Stony Brook University

Mechanistic Studies of Potential Drug Targets against Methicillin-Resistant *Staphylococcus aureus*

A Dissertation Presented

by

Hua Xu

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Chemistry

(Chemical Biology)

Stony Brook University

August 2009

Stony Brook University

The Graduate School

Hua Xu

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Peter J. Tonge - Dissertation Advisor
Professor, Department of Chemistry

Dale G. Drueckhammer - Chairperson of Defense
Professor, Department of Chemistry

Iwao Ojima – Third Member of Defense
Professor, Department of Chemistry

Stephen G. Walker, Outside Member
Associate Professor, School of Dental Medicine, Stony Brook University

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

Mechanistic Studies of Potential Drug Targets against Methicillin-Resistant *Staphylococcus aureus*

by

Hua Xu

Doctor of Philosophy

in

Chemistry

(Chemical Biology)

Stony Brook University

2009

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is one of the most common causative agents for hospital-acquired infections. The recent emergence of MRSA strains that are resistant to all current antibiotics, has caused a further increase in the pressing need to discover new drugs against this bacterium.

Fatty acids are essential components of the bacterial cell membrane. saFabI, the enoyl ACP reductase from *S. aureus,* catalyzes the rate-limiting step in the fatty acid biosynthesis pathway, and is a validated target for drug discovery. Detailed kinetic studies reveal that saFabI is NADPH-dependent, and has a preference for ACP-linked substrates with a long acyl chain. The preference for NADPH is determined by residues R40 and K41, while product inhibition studies

indicate that the reaction proceeds via an ordered sequential mechanism. According to the preincubation inhibition analysis, triclosan and two related diphenyl ethers are all nanomolar slow-onset inhibitors of saFabI, and bind preferentially to the enzyme-$NADP^+$ complex. Three saFabI mutations, A95V, I193S, and F204S, were identified upon selection for resistance. Strains containing these mutations have MIC values 100-fold higher than that of the wild-type strain, whereas the mutant enzymes have K_i values 5 to 3000-fold larger than that of wild-type enzyme, supporting the hypothesis that saFabI is the intracellular target of the inhibitors.

The redox active lipid soluble cofactor menaquinone is an essential component of the electron transport chain and oxidative phosphorylation in some prokaryotes, including *S. aureus*. Disruption of *menD*, one of the genes hypothesized to function in the *S. aureus* menaquinone biosynthetic pathway, resulted in a small colony variant (SCV) phenotype, suggesting that *menD* functions in menaquinone biosynthesis. Ultrastructural studies of this mutant strain reveal that menaquinone may participate directly or indirectly in lipoteichoic acid. It is reasonable since the synthesis of phosphatidyl glycerol, a precursor of lipoteichoic acid, requires electron transport. The mechanistic properties of MenD from *S. aureus* (saMenD), a thiamin-diphosphate enzyme, have been explored. 2-Succinyl-5-enoylpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid is shown to be the product of the enzymatic reaction, instead of the earlier proposed 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid. In addition, Mn^{2+} inhibits the saMenD reaction at high concentrations while Mg^{2+} does not,

probably due to different effects of the metal ions on the enzyme secondary structure, as revealed by the CD spectra. Finally, the role of the residues in the substrate-binding pocket has been explored by site-directed mutagenesis and enzyme kinetics.

Mechanistic studies of MenB, the naphthoate synthase from *S. aureus*, have also been performed. Previous work identified mutations in *menB* genes in clinical *S. aureus* SCV isolates. Kinetic analysis of these mutant saMenB suggests that the mutations abolish enzyme activity, indicating that the menquinone biosynthesis is blocked in clinical SCVs. Inhibitory activities of a series of *O*-succinyl benzoic acid (OSB) analogues against saMenB and *S. aureus* has been studied. Daptomycin, a novel antibiotic, which contains an OSB-like moiety, may target saMenB, since the morphological changes on *S. aureus* caused by daptomycin treatment and defect in menaquinone biosynthesis are similar.

Table of Contents

List of Schemes

List of Figures

List of Tables

List of Abbreviations

MRSA	Methycillin-Resistant *Staphylococcus aureus*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
CHIPS	Chemotaxis inhibitory protein of staphylococci
Eap	Extracellular adherence protein
Sak	Staphylokinase
ClfA	Clumping factor A
MSCRAMM	Microbial-surface components recognizing adhesive matrix molecules
SCVs	Small colony variants
FASI	Type I Fatty acid biosynthesis
FASII	Type II Fatty acid biosynthesis
CoA	Coenzyme A
ACP	Acyl carrier protein
NADH	Nicotinadmie adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
SDRs	Short chain dehydrogenases

IPTG	Isopropyl-β-d-thiogalactopyranoside
NAC	*N*-acetylcysteamine
EPP	5-Ethyl-2-phenoxyphenol
CPP	5-Chloro-2-phenoxyphenol
DDCoA	*trans*-2-Dodecenoyl CoA
DDNAC	*trans*-2-dodecenoyl *N*-acetylcysteamine
DDsaACP	*trans*-2-dodecenoyl *Staphylococcus aureus* ACP
MIC	Minimum inhibitory concentration
IC_{50}	Half maximal inhibitory concentration
OSB	*O*-Succinyl benzoic acid
DHNA	1,4-Dihydroxy-napthoic acid
DHNA-CoA	1,4,-Dihydroxy-2-naphthoyl-CoA
ThDP	Thiamin diphosphate
SHCHC	2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate
SEPHCHC	2-Succinyl-5-enoylpyruvyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate
TEM	Transmission electron microscope
CD	Circular dichroism

APCI	Atmospheric pressure chemical ionization
CS	Crotonase superfamily
CFU	Colony forming unit
RT	Room temperature

CHAPTER 1: *STAPHYLOCOCCUS AUREUS*: INTRODUCTION, TREATMENT AND PREVENTION

1.1 *STAPHYLOCOCCUS AUREUS:* BACKGROUND AND SIGNIFICANCE

Staphylococcus aureus was first discovered in Aberdeen, Scotland in 1880 (*1*). It is a Gram-positive, catalase-positive, and coagulase-positive bacterium. Literally, *S. aureus* means "golden cluster seed", since it appears in pairs, short chains, or bunched, grape-like clusters under microscopic examination (**Figure 1.1**). It forms large yellow colonies on rich medium, and is hemolytic on blood agar plate.

It is estimated that one third of the world population carry *S. aureus*, usually in the anterior nares (*2*). These people have increased risk of becoming infected with this bacterium. *S. aureus* is one of the most common causative agents of hospital-acquired infections, including superficial skin lesions, such as abscesses and impetigo, and more severe infections, such as septic arthritis, osteomyelitis, endocarditis and pneumonia. Most nosocomial infections develop after contact with health care workers' hands, which have been contaminated with *S. aureus* from their own bodies or from direct contact with an infected patient. Notably, the community-acquired staphylococcal infections have been increasing during the last thirty years.

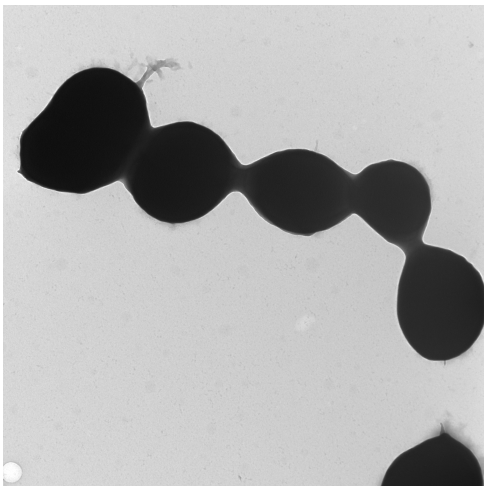


Figure 1.1: Transmission electron microscope image of *Staphylococcus aureus*. The image was taken in the TEM facility of Stony Brook University with 30000x magnification.

S. aureus is infamous for its high adaptability to antibiotic treatments. In 1942, penicillin was first used to treat staphylococcal infections, but the penicillin-resistant strains developed after only a few months of clinical trials (*3*). After methicillin was introduced to treat penicillin-resistant *S. aureus* in 1960, methicillin-resistant *S. aureus* (MRSA) was isolated within one year (*4*). The percentage of MRSA has been increasing continuously in the last few decades. In the USA, MRSA increased from 2.4% in 1975 (*5*) to 35% in 1996 (*6*), and the percentage is still increasing. Currently, glycopeptide antibiotics, such as vancomycin and teicoplanin, are considered to be the only antibiotics for treating MRSA infections. However, vancomycin-resistant *S. aureus* (VRSA) has been reported in Japan and United States (*7, 8*). There is no effective antibiotic to treat this extensive-drug-resistant strain.

According to a recent report, the incidence rate of invasive MRSA is 32 per 100,000 persons. In 2005, MRSA caused nearly 19,000 deaths in the United States, while 16,000 people died from AIDS in the same year (*9*).

One important reason for the widespread *S. aureus* infections is that this bacterium possesses many virulence factors, which promote adhesion to host tissues, diminish or mute the host immune response, aid survival in extreme host environments, such as neutrophils and macrophages, and improve pathogenic efficiency. The factors that help *S. aureus* escape the immune response are critical, and contribute to recurrent infections.

Immediately upon entering the host, *S. aureus* faces the host defense system. The initial response is the so-called complement activation, a process of recruiting effectors to label the bacteria for the host immune cells. During this process, chemoattractants, for instance, small peptide fragments C3a and C5a are released, attracting the phagocytes, such as neutropils and macrophages to the infection site.

The primary host defense against *S. aureus* infection is the innate immunity performed by neutropils. The chemoattractants C3a and C5a, as well as bacterial formylated peptides, can be specifically recognized by the surface receptors in neutrophils, stimulating the immune response. However, the bacterium has several ways to inhibit the neutrophil response (**Figure 1.2**). First, the chemotaxis inhibitory protein (CHIPS) of staphylococci is secreted to bind to both the formyl peptide receptor and the C5a receptor to eliminate the signal transfer from complement activation and bacterial growth (*10*). *S. aureus* also produces the

extracellular adherence protein (Eap), which can bind to the intercellular adhesion molecule-1 (ICAM-1) on the surface of endothelial cells, blocking binding of ICAM-1 with LFA-1, a surface adhesion molecule of neutrophils involved in the recruitment of neutrophils to the infection site (*11*). In addition to the passive defense by CHIPS and Eap, some *S. aureus* strains produce the pore-forming leukotoxins to attack the cytoplasmic membrane of neutrophils, causing cell leakage and death (*12*).

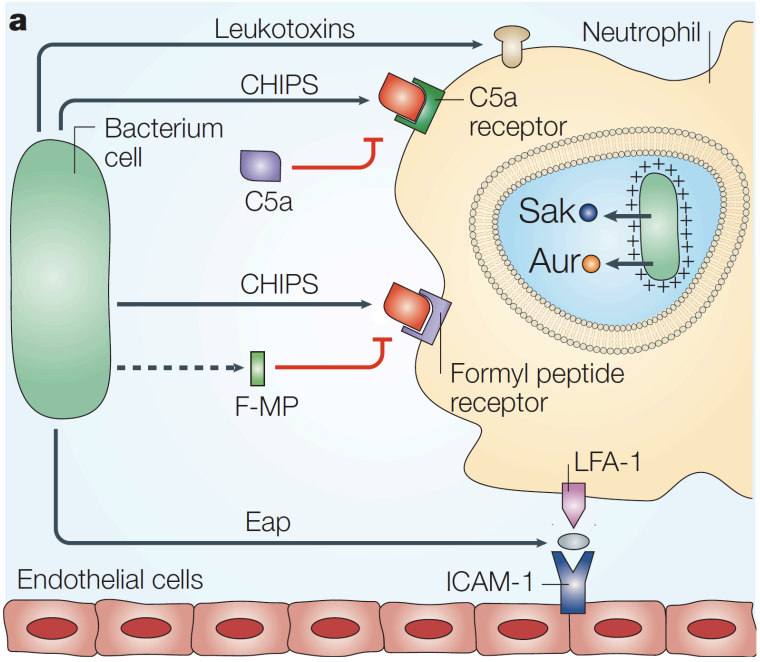


Figure 1.2: Inhibition against the neutrophil response. CHIPS and Eap block neutrophil recruitment to the infection site. Leukotoxins are secreted to disrupt the neutrophil membrane. Sak, Aur and cell wall modifications (indicated by "+") prevent the killing in the phagosome of neutrophils. Figure is from Ref (*13*)**.**

The successful staphylococcal infection is also attributed to the avoidance of phagocytosis by neutrophils. Several components are involved in this process,

including capsule, staphylokinase (Sak), protein A, extracellular fibrinogen-binding protein (Efb) and clumping factor A (ClfA).

Most clinical isolates of *S. aureus* possess a capsule consisting of polysaccharides. *In vitro* studies suggest that the presence of this polysaccharide capsule reduced the phagocytic uptake of bacterial cells by neutrophils, indicating that the capsule is anti-phagocytic (*14*).

IgG molecules are usually bound to the surface of bacterial cells, facilitating opsonophagocytosis. However, in *S. aureus,* Sak is secreted to bind to the host plasminogen molecules, which attach to the bacterial cell surface. Subsequently, Sak activates plasminogen, producing a potent serine protease, plasmin, which cleaves the bacterial surface-bound IgG, significantly decreased phagocytosis by neutrophils (*15*).

Protein A, a surface protein in *S. aureus*, also shows an antiphagocytic effect. This is probably due to its binding to the Fc region of IgG (*16*). Consequently, this interaction creates an incorrect orientation of bacterial surface-bound IgG molecules, which disrupts the recognition of IgG by the Fc-receptor in neutrophils to initiate phagocytosis.

ClfA is the major fibrinogen-binding surface protein in *S. aureus* during the stationary phase. After coating with fibrinogen, the bacterial cell surface becomes inaccessible to the phagocytic-enhancing moleclues, thus preventing phagocytosis.

Recently it has been reported that Efb, an extracellular fibrinogen-binding protein, also contributes to the prevention of phagocytosis and opsonization of *S.*

aureus, due to its binding to the complement factor C3b and to block C3b deposition on the bacterial cell surface (*17, 18*).

Even if *S. aureus* is engulfed into the neutrophils, it still shows resistance to killing by antimicrobial peptides in the phagosome. Most of the antimicrobial peptides in neutrophils are cationic peptides, such as cathelicidin LL-37 and α-defensins, which function by interrupting the integrity of the bacterial lipid bilayer. The resistance to these peptides is promoted by D-alanine and L-lysine modifications to the bacterial cell-wall components, such as wall teichoic acid and lipoteichoic acid (**Figure 1.2**) (*19, 20*). Both modifications reduce the negative charge of the cell wall, causing decreased affinity to the cationic peptides. *S. aureus* also secretes proteins to neutralize the cationic antimicrobial peptides. For instance, in addition to its role in preventing phagocytosis described earlier, Sak is secreted to bind the cationic defensins in the phagosome, facilitating the survival of the bacterium in the extreme environment *(21)*.

After escaping the immune response, the bacteria can adhere to the endothelial cells, and then initiate endothelial-cells phagocytosis (**Figure 1.3**). Once *S. aureus* is engulfed by these host cells, a number of proteolytic enzymes are produced by the bacteria to facilitate breakdown of the host cells and the spread of bacteria to the neighboring tissues. After getting into the subepithelial tissues, the bacteria evoke an inflammatory response, which causes abscess formation. The whole process described above establishes the metastasis of infection. When cardiac endothelium is involved during the infection, endocarditis will develop.

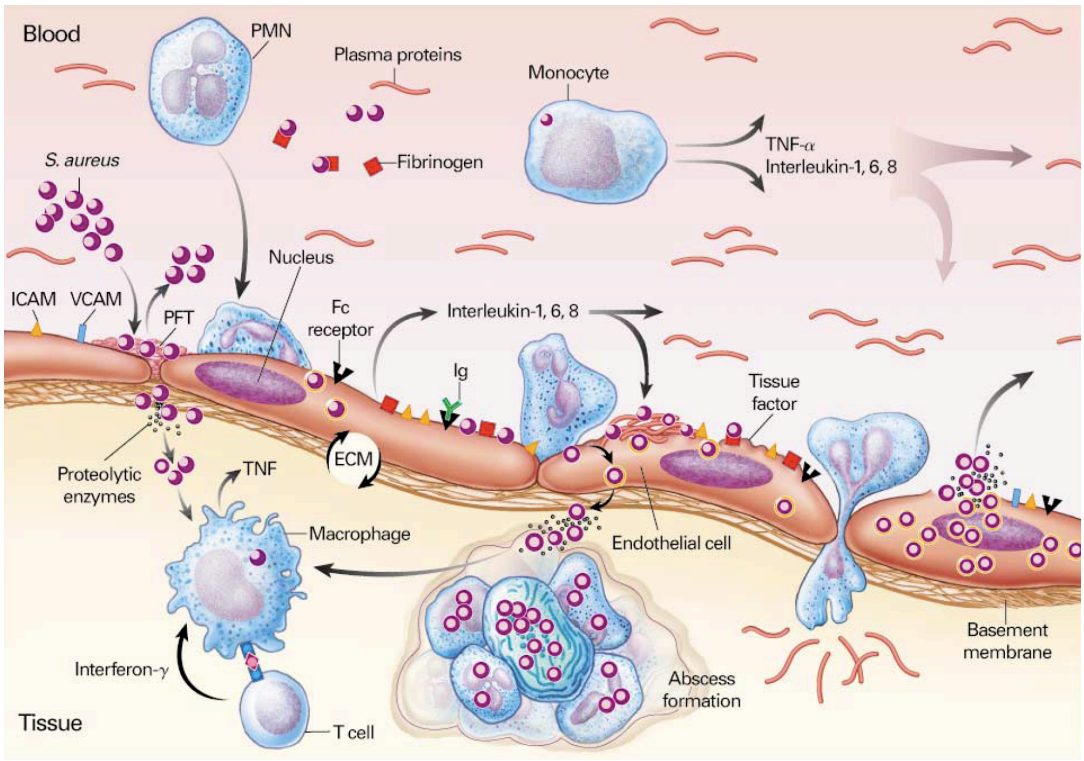


Figure 1.3: Pathogenesis procedure of invasive *Staphylococcus aureus* infections. Figure is from Ref (*22*).

1.2 SMALL COLONY VARIANTS

Bacterial small colony variants (SCVs), which are one-tenth the size of the colonies formed by wild type bacteria, were first reported in 1910 (*23*). The variant strains have been described in a wide range of bacterial species, such as *E. coli, S. aureus*, *S. epidermidis*, *B. cepacia*, *V. cholera* and *Shigella spp (24-27)*. Many of them were isolated from clinical specimens.

In 1995, the first model to correlate the phenotypic changes in *S. aureus* SCVs to the persistent and recurrent staphylococcal infections was described (*28*). Persistent or antibiotic-resistant infections due to *S. aureus* SCVs were described clinically.

There are two types of SCVs in *S. aureus* consistently recovered from clinical specimens: electron-transport defective SCVs, and thymidine-defective SCVs. The former ones are auxotrophic in menadione or haemin biosynthesis, and the phenotype could be reversed by the addition of menadione or haemin. Due to the instability and high reversal rate of clinical SCVs, several genetically defined mutant strains, like *hemB, menD, and ctaA*, which also show electron-transport defective SCV phenotypes, were constructed for detailed phenotype analysis (*29-31*). All of the three genes are involved in either the biosynthesis of menaquinone or cytochrome, which are both components of the electron-transport system. The proteomic and phenotype microarray studies of electron-transport defective SCVs demonstrated that the enzymes in the glycolytic and fermentation pathways are upregulated, while the enzymes in the tricarboxylic acid (TCA) cycle enzymes were significantly downregulated, suggesting that ATP is produced by substrate phosphorylation instead of the electron transport pathway (*32, 33*). Recently the genetic basis of this class of SCVs was also reported to be associated with mutations of the *menB* gene in the menaquinone biosynthesis (*34*).

The second class of SCVs is defective in thymidine biosynthesis. These SCVs were isolated in the patients after long-term treatment of trimethoprim sulphamethoxazole (SXT). The derived SCV strains were resistant to SXT, while the parent strains were susceptible. It was found that a clinical thymidine-defective SCV strain can be complemented with a functional copy of *thyA* gene, which encodes thymidylate synthase (*35*). Later the genetic basis of this type of

clinical SCVs was shown to correlate with *thyA* mutations, which cause a truncation of this enzyme (*36*).

A number of studies suggest that the SCV strains can escape from host defense, and are responsible for recurrent and persistent infections. The transport of aminoglycosides across the cell membrane requires ATP, which is significantly reduced in SCVs, making them less susceptible to the aminoglycoside antibiotics, such as gentamicin and kanamycin (*29*). Resistance to the antimicrobial peptide lactoferricin B was also reported in SCVs (*37*).

In addition, it is reported that the *hemB* SCV persists in host tissues, and expresses a higher level of surface adhesins than the wild type strain, facilitating biofilm formation. SCVs from other bacteria species, such as *P. aeruginosa* and *S. epidermidis,* also show increased biofilm formation capability, which contributes to drug resistance (*38, 39*). It has been shown that physiological strains, like SCVs could not be treated by traditional approaches (*40, 41*), and novel therapies are urgently needed.

1.3 DRUG RESISTANCE MECHANISMS IN *STAPHYLOCOCCUS AUREUS*

Antibiotics have revolutionalized the treatment of infectious diseases but have also rapidly selected for the emergence of resistant pathogen strains. Nowadays, drug resistance has become a serious concern around the world. Generally the bacterial resistance is intrinsic or acquired after exposure to antibiotics.

Several mechanisms are responsible for the bacterial resistance to antibiotics.

(a) Enzymatic degradation and modification of antibiotics so that the antibiotics can no longer be effective.

(b) Modification of the target site of antibiotic so that the binding affinity between antibiotic and its target is reduced.

(c) Removal of antibiotics by overexpression of the efflux pump on the bacterial cell surface.

(d) Reduction of antibiotic permeability by modification of cell wall components and structure.

Most of the resistance mechanisms listed above involve genetic changes within the bacteria. Simple changes are derived from spontaneous or induced mutations, while complex genetic arrays, such as chromosomal cassette *mec* elements and the *vanA* operon in drug-resistant *S. aureus* strains, are obtained through horizontal gene transfer from other microbial species.

Notably, for several clinically important antibiotics, including the fluoroquinolones and rifampicin, point mutations of chromosomally encoded proteins are the major mechanism of resistance, and lead to clinical treatment failure (*42, 43*). Two types of point mutations may be involved during clinical therapy. The first type is called spontaneous mutations, which are generated in the process of bacterial genome replication. Spontaneous mutations produce a subpopulation of bacteria, which have low-level resistance and hence gain survival advantage during the therapy. Furthermore, additional mutations, which

confer to high-level resistance, may be acquired during successive cycles of growth. The second type of point mutations that may contribute to the emergence of resistance during therapy is induced mutation, which allows for a regulated increase of mutation rates during antibiotic treatment. Unlike spontaneous mutations, induced mutations depend on homologous recombination and also the induction of the bacterial SOS response (*46*), which is usually initiated by the presence of single-stranded DNA accumulated as a result of either DNA damage or problematic replication. LexA and RecA are important components in the SOS response system, and have been proposed to be attractive targets for inhibiting the development of drug resistance in *S. aureus (47)*.

In addition to the innate mutations, the resistance genes can be transferred among different bacteria species, which further complicates the antibiotic resistance.

As described earlier, *S. aureus* is extremely adaptive to antibiotics. Penicillin is inactivated by β-lactamase, a serine protease that hydrolyzes the β-lactam ring in penicillin. Now most clinical isolates of *S. aureus* are no longer sensitive to penicillin. The resistance against methicillin requires the *mec* gene that encodes penicillin-binding protein 2a (*48*). The *mec* genes probably originated from a different species of staphylococci (*49*). Other staphylococcal genes, including *bla* (*β*-lactamase) and *fem* (factors essential for methicillin resistance), may also be involved in the resistance.

The mechanism of vancomycin-resistance in *S. aureus* is not elucidated yet. Two possible mechanisms have been suggested, including increased cell wall

synthesis and cell wall alterations, which prevent vancomycin from reaching its target site (*50, 51*).

1.4 SEARCH FOR NOVEL DRUGS AND VACCINES

The products of essential genes are good targets for drug discovery, since the inhibition would be fatal to the bacteria. Genes that are refractory to deletion or insertional inactivation are probably essential. On the basis of this hypothesis, a systematic genetic study by targeted mutagenesis was carried out in *B. subtilis*, a model organism for Gram-positive bacteria (*52*). Most of the essential genes revealed by this study have homologues in *S. aureus.* Several genes in *S. aureus*, such as Pdf1, MurE, and FmhB, have been proven to be essential by conditional lethal construct studies (*53-56*). The products of these genes are considered to be attractive antimicrobial targets.

The antisense RNA expression technology is another important tool to investigate the essentiality of genes. In this technology, the expression of antisense RNA molecules interferes with the corresponding cognate genes. It has been used to examine the genome of *S. aureus*, identifying 658 essential genes, many of which are also essential in other organisms (*57, 58*). However, false positives may arise due to the non-specific RNA interactions. False negatives also exist if the controlled expression of the antisense RNA is not enough to counteract the amount of mRNA cognate essential gene.

A number of studies have focused on the characterization of potential drug targets and the discovery of novel inhibitors, as listed in **Table 1.1**. The targets

are quite diverse, ranging from extracellular proteins (SE), membrane enzymes (MenA, PutP, SrtA, SpsB and AgrC), to cytoplasm enzymes (Pdf1, FabI, MurB and phenylalanyl-RS). Notably, InhA, the FabI homologue in *M. tuberculosis*, is the target of isoniazid, the most effective front line drug against tuberculosis (*59*). In addition, menaquinone biosynthesis has been reported as a novel target against MRSA (*60*).

Table 1.1: Inhibitors of Novel targets in *Staphylcoccus aureus*

Target	Function	Inhibitor
SE	Enterotoxin superantigen	DRα1-linker-TcRVβ chimera (*61*)
MenA	Isoprenyl transferase	Allylaminomethanone (*60*)
PutP	Hign affinity proline permease	Proline analogues (*62*)
SrtA	Cysteine-protease transpeptidase	Methanethiosulfonates and p-hydroxymercuribenzoic acid (*63*) Peptidyl-diazomethane, peptidyl-chloromethane (*64*) Phosphinic peptidomimetics (*65*)
SpsB	Signal peptidase	α-ketoamides (*66*)
AgrC	Receptor histidine kinase	Truncated autoinducer (*67*)
Phenylalanyl-RS	Phenylalanyl-tRNA synthetase	Phenyl-thiazolylurea-sulfonamides (*68*)
MurB	UDP-N-acetylmuramate dehydrogenase	4-thiazolidinones (*69*) Imidazolinones (*70*)
FabI	Enoyl ACP reductase	Pyridone derivatives (*71*) Naphthyridin acylamide (*72*)
Pdf1	Polypeptide deformylase	Hydroxamic acid derivatives (*53*)

Efforts have also been put to develop vaccines against the bacterium, especially for the people with high risk of getting staphylococcal infections, such as haemodialysis patients, HIV patients and low birthweight neonates. However, due to multiple mechanisms of avoiding human innate immunity and lack of a clear understanding of the host-pathogen interaction, the development of effective vaccines against staphylococcal infections is still not successful yet. No vaccine is currently available to stimulate active immunity against staphylococcal infections in humans.

Since capsule-directed vaccines have been in the market for other bacterial pathogens, capsular antigens are good targets for vaccine development against *S. aureus*. Most *S. aureus* produce capsular saccharide to protect the bacterium. Capsular polysaccharide seroptype 5 (CP5) and serotype 8 (CP8) are most commonly found among clinical strains. The antibodies produced by immunization with CP5 and CP8 conjugated with exoprotein A from *P. aeruginosa* dramatically increased the opsonphagocytosis of encapsulated *S. aureus* (*73*). Later Nabi Biopharmaceuticals includes both CP5 and CP8 conjugates in a bivalent vaccine called StaphVAX. However, its failure in Phase III trials suggests that a vaccine that targets only the capsular saccharide is not enough to protect against in *S. aureus*.

In a systematic evaluation of *S. aureus* adhesins for their vaccine potential, four cell-wall anchored proteins, including IsdA, IsdB, SdrD and SdrE, were identified (*74*). Animals immunized with a combined vaccine of all four antigens had higher survival rate when challenged with invasive staphylococcal infections,

compared with the control. The functions of SdrD and SdrE are not known yet. The expression of IsdA and IsdB is triggered by limited iron in the environment. Both proteins are responsible for acquiring iron from haemoglobins. Mice immunized with recombinant IsdB showed improved survival following challenge with five of six clinical strains (*75*). Vaccine V710 from Merck was designed based on IsdB, and completed phase I testing. It is being tested in phase II trials for the prevention of *S. aureus* infection in patients undergoing cardiothoracic surgery and also in haemodialysis patients.

Poly-N-acetylglucosamine (PNAG), alpha-haemolysin and staphylococcal enterotoxin B were also reported as potential targets for vaccine development (*76-78*).

When the precise molecular basis of the interactions between staphylococcal adhesins and host tissue receptors is elucidated, it might be possible to design compounds that block the interactions and thus prevent bacterial colonization. This would also faciliate the vaccine development significantly.

1.5 SUMMARY

Staphylococcus aureus is one of the most causative agents for hospital- and community-acquired infections. Several virulent factors of this bacterium help evade the immune response. In addition, *S. aureus* possesses various mechanisms to fight against antibiotic treatment, causing antibiotic resistance. Small colony variants further complicate the clinical treatment of staphylococcal

infections. Novel drugs are urgently needed to keep the pace with the fight against *S. aureus*.

My thesis research is focused on three enzymes from *S. aureus*, FabI, MenD and MenB, which participate in fatty acid biosynthesis and menaquinone biosynthesis, respectively. It is worthwhile to study these enzymes, since they are involved in pathways proposed to be essential for bacterial survival. Mechanistic characterizations and preliminary inhibition studies are carried out here, and provide useful information for further optimization of inhibitors.

References

1. Ogston, A. (1984) Classics in infectious diseases. "On abscesses". (1844-1929), *Rev. Infect. Dis. 6*, 122-128.
2. Peacock, S. J., de Silva, I., and Lowy, F. D. (2001) What determines nasal carriage of *Staphylococcus aureus*?, *Trends Microbiol. 9*, 605-610.
3. Bradley, S. F. (1992) Methicillin-resistant *Staphylococcus aureus* infection, *Clin. Geriatr. Med. 8*, 853-868.
4. Barbar, M. (1961) Methicillin-resistant staphylococci, *J. Clin. Pathol. 14*, 385-393.
5. Panlilio, A. L., Culver, D. H., Gaynes, R. P., Banerjee, S., Henderson, T. S., Tolson, J. S., and Martone, W. J. (1992) Methicillin-resistant *Staphylococcus aureus* in U.S. hospitals, 1975-1991, *Infect. Control Hosp. Epidemiol. 13*, 582-586.
6. Gaynes, R., and Culver, D. (1997) Nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) in the United States, 1975-1996. National Nosocomial Infection Surveillance (NNIS) System., in *Annual Meeting of the Infectious Desease Society of America*, IDSA, San Francisco.
7. (2002) Vancomycin resistant *Staphylococcus aureus*-Pennsylvania, 2002, *JAMA*, 2116.
8. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility, *J. Antimicrob. Chemother. 40*, 135-136.

9. Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Fridkin, S. K. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States, *JAMA 298*, 1763-1771.

10. de Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P., and van Strijp, J. A. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent, *J. Exp. Med. 199*, 687-695.

11. Chavakis, T., Hussain, M., Kanse, S. M., Peters, G., Bretzel, R. G., Flock, J. I., Herrmann, M., and Preissner, K. T. (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes, *Nat. Med. 8*, 687-693.

12. Gravet, A., Colin, D. A., Keller, D., Girardot, R., Monteil, H., and Prevost, G. (1998) Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family, *FEBS Lett. 436*, 202-208.

13. Foster, T. J. (2005) Immune evasion by staphylococci, *Nat. Rev. Microbiol. 3*, 948-958.

14. Thakker, M., Park, J. S., Carey, V., and Lee, J. C. (1998) *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model, *Infect. Immun. 66*, 5183-5189.

15. Rooijakkers, S. H., van Wamel, W. J., Ruyken, M., van Kessel, K. P., and van Strijp, J. A. (2005) Anti-opsonic properties of staphylokinase, *Microbes Infect. 7*, 476-484.

16. Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., and Lindberg, M. (1984) Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications, *J. Biol. Chem. 259*, 1695-1702.

17. Lee, L. Y., Hook, M., Haviland, D., Wetsel, R. A., Yonter, E. O., Syribeys, P., Vernachio, J., and Brown, E. L. (2004) Inhibition of complement activation by a secreted *Staphylococcus aureus* protein, *J. Infect. Dis. 190*, 571-579.

18. Lee, L. Y., Liang, X., Hook, M., and Brown, E. L. (2004) Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb), *J. Biol. Chem. 279*, 50710-50716.

19. Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., and Gotz, F. (1999) Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides, *J. Biol. Chem. 274*, 8405-8410.

20. Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W. F., Jung, G., Tarkowski, A., van Kessel, K. P., and van Strijp, J. A. (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the

novel virulence factor MprF is based on modification of membrane lipids with l-lysine, *J. Exp. Med. 193*, 1067-1076.

21. Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., and Tarkowski, A. (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism, *J. Immunol. 172*, 1169-1176.

22. Lowy, F. D. (1998) *Staphylococcus aureus* infections, *N. Engl. J. Med. 339*, 520-532.

23. Proctor, R. A., Bates, D. M., and McNamara, P. J. (2001) *Emerging infections*, Vol. 5, American Society for Microbiology Press, Washington, D. C.

24. Bulger, R. J. (1967) A methicillin-resistant strain of *Staphylococcus aureus*. Clinical and laboratory experience, *Ann. Intern. Med. 67*, 81-89.

25. Baddour, L. M., Barker, L. P., Christensen, G. D., Parisi, J. T., and Simpson, W. A. (1990) Phenotypic variation of *Staphylococcus epidermidis* in infection of transvenous endocardial pacemaker electrodes, *J. Clin. Microbiol. 28*, 676-679.

26. Haussler, S., Lehmann, C., Breselge, C., Rohde, M., Classen, M., Tummler, B., Vandamme, P., and Steinmetz, I. (2003) Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia* complex, *Eur. J. Clin. Microbiol. Infect. Dis. 22*, 249-253.

27. Colwell, C. A. (1946) Small Colony Variants of *Escherichia coli*, *J. Bacteriol. 52*, 417-422.

28. Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N., and Arbeit, R. D. (1995) Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*, *Clin. Infect. Dis. 20*, 95-102.

29. von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G., and Gotz, F. (1997) A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly, *J. Bacteriol. 179*, 4706-4712.

30. Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S., and Proctor, R. A. (2003) *Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney, *J. Infect. Dis. 187*, 1654-1661.

31. Clements, M. O., Watson, S. P., Poole, R. K., and Foster, S. J. (1999) CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis, *J. Bacteriol. 181*, 501-507.

32. Kohler, C., von Eiff, C., Liebeke, M., McNamara, P. J., Lalk, M., Proctor, R. A., Hecker, M., and Engelmann, S. (2008) A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*, *J. Bacteriol. 190*, 6351-6364.

33. von Eiff, C., McNamara, P., Becker, K., Bates, D., Lei, X. H., Ziman, M., Bochner, B. R., Peters, G., and Proctor, R. A. (2006) Phenotype

microarray profiling of *Staphylococcus aureus* menD and hemB mutants with the small-colony-variant phenotype, *J. Bacteriol. 188*, 687-693.

34. Lannergard, J., von Eiff, C., Sander, G., Cordes, T., Seggewiss, J., Peters, G., Proctor, R. A., Becker, K., and Hughes, D. (2008) Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 52*, 4017-4022.

35. Kahl, B. C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R. A., and Peters, G. (2003) Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation, *J. Clin. Microbiol. 41*, 410-413.

36. Chatterjee, I., Kriegeskorte, A., Fischer, A., Deiwick, S., Theimann, N., Proctor, R. A., Peters, G., Herrmann, M., and Kahl, B. C. (2008) In vivo mutations of thymidylate synthase (encoded by thyA) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*, *J. Bacteriol. 190*, 834-842.

37. Samuelsen, O., Haukland, H. H., Kahl, B. C., von Eiff, C., Proctor, R. A., Ulvatne, H., Sandvik, K., and Vorland, L. H. (2005) *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B, *J. Antimicrob. Chemother. 56*, 1126-1129.

38. Haussler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B., and Steinmetz, I. (2003) Highly

adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection, *J. Med. Microbiol. 52*, 295-301.

39. Al Laham, N., Rohde, H., Sander, G., Fischer, A., Hussain, M., Heilmann, C., Mack, D., Proctor, R., Peters, G., Becker, K., and von Eiff, C. (2007) Augmented expression of polysaccharide intercellular adhesin in a defined *Staphylococcus epidermidis* mutant with the small-colony-variant phenotype, *J. Bacteriol. 189*, 4494-4501.

40. Baumert, N., von Eiff, C., Schaaff, F., Peters, G., Proctor, R. A., and Sahl, H. G. (2002) Physiology and antibiotic susceptibility of *Staphylococcus aureus* small colony variants, *Microb. Drug Resist. 8*, 253-260.

41. Brouillette, E., Martinez, A., Boyll, B. J., Allen, N. E., and Malouin, F. (2004) Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo, *FEMS Immunol. Med. Microbiol. 41*, 35-41.

42. Drlica, K., and Zhao, X. (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones, *Microbiol. Mol. Biol. Rev. 61*, 377-392.

43. Jin, D. J., and Gross, C. A. (1988) Mapping and sequencing of mutations in the *Escherichia coli* rpoB gene that lead to rifampicin resistance, *J. Mol. Biol. 202*, 45-58.

44. Woodford, N., and Ellington, M. J. (2007) The emergence of antibiotic resistance by mutation, *Clin. Microbiol. Infect. 13*, 5-18.

45. Zhang, Y., and Young, D. (1994) Molecular genetics of drug resistance in *Mycobacterium tuberculosis*, *J. Antimicrob. Chemother. 34*, 313-319.

46. McKenzie, G. J., Harris, R. S., Lee, P. L., and Rosenberg, S. M. (2000) The SOS response regulates adaptive mutation, *Proc. Natl. Acad. Sci. U S A 97*, 6646-6651.

47. Cirz, R. T., Jones, M. B., Gingles, N. A., Minogue, T. D., Jarrahi, B., Peterson, S. N., and Romesberg, F. E. (2007) Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin, *J. Bacteriol. 189*, 531-539.

48. Chambers, H. F. (1997) Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications, *Clin. Microbiol. Rev. 10*, 781-791.

49. Archer, G. L., and Niemeyer, D. M. (1994) Origin and evolution of DNA associated with resistance to methicillin in staphylococci, *Trends Microbiol. 2*, 343-347.

50. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility, *J. Antimicrob. Chemother. 40*, 135-136.

51. Sieradzki, K., and Tomasz, A. (1997) Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*, *J. Bacteriol. 179*, 2557-2566.

52. Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S. C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L. C.,

Danchin, A., Debarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S. K., Dreesen, O., Errington, J., Fillinger, S., Foster, S. J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C. R., Hecker, M., Hosoya, D., Hullo, M. F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauel, C., Meima, R., Mellado, R. P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H. M., Rapoport, G., Rawlins, J. P., Rivas, L. A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H. H., Scanlan, E., Schumann, W., Seegers, J. F., Sekiguchi, J., Sekowska, A., Seror, S. J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H. B., Vagner, V., van Dijl, J. M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U., and Ogasawara, N. (2003) Essential *Bacillus subtilis* genes, *Proc. Natl. Acad. Sci. U S A 100*, 4678-4683.

53. Zhang, L., Fan, F., Palmer, L. M., Lonetto, M. A., Petit, C., Voelker, L. L., St John, A., Bankosky, B., Rosenberg, M., and McDevitt, D. (2000) Regulated gene expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action, *Gene 255*, 297-305.

54. Jana, M., Luong, T. T., Komatsuzawa, H., Shigeta, M., and Lee, C. Y. (2000) A method for demonstrating gene essentiality in *Staphylococcus aureus*, *Plasmid 44*, 100-104.

55. Zalacain, M., Biswas, S., Ingraham, K. A., Ambrad, J., Bryant, A., Chalker, A. F., Iordanescu, S., Fan, J., Fan, F., Lunsford, R. D., O'Dwyer, K., Palmer, L. M., So, C., Sylvester, D., Volker, C., Warren, P., McDevitt, D., Brown, J. R., Holmes, D. J., and Burnham, M. K. (2003) A global approach to identify novel broad-spectrum antibacterial targets among proteins of unknown function, *J. Mol. Microbiol. Biotechnol. 6*, 109-126.

56. Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H., and Berger-Bachi, B. (1999) The essential *Staphylococcus aureus* gene fmhB is involved in the first step of peptidoglycan pentaglycine interpeptide formation, *Proc. Natl. Acad. Sci. U S A 96*, 9351-9356.

57. Ji, Y., Zhang, B., Van, S. F., Horn, Warren, P., Woodnutt, G., Burnham, M. K., and Rosenberg, M. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA, *Science 293*, 2266-2269.

58. Forsyth, R. A., Haselbeck, R. J., Ohlsen, K. L., Yamamoto, R. T., Xu, H., Trawick, J. D., Wall, D., Wang, L., Brown-Driver, V., Froelich, J. M., C, K. G., King, P., McCarthy, M., Malone, C., Misiner, B., Robbins, D., Tan, Z., Zhu Zy, Z. Y., Carr, G., Mosca, D. A., Zamudio, C., Foulkes, J. G., and Zyskind, J. W. (2002) A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*, *Mol. Microbiol. 43*, 1387-1400.

59. Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G., and Jacobs, W. R., Jr. (1994) inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*, *Science 263*, 227-230.

60. Kurosu, M., Narayanasamy, P., Biswas, K., Dhiman, R., and Crick, D. C. (2007) Discovery of 1,4-dihydroxy-2-naphthoate [corrected] prenyltransferase inhibitors: new drug leads for multidrug-resistant gram-positive pathogens, *J. Med. Chem. 50*, 3973-3975.

61. Hong-Geller, E., Mollhoff, M., Shiflett, P. R., and Gupta, G. (2004) Design of chimeric receptor mimics with different TcRVbeta isoforms. Type-specific inhibition of superantigen pathogenesis, *J. Biol. Chem. 279*, 5676-5684.

62. Schwan, W. R., Coulter, S. N., Ng, E. Y., Langhorne, M. H., Ritchie, H. D., Brody, L. L., Westbrock-Wadman, S., Bayer, A. S., Folger, K. R., and Stover, C. K. (1998) Identification and characterization of the PutP proline permease that contributes to in vivo survival of *Staphylococcus aureus* in animal models, *Infect. Immun. 66*, 567-572.

63. Ton-That, H., and Schneewind, O. (1999) Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction, *J. Biol. Chem. 274*, 24316-24320.

64. Scott, C. J., McDowell, A., Martin, S. L., Lynas, J. F., Vandenbroeck, K., and Walker, B. (2002) Irreversible inhibition of the bacterial cysteine

protease-transpeptidase sortase (SrtA) by substrate-derived affinity labels, *Biochem J. 366*, 953-958.

65. Kruger, R. G., Barkallah, S., Frankel, B. A., and McCafferty, D. G. (2004) Inhibition of the *Staphylococcus aureus* sortase transpeptidase SrtA by phosphinic peptidomimetics, *Bioorg. Med. Chem. 12*, 3723-3729.

66. Bruton, G., Huxley, A., O'Hanlon, P., Orlek, B., Eggleston, D., Humphries, J., Readshaw, S., West, A., Ashman, S., Brown, M., Moore, K., Pope, A., O'Dwyer, K., and Wang, L. (2003) Lipopeptide substrates for SpsB, the *Staphylococcus aureus* type I signal peptidase: design, conformation and conversion to alpha-ketoamide inhibitors, *Eur. J. Med. Chem. 38*, 351-356.

67. Lyon, G. J., Mayville, P., Muir, T. W., and Novick, R. P. (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13330-13335.

68. Beyer, D., Kroll, H. P., Endermann, R., Schiffer, G., Siegel, S., Bauser, M., Pohlmann, J., Brands, M., Ziegelbauer, K., Haebich, D., Eymann, C., and Brotz-Oesterhelt, H. (2004) New class of bacterial phenylalanyl-tRNA synthetase inhibitors with high potency and broad-spectrum activity, *Antimicrob. Agents Chemother. 48*, 525-532.

69. Andres, C. J., Bronson, J. J., D'Andrea, S. V., Deshpande, M. S., Falk, P. J., Grant-Young, K. A., Harte, W. E., Ho, H. T., Misco, P. F., Robertson, J. G., Stock, D., Sun, Y., and Walsh, A. W. (2000) 4-Thiazolidinones: novel

inhibitors of the bacterial enzyme MurB, *Bioorg. Med. Chem. Lett. 10*, 715-717.

70. Bronson, J. J., DenBleyker, K. L., Falk, P. J., Mate, R. A., Ho, H. T., Pucci, M. J., and Snyder, L. B. (2003) Discovery of the first antibacterial small molecule inhibitors of MurB, *Bioorg. Med. Chem. Lett. 13*, 873-875.

71. Kitagawa, H., Kumura, K., Takahata, S., Iida, M., and Atsumi, K. (2007) 4-Pyridone derivatives as new inhibitors of bacterial enoyl-ACP reductase FabI, *Bioorg. Med. Chem. 15*, 1106-1116.

72. Payne, D. J., Miller, W. H., Berry, V., Brosky, J., Burgess, W. J., Chen, E., DeWolf Jr, W. E., Jr., Fosberry, A. P., Greenwood, R., Head, M. S., Heerding, D. A., Janson, C. A., Jaworski, D. D., Keller, P. M., Manley, P. J., Moore, T. D., Newlander, K. A., Pearson, S., Polizzi, B. J., Qiu, X., Rittenhouse, S. F., Slater-Radosti, C., Salyers, K. L., Seefeld, M. A., Smyth, M. G., Takata, D. T., Uzinskas, I. N., Vaidya, K., Wallis, N. G., Winram, S. B., Yuan, C. C., and Huffman, W. F. (2002) Discovery of a novel and potent class of FabI-directed antibacterial agents, *Antimicrob. Agents Chemother. 46*, 3118-3124.

