KRITIKA SHARMA 2021

MS.c Biotechnology

To Decipher The Role Of TA Systems In Mycobacterium Tuberculosis

Project Report Submitted In Partial Fulfillment For
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DATE : MAY 30, 2021

CERTIFICATE

This is to certify that the project entitled as " To Decipher the role of Toxin-Antitoxin system in the tuberculosis" submitted by Kritika Sharma, student of M.Sc. Biotechnology, IV Semester of Delhi Technological University, in the partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology, was completed under guidance of Dr. Asmita Das and co-guidance of Dr. Uddipan Das at All India Institute Of Medicine And Science, New Delhi for the duration of three months (1st of January to 30th of March. 2021).

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To whomsoever it may concern

This is to certify that Ms. Kritika Sharma has undergone a short-term training under my mentorship w.e.f. 1st January 2021 to 31st March 2021 (3 months). During this period Ms. Kritika worked on the "Cloning, expression and Purification of Mycobacterial Toxin". The work involved basic molecular biology, gene cloning and protein purification techniques.

Dr. Uddipan Das
7/4/2021

CANDIDATE'S DECLARATION

I, hereby certify that the work is which is presented in Major Project- Ⅱ entitled "To Decipher The Role Of TA Systems In Mycobacterium Tuberculosis" in fulfilment of the requirement for the award of the degree of Masters of Science (M.Sc) in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi, is an authentic record of my own, carried out during a period from Department Of Biotechnology, Delhi Technological University, under the supervision of Dr. Asmita Das and Co-supervision of Dr. Uddipan Das.

The matter presented in this thesis has not been submitted by me for the award of any degree of this or any Institute. The work has been communicated in SCI expanded journal.

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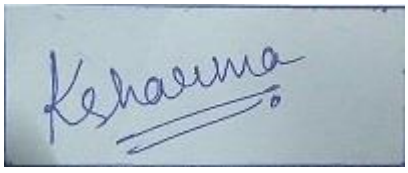

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SUPERVISOR CERTIFICATE

To the best of my knowledge this work has not been submitted in part or full for any degree or diploma to the University or elsewhere. I, further certify that the publication and indexing information given by the student is correct.

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Kritika Sharma

ABBREVIATIONS

Abs	Absorbance
APS	Ammonium persulfate
Bps	Base pairs
BME	□- Mercaptoethanol
BNPAGE	Blue native PAGE
BSA	Bovine Serum Albumin
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E.Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic
Gm	Gram
Hrs	Hour
HRP	Horseradish peroxidase
Hs	Homo sapiens
IPTG	Isopropyl thiogalactoside
Kan	Kanamycin
kDa	Kilo Dalton
Kbp	Kilobase pairs
LB	Luria bertani
Mg	Milligram
mg/ml	Milligram per Milliliter
mM	Milli molar
Ni-NTA	Nickel- nitriloacetate
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate bovine saline
PBST	Phosphate Bovine saline tween 20
PMSF	Phenyl Methyl Sulphonyl fluoride
Rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
TEMED	N,N,N,N Tetramethyl-ethylene diamine
tRNA	Transfer ribonucleic acid
WHO	World health organisation
µg	Microgram
µl	Microliter
µM	Micro mol

CONTENTS

ABSTRACT

Mycobacterium tuberculosis is the bacteria responsible for the disease of the infamous Tuberculosis (TB). mtb is the major human pathogens prevalent in developing countries, with an appropriate number of 2 million deaths per year. This disease is treatable but the hurdles we face, this project helps in giving the new approach and better perspective. Latent Tb is the phase when the bacteria exits in one person's body and unable to replicate properly due to healthy and good immunity response. But it shows its pathogenicity when the immune response gets degraded. The reason can be any other physiological condition or aging. Even after any person is responding regardless having any physiological conditions, the case of patient can be treated. But problem arises when patient's body stop responding regarding the treatment. To overcome the growing problem of MDR, XDR varieties and eradication of the latent form of TB, novel ways of more rapid treatment are the need of the hour. The complete sequence of the genome of Mtb (H37Rv) has been achieved, but more deep knowledge and understanding of different classes of toxin and antitoxin systems needs to be studies. Mycobacterium tuberculosis is known to be the causative agent in tuberculosis. Tuberculosis is the disease in which lungs get affected along with other parts. 87% new cases had been recorded in TB burden countries. Among which out of 30 countries, 8 countries hold the two thirds of the cases. India was among the 8 countries. More than 2 million cases were recoded in past two years. And this data is keep increasing in recent years. Immunology plays a paramount role in this disease. The person who gets affected by the mtb infection is expected to express inflammatory response which causes the breakdown of the lungs matrix and formation of lung cavities. But many underlying immunological dysfunction like aids and diabetes etc. creates variation in formation of lung cavities. The fatality in TB is increased by the drug resistant TB. It means that the person stops responding to the treatment. Over the past decade, there has been a sharp increase in the disease, with the emergence of MDR and EDR varieties of TB. Toxin-Antitoxin systems are potential therapeutic domain in antibiotic resistance. TA systems are present in the bacteria. These TA systems can be classified into six classes. They are prone to the unfavorable conditions. We can manipulate these TA regions to loosen up its harmful effect and will make it less fatal in the cause of tuberculosis.

