The Journal of Microbiology (2011) Vol. 49, No. 6, pp. 956-964
DOI 10.1007/s12275-011-1115-0

Responses in the Expression of Extracellular Proteins in Methicillin-Resistant *Staphylococcus aureus* Treated with Rhodomyrtone

Monton Visutthi[1], Potjanee Srimanote[2], and Supayang Piyawan Voravuthikunchai[1,3]*

[1]*Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*
[2]*Faculty of Allied Health Sciences, Thammasat University, Prathumthani 12120, Thailand*
[3]*Natural Products Research Center, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand*

(Received March 9, 2011 / Accepted August 1, 2011)

Rhodomyrtone from a medicinal plant species, *Rhodomyrtus tomentosa*, is a challenged effective agent against Gram-positive bacteria, especially methicillin-resistant *Staphylococcus aureus* (MRSA). The present study was undertaken to provide insight into MRSA extracellular protein expression following rhodomyrtone treatment. Secreteomic approach was performed on a representative clinical MRSA isolate exposing to sub-inhibitory concentration rhodomyrtone (0.174 μg/ml). The identified extracellular proteins of a response of MRSA to rhodomyrtone treated condition were both suppressed and overexpressed. Staphylococcal antigenic proteins, immunodominant antigen A (IsaA) and staphylococcal secretory antigen (SsaA) involved in cell wall hydrolysis were downregulated after the treatment. The results suggested that rhodomyrtone may interfere with WalK/WalR (YycG/YycF) system. Other enzymes such as lipase precursor and another lipase, glycerophosphoryl diester phosphodiesterase, were absent. In contrast, cytoplasmic proteins such as SpoVG and glycerol phosphate lipoteichoic acid synthase, and ribosomal proteins were found in the treated sample. Appearance of several cytoplasmic proteins in the treated culture supernatant revealed that the bacterial cell wall biosynthesis was disturbed. This finding provides a proteomic mapping of extracellular proteins after rhodomytone treatment. Extensive investigation is required for this natural compound as it has a great potency as an alternative anti-MRSA drug.

***Keywords*:** *Rhodomyrtus tomentosa*, methicillin-resistant *Staphylococcus aureus*, rhodomyrtone, anti-MRSA, extracellular proteins

Staphylococcus aureus is a human pathogen of strong clinical significance due to increasing infections with multi-resistant isolates not only in the hospitals but also in the community. This bacterium has currently developed resistance to all β-lactam antibiotics (Crawford *et al*., 2007), erythromycin, clindamycin, tobramycin (Lindqvist *et al.*, 2009), fluoroquinolones (van der Mee-Marquet *et al.*, 2007), linezolid (Tsiodras *et al.*, 2001), as well as glycopeptides including vancomycin and teicoplanin (Vaudaux *et al.*, 2001), In recent years, clinical efficacy of daptomycin, a cyclic lipopeptide antibiotic, against methicillin-resistant *S. aureus* (MRSA) and glycopetide intermediate-resistant *S. aureus* (GISA) has been described (Fowler *et al.*, 2006; Marco *et al.*, 2008). However, treatment failures associated with daptomycin-resistant strains have been reported (Enoch *et al*., 2007). Concomitant microbial resistance to virtually all available antibiotics raises the specter of untreatable infections caused by this organism, which had been thought to be defeated by modern medicine. This unsolved problem demands new antimicrobial strategies to be developed urgently.

Several acylphloroglucinol compounds have been characterized with anti-staphylococcal activity such as hyperforin (Schempp *et al.*, 1999), myrtucommulone A (Appendino *et al.*, 2002), and rhodomyrtone (Dachriyanus *et al.*, 2002; Saising *et al.*, 2008; Limsuwan *et al.*, 2009). Dachriyanus *et al.* (2002) firstly reported an antibacterial substance, rhodomyrtone, from *Rhodomyrtus tomentosa* (Downy rose myrtle) but without supportive evidence. In recent years, our research group reported rhodomyrtone with pronounced antibacterial activity, an MIC of 0.5 μg/ml against *S. aureus* (Saising *et al.*, 2008) and 0.39-0.78 μg/ml against MRSA (Limsuwan *et al.*, 2009). Many antibiotics can affect virulence factors and release of these factors from bacterial cells. For example, clindamycin and linezolid have been shown to suppress virulence proteins of *S. aureus* (Herbert *et al.*, 2001; Bernardo *et al.*, 2004). Subinhibitory concentrations of β-lactam antibiotics led to an increase in α-toxin expression of *S. aureus* (Ohlsen *et al.*, 1998). In contrast, there have been very few reports on the effect of natural compounds on extracellular protein secretion. Therefore, in the present communication, antibacterial activity of the ethanolic extract and rhodomyrtone was assessed against both reference strain of *S. aureus* and a representative clinical MRSA isolate. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanolic extract of *R. tomentosa* leaves as well as its purified principle, rhodomyrtone, against clinical MRSA isolates were investigated. We then determined the effects of rhodomyrtone on the extracellular proteins of MRSA by the proteomic approaches. Profiles of extracellular MRSA proteins following treatment with rhodomyrtone were investigated to explain drug-bacteria interaction.

* For correspondence. E-mail: supayang.v@psu.ac.th; Tel./Fax: +66-744-46661

Materials and Methods

Preparation of the ethanolic extract of *R. tomentosa* leaves and rhodomyrtone

R. tomentosa leaves were dried in an oven at 60°C for 48 h and ground in an electrical blender. The powder was extracted with 95% ethanol for 7 days at room temperature. The extract was evaporated using a rotary evaporator (BUCHI Rotavapor R-114, Switzerland) and kept at 4°C. For rhodomyrtone purification, the extract was fractionated as earlier described by our research group (Saising *et al.*, 2008; Limsuwan *et al.*, 2009).

Tested bacterial strains

Methicillin-resistant *S. aureus*, MRSA (NPRC 001R-NPRC 024R) isolated from a patient at Hat Yai hospital, Hat Yai, Songkhla were used in this study. *S. aureus* ATCC 29213 was included as a reference strain. The bacterial culture was grown in Tryptic soy broth (TSB, Difco, France) at 37°C for 18 h. The bacterial strains were maintained in TSB containing 20% glycerol at -80°C.

