Distinct Properties of Hexameric but Functionally Conserved *Mycobacterium tuberculosis* Transcription-Repair Coupling Factor

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Abstract

Transcription coupled nucleotide excision repair (TC-NER) is involved in correcting UV-induced damage and other road-blocks encountered in the transcribed strand. Mutation frequency decline (Mfd) is a transcription repair coupling factor, involved in repair of template strand during transcription. Mfd from *M. tuberculosis* (MtMfd) is 1234 amino-acids long harboring characteristic modules for different activities. Mtbf comes complemented *Escherichia coli* mfd (EcoMfd) deficient strain, enhanced survival of UV irradiated cells and increased the road-block repression *in vivo*. The protein exhibited ATPase activity, which was stimulated ~1.5-fold in the presence of DNA. While the C-terminal domain (CTD) containing amino acids 630 to 1234 showed ~2-fold elevated ATPase activity than Mtbf-md. The N-terminal domain (NTD) containing the first 433 amino acid residues was able to bind ATP but deficient in hydrolysis. Overexpression of NTD of Mtbf-md led to growth defect and hypersensitivity to UV light. Deletion of 184 amino acids from the C-terminal end of Mtbf-md increased the ATPase activity by ~10-fold and correspondingly exhibited efficient translocation along DNA as compared to the Mtbf-md and CTD. Surprisingly, Mtbf-md was found to be distributed in monomer and hexamer forms both *in vivo* and *in vitro* and the monomer showed increased susceptibility to proteases compared to the hexamer. Mtbf-md, on the other hand, was predominantly monomeric in solution implicating the extreme C-terminal region in oligomerization of the protein. Thus, although the Mtbf-md resembles EcoMfd in many of its reaction characteristics, some of its hitherto unknown distinct properties hint at its species specific role in mycobacteria during transcription-coupled repair.

Introduction

DNA is a dynamic molecule and is constantly exposed to various types of damaging agents such as mutagenic chemicals, radiation and reactive oxygen. A number of DNA repair systems exist which specialize in the repair of certain types of damage. Nucleotide excision repair (NER) is a highly conserved pathway involved in repair of a wide variety of structurally unrelated DNA lesions [1]. One of the well characterized NER systems is the UvrABC nuclease from *E. coli* [2,3]. NER consists of two related sub-pathways; global genomic repair (GGR), which removes lesions from the overall genome, and transcription coupled repair (TCR), which removes lesions from the transcribed strand of active genes [4–6]. Bulky DNA lesions such as cyclo pyrimidine photodimers (CPD) induced by UV irradiation block RNA polymerase during transcription [6]. In bacteria a product of *mfd* called transcription repair coupling factor (TRCF) or Mfd protein is required for TCR [7–9]. Bacterial Mfd interacts with the stalled RNA polymerase, displaces it from the DNA and recruits NER proteins at the site of damage [10,11]. Mfd thus clears the steric hindrance from the site of damage and loads UvrA protein, resulting in ~10-fold faster repair of the transcribed strand compared to the non-transcribed strand for similar kind of lesions [12]. In addition, Mfd rescues arrested or backtracked transcription elongation complexes by promoting forward translocation of RNA polymerase in ATP dependent manner leading to productive elongation [13]. Additionally, Mfd can release the RNA polymerase when the enzyme cannot continue elongation [13]. Apart from DNA repair, Mfd has other physiological roles in regulation of gene expression, including carbon catabolite repression in *Bacillus subtilis* [14] and transcription termination by bacteriophage HK022 Nup protein [15]. A key role for Mfd as an enhancer of UvrA turnover in *E. coli* cells has also been recently demonstrated [16].

The well characterized Mfd from *E. coli* is a 130 kDa monomeric protein having modular architecture specialized for different functions [8]. The N-terminal domain (NTD) shares a high degree of structural homology with UvrB protein of NER pathway [17,18]. The NTD is known to interact with UvrA protein, which is molecular matchmaker of NER pathway, and this interaction is responsible for enhanced rates of repair [8]. The central portion of Mfd consists of RNA polymerase interacting domain (RID) which binds to subunit of RNA polymerase [13,17]. The C-terminal domain (CTD) of Mfd harbors seven signature motifs of super-family 2 helicases including ATPase motifs. In addition, CTD contains a TRG motif [translation in...
RecG) required for translocation along the DNA. TRG motif as the name implies, is highly homologous to RecG protein, which is known to be involved in branch migration of Holliday junctions during recombination [19,20].

Pathogenic bacteria continuously encounter multiple forms of stress in their hostile environments, which leads to DNA damage. Genes involved in DNA repair and recombination may play an important role in the virulence of pathogenic organisms [21]. M. tuberculosis is a gram positive, acid fast bacterium and one of the most formidable human pathogen. DNA repair pathways in mycobacteria appear to be crucial for their survival at different stages of infection [22]. Sequencing of M. tuberculosis genome revealed the presence of NER associated genes including a putative mfd. In this work, we describe the functional characterization of MtbMfd and discuss its unusual properties. This is the first detailed analysis of the biochemical properties of Mfd from actinomycetes and more importantly from a human pathogen.

Results

Cloning, expression and functional characterization of MtbMfd

Genome analysis of M. tuberculosis revealed that MtbMfd is 1234 amino acids long encoded by 3.7 Kb DNA fragment. Cloning of the Mtbmfd was carried out by reconstructing the full length gene from three PCR amplified fragments using gene specific primers (Table 1) and genomic DNA as a template. The strategy is depicted in Figure 1A and details are described in Materials and Methods. Mfd cloned under the control of T7 promoter (pETmfd) was used for overexpression and purification, whereas the gene cloned under trc promoter (pTrcmfd) was used for in vitro assays (Fig. 1A). Heterologous expression of MtbMfd protein was achieved in E. coli strain BL21 (pLysS) TUNER as N-terminal histidine tagged protein using pETmfd construct. The overexpressed protein had the molecular mass of ~133 kDa (Fig. 1B, lane 6) corresponding to the theoretical molecular weight of MtbMfd calculated from amino acid sequence. Whereas, no such protein was seen in vector control cell lysate (Fig. 1B, lanes 1–3) as well in uninduced cell lysate (Fig. 1B, lane 5).

