Journal of Communicable Diseases
Volume 57, Issue 1 - 2025, Pg. No. 44-54
Peer Reviewed & Open Access Journal

Research Article

Biofilm Feast: Stringent Response-Induced Changes in MRSA and MSSA Isolates, Examining *icaA/ icaD* Gene Expression

Ali A Mussa[1], Harith Jabbar Fahad Al Mathkhury[2]

[1,2]Department of Biology, College of Science, University of Baghdad, Iraq
DOI: https://doi.org/10.24321/0019.5138.202505

I N F O

Corresponding Author:
Harith Jabbar Fahad Al Mathkhury, Department of Biology, College of Science, University of Baghdad, Iraq
E-mail Id:
harith.fahad@sc.uobaghdad.edu.iq
Orcid Id:
https://orcid.org/0000-0002-5414-9834
How to cite this article:
Mussa A A, Mathkhury H J F A. Biofilm Feast: Stringent Response-Induced Changes in MRSA and MSSA Isolates, Examining *icaA/ icaD* Gene Expression. J Commun Dis. 2025;57(1):44-54.

Date of Submission: 2024-05-11
Date of Acceptance: 2024-10-18

A B S T R A C T

Introduction: The stringent response is a bacterial adaptation mechanism triggered by stress conditions, including nutrient limitation. This response helps bacteria survive under harsh conditions, such as those encountered during infection. A key feature of the stringent response is the synthesis of the alarmone (p)ppGpp, which influences various bacterial phenotypes. In several bacterial species, stringent response activation significantly affects biofilm formation and maintenance.

Methods: Clinical specimens were collected from multiple hospitals in Baghdad, Iraq. *Staphylococcus aureus* was identified using conventional biochemical tests. The PCR technique was applied to detect mecA, *icaA*, and *icaD* genes, while the Vitek 2 compact system confirmed Methicillin sensitivity in mecA-negative isolates. Biofilm intensity of all *S. aureus* isolates was assessed under normal and starved conditions. Additionally, the gene expression levels of *icaA* and *icaD* were measured in five MRSA and five MSSA strains under both conditions.

Results: The mecA, *icaA*, and *icaD* genes were detected in 94%, 96.3%, and 100% of *S. aureus* isolates, respectively. Biofilm production analysis showed that 24% of isolates were strong producers, 49% were moderate producers, and 9% were weak producers. Statistical analysis indicated that biofilm intensity significantly decreased under nutrient limitation ($p < 0.0001$) compared to normal conditions across all isolates. Furthermore, *icaA* and *icaD* genes were upregulated under stringent response conditions, regardless of Methicillin resistance status.

Conclusion: The stringent response influences *S. aureus* biofilm formation, with biofilm intensity decreasing under nutrient-limited conditions. However, the upregulation of *icaA* and *icaD* genes suggests a regulatory role of the stringent response in biofilm-related gene expression. These findings highlight the potential impact of stress adaptation mechanisms on bacterial persistence and pathogenicity.

Keywords: Biofilm, *icaADBC*, *S. aureus*, Stringent Response

Introduction

Staphylococcus aureus is a major pathogenic microorganism that is responsible for a diverse range of infections[1] Several investigations have been done to elucidate the structures and pathogenic mechanisms via which *S. aureus* is capable of causing severe infections[2].

Staphylococcal pathogenesis is a complex process that involves both adhesion and biofilm formation[3]. Complex communities of bacteria can adhere permanently to surfaces and create biofilms[4]. Bacteria that produce biofilms are responsible for causing persistent or chronic infections. Cell aggregation and biofilm formation in *Staphylococcus* spp. are facilitated by the products of the *icaADBC* operon. These genes encode the crucial proteins necessary for the synthesis of polysaccharide intercellular adhesion (PIA) and capsular polysaccharide/adhesion (PS/A)[5]. PIA is composed mainly of N-acetylglucosamine and plays a critical role in the invasiveness of *S. aureus*[6].

The emergence of hospital-adapted Methicillin-Resistant *S. aureus* (MRSA) clones worldwide has posed significant challenges[7]. Methicillin resistance occurs when a bacterium acquires a specific protein called penicillin-binding protein 2A (PBP2A), which is expressed by a gene called *mecA*. This gene is found on a mobile genetic element called staphylococcal cassette chromosome (SCC) *mec*. There are 15 SCC*mec* types that have been officially recognised and approved by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements[8,9]. The incidence of MRSA infections has risen in recent years, and these infections are more commonly linked to death compared to infections caused by Methicillin-Sensitive *S. aureus* (MSSA) strains. MRSA exhibits resistance to β-lactam antibiotics. However, MRSA isolates often display multidrug resistance (MDR) by showing resistance to other commonly used antimicrobial agents such as macrolides, tetracycline, aminoglycosides, chloramphenicol, and fluoroquinolones. These microorganisms are frequently associated with infections and their resistance poses challenges in therapeutic interventions[10,11]. Most bacteria undergo the so-called stringent response when they experience a limitation in nutrient supply. This reaction is triggered by the rapid production of alarmones called pppGpp and/ or ppGpp, which will be referred to as (p) ppGp. Under stress, the presence of (p)ppGpp leads to the cessation of activities related to cell proliferation. This includes the suppression of gene transcription for important components of protein synthesis, such as rRNA, ribosomal proteins, and translation factors. Additionally, replication is inhibited[12]. Studies have shown that the activation of the stringent response in certain bacterial species also impacts the production of biofilms[13,14]. In numerous pathogenic bacteria, the presence of (p)ppGpp is a decisive factor in determining their virulence or their ability to tolerate and persist against antibiotics[15]

To the best of our knowledge, little is known about the influence of starvation on *icaA* and *icaD* gene expression *in S. aureus* and whether it is affected by methicillin-resistant phenotype. Therefore, this study aims to examine the impact of starvation-induced stringent response on the expression levels of *icaA* and *icaD* genes, as well as on the thickness of biofilm in locally isolated *S. aureus* strains, including MRSA and MSSA strains.

