ORIGINAL ARTICLE

Expression analysis of homologous non- protein coding RNA in *Providencia stuartii* by Comparative Transcriptomics

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ABSTRACT

Introduction: The Gram-negative bacillus, *Providencia stuartii*, has established itself as a notorious player in catheter-associated urinary tract infections (CAUTIs) by colonising indwelling catheters. Conventional therapeutics are limited by *P. stuartii*'s escalating antibiotic resistance. In search of alternate therapeutic options, this study explores non-coding regulatory RNAs (npcRNAs) to identify homologous npcRNAs and predict their post-transcriptional regulatory roles that could target virulence-associated mRNAs in *P. stuartii*. **Methods:** A comparative genomics approach was used to identify conserved npcRNA homologs of *Escherichia coli, Salmonella typhi* and *Yersinia pestis* in *P. stuartii* genome. Unannotated conserved transcripts in *P. stuartii* were identified through BLASTn and Rfam analysis. Transcripts conserved specifically in the source organisms and *P. stuartii* were selected for differential expression analysis across three bacterial growth phases after target mRNA prediction using the TargetRNA2 web tool. **Results:** In this study, 8 potential homologous npcRNA candidates conserved within the *P. stuartii* genome were identified, of which 3 were specific to *P. stuartii* and the source organism. Notably, StyR-29, a npcRNA from *S. typhi*, displayed a distinctive expression pattern with a steady decline from the lag phase to the stationary phase. It is predicted to regulate fimbrial adhesions, enhancing bacterial attachment and biofilm formation, which suggests a survival strategy for *P. stuartii* on catheters during UTI pathogenesis. **Conclusion:** This study is significant as it successfully identified the virulence-associated homologous npcRNA, StyR-29, in *P. stuartii*. The differential expression study suggests that StyR-29 is growth phase-dependent, thereby deepening our understanding of *P. stuartii* pathogenesis. This could be a breakthrough for RNA-based therapeutics.
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Keywords: npcRNA, virulence, *P. stuartii*, comparative genomics, differential expression

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INTRODUCTION

As the global incidence of urinary tract infections (UTIs) grows more prevalent, the looming threat of multidrug resistance in pathogenic organisms poses a formidable challenge to modern healthcare. This widespread infection, with the capacity to affect individuals across all age groups and genders, can present in various forms (1). Within the spectrum of UTI presentations, a rare yet intriguing phenomenon known as Purple Urine Bag Syndrome has captured the attention of physicians. This benign occurrence often serves as an indicator for urinary infections caused by *Providencia stuartii* in long-term indwelling catheter users (2).

Providencia stuartii belongs to the Gram-negative Enterobacteriaceae family, notorious for its uropathogens including *Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae*, and *Shigella flexneri*. *P. stuartii* has been listed as a critical priority pathogen by WHO due to its increasing drug resistance (3). This facultative anaerobe appears as a straight rod with rounded ends (4). This opportunistic pathogen owes its motility to its peritrichous flagella. Being the most pathogenic species of the genus, *P. stuartii* grows optimally at the average human body temperature of 35-37^{0}C. Its remarkable pH tolerance, ranging from pH 6 to 9, also allows it to survive in urine (5). *P. stuartii* is commonly isolated from sewage water, contaminated soil, and livestock

reservoirs, however, it could also find breeding grounds in hospitals by contaminating dispensers, medical apparatus, and indwelling instruments, including urinary catheters (6).

The nosocomial environment promotes the transmission of this invasive pathogen to immunocompromised individuals including the elderly and patients in intensive care units (ICU). Aside from UTI, *P. stuartii* has also been known to cause numerous other infections including cerebral meningitis, wound infections in burn victims and endocarditis (7). Hospital-acquired *P. stuartii* infections are on the rise due to the spread of multidrug-resistant strains. In addition to the intrinsic resistance to first and second generation of cephalosporins, conferred to by the overexpression of AmpC β-lactamase(8) and downregulation of porins, Omp-Pst1 and Omp-Pst2 (9), this bacterium has been recently discovered to have developed resistance to multiple antibiotics including tigecycline and colistin, which are typically used as final-resort treatments. According to a recent study conducted in Huainan, 92.1% of *P. stuartii* isolates from patients in intensive care exhibited multidrug resistance, with 26% of isolates being extremely fatal due to their resistance to 21 types of antibiotics (10). Currently, *P. stuartii*-associated UTIs are being treated with imipenem and cefepime (11). However, it is likely that *P. stuartii* may soon develop resistance to these antibiotics as well.