Determination of minimal inhibitory concentration (MIC)

The MICs of the ethanolic extract and rhodomyrtone were carried out by a modified broth microdilution method according to CLSI guideline (CLSI, 2006). The extract and rhodomyrtone were serially diluted twofold in Mueller Hinton broth (MHB, Difco) in 96-well microtitre plates to obtain final concentrations ranged from 9-625 μg/ml and 0.125-8 μg/ml, respectively. An equal volume of 100 μg/ml of log phase culture of *S. aureus*, approximately 10^6 CFU/ml, was then added to each well. After incubation at 37°C for 16-18 h, the $OD_{600\ nm}$ of each well was measured with an ELISA reader (Multiscan EX, Labsystems, Finland) against a blank well to which dimethyl sulphoxide, DMSO (Sigma, USA) was added. The MIC was read as the lowest concentration that completely inhibited the bacterial growth. All experiments were done in triplicate.

Time-kill study

Time-kill kinetic experiments of the ethanolic extract and rhodomyrtone were performed in MHB. An inoculum of 10^6 CFU/ml of *S. aureus* was added to MHB containing 1/4 MIC, 1/2 MIC, MIC, and 2 MIC of the ethnolic extract and rhodomyrtone. After the cultures were incubated at 37°C, the samples were collected at time intervals. Ten microliter aliquots (and serial 10-fold dilutions thereof) were plated on nutrient agar (NA, Difco) to determine total numbers of viable bacteria. Assays were carried out in triplicate and the results were expressed as Means±Standard Errors.

Preparation of rhodomyrtone-treated *S. aureus* secreted proteins

A common endemic MRSA isolate with *mecA* gene, NPRC 001R, was used as a representative isolate in proteome analyses. Approximately 5×10^5 CFU/ml from the log-phase culture was inoculated into sterile 500 ml Duran glass bottles containing 50 ml MHB supplemented with rhodomyrtone at sub-MIC concentration (0.174 μg/ml), and incubated at 37°C for 18 h. The bacterial culture in MHB supplemented with a final concentration of 1% DMSO was included as control.

The culture supernatant was precipitated by adding 10% trichloroacetic acid (Merck, Germany). After overnight incubation at 4°C, the precipitate was centrifuged at 8,000×g at 4°C for 30 min. The secreted protein pellets were washed 3 times with ice-cold absolute ethanol and dried under vacuum, and then dissolved in phosphate-buffered saline (PBS) (0.01 M, pH 7.4). The protein concentration was determined using Bradford protein assay kit (Bio-Rad Laboratories, USA) according to the manufacturer's instruction. The protein samples were subjected to SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blot analysis as described below.

SDS-PAGE

One-dimensional denaturating SDS-PAGE was carried out essentially as earlier described by Laemmli (1970). Suspended samples (5-10 mg) were performed with 12% polyacrylamide gels on Mighty® Small II SE250 gel apparatus (Hoefer®, Pharmacia Biotech, USA). Proteins were stained by overnight incubation at room temperature 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 and 8% w/v ammonium sulphate) to visualize the protein bands. A prestained protein ladder (Bio-Rad Laboratories) was used for SDS-PAGE molecular weight markers.

Western blot analysis

A modification of Western blot method (Towbin *et al*., 1979) was carried out. The samples were run on 12% polyacrylamide gels and transferred onto nitrocellulose membrane (Hybond, Amersham Bioscience) at 100 V 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, Germany). Nitrocellulose sheet was blocked by incubating in 5% w/v skim milk powder in PBST (PBS with 0.05% v/v Tween 20) for 30 min followed by incubation in anti-*S. aureus* antiserum at 1:3,000 dilution as primary antibody for 1-16 h. Horse radish peroxidase-conjugated goat anti-mouse (Southern Biotechnology) at a dilution of 1:3,000 was employed as the secondary antibody, and further incubated for 1-2 h. antigen-antibody complexes were detected by the addition of the colour detection solution [phosphate buffer (67 mM, pH 7.6) containing of 0.2% w/v 2,6-dichloroindophenol (Sigma) and 0.03% H_2O_2]. The colour reaction was stopped with deionized water.

Two-dimensional gel electrophoresis (2DE)

Rhodomyrtone-treated secreted protein samples were prepared from 2 L of culture. Samples were resuspended in standard 2DE-buffer (30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS) containing protease inhibitors (Roche Diagnostics GmBH, Germany). The protein mixtures were stored in aliquots at -70°C.

S. aureus protein preparations were cleaned with the 2D-Clean-up kit (PlusOne, GE Healthcare Biosciences, USA) to eliminate detergent, salts, lipids, phenolics, and nucleic acids. After the treatment, the preparations were resuspended in DeStreak Rehydration solution (GE Healthcare Biosciences). The protein concentration in the sample was determined using a 2D-Quant kit (PlusOne, GE Healthcare Biosciences) with bovine serum albumin (BSA) as the standard before subjecting them to isoelectric focusing. For the first dimension separation, bacterial protein samples (400 μg) in DeStreak Rehydration solution containing 1% pH 3-10 IPG buffer (GE Healthcare Biosciences) were loaded onto the center of a slot in the 18 cm strip holder of the IPG phor (GE Healthcare Biosciences). The 18 cm-IPG strip (pH 3-10, non linear, Immobiline™ DryStrip, GE Healthcare Biosciences) was placed into the strip holder containing the sample. The PlusOne™ DryStrip Cover Fluid (PlusOne, GE Healthcare Biosciences) was overlayed onto each IPG strip to prevent evaporation and urea crystallization. The strip holder was then placed into

the Ettan IPG Phor Electrofocusing System (GE Healthcare Biosciences) and the IPG strip was allowed to rehydrate at 20°C for 12 h. The isoelectric focusing parameters were set as followed, the current was applied at 300 V until 200 V h, 1,000 V until 300 V h, a gradient step at 5,000 V until 4,000 V h, and step and hold at 5,000 V until 2,000 V h were obtained.

