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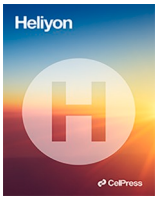

Research article

Differential expression of small RNAs in biofilm-producing clinical methicillin-susceptible *Staphylococcus aureus* recovered from human urine

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ABSTRACT

Bacterial small RNAs (sRNAs) play crucial roles in coordinating gene regulatory networks in various physiological processes, including biofilm formation. In this study, RNA sequencing was performed on biofilm ($n = 4$) and planktonic ($n = 4$) cells harvested at 10 h (pre-stationary phase of biofilm development) to identify biofilm-associated sRNAs in human methicillin-susceptible *Staphylococcus aureus* (MSSA) recovered from urine isolate. A total of 56 highly expressed sRNAs were identified with 15 overlapping sRNA genes (srn_9348, sprD, sRNA205, sRNA288, srn_2467, Sau-25, srn_2468, sRNA260, sRNA200, RsaE, sRNA397, Teg55, Teg60, RsaX05 and Teg140). Further validation through RT-qPCR analysis of nine sRNAs revealed that srn_9348 and sRNA260 were significantly expressed in the biofilm cells of urine sample. Both sRNAs were predicted to interact with mRNA genes including intracellular adhesin A (*icaA*) and host factor protein (*hfq*) involved in biofilm formation via *cis*-acting and *trans*-acting using CopraRNA analysis. Therefore, both sRNAs merit further investigations via reverse genetic approaches to elucidate their mechanism of translational regulation. In summary, the transcriptomic analysis conducted in this study offers new insights into the potential regulatory roles of sRNAs in MSSA biofilm development within the urinary environment.

1. Introduction

Staphylococcus aureus is a leading bacterial pathogen responsible for approximately one million deaths annually. Current prevention initiatives predominantly focus on strategies to combat antimicrobial resistance in multidrug-resistant strains, particularly methicillin-resistant *S. aureus* (MRSA). While MRSA has obtained significant attention due to its resistance profiles, it represents only a subset of the broader *S. aureus* burden. Methicillin-susceptible *S. aureus* (MSSA) remains prevalent in hospital settings, particularly in patients undergoing long-term catheterization with medical instruments [1]. MSSA infections are often associated with bloodstream infections, skin and soft tissue infections, pneumonia and urinary tract infections [2]. It can be found in urine samples, especially in

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patients with indwelling urinary catheters or immunocompromised [3]. Isolation of *S. aureus* from the human bladder could be due to subsequent episodes of *S. aureus* bacteraemia arising from different sites of infection or long-term use of indwelling medical devices such as urinary catheter which accounts for 63–82 % positive *S. aureus* cultures [4,5].

Insertion of the catheter often causes localised inflammatory response releasing host proteins such as fibrinogen [5]. During the growth of *S. aureus* in urine, the ability to form biofilm is one of the virulence factors that aid in disease development. The flow of urine permits continuous exchange of the deposition of organic molecules such as proteins and electrolytes from the urine components which in turn facilitate the development of biofilm [6]. The formation of biofilm is regulated by multiple regulatory signals, including the accessory gene regulator (*agr*) quorum sensing system, which controls the expression of virulence factors, two-component regulatory systems such as SaeSR and ArlSR by modulating gene expression in response to environmental stimuli and alternative sigma factors such as SigB which regulates gene expression in response to environmental stresses [7–9].

The regulatory small RNAs (sRNAs) have emerged as key regulators of bacterial gene expression involved in the formation of biofilm. sRNAs regulate multiple gene expression post-transcriptionally in response to environmental cues and metabolic changes [8]. Regulatory sRNAs is approximately 50–500 nucleotides in length and often are embedded in complex regulatory networks which act as repressors or activators [10]. They perform various functions, such as metabolism, virulence and antibiotic resistance in the cell [10, 11]. Many of these sRNAs do not encode proteins, but they influence gene expression by interacting with mRNA transcripts and/or proteins.

The sRNAs in *S. aureus* regulate biofilm formation via two mechanisms: (i) by base pairing with other RNAs and (ii) protein binding [12]. To date, RNAIII and RsaA were the only regulators found to have impacts on *S. aureus* biofilms [13,14]. The RsaA repressed *mgrA* gene while RNAIII stimulates the *agr* quorum sensing mediated pathway, thereby enhancing the biofilm formation [8]. Hence, there has been significant interest in studying sRNAs associated with the regulatory network of biofilm in this pathogen. The use of RNA sequencing has advanced over the years, both experimental and computational methods were used for sRNA identification. RNA sequencing is a popular method because it is not limited to known transcripts and can also detect the expression of low abundance transcripts and subtle changes under different conditions as compared to microarray-based profiling [15]. However, little attention has been given to the regulation of biofilm formation in MSSA. Therefore, this study aims to identify the sRNA gene in regulating biofilm formation in the urinary environment through a transcriptomic approach, using two populations, i.,e biofilm and planktonic cells collected from the preformed biofilm.

2. Methodology

2.1. Bacteria isolates

Four clinical MSSA isolates that demonstrated their biofilm ability via crystal violet staining were selected for this study (Table 1). These isolates were collected from Hospital Sultanah Nur Zahirah, Terengganu between July 2016 and 2017 with the approval number: NMRR-15-2369-28130 (IIR). The urine isolate S140 was collected from the emergency unit from a 31-year-old female patient hospitalised due to urinary tract infection. Another three non-urine isolates: pus isolate S82 was recovered from a deceased male patient aged 70 years old who suffered from pleuropulmonary infection whereas two blood isolates S44 and S156 were obtained from male patients (aged 55 and 41 years old). The case by isolate S44 was diagnosed as pleuropulmonary infection with a good treatment outcome while another fatal case caused by isolate S156 was due to bacteraemia infection (Table 1). The ability of biofilm formation was re-assessed using a real-time cell analysis according to previously established methods [16].