REVIEW OF LITERATURE

2.1 INTRODUCTION

TB is taking the lives of so many people. It is one the leading causes of deaths in developing countries like India, Bangladesh, Nepal, Pakistan etc. In this disease, the causative agent mycobacterium tuberculosis (mtb) affects the lungs and immunity of the body.Mycobacterium tuberculosis is the bacteria responsible for the disease of the infamous Tuberculosis (TB). mtb is the major human pathogens prevalent in developing countries, with an appropriate number of 2 million deaths per year.
This disease is treatable but the hurdles we face, this project helps in giving the new approach and better perspective. Latent Tb is the phase when the bacteria exits in one person's body and unable to replicate properly due to healthy and good immunity response. But it shows its pathogenicity when the immune response gets degraded. The reason can be any other physiological condition or aging. Even after any person is responding regardless having any physiological conditions, the case of patient can be treated. But problem arises when patient's body stop responding regarding the treatment. To overcome the growing problem of MDR, XDR varieties and eradication of the latent form of TB, novel ways of more rapid treatment are the need of the hour.
The complete sequence of the genome of Mtb (H37Rv) has been achieved, but more deep knowledge and understanding of different classes of toxin and antitoxin systems needs to be studies. Mycobacterium tuberculosis is known to be the causative agent in tuberculosis. Tuberculosis is the disease in which lungs get affected along with other parts. 87% new cases had been recorded in TB burden countries. Among which out of 30 countries, 8 countries hold the two thirds of the cases. India was among the 8 countries. More than 2 million cases were recoded in past two years. And this data is keep increasing in recent years. Immunology plays a paramount role in this disease.. The person who gets affected by the mtb infection is expected to express inflammatory response which causes the breakdown of the lungs matrix and formation of lung cavities.[2] But many underlying immunological dysfunction like aids and diabetes etc.[10] creates variation in formation of lung cavities. The fatality in TB is increased by the drug resistant TB. It means that the person stops responding to the treatment.[5] Over the past decade, there has been a sharp increase in the disease, with the emergence of MDR and EDR varieties of TB.[1]

Toxin-Antitoxin systems are potential targets for novel prophylactic and therapeutic approaches that are required urgently in antibiotic resistance. TA systems are present in the bacteria. These TA systems can be classified into six classes. They are prone to unfavorable conditions. We can manipulate these TA regions to loosen up its harmful effect and will make it less fatal in the cause of tuberculosis.

2.2 ANTIBIOTIC RESISTANCE IN MTB

In 1928 Fleming Alexander first discovered penicillin, the antibiotic which helped bacterium in creating further infection. It opened the gateway for many other antibiotic treatments which saved so many lives. The minor infection which used to be life threatening would be able to cure it. But with time bacteria also adapted these antibiotics. Antibiotics and bacteria are co-evolving. Therefore with gradual evolution of bacteria resistance towards these bacteria has also increased. Several drugs are being used in the treatment of tuberculosis like : diarylquinolines;oxazolidinonesnitroimidazoles;ethylenediamines, benzothiazinones;rifaquin fluoroquinolone. Many TB strains get resistant to both the first line and second line of TB drugs. They show poor or no response towards these drugs and is unable to prevent. These strains are known as TDR TB. Many countries shows this type of mutation in different strains. On the other hand, XDR stands for limited drug resistance. This strains of TB are majorly resistant to first line of TB drugs. In this case the situation is not hopeless, we can treat a person with different cocktails of drugs which might effect the patient.
To eradicate TB and to prevent antibiotic resistance we need to find other novel methods by which we can kill the causative agent. Here comes the role of TA systems.[16,19]

2.3 TOXIN-ANTITOXIN SYSTEMS

TA systems are present in prokaryotic genomes. Firstly it was thought that TA domains were presently only plasmids of bacteria. But, later it was deduced that it is also present in the chromosomal genome. Here it stabilizes the chromosome of bacteria. TA systems regulates cells in the condition of stress and any under supply of necessary nutrients.[3]
It help the cell in surviving the extreme situation. The toxin part is stable and the antitoxin part is unstable and can be degraded by minor stimus from external

environment. When unfavourable circumstances arise, antitoxin mRNA is destroyed selectively. Type 6: antitox is required, T i.e. the toxin portion adheres to the sliding clamp and inhibits DNA replication effectively.[6,14] We can manipulate these domain with the help of bioinformatics and molecular biology and can generate the phenomenon like apoptosis in mtb.