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, 0.002% bromphenol blue) containing 50 mg dithiothreitol (DTT) for 15 min. Subsequently, the strip was placed in 10 ml of the equilibration buffer containing 250 mg iodoacetamide (IAA) for 15 min. The strip was washed by submerging in a cylinder containing electrode buffer and then placed onto a 1 mm thick, 12% polyacrylamide gel (25 cm×20 cm) precasted in the HoeferTMDual Gel Caster (GE Healthcare Biosciences). The warm molten agarose (0.5%) was then placed onto the gel and SDS-PAGE was carried out at 25 mA/gel until the tracking dye reached the lower edge of the gel. After SDS-PAGE, the gel was subjected to Coomassie brilliant blue G-250 staining. Molecular weight was estimated according to the internal running calibration marker. For visualization, gels were briefly rinsed with 1% acetic acid to reduce background and imaged by Image ImageScannerII and ImageMaster™ Software (GE Healthcare Biosciences). 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 twofold ($p>0.05$). Overexpressed or downregulated protein spots occurred after rhodomyrtone treatment were recorded from representative gels.

Spot picking, excision and spot handling work station

The Coomassie brilliant blue G-250 stained 2-DE gels were scanned and the protein spots detected in rhodomyrtone-treated *S. aureus* supernatant and not in the untreated control, and *vice versa*, were selected and carefully excised individually from the Commassie brilliant blue-stained-gels.

In-gel digestion

After the protein spots were excised, the gel pieces were subjected to in-gel digestion using an in-house method developed by the Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand. The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM IAA in 10 mM ammonium bicarbonate. 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) was added to the gels, followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C overnight. To extract the peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid was added, the gels were then incubated at room temperature for 10 min. The extracted peptides were collected, pooled, and dried by vacuum centrifuge they were kept at -80°C for further mass spectrometric analysis.

NanoLiquid chromatography-mass spectrometry (nanoLC-MS) analysis

The protein digest was injected into an Ultimate 3000 LC System (Dionex, USA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with an electrospray at a flow rate of 300 nl/min in a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100 A, 75 µm i.d.×150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid in 80% ACN) was run for 40 min. The data were processed using the analytic software (Bruker).

Database searching

The spectral data were processed using detailed fine structure analysis. The MS/MS spectra were searched by NCBI and Swiss Prot database using Mascot webpage, after converting the acquired MS-MS spectra into MASCOT generic file format. The MASCOT search parameters were set at taxonomy of staphylococcus, with an allowed number of missed cleavages 1, enzyme trypsin, fixed modifications: Carbamidomethyl, Variable modifications: methionine oxidation, Mass values: Monoisotopic, Protein Mass: Unrestricted, Peptide Mass Tolerance: ± 1.2 Da, Fragment Mass Tolerance: ± 0.6 Da. Any MASCOT score less than 25 having low quality were rejected.

Results

Determination of minimal inhibitory concentration (MIC)

The MICs and MBCs of the ethanolic extract from *Rhodomyrtus tomentosa* leaves on *S. aureus* and 24 clinical MRSA isolates ranged from 39-78 µg/ml and 312-624 µg/ml, respectively. The MIC and MBC of the pure compound, rhodomyrtone, against *S. aureus* and MRSA NPRC 001R were 0.5 and 1 µg/ml, respectively (Table 1). The values were comparable to those of vancomycin. The results supported that rhodomyrtone is an active compound in the ethanolic extract of *R. tomentosa* leaves. It produced marked antibacterial activity against all clinically MRSA isolates. A representative strain, MRSA NPRC 001R, which demonstrated multiple antibiotic resistance was selected for further studies.

Time-kill study of the ethanolic extract and rhodomyrtone-treated *S. aureus*

Time-kill study was performed to investigate the effect of the

Table 1. MIC of the ethanolic extract of *R. tomentosa* leaves and rhodomyrtone on *S. aureus* and methicillin-resistant *S. aureus* isolates

Antibacterial agents	*S. aureus* ATCC 29213		MRSA NPRC 001R		MRSA (n=24)
	MIC	MBC	MIC	MBC	MIC ranges (µg/ml)
R. tomentosa extract	78	312	78	312	39-78
Rhodomyrtone	0.5	1	0.5	1	Not done
Vancomycin	0.5	1	0.5	1	Not done

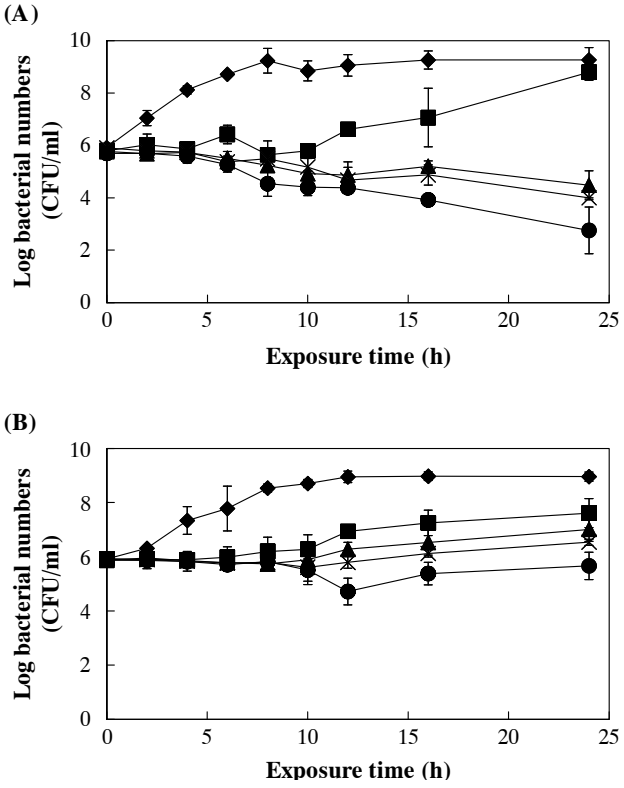

Fig. 1. Effect of the ethanolic extract of *R. tomentosa* leaves on *S. aureus* ATCC 29213 (A) and MRSA 001R (B). Bacterial cells were treated with 1/4MIC (square), 1/2MIC (triangle), MIC (cross) and 2MIC (circle) of the extract, untreated control cells (diamond).

ethanolic extract of *R. tomentosa* and rhodomyrtone on the bacterial growth at different time intervals. As shown in Fig. 1, all concentrations of the extract inhibited the growth of both *S. aureus* and MRSA. At 1/2 MIC, MIC, and 2 MIC, the level of *S. aureus* decreased 4-6 logfolds after 24 h. At MIC and 2 MIC, the numbers of viable MRSA decreased 2-3 logfolds after 24 h, compared with the untreated culture.

