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High hemolytic activity in *Staphylococcus aureus* t1081/ST45 due to increased hla protein production and potential RNAIII-independent regulation

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ABSTRACT

Background: α-Hemolysin, encoded by *hla*, is a major virulence factor of *Staphylococcus aureus*. Sequence type (ST) 45 is a globally spread clone with increasing clinical prevalence in Taiwan. Our previous study showed that among the CC45 isolates, the *spa* type t1081 isolates presented greater hemolytic activity.
Materials and methods: The hemolytic activity of 67 CC45 isolates (44 t1081 and 23 non-t1081) from clinical blood cultures was assessed using rabbit red blood cells. The sequences of *hla* and its upstream regulatory regions and RNAIII were compared between the two groups. The expression of *hla* and its regulators RNAIII, *mgrA*, and *saeR* was analyzed via qRT−PCR, while Hla protein levels were measured via Western blotting.
Results: Compared with non-t1081 isolates, t1081 isolates presented increased hemolytic activity. No significant differences in *hla* sequences, upstream regulatory regions, or gene expression levels were detected between the two groups. The expression of the transcriptional regulators *mgrA* and *saeR* was also similar between the two groups. Western blotting revealed increased Hla protein in the t1081 isolates. However, neither the sequence or expression of RNAIII, a regulator of *hla* at both the transcriptional and posttranscriptional levels, differed between the groups.
Conclusion: Our study revealed that, compared with other CC45 isolates, the t1081/ST45 isolates presented greater hemolytic activity. This heightened activity was due mainly to increased Hla protein levels. Moreover, the higher translation levels may be independent of the known regulator RNAIII, indicating a potential RNAIII-independent mechanism for Hla regulation.

1. Introduction

Staphylococcus aureus, a common commensal bacterium, is also a significant opportunistic pathogen capable of causing a broad range of infections. This bacterium is notorious in clinical settings because of its prevalence, colonizing approximately 30 % of healthy individuals, and its ability to thrive in both healthcare environments and among human and animal hosts.[1] *S. aureus* can opportunistically lead to numerous infections, affecting both community and healthcare settings, including those of the skin, soft tissues, as well as bones and joints.[2] The role of virulence factors in *S. aureus* infections is crucial for its pathogenicity.[1,3,4] These factors enable the bacterium to adhere to host tissues effectively, secrete exotoxins and enzymes that damage host cells, and evade or neutralize the host immune response. By facilitating initial colonization, supporting bacterial growth and spread, and helping bacteria persist within the host, these virulence factors significantly contribute to the development and progression of infections.

Among the key virulence factors are polypeptides that damage host cell membranes, including four hemolysins (α, β, γ, and δ). The α-hemolysin and various bicomponent leukocidins, such as γ-hemolysin,

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Panton-Valentine leukocidin (PVL), and LukED, are notable pore-forming toxins (PFTs). Additionally, *S. aureus* produces β-hemolysin, which functions as a neutral sphingomyelinase, and phenol-soluble modulins (PSMs), a family of small amphipathic peptides. In addition, these bacteria secrete a range of enzymes, including nucleases, proteases, lipases, hyaluronidases, and collagenase, which are involved primarily in breaking down host tissues into nutrients that support bacterial growth. Furthermore, certain strains of *S. aureus* produce additional exoproteins, such as toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins, exfoliative toxins, and leukocidin. These toxins are known for their potent effects on immune cells and are believed to play a role in suppressing the host immune response.

α-Hemolysin, also referred to as α-toxin and encoded by *hla*, is widely regarded as the most significant and well-characterized toxin produced by *S. aureus*, particularly because of its role in pathogenesis. It forms pores with heptameric structures on the target cell membrane. These pores allow rapid efflux of K^+ and other small molecules, as well as influx of Na^+, Ca^{2+}, and molecules with a molecular weight less than 1000.[5] The osmotic swelling of cells ultimately leads to their rupture. It also disrupts epithelial and endothelial integrity by dismantling adherens junctions and impairing the cytoskeleton.[6] α-Hemolysin is known for its pronounced ability to lyse rabbit red blood cells (rRBCs) while having a comparatively mild impact on human erythrocytes.[4] This toxin serves as a potent cytolysin, impacting a wide range of human cell types, including leukocytes, epithelial cells, endothelial cells, and various hematopoietic lineage cells to contribute to immune evasion and pathogenicity.[7–9] The importance of α-hemolysin in *S. aureus* infections has been confirmed through research using animal models, including those for pneumonia, skin infections, brain abscesses, and sepsis.[4,10–13]

