Exploring Rhodomyrtone, A Novel Antibiotic, on Methicillin-Resistant *Staphylococcus aureus*: A Combined Proteomics and Transcriptomics Approach

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ชื่อวิทยานิพนธ์ การศึกษาฤทธิ์ของ rhodomyrtone ต่อเซลล์แบคทีเรีย methicillin-resistant *Staphylococcus aureus* โดยใช้ เทคนิคทางโปรตีโอมิกส์และทรานสคริปโตมิกส์
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บทคัดย่อ

Methicillin-resistant *Staphylococcus aureus* (MRSA) เป็นเชื้อแบคทีเรียดื้อยาที่เป็นสาเหตุสำคัญของการติดเชื้อทั้งในโรงพยาบาลและชุมชน จากการทดสอบฤทธิ์ของ rhodomyrtone ซึ่งเป็นสารบริสุทธิ์ในกลุ่ม acylphloroglucinol ที่สกัดจากใบของต้นกระทุ พบว่าค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อ (MIC) MRSA และ *S. aureus* คือ 0.5-1.0 µg/ml และค่าความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อ (MBC) คือ 2.0-4.0 µg/ml การศึกษาฤทธิ์ของ rhodomyrtone ต่อลักษณะการเจริญเติบโตของเชื้อ MRSA พบว่าทุกความเข้มข้นของ rhodomyrtone ได้แก่ 0.125MIC 0.25MIC 0.5MIC MIC และ 2MIC มีฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อในช่วง 5 ชั่วโมงแรก และหลังจาก 18 ชั่วโมงพบว่า rhodomyrtone ที่ความเข้มข้น MIC และ 2MIC สามารถลดจำนวนของเชื้อได้อย่างน้อย 2 log เมื่อเทียบกับชุดควบคุม ในการศึกษานี้ได้ประยุกต์ใช้เทคนิคทางด้าน proteomics และ transcriptomics เพื่อใช้ในการวิเคราะห์กลไกการออกฤทธิ์ของ rhodomyrtone ต่อรูปแบบการแสดงออกของโปรตีนและการตอบสนองของยีนในเชื้อ MRSA พบว่า rhodomyrtone มีผลต่อรูปแบบการแสดงออกของโปรตีนหลายกลุ่มภายในเซลล์แบคทีเรีย โปรตีนดังกล่าวได้แก่ โปรตีนที่เกี่ยวข้องกับการสร้างผนังเซลล์และการแบ่งตัวของเซลล์, การสลายตัวของโปรตีนในเซลล์, stress response และ oxidative stress, antigen บนผิวเซลล์, ปัจจัยก่อความรุนแรงของเชื้อ, และโปรตีนที่เกี่ยวข้องในเมแทบอลิซึมต่างๆ ได้แก่ โปรตีนที่เกียวกับการสังเคราะห์กรดอะมิโน คาร์โบไฮเดรต พลังงาน ไขมัน และนิวคลีโอไทด์ ภาพจากกล้องจุลทรรศน์อิเล็คตรอนยืนยันฤทธิ์ของ rhodomyrtone ที่มีต่อการเปลี่ยนแปลงรูปร่าง ลักษณะภายในเซลล์ และผิวเซลล์แบคทีเรีย โดยมีผลในการยับยั้งหรือรบกวนกระบวนการสร้างผนังเซลล์และการแบ่งตัวของเซลล์ นอกจากนี้ยังมีผลทำให้เซลล์แบคทีเรียมีขนาดและรูปร่างผิดปกติ ผลการศึกษาการแสดงออกของยีนส์ทั้งหมดในเซลล์ MRSA โดยใช้

microarray assay พบว่า rhodomyrtone มีผลต่อการแสดงออกของยีนส์ในหลายกลุ่ม (การแสดงออกแตกต่างมากกว่าสองเท่า, *p*-value $\leq$ 0.05) นอกจากนี้ยังพบว่ากลุ่มของยีนส์ที่ไม่สามารถระบุหน้าที่ (hypothetical protein) จำนวนอย่างน้อย 48.5% ของยีนส์ทั้งหมดที่มีการแสดงออกเพิ่มขึ้น ยีนส์ที่มีการแสดงออกเพิ่มขึ้นอย่างเด่นชัดได้แก่ ยีนส์ที่เกี่ยวข้องกับการสร้างกรดอะมิโนในกลุ่ม aspartate family กรดอะมิโน Lysine สร้างจาก aspartate โดยผ่านทาง diaminopimelate (DAP) pathway นอกจากนี้ Lysine ยังมีความสำคัญต่อการสังเคราะห์ peptidoglycan ของผนังเซลล์แบคทีเรีย จากนั้นได้ศึกษาฤทธิ์ของ rhodomyrtone ต่อการแสดงออกของยีนส์ในแบคทีเรีย เมื่อบ่มในเวลาต่างๆกัน โดยใช้เทคนิค quantitative reverse transcription-PCR (qRT-PCR) โดยยีนส์ที่นำมาทดสอบประกอบด้วย ยีนส์ 8 ยีนส์ที่เลือกจากผล microarray ได้แก่ *asd dapA thrC scdA rpoB* SAR0996 SAR0437 และ SAR0761 และยีนส์ 4 ยีนส์ (ไม่แสดงผลใน microarray) ได้แก่ *lysA ftsH ftsZ* และ *murE* นอกจากนี้ยังพบว่า rhodomyrtone มีผลทำให้ค่าความเข้มข้นต่ำสุดของ lysostaphin ที่สามารถยับยั้งเชื้อ MRSA เพิ่มขึ้น 4-8 เท่า จากผลดังกล่าว rhodomyrtone อาจจะออกฤทธิ์ต่อโครงสร้าง pentaglycine cross-bridge ของ peptidoglycan อย่างไรก็ตามเมื่อศึกษาฤทธิ์ของ rhodomyrtone ต่อส่วนประกอบของกรดอะมิโนใน peptidoglycan ที่แยกได้จากเชื้อแบคทีเรียโดยเทียบกับชุดควบคุมพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญ แม้ว่าผลการวิเคราะห์รูปแบบการแสดงออกของโปรตีนแสดงให้เห็นว่าโปรตีน autolysin หายไปหลังการกระตุ้นเชื้อ MRSA ด้วย rhodomyrtone แต่เมื่อทดสอบโดยใช้ Triton-X 100 กระตุ้นการเกิด autolysis พบว่าฤทธิ์ของ rhodomyrtone ต่อ autolysis ของเชื้อเมื่อเทียบกับชุดควบคุมไม่มีความแตกต่างอย่างมีนัยสำคัญ จากการใช้เทคนิคด้าน proteomics ร่วมกับ transcriptomics ได้ช่วยบ่งชี้โปรตีนและยีนส์ที่อาจจะเป็นเป้าหมายของกลไกการออกฤทธิ์ของ rhodomyrtone ในเชื้อ MRSA ข้อมูลการแสดงออกของยีนส์จากเชื้อ MRSA ภายหลังกระตุ้นด้วย rhodomyrtone ที่ได้จาก microarray และ qRT-PCR แสดงให้เห็นว่า rhodomyrtone มีผลต่อกระบวนการสังเคราะห์ lysine และ peptidoglycan ซึ่งอาจจะเป็นส่วนหนึ่งของกลไกการต้านเชื้อแบคทีเรียของ rhodomyrtone นอกจากนี้การศึกษาลักษณะที่แสดงออกของเชื้อ (phenotypical assay) ส่วนประกอบทางเคมี (biochemical assay) และภาพจากกล้องจุลทรรศน์อิเล็คตรอน มีส่วนช่วยในการอธิบายกลไกการออกฤทธิ์ของ rhodomyrtone

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial infections and has also become a serious problem of community-acquired infections. Rhodomyrtone, an acylphloroglucinol derivative isolated from leaf of *Rhodomyrtus tomentosa* (Aiton) Hassk., exhibited minimal inhibitory concentration (MIC) and minimal inhibitory concentration (MBC) at 0.5-1.0 μg/ml and 2.0-4.0 μg/ml against both MRSA and *S. aureus* ATCC 29213, respectively. Time-kill study on MRSA was assessed at 0.125MIC, 0.25MIC, 0.5MIC, MIC, and 2MIC of rhodomyrtone by counting viable cells at time intervals. Rhodomyrtone at all concentrations inhibited the bacterial growth over the first approximately 5 h. At MIC and 2MIC, numbers of viable cells were at least two log-fold reductions relative to untreated control within 18 h. In this study, proteomic and transcriptomic techniques were applied to provide insights into antibacterial mechanism of rhodomyrtone involved on cellular protein expression and transcriptional response in MRSA. The proteomic profiling indicated that rhodomyrtone affected the expression of several major functional classes of cellular proteins. Some of these proteins involve in cell wall biosynthesis and cell division, protein degradation, stress response and oxidative stress, cell surface antigen and virulence factor, and various metabolic pathways such as amino acid, carbohydrate, energy, lipid, and nucleotide metabolism. Electron micrographs confirmed effects of rhodomyrtone on morphology, ultrastructure, and cell surface alterations of the bacterial cells. Biological processes

in cell wall biosynthesis and cell division were seemingly interrupted. Unusual sizes and shapes of staphylococcal cells were obvious in treated MRSA samples. Genome-wide expression by microarray analysis revealed that rhodomyrtone rendered many groups of differentially expressed genes (more than 2-fold change at *p* value of ≤0.05). A gene encoding protein of unknown function, hypothetical protein, was the largest proportion of up-regulated genes (at least 48.5%). Of particular interest was prominent up-regulation of a group of genes involve in biosynthetic pathway of amino acids belonging to aspartate amino acid family. Lysine is produced from aspartate through the diaminopimelate (DAP) pathway that is also essential in cell wall peptidoglycan biosynthesis. Time course analysis of eight genes that selected from microarray data including *asd, dapA*, *thrC*, *scdA*, *rpoB*, SAR0996, SAR0437, and, SAR0761, and four genes of interest including *lysA*, *ftsH*, *ftsZ*, and *murE* (not detected by microarray), were examined using quantitative reverse transcription-PCR (qRT-PCR). In a lysostaphin MIC assay, rhodomyrtone produced a 4-8 fold increase in lysostaphin resistance in MRSA suggested that pentaglycine cross-bridge structure of peptidoglycan of MRSA might be affected by rhodomyrtone. However, the analysis of the peptidoglycan amino acid contents in rhodomyrtone-treated cells demonstrated relatively no significant difference, when compared to the control. Even though the production of autolysin was suppressed after rhodomyrtone challenge (from protein analysis data), the staphylococcal cells in the presence of rhodomyrtone exhibited no significant difference to Triton-X 100 induced autolysis, when compared to the control. Based on a combined proteomics and transcriptomics approach, cellular responses of MRSA to rhodomyrtone were assigned to possible potential proteins and specific molecular targets. Protein profiling revealed the effects of rhodomyrtone on several function classes of MRSA cellular protein. The gene expression data from microarray and qRT-PCR of MRSA to rhodomyrtone challenge obviously suggested that rhodomyrtone acts through lysine and/or peptidoglycan biosynthetic pathway that might be the parts of its antimicrobial mode of actions. Together with supporting phenotypical assay, biochemical assay, and electron microscopic examination, the results do provide some guidance on anti-MRSA mode of actions of rhodomyrtone.

CONTENTS

LIST OF TABLES

Tables **Page**

LIST OF FIGURES

Figures **Page**

Figures **Page**

LIST OF ABBREVIATIONS

%	=	percent
°C	=	degree celcius
µg	=	microgram
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
2DE	=	two-dimemsional ge electrophoresis
ATP	=	Adenosine triphosphate
ATCC	=	American Type Culture Collection
bp	=	base pairs
cDNA	=	complementary deoxyribonucleic acid
cfu	=	colony-forming units
CLSI	=	Clinical and Laboratory Standards Institute
cm	=	centimeter
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
g	=	gram
G+C	=	guanine and cytosine
h	=	hour
HPLC	=	high-performance liquid chromatography
kDa	=	kiloDalton
l	=	liter
M	=	molar
mg	=	milligram
MIC	=	minimal inhibitory concentration
MBC	=	minimal bactericidal concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter

mRNA	=	messenger RNA
MRSA	=	methicillin-resistant *Staphylococcus aureus*
NA	=	not available
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
NPRC	=	natural product research centre
OD	=	optical density
ORF	=	open reading frame
PCR	=	polymerase chain reaction
pI	=	isoelectric point
qRT-PCR	=	qualtitaive reverse transcription-polymerase chain reaction
RNA	=	ribonucleic acid
SCC	=	staphylococcal cassette chromosome
SD	=	standard deviation
SDS	=	sodium dodecylsulfate
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel
SEM	=	scanning electron microscope / microscopy
TEM	=	transmission electron microscope / microscopy
U	=	unit
UV	=	ultraviolet
VISA	=	vancomicin intermediate-resistant *Staphylococcus aureus*

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

Staphylococcus aureus is well-evidenced as a major human pathogen. The organism commonly involves in skin and soft tissue infections such as pimples, boils, furuncles, cellulites, folliculitis, impetigo, carbuncles, scalded skin syndrome, and abscesses (Archer and Climo 2001; Lowy 1998). In addition, it can cause some serious infections including bacteremia, pneumonia, acute endocarditis, meningitis, osteomyelitis, toxic shock syndrome, and fatal invasive diseases (Klevens *et al.* 2007). Discovery of penicillin in early 1940s was expected for treatment of life-threatening bacterial diseases including staphylococcal infection (Rolinson 1979). However, the prevalence of penicillinase-producing *S. aureus* was rapidly evidence within a few years (Neu 1983). In the 1960s, one year after the introduction of methicillin in the clinical practice, the emergence and spread of methicillin-resistant *S. aureus* (MRSA) has been originated as an important clinical and epidemiological problem in hospital environments (Parker and Jevons 1964; Jevons, Coe, and Parker 1963). MRSA was first discovered in United Kingdom and developed in to a worldwide health problem. Additionally, *S. aureus* has been a crucial cause of community-acquired infections which may result in morbidity and mortality (Cookson 2000; Zetola *et al.* 2005).

The introduction of glycopeptide antibiotics, a current resort for treatment MRSA infection, was followed by the isolation of either vancomycin-intermediate *S. aureus* (VISA) or vancomycin-resistant *S. aureus* (VRSA) (Chang *et al.* 2003; Hiramatsu 2001). The occurrence of a fully vancomycin resistant strain of MRSA has been reported in 2002, indicated that the successful treatment of MRSA strains by use of glycopeptides antibiotic is not affirmed. MRSA strains that have a tendency to acquire multiple drug resistant determinants are now very common not

only in hospitals and intensive care units around the world but also in the communities (Zetola *et al.* 2005).

Many new drugs such as linezolid (Tsiodras *et al.* 2001), daptomycin (Steenbergen *et al.* 2005), and tigecycline have been approved for clinical use for treatment of MRSA infection (Bradley 2005), however, the isolation of resistant microorganisms have been being isolated (Chang *et al.* 2003; Hiramatsu 2001). In currently medical use, almost all antimicrobial agents are the products of semirational optimization programs based on natural compounds expressed to possess antimicrobial activity in whole cell screening assays (Rosamond and Allsop 2000). A major challenge for pharmaceutics industry is the discovery and development of new drug classes with novel mechanisms of action that will provide effective therapy. Several active researches have documented new antibiotics and semi-synthetic analogs with improved antimicrobial properties (Higgins *et al.* 2005; Ryan *et al.* 2006; Coates *et al.* 2002). Various plants worldwide have been used in traditional medicine as alternative treatments of bacterial infections (Martin and Ernst 2003; Tomczykowa *et al.* 2008; Samy and Gopalakrishnakone 2008; Zhao *et al.* 2001). Plants have been considered as biologically diverse group that deserves a source of chemical diversity for applying as medicine because of the exceptional capability to generate cytotoxicity agents, and the ecological rational that antimicrobial natural compounds should be produced following the organism assault to protect the plants from invasive and pathogenic microorganisms around them.

Rhodomyrtone, an acylphloroglucinol derivative isolated from the leaf of *Rhodomyrtus tomentosa* (Aiton) Hassk., has been briefly reported to produce antibacterial effects against *Escherichia coli* and *S. aureus* (Dachriyanus *et al.* 2002). Moreover, *R. tomentosa* leaf has been pharmacologically documented as a folk medicine in Asia on treatment of hepatitis, gastroenteritis, dyspepsia, dysentery, rheumatic arthritis, and wound bleeding (Sung *et al.* 1998). The preliminary studies from our research group suggested that the ethanolic extract of *R. tomentosa* and rhodomyrtone presented strong antimicrobial activity against a wide range of Gram-positive bacteria such as *Bacillus cereus, B. subtilis*, *Enterococcus faecalis*, *S. aureus*, *S. epidermidis*, and *Streptococcus* spp. (Saising *et al.* 2008; Limsuwan and Voravuthikunchai 2008; Limsuwan *et al.* 2009) (Voravuthikunchai, Dolah, and

Charernjiratrakul 2010). In addition, rhodomyrtone also exhibited significant antimicrobial activity against *S. epidermidis* biofilm-forming, *S. pneumoniae* capsule-producing strains (Limsuwan *et al.* 2009). Several previous studied have attempted to examine the antimicrobial activities of rhodomyrtone against several pathogenic bacteria, however cellular protein expression and transcriptional gene targets have not been defined.

The beginning of proteomics has been established since the invention of the highly sensitive two-dimensional polyacrylamide gel electrophoresis (2DE) by Klose (1975) and O'Farrell (1975). The technique was recently adopted to various microbiology works. 2DE are proved to be a valuable tool for providing a proteome-wide view of the alterations in the protein profile triggered by environmental stimuli such as stress or starvation and drugs (Segura *et al.* 2005; Chatterjee *et al.* 2009; Sibbald *et al.* 2006; Hecker *et al.* 2009; Sianglum *et al.* 2011). Development of several concurrence techniques is not only dramatically induced the widespread of proteomic expression profiling but also launched a novel era of cellular physiology. Moreover, genome sequencing arrival rendered the highly sensitive and large-scale identification of proteins by mass spectrometry (MS). Bioinformatics tools such as MASCOT or SEQUEST allowed the productive cross-examination of databases with MS data. Significant proteome analyses have also been achieved for many pathogenic bacteria. In recent years, 2DE reference maps of cellular or extracellular proteins from various *S. aureus* strains in different growth conditions have been established (Cordwell *et al.* 2002; Kohler *et al.* 2005; Scherl *et al.* 2005). The identification of cell surface, cell membrane, and cytoplasmic proteome map of *S. aureus* provides essential tool for better understanding of biological, pathological, and physiological significance of the bacteria. Furthermore, using 2DE separation technique combined with tryptic peptide mass mapping via mass spectrometry, the global view of the synthesis and distribution of various *S. aureus* protein networks were obtained (Becker *et al.* 2007; Cordwell *et al.* 2002).

Using a global approach to study transcriptional responses, are a powerful indicator of the effects of therapeutic agents on whole cells or tissue of animals. Cell-specific gene expression and the preferential transcription of the gene in microorganisms during exposure to antimicrobials can implicate the potential

relevance of the genes to antimicrobial drug targets (Utaida *et al.* 2003; Muthaiyan *et al.* 2008; Petek *et al.* 2010). The information provides by microarray (DNA-chip) based expression profiling is expected to be significant in the cellular response to curtain environment or antibiotics.

To characterize the antibacterial mechanism of actions of rhodomyrtone to MRSA, proteomic and transcriptomic approach, and supporting biochemical and physiological assay were utilized. Achievements in protein analysis were acquired by the combination of mass spectrometry techniques and bioinformatic tools. Gene expression profiling using full genome *S. aureus* microarray and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were performed to elucidate the global transcriptional response of MRSA to rhodomyrtone. The effects of rhodomyrtone on morphological, ultrastructural, and surface changes in the treated staphylococcal cells were illustrated using electron microscopy.

OBJECTIVES OF THE RESEARCH

The major objective of this project was to study rhodomyrtone's mechanism of action on methicillin-resistant *Staphylococcus aureus* (MRSA). Particular emphasis was placed on cellular protein profiling and transcriptional responses on MRSA analysis after rhodomyrtone challenge.

The specific objectives of the project were:

1. To determine the antimicrobial activity of *Rhodomyrtus tomentosa* leaf ethanolic extract and rhodomyrtone against MRSA and *S. aureus*.
2. To investigate cellular protein expression profiles upon challenge MRSA with rhodomyrtone proteomic technologies.
3. To identify differentially expressed genes in MRSA cellular response to rhodomyrtone on global scale using microarray analysis.
4. To validate expression of selected genes of interest using quantitative reverse transcription-PCR (qRT-PCR).
5. To illustrate effects of rhodomyrtone on morphology and ultrastructure of MRSA and *S. aureus* using electron microscopy
6. To examine effects of rhodomyrtone on phenotypical consequences of MRSA.

CHAPTER 2

LITERATURE REVIEW

1. *Staphylococcus aureus*

1.1 Characterization of the staphylococci

Staphylococci are Gram-positive spherical bacteria, 0.7-1.2 μm in diameter, occurring singly, in pairs, and microscopic grape-like clusters. The genus *Staphylococcus* belongs to the bacterial family "*Staphylococcaceae*". They are facultative anaerobes that grow under aerobic condition or in the presence of CO_2. Colonies of *S. aureus* are β-hemolytic due to the production of several hemolysins including α-hemolysin, β-hemolysin, γ-hemolysin, and δ-hemolysin (Cheung *et al.* 2004). *S. aureus* is differentiated from other species on the basis of the golden pigmentation of colonies, positive results of coagulase, mannitol fermentation, and deoxyribonuclease test (Betley, Borst, and Regassa 1992). The golden pigmentation of *S. aureus* colonies (Figure 1A and 1 B) is caused by the presence of carotenoids that has been reported to be a virulence factor protecting the organism against oxidants produced by host immune system (Liu *et al.* 2005). *Staphylococcus* sp. demonstrates catalase-positive, a feature discriminating them from *Streptococcus* sp. and the organisms are oxidase-negative and require complex nutrients for growth. Staphylococci can grow in a wide pH range (4.8-9.4), tolerance drying and NaCl concentrations as high as 15%, and survive at a temperature range of 15-45°C.

Although *S. aureus* is an important pathogen, many healthy people carry it as part of the normal population of microorganism associated with the nose, throat, perineum, or skin. Among staphylococci, *S. aureus* is usually regarded as a transient, pathogenic organism in the skin, and approximately 20% of the general population always harbours it on the mucosa without any pathogenic event (Peacock

et al., 2001). However, it is known to cause a wide range of infections in human including bacteraemia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, encephalitis, meningitis, choriamnionitis, mastitis, scalded skin syndrome, toxic shock syndrome (TSS), abscesses of the muscle, urinogenital tract, and various intra-abdominal organs (Murray *et al.* 2003; Lowy 1998) (Iwatsuki *et al.* 2006).

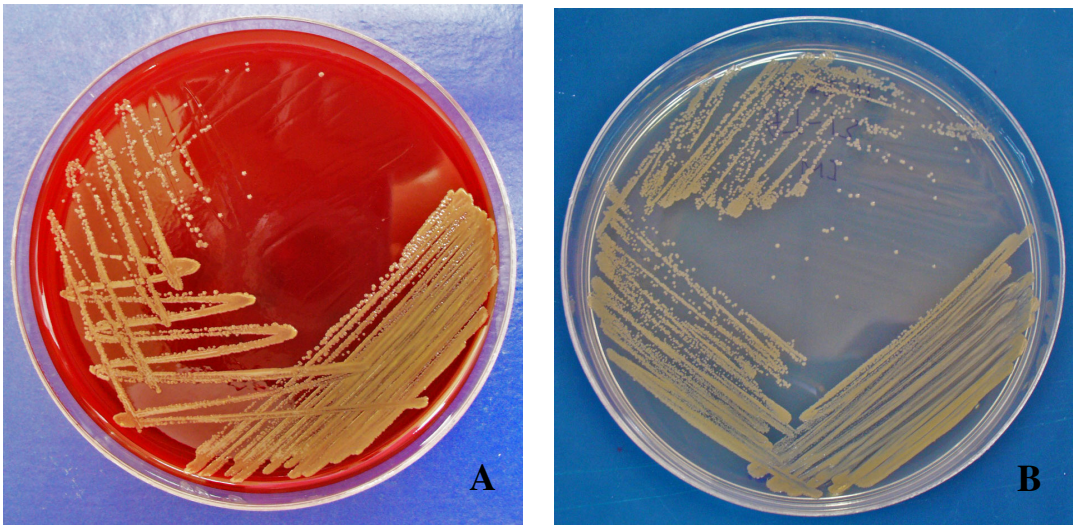


Figure 1. Golden pigment of *Staphylococcus aureus* colonies on blood agar (A) and on Luria-Bertani agar (B). The colonies are 6-8 mm diameter with yellow color, opaque, and smooth entire edge. The plates were incubated at 37°C for 24 h.

