

Expression of Type II Toxin/Antitoxin systems and ClpP protease of Methicillin-Resistant *Staphylococcus aureus* under stress condition

Samira Karimaei

Tehran University of Medical Sciences

Behrooz Sadeghi Kalani

Iran University of Medical Sciences

Nader Shahrokhi

Pasteur Institute of Iran

Rahil Mashhadi

Tehran University of Medical Sciences

Mohammad Reza Pourmand (✉ mpourmand@tums.ac.ir)

Tehran University of Medical Sciences <https://orcid.org/0000-0003-1280-5765>

Research article

Keywords: Staphylococcus aureus, MRSA, Gene expression, Toxin -antitoxin system, qRT-PCR

Posted Date: April 21st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-21867/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Staphylococcus aureus is a major human pathogen causing chronic to persistent infections. Amongst diverse factors of pathogenesis in bacteria, toxin-antitoxin (TA) systems have a potential to be presented as an antibacterial target due to their participation in cell physiology including stress responses. This study was conducted to determine the effects of thermal and oxidative stresses on expression of type II Toxin/Antitoxin systems and ClpP protease in **Methicillin-Resistant** *Staphylococcus aureus* (**MRSA**).

Materials/methods

Expression of type II TA genes (*mazF*, *relE1*, *relE2* and *immA*) and *clpP* gene in MRSA strain were evaluated following thermal and oxidative stresses by qRT-PCR techniques.

Results

The cell viability was constant across thermal stress, whereas oxidative stress induction resulted in a significant reduction in the growth of **MRSA** strain. The result of RT-qPCR revealed that TA genes were expressed in stress conditions and expression of *mazF* gene increased under both thermal and oxidative stresses in MRSA strain.

Conclusions

Based on our results, the MRSA strain responded to stress by altering the expression level of TA genes. In overall, TA system could be an antibacterial target in *S. aureus* that can revitalize the research on TA systems in this pathogen.

Background

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen that causes a wide spectrum of diseases, ranging from minor skin infections to life-threatening diseases such as fatal necrotizing pneumonia, endocarditis and osteomyelitis[1, 2]. Numerous factors contribute to the capacity of *S. aureus* to be a tenacious pathogen. One contributing factor is likely acquisition of antibiotic resistance, which causes patients need prolonged treatment and many experience difficulties as a result of antibiotic-resistance or shift to dormant state that causes a high level of tolerance to most conventional antibiotics[1]. In addition, other factors including responding to various environmental stimuli such as nutrient starvation, stress, biofilm formation, has made this bacteria to a recalcitrant pathogen[3]. *S. aureus* can respond to these stimuli to increase its fitness by shifting the expression of various genes such as the toxin-antitoxin (TA) system[4].

The TA system, which typically comprises a bicistronic operon encoding a stable protein 'toxin' and a labile protein 'antitoxin', have received much attention because of their vital roles in bacterial

physiology[5]. As yet, six types of TA systems have been identified based on their mechanism of action and their nature of the antitoxin (RNA or protein), while the toxin is in all of them a protein[6]. The toxins and antitoxins of all considered bacterial type II TA systems are proteins and are the most prevalent and best-described TAs[6].

TA systems may involve stress response by shifting to dormant state which permits cells to cope with stress conditions[5]. Unfavorable conditions such as starvation, antibiotic stresses and heat shock, resulting in degradation of unstable antitoxin, and the imbalance between toxin and antitoxin, lead to toxin interfering with vital cellular processes such as replication, transcription, translation and cell wall synthesis[4, 7]. Therefore, cellular growth decreases and induces diverse responses to stress conditions[8].

TA systems have been investigated in almost all bacterial species, which are found on plasmids or chromosomes[9]. The number of TA systems varies significantly and can range from none in some species (e.g., in *Mycobacterium leprae*) to highly abundant in others (e.g., in *M. tuberculosis*)[6, 9]. About 80 TA systems are studied in *Mycobacterium tuberculosis* [9] while the study on TA systems in *S. aureus* is less advanced [4]. *S. aureus* has two different type II families and two type I TA systems. It is supposed that rest of TA systems still await for discovery and analysis[4, 10].