Materials and Methods

Microorganisms

Different clinical specimens were collected from patients attending different hospitals in Baghdad; sputum samples, as well as swabs, were collected from wounds, burns, anterior nares, and ears. All these specimens were inoculated onto Mannitol Salt Agar plates (MSA) and then incubated at 37 °C for 24 hours. Afterwards, the colonies were subsequently subjected to conventional biochemical tests, including haemolysin production, acetoin production, catalase, coagulase and oxidase tests in order to identify *S. aureus* isolates (Tille, 2021).

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 the primers used for PCR are listed in Table 1.

PCR was used to identify methicillin resistance by detecting the presence of the *mecA* gene among all isolates, the following programme by Parvin et al. (2021) followed: Initial denaturation at 94 °C for 10 min followed by 10 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 75 s, then followed by 25 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 75 s. Moreover, the Vitek 2 compact system was employed to confirm that mecA-negative isolates were methicillin-sensitive.

All *S. aureus* isolates were screened for the presence of *icaA* and *icaD* genes using the primers listed in Table 1 to amplify 102 bp and 82 bp segments of the *icaA* and *icaD* genes, respectively. The reaction protocol was as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; followed by cycles for 5 min at 72 °C for final extension.

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

Table 1.List of Primers Used in This Study

Target Gene	Primer Name	Sequence 5' 3'	Amplicon Size (bp)	Reference
mecA	MecA1	GTAGAAATGACTGAACGTCCGATAA	310	(Parvin et al., 2021)
	MecA2	CCAATTCCACATTGTTTCGGT CTAA		
icaA	icaA -F	CAATACTATTTCGGGTGTCTTCACTCT	102	(Peyrusson et al., 2020)
	icaA -R	CAAGAAACTGCAATATCTTCGGTAATCAT		
icaD	icaD -F	TCAAGCCCAGACAGAGGGAATA	82	(Peyrusson et al., 2020)
	icaD -R	ACACGATATAGCGATAAGTGCTGTTT		
rpoB	rpoB -F	CAGCTGACGAAGAAGATAGCTATGT	82	(Peyrusson et al., 2020)
	rpoB -R	ACTTCATCATCCATGAAACGACCAT		

Biofilm Formation Assay

The influence of starvation-induced stringent response on biofilm growth was investigated using the microtiter plate method. The quantification of the biofilm under normal conditions was performed according to the method given by Nakao et al. (2012)[16]. Concisely, 200 µL of an overnight Tryptic Soy Broth (TSB) supplemented with 1% glucose culture (bacterial concentration adjusted to be equal with McFarland standard no. 0.5) was added to the wells of sterile 96-well polystyrene microplates. The plates were then covered and incubated aerobically at 37 °C for 24 hours. Every isolate was tested three times. Control wells were established by using bacteria-free TSB. The wells were decanted and rinsed three times with 200 µL of sterile phosphate-buffered saline (PBS); the adhered bacteria were treated with 200 µL of methanol for 15 minutes for fixation. Following air drying, the wells were treated with 200 µL of a 0.1% crystal violet solution for 15 minutes at room temperature. The surplus stain was washed away; thereafter, the plates were dried. Afterwards, the stained attached cells were dissolved again using 200 µL of 33% glacial acetic acid for a duration of 15 minutes. Ultimately, the optical density (OD) of each well was measured at a wavelength of 600 nm using a microplate reader (Biotek, UK). The cut-off value (ODc) was determined by calculating the mean of the optical density (OD) values of the control wells, and then 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 twice ODc), moderate producer (OD values greater than twice ODc but less than or equal to four times ODc), or strong producer (OD values greater than four 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 (D.W.) 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:[17]

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

Gene Expression

The levels of *icaA* and *icaD* gene expression were assessed for 5 MSSA (Sa30, Sa35, Sa41, Sa44 and Sa72) and 5 MRSA (Sa27, Sa34, Sa40, Sa70 and Sa80) isolates under normal and starvation conditions using the primers listed in Table 1; *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 µL of each primer were used in the reactants, while the Rt volume was 1 µL. Moreover, about 50 mg of RNA was added, and the volume was increased to 20 µL using nuclease-free water. The adopted protocol is shown in Table 2.

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

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

Table 2.RT-qPCR Protocol

Cycle Step	Temperature (°C)	Time	Cycles
Reverse transcription	55	10 minutes	1
Initial denaturation	95	1 minute	1
Denaturation	95	10 seconds	40–45
Extension	60	30 seconds	
Melt curve	60–95	Various	1

Ethics Statements

This work has been approved by the College of Science Research Ethics Committee (ref. CSEC/0422/0159). All the participants were allowed to provide the researchers with the specimens. Informed consent was obtained from all participants according to the Declaration of Helsinki.

Results and Discussion

Biofilm Formation under Normal Conditions

The microtiter plate assay stands as the predominant method as it is known to be the gold standard method for detecting biofilm formation ability among bacteria. This approach has been reported to be the most precise, sensitive and replicable screening technique for the detection of biofilm production by *S. aureus* clinical isolates. It also possesses the distinct advantage of serving as a quantitative method for the evaluation of adherence among various strains[18].