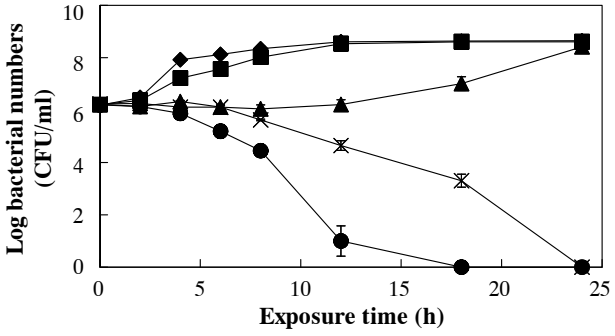

Fig. 2. Effect of rhodomyrtone on MRSA NPRC 001R. The bacterial cells were treated with 1/4MIC (square), 1/2MIC (triangle), MIC (cross) and 2MIC (circle) of the compound, untreated control cells (diamond).

In order to determine protein changes in rhomyrtone-treated MRSA, the killing kinetics of rhodomyrtone at different concentrations were evaluated at time intervals (Fig. 2). At MIC and 2 MIC of rhodomyrtone, the numbers of viable MRSA reduced more than 6 logfolds after 24 and 12 h, respectively.

SDS-PAGE and Western blot analysis of proteins secreted by MRSA and *S. aureus* after treatment with the ethanolic extract and rhodomyrtone

SDS-PAGE demonstrated both overexpressed and down-regulated protein bands from the culture supernatant after treatment with sub-MIC of the ethanolic extract (Fig. 3A). Western immunoblotting using mouse anti-*S. aureus* antisera as a probe confirmed that the ethanolic extract altered the profiles of the immunogenic *S. aureus* proteins in the culture supernatant (Fig. 3B).

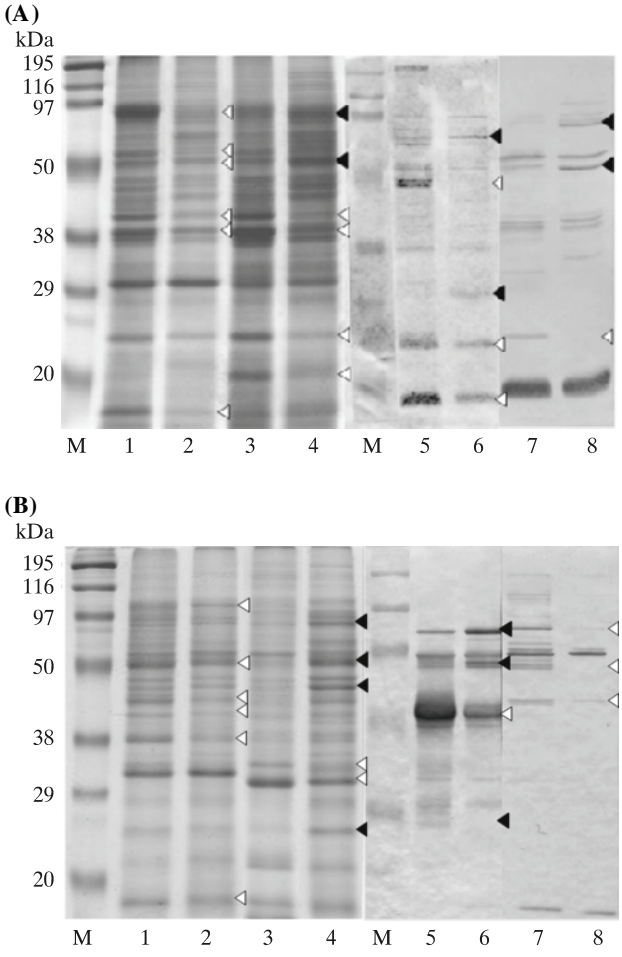

Fig. 3. Profile of proteins from the culture supernatant of *S. aureus* treated with the ethanolic extract of *R. tomentosa* leaves (A) and rhodomyrtone (B) at 37°C for 18 h. Secretome proteins in the supernatant were precipitated and analyzed by 12% SDS-PAGE (Left) and immunoblotting (Right). M (marker), 1 and 5 (untreated *S. aureus* ATCC 29213), 2 and 6 (treated *S. aureus* ATCC 29213), 3 and 7 (untreated MRSA NPRC 001R), 4 and 8 (treated-MRSA NPRC 001R). The arrows indicate the expression of protein bands that were up-regulated (black) or down-regulated (white).