The production of α-hemolysin in *S. aureus* is intricately regulated by multiple factors, ensuring that the bacteria can adapt to varying environmental conditions and host defenses.[14] The *agr* (accessory gene regulator) system is a primary regulator, where RNAIII, the main effector molecule, enhances *hla* at the transcriptional level by modulating transcription initiation or at the posttranscriptional level by stabilizing its mRNA and promoting ribosome binding.[15,16] The SaeRS two-component system (TCS) also influences *hla* expression by directly binding to the consensus SaeR-binding site located upstream of the *hla* promoter.[17] Additionally, MgrA regulates *hla* expression either through direct binding to the *hla* promoter or via an RNAIII-dependent pathway.[18] Moreover, several regulators repress *hla*, such as SarA, SarR, SarT, SarH1, and Rot, either through RNAIII-dependent or RNAIII-independent pathways.[14]

Our previous study indicated that t1081 isolates presented stronger hemolytic activity than the other CC45 isolates did.[19] To investigate its potential mechanisms, this study further analyzed the hemolysin genes and their regulators.

2. Materials and methods

2.1. Bacterial isolates

A total of 67 *S. aureus* CC45 isolates isolated from blood cultures were obtained from the Bacteriology Laboratory at National Taiwan University Hospital (NTUH) as previously described.[19] Among these isolates, 56 were collected between January 2010 and August 2014, 4 were from the year 2005, and 7 were from the year 2000. All the isolates were identified using the VITEK 2 automated system (bioMérieux, France). Among these isolates, 45 were ST45, including 43 of *spa* type t1081, one of t026, and one of unknown type (UT2, which is one nucleotide different from t1081). Additionally, 21 isolates were ST508, and one was ST929. According to the results of previous studies, the isolates were separated into two groups: t1081 (containing UT2) and non-t1081.[19]

2.2. Hemolytic activity

The hemolytic activity assay was carried out as previously described.[20] Supernatants from 500 μl of overnight cultures were collected and mixed with an equivalent volume of 2 % rRBCs suspended in PBS in 96-well V-bottom plates. After a 15-min incubation at 37 °C, the rRBCs were centrifuged at 1000 rpm for 5 min, and the resulting supernatant was measured according to the OD410. Triton X-100 (1 %) and PBS containing 2 % bovine serum albumin were used to induce 100 % and 0 % hemolysis, respectively. A hemolytic unit (HU) was determined as the reciprocal of the highest dilution at which complete hemolysis was observed.

2.3. Detection and sequencing of hemolysin genes and RNAIII

The *hla*, *hlb*, *hld*, *hlg*, and *hlg-2* genes encoding the α-, β-, δ-, γ-, and γ variant hemolysins were detected via PCR as previously described[21] with the primers shown in Table 1. The full-length *hla*, along with its upstream region, was amplified via the primers hla_u233F and hla_d480R, and then sequenced. RNAIII was also amplified via PCR and subsequently sequenced using the primers 10845_722F and agrB_113R.

2.4. RNA extraction and quantitative reverse transcription PCR (qRT–PCR)

The isolates were cultured on tryptic soy agar (TSA) at 37 °C for 16 h, and the cultures were used to inoculate tryptic soy broth (TSB) at an OD600 of 0.05. The bacteria were then grown at 37 °C with shaking until they reached an OD600 of 0.4 in the early exponential phase. Bacteria were harvested and lysed with 10 μg/ml lysostaphin (Sigma–Aldrich, USA) at 37 °C for 1 h, after which RNA was extracted via the Invitrogen Purelink™ RNA Mini Kit (Thermo Fischer Scientific, USA). Two micrograms of total RNA was reverse transcribed with a TOOLS Quant II fast RT kit (BIOTOOLS Co., Ltd., Taiwan) according to the manufacturer's recommendations. qRT–PCR was performed with TOOLS 2X SYBR qPCR Mix (BIOTOOLS) using the primers described previously and listed in Tables 1.[22–24] To determine the relative expression, the cycle threshold (Ct) values of the housekeeping gene *gyrB* were used.[22] The expression level was calculated as $2^{-\Delta Ct}$, where ΔCt is the difference between the Ct values of the target gene and *gyrB* for each isolate.

