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ABSTRACT

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BACTERIAL HUNGER GAMES: SMALL ALARMONE SYNTHETASES IN MRSA VS. MSSA

Introduction. *Staphylococcus aureus* is a significant global human pathogen associated with opportunistic infections. The stringent response in bacteria triggers the synthesis of alarmones (p(ppGpp)) upon encountering stress conditions like starvation, impacting the bacterial transcriptome. *S. aureus* synthesizes these alarmones using either the Rel enzyme (RelA/SpoT homolog) or the small alarmone synthetases RelP and RelQ.

Aim. This investigation aimed to (1) highlight the impact of starvation on biofilm intensity, cell count, and matrix composition (protein, polysaccharide, and DNA) in both MRSA and MSSA isolates and (2) analyze the expression levels of *relP* and *relQ* genes under normal and starved conditions in both MRSA and MSSA isolates.

Methods: *S. aureus* isolates were obtained from patients attending hospitals in Baghdad. Methicillin resistance was determined using the cefoxitin disc diffusion method. PCR confirmed the presence of *relP* and *relQ* genes in all isolates. Additionally, *16SrRNA* gene segments from 10 isolates were amplified for sequencing. Biofilm intensity, matrix composition, and cell count were measured for 10 isolates (5 MRSA and 5 MSSA) under normal and starvation conditions. Finally, *relP* and *relQ* gene expression was compared under both conditions.

Results: Methicillin resistance was detected in 94% of *S. aureus* isolates. *relP* and *relQ* genes were present in 100% and 98.7% of isolates, respectively.

Biofilm thickness and cell count significantly decreased ($P = 0.0020$) after starvation and with treatment involving proteinase K, DNase, and sodium periodate ($P < 0.0001$). relP and relQ genes displayed upregulation after starvation.

Conclusion: Starvation significantly reduced biofilm formation and altered its composition, suggesting the potential involvement of the

stringent response in biofilm regulation; both *relP* and *relQ* were upregulated in both MRSA and MSSA.

Keywords: biofilm, *s. aureus*, small alarmone synthetases, stringent response.

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ABBREVIATIONS

methicillin-resistant *S. aureus* – MRSA
methicillin-sensitive *S. aureus* – MSSA
extracellular polymeric substances – EPS
nucleotides guanosine tetra- and pentaphosphate – (p)ppGpp
tryptic soy broth – TSB
phosphate-buffered saline – PBS
optical density – OD
colony-forming units – CFU
polysaccharide intercellular adhesin – PIA

INTRODUCTION

The modified penicillin-binding protein (PBP) produced by MRSA strains is associated with a lowered affinity for the majority of semisynthetic penicillins, where the acquired *mecA* encodes the modified PBP [1]. The staphylococcal cassette chromosome mec is a mobile genetic element that carries this methicillin-resistant genetic component [2]. Therefore, the acquisition and insertion of these mobile genetic components into the chromosomes of susceptible strains is responsible for the rise of staphylococci strains that are resistant to methicillin. The medical community is facing difficulties in treating and managing staphylococcal infections as a result of this antibiotic resistance [3].

The term "biofilm" describes a population of structured bacterial community as well as the membrane-like extracellular matrix that is created when bacterial colonies adhere to one another and extracellular polymeric substances (EPS) such proteins, polysaccharides, and nucleic acids that are produced by the bacteria during their growth [4]. The biofilm is supported by cohesion and viscoelasticity through the interaction of EPS with bacterial aggregates [5]. Bacteria can therefore adhere to both biotic and abiotic surfaces. Thus, a major factor contributing to the development of chronic, persistent infection is the production of biofilm [6, 7]. Therefore, many scientists come to agree that bacterial biofilms mediate about 80% of chronic illnesses [8].

The biofilm that forms, which increases *S. aureus* resistance to antibiotics, is making it harder and harder to treat the accompanying infections [9]. Furthermore, the production of biofilms is thought to be a protected growth mode that allows bacteria to adapt to more hostile conditions [10]. In addition to sheltering bacterial cells from many environmental stresses such as intense heat, starvation, dehydration, and even antibacterial agents, the biofilm serves as a barrier to sustain a steady internal environment for bacterial cell activity [11].

S. aureus, along with many pathogens, processes sophisticated gene regulation systems to help survive in dynamic environments like the human host. Bacteria have adopted a global stress response called the stringent response to deal with a variety of advert environmental cues, such as temperature and pH changes, nutrient limitation, and environmental stressors [12]. This response is portrayed by the quick synthesis of the nucleotides guanosine tetra- and pentaphosphate, or (p)ppGpp, which adjusts many cellular processes that cause slower development and stress adaption [13]. The stringent response has been linked to *S. aureus* pathogenicity and its participation in chronic and persistent infections, according to many researchers [14, 15].

Three synthetase enzymes (Rel, RelP, and RelQ) are responsible for the synthesis of (p)ppGpp in *S. aureus* [16]. Rel synthesizes (p)ppGpp during amino acid depletion, causing significant transcriptional changes, including the downregulation of protein synthesis machinery and the upregulation of amino acid transport and metabolism genes [17]. On the other hand, RelP and RelQ are thought to respond to stresses such as cell-wall-targeting antibiotics and pH changes [18]. About one-third of the genome is expressed differently in proteobacteria as a result of (p)ppGpp's modulation of transcriptional modifications in these genes by binding

to two locations around the β′ subunit of the RNA polymerase enzyme [19]. Nevertheless, these interactions with the polymerase core enzyme are absent in *S. aureus* and other Firmicutes [20]. Rather, by active GTP depletion and inhibition of several proteins, including those involved in DNA replication, ribosome assembly, and translation, (p)ppGpp causes many alterations [21].

GTP levels decrease with the induction of (p)ppGpp synthesis as it serves as a substrate for pppGpp production. This leads to many cellular processes being significantly impacted, as this affects transcription from promoters that employ GTP as their starting nucleotide, such as promoters for rRNA production [22]. Furthermore, the reduction in GTP levels in Firmicutes permits the derepression of genes governed by the CodY transcriptional repressor. As a global regulator, CodY represses genes related to the production of amino acids, their absorption, and some virulence genes [23].

Objective: To examine the impact of starvation-induced stringent response on the expression levels of *relP* and *relQ* genes, as well as on the thickness of biofilm in locally isolated MRSA and MSSA strains.

MATERIALS AND METHODS

Microorganisms

Different specimens were collected from patients referred to different hospitals in Baghdad; these specimens encompassed anterior nares, wound swabs, burns, ear swabs, and sputum. All specimens were inoculated onto Mannitol Salt Agar plates and incubated at 37 ºC for 24 hours. Afterward, the colonies were subsequently subjected to conventional biochemical tests, including Catalase, Oxidase, Coagulase, Acetoin production, and Haemolysin Production Test to identify *Staphylococcus aureus* isolates [24]. Moreover, the Cefoxitin disc diffusion method was employed for the identification of methicillin-resistant isolates according to CLSI [25].

