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Abstract

Mycobacterium spp belongs to family Mycobacteriaceae, which includes pathogens (*Mycobacterium tuberculosis* & *Mycobacterium leprae*) and nonpathogens (*Mycobacterium phlei*). *Mycobacterium tuberculosis* is the leading cause of death by infections reported in many developing countries. *Mycobacterium marinum* is a model organism for *M. tuberculosis* and it causes same cellular pathology in fish. The *rnpA* gene which encodes RNase P protein has been found to be present downstream of the *rpmH* gene which encodes ribosomal protein L34 in general in bacteria. These genes are essential for survival of bacteria. *rpmH* and *rnpA* were shown to be part of same operon and L34 shows excess expression over RNase P protein in *Escherichia coli*. The purpose of this experiment was to observe the expression levels of *rpmH, rnpA* and *rnpB* in *M. marinum* and *E. coli* on the transcriptional and translational level. Transcription analyses in both bacteria showed that *rpmH* is produced in excess over *rnpA* with down regulation in stationary phase relative to exponential phase but *rnpB* showed up regulation in *M. marinum* and down regulation in *E. coli* in stationary phase relative to exponential phase. Only the *rnpA* gene was cloned successfully while *rpmH* and *rnpB* failed to be cloned.

Contents

1. Introduction

Mycobacterium spp belongs to the family Mycobacteriaceae. *Mycobacterium* spp are acid fast (Ryan, Ray, & Sherris, 2004). They demonstrate an incredible flexibility against ecological change and they occupy different natural niches e.g. ground and surface water, soil, animals and also humans. They are phylogenetically positioned among the actinomycetes which include both non-pathogens and also severe pathogens like *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium ulcerans* (Demangel et al., 2005; Russell, 2007). *M. tuberculosis* infects humans and it is similar to *M. bovis* which can infect both animals and humans which leads to Tuberculosis (TB). *M. avium* subspecies *paratuberculosis* is another *Mycobacterium* spp which is a chronic intestinal pathogen that causes diarrhea in humans. Mostly *M. avium* is present in water (fresh or salt), household dust, soil and pasteurized bovine milk (Chacon et al., 2004). Previous studies suggested that *M. avium* subspecies *paratuberculosis* can cause Crohn's disease (Uzoigwe et al., 2007). *Mycobacteria* can grow in the water where there is disinfects like chlorine also present.

1.1 *Mycobacterium marinum*

Mycobacterium marinum is a Gram-positive bacterium that causes intrinsic TB in ectothermic animals like fish and frog. *Mycobacterium marinum* has close relations to *Mycobacterium tuberculosis* which causes tuberculosis disease in humans. *Mycobacterium marinum* is considered to be an important model system to study molecular mechanisms involving in the development of tuberculosis disease in humans (Ramakrishnan et al., 2004). They are easy to handle when compared to *M. tuberculosis* because of their growth and doubling time. Their growth is limited at high temperatures (37°C) and the doubling time of *M. marinum* is shorter than of *M. tuberculosis*, which explains why they are easier to work with in the laboratories. *M. marinum* is highly similar at the genome sequence level to *M. tuberculosis* and *M. ulcerans* (Tønjum et al., 1998)(Ramakrishnan & Falkow, 1994). They also have the ability to replicate in protozoans and also mammalian macrophages(Ramakrishnan & Falkow, 1994).

1.2 *Escherichia coli*

Escherichia coli is a Gram-negative, non-sporulating, facultative anaerobic bacterium that makes ATP by respiration if oxygen is available; however, it is fit for changing to fermentation or anaerobic respiration if oxygen is not present. Cells are commonly rod-shaped, around 2.0 micrometers (μm) long and 0.25-1.0 μm width and the cell volume is around 0.6-0.7 μm (Kubitschek, 1990). *E. coli* is usually present in the lower digestive tract of endotherms(Singleton, 1999). *E. coli* can utilize various substrates. Most *E. coli* strains are human friendly and safe. However some serotypes can bring severe harm like food poisoning to their hosts (Vogt & Dippold, 2005). The harmless strains are involved in generation of vitamin K2, (Bentley & Meganathan, 1982) and harmless strains helps to healthy human intestinal tract. (Reid, Howard, & Gan, 2001).

E. coli and other facultative anaerobes constitute around 0.1% of the gut flora (Eckburg et al., 2005) and fecal-oral transmission is the significant route through which pathogenic strains of the bacterium cause infection. Cells have the capacity to live outside of the body for some time, which makes them potential markers to test natural samples for soil contamination. A developing body of exploration, however, has analyzed environmentally constant *E. coli* which can survive for extended periods outside of a host (Ishii & Sadowsky, 2008).

E. coli can be easily cultured and grown effectively in a research lab setting, and it has been studied for more than 60 years. *E. coli* is a prokaryotic model organism which is an essential species in the fields of biotechnology and microbiology for a wide range of studies, where the majority of work with recombinant DNA is done, by serving as a host organism. Under favorable conditions it takes just 20 minutes to reproduce (Redorbit, 2013).

E. coli uses mixed fermentation in anaerobic conditions, creating lactate, succinate, ethanol, acetic acid, and carbon dioxide. Since numerous pathways in mixed fermentation produce hydrogen gas, these pathways oblige the levels of hydrogen to be low, similar to the situation

when *E. coli* grow in the presence of hydrogen-devouring organic organisms, for example, methanogens or sulfate reducing bacteria.

1.3 RNase P

RNase P is an enzyme that is responsible for maturation of the 5'-end of precursor tRNAs by endonucleolytic cleavage. RNase P is essential to generate functional tRNA. Importantly, RNase P enzymes in bacteria contain both an RNA subunit and a protein subunit where it is the RNA subunit that is catalytically active. RNase P RNA is about 400 nt long and comprised of two major classes based on their structural differences i.e. type A and type B. Both types have two distinct domains namely a catalytic domain(C) and a specificity domain (S) which are involved in precisely cleaving and recognizing substrates, respectively (Guerrier-Takada et al., 1983 ; Harris & Christian, 2003). The C domain consists of 4 helical subunits connected to each other by nucleotide sequences and this is responsible for substrate binding or catalytic function (Torres-Larios, Swinger, Pan, & Mondragón, 2006). The protein part works as a cofactor and it is needed in vivo (Reich et al., 1988).