One of the mfd mutants of E. coli (UNCNOMFD) was shown to confer moderate ultraviolet sensitivity to the E. coli cells [8]. To determine the cellular function of MtbMfd, in vivo complementation assay was carried out using the above mentioned mfd deficient strain of E. coli. The effect of UV irradiation on survival (S/So) of wild-type E. coli (AB1157), UNCNOMFD and UNCNOMFD transformed with Mtbmfd construct (pTrcmfd) was determined as described in Materials and Methods. First, the expression of Mfd protein in E. coli was verified by western blot analysis using anti-MtbMfd polyclonal antibodies. UNCNOMFD strain did not express any detectable level of Mfd protein (Fig. 1C, lane 2) compared to AB1157 (Fig. 1C, lane 1). When UNCNOMFD was transformed with pTrcmfd construct, a considerable amount of MtbMfd protein was expressed (Fig. 1C, lane 4) which increased further upon addition of 0.5 mM of IPTG (Fig. 1C, lane 3). Next, AB1157, UNCNOMFD and pTrcmfd cells were exposed to UV for varied time and survival was determined. A ~10 fold decrease in survival was observed in UNCNOMFD compared to AB1157 after irradiation. When UNCNOMFD strain was complemented with plasmid pTrcmfd, the survival was restored to the wild-type level (Fig. 1D) indicating that Mtbmfd complements E. coli counterpart in mfd deficient strain.

MtbMfd increases the road-block repression in vivo

Transcription elongation complexes tend to pause or stall when they encounter a protein road-block or DNA damage on the template strand, reducing the transcription of downstream sequences. This has been observed in vivo, where formation of the protein road-blocks influences regulation of gene expression as in the case of the carbon catabolite repression of hut and gnt operons of Bacillus subtilis [14]. An in vitro assay has been developed in which expression of a reporter gene could be monitored in presence and absence of Mfd when DNA polymerase is stalled by Lac repressor [20]. The rationale behind this assay is that when operator site is occupied by Lac repressor, it blocks RNA polymerase engaged in transcription elongation. Mfd recognizes stalled RNA polymerase and removes it from the site of transcription resulting in lower cat transcription and CAT activity. However, in the absence of Mfd, paused RNA polymerase leads to high level of transcription as well as CAT activity after dissociation of the Lac repressor from the operator. To further confirm the functionality of MtbMfd, road-block reporter assays were carried out using pRCB-CAT1 construct [20] (Fig. 2A). AB1157 (mfd+) showed lowest CAT activity followed by UNCNOMFD cells complemented with pTrcmfd construct (Mtbmfd+) compared to UNCNOMFD (mfd–) and vector control, pTrc99A (mfd–) (Fig. 2B). These results suggested that MtbMfd complements E. coli counterpart and when MtbMfd was present in the system, road-block repression increased significantly. From these results it is apparent that MtbMfd interacts with E. coli RNA polymerase leading to its dissociation from the site of transcription.

Table 1. List of oligonucleotides (primers) used in this study.

| Oligonucleotide | Sequence |
|----------------|----------|
| PF1 | 5’TGGGACATATGACCGAAGGGGCAGC3’ |
| PR1 | 5’CAAGGTACCGTGGGCAAGGGGCAGC3’ |
| PF2 | 5’CGAGCTACCGACACCCGCTG3’ |
| PR2 | 5’CCCCGCCTCGACAGTCAAGCTTGGGCG3’ |
| PF3 | 5’CAGGAGCCCGGATCTC3’ |
| PR3 | 5’CCAGAAGCTGCATCGGTGTCG3’ |
| PRdel | 5’CATAGGTTCCAGGGTTCTCGGCGT3’ |
| WBF | 5’CAGCTTGTCGCTTGGGCG3’ |
| WbM | 5’CTCCAGCGGACACCCACAG3’ |
| WbR | 5’GTCGACAGTCTCTGCGCTGGAG3’ |

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Overexpression and purification of MtbMfd proteins

The homology between MtbMfd and EcoMfd is 38% over the entire length. The important domains and their linear organization along the sequence are conserved between the two proteins. MtbMfd (1234 aa) contains UvrB homology domain at the N terminal, a RNA polymerase interacting domain (RID) in the central part and ATPase and translocase domains in the C-terminal. The schematic representation in Fig. 3A depicts the full length and other constructs of MtbMfd generated for this study. In addition to full-length protein, a mutant MtbMfd, MfdD778A was generated by changing a single amino acid in Walker B motif of ATPase domain. A construct referred to as MfdAC was generated which harbors amino acids 1–1050 but lacking the extreme 184 residues from the C-terminal. Another construct referred to as CTD, spanning amino acids 630–1234 having intact C-terminal region was generated. A third construct named as NTD comprising amino acids 1–433 was also generated. Mfd
and its variants were purified to apparent homogeneity as described in Materials and Methods. Purified MtbMfd was subjected to mass spectrometry analysis to confirm the authenticity of the protein, and the result obtained matched with that of the theoretical amino acids sequence of the MtbMfd (data not shown). The purity of the proteins were checked on SDS-PAGE and the experimental molecular masses were in agreement with their predicted molecular weights (full length MtbMfd and MfdD778A are 133 kDa each, MfdC is 115 kDa, CTD is 67 kDa and NTD is 48 kDa) (Fig. 3B).

Western blot analysis of the purified proteins using anti-MtbMfd polyclonal antibodies detected full length MtbMfd as well as its variants (Fig. 3C).

Unusual behavior of MtbMfd in solution

To determine the oligomeric status of MtbMfd, gel filtration analysis was carried out. Surprisingly, MtbMfd eluted as two peaks (Fig. 4A) at positions corresponding to a globular protein of ~790 kDa (peak 1) and ~130 kDa (peak 2) respectively (Fig. 4B). The presence of MtbMfd in both the peaks was confirmed by SDS-PAGE analysis (Inset of Fig. 4A, panel showing SDS-PAGE). The molecular weight of peak 1 and peak 2 corresponded to the hexamer and monomer size of MtbMfd, respectively. Chemical cross-linking of MtbMfd with glutaraldehyde was carried out to determine the oligomeric nature of the protein. Glutaraldehyde is a homo bifunctional cross-linking reagent that cross-links N-terminal primary amines of lysine residues, resulting in the

