Expression of type II toxin-antitoxin systems and ClpP protease of methicillin-resistant *Staphylococcus aureus* under thermal and oxidative stress conditions

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ABSTRACT

Background and Objectives: *Staphylococcus aureus* is a main human pathogen that causes a variety of chronic to persistent infections. Across the diverse factors of pathogenesis in bacteria, Toxin-Antitoxin (TA) systems can be considered as an antibacterial target due to their involvement in cellular physiology counting stress responses. Here, the expression of TA system genes and ClpP protease was investigated under the thermal and oxidative conditions in *S. aureus* strains.

Materials and Methods: The colony-forming unit (CFU) was used to determine the effects of thermal and oxidative stresses on bacterial survival. Moreover, the expressions of TA system genes in *S. aureus* strains were evaluated 30 min and 1 h after thermal and oxidative stresses, respectively, by quantitative reverse transcriptase real-time PCR (qRT-PCR).

Results: The cell viability was constant across thermal stress while oxidative stress induction showed a significantly decrease in the growth of Methicillin-Resistant *S. aureus* (MRSA) strain. Based on the qRT-PCR results, the expression of *mazF* gene increased under both thermal and oxidative stresses in the MRSA strain.

Conclusion: A putative TA system (namely *immA/irrA*) most likely has a role under the stress condition of *S. aureus*. The MRSA strain responds to stress by shifting the expression level of TA genes that has diverse effects on the survival of the pathogen due to the stress conditions. The TA systems may be introduced as potential targets for antibacterial treatment.

Keywords: *Staphylococcus aureus*; Oxidative stress; Toxin-antitoxin systems; Cold-shock response; Heat-shock response

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INTRODUCTION

Staphylococcus aureus is the main human pathogen that causes a varied range of disease, such as skin infections to fatal necrotizing pneumonia, endocarditis, and osteomyelitis (1). The capacity of *S. aureus* to be a tenacious nosocomial pathogen is associated with numerous factors such as the acquisition of an-

tibiotic resistance, response to environmental stimulators, and nutrient starvation (2, 3). *S. aureus* can respond to these stimulants by shifting the expression of various genes such as the TA system (4).

The TA system typically comprises a bicistronic operon encoding a constant protein 'toxin' and an unstable protein 'antitoxin' which has vital roles in bacterial physiology (5). Six types of TA systems have been recognized in bacteria according to the antitoxin nature (RNA or protein) and mechanism of TA systems (6). These systems may have a stress response by shifting to a dormant state, which permits cells to cope with stress conditions (5). Unfavorable conditions such as starvation, antibiotic stresses, and heat shock result in degrading antitoxin. The toxin is then released and inhibits the vital cellular processes like replication, cell wall synthesis, transcription, and translation (4, 7). Therefore, the cellular processes decrease and it makes different reactions to stress situations (8).

TA systems have been investigated in approximately all bacterial species and they are located on chromosomes or plasmids (9). The number of TA systems that are determined in the genome of a specific species of bacteria differs significantly and can vary from none (e.g., in *Mycobacterium leprae*) to a few dozen (i.e., *M. tuberculosis*). *S. aureus* has two different type II TA families and two type I TA systems. The rest of the TA systems has not been discovered and analyzed in this bacterium (4, 10). Moreover, regulation of TA systems through ClpPC was examined in *S. aureus* and it shows a vital role in the degradation of all identified *S. aureus* antitoxins (3). Although it is obvious that stimulation of ClpP proteolysis is effective in the dormant state of bacterial cells in stress conditions.

To provide extra evidence for the characterization of type II TA systems in MRSA under thermal and oxidative stresses, the existence and expression of the systems were evaluated. Furthermore, given the significance of TA systems in pathogen's survival, this study may propose a potential target to introduce novel anti-infectious treatments.

MATERIALS AND METHODS

Bacterial isolates. Overall, 42 isolates that have been previously identified and were offered kindly to this study by the Department of Pathobiology,

School of Public Health (TUMS). All isolates were confirmed as *S. aureus* based on phenotypic and biochemical methods including Gram-stain, coagulase, catalase, DNase, and Mannitol Salt Agar test.

MRSA identification. To detect MRSA strains, bacterial suspensions equal to 0.5 McFarland standard turbidity were prepared and then streaked on Mueller-Hinton agar (Merck, Germany). Then, a cefoxitin disk (30 μg; MAST Diagnostics, Merseyside, U.K) was placed on the plate and incubated at 37°C for 24 h. The inhibition zones diameter were analyzed based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (11). *S. aureus* ATCC 25923 was used as quality control. Furthermore, PCR assay for the *mecA* gene was performed for the molecular detection of MRSA (12).