In comparison with bacterial RNase P RNA (RPR), structures of archaeal and eucaryal RPR are not resolved in high resolution have identified 50 sequences from each domain. Moreover, the size of eucaryal and archaeal RPR is 10-20% smaller than bacterial. RPR structural differences could explain the lack of enzyme substrate interaction (Li, 2004 ; Marquez, 2005).

The protein subunit of RNase P is encoded by the rnpA gene in bacteria. The rnpA gene has been found to be present downstream of the rpmH gene (encoding ribosomal protein L34) which is near to the origin of replication oriC (5-7). These genes are essential for survival. Both the rpmH, rnpA genes were found to be part of same operon and L34 is produced in excess over the RNase P protein (C5)(Feltens et al., 2003). It was suggested that the differential expression of these genes is regulated at the transcriptional and translational level.

1.4 Transcriptome studies

Previous studies of transcriptome of the M. marinum rnpB, rnpA and rpmH (Fig. 1) shows that the expression of both rnpA and rpmH genes increases in the exponential phase in relation to the stationary phase, while the expression of the rnpB gene (RNase P RNA) decreases in the exponential phase compared to the stationary phase.

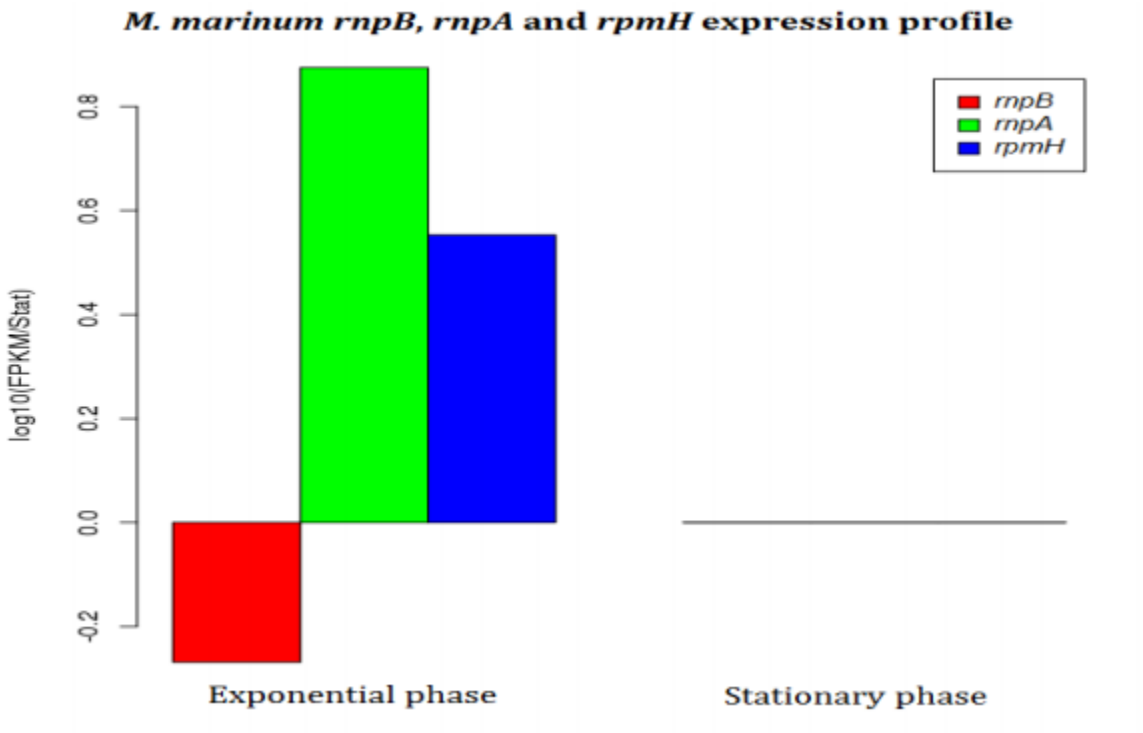

Figure 1: M. marinum rnpB, rnpA and rpmH genes Expression profiles in exponential phase compared to stationary phase. Credits to Fredrik Pettersson and Phani Rama Krishna Behra, Uppsala University, (unpublished).

2. Aim

My main objective of this study is to get confirmations with the previous transcriptome studies and to understand the expression pattern of *rpmH, rnpA, rnpB* genes and growth pattern in *M. marinum & E. coli*.

3. Materials and Methods

3.1 Data mining:

Nucleotide sequences of *rpmH, rnpA, rnpB* were extracted from the NCBI database for selected *Mycobacterium* and *E. coli* species. A multiple sequence alignment of the nucleotide sequences was performed using ClustalW2 with default settings to identify the conserved and differential

regions. Promoter regions and overlaps between *rpmH* and *rnpA* were checked at the nucleotide level for all selected species.

3.2. Bacterial strains, plasmids and growth conditions

Wild type M. marinum CCUG 20988 bacteria were grown at 30°C in 7H9 media (Sechi et al., 2007). The media was supplemented with 2 ml/l glycerol, 10 ml/l oleic acid albumin dextrose catalase (OADC), and 0.5 g/l of Tween80. *E. coli* MG1655 was cultured in two different media containing (i) LB (Tryptone 10g/L, yeast extract 5g/L, Sodium chloride 5g/L, Final pH 7.2 at 37°C) supplemented with 0.4% glucose. (ii) M9 media supplemented with sodium succinate. The cells were grown until they attained exponential (OD= 0.04 to 0.1) and stationary phases (OD= 4.02). To determine cell growth the optical density was measured at 540nm for *M. marinum* and 600nm for *E. coli*. Then duplicates were harvested by performing centrifugation at 1790 g at room temperature. The obtained pellets were snap frozen in liquid nitrogen and shifted to -80°C for storage.