73. Fattom, A., Schneerson, R., Szu, S. C., Vann, W. F., Shiloach, J., Karakawa, W. W., and Robbins, J. B. (1990) Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin A, *Infect. Immun. 58*, 2367-2374.

74. Stranger-Jones, Y. K., Bae, T., and Schneewind, O. (2006) Vaccine assembly from surface proteins of *Staphylococcus aureus*, *Proc. Natl. Acad. Sci. U.S.A. 103*, 16942-16947.

75. Kuklin, N. A., Clark, D. J., Secore, S., Cook, J., Cope, L. D., McNeely, T., Noble, L., Brown, M. J., Zorman, J. K., Wang, X. M., Pancari, G., Fan, H., Isett, K., Burgess, B., Bryan, J., Brownlow, M., George, H., Meinz, M., Liddell, M. E., Kelly, R., Schultz, L., Montgomery, D., Onishi, J., Losada, M., Martin, M., Ebert, T., Tan, C. Y., Schofield, T. L., Nagy, E., Meineke, A., Joyce, J. G., Kurtz, M. B., Caulfield, M. J., Jansen, K. U., McClements, W., and Anderson, A. S. (2006) A novel *Staphylococcus aureus* vaccine: iron surface determinant B induces rapid antibody responses in rhesus macaques and specific increased survival in a murine *S. aureus* sepsis model, *Infect. Immun. 74*, 2215-2223.

76. Kelly-Quintos, C., Kropec, A., Briggs, S., Ordonez, C. L., Goldmann, D. A., and Pier, G. B. (2005) The role of epitope specificity in the human opsonic antibody response to the staphylococcal surface polysaccharide poly N-acetyl glucosamine, *J. Infect. Dis. 192*, 2012-2019.

77. Adlam, C., Ward, P. D., McCartney, A. C., Arbuthnott, J. P., and Thorley, C. M. (1977) Effect immunization with highly purified alpha- and beta-toxins on staphylococcal mastitis in rabbits, *Infect. Immun. 17*, 250-256.

78. Lowell, G. H., Colleton, C., Frost, D., Kaminski, R. W., Hughes, M., Hatch, J., Hooper, C., Estep, J., Pitt, L., Topper, M., Hunt, R. E., Baker, W., and Baze, W. B. (1996) Immunogenicity and efficacy against lethal aerosol

staphylococcal enterotoxin B challenge in monkeys by intramuscular and respiratory delivery of proteosome-toxoid vaccines, *Infect. Immun. 64*, 4686-4693.

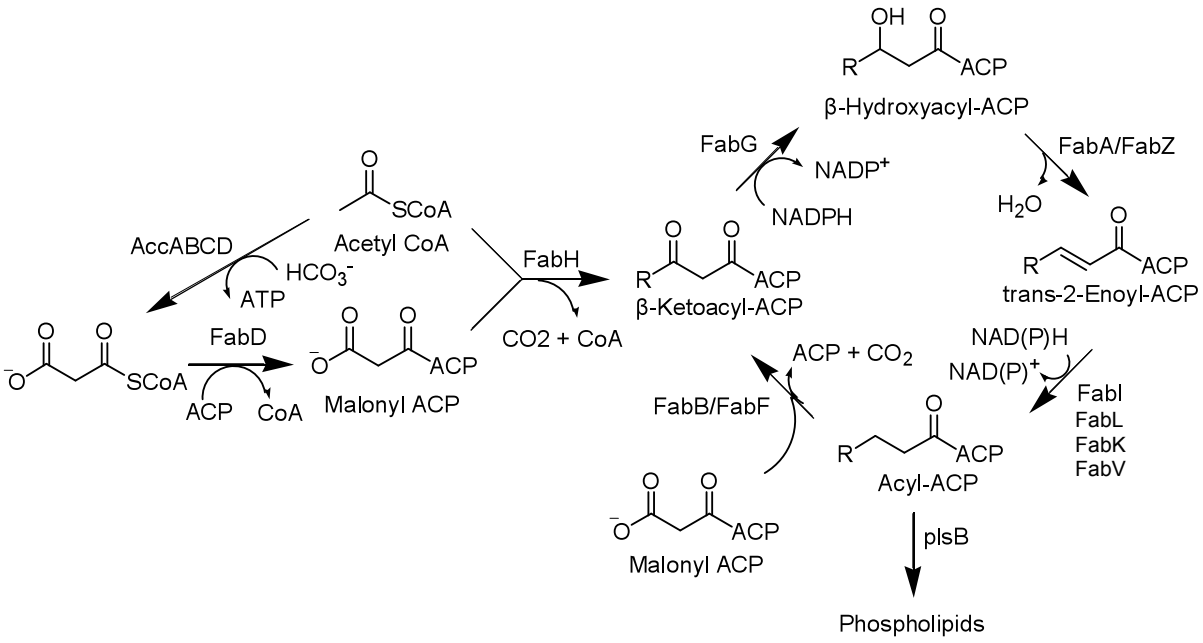
CHAPTER 2: MECHANISM AND INHIBITION OF SAFABI, THE ENOYL ACP REDUCATSE FROM *STAPHYLOCOCCUS AUREUS*

2.1 FATTY ACID BIOSYNTHESIS

Fatty acids are important components of cell membrane. Fatty acid biosynthesis is divided into two types, FASI and FASII, based on whether the reactions are performed by a single polypeptide or by individual enzymes. The FASI pathway usually exists in vertebrates, fungi and certain bacteria, whereas the FASII pathway, as shown in **Scheme 2.1**, is usually found in plants and bacteria. The individual genes of FASII pathway from the model organism *E. coli* have been cloned and studied.

To initiate the fatty acid synthesis, interacting complexes AccAD and AccBD catalyze the carboxylation of acetyl-CoA to form malonyl-CoA (*1*), which is then transferred to acyl carrier protein (ACP) to form malonyl-ACP by the malonyl-CoA:ACP transacylase, FabD (*2*). Then the FASII pathway enters an elongation cycle. In each cycle, the reactions go through condensation, reduction, dehydration, and reduction, and the fatty acids are elongated by two-carbon

atoms. The cycle starts from the condensation between acetyl-CoA and malonyl-ACP, which is catalyzed by β-ketoacyl-ACP synthase III (FabH) (*3*). The intermediate β-ketoacyl-ACP is then reduced by β-ketoacyl-ACP reductase (FabG), forming β-hydroxyacyl-ACP (*4*), which is dehydrated by either FabA or FabZ to form *trans*-2-enoyl-ACP (*5, 6*). FabA also catalyzes a further isomerization of the double bond of the decanoyl ACP to divert into the synthesis of unsaturated fatty acids. The final step and also rate-limiting step in the cycle is catalyzed by the *trans*-2-enoyl-ACP reductase (*4*). There are four forms of this enzyme reported so far, including FabI, FabK, FabL and FabV. The condensation steps in the following cycles are executed by the condensing enzymes FabB and FabF (*7*).



Scheme 2.1: The Type II Fatty Acid Biosynthesis Pathway.

The length of the fatty acids varies among bacteria. For *M. tuberculosis*, which has a waxy cell wall, the fatty acids are elongated up to 60 carbon atoms. It is believed that the substrate specificities and expression levels of the enzymes in the FASII pathway, especially the condensing enzymes, determine the structure and distribution of the final fatty acid products.

The acyl-ACPs from fatty acid synthesis, especially long-chain acyl-ACPs, are also used for the synthesis of membrane phospholipids. The acyl group is transferred from acyl-ACP to glycerol phosphate by *sn*-glycerol-3-phosphate acyltransferase (PlsB) (*8*).

The synthesis of unsaturated fatty acids is diverted by FabA/FabB or FabM. FabA can not only catalyze the formation of *trans*-2-decenoyl ACP, but also facilitate the isomerization from the *trans* to the *cis* conformation. The *cis*-3-unsaturated acyl ACP is then taken by FabB, a condensing enzyme, to enter the synthesis of unsaturated fatty acids (*7*). In a number of bacteria, FabA/FabB pair is not present in the genome, although they produce unsaturated fatty acids. This leads to the discovery of another enzyme involved in unsaturated fatty acid synthesis FabM, which catalyzes the conversion of *trans*-2-unsaturated acyl ACP to *cis*-3-unsaturated acyl ACP (*9*). Then the *cis* intermediate is further elongated by FabF.

In previous studies, it has been shown that the inhibition of the FASII pathway in bacteria causes the breakdown of the cell wall and the disruption of the cell membrane (*10, 11*), demonstrating its importance for bacterial survival. Due to the essentiality of FASII pathway, and the dramatic difference between FASII and

the mammalian FASI pathway, FASII is an attractive target for the development of novel antimicrobials. Inhibitors that target various steps in FASII pathway have been discovered. The condensation step is inhibited by cerulenin, and thiolactomycin (*12, 13*), while NAS-21 and NAS-91 show inhibition against dehydratase FabZ (*14*). The inhibitors of the enoyl ACP reductase will be discussed later.

2.2 ENOYL ACP REDUCTASES AND THEIR INHIBITORS

FabI, the enoyl ACP reductase in the FASII pathway, catalyzes the reduction of enoyl ACP with the cofactor NADH or NADPH. A number of studies suggest that the enoyl reductase, which catalyzes the final and rate-limiting step in each cycle, is a regulator of the FASII pathway, and is essential for the viability of bacteria (*15*). Due to its necessity and low protein sequence homology to the mammalian FASI reductase, FabI is an attractive target for novel antibiotic discovery.

FabI belongs to the family of short-chain dehydrogenase/reductases (SDRs). The SDR family consists of at least 140 different enzymes, which have essential roles in lipid, amino acid, carbohydrate and hormone metabolisms (*16-18*). Most enzymes in this family are NAD(H)- or NADP(H)-dependent oxidoreductases, which contain at least two domains, an NAD(P)-binding domain and a substrate-binding domain. It was suggested that these dehydrogenases evolved from a common ancestral coenzyme nucleotide sequence with various substrate specific domains.

Initially two subfamilies of SDRs were identified, the “classical” subfamily with about 250 residues and “extended” subfamily with 350 residues (*19, 20*). Then three other types, “intermediate”, “complex” and “divergent” are distinguished based on the characteristic sequence motifs of the cofactor binding site and active site (**Table 2.1**) (*21*).

Table 2.1: Five SDR subfamilies.

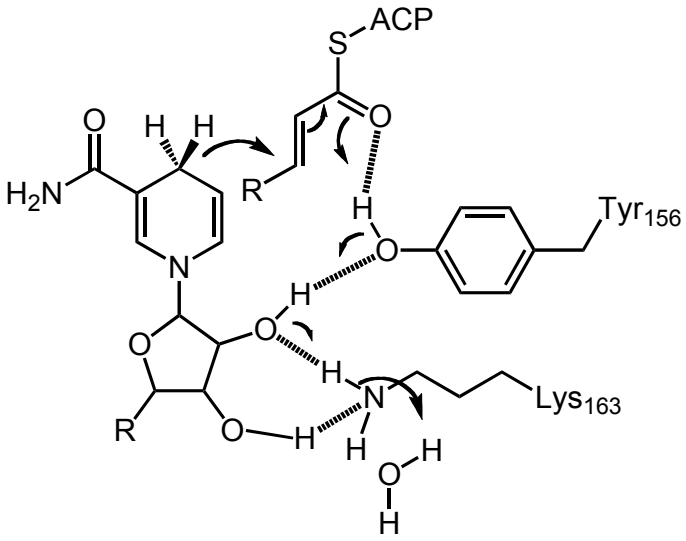
Subfamily	Cofactor binding	Active site
Classical	TGxxx[A/G]xG	YxxxK
Extended	[S/T]GxxGxxG	YxxxK
Intermediate	[G/A]xxGxx[G/A]	YxxxK
Divergent	GxxxxxSxA	YxxMxxxK
Complex	GGxGxxG	YxxxN

Enonyl ACP reductases, such as FabIs from bacteria and plants, belong to the “divergent” subfamily, which shows two distinct patterns. The cofactor-binding Gly-motif is differently spaced compared to the corresponding motifs in other subfamilies, and the second and third glycines are replaced with serine and alanine, respectively. In addition, the active site motif is YxxMxxxK instead of YxxxK. However, according to the crystal structures, different spacing patterns of the conserved tyrosine and lysine residues do not affect their relative positions in the active site (*22*).

The enzymatic reaction mechanisms of FabIs from various organisms have been investigated. FabIs from *E. coli*, *B. subtilis* and *M. tuberculosis* were

reported to catalyze the reaction by a sequential bi-bi mechanism (*23-25*), while surprisingly, a ping-pong mechanism was proposed for the reaction catalyzed by *H. influenzae* FabI (*26*), although it seems unlikely that NADH can transfer a hydride directly to the enzyme.

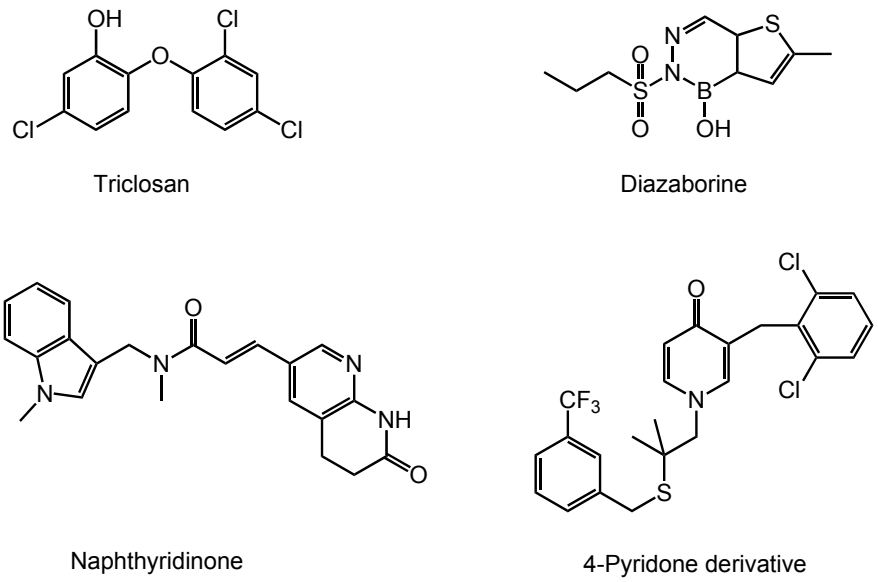
Structural studies as well as the kinetic analysis of oilseed rape FabI lead to a proposal of the reaction mechanism that a hydride transfer from coenzyme NADH to the C3 carbon in the C2-C3 double bond of enoyl ACP, as shown in **Scheme 2.2** (*27*). The resulting enolate anion abstracts a proton from Y156, forming an enol, which then undergoes a tautomerization to produce the final product. The major function of the conserved K163 is to facilitate the cofactor binding by interacting with the ribosyl group in NADH.



Scheme 2.2: Proposed *E. coli* FabI reaction mechanism. A hydride is transferred from NADH to the C3 carbon of the enoyl substrate. Tyr156 is responsible for protonation of the resulting enolate, while Lys163 is hydrogen bonded with the ribosyl ring of NADH.

InhA, the FabI homolog in *M. tuberculosis*, has drawn the attention of several research groups (*22, 28*), since it is a target of isoniazid, one of the front line drugs against tuberculosis (*29*). The crystal structure ternary complex comprising InhA, NAD^+, and a 16-carbon fatty acid substrate reveals important insights of FabI reaction mechanism (*22*). In this complex, the thioester carbonyl oxygen forms a hydrogen bond with the hydroxyl group of the conserved tyrosine residue, while the C3 carbon of the double bonds ideally is positioned for hydride transfer adjacent to the C4 carbon of the ring.

In addition to isoniazid, a number of inhibitors against FabI ranging from thiazoles to pyridone derivatives have been reported recently (*30-35*) (**Scheme 2.3**), and they have shown promising antimicrobial properties against various pathogens, such as *P. aeruginosa*, *S. aureus*, and *M. tuberculosis*.



Triclosan

Diazaborine

Naphthyridinone

4-Pyridone derivative

Scheme 2.3: Chemical structures of FabI inhibitors.

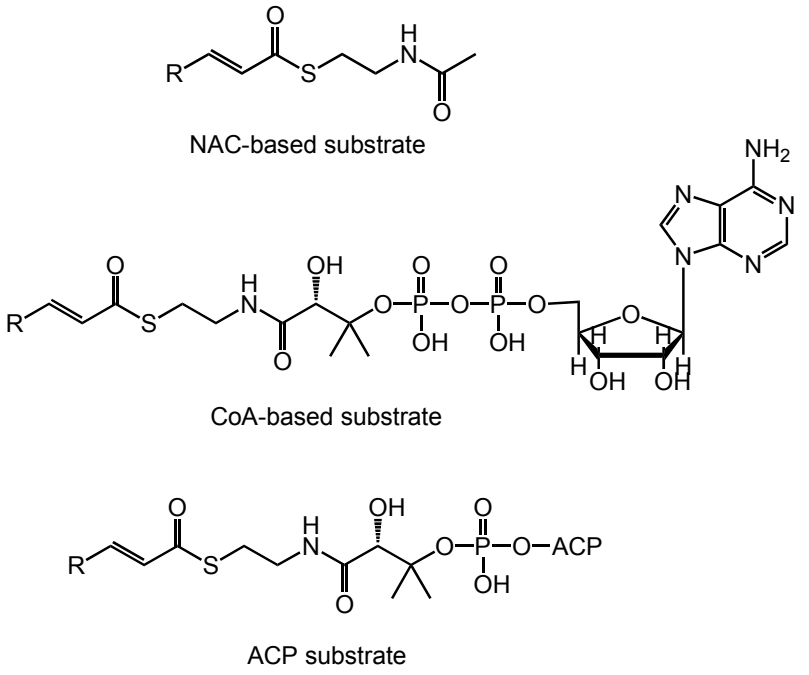
FabI is highly conserved in FASII pathway. However, no FabI homologue could be found in *S. pneumoniae (36)*. Instead, FabK has been reported as the enoyl ACP reductase in this organism. Later, FabK homologues were also identified in other bacteria. Enoyl-ACP reductase III (FabL) was identified in *B. subtilis*, although FabI is present in this bacterium (*24*). Recently, a fourth enoyl-ACP reductase from *Vibrio Cholerae,* termed FabV, was characterized (*37*). Some bacteria have both FabI and FabK/FabL/FabV. It is still not clear why the bacteria need two enoyl ACP reductases.

Generally FabI-directed inhibitors show no or less activity against FabK, FabL or FabV. Recently, Kitagawa et al. designed a class of 4-pyridone inhibitors, which showed good inhibitions against both FabI and FabK (*38*).

2.3 ENOYL ACP REDUCTASE FROM *STAPHYLOCOCCUS AUREUS*

FabI has been identified as the sole enoyl ACP reductase in *S. aureus*. Hence, it is worthwhile to examine the mechanism of this enzyme, as a prelude to the development of potent inhibitors of this enzyme. Although kinetic studies on saFabI have been conducted previously, a detailed mechanistic analysis of the enzyme has so far not been reported. In addition, existing studies are based on *N*-acetylcysteamine (NAC) or CoA based-substrates, rather than the natural acyl carrier protein (ACP) substrates (*39, 40*). As shown in **Scheme 2.4**, NAC-based substrate mimics the end of ACP substrate, but possesses poor aqueous solubility due to lack of charged moiety. CoA-based substrate solves the solubility issue with the addition of a highly hydrophilic CoA portion, but it has an

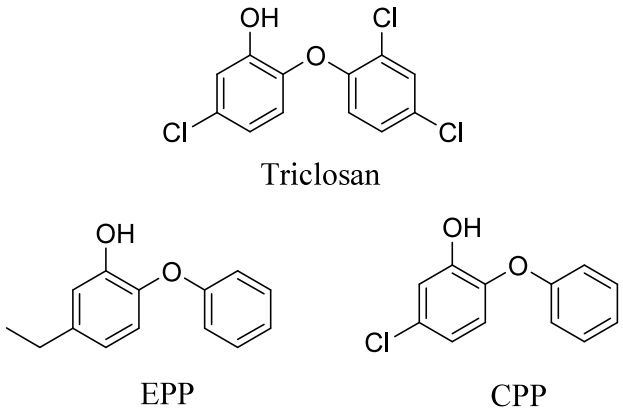
extra adenosine moiety, which may cause nonspecific binding of the substrate. Hence, the ACP substrate is necessary for a precise characterization of the FabI enzyme.



Scheme 2.4: Structures of FabI substrate and substrate analogues.

In the present work we have compared the kinetic properties of NAC, CoA and ACP-based substrates with saFabI, explored the molecular basis for cofactor specificity, and also studied the mechanism of the saFabI-catalyzed reaction. In addition, we have also analyzed the inhibition of saFabI by triclosan and two related diphenyl ethers, 5-ethyl-2-phenoxyphenol (EPP) and 5-chloro-2-phenoxyphenol (CPP) (**Scheme 2.5**). Triclosan is a broad-spectrum antimicrobial that is present in a wide variety of consumer products, such as toothpaste, mouthwashes and hand soaps (*41*). It has been reported that triclosan can inhibit

the growth of *S. aureus* (*42*), and this compound is recommended as a method of controlling MRSA in hospitals (*43, 44*). In order to extend previous studies concerning the mode of action of triclosan (*39, 40, 45, 46*), we performed selection experiments and identified several saFabI mutations that correlated with an increase in resistance to the three inhibitors. Analysis of the impact of these mutations on cell growth and enzyme inhibition strongly suggests that the diphenyl ether based inhibitors target saFabI within the bacterium.



Scheme 2.5: The Diphenyl Ether saFabI Inhibitors

2.4 MATERIALS AND METHODS

Materials.

trans-2-Dodecenoic acid was purchased from TCI (Wellesley Hills, MA). His-bind Ni^{2+}-NTA resin was obtained from Invitrogen (Carlsbad, CA), and centriplus units were from Millipore (Billerica, MA). Vectors were from EMD (Madison, WI). QuikChange site-directed mutagenesis kit was obtained from Stratagene. Triclosan was a gift from Ciba whereas 5-ethyl-2-phenoxyphenol (EPP) and 5-

chloro-2-phenoxyphenol (CPP) were available from a previous study (*47, 48*). All other chemical reagents were purchased from Sigma-Aldrich.

Synthesis of trans-2-dodecenoyl CoA and trans-2-dodecenoyl N-acetylcysteamine.

trans-2-Dodecenoyl CoA (DDCoA) was synthesized by using the mixed anhydride method as described previously (*49*). This method was also used to synthesize *trans*-2-dodecenoyl *N*-acetylcysteamine (DDNAC) with minor modifications. Briefly, 4 mmol *N*-acetylcysteamine was dissolved in 10 mL anhydrous THF, and then 4 mmol of the mixed anhydride was added. After stirring at room temperature for 2 hours, DDNAC was purified by silica gel chromatography using 1:1 ethyl acetate:hexane as the eluant. The identity of the product was confirmed by NMR spectroscopy and ESI mass spectrometry. ^{1}H NMR: δ 0.880 (3H, t), 1.265 (12H, m), 1.964 (3H, s), 2.165-2.237 (2H, q), 3.090 (2H, t), 3.435-3.496 (2H, q), 6.094-6.156 (1H, m), 6.886-6.984 (1H, m). ESI Mass: calcd for $C_{16}H_{30}NO_2S$ ($[M+H]^+$) 300.19, found 300.2.

Expression and Purification of saFabI.

The *fabI* gene from the methicillin-sensitive *S. aureus* strain NCTC 8325 was amplified using the KOD DNA polymerase (Novagen) and the primers listed in **Table 2.2**. The 0.77 kb PCR product was digested with NdeI and BamHI, and cloned into the same restriction sites in pET-16b. The sequence of the construct

was confirmed by DNA sequencing (Sequetech) and the plasmid was then transformed into *Escherichia coli* strain BL21(DE3)plysS.

A single colony was used to inoculate 10 ml of LB medium containing 50 μg/ml ampicillin (LB/AMP) and the culture was grown at 37 ºC overnight. The cells in the starter culture were collected by centrifugation, resuspended in fresh media, transferred to 500 ml LB/Amp medium, and grown at 37 ºC until an optical density of 0.8 at 600 nm had been reached. Subsequently, 0.5 mM IPTG was added to induce the expression of saFabI and the culture was shaken overnight at 25 ºC.

The cells were harvested, and resuspended in 30 ml His binding buffer (20 mM Tris HCl, 500 mM NaCl, and 5 mM imidazole, pH 7.9). After cell disruption by three passages through a French press cell (1000 psi), the cell lysate was centrifuged at 120,000 x g for 1 h, and the supernatant was loaded onto a 5 ml Ni^{2+}-NTA His-bind column, which had been preincubated with 20 ml binding buffer. The His-bind column was washed with 40 ml binding buffer, followed by 30 ml of wash buffer (20 mM Tris HCl, 500 mM NaCl, and 60 mM imidazole, pH 7.9), and saFabI was then eluted using 30 ml of elute buffer (20 mM Tris HCl, 500 mM NaCl, and 500 mM imidazole, pH 7.9). SDS-PAGE was used to identify those fractions containing saFabI, and these fractions were combined and subjected to size exclusion chromatography on a G25 column using 30 mM PIPES, 150 mM NaCl, and 100 mM EDTA, pH 7.8 buffer as the mobile phase. Fractions containing saFabI were pooled, concentrated using a centriplus 30,

and stored in -80 °C. The enzyme concentration was determined by the absorbance at 280 nm with an extinction coefficient of 11520 $M^{-1}cm^{-1}$.

Table 2.2 Nucleotide primers

Name	Sequence [a]
saFabI forward	5'GAGA*CATATG*TTAAATCTTGAAAACAAAACTTATGTCA TCATGGG 3'
saFabI reverse	5' CTC*GGATCC*AATAACGTGAACAAAGCTGTTGAATG 3'
saACP forward	5' GGAATTC*CATATG*GTGGAAAATTTCGATAAAG 3'
saACP reverse	5' CCG*CTCGAG*TTTTTCAAGACTGTTAATAAA 3'
A95V forward	5' GTGTATATCATTCAATCGTATTTGCTAATATGGAAG 3'
A95V reverse	5' CTTCCATATTAGCAAATACGATTGAATGATATACAC 3'
I193S forward	5' CAGCTAGTCCAAGCCGTACATTAAG 3'
I193S reverse	5' CTTAATGTACGGCTTGGACTAGCTG 3'
F204S forward	5' CAAAAGGTGTGGGTGGTTCCAATACAATTCTTAAAG 3'
F204S reverse	5' CTTTAAGAATTGTATTGGAACCACCCACACCTTTTG 3'
R40Q forward	5' GTATTTACTTACCAGAAAGAACGTAGCCG 3'
R40Q reverse	5' CGGCTACGTTCTTTCTGGTAAGTAAATAC 3'
K41N forward	5' GTATTTACTTACCGTAACGAACGTAGCCGTAAAG 3'
K41N reverse	5' CTTTACGGCTACGTTCGTTACGGTAAGTAAATAC 3'
R40Q/K41N forward	5' GTATTTACTTACCAGAACGAACGTAGCCGTAAAG 3'
R40Q/K41N reverse	5' CTTTACGGCTACGTTCGTTCTGGTAAGTAAATAC 3'

[a] Restriction sites are italicized, and mutated sites are shown in underline.

Site-directed mutagenesis, expression and purification of FabI mutants.

Site-directed mutagenesis was performed using the QuikChange mutagenesis kit from Stratagene using the primers listed in **Table 2.2**. The sequence of each mutant plasmid was confirmed by ABI DNA sequencing, and the expression and purification of each saFabI mutant followed the same protocol as that described above for the wild type saFabI protein.

Cloning, expression and purification of S. aureus acyl carrier protein (saACP).

The *S. aureus acp* gene was PCR amplified from NCTC 8325 genomic DNA, digested with NdeI and XhoI, then ligated into the pET23b expression vector so that the coding region of the gene was in-frame with a C-terminal His tag. After DNA sequencing, saACP was expressed and purified using the procedure described above for saFabI. After purification, saACP was dialyzed into 75 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5 and the protein concentration determined using the Bradford method (*50*).

Enzymatic preparation of crotonyl saACP and dodecenoyl saACP (DDsaACP).

Conformationally sensitive SDS PAGE and ESI-MS revealed that saACP expressed and purified from *E. coli* was obtained primarily in the apo form. This protein was converted to the required acyl-saACP using sfp, a phosphopantetheinyl transferase from *Bacillus subtilis* (*51*). Briefly, 25 mg/l apo-saACP was incubated with 80 μM crotonyl CoA or dodecenoyl CoA in the

presence of 1 μM Sfp for 2 h at 37 ºC. The reaction mixture was then loaded onto a Q-sepharose column, and eluted with a gradient consisting of buffer A (20 mM Bis-Tris, pH 7.0) and buffer B (20 mM Bis-Tris, 800 mM NaCl, pH 7.0). Fractions were analyzed by SDS-PAGE and those containing crotonyl-saACP or DDsaACP were pooled and dialyzed into 20 mM Tris-HCl, pH 7.0 buffer. The final products were characterized by ESI mass spectrometry.

Steady-state kinetic assays.

Steady-state kinetics were performed at 25 °C in 100 mM Na_2HPO_4 buffer, pH 7.8. Initial velocities were measured after addition of 50 nM saFabI to give a final assay volume of 500 μl.

For DDsaACP, the reactions were carried out by varying the concentration of DDsaACP at several fixed concentrations of NADPH (50, 100 and 250 μM) or by varying the NADPH concentration at several fixed concentrations of DDsaACP (4.4, 8.2 and 15.0 μM). Kinetic parameters were obtained by fitting the data to equation 1 for a sequential mechanism with GraFit 4.0.

$$v = V_{max} [A] [B]/(K_{ia} K_{mb} + K_{ma} [B] + K_{mb} [A] + [A] [B]) \quad (1)$$

K_{ma} and K_{mb}, are the K_m values of DDsaACP and NADPH, respectively, whereas K_{ia} is the dissociation constant for DDsaACP.

For crotonyl-saACP, as well as the NAC and CoA-based substrates, the kinetic studies employed a concentration range for one substrate (0.3 K_m – 4 K_m) at near saturating concentration of the second substrate. Kinetic parameters

were calculated by fitting the data to the Michaelis-Menten equation (2) using GraFit 4.0.

$$v = V_{max} [S]/ (K_m + [S]) \quad (2)$$

k_{cat} values were obtained by using the relationship between k_{cat} and V_{max}: $V_{max} = k_{cat} [E]$.

Product inhibition assays.

Product inhibition studies were conducted by varying the concentration of DDsaACP at a fixed concentration of NADPH (250 μM) and at different fixed concentrations of $NADP^+$ (0, 1.4 and 2.7 mM) or by varying the concentration of NADPH at a fixed concentration of DDsaACP (20 μM) and at different fixed concentrations of $NADP^+$ (0, 0.7 and 1.4 mM).

The type of inhibition was determined using Lineweaver-Burk plot analysis. Inhibition constants for competitive, uncompetitive, and non-competitive inhibitor were determined by globally fitting all the data points to the equations 3-5, respectively, by using GraFit 4.0.

$$v = V_{max} [S] / (K_m (1 + [I]/K_{is}) + [S]) \quad (3)$$

$$v = V_{max} [S] / (K_m + [S] (1 + [I]/K_{ii})) \quad (4)$$

$$v = V_{max} [S]/ (K_m(1 + [I]/K_{is}) + [S](1 + [I]/K_{ii})) \quad (5)$$

The abbreviations K_{is} and K_{ii} represent the K_i slope and intercept in the double-reciprocal plot according to the nomenclature of Cleland (*52*).

Fluorescence titration.

Equilibrium fluorescence titrations were performed using a Fluorolog-3-21 fluorimeter (Spex) at 25˚C by making microliter additions of ligand to a solution containing 1 μM saFabI. In the fluorescence titration of saFabI with ACP or the diphenyl ethers, the excitation wavelength was 290 nm and the emission wavelength was 335 nm. When titrating saFabI with NADPH, the excitation wavelength was 350 nm, and the emission wavelength was 460 nm. A control experiment was performed using an identical procedure except that enzyme was omitted from the cuvette. K_d values were calulated by fitting the data to equation 6.

$$\Delta F = \Delta F_{max}((K_d + E + L) - [(K_d + E + L)^2 - 4 K_d L]^{1/2})/(2*E) \quad (6)$$

Selection for resistance.

This work was done by Dr. Teruo Kirikae. In brief, Cells from the methicillin-resistant *S. aureus s*train N315 were incubated at 37°C in Mueller-Hinton broth (Becton-Dickinson) containing 0.02 μM triclosan, 0.08 μM EPP, or 0.08 μM CPP, respectively. After 48 h, 100 μl of each culture was plated onto Mueller-Hinton agar medium containing 0.08 μM triclosan, 0.15 μM EPP, and 0.15 μM CPP, respectively. The plates were incubated at 37 °C for 24 h. Resistant colonies were picked and their phenotype confirmed by regrowth on the same media containing selective concentrations of triclosan, EPP, and CPP, respectively. The *S. aureus fabI* gene from the diphenyl ether-resistant mutants was characterized by double-strand nucleotide sequencing of PCR products amplified with *Ex-taq* polymerase (Takara Bio, Shiga, Japan) and the following primers: SafabI-F (5'-

GTCATCATGGGAATCGCTAA-3') and SafabI-R (5'-GCGTGGAATCCGCTATCTAC-3'). Sequencing reactions were performed using the above SafabI-F and SafabI-R primers with the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif), and sequencing data were obtained using an Applied Biosystems 3100 DNA sequencer (Applied Biosystems).

MIC measurement.

The MIC values were determined by the microbroth dilution assay according to the Clinical and Laboratory Standards Institute methods for antimicrobial susceptibility tests for aerobically growing bacteria (*53*).

Preincubation inhibition assays for slow-binding inhibitors.

Kinetic assays were performed essentially as described previously (*47*). Briefly, a 500 µL reaction mixture containing 50 nM saFabI, 250 µM NADPH, and various concentrations of diphenyl ether inhibitors (0 – 2 µM) at different fixed concentrations of $NADP^+$ (20 – 800 µM) were preincubated at 4 ºC for 3 hours. The reaction mixture was then warmed to room temperature, and the reaction was initiated by the addition of 20 µM DDsaACP. Apparent inhibition constants ($K_{i,app}$) were calculated by fitting the inhibition to equation 7 at each concentration of $NADP^+$

$$v_i/v_0 = 1/ (1 + [I]/ K_{i,app}) \quad (7)$$

Subsequently, the series of $K_{i,app}$ values was fitted to equation 8 to obtain K_1 and K_2, which represent the inhibition constants for inhibitor binding to $NADP^+$ bound or NADPH bound forms of the enzyme, respectively.

$$K_{i,app} = K_2 (1 + [NADP^+]/K_{mNADP})/(1 + [NADP^+]/(K_{mNADP}K_1/K_2) \quad (8)$$

Steady-state inhibition studies of diphenyl ethers.

Steady-state inhibition experiments were performed by varying the concentration of NADPH at a fixed concentration of DDsaACP (20 μM) and at different fixed concentrations of triclosan, EPP or CPP. Reactions were initiated by the addition of the enzyme. Unless noted, the enzyme concentration was 50 nM. The data analysis was performed as described for the product inhibition assays.

2.5 RESULTS AND DISCUSSION

Expression and purification of wild-type and mutant saFabI proteins.

The protein was purified using His-tag affinity chromatography, providing homogeneously pure recombinant protein with the predicted molecular mass (~30 kDa) as determined by SDS-PAGE. As previously reported (*39*), the protein precipitated if 100 mM EDTA was omitted from the G25 buffer. In addition, we also observed that saFabI would precipitate even in 100 mM EDTA when the enzyme concentration was higher than 40 μM. Consequently, the enzyme stocks were kept at 30 μM.

Preparation of ACP substrates.

saACP was purified by His-tag affinity chromatography, and only one form of ACP was observed on a 15% Tris-glycine gel, unlike the ACP (AcpM) from *M. tuberculosis* which is obtained in three forms (apo, holo and acyl form) (*54*). The ESI mass of saACP after deconvolution was 9711.6 ± 0.4, which is close to the calculated mass of apo-saACP (9707.7), suggesting that only the apo form was expressed. Subsequently, apo-saACP was converted into crotonyl saACP or dodecenoyl saACP (DDsaACP) by sfp, the phosphopantetheine transferase from *Bacillus subtilis*. According to analysis by SDS-PAGE (**Figure 2.1**), the apparent molecular mass of DDsaACP is smaller than apo-ACP, probably due to favorable hydrophobic interactions between the dodecenoyl acyl chain in DDsaACP and the SDS. The molecular weight of DDsaACP was confirmed as correct by ESI mass analysis.

Figure 2.1: SDS-PAGE analysis of ACP conversion. Samples from left to right: apo-ACP, dodecenoyl ACP, and protein ladder (from top to bottom: 170.8, 109.5, 78.9, 60.4, 47.2, 35.1, 24.9, 18.3, 13.7, and 5.7 kDa).

Substrate specificity.

As shown in **Table 2.3**, saFabI has a 180-fold higher k_{cat}/K_m with NADPH than with NADH, suggesting that saFabI is an NADPH-dependent enzyme, which is consistent with previous results when *trans*-2-octenoyl N-acetylcysteamine was used as the substrate (*39*). The unusual cofactor specificity compared with FabIs from other organisms will be discussed later.

As expected, saFabI also has a preference for ACP-linked substrates compared to those attached to NAC or CoA, indicating that saFabI is specific for its natural substrate. While we were able to detect binding of apo-saACP to saFabI (K_d = 18.2 μM) by fluorescence titration (**Figure 2.2**), no change in fluorescence could be detected upon addition of NAC or CoA, suggesting that these carriers do not bind strongly to the enzyme in agreement with the kinetic data. In addition, the enzyme also had a preference for a long chain (C12) substrate compared to the shorter (C4) acyl group, with DDsaACP having a k_{cat}/K_m value ~30 fold larger than for the corresponding crotonyl-ACP substrate. This effect is also observed for the NAC or CoA-based substrates (**Table 2.3**). Interestingly, the effect of the acyl chain length on k_{cat}/K_m is primarily an effect on catalytic efficiency (k_{cat}) rather than K_m, suggesting that remote interactions between the longer acyl chain and the enzyme modulate the precise orientation of the catalytic groups in the active site.

Finally, both the NAC and CoA-based substrates suffered from some disadvantages, the NAC substrates possessing limited aqueous solubility even in the presence of 5% DMSO, whereas DDCoA exhibited substrate inhibition at

high concentrations (60 μM). Substrate inhibition was not observed with DDNAC or DDsaACP, which leads to the hypothesis that the adenosine portion of CoA can bind non-productively in the NADPH binding-site.

Table 2.3: Kinetic Parameters of Different Substrates with saFabI

Substrates	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
Crotonyl NAC [a]	1.5 ± 0.5	8.0 ± 0.3	0.20 ± 0.06
Crotonyl CoA[b]	- [c]	- [c]	- [c]
Crotonyl saACP [c]	11.6 ± 1.1	11.5 ± 2.0	1.1 ± 0.2
Dodecenoyl NAC [c]	30.4 ± 1.6	23.3 ± 2.9	1.3 ± 0.2
Dodocenoyl CoA [c]	18.0 ± 0.8	24.1 ±2.5	0.8 ± 0.1
Dodecenoyl saACP [d]	130 ± 9	4.5 ± 0.5	29.5 ± 5.0
NADH [e]	-	-	0.010 ± 0.001
NADPH [d, e]	130 ± 9	71 ± 6	1.84 ± 0.31

[a] Parameters were measured by Heath et al. (*55*); [b] reactions were carried out with 300 μM NADPH; [c] no enzymatic activity was observed; [d] K_m and k_{cat} values were obtained by fitting data into the sequential mechanism equation; [e] reactions were carried out with 20 μM DDsaACP.

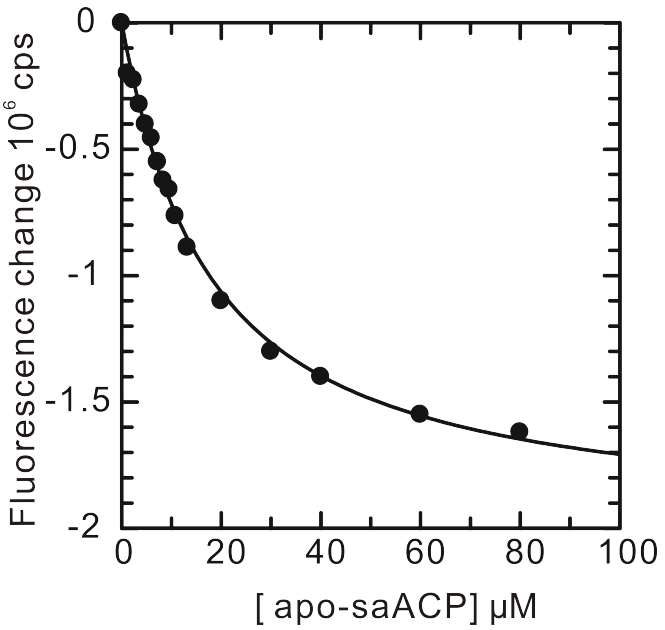


Figure 2.2: Fluorescence titration of saFabI with apo-ACP. Excitation wavelength is 290 nm, and emission wavelength is 336 nm.

It is interesting that saFabI is NADPH-dependent, whereas the FabI homologues from *E. coli, B. subtilis*, *H. influenzae* and *M. tuberculosis*, are all reported to be NADH-dependent ACP reductases (*23, 26, 56*). According to the ecFabI-NAD^+ crystal structure (**Figure 2.3**, pdb code 1DFI), Q40 is very close (2.8 Å) to the 2'-hydroxyl group of the NAD^+ adenosine moiety.

Sequence alignment of the FabI proteins from different organisms (**Figure 2.4**), indicates that two positively-charged residues, R40 and K41, appear close to the position of Q40 in saFabI, whereas nonpolar residues (L, F), or a negatively charged residue (E) is present in the cases of the FabIs from *B. subtilis*, *H. influenzae* and *M. tuberculosis*, respectively.

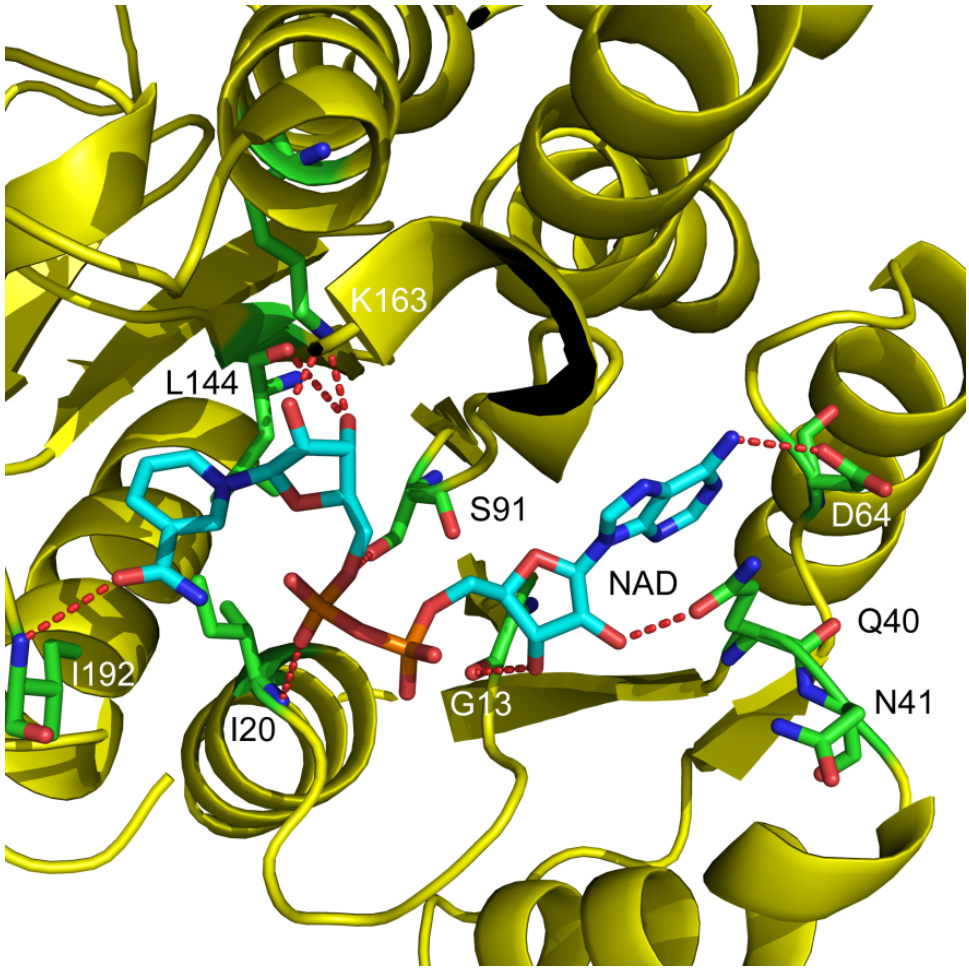


Figure 2.3: Structure of the ecFabI:NAD$^+$ Complex. Structure of ecFabI complexed with NAD$^+$ (pdb code 1DFI) showing interactions between the protein and the NAD$^+$ ribose. ecFabI is colored *yellow* and polar interactions between the protein residues (*green*) and NAD$^+$ (*cyan*) are indicated with *red* dashed lines. Q40 interacts with the 2'-hydroxyl group in the adenosine moity of NAD$^+$. The Figure was made using pymol (*57*).

Figure 2.4: FabIs Sequence Alignment. Sequence alignment of FabIs from *E. coli*, *S. aureus*, *H. influenzae*, *M. tuberculosis* and *B. subtilis* performed using ClustalW. The red-colored amino acids are proposed to interact with the cofactor, according to the X-ray structure of ecFabI:NAD^+ complex.

Basic residues, such as R or K, in this position are expected to interact strongly with the 2'-phosphate of NADPH. In order to investigate the importance of R40 and K41 in the interaction of NADPH with saFabI, we constructed two single-mutants, R40Q and K41N, and found that both mutants exhibit at least a 50-fold decrease in k_{cat}/K_m for NADPH, whereas k_{cat}/K_m for NADH increases by 5 to 7 fold (**Table 2.4**). The double mutation R40Q/K41N further decreases k_{cat}/K_m for NADPH by 10-fold compared with the single mutants, proving that both R40 and K41 are involved in interactions with 2'-phosphate of NADPH. As shown in **Figure 2.4**, all the residues apart from Q40 that have polar interactions with NAD^+ in the ecFabI-NAD^+ crystal structure are highly conserved in saFabI. Hence, it might be expected that the cofactor specificity would be completely reversed in the saFabI double mutant. However, the NADH-specificity of the double mutant remains almost the same as the single mutants, only 7 fold higher than that of the wild type saFabI, indicating that other factors, such as the hydrophobic interactions between the enzyme and the cofactor, are critical for optimal binding of NADH.

