations in SecA NBD1 have been identified as A169D and Y170D. When grown at the non-permissive temperature, both *secA A169D* and *secA Y170D* mutants have a defect in exporting OmpA and MalE proteins (28, 42). Both substitutions result in amino acids with significantly different properties, and are predicted to destabilize SecA structure. It follows then that both of these SecA proteins cannot support protein export at high temperatures.

According to our proposed model, the intragenic suppressor mutations we identified suppress SecA2 K129R by rendering it inactive. If this is true, when the Walker Box mutation is repaired by changing residue 129 back to lysine, the suppressor mutations should still be nonfunctional. We performed this experiment on intragenic suppressor alleles from three different subdomains (NBD1, IRA1, and IRA2). As expected, the resulting alleles could not complement the rich agar growth defect and azide hypersensitivity phenotype displayed by the Δ*secA2* mutant. In addition, these alleles were recessive to wild-type *secA2*, as shown by the absence of any effect on wild-type *M. smegmatis*. This analysis allowed us to identify new *secA2* null alleles, and demonstrate the importance of each subdomain for normal SecA2 function in vivo.

Extragenic suppressors. Suppressor analysis has a long history of being used to identify interacting proteins and proteins that work together in a pathway. Notably, suppressor analysis has been used in studies of the *E. coli* Sec pathway(5, 6, 45). In *E. coli*,

prl alleles are dominant suppressors that were identified because they allow export of proteins with defective signal sequences. The Prl mutations map to components of the canonical Sec pathway including SecA (PrlD), SecY (PrlA), and SecE (PrlG), and are thought to support protein export because of a diminished specificity for aberrant signal sequences. Combinations of some *prlA* and *prlG* alleles lead to synthetic phenotypes; these allele-specific phenotypes are indicative of contact sites between SecY and SecE (3). We hope that by identifying extragenic suppressors of SecA2 K129R, it will similarly help identify components involved in SecA2-mediated protein export.

Both the intragenic and extragenic suppressors we identified exhibited the remarkable property of shifting the localization of SecA2 K129R away from the cell envelope to a predominantly soluble location, like that exhibited by wild-type SecA2. We believe this result to have functional significance. First, it supports our proposal that the membrane localization of SecA2 K129R is relevant to the dominant negative phenotype. Second, it argues strongly for the suppressor mutations acting by alleviating non-functional interactions with a membrane complex. Particularly, for the extragenic suppressors 7S and 20B the shift in SecA2 K129R localization suggests that the mutation in these strains eliminates an interaction with a component of the SecA2 protein export pathway as opposed to some indirect effect alleviating the dominant negative phenotype. As stated previously, one possibility is that the suppressor mutations in 7S and 20B are themselves in a component of the membrane-embedded translocase. Although it is not obvious why the intragenic and extragenic suppressors of SecA2 K129R alter the localization of SecA1, it is suggestive of a relationship between SecA2 and SecA1 and/or other components of the general Sec pathway. For these reasons, we are particularly interested in identifying the mutations in these strains.

We are currently using two approaches to locate the suppressor lesion in 7S and 20B by screening cosmid libraries (described in Results) and by sequencing the genome of the suppressor strains.

Summary. This study represents an important step in understanding how the accessory SecA2 protein export system of mycobacteria functions. By mutating the Walker Box of SecA2, we showed that this ATP binding motif is absolutely required for normal SecA2 function in both *M. tuberculosis* and *M. smegmatis*. We took advantage of the dominant negative properties of the Walker Box mutant SecA2 protein as a tool to study protein export. The shifted localization of SecA2 K129R from the cytosol to the cell envelope reveals a role for SecA2 at the membrane. Presumably, SecA2 associates with the membrane during delivery of pre-proteins to a membrane-embedded translocase. Such an association, particularly if the membrane-embedded translocase is essential like SecYEG, could also explain the exacerbated phenotypes associated with expression of SecA2 K129R in a Δ*secA2* mutant. We collected intragenic and extragenic suppressors of the exacerbated rich agar growth defect. Our analysis of the intragenic suppressors identified several key regions that are required for normal SecA2 function. While we have not yet identified any of the extragenic suppressors, we believe that doing so has the potential to reveal SecA2-interacting proteins. It is our hope that a thorough understanding of all the factors involved in SecA2-mediated protein export will aid in the identification of new targets for anti-mycobacterial therapies.

Acknowledgements

We would like to acknowledge Dr. Sabine Ehrt for providing the Tet ON system, Dr. Martin Pavelka for providing pMP715, and Sherry Kurtz for sharing her unpublished data. We also thank Henry Gibbons for help optimizing the subcellular fractionation assays. This work was supported by an award to Miriam Braunstein from NIAID (AI53530-01).

Attributions

The experiments in this work were performed by me with the following exception. Jessica R. McCann performed the macrophage infections.

Table 3.1. Intragenic suppressors of *M. smegmatis secA2 K129R* and *M. tuberculosis secA2 K115R*

isolate number	subdomain location	sequence details
6S, 9S	NBD1	deletion of residues 182-185
23S*	NBD1	duplication of residues 182-185
34S*	IRA2	Thr 459 → Ile
2S	PPXD	Asp 326 → His
25S*	PPXD	Glu insertion at residue 364
21B*	IRA1	deletion of residues 734-741
38S	IRA1	deletion of residues 732-739
SSW[†]	IRA1	deletion of residues 681-688

* indicates suppressors were subcloned and retested in *M. smegmatis*.
[†] indicates suppressor of M. *tuberculosis secA2 K115R*.

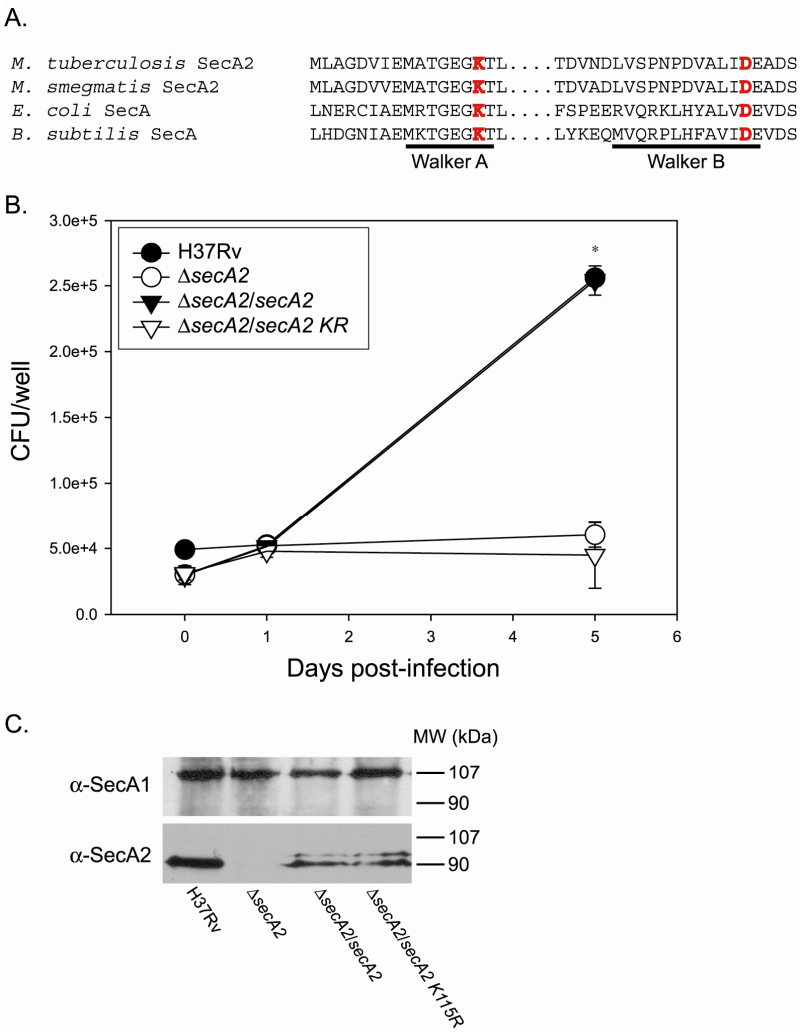

Figure 3.1. The *M. tuberculosis secA2 (K115R)* allele does not complement the *M. tuberculosis* Δ*secA2* mutant phenotype in macrophages. **(A)** ClustalW sequence alignment of mycobacterial SecA2 proteins with *E. coli* and *B. subtilis* SecA. The ATP-binding Walker A lysine and Walker B aspartic acid are indicated in red. **(B)** Murine bone marrow-derived macrophages were infected at a MOI of 1.0 with H37Rv, Δ*secA2* mutant, Δ*secA2* mutant complemented with wild-type *secA2, (*Δ*secA2*/*secA2*), and Δ*secA2* mutant complemented with *secA2 K115R* (Δ*secA2*/*secA2 KR*). CFU were determined by plating macrophage lysates at various times post-infection. The infection was performed with triplicate wells for each strain per time point, and the error bars represent means +/- standard deviations. Symbols for H37Rv and the complemented strain are overlapping in most time points in the graph presented. Data are representative of three independent experiments. *, (P<0.05). (**C**) Expression of SecA1 and SecA2 in strains with different *secA2* alleles. Equal amounts of formalin-fixed whole-cell lysates of the wild-type H37Rv, Δ*secA2* mutant, Δ*secA2* mutant complemented with wild-type *secA2*, and Δ*secA2* mutant complemented with *secA2 K115R* were run on SDS-polyacrylamide gels and subjected to Western blot analysis with anti-SecA1 or anti-SecA2 antibodies. The top panel shows SecA1 protein and the lower panel shows SecA2 protein. MW, molecular weight (in thousands).

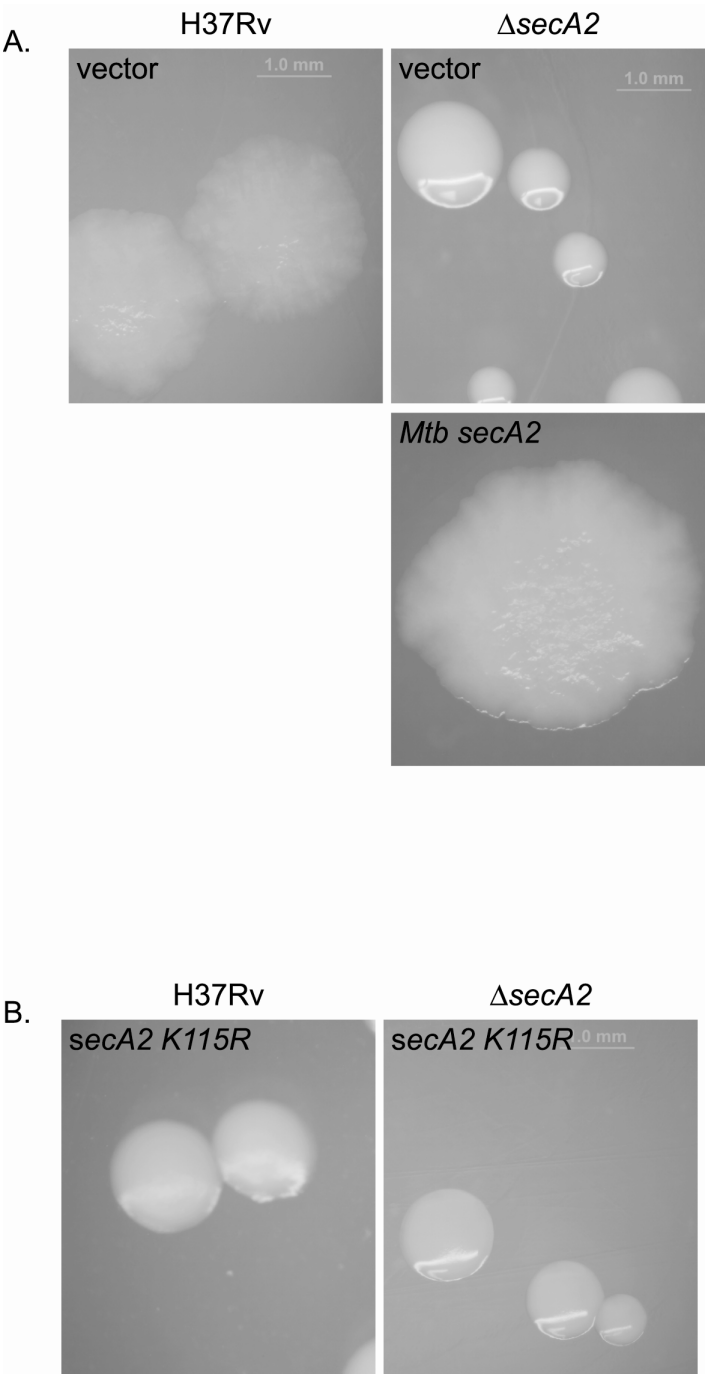

Figure 3.2. SecA2 Walker box mutants fail to restore the rough colony morphology in Δ*secA2 M. tuberculosis*. **(A)** Wild-type (H37Rv) and Δ*secA2* mutant (mc^{2}3112) *M. tuberculosis* strains were transformed with empty vector or wild-type *M. tuberculosis secA2* (*Mtb secA2*). **(B)** Wild-type and Δ*secA2 M. tuberculosis* strains were transformed with a Walker Box mutant *secA2* allele from *M. tuberculosis* (*secA2 K115R*). All transformants were plated onto 7H10 agar plates containing 0.05% Tween 80. Images of individual colonies were obtained after 3 weeks of growth at 37˚C. Scale bar = 1.0 mm.

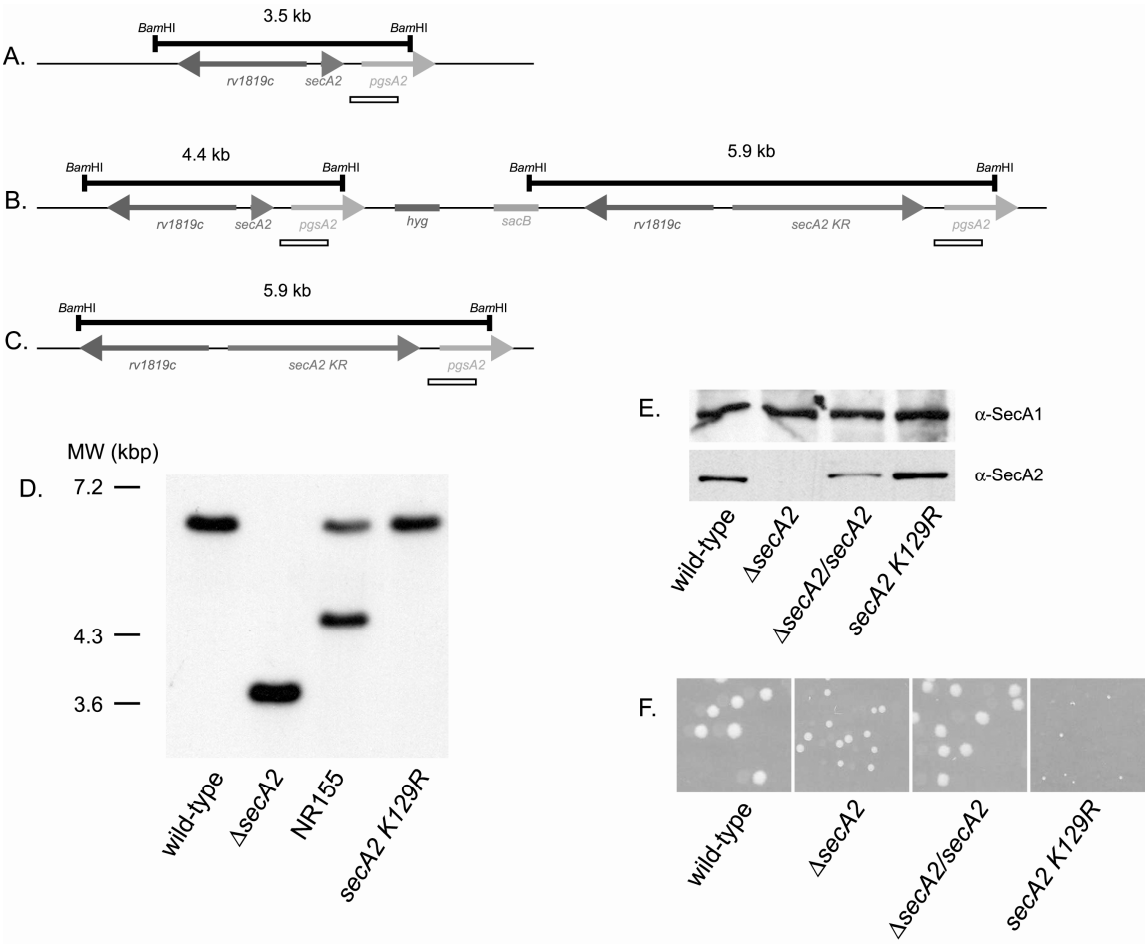

Figure 3.3. Construction and analysis of a *M. smegmatis* strain expressing *secA2 K129R* from the native chromosomal locus. Diagram of the *secA2* locus in a **(A)** Δ*secA2* mutant NR116, **(B)** single crossover strain NR155, and **(C)** *secA2 K129R* strain NR179. Bars below the line indicate where the probe hybridizes. **(D)** Southern blot of *Bam*HI digested genomic DNA. *Bam*HI restriction sites and predicted fragment sizes are indicated in A-C. Lanes 1: mc^2155, 2: NR116, 3: NR155, 4: NR179. **(E)** Whole-cell lysates were prepared from each strain and equal amounts of protein were analyzed by SDS-PAGE and immunoblot with anti-SecA1 and anti-SecA2 antibodies. NR172, Δ*secA2* mutant complemented with wild-type *secA2* encoded by pYA810. **(F)** *M. smegmatis* strains were plated on Mueller Hinton agar plates. Images were taken after 4 days of growth at 37˚C.

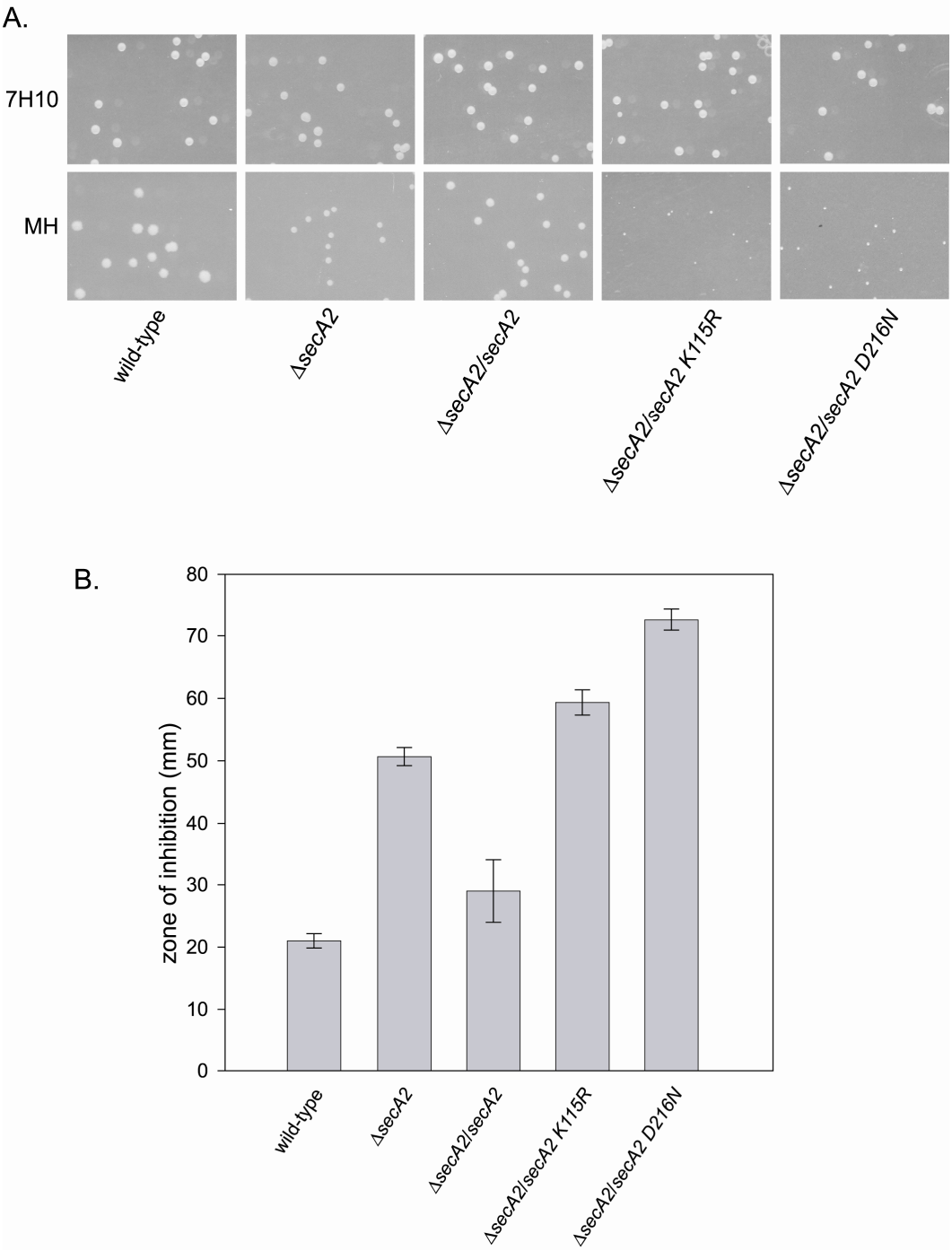

Figure 3.4. ***M. tuberculosis*** **Walker Box mutant** ***secA2 K115R*** **allele fails to complement and exacerbates the** ***M. smegmatis*** **Δ*secA2* mutant rich agar growth defect and azide hypersensitivity. (A)** *M. smegmatis* strains were transformed with empty vector, wild-type *secA2* (*secA2*), or *M. tuberculosis* Walker Box mutant *secA2* (*secA2 KR*) plasmids and plated on 7H10 (top row) and Mueller Hinton (bottom row) plates and incubated at 37˚C. **(B)** Sensitivity to sodium azide was measured as described in Methods. Each strain was tested in triplicate. Error bars represent standard error of the mean.

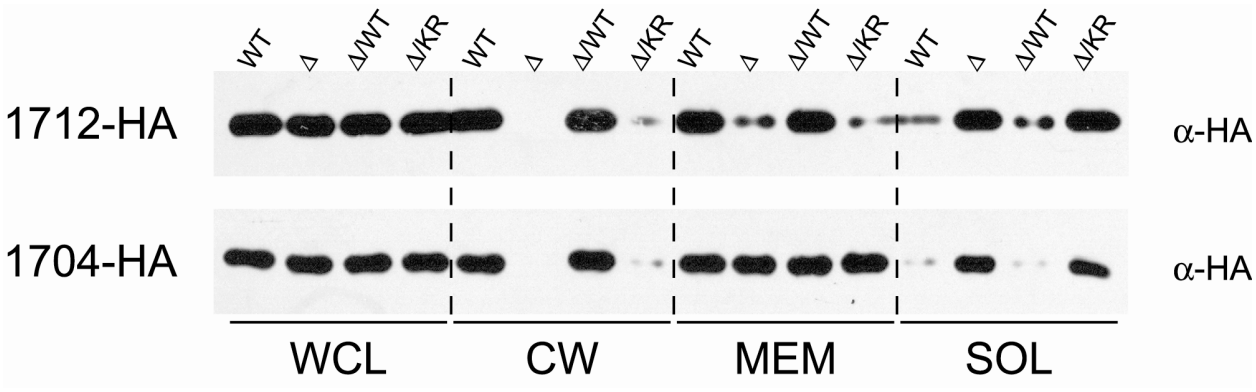

Figure 3.5. *M. smegmatis* SecA2 K129R is unable to support the export of Msmeg1704-HA and Msmeg1712-HA. *M. smegmatis* strains carrying empty vector, wild-type *secA2* (*secA2*), or *M. smegmatis* Walker Box mutant *secA2* (*secA2 K129R*) plasmids were transformed with vectors expressing HA-tagged Msmeg1704 or Msmeg1712. Each strain was grown in 100 ml cultures of Mueller Hinton, harvested, lysed in a French pressure cell, and then fractionated into cell wall (CW), membrane (MEM), and soluble (SOL) fractions. Protein derived from an equal number of starting cells was analyzed by SDS-PAGE and immunoblot using anti-HA antibodies (Covance). Goat anti-mouse secondary antibodies (Biorad) were used according to the manufacturer. Blots were developed using ECL (Perkin Elmer) and visualized on film (Denville).

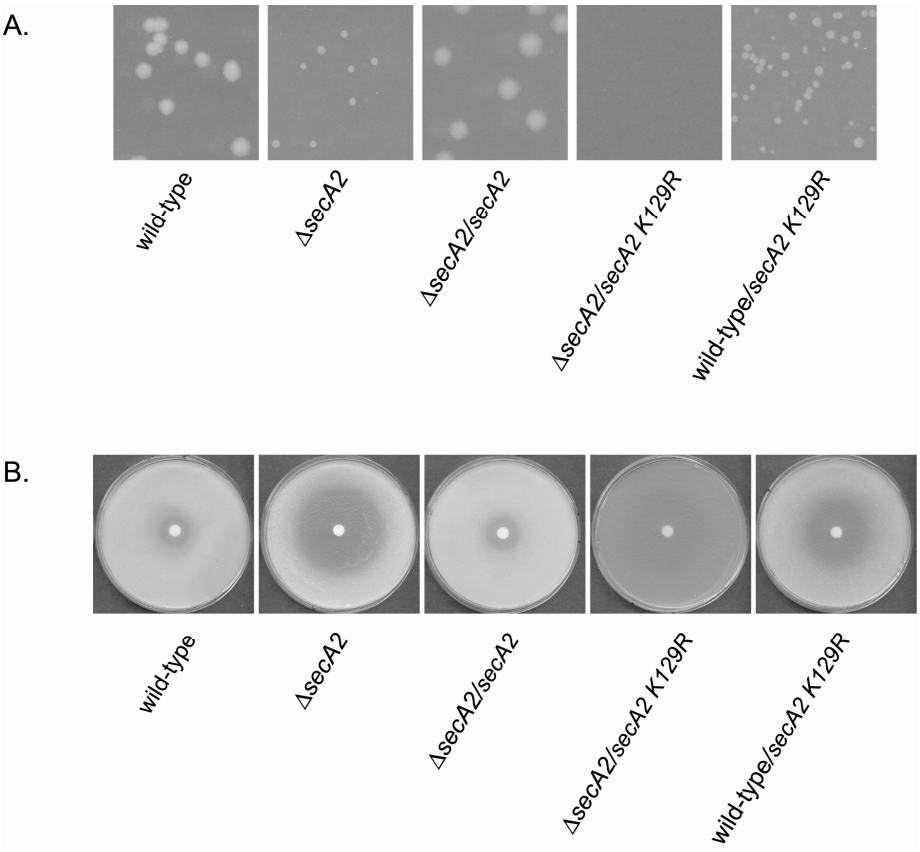

Figure 3.6. *M. smegmatis* Walker Box mutant allele *secA2 K115R* fails to complement and exacerbates the *M. smegmatis* Δ*secA2* mutant rich agar growth defect and azide hypersensitivity. (A) *M. smegmatis* strains carrying empty vector, wild-type *secA2* (*secA2*), or *M. smegmatis* Walker Box mutant *secA2* (*secA2 K129R*) plasmids were plated onto Mueller Hinton agar plates. Images were taken after 4 days of growth at 37˚C. While not visible here, colonies of the Δ*secA2* mutant expressing *secA2 K129R* will appear upon extended incubation. Each strain was also tested for azide sensitivity in **(B)**. Note the exacerbated phenotype observed when *secA2 K129R* is expressed in wild-type *M. smegmatis*.