3.3. RNA extraction

3.3.1. *M. marinum*

Total RNA was extracted from *M. marinum* by the following procedure. Cells were thawed and re-suspended on ice by adding 2 ml Trizol (Invitrogen). 1 ml of re-suspended cells was taken into a 2ml vial of lysing matrix B (0.1 mm beads from Q-BIO gene). 200 µl of chloroform was added and the tubes were bead beaten in a Fast Prep instrument for vigorous shaking at 6.5m/s for 30 sec (4 times), samples were placed on ice for 3 minutes after each time. After the shaking process, samples were spun at 12000 rpm for 30 minutes at 4 °C. 500 µl of the upper liquid phase was separated into a new 2 ml Eppendorf tube and an equal volume of chloroform was added and the tubes were vortexed then spun for 5 minutes at 4 °C, then the upper liquid phase was transferred into new a Eppendorf tube and an equal volume of isopropanol was added and mixed well and incubated overnight at -20°C to precipitate the RNA. Precipitated samples were spun at 12000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet was washed with 500 µl of 80% ethanol. The supernatant was removed carefully and the pellet was air-dried for 10 minutes and dissolved in 40 µl of distilled water.

3.3.2. *E. coli*

RNA was extracted using the TRIzol (Invitrogen) reagent. In this method, the pellet was re-suspended in 1ml of TRIzol reagent and incubated at RT for 5 minutes. The re-suspended pellet was mixed vigorously with 200 µl of chloroform and incubated at room temperature for 3 minutes. The samples were spun at 12000 rpm for 15 minutes. To the aqueous phase 500 µl 100% isopropanol was added and incubated at RT for 10 minutes. Again the samples were centrifuged for 10 minutes at 2-8 °C. The supernatant was discarded and the pellets were washed by vortexing in 1ml of 75% ethanol. Then the samples were centrifuged at 7500 rpm for 5 mins at 2-8 °C. The pellets were air-dried by placing the tubes with the caps opened. The obtained RNA pellet was dissolved in 40 µl of distilled water.

From *M. marinum*, and *E. coli* total RNA was quantified by an ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, US). Purity was checked by running a gel electrophoresis and if DNA contamination was observed the samples was treated with DNase enzyme (Turbo DNA free kit, Ambion Company) following the manufacturer's instructions).

3.4. cDNA synthesis

Two µg of total RNA was reverse transcribed to cDNA by adding 2µl 10X RT buffer, 0.8 µl 25 X dNTPs mix (100 mM), 2µl 10 X random primers, 1µl multiscribe reverse transcriptase (Applied Bio systems Inc. Foster City, CA), and 4.2 µl of nuclease-free H2O. The reaction mix was incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5minutes followed by 4°C. The cDNA was serially diluted 1:1, 1:10, 1:100, 1:1000, and 1:10000 in H2O.

3.5. Quantitative Real-Time PCR (qRT-PCR)

Real-time PCR was performed using a 7300 Real-Time PCR System (Applied Bio systems Inc., Foster City, CA) with 20 µl of reaction volume. 5 µl buffer, 1 µl of each primer (10 µm stock), 2.5 µl probe (2.5 µm stock), 10.5 µl H2O were added in a reaction volume further 5 µl of cDNA was added to 20 µl reaction mixture to make 25 µl total volume. Reaction mixture was loaded in triplicates on ABI 96 well PCR plates and spun down at 4000 rpm for 1 min. The following RT-PCR conditions were used: 95°C for 15 min follow by 40 cycles of 95°C for 15 sec, 60°C for 60 sec. Gene expression quantification was performed by absolute quantification of the Ct value.
Probes used in the study were:

Ec-16Spb FAM-AAT GGC GCA TAC AAA- MGB

Ec- RnpB FAM-CGG CCC GTA CTG AA-MGB

Ec- RpmH FAM-ACCGTT CTC ACG GCT T-MGB

Ec- RnpA FAM-CCA TTC TCG GCC GCC - MGB

Mm- rnpA - FAM-CCG TGC GAT CGA GT- MGB

Mm- rpmH- FAM-CG AAC AAC CGG XG - MGB

Mm-16s FAM-CCA GGG CTA CAC ACG TGC TA-MGB

3.6. Isolation of Genomic DNA from *Mycobacterium*

Two ml o-ring sealed tubes, containing sterilized 0.1mm silica beads were filled with 200 µl of TE (TRIS EDTA) and 200 µl of DNAZol. A large amount of cells from plate growth was added to the tubes and mixed well to make it a homogeneous mixture. Later the samples were lysed using the FastPrep FP 120 rotating device at speed of 6.5m/s for 30 seconds. This was carried out twice for 30 seconds. After incubating for 1 minute on ice, the previous step was repeated. After one minute on ice, samples were spun for 2 minutes at 700×g at room temperature followed by addition of 200 µl of chloroform. The samples were mixed in a rotational mixing device at 50 rpm for 30 minutes. All the samples were spun for 10 minutes at 16000×g at room temperature. The liquid phase was collected and 750µl of 99% cold EtOH was added and the samples were kept at -20°C overnight. Precipitated DNA was taken out from the bottom of the tube with inoculation loop and the supernatant was discarded. The DNA was returned to the tube and washed with 70% EtOH and centrifuged for 10 minutes at 16000×g. After repeating this step two more times, the pellet was dried at room temperature for 10 minutes by discarding supernatant. 100µl of TE was added to dissolve the dried pellet.