Table 2.4: Effect of Mutagenesis on the Specificity of saFabI for NADH and NADPH

Enzyme	k_{cat}/K_m (min^{-1} μM^{-1})	
	NADPH	NADH
WT	1.840 ± 0.311	0.010 ± 0.001
R40Q	0.020 ± 0.001	0.053 ± 0.003
K41N	0.036 ± 0.002	0.072 ± 0.002
R40Q/K41N	0.004 ± 0.001	0.076 ± 0.005

Enzymatic mechanism of the saFabI-catalyzed reaction.

As expected, double-reciprocal plots of the enzyme kinetic data generated intersecting lines (**Figure 2.5A and 2.5B**), which is a characteristic of a ternary complex mechanism in which NADPH transfers a hydride directly to the substrate. In order to determine the order of substrate binding, the effect of the product $NADP^+$ on the reaction was analyzed. Initial velocities were measured at various concentrations of DDsaACP and a fixed concentration of NADPH and at different fixed concentrations of $NADP^+$. The parallel lines in the double reciprocal plot that were obtained (**Figure 2.6A**) suggest that $NADP^+$ is an uncompetitive inhibitor with respect to DDsaACP. However, the assays were also performed with NADPH as the varied substrate. As shown in **Figure 2.6B**, the lines intersect to the left of the y-axis, indicating that the $NADP^+$ is a non-competitive inhibitor with respect to NADPH. The inhibition patterns of $NADP^+$

against the two substrates, are only consistent with an ordered bi-bi mechanism, in which DDsaACP binds first, followed by NADPH.

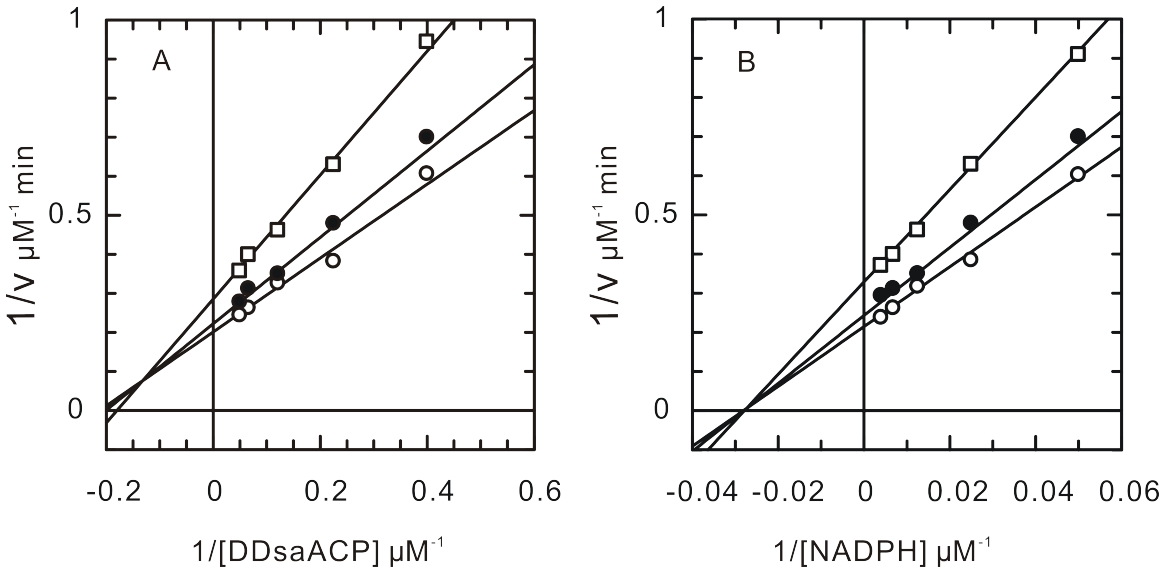


Figure 2.5: Ternary complex mechanism. (A) Double reciprocal plot by varing DD-saACP in the presence of 250 μM NADPH (○), 100 μM NADPH (●) and 50 μM NADPH (□). (B) Double reciprocal plot by varying NADPH in the presence of 15 μM DDsaACP (○), 8.2 μM DDsaACP (●), and 4.4 μM DDsaACP (□).

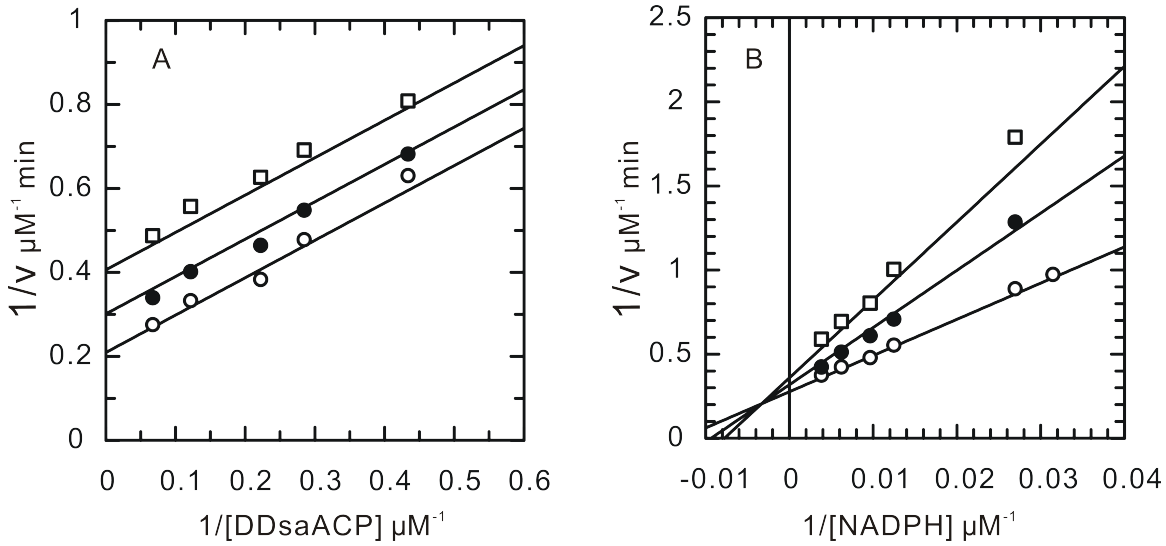


Figure 2.6: Double reciprocal plots of product inhibition studies. (A) Reactions were carried out in the absence of $NADP^+$ (○), or in the presence of 1.4 mM $NADP^+$ (●) and 2.7 mM $NADP^+$ (□). (B) Reactions were carried out in the absence of $NADP^+$ (○), or in the presence of 0.7 mM $NADP^+$ (●) and 1.4 mM $NADP^+$ (□).

In fluorescence titration experiments, no fluorescence change was observed when titrating saFabI with NADPH, suggesting that NADPH does not bind to saFabI, or that binding does not affect the fluorescence of the NADPH fluorophore. In contrast, a K_d value of 2.3 μM was obtained when saFabI was titrated with DDsaACP (**Figure 2.7**). These results provide further support for the proposed ordered bi-bi mechanism.

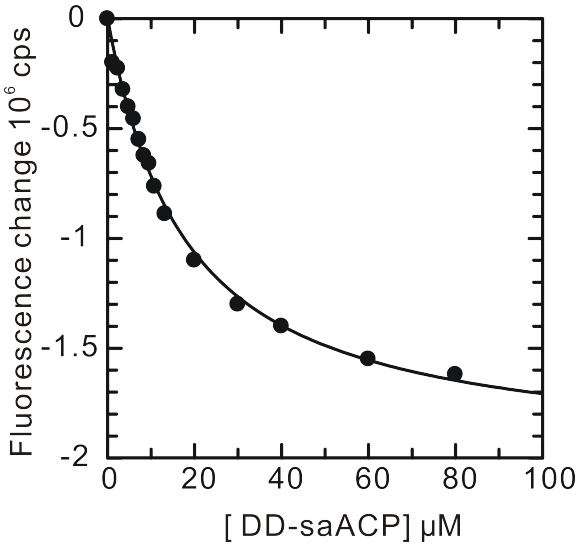


Figure 2.7: Fluorescence titration of saFabI with DDsaACP. Exitation wavelength is 290 nm, and emission wavelength is 336nm.

Antimicrobial activity of the diphenyl ether saFabI inhibitors.

The molecular basis for the antibacterial activity of triclosan against *S. aureus* has attracted much attention. It was proposed that triclosan inhibits *S. aureus* by targeting the enoyl reductase in this organism (*39, 40, 45*), since it is known that triclosan inhibits the FabI homologue in *E. coli* (*58-60*), and that saFabI shows 43% sequence identity to ecFabI. However, the observation that small-colony variants of *S. aureus* have increased resistance to triclosan suggests a more complex underlying mode of action of this antimicrobial agent (*46*).

As a first step in developing novel inhibitors against *S. aureus*, we synthesized two diphenyl ether analogues of triclosan: 5-ethyl-2-phenoxy-phenol (EPP) and 5-chloro-2-phenoxy-phenol (CPP). Both compounds have similar MIC values to triclosan against different strains of *S. aureus* (**Table 2.5**). It is very encouraging that the MIC values are similar for both methicillin-sensitive (ATCC

29213) and methicillin-resistant *S. aureus* strains (N315, Mu50), suggesting that the diphenyl ethers are orthogonal to current antibiotics.

Table 2.5: MICs of diphenyl ethers against different strains of *S. aureus*

Strains	MIC μg/ml (μM)		
	Triclosan	EPP	CPP
ATCC29213	0.03 (0.10)	0.03 (0.14)	0.03 (0.14)
N315	0.03 (0.10)	0.03 (0.14)	0.06 (0.27)
Mu50	0.06 (0.20)	0.06 (0.27)	0.12 (0.54)

In order to probe the mechanism of action of the diphenyl ether inhibitors, selection experiments resulted in the identification of three novel mutations in the *fabI* gene, A95V, I193S and F204S (**Table 2.6**), suggesting that saFabI is the intracellular target for these compounds.

Table 2.6: Selection for Resistance to the Diphenyl Ether saFabI Inhibitors

Mutants derived from *S. aureus* N315	Selections for mutants resistant to compound	*fabI* gene	
		Nucleotide change [a]	Amino acid change
N315Tr.4	Triclosan	GCA → G<u>T</u>A	Ala95 → Val
N315Tr.5	Triclosan	GCA → G<u>T</u>A	Ala95 → Val
N315PT01.1	EPP	GCA → G<u>T</u>A	Ala95 → Val
N315PT01.2	EPP	GCA → G<u>T</u>A	Ala95 → Val
N315PT01.3	EPP	GCA → G<u>T</u>A	Ala95 → Val
N315PT01.4	EPP	GCA → G<u>T</u>A	Ala95 → Val
N315PT52.1	CPP	ATC → A<u>G</u>C	Ile193 → Ser
N315PT52.2	CPP	TTC → T<u>C</u>C	Phe204 → Ser
N315PT52.3	CPP	TTC → T<u>C</u>C	Phe204 → Ser
N315PT52.4	CPP	TTC → T<u>C</u>C	Phe204 → Ser

[a] Mutation sites are shown in underline.

Based on the ecFabI/NAD/triclosan crystal structure (*61*), the three residues are thought to lie in the cofactor binding pocket of saFabI (**Figure 2.8**). Interestingly, the residues corresponding to A95 and F204 in ecFabI (G93 and F203), were also found to be mutated in triclosan-resistant *E. coli*. In addition, the residue corresponding to I193 in ecFabI (I192) interacts with the cofactor, as shown in **Figure 2.8**.

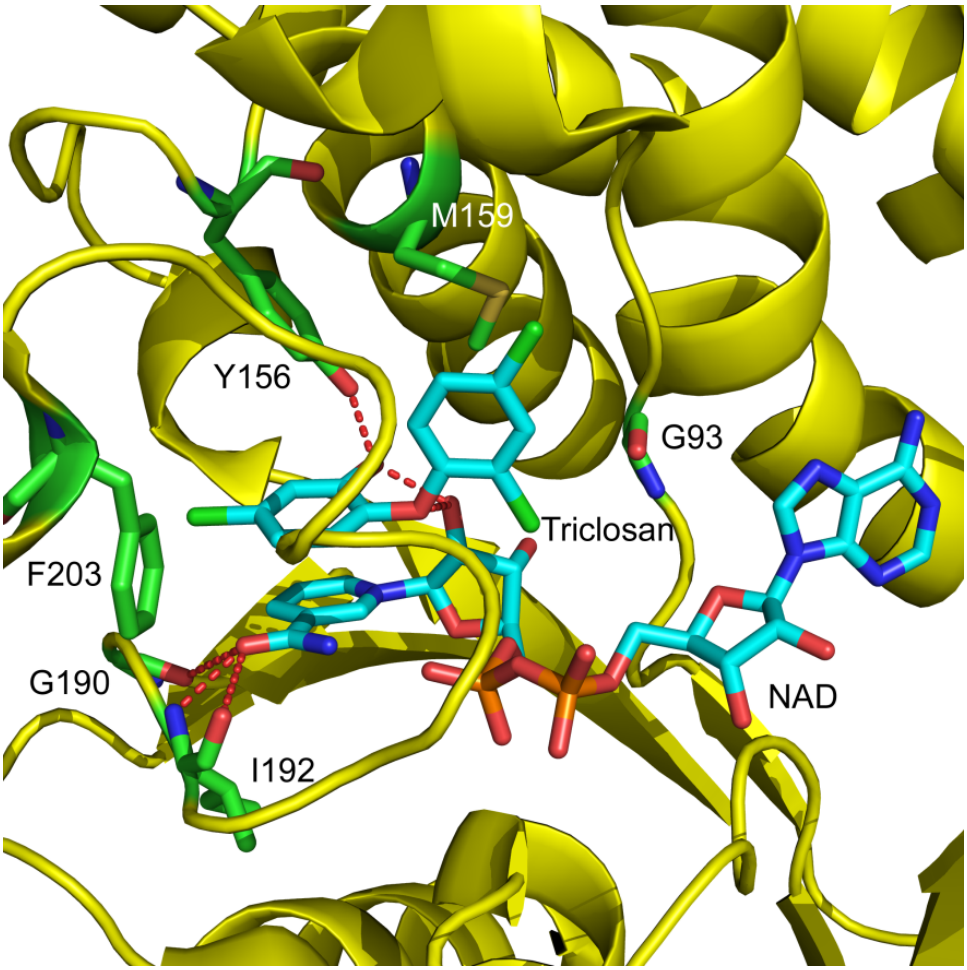


Figure 2.8: Structure of ecFabI Complexed with NAD^+ and Triclosan. Structure of triclosan bound to ecFabI (pdb code 1D8A) showing the proximity of residues G93, I192 and F203 to the inhibitor binding site. The corresponding residues in saFabI were found to be mutated in the diphenyl ether resistant *S. aureus* strains. ecFabI is colored *yellow*, while the polar interactions between the residues (*green*) in ecFabI and NAD/triclosan (*cyan*), are indicated with *red* dashed lines. The Figure was made using pymol (*57*).

As expected, the three saFabI mutations affected the kinetic parameters associated with NADPH more significantly than the parameters for the ACP substrate (**Table 2.7**). In particular, the k_{cat}/K_m value for NADPH decreased by 240 fold for the A95V mutant, while the k_{cat}/K_m value for DDsaACP decreased

only 18 fold, confirming that the mutation has a major impact on the interaction of the cofactor with the enzyme.

Table 2.7: Kinetic Parameters for the Reduction of DDsaACP by Wild Type and Mutant FabIs

Enzyme	k_{cat} (min^{-1})	NADPH		DDsaACP	
		K_m (μM) [a]	k_{cat}/K_m ($min^{-1}μM^{-1}$) [a]	K_m (μM) [b]	k_{cat}/K_m ($min^{-1}μM^{-1}$) [b]
WT	130.2 ± 9.1	70.8 ± 6.0	1.8 ± 0.3	4.5 ± 0.5	29.5 ± 5.0
A95V	18.4 ± 0.1	269.4 ± 29.2	0.07 ± 0.001	11.4 ± 1.6	1.7 ± 0.2
I193S	-	> 1000	0.13 ± 0.02	-	5.1 ± 0.4
F204S	157.3 ± 7.2	429.6 ± 45.0	0.17 ± 0.02	18.4 ± 1.3	7.8 ± 0.7

[a] Reactions were carried out with 20 μM DDsaACP;

[b] reactions were carried out with 300 μM NADPH.

To analyze further the effect of the mutations on enzyme inhibition, we quantified the interaction of the three diphenyl ether inhibitors with both wild-type and mutant saFabIs. In the case of wild-type saFabI, all three compounds were slow-onset inhibitors, binding preferentially to the enzyme-$NADP^+$ product complex. The slow step in the formation of the final enzyme-inhibitor complex is believed to result from the ordering of a flexible loop that covers the active site, as seen for the inhibition of ecFabI by triclosan. Since the loop in ecFabI (residues 193-202) is highly conserved in saFabI, we propose that the slow step

during the inhibition of saFabI by the diphenyl ethers is also caused by the loop ordering. We used the preincubation method to measure K_1 and K_2, which represent the equilibrium dissociation constants of the compounds from the E-$NADP^+$ and E-NADPH forms of the enzyme, respectively.

According to the inhibition results (**Figure 2.9**), the compounds are uncompetitive inhibitors of $NADP^+$, and competitive inhibitors of NADPH. K_1 is 20 to 40 fold smaller than K_2, suggesting that the diphenyl ethers bind preferentially to E-$NADP^+$. In addition, the K_1 values correlate well with the MIC data, suggesting that saFabI is the target of this class of inhibitor.

The use of equation 6 to analyze the inhibition data is based on the assumption that the inhibitors do not bind tightly to the free enzyme. In agreement with this assumption, fluorescence titrations of saFabI with the three inhibitors (**Figure 2.10**) gave K_d values between 50 and 200 μM, at least 200-fold higher than either K_1 or K_2.

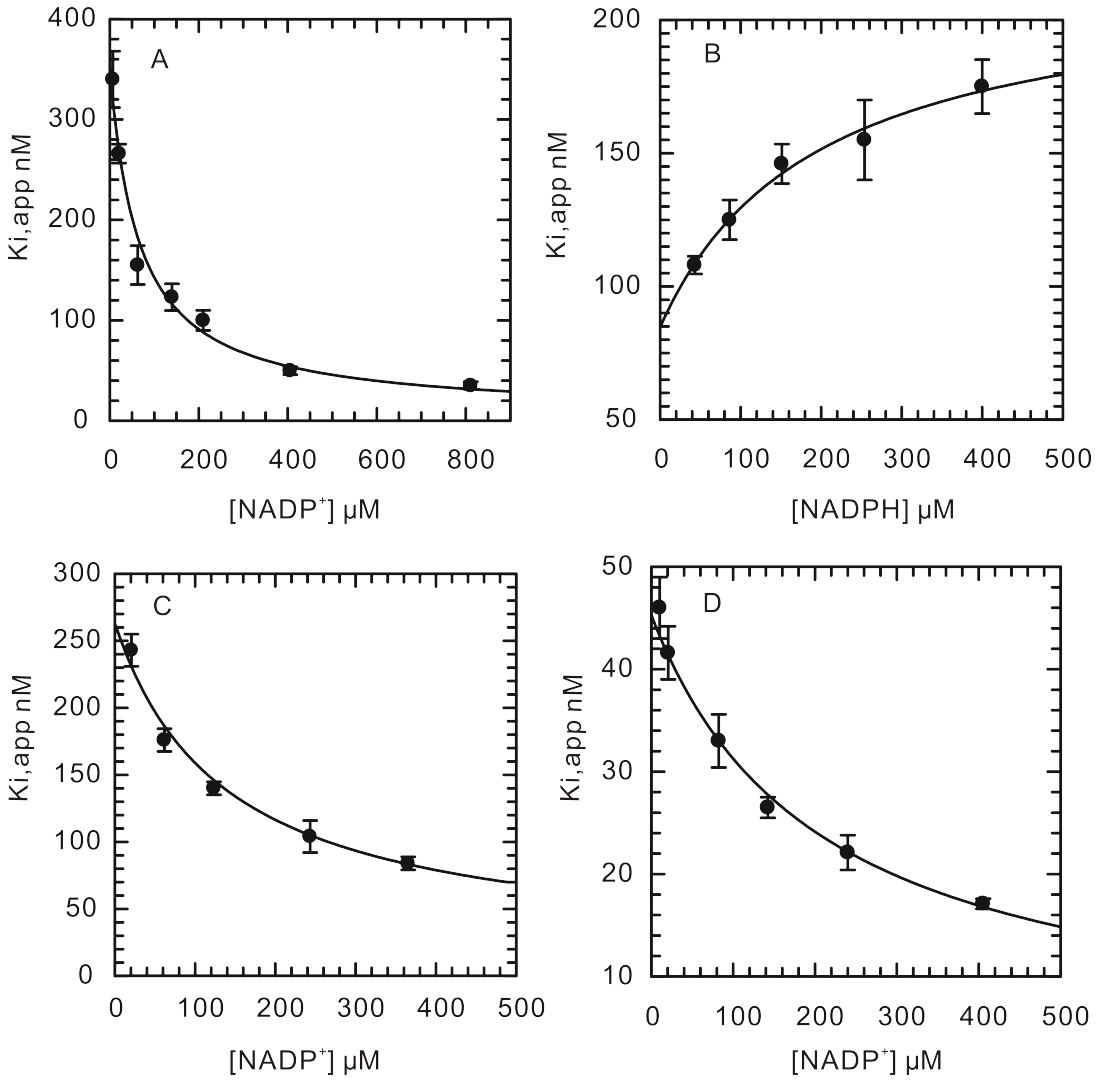


Figure 2.9: Inhibition of Wild Type saFabI by the Diphenyl Ether Inhibitors. (A) Dependence of $K_{i,app}$ on [$NADP^+$] for inhibition by triclosan. (B) Dependence of $K_{i,app}$ on [NADPH] for inhibition by triclosan. (C) Dependence of $K_{i,app}$ on [$NADP^+$] for inhibition by EPP. (D) Dependence of $K_{i,app}$ on [$NADP^+$] for inhibition by CPP.

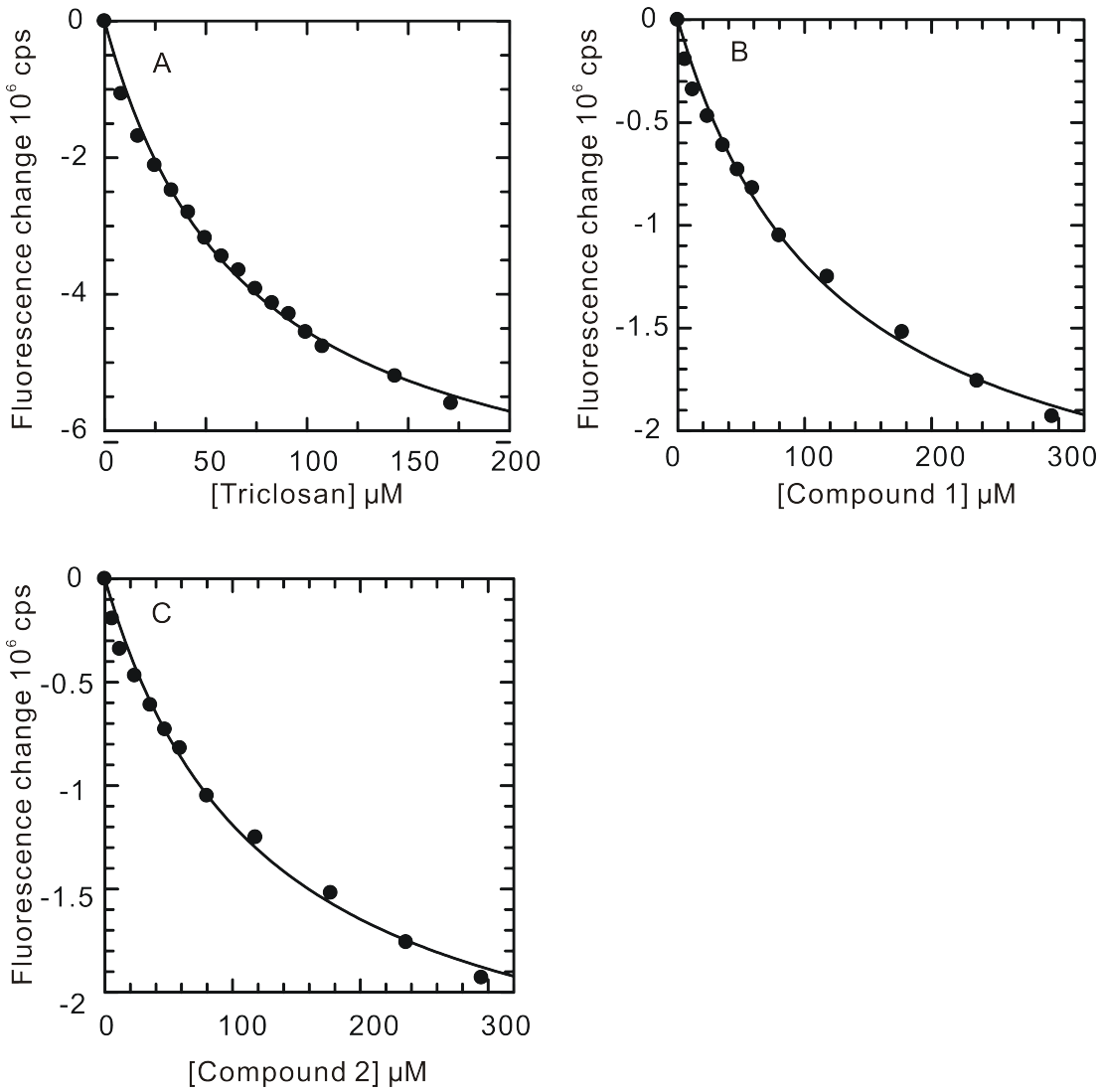


Figure 2.10: Fluorescence titrations of saFabI with diphenyl ethers. Exitation wavelength is 290 nm, and emission wavelength is 336nm. Fluorescence titration of saFabI was done with triclosan (A), EPP (B) and CPP (C), respectively.

In contrast to the wild-type enzyme, the diphenyl ether compounds are classical reversible inhibitors of the saFabI mutant enzymes. Triclosan, CPP and EPP are uncompetitive or noncompetitive inhibitors of the mutant saFabIs with respect to NADPH, with K_i values 5 to 3000 fold higher than for the wild-type

enzyme. This increase in K_i correlates with the increase in the MIC values observed for the *S. aureus* strains harboring the *safabI* mutations (**Table 2.8**). This further substantiates the hypothesis that saFabI is the target of diphenyl ethers within this bacterium. Interestingly, for the mutant I193S, K_i values of triclosan, EPP and CPP increase only by 5 to 25 fold, but the MICs increase by more than 60 fold. This suggests that additional mechanisms of resistance are operative, such as the overexpression of the mutant saFabI as reported by Fan et al. (*45*). We also observed that the frequency with which each mutation occurred also correlated with the effect of that mutation on enzyme inhibition. Thus, A95V, the most frequent mutation in the resistant strains, had the largest impact on inhibition of saFabI by the diphenyl ether inhibitors.

Table 2.8: Inhibition of Wild Type and Mutant saFabIs by Diphenyl Ethers

E	I	K_1 (nM)	K_2 (nM)	Inhibition pattern	MIC µg/ml (µM)
WT	Triclosan	4.9 ± 0.6 [a] 5.6 ± 0.2 [b]	375.1 ± 20.3 [a] 216.4 ± 7.5 [b]	UC with respect to $NADP^+$. C with respect to NADPH.	0.03(0.10)
	EPP [a]	8.3 ± 0.8	273.1 ± 13.2	UC with respect to $NADP^+$.	0.03(0.14)
	CPP [a]	2.0 ± 0.2	48.7 ± 1.6	UC with respect to $NADP^+$.	0.06(0.27)
A95V	Triclosan [c]	8100 ± 1190	-	UC with respect to NADPH.	2.8 (10)
	EPP [c]	1660 ± 50	-	UC with respect to NADPH.	4.40 (20)
	CPP [c]	6150 ± 160	-	UC with respect to NADPH.	8.80 (40)
I193S	Triclosan[d]	35 ± 18	473 ± 196	NC with respect to NADPH.	2.80 (10)

	EPP [d]	41 ± 5	783 ± 281	NC with respect to NADPH.	8.80 (40)
	CPP [d]	52 ± 2	690 ± 320	NC with respect to NADPH.	17.60 (80)
	Triclosan [c]	210 ± 20	-	UC with respect to NADPH.	0.70 (2.5)
F204S	EPP [d]	160 ± 60	300 ± 110	NC with respect to NADPH.	2.20 (10)
	CPP [c]	570 ± 30	-	UC with respect to NADPH.	8.80 (40)

[a] $NADP^+$ was varied; [b] NADPH was varied; [c] Uncompetitive. K_{ii} has been listed under K_1. Enzyme concentration was 200 nM; [d] noncompetitive inhibition. K_{ii} has been listed under K_1, and K_{is} has been listed under K_2. K_{ii} and K_{is} were defined by Cleland previously (*52*).

Finally, we also analyzed the ability of the three compounds to inhibit the growth of *Enterococcus faecalis.* This organism contains both a FabI enoyl reductase as well as a FabK homologue that is not sensitive to triclosan (*36*). In **Table 2.9,** it can be seen that the three diphenyl ethers have MIC values against *E. faecalis* that are ~1000-fold higher than for *S. aureus* again supporting the contention that the antibacterial action of these compounds is directed against FabI.

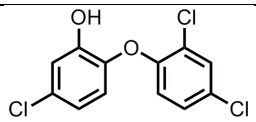
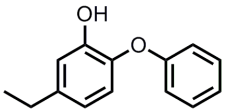
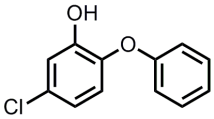
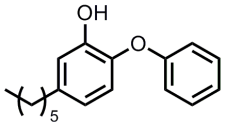
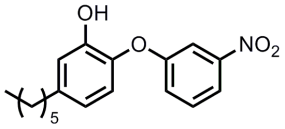
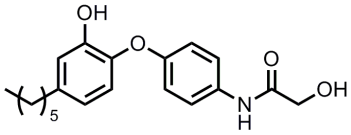
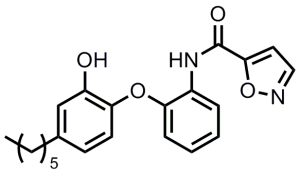
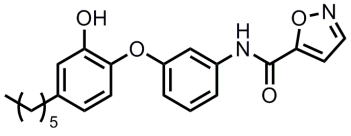
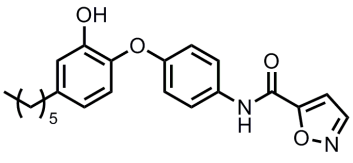
Table 2.9: MICs of diphenyl ethers against *S. aureus* and *E. faecalis*

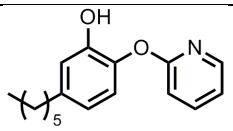
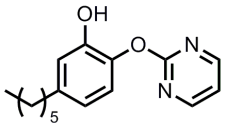
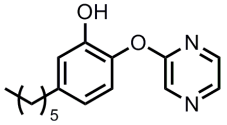
Compound	MIC (μg/ml)	
	S. aureus ATCC25923	*E. faecalis* ATCC19433
Triclosan	0.03	>32
EPP	0.03	>32
CPP	0.06	>32

Inhibition of diphenyl ethers against S. aureus.

A series of diphenyl ethers inhibitors that have improved aqueous solubility were synthesized, as described previously (*62*). Their antibacterial activity was tested against *S. aureus*. The results are shown in **Table 2.10**. Comparing EPP and PT04, it is obvious that short acyl chain substitution in A-ring *para*-position is preferable. In contrast, longer acyl chain (C6 to C9) is preferred to inhibit *M. tuberculosis (63)*, probably because *M. tuberculosis* produces much longer fatty acids than *S. aureus*. In addition, bulky substitutions on B-ring are only tolerated in the *meta*-position (PT28-30). PT42 exhibits 4-fold smaller MIC than PT04, suggesting that pyrazine might serve as a better B-ring scaffold than benzene ring. However, the inhibitory potencies of all these compounds are at least 10-fold weaker than that of triclosan. It may be due to the unsatisfactory inhibition against saFabI, or undesirable drug metabolism in the bacterium. Determination of inhibition constants is needed for guidance of further design.

Table 2.10: Inhibition of diphenyl ethers against *S. aureus*.

Inhibitor	Structure	MIC against ATCC25923 (μg/ml)
Triclosan		0.03
EPP		0.03
CPP		0.06
PT04		2.0
PT11		1.0
PT24		1.0
PT28		>32
PT29		1.0
PT30		>32

PT40		1.0
PT41		2.0
PT42		0.5

2.6 CONCLUSIONS

In summary, we have studied the mechanism of the reaction catalyzed by saFabI using substrates based on the natural ACP carrier molecule. From the results of the kinetic assays, we propose that the binding of substrates in the ternary complex mechanism is ordered, with DDsaACP binding first followed by NADPH. Two basic residues, R40 and K41 are shown to be critical for cofactor specificity.

In addition, we investigated the mechanism of action of three diphenyl ether-based saFabI inhibitors. All three compounds are slow-onset inhibitors of saFabI *in vitro*, binding preferentially to the E-NADP$^+$ product complex with K_1 values of 2-8 nM. These compounds have potent antibacterial activity, with MIC values against *S. aureus* of 0.03-0.06 μg/mL. Selection experiments led to the identification of three mutations in the *safabI* gene, A95V, I193S and F204S, which correlate with resistance to the diphenyl ethers and cause a significant

reduction in the affinity of the inhibitors for the enzyme. These experiments confirm the hypothesis that the diphenyl-ether-based inhibitors target saFabI in live cells, and substantiate the view that saFabI is a novel target for antibacterial drug discovery.

A series of diphenyl ethers have been synthesized and tested against *S. aureus*. However, all the diphenyl ethers are at least 10-fold less potent than the starting compound triclosan. More modifications, especially the substitutions on the *meta*-position of B-ring, are needed to improve the inhibition potency.

References

1. Cronan, J. E., Jr., and Waldrop, G. L. (2002) Multi-subunit acetyl-CoA carboxylases, *Prog. Lipid Res. 41*, 407-435.
2. Shimakata, T., and Stumpf, P. K. (1983) The purification and function of acetyl coenzyme A:acyl carrier protein transacylase, *J. Biol. Chem. 258*, 3592-3598.
3. Heath, R. J., and Rock, C. O. (1996) Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*, *J. Biol. Chem. 271*, 1833-1836.
4. Heath, R. J., and Rock, C. O. (1995) Enoyl-acyl carrier protein reductase (fabI) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*, *J. Biol. Chem. 270*, 26538-26542.
5. Heath, R. J., and Rock, C. O. (1996) Roles of the FabA and FabZ beta-hydroxyacyl-acyl carrier protein dehydratases in *Escherichia coli* fatty acid biosynthesis, *J. Biol. Chem. 271*, 27795-27801.
6. Mohan, S., Kelly, T. M., Eveland, S. S., Raetz, C. R., and Anderson, M. S. (1994) An Escherichia coli gene (FabZ) encoding (3R)-hydroxymyristoyl acyl carrier protein dehydrase. Relation to fabA and suppression of mutations in lipid A biosynthesis, *J. Biol. Chem. 269*, 32896-32903.
7. Garwin, J. L., Klages, A. L., and Cronan, J. E., Jr. (1980) Structural, enzymatic, and genetic studies of beta-ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli*, *J. Biol. Chem. 255*, 11949-11956.

8. Larson, T. J., Lightner, V. A., Green, P. R., Modrich, P., and Bell, R. M. (1980) Membrane phospholipid synthesis in *Escherichia coli*. Identification of the sn-glycerol-3-phosphate acyltransferase polypeptide as the plsB gene product, *J. Biol. Chem. 255*, 9421-9426.

9. Marrakchi, H., Choi, K. H., and Rock, C. O. (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*, *J. Biol. Chem. 277*, 44809-44816.

10. Egan, A. F., and Russell, R. R. (1973) Conditional mutations affecting the cell envelope of *Escherichia coli* K-12, *Genet. Res. 21*, 139-152.

11. Turnowsky, F., Fuchs, K., Jeschek, C., and Hogenauer, G. (1989) envM genes of *Salmonella typhimurium* and *Escherichia coli*, *J. Bacteriol. 171*, 6555-6565.

12. Tsay, J. T., Rock, C. O., and Jackowski, S. (1992) Overproduction of beta-ketoacyl-acyl carrier protein synthase I imparts thiolactomycin resistance to *Escherichia coli* K-12, *J. Bacteriol. 174*, 508-513.

13. Waller, R. F., Ralph, S. A., Reed, M. B., Su, V., Douglas, J. D., Minnikin, D. E., Cowman, A. F., Besra, G. S., and McFadden, G. I. (2003) A type II pathway for fatty acid biosynthesis presents drug targets in *Plasmodium falciparum*, *Antimicrob. Agents Chemother. 47*, 297-301.

14. Sharma, S. K., Kapoor, M., Ramya, T. N., Kumar, S., Kumar, G., Modak, R., Sharma, S., Surolia, N., and Surolia, A. (2003) Identification, characterization, and inhibition of *Plasmodium falciparum* beta-

hydroxyacyl-acyl carrier protein dehydratase (FabZ), *J. Biol. Chem. 278*, 45661-45671.

15. Heath, R. J., and Rock, C. O. (1995) Enoyl-acyl carrier protein reductase (fabI) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*, *J. Biol. Chem. 270*, 26538-26542.

16. Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kallberg, Y., Persson, B., and Jornvall, H. (2003) Short-chain dehydrogenases/reductases (SDR): the 2002 update, *Chem. Biol. Interact. 143-144*, 247-253.

17. Lukacik, P., Kavanagh, K. L., and Oppermann, U. (2006) Structure and function of human 17beta-hydroxysteroid dehydrogenases, *Mol. Cell. Endocrinol. 248*, 61-71.

18. Wu, X., Lukacik, P., Kavanagh, K. L., and Oppermann, U. (2007) SDR-type human hydroxysteroid dehydrogenases involved in steroid hormone activation, *Mol. Cell. Endocrinol. 265-266*, 71-76.

19. Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., and Ghosh, D. (1995) Short-chain dehydrogenases/reductases (SDR), *Biochemistry 34*, 6003-6013.

20. Labesse, G., Vidal-Cros, A., Chomilier, J., Gaudry, M., and Mornon, J. P. (1994) Structural comparisons lead to the definition of a new superfamily of NAD(P)(H)-accepting oxidoreductases: the single-domain reductases/epimerases/dehydrogenases (the 'RED' family), *Biochem. J. 304 (Pt 1)*, 95-99.

21. Persson, B., Kallberg, Y., Oppermann, U., and Jornvall, H. (2003) Coenzyme-based functional assignments of short-chain dehydrogenases/reductases (SDRs), *Chem. Biol. Interact. 143-144*, 271-278.

22. Rozwarski, D. A., Vilcheze, C., Sugantino, M., Bittman, R., and Sacchettini, J. C. (1999) Crystal structure of the *Mycobacterium tuberculosis* enoyl-ACP reductase, InhA, in complex with NAD+ and a C16 fatty acyl substrate, *J. Biol. Chem. 274*, 15582-15589.

23. Bergler, H., Wallner, P., Ebeling, A., Leitinger, B., Fuchsbichler, S., Aschauer, H., Kollenz, G., Hogenauer, G., and Turnowsky, F. (1994) Protein EnvM is the NADH-dependent enoyl-ACP reductase (FabI) of *Escherichia coli*, *J. Biol. Chem. 269*, 5493-5496.

24. Heath, R. J., Su, N., Murphy, C. K., and Rock, C. O. (2000) The enoyl-[acyl-carrier-protein] reductases FabI and FabL from *Bacillus subtilis*, *J. Biol. Chem. 275*, 40128-40133.

25. Quemard, A., Sacchettini, J. C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W. R., Jr., and Blanchard, J. S. (1995) Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*, *Biochemistry 34*, 8235-8241.

26. Marcinkeviciene, J., Jiang, W., Kopcho, L. M., Locke, G., Luo, Y., and Copeland, R. A. (2001) Enoyl-ACP reductase (FabI) of *Haemophilus influenzae:* steady-state kinetic mechanism and inhibition by triclosan and hexachlorophene, *Arch. Biochem. Biophys. 390*, 101-108.

27. Rafferty, J. B., Simon, J. W., Baldock, C., Artymiuk, P. J., Baker, P. J., Stuitje, A. R., Slabas, A. R., and Rice, D. W. (1995) Common themes in redox chemistry emerge from the X-ray structure of oilseed rape (*Brassica napus*) enoyl acyl carrier protein reductase, *Structure 3*, 927-938.

28. Rozwarski, D. A., Grant, G. A., Barton, D. H., Jacobs, W. R., Jr., and Sacchettini, J. C. (1998) Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*, *Science 279*, 98-102.

29. Rawat, R., Whitty, A., and Tonge, P. J. (2003) The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the *Mycobacterium tuberculosis* enoyl reductase: adduct affinity and drug resistance, *Proc. Natl. Acad. Sci. U.S.A. 100*, 13881-13886.

30. Baldock, C., Rafferty, J. B., Sedelnikova, S. E., Baker, P. J., Stuitje, A. R., Slabas, A. R., Hawkes, T. R., and Rice, D. W. (1996) A mechanism of drug action revealed by structural studies of enoyl reductase, *Science (New York, NY) 274*, 2107-2110.

31. Karlowsky, J. A., Laing, N. M., Baudry, T., Kaplan, N., Vaughan, D., Hoban, D. J., and Zhanel, G. G. (2007) In vitro activity of API-1252, a novel FabI inhibitor, against clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Antimicrob. Agents Chemother. 51*, 1580-1581.

32. Kitagawa, H., Kumura, K., Takahata, S., Iida, M., and Atsumi, K. (2007) 4-Pyridone derivatives as new inhibitors of bacterial enoyl-ACP reductase FabI, *Bioorg. Med. Chem. 15*, 1106-1116.

33. Ling, L. L., Xian, J., Ali, S., Geng, B., Fan, J., Mills, D. M., Arvanites, A. C., Orgueira, H., Ashwell, M. A., Carmel, G., Xiang, Y., and Moir, D. T. (2004) Identification and characterization of inhibitors of bacterial enoyl-acyl carrier protein reductase, *Antimicrob. Agents Chemother. 48*, 1541-1547.

34. Payne, D. J., Miller, W. H., Berry, V., Brosky, J., Burgess, W. J., Chen, E., DeWolf Jr, W. E., Jr., Fosberry, A. P., Greenwood, R., Head, M. S., Heerding, D. A., Janson, C. A., Jaworski, D. D., Keller, P. M., Manley, P. J., Moore, T. D., Newlander, K. A., Pearson, S., Polizzi, B. J., Qiu, X., Rittenhouse, S. F., Slater-Radosti, C., Salyers, K. L., Seefeld, M. A., Smyth, M. G., Takata, D. T., Uzinskas, I. N., Vaidya, K., Wallis, N. G., Winram, S. B., Yuan, C. C., and Huffman, W. F. (2002) Discovery of a novel and potent class of FabI-directed antibacterial agents, *Antimicrob. Agents Chemother. 46*, 3118-3124.

35. Zhang, Y. M., and Rock, C. O. (2004) Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI reductases of bacterial type II fatty-acid synthase, *J. Biol. Chem. 279*, 30994-31001.

36. Heath, R. J., and Rock, C. O. (2000) A triclosan-resistant bacterial enzyme, *Nature 406*, 145-146.

37. Massengo-Tiasse, R. P., and Cronan, J. E. (2008) *Vibrio cholerae* FabV defines a new class of enoyl-acyl carrier protein reductase, *J. Biol. Chem. 283*, 1308-1316.

38. Kitagawa, H., Ozawa, T., Takahata, S., Iida, M., Saito, J., and Yamada, M. (2007) Phenylimidazole derivatives of 4-pyridone as dual inhibitors of bacterial enoyl-acyl carrier protein reductases FabI and FabK, *J. Med. Chem. 50*, 4710-4720.

39. Heath, R. J., Li, J., Roland, G. E., and Rock, C. O. (2000) Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene, *J. Biol. Chem. 275*, 4654-4659.

40. Slater-Radosti, C., Van Aller, G., Greenwood, R., Nicholas, R., Keller, P. M., DeWolf, W. E., Jr., Fan, F., Payne, D. J., and Jaworski, D. D. (2001) Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*, *J. Antimicrob. Chemother. 48*, 1-6.

41. Russell, A. D. (2004) Whither triclosan?, *J. Antimicrob. Chemother. 53*, 693-695.

42. Regos, J., Zak, O., Solf, R., Vischer, W. A., and Weirich, E. G. (1979) Antimicrobial spectrum of triclosan, a broad-spectrum antimicrobial agent for topical application. II. Comparison with some other antimicrobial agents, *Dermatologica. 158*, 72-79.

43. Ayliffe, G. A., Buckles, A., Casewell, M. W., Cookson, B. D., Cox, R. A., Duckworth, G. J., Griffiths-Jones, A., Heathcock, R., Humphreys, H., Keane, C. T., Marples, R. R., Shanson, D. C., Slack, R., and Tebbs, E. (1998) Revised guidelines for the control of methicillin-resistant *Staphylococcus aureus* infection in hospitals. British Society for

Antimicrobial Chemotherapy, Hospital Infection Society and the Infection Control Nurses Association, *J. Hosp. Infect. 39*, 253-290.

44. Bamber, A. I., and Neal, T. J. (1999) An assessment of triclosan susceptibility in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, *J. Hosp. Infect. 41*, 107-109.

45. Fan, F., Yan, K., Wallis, N. G., Reed, S., Moore, T. D., Rittenhouse, S. F., DeWolf, W. E., Jr., Huang, J., McDevitt, D., Miller, W. H., Seefeld, M. A., Newlander, K. A., Jakas, D. R., Head, M. S., and Payne, D. J. (2002) Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 46*, 3343-3347.

46. Seaman, P. F., Ochs, D., and Day, M. J. (2007) Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*, *J. Antimicrob. Chemother. 59*, 43-50.

