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CHARACTERIZATION OF SECA1 AND SECA2 FROM GRAM-POSITIVE PATHOGENS
AND DISCOVERY OF NOVEL SECA INHIBITORS

by

JINSHAN JIN

Under the Direction of Phang C. Tai

ABSTRACT

Due to the emergence and dissemination of multidrug resistance, bacterial pathogens have been causing a serious public health problem in recent years. To address the existing drug resistant problem, there is an urgent need to find new antimicrobials, especially those against drug-resistant bacteria. SecA is the central component of Sec-dependent secretion pathway, which is responsible for the secretion of many essential proteins as well as many toxins and virulence factors. Two SecA homologues are identified in some important Gram-positive pathogens. SecA1 is involved in general secretion pathway and essential for viability, whereas SecA2 contribute to secretion of specific virulence factors. The high conservation among a wide range of bacteria and no human counterpart make SecA homologues attractive targets for exploring novel antimicrobials. We hypothesize that inhibition of these SecA homologues could reduce virulence, inhibit bacteria growth, and kill bacteria. SecA1 and SecA2 from four different species

were cloned, purified, and characterized. All these SecA homologues show ATPase activities, thus screening ATPase inhibitors might help to develop new antimicrobials. In this study, three structurally different classes of SecA inhibitors were developed and optimized: 1) Rose Bengal (RB) and RB analogs derived from systematical dissection RB and Structure-Activity relationship (SAR) study; 2) pyrimidine analogs derived from virtual screening based on the ATP binding pocket of EcSecA and SAR study; and 3) bistriazole analogs derived from random screening and SAR study. Several potent SecA inhibitors show promising enzymatic inhibition against SecA homologues as well as bacteriostatic and bactericidal effects. Two major efflux pumps of *S. aureus*, NorA and MepA, have little negative effect on the antimicrobial activities of SecA inhibitors, suggesting that targeting SecA could by-pass efflux pumps. Moreover, these inhibitors impair the secretion of important toxins of *S. aureus* and *B. anthracis*, indicating the inhibition of *in vivo* SecA function could reduce virulence. Target identification assays confirm that these inhibitors could directly bind to SecA homologues, and specifically identify SecA from whole cell lysate of *E. coli* and *S. aureus*, suggesting that these inhibitors are really targeting on SecA. These studies validate that SecA is a good target for development antimicrobials.

INDEX WORDS: SecA, SecA1, SecA2, SecA inhibitors, Antimicrobials, Rose bengal, Rose bengal analogs, Structure-Activity relationship, Pyrimidine analogs, Bistriazole analogs, Bacteriostatic effect, Bactericidal effect, Virulence factor secretion, and Efflux pump

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JINSHAN JIN

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

In the College of Arts and Sciences

Georgia State University

2011

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AND DISCOVERY OF NOVEL SECA INHIBITORS

by

JINSHAN JIN

Committee Chair: Phang C. Tai

Committee: Zehava Eichenbaum

Parjit Kaur

Chung-Dar Lu

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2011

DEDICATION

This dissertation is dedicated to my wonderful family:

the memory of my father, Chengjie Jin, who had been my model for hard work and persistence, and who encouraged me to set high goals and inspired my confidence to achieve them.

my loving mother, Huashu Shen, who has instilled in me the importance of higher education and given me her fullest support.

my dearest husband, Jin Wu, who has been understanding, patient, and has put up with these many years of research.

my precious son, Shangyu Wu, who is the joy of our life and has grown into a wonderful 3 year old despite his mother spending so much time on this dissertation.

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LIST OF ABBREVIATION

a.a	Amino acid
AFM	Atomic force microscopy
Amp	Ampicillin
ATP	Adenosine 5'-triphosphate
Bp	Base pair
BaSecA1	<i>B. anthracis</i> SecA1
BaSecA2	<i>B. anthracis</i> SecA2
BsSecA	<i>B. subtilis</i> SecA
Cm	Chloramphenical
DARTS	Drug affinity responsive target stability
DTT	Dithiothreitol
EB	Erythrosine B
EcSecA	<i>E. coli</i> SecA
EcSecAN68	<i>E. coli</i> SecA mutant with N-terminal 1-609 a.a.
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
IPTG	Isoprophyl- β -D-thiogalactopyranoside
Km	Kanamycin
kb	kilo base pairs
kDa	kilo Dalton
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MS	<i>M. smegmatis</i>

MsSecA1	<i>M. smegmatis</i> SecA1
MsSecA2	<i>M. smegmatis</i> SecA2
MTB	<i>M. tuberculosis</i>
MtbSecA1	<i>M. tuberculosis</i> SecA1
MtbSecA2	<i>M. tuberculosis</i> SecA2
MW	Molecular weight
NBD	Nucleotide-binding domain
OD	Optical density
OG	n-octyl p-D-glucopyranoside
PAGE	Polyacrylamide gel electrophoresis
PBD	Preprotein binding domain
PMF	Proton Motive Force
PMSF	Phenylmethanesulfonyl fluoride
RB	Rose bengal
SAR	Structure-Activity relationship
SaSecA1	SecA1 of <i>S. aureus</i>
SaSecA2	SecA2 of <i>S. aureus</i>
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEM	Transmission electron microscopy
Tet	Tetracycline
Tn	Translocation ATPase
TSST-1	Toxic shock syndrome toxin 1

INTRODUCTION

In the past sixty year, antibiotics have dramatically improve the prognoses of bacterial infectious diseases, and made a major contribution to on the improvement of public health [1]. However, drug resistance has co-developed with the discovery of new antibiotics [1-3]. In recent years, due to the misuse of antibiotics, multidrug resistance has been increased and widely spread [4-5]. In recent years, multi drug-resistant bacteria pathogens cause a serious public health problem, with high mortality rates, prolonged hospital stays, and increased healthcare costs [6]. Therefore, there is an urgent need to find new antimicrobial drugs against those drug-resistant bacterial pathogens [7]. Common mechanisms of antibiotics are inhibition on essential enzymatic processes in the bacterium to interrupt replication, or transcription, or translation, or cell wall synthesis [8]. Because the emergence of drug resistance against all these antibacterial mechanisms [9-10], there is an urgent need to develop new antimicrobials with novel mechanisms.

In our study, we target on protein translocation system, because bacterial exported proteins play important roles in bacterial physiology, viability, or/and pathogenicity [11-16]. Some exported proteins function as essential proteins, required for bacterial growth, cell wall maintenance, and cell division [17-18]; moreover, some exported proteins function as toxins or virulence factors required for establishment of infection, invasion in human tissue, protection from host defense system, or inducing toxic syndrome [19-22] . Therefore, further understanding of protein secretion mechanism may provide opportunities to target therapies or to create a vaccine candidate by interrupting components of the secretion pathway [23]. In both Gram-positive and Gram-negative bacteria, two major conserved protein export systems requiring N-terminal signal peptides are responsible for transporting proteins across cytoplasmic membrane [24-26]: General secretion (Sec) pathway and Twin-arginine translocation (Tat) pathway. Most export proteins are

transported unfolded through Sec-pathway [25]; some folded proteins and cofactor-containing proteins are transported through Tat pathway [27]. In some bacteria, there are also specialized export systems responsible for secretion specific virulence factors [28-30].

In this study, we focus on Sec pathway, because it plays an essential role in bacteria and highly conserved in bacteria. Sec pathway is responsible for export precursor proteins, which contain classical amino-terminal signal sequences across the cytoplasmic membrane [25, 31-32]. Majority of exported proteins are translocated through this pathway. Sec system is not only responsible for the secretion of many essential proteins, and it is also responsible for the secretion of many toxins and virulence factors [15, 33-35]. In *S. aureus*, there are many secretory toxin precursors containing Sec-dependent signal peptide [34]. The table 0.1 showed these toxins play important roles in the pathogenesis of *S. aureus* infection, involving in colonization or spread in human tissue, protecting bacteria from host defensive attack, eliciting immune responses.

The Sec pathway is well-characterized through studies with *E. coli* and *B. subtilis*. In these two model systems, SecA is an essential protein, which interacts with virtually all the other components of the system, acting as a molecular chaperone and a molecular motor providing driving force for translocation and pushing preprotein export through transmembrane channel [32, 36-37], and being involved in forming protein-conducting channel [38-39]. SecA is highly conserved in bacteria and essential for viability, moreover it has no counterpart in mammalian cell [40]. Thus SecA might be an ideal target for developing antimicrobial agents [13, 23]. It was long believed that all bacteria have a single SecA [41]. However, in recent years, two SecA homologues are identified in over thirty Gram-positive bacteria [28, 42-48] (Fig. 0.1). Majority of these bacteria are human pathogens, including some pathogens lethal to human or causing serious multi drug resistant problems, such as *B. anthracis*, *S. aureus*, and *M. tuberculosis*.

Table 0.1 List of toxins with Sec-dependent signal peptide in *S. aureus* (Adapted from [34])

Pathogenic action	Virulence factors
Colonization of host tissues	ClfA, Fibrinogen-binding protein A
	ClfB, Clumping factor B
	IsdA, Cell surface protein
	SdrC, Ser-Asp-rich, fibrinogen-binding bone, sialoprotein-binding protein
	SdrE, Ser-Asp-rich, fibrinogen-binding bone, sialoprotein-binding protein
Lysis of eukaryotic cell membranes and bacterial spread	Geh, Glycerol ester hydrolase
	Hla, α -hemolysin
	Hld, β -hemolysin /phospholipase C
	HlgA, γ -hemolysin chain A-C precursor
	HlgB-C, γ -hemolysin B and C component
	Lip, Triacylglycerol lipase precursor
	LukD, Leukotoxin D
	LukE, Leukotoxin E
	LukF, Pantone-valentine leukocidin chain F precursor
	LukS, Pantone-valentine leukocidin chain S precursor
Survival in phagocytes	Efb, Hypothetical protein
	Spa, Immunoglobulin G binding protein A precursor
Immunological disguise and modulation	ClfA, Fibrinogen-binding protein A
	ClfB, Clumping factor B, have signal peptide
	Coa, Staphylocoagulase precursor
	Spa, Immunoglobulin G binding protein A precursor
Septic shock symptom	SEB, Staphylococcal enterotoxin B
	TSST-1, Toxic shock syndrome toxin 1

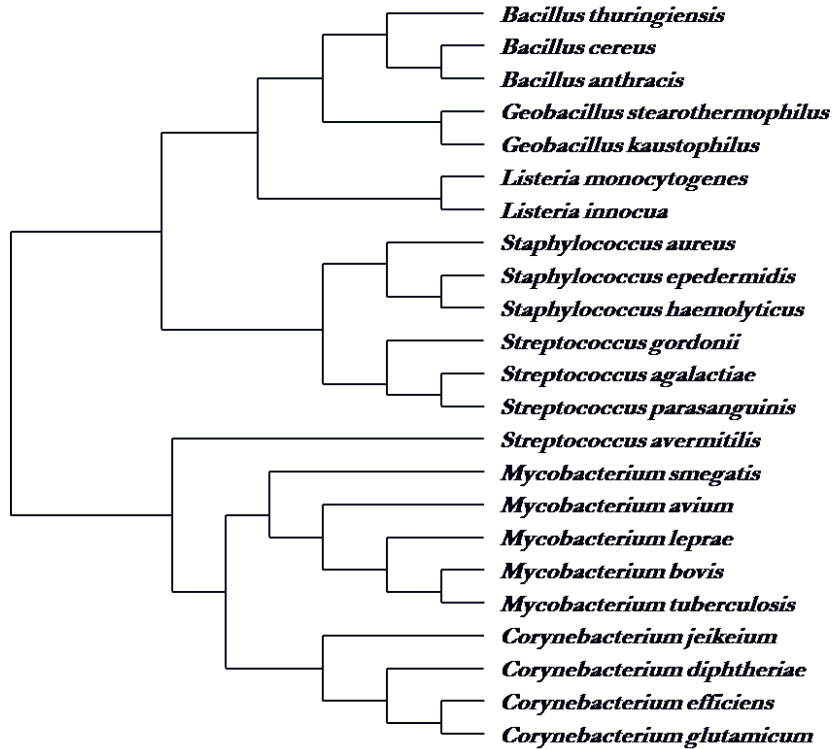


Figure 0.1 Bacteria containing two SecA homologues

For those two SecA homologues, the one with the higher homology to *E. coli* SecA (Ec-SecA) was named SecA1, and the other one with lower homology to EcSecA was named SecA2. Previous genetic studies show that SecA1 essential for viability and might be involved in general secretion pathway [28, 49-50]; while SecA2 is not essential, except in *Corynebacterium glutamicum* [51] and *Clostridium difficile* [52]. In *C. difficile* and *C. corynebacterium*, both SecA1 and SecA2 are essential protein required for growth [51-52]. In *C. difficile*, SecA2 is required for the export of the S-layer proteins and a cell wall protein [52] (Table 0.2). SecA2 is not essential for the viability in other strains, but they are involved in secretion of specific proteins related with virulence, such as protective enzyme, adhesion, autolysin [44-46, 48, 53-54] (Table 0.2). In *S. gordonii* [42] and *S. aureus* [48], SecA2 is required for virulence by mediating export of adhesions, GspB [42] and SraP [48], which are responsible for human platelet binding. In *L. monocytogenes*, SecA2 is required for persistent colonization in host tissues by mediating secretion of

two autolysins, p60 and NamA [44, 53]; and SecA2 is also required for secretion of SodA [55]. In *S. parasanguis* FW213, SecA2 mediate secretion of two adhesions, FimA and Fap1 [45]. All these findings support SecA2 contribute to the virulence of some important Gram-positive pathogen by playing a role in a specialized secretion pathway. SecA2 also plays a role in creating immune response. In *M. tuberculosis*, SecA2 is required for secretion of SodA [54] and restricts maturing of antigen specific CD⁸⁺ T cells and protection *in vivo* [56]; while in *L. monocytogenes*, SecA2 promotes protective immune response [57]. Thus, targeting SecA2 might lead to identify drugs that specifically target pathogenic bacteria without disrupting the normal microflora [8, 58].

Table 0.2 The summary of SecA2's function from previous studies

Bacteria	Protein	Pathogenic action
<i>M. tuberculosis</i> , [54]	SodA , manganese superoxide dismutase	Protect from oxidative attack
<i>M. smegmatis</i> , [59]	Msmeg1704, lipoprotein	Periplasmic sugar binding
	Msmeg1712, lipoprotein	Periplasmic sugar binding
<i>S. aureus</i> , [48]	SraP, adhesin	Colonization of host tissues
<i>S. gordonii</i> , [42]	GspB, adhesin	Colonization of host tissues
<i>S. parasanguis</i> , [45]	Fap1, fimbrial adhesin	Colonization of host tissues
	FimA, adhesin	Colonization of host tissues
<i>L. monocytogenes</i> , [44]	p60, autolysin	Bacterial spread
	NamA, autolysin	Bacterial spread
	SodA, manganese superoxide dismutase	Protect from oxidative attack
<i>C. difficile</i> , [52]	SLPs, S-layer proteins	Protection
	CwpV, Cell wall protein	

A good antimicrobial target should be involved in essential process of bacteria life cycle, interacting with it could inhibit bacteria growth or cause bacteria death, and it should be specific for pathogen without human counterpart. The potential target should contain ligand binding domain(s), be assayable for high throughput investigating *in vitro* and *in vivo* function. SecA homo-

logues fit all these criteria pretty well. **1)** They are required for bacterial viability and/or virulence; highly conserved in bacteria with no human counterpart [40], therefore targeting these proteins may represent a way to combat bacterial pathogens with minimal human toxicity. **2)** SecA could bind with ATP, preproteins, SecYEG, and molecular chaperones. And ligand binding pockets of SecA were identified from crystal structure [60] and NMR structure [61-62], supporting SecA is reasonable for drug targeting by small molecules. **3)** Malachite green is used to measure the ATPase activity of SecA, and this method is applied for high-throughput screening for SecA inhibitors [63-64]. High-throughput-screening compatible fluorescence and surface plasmon resonance techniques were used to investigate the interaction between SecA and other proteins [62, 65-67]. *In vivo* techniques using β -galactosidase fusion reporter [68] and anti-sense interference [69], were used to study the *in vivo* inhibition of SecA. **4)** SecA homologues plays important role during all infection life stages. They have important functions not only in exponential phase but also in stationary phase [34, 70-71]; they are required for colonization and spread in human tissue; they can protect bacteria from host defensive attack; also play roles in eliciting immune responses [56-57]. Thus, inhibition SecA could have dramatically impact on bacteria, causing growth inhibition, death, and virulence reduction. **5)** For those Gram-positive pathogens containing two SecA homologues, like MRSA, MTB, *B. anthracis*, and *C. diphtheria*, drug targeting SecA might have two targets making SecAs more attractive targets for the development of novel antimicrobials. Dual target inhibition could increase the change of combat infection and reduce the occurrence of drug resistance. **6)** SecA is involved in forming transmembrane channel [38-39], implying that it might be accessible directly from the extracellular environment in Gram-positive bacteria. Thus, targeting SecA might be able to by-pass the effect of efflux, which is one of the major and common mechanisms for bacterial drug-resistance devel-

opment [72-76]. Based on all these features of SecA homologues, **we hypothesize that SecA is an excellent target for antimicrobial development. Inhibition of these SecA homologues could reduce bacteria virulence, inhibit bacteria growth, and/or kill bacteria.**

Although the critical role of SecA in bacteria has been noted for a long time, a few small-molecule inhibitors of SecA have been reported [62, 64, 69, 77-82]. Sodium azide is the first reported SecA inhibitors [77]. Sodium azide could only inhibit the translocation ATPase activity of EcSecA and BsSecA [77], but not for intrinsic ATPase activity. Although azide-resistant mutants have been mapped on SecA [78], azide could inhibit activities of many other enzymes, like cytochrome c oxidase [83], proton ATPase [84], superoxide dismutase [85], alcohol dehydrogenase [86], and ceruloplasmin [87]. Previous studies demonstrated that azide stabilize the membrane-inserted and ADP-bound state of SecA, and inhibit the function of SecA by trapping SecA in this transitional state [37, 62, 88-89]. In 2002, an organic compound (CJ-21058), isolated from the fermentation broth of fungus, was reported as an inhibitor against SecA translocation ATPase activities [79]. Pannomycin is the second SecA inhibitor isolated from the fermentation broth of fungus [69], which had weak antibacterial activity against Gram-positive bacteria [62, 69]. Both CJ-21058 and pannomycin contain the decalin-tetramic acid scaffold with a quaternary carbon center at position C-6 [62], which might be important for SecA inhibition. In 2008, two SecA inhibitors were discovered from virtual ligand screening against EcSecA crystal structure [80]. Two pyrimidine analogs were developed from optimization of those two compounds, with improved inhibition effect against EcSecAN68 [81]. Several SecA inhibitors were developed from high-throughput screening or virtual screening, with IC_{50} values between 50-150 μ M [64, 82].

Although the function of SecA is well studied in *E. coli* and *B. subtilis*, and genetic studies have been demonstrated that SecA1 and SecA2 are involved in translocation, the biochemical properties of SecA1 and SecA2 have not been well characterized. It is not clear whether these proteins have similar functions as EcSecA and BsSecA. Alignment result showed that nine motifs of DEAD helicase were highly conserved in all SecA homologues (Fig. 0.2). In EcSecA, these nine motifs form two nucleotide binding domains (NBD); NBD1 has high affinity to nucleotide, and contains minimal ATPase catalytic machinery; NBD2 has low affinity to nucleotide, and acts as an activator of ATP hydrolysis by controlling ADP release and optimal ATP catalysis at NBD1 [90]. Thus, NBD1 and NBD2 are very important to the translocase activity of EcSecA. The conservation of these domains suggests SecA1 and SecA2 might have similar translocase activity as EcSecA. Thus, we hypothesize that SecA1 and SecA2 are ATPase that hydrolyses ATP to promote translocation of specific proteins; and drugs targeting those two nucleotide binding domains might have dual targets in those Gram positive pathogens containing two SecA homologues.

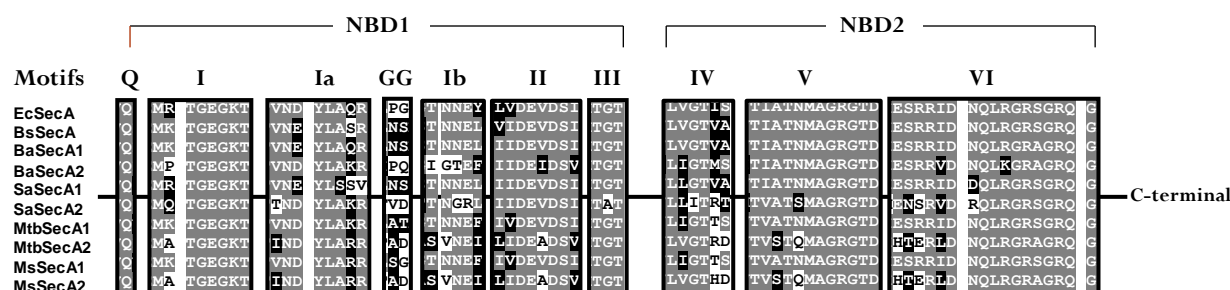


Figure 0.2 Alignment SecA1s and SecA2s with EcSecA and BsSecA

In this study, SecA1 and SecA2 were cloned from *M. tuberculosis* H₃₇Rv, *M. smegmatis* mc²155, *S. aureus* 35556, and *B. anthracis* Sterne, over-expressed in *E. coli*, and purified. The biochemical properties of these SecA homologues were comparatively characterized. To determine whether these SecA1 and SecA2 could be molecular motor, ATPase assay was used to in-

investigate their ATPase and GTPase activities. To determine whether SecA1 and SecA2 could interact with membrane in the same way as EcSecA, the ATPase activity of SecA homologues were investigated in presence of liposomes; proteolysis assay was used to identify specific transmembrane domain of MtbSecA1; transmission electron microscopy (TEM) and atomic force microscopy (AFM) were used to investigate whether SecA1 and SecA2 could change structure in presence of liposomes; the subcellular localization of MsSecA1 and MsSecA2 were investigated.

SecA homologues were used to develop and optimize three structurally different classes of potential SecA inhibitors developed from three different approaches: 1) RB and RB analogs derived from systematical dissection RB and SAR study; 2) pyrimidine analogs derived from virtual screening based on ATP binding pocket of EcSecA and SAR study [80-81]; 3) bistriazole analogs derived from random screening and SAR study. This project is in collaboration with Dr. Binghe Wang's lab. SecA inhibitors were screened and optimized based on the Structure-Activity relationship study. The *in vitro* inhibition effects of inhibitors against the ATPase activity of SecA homologues were investigated as well as the antimicrobial activities against *B. subtilis* 168, *S. aureus* 6538, *B. anthracis* Sterne, *E. coli* NR698, and *S. aureus* Mu50. Several potent SecA inhibitors were subjected to killing assay to investigate their bactericidal effects.

In *E. coli* and *B. subtilis* Sec system, SecYEG complex form the translocation core channel in membrane, while SecA undergo cycles of membrane insertion and dissociation, guiding and pushing pre-protein export through transmembrane channel formed by SecYEG [38, 91]. However, SecY and SecE are not required for protein translocation in some *in vitro* assay [92-93]; and in activated state, some domain of SecA is exposed to the surface of *E. coli* inner membrane [94-95]. Previous studies of our lab demonstrated that EcSecA exists as soluble form and

membrane form [96-97]; SecA alone could form ring-like structures upon interaction with anionic phospholipids [38-39]; and EcSecA is sufficient to export preOmpA across liposomes (Shieh et al, 2012), suggesting that SecA might be involved in forming a protein conducting-channel that spans the entire membrane. Thus, drugs targeting SecA might be directly accessible from the extracellular matrix and exert their effects without entering the cell, and bypassing the negative effect of efflux transporters in bacteria, which is a major mechanism for the development of current drug-resistance [72-76, 98-99]. *S. aureus* Mu50 and *S. aureus* N315 are resistant to QacA efflux-mediated antibiotics [100]. NorA and MepA are two *S. aureus* efflux pumps, belong to the major facilitator superfamily (MFS) or the multidrug and toxic compound extrusion (MATE) family [98, 101]. In this study, we further investigated whether our inhibitors have antimicrobial activities against *S. aureus* Mu50 and *S. aureus* N315, and whether NorA and MepA could affect the antimicrobial activities of our SecA inhibitors.

Sec system is not only responsible for the secretion of many essential proteins, but also responsible for the secretion of many toxins and virulence factors [33-35]. Because both SecA1 and SecA2 are involved in secretion of virulence factors, dual targeting inhibition on these two SecA homologues would dramatically reduce virulence. Virulence target-based therapies are not sufficient to combat infection, but targeting SecA homologues is not only reduces virulence but also decreases viability, dramatically increasing the chance of control bacterial infection and reducing the occurrence of drug resistance. To determine whether inhibitors could inhibit the *in vivo* function of SecA and whether inhibitors could reduce virulence of bacteria pathogen, we investigated the inhibition effect of SecA inhibitors on the secretion of three *S. aureus* toxins and three *B. anthracis* toxins, in which precursors contain N-terminal signal peptide required for secretion through Sec-pathway [34-35].

To validate SecA as the target of those inhibitors developed in our study, we investigated inhibition on *in vivo* function of SecA and interaction between SecA and inhibitors. And two different target identification assays were used to determine whether inhibitors could specifically bind with SecA at whole cell level.

In this research, we study the biochemistry properties of SecA homologues, develop novel SecA inhibitors, and further demonstrate that SecA is a real target of those inhibitors. The dissertation is composed of three parts: 1) Comparative characterization of SecA1 and SecA2 of Gram-positive bacteria. 2) Development and optimization of novel SecA inhibitors. 3) Validation of SecA as a real target.

**CHAPTER 1 COMPARATIVE CHARACTERIZATION OF SECA1 AND SECA2 OF
GRAM POSITIVE PATHOGENS**

Summary

Although majority of bacteria contain only one SecA homologue, two SecA homologues are identified in some important Gram-positive pathogenic bacteria. SecA1 is involved in general protein secretion and essential for viability, whereas SecA2 contribute to the virulence of some important Gram positive pathogen by playing a role in a specialized secretion pathway. The biochemical properties of SecA1 and SecA2 from Gram-positive bacterial pathogens have not been well characterized. Alignment showed that nine motifs of DEAD helicase are highly conserved in all SecA homologues. In EcSecA, these nine motifs form two nucleotide binding domains (NBD); NBD1 acts as the ATPase catalytic machinery; NBD2 is a regulator of ATP hydrolysis at NBD1. The conservation of these motifs suggested that SecA1 and SecA2 might be ATPase and have direct role on translocation. In this study, four different species of SecA1 and SecA2 were cloned, over-expressed in *E. coli*, and purified. All these SecA homologues possess ATPase activity, suggesting that they could be molecular motor. The ATPase activity of SecA1 can be stimulated by liposomes, while the ATPase activity of SecA2 cannot be stimulated by liposomes. The optimal temperature for ATPase activity is varied in different SecA proteins. Only mycobacteria SecA1 showed high ATPase activity at 0°C. The ATPase activity of MsSecA1 could be obviously stimulated by BA13 membrane and the preOmpA of *E. coli*, while other SecA homologues do not show similar response. Because the membrane and precursor were from *E. coli*, they might not be recognized by translocase from other species. *Mycobacteria* SecA1 and SecA2 failed to complement the temperature-sensitive defect EcSecA, suggesting that interaction between secretion factors of Sec system might be specific for different organisms. Majority of MsSecA1 are located on membrane, while majority of MsSecA2 are located in cytosol. Liposomes could induce conformational change of MtbSecA1 and form ring-like structures un-

der TEM and AFM. All these results suggest that mycobacteria SecA1 might interact with membrane in the same way as EcSecA and might be involved in forming transmembrane channel.

Introduction

Bacterial exported proteins play important roles in bacterial physiology, viability, or/and pathogenicity [11-13]. Some exported proteins function as essential proteins, required for bacterial growth, cell wall maintenance, and cell division [17-18]; moreover, some exported proteins function as toxins or virulence factors required for establishment of infection, invasion in human tissue, protection from host defense system, or inducing toxic syndrome [19-22]. Therefore, further understanding of protein secretion mechanism may provide opportunities to target therapies or to create a vaccine candidate by interrupting components of the secretion pathway [23]. In both Gram⁺ bacteria and Gram⁻ bacteria, two major conserved protein export systems are responsible for transporting proteins across cytoplasmic membrane [24-26]: General secretion (Sec) pathway and Twin-arginine translocation (Tat) pathway. In some bacteria, there are also specialized export systems responsible for secretion specific virulence factors [28]. Sec system is not only responsible for the secretion of many essential proteins, and it is also responsible for the secretion of many toxins and virulence factors [33-34].

The Sec pathway is well-characterized through studies with *E. coli* and *B. subtilis*. In these two model systems, an essential protein is the SecA ATPase, which interacts with virtually all the other components of the system, acting as a molecular chaperone, providing driving force for translocation, pushing preprotein export through transmembrane channel [36], and being involved in forming protein conducting channel [38-39]. SecA is highly conserved in bacteria and essential for viability, moreover it has no counterpart in mammalian cell [40]. Thus SecA might be an ideal target for developing antimicrobial agents [13, 23]. It was long believed that all bacteria have a single SecA [41]. However, in recent 9 years, two SecA homologues are identified in some important Gram positive bacteria [28, 42-48]. Majority of these bacteria are human patho-

gens, including some pathogens lethal to human or causing serious multi drug resistant problems. For those two SecA homologues, the one with the higher homology to EcSecA was named SecA1, and the other one with lower homology to EcSecA was named SecA2. Previous genetic studies show that SecA1 essential for viability and might be involved in general secretion pathway [28, 49-50]; while SecA2 is not essential, except in *C. glutamicum* [51] and *C. difficile* [52]. Although SecA2 is not essential for the viability but it is involved in secretion of specific proteins related with virulence, such as protective enzyme, adhesion, autolysin [44-46, 48, 53-54]. SecA2 also plays a role in creating immune response. In MTB, SecA2 restricts maturing of antigen specific CD8⁺ T cells and protection *in vivo* [56]; while in *L. monocytogenes*, SecA2 promotes protective immune response [57].

Although the function of SecA is well studied in *E. coli* and *B. subtilis*, and the functions of SecA1 and SecA2 from Gram⁺ bacteria pathogen were studied with genetic methods, the biochemical properties of SecA homologues not investigated. We don't know whether these proteins have similar functions as EcSecA and BsSecA. Alignment result showed that nine motifs of DEAD helicase were highly conserved in all SecA homologues (Fig. 0.2). In EcSecA, these nine motifs form two nucleotide binding domains (NBD); NBD1 has high affinity to nucleotide, and contains minimal ATPase catalytic machinery; NBD2 has low affinity to nucleotide, and acts as an activator of ATP hydrolysis by controlling ADP release and optimal ATP catalysis at NBD1 [90, 102]. Thus, NBD1 and NBD2 are very important to the translocase activity of EcSecA. The conservation of these domains suggests SecA1 and SecA2 might have similar translocase activity as EcSecA. Thus, we hypothesize that SecA1 and SecA2 are ATPase that hydrolyses ATP to promote translocation of specific proteins. Moreover, drugs targeting NBD domains might have dual targets in those Gram-positive pathogen containing two SecA homologues.

In this study, SecA1 and SecA2 were cloned from *M. tuberculosis* H₃₇Rv, *M. smegmatis* mc²155, *S. aureus* 35556, and *B. anthracis* Sterne, over-expressed in *E. coli*, and purified. The biochemical properties of these SecA homologues were comparatively characterized.

Materials and Methods

Medium and Reagents: LB and TAG were used as liquid and solid (with 1.5% agar) medium to grow bacteria. 1 liter TAG medium contains 10 g tryptone, 5 g NaCl, 10 g glucose, 40 mM potassium phosphate buffer (pH 7.0), 7.6 mM ammonium sulfate, and 1.6 mM sodium citrate. T4 DNA ligase (triple-master) was purchased from Eppendorf. Plasmid and Gel extraction kits were purchased from Qiagen. All other chemicals were purchased from Sigma-Aldrich. Middlebrook 7H9 broth with 0.2% glycerol, 1×ADC (0.5% bovine serum albumin, fraction V, 0.2% dextrose, 0.85% NaCl), and 0.1% Tween 80 was used to grow *M. smegmatis* Mc²155.

Bacterial Strains: *E. coli* DH5α was used as host for cloning. *E. coli* BL21.19 and BL21λDE3 were used for overexpression. *E. coli* BL21.19 and BA13 were used for complementation assay. BL21.19: λDE3, *SecA13(Am)* *supF(Ts)* *trp(AM)* *Zch::Tn10*, *recA::CAT* *claA319::KAN* [103]. BA13: *secA13(Am)* *supF(Ts)* mutant of MC4100, *secA(am)*, *supF(ts)*, *F⁻*, *lacU169*, *araD136*, *relA⁻*, *rps150*, *febB5301*, *deoC7*, *ptsF25thi* [96, 104]. Genes from *M. tuberculosis* H₃₇Rv, *M. smegmatis* mc²155, *S. aureus* 35556, and *B. anthracis* Sterne were used to clone *SecA1* and *SecA2*. *M. smegmatis* mc²155 was from Dr. Miriam Braunstein at University of North Carolina at Chapel Hill. *M. tuberculosis* H₃₇Rv gene was from TB vaccine testing and research materials contract program of Colorado State University.

Primers and Plasmids: The primers and plasmids used in this work were listed in Table 1.1.

Cloning: PCR amplification was carried out in Eppendorf Mastercycler using Triple master DNA polymerase of Eppendorf. 2-3% DMSO was added into amplification mixture when cloning *SecA* homologues from *M. tuberculosis* H₃₇Rv, *M. smegmatis* mc²155. PCR products were purified by gel extraction; vector DNA and PCR product were double digested by restric-

tion enzymes, then purified by gel extraction; then the digested DNA of vector and insert were ligated using T4 DNA ligase at 14 °C overnight; ligation mixture were transformed into *E. coli* DH5 α ; positive cloning was selected and transform into BL21.19 or BL21 λ DE3.

Table 1.1 Plasmids and primers used for cloning *secA1* and *secA2*

Gene	primer (5'-3')	Restriction site	plasmid
<i>MtbsecA1-1</i> (his-)	5'primer GCGGGGACATAC <u>CATATG</u> CTGTCTGAAG	Nde I	pET-20b (+)
	3'primer TCTGCAACC <u>AAGCTT</u> CTAACGCTT	Hind III	
<i>MtbsecA1-2</i> (his+)	5'primer GCGGGGACATAC <u>CATATG</u> CTGTCTGAAG	Nde I	pET-20b (+)
	3'primer GCAACCTAC <u>AAGCTT</u> ACGCTTCTT	Hind III	
<i>MtbsecA2-3</i> (his+)	5'primer CGTCCAGCC <u>CCATGG</u> TGA ACGTG	Nco I	pET-33b (+)
	3'primer CTTACGCTG <u>GAATTCT</u> CAGCGGAA	EcoR I	
<i>MssecA1</i> (his+)	5'primer ACT <u>CATATG</u> CTGTCTGAAGTTGGTC	Nde I	pET-20b (+)
	3'primer ATAG <u>GAGCTC</u> GAGCCCTTGGGGTGC	Sac I	
<i>MssecA2</i> (his+)	5'primer ACT <u>CATATG</u> GCGAATGAGTCCTG	Nde I	pET-33b (+)
	3'primer ATAG <u>GAATTC</u> GAGCGGAACACACCC	EcoR I	
	3'primer ATAA <u>AAGCTT</u> GCGGAACACACCCGG	Hind III	
<i>SasecA1</i> (his+)	5'primer AGCG <u>GCTAGC</u> GGATTTTATCAAAAATTC	Nhe I	pET-21d (+)
	3'primer TCGC <u>AAGCTT</u> TTTTCCATGGCAATTTTGT	Hind III	
<i>SasecA2</i> (his+)	5'primer GAACCATGGGCAAACATAAACTAG	Nco I	pET-33b (+)
	3'primer TAT <u>GAATTC</u> CCTGGAAAATGTATAAC	EcoR I	
	3'primer TAT <u>GAGCTC</u> CCTGGAAAATGTATAAC	Sal I	
<i>BasecA1</i> (his+)	5'primer TCTCC <u>CATATG</u> ATCGGTATTTTAAAAAAGGTG	Nde I	pET-20b (+)
	3'primer TATAG <u>GTCGAC</u> CTTCCCGATACCACAGCAG	Sal I	
	3'primer TATA <u>AAGCTT</u> CTTCCCGATACCACAGCAG	Hind III	
<i>BasecA2</i> (his+)	5'primer TATAC <u>CCATGG</u> TGCTGAATTCGGTAAAAAAGC	NcoI	pET-33b (+)
	3'primer TATAG <u>GTCGAC</u> TTGTACGTTTTTCAGGAACACC	Sal I	

Overexpression: BL21.19 or BL21 λ DE3 containing the respective plasmids were streaked on TAG medium plate and incubated at 30°C or 37°C overnight. Single colony was inoculated into 3 ml of TAG medium and shook at 30°C or 37°C overnight. Then, the overnight culture was diluted into 50 ml of the same medium and continued to grow at 30°C or 37°C until OD₆₀₀ reached 1. The freshly grown-up culture was further diluted into 1.5 L of the same medium and grew until OD₆₀₀ reached 0.8, then the culture was cooled down in tap water and put back to shaker at 20°C. 0.1 mM to 0.5 mM IPTG was added to the culture and the cell culture continued to grow at 20°C overnight and the cells were collected by centrifugation.

ATPase Activity Assay: Malachite green colorimetric assay [105] was used to investigate ATPase or GTPase activity of SecA homologues. For the intrinsic ATPase activity, appropriate amount of SecA was mixed with 2 mM ATP in reaction buffer (50 mM Tris-HCl, pH 7.6, 20 mM KCl, 2 mM Mg(OAc)₂ and 1 mM DTT). For the lipid ATPase activity, different amount of liposomes was added into the intrinsic ATPase assay. To make liposomes, *E. coli* total lipid was dried in a speed vacuum, resuspended in TK buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl), then sonicated for 10 min on ice. For membrane ATPase activity, *E. coli* BA13 membrane fraction II (washed by 6 M Urea) was added into the intrinsic ATPase activity. For translocation ATPase activity, *E. coli* BA13 membrane fraction II (washed by 6M Urea) and proOmpA were added into the intrinsic ATPase assay. The components were mixed at 0°C, and incubated at different temperature for different time; the reactions were stopped by the subsequent addition of 800 µl of color reagent (0.045% malachite green mixed with 4.2% ammonium molybdate in 4N HCl at a ratio of 3:1, 0.1% Triton X-100 was added after filtration of the mixture) and 100 µl of 34% sodium citrate; the mixture was incubated at room temperature for 40 min, then its absorption was measured at 660 nm. The specific activity was determined based on a standard curve which was prepared with phosphate salt.

Complementation Test: Plasmids harboring different mycobacteria SecA homologues genes were transformed into BL21.19 strain or BA13, and grew on TAG/agar plates at 30°C overnight. The single colony was picked up and streaked on two new TAG/agar plates with different concentration of IPTG or arabinose, and incubated at 30°C and 42°C overnight respectively.

Subcellular distribution of mycobacteria SecA2 and SecA1: *M. smegmatis* Mc²155 were grown in Middlebrook 7H9 broth with 0.2% glycerol, 1×ADC (0.5% bovine serum albu-

min, fraction V, 0.2% dextrose, 0.85% NaCl), and 0.1% Tween 80. The cell pellet was French pressed at 18,000 psi, and unbroken cells and large cellular debris are removed by centrifugation at $3,000 \times g$. The supernatant of $3,000 \times g$ was centrifuged at $27,000 \times g$ for 1h to generate a fraction enriched in cell wall. The supernatant of $27,000 \times g$ was centrifuged at $258,000 \times g$ for 1.5 hrs to sediment the remaining cytoplasmic membrane.

Preparation of sample for TEM: 15-20 μ l samples of protein (25-200 μ g/ml) in 20 mM Tris-HCl buffer (pH 7.4) were added to small Teflon wells (4 mm in diameter and 0.5 mm deep) until the liquid surface bulged up. Add 0.5-1.0 μ l of *E. coli* phospholipids (from Avanti Polar Lipids) solutions (1 mg/ml) in chloroform/methanol (3:1 vol/vol) with a syringe. The wells were incubated in a sealed humid atmosphere at 4°C for 24 hours. Then, the lipid monolayers at the air/liquid interfaces were picked off with hydrophobic carbon coated gold grids. After washing with several drops of incubation buffer, grids were negatively stained with uranyl acetate solution (0.5-1% wt/vol). The grids were examined with TEM.

Preparation of sample for AFM: To prepare lipid bilayers, extracted *E. coli* total lipid was vacuum dried, resuspended in TKM buffer (10 mM Tris-HCL, pH 8.0, containing 50 mM KCl and 2 mM $MgCl_2$) and sonicated for 15 min in an ice bath. The mixture of SecA with or without lipid bilayer in 10 μ l TKM buffer was vortexed for 10 sec and applied to freshly cleaved mica, which were held at room temperature for 10-15 min, rinsed four times with deionized water, and dried in a dessicator. Goat anti-rabbit IgG gold at concentration of 15 μ g/ml in the same TKM buffer, was used as a reference for measuring the sizes.

Results

Cloning, Expression, and Purification of SecA Homologues

Cloning, Expression, and Purification of SecA Homologues of *M. tuberculosis* H₃₇Rv

Cloning and Over-expression *M. tuberculosis secA1* (*MtbsecA1*) The *MtbSecA1* gene was amplified from *M. tuberculosis* H₃₇Rv, cloned into pET-20(+), and overexpressed in BL21.19. SecA1 can be expressed very well at 20°C for 4-6 hours induced by 0.5 mM IPTG, and in the supernatant of 50k rpm supernatant (Fig. 1.1).

Purification of MtbSecA1 The PI/MW of MtbSecA1 (5.29/10.6 kDa) is very close to the PI/MW of EcSecA (5.4/10.2 kDa), causing difficulty to separate these two proteins. To improve purity of MtbSecA1, EcSecA could be depleted by culturing BL21.19 at 41°C for more than three generation. However, our result showed that depletion EcSecA at 41°C will cause much lower expression of MtbSecA1-1 (Fig. 1.1). Therefore, instead of depletion, Nickle column was used to separate those two proteins (Fig. 1.2a). The elution from Nickle column was load to SP-Sepharose column, and further purified by size column (Fig. 1.2b-1.2c).

Cloning and Over-expression *M. tuberculosis secA2* (*MtbsecA2*) The *MtbSecA2* gene were amplified from *M. tuberculosis* H₃₇Rv, cloned into pET-33b with 6X his-tag at C-terminal, and overexpressed in *E. coli* BL21λDE3. MtbSecA2 was expressed at three temperature, different time, and different concentration of IPTG. Our results showed that TAG medium was better than LB medium, and 37°C was better than 30°C and 18°C. However, at 30°C and 37°C, majority of MtbSecA2 formed inclusion body or aggregated; while at 18°C, MtbSecA2 expression was not very good, but majority of MtbSecA2 remained at the supernatant (Fig. 1.3a). To increase expression of MtbSecA2, rifampicin was used to shut down the expression of *E. coli* host protein. The cell was grown and expressed as Fig. 1.3b. When expressed at 30°C, the expression of

MtbSecA2 was very good (Fig. 1.3c), however there was no soluble MtbSecA2 (Fig. 1.3d). To induce enough T7 RNA polymerase, 1 mM IPTG was added 25 min earlier before adding rifampicin at 20°C. The results showed that the expression of MtbSecA2 at 20°C is not as good as expression at 30°C (Fig. 1.3e), but adding rifampicin at 20°C without up-regulate temperature, majority of MtbSecA2 are soluble (Fig. 1.3f). To enhance expression amount of soluble MtbSecA2, yeast extract or MgSO₄ was added in TAG medium when adding IPTG to induce expression. However, the expression of Mtb SecA2 didn't affected by adding yeast extract and Mg²⁺ (data not shown). To express MtbSecA2 at a large scale, MtbSecA2 was grown at 37°C in 1.5 L TAG with 6L bottle. When OD₆₀₀ reach 0.9, cell culture was cooled down to 20°C, then added IPTG to 1 mM; 25 min later adding 200 µg/ml rifampicin, then expressed at 20°C overnight or 5.5 hours. A lot of soluble MtbSecA2 remain in the supernatant of 50K rpm (Fig. 1.3g).

Purification of MtbSecA2 Cell pellet was resuspended, and French pressed at 18k PSI, then centrifuged at 4k rpm, 8k rpm, and 37k rpm to remove unbroken cell, inclusion body, and membranes. The supernatant of 37k rpm were loaded to His-trap column and further washed with imidazole gradient by using FPLC. MtbSecA2 bound His-trap very well, and majority of MtbSecA2 was eluted out with 50 mM to 100 mM imidazole (Fig. 1.4a). To avoid denaturation, the eluents from His-trap column were directly loaded to HisprepTM 26/10 desalting column instead of dialysis. The eluted fraction of desalting column was loaded to Q-sepharose column. The result showed that Q-Sepharose column could further remove some junk protein and increase purity of MtbSecA2 (Fig. 1.4b). The appropriate fractions from Q-Sepharose column were concentrated with Amicon Ultra centrifugal filter, then, loaded to Superose-6 column. The column was washed with 100 mM NH₄HCO₃. The result showed the purity is very high, but there is some degradation (Fig. 1.4c).

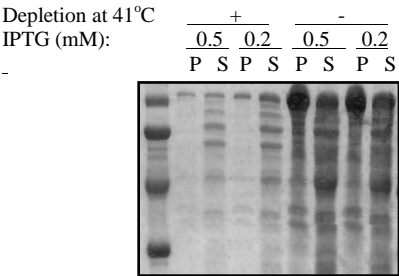


Figure 1.1 Expression of MtbSecA1
P: pellet of 50k rpm 30min; S: supernatant of 50k rpm 30 min

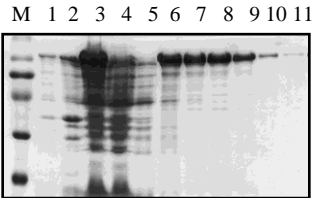


Figure 1.2a Purification of MtbSecA1 with Ni-NTA resin
lane 1: pellet of 4k rpm; lane 2: pellet of 36k rpm; lane 3: supernatant of 36k rpm; lane 4: unbinding sample; lane 5: buffer wash; lane 6: 20 mM imidazole; lane 7: 40 mM imidazole; lane 8: 60 mM imidazole; lane 9: 250 mM imidazole; lane 10: 500 mM imidazole.

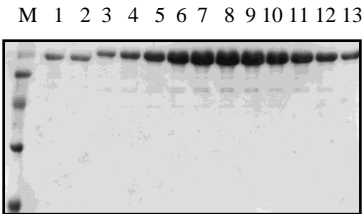


Figure 1.2b Purification of MtbSecA1 with SP-sepharase column
Lane 1-2: fraction 40-41, confirmed to be EcSecA by western blot; lane 3-13: fraction 59-69. PBS buffer: pH 6.4.



Figure 1.2c Purification MtbSecA1 with size column
Buffer: 100 mM NH₄HCO₃; lane 1-14: different fraction from Superose-6 column; lane 15: loading sample of Superdex-300 column; lane 16-26: different eluent fraction from Superdex-300 column.

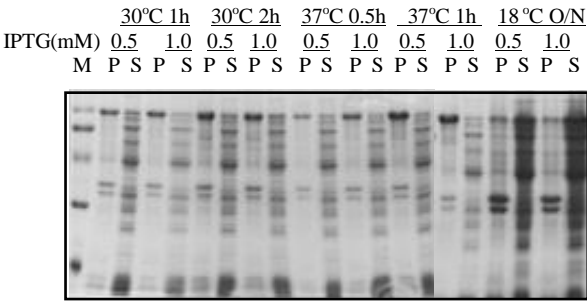


Figure 1.3a Expression of MtbSecA2
P: pellet of 50k rpm; S: supernatant of 50k rpm

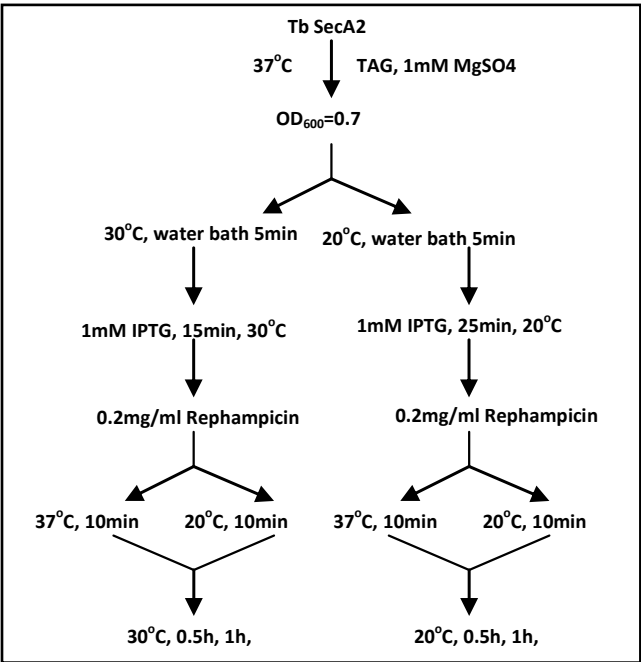


Figure 1.3b Expression of MtbSecA2 with rifampicin at different temperature

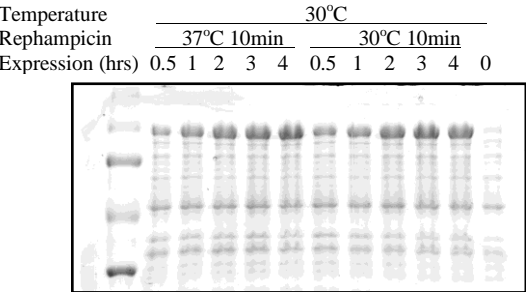


Figure 1.3c Expression of MtbSecA2 with rifampicin at 30°C

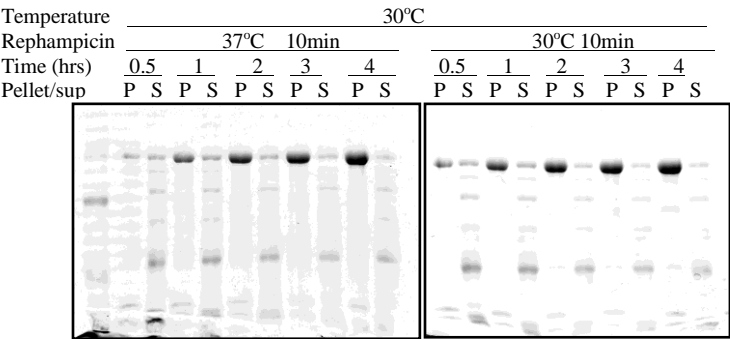


Figure 1.3d Solubility of MtbSecA2 expressed at 30°C
P: pellet of 50K rpm 30 min; S: supernatant of 50K rpm 30 min.

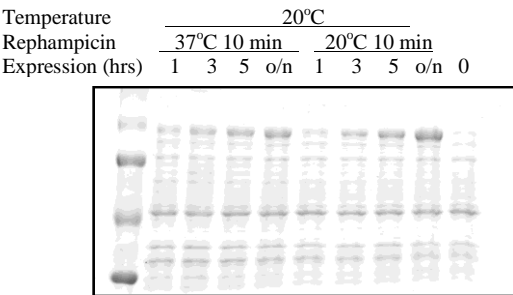


Figure 1.3e Expression of MtbSecA2 and rifampicin at 20°C
o/n: overnight.

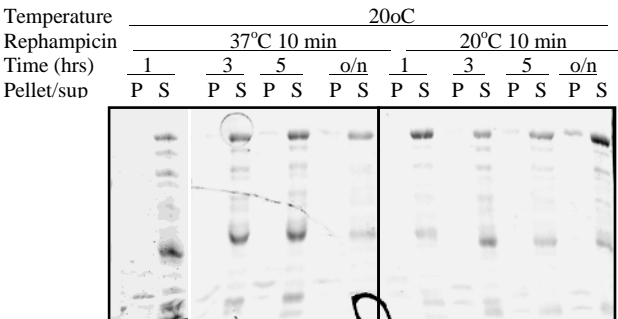


Figure 1.3f Solubility of MtbSecA2 expressed at 20°C
P: pellet of 50K rpm 30 min; S: supernatant of 50K rpm 30 min

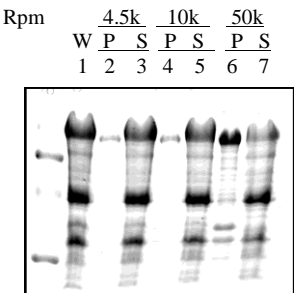


Figure 1.3g Expression of MtbSecA2 at large scale
W: crude cell lysate; P is pellet; S is supernatant.

