Special Issue: International Conference on Contemporary Issues 2022
MJSAT 2 (S1) 12-15, December 2022

MJSAT

Malaysian Journal of Science and Advanced Technology

journal homepage: https://mjsat.com.my/

Identification and Characterization of Non-Protein Coding RNA Homologs in *Citrobacter rodentium* by Comparative Genomics

Agilandeswarie Kavin Selvam[1], Kishan Raj Selva Raju [1*]

[1]*Department of Biotechnology, School of Biotechnology, Manipal International University, No 1, MIU Boulevard, Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia.*

KEYWORDS

Non-protein Coding RNA
In silico
Reverse Transcriptase Polymerase Chain

ABSTRACT

This experiment was conducted to identify homologous non-protein coding RNAs (npcRNA) in *Citrobacter rodentium* as an alternate for solving health issues caused by this bacteria. Abuse of antimicrobial leads to the emergence of antimicrobial resistance pathogen. Aim of this research is to screen homologous npcRNAs from known bacteria in *C. rodentium,* to study the expression of homologous npcRNAs in *C. rodentium* and to predict the regulatory roles of identified homologous npcRNA in *C. rodentium*. Firstly, screening for presence of homologous npcRNA in *Citrobacter rodentium* was conducted by collecting known npcRNAs from *S. typhi*, *E. coli* and *Y. pestis* falling under the same genus of *Enterobacteriaceae*. Secondly, screening the homolog npcRNA identified from previous step (BLASTn) through Rfam (RNA family) to identify unannotated homologs. Then, target mRNA prediction for homologous npcRNA was done using TargetRNA2 webtool to find the compliment mRNA binding of the homologous sequence and the regulatory activities of this transcript. The npcRNA homologs which predicted to regulate virulence target mRNA were assessed for its expression profile at different growth stages via reverse transcription PCR and the band intensity was quantitatively analysed using Image J tool. The known npcRNA Styr-296 from *S. typhi* showed expression in *C. rodentium* during three growth stages (lag, log and stationary). The expression was observed to be high during lag phase followed by no expression during log and stationary phase. This Styr-296 homolog was predicted to regulate mRNA translating modulator drug activity B which is associated with the bacterial virulence. Hence, this is a preliminary study promising for further elucidation of more virulence associated npcRNAs that are yet to be discovered from *C. rodentium* developing strategy to unveil alternate therapeutic options for diseases caused by this bacterium.

1. INTRODUCTION

Citrobacter rodentium is a gram-negative bacteria of the family *Enterobacteriaceae*, which causes infectious mouse colonic hyperplasia and causes high mortality in lactating mice [1]. In mice, *enteropathogenic Escherichia coli* (EPEC) and *enterohaemorrhagic E. coli* (EHEC), two clinically significant human gastrointestinal pathogens, share similar pathogenic pathways with *Citrobacter rodentium* [2]. *Citrobacter rodentium* is a mouse-specific adherent and reflex pathogen that colonizes the gastrointestinal system of mouse model. Mitochondria-related proteins, also known as MAPs, are multifunctional effector proteins that target the mitochondria of host cells and contribute to infection-induced epithelial barrier dysfunction *in vitro* [3].

Like its human counterpart, EHEC, the mouse enteric pathogen *C. rodentium* generates attaching and effacing lesions in the intestinal epithelium of its host. The virulence factors expressed by the Locus for Enterocyte Effacement (LEE) pathogenicity island are required for this trait (Franzin & Sircili, 2015). The type III secretion system (T3SS), which is encoded by a pathogenic island called the enterocyte loss (LEE) locus, is central to their aetiology. Type III Secretion System is used to inject effector proteins that are both LEE and non-LEE encoded into host cells. The signalling pathways and

*Corresponding author:
E-mail address: Kishan Raj Selva Raju <kishan.raj@miu.edu.my>.