MATERIALS AND METHODS

3.1 E. coli Competent cells preparation

Sambrook et al. (1989) described a procedure for making chemically competent cells. E. coli DH5 cells were streaked on an LB agar plate and incubated overnight at 37o C from their glycerol stock. A single colony was chosen and inoculated in 5 mL of LB broth, then incubated overnight at 37o C in a 200 rpm incubator shaker. 500 l of the culture (1 percent v/v of primary culture) was utilised as inoculum for a 50 ml LB broth after 16 hours. After growing the culture to an OD600 of 0.3-0.5, the cells were cooled on ice for 30 minutes before being centrifuged at 2,500 x g for 10 minutes at 4o C. The supernatant was discarded, and the pellet was resuspended in 25 ml of cold 100 mM calcium chloride and incubated for an 30 mins on ice. The cells were gently swirled and then centrifuged at 2500 x g for 10 minutes at 4º C. The pellet obtained was resuspended in 2 ml of 100 mM calcium chloride and 100 % glycerol was added to it to a finalconcentration of 15%. The volume was divided into 100 µl aliquots in 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen and stored at -80º C.

3.2 Transformation of E. coli competent cells

E. coliDH5 competent cells were maintained on ice for about 30 minutes before being transformed. The ligation mixture or 10 ng of plasmid DNA were added to thawed cells and incubated for 30 minutes on ice. The cells were then treated to a 90-second heat shock treatment by moving each microcentrifuge tube to a water bath set at 42°C for 90 seconds, followed by a 5-minute incubation on ice. 500 l of autoclaved sterile LB broth was added and incubated in an incubator shaker at 200 rpm for 1 hour 20 minutes at 37o C. The cells were then centrifuged for 3-5 minutes at 2500 x g before being resuspended in 100 l of LB broth. The cells were placed on LB agar plates with the appropriate antibiotic selection. The LB plates were incubated overnight at 37°C and colonies were checked the next day.

3.3 Protein expression

The approach was used to transform the plasmid containing the gene of interest into the E.coli BL21 (DE3) strain. A single colony was taken from the changed plate and inoculated in 10 mL LB broth supplemented with appropriate antibiotics. The cells were cultivated at 37°C and 200 rpm in an incubator shaker until the OD600

reached 0.6. 1 ml culture was taken out as a control and 1mM final concentration of IPTG was added. After 3 hours incubation, the cells were harvested using centrifugation and stored at -20º C.

3.4 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS gels were cast using Bio-Rad mini protein apparatus. The gel consisted of 5 % of stacking overlaid on 12.5 % of resolving gel. 29:1 ratio of acrylamide:bisacrylamide was used to make 30 % stock concentration of acrylamide solution. The gel was polymerized using final concentration of 0.05 % of APS and 0.05 % TEMED. The protein samples to be analyzed were mixed with 5X SDS loading dye to the final concentration of 1X. The samples were then boiled at 95ºC for 5 minutes, followed by high speed centrifugation for 5 minutes. 20 µl of protein samples and 4 µl protein marker were loaded in the SDS PAGE gels, submerged in 1X SDS running buffer (Tris-Glycine-SDS). The gels were allowed to run at a constant voltage of 200V.

3.5 Coomassie blue staining

The SDS gels were washed with double distilled water and stained using freshly prepared Coomassie stain (0.25 % of coomassie dye, 40 % of methanol and 10 % of acetic acid) for 10 minutes. The excess stain was washed away with double distilled water, and the gels were destained with 40% methanol and 10% acetic acid until protein bands could be seen clearly. The gels were scanned and stored using a standard picture scanner.

3.6 Protein purification

After the protein expression was successful, protein purification was carried out. The materials and methods employed in protein purification experiments are described into following subsections.