47. Sivaraman, S., Sullivan, T. J., Johnson, F., Novichenok, P., Cui, G., Simmerling, C., and Tonge, P. J. (2004) Inhibition of the bacterial enoyl reductase FabI by triclosan: a structure-reactivity analysis of FabI inhibition by triclosan analogues, *J. Med. Chem. 47*, 509-518.

48. Sullivan, T. J., Truglio, J. J., Boyne, M. E., Novichenok, P., Zhang, X., Stratton, C. F., Li, H. J., Kaur, T., Amin, A., Johnson, F., Slayden, R. A., Kisker, C., and Tonge, P. J. (2006) High affinity InhA inhibitors with activity against drug-resistant strains of *Mycobacterium tuberculosis*, *ACS Chem. Biol. 1*, 43-53.

49. Parikh, S., Moynihan, D. P., Xiao, G., and Tonge, P. J. (1999) Roles of tyrosine 158 and lysine 165 in the catalytic mechanism of InhA, the enoyl-ACP reductase from *Mycobacterium tuberculosis*, *Biochemistry 38*, 13623-13634.

50. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248-254.

51. Quadri, L. E., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., and Walsh, C. T. (1998) Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases, *Biochemistry 37*, 1585-1595.

52. Cleland, W. W. (1979) Statistical analysis of enzyme kinetic data, *Methods Enzymol. 63*, 103-138.

53. NCCLS. (2003) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 6th ed. Approved Standard M7-A6, *National Committee for Clinical Laboratory Standards, Wayne, PA*.

54. Schaeffer, M. L., Agnihotri, G., Kallender, H., Brennan, P. J., and Lonsdale, J. T. (2001) Expression, purification, and characterization of the *Mycobacterium tuberculosis* acyl carrier protein, AcpM, *Biochim. Biophys. Acta 1532*, 67-78.

55. Heath, R. J., Li, J., Roland, G. E., and Rock, C. O. (2000) Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein

reductase by triclosan and hexachlorophene, *J. Biol. Chem. 275*, 4654-4659.

56. Heath, R. J., Su, N., Murphy, C. K., and Rock, C. O. (2000) The enoyl-[acyl-carrier-protein] reductases FabI and FabL from *Bacillus subtilis*, *J. Biol. Chem. 275*, 40128-40133.

57. Delano, W. L. (2002) The PyMOL Molecular Graphics System, *DeLano Scientific LLC, San Carlos, CA, USA.* http://www.pymol.org.

58. Heath, R. J., Rubin, J. R., Holland, D. R., Zhang, E., Snow, M. E., and Rock, C. O. (1999) Mechanism of triclosan inhibition of bacterial fatty acid synthesis, *J. Biol. Chem. 274*, 11110-11114.

59. Heath, R. J., Yu, Y. T., Shapiro, M. A., Olson, E., and Rock, C. O. (1998) Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis, *J. Biol. Chem. 273*, 30316-30320.

60. McMurry, L. M., Oethinger, M., and Levy, S. B. (1998) Triclosan targets lipid synthesis, *Nature 394*, 531-532.

61. Levy, C. W., Roujeinikova, A., Sedelnikova, S., Baker, P. J., Stuitje, A. R., Slabas, A. R., Rice, D. W., and Rafferty, J. B. (1999) Molecular basis of triclosan activity, *Nature 398*, 383-384.

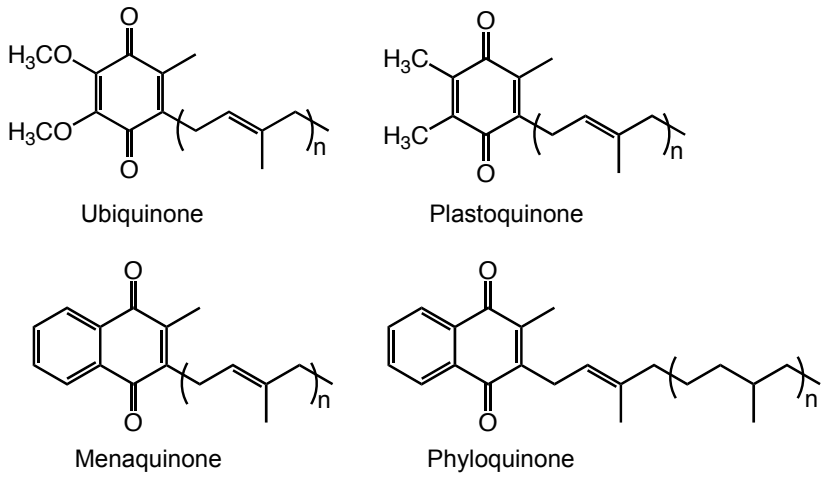
62. am Ende, C. W., Knudson, S. E., Liu, N., Childs, J., Sullivan, T. J., Boyne, M., Xu, H., Gegina, Y., Knudson, D. L., Johnson, F., Peloquin, C. A., Slayden, R. A., and Tonge, P. J. (2008) Synthesis and in vitro antimycobacterial activity of B-ring modified diaryl ether InhA inhibitors, *Bioorg. Med. Chem. Lett. 18*, 3029-3033.

63. Sullivan, T. J., Truglio, J. J., Boyne, M. E., Novichenok, P., Zhang, X., Stratton, C. F., Li, H. J., Kaur, T., Amin, A., Johnson, F., Slayden, R. A., Kisker, C., and Tonge, P. J. (2006) High affinity InhA inhibitors with activity against drug-resistant strains of *Mycobacterium tuberculosis*, *ACS Chem. Biol. 1*, 43-53.

CHAPTER 3: MECHANISTIC AND FUNCTIONAL STUDIES OF MEND FROM *STAPHYLOCOCCUS AUREUS*

3.1 ISOPRENOID QUINONES

Isoprenoid quinones are important constituents of the bacterial plasma membrane, and are involved in electron transport and oxidative phosphorylation. Two classes of isoprenoid quinones exist in bacteria, benzoquinones and naphthoquinones.



Scheme 3.1: Isoprenoid quinones in bacteria.

Benzoquinones include plastoquinones and ubiquinones (coenzyme Q), as shown in **Scheme 3.1**. Plastoquinones are present not only in photosynthetic tissues of plants, but also in algae and cyanobacteria (*1, 2*). Ubiquinones are

only found in Gram-negative bacteria, such as *E. coli (3)*. A wide range of isoprenologs (n = 1 - 12) are found in bacteria. In addition to normal analogues, modifications of the isoprenyl chain of ubiquinones, such as hydrogenation and epoxidation (*2, 4*), have been reported.

The second major class of bacterial isoprenoid quinones are the naphthoquinones, which are further divided into two main types, phyloquinones and menaquinones (vitamine K2). Phyloquinones were first isolated from alfalfa (*5*). Generally they are associated with plants, but less commonly found in bacteria. Menaquinones are usually found in Gram-positive bacteria, such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* (*6, 7*). Like ubiquinones, similar modifications of the isoprenyl chain of menaquinones are observed.

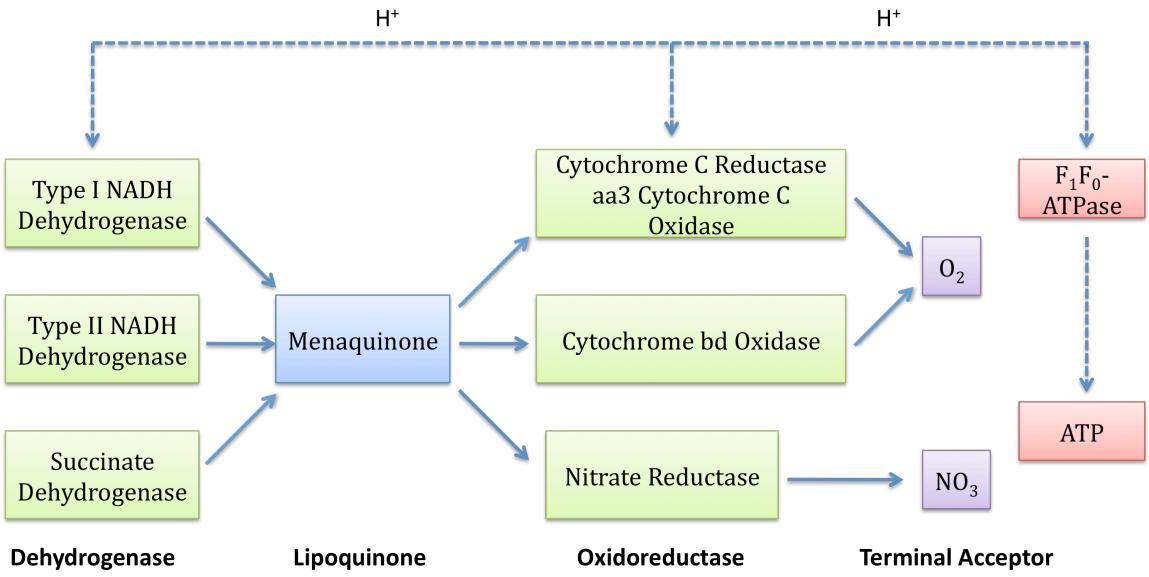


Figure 3.1: Electron transport in bacteria. Menaquinone is utilized by both aerobic electron transport and anaerobic nitrate reductase complex (*8*), where oxygen and nitrate is used as the terminal acceptor, respectively.

Isoprenoid quinones are involved in bacterial electron transport, which produces adenosine triphosphate (ATP), the main energy intermediate. To initiate the process of electron transport (**Figure 3.1)**, an electron donor, such as NADH and succinate, is oxidized by dehydrogenases. Then electrons are transferred successively through quinones, cytochromes, and finally to the terminal acceptors, such as O_2 and nitrate. Meanwhile, the efflux of H^+ ions is coupled with the electron transport, generating an electrochemical gradient, which is utilized by F_1F_0-ATP synthase to produce ATP.

In addition to the role in bacterial respiration, menaquinone is also found in quinoproteins (*9*), malate quinol oxidoreductase, dihydroorotate dehydrogenase and NAD(P)H:quinone oxidoreductase, which also play important metabolic functions.

In humans, the most abundant isoprenoid quinones are phylloquinones and menaquinones, also called vitamin K. Vitamin K plays an important role in haemostasis, and acts as cofactors for γ-glutamyl carboxylase (*10*). In several studies, it is claimed that an inverse relationship exists between vitamin K and an inflammatory response (*11, 12*). In addition, it can prevent the oxidative damage to oligodendrocyte precursor cells and foetal cortical neurons (*13*), and is involved in the synthesis of sphingolipids, a group of lipids found in large amounts in the brain (*14*). The major dietary source of vitamin K is phylloquinone from plant sources. In addition, the quinones synthesized by bacteria that reside in human intestine also contribute to human nutrition. But it is believed that gut menaquinones are much less important than those from dietary sources.

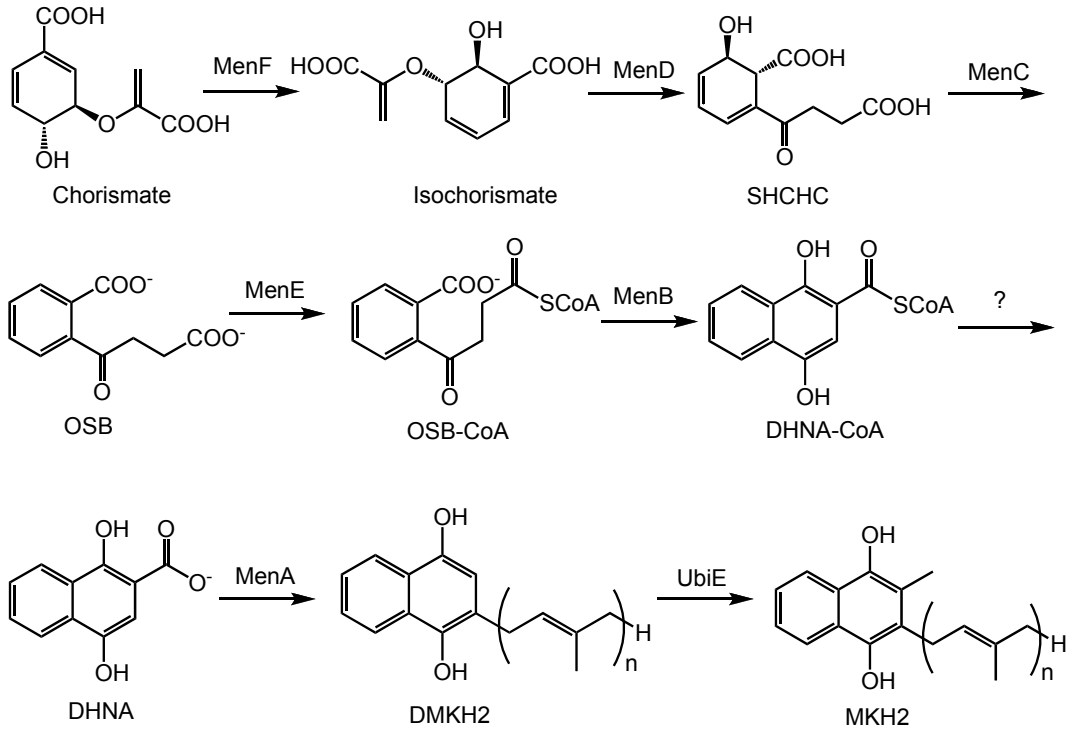
3.2 MENAQUINONE BIOSYNTHESIS

The biosynthetic pathway of menaquinone has been well characterized in the model organism *E. coli* by radioactive feeding and genetic studies. In 1964, the conversion of radioactive shikimate into ubiquinone and menaquinone was observed (*15*). It was also suggested that shikimate was first converted to chorismate. Later it was found that all seven carbon atoms of shikimate were incorporated into menaquinone (*16*), while the remaining three carbon atoms in the aromatic nucleus of menaquinone were obtained from α-ketoglutarate (*17*). The methyl and isoprenoid side chain were acquired from *S*-adenosylmethionine and isoprenoid pyrophosphate, respectively.

In 1975, Young et al. isolated two types of menaquinone-defective *E. coli* mutants (*18, 19*). One group of the mutants was *menA* mutants in which 1,4-dihydroxy-napthoic acid (DHNA) accumulated. The other group of mutants were originally termed *menB*, and the conversion from *O*-succinyl benzoic acid (OSB) to DHNA was blocked. These mutants were later shown to include two groups, termed *menE* and *menB*. Then two further groups of mutants, *menC* and *menD* were identified. These mutants were OSB auxotrophic, indicating that they might be involved in the transformation from chorismate to OSB (*20, 21*).

Based on the genetic studies, the proposed menaquinone biosynthetic pathway is shown in **Scheme 3.2**. Putative genes in *E. coli* and other organisms were isolated, and characterized *in vitro*. Most reports support this pathway. Initially MenF catalyzes the formation of isochorismate (*22*), which is then

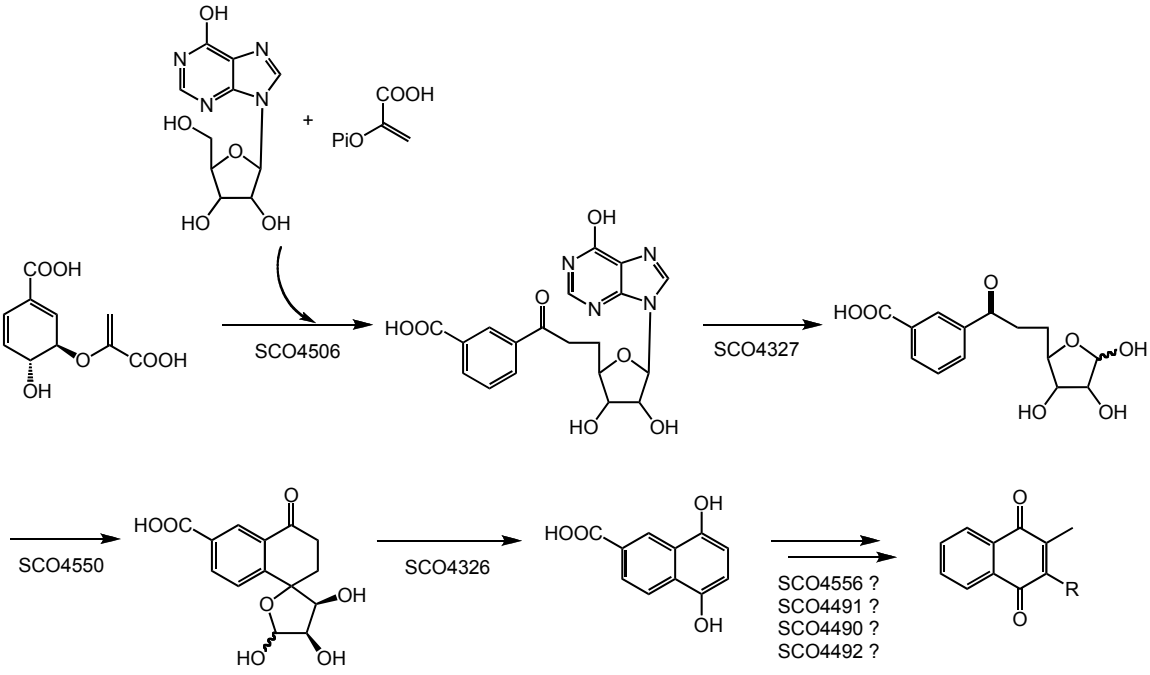
converted into OSB, the first aromatic intermediate in the pathway, by MenD and MenC (*23, 24*). Then MenE and MenB convert OSB to DHNA-CoA (*25, 26*). DHNA-CoA is then hydrolyzed into DHNA. However, the enzyme responsible for this conversion has not been identified yet. Finally an isoprenyl chain is transferred to DHNA with the help of MenA (*27*), followed by a methyl addition catalyzed by UbiE, forming the final product menaquinone.



Scheme 3.2: Menaquinone biosynthetic pathway in *E. coli*. n = 8 in both *S. aureus* and *E. coli*.

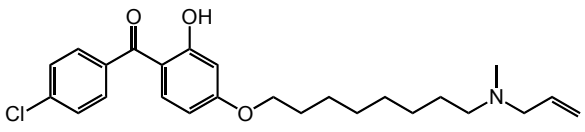
Some organisms synthesize menaquinones, but lack the *men* gene homologues (*28-30*). Recently, an alternative pathway for menaquinone biosynthesis (**Scheme 3.3**) was discovered in *Streptomyces coelicolor*, a soil bacterium (*31, 32*). This pathway is predicted to exist in several Gram-negative

bacteria (such as Chlamydia and Spirochetes) and Archaea. However, it is not found in the bacteria, which already have a *ubi* or *men gene* cluster.

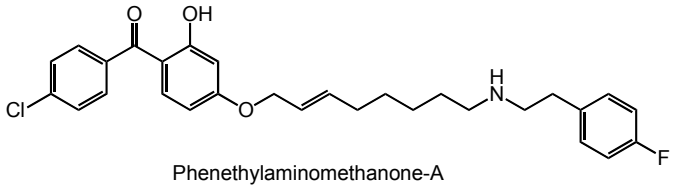


Scheme 3.3: A proposed alternative menaquinone biosynthetic pathway.

It is hypothesized that the menaquinone biosynthetic pathway is an attractive target against Gram-positive pathogens. Notably, several MenA inhibitors were discovered (*33, 34*) (**Scheme 3.4**), and they showed good inhibition against *S. aureus* and *M. tuberculosis*. It is even more encouraging that they are active against non-replicating persistent (NRP) *M. tuberculosis*, which might help to eradicate the bacterium in latent TB patients. About 10% of the patients with latent TB will develop active tuberculosis during their lives. Therefore, inhibitors targeting menaquinone biosynthesis would be quite helpful in TB chemotherapy, especially in preventing the progression of latent TB into active TB.



Allylaminomethanone-A



Phenethylaminomethanone-A

Scheme 3.4: MenA inhibitors.

Defective menaquinone biosynthesis has been reported to lead to small colony variant phenotype in *S. aureus* (*35*). Comparative proteomic studies of the wild type and a genetically defined SCV strain reveal that the proteins involved in glycolytic as well as in fermentation pathways were upregulated, whereas tricarboxylic acid (TCA) cycle enzymes were significantly downregulated, suggesting that SCV *S. aureus* generates ATP from glucose or fructose mainly by substrate phosphorylation and might be defective in utilizing a variety of carbon sources, including TCA cycle intermediates and compounds that generate ATP only via electron transport phosphorylation (*36*).

3.3 THIAMIN DIPHOSPHATE-DEPENDENT ENZYMES

MenD, the enzyme that catalyzes the second step in the menaquinone biosynthetic pathway, is thiamin diphosphate-dependent. It has been suggested that a highly conserved glutamate residue in thiamin diphosphate-dependent enzymes interacts with N1' of the pyrimidine moiety of ThDP, facilitating the

formation of ylide (**Figure 3.2**), which is essential for initiating the enzymatic reaction. However, a recent study on glyoxylate carboligase reveals that the glutamate residue is absent in this ThDP-dependent enzyme, suggesting that the role of Glu is not strictly required (*37*).

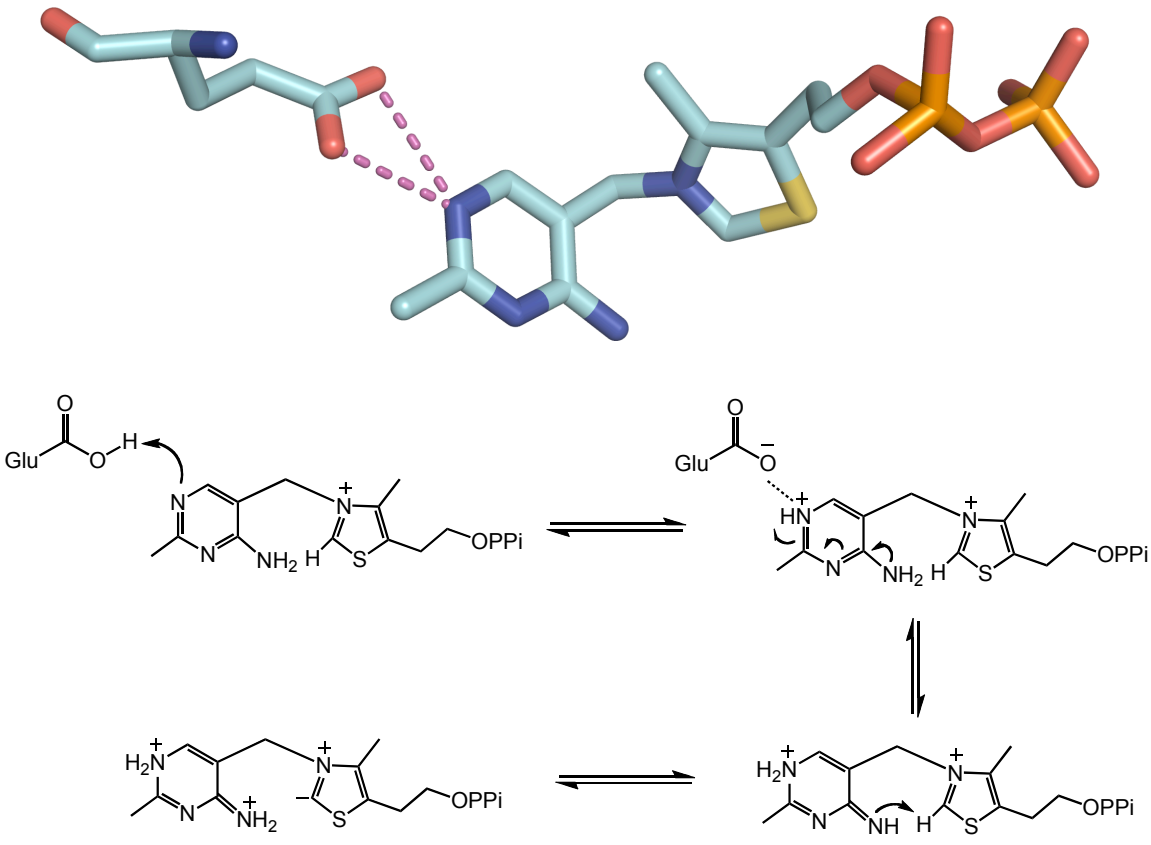
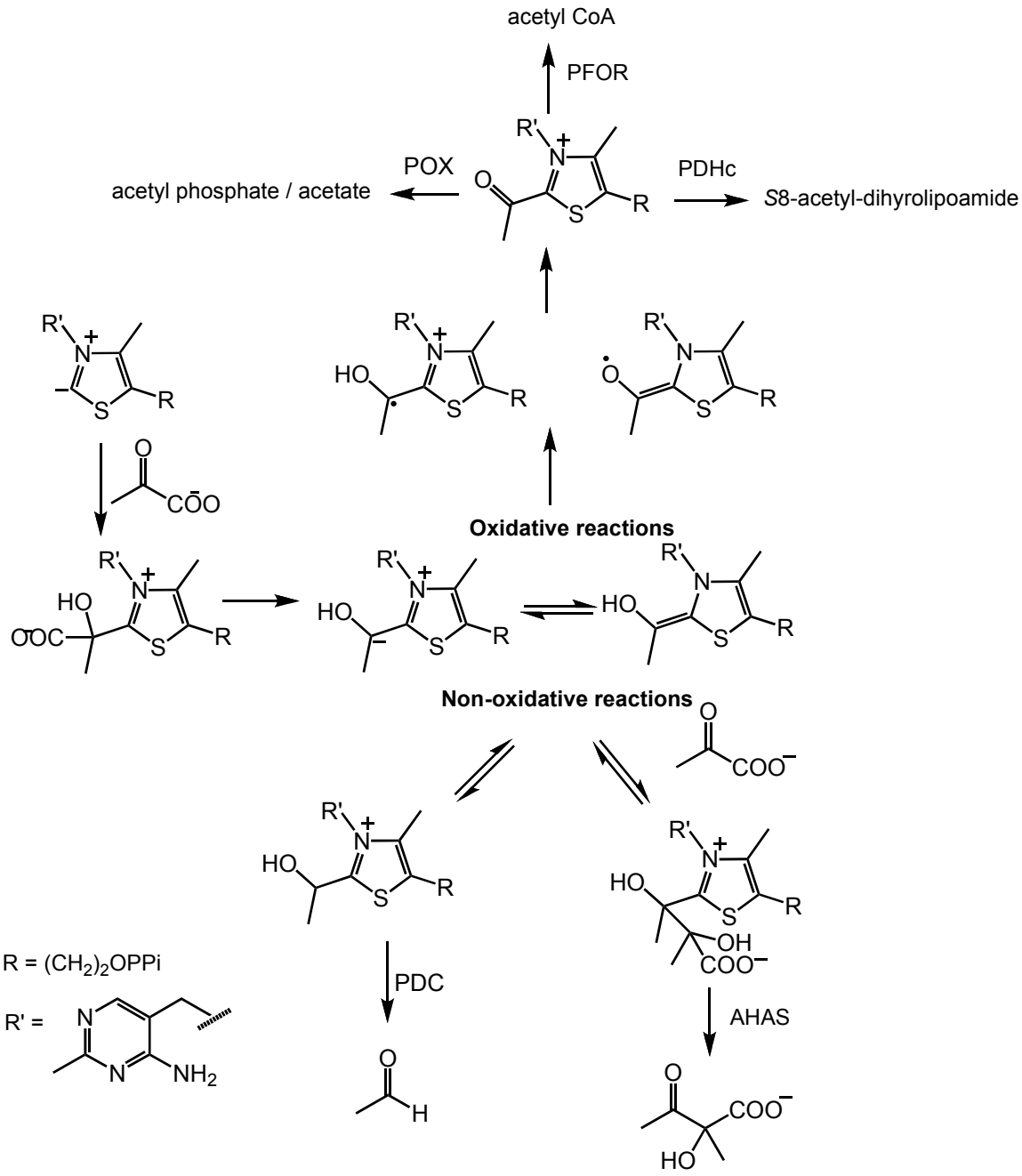


Figure 3.2: The role of ThDP in the reaction. Top: crystal structure of ecMenD showing the interaction between the conserved glutamate residue and ThDP. Bottom: proposed mechanism for cofactor activation by Glu. A proton from the carboxyl group of glutamate side chain is transferred to N1' of ThDP, producing 4'-aminopyrimidinium. After losing one proton to the solvent, the resulting 1',4'-iminopyrimindine abstracts a proton from C2 in the thiazolium ring, generating the C2 carbanion.

All ThDP-dependent enzyme-catalyzed reactions involve the formation of an enamine intermediate, which is produced by decarboxylation, such as pyruvate-processing ThDP enzymes, or other fragmentation of the first substrate-ThDP adduct, such as transketolase. The resulting enamine intermediate then undergoes either oxidative or nonoxidative reactions, generating various products. As shown in **Scheme 3.5**, in oxidative reactions, the enamine is oxidized to form 2-acetyl-ThDP by neighboring redox-active cofactors, such as flavin adenine dinucleotide (FAD) and Fe_4S_4 cluster. Radical intermediates may participate in the reaction. Then the carbonyl in the resulting 2-acetyl-ThDP is attacked by a nucleophile, such as phosphate and CoA, releasing the final product.

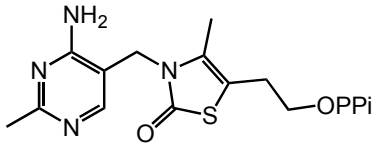
In non-oxidative reactions, the enamine intermediate can be protonated at C2α position, producing an aldehyde after cleavage from the product-ThDP adduct, such as pyruvate decarboxylase. Alternatively, the enamine/carbanione can attach another 2-ketoacid, forming a carbon-carbon bond between the two substrates, such as acetohydroxy acid synthase.



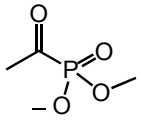
Scheme 3.5: Reaction patterns of pyruvate-processing ThDP enzymes (*38*)**.**

As shown above, the enzyme reactions involve a series of ThDP adduct intermediates. Several techniques have been utilized to detect the formation of these intermediates. Analysis of the reaction mixture by ^{1}H NMR after chemical quenching is quite appealing, since the rate constants of all the steps during the reaction could be measured (*37, 39*). Time-resolved absorbance spectroscopy has also been used to monitor the reaction course (*40*).

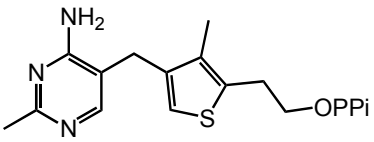
Most ThDP-dependent enzymes exist as a dimer or dimer of dimers, according to the reported crystal structures (*41-43*). Another common feature is that ThDP as well as the active site lies in the interface of two monomers.



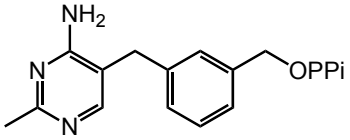
Thiamine-thiazolone diphosphate



Methyl acetylphosphonate



3-Deazathiamine diphosphate



4-Amino-5-[3-(2-hydroxyethyl)benzyl]-
2-methylpyrimidine diphosphate

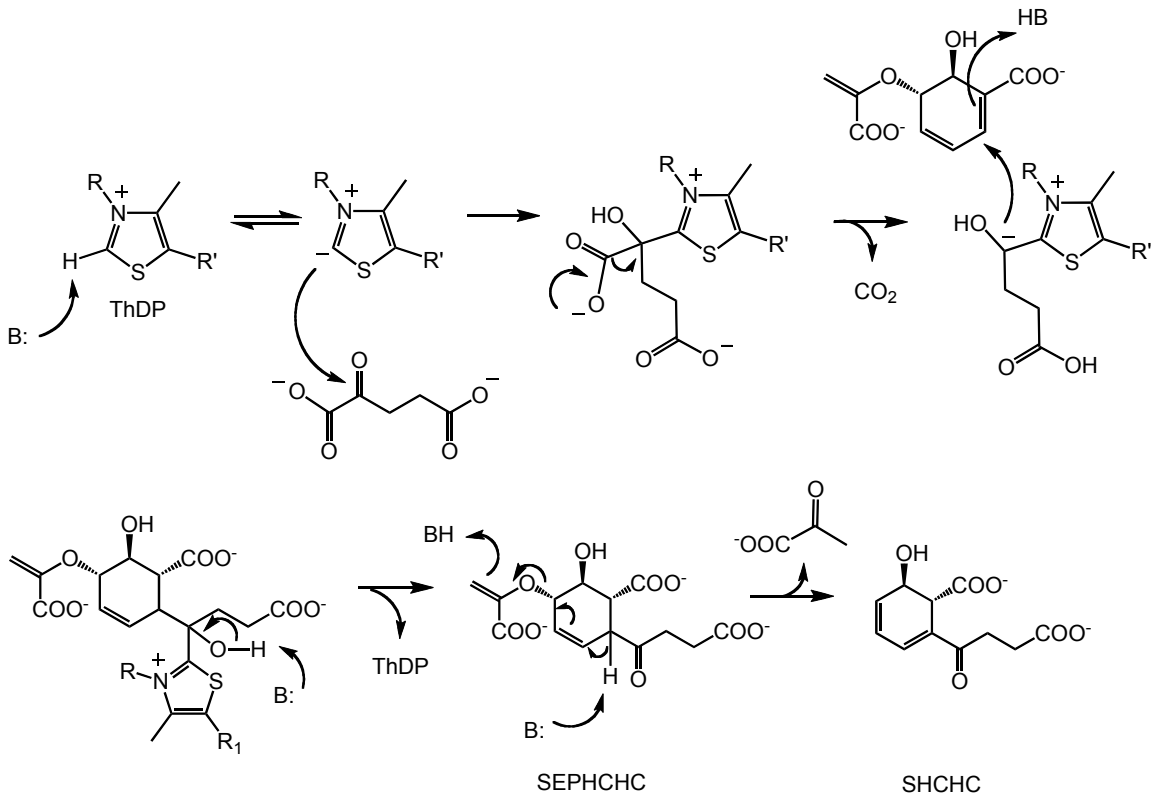
Scheme 3.6: Inhibitors of ThDP enzymes.

Efforts have been put into the inhibitor development on ThDP-dependent enzymes. Some intermediate-related inhibitors have been reported, as shown in **Scheme 3.6**. Thiamin-thiazolone diphosphate, a transition state analogue, bound very tightly to pyruvate dehydrogenase (*44*). Later, a suicide inhibitor, methyl acetylphosphonate, which could be catalyzed by the enzyme itself to react with

ThDP to form α-(methyl)phosphonolactyl-ThDP, showed good inhibition (*45*). Recently Leeper et al. synthesized 3-deazathiamine and a related analogue, both showing strong inhibition against ThDP enzymes (*46, 47*). One issue about these inhibitors is their selectivity. Presumably the problem could be solved by attaching a specific side chain to the C2 position of the thiazole or thiazolium ring to match the reaction intermediate of the target enzyme.

3.4 PREVIOUS STUDEIS ON MEND FROM *E. COLI*

Based on previous genetic studies, the product of *menD* gene in *E. coli* was proposed to catalyze the conversion from isochorismate and α-ketoglutarate into 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) (*48*). Similar studies performed in *B. subtilis,* a model organism for Gram-positive bacteria, also support *menD* as the SHCHC synthase (*49, 50*). A reaction mechanism was proposed based on the knowledge of the ThDP-dependent enzymes, as shown in **Scheme 3.7** (*24*). The carbanion in the thiazolium ring of ThDP attacks α-ketoglutarate, followed by a decarboxylation step. The resulting carbanion on C2α attacks isochorismate, forming the SEPHCHC-ThDP adduct. The product is released after dissociation of the adduct and pyruvate elimination. However, the recent studies by Jiang et al. (*51*) suggest that SEPHCHC, the intermediate before pyruvate elimination, is the actual product of ecMenD-catalyzed reaction.



Scheme 3.7: Proposed *E. coli* MenD reaction mechanism.

Two crystal structures of ecMenD have been reported, one apo form, the other bound with the cofactor ThDP. The structural studies reveal that MenD enzyme belongs to the pyruvate oxidase family. Results from circular dichroism spectra and isothermal calorimetry suggest that ThDP helps stabilize the ecMenD structure (*52, 53*).

Despite a number of studies on *E. coli* MenD, no kinetic analysis has been performed to investigate the isochorismate-binding pocket. More importantly, little is known about the MenD enzyme from Gram-positive pathogens. Therefore, it is worthwhile to characterize the enzymatic properties of *S. aureus* MenD (saMenD). In addition, genetic and chemical approaches are utilized here to

probe the essentiality of *menD* gene as well as menaquinone in Gram-positive bacteria.

3.5 MATERIALS AND METHODS

Materials.

HiLoad 26/60 Superdex 200 column was from GE Healthcare. Restriction enzymes were from New England Biolabs. Quikchange mutagenesis kit was obtained from Stratagene. Other chemicals and reagents were purchased from Sigma.

Cloning, expression, and purification of menD from S. aureus.

The *menD* gene was cloned from the genome from *S. aureus* ATCC 25923 by using the primers listed in **Table 3.1**. After digest with BamHI and HindIII, *menD* gene was inserted into pET23b vector. The insert was confirmed by ABI DNA sequencing. MenD protein was expressed and purified as described earlier for saFabI. After analyzed by SDS-PAGE, MenD was dialyzed into 50 mM Tris-HCl buffer containing 50 mM NaCl, 25% glycerol (pH 8.5).

Gel filtration chromatography of saMenD.

This experiment was performed by John Angiolillo in Professor Kisker's lab. Briefly, saMenD was analyzed with HiLoad 26/60 Superdex 200 column. The retention volume was used to calculate K_{av} by the following equation

$K_{av} = (V_e - V_o)/(V_t - V_o)$

where V_o = 115 (empirically determined), V_t = 330.

Apparent molecular weight was obtained by using the equation

K_{av} = -0.36(logMW)+2.1375.

Table 3.1: Nucleotide sequences

Primer Names	Sequence [a]
MenD_Forward	5'-CGC*GGATCC*ATGGGAAATCATAAAGCA-3'
MenD_Reverse	5'-CCC*AAGCTT*TAATGTGTCATGAATCAT-3'
K288M_F	5'-CCAGTGATTTCTATGAAATTGAATCAATG-3'
K288M_R	5'-CATTGATTCAATTTCATAGAAATCACTGG-3'
K289M_F	5'-CCAGTGATTTCTAAAATGTTGAATCAATGGTTA-3'
K289M_R	5'-TAACCATTGATTCAACATTTTAGAAATCACTGG-3'
K288M/K289M_F	5'-GTGATTTCTATGATGTTGAATCAATGG-3'
K288M/K289M_F	5'-CCATTGATTCAACATCATAGAAATCAC-3'
K288E_F	5'-GAAACCAGTGATTTCTGAAAAGTTGAATCAATG-3'
K288E_R	5'-CATTGATTCAACTTTTCAGAAATCACTGGTTTC-3'
K411M_F	5'-GATGTCTATGCGAATATGGGTGCGAATGGTATTG-3'
K411M_R	5'-CAATACCATTCGCACCCATATTCGCATAGACATC-3'

[a] Restriction sites are italicized, while mutation nucleotides are underline.

Enzymatic synthesis of isochorismate.

Isochorismate was synthesized from chorismate enzymatically by *E. coli* MenF as described previously (*54*). After purification with HPLC, it was dissolved in H_2O and stored at -80 °C.

Site-directed Mutagenesis, expression and purification of saMenD mutants.

Mutagenesis on saMenD was performed using the QuikChange mutagenesis kit from Stratagene using the primers as listed in **Table 3.1**. The mutations were confirmed by DNA sequencing. The expression and purification of the saMenD mutants followed the same protocol as that for the wild type saMenD protein.

Circular dichroism spectra of saMenD with bivalent ions.

The effects of bivalent ions on the protein secondary structures were examined using AVIV 62 DS spectrometer equipped with a Peltier temperature control unit. Briefly, the far-UV CD spectra of the protein solution with various concentrations of Mg^{2+} or Mn^{2+} (0 - 1.2 mM) were collected at 25 °C at a scan speed of with a 1 nm step resolution. Six measurements were taken and averaged for each sample. The concentration of saMenD was 20 μM.

HPLC analysis of MenD reaction.

An 150 μl aliquot of the reaction mixture was taken at 10, 30 and 90 minutes, respectively, quenched with 0.5 ml of 5% acetic acid, and then analyzed with Xterra C18-MS column at 293 nm. The buffer gradient was described as follows:

0-20 minutes, 100% solution A (5% acetic acid); 20-30 minutes, 100% solution A to 100% B (95% acetonitrile with 5% H2O); 30-35 minutes, 100% B to 100% A. The flow rate was 1.2 ml/minute.

Scan kinetic studies of MenD reaction.

The absorbance spectra of the reaction mixture between 200 nm and 400 nm were collected every minute with Varian 300 UV-Vis Spectrometer. Typically the reaction mixture contained 40 μM isochorimate, 50 μM thiamine diphosphate, 660 μM α-ketoglutarate, 1 mM $MgCl_2$, 1 μM saMenD, 50 mM Tris-HCl, and 50 mM NaCl (pH 8.0). The reaction was initiated by the addition of the enzyme.

Steady state kinetic assays.

The kinetic assays were performed at 25 °C in 50 mM Tris-HCl buffer (pH 7.5) with Varian Cary300 spectrometer. The initial velocities were measured after initiating the reactions with the addition of α-ketoglutarate. The kinetic studies were performed with a concentration range for one substrate (0.3 K_m – 4 K_m) at near saturating concentration of other reaction components. Kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation using GraFit 4.0.

$v = V_{max}$ [S]/ (K_m + [S])

k_{cat} values were calculated by using the equation: $k_{cat} = V_{max}$ / [E].

Synthesis of ThDP analogues.

Thiamin (compound **3.1**) and oxythiamin (compound **3.2**) were commercially available. The synthetic procedures for other thiamine or oxythiamine derivatives were as described previously with minor modifications (*55*). Briefly, 1 mmol of thiamine or oxythiamine was dissolved in 10 ml dry DMF, and cooled to -5 °C under dry nitrogen. After 4 mmol of sodium ethoxide dissolved in DMF was added to the solution and stirred for 10 min, 1.5 mmol of ethyl succinate chloride was added dropwise. After 30 min, the reaction was quenched with HCl. The precipitate was removed by filtration. Solvent was removed from the filtrate by rotary evaporation. The resulting solid was crystallized in ethyl acetate.

For the synthesis of thiazole or benzothiazole derivative, 1 mmol thiazole or benzothiazole was dissolved in 5 mL of dry THF at -78 °C under nitrogen. Then 1.1 mmol of n-BuLi was added to the solution. After 30 minutes, 1.2 mmol of succinic anhydride was added and reacted for 2 h. Then the solution was slowly brought up to -20 °C, and quenched with 5 ml HCl(aq). The reaction mixture was extracted with 10 ml of ethyl acetate twice. Then the organic phase was collected and the solvent was removed by rotary evaporation. The residual solid was crystallized in methanol.

Products were analyzed with ^{1}H NMR and ESI mass spectrometry. Compound **3.3**, ^{1}H NMR: δ 7.86 (1H, s), 5.32 (2H, s), 4.23 (2H, t), 3.99-3.93 (4H, m), 3.20 (2H, t), 2.51-2.42 (2H, m), 2.40 (3H, t), 2.06 (3H, s), 1.79 (3H, s). ESI mass: calcd for $C_{18}H_{25}N_4O_4S^+$ ($[M]^+$) 393.16, found 393.2. Compound **3.4**, ^{1}H NMR: 7.87 (1H, s), 5.32 (2H, s), 4.24 (2H, t), 3.99-3.94 (4H, m), 3.20 (2H, t),

2.51-2.42 (2H, m), 2.40 (3H, t), 2.06 (3H, s), 1.82 (3H, s); ESI mass: calcd for $C_{18}H_{24}N_3O_5S$ ($[M]^+$) 394.14, found 394.1. Compoud **3.5**, ^1H NMR: 6.89 (2H, s), 2.75 (2H, t), 2.49-2.44 (8H, m). ESI mass: calcd for $C_{12}H_{15}N_2O_3S_2$ ($[M+H]^+$) 299.04, found 299.1. Compound **3.6**, ^1H NMR: 7.70-7.35 (4H, m), 6.89 (2H, s), 2.75 (2H, t), 2.48 (6H, s). ESI mass: calcd for $C_{16}H_{13}N_2O_2S_2$ ($[M+H]^+$) 329.03, found 329.0.

Determination of MIC and IC_{50}.

The MIC values were determined by the microbroth dilution assay according to the CLSI protocol for antimicrobial susceptibility tests for aerobically growing bacteria (*56*).

To determine IC_{50}, the reaction velocities were measured with the reaction mixture containing 1 μM saMenD, 30 μM isochorismate, 600 μM α-ketoglutarate, 50 μM ThDP, 2 mM Mg^{2+} and various concentrations of inhibitors at 25 ˚C. IC_{50} values were calculated by fitting the data to the following equation at each concentration of inhibitor.

$$v_i/v_0 = 1/ (1 + [I]/ IC_{50})$$

Bacterial strains and media.

The standard laboratory strains NCTC 8325 and RN 4220 were obtained from NARSA. ATCC 25923 was from ATCC. The *menD*-disrupted *S. aureus* strain was a generous gift from Dr. Becker. It was constructed as described previously

(*35*). MH broth was bought from Fisher Scientific Inc., while AOAC synthetic broth was purchased from Bacto-Difco.

Extraction of menaquinones from S. aureus.

One single colony of *S. aureus* ATCC 25923 was selected and grown overnight at 37 ˚C at 250 rpm in 10 ml LB media, and then transferred to 1 l LB media and grown at 37 ˚C at 250 rpm until OD600 reaches 0.8. Then the cells were collected by centrifugation. Extraction of menaquinone from *S. aureus* cells was performed as described earlier by White et al (*57*). The choloroform fraction from the silica gel chromatography was analyzed by APCI-MS, as described before (*58*).

Ultrastructural analysis of S. aureus.

For whole cell imaging, the cells were collected by centrifugation, washed with PBS twice, and then resuspended in PBS. The formvar coated copper mesh grids were soaked in a series of droplets consisting of 1% glutaraldehyde/0.1 M sodium phosphate buffer (pH 7.4), then washed with phosphate buffer and water, and finally stained with 0.5% phoshotungstic acid.

For thin-sectioned imaging, samples were fixed in 2.5% glutaraldehyde/ 0.1 M sodium phosphate buffer (pH 7.4) and were then processed using standard TEM techniques. Briefly, after fixation, samples were placed in 2% osmium tetroxide in 0.1 M sodium phosphate buffer, dehydrated in a graded series of ethyl alcohol and embedded in Epon resin. Ultrathin sections were cut with a Reichert-Jung

UltracutE ultramicrotome and placed on formvar coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate.