3.7. RNase treatment of isolated DNA samples

To avoid RNA and protein contamination, first RNase A and then after 1hour incubation at 37°C proteinase K with final concentration 50µg/ml was added by following dilution method $V_1 = (C_2*V_2)/C_1$ (c1=initial concentration or molarity, V1=initial volume, c2=final concentration or

molarity, V2=final volume). The tube was placed for incubation for another hour at 37°C. Equal volumes of chloroform/phenol (1:1) was added to the sample and mixed in the rotating machine for 30 minutes. Then the tube was centrifuged at 4000×g for 5 minutes in the cold room. Chloroform was added twice more and the rotating step was repeated. The water phase was transferred to new tubes to which 2 volumes of chilled (-20°C) 99% EtOH was added together with 1/10 volume of NaOAc (pH 5.3) and the samples were mixed well. After incubation overnight at -20°C to precipitate DNA, the DNA was recovered by centrifugation at 25.000×g at 4°C for 15 minutes in the cold room. The same amount of chilled 70%EtOH was added to the tubes after discarding the supernatant and mixed well followed by centrifugation for 10 minutes. As above this step was repeated twice and the supernatant was discarded. 50µl of warm sterile water was added to the pellet to dissolve the obtained DNA and its concentration was checked at A260/A280 using a Nanodrop machine. The samples were saved at -20°C for further use.

3.8. DNA amplification by Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify the rnpA gene with the primer set 5'TTTTCTTAAGTGAGTACCACCGAAGGGGTGTATA3',5'TTACTAGTTCGAGCGACCT CGCGTGAGCGAGGCGCTCGATCACCGGTCGGC 3'. 23 µl of master mix was prepared for each sample with 0.5 µl of dNTP mix, 0.5 µl of forward and reverse primer, 2.5 µl of buffer and 0.25 µl of phusion polymerase enzyme was used. 18.75 µl of water and finally 2 µl of cDNA containing 100ng was added and mixed well. A master mix without template was used as negative control. All the PCR tubes were placed in thermal cycler (Bio Rad, CA).

Conditions used in the PCR

Heated lid 100°C

1. First Denaturation 94°C for 2 minutes.
2. Denaturation 94°C for 30 sec.
3. Annealing 52 °C for 1 minute.
4. Elongation 72°C for 2 minute.

Steps 2, 3 and 4 were repeated for 35 cycles followed by an extension at 72°C for 10 minutes. Then the amplified products were analyzed by 1 % agarose gel electrophoresis.

3.9. Gel extraction

Positive PCR products were visualized in agarose gel under UV light and were collected in 1.5 ml Eppendorf tubes. Identified bands were cut from the gel and collected into new 1.5 ml Eppendorf tubes by using sterile razor blades. Then the DNA was extracted from the gel using Gene JET gel extraction kit by following the manufacturer's directions. Purified DNA product was quantified in the Nano drop machine (Nano drop technologies).

3.10. Cloning of the *rnpA* gene

3.10.1. Ligation

Vector pET19b was used for the ligation process. The reaction was set up as follows. In a microcentrifuge tube 2 µl of 10X T4 DNA Ligase Buffer, 1 µl of vector (50ng/ µl (0.020pmol)), 1 µl of purified amplicon (40 ng (0.060pmol)) and nuclease free water was added to 20 µl. Finally, 1 µl of T4 DNA ligase enzyme was added and the contents were mixed gently with the pipet and spun down briefly, the ligation mixture was incubated at 16° overnight.

3.10.2. Bacterial growth and transformation

For *E. coli* bacterial transformation, agar plates were prepared and sub-cloning efficiency DH5-α competent *E. coli* cells were taken from -70°C and thawed on ice for 10 minutes. 5 µl of ligation mixture was added to the competent cells (100 µl) and incubated for 30 minutes on ice followed by heat shock at 42°C for one minute. Then the mixture was incubated on ice for 2 minutes. 900 µl of LB was added to the mixture and incubated on shakers with 225rpm at 37°C for one hour. LB agar plates supplemented with 50µg/ml ampicillin were used. The incubated mixture was spread on plates using a sterile glass rod. Then the LB agar plates were kept for incubation at 37°C for overnight.

For *M. marinum* 7H10 media agar plates were used. Plates were kept for incubation at 30°C for 5-6 days to allow bacteria to grow.

3.11. Preparation of cultures & plasmid isolation

Selected colonies were inoculated in 4 ml of sterile LB in culture tubes in the presence of 50-µg/ml of ampicillin. The tubes were incubated at 37°C overnight to allow bacteria to grow. 2 ml of overnight cultures were transferred into 2ml Eppendorf tubes and pelleted by centrifugation

for 4 minutes at 8000 rpm. The obtained pellet was used in plasmid isolation. To isolate the plasmid Fermentas GeneJET miniprep kit (#k0503) was used following the manufacturer's instructions. To check the presence of insert, restriction analysis was performed. Two enzymes *Eco*R1 and *Bam*HI were selected to cut the plasmid at two different sites. 2 µl DNA was taken into a new 1.5 ml Eppendorf tube and 2 µl of 10x Fast Digest green buffer, 1 µl of both enzymes, and 14 µl of water making up 20 µl was added to the Eppendorf tube followed by incubation at 37°C for 30 minutes. The restriction digestion products were analyzed by gel electrophoresis and the gel was run at 80V for one hour.

3.12. Sequencing

25µl of positive samples of isolated plasmid DNA (~100ng/µl) was transferred to new tubes and sent for sequencing to the Macrogen sequencing company. Obtained sequences were analyzed by BLASTn technique.

4. Results

4.1. Data mining results of *rmpH-rnpA* genes in *M. marinum*

The nucleotide sequence of the *rpmH-rnpA* genes in *M. marinum* was analyzed. The *rpmH* gene was identified upstream of the *rnpA* gene. A sequence overlap of 23 nucleotides was found between the *rnpA* and the *rpmH* genes. Downstream of the *rpmH* gene, we observed a stop codon (TGA). A potential start codon GTG was observed in the *rnpA* and the *rpmH* genes *of M. marinum* (Fig 2).