Here, to provide extra evidence about the role of type II TA systems in thermal and oxidative stresses, we analyzed available staphylococcal genomes in silico and wet lab to assess the distribution of type II TA system operons and their prevalence in clinical isolates. Then, the expression levels of Type II TA and ClpP protease in the stress conditions were evaluated.

Results

Identification of type II TA systems

The type II TA loci in *S. aureus* MU50 were assessed with TAFinder, a developed online tool in TADB Database, which can quickly detect the TA prediction. Five type II TA systems and their TA family are shown in Table 1. The existence of type II TA systems was investigated in *S. aureus* isolates. Results revealed the presence of all of the studied genes coding for type II TA system except omega, epsilon/zeta in *S. aureus* isolates. In this context, *mazF/RHH*, *relE1/PHD*, *relE2/PHD* and *ImmA/IrrA* were present in all of the isolates.

Cold shock response

To evaluate the ability of *S. aureus* to adapt to variations in temperature, MRSA and MSSA isolates were grown at 37°C to the mid-log phase of growth, at which time bacteria were incubated at 10°C for an additional 30 min to induce the cold shock response. Then, qRT-PCR was used to compare the transcript profile of cold-shocked and control cells (grown at 37°C).

Induction of cold shock did not appreciably affect cell viability (data not shown) but changed the expression level of TA genes.

Our results displayed the reduced expression levels of genes coding for the *relE1* and *ImmA* in both MRSA and MSSA isolates. Moreover, in MRSA isolate, the expression value of *mazF* and *relE2* genes were 1.4-fold and 3.8-fold higher in treated subjects than the control sample; however, the expression of these genes, 9-fold and 2.5-fold decreased in MSSA isolate compared to the control sample. The cold shock demonstrated the upregulation of ClpP protease gene in MSSA isolate and decreased expression levels of this gene in MRSA isolate (Fig 1 A).

Heat shock response.

Following, transcripts were investigated which are induced in heat-shocked cells. To do so, mid-log phase cells of *S. aureus* (grown at 37°C) were then incubated for 30 min at 42°C to induce the heat shock response. The transcript profile of heat-shocked cells was then compared to that of unshocked cells. Induction of the heat shock response did not change cell viability (data not shown) but induced transcription of some TA genes.

As shown in figure 1.B, in MRSA isolate, two TA genes, *mazF* and *relE2*, were upregulated 28.2-fold and 1.74-fold, respectively, during growing at the elevated temperature. In this isolate, the expression of other genes (*relE1*, *immA* and *clpP*) decreased in heat shock condition. MSSA isolate exposure to heat shock slightly increased the expression level of *relE2* and *immA* (4 and 11-fold higher in treated subjects than the control sample) while reducing the expression levels of other genes (*mazF*, *relE1* and *clpP*).

Growth curves and viability assessments

To estimate the effects of oxidative stress on cell survival, the exponential phase of *S. aureus* was exposed with H₂O₂. After numerous trials, two concentration of H₂O₂ was chosen (5 mM and 10 mM H₂O₂) in which a small decrease, if any (~90%) in the colony-forming unit (CFU) counts in the *S. aureus* isolates were found. Both the viable counts and OD600 values were measured in five sequential hours and 24 h post exposure.

As shown in Figure 2, oxidative stress induction resulted in a significant reduction in the growth of both MRSA and MSSA isolates, upon treatment with 5 mM and 10 mM H₂O₂. After 24 h exposure to H₂O₂, in MRSA isolate notably 9.2 log decrease with 5 mM, and 11 logs with 10 mM H₂O₂. In the case of MSSA isolate, after 24 h, a notable decrease in the number of bacterial cells was observed. In this isolate, 5 mM and 10 mM H₂O₂ gave diminish to 5 logs, and 12 logs decrease in the number of bacterial cells, respectively. After 24 h exposure to 10 mM H₂O₂ in MSSA isolate, log₁₀ cfu/ml reduced from 8.5 to 0 and the value of OD600 reached 0.04, showing the lowest value among the studied concentrations. Further, we performed qRT-PCR for to evaluate the expression of ClpP protease and type II TA system genes under thermal and oxidative stresses. Our results presented the reduced expression levels of genes

coding for the ClpP protease and type II TA systems except *mazF* in the presence of 10 mM H₂O₂. In this context, MRSA exposure to 10 mM H₂O₂ slightly increased the expression level of *mazF* gene.

