A NusG paralogue from *Mycobacterium tuberculosis*, Rv0639, has evolved to interact with ribosomal protein S10 (Rv0700) but not to function as a transcription elongation–termination factor

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NusG, a well-conserved protein in all the three forms of life, is involved in transcription elongation and termination, as well as in the process of transcription–translation coupling. The existence of species-specific functional, as well as conformational, divergences in NusG makes it an attractive transcription factor to study, especially if it originates from a pathogen. Here, we report functional and conformational characterizations of the *Mycobacterium tuberculosis* (Mtb) protein Rv0639 that has been annotated as a homologue of *Escherichia coli* NusG. Rv0639 failed to complement the *in vivo* functions of *E. coli* NusG (Ec NusG) and did not exhibit any signature of a transcription elongation–termination factor. However, it retained the ability to bind to its cognate ribosomal protein S10 (Rv0700). Compared with Ec NusG, Rv0639 possesses unique conformational features characterized by altered secondary structures in the C-terminal domain (CTD), an unusually long and disordered linker region between the N-terminal domain (NTD) and CTD, and a folding of its NTD over its CTD. This unusual folded conformation could have imparted specialized functions to this protein, required to adapt the physiology of Mtb. We speculate that in the absence of a bona fide RfaH, a NusG paralogue that is involved in pathogenicity in *E. coli*, Rv0639 functions as an RfaH-like factor and is involved in pathogenicity using unidentified opslike sequences in the Mtb genome. And hence, we reannotate Rv0639 as a paralogue of NusG, instead of a homologue.

### INTRODUCTION

The NusG (in bacteria)/Spt5 (in archaea and eukarya) family of transcription regulators are conserved in all the three forms of life. *Escherichia coli* NusG (Ec NusG) loads onto the transcription elongation complex (EC) after the release of sigma factors and remains with it until the end of the transcription cycle (Yakhnin & Babitzke, 2014; Tomar & Artsimovitch, 2013). NusG increases the elongation rate both *in vitro* (Burns et al., 1998) and *in vivo* (Zellars & Squires, 1999), and prevents backtracking of the EC at certain pause sites (Artsimovitch & Landick, 2000). It is an important factor in the transcription antitermination complex formed by both ribosomal- (Squires et al., 1993) and bacteriophage-derived (Weisberg & Gottesman, 1999) N-anti-termination machineries. Being bound to the EC, it facilitates bacterial transcription termination by interacting with the terminator, Rho (Li et al., 1993), and also couples translation with transcription by binding to the ribosomal protein S10 (Burmann et al., 2010).

*E. coli* NusG is a 21 kDa monomeric protein having two functionally independent domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), connected via a flexible linker (Yakhnin & Babitzke, 2014). The NTD comprises three α-helices and four β-strands, whereas the CTD contains five β-strands forming an anti-parallel β-barrel structure (Mooney et al., 2009; Fig. 1b,c). The NTD interacts with the β-clamp helices of the RNA polymerase (Mooney et al., 2009), whereas the CTD interacts with Rho and ribosomal protein S10 (Li et al., 1993; Chalissery et al., 2011). The motif present in its CTD (Kyrpides et al., 1996) is predicted to interact with different protein domains, and its number may determine various interacting partners of NusG-CTD.

While most of the *in vivo* and *in vitro* studies of this protein were based on Ec NusG, a few of its variants have been...
Fig. 1. Domain organization, structural homology models and in vivo complementation assays of Rv0639. (a) Domain organization of Rv0639 was obtained using pBLAST. (b, c) Three homology models of Rv0639 were generated using the crystal structure of *Aquifex aeolicus* NusG (PDB: 1npp for model i and 1npr for model ii) and the NMR structure of *Thermotoga maritima* NusG (PDB: 2LQ8 for model iii). NMR structures of the NTD (PDB: 2K06) and CTD (PDB: 2JVV) of Ec NusG are shown in the same orientation as model ii of Rv0639. The homology models were obtained by submitting the amino acid sequence of Rv0639 to an automated protein modelling server, SWISS-MODEL (swissmodel.expasy.org/workspace). The structures were made by PyMol software. (d) In vivo termination assays of Rv0639 and Ec NusG. \(\beta\)-Galactosidase activities of lacZ reporter fused to \(t_{ac}\) terminator of different MC4100*nusG G146D* strains transformed with pHYD3011 plasmids expressing the indicated proteins. AU, arbitrary units. (e) *E. coli* MG1655 \((rac^+\) and \(rac^-\) strains having the pHYD3011 plasmid
expressing the indicated proteins were transduced with a nusG::kanR cassette by P1 transduction. Transductants on LB-kanamycin plates are shown. (f) MG1655 rac+ and rac- strains were transformed with pHYD3011 plasmid expressing either full-length (FL) or NTD fragments of Rv0639 or E. coli NusG, and overnight cultures were spotted onto 0 and 0.2% arabinose plates. Vector denotes empty pHYD3011.

described recently. RfaH, a parologue of NusG, has evolved functionally and structurally to load onto RNAP only at specialized sequences, called ops (operon polarity suppressor), and is not capable of binding to Rho but can interact with S10 (Belogurov et al., 2009; Burmann et al., 2012). Bacillus subtilis NusG can stimulate pauses at the untranslated regions of the trp operon (Yakhnin et al., 2008), which contrasts with the anti-pausing properties of E. coli NusG. In a recent report, Mycobacterium bovis NusG was shown to stimulate the hairpin-dependent transcription terminations in vitro (Czyz et al., 2014). These examples predict the existence of functional, as well as conformational, divergences in NusG depending on the lifestyle of the species, especially in the pathogens.

Previously, using E. coli RNAP, we have reported that the transcription factor, Rho, from Mycobacterium tuberculosis (MtB) does not require NusG in in vitro transcription reactions (Kalarickal et al., 2010). Also, from sequence analyses of the MtB genome, we noticed that it does not have a RfaH homologue (Tuberculist database: http://tuberculist.epfl.ch). These led us to postulate that Rv0639 may not be a canonical NusG, but could possess specialized functions; hence, we undertook its characterization.