The results revealed that only 24% of *S. aureus* isolates were strong biofilm producers; while 49% and 9% of the isolates were moderate and weak producers, respectively (Table 3).

Furthermore, statistical analysis revealed that there was a significant difference (p value < 0.0001) between the intensity of the biofilm that was developed by the different *S. aureus* isolates.

The result of the current study was in agreement with that of a study conducted by[19]who found that 37.93%, 48.28% and 13.79% of *S. aureus* isolates were strong, moderate and weak biofilm producers, respectively.

It also agreed to some extent with the findings of Abdrabaa and Abd Aburesha (2023)[20] in the sense that all *S. aureus* isolates included in their study developed biofilm; however, about 42% of the isolates were strong biofilm producers while 20% and 38% were moderate and weak biofilm producers, respectively.

There is an increasing belief by many scientists that bacterial biofilms are responsible for over 80% of chronic illnesses. *S. aureus* resides within the hospital surroundings, and from there it can adhere to host tissues and medical equipment that are inserted into the body[21], leading to many illnesses including skin and soft tissue infection, osteomyelitis, endocarditis, pneumonia, and bacteraemia. The capacity to form biofilm further contributes to the heightened antibiotic resistance of *S. aureus*, making it challenging to treat[22].

Moreover, biofilm formation is regarded as a safeguarded mechanism for bacteria to acclimate to challenging environments. The biofilm functions as a protective shield, ensuring a steady internal milieu for bacterial cellular processes. It safeguards bacterial cells from hostile circumstances such as severe temperatures, limited nutrients, desiccation, and even antibacterial agents[23].

Consequently, combating biofilm infection typically necessitates the prolonged administration of antibiotics at higher dosages (Beloin et al., 2014). However, prolonged administration of these antibiotics can result in the spread of resistance and may cause drug toxicity[24].

Biofilm Formation under Nutrient Limitation

The ability of all *S. aureus* isolates to develop biofilm under nutrient limitation was measured using the microtiter plate method. The results summarised in Table 3 revealed that all *S. aureus* isolates were able to develop biofilm under nutrient limitations; moreover, the majority of the isolates (97.5%) developed weak biofilm layers under these conditions.

Additionally, 2.5% of the isolates (Sa41 and Sa44) were able to develop a moderate biofilm.

Statistical analysis revealed that the biofilm intensity that was developed under nutrient limitation decreased significantly (p value < 0.0001) when compared with the biofilm that was developed under normal conditions for all isolates (Figure 1).

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

Table 3.Biofilm Forming Capacity of Bacterial Isolates

Isolate	Mean OD_{600}		Isolate	Mean OD_{600}		Isolate	Mean OD_{600}	
	Normal	Starvation		Normal	Starvation		Normal	Starvation
Sa1	00.348	00.134	Sa29	00.464	00.216	Sa57	00.282	00.117
Sa2	00.924	00.160	Sa30	00.451	00.152	Sa58	00.308	00.162
Sa3	00.162	00.150	Sa31	00.476	00.181	Sa59	00.236	00.145
Sa4	00.213	00.123	Sa32	00.284	00.134	Sa60	00.296	00.156
Sa5	00.357	00.137	Sa33	00.305	00.199	Sa61	00.302	00.145
Sa6	00.188	00.138	Sa34	00.727	00.183	Sa62	00.250	00.116
Sa7	00.566	00.154	Sa35	00.334	00.206	Sa63	00.210	00.145
Sa8	00.529	00.148	Sa36	00.445	00.149	Sa64	00.325	00.175
Sa9	00.246	00.131	Sa37	00.638	00.187	Sa65	00.314	00.180
Sa10	00.437	00.135	Sa38	00.509	00.141	Sa66	00.380	00.150
Sa11	00.384	00.131	Sa39	00.526	00.153	Sa67	00.376	00.152
Sa12	00.290	00.133	Sa40	00.549	00.207	Sa68	00.316	00.179
Sa13	00.299	00.118	Sa41	00.663	00.257	Sa69	00.406	00.179
Sa14	00.365	00.132	Sa42	00.292	00.149	Sa70	00.521	00.222
Sa15	00.193	00.156	Sa43	00.399	00.138	Sa71	00.374	00.155
Sa16	00.396	00.140	Sa44	00.377	00.227	Sa72	00.809	00.178
Sa17	00.374	00.140	Sa45	00.250	00.155	Sa73	00.365	00.127
Sa18	00.496	00.155	Sa46	00.341	00.180	Sa74	00.344	00.146
Sa19	00.615	00.181	Sa47	00.272	00.188	Sa75	00.237	00.125
Sa20	00.496	00.141	Sa48	00.347	00.162	Sa76	00.420	00.120
Sa21	00.240	00.178	Sa49	00.273	00.178	Sa77	00.378	00.154
Sa22	00.364	00.127	Sa50	00.317	00.210	Sa78	00.335	00.134
Sa23	00.388	00.204	Sa51	00.195	00.158	Sa79	00.321	00.149
Sa24	00.277	00.192	Sa52	10.008	00.199	Sa80	10.205	00.126
Sa25	00.237	00.132	Sa53	00.314	00.174	Sa81	00.223	00.178
S26	00.233	00.159	S54	00.396	00.130	Sa82	00.212	00.131
S27	00.897	00.146	S55	00.294	00.160	C	0.099	
S28	00.315	00.170	S56	00.405	00.152	-		