In contrast to this result, we found that *mazF*, *ImmA* and ClpP protease expression was increase under 5 mM H₂O₂ in MRSA isolate. This was shown on the transcriptional level of *mazF* wherein both MRSA and MSSA were significantly increased throughout growth under oxidative to control conditions.

Discussion

Infections are dynamic processes, during which the invading microorganisms have developed processes to respond to environmental pressures. This ability when elicited alter the cellular physiology which enhances the organism's survival and its capability to cause disease [16]. *S. aureus* being an important cause of nosocomial and community-acquired infections, but unexpectedly slight is known about *S. aureus* responses to stress conditions [16].

Here, we have studied type II TA systems of MRSA and MSSA isolates namely *mazEF*, *relBE1*, *relBE2*, *immA* and omega, epsilon/zeta. Results discovered the presence of all of the studied genes coding for type II TA system except omega, epsilon/zeta in *S. aureus* isolates. According to previous studies, *S. aureus* strain CM05 comprises a three-component type II TA system, namely omega-epsilon-zeta but remained unknown, although it was reported that the zeta toxin can inhibit peptidoglycan synthesis in pneumococci [17]. In our study, none of isolate had this type II TA system, that indicates this locus has low frequency in *S. aureus* strains.

One of the aims of this study was to determine the effects of thermal and oxidative stresses on growth and expression of the type II TA system genes in *S. aureus*. Thermal stress stimulates the transcription of numerous genes that enable adaptation to this stress [18]. To determine the potency of *S. aureus* to adapt to variations in temperature, we first surveyed the cold shock response. Accordingly, MRSA and MSSA isolates were grown at 37 °C to the mid-log phase of growth, at this time, cell cultures were incubated at 10 °C for an additional 30 min to induce the cold shock response. The cold shock condition considered did not substantially affect cell viability (data not shown) but did enhancement the expression level of three genes. In MRSA isolate, transcription of the *mazF* and *relE2* were upregulated, and transcription of the ClpP protease gene was induced 1.03-fold at the lower temperature in MSSA isolate, approving that the conditions used were suitable for investigating the *S. aureus* cold shock response. Other transcript titers decreased in response to low temperature (see Fig. 1). Further, we identified *mazF* is induced in heat-shocked MRSA cell and *RelE2* and *immA* are induced in heat-shocked MSSA cell. To do so, mid-log phase cells were incubated for 30 min at 42 °C to induce heat shock response. Induction of the heat shock response did not distress cell viability (data not shown). In the study of Janssen et al. (2015), *YefM/YoeB* functions was reported in adaptation to temperature stress in *Escherichia coli*. Moreover, this group reported that Lon protease levels do not increase during thermal stress which was similar to the results of our study [18]. Our result indicate that ClpP protease not increase in the heat stress which indicating that another mechanism accounts for temperature-induced TA activation. In another study,

Curtis et al. (2017), was reported upregulation of *maz* genes under heat shock in *Listeria monocytogenes*. They suggested that This system may be complicated in the survival and growth abilities of *L. monocytogenes* between 0 and 42 °C [19].

Further, effects of oxidative stress on growth and expression of the type II TA system genes was determined in *S. aureus* isolates. Results shown significant reduction in the survival to H₂O₂ treatments in both MRSA and MSSA isolates. According to our results, only heat shock was able to upregulate the expression of the *immA* gene in MSSA isolate, while 5 mM H₂O₂ upregulate the expression of this gene in MRSA isolate. Moreover, the concentration of 5 mM of H₂O₂ slightly affected the *clpP* expression in MRSA isolate, while H₂O₂ downregulated the expression of *clpP* gene in MSSA isolate.