Here, we report functional and conformational characterization of the protein Rv0639, which has been annotated as a homologue of E. coli NusG (Ec NusG; Tuberculist database). Rv0639 failed to complement the in vivo functions of Ec NusG, was not able to bind either to MtB or E. coli Rho, and was not capable of enhancing the elongation rate of MtB RNAP. However, it retains the ability to bind the ribosomal protein, S10 (Rv0700). Compared with E. coli NusG CTD, its CTD comprised a unique secondary structure that is trypsin resistant and its 50 aa extra NTD region appeared to fold over its CTD. Rv0639 also possesses a long and highly disordered linker region that is likely to produce a higher degree of rotational freedom to the NTD and CTD about the linker axis. This unusually folded conformation could have imparted specialized functions to this NusG parologue (rather than homologue), adapted for the physiology of MtB. We speculate that in the absence of a bona fide RfaH, Rv0639 functions as an RfaH-like factor and is involved in pathogenicity using unidentified ops-like sequences in the MtB genome.

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<td><strong>Strains and plasmids.</strong> These are described in Table 1.</td>
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**In vivo complementation assays.** MG1655rac+ and rac- (RS862) strains were transformed with a modified pBAD plasmid (pHYD3011) expressing either Ec NusG or Rv0639 from the pBAD promoter. Subsequently, the chromosomal nusG in both strains was deleted by P1 (nusG::kanR) transduction. Transductants were plated on LB plates supplemented with appropriate antibiotics. Under this condition, the transductants will grow only if a functional nusG is expressed.

To check the in vivo complementation of the Rho-dependent terminations in E. coli, pHYD3011 expressing either Ec NusG or Rv0639 was used to transform the MC4100 rac- strain having a tem terminator cassette as a lysogen and a nusG mutation (G146D) inserted into its chromosome (RS1514; Pr35×racR1tem- lacZYA). Transformants were plated on LB plates with appropriate antibiotic. Six colonies from each of the above-mentioned transductants were streaked on LB plates and were subsequently used to measure β-galactosidase activity, following published methods (Shashni et al., 2014).

For the NusG-NTD toxicity assays, MG1655rac+ strain was transformed with pHYD3011 expressing either MtB NusG-NTD or E. coli NusG-NTD. From each transformed plate, two colonies were streaked on LB plates and then single colonies were inoculated for overnight cultures. Serial dilutions of the overnight cultures were spotted on LB with or without 0.2% arabinose.

**Cloning of WT Rv0639 and its different derivatives.** The Rv0639 gene was PCR amplified from a cosmid library of the MtB genome, and was cloned at the Xhol–NdeI site of pET28b and pET21b to make different tagged derivatives. For in vivo assays, the WT and NTD fragments of Rv0639 were cloned at the NdeI–SalI sites of pHYD3011.

To make the deletion derivatives of Rv0639, DNA fragments with Δ20, Δ30, Δ50 and Δ184 deletions were prepared by PCR and were cloned at the Xhol–NdeI site of the pET21b vector. Cysteine derivatives S44C, S44C-S190C and S44C-S220C of Rv0639 were made by site-directed mutagenesis. WT Rv0639 is a cysteine-less protein.

**Purification of His-tagged and non-His-tagged Rv0639 proteins.** His-tagged Rv0639 proteins were purified using Ni-NTA beads (Qiagen) as per the manufacturer’s protocol. The elution fraction was dialysed against storage buffer [10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM DTT, 150 mM NaCl, 5 % (v/v) glycerol], and was concentrated and stored in the presence of 50 % (v/v) glycerol.

To purify non-His Rv0639 proteins, before applying the crude protein mixture to different columns we followed the procedures described elsewhere for the purification of non-His MtB Rho protein (Kalarickal et al., 2010). Following ammonium sulphate precipitation, protein was passed through a Resource Q column (GE Healthcare). The elution fractions containing Rv0639 were pooled and dialysed against TGED buffer. The protein was then passed through a Resource-S column (GE Healthcare), and Rv0639 was collected from the flow-through fractions. It was further purified by passing through Superdex 75 (GE Healthcare). The fractions containing pure Rv0639 were concentrated by Amicon YM-10 and stored in the storage buffer [20 mM Tris/HCl (pH 7.9), 0.1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, and 50 % (v/v) glycerol].
at the XhoI–NdeI site of pET33b. These were then purified using Ni-NTA beads (Qiagen) using buffers containing 20 mM β-mercaptoethanol. The purified protein was stored in a buffer containing 10 mM Tris pH 8.0, 0.1 mM EDTA, 20 mM DTT, 0.4 M NaCl and 50% (v/v) glycerol.

**In vitro Rho-NusG pull-down assays.** In vitro pull-down assays were performed following procedures described elsewhere (Kalarickal et al., 2010).

**In vivo NusG-S10 pull-down assays.** Plasmids pET28 (kan<sup>R</sup>), expressing S10 proteins, and pET21b (amp<sup>R</sup>), expressing NusG, were co-transformed into *E. coli* BL21(DE3) strain. S10 proteins were His-tagged at the N-terminus. Transformants were inoculated in 50 ml LB medium and grown at 37°C until the OD<sub>600</sub> reached ~0.4 were then induced with 0.1 mM IPTG for protein expression, and the induction was continued for 3 h. The rest of the procedures for the pull-down assay were performed by following published methods (Pani et al., 2009).

**CD spectroscopy.** CD spectroscopy of WT Rv0639 and Ec NusG, together with their different derivatives, was performed using a JASCO 810 spectro-polarimeter at 25°C. Scanning was done from 200 to 400 nm.
250 to 200 nm in a cuvette with 0.1 cm path length. The following settings were used for the measurements: 1 nm data pitch, 2 nm bandwidth, 2 s response time and 50 nm min⁻¹ scan speed. All the recordings were made in phosphate buffer (pH 7.0; 20 mM sodium phosphate, 50 mM NaCl). The estimations of secondary structures were carried out according to a previously published method (Yang et al., 1986), inbuilt in the CD software.

**Gluteraldehyde cross-linking.** Intra-molecular cross-linking reactions were performed following the procedures described by Kalarickal et al. (2010). For intermolecular gluteraldehyde cross-linking, the reaction conditions were the same as above, except that the HMK-tagged Rv0639 was radiolabelled with γ-ATP and was desalted. To that, Rv0700 was added and the cross-linking was performed. After running the samples on 12 % SDS-PAGE gels, the gels were scanned in a phosphorimager.