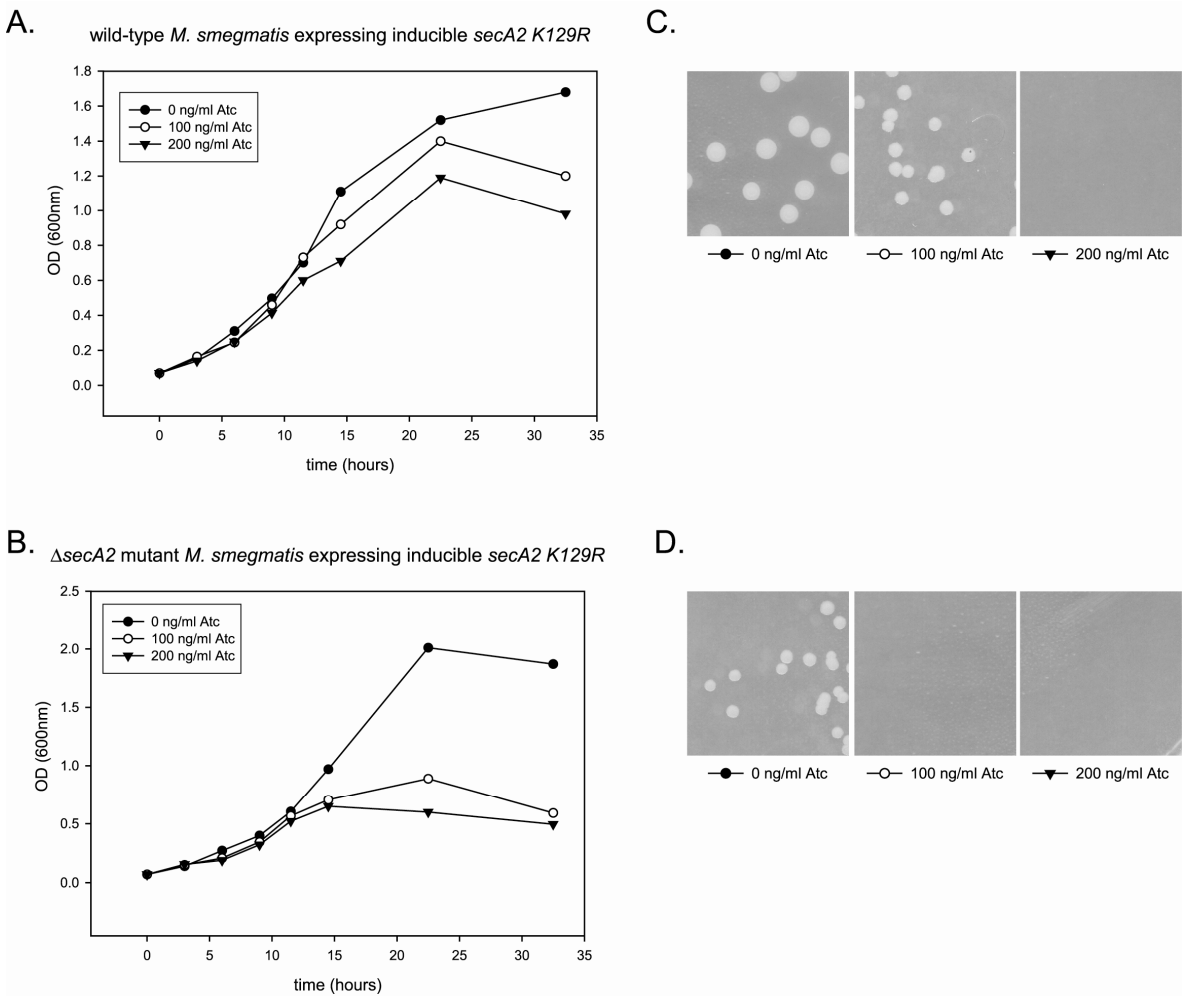

Figure 3.7. SecA2 K129R is dominant negative. Wild-type and Δ*secA2* mutant *M. smegmatis* strains were transformed with pNR54 (*secA2 K129R* under control of an inducible promoter). Transformants were grown in Mueller Hinton broth with various concentrations of Atc (closed circle, 0 ng/ml; open circle, 100 ng/ml; closed triangle, 200 ng/ml). Growth of wild-type (**A**) and Δ*secA2* mutant (**C**) was monitored by measuring OD_{600nm}. At 21 hours post inoculation, serial dilutions of the wild-type (**B**) and Δ*secA2* mutant (**D**) cultures were plated onto Mueller Hinton plates containing 500 ng/ml Atc. The plates were incubated at 37˚C for 3-4 days.

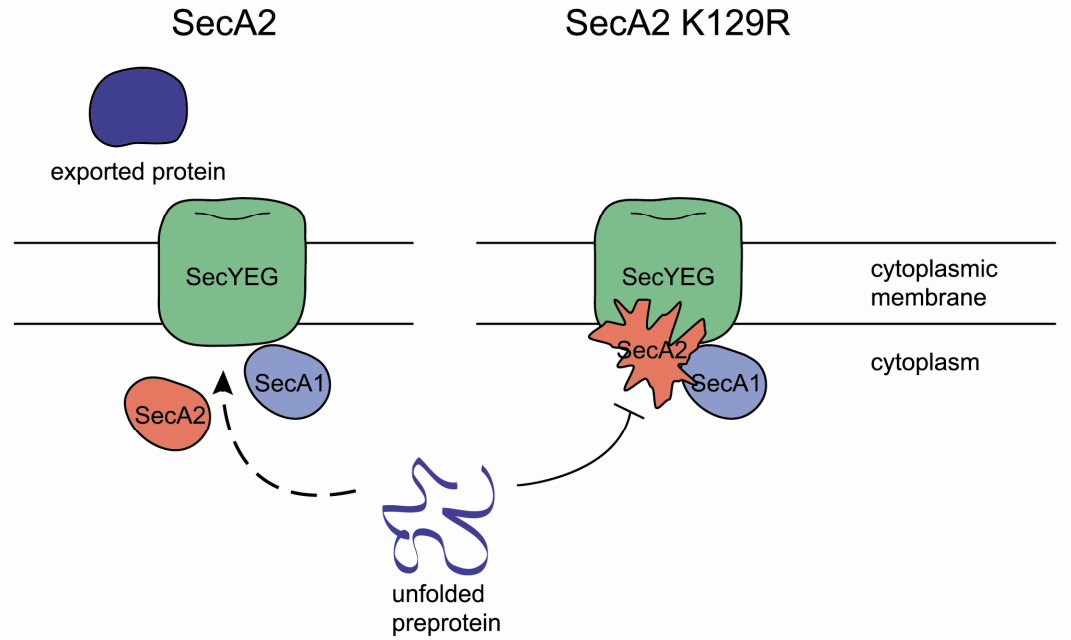

Figure 3.8. Model depicting SecA2 K129R interfering with the essential Sec pathway. Normally, SecA2 exports proteins across the cytoplasmic membrane by transiently interacting with the SecYEG translocase. Upon mutation of the Walker A motif, SecA2 K129R becomes permanently bound to the SecYEG translocase, compromising essential protein export and leading to a rich agar growth defect.

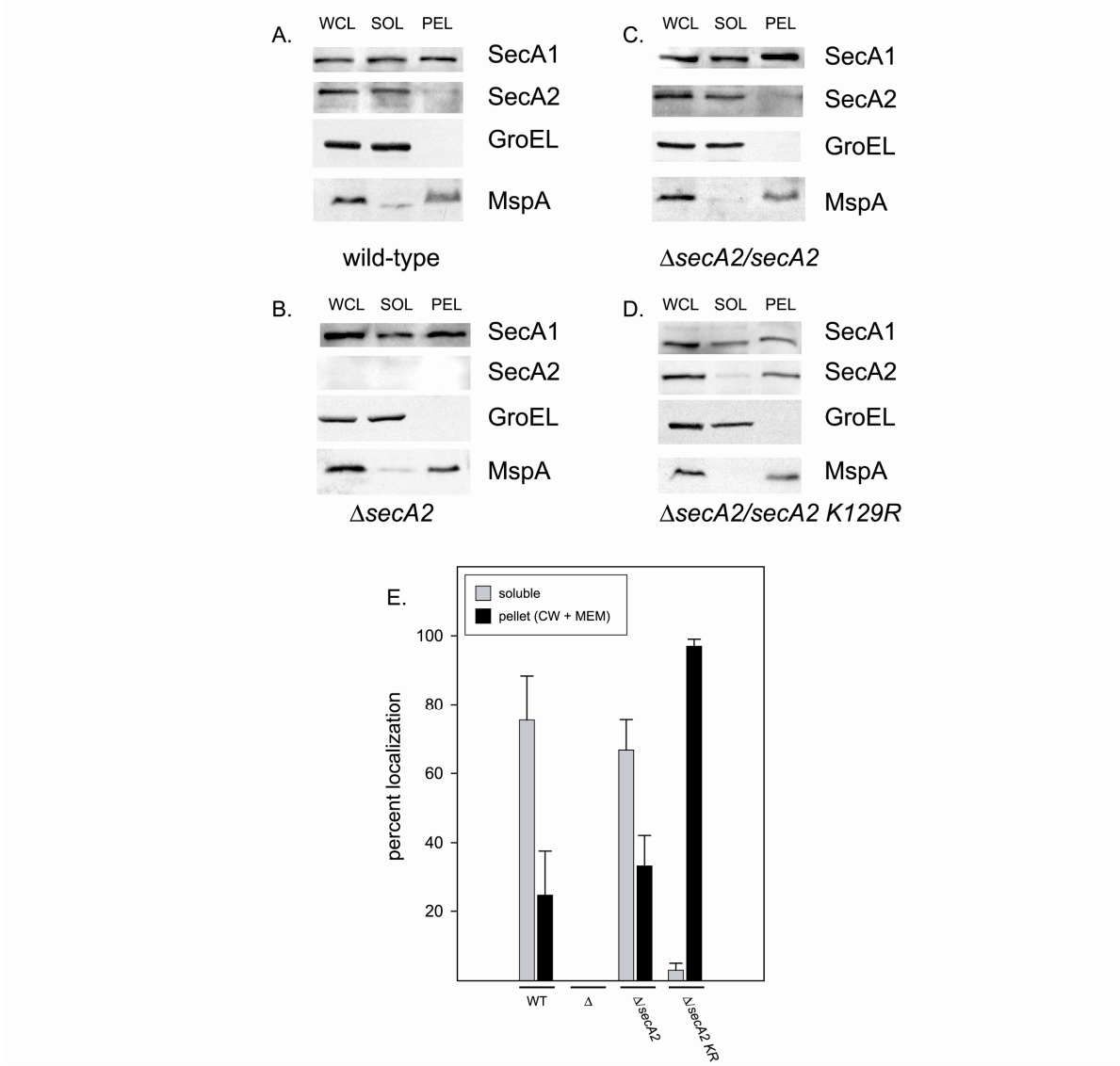

Figure 3.9. Mutation of the SecA2 Walker Box changes localization of SecA2 from the cytoplasm to the cell envelope, while the localization of SecA1 remains unchanged. Cultures of **(A)** wild-type, **(B)** Δ*secA2* mutant, **(C)** Δ*secA2* mutant complemented with wild-type *secA2*, and **(D)** Δ*secA2* mutant carrying *secA2 K129R* were grown in Mueller Hinton until an OD_{600nm} of 1.0. Whole-cell lysates were used to make subcellular fractions as described in the Methods. To analyze each fraction, protein derived from an equal number of cells was analyzed by quantitative immunoblot. Anti-SecA1 and anti-SecA2 antibodies were used at a 1:50,000 dilution and 1:20,000 dilution, respectively. The cell wall porin MspA and cytoplasmic chaperone GroEL were used as quality controls for the fractionation. Goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase were used according to the manufacturer. Immune complexes were developed using ECF and visualized using a phosphorimager. All strains were assayed in triplicate. Error bars indicate the standard error from the mean of three independent replicates. Figures 3.9 A-C are the same as Figures 2.6 A-C.

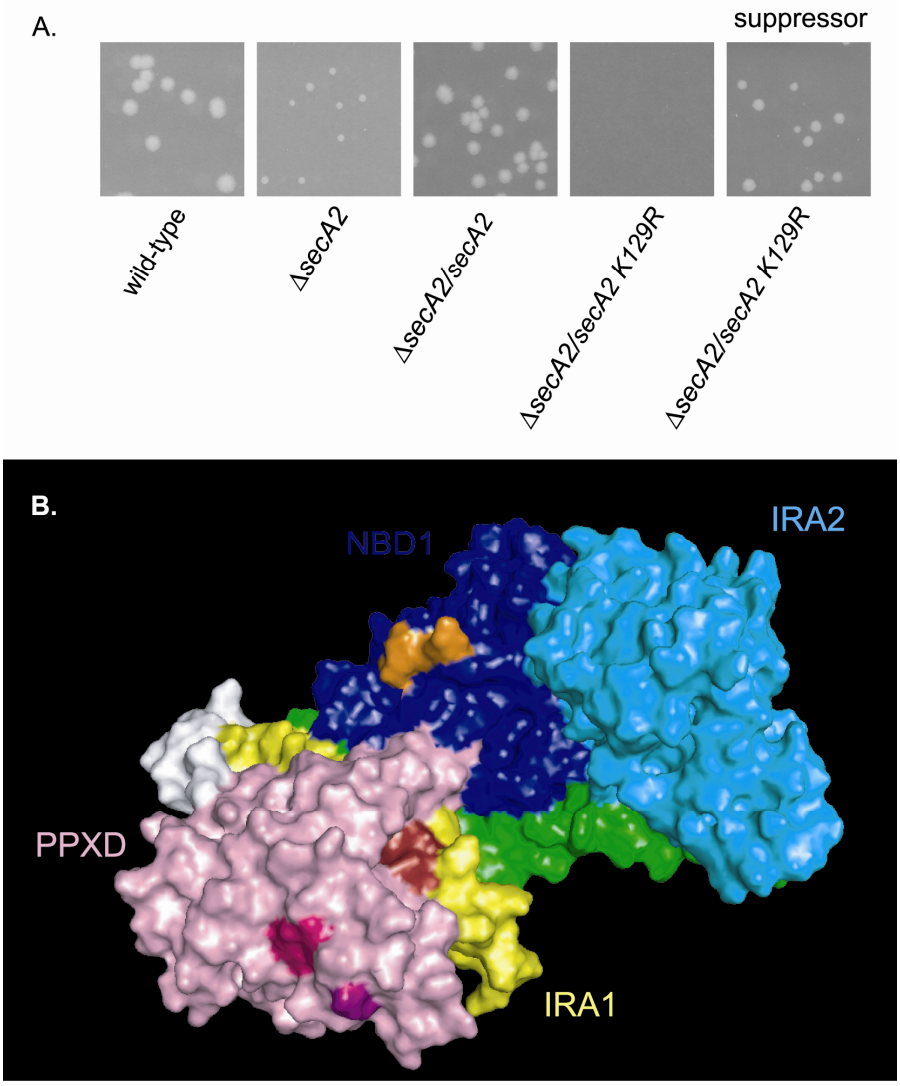

Figure 3.10. Spontaneous suppressors of the SecA2 KR rich agar growth defect can be isolated. (A) *M. smegmatis* strains expressing different *secA2* alleles after 4 days of growth on Mueller Hinton agar plates at 37˚C. A typical suppressor is pictured at the right. **(B)** Space-filling model of SecA2 homology model showing the location of identified intragenic suppressors. The suppressors cluster in four different subdomains of SecA2: NBD1 (dark blue), IRA2 (cyan), IRA1 (yellow), and PPXD (pink). Within each domain, the location of each suppressor mutation is indicated by different colors (orange, red, pink, and purple).

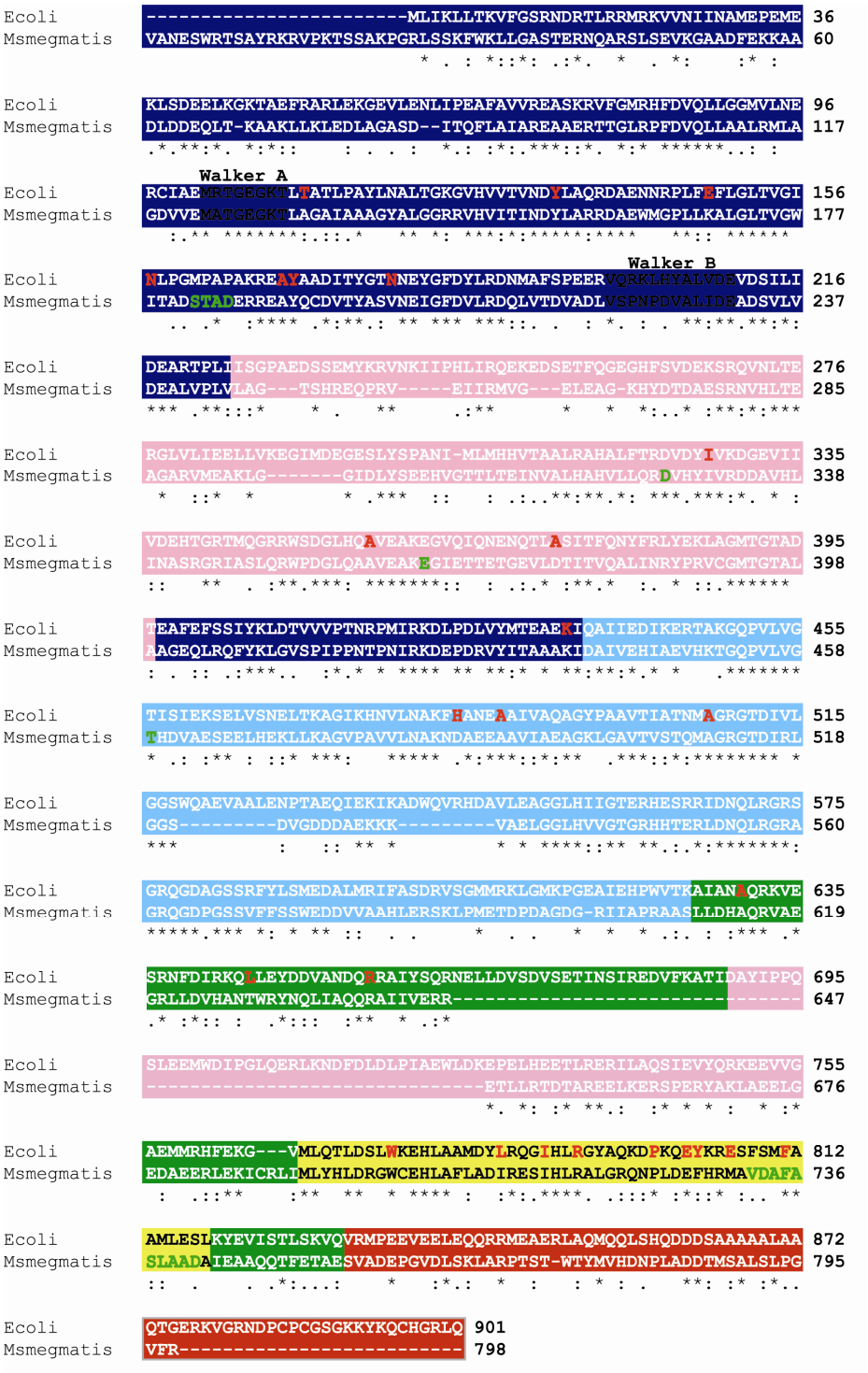

Figure 3.11. ClustalW alignment of *E. coli* SecA and *M. smegmatis* SecA2. Suppressors of protein export defects in *E. coli* are indicated in red. The location of the SecA2 K129R intragenic suppressors identified in this study is indicated in green. The Walker A and Walker B ATP-binding motifs are indicated in bold. Colored shading is the same as in Figure 3.10.

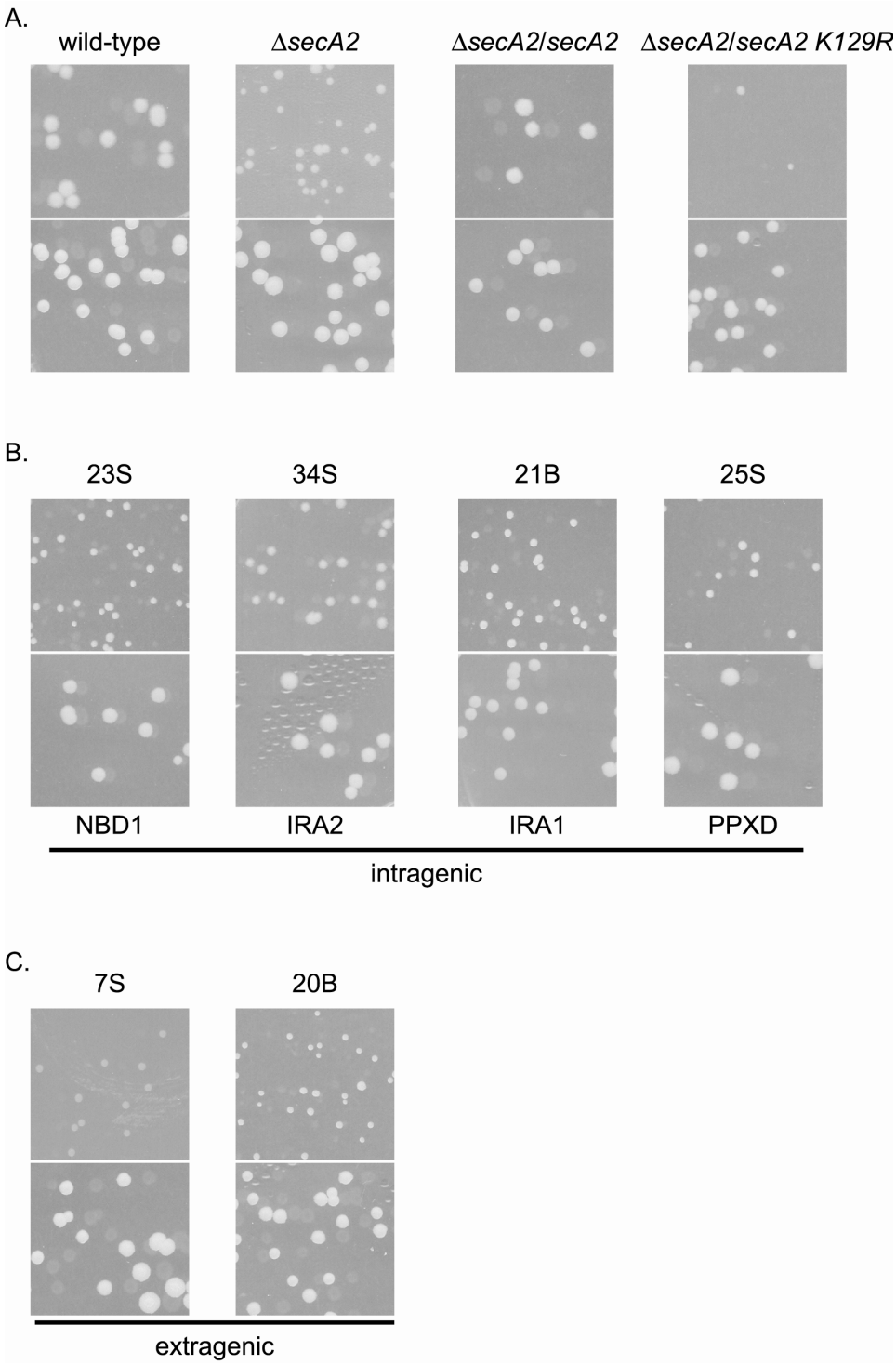

Figure 3.12. Phenotypes of representative suppressors of SecA2 K129R. (A) Wild-type, Δ*secA2* mutant, Δ*secA2* mutant complemented with *secA2*, and Δ*secA2* mutant expressing *secA2 K129R* grown on Mueller Hinton (top) and 7H10 (bottom) plates. **(B)** Intragenic suppressors of *secA2 K129R* representing the four different subdomains of SecA2. **(C)** Two extragenic suppressors also suppress the exacerbated rich agar growth defect caused by SecA2 K129R.

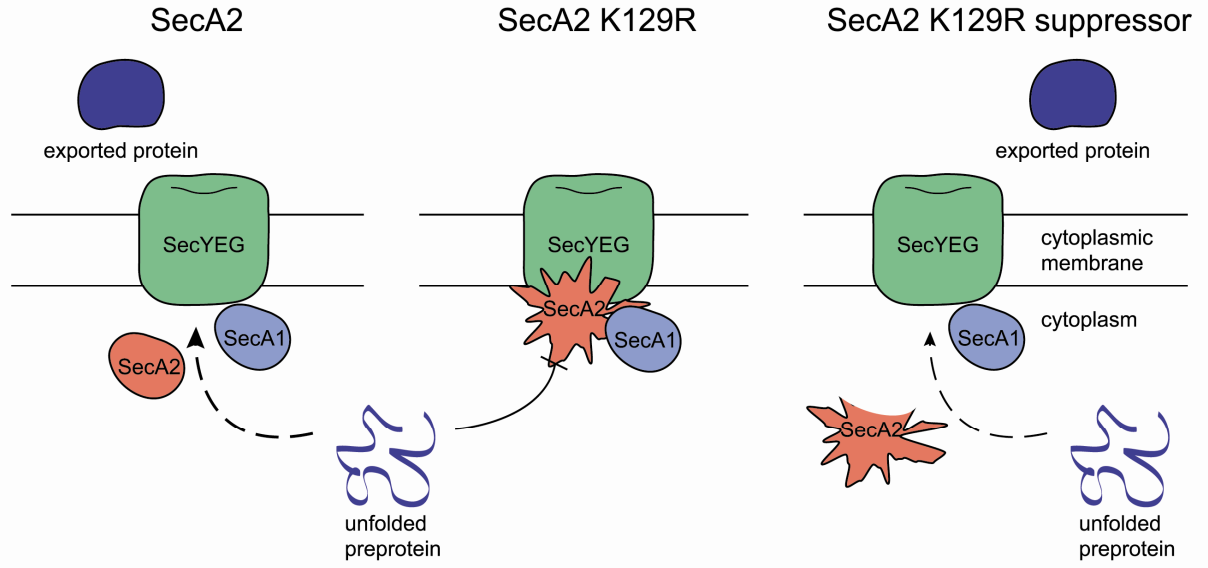

Figure 3.13. A model to explain how intragenic suppressors of SecA2 K129R alleviate the exacerbated rich agar growth defect caused by SecA2 K129R. As described in the text, one role of SecA2 might be to increase the efficiency of protein export through the SecYEG translocase. SecA2 K129R becomes associated with the cell envelope, possibly interfering with the essential Sec pathway. Mutations in SecA2 K129R, particularly those that cause structural distortions, might release SecA2 K129R from the cell envelope. In the resulting strain, export of proteins across the cytoplasmic membrane would be restored, although less efficient than in wild-type mycobacteria.

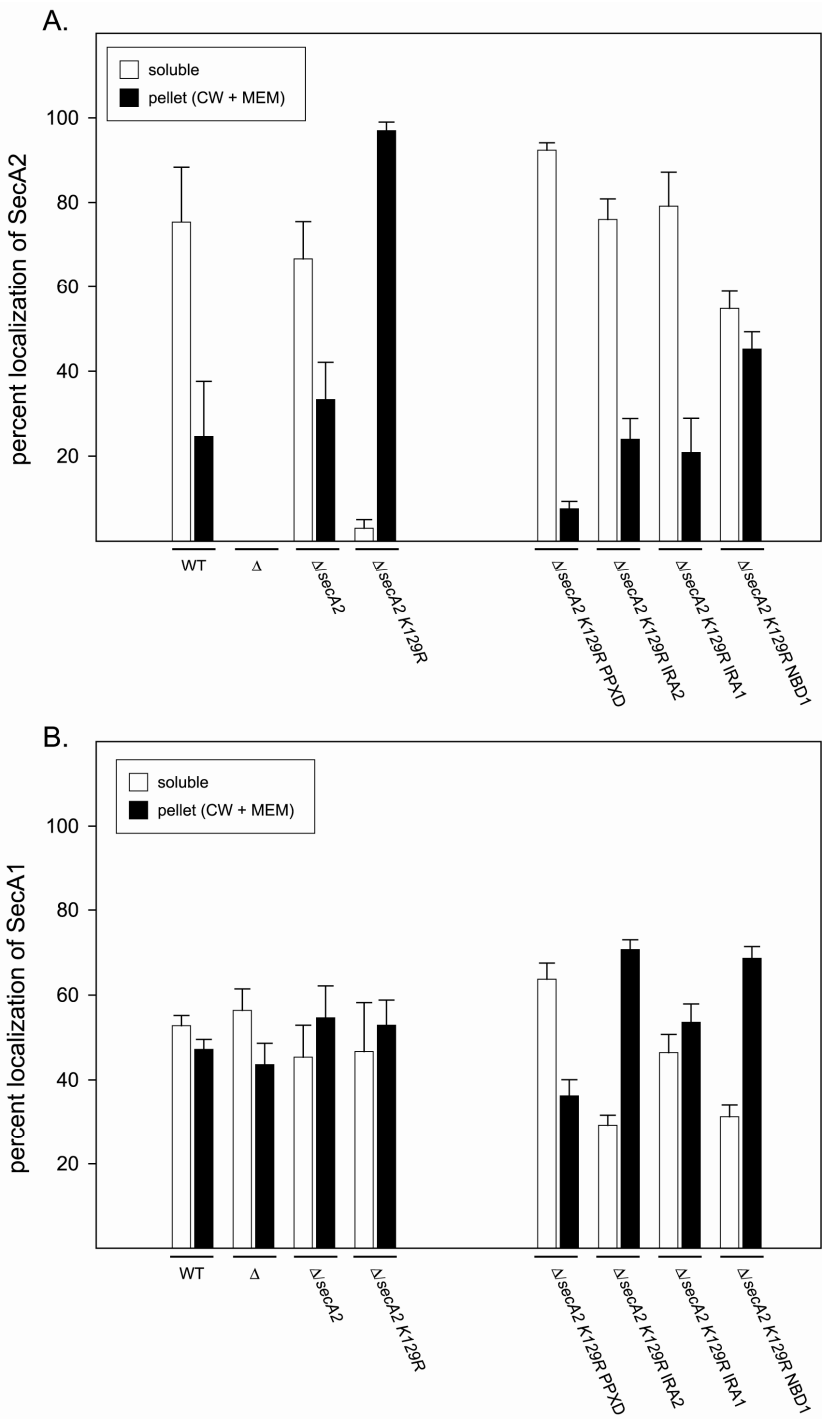

Figure 3.14. Subcellular localization of SecA1 and SecA2 is altered in intragenic suppressors of SecA2 K129R. Mueller Hinton-grown cultures of *M. smegmatis* were fractionated as described above. Intragenic suppressors (PPXD, IRA2, IRA1, and NBD1) are indicated. Protein derived from an equal number of cells was analyzed by SDS-PAGE and quantitative immunoblot with anti-SecA2 **(A)** or anti-SecA1 **(B)** antibodies. Error bars represent the mean of three independent replicates of each strain.