In order to address the antibiotic resistance of this bacteria, a closer examination of the pathogenesis pathway becomes crucial, particularly within the context of UTIs. *P. stuartii* gains access to the urogenital tract by colonising the surfaces of indwelling urinary catheters (12). *P. stuartii* is able to attach firmly to plastic surfaces, such as that of catheters, due to the presence of hair-like appendages known as fimbriae. Upon attachment, this bacterium forms a crystalline biofilm, obstructing catheter flow and facilitating penetration into the bladder (13). Biofilm also contributes to antibiotic resistance and aids the bacterium in withstanding high concentrations of urea, up to 500mM, in the urine. Planktonic cells of the biofilm are released to enable the colonisation of other parts of host cells. These planktonic cells utilise swarming motility to invade bladder and kidney tissues. *P. stuartii* employs the type 6 secretion system to inject virulence factors directly into host cells, inducing oxidative stress and subsequent cell death (14).

The invasion of these bacterial cells, coupled with the breakdown of urea into ammonia and carbon dioxide, results in pyelonephritis. If left untreated, a *P. stuartii*-induced UTI may lead to complications due to urease activity. By-products of urea breakdown, such as ammonia and carbon dioxide, can bind with magnesium and calcium ions in the urine, forming hard plaques and potentially causing kidney stones (15). Additionally, untreated UTI, especially in immunocompromised individuals, poses the risk of systemic infections like bacteraemia or sepsis as bacteria spread through the bloodstream.

The underlying molecular mechanisms behind the pathogenesis pathway involved in *P. stuartii* UTI is an intricate interplay between bacterial virulence genes and non-protein coding RNA - regulatory molecules that do not encode proteins (16). Transcribed from intergenic regions between genes, *cis*-acting and *trans*-acting npcRNA regulate the expression of their target mRNAs post-transcriptionally (17). The npcRNA is involved in tasks that contribute to cellular regulatory processes, stress responses, and antibiotic resistance. For example, npcRNAs can bind to mRNAs to either enhance or inhibit their translation, to aid bacteria in adapting quickly to stressors like nutrient scarcity (18) or oxidative stress (19). Additionally, certain npcRNAs are implicated in antibiotic resistance by regulating the expression of genes that encode efflux pumps, among other mechanisms (20). By detecting variations in temperature and pH, npcRNAs enable bacteria to respond rapidly to ensure survival in harsh conditions encountered within a host organism (21). On top of promoting bacterial resilience within a host, npcRNAs play a crucial role in regulating the expression of virulence factors, contributing to the development of persistent and severe infections.

An intense literature search was conducted to explore previous studies on the identification of non-protein-coding RNAs in *P. stuartii* leading to limited results. In fact, there was no literature on homologous npcRNA differential expression studies. Hence, in this study, a comparative genomics approach is employed to screen known npcRNAs within the Enterobacteriaceae family, specifically from the selected reference organisms (*E. coli, S. typhi* and *Y. pestis*) aiming to identify putative homologous npcRNAs within the *P. stuartii* genome predicted to regulate *P. stuartii* virulence factors at the post-transcriptional level.

This screening was performed through BLASTn, a nucleotide sequence alignment tool, by comparing known npcRNA sequences in the reference organisms to *P. stuartii*. The transcripts identified through BLASTn were screened for annotated sequences using the non-coding RNA database, Rfam. Further, differential expression of identified homologous npcRNAs was determined via reverse transcription PCR to understand the post-transcriptional regulatory level towards its predicted target mRNA. TargetRNA2, a program for predicting npcRNA-mRNA interactions, was used to identify potential mRNA targets for the npcRNA candidates based on sequence complementarity and lowest hybridisation enthalpy. The RNA secondary structure prediction tool, RNAfold was then used to predict the possible folding between the npcRNA sequence forming the stem-loop secondary structure.

The predicted regulatory roles of homologous npcRNAs lay the groundwork for future studies to validate these roles via experimental methods. This valid information can lead to an alternate option of npcRNA-based therapeutics, offering a potential solution to combat multidrug resistance issues in the future.