Figure 1. Cloning, expression and functional analysis of M. tuberculosis mfd (Mtbmfd). A. Schematic showing the strategy used for cloning of Mtbmfd; F1, F2 & F3 represents three fragments of Mtbmfd obtained by PCR amplification from genomic DNA of M. tuberculosis by using set of specific primers. F1 was cloned into pET32a in MscI-KpnI sites and F2 was cloned into pGPS3 vector using KpnI-BsrB1 sites. F2+F3 fragment was obtained by ligation of F3 into pGPS3 containing F2 using BsrB1-HindIII sites. A 2.4 kb fragment containing F2+F3 was released using KpnI-HindIII sites from pGPS3 clone and ligated into pET32a F1 clone to obtain full length Mtbmfd gene. Sub cloning of Mtbmfd in pET28a and pTrc99A vectors were used for overexpression and in vivo assays respectively. B. SDS-PAGE analysis of overexpression of His-tagged MtbMfd in E. coli expression strain (Tuner) using pETmfd construct. Lane 1, total cell extracts of Tuner cells; lane 2, total cell extracts of uninduced Tuner cells carrying pET28a vector alone; lane 3, induced cell extracts of Tuner cells harboring pET28a vector; lane 4, protein molecular weight marker; lane 5, total cell extracts of uninduced Tuner cells carrying pETmfd construct and lane 6, total cell extract of induced cell extracts (0.3 mM IPTG) of Tuner cells carrying pETmfd construct. C. Western blot analysis using anti-MtbMfd polyclonal antibody for expression of Mfd in E. coli stains used for complementation studies; lane 1, AB1157; lane 2, UNCNOMFD; lane 3, pTrcmfd in presence of 0.5 mM of IPTG and lane 4, pTrcmfd. D. Effect of UV on survival (S/S0) of E. coli strains; AB1157 (red, ■); UNCNOMFD (blue, □); pTrcmfd (brown, ▼) and pTrc99A (black, ◆). Each value represents the average from three independent experiments. (Survival = S/S0, where S0 = number of bacterial colonies obtained without UV irradiation and S = number of bacterial colonies obtained after UV irradiation). (AB1157, E. coli wild-type strain; UNCNOMFD, mfd deficient strain of E. coli; pTrcmfd, UNCNOMFD transformed with Mtbmfd construct and pTrc99A, UNCNOMFD transformed with pTrc99A vector alone).

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formation of amidine cross-links between protein subunits. Glutaraldehyde treated MtbMfd migrated with a lower mobility than the monomer of MtbMfd (133 kDa). Two reduced mobility crosslinked products were observed on SDS-PAGE (oligomer 1 and 2, Fig. 4C). The majority of the crosslinked species was the slowest migrating species (oligomer 2) suggesting that the oligomer 1 form may be an intermediate product of cross linking reaction.

The hydrodynamic radius \( R_H \) of MtbMfd was calculated by dynamic light scattering experiments (DLS). 100 nM–1 \( \mu \)M purified MtbMfd protein was subjected to DLS analysis and a \( R_H \) value of 9.2 \( \pm \)1 nm obtained corresponded approximately to the hexamer of MtbMfd as estimated by comparison with typical globular proteins (Fig. 4D).

EcoMfd is known to be monomeric in nature [17]. In spite of its structural and functional similarity to the EcoMfd, the existence of MtbMfd in hexameric form prompted us to carry out further analysis to confirm the oligomeric nature of the protein. The behavior of the protein was analyzed by gel filtration chromatography under different conditions. Equimolar concentrations of MtbMfd \((\sim 133 \text{ kDa})\) and thyroglobulin \((669 \text{ kDa})\) were mixed and co injected into the column. The two peaks obtained were analyzed on SDS-PAGE and noticeably, the fraction corresponding to peak 1 retained both MtbMfd and thyroglobulin whereas peak 2 retained only MtbMfd (Fig. S1A). Co-elution of hexameric MtbMfd with thyroglobulin and the elution of monomeric MtbMfd separately support the existence of two forms of MtbMfd as estimated by comparison with typical globular proteins (Fig. 4D).

In order to check whether oligomeric forms of MtbMfd exist in vivo, the following experiments were carried out using cell lysate of \( M. \text{tuberculosis} \) H37Ra. First, native-PAGE Western blot analysis was carried out with the crude cell lysate using anti-MtbMfd antibody and it was found that MtbMfd exists as predominantly in two forms in the cell (Fig. 5A). Second, cell lysate of \( M. \text{tuberculosis} \) was subjected to gel filtration chromatography under non-denaturing conditions (Fig. S2A) and eluted fractions were analyzed by Western blot using anti-MtbMfd antibody. The hexameric fractions eluted between 10 to 11.5 ml while the monomeric MtbMfd was present in the 14 to 15 ml fractions (Fig. 5B). Gel filtration chromatography of purified native MtbMfd (Fig. S2B) showed a similar profile as described for His-tagged MtbMfd.

Limited proteolysis is often employed to determine the domainal organization, stability and conformational changes within the protein. The hexamer and monomer fractions of MtbMfd obtained by gel filtration chromatography were subjected to limited digestion by trypsin and V8 protease to further explore the characteristics of the two species of the protein. Trypsin cleaves peptide bonds exclusively at C-terminal of arginine and lysine residues and V8 protease cleaves on the carboxyl side of glutamic acid. The digestion with trypsin gave multiple bands with both the forms of MtbMfd in a time dependent manner but monomeric
fraction showed more sensitivity (Fig. 6A). Similarly, V8 protease digestion showed that the hexameric fraction was significantly more resistant compared to the monomer fraction (Fig. 6B). To assess the functional significance of oligomerization of MtbMfd, ATPase assay was carried out with the hexamer and monomer fractions of MtbMfd after separating them by size exclusion chromatography. It was found that both the forms of MtbMfd were able to hydrolyze ATP. However, the specific ATPase activity of monomer (156.78 pmoles ATP hydrolyzed/min/mg of protein) was 3-fold higher compared to the hexamer (58.1 pmoles of ATP hydrolyzed/min/mg of protein) (Fig. S3).

Since both the forms of MtbMfd showed ATPase activity, next we considered possible ligand mediated transition between these forms. Gel filtration was carried out in the presence of ATP and DNA and it was observed that the elution profile of MtbMfd did not alter in presence of these two substrates (Fig. S4 A–E). In addition, the oligomeric status of the purified individual domains of MtbMfd (NTD and CTD) was determined and they were found to be monomeric in nature (data not shown). When MfdΔC was subjected to gel filtration chromatography and compared with full-length MtbMfd (Fig. 6C), surprisingly a majority of MfdΔC eluted as a monomer even at higher concentrations (Fig. 6D and E). These results indicate that the extreme C-terminus region could be important for MtbMfd to acquire an oligomeric form.