Polymerase Chain Reaction

Bacterial genomic DNA was extracted using Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan), and all amplifications were carried out using AccuPower® PCR PreMix (Bioneer, USA) and Gradient master cycler (Eppendorf, Germany). All of the primers used for PCR are listed in Table 1

Table 1 – Primers used in this study

Target gene	Primer name	Sequence 5' ⟶ 3'	Amplicon size (bp)	Reference
16SrRNA	Staph 756F	AACTCTGTTATTAGGGAAGAACA	756	[50]
	Staph 750R	CCAACCTTCCTCCGGTTTGTCACC		
relP	*relP* -F	AAGCAAGCGGTTGATGAGTT	112 bp	[51]
	relP -R	CGATTGGTTTAACACGACCAG		
relQ	*relQ* -F	TATGGAGCGACGTGTGAAAG	113 bp	[51]
	relQ -R	GAATTCCGGCAATATCCAGT		
rpoB	*rpoB* -F	CAGCTGACGAAGAAGATAGCTATGT	82 bp	[51]
	rpoB -R	ACTTCATCATCCATGAAACGACCAT		

To confirm the identity of *S. aureus* isolates, amplification of 756bp 16srRNA gene segment using genus-specific primers was carried out on 5 MSSA strains (S30, S35, S41, S44 and S72) and 5 MRSA strains (S27, S34, S40, S70 and S80); The reaction protocol was as followed: initial denaturation at 95 ºC for 10 min followed by 40 cycles of 95 ºC 30 sec, 58 ºC 1 min, and 72 ºC 30 sec; following that 5 min at 72 ºC for final extension; the sequences of the PCR products were then obtained using Sanger method, the obtained sequences were then aligned with gene sequences from NCBI using BLAST analysis. Moreover, all *S. aureus* isolates were screened for the presence of the genes encoding for the small alarmone synthases relP and relQ to amplify 112 bp and 113 bp segments of the *relP* and *relQ* genes, respectively. The reaction protocol was as follows: initial denaturation at 94 ºC for 2 min followed by 35 cycles of 94 ºC 60 sec, 55 ºC 30 sec, and 72 ºC 90 sec; following that 4 min at 70 ºC for final extension.

Biofilm Formation Assay

In order to investigate the influence of starvation-induced stringent response on biofilm formation, 5 MSSA (S30, S35, S41, SA44, and S72) and 5 MRSA (S27, S34, S40, S70, and SA80) *S. aureus* isolates were chosen in this study.

Initially, the biofilm was quantified using the same method as stated in a prior study [26]. Concisely, 200 µl of an overnight Tryptic Soy Broth (TSB) supplemented with 1% glucose (bacterial concentration adjusted to match McFarland standard no. 0.5) were added to the

wells of sterile 96-well polystyrene microplates. The plates were then covered and incubated aerobically at 37 ºC for 24 hours. Every individual isolate was tested three times. Control wells were established by using bacteria-free TSB. Following decantation, the wells were washed three times using 200 µl of sterile phosphate-buffered saline (PBS); any bacteria that remained adhered were fixed for 15 minutes using 200 µl of methanol. Following air drying, the wells were treated with 200 µl of a 0.1% crystal violet solution for 15 minutes at room temperature. The stain was then washed away; thereafter, the plates were dried. Afterward, the stained attached cells were dissolved again using 200 µl of 33% glacial acetic acid for 15 minutes. Ultimately, the optical density (OD) of each well was measured at 600 nm using a microplate reader (Biotek, UK). The cut-off value (ODc) was determined by calculating the average optical density (OD) of the control wells and adding three times the standard deviation. The isolates were subsequently classified as Non–producer (OD values less than or equal to ODc), weak producer (OD values greater than ODc but less than or equal to 2 times ODc), moderate producer (OD values greater than 2 times ODc but less than or equal to 4 times ODc), or strong producer (OD values greater than 4 times ODc).

Furthermore, to assess the biofilm under nutrient limitation, the same procedure was adopted with only one exception: the tryptic soy broth + glucose was diluted with distilled water up to 100-fold (1:100); the obtained OD values for each isolate were then compared to the corresponding values that were observed in the previous experiment.

The percentage of biofilm inhibition after starvation was calculated according to the following formula:

Percentage of Inhibition = [(OD Normal – OD Starved)/OD Normal] * 100 [27].

Effect of starvation on bacterial count in the biofilm

The number of bacterial cells within the biofilm was determined under normal and starvation conditions using the viable plate count method [28, 29].

The media was extracted from each well following the designated incubation periods. The developed biofilm was rinsed once with 200 µl of PBS. Subsequently, a volume of 100 µl of PBS solution was pipetted into the wells containing the biofilm, followed by the agitation of the biofilm cells using pipetting. The biofilm that was suspended was moved to a fresh microplate with a flat bottom that had 96 wells. Then, dilutions were made in PBS, with each dilution being ten times less concentrated than the previous one. Approximately 100 µl from each dilution was dispensed onto Tryptic soy agar. Colony-forming units (CFU) were counted after 24 hours of incubation at a temperature of 37 ºC. The experiment was conducted with three repetitions.

Biofilm composition

The biofilm composition (protein, polysaccharide, and/or DNA) under both normal and nutrient limitation conditions was assessed as follows:

Biofilms were washed twice with PBS and treated with 1 mg/ml proteinase K (AppliChem, 37 ºC, 4 h), 0.1 mg/ml DNase (Sigma-Aldrich, 37 ºC, 4 h) or with 40 mM sodium periodate ($NaIO_4$) (24 h, 4 ºC). The biofilms were then washed twice with PBS, dried, and stained as described above [30, 31].

Gene expression

The levels of *relP* and *relQ* gene expression were assessed for the previously selected isolates (MRSA and MSSA) under normal and starvation conditions using the primers listed in Table 1; the *rpoB* gene was used as a housekeeping gene.

RNA was extracted from biofilm cells (grown under both conditions) using Genezol Reagent according to the manufacturer's instructions (Geneaid, Taiwan). The concentration of the extracted RNA was determined using a nanodrop instrument.

The extracted RNA and primers were combined with a qPCR master mix (New England Biolabs, USA) and were vortexed to ensure homogeneous contents, resulting in a qPCR mixture with a final volume of 20 µl. Ten microliters of master mix and 0.8 microliters of each primer were used in the reactants, while the Rt volume was 1µl. Moreover, about 50 mg of RNA was added, and then the volume was completed to 20 µl using nuclease-free water. The protocol is illustrated in Table 2.