S1–S82: *S. aureus* isolates 1–82; C: Control; Cut-off value: 0.111

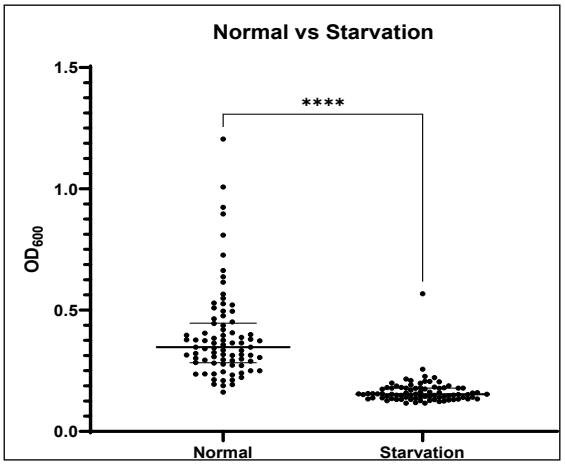

Figure 1.Biofilm Formation: Normal Conditions vs Starvation

Furthermore, there was a significant difference (p value < 0.0001) among the biofilm-forming capabilities of the isolates under starvation conditions. Such a decrease in biofilm intensity could be attributed to biofilm dispersal that is influenced by nutrient starvation.

The study conducted by Huynh et al. (2012)[25] investigated the impact of glucose deprivation on *P. aeruginosa* biofilm. The findings revealed that glucose limitation significantly affected biofilm dispersal. Dispersal began within five minutes of glucose deficiency, reached its peak after 2 hours, and resulted in the dispersion of up to 60% of the initial biomass after 24 hours of starvation. Furthermore, the biofilm that experienced glucose starvation exhibited a decrease in optical density (OD) from 1.11 to 0.43 over a span of two days. In addition, a study conducted by Thormann et al. (2005)[26] highlighted that the separation

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

of cells from biofilms of *Shewanella oneidensis* can be triggered by halting the flow of the medium in a hydrodynamic biofilm system. In a similar manner, They also demonstrated that *Serratia marcescens* developed denser and more elongated biofilms when exposed to high nutrient concentrations, while biofilms became thinner under low nutrient conditions.[27]

PCR Study

A set of primers was used for *mecA* gene detection in 82 isolates of *S. aureus* by using the monoplex PCR technique. The result showed that 77 out of the 82 *S. aureus* isolates (94%) that were included in this study harboured mecA as depicted in Figure 2.

This finding indicates that these isolates are methicillin-resistant as there is no *mecA* gene in MSSA strains; thus the detection of this gene in any isolate of *S. aureus* is indicative of MRSA[28,29].

The result of this study was in agreement with one of the study who found that about 94.29% of *S. aureus* isolates that were included in their study harboured *mecA* gene, and hence were regarded as being methicillin-resistant.[30]

The results of the study were similar to those of a study conducted by Ibraheem & Al-Mathkhury (2018)[31]who found that about 80% of *S. aureus* isolates were methicillin-resistant by virtue of harbouring the *mecA* gene.

The findings of the research corroborated the conclusions drawn by Ibraheem & Al-Mathkhury (2018)[31] as well, wherein approximately 80% of the *S. aureus* isolates exhibited resistance to methicillin by virtue of harbouring the mecA gene.

The results also agreed with those of Jabur and Kandala (2022)[32]who conducted an experiment on 68 *S. aureus* isolates that were isolated from post-surgical wound infections and found that about 98.5% of the isolates contained the *mecA* gene. However, the results differed from their findings concerning *S. aureus* isolates collected from women with bacterial vaginosis, as they reported that only 55% of these isolates harboured the *mecA* gene.

Moreover, the results also disagreed with those of a study conducted by Al-Halaq and Utba (2023)[33]who discovered that only 60% of *S. aureus* isolates from patients with furunculosis contained the *mecA* gene.

The differences in *mecA* distribution percentage between the differing studies could be attributed to the difference in the infection site from where the isolates were collected.[34]

For the detection of *icaA* and *icaD* genes in all of the 82 *S. aureus* isolates, two sets of primers were employed in a monoplex PCR pattern. The findings from this experiment revealed that the *icaA* gene was present in 79 (96.3%) isolates while the *icaD* gene was located in all isolates that were included in the study, as illustrated in Figures 3 and 4.

The results agreed to some extent with a local study published by the researcher who found that the *icaA* gene was present in 81% while *icaD* was located in 84.6% of the tested *S. aureus* isolates that were included in their research (13 isolates).[35] Likewise, another local study conducted by Abdrabaa and Abd Aburesha (2023) showed that *icaA* was observed in 73.3% while *icaD* was found in 84.4% of the 45 *S. aureus* isolates that were screened.[36]

Interestingly, the *icaA* gene was absent in the genome of Sa2, Sa10 and Sa46 isolates; however, Sa2 developed strong biofilm while Sa10 and Sa46 developed moderate biofilm when tested using the microtiter plate method. This suggests that the developed biofilm by the aforementioned isolates was ica independent and it was reliant on other mechanisms.

