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Hypothesis

Cloning and over expression of non-coding RNA *rprA* in *E.coli* and its resistance to Kanamycin without osmotic shock

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Abstract
Recent reports have indicated that small RNAs have key roles in the response of the *E.coli* to stress and also in the regulating of virulence factors. It seems that some small non-coding RNAs are involved in multidrug resistance. Previous studies have indicated that *rprA* can increase the tolerance to Kanamycin in RcsB-deficient Escherichia coli K-12 following osmotic shock. The current study aims to clone and over-express the non-coding RNA *rprA* in E.coli and investigate its effect on the bacterial resistance to Kanamycin without any osmotic shock. For this purpose, *rprA* gene was amplified by the PCR and then cloned into the PET-28a (+) vector. The recombinant plasmid was transformed into wild type E.coli BL21 (DE3). The over expression was induced by IPTG and confirmed by qRT-PCR. The resistance to the kanamycin was then measured in different times by spectrophotometry. The statistical analysis showed that the *rprA* can increase the resistance to Kanamycin in Ecoli K12. The interaction between *rprA* and *rpoS* was reviewed and analyzed by *in silico* methods. The results showed that the bacteria with over-expressed *rprA* were more resistant to Kanamycin. The present study is an important step to prove the role of non-coding RNA *rprA* in bacterial resistance. The data can be the basis for future works and can also help to develop and deliver next-generation antibiotics.

Key words: Escherichia coli, non-coding RNA, *rprA*, Kanamycin

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Background:
Bacteria must constantly adapt to the external and environmental conditions by adjusting their physiology and behavior. It appears that the phenotype of bacterial resistance to antibiotics is frequently regulated through some biochemical pathways. The prevalence of antibiotic resistance in infections caused by *E.coli* is high **[1, 2, 3].** "Small non-coding RNAs" (sRNAs) are molecules, which have gained much attention in recent studies due to their potential roles in antibiotic resistance **[4]**. The reports have indicated that sRNAs have key roles in the response to different stresses and regulation of bacterial virulence factors **[5].** The first Small non-coding RNA regulator in *E.coli* was named *MicF* RNA, which has 174 nucleotides. This small non-coding RNA inhibits the translation of *ompF* mRNA encoding the major outer membrane purine **[6].** This discovery persuaded other researchers to identify small non-coding RNAs and find their characteristics in a variety of bacteria with different methods **[6].** In bacteria, non-coding RNAs (ncRNAs) are regulated by different ways. The RNAs can affect the function of the protein, transcription initiation, stability, and initiation/elongation of mRNA translation. NcRNA transcripts are categorized into two groups including anti sense RNAs (asRNAs) and trans encoded RNAs. The asRNAs are fully complementary for mRNA targets, while trans-encoded sRNAs are much shorter than their complementary regions **[7].**

RprA is a small non-coding RNA with 106 nucleotide length. It seems that *rprA* forms three stem-loop structures and is highly conserved. It is also suggested that *rprA* contributes in the

positive regulation of *RpoS* translation. *RprA* promoter is positively regulated by *RcsB* **[8],** which is a capsule synthesis response regulator. Under stressful stimulus signal, *RcsB* is activated and so increases the *rprA* expression. Then, *RprA* expression seems to increase the translation of RpoS **[9].** Amirault et al. reported the importance of this gene in Kanamycin resistance in *E.coli* after osmotic shock **[10].** Since it is important to analysis the effect of *rprA* in different conditions, the current study was designed to clone and over express this gene in *E.coli*. In order to find the role of *rprA* in different conditions, we tried to analyze the overexpression of *rprA* in the wild type Bl21 *E.coli* in different times. In the current study, we analyzed this effect by pET expression system and without any osmotic shock. Also, we tried to analysis and review the interaction of *rpoS-rprA* RNAs through in silico methods. The results can provide a context to prove the role of the *rprA* in antibiotic resistance, which can be a basis for future works. It can also help to develop and deliver next-generation antibiotics.

Methodology

Gene Amplification

After Bacterial DNA extraction by boiling method, the *rprA* gene was amplified using polymerase chain reaction (PCR) through specific primers **(Table1).** PCR process was performed under the following conditions: The initial denaturation step at 94 °C for 3 minutes, then 35 consecutive cycles of denaturation steps at 94 °C for 30 seconds, annealing at 58 °C for 30 seconds, extension at 72 °C for 30 seconds, final extension for 10 min at 72 °C. Finally, the PCR product was loaded on 1% agarose.

Cloning of *rprA* and over-expression in *E.coli*

The PCR product and pET 28a plasmid were digested by *Xba*1 and *Hind*III enzymes (Fermentas Company). The products were loaded on the gel and purified by gel extraction kit (GENALL Company*). RprA* gene was cloned into the pET28a plasmid using T4 ligase enzyme (Sinaclone) and the resulting recombinant plasmid was called pET28a/*rprA*. Recombinant plasmid was sequenced to verify the gene insertion. Recombinant plasmid was transformed into the competent *E.coli BL21* (*DE3*) by chemical methods using heat shock **[11].** The transformed bacteria were then cultured in *Luria-Bertan*i medium (LB) containing kanamycin (30µg/ml) in shaker incubator at 37 °C with 200 rpm for 2 hrs. When the OD reached to 0.4-0.6, the induction was done by IPTG at a final concentration of 0.5 mM. The culture was again continued with previous condition, and sedimentation was performed 6 hours after the induction with centrifugation at rpm4000 for 4 minutes.