3.7 Immobilised metal affinity chromatography (IMAC)

Immobilized metal affinity chromatography was used to purify recombinant Histidine tagged proteins (IMAC). Poly- His tagged proteins have high affinity for various transition state metal ions. Usually, six to eight histidine residues are coded

by the various vectors either at their N- or C- terminal of the translated proteins. When these proteins are passed through IMAC resin, they get attached to the metals in the resin to their tags and untagged proteins flow Through. TALON (Clonetach) IMAC resins were used in this work which chelate the Cobalt ions (Co2+) to the Sepharose matrix. Usually, 1 ml of resin has the affinity to bind 5 mg of protein. Therefore 1 ml of resin (in 20 % EtOH) was taken in disposable BIO-RAD econo gravity columns. The binding buffer equilibrated the resin. The resin was washed with 50 CV wash buffer after protein immobilisation, and the immobilised protein was eluted using 15 CV elution buffer.

3.8 Gel Permeation Chromatography (GPC)

Gel permeation chromatography (GPC) was used as a final step of purification of recombinant proteins. The Hi load Superdex S-200 16/60 (GE healthcare) column was used in GPC, set up on an AKTA Prime Plus (GE healthcare). The GPC column was equilibrated using GPC buffer at low rate of 1 ml/minute. The protein sample was concentrated to 2 ml and put into the AKTA Prime Plus via a 2 ml loop. 1 CV of GPC buffer was used to wash the column. UNICORN software was used to examine the chromatograms (GE healthcare). For additional investigation, the peak fractions were collected and run on an SDS gel.

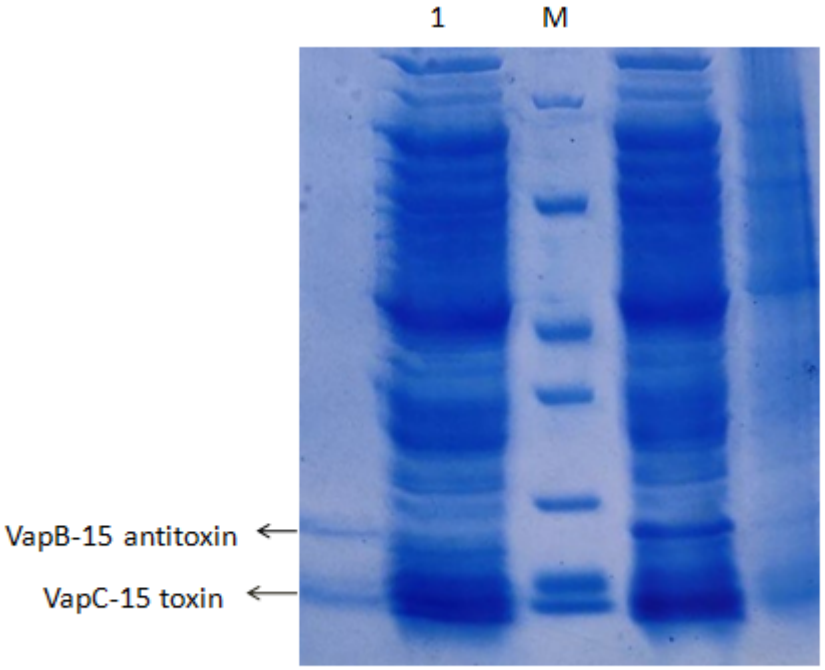
Concentration of protein samples

Amicon Ultra 15 kDa cut-off filters were used to concentrate the protein (Millipore). The 10 mL filters were centrifuged at maximum of 2 L till the protein samples reached the desired volume.