**Gel filtration.** Size-exclusion chromatography was performed using a Superdex 200 PC (Kalarickal et al., 2010) and an AKTA (GE Healthcare) protein purification system, in buffer containing 20 mM Tris/HCl (pH 8.0) and 100 mM NaCl at 25 °C. The flow rate for each run was 0.04 ml min⁻¹ and the sample volume was 100 µl (50 µg protein). Elution profile of protein markers (Sigma) were used to obtain the calibration curve. To check the NusG–NusE complex formation by gel-filtration technique, both proteins were incubated at 25 °C for 10 min prior to application to the above column, and the separation procedures were the same as above.

**In vitro Rho-dependent transcription termination assays.** The T7A1-X5g template was PCR amplified from the plasmid pRS604 (Pani et al., 2009) using primers RS58 and RS333. Reactions were performed in E. coli transcription buffer [25 mM Tris/HCl (pH 8.0), 5 mM MgCl₂, 50 mM KCl, 1 mM DTT and 0.1 mg BSA ml⁻¹] at 37 °C. In the case of Mtb, RNA polymerase reactions were carried out in the Mtb transcription buffer [45 mM Tris/HCl (pH 8.0), 5 mM MgCl₂, 70 mM KCl, 1 mM DTT, 10 % (v/v) glycerol and 1.5 mM MnCl₂] at 37 °C. The reactions were initiated with 5 nM DNA template, 25 nM E. coli RNA polymerase or 50 nM Mtb RNA polymerase, 175 mM AmU, 5 µM each of GTP and ATP, and 2.5 µM CTP to make a 23-mer EC. [α-³²P]CTP [10 µCi ml⁻¹; with a specific activity of ~3000 Ci mmol⁻¹ (111 TBq mmol⁻¹)] was added to the reaction to radiolabel the EC23. The complex was chased with 20 µM NTPs in the presence of 10 µg rifampicin ml⁻¹ for 10 min at 37 °C. When required, 50 nM E. coli Rho or 100 nM Mtb Rho and 200 nM of either E. coli or Mtb NusG were added to the chase solution.

**In vitro transcription elongation assays.** For the transcription elongation assays, the same template as mentioned above was used. Also, the same Ec and Mtb transcription reaction buffers as mentioned above were used in these cases. Radiolabelled EC23 was prepared in the same way as described above, and was then chased with 20 µM NTPs in the presence of 10 µg rifampicin ml⁻¹ and 200 nM E. coli NusG or Mtb NusG, to measure the general elongation rates. In case of elongation through the opa pause sequence, the EC23 was chased in the presence of 100 µM each of UTP, CTP and ATP, and 10 µM GTP. Aliquots (5 µl) were removed at the indicated time points and mixed with an equal volume of formamide loading dye. Products were analysed on an 8 % sequencing gel and exposed to a phosphorimager screen, which was scanned using a Fujifilm phosphorimager. The transcript intensities were quantified using Image QuantTL software. The fraction of full-length RNA at different time points was calculated by measuring the band intensity of full-length transcript (RO) divided by total intensity in each lane from position 23 to the full-length transcript (RO).

**Copper phenanthroline (Cu-P) cross-linking.** These experiments were performed following published procedures (Pani et al., 2009).

**Footprinting experiment.** Mtb and E. coli NusG were subjected to limited proteolysis by trypsin and V8. For this purpose, C-terminal HMK-tagged Rv0639 and Ec NusG proteins were purified using Ni-NTA columns. These proteins were end-labelled with [³²P]ATP [10 µCi ml⁻¹; with a specific activity of ~3000 Ci mmol⁻¹ (111 TBq mmol⁻¹)] by phosphorilating the serine residue in the HMK-tag sequence using protein kinase A (Sigma). Both labelled proteins were proteolysed by trypsin and V8. The amounts of trypsin and V8 used were 0.0006 and 0.0003 U µl⁻¹, respectively, and the incubation times were 15 and 30s, respectively. Proteolysis reactions were performed in transcription buffer [250 mM Tris/HCl (pH 8.0), 500 mM KCl and 50 mM MgCl₂].

**Site-specific chemical cross-linking.** Surface-exposed primary amines of Rv0639 and Ec NusG having no cysteine were first labelled with SPDP. Concentrations of NusG and cross-linker were 10 and 50 µM, respectively, and they were mixed in phosphate buffer (100 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA, pH 7.5) and incubated at 25 °C for 30 min. After incubation, the reaction mixture was passed through a protein desalting column (Pierce) to remove excess SPDP. HMK-tagged WT and C50S Mtb NusE (Rv0700) proteins were end-labelled with [³²P]ATP [10 µCi ml⁻¹; with a specific activity of ~3000 Ci mmol⁻¹] before performing cross-linking reaction with SPDP derivatives of Rv0639 and Ec NusG. Final concentrations of NusG and Rv0700 were ~0.5 and ~2.5 µM. The cross-linking reactions were performed in the same phosphate buffer and incubated for 2 and 5 min at 37 °C. Non-reducing SDS-sample buffer was added to the reactions, and they were loaded onto a 12 % polyacrylamide gel for non-reducing PAGE and the products were analysed with phosphorimager.

To measure the dissociation constant (Kd), the same cross-linking experiment was repeated by mixing ~0.5 µM radiolabelled Rv0700 and increasing concentrations (0.25–5.0 µM) of SPDP-labelled NusG. The fraction of cross-linked species was plotted against the concentration of SPDP-labelled NusG. The binding curve was fitted to the hyperbolic equation of the form: $y = a x / (b + x)$, where $b = K_d$ and ‘a’ is the amplitude of the curve. Error bars were calculated from three independent measurements.

**Site-specific chemical cross-linking in the presence of Mycobacterium smegmatis cell lysate.** M. smegmatis, mc²155, was grown in 7H9 medium supplemented with 10 % (v/v) glycerol, 10 % ADC (albumin dextrose catalase), 0.05 % Tween 80, 50 µg carbencillin ml⁻¹ and 10 µg cycloheximide ml⁻¹ at 37 °C for 48 h to obtain a saturating culture. From this culture, 500 µl was subcultured in 50 ml 7H9 media together with the above-mentioned components except Tween 80. The culture was grown at 37 °C until its OD₆₀₀ reached ~0.9 and then the cells were pelleted down by centrifuging at 6000 r.p.m. for 10 min at 4 °C. The pellet thus obtained was suspended in 5 ml lysis buffer (100 mM NaH₂PO₄, 150 mM NaCl and 1 mM EDTA, pH 7.5). Cells were lysed by sonicator along with glass beads, and centrifuged at 20217 g for 30 min at 4 °C to obtain the lysate. Different amounts of this lysate were mixed separately with either 0.5 µM radiolabelled WT or C50S Rv0700 and 50 µM SPDP-labelled Rv0639, and incubated at 37 °C for 3 min. Non-reducing SDS-sample buffer was added to the reactions, they were loaded onto a 12 % polyacrylamide gel for non-reducing PAGE and the products were analysed with phosphorimager.