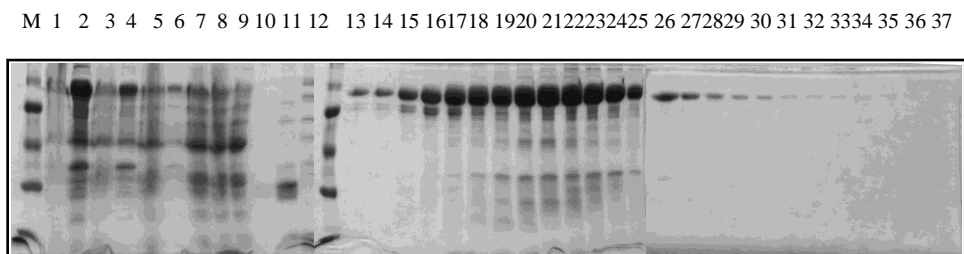


Figure 1.4a Purification of MtbSecA2 with His-trap column

Lane 1: 4k rpm pellet; lane 2: 4k rpm supernatant; lane 3-4: 8k rpm pellet; lane 5: 8k rpm supernatant; lane 6: 37k rpm pellet; lane 7: 37k rpm supernatant; lane 8: loading sample; lane 9: unbinding sample; lane 10-37: different eluent fractions.

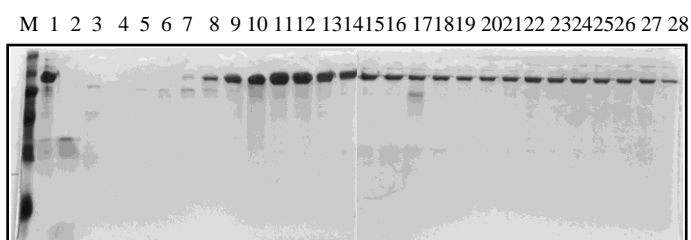


Figure 1.4b Purification of MtbSecA2 with Q-sepharose column

Buffer: PBS buffer pH 7.6; lane 1: loading sample; lane 2: unbinding sample; lane 3-28: different eluent fractions.

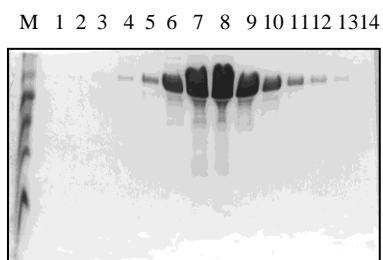


Figure 1.4c Purification of MtbSecA2 with Superose-6 column

Buffer: 100 mM NH_4HCO_3 ; lane 1-14: different eluent fractions.

Cloning, Expression, and Purification of SecA Homologues of *M. smegmatis*

Cloning and overexpression of *M. smegmatis* *secA1* (*MssecA1*) The gene of *MsSecA1* was amplified from *M. smegmatis* mc²155, cloned into pET-20b (+) with 6X his-tag at C-terminal, and overexpressed well in *E. coli* BL21λDE3 at 20°C for 10 hours (Fig. 1.5).

Purification of MsSecA1 The harvested cells were French pressed at 18,000 PSI and centrifuged at 5,000 g for 10 min and 36K rpm for 30 min. Then, the supernatant of 36k rpm was purified with His-trap column. MsSecA1 could bind with His-trap column very well (Fig. 1.6a). MsSecA1 could be washed out with 80 mM imidazole, and majority of the protein came out between 100 mM-250 mM imidazole. The elution from His-trap column was mixed and dialyzed over night with 25 mM potassium phosphate buffer (pH 6.4), then further purified with SP-sepharose column (Fig.1.6b) and size column (Fig. 1.6c). The results of SP column and size column showed that there was some degradation.

Cloning and overexpression of *M. smegmatis* *secA2* (*MssecA2*) The gene of *MsSecA2* (His tag +) was amplified from *M. smegmatis* mc²155, cloned into pET-33b with 6X his-tag at C-terminal, and overexpressed in *E. coli* BL21λDE3 at 20°C for 10 hours. The expression was very good (Fig. 1.5). The harvested cell was French pressed at 18,000 PSI and centrifuged at 5,000 X g for 10 min and 36K rpm for 30 min. Then, the supernatant of 36k rpm was purified with His-trap column with 25 mM potassium phosphate buffer (pH 7.6) containing 0.5 M NaCl. MsSecA2 could bind with His-trap column, but MsSecA2 could be washed out with 10 mM imidazole, and majority of the protein came out between 20 mM-100 mM imidazole (Fig. 1. 7a). Some appropriate fraction of His-trap column was mixed and dialyzed over night with 25 mM potassium phosphate buffer (pH 8.0), then further purified with Q-Sepharose column (Fig. 1.7b) and Superdex-200 column (Fig. 1.7c).

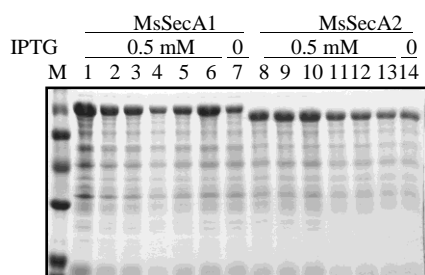


Figure 1.5 Expression of MsSecA1 and MsSecA2

Lane 1-6 and lane 8-13: different colonies.

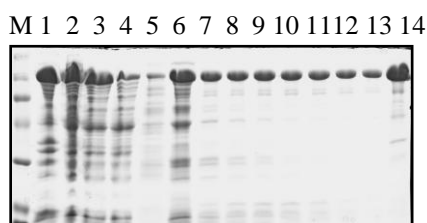


Figure 1.6a Purification of MsSecA1 with His-trap column

Lane 1: the pellet of 36k rpm; lane 2: the supernatant of 36k rpm; lane 3: loading sample; lane 4: unbinding sample; lane 5-14 is different eluent fraction.

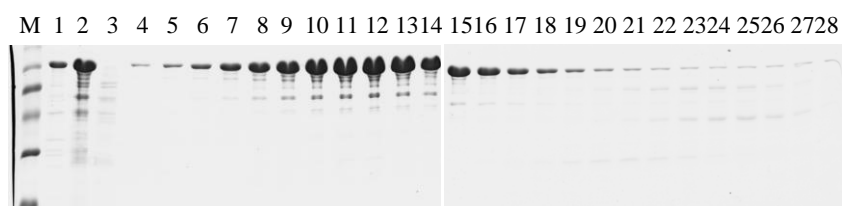


Figure 1.6b Purification of MsSecA1 with SP-sepharose column

Lane 1 is the precipitated pellet of dialysis; lane 2 is loading sample; lane 3 is unbinding sample; lane 4-28 are different eluent fraction.

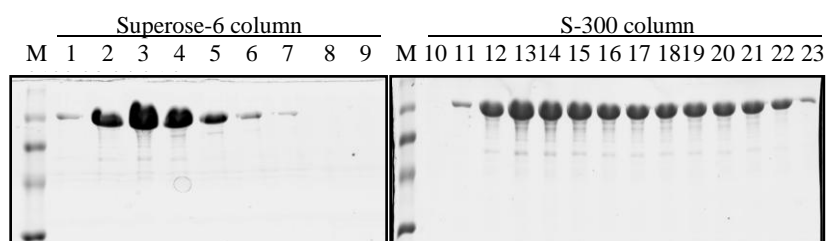


Figure 1.6c Purification of MsSecA1 with size column.

Buffer: 100 mM NH_4HCO_3 ; lane 1-9: different fraction from Superose-6 column; lane 10-23: different eluent fraction.

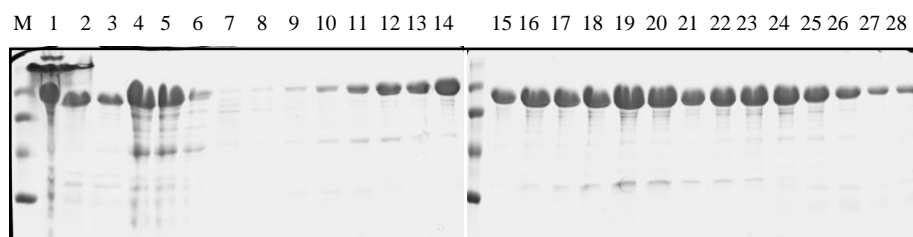


Figure 1.7a Purification of MsSecA2 with His-trap

Lane 1: crude cell lysate; lane 2: the pellet of 4k rpm; lane 3: the pellet of 36k rpm; lane 4: the supernatant of 36k rpm; lane 5: loading sample; lane 6: unbinding sample; lane 7-28 is different eluent fraction.

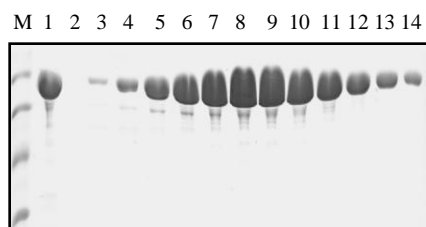


Figure 1.7b Purification of MsSecA2 with Q-sepharose column

Lane 1: loading sample; lane 2: unbinding sample; lane 3-14: different eluent fractions.

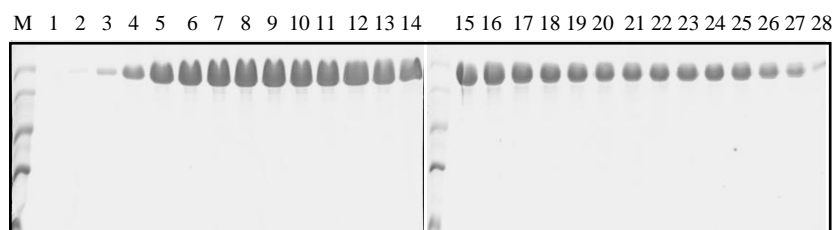


Figure 1.7c Purification of MsSecA2 with S-200

Buffer: 100 mM NH_4HCO_3 ; lane 1-28: different eluent fractions.

Cloning, Expression, and Purification of SecA Homologues of *S. aureus*

Cloning and expression of *S. aureus secA1* (*SasecA1*) The *SasecA1* gene was amplified from *S. aureus* ATCC35556, cloned into pET-21d with 6X his-tag at C-terminal (Fig. 1.8), then over-expressed in BL21λDE3 at 20°C or 37°C with 0.5 mM IPTG. The gel result showed that the expression of SaSecA1 at 20°C was better than at 37°C (Fig. 1.9).

Purification of SaSecA1 Cell pellet was French pressed at 18,000 psi. The supernatant of 37k rpm was loaded onto 5 ml His-trap column, and washed with different imidazole gradient by FPLC. The results show that SaSecA1 bind to His-trap column very well, and it could be eluted with >60 mM imidazole, and there were two peaks. The SDS-PAGE gel result showed the purity of some fraction was very high (Fig. 1.10a). The elution of his-trap column was concentrated with centrifugal filter to 10 mg/ml, then loaded to Superdex-200 column. The result of Superdex-200 column showed that SaSecA1 was very big and had degradation (data not shown). Some appropriate fractions of His-trap column were mixed, and concentrated with centrifugal filter, then diluted and loaded to Q-Sepharose column. SaSecA1 was further purified by using Q-Sepharose column (Fig. 1.10b). Then the fraction 59-71 were concentrated, and loaded to S-200 column. The result of Superdex-200 column showed that the purity was very high and the protein was shown as dimers when compared with other previous data (Fig. 1.10c). This data were different with previous result of Superdex-200 column. The possible explanation is that when eluted fraction of his-trap column was concentrated, the protein was aggregated and formed large molecule, which might be caused by high salt and high concentration of imidazole.

Cloning and expression *S. aureus secA2* (*SasecA2*) The *SasecA2* gene had been cloned into pET-33b, and expressed in BL21λDE3 at 20°C overnight with 0.5 mM IPTG. The expression was very good (Fig. 1.11).

Purification of SaSecA2 Cell pellet was French pressed at 10,000 psi. The supernatant of 37k rpm was loaded onto 5 ml His-trap column, and washed with different imidazole gradient by FPLC. The results showed that SaSecA2 bound to His-trap column very well, and it could be eluted with >50 mM imidazole. The SDS-PAGE gel result showed the purity of some fraction was very high (Fig. 1.12a). Some fractions were precipitated by ammonium sulfate, however after precipitation, the protein couldn't be re-solubilized. Some fraction was concentrated to 10 mg/ml with centrifugal filter, but the concentrated protein precipitated very quickly during loading to sample loop at room temperature, while loading sample on ice prevents precipitation. The buffer for his-trap column was 25 mM K_3PO_4 pH 8.0 with 0.5 M NaCl, 1 mM DTT, and imidazole. We don't know why high concentration of NaCl and imidazole will cause non-recoverable protein precipitate at room temperature, however if the imidazole was removed by desalting column or Q-column, then concentrated SaSecA2 did not precipitate. Superose-6 column could further separate small proteins around 29 kDa from SaSecA2 (Fig. 1.12b).

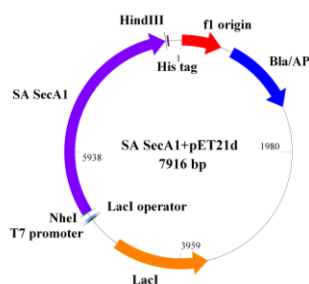


Figure 1.8 Map of SaSecA1/pET-21d

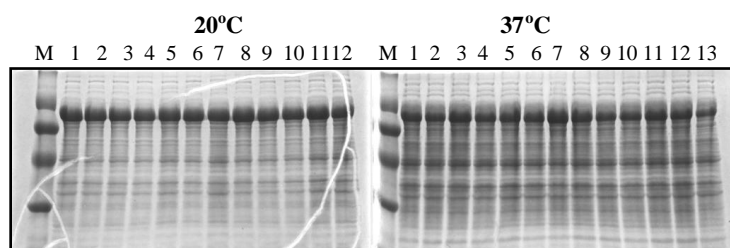


Figure 1.9 Expression of SaSecA1

Lane 1-13: different colonies.

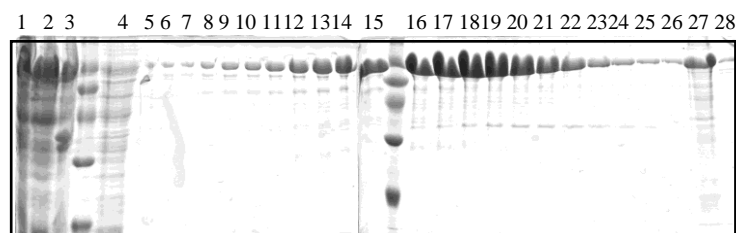


Figure 1.10a Purification of SaSecA1 with His-trap column

Lane 1: pellet of 4k rpm; lane 2: supernatant of 37k rpm; lane 3: pellet of the 37k rpm; lane 4: unbinding sample; lane 5-28: different eluent fraction of his-trap column.

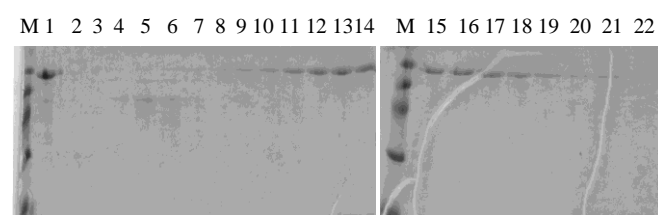


Figure 1.10b Purification of SaSecA1 with Q-sepharose column

Lane 1: loading sample; lane 2: unbinding sample; lane 3-22: different eluent fractions.

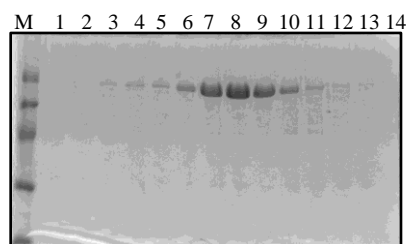


Figure 1.10c Purification of SaSecA1 with S-200

Lane 1-14: different eluent fractions.

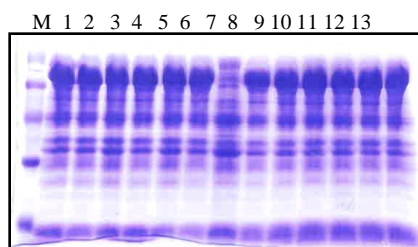


Figure 1.11 Expression of SaSecA2

Lane 1-6 and 8-13: different colonies; lane 7: control without IPTG induction.

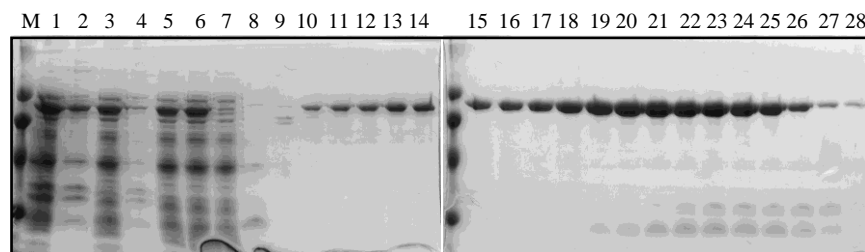


Figure 1.12a Purification of SaSecA2 with His-trap column

Lane 1: whole cell lysis; lane 2: pellet of 5k rpm; lane 3: supernatant of 5K rpm; lane 4: pellet of 37k rpm; lane 5: supernatant of 37k rpm; lane 6: loading sample; lane 7: unbinding sample; lane 8-28: different eluent fractions.

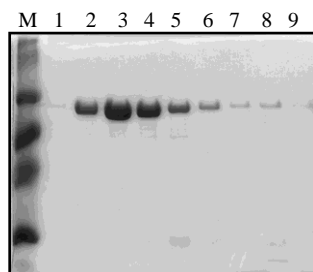


Figure 1.12b Purification SaSecA2 with Superos-6 column

Buffer: 100 mM NH_4HCO_3 ; lane 1-9: different eluent fractions.

Cloning, Expression, and Purification of SecA Homologues of *B. anthracis*

Cloning and expression of *B. anthracis* *secA1* (*BasecA1*) The *BasecA1* gene was amplified from *B. anthracis* Sterne, cloned into pET-20b (Fig. 1.13). Then, it was over-expressed in BL21λDE3 at 20°C or 37°C with 0.5 mM IPTG. The gel result showed that the expression of BaSecA1 was better at 20°C than at 37°C (Fig. 1.14).

Purification of BaSecA1 Cell pellet was French pressed at 10,000 psi. The supernatant of 37k rpm was loaded onto 5 ml His-trap column, and washed with different imidazole gradient by FPLC. BaSecA1 bind to His-trap column very well, and it could be eluted with >60 mM imidazole (Fig. 1.15a). The eluent from His-trap column was desalted, then loaded to SP-sepharose column. The result showed that BaSecA1 was further purified by using SP-sepharose column (Fig. 1.15b). Some appropriate fraction were concentrated, and loaded to Superdex-200 column. The result of S-200 column showed that the purity was high, but there were some degradation (Fig. 1.15c). And the result of Superose-6 column was shown as dimmer when compared with other previous data (data not shown).

Cloning and expression of *B. anthracis* *secA2* (*BasecA2*) The *secA2* gene of *B. anthracis* was amplified from *B. anthracis* Sterne, cloned into pET-33b (Fig. 1.16). Then, it was over-expressed in BL21λDE3 at 20°C or 37 °C with 0.5 mM IPTG. The gel result showed that the expression of BaSecA2 was good at 20°C and 37 °C (Fig. 1.17a), however majority of BaSecA2 was in insoluble form (Fig. 1.17b). Thus, IPTG concentration was decrease to 0.05 mM, 0.075 mM, or 0.1 mM to induce expression of BaSecA2. The result showed that 0.075 mM IPTG induce very little amount of BaSecA2 expression, while 0.1 mM IPTG could induce a lot, but BaSecA2 was become insoluble as soon as it was expressed (data not show).

Purification of BaSecA2 Cell pellet was French pressed at 18k PSI for three times, and centrifuged at 3.5K rpm twice and 8k rpm twice to separate unbroken cell and inclusion body. However, a lot inclusion body was centrifuged down in 3.5K rpm. The SDS-PAGE gel result showed that 4 preps had similar purity of BaSecA2, thus, 4 pellets were combined, washed with 0.5 M and 1 M Urea, then was resuspended with 6 M urea (Fig. 1.18a-1.18b). The 90k rpm supernatant of 6M urea suspension was slowly diluted to 20 times with Tris buffer. After dilution, BaSecA2 remained soluble and have ATPase activity. Then the 20 times diluted sample was loaded to His-trap column and washed with imidazole gradient. The result showed that renatured BaSecA2 could bind to the His-trap column, and the purity was very high (Fig. 1.19a). The some elution fractions of his-trap column were desalted with desalting column, then loaded to Q-sepharose column. The result showed that BaSecA2 was further purified through Q column (Fig. 1.19b). The some elution fractions were combined and concentrated, then loaded to Superdex-200 column. The result showed that the purity of BaSecA2 was very high (Fig. 1.19c).

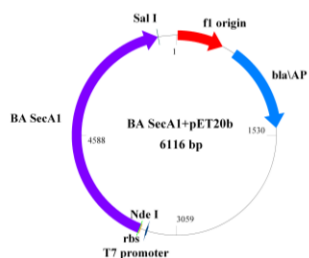


Figure 1.13 Map of BaSecA1/pET20b

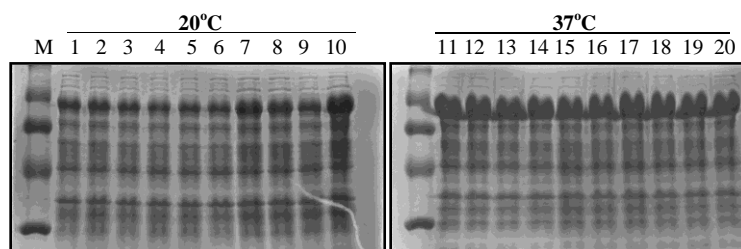


Figure 1.14 Expression of BaSecA1

Lane 1-20: different colonies.

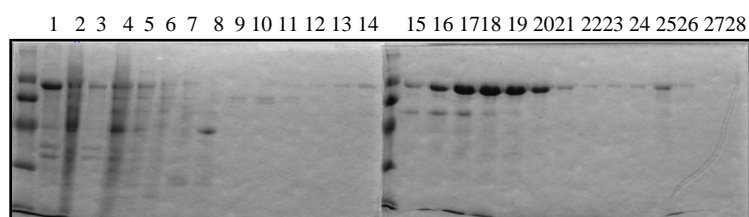


Figure 1.15a Purification of BaSecA1 with His-trap column

Lane 1: pellet of 4k rpm; lane 2: supernatant of 4k rpm; lane 3: pellet of 37k rpm; lane 4: supernatant of 37k rpm; lane 5-28: different eluent fractions.

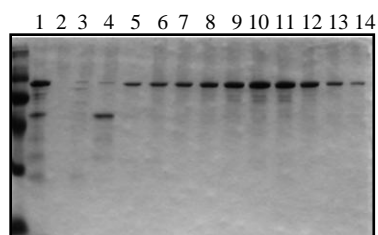


Figure 1.15b Purification of BaSecA1 with S-sepharose column

Lane 1: loading sample; lane 2: unbinding sample; lane 3-14: different eluent fractions.

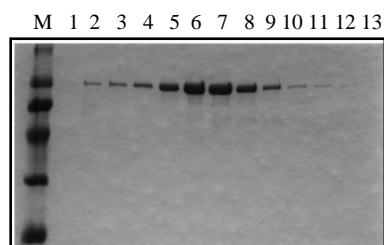


Figure 1.15c Purification of BaSecA1 with S-200 column

Buffer : 100 mM NH_4HCO_3 . Lane 1-13: different eluent fractions.

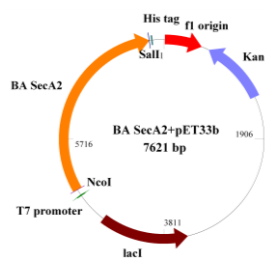


Figure 1.16 Map of BaSecA2/pET20b

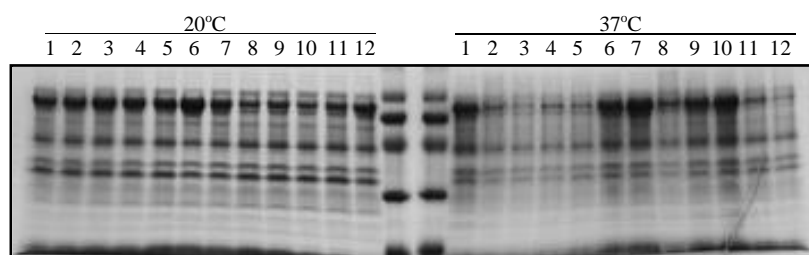


Figure 1.17a Expression of BaSecA2

Lane 1-12: different colonies.

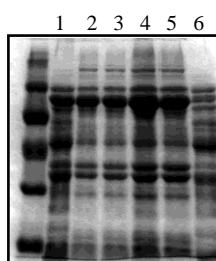


Figure 1.17b Over-expression of BaSecA2 with 0.5mM IPTG

Lane 1: whole cell lysate; lane 2-3: first and second pellets of 3.5k rpm; lane 4-5: first and second pellets of 8k rpm; lane 6: the supernatant of 8k rpm.

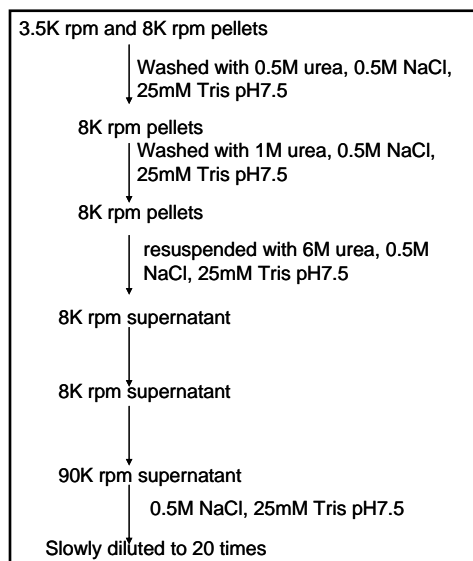


Figure 1.18a Denature and renature of BaSecA2

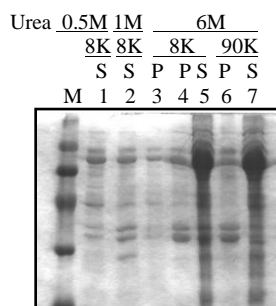


Figure 1.18b Denature of BaSecA2

S: supernatant; P: pellet; Lane 1: 8K rpm supernatant of 0.5M urea suspension; lane 2: 8K rpm supernatant of 1M urea suspension; lane 3-4: first and second 8K rpm pellet of 6M urea suspension; lane 5: 8K rpm supernatant of 6M urea suspension; lane 6-7: 90K rpm pellet and supernatant of 6M urea suspension.

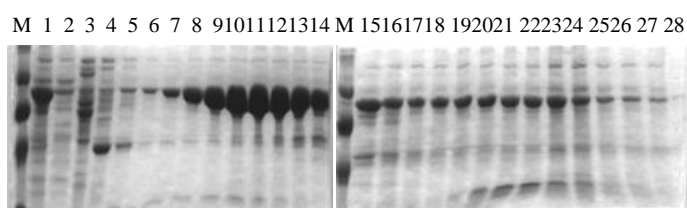


Figure 1.19a Purification BaSecA2 with His-trap column

Lane 1: loading sample; lane 2: unbinding sample; lane 3-28: different elution fraction.

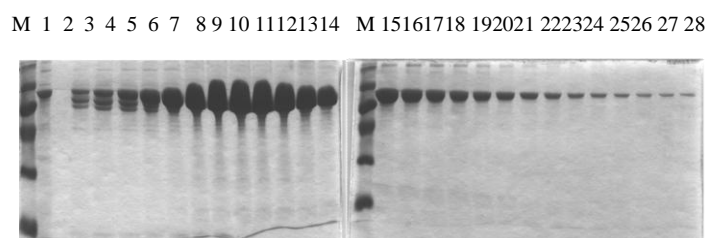


Figure 1.19b Purification of BaSecA2 with Q-sepharose column

Lane 1: loading sample; lane 2: unbinding sample; lane 3-28: different elution fraction.

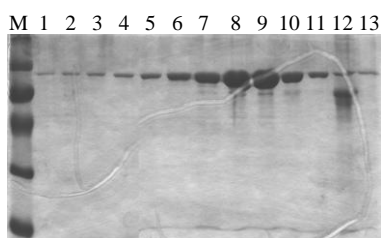


Figure 1.19c Purification of BaSecA2 with Superdex-200 column

Lane 1-13: different elution fraction.

Immunospecificity of SecA homologues

Making the Antibody of MtbSecA1 Purified MtbSecA1 was cut from SDS-PAGE Gel. At the first time, 1 mg MtbSecA1 mixed with Freund's complete adjuvant was injected into each of Rabbit No. 128 and No. 129. Two weeks later, 0.5 mg MtbSecA1 mixed with Freund's incomplete adjuvant was injected into those two rabbits. Two weeks after second injection, 20 ml blood was collected from each rabbit and serum was separated, and 0.5 mg MtbSecA1 mixed with Freund's incomplete adjuvant was injected again. Injection was repeated 5 times for each rabbit. Antibody appeared from the first antiserum and the sensitivity was increasing with the injection times. Fig. 1.20a shows that even the first bleed can recognize 30 ng Mtb secA1 at 5,000 times dilution. The forth bleed can recognize 30 ng MtbSecA1 at 10,000 times dilution.

Making the Antibody MtbSecA2 To make of MtbSecA2 antibody, denatured MtbSecA2-2 (his tag -) was purified from inclusion body and aggregated protein. MtbSecA2-2 cell pellet was French pressed at 18,000 psi., then centrifuged at 4K rpm, 12K rpm, 20K rpm, 50K rpm consecutively to separate unbroken cell, inclusion body, and membrane. The pellets of 12K rpm, 20K rpm, 50K rpm were washed with 20 mM Tris-HCl buffer, then centrifuge at 12K rpm or 20K rpm, repeat 4-5 times. The pellets were dissolved in 8 M urea, centrifuged at 38K rpm, and then the supernatant was loaded to Q sepharose column. Majority of MtbSecA2-2 didn't bind to Q-sepharose column, and the purity of MtbSecA2 was very high in the unbinding sample. Thus, MtbSecA2-2 band in unbinding sample was cut from SDS-PAGE, and injected into two rabbits. At the first time, 1 mg MtbSecA1 mixed with Freund's complete adjuvant was injected into each of Rabbit No. 120 and No. 121. Two weeks later, 0.5 mg MtbSecA2 mixed with Freund's incomplete adjuvant was injected into those two rabbits. Two weeks after second injection, 20 ml blood was collected from each rabbit and serum was separated, and 0.5 mg MtbSecA2 mixed

with Freund's incomplete adjuvant was injected again. Injection was repeated 7 times for each rabbit. Antibody appeared from the first antiserum and the sensitivity was increasing with the injection times. 300 ng MtbSecA2 was detected out with 1:5000 dilution of the first and second bleed, with 1:10000 dilution of the third bleed, and with 1:20000 dilution of the 4th bleed (data not shown). For the 5th and 6th bleed, 25ng MtbSecA2 can be detected with 1:10000 dilution of serum (Fig. 1.20b). The antibody sensitivity of rabbit No. 120 is higher than rabbit No. 121.

Making the antibody of SaSecA2 Purified SaSecA2 protein was denatured and separated by SDS-PAGE Gel, then corresponding protein band was cut out and mixed with adjuvant, then injected into two rabbits. At the first time, 1mg SaSecA2 mixed with Freund's complete adjuvant was injected into each of Rabbit No. 130 and No. 131. Two weeks later, 0.5 mg SaSecA2 mixed with Freund's incomplete adjuvant was injected into those two rabbits. Two weeks after second injection, 20 ml blood was collected from each rabbit and serum was separated. And one week later, 0.5 mg MtbSecA1 mixed with Freund's incomplete adjuvant was injected again. Injection was repeated 4 times for each rabbit. Antibody appeared from the first antiserum and the sensitivity was increasing with the injection times. The Western blot result showed that those rabbit produce very good antibody (Fig. 1.20c). Ten thousand times diluted third serum could detect 100 ng of SaSecA2 in 3 min development.

Specificity of SecA antibodies Western blot results demonstrated that 50ng MtbSecA1 could be recognized by BsSecA antibody and EcSecA antibody at 5000 times dilution, and BsSecA antibody is more sensitive than EcSecA antibody (Fig. 1.21). The specificity of MtbSecA2 antibody was checked by western blot with different SecA proteins. Although 300 ng of SecA proteins were loaded to each lane, the SDS-PAGE gel result showed the actual amounts of those proteins were different (Fig. 1.22a). This result may caused by degradation. The western

blot result showed the specificity of MtbSecA2 is very high (Fig. 1.22a). We also investigated that specificity of EcSecA antibody, BsSecA antibody, MtbSecA1 antibody, and SaSecA2 antibody. EcSecA antibody showed high sensitivity to SaSecA1 and BaSecA2; it could cross react with BsSecA and BaSecA1, but not with SaSecA2 (Figure 1.22b). BsSecA antibody showed high sensitivity to BaSecA1 and SaSecA1, while it showed low sensitivity to EcSecA, SaSecA2, and BaSecA2 (Figure 1.22c). SaSecA2 antibody showed high sensitivity to SaSecA1, good sensitivity to BaSecA1, low sensitivity to BaSecA2, EcSecA, and BsSecA (Figure 1.22d). MtbSecA1 showed low sensitivity to EcSecA, BsSecA, BaSecA and BaSecA2 (Figure 1.22e).

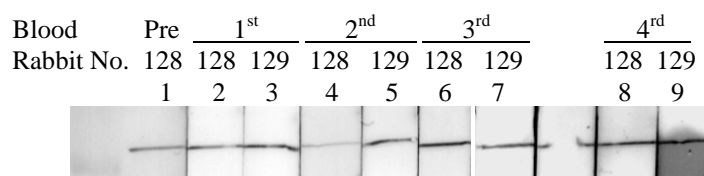


Figure 1.20a Sensitivity of MtbSecA1 antibody

Each lane contains 30 ng MtbSecA1. Lane 1-7: 1:5000 dilution; lane 8-9: 1:10000 dilution.

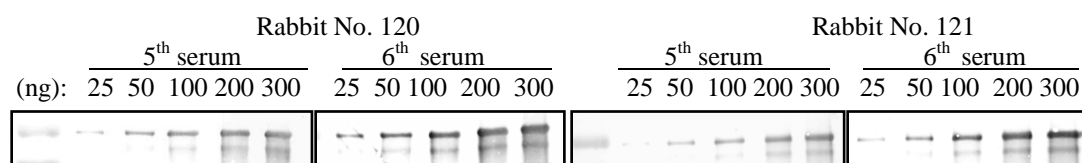


Figure 1.20b Sensitivity of MtbSecA2 antibody

Serum was diluted 1:10000 times.

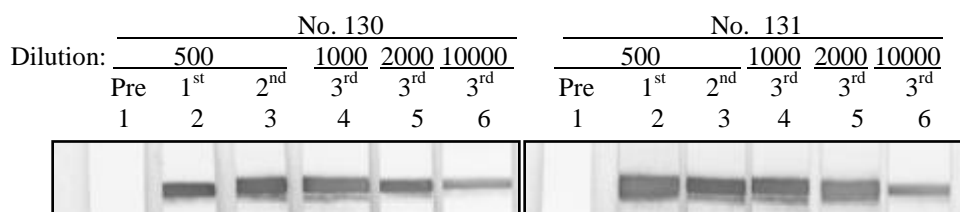


Figure 1.20c Sensitivity of SaSecA2 antibody

Lane 1: pre-serum (1:500, 5min); lane 2: 1st bleeding (1:500, 5min); lane 3: 2nd bleeding (1:500, 5min); lane 4: 3rd bleeding (1:1000, 5min); lane 5: 3rd bleeding (1:2000, 5min); Lane 6: 3rd bleeding (1:10000, 3min). Each lane contains 100ng.

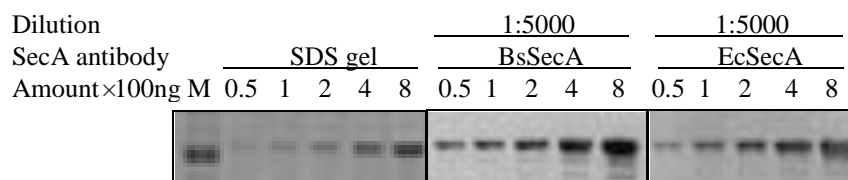


Figure 1.21 Identification of MtbSecA1 with BSecA antibody and ECSecA antibody

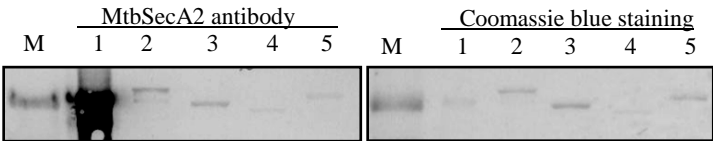


Figure 1.22a Specificity of MtbSecA2 antibody
Lane 1: 300 ng MtbSecA2; lane 2: 300 ng MtbSecA1; lane 3: 300 ng EcSecA; lane 4: 300 ng BsSecA; Lane 5: 300 ng PaSecA.

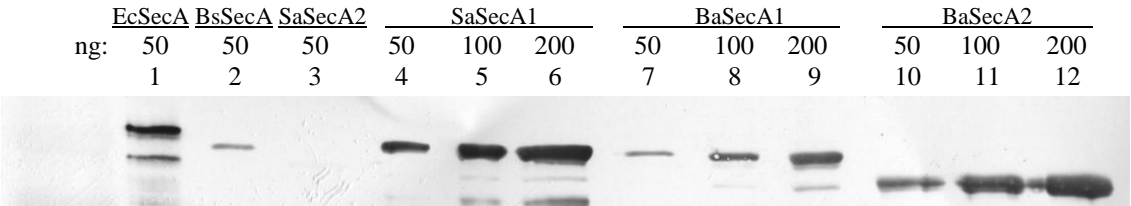


Figure 1.22b Specificity of EcSecA antibody
Serum was diluted 1:5000 times.

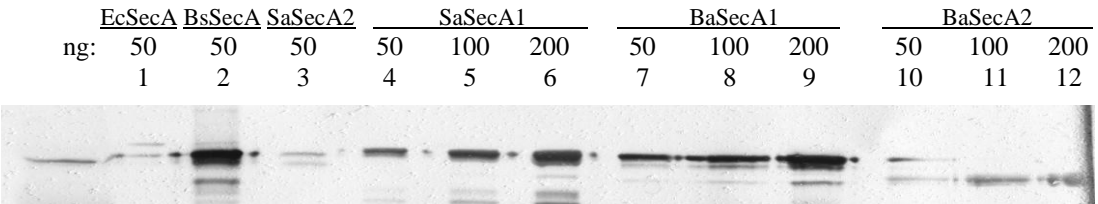


Figure 1.22c Specificity of BsSecA antibody
Serum was diluted 1:5000 times.

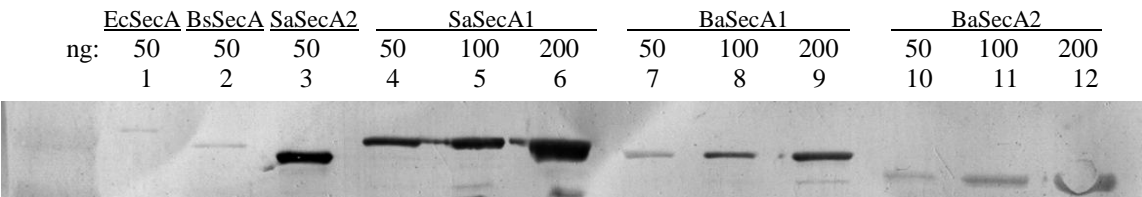


Figure 1.22d Specificity of SaSecA2 antibody
Serum was diluted 1:5000 times.

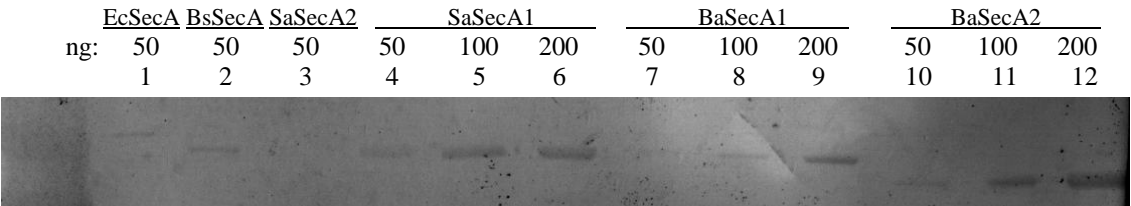


Figure 1.22e Specificity of MtbSecA1 antibody
Serum was diluted 1:5000 times.

The Intrinsic ATPase Activity of SecA1 and SecA2

Although the function of SecA is well studied in *E. coli* and *B. subtilis*, and the functions of SecA1 and SecA2 from Gram-positive bacteria pathogen were studied with genetic methods, the biochemical properties of SecA homologues are not clear. Alignment result showed that nine motifs of DEAD helicase were highly conserved in all SecA homologues (Figure 0.2). In EcSecA, these nine motifs form two nucleotide binding domains (NBD); NBD1 has high affinity to nucleotide, acting as an ATPase catalytic machinery; NBD2 has low affinity to nucleotide, and acting as a an activator of ATP hydrolysis [90]. Thus, NBD1 and NBD2 are very important to the translocase activity of EcSecA. The conservation of these domains suggests SecA1 and SecA2 might have similar ATPase activity as EcSecA. **Thus, we hypothesize that SecA1 and SecA2 are ATPases that hydrolyses ATP to promote translocation of specific proteins.** To test our hypothesis, Malachite Green colorimetric assay was used to investigate the ATPase and GTPase activity of SecA1 and SecA2 from *M. tuberculosis*, *M. smegmatis*, *S. aureus*, and *B. anthracis*.

Our results showed that the ATPase activity of MtbSecA1 was much higher than its GTPase activity (Fig. 1.23), indicating MtbSecA1 is an ATPase. Although two nucleotide binding domains were highly conserved in MtbSecA2, MtbSecA2 had low ATPase and GTPase activity at 30°C, 37°C, and 42°C (Fig. 1.24). Similar to MtbSecA1, MsSecA1 and MsSecA2 had much higher ATPase activity than GTPase activity, suggesting they are ATPases (Fig. 1.25).

For EcSecA, Mg^{2+} is required for its ATPase activity. To determine whether metal ions required for the activity of MtbSecA2 are different with ion required for the activity of MtbSecA1 and EcSecA, NTPase activity of MtbSecA1 and MtbSecA2 was determined in presence of different metal ions. Our results showed that Mg^{2+} and $Zinc^{2+}$ were required for ATPase activity

of MtbSecA1 (Fig. 1.26), which is similar to the result of EcSecA. At 30°C in presence of Mg^{2+} and $Zinc^{2+}$, liposomes could stimulate ATPase activity of MtbSecA1 up to 10 fold (Fig. 1.26); however, when Mg^{2+} and $Zinc^{2+}$ were mixed together, the ATPase activity of MtbSecA1 was lower than with single metal ion (Fig. 1.26). These results might due to competition between these two ions on the binding sites. The NTPase activity of MtbSecA2 couldn't be significantly stimulated by Mg^{2+} , Mn^{2+} , Zn^{2+} and liposomes (Fig. 1.27a-1.27b). MsSecA2 share more than 80% homologue with MtbSecA2. However, Mg^{2+} could dramatically increase ATPase activity of MsSecA2 (Fig. 1.28). The alignment result show that SecA2 is highly conserved in mycobacteria, the mainly different is located on N-terminal. Mycobacteria use GTG as start codon. There are three GTG located at N-terminal of MTB SecA2 gene. In our study, *MtbsecA2* gene was cloned from the first GTG. In a paper published on JB 2008 [106], *MtbsecA2* was cloned from the third GTG, because the protein sequence of the shorter MtbSecA2 more closely resemble SecA2 from other mycobacteria (Fig. 1.29). The shorter MtbSecA2 have higher intrinsic ATPase activity than the full length MtbSecA2. In the genome sequence of *M. tuberculosis*, there is no obvious ribosome binding site in front of either first or third GTG. Both the full length and the shorter MtbSecA2 can complement *secA2* mutant phenotypes in *M. smegmatis* and *M. tuberculosis* [54, 106-107], suggesting that the full length MtbSecA2 function *in vivo*. We don't know why the N-terminal 30 amino acid could inhibit the ATPase activity of MtbSecA2. Three cystine residues in the N-terminal of MtbSecA2 might cause the protein very unstable.

Similar to mycobacteria SecA1, SecA1 and SecA2 from *S. aureus* and *B. anthracis* have very high endogenous ATPase activity (Fig. 1.30-1.31), indicating that they are ATPase. The intrinsic ATPase activity of SaSecA2 and BaSecA2 are higher than SaSecA1 and BaSecA1 (Fig. 1.30-1.31).

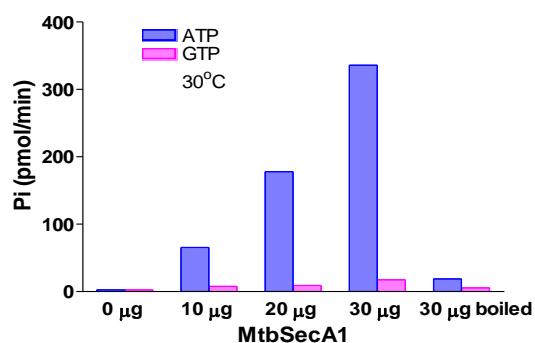


Figure 1.23 The intrinsic ATPase and GTPase activity of MtbSecA1
30°C, 40 min, 2 mM ATP or GTP, 2 mM Mg²⁺.

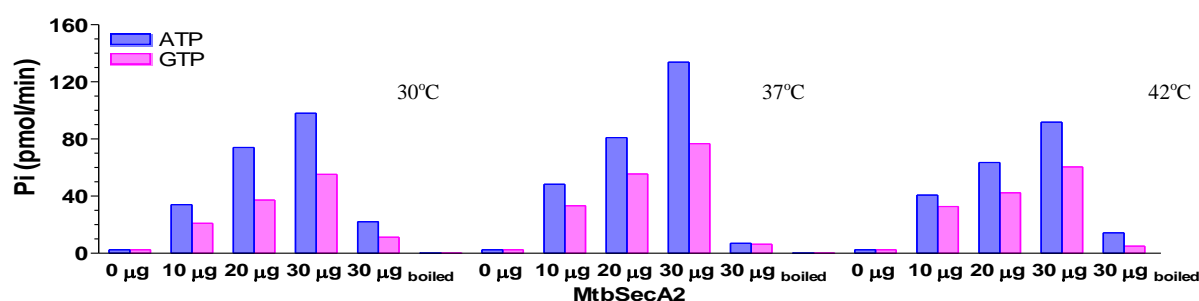


Figure 1.24 The intrinsic ATPase and GTPase activity of MtbSecA2
40 min, 2 mM ATP or GTP, 2 mM Mg²⁺.

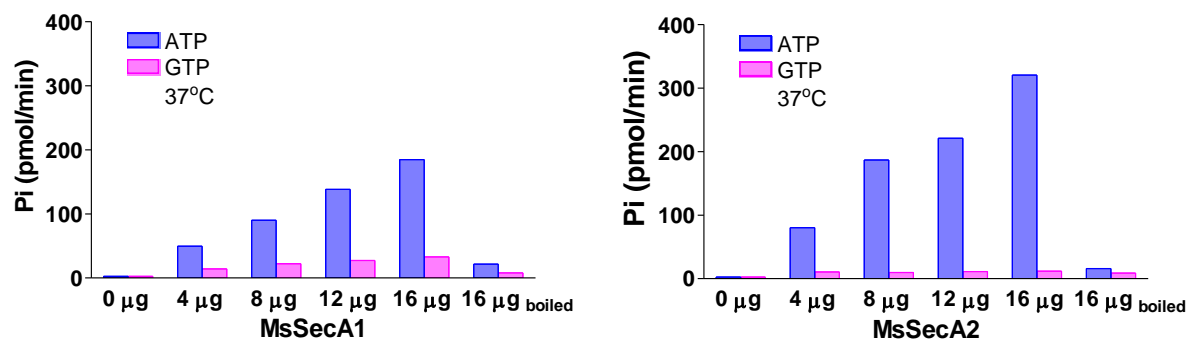


Figure 1.25 The intrinsic ATPase activities of MsSecA1 and MsSecA2
37°C, 40 min, 2 mM ATP or GTP, 2 mM Mg²⁺.

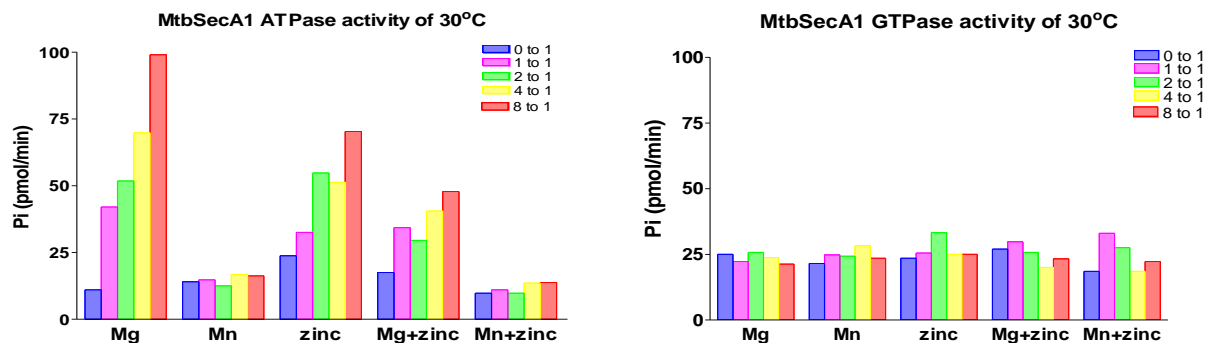


Figure 1.26 NTPase activity of MtbSecA1 with different metal ion
30°C, 40 min, 2 mM ATP or GTP, 2 mM metal ion.

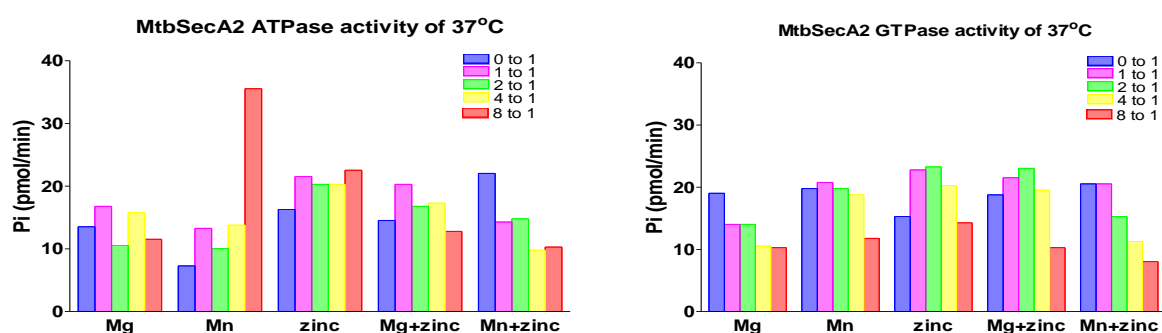


Figure 1.27a NTPase activity of MtbSecA2 with different metal ion
37°C, 40 min, 2 mM ATP or GTP, 2 mM metal ion.