2.5. SDS–PAGE and western blotting

The supernatants of overnight cultures were filtered through a 0.45 μm pore size syringe filter (Millipore Corporation, USA) and concentrated using an Amicon Ultra centrifugal filter (3k MWCO, Millipore). Equal amounts of protein were separated by 12 % SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5 % skim milk dissolved in 0.1 % Tween-20 Tris-buffered saline solution (TBST) for 1 h at RT and then incubated at RT with a 1:1000 dilution of mouse anti-Hla primary antibody (ab190467; Abcam, UK) or a 1:10000 dilution of anti-mouse IgG (ab205719; Abcam) secondary antibody conjugated to horseradish peroxidase for 1 h. Protein bands were visualized using the WesternBright ECL kit (Advansta Inc., USA), and the resulting images were analyzed with ImageJ version 1.54 software (National Institutes of Health). The levels of protein expression were quantified and corrected with a loading control with CL06 as a baseline.

2.6. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8 software (GraphPad Software Inc., USA). To assess the differences between the t1081 and non-t1081 isolates, an unpaired two-tailed Student's *t*-test was applied. Owing to the small sample size, the protein expression levels were analyzed using the Mann–Whitney *U* test. A p value of less

Table 1
Primers used in this study.

Primer	Sequences (5′ to 3′)	Target	References
Detection			
HLA-1	CTGATTACTATCCAAGAAATTCGATTG	*hla*	[21]
HLA-2	CTTTCCAGCCTACTTTTTTATCAGT		
HLB-1	GTGCACTTACTGACAATAGTGC	*hlb*	[21]
HLB-2-2	GTTGATGAGTAGCTACCTTCAGT		
HLD-1	AAGAATTTTTATCTTAATTAAGGAAGGAGTG	*hld*	[21]
HLD-2	TTAGTGAATTTGTTCACTGTGTCGA		
mpHLG-1	GTCAYAGAGTCCATAATGCATTTAA	*hlg*	[21]
mpHLG-2	CACCAAATGTATAGCCTAAAGTG		
mpHLG2-1	GACATAGAGTCCATAATGCATTYGT	*hlg-2*	[21]
mpHLG2-2	ATAGTCATTAGGATTAGGTTTCACAAAG		
Sequencing			
hla_u233F	TCAACTTTGACTAACCCTCG	*hla*	This study
hla_d480R	CCATTTGTCATCCTCCTTG		
10845_722F	AAGTAGAACAGCAACGCG	RNAIII	This study
agrB_113R	ACTGCTAAGACCTGCATC		
qRT-PCR			
RT-gyrB-F	CGACTTTGATCTAGCGAAAG	*gyrB*	[22]
RT-gyrB-R	ATAGCCTGCTTCAATTAACG		
RT-hla-F	CAATTTGTTGAAGTCCAATG	*hla*	[22]
RT-hla-R	GATCCTAACAAAGCAAGTTCTC		
OL4047	GCTCAAAGACAAGTTAATCGCTAC	*mgrA*	[23]
OL4048	CGTTTACAGGAGATTCATCCCA		
OL3116	ACCACAATAACTCAAATTCCTTAATACG	*saeR*	[23]
OL3117	GTTGAACAACTGTCGTTTGATGA		
RNAIII-RT-FWD	TAATTAAGGAAGGAGTGATTTCAATG	RNAIII	[24]
RNAIII-RT-REV	TTTTTAGTGAATTTGTTCACTGTGTC		