**RESULTS**

**Rv0639 does not functionally complement E. coli NusG**

A pBLAST analysis of 238 aa of Rv0639 revealed that it contains an extra N-terminal region in addition to the
conserved NGN and KOW motifs present in all the NusG/SpT5 family of proteins (Fig. 1a). This extra N-terminal region is present in most of the mycobacterial NusG homologues and is predicted to form a homo–dimer interface. However, the sequence conservation of this region is poor among the mycobacterial NusGs as compared with more conserved NGN and KOW domains (data not shown).

Three structural homology models of Rv0639 could be obtained using the co-ordinates of the crystal and NMR structures of NusG from other bacteria (Fig. 1b). Grossly these structures have similarities with the 3D structure of Ec NusG, characterized by two specific globular domains, the NTD and the CTD, connected by an unstructured linker region (Fig. 1c). However, in the predicted structures of Rv0639, the linker region is longer and the stem part of NTD is more disordered compared with Ec NusG. Also, the orientations of the CTD β-sheets are different from those of Ec NusG. The predicted domain and structural analyses suggest that Rv0639 is a NusG homologue.

To test this prediction, we at first tested the in vivo functions of Rv0639 in E. coli. We measured the ability of this protein to suppress the defects of Rho-dependent termination of a well-known Ec NusG mutant, G146D, which is defective for Rho binding (Chalissery et al., 2011). We used a MC4100 strain having G146D nusG in the chromosome, and a lacZ reporter cassette fused downstream of a Rho-dependent terminator, trac. This terminator was recently shown to be highly dependent on NusG (Shashni et al., 2014). This strain was transformed with pHYD3011 expressing either Rv0639 or Ec NusG, and the β-galactosidase activities were measured (Fig. 1d). Due to the presence of the G146D mutant of Ec NusG, this activity will be high and the defect will be suppressed only in the presence of an exogenous functional NusG. As expected, WT Ec NusG suppressed the defect, but in spite of having a structural homology with Ec NusG, Rv0639 failed to suppress the defect of G146D NusG.

We next checked whether the growth defect of MG1655 strains [either having a rac prophage (rac+) or not (rac−)] caused by the deletion of the chromosomal copy of nusG could be suppressed by Rv0639 (Fig. 1e). The presence of a rac prophage in this strain makes it more dependent on Rho-dependent termination, which is required to suppress the expressions of toxic genes from this prophage (Shashni et al., 2014; Cardinale et al., 2008). We observed that the transductants could be observed when the nusG::kanR cassette was P1 transduced in the presence of WT Ec NusG expressed from pHYD3011, whereas expression of Rv0639 was unable to produce these transductants. So Rv0639 cannot replace the functional Ec NusG.

Overexpression of the NTD fragment of Ec NusG (residues 1–123) is capable of replacing WT NusG bound to the EC, which prevents Rho from interacting with the EC-bound NusG, and thereby causes a termination defect as well as lethality (Mooney et al., 2009). We tested whether overexpression of Rv0639-NTD (residues 1–172) is capable of producing this toxicity. Both NTD fragments of Ec NusG and Rv0639 were overexpressed from an arabinose-inducible pBAD promoter. The NTD fragment of Ec NusG caused toxicity in the presence of 0.2% arabinose (Fig. 1f), whereas the same fragment of Rv0639 did not show any effect.

The aforementioned results strongly indicated that Rv0639 was not capable of complementing the functions of Ec NusG under the in vivo conditions of E. coli, with no participation in the latter’s Rho-dependent termination process. However, these data do not rule out the possibility of its role in Rho-dependent termination under the in vivo conditions of Mycobacterium.

Rv0639 protein

The highly unusual behaviour of Rv0639 led us to biochemically characterize the protein. The protein sequence is given in Fig. S1 (available in the online Supplementary Material). We cloned and purified the full-length protein and different N-terminal deletion derivatives devoid of different parts of the N-terminal region. We observed that the full-length Rv0639 migrates like a 35 kDa protein even though it is 27 kDa (Fig. 2a, left panel). This anomalous mobility was also observed for its N-terminal deletion derivatives (Fig. 2a, right panel). This indicates that either Rv0639 has an unusual shape or it occurred due to suboptimal binding of SDS during denaturation.

To further probe the unusual hydrodynamic volume, we checked the elution profile of Rv0639 using a gel-filtration column and compared the same to Ec NusG (Fig. 2b). While the elution profile of Ec NusG was consistent with its molecular mass, Rv0639 eluted as a 42 kDa protein, which indicates that the latter either has a significantly bigger hydrodynamic volume than predicted by its molecular mass or exists as a higher oligomer in solution. However, glutaraldehyde cross-linking (Fig. 2c) revealed that, like Ec NusG, Rv0639 also exists as a monomer.

Next, we compared the secondary structure contents of Rv0639 to Ec NusG from CD spectra. Rv0639 appeared to have more β-sheet content and was composed of a less disordered region compared with Ec NusG; hence, it was likely to have a more compact structure (Fig. 2d). Larger hydrodynamic volume and more structured conformation suggest a significantly different conformation of Rv0639 compared with its E. coli counterpart, even though the homology models predict similar structures between them.

Extra NTD domain of Rv0639

So far we have observed that Rv0639 has not only altered function but also significant differences in structure as compared with Ec NusG, which led us to probe the importance of the extra N-terminal 50 aa region characteristic of mycobacterial NusGs. We deleted 20 (Δ20), 30 (Δ30) or 50 (Δ50) amino acids sequentially from the N-terminus...
**Fig. 2.** Biochemical properties of purified Rv0639 protein. (a) SDS-PAGE gels showing anomalous migration of purified Rv0639 and its different deletion derivatives. Actual molecular masses of different fragments are indicated above each lane. (b) Size exclusion chromatography showing the elution profiles of Ec NusG and Rv0639 obtained using Superdex 200 PC (3.2/30) gel-filtration columns. Elution volumes are indicated on the x-axis. Molecular mass values corresponding to the peaks were estimated from the calibration curve derived from the molecular mass markers. (c) Migration pattern of glutaraldehyde cross-linked Ec NusG and Rv0639 on SDS-PAGE gels. Time (0, 15, 30, 60, 90 and 120 s) indicates the duration of cross-linking reactions. Each lane contains 5 μg protein. (d) Far-UV CD spectra of Ec NusG and Rv0639. The estimation of the secondary structure was carried out by the software supplied with the machine. M, molecular mass markers.
of Rv0639 to generate intermediary length constructs. We observed that deletions in this region decreased the solubility of the protein, as was evidenced from the appearance of the majority of the protein in the pellet (P) fractions as compared with the full-length one (Fig. 3a). It is possible that this region of all the mycobacterial NusG homologues is required to mask some of the solution exposed hydrophobic surfaces.