1.2 Pathogenesis

Staphylococcus aureus is clearly predominant pathogen among all staphylococcal species due to many combined factors rather than a single factor (Cheung *et al.* 2004). The pathogenic capacity of *S. aureus* is multifactorial (Bronner, Monteil, and Prevost 2004). The large number of newly characterized genes displays the complexity of regulatory networks, which is consistent with the extraordinary capability of *S. aureus* to adapt to human host. Moreover, *S. aureus* contains genes encoding toxins, cell surface proteins, and resistance to antimicrobials, which might strengthen its virulence and confer resistance to all families of antibiotics. Virulence factors of *S. aureus* comprise cell surface structural components such as proteins, lipids, carbohydrates, proteoglycans, glycolipids, and secretory products. Based on their biological activities, the *S. aureus* virulence factors can be divided into three general functional categories: including mediate adhesion of bacteria to host cells or tissue (adhesins), promote tissue damage and spread (invasins), and protect the bacteria from the host immune system. Therefore, the pathogenicity of *S. aureus* depends on the combined action of cell surface structural components, different extracellular toxins, and enzymes. The expression of virulence factors of *S. aureus* is dependent on the bacterial growth phase. The cell surface components are expressed during exponential growth phase whereas the secretory factors are expressed during post exponential growth phase (Cheung *et al.* 2004). The virulence factors are also differently circulated among strains, they are not always regulated in the same way in different strains.

1.3 Cellular structure

The staphylococcal cell envelope is a complex structure that consists of a cell membrane composed of lipids and proteins, a cell wall made peptidoglycan and teichoic acids, and polysaccharides. Peptidoglycan, present in all bacteria, provides shape and structural integrity (Scheffers and Pinho 2005; Pinho and Errington 2003). Cell membrane integrity is necessary to maintain a boundary between the external environment and the cytoplasm. The membrane also holds a number of proteins that transport solutes across chemical gradients, expending ATP or membrane potential. The electron transport machinery including NADH oxidase, cytochrome, and F_0F_1-

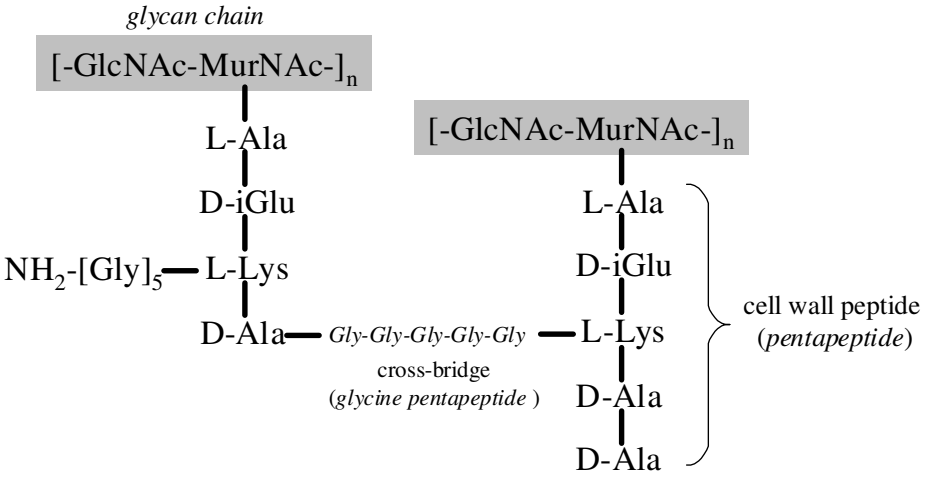
ATPase is also placed to the cell membrane, producing ATP and establishing electrochemical gradient across the membrane that powers abundance activities. In addition, the cell wall envelopes of gram-positive bacteria also promote interactions between bacteria and their environment (Marraffini, Dedent, and Schneewind 2006).

1.3.1 Cell wall

Major components of staphylococcal cell wall are peptidoglycan (also called murein) and teichoic acid, which are approximately 50-60% and 30-50% of the dry weight. It plays an important role in maintaining the spherical shape of the bacterial cells (Koch 2003). Peptidoglycan consists of alternating polysaccharide subunits of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) with β-1,4 linkages. Peptidoglycan chains is cross-linked by a chain of a pentaglycine-cross bridge from the &-amino group of L-lysine in one peptide to the D-alanine residue in position 4 of an adjacent peptide (Figure 2.) (Lowy 1998; Scheffers and Pinho 2005). Peptidoglycan possesses endotoxin-like activity, stimulating the release of cytokines by macrophages, activation of complement, and aggregation of platelets (Dziarski and Dziarski 1979; Timmerman *et al.* 1993). Variations in the peptidoglycan composition among staphylococcal strains may contribute to differences in their capacity to cause disseminated intravascular coagulation (Kessler, Nussbaum, and Tuazon 1991).

1.3.2 Protein A

Protein A is a major surface protein of *S. aureus* strains that covalently anchored to the peptidoglycan and belongs to the cell wall-associated virulence factors of *S. aureus*. It encoded by *spa* which is one of the most widely studied and characterized staphylococcal cell wall-anchored surface components (DeDent, McAdow, and Schneewind 2007). It binds to the Fc portion of IgG molecule (IgG1, IgG2, and IgG4) except IgG3. The Fab portion of IgG bound to protein A is free to combine with a specific antigen. Protein A has become an important reagent in immunology and diagnostic laboratory technology for example, protein A with attached IgG molecules directed against a specific bacteria antigen will agglutinate bacteria that have that antigen (coagglutination).



(modified from Navarre and Schneewind 1999)

Figure 2. Diagram of peptidoglycan structures of *Staphylococcus aureus*. Glycan chains composed of a heteropolymer of a repeating disaccharide, N-acetyl-D-glucosamine (GlcNAc) and N-Acetylmuramic acid (MurNAc), are linked to short wall peptides through the lactyl moeity of MurNAc. Adjacent wall peptides can be linked through crossbridge peptides, glycine pentapeptide.

1.3.3 Capsules

Staphylococcal capsules are located external to the cell wall and they are primarily antigenic (capsule) polysaccharides. Most staphylococci produce microcapsules, which inhibit phagocytosis by polymorphonuclear leukocytes unless specific antibodies are present. Serotyping studies of staphylococcal isolates revealed that serotypes 5 and 8 isolates (of 13 capsular serotypes identified) account for approximately 80% of isolates recovered from humans. Most methicillin-resistant *S. aureus* isolates are type 5 (Roghmann *et al.* 2005). The chemical composition of four of these antiphagocytic polysaccharides, including types 5 and 8, has been determined, and all four have been shown to be chemically related (Lee 1996).

1.3.4 Toxins

Staphylococcus aureus produces many toxins that are grouped on the basis of their mechanisms of action. The staphylococcal α-toxin is a major virulence determinant encoded by the *hla* gene. It can cause pore formation and induce proinflammatory changes in mammalian cells (Bhakdi and Tranum-Jensen 1991). The α-toxin (hemolysin) is the heterogenous protein that can lyses erythrocytes and damage platelets and is propably identical with the lethal and dermonecrotic facteors of exotoxin. *Staphylococcus aureus* also produces a toxin called the Panton-Valentine leucocidin (PVL), now an established virulence factor linked to community-acquired MRSA strains (Boyle-Vavra and Daum 2007). PVL is a bicomponent, poreforming leukotoxin designated initially 'substance leukocidine' by Van deVelde in 1894 due to its ability to lyse leukocytes. Panton and Valentine first associated the leukotoxin with skin and soft tissue infection (Boyle-Vavra and Daum 2007).

Toxic shock syndrome toxin (TSST) acts as superantigens, which are protein toxins that share the ability to trigger excessive and non-conventional T-cell activation with consequent downstream activation of other cell types, and cytokine/chemokine release (Lappin and Ferguson 2009). Staphylococcal TSST-1, encoded by the *tst* gene, is a major virulence factor in toxic shock syndrome, responsible for nearly all (95%) menstrual-related TSS cases (Parsonnet *et al.* 2005). *tst*-positive MRSA strains appear to be highly virulent and to cause a variety of illness, ranging from toxic shock syndrome to various suppurative infections (Tristan *et al.* 2007).

The exfoliative toxins cause skin erythema and separation, as seen in the staphylococcal scalded skin syndrome. The study of Yamaguchi *et al.* in 2002 raises the question of whether new exfoliative toxin A (with the *eta* gene) and exfoliative toxin B (with the *etb* gene) positive MRSA groups are emerging worldwide.

1.3.5 Enzymes

Staphylococci produce a number of exoproteins that are widely presumed to contribute to pathogenesis by promoting nutrient acquisition and host tissue invasion, such as protease, lipase, nuclease, and hyaluronidase; or spreading factor, and a staphylokinase resulting in fibrinolysis but acting much more slowly than streptokinase. Staphylococci can produce catalase, which convert hydrogen peroxide into water and oxygen. The catalase test differentiates staphylococci, which positive, from streptococci, which are negative. Coagulase, an enzyme like-protein that clots oxalated and citated plasma in the presence of factor contained in many sera, is secreted by virtually all *S.aureus* isolates. The serum factor reacts with coagulase to generate both esterase and clotting activities, in a manner similar to the activation of prothrombin to thrombin. The action of coagulase circumvents the normal plasma clotting cascade. Coacgulase production is considered synonymous with invasive pathogenic potential. β-Lactamase is an enzyme that hydrolyzes the β-Lactam ring, rendering β-Lactam inactive. Penicillin-binding proteins are enzymes located in the cytoplasmic membrane that are involved in cell-wall assembly.

Staphylococcus aureus also expresses many other surface proteins including extracellular adherence protein (Eap), fibronectin binding proteins (FnBPs), collagen adhesin protein (*can*), fibrinogen binding proteins (FgBPs), a vitronectin binding protein, and elastin binding protein. These proteins serve as adhesins to allow the bacterium to attach to the host cell surface during early stage of infection to achieve colonization of host tissue (Lee 1996).

2. Genomics of the *Staphylococcus aureus*

Genome sequencing of the staphylococci has provided more understanding into its virulence, resistance, physiology, and host interactions (Baba *et al.* 2002). Whole cell genome sequences of 14 *S. aureus* have been available in the public domain (www.ebi.ac.uk/genomes/bacteria. html) (Lindsay 2010) that widely used for comparative analyses to identify both conserved metabolic and structural functions as well as species-specific functions enabling virulence and host-specific adaptation (Cheng *et al.* 2011; Kuroda *et al.* 2003; Delaune *et al.*). The genome sequences have also been used as the basis for the application and development of postgenomic tools including microarray, protein arrays, and other high-throughput approaches that are valuable to inspect genomic diversity and transcriptional responses, identify small RNA, and investigate the *S. aureus* proteome (Nagarajan and Elasri 2007; Sergeev *et al.* 2004).

The core *S. aureus* genome is composed of approximately 80%, 1930, of the genes conserved among strains, encoding essential metabolic and regulatory functions as well as surface proteins with roles in tissue adhesion and surface architecture. The remaining 20% of the *S. aureus* genome, often referred to as the accessory genome or unessential genetic material, is assembled from mobile genetic elements (MGEs) that are integrated throughout the genome and carry about 50% of known *S. aureus* virulence factors. The major elements which include pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmid and transposons, transfer horizontally between strains and though to be play important roles in the evolution of *S. aureus* virulence and emergence of new strains with clinical implications (Lindsay). Particular lineages may dominate in carriage and disease as a result of MGE-mediated movement of toxin genes associated with these lineages. The regulation of virulence in *S. aureus* is a complex and coordinated process involving with the expression and interaction of several genes and enables the pathogen to establish and maintain infection.

3. Resistance to antimicrobial agents

Antimicrobial resistance has been considered as a major problem in Public Health world wide. In 2005, 18,650 deaths were estimated in patients with invasive MRSA disease in the USA. Methicillin-resistant *S. aureus* human isolates are frequently resistant to the penicillinase-resistant penicillins (Kloos and Bannerman, 1995; Tenover and Gaynes, 2000). Introduction of methicillin into medical practice in the early 1960s quickly resulted in the isolation of methicillin-resistant staphylococci. The first clone in hospitals were reportted in 1961, however, MRSA detection rates were relatively low (1-2%) in most hospitals until epidemic MRSA emerged. Methicillin, a β-lactam antibiotic, acts by inhibiting penicillin-binding proteins (PBPs) which are involved in the synthesis of peptidoglycan, an essential cell wall component. Methicillin resistant- *S. aureus* (MRSA) is resistant to most antibiotics derived from penicillin, including methicillin. Low-level resistance is generally the result of β-lactamase overproduction, increased levels of intrinsic PBPs, or reduction of the binding-affinity (Tomasz *et al.* 1989; Barg, Chambers, and Kernodle 1991). High-level resistance is always dependent on the expression of an alternative low-affinity PBP (PBP2a) encoded by the *mecA* gene that can function as the original PBP but is resistant to methicillin and all other β-lactam antibiotics (Hartman and Tomasz 1984) (Stapleton and Taylor 2002) and they allow the strains to grow in the presence of drug concentrations sufficient to inactivate the PBP modules of all other multimodular PBPs.

The *S. aureus* genome revealed the existence of many mobile genetic elements, including insertion sequences, transposons, bacteriophages and pathogenicity islands (Novick 2003; Baba *et al.* 2002), which contain specific determinants responsible for disease and antibiotic resistance.

The *mecA* gene is located on a mobile genetic element known as the staphylococcal cassette chromosome *mec* element (SCC*mec*) (Hiramatsu *et al.* 2001). SCC*mec* is a basic mobile genetic element that serves as a vehicle for gene exchange among staphylococcal species. SCC can also carry other antibiotic resistance genes which may be integrated small plasmids or on small transposon. SCC is probably mobilized by proteins encoded by *ccr* (recombinase genes) (Katayama, Ito, and

Hiramatsu 2000), the process does not seem to be very efficient. They have been typed according to the sequences of the *ccr* (*ccrA/ccrB,* or *ccrC*) and *mec* complexes with five cores (types I to V) being prevalent but with considerable variation in the genetic organization within each element (Ito *et al.* 2004; Ito *et al.* 2001).

The type I SCC*mec* contains the *mecA* gene as the only resistance element, while the type II and III elements contain, besides *mecA*, multiple determinants for resistance against non-β-lactam antibiotics. Accordingly, type II and III SCC*mec* elements are responsible for multidrug resistance in nosocomial MRSA isolates. Type IV SCC*mec* elements, like type I elements, contain no resistance genes other than *mecA*, and they are significantly smaller than the type II and III elements. This might serve as an evolutionary advantage, making it easier for these mobile genetic elements to spread across bacterial populations. Type V SCC*mec* elements are also small compared to the other elements and differ in their set of recombinase genes (Ito *et al.* 2004). Whereas the type I to IV SSC*mec* elements contain the two recombinase genes *ccrA* and *ccrB*, the type V elements contain a single copy of a gene, *ccrC*, homologous to a cassette chromosome recombinase gene.

4. Antibiotics used for treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections

4.1 Vancomycin

Vancomycin is a first-generation glycopeptide antibiotic. The mode of action is inhibiting growth of the microorganism by binding to cell wall precursors, specifically the terminal two alanine peptides in a pentapeptide-carbohydrate complex, to prevent transglycosylation. Vancomycin has been the mainstay to treat serious infections cause by methicillin- and other β-lactam-resistant staphylococci. Resistance to vancomycin by *S. aureus* is relatively new and still quite limited. The first cases of intermediate resistance being reported in the United States in 1997 (Smith *et al.* 1999), and complete resistance being reported in 2002 have been recently reviewed by Appelbaum and Bozdogan. The resistance mechanisms in vancomycin intermediate *S. aureus* (VISA) strains with decreased susceptibility are different from those having complete resistance. For intermediate-resistance strains,

the two terminal amino acids remain capable of binding vancomycin, but preliminary data suggest that the precursor structures on which the pentapeptides are presented are increased in number and are more varied in structure. This results in an increased number of vancomycin binding sites available, resulting in the "trapping" of vancomycin in the upper layers of the cell wall, preventing the antibiotic from reaching a sufficient number of critical substrates that would then lead to cell death (Lowy 2003). Of great importance is the observation that within every population of *S. aureus*, a low frequency of these intermediate resistant organisms exist, which may become selected out in patients with prolonged exposure to vancomycin.

Additionally, vancomycin-resistant MRSA have recently emerged in United States (Chang *et al.* 2003; Hiramatsu 2001). Vancomycin resistance is produced by acquisition of a set of resistance genes from vancomycin-resistant *Enterococcus* (Showsh, De Boever, and Clewell 2001). The protein encoded by these genes are responsible for the C-terminal D-alanyl–D-alanine (D-Ala–D-Ala) of the disaccharide pentapeptide cell wall precursor with a depsipeptide, D-alanyl–D-lactate (D-Ala–D-Lac), thereby lowering the cell wall affinity for vancomycin (Gonzalez-Zorn and Courvalin 2003). Chemical modification of glycopeptides has been used as a strategy to overcome this resistance to introduce additional transpeptidase and membrane binding sites. Therefore, second-generation glycopeptides, dalbavancin, and oritavancin have been produced (Kim *et al.* 2008; Jabes *et al.* 2004)

4.2 New anti-methicillin resistant-*S. aureus* agents

As resistance to methicillin and vancomycin increase in *S. aureus*, many researchers were challenged to find the alternative and efficient antibiotics. Linezolid is a new class of antibiotics of a protein synthesis inhibitor, with activity against MRSA. Linezolid is bacteriostatic against *S. aureus*. It binds to 23S rRNA of the 50S ribosomal subunit and prevents formation of the 70S initiation complex (Murray *et al.* 2003). It is active against a wide range of gram-positive cocci, including streptococci that are penicillin- and macrolide resistant, and coagulase-negative staphylococci, but it has very limited activity against gram-negative

organisms. Most of these multiply resistant strains of MRSA are still susceptible to linezolid, the first of the oxazolidinone antibiotics. However, resistance to linezolid has already been documented to develop in a patient receiving therapy for a bacteremic MRSA infection as a function of a mutation in the 23S rRNA (Haddadin, Fappiano, and Lipsett 2002).

The streptogramins are a series of antibiotic derivatives of natural products of *Streptomyces pristinaespiralis*. Two of these streptogramins, quinupristin and dalfopristin, when used in combination have been shown to be bactericidal against many gram-positive organisms. They bind to complementary sites on the 23S rRNA of the 50S ribosomal subunit. Each antibiotic is bacteriostatic, but when they are used together in a 30:70 ratio the combination is bactericidal (Murray *et al.* 2003). It is also approved for the treatment of skin and skin structure infections caused by *S. aureus* (methicillin susceptible strains) and by group A *Streptococcus*. *In vitro,* quinupristin/dalfopristin is also active against MRSA and vancomycin-resistant *S. aureus*, although clinical data on treatment of these infections are currently lacking.

Daptomycin is a novel bactericidal agent for Gram-positive organisms, including *S. aureus*, coagulasenegative staphylococci, enterococci, and streptococci . Structurally, daptomycin is a lipopeptide composed of a 13 amino acid cyclic peptide with a lipophilic tail that inserts into the cell membrane (Cottagnoud 2008). Daptomycin is a new lipopeptide antibiotic. The mechanism of action of daptomycin is poorly understood, but it seems that depolarization of the membrane occurs as the antibiotic polymerizes in the membrane, producing channels that result in leakage of cell contents, inhibition of protein, DNA, and RNA synthesis, and cell death of many Gram positive pathogens (Steenbergen *et al.* 2005; Hobbs *et al.* 2008). Daptomycin shows concentration-dependent killing pharmacodynamics. Daptomycin is approved by the FDA for the treatment of complicated skin and skin structure infections caused by *S. aureus* (including MRSA), streptococci, and enterococci. Daptomycin also shows *in vitro* activity against vancomycin-resistant *S. aureus* and should represent an effective agent should these strains become more widespread.

Oritavancin and dalbavancin are glycopeptide antibiotics structurally related to vancomycin, and also to teicoplanin, a glycopeptide that has been available in Europe. With chemical modifications of the primary structure, improved binding

to the target sites within the organisms and increased antibacterial activity have been achieved (Kim *et al.* 2008). Its capacity to interact with the bacterial cell membrane, leading to loss of membrane potential and increased membrane permeability (McKay, *et al,* 2007), confers rapid bactericidal activity (NCCLS, 1999) against exponentially growing MRSA within 15 min to 2 h. (McKay, *et al,* 2006).

Several companies have developed cephalosporins with the capability to bind to PBP2a, the *mec* gene product that makes *S. aureus* methicillin- and cephalosporin resistant. At least four new compounds have been investigated in the laboratory, with excellent activity against both MSSA as well as MRSA. Some of these compounds also retain activity against *Pseudomonas* and the Enterobacteriaceae. Given the long-standing safety and efficacy profile of β-lactam compounds in children, it is likely that one or more of these agents will enter clinical trials within the next few years.

5. *Rhodomyrtus tomentosa* (Aiton) Hassk.

5.1 Botanical description

Rhodomyrtus tomentosa, a flowering plant in the family Myrtaceae, is an evergreen shrub or small tree growing up to 4-5 m tall. The leaves are opposite, leathery, 5-7 cm long and 2-3.5 cm broad, three-veined from the base, oval, obtuse to sharp pointed at the tip, glossy green above, densely grey or rarely yellowish-hairy beneath, with a wide petiole and an entire margin. The flowers are solitary or in clusters of two or three, 2.5-3 cm diameter, with five petals which are tinged white outside with purplish-pink or all pink (Figure 3A.). The fruit is edible, 10-15 mm long, purple, round, three or four-celled with many seed (Figure 3B). The common names including Ceylon hill gooseberry (English), Downy myrtle (English-Florida), Downy rose myrtle (English-Florida), Feijoa (French), Hill gooseberry (English), Hill guava (English), Isenberg bush (English-Hawaii), Myrte-groseille (French), and Rose myrtle (English-Florida) (Grin, 2002; Wagner *et al.,* 1999).

5.2 Habitat description

It is native to South-East Asia. The plant is growing wide and cultivated in Southeast Asia, India, Sri Lanka, and Southern China. However, it has been reported as serious an invasive species in some countries such as Florida, spreading to form large, monospecific thickets that displace native flora and fauna. Areas especially affected include Florida, Hawaii, and French Polynesia (Janick and Paull 2008). It thrives in open, often in degraded sandy sites, along the shore and river banks. It tolerates full sun and flooding. In Thailand, it has been often found in coastal sandy soils on coasts of the Southern and Eastern Thailand.

A

B

Figure 3. ***Rhodomyrtus tomentosa*****'s flowers and fruits**

5.3 Traditional uses

In Malaysia, the fruits have been employed as a cure for dysentery and diarrhea. A decoction of its roots or leaves is used for diarrhea and stomach-ache and as a prospective medicine after birth. In Indonesia, the crushed leaves are used to dress wounds (Sung *et al.* 1998). It has been used in Vietnamese traditional medicine for diarrhea and wound treatments (Ho, 1999).

5.4 Chemical components

The chemical constituents of *R. tomentosa* have been reported including triterpenoids, steroids, tannins, flavone glycosides, and acylphloglucinols (Dachriyanus *et al.* 2002; Hiranrat and Mahabusarakam 2008; Li 2006).

5.5 Antibacterial activity

In 2002, Dachriyanus (Dachriyanus *et al.* 2002) has been showed the antimicrobial activity against *E. coli* and *S. aureus* of the ethyl acetate extract of *Rhodomyrtus tomentosa* leaf. Recently, the ethanolic extract of *Rhodomyrtus tomentosa* leaf has been reported the good activity against many Gram positive bacteria including *Bacillus* cereus, *Enterococcus faecalis*, *Listeria monocytogenes*, *S. aureus*, *Streptococcus pyogenes* (Limsuwan and Voravuthikunchai 2008; Limsuwan *et al.* 2009; Saising *et al.* 2008; Voravuthikunchai, Dolah, and Charernjiratrakul 2010). Research works with this plant species have not well been established and its antimicrobial mechanisms have not yet published. An acylphloroglucinal derivative isolated from this plant has been proposed the name as rhodomyrtone by Dachriyanus *et al.* (Dachriyanus *et al.* 2002). Acylphloroglucinals are natural products based on an aromatic ring that in many cases has been reduced or has a keto-enol form. The prenylated and/or farnesylated are reported as the majority of these products and possess simple acyl groups such as 2-methylpropanoyl which is found in hyperforin (Gibbons 2004). The metabolite appears in *Hypericum perforatum* (St John's Wort) and is commonly used as an herbal antidepressant product (Hippius 1998).

Detailed study on antimicrobial mechanisms of this plant and its bioactive compounds is on going to examine. The further investigation of rhodomyrtone potentials could provide the scientific data in order to develop and define a novel alternative antibiotic for treatment of MRSA infections.

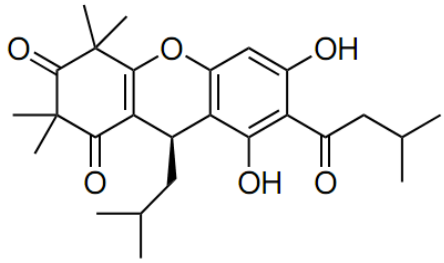


Figure 4. Chemical structure of rhodomyrtone, [6,8-dihydroxy-2,2,4,4-tetramethyl -7-(3-methyl-1-oxobutyl)-9-(2-methylpropyl)-4,9-dihydro-1H-xanthene-1,3(2H)-di-one] (Dachriyanus *et al.* 2002), an acylphloroglucinal derivative isolated from *Rhodomyrtus tomentosa* (Hiranrat and Mahabusarakam 2008).

CHAPTER 3

MATERIALS AND METHODS

1. Materials and equipments

1.1 Bacterial strains

1.1.1 Methicillin-resistant *Staphylococcus aureus* NPRC 001R (MRSA NPRC 001R)

1.1.2 Epidemic methicillin-resistant *Staphylococcus aureus*-16 (EMRSA-16)

1.1.3 *Staphylococcus aureus* ATCC 29213

1.2 Medicinal plant

1.2.1 *Rhodomyrtus tomentosa* (Aiton) Hassk.

