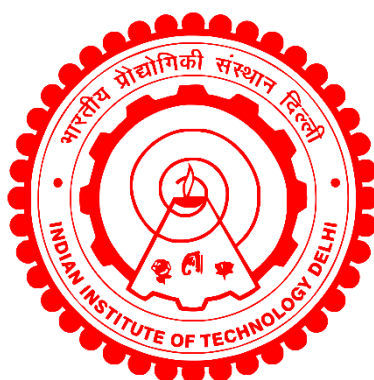


**UNRAVELLING THE TRANSCRIPTION ACTIVATION MECHANISM
OF A VIRULENCE-ASSOCIATED SMALL RNA MTS1338 & ITS
FUNCTIONS IN *MYCOBACTERIUM TUBERCULOSIS***

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INDIAN INSTITUTE OF TECHNOLOGY DELHI
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OF A VIRULENCE-ASSOCIATED SMALL RNA MTS1338
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by

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Department of Chemistry

submitted

in fulfillment of the requirement of the degree of

DOCTOR OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY (IIT) DELHI

FEBRUARY 2026

CERTIFICATE

This is to certify that the thesis entitled “**Unravelling the transcription activation mechanism of a virulence-associated small RNA MTS1338 and its functions in *Mycobacterium tuberculosis***” being submitted by **Mr. Krishan Kumar** to the **Indian Institute of Technology Delhi** for the award of the degree of “*Doctor of Philosophy*” (PhD) in Chemistry, is a record of bonafide research work carried out by him. Krishan joined the **RNA biology lab** on December 27, 2019. Under my guidance and supervision at the **Department of Chemistry, IITD**. He worked diligently, published two first-author research papers, and fulfilled the requirements for thesis submission, meeting the requisite standard to my knowledge and demonstrating the ability to work independently. The results of this thesis have not been submitted in part or whole to any other university or institute for the award of any degree or diploma.

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Abstract

MTS1338 (ncRv11733), a distinctive small non-coding RNA (sRNA) in pathogenic mycobacteria, is crucial in host-pathogen interactions during infection. Mycobacterial cells encounter heterogeneous stresses in macrophages upon infection. MTS1338 has been shown to be highly abundant in response to various stress conditions, including exposure to low pH, H₂O₂, hypoxia, and NO. The dormancy regulatory factor DosR is a part of two-component system that regulates ~50 genes in *Mycobacteria* and has only recently been identified as involved in MTS1338 abundance. Little is known about sRNA-mediated regulation in *Mycobacterium tuberculosis*. We have taken a biophysicochemical approach in the current study to investigate how DosR interacts with the upstream promoter region of the MTS1338 gene, also known as the DosR-regulated sRNA (DrrS). We also examined whether PhoP, a response regulator that controls the expression of a wide range of low-pH-inducible genes, interacts with the promoter of the MTS1338 gene. We identified that DosR can strongly bind to the two regions upstream of the MTS1338 gene. The proximal region, though individually, possesses a 3-fold higher affinity than the distal site; however, the presence of both regions increased the affinity of the MTS1338 gene for DosR by >10-fold. Although PhoP did not directly bind to the MTS1338 gene, it binds to the DosR-bound MTS1338 gene, which is evocative of a concerted regulation of MTS1338 expression under stresses. *whiB6* (Rv3862c) product is also a part of this virulence network. It controls metabolism, cell division, and virulence, and its gene is under the control of PhoP. The ESX-1 (Type VII) secretion system in *M. tuberculosis* delivers virulence factors like ESAT-6 (EsxA) to harm host cells, causing tuberculosis. Once *WhiB6* is translated as a stress signal, it can modulate the expression of DosR and ESX-1 differentially, thus making the understanding more complex. We found that *whiB6* expression was upregulated >3-fold under pH 4.5 stress and ~10-fold under the MTS1338 overexpression conditions compared to the wild-type. Furthermore, we utilized *in vitro* transcribed RNAs to validate our theory using gel-shift assays. MTS1338 binds *whiB6* mRNA at 3' end. Our findings indicate that PhoP phosphorylation enhanced its binding to the *whiB6* upstream promoter, where the PhoP box is crucial for the binding. On the other hand, DosR binds only PhoP:DNA binary complex, and thus, a super-shifted band was observed. This research highlights the PhoP network as a part of sRNA regulation at low pH and may significantly aid in targeting such seed interactions. This study substantially adds to our knowledge of regulating MTS1338 expression in *M. tuberculosis*. Further research can lead to novel strategies and antibiotics against devastating human diseases.