It has been reported that the NTD of E. coli RfaH (a NusG paralogue) masks the hydrophobic interface of its CTD (Burmann et al., 2012). We hypothesized that this N-terminal extra region of Rv0639 might fold over the other part of the protein to improve the solubility. To test this, we engineered cysteine pairs, one each in the extra N-terminal region and in CTD, 44C-190C and 44C-220C, respectively, in Rv0639 (Fig. 3b), and di-sulphide bridges (Cys–Cys) were induced between them using an oxidizing agent, Cu-P (Chalissery et al., 2011). Cysteine residues were introduced in the place of serines. These bridges will form only when the distance between the two cysteines is within 6 Å. We observed two kinds of disulphide-bonded species (Fig. 3c): one migrating as ~95 kDa, which is likely to be a dimer of Rv0639, formed due to inter-cysteine linkages (dimer), and a ~25 kDa species migrating faster than the un-cross-linked Rv0639 (C-C X-linked). The latter formed due to the intra-molecular Cys–Cys disulphide formation.

**Fig. 3.** The extra N-terminal region. (a) In vivo solubility profiles of full-length (FL) and different N-terminal deletion derivatives (20 and 50 aa deletions). Pellet (P) and supernatant (S) fractions of the cell lysate were loaded after separating these fractions by centrifugation. (b) Position of cysteines in different derivatives of Rv0639 shown on its homology model. Serine residues were changed to cysteine to minimize the change. The location of Cys 44 (44C) is on the extra N-terminal region of Rv0639, shown as a solid curve. (c) Intra-molecular Cu-P cross-linking between cysteine pairs, 44C-190C and 44C-220C performed under different conditions as indicated. Different products are indicated, and they were identified based on their molecular mass determined from molecular mass markers. (d) Cartoon showing that the N-terminal extra region of Rv0639 is folded over its CTD, where 44C comes within Cu-P cross-linking distances with 190C and 220C located in the CTD. The extra region is shown as a curved line. M, molecular mass markers.
between the 44C-190C and 44C-220C pairs. These species disappear in the presence of the reducing agent, DTT; hence, they were disulphide linked.

Formation of disulphide linkages between 44C, an N-terminal residue, and those from CTD strongly indicates that the extra N-terminal region folds over the globular CTD in Rv0639 (Fig. 3d), which echoes the ‘auto-inhibited’ state of E. coli RfaH (Burmann et al., 2012) and T. maritima NusG (Drögemüller et al., 2013).

**Rv0639 is not capable of binding to Rho**

As NusG-CTD interacts with Rho (Burmann et al., 2010; Chalissery et al., 2011) and facilitates the latter’s termination function (Burns et al., 1998; Chalissery et al., 2007), the failure of Rv0639 to complement the in vivo functions of Ec NusG (Fig. 1), and the folding of its NTD over its CTD to form a putative auto-inhibited state (Fig. 3d), led us to question its capability of interacting with the transcription terminator, Rho. We immobilized His-tagged derivatives of either full-length Rv0639 or CTD-Rv0639 on Ni-NTA agarose beads, and passed over them solutions of either Mtb or E. coli non-His-tagged Rho. For comparison, E. coli Rho solution was also passed over immobilized Ec NusG-CTD (Fig. 4a). In these assays, we consistently observed that >80% E. coli Rho molecules remain associated with Ec NusG-CTD (Fig. 4a, right-most panel; Chalissery et al., 2011); hence, the amount of Rho eluted with His-tagged Rv0639 or NusG will provide the measure of Rho-Rv0639 or Ec NusG binding. We observed that association of Rho proteins from both E. coli and Mtb with either full-length Rv0639 or its CTD (185–238 amino acids) was much less obvious. These data strongly suggest that neither Mtb nor E. coli Rho can interact with the full-length Rv0639 or CTD-Rv0639.

The above method of probing the interaction requires stable binding between the partners. Hence, it is possible that Rv0639–Rho interaction could not be detected due to their weak interactions, and the stability of complex formation may improve in the presence of elongating RNAP. Interaction of NusG-NTD with the RNAP (Mooney et al., 2009) induced more stable interaction between its CTD and Rho (Burmann et al., 2010; Chalissery et al., 2011). We performed in vitro transcription termination assays with RNAPs from both E. coli and Mtb, in the presence of Rho and NusG proteins from both these bacteria (Fig. 4b). The transcription reaction was initiated from a strong E. coli promoter, T7A1, and the elongation was allowed to go through the termination zone of a well-known terminator of E. coli Rho, ς₃₉. Interestingly, Mtb RNAP and Rho were observed to utilize the E. coli promoter and the terminator very efficiently (Fig. 4b). E. coli and Mtb Rho proteins terminated the elongation of both RNAPs very efficiently (Fig. 4b, lanes 2, 4, 7 and 9). However, the termination pattern was different for each of the RNAPs, which could be due to either the variable elongation rates of RNAP, a different translocation speed of Rho or differential ways of utilizing the terminator sequences. Mtb Rho induced early termination for both RNAPs (Fig. 4b, lanes 4 and 9; Kalarickal et al., 2010). The facilitation of Rho-induced RNA release by NusG, due to their interactions, was manifested as early termination in in vitro transcription assays (Burns et al., 1998, Chalissery et al., 2007). Consistent with this fact, Ec NusG was observed to induce early termination not only for E. coli RNAP (Fig. 4b, lane 3) but also for its Mtb counterpart (Fig. 4b, lane 8). However, Rv0639 failed to bring further forward the termination zone by Mtb Rho (Fig. 4b, lanes 5 and 10), which indicates that Rv0639 is unable to facilitate the termination process by interacting with its cognate Rho. Based on these results and those reported by us previously on Mtb Rho (Kalarickal et al., 2010), we can now more convincingly suggest that unlike E. coli Rho, Mtb Rho can function without the help of a NusG homologue, and Rv0639 has evolved not to interact with Rho.

