Proteome Analyses of Cellular Proteins in Methicillin-Resistant Staphylococcus aureus Treated with Rhodomyrtone, a Novel Antibiotic Candidate

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Abstract

The ethanolic extract from Rhodomyrtus tomentosa leaf exhibited good antibacterial activities against both methicillin-resistant Staphylococcus aureus (MRSA) and S. aureus ATCC 29213. Its minimal inhibitory concentration (MIC) values ranged from 31.25–62.5 μg/ml, and the minimal bactericidal concentration (MBC) was 250 μg/ml. Rhodomyrtone, an acylphloroglucinol derivative, was 62.5–125 times more potent at inhibiting the bacteria than the ethanolic extract, the MIC and MBC values were 0.5 μg/ml and 2 μg/ml, respectively. To provide insights into antibacterial mechanisms involved, the effects of rhodomyrtone on cellular protein expression of MRSA have been investigated using proteomic approaches. Proteome analyses revealed that rhodomyrtone at subinhibitory concentration (0.174 μg/ml) affected the expression of several major functional classes of whole cell proteins in MRSA. The identified 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. Transmission electron micrographs confirmed the effects of rhodomyrtone on morphological and ultrastructural alterations in the treated bacterial cells. Biological processes in cell wall biosynthesis and cell division were interrupted. Prominent changes including alterations in cell wall, abnormal septum formation, cellular disintegration, and cell lysis were observed. Unusual size and shape of staphylococcal cells were obviously noted in the treated MRSA. These pioneer findings on proteomic profiling and phenotypic features of rhodomyrtone-treated MRSA may resolve its antimicrobial mechanisms which could lead to the development of a new effective regimen for the treatment of MRSA infections.

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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, cellulitis, impetigo, carbuncles, scalded skin syndrome, and abscesses. In addition, it can cause some serious infections including bacteremia, pneumonia, acute endocarditis, meningitis, osteomyelitis, toxic shock syndrome, and fatal invasive diseases [1]. The emergence and spread of methicillin-resistant S. aureus (MRSA) originated in the 1960s as an important clinical and epidemiological problem in hospital environments. Additionally, S. aureus has been a crucial cause of community-acquired infections which may result in morbidity and mortality [2,3]. The introduction of glycopeptide antibiotics, a last resort to treat such infections, was followed by the isolation of either vancomycin-intermediate S. aureus (VISA) or vancomycin-resistant S. aureus (VRSA) [4,5].

Several active researches have documented new antibiotics and semi-synthetic analogs with improved antimicrobial properties [6,7,8]. Various plants worldwide have been used in traditional medicine as alternative treatments of bacterial infections [9,10,11]. 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 [12]. From our preliminary studies, the data 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 subtilis, Enterococcus faecalis, S. aureus, S. epidermidis, and Streptococcus spp. [13,14,15]. In addition, rhodomyrtone also exhibited significant antimicrobial activity against S. epidermidis biofilm-forming and S. pneumoniae capsule-producing strains [15].

In recent years, two dimensional gel electrophoresis (2DE) reference maps of cellular or extracellular proteins from various S. aureus strains in different growth conditions have been established [16,17,18]. 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
rhodomyrtone inhibited the synthesis of six proteins functionally identified in the global transcriptome data of MRSA treated with 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™ Software. Most of the protein spots were observed in acidic range as illustrated in Figures 2A and 2B. 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. Of these, 203 spots were shared (unchanged expression) between the two protein profiles. The investigation was focused on the more/less intense proteins. Of the overexpressed or downregulated protein spots in rhodomyrtone-treated MRSA, 18 proteins that presented only in rhodomyrtone treated-sample and 28 proteins that disappeared after the treatment were the proteins of interest. The presence or the absence of protein spots in the treated MRSA proteomes were denoted in Figures 2A and 2B. Protein spot number, protein annotation, functional category (NCBI database) are listed in Tables 1 and 2. The potential roles of these proteins are discussed below.

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-phosphohexulonate 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 [24]. Protein members of chromosome partition, cell wall metabolism, and cell division such as endo-1, 4-β-glucanase and RNA (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 osmoregulated transport system permease protein involved in osmotic adjustment in bacteria [25], 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 catalysis 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 [26], cold shock response [27], and the post-transcriptional modification [28] 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, dihydriopipamide
dehydrogenase, and triosephosphate isomerase. Some of absent proteins have been reported to be participated with chromosome partition such as GTPase ObgE [29], cell wall biosynthesis, cell division, and cell wall turnover including cell wall biosynthesis protein ScdA [30], tubulin-liked FtsZ [31], and autolysin [32].