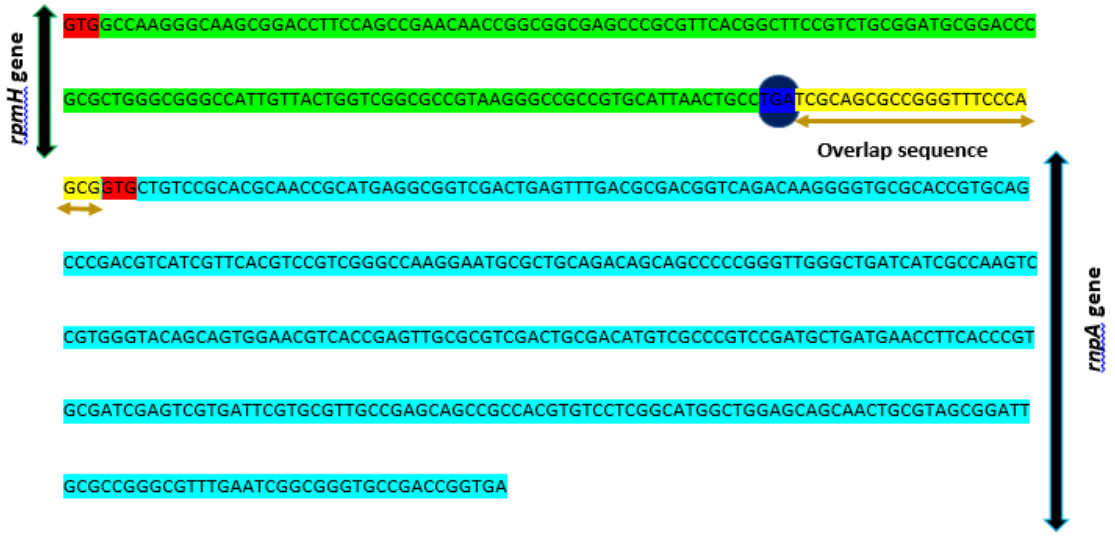

Figure 2: Genomic context of the *rpmH-rnpA* genes in *M. marinum*. Red shades represent the start codon of *rpmH* and *rnpA* gene. *rpmH* gene is represented in green shades and it is followed by a stop codon (TGA) which is shown is blue box. *rnpA* reading frame is shaded in cyan blue. The overlap sequence between *rnpA* and *rpmH* gene is represented in yellow shade.

4.2. Bacterial growth curve

M. marinum CCUG 20998 was cultured until it attained stationary phase (OD = 4.1). During the culturing process OD values were recorded for every 6 hours. Samples were collected in exponential phase and stationary phase for RNA isolation. The growth curve was analyzed in two experiments (Exp1-10^4, Exp2-10^2). The growth curve from experiment 2 shows that the culture entered into stationary phase (OD=3.7 at 72 hrs.), very rapidly compared to experiment 1 (OD=4.8 at 102 hrs.) (Fig 3).

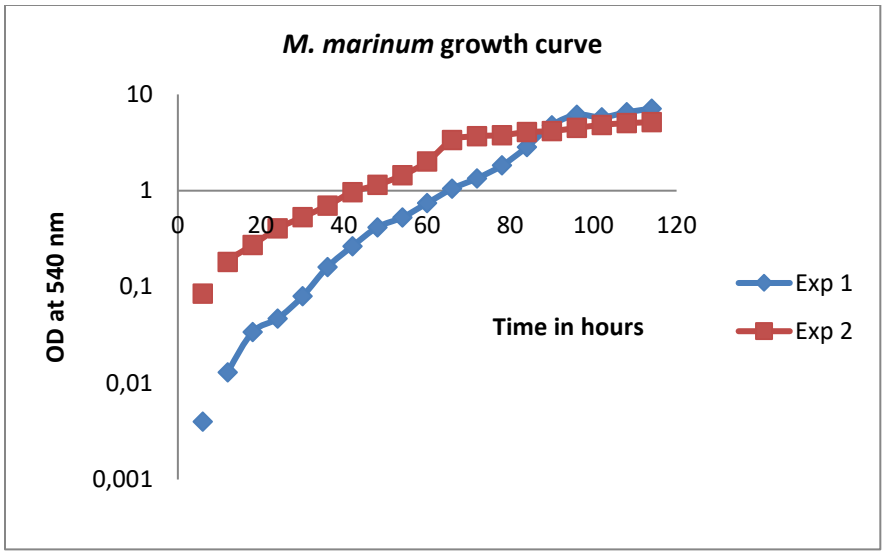

Figure 3: *M. marinum* growth curve with 7H9 liquid broth at 30°C temperature with tween. Initial dilutions of culture is exp1-1× 104: exp2-1× 102

For *E. coli* in LB supplemented with glucose there was a uniform increase with a flattening beginning by 0.03 OD at 600nm. Values were recorded every 20 minutes and it entered into stationary phase at approximately 300 minutes by 4.2 OD (Fig 4A)

For *E. coli* in M9 medium supplemented with succinate, there was a slow growth rate with a beginning OD of 0.08 at 600nm. Values were recorded every 20 minutes and it entered into stationary phase after 785minutes by 1.7 OD (Fig 4B).

