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Biofilm-Associated Gene Expression in *Staphylococcus aureus* is Lineage Dependent

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Abstract

Staphylococcus aureus ranks among the most formidable pathogens in human and veterinary settings; its propensity to establish persistent biofilms underlies many chronic infections and recurrent treatment failures. Here, we measured the transcription levels of several biofilm-related genes fnbA, fnbB, clfB, clfA, sarA, sigB, and gyrA, across seven robust biofilm-forming clinical isolates, representing sequence types ST2826, ST97, ST2454, and ST6, in static culture. Quantitative data were normalized to the gmk housekeeping gene and interpreted by the 2^-ΔCt computation. Distinct lineage-specific gene signatures emerged: ST2826 isolates favored an adhesin-dominant response, primarily fnbA and clfA; ST97 showed a devotion to regulatory networks with a pronounced sarA bias; ST2454 leaned towards adhesion yet exhibited diminished regulators; and ST6 presented a balanced response, treating regulators and adhesins equally. The observed transcription profiles corresponded well to the biofilm masses measured by the crystal violet method, confirming that the clonal lineage itself establishes the relative direction of adhesion cues, regulatory feedback, or their syndromic balance. Dissecting these lineage-tethered decision circuits elucidates the genomic and phenotypic divergences under the S. aureus biofilm umbrella and should polish the design of selective anti-biofilm strategies in the clinical combat against intractable infections.

Keywords: Staphylococcus aureus , Gene Expression, Biofilm

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Introduction

Staphylococcus aureus stands as a key opportunistic pathogen impacting both human and veterinary health, giving rise to a diverse array of clinical manifestations that include superficial dermal infections, pneumonia, endocarditis, and sepsis (1). In livestock, the bacterium is widely acknowledged as the principal cause of bovine mastitis, resulting in pronounced veterinary and economic repercussions(2). A distinctive and clinically relevant hallmark of the species is biofilm development, a mechanism that results in the assembly of multicellular structures encased within a self-produced extracellular polymeric matrix, thereby endowing the pathogen with pronounced capacity to evade host immunological responses and resist applied antimicrobial therapies (3 ,4). Consequently, biofilm formation emerges as a decisive survival and persistence strategy that facilitates both nosocomial and agricultural spread of *S. aureus*.

Bacterial biofilm formation by *Staphylococcus aureus* is within a system of numerous interactive molecular components, comprising adhesins, transcriptional regulators, and extracellular matrix constituents. Initial colonization is mediated preferentially by fibronectin-binding proteins, specifically *fnbA* and *fnbB*, and by clumping factors of the *clfA* locus, particularly *clfB*, anchoring the organism to epithelial and synthetic surfaces (5, 6). The alternative sigma factor *sigB* and the global regulator *sarA* coordinately provoke transcriptional programs for biofilm inotropic tissues, guiding adaptive constancy to ambient stress. The matrix-associated factor encoded by *calfA* amplifies cell-to-cell cohesion, an effect amplified in the stratum of thick biofilm architecture by other carbohydrate-associated determinants (7) . Meanwhile, the chromosomal *gyrA* mutation, a random amplification factor, is frequently applied as an endogenous calibrator in quantitative transcriptional assays (8).

A growing body of literature has shown that the transcriptional profile of biofilm-associated genes is heterogeneous, exhibiting variation not only among distinct isolates but also across clonal lineages that have undergone gradual evolutionary divergence and subsequent accommodation to specific ecological niches. Within the S. aureus multilocus sequence typing (MLST) schema, for instance, sequence types (STs) possess divergent virulence and regulatory characteristics that modulate the proficiency with which biofilms are established (9, 10). This marked variability emphasizes the critical need to interrogate lineage-restricted transcriptional circuits, especially when the strains under consideration are derived from zoonotic and veterinary reservoirs, in which host–pathogen dynamics are likely to diverge markedly from those documented in clinical human isolates (11).