**Characterization of ATPase activity of MtbMfd and its derivatives**

Mfd hydrolyses ATP in order to displace RNA polymerase from the site of damage [13]. It possesses a typical ATPase active site having Walker A and B motifs towards its C-terminal region. To analyze the kinetics of ATP hydrolysis of MtbMfd and its truncated proteins, reactions were carried out using radiolabeled ATP as a tracer along with unlabeled ATP. Wild-type MtbMfd protein exhibited ATPase activity which was stimulated, 1.5-fold in presence of dsDNA. The mutant MtbMfd (D778A) which harbors mutation in one of the key residues of Walker B motif of the ATPase domain, showed negligible ATPase activity indicating the importance of residue D778 for ATP hydrolysis (Fig. 7A). The kinetic parameters for MtbMfd were determined under steady state conditions. The turnover number (kcat) of 3.3±0.2 min⁻¹ and a Kₘ (ATP) of 1.1±0.3 mM obtained at pH 8.0 and 37°C are comparable to those for EcoMfd. However, the turnover number for EcoMfd ATPase reported by different groups varies from 2.3–8.0 min⁻¹ [8,23,24]. It can be seen from Table 2 that while the turnover number of MtbMfd did not significantly increase in the
presence of DNA, the affinity for ATP ($K_m$) increased by 2-fold. Next, the turnover number of ATP hydrolysis for the CTD of MtbMfd was determined to be $5.2 \pm 0.5 \text{ min}^{-1}$ (Table 2). In contrast, a higher level of ATPase activity was reported for CTD of EcoMfd (turnover number, 190 min$^{-1}$) [24]. The huge difference in the rate of ATP hydrolysis between the two CTD proteins could account for their differences in translocase activity (see the later section). In the presence of DNA, the affinity of MtbMfd CTD towards the ATP increased by 2-fold (Table 2) similar to the full length MtbMfd suggesting that the deletion of first 600 residues did not alter the DNA binding properties. In contrast to full length MtbMfd, MfdD showed robust ATPase activity with a 10-fold higher turnover number for ATP hydrolysis ($27.6 \pm 1.2 \text{ min}^{-1}$) (Table 2). This result is similar to the one obtained for the E. coli MfdD [23], implicating an auto regulatory function for the extreme C-terminus of MtbMfd.

Unlike the classical ATPase motif which is present at the C-terminal of Mfd, an additional RecA like domain is located at the N-terminal of Mfd which resembles the one found in UvrB protein [18]. In order to check whether the purified NTD of MtbMfd can perform ATP binding and hydrolysis, assays were carried out as described in Materials and Methods. Notably, fluorescence quenching studies demonstrate that the NTD of MtbMfd binds ATP in a concentration dependent manner. Quenching of intrinsic fluorescence was observed in presence of ATP with $K_s$ constant of $526 \mu\text{M}$ (Fig. 7B). However, NTD was deficient in ATP hydrolysis (Fig. 7A). Previous studies with EcoMfd revealed that the degenerate ATPase motif in its NTD to be deficient for the nucleotide binding and hydrolysis [18].

Translocation of MtbMfd along DNA

Mfd belongs to super-family 2 (SF2) helicases and is known to translocate along the DNA to displace RNA polymerase in an ATP dependent manner [13]. The translocase activity of MtbMfd was measured on linear triplex DNA substrate by carrying out TFO (Triplex Forming Oligonucleotide) displacement assay described previously for EcoMfd [23]. The triplex linear DNA was separately incubated with MtbMfd, CTD and MfdD and the...
displacement of radiolabeled TFO was monitored on the polyacrylamide gel. MtbMfd did not exhibit significant translocase activity under these assay conditions (Fig. 8A, lanes 2–8). This is similar to the data obtained with EcoMfd [23]. Interestingly, the CTD of MtbMfd did not show detectable level of translocase activity (Fig. 8B, lanes 2–8) unlike the CTD of E. coli which was shown to efficiently translocate along DNA possibly because of its high ATPase activity [24]. Unlike CTD, MfdC from M. tuberculosis exhibited efficient ATPase activity and also showed robust translocase activity (Fig. 8C, lanes 4–10) in an ATP dependent manner. About 80% of the ssDNA was displaced from the triplex substrate (Fig. 8D) and these results are similar to those obtained for E. coli MfdC [23]. In the absence of ATP or in the presence of a non-hydrolysable form of ATP (ATPγS) in the reaction, the translocase activity of MtbMfdC was found to be negligible (Fig. 8C, lanes 1 & 2). These results provide a direct correlation between translocase and ATPase activity of MtbMfd, and suggest the dependence of the former on the later reaction.

Although Mfd possesses helicase motifs in the C-terminal region, the purified MtbMfd did not appear to unwind DNA: DNA hybrids (data not shown) reiterating the notion that all translocases do not necessarily function as helicases.

NTD overexpression affects cellular function

Sequence comparisons of NTD of Mfd and UvrB showed that NTD retains intact UvrA interacting domain of UvrB and probably recruits UvrA during TCR [18]. Overexpression of NTD of MtbMfd in E. coli resulted in a delayed growth phenotype. To explore this further, growth of wild-type and the NTD expressing cells was monitored on solid and liquid medium. The NTD expressing cells showed growth defects on solid medium (Fig. 9A) whereas delayed growth phenotype was observed in liquid medium (Fig. 9B). On the other hand, no such defects were observed when other truncated MtbMfd proteins viz MfdC, CTD and RID were overexpressed (data not shown). Since NTD harbors an UvrA interacting domain, when it is expressed it may sequester the cellular pool of UvrA leading to dominant negative phenotype. When UV survival assays were carried out, cells expressing NTD showed hyper-sensitivity (Fig. 9C) to UV light – a typical characteristic of NER deficiency, indicating that NTD expression could influence the NER pathway.