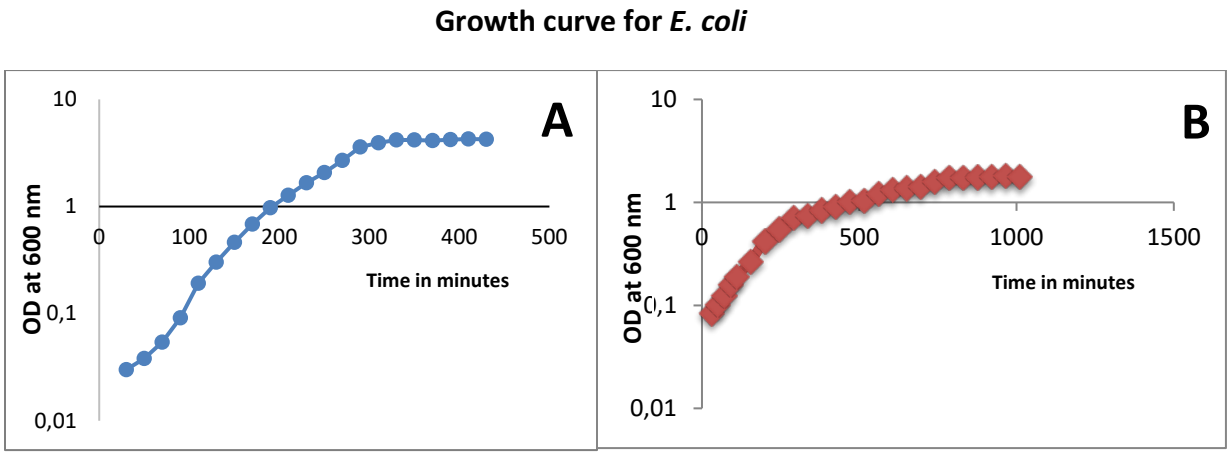

Figure 4: A) Growth curve of *E. coli* MG1655, cultured in LB supplemented with 0.4 % Glucose as carbon source B) Growth curve of *E. coli* MG1655, cultured in M9 medium supplemented with 0.45% succinate as carbon source.

4.3. RNA isolation & DNase treatment

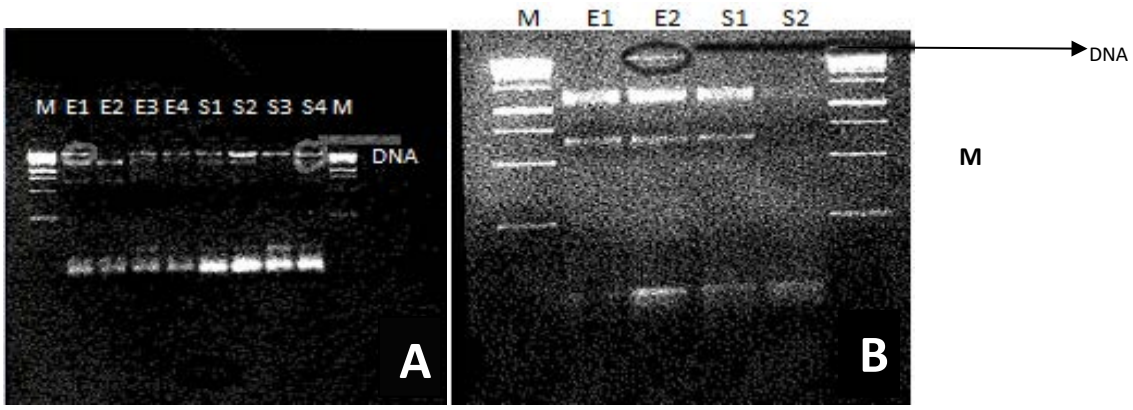

Figure 5: A) Isolation of total RNA from *M. marinum* before DNase treatment. Here E1, E2, E3, E4 represents Isolation in exponential phase and S1, S2, S3, S4 represents stationary phase, M represents 1kb ladder. B) Total RNA from *M. marinum* after DNase treatment. Here E1, E2 represents exponential phase and S1, S2 represents stationary phage, M represents 1kb ladder.

The RNA isolation without DNase treatment retained the DNA fragments in the sample, which leads to contamination (Fig5A). To prevent the contamination, DNase treatment was performed (Fig 5B). DNA-free samples were used for further analysis.

4.4. Quantification

In *M. marinum*, the relative expression of *rpmH*, *rnpA* and *rnpB* genes in stationary vs exponential phases were analyzed using the comparative Ct method. Negative control was used as without cDNA, 16S gene was used as a reference gene and 3 individual cultivators were run for quantification. This quantification is made to confirm previous transcriptome results. The expressions of rpmH and rnpA genes were down-regulated whereas the expression of rnpB was up-regulated were identified. In my experiment I have tried to confirm previous results with invitro. The expressions of rpmH and rnpA genes were down-regulated by 12.5 and 72 fold respectively whereas the expression of *rnpB* was up-regulated by 3 fold (Fig 6).

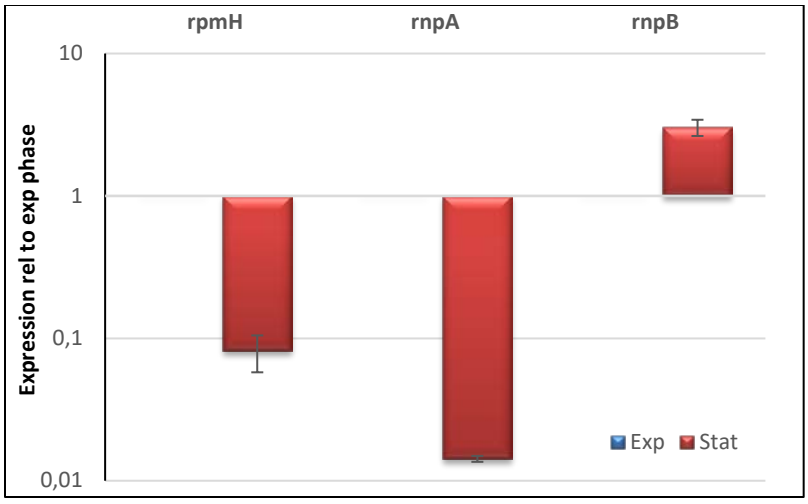

Figure 6: qRT-PCR analysis in stationary phase relative to exponential phase of *rpm H, rnpA, rnpB* genes in *M. marinum.* Exp represents exponential phase and Stat represents stationary phase. Error bars indicate one standard error of the mean.

During growth of *E. coli* in LB medium, which was supplemented with glucose as carbon source, the relative expression of *rpmH*, *rnpA* and *rnpB* genes in stationary vs exponential phases were down-regulated by 52.6, 333 and 2.3 fold respectively (Fig 7A) whereas the bacteria which was supplemented with succinate in M9 medium showed down-regulation of *rpmH*, *rnpA* and *rnpB* genes by 2.3, 3.90 and 1.5 fold respectively (Fig 7B).