Table 2 – RT- qPCR protocol

Cycle Step	Temperature	Time	Cycles
Reverse Transcription	55 °C	10 minutes	1
Initial Denaturation	95 °C	1 minute	1
Denaturation	95 °C	10 seconds	40-45
Extension	60 °C	30 seconds	
Melt Curve	60-95 °C	various	1

A melting curve was obtained with temperatures ranging from 95 ºC to 60 ºC with a 0.5 ºC C increment every 15 seconds.

Statistical analysis

Biofilm data (before and after starvation) were analyzed using the Shapiro-Wilk test to determine their normality; then, using Wilcoxon matched-pairs signed rank test to determine the influence of starvation on biofilm thickness; the data from biofilm cell count were also analyzed using Wilcoxon matched-pairs signed rank test to assess the effect of starvation on biofilm cell count.

The data obtained from the biofilm composition experiment were analyzed first using an ANOVA test to determine the difference between each treatment on biofilm thickness under each condition, followed by a Wilcoxon matched-pairs signed rank test to determine the difference in each treatment before and after starvation.

RESULTS

Microorganisms

About 82 *S. aureus* isolates were identified using conventional biochemical tests. Among these, 77 (94%) were resistant to Cefoxitin (MRSA), while the rest (5 isolates) were regarded as MSSA.

PCR study

S. aureus 16S rRNA

PCR Amplification of the *16srRNA* gene yielded a product with approximately 756bp (Figure 1); the product obtained sequences were aligned with reference *S. aureus 16S rRNA* gene using Blast analysis, which yielded similarity ratios between 99-100%. The sequences were registered in the GenBank with accession numbers: PP087404, PP100137, PP100138, PP100139, PP100140, PP100141, PP100142, PP100143, PP100144, PP100145.

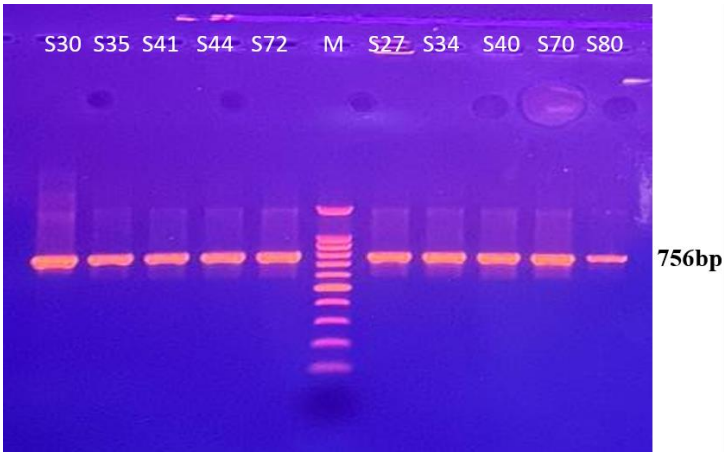

Figure 1 – Visualization of 16srRNA gene by 1.5% agarose gel analysis. The shown bands are representative of PCR products (756bp) amplified from S. aureus isolates (S30, S35, S41, S44, S72, S27, S34, S40, S70 and S80), and lane M represents the 100 bp DNA ladder

relP* and *relQ

The findings from this experiment revealed that the *relP* gene was present in all the 82 tested isolates of *S. aureus*, while the *relQ* gene was present in 81 (98.7 %) out of the 82 isolates that were included in the study, as represented in Figures 2 and 3.

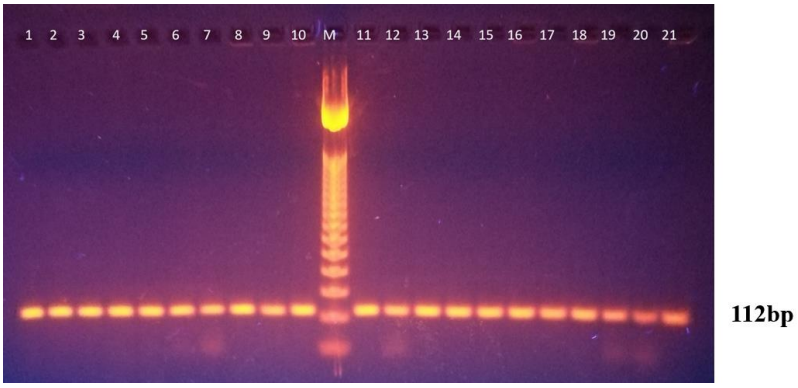

Figure 2 – Visualization of relP gene by 1.5% agarose gel analysis. The shown bands are representative of PCR products (112bp) amplified from S. aureus isolates (lanes 1 - 21), and lane M represents the 100 bp DNA ladder

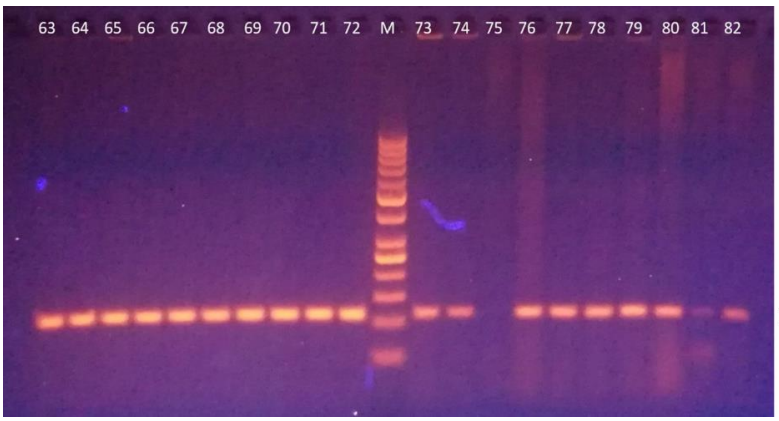

Figure 3 – Visualization of relQ gene by 1.5% agarose gel analysis. The shown bands are representative of PCR products (113bp) amplified from S. aureus isolates (lanes 63 - 77), and lane M represents the 100 bp DNA ladder

Biofilm

The results summarized in Table 3 revealed that under normal growth conditions, 70 % of the tested isolates developed strong biofilm, where all MRSA strains were among the strong biofilm-producing isolates; moreover, 30% were moderate biofilm producers.

Furthermore, under nutrient limitation, 30% of the tested isolates were moderate biofilm producers, while the rest (70%) were weak in their biofilm-forming capabilities.

It also can be noted that the biofilm decreased significantly (P value = 0.0020) in all the tested isolates regardless of their type (MRSA or MSSA) (Figure 4).

The result also demonstrates that the mean percentage of inhibition for MRSA strains was (73.64%), while it was (56.65%) for MSSA strains.