We show that under oxidative conditions MRSA possess a fitness advantage. Interestingly, the MRSA is not lost but remains in the culture at low numbers, in other hand MSSA is vanished from population in 10 mM H₂O₂ (Fig. 2). This is likely because *mazF* expression increased in oxidative stress for MRSA. In the study of Chan et al (2018) indicate *yefM-yoeB* and *relBE* participate in oxidative stress in *Streptococcus pneumoniae* [20]. It was proposed that the fitness advantage of most bacteria is due to the costs associated with expression of TA genes. TA systems are abundantly existing in bacterial genomes and under stress conditions, toxin can target diverse cellular processes, such as translation, transcription, replication, and cell wall synthesis [8, 21]. This process leading to the inhibition of cell growth, and switch to a dormant state, which cause to responding to stress conditions[22, 23]. Most of TA systems comprise a liable antitoxin, under stress conditions, antitoxin is either degraded by cellular proteases or suppressed by enhance toxin expression, therefore proposing that the antitoxin can be a positive or a negative regulator of the TA system [4]. Six different types of Clp proteases were detected in *S. aureus*, among them ClpP is involved in the antitoxin homeostasis by either conditional degradation or disposing of the unwanted proteins [4]. In the current study, ClpP protease gene expression exhibited 1.07 and 2-fold increase in the cold shock (in MSSA isolate) and 5 mM H₂O₂ (in MRSA isolate) respectively, compared with the control samples. Expression of this protease displayed reduction after exposure to heat shock and 10 mM H₂O₂ in both MRSA and MSSA isolate, indicating the essential role in keeping the toxin level in the balanced state in order not to severely damage the bacterial cells.

Conclusions

In summary, we have identified a new TA system in *S. aureus* (namely *immA/irrA*). We also studied the expression levels of the TA system genes in *S. aureus* under oxidative and thermal stress. Our result indicates diverse expression of these gene in stress condition, for example the expression levels of some of these systems increased, while the expression levels of other the studied TA system genes decreased in the stress conditions. Therefore, these results indicate the diverse effects of these systems depending on the stress condition, which may open a new window for further studies of the importance of these TA systems, particularly, their roles in *S. aureus* pathogenicity and virulence.

Materials And Methods

Bacterial Strains and Growth Conditions

In this study, 42 clinical *S. aureus* isolates were collected from urology research center of Sina hospital, Iran. All isolates were confirmed as *S. aureus* according to phenotypic and biochemical method including Gram-stain, catalase, coagulase, DNase, and mannitol fermentation tests. All isolates were stored in Tryptic Soy Broth (TSB) (Merck, Germany) with 20% glycerol at -80°C for additional study.

Methicillin-resistant *Staphylococcus aureus* (MRSA) identification

For detection of MRSA isolates, bacterial suspensions of *S. aureus* isolates equivalent to 0.5 McFarland were prepared and cultured on Mueller-Hinton agar. Then, a cefoxitin disk (30µg; MAST Diagnostics, Merseyside, U.K) were placed on the plate and incubated overnight at 37 °C. The growth inhibition zones were analyzed by the Clinical and Laboratory Standards Institute (CLSI) guidelines [11]. As a quality control, *S. aureus* ATCC 25923 was used. Moreover, for the molecular recognition of MRSA, PCR assay for the *mecA* gene was done [12].

DNA extraction

S. aureus isolates were grown overnight at 37°C on Trypticase soy agar (TSA) (Merck; Germany). Then genomic DNA was isolated using the High Pure PCR Template Preparation kit (Roche, Germany)[13, 14]. Evaluation of quality and concentration of each extracted DNA was examined using NanoDrop (Thermo Fisher Scientific; USA).

Identification of type II TA systems

The whole genome of *S. aureus* MU50 was retrieved from the NCBI database to identify the type II TA genes in *S. aureus* isolates. Type II TA systems were determined by TADB database (Toxin-Antitoxin database) and specific primers were designed using OLIGO software V. 7.56 [15]. The presence of gene coding ClpP and type II TA system (*mazE/mazF*, *relE1/relB1*, *relE2/relB2*, *immA/irrA* and *omega/epsilon-zeta*) was evaluated by PCR. The PCR was performed in a DNA thermal cycler (Bio-Rad, USA) in a final volume of 25 µl. The PCR program comprised of an initial denaturation step at 94 °C for 4 min; 35 (94 °C for 35 s; annealing for 45 s (Table 1); with a final extension at 72 °C for 20 s) [15]. Finally, two MRSA and MSSA (Methicillin-sensitive *Staphylococcus aureus*) isolates were selected to evaluate the expression levels of mRNA of Type II TA and ClpP protease in thermal and oxidative stresses.