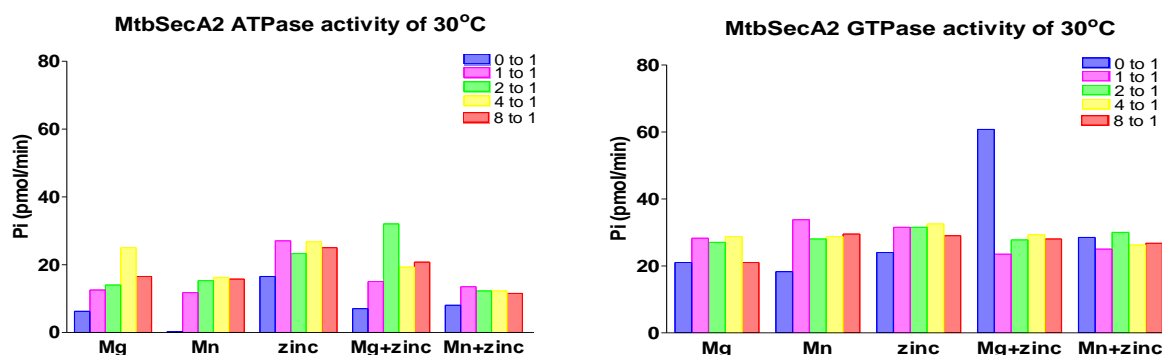


Figure 1.27b NTPase activity of MtbSecA2 with different metal ion
30°C, 40 min, 2 mM ATP or GTP, 2 mM metal ion.

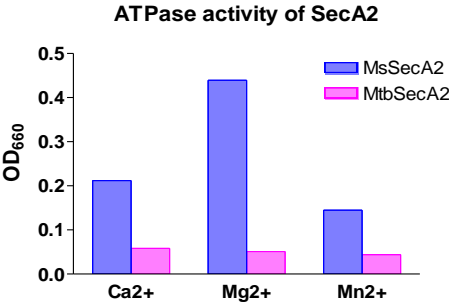


Figure 1.28 ATPase activities of mycobacteria SecA2 with different metal ion
30oC, 40 min, 2 mM ATP, 2 mM Ca²⁺, or 2 mM Mg²⁺, or 2 mM Mn²⁺.

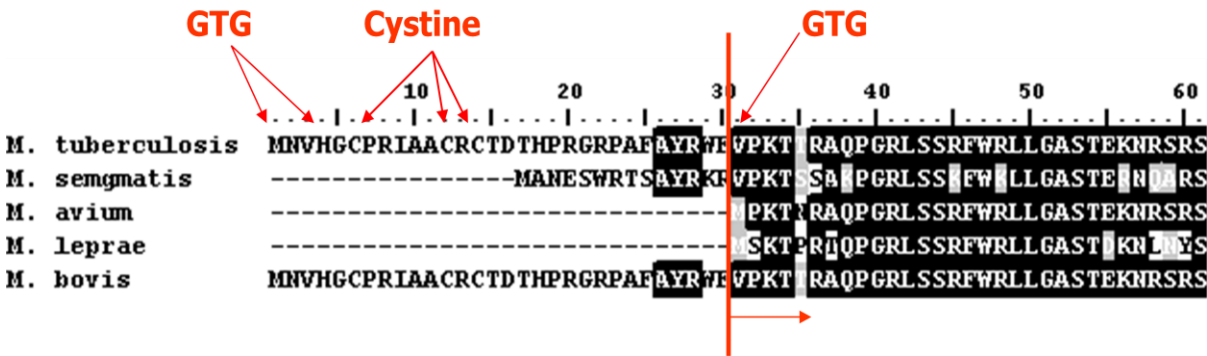


Figure 1.29 N-terminal alignment of mycobacteria SecA2 (Adapted from)

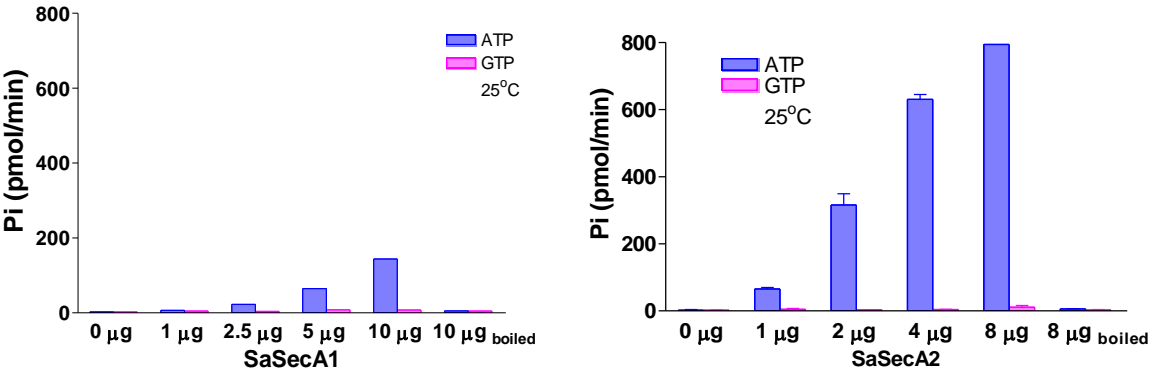


Figure 1.30 The intrinsic ATPase activities of SaSecA1 and SaSecA2
25°C, 40 min, 2 mM ATP or GTP, 2 mM Mg²⁺.

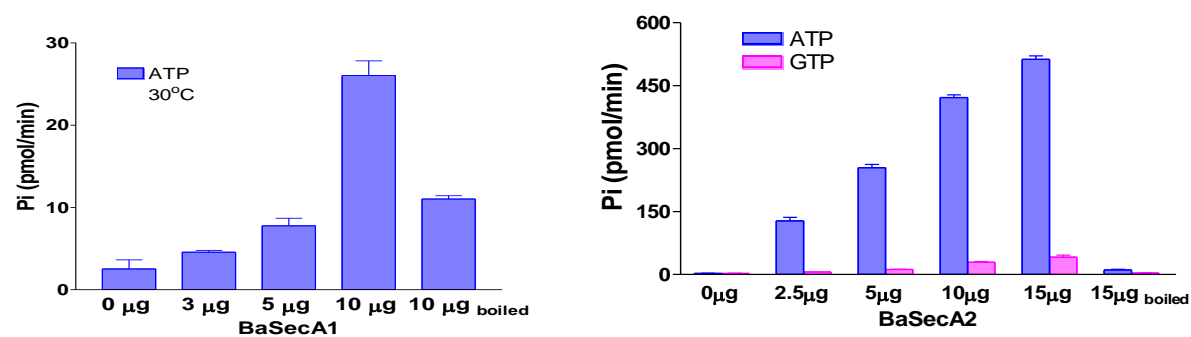


Figure 1.31 The intrinsic ATPase activities of BaSecA1 and BaSecA2
 30°C, 40 min, 2 mM ATP or GTP, 2 mM Mg^{2+} .

The Lipid, Membrane, and Translocation ATPase Activity of SecA1 and SecA2

In *E. coli* Sec-system, SecA undergo cycles of membrane insertion and dissociation, guiding and pushing preprotein export through transmembrane channel [38, 91]. And lipid could induce conformational change of EcSecA forming ring-like structure, indicating it might be involved in forming transmembrane channel [38]. Interacting with anionic phospholipids, membrane, and precursor protein could stimulate the ATPase activity of EcSecA (Fig. 1.36). To determine whether SecA1 and SecA2 could interact with membrane in the same way as EcSecA, the ATPase activity of SecA homologues were investigated in presence of liposomes, membrane and precursor proteins. Our results showed that the ATPase activity of MtbSecA1, MsSecA1, SaSecA1, and BaSecA1 could be stimulated by liposomes (Fig. 1.32-1.35), however the ATPase activity of MtbSecA2, MsSecA2, SaSecA2, and BaSecA2 could not be stimulated by liposomes (Fig. 1.32-1.35). These results suggested that SecA1 might have similar function as EcSecA and SecA2 might have similar function as BsSecA. The ATPase activities of SecA1 and SecA2 were further investigated in presence of *E. coli* BA13 membrane washed with 6 M urea, or/and preOmpA of *E. coli*. The ATPase activity of MsSecA1 could be stimulated by BA13 membranes (Fig. 1.37a-c) and preOmpA (Fig. 1.38). However, the ATPase activities of MsSecA2, SaSecA1, SaSecA2, BaSecA1, and BaSecA2 were not obviously stimulated by BA13 membrane and preOmpA (Fig. 1.39-1.42). These results might due to specific interactions required for recognition between precursor and translocase. Because the membrane and precursor were from *E. coli*, they might not be recognized by translocase from other species.

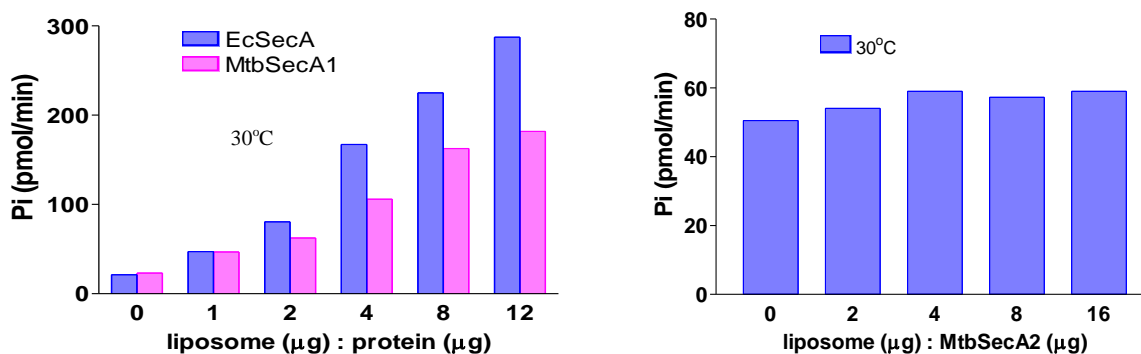


Figure 1.32 The ATPase activities of MtbSecA1 and MtbSecA2 in presence of liposomes
30°C, 40 min, 2 mM ATP, 2 mM Mg²⁺.

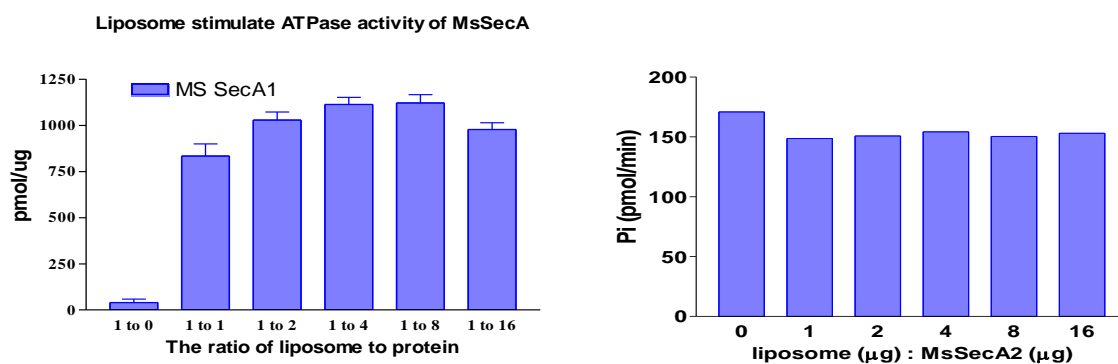


Figure 1.33 The ATPase activities of MsSecA1 and MsSecA2 in presence of liposomes
30°C, 40 min, 2 mM ATP, 2 mM Mg²⁺.

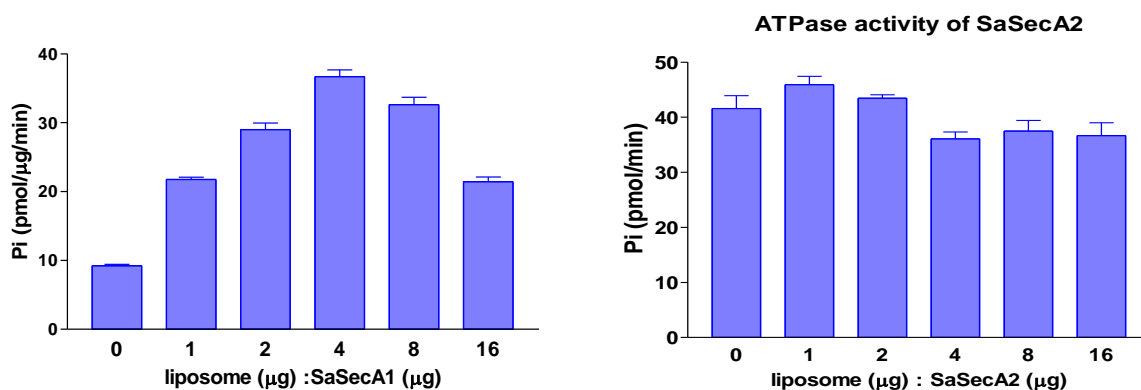


Figure 1.34 The ATPase activities of SaSecA1 and SaSecA2 in presence of liposomes
25°C, 40 min, 2 mM ATP, 2 mM Mg²⁺. SaSecA2: 1 μg.

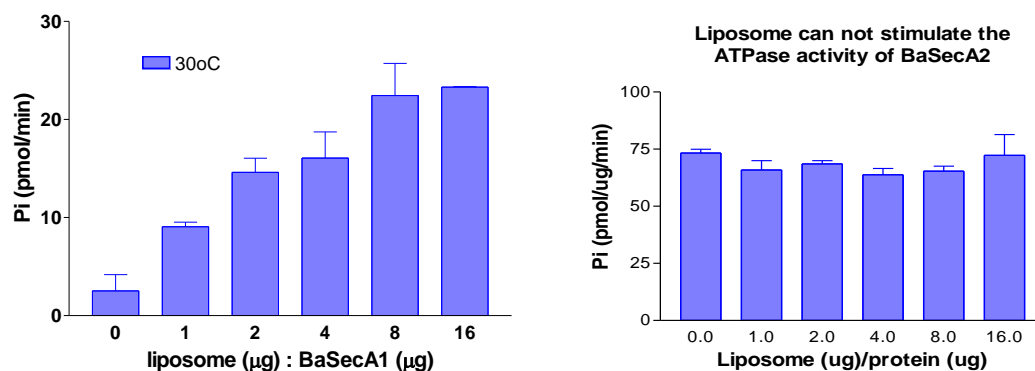


Figure 1.35 The ATPase activities of BaSecA1 and BaSecA2 in presence of liposomes
30°C, 40 min, 2 mM ATP, 2 mM Mg^{2+} .

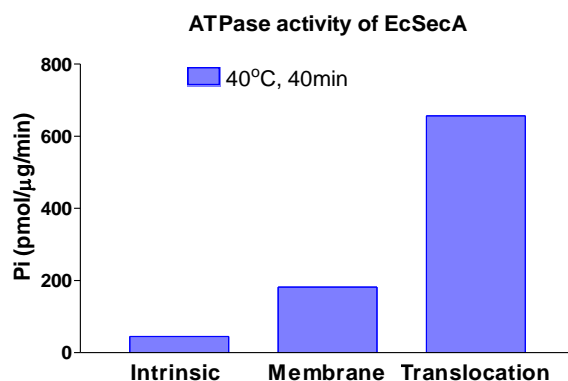


Figure 1.36 Intrinsic, membrane, and translocation ATPase activities of EcSecA

Intrinsic ATPase activity: EcSecA 5 μg; membrane ATPase activity: EcSecA 1 μg, membrane 3 μg; translocation ATPase activity: EcSecA 0.5 μg, membrane 1.5 μg, preOmpA 0.5 μg; membrane: 6M urea washed BA13 membrane; 40°C 40 min; 2 mM Mg^{2+} ; 2 mM ATP.

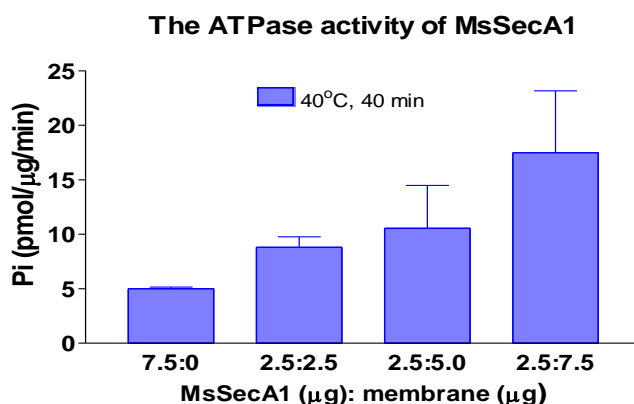


Figure 1.37a Stimulation of membrane on the ATPase activity of MsSecA1 at 40°C
Membrane: 6M urea washed BA13 membrane; 40°C 40 min; 2 mM Mg^{2+} ; 2 mM ATP.

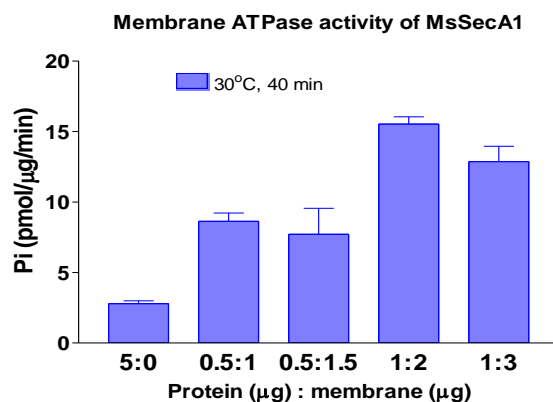


Figure 1.37b Stimulation of membrane on the ATPase activity of MsSecA1 at 30°C
 Membrane: 6M urea washed BA13 membrane; 30°C 40 min; 2 mM Mg^{2+} ; 2 mM ATP.

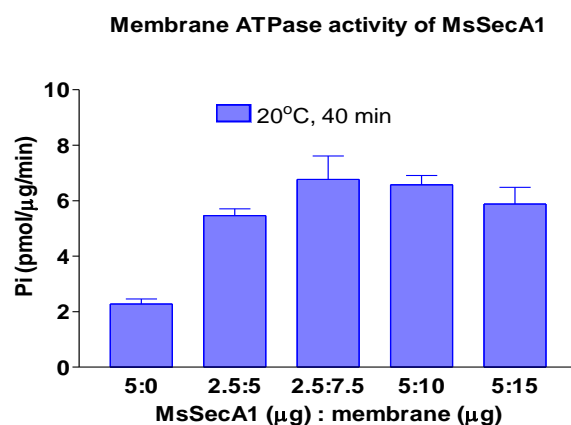


Figure 1.37c Simulation of membrane on the ATPase activity of MsSecA1 at 20°C
 Membrane: 6M urea washed BA13 membrane; 40°C 40 min; 2 mM Mg^{2+} ; 2 mM ATP.

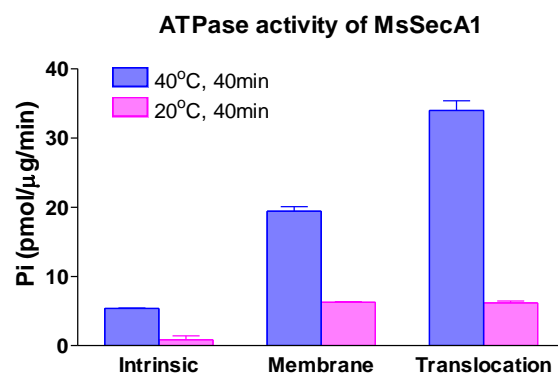


Figure 1.38 Intrinsic, membrane, and translocation ATPase activities of MsSecA1
 Intrinsic ATPase activity: MsSecA1 5 μg; membrane ATPase activity: MsSecA1 2.5 μg, membrane 7.5 μg; translocation ATPase activity: MsSecA1 2.5 μg, membrane 7.5 μg, preOmpA 0.5 μg. 40°C 40 min; 2 mM Mg^{2+} ; 2 mM ATP.

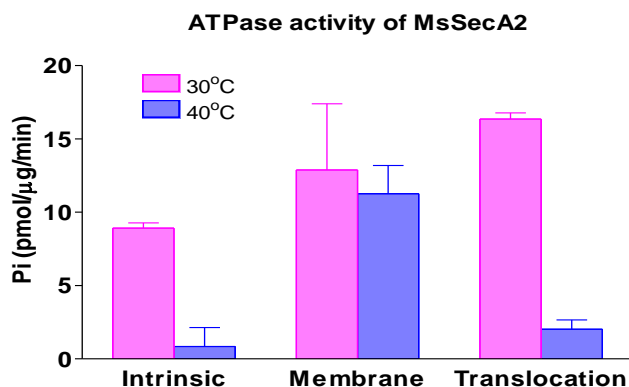


Figure 1.39 Intrinsic, membrane, and translocation ATPase activities of MsSecA2

Intrinsic ATPase activity: MsSecA2 5 μg; membrane ATPase activity: MsSecA2 2.5 μg, membrane 7.5 μg; translocation ATPase activity: MsSecA2 2.5 μg, membrane 7.5 μg, preOmpA 0.5 μg.

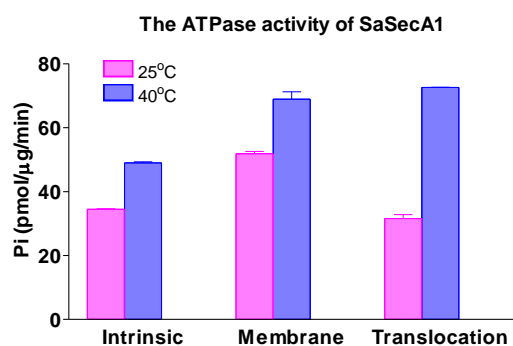


Figure 1.40 Intrinsic, membrane, and translocation ATPase activities of SaSecA1

Intrinsic ATPase activity: SaSecA1 5 μg; membrane ATPase activity: SaSecA1 2.5 μg, membrane 7.5 μg; translocation ATPase activity: SaSecA1 2.5 μg, membrane 7.5 μg, preOmpA 0.5 μg.

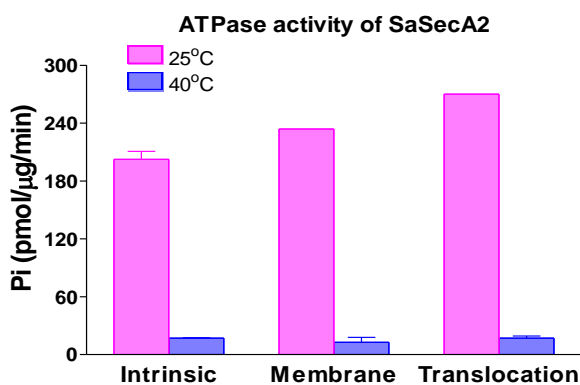


Figure 1.41 Intrinsic, membrane, and translocation ATPase activities of SaSecA2

Intrinsic ATPase activity: SaSecA2 2.5 μg; membrane ATPase activity: SaSecA2 1.25 μg, membrane 3.75 μg; translocation ATPase activity: SaSecA2 1.25 μg, membrane 3.75 μg, preOmpA 0.5 μg.

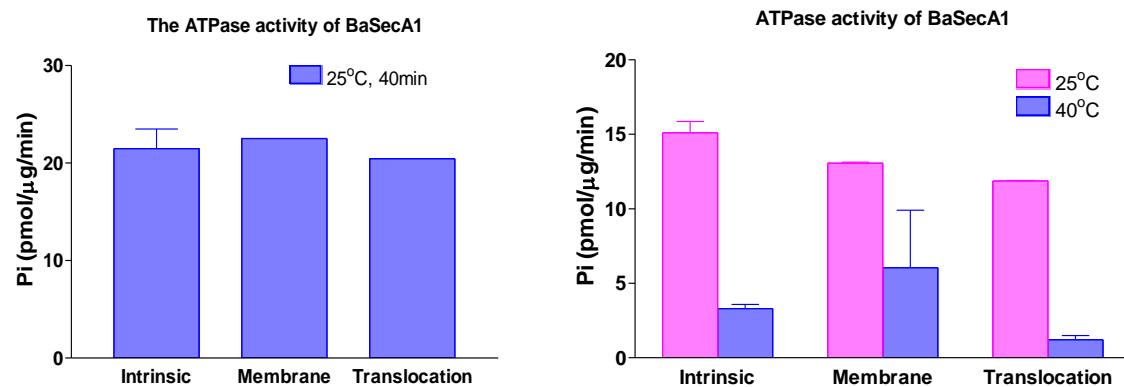


Figure 1.42 Intrinsic, membrane, and translocation ATPase activities of BaSecA1

Intrinsic ATPase activity: BaSecA1 5 μg; membrane ATPase activity: BaSecA1 2.5 μg, membrane 7.5 μg; translocation ATPase activity: BaSecA1 2.5 μg, membrane 7.5 μg, preOmpA 0.5 μg.

Temperature Effect on the ATPase Activity of SecA1 and SecA2

To investigate whether temperature could affect the ATPase activity of mycobacteria SecA homologues, the ATPase activity of SecA homologues was investigated at different temperature. The optimal temperature for the intrinsic ATPase activity of EcSecA is around 42°C, while for ATPase activity of EcSecA in presence of liposomes is around 30°C, which is good for the function of liposomes (Fig. 1.36). To our surprise, MtbSecA1 has highest ATPase activity at 0°C with or without liposomes (Fig. 1.37a). To confirm whether the ATPase activity of MtbSecA1 at 0°C was real, kinetic assay was carried at different temperature, and boiled MtbSecA1 was used at negative control. The result showed that the ATPase activity of MtbSecA1 is increasing with time, activity at 0°C is higher than that at 30°C, and the denatured boiled protein lose its activity (Fig. 1.37b). We don't know why and how these characteristics of MtbSecA1 could help for the growth or physiology of MTB. To investigate whether all SecA1 of mycobacteria have similar high ATPase activity at low temperature, the ATPase activity of MsSecA1 was investigated. The results showed that MsSecA1 has high ATPase activity at 0°C (Fig. 1.38a). The result of kinetic assay showed that its ATPase activity was increasing with time at 0°C (Fig. 1.38b). The intrinsic ATPase activity of MtbSecA2 at 37°C is higher than that at 30°C and 42°C (Fig. 1.24). To investigate whether temperature has similar effect on MsSecA2, its ATPase assay was carried at different temperatures. The results showed that MsSecA2 has highest intrinsic ATPase activity at around 33°C (Fig. 1.39). This result was different with the activity of mycobacteria SecA1. Does this result indicate SecA1 and SecA2 might have different functions at different growth condition of mycobacteria? The optimal temperatures for the ATPase activities of SaSecA1, SaSecA2, BaSecA1, and BaSecA2 are all between 20°C-30°C (Fig. 1.40-1.43), while the optimal temperature for BsSecA is above 40°C (Fig. 1.44).

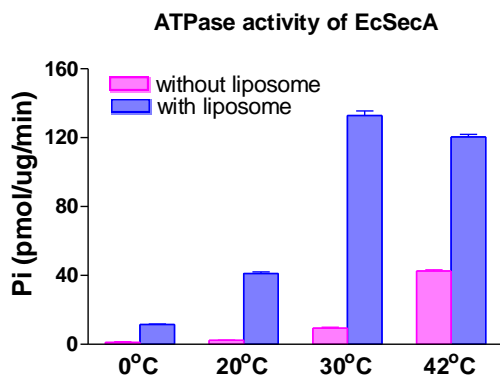


Figure 1.43 Temperature effect on the ATPase activity of EcSecA
40 min; 2 mM ATP; 2 mM Mg^{2+} .

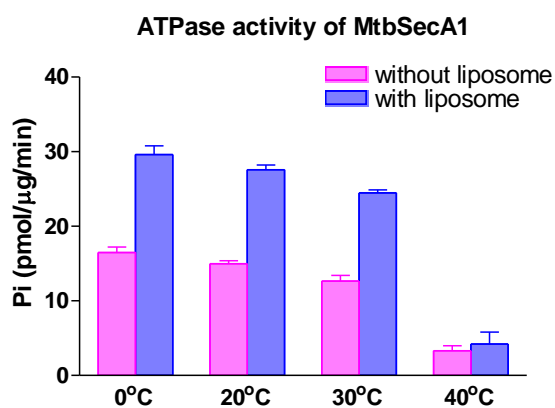


Figure 1.44a Temperature effect on the ATPase activity of MtbSecA1
40 min; 2 mM ATP; 2 mM Mg^{2+} .

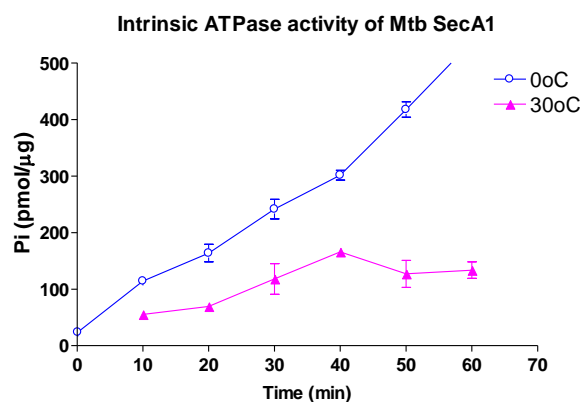
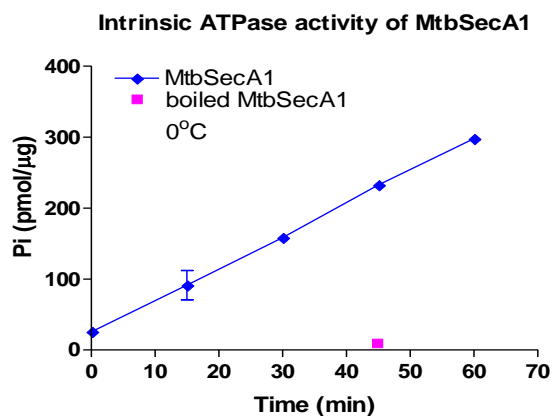


Figure 1.44b Temperature effect on the ATPase activity of MtbSecA1
0°C or 30°C; 2 mM ATP; 2 mM Mg^{2+} .

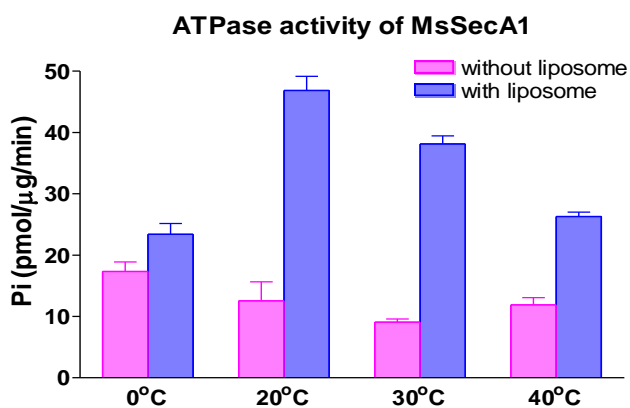


Figure 1.45a Temperature effect on the ATPase activity of MsSecA1
40 min; 2 mM ATP; 2 mM Mg^{2+} .

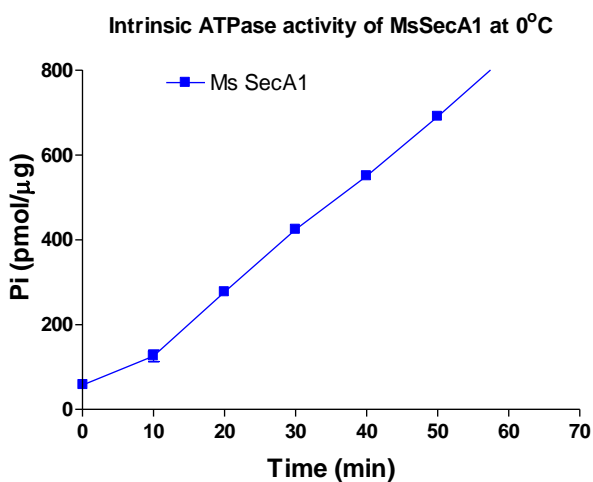


Figure 1.45b Temperature effect on the ATPase activity of MsSecA1
0°C; 2 mM ATP; 2 mM Mg^{2+} .

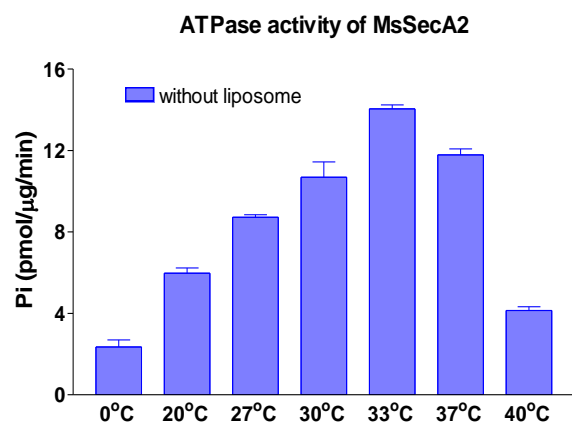


Figure 1.46 Temperature effect on the ATPase activity of MsSecA2
40 min; 2 mM ATP; 2 mM Mg^{2+} .

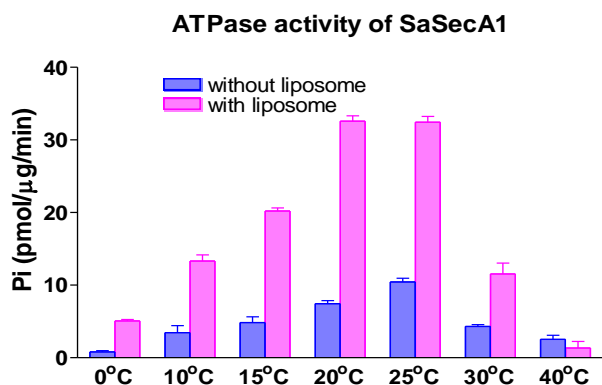


Figure 1.47 Temperature effect on the ATPase activity of SaSecA1
40 min; 2 mM ATP; 2 mM Mg^{2+} .

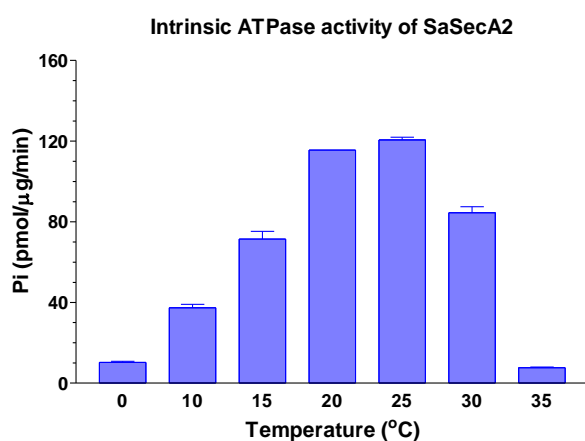


Figure 1.48 Temperature effect on the ATPase activity of SaSecA2
40 min; 2 mM ATP; 2 mM Mg^{2+} .

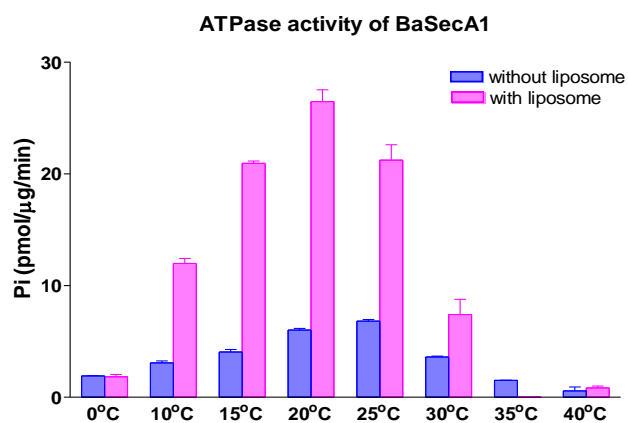


Figure 1.49 Temperature effect on the ATPase activity of BaSecA1
40 min; 2 mM ATP; 2 mM Mg^{2+} .

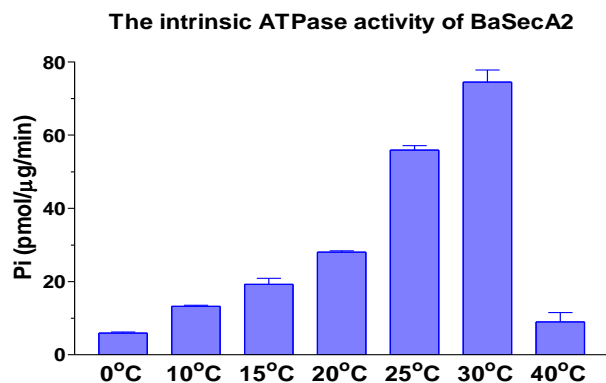


Figure 1.50 Temperature effect on the ATPase activity of BaSecA2
40 min; 2 mM ATP; 2 mM Mg^{2+} .

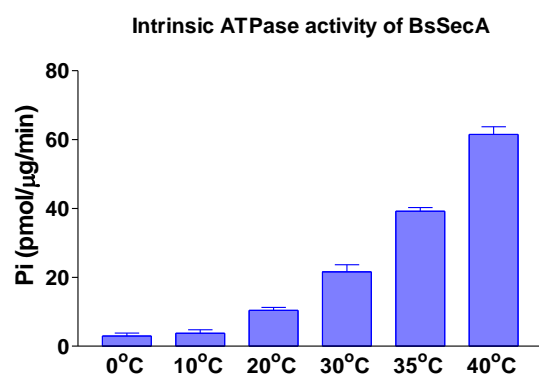


Figure 1.51 Temperature effect on the ATPase activity of BsSecA
40 min; 2 mM ATP; 2 mM Mg^{2+} .

Subcellular Distribution of Mycobacteria SecA2 and SecA1

To determine the distribution of mycobacteria SecA homologues, *M. smegmatis* Mc²155 were grown in Middlebrook 7H9 broth with 0.2% glycerol, 1×ADC(0.5% bovine serum albumin, fraction V, 0.2% dextrose, 0.85% NaCl), and 0.1% Tween 80. The cell pellet was French pressed at 18,000 psi, and unbroken cells and large cellular debris are removed by centrifugation at 3,000 × g. The supernatant of 3,000 × g was centrifuged at 27,000 × g for 1h to generate a fraction enriched in cell wall. The supernatant of 27,000 × g was centrifuged at 258,000 × g for 1.5 hrs to sediment the remaining cytoplasmic membrane. The remaining supernatant contains soluble components of the cytoplasm. Western blot was used to determine the distribution of *M. smegmatis* SecA1 and SecA2 by using the antibodies of MtbSecA1 and MtbSecA2. The results show that both SecA1 and SecA2 could be detected in the supernatant of 285,000 × g and the pellet of 258,000 × g (Fig.1.52a). 80% of MtbSecA1 are in the pellet, while 60% of MtbSecA2 are in the supernatant, suggestion that MtbSecA2 are more soluble than MtbSecA1. To investigate whether SecA1 and SecA2 are real membrane protein, those two pellets were extracted with 1% OG, 1% Triton-100, 6 M urea. The result show that some of SecA1 and SecA2 in those pellets are real membrane protein, because they could be extracted with OG and Triton-100, but not be 6 M urea (Fig. 1.52b). Some of SecA1 and SecA2 in those pellets are aggregated, or big oligmer, or membrane bounded, because they could be extracted by 6 M urea, but not be OG and Triton (Fig. 1.52b). Our data suggested that mycobacteria SecA1 and SecA2 are both located on membrane and in cytosol, and SecA2 are more soluble than SecA1. These results are similar to the subcellular distribution of EcSecA, suggesting mycobacteria SecA homologues might have similar working mechanism as EcSecA.

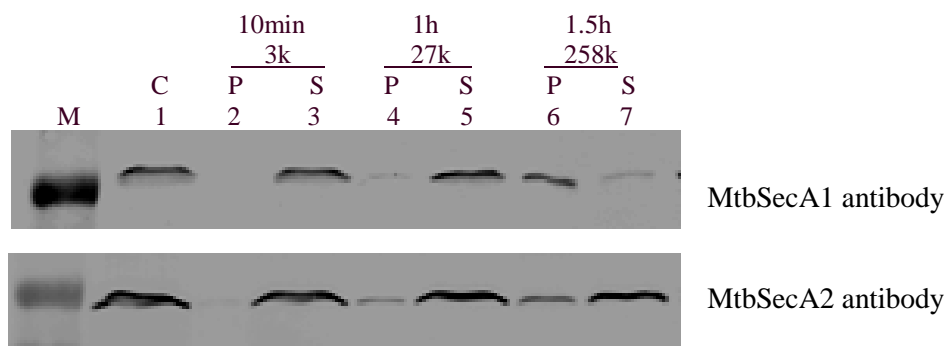


Figure 1.52a The distribution of SecA1 and SecA2 in *M. smegmatis*

Lane 1 is the crude cell lysate; Lane 2 and lane 3 are the pellet and supernatant of 3,000 g for 10 min. Lane 4 and lane 5 are the pellet and supernatant of 27,000 g for 1 hour. Lane 6 and lane 7 are the pellet and supernatant of 258,000 g for 1.5 hours.

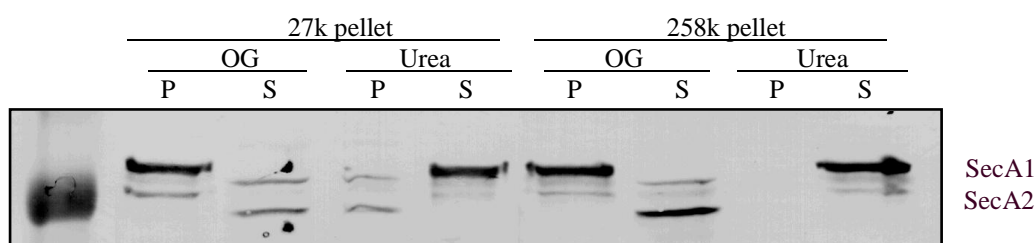


Figure 1.52b The distribution of SecA1 and SecA2 in *M. smegmatis*

P: pellet; S: supernatant; OG: 0.1% octyl glucoside; Urea: 6 M urea.

Membrane Interaction of SecA1 and SecA2

Trypsin digestion of MtbSecA1 In *E. coli* Sec-system, SecA undergo cycles of membrane insertion and dissociation, guiding and pushing pre-protein export through transmembrane channel [38, 91]. Previous studies of our lab showed the EcSecA has soluble and membrane-integral forms. It can form a ring-like pore structure in the presence of anionic phospholipids. These results indicated that EcSecA may form the central core of protein-conducting channels [38]. Our preliminary data show that MtbSecA1 was located on membrane and cytosol, and liposomes could stimulate its ATPase activity up to 9 fold. These results suggest they might have similar function and working model as EcSecA. To determine whether mycobacteria SecA homologues could interact with membrane in the same way as EcSecA, proteolysis assay was used to identify specific transmembrane domain. MtbSecA1 was incubated with liposomes on ice for 10 min, then, after adding trypsin, put on ice for 15 min. The result shows that the digestion pattern of MtbSecA1 was not changed by liposomes, while liposomes can decrease 66 kDa fragments and increase 45 kDa and 39 kDa fragments of EcSecA (Fig. 1.53a-b). To find whether the temperature could affect the correlation of SecA and liposomes, the mixture of SecA and liposomes was pre-incubated on ice or 30°C, then, digested by trypsin for 15 min on ice. The result shows no change was caused by different pre-incubation temperature (Fig. 1.53c). Liposomes used in this study was made from *E. coli* Avanti polar lipid, which might be different with lipid in the membrane of *M. tuberculosis*.

Study the structure of MtbSecA1 with TEM and AFM. The two-dimensional crystallization technique was applied to MtbSecA1. Some ring-like structures were observed in MtbSecA1's sample with around 11 nm outer diameter and 7 nm inner hole (Fig. 1.54). The ring-like structure is bigger than EcSecA which formed 8 nm outer diameter with a 2 nm inner hole [38].

AFM results showed that MsSecA1 could form ring-like structure (Fig. 1.55), while MsSecA2 didn't (Fig. 1.56). These results indicated that lipid could induce conformational change of mycobacteria SecA1, suggesting SecA1 might interact with membrane in the same way as EcSecA and it might be involved in forming transmembrane channel.

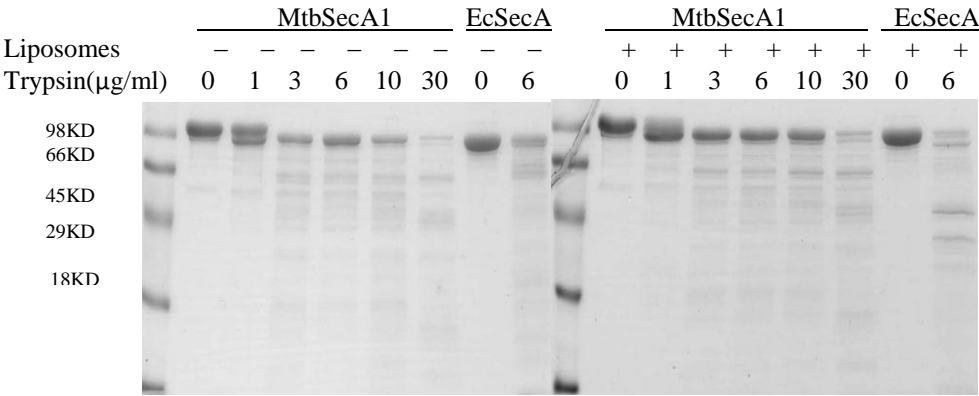


Figure 1.53a Trypsin resistance of MtbSecA1 with or without liposomes
MtbSecA: 10 μg; EcSecA: 10 μg.

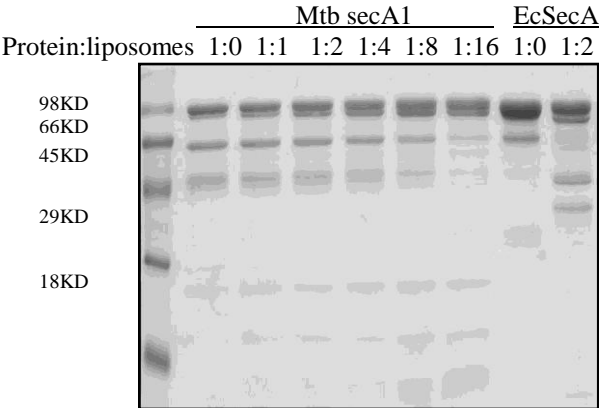


Figure 1.53b Trypsin resistance of MtbSecA1
MtbSecA: 10 μg; EcSecA: 10 μg.

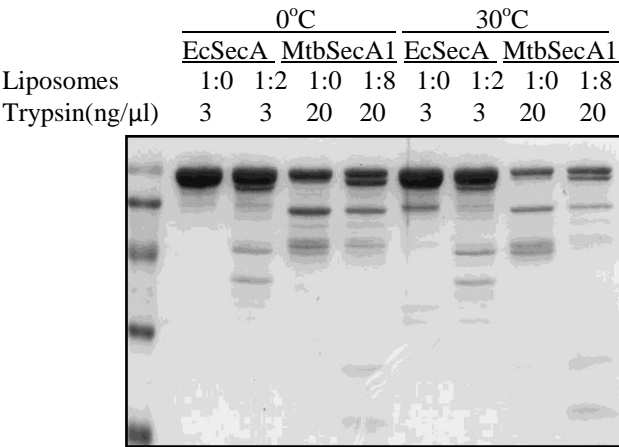


Figure 1.53c Trypsin resistance of MtbSecA1
MtbSecA: 10 μg; EcSecA: 10 μg.

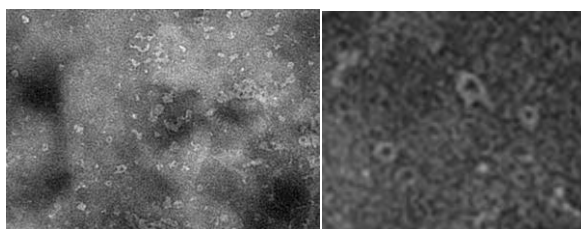


Figure 1.54 TEM structure of MtbSecA1 in presence of liposomes

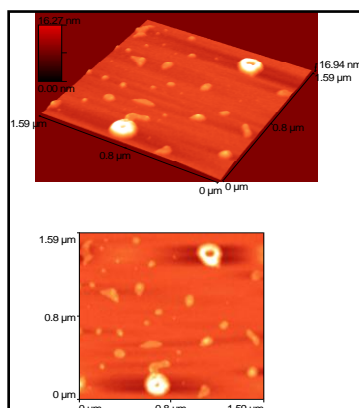


Figure 1.55a AFM structure of MsSecA1 in presence of liposomes

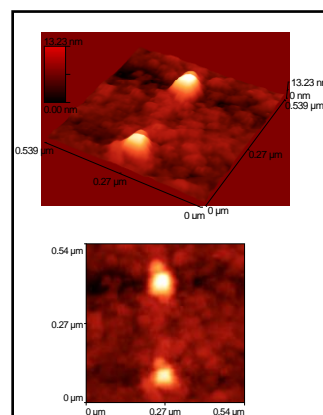


Figure 1.55b AFM structure of MsSecA1 without liposomes

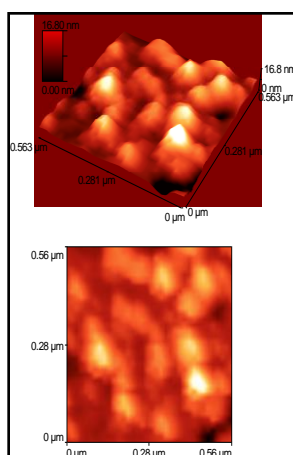


Figure 1.56a AFM structure of MsSecA2 in presence liposomes

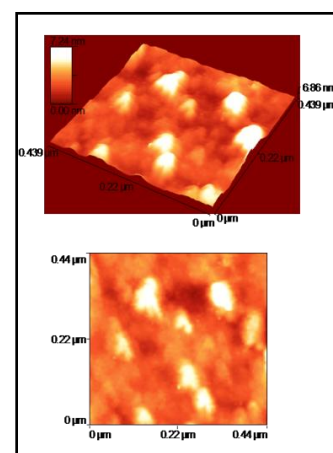


Figure 1.56b AFM structure of MsSecA2 without liposomes

Conclusions and Discussion

SecA is the central component of Sec-dependent secretion pathway, which is responsible for the secretion of many essential proteins as well as some toxins and virulence factors. Two SecA homologues are identified in some important Gram-positive pathogens. SecA1 is involved in general protein secretion and essential for viability, whereas SecA2 contribute to secretion of specific virulence factors. Alignment result showed that nine motifs of DEAD helicase were highly conserved in all SecA homologues (Fig. 0.2). In EcSecA, these nine motifs form two nucleotide binding domains (NBD); NBD1 has high affinity to nucleotide, and contains minimal ATPase catalytic machinery; NBD2 has low affinity to nucleotide, and acts as an activator of ATP hydrolysis [90]. Thus, NBD1 and NBD2 are very important to the translocase activity of EcSecA. The conservation of these domains suggests SecA1 and SecA2 might have similar translocase activity as EcSecA.

In this study, SecA1 and SecA2 from different four species were cloned, over-expressed, and purified (Table 1.2). The biochemistry properties of these SecA homologues were characterized. The results were summarized in Table 1.3. All these SecA homologues had ATPase activity, suggesting that they could be molecular motor like EcSecA. SaSecA2 and BaSecA2 showed very high endogenous ATPase activity, while MtbSecA2 showed very low ATPase activity and low GTPase activity; Mg^{2+} and $Zinc^{2+}$ are required for ATPase activity of MtbSecA1, which is similar to the result of EcSecA. The results confirmed that MtbSecA2 has low NTPase activity in presence of Mg^{2+} and Ca^{2+} . The optimal temperature for ATPase activity is varied in different SecA proteins. Only mycobacteria SecA1 showed high ATPase activity at 0°C.

In *E. coli* Sec-system, SecA undergo cycles of membrane insertion and dissociation, guiding and pushing preprotein export through transmembrane channel [38, 91]. Interacting with lipid, membrane, and precursor protein could further stimulate the ATPase activity of EcSecA. And the previous data from our lab show that EcSecA can form ring-like structure in presence of lipid, indicating it might be involved in forming transmembrane channel [38]. To determine whether SecA1 and SecA2 could interact with membrane in the same way as EcSecA, the ATPase activity of SecA homologues were investigated in presence of liposomes, membrane, and precursor. The ATPase activity of SecA1 could be stimulated by liposomes, while the ATPase activity of SecA2 couldn't be stimulated by liposomes, suggesting that SecA1 might have similar function as EcSecA and SecA2 might have similar function as BsSecA. The ATPase activity of MsSecA1 could be stimulated by BA13 membrane and the preOmpA, while other SecA homologues didn't show similar response. Because the membrane and precursor were from *E. coli*, they might not be recognized by translocase from other species. The complementation assay results showed that mycobacteria SecA1 and SecA2 failed to complement the temperature-sensitive defect of EcSecA (Table 1.4), suggesting that interaction between secretion factors of Sec system might be specific for different organisms.