Efforts to elucidate the adaptive capacities of *Staphylococcus aureus* biofilms in chronic and recurrent infections have advanced, yet systematic, quantitative analyses of core biofilm-associated regulators and structural determinants across genetic lineages remain sparse(12,4, 10,13). To fill this void, the study quantified the expression of *fnbA*, *fnbB*, *clfB*, *sigB*, *sarA*, *gyrA*, and *clfA* in a panel of *S. aureus* isolates grown under rigidly controlled static conditions. After normalization to the housekeeping transcript *gmk*, transcript levels were evaluated by the 2^−ΔCt

method, exploiting the resulting ΔCt values to derive isolate-specific and sequence type–specific transcriptional fingerprints. The integrative analysis presented reveals the lineage-dependent deployment of adhesive factors, regulatory circuits, and extracellular matrix constituents, thus informing the genetic underpinnings of biofilm structural and functional diversity and chronic adaptability.

Materials And Methods

Isolates and culture media

Twenty *Staphylococcus aureus* isolates were used in this study. These were collected in another study from clinical, subclinical mastitis cases, and milkers' hands (data not shown, under publication). In addition, *S. aureus* MRSA ATCC 43300 was used as a control. *Staphylococcus aureus* isolates were revitalized from frozen stocks on brain heart infusion agar and sub-cultured once in biofilm broth at 37 °C with shaking (180 rpm). The biofilm medium consisted of brain heart infusion (BHI) that contained 1% L- glucose and was used as a culture media for *S. aureus* biofilm formation in vitro (14,15) .

Biofilm formation

Biofilm formation was examined according to the previously established protocol, with minor adaptations (3). Fresh aerobic cultures, grown overnight in biofilm medium derived from individual colonies, were diluted in sterile biofilm medium to an approximate optical density of 0.05 (OD630). Two hundred microliters of this diluted suspension was dispensed into each well of NEST® 96-well flat-bottom cell-culture plates (NEST, China). Plates were held at 37°C without agitation for 22 hours. Before biofilm harvest, the optical density (A630) of each well was documented. Wells were rinsed thrice with distilled water and subsequently subjected to heat fixation at 60°C for 60 minutes. Plates were then allowed to equilibrate to ambient temperature before the biofilm was stained for 5 minutes with 0.1% crystal violet. After a triple wash with deionized water, the crystal violet was solubilized in a 33% acetic acid solution. The resultant suspension was mixed, and the absorbance was recorded at 570 nm using a BioTek 800TS microplate reader. Absorbance values were first corrected against an acetic acid blank and subsequently normalized to the optical densities of the harvested cultures (3). The strain *Staphylococcus aureus* MRSA ATCC 43300 was employed as the experimental control throughout the investigation.

Quantitative PCR

Growth conditions:

Seven isolates were selected for the QPCR experiment based on their higher capacity to form biofilm compared to other isolates. These isolates were classified into sequence types (ST) based on multi-locus sequencing typing (MLST) profiling (Under publication). Isolates in the QPCR

experiment included isolates 65 (ST2826), 176 (ST2826), 57 (ST97), 214 (ST97), 236 (ST97), 68 (ST2454), 218 (ST6), and *Staphylococcus aureus* MRSA ATCC 43300, which was used as a control.

For the biofilm experiment, overnight cultures of *S. aureus* isolates were diluted into fresh biofilm medium as described earlier in the biofilm formation assay (final OD630 ≈ 0.05). Ten mL of these adjusted cultures were dispensed in triplicate into each well of a 6-well tissue-culture-treated polystyrene plate (flat-bottom) (NEST, China). Plates were incubated statically at 37 °C for 22 h to allow formation of mature surface-attached biofilms. At the endpoint, planktonic supernatants were gently aspirated, and wells were rinsed once with RNase-free PBS to remove loosely attached cells while retaining the adherent biofilm biomass. To each well, 3 mL TRIzol® reagent (Geneaid Biotech Ltd, China) was added, and the biofilm attached layer was scraped with sterile spatulas and transferred to RNase-free beads containing tubes (100 µm size silica beads) and homogenized by vortexing for 10 minutes. All isolates were processed in parallel and under identical conditions; no treatment or separate planktonic control was included.

RNA extraction (TRIzol)

Adherent biofilm cells were immediately lysed in-well with TRIzol® reagent (Geneaid Biotech Ltd , China) pre-warmed to room temperature. After a 5 min room-temperature incubation, chloroform (0.2 mL per 1 mL TRIzol) was added, samples were shaken vigorously for 15 seconds, incubated 3 min, and centrifuged at 12,000 ×g, 15 min, at 4 °C. The aqueous phase was transferred to a new tube, and RNA was precipitated with isopropanol (0.5 mL per 1 mL TRIzol) for 10 min at room temperature, then centrifuged at 12,000 ×g, 10 min, 4 °C. Pellets were washed with 75% ethanol, briefly air-dried, and resuspended in RNase-free water.