Keywords: Small RNA; Mycobacteria; Dormancy regulatory factor; Cooperative binding; DNA-protein interaction; Two-component system

सार

MTS1338, रोगजनक माइकोबैक्टीरिया में एक विशिष्ट छोटा गैर-कोडिंग RNA (sRNA) है, जो संक्रमण के दौरान मेजबान-रोगजनक अंतःक्रियाओं में महत्वपूर्ण है। माइकोबैक्टीरिया कोशिकाएँ संक्रमण के बाद मैक्रोफेज में विषम तनावों का सामना करती हैं। विभिन्न तनाव स्थितियों, जैसे कि कम pH, H₂O₂, हाइपोक्सिया और NO के संपर्क में आने पर MTS1338 की मात्रा अत्यधिक पाई गई है। डॉर्मैसी रेगुलेटरी फैक्टर D_{os}R एक दो-घटक प्रणाली का हिस्सा है जो माइकोबैक्टीरिया में लगभग 50 जीनों को नियंत्रित करता है और हाल ही में MTS1338 की प्रचुरता में इसकी भूमिका की पहचान की गई है। *माइकोबैक्टीरियम ट्यूबरकुलोसिस* में sRNA-मध्यस्थ विनियमन के बारे में बहुत कम जानकारी है। हमने वर्तमान अध्ययन में यह जांचने के लिए एक जैव रासायनिक दृष्टिकोण अपनाया है कि D_{os}R MTS1338 जीन के अपस्ट्रीम प्रमोटर क्षेत्र के साथ कैसे अंतःक्रिया करता है, इसे D_{os}R-विनियमित sRNA (D_{rr}S) भी कहा जाता है। हमने यह भी जांच की कि क्या PhoP, एक प्रतिक्रिया विनियामक जो कम pH-प्रेरित जीनों की अधिकता की अभिव्यक्ति को नियंत्रित करता है, MTS1338 जीन के प्रमोटर के साथ अंतःक्रिया करता है। हमने पहचाना कि D_{os}R MTS1338 जीन के अपस्ट्रीम के दो क्षेत्रों से मजबूती से जुड़ सकता है। समीपस्थ क्षेत्र, हालांकि व्यक्तिगत रूप से, दूरस्थ साइट की तुलना में 3 गुना अधिक आत्मीयता रखता है; हालांकि, दोनों क्षेत्रों की उपस्थिति ने D_{os}R के लिए MTS1338 जीन की आत्मीयता को >10 गुना बढ़ा दिया। हालांकि PhoP सीधे MTS1338 जीन से नहीं जुड़ा, लेकिन यह D_{os}R-बद्ध MTS1338 जीन से जुड़ता है, जो तनाव के तहत MTS1338 अभिव्यक्ति के एक समन्वित विनियमन का संकेत देता है। *whiB6* (Rv3862c) उत्पाद भी इस विषाणु नेटवर्क का एक हिस्सा है। यह चयापचय, कोशिका विभाजन और विषाणुता को नियंत्रित करता है, और इसका जीन PhoP के नियंत्रण में है। *M. ट्यूबरकुलोसिस* में ESX-1 (टाइप VII) साव प्रणाली ESAT-6 (EsxA) जैसे विषाणु कारकों को मेजबान कोशिकाओं तक पहुंचाती है, जिससे तपेदिक होता है। एक बार जब *WhiB6* को तनाव संकेत के रूप में अनुवादित किया जाता है, तो यह D_{os}R और ESX-1 की अभिव्यक्ति को अलग-अलग तरीके से संशोधित कर सकता है, जिससे समझ और अधिक जटिल हो जाती है। *WhiB6* (Rv3862c) चयापचय, कोशिका विभाजन और विषाणु को नियंत्रित करता है। हमने पाया कि *whiB6* की अभिव्यक्ति pH 4.5 तनाव के तहत > 3 गुना और MTS1338 ओवरएक्सप्रेसन स्थितियों के तहत वाइल्ड-टाइप की तुलना में ~ 10 गुना अधिक थी। इसके अलावा, हमने जेल-शिफ्ट परख का उपयोग करके अपने सिद्धांत को मान्य करने के लिए इन विट्रो ट्रांसक्राइब किए गए RNA का उपयोग किया। MTS1338 3' छोर पर *whiB6* mRNA को बांधता है। हमारे निष्कर्षों से पता चलता है कि PhoP फॉस्फोरिलेशन ने *whiB6* अपस्ट्रीम प्रमोटर से इसके बंधन को बढ़ाया, जहां PhoP बॉक्स बंधन के लिए महत्वपूर्ण है। दूसरी ओर, D_{os}R केवल PhoP:DNA बाइनरी कॉम्प्लेक्स को बांधता है, और इस प्रकार, एक सुपर-शिफ्टेड बैंड देखा गया। यह शोध कम pH पर sRNA विनियमन के एक भाग के रूप में PhoP नेटवर्क पर प्रकाश डालता है और इस तरह के बीज इंटरैक्शन को लक्षित करने में महत्वपूर्ण रूप से सहायता कर सकता है। यह अध्ययन *M. ट्यूबरकुलोसिस* में MTS1338 अभिव्यक्ति को विनियमित करने के हमारे ज्ञान में काफी वृद्धि करता है। आगे के अनुसंधान से विनाशकारी मानव रोगों के विरुद्ध नवीन रणनीतियां और एंटीबायोटिक्स विकसित हो सकते हैं।