1.3 Antibiotic

1.3.1 Oxacillin (Sigma)

1.3.2 Vancomycin (Sigma)

1.4 Media

1.4.1 Luria-Bertan agar (LB) (Difco)

1.4.2 Mueller-Hinton agar (MHA) (Difco)

1.4.3 cation-adjusted Mueller Hilton broth (CAMHB) (Difco)

1.5 Chemicals

1.5.1 Acetone (Fisher Scientific)

1.5.2 CHAPS (Amersham Bioscience)

1.5.3 Coomassie Brilliant Blue G-250 (Sigma)

1.5.4 Dichloromethane (Lab-Scan Analytical Science)

1.5.5 Dimethylsulfoxide (Merck)

1.5.6 Dithiothreitol (USB)

1.5.7 Dnase I (QIAGEN)

1.5.8 Ethylenediaminetetraacetic acid (Sigma)

1.5.9 95% Ethanol (Lab-Scan Analytical Science)

1.5.10 Glass bead, acid washed (Sigma)

1.5.11 Glycerol (Vidhyasom)

1.5.12 Glutaraldehyde (Merck)

1.5.13 Hexane (Lab- Scan Analytical Science)

1.5.14 Hydrochloric acid (HCI) (BDH Laboratory)

1.5.15 Hydrogen peroxide (BDH)

1.5.16 Lysostaphin (Sigma)

1.5.17 Lysozyme (Sigma)

1.5.18 Methanol (BDH)

1.5.19 Sodium carbonate (Na_2CO_3) (Merck)

1.5.20 Sodium chloride (NaCl) (Merck)

1.5.21 Sodium dodecyl sulfate (SDS) (BDH)

1.5.22 Sodium hydroxide (NaOH) (Lab- Scan Analytical Science)

1.5.23 *Taq* polymerase (Fermentus)

1.5.24 Thiourea (Amersham Bioscience)

1.5.25 Trichloroacetic acid (TCA) (Sigma)

1.5.26 Trypsin (Sigma)

1.5.27 Tween20 (Sigma)

1.5.28 Urea (Amersham Bioscience)

1.6 Equipments

1.6.1 Autoclave (Tomy, ES 315)

1.6.2 Balance (Sartorius, BP 210S)

1.6.3 Cell Disrupter (Qbiogene, FastPrep® FP120)

1.6.4 Centrifuge (Hitachi)

1.6.5 Centrifuge (Eppendorf, 5417R)

1.6.6 Centrifuge (Beckman Coulter, Avanti J-20 XPI)

1.6.7 ELISA reader (Labsystems)

1.6.8 Freeze Dryer (Christ, ALPHA 1-2 LD plus)

1.6.9 Hot air oven (Binder, T410340)

1.6.10 Hot plate stirrer (Lab. Companion, HP 3000)

1.6.11 Incubator (Heraeus, B 5100E)

1.6.12 Laminar air flow cabinet (Gelman, BH 143AS)

1.6.13 Light microscope (Olympus, CX31RBSFA)

1.6.14 pH Meter (Beckman)

1.6.15 Portable Autoclaves (Dixons)

1.6.16 Refrigerator (Sanyo)

1.6.17 Rotor Gene 3000 (Corbett Research)

1.6.18 Scanning electron microscope (HITASHI S-236ON)

1.6.19 Scanning electron microscope (FEI Quanta 200 FEG)

1.6.20 Double sided carbon adhesive discs (Taab Laboratories)

1.6.21 Spectronic Biomate 5 (Thermo Electron)

1.6.22 Spectrophotometer (PerkinElmer)

1.6.23 Sputter Coater (Q150T, Quorum Technologies)

1.6.24 Thermal cycler GeneAmp PCR System 2400 (Applied Biosystems)

1.6.25 Transmission electron microscope (Hitashi H7000)

1.6.26 Water bath (Julabo, TW 20)

2. Methods

2.1 Preparation of *Rhodomyrtus tomentosa* extract

2.1.1 Preparation of an ethanolic extract

The plant leaves were cut into small pieces and dried overnight at 60°C. Samples were crushed in a mechanical mortar and soaked with extracted solvents (95% ethanol) for 7 days (3 times). The solvent was then removed under reduced pressure in a rotary evaporator until it became completely dry (Limsuwan and Voravuthikunchai 2008). The extract was dissolved in dimethyl sulphoxide (DMSO, Merck, Germany) before use.

2.1.2 Isolation of rhodomyrtone

Rhodomytone was isolated from *R. tomentosa* leaf using antibacterial bioguided fractionation by Dr. Asadhawut Hiranrat and Assoc. Prof. Dr. Wilawan Mahabusarakam (Hiranrat and Mahabusarakam 2008). The dried ground leaves of *R. tomentosa* (2.1 kg) were sequentially extracted at room temperature with dichloromethane and acetone. The acetone extract (46.6 g) was further fractionated by dissolving in hexane. The soluble fraction (21.4 g) was then separated on quick column chromatography (QCC) using silica gel 60H and eluted with hexane-dichloromethane, dichloromethane, dichloromethane-acetone, and acetone gradient solvent system to give 20 fractions. Fraction 6 (606.0 mg) was subjected to column chromatography (CC) using silica gel 100 and eluted with dichloromethane and dichloromethane-methanol gradient to give fractions 6A-6G. Fraction 6C (183.6 mg) was further fractionated by CC and eluted with dichloromethane to give fractions 6C1-6C6. Fraction 6C3 (138.1 mg) was subjected to CC, and eluted with hexane-acetone (97:3) to give fractions 6C3A-6C3G. Bioassayguided chromatography of this extract allowed the isolation of the active principal. Rhodomyrtone (23.0 mg) was obtained from fraction 6C3F (31.4 mg) by crystallization from hexane-acetone (5:1). The elucidated structure was in accordance with the previously published data (Hiranrat and Mahabusarakam 2008; Dachriyanus *et al.* 2002). Purity of rhodomyrtone has been confirmed by referring to nuclear magnetic resonance (NMR)

and mass spectrometry (MS) reference (Limsuwan *et al.* 2009; Dachriyanus *et al.* 2002).

2.2 Bacterial strains and growth conditions

Methicillin-resistant *Staphylococcus aureus* NPRC 001R (MRSA NPRC 001R) was a clinical isolate obtained from Hat Yai Hospital, Songkhla, Thailand (Chusri and Voravuthikunchai 2009). This bacterial strain has been reported to be strongly resistant to several classes of antibiotics including β-lactam, aminoglycoside, tetracycline, macrolide, fluoroquinolone, and clindamycin. Methicillin-susceptible *S. aureus* strain ATCC 29213 was obtained from the American Type Culture Collection. Epidemic MRSA isolate-16 (EMRSA-16) was from a clinical sample obtained at the Royal Free Hospital, London, United Kingdom (Bernal, Zloh, and Taylor 2009). All bacterial strains were stored as 20% glycerol stocks at -80°C until required. Bacteria were precultured overnight from glycerol stock on Luria-Bertani agar. One colony was inoculated into 3 ml of Mueller-Hinton broth (MHB) at 37°C with constant shaking until reach mid-exponential phase growth.

2.3 Anti-*Staphylococcus aureus* activities

2.3.1 Determination of minimal inhibitory concentration (MIC)

MICs of the ethanolic extract and rhodomyrtone against *S. aureus* were carried out by a modified broth microdilution method recommended by Clinical Laboratory Standardization Institute (CLSI) guideline (CLSI 2005). Two fold-serially dilution in a 96-well microtiter plate of the extract and rhodomyrtone was prepared to obtain final concentration ranged from 0.24 to 500 μg/ml and 0.0625 to 128 μg/ml, respectively. Briefly, an exponential phase growth of *S. aureus* was diluted to a density of 10^8 cfu/ml in cation-adjusted Mueller Hilton broth (CAMHB). The bacterial suspension was then added to each well to provide a final inoculum density of $5x10^5$ cfu/ml. After incubation at 37°C for 16-18 h, the bacterial suspension in each well was measured at OD_{600nm} against a blank well (the broth containing 1% dimethyl sulphoxide, DMSO) with an ELISA reader (Labsystems). The MIC was

defined as the lowest concentration of the agent that completely inhibited the bacterial growth. Three independent experiments were performed with triplicate.

2.3.2 Time-kill study

Time-kill kinetic studies of the rhodomyrtone were performed in CAMHB. An overnight culture was used to inoculate in CAMHB containing rhodomyrtone at 0.125, 0.25, 0.5, 1, and 2MIC. The initial inoculum was approximately 10^8 cfu /ml. A culture containing 1% DMSO was included in each assay as a growth control. Sampling was done at 0, 1, 2, 3, 4, 5, 6, 8, 18, and 24 h. Viable bacteria were determined by plate counts on Mueller Hilton agar (MHA). The activity of rhodomyrtone was determined by plotting $\log_{10}$ colony count (cfu/ml) against time. Values shown are the means of duplicate determinations from the experiments repeated three times.

2.4 Detection of *mecA* gene by polymerase chain reaction (PCR)

2.4.1 Genomic DNA extraction

The bacterial cells were collected from overnight culture by centrifugation. The supernatant was removed and the pellets were then resuspended in lysis buffer (10 mM Tris-1 mM EDTA and 20 mg/ml egg white lysozyme). The mixtures were further incubated at 37ºC for 30 min with constant shaking. Genomic DNA extraction was performed with a commercially available kit, DNeasy Blood and Tissue Kit (QIAGEN), using the manufacturer's protocols. The quality of extracted DNA was examined by agarose gel electrophoresis and ethidium bromide staining. The DNA concentrations were evaluated by spectrophotometer at OD_{260nm} (Spectronic Biomate 5, Thermo Electron).

2.4.2 Polymerase chain reaction amplification

The PCR amplification to detect *mecA* encoding the MRSA-specific penicillin binding protein, PBP2A, was performed using a pair of primers, mecA1, 5′-TGTCCGTAACCTGAATCAGC -3′ and mecA2, 5′- TGCTATCCACCCTCAAA CAG-3′ (position 25982-25963 and position 25464-25483 of MRSA strain N315, 85/2082 (Ishino *et al.* 2002). Each PCR reaction contained 1 µl of genomic DNA ,

0.5 µM of each primer, 200 µM of deoxynucleoside triphosphate, 1x PCR buffer containing 1.5 µM of $MgCl_2$ and 2.5 U of *Taq* polymerase in a total volume of 50 µl. PCR amplification was performed with following condition; predenaturation for 5 min at 95°C, 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min, for a total of 30 cycles, and post extension at 72°C for 7 min with a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems). PCR products were run in 1% agarose gel in 1x Tris-acetate-EDTA (TAE) buffer at 100 V. Gels were stained by ethidium bromide solution and visualized under ultra-violet light.

2.5 Proteome analysis of rhodomyrtone-treated cells

2.5.1 One-Dimensional Denaturating SDS-PAGE

2.5.1.1 Bacterial cell protein preparation

The preliminary study suggested that concentration of rhodomyrtone that allow the bacteria growth to approximately $5x10^7$ cfu/ml after incubation for 18 h was 0.174 µg/ml. Rhodomyrtone concentration at 0.174 µg/ml (0.35MIC) was added in the bacterial cultures to prepare *S. aureus* whole cell homogenate in this study. Following the 18 h incubation, staphylococcal cells were collected by centrifugation at 10,000 x *g* at 4°C for 20 min. The culture supernatant was removed and the pellets were kept at -80°C until used. To prepare cellular proteins, the lysate was washed twice with 0.85% (w/v) NaCl and resuspended in 0.01 M phosphate buffer saline (PBS). The cells were agitated by a 750-Watt Ultrasonic processor at 30% amplitude for 15 min on ice, pulse durations of 9 sec on and 9 sec off. The lyzed cells were centrifuged at 10,000 x *g* at 4°C for 20 min to remove the cellular debris and aggregate. Protein concentrations were quantified by Bradford protein assay kit (Bio-Rad) according to the manufacturer's instruction. Bovine serum albumin (BSA) was used as protein standard. The supernate containing solubilized proteins was used directly or stored at -80°C until required.

2.5.1.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples were suspended in SDS sample buffer (62.5 mM Tris (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) mercaptoethanol, 0.05% (w/v) bromphenol blue) and heated at 100°C for 3-5 min prior to loading. Each 200 µg protein (5-10 µl) was applied on acrylamide gels on Mighty® Small II SE250 gel apparatus (Hoefer® Pharmacia Biotech). Proteins were separated based on their molecular weight by electrophoresis at 25 mA through a stacking gel (4% acrylamide) and a separating gel (12.5% acrylamide). The gels were then stained by overnight incubation with gentle agitation in staining solution (0.5% (w/v) Coomassie Brilliant Blue G-250 in 20% (v/v) methanol, 2% (v/v) *O*-phosphoric acid, 8% (w/v) ammonium sulphate). Prestained protein ladder (Bio-Rad) was used for SDS-PAGE molecular weight markers.

2.5.2 Western blot analysis

The method used was a modification of that described by Towbin *et al*. (Towbin, Staehelin, and Gordon 1979). The samples were run on 12.5% polyacrylamide gels and transferred onto nitrocellulose membrane (Amersham bioscience) at 100V for 1 h in transfer buffer (25 mM Tris-HCl (pH 8.3), 192 mM glycine, 10% (v/v) methanol) using a Transblot unit (Bio-Rad). Nitrocellulose sheet was blocked by incubating for 30 min in 5% (w/v) skim milk powder in PBST (PBS with 0.05% (v/v) Tween-20), and followed by incubation in mouse anti-*S. aureus* antiserum (1:3,000 dilution in PBST containing 0.2% (w/v) BSA and 0.2% (w/v) gelatin) at 4°C for 1-16 h. The antiserum was removed and the nitrocellulose membrane was washed three times for 5 min in PBST with gentle agitation. Horse radish peroxidase-conjugated goat anti-mouse (Southern biotechnology) was added at a dilution of 1:3,000 in PBST containing 0.2% (w/v) BSA and 0.2% (w/v) gelatin, and incubated for 1-2 h with gentle agitation at room temperature. The sheet was then washed four times in PBST and equilibrated in 67 mM phosphate buffer (pH 7.6). Antigen-antibody complexes were detected by the addition of color detection solution (67 mM phosphate buffer (pH 7.6) containing of 0.2% (w/v) 2,6-dichloroinophenol

and 0.03% hydrogen peroxide) and incubated in the dark. The color reaction was stopped with deionized water.

2.5.3 Two-Dimensional Gel Electrophoresis (2DE)

2.5.3.1 Preparation of *Staphylococcus aureus* whole-cell homogenate

The bacterial cell samples were prepared from four liter cultures in the presence or absence of subinhibitory concentration of rhodomyrtone (0.174 µg/ml) after incubation for 18 h. Untreated control cultures were grown in MHB containing 1% DMSO. The pellets were then resuspended in standard cell lysis buffer (30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS) containing protease inhibitors (Roche Diagnostics). Each sample was subjected at 30% amplitude, 2 sec pulse-on, 2.5 sec pulse-off, for a total of 5 min in an ice water bath. After sonication, the lyzed cells were centrifuged and the supernatant was transferred to a new tube. The pH of the homogenate was adjusted to 8.5 by slow and careful addition of 50 mM sodium hydroxide. The mixtures were stored in aliquots at -70°C until required. The proteins were cleaned with 2D-Clean-up kit (Amersham Biosciences) to eliminate detergents, salts, lipids, phenolics, and nucleic acids. After the treatment, the samples were solubilized with DeStreak Rehydration solution (Amersham Biosciences). Protein concentrations were quantitated by 2D-Quant kit (Amersham Biosciences) with BSA as the protein standard before subjecting to isoelectric focusing.

2.5.3.2 2D-gel electrophoresis

To separate the cellular proteins in first dimension, the samples (200-500 µg) in DeStreak Rehydration solution containing 1% pH range of 3-10 immobilized pH gradient (IPG) buffer (Amersham Biosciences) were applied onto the center of a slot in the 7 or 18 cm strip holder of the IPG phor (GE Healthcare Biosciences). The IPG strip (pH 3-10, non linear) (GE Healthcare Biosciences) was placed into the strip holder containing the sample. The PlusOne™ DryStrip Cover Fluid (Amesham Biosciences) was overlaid onto each IPG strips to prevent evaporation and urea crystallization. The strip holder was then placed into the Ettan IPG Phor Electrofocusing System (Amersham Biosciences) and the IPG strip was allowed to rehydrate at 20°C for 12 h. The isoelectric focusing parameters were as

followed; the voltage was set at 300 V (Volt) until 200 Volt-hours (Vh), 1,000 V until 300 Vh, 5,000 V until 4,000 Vh, and 5,000 V until 2,000 Vh.

For the second dimension gel electrophoresis, each electrofocused-IPG strip was equilibrated in 5 ml SDS-equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromphenol blue) containing 50 mg dithiothreitol (DTT) for 15 min at room temperature. The strip was then placed in 10 ml of the equilibration buffer containing 250 mg iodoacetamide (IAA) for 15 min, washed by 1x electrode buffer, and placed onto a 1 mm thick 12.5% polyacrylamide gel (10x11 cm^2) precasted in HoeferTMDual Gel Caster (Amersham Biosciences). The second dimensional gel was carried out at 25 mA/gel until the tracking dye reached the lower edge of the gel. After electrophoresis, the gel was subjected to colloidal Coomassie brilliant blue staining. The molecular weight was estimated according to the internal running calibration marker.

2.5.3.3 Image analysis

For visualization, gels were briefly rinsed with 1% acetic acid to reduce background. The comparative analysis using ImageScannerII and ImageMaster™ Software (Amersham Biosciences) was performed to detect differences in protein productions between gel images of protein profiles obtained from rhodomyrtone-treated cell proteins and the untreated cell proteins. Protein changes in 2D-gels were analyzed from three independent experiments (biological replicates) and similar protein patterns were observed among the gels. Unchanged expression of protein spot was considered if protein intensity was less than two-fold (p>0.05). Overexpressed or downregulated protein spots occurred after rhodomyrtone treatment were excised recorded from representative gels. Selected spots that presented only in rhodomyrtone-treated *S. aureus* samples and those that disappeared after the treatment were individually excised from the respective Coomassie dye stained-gels.

2.5.3.4 In-gel digestion and peptide extraction

Protein spots were destained using washing solution containing 50% methanol and 5% acetic acid in ultra deep water at 25°C for 18 h. Gel plugs were rinsed a few times in a period of 2-3 h with fresh washing solution. The gel pieces were dehydrated with 100% acetonitrile (ACN) and reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h. For alkylation, 100 mM IAA in 10 mM ammonium bicarbonate was added to the gel plugs and incubated at room temperature for 1 h in the dark. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) were added to the gels and incubated at room temperature for 20 min. After incubation, 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid was added into the gels, and the gels were then incubated at room temperature for 10 min in a shaker. Extracted peptides were collected and pooled. The peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

2.5.4 Mass spectrometry

2.5.4.1 Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Identification of interested protein spots was performed using MALDI-TOF/MS. The tryptic digests were spotted with α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich, St.Louis, MO) matrix solution, and analyzed with an Ultraflex III MALDI-TOF-TOF MS (Bruker Daltonics, Bremen, Germany). Mass spectra were recorded in the positive-ion, delayed extraction mode. All spectra were acquired with 20 kV accelerating voltage, 150 ns delayed extraction time, and 900 Da low-mass gate. All spectra were externally mass calibrated with ProteoMassTM Peptide and Protein MALDI-MS Calibration Kit (Sigma-Aldrich).

2.5.4.2 Liquid chromatography-mass spectrometry (LC-MS)

The protein digested from rhodomyrtone-treated and untreated MRSA were injected into Ultimate 3000 LC System (Dionex) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System (Bruker)) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 80% acetonitrile) was run in 40 min**.**

2.5.4.3 Database searching

All of the tandem mass spectra were analyzed by a public database (National Center for Biotechnology Information) or Swiss-Prot from MALDI fingerprint data by MASCOT (http://www.matrixscience.com). Protein identification was performed on the basis of statistically significant Mowse score (p<0.05).

2.6 Transcriptome analysis

2.6.1 Microarray analysis

2.6.1.1 RNA isolation

Total RNA samples were extracted using FastRNA® Pro Blue kit (Q-biogen) according to the manufacturer's instruction. For microarray analysis, flasks containing MHB were inoculated with EMRSA-16 overnight culture to provide an initial inoculum of approximately 10^6 cfu/ml. The bacterial cultures were incubated at 37°C until reached exponential phase growth (optical density at 600_{nm} = 0.15). Rhodomyrtone was added to the cultures to give a final concentration of 0.5 µg/ml (0.5MIC). DMSO at the same value as used in treated-cells was also added for the untreated controls. The cultures were further incubated at 37°C for 1 h. The bacterial pellets were collected by centrifugation at 8,000 rpm for 30 min and washed twice with phosphate-buffer saline (PBS). A double volume of RNAprotect Bacteria reagent (QIAGEN) was added to the pellets. The mixture was incubated at room temperature for 5 min and the bacterial cells were centrifuged at 3,500 rpm at 4°C for 10 min. The samples were kept at -80°C until needed for RNA preparation. Total RNA was extracted from the bacterial cells using the manufacturer's "Fast prep" protocol for the RNeasy Mini-Prep Kit (QIAGEN). The bacterial DNA was removed

by DNase digestion steps with RNase-free DNase (QIAGEN) and RNA was further purified using RNeasy Mini protocol as recommended by the manufacturer. RNA quality and quantity was determined by agarose gel electrophoresis, NanoDrop 2000 spectrophotometer (Nanodrop Technologies), and Bioanalyzer 2100 (Agilent Technologies), all samples were stored at -80°C until required.

2.6.1.2 Microarray analysis

Microarray analysis procedure used in this study has been previously described by Doyle *et. al.* (Doyle *et al.* 2009). It contains PCR products representing all predicted open reading frames (ORFs) from the initial seven *S. aureus* genome sequencing projects (Witney *et al.* 2005). The array design is available in BμG@Sbase and also ArrayExpress. RNA was transcribed into cDNA and concomitantly labeled by incorporation of Cy3 and Cy5 (GE Healthcare) using superscript III reverse transcriptase (Invitrogen). cDNA was purified using a MinElute kit (QIAGEN), probes pooled, hybridized overnight to the *S. aureus* microarrays at 65 °C and subjected to stringent washing. Hybridization data was analyzed by Affymetrix 428 scanner then quantified using Bluefuse for Microarrays 3.5 software (BlueGnome). Data analysis was performed in GeneSpring GX 7.3 (Agilent Technologies) by median-normalized Cy5/Cy3 ratio intensity for three biological replicates. Only genes whose expression ratio showed at least a two-fold difference with Benjamini and Hochberg false discovery rate ≤0.05% in the presence of rhodomyrtone were regarded as being significantly different from the control. Microarray analysis data of selected genes of interest were further validated by qRT-PCR.

2.6.2 Quantitative reverse transcription-PCR (qRT-PCR)

2.6.2.1 RNA extraction

Total RNA was extracted using RiboZol Reagent (Amresco) according to the manufacturer's protocol. In brief, the bacterial cells were harvested by centrifugation at 8000 x *g* for 30 min. To avoid the degradation of mRNA, washing samples before the addition of Ribozol was not carried on. Before adding RiboZol, the cells were added with lysis buffer (10 mM Tris-1 mM EDTA and 20 mg/ml egg

white lysozyme) and shaked at 37°C for 30 min. Approximatly $1x10^7$ cfu/ml of the bacterial cells was resuspended in 1 ml of RiboZol at room temperature for 5-10 min. Subsequently, 200 ml of cholorform per 1 ml of TRIzol was added and then incubated at room temperature for 3 minutes. This was followed by a centrifugation step at 12,000 x *g* for 15 minutes at 4° which separated the mixture into three phases and the RNA remained in the clear upper aqueous phase. The aqueous phase was carefully removed into a new tube and RNA was then precipitated with the addition of 0.5 ml of isopropanol. The RNA sample was incubated at room temperature for 5-10 min and centrifuged at 12,000 x *g* for 10 min at 4°C. The RNA pellet which was nearly transparent after the centrifugation step was washed with 1 ml of 75% ethanol per 1 ml of RiboZol and then centrifuged at 7,500g for 5 min at 4°C. The RNA pellet was air-dried, resuspended in 40 ml of RNase free water (DEPC-treated water), and stored at -80°C until used. RNA was removed DNA contaminants using RNase free DNase I (QIAGEN) according to the manufacturer's protocol. The quality of extracted RNA was examined by agarose gel electrophoresis and ethidium bromide staining. RNA was evaluated using absorption of light at 260 and 280 nm ($A260_{nm}/280_{nm}$) with an ultraviolet-visible spectrophotometer (Spectronic Biomate 5, Thermo Electron).

2.6.2.2 Oligonucleotide primer design

Genes of interest were selected from proteomic and microarray analysis data as were listed in Table 1. RNA samples of EMRSA-16 after treatment with rhodomyrtone for 1, 4, and 18 h, were isolated and used for time course analysis using qRT-PCR. Specific-gene primers were designed with Clone manager software version 6.0 (Sci-Ed Software, USA) to yield amplicon size of 100–200 bp. Primers were 18-20 bp length, with a guanine-cytosine (GC) content of 50-60 and a melting temperature between 55-60 °C.