Samples were viewed with a FEI Tecanai BioTwinG^2 transmission electron microscope. Digital images were acquired with an AMT XR-60 CCD digital camera system.

Effect of menD disruption on growth.

Wild type and *menD*-disrupted S. aureus were plated on trypticase-soy agar or AOAC synthetic agar plates, and incubated at 37 °C for 48 hours. In addition, bacteria were also grown in trypticase-soy broth as well as AOAC synthetic broth supplemented with 0.15, 0.60, or 1.80 µg/mL menadione at 37 °C.

Oxygen consumption assay.

S. aureus cells were grown until reaching mid-log phase. Then 0.02% methylene blue was added to the culture, and the cells were further incubated at 37 °C up to 24 h.

3.6 RESULTS AND DISCUSSION

Cloning, expression and purification of saMenD.

saMenD was successfully cloned into a pET23b vector. The fractions during the purification were analyzed by SDS-PAGE, as shown in **Figure 3.3**, indicating that MenD was purified homogeneously.

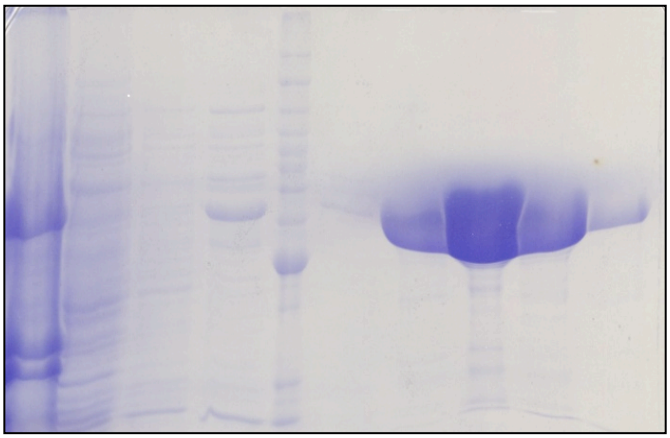


Figure 3.3: SDS-PAGE analysis of saMenD. Samples from left to right: cell pellets, flow through, binding buffer, washing buffer, protein ladder and eluting fractions 1-5.

According to the gel filtration chromatography (**Figure 3.4**), saMenD mainly exists as a hexamer (V_e = 141 ml). This differs significantly with previous studies of *E. coli* MenD, which claim that it exist in solution as a dimer and tetramer in a 3:1 ratio. The even earlier fraction (V_e = 121 ml) is probably saMenD aggregates, since it is quite close to the void volume (V_o = 115 ml) and it is also confirmed to be saMenD based on SDS-PAGE analysis. The crystal structure of saMenD is being analyzed. The attempt to solve its structure by molecular replacement using the reported ecMenD structure failed, due to insufficient similarity between the two enzymes. Multiple anomalous diffraction of SeMet saMenD crystals will be used for further phase determination by Shambhavi Mishra from Dr. Kisker's group.

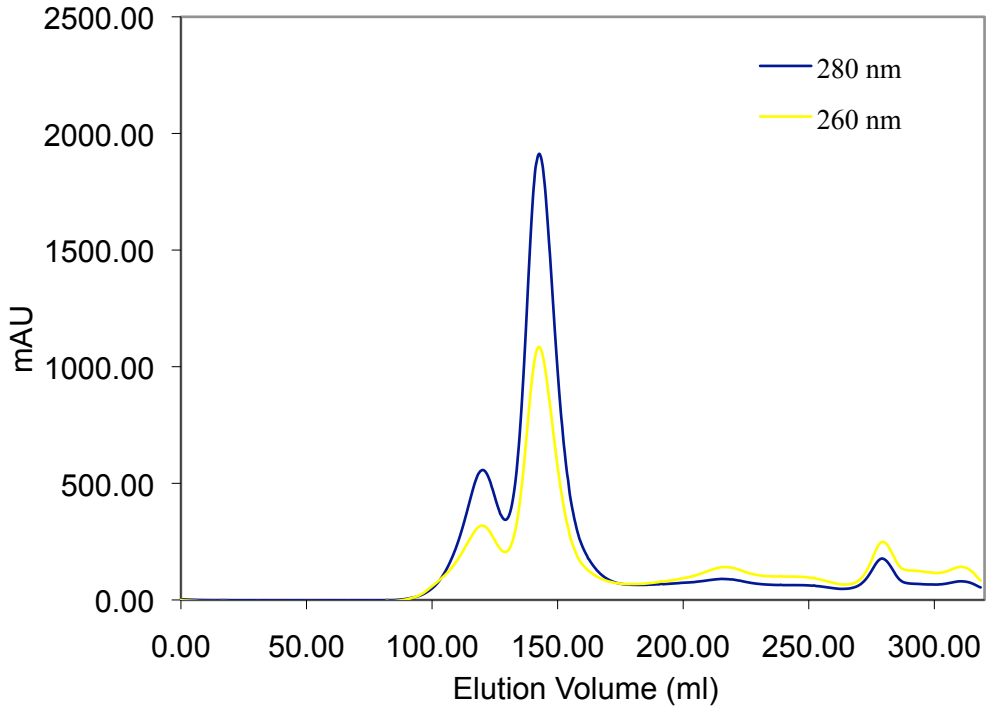


Figure 3.4: Gel filtration chromatography of saMenD. Superdex 200 26/60 elution of saMenD monitored at 280 nm (blue) and 260 nm (blue).

The Product of saMenD-Catalyzed Reaction.

When incubating saMenD with isochorismate, ThDP, Mg^{2+} and α-ketoglutarate at 25 °C, a continuous decrease in absorbance at 278 nm was observed (**Figure 3.5**), indicative of consumption of isochorismate. However, no absorbance increase was observed at 293 nm, at which SHCHC has the maximum absorbance, implying that SHCHC is not the product of saMenD-catalyzed reaction. However, a small increase at the 290 nm region was observed after 2 hours.

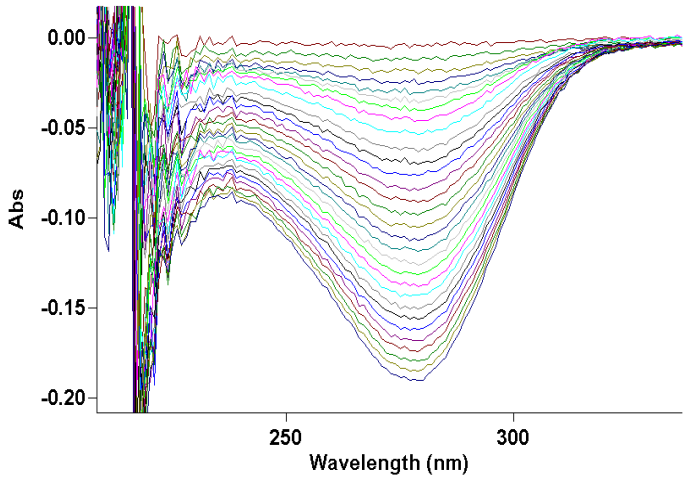
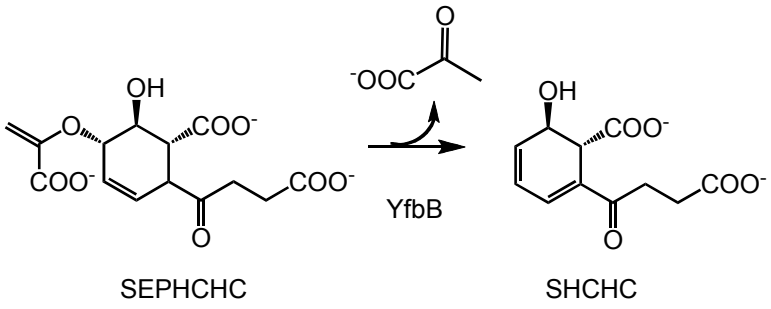


Figure 3.5: UV absorbance spectra of MenD reaction. The reaction was carried out under pH 7.9 at 25 °C. The spectra were collected every minute.

To further explore whether SHCHC is the product, the reaction was carried out at 37 °C to increase the conversion rate, and then monitored simultaneously by HPLC and absorption spectrometer. In the initial 10 min, isochorismate was consumed, while no new peak was observed at 293 nm (**Figure 3.6(a), 6(d)**). An aliquot of the reaction mixture was acidified with HCl, and extracted with ethyl acetate. The extract contains an ion species with m/z of 163.1 under negative ESI mass, which corresponds to SEPHCHC with two negative charges. After 30 min, a new peak with a retention time of 7.0 min was identified, and kept increasing up to 90 min (**Figure 3.6(d)**), which is also consistent with the absorbance spectra (**Figure 6(b), 6(c)**). This peak was collected, and ESI Mass spectroscopy in negative mode gave a 118.9 ion, corresponding to SHCHC with two negative charges. The results from HPLC, absorbance spectra as well as

mass spectrometry suggest that the consumption of isochorismate is synchronized with the production of SEPHCHC instead of SHCHC.

In addition, it was found that the production of SHCHC only occurred in basic condition but not in acidic condition, suggesting that the pyruvate elimination of SEPHCHC is base-catalyzed, instead of enzyme-catalyzed, which is consistent with early report (*51*). Actually, YfbB, another enzyme in the menaquinone biosynthetic pathway in *E. coli*, which was previously annotated as a thioesterase to hydrolyze DHNA-CoA to form DHNA, has been demonstrated to catalyze the conversion from SEPHCHC into SHCHC (**Scheme 3.8**) (*59*).



Scheme 3.8: Reaction catalyzed by YfbB.

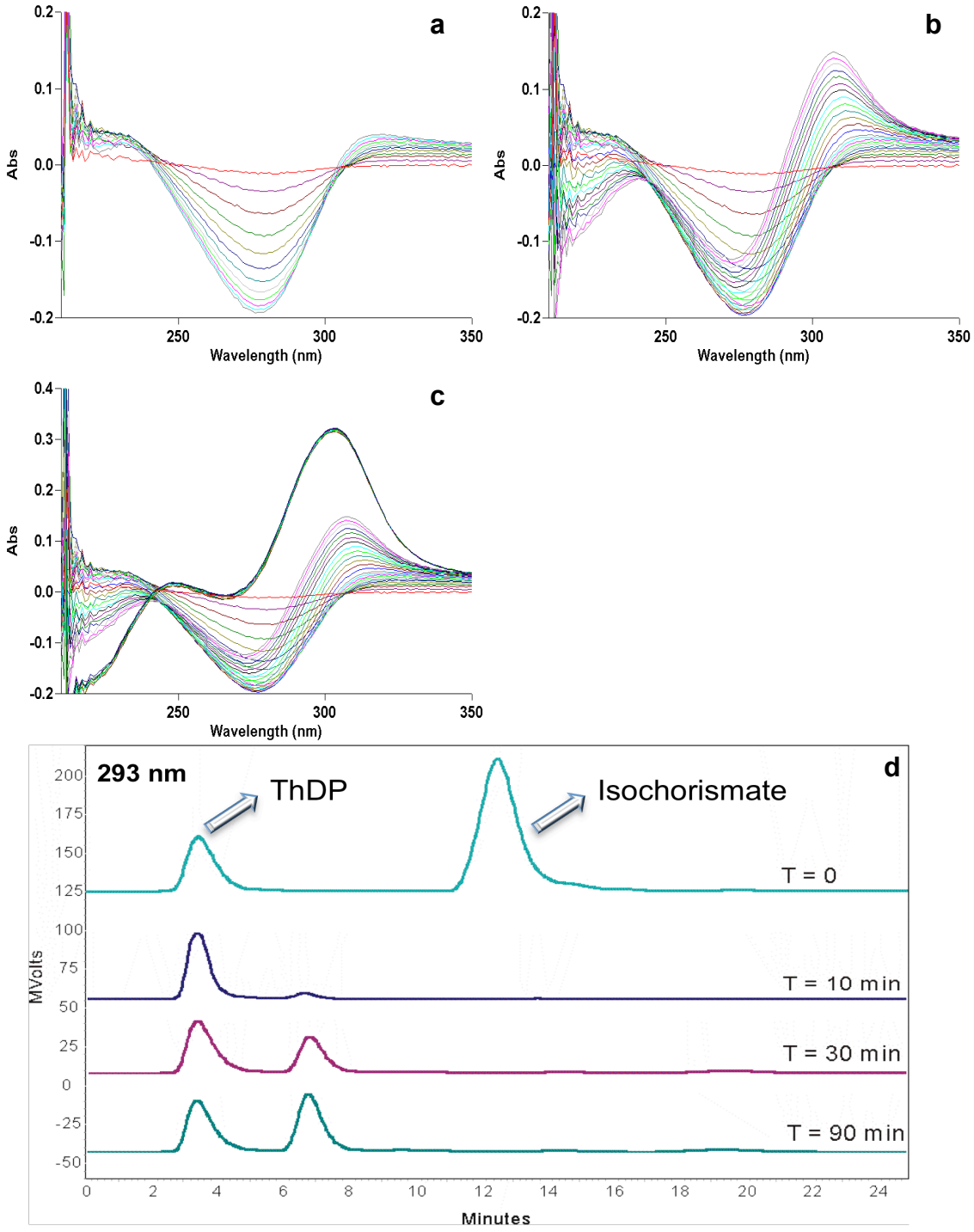


Figure 3.6: Monitoring of saMenD reaction by UV-Vis absorbance spectra (a-c) and HPLC (d). UV-Vis spectra of the reaction mixture were collected in 10 minutes (a), 30 minutes (b) and 90 minutes (c).

Kinetic parameters of substrates and cofactors.

Some enzymes in the pyruvate oxidase family showed a slow-approach to the steady-sate kinetic rates, such as AHAS and PDC (*60, 61*). saMenD exhibited this “lag-phase” kinetics when the reaction was initiated with the addition of the enzyme. But with 8-minute preincubation of saMenD with the cofactors, the initial rates became normal. We hypothesize that the binding of ThDP with the enzyme is relatively slow. Consequently, preincubation is required to obtain the normal initial rate behavior. In addition, the cooperativity observed in other enzymes including ecMenD, were not found in saMenD, suggesting that there is dramatic structural difference between ecMenD and saMenD.

The kinetic parameters of substrates and cofactors were determined by steady-state kinetics. As shown in **Table 3.2**, K_m values of the substrates (α-ketoglutarate and isochorismate) of saMenD are comparable to those of *E. coli* MenD reported by Bhasin et al (*24*). However, the “K_m” of the cofactor ThDP is about 5-fold higher in saMenD than in ecMenD, indicative of differences in cofactor binding.

Table 3.2: Kinetic parameters of saMenD-catalyzed reaction.

Enzymes	Reactant	k_{cat} (min^{-1})	K_m (μM)
saMenD	Isochorismate	10.8 ± 0.5	23.9 ± 3.1
	α-Ketoglutarate		8.8 ± 0.2
	Mg^{2+}		330.4 ± 45.2
	ThDP		21.2 ± 1.9
ecMenD [a]	Isochorismate	10.2 ± 1.1	15.5 ± 0.3
	α-Ketoglutarate		17.1 ± 0.2
	Mg^{2+}		950 ± 30
	ThDP		4.28 ± 0.09

[a] Kinetic parameters were measured by Bhasin et al (*24*).

Bivalent metal ion effect.

In the cofactor-binding pocket of ThDP-dependent enzymes, a bivalent metal ion, such as Mg^{2+} and Mn^{2+}, has favorable interactions with the pyrophosphate group on ThDP as well as several residues in the enzyme, facilitating the cofactor binding. Interestingly, Mn^{2+} and Mg^{2+} behave differently in the saMenD reaction. As shown in **Table 3.3**, Mn^{2+} possesses 10-fold stronger binding affinity than Mg^{2+} does, but it shows an inhibitory effect at high concentrations. The K_i value was determined to be 524.3 μM.

Table 3.3: Effect of Mg^{2+} and Mn^{2+} on saMenD reaction.

	k_{cat} (min^{-1})	K_m (μM)	K_i (μM)
Mg^{2+}	10.8 ± 0.5	330.4 ± 45.2	-
Mn^{2+}	5.4 ± 0.6	36.5 ± 9.6	524.3 ± 131.6

In order to investigate the basis of effects of metal ions on the reaction, the CD spectra of the enzyme was monitored while increasing the ion concentration. As shown in **Figure 3.7**, Mn^{2+} causes more severe loss of CD signals than Mg^{2+} does, indicative of a more dramatic change in the secondary structure. We hypothesize that Mn^{2+} binds to a nonspecific site in the enzyme at high concentrations, leading to the loss of the secondary structures. The observation is quite different to ecMenD, which shows preference of Mn^{2+}, but is not inhibited by either Mg^{2+} or Mn^{2+}, further indicating that the structures of ecMenD and saMenD might be significantly different.

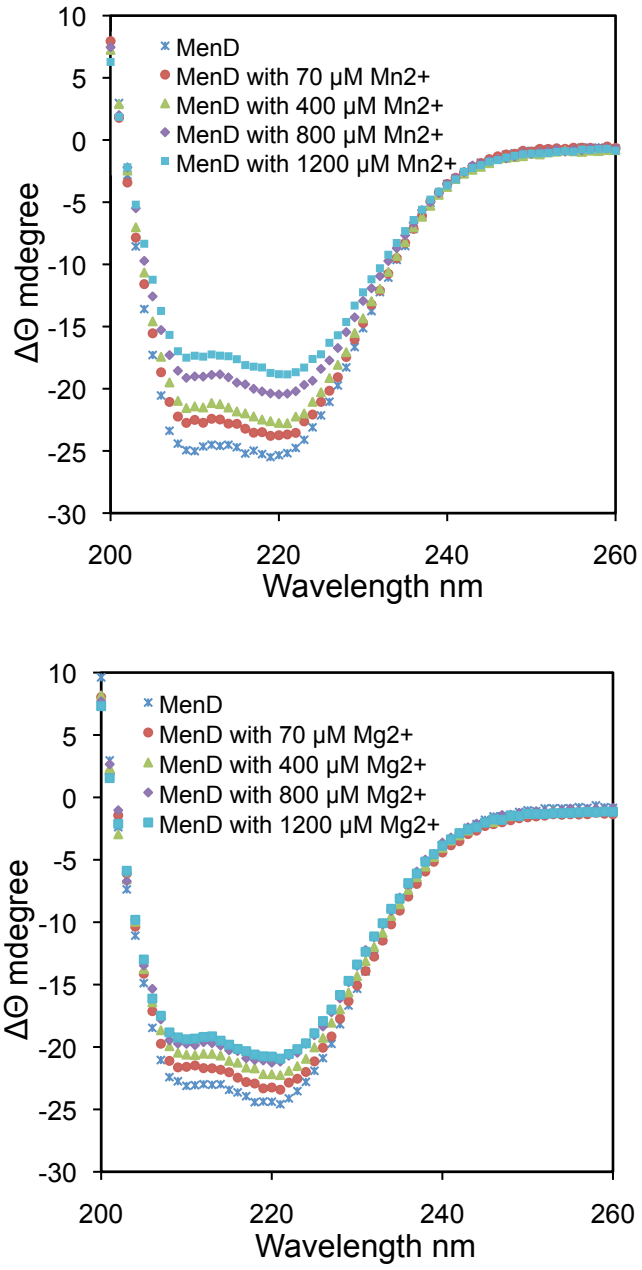


Figure 3.7: CD spectra of saMenD with the addition of Mg^{2+} or Mn^{2+}. The spectra were collected at 25 °C with 10 µM of saMenD.

ThDP activation in saMenD.

A highly conserved glutamate residue is identified in all the ThDP-dependent enzymes, except the recently characterized glyoxylate carboligase, which has a glycine residue instead in this position (*37*). The glutamate residue is believed to activate the cofactor by facilitating the formation of a carbanion on the C2' position in the thiazolium ring, which has been described earlier in this chapter. The sequence alignment with other ThDP-dependent enzymes (**Figure 3.8**) suggests that E56 in saMenD may be responsible for cofactor activation. Kinetic analysis reveals that E56Q mutation abolishes the enzyme activity, supporting the role of E56 during the reaction.

The role of FAD in the reaction.

It was described that ecMenD belongs to the pyruvate oxidase family, and retains the flavin adenine dinucleotide (FAD)-binding domain. It is similar to acetohydroxy acid synthase (AHAS), another enzyme in this family, which also retains the FAD-binding ability. Although FAD does not participate in the reaction directly, it facilitates the reaction of AHAS, probably due to a conformational change in the active site upon FAD binding. Hence, FAD was examined to see whether it has a similar role in the MenD reaction. However, saMenD reaction rates were not significantly affected by FAD, suggesting that FAD is not involved in the reaction.

Figure 3.8: Sequence alignment of ThDP-dependent enzymes by ClustalW. The conserved Glu residues are in red. POX, pyruvate oxidase; AHAS, acetohydroxy acid synthase; BFD, benzoylformate decarboxylase.

Isochorismate binding pocket.

Little information of the isochorismate binding pocket is known due to the instability of isochorismate, although the crystal structure of *E. coli* MenD bound with ThDP was published recently (*52*). A possible isochorismate-binding pocket was generated by molecular modeling, as shown in **Figure 3.9**. Hydrophobic residues F475 and I474 from chain A of the ecMenD dimer were proposed to interact with the hydrophobic side of isochorismate, while several positively-charged residues, such as K292, R293 and R413 from chain A and R33 and R107 from chain B interact with the highly hydrophilic side.

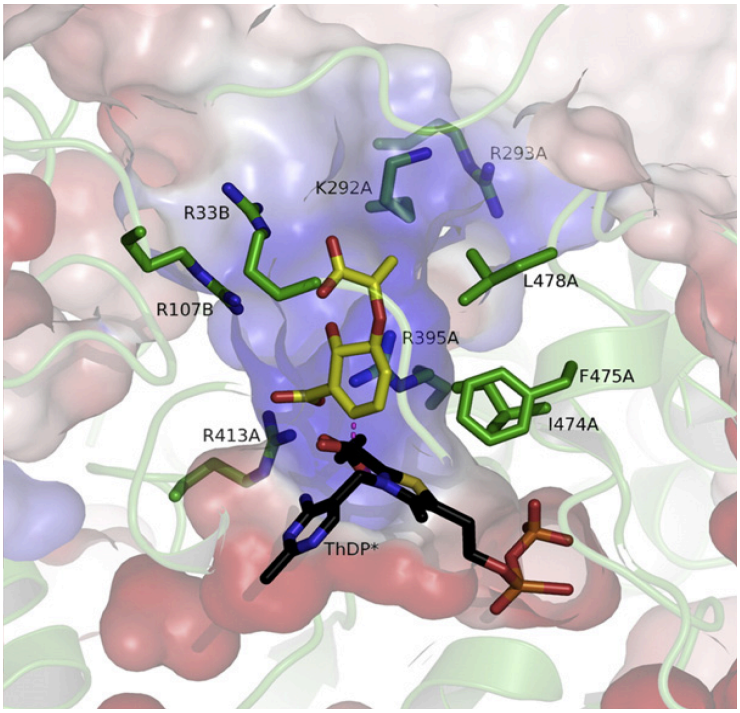


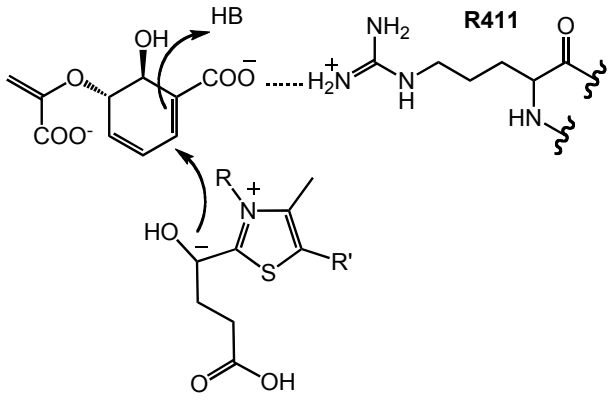
Figure 3.9: Proposed isochorismate-binding pocket in ecMenD from molecular modeling (*52*).

The role of the corresponding residues in saMenD was probed by site-directed mutagenesis. The single mutant K289M causes a 4-fold increase on K_m of isochorismate, while the K288M/K289M double mutant leads to a further 2-fold increase, indicating that both K288 and K289 are involved in the binding of isochorismate. The interaction between K288 and isochorismate may be weak, since the mutation K288M barely affects the K_m value. But the introduction of a negatively charged residue in this position by K288E causes a 6-fold increase on K_m, indicating that K288 still contributes to the positively-charged binding pocket for isochorismate. In addition, the fact that k_{cat} is not significantly affected suggests that isochorismate is not rate-limiting during the reaction. Attempts to purify the saMenD R394M mutant failed, due to the low expression level.

Table 3.4: Kinetic analysis of residues in isochorismate-binding pocket.

	k_{cat} (min^{-1})	$K_{m,\ isochorismate}$ (μM)	$k_{cat}/K_{m,\ isochorismate}$ ($min^{-1}\ \mu M^{-1}$)
WT	10.8 ± 0.5	23.9 ± 3.1	0.46 ± 0.08
K288M	4.7 ± 0.2	22.5 ± 2.4	0.21 ± 0.03
K289M	7.0 ± 0.1	90.1 ± 3.9	0.078 ± 0.005
K288M/K289M	3.9 ± 0.2	194.1 ± 19.4	0.013 ± 0.003
K288E	11.6 ± 0.4	136.1 ± 11.6	0.083 ± 0.010
R411M	0.15 ± 0.01	38.5 ± 4.5	0.0040 ± 0.0007

Interestingly, k_{cat} of the mutant R411M decreases by about 60-fold compared with the wild type saMenD, while K_m of isochorismate slightly increases by less than 2-fold. The result suggests that the residue R411 might be involved in a rate-limiting step during the catalysis. We hypothesize that it might help to modulate the precise orientation of isochorismate for the carboligation step (**Scheme 3.9**).



Scheme 3.9: Possible role of R411 in catalysis.

Mass spectrometry analysis of menaquinones from S. aureus.

The chloroform fraction from the silica gel fractionation was characterized by APCI-MS (**Figure 3.10**). Ions with m/z of 717.7 and 649.5, which corresponds to the molecular ion of MK-8 and MK-7, respectively, were identified. They were confirmed to be menaquinones by MS-MS analysis, since a characteristic peak of 187 corresponding to the napthoate moiety was observed. Multiple reaction monitoring analysis reveals that MK-7 and MK-8 are the major quinones in *S. aureus*, and MK-8 is about 5-fold more redundant than MK-7 (**Table 3.5**),

assuming that all menaquinones have the same fragmentation tendency in the APCI mode. The results are in agreement with previous studies (*62*).

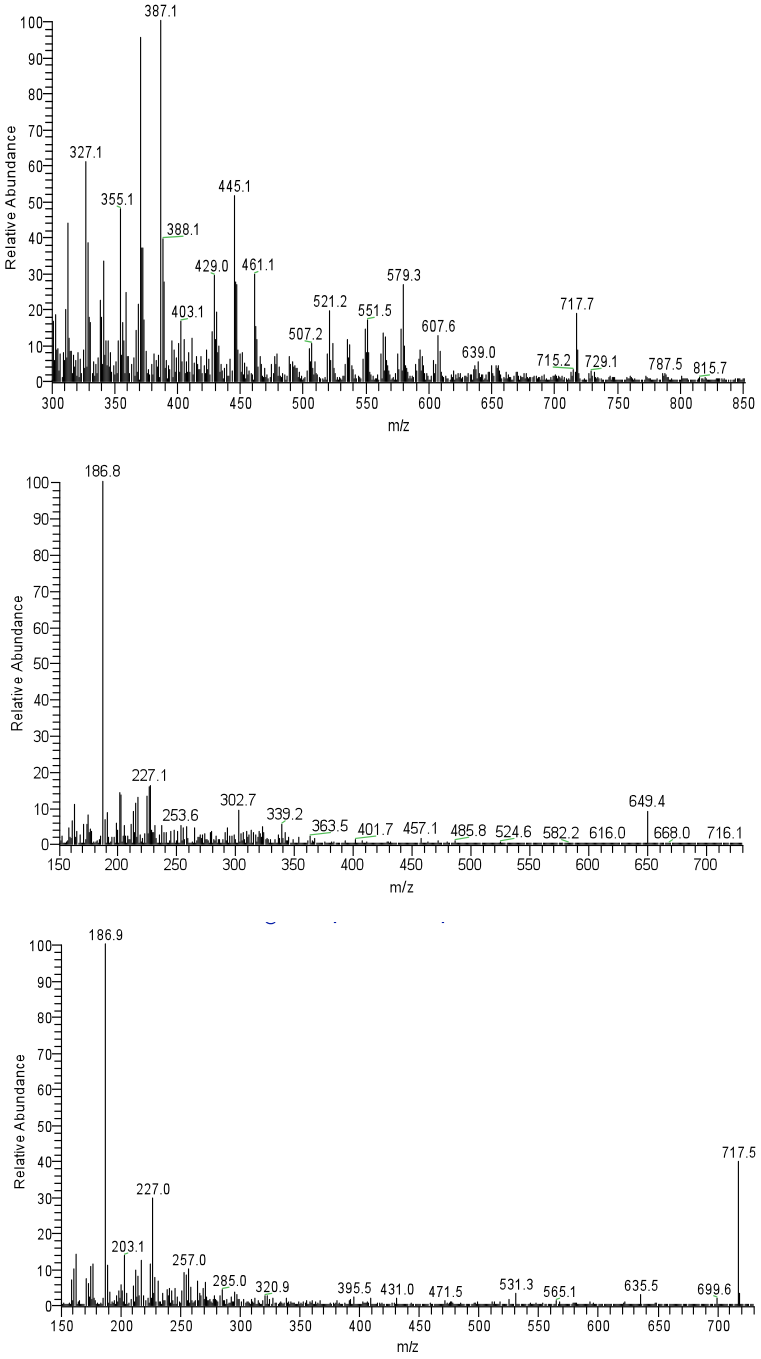


Figure 3.10: Mass spectra of the chloroform fraction. (a) Analysis in APCI mode; (b) MS-MS analysis of ion 649.4; (c) MS-MS analysis of ion 717.7.

Table 3.5: Multiple-Reaction-Monitoring analysis.

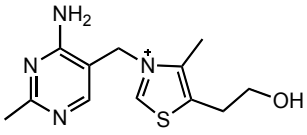
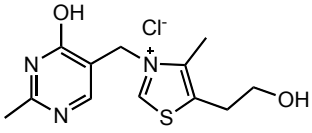
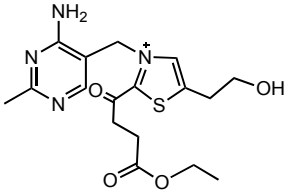
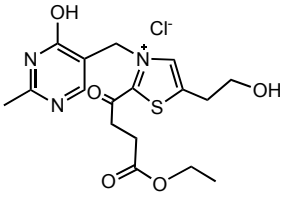
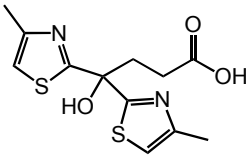
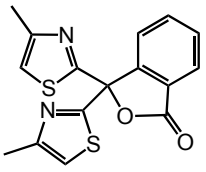
	Area MK7	Area MK8
	649.6 → 186.9	717.7 → 186.9
Chloroform fraction	1,036,765	5,098,995

Inhibitory activities of ThDP analogues.

Two ThDP analogues, thiamine (**3.1**) and oxythiamine (**3.2**) exhibited inhibition against the enzyme saMenD (**Table 3.6**). But only oxythiamine inhibits the growth of *S. aureus*. The oxythiamine or thiamine derivatives (compound **3.3** and **3.4**) with an ethyl succinate group attached to the C2α position of the thiazolium ring, which mimic one of the MenD reaction intermediates, were synthesized. However, the compounds do not inhibit bacterial growth, although they show 3 to 4-fold increased inhibition against the enzyme. The poor inhibition of these intermediate analogues against *S. aureus* could be due to their instability, since the positively-charged neighboring nitrogen atom increase the susceptibility of the side chain carbonyl group to hydrolysis. In order to overcome this disadvantage, we attempted to synthesize thiazole derivatives with a succinate or benzoyl side chain. However, the major products resulted from a 2:1 reaction of thiazole and succinic anhydride/phthalic anhydride (compound **3.5** and **3.6**), although one equivalent of succinic anhydride or phthalic anhydride was added. This is probably because of the strong base n-BuLi, which facilitates

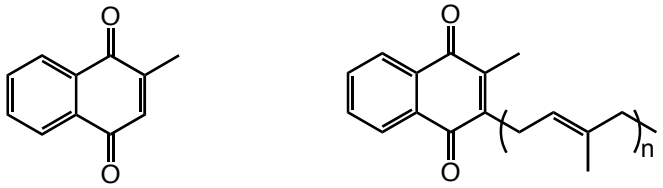
the second addition. Further optimization of the reaction condition is needed to obtain the desired products. Due to the bulky side chain, compound **3.5** and **3.6** showed no inhibition.

Table 3.6: Inhibition of thiamine derivatives.

Compound	Compound	IC_{50} against saMenD (μM)	MIC against *S. aureus* ATCC25923 (μM)
3.1		100.4 ± 15.5	> 3000
3.2		90.2 ± 10.3	200
3.3		30.4 ± 3.8	> 600
3.4		25.4 ± 2.8	> 600
3.5		No inhibition	> 600
3.6		Not Determined	> 600

Effect of menD-disruption on growth

As shown in **Table 3.7**, the growth of *menD*-disrupted strain in the chemically defined medium that contains no quinones requires the supplementation of a low concentration of menadione (**Scheme 3.10**), a precursor of menaquinone, indicating that menaquinone is essential for the growth of *S. aureus*. However, at higher concentration of menadione (1.8 μg/mL), medadione showed inhibitory effect.



Menadione Menaquinone

Scheme 3.10: Structures of menadione and menaquinone

menD-disrupted strain could grow in trypticase soy broth, although it grew much slower than the wild type strain. Also small colony phenotype was observed on trypticase soy agar. The results suggest that trace amount of menaquinone or its precursors might be present in the rich medium, supporting a slow metabolism of the bacteria even if the menaquinone biosynthesis in the bacterium is impaired. This raises a concern for targeting menaquinone biosynthesis as a novel chemotherapy, since *S. aureus* could scavenge menaquinone or its precursors from the environment, and persist as SCV.

Table 3.7: Growth requirement of *menD-disrupted strain*

Agar plate		Liquid medium			
TSA	CDM	CDM + 0.15 μg/mL menadione	CDM + 0.60 μg/mL menadione	CDM + 1.8 μg/mL menadione	TSB
+	-	+	+	-	+

"+" indicates turbidity or colonies observed after 48 hours incubation;

"-" indicates culture remains clear or no colony was observed after 48 hours.

Oxygen consumption assay.

Methylene blue, a redox dye, has been reported as a good indicator of the respiration level in *M. tuberculosis (63)*. It was used here to compare the difference in respiration between the wild type *S. aureus* strain and *menD*-disrupted strain. As shown in **Figure 3.11**, wild type *S. aureus* already decolorized the dye after 20 minutes incubation of cells with methylene blue, while *menD*-disrupted strain did not. After two hours incubation, *menD*-disrupted strain also began to decolorize the dye. The results indicate the respiration is significantly reduced but not eliminated by the disruption of *menD* gene.

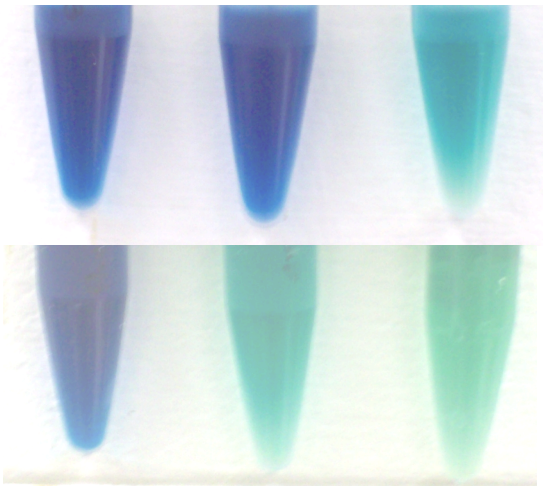


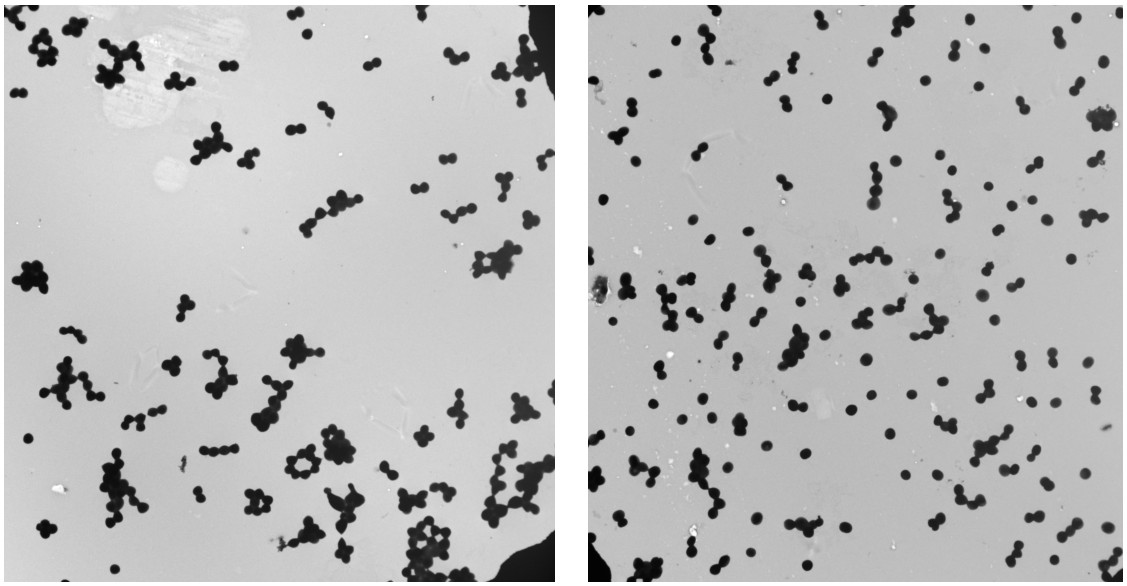
Figure 3.11: Oxygen consumption assay in wild type and *menD*-disrupted *S. aureus* after 30 minutes (top) and 2 hours (bottom) incubation with 0.02% methylene blue. The samples were blank (no bacteria), *menD*-disrupted strain and wild type strain, respectively, from left to right.

Effect of menD-disruption on morphology of S. aureus.

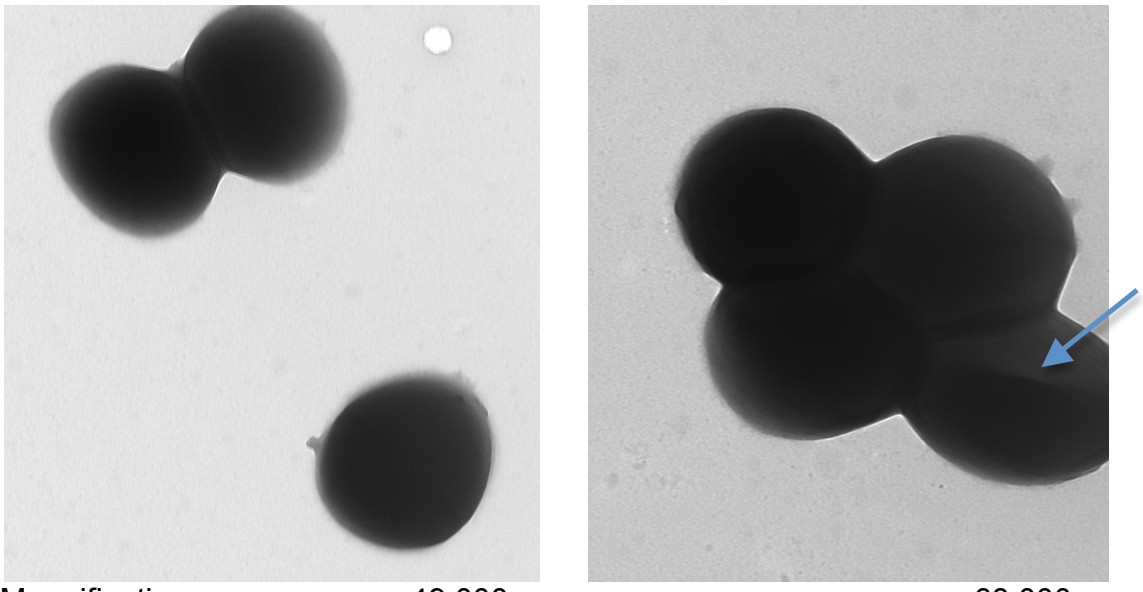
According to the TEM images, it seems that the wild type strain forms larger clusters than the *menD* mutant strain (**Figure 3.12**), indicating that the intercellular adhesins may be downregulated in the mutant strain. Some *menD* mutant cells have a slightly elevated center, while wild type cells do not. Similar observation was also found in thymidine-defective SCV strains (*64*). This may be correlated with incomplete cell membrane separation during division. The size of thymidine-defective SCV cells was enlarged, but this was not observed in *menD*-disrupted strain. Interestingly, the cell wall of the *menD* mutant strain, about 40-80 nm away from the plasma membrane (**Figure 3.13**), binds much less of the heavy metal stain than the wild type strain, indicating that the amount of anionic cell wall polymers decrease significantly in the mutant strain. In addition, the furry cell wall structure in the wild type strain, which probably consists of surface

proteins, (**Figure 3.13**) was absent in the mutant strain. This observation is contrary to a previous study on *hemB*-disrupted SCV strain claiming that the expression level of surface adhesins was higher than the wild type strain (*65*). The difference suggests that defect in menaquinone causes more significant damage to the cell wall-assocaited proteins. The anchoring of the surface proteins requires a transamidase called “sortase”, which recognizes and cleaves the LPXTG motif in the C-terminus of the surface proteins, and then links to the peptidoglycans in the cell wall. The absence of surface proteins in the *menD* mutant strain suggests that menaquinone participates either in the production or the anchoring of surface proteins.

Aberrant cell divisions and cell shapes, such as multiple cell membranes within the bacterium and abnormal binary fission segregation site, were also observed, as shown in **Figure 3.13**. Similar abnormal cell division was also observed in another electron transport-defective *S. aureus* strain, in which *hemB* was disrupted (*67*). The morphological change is similar to that caused by the depletion of lipoteichoic acid (**Figure 3.14**) (*66*), indicating that lipoteichoic acid production is affected by *menD*-disruption. It is reasonable, since the synthesis of phosphatidyl glycerol, a precursor of lipoteichoic acid, requires ATP, which is limited when electron transport is defective.

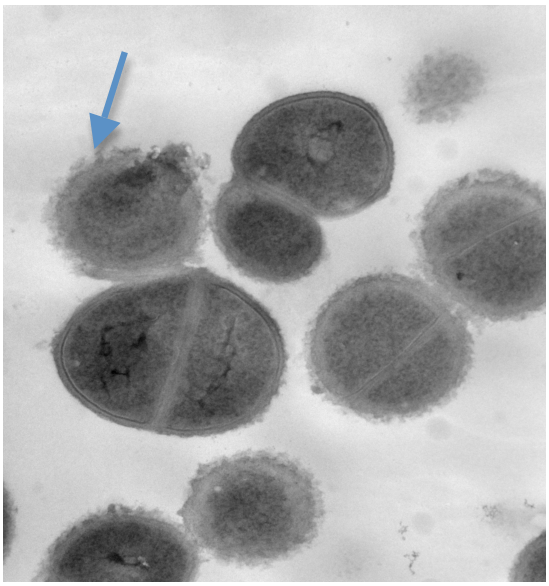


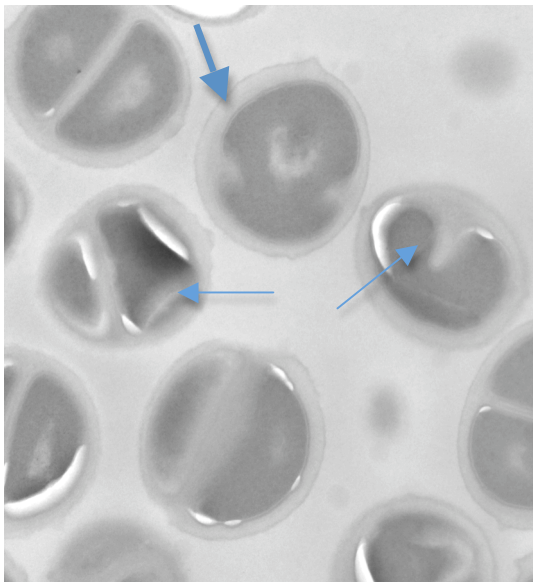
Magnification	2,900 x	2,900 x



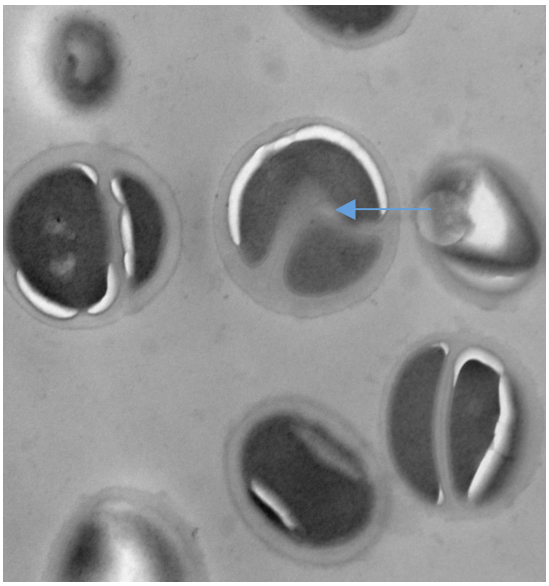
Magnification	49,000 x	68,000 x

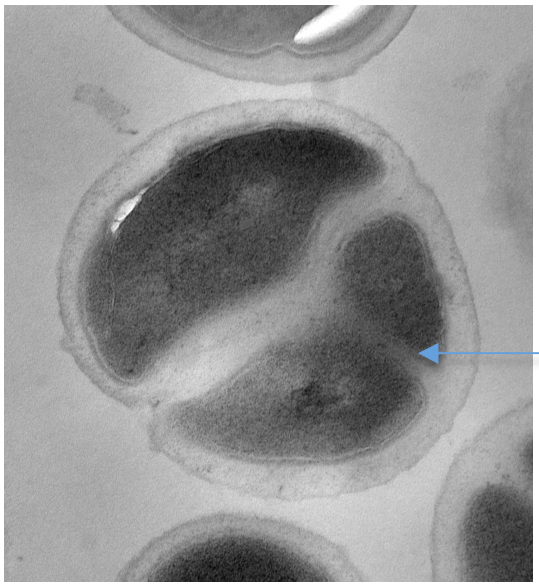
Figure 3.12: Transmission electron microscope images of wild type *S. aureus* (left) and *menD*-disrupted *S. aureus* strain (right). The arrow indicates the elevated edge in the *menD*-disrupted strain.