Alterations in the levels of NER components or Mfd have an effect on generation of spontaneous mutations [25,26]. To analyze the frequency of spontaneous mutations, mutator assays were carried out using E. coli cells expressing NTD of MtbMfd and mfd deficient strain (UNCNOMFD) and mutation frequencies were calculated as described in Materials and Methods. The reduction in mutation frequency in NTD expressing cells (0.32) and UNCNOMFD (0.72) compared to wild-type (1) (Fig. 9D) indeed supports the dominant negative effect of NTD on NER.
Discussion

Every genome invests significant effort in ensuring genomic integrity and stability. A plethora of repair mechanisms which exist in the organisms, functionally cooperate to safeguard the genomes by repairing the diverse range of damages inflicted on the DNA. Most bacterial genomes harbor a full arsenal of repair pathways viz photoreactivation, base excision repair, nucleotide excision repair, mismatch repair, recombination repair and SOS response. Sequencing of *M. tuberculosis* genome facilitated the identification of the various repair processes operational in mycobacteria. Although several DNA repair pathways were found in mycobacteria, surprisingly mismatch repair genes were absent. However, the components needed for NER and transcription coupled repair were present. In this study, we have carried out detailed characterization of MtbMfd. At first glance, the primary sequence analysis of MtbMfd revealed significant similarity to EcoMfd with respect to size, domainal organization and conservation of the motifs. Thus, as one could predict, the purified MtbMfd protein had typical activities of Mfd viz DNA binding, ATPase and translocase. Moreover, the MtbMfd complemented mfd deficiency in an *E. coli* strain in two different assays viz UV survival and roadblock repair (Figs. 1 and 2). Thus, although overall similarity is about 38%, the Mfd function seems to be functionally conserved across these two widely divergent species. However, most surprisingly, the MtbMfd was found to occur in an oligomeric form in contrast to the monomeric form of EcoMfd (Figs. 4 and 5). The MtbMfd existed in both monomeric and hexameric form and deletion of the extreme C-terminus resulted in shifting of the equilibrium mostly towards the monomeric form. The higher stability of the hexameric form under various conditions as well as resistance to limited proteolysis suggests functional importance for the oligomeric MtbMfd (Fig. 6).

What could be the physiological significance of hexameric MtbMfd? This has turned out to be a very challenging question as both forms are found both in vivo and in vitro (Figs. 4 and 5). Since EcoMfd was found only in monomeric form, the monomer of MtbMfd could be the active form considering the similar
domainal architecture. If so, hexameric form of MtbMfd could be non functional. However, the hexameric form was found to have ATPase activity (Fig. S3). Next, the possibility of ligand mediated transition in the protein as a mechanism for multimerization was considered. For instance, changes in the conformation stabilizes the oligomeric form of prokaryotic enhancer binding protein, NtrC1; binding of ATP analogues stabilize oligomeric form of the protein and facilitate its binding to Sigma 54 [27]. Further, the McrBC restriction endonuclease assembles into a ring structure in the presence of GTP and its analogues [28]. However, in case of MtbMfd, the presence of DNA or ATP did not alter the oligomerization status indicating that it is rather an intrinsic property of the protein (Fig. S4 D & E). The increased sensitivity of the monomer and the relative stability of the hexamer to protease digestion (Fig. 6A & B) suggested that the hexamer was a stable form of MtbMfd probably serving as a reservoir inside the cell for its ready availability at the repair site during transcription. Intracellular factors may trigger the monomerization of the protein prior to its action at the stalled transcription site. One scenario that could be envisioned is that upon encountering DNA damage, the hexameric MtbMfd is available for immediate recruitment to the stalled transcription complex.

Table 2. Kinetic parameters of M. tuberculosis Mfd ATPase.

| Proteins | $k_{\text{cat}}$ (min$^{-1}$) | $K_{\text{m}}$ (mM) |
|----------|------------------|------------------|
| MtbMfd   | $3.3 \pm 0.7$    | $5.3 \pm 0.5$    |
| CTD      | $5.2 \pm 0.8$    | $3.3 \pm 0.4$    |
| MfdΔC    | $27.6 \pm 1.2$   | $43.7 \pm 2$     |

Notes: Values represent average of three replicates. $K_{\text{cat}}$ and $K_{\text{m}}$ values were calculated using Lineweaver-Burk plots. The standard errors were calculated from the replicate data. The $K_{\text{cat}}$ and $K_{\text{m}}$ values for MtbMfd with DNA were calculated using a double reciprocal plot and the values for CTD and MfdΔC were calculated using a single reciprocal plot. The $K_{\text{cat}}$ and $K_{\text{m}}$ values for MtbMfd with DNA were calculated using a double reciprocal plot and the values for CTD and MfdΔC were calculated using a single reciprocal plot.
The studies described here allow us to compare the properties of MtbMfd with very well studied EcoMfd. In addition to the differences in oligomerization properties described above, the enzymes seem to differ significantly in a few other properties. While the full length proteins do not reveal significant differences in their catalytic properties, the CTDs show vast differences in their translocase activities, which correlate well with their respective ATPase activities (Table 2). This would mean that the sequences in CTD are responsible for the distinct properties of the two Mfds with respect to oligomerization potential and control of translocase activity. One important finding of the present study is the binding of ATP to NTD of MtbMfd. All Mfd NTDs resemble UvrB and possess the degenerate ATPase motifs. Indeed, on the basis of sequence and structural similarities, it has been suggested that Mfds have evolved from UvrB incorporating an additional translocase activity [18]. UvrB has cryptic ATPase activity while the NTD of Mfd may have lost the activity as it possesses degenerate Walker motifs. Structural analysis of EcoMfd and ATPase assays revealed that NTD of EcoMfd lacks functional ATP binding sites [18]. In contrast, NTD of MtbMfd binds ATP but is hydrolysis deficient (Fig. 7). A closer comparison of the amino acid sequences in the Walker A motif reveal that conserved lysine 43 of UvrB has been replaced by arginine in case of NTD of MtbMfd. It has been shown previously that mutation of lysine 43 to alanine, aspartate and arginine led to a loss of ATPase activity of UvrB [29]. Thus, MtbMfd seems to be a natural mutant of UvrB. B. subtilis Mfd also has arginine in this location while EcoMfd has cysteine [18]. These differences could account for the observed difference in ATP binding in case of EcoMfd and MtbMfd. Single amino acid change appears to be one of the determinants in evolving an ATPase deficient NTD from ATP hydrolyzing UvrB although other residues in both Walker A and B motifs could also contribute to the loss of function. What could be the physiological basis of ATP binding to the NTD of MtbMfd? It could serve as an ATP reservoir for ATPase/translocase activity. Alternatively, it may have a role in altering the stability or conformation of the protein or may be just vestigial. The C-terminal domain of Mfd has dedicated ATPase and the role of the NTD appears to be only in UvrA recruitment. Having ATPase activity at NTD may interfere with the recruitment process and the loss of ATPase activity in evolving Mfd seems to be crucial for its function. The role in UvrA recruitment is amply evident from the dominant negative effect of NTD expression on NER pathway.