**Interaction of Rv0639 with transcription EC**

The NTD of Ec NusG interacts with the clamp helix domain of the β'-subunit of elongating RNAP and increases the transcription elongation rate by suppressing pauses via stabilizing the closed conformation of the RNAP clamp domain (Weixlbaumer et al., 2013). In vitro elongation rate enhancement by NusG is also a measure of NusG–EC interaction. We checked whether Rv0639 is capable of increasing the elongation rate of Ec or Mtb RNAP. We initiated transcription of both the RNAPs from the T7A1 promoter using a DNA template coding for a 23 nt U-less sequence, so that by withdrawing UTP from the reactions one can make an EC₂₃₁-mer complex (Fig. 5a). This complex was chased with all the NTPs in the absence and presence of either Ec NusG or Rv0639 and samples were removed at different time points (Fig. 5b–e). We observed the following: (i) Ec RNAP did not exhibit significant pause or arrest on this template, whereas the elongation rate of Mtb RNAP was quite slow (compare the plots in Fig. 5d, e) and it paused and became arrested at several positions of this template (Fig. 5c); (ii) the elongation rate of both RNAPs increased in the presence of Ec NusG, which was evident from the early formation of full-length RNA (RO; see the plots in Fig. 5d, e); and (iii) this enhancement of elongation rate of either of the RNAPs was not observed in the presence of Rv0639, rather it moderately slowed Mtb RNAP (compare the plots). These results indicate that Rv0639 has a different mode of interaction with the EC. Either its property of enhancing elongation rate is very specific to some unknown sequences of Mtb or, like B. subtilis NusG (Yakhnin et al., 2008), Rv0639 induces pauses rather than suppressing it.

**Rv0639 forms a stable complex with its cognate NusE**

NusG has been shown to form a complex with the ribosomal protein NusE (S10), and the structure of the
complex was solved by NMR (Burmann et al., 2010), which suggests a new mode of transcription–translation coupling in bacteria. We tested the existence of this property of Rv0639. For this purpose, we cloned and purified Rv0700 protein, a S10 homologue of Mtb. The recombinant Rv0700 protein was purified, its interaction with Mtb NusB (Rv2533c) having been studied elsewhere (Gopal et al., 2001).

Initially we tested the complex formation by following the elution profiles of Rv0639 and Rv0700 separately and when mixed together using gel-filtration techniques at room temperature (25 °C). The elution peaks of free Rv0639 and Rv0700 were at 1.63 and 2.39 ml, respectively (Fig. 6a). The presence of each of the proteins was further confirmed by running these elution fractions in SDS-PAGE gels (Fig. 6b). When both were mixed in a 1:3 ratio (NusG:NusE), a single elution peak at 1.6 ml was observed. Composition of this peak fraction, as observed in SDS-PAGE, revealed the presence of both Rv0639 and Rv0700, which suggests a

**Fig. 4.** (a) In vitro pull-down assays for various combinations of full-length and CTD domain His-tagged Ec NusG and Rv0639, and full-length non-His-tagged Rho. In the ‘–’ NusG panels Rho was incubated with only Ni-NTA beads and it demonstrates the extent of non-specific adsorption. FT, W and E denote flow-through, wash and eluted fractions, respectively. Different proteins are indicated. M, molecular mass markers. (b) Autoradiograms showing the in vitro Rho-dependent transcription termination assays in the presence and absence of NusG using both E. coli and Mtb RNAP. Different combinations of Rho and NusG from E. coli and Mtb were used as indicated. The cartoon drawn above the autoradiograms indicates that the transcription was initiated from the T7A1 promoter and proceeded through the tR1 termination region. Termination zones are indicated by dashed lines adjacent to the lanes.
complex formation between the two proteins. Interestingly, the complex eluted even earlier than the free Rv0639, which indicates that it has a very compact shape with low hydrodynamic volume. In a published study, the Rv0700 protein was noticed to be unfolded at lower temperatures (Gopal et al., 2001), so we checked its secondary structure contents at different temperatures by CD (data not shown) and found that they were not significantly different at 25 °C (room temperature) and 30 °C.

To further confirm the complex formation between Rv0639 and Rv0700, we employed SPDP and glutaraldehyde cross-linking techniques. The bi-functional cross-linker SPDP (see Methods) cross-links between cysteine and amine-containing amino acids if the residues are within ~15 Å. Rv0639 and Ec NusG derivatives, having no cysteines, were initially labelled with SPDP, were then mixed with either WT or C50S (no-cysteine) Rv0700 and the reactions were followed under different conditions (Fig. 6c). We observed that the
Fig. 6. Rv0639–NusE interaction. (a) Elution profiles of Rv0639 and Mtb NusE (Rv0700) separately and as a complex obtained using a Superdex-200 column. Different elution peaks are indicated by vertical lines. (b) The peak fractions thus obtained were loaded onto SDS-PAGE gels to identify each of the proteins as indicated. (c) Autoradiograms of the $^{32}$P-labelled WT Mtb NusE (Rv0700; left panel) and C50S Mtb NusE (right panel) are shown after performing SPDP cross-linking under different conditions in the presence and absence of Rv0639 and Ec NusG. Cross-linking was performed for the indicated time periods. Different species were identified from their migration pattern and by comparison to molecular mass markers. Fractions of cross-linked species were calculated as: (Mtb NusE–Rv0639)/(NusE monomer) + (NusE dimer), whereas the same for monomer NusE was calculated as: (monomer) + (monomer) + (dimer) + (cross-linked species). No detectable cross-linked products were observed for C50S NusE. (d) Amounts of cross-linked (Rv0639–Rv0700) species were plotted against the increasing concentrations of SPDP-labelled Rv0639. $K_d$ was calculated from the hyperbolic binding curve. Error bars were obtained from three independent measurements. (e) In vitro pull-down assays for Mtb non-His Rv0639 and His-Rv0700. Non-specific adsorption was measured by incubating non-His Rv0639 only with Ni-NTA beads. Flow-through (FT), wash (W) and eluted (E) fractions are indicated. Both protein bands are also indicated. (f) In vivo complex formation between NusG and NusE of Mtb and E. coli. Both His-S10 and non-His NusG proteins of E. coli and Mtb were overexpressed from two different plasmids, and the cell lysates were passed over Ni-NTA beads. Different fractions are indicated. Both protein bands are also indicated. (g) SPDP cross-linking experiments in the presence of the cell lysate of M. smegmatis. Experimental conditions are as in (c). Amounts of lysate are indicated. Reactions were performed for 5 min. As indicated, the Rv0639–Rv0700 cross-linked product migrated as a high-molecular mass species. (h) Autoradiogram showing $^{32}$P-labelled Rv0639 cross-linked to Mtb NusE following addition of glutaraldehyde for the indicated time period. Different species are indicated.