RNA extraction, cDNA synthesis, and RT-PCR

The expression analysis was done by Reverse Transcriptase PCR method. The RNA was extracted using Sina pure kit and the cDNA was synthesized from colonies by Omni script reverse transcription kit. DNAase was used in order to remove any genomic DNA. Semi quantitative RT-PCR reaction was finally performed using the cDNA as template. 16srRNA was also used as endogenous control in RT-PCR (Primers are in **Table 2**).

Study of bacterial resistance to Kanamycin

To check the bacterial resistance to kanamycin, the new Luria-Bertani (LB) medium was made and divided into the equal amounts. Kanamycin and overnight-cultured recombinant bacteria were equally added into each falcon tube containing the medium and placed into the shaker incubator at 200 rpm at 37 °C for 2 hrs. When the OD reached to about 0.5, the IPTG with the final concentration of 0.5 mM was added into the falcon tubes assigned as "over-expressed bacteria", whereas the remaining tubes were not treated with IPTG (assigned as controls). Cultures were then incubated and culture turbidity was read after 2, 4, 6, and 8 hours **[10, 12].**

Statistical analysis

The data for growth of bacteria were analyzed by spectrophotometry. The difference between growth of over expressed bacteria and control bacteria was analyzed by two-way analysis of variance (ANOVA) method through Graphpad Prism5 software.

In silico prediction of RNA-RNA interaction and folding energy

The EcoCyc database (ecocyc.org) was used for describing the structure of *rpoS* operon and interactions. Then, the sequence of *rpoS* (from the transcription start site 4) was retrieved from the Genebank. The interaction between *rpoS* AND *rprA* RNAs was analyzed by IntraRNA program (rna.informatik.uni-freiburg.de/). The hybridization energy was calculated through this software.

Results

After the cloning, the accuracy of pET28a/*rprA* recombinant plasmid was examined. The screening of the colonies was done by direct PCR and sequencing. After the IPTG using RT-PCR with 16srRNA as the endogenous control performed induction the expression analysis at the RNA level. The analysis showed that the overexpressed bacteria have the higher amount of *rprA* level compared to the control bacteria. The spectrophotometry and statistical analysis showed that the growth rate of over-expressed bacteria is increased compared to control bacteria (P-value<0.001) in different times after the IPTG treatment (**Figure 1**). The results show that the overexpression of *rprA* can increase the resistance to Kanamycin in E.coli.

Furthermore, we also checked the interaction of *rprA* and *rpoS* through EcoCyc. We got the operon structure and the mechanism of action of RNA-RNA interactions (**Figure 2 a, b**). The sequence of the *rpoS* RNA transcribed from the transcription start site4 was retrieved from GeneBank. Then, the RNA-RNA interaction between *rprA* and *rpoS* was analyzed and reviewed by IntraRNA software (**Figure 3**). The analysis showed that the energy is about -16.63340 Kcal/mol. The details of the interaction are depicted in **Figure 3.**

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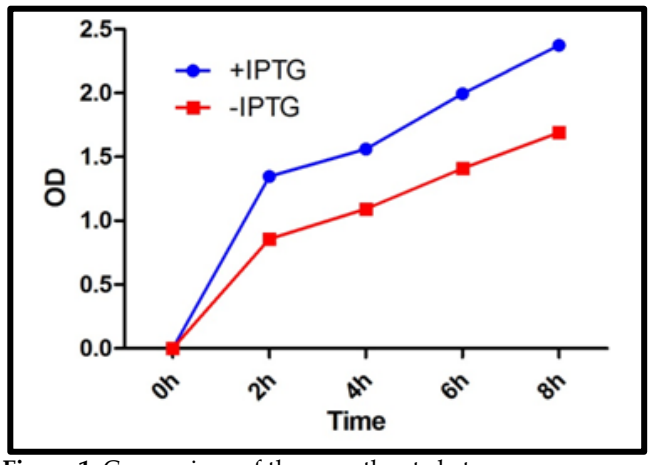

Figure 1: Comparison of the growth rate between overexpressed bacteria and negative control bacteria with LB and Kanamycin culture. The lines show the OD of two groups of bacteria (+IPTG : rprA overexpressed; -IPTG: Non over expressed bacteria) in different times. Two Way ANOVA shows the significant difference between growth rate of two groups.