Table 1. Oligonucleotide primers used for quantitative reverse transcription-PCR (qRT-PCR) analysis

Primer target[b]	Category		Nucleotide sequence (5' to 3')[a]
asd	Aspartate family	F	CAAGTCAATGGCGTATGG
		R	GGCACAACAGACTGAATC
dapA	Aspartate family	F	ACTACTGCTGAGAGCCCTAC
		R	GTTCCAGTTCCAGCTATGAC
lysA	Aspartate family	F	TTAGACTGCCGGTATCTTGG
		R	CAATAACGTCGCCTGTACTG
thrC	Aspartate family	F	TTTGGCTTCCAAGCTGAAGG
		R	TCCCAACTAGCAGGATTACC
ftsH	Cell wall biosynthesis	F	TAGAGCGGTTGCAGGTGAAG
		R	ACCACGTTGACGACCAACAG
ftsZ	Cell wall biosynthesis	F	TTACTGGTGGCGAGTCATTG
		R	TTTACGCTTGTTCCGAATCC
murE	Cell wall biosynthesis	F	TGATTCACGTACAGCGAGAG
		R	CTTAATGTGTCCGGCACAAC
rpoB	Nucleotide metabolism	F	CGCCTAAAGGTGTAACTGAG
		R	GATAATGTGTCGTCGCCTTC
SAR0996	Putative membrane protein	F	GAAGCGTTTGTCACAACC
		R	ATTGACGAGTGCGTCATC
SAR0437	Putative exported protein	F	CCGTCTTGCTTAGCTTTG
		R	GAAGCAGATGCGAAAGTG
SAR0761	Putative lipoprotein	F	TTAATTTAGCGCCGCCGAAG
		R	TCGCAATGGTTGACTACG
16s RNA	House keeping gene	F	GACGGTCTTGCTGTCACTTA
		R	AGTTCCAGTGTGGCCGATCA

[a] Based on the annotation of MRSA252 genome

[b] Forward (F) and reverse (R) primers

2.6.2.3 Quantitative reverse transcription-PCR

The reaction was performed on cDNA generated from RNA samples that were prepared independently of the RNA used for microarray analysis. qRT-PCR was carried out using the Brilliant II SYBR® Green qRT-PCR one-step kit (Stratagene) according to the manufacturer's instructions. The reaction was run in Rotor gene 3000 (Corbett Research) using primer pairs listed in Table 1. The conditions used for all the PCR reaction were 95°C for 10 min, followed by 55 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s (Table 2). The comparative threshold method was used to determine relative quantification of mRNA abundance and changes in mRNA expression level were calculated after normalization to the internal control, 16S rRNA, the bacterial house-keeping gene.

Bacteria grown in different time point of incubation (1, 4, and 18 h) in the presence or absence of rhodomyrtone were prepared in two independent cultures (biological replicates). Four qRT-PCR experiments per each time point (duplicated for each replicate) were performed. Replicate was combined to calculate mean of expression values. Normalization and analysis were carried out using the comparative Ct method to identify genes with at least two-fold difference in gene expression.

The formula used for calculation of the relative expression level for each gene $(2^{(-\Delta\Delta Ct)})$.

ΔCt = Ct (GOI) - average (Ct (16s rRNA))

Ct-threshold cycle

GOI-Gene of interest

16s rRNA - housekeeping gene

Table 2. The temperature cycling profile used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Cycle	Cycle Point
Hold 1 at 50°C, 30 min 0 secs	
Hold 2 at 95°C, 10 min 0 secs	
Cycling (55 repeats)	Step 1: at 95°C, hold 30 secs
	Step 2: at 57°C, hold 30 secs
	Step 3: at 72°C, hold 30 secs,
	acquiring to Cycling A (FAM/Sybr)
Melt (60-95°C) ,	
hold 45 secs on the 1st step,	
hold 5 secs on next steps,	
Melt A (FAM/Sybr)	

2.7 Electron microscopy

Bacterial cultures grown in the presence or absence of rhodomyrtone at 0.174 µg/ ml for 18 h were harvested by centrifugation at 10,000 x *g* for 5 min. The cells were washed twice, resuspended in PBS, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate. After incubation, the cells were recovered by centrifugation at 10,000 x *g* for 5 min and washed twice with PBS.

For transmission electron microscopy (TEM), the pellets were further fixed with 1% osmium tetraoxide and were then left at room temperature for 1 h. The samples were dehydrated with graded ethanol solutions such as 30% ethanol for 10 min, 50% ethanol for 10 min, 70% ethanol for 10 min, 90% ethanol for 10 min, and twice in 100% ethanol for 15 min. They were fixed in Epon 812 resin and left to polymerize for 3 days. Each sample was cut into thin slices, approximately 90 nm, with a glass knife, stained with uranyl acetate, and lead citrate on grids. Mophological and ultrastructural of the bacteria were observed and photographed by a Transmission electron microscope, Hitashi H7000, at 75 kV.

For scanning electron microscopy (SEM) examination of the bacterial cells treated with rhodomyrtone at 0.174 µg/ ml (0.35MIC) for 18 h, the bacterial cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate (as described in TEM). The fixed samples on the membranes of the transwells were gradually dehydrated through a graded series of ethanol (30-100%), dried by using the critical point dryer, and coated with gold particles. Samples were examined by a HITASHI S-236ON scanning electron microscope using an accelerating voltage of 15-20 kV.

For SEM characterization of EMRSA-16 cells collected after treatment with rhodomyrtone at 0.5 µg/ ml (0.5MIC) for 1 and 4 h, the materials and equipments used were described as follows. The cells were washed twice with PBS and fixed in 1.5% glutaraldehyde for at least 2 h at room temperature. Samples were washed one with 70% ethanol, twice with 100% ethanol, air dried, and adhered to a SEM stub using double sided carbon adhesive discs (Taab Laboratories). The sample were given gold coating in a Q150T Sputter Coater (Quorum Technologies) and viewed under a FEI Quanta 200 FEG Scanning Electron Microscope using an accelerating voltage of 5 kV.

2.8 Phenotypical analysis

2.8.1 Triton X-100-induced autolytic assay

Triton X-100 stimulated autolysis was measured as previously described (Mani, Tobin, and Jayaswal 1993). *S. aureus* strains were grown exponentially to an optical density at $OD600_{nm}$ of 0.3 in MHB containing 1 M NaCl. The bacterial cells were collected by centrifugation at 10,000 x g, 4°C for 20 min. Cells were washed twice with 50 ml of ice-cold water and resuspended in 50 ml of 0.05 M Tris-HCl (pH 7.2) containing rhodomyrtone at 0, 0.0625, 0.125, 0.25, 0.5, 1.0, and 2 µg/ml (untreated control, 0.125MIC, 0.25MIC, 0.5MIC, MIC, 2MIC, and 4MIC, respectively) and 0.05% (vol/vol) Triton X-100 (Sigma) to an $OD600_{nm}$ of 1. The cell suspension was incubated at 30°C with shaking and the $OD600_{nm}$ was determined 30 min intervals for 3 h.

2.8.2 Lysostaphin MIC

Serial two-fold dilution of lysostaphin was prepared in MHB containing rhodomyrtone at a final concentration of 0.125MIC (0.125 µg/ml for EMRSA-16) in a 96-well microtiter plate. The exponential phase bacteria culture was added to each well to provide a final inoculum of $5x10^5$ cfu/ml in a total volume of 200 µl. After incubation at 37°C for 16-18 h, the bacterial suspension was read against a blank well (MHB containing 1% DMSO). The MIC was defined as the lowest concentrations of the agent that completely inhibited the bacterial growth. Values shown are the means of triplicate determinations from three experiments. (Stapleton *et al.* 2007)

2.8.3 Peptidoglycan composition analysis

2.8.3.1 Preparation of bacterial peptidoglycan

Peptidoglycan was extracted and purified was a modification methods described earlier (Stranden *et al.* 1997). Briefly, bacteria at log phase growth were inoculated in 1,000 ml (x3 flasks) of MHB with 0.5 µg/ml of rhodomyrtone and 1,000 ml for untreated culture, and were then incubated for 4 h. The cells were rapidly chilled, harvested by centrifugation at 10,000 x g at 4°C for 20 min, and further

washed with 0.01 M PBS. The cells were broken with glass beads (0.2 mm) by a cell homogenizer (FastPREP FP120, Savant) at a speed setting of 6.0 for 45 s. The samples were placed on ice for 2 min and centrifuged at 14,100 x g for 5 min. After removal of the supernatant, the pellet was washed with 2M NaCl and the supernatant was removed. The collected cell suspension was then washed with 0.5% SDS and incubated at 60°C for 30 min with general stirring to remove noncovalently bound components. The bacterial cell wall was isolated by centrifugation at 5,100 x g at 25°C for 15 min and washed with water. Protein A was removed by incubation with 0.2 mg of trypsin per ml in 1 M Tris-HCl (pH 7.2) at 37°C for 24 h. The samples were centrifuged, washed several times with buffer and water, and then resuspended in 1 ml of 10% (wt/vol) aqueous trichloroacetic acid at 4°C for 18 h to remove teichoic acids. Purified murein was isolated by centrifugation at 14,100 x g for 5 min, washed with water, and lyophilized using Feeze Dryer (ALPHA 1-2 LDplus, Christ).

2.8.3.2 Peptidoglycan amino acid analysis

Determination of amino acid contents on the bacterial peptidoglycan was preformed by Alta Bioscience (University of Birmingham, UK). The lyophilized isolated peptidoglycan was hydrolyzed with 5.8M HCl under vacuum at 110°C for 24 h. The amino acid was analyzed in amino acid analyzer based on Waters HPLC hardware and using a sodium citrate buffer system. The amino acids were detected by means of the ninhydrine reagent.

CHAPTER 4

RESULTS

1. Determination of *mecA* gene in a methicillin-resistant *Staphylococcus aureus* isolate

MRSA NPRC 001R isolate was a representative clinical strain used in this study that has been identified to be strongly resistant to several classes of antibiotics including *β*-lactams (oxacillin and penicillin), aminoglycoside (gentamicin), tetracycline, macrolide (erythromycin), fluoroquinolone (ciprofloxacin), clindamycin, and trimethoprim-sulfamethoxazole. To determine weather MRSA NPRC 001R contain *mecA* encoding a low-affinity penicillin binding protein (PBP2a or PBP2'), polymerase chain reaction (PCR) amplification was carried out. The PCR product size of 519 bp was examined from the isolate as shown in Figure 5. EMRSA-16 was used as a positive control strain, and *S. aureus* ATCC 29213 was used as a negative control strain.

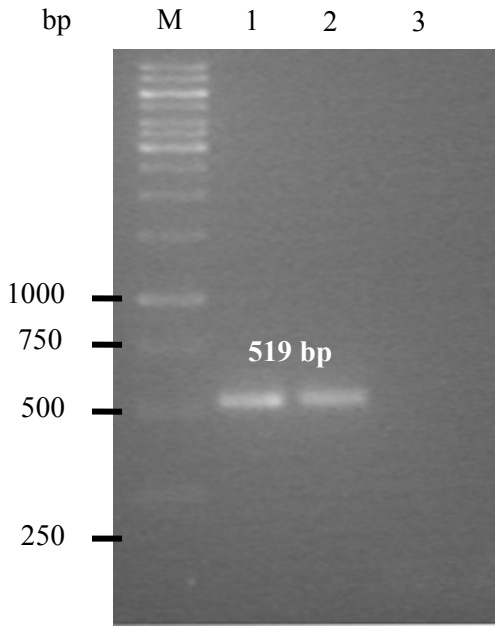


Figure 5. Agarose gel electrophoresis of PCR products of *mecA* gene.

The amplicon size is 519 bp.

Lane M, 1 kb DNA ladder.

Lane 1, MRSA NPRC 001R

Lane 2, EMRSA-16 (Positive control)

Lane 3, *S. aureus* ATCC 29213 (Negative control)

2. Antibacterial activity of *Rhodomyrtus tomentosa*

2.1 Determination of antibacterial activity of ethanolic extract of *Rhodomyrtus tomentosa* and rhodomyrtone

In order to assess the antibacterial activity of the crude ethanolic extract of *R. tomentosa* and rhodomyrtone against *S. aureus*, susceptibility test by broth microdilution method was employed according to CLSI guidelines (CLSI 2005). The ethanolic extract exhibited good antibacterial activities against MRSA NPRC 001R, EMRSA-16, and *S. aureus* ATCC 29213 (Table 3). For MRSA NPRC 001R and *S. aureus* ATCC 29213, the minimal inhibitory concentration (MIC) values were 31.25-62.5 μg/ml, and the minimal bactericidal concentration (MBC) was 250 μg/ml. Rhodomyrtone was 62.5-125 times more potent at inhibiting for both strains than the crude extract, its MIC and MBC were 0.5 μg/ml and 2 μg/ml, respectively. The MIC and MBC values of rhodomyrtone against EMRSA-16 were 1 μg/ml and 4 μg/ml, respectively.

Table 3. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the crude ethanolic extract and rhodomyrtone against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA)

Bacterial strains	Crude (μg/ml)		Rhodomyrtone (μg/ml)		Vancomycin (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
MRSA NPRC 001R	31.3	250	0.5	2	1	1
EMRSA-16	NA	NA	1	4	1	1
S. aureus ATCC 29213	62.5	250	0.5	2	0.5	1

NA=not available

2.2 Time-kill assay

Time-kill kinetic was carried out with EMRSA-16 strain. The result of the time-kill curves was shown in Figure 6. Within 4-5 h, the bacterial growth was inhibited at initial inoculum following treatment with rhodomyrtone at 0.125, 0.25, 0.5, 1, and 2MIC. Following this, over the next 18 h, the number of viable cells after exposure to MIC and 2MIC of rhodomyrtone decreased by at least a two $\log_{10}$-fold, when compared to the untreated control.

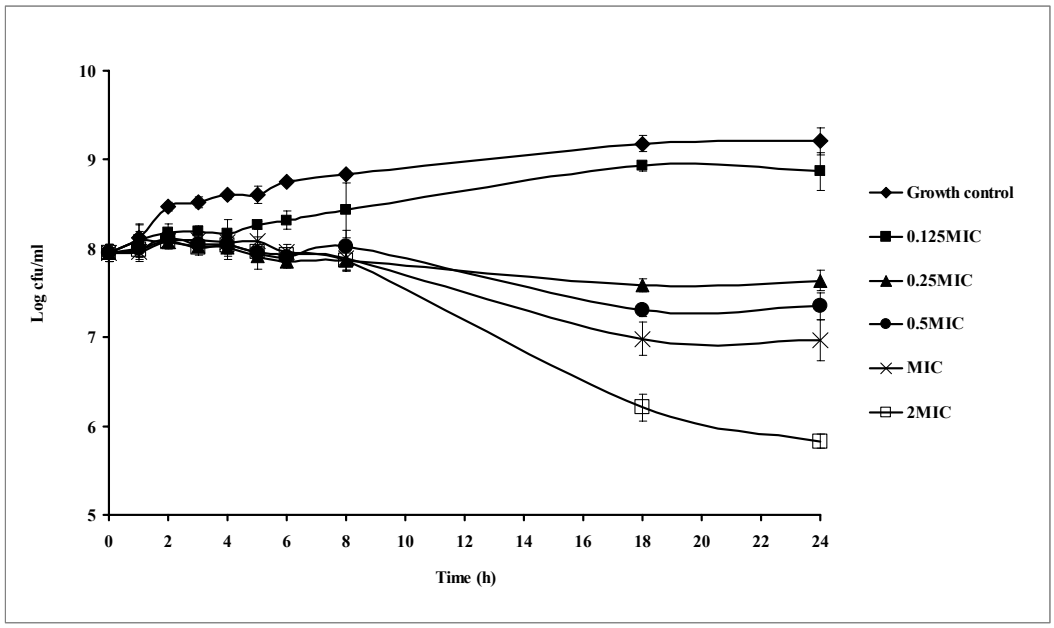


Figure 6. Time-kill curves of rhodomyrtone against EMRSA-16. Viable cell counts were performed at the indicated time points including 0, 1, 2, 3, 4, 5, 6, 8, 18, and 24 after exposure to rhodomyrtone at different concentrations (0.125MIC, 0.25MIC, 0.5MIC, MIC, 2MIC, and the untreated control) by serial dilution plating. Each point represented the mean of $\log_{10}$ ± standard deviations (SD) of three different experiments performed in duplicate.

3. Protein profiling analysis of MRSA and *Staphylococcus aureus* treated with the crude extract and rhodomyrtone

3.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

To preliminary address whether rhodomyrtone affected staphylococcal cellular protein patterns, SDS-PAGE and Western immunoblotting were performed. Subinhibitory concentration of rhodomyrtone at 0.174 μg/ml (0.35MIC) was used in this study to allow the bacterial growth at approximately $5x10^7$ cfu/ml after incubation for 18 h that. Rhodomyrtone-treated staphylococcal cells exhibited several protein bands with increase or decrease in intensity, or with the presence or absence of the protein bands, compared with the untreated controls. The expression of up- and down-regulated proteins was further assessed by immunoblot probed with mouse anti-*S. aureus* antisera (Figure 7). The protein patterns observed after immunoblotting clearly confirmed SDS-PAGE that both the extract and rhodomyrtone disturbed the profiles of immunogenic *S. aureus* cellular proteins in MRSA and *S. aureus* ATCC 29213.

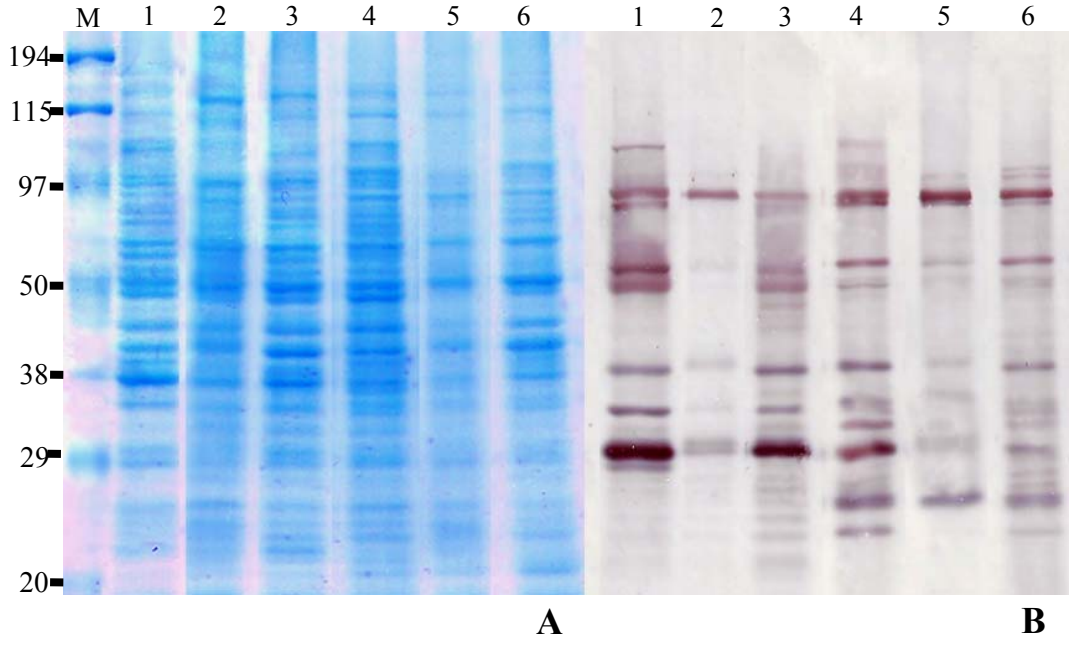


Figure 7. Profiles of *Staphylococcus aureus* cellular proteins. *S. aureus* ATCC 29213 (lane 1-3) and methicillin-resistant *Staphylococcus aureus* NPRC 001R (lane 4-6) were treated with the ethanolic extract of *Rhodomyrtus tomentosa* leaf and rhodomyrtone at 37^{o}C for 18 h. Samples were analyzed by 12.5% SDS-PAGE (A) and immunoblotting (B). M (marker), lane 1 - untreated *S. aureus*, lane 2 - the crude-treated *S. aureus*, lane 3 - rhodomyrtone-treated *S. aureus*, lane - 4 untreated MRSA NPRC 001R, lane 5 - the extract-treated MRSA NPRC 001R, and lane 6 - rhodomyrtone-treated MRSA NPRC 001R.

3.2. Protein variation identification of MRSA cells grown in the presence or absence of rhodomyrtone

To characterize the effect of rhodomyrtone on whole-cell protein expression in MRSA, we further identified cellular proteins that appeared or disappeared following the exposure to 0.35MIC rhodomyrtone at 0.174 μg/ml in CAMHB for 18 h by 2DE in combination with mass spectrometry. Image analysis of 2DE patterns was performed by ImageScannerII and ImageMaster^{TM} Software. Most of the protein spots were observed in acidic range as illustrated in Figure 8A and 8B. The mapping of cellular protein expression in MRSA revealed distinct alterations between the two proteomes. A total of 380 MRSA protein spots were observed in the cultures after treatment with rhodomyrtone while 301 spots were detected in cells grown in compound-free cultural media. The investigation was focused on the more/less intense proteins. Total number of excised spots from both proteomes to further identify was 275. Of these, 203 spots were shared (unchanged expression) between the two protein profiles. The protein spots that present only in whole cell preparation of rhodomyrtone-treated MRSA but absent in untreated-MRSA were 177 (18 identified proteins, 83 unidentified proteins, and 76 polyethylene glycol-contaminated proteins). On the other hand, the presence proteins in untreated-MRSA which were absent in rhodomyrtone-treated MRSA were 98 (28 identified proteins and 70 unidentified proteins). The presence or absence of protein spots in the treated MRSA proteomes were denoted in Figure 8A and 8B. Protein spot number, protein annotation, functional category (NCBI database) are listed in Table 4-5. The potential roles of these proteins were discussed below.

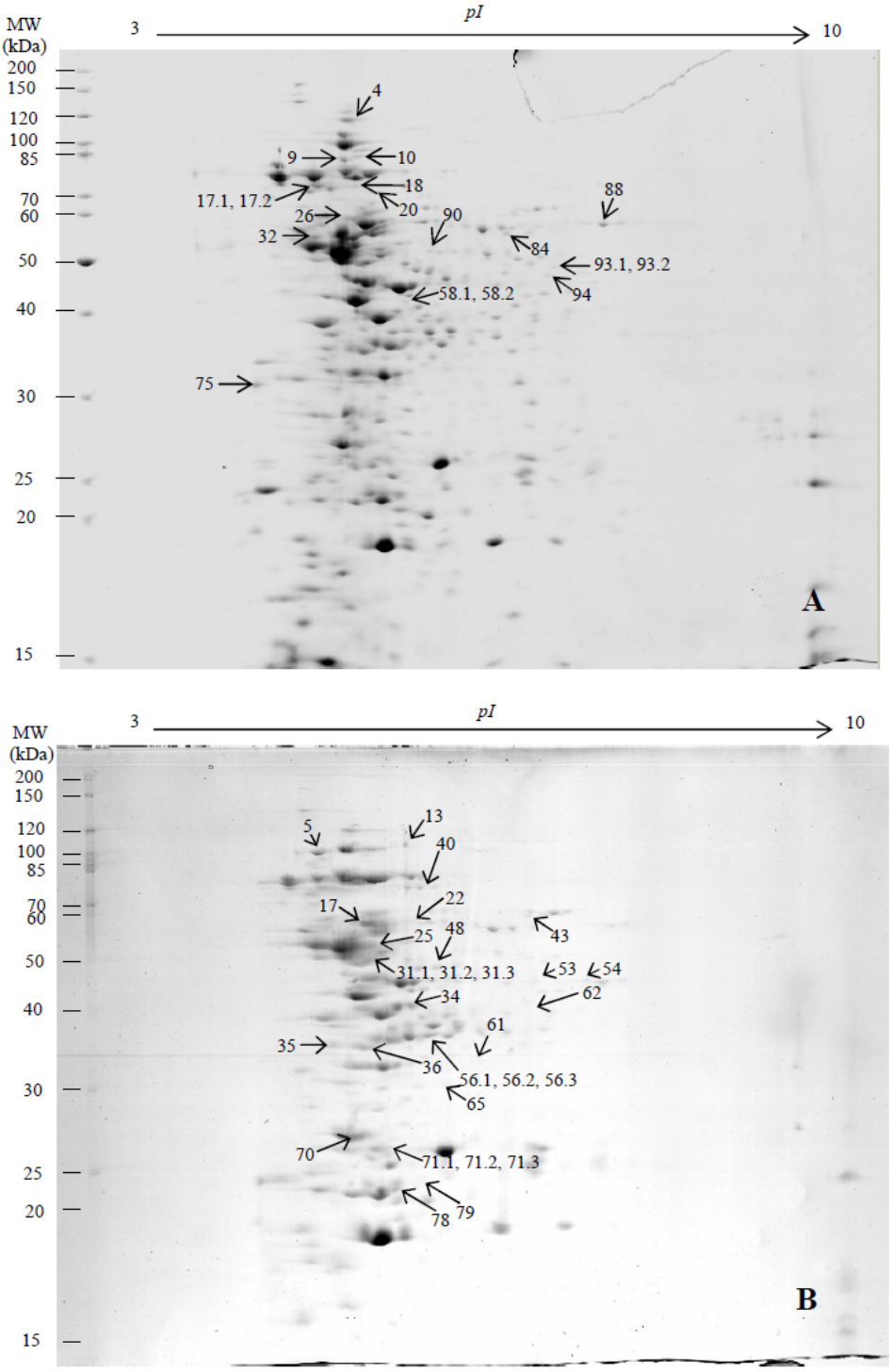


Figure 8. Two dimensional gel electrophoresis of whole cell proteins of methicillin-resistant *Staphylococcus aureus*. Protein extracts were prepared from rhodomyrtone-treated cells (A) and untreated control cells (B).

