ORIGINAL ARTICLE

Discovery and Expression Profiling of Novel Non-Coding RNAs, AbaR-43 in *Acinetobacter baumannii*

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ABSTRACT

Introduction: One of the most persistent bacteria associated with nosocomial infections is *Acinetobacter baumannii*. The World Health Organisation (WHO) had list the *A. baumannii* in critical group for research on drug-resistant bacteria and antimicrobial resistance due to the emergence of multidrug resistant strains and the considerable morbidity and death caused by this infection. Finding new strategies for the effective treatment of diseases is essential for in *A. baumannii* infections. Non-protein coding RNAs have evolved into significant mediators in the regulation of bacterial pathogenicity. The ncRNA regulates the mRNA expression by binding to the RNA chaperone Hfq protein and producing ribonucleoprotein complexes. Although, several studies have identified and characterized a large number of ncRNAs from many organisms, there is lacking of detailed investigation for functional characterization of ncRNAs in *A. baumannii*. **Methods:** In this study, the ncRNA AbaR-43 was identified through transcriptome analysis of *A. baumannii* and the predicted target mRNA was identified. The total RNA from *A. baumannii* was extracted at 3 different growth conditions and this RNA was proceed with Northern blot analysis to confirm the expression of AbaR-43. **Results:** In this study, we described the novel ncRNA of AbaR-43 might help in understanding the regulatory pathways of the pathogen as the predicted target mRNA involved in the production of flavin reductase-like protein which involved in dNTP synthesis and iron metabolism. **Conclusion:** This finding will contribute to further research in developing ncRNA based detection method, exploring new potential therapeutic targets and development of RNA based vaccines.
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Keywords: *Acinetobacter baumannii*, ncRNA, AbaR-43, transcriptome, Antimicrobial resistance

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INTRODUCTION

Acinetobacter baumannii is classified as a Gram-negative bacterium that is non-motile and aerobic. It is a member of the Moraxellaceae family which is known for causing opportunistic infections. The bacterium exhibits adaptability to many environmental circumstances, including hypoxic environments. Acinetobacter baumannii has metabolic versatility, enabling it to effectively utilize a wide range of carbon sources, hence enhancing its adaptability and survival in various conditions. The organism is widely recognized for its ability to withstand dehydration, numerous disinfectants, and multiple antibiotics, including ones that are typically reserved as final options for treatment (1).

Acinetobacter baumannii is an important pathogen associated with healthcare and is the primary major source of nosocomial (hospital-acquired) infections (2). It is associated with ventilator-associated pneumonia, skin and soft-tissue infections, secondary meningitis, urinary tract infections, wound and blood stream infections, endocarditis, intra-abdominal abscess, and surgical site infections (3). Its ability to survive in hospital environments for extended periods of time, resistance to wide range of antimicrobial compounds, acquire antibiotic resistance genes by horizontal transfer has led to high mortality in patients infected with this organisms (25-28). This become a challenge for the clinicians and healthcare providers in controlling and eliminating the spread of *A. baumannii* (26, 29).

This organism is also known for its multidrug resistance. In 2024, the World Health Organization (WHO) released a priority list of bacteria, with *Acinetobacter baumannii* classified under critical group due to its urgent need

for new antibiotics or treatment. The organism has gained genetic elements that confer resistance, resulting in its ability to withstand the effects of many types of antibiotics, including carbapenems (2,4–8) . *Acinetobacter baumannii* poses a significant threat to healthcare institutions due to its antibiotic resistance and propensity to cause healthcare-associated illnesses. Consequently, extensive research efforts are dedicated to investigating infection control techniques and innovative treatment strategies for this pathogen. Understanding and combating this virus necessitates a comprehensive examination of its gene regulation, virulence factors, and mechanisms of antibiotic resistance.