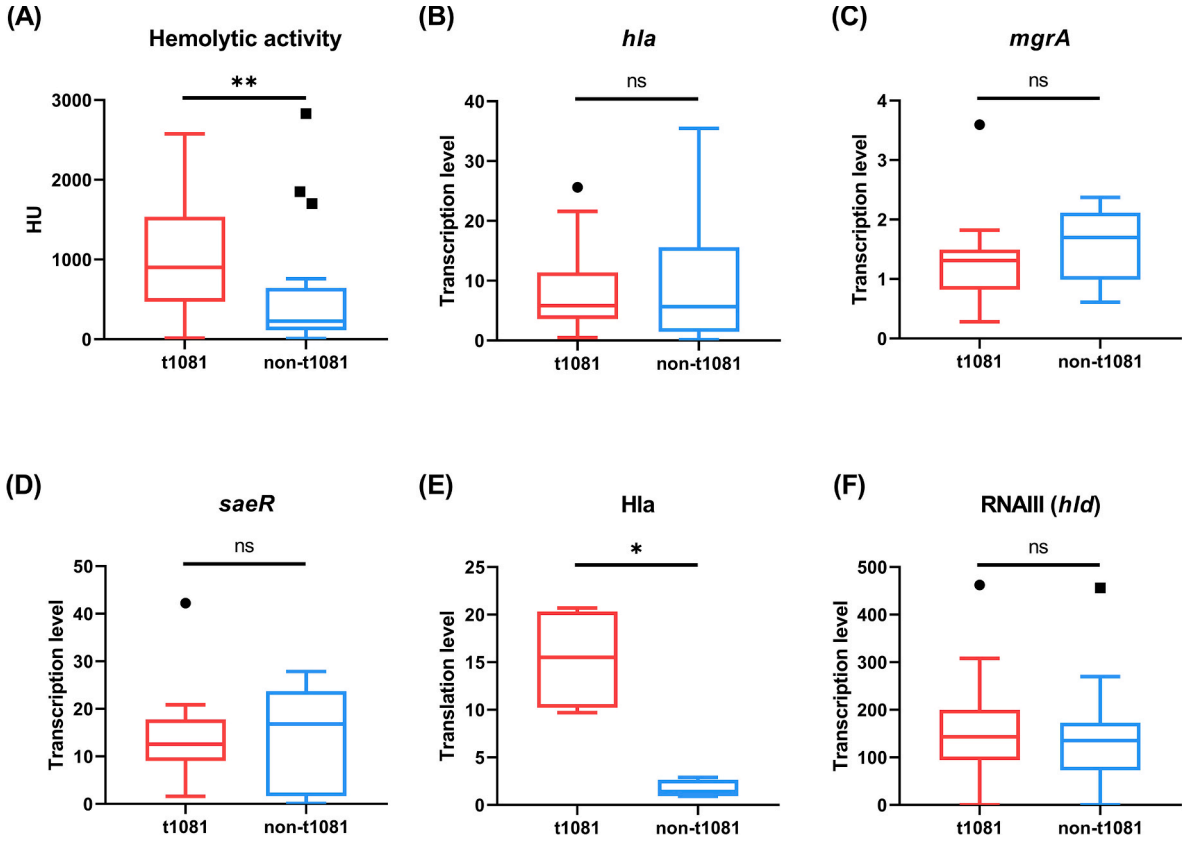

Fig. 1. Hemolytic activities and expression analysis of *hla* and regulators among the t1081 and non-t1081 isolates. (A) Hemolytic activity assay with rRBCs. (B) mRNA levels of *hla*. (C) mRNA levels of *mgrA*. (D) mRNA levels of *sarR*. (E) Protein levels of Hla detected by western blotting with anti-Hla antibody and normalized against the loading control for 8 selected isolates. The levels of Hla expression are reported as fold changes compared with those of the CL06 isolate. (F) mRNA levels of RNAIII. The expression levels of *hla*, *mgrA*, *saeR* and RNAIII were detected via qRT−PCR and normalized against *gyrB*. The box plots illustrate the minimum, maximum, median, and interquartile range of the data from all participants, as determined by Tukey's boxplot method. The red boxes and circles represent the t1081 isolates, and the blue boxes and squares represent the non-t1081 isolates. ns, not significant. *, $p < 0.05$. **, $p < 0.01$.

than 0.05 was considered statistically significant.