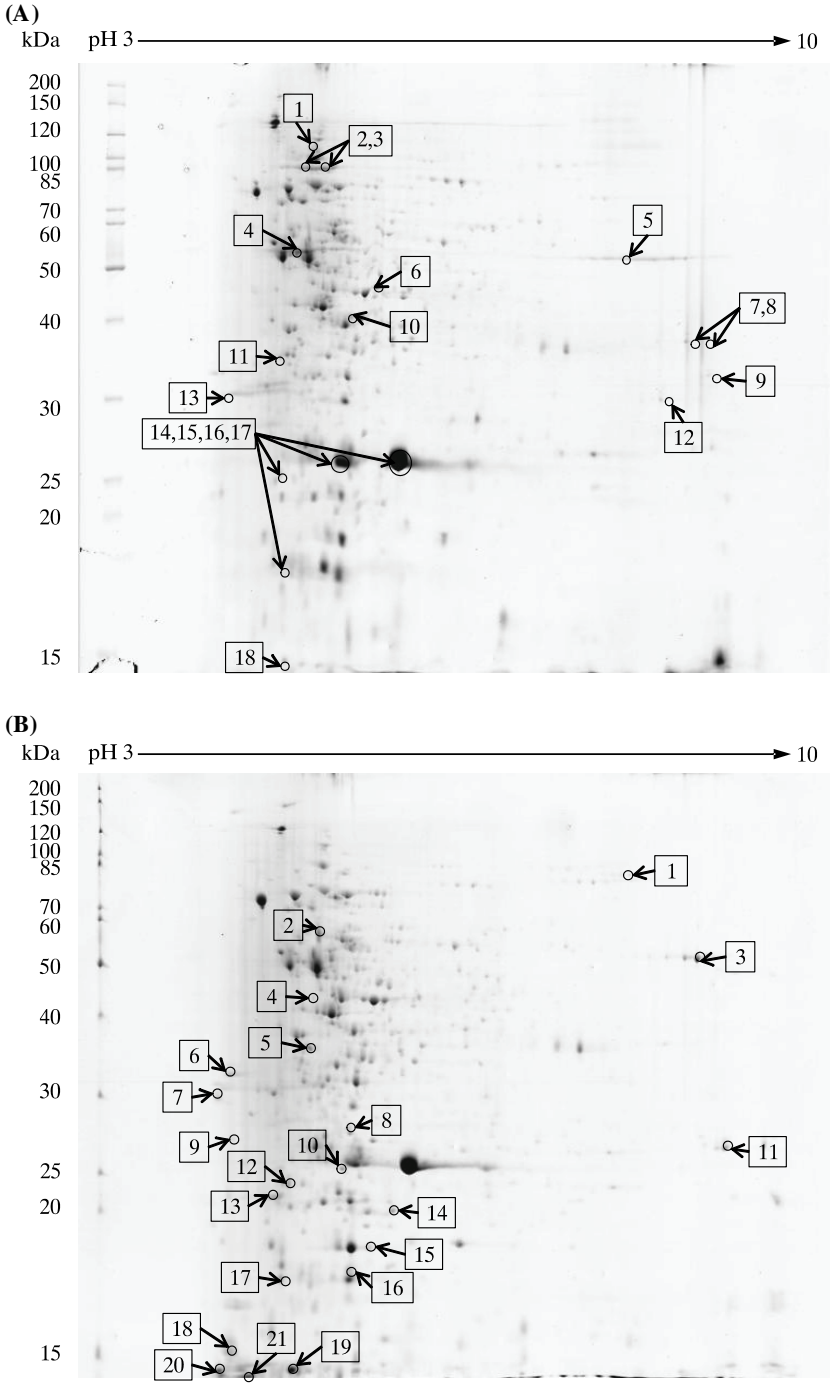

Fig. 4. Extracellular proteins profiling of culture supernatant from rhodomyrtone-untreated (A) and sub-MIC rhodomyrtone-treated (B) MRSA NPRC 001R. The extracellular proteins of MRSA, approximately 400 μg protein samples were separated on Immobiline™ DryStrip non linear pH 3-10 and 12% polyacrylamide gel. The 2DE-gels were stained by Coomassie dye. The tag number indicated spot that selected for identified protein by LC-MS/MS. Selected protein spots, either overexpressed or downregulated, were indicated.

Two-dimensional polyacrylamide gel electrophoresis (2DE) profile of proteins prepared from sub-MIC rhodomyrtone-treated MRSA

The effect of rhodomyrtone on MRSA NPRC 001R secretome was characterized using proteomic approach. Approximately 452 protein spots on the 2DE-gel from secretomes of the untreated (Fig. 4A) and 556 protein spots rhodomyrtone-treated MRSA (Fig. 4B) were observed respectively. Numerous protein spots differed with 141 spots present in the secretome from the untreated MRSA that were absent in that of the rhodomyrtone-treated MRSA. In contrast, there were 233 spots present in the secretome of the rhodomyrtone-treated

Table 2. Identification of downregulated extracellular proteins in MRSA after treatment with rhodomyrtone

Spot No.	Protein name	Accession no.	Mascot score	Sequence recovery (%)	Molecular mass (Da)/p*I* (theoretical)
Non-secretory proteins					
13	30 kDa neutral phosphatase; NPTase/30 kDa neutral phosphatase	gi|129123	122	54%	3768/8.48
1	Elongation factor G	gi|15923537	540	6%	76854/4.80
10	Ornithine carbamoyltransferase	gi|49484831	274	13%	37823/5.14
6	Phosphoglycerate kinase	gi|15923763	86	6%	42633/5.17
18	Regulatory protein SpoVG	gi|21282182	209	58%	11271/4.64
Signal peptides					
4, 5	Glycerol phosphate lipoteichoic acid synthase	gi|15923709	537, 308	15%, 11%	74353/9.04
12	Glycerophosphoryl diester phosphodiesterase	gi|15923949	525	34%	35289/8.67
14-16	Immunodominant antigen A/IsaA	gi|49484768	242, 173, 274	19%, 15%, 19%	24198/6.11
17	Immunodominant antigen A/IsaA	gi|15925559	105	9%	24198/5.90
2, 3	Lipase precursor	gi|49484866	812	28%, 26%	76686/7.75
7, 8	Staphylococcal secretory antigen SsaA2	gi|15925289	429, 390	28%, 10%	29367/8.96
9	Staphylococcal secretory antigen SsaA3	gi|15925289	79	10%	29367/8.96
11	Serine protease/Glutamyl endopeptidase	gi|15924038	322	13%	36955/5.00

MRSA that were not present in the secretome of the untreated MRSA. Selected protein spots that were clearly overexpressed or downregulated in another preparation were subsequently picked. Thirty nine protein spots of interest were further subjected to protein identification by LC-MS.

Identification of rhodomyrtone-sensitive proteins by LC-MS

Thirty four extracellular proteins were identified by signalP. Significant downregulation or overexpression of the untreated and rhodomyrtone-treated secretome are reported in Table 2 and 3, respectively. As shown in Table 2, eight amino acid sequences of 18 protein spots from the untreated MRSA and four of 21 rhodomyrtone-treated protein spots were predicted as signal peptide sequences (Table 3).