Although the *icaADBC* locus and the regulatory channels that direct PIA/ PNAG production play a crucial role in staphylococcal biofilm development, multiple studies have shown the presence of biofilm processes in *S. aureus* that are not dependent on PIA/ PNAG.[37]

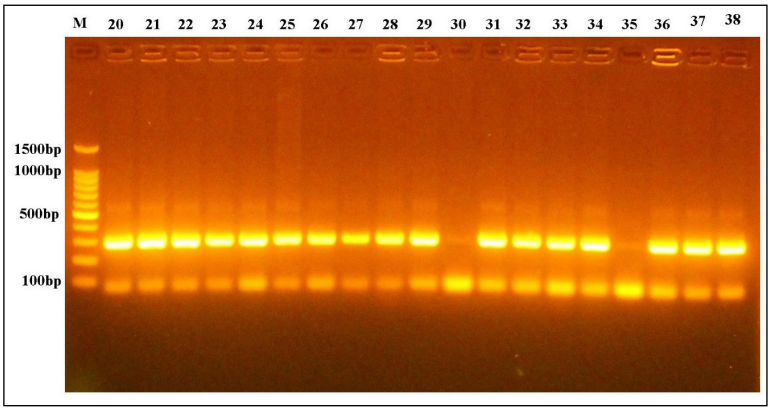

Figure 2.Visualisation of mecA Gene by 1.5% Agarose Gel Analysis. The shown bands are representative of PCR products (310 bp) amplified from *S. aureus* isolates (lanes 20–38), with lane M representing the 100 bp DNA ladder

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

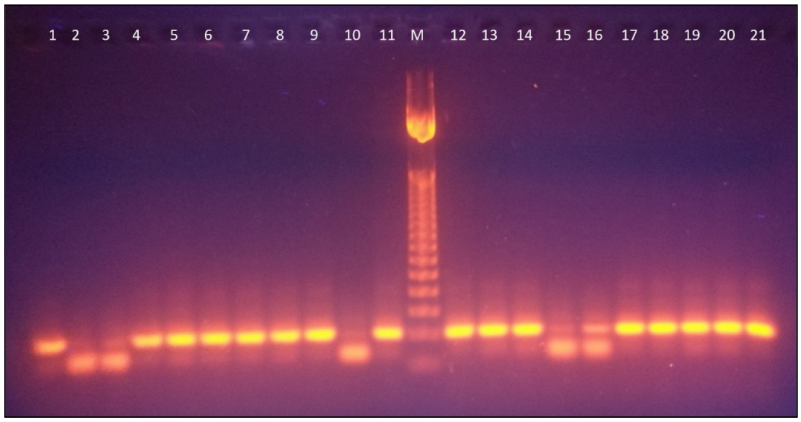

Figure 3.Visualisation of icaA Gene by 1.5% Agarose Gel Analysis. The shown bands are representative of PCR products (102 bp) amplified from *S. aureus* isolates (lanes 1– 21), with lane M representing the 50 bp DNA ladder

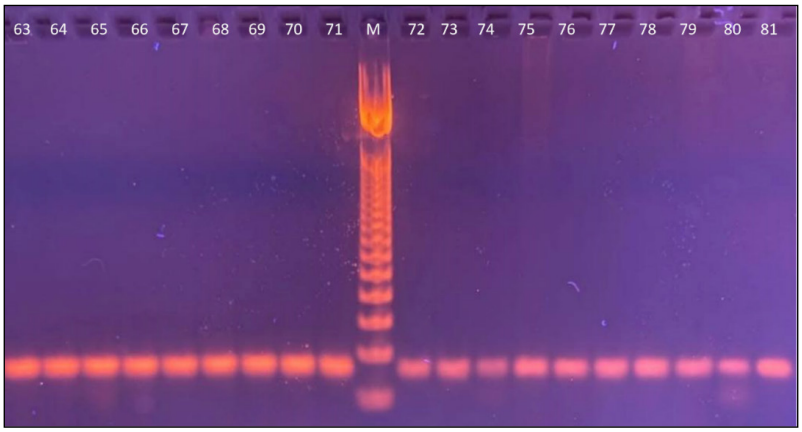

Figure 4.Visualisation of icaD Gene by 1.5% Agarose Gel Analysis. The shown bands are representative of PCR products (82 bp) amplified from *S. aureus* isolates (lanes 63–81), with lane M representing the 50 bp DNA ladder

The processes involved in biofilms that do not rely on the *ica* gene are complex and have not been completely comprehended yet. [38]Nevertheless, the protein known as biofilm-associated protein (Bap) was recognised among the primary tools by which *S. aureus* can form an *ica*-independent biofilm.[39] A significant number of other staphylococcal proteins were later linked to ica-independent biofilms, including fibronectin-binding proteins A and B [40], as well as *S. aureus* surface protein G, among many others.[41]

Surprisingly, the alternative transpeptidase PBP2a or 2', which is produced by the *mecA* gene, is closely linked to the formation of biofilms in many recent MRSA isolates.[42]

Gene Expression

To study the effect of stringent response initiated by nutrient limitation on the expression of *icaA* and *icaD* 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.[43]

The results summarised in Table 4 and illustrated in Figure 5 demonstrate that the level of expression for all the aforementioned genes was upregulated under nutrient limitation as compared to that with normal growth conditions. Furthermore, there was no difference observed in gene expression profile between MRSA and MSSA strains.

The data presented in Table 4 clearly demonstrates that in isolates Sa30, Sa35 and Sa41, the upregulation in gene expression levels was much higher when compared with other isolates, all of which were MSSA, suggesting that *icaA* and *icaD* genes play an important role in their biofilm formation under the control of the stringent response. On the other hand, concerning the remaining isolates, since the levels of gene expression were not increased considerably; other genes could be involved under nutrient limitation.