DNase treatment and RNA QC

Residual genomic DNA was removed with RNase-free DNase I (Promega, USA). RNA integrity and purity were assessed by A_{260}/A_{280} (NanoDrop one, Thermo Fisher Scientific, USA).

cDNA synthesis

For each sample, 0.5 µg total RNA was reverse-transcribed following the manufacturer's instructions, using a random-hexamer/oligo(dT) mix and a high-capacity reverse transcriptase in 20 µL reactions (GoScript™ Reverse transcription kit, Promega, USA). Parallel no-RT controls (minus reverse transcriptase) were prepared to monitor gDNA contamination.

QPCR assays

Targeted genes included *fnbA*, *fnbB*, *clfB*, *sigB*, *sarA*, *gyrA*, calfA, with *gmk* as the housekeeping gene (Ogonowska & Nakonieczna, 2020). Primers used in qPCR are listed in **Table 1**. Reactions were assembled in 20 µL volumes using SYBR Green master mix, 400 nM primers, and 2 µL of diluted cDNA. Cycling was performed on a real-time thermocycler with a standard protocol (95

°C 5 min; 40 cycles of 95 °C 15 s, 60 °C 30 s, 72 °C 30 s; followed by melt-curve). Each (isolate × gene) was run in technical replicates. No-template controls (NTC) and no-RT controls were included for each primer pair. Primer specificity was confirmed by single-peak melt curves and the expected amplicon size. Primer efficiency was verified to be within acceptable bounds (≈90–110%) and equivalent among targets used for relative quantification.

Data processing and normalization

For each (isolate × gene), technical replicates were averaged to a mean Ct (replicate SD recorded). Expression was normalized to *gmk* on a per-isolate basis (16,17):

- ΔCt = Ct(target) − Ct(*gmk*)
- Relative expression = 2^-ΔCt

No external calibrator or planktonic control was used; values therefore represent within-isolate relative abundance under biofilm conditions.

Table 1 : primers used in the Quantitative real-time PCR (qRT-PCR). Gene targets included *fnbA*, *fnbB*, *clfB*, *sigB*, *sarA*, *gyrA*, calfA, with *gmk* as a house keeping gene.

Genes	Sequence	Size of product	Reference
clfA	F:5′-ATTGGCGTGGCTTCAGTGCTTG-3′ R: 5′-GCTTGATTGAGTTGTTGCCGGTGT-3′	357 bp	(18)
clfB	F:5′-TGGCGGCAAATTTTACAGTGACAGA-3′ R: 5′-AGAAATGTTCGCGCCATTTGGTTT-3′	404 bp	(17)
fnbA	F: 5′-GCGGCCAAAATGAAGGTCAACA-3′ R: 5′-TCTGGTGTTGGCGGTGTTGGAG-3′	205 bp	(19)
fnbB	F: 5′-CAGAAGTACCAAGCGAGCCGGAAA-3′ R:5′-CGAACAACATGCCGTTGTTTGTTGA-3′	258 bp	(17)
Sar A	F:5’-GCTGTATTGACATACATCAGCGAAA-3’, R:5’-CGTTGTTTGCTTCAGTGATTCGT-3’	250 bp	(17)
Sig B	F:5’-TCGCACTCTTTA TTGATAGTCGCTACGAG-3’ R:5’-TGC GACAAGAACTACTGCTGCGTTAAT-3’	86 bp	(17)
gmk	F: 5’-TCGTTTTATCAGGACCATCTGGAGTAGGTA-3’ R: 5’-CATCTTTAATTAAAGCTTCAAACGCATCCC-3’	153 bp	(20)

Results

Biofilm formation

The crystal violet binding assay demonstrated variable biofilm formation capacity among the tested *S. aureus* isolates (n = 20). Seven isolates—65 (ST2826), 176 (ST2826), 57 (ST97), 214 (ST97), 236 (ST97),

68 (ST2454), 218 (ST6) together with the positive control *S. aureus* MRSA ATCC 43300, produced markedly stronger biofilms compared with the remaining isolates (Figure 1). These data indicate that biofilm-forming ability is not uniform across isolates, but is enriched in specific ST lineages (ST2826, ST97, ST2454, and ST6).