Biofilm composition

The results summarized in Table 4 revealed that the biofilm thickness decreased significantly after treatment with DNase (P<0.0001). Similarly, it decreased in biofilms under starvation as well (P<0.0001); moreover, there was a significant difference (P = 0.0020) in the effect of DNase enzyme before and after starvation (Figure 5-A).

Regarding proteinase K treatment, the result also demonstrated a significant decrease in the biofilm after treatment with proteinase K before starvation (P<0.0001) and also after starvation (P<0.0001); furthermore, there was a significant difference (P = 0.0020) in the effect of proteinase K before and after starvation (Figure 5-B).

Concerning Na periodate, the result showed that the biofilm intensity decreased significantly after treatment with Na periodate before (P<0.0001) and after starvation (P<0.0001); additionally, there was a significant difference (P = 0.0020) in the effect of Na periodate before and after starvation (Figure 5-C).

Table 3 – Biofilm forming capacity of bacterial isolates

Isolate	Mean OD		Percentage of inhibition %
	Normal	Starvation	
S30	0.436	0.150	65.60
S35	0.365	0.221	39.45
S41	0.679	0.268	60.53
S44	0.388	0.241	37.89
S72	0.851	0.172	79.79
S27	0.868	0.152	82.49
S34	0.754	0.171	77.32
S40	0.534	0.220	58.80
S70	0.566	0.232	59.01
S80	1.233	0.116	90.59
C	0.099		

Note. S1-S10: S. aureus isolates 1-10; C: Control; cut off value: 0.111

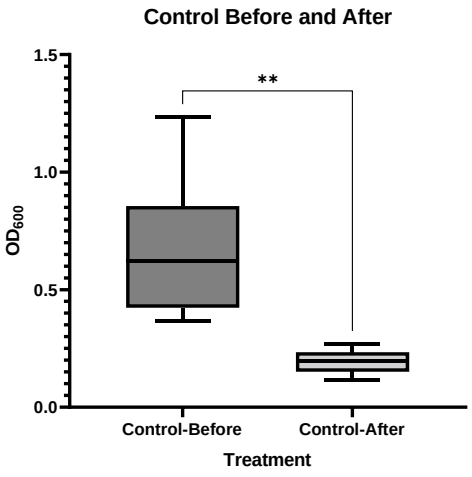

Figure 4 – Biofilm before and after starvation

Table 4 – Biofilm composition for normal and starved cells

Isolate code	Control	DNase	Proteinase K	Na periodate
Before starvation				
S30	0.436	0.226	0.273	0.194
S35	0.365	0.311	0.331	0.148
S41	0.679	0.663	0.548	0.266
S44	0.388	0.278	0.336	0.185
S72	0.851	0.686	0.595	0.342
S27	0.868	0.540	0.262	0.339
S34	0.754	0.503	0.633	0.232
S40	0.534	0.347	0.384	0.168
S70	0.566	0.362	0.400	0.214
S80	1.233	0.988	0.647	0.342
After starvation				
S30	0.150	0.130	0.149	0.114
S35	0.221	0.178	0.176	0.141
S41	0.268	0.207	0.247	0.153
S44	0.241	0.203	0.215	0.116
S72	0.172	0.151	0.156	0.095
S27	0.152	0.107	0.139	0.096
S34	0.171	0.145	0.165	0.096
S40	0.220	0.182	0.203	0.102
S70	0.232	0.187	0.215	0.118
S10	0.116	0.083	0.096	0.096

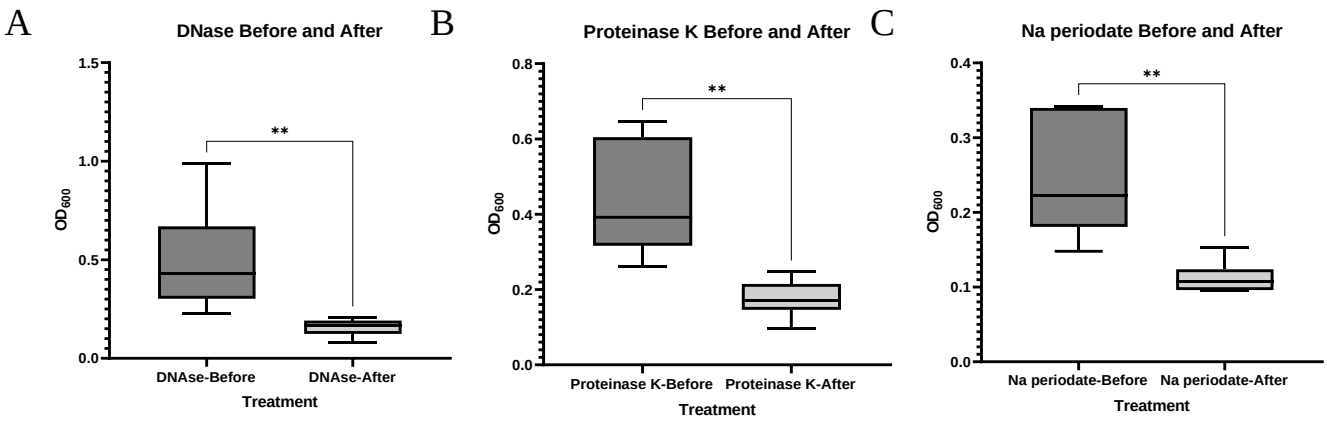

Figure 5 – Biofilm treatment with DNase (A), Proteinase k (B) and Na periodate (C) before and after starvation

Likewise, statistical analysis using ANOVA revealed that there was a significant difference across the activity of each treatment before starvation (P<0.0001) and also after starvation (P<0.0001) (Figure 6).

Moreover, when calculating the percentage of biofilm inhibition after each treatment under normal culture conditions, the results summarized in Table 5 revealed that Na periodate led to a much higher inhibition percentage (62.10%) followed by proteinase K (30.01%) than DNase (27.46%).

Similarly, under starved conditions, a higher percentage of biofilm inhibition was achieved by Na periodate treatment (40.05%). However, this time the inhibition was higher in DNase treatment (19.4%) when compared with proteinase K treatment (9.4%)

Biofilm cell count

In order to determine the effect of stringent response on the number of bacterial cells within biofilms, the biofilm cells were resuspended and plated on TSA agar plates.