In bacteria, the of the 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 [33]. 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 [33]. Bacteria with compact peptidoglycan produce cell wall hydrolase, N-acetyl-glucosaminidase, N-acetylmuramidase, and endopeptidase for cell division [34]. It has been observed that rhodomyrtone suppressed the expression of autolysin with bifunctional activities, N-acetylmuramyl-L-alanine-amidase and N-acetylg glucosaminidase. The bacterial cell division machinery is triggered with a formation of Z ring which consists of polymers of FtsZ protein at the division site [35]. FtsZ is the bacterial tubulin homologue which plays a key role for the recruitment of other proteins to the cell division septum [36]. 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

**Figure 1. Time-kill curves of rhodomyrtone against MRSA.** Viability was counted at the indicated time points by serial dilution plating. Each point represented the mean of log_{10} ± standard deviations of three different experiments performed in duplicate. doi:10.1371/journal.pone.0016628.g001

**Figure 2. Two dimensional electrophoresis of the of cellular methicillin-resistant S. aureus proteomes.** Protein extracts were prepared from rhodomyrtone-treated cells (A) and untreated control cells (B). doi:10.1371/journal.pone.0016628.g002
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. However, many of the enzymes involved in cell wall synthesis were not covered by the 2DE approach. An important challenge for our further studies will be to validate distinct alterations and clarify changes in gene expression following rhodomyrtone treatment. Thus, DNA microarray experiments would be extremely helpful to study effects on the transcription of genes involved in these processes, to provide more insight in potential mode of actions of rhodomyrtone.

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-acetylserylserine sulfhydrylase)-like protein which related with three metabolic pathways including cysteine, seleno amino acid, and sulfur metabolism; (ii), carbamoylase 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 [37,38]. 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 [39].

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

| Table 1. 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|15925517 | CTP synthetase | 60297 | 5.00 | 191 | 8 |
| | 94 | gi|15924727 | bifunctional 3-deoxy-7-phosphohexulonate 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-beta-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 alpha | 54608 | 4.91 | 595 | 18 |
| Nucleotide metabolism | 10 | gi|15924264 | polynucleotide phosphorylase/polyadenylase | 77428 | 4.89 | 1533 | 42 |
| Protein degradation | 9 | gi|15925538 | ATP-dependent Clp protease 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 | 46463 | 5.93 | 108 | 5 |

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cellular functions such as replication of DNA, control of gene expression, heat stress tolerance, protein degradation, and protein folding as molecular chaperones [40,41]. In addition, they are related to various biological processes such as cell division, development of sporulation, genetic competence, and quality control of protein translation [42,43]. 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

| 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) | 54861 | 5.70 | 65 | 19 |
| 48       | gi|156978879  | 2-amino-3-keto butyrate coenzyme A 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 | 33 | 7 |
| 62       | gi|15923503   | cysteine synthase (O-acetylserine sulfhydrylase)-like protein | 33012 | 5.39 | 289 | 40 |
| 65       | gi|49484892   | putative N-acetyltansferase | 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 |
| 54       | gi|221137774  | glyceraldehyde 3-phosphate 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   | GT-Pase 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 |
| 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 |
in non-growing or slow-growing cells [44]. In this study, ATP-dependent Clp protease proteolytic subunit-like protein (ClpP, proteolytic subunit) was suppressed whereas ATP-dependent Clp protease chain (ClpB, ATPase subunit) was induced. In *E. coli*, ClpP has a crucial function in cell survival at high temperatures [45] and participates in de novo protein folding in mildly stressed [46]. 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 immunomodulatory antigen A, a virulence factor which elicits immune response during septicemia [47].

**Effect of rhodomyrtone on *S. aureus* cell morphology**

To elucidate the physiological effects of rhodomyrtone against *S. aureus*, transmission electron microscopy was utilized. As illustrated in Figure 3 and Figure 4, the micrographs clearly demonstrated that the growth of both MRSA and *S. aureus* ATCC 29213 in media containing rhodomyrtone at 0.174 μg/ml for 18 h generated profound changes in both *S. aureus* cell morphology and ultrastructure. 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 3C), thickened cell wall (Figure 3D, arrow), and thickened septa with irregular features (Figures 3E, 3F and 4E, arrows). Increasing in staphylococcal cell wall thickness has been previously reported due to the mechanism of actions of some antibiotics such as chloramphenicol [34], erythromycin [48], penicillin [34], and vancomycin [49]. Furthermore, the arc-like shape of fibrillar wall material occurring from turning of compressed cross wall and septal formation (Figures 3E and 4E, arrows) were also observed in rhodomyrtone exposed cells. This effect of the compound was similar to staphylococcal cells grown in the presence of penicillin at a concentration of 0.05 μg/ml [34]. After treatment with low dose of penicillin, staphylococcal cells always affect the penicillin binding protein which is considered to be located in the splitting structure of rhodomyrtone, a major bioactive principle, have been described previously [15]. Purification protocol and the structure of rhodomyrtone, a major bioactive principle, have been previously published by our research group [15,51]. Purity of rhodomyrtone has been confirmed by referring to nuclear magnetic resonance (NMR) and mass spectrometry (MS) reference [12,51].