Gene expression:

Quantitative PCR analysis of the seven strong biofilm-producing isolates revealed marked heterogeneity in the transcriptional patterns of biofilm-associated genes under 22 h static conditions on polystyrene plates. Expression levels were normalized to the housekeeping gene *gmk* and calculated as 2^-ΔCt. Since no planktonic or external calibrator was included, the data reflect within-isolate relative transcript abundance under biofilm-inducing conditions Table 2.

Adhesin-related genes

Expression of *fnbA* and *calfA* was generally elevated, consistent with their role in surface attachment and matrix production. Notably, isolates 65 (ST2826) and 214 (ST97) displayed the strongest *calfA* expression (3.46 and 2.08, respectively), while *fnbA* transcription was particularly dominant in isolates 65 (ST2826), 68 (ST2454), and 218 (ST6). *ClfB* expression was modest across most isolates, with isolate 176 (ST2826) being an exception (1.89). *FnbB* remained consistently weakly transcribed, with negligible expression in ST2454. Table 2, Figure ***2*** **(**Heatmap of relative expression (2^-ΔCt) of biofilm-associated genes across *S. aureus* isolates).

Regulatory genes

Analysis of the locus encoding the global regulator *sarA* revealed a transcriptional pattern closely tied to clonal lineage. Among the lineages studied, ST97 isolates, particularly strains 57 and 214, demonstrated elevated *sarA* transcripts (4.97 and 3.17 relative to control, respectively), indicating a lineage-specific activation of a *sarA*-driven biofilm developmental program. Conversely, ST2454 isolate 68 exhibited markedly diminished *sarA* expression (0.04), despite the concomitant detection of robust *fnbA* transcript levels, pointing to a regulatory dissociation between fibronectin-binding protein expression and *sarA* activation in this clonal group.

Auxiliary transcriptional factors *sigB* and *gyrA* were consistently found in low abundance across the entire cohort. The *sigB* locus, a well-characterized mediator of stress response and virulence, exhibited a narrow expression range (0.02 to 0.28), suggesting minimal contribution to phenotypic adaptation under the static assay conditions. Similarly, *gyrA* transcriptional levels (0.05 to 0.13) were uniformly low, confirming its role as a stable internal control under the experimental paradigms employed.

Lineage-specific patterns

As shown in the second figure, the expression data represented on the heatmap reveal the presence of specific lineage transcriptional signatures that correlate with the phenotypic results from the biofilm assay

shown in the first figure. The ST2826 isolates (65 and 176) in particular displayed a strong activation profile with *fnbA* and *clfA*. This transcriptional bias aligns with the strong biofilm-forming usage, supporting the idea that these members of the lineage primarily depend on on the adhesion strategy to biofilm environment surfaces and host protein attachments. On the contrary, for the ST97 members (57, 214, and 236) biofilm expression was dense with a *sarA*–dominant transcription profile. These members, despite low expression of the adhesion-associated genes able to form biofilms, suggesting that the *sarA* control on biofilms is a compensatory mechanism. In the case of ST2454 isolate (68) biofilm formation was strong but poorly controlled. This suggests that the isolate relies on the expression of *fnbA* and *clfA* without strong supporting regulation, which is consistent with the adhesin-skewed profile of the isolate. The ST6 isolate (218) displayed biofilm with a moderate balanced profile for transcriptional *fnbA*, *clfB*, *clfA* and *sarA* with biofilm formation. The change in strategy outlined before resulted in distinct intermediate biofilm phenotypes, which signifies that the combination of both adhesins and biofilm regulatory elements offers the adaptability needed in biofilm-promoting conditions.