Magnification 49,000 x 49,000x





Magnification 49,000x 120,000x

Figure 3.13: TEM images of thin-sectioned wild type *S. aureus* (top left) and *menD*-disrupted *S. aureus* strain (top right and bottom). Thick arrowhead in top left image indicates the capsular polysaccharide, while thick arrowhead in top right indicates the outer membrane-like structure. Thin arrows point to the abnormal cell shape or cell separation.

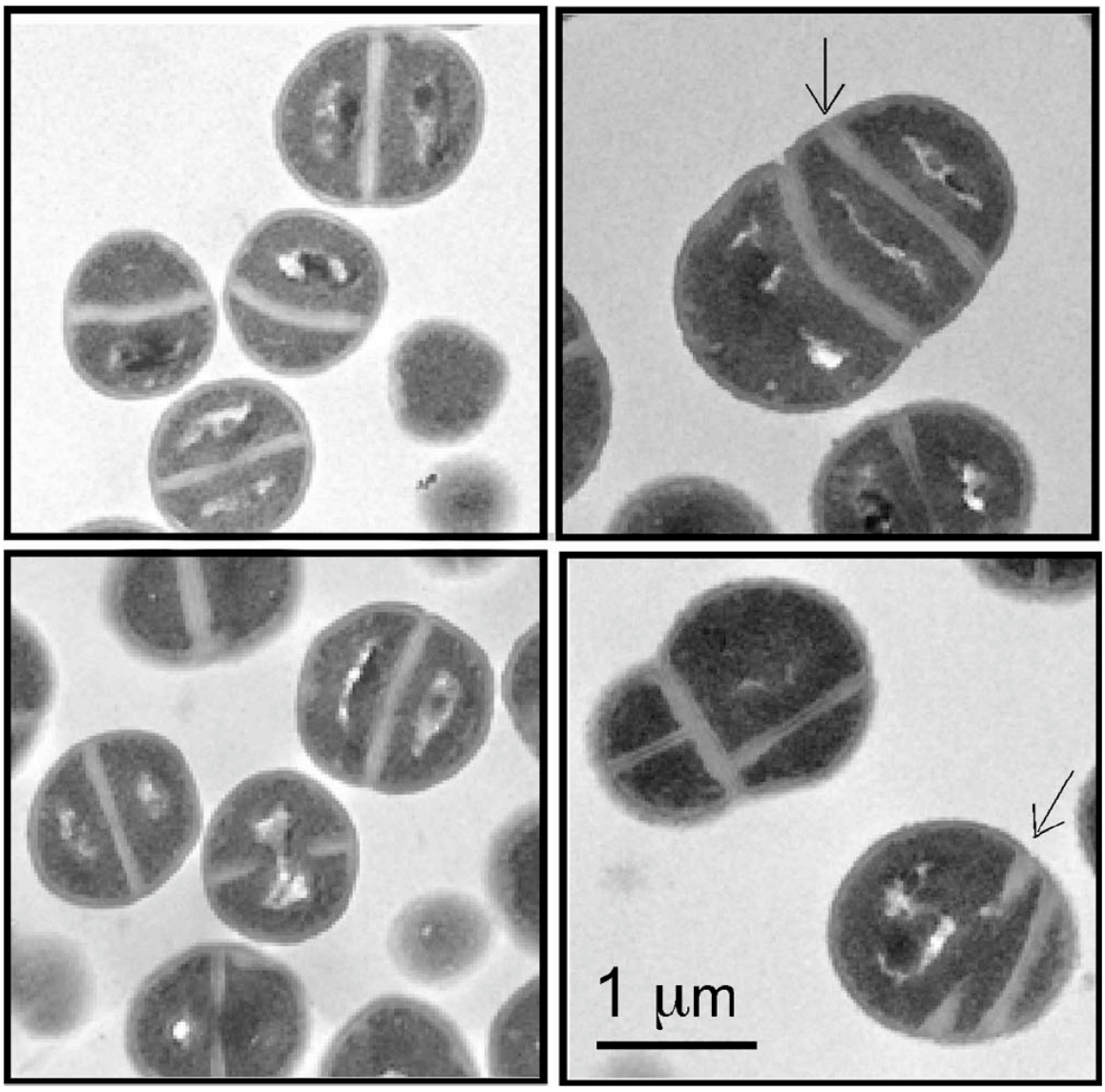


Figure 3.14: Cell envelope assembly of *S. aureus* with (left) or without (right) the expression of lipoteichoic acid. TEM images are from Ref (*66*).

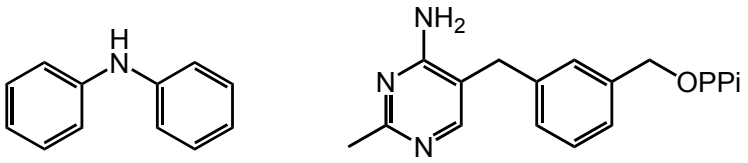
3.7 CONCLUSIONS AND FUTURE DIRECTIONS

Mechanistic studies of saMenD confirm that SEPHCHC is the actual product instead of SHCHC. Mutagenesis analysis reveals that E56 is responsible for the cofactor activation, and residues K288, K289 are involved in the binding of isochorismate. R411 participates in the rate-limiting step of the reaction, which may be the carboligation step or the dissociation of the product-ThDP adduct.

MenD is essential for the production of menaquinone in *S. aureus*. The *menD*-disrupted strain cannot grow in the absence of menadione, suggesting that menaquinone is essential for the growth of *S. aureus*. In addition, *menD*-disrupted strain also showed reduced respiration compared with the wild type strain. The lack of menaquinone affects the morphology dramatically, including reduction in the surface protein production and abnormal cell separation. The morphological change indicates the lipoteichoid acid synthesis may be blocked in the *menD*-disrupted strain. This may also explain why the mutant strain binds much less heavy metal ions, since lipoteichoic acids are anionic cell wall polymers. The lipid profile comparison between the wild type strain and *menD* mutant strain will be performed by mass spectrometry to see whether the lipoteichoic acid level is reduced.

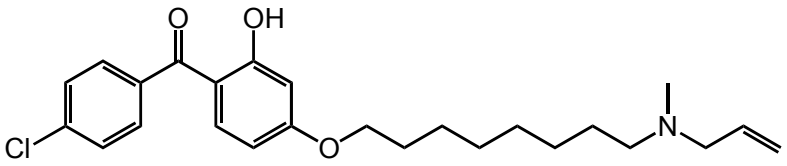
Some ThDP analogues were synthesized and tested against *S. aureus*. Unfortunately, none of them showed strong inhibition against the bacterium. Interestingly, it was reported that diphenyl amine showed inhibition in menaquinone and carotenoid biosynthesis in *S. aureus (68, 69)*. But the mode of action remains unclear. We hypothesize that diphenyl amine targets MenD and

MenA in *S. aureus*, since it resembles the structures of 4-amino-5-[3-(2-hydroxyethyl)benzyl]-2-methylpyrimidine diphosphate and allyaminomethanone-A (**Scheme 3.11**). The former compound has been reported to inhibit ThDP-dependent enzyme (*47*), while the second one inhibits MenA activity (*33*). *In vitro* inhibition assays will be performed to confirm whether diphenyl amine inhibits enzyme activities of MenD and MenA, and whether it can serve as a scaffold for further design of inhibitors against menaquinone biosynthesis.



Diphenyl amine

4-Amino-5-[3-(2-hydroxyethyl)benzyl]-2-methylpyrimidine diphosphate



Allylaminomethanone-A

Scheme 3.11: Chemical structures of diphenyl amine, 4-amino-5-[3-(2-hydroxyethyl)benzyl]-2-methylpyrimidine diphosphate and a MenA inhibitor.

References

1. Lester, R. L., and Crane, F. L. (1959) The natural occurrence of coenzyme Q and related compounds, *J. Biol. Chem. 234*, 2169-2175.
2. Threlfall, D. R., and Whistance, G. R. (1971) Biosynthesis of isoprenoid quinones and chromanols, in *Aspects of terpernoid chemistry and biochemistry* (Goodwin, T. W., Ed.), Academic Press Inc., London.
3. Bishop, D. H., Pandya, K. P., and King, H. K. (1962) Ubiquinone and vitamin K in bacteria, *Biochem. J. 83*, 606-614.
4. Friis, P., Daves, G. D., Jr., and Folkers, K. (1967) New epoxyubiquinones, *Biochemistry 6*, 3618-3624.
5. MacCorquodale, D. W., Cheney, L. C., Binkley, S. B., Holcomb, W. F., McKee, R. W., Thayer, S. A., Doisy, E. A. (1939) The constitution and synthesis of vitamin K, *J. Biol. Chem. 131*, 357-370.
6. Collins, M. D., Pirouz, T., Goodfellow, M., and Minnikin, D. E. (1977) Distribution of menaquinones in actinomycetes and corynebacteria, *J. Gen. Microbiol. 100*, 221-230.
7. Hammand, R. K. a. W., D. C. (1969) Separation of vitamin K2 isorenologues by reverse-phase thin-layer chromatography, *J. Chromatogr. 45*, 446-452.
8. Sasarman, A., Purvis, P., and Portelance, V. (1974) Role of menaquinone in nitrate respiration in *Staphylococcus aureus*, *J. Bacteriol. 117*, 911-913.
9. Davidson, V. L. (2004) Electron transfer in quinoproteins, *Arch. Biochem. Biophys. 428*, 32-40.

10. Suttie, J. W. (1995) The importance of menaquinones in human nutrition, *Annu. Rev. Nutr. 15*, 399-417.

11. Reddi, K., Henderson, B., Meghji, S., Wilson, M., Poole, S., Hopper, C., Harris, M., and Hodges, S. J. (1995) Interleukin 6 production by lipopolysaccharide-stimulated human fibroblasts is potently inhibited by naphthoquinone (vitamin K) compounds, *Cytokine 7*, 287-290.

12. Ohsaki, Y., Shirakawa, H., Hiwatashi, K., Furukawa, Y., Mizutani, T., and Komai, M. (2006) Vitamin K suppresses lipopolysaccharide-induced inflammation in the rat, *Biosci. Biotechnol. Biochem. 70*, 926-932.

13. Li, J., Lin, J. C., Wang, H., Peterson, J. W., Furie, B. C., Furie, B., Booth, S. L., Volpe, J. J., and Rosenberg, P. A. (2003) Novel role of vitamin k in preventing oxidative injury to developing oligodendrocytes and neurons, *J. Neurosci. 23*, 5816-5826.

14. Sundaram, K. S., and Lev, M. (1988) Warfarin administration reduces synthesis of sulfatides and other sphingolipids in mouse brain, *J. Lipid Res. 29*, 1475-1479.

15. Cox, G. B., and Gibson, F. (1964) Biosynthesis of Vitamin K and Ubiquinone. Relation to the Shikimic Acid Pathway in *Escherichia Coli*, *Biochim. Biophys. Acta 93*, 204-206.

16. Campbell, I. M., Coscia, C. J., Kelsey, M., and Bentley, R. (1967) Origin of the aromatic nucleus in bacterial menaquinones, *Biochem. Biophys. Res. Commun. 28*, 25-29.

17. Robins, D. J., Bentley, R. (1972) Biosynthesis of bacterial menaquinones-evidence for the involvement of 2-oxoglutaric acid, *J. Chem. Soc. Chem. Commun.*, 232-233.

18. Young, I. G. (1975) Biosynthesis of bacterial menaquinones. Menaquinone mutants of *Escherichia coli*, *Biochemistry 14*, 399-406.

19. Shineberg, B., and Young, I. G. (1976) Biosynthesis of bacterial menaquinones: the membrane-associated 1,4-dihydroxy-2-naphthoate octaprenyltransferase of *Escherichia coli*, *Biochemistry 15*, 2754-2758.

20. Guest, J. R. (1977) Menaquinone biosynthesis: mutants of *Escherichia coli* K-12 requiring 2-succinylbenzoate, *J. Bacteriol. 130*, 1038-1046.

21. Guest, J. R. (1979) Anaerobic growth of *Escherichia coli K12* with fumarate as terminal electron acceptor. Genetic studies with menaquinone and fluoroacetate-resistant mutants, *J. Gen. Microbiol. 115*, 259-271.

22. Daruwala, R., Bhattacharyya, D. K., Kwon, O., and Meganathan, R. (1997) Menaquinone (vitamin K2) biosynthesis: overexpression, purification, and characterization of a new isochorismate synthase from *Escherichia coli*, *J. Bacteriol. 179*, 3133-3138.

23. Sharma, V., Meganathan, R., and Hudspeth, M. E. (1993) Menaquinone (vitamin K2) biosynthesis: cloning, nucleotide sequence, and expression of the menC gene from *Escherichia coli*, *J. Bacteriol. 175*, 4917-4921.

24. Bhasin, M., Billinsky, J. L., and Palmer, D. R. (2003) Steady-state kinetics and molecular evolution of *Escherichia coli* MenD [(1R,6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase], an anomalous

thiamin diphosphate-dependent decarboxylase-carboligase, *Biochemistry 42*, 13496-13504.

25. Sharma, V., Hudspeth, M. E., and Meganathan, R. (1996) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the menE gene from *Escherichia coli*, *Gene 168*, 43-48.

26. Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J., and Kisker, C. (2003) Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in vitamin K2 biosynthesis, *J. Biol. Chem. 278*, 42352-42360.

27. Suvarna, K., Stevenson, D., Meganathan, R., and Hudspeth, M. E. (1998) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the menA gene from *Escherichia coli*, *J. Bacteriol. 180*, 2782-2787.

28. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*, *Nature 388*, 539-547.

29. Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A., Rutherford, K. M., van Vliet, A. H., Whitehead, S., and Barrell, B. G. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences, *Nature 403*, 665-668.

30. Marcelli, S. W., Chang, H. T., Chapman, T., Chalk, P. A., Miles, R. J., and Poole, R. K. (1996) The respiratory chain of *Helicobacter pylori*: identification of cytochromes and the effects of oxygen on cytochrome and menaquinone levels, *FEMS Microbiol. Lett. 138*, 59-64.

31. Hiratsuka, T., Furihata, K., Ishikawa, J., Yamashita, H., Itoh, N., Seto, H., and Dairi, T. (2008) An alternative menaquinone biosynthetic pathway operating in microorganisms, *Science 321*, 1670-1673.

32. Seto, H., Jinnai, Y., Hiratsuka, T., Fukawa, M., Furihata, K., Itoh, N., and Dairi, T. (2008) Studies on a new biosynthetic pathway for menaquinone, *J. Am. Chem. Soc. 130*, 5614-5615.

33. Kurosu, M., Narayanasamy, P., Biswas, K., Dhiman, R., and Crick, D. C. (2007) Discovery of 1,4-dihydroxy-2-naphthoate [corrected] prenyltransferase inhibitors: new drug leads for multidrug-resistant gram-positive pathogens, *J. Med. Chem. 50*, 3973-3975.

34. Dhiman, R. K., Mahapatra, S., Slayden, R. A., Boyne, M. E., Lenaerts, A., Hinshaw, J. C., Angala, S. K., Chatterjee, D., Biswas, K., Narayanasamy,

P., Kurosu, M., and Crick, D. C. (2009) Menaquinone synthesis is critical for maintaining mycobacterial viability during exponential growth and recovery from non-replicating persistence, *Mol. Microbiol. 72*, 85-97.

35. Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S., and Proctor, R. A. (2003) *Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney, *J. Infect. Dis. 187*, 1654-1661.

36. Kohler, C., von Eiff, C., Liebeke, M., McNamara, P. J., Lalk, M., Proctor, R. A., Hecker, M., and Engelmann, S. (2008) A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*, *J. Bacteriol. 190*, 6351-6364.

37. Kaplun, A., Binshtein, E., Vyazmensky, M., Steinmetz, A., Barak, Z., Chipman, D. M., Tittmann, K., and Shaanan, B. (2008) Glyoxylate carboligase lacks the canonical active site glutamate of thiamine-dependent enzymes, *Nat. Chem. Biol. 4*, 113-118.

38. Kluger, R., and Tittmann, K. (2008) Thiamin diphosphate catalysis: enzymic and nonenzymic covalent intermediates, *Chem. Rev. 108*, 1797-1833.

39. Schutz, A., Golbik, R., Konig, S., Hubner, G., and Tittmann, K. (2005) Intermediates and transition states in thiamin diphosphate-dependent decarboxylases. A kinetic and NMR study on wild-type indolepyruvate

decarboxylase and variants using indolepyruvate, benzoylformate, and pyruvate as substrates, *Biochemistry 44*, 6164-6179.

40. Chakraborty, S., Nemeria, N. S., Balakrishnan, A., Brandt, G. S., Kneen, M. M., Yep, A., McLeish, M. J., Kenyon, G. L., Petsko, G. A., Ringe, D., and Jordan, F. (2009) Detection and time course of formation of major thiamin diphosphate-bound covalent intermediates derived from a chromophoric substrate analogue on benzoylformate decarboxylase, *Biochemistry 48*, 981-994.

41. Berthold, C. L., Gocke, D., Wood, M. D., Leeper, F. J., Pohl, M., and Schneider, G. (2007) Structure of the branched-chain keto acid decarboxylase (KdcA) from *Lactococcus lactis* provides insights into the structural basis for the chemoselective and enantioselective carboligation reaction, *Acta Crystallogr. D Biol. Crystallogr. 63*, 1217-1224.

42. Neumann, P., Weidner, A., Pech, A., Stubbs, M. T., and Tittmann, K. (2008) Structural basis for membrane binding and catalytic activation of the peripheral membrane enzyme pyruvate oxidase from *Escherichia coli*, *Proc. Natl. Acad. Sci. U S A 105*, 17390-17395.

43. Pang, S. S., Duggleby, R. G., and Guddat, L. W. (2002) Crystal structure of yeast acetohydroxyacid synthase: a target for herbicidal inhibitors, *J. Mol. Biol. 317*, 249-262.

44. Gutowski, J. A., and Lienhard, G. E. (1976) Transition state analogs for thiamin pyrophosphate-dependent enzymes, *J. Biol. Chem. 251*, 2863-2866.

45. Kluger, R., and Pike, D. C. (1977) Active site generated analogues of reactive intermediates in enzymic reactions. Potent inhibition of pyruvate dehydrogenase by a phosphonate analogue of pyruvate1, *J. Am. Chem. Soc. 99*, 4504-4506.

46. Hawksley, D. G., D. A. and Leeper, F. J. (2001) Synthesis of 3-deazathiamine, *J. Chem. Soc., Perkin Trans 1*, 144-148.

47. Mann, S., Perez Melero, C., Hawksley, D., and Leeper, F. J. (2004) Inhibition of thiamin diphosphate dependent enzymes by 3-deazathiamin diphosphate, *Org. Biomol. Chem. 2*, 1732-1741.

48. Emmons, G. T., Campbell, I. M., and Bentley, R. (1985) Vitamin K (menaquinone) biosynthesis in bacteria: purification and probable structure of an intermediate prior to o-succinylbenzoate, *Biochem. Biophys. Res. Commun. 131*, 956-960.

49. Taber, H. W., Dellers, E. A., and Lombardo, L. R. (1981) Menaquinone biosynthesis in *Bacillus subtilis*: isolation of men mutants and evidence for clustering of men genes, *J. Bacteriol. 145*, 321-327.

50. Palaniappan, C., Taber, H., and Meganathan, R. (1994) Biosynthesis of o-succinylbenzoic acid in *Bacillus subtilis*: identification of menD mutants and evidence against the involvement of the alpha-ketoglutarate dehydrogenase complex, *J. Bacteriol. 176*, 2648-2653.

51. Jiang, M., Cao, Y., Guo, Z. F., Chen, M., Chen, X., and Guo, Z. (2007) Menaquinone biosynthesis in *Escherichia coli*: identification of 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate as a novel

intermediate and re-evaluation of MenD activity, *Biochemistry 46*, 10979-10989.

52. Dawson, A., Fyfe, P. K., and Hunter, W. N. (2008) Specificity and reactivity in menaquinone biosynthesis: the structure of *Escherichia coli* MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate synthase), *J. Mol. Biol. 384*, 1353-1368.

53. Priyadarshi, A., Saleem, Y., Nam, K. H., Kim, K. S., Park, S. Y., Kim, E. E., and Hwang, K. Y. (2009) Structural insights of the MenD from *Escherichia coli* reveal ThDP affinity, *Biochem. Biophys. Res. Commun. 380*, 797-801.

54. Kolappan, S., Zwahlen, J., Zhou, R., Truglio, J. J., Tonge, P. J., and Kisker, C. (2007) Lysine 190 is the catalytic base in MenF, the menaquinone-specific isochorismate synthase from *Escherichia coli*: implications for an enzyme family, *Biochemistry 46*, 946-953.

55. Kluger, R., Chin, J. and Smyth, T. (1981) Thiamin-catalyzed decarboxylation of pyruvate. Synthesis and reactivity analysis of the central, elusive intermediate, α-lactylthiamin, *J. Am. Chem. Soc. 103*, 884-888.

56. CLSI. (2006) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 7th ed. Approved Standard M7-A7, *Clinical and Laboratory Standards Institute, Wayne, PA.*

57. White, D. C., and Frerman, F. E. (1967) Extraction, characterization, and cellular localization of the lipids of *Staphylococcus aureus*, *J. Bacteriol. 94*, 1854-1867.

58. Geyer, R., Peacock, A. D., White, D. C., Lytle, C., and Van Berkel, G. J. (2004) Atmospheric pressure chemical ionization and atmospheric pressure photoionization for simultaneous mass spectrometric analysis of microbial respiratory ubiquinones and menaquinones, *J. Mass Spectrom. 39*, 922-929.

59. Jiang, M., Chen, X., Guo, Z. F., Cao, Y., Chen, M., and Guo, Z. (2008) Identification and characterization of (1R,6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase in the menaquinone biosynthesis of *Escherichia coli*, *Biochemistry 47*, 3426-3434.

60. Bar-Ilan, A., Balan, V., Tittmann, K., Golbik, R., Vyazmensky, M., Hubner, G., Barak, Z., and Chipman, D. M. (2001) Binding and activation of thiamin diphosphate in acetohydroxyacid synthase, *Biochemistry 40*, 11946-11954.

61. Sergienko, E. A., and Jordan, F. (2002) New model for activation of yeast pyruvate decarboxylase by substrate consistent with the alternating sites mechanism: demonstration of the existence of two active forms of the enzyme, *Biochemistry 41*, 3952-3967.

62. Nahaie, M. R., Goodfellow, M., Minnikin, D. E., and Hajek, V. (1984) Polar lipid and isoprenoid quinone composition in the classification of Staphylococcus, *J. Gen. Microbiol. 130*, 2427-2437.

63. Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., and Barry, C. E., 3rd. (2004) The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism: novel insights into drug mechanisms of action, *J. Biol. Chem. 279*, 40174-40184.

64. Kahl, B. C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R. A., and Peters, G. (2003) Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation, *J. Clin. Microbiol. 41*, 410-413.

65. Vaudaux, P., Francois, P., Bisognano, C., Kelley, W. L., Lew, D. P., Schrenzel, J., Proctor, R. A., McNamara, P. J., Peters, G., and Von Eiff, C. (2002) Increased expression of clumping factor and fibronectin-binding proteins by hemB mutants of *Staphylococcus aureus* expressing small colony variant phenotypes, *Infect. Immun. 70*, 5428-5437.

66. Grundling, A., and Schneewind, O. (2007) Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*, *Proc. Natl. Acad. Sci. U S A 104*, 8478-8483.

67. Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., and Peters, G. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections, *Nat. Rev. Microbiol. 4*, 295-305.

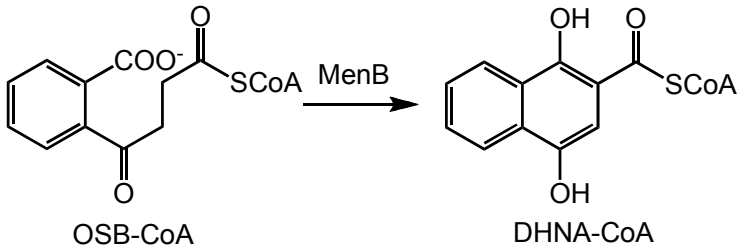
68. Hammond, R. K., and White, D. C. (1970) Inhibition of vitamin K2 and carotenoid synthesis in *Staphylococcus aureus* by diphenylamine, *J. Bacteriol. 103*, 611-615.

69. Salton, M. R., and Schmitt, M. D. (1967) Effects of diphenylamine on carotenoids and menaquinones in bacterial membranes, *Biochim. Biophys. Acta 135*, 196-207.

CHAPTER 4: MECHANISTIC STUDIES OF MENB AND THE CORRELATION TO CLINICAL SMALL COLONY VARIANTS

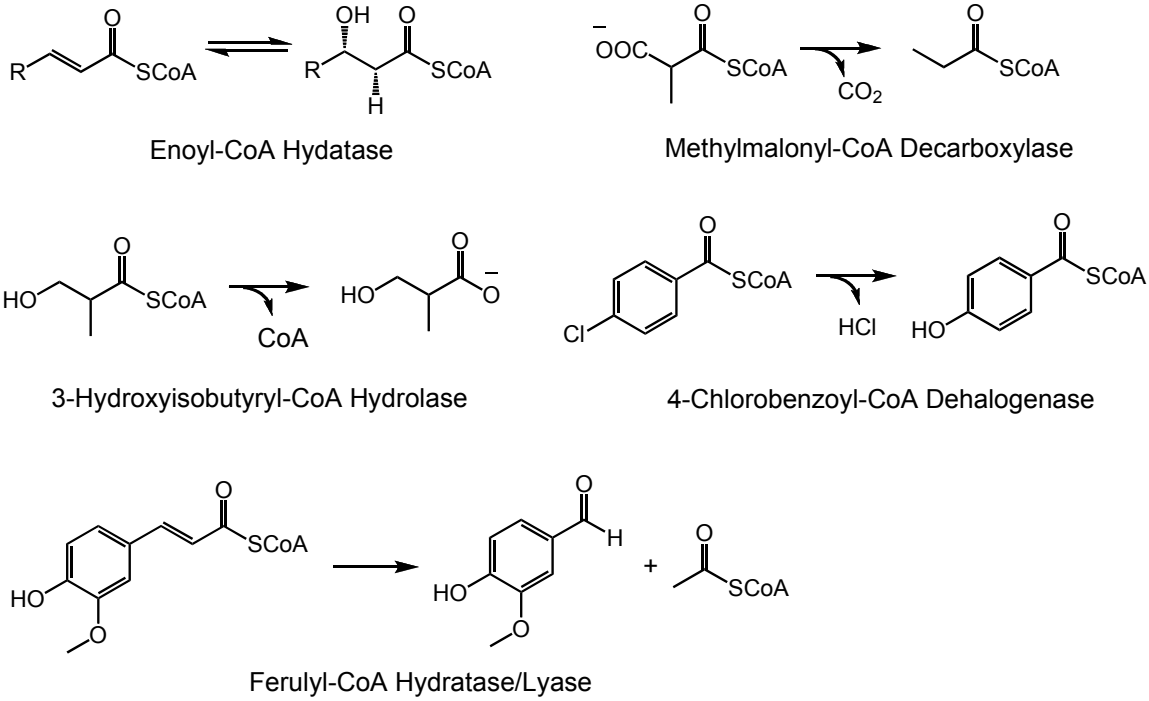
4.1 MENB IN MENAQUINONE BIOSYNTHESIS

MenB, one enzyme involved in menaquinone biosynthesis, catalyzes the production of 1,4,-dihydroxy-2-naphthoyl-CoA (DHNA-CoA) from *O*-succinylbenzoyl-CoA (OSB-CoA) via an intramolecular Claisen/Dieckmann condensation reaction (*1, 2*) (**Scheme 4.1**).



Scheme 4.1: MenB-catalyzed reaction.

MenB belongs to the crotonase superfamily (CS). The enzymes in this superfamily catalyze diverse reactions (*3-7*), including decarboxylation, thioester hydrolysis, dehalogenation, Claisen/Dieckmann condensation, and reverse aldol condensation (**Scheme 4.2**). A common feature of the reaction mechanisms is the stabilization of an enolate intermediate by two backbone NH groups, which is similar to the oxyanion hole in proteases.



Scheme 4.2: Reactions catalyzed by crotonase superfamily enzymes.

MenB reaction is initiated by removing one α-proton to the thioester of OSB-CoA, producing a thioester enolate intermediate, which is stabilized by two backbone NH groups. The enolate then reacts with the C-2 carboxylic acid to form a bicyclic structure with two ketones. Tautormerization of both ketones leads to the final product DHNA-CoA.

The crystal structures of MenB from *M. tuberculosis* (mtbMenB) and *S. aureus* (saMenB) have been reported (*8, 9*). mtbMenB folds as a dimer of trimers, and the C-terminus crosses the trimer-trimer interface, which is unusual in the crotonase superfamily. saMenB structure is quite similar to mtbMenB, except that it folds as a dimer.

Recently, three mutations in *menB* gene were identified in the small colony variant *S. aureus*, a clinical strain that is responsible for persistent infections (*10*). The authors claimed that these mutations were the genetic basis for SCV phenotype.

As menaquinone is essential for the growth of *S. aureus*, and *menB* mutations are observed in SCV strain, it is worthwhile to study the enzymatic properties of MenB, and the impact of mutations on the enzyme activity.

4.2 MATERIALS AND METHODS

Materials.

His-bind Ni^{2+}-NTA resin was from Invitrogen, and centriplus units from Millipore. QuikChange site-directed mutagenesis kit was obtained from Stratagene. Restriction enzymes were purchased from NEB. All other chemicals were from Sigma.

Cloning, expression and purification of MenB from Staphylococcus aureus.

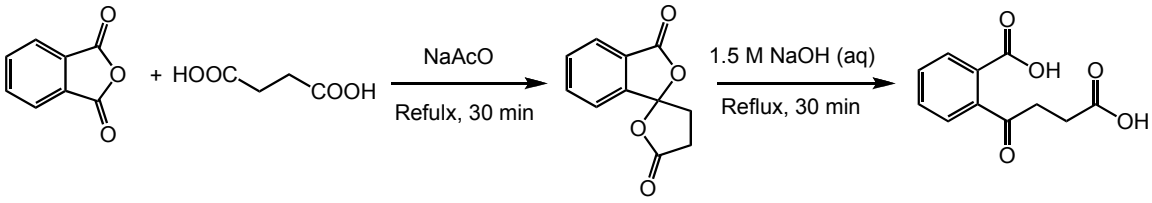
The *menB* gene was amplified from the genomic DNA of *S. aureus* strain NCTC8325 by PCR with the primers listed in **Table 4.1**. The PCR fragment was digested with resctriction enzymes NdeI and BamHI simultaneously. Then the *menB* gene was ligated into pET15b, which was also treated with the same restriction enzymes, by T4 DNA ligase. The plasmid sequenced was confirmed by DNA sequencing, and then transformed into BL21(DE3) cells.

A single colony was used to inoculate 10 ml of LB medium containing 200 μg/ml ampicillin (LB/Amp) and the culture was grown at 37 ºC overnight. The cells in the starter culture were collected by centrifugation, resuspended in fresh LB media, transferred to 500 ml LB/Amp medium, and grown at 37 ºC until an optical density of 0.8 at 600 nm had been reached. Subsequently, 0.5 mM IPTG was added to induce the expression of saMenB overnight at 25 ºC.

The cells were harvested, and resuspended in 30 ml binding buffer (50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole, pH 8.0), and disrupted by three passages through a French press cell (1000 psi). Then the cell lysate was centrifuged at 120,000 x g for 1 h, and the supernatant was loaded onto a 5 ml Ni^{2+}-NTA His-bind column, which had been preincubated with 20 ml binding buffer. The His-bind column was washed with 40 ml binding buffer, followed by 30 ml of wash buffer (50 mM sodium phosphate, 300 mM NaCl, and 20 mM imidazole, pH 8.0), and the enzyme was then eluted using 30 ml of elute buffer (50 mM sodium phosphate, 300 mM NaCl, and 250 mM imidazole, pH 8.0). SDS-PAGE was used to identify those fractions containing saMenB, and these fractions were combined and immediately exchanged into 50 mM Tris-HCl buffer containing 50 mM NaCl, 20% glycerol (pH 8.5). The enzyme was concentrated using Centriplus 10, and stored in -80 °C. The enzyme concentration was determined by the absorbance at 280 nm with an extinction coefficient of 31150 $M^{-1}cm^{-1}$.

Synthesis of O-succinylbenzoic acid.

Briefly, 40.0 g of succinic acid (0.34 mol) was heated to about 200 °C until it melted. Then 30.0 g of phthalic anhydride (0.20 mol) and 16.4 g of sodium acetate (0.20 mol) were added. After refluxing at 200-220 °C for 30 min, the reaction mixture was extracted with 100 ml of boiling water four times. The crude spirodilactone precipitated after cooling to RT, and was further purified with recrystalization from methanol. Subsequently, 10.3 g (0.05 mol) of spirodilactone was added to 500 ml of 1.5 M NaOH solution, and refluxed for 30 min. The reaction mixture was cooled to RT, adjusted to pH 1-2 with HCl (aq), and then extracted with 100 ml of ethyl acetate three times. OSB was obtained after removing the organic solvent by rotary evaporation. The overall yield was 20%. The product was analyzed with ^{1}H NMR and mass spectrometry. ^{1}H NMR: δ 7.85-7.62 (4H, m), 2.61 (2H, t), 2.49 (2H, t). ESI MS: calcd for $C_{11}H_9O_5$ ($[M-H]^-$) 221.05, found 221.1.

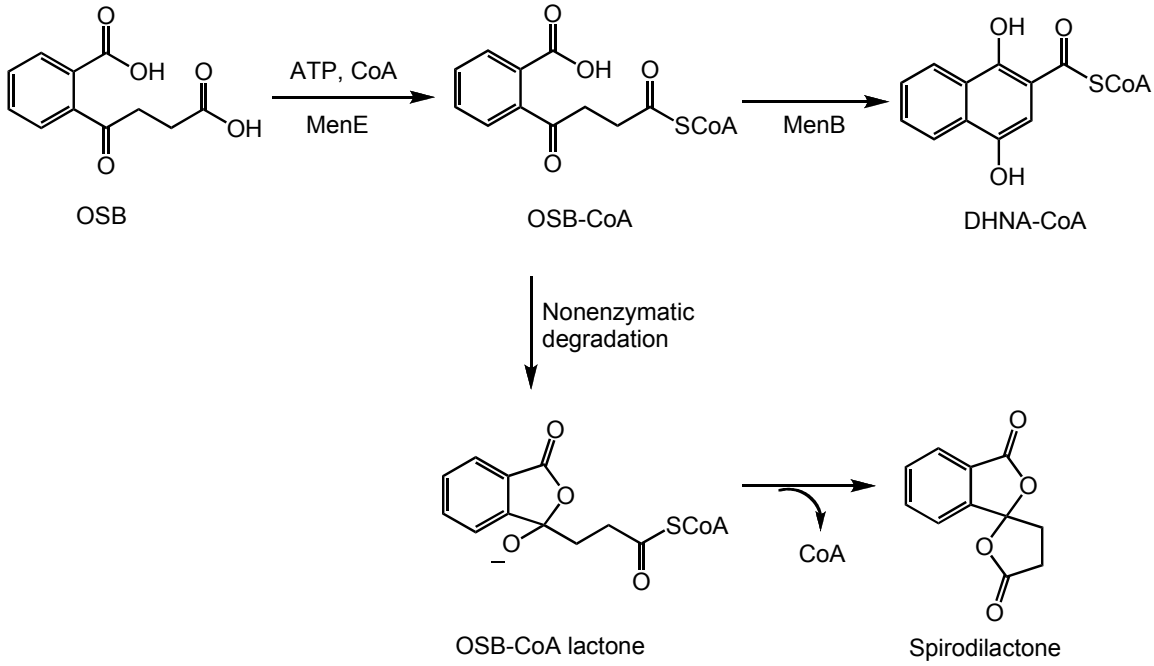


Scheme 4.3: Synthesis of *O*-succinylbenzoic acid.

Kinetic assay of the MenB reaction.

The MenE-coupled assay was used to characterize the MenB reaction, since OSB-CoA, the substrate of MenB, is unstable, as shown in **Scheme 4.4** (*2*). A

typical reaction mixture contained 2 μM saMenB, 5 μM ecMenE or saMenE, 600 μM ATP, 600 μM CoA, and various concentrations of OSB. The reaction was performed in 20 mM Tris-HCl buffer (pH 7.0) with 150 mM NaCl and 1 mM $MgCl_2$. After initiating the reaction with the addition of saMenB, the absorbance increase at 392 nm was monitored with a Cary-300 spectrometer. The reaction rates were calculated using an extinction coefficient of 4000 $M^{-1}cm^{-1}$ of DHNA-CoA.



Scheme 4.4: MenE-coupled assay and degradation of OSB-CoA.

Table 4.1 Primer sequences

Name	Nucleotide Sequence [a]
MenB_Forward	5'-GGAATTC*CATATG*ATGACTAACAGACAATGGGAAAC-3'
MenB_Revserse	5'-CG*GGATCC*TTATGGGAATTTAGGGAATTGA-3'
G233V_Forward	5'-CACAGATGGTTTAGCTGTTTTACAACAAATGGCTG-3'
G233V_Revserse	5'-CAGCCATTTGTTGTAAAACAGCTAAACCATCTGTG-3'
Trunc_Forward	5'-GAATGCTGACACAGA(T)GGTTTAGCTGGTTTAC-3'
Trunc_Reverse	5'-GTAAACCAGCTAAACC(A)TCTGTGTCAGCATTC-3'

[a] Restriction sites are italicized, while mutating sites are indicated in underline, and deleted nucleotides were in parenthesis.

Circular dichroism analysis.

Far UV CD spectra of saMenB and its mutants were collected at 25 °C using AVIV 62 DS spectrometer equipped with a Peltier temperature control unit. The enzyme concentration was 40 μM.

Site-directed mutagenesis, expression and purification of saMenB.

Site-directed mutagenesis was performed using the QuikChange mutagenesis kit from Stratagene using the primers in **Table 4.1**. The procedure of expression and purification of saMenB mutants was the same as that of the wild type saMenB.

Kinetic data analysis.

The kinetic studies employed a substrate concentration range of 0.3 K_m to 4 K_m. Kinetic parameters were calculated by fitting the data to the Michaelis-Menten equation using GraFit 4.0.

$v = V_{max}$ [S]/ (K_m + [S])

k_{cat} values were calculated by the equation $k_{cat} = V_{max}$ / [E].

Inhibition studies.

The assay mixture contained 2 μM saMenB, 5 μM saMenE, 600 μM ATP, 600 μM CoA, 60 μM OSB, and various concentrations of inhibitor. The reaction rates at various inhibitor concentrations were recorded and fitted into the following reaction to calculate IC_{50}.

$v_i/v_0 = 1/ (1 + [I]/ IC_{50})$

MIC measurement.

The MIC values were determined by the microbroth dilution assay according to the Clinical and Laboratory Standards Institute methods for antimicrobial susceptibility tests for aerobically growing bacteria (*11*).

Time-kill assay.

The assay was performed according to the protocol of NCCLS (*12*). Briefly, 10^5 CFU *S. aureus* was inoculated and treated with MIC, 2*MIC and 4*MIC

concentration of compound **4.4**. Samples were taken at 0, 4, 8, 24 h, and plated on blood agar plates with a series of dilutions. Colonies on the agar plates were counted after 24 hours incubation. CFU was calculated based on the colony number and dilution fold.

4.3 RESULTS AND DISCUSSION

Kinetic parameters of saMenB substrates.

The kinetic parameters were measured with the assays coupled with ecMenE or saMenE. An excess of MenE is used to produce OSB-CoA *in situ*, and a 5-minute preincubation allows the complete conversion from OSB to OSB-CoA. In **Figure 4.1**, a peak with maximum absorbance at 392 nm was observed after the addition of saMenB, corresponding to the formation of DHNA-CoA. The kinetic assays were monitored continuously at 392 nm, and the kinetic parameters were calculated. As shown in **Table 4.2**, the K_m value determined from saMenE-coupled assay is 3-fold smaller than that from the ecMenE-coupled assay, indicative of favorable interactions between saMenE and saMenB. In addition, it is notable that saMenB possesses the smallest k_{cat}/K_m compared with ecMenB and mtbMenB.

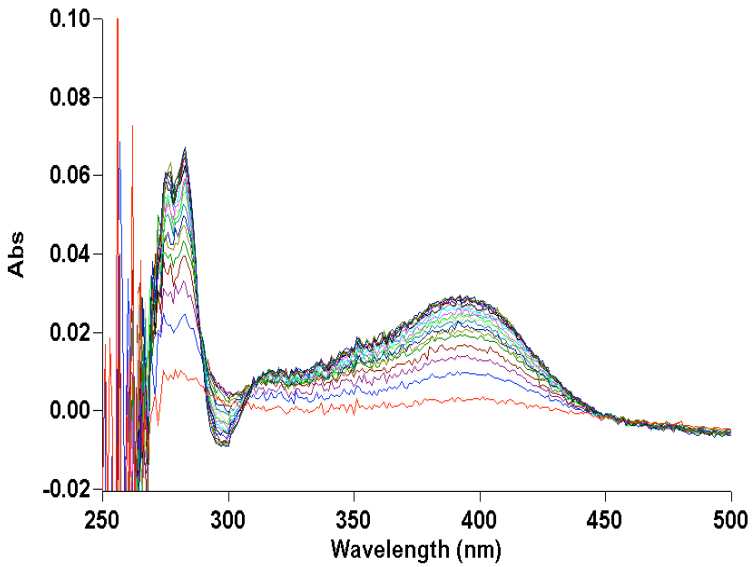


Figure 4.1: Formation of DHNA CoA by saMenB. The absorbance spectra of saMenB reaction mixture between 250 and 500 nm were collected every minute by Cary 300 spectrometer.

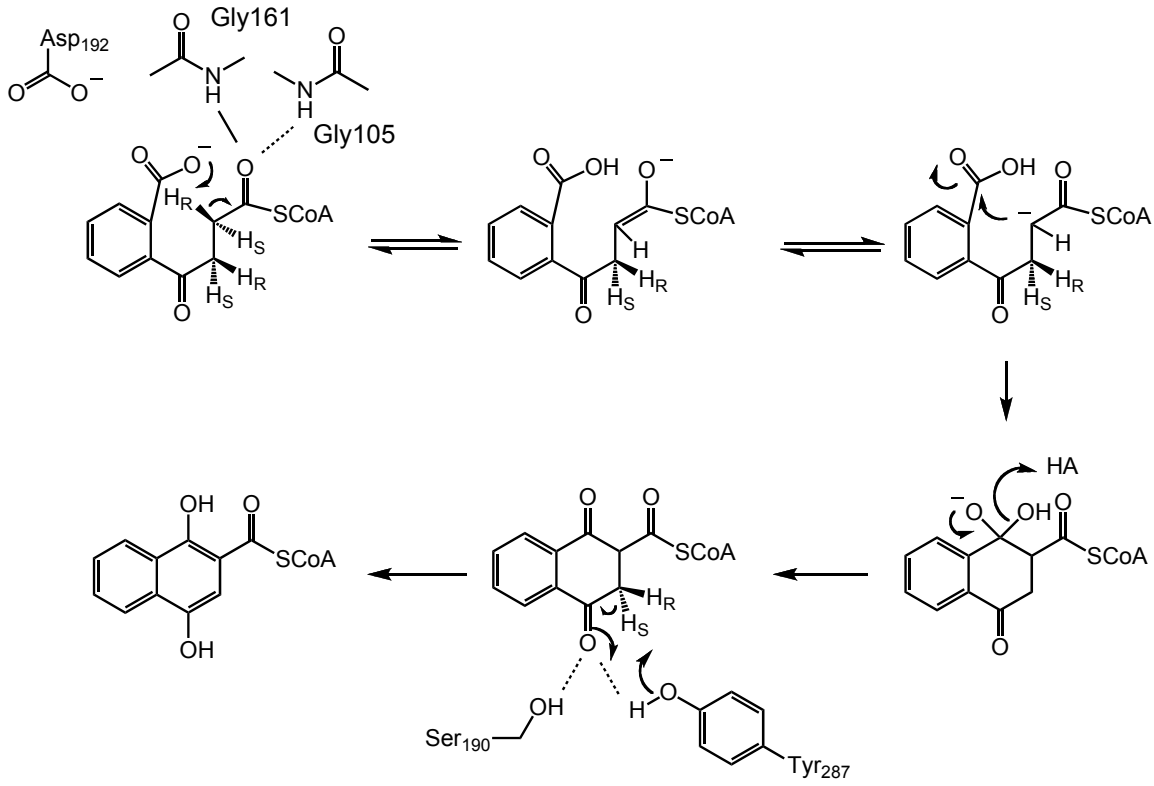
Table 4.2: Kinetic parameters of MenBs from different organisms.

Enzyme	Assay	k_{cat} (min^{-1})	$K_{m,OSB\text{-}CoA}$ (μM)	k_{cat} / $K_{m,OSB\text{-}CoA}$ ($min^{-1}μM^{-1}$)
saMenB	ecMenE-coupled assay	1.0 ± 0.1	205.3 ± 24.9	0.005 ± 0.001
	saMenE-coupled assay	1.1 ± 0.1	63.3 ± 6.0	0.018 ± 0.003
ecMenB[a]	ecMenE-coupled assay	3.7 ± 0.1	25.9 ± 3.3	0.14 ± 0.03
mtbMenB[a]	ecMenE-coupled assay	27.7 ± 0.9	22.4 ± 2.1	1.2 ± 0.2

[a] Unpublished data by Dr. Huaning Zhang.