कीवर्ड: लघु आरएनए; माइकोबैक्टीरिया; सुप्तता नियामक कारक; सहयोगात्मक बंधन; डीएनए-प्रोटीन अंतःक्रिया; द्विघटक प्रणाली

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Figure 5.3. Effect of MTS1338 overexpression, acidic pH, and tert-butyl hydroperoxide (t-BHP) oxidation on *whiB6*. (A) *whiB6* expression (RT-qPCR) during MTS1338 overexpression versus empty vector (Tet+). Data plotted as Mean±SD (*p<0.05). (B) Northern blot analysis and 5S loading control, the number represented under each lane *whiB6* amount compared to empty vector or untreated WT (pH 7), set as 1. (C) Moderate effect of low pH (4.5) on *whiB6* expression (RT-qPCR data), resultant expression of *whiB6* (mean±SD, *p<0.05). (D) Effect of tBHP oxidative stress on *whiB6* expression (RT-qPCR data), resultant expression of *whiB6* (mean±SD, *p<0.05) 73

Figure 5.4. 19 bp direct interaction leads to sRNA:mRNA binary complex (A). IntaRNA results in *MTS1338:whiB6* interaction. (B) *In vitro* transcription followed by gel-shift assay confirms the sRNA:mRNA binary complex formation. MTS1338 constant, while *whiB6* concentration exponentially increased up to 1:1 interaction. (C) DNA Oligo (24 nt) complementary to the *whiB6* 3' was utilized in increasing concentrations to disrupt the sRNA:mRNA interaction 74

Figure 5.5. PCR amplified constructs 1 (489 bp) binding assay with PhoP ($K_d \sim 42.5 \pm 5$ nM). PhoP-p binding to construct 1 is cooperative in nature and observed with increased PhoP-p concentration via gel-shift assay (EMSA). K_d represents the ligand (PhoP-p) concentration at which half of the fraction of dsDNA construct 1 is already bound (arrowhead) 74