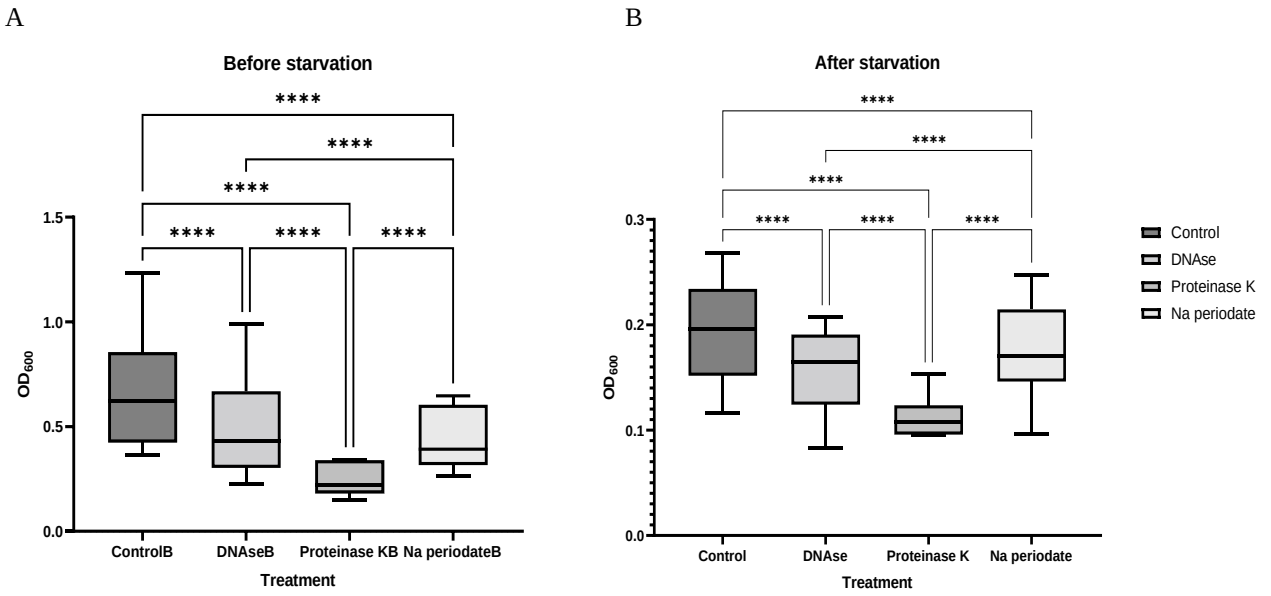

Figure 6 – DNase, Proteinase k and Na periodate treatment of biofilm formed under normal (A) and starved (B) conditions

Table 5 – Effect of starvation on biofilm composition

	Biofilm inhibition before starvation (%)			**Biofilm inhibition after starvation (%)**		
Id	DNase	Na periodate	Proteinase K	DNase	Na periodate	Proteinase K
S30	48.05	55.39	37.26	13.14	23.83	0.67
S35	14.72	59.41	9.41	19.73	36.14	20.33
S41	2.31	60.81	19.35	22.54	42.84	7.47
S44	28.20	52.36	13.41	16.02	51.80	10.77
S72	19.39	59.85	30.16	12.04	44.47	9.13
S27	37.83	60.94	69.74	29.54	36.98	8.75
S34	33.30	69.17	16.05	15.37	44.16	3.70
S40	34.98	68.46	27.92	17.25	53.86	8.17
S70	35.98	62.25	29.27	19.42	49.21	7.48
S80	19.89	72.30	47.54	28.94	17.19	17.48
Mean	27.46 ±13.39	62.10 ±6.23	30.01 ±18.11	19.40 ±6.05	40.05 ±11.86	9.40 ±5.52

The results summarized in Figure (7) clearly demonstrate a significant drop (P value = 0.002) in the number of cells within the biofilm under the influence of starvation.

Gene expression

To study the effect of stringent response initiated by nutrient limitation on the expression of *relP* and *relQ* genes, the RNA was extracted from the established biofilm under normal and starved conditions to measure the expression of the genes using qRT-PCR.

Melting curve analysis revealed a single distinct peak representing a pure single discrete amplicon [32].

The results summarized in Figure (8) demonstrate that the level of expression for all of the aforementioned genes was upregulated under nutrient limitation when compared with normal growth conditions. Furthermore, no difference was observed in the gene expression profile between MRSA and MSSA strains.

DISCUSSION

It is becoming evident that Methicillin resistance is increasing in prevalence among *S. aureus* strains; such increase of Methicillin resistance phenotype in Iraq can be attributed to many reasons, including a destroyed healthcare infrastructure, inappropriate microbial therapies, limited resources, high heavy metal contamination in humans and the environment and lack of Wash [33]; the results in this study demonstrated a high prevalence of Methicillin resistance which came in

agreement with our previous works where a high prevalence of MRSA was detected among local isolates of *S. aureus* [34, 35].

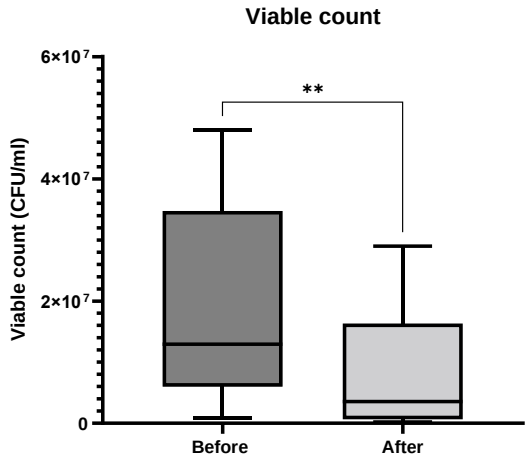

Figure 7 – Biofilm cell count under normal vs starvation conditions

Firmicutes, including *S. aureus*, have one bifunctional RSH enzyme and one or two SAS enzymes, namely RelP and RelQ [36]. *S. aureus* produces (pp)pGpp 'alarmones' through the joint actions of the bifunctional long-Rel protein (Sa-Rel) and two SAS proteins: Sa-RelP and Sa-RelQ [37]. Although Sa-Rel is the primary producer of (p)ppGpp in *S. aureus* cells when the stringent response is activated [38], the results revealed that genes encoding relP and relQ are found in almost all of the tested isolates. Many studies have shown that deleting relP and relQ renders the rel gene unnecessary, as it prevents the synthesis of (pp)pGpp in the cell. Mutant strains that lack both relP and relQ have diminished survival capabilities when exposed to antibiotic drugs that cause cell-envelope 'stresses,' such as ampicillin and vancomycin [39].