The subcellular distribution of *M. smegmatis* SecA1 and SecA2 were investigated by using the antibodies of MtbSecA1 and MtbSecA2. Our data suggested that mycobacteria SecA1 and SecA2 are both located on membrane and in cytosol. These results are similar to the subcellular distribution of EcSecA, suggesting mycobacteria SecA homologues might have similar working mechanism as EcSecA. Thus, proteolysis assay was used to identify specific transmembrane domain. However, liposomes didn't induce the formation of trypsin resistant fragment of MtbSecA1, while liposomes can decrease 66KD fragments and increase 45KD and 39KD frag-

ments of EcSecA (Fig. 1.53a-b). TEM result and AFM result showed that liposomes could induce conformational change of MtbSecA1 and form ring-like structures. Liposomes could stimulate the ATPase activity of MtbSecA1 and MsSecA1, and the ATPase activity of MsSecA1 could be stimulated by membrane and pre-protein. All these results suggest that mycobacteria SecA1 might interact with membrane in the same way as EcSecA and might be involved in forming transmembrane channel.

Table 1.2 Summary of cloning, expression, and purification of SecA1 and SecA2

Protein	Homology to EcSecA	Size (a.a.)	Vector	Expression (20°C)	His-tag	Purification		
						Ni	Ion	Size
MtbSecA1	44.7/59.9	949	pET-20b	BL21.19	+	Ni-NTA	S	S200
MtbSecA2	31.8/45.6	808	pET-33b	BL21λDE3	+	His-trap	Q	Superose6
MsSecA1	45.1/61.9	957	pET-20b	BL21λDE3	+	His-trap	SP	S200 or Superose6
MsSecA2	32.8/45.1	789	pET-20b	BL21λDE3	+	His-trap	Q	S200 or Superose6
SaSecA1	44.3/63.6	843	pET-20b	BL21λDE3	+	His-trap	/	S200 or S300
SaSecA2	31.5/50.5	796	pET-33b	BL21λDE3	+	His-trap	/	S200 or Superose6
BaSecA1	50.6/66.9	835	pET-21d	BL21λDE3	+	His-trap	/	S200 or S300
BaSecA2	39.4/56.7	788	pET-33b	BL21λDE3	+	His-trap	/	S200 or S300

Homology: identity/similarity; '+': with his-tag; '/': without; S200: Superdex 200; S300: Superdex 300.

Table 1.3 ATPase activity of SecA1 and SecA2

Protein	Intrinsic ATPase	Liposomes stimulation	Membrane ATPase	Translocation ATPase	Optimal temperature	
					Intrinsic	With liposomes
Ec SecA	Pi ≈ 36	11 fold ↑	4 fold ↑	18.3 fold ↑	42°C (Pi ≈ 36)	30°C (Pi ≈ 137)
Bs SecA	Pi ≈ 67	-	ND	ND	40°C (Pi ≈ 67)	ND
MtbSecA1	Pi ≈ 16	7 fold ↑	ND	ND	0°C (Pi ≈ 16)	0°C (Pi ≈ 30)
MtbSecA2	Pi ≈ 4	-	ND	ND	37°C (Pi ≈ 16)	
MsSecA1	Pi ≈ 18	3 fold ↑	3.3 fold ↑	5.5 fold ↑	0°C (Pi ≈ 18)	20°C (Pi ≈ 47)
MsSecA2	Pi ≈ 12	-	1.4 fold	1.8 fold	30°C (Pi ≈ 9)	ND
SaSecA1	Pi ≈ 10	3.5 fold ↑	1.3 fold	1.3 fold	25°C (Pi ≈ 10)	25°C (Pi ≈ 32)
SaSecA2	Pi ≈ 120	-	1.1 fold	1.3 fold	25°C (Pi ≈ 120)	ND
BaSecA1	Pi ≈ 25	1.5 fold ↑	1 fold	1 fold	25°C (Pi ≈ 8)	20°C (Pi ≈ 28)
BaSecA2	Pi ≈ 74.4	-	ND	ND	30°C (Pi ≈ 74)	ND

ATPase activity: Pi (pmol/μg/min); '↑': increase; '-': negative result; membrane: 6M urea washed *E. coli* BA13 membrane; precursor: *E. coli* preOmpA; ND: not determined.

Table 1.4 Complementation activity of mycobacteria SecA1 and SecA2

Protein	Vector	<i>E. Coli</i> Strain	TAG Plate	Growth at 30°C	Growth at 42°C
MtbSecA1	pET-20b	BL21.19	0.01 μM-1 mM IPTG	+++	-
MtbSecA2	pBAD	BA13	0.00002%-2% arabinose	+++	-
MsSecA1	pET-20b	BL21.19	0.01 μM-1 mM IPTG	+++	-
MsSecA2	pET-20b	BL21.19	0.01 μM-1 mM IPTG	+++	-

CHAPTER 2 DEVELOPMENT AND OPTIMIZATION OF NOVEL SECA INHIBITORS

Summary

Two SecA homologues are identified in some important Gram-positive pathogens. SecA1 is involved in general protein secretion and essential for viability, whereas SecA2 contribute to secretion of specific virulence factors. SecA is conserved in bacteria, required for viability and virulence; moreover it has no counterpart in mammalian cells. Inhibition on the function of SecA homologues might reduce the virulence, inhibit bacteria growth, or/and kill bacteria, and with minimal human toxicity. Therefore these SecA homologues are potential good antibacterial drug targets, and identification SecA inhibitor might help to develop new anti-bacteria strategy. SecA proteins have ATPase activities, thus screening ATPase inhibitors might help to develop new antimicrobial agents. In this study, in collaboration with Dr. Binghe Wang's lab, three structurally different classes of SecA inhibitors were developed and optimized. (1) RB is a membrane permeant ATPase inhibitor. RB could noncompetitively inhibit the ATPase activity of SecA1 and SecA2, and SecA2 is more sensitive to RB than SecA1. RB show bactericidal and bacteriostatic effects against *M. smegmatis* without affecting H⁺-ATPase activity. The bactericidal effect of RB is independent of protein synthesis and growing phase. In *M. smegmatis*, SecA2 might be the first target. To develop more potent SecA inhibitors with less side effects and molecular weight, RB was systematically dissected to understand the importance of structural feature. The SAR studies lead to the development of two potent RB analogs, which molecular weight are less than one third of RB, and show better bacteriostatic effects and bactericidal effect than RB. (2) Pyrimidine analogs were derived from virtual screening based on the ATP binding pocket of Ec-SecA and SAR study. In this study, new pyrimidine analogs were developed and optimized from SAR studies, which show better bacteriostatic effects and better bactericidal effects. (3) Bistriazole analog was derived from random screening, with good antimicrobial activities against

Gram-positive bacteria. New bistriazole analogs were developed and optimized from SAR studies, with better bacteriostatic effects. In our study, several potent SecA inhibitors show promising bacteriostatic effect and bactericidal effect against one *E. coli* leaky mutant strain and some Gram-positive bacteria, including *MRSA* and *B. anthracis*. These novel inhibitors with structural diversity will aid the understanding of the pharmacophore required for SecA inhibition. Our study will provide a starting point for developing more potent antimicrobials.

Introduction

Due to the emergence and dissemination of multidrug resistance, bacterial pathogens have been causing a serious public health problem in recent years [6]. To address the existing drug resistant problem, there is an urgent need to find new antimicrobials, especially those against drug-resistant strains of bacteria. SecA is conserved in bacteria, required for viability and virulence [33-34]; moreover it has no counterpart in mammalian cells [40]. Thus, Inhibition on the function of SecA homologues might reduce the virulence, inhibit bacteria growth, or/and kill bacteria, and with minimal human toxicity. Therefore these SecA homologues are potential good antibacterial drug targets, and identification SecA inhibitor might help to develop new anti-bacteria strategy. SecA proteins have ATPase activities and contain ATP binding domains, thus targeting ATP binding pocket of SecA might help to develop new antimicrobial agents. In this study, three different strategies were applied to develop SecA inhibitors: screening known ATPase, virtual docking on ligand binding pocket, and random screening. This project is in collaboration with Dr. Binghe Wang's lab.

Rose Bengal (RB) is a polyhalogenated fluorescein derivative (Fig. 2.1). Previous studies demonstrated that RB can bind and inhibit the function of DNA polymerase and RNA polymerase [108-110].

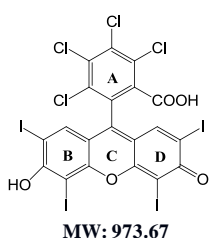


Figure 2.1 RB structure

Rose Bengal is used as an ingredient in agar media to separate fungal from bacteria, and it could inhibit the growth of *Staphylococcus aureus* [111-112]. Our lab's previous data showed that RB could inhibit the ATPase activity of EcSecA and BsSecA (Fig. 2.4). To investigate

whether RB could inhibit other SecA homologues, especially SecA homologues from pathogens causing drug resistant problems, SecA1 and SecA2 from *M. smegmatis*, *S. aureus*, *B. anthracis*

were subjected to *in vitro* RB sensitivity assay; and *M. smegmatis* was used as surrogates of *M. tuberculosis* to do *in vivo* assay.

RB is known to inhibit other ATPases [113] and to undergo photo-oxidation leading to DNA polymerase [108-110]. Moreover RB has been banned as a food dye because of its cytotoxicity [114]. 10 mg/kg RB could cause DNA damage to the glandular stomach, colon, and urinary bladder in 3 hours [115]. Thus, in collaboration with Dr. Binghe Wang's lab, we want to modify the structure of RB, increasing inhibition effect, and decreasing side effects. RB is a large molecule, molecular weight is 973.67, and it consists of a polyiodinated xanthene moiety and a polychlorinated benzoate moiety. We don't know whether chloro group, or carboxyl group, or the number and position of iodo group are essential for the inhibition effect of RB. In this study, new SecA inhibitors were developed from systematical dissection of RB and Structure-Activity Relationship study. This approach allowed us to determine the importance of iodo group, hydroxyl group, carboxyl group, and the necessity of ring A /B/D. Furthermore, the most effective RB analogues were used to study the inhibition mechanism of RB analogues.

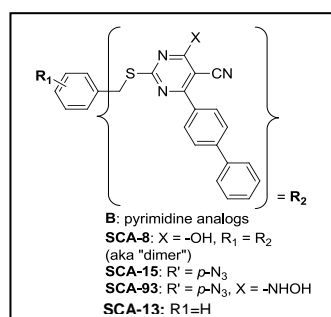


Figure 2.2 Structure of pyrimidine analogs

Pyrimidine analogues were developed from virtual screening based on the ATP binding pocket of *E. coli* SecA in our previous study [81]. SCA-8 showed strong inhibitory effect on the endogenous ATPase activity of EcSecAN68, however it showed low bacteriostatic effects. SCA-8 is an aka dimer (Fig.2.2), and it might be too big to get through bacteria cell membrane. SCA-13 contains half

structure of SCA-8; although inhibition effect on the ATPase activity of SecA was 10 times reduced, bacteriostatic effect was increased more than 5 to 50 times. In this study, new SecA inhibitors were developed from further optimizing of structure SCA-13. Among tested compounds,

SCA-15 and SCA-93 showed promising bacteriostatic effects and bactericidal effects against *B. anthracis* Sterne and numerous drug resistant *S. aureus* strains.

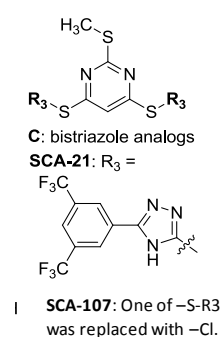


Figure 2.3 Structure of bistriazole analogs

Bistriazole analogs were derived from random screening. SCA-21 showed good bacteriostatic effect and bactericidal effect on gram positive bacteria. Replacing half part of SCA-21 with chloro group, slightly increased *in vitro* inhibition effect, but significantly increased *in vivo* inhibition; further removing the fluoro group or methylthio group, decreased inhibition effect; the methyl group could be replaced with benzene ring.

In our study, several potent SecA inhibitors had varying degrees of inhibitory effects against SecA homologues; and showed promising bacteriostatic effect and bactericidal effect against some gram positive bacteria and one *E. coli* leaky mutant strain. These new inhibitors with structural diversity will aid the understanding of the pharmacophore required for SecA inhibition. Our study will provide a starting point for developing more potent antimicrobials.

Materials and Methods

Medium for *M. smegmatis*: Middlebrook 7H9 broth with 0.2% glycerol, 1×ADC (0.5% bovine serum albumin, fraction V, 0.2% dextrose, 0.85% NaCl), and 0.1% Tween 80 was used to grow *M. smegmatis*. Muller Hinton Agar was used to grow *M. smegmatis* on plate.

Medium: LB medium was used to grow bacteria in this study.

SecA inhibitors: were synthesized by Dr. Binghe Wang's lab.

Strains: *M. smegmatis* mc²155 and *M. smegmatis* mc²2522 (SecA2 deletion mutant) were from Dr. Miriam Braunstein in Univ. of North Carolina. *E. coli* NR698, *B. subtilis* 168, *S. aureus* N315, *S. aureus* 6538, *S. aureus* Mu50, *B. anthracis* Sterne.

***In vitro* inhibition assay:** Because SecA homologues have ATPase activity, Malachite green colorimetric assay was used to investigate the inhibition of inhibitors on the ATPase activity of SecA homologues. In this assay, ATPase assay was carried in different concentration of inhibitors, and IC₅₀ is the concentration of RB which could inhibit 50% ATPase activity of SecA homologues. Because all compounds were not soluble except for RB, thus compounds were dissolved in 100% DMSO. In the final *in vitro* assay, there were 1-5% DMSO.

Inhibition Assay on Plates: *M. smegmatis* wild type and *M. smegmatis* SecA2 deletion mutant were grown in 3 ml M7H9 broth (containing 0.5% Dextrose, 0.1% Tween 80, 0.2% glycerol) in 12 ml culture tube; when OD₆₀₀ ≈ 0.5, 500 µl culture was taken and mixed with 9.5 ml of 70% Muller Hinton Agar, then 5 ml mixture was spread on the top of 100% Muller Hinton Agar plates. 1 µl RB (different concentration) was dropped on those plates, then the plates were incubated at 37°C for one day.

***In vivo* inhibition assay in medium:** *M. smegmatis* wild type and *M. smegmatis* ΔSecA2 were grown in 3 ml M7H9 broth (containing 0.5% Dextrose, 0.1% Tween 80, 0.2% glycerol) in

12 ml culture tube; when $OD_{600} \approx 1.0$, they are diluted into 50 ml M7H9 medium; when $OD_{600} \approx 1.0$, 100 μ l culture were inoculated into 3 ml M7H9 medium which contains different concentration of RB; then incubate at 37°C, 240 rpm. OD_{600} was measured at different time point. For strains other than MS, Single colony of bacteria was inoculated in 3 ml LB medium in 12 ml culture tube, then grow at 37°C; when $OD_{600} \approx 0.5$, 300 μ l culture were diluted into 3 ml LB medium, then 97.5 μ l culture was aliquated into 1.5 ml eppendorf tube, then adding 2.5 μ l of different concentration of RB analogues; incubated at 37°C, 200 rpm for 22 hours. OD_{600} was measured to determine inhibition effect.

Killing assay: *M. smegmatis* wild type and *M. smegmatis* Δ SecA2 were grown in 3 ml M7H9 broth (containing 0.5% dextrose, 0.1% tween 80, 0.2% glycerol) in 12 ml culture tube; when $OD_{600} \approx 1.0$, they are diluted into 50 ml M7H9 medium; when $OD_{600} \approx 0.5$, 3 ml cell culture were aliquated into 12 ml culture tube, then add different concentration of RB into the cell culture; then incubate at 37°C, 240 rpm for 2 hours. The cell cultures were serially diluted with dd H₂O and 100 μ l or 200 μ l was spread on plates or 1 μ l was dropped onto plates. For strains other than *M. smegmatis*, single colony of bacteria was inoculated in 3 ml LB medium in 12 ml culture tube, then grow at 37°C; when $OD_{600} \approx 0.5$, 97.5 μ l cell culture was aliquated into 1.5 ml eppendorf tube, then 2.5 μ l inhibitors was added into tube; incubated at 37°C, 1000 rpm for 1-2 hours. The cell cultures were diluted with dd H₂O and spread 150 μ l on LB plates.

Results

The Inhibition Effect of Rose Bengal

SecA is conserved in bacteria, required for viability and virulence; moreover it has no counterpart in mammalian cells. Thus, inhibition on the function of SecA homologues might reduce the virulence, inhibit bacteria growth, or/and kill bacteria, and with minimal human toxicity. Therefore these SecA homologues are potential good antibacterial drug targets, and identification SecA inhibitor might help to develop new anti-bacteria strategy. Two NBDs are highly conserved in SecA proteins and enzymatic assay demonstrated that SecA proteins have ATPase activities, thus screening ATPase inhibitors might help to develop new antimicrobials. RB is a polyhalogenated fluorescein derivative. Previous studies demonstrated that RB is a membrane-permeant ATPase inhibitor. Our lab's previous data showed that RB could inhibit the ATPase activity of EcSecA and BsSecA (Fig. 2.4). In this study, SecA1 and SecA2 from *M. smegmatis*, *S. aureus*, *B. anthracis* were subjected to *in vitro* RB sensitivity assay; and *M. smegmatis* was used as surrogates of *M. tuberculosis* to do *in vivo* assay.

The Inhibition of RB on MtbSecA1 RB could inhibit intrinsic ATPase activity of MtbSecA1, and the IC_{50} is around 60 μ M (Fig. 2.5a). Because the intrinsic ATPase activity of MsSecA1 is low, liposomes were used to stimulate the ATPase activity. The results show that RB also could inhibit the ATPase activity of MsSecA1 in the presence of liposomes, and IC_{50} is around 130 μ M at 30°C (Fig. 2.5b), while at 20°C, IC_{50} is around 75 μ M (Fig. 2.5c). These results indicated that lower temperature is good for RB binding with MtbSecA1.

The Inhibition Effect of RB on MsSecA1 and MsSecA2 RB could inhibit the ATPase activity of MsSecA1 in presence of liposomes; IC_{50} is around 45 μ M at 20°C and 125 μ M at 30°C (Fig. 2.6a). These results indicated that lower temperature is good for RB binding with

MsSecA1. In the presence of liposomes, 100 μM RB could noncompetitively inhibit ATPase activity of MsSecA1 (Fig. 2.6b-c). MsSecA2 is more sensitive to RB than MsSecA1, and IC_{50} is 2 μM (Fig. 2.7a). Kinetic results show that RB noncompetitively inhibited the endogenous ATPase activity of MsSecA2; K_m is 3.88 mM, and K_i is 4.66 μM (Fig. 2.7b).

The Inhibition of RB on SaSecA1 and SaSecA2 To do kinetic ATPase assay, the ATPase activity of SaSecA1 was investigated at different time points. The result showed that its ATPase activity was linear within 60 min at 25°C (Fig. 2.8a). Thus, we chose 40 min as reaction time. RB could inhibit the ATPase activity of SaSecA1 in the presence of liposomes, and IC_{50} is around 2.5 μM (Fig. 2.8b). Kinetic assay results show that RB noncompetitively inhibited the ATPase activity of SaSecA1 and it may have two noncompetitive ATP binding sites (Fig. 2.8c).

The ATPase activity of SaSecA2 was investigated at different time points. The result showed that its ATPase activity was linear at first 25 min at 25°C (Fig. 2.9a). Thus, we chose 20 min as reaction time. RB could inhibit the endogenous ATPase activity of SaSecA2, and IC_{50} is around 1 μM , which is lower than SaSecA1 (Fig. 2.9b). And this result was similar to RB sensitivity of MsSecA2, suggesting that SecA2 might very sensitive to RB. Kinetic assays results show that RB has non competitive inhibition on the ATPase activity of SaSecA2 (Fig. 2.9c).

The Inhibition Effect of RB on BaSecA1 and BaSecA2 The ATPase activity of BaSecA1 was investigated at different time points. The result showed that its ATPase activity was linear within 60 min at 20°C (Fig. 2.10a). Thus, we chose 40 min as reaction time. RB could inhibit the ATPase activity of BaSecA1 in presence of liposomes, and IC_{50} is around 10 μM at 20°C (Fig. 2.10b). To determine the inhibition type of RB on BaSecA1, kinetic ATPase assays were carried out at different concentration of RB. The results show that RB noncompetitively inhibited

the ATPase activity of BaSecA1, and it might have two non-competitive binding sites (Fig. 2.10c).

The ATPase activity of BaSecA2 was investigated at different time points. The result showed that its ATPase activity was linear within 60 min at 30°C (Fig. 2.11a). Thus, we chose 40 min as reaction time. To determine whether RB could inhibit the ATPase activity of BaSecA2, ATPase assay was carried in presence of different RB concentration. The result showed that RB could inhibit the endogenous ATPase activity of BaSecA2, and IC_{50} is around 3.5 μ M at 30°C (Fig. 2.11b). To determine the inhibition type of RB on BaSecA2, kinetic ATPase assays were carried out at different concentration of RB. The results show that RB noncompetitively inhibited the ATPase activity of BaSecA2 (Fig. 2.11c).

Bacteriostatic Effect of RB against *M. smegmatis* MS wild type and MS Δ SecA2 were grown in M7H9 broth which contain different concentration of RB and OD_{600} was measured at different time point. Our results showed that RB could inhibit the growth of Ms wild type and MS Δ SecA2 (Fig. 2.12a-b). The bacteriostatic effect against of MS wild type was compared with the result of MS Δ SecA2. For wild type, MIC_{50} is 30 μ M and MIC_{99} is 130 μ M; but for MS Δ SecA2 MIC_{50} is 35 μ M and MIC_{99} is 150 μ M (Fig. 2.12c). MS Δ SecA2 was slightly more resistant to RB than wild type. MS was grown in the M7H9 broth which contains 0.5% dextrose, the bactericidal effect of RB was not caused by inhibition the ATPase activity of H^+ -ATPases. Because MS is very easy to aggregate, OD reading might not be accurate. Therefore, we did plate inhibition assay. The result of plate inhibition assay confirmed that MS Δ SecA2 was more resistant to RB than wild type. For wild type, MIC is 20 μ M; while for MS Δ SecA2 MIC is 60 μ M (Fig. 2.13).

Bactericidal Effect of RB against *M. smegmatis* After 2.5 hours incubation with different concentration of RB, the cell culture was diluted and 1ul was put onto plates. The results showed that MS Δ SecA2 was more resistant to RB than wild type (Fig. 2.28). To kill two log number of cell die, for MS wild-type only need 3.9 μ M RB, but for MS Δ SecA2 need 15.6 μ M RB was needed (Fig. 2.14).

The Bactericidal Effect of RB on Growing MS To investigate the effects of different dilution buffers on the cell number grown on the plates, 1X A buffer and dd H₂O with or with Tween 80 were used to dilute the same cell culture ($OD_{600} \approx 0.5$), then, 200ul was spread on the plates. The result showed that the 1X A buffer with Tween 80 had the best effect to inhibit the clump formation of MS (Fig. 2.15). Thus, this buffer was used to dilute the cell culture in the following experiment. MS was grown at 37°C to $OD_{600} \approx 0.5$, then, different concentration of RB was added into the medium. After 1 hour incubation with RB, the MS cultures were diluted and spread onto plates, which were incubated at 37°C for three days then subjected to count the viable cell colonies. The result showed that RB had bactericidal effect on growing MS (Fig. 2.16). Because MS was grown in the M7H9 broth which contains 0.5% dextrose, the bactericidal effect of RB was not caused by inhibition the ATPase activity of H⁺-ATPase. The result also showed that MS SecA2 mutant was more resistant to RB than MS wild type, suggesting SecA2 might be the first target of RB in MS.

The Bactericidal Effect of RB on Non-growing MS To determine whether RB has bactericidal effect on non-growing MS, different concentration of RB was added into the medium when MS grew into stationary phase. The results show that RB had bactericidal effect on non-growing MS (Fig. 2.17). The result confirmed that MS SecA2 mutant was more resistant to RB than MS wild type. To compare the RB effect on growing and non-growing MS, the growth of

MS was monitored (2.18a), and culture was taken out at different growing phase to do the killing assay with or without dilution. The results showed that the bactericidal effect of RB was independent of growing phase (Fig. 2.18b).

The Bactericidal Effect of RB is Independent of Protein Synthesis Previous study demonstrated that RB could inhibit the function of RNA polymerase. To determine whether the bactericidal effect of RB related to protein synthesis, when MS was grown at 37°C to $OD_{600} \approx 1.0$, 20 µg/ml or 50 µg/ml Chromphenical (CM) was added into the medium; then after 1 hour incubation, 320 µM RB was added into the medium; after additional 1 hour incubation, the MS culture were diluted and spread onto plates, which were incubated at 37°C for three days then subjected to count the viable cell colonies. The result showed that 20 µg/ml CM was sufficient to inhibit MS growth (Fig. 2.25). And the bactericidal effect of RB on MS was in respect of the presence of CM in the medium. The result show that CM do not have negative effect on the result of killing assay, thus the bactericidal effect of RB is independence of protein synthesis (Fig. 2.19). The result also showed that MS Δ SecA2 was slightly more resistant to RB than MS wild type.

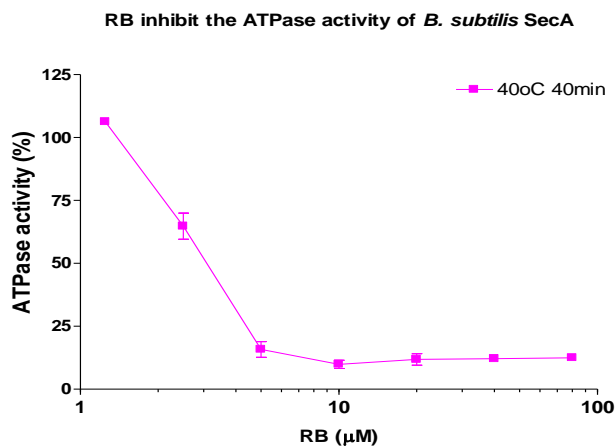


Figure 2.4 Inhibition of RB on the intrinsic ATPase activity of BsSecA
40 min reaction at 40°C; 2 mM Mg²⁺ and 2 mM ATP; without liposomes; IC₅₀ ≈ 3 μM.

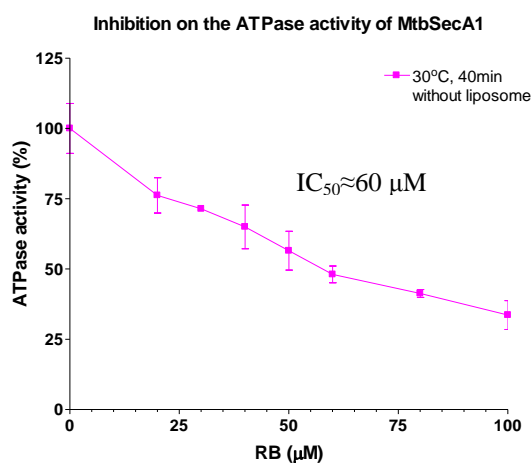


Figure 2.5a Inhibition of RB on the intrinsic ATPase activity of MtbSecA1
40 min reaction at 30°C; 2 mM Mg²⁺ and 2 mM ATP; IC₅₀ ≈ 60 μM.

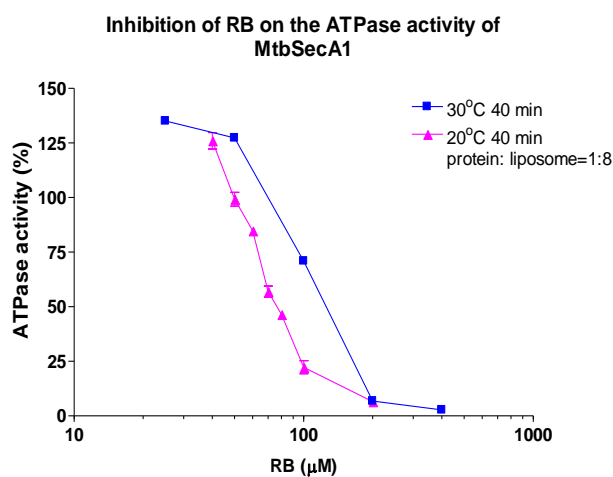


Figure 2.5b Inhibition of RB on the ATPase activity of MtbSecA1 in presence of liposomes
2 mM Mg²⁺ and 2 mM ATP; protein : liposomes = 1:8; 40 min reaction at 30°C, IC₅₀ ≈ 130 μM; 40 min reaction at 20°C, IC₅₀ ≈ 75 μM.

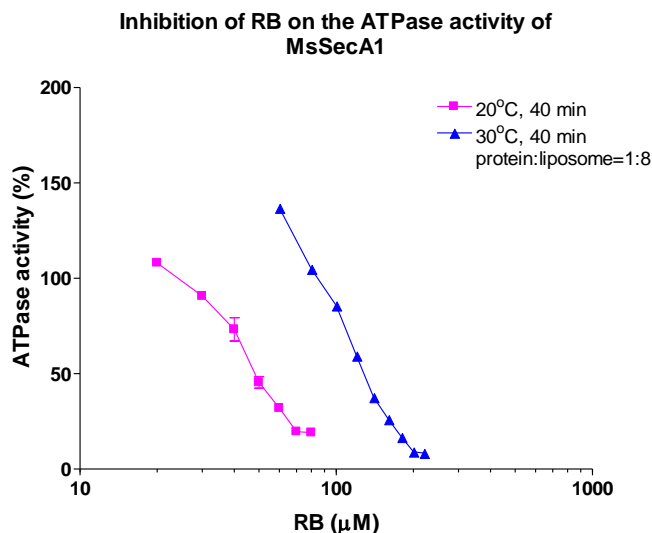


Figure 2.6a Inhibition of RB on the ATPase activity of MsSecA1

2 mM Mg^{2+} and 2 mM ATP; protein : liposomes = 1:8; 40 min reaction at 20°C, $\text{IC}_{50} \approx 45 \mu\text{M}$; 40 min reaction at 30°C; $\text{IC}_{50} \approx 125 \mu\text{M}$.

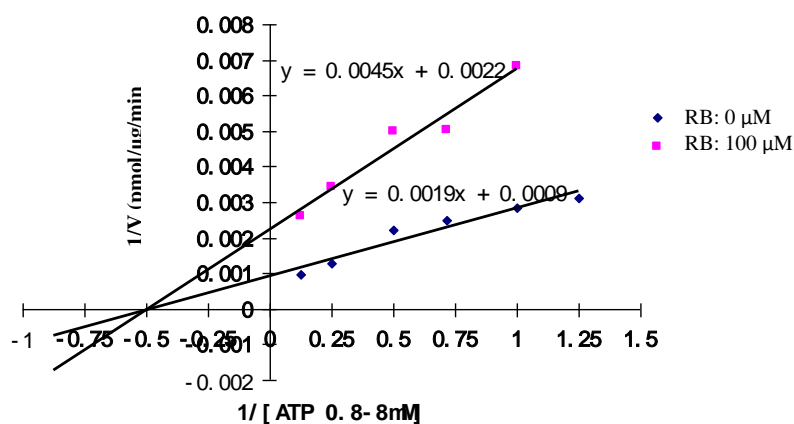


Figure 2.6b Non-competitive inhibition of RB on the ATPase activity of MsSecA1

20 min reaction at 37°C; 2 mM Mg^{2+} ; ATP: 0.8-8 μM ; protein: liposomes=1:8; $K_m \approx 2.07 \text{ mM}$ and $K_i \approx 69.23 \mu\text{M}$

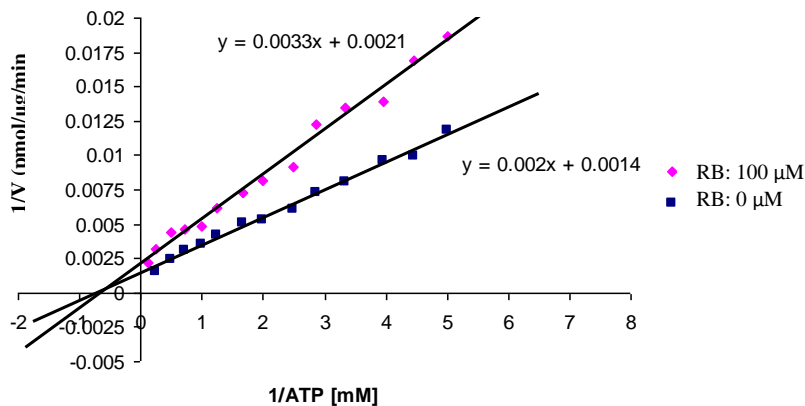


Figure 2.6c Non-competitive inhibition of RB on the ATPase activity of MsSecA1

40 min reaction at 37°C; protein : liposomes = 1 : 8; ATP: 0.8-8 μM ; 2 mM Mg^{2+}

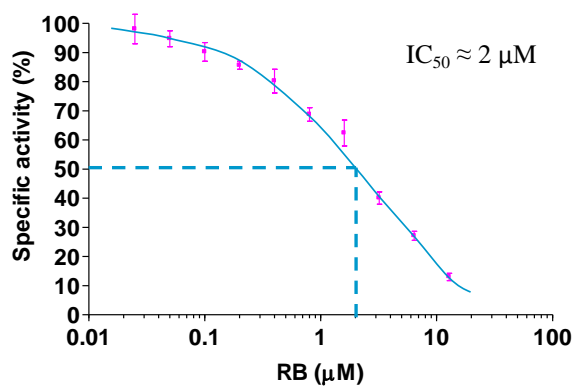


Figure 2.7a Inhibition of RB on the intrinsic ATPase activity of MsSecA2
40 min reaction at 30°C; 2 mM Mg^{2+} and 2 mM ATP; without liposomes; $\text{IC}_{50} \approx 2 \mu\text{M}$.

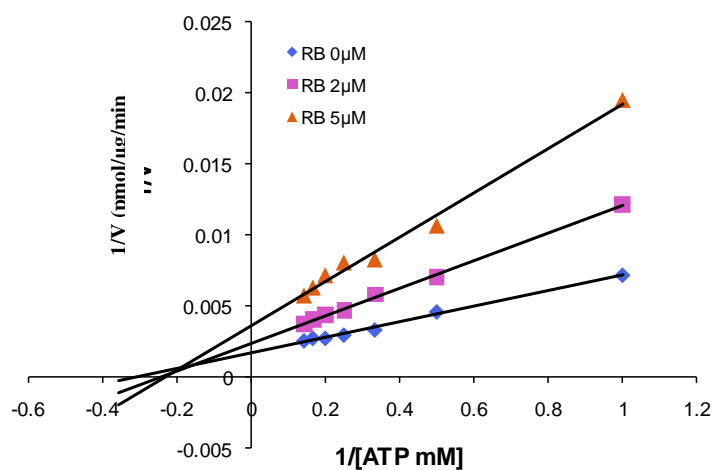


Figure 2.7b Non-competitive inhibition of RB on the intrinsic ATPase activity of MsSecA2
40 min reaction at 30°C; without liposomes; 2 mM Mg^{2+} ; $K_m \approx 3.88 \text{ mM}$; $K_i \approx 4.66 \mu\text{M}$.

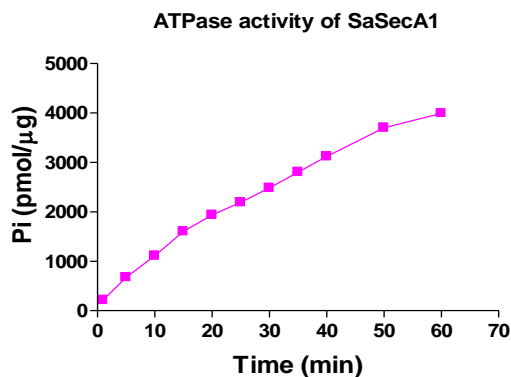


Figure 2.8a Time dependent ATPase activity of SaSecA1
40 min reaction at 25°C; protein: liposomes = 1:8; 2 mM Mg^{2+} ; 2 mM ATP

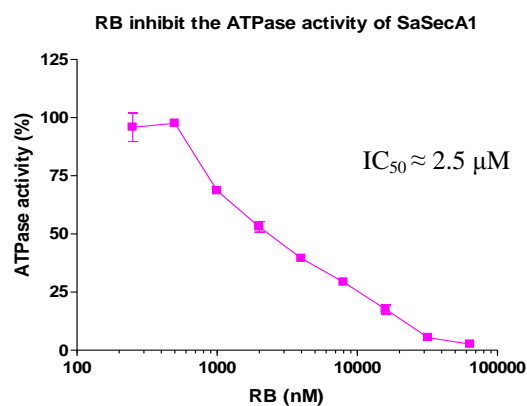


Figure 2.8b Inhibit of RB on the ATPase activity of SaSecA1
40 min reaction at 25°C; protein: liposomes = 1:8; 2 mM Mg^{2+} ; 2 mM ATP; $IC_{50} \approx 2.5 \mu M$

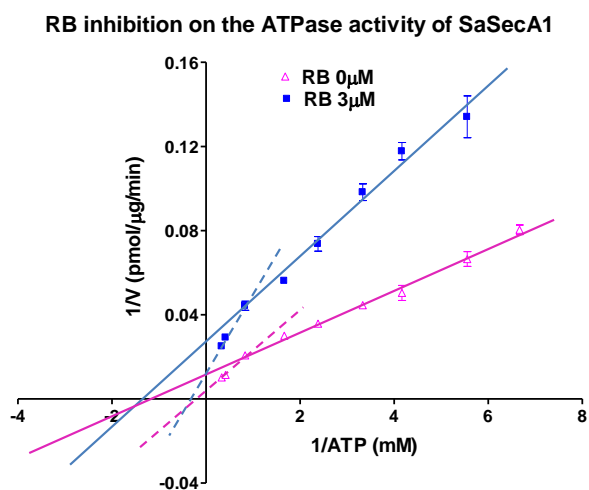


Figure 2.8c Non-competitively inhibition of RB on the ATPase activity of SaSecA1 in presence of liposomes
40 min reaction at 25°C; protein: liposomes = 1:8; 2 mM Mg^{2+} ; 2 mM ATP

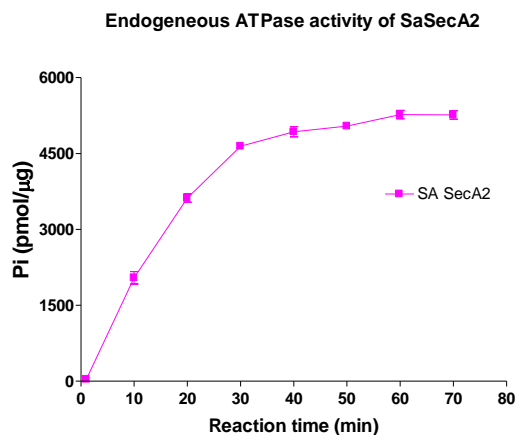


Figure 2.9a Time dependent ATPase activity of SaSecA2
25°C; without liposomes; 2 mM Mg^{2+} ; 2 mM ATP.

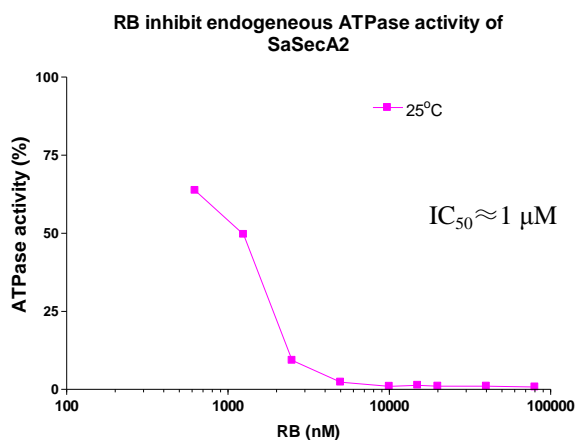


Figure 2.9b Inhibition of RB on the intrinsic ATPase activity of SaSecA2
20 min reaction at 25°C; without liposomes; 2 mM Mg^{2+} ; 2 mM ATP; $IC_{50} \approx 1 \mu M$.

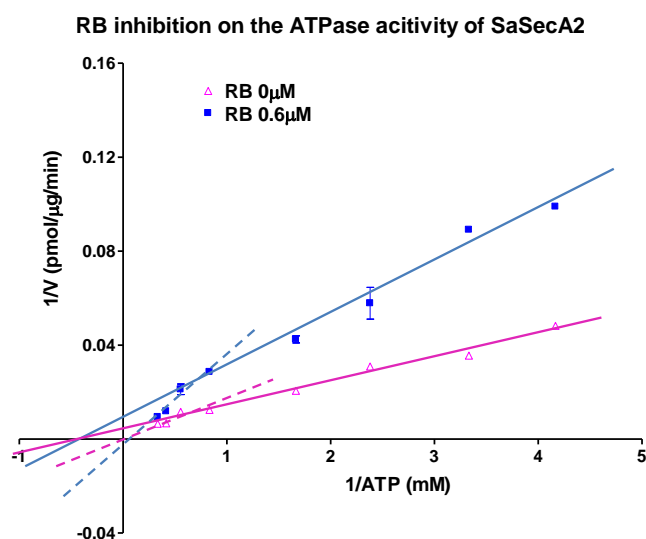


Figure 2.9c Non-competitive inhibition of RB on the intrinsic ATPase activity of SaSecA2
20 min reaction at 25°C; without liposomes; 2 mM Mg^{2+} ; 2 mM ATP.

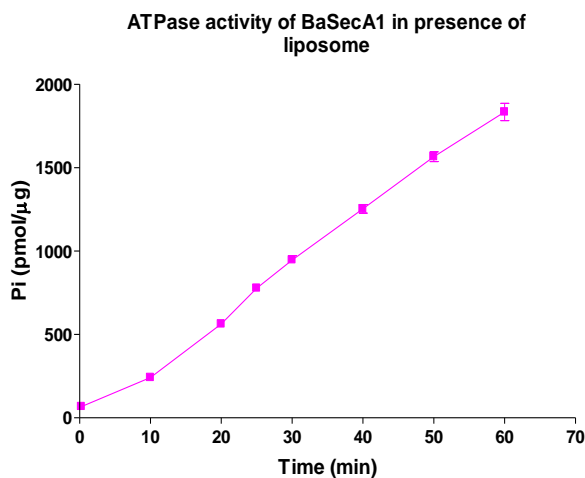


Figure 2.10a Time dependent ATPase activity of BaSecA1 in presence of liposomes
 20°C; BaSecA1: 5 μg; protein : liposomes=1 : 8; 2 mM Mg^{2+} ; 2 mM ATP.

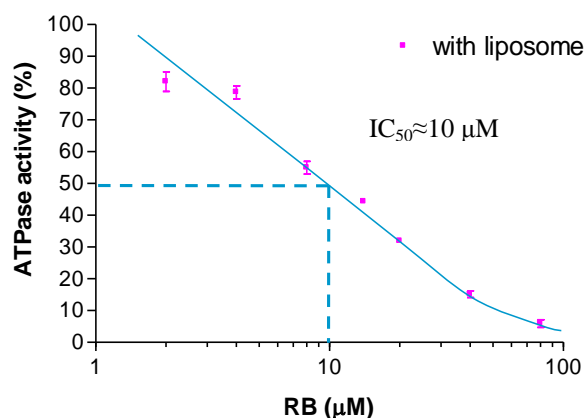


Figure 2.10b Inhibition of RB on the ATPase activity of BaSecA1
 40 min reaction at 20°C; BaSecA1: 5 μg; protein : liposomes=1:8; 2 mM Mg^{2+} ; 2 mM ATP; $IC_{50} \approx 10 \mu M$.

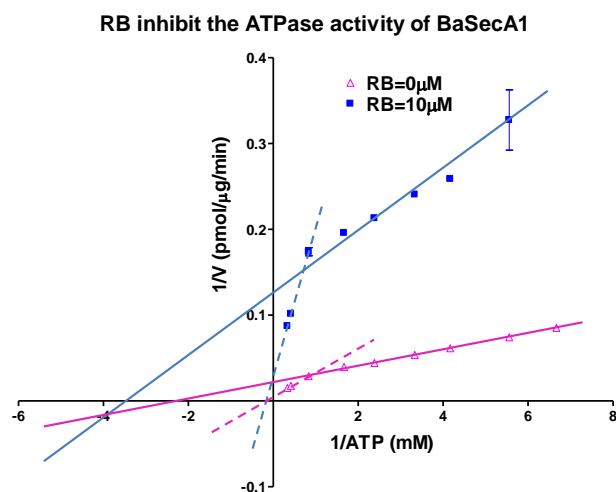


Figure 2.10c Non-competitive inhibition of RB inhibition on the ATPase activity of BaSecA1
 40 min reaction at 20°C; BaSecA1: 5 μg; protein : liposomes = 1 : 8; 2 mM Mg^{2+} ; 2 mM ATP.

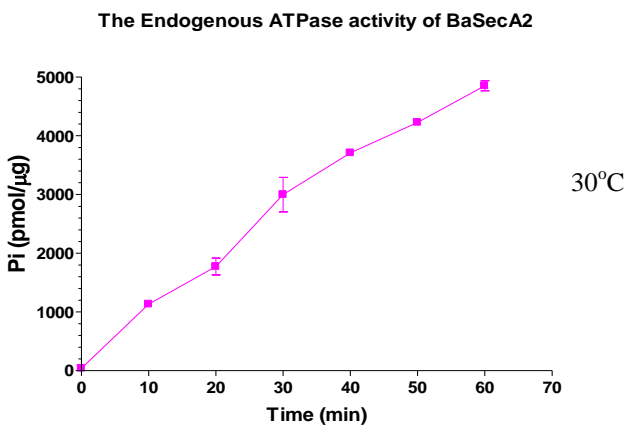


Figure 2.11a Time dependent ATPase activity of BaSecA2
30°C; without liposomes; 2 mM Mg^{2+} ; 2 mM ATP.

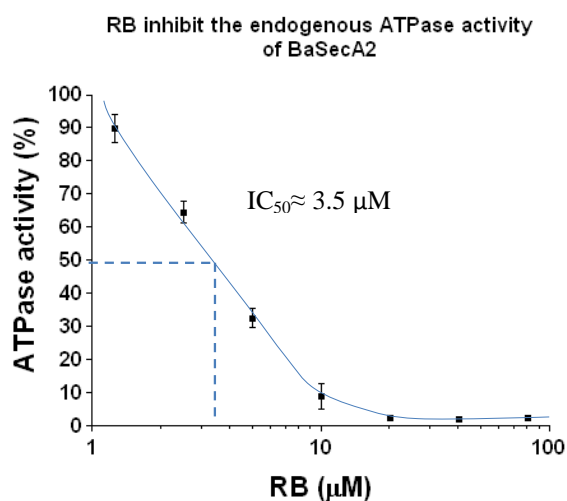


Figure 2.11b Inhibition of RB on the intrinsic ATPase activity of BaSecA2
40 min reaction at 30°C; without liposomes; 2 mM Mg^{2+} , 2 mM ATP.

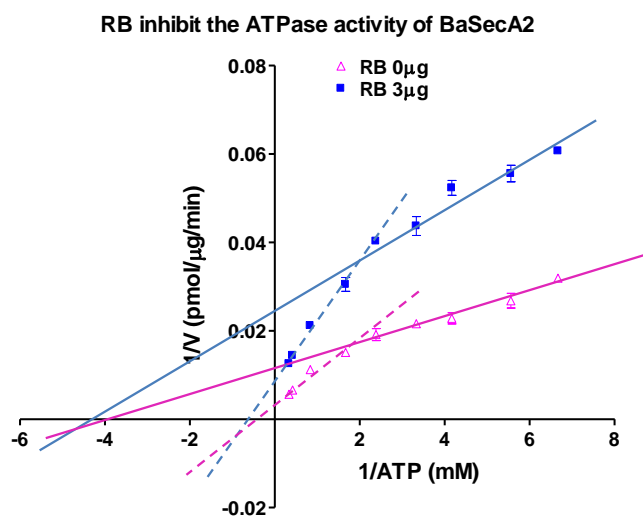


Figure 2.11c Non-competitive inhibition of RB on the ATPase activity of BaSecA2
40 min reaction at 30°C; without liposomes; 2 mM Mg^{2+} .

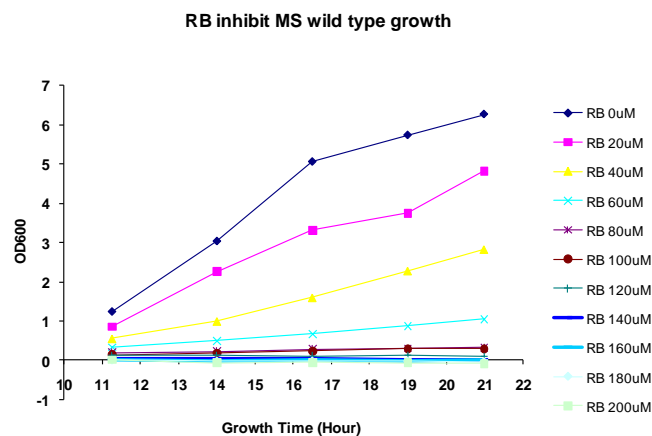


Figure 2.12a Bacteriostatic effect of RB against MS wild type

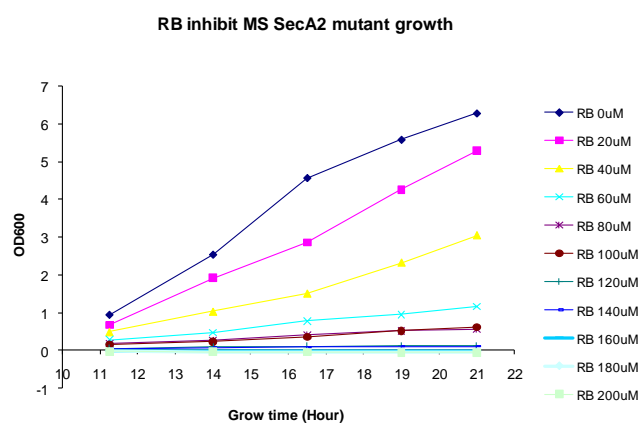


Figure 2.12b Bacteriostatic effect of RB against MS Δ SecA2 mutant

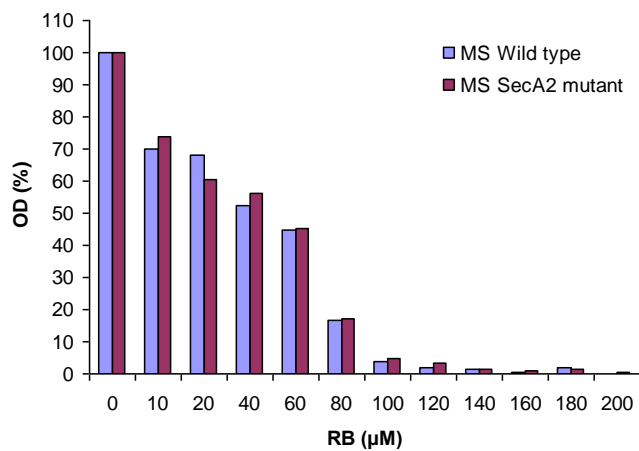


Figure 2.12c Bacteriostatic effect of RB against Ms wild type and Ms Δ SecA2 mutant
 MS wild type: MIC₅₀ \approx 30 μ M, MIC₉₉ \approx 130 μ M; MS Δ SecA2: MIC₅₀ \approx 35 μ M, MIC₉₉ \approx 150 μ M.

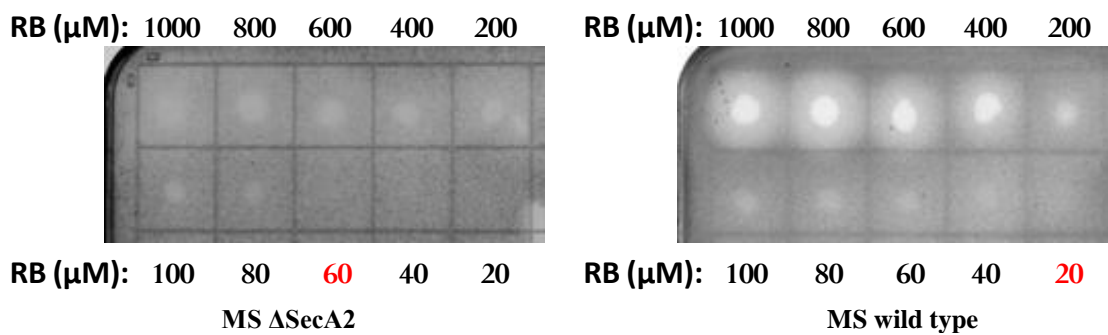


Figure 2.13 Result of RB plate inhibition assay against *M. smegmatis*

Ms wild type: MIC \approx 20 μM ; MS ΔSecA2 : MIC \approx 60 μM .

