Abstract

Toxin-antitoxin (TA) systems have been implicated in bacterial cell survival during various stress conditions and help them to undergo a state of dormancy. TA systems are organized in an operon, where the gene encoding antitoxin precedes the toxin gene. These genes are co-transcribed to form a stable non-toxic protein-protein complex. The antitoxin binds the promoter-operator region and represses the transcription of the TA operon. During stress, TA systems are triggered. The labile antitoxin gets proteolytically cleaved and the toxin is set free which targets essential cellular processes such as replication and translation. This allows the bacteria to undergo a state of dormancy. As these systems targets important biological processes they serve as attractive therapeutic targets. TA systems are widely distributed in the genomes of pathogenic bacteria. *Mycobacterium tuberculosis* (Mtb), responsible for millions of death worldwide encodes 88 putative TA systems, among which 47 of them belong to VapBC TA family. Until now seven VapBC TA systems have been structurally and functionally characterized, while remaining still needs to be explored. A detailed investigation of the structures at the atomic level of putative VapBC TA system will help to elucidate their functional mechanism at the molecular level. In this work, attempts have been made to characterize mycobacterial VapBC46 TA system using NMR spectroscopy, X-ray crystallography, biochemical and biophysical methods. Genes encoding antitoxin VapB46, its N-terminal domain ($VapB46^{1-54}$) and VapC46 toxin have been cloned, overexpressed and purified. VapB46 antitoxin has a well folded N-terminal domain, while the C-terminal domain is unstructured as shown by CD and NMR spectroscopy. Size exclusion chromatography indicates that full length VapB46 exists as a tetramer while $VapB46^{1-54}$ exists as a dimer in solution. Hence it is assumed that tetramerization of VapB46 is mediated by the presence of unstructured C-terminal domain. EMSA and DNase I footprinting experiments show that VapB46 specifically binds two sites (Site1- 5'-CCAGCTCAGC-3') and (Site2- 5'-AACGCGCCGACTT-3') in the upstream promoter-operator region. Using solution NMR spectroscopy, chemical shift based secondary structure analysis reveals that $VapB46^{1-54}$ resembles Phd/YefM DNA binding domain and interacts with Site1 and Site2 DNA sequences. DNA binding residues were identified using chemical shift perturbation study. Dissociation constants calculated from the chemical shift perturbation study show that $VapB46^{1-54}$ binds Site1 DNA sequence with more affinity (K_d, ~12 μM) than Site2 (K_d, ~24 μM) DNA sequence. Chemical shift based model of $VapB46^{1-54}$ docked to Site1 DNA sequence shows that $VapB46^{1-54}$ adopts a conserved

mode of DNA recognition similar to Phd antitoxin from *Escherichia coli* P1 bacteriophage. As the yield of VapB46$^{1-54}$ was not enough for structure determination by solution NMR spectroscopy, crystallization was performed. Crystals appeared after five weeks. Mass spectroscopic analysis of the crystals reveals that VapB46$^{1-54}$ crystallized from T11 to Q54. The crystal diffracted upto 1.64 Å and the phase of the reflections was obtained by molecular replacement method. After successive refinements, the structure was solved with R_{work}/R_{free} of 0.19/0.23. The crystal structure shows a compact homotetrameic assembly, where each monomer adopts a Phd/YefM DNA binding fold with two alpha helices and three beta strands. Secondary structures obtained from the crystal structure corroborate well with the NMR spectroscopy data.

It is shown that overexpression of VapC46 toxin in *E. coli* exhibits bacteriostasis behaviour. Surface morphology analysis using SEM shows that over expression of toxic VapC46 leads to rough, crinkled and uneven cell surface. VapC46 toxin behaves as a ribonuclease in a magnesium dependent manner, and the RNase activity is inhibited by VapB46 antitoxin. *In vitro* interaction study shows that VapC46 interacts with VapB46 specifically. A three dimensional model of VapC46 shows that it possesses a conserved PIN domain architecture with four conserved acidic residues in close proximity to each other.

Keywords: *Mycobacterium tuberculosis*, toxin-antitoxin systems, VapBC46, NMR spectroscopy, X-ray crystallography, Phd/YefM, SEM, bacteriostasis