The presence of rhodomyrtone in MRSA cultures remarkably induced the expression of some general metabolic pathway related proteins based on their functions. Three proteins including D-fructose-6-phosphate amidotransferase, CTP synthetase, and bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase in amino acid metabolism and three proteins involved in carbohydrate metabolism such as aconitate hydratase, pyruvate oxidase, and malate:quinone oxidoreductase were identified. Two other proteins including coenzyme A disulfide reductase and pantothenate metabolism flavoprotein that related in cofactor metabolism were noted.

F0F1 ATP synthase subunit α was induced after treatment. The protein is partly embedded in the cell membrane and required for the regeneration of ATP from during the transfer of protons down the electrochemical gradient (Weber and Senior 1997). Protein members of chromosome partition, cell wall metabolism, and cell division such as endo-1, 4-*β*-glucanase and tRNA (uracil-5-)-methyltransferase Gid also up-regulated. Increase in the expression of three membrane transport systems-related proteins including phosphoenolpyruvate-protein phosphatase, ATP-binding protein Mrp-like protein, and glycine betaine/carnitine/choline ABC transporter, an osmoprotectant transport system permease protein involves in osmotic adjustment in bacteria (Ly *et al.* 2004), were observed.

Polynucleotide phosphorylase/polyadenylase (PNPase) was also up-regulated in rhodomyrtone-treated MRSA. This is an enzyme conserved in both bacteria and eukaryotic organelles that play roles in catalyzation of the phosphorolysis of RNA, releasing nucleotide diphosphates, and the reverse polymerization reaction. PNPase seems also to be participated in the control of several processes such as mRNA decay (Jarrige, Mathy, and Portier 2001), cold shock response (Gualerzi, Giuliodori, and Pon 2003), and the post-transcriptional modification (Sohlberg, Huang, and Cohen 2003) of mRNAs in a variety of prokaryotes. The induction of transcription related proteins including transcription elongation factor NusA and elongation factor P, which are associated in protein biosynthesis were noted after the treatment. The bacteria may utilize these up-regulated proteins to compensate for the

decrease in cell metabolic activities and to provide more efficient response to high-osmolarity stress due to rhodomyrtone challenge.

Rhodomyrtone inhibited the synthesis of six proteins functioning in glycolysis metabolic pathway such as fructose-1, 6-bisphosphate aldolase, pyruvate kinase, glyceraldehyde 3-phosphate dehydrogenase 2, D-lactate dehydrogenase, dihydrolipoamide dehydrogenase, and triosephosphate isomerase.

Some of absent proteins have been reported to be participated with chromosome partition such as GTPase ObgE (Foti *et al.* 2005), cell wall biosynthesis, cell division, and cell wall turnover including cell wall biosynthesis protein ScdA (Brunskill, de Jonge, and Bayles 1997), tubulin-liked FtsZ (Bramhill 1997), and autolysin (Sugai *et al.* 1997). In bacteria, cell wall is composed of the cross-linked polymer peptidoglycan, also known as murein. Peptidoglycan architecture consists of a disaccharide backbone built of alternating *β*-1-4-*N*-acetylglucosamines and *N*-acetylmuramic acids (Schleifer and Kandler 1972). In *S. aureus*, the tetrapeptide chains allow the formation of peptide cross-bridge including L-alanine, D-isoglutamine, L-lysine, and D-alanine, which are attached to the *N*-acetylmuramic acid. This cross-linking of peptide cross-bridge is made up of five glycines (Schleifer and Kandler 1972). Bacteria with compact peptidoglycan produce cell wall hydrolase, *N*-acetyl-glucosaminidase, *N*-acetylmuramidase, and endopeptidase for cell division (Giesbrecht *et al.* 1998). It has been observed that rhodomyrtone suppressed the expression of autolysin with bifunctional activities, *N*-acetylmuramyl-L-alanine-amidase and *N*-acetylglucosaminidase. The bacterial cell division machinery is triggered with a formation of Z ring which consists of polymers of FtsZ protein at the division site (van den Ent, Amos, and Lowe 2001). FtsZ is the bacterial tubulin homologue which plays a key role for the recruitment of other proteins to the cell division septum (Scheffers and Pinho 2005). The reduction of the cell wall-related proteins (GTPase ObgE, ScdA, FtsZ) following treatment with rhodomyrtone likely contributes to the reduced rate of cell wall biosynthesis, cell division, and cell wall morphogenesis and turnover. Additionally, suppressed production of autolysin might be the result as the bacterial cells react to prevent the destruction of peptidoglycan by autolytic enzymes.

A number of enzymes related in metabolism of some amino acids such as ornithine carbamoyltransferase and arginase in arginine biosynthesis, alanine dehydrogenase in alanine biosynthesis, glycine dehydrogenase subunit 2 (decarboxylating) and 2-amino-3-ketobutyrate coenzyme A ligase in glycine, serine, and threonine biosynthesis, formimidoylglutamase in histidine biosynthesis, and glutamine-ammonia ligase in glutamine biosynthesis were down-regulated. Other enzymes in several amino acid and energy metabolism were down regulated by subinhibitory concentration of rhodomyrtone were (i), cysteine synthase (*O*-acetylserine sulfhydrylase)-like protein which related with three metabolic pathways including cysteine, seleno amino acid, and sulfur metabolism; (ii), carbamate kinase in four metabolic pathways including purine, glutamate, arginine and proline, and nitrogen metabolism; and (iii), putative *N*-acetyltransferase. The reduction of alanine, glycine, and glutamine expression in rhodomyrtone-treated MRSA may be associated with the decrease in protein and peptidoglycan synthesis since those amino acid are crucial in both biological processes (de Jonge *et al.* 1992; Maidhof *et al.* 1991). Particularly, the lower glycine content which is an amino acid composition of peptidoglycan cross-bridges might excite the aberrant cell septum formation and retard cell division (Gustafson *et al.* 1992).

Other proteins involved in protein degradation, oxidative stress, putative hydrolase, and hypothetical protein were also inhibited after treatment. The stress response proteins were evidenced by the suppressing of the expression of heat-shock proteins including DnaK, ATP-dependent Clp protease proteolytic subunit-like protein, and oxidative stress-related protein such as superoxide dismutase. The proposed stress-related protein obviously reflected an adaption of the bacteria to survival under stress condition. In *S. aureus*, Clp proteins play significant roles in the regulation of many cellular functions such as replication of DNA, control of gene expression, heat stress tolerance, protein degradation, and protein folding as molecular chaperones (Singh *et al.* 2007; Squires and Squires 1992). In addition, they also related to various biological processes such as cell division, development of sporulation, genetic competence, and quality control of protein translation (Jenal and Fuchs 1998; Turgay *et al.* 1998). Clp proteinase are composed of two distinct

subunits, proteolytic part is assembled with ATPase subunit into a multimeric structure. In *E. coli,* Clp protease is known to play a role in cytoplasmic quality control and participate in numerous regulatory mechanisms that are important in non-growing or slow-growing cells (Weichart *et al.* 2003). In this study, ATP-dependent Clp protease proteolytic subunit-like protein (ClpP, proteolytic subunit) was suppressed whereas ATP-dependent Clp proteinase chain (ClpB, ATPase subunit) was induced. In *E. coli*, ClpB has a crucial function in cell survival at high temperatures (Squires *et al.* 1991) and participates in *de novo* protein folding in mildly stressed (Thomas and Baneyx 2000). Our data suggested that MRSA responded to stress and degradation of many intracellular proteins after exposure to rhodomyrtone at subinhibitory concentration. The compound also inhibited the expression of immunodominant antigen A, a virulence factor which elicits immune response during septicaemia (Lorenz *et al.* 2000).

Table 4. Identification of *Staphylococcus aureus* cellular proteins presented after rhodomyrtone treatment

Spot no.	Accession no.	Identified proteins	Mw (KDa)	*pI*	Mascot score	Sequence coverage (%)
Amino acid metabolism						
18	gi|21283809	D-fructose-6-phosphate amidotransferase	65925	4.93	460	17
20	gi|15925117	CTP synthetase	60297	5.00	191	8
94	gi|15924727	bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase	40709	5.83	266	16
Carbohydrate metabolism						
4	gi|15924340	aconitate hydratase	99136	4.80	139	21
17.1	gi|15925529	pyruvate oxidase	63932	6.88	332	14
88	gi|15925597	malate:quinone oxidoreductase	56135	6.12	701	34
Cell wall metabolism						
58.1	gi|15925445	endo-1,4-β-glucanase	39212	5.21	326	16
Cell division						
84	gi|15924241	tRNA (uracil-5-)-methyltransferase Gid	48515	5.57	1012	40
Cofactor metabolism						
90	gi|15923960	coenzyme A disulfide reductase	49374	5.28	453	21
93.2	gi|15924201	pantothenate metabolism flavoprotein	44171	5.68	60	4
Energy metabolism						
26	gi|15925095	F0F1 ATP synthase subunit α	54608	4.91	595	18
Nucleotide metabolism						
10	gi|15924264	Polynucleotide phosphorylase/polyadenylase	77428	4.89	1533	42

Table 4. Identification of *Staphylococcus aureus* cellular proteins presented after rhodomyrtone treatment (2)

Spot no.	Accession no.	Identified proteins	Mw (KDa)	*pI*	Mascot score	Sequence coverage (%)
Protein degradation						
9	gi15925538	ATP-dependent Clp proteinase chain	77926	4.83	1256	33
Transcription						
32	gi|15924256	transcription elongation factor NusA	43787	4.60	267	14
75	gi|15924518	elongation factor P	20541	4.75	263	23
Transport system membrane						
17.2	gi|15924074	phosphoenolpyruvate-protein phosphatase	63394	4.62	266	11
58.2	gi|15925155	ATP-binding protein Mrp-like protein	38334	5.29	86	3
93.1	gi|15925438	glycine betaine/carnitine/choline ABC transporter transporter	46463	5.93	108	5

Table 5. Identification of *Staphylococcus aureus* cellular proteins absented after rhodomyrtone treatment

Spot no.	Accession no.	Identified proteins	Mw (KDa)	*pI*	Mascot score	Sequence coverage (%)
Amino acid and energy metabolisms						
22	gi|15924300	glutamine-ammonia ligase	51108	5.09	137	5
34	gi|49484831	ornithine carbamoyltransferase	37867	5.15	42	37
43	gi|57651929	glycine dehydrogenase subunit 2 (decarboxylating) (decarboxylating)	54861	5.70	65	19
48	gi|156978879	2-amino-3-ketobutyrate coenzyme A ligase ligase	42935	5.14	79	43
53	gi|21283381	alanine dehydrogenase	40080	5.58	152	13
56.1	gi|49242958	carbamate kinase	35425	5.36	1086	35
56.2	gi|3892892	arginase	33170	5.29	165	8
61	gi|15925324	formimidoylglutamase	34605	5.35	53	7
62	gi|15923503	cysteine synthase (*O*-acetylserine sulfhydrylase)-sulfhydrylase)-like protein	33012	5.39	289	40
65	gi|49484892	putative *N*-acetyltransferase	30603	5.26	299	38
Carbohydrate metabolism						
17	gi|15924086	dihydrolipoamide dehydrogenase	49592	4.95	143	27
36	gi|49484802	fructose-1,6-bisphosphate aldolase	32893	4.96	46	28
40	gi|15924687	pyruvate kinase	63291	5.23	523	13

Table 5. Identification of *Staphylococcus aureus* cellular proteins disappeared after rhodomyrtone treatment (2)

Spot no.	Accession no.	Identified proteins	Mw (KDa)	*pI*	Mascot score	Sequence coverage (%)
Carbohydrate metabolism (continuted)						
54	gi|221137774	glyceraldehyde 3-phosphate dehydrogenase 2 dehydrogenase 2	37126	6.07	137	9
56.3	gi|15925514	D-lactate dehydrogenase	39297	5.35	57	2
70	gi|15923764	triosephosphate isomerase	27417	4.81	71	39
Cell wall biosynthesis and metabolism						
13	gi|21282665	autolysin	137323	9.61	567	8
31.2	gi|15924634	GTPase ObgE	47263	5.05	190	11
71.2	gi|4948245	cell wall biosynthesis protein ScdA	25672	5.09	378	30
Lipid metabolism						
31.1	gi|21282595	3-oxoacyl- synthase	43883	5.03	1125	49
Cell division						
25	gi|15924176	cell division protein FtsZ	43135	4.74	523	32
Oxidative stress						
78	gi|15924543	superoxide dismutase	22697	5.08	221	18
Pyrimidine metabolism						
31.3	gi|15924191	dihydroorotase	46742	5.06	114	4

Table 5. Identification of *Staphylococcus aureus* cellular proteins disappeared after rhodomyrtone treatment (3)

Spot no.	Accession no.	Identified proteins	Mw (KDa)	*pI*	Mascot score	Sequence coverage (%)
Protein degradation and Stress response						
5	gi|15924570	DnaK protein	66378	4.65	538	17
79	gi|15923758	ATP-dependent Clp protease proteolytic subunit-like protein	21558	5.13	76	11
Surface antigen and virulence factor						
71.3	gi|4948478	immunodominant antigen A	24198	6.11	189	15
Others						
35	gi|49292908	putative hydrolase	30956	4.76	49	29
71.1	gi|4126674	hypothetical protein	22954	5.21	414	33

4. Effect of rhodomyrtone on *Staphylococcus aureus* cell morphology and ultrastructure

To elucidate the physiological effects of rhodomyrtone on morphology and ultrastructure of *S. aureus*, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were utilized. As shown in Figure 9 and Figure 10, the TEM clearly demonstrated that the growth of both MRSA NPRC 001R and *S. aureus* ATCC 29213 in media containing rhodomyrtone at 0.174 μg/ml for 18 h generated profound changes in *S. aureus* cell morphology and ultrastructure. SEM illustrated no significant different of morphological and surface change between treated bacteria and untreated control cells after incubation for 1 h while a greater number of debris, lyzed cells, and wrinkled cells have been observed at incubation period of 4 h.

4.1 Transmission electron microscopy

Almost all rhodomyrtone-treated cells were obviously affected on the cell envelope, cytoplasmic contents, cell ultrastructure, and cell separation process. Interestingly, we found that rhodomyrtone induced cell wall deformation (Figure 9C), thickened cell wall (Figure 9D, arrow), and thickened septa with irregular features (Figure 9E, 9F and 10E, arrows). Furthermore, the arc-like shape of fibrillar wall material occurring from turning of compressed cross wall and septal formation (Figure 9E and 10E, arrows) were also observed in rhodomyrtone exposed cells. Cell separation system is inhibited and only the fibrillar wall material is organized instead of a compact cross wall. The results might suggest that cells with thickened walls may represent a defensive response to rhodomyrtone. Cells with broken wall, protoplast, cell wall shreds, and cell debris were generally found following rhodomyrtone treatment (Figure 9C, 9F, 9G, 9H, and 10C-10H). Indeed, various atypical size and cell shape alterations, for example, irregular, swollen, oval-shaped, and defective cells were obviously noticeable, especially in the treated MRSA (Figure 9C), while these aberrations were not observed in the untreated cells (Figure 10C). Overnight exposure of *S. aureus* ATCC 29213 to rhodomyrtone resulted in almost complete lysis of intact bacteria to cellular debris (Figure 10C). In this study, rhodomyrtone was found to cause cell wall and cytoplasmic membrane damage in *S. aureus*.

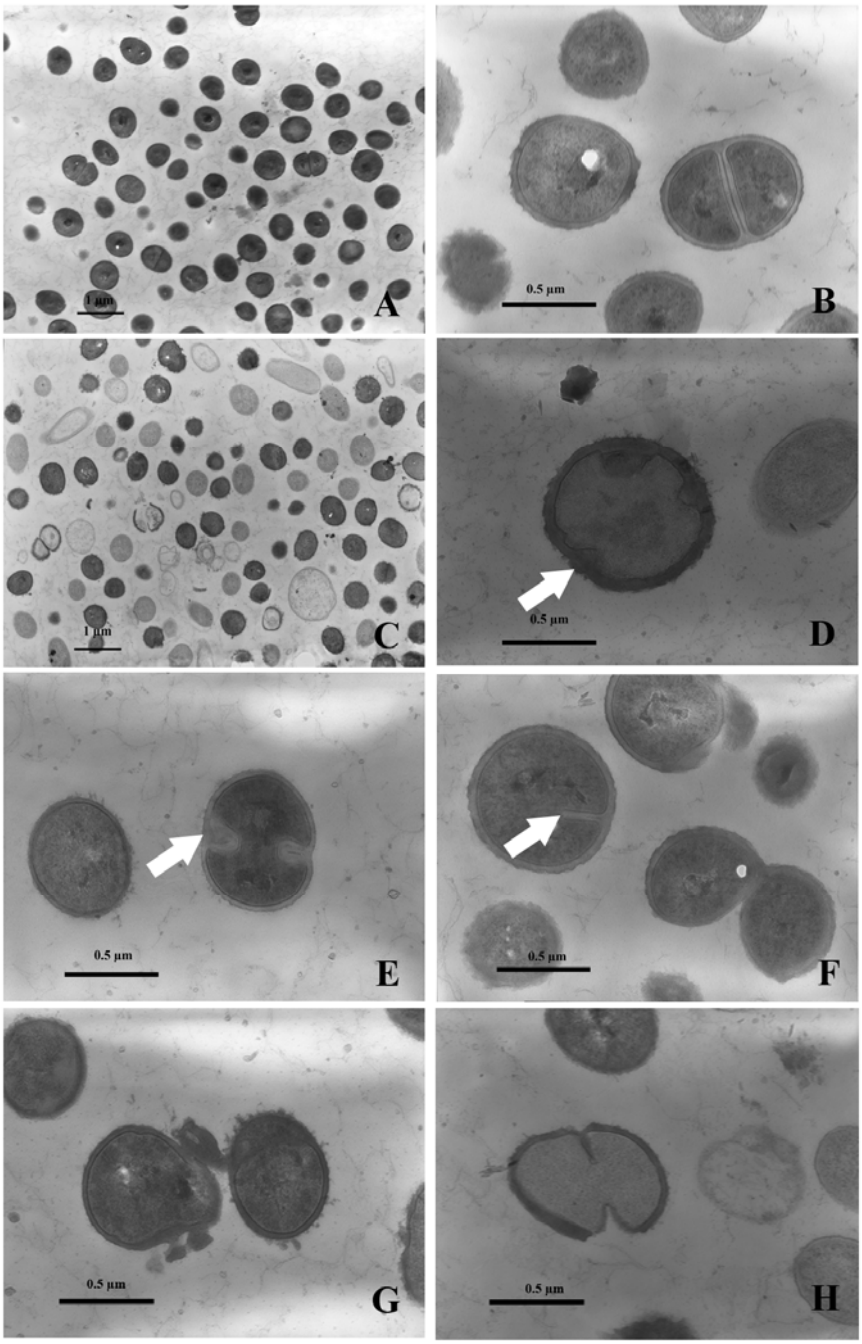


Figure 9. Transmission electron micrographs demonstrate effect of rhodomyrtone on MRSA NPRC 001R morphology. The bacteria were incubated in CAMHB supplemented with DMSO, untreated control culture (A, B) and CAMHB containing 0.174 µg/ml rhodomyrtone (MIC=0.5 µg/ml) (C, D, E, F, G, H) for 18 h. Scale bars=1 µm (A, C) and 0.5 µm (B, D, E, F, G, H), respectively.

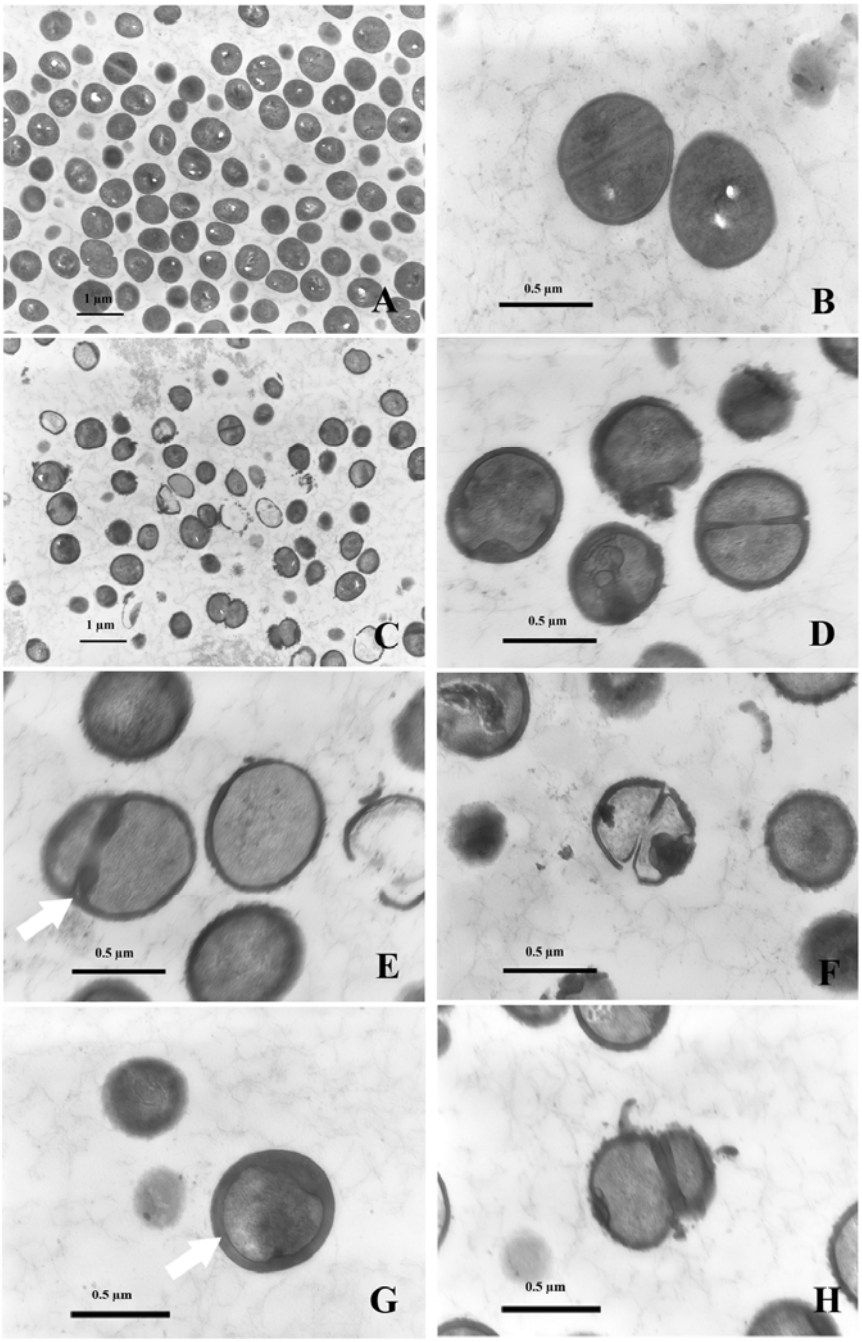


Figure 10. Transmission electron micrographs demonstrate effect of rhodomyrtone on *Staphylococcus aureus* ATCC 29213 morphology. The bacteria were incubated in CAMHB supplemented with DMSO, untreated control culture (A and B) and CAMHB containing 0.174 µg/ml rhodomyrtone (MIC=0.5 µg/ml) (C, D, E, F, G, H) for 18 h. Scale bars=1 µm (A, C) and 0.5 µm (B, D, E, F, G, H), respectively.

4.2. Scanning electron microscopy

SEM examination of staphylococcal cells illustrated the effect of the rhodomyrtone on the bacterial cell morphology and surface. SEM micrographs demonstrated insignificant alteration on MRSA NPRC 001R (Figure 11) and *S. aureus* ATCC 29213 cells (Figure 12) after exposure to rhodomyrtone at 0.174 µg/ml for 18 h. This result was inconsistent from TEM examination that showed pronounced changes on rhodomyrtone-treated cells (Figure 9 and Figure 10). A few number of abnormal size cells were observed on rhodomyrtone-treated MRSA NPRC 001R (Figure 11B and 11C) while a large number of lyzed and wrinkle cells were found in treated *S. aurues* ATCC 29213 (Figure 12), when compared to untreated control (Figure 11A).

The cultures of EMRSA-16 containing rhodomyrtone at 0.5MIC (0.5 µg/ml) was investigated by SEM. An incubation time point used for treatment in this study, was 1 h according to microarray procedure. Even though microarray data displayed some differentially expressed genes encoding biosynthesis of the bacterial cell wall, the MRSA cells after exposure to rhodomyrtone at 0.5MIC for 1 h exhibited no significant alteration of morphology and surface when compared to untreated control (Figure 13). SEM of the cells in the presence or absence of rhodomyrtone treatment for 4 h was further observed (Figure 14). A high numbers of lyzed- and wrinkle cells were obviously found in treated cells when compared to untreated control cells. Therefore, incubating for 4 h after exposure to rhodomyrtone was selected as the condition used for analysis of peptidoglycan amino acid composition.