3. Results

3.1. Higher hemolytic activities of the t1081 isolates

The hemolysis test with rRBCs revealed that the t1081 isolates presented significantly greater hemolytic activity than the non-t1081 isolates did ($p = 0.006$) (Fig. 1A). Although a few non-t1081 isolates exhibit high hemolytic activity, the majority of these isolates have lower hemolytic activity than do the t1081 isolates. The average HU for the t1081 isolates was 1017.6, with 40 % (18/45) of the isolates having an HU greater than 1000 (data not shown). In contrast, the average HU for non-t1081 isolates was 523.9, with only 3 isolates (3/22, 13.6 %) having an HU above 1000.

3.2. Detection of hemolysin genes

To determine whether there are differences in hemolysin genes among the isolates, we analyzed the genes encoding α-, β-, δ-, γ-, and γ-variant hemolysins, specifically *hla*, *hlb*, *hld*, *hlg*, and *hlg-2*. The results indicated that all the isolates carried *hld* (encoded by RNAIII), but none of the isolates possessed *hlg-2* (Table 2). Additionally, only 3 isolates lacked *hla*, and all of these were t1081 isolates. These isolates also had relatively lower HU values than the other t1081 isolates did. Among the 67 isolates, 61.2 % (41/67) had *hlg*, with no significant difference between the t1081 isolates (26/44, 59.1 %) and non-t1081 isolates (15/23, 65.2 %). The notable difference between the two groups was in *hlb*, where most t1081 isolates (41/44, 93.2 %) had a truncated *hlb* due to phage insertion, whereas 34.8 % (8/23) of non-t1081 isolates retained the complete *hlb*. However, since rRBCs are insensitive to β-hemolysin,[25] the truncation of *hlb* may not be the primary reason for the increased hemolytic activity in the t1081 isolates.

3.3. Sequences and expression of hla

Since only *hla* and *hld* were present in most isolates, and α-hemolysin has a more pronounced hemolytic effect on rRBCs,[26] we first analyzed the sequences of *hla* and its upstream regulatory region in all the isolates, with some results presented in Table 3. Using the well-studied strain wood 46 as a reference, we analyzed the nucleotide and amino acid sequence differences in the *hla*-coding region. In the full-length 960 bp of *hla*, 53 nucleotide differences were identified compared with the reference strain, with some being silent mutations, ultimately resulting in 5 amino acid changes. Among these, 50 nucleotide variants were present in all 67 isolates. The three inconsistent variants among the isolates were G339A, C911G, and A915T. G339A and A915T are silent mutations, while only A915T leads to an amino acid change from S304 to C. Thus, among the 67 isolates, the amino acid sequence of Hla differs only at position 304. S304C is present in most non-t1081 isolates but was also found in some t1081 isolates. Additionally, this amino acid is not a critical site for Hla activity,[27] and there is no clear correlation with higher HUs (Table 3).

In the upstream regulatory region of *hla*, there was only a difference at position −190 among the 67 isolates. Most isolates had an A at this position, while a few t1081 isolates had a T. Similarly, there was no significant correlation with the HU level (Table 3).

Since no significant differences were detected in the nucleotide sequences between the two sets of isolates, we further investigated whether there were differences in the expression levels of *hla* via qRT–PCR. The results revealed that there was no significant difference in the expression levels of *hla* between the t1081 isolates and non-t1081 isolates (Fig. 1B).

Table 2
Prevalence of hemolysin genes among the CC45 isolates.

spa types (No.)	No. of hemolysin genes (%)				
	hla	*hlb*	*hld*	*hlg*	*hlg-2*
t1081[a] (44)	41 (93.2)	3 (6.8)	44 (100)	26 (59.1)	0 (0)
Non-t1081 (23)	23 (100)	8 (34.8)	23 (100)	15 (65.2)	0 (0)
Total (67)	64 (95.5)	11 (16.4)	67 (100)	41 (61.2)	0 (0)

[a] One UT2 isolate was included.

3.4. Expression of mgaA and saeR

To confirm that the transcription of *hla* was not affected between the two groups of isolates, the two transcriptional regulators of *hla*, *mgrA* and *saeR*, were analyzed via qRT–PCR. The results, shown in Fig. 1C and D, indicated that there were no significant differences in the expression levels of either *mgrA* or *saeR* between the two groups of isolates.