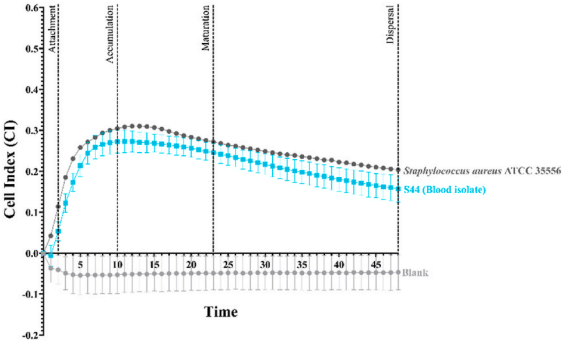
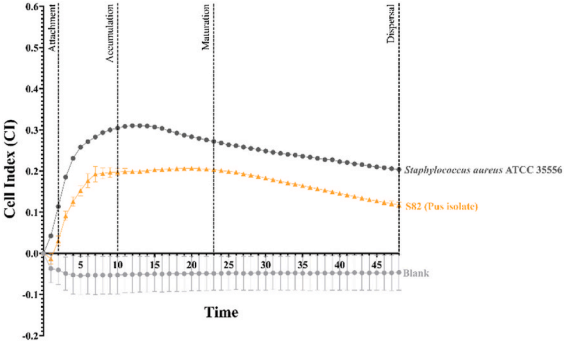
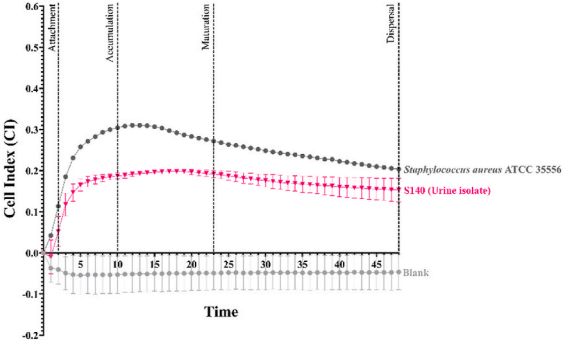
2.2. Growth of bacterial biofilm and planktonic phases

Two cell populations were investigated in this study, biofilm- and planktonic cells, which were harvested from the MSSA isolates (S44, S82, S140 and S156) based on the time point identified from impedance-based real-time cell analyser (RTCA) experiment. A single colony was transferred into sterile TSB and incubated overnight at 37 °C. On the next day, bacterial cultures were diluted 1:100 into 40 mL of TSB supplemented with 0.4 % glucose and were incubated for approximately 2 h at 37 °C until mid-log phase (OD_{600} = 0.6) was achieved. The mid-log phase culture was diluted to OD_{600} of 0.1, transferred into tissue flask T75 cm^2 and incubated statically for 10 h at 37 °C. Planktonic samples (n = 4) were collected from the suspension while biofilm samples (n = 4) were collected from the bottom of the tissue flasks. The samples were subjected to centrifugation at 13, 000×*g* for 10 min at 4 °C. The cell pellets were treated with 500 μL of RNAprotect™ Bacteria Reagent (Qiagen, Germany) and were incubated for 5 min at room temperature for RNA stabilisation. Bacteria were then harvested by centrifugation at 13, 000×*g* for 1 min at 4 °C.

2.3. Total RNA extraction and library preparation

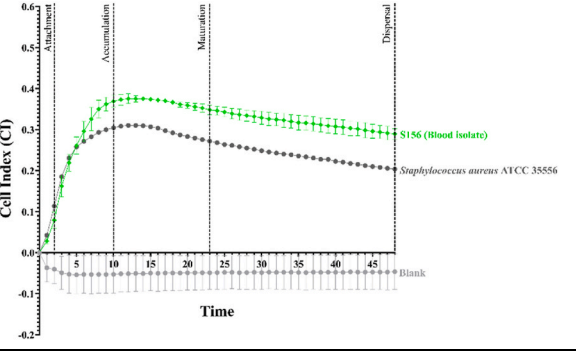
For each MSSA isolate, total RNA from planktonic and biofilm cells was extracted separately using RNeasy PowerBiofilm Kit (Qiagen, Germany) according to the manufacturer's instructions, including DNase I treatment for DNA removal. Briefly, approximately 0.2 g amount of pellet cells were added into the PowerBiofilm bead tube and then heated to activate lysis components. The biofilm matrix was lysed chemically (lysis buffers) and mechanically (bead beating). Each RNA sample was suspended in 50 μL of RNase-free water. The removal of 5S, 16S and 23S rRNA of the extracted total RNA was performed using QIAseq Fast Select Bacterial – 5S/16S/23S kit (Qiagen, Germany). The quantity and quality of total RNA were determined using a Qubit RNA assay kit and High Sensitivity RNA ScreenTape (Agilent Tapestation), respectively. The library preparation was performed using QIAseq Stranded RNA Library kit

Table 1
Clinical background of four methicillin-susceptible *Staphylococcus aureus* isolates selected for RNA-sequencing.