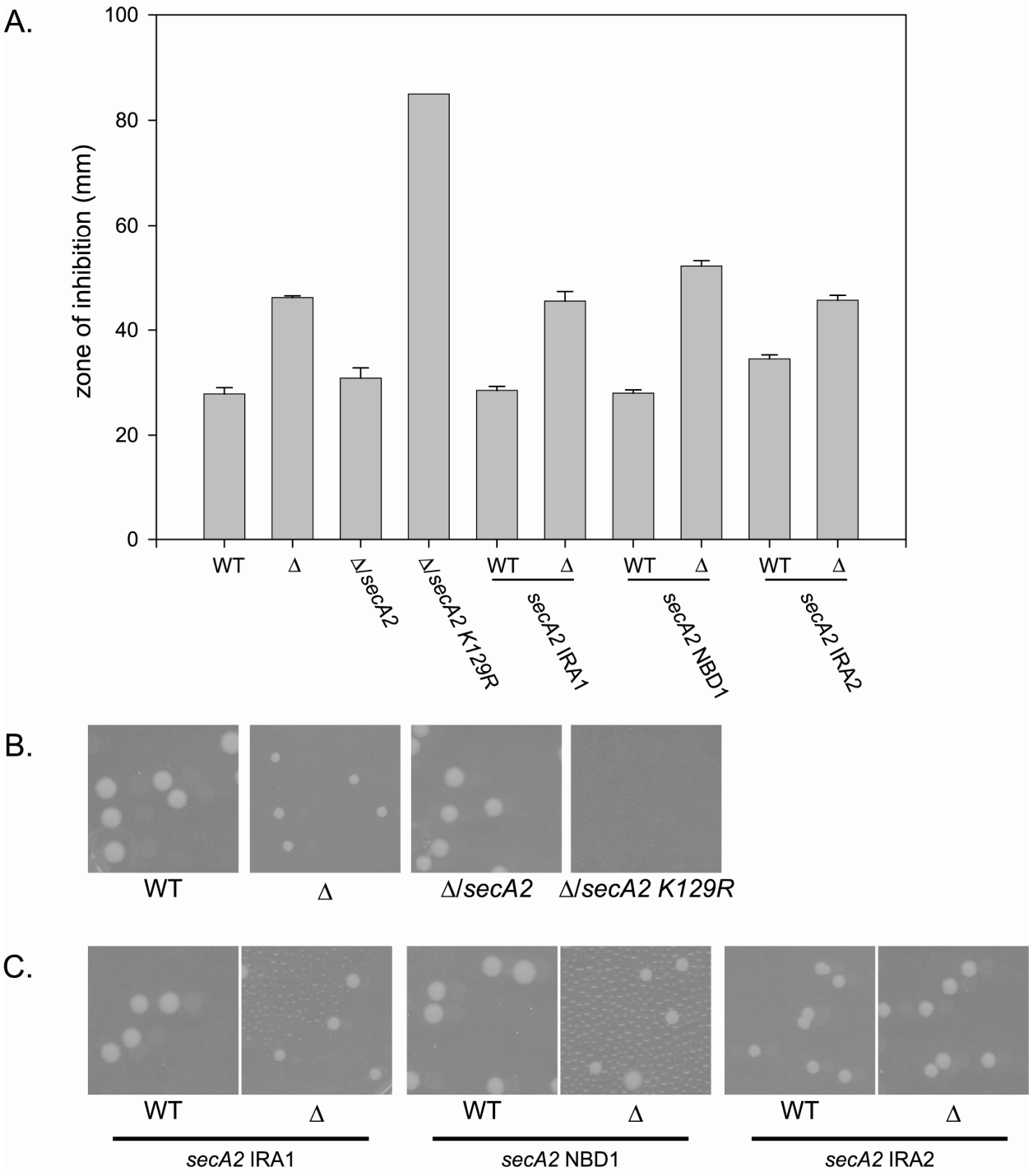

Figure 3.15. Intragenic suppressor mutations of SecA2 K129R also render wild-type SecA2 nonfunctional. Wild-type (mc^2155) and Δ*secA2* mutant (NR116) *M. smegmatis* strains were transformed with plasmids containing intragenic suppressor mutations in SecA2 domains (IRA1, IRA2, and NBD1) with a normal, wild-type Walker Box. Each strain was assayed for sensitivity to sodium azide as described above. Error bars represent the standard error from the mean of three replicates.

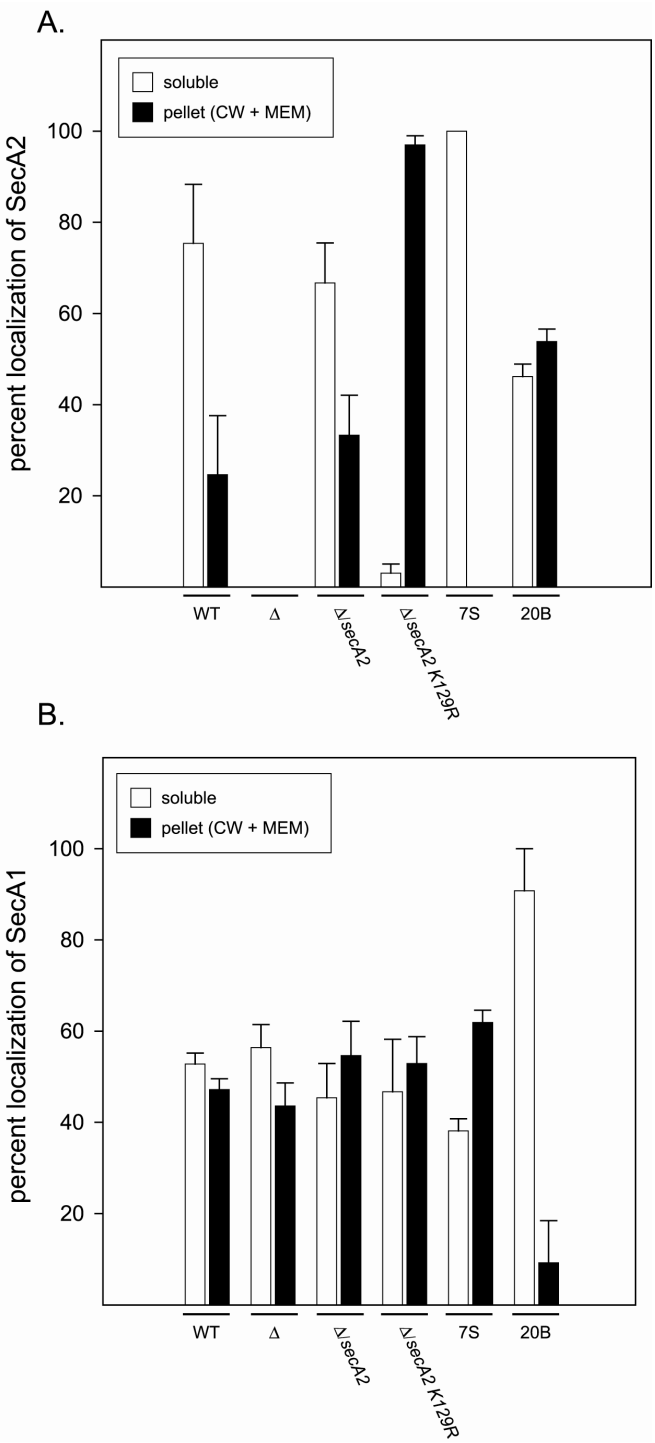

Figure 3.16. The localization of SecA1 and SecA2 is altered in two different extragenic suppressors compared to wild-type *M. smegmatis*. Subcellular fractions of *M. smegmatis* strains were generated and analyzed by SDS-PAGE and immunoblot for SecA2 **(A)** or SecA1 **(B)** as described above. Error bars represent the standard error from the mean of three replicates.

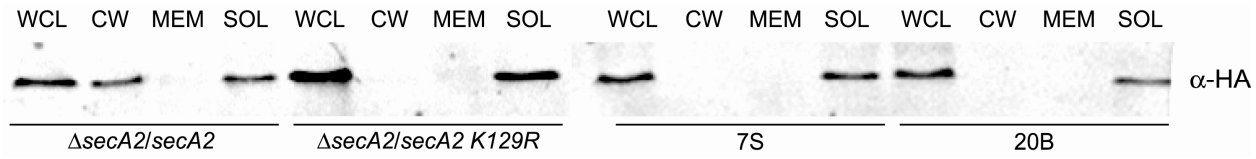

Figure 3.17. Msmeg1712-HA is not exported to the cell wall by two extragenic suppressors. The export of Msmeg1712-HA was monitored as described above. For each blot, equal proteins derived from the same number of starting cells were analyzed. Blots were probed with anti-HA antibodies, and the proteins were detected using ECF. The blots shown are representative of triplicate experiments.

References

1. **Abdallah, A. M., N. C. Gey van Pittius, P. A. Champion, J. Cox, J. Luirink, C. M. Vandenbroucke-Grauls, B. J. Appelmelk, and W. Bitter.** 2007. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol **5:**883-91.

2. **Appling, D. R.** 1999. Genetic approaches to the study of protein-protein interactions. Methods **19:**338-49.

3. **Bankaitis, V. A., and P. J. Bassford, Jr.** 1985. Proper interaction between at least two components is required for efficient export of proteins to the *Escherichia coli* cell envelope. J Bacteriol **161:**169-78.

4. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol **44:**1081-94.

5. **Bieker, K. L., G. J. Phillips, and T. J. Silhavy.** 1990. The *sec* and *prl* genes of *Escherichia coli*. J Bioenerg Biomembr **22:**291-310.

6. **Bieker, K. L., and T. J. Silhavy.** 1990. PrlA (SecY) and PrlG (SecE) interact directly and function sequentially during protein translocation in *E. coli.* Cell **61:**833-42.

7. **Braunstein, M., S. S. Bardarov, and W. R. J. Jacobs.** 2002. Genetic methods for deciphering virulence determinants of *Mycobacterium tuberculosis*, p. 67-99. *In* V. L. Clark and P. M. Bavoil (ed.), Methods in Enzymology, vol. 358. Academic Press, London.

8. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J Bacteriol **183:**6979-90.

9. **Braunstein, M., B. Espinosa, J. Chan, J. T. Belisle, and W. R. J. Jacobs.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol **48:**453-64.

10. **Budd, M. E., W. Choe, and J. L. Campbell.** 2000. The nuclease activity of the yeast DNA2 protein, which is related to the RecB-like nucleases, is essential in vivo. J Biol Chem **275:**16518-29.

11. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch Microbiol **189:**605-10.

12. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol Microbiol **53:**843-56.

13. **Driessen, A. J., and N. Nouwen.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem **77:**643-67.

14. **Ehrt, S., X. V. Guo, C. M. Hickey, M. Ryou, M. Monteleone, L. W. Riley, and D. Schnappinger.** 2005. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res **33:**e21.

15. **Erlandson, K. J., S. B. Miller, Y. Nam, A. R. Osborne, J. Zimmer, and T. A. Rapoport.** 2008. A role for the two-helix finger of the SecA ATPase in protein translocation. Nature **455:**984-7.

16. **Fikes, J. D., and P. J. Bassford, Jr.** 1989. Novel *secA* alleles improve export of maltose-binding protein synthesized with a defective signal peptide. J Bacteriol **171:**402-9.

17. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev **61:**136-69.

18. **Fortin, Y., P. Phoenix, and G. R. Drapeau.** 1990. Mutations conferring resistance to azide in *Escherichia* coli occur primarily in the *secA* gene. J Bacteriol **172:**6607-10.

19. **Gerlach, R. G., and M. Hensel.** 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int J Med Microbiol **297:**401-15.

20. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J Bacteriol **189:**5090-100.

21. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* using tetracycline repressors. J Bacteriol **189:**4614-23.

22. **Hendrick, J. P., and W. Wickner.** 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:**24596-600.

23. **Hinchey, J., S. Lee, B. Y. Jeon, R. J. Basaraba, M. M. Venkataswamy, B. Chen, J. Chan, M. Braunstein, I. M. Orme, S. C. Derrick, S. L. Morris, W. R. Jacobs, Jr., and S. A. Porcelli.** 2007. Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. J Clin Invest **117:**2279-88.

24. **Hou, J. M., N. G. D'Lima, N. W. Rigel, H. S. Gibbons, J. R. McCann, M. Braunstein, and C. M. Teschke.** 2008. ATPase activity of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins and its importance for SecA2 function in macrophages. J Bacteriol **190:**4880-7.

25. **Huie, J. L., and T. J. Silhavy.** 1995. Suppression of signal sequence defects and azide resistance in *Escherichia coli* commonly result from the same mutations in *secA*. J Bacteriol **177:**3518-26.

26. **Jarosik, G. P., and D. B. Oliver.** 1991. Isolation and analysis of dominant *secA* mutations in *Escherichia coli*. J Bacteriol **173:**860-8.

27. **Jilaveanu, L. B., C. R. Zito, and D. Oliver.** 2005. Dimeric SecA is essential for protein translocation. Proc Natl Acad Sci U S A **102:**7511-6.

28. **Khatib, K., and D. Belin.** 2002. A novel class of *secA* alleles that exert a signal-sequence-dependent effect on protein export in *Escherichia coli*. Genetics **162:**1031-43.

29. **Kourtz, L., and D. Oliver.** 2000. Tyr-326 plays a critical role in controlling SecA-preprotein interaction. Mol Microbiol **37:**1342-56.

30. **Kurtz, S.** 2007. SecA2 of *M. tuberculosis* contributes to intracellular survival, immune modulation, and surface properties of the bacillus. Masters of Science. University of North Carolina at Chapel Hill, Chapel Hill.

31. **Kurtz, S., K. P. McKinnon, M. S. Runge, J. P. Ting, and M. Braunstein.** 2006. The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. Infect Immun **74:**6855-64.

32. **Lenz, L. L., S. Mohammadi, A. Geissler, and D. A. Portnoy.** 2003. SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. Proc Natl Acad Sci U S A **100:**12432-7.

33. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol Microbiol **45:**1043-56.

34. **McNicholas, P., T. Rajapandi, and D. Oliver.** 1995. SecA proteins of *Bacillus subtilis* and *Escherichia coli* possess homologous amino-terminal ATP-binding domains regulating integration into the plasma membrane. J Bacteriol **177:**7231-7.

35. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol Microbiol **10:**483-97.

36. **Papanikou, E., S. Karamanou, C. Baud, M. Frank, G. Sianidis, D. Keramisanou, C. G. Kalodimos, A. Kuhn, and A. Economou.** 2005. Identification of the preprotein binding domain of SecA. J Biol Chem **280:**43209-17.

37. **Pashley, C. A., and T. Parish.** 2003. Efficient switching of mycobacteriophage L5-based integrating plasmids in *Mycobacterium tuberculosis*. FEMS Microbiol Lett **229:**211-5.

38. **Pavelka, M. S., Jr., and W. R. Jacobs, Jr.** 1999. Comparison of the construction of unmarked deletion mutations in *Mycobacterium smegmatis, Mycobacterium bovis* bacillus Calmette-Guerin, and *Mycobacterium tuberculosis* H37Rv by allelic exchange. J Bacteriol **181:**4780-9.

39. **Rigel, N. W., and M. Braunstein.** 2008. A new twist on an old pathway--accessory Sec systems. Mol Microbiol **69:**291-302.

40. **Sakai, D., T. Horiuchi, and T. Komano.** 2001. ATPase activity and multimer formation of PilQ protein are required for thin pilus biogenesis in plasmid R64. J Biol Chem **276:**17968-75.

41. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

42. **Schmidt, M. G., E. E. Rollo, J. Grodberg, and D. B. Oliver.** 1988. Nucleotide sequence of the *secA* gene and *secA*(*Ts*) mutations preventing protein export in *Escherichia coli*. J Bacteriol **170:**3404-14.

43. **Sexton, J. A., J. S. Pinkner, R. Roth, J. E. Heuser, S. J. Hultgren, and J. P. Vogel.** 2004. The *Legionella pneumophila* PilT homologue DotB exhibits ATPase activity that is critical for intracellular growth. J Bacteriol **186:**1658-66.

44. **Siboo, I. R., D. O. Chaffin, C. E. Rubens, and P. M. Sullam.** 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. J Bacteriol **190:**6188-96.

45. **Stader, J., L. J. Gansheroff, and T. J. Silhavy.** 1989. New suppressors of signal-sequence mutations, *prlG*, are linked tightly to the *secE* gene of *Escherichia coli*. Genes Dev **3:**1045-52.

46. **Sun, Y., Z. Dong, K. Nakamura, and N. H. Colburn.** 1993. Dosage-dependent dominance over wild-type p53 of a mutant p53 isolated from nasopharyngeal carcinoma. FASEB J **7:**944-50.

47. **Vrontou, E., and A. Economou.** 2004. Structure and function of SecA, the preprotein translocase nanomotor. Biochim Biophys Acta **1694:**67-80.

48. **Vrontou, E., S. Karamanou, C. Baud, G. Sianidis, and A. Economou.** 2004. Global co-ordination of protein translocation by the SecA IRA1 switch. J Biol Chem **279:**22490-7.

49. **Walker, J. E., A. Eberle, N. J. Gay, M. J. Runswick, and M. Saraste.** 1982. Conservation of structure in proton-translocating ATPases of *Escherichia coli* and mitochondria. Biochem Soc Trans **10:**203-6.

50. **Zimmer, J., Y. Nam, and T. A. Rapoport.** 2008. Structure of a complex of the ATPase SecA and the protein-translocation channel. Nature **455:**936-43.

Chapter 4

ATPase Activity of *Mycobacterium tuberculosis* SecA1 and SecA2 Proteins and Its Importance for SecA2 Function in Macrophages

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The Sec-dependent translocation pathway that involves the essential SecA protein and the membrane-bound SecYEG translocon is used to export many proteins across the cytoplasmic membrane. Recently, several pathogenic bacteria, including *Mycobacterium tuberculosis*, were shown to possess two SecA homologs, SecA1 and SecA2. SecA1 is essential for general protein export. SecA2 is specific for a subset of exported proteins and is important for *M. tuberculosis* virulence. The enzymatic activities of two SecA proteins from the same microorganism have not been defined for any bacteria. Here, *M. tuberculosis* SecA1 and SecA2 are shown to bind ATP with high affinity, though the affinity of SecA1 for ATP is weaker than that of SecA2 or *Escherichia coli* SecA. Amino acid substitution of arginine or alanine for the conserved lysine in the Walker A motif of SecA2 eliminated ATP binding. We used the SecA2 (K115R) variant to show that ATP binding was necessary for

the SecA2 function of promoting intracellular growth of *M. tuberculosis* in macrophages. These results are the first to show the importance of ATPase activity in the function of accessory SecA2 proteins.

Introduction

Mycobacterium tuberculosis is the causative agent of the disease tuberculosis (TB). TB kills about 2 million people annually, and approximately one-third of the world's population is currently infected with *M. tuberculosis* (40). A serious problem in the worldwide fight against TB is the emergence of multidrug-resistant strains of *M. tuberculosis*. To develop logical targets for new, effective drugs, the physiology of mycobacteria must be better understood.

As with all bacterial pathogens, the majority of *M. tuberculosis* virulence factors are extracytoplasmic proteins exported to the bacterial cell surface or secreted further to the extracellular milieu (11, 21, 24). Bacteria possess several different pathways for exporting proteins from the cytoplasm, including the highly conserved Sec pathway (8, 24). The Sec pathway uses the SecA protein and the membrane-integrated SecYEG translocon to transport precursor proteins that contain a characteristic amino-terminal signal sequence across the cytoplasmic membrane (18). SecA, an essential ATPase found in all bacteria, undergoes conformational changes upon ATP binding and hydrolysis that drive the transport of unfolded precursor proteins through the SecYEG translocon (36, 37). The well-characterized ATPase activity of *Escherichia coli* SecA is absolutely required for protein export and is stimulated by the addition of phospholipids and by the presence of precursor protein (27, 29, 39).

Most bacteria, including the model organisms *E. coli* and *Bacillus subtilis*, possess a single, essential SecA protein. Recently, several microorganisms, including *M. tuberculosis*, *Listeria monocytogenes*, corynebacteria, and some streptococci, were found to carry two SecA proteins, SecA1 and SecA2 (2–5, 7, 26). At the amino acid level, SecA1 and SecA2 of *M. tuberculosis* are about 50% similar to each other and 61% and 50% similar to *E. coli* SecA, respectively (3). The mycobacterial SecA1 protein is essential and is thought to function similarly to the single SecA proteins of *E. coli* and *B. subtilis* (3, 16). The mycobacterial SecA2 protein is not essential for growth in culture but is required for exporting a subset of proteins (3, 4, 14). Furthermore, the Δ*secA2* deletion mutant of *M. tuberculosis* is attenuated in virulence, suggesting that some of the SecA2-dependent exported proteins are virulence factors (4, 22). Interestingly, the proteins exported by SecA2 systems in different bacteria include examples with and without amino-terminal signal sequences (1, 2, 4, 7, 22, 25).

The features that distinguish the function of SecA1 from that of SecA2 in a single bacterial species are not known, nor have the biochemical properties of each SecA been studied previously. Here, we report that the SecA1 and SecA2 proteins of *M. tuberculosis* are present at comparable levels, indicating that expression levels do not explain the different functions of these proteins.

Both SecA1 and SecA2 exhibit high sequence homology with other SecA proteins in the Walker A and B motifs commonly found in ATPases (38). The Walker motifs are part of the motor domain of SecA. In structural studies, the motor domain of *M. tuberculosis* SecA1 also shows the highest similarity to that of *B. subtilis* SecA (32). Using purified *M. tuberculosis* SecA1 and SecA2 proteins, we show that both proteins exhibit fully functional

ATPase activities. Moreover, replacement of the conserved lysine residue in the Walker A motif of SecA2 to produce the SecA2 (K115R) or the SecA2 (K115A) variant eliminates ATP binding. This amino acid replacement in the Walker A motif also affects the biological activity of SecA2, as the *secA2* (*K115R*) variant fails to complement the intracellular growth defect of the *M. tuberculosis* Δ*secA2* mutant in macrophages. Our data present the first report of the characterization of the ATPase activity for any SecA2 protein and show that ATP binding is necessary for SecA2 function. This work represents an important first step toward understanding how the two SecA proteins in mycobacteria function in protein translocation.

Materials and Methods

Plasmids and strains. The *E. coli* SecA expression vector was a gift from Linda Randall (31). The *M. tuberculosis* SecA1 expression vector was a gift from James Sacchettini. The SecA1 plasmid was generated by PCR amplification of the *secA1* gene from *M. tuberculosis* H37Rv genomic DNA into pET29a (Novagen). A stop codon was added to the 3' end of the gene to avoid addition of the C-terminal hexahistidine tag from the plasmid. The SecA2 expression plasmid was constructed by PCR amplification of the *secA2* gene from *M. tuberculosis* H37Rv genomic DNA, using the primers 5' CATTAATGGTGCCCAAGACCACCCGCGCTCA 3' and 5' TAAGCTTCAGCGGAACACCCCGGGCAGACT 3'. The resulting PCR product was cloned into pET41b (Novagen) to create pNR14. This SecA2 expression vector uses an internal GTG start codon located at nucleotide position 91 relative to the annotated start site (NCBI accession number NP_216337). We believe this represents the true start codon.

Translation from this codon results in a protein whose sequence more closely resembles that of SecA2 from other mycobacteria, and the corresponding shorter SecA2 sequence can complement the Δ*secA2* mutant phenotypes in *Mycobacterium smegmatis* and *M. tuberculosis* (data not shown). Plasmids that overexpress SecA1 or SecA2 were transformed into *E. coli* BL21(DE3).

The Walker A motif of *M. tuberculosis* SecA2 was mutated in plasmid pNR14, using a QuikChange site-directed mutagenesis kit (Stratagene). The primers used to change lysine 115 (AAG) to arginine (AGG) (underlined) were 5' CGGTGAGGGCAGAACCCTTGCC 3' and 5' CGGCAAGGGTTCTGCCCTCACC 3'. The resulting SecA2 (K115R) expression vector for *E. coli* is plasmid pNR24. To create the same substitution in a mycobacterial expression vector, pMB162 was used as a template to create pNR7. Plasmid pMB162 contains the *M. tuberculosis secA2* open reading frame, as annotated in the genome database, under control of the constitutive mycobacterial *hsp60* promoter and an attachment site that enables stable integration into the mycobacterial genome in single copy (4). To generate the SecA2 (K115A) pNR60 expression vector, site-directed mutagenesis was performed with pNR24 with the primers 5' CGGTGGCGGCAGAACCCTTGCC 3' and 5' CGGCAAGGGTTCTGCCGCCACC 3'. Sequencing was used to confirm the appropriate *secA2* sequence in all vectors used in this study (Eton Biosciences).

Purification of proteins. *E. coli* SecA was expressed and purified as described previously (10). Purification of *M. tuberculosis* SecA1 and SecA2 followed essentially the same protocol. Freshly transformed cells were selected for the ability to overproduce each protein. The cultures that overproduced the SecA proteins were pooled and used for the protein preparation. The induced cells were suspended in 25 mM Tris (pH 7.6), 10 mM magnesium

acetate, and 10 mM NaCl and lysed, using a French pressure cell at 15,000 lb/in^2, into a 2 μg/ml final concentration of pepstatin A, leupeptin, and aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma). The lysate was clarified by low-speed centrifugation, and the supernatants were centrifuged in a Sorvall T-865 rotor at 115,000 x *g* for 3 h to remove the cell membranes.

For the purification of SecA1 and SecA2, the supernatant was applied to a Blue-Sepharose CL-6B column (Pharmacia Biotech) equilibrated with 25 mM Tris at pH 7.6, 0.5 mM EDTA, 100 μM dithiothreitol, and 0.5 mM PMSF. The protein was eluted with a step gradient consisting of a low-salt (the same buffer supplemented with 10 mM NaCl), a medium-salt (0.4 M NaCl), and a high-salt (1.3 M NaCl) buffer. Fractions containing SecA1 were collected, precipitated with ammonium sulfate at 60%, and dialyzed against 10 mM HEPES (pH 7.6), 25 mM potassium acetate, 0.5 mM EDTA, and 100 μM tris(2-carboxyethyl)phosphine (TCEP)-HCl (Pierce). SecA2 was concentrated by using an Amicon ultrafiltration stirred cell and a membrane with a molecular weight (MW) cutoff of 30,000. The purified protein was stored in aliquots at -80°C. The SecA2 (K115R) and SecA2 (K115A) variant proteins were purified by the same method as that used for the wild-type (WT) SecA2.

Quantification of the ratio of SecA2 to SecA1 in vivo. Whole-cell lysates of exponential phase *M. tuberculosis* cultures grown in Middlebrook 7H9 medium were prepared following fixation in an equal volume of 10% formalin for 1 h. Fixed cells were pelleted by centrifugation, resuspended in extraction buffer, and lysed by bead beating. For quantitative SecA1 and SecA2 Western blot analysis, 100 μg of formalin-fixed whole-cell lysates were run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel alongside a standard curve

of known amounts of purified SecA1 or SecA2 protein. Proteins were transferred to nitrocellulose membranes and probed with rabbit polyclonal anti-SecA1 (1:50,000 dilution) or anti-SecA2 (1:20,000 dilution) antibody (16). The secondary antibody was goat anti-rabbit conjugated to alkaline phosphatase (1:20,000 dilution), and detection of the fluorescence from ECF (Amersham/GE Healthcare) was done with a PhosphorImager (Molecular Dynamics). The signal intensity values from six independently prepared whole-cell lysates were quantified by comparison with standard curve values to determine the number of moles of SecA1 and SecA2 per mg of cellular protein.

Circular dichroism spectra. Circular dichroism (CD) was measured with an Applied Photophysics Pi-Star 180 circular dichroism spectrapolarimeter, with the cuvette maintained at 20°C with a circulating water bath. A 1-cm-path-length quartz cuvette was used. The protein concentration was 0.1 mg/ml in 20 mM phosphate buffer. The slits were set at 2 nm. The CD spectrum was taken from 250 to 200 nm with a 1-nm step size. The signal was averaged for 500,000 samples for a total of 12.5 s at each wavelength.

Determination of melting temperature. CD at 222 nm was used to monitor the secondary structure of the SecA proteins. The protein concentration was 0.1 mg/ml. To determine the melting temperature, the temperature was ramped from 8°C to 65°C with a step size of 0.5°C. At each temperature, the signal at 222 nm was averaged for 100,000 points, which required around 10 s. The raw data were normalized (12) to show the fraction unfolded at each temperature.