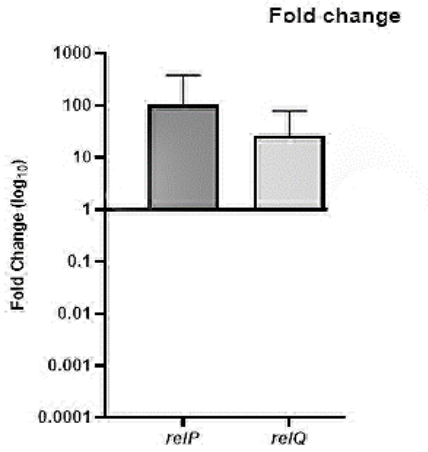

Figure 8 – Fold change in gene expression

To understand the influence of starvation-induced stringent response on biofilm, *S. aureus* biofilms were allowed to be formed in normal and starved conditions; this study demonstrated a significant reduction in the thickness of the biofilm layer in both MRSA and MSSA isolates. The biofilm composition under normal and starved conditions was also evaluated, and the results disagreed with Salzer et al. [31], who studied the biofilm composition of *S. aureus* strain HG001 under normal conditions and the influence of the stringent response induced under cell wall stress conditions by vancomycin treatment and found that under all conditions, the biofilm was almost not affected by Sodium periodate treatment and that the dominant composition of the biofilm was protein and DNA. However, our results agreed with the same study in the context that the biofilm composition was not affected by the stringent response. The results agreed with Seidl et al. [30], who found that the biofilm of *S. aureus* was sensitive to sodium periodate, proteinase K, and DNase I treatments, illustrating the importance of Polysaccharide intercellular adhesin (PIA), proteins and genomic DNA respectively as important structural components of *S. aureus* strain SA113 biofilm matrix. The decrease in biofilm after treatment with the DNase enzyme signifies the importance of DNA as a major composition of the biofilm matrix. It seems that eDNA has an important role in the development of the biofilm matrix in many bacterial and fungal species [40]. This component holds physicochemical characteristics that are well-suited for fulfilling the structural and functional needs of microbial cells to survive in various environments, such as the challenging niches within the human body [41]. Likewise, the decrease in biofilm thickness after proteinase k treatment in our study highlights the significance of proteins as important structural components in the biofilm matrix. Fibronectin-binding proteins and biofilm-associated proteins are present in the EPS matrix of Staphylococcal biofilms [42]. The research conducted by Houston et al. [43] revealed that the aforementioned proteins play a crucial role in the formation of *S. aureus* biofilms. This process is facilitated by the main autolysin, as well as the regulation of agr and sigB. Nevertheless, the accumulation-associated protein has a crucial role in the formation of biofilms in *Staphylococcus epidermidis*, which is reliant on the production of PIA [44]. Additionally, it comes as no surprise that treatment with Na periodate led to a significant decrease in the biofilm since it has long been recognized that polysaccharides are essential constituents of Staphylococcal biofilms. PIA is widely recognized as the primary exopolysaccharide constituent of the staphylococcal biofilm matrix. PIA has long been deemed essential for staphylococcal biofilms. However, strains that rely on PIA-independent biofilm

production tend to produce weaker and less stable biofilms compared to those that employ PIA [45]. In addition, the synthesis of PIA leads to the formation of dense and rough colonies, in contrast to the smooth colonies formed by PIA-negative biofilm-forming strains [46, 47].

To understand the decrease in the biofilm thickness under starvation, the biofilm cell count was measured under normal and starved conditions; the results indicated a decrease in the cell count, which can explain the decrease in the biofilm thickness. Modified cellular proliferation is arguably one of the most crucial elements of bacterial viability and adjustment to novel habitats. The stringent response involves the utilization of (p)ppGpp to reorganize many cellular activities, resulting in a decrease in growth [48].

Weaver et al. [49] conducted a study on *Escherichia coli* (p)ppGpp-mutant strains and found that these strains do not have growth rate control. This emphasizes the significant role of (p)ppGpp in regulating growth rate by inhibiting various cellular processes, such as DNA replication, ribosome synthesis, and translation.

The level of expression for relP and relQ genes was upregulated under starvation, while no difference was observed in the gene expression profile between MRSA and MSSA strains. However, in isolates S30, S35, and S41, the upregulation in gene expression levels was much higher when compared with other isolates, and since the aforementioned isolates are all methicillin-sensitive, their susceptibility to methicillin and hence the lack of *mecA* gene and retaining the original structure of penicillin-binding proteins in their cell wall could be the reason why the starvation caused their gene expression levels to be much higher when compared with other isolates. On the other hand, concerning the remaining isolates, since the levels of gene expression did not increase considerably, other genes could be involved under nutrient limitation, such as the gene coding for the bifunctional enzyme Rel.

Salzer et al. [31] conducted an experiment to investigate the production of biofilm by *S. aureus* in the presence of subinhibitory levels of vancomycin, which induce the stringent response due to cell wall stress situations. At a concentration of vancomycin that is below the level that inhibits growth, researchers discovered that the relPQ double mutant (with non-functioning relP and relQ enzymes) and the (p)ppGpp0 mutant (with non-functioning RelP, RelQ, and RSH enzymes) exhibited a substantial decrease in biofilm formation compared to the wild type and the relsyn mutant (with non-functioning RSH). The wild type developed a nearly homogeneous and dense biofilm layer during vancomycin treatment. Conversely, the relPQ mutant and the (p)ppGpp0 strain exhibited a substantial reduction in biofilm production.

The synthesis of (p)ppGpp is anticipated to take place via the activity of relP and relQ enzymes upon activation; (p)ppGpp has the ability to promote biofilm formation. In general, the molecular technique is applied in different scopes of medical microbiology [52-56].

CONCLUSIONS

Nearly all isolates of *S. aureus* harbored the genes encoding for the small alarmone synthetases relP and relQ, the function of which is crucial for the proper functioning of the stringent response that is driven by starvation regardless of their Methicillin resistance status; moreover, starvation *per se* resulted in a significant decrease in the biofilm thickness along with a sharp decrease in the number of bacterial cells within the biofilm.

AUTHOR CONTRIBUTIONS

Both of the authors, Ali A. Mussa and Jabbar Fahad Al-Mathkhury, have equal contributions in all aspects of this study, including study Conceptualization, data curation, analysis and interpretation of results, draft manuscript preparation ending with revisions, and editing of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Utsui Y, Yokota T. Role of an altered penicillin-binding protein in methicillin-and cephem-resistant Staphylococcus aureus. Antimicrobial agents and chemotherapy. 1985;28(3):397-403. https://doi.org/10.1128/aac.28.3.397
2. Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrobial agents and chemotherapy.