immunological responses of the host are regulated by these effectors. In some infections, T3SS is used to secrete some expression and secretion regulatory proteins. Because effectors regulate or disrupt the host's immune response as well as cell structure and function, identifying effectors is critical for understanding the pathogen's etiologic mechanisms. There are few antibiotics left to treat patients because the rise of significant and multi-drug resistant bacteria, so novel RNA-primarily based therapeutic approaches are being examined [4]. Some Gram-negative bacteria are classified as an emergency or a major public health danger by the Centers for Disease Control and Prevention. *Enterobacteriaceae* resistant to carbapenem (CRE) or extended spectrum beta-lactamase (EBSL), as well as multidrug-resistant *Acinetobacte*r and *Pseudomonas* species, are among the most dangerous. As a result, new approaches to combating these pathogens are being promoted (World Health Organization [WHO], 2014). The use of RNA-based treatments is a promising method looks at the current state of non-coding RNA (npcRNA) elements as antibacterial agents, as well as various implementation options and constraints. From animal stool samples, four *Citrobacter spp.* bacteria were identified so far [5]. Three of these *Citrobacter* spp. isolates have ability to synthesis ESBL. All isolates have multiple antimicrobial resistance (MDR), making them all multidrug resistant (MDR). Our phenotypic and genetic results reinforce the need for enhanced effort to monitor the potentially quick spread of ESBL-producers from a single health perspective and highlight the danger of rising ESBL prevalence in animals. Thus, it also predicted that *C. rodentium* also might have the same ability to synthesis ESBL. The npcRNA is a type of RNA that does not get translated into proteins. The RNA gene refers to the DNA sequence to which a functional non-coding RNA is transcribed. Transfer RNA (tRNA) and ribosomal RNA (rRNA), as well as short RNAs like microRNA, siRNA, piRNA, snoRNA, snRNA, exRNA, and scaRNA, and Xist, are all functionally significant non-coding RNA [6]. The function of many of the recently discovered npcRNAs has yet to be determined. Initially these npcRNAs are classified as junk RNAs as not much importance have been given to its functional roles. Recently, non-coding RNA has been discovered to have post-transcriptional regulatory functions which controls the expression of diseases such as cancer and Alzheimer's. As a whole, npcRNA is now known to regulate the expression level of its target mRNA by either activating or inhibiting the translation of the mRNA [7].

2. METHODOLOGY

2.1 Identification and screening for Citrobacter rodentium npcRNA homologs

From all of the homologs in *C. rodentium*, the npcRNA belonging to the same *Enterobacteriaceae* family was chosen for screening. In a comparison study applying the BLASTn program, the known npcRNA sequences from *Salmonella* Typhi (133 npcRNAs), *E. coli* (26 npcRNAs), and *Yersinia pestis* (5 npcRNAs) were gathered. The criteria that must be met for the identification of very likely homologs includes that the corresponding sequence selected must be the best hit and that the E-values must be less than 0.001. To search for homologs conserved within *C. rodetium* and the source organism only, the identified npcRNA homologs were examined using the Rfam online database. The TargetRNA2 online database was used to screen the identified npcRNA homologs in order to determine potential mRNA targets and their binding region based on the lowest binding energy [8].

2.2 Bacterial culture conditions and total RNA extraction from C. rodentium

Conical flask containing Luria Bertani medium (Himedia, India) (100 ml) was prepared and inoculated with *C. rodentium* ATCC 1388 glycerol stock (2ul). At 37°C, the inoculated LB medium was incubated along with 180 rpm of agitation. The bacterial cells were harvested at lag phase (OD_{600} 0.2), exponential phase (OD_{600} 0.6), and stationary phase (OD_{600} 0.8). Following the manufacturer's instructions, the total RNA from the collected bacterial cells was extracted using the TRIzol technique. The final concentrations of total RNA were determined using Nanodrop

2.3 Gradient PCR amplification

Oligos were designed to fix annealing temperature to carry out PCR amplification (Table 1). The thermal cycle procedure involved an initial single cycle of denaturation step at 95°C for 5 minutes, followed by 38 cycles of denaturation at 95°C for 2 minutes, annealing temperature range of 56°C - 69°C for 90 seconds, extension at 72°C for 2 minutes, and final single cycle extension at 72°C for 5 minutes. *C. rodentium* DNA was extracted using boiling lysate technique. To standardize the annealing temperature of specially designed oligos, gradient PCR amplification was carried out to identify the ideal annealing temperature.

Table 1. List of primers designed for reverse transcription PCR

npcRNA	Forward primers (5' to 3')	Reverse primers (3' to 5')
Styr-296	GGAAAGAGCCTC CGTTGTCT	GCAGCCCGGATGTAAG ACTC
Styr-281	CCACCTCAAAGT AGACGGCGCAT	TAAGAGGTTGATGCGG AGAGCGG

2.4 RT-PCR for expression analysis of selected npcRNA homologs

Using specifically designed oligonucleotides, reverse transcription PCR was performed on total RNAs isolated from *C. rodentium.* The first strand of cDNA was synthesized using a two-step PCR technique, at which the first step involved incubation at 70 °C for 5 minutes, the second stage involved incubation at 25 °C for 5 minutes, 50 °C for 1 hour, and lastly 70 °C for 5 minutes. Following a standardized annealing temperature of 60.9°C, this cDNA was amplified using the same PCR cycle as described in the gradient PCR methodology in section 2.3.