Analysis of sequenced bacterial genomes revealed that Mfd is found in most of the genomes highlighting the importance of transcription coupled DNA repair for ensuring error free gene expression. In spite of this evolutionary conservation and the high degree of relatedness, the present findings reveal differences in Mfd between the organisms. These differences may indicate the
appropriate tailoring of the functions based on the nature of the genome (size, G+C content) and the transcription process. The high G+C content of mycobacterial genome [30], presence of a large number of sigma factors [31], varied promoter architecture [32] and slow rate of transcription [33,34] hint at some distinctive role of Mfd in \textit{M. tuberculosis}. Thus, the differences in the properties of MtbMfd could be due to mycobacteria specific optimization in its function. All mycobacterial genomes sequenced have Mfd, highly homologous to that of MtbMfd and hence, are likely to display similar properties. Moreover, the gene is located at a fixed location between TetR and MazG regulatory proteins in most of the mycobacterial genomes indicating its early existence in the genus pointing at its crucial intracellular role.

Materials and Methods

Bacterial strains and plasmids

\textit{Mycobacterium tuberculosis} H37Rv (\textit{M. tuberculosis}) strain was used as a source of genomic DNA for polymerase chain reaction (PCR) to amplify \textit{mfd} gene. AB1157 (thr leu thi lacY1 galK2 ara14 xyl5 metII kbglK1 proA2 his4 argE3 str31 tcrZ3 supE44) and its derivative UNCNOTMFD (same as AB1157 but mfd\textsuperscript{D}, kan\textsuperscript{R}) was obtained from New England Biolabs and used for transformation and purification of plasmid DNA. BL21 (DE3) pLysS (F\textsuperscript{ompT} hsdSB (rB\textsuperscript{2} mB\textsuperscript{2}) gal dcm (DE3) pLysS (Cam\textsuperscript{R}) and its derivatives TUNER and ER2655 were used for overexpression and purification of proteins. pET32a, pET28a, pET21a, pET14b (Novagen) pRSETA (Invitrogen) and pTrc99A (New England Biolabs) plasmids were used for cloning of \textit{Mtbmfd} gene and its derivatives.

Chemicals, oligonucleotides and radiolabeling

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase were obtained from New England Biolabs. Ampicillin, kanamycin, tetracycline, streptomycin, protease \textit{K}, trypsin, V8 protease, protease inhibitor cocktail, Coomassie brilliant blue (R-250), IPTG were obtained from Sigma (USA). \textsuperscript{14}C chloramphenicol (57.0 mCi/mmol) and \textsuperscript{32}P-ATP (3500 Ci/mmol) were procured from GE healthcare (Uppsala, Sweden) and BRIT India respectively. All other reagents used were ultra pure, analytical or molecular biology grade.
Oligonucleotides used in this study were synthesized by Sigma Genosys, and their sequences given in Table 1. Oligonucleotides were labeled at the 5’-end with [γ-32P] ATP (20 μCi) using T4 polynucleotide kinase. The labeled oligonucleotides were purified by nucleotide removal kit (Qiagen, USA) and DNA was further purified from native polyacrylamide gels.

Amplification and cloning of M. tuberculosis mfd (Mtbmfd)

The ~5.7 kb long Mtbmfd was amplified in three different fragments F1 (1.3 kb), F2 (1.5 kb) and F3 (0.9 kb) by PCR. M. tuberculosis H37Rv genomic DNA was taken as a template and primer pairs used were PF1-PR1, PF2-PR2 and PF3-PR3 respectively (Table 1). The primers were designed based on the annotated complete genome sequence of M. tuberculosis [30]. All three fragments were cloned sequentially into pET32a vector to obtain full length product without altering the nucleotide sequence of Mtbmfd gene. The full length Mtbmfd was sub-cloned into the bacterial expression vector pET28a (pETmfd) and pTRc99A (pTrcmfd) using the restriction enzyme sites NdeI-HindIII and BamHI-HindIII respectively. A construct containing the NTD of Mtbmfd was generated by cloning the F1 fragment into pET14b using Ndel-KpnI sites. CTD was generated by releasing 1.9 kb Mtbmfd (pTrcmfd) using the restriction enzyme sites NdeI-HindIII and BamHI-HindIII respectively. A construct containing the C-terminal deletion (104 aa) was generated using specific primer set PF3-PR3A (Table 1) and replaced in place of wild type F3 fragment in pETmfd clone. MidD778A having point mutation in Walker B motif of the ATPase domain was generated by meganuclease digestion of pETmfd clone. MfdD778A having C-terminal deletion (104 aa) was generated using specific primer set PF3-PR3A (Table 1) and replaced in place of wild type F3 fragment in pETmfd clone. MidD778A having point mutation in Walker B motif of the ATPase domain was generated by meganuclease digestion of pETmfd clone.

UV-survival assay

Wild-type E. coli (AB1157) was used as a control and mfd deficient E. coli strain (UNCNOMFD) was transformed with either pTrcmfd construct containing Mycobacterium mfd or pTrc99A vector alone. An overnight grown culture was inoculated into LB medium and grown to 0.6 OD600 nm. 10 ml of each culture were pelleted down, dissolved in half the volume of ice cold normal saline (0.9% NaCl) and irradiated with UV light (1 J/m2) for different time points. Different dilutions of each culture (± UV) were plated on LB agar with appropriate antibiotics. Twelve hours later, colonies were counted and survival (S/S0) was measured and plotted against time. All the procedures after UV irradiation were carried out in dark.

To analyze the effect of NTD expression on UV survival, similar methodology was used with minor modification. After UV irradiation different dilutions of culture (1 μl) were spotted on agar plate containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol and incubated overnight at 37 C. Formation of colonies were observed and documented.