ATP binding assay. Nitrocellulose membrane filtration assays were used to measure the ATP binding affinities of the SecA proteins as described in reference 42. The 0.2-μM-pore-size nitrocellulose membranes (Whatman) were incubated briefly with 0.5 N NaOH, rinsed

extensively with deionized H_2O, and then equilibrated with binding buffer [50 mM HEPES-NaOH (pH 7.5), 30 mM KCl, 10 mM $Mg(OAc)_2$] for at least 1 h. ATP stocks were prepared using Sigma Ultrapure ATP and GE Healthcare [α-^{32}P]ATP. The protein concentration in each assay was held between 3.7 and 4.5 μM of monomer, and increasing concentrations of ATP were added. The protein-ATP mixtures were incubated on ice, which slows the ATPase activity to a rate undetectable by the use of the malachite green assay, as described below. The solutions were filtered through the nitrocellulose membrane to separate the bound ATP from the free ATP. Because the apparent K_d (dissociation constant) values of *E. coli* SecA and *M. tuberculosis* SecA2 for ATP were lower than the protein concentrations used in the assays, the binding data were fitted with an equation used when there is ligand depletion (15, 33), using the following equation: $[LR] = [(K_d + [R_t] + [L_t]) - \sqrt{\{(K_d + [R_t] + [L_t])^2 - 4[R_t][L_t]\}}]/2$, where R_t is the total protein concentration and L_t is the total ATP added. The K_d value for SecA1 was obtained by fitting the data to the standard equation for binding data, since here the apparent K_d value was above the protein concentration used in the assay equation $[LR] = (R_t [L]) / (K_d + [L])$, where L is the free ligand. The data were fitted with KaleidaGraph software (Synergy Software). The K_d value was determined for each assay performed in triplicate, and the average K_d value and the standard error were determined.

Analysis of ATPase activity. The protocol used to measure SecA ATPase activity was a modification of the malachite green assay described previously (23, 27). A reaction mixture contained 40 μg/ml of the SecA proteins, 1 mg/ml bovine serum albumin, and 4 mM ATP in reaction buffer [50 mM Tris-HCl (pH 7.0), 30 mM KCl, 30 mM NH_4Cl, 1 mM dithiothreitol, and 5 mM $Mg(OAc)_2$]. The assay was conducted at 25°C. Formation of inorganic phosphate was monitored spectrophotometrically by the increase in absorbance at 660 nm at each time

point. The inorganic phosphate concentration generated in the reaction mixture with time was calculated using a standard curve. Each assay was done at least four times with each protein. The rate of hydrolysis was determined for each assay done in duplicate, and the averages and standard errors were determined.

Macrophage infections. Bone marrow macrophages were elicited from femurs of C57BL/6 mice, as described previously (22, 28), and 2.5 x 10^5 macrophages were seeded into wells of 8-well-chamber slides 24 h prior to infection. The *M. tuberculosis* strains were grown to mid-exponential phase, washed with phosphate-buffered saline containing 0.05% Tween 80, diluted in tissue culture medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 1x nonessential amino acids [Gibco]) and added to the macrophage monolayer to achieve a multiplicity of infection of 1.0. Macrophage monolayers were infected with *M. tuberculosis* strains for 4 h at 37°C in 5% CO_2. On days 0, 1, and 5 post infection, the contents of triplicate wells for each infection were washed and then lysed with 0.05% SDS. The resulting lysates were diluted and plated on Middlebrook 7H10 agar to enumerate intracellular bacteria during the course of infection.

Results

SecA1 and SecA2 proteins are present in equivalent amounts in *M. tuberculosis*.

The identification of bacteria that possess two SecA proteins, such as *M. tuberculosis*, is a relatively new discovery. As a starting point to understanding the contribution of each SecA protein to the process of protein export in *M. tuberculosis*, we determined the relative amounts of SecA1 and SecA2 inside the bacterial cell. Quantitative immunoblot analysis was performed with whole-cell lysates prepared from the virulent *M. tuberculosis* strain

H37Rv, using antibodies specific to each SecA protein. These antibodies were raised against peptides specific for SecA1 or SecA2, and they recognize only the respective protein (16). These antibodies were used to determine the number of moles of SecA1 or SecA2 protein per mg of cellular protein by comparison to a standard curve for the purified *M. tuberculosis* SecA1 protein or to that of the SecA2 protein. The results, from an evaluation of six independent whole-cell lysates, revealed nearly equivalent amounts of each protein. The ratio of the average number of moles per mg of protein of SecA2 (2.07×10^{-11}) to that of SecA1 (2.25×10^{-11}) across these experiments was 0.93 +/- 0.12 (standard error). This indicates that the differences between the functions of SecA1 and those of SecA2 in *M. tuberculosis* are not due to different levels of expression, at least under standard laboratory conditions.

Purification of *M. tuberculosis* SecA1 and SecA2. The two SecA proteins from any bacteria have not been biochemically characterized. The SecA1 and SecA2 proteins of *M. tuberculosis* were expressed from a plasmid and purified from *E. coli* as soluble proteins. SecA1 has a predicted MW of 106,000/monomer, while SecA2 is smaller, with a predicted MW of 85,400/monomer (3). *E. coli* SecA has a MW of 101,000/monomer. All the proteins were purified to >90% using Blue-Sepharose column chromatography (Fig. 1A). SecA2 bound less tightly to this column than SecA1 or *E. coli* SecA did, eluting with 0.5 M salt instead of 1.2 M. This change in binding to a pseudoaffinity column for nucleotide-binding proteins suggested that SecA2 might have an altered affinity for nucleotides.

We next determined if the purified *M. tuberculosis* SecA1 and SecA2 proteins had CD spectra consistent with folded secondary and tertiary structures (Fig. 1B). Each of the proteins exhibited the characteristic minima at 209 and 222 nm associated with proteins with

significant levels of helical structure. Tryptophan fluorescence spectra for all the proteins were also consistent with a native tertiary structure with emission maxima around 340 nm, which is typical for folded proteins (data not shown). These results were expected for properly folded SecA1 and SecA2 proteins, as the three-dimensional structure of *M. tuberculosis* SecA1 is similar to that of *B. subtilis* SecA and *E. coli* SecA (17, 30, 32) and the sequences of the mycobacterial SecA proteins are highly homologous.

SecA1 and SecA2 are less stable to heat denaturation than *E. coli* SecA. At the standard temperature (37°C) used for ATP hydrolysis assays, we saw little ATPase activity for the *M. tuberculosis* SecA proteins. Since the *E. coli* SecA ATPase activity is temperature dependent because the protein is thermolabile (27), we reasoned that the low activity levels of SecA1 and SecA2 proteins could be due to lower thermal stability. To characterize the thermal stability of the *M. tuberculosis* SecA1 and SecA2 proteins, we denatured the proteins with heat and followed the melting transition by CD at 222 nm (Fig. 2). The data were normalized to show the fraction unfolded at each temperature (12). The melting temperature (T_m) for *E. coli* SecA was ~43°C, as shown previously (34). Both *M. tuberculosis* SecA1 and SecA2 were less stable to heat denaturation, with T_ms of ~35°C, indicating a moderate decrease in the stability of the *M. tuberculosis* SecA proteins compared to that of *E. coli* SecA. Our data show that measurements of activity of the recombinant *M. tuberculosis* SecA proteins must be done at temperatures lower than 30°C to maintain their fully native structures.

***M. tuberculosis* SecA1 and SecA2 bind ATP with different affinities.** To determine if SecA1 and SecA2 bind ATP as anticipated for proteins carrying canonical Walker A motifs, we used a standard nitrocellulose membrane binding assay with [α-

^{32}P]ATP to determine the affinity of each protein for ATP (42). The protein concentration in each assay was held constant, and increasing concentrations of ATP were added. The solutions were filtered through a nitrocellulose membrane to separate the bound ATP from the free ATP. Because the apparent K_d values of *E. coli* SecA and *M. tuberculosis* SecA2 for ATP were lower than the protein concentrations used in the assays, the binding data were fitted with an equation used when there is ligand depletion, as described in Materials and Methods and previously (15, 33). The K_d value of SecA1 for ATP was obtained by fitting the data to the standard equation for binding data, since here the apparent K_d value was above the protein concentration used in the assay. Analysis of the binding data (Fig. 3A) showed that *E. coli* SecA bound ATP with a K_d value of 0.46 +/- 0.11 μM, which is within the standard error for a previously published value (42). *M. tuberculosis* SecA2 bound ATP with a somewhat weaker affinity of 1.9 +/- 0.3 μM. *M. tuberculosis* SecA1 bound ATP ~10-fold more weakly than SecA2, with a K_d value of 21.8 +/- 4.0 μM.

An interesting observation is that SecA2 binds more weakly to the Blue-Sepharose column than either *E. coli* SecA or SecA1, even though its K_d value for ATP binding is similar to that of *E. coli* SecA, which binds tightly to this column. This observation suggests that the SecA2 binding site for the Cibacron Blue dye, presumably the ATP binding site, may have a different conformation than SecA1 or *E. coli* SecA.

SecA1 and SecA2 exhibit ATPase activity. To determine if *M. tuberculosis* SecA1 and SecA2 hydrolyze ATP, we used a malachite green assay, which measures the amount of free inorganic phosphate produced over time (6, 9, 23, 29) (Fig. 3B). Because each of the T_ms of the *M. tuberculosis* SecA proteins was lower than that of *E. coli* SecA, all ATPase assays were done at 25°C. The ATPase activity level of each protein was determined in

duplicate from at least four separate experiments. While we did see some variability in the results of the ATPase assays, the trends were the same for all assays. With assays done at 25°C, each of the SecA proteins was able to hydrolyze ATP; the endogenous ATPase activity level of *M. tuberculosis* SecA1 was similar to that of *E. coli* SecA, while that of SecA2 was about threefold higher than *E. coli* SecA.

Amino acid substitutions in the Walker A motif of SecA2 affect ATP binding in vitro. Alignment of *M. tuberculosis* SecA1 and SecA2 with the SecA of *E. coli* and *B. subtilis* reveals high sequence identity across the characteristic Walker A motif of ATPases (Fig. 4A) (3). Structural studies demonstrate that the amino acids of this motif participate in ATP binding (20, 37). In previous studies, site-directed mutagenesis of the conserved lysine in the Walker A motif (K108 in *E. coli* SecA and K106 in *B. subtilis* SecA) has shown it to be important for SecA ATP binding (29, 35). The *E. coli* SecA ATPase K108R mutant is defective in ATP binding and protein translocation in vitro, as well as biologically inactive in vivo as demonstrated by the inability to complement an *E. coli* temperature-sensitive *secA* allele (29). The *B. subtilis* SecA (K106N) ATPase mutant is also unable to translocate precursor proteins in vivo (20).

To determine if ATP binding and ATPase activity in *M. tuberculosis* SecA2 depend similarly on the Walker A motif, we substituted arginine or alanine for the conserved lysine K115 in the Walker A motif of *M. tuberculosis* SecA2 and purified the corresponding proteins as described above. We tested the ability of the SecA (K115R) and SecA2 (K115A) variants to bind ATP (Fig. 4B). Both the K115R and the K115A substitutions significantly decrease the ability of SecA2 to bind ATP. We then tested the ATP hydrolysis activity of the SecA2 (K115R) variant, using the malachite green assay. The endogenous ATPase activity

for the SecA2 (K115R) variant was somewhat higher than that for the WT SecA2. The SecA2 (K115A) variant had an ATP hydrolysis rate lower than that of WT SecA2 (Fig. 4C). These in vitro results show that the Walker box of SecA2 is required for efficient ATP binding, as predicted and shown to be the case for other SecA proteins (20, 29, 35). These data also indicate that a SecA2 variant in which lysine, K115, has been replaced can be used to assess the importance of ATP binding for the biological functions of SecA2 in *M. tuberculosis*.

A substitution in the Walker A motif of SecA2 affects biological activity in *M. tuberculosis*. A Δ*secA2* mutant of *M. tuberculosis* is attenuated for growth in macrophages and in mice (3, 4). The biochemical data presented above and the results of previous studies suggest that *M. tuberculosis* SecA2 functions as an ATPase to promote the export of a specific subset of proteins that are important to pathogenesis. To test this idea, we asked whether the *secA2* (*K115R*) allele encodes a functional protein that is able to fulfill the function of SecA2 in promoting the growth of *M. tuberculosis* in macrophages. The *secA2* (*K115R*) allele was introduced into the *M. tuberculosis* Δ*secA2* mutant in a single copy at the chromosomal *attB* site. Murine bone marrow-derived macrophages were infected in parallel with the parental *M. tuberculosis* strain H37Rv, the Δ*secA2* mutant of *M. tuberculosis*, the Δ*secA2* mutant complemented with the wild-type *secA2* gene inserted at the *attB* locus, or the Δ*secA2* mutant with *secA2* (*K115R*) (Fig. 5A). As shown previously, H37Rv and the *secA2*-complemented strain grew at similar rates in macrophages over a 5-day period of infection, while the Δ*secA2* mutant failed to grow in macrophages (22). Unlike introduction of the WT *secA2* gene at the *attB* site, which promoted *M. tuberculosis* growth in macrophages, the introduction of *secA2* (*K115R*) in the Δ*secA2* mutant failed to promote this growth.

Importantly, Western blot analysis confirmed that the Δ*secA2* mutant strains carrying WT *secA2* or *secA2* (*K115R*) expressed the SecA2 protein at similar levels (Fig. 5B). In addition, the growth defect of the *secA2* (*K115R*) strain in macrophages was specific, as this strain did not exhibit a general growth defect when tested in 7H9 liquid medium (data not shown). These results indicate that replacing the conserved lysine in the Walker A motif of SecA2 renders SecA2 inactive in its biological role of promoting *M. tuberculosis* growth in macrophages.

Discussion

Here we report the first description of ATPase activities for two SecA proteins from the same bacterium and the first description of the ATPase activity for any SecA2 protein. Based on amino acid sequence analysis, *M. tuberculosis* SecA1 and SecA2 possess the consensus Walker A and B motifs (3) that are found in ATP hydrolyzing enzymes and are essential to *E. coli* and *B. subtilis* SecA functions (20, 29). However, for SecA2, which currently has an undefined role in protein export and differs from the well-characterized *E. coli* SecA protein in being nonessential in mycobacteria, the question of whether it can function as an ATPase remains unanswered (4).

Here we demonstrated that both SecA proteins of *M. tuberculosis* are ATP binding and hydrolyzing proteins. SecA2 bound ATP with an affinity that was somewhat weaker than that of *E. coli* SecA, and SecA1 bound ATP about 10-fold more weakly than SecA2. Since the ATP concentration within an *M. tuberculosis* cell is approximately 1 mM (13, 19), even with these different affinities, both SecA proteins should be fully saturated with ATP in vivo. Therefore, the physiological significance of these differences in affinity is unclear.

The *M. tuberculosis* SecA proteins showed a lower melting temperature than *E. coli* SecA under our buffer conditions, suggesting that the *M. tuberculosis* SecA proteins are less thermostable than *E. coli* SecA. Therefore, all of the ATP hydrolysis assays were done at a temperature at which the proteins maintained their native state. At 25°C, each of the SecA proteins was able to hydrolyze ATP. Although it is difficult to directly compare results of ATP hydrolysis activities of SecA proteins from different species because of the use of different experimental conditions, our results are in general agreement with the ATP hydrolysis rates of SecA proteins from *Pseudomonas aeruginosa* (41), *E. coli* (29), and *B. subtilis* (35). Nevertheless, our data suggest that there may be some functional differences between SecA1 and SecA2 with regard to ATP affinities and hydrolysis rates. The endogenous ATPase activity of *M. tuberculosis* SecA2 was found to be higher than that of *M. tuberculosis* SecA1 and *E. coli* SecA.

The K115R substitution in the SecA2 Walker A motif clearly establishes the fact that ATP binding of SecA2 is vital for its function in vivo, as this substitution in SecA2 abolished the ability of *M. tuberculosis* to grow within macrophages. In an ATP binding assay, the SecA2 (K115R) and SecA2 (K115A) variants exhibited significantly reduced ATP binding, as anticipated when the conserved lysine of Walker A motif was replaced. However, neither the substitution of R for K nor A for K eliminated the endogenous ATPase activity. The K115R substitution slightly increased the endogenous rate of ATP hydrolysis, while the SecA2 (K115A) variant showed about 60% of the activity of WT SecA2. These results are consistent with those of Mitchell and Oliver (29), where the *E. coli* SecA (K108R) substitution in the Walker A motif affected biological activity and eliminated effective ATP

binding but did not eliminate—and even somewhat increased—the endogenous ATPase activity (29).

The mycobacterial SecA proteins have been shown to be functionally dissimilar from one another in that SecA2 participates only in the export of a specific subset of proteins (3, 4, 14). The data presented here indicate that both *M. tuberculosis* SecA1 and SecA2 are ATPases. Our data suggest that each *M. tuberculosis* SecA protein is likely to function in a manner similar to that of *E. coli* SecA in undergoing cycles of ATP binding and hydrolysis-promoted conformational changes that drive protein export across the cytoplasmic membrane. In addition, we show that the levels of SecA1 and SecA2 in the cell are equivalent. Therefore, the functional differences between SecA1 and SecA2 may be at the level of the specific proteins they recognize for export or the proteins with which they interact to form a translocation complex. For SecA1, we believe the protein works in concert with the membrane-localized SecYEG translocon. The SecA2 protein may also work with SecYEG or with an as-yet unidentified translocation complex to selectively export a subset of proteins. Alternatively, differences in the function of the SecA proteins might be in the regulation of their enzymology. The studies here lay the groundwork for further investigation of the SecA2 export pathway of mycobacteria.

Acknowledgements

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Attributions

The work described here was performed by me with the following exceptions. SecA1 and SecA2 purification, circular dichroism, thermostability, and ATP binding/hydrolysis assays were performed by Jie M. Hou and Nadia G. D'Lima. Jessica R. McCann performed the macrophage infections. This work has been previously published (*J Bacteriol*. 2008. Jul; 190 (14):4880-7). Permission to reprint this work has been granted by the publisher.

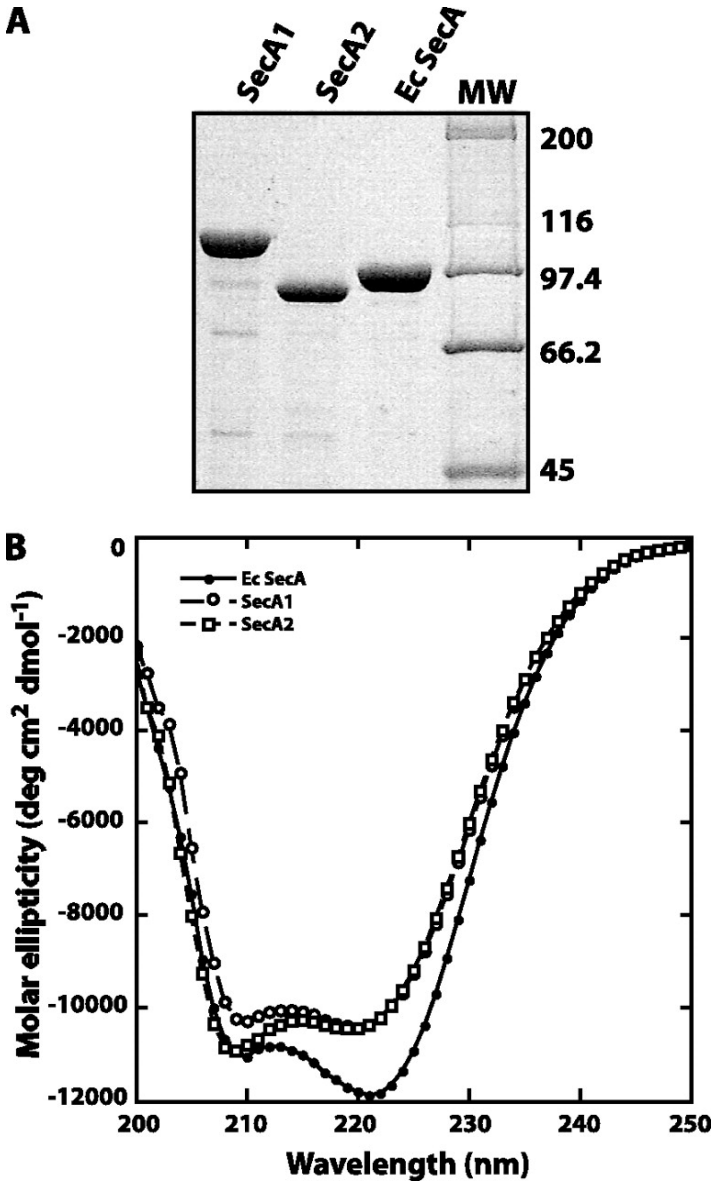

Figure 4.1. Purification of *M. tuberculosis* SecA1 and SecA2. **(A)** SDS gel of the purified SecA proteins. SecA1, SecA2, and *E. coli SecA* were purified as described in Materials and Methods. The proteins were separated on a 10% SDS polyacrylamide gel. Molecular weight markers (MW, in thousands) are indicated on the right side of the gel. **(B)** CD spectra of each SecA protein. The spectra were obtained as described in Materials and Methods. The protein concentration was 0.1 mg/ml in 20 mM phosphate buffer (pH 7.6).

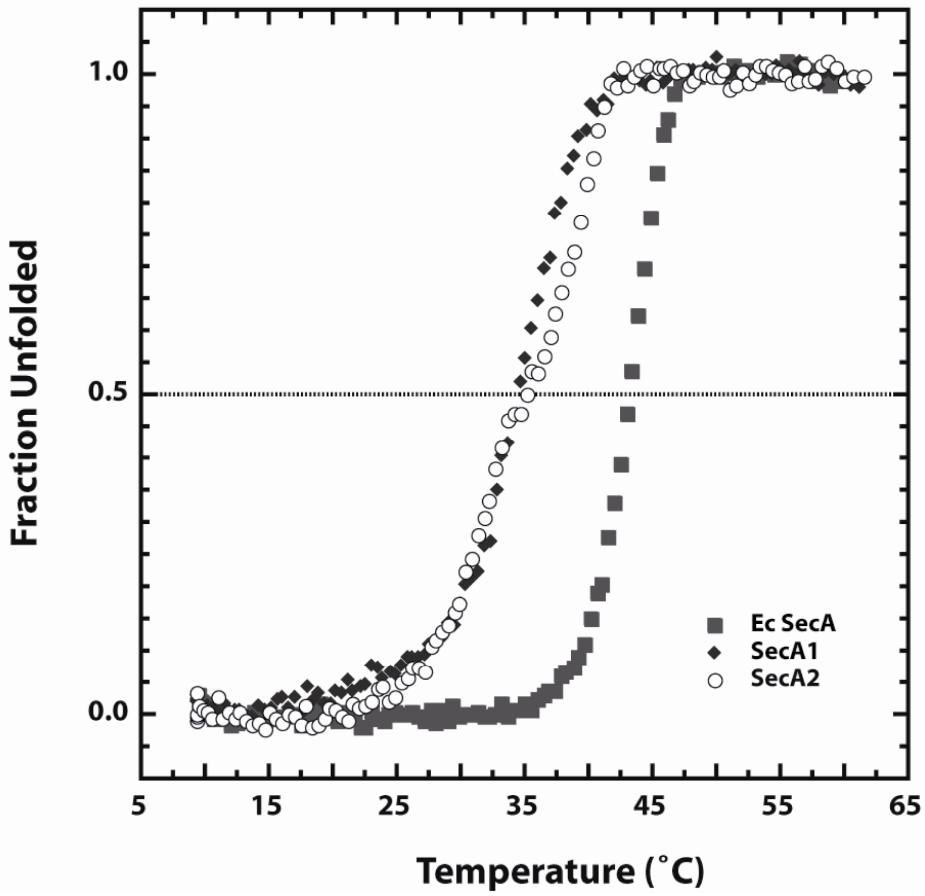

Figure 4.2. ***M. tuberculosis*** **SecA proteins have decreased thermostability compared to that of** ***E. coli*** **SecA.** To determine the melting temperatures, the SecA proteins (0.1 mg/ml) were ramped from 8 to 65°C. At each 0.5 °C increment in temperature, the CD at 222 nm was measured. The CD signal was normalized to show the fraction unfolded at each temperature.

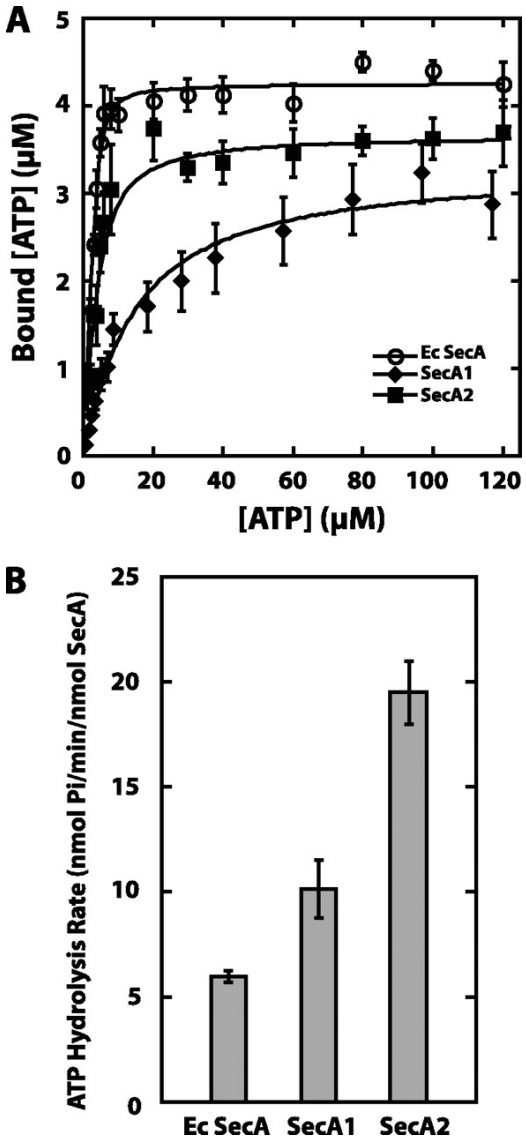

Figure 4.3. ***M. tuberculosis*** **SecA1 and SecA2 bind and hydrolyze ATP.** **(A)** SecA2 binds ATP with high affinity. The affinity of ATP binding by SecA proteins was determined by a nitrocellulose membrane filtration assay. The concentrations of bound and free ATP were determined. The data were fitted using the binding equations described in Materials and Methods to determine the K_d values. Since the data were fitted using two different equations, the ATP concentration on the x axis is the total ATP for the *E. coli* SecA and SecA2 proteins and the free ATP for SecA1. The binding assays were repeated at least three times for each protein. Shown here are the averages and standard errors of the averages determined for the combined data. **(B)** Endogenous ATPase activities of the *M. tuberculosis* SecA proteins. The endogenous activity levels of the SecA proteins were determined using a malachite green assay for the formation of free inorganic phosphate. Each assay was done in duplicate and repeated at least four times to determine the average rates and standard errors of the averages.

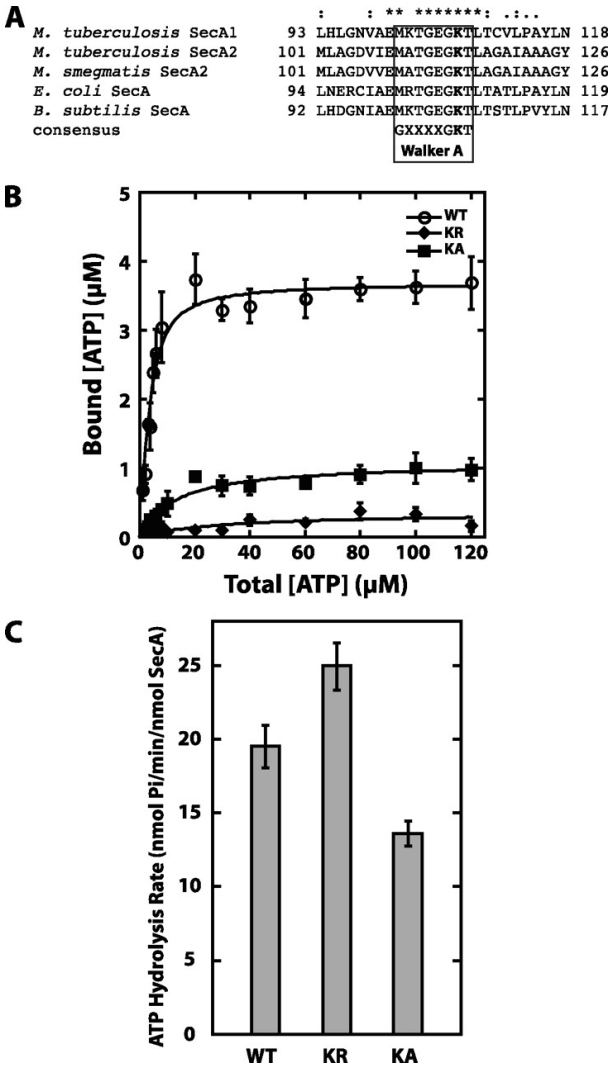

Figure 4.4. **Effect of the K115R and K115A substitutions in the Walker A motif of SecA2 in vitro. (A)** Alignment of Walker A motifs in SecA proteins. Sequence alignment of the highly conserved Walker A motif from mycobacterial SecA1 and SecA2 to the well-characterized SecA proteins of *E. coli* and *B. subtilis*. The conserved lysine residue that was mutated to generate SecA2 (K115R) or SecA2 (K115A) is shown in bold. **(B)** ATP binding by SecA2 (K115R) (KR) and SecA2 (K115A) (KA). The ATP binding was measured as described in the legend to Fig. 4.3. The binding data for WT SecA2 is the same as that shown in Fig. 4.3. **(C)** Endogenous ATPase activity of SecA2 (K115R) and SecA2 (K115A). The average rates and standard deviations of the ATP hydrolysis by each protein were determined by using the malachite green assay for determination of free inorganic phosphate, as described in the legend to Fig. 4.3. The WT SecA2 hydrolysis rate is the same as that shown in Fig. 4.3. The average rates and standard deviations from all of the experiments are shown.