MATERIALS AND METHODS

Screening for conserved homologous npcRNAs in *P. stuartii*

Conserved homologous non-protein coding RNAs (npcRNAs) in *Providencia stuartii* were identified by conducting a Basic Local Alignment Search Tool (BLASTn version: BLAST+ 2.16.0) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) search using 133 known npcRNAs from *Escherichia coli* (22), 96 npcRNAs from *Salmonella typhi* (23), and 5 npcRNAs from *Yersinia pestis* against the genome of *Providencia stuartii* strain MRSN 2154. The BLASTn tool was utilised in this comparative analysis of bacterial species in the Enterobacteriaceae family. The criteria for identifying conserved homologous npcRNAs in *Providencia stuartii* included a query coverage greater than 80%, E-values less than 0.001, and complete genome coverage (24). The homologous npcRNAs were also used to derive additional information, such as the 5' coordinate, 3' coordinate, strand (plus/minus), and Max Score. The possible homologs identified from BLASTn analysis were subjected to Rfam (https://rfam.org/) analysis to screen for any annotated transcripts in *P. stuartii*. The potential mRNA targets of the putative homologs were predicted using Target RNA2 (https://cs.wellesley.edu/~btjaden /TargetRNA2/userguide.html) analysis based on the lowest energy of hybridisation and removal of possible putative and hypothetical genes.

Total RNA extraction from three growth phases of *P. stuartii*

Overnight inoculum of the bacteria was prepared reviving 5µl of glycerol stock of *Providencia stuartii* strain MRSN 2154 in 5 ml of Luria-Bertani (LB) broth. Subsequently, a culture flask containing 100 ml of LB broth was inoculated with 1% volume of the overnight inoculum and allowed to incubate at 37^{0}C with agitation at 180 rpm. The bacterial culture was harvested at the lag (OD_{600} 0.2), exponential (OD_{600} 0.6) and stationary phase (OD_{600} 1.0) (25). These OD_{600} values for respective lag (0.2) log (0.6) and stationary (1.0) were fixed upon literature search which reveals the growth curve generated for *P. stuartii* (5). The wavelength of 600 nm was specifically chosen for measuring bacterial growth phases because it is minimally absorbed by Luria-Bertani (LB) broth, which is used for cultivating *P. stuartii*. Total RNA was extracted using the Trizol method and washed with 80 percent ethanol after being allowed to precipitate overnight. Total RNA from the three bacterial growth phases were eluted with nuclease-free water before checking their concentrations using Nanodrop. Following that, Urea-PAGE gel electrophoresis was used to analyse the total RNA fragmentation in the sample. The fragmented RNA sample bands were observed under the UV fluorescent light using a gel documentation system. DNase treatment was then carried out with 1000 ng of total RNA to remove contaminating DNA. Following DNase treatment, the total RNA extracted from each growth phase was validated for DNA contamination through normal PCR amplification. The concentrations of DNase-treated lag, exponential and stationary phase RNA were determined using Nanodrop.

Optimising annealing temperatures of custom-designed primers through gradient PCR

The optimal annealing temperatures for the custom-designed forward and reverse primers for S15 from *E. coli* (forward primer: TGAGATGCTAAATTAGAGATTGGCT, reverse primer: ATTAACGACGCAGACCCAGA), StyR-29 from *S. typhi* (forward primer: GACGTTTATTTGCACAAATCCATTG, reverse primer: ACTTTCTTGATAAAAGTAC GCACC) and StyR-64 from *S. typhi* (forward: AAACTACGAAAACCATCG, reverse: TTTCTTAACGCGTTCTTC), were standardised by performing gradient PCR amplification of genomic DNA of *P. stuartii* extracted through the boiling lysate method. The PCR reaction was performed using the reaction mix comprised of the forward and reverse primers, Taq DNA Polymerase, Taq DNA Polymerase PCR buffer, deoxynucleoside triphosphate and nuclease-free water. The thermal cycler (Bio-Rad T100 Thermal Cycler) condition used in the PCR started with an initial denaturation step at 95^{0}C for 5 minutes followed by 38 cycles of denaturation at 95^{0}C for 2 minutes, annealing at temperatures between 56 and 69^{0}C for 90 seconds and extension at 72^{0}C for 2 minutes. This was followed by a final extension at 72^{0}C for 5 minutes and final hold at 4^{0}C. The amplified products were visualised using a gel documentation system after resolving in 1% agarose gel.

Differential expression analysis of selected npcRNA homologs by reverse transcription PCR

DNase-treated RNA from the lag, exponential and stationary growth phases were reverse transcribed with the reverse transcription reaction mix consisting of reverse transcriptase enzyme, 5x reverse transcription buffer, PCR nucleotide mix, magnesium chloride and nuclease-free water to obtain complementary DNA (26). The reverse transcription step was carried out by incubating at 25°C for 5 minutes, followed by incubation at 50°C for an hour, and final incubation at 75°C for 5 minutes. The cDNA synthesised was subjected to PCR amplification at an annealing temperature of 60^{0}C. The products of PCR amplification were resolved in 1% agarose gel and visualised in a gel documentation system. ImageJ software was used to examine the mean band intensity of the amplified products from digital photographs of the gel.