DNA extraction. Genomic DNA was extracted using the High Pure PCR Template Preparation kit (Roche, Germany) consistent with the manufacturer's instructions (13). Assessment of quantity and quality of each obtained DNA was examined using Nano-Drop (Thermo Fisher Scientific; USA).

Identification of type II TA systems genes by PCR assay. The whole-genome sequence of *S. aureus* MU50 was obtained from the NCBI to recognize the type II TA system in *S. aureus* isolates. TA systems were found through the TADB database (14), and specific PCR primers were designed by OLIGO software V. 7.56. The presence of gene coding *clpP* and type II TA system toxins (*mazF, relE1, relE2, immA,* and *zeta*) were evaluated by PCR. The PCR was done in a thermocycler (Bio-Rad, USA). The PCR protocol comprised a primary denaturation step at 95°C for 5 min, followed by 35 cycles (94°C for 35 s; annealing for 45 s (Table 1), and extension at 72°C for 30 s), with a final extension at 72°C for 5 min) (14). Finally, two strains (MRSA and Methicillin-sensitive *S. aureus* (MSSA)) were selected to evaluate the expression of type II TA genes and ClpP protease in thermal and oxidative stresses.

Growth curve and viability assessment. The growth curve of MRSA and MSSA strains were determined in the presence of 5 mM and 10 mM of hydrogen peroxide (H_2O_2), according to the methods of Chan et al. (15) with the following modifications. Briefly, overnight cultures were diluted 100-fold in

Table 1. Characteristics of designed primers for both PCR and qRT-PCR assays

Gene	Sequence (5'-3')	TM (°C)	Amplicon size (bp)
mazF	F AACAAGGGGGAGTCAGACCT	60	294
	R GAGCTACTGCATTCAGCCCTA		
relE1	F TACGTTTTCGCCTCAAGCCT	60	117
	R CCTTCCAATGCACCATTTCTGT		
relE2	F TTAACCATCAGCACAGGGTCG	58	84
	R AATCATAATGTGACCATGCCGAT		
Zeta	F ACAGGACGAACAACAGACGTT	60	177
	R TTGTTTTGGTGTTGCCCTGG		
immA	F GGGTCTCTATGACAATGGGGT	60	98
	R TCAACTTGTGGTGGGCAAGT		
clpP	F GCGGTGAACGTGCATATGAT	59	135
	R TCTCTGAGTCTTGCGCTTGT		
16s rRNA	F TGTTGCAAACCTGCCGTACT	60	116
	R TTAAGCGTTCGCCCACTTCT		

50 ml TSB medium (Merck, Germany) and incubated at 37°C with shaking at 200 rpm until reaching an optical density of 0.4 at 600 nm (OD_{600}). Then, bacterial suspensions were diluted 3-fold to reached OD of 0.08-0.1 at 600 nm. Next, each suspension was divided, and two concentrations (5 mM and 10 mM) of H_2O_2 were added. Following the incubation of each concentration, 1 ml of suspension was removed at 0, 1, 2, 3, 4, and 24 h after the addition of H_2O_2, and then the growth curves were assessed turbidimetrically. Moreover, in each time point, 1 ml of suspension was washed twice with sterile sodium chloride solution 0.9%, and the serial dilution was provided, poured into TSA plate (Merck, Germany), and incubated at 37°C. Colony count was performed after 24 h incubation. Bacterial cells without H_2O_2 were used as control in various time intervals. All experiments were conducted triplicate independently.

Thermal stresses response. Overnight cultures of MRSA and MSSA cells diluted 1:100 in 50 ml fresh TSB medium (Merck, Germany) and incubated at 37°C with shaking at 200 rpm until reaching to mid-log phase (OD_{600} nm = 0.25). To induce the cold and heat shock responses, bacterial cultures were incubated for an additional 30 min at 10°C and 42°C, respectively. The control sample was located at 37°C (16). Then, the gene expression of *clpP* and toxins of type II TA systems (*mazF, relE1, relE2,* and *immA*) were evaluated.

RNA isolation and qRT-PCR. The gene expression of *clpP* and toxins of type II TA systems (*mazF, relE1, relE2,* and *immA*) upon oxidative and thermal stresses were evaluated using qRT-PCR method. RNA was extracted using a high pure RNA isolation kit (Roche; Germany) one hour after adding H_2O_2 and 30 min after the thermal stresses.