**Materials and Methods**

**Preparation of ethanolic extract and rhodomyrtone**

Dried leaves of *R. tomentosa* were extracted with 95% ethanol as has been described previously [15]. Purification protocol and the structure of rhodomyrtone, a major bioactive principle, have been previously published by our research group [15,51]. Purity of rhodomyrtone has been confirmed by referring to nuclear magnetic resonance (NMR) and mass spectrometry (MS) reference [12,51].

**Bacterial strains and growth conditions**

MRSA NPRC 001R, a common endemic isolate with mecA gene obtained from Hat Yai hospital, Thailand [52] and *S. aureus* ATCC 29213 were used throughout this study. The bacterial strains were stored as a 20% glycerol stock at −80°C until required. Bacteria were precultured overnight from glycerol stock on Luria-Bertani agar. The bacteria were inoculated into 2 ml of trypticase soy broth at 37°C with constant shaking to mid-exponential phase growth.

**Determination of minimal inhibitory concentration (MIC)**

MICs of the crude extract and rhodomyrtone against *S. aureus* were carried out by a modified broth microdilution method recommended by Clinical Laboratory Standardization Institute (CLSI) guideline [53]. 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 CAMHB (Difco, Detroit, MI). The bacterial suspension was then added to each well to provide a final inoculum density of 5×10^7 cfu/ml. After incubation at 37°C for 16–18 h, the bacterial suspension in each well was measured at OD_600 nm 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. All experiments were carried out in triplicate.

**Time-kill study**

Time-kill kinetic studies of the rhodomyrtone were performed in cation-adjusted Mueller Hilton broth (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 10^6 cfu/ml. A culture containing 1% DMSO and no rhodomyrtone 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. 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.
Staphylococcal cells were collected by centrifugation at 10,000 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 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 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, Hercules, CA) according to the manufacturer's instruction. Bovine serum albumin (BSA) was used as protein standard. The supernate containing solubilized proteins 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.

Western blot analysis

The method used was a modification of that described by Towbin et al. [54]. 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

**Figure 3.** Transmission electron microscopy demonstrating the effects of rhodomyrtone on methicillin-resistant *S. aureus* NPRC 001R morphology and ultrastructure. The bacteria were incubated in CAMHB for 18 h, media containing 0.174 µg/ml of rhodomyrtone (C, D, E, and F) and untreated control cultures (A and B). Scale bars = 1 µm (A and C) and 0.5 µm (B, D, E, and F), respectively.
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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 0.2% (w/v) 2,6-dichloroindophenol and 0.03% hydrogen peroxide) and incubated in the dark. The color reaction was stopped with deionized water.

Preparation of 2D-gel samples

The bacterial cell samples were prepared from four liter cultures in the presence or the absence of subinhibitory concentration of rhodomyrtone (0.174 mg/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, Mannheim, Germany). 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 lysed 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.

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
incubated for 20 min. After incubation, 10 mM ammonium bicarbonate was added to the gels and 50% ACN in 0.1% formic acid was added into the gels, and the dye stained gels. Overexpressed or downregulated protein spots occurred after rhodomyrtone treatment were excised and recorded from representative gels. Selected spots that presented only in rhodomyrtone-treated samples and those that disappeared after the rhodomyrtone treatment were excised and recorded from representative gels. Select spots that presented only in rhodomyrtone-treated S. aureus gels and those that disappeared after the rhodomyrtone treatment were individually excised from the respective Coomassie dye stained gels.

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 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.

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 trypic digests were spotted with 2-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).

Liquid chromatography-mass spectrometry (LC-MS)

The protein digests 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 (Accliam PepMap 100 C18, 3 μm, 100A, 75 μm id×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.

Database searching

All of the tandem mass spectra were analyzed against 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).

Transmission electron microscopic analysis

Overnight cultures grown in the presence or the absence of rhodomyrtone at 0.174 μg/ml were harvested by centrifugation at 10,000×g for 5 min. The cells were washed twice, resuspended in PBS, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate. The samples were then incubated at 4°C for at least 1 h. After incubation, the cells were recovered by centrifugation at 10,000×g for 5 min and washed twice with PBS. The pellets were fixed with 1% osmium tetroxide and were then left at room temperature for 1 h. The samples were dehydrated with gradient 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. Morphological and ultrastructural of the bacteria were observed and photographed by a Hitachi H7000 at 75 kV.

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Author Contributions

Conceived and designed the experiments: WS PS SPV. Performed the experiments: WS KK. Analyzed the data: PS WW. Contributed reagents/materials/analysis tools: SPV. Wrote the paper: WS SPV.
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