RESULTS

TargetRNA2 analysis

The analysis was conducted using the TargetRNA2 tool to predict the mRNA targets of the identified homologous npcRNAs. Targets with the lowest predicted hybridisation energy, specifically those associated with virulence were selected. Putative or hypothetical targets were eliminated to ensure the results reflect biologically relevant interactions. This approach enhances the reliability of predictions and provides a clearer understanding of how these npcRNAs may influence the pathogenesis pathway in *P. stuartii* (Figure 1).

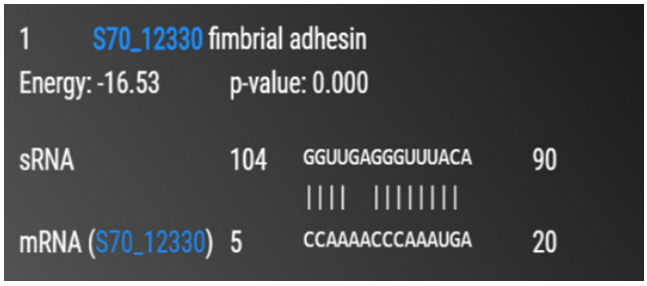

Figure 1:. Target mRNA (fimbrial adhesin) for StyR-29 homolog in P. stuartii predicted using TargetRNA2 webtool.

TargetRNA2 prediction of StyR-29 reveals its potential role in mRNA encoding for fimbrial adhesin at the lowest hybridisation energy of -16.53 kcal/mol (Figure 2).

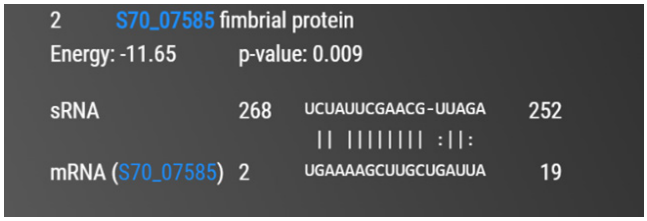

Figure 2: Target mRNA (fimbrial protein) for S15 homolog in *P. stuartii* predicted using TargetRNA2 webtool.

Using TargetRNA2, the npcRNA S15 was predicted to regulate mRNA encoding virulence factor fimbrial protein with the lowest hybridisation energy of –11.65 kcal/mol. The S15 gene has a specific binding site to its target mRNA at the blue highlighted region (Figure 3).

The StyR-64 gene was predicted to bind to its target mRNA flhA at the blue highlighted region to regulate the virulence function of *P. stuartii*. Using TargetRNA2, the npcRNA StyR-64 was predicted to regulate mRNA encoding virulence factor fimbrial protein with the lowest hybridisation energy of –9.92 kcal/mol.

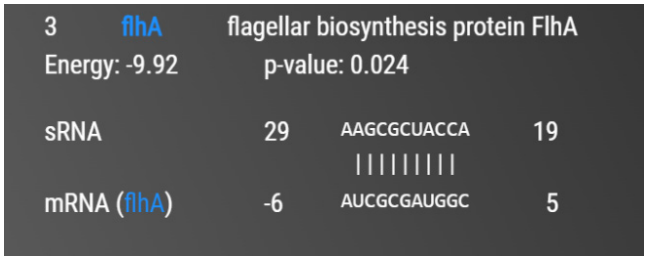

Figure 3: Target mRNA (flhA) for StyR-64 homolog in *P. stuartii* predicted using TargetRNA2 webtool.

DNA gel electrophoresis and quantification of the expression level of StyR-29 using ImageJ

The gel electrophoresis result showing the expression levels of StyR-29 in *P. stuartii* was quantified using ImageJ. The band intensity provides insights into the abundance of StyR-29 and its potential regulatory functions in *P. stuartii* pathogenesis (Figure 4).

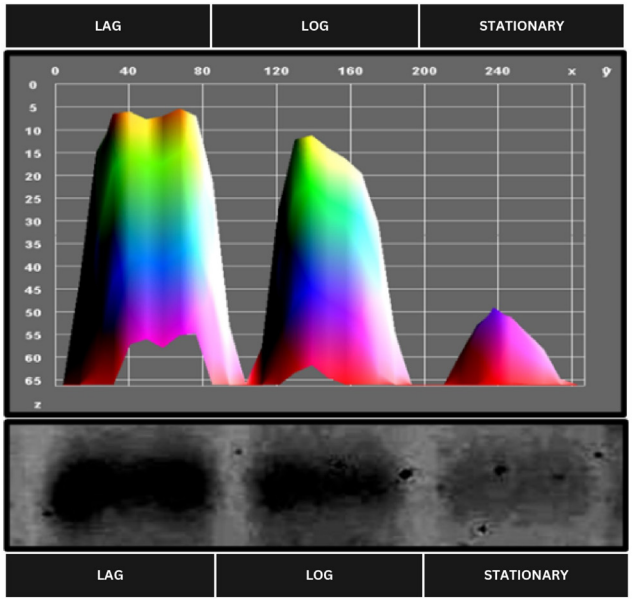

Figure 4: Expression profile of StyR-29 homolog in P. stuartii during different growth stages.