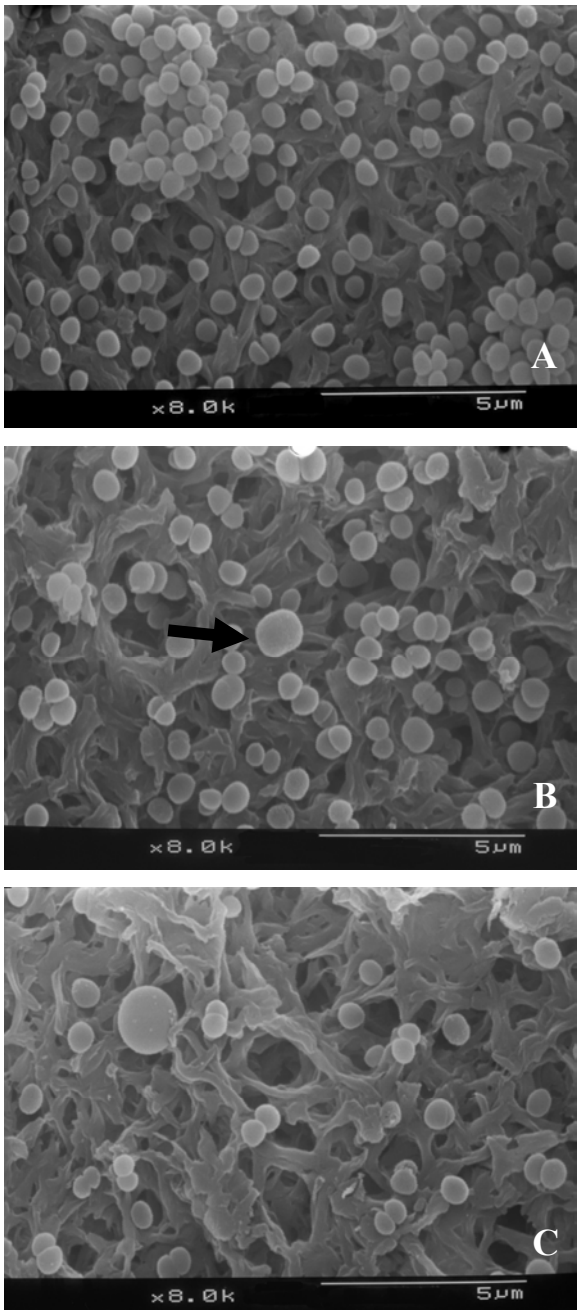


Figure 11. Scanning electron micrographs demonstrate effect of rhodomyrtone on MRSA NPRC 001R morphology. The bacteria were incubated in CAMHB supplemented with DMSO, untreated control culture (A) and CAMHB containing 0.174 µg/ml rhodomyrtone (MIC=0.5 µg/ml) (B, C) for 18 h. Scale bars= 5 µm.

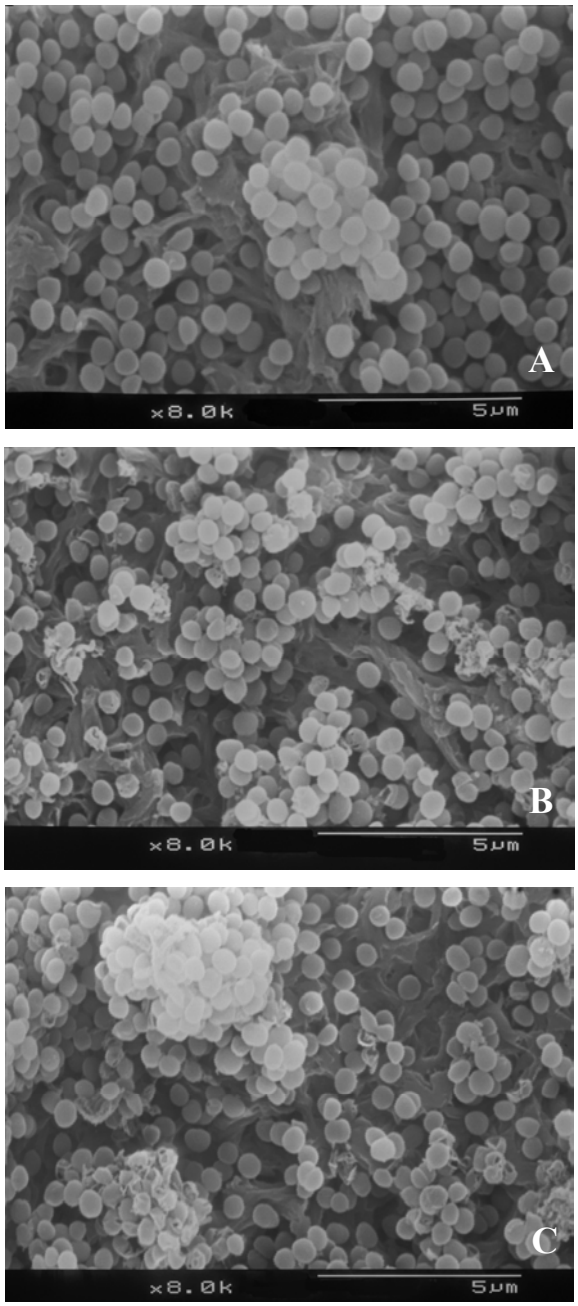


Figure 12. Scanning electron micrographs demonstrate effect of rhodomyrtone on *S. aureus* ATCC 29213 morphology. The bacteria were incubated in CAMHB supplemented with DMSO, untreated control culture (A) and CAMHB containing 0.174 μg/ml rhodomyrtone (MIC=0.5 μg/ml) (B, C) for 18 h. Scale bars= 5 μm.

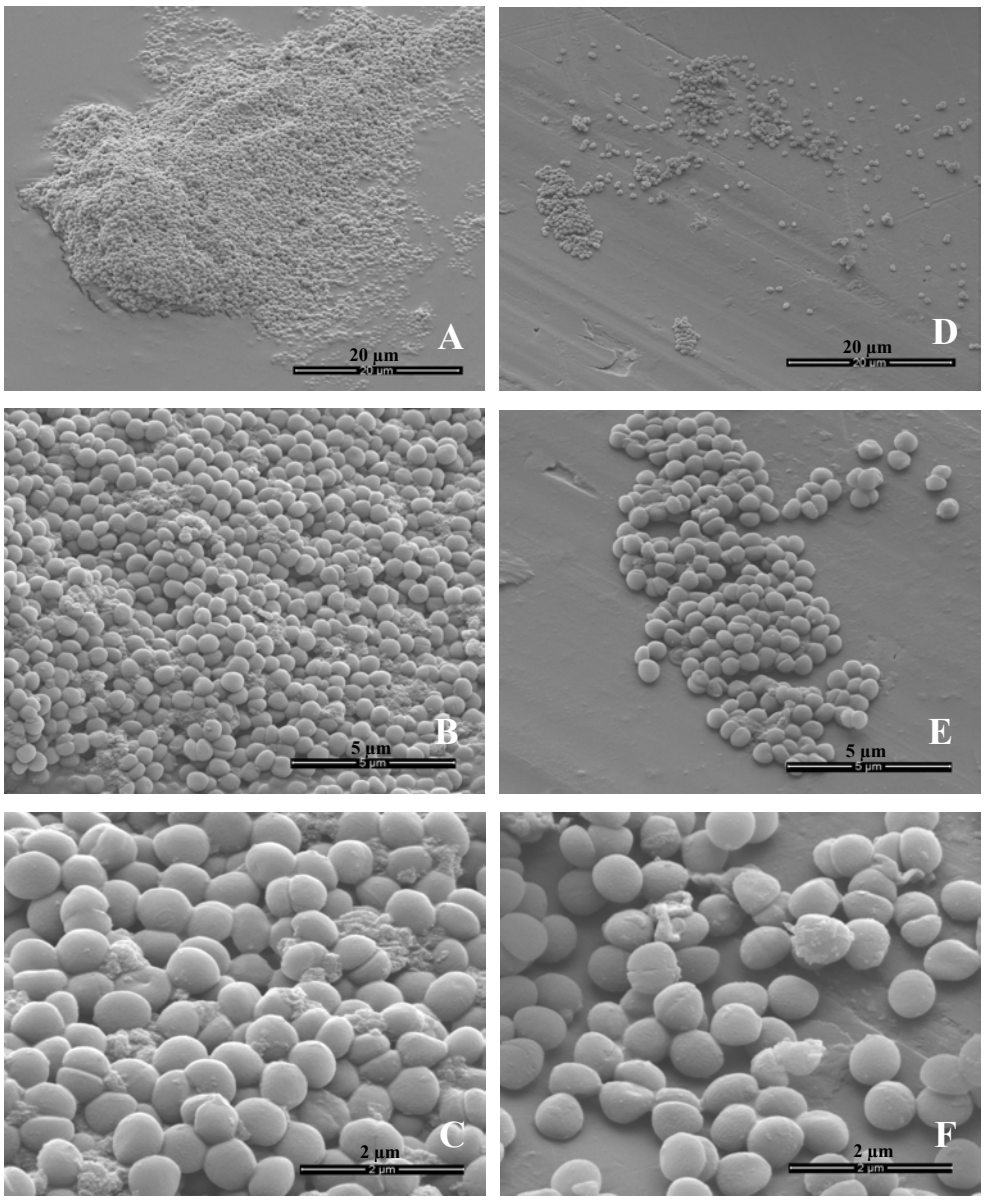


Figure 13. Scanning electron micrographs demonstrate effect of rhodomyrtone on EMRSA-16 cell morphology after treatment for 1 h. The bacteria were incubated in MHB containing 0.5 µg/ml rhodomyrtone (MIC=1.0 µg/ml) (A, B, C) and MHB supplemented with DMSO, untreated control culture (D, E, F) for 1 h. Scale bars=20 µm (A, D), 5 µm (B, F), and 2 µm (C, F), respectively.

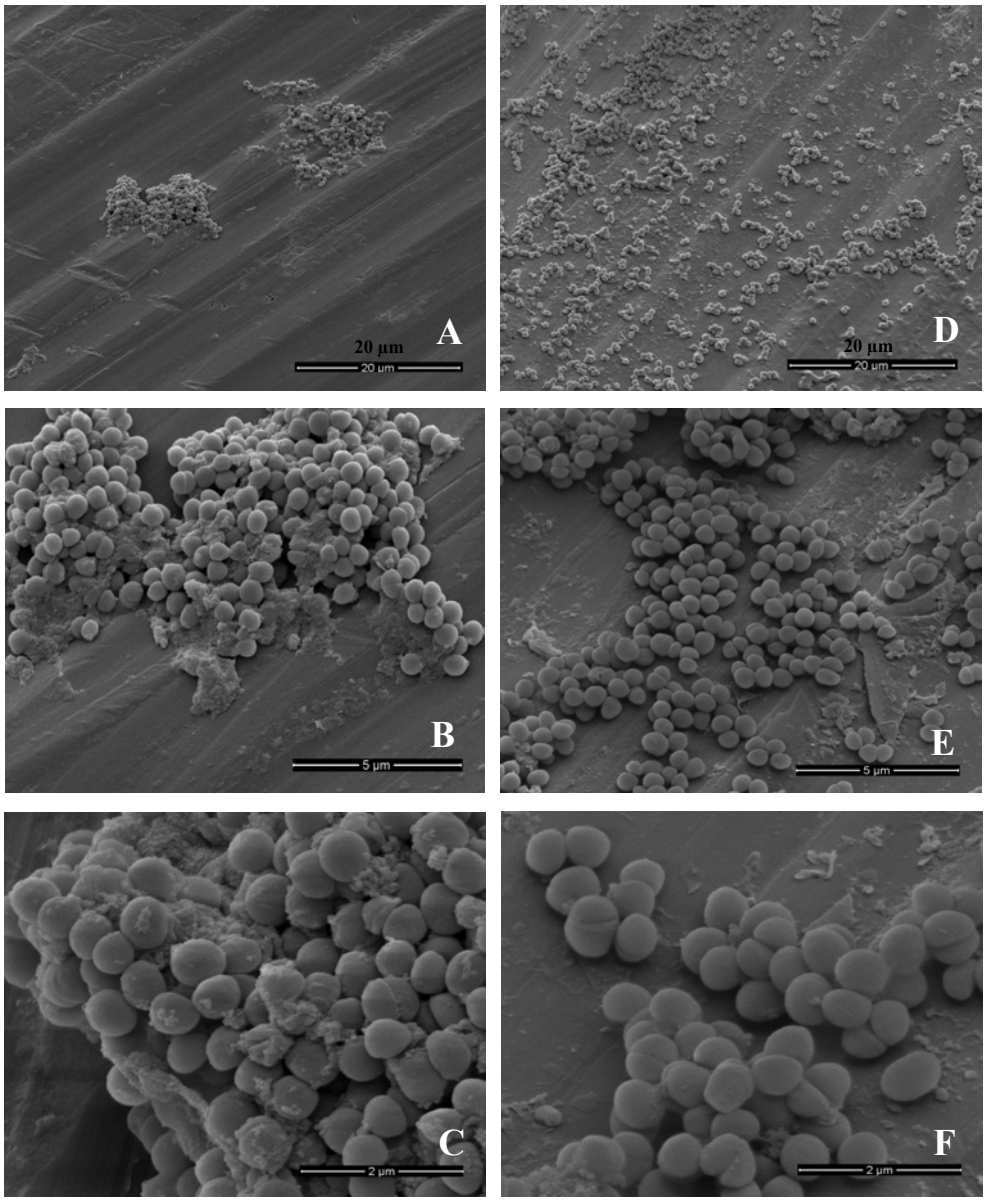


Figure 14. Scanning electron micrographs demonstrate effect of rhodomyrtone on EMRSA-16 cell morphology after treatment for 4 h. The bacteria were incubated in MHB containing 0.5 µg/ml rhodomyrtone (MIC=1.0 µg/ml) (A, B, C) and MHB supplemented with DMSO, untreated control culture (D, E, F) for 4 h. Scale bars=20 µm (A, D), 5 µm (B, F), and 2 µm (C, F), respectively.

5. Transcriptional response of methicillin-resistant *Staphylococcus aureus* in the presence of subinhibitory concentration of rhodomyrtone

5.1. Microarray analysis

In order to have a global understanding of the transcriptional response of MRSA to rhodomyrtone, a microarray based approach was used to profile genes that were differently expressed after treatment. The incubation period at 1 h following rhodomyrtone treatment induced a dependent early transcriptional response of EMRSA-16. Transcript abundance from mid log-phase cells grown in MHB in the presence or absence of rhodomyrtone were compared by Affymetrix microarray analysis. Genes presenting more than two-fold up- or down- regulation upon rhodomyrtone challenge were shown in Table 5 and Table 6, respectively. Microarray analysis data were validated by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Twelve gene-specific primer pairs used for qRT-PCR (Figure 15) including (i), eight genes of interest from microarray data, *asd*, *dapA*, *thrC*, *scdA*, SAR0996, SAR0437, SAR0761, and *rpoB*, and (ii), Four negative control genes, *lysA*, *ftsH*, *ftsZ*, and *murE*, that were not defined to be differentially expressed by microarray analysis. Gene expression data obtained for *asd*, *dapA*, *thrC*, *scdA*, SAR0996, SAR0437, SAR0761, and *rpoB* was similar from both microarray analysis and qRT-PCR. Although, the fold changes values of almost all genes obtained from qRT-PCR were much higher than that obtained from microarrays in this study.

Total number of 66 rhodomyrtone-induced genes and 36 of rhodomyrtone-repressed genes that showed more than two-fold change in expression by microarray analysis were listed (Table 5 and 6). The significant proportions of these differentially expressed genes were responsible for a group of genes encoding protein of unknown function (hypothetical protein), amino acid metabolism, membrane protein, transporter protein, and nucleotide metabolism, respectively. Overall, the genes expression changes were ranged into three groups; <10 fold change were 64 genes (52.9%), 10-50 fold change were 22 genes (21.6%), and >50 fold change were 16 genes (15.7%). Of these, a very large propotion of genes annotated as

hypothetical proteins were up-regulated (at least 48.5%) and down-regulated (22.2%). Interestingly, a gene encoding conserved hypothetical protein, SAR0996 was most prominent up-regulated (up to 97.3-fold) consisting of 75 amino acids (288 bp). It has the following amino acid sequence:
"mvsmkyntnvkhttleafvttvndlgieliinealrevrkrqlieliddalvnkdeaafnqymaeyknleaflge"

Indeed, rhodomyrtone showed significantly up-regulated a groups of genes involved with amino acid biosynthetic pathway such as lysine biosynthesis; aspartate semialdehyde dehydrogenase *(asd*), dihydrodipicolinate synthase (*dapA*), dihydrodipicolinate reductase (*dapB*), and putative tetrahydrodipicolinate (*dapD*), threonine biosynthesis; threonine synthase (*thrC*), and arginine biosynthesis; argininosuccinate synthase (*argG*). The obvious up-regulating of four ATP-binding cassette (ABC) transporter suggested possibly action in exportation toxic agents to outer side of the bacterial membrane. Moreover, rhodomyrtone also significantly affects many genes encoding bacterial membrane protein including seven (10.6%) were up-regulated genes and five (13.9%) down-regulated genes. There was up-regulation of two genes including SAR2030 (encoding MHC class II analog) and *sbi* (encoding IgG-binding protein), which involved in the bacterial virulence factor. MHC class II analog and IgG binding protein is bacterial "analog" to eukaryotic major histocompatibility complex (MHC) class II molecules and may potentially interfere with the immune defense system of the host (Jonsson *et al.* 1995). Major functional categories of down-regulated genes were nucleotide metabolism, membrane proteins, hypothetical proteins, amino acid metabolism, and transporter protein. A reduction of expression of genes associated in nucleotide metabolism was observed in many genes including *gyrB*, *holB*, *purR*, *nusG*, *rpoB*, *sdhC*, *rluB*, *tgt*, *sigB*, *rho*, *truA*, and. SAR1833. A phosphor transferase system (PTS) gene, *mtlA*, was expressed at a decreased level of 12.44 fold. The down-regulation of *ldh*1, gene encoding L-lactate dehydrogenase 1, was > 15 fold change in the presence of rhodomyrtone. Down-regulation of this gene involved in pyruvate utilization by converting pyruvate to lactate.

5.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Time course analysis of 12 selected genes of interest was carried out using qRT-PCR. The RNA samples of rhodomyrtone-treated cells were prepared from the bacterial samples after treated with rhodomyrtone at different time point including 1, 4, and 18 h (Figure 15). Gene-specific primers were listed in Table 1. For analysis, genes were divided into three groups including group I (Figure 17); *asd*, *dapA*, *thrC*, and *lysA*, group II (Figure 18); SAR0996, SAR0437, and SAR0761, and group III (Figure 19), *ftsH*, *ftsZ*, *murE, scdA,* and *rpoB*.

Gene of group I (Figure 17), the genes that related in DAP pathway *asd* and *dapA*, and threonine pathway including *thrC*, were up-regulated (the trend was similar to microarray data) while only *lysA* (DAP pathway) was down-regulated (no significantly presented in microarray data). After incubation for 4 h, *asd* and *thrC* were still up-regulated with the lower fold change values than at 1 h while *dapA* and *lysA* were no significant change in expression. At 18 h, no statistically significant change was detected of all four genes.

Genes of group II (Figure 18), hypothetical proteins, were highly up-regulated both at 1 and 4 h. After 18 h, the significant down-regulation was presented in only SAR0996 (9.56 fold) SAR0437 (encoding putative exported protein) and SAR0761 (putative lipoprotein) were no significant different change displayed

Genes of group III (Figure 19), *ftsH*, *ftsZ*, *murE,* and *scdA* are genes involved in bacterial cell wall biosynthesis, and *rpoB* is the β subunit of RNA polymerase. At 1h incubation time, only *murE* was up-regulated (>19 fold), *ftsZ* and *rpoB* were down-regulated, and *ftsZ* and *scdA* were no significant different change of expression. At 4 h, *rpoB* was still down-regulated while other genes were no different change. Only *ftsH* was down-regulated at 18 h incubation time, while all other genes were no significant different change.

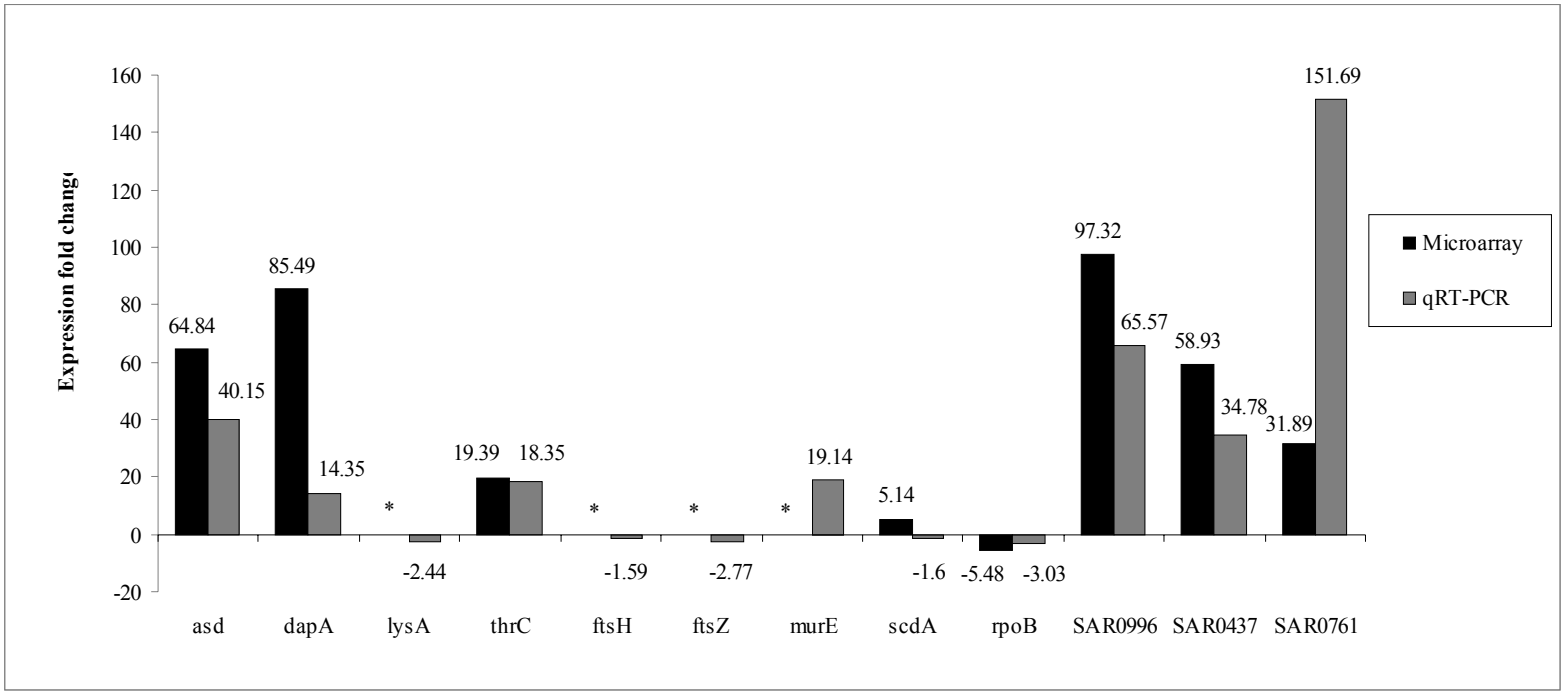


Figure 15. Validation of microarray data using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) Differentially expressed genes were analyzed in methicillin-resistant *Staphylococcus aureus* strain EMRSA-16 after treatment with 0.5µg/ml (0.5MIC) of rhodomyrtone at 37^{o}C for 18 h. * - Gene expression < 2.0 fold change (*p* value of ≤0.05)

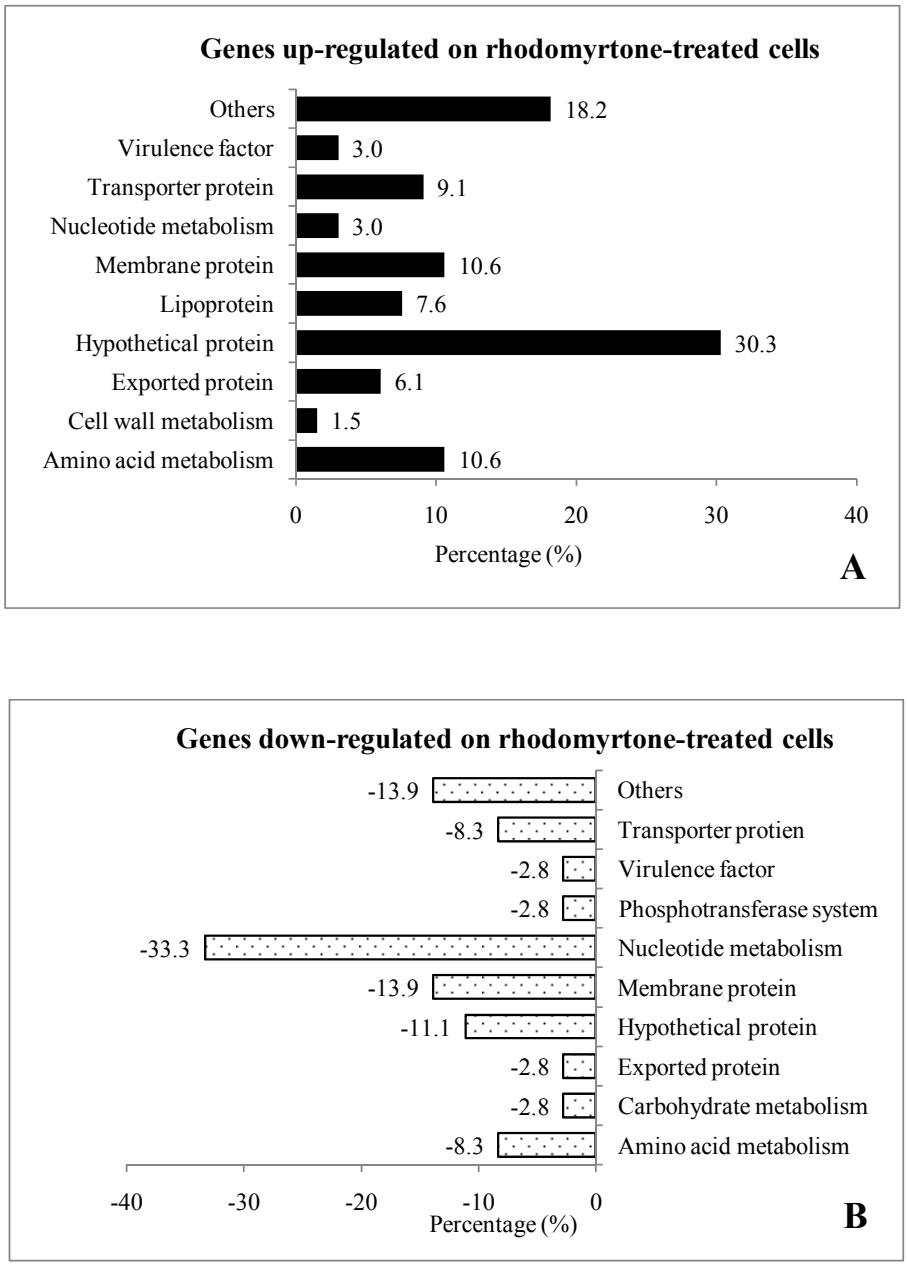


Figure 16. List of biological pathways identified from microarray analysis of rhodomyrtone-treated EMRSA-16, (A); up-regulated genes and (B); down-regulated genes. The bacteria were treated with rhodomyrtone at 0.5µg/ml (0.5MIC) at 37^{o}C for 18 h. Differentially expressed genes have more than two-fold change ($p \leq 0.05$).