At sub-MIC, expression of rhodomyrtone-treated MRSA on the secreted proteins such as exo-enzymes and antigenic proteins was suppressed (Fig. 4A and Table 2). Staphylococcal

Table 3. Identification of MRSA overexpressed extracellular proteins only when treated with rhodomyrtone

Spot No.	Protein name	Accession no.	Mascot score	Sequence recovery (%)	Molecular mass (Da)/p*I* (theoretical)
Non-secretory proteins					
6	30S ribosomal protein S2	gi|15924246	230	16%	29133/5.44
11	50S ribosomal protein L1	gi|15923528	590	34%	24693/9.00
13	50S ribosomal protein L4	gi|15925239	417	33%	22451/9.90
21	50S ribosomal protein L18	gi|15925224	391	49%	13089/9.95
17	50S ribosomal protein L20	gi|15924668	55	16%	13678/11.26
18	50S ribosomal protein L22	gi|15925235	467	59%	12827/9.92
20	50S ribosomal protein L24	gi|15925229	128	10%	11529/9.82
8	Acetoin reductase	gi|15923116	630	39%	27256/5.04
15	Alkaline shock protein 23	gi|15925172	158	15%	19180/5.13
4	Cell division protein FtsZ	gi|15924176	235	23%	41012/4.87
7	Elongation factor P	gi|15924518	167	23%	20541/4.75
2	Elongation factor Tu	gi|15923538	741, 932	42%, 46%	43135/4.74
10	Hypothetical protein SACOL2136	gi|57652166	755	66%	24017/4.94
14	Hypothetical protein SAV1854	gi|15924844	845	64%	22330/5.33
12	Peptidyl-prolyl cis-trans isomerase-like protein	gi|15923944	88	9%	21605/4.57
19	Regulatory protein SpoVG	gi|21282182	342	56%	9742/5.28
9	Ribosomal subunit interface protein	gi|15923742	137	24%	22199/5.15
Signal peptides					
3	Glycerol phosphate lipoteichoic acid synthase	gi|15923709	1327	41%	74353/9.04
16	Hypothetical protein SAV2304	gi|15925294	102	9%	17445/5.77
1	Lipase precursor	gi|221137507	309	13%	74395/8.14
5	Serine protease	gi|15924038	448	25%	36955/5.00

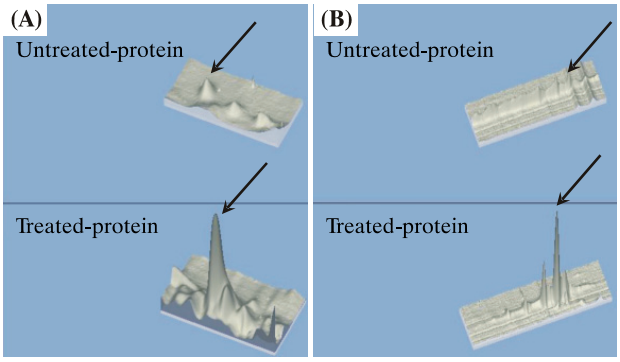

Fig. 5. Differential expression of regulatory protein SpoVG/putative septation protein spoVG (A) and glycerol phosphate lipoteichoic acid synthase (B) of MRSA NPRC 001R in extracellular proteins untreated and treated with rhodomyrtone.

antigenic protein, immunodominant antigen A (IsaA) (spot 14-17) and many other protein spots related to staphylococcal secretory antigen (SsaA) (spot 7-9) were downregulated. Certain enzymes such as lipase precursor (spot 2-3) and glycerophosphoryl diester phosphodiesterase (spot 12) were absent and glycerol phosphate lipoteichoic acid synthase (spot 4-5) was downregulated. On the other hand, expression of extracellular proteins including many cytoplasmic proteins and four of signal proteins were upregulated (Fig. 4B and Table 3). The four proteins such as glycerol phosphate lipoteichoic acid synthase (spot 3), hypothetical protein SAV2304 (spot 16), lipase precursor (spot 1), serine protease (spot 5) were upregulated. An abundance of cytoplasmic proteins in protein synthesis such as ribosomal proteins (Fig. 4B, spot 6, 11, 13, 17, 18, 20, 21) were upregulated in the treated sample. In addition, other proteins involved in cell wall synthesis including a regulatory protein SpoVG (Fig. 5B) and glycerol phosphate lipoteichoic acid synthase (Fig. 5A) were markedly overexpressed, compared with the untreated sample.

Discussion

Secretomic results revealed that staphylococcal secretory antigen, SsaA2, SsaA3, and partial isoforms of IsaA protein were reduced in response to rhodomyrtone treatment. The results suggested that rhodomyrtone may interfere with WalK/WalR (YycG/YycF) system. Proteins associated with cell wall hydrolases such as SsaA and IsaA were regulated by this system influencing the staphylococcal cell wall degradation (Dubrac and Msadek, 2004). WalK/WalR system is a two-component system in low-G+C Gram-positive bacteria. In *S. aureus*, it controlled autolysin synthesis related to cell wall metabolism and biofilm formation (Dubrac *et al.*, 2007). Inhibition of this system by WalK/WalR depletion, not only was autolysin production inhibited, but also staphylococcal cell wall could not separate, leading to cell death (Dubrac *et al.*, 2007). *SsaA* is composed of five paralogues (*SsaA*1, 2, 3, 4, and 5). The genes have been reported to share a common cysteine, histidine-dependent amidohydrolases/peptidases-amidase domain and amino-terminal signal sequences. These conserved regions have been suggested to play a role in cell wall hydrolysis (Dubrac *et al.*, 2007). A highly antigenic protein, SsaA was detected in sera of patients with *S. epidermidis* endocarditis (Lang *et al.*, 2000). Other workers reported IsaA as an immunodominant antigen in *S. aureus* (Lorenz *et al.*, 2000). SsaA2 exhibited a distinct change for the full expression of resistance to macrolide-lincosamide-streptogramin B antibiotics (Martin *et al.*, 2002). However, the role of SsaA3 in staphylococci has not yet been established and further investigation is required.