The field of bacterial ncRNA research is expanding, and gaining knowledge about their functions offers valuable insights into the complex systems that regulate bacterial activity and their interactions with the surrounding environment and host species. At every stage of the gene regulation of biological events, ncRNA uses molecular strategies to regulate the expression of genes in bacteria, which may have both positive and negative effects (11). Non-coding RNAs played an essential role in bacteria environmental changes, their ability to adapt to stressful conditions, the regulation of their pathogenicity, physiology and pathophysiology of bacteria (30, 31). These ncRNAs regulate gene expression in response to environmental cues, which affects bacterial survival, adaptation, and virulence (32). In *A. baumannii*, the ncRNA AbsR25 was showed to played a crucial role in pathogenicity, biofilm formation, and fosfomycin resistance (9). The expression of this AbsR25 was shown to downregulate genes that are linked to virulence and antibiotic resistance by suppressing the abaF gene (9). Moreover, sRNA 13573 has been associated with the development of biofilms and host-pathogen interactions, suggesting a possible function in infection development (10).

The objective of this study is to discover the novel ncRNA and investigate its possible regulatory role by predicting the mRNA targets that it may affect by transcriptome analysis. Besides that, the differential expression of ncRNA will be validated through the Northern blot analysis.

MATERIALS AND METHODS

Data mining

The transcriptome data of *A. baumannii* with the accession number of SRA157695, SRA230506 and SRA439454 were retrieved from DNA Database of Japan (DDBJ). The genomic sequence used in this study was *Acinetobacter baumannii* 1656-2 chromosome.

Transcriptome analysis of *Acinetobacter baumannii*

Transcriptome analysis was performed on Ubuntu platform based on the software developer's protocol, unless stated otherwise. The quality of the sequencing results was analyzed using FastQC.(12). Softwares such as Trimmomatic (13) and Bowtie2 (14) were used for pre-processing analysis of the RNA raw sequences. Trimmomatic was used to filter the low-quality reads before assembly and alignment. Following the building of reference files in Bowtie2, the trimmed sequences were genome-aligned and saved as SAM format file. After the SAM file was converted to BAM format, it was sorted. Parallelly, the .gff file was created with all ncRNA candidates identified in *A. baumannii*. Artemis, a viewing software was used to obtain the peaks of these ncRNA candidates and their RPKM values (Reads per Kilobase of transcript per Million mapped reads) (15). The BLASTn (16) search and Rfam (17) database were used to identify known protein coding and ncRNA genes respectively. The nocoRNAc software was used to predict the possible ncRNAs and its direction in *A. baumannii* (18). To predict its target mRNA genes for the identified ncRNAs, targetRNA2 (19) servers was used. The default parameters were used.

Total RNA extraction from *Acinetobacter baumannii* ATCC 17978

Acinetobacter baumannii was cultured in Luria-Bertani broth (LB); 100 µl of *A. baumannii* ATCC 17978 from glycerol stock was inoculated into 10 ml of LB broth and incubated overnight at 37°C with 220 rpm. The 0.25 ml aliquot of the overnight culture was inoculated into 250 ml fresh LB broth. Based on the growth curve of *A. baumannii*, the growth phase of lag, exponential and stationary phases were identified. At lag (OD_{600} 0.2), exponential (log) (OD600 0.6–0.7) and stationary (OD_{600} 1.0) phases, cells were harvested. Oxidative stress was applied to the cells when the *A. baumannii* culture reached an OD_{600} of 0.6-0.7. The culture was incubated at 50 µg/ml of hydrogen peroxide for 30 mins. Total RNA was extracted using Trizol reagent (Gibco BRL, Eggenstein, Germany) according to the manufacturer's instructions and the final RNA concentrations were determined using UV-VIS spectrophometer (Beckman Coulter, U.S.) at 260nm.

Northern blot analysis

Total RNA (8–10 µg) was separated on 7M urea, 8% polyacrylamide denaturing gels and transblotted onto positively charged nylon membranes (Ambion Ltd, Cambridgeshire, UK). Northern blots were prepared as described previously (20) only after hybridizing with specific oligonucleotide that complementary to novel ncRNAs (Table I).