Subject	Gender/ Age	Isolation source/ Ward	Clinical feature	Length of stay	Outcome	Real-time biofilm (CI value at 10h)	RTCA
S44	Male/55	Blood/Emergency	Pleuropulmonary	3 days	Alive	0.2724	
S82	Male/70	Pus/Medical	Pleuropulmonary	23 days	Dead	0.1755	
S140	Female/31	Urine/Emergency	Urinary Tract Infection	3 days	Alive	0.1773	

(*continued on next page*)

Table 1 (*continued*)

Subject	Gender/ Age	Isolation source/ Ward	Clinical feature	Length of stay	Outcome	Real-time biofilm (CI value at 10h)	RTCA
S156	Male/41	Blood/Medical	Bacteraemia	11 days	Dead	0.3342	

(Qiagen, Germany). RNA was fragmentised to a size ranging from 300 bp to 500 bp by heat fragmentation, end-repaired by 5′ phosphorylated random primers (Qiagen, Germany) before addition of adapters. The cDNA libraries were generated using Second-Strand Master Mix (Qiagen, Germany) and enriched with CleanStart PCR Mix (Qiagen, Germany). The library quantity was measured with a Qubit dsDNA assay kit and the quality was assessed on D5000 ScreenTape (Agilent Tapestation). The concentration of the final libraries pool was then measured with a Qubit dsDNA assay kit and the quality was assessed on D5000 ScreenTape (Agilent Tapestation). QIAseq Library Quant assay kit (Qiagen, Germany) was then performed with real-time qPCR using Applied Biosystems (ABI) 7500 Fast real-time PCR (ThermoFisher, USA) for accurate identification of final libraries before proceeding to sequencing, which was carried out on an Illumina HiSeq 2500 sequencing platform by a commercial service provider (GENEWIZ, China).

2.4. Assembly and quality check of RNA-sequencing data

Raw reads were imported in FastQ format into CLC Genomics Workbench version 22.0.2 (Qiagen Bioinformatics) and pre-processed by filtering out failed reads with the quality score parameter option set to Illumina Pipelines 1.8. The basic quality statistics were determined to assess the quality of the libraries sequenced which consisted of the calculation of sequence lengths distribution, GC-content, ambiguous base-content, PHRED quality score distribution, nucleotide contributions, *k*-mers distribution analysis and sequence duplication levels. Quality-based trimming was performed to remove the low-quality reads with a quality limit set to 0.01 and a maximum number of ambiguities set to 2. In addition, adapter sequences were also removed automatically when found outside of the high-quality region [17].

2.5. Read mapping

Before mapping the clean reads, a custom-built reference database that consisted of sRNAs data and reference genome was prepared. The known sRNA genes for *S. aureus* were obtained from the *Staphylococcus* Regulatory RNA Database (SRD, http://srd.genouest.org/). The sRNA annotation files provided details such as the name, the orientation of the strand, start and end position of each sRNA. Additionally, a complete *S. aureus* NCTC8325 genome sequence (GenBank accession number: CP000253.1) and annotation files (mRNA genes, tRNA, rRNA and pseudogene) were retrieved from NCBI. The pre-built reference database in GFF format was imported into CLC Genomics Workbench version 22.0.2 (Qiagen Bioinformatics) under gene track [18,19]. The read files in FastQ format from our study were then mapped with a custom-built reference database. The mapping parameters were set as follows: mismatch cost 2; insertion cost 3, deletion cost 3; length fraction 0.8 and similarity fraction 0.8. The total counts of the gene were normalised according to the weighted trimmed mean of M values method.

2.6. Visualisation and functional enrichment analysis of sRNAs

The comparative prediction algorithm for small RNA targets (CopraRNA) tool (https://rna.informatik.uni-freiburg.de/CopraRNA/) was used for the prediction of interactions between the sRNAs of interest and their target genes. The sRNAs sequence was submitted using a background model built on the *S. aureus* NCTC8325 genome sequence with default settings. The output dataset queried through the CopraRNA for each sRNA with their predicted interaction energies and respective *p*-values (<0.05) were imported to Cytoscape, a software that can link genes to its biological pathways through KEGG database for enrichment [20]. The sRNA-predicted target genes and biological pathways were visualised with Cytoscape software integrated with ClueGo + CluePedia plugins (https://cytoscape.org). The secondary structure of sRNA was visualised with mFold software (http://www.mfold.org/).

2.7. Validation of RNA-sequencing by quantitative real-time qPCR

Nine sRNAs genes (srn_9348, sRNA205, srn_2467, sRNA288, Sau-25, srn_2468, sRNA260, sRNA200 and sRNA397) that have not been experimentally characterised previously by other researchers were selected for RT-qPCR analysis. The expression level of these sRNA genes was quantified by RT-qPCR using SensiFAST SYBR Lo-Rox Kit (Bioline, UK) conducted on ABI 7500 Fast instrument. Specific primers were designed and were listed in Supplementary Table S1 while *ftsZ* gene was used as a housekeeping gene. The experiment was performed with three technical replicates and two independent biological samples using (i) the same total RNA sent for RNA sequencing and (ii) newly extracted RNA obtained from the same samples performed under the same biological conditions. All extracted RNA samples with a total of 1 μg were then used to synthesise first-strand cDNA using Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen Biotech, China) following the manufacturer's instructions.

Each reaction was prepared in 10 μL total volume containing 100 ng of cDNA and 400 nM of specific primers under the following conditions: 50 °C for 20 s, 95 °C for 2 min, 40 cycles of 95 °C for 5 s and 60 °C for 1 min. Melt curve analyses were performed at the end of the run by heating from 60 to 95 °C to confirm the single PCR product. The relative expression levels of each gene were calculated based on the cycle threshold (Ct) and the fold change was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) [21]. A difference of ≥ 7 between the cDNA sample and no-template PCR control was considered negligible for relative quantification analysis.

2.8. Statistical analysis

The analysis of differentially expressed sRNAs was conducted by comparing biofilm cells produced by urine isolate S140 (control group) with three experimental groups: Experimental Group 1 consisted of planktonic cells from urine isolate S140, Experimental

Group 2 consisted of biofilm cells from non-urine isolates [blood (S44 and S156) and pus isolate (S82)] and Experimental Group 3 consisted of planktonic cells from non-urine isolates [blood (S44 and S156) and pus (S82)]. The analysis of differentially expressed genes were performed using a two-way analysis of variance (ANOVA). Specifically, genes with $p < 0.05$ and $\log_2$ fold change ≥ 1 were extracted and considered differentially expressed. All data were analysed using Graph Pad Prism 7.