Radiolabelled WT Rv0700 was efficiently cross-linked to Rv0639 with ~50% efficiency (Fig. 6c, left panel, lanes 3 and 4) indicating a stable in vitro complex formation. However, it was also capable of interacting weakly with Ec NusG (Fig. 6c, lanes 6 and 7). Disappearance of these products in the presence of DTT (Fig. 6c, lanes 5 and 8), and their absence when C50S NusE was used (Fig. 6b, right panel), strongly suggest that these specific complexes were formed between the cysteine 50 of NusE and the nearby lysine residues of Rv0639 and Ec NusG.

We used the extent of SPDP–NusG concentration-dependent formation of the Rv0700–Rv0639 cross-linked species to measure the $K_d$ of their interaction. The hyperbolic binding isotherm, depicted in Fig. 6d, yielded a mean $K_d$ value of 0.47 μM. This value indicates that these two species, under in vitro experimental conditions, do not form a very high-affinity complex, which might be due to a higher dissociation rate of the reaction. In vivo the affinity may increase when they are part of transcription and translation machineries. It should also be noted that we were unable to observe stable complex formation between these two proteins in either the in vitro pull-down assays (Fig. 6e) or when they were expressed in E. coli (Fig. 6f, left panel), which could also be due to a higher dissociation rate. Under the same in vivo conditions, however, E. coli S-10 and Ec NusG formed a stable complex (Fig. 6f, right panel). Therefore, we concluded that the cross-linking reaction might have favoured the association rate and pushed the binding equilibrium towards the bound state.

Next, we repeated the aforementioned cross-linking experiments in the presence of cellular lysates of M. smegmatis to check whether Rv0700–Rv0639 complex formation occurs in the presence of other cellular machineries (Fig. 6g). We observed that the Rv0700–Rv0639 cross-linked species migrated as a high-molecular mass product. This aggregate was seen in the presence of neither DTT nor a non-cysteine (C50S) derivative of Rv0700, which suggested that the formation of these species was dependent on the sole cysteine residue of Rv0700. The slower migration could have arisen due to the presence of components of the transcription and translation machineries in the aggregate. It is likely that the non-cross-linked SPDP moieties of Rv0639 may have interacted with cysteines available from other proteins present in the aggregate. We concluded that the Rv0700–Rv0639 complex could also occur in vivo.

We further confirmed the complex formation between these two proteins using glutaraldehyde cross-linking (Fig. 6h). We observed that in addition to the formation of intra-molecular cross-linking, Rv0639–NusE cross-linked products were also formed (Fig. 6h, lanes 5 and 6). However, weak interaction with Ec NusG was not visible using this technique (Fig. 6h, lanes 8 and 9).

Therefore, Rv0639–Rv0700 interaction could be detected using three techniques and under various conditions, which strongly suggests the formation of a stable complex between these two proteins. We propose that Rv0639 has evolved in a unique way to interact with NusE but not with Rho.

**Rv0639 has altered conformations**

Failure to complement the functions of Ec NusG, inability to bind to Rho or to stimulate transcription elongation rate by Rv0639 and likely folded conformations of the NTD over its CTD led us to further probe the conformational properties of the Rv0639 protein and compare them to Ec NusG.

Initially we probed the conformations of the CTD domains of both Ec NusG (residues 117–181) and Rv0639 (residues 185–238) by CD spectroscopy (Fig. 7a) and by measuring trypsin sensitivity (Fig. 7b). The CD spectrum of Rv0639...
showed distinctly different conformations of the CTD compared with Ec NusG. The former has more helix as well as $\beta$-sheet elements, at the expense of disordered structures like turns and random coils, which is likely to give its CTD a more compact conformation. Ec NusG CTD had many disordered regions in addition to its structured core, consistent with the compact conformation, Rv0639 showed more resistance to trypsin digestion compared with Ec NusG (Fig. 7b). However, the recently solved co-ordinates are available in the RCSB protein database (PDB; www.rcsb.org) NMR structure of Rv0639 resembles more its Ec NusG counterpart and does not match with what we observed (Fig. 7c). At this point in time, we do not have an adequate explanation for this discrepancy other than stating that the experimental conditions of NMR and CD (buffer composition, pH, etc.) are different and the CTD of Rv0639 was His-tagged.

Next, we probed the tertiary structures of these two proteins by protein footprinting using trypsin (arginine- and lysine-specific) and V8 proteases (glutamic acid-specific; Fig. 7d–g). We subjected radiolabelled Rv0639 (Fig. 7d, e) and Ec NusG (Fig. 7f, g) to partial trypsin and V8 digestion to obtain the footprinting patterns. The HMK-tags present in either the CTD (for Rv0639) or NTD (for Ec NusG) were labelled with $^{32}$P. Under our experimental conditions, it was expected that most surfaces exposed and accessible sites would be cleaved only by the proteases. We observed the following. (i) Other than the 43R residue, no cleavage sites were visible in the extra NTD of Rv0639, which indicates that either this region is folded or is protected by other parts of the protein. The latter proposal is consistent with the intra-molecular Cys–Cys disulphide formation patterns described in Fig. 3c, d. (ii) Like Ec NusG-NTD, the stem part of Rv0639 NTD is also quite disordered as evidenced from the sensitivity towards proteases. (iii) In contrast to Ec NusG, the linker region of Rv0639 is an easier target for both proteases, which is indicative of a highly disordered structure. The homology model of Rv0639 also showed the presence of a longer linker. These data predict higher degrees of freedom for both NTD and CTD relative to the axis of the linker region in Rv0639. (iv) Ec NusG-CTD exhibited three distinct sites of cleavage by trypsin and V8, whereas there is only one strong V8 susceptible site at position 207 of Rv0639, which indicates that the latter has either a more compact CTD or the susceptible residues are protected by the extra NTD region. The CTD cleavage pattern is more consistent with our CD analyses rather than the NMR structure. Based on these tertiary structure analyses, we suggest that Rv0639 has distinct and different conformational features compared with Ec NusG, which might have contributed to its altered functions.