Another virulence factor, lipase precursor was suppressed at sub-MIC rhodomyrtone. The compound may interfere with lipase belongs to Agr and SarA two component system in *S. aureus* (Ziebandt *et al.*, 2001, 2004). Similarly, the study of the effects of linezolid on exoprotein of *S. aureus* demonstrated that the agent downregulated lipase after the bacteria was treated with sub-MIC linezolid (Bernardo *et al.*, 2004). On the other hand, the effect of quinupristin-dalfopristin on lipase revealed a biphasic response. The enzyme was increased at concentration below 50% MIC, at 90% MIC, it was downregulated (Koszczol *et al.*, 2006).

SpoVG and glycerol phosphate lipoteichoic acid synthase which are primarily implicated in bacteria cell defense, were remarkably overexpressed in sub-MIC rhodomyrtone-treated culture supernatant. The results suggested that MRSA attempted to prevent themselves from rhodomyrtone via capsular formation and cell division process. SpoVG has been reported as a major product from *yabJ-spoVG* operon required for capsular polysaccharide production and antibiotic resistance in *S. aureus* (Schulthess *et al.*, 2009). This protein was known to be involved in spore formation of *B. subtilis* (Rosenbluh *et al.*, 1981). SpoVG in *B. subtilis* was found in genomes of several nonsporulating bacteria. Lipoteichoic acid (LTA) is an important cell wall polymer and consists of a polyglycerolphosphate backbone chain linked to the membrane by a glycolipid. Glycerol phosphate lipoteichoic acid synthase is an enzyme responsible for polyglycerolphophate backbone chain formation. The enzyme catalyzes the polymerization of LTA polyglycerol phosphate, a reaction that presumably uses phosphatidylglycerol as substrate (Gründling and Schneewind, 2007). Two subsequent studies on *S. aureus* and *B. subtilis* revealed that LTA is important for normal bacterial growth. Observed morphological alterations indicate a crucial role of LTA in cell division and sporulation process in *B. subtilis* (Oku *et al.*, 2009; Schirner *et al.*, 2009).

In addition, there were several non-secretory proteins including ribosomal proteins, transcription factors, and other cellular proteins related to cell wall biosynthesis present in the culture supernatant of MRSA treated with rhodomyrtone. MRSA cell wall damage resulted from rhodomyrtone treatment was recently reported (Sianglum *et al.*, 2011). We suggest that the presence of the proteins in the culture supernatant may be the results of dysfunction of cell membrane, cell wall or protein export machinery of the bacterial cell. During cell cycle, many of the non-secretory proteins are normally found in culture medium (Sibbald et *al.*, 2006). Linezolid and quinupristin-dalfopristin are both ribosome-targeted compounds that inhibit bacterial protein synthesis. Their effects on exoprotein secretion had been reported. Ribosomal proteins were present in culture supernatant in the antibiotic-treated condition (Bernardo *et al.*, 2004; Koszczol *et al.*, 2006).

In summary, this paper defines the effects of rhodomyrtone on MRSA by secretomic approach. The finding reveals a response of the bacterial cell against rhodomyrtone by down-regulated the antigenic proteins related to WalK/WalR two component system. Appearance of several cytoplasmic proteins in the treated culture supernatant revealed that the bacterial cell wall biosynthesis was disturbed. This study presents the establishing of the framework for further examination on the modes of action of rhodomyrtone. Extensive investigation is required for this natural compound as it has a great potency as an alternative anti-MRSA drug.

Acknowledgements

We would like to thank the National Research University Project of Thailand's Office of the Higher Education Commission for financial support and Office of the Higher Education Commission, Thailand for supporting by grant fund under the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree for this research.

References

Appendino, G., F. Bianchi, A. Minassi, O. Sterner, M. Ballero, and S. Gibbons. 2002. Oligomeric Acylphloroglucinols from Myrtle (*Myrtus communis*). *J. Nat. Prod*. 65, 334-338.

Bernardo, K., N. Pakulat, S. Fleer, A. Schnaith, O. Utermöhlen, O. Krut, S. Müller, and M. Krönke. 2004. Subinhibitory concentrations of linezolid reduce *Staphylococcus aureus* virulence factor expression. *Antimicrob. Agents Chemother*. 48, 546-555.

Clinical and Laboratory Standards Institute (CLSI). 2006. Methods for dilution antimicrobial susceptibility tests for bacterial that grow aerobically; Approved standards, 7th ed. Clinical and Laboratory Standards Institute Document M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

Crawford, S.E., S. Boyle-Vavra, and R.S. Daum. 2007. Community associated methicillin-resistant *Staphylococcus aureus*, p. 153-79. *In* D.C. Hooper, M.W. Scheld, and J.M. Hughes (eds.), Emerging infections, Vol. 7. American Society for Microbiology, Washington, D.C., USA.

Dachriyanus, Salni, M.V. Sargent, B.W. Skelton, I. Soediro, M. Sutisna, A.H. White, and E. Yulinah. 2002. Rhodomyrtone, an antibotic from *Rhodomyrtus tomentosa*. *Aus. J. Chem*. 55, 229-232.

Dubrac, S., I.G. Boneca, O. Poupel, and T. Msadek. 2007. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *J. Bacteriol*. 189, 8257-8269.

Dubrac, S. and T. Msadek. 2004. Identification of genes controlled by the essential YycG/YycF two-component system of *Staphylococcus aureus*. *J. Bacteriol*. 186, 1175-1181.

Enoch, D.A., J.M. Bygott, M.L. Daly, and J.A. Karas. 2007. Daptomycin. *J. Infect*. 55, 205-213.

Fowler, V.G., H.W. Boucher Jr., G.R. Corey, E. Abrutyn, A.W. Karchmer, M.E. Rupp, D.P. Levine, and *et al.* 2006. Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. *N. Engl. J. Med.* 355, 653-665.

Gründing, A. and O. Schneewind. 2007. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* 104, 8478-8483.

Herbert, S., P. Barry, and R.P. Novick. 2001. Subinhibitory clindamycin differentially inhibits transcription of exoprotein genes in *Staphylococcus aureus*. *Infect. Immun.* 69, 2996-3003.