3. Results

3.1. Real-time monitoring of biofilm formation using a real-time cell analyser (RTCA)

The RTCA reveals four distinct phases of biofilm development throughout 48 h: (I) attachment, (II) accumulation, (III) maturation and (IV) dispersal. The biofilm formation was detected within 10 h where the cell index (CI) value reached its peak before gradually showing a decreasing pattern (Table 1). At 10 h, the highest peak was observed for blood isolate S156 with the CI value of 0.3342, followed by blood isolate S44 with the CI value of 0.2724, urine isolate S140 with the CI value of 0.1773 and pus isolate S82 with the CI value of 0.1755. Overall, the highest peaks of the CI value were observed at 10 h for all isolates indicating that biofilm has matured, therefore, both biofilm and planktonic cells were harvested at this time point for further investigations.

3.2. Quality of RNA-sequencing data

Illumina reads resulted in an average of 18.2 million 150-bp paired-end sequence reads per sample with a range of 15.7–20.1 million sequence reads. More than 88.9 % of the Illumina reads have PHRED scores above 30 (Q > 30), a conventional threshold denoting high-quality base calls, with a low percentage of ambiguous bases and sequence duplication levels detected.

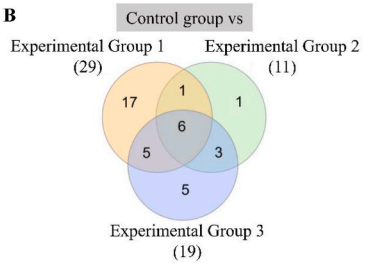
3.3. sRNA upregulated in biofilm for MSSA

Analysis of biofilm cells of urine isolate S140 (control group) compared to three experimental groups consisting of planktonic cells of urine isolate, biofilm and planktonic cells of non-urine isolates showed differentially expressed sRNAs were 434, 491 and 483, respectively (Fig. 1A). sRNAs with $\log_2$ fold change of more than 1 and significant p-value (<0.05) exhibited 59 highly expressed sRNAs in the biofilm population produced by urine isolate S140 compared to three experimental groups as shown in Fig. 1B. Among these highly expressed sRNAs, 15 sRNAs were observed present in more than one experimental group, thus were selected for further investigations (Fig. 1C). These 15 highly expressed sRNAs are of different type of sRNA which consisted of 5 *trans*-encoded and 10 *cis*-

A

$\log_2$ fold change	Number of differentially expressed genes identified in biofilm cells of S140 when compared to the different experimental groups:		
	Experimental group 1 (vs. planktonic cells of S140)	Experimental group 2 (vs. biofilm cells of S44, S82, S156)	Experimental group 3 (vs. planktonic cells of S44, S82, S156)
<−2	50	112	87
<−1	30	80	103
−0.9 > 0 < 0.9	258	228	202
> 1	37	47	56
> 2	59	24	35
Total	434	491	483

B



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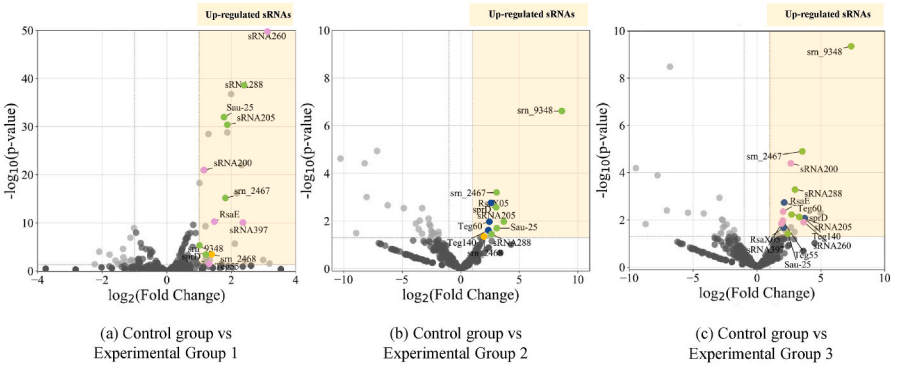

(a) Control group vs Experimental Group 1 (b) Control group vs Experimental Group 2 (c) Control group vs Experimental Group 3

sRNA genes	Control group vs		
	Exp 1	Exp 2	Exp 3
srn_9348	1.02	8.63	7.38
sprD	1.20	3.00	2.70
sRNA205	1.88	3.67	3.32
sRNA288	2.40	2.62	2.98
srn_2467	1.82	3.06	3.54
Sau-25	1.78	3.09	2.43
srn_2468	1.39	1.94	
sRNA260	3.11		3.67
sRNA200	1.16		2.64
RsaE	1.48		2.05
sRNA397	2.36		1.92
Teg55	1.31		2.02
Teg60		2.44	2.12
RsaX05		2.57	2.06
Teg140		2.36	3.74

Fig. 1. Differentially expressed sRNAs in the biofilm cells of methicillin-susceptible *S. aureus* S140 urine isolate (control group). (A) Total number of differentially expressed sRNAs identified in the biofilm cells of S140 when compared to three experimental groups. (B) A Venn diagram of 59 highly expressed sRNAs identified in biofilm cells of S140. (C) Volcano plot of highly expressed sRNAs with overlapping sRNAs across three experimental groups.

acting regulators (Table 2). Nine (srn_9348, srn_2468, sRNA260, sRNA397, RsaE, Teg55, Teg60, RsaX05 and Teg140) were transcribed from the positive strand while 6 (sprD, sRNA205, sRNA288, srn_2467, Sau-25 and sRNA200) from the negative strand. Among the 15 highly expressed sRNAs, 6 (sprD, RsaE, Teg55, Teg160, Teg140 and RsaX05) were previously characterised while 9 (srn_9348, sRNA205, sRNA288, srn_2467, Sau-25, srn_2468, sRNA260, sRNA200 and sRNA397) were uncharacterised.