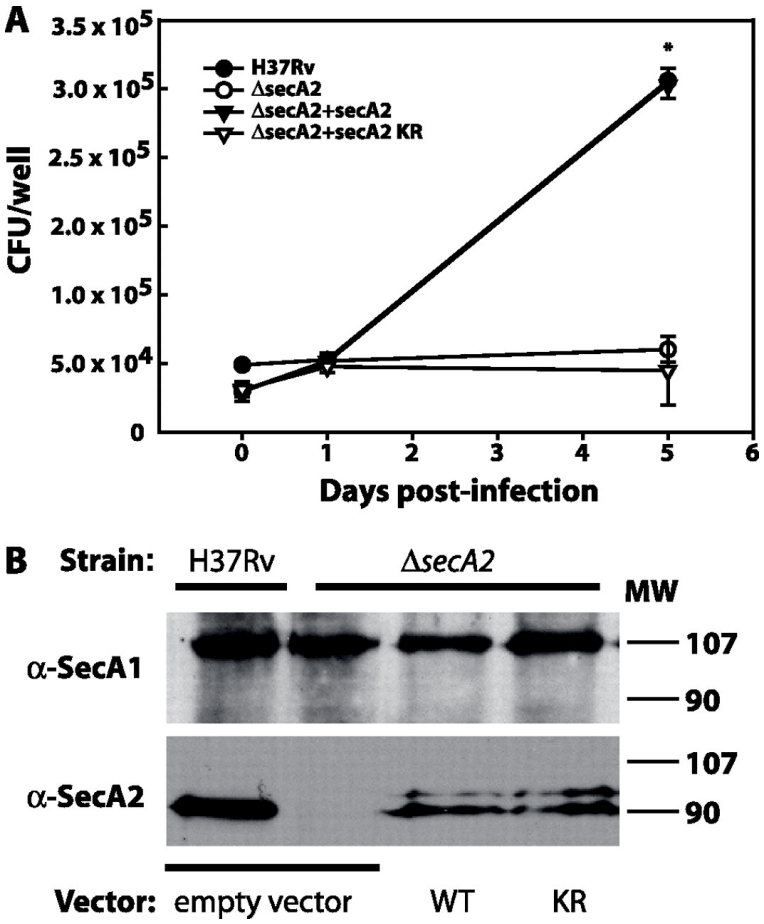

Figure 4.5. **A substitution in the Walker A motif of SecA2 affects biological activity in *M. tuberculosis*. (A)** The *secA2* (*K115R*) allele does not complement the *M. tuberculosis* Δ*secA2* mutant phenotype in macrophages. Murine bone marrow-derived macrophages were infected at a multiplicity of infection of 1.0 with the strain H37Rv, the Δ*secA2* mutant, the Δ*secA2* mutant complemented by the addition of WT *secA2*, (Δ*secA2*+*secA2*), and the Δ*secA2* mutant with *secA2* (*K115R*) (Δ*secA2*+*secA2 KR*). CFU were determined by plating macrophage lysates at various times post infection. The infection was performed with triplicate wells for each strain per time point, and the error bars represent means +/- standard deviations for the triplicate wells. The symbols for H37Rv and the complemented strain are overlapping at most time points in the graph presented. Data are representative of three independent experiments. *, data are statistically significantly different ($P<0.05$). **(B)** Expression of SecA1 and SecA2 in strains with different *secA2* alleles. Equal amounts of formalin-fixed whole-cell lysates of the *M. tuberculosis* H37Rv strain carrying an empty vector, the *M. tuberculosis secA2* mutant with an empty vector, the *secA2* mutant with the WT *secA2* integrated at the chromosomal *attB* site (WT), and the *secA2* mutant with the *secA2* (*K115R*) allele integrated at the *attB* site (*KR*) were run on SDS-polyacrylamide gels and subjected to Western blot analysis with anti-SecA1 or anti-SecA2 antibodies. The top panel shows SecA1 protein and the lower panel shows SecA2 protein. MW, molecular weight (in thousands).

References

1. **Archambaud, C., M. A. Nahori, J. Pizarro-Cerda, P. Cossart, and O. Dussurget.** 2006. Control of Listeria superoxide dismutase by phosphorylation. J. Biol. Chem. **281:**31812–31822.

2. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol. Microbiol. **44:**1081–1094.

3. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J. Bacteriol. **183:**6979–6990.

4. **Braunstein, M., B. J. Espinosa, J. Chan, J. T. Belisle, and W. R. Jacobs, Jr.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol. Microbiol. **48:**453–464.

5. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch. Microbiol. **189:**605–610.

6. **Chan, K. M., D. Delfert, and K. D. Junger.** 1986. A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal. Biochem. **157:**375–380.

7. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol. Microbiol. 53:843–856.

8. **de Keyzer, J., C. van der Does, and A. J. Driessen.** 2003. The bacterial translocase: a dynamic protein channel complex. Cell. Mol. Life Sci. **60:**2034–2052.

9. **de la Cruz, J., D. Kressler, and P. Linder.** 1999. Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families. Trends Biochem. Sci. **24:**192–198.

10. **Doyle, S. M., E. H. Braswell, and C. M. Teschke.** 2000. SecA folds via a dimeric intermediate. Biochemistry **39:**11667–11676.

11. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61:**136–169.

12. **Finn, B. E., X. Chen, P. A. Jennings, S. M. Saal'au-Bethell, and C. R. Matthews.** 1992. Principles of protein stability. Part 1. Reversible unfolding of proteins: kinetic and thermodynamic analysis, p. 168–189. *In* A. R. Rees, M. J. E. Sternberg, and R. Wetzel (ed.), Protein engineering: a practical approach. Oxford University Press, Oxford, United Kingdom.

13. **Franzblau, S. G., and E. B. Harris.** 1988. Biophysical optima for metabolism of *Mycobacterium leprae*. J. Clin. Microbiol. **26:**1124–1129.

14. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J. Bacteriol. **189:**5090–5100.

15. **Goldstein, A., and R. W. Barrett.** 1987. Ligand dissociation constants from competition binding assays: errors associated with ligand depletion. Mol. Pharmacol. **31:**603–609.

16. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* by using tetracycline repressors. J. Bacteriol. **189:**4614–4623.

17. **Hunt, J. F., S. Weinkauf, L. Henry, J. J. Fak, P. McNicholas, D. B. Oliver, and J. Deisenhofer.** 2002. Nucleotide control of interdomain interactions in the conformational reaction cycle of SecA. Science **297:**2018–2026.

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

19. **James, B. W., A. Williams, and P. D. Marsh.** 2000. The physiology and pathogenicity of *Mycobacterium tuberculosis* grown under controlled conditions in a defined medium. J. Appl. Microbiol. **88:**669–677.

20. **Klose, M., K.-L. Schimz, J. P. W. van der Wolk, A. J. M. Driessen, and R. Freudl.** 1993. Lysine 106 of the putative catalytic ATP-binding site of the *Bacillus subtilis* SecA protein is required for functional complementation of *Escherichia coli secA* mutants in vivo. J. Biol. Chem. **268:**4504–4510.

21. **Kurtz, S., and M. Braunstein.** 2005. Protein secretion and export in *Mycobacterium tuberculosis*. Horizon Bioscience, Norfolk, United Kingdom.

22. **Kurtz, S., K. P. McKinnon, M. S. Runge, J. P. Ting, and M. Braunstein.** 2006. The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. Infect. Immun. **74:**6855–6864.

23. **Lanzetta, P. A., L. J. Alvarez, P. S. Reinach, and O. A. Candia.** 1979. An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. **100:**95–97.

24. **Lee, V. T., and O. Schneewind.** 2001. Protein secretion and the pathogenesis of bacterial infections. Genes Dev. **15:**1725–1752.

25. **Lenz, L. L., S. Mohammadi, A. Geissler, and D. A. Portnoy.** 2003. SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. Proc. Natl. Acad. Sci. USA **100:**12432–12437.

26. **Lenz, L. L., and D. A. Portnoy. 2002.** Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol. Microbiol. **45:**1043–1056.

27. **Lill, R., W. Dowhan, and W. Wickner.** 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.

28. **McCann, J. R., J. A. McDonough, M. S. Pavelka, and M. Braunstein.** 2007. Beta-lactamase can function as a reporter of bacterial protein export during *Mycobacterium tuberculosis* infection of host cells. Microbiology **153:**3350–3359.

29. **Mitchell, C., and D. B. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol. Microbiol. **10:**483–497.

30. **Papanikolau, Y., M. Papadovasilaki, R. B. Ravelli, A. A. McCarthy, S. Cusack, A. Economou, and K. Petratos.** 2007. Structure of dimeric SecA, the *Escherichia coli* preprotein translocase motor. J. Mol. Biol. **366:**1545–1557.

31. **Randall, L. L., J. M. Crane, A. A. Lilly, G. Liu, C. Mao, C. N. Patel, and S. J. Hardy.** 2005. Asymmetric binding between SecA and SecB two symmetric proteins: implications for function in export. J. Mol. Biol. **348:**479–489.

32. **Sharma, V., A. Arockiasamy, D. R. Ronning, C. G. Savva, A. Holzenburg, M. Braunstein, W. R. Jacobs, Jr., and J. C. Sacchettini.** 2003. Crystal structure of *Mycobacterium tuberculosis* SecA, a preprotein translocating ATPase. Proc. Natl. Acad. Sci. USA **100:**2243–2248.

33. **Swillens, S.** 1995. Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. Mol. Pharmacol. **47:**1197–1203.

34. **Ulbrandt, N. D., E. London, and D. B. Oliver.** 1992. Deep penetration of a portion of *Escherichia coli* SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding. J. Biol. Chem. **267:**15184–15192.

35. **van der Wolk, J. P. W., M. Klose, E. Breukink, R. A. Demel, B. de Kruijff, R. Freudl, and A. J. M. Driessen.** 1993. Characterization of a *Bacillus subtilis* SecA mutant protein deficient in translocation ATPase and release from the membrane. Mol. Microbiol. **8:**31–42.

36. **Veenendaal, A., C. van der Does, and A. Driessen.** 2004. The protein-conducting channel SecYEG. Biochim. Biophys. Acta **1694:**81–95.

37. **Vrontou, E., and A. Economou.** 2004. Structure and function of SecA, the preprotein translocase nanomotor. Biochim. Biophys. Acta **1694:**67–80.

38. **Walker, J. E., A. Eberle, N. J. Gay, M. J. Runswick, and M. Saraste.** 1982. Conservation of structure in proton-translocating ATPases of *Escherichia coli* and mitochondria. Biochem. Soc. Trans. **10:**203–206.

39. **Wang, L., A. Miller, and D. A. Kendall.** 2000. Signal peptide determinants of SecA binding and stimulation of ATPase activity. J. Biol. Chem. **275:**10154–10159.

40. **World Health Organization.** March 2007, posting date. Fact sheets: tuberculosis, no. 104. WHO, Geneva, Switzerland. http://www.who.int/mediacentre/factsheets/fs104/en.

41. **Yu, L., H. Yang, Q. Ho, and P. C. Tai.** 2006. Expression, purification, and characterization of *Pseudomonas aeruginosa* SecA. Protein Expr. Purif. **50:**179–184.

42. **Zito, C. R., E. Antony, J. F. Hunt, D. B. Oliver, and M. M. Hingorani.** 2005. Role of a conserved glutamate residue in the *Escherichia coli* SecA ATPase mechanism. J. Biol. Chem. **280:**14611–14619.

Chapter 5

Examining the Relationship Between the General Sec Pathway (SecA1/SecYEG) and the Accessory SecA2 Pathway

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Protein export is an essential process in all bacteria. The bulk of proteins exported across the cytoplasmic membrane utilize the essential Sec pathway. SecA is an essential ATPase that drives unfolded pre-proteins through the membrane embedded SecYEG translocase. Increasingly, nonessential accessory SecA homologs are found in several bacterial species, including *Mycobacterium smegmatis*. Unlike the essential SecA1 proteins, these accessory SecA2 proteins are required to export only a small subset of proteins. However, the mechanism behind SecA2-mediated protein export is not known. In particular, it is not known if components of the essential SecA1 pathway or the accessory SecA2 pathway work together. To test this possibility, we examined the role of the essential SecA1 protein in exporting SecA2-dependent protein Msmeg1712 in *M. smegmatis*. Using a conditional SecA1 strain, we showed that depletion of SecA1 impairs the export of Msmeg1712 to the mycobacterial cell wall. This is the first direct demonstration that a SecA2-dependent protein (Msmeg1712) requires components of the essential SecA1 pathway

for export. Using this approach, we can now determine if other components of the essential Sec pathway are involved in exporting SecA2-dependent proteins.

Introduction

In bacteria, most exported proteins cross the cytoplasmic membrane through the highly conserved Sec pathway (11, 18). The Sec pathway specifically exports unfolded proteins through the membrane-embedded translocase composed of the proteins SecYEG (5). Energy for this system is provided by ATP hydrolysis by SecA, an essential ATPase that interacts with both the membrane-embedded SecYEG translocase and cytosolic pre-proteins destined for export (15, 17, 21). Through repeated cycles of ATP binding and hydrolysis, SecA undergoes conformational changes and drives export of substrate proteins across the cytoplasmic membrane (23).

Recently, the existence of two SecA homologs was reported in a small subset of Gram positive bacteria and mycobacteria (1, 3, 4, 6, 7, 16, 22, 25). In *M. smegmatis*, SecA1 is essential for growth and it is believed to function as the "housekeeping" SecA in the canonical Sec pathway (3). In contrast, SecA2 is not essential for growth and is only required for the export of a subset of proteins (13). In *M. smegmatis* there are two cell wall lipoproteins identified as requiring SecA2 for their export, Msmeg1704 and Msmeg1712. Both of these proteins have predicted Sec signal sequences.

The mechanism of SecA2-dependent protein export is not yet understood. A basic question to be answered is whether SecA2 works with a dedicated export apparatus of its own or the canonical Sec pathway (SecA1/SecYEG) to export its select substrates. The accessory SecA2 system in *Streptococcus gordonii* and *S. parasanguinis* includes an

accessory SecY2 protein for export (1, 29). In mycobacteria, there are no accessory SecY homologs (8). Without an obvious secretion apparatus or translocase, it is possible that SecA2 works with the canonical Sec pathway to promote export of its select subset of proteins.

If SecA1 and SecYEG are involved in SecA2 export, then the export of SecA2-dependent proteins should also be SecA1/YEG dependent. Because *secA1* is an essential gene, in order to test the role of SecA1 in the export of SecA2-dependent proteins a conditional *secA1* mutant is required (14). For this purpose we engineered a strain in which we can deplete SecA1 and simultaneously monitor the export of the SecA2-dependent protein Msmeg1712-HA to the cell wall. Here we show that in the absence of SecA1, export of Msmeg1712-HA to the cell wall is severely impaired. The export defect seen upon SecA1 depletion is comparable to the export defect observed for Msmeg1712-HA in a Δ*secA2* mutant. This result indicates that SecA1 is required for SecA2-dependent export of Msmeg1712-HA. This is the first experimental evidence indicating a role for the canonical Sec pathway in the accessory SecA2 pathway of any bacteria.

Materials and Methods

Bacterial strains and culture conditions. Middlebrook 7H9 or 7H10 medium (BD Biosciences), or Mueller Hinton medium (Difco, BD Biosciences) was used for growing mycobacteria. Middlebrook growth medium was supplemented with 0.2% (w/v) glucose, 0.5% (w/v) glycerol, and 0.1% (w/v) Tween 80 (Fisher). Mueller Hinton was supplemented with 0.1% (w/v) Tween 80. When needed, kanamycin (Acros Chemicals) or hygromycin (Roche) was added to medium at 20 μg/ml or 50 μg/ml, respectively. For SecA1-depletion

experiments, saturated *M. smegmatis* cultures were diluted to an OD_{600nm} of 0.1 in 100 ml of Mueller Hinton. Anhydrotetracycline (Sigma) was added to 600 ng/ml and the cultures were grown for ~21 hours at 37˚C. LB was used to grow *Escherichia coli* cultures. When needed, kanamycin or hygromycin was added at 40 μg/ml or 150 μg/ml, respectively. The identity of all vectors used in this study was confirmed by DNA sequencing (UNC-CH automated DNA sequencing facility and Eton Biosciences). The *secA1* depletion strain MSE10 and suicide plasmid pKIsecA1 were kind gifts from Sabine Ehrt (14).

Construction of JM693, a *M. smegmatis* Δ*secA2* mutant with *secA1* under control of P_{myc1}*tetO*. To construct a *secA1* depletion strain in a Δ*secA2* mutant background, NR116 was transformed with the suicide plasmid pKIsecA1, and hygromycin-resistant transformants were selected on 7H10 plates. Transformants were screened by Southern blot to detect integration of pKIsecA1 into the native *secA1* locus in the chromosome. Genomic DNA was isolated from *M. smegmatis* strains and digested with *Bam*HI and *Eco*RI. The probe used was a 410 bp *Sph*I-*Msc*I fragment from pKIsecA1. The probe was labeled with [^{32}P]dCTP using the Ready-to-Go labeling kit (Amersham).

Construction of pNR61. We wanted to construct a vector that encodes Msmeg1712 with a C-terminal HA epitope tag to monitor export of this protein. Additionally, we also wanted this plasmid to encode the rev TetR repressor protein to enable regulation of genes placed under control of the Tet promoter. Upon addition of anhydrotetracycline, rev TetR binds to Tet operator sequences in the Tet promoter, thus preventing expression of downstream genes (Tet OFF). To construct pNR61, a 2.4 kb *Nhe*I fragment containing rev *tetR* (r1.7) was cloned from pTEK-4S0X (14) (gift from Sabine Ehrt) into *Nhe*I-cut pHSG85 (13). pHSG85 encodes Msmeg1712-HA expressed from its native promoter.

Subcellular fractionation. Cell wall, membrane, and soluble fractions were prepared by differential ultracentrifugation as described previously (13). Briefly, 100 ml cultures of *M. smegmatis* grown in Mueller Hinton were harvested by centrifugation at 3,000 x *g*. Cell pellets were resuspended in 4 ml of breaking buffer (PBS, 0.6 μg/ml each of DNase and RNase, and a cocktail of protease inhibitors (2 μg/ml each of aprotinin, E-64, leupeptin, and pepstatin A and 100 μg/ml Pefabloc SC) and then lysed by five passages through a French pressure cell. Unbroken cells were pelleted at 3,000 x *g* for 20 min to generate a clarified whole cell lysate, which was centrifuged at 27,000 x *g* for 30 min to pellet the cell wall. The supernatant was centrifuged at 100,000 x *g* for 2 h to separate the membrane fraction from the soluble fraction. The cell wall and membrane fractions were washed once and then resuspended in PBS.

Immunoblot antibody conditions. The anti-SecA2, anti-GroEL (HAT5/IT-64 from the World Health Organization collection), anti-MspA (gift from Michael Niederweis, (19)), and anti-HA (Covance) antibodies were used at 1:20,000 dilutions. The anti-SecA1 antibody was used at a 1:50,000 dilution. For most experiments, secondary antibodies conjugated to HRP (Bio-Rad) were used along with ECL (Perkin Elmer) and film (Denville) to visualize blots. For quantitative immunoblots, secondary antibodies conjugated to AP (Amersham/GE Healthcare) were used in conjunction with the ECF detection system. The blots were visualized using a phosphorimager and quantified using ImageQuant 5.2 (Molecular Dynamics).

Results

Construction of SecA1 depletion strains. In order to study the potential contribution of SecA1 in the process of SecA2-dependent export, a conditional *secA1* strain was required. Recently, strain MSE10 was constructed in *M. smegmatis* where the chromosomal *secA1* is under the control of a tetracycline operator/repressor system (14). Using the Tet OFF system, when MSE10 is grown in the presence of anhydrotetracycline (Atc) the amount of SecA1 is diminished as shown by western blot (data not shown) (12). In previous studies, MSE10 was used to show depletion of SecA1 prevents export of the cell wall porin protein MspA. MspA has a classical Sec signal sequence, and therefore its export was expected to depend on the canonical Sec pathway. As expected due to the established essential nature of SecA1, depletion of SecA1 protein also inhibits growth of *M. smegmatis*. This strain will allow us to determine if depletion of SecA1 influences export of SecA2-dependent proteins.

For this study, we similarly constructed a SecA1-depletion strain in a Δ*secA2* mutant background. This strain will allow us to determine the combined effect of removing SecA1 and SecA2 on export of SecA2-dependent proteins. As shown in Figure 5.1A, pKIsecA1 (gift from Sabine Ehrt) contains a mutant *secA1* allele with a hygromycin insertion and a wild-type *secA1* allele under control of the Tet operator (TetO). We electroporated pKISecA1 into the Δ*secA2* mutant strain NR116 and selected for transformants on 7H10 plates supplemented with hygromycin (50 μg/ml). Since pKISecA1 does not replicate in mycobacteria, it must integrate into the chromosome at the *secA1* locus (Figure 5.1B and C). Successful construction of the resulting strain, JM693, was confirmed by Southern analysis (Figure 5.1D).

With *secA1* cloned behind the Tet operator in strain MSE10 and JM693, we then further engineered the strains so they expressed Msmeg1712-HA and the revTetR repressor. The revTetR binds to TetO only in the presence of the inducer molecule Atc thereby blocking expression of SecA1 (12). We cloned both these elements, constitutively expressed revTetR and Msmeg1712-HA, onto a single mycobacteria plasmid pNR61. MSE10 and JM693 were electroporated with pNR61 and transformants were recovered on 7H10 plates supplemented with hygromycin (50 μg/ml) and kanamycin (40 μg/ml). Next, these transformants were grown in 100 ml cultures of Mueller Hinton with or without addition of Atc (600 μg/ml) for 21 hours. Cells were harvested by centrifugation and lysed in a French pressure cell. To monitor export of Msmeg1712-HA in these strains, we prepared subcellular fractions by differential ultracentrifugation and analyzed them by SDS-PAGE and immunoblot with anti-HA antibodies.

Addition of Atc resulted in greater than 95% reduction of SecA1, as determined by densitometry (Figure 5.2). This outcome was observed in both wild-type (Figure 5.2A) and Δ*secA2* mutant (Figure 5.2B) backgrounds. Importantly, under the conditions we used depletion of SecA1 did not have any effect on the expression of control proteins GroEL or MspA nor was there a growth defect of the strains. This showed our Atc treatment conditions were specifically influencing SecA1 depletion and not having a general effect on total protein levels. As reported previously, MspA was not exported to the cell wall fraction of *M. smegmatis* when SecA1 was depleted (14). This important control showed that depletion of SecA1 in our experiments had the expected effect on export of a SecA1-dependent protein.

Depletion of SecA1 inhibits export of Msmeg1712-HA to the cell wall. The SecA1-depletion strains allowed us to assess the contribution of SecA1 in exporting SecA2-dependent proteins. Strikingly, when SecA1 was depleted from wild-type *M. smegmatis*, Msmeg1712-HA did not localize to the cell wall fraction (Figure 5.2A). Quantification of our blots showed a 10-fold reduction of Msmeg1712-HA in the cell wall fraction of the SecA1 depleted strain MSE10 compared to the wild-type strain. Furthermore, Msmeg1712-HA accumulated in the soluble cytoplasmic fraction. Accumulation of Msmeg1712-HA in the soluble fraction is consistent with the protein accumulating in the cytosol due to a defect in its export in the absence of SecA1. As a control, we monitored the export of MspA, a known SecA1 substrate (14). Upon SecA1 depletion, export of MspA to the cell wall fraction was abolished. Some MspA was detected in the soluble fraction; we believe this corresponded to newly synthesized MspA that is awaiting export. As a quality control check on our subcellular fractions, we also analyzed the localization of GroEL, a known cytoplasmic chaperone. As expected, GroEL was found in the soluble cytoplasmic fraction, indicating that our cell wall fractions are not contaminated (Figure 5.2).

As shown previously, in the *M. smegmatis* Δ*secA2* mutant Msmeg1712-HA did not localize to the cell wall (Figure 5.2B) (13). By comparing the Δ*secA2* mutant without depletion (-Atc) to the wild-type strain with SecA1 depletion (+ Atc), the amount of Msmeg1712-HA in the cell wall of these two strains is similar (Figure 5.2C). We also tested whether the Msmeg1712-HA export defect of a Δ*secA2* mutant is exacerbated by depletion of SecA1. Depletion of SecA1 in the Δ*secA2* mutant background resulted in an export defect that was no greater than that seen when just Δ*secA2* was deleted (i.e. in absence of Atc treatment) (Figure 5.2B and C). Taken together, these results indicated that SecA1 and

SecA2 are both required for optimal export of Msmeg1712-HA. On their own, neither SecA1 nor SecA2 are sufficient to support export of Msmeg1712-HA. These data are the first to show the canonical SecA1 has a role in exporting a substrate of the accessory SecA2 pathway.

Discussion

In this Chapter, we established a clear role for the essential SecA1 in the SecA2-dependent process of exporting Msmeg1712-HA. Since SecA1 is essential, this analysis required construction of a conditional *secA1* strain (14). When SecA1 expression was depleted in wild-type *M. smegmatis*, Msmeg1712-HA was no longer efficiently exported to the cell wall. This export defect is comparable to that exhibited by a Δ*secA2* mutant (13). Additionally, the effect of depleting SecA1 in the absence of SecA2 did not have an additive effect on Msmeg1712-HA export. Msmeg1712-HA is the first example of a protein that requires both SecA1 and SecA2 for export from the cytoplasm. Because of the need of a conditional *secA1* mutant to ask this question, it has remained unaddressed in any accessory SecA2 system until now.

While our data showed a requirement for the canonical SecA1 in mycobacterial SecA2 export, the precise nature of the role of SecA1 is not clear. We do not know if SecA1 directly interacts with SecA2, or if the role of SecA1 is indirect. One possibility is that SecA1 and SecA2 form a heterodimer. In *E. coli*, SecA forms dimers, although the exact role of dimers in protein translocation remains in dispute (10, 20, 28). It is possible that in mycobacteria, SecA2 forms heterodimers with SecA1 to export a subset of proteins like

Msmeg1712. However, there is no evidence of a physical interaction between SecA1 and SecA2.

In Chapter 3, we showed that SecA1 is equally distributed between cell envelope and cytoplasmic fractions. This localization likely reflects the dynamic role of SecA1 reversibly associating with the SecYEG translocase to export proteins across the cytoplasm. In contrast, SecA2 is found predominantly in the cytoplasmic fraction. This finding could reveal something about the relationship between SecA1 and SecA2. Perhaps the role of SecA2 is to recognize pre-protein substrates in the cytoplasm that are normally overlooked by SecA1. Upon recognition, SecA2 then delivers these pre-proteins to the SecA1/SecYEG translocase complex. Another possibility is that SecA2 works with SecA1 to promote export via a novel translocase. To examine the potential role of the SecYEG translocase in export of Msmeg1712-HA, the construction of a SecY depletion strain followed by experiments to monitor the effect depletion has on export of SecA2-dependent proteins is planned.

Although less likely, another formal possibility is that SecA2-dependent proteins cross the cytoplasmic membrane through a novel translocase that is assembled by SecA1. There are a few experiments suggesting SecA can be involved in the assembly of some domains of multi-spanning integral membrane proteins (26). However, this role of SecA appears rare. There are integral membrane proteins well-demonstrated not to require SecA for localization and it is the SRP GTPase that is the requisite secretion factor for membrane protein localization in bacteria (24, 27). Furthermore, there is no evidence that SecA1 is required for assembling cytoplasmic membrane proteins in mycobacteria.

Unlike the accessory SecA2/Y2 system in Gram positive bacteria like *S. gordonii*, the accessory SecA2 system in mycobacteria lacks an obvious translocase or an accessory SecY

homolog (8, 9, 22). Without other obvious candidates, we think it is an attractive possibility that SecA2-dependent proteins are exported with the assistance of the SecA1/SecYEG translocase. Clearly, identifying proteins that interact with SecA2 will be important for fully understanding the relationship between the essential SecA1/SecYEG pathway and the accessory SecA2 pathway.