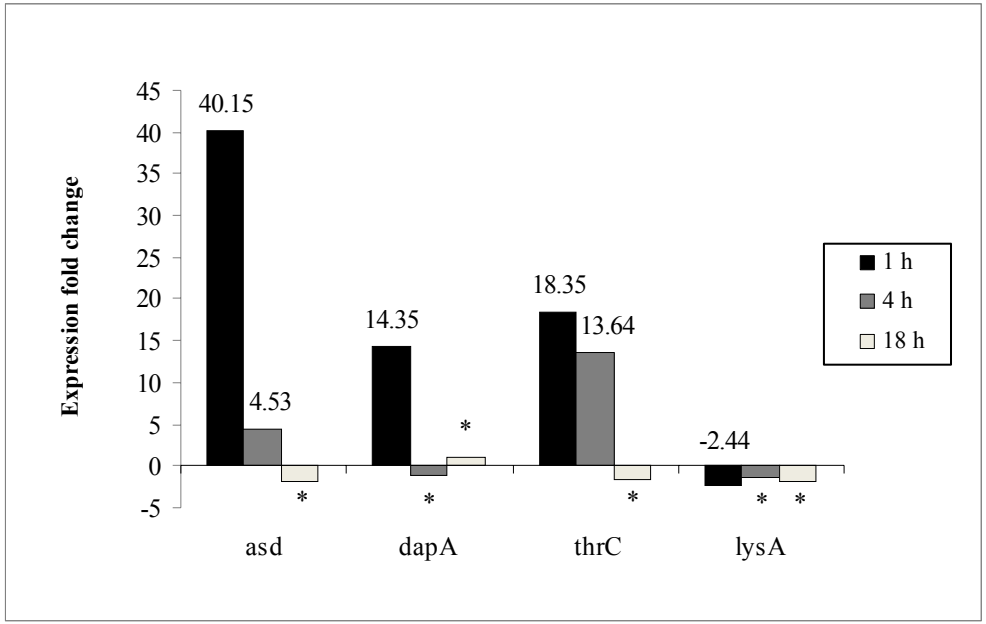


Figure 18. Time course analysis of genes in group I. RNA samples were extracted from rhodomyrtone-treated cells (0.5 µg/ml, 0.5MIC) after incubation at various time points including 1 h, 4 h, and 18 h. Selected genes of interest (Group I)were *asd*, *dapA*, *thrC*, and *lysA*. The expression levels were normalized to 16s rRNA. * - Gene expression < 2.0 fold change (*p* value of ≤0.05)

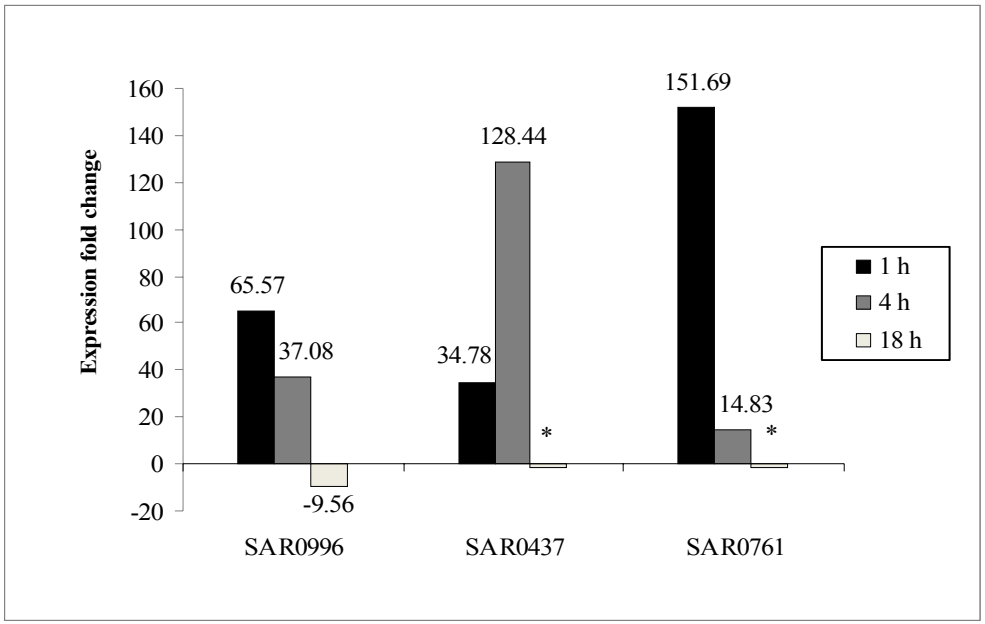


Figure 19. Time course analysis of genes in group II. RNA samples were extracted from rhodomyrtone-treated cells (0.5 µg/ml, 0.5MIC) after incubation at various time points including 1 h, 4 h, and 18 h. Selected genes of interest (Group II) were SAR0996, SAR0437, and SAR0761. The expression levels were normalized to 16s rRNA. * - Gene expression < 2.0 fold change (*p* value of ≤0.05)

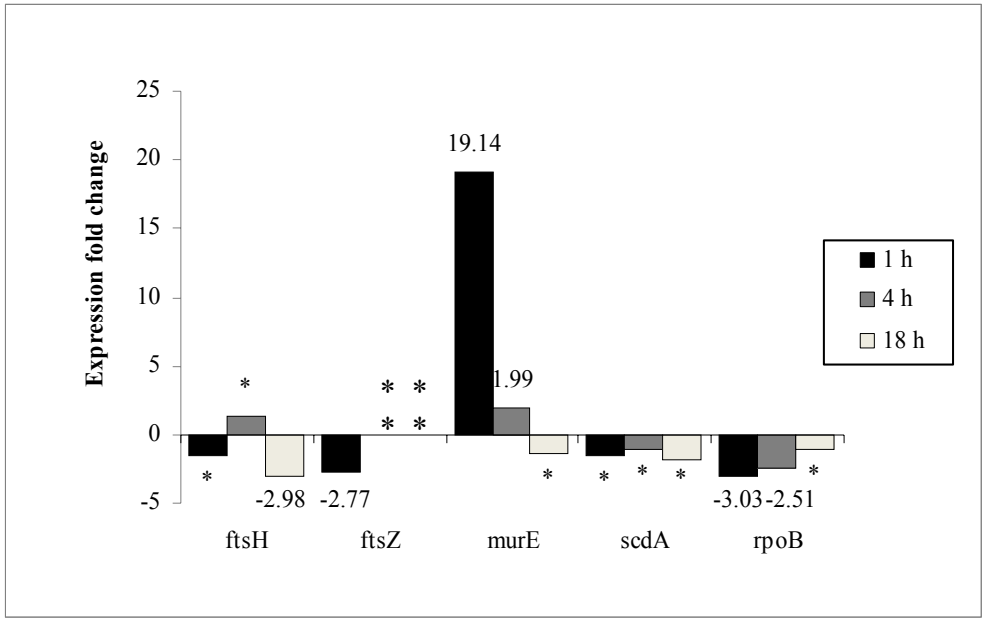


Figure 20. Time course analysis of genes in group III. RNA samples were extracted from rhodomyrtone-treated cells (0.5 µg/ml, 0.5MIC) after incubation at various time points including 1 h, 4 h, and 18 h. Selected genes of interest (Group III) were *ftsZ*, *ftsH*, *murE*, *scdA*, and *rpoB*. The expression levels were normalized to 16s rRNA. * - Gene expression < 2.0 fold change (*p* value of ≤0.05). $\overset{*}{*}$ - more biological replicates were required.

Table 6. Genes up-regulated in rhodomyrtone-treated EMRSA-16.

MRSA252[a] (or annotated in other *S. aureus* strains genome)	Gene	Gene product description	Expression-fold change*	Functional category
SAR0923	*argG*	argininosuccinate synthase	7.35	Amino acid metabolism
SAR1338	*dhoM*	homoserine dehydrogenase	34.65	Amino acid metabolism
SAR1339	*thrC*	threonine synthase	19.39	Amino acid metabolism
SAR1406	*asd*	aspartate semialdehyde dehydrogenase	64.84	Amino acid metabolism
SAR1407	*dapA*	dihydrodipicolinate synthase	85.49	Amino acid metabolism
SAR1408	*dapB*	dihydrodipicolinate reductase	88.30	Amino acid metabolism
SAR1409	*dapD*	tetrahydrodipicolinate acetyltransferase	51.45	Amino acid metabolism
SAR0256	*scdA*	cell wall metabolism protein	5.14	Cell wall metabolism
SAR0437		putative exported protein	58.93	Exported protein
SAR1094		putative exported protein	2.25	Exported protein
SAR2295		putative exported protein	7.49	Exported protein
SAR2615		putative exported protein	5.35	Exported protein
	lpl5	hypothetical protein	2.99	Hypothetical protein
NA (MW0754)		hypothetical protein	2.10	Hypothetical protein
NA (SACOL1336)		hypothetical protein	2.27	Hypothetical protein
NA (SACOL2406)		hypothetical protein	3.67	Hypothetical protein
NA (SACOL2454)		hypothetical protein	3.05	Hypothetical protein

NA (SAOUHSC_01090)	hypothetical protein	2.10	Hypothetical protein
SAR0090	hypothetical protein	2.50	Hypothetical protein
SAR0421	conserved hypothetical protein	9.90	Hypothetical protein
SAR0448	hypothetical protein	2.05	Hypothetical protein
SAR0996	conserved hypothetical protein	97.32	Hypothetical protein
SAR1342	hypothetical protein	4.14	Hypothetical protein
SAR1415	hypothetical protein	3.02	Hypothetical protein
SAR1572	conserved hypothetical protein	3.71	Hypothetical protein
SAR1682	hypothetical phage protein (pseudogene)	2.09	Hypothetical protein
SAR2097	hypothetical phage protein	3.02	Hypothetical protein
SAR2098	hypothetical phage protein	2.53	Hypothetical protein
SAR2228	conserved hypothetical protein	3.71	Hypothetical protein
SAR2380	hypothetical protein	2.12	Hypothetical protein
SAR2561	conserved hypothetical protein	6.88	Hypothetical protein
SAR2617	hypothetical protein	3.45	Hypothetical protein
SAR0445	putative lipoprotein	2.66	Lipoprotein
SAR0761	putative lipoprotein	31.89	Lipoprotein
SAR0872	putative lipoprotein	10.68	Lipoprotein
SAR0997	putative lipoate-protein ligase A	2.72	Lipoprotein
SAR1402	phosphate-binding lipoprotein	13.74	Lipoprotein
SAR0128	putative membrane protein	4.45	Membrane protein
SAR0420	putative membrane protein	5.20	Membrane protein

SAR0442		putative membrane protein	3.18	Membrane protein
SAR0760		putative membrane protein	8.79	Membrane protein
SAR2451		putative membrane protein	3.11	Membrane protein
SAR2613		putative membrane protein	15.76	Membrane protein
SAR2783		putative membrane protein	2.88	Membrane protein
SAR2372	*ureA*	urease gamma subunit	3.90	Nucleotide metabolism
SAR2374	*ureC*	urease alpha subunit	2.74	Nucleotide metabolism
NA (SACOL2069)	*kdpF*	K+-transporting ATPase, F subunit	4.08	Transport protein
SAR0870		ABC transporter ATP-binding protein	9.51	Transport protein
SAR0871		ABC transporter permease protein	8.73	Transport protein
SAR1079		putative manganese transport protein	5.34	Transport protein
SAR2701		ABC transporter ATP-binding protein	3.67	Transport protein
SAR2781	*vraD*	ABC transporter ATP-binding protein	11.01	Transport protein
SAR2030		MHC class II analog	27.82	Virulence factor
SAR2508	*sbi*	IgG-binding protein	3.24	Virulence factor
SAR1280		putative glutathione peroxidase	2.39	Others
SAR1410		putative peptidase	6.38	
SAR2121		putative carbon-nitrogen hydrolase	8.15	
SAR2290		aldo/keto reductase family protein	4.04	
SAR2522		putative glycerate kinase	6.32	

SAR2628	*clpL*	putative ATP-dependent protease ATP-binding subunit ClpL	7.56
SAR2661		putative hydrolase	5.54
SAR2708		putative esterase	2.91
SAR2779		putative N-acetyltransferase	3.58
SAR2784		transposase (pseudogene)	6.50

[a] Based on the annotation of MRSA252 genome, a EMRSA-16 clone

NA=not annotated in MRSA252 genome

* Threshold ratio value set at ≥2.0-fold change

Table 6. Genes down-regulated in rhodomyrtone-treated EMRSA-16

MRSA252[a] (or annotated in other *S. aureus* strains genome)	Gene	Gene product description	Expression-fold change*	Functional category
SAR0459	*cysM*	pyridoxal-phosphate dependent enzyme	3.87	Amino acid metabolism
SAR0589		amino acid permease	4.55	Amino acid metabolism
SAR2400		amino acid permease	2.85	Amino acid metabolism
SAR0234	*ldh1*	L-lactate dehydrogenase 1	15.35	Carbohydrate metabolism
SAR1834		conserved hypothetical protein	3.63	Hypothetical protein
SAR1857		putative exported protein	3.29	Others
SAR2243		hypothetical protein	2.94	Hypothetical protein
SAR2569		hypothetical protein	2.86	Hypothetical protein
SAR1051		putative membrane protein	4.26	Membrane protein
SAR1091		putative membrane protein	3.44	Membrane protein
SAR1493		putative membrane protein	2.44	Membrane protein
SAR2179		putative membrane protein	8.96	Membrane protein
SAR2647		putative membrane protein	2.83	Membrane protein
SAR0005	*gyrB*	DNA gyrase subunit B	2.41	Nucleotide metabolism
SAR0485	*holB*	putative DNA polymerase III, delta' subunit	2.23	Nucleotide metabolism

SAR0497	*purR*	putative pur operon repressor	2.10	Nucleotide metabolism
SAR0540	*nusG*	transcription antitermination protein	2.55	Nucleotide metabolism
SAR0547	*rpoB*	DNA-directed RNA polymerase beta chain protein	5.48	Nucleotide metabolism
SAR1120	*sdhC*	putative succinate dehydrogenase cytochrome b558	3.65	Nucleotide metabolism
SAR1569	*rluB*	ribosomal large subunit pseudouridine synthase B	2.05	Nucleotide metabolism
SAR1701	*mnmA*	tRNA-specific 2-thiouridylase MnmA	2.61	Nucleotide metabolism
SAR1719	*tgt*	queuine tRNA-ribosyltransferase	3.15	Nucleotide metabolism
SAR1833		putative methyltransferase	3.71	
SAR2152	*sigB*	RNA polymerase sigma-B factor	5.87	Nucleotide metabolism
SAR2209	*rho*	transcription termination factor	2.35	Nucleotide metabolism
SAR2302	*truA*	putative tRNA pseudouridine synthase	2.74	Nucleotide metabolism
SAR2244	*mtlA*	PTS system, mannitol-specific IIBC component	12.44	Phosphotransferase system
SAR0648	*tagG*	teichoic acid ABC transporter	2.55	Transporter protien
		Permease protein		Transporter protien
SAR1049		putative cobalt transport protein	3.86	Transporter protien
SAR1050		ABC transporter ATP-binding protein	5.79	Transporter protien
SAR0033	*knt*	kanamycin nucleotidyltransferase	6.94	Others

SAR0842	*clfA*	clumping factor	4.53	Others
SAR0911	*mnhD*	Na+/H+ antiporter subunit	2.21	Others
SAR1018		putative hydrolase	2.27	Others
SAR0644	*sirR*	putative metalloregulator	2.25	Others

[a] Based on the annotation of MRSA252 genome, a EMRSA-16 clone

NA=not annotated in MRSA252 genome

* Threshold ratio value set at ≥2.0-fold change

6. Effect of rhodomyrtone on autolysis of MRSA

The effect of rhodomyrtone one autolytic activity of MRSA NPRC 001R and *S. aureus* ATCC29213 in the presence of Triton X-100 was determined. The cells were grown with rhodomyrtone at various concentrations ranged from 0.125MIC-4MIC (0.125-4 μg/ml) and the rate of Triton X-100-induced lysis was compared with an untreated control cultures. Percentage lysis was determined from $OD600_{nm}$ measurement and compared to the values at time point 0 h. The rates of Triton X-100-induced autolysis seemed to be equivalent among the concentrations over the 180 min of the assay. Therefore, rhodomyrtone had no effect on the rate of Triton X-100 stimulated autolysis, when compared with untreated control (Figure 21).

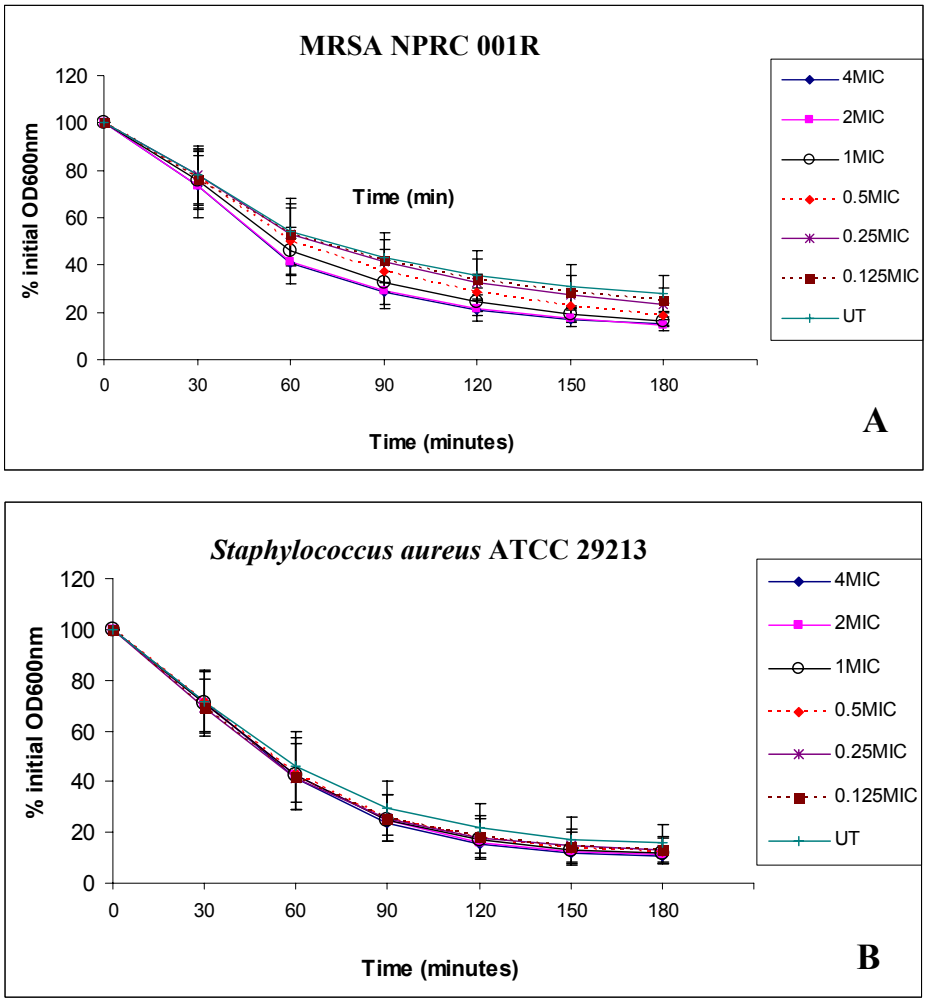


Figure 20. The effect of rhodomyrtone on whole cell lysis of *Staphylococcus aureus*. Autolysis rate was measured as the decline in optical density on MRSA NPRC 001R (A) and *S. aureus* ATCC29213 (B). The concentrations of rhodomyrtone were 0MIC, 0.125MIC, 0.25MIC, 0.5MIC, MIC, 2MIC, and 4MIC, respectively. Data points are the mean ± standard deviations (SD) of three replicate samples. $OD600_{nm}$, optical density at 600_{nm}.

7. Effect of rhodomyrtone on susceptibility to lysostaphin of methicillin-resistant *Staphylococcus aureus* cells

Lysostaphin is a peptidoglycan hydrolase that specifically cleaves the pentaglycine cross-bridge joining glycan strands within peptidoglycan of *S. aureus* (Figure 21). To determine the effect of rhodomyrtone on the susceptibility of the cell wall to lysostaphin, the minimal concentrations of lysostaphin required to inhibit MRSA growth in the presence or absence of 0.125MIC (0.125 μg/ml) of rhodomyrtone were examined in MRSA NPRC 001R, EMRSA-16, and *S. aureus* ATCC 29213 strains. In the presence of sub-MIC of rhodomyrtone, bacterial growth was four-fold increase, eight-fold increase, and no significant difference in the MIC of lysostaphin, when compared to the untreated control. The values were increased from 0.25 to 1.0 μg/ml and 0.25 to 2.0 μg/ml in rhodomyrtone treated-cells of MRSA NPRC 001R and EMRSA-16, respectively while the lysostaphin MIC of *S. aureus* ATCC 29213 was unchanged at 0.125 μg/ml (Table 7). The results suggested that rhodomyrtone may inhibit the lysostaphin efficiency or alter the structure of glycine pentapeptide cross-linking of the bacterial peptidoglycan that is the target of lysostaphin.

Table 7. Minimal inhibitory concentration of lysostaphin in the presence of rhodomyrtone

Bacterial strains	Rhodomyrtone (μg/ml)	
	Treated cells	Untreated control
MRSA NPRC 001R	1.0	0.25
EMRSA-16	2.0	0.25
S. aureus ATCC 29213	0.125	0.125

8. Effect of rhodomyrtone on peptidoglycan amino acid composition of methicillin-resistant *Staphylococcus aureus* cells

To determine whether rhodomyrtone rendered the modification in peptidoglycan structure of rhodomyrtone-treated cells, peptidoglycan of EMRSA-16 in the presence or absence of rhodomyrtone at 0.5MIC (0.5 μg/ml) were purified and examined using amino acid analyzer (Alta Bioscience, University of Birmingham, UK). The results from SEM examination (Figure 13 and 14) of the bacterial cells grown in the presence or absence of rhodomyrtone at 0.5 μg/ml (0.5MIC) for 1 h demonstrated no significant alteration of the cell surface and morphology. More effects were observed at 4 h after treatment. Therefore, analysis of isolated peptidoglycan of EMRSA-16 either with or without treatment for 4 h has been further elucidated. Molar mass ratio of the amino acid in the bacterial peptidoglycan including serine, glycine, alanine, and lysine were normalized with respect to glutamic acid residue. Rhodomyrtone-treated cells were not influenced and conformed which the one would expect on the basis of the well-known staphylococcal peptidoglycan amino acid composition. No significant difference of any amino acid contents has been observed between rhodomyrtone-treated cells and untreated control (Table 8 and appendix 2, 3).

Table 8. Amino acid composition analysis of the purified peptidoglycan of EMRSA-16 in the presence or absence of rhodomyrtone.

	Molar mass ratio relative to glutamic acid*	
Amino acid	Rhodomyrtone-treated cells	Untreated control cells
Glutamic acid	1.00	1.00
Glycine	3.33	3.94
Alanine	1.70	2.13
Lysine	0.97	1.05
Serine	0.65	0.55

* Full data are listed in appendix 2 and 3.

CHAPTER 5

DISCUSSION

This study was conducted to apply two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) based proteomic methods, and microarray database and quantitative reverse transcription-PCR (qRT-PCR) approach for exploration of proteomic profiling and transcriptional response of MRSA following treatment of rhodomyrtone at subinhibitory concentration. Proteomic analysis of MRSA after treatment was carried out using a representative clinical isolate, MRSA NPRC 001R, which was a common endemic isolate with *mecA* gene obtained from Hat Yai Hospital, Songkhla, Thailand. This bacterial strain was observed to be resistant to several classes of antimicrobials including β-lactam, aminoglycoside, tetracycline, macrolide, fluoroquinolone, clindamycin, and trimethoprim-sulfamethoxazole (Chusri and Voravuthikunchai 2009). After exposure of MRSA to rhodomyrtone there was alteration of several cellular protein expressions. The most affected proteins belong to many functional categories such as amino acid metabolism, carbohydrate metabolism, and cell wall biosynthesis, suggested that these might be the proteins affected most by rhodomyrtone.