The bacterial cells were precipitated by centrifugation at 7000×g for 10 minutes and the sediment was re-suspended in Tris-EDTA (TE) buffer (pH 8). Then, 50 µg/ml lysozyme (Yekta -Tajhiz- Azma; Iran) and 300 µg/ml lysostaphin (Sigma-Aldrich; Germany) was added, followed by an incubation step at 37°C for 10 min and 15 min, respectively (13). The RNA extraction was carried out by a high pure RNA isolation kit (Roche; Germany) based on the manufacturer's instructions. The quantity and quality were evaluated by gel electrophoresis and NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). DNA contamination was resolved by treating all RNA isolates with DNase I RNase free (Thermo Fisher Scientific, USA) treatment. Reverse transcription reaction was performed by the FIREScript RT cDNA synthesis kit (Solis BioDyne; Estonia) based on the manufacturer's instructions. Finally, qRT-PCR was carried out on the QIAGEN's real-time PCR cycler (Corbett 6000; Germany) with SYBR Green protocol (Amplicon; Denmark) by specific primers. A final volume of 20 µl reaction, comprising 4.5 µl nuclease-free water, 12.5 µl SYBR Green master mix, 1 µl of cDNA (100 ng/ml) and 1 µl of each primer (5 pmol) was performed as follows: a primary activation step at 94°C for 10 min, 40 cycles of denaturation step at 95°C for 20 s,

and one cycle of 60°C for 45 s. The 16s rRNA was used as an internal control for normalizing mRNA expression levels and fold changes. Calculation of expression was by the $2^{-\Delta\Delta CT}$ method (14).

Statistical analysis. The data obtained from viability evaluation and growth curves were explained as the average of three independent experiments: The results of mRNA expression study were provided as means ± standard error of three independent experiments. All of the statistics were done using Graph pad prism 8 (GraphPad Software, Inc). Student's t-test (for two groups) and examination of variance (two-way ANOVA) were run.

RESULTS

MRSA identification. Using both cefoxitin susceptibility and PCR assay, the frequency of MRSA and MSSA were 64.2% (27 isolates) and 35.8% (15 isolates), respectively.

Identification of TA systems genes by PCR assay. The type II TA loci in *S. aureus* MU50 were assessed with TA finder, an online tool developed in the TADB Database which can rapidly identify the TA prediction. Finally, one putative TA system was predicted which was characterized as *immA/irrA* belonging to the Metalloendopeptidase family. Five type II TA systems examined in this study are shown in Table 2. The attendance of type II TA systems toxins was investigated in *S. aureus* isolates. Unexpectedly, *mazF, relE1, relE2,* and *immA* were present in all the isolates and zeta toxin was found in one isolate (Fig. 1).

Growth curve and viability assessment. Both the
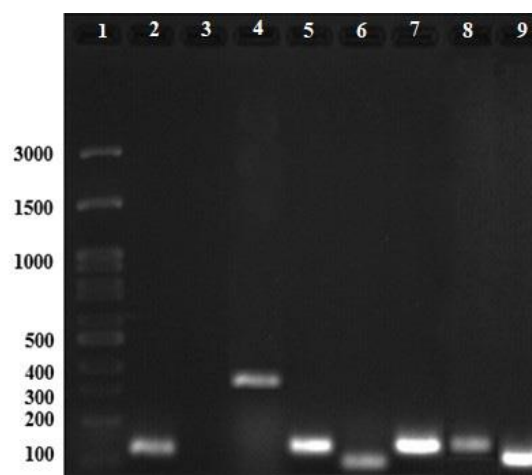
Fig. 1. Presence of ClpP protease and TA system toxins genes using PCR assay on 1.5% gel agarose. Lanes 1-9, comprises, (1) 100 bp DNA ladder; (2) positive control, 16S rRNA gene (116 bp); (3) Negative control; (4) *mazF* (294 bp); (5) *relE1* (117bp); (6) *relE2* (84 bp); (7) *clpP* (135 bp), (8) *zeta* (117 bp); (9) *immA* (98 bp).

cell viability counts and OD_{600} amount was evaluated in five sequential hours and 24 h after exposure to H_2O_2. Oxidative stress induction led to a significant decrease in the growth of both MRSA and MSSA strains upon exposure to H_2O_2. After 24 h of H_2O_2 exposure, in MRSA strains, 9.2 and 11-log reductions were observed in 5 mM and 10 mM concentrations, respectively. Moreover, a significant reduction in the number of MSSA viable cells was detected under exposure to 10 mM H_2O_2 (Fig. 2).