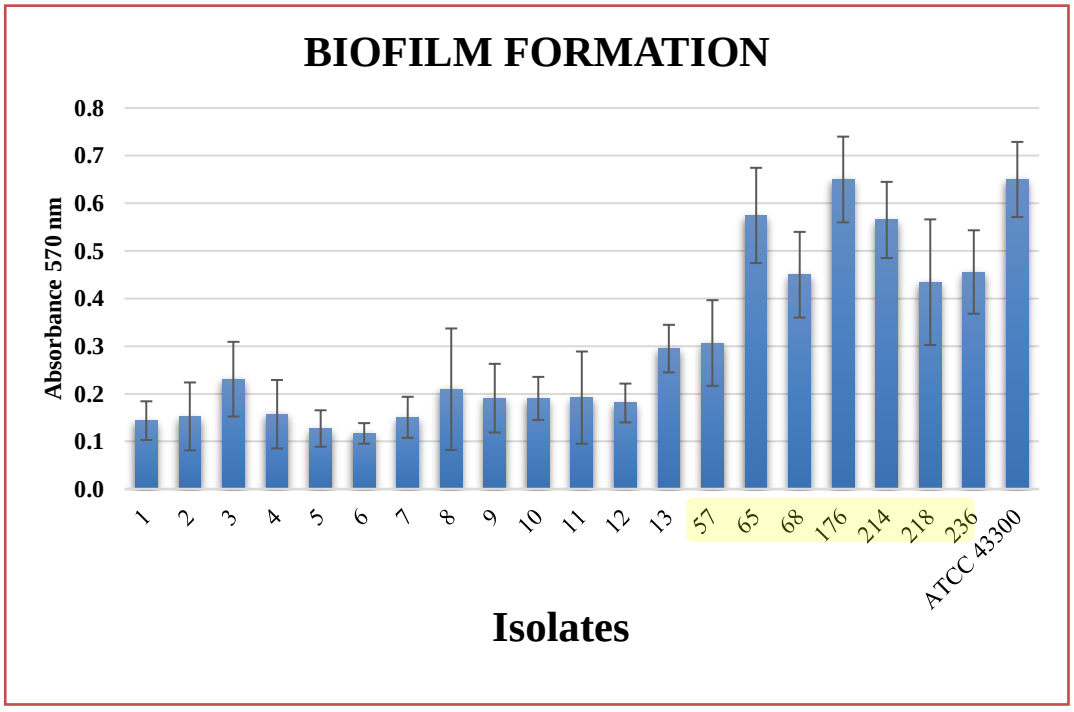

Figure (1): Crystal violet binding assay displaying biofilm formation capability of different *S. aureus* local isolates (n=20). Strains used in the QPCR experiment are highlighted in yellow. Values represent the mean of two independent experiments; error bars indicate the standard deviation. *S. aureus* ATCC 29213, ATCC 43300 were used as a control.

Table 2: Gene expression patterns of *fnbA, fnbB, clfB, sigB, sarA, gyrA*, and calfA across multiple *S. aureus isolates* cultivated under standardized static biofilm conditions. Expression levels were normalized to the housekeeping gene *gmk* and calculated as relative expression 2^-ΔCt .

Isolate (ST)	calfA	*clfB*	*fnbA*	*fnbB*	*sarA*	*sigB*	*gyrA*
65 (ST2826)	3.46	0.16	3.21	0.09	0.45	0.02	0.10
176 (ST2826)	0.33	1.89	1.50	0.22	0.51	0.24	0.11
57 (ST97)	0.40	0.11	0.25	0.08	4.97	0.06	0.13
214 (ST97)	2.08	0.14	0.61	0.05	3.17	0.04	0.09
236 (ST97)	0.30	0.23	0.30	0.04	1.06	0.09	0.07
68 (ST2454)	0.61	0.15	2.23	0.00	0.04	0.10	0.05
218 (ST6)	1.17	1.25	2.46	0.17	1.53	0.28	0.12