3.4. Prediction and visualisation of sRNA target genes

These 15 sRNAs were further examined to identify their targeted mRNAs and the interactions demonstrated that single sRNAs has the capability to affect the regulation of a large number of genes (Supplementary Data 1). The mRNA targets list was further refined to include only those with p-values <0.05. Through KEGG pathway enrichment analysis, 620 predicted target genes were identified in 12 KEGG pathways (Fig. 2). Among these, two-component systems (TCSs), ABC transporter, purine metabolism, ribosome, pyruvate metabolism, aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, glycine, serine and threonine metabolism, amino sugar and nucleotide sugar metabolism were the most representative KEGG pathways.

Furthermore, 6 uncharacterised sRNAs were found to target mRNA genes involved in the biofilm formation of *S. aureus* as identified by CopraRNA tools. Among them, 3 sRNAs (srn_9348, sRNA288 and srn_2468) were found to target intercellular adhesin A (*icaA*). Meanwhile, 2 sRNAs (sRNA397 and sRNA260) were found to target RNA-binding protein Hfq and one sRNA (Sau-25) was found to target staphylokinase (*sak*).

3.5. Validation of RNA-sequencing by quantitative real-time qPCR

Eight studied sRNAs showed sRNA expression across all isolates (Fig. 3). Of these, only sRNA260 showed high expression value ($\log_2$ fold change>1) across all urine (S140) and non-urine (S44, S82 and S156) isolates. Another studied sRNA srn_9348 was only expressed in biofilm cell of urine sample S140.

4. Discussion

In MSSA, the primary event in establishing biofilm is crucial for causing persistent infections. In this study, the maximum CI value was observed in the accumulation phase when the entire surface of the microelectrode was covered with the biofilm layer. The flow of electrical signals was impeded when cells started to form a dense layer on the microelectrode as reflected in the CI value. A high CI value indicates significant impedance changes caused by cells obstructing the electrode flow [22]. Biofilm begins to develop when free-floating planktonic cells adhere to a surface and produce a self-generated polymeric matrix where initially forming a preformed biofilm. At this stage, bacteria cells are loosely embedded within the matrix, readily able to detach from the surface, called a reversible attachment. Bacteria cells enter the irreversible attachment when the accumulation of bacteria is structured in a multilayered cell cluster forming a mature biofilm [23]. Mature biofilms are harder to eradicate, while preformed biofilms are more susceptible to treatment. Therefore, it is crucial to consider strategies for structurally weakening the biofilms or inhibiting biofilm formation during

Table 2
List of highly expressed sRNAs found in the biofilm cells of MSSA isolate (S140) across three experimental groups.

Name (SRD)	Length	Strand	Type	Others name (from publications)	Remarks
Control group vs. Experimental Group 1, 2, 3					
srn_9348	201	+	*cis*-acting regulator		Not done
sRNA205	321	–	*cis*-acting regulator	srn_2480	Not done
srn_2467	205	–	*cis*-acting regulator		Not done
sRNA288	207	–	*cis*-acting regulator	srn_3640	Not done
Sau-25	167	–	*trans*-encoded	sRNA340	Not done
sprD	145	–	*trans*-encoded	Teg14, sRNA300	Confirmation by Northern blot [28]
Control group vs. Experimental Group 1 & 2					
srn_2468	206	+	*cis*-acting regulator		Not done
Control group vs. Experimental Group 1 & 3					
sRNA260	259	+	*trans*-encoded	srn_3280	Not done
sRNA397	307	+	*trans*-encoded	srn_4950	Not done
sRNA200	280	–	*cis*-acting regulator	srn_2390	Not done
RsaE	459	+	*cis*-acting regulator	Teg92, Sau-20, sRNA183	Confirmation by Northern blot [32]
Teg55	125	+	*cis*-acting regulator	sRNA198	Confirmation by RT-qPCR [30]
Control group vs. Experimental Group 2 & 3					
Teg140	77	+	*cis*-acting regulator	sRNA23	Confirmation by RT-qPCR [32]
RsaX05	205	+	*trans*-encoded	Teg41, sRNA95	Confirmation by RT-qPCR [30]
Teg60	216	+	*cis*-acting regulator	sRNA208, tsr22	Confirmation by RT-qPCR [32]

Control group = Dataset for biofilm cells from urine sample (S140).
Experimental group 1 = Dataset for planktonic cells from urine sample (S140).
Experimental group 2 = Dataset for biofilm cells from non-urine samples (combination of S44, S82 and S156).
Experimental group 3 = Dataset for planktonic cells from non-urine samples (combination of S44, S82 and S156).