2.5 Quantification of npcRNA expression level

From digital images of the gel, ImageJ software was used to quantify the bands visualized after PCR gel electrophoresis. Bands were defined and recognised visually. After grayscale image processing, the PCR band's signals and its background were recorded in triplicate to determine the band's mean intensity [9].

3. RESULTS AND DISCUSSIONS

3.1 Homologous conservation of npcRNAs in Citrobacter rodentium

As observed from Table 2, a total of 164 npcRNAs from *Salmonella* Typhi, *E. coli* and *Y. pestis* were retrieved and screened against *C. rodentium.* It is observed that from the 164

npcRNAs, a total of 98 transcripts respectively 80 from *Salmonella typhi,* 15 from *Escherichia coli*, and 3 from *Yersinia pestis* were found to be homologous to *C. rodentium*. Out of these 98 transcripts, 28 were identified to be conserved only in *C. rodentium* and also in respective three above mentioned source organisms whereas the remaining 70 were conserved in a wide range of other bacterial species. As a whole, this screening pathway justified there were only 2 candidates that were potential for further target mRNA search and expression analysis to understand its possible regulatory roles as shown in Table 2.

3.2 *Expression profiling of selected homologous npcRNA of C. rodentium at different growth stages*

The orderly screening of the identified homologous npcRNAs from *C. rodentium* reveals only 28 transcripts not conserved in any other organisms wheras only conserved in *C. rodentium* and the source organism (*S. Typhi* / *E. coli* / *Y. pestis*). Screening through TargetRNA2 webtool reveals that Styr-296 and Styr-281 is involved in the regulation of virulence activities of *C. rodentium* (Figure 2 and 4). The secondary structure predicted for Styr-296 and Styr-281 (Figure 3 and 5 respectively) show lesser folding within the binding region which can be deduced that high possibility for compliment binding between this npcRNA and its target mRNA. It was our interest to identify homologous npcRNAs that regulates target mRNAs associated with the virulence factors of *C. rodentium*. Hence, from Figure 2 and Figure 4 it is observed that through partial compliment binding, Styr-296 regulates modulator drug activity B whereas Styr-281 regulates fimbrial protein both associated with the virulence of the bacteria. These two transcripts were selected for expression profiling to understand the regulatory pathway and only Styr-296 was showing expression meanwhile Styr-281 did not show any expression. However, Styr-296 npcRNA was observed to be expressing only in the lag phase of *C. rodentium* as shown in Figure 1. High expression level was found during lag phase. The assumption that can be made here is that the lag phase enables the adaptability needed for bacterial cells to start taking advantage of changing environmental conditions. These adaptability process could involve the production of the cellular elements required for development as well as the repair of macromolecular damage that accrued during stationary phase. The present physiological data just demonstrate that lag-phase bacteria are metabolically active, therefore these remain as hypothetical possibilities. As a result, there are no physiological or biochemical standards for defining lag phase at this time.

3.3 *Expression profiling of selected homologous npcRNA of C. rodentium at different growth stages*

The orderly screening of the identified homologous npcRNAs from *C. rodentium* reveals only 28 transcripts not conserved in any other organisms wheras only conserved in *C. rodentium* and the source organism (*S.* Typhi / *E. coli* / *Y. pestis*). Screening through TargetRNA2 webtool reveals that Styr-296 and Styr-281 is involved in the regulation of virulence activities of *C. rodentium* (Figure 2 and 4). The secondarystructure predicted for Styr-296 and Styr-281 (Figure 3 and 5 respectively) show lesser folding within the binding region which can be deduced that high possibility for compliment binding between this npcRNA and its target mRNA. It was our interest to identify homologous npcRNAs that regulates target mRNAs associated with the virulence factors of *C. rodentium*. Hence, from Figure 2 and Figure 4 it is observed that through partial compliment binding, Styr-296 regulates modulator drug activity B whereas Styr-281 regulates fimbrial protein both associated with the virulence of the bacteria. These two transcripts were selected for expression profiling to understand the regulatory pathway and only Styr-296 was showing expression meanwhile Styr-281 did not show any expression. However, Styr-296 npcRNA was observed to be expressing only in the lag phase of *C. rodentium* as shown in Figure 1. High expression level was found during lag phase. The assumption that can be made here is that the lag phase enables the adaptability needed for bacterial cells to start taking advantage of changing environmental conditions. These adaptability process could involve the production of the cellular elements required for development as well as the repair of macromolecular damage that accrued during stationary phase. The present physiological data just demonstrate that lag-phase bacteria are metabolically active, therefore these remain as hypothetical possibilities. As a result, there are no physiological or biochemical standards for defining lag phase at this time.