Koszczol, C., K. Bernardo, M. Kronke, and O. Krut. 2006. Subinhibitory quinupristin/dalfopristin attenuates virulence of *Staphylococcus aureus*. *J. Antimicrob. Chemother*. 58, 564-574.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Lang, S., M.A. Livesley, P.A. Lambert, W.A. Littler, and T.S. Elliott. 2000. Identification of a novel antigen from *Staphylococcus epidermidis*. *FEMS Immunol. Med. Microbiol*. 29, 213-220.

Limsuwan, S., E.N. Trip, T.R. Kouwen, S. Piersma, A. Hiranrat, W. Mahabusarakam, S.P. Voravuthikunchai, J.M. van Dijl, and O. Kayser. 2009. Rhodomyrtone, a new candidate as natural antibacterial drug from *Rhodomyrtus tomentosa*. *Phytomedicine 16*, 645-651.

Lindqvist, M., B. Isaksson, A. Samuelsson, L. Nilsson, and A. Hallgren. 2009. A clonal outbreak of methicillin-susceptible *Staphylococcus aureus* with concomitant resistance to erythromycin, clindamycin and tobramycin in a Swedish county. *Scand. J. Infect. Dis*. 41, 324-333.

Lorenz, U., K. Ohlsen, H. Karch, M. Hecker, A. Thiede, and J. Hacker. 2000. Human antibody response during sepsis against targets expressed by methicillin resistant *Staphylococcus aureus*. *FEMS. Immunol. Med. Microbiol*. 29, 145-153.

Marco, F., C.G. de la Mària, Y. Armero, E. Amat, D. Soy, A. Moreno, A. del Río, and *et al.* 2008. Daptomycin is effective in treatment of experimental endocarditis due to methicillin-resistant and glycopeptide-intermediate *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. 52, 2538-2543.

Martin, P.K., Y. Bao, E. Boyer, K.M. Winterberg, L. McDowell, M.B. Schmid, and J.M. Buysse. 2002. Novel locus required for expression of high-level macrolide-lincosamide streptogramin B resistance in *Staphylococcus aureus*. *J. Bacteriol*. 184, 5810-5813.

Ohlsen, K., W. Ziebuhr, K.P. Koller, W. Hell, T.A. Wichelhaus, and J. Hacker. 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (*hla*) gene expression of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob. Agents Chemother*. 42, 2817-2823.

Oku, Y., K. Kurokawa, M. Matsuo, S. Yamada, B.L. Lee, and K. Sekimizu. 2009. Pleiotropic roles of polyglycerolphosphate synthase of lipoteichoic acid in growth of *Staphylococcus aureus* cells. *J. Bacteriol*. 191, 141-151.

Rosenbluh, A., C.D.B. Banner, R. Losick, and P.C. Fitz-James. 1981. Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. *J. Bacteriol*. 148, 341-351.

Saising, J., A. Hiranrat, W. Mahabusarakam, M. Ongsakul, and S.P. Voravuthikunchai. 2008. Rhodomyrtone from *Rhodomyrtus tomentosa* (Aiton) Hassk. as a natural antibiotic for staphylococcal cutaneous infections. *J. Health. Sci*. 54, 589-595.

Schempp, C.M., K. Pelz, A. Wittmer, E. Schopf, and J.C. Simon. 1999. Antibacterial activity of hyperforin from St John's wort, against multiresistant *Staphylococcus aureus* and Gram-positive bacteria. *Lancet* 353, 2129.

Schirner, K., J. Marles-Wright, R.J. Lewis, and J. Errington. 2009. Distinct and essential morphogenic functions for wall- and lipoteichoic acids in *Bacillus subtilis*. *EMBO. J*. 28, 830-842.

Schulthess, B., S. Meier, D. Homerova, C. Goerke, C. Wolz, J. Kormanec, B. Berger-Bächi, and M. Bischoff. 2009. Functional characterization of the sigmaB-dependent *yab*J-*spo*VG operon in *Staphylococcus aureus*, role in methicillin and glycopeptide resistance. *Antimicrob. Agents Chemother*. 53, 1832-1839.

Sianglum, W., P. Srimanote, W. Wonglumsom, K. Kittiniyom, and S.P. Voravuthikunchai. 2011. Proteome analyses of cellular proteins in methicillin-resistant *Staphylococcus aureus* treated with rhodomyrtone, a novel antibiotic candidate. *PLoS ONE* 6, e16628.

Sibbald, M.J., A.K. Ziebandt, S. Engelmann, M. Hecker, A. de Jong, H.J.M. Harmsen, G.C. Raangs, and *et al.* 2006. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. *Microbiol. Mol. Biol. R*. 70, 755-788.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic trans-

fer of proteins from polyacrylamide gels to nitrocellulose sheets, procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.

Tsiodras, S., H.S. Gold, G. Sakoulas, G.M. Eliopoulos, C. Wennersten, L. Venkataraman, R.C. Moellering, and M.J. Ferraro. 2001. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet* 358, 207-208.

van der Mee-Marquet, N., C. Epinette, J. Loyau, L. Arnault, A.S. Domelier, B. Losfelt, N. Girard, R. Quentin, and the Bloodstream Infection Study Group of the Relais d'Hygiène du Centre. 2007. *Staphylococcus aureus* strains isolated from bloodstream infections changed significantly in 2006. *J. Clin. Microbiol*. 45, 851- 857.

Vaudaux, P., P. Francois, B. Berger-Bächi, and D.P. Lew. 2001. *In vivo* emergence of subpopulations expressing teicoplanin or vancomycin resistance phenotypes in a glycopeptide-susceptible, methicillin-resistant strain of *Staphylococcus aureus*. *J. Antimicrob. Chemother*. 47, 163-170.

Ziebandt, A.K., D. Becher, K. Ohlsen, J. Hacker, M. Hecker, and S. Engelmann. 2004. The influence of *agr* and sigmaB in growth phase dependent regulation of virulence factors in *Staphylococcus aureus*. *Proteomics* 4, 3034-3047.

Ziebandt, A.K., H. Weber, J. Rudolph, R. Schmid, D. Hoper, S. Engelmann, and M. Hecker. 2001. Extracellular proteins of *Staphylococcus aureus* and the role of SarA and sigmaB. *Proteomics* 1, 480-493.