Table I: List of the DNA oligonucleotide probes designed for northern hybridization

ncRNA	**Oligonucleotide sequences (5' to 3')**	**Melting temperature (Tm) °C**
AbaR-43	CAAACTCGAACTGACTCAG	60
5s rRNA	GCTGGCGATGACTTACTCTCAC	68

RESULTS

Transcriptome analysis of ncRNAs in *Acinetobacter baumannii*

Total of 395 intergenic region(IGR) of unannotated transcripts were identified from transcriptome data of *A. baumannii* with the accession number of SRA157695 and further examined for their annotation in other organisms as protein coding or ncRNA genes using BLASTn search and Rfam database. From BLASTn search analysis, 76 were annotated as known protein or ncRNA in other species or genus. Those transcripts which were not annotated in GenBank database were then further screened using Rfam database to ensure these transcripts were conserved not for any ncRNA. From 319 transcripts, 27 were known ncRNAs in other bacterial species. After a series of analyses, the remaining 292 transcripts were identified as novel ncRNAs. The genes encoding the ncRNA are denoted as AbaR (1 to 292) for *Acinetobacter baumannii* ncRNA. Out of these 292 ncRNAs, 269 are the novel ncRNAs that identified in *A. baumannii*.

Conservation and specificity analysis of *Acinetobacter baumannii* ncRNA candidates across diverse Acinetobacter strains and species

To determine the specificity of the novel ncRNAs identified in this study, the BLASTn analysis was performed. Out of the 292 ncRNAs, there are 15 novel ncRNAs that are specific to *A. baumannii*, 275 are specific to Acinetobacter species and one novel ncRNA is found conserved in most Gram- negative bacteria. Besides that, out of 292 ncRNAs that identified in *A. baumannii*, 75 of the ncRNAs were repeated more than once on the genome.

Expression analysis of ncRNAs AbaR-43 during different growth phases of *Acinetobacter baumannii*

Based on the high Reads Per Kilobase Per Million Mapped Reads (RPKM) value obtained from the transcriptome analysis, ncRNA AbaR-43 was selected for further confirmation of its differential expression during in the 3 growth phases which is the lag, exponential and stationary phases. The ncRNA AbaR-43 is located at the intergenic region, flanked by downstream gene tRNA-Lys and long-chain-acyl-CoA synthetase at upstream to ST31 in reverse orientation of *A. baumannii* 1656-2 genome as shown in Figure 1. Based on Figure 2, the ncRNA expresses during lag, exponential and oxidative stress conditions.

DISCUSSION

Differential expression profile of AbaR-43 during different growth phases and oxidative stress condition

In the present study, we reported the integration of computational and experimental methods to identify novel ncRNAs in the nosocomial pathogen, *A. baumannii*. Total of 292 IGR transcripts were identified as novel ncRNAs. Out of the 292 identified novel

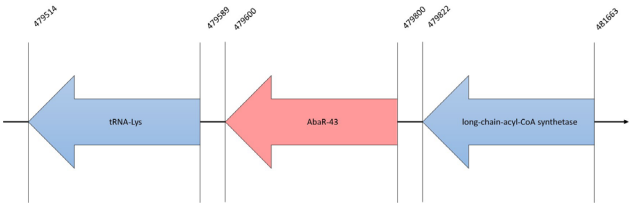

Figure 1: Genomic transcriptional orientation location of ncRNA AbaR-43 in *A. baumannii* 1656-2 genome. The ncRNA gene was indicated in red

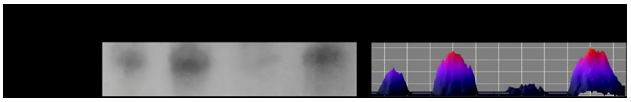

Figure 2: Expression profile of novel ncRNA AbaR-43 in *A. baumannii* at lag, log (exponential) stationary growth phases and oxidative stress condition by northern blot analysis. The intensity of the band as developed by ImageJ software.

ncRNAs, there are 15 novel ncRNAs that are specific to *A. baumannii*, 275 are specific to Acinetobacter species and one novel ncRNA is found conserved in most Gram-negative bacteria. In bacteria, it is very common that the ncRNAs are presence in other closely related bacteria. For example, 60-70% of the ncRNAs that identified in *S. typhi* had homologs in E. coli (20). For novel ncRNAs that are genus specific, they were conserved mainly in three species (*A. pitti, A. calcoaceticus*, and *A. nosocomialis*) that are highly like *A. baumannii* only. Nonetheless, there are still novel ncRNAs that specific to *A. baumannii* at species level which make them special compared to closely related Acinetobacter species.