Heat and cold shock responses

Overnight cultures of MRSA and MSSA cells were diluted 1:100 in 50 ml fresh TSB medium and incubated at 37°C at 200 rpm. Cultures of *S. aureus* were grown to mid-log phase (optical density at 600 nm, 0.25). For induction of the cold shock and heat shock responses, were then incubated for an

additional 30 min with aeration at 10°C and 42°C, respectively. The control sample was placed in the incubator at 37 °C [16].

Growth curve and viability assessment

The Growth curve and the viability of MRSA and MSSA isolates were determined in the presence of 5mM and 10 mM H₂O₂. Overnight cultures were diluted 100 – fold in 50 ml TSB in 250 ml Erlenmeyer flasks. Cultures were placed in an incubator with shaking at 200 rpm at 37 °C until reaching an optical density of 0.4 at 600 nm (OD₆₀₀). Then, cultures were diluted 3-fold to reach an OD of 0.08-0.1 at 600 nm. These cultures were divided and H₂O₂ was added to the two concentration of 5 mM and 10 mM of H₂O₂. Following incubation of each concentration, 1 ml of cell was removed at 0, 1, 2, 3, 4 and 24h, then growth curves were assessed turbidimetrically. Moreover, in each time point, 1 ml of cells were washed twice with 0.9% sterile saline solution and serial dilution was prepared and plated onto TSA plates and incubated in 37 °C. Colony count was done after 24h incubation. Untreated bacterial cells were used as controls at different time intervals. All experiments were done independently at least three times.

RNA isolation and quantitative real-time reverse-transcriptase PCR (qRT-PCR)

The expression levels of gene coding ClpP protease and toxin of type II TA systems (*mazF*, *relE1*, *relE2* and *immA*) upon oxidative and thermal stresses were evaluated by qRT-PCR method. RNA extraction was performed using a high pure RNA isolation kit (Roche; Germany) 1 hour after adding H₂O₂ and 30 min after the heat and cold shock.

Bacteria were pelleted by centrifugation at 7000×g for 10 min, the supernatant was discarded and the pellet was resuspended in Tris-EDTA (TE) buffer (pH 8). Then 50 µg/ml lysozyme (Sigma-Aldrich; Germany) and 250 µg/ml lysostaphin (Sigma-Aldrich; Germany) were added followed by incubation at 37 °C for 10 min and 15 min, respectively[13]. RNA extraction was performed using a high pure RNA isolation kit (Roche; Germany) according to the manufacturer's instructions. The quantity and quality were evaluated by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and gel electrophoresis. DNA contamination was resolved with the addition of an extra DNase I (Thermo Fisher Scientific, USA) treatment. The absence of DNA contamination in RNA samples was confirmed by PCR amplification of the housekeeping 16S rRNA gene. Reverse transcription was performed using the FIREScript RT cDNA synthesis kit (Solis BioDyne; South Korea) according to the manufacturer's instructions. Finally, quantitative real-time PCR was carried out in a Rotor-Gene thermal cycler (Corbett 6000; Germany) using SYBR Green method (Amplicon; Denmark) by specific primers. A total volume of 20 µl reaction containing 2 µl of cDNA, 12.5 µl SYBR Green master mix, 3.5 µl nuclease-free water, and 1 µl of each primer (5 pmol) was performed according to the following program: an initial activation step at 94 °C for 10min, 45 cycles of denaturation at 95 °C for 30 s, and one cycle of 60 °C for 45 s. the 16s rRNA was used as an internal control to normalization of mRNA levels and fold changes. Expression was calculated by the $2^{-\Delta\Delta CT}$ method [8, 15].

Statistical analysis

Data obtained from growth curves and viability assessment were expressed as the mean of the three independent examinations. Data of the mRNA expression analysis were presented as means \pm standard error of three independent experiments. All the statistics were performed by Graph pad prism 8 (GraphPad Software, Inc). Student's t-test (for two groups) and analysis of variance (two-way ANOVA) were used.

Abbreviations

MRSA: Methicillin-resistant *Staphylococcus aureus*; MSSA: Methicillin-sensitive *Staphylococcus aureus*; CFU: Colonies forming units; TA: Toxin – antitoxin.