Thermal stresses response. To evaluate the ability of *S. aureus* to adapt to temperature variations, both strains (MRSA and MSSA) were grown to the mid-log phase of growth, then bacteria were incubated at 10°C and 42°C for an additional 30 min to induce the cold and heat shock response, respectively. Then,

Table 2. Type II toxin-antitoxin (TA) systems in *S. aureus* MU50

TA-No	Toxin	Antitoxin	Family	Domain Pair *
TA-1	SAV2068	SAV2069	MazF - RHH	-
TA-2	SAV2407	SAV2408	*relE1* - PHD	COG2026/ COG2161
TA-3	SAV2456	SAV2457	*relE2* - PHD	COG2026/ COG2161
TA-4	AET37228	AET37227	Omega-epsilon/zeta	-
TA-5	YP_001574753	YP_001574754	ImmA - IrrE	Xre/COG2856

* A domain pair characterizes the TA protein domain pair of each toxin and its cognate toxin, -: it means no TA domain pair is found.

(A) MSSA isolate

(B) MSSA isolate

(C) MRSA isolate

(D) MRSA isolate
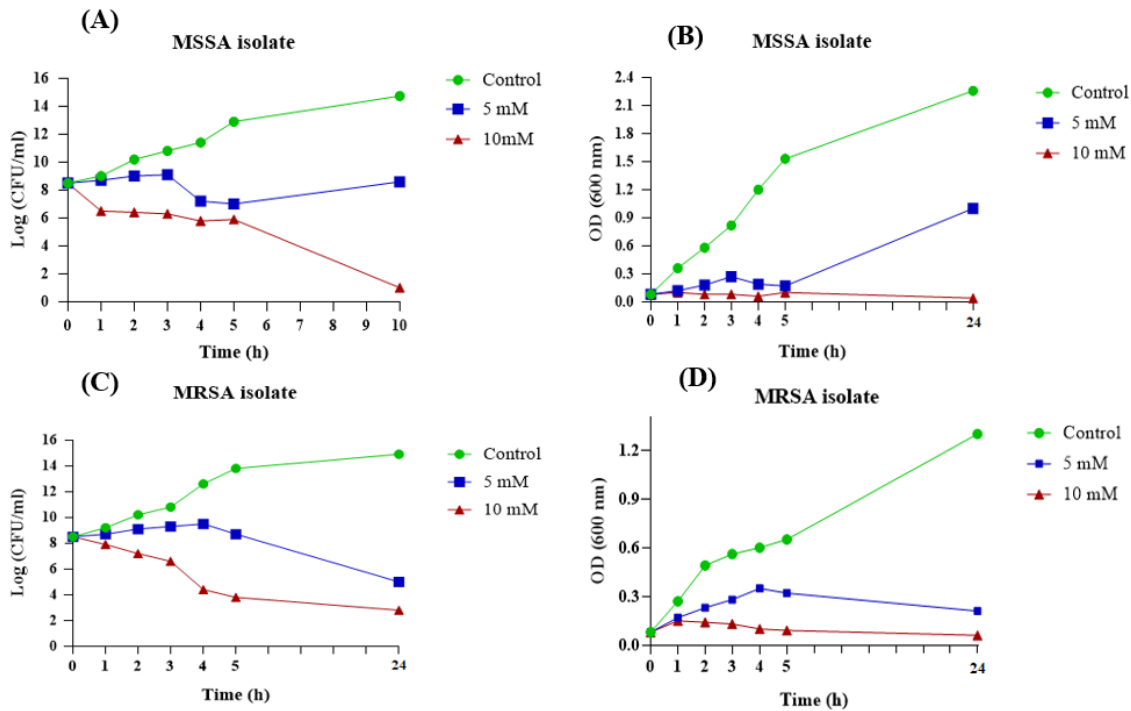
Fig. 2. Effects of 5 mM and 10 mM of H_2O_2 on the survival of MRSA and MSSA strains: A and C for growth curves, and B and D for viability curves (Data are shown as the means ± SD of three independent replicates).

qRT-PCR was applied to compare the transcript profile of thermal shocked and control cells. The induction of thermal shock did not noticeably change cell viability but changed the expression level of toxin genes.