Road-block reporter assay

AB1157 or UNCNOMFD cells were transformed with pRBCGAT1 [20] construct along with pTrcmfd or pTrc99A, grown at 37°C in 2XYT medium. After cells were grown to 0.6 OD600 nm. 3 ml of cultures were harvested by centrifugation and cells lysed with 180 μl of TME buffer (25 mM Tris-Cl pH 8.0, 2 mM β-mercaptoethanol and 1 mM EDTA) supplemented with 20 μl of 1 mg/ml lysozyme and 6 μl of 1 mg/ml DNaseI. The mixture was incubated for 5 minutes at room temperature followed by freeze thawing in liquid nitrogen and spin at 12,000 rpm for 15 minutes. Supernatants were stored in equal amount of storage buffer (20 mM Tris-Cl pH 0.0, 200 mM NaCl, 20 mM mercaptoethanol and 90% glycerol) and snap frozen at −80°C. CAT activity was measured in Tris-Cl buffer pH 0.0 containing 3 mg/ml acetyl coA, 500 ng of protein, 10 mM chloramphenicol (radioactively labeled [14C]chloramphenicol was used as tracer) at 37°C for 30 minutes and stopped by the addition of 500 μl of ethyl acetate. The ethyl acetate phase was separated in fresh tube, dried in speed vac and 5 μl was loaded onto silica plate. The plate was developed in a chamber saturated with chloroform: methanol (95:5) and exposed to phosphor Imager and quantified by image gauge software. CAT activities are expressed as nmol of chloramphenicol acetylated/min/mg of protein.

Purification of MtbMfd

E. coli BL21 (DE3) pLysS TUNER cells harboring the pETmfd construct were grown at 37°C in TB broth containing 30 μg/ml kanamycin and 35 μg/ml chloramphenicol to an OD600 of ~0.6 and induced by the addition of 0.3 mM IPTG. After 10 hrs of incubation at 18°C, bacterial cells were harvested by centrifugation at 8,000 rpm for 10 min, the pellet was resuspended in buffer A (50 mM Tris-Cl pH 8.0, 500 mM NaCl, 5 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol and 0.01% triton X11) supplemented with 1 mM PMSF and EDTA-free protease-inhibitor cocktail (Sigma) and lyzed by sonication. For purification, cell free lysate was obtained by centrifugation of 20,000 rpm for 1 hr. Lysate was loaded onto a Ni2+-NTA column (Amersham Biosciences) and eluted using a 15–300 mM imidazole linear gradient. Fractions containing MtbMfd were pooled and dialyzed against buffer B (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM EDTA and 10 mM β-mercaptoethanol). MtbMfd containing fractions were further purified on a Heparin-sepharose column (Amersham Biosciences) using a linear gradient from 100–400 mM NaCl and finally by size-exclusion chromatography on a Superdex 200 column (Amersham Biosciences) in a buffer B consisting 500 mM NaCl. The purity of the protein was analyzed on SDS-PAGE and by silver-staining and concentration of protein was determined by measuring OD at 280 nm as well as by Bradford method [36]. Variants of MtbMfd protein were essentially purified using the same protocol. For NTD purification Q-sepharose column was used instead of heparin-sepharose.

Generation of polyclonal antibodies against MtbMfd

Antibodies were raised in rabbit by injecting 500 μg of denatured MtbMfd (native protein) with an equal volume of Freund’s complete adjuvant subcutaneously. ~3 ml of pre-immune serum was collected on day 1 prior to injection. Approximately 300 μg of protein with Freund’s incomplete adjuvant was injected after three weeks (21 days) as first booster dose. The second booster dose was given similarly with 300 μg of protein after 15 days interval after the first booster dose. After 7 days of second booster, the rabbit was bled, blood was collected, centrifuged and serum stored in aliquots at −20°C.

Western blot analysis

For Western blot analysis, E. coli lysate or purified recombinant proteins were subjected to 10% SDS-PAGE containing 0.1% SDS after solubilization with buffer B. Proteins were transferred on to a nitrocellulose membrane Hybond-C for 2 hr at 200 mA in transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, and 20% methanol). After the transfer, the membrane was blocked with blocking solution. The membrane was immuno stained with polyclonal rabbit anti-MtbMfd antibodies (1:10000 dilutions) for 2–3 hr at room temperature followed by three times washing with...
PBST (10 min each). The membrane was further stained with secondary antibody, anti rabbit IgG tagged with HRP for one hour followed by washing for three times with PBST [35]. The blot was developed using ECL kit (GE, Amersham).

Size exclusion chromatography

Native molecular mass of MtbMfd and its variants was determined by gel filtration chromatography. Superose 6 column was equilibrated in buffer B. The void volume \( V_0 \) of the column was determined using blue dextran (20,000 kDa) and was found to be 7.5 ml and the bed volume 24 ml. The column was calibrated with suitable molecular weight markers ranging from 66 kDa to 669 kDa; thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (150 kDa) and bovine serum albumin (66 kDa). Fractions (0.5 ml each) were collected and the presence of protein was confirmed by SDS-PAGE and by Bradford's method [36]. The elution volumes \( V_e \) of marker proteins and wild-type or mutant MtbMfd were determined. The molecular mass was calculated from plot of \( V_e / V_0 \) versus log molecular weight. The molecular weights corresponding to the peaks of MtbMfd and its derivatives were calculated from the standards graph using graph pad prism software.

Glutaraldehyde cross-linking

MtbMfd (4 µg/reaction) was incubated with increasing amounts of glutaraldehyde to a final concentration range of 0.01–0.1%, on ice for 20 minutes. Reactions were stopped by adding SDS gel loading dye and products were separated on a 4.5% denaturing polyacrylamide gel by electrophoresis, and visualized by silver-staining.

Analysis of NTD-ATP interaction by Fluorescence Spectroscopy

Fluorescence emission spectra were measured for NTD of MtbMfd on a Shimadzu, RF 5000 spectrophotometer using a 1-cm quartz cuvette at 25°C. The emission spectra were recorded over a wavelength of 300–400 nm with an excitation wavelength of 280 nm. NTD was allowed to equilibrate for 2 min in ATPase buffer (without MgCl₂) before measurements were made. Small aliquots of ATP (final concentration 100 µM-1 mM) were added to NTD (1 µM) before recording the spectra. The binding of ATP to proteins resulted in quenching of tryptophan fluorescence. The slit widths of 10 nm for excitation and emission were used and each spectrum recorded was an average of three scans. Data analyzed according to Stern-Volmer relationship which is represented by

\[
\frac{F_0}{F} = 1 + K_{sv}[Q]
\]

where \( F_0 \) and \( F \) are fluorescent intensities in the absence and presence of ATP respectively, \( K_{sv} \) is the Stern-Volmer constant and \( Q \) is the quencher (ATP) concentration [37].