Alarmones (p)ppGpp synthesised when the stringent response is initiated have the ability to facilitate biofilm formation. The mechanism by which (p)ppGpp enhances biofilm development is yet to be fully comprehended. The presence of (p)ppGpp leads to a rapid reduction in the amount of GTP inside the cell, and the activation of the CodY regulon is relieved.[44]

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

CodY is a regulatory protein that controls gene expression in many bacteria. It responds to the presence of branched-chain amino acids (BCAAs) such as isoleucine, leucine, and valine, as well as GTP. CodY regulates the expression of numerous genes involved in various metabolic pathways that are responsible for the utilisation of alternative nutrient sources. These genes play a role in processes such as nutrient search, uptake, and processing.[45] When the levels of BCAAs and GTP inside a cell are elevated, CodY is stimulated and functions as a DNA-binding protein, usually leading to the suppression of gene expression.[46] When the amounts of BCAAs and GTP fall, the activity of CodY protein in the cell also decreases. This leads to changes in the transcriptome.[47]

Table 4.Gene Expression Profile Represented by Fold Change

Id	icaA	icaD
Sa30	137.1870	1552
Sa35	39.3960	6.4980
Sa41	84.4480	103.9680
Sa44	0.7070	0.3290
Sa72	0.0014	0.0006
Sa27	0.7070	1.5150
Sa34	0.0440	0.0580
Sa40	1.3195	4.2870
Sa70	6.4980	19.6980
Sa80	3.7320	4.9240

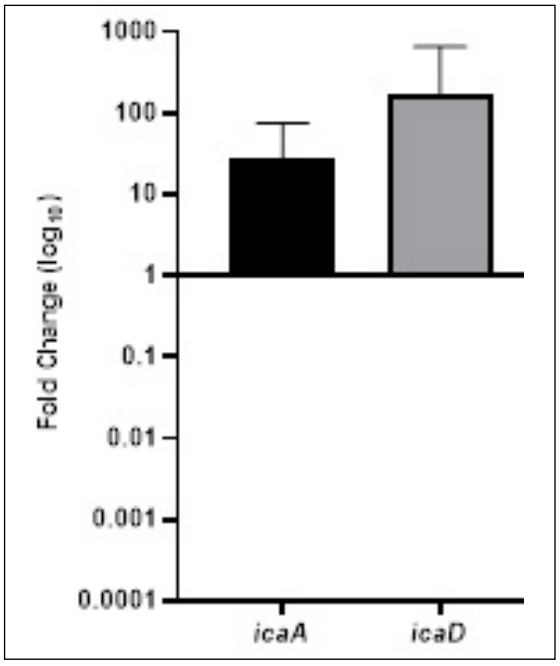

Figure 5.Fold Change of icaA and icaD

In addition, CodY controls the transcription of genes that are involved in the synthesis or modification of the biofilm matrix.[48]CodY has been demonstrated to play a crucial role in the regulation of FnbAB and SasG proteins, which aid in the initial attachment of bacterial cells to host tissue. Additionally, CodY is essential for the production of biofilms.[49]

On the other hand, CodY inhibits the activity of genes that produce proteases that have a detrimental effect on the formation of biofilms. It also inhibits the activity of nuclease (Nuc), which is essential for the final stage of biofilm development.[50] This suggests that CodY plays a crucial role in regulating both PIA-dependent and PIA-independent biofilm formation.[51]

Conclusion

Methicillin-resistant *S. aureus* strains are much more prevalent than their sensitive counterparts in hospital settings and given that almost all these isolates possess both *icaA* and *icaD* genes, it further complicates the situation as treatment options become more limiting; more importantly, while the starvation-driven stringent response leads to the development of a thinner biofilm, it indeed upregulates the *icaA* and *icaD* genes in all *S. aureus* isolates irrespective of their methicillin resistance status.

Authors' Contribution: Both authors equally contributed to all aspects of this study, including study conceptualisation, data curation, analysis and interpretation of results, and draft manuscript preparation ending with revisions and editing of the manuscript.

Conflict of Interest: None

Source of Funding: None

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process: None

References

1. Abdrabaa MK, Aburesha RA. Assessment of ica A and D genes in biofilm producers methicillin-resistant *Staphylococcus aureus* isolates. Iraqi J Sci. 2023;64(6):2696-706.
2. Al-Halaq AA, Utba NM. Prevalence of methicillin-resistant *Staphylococcus aureus* carrying lukS-lukF gene in Iraqi patients with furunculosis. Iraqi J Sci. 2023;64(7):3323-9.
3. Alkhafajy RT, Al-Mathkhury HJ. Gentamicin up regulates the gene expression of hla and nuc in *Staphylococcus aureus*. Iraqi J Sci. 2023;64(3):1079-2.
4. Arciola CR, Baldassarri L, Montanaro L. Presence of *icaA* and icaDgenes and slime production in a collection of staphylococcal strains from catheter-associated infections. J Clin Microbiol. 2001;39(6):2151-6. [PubMed] [Google Scholar]