The cold shock showed the decreased expression values of the gene coding for the *relE1* and *immA* in both MRSA and MSSA strains. Also, in MRSA strain, the expression level of *mazF* and *relE2* genes were 1.4-fold and 3.8-fold higher in the treated sample than the untreated sample. Though, the expression of these genes, 9-fold and 2.5-fold decreased in MSSA strain in comparison to the control sample. The upregulation of the *clpP* gene was observed in MSSA strain (Fig. 3A).

MSSA strain exposure to heat shock slightly increased the expression level of *relE2* and *immA* while reducing the expression levels of other genes. On the other hand, in MRSA strain, *mazF* and *relE2* were upregulated 28.2-fold and 1.74-fold, respectively (Fig. 3B).

Oxidative stress response. To estimate the effects of oxidative stress on TA gene expression, the exponential phase of MRSA and MSSA strains was exposed to H_2O_2. After numerous trials, two concentra-

tions of H_2O_2 (5 mM and 10 mM H_2O_2) were chosen in which a slight decrease was found, if any (~90%) in the colony-forming unit (CFU) amounts in the *S. aureus* strains.

The expression level of ClpP protease and genes of type II TA system under oxidative conditions were evaluated by qRT-PCR assay. In the presence of 10 mM H_2O_2, the expression level of *clpP* and toxins genes were decreased except for *mazF*. Moreover, MRSA strain exposure to 10 mM H_2O_2 slightly increased the expression of *mazF* gene (Fig. 3D). On the other hand, the expression level of *mazF, immA,* and *clpP* increased under 5 mM H_2O_2 in MRSA strain (Fig. 3C).

DISCUSSION

Infections are dynamic processes that pathogens respond to environmental pressures. This ability can alter cellular physiology and enhance the survival of the organism and its capability to cause disease (16). *S. aureus* is an important source of community and hospital-acquired infections. However, a few concepts are identified about *S. aureus* responses to stress conditions (16).
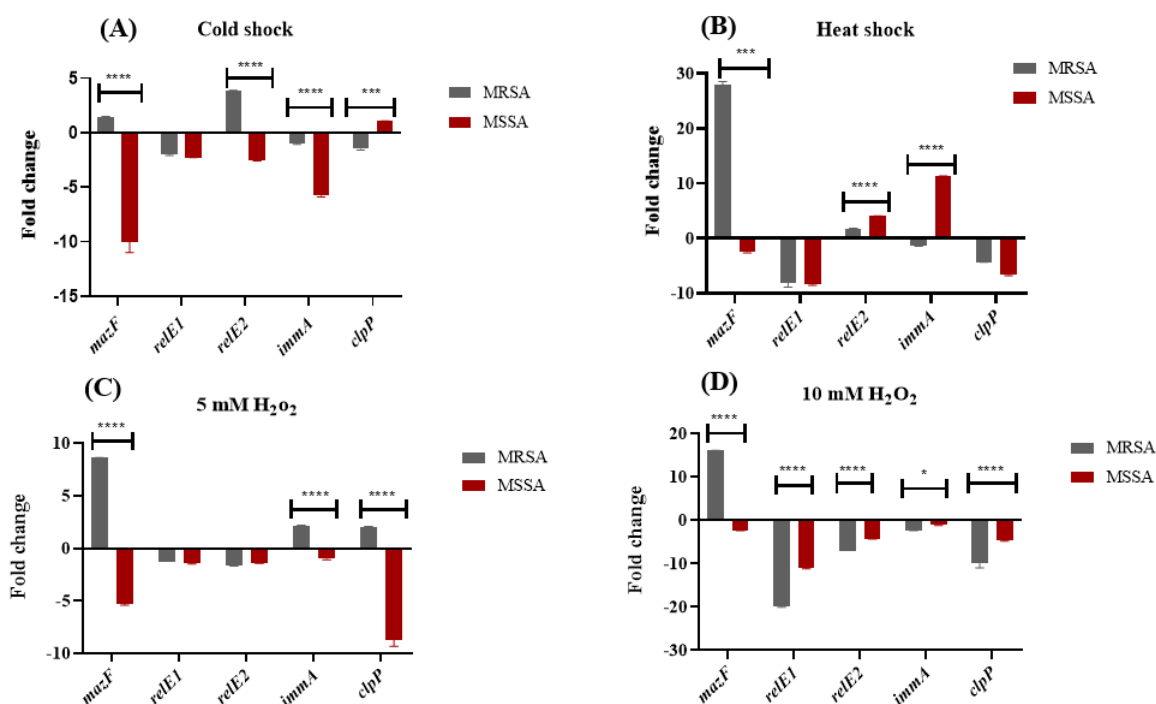
Fig. 3. Analysis of the relative expression level of the ClpP protease and type II TA system genes in the presence of 5 mM and 10 mM of H_2O_2 and thermal stress in MRSA and MSSA strains. Graph data are shown as the means ± SD of three independent replicates (*P<0.05; **** P<0.0001 by two-way ANOVA test for multiple comparisons)