Transmission electron micrographs (TEM) illustrated the morphological and ultrastructural alterations of the treated cells that supported the protein analysis data. Thickened septa with irregular features were also observed in rhodomyrtone-treated *S. aureus* (Figure 9E, 9F, and 10E, arrows). Increasing in staphylococcal cell wall thickness has been previously reported due to the mechanism of actions of some antibiotics such as chloramphenicol (Giesbrecht *et al.* 1998), erythromycin (Nishino 1975), penicillin (Giesbrecht *et al.* 1998), and vancomycin (Belley *et al.* 2009). Another effect of rhodomyrtone that was similar to staphylococcal cells grown in the presence of penicillin at a concentration of 0.05 μg/ml (Giesbrecht *et al.* 1998), was generating the fibrillar wall material that occurred

from turning of compressed cross wall and septal formation (Figure 9E and 10E, arrows). After treatment with low dose of penicillin, staphylococcal cells always affect the penicillin binding protein which is considered to be located in the splitting system (Giesbrecht, Wecke, and Reinicke 1976). TEM micrographs revealed that rhodomyrtone rendered abnormal cell wall, septum formation, and cytoplasmic membrane in MRSA and *S. aureus* indicating that the exposure to rhodomyrtone to staphylococcal cells for 18 h might involve in damage of cell wall turnover or membrane-related proteins. The findings were consistent with the protein analysis data that rhodomyrtone up-regulated some proteins relating to cell division and cell wall biosynthesis such as GTPase ObgE, ScdA, FtsZ, and autolysin. Meanwhile, it exhibited only insignificant morphological effects to *Streptococcus mutans* using phase contrast microscopy, indistinct enlarged cells have been detected after treatment with 5MIC rhodomyrtone for 20 h (Limsuwan *et al.* 2009).

Although proteomic analysis data and TEM indicated that exposure to rhodomyrtone affected the alterations of the bacterial cell wall and cell membrane which might be parts of its mechanisms of action, many of the enzymes involved in cell wall biosynthesis were not covered by the 2DE approach. An important challenge for our further studies was to validate distinct alterations and clarify changes in genome-wide expression of MRSA following rhodomyrtone challenge. In order to identify the molecular target genes that response to rhodomyrtone by altered transcription, and establish the presumably regulatory relations between them, transcriptomic approaches were employed. Thus, DNA microarray analysis was used to study effects on the transcription of genes involved in these processes to provide more understanding in potential mode of actions of rhodomyrtone. The experiment was designed to enable investigation of early effects of rhodomyrtone treatment after 1 h of incubation. The concentration of rhodomyrtone in this study was 0.5 μg/ml (0.5MIC) that not affected the bacterial growth (as shown in Figure 6). In this experiment, EMRSA-16 strain, an epidemic MRSA strain in the UK, was used. Microarray analysis data demonstrated extensive alterations of gene expression profiles in a variety of gene categories exponentially growing cells after exposure to rhodomyrtone (Figure 16A and 16B). Of these, the largest proportion of up-regulated genes (32 open reading frames (ORFs), 48.5%) encoding a protein of unknown

function (genes assigned as encoding hypothetical proteins). Particularly, some of them showed the outstanding increase in fold change of expression by a factor of more than 30 fold (SAR0996, SAR0437, SAR0761), thus all of these genes were chosen for time course analysis by qRT-PCR.

An overview of the key genes responses of MRSA after rhodomyrtone challenge for 1 h was illustrated in Figure 17. The prominent up-regulation is a group of genes in the biosynthesis of amino acids of the aspartate family. This family is comprised of methionine, threonine, isoleucine, and lysine, amino acids whose carbon skeletons are initially obtained from aspartate. These up-regulated genes encoding several enzymes of lysine biosynthetic pathway including *asd* (encoding aspartate semialdehyde dehydrogenase), *dapA* (dihydrodipicolinate synthase), *dapB* (dihydrodipicolinate reductase), and *dapD* (tetrahydrodipicolinate acetyltransferase). Diaminopimelate (DAP) is synthesized by the bacteria via the aspartate amino acid family pathway (Rodionov *et al.* 2003). The essentiality of DAP biosynthesis results from the fact that DAP is being both a component of the peptidoglycan, an essential portion in peptidoglycan synthesis by cross-linking glycan chains to provide rigidity and strength to the peptidoglycan (van Heijenoort 2001), and the direct precursor to lysine (Pavelka and Jacobs 1996)(Rodionov *et al.* 2003; Girish, Sharma, and Gopal 2008). The DAP biosynthetic pathway is an attractive target for antimicrobial agents development since it is neither produced nor required by mammals (Steenbergen *et al.* 2005). Therefore, an increase in expression of DAP pathway related genes suggested that this biosynthetic pathway is one of the major targets of rhodomyrtone. It is possible that rhodomyrtone acts through lysine and/or cell wall synthesis. Other three genes in aspartate amino acids family those were also up-regulated after treatment including threonine synthase (*thrC*) and homoserine dehydrogenase (*dhoM*), and argininosuccinate synthase (*argG*) that belong to threonine and arginine biosynthetic pathway, respectively.

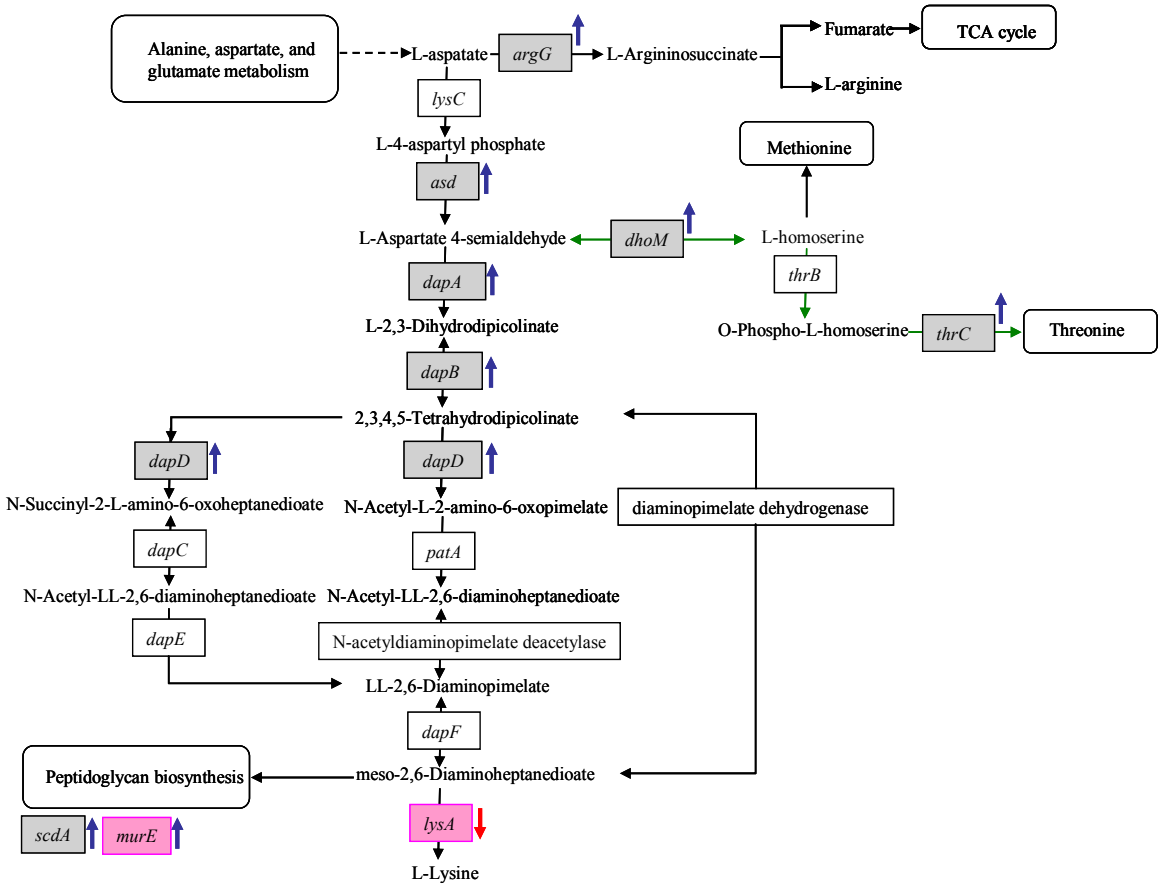


Figure 21. Schematic representation of diaminopimelate (DAP) pathway. The pathway depict expression of DAP biosynthesis related genes induced and reduced in rhodomyrtone-treated MRSA. Gray boxes: genes that are significantly represented by microarray; pink boxes: genes that are significantly represented by qRT-PCR. Blue arrow and red arrow represented an increase or decrease in expression fold change, respectively.

An reduction of several gene expression encoding nucleotide metabolism such as *gyrB*, *holB*, *purR*, *nusG*, *rpoB*, *sdhC*, *rluB*, *tgt*, *sigB*, *rho*, *truA*, and. SAR1833, suggesting that the bacteria might compensate the damage within cell system by a decrease in cell metabolic activities and nucleotide metabolism

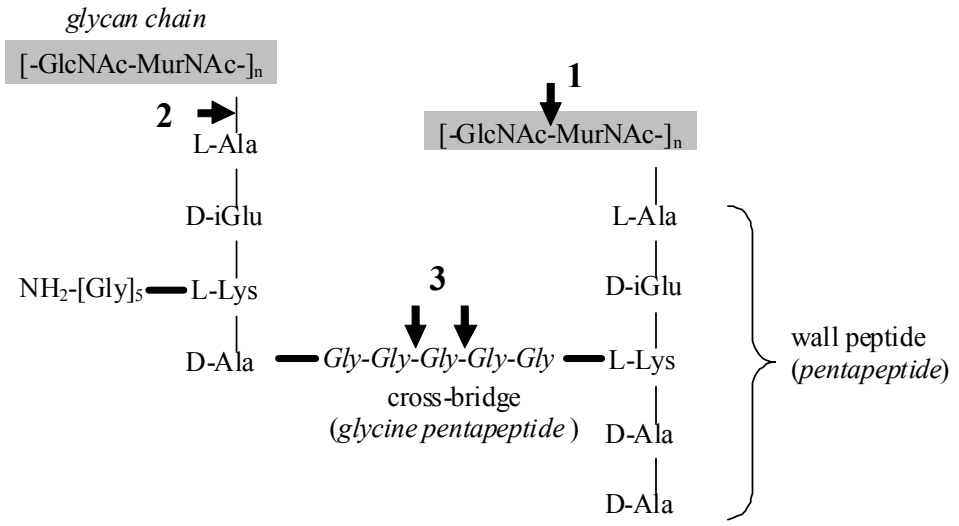
Time course analysis of selected genes of interest were further validated using qRT-PCR (Table 1). Genes related in DAP pathway including *asd*, *dapA*, *thrC*, and l*ysA* (group I) were analyzed (Figure 20). In DAP pathway, *lysA* encoding *meso*-DAP-decarboxylase, mediates the last step of the lysine biosynthesis (Li and Ricke 2003). It is essential under certain conditions with auxotrophs exhibiting attenuated growth, but is not absolutely essential as most growth media containing enough lysine for growth (Gerdes *et al.* 2003). Therefore, *lysA* was included in the study of gene expression at different incubation time. The data from qRT-PCR of rhodomyrtone-treated cells revealed that *asd*, *dapA*, and *thrC* were up-regulated while *lysA* was down-regulated at 1 h incubation time. At 4 h, *asd* and *thrC* still increased in expression while *dapA* showed no significant difference in expression as same as *lysA*. No significant difference in expression was observed in all four genes at 18 h incubation time. These results indicated that treatment with rhodomyrtone for 1 h increased in expression of genes related in both lysine and threonine biosynthetic pathway, but at later time point (4 h) *asd* and *thrC* in threonine pathway were still up-regulated while *dapA* and *lysA* in lysine biosynthetic pathway were no significant difference in expression. Up-regulation of genes in threonine biosynthesis after treatment might related with attempt to increase in biosynthesis of some amino acids such as valine, leucine, and isoleucine, and glycine, serine, and threonine in MRSA.

Time course analysis of genes in group III that encoded cell wall biosynthesis and cell division related proteins including *ftsH*, *ftsZ*, *murE*, *scdA*, and *rpoB*, was observed. The *scdA* (encoding cell wall biosynthesis protein Scd), a gene involved in cell wall biosynthesis and cell division, was induced by rhodomyrtone challenge in microarray data. The gene was up-regulated by the factor of 5.14 fold change in expression after EMRSA-16 exposed to 0.5MIC rhodomyrtone, 0.5 µg/ml, for 18 h in microarray, while proteomic analysis data on MRSA NPRC 001R shown

down-regulation of ScdA (the absented protein) following treatment at 0.174 µg/ml of rhodomyrtone for 18 h. In contrast, expression of *scdA* was no significant change at all time points of incubation (1, 4, and 18 h) when estimated by qRT-PCR. *murE* gene was not significantly presented in microarray data but showed up-regulation (19.14 fold) in qRT-PCR at 1 h incubation time. The *murE* gene, encoding the UDP–*N*-acetylmuramyl tripeptide synthetase that catalyzes the addition of the L-lysine residue to the UDPlinked muramyl dipeptide cell wall precursor, is an essential enzyme in the stepwise assembly of the peptide stem of peptidoglycan (Barreteau *et al.* 2008). A gene encoding cell division protein *ftsZ*, was not significantly presented in microarray analysis but was significantly down-regulated (-2.77 fold) in qRT-PCR at 1 h after rhodomyrtone challenge. The fold change in expression of *ftsZ* at 4 and 18 h of incubation period were not available, more biological replicates for analysis of this gene were required. An reduce in expression of *ftsH* gene encoding cell division protein FtsH (ATP-dependent metalloprotease) (-2.98 fold), was observed at 18 h after treatment while at 1 and 4 h incubation time displayed no significant difference of expression fold change.

Proteomic analysis data revealed that autolysin was absence after treatment with rhodomyrtone. A significant difference in expression of genes such as *murE*, *scdA*, and *ftsZ* coding for regulators of cell wall biosynthesis, cell wall septum, and cell division genes was examined by qRT-PCR. To further elucidate effect of rhodomyrtone on autolysis of MRSA, the experiment was carried out using Triton-X 100 induced assay. Triton X-100 is a non-ionic detergent with solubilizes and thus ruptures the cell membrane, as well as activates the autolysins (Komatsuzawa *et al.* 1994). The result showed that rhodomyrtone had no effect on the rate of Triton-X 100 induced lysis in both MRSA NPRC 001R and *S. aureus* ATCC 29213 strains when compared to untreated control cells. Lysostaphin is an endopeptidase that specifically cleaves the Gly-Gly bond in the peptapeptide glycine interbridge of staphylococcal peptidoglycan (Figure 22) (Iversen and Grov 1973). By lysostaphin MIC assay, MRSA growth with subinhibitory concentration of rhodomyrtone (0.125) was significant increase in the MIC of lysostaphin, eight-fold increase on EMRSA-16 strain and four-fold increase on MRSA NPRC 001R strain. While lysostaphin MIC was no significant difference in *S. aureus* ATCC 29213 when compare to untreated

control cells. This result might indicate that of lysostaphin efficiency was inhibited or the alteration of glycine pentapeptide cross-linking structure of peptidoglycan in MRSA. There are some researches have been reported an increasing in lysostaphin MIC might be explained by mutation of *femA*, the gene responsible for addition of the second and third glycines to the pentaglycine cross bridge. Mutations of *femA* that render this gene nonfunctional resulting in monoglycine crossbridges and cause the *S. aureus* completely resistant to lysostaphin as the enzymatic target for lysostaphin is no longer elicit (Ehlert, Schroder, and Labischinski 1997; Stranden *et al.* 1997). Substitution of serines for glycines in the glycine pentapepide cross-bridges also cause the bacteria resistant to lysostaphin. Transcriptional responses to rhodomyrtone challenge of EMRSA-16 showed an increase in expression of genes related with DAP biosynthetic pathway that is the precursor of lysine and/or peptidoglycan biosynthesis (Figure 22). However, analysis of the cell wall composition with high-performance liquid chromatography (HPLC) revealed no significant difference of peptidoglycan amino acid composition of rhodomyrtone-treated MRSA, when compared to untreated control (Table 8 and appendix 2, 3).



(modified from Kumar 2008)

Figure 22. Enzymatic activities of lysostaphin: Peptidoglycan of *S. aureus* showing positions of cleavage. Lysostaphin has three enzymatic activities, (1) endo-β-*N*-acetyl glucosaminidase, (2) *N*-acetyl-muramyl-L-alanine amidase, and (3) endopeptidase. Endopeptidase causes the solubilization of pentaglycine cross bridges. NAG: *N*-acetyl glucosamine, NAM: *N*-acetyl muramic acid, ala: alanine, lys: lysine, glu: glutamine, and $(Gly)_5$: pentaglycine.

CHAPTER 6

CONCLUSION

1. The results obtained from protein analysis data, electron microscopic examination, and phenotypic assay, suggested that disruption during the processes of cell wall biosynthesis, septum formation, and cell division were parts of rhodomyrtone antibacterial mode of action. However, the obviously alteration in peptidoglycan structure was not observed by peptidoglycan amino acid analysis.

2. A strong increasing in expression of many genes relating with several amino acid biosynthetic pathway might also be parts of mode of action of rhodomyrtone. This transcriptional response might due to the bacteria needed to compensate some metabolic pathway or the acquisition of more energy and/or protein for their survival.

3. A combined proteomic and transcriptomic approach might generate clues to protein and gene functions that can help to identify targets for the therapeutic intervention and to monitor alterations in global protein and gene expression underlying cellular response to rhodomyrtone treatments. The data from proteomic and transcriptomic analysis revealed the global view of various protein and genes that showed significant difference of expression after rhodomyrtone challenge. It might be possible that rhodomyrtone also has other mechanism of actions that should be further characterized

5. Because rhodomyrtone be able to render differentially expressed genes of a large number of genes encoding protein of unknown function (hypothetical proteins). Therefore, it is possible that rhodomyrtone might be a valuable biological tool for characterization of the functions of these genes.

6. Detailed study on antimicrobial mode of actions of rhodomyrtone should be further examined. The additional investigation of rhodomyrtone potentials can provide the scientific data in order to develop and define a novel alternative antibiotic for treatment of MRSA infections.

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APPENDIX

1. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of gene expression in EMRSA-16

ORF ID[a]	Gene	MRSA252 gene product	Incubation time (hour)		
			1	4	18
SAR1406	*asd*	aspartate semialdehyde dehydrogenase	40.15	4.53	-1.95
SAR1407	*dapA*	dihydrodipicolinate synthase	14.35	-1.22	1.06
SAR1339	*thrC*	threonine synthase	18.35	13.64	-1.71
SAR0126	*lysA*	diaminopimelate decarboxylase	-2.44	-1.28	-1.95
SAR0512	*ftsH*	cell division protein FtsH	-1.59	1.40	-2.98
SAR1162	*ftsZ*	cell division protein FtsZ	-2.77	NA	NA
SAR0988	*murE*	UDP-N-acetylmuramoylalanyl-D-glutamate--L-lysine ligase	19.14	1.99	-1.33
SAR0256	*scdA*	cell wall biosynthesis protein ScdA	-1.60	-1.03	-1.78
SAR0547	*rpoB*	DNA-directed RNA polymerase subunit beta	-3.03	-2.51	-1.13
SAR0996	-	hypothetical protein	65.57	37.08	-9.56
SAR0437	-	hypothetical protein	34.78	128.44	-1.14
SAR0761	-	hypothetical protein	151.69	14.83	-1.56

The values correspond to the expression fold change of each gene analyzed following rhodomyrtone treatment at different incubation times when compared to the untreated control.

[a] ORF (Opern reading frame) identification (IDs) refer to *S. aureus* strain MRSA252.

$p<0.05$.

NA, not available

2. Peptidoglycan amino acid analysis of rhodomyrtone-treated cells

Analysis after hydrolysis using 5.8M HCl, 24 h at 110°C

AA	n.mole / mg	u.g / mg	m.g / mg	g / 100g	MW	Molar mass
Alanine	1170.00	82.90	0.0829	8.290	89	2.13
Arginine	10.70	1.68	0.0017	0.168	174	0.02
Aspartic acid	101.00	11.60	0.0116	1.160	133	0.20
Cysteic acid	0.00	0.00	0.0000	0.000		0.00
Cystine	0.00	0.00	0.0000	0.000		0.00
Glutamic acid *	497.00	64.20	0.0642	6.420	147	1.00
Glycine	2250.00	129.00	0.1290	12.900	75	3.94
Histidine	198.00	27.20	0.0272	2.720	155	0.40
Hydroxyproline	0.00	0.00	0.0000	0.000		0.00
Isoleucine	53.50	6.06	0.0061	0.606	131	0.11
Leucine	53.60	6.07	0.0061	0.607	131	0.11
Lysine	520.00	66.70	0.0667	6.670	146	1.05
Methionine	15.60	2.04	0.0020	0.204	149	0.03
Phenylalanine	16.40	2.41	0.0024	0.241	165	0.03
Proline	26.10	2.54	0.0025	0.254	115	0.05
Serine	289.00	25.20	0.0252	2.520	105	0.55
Threonine	35.90	3.63	0.0036	0.363	119	0.07
Tryptophan	0.00	0.00	0.0000	0.000		0.00
Tyrosine	21.60	3.53	0.0035	0.353	181	0.04
Valine	43.60	4.32	0.0043	0.432	117	0.08
Totals	5300	439	0.439	43.9		

Molar mass ratio of each amino acid relative to glutamic acid.

3. Peptidoglycan amino acid analysis of untreated cells.

Analysis after hydrolysis using 5.8M HCl, 24 h at 110°C

AA	n.mole / mg	u.g / mg	m.g / mg	g / 100g	MW	Molar mass
Alanine	931.00	66.20	0.0662	6.620	89	1.70
Arginine	41.50	6.49	0.0065	0.649	174	0.09
Aspartic acid	195.00	22.40	0.0224	2.240	133	0.39
Cysteic acid	0.00	0.00	0.0000	0.000		0.00
Cystine	7.71	1.71	0.0017	0.171		0.00
Glutamic acid *	497.00	64.10	0.0641	6.410	147	1.00
Glycine	1910.00	109.00	0.1090	10.900	75	3.33
Histidine	158.00	21.70	0.0217	2.170	155	0.32
Hydroxyproline	0.00	0.00	0.0000	0.000		0.00
Isoleucine	99.80	11.30	0.0113	1.130	131	0.20
Leucine	115.00	13.00	0.0130	1.300	131	0.23
Lysine	483.00	61.90	0.0619	6.190	146	0.97
Methionine	41.90	5.49	0.0055	0.549	149	0.08
Phenylalanine	44.80	6.60	0.0066	0.660	165	0.09
Proline	53.00	5.14	0.0051	0.514	115	0.10
Serine	342.00	29.80	0.0298	2.980	105	0.65
Threonine	88.00	8.90	0.0089	0.890	119	0.17
Tryptophan	0.00	0.00	0.0000	0.000	204	0.00
Tyrosine	41.30	6.74	0.0067	0.674	181	0.09
Valine	90.80	9.00	0.0090	0.900	117	0.18
Totals	5130	449	0.449	44.9		

Molar mass ratio of each amino acid relative to glutamic acid.

Notes about amino acid analysis:-

Asn and Gln are completely converted to Asp and Glu during the acid hydrolysis of the protein. The values for Thr and Ser have been corrected for hydrolysis losses of 5% and 10% respectively. Trp usually suffers complete loss during acid hydrolysis and is not normally quantified. In proteins, Cys is usually observed as cystine. The recovery of Cys is variable when using standard hydrolysis conditions. The reported values have been rounded off to either 2 or 3 significant figures, depending on peak size.

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List of Publications and Proceedings

Publications

1. **Sianglum, W.**, Srimanote, P., Wonglumsom, W., Kittiniyom, K., and Voravuthikunchai S.P. 2011. Proteome analyses of cellular proteins in methicillin-resistant *Staphylococcus aureus* treated with rhodomyrtone, a novel antibiotic candidate. PLoS One. 6: e16628. doi:10.1371/journal.pone. 0016628.

Proceedings

1. **Sianglum, W.**, Srimanote, P., Wonglumsom, W., Kittiniyom, K., and Voravuthikunchai, S.P. 2008. Study on the effect of *Rhodomyrtus tomentosa* (Aiton) Hassk. leaf on methicillin-resistant *Staphylococcus aureus* cells. International Conference on Natural Products for Health and Beauty, 17-19 December, 2008, Narasuan University, Phayao, Thailand.

2. **Sianglum, W.**, Srimanote, P., Wonglumsom, W., Kittiniyom, K., and Voravuthikunchai, S.P. 2009. Proteomic study on the effects of rhodomyrtone on methicillin-resistant *Staphylococcus aureus.* RGJ-Ph.D. Congress X, "Climate Change and Its Impacts", 3–5 April, 2009, Jomtien Palm Beach Hotel, Pattaya, Thailand.

3. **Sianglum, W.**, Srimanote, P., Wonglumsom, W., Kittiniyom, K., and Voravuthikunchai S.P. 2009. Ultrastructural effects of rhodomyrtone on methicillin-resistant *Staphylococcus aureus.* RGJ Seminar Series LXIV: Science and Technology for the Sustainable Development, 4 September, 2009, Faculty of Science, Prince of Songkla University.