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Crystal Structure of *Mycobacterium tuberculosis* YefM Antitoxin Reveals that it is Not an Intrinsically Unstructured Protein

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Toxin–antitoxin modules are present on chromosomes of almost all free-living prokaryotes. Some are implicated to act as stress-responsive elements, among their many functional roles. The YefM–YoeB toxin–antitoxin system is present in many bacterial species, where YefM belongs to the Phd family antidote of phage P1, whereas YoeB is a homolog of the RelE toxin of the RelBE system, rather than the Doc system of phage P1. YoeB, a ribonuclease, is believed to be conformationally stable, whereas YefM has been proposed to be a member of intrinsically disordered proteins. The ribonucleolytic activity of YoeB is neutralized by YefM upon formation of the YefM–YoeB complex. We report here the crystal structure of *Mycobacterium tuberculosis* YefM from two crystal isoforms. Our crystallographic and biophysical studies reveal that YefM is not an intrinsically unfolded protein and instead forms a well-defined structure with significant secondary and tertiary structure conformations. The residues involved in core formation of the folded structure are evolutionarily conserved among many bacterial species, supporting our observation. The C-terminal end of its polypeptide is highly pliable, which adopts different conformations in different monomers. Since at the physiological level YefM controls the activity of YoeB through intricate protein–protein interactions, the conformational heterogeneity in YefM revealed by our structure suggests that these might act a master switch in controlling YoeB activity.

Keywords: antitoxin; toxin; persistence; *Mycobacterium tuberculosis*; YefM–YoeB

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Many prokaryotes have adopted the strategy of persistence in order to survive under drastically changing environmental conditions. Transcriptome of the persisters of *Escherichia coli* reveals that toxin–antitoxin (TA) modules such as *dinJ–yafQ* and *yefM–yoeB* could be the contributors of dormancy.[1] Interestingly, slow-growing bacteria carry a large number of the TA modules. For example, *Nitrosomonas europea* has 45 TA modules and *Mycobacterium tuberculosis* carries more than 35 TA modules.[2] On the other hand, *Mycobacterium smegmatis*, the fast-growing relative of *M. tuberculosis*, has only 2 TA modules.[2,3] This difference in TA numbers among the different species suggests that these modules might be correlated with the slow growth of these bacteria. Moreover, with persistence being one of the most distinguishing characteristic features of *M. tuberculosis*, study of its TA modules might offer useful insights into the molecular mechanisms of dormancy in *M. tuberculosis*.

TA modules were first discovered on low-copy-number plasmids and were proposed to be a plasmid maintenance system by postsegregational killing of the plasmid free host cell.[4–7] These modules are composed of pairs of genes present within a single operon, generally with the first gene encoding for the

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Abbreviation used: TA; toxin–antitoxin.

antitoxin and the second encoding for the toxin.[5–7] The protein products of the two genes have different life spans, with antitoxin being susceptible for proteases and toxin being a stable protein, less susceptible for proteases. Antitoxin makes a stable physical complex with its cognate toxin by direct protein–protein interactions, thereby inhibiting the toxic effect of the toxin. Toxins possess diverse activities, such as ribonucleolytic activity, act as DNA gyrase inhibitors or mimic the structure of antibiotics and inhibit translation. At the transcriptional level, the TA complex functions as a transcriptional repressor–corepressor, where the antitoxin binds directly to the DNA and the toxin assists the binding.[8–10]

Homologs of the plasmid maintenance systems have been discovered on many prokaryotic chromosomes where these have been implicated in physiological stress conditions, such as nutrient starvation and DNA damage, among others.[11–14] Broadly, the TA modules have been classified into seven categories: RelBE, MazEF, CcdAB, VapBC, HigAB, ParDE and Phd–Doc. Among these, the functional and structural features of MazEF and RelBE have been well characterized. Both MazEF and RelBE are triggered in response to nutritional starvation. RelBE has a role in stringent response, whereas MazEF has been described as a growth arrest or programmed cell death module of bacteria under stress conditions.[12–19]

The YefM–YoeB constitutes an interesting TA module in *E. coli*, where YefM belongs to the Phd antitoxin of the Phd–Doc system of phage P1, whereas YoeB is similar to RelE.[20] Although YefM has a low level of sequence homology to Phd, in an analogous manner to Phd–Doc, it forms a heterotrimeric complex with YoeB. The YefM–YoeB system is known to get activated under amino acid starvation conditions and is involved in Lon protease-dependent translation inhibition.[21] Previous studies have described YefM to be an intrinsically unstructured protein with the assumption that an unstructured linear determinant of YefM, rather than a conformational one, is recognized by the toxin.[22] The structure of YoeB toxin in free form and its complex with YefM antitoxin have been elucidated where it has been shown that it shares a common fold with RelE.[20] The structure of the complex has revealed that the C-terminus of YefM interacts with YoeB and that the stoichiometry of complex formation is $YefM_2$–YoeB. The YefM dimer has a symmetrical N-terminal globular structure and an extended C-terminal region.[20]

Currently, free antitoxin structure is not available for any of the TA systems. Conflicting reports indicate that YefM might be either totally disordered[22] or partially ordered in certain species.[23,24] Conformational transitions required for the association and dissociation of the YefM–YoeB complex are also not understood due to the lack of knowledge on antitoxin structures. In this study, we report the structure of the free YefM antitoxin of *M. tuberculosis* fortuitously crystallized in two conditions.

Structure determination and refinement

Typically, 30 mg of homogenous YefM–YoeB complex was obtained from 1 l of culture pellet. Crystals of the purified complex were obtained under two conditions (named crystal I and crystal II henceforth) where the native crystals appeared in 24 h and grew to a final size of 0.2 mm × 0.2 mm × 0.2 mm in 4 days. Both of these belonged to the same crystal form with the space group $P2_12_12_1$. Se-Met crystals appeared in 3 days, attained their optimum size in around 15 days and belonged to the $P4_32_12$ space group. Crystal I diffracted up to 2.5-Å resolution, whereas crystal II diffracted up to 2.14-Å resolution (Table 1).

Matthew's coefficient for crystal I suggested the presence of 3 or 4 molecules per asymmetric unit, indicating the presence of a heterotrimer of TA complex in the crystal asymmetric unit. The initial model was obtained by single-wavelength anomalous dispersion phasing of the Se-Met crystals, and this model was used to determine the structure of crystal I by molecular replacement. Although the TA complex protein was used for crystallization, its structure revealed that only the YefM antitoxin molecules were present in the crystals. The values of R_{cryst} and R_{free}

Table 1. Data collection statistics (numbers in parentheses represent the value for the highest-resolution shell)

Crystallographic data	Native antitoxin (crystal I)	Native antitoxin (crystal II)	Se-Met antitoxin
Unit cell parameters (Å)			
a	65.0	64.8	65.0
b	64.6	64.8	65.0
c	83.5	83.5	83.0
Space group	$P2_12_12_1$	$P2_12_12_1$	$P4_32_12$
Resolution range (Å)	41.77–2.50	30.86–2.14	51.23–3.5
Completeness (%)	98.2 (97.7)	98.7 (93.9)	100 (100)
Mean $I/\sigma(I)$	22.3 (8.58)	24.6 (3.8)	33.3 (13.5)
R_{merge} (%)	5.9 (18.8)	5.7 (44.8)	8.1 (26.4)
Multiplicity	4.9	4.9	25.6
Anomalous completeness (%)			100 (100)
Anomalous multiplicity			15 (15)

$R_{merge} = \sum | I_i - I_m | / \sum I_i$, where I_i is the intensity of the measured reflection and I_m is the mean intensity of all symmetry-related reflections.