Quantitative analysis of the expression levels during lag, log and stationary phase was done using the ImageJ tool. The expression is the highest during the lag phase followed by a gradual decline log and stationary phase.

The Image J software generated peaks corresponding to the density of the PCR bands for the StyR-29 gene at different growth stages in *P. stuartii*. The PCR band with the maximum density in the Lag phase was visible in Lane 1 which reveals the highest expression of StyR-29.

Secondary structure prediction using RNAfold web tool

The predicted secondary structure was generated for StyR-29 using the RNAfold web tool. This analysis provides insights into optimal folding patterns of this npcRNA revealing important structural motifs that may facilitate interactions with target mRNAs (Figure 5).

The RNA fold analysis reveals the specific binding site of StyR-29 to its target mRNA at the blue highlighted region

Circular representation of whole genome and conserved homologous npcRNAs using DNA plotter

The circular genome map generated by DNA plotter illustrates the complete genome of *P. stuartii*, highlighting the locations of the conserved homologous npcRNAs

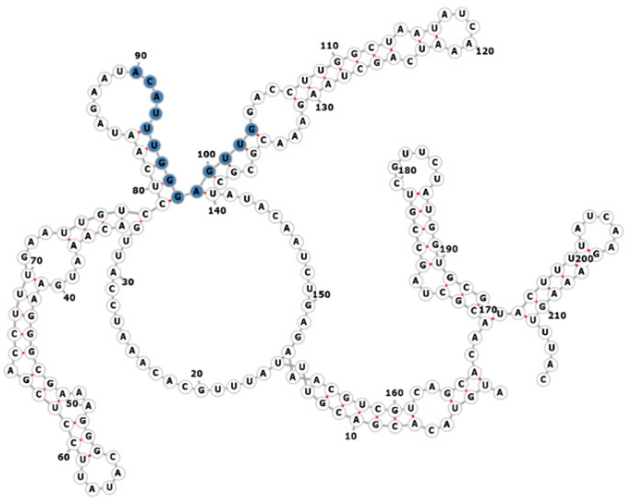

Figure 5: Secondary structure of StyR-29 homolog predicted using RNAfold webtool. The blue-coloured sequence is the binding site of the target mRNA in *P. stuartii*

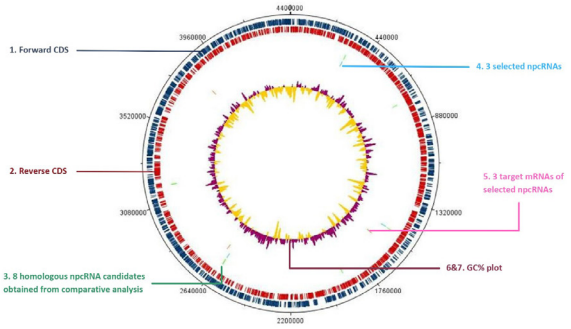

Figure 6: Circular representation of whole genome and conserved homologous npcRNAs identified in Providencia stuartii strain MRSN 2154. The tracks from the outside represent (1) Forward CDS (Dark Blue); (2) Reverse CDS (Red); (3) 8 homologous npcRNA candidates obtained from comparative analysis (Green); (4) 3 selected npcRNAs (Light blue) - (Clockwise) StyR-64, StyR-29 and S15; (5) 3 target mRNAs of selected npcRNAs (Pink) – (Clockwise) fimbrial protein S70_07585, fimbrial adhesin S70_12330 and flagella biosynthesis mRNA, flhA; (6&7) % GC Plot (Purple and Yellow).

identified in *P. stuartii*. It also shows the relative distances between these npcRNAs and their target genes within the genome (Figure 6).