PROTOCOL AND RESULTS

Purification of VapBC15

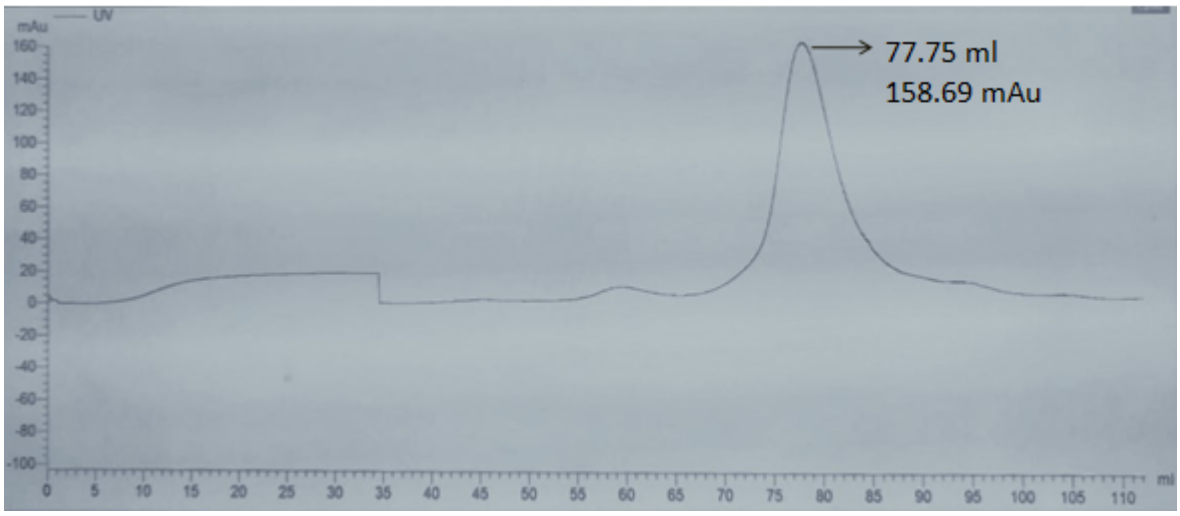
At 4o C, all chromatographic stages were completed. The cell pellet was resuspended in lysis buffer (25 mM Hepes pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol, and 5 mM beta-mercaptoethanol) (BME). Ultrasonication on ice was used to lyse the cells, with pulses lasting 8 seconds on and 9 seconds off with an amplitude of 30- 50A. 10 to 15 cycles of sonication were completed. After that, the cell lysate was centrifuged at 13000 x g for 45 minutes at 4o C. The supernatant was filtered via a 0.45 m filter and run through a 2 ml bed volume Cobalt NTA sepharose (GE healthcare) column.Binding buffer containing 25mM Hepes pH 7.5, 500mM NaCl, 5 mM imidazole, 5% glycerol, and 5mM BME was used to pre-equilibrate the cells. To eliminate non-specific bound proteins, the column was washed with 50 column volume of wash buffer comprising 25mM Hepes pH 7.5, 500mM NaCl, 5mM imidazole, 5% glycerol, and 5mM BME. The protein was then eluted with elution buffer containing 25mM Hepes pH 7.5, 500 mM NaCl, 250mM imidazole, 5% glycerol and 5mM BME. The protein purity was checked on a 12.5% SDS gel and then concentrated using Amicon 15 kDa cut-off filter (Millipore).



SDS- PAGE analysis after affinity chromatography : SDS-PAGE picture representing the purified VapBC- 15 complex. Lane M represent standard protein marker and lane 1 represent affinity purified VapBC-15 complex.

Size exclusion chromatography

After concentrating the protein in 15 kDa centricon. The volume we concentrated was from 10 ml to 2 ml. The Hiload 16/60 superdex- 200 column (GE healthcare) was used for size exclusion chromatography, which was pre-equilibrated with 25 mM HEPES, 150 mM NaCl, and 1 mM DTT. The peak fractions were gathered and examined, using 12.5 % SDS PAGE and protein concentration was determined using Nanodrop Spectrophotometer (DeNovix Inc).



Size Exclusion Chromatography of VapBC. The superdex-200 16/60 gel permeation chromatogram of VapBC15 showing the elution peak.

RESULT

The plan covers the purification of VapBC15. Affinity chromatography was used to purify the VapBC15, which was then followed by Superdex S-200 gel permeation chromatography. The size was consistent with the UNIPROT database's theoretically predicted estimate.

CONCLUSIONS

- TA systems can be the the new approach for the causative agent that is mycobacterium in tuberculosis.

- It is now not widely studied approach but it holds many aspect of by which we can treat the drug resistant TB.

- This endemic can be halt by the new approach and technologies. Bioinformatic and Molecular biology together can give the desired results but for that we need to have the deep understanding of TA systems in mtb.

DISCUSSION

Tuberculosis (TB), is one of the major human pathogens prevalent in developing countries, with an appropriate number of 2 million deaths per year. This disease is treatable but the hurdles we face, this project helps in giving the new approach and better perspective. Latent Tb is the phase when the bacteria exits in one person's body and unable to replicate properly due to healthy and good immunity response. But it shows its pathogenicity when the immune response gets degraded. The reason can be any other physiological condition or aging. Even after any person is responding regardless having any physiological conditions, the case of patient can be treated. But problem arises when patient's body stop responding regarding the treatment. To overcome the growing problem of MDR, XDR varieties and eradication of the latent form of TB, novel ways of more rapid treatment are the need of the hour.

FUTURE PROSPECTUS

- To carry out co-crystallization trials with the drugs using hanging drop vapor diffusion method, for understanding their structural and functional relevance.

- TA module can be the therapeutic approach for the problem we face in the antibiotic resistance.

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