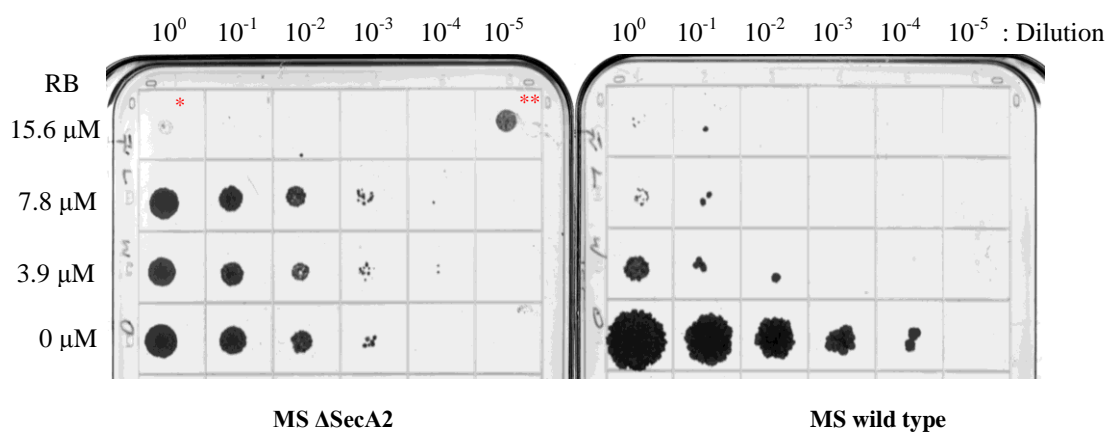


Figure 2.14 Result of RB spot-killing assay against *M. smegmatis*

Culture was incubated with different conc. of RB at 37°C for 2.5 hours, then the cell culture was serially diluted, and 1 μl was dropped onto plates, and incubated at 37°C for 3 days. *: 10⁻¹; **: 10⁻⁵.

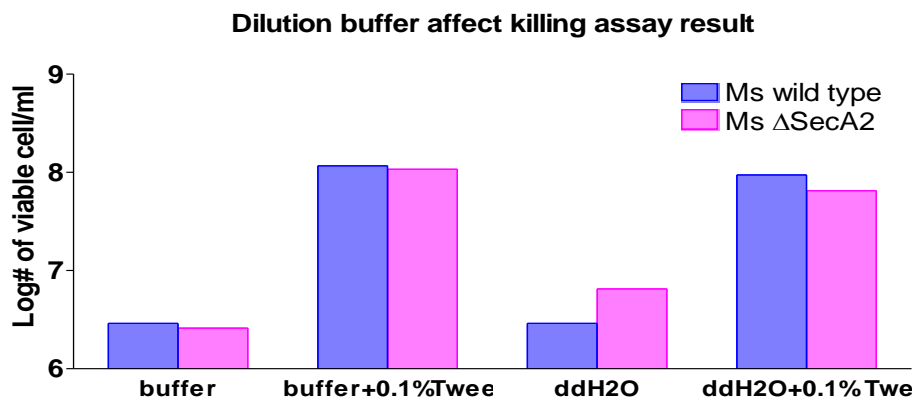


Figure 2.15 Dilution buffer affect the result of killing assay against *M. smegmatis*
RB was added in to culture when $OD_{600} \approx 0.5$; 37°C; 1 hour killing.

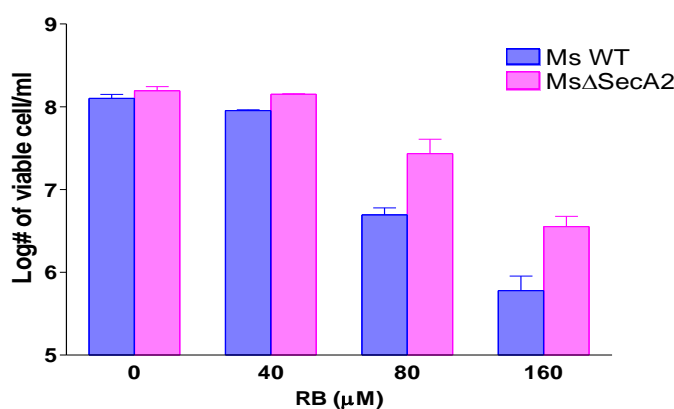


Figure 2.16 Bactericidal effect of RB on growing *M. smegmatis*
RB was added into culture when $OD_{600} \approx 0.5$; 37°C; 1 hour killing.

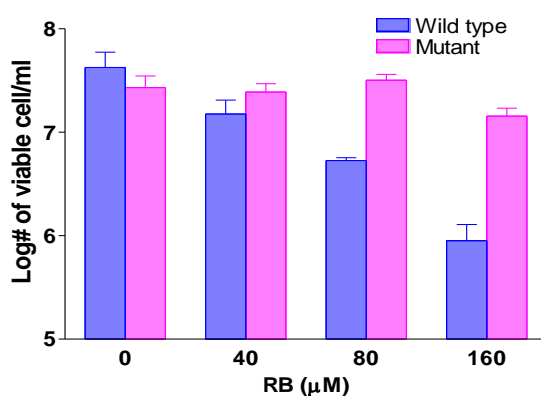


Figure 2.17 Bactericidal effect of RB on non-growing *M. smegmatis*
Stationary phase cell culture was incubated with RB to do killing assay at 37°C for 1 hour.

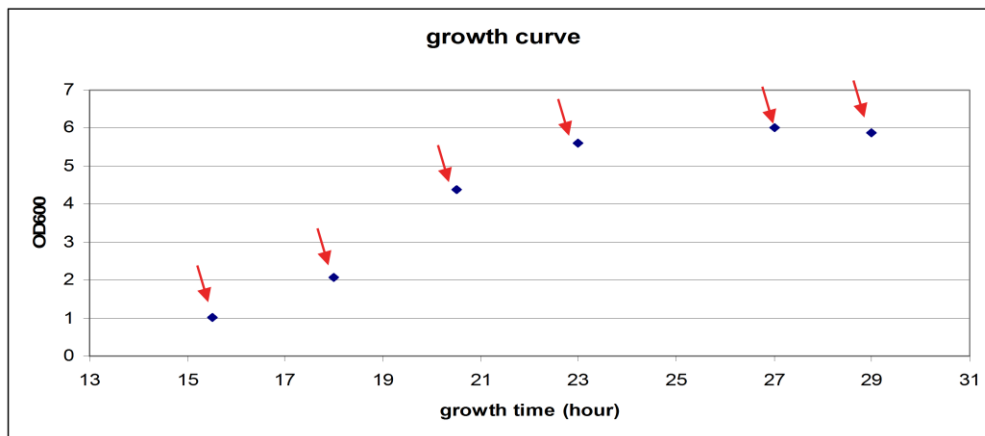


Figure 2.18a The growth curve of *M. smegmatis*

Cell culture was taken out at different time point (labeled with red arrow), and subjected to killing assay.

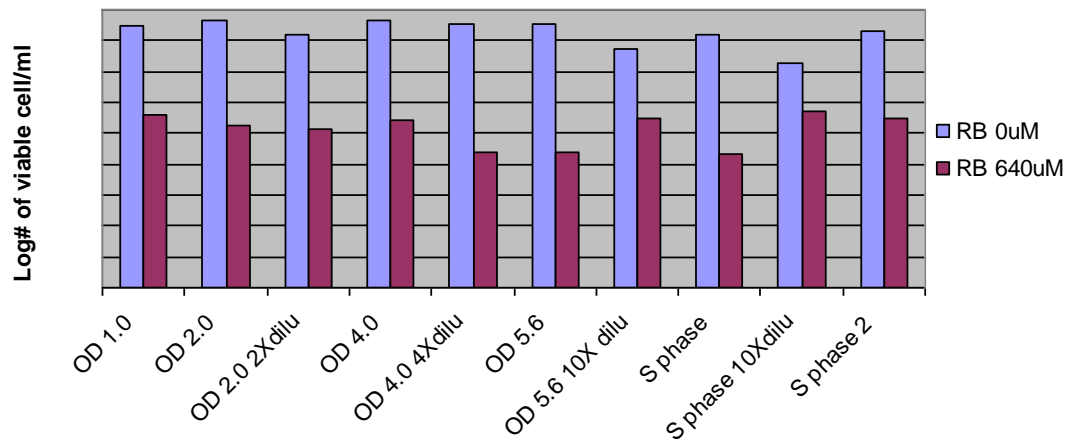


Figure 2.18b Bactericidal effect of RB on *M. smegmatis* is independent of growing phase

Cell culture was taken out at different growing phase, and subjected to killing at 37°C for 1 hour. S phase: stationary phase.

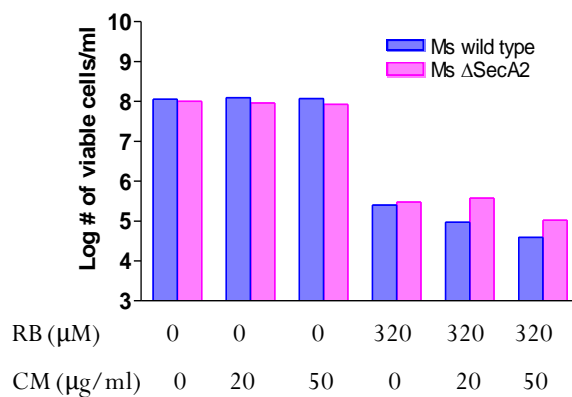


Figure 2.19 Bactericidal effect of RB is independent of protein synthesis

When OD₆₀₀ \approx 1.0, Chromphenical was added into the medium; then after 1 hour incubation, RB was added into the medium to do killing assay at 37°C for 1 hour.

Table 2.1 *In vitro* inhibition effect of RB against on SecA1 and SecA2

	liposome	IC ₅₀ (μM)	Kinetic Assay		
			Binding site	Low ATP conc. (1-0.2 mM)	High ATP conc.(2-8 mM)
MsSecA1	+	45	1	Noncompetitive inhibition	Noncompetitive inhibition
MsSecA2	-	2	ND	ND	Noncompetitive inhibition
SaSecA1	+	2.5	2	Noncompetitive inhibition	Noncompetitive inhibition
SaSecA2	-	1	ND	ND	Noncompetitive inhibition
BaSecA1	+	10	2	Noncompetitive inhibition	Noncompetitive inhibition
BaSecA2	-	3.2	2	Noncompetitive inhibition	Noncompetitive inhibition

‘+’: with liposomes; ‘-’: without liposomes; ‘ND’: not determined;

Development of Novel SecA Inhibitors by Screening RB Analogs

SecA, an essential component of Sec-dependent secretion pathway, is required for viability and virulence of bacteria. Its high conservation among a wide range of bacteria, no human counterpart, and not fully developed properties make it an attractive target for exploring novel antimicrobial agents. Our results showed that RB could noncompetitively inhibit the ATPase activity of SecA1 and SecA2 from different species, and RB showed bactericidal and bacteriostatic effects on the growth of *M. smegmatis* without affecting H⁺-ATPase activity. However RB is known to inhibit other ATPases and to undergo photo-oxidation leading to DNA alkylation [108-110]. Moreover RB is known to be cytotoxic [114] [115], and has been banned as a food dye. Thus, we want to modify the structure of RB, increasing inhibition effect on SecA, and decreasing side effects.

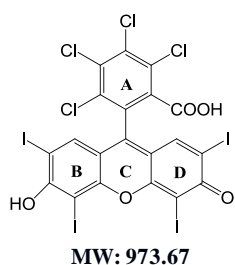


Figure 2.1 RB structure

RB is a large molecule with molecular weight of 973.67. RB consists of a polyiodinated xanthen moiety and a polychlorinated benzoate moiety. There are some commercial fluorescein derivatives, which structure is similar to RB (Fig. 2.20). However, the *in vitro* inhibition effects of these fluorescein derivatives were much worse than RB (Fig. 2.21). We

don't know whether chloro group, or carboxyl group, or the number and position of iodo group are essential for the inhibition effect of RB. To develop new SecA inhibitors, we began with SAR studies. In collaboration with Dr. Binghe Wang's group, RB was systematically dissected to understand the importance of structural feature by investigating the *in vitro* & *in vivo* inhibition effects of new analogues. In this study, we determined the importance of iodo group, hydroxyl group, carboxyl group, and the necessity of ring A/B/D.

4-Phenyl-chromene Might not Be the Basic Effective Structure of RB To determine the importance of D Ring and chloro groups, first batch RB analogues were synthesized with 4-phenyl-chromene or 4-phenyl naphthalene as basic structure (Fig. 2.22). In these compounds, iodo groups were kept, or removed, or replaced with bromo groups. The *in vitro* inhibition assay result showed that 20 μ M RB could inhibit nearly 60% ATPase activity of BaSecA2 and 75% ATPase activity of BsSecA and, but 100 μ M or 200 μ M of these RB analogues couldn't or only slight inhibit activity of BsSecA or BaSecA2 (Fig. 2.23a-b), suggesting that D ring or chloro group might be required for the inhibition on the ATPase activity of SecA proteins. SCA-23, SCA-24, and SCA-25 contain bromo or iodo group, and they didn't show *in vitro* inhibition effect at 200 μ M (Fig. 2.23a-b), however, they had some *in vivo* inhibition effect on the growth of *B. subtilis* 168 and *E. coli* NR698, suggesting that halo group was important for the *in vivo* inhibition (Fig. 2.24a-b, Table 2.2).

Xanthene with a Carbon Ring Might be the Basic Effective Structure of RB To determine the importance of A ring, chloro group, and carboxyl group, second batch of RB analogues were synthesized with xanthenes as basic structure (Fig. 2.25). In these compounds, A-ring was replaced by ketone group or 5-membered aliphatic ring; 4 iodo groups were partially or completely removed, or replaced by bromo group. 200 μ M of these compounds were used to do the *in vitro* inhibition assay. The result showed that all five effective compounds are consisted of xanthene and a 5-membered ring (Fig. 2.26). Thus xanthene with a carbon ring might be the basic effective structure of RB. Among these compounds, SCA-41 had the best *in vitro* inhibition effects and it has simplest structure. Masking the hydroxyl group with methyl group dramatically decreased inhibition activities of SCA-41, suggesting hydroxyl group might be important for *in vitro* inhibition activity (Fig. 2.26). Adding four bromo groups to SCA-41 decrease inhibition

activities (Fig. 2.26), while adding two iodo groups decreased inhibition on BsSecA (Fig. 2.26), but didn't change inhibition activity on BaSecA2, suggesting the number of iodo group might affect *in vitro* inhibition activity (Fig. 2.26).

A-ring and chloro group, carboxyl group, and iodo group were all removed in SCA-36. Our results showed that removing these groups dramatically decreased *in vivo* inhibition effects (Fig. 2.27a-b, Table 2.3). Adding bromo group or iodo group regained some *in vivo* inhibition effect (Fig. 2.27a-b, Table 2.3). Replacing chlorinated benzoate group with a 5-membered ring remained good *in vitro* inhibition, and showed better *in vivo* inhibition effects than RB (Table 2.3). SCA-41 has very simple structure it does not have chloro group, carboxyl group, and iodo group. This result suggests that carboxyl group and chloro group is not necessary for inhibition effect, and the aromatic A-ring could be replaced by an aliphatic 5-membered ring. Masking hydroxyl group with methyl group dramatically decreased inhibition effect, suggesting hydroxyl group is important for the *in vivo* inhibition effect (Fig. 2.27a-b, Table 2.3). These results indicated that xanthene with a carbon ring and hydroxyl group might be essential for inhibitory activity of RB.

The Number of Iodine Groups Affected the Inhibition Effect RB has 4 iodine groups, while SCA-41 doesn't have any iodo group. To investigate whether iodo group could affect inhibition effect, compounds with 2 iodo groups, 3 iodo groups, or 4 iodo groups were synthesized (Table 2.4). Our results showed that iodo group slightly increased *in vivo* inhibition effect, and *in vitro* inhibition against BaSecA2, but inhibition effect on BsSecA and EcSecA was dramatically reduced (Table 2.4). These results suggested that iodo group is not necessary for inhibition. And 100 μM of these three compounds couldn't inhibit the growth of *E. coli* 4100 (Table 2.4), indicating that the outer membrane of Gram-negative bacteria provide a permeability barrier that re-

duces the absorption of the dye by the cells. Replacing 4-iodo groups with 4 bromo groups increased IC_{50} (Table 2.4), suggesting bromo group reduces inhibition effect.

The inhibition effects of these analogues were further investigated on the ATPase activity of *E. coli* F1F0 (EcF1F0). RB and some analogues could inhibit the ATPase activity of EcF1F0 (Table 2.4), suggesting they might be general ATPase inhibitors.

To evaluate the importance of A-ring, it was replaced by cyclopentane, cyclohexane, i-propane, and N-hexyl (Table 2.5). The assay results show that hexyl group has similar effect as cyclopentane; cyclohexane reduces some inhibition effect; i-propane group dramatically reduces inhibition effect, indicating that attaching 5 to 6 carbons to the top of xanthene moiety was necessary for inhibition effect (Table 2.5). To increase solubility, a carboxyl group was added to the 5 carbon ring of SCA-41 (Fig. 2.28a), but our results showed that the carboxy group dramatically reduced inhibition effect both *in vitro* and *in vivo* (Fig. 2.28b-c).

Bactericidal Effect of RB analogs In this study, *B. subtilis* 168 was used as a model to study the bactericidal effect of RB analogues, comparing the results of RB. SCA-41 and SCA-50 showed very good bactericidal effect on *B. subtilis* 168 (Fig. 2.29a). 20 μ M of these two compounds could kill 4 log numbers of *B. subtilis* 168, while 40 μ M of RB only kill 1 log number. And increasing the killing assay time slightly increased the killing effect (Fig. 2.29b). SCA-41 was not soluble in water, and increasing temperature might be able to increase solubility. *B. subtilis* 168 can grow at high temperature, thus, we further investigated the bactericidal effect of SCA-41 at 42°C. However, our results showed that, 10 μ M SCA-41 kill two more log number of bacteria at 42°C than at 37°C, while 20 μ M of SCA-41 showed similar results at both temperatures (Fig. 2.29c). To investigate whether the inhibition effect of RB is growth phase dependent, the growth of *B. subtilis* 168 was monitored (Fig. 2.30a), and cell culture was taken out at different

phase and subjected to killing assay. The result showed that RB has bactericidal effect on growing and no-growing *B. subtilis* 168 (Fig. 2.30b). For non growing cell, higher concentration of SCA-41 was needed because of the increasing of cell number.

Bacteriostatic effect of RB analogs against MRSA Some RB analogs were subjected to *in vivo* inhibition assay on the growth of *S. aureus* Mu50, which is a Methicillin-resistant *S. aureus* strain. Our results showed that SCA-44, SCA-45, SCA-47 and SCA-48 had very good inhibition effects with $IC_{50} < 10 \mu M$ (Table 2.6a). But if comparing the inhibition effect of these compounds with SCA-41, with or without iodo groups, their inhibition effects had no obvious difference, suggesting that iodo group might have inhibition effect, but iodo is not critical for inhibition effect of RB (Table 2.6a). The inhibition effect of SCA-46 was around $75 \mu M$, much worse than SCA-41, suggesting that replacing top five carbon ring with i-propane will reduce inhibition effect (Table 2.6a). We further investigated inhibition effect on two other MRSA strains, *S. aureus* Mu3 and *S. aureus* N315. Our results showed that bacteriostatic effects of SCA-41 on these MRSA strains were similar to their effects on *S. aureus* 6538, and much better than effects of RB (Table 2.6b). Both Mu50 and N315 are resistant to bleomycin, β -lactam, tetracyclines, several aminoglycosides, and MLS. Thus, RB analogs showed promising effect on control these MRSA.

Bactericidal Effect of SCA-50 SCA-50 showed good bactericidal effect against *S. aureus* and *B. anthracis* Sterne. $40 \mu M$ of SCA-50 could kill a clinical strain, *S. aureus* 6538, as well as a MRSA strain-Mu50 (Fig. 2.31). $20 \mu M$ SCA-50 could kill 3 log numbers *B. anthracis* Sterne (Fig. 2.31).

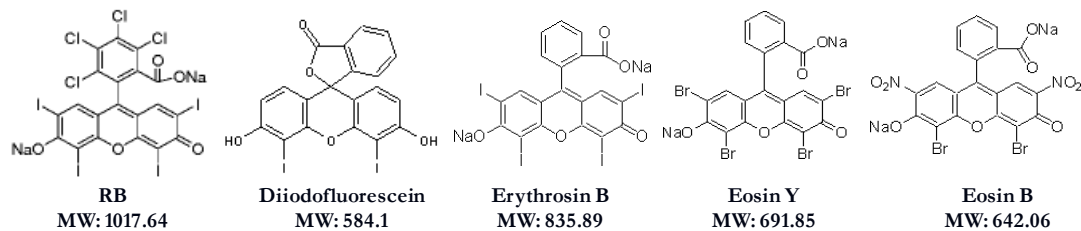


Figure 2.20 Structure of some commercial RB analogs

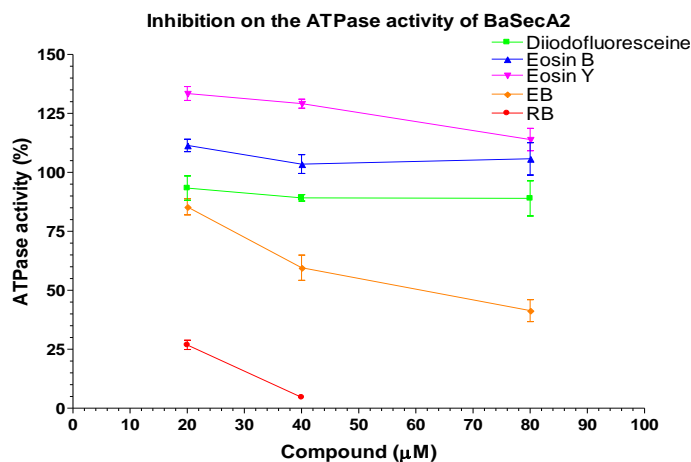


Figure 2.21 *In vitro* inhibition effects of some commercial RB analogs against BaSecA2

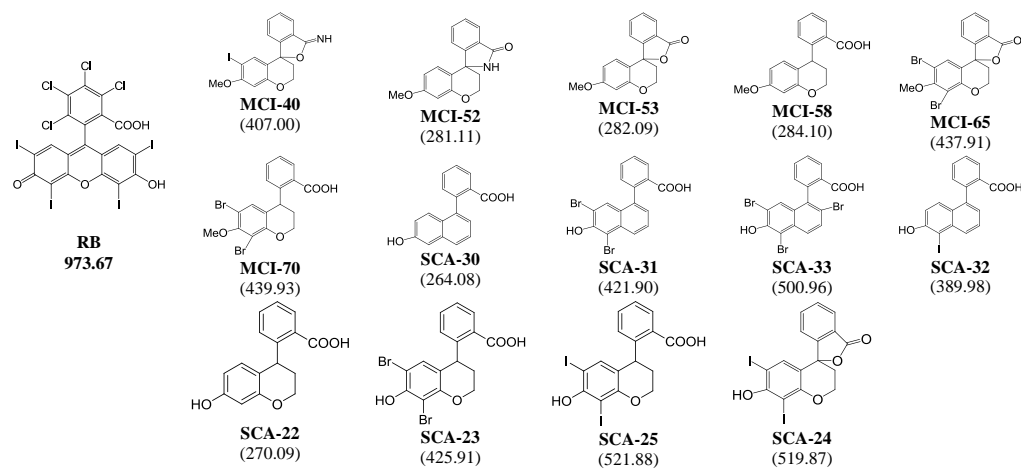


Figure 2.22 Structure of first batch RB analogs

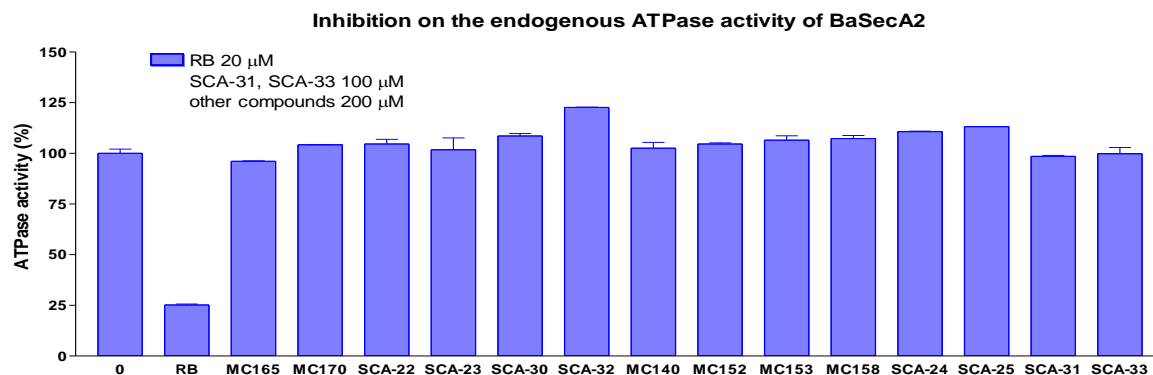


Figure 2.23a *In vitro* inhibition effects of first batch RB analogs against BaSecA2
 30°C, 40 min; 5% DMSO.

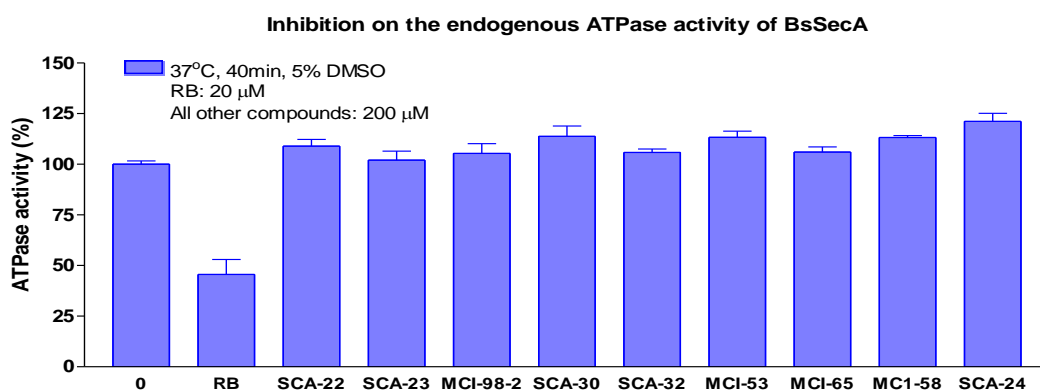


Figure 2.23b *In vitro* inhibition effects of first batch RB analogs against BsSecA
 37°C, 40 min; 5% DMSO.

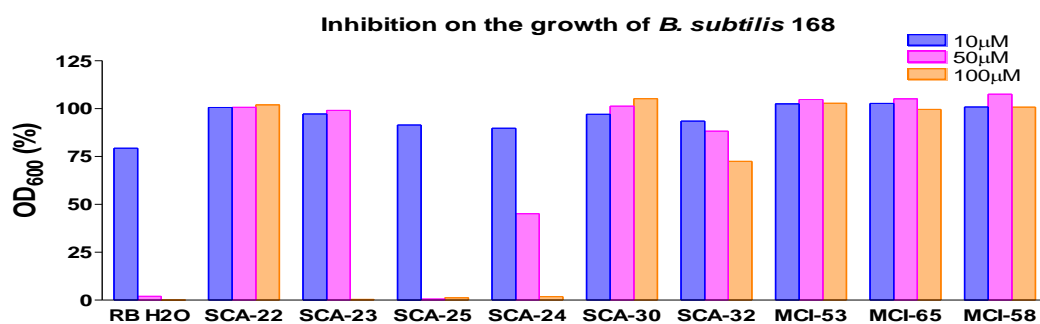


Figure 2.24a Bacteriostatic effects of first batch RB analogs against *B. subtilis* 168
 Incubation at 37°C, 1,000 rpm for 14 hrs; 2.5% DMSO.

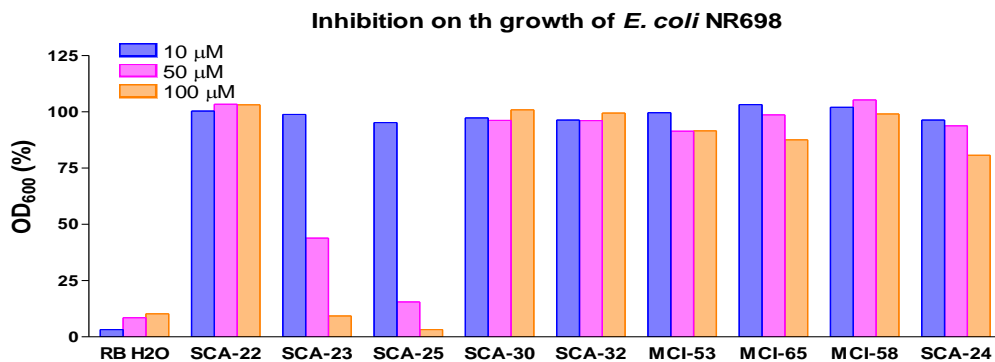
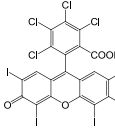
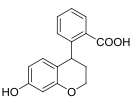
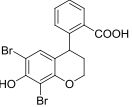
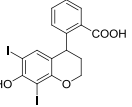
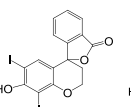
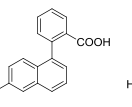
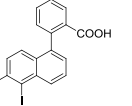


Figure 2.24b Bacteriostatic effects of first batch RB analogs against *E. coli* NR698
Incubation at 37°C, 1,000 rpm for 14 hrs; 2.5% DMSO.

Table 2.2 The importance of D-ring and chloro group for inhibition effects of RB analogs

								
		RB 973.67	SCA-22 (270.09)	SCA-23 (425.91)	SCA-25 (521.88)	SCA-24 (519.87)	SCA-30 (264.08)	SCA-32 (389.98)
In vitro inhibition IC ₅₀ (μM)	EcSecAN68	1	>100	100 (85%↓)	TBD	TBD	100 (75%↓)	100 (55%↓)
	BaSecA2	15	>200	>200	>200	>200	>200	>200
	BsSecA	20	>200	>200	>200	>200	>200	>200
In vivo inhibition IC ₅₀ (μM)	<i>B. subtilis</i> 168	34/78	>100	75	29	45	>100	>100
	<i>E. coli</i> NR698	10/18	>100	45	33	>100	>100	>100

For *in vivo* inhibition, incubation at 37°C, 1,000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, 40 min reaction with 5% DMSO; for EcSecAN68 and BsSecA, reaction at 37°C; for BaSecA2, reaction at 30°C.

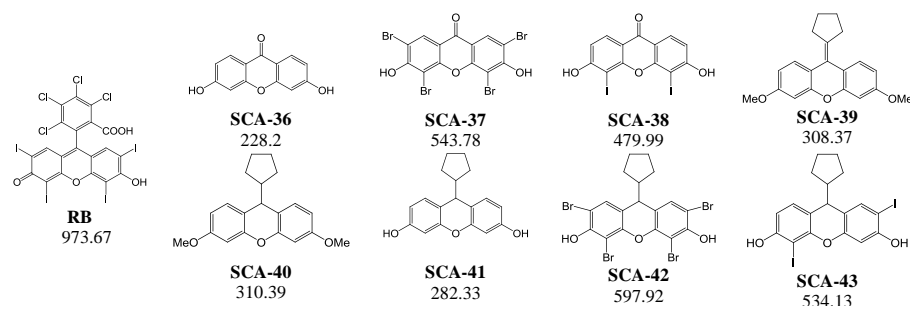


Figure 2.25 The structure of second batch of RB analogues

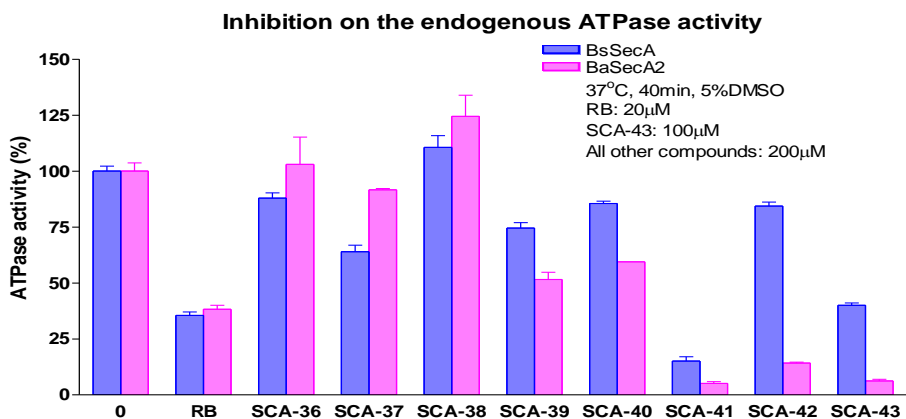


Figure 2.26 *In vitro* inhibition effects of second batch RB analogs

For BsSecA, reaction at 37°C 40 min with 5% DMSO; for BaSecA2, reaction at 30°C 40 min with 5% DMSO.

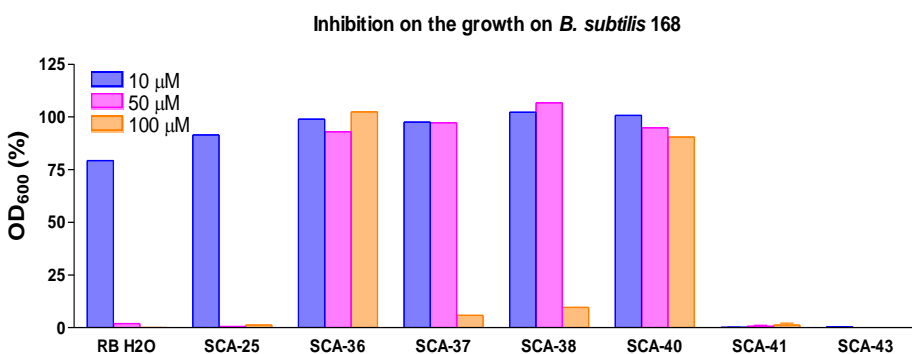


Figure 2.27a Bacteriostatic effects of second batch RB analogs against *B. subtilis* 168

Incubation at 37°C, 1,000 rpm for 14 hrs; 2.5% DMSO.

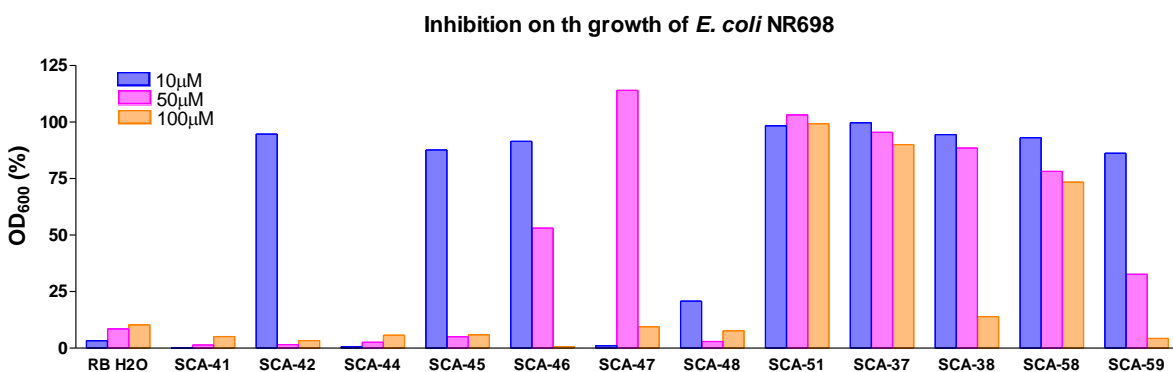


Figure 2.27b Bacteriostatic effects of second batch RB analogs against *E. coli* NR698

Incubation at 37°C, 1,000 rpm for 14 hrs; 2.5% DMSO.

Table 2.3 Importance of xanthene for inhibition effects of RB analogs

		 RB (973.67)	 SCA-36 (228.2)	 SCA-37 (543.78)	 SCA-38 (479.99)	 SCA-40 (310.39)	 SCA-41 (282.33)	 SCA-43 (534.13)
In vitro inhibition IC₅₀ (μM)	EcSecAN68	1	TBD	30	>100	TBD	8	3
	BaSecA2	15	>200	>200	>200	>200	30	13
	BsSecA	20	>200	>200	>200	>200	30	100
In vivo inhibition IC₅₀ (μM)	<i>B. subtilis</i> 168	34/74	>100	75	79	>100	7	4
	<i>E. coli</i> NR698	10/18	>100	>100	75	TBD	9	9

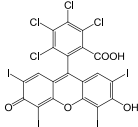
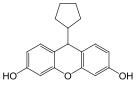
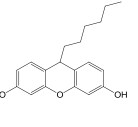
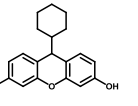
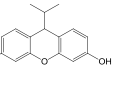
For *in vivo* inhibition, incubation at 37°C, 1,000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, 40 min reaction with 5% DMSO; for EcSecAN68 and BsSecA, reaction at 37°C; for BaSecA2, reaction at 30°C.

Table 2.4 The importance of iodo group for inhibition effect of RB analogs

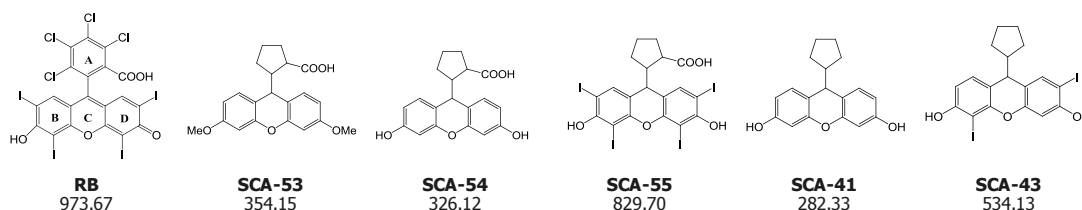
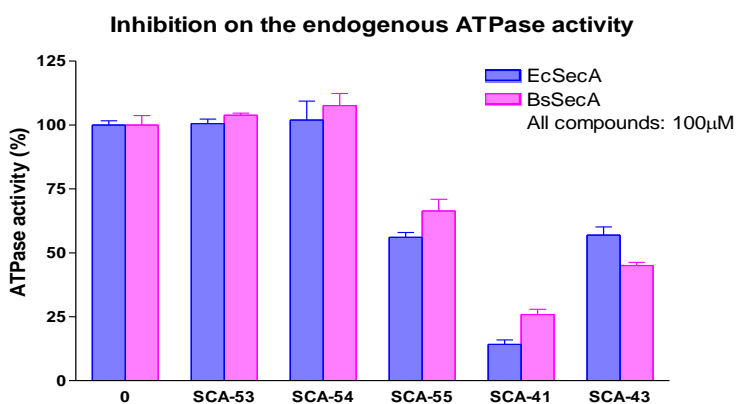
		 RB 973.67	 SCA-41 282.33	 SCA-43 534.13	 SCA-45 660.02	 SCA-44 785.92	 SCA-42 597.92
In vitro inhibition IC₅₀ (μM)	EcSecAN68	1.3	8	3	1.3	1.7	4.5
	BaSecA2	15	30	13	17	18	45
	BsSecA	20	30	100	>100	>100	>100
	EcSecA	60	43	200	>100	>100	TBD
	EcF1F0	14	60	17	<100	>100	100 (70%↓)
In vivo inhibition IC₅₀ (μM)	<i>B. subtilis</i> 168	34/74	7	4	5	5	9
	<i>E. coli</i> NR698	10/18	9	9	18	3	19
	<i>B. anthracis</i> Sterne	7	4	1	2	2	4
	<i>S. aureus</i> 6538	28	7	4	5	18	10
	<i>E. coli</i> MC4100	>100	>100	>100	TBD	TBD	TBD

For *in vivo* inhibition, incubation at 37°C, 1,000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, 40 min reaction with 5% DMSO; for EcSecAN68, BsSecA, EcSecA, and EcF1F0, reaction at 37°C; for BaSecA2, reaction at 30°C.

Table 2.5 Replacing aromatic A-ring of RB with aliphatic ring or hexyl group

						
		RB 973.67	SCA-41 282.33	SCA-50 298.16	SCA-57 296.16	SCA-46 256.3
In vitro inhibition	EcSecAN68	1	8	4	20	60
	BaSecA2	15	30	20	TBD	TBD
	IC₅₀ (μM)	20	30	33	62	>200
	EcSecA	60	43	>100	100	TBD
	EcSecA Tn	1	15	60	TBD	TBD
In vivo inhibition	<i>B. subtilis</i> 168	34/74	7	7	7	70
	MIC₅₀ (μM)	<i>E. coli</i> NR698	10/18	9	14	13
		<i>S. aureus</i> 6538	28	7	8	12
		<i>B. anthracis</i> Sterne	7	4	4	TBD

For *in vivo* inhibition, incubation at 37°C, 1,000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, 40 min reaction with 5% DMSO; for EcSecAN68, BsSecA, EcSecA, and EcF1F0, reaction at 37°C; for BaSecA2, reaction at 30°C; TBD: not determined.

**Figure 2.28a Structure of RB analogs containing carboxyl group****Figure 2.28b Carboxyl group decrease *in vitro* inhibition effect of SCA-41**

For BsSecA, reaction at 37°C 40 min with 5% DMSO; for BaSecA2, reaction at 30°C 40 min with 5% DMSO.

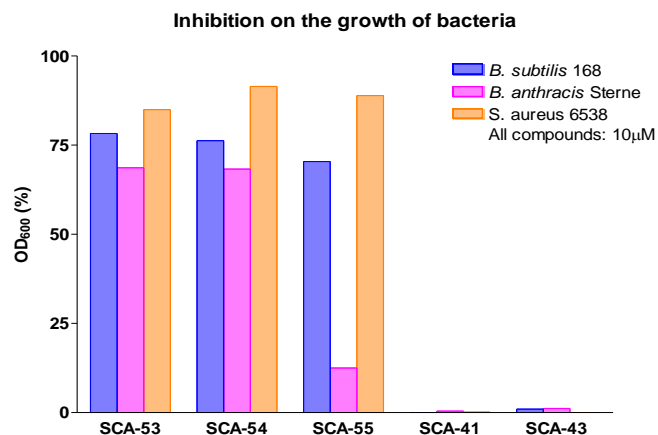


Figure 2.28c Carboxyl group decrease *in vivo* inhibition effect of SCA-41
 Incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO.

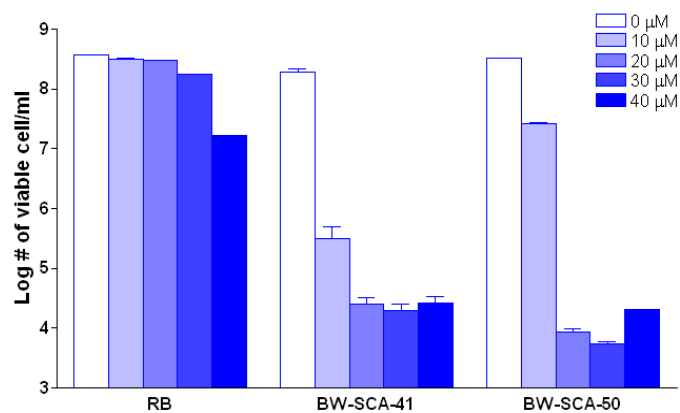


Figure 2.29a Bactericidal effects of SCA-41 and SCA-50 against *B. subtilis* 168
 37°C, 1 hour killing with 2.5% DMSO.

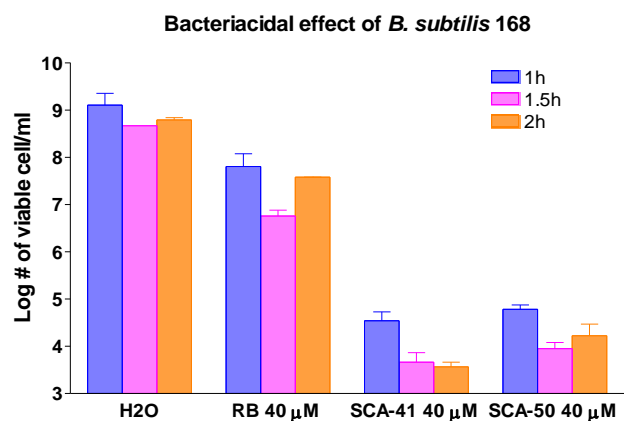


Figure 2.29b Time effect on killing assay results of SCA-41 and SCA-50
 Killing at 37°C for 1 hour, or 1.5 hrs, or 2 hrs, with 2.5% DMSO.

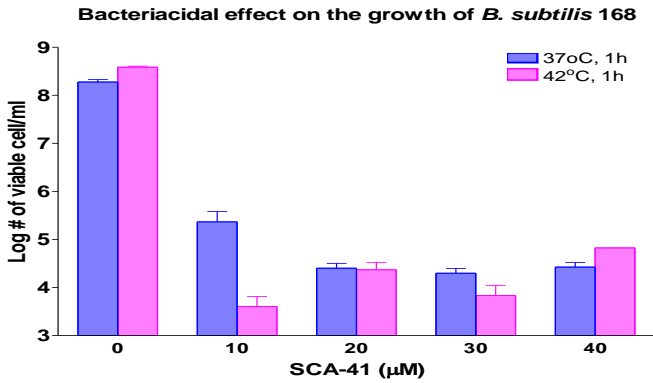


Figure 2.29c Temperature effect on killing assay result of SCA-41
Killing at 37°C or 42°C for 1 hour with 2.5% DMSO.

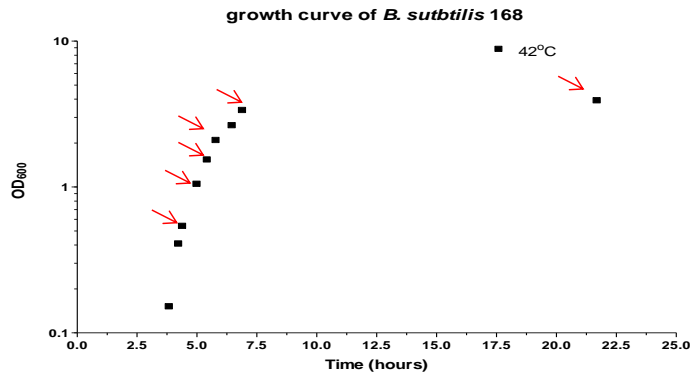


Figure 2.30a Bactericidal effect of SCA-41 is independent of growing phase
Grow at 42°C; cell culture was taken out at labeled time point and subjected to killing assay.

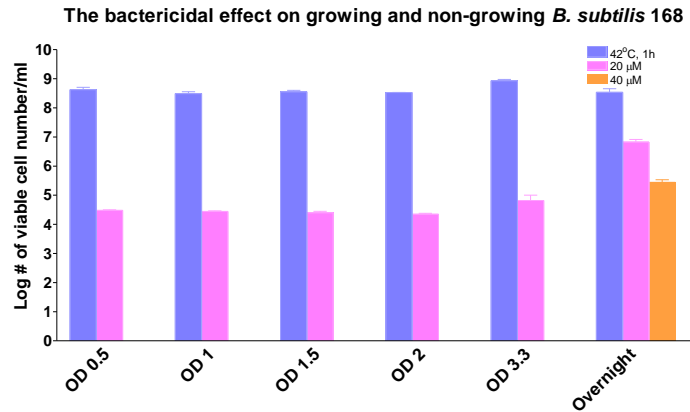
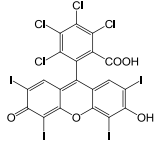
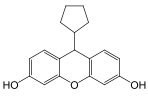
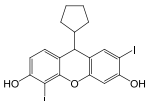
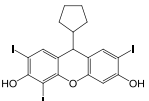
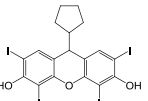
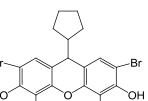
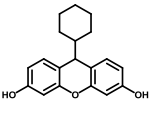
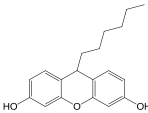
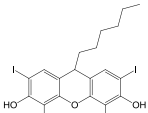
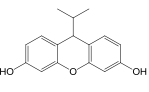
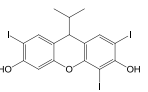
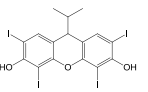


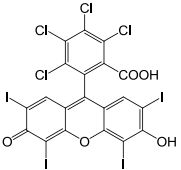
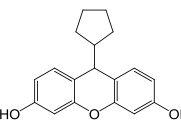
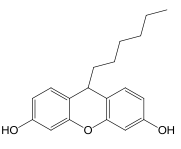
Figure 2.30b Bactericidal effect of SCA-41 is independent of growing phase
Killing at 42°C for 1 hour with 2.5% DMSO.

Table 2.6a Bacteriostatic effects of RB analogs against MRSA

Bacteriostatic effect, IC ₅₀ (μM)						
						
	RB 973.67	MCH-89 282.33	MCH-93 534.13	MCH-99-2 660.02	MCH-99-1 785.92	MCH-92 597.92
<i>S. aureus</i> 6538	28	7	4	5	18	10
<i>S. aureus</i> Mu50	26.5	5.5	2.7	4	7	10
						
	MCH-110 296.16	MCH-10 298.16	MCH-6 801.74	MCH-122 256.3	MCH-135-2 633.99	MCH-135-1 759.88
<i>S. aureus</i> 6538	12	7	/	/	7.4	6.9
<i>S. aureus</i> Mu50	12	7	15	70	5.5	9

Incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO.

Table 2.7b Bacteriostatic effects of RB analogs on MRSA

			
	RB 973.67	SCA-41 282.33	SCA-50 298.16
<i>S. aureus</i> 6538	28	7	7
<i>S. aureus</i> Mu50	26.5	5.5	7
<i>S. aureus</i> Mu3	>50	5	12.5
<i>S. aureus</i> N315	45	5	7.5

Incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO.

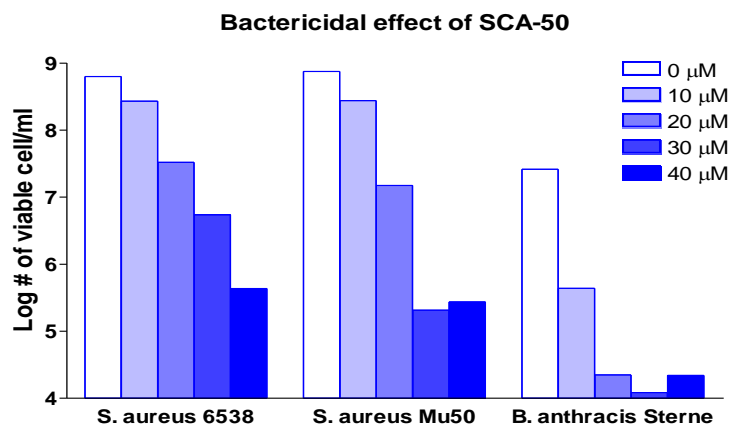
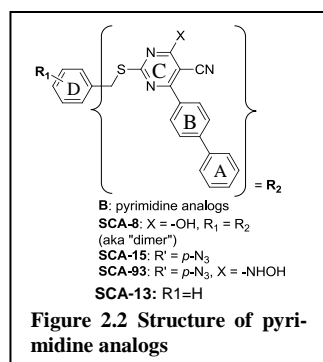


Figure 2.31 Bactericidal effects of SCA-50 against *S. aureus* and *B. anthracis* Killing at 37°C for 1 hour with 2.5% DMSO.