DISCUSSION

Screening for homologs of known npcRNAs in *P. stuartii*

This study employed a combined approach that integrated comparative genomics and differential expression analysis. This approach aimed to identify and shed light on the mRNA targets of conserved homologous npcRNAs associated with *P. stuartii* virulence. Comparative genomics proves invaluable in identifying putative non-protein coding RNAs (npcRNAs) that retain crucial biological significance across evolutionarily related species. This is why a similar approach has been utilised by many researchers to identify conserved homologous non-coding RNA in less-studied organisms. For instance, Kishan et al applied this approach to identify 33 homologous npcRNA in *Proteus vulgaris* (25). The identified npcRNAs undergo differential expression studies across three bacterial growth phases to elucidate the expression pattern across these three phases. Studying the expression of virulence-associated npcRNAs across different growth phases is essential for understanding bacterial pathogenesis and, therefore helps assess the stage of infection, enabling targeted therapies to be administered before the bacteria spread from their initial niche. Each growth phase affects virulence-related npcRNA expression differently. During the lag phase, bacteria may downregulate certain virulence factors as they focus on proliferation and adaptation to their environmental conditions. Hence this phase also confers low or no mutation in the bacterial genome which could be a significant growth phase for molecular detection using the identified homologs as biomarkers (27). The npcRNA highly expressed during this phase warrants close monitoring as it may serve as biomarkers for early detection of infection. In the stationary phase, nutrient depletion shifts the focus to survival, resulting in reduced virulence expression, but factors like biofilm formation are expressed increasingly to enhance resilience and persistent infections (28).

This combined approach of comparative genomics and differential expression led to the prediction of the homologous npcRNA's secondary structural confirmation, regulatory functions and mRNA targets. Identification of novel npcRNAs, especially those modulating the expression of virulence-associated mRNAs, gives insight into the molecular strategies used by pathogens to manipulate host responses. Hence, this study could be a breakthrough in establishing alternate npcRNA-based therapeutics against multidrug resistance *P. stuartii* combating the current global pandemic,

In this study, the BLASTn tool was used to identify the conservation of known npcRNA from three human disease-causing pathogenic bacteria, Escherichia coli, Salmonella typhi and Yersinia pestis against pathogenic *Providencia stuartii* (strain MRSN 2154). The three source organisms selected for this study all belong to the family Enterobacteriaceae, sharing a recent common ancestor with *P. stuartii*. Despite their common ancestry, the three chosen organisms offer enough genomic diversity and are considered model organisms in the field of non-coding RNA, making them valuable reference organisms for identifying and validating homologous conserved npcRNA in the less-characterized *P. stuartii* genome. Identifying npcRNA that are evolutionarily conserved among bacterial species highlights their fundamental importance in bacterial survival (29), which is why there is a strong evolutionary pressure to maintain their structure and function. Comparative genomic studies reveal evolutionary relationships between different

bacterial species through their shared npcRNAs. Conserved npcRNA in distantly related species could also highlight their importance as they could have been passed through horizontal gene transfer to ensure survival (30).

A total of 234 known npcRNA out of which 133 from *E. coli*, 96 from *S. typh*i and 5 from *Y. pestis* were screened against *P. stuartii* MRSN 2154 to detect conserved homologous region. Only 1 out of 133 npcRNAs from E. coli was found to be conserved in *P. stuartii*, followed by 5 out of 96 from *S. typhi* and 2 out 5 from *Y. pestis* which summed to a total of 8 homologous npcRNA homologous to *P. stuartii*. The 8 homologous candidates were further screened for annotations in the Rfam and NCBI databases to neglect annotated transcripts in other organisms as shown in Table I. Interestingly out of the 8 candidates, only 3 candidates were identified to be species specific to *P. stuartii* and origin organisms (*E. coli* and *S. typhi*). Homologs conserved in other organisms were excluded from consideration, as they are likely associated with common cellular processes. The 3 npcRNAs conserved specifically between the source organism and *P. stuartii* are more promising candidates, holding greater potential for novelty and could possibly regulate the virulence-associated mRNAs post-transcriptionally.

Differential Expression studies of three homologous npcRNA in *P. stuartii*

TargetRNA2 tool was used to predict possible target mRNAs for all 8 homologs, to understand their possible post-transcriptional roles either in the upregulation or downregulation of their targets responding to external parameters such as growth condition and environmental cues. Interestingly TargetRNA2 predicted 3 out of the 8 homologous transcripts (S15, StyR-29 and StyR-64) targeting virulence-associated mRNAs in *P. stuartii*. The S15 and StyR-29 were predicted to bind mRNA coding for fimbrial proteins and fimbrial adhesins with the lowest hybridization energy of -11.65 kcal/mol and -16.53 kcal/mol respectively (Fig. 1 and Fig. 2). StyR-64 was predicted to bind mRNA coding for flagella biosynthesis protein, FlhA, with the lowest hybridisation energy of -9.92 kcal/mol (Fig. 3). The thermodynamic energy (kcal/mol) of hybridization is the selection criteria for interaction between the npcRNA and its target mRNA. Lower binding energy indicates a probability of an interaction occurring between the complement nucleotides by chance that is at least as energetically favourable (31).