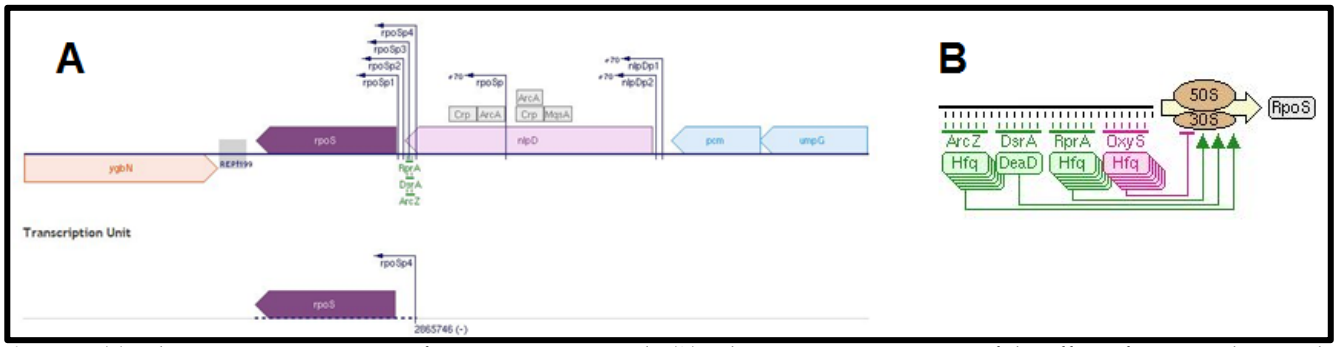

Figure 2: (a) Schematic representation of rpoS operon in E.coli, **(b)** Schematic representation of the effect of *rprA* on the translation of rpoS. The images are achieved from EcoCyc database.

Figure 3: The interaction between rpoS (Target) and *rprA* (Query) RNAs. The data are drawn from IntraRNA software.

Table 1: The primers used for amplification of *rprA* gene from *E.coli* for cloning into PET28 vector.

Primer	Fragment Length	Enzyme cutting sit	Primer Sequence
rprA-F	106	*Xba*1	CTAGACTCTAGAACGGTTATAAATCAAC
rprA-R	106	*HinD*III	GTTGACAAGCTTAAAAAAAAGCCCATCG

Table 2: The primers used for expression analysis of *rprA* by RT-PCR.

Primer	Fragment Length	Primer Sequence
RT-rprA- F	78	5′ATGGAAATCCCCTGAGTG3′
RT-rprA -R		5′AAAAGCCCATCGTGGGAG3′
16SRNA-F	230	5′AGTACTTTCAGCGGGGAGGA3′

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16SRNA -R	5'CGAGACTCAAGCTTGCCAG3'

Discussion

E. coli usually grows slowly in response to changing environmental conditions due to the limitations of food sources needed to protect and survive under stress conditions. To survive in such difficult conditions, E.coli expresses multiple genes involved in both transient and long-term emergency responses. Many of these genes depend on stress / stationary phase sigma factors, namely Rpos, for their transcription **[12].** RpoS translation increases quickly following the stress stimuli. In this response, Hfq, and sRNAs such as DsrA and *rprA* are involved in the activation of rpoS translation. These two sRNAs can activate the RpoS translation by pairing with rpoS mRNA with the help of Hfq **[13].** So, *rprA* is one of the small non-coding RNAs, whose expression increases the RpoS translation. These interactions are reviewed in the Figures 2 and 3. In general, when *RprA* is produced in high levels, it can activate σ^S (RpoS) synthesis by stabilizing the rpoS mRNA [**14, 15**]. Amirault et al. recently showed that *rprA* expression, a regulator for RpoS translation, increases resistance to kanamycin in Escherichia coli K-12 following osmotic shock. They showed that *rprA* expression from inducible arabinose promoter can increase minimum inhibitory concentration (MIC) in ΔrcsB cells **[10].** Although the statistical analysis was not performed on their data, they concluded that it can be some *rprA*-independent factor missing from the ΔrcsB strain which increased the tolerance of wild type cells to Kanamycin. In this study, by statistical analyses, we also showed that the resistance to Kanamycin could be increased without any osmotic shock in Ecoli K12 with inducible beta galactosidase promoter and without any osmotic shock.

Some conditions such as osmotic shock can induce the general stress response genes through the central regulator *RcsB*. Previous studies have indicated that the increased susceptibility to kanamycin in rcsB deficient strain was because of the inability to produce extracellular capsule. Amirault et al. investigated the role of *rpr*A in restoring kanamycin resistance of *E. coli* after osmotic shock. They used the *rcs*B knockout strain transformed with a plasmid containing *rpr*A under an arabinose-inducible promoter. Δ*rcs*B mutants are unable to form a capsule; this result may have been due to an antibiotic-resistance effect conferred by capsule presence **[16, 17].** In this study, we aimed to confirm the effect of *rprA* without any external stress such as osmotic shock. This can support the independency of the function of *rprA* in kanamycin tolerance. We studied this effect through pET-28 vector system, which has the kanamycin resistance gene itself. The statistical analysis confirm the results of Amirault and colleagues. These results can be the fundamental knowledge, which can be used to develop and deliver the next generation of antibiotics.

Conflicts of interest

None

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