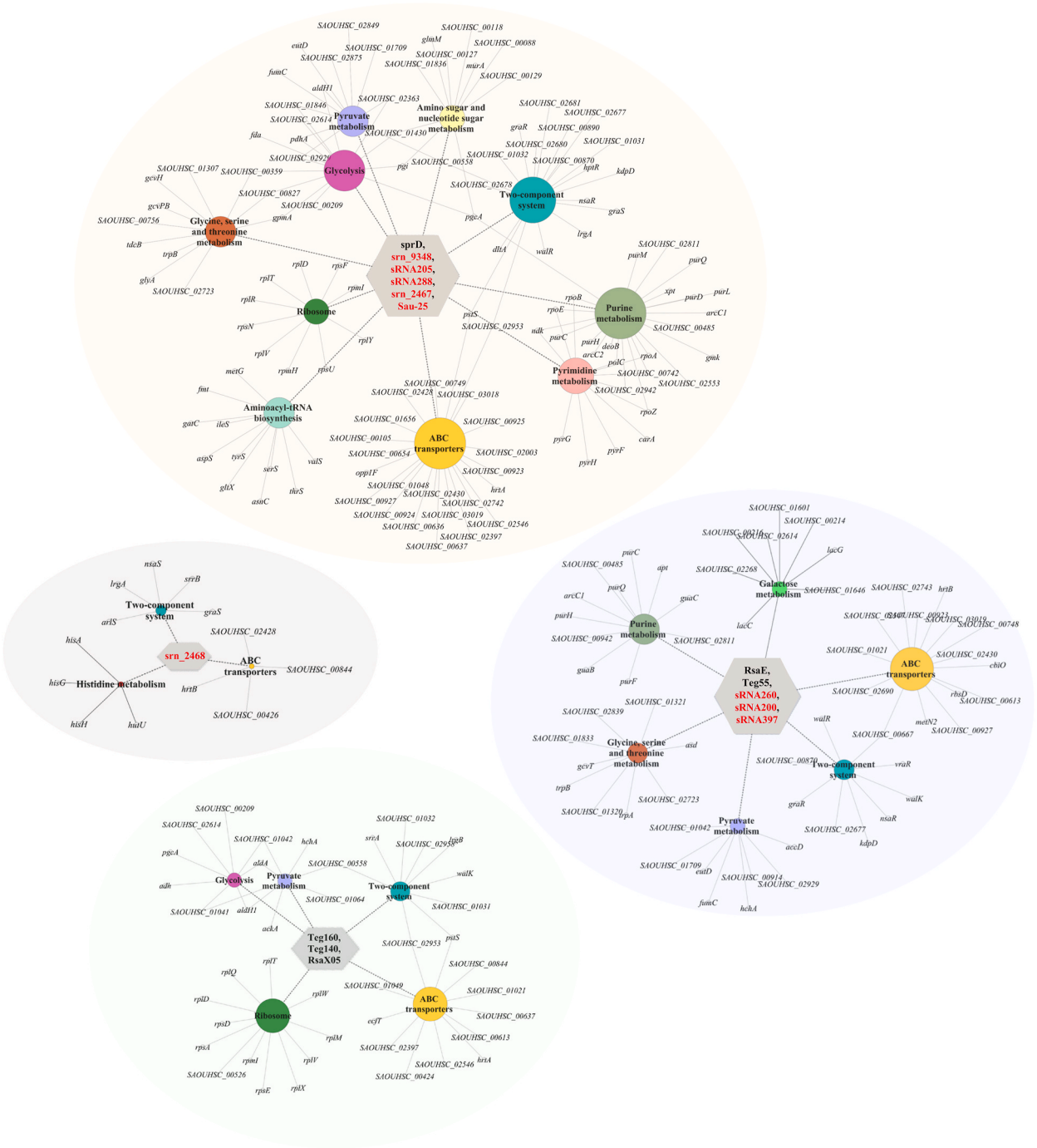

Fig. 2. Target genes of 15 upregulated sRNAs using Cytoscape + ClueGo. Each hexagon denoted the highly expressed sRNAs across three experimental groups presented in Table 2. Uncharacterised sRNAs are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the pre-stationary stage [24]. Our observations in the RTCA experiment indicated that the importance of the initial 10 h of growth in establishing a biofilm for MSSA. So, to investigate the sRNAs governing biofilm formation at this time point, we compared the MSSA biofilm cells isolated from urine isolate S140 to their planktonic cells, as well as to planktonic and biofilm cells from non-urine isolates (blood isolates S44 and S156; pus isolate S82). Using Illumina RNA-sequencing, differentially expressed sRNAs were successfully profiled.

Overall, transcriptome sequencing results revealed 59 highly expressed sRNAs and the three-way Venn diagram (Fig. 1B) showed

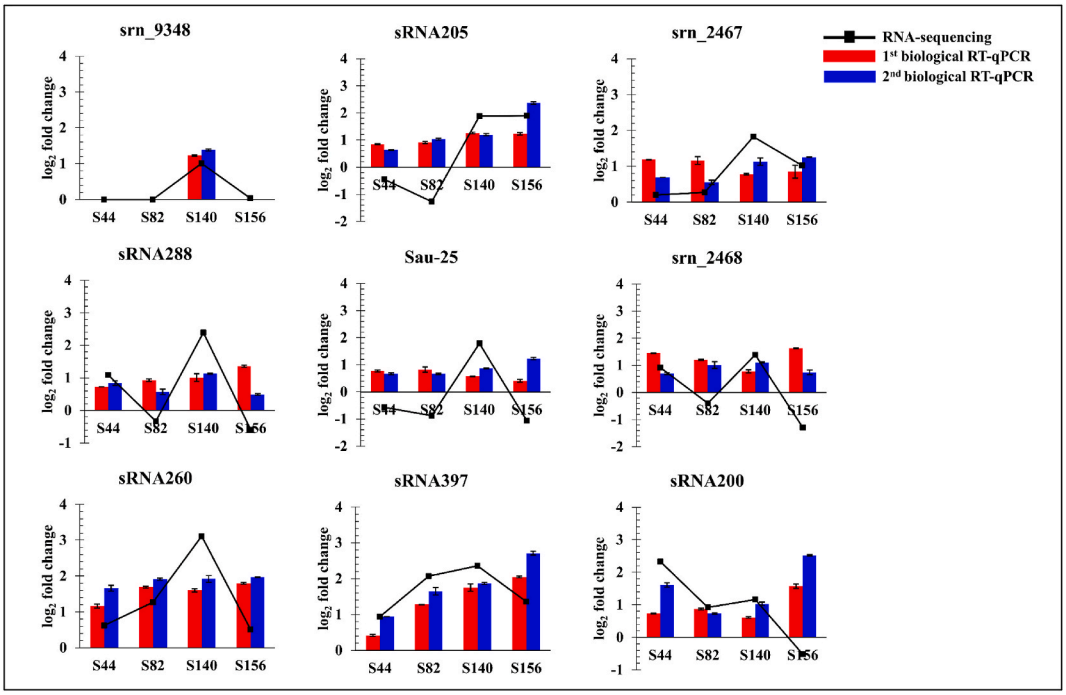

Fig. 3. Validation of selected uncharacterised sRNAs using real-time qPCR (RT-qPCR). Bar charts represent the expression value in $\log_2$ fold change of two biological independent RT-qPCR and line charts represent the expression value in $\log_2$ fold change of RNAsequencing. Error bars represent standard errors of three technical replicates.