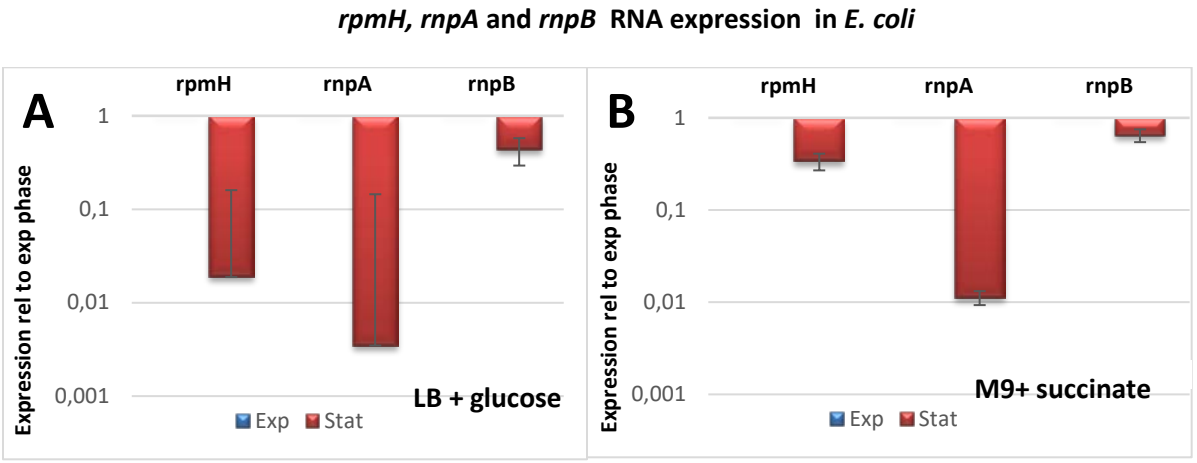

Figure 7: A) qRT-PCR analysis of *rpmH, rnpA, rnpB* genes in *E. coli* grown in LB+ glucose medium. Exp represents exponential phase and stat represents stationary Phase. B) qRT-PCR analysis of *rpmH, rnpA, rnpB* genes in *E. coli* grown in M9 + succinate medium. Exp represents exponential phase and stat represents stationary Phase. Error bars shows technical replicates of the cDNA

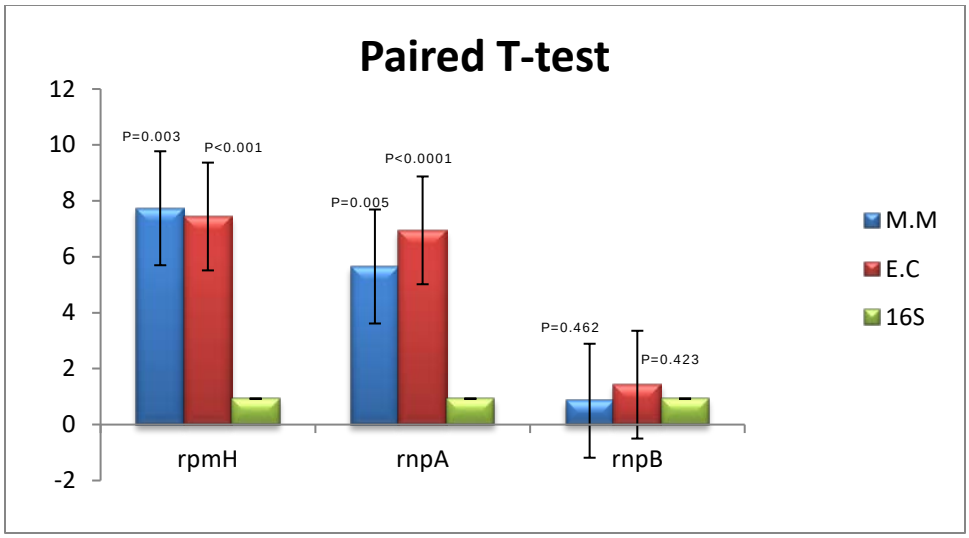

Figure 8: Relative fold expression of rpmH, rnpA and rnpB genes with reference gene 16S from *E. coli* and *M. marinum*. Error bars indicate one standard error of the mean. Statistical differences were analysed by Student's t test. *P < 0.05, significant; **P < 0.01, highly significant; ***P > 0.05, insignificant.

The qRT-PCR data of both E. coli and M. marinum in exponential and stationary phases (Fig. 7) shows that there is a down-regulation of (rnpA gene) at the transcriptional level in the stationary phase in comparison to the exponential phase. The transcriptional level of the RNase P RNA (rnpB gene) is constant in both the stationary and exponential phases in case of E. coli, while, it increases in the stationary phase of M. marinum. Statistical data of qRT-PCR both E. coli and M. marinum in exponential and stationary phases (Fig. 8) shows that *rnpA* ($P < 0.0001$, $P = 0.005$) and *rpmH* ($P = 0.003$, $P < 0.001$) genes are more expressed relative to exponential phase while *rnpB* gene was observed less expression in *M. Marinum* ($P = 0.462$) and in E.coli ($P = 0.423$).

4.5. PCR & Restriction Analysis

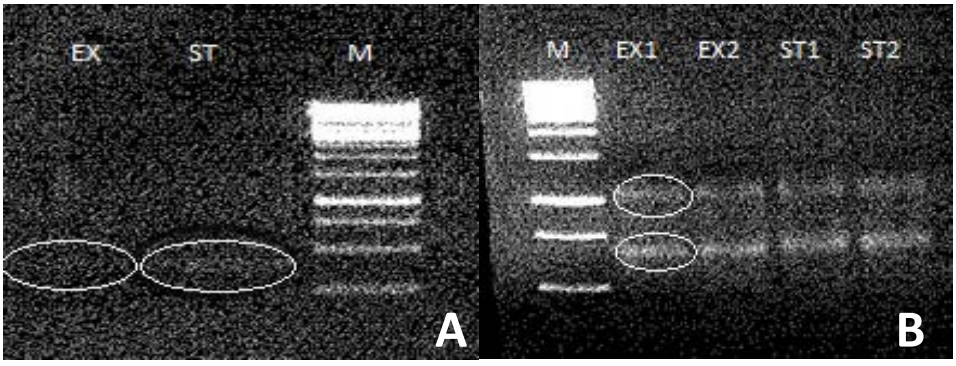

Figure 9.A: PCR amplification of the *rnpA* gene with EX- exponential and ST-stationary phase samples. M represents 1kb Ladder. Amplified DNA is highlighted with circles. **B**) Restriction analysis of the *rnpA* gene with *Eco*RI and *Bam*HI enzymes. EX1, 2 represent exponential phase samples. ST1, 2 represents stationary phase. Insert and vector highlighted with the circles.