2000;44(6):1549-55. https://doi.org/10.1128/aac.44.6.1549-1555.2000
3. Mussa AA, Al-Mathkhury HJF. Incidence of Ciprofloxacin-Resistant of Methicillin Resistant Staphylococcus aureus isolated from Iraqi patients. Iraqi Journal of Science. 2018:1225-30. http://dx.doi.org/10.24996/ijs.2018.59.3A.10
4. Karygianni L, Ren Z, Koo H, Thurnheer T. Biofilm matrixome: extracellular components in structured microbial communities. Trends in microbiology. 2020;28(8):668-81. https://doi.org/10.1016/j.tim.2020.03.016
5. Di Martino P. Extracellular polymeric substances, a key element in understanding biofilm phenotype. AIMS microbiology. 2018;4(2):274. https://doi.org/10.3934%2Fmicrobiol.2018.2.274
6. Guilhen C, Forestier C, Balestrino D. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. Molecular microbiology. 2017;105(2):188-210. https://doi.org/10.1111/mmi.13698
7. Bhattacharya M, Wozniak DJ, Stoodley P, Hall-Stoodley L. Prevention and treatment of Staphylococcus aureus biofilms. Expert review of anti-infective therapy. 2015;13(12):1499-516. https://doi.org/10.1586/14787210.2015.1100533
8. Jamal M, Ahmad W, Andleeb S, Jalil F, Imran M, Nawaz MA, et al. Bacterial biofilm and associated infections. Journal of the chinese medical association. 2018;81(1):7-11. https://doi.org/10.1016/j.jcma.2017.07.012
9. Lee J, Zilm PS, Kidd SP. Novel research models for Staphylococcus aureus small colony variants (SCV) development: co-pathogenesis and growth rate. Frontiers in Microbiology. 2020;11:321. https://doi.org/10.3389/fmicb.2020.00321
10. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nature reviews microbiology. 2004;2(2):95-108. https://doi.org/10.1038/nrmicro821
11. Guo H, Tong Y, Cheng J, Abbas Z, Li Z, Wang J, et al. Biofilm and small colony variants—an update on staphylococcus aureus strategies toward drug resistance. International journal of molecular sciences. 2022;23(3):1241. https://doi.org/10.3390/ijms23031241
12. Irving SE, Choudhury NR, Corrigan RM. The stringent response and physiological roles of (pp) pGpp in bacteria. Nature Reviews Microbiology. 2021;19(4):256-71. https://doi.org/10.1038/s41579-020-00470-y
13. Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. Recent functional insights into the role of (p) ppGpp in bacterial physiology. Nature Reviews Microbiology. 2015;13(5):298-309. https://doi.org/10.1038/nrmicro3448
14. Deventer AT, Bryson D, Shortill M, Boraston AB, Hobbs JK. Molecular characterization of clinical rel mutations and consequences for resistance expression and fitness in Staphylococcus aureus. Antimicrobial Agents and Chemotherapy. 2022;66(12):e00938-22. https://doi.org/10.1128/aac.00938-22
15. Aedo S, Tomasz A. Role of the stringent stress response in the antibiotic resistance phenotype of methicillin-resistant Staphylococcus aureus. Antimicrobial agents and chemotherapy. 2016;60(4):2311-7. https://doi.org/10.1128/aac.02697-15
16. Geiger T, Goerke C, Fritz M, Schäfer T, Ohlsen K, Liebeke M, et al. Role of the (p) ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of Staphylococcus aureus. Infection and immunity. 2010;78(5):1873-83. https://doi.org/10.1128/iai.01439-09
17. Geiger T, Francois P, Liebeke M, Fraunholz M, Goerke C, Krismer B, et al. The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. Plos pathogens. 2012;8(11):e1003016. https://doi.org/10.1371/journal.ppat.1003016
18. Irving SE, Corrigan RM. Triggering the stringent response: signals responsible for activating (p) ppGpp synthesis in bacteria. Microbiology. 2018;164(3):268-76. https://doi.org/10.1099/mic.0.000621
19. Sanchez-Vazquez P, Dewey CN, Kitten N, Ross W, Gourse RL. Genome-wide effects on Escherichia coli transcription from ppGpp binding to its two sites on RNA polymerase. Proceedings of the National Academy of Sciences. 2019;116(17):8310-9. https://doi.org/10.1073/pnas.1819682116
20. Liu K, Bittner AN, Wang JD. Diversity in (p) ppGpp metabolism and effectors. Current opinion in microbiology. 2015;24:72-9. https://doi.org/10.1016%2Fj.mib.2015.01.012
21. Bennison DJ, Irving SE, Corrigan RM. The impact of the stringent response on TRAFAC GTPases and prokaryotic ribosome assembly. Cells. 2019;8(11):1313. https://doi.org/10.3390%2Fcells8111313
22. Kästle B, Geiger T, Gratani FL, Reisinger R, Goerke C, Borisova M, et al. rRNA regulation during growth and under stringent conditions in S taphylococcus aureus. Environmental microbiology. 2015;17(11):4394-405. https://doi.org/10.1111/1462-2920.12867
23. Majerczyk CD, Dunman PM, Luong TT, Lee CY, Sadykov MR, Somerville GA, et al. Direct targets of CodY in Staphylococcus aureus. Journal of bacteriology. 2010;192(11):2861-77. https://doi.org/10.1128%2FJB.00220-10
24. Tille PM. Bailey & Scott's Diagnostic Microbiology - E-Book: Elsevier Health Sciences; 2021.
25. CLSI. Performance Standards for Antimicrobial Susceptibility Testing2023.
26. Nakao R, Ramstedt M, Wai SN, Uhlin BE. Enhanced biofilm formation by Escherichia coli LPS mutants defective in Hep biosynthesis. PloS one. 2012;7(12):e51241. https://doi.org/10.1371/journal.pone.0051241