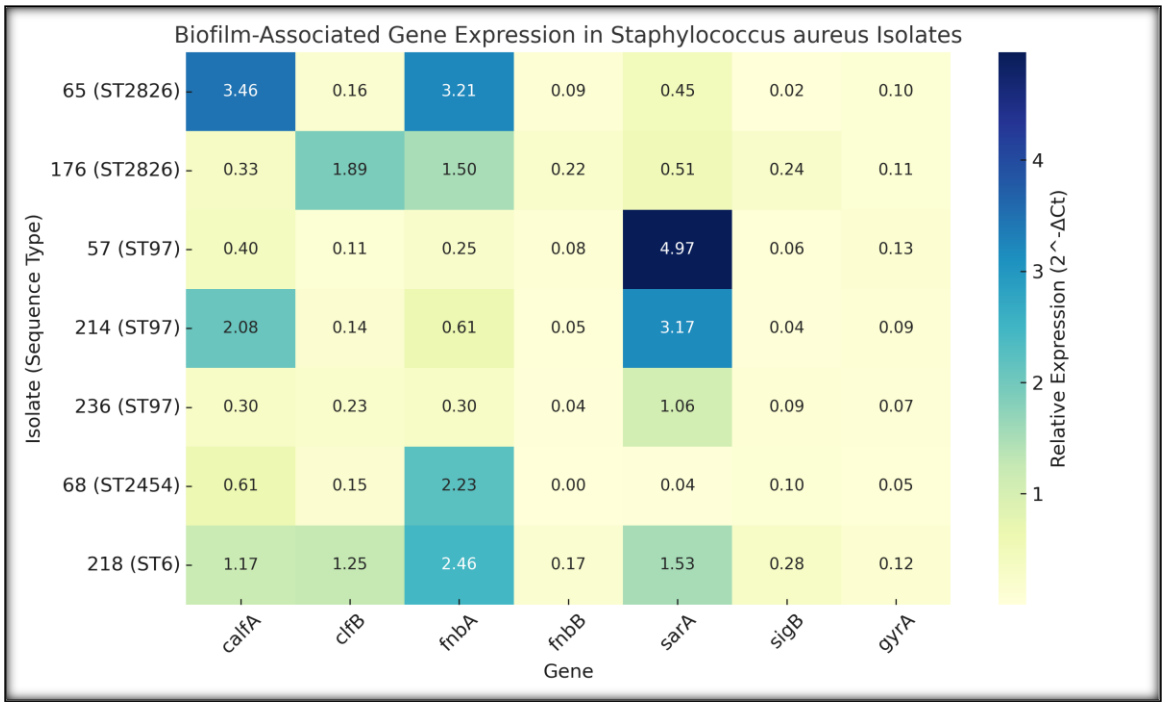

Figure 2. Heatmap showing relative expression (2^-ΔCt) of biofilm-associated genes across *S. aureus* isolates.

Discussion

This study highlights how clonal lineage shapes the transcriptional architecture of biofilm-associated genes in *S. aureus*. By combining phenotypic assays with qPCR data, we demonstrate that biofilm strategies are not uniform but instead vary between adhesin-dominant, regulator-dominant, and balanced profiles.

ST2826 isolates showed high levels of *fnbA* and *clfA* expression, consistent with an adhesion-focused mechanism. This could facilitate strong attachment to host proteins and abiotic surfaces, which is critical during the early phases of biofilm development (21). The ST2454 isolate also showed a similar adhesin profile but with minimal *sarA* activity, signifying a structurally influenced but less tightly structured strategy. While effective at producing biomass, such skewed adhesin-expression may decrease flexibility under environmental stress (16, 14). The ST97 isolates exhibited elevated *sarA* transcription while only recording low-level expression of classical adhesin genes, a notable regulator-over-adhesin model. Such a pattern corroborates prior observations linking *sarA* to biofilm association. *SarA* is a global transcriptional regulator that controls the icaADBC operon gene cluster and other genetic elements related to biofilm formation (7) *SarA* mutation caused reduction of biofilm biomass, highlighting its important role in the Biofilm formation process (16). In this context, the ST97 appears to rely on *sarA* mediated regulatory response, which in turn improves its phenotypic adaptability, routing downstream circuits toward the production of protective extracellular polymeric matrix and thus enhancement of stressful environment tolerances (7,16,22).

The ST6 lineage expressed both adhesions and *sarA* at a moderate level, producing an intermediate phenotypic response. This restrained presentation aligns with a postulated state of trade-off, promoting robust surface colonization and concomitant capacity to modify downstream expression. Such phenotype cession toward moderation echoes tactics found in isolates that can affect multispecies and environments (23).