The current study aimed to characterize the effects of thermal and oxidative stresses on the growth and expression levels of type II TA genes in *S. aureus.* The results indicated the presence of all the considered genes coding for type II TA system toxins except omega/epsilon/zeta in *S. aureus* isolates. Previous studies showed that MRSA strain CM05 comprised a three-module type II TA system omega/epsilon/zeta but remained unknown but it is clear that the *zeta* toxin inhibits peptidoglycan synthesis in pneumococci (17). The presence of omega/epsilon/zeta was identified in just one isolate in our study.

Thermal stress stimulates the transcription of several genes, which enabled bacterium to adapt the stress (18). In this study, the thermal stress did not substantially change cell viability but enhanced the expression level of the genes, suggesting that the applied conditions were suitable to *S. aureus* cold shock response. A study by Anderson et al. also showed that thermal stress conditions did not appreciably affect cell viability but increase the expression of 46 genes (16). Further, *mazF* was highly expressed under heat-shocked stress in MRSA, and *relE2* and *immA* were induced in heat-shocked MSSA cells. The TA system in *Escherichia coli*, *YefM/YoeB,* was involved in ad-

aptation to temperature stress, although Lon protease levels did not increase during thermal stress (18). Our results indicated that the expression of *clpP* did not increase in the heat stress suggesting that another mechanism may account for temperature-induced TA activation. Also, the upregulation of *maz* genes under heat shock in *Listeria monocytogenes* was observed. Thus TA systems have a role in the survival of *L. monocytogenes* under thermal stresses (19).

The survival rate of both MRSA and MSSA strains were sharply declined with the increasing H_2O_2 concentration. Although the concentration of 5 mM of H_2O_2 slightly affected the *clpP* expression in MRSA strain but it was downregulated the gene in MSSA strain. The findings indicated that MRSA possesses a fitness advantage under oxidative conditions. Interestingly, the MRSA remains alive in the exposure to 10 mM H_2O_2 (Fig. 2), suggesting that this may be due to increased *mazF* gene expression. A report showed *yefM-yoeB* and *relBE* participate in oxidative stress in *Streptococcus pneumoniae* (15). Moreover, oxidative stress reduces the expression of type II TA systems genes in *Klebsiella pneumoniae,* which is inconsistent with our results (8). On the other hand, Lon protease expression increased slightly under ox-

idative stress.

TA systems are broadly present in bacterial genomes, and toxin can target various cellular procedures under stress conditions (20). This process can lead to the prevention of cell growth and shift to a dormant state, which results in responding to stress conditions (21, 22). The antitoxin is either destroyed by cellular proteases or suppressed by enhanced toxin expression. Therefore, the antitoxin may have a positive or negative regulatory role for the TA systems (4). Six different types of Clp proteases were detected in *S. aureus*, among which ClpP was involved in the antitoxin homeostasis by either conditional degradation or disposing of the unwanted proteins (4). In the current study, *clpP* gene expression exhibited an increase in the cold shock and at the presence of 5 mM H_2O_2 in MSSA and MRSA strains, respectively. The expression of this protease displayed reduction after exposure to heat shock and 10 mM H_2O_2 in both MRSA and MSSA strains, which indicates the essential role of this protease in keeping the toxin level in the balanced state to prevent severe damages to the bacterial cells. In Gram-positive bacteria, the expression of *clp* genes was controlled by the negative heat-shock regulator CtsR. Remarkably, the CtsR regulon as well comprises the chaperone genes *groESL* and *dnaK* in *S. aureus* (23). Synthesis and expression of the two major *S. aureus* chaperones was shown to be induced during infection of human epithelial cells (23). Moreover, the study by Springer et al. showed that ClpP protease is involved in persister cell formation and their maintenance (24).

CONCLUSION

A putative TA system (namely *immA/irrA*) most likely has a role under the stress conditions of *S. aureus*. The MRSA strain responds to stress by shifting the expression level of TA genes that has diverse effects on the survival of the pathogen due to the stress conditions. The TA systems may be introduced as potential targets for antibacterial treatment.

ACKNOWLEDGEMENTS

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