In mycobacteria, the features of a pre-protein substrate that dictate export via SecA2 remain poorly understood. In *M. smegmatis*, Msmeg1704 and Msmeg1712 were identified as being dependent on SecA2 for export from the cytoplasm (13). Interestingly, Msmeg1704 and Msmeg1712 are both predicted lipoproteins. Perhaps post-translational modifications help direct or limit Msmeg1704 and Msmeg1712 to the accessory SecA2 pathway. In *S. gordonii*, post-translational glycosylation is clearly an important signal for blocking GspB export via the canonical Sec system (2). There is no evidence that Msmeg1704 and Msmeg1712 are similarly glycosylated. However, it is interesting to speculate that the lipid modifications of Msmeg1704 and Msmeg1712 could be unique determinants that specify the need for SecA2 to export these proteins. It is important to point out that not all lipoproteins are SecA2-dependent as *M. tuberculosis* LpqH and *M. smegmatis* PhoA are proven lipoproteins demonstrated to be exported normally in a Δ*secA2* mutant of *M. smegmatis* (13). Experiments are underway to determine the elements that direct pre-proteins for export via SecA2. Taken together, we propose a model where the majority of pre-proteins require SecA1 for export, while a small subset of proteins require both SecA1 and SecA2 for export (Figure 5.3). We have pursued genetic and biochemical strategies in an attempt to identify SecA2-interating proteins. Clearly, identification of SecA2-interacting proteins will be

critical to distinguishing between these different possible models for SecA2-mediated protein export in mycobacteria.

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Attributions

All of the experiments described here were performed by me with the following exception. JM693, a Δ*secA2* mutant with *secA1* under control of P_{myc1}*tetO*, was constructed by Justin McDonough.

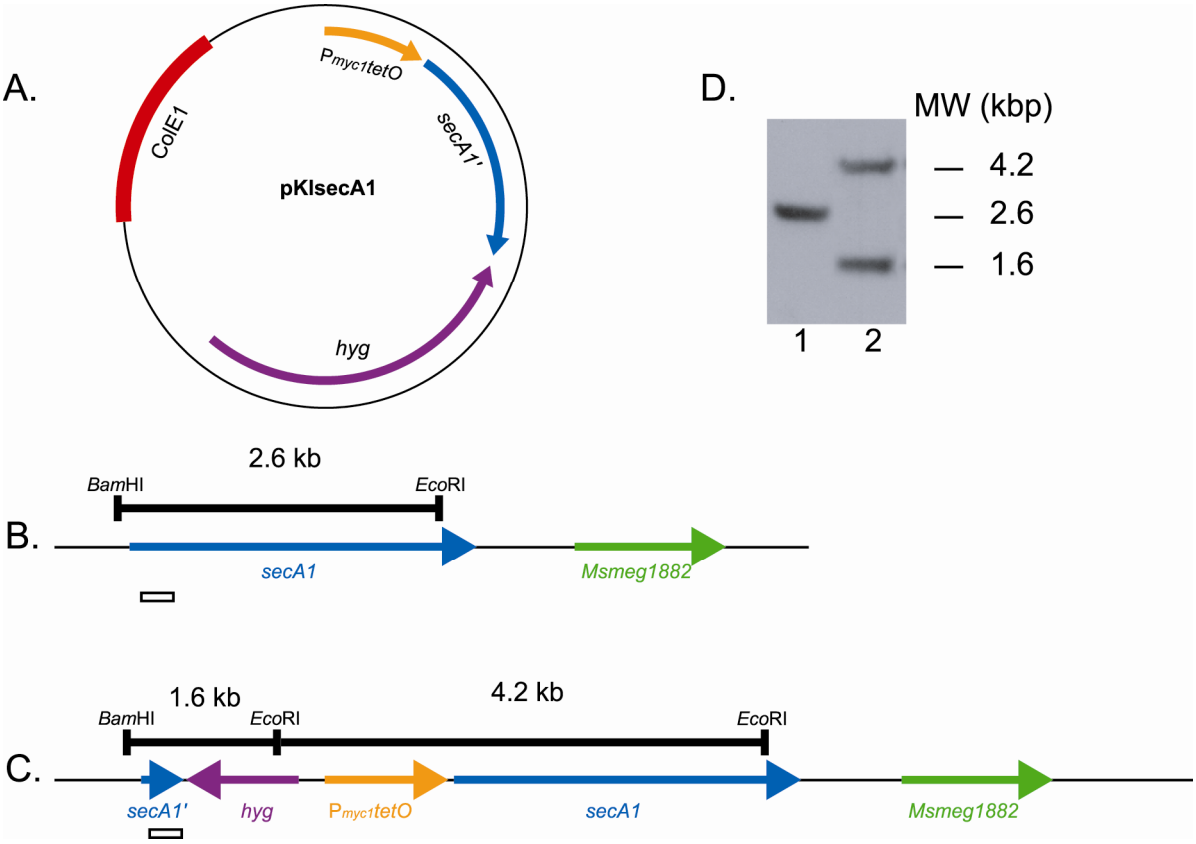

Figure 5.1. A SecA1 depletion strain was constructed in a Δ*secA2* mutant background. **(A)** Map of suicide plasmid pKIsecA1. **(B)** Diagram of the *secA1* locus in mc^{2}155. **(C)** Diagram of the *secA1* locus upon integration of pKIsecA1. The bar below each line indicates site of probe hybridization. **(D)** Southern blot of genomic DNA digested with *Bam*HI and *Eco*RI. Restriction sites and predicted fragment sizes are indicated in B and C. Lane 1: mc^{2}155, Lane 2: JM693.

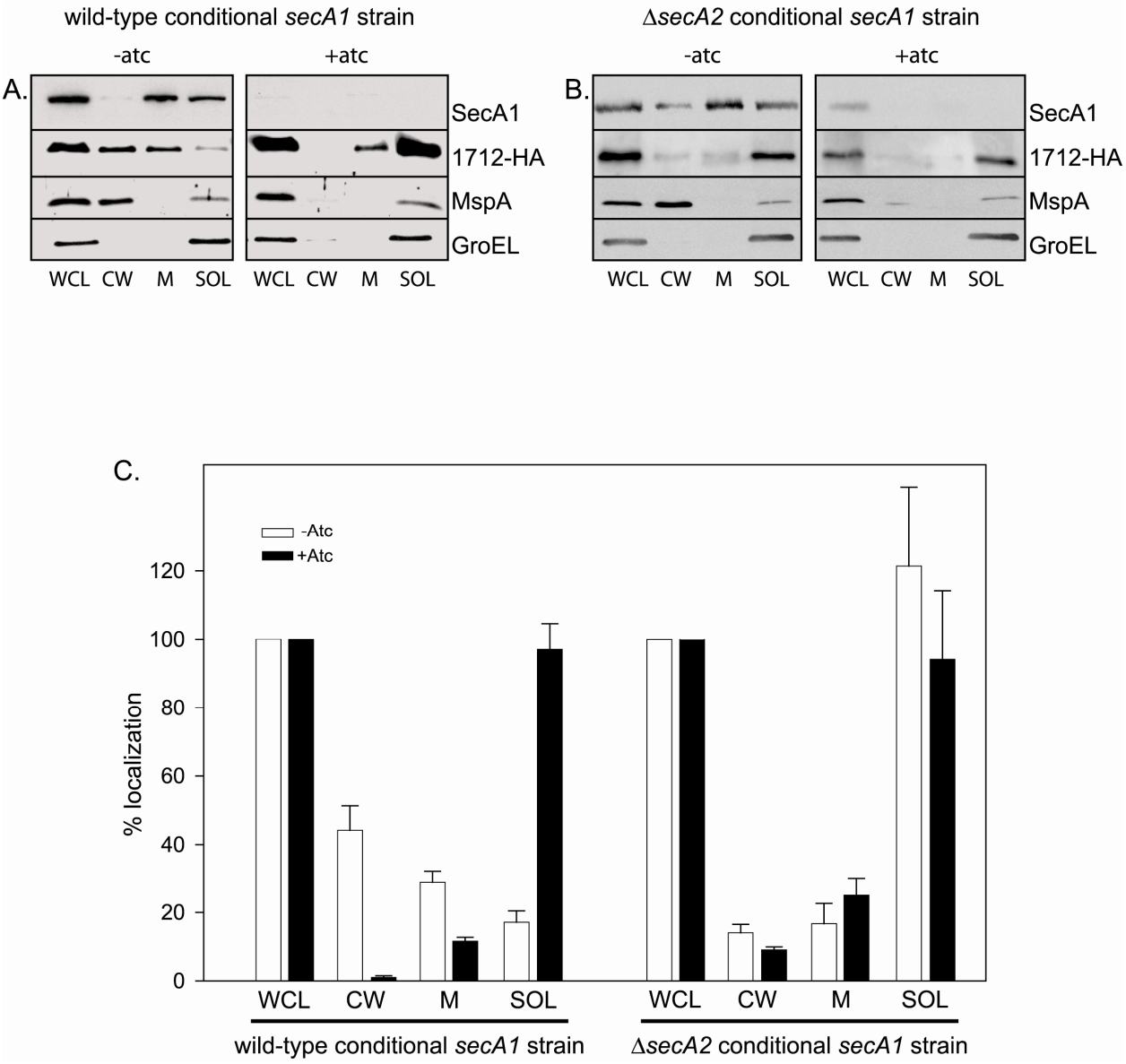

Figure 5.2. Expression of both SecA1 and SecA2 is required for export of Msmeg1712-HA. SecA1 was depleted from cultures of **(A)** wild-type or **(B)** Δ*secA2* mutant *M. smegmatis* by addition of 600 ng/ml Atc for 21 hours. Cell wall (CW), membrane (M), and soluble (SOL) subcellular fractions were prepared from these cultures. For each fraction, protein derived from an equal number of starting cells was analyzed by SDS-PAGE and western blot using the indicated antibodies. **(C)** The percent localization of Msmeg1712-HA from **(A)** and **(B)** was quantified using a phosphorimager and is reported as the percent of starting WCL. The results are the mean of three independent experiments +/- standard error.

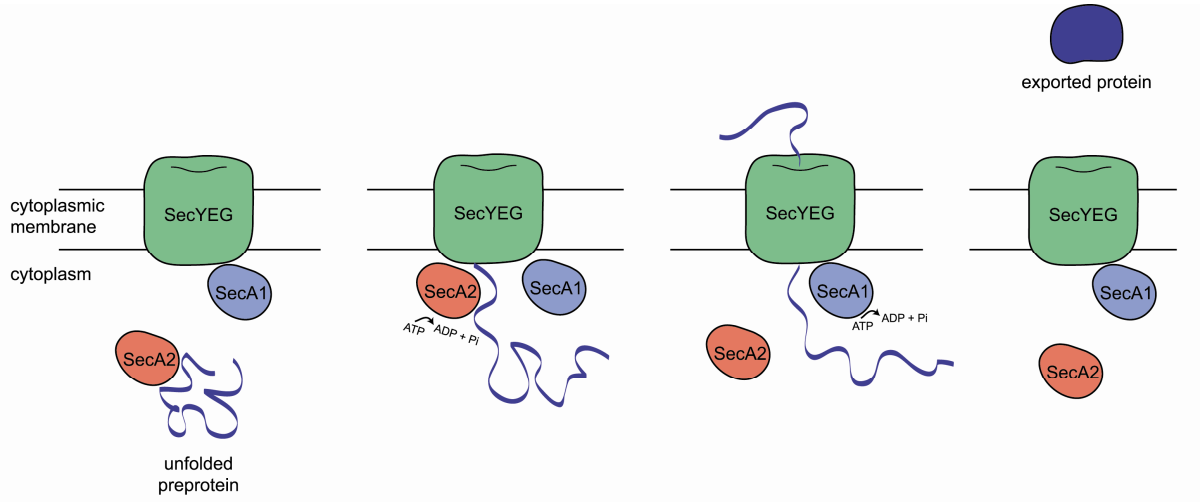

Figure 5.3. Proposed model for SecA2-mediated protein export. SecA2 recognizes pre-proteins in the cytoplasm, and delivers them to the SecA1/SecYEG translocase. Upon ATP binding and hydrolysis, SecA2 undergoes conformational changes to initiate the export cycle. SecA1 finishes protein export through multiple rounds of insertion/deinsertion. Once across the cytoplasmic membrane, the exported protein can fold into its mature conformation.

References

1. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol **44:**1081-94.

2. **Bensing, B. A., D. Takamatsu, and P. M. Sullam.** 2005. Determinants of the streptococcal surface glycoprotein GspB that facilitate export by the accessory Sec system. Mol Microbiol **58:**1468-81.

3. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J Bacteriol **183:**6979-90.

4. **Braunstein, M., B. Espinosa, J. Chan, J. T. Belisle, and W. R. J. Jacobs.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol **48:**453-64.

5. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. Driessen, and W. Wickner.** 1990. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649-57.

6. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch Microbiol **189:**605-10.

7. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol Microbiol **53:**843-56.

8. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.

9. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M. A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward, and B. G. Barrell.** 2001. Massive gene decay in the leprosy bacillus. Nature **409:**1007-11.

10. **Das, S., E. Stivison, E. Folta-Stogniew, and D. Oliver.** 2008. Reexamination of the role of the amino terminus of SecA in promoting its dimerization and functional state. J Bacteriol **190:**7302-7.

11. **Driessen, A. J., and N. Nouwen.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem **77:**643-67.

12. **Ehrt, S., X. V. Guo, C. M. Hickey, M. Ryou, M. Monteleone, L. W. Riley, and D. Schnappinger.** 2005. Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. Nucleic Acids Res **33:**e21.

13. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J Bacteriol **189:**5090-100.

14. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* using tetracycline repressors. J Bacteriol **189:**4614-23.

15. **Hendrick, J. P., and W. Wickner.** 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:**24596-600.

16. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol Microbiol **45:**1043-56.

17. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol Microbiol **10:**483-97.

18. **Murphy, C. K., and J. Beckwith.** 1996. Export of proteins to the cell envelope in *Escherichia coli*., p. 967-978. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*. ASM Press, Washington, D.C.

19. **Niederweis, M., S. Ehrt, C. Heinz, U. Klocker, S. Karosi, K. M. Swiderek, L. W. Riley, and R. Benz.** 1999. Cloning of the *mspA* gene encoding a porin from *Mycobacterium smegmatis*. Mol Microbiol **33:**933-45.

20. **Or, E., and T. Rapoport.** 2007. Cross-linked SecA dimers are not functional in protein translocation. FEBS Lett **581:**2616-20.

21. **Papanikou, E., S. Karamanou, C. Baud, M. Frank, G. Sianidis, D. Keramisanou, C. G. Kalodimos, A. Kuhn, and A. Economou.** 2005. Identification of the preprotein binding domain of SecA. J Biol Chem **280:**43209-17.

22. **Rigel, N. W., and M. Braunstein.** 2008. A new twist on an old pathway--accessory Sec systems. Mol Microbiol **69:**291-302.

23. **Schiebel, E., A. J. Driessen, F. U. Hartl, and W. Wickner.** 1991. Delta mu H+ and ATP function at different steps of the catalytic cycle of preprotein translocase. Cell **64:**927-39.

24. **Serek, J., G. Bauer-Manz, G. Struhalla, L. van den Berg, D. Kiefer, R. Dalbey, and A. Kuhn.** 2004. *Escherichia coli* YidC is a membrane insertase for Sec-independent proteins. EMBO J **23:**294-301.

25. **Siboo, I. R., D. O. Chaffin, C. E. Rubens, and P. M. Sullam.** 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. J Bacteriol **190:**6188-96.

26. **Urbanus, M. L., P. A. Scotti, L. Froderberg, A. Saaf, J. W. de Gier, J. Brunner, J. C. Samuelson, R. E. Dalbey, B. Oudega, and J. Luirink.** 2001. Sec-dependent membrane protein insertion: sequential interaction of nascent FtsQ with SecY and YidC. EMBO Rep **2:**524-9.

27. **Wagner, S., O. Pop, G. J. Haan, L. Baars, G. Koningstein, M. M. Klepsch, P. Genevaux, J. Luirink, and J. W. de Gier.** 2008. Biogenesis of MalF and the MalFGK(2) maltose transport complex in *Escherichia coli* requires YidC. J Biol Chem **283:**17881-90.

28. **Wang, H., B. Na, H. Yang, and P. C. Tai.** 2008. Additional in vitro and in vivo evidence for SecA functioning as dimers in the membrane: dissociation into monomers is not essential for protein translocation in *Escherichia coli*. J Bacteriol **190:**1413-8.

29. **Wu, H., S. Bu, P. Newell, Q. Chen, and P. Fives-Taylor.** 2007. Two gene determinants are differentially involved in the biogenesis of Fap1 precursors in *Streptococcus parasanguis*. J Bacteriol **189:**1390-8.

Chapter 6

Efforts to identify SecA2-interacting proteins: SecA2-HIS and SecA2 K129R-HIS

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The accessory SecA2 pathway is an example of a specialized protein export system in mycobacteria and some Gram positive bacteria. The mechanism of SecA2-mediated protein export is not well understood. For example, it is not known how proteins exported via SecA2 cross the bacterial cytoplasmic membrane. In order to clarify this process, we attempted to identify SecA2-interacting proteins using protein pull-down experiments. We successfully expressed and purified SecA2 with a C-terminal 6xHIS tag from *M. smegmatis*. To preserve and identify proteins that transiently interact with SecA2, we used in vivo and in vitro crosslinking. Crosslinked protein complexes were analyzed by mass spectrometry. While we were unable to identify SecA2-interacting proteins, we provide evidence that SecA2 forms oligomers. We also explored using a dominant negative mutant SecA2 protein (SecA2 K129R) to identify SecA2-interacting proteins. SecA2 K129R localized to the cell envelope

fraction, and was purified upon detergent solubilization. Taken together, our results provide the basis for future experiments to identify SecA2-interacting proteins.

Introduction

In mycobacteria and some Gram positive bacteria, export of a subset of proteins depends on an accessory SecA2 export system (1, 3, 4, 6, 7, 16, 21). The mechanism by which SecA2-dependent proteins are exported from the cytoplasm is only beginning to be investigated. In SecA2/Y2 systems of Gram positive organisms like *Streptococcus gordonii*, SecA2 is encoded near the *secY2* and *asp* genes whose products are involved in SecA2-dependent protein export (1, 20). For example, disruption of *S. gordonii secY2* prevents export the SecA2-dependent protein GspB (1). SecY2 is predicted to possess transmembrane domains, and share sequence similarity with the essential SecY protein that is part of the canonical SecYEG translocase. Asp2 (for *A*ccessory *s*ecretory *p*rotein) also contains a predicted transmembrane domain. Although Asp2 is required for GspB export, the function of this protein has not yet been determined (23). Two other ORFs, *asp4* and *asp5*, encode proteins with homology to SecE and SecG, respectively. Disruption of these two genes also prevents GspB export (24). By working together, SecY2 and the Asp proteins may form a membrane-embedded channel through which SecA2-dependent exported proteins may pass.

In mycobacteria there is no extra SecY (8, 9). Furthermore, the genes adjacent to *secA2* in mycobacteria do not encode proteins with homology to the Asp proteins, and they do not have any known roles in SecA2-dependent protein export (20). Three nearby genes are predicted to encode proteins with transmembrane domains, but the function of these proteins remains untested. Without knowing the components of the mycobacterial SecA2

export pathway, our understanding of this important system is incomplete. Therefore, we sought to identify SecA2-interacting proteins using protein pull-down assays. To date, SecA2-interacting proteins have yet to be identified in any accessory SecA2 system.

SecA2 was engineered to contain a C-terminal histidine (6xHIS) tag, and expressed in *M. smegmatis*. As interactions with SecA2 might be transient, we used a membrane permeable crosslinking reagent to increase the odds of finding an interacting protein. Samples were crosslinked, lysed, and subject to affinity purification using nickel resin. SecA2-HIS crosslinked complexes were eluted and analyzed by SDS-PAGE, western blot, and mass spectrometry.

These experiments demonstrated SecA2-HIS forms oligomers upon crosslinking. The essential SecA of *Escherichia coli* forms dimers, although the importance of dimerization for function of this protein is still a matter of debate (10, 15, 18). This is the first demonstration of oligomerization of an accessory SecA2 protein from any system.

In the above experiments, no proteins other than SecA2 were identified as being bound to SecA2. Therefore, we developed an alternative strategy to identify SecA2-interactors. The approach involves the dominant negative SecA2 K129R protein, which we showed previously to become trapped at the membrane in a presumed protein complex. We believe the SecA2 K129R-HIS tagged system we established will be a valuable tool for future efforts to indentify SecA2-interacting proteins in *M. smegmatis*.

Materials and Methods

Bacterial strains and growth conditions. Middlebrook 7H9 or 7H10 medium (BD Biosciences) was used for growing *M. smegmatis*. This growth medium was supplemented

with 0.2% (w/v) glucose, 0.5% (w/v) glycerol, and 0.1% (w/v) Tween 80 (Fisher). In other experiments, Mueller Hinton (BD Biosciences) was used to grow *M. smegmatis*. When needed, kanamycin (Acros Chemicals) or hygromycin (Roche) was added to the medium at 20 μg/ml or 50 μg/ml, respectively. Luria Bertani (LB) was used to grow *E. coli* cultures. When needed, kanamycin or hygromycin was added at 40 μg/ml or 150 μg/ml, respectively. The identity of all vectors used in this study was confirmed by DNA sequencing (UNC-CH automated DNA sequencing facility and Eton Biosciences).

Construction of HIS-tagged SecA2. During the course of our studies, we had reason to believe that the start codon of *M. smegmatis* SecA2 was different from the one originally annotated. We constructed pHSG86 to reflect this change. A segment of *secA2* was PCR amplified using primers 5'-GAATCACTTCGTGCCGAAGACGTCCAGC-3' and 5'-GCGGAACACACCCGGGAGGCT-3' and cloned into pCR2.1 (Invitrogen) yielding pHSG81. A 1.4 kbp *Xmn*I-*Nar*I fragment of pHSG81 was cloned into similarly digested pYA810 giving plasmid pHSG86. pHSG86 carries *secA2* driven off of the constitutive *hsp*60 promoter, but now has the predicted correct start codon. To C-terminally tag SecA2 with 6xHIS, primers 5'-CGAATTCCTCGGCGCCGTCACCGTGT-3' and 5'-CGAATTCAGTGGTGGTGGTGGTGGTGGCGGAACACACCCGGCAGG-3' were used to amplify a 0.9 kbp fragment of *secA2* (the codons for histidine are underlined). The resulting fragment was cloned into pCR2.1 yielding pHSG82. A 0.9 kbp *Nar*I-*Eco*RI fragment from pHSG82 was cloned into similarly cut pYA810 to yield pHSG87. Lastly, pHSG87 was digested with *Nar*I-*Eco*RI and the resulting 0.9 kbp fragment was cloned into similarly cut pHSG86, yielding plasmid pHSG93. pHSG93 encodes SecA2-HIS using the predicted correct start codon and tagged at the C-terminus with 6xHIS.

Azide assay. 200 µl of saturated (OD_{600nm} = 2.0) *M. smegmatis* culture was mixed with 3.5 ml of molten 7H9 top agar, and then poured onto a 7H10 bottom agar plate. After the top agar cooled, sterile 6 mm filter discs were placed onto the surface. 10 µl of 0.15 M sodium azide was then added to the disc. The plates were inverted and incubated for 2 days at 37˚C. After incubation, any resulting zone of inhibition on the plate was measured. Untreated plates were included as a control. Each strain was tested in triplicate.

Purification of SecA2-HIS from *M. smegmatis*. In a previous study, the cytoplasmic chaperone GroEL1 was shown to bind to nickel resin by virtue of a histidine-rich C-terminus (17). To avoid contamination of SecA2-HIS with GroEL1 and GroEL1-interacting proteins, we purified SecA2-HIS from a Δ*groEL1* mutant strain (kind gift from Dr. Graham Hatfull, University of Pittsburgh). The Δ*groEL1* mutant was electroporated with pHSG93 to generate strain NR151. 100 ml cultures of NR151 grown in Muller Hinton were harvested at OD_{600nm} = 1.0. The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed in a French pressure cell at 20,000 psi. Cellular debris was removed by centrifugation. The resulting lysate was mixed with Ni-NTA resin (QIAGEN) and mixed on a rocker at 4˚C for 1 hour. The beads were collected in a column and washed three times with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, pH 8.0). Proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and concentrated using Microcon spin columns with a 10 kDa cutoff (Millipore).

Crosslinking experiments. To crosslink SecA2-HIS with any interacting proteins, dithiobis(succinimidylpropionate) (DSP, Pierce) was used at a final concentration of 4 mM according to the manufacturer's instructions. DSP was added to the concentrated cell pellet

prior to lysis (in vivo crosslinking) or after lysis (in vitro crosslinking). Crosslinked proteins were purified as described above. To reverse the crosslinks, samples were boiled in SDS-PAGE sample buffer containing 5% β-mercaptoethanol (BME).

Construction and purification of SecA2 K129R-HIS. Since SecA2 K129R is toxic to *M. smegmatis*, SecA2 K129R-HIS was placed under control of an inducible Tet promoter (Tet ON) (13). Primers 5'-AGGATCCATCCGGAGGAATCACTT-3' and 5'-AGGATCCCTAGTGGTGGTGGTGGTGGTGGCGGAACACACCCGGCAGG-3' were used to amplify SecA2 from pHSG86. The resulting 2.4 kbp fragment was cloned into pCC1 yielding plasmid pNR49. A 2.4 kbp *Bam*HI fragment was cloned from pNR49 into similarly cut pMP715 (gift from Dr. Martin Pavelka, University of Rochester) generating plasmid pNR50. This plasmid has wild-type *secA2* cloned downstream of the Tet operator (TetO). Additionally, pNR50 also encodes the TetR repressor. To mutate the Walker A lysine 129 residue to arginine, site-directed mutagenesis was performed using primers 5'-CGGGTGAGGGCAGGACGCTGGC-3' and 5'-GCCAGCGTCCTGCCCTCACCCG-3'to generate pNR54. pNR54 was electroporated into wild-type mc^2155 and Δ*secA2* mutant NR116 to yield strains NR274 and NR275, respectively. To induce expression of SecA2 K129R-HIS, cultures were grown in Mueller Hinton broth at 37˚C and treated with 300 ng/ml anhydrotetracycline for 21 hours. Following induction, bacterial cells were harvested and processed into cell envelope and cytoplasmic fractions by differential ultracentrifugation as described previously (12). Cell envelopes were solubilized using a panel of denaturants and then pooled with the soluble fraction prior to affinity purification.

Mass spectrometric identification of proteins. All analyses were performed by the UNC-Duke Proteomics Core Facility at UNC-Chapel Hill.

Results

SecA2-HIS is functional and highly expressed in *M. smegmatis*. We generated a vector that overexpressed SecA2-HIS protein. Comparison of the annotated *M. smegmatis* SecA2 sequence to other mycobacterial SecA2 proteins revealed an extension at the extreme N-terminus (data not shown). We noticed a GTG codon internal to the currently annotated start codon that would result in a protein 15 amino acids shorter at the N-terminus. By using this internal start codon and putting *secA2* under control of the constitutive *hsp60* promoter, we created pHSG93, a SecA2-HIS expression vector in *M. smegmatis*. When electroporated into wild-type or Δ*secA2* mutant *M. smegmatis*, pHSG93 highly expressed SecA2-HIS compared to SecA2 expression from the chromosome as shown by immunoblot using anti-HIS or anti-SecA2 antibodies (Figure 6.1A). To ensure that SecA2-HIS with the small N-terminal truncation is still functional, we tested the ability of pHSG93 to complement the azide hypersensitivity of the Δ*secA2* mutant. Resistance to azide was restored to wild-type levels when the Δ*secA2* mutant was transformed with pHSG93 (Figure 6.1B). From these data, we conclude that the N-terminal extension is dispensable for normal SecA2 function and that SecA2-HIS is a fully functional protein in *M. smegmatis*.

Mass spectrometric analysis of cross-linked species indicates the existence of SecA2-HIS multimers. We purified SecA2-HIS from whole cell lysates using nickel-affinity columns (Figure 6.2A). In order to increase the chances of identifying SecA2-HIS interacting proteins, we employed the membrane-permeable covalent crosslinking reagent DSP (dithiobis(succinimidylpropionate)). DSP is commonly used to preserve transient protein-protein interactions by reacting with primary amine groups on amino acids.

Crosslinking reagents have been successfully used to identify interactions among components of protein secretion systems. We performed DSP crosslinking either before (in vivo) or after (in vitro) lysing *M. smegmatis* cells that expressed SecA2-HIS. Cultures carrying an empty vector were processed in parallel as a negative control. Crosslinked proteins were purified from soluble whole cell lysates using nickel-affinity columns and analyzed by SDS-PAGE and Coomassie staining (Figure 6.2B). Where indicated, protein samples were treated with 5% BME prior to loading onto a gel; treatment with BME breaks the DSP crosslinks and will liberate any SecA2-HIS-interacting protein.