3.5. High levels of hla protein expression in t1081 isolates

α-Hemolysin is also regulated at the posttranscriptional level, so we further analyzed Hla protein expression. We selected 4 isolates from both the t1081 and non-t1081 isolates, choosing isolates with similar *hla* expression levels but differing HU values. The selected isolates were CL61, CL67, CL65, and CL66 for t1081 and CL25, CL01, CL06, and CL11 for non-t1081 (Table 3). The results from western blotting were corrected using a loading control. These findings indicated that, despite having similar *hla* expression levels, the Hla protein expression in the t1081 isolates was significantly greater than that in the non-t1081 isolates (Fig. 1E and 2, S1 and Table 3). In fact, even an t1081 isolate with relatively low *hla* expression (CL61) presented higher protein levels than did a non-t1081 isolate with relatively high *hla* expression (CL11). This result suggested that the higher hemolytic activity in the t1081 isolates may be due to a greater level of translation of the *hla* mRNA.

3.6. Sequences and expression of RNAIII (hld)

To understand the mechanism underlying the differences in *hla* translation levels between the t1081 and non-t1081 isolates, we analyzed RNAIII, which is known to regulate *hla* at both the transcriptional and posttranscriptional levels. The full-length RNAIII sequences of all the isolates, including the *hld*-coding region, were analyzed. The results revealed that only one isolate (CL63) differed in sequence from the others, with variations A95U and A407U. CL63 was an t1081 isolate and had an HU of only 14.4. Although it cannot be determined whether the lower HU of CL63 was due to variations in RNAIII, it was clear that the different HUs between the two sets of isolates were unrelated to the RNAIII sequence.

We further investigated whether there were differences in the expression levels of RNAIII via qRT–PCR. Since the RNAIII sequence includes the *hld*-coding region and Hld also contributes to hemolysis, primers targeting the *hld*-coding region were chosen for the analysis. The results revealed that there was no significant difference in the expression levels of RNAIII (*hld*) between the t1081 isolates and non-t1081 isolates (Fig. 1F). On the basis of these results, the higher translation levels in t1081 may not be caused by RNAIII.

4. Discussion

CC45, a clonal complex of *S. aureus* that includes ST45 and other closely related sequence types, appears to have diverged early in the evolutionary tree of this species. This lineage is notable for its variety, encompassing both methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) isolates, as well as community-acquired (CA) and healthcare-associated (HA) isolates, alongside clinical and commensal isolates. CC45 has spread globally and has been reported in America, Australia, Europe, Asia, and Africa.[28] The core lineage, ST45, is widely carried but is also frequently responsible for severe invasive diseases,

Table 3
Different characteristics between the t1081 and non-t1081 isolates.

Isolates	HU	Transcription level		Translation level of Hla	Changes in sequence				
					hla				Hla
		hla	RNAIII		−190	339	911	915	304
t1081									
CL61	1435.2	3.0 ± 0.4	98.7 ± 0.9	19.2 ± 2.4	A	G	C	A	S
CL67	857.3	4.0 ± 0.2	94.2 ± 0.8	11.8 ± 1.6	T	G	C	A	S
CL65	1281.0	4.8 ± 0.4	62.5 ± 0.8	9.7 ± 0.8	A	G	C	A	S
CL66	2408.5	6.1 ± 0.1	130.2 ± 0.7	20.7 ± 1.0	T	A	G	T	C
Non-t1081									
CL25	458.5	3.2 ± 0.6	121.0 ± 0.9	1.8 ± 0.1	A	A	G	T	C
CL01	114.5	4.9 ± 0.3	0.2 ± 0.8	0.9 ± 0.3	A	A	G	T	C
CL06	163.2	5.0 ± 0.3	102.4 ± 0.8	1.0 ± 0.0	A	A	G	T	C
CL11	339.7	6.1 ± 0.3	54.4 ± 0.8	2.9 ± 0.1	A	A	G	T	C