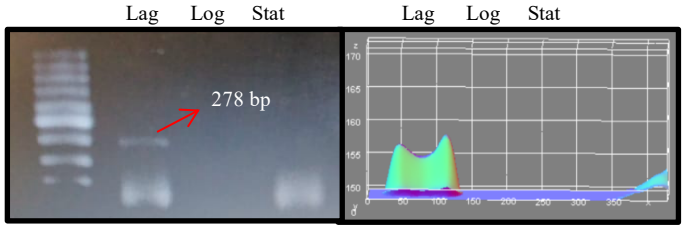

Fig. 1: Expression profile of Styr-296 homolog in *C. rodentium* during different growth stages. Right image shows quantitative analysis of the expression level using Image J tool.

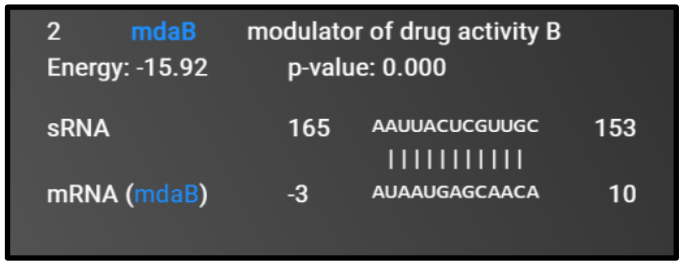

Fig. 1: Target mRNA (modulator of drug activity B) for Styr-296 homolog predicted in *C. rodentium* using TargetRNA2 webtool

Table 2. Total number of homologous npcRNAs conserved in *C. rodentium*

Microorganisms	Number of known npcRNA from source bacteria	Number of npcRNAs homologous to *C. rodentium*	Conservation percentage in *C. rodentium* (%)	Number of npcRNAs that only conserved in source organism and *C. rodentium*
S. Typhi	133	80	60.50	25
E. coli	26	18	69.23	2
Y. pestis	5	3	60.00	1
Total	164	100	60.98	28

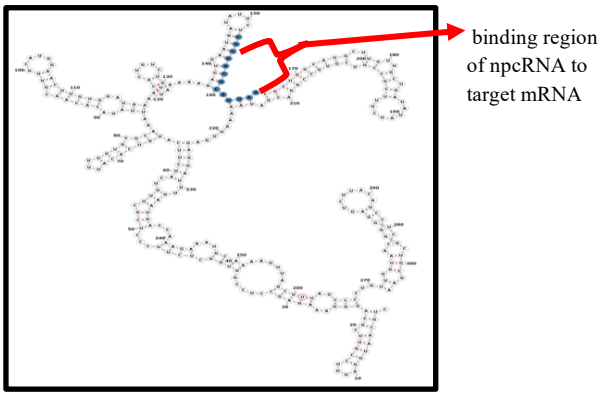

Fig. 2: Secondary structure of Styr-296 homolog predicted using RNAfold webtool. Blue colored sequence is the binding site for target mRNA in *C. rodentium*

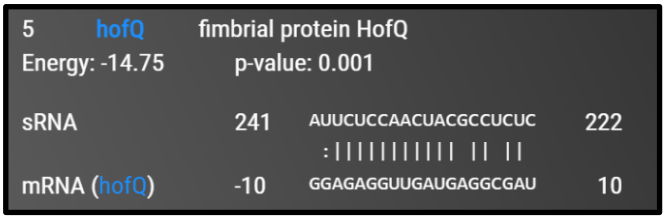

Fig. 3: Target mRNA (fimbrial protein Hofq) for Syr-281 homolog predicted using TargetRNA2 webtool in *C. rodentium*