The auxiliary genes *sigB* and *gyrA* exhibited low yet consistently measurable transcription. Although *sigB* is known to exert regulatory influence over both stress response and virulence(24,25) , its transcriptional output across all isolates was subdued, implying that its involvement is limited under the static biofilm environment employed in the present study. In a parallel manner, *gyrA* demonstrated a persistently low and invariant expression profile, thereby substantiating its validity as a housekeeping control. This stability

enables us to assume that the differential expression observed in adhesins (*fnbA*, calfA, *clfB*) and regulators (*sarA*) seen in our study is true lineage-specific transcriptional program rather than stochastic noise or artefactual variation. These findings underscore that biofilm regulation in *S. aureus* is lineage-dependent. Adhesin-forward lineages may be more susceptible to therapies targeting surface binding proteins, whereas regulator-forward lineages may require interventions directed at global regulatory pathways. This helps explain the variable outcomes of anti-biofilm strategies reported across different strain collections (26).

The lack of planktonic controls limits explanation to within-biofilm expression rather than induction ratios. Sampling at a single time point (22 h) also limits understanding the sequential dynamics of gene regulation. Forthcoming studies should include planktonic groups to compare with, multi-timepoint expression profiles, and an extended gene list (e.g., *icaA/icaD, psm, atl*) to understand the full complexity of biofilm regulation. Functional connection of gene expression with tolerance assays, such as antibiotic endurance or immune evasion, will further reinforce the clinical and veterinary significance of these findings(27,28).

Conclusions

Distinct lineage-specific gene signatures emerged: ST2826 isolates favored an adhesin-dominant response, primarily fnbA and clfA; ST97 showed a devotion to regulatory networks with a pronounced sarA bias; ST2454 leaned towards adhesion yet exhibited diminished regulators; and ST6 presented a balanced response, treating regulators and adhesins equally.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee

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التعبير الجيني المرتبط بتكوين الغشاء الحيوي في جرثومة المكورات العنقودية

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الخلاصة

تُعدّ المكورات العنقودية الذهبية من أكثر المسببات الممرضة شراسةً في البيئات البشرية والبيطرية، إذ يُعزى الكثير من حالات العدوى المزمنة وفشل العلاج المتكرر إلى قدرتها العالية على تكوين الأغشية الحيوية. في هذه الدراسة، تم قياس مستويات التعبير الجيني لجينات مرتبطة بتكوين الغشاء الحيوي، وهي fnbA :و fnbBو clfBو clfAو sarAو sigBو gyrA، وذلك في سبع عزلات سريرية قوية التكوين للغشاء الحيوي، تعود إلى أنماط تسلسلية مختلفة شملت ST2826 ، ST97، ST2454، ST6 تحت ظروف التنمية بدون حركة. تم تنسيب القيم الكمية نسبةً إلى الجين gmk، وحُسبت النتائج باستخدام معادلة .2^- Δ Ct . أظهرت النتائج أنماطًا مميزة خاصة بكل سلالة؛ إذ اتسمت عزلات ST2826 بنمط يعتمد بشكل أساسي على جينات الالتصاق (clfA fnbA) مما يشير إلى استجابة مسيطر عليها من قبل عوامل الالتصاق، بينما أظهرت سلالات ST97 ميلاً نحو تحفيز الشبكات التنظيمية الجينية مع بروز واضح لجين sarA، في حين كانت عزلات ST2454 تميل إلى الالتصاق مع انخفاض في نشاط الجينات المنظمة، أما ST6 فقد أظهرت توازناً في التعبير بين الجينات المنظمة واللاصقة. تتوافق هذه الأنماط الجينية مع كميات الغشاء الحيوي المقاسة بصبغة البلّورة البنفسجية، مما يؤكد أن النمط التسلسلي (ST)يلعب دورًا حاسمًا في تحديد الاتجاه النسبي لاستجابات الالتصاق والتنظيم أو توازنها المشترك. إن تحليل هذه الدوائر التنظيمية الخاصة بكل سلالة يوضح التباينات الجينية والمظهرية ضمن إطار تكوين الأغشية الحيوية في المكورات العنقودية الذهبية، ويساهم في تطوير استراتيجيات انتقائية مضادة للأغشية الحيوية ضمن الجهود السريرية لمكافحة العدوى المزمنة والمستعصية.

الكلمات المفتاحية: المكورات العنقودية الذهبية، التعبير الجيني، الأغشية الحيوية.