Declarations

Acknowledgements

Not applicable

Authors' contributions

SK and MRP designed the study; SK and RH performed the sample processing and the bioinformatics analysis; SK, BSK, NS contributed to the interpretation of the data; SK wrote the first draft of the manuscript and all authors read and contributed to the final version. All authors read and accepted the final manuscript.

Consent for publication

Not applicable

Funding

This study was supported by research committee of Tehran University of Medical Sciences, Tehran, Iran (Grant No. 40630). The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable

Author details

¹Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. ² Department of Microbiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran. ³ Department of Molecular Biology, Pasteur Institute of Iran, Tehran, Iran. ⁴ Urology Research Center, Tehran University of Medical Sciences, Tehran, Iran

References

1. Kim, W., et al., *Strategies against methicillin-resistant Staphylococcus aureus persists*. Future medicinal chemistry, 2018. **10**(7): p. 779-794.
2. Turlej, A., W. Hryniewicz, and J. Empel, *Staphylococcal cassette chromosome mec (Sccmec) classification and typing methods: an overview*. Pol J Microbiol, 2011. **60**(2): p. 95-103.
3. Kato, F., et al., *Deletion of mazF increases Staphylococcus aureus biofilm formation in an ica-dependent manner*. Pathogens and disease, 2017. **75**(5): p. ftx026.
4. Habib, G., Q. Zhu, and B. Sun, *Bioinformatics and Functional Assessment of Toxin-Antitoxin Systems in Staphylococcus aureus*. Toxins, 2018. **10**(11): p. 473.
5. Wang, Y., et al., *The antitoxin MqsA homologue in Pseudomonas fluorescens 2P24 has a rewired regulatory circuit through evolution*. Environmental microbiology, 2019. **21**(5): p. 1740-1756.
6. Bukowski, M., et al., *Identification of novel mazEF/pemIK family toxin-antitoxin loci and their distribution in the Staphylococcus genus*. Scientific reports, 2017. **7**(1): p. 13462.
7. Sierra, R., P. Viollier, and A. Renzoni, *Linking toxin-antitoxin systems with phenotypes: A Staphylococcus aureus viewpoint*. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 2019. **1862**(7): p. 742-751.
8. Narimisa, N., et al., *Effects of Sub-inhibitory Concentrations of Antibiotics and Oxidative stress on the expression of type II TA system genes in Klebsiella pneumoniae*. Journal of global antimicrobial resistance, 2019.

9. Xu, J., et al., *Identification of Three Type II Toxin-Antitoxin Systems in Streptococcus suis Serotype 2*. Toxins, 2018. **10**(11): p. 467.
10. Schuster, C.F., et al., *The MazEF toxin-antitoxin system alters the β -lactam susceptibility of Staphylococcus aureus*. PLoS One, 2015. **10**(5): p. e0126118.
11. Weinstein, M.P., *Performance standards for antimicrobial susceptibility testing*. 2019: Clinical and Laboratory Standards Institute.
12. Moghadam, S.O., et al., *Utilization of PFGE as a powerful discriminative tool for the investigation of genetic diversity among MRSA strains*. Iranian journal of public health, 2017. **46**(3): p. 351.
13. Maleki, D.T., et al., *Molecular analysis of accessory gene regulator functionality and virulence genes in Staphylococcus aureus derived from pediatric wound infections*. Infection, Genetics and Evolution, 2019.
14. Sadeghi, Y., et al., *Real-time PCR followed by high-resolution melting analysis—a new robust approach to evaluate SCC mec typing of methicillin-resistant Staphylococcus aureus*. Future microbiology, 2019. **14**(02): p. 155-164.
15. Kalani, B.S., et al., *Putative type II toxin-antitoxin systems in Listeria monocytogenes isolated from clinical, food, and animal samples in Iran*. Microbial pathogenesis, 2018. **122**: p. 19-24.
16. Anderson, K.L., et al., *Characterization of the Staphylococcus aureus heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover*. Journal of bacteriology, 2006. **188**(19): p. 6739-6756.
17. Mutschler, H., et al., *A novel mechanism of programmed cell death in bacteria by toxin-antitoxin systems corrupts peptidoglycan synthesis*. PLoS biology, 2011. **9**(3).
18. Janssen, B.D., F. Garza-Sánchez, and C.S. Hayes, *YoeB toxin is activated during thermal stress*. Microbiologyopen, 2015. **4**(4): p. 682-697.
19. Curtis, T.D., et al., *The influence of the toxin/antitoxin mazEF on growth and survival of Listeria monocytogenes under stress*. Toxins, 2017. **9**(1): p. 31.
20. Chan, W.T., et al., *The Streptococcus pneumoniae yefM-yoeB and relBE toxin-antitoxin operons participate in oxidative stress and biofilm formation*. Toxins, 2018. **10**(9): p. 378.
21. Behrooz, S.K., et al., *Study of MazEF, sam, and phd-doc putative toxin-antitoxin systems in Staphylococcus epidermidis*. Acta microbiologica et immunologica Hungarica, 2018. **65**(1): p. 81-91.
22. Gerdes, K., S.K. Christensen, and A. Løbner-Olesen, *Prokaryotic toxin-antitoxin stress response loci*. Nature Reviews Microbiology, 2005. **3**(5): p. 371-382.
23. Chan, W.T., D. Balsa, and M. Espinosa, *One cannot rule them all: Are bacterial toxins-antitoxins druggable?* FEMS microbiology reviews, 2015. **39**(4): p. 522-540.