5. Belitsky BR, Sonenshein AL. Genetic and biochemical analysis of CodY-binding sites in *Bacillus subtilis*. J Bacteriol. 2008;190(4):1224-36. [PubMed] [Google Scholar]
6. Beloin C, Renard S, Ghigo JM, Lebeaux D. Novel approaches to combat bacterial biofilms. Curr Opin Pharmacol. 2014;18:61-8. [PubMed] [Google Scholar]
7. Bispo PJ, Sahm DF, Asbell PA. A systematic review of multi-decade antibiotic resistance data for ocular bacterial pathogens in the United States. Ophthalmol Ther. 2022;11(2):503-20. [PubMed] [Google Scholar]
8. Brinsmade SR. CodY, a master integrator of metabolism and virulence in gram-positive bacteria. Curr Genet. 2017;63(3):417-25. [PubMed] [Google Scholar]
9. Colomer-Winter C, Flores-Mireles AL, Kundra S, Hultgren SJ, Lemos JA. (p)ppGpp and CodY promote *Enterococcus faecalis* virulence in a murine model of catheter-associated urinary tract infection. mSphere. 2019;4(4):e00392-19. [PubMed] [Google Scholar]
10. Cortes MF, Beltrame CO, Ramundo MS, Ferreira FA, Figueiredo AM. The influence of different factors including *fnbA* and *mecA* expression on biofilm formed by MRSA clinical isolates with different genetic backgrounds. Int J Med Microbiol. 2015;305(1):140-7. [PubMed] [Google Scholar]
11. Cucarella C, Solano C, Valle J, Amorena B, Lasa I, Penadés JR. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J Bacteriol. 2001;183(9):2888-96. [PubMed] [Google Scholar]
12. Díaz-Salazar C, Calero P, Espinosa-Portero R, Jiménez-Fernández A, Wirebrand L, Velasco-Domínguez MG, López-Sánchez A, Shingler V, Govantes F. The stringent response promotes biofilm dispersal in *Pseudomonas putida*. Sci Rep. 2017;7(1):18055. [PubMed] [Google Scholar]
13. Figueiredo AM, Ferreira FA, Beltrame CO, Côrtes MF. The role of biofilms in persistent infections and factors involved in *ica*-independent biofilm development and gene regulation in *Staphylococcus aureus*. Crit Rev Microbiol. 2017;43(5):602-20. [PubMed] [Google Scholar]
14. Formosa-Dague C, Speziale P, Foster TJ, Geoghegan JA, Dufrêne YF. Zinc-dependent mechanical properties of *Staphylococcus aureus* biofilm-forming surface protein SasG. Proc Natl Acad Sci. 2016;113(2):410-5. [PubMed] [Google Scholar]
15. Frank KL, Patel R. Poly-N-acetylglucosamine is not a major component of the extracellular matrix in biofilms formed by *icaADBC*-positive *Staphylococcus lugdunensis* isolates. Infect Immun. 2007;75(10):4728-42. [PubMed] [Google Scholar]
16. Geiger T, Goerke C, Fritz M, Schäfer T, Ohlsen K, Liebeke M, Lalk M, Wolz C. Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of *Staphylococcus aureus*. Infect Immun. 2010;78(5):1873-83. [PubMed] [Google Scholar]
17. Girish VM, Liang H, Aguilan JT, Nosanchuk JD, Friedman JM, Nacharaju P. Anti-biofilm activity of garlic extract loaded nanoparticles. Nanomedicine. 2019;20:102009. [PubMed] [Google Scholar]
18. Gordon RJ, Lowy FD. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. Clin Infect Dis. 2008;46(Suppl 5):S350-9. [PubMed] [Google Scholar]
19. Gries CM, Biddle T, Bose JL, Kielian T, Lo DD. *Staphylococcus aureus* fibronectin binding protein A mediates biofilm development and infection. Infect Immun. 2020;88(5):e00859-19. [PubMed] [Google Scholar]
20. Guo H, Tong Y, Cheng J, Abbas Z, Li Z, Wang J, Zhou Y, Si D, Zhang R. Biofilm and small colony variants—an update on *Staphylococcus aureus* strategies toward drug resistance. Int J Mol Sci. 2022;23(3):1241. [PubMed] [Google Scholar]
21. Huynh TT, McDougald D, Klebensberger J, Al Qarni B, Barraud N, Rice SA, Kjelleberg S, Schleheck D. Glucose starvation-induced dispersal of *Pseudomonas aeruginosa* biofilms is cAMP and energy dependent. PLoS One. 2012;7(8):e42874. [PubMed] [Google Scholar]
22. Ibraheem H, Al-Mathkhury HJ. pvl-carried methicillin resistant *Staphylococcus aureus* isolated from hospitalized patients in Baghdad, Iraq. Iraqi J Sci. 2018;59(4):1967-72.
23. Ibrahim MA, Deaf EA, Hafez R, Hussein S, Essam R, Elkhawaga AA. PCR-based rapid identification of methicillin-resistant *Staphylococcus aureus* strains and their antibiotic resistance profiles in febrile neutropenic patients. Microbes Infect Dis. 2023;4(4):1998-209. [Google Scholar]
24. Ito T, Hiramatsu K, Tomasz A, de Lencastre H, Perreten V, Holden MT, Coleman DC, Goering R, Giffard PM, Skov RL, Zhang K, Westh H, O'Brien F, Tenover FC, Oliveira DC, Boyle-Vavra S, Laurent F, Kearns AM, Kreiswirth B, Ko KS, Grundmann H, Sollid JE, John Jr JF, Daum R, Soderquist B, Buist G; International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Guidelines for reporting novel mecA gene homologues. Antimicrob Agents Chemother. 2012;56(10):4997-9. [PubMed] [Google Scholar]
25. Jabur EQ, Kandala N. The production of biofilm from methicillin resistant *Staphylococcus aureus* isolated from post-surgical operation inflammation. Iraqi J Sci.