Development of Novel SecA Inhibitors by Optimizing Pyrimidine Analogs

The emergence and dissemination of multidrug resistance has become a clinical issue, thus development of antibacterial agents with new mechanisms is an urgent need. SecA ATPase, an essential component of the Sec translocation system, is required for viability and virulence of bacteria, and is highly conserved among bacteria. Furthermore there is no human counterpart of



this translocase. These properties make it an attractive target for exploring novel antimicrobial agents. Pyrimidine analogues were derived from virtual screening based on the ATP binding pocket of *E. coli* SecA. SCA-8 showed strong inhibitory effect on the endogenous ATPase activity of EcSecAN68, however it showed low bacteriostat-

ic effects (Table 2.9). SCA-8 is an aka dimer (Fig. 2.2), and it might be too big to get through bacteria cell membrane. SCA-13 contains half structure of SCA-8; although inhibition effect on the ATPase activity of SecA was 10 times reduced, bacteriostatic effect was increased more than 5 to 50 times (Table 2.7). In this study, many new pyrimidine analogues were synthesized with modification on R1 position and X-position, and the importance of A-ring and D-ring were determined.

The importance of D-ring To investigate the importance of D-ring, it was replaced to methyl group or carboxyl group. However, these modifications dramatically decreased both *in vitro* and *in vivo* inhibition effects, suggesting ring D was necessary for inhibition effect of pyrimidine analogues (Table 2.7).

The importance of A-ring To investigate the importance of A-ring, it was deleted, or replaced to -OCH₃, CH₃, or was attached with a benzene ring. All these modification dramatically reduced inhibition effect, suggesting that A-ring was required for inhibition effect (Table 2.8).

The effect of modifications on R1-position Modeling result suggested that the R1-position can tolerate further modifications for optimization, improved physicochemical properties. Thus, R1-position was modified with azido group, $-NH_2$, or triazole group. Majority of these modifications could improve inhibition effect on the ATPase activity of EcSecAN68, however only SCA-15 with azido group at R1 position showed good bacteriostatic effects (Table 2.9a-b). Other modifications might be hard to get through cell membrane. Replacing azido group with methyl formate group and ethyl formate group increased inhibition effect on the ATPase activity of EcSecAN68, however these modifications decrease bacteriostatic effects (Table 2.9b). Replacing azido group with carboxylic acid group, boronic group, or pyridine group dramatically reduced both *in vitro* and *in vivo* inhibition effects (Table 2.9b).

The importance of para-position of A-ring and azido group To investigate whether different position of A-ring and azido group could affect inhibition effect, SCA-65 and SCA-90 were synthesized, which contain A-ring or azido group at meta-position. Our result showed that changing A-ring or azido group from para-position to meta-position could abolish inhibition effect (Table 2.10), suggesting that keeping A-ring or azido group at para-position were critical for inhibition effect.

The effect of different modifications at X-position Modeling results suggested the X-position can also be modified with the introduction of polar and ionizable function groups. Thus, the $=O$ group at X-position was replaced to $-NHOH$, $-Cl$, $-NHCH_3$, or $-NH-C_{10}O_3-NH-Boc$. Our results showed that $NHOH$ slightly increased inhibition effect, and other modification reduced inhibition effect (Table 2.11a-b).

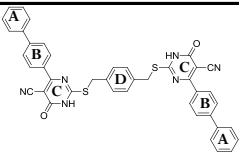
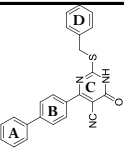
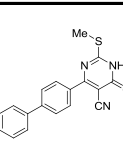
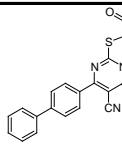
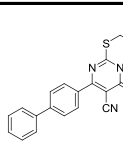
The effect of modifications at A-ring To increase the solubility, two $-OCH_3$ group and one $COOCH_3$ group were added to ring A of SCA-15 and SCA-61. Our result showed that these

modifications could reduce *in vivo* inhibition effect (Table 2.12). But since SCA-82 retained reasonable inhibition activities, it could be further modified for affinity labeling.

Among tested pyrimidine analogs, SCA-15 and SCA-93 showed best bacteriostatic effects. In Table 2.13 & Table 2.14, we summarized of *in vitro* and *in vivo* inhibition of these two compounds comparing with results of SCA-8 and SCA-13. SCA-8 showed best *in vitro* inhibition effects, however SCA-15 and SCA-93 showed much better bacteriostatic effects. Thus, these two compounds were used to further investigate bactericidal effects.

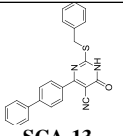
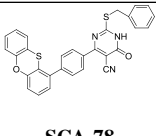
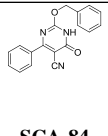
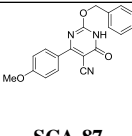
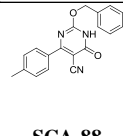
Bactericidal effect of SCA-15 SCA-15 showed good bactericidal effect on *B. anthracis* Sterne, *S. aureus* 6538, and *S. aureus* Mu50. 10 μ M SCA-15 could kill two log numbers of *B. anthracis* Sterne (Fig. 2.32), while 25 μ M could kill three log numbers of the other strains (Figure 2.32).

Table 2.7 Importance of D-ring for the inhibition effects of pyrimidine analogs

						
		SCA-8 712.84	SCA-13 395.48	SCA-89 319.3	SCA-66 363.07	SCA-77
<i>In vitro</i> inhibition, IC ₅₀ (μM)	EcSecA N68	2	20	>200	>100	30
<i>In vivo</i> inhibition, IC ₅₀ (μM)	<i>B. anthracis</i> sterne	>250	5	>500	>250	75
	<i>S. aureus</i> 6538	>250	52	>500	>250	>100
	<i>E. coli</i> NR698	>250	55/20	>500	>250	>100
	<i>B. subtilis</i> 168	>250	7	>500	>250	>100







For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.8 Importance of A-ring for the inhibition effects of pyrimidine analogs

						
		SCA-13	SCA-78 DK-II-16	SCA-84 KW-I-15	SCA-87 DK-II-35	SCA-88 DK-II-36
N68	19	TBD	>100	>100	>100	>100
<i>B. anthracis</i> sterne	5	>250	>250	>250	>250	>250
<i>S. aureus</i> 6538	52	>250	>250	>250	>250	>250
<i>E. coli</i> NR698	55	>250	>250	>250	>250	>250

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.9a Modification on R1-position of pyrimidine analogs

		SCA-13 395.48	SCA-15 436.49	SCA-17	SCA-18	SCA-19	SCA-61 534.59	SCA-62 534.59	SCA-63 520.5	SCA64 492.5
Structure	R1 position	-H	-N3	-NH2						
<i>In vitro</i> inhibition, IC ₅₀ (μM)	EcSecA N68	19	10	11	2.5	3	6	8	ND	ND
<i>In vivo</i> inhibition, IC ₅₀ (μM)	<i>B. anthracis</i> sterne	5	4	>100	19	>100	19	20μM<10%↓	>100	>100
	<i>S. aureus</i> 6538	52	15	>100	100	>100	>100	>100	>100	>100
	<i>E. coli</i> NR698	55	35	>100	>100	>100	>100	>100	>100	>100
	<i>B. subtilis</i> 168	15	7	>100	>100	>100	>100	>100	>100	>100

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.9b Modification on R1-position of pyrimidine analogs

Structure	R1 position	SCA-13	SCA-15	SCA-71	SCA-72	SCA-75	SCA-76
		395.48	436.49	453.5	467.5	437.51	437.32
		-H	-N3	-COOCH3	-COOCH ₂ CH ₃	-COOH	B(OH ₂)
<i>In vitro</i> inhibition, IC ₅₀ (μM)	EcSecA N68	19	10	6	8.5	30	60
	<i>B. anthracis</i> Sterne	5	4	8	15	>100	>100
	<i>S. aureus</i> 6538	52	15	15	90	>100	>100
<i>In vivo</i> inhibition, IC ₅₀ (μM)	<i>E. coli</i> NR698	55	35	200	>250	>100	>100
	<i>B. subtilis</i> 168	15	7	18	180	>100	>100

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.10 Importance of para-position of A Ring and N₃ group for inhibition effects of pyrimidine analogs

		Para	Meta	Para	Mera
		SCA-13	SCA-65	SCA-15	SCA-90
<i>In vitro</i> inhibition, IC ₅₀ (μM)	EcSecA N68	19	70	10	>200
	<i>B. anthracis</i> Sterne	5	>100	4	>500
<i>In vivo</i> inhibition, IC ₅₀ (μM)	<i>S. aureus</i> 6538	52	>100	15	>500
	<i>E. coli</i> NR698	55	>100	35	>500
	<i>B. subtilis</i> 168	15	>100	7	>500

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.11a Modifications at X-position of pyrimidine analogs

Compound		SCA-15	SCA-93	SCA-95	SCA-98	SCA-102	SCA-103	SCA-109	SCA-108
		436.49	451.1	454.9	449.1	481.5	452.5	450.5	450.5
Structure	R1	N3	N3	N3	N3	N3	N3	N3	N3
	X	=O	-NHOH	-Cl	-NHCH ₃	-NH(CH ₂) ₂ OH	-NHNH ₂	-OCH ₃	=O
	Y	H	H	H	H	H	H	H	-CH ₃
<i>In vitro</i> inhibition, IC ₅₀ (μM)	EcSecA N68	10	6	8	3.5	>100	>100	ND	ND
	<i>B. anthracis</i> Sterne	4	2.5	65	>200	>100	>100	>100	>100
<i>In vivo</i> inhibition, IC ₅₀ (μM)	<i>S. aureus</i> 6538	15	9	>200	>200	>100	>100	>100	>100
	<i>E. coli</i> NR698	35	45	>200	>200	>100	>100	>100	>100
	<i>B. subtilis</i> 168	7	5	150	>200	>100	>100	>100	>100

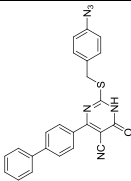
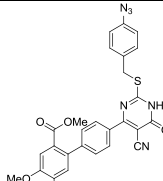
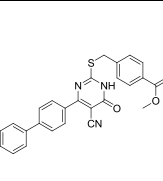
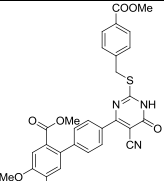
For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.11b Modification at X-position of pyrimidine analogs

Compound		SCA-13 395.48	SCA-94 438.5	SCA-96 409.5	SCA-97 413.0
R1		H	H	H	H
X		=O	-NH(CH ₂) ₂ OH	-OCH ₃	-Cl
<i>In vitro</i> inhibition, IC ₅₀ (μM)	EcSecA N68	19	55	ND	ND
<i>In vivo</i> inhibition, IC ₅₀ (μM)	<i>B. anthracis</i> Sterne	5	>200	>200	>200
	<i>S. aureus</i> 6538	52	>200	>200	>200
	<i>E. coli</i> NR698	55	>200	>200	>200
	<i>B. subtilis</i> 168	15	>200	>200	>200

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.12 Modifications on A-ring of pyrimidine analogs

				
	SCA-15	SCA-82	SCA-71	SCA-81
EcSecA N68	10	20	6	/
BaSecA2	20	14	/	/
<i>B. anthracis</i> Sterne	4	5	8	20
<i>S. aureus</i> 6538	15	55	15	>100
<i>E. coli</i> NR698	35	>100	200	>100
<i>B. subtilis</i> 168	7	50	18	>100

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO.

Table 2.13 *In vitro* inhibition of pyrimidine analogs against SecA homologues

Proteins compound	EcSecAN68	EcSecA	EcSecA Tn	BsSecA	BaSecA2	SaSecA2	Ec-F ₁ F ₀ -H ⁺ - ATPase
SCA-8	2	ND	6	8	7	3	>200
SCA-13	18	ND	TBD	100	>200	TBD	>200
SCA-15	8	>100	30	>100	20	13	>100
SCA-93	4.5	30	25	>100	TBD	TBD	TBD

In vitro inhibition: IC₅₀ (μM); 40 min reaction with 5% DMSO; for EcSecAN68, BsSecA, EcSecA, and EcF₁F₀, reaction at 37°C; for BaSecA2, reaction at 30°C; EcSecA Tn: translocation ATPase activity of EcSecA; TBD: not determined; IC₅₀ of SCA-8: 50 μM, IC₅₀ of SCA-13: 60 μM.

Table 2.14 Bacteriostatic effects of pyrimidine analogs

compounds	SCA-8	SCA-13		SCA-15		SCA-93	
Strains	MIC ₅₀ (μM)	MIC ₅₀ (μM)	MIC ₉₅ (μM)	MIC ₅₀ (μM)	MIC ₉₅ (μM)	MIC ₅₀ (μM)	MIC ₉₅ (μM)
<i>B. anthracis</i> Sterne	>250	5	6	4	5	3	4
<i>B. subtilis</i> 168	>250	15/7	/10	7	10	4	6
<i>E. coli</i> NR698	>250	55	>70	35	50*	70	200*
<i>S. aureus</i> 6538	>250	52	>70	12	15	9	10
<i>S. aureus</i> Mu50	>250	65	>100	22	38	9	10
<i>S. aureus</i> N315	>250	60	>100	9	25	9	18
<i>S. aureus</i> Mu3	230	>100	>100	35	100	50	>100

MIC: 95% inhibition, *90% inhibition; incubation at 37°C, 1,000 rpm for 14 hrs with 2.5% DMSO.

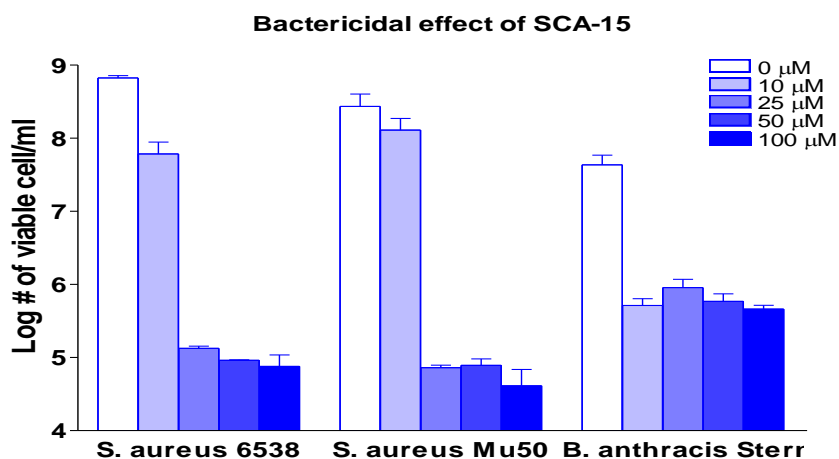


Figure 2.32 Bactericidal effects of SCA-15 against *S. aureus* and *B. anthracis*
37°C, 1 hour killing.

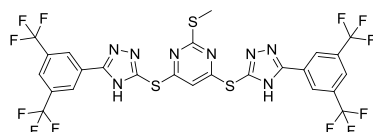
Development of Novel SecA Inhibitors by Optimizing Bistriazole Analogs

***In vitro* Inhibition of SCA-21** SCA-21 (Fig. 2.33) is a bistriazole analog derived from random screening. It could inhibit the ATPase activity of SecA homologues (Table 2.15). The *in vitro* inhibition of SCA-21 was also affected by temperature. Increasing temperature from 40°C to 42°C, increased inhibition effect (Table 2.16); and liposomes could reduce inhibition effect at lower temperature (Table 2.16).

***In vivo* Inhibition of SCA-21** SCA-21 showed good bacteriostatic effect on Gram-positive bacteria (Table 2.17). Although SCA-21 could inhibit the growth of *E. coli* NR 698, and IC₅₀ is around 10 µM, SCA-21 didn't inhibit the growth of *E. coli* RAM1398 at 100 µM (2.34), suggesting the compound had some permeability problem in Gram-negative bacteria.

Optimization of SCA-21 SCA-21 is a dimer (Fig. 2.33). Replacing half part of SCA-21 with Chloro group slightly increased *in vitro* inhibition effect, and significantly increased *in vivo* inhibition (Table 2.17). Removing the chloro group or methylthio group decreased inhibition effects (Table 2.17). The methyl group could be replaced with benzene ring (Table 2.17).

Bactericidal effect of SCA-21 SCA-21 showed good bactericidal effect. 12.5 µM SCA-21 could kill three log numbers of *B. anthracis* Sterne, and 50 µM could kill three log numbers of *S. aureus* 6538 and *S. aureus* Mu50 (Fig. 2.35).



Molecular Weight: 748.59

Figure 2.33 Structure of SCA-21**Table 2.15 *In vitro* inhibition of SCA-21 on the endogenous ATPase activity of SecA homologues**

	EcSecA N68	EcSecA	EcSecA Tn	BsSecA	BaSecA2	SaSecA2
SCA-21, IC ₅₀ (μM)	18	32	20	>100	45	43

All reaction with 5% DMSO; for EcSecAN68, BsSecA, EcSecA, and EcF1F0, reaction at 37°C for 40 min; for BaSecA2, reaction at 30°C for 40 min; for SaSecA2, reaction at 25°C for 20 min .

Table 2.16 Temperature and liposomes affect on *in vitro* inhibition effects of SCA-21

	Liposomes -		Liposomes +	
	40°C	42°C	30°C	40°C
EcSecA, IC ₅₀ (μM)	45 μM	20 μM	>100 μM	45 μM

Table 2.17a *In vitro* and *in vivo* inhibition effects of bistriazole analgs

		SCA-21 748.59	SCA-107 470.98	SCA-110 425.74	SCA-111 317.8	SCA-112 533.9
<i>In vitro</i> inhibition IC ₅₀ (μM)	EcSecAN68	18	30	150	>200	20
<i>In vivo</i> inhibition IC ₅₀ (μM)	<i>B. anthracis</i> Sterne	3	0.73	2.5	80	0.7
	<i>S. aureus</i> 6538	1.5	0.55	2	>100	0.6
	<i>S. aureus</i> Mu50	0.75	0.35	ND	>100	0.5
	<i>E. coli</i> NR698	60	6.3	18	>100	4
	<i>B. subtilis</i> 168	3	0.33	0.8	>100	0.5

Table 2.17b *In vitro* and *in vivo* inhibition effect of bistriazole analgs

		SCA-21 748.59	SCA-106 363.04	SCA-107 470.98	SCA-117 329.09	SCA-123 437.02	SCA-116 351.01
<i>In vitro</i> inhibition IC ₅₀ (μM)	EcSecAN68	18	30	30	ND	ND	ND
<i>In vivo</i> inhibition IC ₅₀ (μM)	<i>B. anthracis</i> Sterne	3	17.5	0.73	>100	1.5	2
	<i>S. aureus</i> 6538	1.5	15	0.55	>100	7	20
	<i>S. aureus</i> Mu50	0.75	ND	0.35	>100	7	15
	<i>E. coli</i> NR698	60	32.5	6.3	>100	35	70
	<i>B. subtilis</i> 168	3	15	0.33	>100	7	30

For *in vivo* inhibition, incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; for *in vitro* inhibition, reaction at 37°C for 40 min with 5% DMSO; 'ND': not determined.

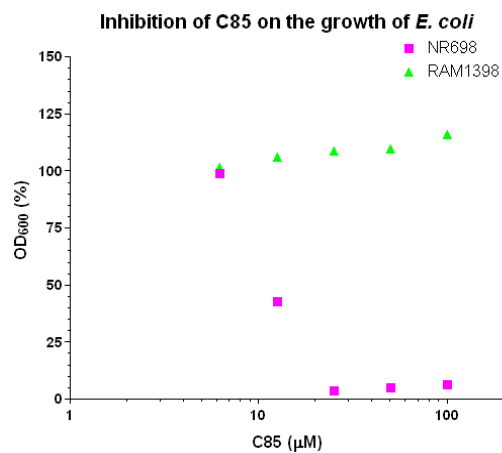


Figure 2.34 Bacteriostatic effect of SCA-21 on the growth of *E. coli*
Incubation at 37°C, 1000 rpm for 14 hrs with 2.5% DMSO; C85: SCA-21.

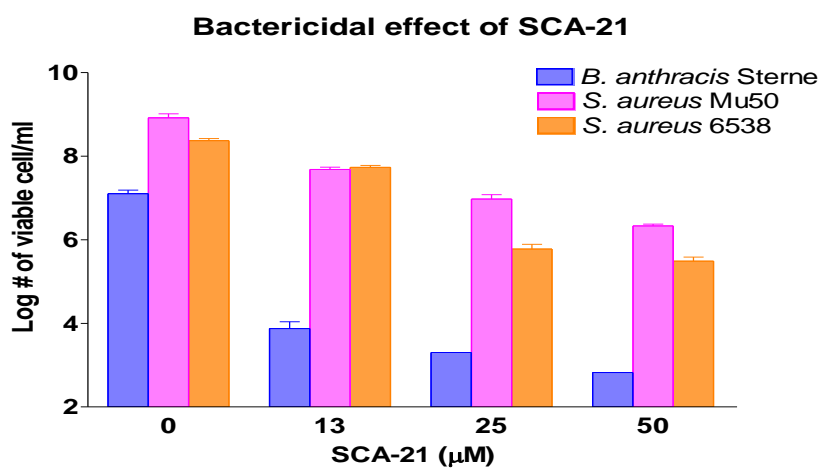


Figure 2.35 Bactericidal effect of SCA-21 against *S. aureus* and *B. anthracis*
37°C, 1 hour killing.

Competition assay

In this study, three structurally different classes of SecA inhibitors were developed and optimized. Several SecA inhibitors show varied degree of *in vitro* inhibition effect on ATPase activities of SecA homologues. To determine whether these three classes of inhibitors bind to the same site or different site of SecA protein, RB, SCA-15, and SCA-21 were subjected to competition assay against the ATPase activity of BaSecA2.

When RB and SCA-15 were added together, inhibition effect is greater than two compounds individually. These two compounds have synergistic effect, suggesting that they might have different binding sites on BaSecA2. When SCA-15 and SCA-21 were added together or when RB and SCA-21 were added together, inhibition effect is the same as the sum of two compounds individually. Thus SCA21 has additive effect with RB and SCA-15, suggesting that they might have different binding sites on BaSecA2.

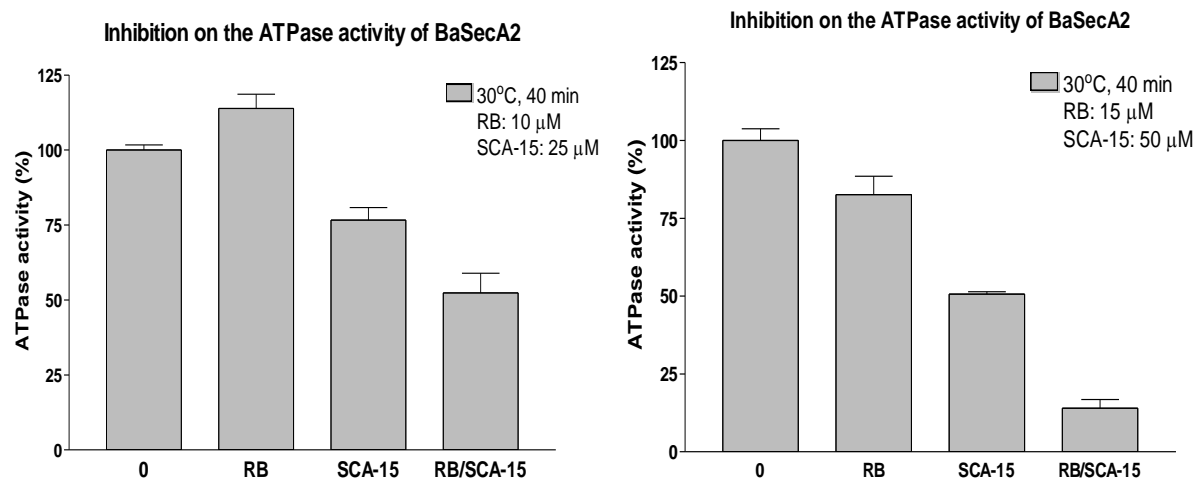


Figure 2.36 Synergistic effect of RB and SCA-15 against the ATPase activity of BaSecA2

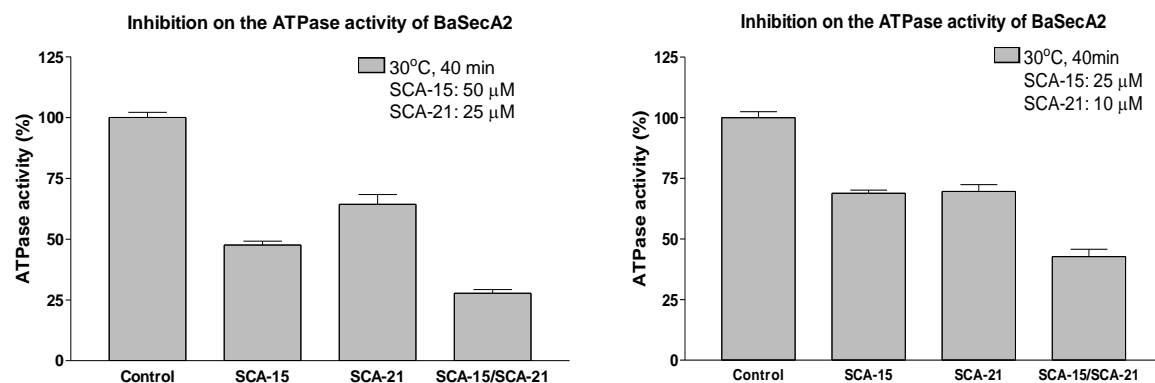


Figure 2.37 Additive effect of SCA-15 and SCA-21 against the ATPase activity of BaSecA2

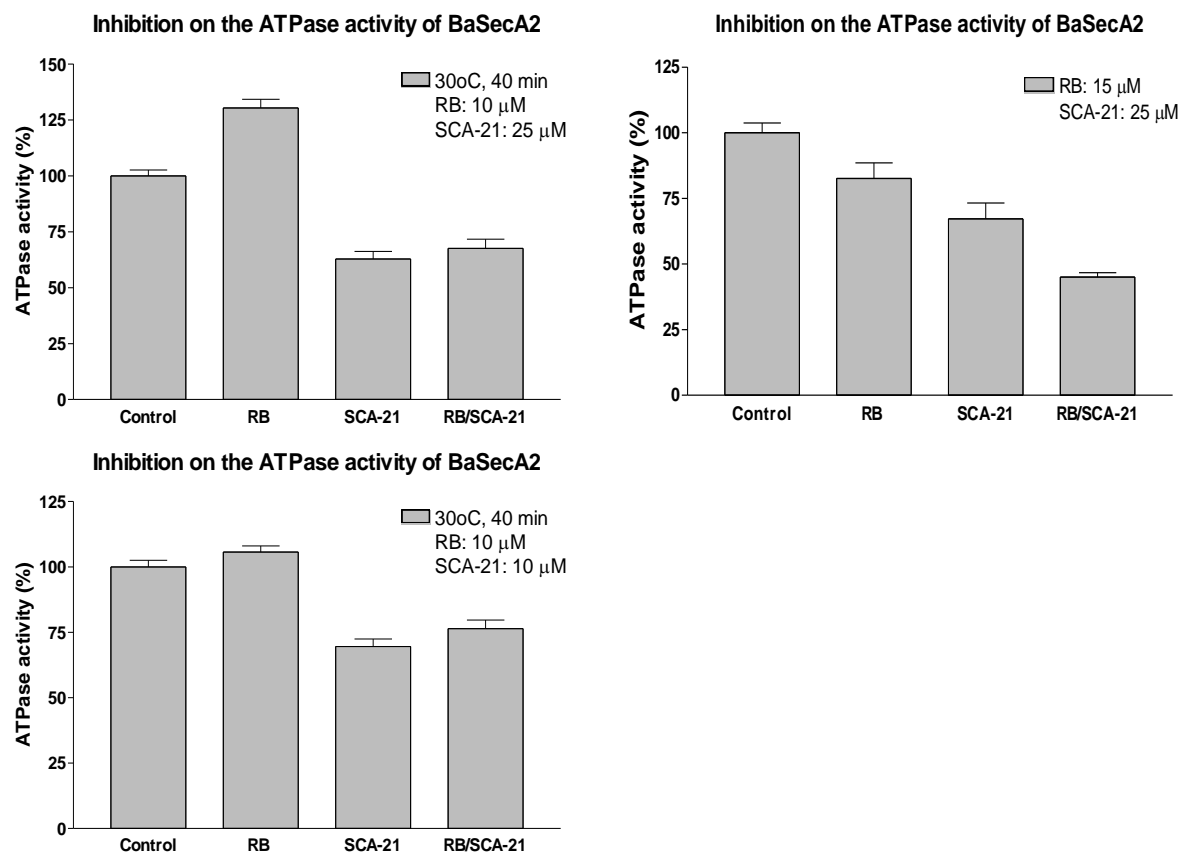


Figure 2.38 The additive effect of RB and SCA-21 against the ATPase activity of BaSecA2

Conclusion and Discussion

SecA is conserved in bacteria, required for viability and virulence; moreover it has no counterpart in mammalian cells. Thus, inhibition on the function of SecA homologues might reduce the virulence, inhibit bacteria growth, or/and kill bacteria, and with minimal human toxicity. Therefore these SecA homologues are potential good antibacterial drug targets, and identification SecA inhibitor might help to develop new anti-bacteria strategy. SecA proteins have ATPase activities, thus screening ATPase inhibitors might help to develop new antimicrobial agents.

Three different methods were used to develop SecA inhibitors: screening known ATPase, virtual docking, and random screening. In this study, three structurally different classes of SecA inhibitors were developed and optimized: (1) Rose Bengal (RB) and RB analogs derived from systematical dissection RB and Structure-Activity relationship (SAR) study; (2) pyrimidine analogs derived from virtual screening based on the ATP binding pocket of EcSecA and SAR study; and (3) bistriazole analog derived from random screening and SAR study.

RB could noncompetitively inhibit the ATPase activity of SecA1 and SecA2, and SecA2 is more sensitive to RB than SecA1. RB showed bacteriostatic and bactericidal effect against MS. Because MS was grown in the M7H9 broth which contains 0.5% dextrose, the antimicrobial activities of RB against MS were probably not caused by inhibition the ATPase activity of H⁺-ATPases. MS Δ SecA2 was more resistant to RB than MS wild type, and MS SecA2 had higher affinity to RB than SecA1, suggesting RB might have dual targets in mycobacteria -SecA1 & SecA2, and SecA2 might be the first target. The bactericidal effect of RB was independent of protein synthesis and growing phase of MS.

To develop more potent SecA inhibitors with less side effects and molecular weight, RB was systematically dissected to understand the importance of structural feature. We found that xanthene with 5 to 6 carbon at C9 position and two hydroxyl group at C3 and C6 position was essential for inhibition effect; the aromatic A-ring could be replaced by an aliphatic 5 or 6 membered ring or hexyl group; carboxyl group decrease inhibition effect; chloro groups are not necessary; and iodo groups slightly increase inhibition effect, but not necessary for inhibition effect. The SAR studies lead to the development of two potent RB analogs, of which molecular weight are less than one third of RB, and show better bacteriostatic effects and bactericidal effect than RB.

Pyrimidine analogues were derived from virtual screening based on the ATP binding pocket of *E. coli* SecA. SAR study results demonstrated that D-ring and A-ring are necessary for inhibition effect; keeping A-ring at para position are critical for inhibition effect; adding azido group to para position of D ring increase inhibition effect; replacing keto group to hydroxylamine at X-position slightly increase inhibition effect. SCA-15 and SCA-93 are the best pyrimidine analogs from our study.

Bistriazole analog was derived from random screening. SCA-21 is a dimer. Replacing half part with chloro group slightly increase *in vitro* inhibition effect, and significantly increase bacteriostatic effects. SAR study results demonstrated that chloro group, methylthio group, and sulfur are important for inhibition effect; the methyl group could be replaced with benzene ring. SCA-107 and SCA-112 are the best triazole analogs from our study.

The *in vitro* inhibition effects of three classes SecA inhibitors were compared in Table 2.18. SCA-8 has the best *in vitro* inhibition effect. The bacteriostatic effects of three classes of

SecA inhibitors were compared in Table 2.19. Bistriazole analogs have the best *in vivo* inhibition effect. Moreover, our SecA inhibitors show promising bacteriostatic effects (Table 2.19) and bactericidal effects (Table 2.20) against MRSA strain(s) and *B. anthracis* Sterne. At Table 2.21, the antimicrobial activities of our inhibitors with other antibiotics. Among the tested antibiotics, vancomycin has the best inhibition on *S. aureus* Mu50, MIC is around 5 µg/ml; five our SecA inhibitors have better bacteriostatic effect than vancomycin against *S. aureus* Mu50. Tetracycline and erythromycin have the best bacteriostatic effect against *B. anthracis* Sterne. Although inhibition effect of our inhibitors are not as good as tetracycline and erythromycin, but our inhibitors have bactericidal effect while these two antibiotics do not have.

Several potent inhibitors developed from this study show different varying degrees of inhibitory effects against the intrinsic ATPase and translocation ATPase activity of SecA homologues; results of other lab members show that these inhibitors also could inhibit the *in vitro* protein translocation ATPase activity of EcSecA (IC₅₀: 1-55 µM), and ion-channel activities of Sa-SecA1, BaSecA1, and EcSecA in oocyte system (IC₅₀: 0.4-5 µM). These SecA inhibitors show promising bacteriostatic effects against *B. subtilis* 168 and one *E. coli* leaky mutant strain NR698; moreover, they have very good bacteriostatic effects and bactericidal effect of *B. anthracis* Sterne, numerous clinical or drug-resistant *S. aureus* strains. However we need to further determine whether the antimicrobial activities of these inhibitors were due to inhibition on SecA.

Table 2.18 *In vitro* inhibition effects of three classes SecA inhibitors

		RB	RB analogs		Pyrimidine analogs		Triazole analogs	
		RB MW: 974	SCA-41 MW: 282	SCA-50 MW: 298	SCA-8 MW: 713	SCA-15 MW: 436	SCA-21 MW: 749	SCA-107 MW: 471
Intrinsic ATPase	BsSecA	20	30	33	8	>100	>100	ND
	BaSecA2	15	30	20	7	20	45	65
	SaSecA2	1	6	ND	3	13	43	50
	EcSecA N68	1	8	4	2	8	18	30
	EcSecA	60	30	60	>100	30	32	ND
Translocation ATPase	EcSecA	1	15	60	6	30	20	28

In vitro inhibition: IC₅₀ (μM); 40 min reaction with 5% DMSO; for EcSecAN68, BsSecA, and EcSecA, reaction at 37°C; for BaSecA2, reaction at 30°C; Translocation ATPase activity of EcSecA: 37°C 40 min reaction with *E. coli* BA13 membrane washed with 6 M Urea and *E. coli* preOmpA; “ND”: not determined;

Table 2.19 Bacteriostatic effects of three classes SecA inhibitors

Class	RB		RB analogs				Primidine analogs				Bistriazole analogs			
Compound	RB MW: 974		SCA-41 MW: 282		SCA-50 MW: 298		SCA-15 MW: 436		SCA-93 MW: 451		SCA-21 MW: 749		SCA-107 MW: 471	
MIC (μM)	50%	95%	50%	95%	50%	95%	50%	95%	50%	95%	50%	95%	50%	95%
<i>B. subtilis</i> 168	74	80	7	8	7	8	7	10	5	6	3	4	0.3	0.8
<i>E. coli</i> NR698	18	25	9	25	14	22	35	50*	45	200*	40	75	6.3	9.5
<i>B. anthracis</i> Sterne	7	13	3	4	4	12.5	4	5	3	4	3	4	0.7	1.5
<i>S. aureus</i> 6538	28	50	7	10	8	12.5	12	15	9	10	1.5	4	0.6	1.9
<i>S. aureus</i> Mu50	27	40	6	7	11	19	23	25	9	10	0.8	2	0.4	2
<i>S. aureus</i> Mu3	>50	>50	5	8	12.5	18.8	35	100	50	>100	1.5	4	0.6	2
<i>S. aureus</i> N315	45	>50	5	6	7.5	9.4	9	25	9	18	0.8	2	0.4	2

Incubation at 37°C, 1,000 rpm for 14 hrs with 2.5% DMSO; *:MIC₉₀

Table 2.20 Bactericidal effects of three classes SecA inhibitors

	RB analogs	Primidine analogs	Bistriazole analogs
	SCA-50 MW: 436	SCA-15 MW: 436	SCA-21 MW: 749
<i>S. aureus</i> 6538	3 (40 μM)	4 (50 μM)	3 (50 μM)
<i>S. aureus</i> Mu50	3 (40 μM)	4 (50 μM)	3 (50 μM)
<i>B. anthracis</i> Sterne	3 (20 μM)	2 (10 μM)	4 (12.5 μM)
<i>B. subtilis</i> 168	4 (20 μM)	ND	ND

Log # in cell killing/ml; 37°C 1 hour killing; ‘ND’: not determined.

Table 2.21 Comparison of the antimicrobial activities of SecA inhibitors with other antibiotics

Class	Strains Antibiotics	<i>S. aureus</i> Mu50		<i>B. anthracis</i> Sterne	
		MIC ₉₅ (µg/ml)	Log #/ ml	MIC ₉₅ (µg/ml)	Log #/ ml
RB & analogs	RB	40.7	+	12.2	ND*
	SCA-41	1.7	ND*	1.1	ND*
	SCA-50	2.4	3.5 (8.9 µg/ml)	1.7	3 (6.0 µg/ml)
Pyrimidine analogs	SCA-15	10.9	3 (10.9 µg/ml)	2.2	2 (4.4 µg/ml)
	SCA-93	4.5	ND*	1.6	ND*
Bistriazole analogs	SCA-21	1.5	2 (19.0 µg/ml)	3.0	3.5 (19.0 µg/ml)
	SCA-107	0.5	ND*	0.7	ND*
	SCA-112	0.4	ND*	0.8	ND*
Glycopeptides	Vancomycin	5	+	2.5	+
Penicillins	Ampicillin	7.8	+	>20	+
Aminoglycosides	Kanamycin	>100	+	1	+
	Gentamycin	>500	+	5	+
Polypeptides	Polymyxin B	15	+	10	+
Tetracyclines	Tetracycline	200	-	0.1	-
Macrolides	Erythromycin	>500	-	0.3	-
others	Chloramphenicol	>40	-	10	-

Incubation at 37°C, 1,000 rpm for 14 hrs; log #/ml: log number in cell killing/ml; “ND”: not determined; “+”: have bactericidal effect; “-”: do not have bactericidal effect.

CHAPTER 3 VALIDATION OF SECA AS A DRUG TARGET

Summary

In this study, three structurally different classes of SecA inhibitors were developed and optimized. Several SecA inhibitors showed promising *in vitro* inhibition effect on ATPase activities of SecA homologues, as well as bacteriostatic effects and bactericidal effects against *B. anthracis* Sterne and numerous drug resistant *S. aureus* strains. However, to validate SecA as a real target of these inhibitors, we need to determine whether their antimicrobial activities are due to inhibition on SecA *in vivo*. SecA is involved in forming a protein conducting channel that spans the entire membrane. Thus, drugs targeting SecA might be directly accessible from the extracellular matrix and exert their effects without entering the cell, and bypassing the major negative effect of efflux transporters in bacteria, which is a major mechanism for the development of current drug-resistance. Two major efflux pumps of *S. aureus*, NorA and MepA, had little negative effect on the antimicrobial activities of SecA inhibitors, suggesting that targeting SecA might be able to by-pass efflux pump. Sec system is responsible for the secretion of many toxins and virulence factors, which play important roles in the pathogenesis of bacterial infection. Our results showed SecA inhibitors could inhibit secretion of important virulence factors of *S. aureus* and *B. anthracis* Sterne, suggesting that these inhibitors could inhibit the function of SecA *in vivo* and reduce virulence of these important bacteria pathogens. Previous studies indicated that photooxidation and halo group contribute to the antimicrobial activities of RB as well as inhibition on specific enzymes. However, our results showed that photooxidation might not contribute to the antimicrobial activities of RB analogs, which do not have halo group and have better antimicrobial activities than RB, suggesting that the ability of inhibition on specific enzyme is increased in RB analogs. The interaction between SecA homologues and inhibitors was confirmed by altered trypsin sensitivity assay. Target identification assays confirmed that SecA

inhibitor could identify SecA homologues from whole cell lysate of *E. coli* and *S. aureus*, suggesting that these inhibitors were really targeting on SecA. These results validated that SecA is a good target for development antimicrobial agents.

Introduction

In this study, three structurally different classes of SecA inhibitors were developed and optimized. (1) RB and RB analogs derived from systematical dissection RB and SAR study; (2) pyrimidine analogs derived from virtual screening based on the ATP binding pocket of EcSecA and SAR study; and (3) bistriazole analog derived from random screening and SAR study. Several SecA inhibitors showed promising *in vitro* inhibition effect on ATPase activities of SecA homologues, as well as bacteriostatic effects and bactericidal effects against *B. anthracis* Sterne and numerous drug resistant *S. aureus* strains. However, to validate SecA as a real target of these inhibitors, we need to determine whether these antimicrobial activities of SecA inhibitors are due to *in vivo* inhibition on SecA. Thus, we further investigated their inhibition effects on secretion of toxins, the interaction between SecA homologues and inhibitors, and the ability of binding with SecA from whole cell lysate.

Previous studies demonstrated that EcSecA exists as soluble form and membrane form [96-97]; SecA alone could form ring-like structures upon interaction with anionic phospholipids [38-39]; SecA alone could export preOmpA across liposomes (Hsieh, et al, 2012), suggesting that SecA indeed is involved in forming a protein conducting channel that spans the entire membrane. Thus, drugs targeting SecA might be directly accessible from the extracellular matrix and exert their effect without entering the cell, and bypassing the negative effect of efflux transporters in bacteria, which is a major mechanism for the development of current drug-resistance [72-76]. Therefore, bacterial efflux pumps may have little negative effect on the ability for these inhibitors to inhibit bacterial growth. *S. aureus* Mu50 and *S. aureus* N315 are resistant to QacA efflux-mediated antibiotics [100]. SCA-41, SCA-50, SCA-15, SCA-93, and SCA-21 are best inhibitors developed from our study. They showed promising bacteriostatic effects or/and bacteri-

cidal effect on *S. aureus* Mu50 or/and *S. aureus* N315, suggesting that these SecA inhibitors might be able to overcome QacA mediated efflux. NorA and MepA are two major efflux pumps of *S. aureus*. In this study, we further investigated whether NorA and MepA could affect bacteriostatic effect and bactericidal effect of our SecA inhibitors.

Sec system is not only responsible for the secretion of many essential proteins, and it is also responsible for the secretion of many toxins and virulence factors [33-35]. In *S. aureus*, there are many secretory toxin precursors containing Sec-dependent signal peptide (Table 0.1) [34]. Table 0.1 showed these toxins play important roles in the pathogenesis of *S. aureus* infection, promoting adhesion, colonization, and spread in host tissue, protecting bacteria from environmental toxic condition or from host immune defense system, causing serious host cell damage or toxic syndrome. Because both SecA1 and SecA2 were involved in secretion of virulence factors, dual targeting inhibition on these two SecA homologues would dramatically reduce virulence. Virulence target-based therapies are not sufficient to combat infection, but targeting SecA homologues is not only reduce virulence but also decrease viability, dramatically increasing the chance of control bacterial infection and reduce the occurrence of drug resistance. Enterotoxin B, α -hemolysin, and toxic shock syndrome toxin 1 (TSST-1) are three toxins of *S. aureus*, which containing Sec-dependent signal peptide (Table 0.1) [34]. Lethal factor (LF), edema factor (EF), and protective antigen (PA), are three major component toxins of *B. anthracis*, and all of them have Sec-dependent signal peptide [35]. In this study, commercial antibodies for these toxins were used to assess the inhibition effect on toxin secretion.

Previous studies indicated that there are three mechanism involved in the antimicrobial activity of RB. RB could specific binding to essential enzyme and inhibit its function, like DNA polymerase [108, 110], RNA polymerase [109]. RB might release chloro and iodo group at cer-

tain condition, these halo groups have antimicrobial activity. RB is a photosensitizer [116], and it could generate singlet oxygen through photooxidation [117-118]. Singlet oxygen could cause cell damage and bacteria death. SCA-41 don't have halo group, but it showed better bacteriostatic and bactericidal effect than RB. We don't know whether its antimicrobial activities are due to inhibition on SecA, other enzyme, or photooxidation. In this study, we investigate whether antimicrobial activity of RB and RB analogs are due to inhibition on proton ATPase activity or due to photooxidation.

To truly validate SecA as the target of those inhibitors developed in our study, we need to investigate interaction between SecA and inhibitors, identify how many proteins interact with inhibitors, and determine whether interactions with other proteins could contribute to observed antimicrobial effect. Affinity chromatography is the most widely used method to identify drug targets [119-120], however the application of affinity chromatography was limited, because of the need of vast structural diversity, the complexity of biologically active small molecules, and high level of nonspecific binding of non-target proteins [121-124]. Drug affinity responsive target stability (DARTS) is a new method to identify drug targets [121, 125]. The principle of this method is that binding with small ligand would stabilize the structure of its target protein and result in changing of protease resistance, which offered the chance of target identification [121, 125]. Affinity chromatography is a positive target selection method by pulling out the target proteins and leaving nontarget proteins behind [121], while DARTS is a negative drug target selection method by digesting away nontarget proteins while leaving behind the target proteins [121]. In this study, these two methods were used to validate that SecA is a real target. Azide is a SecA inhibitor, and previous study showed that azide enhances the formation of a trypsin-resistant 30

kDa fragment, which is related with SecA membrane insertion [37, 62, 88]. In this study, altered trypsin sensitivity assay was used to detect interaction between inhibitors and SecA homologues.

Material and Methods

Medium: LB medium was used to grow bacteria in this study.

SecA inhibitors: All inhibitors were synthesized by Dr. Binghe Wang's lab.

Strains: *E. coli* NR698, *B. subtilis* 168, *S. aureus* N315, *S. aureus* 6538, *S. aureus* Mu50, *B. anthracis* Sterne. Five efflux pump related strains: *S. aureus* 8325, *S. aureus* K1758 (NorA⁻), *S. aureus* K2361 (NorA⁺⁺⁺), *S. aureus* K2068 (MepA⁺⁺⁺), and *S. aureus* K2908 (MepA⁻), were from Dr. GW Kaatz at Wayne State University School of medicine and Jon D. Dingell VA Medical Center.

Antibodies: Antibodies for *S. aureus* α -hemolysin, *S. aureus* Enterotoxin B, *S. aureus* TSST-1, *B. anthracis* lethal factor (LF), *B. anthracis* edema factor (EF), and *B. anthracis* protective antigen (PA), were purchased from abcam.com.

***In vitro* inhibition assay:** Because SecA homologues have ATPase activity, Malachite green colorimetric assay was used to investigate the inhibition of inhibitors on the ATPase activity of SecA homologues. In this assay, ATPase assay was carried in different concentration of inhibitors, and IC₅₀ is the concentration of RB which could inhibit 50% ATPase activity of SecA homologues. Because the compounds were not soluble, so the compounds were dissolved in 100% DMSO. In the final *in vitro* assay there were 1-5% DMSO.

***In vivo* inhibition assay in medium:** Single colony of bacteria was inoculated in 3 ml LB medium in 12 ml culture tube, then grow at 37°C; when OD₆₀₀ \approx 0.5, 300 μ l culture were diluted into 3 ml LB medium, then 97.5 μ l culture was aliquated into 1.5 ml eppendorf tube, then adding 2.5 μ l of different concentration of RB analogs; incubated at 37°C, 1,000 rpm, 14 hours in Thermo-mixer. OD₆₀₀ was measured to determine inhibition effect.

Killing assay: Single colony of bacteria was inoculated in 3 ml LB medium in 12 ml culture tube, then grow at 37°C; when OD₆₀₀ ≈ 0.5, 97.5 µl cell culture was aliquated into 1.5 ml eppendorf tube, then add 2.5 µl different concentration of RB analogs into tube; incubated at 37°C, 1000 rpm for 1-2 hours. The cell culture was diluted with dd H₂O and spread 150 µl on LB plates.

Toxin secretion: Inhibitors were added to the mid-log phase of *S. aureus* Mu50 or *B. anthracis* Sterne. The growth of bacteria was monitored. Culture was collected after treating with inhibitor for 1 h, 3 hrs, and 5 hrs. For *S. aureus* cell culture, the supernatant and cell pellet were separated by centrifugation and 0.45 µm filter; while the supernatant of *B. anthracis* was separated from cell by 0.45 µm filter only. The collected supernatant was subjected to TCA precipitation, and the protein pellet was resuspended in Tris-buffer pH 7.5. Western blots with specific toxin antibodies were used to detect the amount of toxins.

DARTS for purified protein binding: 10 µg protein was incubated at 37°C for 10 min with or without inhibitors, then exposed to UV for 0 sec or 90 sec, followed by digestion with 1.2 µg trypsin on ice for 15 min to 25 min. The digestion was stopped by adding sample buffer and boiling for 20 min. 12% or 15% SDS-PAGE was used to investigate the trypsin digestion pattern.

DARTS for whole cell lysate: *E. coli* MC4100, *S. aureus* Mu50, and *B. anthracis* Sterne were grown in LB medium overnight at 37°C. Bacteria cell were harvested by centrifugation and washed with Tris-buffer pH 7.5. Cell pellet was resuspended with Tris-buffer, which contain cocktail proteinase inhibitor, then French pressed at 10,000 psi. Unbroken cells were separated by 5,000 rpm centrifugation. 100 µg or 200 µg whole cell lysate was incubated on ice for 2 hrs with or without inhibitors, then exposed to UV for 0 sec or 90 sec, followed by digestion with 50

µg trypsin at 25°C for 10 min-20 min. The digestion was stopped by adding sample buffer and boiling for 20 min. 12%, 15%, or 10-20% SDS-PAGE was used to investigate the trypsin digestion result.

Pull down assay: *E. coli* MC4100 and *S. aureus* Mu50 were grown in LB medium overnight at 37°C. Bacteria cell were harvested by centrifugation and washed with binding buffer (0.1 M K₃PO₄ pH 7.2). Cell pellet was resuspended with binding buffer, which contain cocktail proteinase inhibitors, then French pressed at 10,000 psi. Unbroken cells were separated by 5 k rpm centrifugation. Agarose beads and straptavidin beads were used in our study. For agarose beads, whole cell lysate was mixed with beads and rolling with or without inhibitors at 4°C for 1 hour, then rolling at room temperature for 10 min. For straptavidin beads, the whole cell lysate was pretreated with beads at 4°C for 1 hour, then centrifuged for 2 min at 0.5 k rpm; the supernatant was taken out and mixed with clean beads and rolling with or without inhibitors at 4°C for 1 hour, then rolling at room temperature for 10 min. Both agarose beads and straptavidin bead were separated with cell lysate by centrifuged at 0.5 k rpm for 2 min, then beads were washed with binding buffer for 5 times. After washing, beads were mixed with binding buffer and sample buffer, then boiled for 30 min.

Results

Effect of Efflux Pumps on the Antimicrobial Activities of SecA Inhibitors

SecA might be involved in forming a protein-conducting channel that spans the entire membrane [38-39]. Thus, drugs targeting SecA might be directly accessible from the extracellular matrix and exert their effect without entering the cell, and bypassing the major negative effect of efflux transporters in bacteria, which is a major mechanism for the development of current drug-resistance [72-76]. *S. aureus* Mu50 and *S. aureus* N315 are resistant to beomycin, β -lactam, tetracyclines, several aminoglycosides and MLS [100]. And these two strains are resistant to QacA efflux-mediated antibiotics [100]. SCA-41, SCA-50, SCA-15, SCA-93, and SCA-21 are best inhibitors developed from our study. They showed promising bacteriostatic effects or/and bactericidal effect on *S. aureus* Mu50 or/and *S. aureus* N315, suggesting that the SecA inhibitors might be able to overcome QacA mediated efflux. NorA and MepA are two major efflux pumps of *S. aureus*. In this study, we further tested whether NorA and MepA could affect bacteriostatic effect and bactericidal effect of our SecA inhibitors.

Effect of efflux pumps on bacteriostatic effect of RB and RB analogs Deletion NorA significantly increased sensitivity to RB, and over-expression NorA restore resistance to RB, suggesting that NorA was involved in RB resistance (Table 3.1). However, over-expression MepA increased sensitivity to RB, and over-expression NorA or MepA slightly increased sensitivity to SCA-41 (Table 3.1a). For SCA-50, IC_{50} of NorA⁻ and NorA⁺⁺⁺ were similar. Thus, NorA and MepA didn't show negative effect on bacteriostatic effect of SCA-41 and SCA-50. All these results strongly suggest that RB analogs might be able to overcome the effect of efflux pumps in drug-resistance development.