Expression of ncRNA AbaR-43 shows differential pattern during *A. baumannii* growth phases where the expression increased from lag phase to log phase. The expression of this ncRNA is reduced during stationary phase. It also expressed in the oxidative stress condition almost equivalent to log phase (Figure 2). This might indicate that the involvement of ncRNA AbaR-43 in oxidative stress adaptation. Since the expression of the ncRNA is distinct in the log phase, it is understood that it may play a crucial role during the log phase apart from the oxidative stress condition.

The sequence alignment information from TargetRNA2 prediction tool predicted that the ncRNA AbaR-43 interacts with mRNA ABK1_1343. The mRNA ABK1_1343 involved in the production of flavin reductase-like protein which involved in dNTP synthesis (21). As dNTP synthesis is highly needed during the log phase, the interaction of ncRNA AbaR-43 to flavin-reductase mRNA could help the initiation of the expression.

Relationship between Flavin Reductase Family Proteins and Iron Metabolism in Bacterial Physiology

The acquisition of iron is an essential process for the proliferation, endurance, and pathogenicity of numerous

bacterial diseases (33). *Acinetobacter baumannii* necessitates iron for diverse intracellular functions, including DNA replication, respiration, and energy generation (34). Redox reactions play a critical role in facilitating the operation of iron acquisition systems, as the process of iron intake frequently necessitates the reduction of iron from its oxidized form (Fe^{3+}) to its reduced state (Fe^{2+}) (22). The presence of flavin reductase family proteins has been observed throughout a diverse array of species, encompassing bacteria, archaea, and eukaryotes. These proteins play a crucial role in redox processes by facilitating the transport of electrons to flavin coenzymes (35). These enzymes have the ability to affect the accessibility of reduced flavin coenzymes, which in turn can have an impact on the reduction of iron and other redox reactions occurring within the cellular environment (23). It is probable that these proteins provide a significant contribution to the bacterium's capacity to obtain and utilize iron, which, consequently, plays a crucial part in its virulence and pathogenicity (24).

CONCLUSION

The preliminary investigation of novel ncRNAs *A. baumannii* contribute to understand the role of these significant RNA molecules. We have reported the conservation of novel ncRNAs in *A. baumannii* by adapting the integrated approach of computational and experimental methods. Through the transcriptome analysis, 292 ncRNAs are conserved in *A. baumannii*. The ncRNA AbaR-43 was selected for further validation based on its RPKM value, and its expression was confirmed by Northern blot analysis. These ncRNAs have shown either consistant or differential expression during lag, exponential, and stationary phases and oxidative stress condition of *A. baumannii*. At different growth phases of *A. baumannii* , the ncRNA, AbaR-43 expression exhibits a varied pattern, increasing from the lag phase to the log phase. During stationary phase, this ncRNA's expression is reduced. It also showed the same conditions as log phase for oxidative stress. This would suggest that ncRNA, AbaR-43 may have a significant role during the log phase and in oxidative stress condition. AbaR-43 is predicted to interact with the mRNA ABK1_1343, which encodes a flavin reductase-like protein involved in dNTP synthesis which is a crucial process during the log phase. Flavin reductase proteins aid bacterial iron metabolism through redox reactions essential for iron acquisition. These proteins support bacterial survival, virulence, and pathogenicity. AbaR-43 may regulate flavin reductase expression, linking it to bacterial growth and oxidative stress adaptation. Besides that, the potential regulatory influence of AbaR-43 on this gene indicates that it may play a key role in modulating *A. baumannii*'s adaptive responses to hostile environments, including antibiotic-induced stress. Given the importance of iron acquisition and redox balance in bacterial pathogenicity and antibiotic resistance, AbaR-43 may serve as a novel regulatory element contributing to these processes. However, to fully elucidate the function of ncRNAs AbaR-43 further study by CRISPR-Cas or RNA silencing required to validate its regulatory functions in *A. baumannii*. However, other ncRNA candidates need to be studied for their expression and role in *A. baumannii* in future studies. Through this understanding, ncRNAs AbaR-43 could serve as novel therapeutic targets to disrupt key survival pathways. Additionally, these ncRNA can serve as biomarkers to detect stress-responsive or virulence-associated states in *A. baumannii* infections.

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