such as bacteremia. In Taiwan, ST45 has become one of the primary nasal carriage MRSA lineages among healthcare workers, residents of nursing homes and long-term care facilities (LTCFs), and patients.[29–31] At the same time, the prevalence of ST45 among clinically isolated MRSA strains has also increased.[32–35] The major ST of non-ST45 in the present study was ST508, a distinct variant of ST45. ST508 represents one of the primary MSSA clones found in nasal carriers throughout Taiwan.[36] In this study, the ST45 isolates were composed of three distinct *spa* types: t1081, t026, and an unknown type (UT2). Both t026 and UT2 are represented by only a single isolate each. Our previous study demonstrated that t026/ST45 isolates were more similar to those of the ST508 isolates than t1081/ST45 isolates.[19] t1081 is a highly transmissible clone that is predominantly prevalent in Hong Kong, China, and Taiwan, as well as in LTCFs and hospitals.[30,37] In Taiwan, the native ST45 isolates are primarily of the t1081 type, whereas t026 is more commonly isolated from individuals who have migrated from neighboring Southeast Asian countries.[38,39]

α-Hemolysin is the major toxin of *S. aureus*. It exerts its effects by binding to the ADAM10 receptor, which not only allows it to form pores in target cells but also cleaves E-cadherin molecules, actively promoting epithelial disruption and contributing to invasive infections.[9] Monecke et al. reported that there is no significant association between α-hemolysin production and fatal outcomes.[40] However, the study by Monecke et al. categorized outcomes simply as fatal or surviving, while many reports still highlight the crucial role of α-hemolysin in the pathogenicity of *S. aureus*. In previous studies, mice infected with MRSA strains deficient in Hla expression exhibited a significantly reduced ability to cause epidermal necrotic damage.[41] Monecke et al. also investigated the relationship between α-hemolysin production and different clonal complexes. In their study, they reported nearly no α-hemolysin production in 42 CC45 isolates. They did not reveal the STs of the isolates but only mentioned that 40 isolates were classified as *agr*-I and 2 as *agr*-IV. In contrast, the t1081 isolates in our research belong to *agr*-IV, whereas non-t1081 isolates are *agr*-I.[19] Although Monecke et al. concluded that α-hemolysin production is unrelated to the *agr* type, their analysis included isolates from various clonal complexes. On the basis of the differences in *agr* types among the isolates, we speculate that there may be fundamental genetic differences between the isolates used in the two studies. Previous studies have also indicated that the expression rates of *hla* are often strain specific.[42] It is possible that the high α-hemolysin-producing t1081 isolates identified in our study were not or were only minimally represented in Monecke et al.'s research.

To date, several regulators, including *agr*-RNAIII, the SaeRS TCS, the SarA protein family, ArlRS, and ArcR, are known to directly or indirectly modulate the expression of *hla*.[14,43] RNAIII expression is directly regulated by the *agr* system, a quorum-sensing (QS) system that detects environmental signals associated with bacterial density. RNAIII modulates the initiation of transcription of *hla*, thereby directly influencing its expression. SaeR can directly bind to the −35 region of the *hla* promoter, thereby influencing the expression of *hla*.[17] The SarA protein family, which includes SarA, SarR, SarS, SarT, and Rot, can influence *hla* expression through various mechanisms. SarA promotes *hla* expression, whereas SarR inhibits it. SarS acts as a strong repressor of *hla*, and both SarT and Rot affect *hla* expression by inhibiting *saeRS*.[14,44] Additionally, MgrA, another member of the SarA protein family, can either directly bind to the *hla* promoter or facilitate *hla* expression through the *agr*-RNAIII system. In contrast, both ArlRS and ArcR inhibit *hla* expression, with ArlRS repressing *agr*-RNAIII and ArcR binding to the *hla* promoter to suppress its expression.[14,43] However, the mechanisms mentioned above, as well as other known mechanisms, regulate *hla* expression at the transcriptional level. In our study, we measured the expression levels of *saeR* and *mgrA* but found no differences between the two groups of isolates.

Currently, RNAIII, a large regulatory RNA, is the only known factor that specifically regulates *hla* translation. *Hla* mRNA typically adopts a hairpin loop structure, which obstructs ribosome access to its binding site. However, RNAIII interacts with *hla* mRNA, which disrupts this hairpin configuration and facilitates ribosome recognition at the translation initiation site.[15] Small regulatory RNAs, such as *sprY* and RSaX28, influence *hla* expression by affecting RNAIII, whereas Teg41 regulates *hla* expression by impacting *psmα*. However, these mechanisms operate at the transcriptional level.[45–47] Since no differences in RNAIII expression levels were detected between the two groups of isolates in this study, we infer that the higher *hla* translation level observed is likely independent of RNAIII.