Tables

Table 1. Type II toxin-antitoxin (TA) systems in *S. aureus* MU50

| TA-No | Toxin | Antitoxin | Family | Domain Pair ¹ |
|-------|--------------|--------------|--------------------|--------------------------|
| 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 |

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

Table 2. Characteristics of design primers used for both PCR and qRT-PCR studies.

| Gene | Sequence (5'-3') | TM (°C) | Amplicon size | Reference |
|-----------------|---------------------------|---------|---------------|------------|
| <i>mazF</i> | F AACAAAGGGGGAGTCAGACCT | 60 | 294 | This study |
| | R GAGCTACTGCATTCAGCCCTA | | | |
| <i>relE (1)</i> | F TACGTTTTTCGCCTCAAGCCT | 60 | 117 | This study |
| | R CCTTCCAATGCACCATTTCTGT | | | |
| <i>relE (2)</i> | F TTAACCATCAGCACAGGGTCG | 58 | 84 | This study |
| | R AATCATAATGTGACCATGCCGAT | | | |
| <i>Zeta</i> | F ACAGGACGAACAACAGACGTT | 60 | 177 | This study |
| | R TTGTTTTGGTGTGGCCCTGG | | | |
| <i>ImmA</i> | F GGGTCTCTATGACAATGGGGT | 60 | 98 | This study |
| | R TCAACTTGTGGTGGGCAAGT | | | |
| <i>clpP</i> | F GCGGTGAACGTGCATATGAT | 59 | 135 | This study |
| | R TCTCTGAGTCTTGCGCTTGT | | | |
| <i>16s rRNA</i> | F TGTTGCAAACCTGCCGTACT | 60 | 116 | This study |
| | R TTAAGCGTTCGCCCACTTCT | | | |