15 overlapped differentially expressed sRNAs (srn_9348, sRNA205, srn_2467, sRNA288, Sau-25, sprD, srn_2468, sRNA260, sRNA397, sRNA200, RsaE, Teg55, Teg140, RsaX05 and Teg60) present in the biofilm cells of S140. Among the 15 sRNA genes, only 6 sRNAs (sprD, RsaX05, RsaE, Teg55, Teg60 and Teg140) have been characterised previously (Table 2). Those 9 uncharacterised sRNAs have also been reported by other researchers but have yet to undergo experimental validation. These sRNAs were identified via (i) RNA -seq: srn_9348, srn_2467 and srn_2468 by Bronsard et al. [25] while sRNA205, sRNA288, sRNA260, sRNA397 and sRNA200 by Howden et al. [26] (ii) a combination of experimental and computational search strategy: Sau-25 was identified by Abu-Qatouseh et al. [27].

Among characterised sRNAs, the SprD is a small pathogenicity island-encoded RNA D that downregulates the translational expression of Sbi immune evasion molecules in an antisense mechanism during the growth phase [28,29]. Another characterised sRNA RsaX05, also known as Teg41, regulates alpha phenol-soluble modulins in *trans* which is one of the most potent toxins in *S. aureus* that is associated with immune evasion [30]. Meanwhile, RsaE is one of the important sRNAs reported to affect *S. epidermidis* biofilm matrix composition. The overexpression of RsaE caused an increased polysaccharide intercellular adhesin (PIA) production and the release of the extracellular DNA (eDNA) due to enhanced bacterial lysis. Similarly, the PIA production is crucial for the *S. aureus* adhesion which is encoded by the *icaADBC* operon. At the same time, the eDNA is a regular component and an important stabilising factor in biofilm formation controlled by cognate regulators (CidR and LytSR) [31]. There is no direct evidence that reported RsaE participates in *S. aureus* biofilm formation so far, thus the role of RsaE in *S. aureus* should be further investigated using reverse genetic approaches. Other sRNAs Teg55, Teg60 and Teg140 were first reported by Beaume et al. [32] using Illumina RNA-sequencing and their expression was validated by RT-qPCR. The expression of these sRNAs was induced when introduced to various environmental stresses such as oxidative stress, thermal stress and pH stress in the early growth phase. The stress exposure impacts *S. aureus* growth suggesting that bacterial adaptation to drastic environments during the growth phase where their survival is essential for the acquisition of virulence factors such as biofilm formation [32]. Most of the characterised sRNAs identified in this study influence the biofilm formation in various ways from modulating the toxins expression, evasion from the immune system or directly impacting the structural biofilm matrix, highlighting the multifaceted role of sRNAs.

To further understand the regulatory network of those 9 uncharacterised sRNAs, KEGG enrichment analysis was performed. The most presented biological pathways were TCSs, ABC transport and purine metabolism. Among them, two uncharacterised sRNAs (sRNA288 and srn_2468) have been shown to target mRNAs involved in biofilm formation via TCS regulatory system. In this case, the TCS may govern the transcriptional regulation initiating the expression of the target gene while the sRNA exerts post-transcriptional regulation to further fine-tune the expression of the target gene [33]. The sRNA288 and srn_2468 have been shown to target *lrgA* which is involved in the tower structuring of biofilm development under the control of TCS LytSR [34]. The *lrgA* gene encodes antiholin-like proteins that function to induce cell death and lysis during biofilm development and subsequently mature biofilms has led to an increase of eDNA release in response to increased bacterial lysis [35–37]. Previous studies conducted by Mann et al. [35] and Sharma-Kuinkel et al. [36] have reported that *lrgAB* mutant demonstrate increased static biofilm formation along with the release of

eDNA. One of the major components of the biofilm matrix is eDNA thus the increased amount of eDNA resulted in a more stable structure of the biofilm matrix. Furthermore, srn_2468 also targets *arlS*, a member of the TCS ArlSR involved in the regulation of adhesion of bacterial on host cells by controlling the expression of a major surface protein implicated in biofilm formation such as surface protein G [38]. It is also reported that the *arlS* gene controls attachment to polymer surfaces [39]. Such polymer surfaces can be found in implanted catheters therefore srn_2468 may control the primary attachments of *S. aureus* via ArlSR regulatory to initiate the development of biofilm within the urinary tract. However, these hypotheses require further investigation.

Interestingly, only srn_9348 is expressed in the biofilm cells of urine isolate S140 with a $\log_2$ fold change of more than 1 in the two independent biological RT-qPCR. The abundance of srn_9348 only in the biofilm cells of urine isolate may suggest that srn_9348 exhibits the capability to exclusively modulate gene expression within urine sample. On the other hand, sRNA260 is the most stable expressed sRNA across all isolates with a significant expression value $\log_2$ fold change of more than 1. It is noted that expression of sRNA260 was significantly elevated in biofilm-associated cells of urine samples in comparison to both planktonic cells in urine and non-urine samples. The expression of sRNA260 has been reported previously to be involved in antibiotic resistance [26,40]. Yet, the regulation of biofilm formation by sRNA260 has not been reported. In 2013, it was first reported by Howden and co-workers using the vancomycin-susceptible and -intermediate *S. aureus* strain [26]. Its expression was enhanced in vancomycin-intermediate *S. aureus* compared with the susceptible strain after antibiotic exposure (ceftobiprole, linezolid, tigecycline and vancomycin). Both srn_9348 and sRNA260 are unique to *S. aureus* however their roles in biofilm formation are not understood as it has not been experimentally characterised previously. Based on CopraRNA analysis, srn_9348 and sRNA260 were predicted to interact with mRNA genes involved in biofilm formation such as intracellular adhesin A (*icaA*) and host factor protein (*hfq*), respectively. Therefore, further computational analysis was conducted to analyse their secondary structure and base-pairing mechanism (Fig. 4).