The reaction mechanism of MenB from *M. tuberculosis* was proposed previously (*8*). As shown in **Scheme 4.5,** initially the benzoic carboxylate

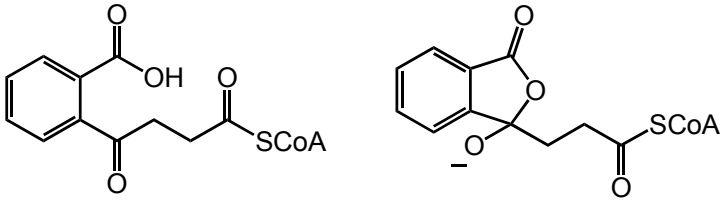
abstracts the pro-2R succinyl proton, forming an enolate that is stabilized by the back amide NH from Gly105 and Gly161. Subsequently the enolate attacks the benzoic carboxyl group, producing a six-member ring. The resulting intermediate loses a water molecule to generate the keto form of DHNA-CoA, which then enolizes to give the final product. In addition, since the pro-3R succinyl proton is stereospecifically retained in the product, it was hypothesized that the keto-enol tautomerization is also catalyzed by the enzyme and involves Tyr-287.



Scheme 4.5: A proposed reaction mechanism for mtbMenB reaction.

Recently, a different reaction mechanism was postulated by Dr. Huaning Zhang from our group (unpublished work). In this mechanism, the spirodilactone form of OSB-CoA (**Scheme 4.6**) is proposed to be the actual substrate of

mtbMenB, based on the kinetic data that the K_m value with preincubation is about 20-fold smaller than that without preincubation. A unique D185 is proposed to facilitate the abstraction of the pro-*2R* proton from the spirodilactone OSB-CoA.



OSB-CoA OSB-CoA lactone

Scheme 4.6: Structures of OSB-CoA and its spirodilactone form.

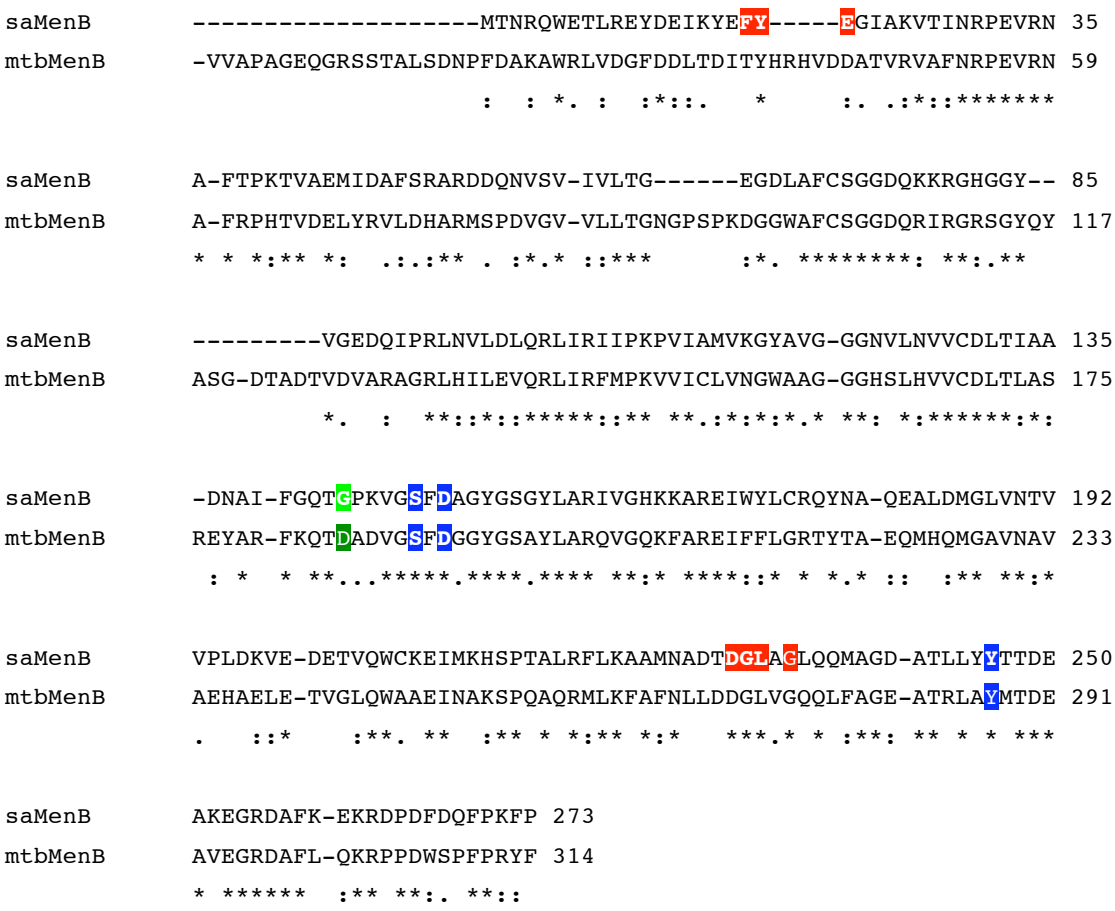
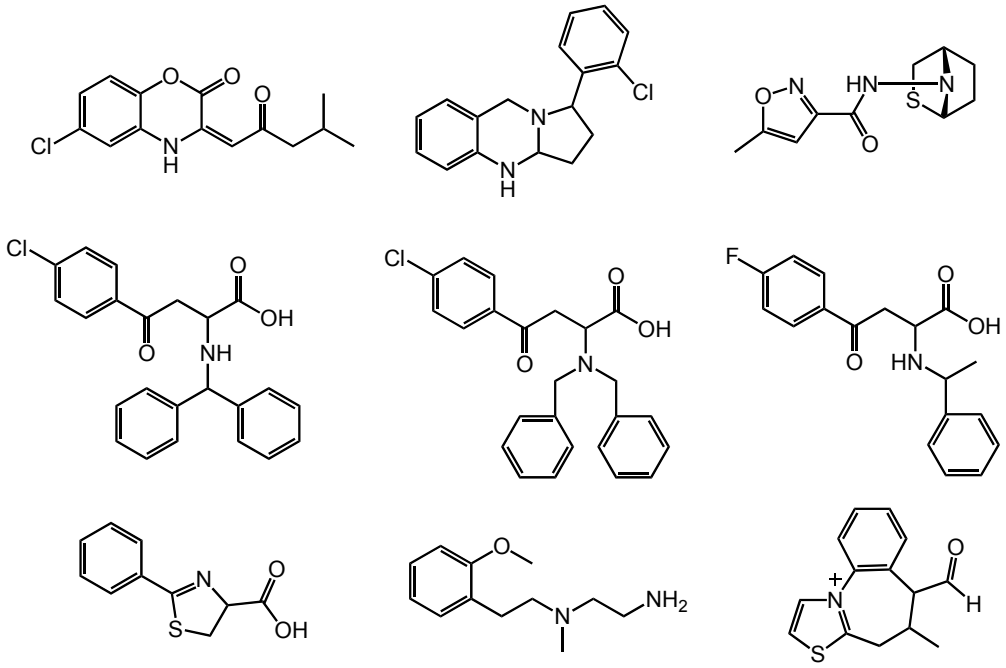


Figure 4.2: Sequence alignment between saMenB and mtbMenB. The conserved catalytic residues are colored blue, while D185 in mtbMenB and G144 in saMenB are colored dark and light green, respectively. The red residues are the mutating sites identified in clinical *S. aureus* SCV isolates.

However, for saMenB, the K_m values of OSB-CoA determined with or without preincubation were not significantly different. In addition, the corresponding residue of D185 in saMenB is a glycine, which is not able to abstract the pro-*2R* proton. Hence, current data suggests that the open form of OSB-CoA rather than the spirodilactone form is the substrate of saMenB.

Antimicrobial activity of OSB analogues.

Compounds exhibiting strong inhibition against mtbMenB were identified from high-throughput screening at the ICCB-Longwood Screening facility at Harvard Medical School. Many of the lead compounds are substrate analogues, which have an OSB core structure, as seen in the second row in **Scheme 4.7**. Accordingly, the OSB scaffold was selected for further inhibitor optimization. The analogues were synthesized as described in Materials and Methods.



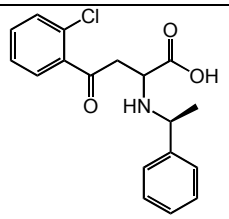
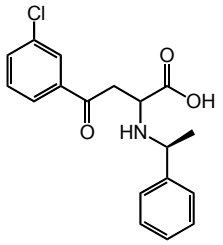
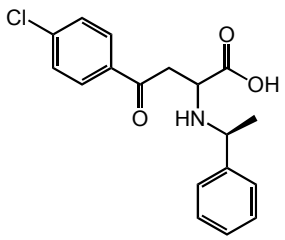
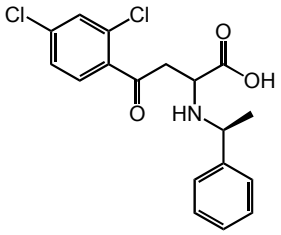
Scheme 4.7: MenB inhibitors identified from HTS.

As shown in **Table 4.3**, the compound with a chlorine in the *ortho* position (compound **4.1**) shows a 5 or 12-fold stronger inhibition than the analogue with a *meta* (compound **4.2**) or *para* chlorine (compound **4.3**), respectively. The inhibitory trend is *ortho* > *para* > *meta*. The compound with chloro substitutions in

both *ortho* and *para* positions (compound **4.4**) showed the strongest *in vitro* activity against *S. aureus*.

In order to investigate the mode of action of these compounds, killing kinetic analysis was performed. As shown in **Figure 4.3**, with MIC or 2*MIC concentrations of the inhibitor **4.4**, the CFU dropped less than 1 log unit, while the CFU slightly decreased in the first 8 hours, and then started to increase with MIC. The killing curve suggests that the inhibitor is bacteriostatic, and the late recovery probably results from the acquired resistance or the degradation of the compound. Stability test of compound **4.4** confirms that it is not stable under basic condition (pH 7.5), and the half-life of the compound is about 12 hours, consistent with the result from the time-kill assay. A possible degradation mechanism is shown in **Scheme 4.8**.

Table 4.3: Inhibition of OSB analogues.

Compound	Structure	IC_{50} (μM)	MIC (μg/ml)
4.1		24.5 ± 2.3	32
4.2		299.4 ± 10.8	32
4.3		125.1 ± 13.4	64
4.4		Not determined	16

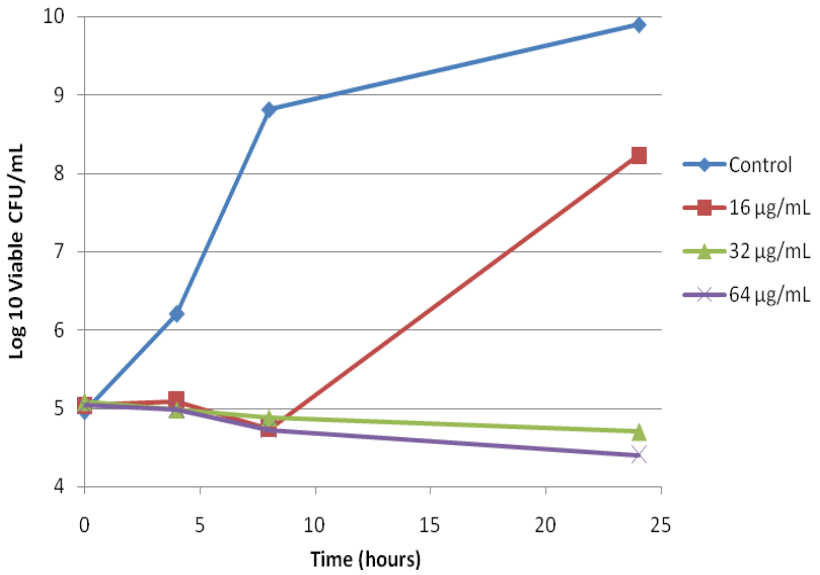
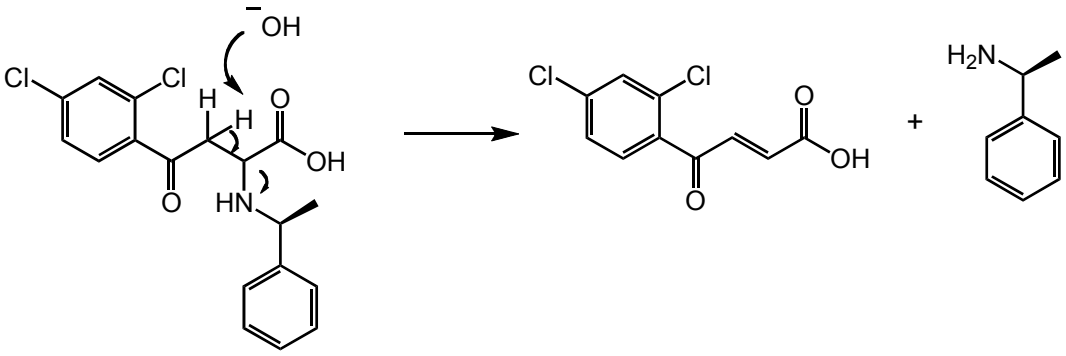
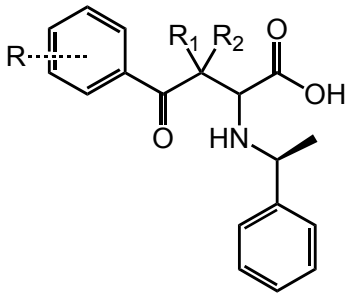


Figure 4.3: Time-kill curve of OSB analogue 4.4 against *S. aureus*.



Scheme 4.8: Possible degradation of OSB analogues.

In order to improve the stability, OSB analogues with no hydrogen on the carbon adjacent to the carbonyl group are being synthesized by Xiaokai Li in our group (**Scheme 4.9**). Since there is no hydrogen atom susceptible to OH^-, the degradation would be blocked.



Scheme 4.9: Compounds with improved stability. $R_1 = R_2 = F$.

Correlation of menB mutations with SCV phenotype in S. aureus.

Small colony variants of *S. aureus* show phenotypes of slow-growth, decreased production of pigments, and small colony size on the agar plate. They cause recurrent infections and drug resistance (*13-15*). The genetic basis of these clinical strains is difficult to explore, since they could easily revert back to the normal phenotype. However, based on the studies of the genetically defined SCV strains, it is believed that defects in electron transport leads to the SCV phenotype (*16-18*).

Recently three mutations were identified in the *menB* gene from clinical SCV *S. aureus* isolates *(10)*: (i) a 9-bp deletion from nucleotides 55 to 63; (ii) a frame shift mutation that resulted in a premature stop codon at position 230; and (iii) a point mutation that caused the amino acid substitution Gly to Val at codon 233.

Two of the three clinically identified saMenB mutants were constructed and purified (**Figure 4.4**). CD spectra indicate that a major conformational change occurs for the truncated saMenB (**Figure 4.5**), and no enzymatic activity was observed. It is not surprising since the C-terminal region was proposed to be essential both for completion of the binding pocket and for stabilization of the

hexamer in mtbMenB (*8, 19*). In addition, the conserved catalytic residue Y246, which facilitates the keto-enol tautomerization, is in the C-terminus. Hence, the removal of the C-terminal region would abolish the activity. However, it is unexpected that G233V mutant is also inactive, since little structural perturbation was observed from CD spectra (**Figure 4.5**). According to the crystal structure of saMenB solved by Hunter et al (*9*), G233 residues lie in the interface of the two monomers (**Figure 4.6**). Native-PAGE analysis showed no difference of the oligomerization states between wild type and G233V saMenB, ruling out the possibility that the inactivation results from the dissociation of the saMenB dimer due to the bulky side chain of Val in the dimer interface. We hypothesize that residue G233 might regulate the active site remotely, and the G233V mutation has an adverse effect on the geometry of the active site. Similarly, G274V mutation in mtbMenB, where G274 corresponds to G233 in saMenB, causes loss of activity.

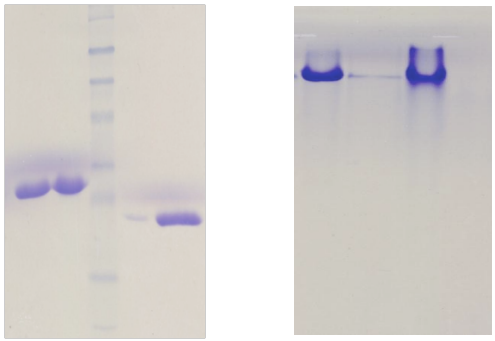


Figure 4.4: SDS-PAGE (left) and Native-PAGE analysis (right) of wild type saMenB and its mutants. Samples in SDS-PAGE from left to right: WT saMenB, G233V, protein marker, and truncated saMenB. Samples in Native-PAGE from left to right: WT saMenB and G233V.

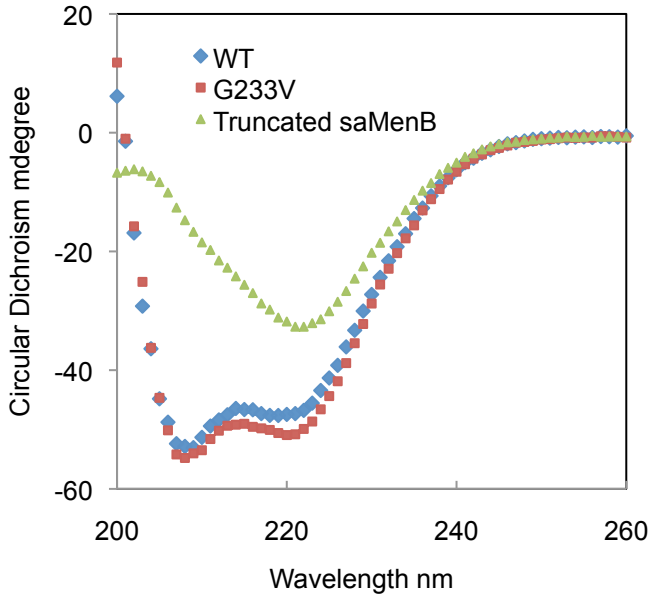


Figure 4.5: Conformational analysis of wild type and mutant saMenB by circular dichroism. The spectra were collected with 40 μM enzymes at 25 °C.

The third *menB* mutation, which is not characterized here, is the deletion of the three residues 19-21. The mutation site is in the C-terminus of a β-strand (**Figure 4.6**), which is also far away from the active site. It might affect the enzyme activity in a similar way to G233V.

The fact that the saMenB mutants are inactive indicates that the menaquinone biosynthetic pathway is blocked in the small colony variant. The loss of internal source of menaquinone might slow down the metabolism of the bacteria. But due to the substrate-level phosphorylation as well as possible scavenge of quinone molecules from the host environment, the bacteria can still sustain its growth.

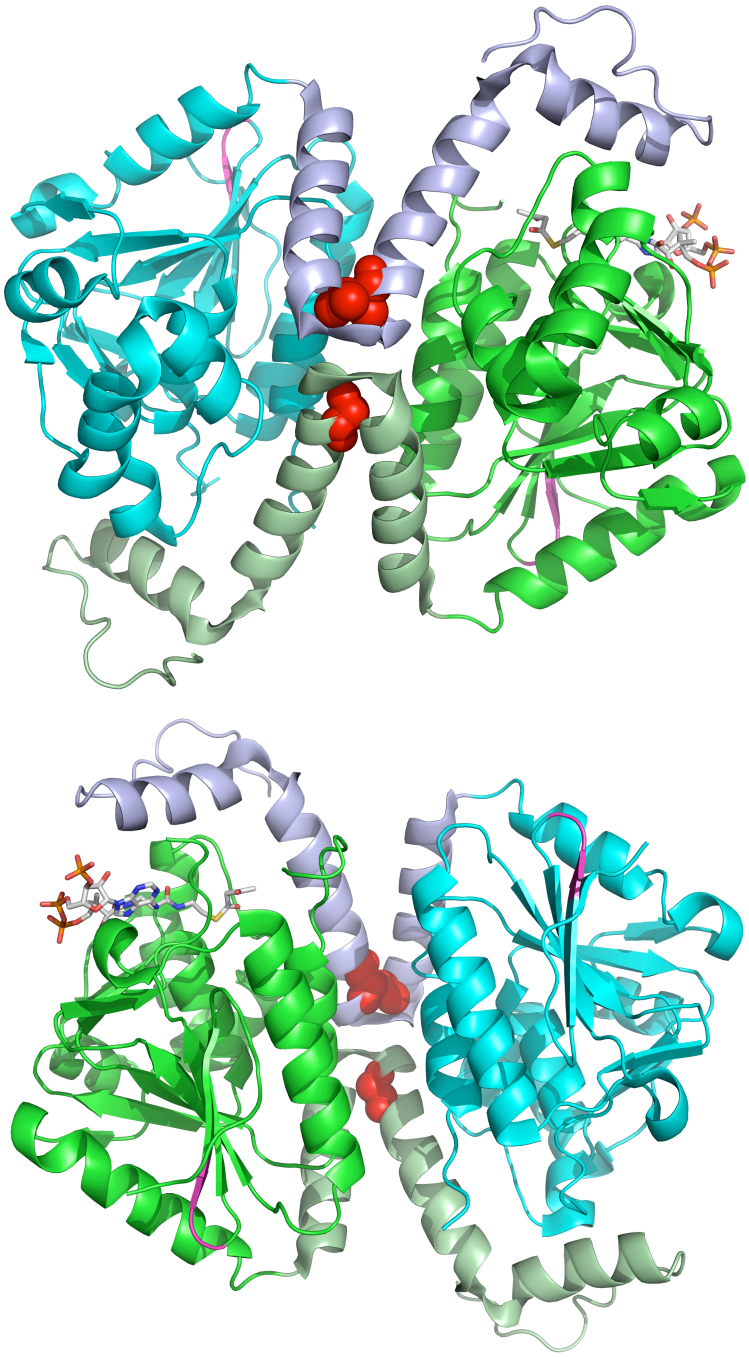
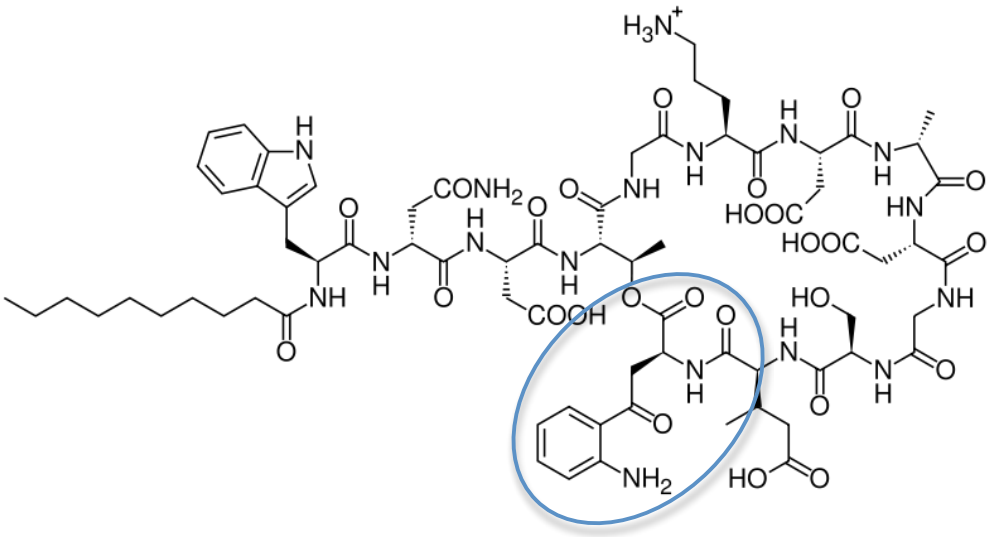


Figure 4.6: Front (top) and back view (bottom) of the crystal structure of saMenB. The picture was generated with PyMol (*20*). Truncated C-terminuses of two chains are shown in light blue and dark green, respectively. G233 is shown in red. Residues 19-21 are in pink. The ligand AcAcCoA is shown in stick.

Daptomycin targets MenB?

Daptomycin is a cyclic 13-residue lipopeptide antibiotic (**Scheme 4.10**), which is synthesized by the nonribosomal peptide synthetase machinery in the soil saprotroph *Streptomyces roseosporus*. It was approved to treat complicated skin and skin structure infections in the United States in 2003 after minimizing the adverse effects on skeletal muscle by one single dose daily (*21*). More importantly, it shows good inhibition against MRSA infections.



Scheme 4.10: Structure of daptomycin.

Daptomycin is active against Gram-positive pathogens, but not on Gram-negative bacteria. It has been suggested that the action of daptomycin is initiated by insertion into the Gram-positive bacterial cytoplasmic membrane (*22*). Once inserted into the membrane, daptomycin undergoes a calcium-dependent

conformational change, which leads to dramatic perturbation of the cell membrane, including depolarization and membrane permeability (*23*).

As shown in **Scheme 4.10**, daptomycin contains a moiety analogous to OSB (circled), an intermediate in menaquinone biosynthesis. It is possible that daptomycin also targets the menaquinone biosynthesis, such as MenE and MenB. This also explains why it is only effective against Gram-positive bacteria, since Gram-negative bacteria either does not produce menaquinone, or they have both menaquinone and ubiquinone.

Daptomycin-resistant *S. aureus* obtained from subculture in a sublethal concentration of daptomycin possess abnormal cell shapes (*24*) under TEM, similar to the observation in *menD*-disrupted strain, in which the menaquinone biosynthesis is blocked (**Figure 4.7**). *In vitro* inhibition assay are needed to further substantiate the hypothesis that menaquinone is a target of daptomycin.

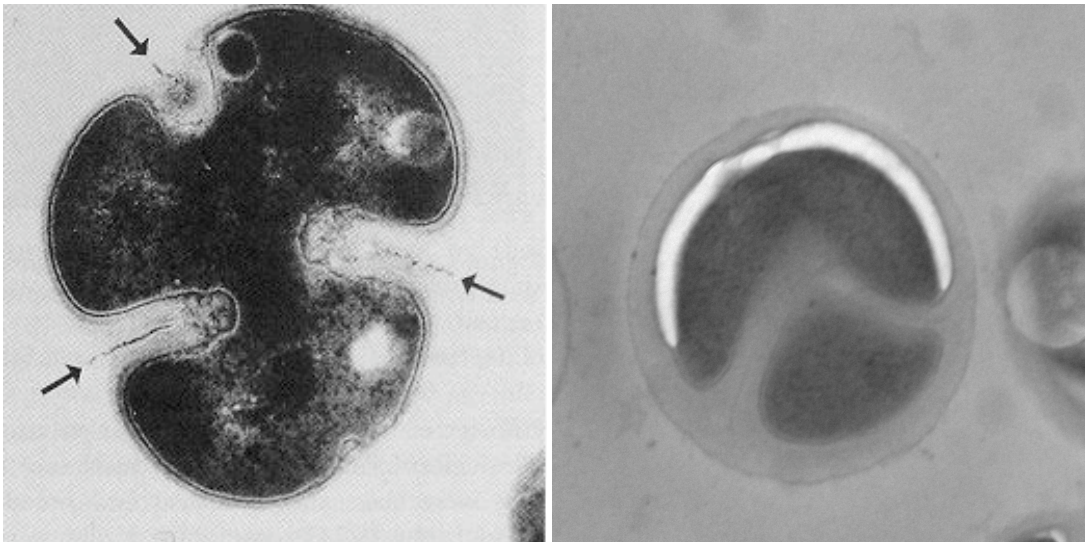


Figure 4.7: Transmission electron microscope images of daptomycin-resistant *S. aureus* (left) and *menD*-disrupted *S. aureus* (right).

4.4 CONCLUSIONS

The enzymatic studies of saMenB support the use of OSB-CoA as the substrate instead of the proposed spirodilactone form for mtbMenB. In addition, there is probably a favorable interaction between saMenB and saMenE.

In the preliminary inhibition studies, a series of OSB analogues were designed and showed fairly good inhibition against the enzyme and *S. aureus*. The major conern is the stability of these compounds, since they degrades when pH is higher than 7.5. Further modifications are being made to solve this problem.

The effects of two of the three *menB* mutations identified in clinical SCV *S. aureus* strains on the saMenB activity were examined. Both saMenB mutants are inactive, indicating that menaquinone biosynthesis is blocked in the clinical SCV isolates. The result implies that clinical persistence and slow growth of SCV is due to the acquisition of menaquinone from host.

It is interesting to find that daptomycin, a novel class of antibiotic, includes an OSB-like moiety. Daptomycin-resistant strains and SCV strains of S*. aureus* both show similar abnormal morphological changes, indicating that menaquinone pathway may be inhibited by daptomycin. It is also consistent with the fact that daptomycin only inhibits Gram-positive bacteria. Further detailed inhibition assays are needed to investigate the hypothesis.

References

1. Bryant, R. W., Jr., and Bentley, R. (1976) Menaquinone biosynthesis: conversion of o-succinylbenzoic acid to 1,4-dihydroxy-2-naphthoic acid and menaquinones by *Escherichia coli* extracts, *Biochemistry 15*, 4792-4796.
2. Heide, L., Arendt, S., and Leistner, E. (1982) Enzymatic synthesis, characterization, and metabolism of the coenzyme A ester of o-succinylbenzoic acid, an intermediate in menaquinone (vitamin K2) biosynthesis, *J. Biol. Chem. 257*, 7396-7400.
3. Bahnson, B. J., Anderson, V. E., and Petsko, G. A. (2002) Structural mechanism of enoyl-CoA hydratase: three atoms from a single water are added in either an E1cb stepwise or concerted fashion, *Biochemistry 41*, 2621-2629.
4. Benning, M. M., Haller, T., Gerlt, J. A., and Holden, H. M. (2000) New reactions in the crotonase superfamily: structure of methylmalonyl CoA decarboxylase from *Escherichia coli*, *Biochemistry 39*, 4630-4639.
5. Wong, B. J., and Gerlt, J. A. (2003) Divergent function in the crotonase superfamily: an anhydride intermediate in the reaction catalyzed by 3-hydroxyisobutyryl-CoA hydrolase, *J. Am. Chem. Soc. 125*, 12076-12077.
6. Benning, M. M., Taylor, K. L., Liu, R. Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D., and Holden, H. M. (1996) Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 A resolution:

an enzyme catalyst generated via adaptive mutation, *Biochemistry 35*, 8103-8109.

7. Gasson, M. J., Kitamura, Y., McLauchlan, W. R., Narbad, A., Parr, A. J., Parsons, E. L., Payne, J., Rhodes, M. J., and Walton, N. J. (1998) Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester, *J. Biol. Chem. 273*, 4163-4170.

8. Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J., and Kisker, C. (2003) Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in vitamin K2 biosynthesis, *J. Biol. Chem. 278*, 42352-42360.

9. Ulaganathan, V., Agacan, M. F., Buetow, L., Tulloch, L. B., and Hunter, W. N. (2007) Structure of *Staphylococcus aureus* 1,4-dihydroxy-2-naphthoyl-CoA synthase (MenB) in complex with acetoacetyl-CoA, *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 63*, 908-913.

10. Lannergard, J., von Eiff, C., Sander, G., Cordes, T., Seggewiss, J., Peters, G., Proctor, R. A., Becker, K., and Hughes, D. (2008) Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 52*, 4017-4022.

11. CLSI. (2006) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 7th ed. Approved Standard M7-A7, *Clinical and Laboratory Standards Institute, Wayne, PA.*

12. NCCLS. (1999) Methods for determining bactericidal activity of antimicrobial agents. Approved guideline M26-A., *NCCLS, Wayne, PA.*

13. Brouillette, E., Martinez, A., Boyll, B. J., Allen, N. E., and Malouin, F. (2004) Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo, *FEMS Immunol. Med. Microbiol. 41*, 35-41.

14. Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N., and Arbeit, R. D. (1995) Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*, *Clin. Infect. Dis. 20*, 95-102.

15. Samuelsen, O., Haukland, H. H., Kahl, B. C., von Eiff, C., Proctor, R. A., Ulvatne, H., Sandvik, K., and Vorland, L. H. (2005) *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B, *J. Antimicrob. Chemother. 56*, 1126-1129.

16. von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G., and Gotz, F. (1997) A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly, *J. Bacteriol. 179*, 4706-4712.

17. Clements, M. O., Watson, S. P., Poole, R. K., and Foster, S. J. (1999) CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis, *J. Bacteriol. 181*, 501-507.

18. Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S., and Proctor, R. A. (2003) *Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney, *J. Infect. Dis. 187*, 1654-1661.

19. Johnston, J. M., Arcus, V. L., and Baker, E. N. (2005) Structure of naphthoate synthase (MenB) from *Mycobacterium tuberculosis* in both native and product-bound forms, *Acta Crystallogr. D Biol. Crystallogr. 61*, 1199-1206.

20. Delano, W. L. (2002) in *Pymol*.

21. Oleson, F. B., Jr., Berman, C. L., Kirkpatrick, J. B., Regan, K. S., Lai, J. J., and Tally, F. P. (2000) Once-daily dosing in dogs optimizes daptomycin safety, *Antimicrob. Agents Chemother. 44*, 2948-2953.

22. Silverman, J. A., Perlmutter, N. G., and Shapiro, H. M. (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 47*, 2538-2544.

23. Jung, D., Rozek, A., Okon, M., and Hancock, R. E. (2004) Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin, *Chem. Biol. 11*, 949-957.

24. Conrad, R. S., Howard, M. J., Garrison, R. C., Winters, S., and Henderson, D. A. (1998) The effects of daptomycin on chemical composition and morphology of *Staphylococcus aureus*, *Proceedings of the Oklahoma Academy of Science 78*, 15-21.

Full List of References

Chapter 1:

1. Ogston, A. (1984) Classics in infectious diseases. "On abscesses". (1844-1929), *Rev. Infect. Dis. 6*, 122-128.
2. Peacock, S. J., de Silva, I., and Lowy, F. D. (2001) What determines nasal carriage of *Staphylococcus aureus*?, *Trends Microbiol. 9*, 605-610.
3. Bradley, S. F. (1992) Methicillin-resistant *Staphylococcus aureus* infection, *Clin. Geriatr. Med. 8*, 853-868.
4. Barbar, M. (1961) Methicillin-resistant staphylococci, *J. Clin. Pathol. 14*, 385-393.
5. Panlilio, A. L., Culver, D. H., Gaynes, R. P., Banerjee, S., Henderson, T. S., Tolson, J. S., and Martone, W. J. (1992) Methicillin-resistant *Staphylococcus aureus* in U.S. hospitals, 1975-1991, *Infect. Control Hosp. Epidemiol. 13*, 582-586.
6. Gaynes, R., and Culver, D. (1997) Nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) in the United States, 1975-1996. National Nosocomial Infection Surveillance (NNIS) System., in *Annual Meeting of the Infectious Desease Society of America*, IDSA, San Francisco.
7. (2002) Vancomycin resistant *Staphylococcus aureus*-Pennsylvania, 2002, *JAMA*, 2116.
8. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with

reduced vancomycin susceptibility, *J. Antimicrob. Chemother. 40*, 135-136.

9. Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Fridkin, S. K. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States, *JAMA 298*, 1763-1771.

10. de Haas, C. J., Veldkamp, K. E., Peschel, A., Weerkamp, F., Van Wamel, W. J., Heezius, E. C., Poppelier, M. J., Van Kessel, K. P., and van Strijp, J. A. (2004) Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent, *J. Exp. Med. 199*, 687-695.

11. Chavakis, T., Hussain, M., Kanse, S. M., Peters, G., Bretzel, R. G., Flock, J. I., Herrmann, M., and Preissner, K. T. (2002) *Staphylococcus aureus* extracellular adherence protein serves as anti-inflammatory factor by inhibiting the recruitment of host leukocytes, *Nat. Med. 8*, 687-693.

12. Gravet, A., Colin, D. A., Keller, D., Girardot, R., Monteil, H., and Prevost, G. (1998) Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family, *FEBS Lett. 436*, 202-208.

13. Foster, T. J. (2005) Immune evasion by staphylococci, *Nat. Rev. Microbiol. 3*, 948-958.

14. Thakker, M., Park, J. S., Carey, V., and Lee, J. C. (1998) *Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and

enhances bacterial virulence in a murine bacteremia model, *Infect. Immun. 66*, 5183-5189.

15. Rooijakkers, S. H., van Wamel, W. J., Ruyken, M., van Kessel, K. P., and van Strijp, J. A. (2005) Anti-opsonic properties of staphylokinase, *Microbes Infect. 7*, 476-484.

16. Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., and Lindberg, M. (1984) Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications, *J. Biol. Chem. 259*, 1695-1702.

17. Lee, L. Y., Hook, M., Haviland, D., Wetsel, R. A., Yonter, E. O., Syribeys, P., Vernachio, J., and Brown, E. L. (2004) Inhibition of complement activation by a secreted *Staphylococcus aureus* protein, *J. Infect. Dis. 190*, 571-579.

18. Lee, L. Y., Liang, X., Hook, M., and Brown, E. L. (2004) Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb), *J. Biol. Chem. 279*, 50710-50716.

19. Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G., and Gotz, F. (1999) Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides, *J. Biol. Chem. 274*, 8405-8410.

20. Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W. F., Jung, G., Tarkowski, A., van

Kessel, K. P., and van Strijp, J. A. (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine, *J. Exp. Med. 193*, 1067-1076.

21. Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J., and Tarkowski, A. (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism, *J. Immunol. 172*, 1169-1176.

22. Lowy, F. D. (1998) *Staphylococcus aureus* infections, *N. Engl. J. Med. 339*, 520-532.

23. Proctor, R. A., Bates, D. M., and McNamara, P. J. (2001) *Emerging infections*, Vol. 5, American Society for Microbiology Press, Washington, D. C.

24. Bulger, R. J. (1967) A methicillin-resistant strain of *Staphylococcus aureus*. Clinical and laboratory experience, *Ann. Intern. Med. 67*, 81-89.

25. Baddour, L. M., Barker, L. P., Christensen, G. D., Parisi, J. T., and Simpson, W. A. (1990) Phenotypic variation of *Staphylococcus epidermidis* in infection of transvenous endocardial pacemaker electrodes, *J. Clin. Microbiol. 28*, 676-679.

26. Haussler, S., Lehmann, C., Breselge, C., Rohde, M., Classen, M., Tummler, B., Vandamme, P., and Steinmetz, I. (2003) Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants

of the *Burkholderia cepacia* complex, *Eur. J. Clin. Microbiol. Infect. Dis. 22*, 249-253.

27. Colwell, C. A. (1946) Small Colony Variants of *Escherichia coli*, *J. Bacteriol. 52*, 417-422.

28. Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N., and Arbeit, R. D. (1995) Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*, *Clin. Infect. Dis. 20*, 95-102.

29. von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G., and Gotz, F. (1997) A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly, *J. Bacteriol. 179*, 4706-4712.

30. Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S., and Proctor, R. A. (2003) *Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney, *J. Infect. Dis. 187*, 1654-1661.

31. Clements, M. O., Watson, S. P., Poole, R. K., and Foster, S. J. (1999) CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis, *J. Bacteriol. 181*, 501-507.

32. Kohler, C., von Eiff, C., Liebeke, M., McNamara, P. J., Lalk, M., Proctor, R. A., Hecker, M., and Engelmann, S. (2008) A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*, *J. Bacteriol. 190*, 6351-6364.

33. von Eiff, C., McNamara, P., Becker, K., Bates, D., Lei, X. H., Ziman, M., Bochner, B. R., Peters, G., and Proctor, R. A. (2006) Phenotype microarray profiling of *Staphylococcus aureus* menD and hemB mutants with the small-colony-variant phenotype, *J. Bacteriol. 188*, 687-693.

34. Lannergard, J., von Eiff, C., Sander, G., Cordes, T., Seggewiss, J., Peters, G., Proctor, R. A., Becker, K., and Hughes, D. (2008) Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 52*, 4017-4022.

35. Kahl, B. C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R. A., and Peters, G. (2003) Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation, *J. Clin. Microbiol. 41*, 410-413.

36. Chatterjee, I., Kriegeskorte, A., Fischer, A., Deiwick, S., Theimann, N., Proctor, R. A., Peters, G., Herrmann, M., and Kahl, B. C. (2008) In vivo mutations of thymidylate synthase (encoded by thyA) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*, *J. Bacteriol. 190*, 834-842.

37. Samuelsen, O., Haukland, H. H., Kahl, B. C., von Eiff, C., Proctor, R. A., Ulvatne, H., Sandvik, K., and Vorland, L. H. (2005) *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B, *J. Antimicrob. Chemother. 56*, 1126-1129.

38. Haussler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B., and Steinmetz, I. (2003) Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection, *J. Med. Microbiol. 52*, 295-301.

39. Al Laham, N., Rohde, H., Sander, G., Fischer, A., Hussain, M., Heilmann, C., Mack, D., Proctor, R., Peters, G., Becker, K., and von Eiff, C. (2007) Augmented expression of polysaccharide intercellular adhesin in a defined *Staphylococcus epidermidis* mutant with the small-colony-variant phenotype, *J. Bacteriol. 189*, 4494-4501.

40. Baumert, N., von Eiff, C., Schaaff, F., Peters, G., Proctor, R. A., and Sahl, H. G. (2002) Physiology and antibiotic susceptibility of *Staphylococcus aureus* small colony variants, *Microb. Drug Resist. 8*, 253-260.

41. Brouillette, E., Martinez, A., Boyll, B. J., Allen, N. E., and Malouin, F. (2004) Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo, *FEMS Immunol. Med. Microbiol. 41*, 35-41.

42. Drlica, K., and Zhao, X. (1997) DNA gyrase, topoisomerase IV, and the 4-quinolones, *Microbiol. Mol. Biol. Rev. 61*, 377-392.

43. Jin, D. J., and Gross, C. A. (1988) Mapping and sequencing of mutations in the *Escherichia coli* rpoB gene that lead to rifampicin resistance, *J. Mol. Biol. 202*, 45-58.

44. Woodford, N., and Ellington, M. J. (2007) The emergence of antibiotic resistance by mutation, *Clin. Microbiol. Infect. 13*, 5-18.

45. Zhang, Y., and Young, D. (1994) Molecular genetics of drug resistance in *Mycobacterium tuberculosis*, *J. Antimicrob. Chemother. 34*, 313-319.

46. McKenzie, G. J., Harris, R. S., Lee, P. L., and Rosenberg, S. M. (2000) The SOS response regulates adaptive mutation, *Proc. Natl. Acad. Sci. U S A 97*, 6646-6651.

47. Cirz, R. T., Jones, M. B., Gingles, N. A., Minogue, T. D., Jarrahi, B., Peterson, S. N., and Romesberg, F. E. (2007) Complete and SOS-mediated response of *Staphylococcus aureus* to the antibiotic ciprofloxacin, *J. Bacteriol. 189*, 531-539.

48. Chambers, H. F. (1997) Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications, *Clin. Microbiol. Rev. 10*, 781-791.

49. Archer, G. L., and Niemeyer, D. M. (1994) Origin and evolution of DNA associated with resistance to methicillin in staphylococci, *Trends Microbiol. 2*, 343-347.

50. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility, *J. Antimicrob. Chemother. 40*, 135-136.

51. Sieradzki, K., and Tomasz, A. (1997) Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*, *J. Bacteriol. 179*, 2557-2566.

52. Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., Asai, K., Ashikaga, S., Aymerich, S., Bessieres, P., Boland, F., Brignell, S. C., Bron, S., Bunai, K., Chapuis, J., Christiansen, L. C., Danchin, A., Debarbouille, M., Dervyn, E., Deuerling, E., Devine, K., Devine, S. K., Dreesen, O., Errington, J., Fillinger, S., Foster, S. J., Fujita, Y., Galizzi, A., Gardan, R., Eschevins, C., Fukushima, T., Haga, K., Harwood, C. R., Hecker, M., Hosoya, D., Hullo, M. F., Kakeshita, H., Karamata, D., Kasahara, Y., Kawamura, F., Koga, K., Koski, P., Kuwana, R., Imamura, D., Ishimaru, M., Ishikawa, S., Ishio, I., Le Coq, D., Masson, A., Mauel, C., Meima, R., Mellado, R. P., Moir, A., Moriya, S., Nagakawa, E., Nanamiya, H., Nakai, S., Nygaard, P., Ogura, M., Ohanan, T., O'Reilly, M., O'Rourke, M., Pragai, Z., Pooley, H. M., Rapoport, G., Rawlins, J. P., Rivas, L. A., Rivolta, C., Sadaie, A., Sadaie, Y., Sarvas, M., Sato, T., Saxild, H. H., Scanlan, E., Schumann, W., Seegers, J. F., Sekiguchi, J., Sekowska, A., Seror, S. J., Simon, M., Stragier, P., Studer, R., Takamatsu, H., Tanaka, T., Takeuchi, M., Thomaides, H. B., Vagner, V., van Dijl, J. M., Watabe, K., Wipat, A., Yamamoto, H., Yamamoto, M., Yamamoto, Y., Yamane, K., Yata, K., Yoshida, K., Yoshikawa, H., Zuber, U., and Ogasawara, N. (2003) Essential *Bacillus subtilis* genes, *Proc. Natl. Acad. Sci. U S A 100*, 4678-4683.

53. Zhang, L., Fan, F., Palmer, L. M., Lonetto, M. A., Petit, C., Voelker, L. L., St John, A., Bankosky, B., Rosenberg, M., and McDevitt, D. (2000) Regulated gene expression in *Staphylococcus aureus* for identifying

conditional lethal phenotypes and antibiotic mode of action, *Gene 255*, 297-305.

54. Jana, M., Luong, T. T., Komatsuzawa, H., Shigeta, M., and Lee, C. Y. (2000) A method for demonstrating gene essentiality in *Staphylococcus aureus*, *Plasmid 44*, 100-104.

55. Zalacain, M., Biswas, S., Ingraham, K. A., Ambrad, J., Bryant, A., Chalker, A. F., Iordanescu, S., Fan, J., Fan, F., Lunsford, R. D., O'Dwyer, K., Palmer, L. M., So, C., Sylvester, D., Volker, C., Warren, P., McDevitt, D., Brown, J. R., Holmes, D. J., and Burnham, M. K. (2003) A global approach to identify novel broad-spectrum antibacterial targets among proteins of unknown function, *J. Mol. Microbiol. Biotechnol. 6*, 109-126.

56. Rohrer, S., Ehlert, K., Tschierske, M., Labischinski, H., and Berger-Bachi, B. (1999) The essential *Staphylococcus aureus* gene fmhB is involved in the first step of peptidoglycan pentaglycine interpeptide formation, *Proc. Natl. Acad. Sci. U S A 96*, 9351-9356.

57. Ji, Y., Zhang, B., Van, S. F., Horn, Warren, P., Woodnutt, G., Burnham, M. K., and Rosenberg, M. (2001) Identification of critical staphylococcal genes using conditional phenotypes generated by antisense RNA, *Science 293*, 2266-2269.