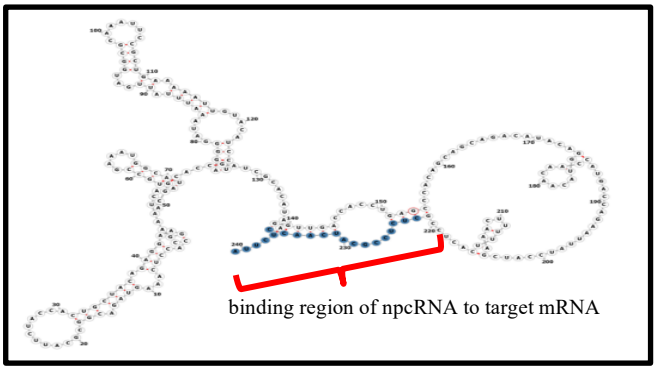

Fig. 4: Secondary structure of Styr-281 homolog predicted using RNAfold webtool. Blue colored sequence is the binding site for target mRNA in *C. rodentium*

When compared to a stationary-phase inoculum, A study on *Saccharomyces cerevisiae* transcriptomic revealed the genes that undergo lag-phase-dependent expression (Bertrand, 2019). Translation, protein folding, modification, translocation, and destruction, ribosome biogenesis, transcription, RNA processing, cell polarity, cell division, and control of the cell cycle were among the genes upregulation activity during the early lag phase shown in the study [10]. The current work demonstrates that the majority of these basic groups of genes also exhibit upregulation during the bacterial lag phase.

4. CONCLUSION

This is the first investigation for conservation of homologous npcRNAs in *C. rodentium*. Twenty eight of the 98 homologous npcRNAs that were conserved in *C. rodentium*. The potential regulatory responsibilities of each of the 28 homologs were also predicted. Reverse transcription PCR was used to detect the differential expression pattern of 1 homolog out of the 28. The expression displayed by Styr-296 npcRNA met the primary objective of this investigation, which was to explore the potential regulatory roles of detected npcRNA at distinct growth phases related with the pathogenicity of the bacteria. To establish their functions in the virulence and drug resistance of *C. rodentium*, all homologous npcRNAs discovered in this study should undergo additional experimental validation using techniques such gene knockout. This experimental validation may offer a foundation for the improvement of existing conventional gene therapies to produce more effective ones.

ACKNOWLEDGEMENT

Sincere gratitude to School of Biotechnology, Manipal International University for providing the platform to conduct this research project.

REFERENCES

[1] G. Bhinder, H. P. Sham, J. M. Chan, V. Morampudi, K. Jacobson, and B. A. Vallance, "The Citrobacter rodentium mouse model: studying pathogen and host contributions to infectious colitis," JoVE J. Vis. Exp., no. 72, p. e50222, 2013.

[2] N. Bouladoux, O. J. Harrison, and Y. Belkaid, "The mouse model of infection with Citrobacter rodentium," Curr. Protoc. Immunol., vol. 119, no. 1, pp. 19–15, 2017.

[3] C. Ma et al., "Citrobacter rodentium infection causes both mitochondrial dysfunction and intestinal epithelial barrier disruption in vivo: role of mitochondrial associated protein (Map)," Cell. Microbiol., vol. 8, no. 10, pp. 1669–1686, 2006, doi: 10.1111/j.1462-5822.2006.00741.x.

[4] G. Parmeciano Di Noto, M. C. Molina, and C. Quiroga, "Insights into non-coding RNAs as novel antimicrobial drugs," Front. Genet., vol. 10, p. 57, 2019.

[5] J. Qin et al., "Comparative genomic characterization of multidrug-resistant Citrobacter spp. strains in Fennec fox imported to China," Gut Pathog., vol. 13, no. 1, pp. 1–7, 2021.

[6] Y. Zhang et al., "A gRNA-tRNA array for CRISPR-Cas9 based rapid multiplexed genome editing in Saccharomyces cerevisiae," Nat. Commun., vol. 10, no. 1, pp. 1–10, 2019.

[7] K. Kingwell, "Small activating RNAs lead the charge to turn up gene expression," Nat Rev Drug Discov, vol. 20, pp. 573–574, 2021.

[8] M. B. Kery, M. Feldman, J. Livny, and B. Tjaden, "TargetRNA2: identifying targets of small regulatory RNAs in bacteria," Nucleic Acids Res., vol. 42, no. W1, pp. W124–W129, Jul. 2014, doi: 10.1093/nar/gku317.

[9] M. Citartan et al., "Asymmetric PCR for good quality ssDNA generation towards DNA aptamer production.," Songklanakarin J. Sci. Technol., vol. 34, no. 2, 2012.

[10] M. Piazzi, A. Bavelloni, A. Gallo, I. Faenza, and W. L. Blalock, "Signal transduction in ribosome biogenesis: a recipe to avoid disaster," Int. J. Mol. Sci., vol. 20, no. 11, p. 2718, 2019.