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

2022;63(9):3688-3702.
26. Jaddoa NT, Al-Mathkhury HJ. Biofilm shows independency form hemolysin genes arsenal in methicillin resistant *Staphylococcus aureus*. Iraqi J Sci. 2018;59(4C):2184-94.
27. Klug D, Wallet F, Kacet S, Courcol RJ. Involvement of adherence and adhesion *Staphylococcus epidermidis* genes in pacemaker lead-associated infections. J Clin Microbiol. 2003;41(7):3348-50. [PubMed] [Google Scholar]
28. Lee J, Zilm PS, Kidd SP. Novel research models for *Staphylococcus aureus* small colony variants (SCV) development: co-pathogenesis and growth rate. Front Microbiol. 2020;11:321. [PubMed] [Google Scholar]
29. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol. 2006;24(1):25-9. [PubMed] [Google Scholar]
30. Mlynek KD, Bulock LL, Stone CJ, Curran LJ, Sadykov MR, Bayles KW, Brinsmade SR. Genetic and biochemical analysis of CodY-mediated cell aggregation in *Staphylococcus aureus* reveals an interaction between extracellular DNA and polysaccharide in the extracellular matrix. J Bacteriol. 2020;202(8):e00593-19. [PubMed] [Google Scholar]
31. Mohammed RS, Al-Mathkhury HJ. The ability of *Staphylococcus aureus* to establish biofilm on acrylic, plastic, and metallic denture materials. Iraqi J Sci. 2023;64(2):546-59.
32. Mussa AA, Al-Mathkhury HJ. Incidence of ciprofloxacin-resistant of methicillin resistant *Staphylococcus aureus* isolated from Iraqi patients. Iraqi J Sci. 2018;59(3A):1225-30.
33. 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. [PubMed] [Google Scholar]
34. O'Gara JP. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and *Staphylococcus aureus.* FEMS Microbiol Lett. 2007;270(2):179-88. [PubMed] [Google Scholar]
35. Parvin MS, Ali MY, Talukder S, Nahar A, Chowdhury EH, Rahman MT, Islam MT. Prevalence and multidrug resistance pattern of methicillin resistant *S. aureus* isolated from frozen chicken meat in Bangladesh. Microorganisms. 2021;9(3):636. [PubMed] [Google Scholar]
36. Peyrusson F, Varet H, Nguyen TK, Legendre R, Sismeiro O, Coppée JY, Wolz C, Tenson T, Van Bambeke F. Intracellular *Staphylococcus aureus* persisters upon antibiotic exposure. Nat Commun. 2020;11(1):2200. [PubMed] [Google Scholar]
37. Pohl K, Francois P, Stenz L, Schlink F, Geiger T, Herbert S, Goerke C, Schrenzel J, Wolz C. CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression. J Bacteriol. 2009;191(9):2953-63. [PubMed] [Google Scholar]
38. Pournajaf A, Ardebili A, Goudarzi L, Khodabandeh M, Narimani T, Abbaszadeh H. PCR-based identification of methicillin-resistant *Staphylococcus aureus* strains and their antibiotic resistance profiles. Asian Pac J Trop Biomed. 2014;4(Suppl 1):S293-7. [PubMed] [Google Scholar]
39. Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S. Biofilm formation and sloughing in Serratia marcescens are controlled by quorum sensing and nutrient cues. J Bacteriol. 2005;187(10):3477-85. [PubMed] [Google Scholar]
40. Rivera FE, Miller HK, Kolar SL, Stevens Jr SM, Shaw LN. The impact of CodY on virulence determinant production in community-associated methicillin-resistant *Staphylococcus aureus*. Proteomics. 2012;12(2):263-8. [PubMed] [Google Scholar]
41. Rohde H, Frankenberger S, Zähringer U, Mack D. Structure, function and contribution of polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. Eur J Cell Biol. 2010;89(1):103-11. [PubMed] [Google Scholar]
42. Scientific TF. Real-time PCR handbook.(2016). Dostupno na; 2017.
43. Shinde S, Lee LH, Chu T. Inhibition of biofilm formation by the synergistic action of EGCG-S and antibiotics. Antibiotics (Basel). 2021;10(2):102. [PubMed] [Google Scholar]
44. Shrestha LB, Bhattarai NR, Rai K, Khanal B. Antibiotic resistance and mecA gene characterization of coagulase-negative *Staphylococci* isolated from clinical samples in Nepal. Infect Drug Resist. 2020;13:3163-9. [PubMed] [Google Scholar]
45. Stobie N, Duffy B, McCormack DE, Colreavy J, Hidalgo M, McHale P, Hinder SJ. Prevention of *Staphylococcus epidermidis* biofilm formation using a low-temperature processed silver-doped phenyltriethoxysilane sol–gel coating. Biomaterials. 2008;29(8):963-9. [PubMed] [Google Scholar]
46. Thormann KM, Saville RM, Shukla S, Spormann AM. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. J Bacteriol. 2005;187(3):1014-21.

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505

[PubMed] [Google Scholar]
47. Tille PM. Bailey & Scott's diagnostic microbiology - e-book. Elsevier Health Sciences; 2021.
48. Udo EE. Community-acquired methicillin-resistant *Staphylococcus aureus*: the new face of an old foe? Med Princ Pract. 2013;22(Suppl 1):20-9. [PubMed] [Google Scholar]
49. Urushibara N, Aung MS, Kawaguchiya M, Kobayashi N. Novel staphylococcal cassette chromosome mec (SCC*mec*) type XIV (5A) and a truncated SCC*mec* element in SCC composite islands carrying *speG* in ST5 MRSA in Japan. J Antimicrob Chemother. 2020;75(1):46-50. [PubMed] [Google Scholar]
50. Vestby LK, Grønseth T, Simm R, Nesse LL. Bacterial biofilm and its role in the pathogenesis of disease. Antibiotics (Basel). 2020;9(2):59. [PubMed] [Google Scholar]
51. Waters NR, Samuels DJ, Behera RK, Livny J, Rhee KY, Sadykov MR, Brinsmade SR. A spectrum of CodY activities drives metabolic reorganization and virulence gene expression in *Staphylococcus aureus*. Mol Microbiol. 2016;101(3):495-514. [PubMed] [Google Scholar]

ISSN: 0019-5138
DOI: https://doi.org/10.24321/0019.5138.202505