Effect of efflux pumps on antimicrobial effect of pyrimidine analogs Deletion NorA or MepA slightly reduced bacteriostatic effect of SCA-15 and SCA-93, and over-expression NorA or MepA slightly increase bacteriostatic effect (Table 3.1b). Deletion NorA and MepA also slightly reduced bactericidal effects comparing to wild type, while over-expression MepA slightly increased killing effect comparing to MepA deletion mutant (Fig. 3.1). Thus, NorA and MepA didn't showed negative effect on the ability for these inhibitors to inhibit bacterial growth or kill bacteria.

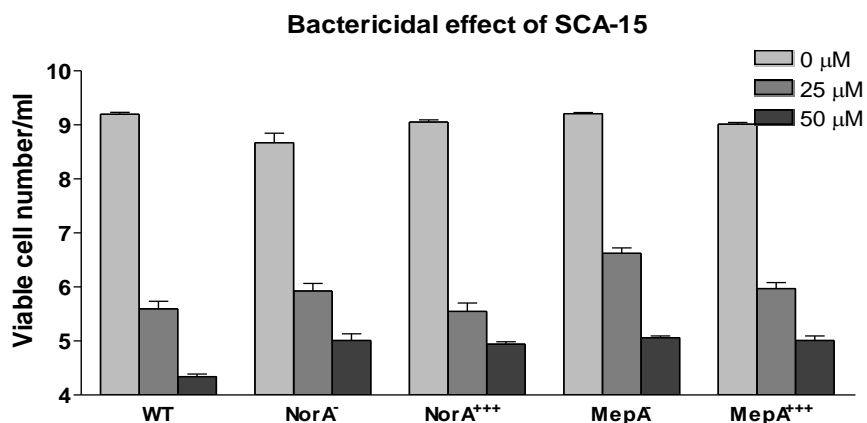
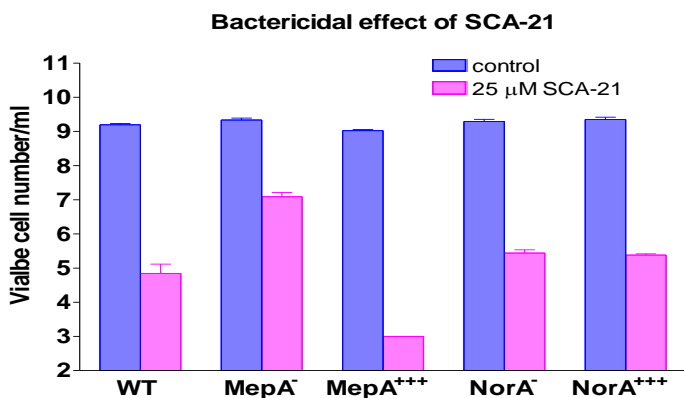
Effect of efflux pumps on antimicrobial effect of bistriazole analogs Deletion or over-expression NorA or MepA didn't affect bacteriostatic effect of SCA-21 (Table 3.1b). Deletion or overexpression NorA didn't affect bactericidal effect of SCA-21, but deletion MepA reduced bactericidal effect and over-expression MepA increased bactericidal effect of SCA-21 (Fig. 3.2).

Table 3.1a Bacteriostatic effects of RB and RB analogs against *S. aureus* efflux strains

Strains		WT	NorA ⁻	NorA ⁺⁺	MepA ⁻	MepA ⁺⁺
compounds		8325-4	K1758	K2361	K2908	K2068
RB	MIC ₅₀ (μM)	15	5	18	19	7
	MIC ₉₅ (μM)	25	10	25	25	25
SCA-41	MIC ₅₀ (μM)	7	8	5	8	5
	MIC ₉₅ (μM)	8	10	8	10	8
SCA-50	MIC ₅₀ (μM)	9	7	8	13	9
	MIC ₉₅ (μM)	15	8	15	15	15

Table 3.1b Bacteriostatic effects of pyrimidine analogs and bistrizazole analogs against *S. aureus* efflux strains

Strains		WT	NorA ⁻	NorA ⁺⁺	MepA ⁻	MepA ⁺⁺
compounds		8325-4	K1758	K2361	K2908	K2068
SCA-15	MIC ₅₀ (μM)	22	25	23	30	19
	MIC ₉₅ (μM)	64	50	40	75	50
SCA-93	MIC ₅₀ (μM)	27	33	21	32	19
	MIC ₉₅ (μM)	50*	>50	50*	>50	50*
SCA-21	MIC ₅₀ (μM)	3.0	3.0	3.0	3.0	2.5
	MIC ₉₅ (μM)	4	4	4	4	4

**Figure 3.1 Bactericidal effects of SCA-15 against *S. aureus* efflux strains****Figure 3.2 Bactericidal effects of SCA-21 against *S. aureus* efflux strains**

Inhibition on the Secretion of Bacterial Toxins

Being conserved throughout bacteria, the Sec-dependent protein export pathway is responsible for export precursor proteins, which contain classical amino-terminal signal sequences across the cytoplasmic membrane [31]. Sec system is not only responsible for the secretion of many essential proteins, but also responsible for the secretion of many toxins and virulence factors, promoting adhesion, colonization, and spread in host tissue, protecting bacteria from environmental toxic condition or from host immune defense system, causing serious host cell damage or toxic syndrome. Because both SecA1 and SecA2 are involved in secretion of virulence factors, dual targeting inhibition on these two SecA homologues could dramatically reduce virulence. Virulence target-based therapies are not sufficient to combat infection, but targeting SecA homologues is not only reduce virulence but also decrease viability, dramatically increasing the chance of control bacterial infection and reduce the occurrence of drug resistance. Antibodies for several important extracellular toxins in both bacteria are commercially available, and were used for the assessment of the effects of the inhibitors on the SecA inhibition in this study.

Inhibition of RB on the secretion of *S. aureus* Toxins RB was added to the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 3$. RB could inhibit the growth of *S. aureus* Mu50 at 15 μ M-60 μ M (Fig. 3.3a). SDS-PAGE gel results showed that the general secretion of *S. aureus* Mu50 was inhibited by RB (Fig. 3.3b). Western Blot results showed that the amount of α -hemolysin, enterotoxin B, and TSST-1 were dramatically decreased by RB (Fig. 3.3c). To investigate whether these results were due to inhibition on secretion or due to inhibit on synthesis or stability, this experiment was repeated and the toxins in medium and inside the bacteria were detected by antibody side by side. The results confirmed that 10 μ M RB could inhibit the growth of *S. aureus* Mu50, while 4.5 mM sodium azide had more strong inhibition on the growth of *S. aureus*

Mu50 than 10 μ M RB (Fig. 3.4a). Western blot results showed that 10 μ M RB almost totally decreased the amount of α -hemolysin, enterotoxin B, and TSST-1 not only in medium but also inside of cell (Fig. 3.4b-c), suggesting that RB could affect the synthesis or stability of these toxins; 4.5 mM sodium azide decreased the amount of these two toxins in medium, however the amount of these toxins were not obviously changed inside the cells (Fig. 3.4b-c). Because previous study showed that RB could bind to RNA polymerase, so it is possible the decreasing amount of toxins in the supernatant was due to inhibition on protein synthesis. But because our results showed that RB could inhibit the ATPase activity of SaSecA1 and SaSecA2, we couldn't exclude the possibility that the decreasing amount of toxins in the supernatant was due to inhibition on secretion and that there are might have some feed-back regulation system which control the amount of toxins inside bacteria.

Inhibition of RB on the secretion of *B. anthracis* Toxins RB was added to the mid-log phase of *B. anthracis* Sterne at $OD_{600} \approx 3$. RB could inhibit the growth of *B. anthracis* Sterne at 10 μ M-20 μ M, while 5 μ M RB didn't obviously change its growth (Fig. 3.5a). SDS-PAGE gel results showed that the general secretion of *S. aureus* Mu50 was inhibited by RB (Fig. 3.5b). Western Blot results showed that the amount of lethal factor, edema factor, and protective antigen were dramatically decreased by RB (Fig. 3.5c).

Inhibition of RB analogs on the secretion of *S. aureus* Toxins When 10 μ M SCA-41 was added into the mid-log phase of *S. aureus* Mu50, the OD reading was slightly lower than control in 15 hours (Fig. 3.6a). Increasing concentration, OD became lower. 10 μ M SCA-41 was enough to significantly reduce the total secretion profile (Fig. 3.6b). Western blot with specific toxin antibody showed that 10 μ M SCA-41 was enough to significantly decrease the amount of α -hemolysin, enterotoxin B, and toxin shock syndrome toxin-1 (TSST-1) in the supernatant (Fig.

3.6c). To test whether this result was due to decreasing protein synthesis, the amount of three toxins inside and outside was compared side by side. 10 μ M SCA-50 didn't obviously change OD reading (Fig. 3.7a), however it significantly decreased the amount of three toxins in the supernatant and inside cell (Fig. 3.7b); and these toxins were not stable in the supernatant (Fig. 3.7c). Although adding RB analogues didn't increase the amount of toxins in cytosol, we couldn't exclude the possibility that there are might have some feed-back regulation system which control the amount of toxins inside bacteria. Because previous study showed that RB could bind to RNA polymerase, so it is possible the decreasing amount of toxins in the supernatant was due to inhibition on protein synthesis.

Inhibition of RB analogs on the secretion of *B. anthracis* Toxins When 5 μ M to 10 μ M SCA-41 was added into the mid-log phase of *B. anthracis* Sterne., the OD reading was not obviously changed comparing with control in 5 hours, while 20 μ M SCA-41 slightly inhibited bacteria growth and drop OD reading (Fig. 3.8a). 5-10 μ M SCA-41 slightly decreased the amount of secretory proteins in the supernatant in 1 hour sample (Fig. 3.8b). Western blot results showed that 5 μ M SCA-41 was enough to significantly reduce the amount of lethal factor, edema factor, and protective antigen in the supernatant (Fig. 3.8c).

Inhibition of pyrimidine analogs on secretion of *S. aureus* toxins When 10 μ M to 30 μ M SCA-15 was added into the mid-log phase of *S. aureus* Mu50, the OD reading was not obviously changed comparing without compound in 15 hours (Fig. 3.9a), however 10 μ M SCA-15 was enough to significantly reduce the total secretion profile, and increasing concentration of SCA-15 reduced more (Fig. 3.9b). Western blots with specific toxin antibodies showed that 10 μ M SCA-15 was enough to significantly decrease the amount of α -hemolysin, enterotoxin B, and TSST-1 in the supernatant, and increasing concentration of SCA-15 decreased more (Fig. 3.9c).

To test whether this result was due to decreasing protein synthesis, the amount of three toxins inside and outside was compared side by side. 10 μ M SCA-15 and 10 μ M SCA-93 didn't obviously change OD reading (Fig. 3.10a), however these compounds significantly decreased the amount of three toxins in the supernatant, while the amount inside were not obviously changed (Fig. 3.10b); and these toxins were not stable in the supernatant (Fig. 3.10b). Although adding SecA inhibitors didn't increase the amount of toxins in cytosol, we could not exclude the possibility that there might have some feed-back regulation systems which control the amount of toxins inside bacteria.

Inhibition of pyrimidine analogs on secretion of *B. anthracis* toxins When 2.5 μ M to 5 μ M SCA-15 was added into the mid-log phase of *B. anthracis* Sterne., the OD reading was not obviously changed comparing with control in 5 hours, while 10 μ M SCA-15 slightly inhibited bacteria growth (Fig. 3.11a). 2.5 μ M SCA-15 slightly decreased the amount of secretory proteins in the supernatant in 1 hour sample (Fig. 3.11b); 5 μ M decreased a little more amount of protein in 5 hour samples (Fig. 3.11b); and 10 μ M significantly decreased the amount of secretory protein in the supernatant (Fig. 3.11b). Western blot results showed that 2.5 μ M SCA-15 was enough to significantly reduce the amount of lethal factor, edema factor, and protective antigen in the supernatant, and increasing concentration of SCA-15 reduced a little more (Fig. 3.11c).

Inhibition of bistriazole analogs on secretion of *S. aureus* toxins When 2.5 μ M SCA-21 was added into the mid-log phase of *S. aureus* Mu50, the growth of bacteria was obviously inhibited (Fig. 3.12a), 10 μ M SCA-21 totally stopped bacteria growth. 2.5 μ M SCA-21 was enough to significantly reduce the total secretion profile (Fig. 3.12b). Western blot with specific toxin antibody showed that 2.5 μ M SCA-21 was enough to significantly decrease the amount of α -hemolysin, enterotoxin B, and TSST-1 in the supernatant (Fig. 3.12c). To test whether this re-

sult was due to decreasing protein synthesis, the amount of three toxins inside and outside was compared side by side. 2.5 μ M SCA-21 inhibited the bacteria growth (Fig. 3.13a), decreased the amount of three toxins in the supernatant and inside cell (Fig. 3.13b); and these toxins were not stable in the supernatant (Fig. 3.13b). Because bacteria growth was obvious inhibited by SCA-21, it is hard to conclude that SCA-21 inhibit the secretion of *S. aureus* toxins.

Inhibition of bistriazole analogs on secretion of *B. anthracis* toxins 1.25 μ M SCA-21 slightly inhibit the growth of *B. anthracis* Sterne. (Fig. 3.14a), while 2.5 μ M SCA-21 totally stopped bacteria growth (Fig. 3.14a). 1.25 μ M SCA-21 slightly decreased the amount of secretory proteins in the supernatant (Fig. 3.14b); 2.5 μ M SCA-21 blocked almost everything in the supernatant (Fig. 3.14b). Western blot results showed that 1.25 μ M SCA-15 was enough to significantly reduce the amount of lethal factor, edema factor, and protective antigen in the supernatant (Fig. 3.14c).

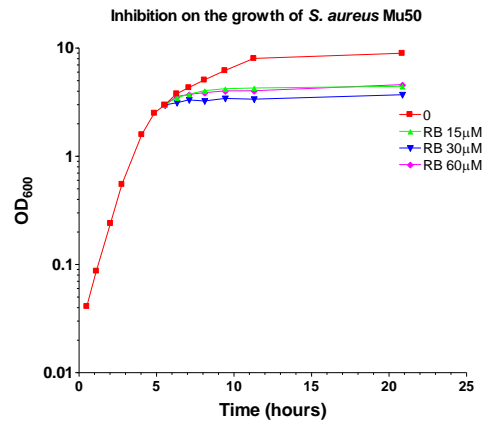


Figure 3.3a Inhibition of RB on the growth of *S. aureus* Mu50

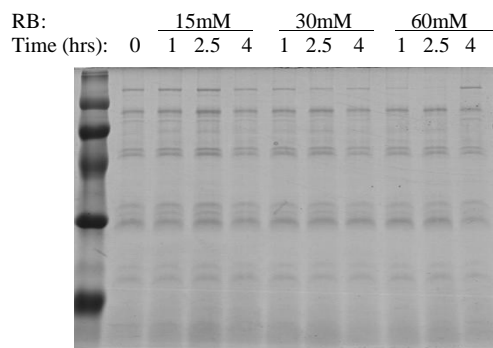


Figure 3.3b Inhibition of RB on the secretion of *S. aureus* Mu50

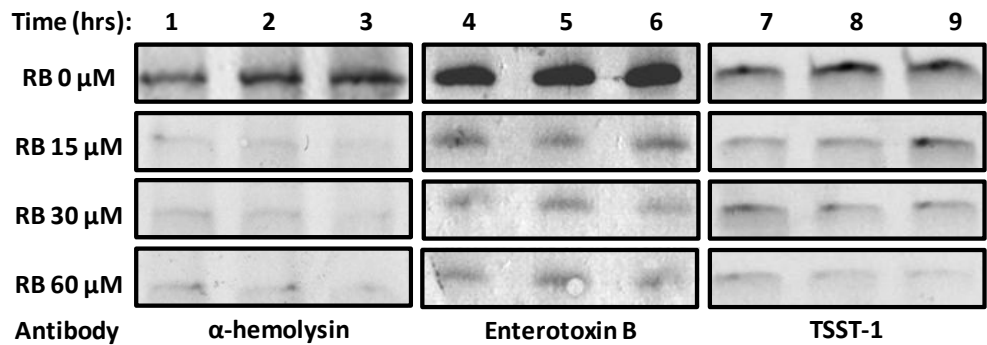


Figure 3.3c Inhibition of RB on secretion of specific toxins of *S. aureus* Mu50

Figure 3.3 Inhibition of RB on the secretion of *S. aureus* toxins

Different conc. of RB was added to the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 2.6$; the growth of bacteria was monitored; 2 ml culture was collected after 1 h, 2.5 hrs, and 4 hrs, and the supernatant and cell pellet were separate; 200 μ l TCA was added into 1.8 ml supernatant to precipitate protein, and the protein pellet was suspended in 200 μ l Tris-buffer; specific antibodies were used to detect α -hemolysin, enterotoxin B, and TSST-1.

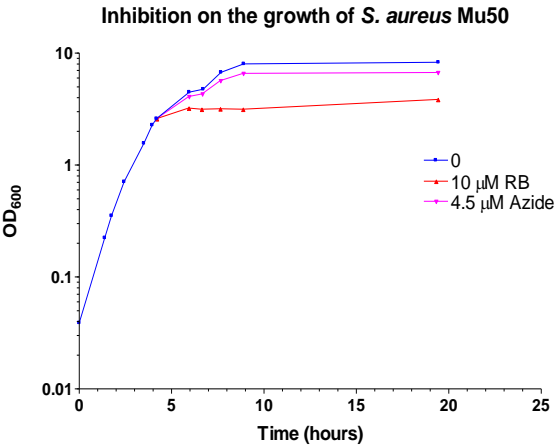


Figure 3.4a Inhibition of RB and azide on the growth of *S. aureus* Mu50

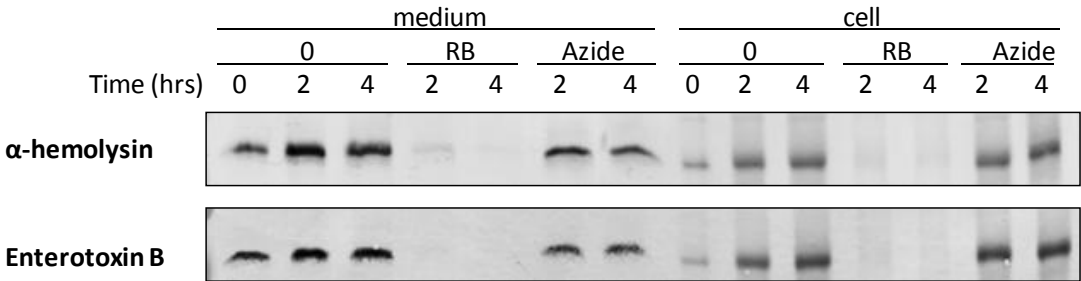


Figure 3.4b Inhibition of RB and azide on the secretion of α -hemolysin and enterotoxin B

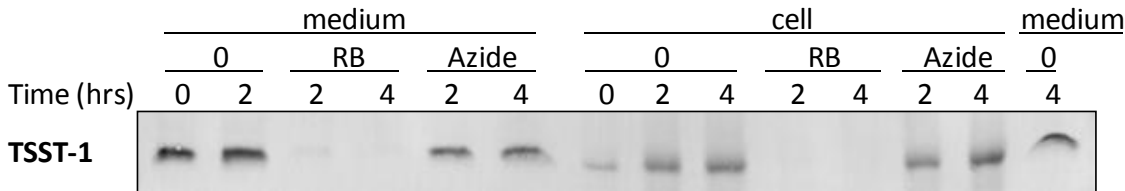


Figure 3.4c Inhibition of RB and azide on the secretion of TSST-1

Figure 3.4 Inhibition of RB and azide on the secretion of *S. aureus* toxins
10 μ M RB and 4.5 mM Sodium Azide were added to the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 3$; the growth of bacteria was monitored; then 2 ml culture was collected after 2 hrs and 4 hrs, and the supernatant and cell pellet were separated; 200 μ l TCA was added into 1.8 ml supernatant to precipitate protein, and the protein pellet was suspended in 200 μ l Tris-buffer; specific antibodies were used to detect α -hemolysin, enterotoxin B, and TSST-1.

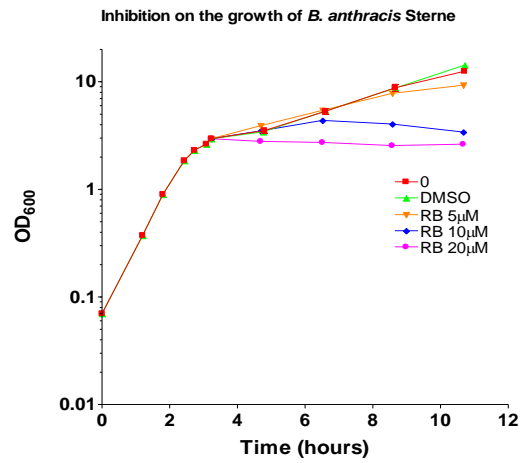


Figure 3.5a Inhibition of RB on the growth of *B. anthracis* Sterne

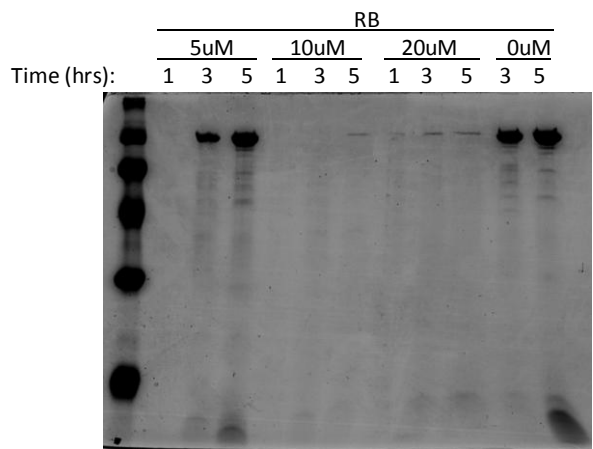


Figure 3.5b Inhibition of RB on the general secretion of *B. anthracis* Sterne

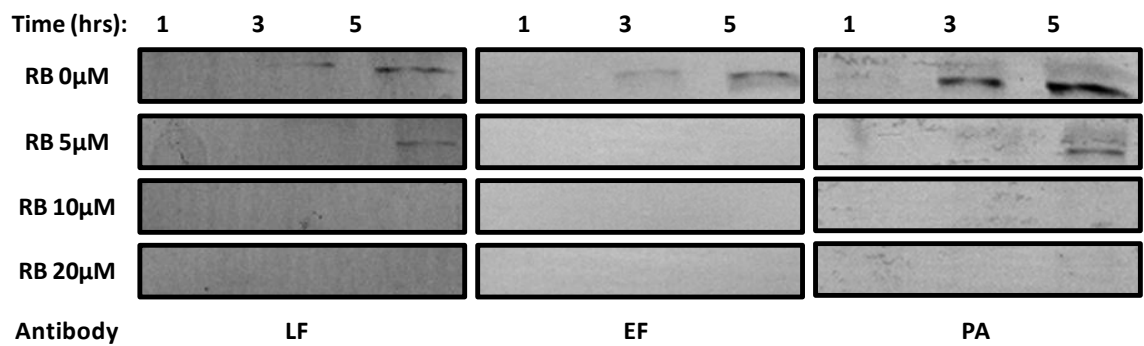


Figure 3.5c Inhibition of RB on the secretion of specific toxins of *B. anthracis* Sterne.

Figure 3.5 Inhibition of RB on the secretion of *B. anthracis* toxins

Different conc. of RB was added to the mid-log phase of *B. anthracis* Sterne at OD₆₀₀ ≈ 3; the growth of bacteria were monitored; then 2 ml culture was collected after 1 h, 3 hrs, and 5 hrs, then the supernatant and cell pellet were separated. 110 μl TCA was added into 1 ml supernatant to precipitate proteins; the protein pellet was suspended in 100 μl Tris-buffer. Specific antibodies were used to detect lethal factor (LF), edema factor (EF), and protective antigen (PA).

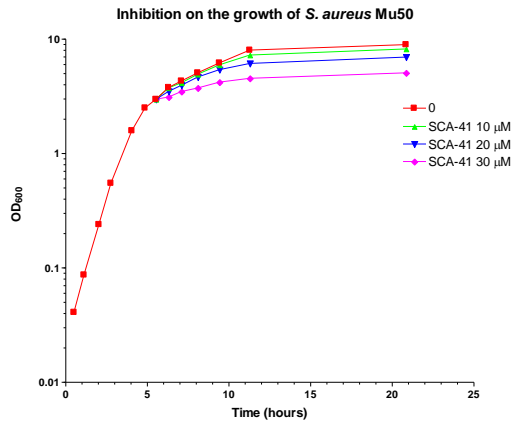


Figure 3.6a Inhibition of SCA-41 on the growth of *S. aureus* Mu50

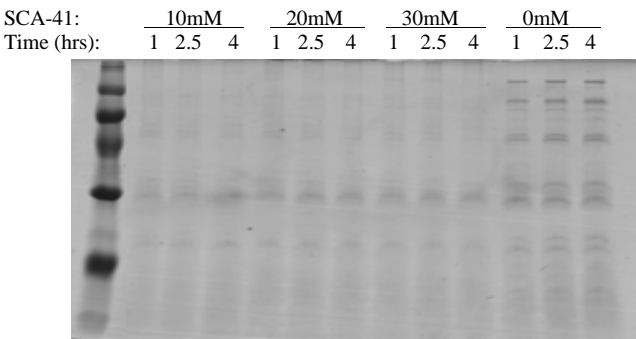


Figure 3.6b Inhibition of SCA-41 on the secretion of *S. aureus* Mu50

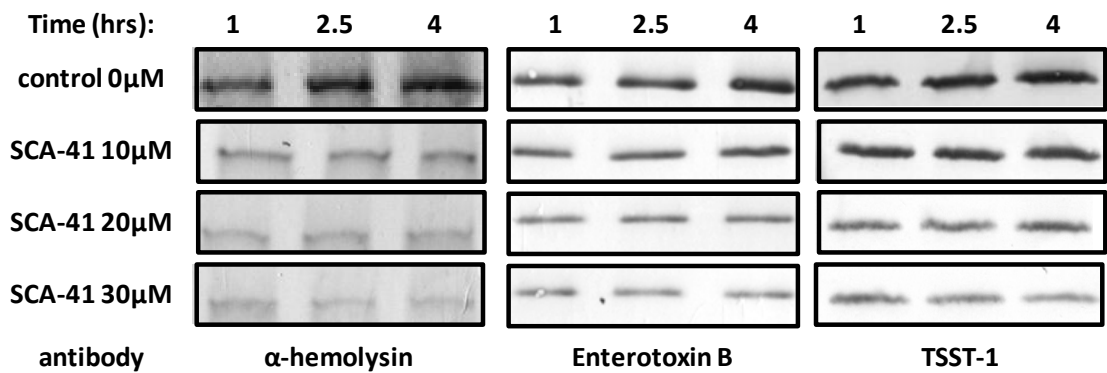


Figure 3.6c Inhibition of SCA-41 on the secretion of specific toxins of *S. aureus* Mu50

Figure 3.6 Inhibition of SCA-41 on the secretion of *S. aureus* toxins
Different conc. of SCA-41 were added to the mid-log phase of *S. aureus* Mu50 at OD₆₀₀ ≈ 3; the growth of bacteria was monitored; 200 μl TCA was added into 1.8 ml supernatant to precipitate protein; the protein pellet was resuspended in 200 μl Tris-buffer; specific antibodies were used to detect α-hemolysin, enterotoxin B, and TSST-1.

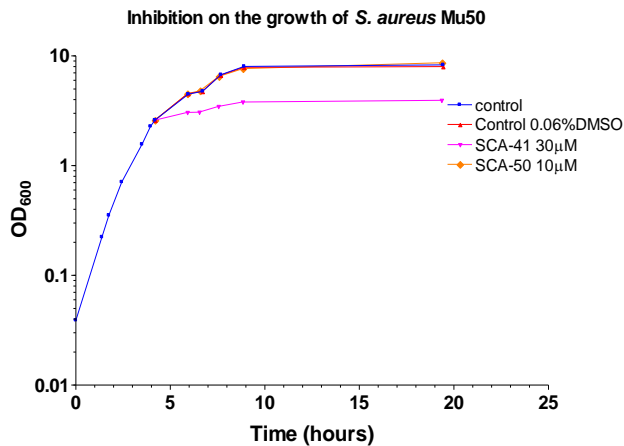


Figure 3.7a Inhibition of SCA-41 and SCA-50 on the growth of *S. aureus* Mu50

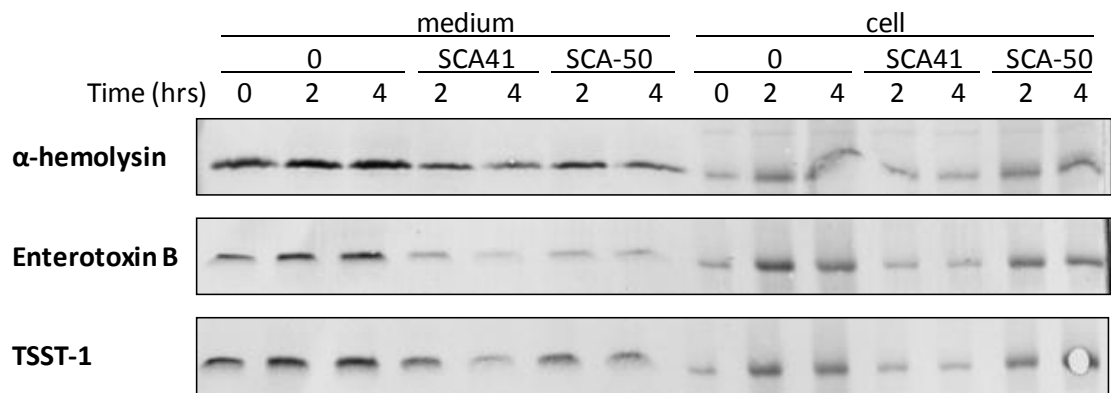


Figure 3.7b Inhibition of SCA-41 and SCA-50 on the secretion of α-hemolysin, Enterotoxin B, and TSST-1

Figure 3.7 Inhibition of SCA-41 and SCA-50 on the secretion of *S. aureus* toxins
30 μM SCA-41 or 10 μM SCA-50 was added to the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 2.6$; the growth of bacteria was monitored; then 2 ml culture was collected after 2 hrs and 4 hrs, and the supernatant and cell pellet fraction were separated; specific antibodies were used to detect α-hemolysin, Enterotoxin B, and TSST-1.

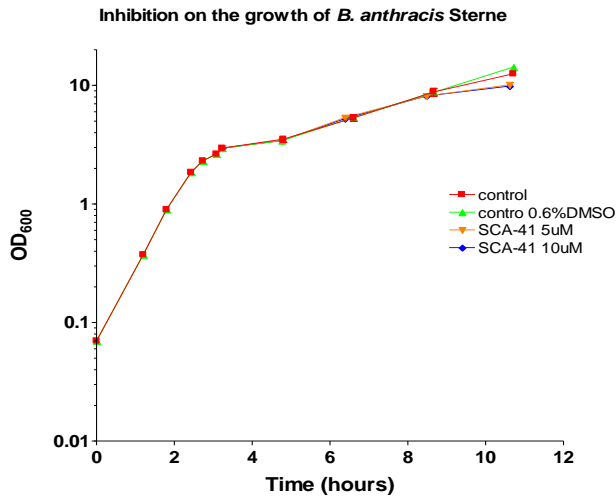


Figure 3.8a Inhibition of SCA-41 on the growth of *B. anthracis* Sterne

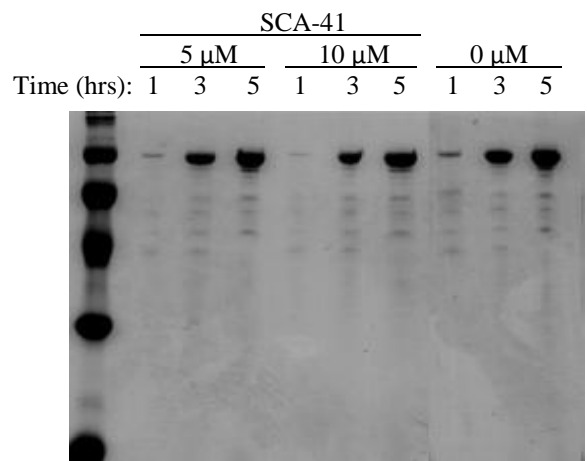


Figure 3.8b Inhibition of SCA-41 on the general secretion of *B. anthracis*

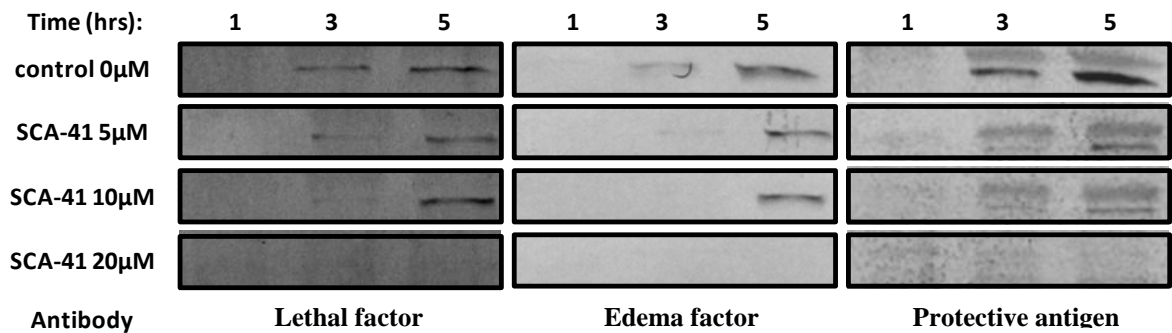


Figure 3.8c Inhibition of SCA-41 on the secretion of specific toxins of *B. anthracis*

Figure 3.8 Inhibition of SCA-41 on the secretion of *B. anthracis* toxins
Different conc. of RB was added to the mid-log phase of *B. anthracis* Sterne at $OD_{600} \approx 2.6$; the growth of bacteria was monitored; then 2ml culture was collected after 1 hr, 3 hrs, and 5 hrs, and the supernatant and cell pellet were separated; 110 μ l TCA was added into 1 ml supernatant to precipitate protein; the protein pellets were suspended in 100 μ l Tris-buffer; specific antibodies were used to detect lethal factor, edema factor, and protective antigen.

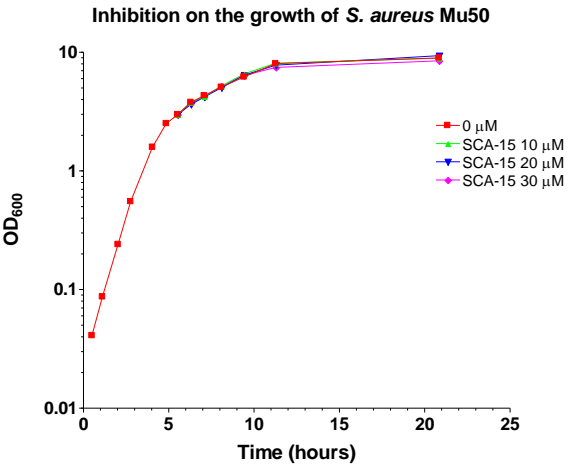


Figure 3.9a Inhibition of SCA-15 on the growth of *S. aureus* Mu50

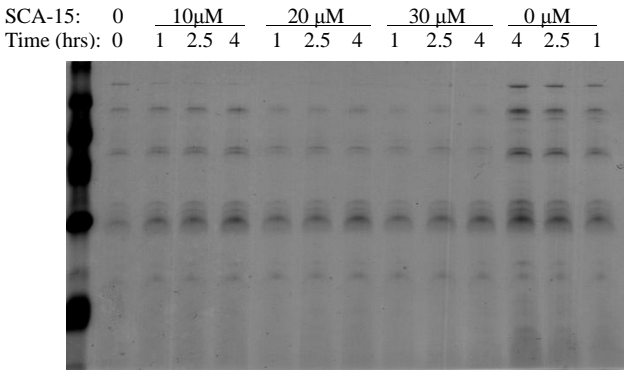


Figure 3.9b Inhibition of SCA-15 on the general secretion of *S. aureus* Mu50

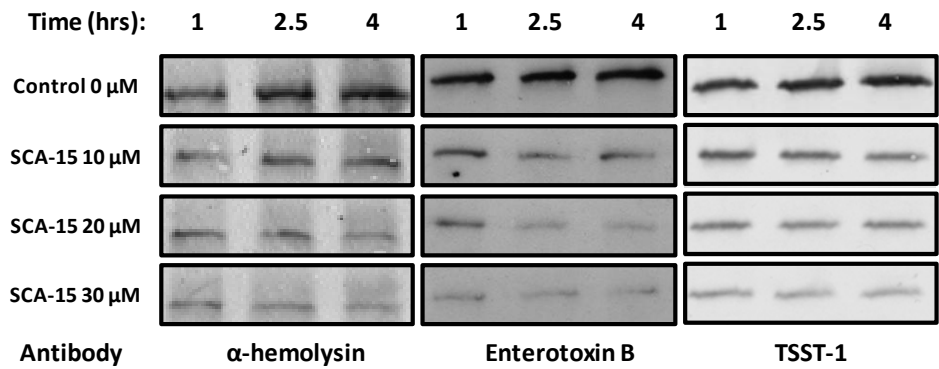


Figure 3.9c Inhibition of SCA-15 on secretion of specific toxins of *S. aureus* Mu50

Figure 3.9 Inhibition of SCA-15 on the secretion of *S. aureus* toxins
Different conc. of SCA-15 was added into the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 3$; the growth of bacteria was monitored; 2 ml cultures were collected after 1hr, 2.5 hrs, and 4 hrs, and the supernatant and cell pellet were separate; 200 μ l TCA was added into 1.8ml supernatant to precipitate protein; the protein pellet was suspended in 200 μ l Tris-buffer; specific antibodies were used to detect α -hemolysin, Enterotoxin B, and TSST-1.

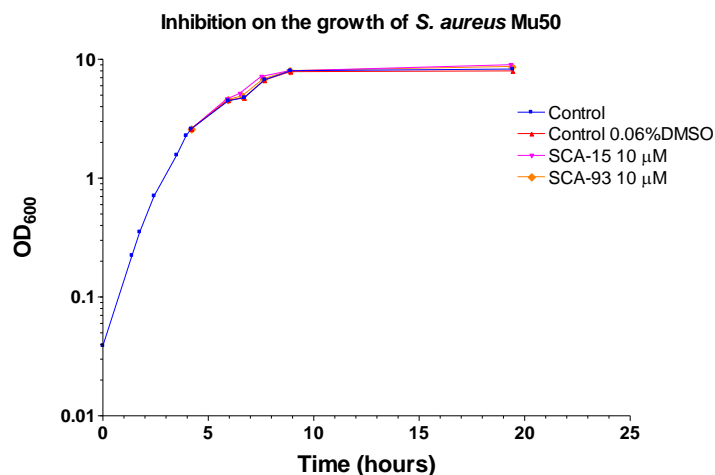


Figure 3.10a Inhibition of SCA-15 and SCA-93 on the growth of *S. aureus* Mu50

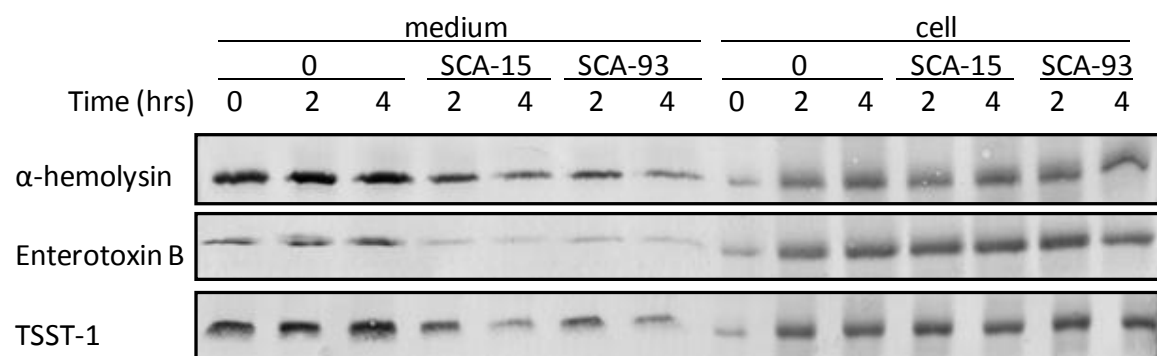


Figure 3.10b Inhibition of SCA-15 and SCA-93 on the secretion of α -Hemolysin, Enterotoxin B, and TSST-1

Figure 3.10 Inhibition of SCA-15 and SCA-93 on the secretion of *S. aureus* toxins

10 μ M SCA-15 or 10 μ M SCA-93 was added to the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 2.6$; the growth of bacteria was monitored; 2 ml culture was collected after 2 hrs and 4 hrs, and the supernatant and cell pellet were separated; 200 μ l TCA was added into 1.8 ml supernatant to precipitate protein; the protein pellet was suspended in 200 μ l Tris-buffer; specific antibodies were used to detect α -hemolysin, enterotoxin B, and TSST-1.

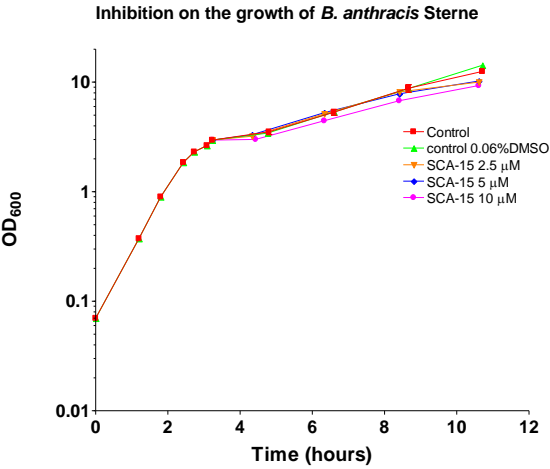


Figure 3.11a Inhibition of SCA-15 on the growth of *B. anthracis* Sterne

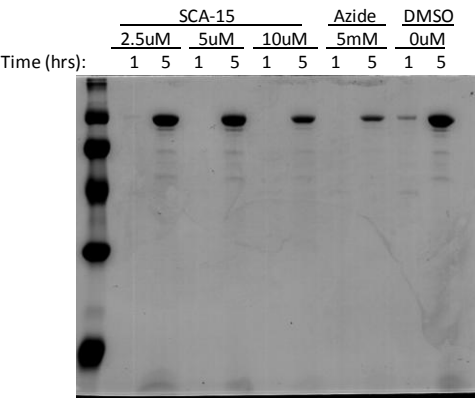


Figure 3.11b Inhibition of SCA-15 on the general secretion of *B. anthracis* Sterne

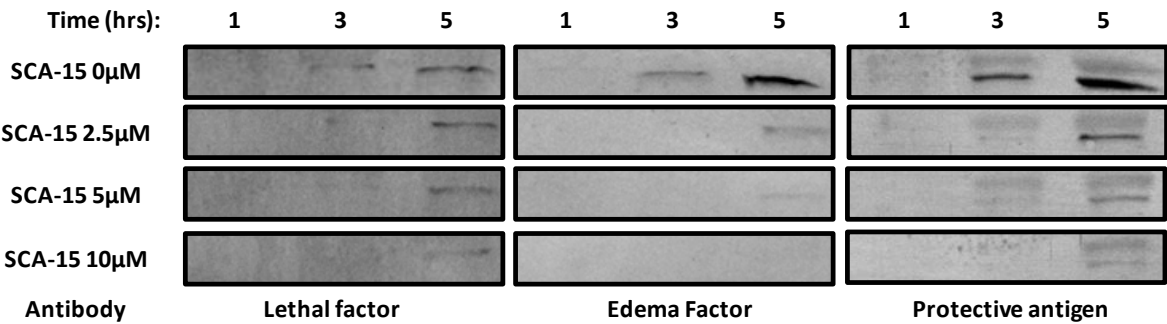


Figure 3.11c Inhibition of SCA-15 on the secretion of specific toxins of *B. anthracis*

Figure 3.11 Inhibition of SCA-15 on the secretion of *B. anthracis* toxins

Different conc. of SCA-15 was added to the mid-log phase of *B. anthracis* Sterne at $OD_{600} \approx 2.6$; the growth of bacteria was monitored; 2 ml culture was collected after 1 h, 3 hrs, and 5 hrs, then the supernatant and cell pellet were separated; 110 μ l TCA was added into 1 ml supernatant to precipitate protein; the protein pellet was resuspended in 100 μ l Tris-buffer; specific antibodies were used to detect lethal factor, edema factor, and protective antigen.

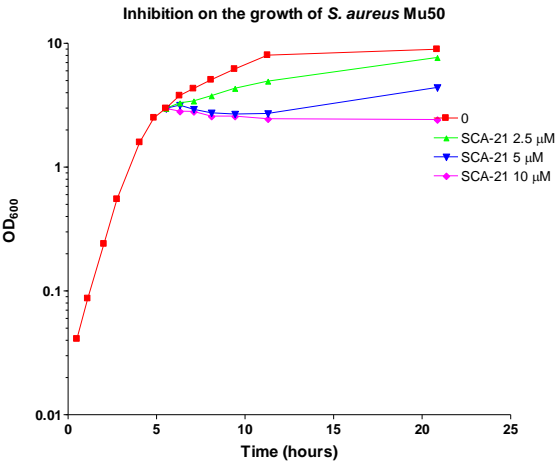


Figure 3.12a Inhibition of SCA-21 on the growth of *S. aureus* Mu50

SCA-21: 0 2.5 μ M 5 μ M 10 μ M 0 μ M
Time (hrs): 0 1 2.5 4 1 2.5 4 1 2.5 4 4 2.5 1

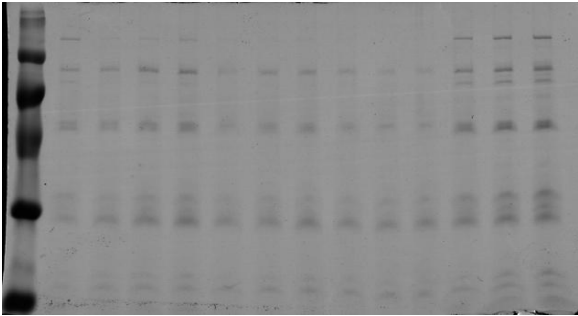


Figure 3.12b Inhibition of SCA-21 on general secretion of *S. aureus* Mu50

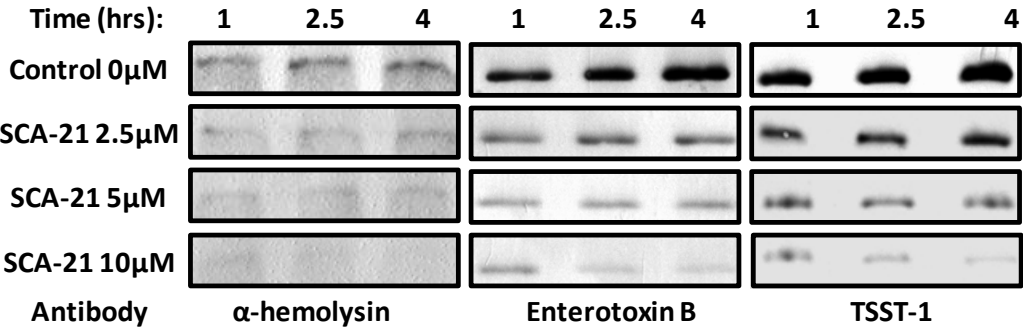


Figure 3.12c Inhibition of SCA-21 on the secretion of α -hemolysin, enterotoxin B, and TSST-1

Figure 3.12 Inhibition of SCA-21 on the secretion of *S. aureus* toxins

Different conc. of SCA-21 was added to the mid-log phase of *S. aureus* Mu50 at OD₆₀₀ \approx 3; the growth of bacteria was monitored; 2 ml culture was collected after 1 h, 2.5 hrs, and 4 hrs, then the supernatant and cell pellet were separated; 200 μ l TCA were added into 1.8 ml supernatant to precipitate protein; the protein pellet was resuspended in 200 μ l Tris-buffer; specific antibodies were used to detect α -hemolysin, enterotoxin B, and TSST-1.

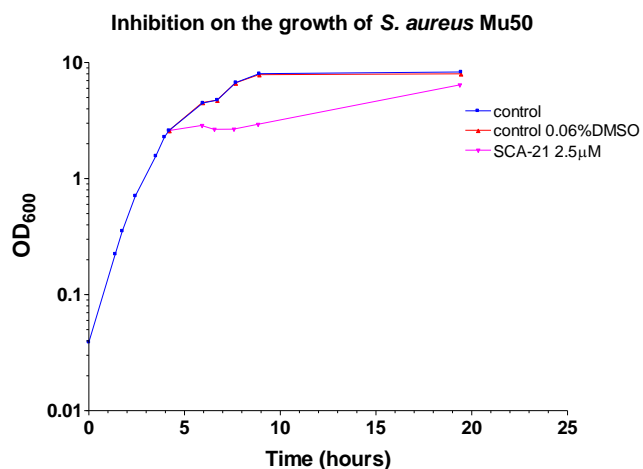


Figure 3.13a Inhibition of SCA-21 on the growth of *S. aureus* Mu50

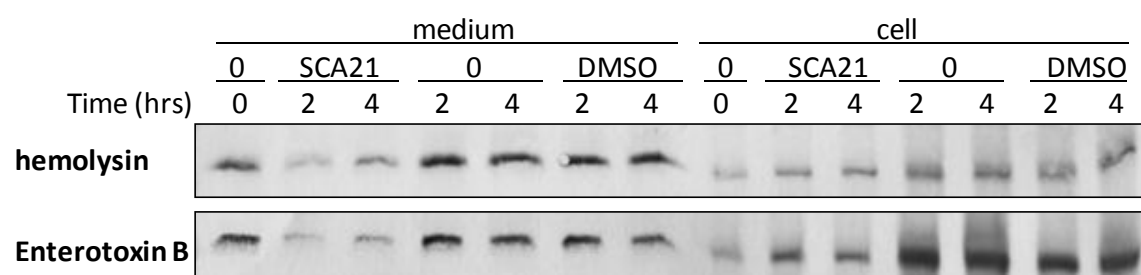


Figure 3.13b Inhibition of SCA-21 on the secretion of α -Hemolysin and enterotoxin B

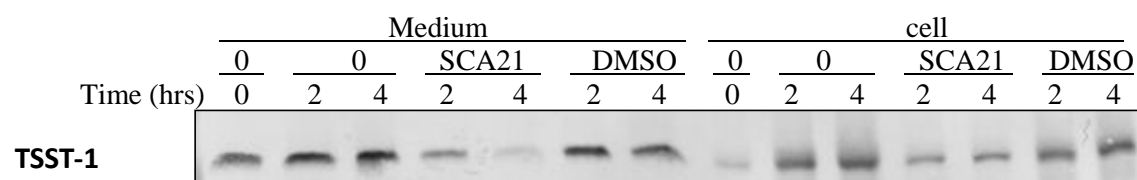


Figure 3.13c Inhibition of SCA-21 on the secretion of TSST-1

Figure 3.13 Inhibition of SCA-21 on the secretion of *S. aureus* toxins

2.5 μ M SCA-21 was added to the mid-log phase of *S. aureus* Mu50 at $OD_{600} \approx 2.6$; the growth of bacteria was monitored; 2 ml culture was collected after 2 hrs and 4 hrs, then the supernatant and cell pellet was separated; 200 μ l TCA were added into 1.8 ml supernatant to precipitate protein; the protein pellet was resuspended in 200 μ l Tris-buffer; specific antibodies were used to detect α -hemolysin, enterotoxin B, and TSST-1.