SecA2-HIS is readily visible at the predicted molecular weight of 89 kDa. We also observed multiple bands larger than monomeric SecA2-HIS in the crosslinked samples (Figure 6.2B). DSP crosslinking yielded three SecA2-containing products, as shown by immunoblotting with anti-SecA2 antibody (Figure 6.2C). We repeated this experiment with both in vivo and in vitro DSP crosslinking on strains that carried the SecA2-HIS expression plasmid or an empty vector for analysis by mass spectrometry. In an effort to avoid false positives, we selected bands present in the SecA2-HIS lanes but absent in the vector control for further analysis (band marked with * in Figure 6.2D). These bands were excised and digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF MS and by LC-ESI-MS/MS (UNC-Duke Proteomics Core). The data was matched to *M. smegmatis* proteins with the MASCOT search engine (19). In all cases, SecA2 was the only protein identified. This suggests the crosslinked products are SecA2 oligomers. *E. coli* SecA is reported to dimerize although it remains controversial if SecA functions as a monomer or dimer during protein export (10, 15, 18). Combined, these data argue that SecA2 is able to

interact with itself. Some of the bands where SecA2 was found are much larger than the predicted dimer size; this could indicate that SecA2 forms much larger multimers.

SecA2 K129R-HIS can be purified from cell envelope fractions of *M. smegmatis*. The above affinity purification of SecA2-HIS from whole cell lysates under non-denaturing conditions led to the apparent identification of SecA2-SecA2 interactions, but it did not identify other SecA2-interacting proteins. Furthermore, any SecA2-interacting integral membrane proteins, including a membrane-embedded translocase, would have been missed because of their under representation in the whole cell lysate and because membrane solubilization was not performed. In continuation of our efforts to co-purify SecA2-interacting proteins, we decided to take advantage of the SecA2 K129R dominant negative protein that we found localized to the membrane in what we believe to be non-functional protein complexes (Chapter 3). We proposed to purify SecA2 K129R-HIS from a membrane fraction in conjunction with DSP crosslinking as new approach to identify SecA2-interacting proteins. Our model to explain the dominant negative phenotypes associated with SecA2 K129R is that this mutant protein forms stable, inactive complexes with an essential membrane-associated protein. The rich agar growth defect is more severe when SecA2 K129R is expressed in a Δ*secA2* mutant than in wild-type *M. smegmatis*; this is probably because some of the wild-type SecA2 molecules in this strain are still functional (Chapter 3). Therefore, we used expressed SecA2 K129R-HIS in the Δ*secA2* mutant strain for our analysis. One drawback to this approach is that GroEL1 is still expressed in the Δ*secA2* mutant; thus by using nickel-affinity columns we expected to not only purify SecA2 KR-HIS but GroEL1 as well. Since SecA2 K129R is toxic to *M. smegmatis*, we used a system where expression of SecA2 K129R-HIS could be controlled by an inducible tetracycline promoter

(Tet ON) (13). When induced by Atc treatment, the TetR repressor protein dissociates from the TetO operator and allows expression of SecA2 K129R-HIS. SecA2 K129R-HIS can then be purified from cell lysates using nickel-affinity columns. Upon subcellular fractionation, the inducible SecA2 K129R-HIS is predominantly localized to the cell envelope fraction, indicating that the C-terminal HIS tag does not affect the localization of SecA2 K129R. Furthermore, SecA2 K129R-HIS also causes a dominant negative phenotype on rich agar growth defect as expected (data not shown).

Cell envelope proteins are relatively insoluble in non-denaturing conditions (26). Therefore, we resuspended cell envelope fractions in different denaturants including urea, Triton X-100, CHAPS, and NP-40 to solubilize the cell envelope proteins which hopefully include SecA2 K129R-HIS complexes (Figure 6.3). First, SecA2 K129R expression was induced by adding Atc to the cultures. After induction, the cultures were in vivo crosslinked by adding DSP. The crosslinked cells were then lysed, and the resulting whole cell lysate was fractionated by differential ultracentrifugation to purify the cell envelope fraction. The cell envelopes were solubilize using a variety of denaturants, and then purified using nickel resin as before. We were able to easily purify SecA2 K129R-HIS from the cell envelope of *M. smegmatis*. After repeated experiments using the panel of denaturants, no reproducible SecA2 K129R-HIS interacting protein complexes were observed, other than those likely to be SecA2 multimers. Other than these SecA2 multimers, every other band in the SecA2 K129R-HIS lanes was also found in the vector control.

Discussion

One commonly used approach to understand how a protein functions is to identify interacting proteins by methods like coimmunoprecipitation and protein pull-down assays (2, 5). These methods have been successful in characterizing the components of specialized secretion systems, bacterial surface structures, and other multi-subunit complexes (14, 22, 25). We attempted to use protein pull-down experiments to identify proteins that interact with the accessory SecA2 protein of *M. smegmatis*. Our results showed that SecA2 is able to form multimers. In *E. coli* and *B. subtilis*, the single essential SecA protein has been shown to form dimers (11, 15, 18). There is still controversy over whether SecA dimerization is required for function (10).

The oligomeric state of accessory SecA2 proteins has not been explored in any bacteria that possess two SecA homologs until now. Our results support the existence of SecA2 oligomers and they are an important step in understanding SecA2 function. However, a much more thorough biochemical analysis is required to understand how and why SecA2 exports only a select subset of proteins from the cytoplasm.

During our studies, we came to appreciate the technical hurdles associated with mass spectrometric analysis of crosslinked proteins. For example, not all peptides ionize equally well with MALDI. Ionized peptides that are more abundant are preferentially represented in the mass spectra that are generated. This could be a limitation with our analysis since SecA2-HIS was so highly overexpressed. To get around this issue, we also performed liquid chromatography (LC)/MS/MS on the trypsinized bands. By including the LC step, we could separate out the SecA2-HIS peptides from the other, less abundant peptides to aid in their detection. With this method, the peptides were ionized by electrospray, which also ionizes

different peptides with variable efficiency. However, even with this more sensitive approach we did not identify proteins other than SecA2 in our complexes. Another issue was that we did not see new bands appear when the crosslinking was reversed as we would expect. This might indicate that while DSP is effective at crosslinking SecA2 to itself, it is not suitable for crosslinking SecA2 to other proteins. Despite our extensive efforts to identify novel SecA2 protein interactions, we only uncovered SecA2-SecA2 multimers.

We think the association of SecA2 K129R with the cell envelope provides a useful tool for identifying SecA2-interacting proteins. As described in Chapter 3, wild-type SecA2 localized in the cytoplasm, but the Walker box mutant SecA2 K129R protein became predominantly cell envelope-associated. We think this indicates SecA2 K129R is locked in a complex with a membrane-embedded protein. This finding help explain the rich agar growth defect associated with expression of SecA2 K129R. It is possible that SecA2 K129R impairs the function of components of the essential Sec pathway. In Chapter 5, we showed that SecA1 is required for the export of SecA2-dependent proteins. Because we have been unable to show a direct physical interaction between SecA1 and SecA2, we think it is possible that SecA2 indirectly delivers pre-proteins to SecA1 or maintains them in a translocation competent state and thus does not need to physically associate with SecA1. Alternatively, SecA2 might deliver pre-proteins directly to the SecYEG translocase. On the other hand, SecA2 may work with another translocase and SecA1292 KR is locked in a complex with this other export machine. Experiments are needed to distinguish between these possible models. It is our hope that by further exploration of different detergents and crosslinkers with the inducible SecA2-K129R-HIS inducible system the important goal of identifying proteins that work with SecA2 in the accessory export pathway will one day be achieved.

Acknowledgements

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Attributions

All experiments described in this work were performed by me with the following exception. The pHSG series of plasmids were all constructed by Henry S. Gibbons.

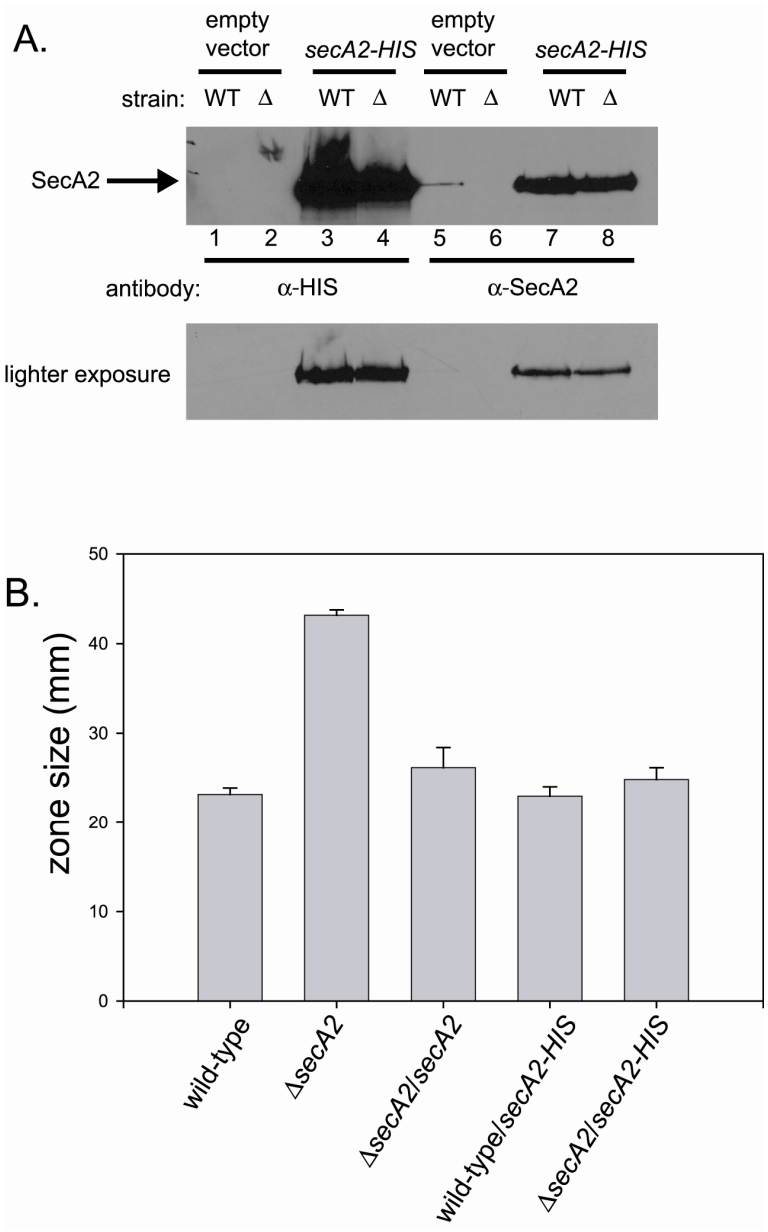

Figure 6.1. SecA2-HIS is highly expressed and fully functional in *M. smegmatis*. (A) Whole cell extracts from wild-type and Δ*secA2* mutant *M. smegmatis* strains expressing SecA2-HIS were subjected to SDS-PAGE and immunoblot with anti-HIS (lanes 1-4) or anti-SecA2 antibodies (lanes 5-8). Note the increased expression of SecA2-HIS (lanes 7 and 8) compared to SecA2 encoded on the chromosome (lane 5). **(B)** SecA2-HIS restores resistance to azide in a Δ*secA2* mutant. Wild-type and Δ*secA2* mutant were transformed with empty vector and pHSG93 (*secA2-HIS*; SecA2 with corrected translational start and a C-terminal 6xHIS tag). The Δ*secA2* mutant was also transformed with pHSG86 (*secA2*; SecA2 with corrected translational start). Each strain was tested in triplicate, and the data is presented as the mean plus standard error.

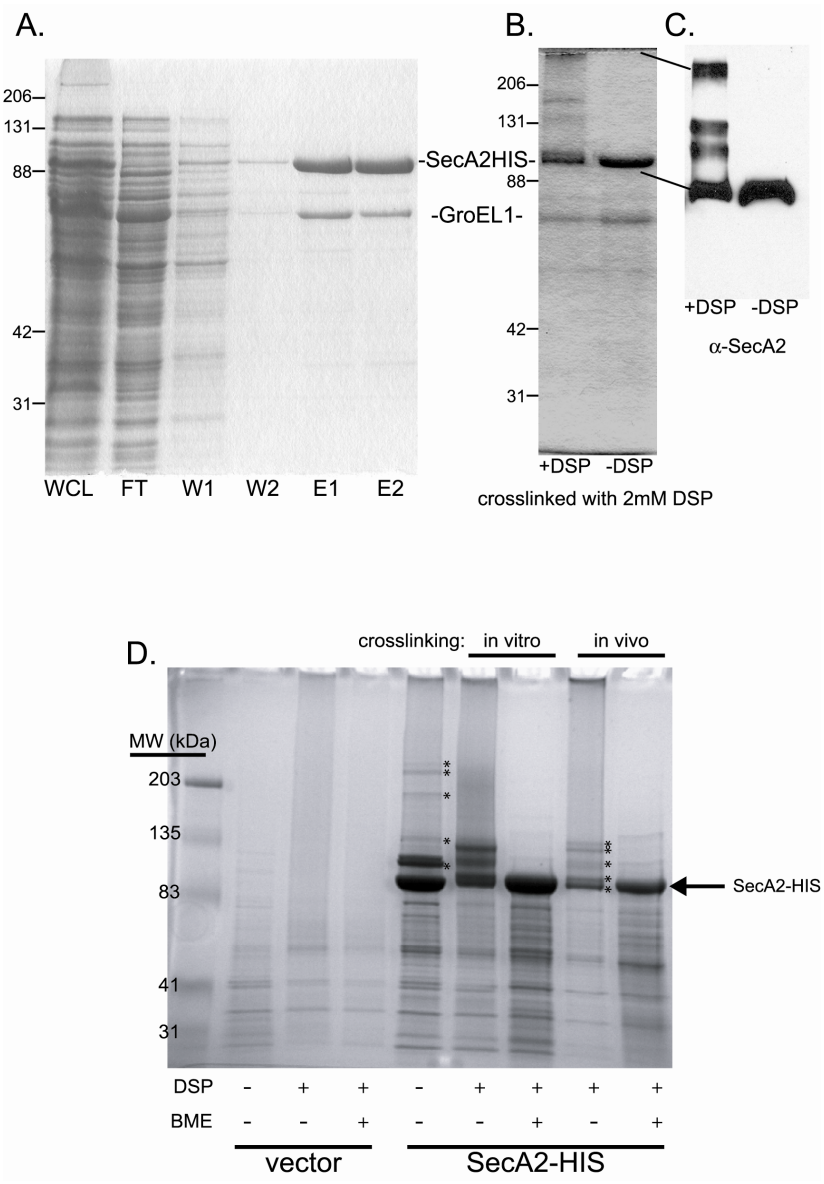

Figure 6.2. SecA2-HIS forms multimers. **(A)** SecA2-HIS was purified from *M. smegmatis* whole cell lysates (WCL) using nickel-affinity columns. Flow through (FT), wash (W1 and W2), and elution (E1 and E2) fractions were analyzed by SDS-PAGE and Coomassie staining. **(B)** Coomassie stained gel of DSP crosslinking experiment. DSP crosslinking was performed where indicated. **(C)** Anti-SecA2 immunoblot shows formation of high molecular weight SecA2-reactive complexes upon DSP crosslinking. **(D)** Crosslinked proteins for analysis by mass spectrometry. Bands marked with * were isolated from the Coomassie stained gel, digested by trypsin, and subjected to peptide mass fingerprinting. For some samples, crosslinking was reversed by treatment with 5% BME prior to gel analysis.

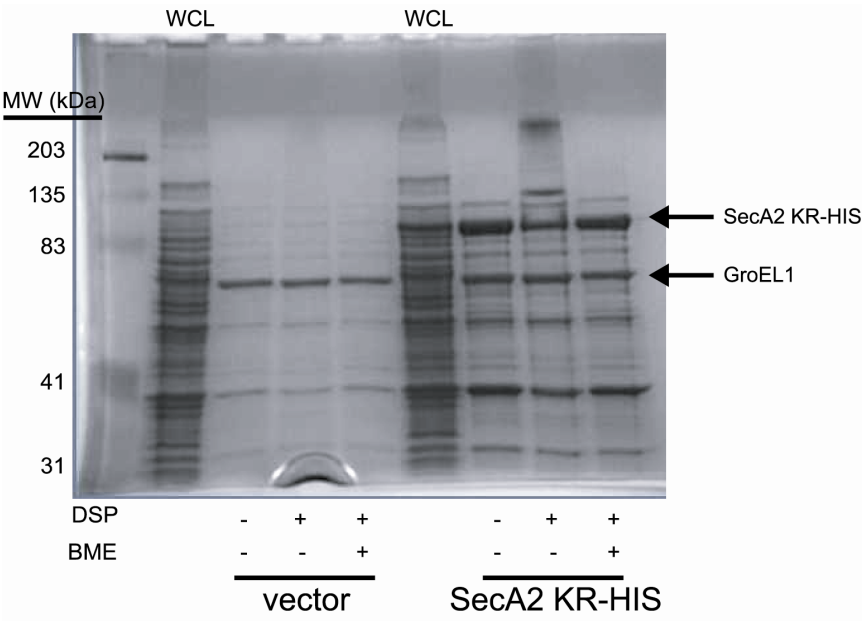

Figure 6.3. SecA2 K129R-HIS can be purified from cell envelope fractions of *M. smegmatis*. SecA2 K129R-HIS was purified from *M. smegmatis* cell envelope fractions and crosslinked with DSP where indicated. Crosslinking was reversed by treatment with 5% BME where indicated. Several different denaturants were used for solubilization and showed similar results; shown is a representative Coomassie stained gel of protein samples solubilized with 8M urea. The position of SecA2 KR-HIS and GroEL are indicated by the arrows.

References

1. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol **44:**1081-94.

2. **Berggard, T., S. Linse, and P. James.** 2007. Methods for the detection and analysis of protein-protein interactions. Proteomics **7:**2833-42.

3. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J Bacteriol **183:**6979-90.

4. **Braunstein, M., B. Espinosa, J. Chan, J. T. Belisle, and W. R. J. Jacobs.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol **48:**453-64.

5. **Brymora, A., V. A. Valova, and P. J. Robinson.** 2004. Protein-protein interactions identified by pull-down experiments and mass spectrometry. Curr Protoc Cell Biol **Chapter 17:**Unit 17 5.

6. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch Microbiol **189:**605-10.

7. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol Microbiol **53:**843-56.

8. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.

9. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M. A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward, and B. G. Barrell.** 2001. Massive gene decay in the leprosy bacillus. Nature **409:**1007-11.

10. **Das, S., E. Stivison, E. Folta-Stogniew, and D. Oliver.** 2008. Reexamination of the role of the amino terminus of SecA in promoting its dimerization and functional state. J Bacteriol **190:**7302-7.

11. **Ding, H., J. F. Hunt, I. Mukerji, and D. Oliver.** 2003. *Bacillus subtilis* SecA ATPase exists as an antiparallel dimer in solution. Biochemistry **42:**8729-38.

12. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J Bacteriol **189:**5090-100.

13. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* using tetracycline repressors. J Bacteriol **189:**4614-23.

14. **Jakubowski, S. J., V. Krishnamoorthy, and P. J. Christie.** 2003. *Agrobacterium tumefaciens* VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. J Bacteriol **185:**2867-78.

15. **Jilaveanu, L. B., C. R. Zito, and D. Oliver.** 2005. Dimeric SecA is essential for protein translocation. Proc Natl Acad Sci U S A **102:**7511-6.

16. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol Microbiol **45:**1043-56.

17. **Ojha, A., M. Anand, A. Bhatt, L. Kremer, W. R. Jacobs, Jr., and G. F. Hatfull.** 2005. GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. Cell **123:**861-73.

18. **Or, E., and T. Rapoport.** 2007. Cross-linked SecA dimers are not functional in protein translocation. FEBS Lett **581:**2616-20.

19. **Perkins, D. N., D. J. Pappin, D. M. Creasy, and J. S. Cottrell.** 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20:**3551-67.

20. **Rigel, N. W., and M. Braunstein.** 2008. A new twist on an old pathway--accessory Sec systems. Mol Microbiol **69:**291-302.

21. **Siboo, I. R., D. O. Chaffin, C. E. Rubens, and P. M. Sullam.** 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. J Bacteriol **190:**6188-96.

22. **Sklar, J. G., T. Wu, L. S. Gronenberg, J. C. Malinverni, D. Kahne, and T. J. Silhavy.** 2007. Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. Proc Natl Acad Sci U S A **104:**6400-5.

23. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2004. Genes in the accessory *sec* locus of *Streptococcus gordonii* have three functionally distinct effects on the expression of the platelet-binding protein GspB. Mol Microbiol **52:**189-203.

24. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2005. Two additional components of the accessory *sec* system mediating export of the *Streptococcus gordonii* platelet-binding protein GspB. J Bacteriol **187:**3878-83.

25. **Yahr, T. L., and W. T. Wickner.** 2000. Evaluating the oligomeric state of SecYEG in preprotein translocase. Embo J **19:**4393-401.

26. **Zuobi-Hasona, K., and L. J. Brady.** 2008. Isolation and solubilization of cellular membrane proteins from bacteria. Methods Mol Biol **425:**287-93.

Chapter 7

CONCLUSION

The global burden of tuberculosis (TB) is staggering – worldwide, 2 billion people are believed to be infected with *Mycobacterium tuberculosis*, the causative agent of TB (65). On average, each person with an active case of TB infects 10-15 people per year. While antibiotic treatment for TB exists, the drug regimen is lengthy (up to 6 months). Full patient compliance is essential to prevent treatment failure. Furthermore, misuse of antibiotics contributes to drug resistance. The emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) TB strains represents a major problem for disease treatment (43, 65). It is therefore critically important that new therapies be designed to combat TB. Understanding the physiology of *M. tuberculosis* could aid in the discovery of novel drug targets. To that end, we study mycobacterial protein export pathways and how they contribute to the success of *M. tuberculosis* as a pathogen of global importance (38).

Specialized protein export and secretion pathways are often used by bacterial pathogens to initiate and establish infection (24). Gram negative bacteria possess a wide variety of specialized secretion systems, including the well-characterized Type III secretion system (T3SS) (32). Organisms like *Yersinia pestis* and *Salmonella typhimurium* use a T3SS to directly inject virulence factors into the host cell cytoplasm. Gram negative bacteria have multiple specialized secretion systems (Type I-VI) (25).

There are comparatively few specialized secretion systems in Gram positive and mycobacteria. The ESX-1 system is one example of specialized secretion in Gram positive and mycobacteria (1). This system was initially described as being required for *M. tuberculosis* virulence (41). Subsequently, ESX-1 systems have been discovered in *Staphylococcus aureus* and *Listeria monocytogenes* (1, 57). In *M. tuberculosis*, ESX-1 is required for the export of two small protein virulence factors, ESAT-6 and CFP-10 (9).

All bacteria use the essential canonical Sec pathway to transport the bulk of exported proteins across the bacterial cytoplasmic membrane (17, 45). SecA is an ATPase that powers the export of unfolded pre-proteins through the membrane-embedded SecYEG translocase (44). Through repeated cycles of ATP binding and hydrolysis, SecA undergoes conformational changes and ratchets pre-proteins across the central channel formed by SecYEG (18, 19). SecA is an essential protein in all tested cases. A small subset of Gram positive bacteria and mycobacteria contain a second accessory SecA protein (3, 6, 7, 13, 14, 40, 49, 54). Recent studies lead to the identification of accessory SecA2 pathways as new specialized protein export systems. In the cases of bacteria with two SecAs, the general trend is that SecA1 is the essential, housekeeping secretion factor. SecA2 is the accessory secretion factor that is only required to export a small subset of proteins. SecA2 is not essential for growth. Accessory SecA2 homologs exist in all species of mycobacteria, and are found in some Gram positive bacteria including *Streptococcus gordonii* and *Listeria monocytogenes* (49).

In mycobacteria, the accessory SecA2 system is required for exporting a subset of proteins (7, 26, 31). However, almost nothing is known about the mechanism of SecA2-mediated protein export in mycobacteria. The work described in this dissertation represents

the first attempts to define how SecA2 functions to export proteins in mycobacteria. Our studies started by asking basic questions. In the process, we uncovered structural and functional differences between SecA1 and SecA2. We also developed genetic and biochemical tools that will enable further study and identification of other important components of the accessory SecA2 protein export system. Through the course of our experiments, we also provided evidence that *M. smegmatis* is a legitimate model system for studying the mycobacterial SecA2 protein export pathway.

Similarities and differences exist between SecA1 and SecA2.

Sequence alignments show *M. tuberculosis* SecA1 and SecA2 are 50% similar to each other (Chapter 2). This similarity is spread throughout much of the two proteins, even though SecA1 is 20 kDa larger than SecA2. Both SecA1 and SecA2 possess Walker Box motifs which are the site of ATP binding for many ATPases (6, 49). The difference in size is attributed to two deletions within the SecA2 sequence. To better understand the differences between SecA1 and SecA2, we created a computer-generated homology model of SecA2 based on the experimentally determined crystal structure of SecA1. We found that the deletions in SecA2 occur in two different subdomains: the Gram negative loop and HWD/CTL (*H*elix *w*ing *d*omain/*C*-*t*erminal *l*inker) (17). The Gram negative loop protrudes from a subdomain called IRA2 (*I*ntramolecular *r*egulator of *A*TPase). As defined in *E. coli* SecA, IRA2 forms part of the ATPase motor domain along with NBD1 (*N*ucleotide *b*inding *d*omain) (46, 62). Interestingly, the Gram negative loop is also absent in Gram positive SecA proteins, but is present in Gram negative SecA proteins (27). The function of this Gram negative loop is unknown. Perhaps this loop confers substrate specificity to SecA1 versus

SecA2. Mutagenesis of the Gram negative loop in SecA1 will help to understand the function of this substructure.

The other SecA2 deletion is in HWD/CTL. The function of the HWD is not understood, even in the well-studied SecA of *E. coli* (17). Activities that map to the CTL of *E. coli* include binding to phospholipids, binding to the chaperone protein SecB, and binding to a Zn^{2+} ion (8, 21, 22, 29). It is worth noting that the corresponding CTL region of SecA1 in mycobacteria does not contain residues for a Zn^{2+} binding motif. In *E. coli*, the Zn^{2+} binding site is also part of the SecB binding site. There are no SecB homologs in mycobacteria, so the absence of a SecB binding site is not surprising (15). The functional significance of the missing subdomains in SecA2 is not clear. Perhaps these subdomains are involved in recognizing pre-proteins that are compatible with SecA1 versus SecA2. Alternatively, these domains could be important for specifying interactions with different translocases. We think that the SecA2 homology model will be a useful tool for designing mutations to characterize the functional subdomains of SecA1 and SecA2.

SecA1 and SecA2 have unique functions; the two proteins are not functionally redundant. This is shown in *M. smegmatis* where overexpression of SecA1 does not compensate for the absence of SecA2 and vice versa (6). However, it is not obvious how these similar proteins play different roles inside the cell. To begin our analysis, we determined that both in *M. tuberculosis* and *M. smegmatis*, SecA1 and SecA2 are expressed at the same level, at least under standard laboratory growth conditions (Chapter 2). This shows that the difference between SecA1 and SecA2 is not due to expression level. Rather, the difference between SecA1 and SecA2 is more likely due to different functions and/or different proteins with which they interact.

Since components of bacterial protein export systems are found in different subcellular compartments(10, 25, 52), we performed experiments to determine where SecA1 and SecA2 function in the mycobacterial cell. *E. coli* SecA cycles between the cell envelope and the cytoplasm (12, 18, 19). This dynamic localization reflects the ability of SecA to interact with newly synthesized pre-proteins as well as the membrane-embedded SecYEG translocase (29, 47). In Chapter 2, we performed subcellular localization of *M. smegmatis* to determine which subcellular fractions contain SecA1 and SecA2. Similar to *E. coli* SecA, we found that SecA1 is equally represented in the cytoplasm and the cell envelope. This is not surprising since SecA1 is the essential SecA of mycobacteria (6, 28, 51). In contrast, we found SecA2 enriched in the cytosolic fraction. We suspect this difference between SecA1 and SecA2 is functionally significant. The cytoplasmic localization of SecA2 might indicate that the accessory SecA plays a supportive role in protein export, perhaps functioning as a chaperone analogous to the role of SecB in *E. coli*. Of note, SecB is only required for the export of a subset of proteins in *E. coli* and there are no SecB homologs in mycobacteria (15, 16).

While SecA1 and SecA2 certainly have distinct roles inside the cell, we also wanted to explore whether SecA1 and SecA2 work together in protein export. The absence of SecA2 only affects a subset of exported proteins (7, 26). We wanted to determine if SecA1 plays a role in exporting SecA2-dependent proteins. Using a tetracycline repressible system (28), we depleted SecA1 from *M. smegmatis* and followed the export of the SecA2-dependent lipoprotein Msmeg1712-HA to the mycobacterial cell wall. This experiment showed depletion of SecA1 prevents Msmeg1712-HA from reaching the cell wall; furthermore, Msmeg1712-HA accumulated in the cytoplasm in the absence of its export. This export

defect revealed that the effect of SecA1 depletion on Msmeg1712-HA export is comparable to the defect exhibited by the Δ*secA2* mutant. This is the first demonstration of the canonical SecA1 being involved in exporting substrates that require the accessory SecA2. In the other accessory SecA2 systems being studied, this exact question has yet to be asked.