Figure 5.6. Schematic description of Mechanism of MTS1338: DosR and PhoP overexpression promotes intracellular MTS1338 levels in *M. tuberculosis*. This elevated MTS1338 accumulation likely making dsRNA with *whiB6* target RNA (sRNA-mRNA complex), protecting it from cellular ribonucleases and thus ESX-1 mediated regulation of virulence factor (EsxA) secretion..... 76

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ABBREVIATIONS

AcP : Lithium potassium acetyl phosphate
AG-PG : Arabinogalactan-peptidoglycan
CD : Circular dichroism
CO : Carbon monoxide
CO₂ : Carbon dioxide
COVID-19 : Coronavirus disease 2019
CTD : C-terminal domain (His₆-tag)
DBS : DosR binding site
DBS₁ : Proximal (strong) DosR binding site
DBS₂ : Distal (weak) DosR binding site
DNA : Deoxyribonucleic acid
DosR : Dormancy survival regulator
DOT : Directly observed therapy
DOTS : Directly observed therapy, short course
DrrS : DosR-regulated sRNA
ds : Double-stranded
E. coli : *Escherichia coli*
EDTA : Ethylenediaminetetraacetic acid
EMSA : Electrophoretic mobility gel-shift assay
ESX-1 : ESAT-6 (EsxA) secretion system-1
FAM : Fluorescein amidite dye (bright green)
gDNA : Genomic DNA
HICs : High-income countries
HIV : Human immunodeficiency virus
IPTG : Isopropyl β-D-thiogalactopyranoside
 K_d : Dissociation equilibrium constant
LB : Luria-Bertani growth media
LMICs : Low and middle-income countries
MA : Mycolic acids
MDGs : Millennium development goals
MDR-TB : Multi-drug resistance
MHC : Major histocompatibility complex (1 and 2)
miRNAs : microRNAs
MOM : Mycobacterial outer membrane
MOPS : 3-(N-morpholino) propane sulfonic acid

MST : Microscale thermophoresis
MTB : *Mycobacterium tuberculosis*
MTS1338 : *Mycobacterium tuberculosis* small RNA 1338
M ϕ : Pulmonary (Lung) macrophages
NFW : Nuclease-free water
NO : Nitric oxide
NRP : Non-replicating persistence
nt : Nucleotide
NTA : Nitrilotriacetic acid
OADC : Oleic acid, Bovine albumin, Sodium chloride, Dextrose, and Catalase supplement
OD : Optical density
PAMPs : Pathogen-associated molecular patterns
PBS : PhoP binding (TCACAGC) sites
PCR : Polymerase chain reaction
pH : Potential of hydrogen
PhoP : Phosphate regulon response regulator protein
RBS : Ribosome-binding site
RDD : Restriction double digestion
RE : Restriction endonuclease
RNA : Ribonucleic acid
ROS : Reactive oxygen species
rpm : Revolution per minute
RR : Response regulator
SD : Standard deviation
SDGs : Sustainable development goals
SDS-PAGE : Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis
SGLs : Sulfoglycolipids
siRNAs : Short interfering RNAs
sRNA : Regulatory small non-coding RNA
ss : Single-stranded
SUF : Sulfur utilization factor
T7SS : Type VII secretion system
TB : Tuberculosis
tBHP : Tert-butyl Hydroperoxide
TCA : Tricarboxylic acid
TCR : T cell receptors
TCS : Two-component system *i.e.*, sensor histidine kinase (HK), an effector response regulator (RR).

TEMED : N,N,N',N'-Tetramethylethylenediamine

TF : Transcription factor

TLR2 : Toll-like receptor 2

TSS : Transcription start site

UN : United Nations

UV : Ultraviolet

WHO : World health organization

WT : Wild-type

XDR-TB : Extreme-drug resistance