27. Shinde S, Lee LH, Chu T. Inhibition of biofilm formation by the synergistic action of EGCG-S and antibiotics. Antibiotics. 2021;10(2):102. https://doi.org/10.3390/antibiotics10020102
28. Cruz CD, Shah S, Tammela P. Defining conditions for biofilm inhibition and eradication assays for Gram-positive clinical reference strains. BMC microbiology. 2018;18:1-9. https://doi.org/10.1186/s12866-018-1321-6
29. Morin V, Dawson A, Nandi B, Subacz K, Fleming E. Introductory Medical Microbiology Ancillary Materials (2021 Update). 2021.
30. Seidl K, Goerke C, Wolz C, Mack D, Berger-Bächi B, Bischoff M. Staphylococcus aureus CcpA affects biofilm formation. Infection and immunity. 2008;76(5):2044-50. https://doi.org/10.1128%2FIAI.00035-08
31. Salzer A, Keinhörster D, Kästle C, Kästle B, Wolz C. Small alarmone synthetases RelP and RelQ of Staphylococcus aureus are involved in biofilm formation and maintenance under cell wall stress conditions. Frontiers in Microbiology. 2020;11:575882. https://doi.org/10.3389/fmicb.2020.575882
32. Scientific TF. Real-Time PCR Handbook.(2016). Dostupno na. 2017.
33. Abou Fayad A, Rizk A, El Sayed S, Kaddoura M, Jawad NK, Al-Attar A, et al. Antimicrobial resistance and the Iraq wars: armed conflict as an underinvestigated pathway with growing significance. BMJ Global Health. 2023;7(Suppl 8):e010863. https://doi.org/10.1136/bmjgh-2022-010863
34. Jaddoa N, Al-Mathkhury HJF. Biofilm shows independency form hemolysin genes arsenal in methicillinresistant Staphylococcus aureus. Iraqi Journal of Science. 2018;59(4C):2184-94. http://dx.doi.org/10.24996/ijs.2018.59.4C.5
35. Ibraheem H, Al-Mathkhury HJF. pvl-carried methicillin resistant Staphylococcus aureus isolated from hospitalized patients in Baghdad, Iraq. Iraqi Journal of Science. 2018;59(4):1967-72.
36. Horvatek P, Salzer A, Hanna AMF, Gratani FL, Keinhörster D, Korn N, et al. Inducible expression of (pp) pGpp synthetases in Staphylococcus aureus is associated with activation of stress response genes. PLoS Genetics. 2020;16(12):e1009282. https://doi.org/10.1371/journal.pgen.1009282
37. Gratani FL, Horvatek P, Geiger T, Borisova M, Mayer C, Grin I, et al. Regulation of the opposing (p) ppGpp synthetase and hydrolase activities in a bifunctional RelA/SpoT homologue from Staphylococcus aureus. PLoS genetics. 2018;14(7):e1007514. https://doi.org/10.1371/journal.pgen.1007514
38. Mwangi MM, Kim C, Chung M, Tsai J, Vijayadamodar G, Benitez M, et al. Whole-genome sequencing reveals a link between β-lactam resistance and synthetases of the alarmone (p) ppGpp in Staphylococcus aureus. Microbial Drug Resistance. 2013;19(3):153-9. https://doi.org/10.1089%2Fmdr.2013.0053
39. Geiger T, Kästle B, Gratani FL, Goerke C, Wolz C. Two small (p) ppGpp synthases in Staphylococcus aureus mediate tolerance against cell envelope stress conditions. Journal of bacteriology. 2014;196(4):894-902. https://doi.org/10.1128/jb.01201-13
40. Campoccia D, Montanaro L, Arciola CR. Extracellular DNA (eDNA). A major ubiquitous element of the bacterial biofilm architecture. International Journal of Molecular Sciences. 2021;22(16):9100. https://doi.org/10.3390/ijms22169100
41. Xia P, Wu Y, Lian S, Yan L, Meng X, Duan Q, et al. Research progress on Toll-like receptor signal transduction and its roles in antimicrobial immune responses. Applied Microbiology and Biotechnology. 2021;105:5341-55. https://doi.org/10.1007/s00253-021-11406-8
42. Rahman MA, Amirkhani A, Chowdhury D, Mempin M, Molloy MP, Deva AK, et al. Proteome of Staphylococcus aureus biofilm changes significantly with aging. International Journal of Molecular Sciences. 2022;23(12):6415. https://doi.org/10.3390%2Fijms23126415
43. Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP. Essential role for the major autolysin in the fibronectin-binding protein-mediated Staphylococcus aureus biofilm phenotype. Infection and immunity. 2011;79(3):1153-65. https://doi.org/10.1128%2FIAI.00364-10
44. Hennig S, Wai SN, Ziebuhr W. Spontaneous switch to PIA-independent biofilm formation in an ica-positive Staphylococcus epidermidis isolate. International Journal of Medical Microbiology. 2007;297(2):117-22. https://doi.org/10.1016/j.ijmm.2006.12.001
45. Fitzpatrick F, Humphreys H, O'Gara JP. Evidence for icaADBC-independent biofilm development mechanism in methicillin-resistant Staphylococcus aureus clinical isolates. Journal of clinical microbiology. 2005;43(4):1973-6. https://doi.org/10.1128%2FJCM.43.4.1973-1976.2005
46. Schommer NN, Christner M, Hentschke M, Ruckdeschel K, Aepfelbacher M, Rohde H. Staphylococcus epidermidis uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A. 1. Infection and immunity. 2011;79(6):2267-76. https://doi.org/10.1128%2FIAI.01142-10
47. Abdrabaa MK, Abd Aburesha R. Assessment of ica A and D Genes in Biofilm producers methicillin-resistant Staphylococcus aureus isolates. Iraqi Journal of Science. 2023:2696-706.
48. Potrykus K, Cashel M. (p) ppGpp: still magical? Annu Rev Microbiol. 2008;62:35-51. https://doi.org/10.1146/annurev.micro.62.081307.162903
49. Weaver JW, Proshkin S, Duan W, Epshtein V, Gowder M, Bharati BK, et al. Control of transcription elongation and DNA repair by alarmone ppGpp. Nature Structural & Molecular Biology. 2023;30(5):600-7. https://doi.org/10.1038/s41594-023-00948-2

50. Valle DL, Paclibare PAP, Cabrera EC, Rivera WL. Molecular and phenotypic characterization of methicillin-resistant Staphylococcus aureus isolates from a tertiary hospital in the Philippines. Tropical medicine and health. 2016;44(1):1-9. https://doi.org/10.1186/s41182-016-0003-z
51. Peyrusson F, Varet H, Nguyen TK, Legendre R, Sismeiro O, Coppée J-Y, et al. Intracellular Staphylococcus aureus persisters upon antibiotic exposure. Nature communications. 2020;11(1):2200. https://doi.org/10.1038/s41467-020-15966-7
52. Mohsin MR, Al-Rubaii BA. Bacterial growth and antibiotic sensitivity of Proteus mirabilis treated with anti-inflammatory and painkiller drugs. Biomedicine. 2023;43(2):728-34.
53. Sabah Fakhry S, Noori Hammed Z, Abdul-elah Bakir W, Abdullah Laftaah ALRubaii B. Identification of methicillin-resistant strains of Staphylococcus aureus isolated from humans and food sources by Use of mecA 1 and mecA 2 genes in Pulsedfield gel electrophoresis (PFGE (technique. Revis Bionatura 2022; 7 (2) 44.
54. Ali MA, Al-Rubaii BA. Study of the Effects of Audible Sounds and Magnetic Fields on Staphylococcus aureus Methicillin Resistance and mecA Gene Expression: Tropical Journal of Natural Product Research (TJNPR). 2021 ;5(5):825-30.
55. Husain AG, Alrubaii BA. Molecular detection and expression of virulence factor encoding genes of Pseudomonas aeruginosa isolated from clinical samples. Biomedicine. 2023;43(5):1514-9.
56. Bassi AG, Al-Rubaii BA. Detection of Pyocin S and the Effects of Lactobacillus Acidophilus Cell-Free Supernatants on Multi-Drug Resistant Pseudomonas Aeruginosa Isolated from Patients of Baghdad Hospitals. Journal of Communicable Diseases 2024;56(1):135-144. https://doi.org/10.24321/0019.5138.202418

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