We do not yet understand the nature of the relationship between SecA1 and SecA2. SecA2 could directly interact with SecA1 or other Sec components to export Msmeg1712-HA. In this model, discussed again below, the role of SecA2 may be to deliver pre-proteins to the SecYEG translocase that are normally overlooked by the general Sec apparatus. To further explore this idea, we can test whether depletion of other Sec components will impair Msmeg1712-HA export. It is also possible that SecA1 works with SecA2 in conjunction with a novel translocase. As noted in Chapter 1, there are several putative transmembrane proteins encoded by genes that neighbor *secA2* (49). In *S. gordonii* and *S. parasanguinis*, the genes that neighbor *secA2* are involved in accessory SecA2 export (3, 14, 58-60, 66). It is worth exploring the functions of the genes that neighbor *secA2* in mycobacteria in case they comprise a unique SecA2-dependent translocase. Although less likely, a final possibility is that SecA1 assembles a novel translocase used by SecA2 to export Msmeg1712-HA. However, it is only in rare cases that *E. coli* SecA influences integral membrane protein assembly and there is no evidence SecA1 does this in mycobacteria (52, 61, 64). The identification of SecA2-interacting proteins will be critical to defining the role of SecA1/SecYEG in the process of SecA2 export.

ATP binding is required for normal SecA2 function in *M. tuberculosis* and *M. smegmatis*.

The single, essential SecA protein of *E. coli* and *B. subtilis* binds and hydrolyzes ATP (42, 44). This ATPase activity is absolutely required for SecA function. Sequence alignments show that *M. tuberculosis* SecA1 and SecA2 both have predicted Walker Box motifs. We hypothesized that SecA1 and SecA2 could bind ATP. In Chapter 4, we showed that *M. tuberculosis* SecA1 and SecA2 proteins both bind and hydrolyze ATP in vitro. This was the first demonstration of ATPase activity for an accessory SecA2 protein from any bacterial species. In support of this finding, we also showed that mutating a conserved lysine residue in the Walker A motif of SecA2 prevents ATP binding in vitro.

We assessed the biological importance of ATP binding for SecA2 function by testing the ability of Walker Box mutant *secA2* alleles to complement Δ*secA2* mutant phenotypes (Chapter 3). *M. tuberculosis* is an intracellular pathogen and as such is able to grow inside macrophages (30). However, the *M. tuberculosis* Δ*secA2* mutant is unable to grow inside macrophages, indicating that SecA2 is an important virulence factor (39). This macrophage growth defect can be overcome by expressing wild-type *secA2* from a plasmid in the Δ*secA2* mutant. We transformed the Δ*secA2* mutant with a plasmid carrying *secA2 K115R*, and showed the resulting transformants are unable to grow inside macrophages. This indicates that the Walker Box residue K115 is required for normal SecA2 function during intracellular growth.

We also tested the ability of Walker Box mutant *secA2* alleles to complement the smooth colony morphology of the *M. tuberculosis* Δ*secA2* mutant (37). Expression of SecA2 K115R in the Δ*secA2* mutant does not complement the colony morphology. Furthermore, expression of SecA2 K115R in wild-type H37Rv causes the colonies to appear smooth. This was our first indication that SecA2 K115R was dominant negative. Taken together, these

results confirm that ATP binding is absolutely required for normal SecA2 function in *M. tuberculosis*.

We also tested Walker Box mutant *secA2* alleles in *M. smegmatis*. The *M. smegmatis* Δ*secA2* mutant has a rich agar growth defect and is hypersensitive to sodium azide (6). As described in Chapter 3, Walker Box mutant *secA2* alleles from *M. tuberculosis* (*secA2 K115R* and *secA2 D216N*) and *M. smegmatis* (*secA2 K129R*) failed to complement these phenotypes. In fact, we found that these alleles actually exacerbated the Δ*secA2* mutant phenotypes. Furthermore, when the *secA2 K129R* allele is expressed in wild-type *M. smegmatis*, the resulting strain had a rich agar growth defect equivalent to a Δ*secA2* null mutant. This result also indicated SecA2 K129R is dominant negative.

SecA2 K129R is dominant negative.

Dominant negative mutants have a wide history of use as a tool to understand biological processes, including protein export pathways (50). In fact, the Walker Box mutant SecA K108R of *E. coli* has dominant negative effects on Sec-dependent protein export in vitro (44). Other Walker Box mutants also have dominant negative effects (11, 50, 53). Dominant negative phenotypes are often dosage dependent (2, 55, 56). We confirmed that SecA2 K129R was dominant negative by showing that the phenotype associated with this protein is dosage dependent; increased expression of SecA2 K129R correlates with a worsened rich agar growth defect in wild-type *M. smegmatis* (Chapter 3). By discovering this dominant negative phenotype, we now have another tool to dissect the mechanism of the accessory SecA2 pathway.

We think SecA2 K129R is dominant negative because it is locked in a non-functional complex with SecA2-interacting proteins. This interaction appears to disable components of a process that is essential for normal cell growth, as shown by the growth inhibitory effects of high level expression of SecA2 K129R. Therefore, we consider essential Sec components strong candidates for being the proteins affected by SecA2 K129R. As already mentioned, we think it is a strong possibility that the role for SecA2 is to work with SecA1 to enable efficient export of a particular subset of proteins via the canonical Sec pathway. In a Δ*secA2* mutant, this subset of proteins might still be exported, but much less efficiently if at all. We believe some of these exported proteins might be important for metabolizing certain nutrients or for resisting stress on the cell envelope. This would explain why the Δ*secA2* mutant has a growth defect on rich agar and high salt agar plates. When the only SecA2 molecule inside the cell contains the K129R mutation, the plate phenotypes are greatly exacerbated.

SecA2 K129R is a tool to identity residues critical for normal SecA2 function.

During our studies, we isolated spontaneous intragenic and extragenic suppressors of the exacerbated rich agar growth defect of the *M. smegmatis* Δ*secA2*/*secA2 K129R* strain. As discussed in Chapter 3, the intragenic suppressors mapped to different subdomains of SecA2, including surface exposed regions of the NBD1, IRA2, IRA1, and PPXD subdomains. Together, NBD1 and IRA2 constitute the N-domain while IRA1 and PPXD are components of the C-domain (17, 62). If SecA2 normally interacts with a membrane-embedded translocase, and SecA2 K129R irreversibly associates with this translocase, then suppressors could alleviate the dominant negative phenotype by disrupting interactions with the translocase. A recent structural study showed that the IRA1 and PPXD subdomains are

important for interactions between SecA and SecY in *Thermotoga maritima* (20, 67). Thus, we predicted that suppressor mutations in IRA1 and PPXD could alter how SecA2 K129R interacts with other proteins. To test this hypothesis, we localized SecA2 K129R in the intragenic suppressor strains. We found that the mutations in IRA1 and PPXD released SecA2 from the cell envelope. This finding was important because it provides support for our model of why SecA2 K129R is dominant negative, and how suppressor mutations alleviate this phenotype.

Additionally, by isolating these intragenic suppressor mutants we uncovered residues of SecA2 that are important for function. When the suppressor mutations are recreated in SecA2 with a normal, wild-type Walker Box, the resulting proteins are unable to complement the Δ*secA2* mutant phenotypes. Said another way, the recreated suppressors were equivalent to *secA2* null alleles. We think these results are particularly exciting since to date, no mutational analysis has been performed on accessory SecA2 proteins in any bacteria. Furthermore, the residues that we found important for SecA2 function have not been identified in previous mutational analyses of the essential *E. coli* SecA protein (23, 33-36, 63).

Extragenic suppressors of SecA2 K129R can be used to identify SecA2-interacting proteins.

In addition to the intragenic suppressors, we also collected extragenic suppressors of SecA2 K129R. Suppressor analysis has been successfully used to identify interactions between proteins that function in the same pathway (2). Indeed, components of the *E. coli* Sec pathway were identified by suppressor analysis (4, 5). We think using these extragenic

suppressors represents an excellent opportunity to identify components that interact with SecA2 during protein export. SecA2 K129R is released from the cell envelope in two extragenic suppressors we tested. This result makes us think that these suppressor mutations function by preventing formation of inactive complexes with SecA2 K129R. Therefore the suppressor mutation must be in a protein that normally interacts with SecA2. We have not yet identified where the suppressor mutations are in these strains, but we are currently pursuing several strategies to do so. First, we have started to sequence the genomes of two representative suppressors. The cost of whole genome sequencing is rapidly decreasing, making this approach a feasible option. Second, we are attempting a more traditional library approach to clone the suppressor mutation. Until recently, we were unable to pursue this strategy due to technical limitations. However, using genomic libraries in a newly created cosmid vector this is also now a valid option. We believe that one of these strategies will enable us to identify the extragenic suppressor mutations in our strains.

A model for SecA2-dependent protein export in mycobacteria that involves components of the canonical Sec pathway.

We propose that SecA2-mediated protein export involves some or all of the components of the canonical Sec pathway. We favor this model over others that invoke the existence of a completely novel set of translocase components because several results point in this direction. First, mycobacteria do not have an accessory SecY2 homolog (15). Perhaps SecA2 is capable of functioning with the essential SecY, so the presence of an accessory SecY2 protein is not necessary.

Second, SecA2 K129R is dominant negative and has a negative impact on growth of wild-type and Δ*secA2 M. smegmatis* strains. This implies that SecA2 K129R inactivates an essential process. It is consistent with SecA2 K129R disrupting the canonical Sec pathway through formation of nonfunctional SecA2 K129R/SecYEG complexes. We showed that the K129R substitution changes the localization of SecA2 from the cytoplasm to the cell envelope (Chapter 3). This is consistent with SecA2 K129R becoming irreversibly associated with a membrane-embedded complex such as the Sec translocase.

Third, in some of our suppressors of SecA2 K129R phenotypes the localization of SecA1 changed. Even though the suppressor mutations are not in *secA1* in these strains, we think this altered localization indicates that the suppressor mutation is in another component of the canonical Sec pathway which in turn influences the localization of SecA1.

Fourth, we showed that SecA1 is required for the export of the SecA2-dependent protein Msmeg1712-HA (Chapter 5). Although it is not yet possible to rule out indirect roles for SecA1 in Msmeg1712-HA export, this result is consistent with the proposal that SecA2 works with SecA1 to export a subset of proteins across the membrane. The fact that SecA in *E. coli* is known to dimerize suggested to us that SecA1 and SecA2 may work together as a heterodimer. However, we have been unable to demonstrate a physical interaction between SecA1 and SecA2. Another possibility is that SecA2 binds to components of the Sec pathway other than SecA1.

Fifth, there are synthetic phenotypes that link SecA1 and SecA2. Overexpression of SecA1 in wild-type *M. smegmatis* has no detrimental effect, but overexpression of SecA1 in a Δ*secA2* mutant exacerbates the rich agar growth defect and azide hypersensitivity phenotypes (6). Perhaps an overabundance of SecA1 and the dearth of SecA2 in a Δ*secA2*

mutant alter the normal stoichiometry of and interaction with the canonical Sec pathway. Although the explanation for this synthetic phenotype remains a mystery, this synthetic phenotype is further evidence of a relationship between SecA1 and SecA2.

The above results are all consistent with there being a functional relationship between SecA2 and the canonical Sec pathway. We think that in mycobacteria, the primary role of SecA2 is to recognize and/or deliver a subset of pre-proteins to the essential SecYEG translocase (Figure 7.1). ATPase activity is required for SecA2 function. Perhaps energy derived from the hydrolysis of ATP allows SecA2 to initially engage pre-proteins with the SecYEG channel, but SecA1 is required for subsequent cycles of export. However, more studies are obviously needed to definitively demonstrate a role for SecA1 and/or SecYEG in the mechanism of SecA2-dependent export. The identification of SecA2-interacting proteins will be a critical step in proving the mechanism of SecA2-mediated protein export.

***M. smegmatis* is a valid model for studying cellular processes of mycobacteria.**

Most of our studies of the SecA2-dependent pathway used *M. smegmatis*. Fast growing, nonpathogenic *M. smegmatis* is much more amenable to genetic and biochemical analyses than slow growing, pathogenic *M. tuberculosis* (48). During the course of our studies, we performed several experiments to show that *M. smegmatis* is a valid model system to study processes that are conserved in *M. tuberculosis*. Using cross-species complementation experiments, we showed that *M. smegmatis secA2* can complement the macrophage growth defect and the smooth colony phenotypes of the *M. tuberculosis* Δ*secA2* mutant. It is also possible to complement phenotypes of the *M. smegmatis* Δ*secA2* mutant by

expressing *M. tuberculosis secA2*. These findings clearly demonstrate functional conservation of SecA2 between the two mycobacterial species.

Our studies also identified several other similarities between *M. tuberculosis* and *M. smegmatis*. Expression of SecA1 and SecA2 is the same in both organisms. Our analysis also showed ATP binding is required for function of SecA2 in *M. tuberculosis* and *M. smegmatis*. We also showed that expression of *secA2* Walker Box mutant alleles was dominant negative in both species. One discrepancy is that all the known SecA2-dependent proteins of *M. tuberculosis* lack signal sequences, while all the known SecA2-dependent protein of *M. smegmatis* contain signal sequences. This could indicate a slight difference in how pre-proteins are recognized between these two mycobacteria. It is also possible that we have not yet identified SecA2 substrates in *M. tuberculosis* that possess signal sequences, or SecA2 substrates in *M. smegmatis* that lack signal sequences. It will be important to identify more SecA2-dependent proteins to fully understand this difference. Taken together, we think our results provide a rationale for continued studies of the accessory SecA2 pathway of *M. smegmatis* as a key to understanding how specialized secretion systems contribute to pathogenesis of *M. tuberculosis*.

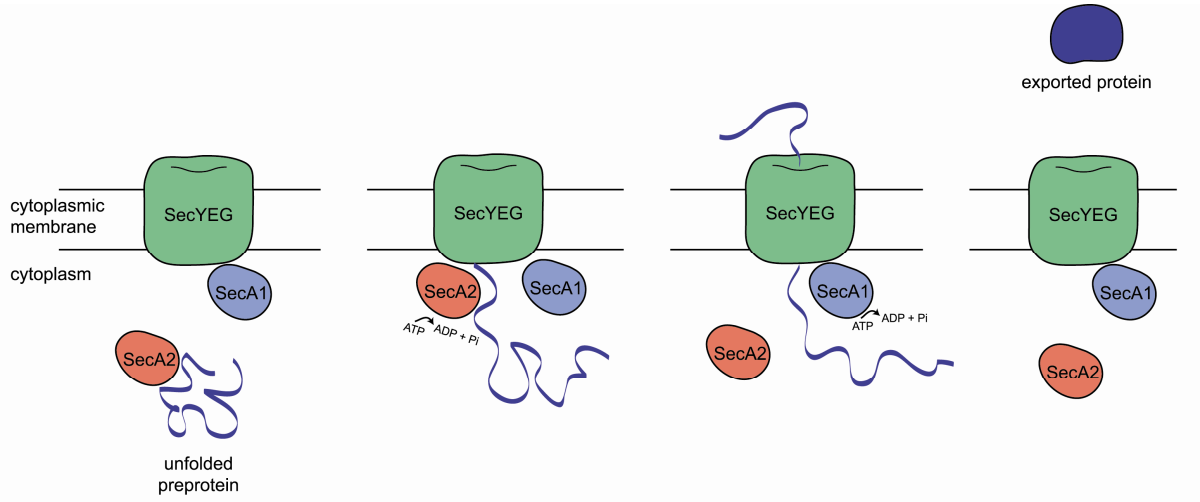

Figure 7.1. Proposed model for SecA2-mediated protein export. SecA2 recognizes pre-proteins in the cytoplasm, and delivers them to the SecA1/SecYEG translocase. Upon ATP binding and hydrolysis, SecA2 undergoes conformational changes to initiate the export cycle. SecA1 finishes protein export through multiple rounds of insertion/deinsertion. Once across the cytoplasmic membrane, the exported protein can fold into its mature conformation.

References

1. **Abdallah, A. M., N. C. Gey van Pittius, P. A. Champion, J. Cox, J. Luirink, C. M. Vandenbroucke-Grauls, B. J. Appelmelk, and W. Bitter.** 2007. Type VII secretion--mycobacteria show the way. Nat Rev Microbiol **5:**883-91.

2. **Appling, D. R.** 1999. Genetic approaches to the study of protein-protein interactions. Methods **19:**338-49.

3. **Bensing, B. A., and P. M. Sullam.** 2002. An accessory *sec* locus of *Streptococcus gordonii* is required for export of the surface protein GspB and for normal levels of binding to human platelets. Mol Microbiol **44:**1081-94.

4. **Bieker, K. L., G. J. Phillips, and T. J. Silhavy.** 1990. The *sec* and *prl* genes of *Escherichia coli*. J Bioenerg Biomembr **22:**291-310.

5. **Bieker, K. L., and T. J. Silhavy.** 1990. PrlA (SecY) and PrlG (SecE) interact directly and function sequentially during protein translocation in *E. coli*. Cell **61:**833-42.

6. **Braunstein, M., A. M. Brown, S. Kurtz, and W. R. Jacobs, Jr.** 2001. Two nonredundant SecA homologues function in mycobacteria. J Bacteriol **183:**6979-90.

7. **Braunstein, M., B. Espinosa, J. Chan, J. T. Belisle, and W. R. J. Jacobs.** 2003. SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol **48:**453-64.

8. **Breukink, E., N. Nouwen, A. van Raalte, S. Mizushima, J. Tommassen, and B. de Kruijff.** 1995. The C terminus of SecA is involved in both lipid binding and SecB binding. J Biol Chem **270:**7902-7.

9. **Brodin, P., L. Majlessi, L. Marsollier, M. I. de Jonge, D. Bottai, C. Demangel, J. Hinds, O. Neyrolles, P. D. Butcher, C. Leclerc, S. T. Cole, and R. Brosch.** 2006. Dissection of ESAT-6 system 1 of *Mycobacterium tuberculosis* and impact on immunogenicity and virulence. Infect Immun **74:**88-98.

10. **Brundage, L., J. P. Hendrick, E. Schiebel, A. J. Driessen, and W. Wickner.** 1990. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell **62:**649-57.

11. **Budd, M. E., W. Choe, and J. L. Campbell.** 2000. The nuclease activity of the yeast DNA2 protein, which is related to the RecB-like nucleases, is essential in vivo. J Biol Chem **275:**16518-29.

12. **Cabelli, R. J., K. M. Dolan, L. P. Qian, and D. B. Oliver.** 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:**24420-7.

13. **Caspers, M., and R. Freudl.** 2008. *Corynebacterium glutamicum* possesses two *secA* homologous genes that are essential for viability. Arch Microbiol **189:**605-10.

14. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol Microbiol **53:**843-56.

15. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537-44.

16. **Collier, D. N.** 1993. SecB: a molecular chaperone of *Escherichia coli* protein secretion pathway. Adv Protein Chem **44:**151-93.

17. **Driessen, A. J., and N. Nouwen.** 2008. Protein translocation across the bacterial cytoplasmic membrane. Annu Rev Biochem **77:**643-67.

18. **Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner.** 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell **83:**1171-81.

19. **Economou, A., and W. Wickner.** 1994. SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. Cell **78:**835-43.

20. **Erlandson, K. J., S. B. Miller, Y. Nam, A. R. Osborne, J. Zimmer, and T. A. Rapoport.** 2008. A role for the two-helix finger of the SecA ATPase in protein translocation. Nature **455:**984-7.

21. **Fekkes, P., J. G. de Wit, A. Boorsma, R. H. Friesen, and A. J. Driessen.** 1999. Zinc stabilizes the SecB binding site of SecA. Biochemistry **38:**5111-6.

22. **Fekkes, P., C. van der Does, and A. J. Driessen.** 1997. The molecular chaperone SecB is released from the carboxy-terminus of SecA during initiation of precursor protein translocation. EMBO J **16:**6105-13.

23. **Fikes, J. D., and P. J. Bassford, Jr.** 1989. Novel *secA* alleles improve export of maltose-binding protein synthesized with a defective signal peptide. J Bacteriol **171:**402-9.

24. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev **61:**136-69.

25. **Gerlach, R. G., and M. Hensel.** 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. Int J Med Microbiol **297:**401-15.

26. **Gibbons, H. S., F. Wolschendorf, M. Abshire, M. Niederweis, and M. Braunstein.** 2007. Identification of two *Mycobacterium smegmatis* lipoproteins exported by a SecA2-dependent pathway. J Bacteriol **189:**5090-100.

27. **Guina, T., D. Helfet-Hilliker, V. Ramamurthy, and D. Oliver.** 1998. Sequence and phylogenetic analysis of the *Borrelia burgdorferi secA* gene. Biochim Biophys Acta **1371:**24-30.

28. **Guo, X. V., M. Monteleone, M. Klotzsche, A. Kamionka, W. Hillen, M. Braunstein, S. Ehrt, and D. Schnappinger.** 2007. Silencing essential protein secretion in *Mycobacterium smegmatis* using tetracycline repressors. J Bacteriol **189:**4614-23.

29. **Hendrick, J. P., and W. Wickner.** 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:**24596-600.

30. **Hestvik, A. L., Z. Hmama, and Y. Av-Gay.** 2005. Mycobacterial manipulation of the host cell. FEMS Microbiol Rev **29:**1041-50.

31. **Hinchey, J., S. Lee, B. Y. Jeon, R. J. Basaraba, M. M. Venkataswamy, B. Chen, J. Chan, M. Braunstein, I. M. Orme, S. C. Derrick, S. L. Morris, W. R. Jacobs, Jr., and S. A. Porcelli.** 2007. Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. J Clin Invest **117:**2279-88.

32. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev **62:**379-433.

33. **Huie, J. L., and T. J. Silhavy.** 1995. Suppression of signal sequence defects and azide resistance in *Escherichia coli* commonly result from the same mutations in *secA*. J Bacteriol **177:**3518-26.

34. **Jarosik, G. P., and D. B. Oliver.** 1991. Isolation and analysis of dominant *secA* mutations in Escherichia coli. J Bacteriol **173:**860-8.

35. **Khatib, K., and D. Belin.** 2002. A novel class of *secA* alleles that exert a signal-sequence-dependent effect on protein export in *Escherichia coli*. Genetics **162:**1031-43.

36. **Kourtz, L., and D. Oliver.** 2000. Tyr-326 plays a critical role in controlling SecA-preprotein interaction. Mol Microbiol **37:**1342-56.

37. **Kurtz, S.** 2007. SecA2 of *M. tuberculosis* contributes to intracellular survival, immune modulation, and surface properties of the bacillus. Masters of Science. University of North Carolina at Chapel Hill, Chapel Hill.

38. **Kurtz, S., and M. Braunstein.** 2005. Protein secretion and export in *Mycobacterium tuberculosis*, p. 71-138. *In* T. Parish (ed.), *Mycobacterium* molecular biology. Horizon bioscience, Norfolk, UK.

39. **Kurtz, S., K. P. McKinnon, M. S. Runge, J. P. Ting, and M. Braunstein.** 2006. The SecA2 secretion factor of *Mycobacterium tuberculosis* promotes growth in macrophages and inhibits the host immune response. Infect Immun **74:**6855-64.

40. **Lenz, L. L., and D. A. Portnoy.** 2002. Identification of a second Listeria *secA* gene associated with protein secretion and the rough phenotype. Mol Microbiol **45:**1043-56.

41. **Lewis, K. N., R. Liao, K. M. Guinn, M. J. Hickey, S. Smith, M. A. Behr, and D. R. Sherman.** 2003. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. J Infect Dis **187:**117-23.

42. **McNicholas, P., T. Rajapandi, and D. Oliver.** 1995. SecA proteins of *Bacillus subtilis* and *Escherichia* coli possess homologous amino-terminal ATP-binding domains regulating integration into the plasma membrane. J Bacteriol **177:**7231-7.

43. **Meya, D. B., and K. P. McAdam.** 2007. The TB pandemic: an old problem seeking new solutions. J Intern Med **261:**309-29.

44. **Mitchell, C., and D. Oliver.** 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol Microbiol **10:**483-97.

45. **Murphy, C. K., and J. Beckwith.** 1996. Export of proteins to the cell envelope in *Escherichia coli*., p. 967-978. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella*. ASM Press, Washington, D.C.

46. **Papanikolau, Y., M. Papadovasilaki, R. B. Ravelli, A. A. McCarthy, S. Cusack, A. Economou, and K. Petratos.** 2007. Structure of dimeric SecA, the *Escherichia coli* preprotein translocase motor. J Mol Biol **366:**1545-57.

47. **Papanikou, E., S. Karamanou, C. Baud, M. Frank, G. Sianidis, D. Keramisanou, C. G. Kalodimos, A. Kuhn, and A. Economou.** 2005. Identification of the preprotein binding domain of SecA. J Biol Chem **280:**43209-17.

48. **Reyrat, J. M., and D. Kahn.** 2001. *Mycobacterium smegmatis*: an absurd model for tuberculosis? Trends Microbiol **9:**472-4.

49. **Rigel, N. W., and M. Braunstein.** 2008. A new twist on an old pathway--accessory Sec systems. Mol Microbiol **69:**291-302.

50. **Sakai, D., T. Horiuchi, and T. Komano.** 2001. ATPase activity and multimer formation of PilQ protein are required for thin pilus biogenesis in plasmid R64. J Biol Chem **276:**17968-75.

51. **Sassetti, C. M., D. H. Boyd, and E. J. Rubin.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

52. **Serek, J., G. Bauer-Manz, G. Struhalla, L. van den Berg, D. Kiefer, R. Dalbey, and A. Kuhn.** 2004. *Escherichia coli* YidC is a membrane insertase for Sec-independent proteins. EMBO J **23:**294-301.

53. **Sexton, J. A., J. S. Pinkner, R. Roth, J. E. Heuser, S. J. Hultgren, and J. P. Vogel.** 2004. The *Legionella pneumophila* PilT homologue DotB exhibits ATPase activity that is critical for intracellular growth. J Bacteriol **186:**1658-66.

54. **Siboo, I. R., D. O. Chaffin, C. E. Rubens, and P. M. Sullam.** 2008. Characterization of the accessory Sec system of *Staphylococcus aureus*. J Bacteriol **190:**6188-96.

55. **Somers, D. E., W. Y. Kim, and R. Geng.** 2004. The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. Plant Cell **16:**769-82.

56. **Sun, Y., Z. Dong, K. Nakamura, and N. H. Colburn.** 1993. Dosage-dependent dominance over wild-type p53 of a mutant p53 isolated from nasopharyngeal carcinoma. Faseb J **7:**944-50.

57. **Sundaramoorthy, R., P. K. Fyfe, and W. N. Hunter.** 2008. Structure of *Staphylococcus aureus* EsxA suggests a contribution to virulence by action as a transport chaperone and/or adaptor protein. J Mol Biol **383:**603-14.

58. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2004. Four proteins encoded in the *gspB-secY2A2* operon of *Streptococcus gordonii* mediate the intracellular glycosylation of the platelet-binding protein GspB. J Bacteriol **186:**7100-11.

59. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2004. Genes in the accessory *sec* locus of *Streptococcus gordonii* have three functionally distinct effects on the expression of the platelet-binding protein GspB. Mol Microbiol **52:**189-203.

60. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2005. Two additional components of the accessory *sec* system mediating export of the *Streptococcus gordonii* platelet-binding protein GspB. J Bacteriol **187:**3878-83.

61. **Urbanus, M. L., P. A. Scotti, L. Froderberg, A. Saaf, J. W. de Gier, J. Brunner, J. C. Samuelson, R. E. Dalbey, B. Oudega, and J. Luirink.** 2001. Sec-dependent membrane protein insertion: sequential interaction of nascent FtsQ with SecY and YidC. EMBO Rep **2:**524-9.

62. **Vrontou, E., and A. Economou.** 2004. Structure and function of SecA, the preprotein translocase nanomotor. Biochim Biophys Acta **1694:**67-80.

63. **Vrontou, E., S. Karamanou, C. Baud, G. Sianidis, and A. Economou.** 2004. Global co-ordination of protein translocation by the SecA IRA1 switch. J Biol Chem **279:**22490-7.

64. **Wagner, S., O. Pop, G. J. Haan, L. Baars, G. Koningstein, M. M. Klepsch, P. Genevaux, J. Luirink, and J. W. de Gier.** 2008. Biogenesis of MalF and the MalFGK(2) maltose transport complex in *Escherichia coli* requires YidC. J Biol Chem **283:**17881-90.

65. **WorldHealthOrganization** 2007, posting date. WHO Information tuberculosis fact sheet. [Online.]

66. **Wu, H., S. Bu, P. Newell, Q. Chen, and P. Fives-Taylor.** 2007. Two gene determinants are differentially involved in the biogenesis of Fap1 precursors in *Streptococcus parasanguis*. J Bacteriol **189:**1390-8.

67. **Zimmer, J., Y. Nam, and T. A. Rapoport.** 2008. Structure of a complex of the ATPase SecA and the protein-translocation channel. Nature **455:**936-43.