These three promising candidates were subjected to reverse transcription PCR to understand their differential expression levels across three growth stages in *P. stuartii*. Interestingly, StyR-29 showed expression in all three growth stages (Fig. 4). A very high expression level could be observed during the lag phase followed by mid-level expression during the log phase and reduced expression during the stationary phase as quantitatively calculated by the ImageJ tool. As mentioned previously, StyR-29 was predicted to be associated with fimbrial adhesin, which is implicated in bacterial adhesion to host cells for colonisation as well formation of strong non-destabilizing biofilms leading to prolonged and antimicrobial resistance, especially in hospital-acquired infections. Fimbrial adhesins assist in the attachment of *P. stuartii* in the urinary tracts as well as indwelling urinary catheters via forming a sticky biofilm layer which acts like a glue (32). Previous study shows fimbriae mRNA expression to be the highest at the stationary phase (33). An assumption could be made that the overexpression of fimbrial adhesins during the lag phase, characterised by environmental adaptation, is most likely due to the

Table I: List of npcRNA homolog candidates in *P. stuartii* screened using NCBI BLASTn tool.

Organisms of known npcRNA	Name of known npcRNA	Conservation in *P. stuartii MRSN 2154*						
		Query coverage (%)	E-value	Identities (%)	Strand	5′ coordinate	3′ coordinate	Known function of npcRNA in reference organism
E. coli	S15	91	5e-73	81.68	+	2617680	2618009	Translation regulation of the ribosomal S15 protein (43)
S. typhi	StyR-29	87	5e-66	87.5	+	1510295	1510509	Masks the S20 ribosomal binding site of *rpsT* mRNA reducing the growth rate of *S. typhi* (23)
S. typhi	StyR-64	80	8e-36	97	-	330985	331071	Regulates housekeeping mRNAs in *S. typhi* (23)
S. typhi	ssrS	98	2e-28	79	+	752352	752529	Promotes the survival of *S. typhi* in acidic environment (pH 3.0) (44)
S. typhi	GcvB	93	2e-26	77	-	3179234	3179007	Regulates periplasmic substrate-binding proteins of ABC uptake systems for amino acids and peptides (45)
S. typhi	S15	91	3e-75	82	+	2617680	2618009	Regulates mRNAs with housekeeping functions in *S. typhi* (46)
Y. pestis	GcvB	94	1e-73	85	-	3179282	3179008	Represses periplasmic-binding protein component of the dipeptide transport system (47)
Y. pestis	Spot_42	91	9e-36	91	-	1888852	1888744	Regulates ribosome biogenesis GTP-binding protein YsxC (48)

initial steps of establishing the infection (34). Pathogenic bacteria, like *P. stuartii*, may prioritise attaching to host cells in these early stages before forming biofilm colonies. Aside from adhesion, this initial interaction with the host also provides optimal physiological conditions and access to nutrients (35). By analysing the differential expression pattern of StyR-29, clinicians may be able to monitor the specific growth phase during which the fimbrial adhesin gene is highly expressed. This could lead to the development of biomarkers for detecting infection progression, allowing for targeted intervention against *P. stuartii* before it reaches its peak virulence in CAUTIs.

It remains unclear whether StyR-29 upregulates or downregulates the fimbrial adhesin. Hence, understanding the post-transcriptional regulatory interaction between StyR-29 and its predicted target mRNA requires a separate experimental study. Nevertheless, it is important to note that similar recent studies involving other npcRNAs, namely RpoS (36) and SdiA (37), which regulate type 1 fimbriae in *E. coli* and *Klebsiella pneumoniae*, respectively, have indicated a trend of these npcRNAs repressing fimbrial expression. A previous study conducted by Chinni et al revealed StyR-29 had differential expression in three different loci in the genome of *S. typhi* during the stationary phase via northern blot analysis. Two out of three expressions were high while the other was low. Hence, this finding could deduce that StyR-29 can confer either high or low post-transcriptional regulation towards its target mRNA in *P. stuartii* in a similar fashion as in *S. typhi*.