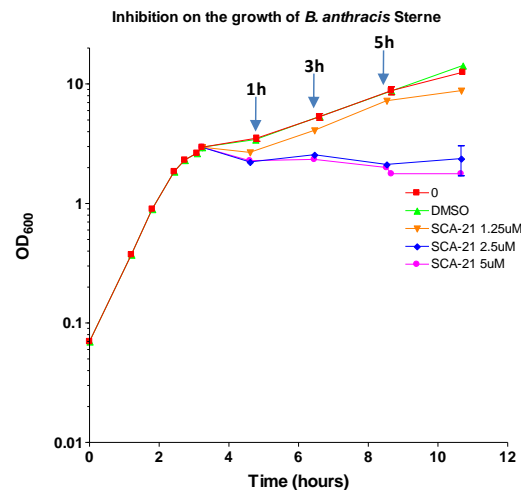


Figure 3.14a Inhibition of SCA-21 on the growth of *B. anthracis* Sterne

SCA-21 (μM): $\frac{1.25}{1}$ $\frac{2.5}{3}$ $\frac{5}{5}$ $\frac{0}{3}$ $\frac{0}{5}$
Time (hrs):

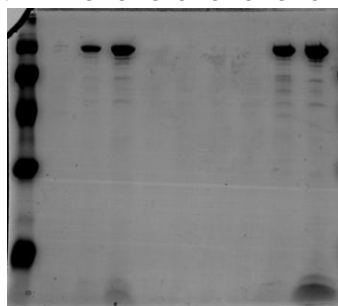


Figure 3.14b Inhibition of SCA-21 on the general secretion of *B. anthracis*

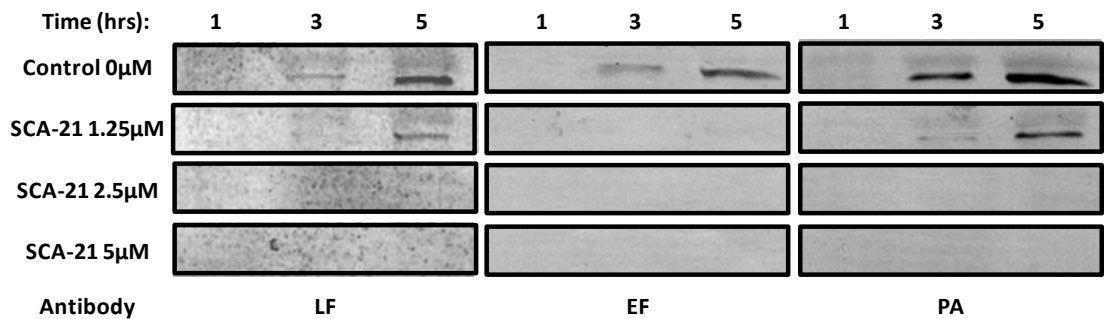


Figure 3.14c Inhibition of SCA-21on the secretion of specific toxins of *B. anthracis*

Figure 3.14 Inhibition of SCA-21 on the secretion of *B. anthracis* toxins

Different conc. of SCA-21 was added to the mid-log phase of *B. anthracis* Sterne at $\text{OD}_{600} \approx 2.6$; the growth of bacteria was monitored; 2 ml culture was collected after 1 hr, 3 hrs, and 5 hrs, then the supernatant and cell pellet were separated; 110 μl TCA was added into 1ml supernatant to precipitate protein; the protein pellet was suspended in 100 μl Tris-buffer; specific antibodies were used to detect lethal factor, edema factor, and protective antigen.

The Antimicrobial Mechanism of RB analogs

Previous studies indicated that there are three mechanism involved in the antimicrobial activity of RB. RB could specific binding to essential enzyme and inhibit its function, like DNA polymerase [108, 110], RNA polymerase [109]. RB might release chloro and iodo group at certain condition, these halo groups have antimicrobial activity. Moreover, iodine could enhance yield of singlet oxygen via photooxidation [126]. RB is a photosensitizer [116], and it could generate singlet oxygen through photooxidation [117, 126]. Singlet oxygen could cause cell damage and bacteria death. SCA-41 don't have halo group, but it showed better bacteriostatic and bactericidal effect than RB. We don't know whether its antimicrobial activities are due to inhibition on SecA, other enzyme, or photooxidation. In this study, different scavengers were used to investigate whether the antimicrobial activity of RB and RB analogs were due to photooxidation.

Bacteriostatic Effect was not due to Inhibition on H^+ -ATPase Activity Because RB, SCA-41, and SCA-43 could inhibit the ATPase activity of EcF1F0 (Table 3.2), therefore 0.25% glucose was added into growth medium to bypass affecting the H^+ ATPase activity. Our result showed that with or without glucose, the MIC_{50} of RB and RB analogs were the same (Table 3.3), indicating the bacteriostatic effect of RB and RB analogs were independent of proton ATPase activity.

The Negative Effect of DMSO on the Enzymatic Effect of RB Because synthesized RB analogs were not soluble in water, they were dissolved in 100% DMSO. To investigate whether DMSO could affect the ATPase activity, BaSecA2 was incubate with 10% DMSO for 1 hour, 30 min and 4 min, then subjected to ATPase assay. Although 30 min incubation result showed DMSO slightly increased ATPase activity (Fig. 3.15), 1 h and 4 min incubation result showed DMSO slightly decreased ATPase activity (Fig. 3.15), suggesting that 10 % DMSO do

not significantly affect the ATPase activity of BaSecA2. Interestingly, Fig. 3.15 showed that the ATPase activity of BaSecA2 was increased with pre-incubation time. Previous study demonstrated that RB could bind with DNA polymerase and inactivate its function via photooxidation [110]. DMSO could act as hydroxyl radical scavenger [118] and could protect cells from photooxidation damage. To test whether DMSO could affect inhibition effect of RB on the ATPase activity of BaSecA2, *in vitro* RB inhibition assay was carried with or without DMSO. Our results showed that DMSO could decrease the *in vitro* inhibition of RB (Fig. 3.16). Without DMSO, IC_{50} is around 3 μ M, but in presence of 10 % DMSO, IC_{50} is around 25 μ M (Fig. 3.16). Although our ATPase inhibition assay was carried at normal room light condition, this result suggested that photooxidation might also contribute to inhibition on ATPase activity.

The Negative Effect of DMSO on Bacteriostatic Effect of RB To determine whether DMSO affects the *in vivo* inhibition assay result, RB was subjected to do inhibition assay with 2.5% DMSO or without DMSO. Our result showed that without DMSO, MIC_{50} of RB on the growth of *E. coli* NR698 and *B. subtilis* 168 were 9 μ M and 42 μ M (Fig. 3.17), but in presence of 2.5% DMSO, MIC_{50} were reduced to 18 μ M and 72 μ M (Fig. 3.17). DMSO could act as hydroxyl radical scavenger [118] and could protect cells from photooxidation damage. Although our inhibition assay was carried at normal room light condition, this result suggested that photooxidation might also contribute to the bacteriostatic effect of RB.

The Negative Effect of DMSO on Bactericidal Effect of RB To determine whether DMSO affects the bactericidal effect, RB was subjected to killing assay with 2.5% DMSO or without DMSO. Our result showed that 40 μ M RB could kill two log *B. subtilis* 168 without DMSO, however in presence of 2.5% DMSO could not kill even half log number of bacteria (Fig. 3.18). DMSO could act as hydroxyl radical scavenger [118] and could protect cells from

photooxidation damage. Although our inhibition assay was carried at normal room light condition, this result suggested that photooxidation might also contribute to the bactericidal effect of RB.

The Effect of Light on Bactericidal Effect of RB and SCA-41 The growth of *S. aureus* 6538 was monitored, SCA-41 or RB was added into cell culture when OD₆₀₀ reach 1, or added into overnight culture, and the cell was growth with or without light. After adding RB or SCA-41, cell culture was taken out at different time point and spread on LB plates with different dilution. Plate assay results showed that RB and SCA-41 had bactericidal effect on both log phase and stationary phase *S. aureus* 6538 (Fig. 3.19a-b). Light could dramatically increase bactericidal effect of 80 μ M RB; while light slightly affect bactericidal result of 40 μ M SCA-41 (Fig. 3.19a-b). And in presence of 80 μ M RB, bacteria can grow back after overnight incubation; however, in presence of 40 μ M SCA-41, bacteria couldn't survive even after overnight incubation (Fig. 3.19a). Thus, SCA-41 had much better bactericidal effect than RB, and photooxidation might contribute to the bactericidal effect of RB, not for SCA-41.

Bactericidal effect of SCA-41 might be independent with Photooxidation Histidine is a scavenger of singlet oxygen [117-118], therefore it could protect bacteria against photo-damage which is involved reaction with singlet oxygen [117]. Therefore, histidine was used to investigate whether singlet oxygen plays important role in antimicrobial activity of RB and SCA-41. Killing assay results showed that histidine could dramatically reduce bactericidal effect of RB both in light (3 log numbers) and in dark (1 log number) (Fig. 3.20). Light slightly increased bactericidal effect of SCA-41, and histidine dramatically increased bactericidal effect of SCA-41 (Fig. 3.20), suggesting that singlet oxygen might not contribute to the antimicrobial activity of SCA-41. Phloxine B is a RB analog, with four bromo groups instead of four iodo groups. And

previous study demonstrated that phloxine B had phototoxicity [117]. It could cause membrane and DNA damage through ROS pathway and free hydroxyl pathway [117]. The reaction of both pathway required Fe^{2+} . In this study, 100 μM phenanthroline was used to chelate Fe^{2+} and block these two pathways. Phenanthroline increased the bacteriostatic effect of SCA-41 (Fig. 3.21), while it decreased the bacteriostatic effect of RB (Fig. 3.21), indicating that photooxidation might not be the antimicrobial mechanism of SCA-41.

Table 3.2 *In vitro* inhibition effect of RB analogs against EcF1F0

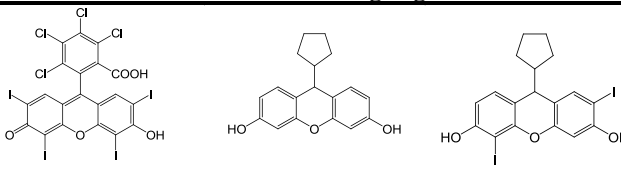
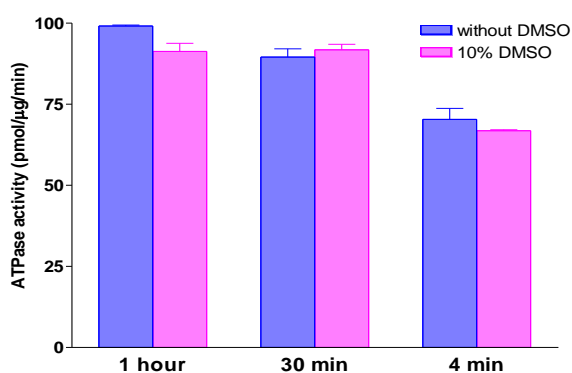
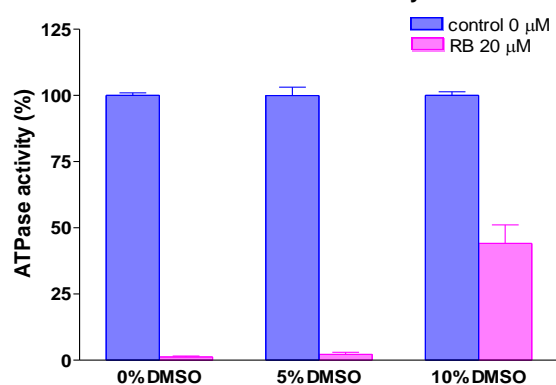
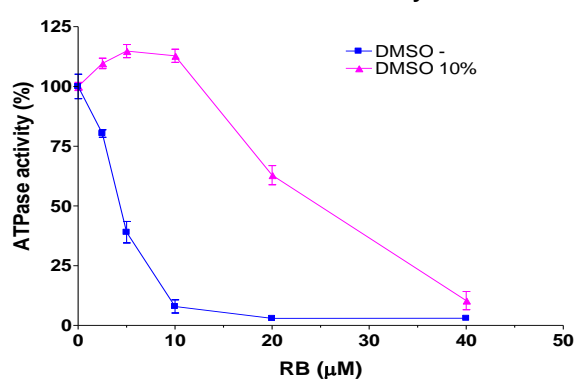
			
	RB	SCA-41	SCA-43
	973.67	282.33	534.13
EcF1F0, IC₅₀ (μM)	14	60	17

Table 3.3 Bacteriostatic effects of RB and RB analogues were independent of inhibition on proton ATPase

	Inhibition on the growth of bacteria, MIC ₅₀ (μM)					
	<i>S. aureus</i> 6538		<i>B. anthracis</i> Sterne		<i>E. coli</i> NR698	
	0% Glu	0.25% Glu	0% Glu	0.25% Glu	0% Glu	0.25% Glu
RB	26.5	25	6	5	13.5	13
SCA-41	6	5.5	4	4	7.5	6
SCA-43	3	3	1.5	1.5	9	5

The endogenous ATPase activity of BaSecA2**Figure 3.15** The effect of DMSO on the intrinsic ATPase activity of BaSecA2

BaSecA2 was incubated with reaction buffer (Tris-HCl pH 7.5, 2 mM Mg²⁺), or/and 10% DMSO for 4 min, 30 min, or 1 hour, then add 2 mM ATP to do ATPase assay at 30°C for 40 min.

Inhibition on the ATPase activity of BaSecA2**Inhibition on the ATPase activity of BaSecA2****Figure 3.16** DMSO decrease the *in vitro* inhibition effect of RB against BaSecA2

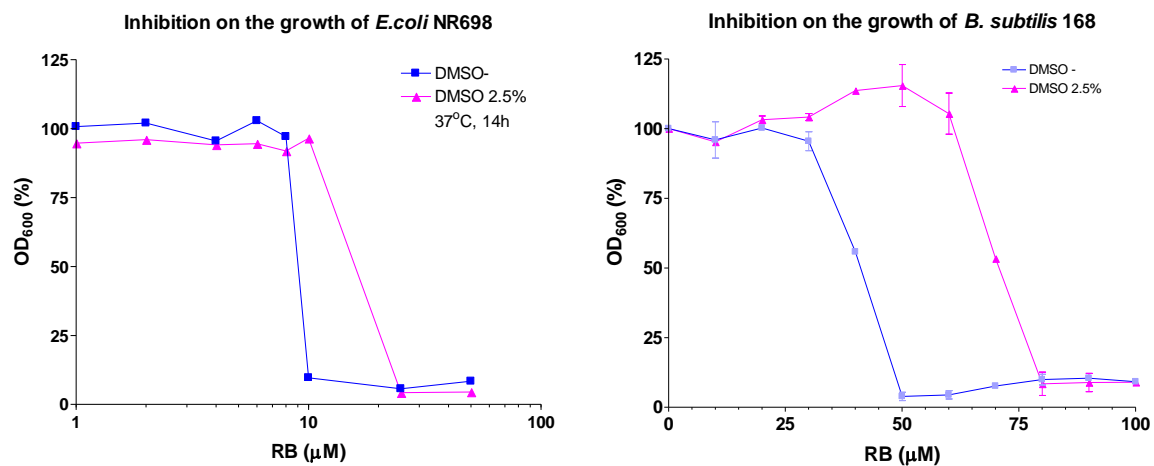


Figure 3.17 DMSO decrease the bacteriostatic effect of RB

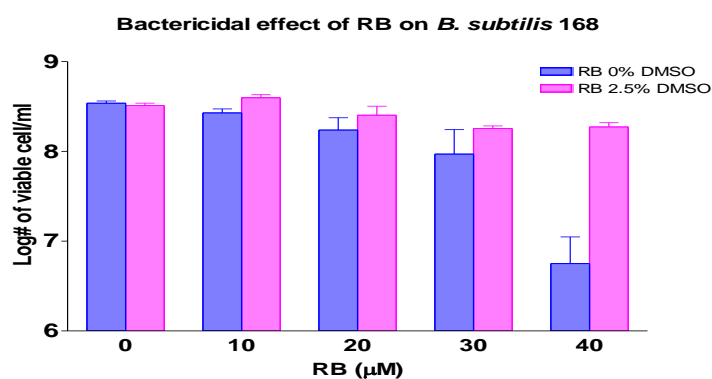


Figure 3.18 DMSO decrease bactericidal effect of RB against *B. subtilis* 168

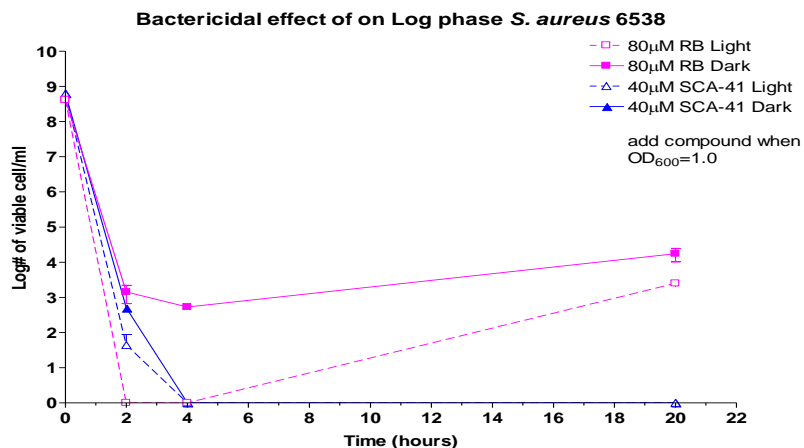


Figure 3.19a The effect of light on the bactericidal effects of RB and SCA-41 against *S. aureus*

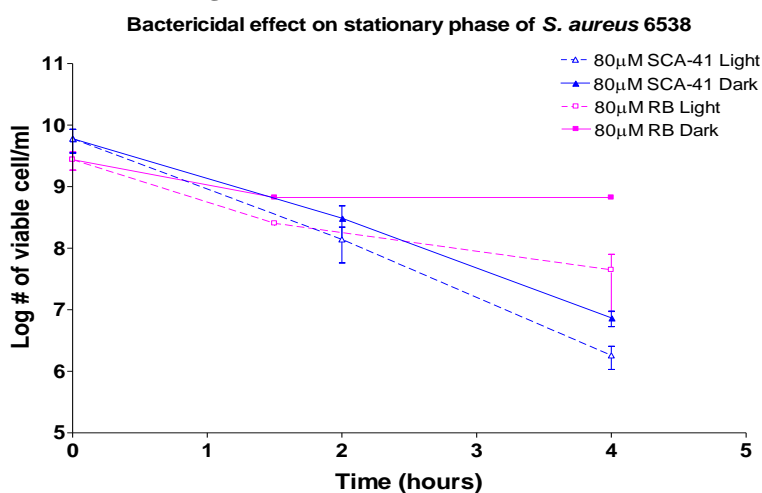


Figure 3.19b The effect of light on bactericidal effects of RB and SCA-41 against *S. aureus*

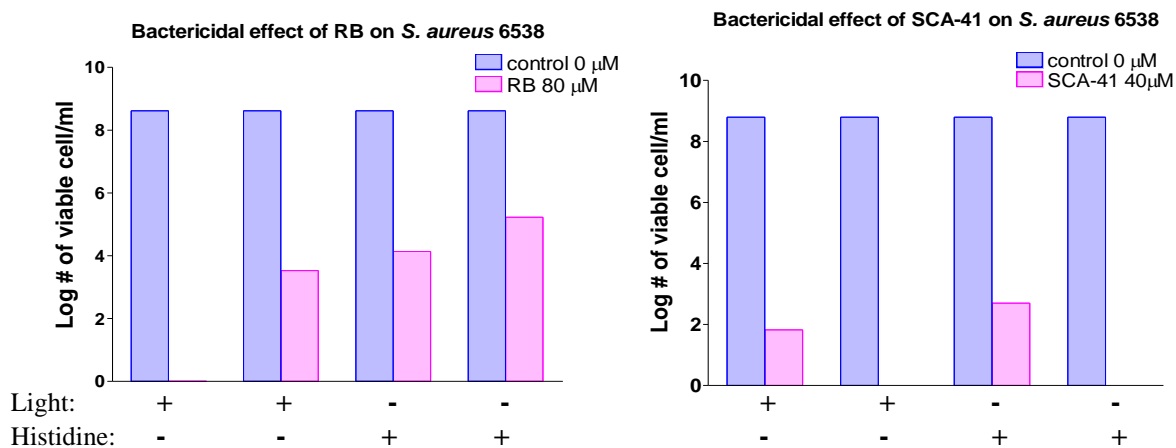


Figure 3.20 The effect of singlet oxygen on the bactericidal effects of RB and SCA-41
37°C, 2 hrs killing; histidine: 10 mM; light: 15 Watt fluorescent light.

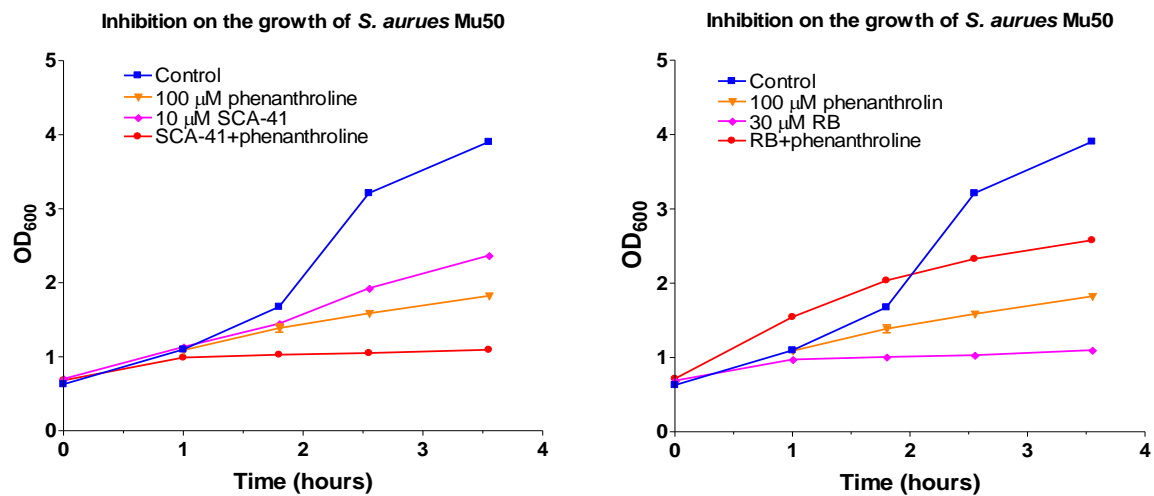


Figure 3.21 The effect of photooxidation on bacteriostatic effects of RB and SCA-41

Interaction between Inhibitors and SecA Homologues

To truly validate SecA as the target of our inhibitors, we need to investigate interaction between SecA and inhibitors, identify how many proteins interact with inhibitors, and determine whether interactions with other proteins could contribute to observed antimicrobial effect.

Azide is a SecA inhibitor, and previous study showed that azide enhances the formation of a trypsin-resistant 30 kDa fragment, which is related with SecA membrane insertion [37, 62, 88]. If compound could bind with target protein, it might induce conformational change and result in different trypsin digestion pattern. In this study, altered trypsin sensitivity assay was used to detect interaction between inhibitors and SecA homologues. First, we investigate whether interaction between SCA-15 and EcSecAN68 could induce conformational change and result in different trypsin digestion pattern. Our result showed that adding 80 μ M SCA-15 could dramatically change the trypsin digestion pattern of EcSecAN68 (Figure 3.22a), suggesting that the interaction between the compound and protein induced conformational change of protein and changed protease sensitivity. Adding ATP alone slightly changed trypsin digestion pattern, which is different with the pattern resulted by adding SCA-15 (Fig. 3.22a). And adding SCA-15 in addition to ATP resulted in similar trypsin digestion pattern as by adding SCA-15 alone (Fig. 3.22a). These results suggested that SCA-15 and ATP might bind to different sites of EcSecAN68. Fig. 3.22b confirmed that SCA-15 could change trypsin resistance, and there were three distinct trypsin resistant bands showed in the presence of 80 μ M SCA-15. Increasing SCA-15 concentration to 160 μ M caused similar results, and the trypsin resistant band around 15 kDa became more prominent (Fig. 3.22b). Since SCA-15 contains azido group, it might be able to photo-crosslink to protein by UV exposure, thus subsequent MS/MS results could identify binding domain of SCA-15. However, with or without UV showed similar MS/MS results, suggesting that photo

cross-linking rate might be too low (data not shown). Moreover, SCA-15 also could induce conformational change of SaSecA1 and SaSecA2 and change trypsin resistance (Fig. 3.23). High concentration of SCA-41 and RB could protect wild type EcSecA from trypsin digestion (Fig. 3.24-3.25). All these results supported that DARTS might be a good method to identify the drug targets of SCA-15.

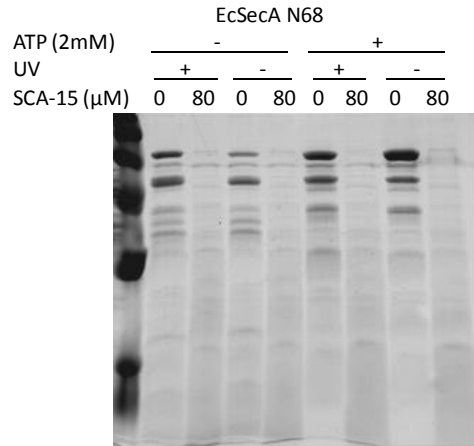


Figure 3.22a SCA-15 change trypsin resistance of EcSecAN68
10 μg EcSecA N68 was incubated at 37°C for 10 min with or without SCA-15, then added 1.2 μg trypsin and incubate on ice for 25 min. 15% SDS-PAGE

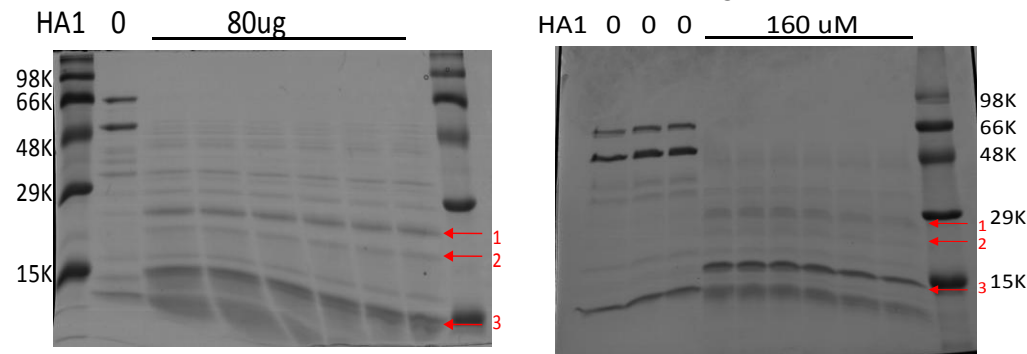


Figure 3.22b SCA-15 change trypsin resistance of EcSecAN68
10 μg EcSecA N68 was incubated at 37°C for 10 min with or without SCA-15, then added 1.2 μg trypsin and incubate on ice for 25 min. 15% SDS-PAGE

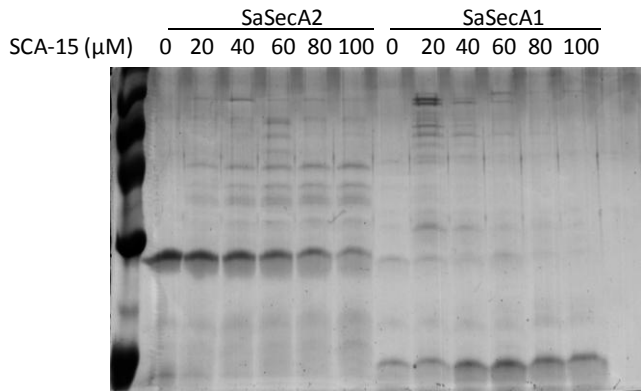


Figure 3.23 SCA-15 change trypsin resistance of SaSecA1 and SaSecA2
10 μg SaSecA1 and SaSecA2 was incubated at 25°C for 10 min with or without SCA-15, then added 1.2 μg trypsin and incubate on ice for 25 min. 15% SDS-PAGE

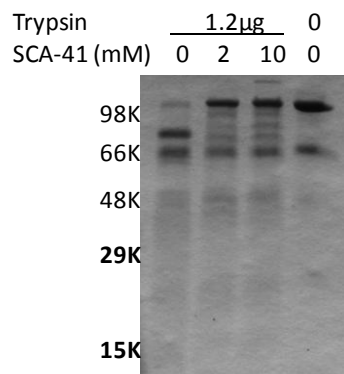


Figure 3.24 SCA-41 could protect EcSecA from trypsin digestion

10 μ g EcSecA was incubated at 30°C for 30 min with or without SCA-41, then added 1.2 μ g trypsin and incubate on ice for 20 min.

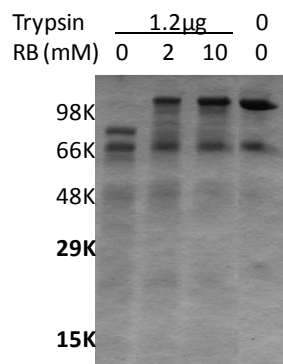


Figure 3.25 RB could protect EcSecA from trypsin digestion

10 μ g EcSecA was incubated at 30°C for 30 min with or without RB, then added 1.2 μ g trypsin and incubate on ice for 20 min.

Target Identification at Whole Cell Extract Level

To truly validate SecA as the target of those inhibitors developed in our study, we need to investigate interaction between SecA and inhibitors, identify how many proteins interact with inhibitors, and determine whether interactions with other proteins could contribute to observed antimicrobial effect.

Affinity chromatography is the most widely used method to identify drug target, however the application of affinity chromatography was limited, because of the need of vast structural diversity, the complexity of biologically active small molecules, and high level of nonspecific binding of non-target proteins [121-124]. Drug affinity responsive target stability (DARTS) is a new method to identify drug targets [121, 125]. The principle of this method is that a small ligand would stabilize its target protein's structure and result in changing of protease resistance, which offer the chance of target identification [121, 125]. Affinity chromatography is a positive drug target selection method by pulling out the target proteins and leaving non-target proteins behind [121], while DARTS is a negative drug target selection method by digesting away non-target proteins while leaving behind the target proteins [121]. In this study, these two methods were both used to validate that SecA is a real target of pyrimidine analogues.

Targets of SCA-15 identified by using DARTS Next, DARTS was used to identify drug targets of SCA-15 from whole cell lysate of *E. coli* MC4100. Our results identified three trypsin resistant bands in 1-D Gel (Fig. 3.26a-b), and western blot result showed that one band was positive with EcSecA antibody (Fig. 3.26a). That band was cut and subjected to Maldi-MS/MS, however MS/MS result didn't prove *E. coli* SecA was there. This result might because larger amount of protein might be required for MS/MS than Western blot.

Targets of SCA-21 identified by using DARTS DARTS was used to identify drug targets of SCA-21 from whole cell lysate of *E. coli* MC4100. Our results identified that three trypsin resistant bands in 1-D Gel (Fig. 3.27a-b), and Western blot results showed that one band was positive with EcSecA antibody (Fig. 3.27a). That band was cut and subjected to Moldi-MS/MS, however MS/MS result didn't prove *E. coli* SecA was there. This result might because larger amount of protein required for MS/MS than western blot.

Pull down assay To truly validate SecA as the target for the proposed inhibitors, we need to do protein pull-down assay to identify potential target, and further determine whether interactions with proteins other than designed target could contribute to the observed antimicrobial effect. For this purpose, a long linker was attached to pyrimidine analogs. Our results showed that SCA-91 and SCA-101 retained reasonable inhibition effect on the ATPase activity of SecA homologues (Table 3.4). Thus, these compounds were further immobilized on beads or conjugated to biotin (Table 3.5). Conjugation with biotin slightly increased inhibition effect of SCA-91 (Table 3.4). Western blot results showed that these compounds could pull down EcSecA (Fig. 3.28) and SaSecA1 (Fig. 3.29) from whole cell lysate, supporting that SecA is a real target of SCA-15.

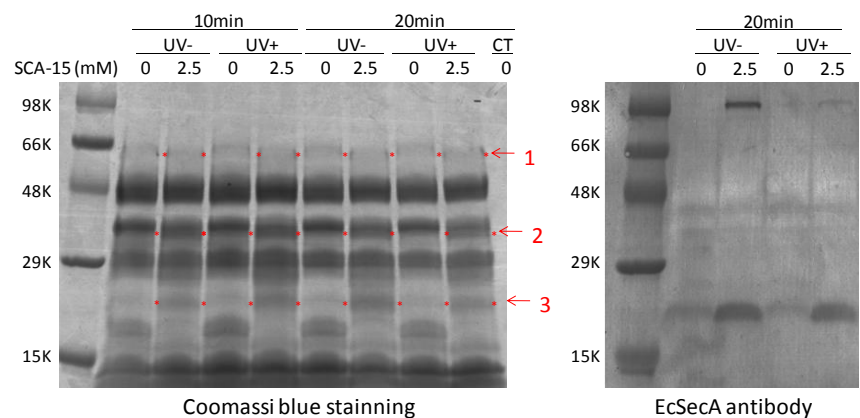


Figure 3.26a DARTS results confirm EcSecA as a target of SCA-15

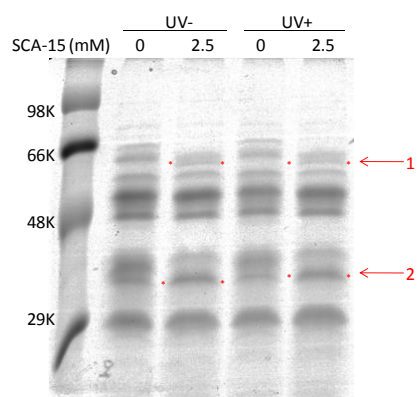


Figure3.26b DARTS results confirm EcSecA as a target of SCA-15
200 µg whole cell lysate of *E. coli* MC4100 was incubated on ice for 2 hrs with or without SCA-15, then exposed to UV for 0 sec or 90 sec, followed by digesting with 50 µg trypsin at 25°C for 10-20 min. The digestion was stopped by adding sample buffer and boiling for 20 min. 10-20% SDS-PAGE.

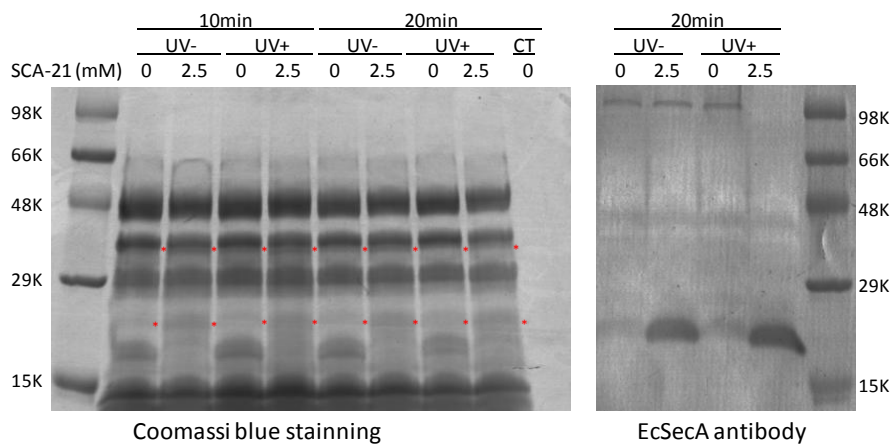


Figure 3.27a DARTS results confirmed EcSecA as a target of SCA-21

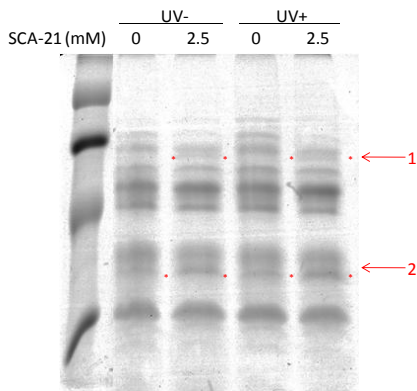


Figure 3.27b DARTS results confirmed EcSecA as a target of SCA-21
Whole cell lysate of *E. coli* MC4100 was incubated on ice for 2 hrs with or without SCA-21, then exposed to UV for 0 sec or 90 sec, followed by digesting with 50 μ g trypsin at 25°C for 10-20 min. The digestion was stopped by adding sample buffer and boiling for 20 min. 10-20% SDS-PAGE.

Table 3.4 The effect of linker on *in vitro* inhibition effect of pyrimidine analogs

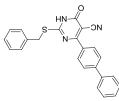
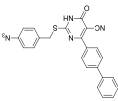
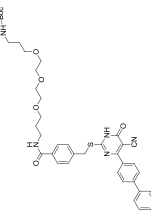
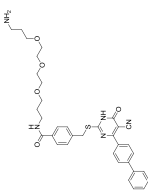
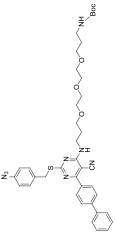
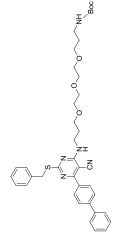
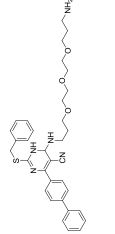
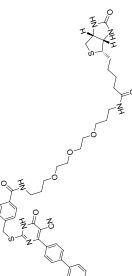
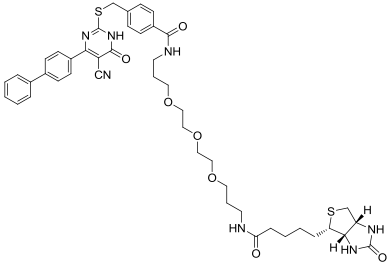
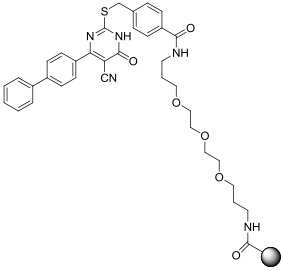
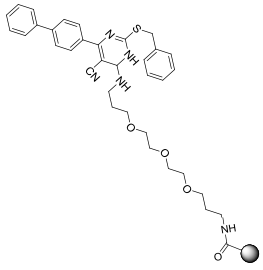
								
	BW-SCA-13	BW-SCA-15	SCA-91	SCA-92	SCA-99	SCA-100	SCA-101	SCA-104
EcSecA N68	20	10	25	45	50	60	25	20
BaSecA2	140	20	40	140	>200	>200	20	33

Table 3.5 Pyrimidine analogs were conjugated to biotin or immobilized on beads

		
SCA-104	SCA-105	SCA-113

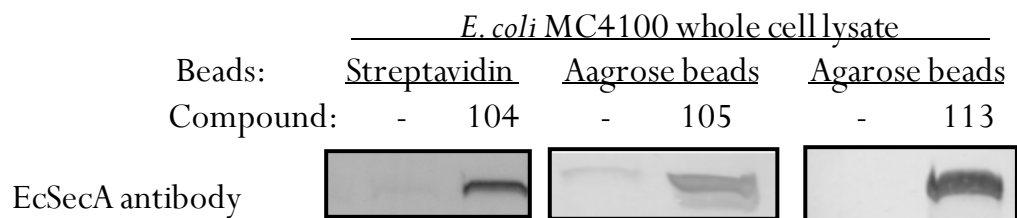


Figure 3.28 EcSecA as a target of pyrimidine analogs

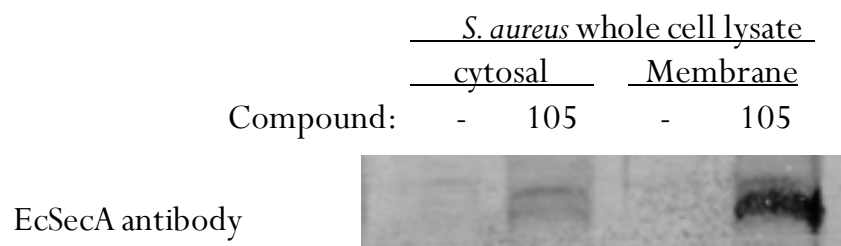


Figure 3.29 SaSecA1 as a target of pyrimidine analogs

Conclusion and Discussion

In this study, three structurally different classes of SecA inhibitors were developed and optimized. Several SecA inhibitors showed promising *in vitro* inhibition effect on ATPase activities of SecA homologues, as well as bacteriostatic effects and bactericidal effects against *B. anthracis* Sterne and numerous drug resistant *S. aureus* strains. However, to validate SecA as a real target of these inhibitors, we need to determine whether their antimicrobial activities are due to inhibition on SecA *in vivo*.

SecA is involved in forming a protein-conducting channel that spans the entire membrane [38-39] [96-97]. Thus, drugs targeting SecA might be directly accessible from the extracellular matrix and exert their effects without entering the cell, and bypassing the major negative effect of efflux transporters in bacteria, which is a major mechanism for the development of current drug-resistance [72-76]. *S. aureus* Mu50 and *S. aureus* N315 are resistant to QacA efflux-mediated antibiotics [100]. SCA-41, SCA-50, SCA-15, SCA-93, and SCA-21 are best inhibitors developed from our study. They showed promising bacteriostatic effects or/and bactericidal effect on *S. aureus* Mu50 or/and *S. aureus* N315, suggesting that these SecA inhibitors might be able to overcome QacA mediated efflux. NorA and MepA are two major efflux pumps of *S. aureus*, and they have little negative effect on the antimicrobial activities of SecA inhibitors, suggesting that targeting SecA might be able to by-pass efflux pump.

Sec system is responsible for the secretion of many toxins and virulence factors, which play important roles in the pathogenesis of bacterial infection [33-35]. Enterotoxin B, α -hemolysin, and toxic shock syndrome toxin 1 (TSST-1) are three toxins of *S. aureus*, which containing Sec-dependent signal peptide (Table 0.1) [34]. Lethal factor (LF), edema factor (EF), and protective antigen (PA), are three major component toxins of *B. anthracis*, and all of them have

Sec-dependent signal peptide [35]. Our results showed SecA inhibitors could inhibit secretion of these important toxins of *S. aureus* and *B. anthracis* Sterne, suggesting that these inhibitors could inhibit the function of SecA *in vivo* and reduce virulence of these important bacteria pathogens. Virulence target-based therapies are not sufficient to combat infection, but targeting SecA homologues is not only reduce virulence but also decrease viability, dramatically increasing the chance of control bacterial infection and reduce the occurrence of drug resistance.

Previous studies indicated that photooxidation and halo groups contribute to the antimicrobial activities of RB as well as inhibition on specific enzymes. However, our results showed that photooxidation might not contribute to the antimicrobial activities of RB analogs, which do not have halo group and have better antimicrobial activities than RB, suggesting that the ability of inhibition on specific enzyme is increased in RB analogs. And our results showed that the bacteriostatic effects of SCA-41 and SCA-43 were independent of inhibition on proton ATPase activity.

Binding with small ligand could stabilize the structure of its target protein and results in changing of protease resistance, which offered the chance of target identification. In this study, partial trypsin digestion was used to investigate whether inhibitors could bind with SecA homologues. Our results showed that inhibitors could induce the change of trypsin resistance of SecA homologues, suggesting that inhibitors could bind with SecA homologues and change their conformation and stability. Affinity chromatography is a positive drug target selection method by pulling out the target proteins and leaving non-target proteins behind [121], while DARTS is a negative drug target selection method by digesting away non-target proteins while leaving behind the target proteins [121]. Target identification assays confirmed that SecA inhibitor could identify SecA homologues from whole cell lysate of *E. coli* and *S. aureus*, supporting that SecA

homologues are the targets of these inhibitors. However, other proteins were also pulled down from affinity chromatography, including D-ribosomal binding protein, tryptophanase, Trp repressor binding protein, Glutamate dehydrogenase, Acyl-CoA carboxylase, Acyl-CoA dehydrogenase, and Enoyl-(acyl-carrier protein) reductase. It's possible that inhibitors have multiple targets inside bacteria. But when we analyze pull down assay data, we have to consider about the limitation of affinity chromatography, which is based on binding of the drug to its target proteins. When we modify small molecules with long linker or conjugated with biotin, the binding ability and bioactivity of the small molecule might be changed, resulting in nonspecific binding with a lot of non-target proteins. Therefore, we need to evaluate whether inhibition on those proteins could result in the antimicrobial phenotype of those inhibitors. The proteins identified from pull down assay might not be essential for bacteria, except for SecA and Enoyl-(acyl-carrier protein) reductase. Our inhibitors could inhibit the enzyme activity of SecA, have bacteriostatic effect and bactericidal effect against Gram-positive bacteria, inhibit the secretion of bacteria toxin, and they could bind with SecA homologues and pull down SecA homologues from whole cell lysate. All these results validate SecA homologues as drug targets of our inhibitors. Enoyl-(acyl-carrier protein) reductase is an attractive target for developing antimicrobials, because it is a key enzyme of the type II fatty acid synthesis system, essential for bacteria, and the conservation across bacteria without human counterpart. We need further investigate whether our inhibitors could inhibit its enzyme activity. If a single small molecule could target two essential and specific bacteria proteins, that would be an exciting discovery.

GENERAL DISCUSSION

The emergence and dissemination of multidrug resistance, bacterial pathogens have been causing a serious public health problem in recent years. To overcome the existing drug resistant problem, there is an urgent need to find new antimicrobials, especially those against drug-resistant strains of bacteria. SecA is the central component of Sec-dependent secretion pathway, which is responsible for the secretion of many essential proteins as well as some toxins and virulence factors. Two SecA homologues are identified in some Gram-positive pathogenic bacteria. SecA1 is involved in general secretion pathway and essential for viability, whereas SecA2 contribute to the virulence of some important Gram-positive pathogen by playing a role in a specialized secretion pathway. SecA homologues are required for bacterial viability and virulence, moreover, highly conserved in bacteria with no human counterpart, therefore targeting these proteins may represent a way to combat bacterial pathogens with minimal human toxicity. SecA contains small ligand binding domains, indicating that it is amenable for drug targeting. *In vitro* and *in vivo* assays have been developed to investigate the function of SecA, and those assays can be applied for high-throughput screening for SecA inhibitors. SecA homologues plays important role during all infection life stages. They have important functions not only in exponential phase but also in stationary phase; they are required for colonization and spread in human tissue; they can protect bacteria from host defensive attack; also play roles in eliciting immune responses [56-57]. Thus, SecA homologues are ideal targets for exploring novel antimicrobials. We hypothesize that inhibition of SecA homologues could reduce virulence, inhibit bacteria growth, and kill bacteria. In those Gram-positive pathogens containing two SecA homologues, like MRSA, *M. tuberculosis*, *B. anthracis*, and *C. diphtheria*, dual targets inhibition could increase the chances of combat infection and reduce the occurrence of drug resistance. SecA is involved in

forming transmembrane channel. That means that it might be accessible directly from the extracellular environment in Gram-positive bacteria. Thus, targeting SecA might be able to by-pass the effect of efflux, which is one of the major and common mechanisms for bacterial drug resistance development. Antimicrobials which could by-pass the effect of efflux could have very significant implications. Thus, SecA might be an excellent target for antimicrobial development.

Alignment results showed that nine motifs of DEAD helicase were highly conserved in all SecA homologues. In *E. coli* SecA, these nine motifs form two nucleotide binding domains (NBD); NBD1 acts as the ATPase catalytic machinery; NBD2 is a regulator of ATP hydrolysis at NBD1. The conservation of these motifs suggested that SecA1 and SecA2 might be ATPase and have direct role on translocation, and targeting NBD of SecA homologues might result in dual target in those Gram-positive bacteria pathogens containing two SecA homologues. SecA1 and SecA2 were cloned from *M. tuberculosis* H₃₇Rv, *M. smegmatis* mc²155, *S. aureus* 35556, and *B. anthracis* Sterne, over-expressed in *E. coli*, and purified. All these SecA homologues showed ATPase activities, suggesting that they could be molecular motor. The ATPase activity of SecA1 can be stimulated by liposomes, while the ATPase activity of SecA2 cannot be stimulated by liposomes. The ATPase activity of MsSecA1 could be obviously stimulated by BA13 membrane and the preOmpA of *E. coli*. Majority of MsSecA1 are located on membrane, while majority of MsSecA2 are located in cytosol. Liposomes could induce conformational change of MtbSecA1 and form ring-like structures under TEM and AFM. All these results suggest that mycobacteria SecA1 might interact with membrane in the same way as EcSecA and might be involved in forming transmembrane channel. Mycobacteria SecA1 and SecA2 failed to complement the temperature-sensitive defect EcSecA, suggesting that interaction between secretion factors of Sec system might be specific for different organisms.

All these SecA homologues showed ATPase activities, thus screening ATPase inhibitors might help to develop new antimicrobial agents. In collaboration with Dr. Beinghe Wang's lab, three structurally different classes of SecA inhibitors were developed and optimized in this study: (1) Rose Bengal (RB) and RB analogs derived from systematical dissection RB and Structure-Activity relationship (SAR) study; (2) pyrimidine analogs derived from virtual screening based on the ATP binding pocket of EcSecA and SAR study; and (3) bistriazole analog derived from random screening and SAR study. Several SecA inhibitors showed promising bacteriostatic effects and bactericidal effects against *B. anthracis* Sterne and numerous drug-resistant *S. aureus* strains. Our SecA inhibitors were compared with some other antibiotics for the antimicrobial activities of against *S. aureus* Mu50 and *B. anthracis* Sterne. Among these antibiotics, vancomycin had the best inhibition on *S. aureus* Mu50, $MIC_{95} \approx 5 \mu\text{g/ml}$. Five SecA inhibitors developed from this study have better bacteriostatic effect than vancomycin. Tetracycline and erythromycin showed the best inhibition on *B. anthracis* Sterne, MIC_{95} are around 0.1-0.3 $\mu\text{g/ml}$. Although inhibition effect of our inhibitors (The best with $MIC_{95} \approx 0.7 \mu\text{g/ml}$) are not as good as tetracycline and erythromycin, but our inhibitors had bactericidal effect while these two antibiotics do not.

SecA is involved in forming a protein conducting-channel that spans the entire membrane [38-39, 96-97]. Thus, drugs targeting SecA might be directly accessible from the extracellular matrix and exert their effects without entering the cell, and bypassing the major negative effect of efflux transporters in bacteria, which is a major mechanism for the development of current drug-resistance [72-76]. *S. aureus* Mu50 and *S. aureus* N315 are resistant to QacA efflux-mediated antibiotics [100]. SCA-41, SCA-50, SCA-15, SCA-93, and SCA-21 are best inhibitors developed from our study. They show promising bacteriostatic effects or/and bactericidal effect

on *S. aureus* Mu50 or/and *S. aureus* N315, suggesting that these SecA inhibitors might be able to overcome QacA mediated efflux. Two major efflux pumps of *S. aureus*, NorA and MepA, have little negative effect on the antimicrobial activities of SecA inhibitors, suggesting that targeting SecA might be able to by-pass efflux pump. Antimicrobials, which could by-pass the effect of efflux, could have very significant implications.

Sec system is not only responsible for the secretion of many essential proteins, and it is also responsible for the secretion of many toxins and virulence factors [33-35]. Because both SecA1 and SecA2 are involved in secretion of virulence factors, dual targeting inhibition on these two SecA homologues would dramatically reduce virulence. Moreover SecA inhibitors could inhibit secretion of important toxins of *S. aureus* and *B. anthracis* Sterne, which precursor contain N-terminal signal peptide required for secretion through Sec-pathway, suggesting that these inhibitors could inhibit the function of SecA *in vivo* and reduce virulence of these important bacteria pathogens. Virulence target-based therapies are not sufficient to combat infection, but targeting SecA homologues is not only reduce virulence but also decrease viability, dramatically increasing the chance of control bacterial infection and reduce the occurrence of drug resistance.

Partial trypsin digestion results showed that inhibitors could induce the change of trypsin resistance of SecA homologues, suggesting that inhibitors could bind with SecA homologues and change their conformation and stability. Target identification assays confirmed that SecA inhibitor could identify SecA homologues from whole cell lysate of *E. coli* and *S. aureus*, supporting that SecA homologues are drug targets of these inhibitors. Those inhibitors developed in our study could inhibit the enzyme activity of SecA, have bacteriostatic effect and bactericidal effect against Gram positive bacteria, inhibit the secretion of bacteria toxins, and they could bind with

SecA homologues and pull down SecA homologues from whole cell lysate. All these results validate SecA homologues as drug targets of our inhibitors.

These novel inhibitors with structural diversity will aid the understanding of the pharmacophore required for SecA inhibition. This study will provide a starting point for developing more potent antimicrobials. To develop more potent SecA inhibitors with improved water solubility, affinity-labeling and crystallographic analysis will be used to identify and characterize the SecA1 and SecA2 binding sites for selected inhibitors developed from this study. Since SecA is essential for the secretion of many proteins including virulent factors/toxins, SecA inhibitors are expected to inhibit protein secretion with no effect on protein synthesis in general. However, blockage of SecA-dependent protein secretion may trigger other physiological responses. Thus, exploration of these cellular responses with secretome and transcriptome analysis is important for the clear understanding on how these inhibitors work other than the proposed mechanism, and may lead to the identification of potential targets of these inhibitors other than SecA. Data collected from this study will be beneficial in understanding the molecular mechanism through which SecA inhibition achieves antimicrobial effect and for the future refinement of these compounds.

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