Many sRNAs regulate gene expression through base pairing with mRNA molecules of their target or interaction with modulating proteins for either positive or negative regulation mechanisms [41]. The base pairing mechanism of srn_9348 is *cis*-encoded, which is directly transcribed from the target mRNA, showing a high level of complementarity potential. It is hypothesised that srn_9348 directly regulates the *icaA* gene to produce polysaccharide intracellular adhesin (Fig. 4A). On the other hand, sRNA260 is a *trans*-encoded sRNA that can regulate the expression of multiple target mRNAs separated from its loci, and the base pairing interaction of *trans*-encoded sRNA is interrupted by gaps in the pairing, indicating a low level of complementarity [42]. The Hfq protein is required when the level of complementarity is low. It is hypothesised that *trans*-acting sRNA260 interacts with Hfq protein (Fig. 4B) to help to stabilise the sRNA and enhance the binding between sRNA260 and its mRNA targets [43,44]. Therefore, the RT-qPCR results combined with computational analysis suggested that sRNA260 and srn_9348 are warranted for further investigations to verify their functional roles in MSSA biofilm formation arising from a urine sample.

It is postulated that base pairing between the aforementioned sRNAs and target mRNAs may alter mRNA translation and stability by affecting ribosome binding sites or enhancing ribonuclease-mediated degradation, thus influencing target gene expression involved in biofilm matrices development. Therefore, a therapeutic strategy targeting sRNA260 and srn_9348 may offer a potential solution for disrupting biofilm formation and treating biofilm-associated infections caused by MSSA. This leads to the exploration of antimicrobial compounds designed to target sRNAs, thereby disrupting biofilms by interfering with sRNA-mRNA interactions. For instance, antimicrobial compounds such as pyrithiamine have been shown to inhibit the translation by binding to the flavin mononucleotide riboswitch in *Bacillus subtilis*, inducing the sequester loop formation that hides the start codon [45]. In the battle against biofilms, RNA-based drug targets hold significant promise for effective interventions.

5. Conclusion

This study successfully elucidated the regulatory mechanism of biofilm cells isolated from urine sample and identified 59 highly expressed sRNAs using RNA-sequencing. Among these sRNAs, 15 sRNAs (srn_9348, sprD, sRNA205, sRNA288, srn_2467, Sau-25,

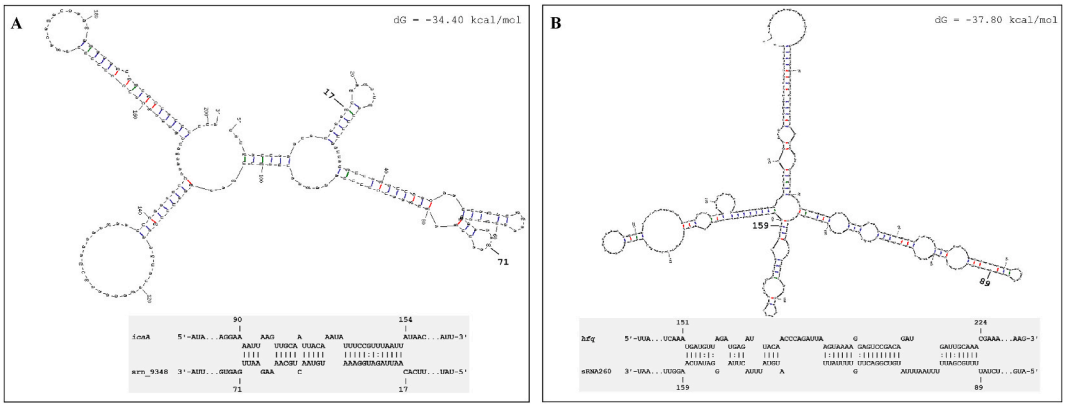

Fig. 4. Secondary structure of sRNA srn_9348 and sRNA260 with their target mRNA visualised by mFold and CopraRNA. (A) represents the interaction of srn_9348 and *icaA*. (B) represents the interaction of sRNA260 and *hfq*.

srn_2468, sRNA260, sRNA200, RsaE, sRNA397, Teg55, Teg60, RsaX05 and Teg140) were found to overlap for more than one experimental group. Validation using RT-qPCR showed significant expression of two uncharacterised sRNAs srn_9348 and sRNA260 in the biofilm cells of urine sample. These two sRNAs are the potential candidates to be further investigated using a reverse genetic approach to elucidate their roles in biofilm formation and these findings will contribute to our understanding of the role of sRNAs in patients with urinary tract infection.

CRediT authorship contribution statement

Sherry Usun Jones: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Boon Pin Kee:** Writing – review & editing, Supervision, Funding acquisition. **Ching Hoong Chew:** Writing – review & editing, Resources, Funding acquisition. **Chew Chieng Yeo:** Writing – review & editing, Visualization, Resources. **Kek Heng Chua:** Writing – review & editing, Supervision, Funding acquisition. **Suat Moi Puah:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Compliance with ethical standards

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Ethical approval

The study was approved by the Malaysian Ministry of Health, National Medical Research Registry (NMRR) and ethics committee with a reference number: NMRR-15-2369-28130 (IIR).

Data and code availability statement

The data are contained within the article and supplementary materials. The reported sequence data have been deposited in the NCBI Sequence Read Archive (SRA) database under BioProject ID: PRJNA1173362, with the accession numbers, SRX26397594 - SRX26397601.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Puah Suat Moi reports financial support was provided by Malaysian Ministry of Higher Education. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39634.

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