ATPase assay

ATPase activity of MtbMfd and its mutants were assayed as described previously [38] with minor modifications in 10 µl of buffer containing 40 mM HEPES pH 8.0, 50 mM KCl, 5 mM DTT, 8 mM MgCl₂ and 2 mM ATP, 100 µg of bovine serum albumin per ml, 4% glycerol and 6% polyethylene glycol 6000. pUC 19 DNA (1 µM) was used in the reaction mixture unless otherwise specified, MtbMfd and its mutant proteins were included at a concentration of 250 nM and \([\gamma^{32}P]\) ATP was used as a tracer. Reactions were carried out at 37°C for 30 minutes, terminated by the addition of 2 µl of 50 mM EDTA and 0.5 µl of each reaction mix was spotted on a PEI-Cellulose TLC sheet. TLC sheet were developed in 1.2 M LiCl and 0.1 mM EDTA, exposed to Fuji BAS phosphor screen, scanned using Fuji Phosphor Imager and quantified by image gauge software. All kinetic parameters were measured under steady state conditions using linear regression analysis with the help of Prism v.5 software. All enzymatic assays were carried out at least three times.

Translocase or TFO (triplex forming oligonucleotide) displacement assay

Translocase assays were carried out essentially as described previously [23] with minor modifications. A 72 mer oligonucleotide containing triplex forming region was cloned into pUC19.
vector in EcoRI-HindIII sites. A 300 base pair fragment was released from pUC19 using PvuII enzyme. End labeled \(([^{32}p]-\gamma\text{ATP}\)) 22-mer oligonucleotide was incubated with 300 mer dsDNA in MES buffer pH 5.5 containing 10 mM MgCl₂ at 20°C overnight. The tripled forms was purified from native polyacrylamide gel and used as a substrate in the translocase assay. 250 nM of each protein (MtMfd, CTD and MfdΔC) were separately incubated with tripled DNA in 50 mM Tris-Cl (pH 8.0) containing 10 mM MgCl₂, 2 mM ATP and 1 mM DTT at 20°C and aliquots were taken at different time points. Reactions were stopped by adding GSMB buffer (15% glucose, 3% SDS, 250 mM MOPS pH 5.5 and 0.4 mg/ml bromphenol blue) and separated on 5% native polyacrylamide gel in TAE buffer (pH 5.5) containing 5 mM MgCl₂ and 5 mM sodium acetate at 4°C and visualized by Fuji Phosphor Imager. Quantitation was carried out using image gauge software.

Growth curve analysis of NTD expressing E. coli cells

pET14b vector carrying NTD construct (pETNTD) was transformed in BL21 pLysS cells. Colonies were picked and grown overnight at 37°C. Cells transformed with empty vector were taken as control. The overnight grown culture was used as a primary inoculum (1%) and inoculated in to 100 ml of LB media containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol and allowed to grow at 37°C. Aliquots were taken at every 30 min time intervals and OD was monitored at 600 nm. Growth curves were obtained by plotting OD₆₀₀ nm in Y-axis and time in X-axis.

For growth on the solid medium, equal number of cells from log phase cultures were taken from each sample and streaked on the agar plates having suitable antibiotics. Plates were incubated overnight at 37°C, formation of colonies was observed and documented.

Determination of mutation frequencies for Rif⁰→Rif⁰ι spontaneous mutations

The frequencies of rifampicin resistant mutants were determined by plating the overnight grown culture on plates containing ampicillin plus rifampicin (100 μg/ml). Duplicate samples were also plated on LB agar with ampicillin (100 μg/ml) to determine the cell viability. Plates were incubated at 37°C overnight, for scoring the rifampicin resistant colonies. Fold change was calculated by dividing the number of Rif⁰ colonies by that of total number of colonies [25].

Supporting Information

Figure S1 Gel filtration chromatography of MtMfd under different conditions and their elution profiles. A. Profile of MtMfd and Thryoglobulin, when both the proteins were co-injected into the column. 1 & 2 represents peak 1 & peak 2 and their retention volume are indicated on top of the peaks. The panel below shows the SDS-PAGE profile of the same, where peak 1 retains both the proteins and peak 2 contains only MtMfd. B. MtMfd (250 μg). C. MtMfd (700 μg). D. MtMfd (700 μg) in presence of 500 mM NaCl. Retention volumes are indicated on the top of the respective peaks; where 7.5 ml corresponds to void volume of the column; 11.3 ml is the retention volume of hexameric species and 14.6 ml is the retention volume of the monomeric species of the MtMfd.

(TIF)

Figure S2 Gel filtration chromatography of crude cell lysate of M. tuberculosis Ra and native MtMfd.

A. Elution profile of total proteins present in crude cell lysate of M. tuberculosis Ra. B. Elution profile of purified untagged or native MtMfd (400 μg), peak 1 & 2 corresponding to hexamer and monomer of MtMfd respectively. Retention volumes of respective peaks are indicated on the top of each peak. Peak at 7.5 ml corresponds to void volume of the column.

(TIF)

Figure S3 Comparison of ATPase activity of hexamer and monomer of MtMfd. Hexamer and monomer fractions of MtMfd was separated by gel filtration chromatography and subjected to ATPase assay, data was quantified by image gauge software. Specific ATPase activity was expressed as pmoles ATP hydrolyzed per min per μg of protein.

(TIF)

Figure S4 Gel filtration chromatography of MtMfd in presence of ATP and DNA. 700 μg of MtMfd was incubated with excess of ATPyS or DNA or both ATPyS and DNA for 30 min on ice and then co-injected into the column. Elution profiles were then monitored. A. MtMfd alone. B. ATPyS. C. pUC19 DNA. D. MtMfd+ATPyS. E. MtMfd+ATPyS+S+UCDNA. Retention volumes are indicated on top of the respective peaks.

(TIF)

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Author Contributions

Conceived and designed the experiments: SP DNR VN. Performed the experiments: SP. Analyzed the data: SP DNR VN. Contributed reagents/materials/analysis tools: DNR VN. Wrote the paper: SP DNR VN.

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