58. Forsyth, R. A., Haselbeck, R. J., Ohlsen, K. L., Yamamoto, R. T., Xu, H., Trawick, J. D., Wall, D., Wang, L., Brown-Driver, V., Froelich, J. M., C, K. G., King, P., McCarthy, M., Malone, C., Misiner, B., Robbins, D., Tan, Z., Zhu Zy, Z. Y., Carr, G., Mosca, D. A., Zamudio, C., Foulkes, J. G., and

Zyskind, J. W. (2002) A genome-wide strategy for the identification of essential genes in *Staphylococcus aureus*, *Mol. Microbiol. 43*, 1387-1400.

59. Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G., and Jacobs, W. R., Jr. (1994) inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*, *Science 263*, 227-230.

60. Kurosu, M., Narayanasamy, P., Biswas, K., Dhiman, R., and Crick, D. C. (2007) Discovery of 1,4-dihydroxy-2-naphthoate [corrected] prenyltransferase inhibitors: new drug leads for multidrug-resistant gram-positive pathogens, *J. Med. Chem. 50*, 3973-3975.

61. Hong-Geller, E., Mollhoff, M., Shiflett, P. R., and Gupta, G. (2004) Design of chimeric receptor mimics with different TcRVbeta isoforms. Type-specific inhibition of superantigen pathogenesis, *J. Biol. Chem. 279*, 5676-5684.

62. Schwan, W. R., Coulter, S. N., Ng, E. Y., Langhorne, M. H., Ritchie, H. D., Brody, L. L., Westbrock-Wadman, S., Bayer, A. S., Folger, K. R., and Stover, C. K. (1998) Identification and characterization of the PutP proline permease that contributes to in vivo survival of *Staphylococcus aureus* in animal models, *Infect. Immun. 66*, 567-572.

63. Ton-That, H., and Schneewind, O. (1999) Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction, *J. Biol. Chem. 274*, 24316-24320.

64. Scott, C. J., McDowell, A., Martin, S. L., Lynas, J. F., Vandenbroeck, K., and Walker, B. (2002) Irreversible inhibition of the bacterial cysteine protease-transpeptidase sortase (SrtA) by substrate-derived affinity labels, *Biochem J. 366*, 953-958.

65. Kruger, R. G., Barkallah, S., Frankel, B. A., and McCafferty, D. G. (2004) Inhibition of the *Staphylococcus aureus* sortase transpeptidase SrtA by phosphinic peptidomimetics, *Bioorg. Med. Chem. 12*, 3723-3729.

66. Bruton, G., Huxley, A., O'Hanlon, P., Orlek, B., Eggleston, D., Humphries, J., Readshaw, S., West, A., Ashman, S., Brown, M., Moore, K., Pope, A., O'Dwyer, K., and Wang, L. (2003) Lipopeptide substrates for SpsB, the *Staphylococcus aureus* type I signal peptidase: design, conformation and conversion to alpha-ketoamide inhibitors, *Eur. J. Med. Chem. 38*, 351-356.

67. Lyon, G. J., Mayville, P., Muir, T. W., and Novick, R. P. (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13330-13335.

68. Beyer, D., Kroll, H. P., Endermann, R., Schiffer, G., Siegel, S., Bauser, M., Pohlmann, J., Brands, M., Ziegelbauer, K., Haebich, D., Eymann, C., and Brotz-Oesterhelt, H. (2004) New class of bacterial phenylalanyl-tRNA synthetase inhibitors with high potency and broad-spectrum activity, *Antimicrob. Agents Chemother. 48*, 525-532.

69. Andres, C. J., Bronson, J. J., D'Andrea, S. V., Deshpande, M. S., Falk, P. J., Grant-Young, K. A., Harte, W. E., Ho, H. T., Misco, P. F., Robertson, J. G., Stock, D., Sun, Y., and Walsh, A. W. (2000) 4-Thiazolidinones: novel inhibitors of the bacterial enzyme MurB, *Bioorg. Med. Chem. Lett. 10*, 715-717.

70. Bronson, J. J., DenBleyker, K. L., Falk, P. J., Mate, R. A., Ho, H. T., Pucci, M. J., and Snyder, L. B. (2003) Discovery of the first antibacterial small molecule inhibitors of MurB, *Bioorg. Med. Chem. Lett. 13*, 873-875.

71. Kitagawa, H., Kumura, K., Takahata, S., Iida, M., and Atsumi, K. (2007) 4-Pyridone derivatives as new inhibitors of bacterial enoyl-ACP reductase FabI, *Bioorg. Med. Chem. 15*, 1106-1116.

72. Payne, D. J., Miller, W. H., Berry, V., Brosky, J., Burgess, W. J., Chen, E., DeWolf Jr, W. E., Jr., Fosberry, A. P., Greenwood, R., Head, M. S., Heerding, D. A., Janson, C. A., Jaworski, D. D., Keller, P. M., Manley, P. J., Moore, T. D., Newlander, K. A., Pearson, S., Polizzi, B. J., Qiu, X., Rittenhouse, S. F., Slater-Radosti, C., Salyers, K. L., Seefeld, M. A., Smyth, M. G., Takata, D. T., Uzinskas, I. N., Vaidya, K., Wallis, N. G., Winram, S. B., Yuan, C. C., and Huffman, W. F. (2002) Discovery of a novel and potent class of FabI-directed antibacterial agents, *Antimicrob. Agents Chemother. 46*, 3118-3124.

73. Fattom, A., Schneerson, R., Szu, S. C., Vann, W. F., Shiloach, J., Karakawa, W. W., and Robbins, J. B. (1990) Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5

and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin A, *Infect. Immun. 58*, 2367-2374.

74. Stranger-Jones, Y. K., Bae, T., and Schneewind, O. (2006) Vaccine assembly from surface proteins of *Staphylococcus aureus*, *Proc. Natl. Acad. Sci. U.S.A. 103*, 16942-16947.

75. Kuklin, N. A., Clark, D. J., Secore, S., Cook, J., Cope, L. D., McNeely, T., Noble, L., Brown, M. J., Zorman, J. K., Wang, X. M., Pancari, G., Fan, H., Isett, K., Burgess, B., Bryan, J., Brownlow, M., George, H., Meinz, M., Liddell, M. E., Kelly, R., Schultz, L., Montgomery, D., Onishi, J., Losada, M., Martin, M., Ebert, T., Tan, C. Y., Schofield, T. L., Nagy, E., Meineke, A., Joyce, J. G., Kurtz, M. B., Caulfield, M. J., Jansen, K. U., McClements, W., and Anderson, A. S. (2006) A novel *Staphylococcus aureus* vaccine: iron surface determinant B induces rapid antibody responses in rhesus macaques and specific increased survival in a murine *S. aureus* sepsis model, *Infect. Immun. 74*, 2215-2223.

76. Kelly-Quintos, C., Kropec, A., Briggs, S., Ordonez, C. L., Goldmann, D. A., and Pier, G. B. (2005) The role of epitope specificity in the human opsonic antibody response to the staphylococcal surface polysaccharide poly N-acetyl glucosamine, *J. Infect. Dis. 192*, 2012-2019.

77. Adlam, C., Ward, P. D., McCartney, A. C., Arbuthnott, J. P., and Thorley, C. M. (1977) Effect immunization with highly purified alpha- and beta-toxins on staphylococcal mastitis in rabbits, *Infect. Immun. 17*, 250-256.

78. Lowell, G. H., Colleton, C., Frost, D., Kaminski, R. W., Hughes, M., Hatch, J., Hooper, C., Estep, J., Pitt, L., Topper, M., Hunt, R. E., Baker, W., and Baze, W. B. (1996) Immunogenicity and efficacy against lethal aerosol staphylococcal enterotoxin B challenge in monkeys by intramuscular and respiratory delivery of proteosome-toxoid vaccines, *Infect. Immun. 64*, 4686-4693.

Chapter 2:

1. Cronan, J. E., Jr., and Waldrop, G. L. (2002) Multi-subunit acetyl-CoA carboxylases, *Prog. Lipid Res. 41*, 407-435.
2. Shimakata, T., and Stumpf, P. K. (1983) The purification and function of acetyl coenzyme A:acyl carrier protein transacylase, *J. Biol. Chem. 258*, 3592-3598.
3. Heath, R. J., and Rock, C. O. (1996) Regulation of fatty acid elongation and initiation by acyl-acyl carrier protein in *Escherichia coli*, *J. Biol. Chem. 271*, 1833-1836.
4. Heath, R. J., and Rock, C. O. (1995) Enoyl-acyl carrier protein reductase (fabI) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*, *J. Biol. Chem. 270*, 26538-26542.
5. Heath, R. J., and Rock, C. O. (1996) Roles of the FabA and FabZ beta-hydroxyacyl-acyl carrier protein dehydratases in *Escherichia coli* fatty acid biosynthesis, *J. Biol. Chem. 271*, 27795-27801.

6. Mohan, S., Kelly, T. M., Eveland, S. S., Raetz, C. R., and Anderson, M. S. (1994) An Escherichia coli gene (FabZ) encoding (3R)-hydroxymyristoyl acyl carrier protein dehydrase. Relation to fabA and suppression of mutations in lipid A biosynthesis, *J. Biol. Chem. 269*, 32896-32903.

7. Garwin, J. L., Klages, A. L., and Cronan, J. E., Jr. (1980) Structural, enzymatic, and genetic studies of beta-ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli*, *J. Biol. Chem. 255*, 11949-11956.

8. Larson, T. J., Lightner, V. A., Green, P. R., Modrich, P., and Bell, R. M. (1980) Membrane phospholipid synthesis in *Escherichia coli*. Identification of the sn-glycerol-3-phosphate acyltransferase polypeptide as the plsB gene product, *J. Biol. Chem. 255*, 9421-9426.

9. Marrakchi, H., Choi, K. H., and Rock, C. O. (2002) A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*, *J. Biol. Chem. 277*, 44809-44816.

10. Egan, A. F., and Russell, R. R. (1973) Conditional mutations affecting the cell envelope of *Escherichia coli* K-12, *Genet. Res. 21*, 139-152.

11. Turnowsky, F., Fuchs, K., Jeschek, C., and Hogenauer, G. (1989) envM genes of *Salmonella typhimurium* and *Escherichia coli*, *J. Bacteriol. 171*, 6555-6565.

12. Tsay, J. T., Rock, C. O., and Jackowski, S. (1992) Overproduction of beta-ketoacyl-acyl carrier protein synthase I imparts thiolactomycin resistance to *Escherichia coli* K-12, *J. Bacteriol. 174*, 508-513.

13. Waller, R. F., Ralph, S. A., Reed, M. B., Su, V., Douglas, J. D., Minnikin, D. E., Cowman, A. F., Besra, G. S., and McFadden, G. I. (2003) A type II pathway for fatty acid biosynthesis presents drug targets in *Plasmodium falciparum*, *Antimicrob. Agents Chemother. 47*, 297-301.

14. Sharma, S. K., Kapoor, M., Ramya, T. N., Kumar, S., Kumar, G., Modak, R., Sharma, S., Surolia, N., and Surolia, A. (2003) Identification, characterization, and inhibition of *Plasmodium falciparum* beta-hydroxyacyl-acyl carrier protein dehydratase (FabZ), *J. Biol. Chem. 278*, 45661-45671.

15. Heath, R. J., and Rock, C. O. (1995) Enoyl-acyl carrier protein reductase (fabI) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*, *J. Biol. Chem. 270*, 26538-26542.

16. Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kallberg, Y., Persson, B., and Jornvall, H. (2003) Short-chain dehydrogenases/reductases (SDR): the 2002 update, *Chem. Biol. Interact. 143-144*, 247-253.

17. Lukacik, P., Kavanagh, K. L., and Oppermann, U. (2006) Structure and function of human 17beta-hydroxysteroid dehydrogenases, *Mol. Cell. Endocrinol. 248*, 61-71.

18. Wu, X., Lukacik, P., Kavanagh, K. L., and Oppermann, U. (2007) SDR-type human hydroxysteroid dehydrogenases involved in steroid hormone activation, *Mol. Cell. Endocrinol. 265-266*, 71-76.

19. Jornvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., and Ghosh, D. (1995) Short-chain dehydrogenases/reductases (SDR), *Biochemistry 34*, 6003-6013.

20. Labesse, G., Vidal-Cros, A., Chomilier, J., Gaudry, M., and Mornon, J. P. (1994) Structural comparisons lead to the definition of a new superfamily of NAD(P)(H)-accepting oxidoreductases: the single-domain reductases/epimerases/dehydrogenases (the 'RED' family), *Biochem. J. 304 (Pt 1)*, 95-99.

21. Persson, B., Kallberg, Y., Oppermann, U., and Jornvall, H. (2003) Coenzyme-based functional assignments of short-chain dehydrogenases/reductases (SDRs), *Chem. Biol. Interact. 143-144*, 271-278.

22. Rozwarski, D. A., Vilcheze, C., Sugantino, M., Bittman, R., and Sacchettini, J. C. (1999) Crystal structure of the *Mycobacterium tuberculosis* enoyl-ACP reductase, InhA, in complex with NAD+ and a C16 fatty acyl substrate, *J. Biol. Chem. 274*, 15582-15589.

23. Bergler, H., Wallner, P., Ebeling, A., Leitinger, B., Fuchsbichler, S., Aschauer, H., Kollenz, G., Hogenauer, G., and Turnowsky, F. (1994) Protein EnvM is the NADH-dependent enoyl-ACP reductase (FabI) of *Escherichia coli*, *J. Biol. Chem. 269*, 5493-5496.

24. Heath, R. J., Su, N., Murphy, C. K., and Rock, C. O. (2000) The enoyl-[acyl-carrier-protein] reductases FabI and FabL from *Bacillus subtilis*, *J. Biol. Chem. 275*, 40128-40133.

25. Quemard, A., Sacchettini, J. C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W. R., Jr., and Blanchard, J. S. (1995) Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*, *Biochemistry 34*, 8235-8241.

26. Marcinkeviciene, J., Jiang, W., Kopcho, L. M., Locke, G., Luo, Y., and Copeland, R. A. (2001) Enoyl-ACP reductase (FabI) of *Haemophilus influenzae:* steady-state kinetic mechanism and inhibition by triclosan and hexachlorophene, *Arch. Biochem. Biophys. 390*, 101-108.

27. Rafferty, J. B., Simon, J. W., Baldock, C., Artymiuk, P. J., Baker, P. J., Stuitje, A. R., Slabas, A. R., and Rice, D. W. (1995) Common themes in redox chemistry emerge from the X-ray structure of oilseed rape (*Brassica napus*) enoyl acyl carrier protein reductase, *Structure 3*, 927-938.

28. Rozwarski, D. A., Grant, G. A., Barton, D. H., Jacobs, W. R., Jr., and Sacchettini, J. C. (1998) Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*, *Science 279*, 98-102.

29. Rawat, R., Whitty, A., and Tonge, P. J. (2003) The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the *Mycobacterium tuberculosis* enoyl reductase: adduct affinity and drug resistance, *Proc. Natl. Acad. Sci. U.S.A. 100*, 13881-13886.

30. Baldock, C., Rafferty, J. B., Sedelnikova, S. E., Baker, P. J., Stuitje, A. R., Slabas, A. R., Hawkes, T. R., and Rice, D. W. (1996) A mechanism of drug action revealed by structural studies of enoyl reductase, *Science (New York, NY) 274*, 2107-2110.

31. Karlowsky, J. A., Laing, N. M., Baudry, T., Kaplan, N., Vaughan, D., Hoban, D. J., and Zhanel, G. G. (2007) In vitro activity of API-1252, a novel FabI inhibitor, against clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Antimicrob. Agents Chemother. 51*, 1580-1581.

32. Kitagawa, H., Kumura, K., Takahata, S., Iida, M., and Atsumi, K. (2007) 4-Pyridone derivatives as new inhibitors of bacterial enoyl-ACP reductase FabI, *Bioorg. Med. Chem. 15*, 1106-1116.

33. Ling, L. L., Xian, J., Ali, S., Geng, B., Fan, J., Mills, D. M., Arvanites, A. C., Orgueira, H., Ashwell, M. A., Carmel, G., Xiang, Y., and Moir, D. T. (2004) Identification and characterization of inhibitors of bacterial enoyl-acyl carrier protein reductase, *Antimicrob. Agents Chemother. 48*, 1541-1547.

34. Payne, D. J., Miller, W. H., Berry, V., Brosky, J., Burgess, W. J., Chen, E., DeWolf Jr, W. E., Jr., Fosberry, A. P., Greenwood, R., Head, M. S., Heerding, D. A., Janson, C. A., Jaworski, D. D., Keller, P. M., Manley, P. J., Moore, T. D., Newlander, K. A., Pearson, S., Polizzi, B. J., Qiu, X., Rittenhouse, S. F., Slater-Radosti, C., Salyers, K. L., Seefeld, M. A., Smyth, M. G., Takata, D. T., Uzinskas, I. N., Vaidya, K., Wallis, N. G., Winram, S. B., Yuan, C. C., and Huffman, W. F. (2002) Discovery of a novel and potent class of FabI-directed antibacterial agents, *Antimicrob. Agents Chemother. 46*, 3118-3124.

35. Zhang, Y. M., and Rock, C. O. (2004) Evaluation of epigallocatechin gallate and related plant polyphenols as inhibitors of the FabG and FabI

reductases of bacterial type II fatty-acid synthase, *J. Biol. Chem. 279*, 30994-31001.

36. Heath, R. J., and Rock, C. O. (2000) A triclosan-resistant bacterial enzyme, *Nature 406*, 145-146.

37. Massengo-Tiasse, R. P., and Cronan, J. E. (2008) *Vibrio cholerae* FabV defines a new class of enoyl-acyl carrier protein reductase, *J. Biol. Chem. 283*, 1308-1316.

38. Kitagawa, H., Ozawa, T., Takahata, S., Iida, M., Saito, J., and Yamada, M. (2007) Phenylimidazole derivatives of 4-pyridone as dual inhibitors of bacterial enoyl-acyl carrier protein reductases FabI and FabK, *J. Med. Chem. 50*, 4710-4720.

39. Heath, R. J., Li, J., Roland, G. E., and Rock, C. O. (2000) Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene, *J. Biol. Chem. 275*, 4654-4659.

40. Slater-Radosti, C., Van Aller, G., Greenwood, R., Nicholas, R., Keller, P. M., DeWolf, W. E., Jr., Fan, F., Payne, D. J., and Jaworski, D. D. (2001) Biochemical and genetic characterization of the action of triclosan on *Staphylococcus aureus*, *J. Antimicrob. Chemother. 48*, 1-6.

41. Russell, A. D. (2004) Whither triclosan?, *J. Antimicrob. Chemother. 53*, 693-695.

42. Regos, J., Zak, O., Solf, R., Vischer, W. A., and Weirich, E. G. (1979) Antimicrobial spectrum of triclosan, a broad-spectrum antimicrobial agent

for topical application. II. Comparison with some other antimicrobial agents, *Dermatologica. 158*, 72-79.

43. Ayliffe, G. A., Buckles, A., Casewell, M. W., Cookson, B. D., Cox, R. A., Duckworth, G. J., Griffiths-Jones, A., Heathcock, R., Humphreys, H., Keane, C. T., Marples, R. R., Shanson, D. C., Slack, R., and Tebbs, E. (1998) Revised guidelines for the control of methicillin-resistant *Staphylococcus aureus* infection in hospitals. British Society for Antimicrobial Chemotherapy, Hospital Infection Society and the Infection Control Nurses Association, *J. Hosp. Infect. 39*, 253-290.

44. Bamber, A. I., and Neal, T. J. (1999) An assessment of triclosan susceptibility in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*, *J. Hosp. Infect. 41*, 107-109.

45. Fan, F., Yan, K., Wallis, N. G., Reed, S., Moore, T. D., Rittenhouse, S. F., DeWolf, W. E., Jr., Huang, J., McDevitt, D., Miller, W. H., Seefeld, M. A., Newlander, K. A., Jakas, D. R., Head, M. S., and Payne, D. J. (2002) Defining and combating the mechanisms of triclosan resistance in clinical isolates of *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 46*, 3343-3347.

46. Seaman, P. F., Ochs, D., and Day, M. J. (2007) Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*, *J. Antimicrob. Chemother. 59*, 43-50.

47. Sivaraman, S., Sullivan, T. J., Johnson, F., Novichenok, P., Cui, G., Simmerling, C., and Tonge, P. J. (2004) Inhibition of the bacterial enoyl

reductase FabI by triclosan: a structure-reactivity analysis of FabI inhibition by triclosan analogues, *J. Med. Chem. 47*, 509-518.

48. Sullivan, T. J., Truglio, J. J., Boyne, M. E., Novichenok, P., Zhang, X., Stratton, C. F., Li, H. J., Kaur, T., Amin, A., Johnson, F., Slayden, R. A., Kisker, C., and Tonge, P. J. (2006) High affinity InhA inhibitors with activity against drug-resistant strains of *Mycobacterium tuberculosis*, *ACS Chem. Biol. 1*, 43-53.

49. Parikh, S., Moynihan, D. P., Xiao, G., and Tonge, P. J. (1999) Roles of tyrosine 158 and lysine 165 in the catalytic mechanism of InhA, the enoyl-ACP reductase from *Mycobacterium tuberculosis*, *Biochemistry 38*, 13623-13634.

50. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248-254.

51. Quadri, L. E., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P., and Walsh, C. T. (1998) Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases, *Biochemistry 37*, 1585-1595.

52. Cleland, W. W. (1979) Statistical analysis of enzyme kinetic data, *Methods Enzymol. 63*, 103-138.

53. NCCLS. (2003) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 6th ed. Approved Standard M7-A6, *National Committee for Clinical Laboratory Standards, Wayne, PA*.

54. Schaeffer, M. L., Agnihotri, G., Kallender, H., Brennan, P. J., and Lonsdale, J. T. (2001) Expression, purification, and characterization of the *Mycobacterium tuberculosis* acyl carrier protein, AcpM, *Biochim. Biophys. Acta 1532*, 67-78.

55. Heath, R. J., Li, J., Roland, G. E., and Rock, C. O. (2000) Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene, *J. Biol. Chem. 275*, 4654-4659.

56. Heath, R. J., Su, N., Murphy, C. K., and Rock, C. O. (2000) The enoyl-[acyl-carrier-protein] reductases FabI and FabL from *Bacillus subtilis*, *J. Biol. Chem. 275*, 40128-40133.

57. Delano, W. L. (2002) The PyMOL Molecular Graphics System, *DeLano Scientific LLC, San Carlos, CA, USA.* http://www.pymol.org.

58. Heath, R. J., Rubin, J. R., Holland, D. R., Zhang, E., Snow, M. E., and Rock, C. O. (1999) Mechanism of triclosan inhibition of bacterial fatty acid synthesis, *J. Biol. Chem. 274*, 11110-11114.

59. Heath, R. J., Yu, Y. T., Shapiro, M. A., Olson, E., and Rock, C. O. (1998) Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis, *J. Biol. Chem. 273*, 30316-30320.

60. McMurry, L. M., Oethinger, M., and Levy, S. B. (1998) Triclosan targets lipid synthesis, *Nature 394*, 531-532.

61. Levy, C. W., Roujeinikova, A., Sedelnikova, S., Baker, P. J., Stuitje, A. R., Slabas, A. R., Rice, D. W., and Rafferty, J. B. (1999) Molecular basis of triclosan activity, *Nature 398*, 383-384.

62. am Ende, C. W., Knudson, S. E., Liu, N., Childs, J., Sullivan, T. J., Boyne, M., Xu, H., Gegina, Y., Knudson, D. L., Johnson, F., Peloquin, C. A., Slayden, R. A., and Tonge, P. J. (2008) Synthesis and in vitro antimycobacterial activity of B-ring modified diaryl ether InhA inhibitors, *Bioorg. Med. Chem. Lett. 18*, 3029-3033.

63. Sullivan, T. J., Truglio, J. J., Boyne, M. E., Novichenok, P., Zhang, X., Stratton, C. F., Li, H. J., Kaur, T., Amin, A., Johnson, F., Slayden, R. A., Kisker, C., and Tonge, P. J. (2006) High affinity InhA inhibitors with activity against drug-resistant strains of *Mycobacterium tuberculosis*, *ACS Chem. Biol. 1*, 43-53.

Chapter 3:

1. Lester, R. L., and Crane, F. L. (1959) The natural occurrence of coenzyme Q and related compounds, *J. Biol. Chem. 234*, 2169-2175.

2. Threlfall, D. R., and Whistance, G. R. (1971) Biosynthesis of isoprenoid quinones and chromanols, in *Aspects of terpernoid chemistry and biochemistry* (Goodwin, T. W., Ed.), Academic Press Inc., London.

3. Bishop, D. H., Pandya, K. P., and King, H. K. (1962) Ubiquinone and vitamin K in bacteria, *Biochem. J. 83*, 606-614.

4. Friis, P., Daves, G. D., Jr., and Folkers, K. (1967) New epoxyubiquinones, *Biochemistry 6*, 3618-3624.

5. MacCorquodale, D. W., Cheney, L. C., Binkley, S. B., Holcomb, W. F., McKee, R. W., Thayer, S. A., Doisy, E. A. (1939) The constitution and synthesis of vitamin K, *J. Biol. Chem. 131*, 357-370.

6. Collins, M. D., Pirouz, T., Goodfellow, M., and Minnikin, D. E. (1977) Distribution of menaquinones in actinomycetes and corynebacteria, *J. Gen. Microbiol. 100*, 221-230.

7. Hammand, R. K. a. W., D. C. (1969) Separation of vitamin K2 isorenologues by reverse-phase thin-layer chromatography, *J. Chromatogr. 45*, 446-452.

8. Sasarman, A., Purvis, P., and Portelance, V. (1974) Role of menaquinone in nitrate respiration in *Staphylococcus aureus*, *J. Bacteriol. 117*, 911-913.

9. Davidson, V. L. (2004) Electron transfer in quinoproteins, *Arch. Biochem. Biophys. 428*, 32-40.

10. Suttie, J. W. (1995) The importance of menaquinones in human nutrition, *Annu. Rev. Nutr. 15*, 399-417.

11. Reddi, K., Henderson, B., Meghji, S., Wilson, M., Poole, S., Hopper, C., Harris, M., and Hodges, S. J. (1995) Interleukin 6 production by lipopolysaccharide-stimulated human fibroblasts is potently inhibited by naphthoquinone (vitamin K) compounds, *Cytokine 7*, 287-290.

12. Ohsaki, Y., Shirakawa, H., Hiwatashi, K., Furukawa, Y., Mizutani, T., and Komai, M. (2006) Vitamin K suppresses lipopolysaccharide-induced inflammation in the rat, *Biosci. Biotechnol. Biochem. 70*, 926-932.

13. Li, J., Lin, J. C., Wang, H., Peterson, J. W., Furie, B. C., Furie, B., Booth, S. L., Volpe, J. J., and Rosenberg, P. A. (2003) Novel role of vitamin k in preventing oxidative injury to developing oligodendrocytes and neurons, *J. Neurosci. 23*, 5816-5826.

14. Sundaram, K. S., and Lev, M. (1988) Warfarin administration reduces synthesis of sulfatides and other sphingolipids in mouse brain, *J. Lipid Res. 29*, 1475-1479.

15. Cox, G. B., and Gibson, F. (1964) Biosynthesis of Vitamin K and Ubiquinone. Relation to the Shikimic Acid Pathway in *Escherichia Coli*, *Biochim. Biophys. Acta 93*, 204-206.

16. Campbell, I. M., Coscia, C. J., Kelsey, M., and Bentley, R. (1967) Origin of the aromatic nucleus in bacterial menaquinones, *Biochem. Biophys. Res. Commun. 28*, 25-29.

17. Robins, D. J., Bentley, R. (1972) Biosynthesis of bacterial menaquinones-evidence for the involvement of 2-oxoglutaric acid, *J. Chem. Soc. Chem. Commun.*, 232-233.

18. Young, I. G. (1975) Biosynthesis of bacterial menaquinones. Menaquinone mutants of *Escherichia coli*, *Biochemistry 14*, 399-406.

19. Shineberg, B., and Young, I. G. (1976) Biosynthesis of bacterial menaquinones: the membrane-associated 1,4-dihydroxy-2-naphthoate octaprenyltransferase of *Escherichia coli*, *Biochemistry 15*, 2754-2758.

20. Guest, J. R. (1977) Menaquinone biosynthesis: mutants of *Escherichia coli* K-12 requiring 2-succinylbenzoate, *J. Bacteriol. 130*, 1038-1046.

21. Guest, J. R. (1979) Anaerobic growth of *Escherichia coli K12* with fumarate as terminal electron acceptor. Genetic studies with menaquinone and fluoroacetate-resistant mutants, *J. Gen. Microbiol. 115*, 259-271.

22. Daruwala, R., Bhattacharyya, D. K., Kwon, O., and Meganathan, R. (1997) Menaquinone (vitamin K2) biosynthesis: overexpression, purification, and characterization of a new isochorismate synthase from *Escherichia coli*, *J. Bacteriol. 179*, 3133-3138.

23. Sharma, V., Meganathan, R., and Hudspeth, M. E. (1993) Menaquinone (vitamin K2) biosynthesis: cloning, nucleotide sequence, and expression of the menC gene from *Escherichia coli*, *J. Bacteriol. 175*, 4917-4921.

24. Bhasin, M., Billinsky, J. L., and Palmer, D. R. (2003) Steady-state kinetics and molecular evolution of *Escherichia coli* MenD [(1R,6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase], an anomalous thiamin diphosphate-dependent decarboxylase-carboligase, *Biochemistry 42*, 13496-13504.

25. Sharma, V., Hudspeth, M. E., and Meganathan, R. (1996) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the menE gene from *Escherichia coli*, *Gene 168*, 43-48.

26. Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J., and Kisker, C. (2003) Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in vitamin K2 biosynthesis, *J. Biol. Chem. 278*, 42352-42360.

27. Suvarna, K., Stevenson, D., Meganathan, R., and Hudspeth, M. E. (1998) Menaquinone (vitamin K2) biosynthesis: localization and characterization of the menA gene from *Escherichia coli*, *J. Bacteriol. 180*, 2782-2787.

28. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*, *Nature 388*, 539-547.

29. Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., Chillingworth, T., Davies, R. M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A. V., Moule, S., Pallen, M. J., Penn, C. W., Quail, M. A., Rajandream, M. A., Rutherford, K. M., van Vliet, A. H., Whitehead, S., and Barrell, B. G. (2000) The genome sequence of the food-borne pathogen

Campylobacter jejuni reveals hypervariable sequences, *Nature 403*, 665-668.

30. Marcelli, S. W., Chang, H. T., Chapman, T., Chalk, P. A., Miles, R. J., and Poole, R. K. (1996) The respiratory chain of *Helicobacter pylori*: identification of cytochromes and the effects of oxygen on cytochrome and menaquinone levels, *FEMS Microbiol. Lett. 138*, 59-64.
31. Hiratsuka, T., Furihata, K., Ishikawa, J., Yamashita, H., Itoh, N., Seto, H., and Dairi, T. (2008) An alternative menaquinone biosynthetic pathway operating in microorganisms, *Science 321*, 1670-1673.
32. Seto, H., Jinnai, Y., Hiratsuka, T., Fukawa, M., Furihata, K., Itoh, N., and Dairi, T. (2008) Studies on a new biosynthetic pathway for menaquinone, *J. Am. Chem. Soc. 130*, 5614-5615.
33. Kurosu, M., Narayanasamy, P., Biswas, K., Dhiman, R., and Crick, D. C. (2007) Discovery of 1,4-dihydroxy-2-naphthoate [corrected] prenyltransferase inhibitors: new drug leads for multidrug-resistant gram-positive pathogens, *J. Med. Chem. 50*, 3973-3975.
34. Dhiman, R. K., Mahapatra, S., Slayden, R. A., Boyne, M. E., Lenaerts, A., Hinshaw, J. C., Angala, S. K., Chatterjee, D., Biswas, K., Narayanasamy, P., Kurosu, M., and Crick, D. C. (2009) Menaquinone synthesis is critical for maintaining mycobacterial viability during exponential growth and recovery from non-replicating persistence, *Mol. Microbiol. 72*, 85-97.
35. Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S., and Proctor, R. A. (2003) *Staphylococcus aureus* menD and

hemB mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney, *J. Infect. Dis. 187*, 1654-1661.

36. Kohler, C., von Eiff, C., Liebeke, M., McNamara, P. J., Lalk, M., Proctor, R. A., Hecker, M., and Engelmann, S. (2008) A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*, *J. Bacteriol. 190*, 6351-6364.

37. Kaplun, A., Binshtein, E., Vyazmensky, M., Steinmetz, A., Barak, Z., Chipman, D. M., Tittmann, K., and Shaanan, B. (2008) Glyoxylate carboligase lacks the canonical active site glutamate of thiamine-dependent enzymes, *Nat. Chem. Biol. 4*, 113-118.

38. Kluger, R., and Tittmann, K. (2008) Thiamin diphosphate catalysis: enzymic and nonenzymic covalent intermediates, *Chem. Rev. 108*, 1797-1833.

39. Schutz, A., Golbik, R., Konig, S., Hubner, G., and Tittmann, K. (2005) Intermediates and transition states in thiamin diphosphate-dependent decarboxylases. A kinetic and NMR study on wild-type indolepyruvate decarboxylase and variants using indolepyruvate, benzoylformate, and pyruvate as substrates, *Biochemistry 44*, 6164-6179.

40. Chakraborty, S., Nemeria, N. S., Balakrishnan, A., Brandt, G. S., Kneen, M. M., Yep, A., McLeish, M. J., Kenyon, G. L., Petsko, G. A., Ringe, D., and Jordan, F. (2009) Detection and time course of formation of major thiamin diphosphate-bound covalent intermediates derived from a

chromophoric substrate analogue on benzoylformate decarboxylase, *Biochemistry 48*, 981-994.

41. Berthold, C. L., Gocke, D., Wood, M. D., Leeper, F. J., Pohl, M., and Schneider, G. (2007) Structure of the branched-chain keto acid decarboxylase (KdcA) from *Lactococcus lactis* provides insights into the structural basis for the chemoselective and enantioselective carboligation reaction, *Acta Crystallogr. D Biol. Crystallogr. 63*, 1217-1224.

42. Neumann, P., Weidner, A., Pech, A., Stubbs, M. T., and Tittmann, K. (2008) Structural basis for membrane binding and catalytic activation of the peripheral membrane enzyme pyruvate oxidase from *Escherichia coli*, *Proc. Natl. Acad. Sci. U S A 105*, 17390-17395.

43. Pang, S. S., Duggleby, R. G., and Guddat, L. W. (2002) Crystal structure of yeast acetohydroxyacid synthase: a target for herbicidal inhibitors, *J. Mol. Biol. 317*, 249-262.

44. Gutowski, J. A., and Lienhard, G. E. (1976) Transition state analogs for thiamin pyrophosphate-dependent enzymes, *J. Biol. Chem. 251*, 2863-2866.

45. Kluger, R., and Pike, D. C. (1977) Active site generated analogues of reactive intermediates in enzymic reactions. Potent inhibition of pyruvate dehydrogenase by a phosphonate analogue of pyruvate1, *J. Am. Chem. Soc. 99*, 4504-4506.

46. Hawksley, D. G., D. A. and Leeper, F. J. (2001) Synthesis of 3-deazathiamine, *J. Chem. Soc., Perkin Trans 1*, 144-148.

47. Mann, S., Perez Melero, C., Hawksley, D., and Leeper, F. J. (2004) Inhibition of thiamin diphosphate dependent enzymes by 3-deazathiamin diphosphate, *Org. Biomol. Chem. 2*, 1732-1741.

48. Emmons, G. T., Campbell, I. M., and Bentley, R. (1985) Vitamin K (menaquinone) biosynthesis in bacteria: purification and probable structure of an intermediate prior to o-succinylbenzoate, *Biochem. Biophys. Res. Commun. 131*, 956-960.

49. Taber, H. W., Dellers, E. A., and Lombardo, L. R. (1981) Menaquinone biosynthesis in *Bacillus subtilis*: isolation of men mutants and evidence for clustering of men genes, *J. Bacteriol. 145*, 321-327.

50. Palaniappan, C., Taber, H., and Meganathan, R. (1994) Biosynthesis of o-succinylbenzoic acid in *Bacillus subtilis*: identification of menD mutants and evidence against the involvement of the alpha-ketoglutarate dehydrogenase complex, *J. Bacteriol. 176*, 2648-2653.

51. Jiang, M., Cao, Y., Guo, Z. F., Chen, M., Chen, X., and Guo, Z. (2007) Menaquinone biosynthesis in *Escherichia coli*: identification of 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate as a novel intermediate and re-evaluation of MenD activity, *Biochemistry 46*, 10979-10989.

52. Dawson, A., Fyfe, P. K., and Hunter, W. N. (2008) Specificity and reactivity in menaquinone biosynthesis: the structure of *Escherichia coli* MenD (2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexadiene-1-carboxylate synthase), *J. Mol. Biol. 384*, 1353-1368.

53. Priyadarshi, A., Saleem, Y., Nam, K. H., Kim, K. S., Park, S. Y., Kim, E. E., and Hwang, K. Y. (2009) Structural insights of the MenD from *Escherichia coli* reveal ThDP affinity, *Biochem. Biophys. Res. Commun. 380*, 797-801.

54. Kolappan, S., Zwahlen, J., Zhou, R., Truglio, J. J., Tonge, P. J., and Kisker, C. (2007) Lysine 190 is the catalytic base in MenF, the menaquinone-specific isochorismate synthase from *Escherichia coli*: implications for an enzyme family, *Biochemistry 46*, 946-953.

55. Kluger, R., Chin, J. and Smyth, T. (1981) Thiamin-catalyzed decarboxylation of pyruvate. Synthesis and reactivity analysis of the central, elusive intermediate, α-lactylthiamin, *J. Am. Chem. Soc. 103*, 884-888.

56. CLSI. (2006) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 7th ed. Approved Standard M7-A7, *Clinical and Laboratory Standards Institute, Wayne, PA.*

57. White, D. C., and Frerman, F. E. (1967) Extraction, characterization, and cellular localization of the lipids of *Staphylococcus aureus*, *J. Bacteriol. 94*, 1854-1867.

58. Geyer, R., Peacock, A. D., White, D. C., Lytle, C., and Van Berkel, G. J. (2004) Atmospheric pressure chemical ionization and atmospheric pressure photoionization for simultaneous mass spectrometric analysis of microbial respiratory ubiquinones and menaquinones, *J. Mass Spectrom. 39*, 922-929.

59. Jiang, M., Chen, X., Guo, Z. F., Cao, Y., Chen, M., and Guo, Z. (2008) Identification and characterization of (1R,6R)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase in the menaquinone biosynthesis of *Escherichia coli*, *Biochemistry 47*, 3426-3434.

60. Bar-Ilan, A., Balan, V., Tittmann, K., Golbik, R., Vyazmensky, M., Hubner, G., Barak, Z., and Chipman, D. M. (2001) Binding and activation of thiamin diphosphate in acetohydroxyacid synthase, *Biochemistry 40*, 11946-11954.

61. Sergienko, E. A., and Jordan, F. (2002) New model for activation of yeast pyruvate decarboxylase by substrate consistent with the alternating sites mechanism: demonstration of the existence of two active forms of the enzyme, *Biochemistry 41*, 3952-3967.

62. Nahaie, M. R., Goodfellow, M., Minnikin, D. E., and Hajek, V. (1984) Polar lipid and isoprenoid quinone composition in the classification of Staphylococcus, *J. Gen. Microbiol. 130*, 2427-2437.

63. Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., and Barry, C. E., 3rd. (2004) The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism: novel insights into drug mechanisms of action, *J. Biol. Chem. 279*, 40174-40184.

64. Kahl, B. C., Belling, G., Reichelt, R., Herrmann, M., Proctor, R. A., and Peters, G. (2003) Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural

changes consistent with impaired cell separation, *J. Clin. Microbiol. 41*, 410-413.

65. Vaudaux, P., Francois, P., Bisognano, C., Kelley, W. L., Lew, D. P., Schrenzel, J., Proctor, R. A., McNamara, P. J., Peters, G., and Von Eiff, C. (2002) Increased expression of clumping factor and fibronectin-binding proteins by hemB mutants of *Staphylococcus aureus* expressing small colony variant phenotypes, *Infect. Immun. 70*, 5428-5437.

66. Grundling, A., and Schneewind, O. (2007) Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*, *Proc. Natl. Acad. Sci. U S A 104*, 8478-8483.

67. Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., and Peters, G. (2006) Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections, *Nat. Rev. Microbiol. 4*, 295-305.

68. Hammond, R. K., and White, D. C. (1970) Inhibition of vitamin K2 and carotenoid synthesis in *Staphylococcus aureus* by diphenylamine, *J. Bacteriol. 103*, 611-615.

69. Salton, M. R., and Schmitt, M. D. (1967) Effects of diphenylamine on carotenoids and menaquinones in bacterial membranes, *Biochim. Biophys. Acta 135*, 196-207.

Chapter 4:

1. Bryant, R. W., Jr., and Bentley, R. (1976) Menaquinone biosynthesis: conversion of o-succinylbenzoic acid to 1,4-dihydroxy-2-naphthoic acid and menaquinones by *Escherichia coli* extracts, *Biochemistry 15*, 4792-4796.
2. Heide, L., Arendt, S., and Leistner, E. (1982) Enzymatic synthesis, characterization, and metabolism of the coenzyme A ester of o-succinylbenzoic acid, an intermediate in menaquinone (vitamin K2) biosynthesis, *J. Biol. Chem. 257*, 7396-7400.
3. Bahnson, B. J., Anderson, V. E., and Petsko, G. A. (2002) Structural mechanism of enoyl-CoA hydratase: three atoms from a single water are added in either an E1cb stepwise or concerted fashion, *Biochemistry 41*, 2621-2629.
4. Benning, M. M., Haller, T., Gerlt, J. A., and Holden, H. M. (2000) New reactions in the crotonase superfamily: structure of methylmalonyl CoA decarboxylase from *Escherichia coli*, *Biochemistry 39*, 4630-4639.
5. Wong, B. J., and Gerlt, J. A. (2003) Divergent function in the crotonase superfamily: an anhydride intermediate in the reaction catalyzed by 3-hydroxyisobutyryl-CoA hydrolase, *J. Am. Chem. Soc. 125*, 12076-12077.
6. Benning, M. M., Taylor, K. L., Liu, R. Q., Yang, G., Xiang, H., Wesenberg, G., Dunaway-Mariano, D., and Holden, H. M. (1996) Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 A resolution: an enzyme catalyst generated via adaptive mutation, *Biochemistry 35*, 8103-8109.

7. Gasson, M. J., Kitamura, Y., McLauchlan, W. R., Narbad, A., Parr, A. J., Parsons, E. L., Payne, J., Rhodes, M. J., and Walton, N. J. (1998) Metabolism of ferulic acid to vanillin. A bacterial gene of the enoyl-SCoA hydratase/isomerase superfamily encodes an enzyme for the hydration and cleavage of a hydroxycinnamic acid SCoA thioester, *J. Biol. Chem. 273*, 4163-4170.

8. Truglio, J. J., Theis, K., Feng, Y., Gajda, R., Machutta, C., Tonge, P. J., and Kisker, C. (2003) Crystal structure of *Mycobacterium tuberculosis* MenB, a key enzyme in vitamin K2 biosynthesis, *J. Biol. Chem. 278*, 42352-42360.

9. Ulaganathan, V., Agacan, M. F., Buetow, L., Tulloch, L. B., and Hunter, W. N. (2007) Structure of *Staphylococcus aureus* 1,4-dihydroxy-2-naphthoyl-CoA synthase (MenB) in complex with acetoacetyl-CoA, *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 63*, 908-913.

10. Lannergard, J., von Eiff, C., Sander, G., Cordes, T., Seggewiss, J., Peters, G., Proctor, R. A., Becker, K., and Hughes, D. (2008) Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 52*, 4017-4022.

11. CLSI. (2006) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, 7th ed. Approved Standard M7-A7, *Clinical and Laboratory Standards Institute, Wayne, PA.*

12. NCCLS. (1999) Methods for determining bactericidal activity of antimicrobial agents. Approved guideline M26-A., *NCCLS, Wayne, PA.*

13. Brouillette, E., Martinez, A., Boyll, B. J., Allen, N. E., and Malouin, F. (2004) Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo, *FEMS Immunol. Med. Microbiol. 41*, 35-41.

14. Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N., and Arbeit, R. D. (1995) Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*, *Clin. Infect. Dis. 20*, 95-102.

15. Samuelsen, O., Haukland, H. H., Kahl, B. C., von Eiff, C., Proctor, R. A., Ulvatne, H., Sandvik, K., and Vorland, L. H. (2005) *Staphylococcus aureus* small colony variants are resistant to the antimicrobial peptide lactoferricin B, *J. Antimicrob. Chemother. 56*, 1126-1129.

16. von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G., and Gotz, F. (1997) A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly, *J. Bacteriol. 179*, 4706-4712.

17. Clements, M. O., Watson, S. P., Poole, R. K., and Foster, S. J. (1999) CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis, *J. Bacteriol. 181*, 501-507.

18. Bates, D. M., von Eiff, C., McNamara, P. J., Peters, G., Yeaman, M. R., Bayer, A. S., and Proctor, R. A. (2003) *Staphylococcus aureus* menD and hemB mutants are as infective as the parent strains, but the menadione

biosynthetic mutant persists within the kidney, *J. Infect. Dis. 187*, 1654-1661.

19. Johnston, J. M., Arcus, V. L., and Baker, E. N. (2005) Structure of naphthoate synthase (MenB) from *Mycobacterium tuberculosis* in both native and product-bound forms, *Acta Crystallogr. D Biol. Crystallogr. 61*, 1199-1206.
20. Delano, W. L. (2002) in *Pymol*.
21. Oleson, F. B., Jr., Berman, C. L., Kirkpatrick, J. B., Regan, K. S., Lai, J. J., and Tally, F. P. (2000) Once-daily dosing in dogs optimizes daptomycin safety, *Antimicrob. Agents Chemother. 44*, 2948-2953.
22. Silverman, J. A., Perlmutter, N. G., and Shapiro, H. M. (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 47*, 2538-2544.
23. Jung, D., Rozek, A., Okon, M., and Hancock, R. E. (2004) Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin, *Chem. Biol. 11*, 949-957.
24. Conrad, R. S., Howard, M. J., Garrison, R. C., Winters, S., and Henderson, D. A. (1998) The effects of daptomycin on chemical composition and morphology of *Staphylococcus aureus*, *Proceedings of the Oklahoma Academy of Science 78*, 15-21.