Previous research has demonstrated that RNAIII can affect the translation of *hla* via the use of the K6812-1 strain, which lacks RNAIII but still produces sufficient *hla* transcripts; however, the mechanism remains unclear.[15] In our study, two t1081 isolates presented notably low RNAIII expression levels (0.002 and 0.003), and their *hla* expression levels were also significantly below average (0.49 and 2.62). Further testing is needed to determine whether we can generate derivatives with RNAIII deletion but still maintain substantial *hla* expression among our isolates, which would allow us to understand the effects of other regulators on *hla* translation in the absence of RNAIII.

In the Western blotting experiment, since the supernatants of overnight cultures were used, protein quantification was influenced by the culture medium. We observed that the total protein amount from isolate CL01 was significantly lower according to the SDS–PAGE results under the same loading conditions (Fig. 2). Therefore, we corrected the relative protein levels obtained from Western blotting via SDS–PAGE. Generally, a lack of δ-hemolysin synergism can be used as an indicator of *agr* dysfunction. We observed that isolate CL01 had very low RNAIII expression levels (Table 3), which may suggest *agr* dysfunction. Previous studies have shown that *agr* dysfunction strains often exhibit reduced overall protein secretion.[48] Therefore, we hypothesize that the lower protein content in the supernatants of overnight cultures from CL01 may be due to *agr* dysfunction.

Previous studies have indicated a significant relationship between hemolysin genes and antibiotic resistance patterns.[49,50] Our previous

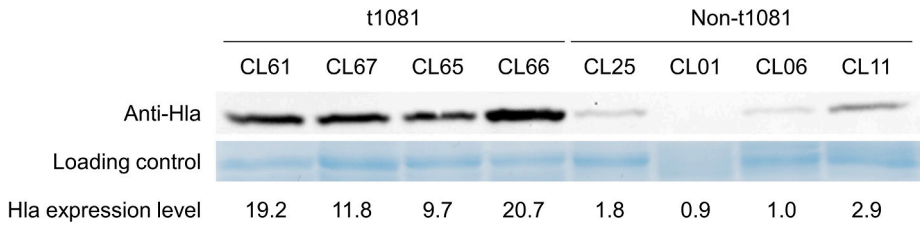

Fig. 2. Western blot analysis of α-hemolysin production. The supernatants of the cultures were analyzed via SDS−PAGE and blotted with an anti-Hla antibody. The numbers below the blot and loading control represent the levels of Hla expression normalized against the loading control quantified with CL06 as a baseline.

research also showed that t1081 isolates had higher ciprofloxacin resistance.[19] However, we also found distinct genetic backgrounds between t1081 and non-t1081 isolates. It suggests that the relationship between ciprofloxacin resistance and hemolytic activity may be associated with these genetic differences.

This study has several limitations. First, we analyzed only hemolysin and did not investigate other virulence factors that might affect hemolytic activity. Second, not all known regulators of α-hemolysin production were examined. Third, the mechanisms underlying the relatively high levels of Hla protein have not been fully elucidated.

In conclusion, we found that among the CC45 isolates obtained from clinical blood samples, the t1081 isolates exhibited greater hemolytic activity than the other isolates did. This enhanced hemolytic activity is attributed primarily to increased Hla protein production rather than to differences in the *hla* sequence or its transcription levels. Furthermore, the elevated translation levels may not be caused by the known transcriptional regulator RNAIII, suggesting the existence of an RNAIII-independent posttranscriptional regulatory pathway for Hla production.

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Data availability statement

The data sets used during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Yu-Tzu Lin: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ngoc-Niem Bui:** Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Yu-Syuan Cheng:** Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Cheng-Wen Lin:** Writing – original draft, Supervision, Resources, Methodology. **Chun-Li Lee:** Writing – original draft, Visualization, Investigation, Formal analysis. **Tai-Fen Lee:** Writing – original draft, Resources, Investigation. **Po-Ren Hsueh:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.09.005.

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