Figures

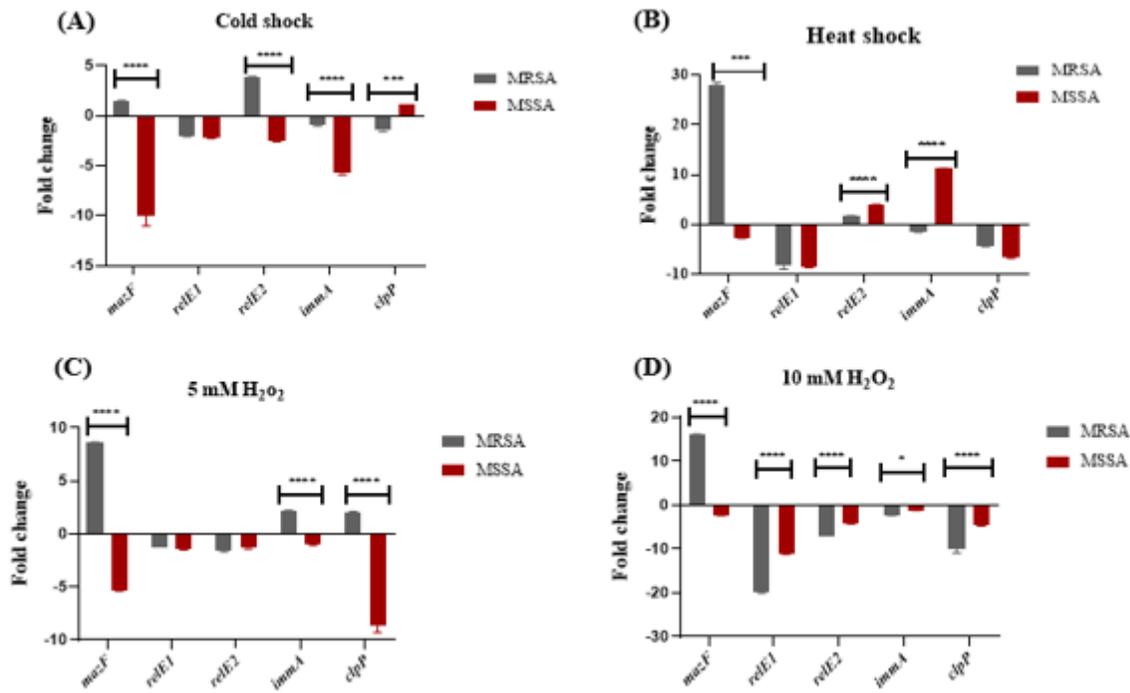


Figure 1

Analysis of relative expression level of the ClpP protease and type II TA system genes in the presence of 5mM and 10mM of H₂O₂ and thermal stress in MRSA and MSSA isolates. Graph data are indicated as the means±SD of three independent replicates. *P<0.05; **** P<0.0001 by Two-way ANOVA test for multiple comparisons.

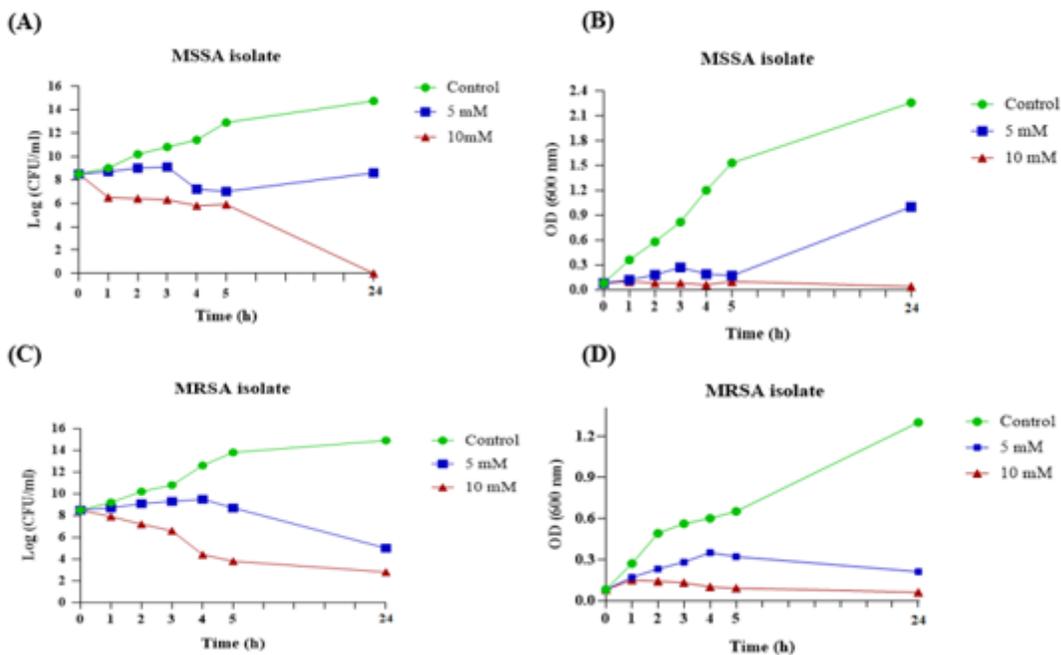


Figure 2

Effects of 5mM and 10mM of H₂O₂ on the survival of MRSA and MSSA isolates. (A and C) Growth curves (B and D) viability curves. Data are showed as the means±SD of three independent replicates.