The S15 originating from *E. coli* also targets fimbrial proteins similar to StyR-29. It is possible to hypothesise that S15 performs a similar role as StyR-29 regulating bacterial virulence by promoting bacterial adhesion to host cells. Nevertheless, S15 might have distinct functions in different organisms. For example, S15 in *E. coli* participates in the assembly of the small ribosomal subunit and can autoregulate its synthesis through a conformational change in its binding site. In contrast, in another bacterium—*T. thermophilus*—this npcRNA employs a unique mechanism with the leader segment of its mRNA to hinder its own translation (38). The secondary structure prediction of Styr-29 as shown in Fig. 5 reveals the mRNA binding region to be almost linear with less folding. Lesser folding correlates with lesser binding energy required for annealing both npcRNA and its target due to the complementary sequences (Blue-coloured sequence as in Fig. 5) are not hindered by any intramolecular secondary folding which requires more energy to break the hydrogen bonding between the nucleotides.

Furthermore, StyR-64 is predicted to control the expression of the flagella biosynthesis protein, FlhA. FlhA, along with the FliHIJ complex, helps coordinate the export of flagellar proteins, ensuring they are properly directed through the export apparatus for the assembly of the flagellar structure. FlhA is also a component of the export gate of the flagellar Type 3 Secretion System (T3SS) which is responsible for exporting proteins associated with virulence to host cells (39).

With the exception of StyR-64, the two other identified npcRNAs regulate the expression of fimbrial proteins, despite their varying roles in the source organisms. However, only StyR-29 had differential expression in the three growth phases. This could be due to the experimental conditions not being favourable for the expression of the S15 and StyR-64 npcRNAs. In further experiments, the expression of these transcripts could be induced under other conditions such as temperature, pH, nutrient or antibiotic stressors. When the critical role of adherence as a key virulence factor is emphasised, it becomes evident that bacteria's ability to establish infection is highly dependent on their ability to attach to host cells. In the context of *P. stuartii*, which is known to cause infections via catheters, the survival of the bacterium heavily relies on fimbrial proteins facilitating its attachment to plastic surfaces. Nevertheless, the predicted targets from TargetRNA2 are not definitive, as the tool relies solely on sequence complementarity between npcRNAs and target mRNAs. Factors like RNA secondary structures and interactions with chaperone proteins are often overlooked by TargetRNA2 (40). Therefore, a separate experimental validation through RT-PCR or Northern blot of both the npcRNAs and their predicted target gene is necessary to accurately determine the expression level of these two transcripts. Furthermore, another separate gene knock-out study could be done to understand the exact post-transcriptional roles of the homologous npcRNAs either as housekeeping genes or regulating virulence activity of *P. stuartii*.

The locus of all 3 putative homologous npcRNAs are illustrated in Fig. 6 showing that all three are distantly located from each other in the *P. stuartii* genome. The genomic locations of the mRNA targets of these npcRNAs are also depicted in Fig. 6. Based on the distance of the npcRNAs StyR-29, StyR-64 and from their target mRNAs which are the fimbrial protein-coding mRNA S70_12330, flhA and S70_07585 fimbrial protein, respectively, we can conclude that these npcRNAs are likely *trans*-acting npcRNAs that interact with mRNA through imperfect base pairing. *Trans*-acting npcRNAs often require the help of RNA chaperones, like Hfq (41) or ProQ(42), to facilitate binding to their mRNA targets.

Building upon these findings on the genomic proximity of putative npcRNAs and their target mRNAs, we are not only able to dive deeper into the role of npcRNAs in bacterial virulence but also the specific mechanisms involved in virulence regulation. Advancing this research on npcRNA expression associated with virulence, particularly considering the antigenic nature of fimbriae,

this discovery could set a platform for future experimental approaches unveiling exciting innovations such as RNA vaccines targeting uropathogenic *P. stuartii*. Since there is a lack of accurate and robust detection of infectious disease-causing bacteria and increasingly critical levels of antibiotic-resistant bacteria in the clinical setting, these findings lay the groundwork for the potential application in a later developmental stage whereby StyrR-29 can be used for accurate gene-based detection of *P. stuartii* to prime the human immune system against pathogens as bacterial non-protein-coding RNAs are very rare for mutation compared to mRNAs.

CONCLUSION

In this study, we successfully identified three potential npcRNAs; S15, StyR-29 and StyR-64 in the uropathogenic *P. stuartii* through comparative genomic studies involving 234 known npcRNAs from *E. coli*, *S. typhi* and *Y. pestis*. The identified npcRNA are conserved homologous transcripts that are unannotated in other organisms. A study on the expression patterns of the putative npcRNA candidates across different growth phases revealed that only StyR-29 exhibited differential expression, being highly expressed in the lag phase, gradually decreasing in the log phase, and further declining in the stationary phase. The predicted role of StyR-29 in regulating the expression of fimbrial adhesin, crucial for bacterial adherence in the early stages of infection, suggests its significance. The homologs found in this study could also be the source for the future development of RNA-based vaccines and RNA-based antibiotics upon further experimental evaluations.

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