DISCUSSION

The parasitic life style of Mtb in the human host is likely to offer in vivo conditions different to its transcription machinery, which may impose evolutionary pressure on this machinery to acquire altered functions and conformations. The basic catalytic reactions during the transcription process may remain the same, and the regulatory molecules associated with different steps of transcription are usually the targets of evolutionary plasticity (Merino et al., 2008). This makes it important to characterize these RNAP-associated transcription factors, especially from the pathogens, not only to understand the mechanistic details but also to evaluate their importance as anti-microbial drug targets.

Here, we present a detailed analysis of one such regulatory transcription factor of Mtb, Rv0639, annotated as a homologue of the transcription elongation–termination factor NusG. We provide the following evidence revealing that Rv0639 has acquired divergent functions and conformations. (i) Rv0639 failed to complement NusG functions under the in vivo conditions of E. coli, even though it was predicted to have similar domain organization to Ec NusG (Fig. 1). (ii) Rv0639 did not show appreciable interactions with the transcription terminator Rho of Mtb (Rv1297). Mtb Rho, however, had a robust termination function in the absence of Rv0639 (Fig. 4). (iii) Rv0639 also failed to induce elongation rate enhancement of either E. coli or Mtb RNAPs (Fig. 5). (iv) Interestingly, Rv0639 can form a moderately stable complex with the Mtb ribosomal protein S10 (Rv0700) both in vitro and in the presence of mycobacterial cellular machineries (Fig. 6). (v) Rv0639 has significantly different conformational features characterized by shape, secondary structure content, folding of the extra N-terminal region over its CTD, an unusually long and disordered linker region and conformation of the CTD (Figs 2, 3 and 7). Based on this evidence, we propose that
Rv0639 has ceased to function as a transcription elongation–termination factor but has retained the function of interacting with ribosomal protein S10, and thereby participates in transcription–translation coupling. The aforementioned results also led us to redefine Rv0639 as a parologue of NusG, and not a homologue as annotated (http://tuberculist.epfl.ch). In this context, it is worth mentioning that Rv0639 exhibits sequence homology mostly with its counterparts from other mycobacterial species only (data not shown).

The Cu-P-induced disulphide formation between the Cys residues of the N- and C-terminals of Rv0639 indicates physical proximity and probable interactions between these two domains (Fig. 3d). This type of ‘closed’ conformation is similar to that observed for another NusG parologue, RfaH (Belogurov et al., 2009; Burmann et al., 2012), and for T. maritima NusG (Drögemüller et al., 2013). These closed conformations produce an auto-inhibitory state that prevents RfaH from interacting with Rho, opening of which occurs upon interaction with the ops sequence in the DNA. Our conformational (Fig. 3) and functional probing indicates a similar behaviour for Rv0639. However, we do not know the stability of this ‘auto-inhibited’ state of Rv0639, nor is the nature of the ‘trigger’ required to open this closed structure known. We predict that Rv0639, like RfaH, can also recognize unidentified ops-like sequences in the Mtb genome, and take part in pathogenicity.

The secondary structure of full-length Rv0639 has a very high β-sheet content (~61%; Fig. 2), and if we assume that the random structures (~27–28%) arise mostly from the linker region, then its CTD, when a part of the whole protein, is likely to be made predominantly of β-sheets. This is starkly in contrast to the α-helical CTD of RfaH (Burmann et al., 2012). Interestingly, the free CTD of Rv0639 (Fig. 7) seems to have significantly high α-helical (~41%) and decreased β-sheet content (~48%), which is suggestive of moderate structural transition. This could be similar to the structural refolding of the α-helical CTD of RfaH into a β-sheet when it is released from its NTD (Burmann et al., 2012), but is less drastic and opposite in nature.

Unlike the anti-pausing activity of Ec NusG, NusG proteins of B. subtilis (Yakhnin et al., 2008) and Thermus thermophilus (Sevostyanova & Artsimovich, 2010) exhibit pausing activity in in vitro transcription assays, and that from M. bovis showed moderate stimulation of transcription termination at some hairpin-dependent terminators (Czyz et al., 2014). We found that Rv0639 was unable to increase the transcription elongation rate, which indicates that it could very well belong to the family of these aforementioned NusGs. It is likely that the NusG-NTDs of these species have altered interactions at the central cleft of the RNAP. However, we have no evidence as to whether Rv0639 or other pause-inducing NusGs interact with the same structural elements of RNAP as Ec NusG (Sevostyanova et al., 2011).

Rv0639-CTD retained the capability of forming a stable complex with Mtb S10, Rv0700, even though it had lost all the other functions. In this regard too, it is similar to the NusG parologue, RfaH (Burmann et al., 2012). These data suggest that in Mtb, like E. coli, coupling of transcription and translation through NusG-S10 interaction is still an active process. The slow transcription elongation rate of Mtb RNAP (Fig. 5) is not enough to maintain this coupling process. However, in the case of Mtb, Rv0639–S10 interaction does not face competition from Rho for the same CTD; hence, Rho–ribosome competition occurs only at the RNA-binding step(s).

In regard to the aforementioned conformational and functional diversities of a conserved transcription factor like Rv0639, it is clear that knowledge gathered for E. coli transcription machinery may not be directly applicable to understanding the transcription factors of a pathogen like Mtb. Therefore, it is now more important to understand the behaviour of these factors in the in vivo conditions of Mtb when grown in laboratory conditions, as well as when it is residing within the human macrophage, to establish them as effective drug targets.

ACKNOWLEDGEMENTS

Dr Nisha C. Kalarickal was involved in the initial stages of the work. Mr Madhav Rao of Dr Sekhar Mande’s laboratory, NCCS, Pune, India, provided us with the WT Rv0639 clone in pET28a vector (pRS552). We thank Dr Sanjeev Khosla’s laboratory, Center for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India, for providing the genomic DNA of Mtb H37Rv and DrJayanta Mukhopadhyay of Bose Institute, Kolkata, India, for gifting us the Mtb RNA polymerase. We thank Ms Richa Gupta and Ms Gairika Ghosh for carefully reading the manuscript. This work is supported by CDFD intramural funding.

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Edited by: R. Manganelli