The *rnpA* gene was successfully amplified in exponential phase and in stationary phase (Fig 9A). Successful cloning of the *rnpA* gene into the pBS401 vector was confirmed by restriction analysis with *Eco*RI and *Bam*HI *enzymes* (Fig 9B). *rnpB* and *rpmH* genes were observed as no amplification in PCR.

5. Discussion

Genes which are encoding the ribosomal protein (L34) and the RNase P protein are co-localized in most bacterial genomes and it demonstrates co-transcription as noticed in *E. coli* (Panagiotidis et al.,1992). This study reveals the expression levels of *rnpA, rnpB* and *rpmH* in *M. marinum* at RNA level. To confirm the previous studies of expression levels *rnpA, rnpB* and *rpmH* in *E. coli* was analyzed. From previous studies of *E. coli* cells, a higher amount of ribosomes is noticed than RNase P RNA with 60- to 100-fold excess (Dong et al.,1996).

A stop codon (TGA) was identified in the 3′-end of the *rpmH* gene. In this study it shows that *rpmH* overlaps the *rnpA* gene, which is observed immediately downstream to *rpmH* (Fig 1). From a previous study, it was observed that the premature termination of translation is caused by hairpin structures. Similar hairpin structures are observed as encoded in other bacterial *rpmH-rnpA operons* (Feltens et al., 2003).

During the culturing, OD values were observed every 6 hours with 1:100 dilutions and 1:1000 dilutions. A clear difference in the growth of the cells was noticed in both the dilution concentration in which 1:100 showed a higher OD value of 0.08 in a short time while the other one showed an OD value of 0.004 for the same time point. At the same time, the cells in high dilution concentration reached the stationary point soon when compared to low-density culture. The reason behind both the observation could be explained by cell density. The cell density is high in high dilution concentration whereas lower cell density in low dilution concentration.

The growth of cells was studied in *E. coli* with different media. The media were LB+glucose and M9+succinate as carbon source. The difference in cell growth between the two media could be explained by the difference in supplement of nutrients and the carbon source supplied to the cells (Lee et al., 2014). Firstly, the growth of bacteria in M9 was very slow when compared to LB

medium because M9 medium is a minimal nutrient medium and it is deficit of amino acid supplement, The cells are growing slower since they have to synthesize all organic compounds by themselves. Secondly the carbon supplement succinate has lower energy content per C than glucose in M9 medium. LB medium is a rich nutrient medium with contents like peptides, vitamins and minerals and additional rich carbon sources like glucose, provides higher energy for the cells and promotes rapid cell growth(Lee et al., 2014) .

At different time points after inoculation, total RNA was prepared and used as the template for RT-PCR analysis with primers specific for *rnpA* and *rnpB and rpmH genes*, respectively in *E. coli* and *M. marinum*. qRT-PCR results of *M. marinum* shows down-regulation in expression of *rnpA* ($P = 0.005$) and *rpmH* ($P = 0.003$) whereas *rnpB* ($P = 0.423$) shows up-regulation of 3 fold in stationary phase relative to exponential phase. The expression of all the three genes was studied in *E.coli* and *M.Marinum* (Fig. 8). The expression of *rnpA* ($P < 0.001$) and *rpmH* ($P < 0.001$) observed in the *E. coli* system is in agreement with the previous studies in *E. coli*. Also a similar down regulation in gene expression of *rnpA* ($P = 0.001$) and *rpmH* ($P = 0.003$) was noticed in *M. marinum*. In contrast, *rnpB* shows up-regulation in *M. marinum* while down-regulation in *E. coli* with a 95% confidence interval. Previous transcriptome studies of the M. marinum *rnpB, rnpA* and *rpmH* (Fig. 1) shows that the expression of both *rnpA* and *rpmH* genes increases in the exponential phase in relation to the stationary phase, while the expression of the *rnpB* gene (RNase P RNA) decreases in the exponential phase compared to the stationary phase. Therefore, both the qRT-PCR and the transcriptome data are in agreement with each other. qRT-PCR data and the transcriptome data indicate the possibility for the presence of post-transcriptional (translational level) regulation.

To test the above results at the translation level, total genomic DNA was isolated with RNA free treatment from *M. marinum*. The C5 gene was successfully amplified while *rpmH* and *rnpB* failed. The factual reasons for amplification failures is uncertain to explain in principle but the possibilities could be i) Inappropriate transfer of isolated cells into PCR tubes ii) unusual changes in structure or function of the cell due to change in physical or chemical conditions iv) DNA is not isolated properly during the process of cell lysis, by which it fails to interact with PCR reagents (Piyamongkol, 2003).

6. Conclusion and Future Aspects

At the RNA level, the expression of *rnpA* and *rpmH* in *M. marinum* was similar to expression in *E. coli*. However *rnpB* shows a difference in expression in *M. marinum* and *E. coli*. Expression of the genes will depend on the source of medium used in the culture. If medium is rich in energy source provided in the form of carbon, the growth seems quite rapid also rich in energy source that impacts the levels of gene expression. However this phenomenon was investigated at the transcriptional level but to get a clearer idea the study should be conducted at the protein level. Thus this study should be extend to study the gene expression in depth.

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