Structural analyses of the MazEF4 toxin-antitoxin pair in Mycobacterium tuberculosis provide evidence for a unique extracellular death factor

Do-Hwan Ahn1, Ki-Young Lee2, Sang Jae Lee1, Sung Jean Park1, Hye-Jin Yoon3, Soon-Jong Kim1, and Bong-Jin Lee1

From the 1Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Gwanak-gu, Seoul 151-742, the 2College of Pharmacy, Gachon University, 534-2 Yeonsu-dong, Yeonsu-gu, Incheon, the 3Department of Biophysics and Chemical Biology, College of Natural Sciences, Seoul National University, Seoul 151-742, and the 4Department of Chemistry, Mokpo National University, Chonnam 534-729, Republic of Korea

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The bacterial toxin-antitoxin MazEF system in the tuberculosis (TB)-causing bacterium Mycobacterium tuberculosis is activated under unfavorable conditions, including starvation, antibiotic exposure, and oxidative stress. This system contains the ribonucleolytic enzyme MazF and has emerged as a promising drug target for TB treatments targeting the latent stage of M. tuberculosis infection and reportedly mediates a cell death process via a peptide called extracellular death factor (EDF). Although it is well established that the increase in EDF-mediated toxicity of MazF drives a cell-killing phenomenon, the molecular details are poorly understood. Moreover, the divergence in sequences among reported EDFs suggests that each bacterial species has a unique EDF. To address these open questions, we report here the structures of MazF4 and MazEF4 complexes from M. tuberculosis, representing the first MazEF structures from this organism. We found that MazF4 possesses a negatively charged MazE4-binding pocket in contrast to the positively charged MazE-binding pockets in homologous MazEF complex structures from other bacteria. Moreover, using NMR spectroscopy and biochemical assays, we unraveled the molecular interactions of MazF4 with its RNA substrate and with a new EDF homolog originating from M. tuberculosis. The EDF homolog discovered here possesses a positively charged residue at the C terminus, making this EDF distinct from previously reported EDFs. Overall, our results suggest that M. tuberculosis evolved a unique MazF and EDF and that the distinctive EDF sequence could serve as a starting point for designing new anti-tuberculosis drugs. We therefore conclude that this study might contribute to the development of a new line of anti-tuberculosis agents.

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This article contains supplemental Table S1 and Figs. S1–S6.

The atomic coordinates and structure factors (codes 5XE2 and 5XE3) have been deposited in the Protein Data Bank (http://wwpdb.org/).

The chemical shift assignments were deposited in BioMagResBank (http://www.bmrb.wisc.edu) under the accession number 27233.

1 To whom correspondence should be addressed. Tel.: 82-2-880-7869; Fax: 82-2-889-1568; E-mail: ljbj@nmr.snu.ac.kr.

Approximately one-third of the world’s population is infected with tuberculosis (TB), which is the second most common cause of death from infectious disease after HIV (1). Notably, only 5–15% of the infected population develops clinical manifestations of active TB disease (2), whereas the remaining infected population manifests the latent tuberculosis infection (LTBI), which is typified by the slow-growing and/or non-replicating state of Mycobacterium tuberculosis in host cells (3). In this state, the pathogen is less susceptible to the host immune system and conventional anti-tuberculosis agents that target the biologically active M. tuberculosis (4). For this reason, it is necessary to develop drugs that specifically target proteins involved in LTBI. The toxin-antitoxin (TA) system is a promising drug target for TB treatments, as it can induce LTBI that is characterized by growth arrest and bacterial persistence (5). The TA system is activated under unfavorable conditions such as starvation, antibiotic treatments, high temperatures, DNA damage, and oxidative stress (6–8). Currently, TA systems are classified into 6 types, mainly based on how antitoxins neutralize the activities of the toxins (9). Among these TA systems, the type II TA system is the most common system in bacteria and archaea, especially in M. tuberculosis (10). In this system, the protein toxin is inhibited by binding of the protein antitoxin. Under normal conditions, the stable toxin is suppressed by its cognate labile antitoxin. However, stress conditions induce degradation of the antitoxin, and the liberated toxin subsequently exhibits its ribonucleolytic activity (11–13). Generally, type II TA systems are further categorized into subfamilies such as ccd, parDE, mazEF, yafNO, higAB, kid/kis, RelBE, and vapBC (14–17). M. tuberculosis possesses abundant type II TA systems, pertaining to the following families: vapBC, mazEF, yefM/yoeB, higBA, relBE, and parDE (10). At least seven MazF homologs have been identified in M. tuberculosis (18), and four MazF homologs have been confirmed to exhibit an endoribonuclease activity (19, 20). Unlike other type II toxins, MazF toxin recognizes and cleaves a specific sequence of intracellular
RNAs in a ribosome-independent manner (21). Due to its sequence specificity, MazF suppresses translation to a lesser degree than non-sequence-specific toxins and triggers a quasidormant state, during which cells are non-dividing but still are capable of protein synthesis (22). Recent studies have revealed that MazF is also involved in extracellular death factor (EDF)-mediated quorum sensing (23, 24). MazF activity (26), is conserved.

A β-barrel-like structure composed of six anti-parallel β strands, which is flanked by three helices defines the global structure of M. tuberculosis MazF4 in MazEF4 complex. Two MazF4 monomers interact with each other through hydrogen bonds and close hydrophobic contacts, mainly formed by residues placed on strands β3, β6 (β4 for chains B and D) and helix α2 (Fig. 2B). The homodimer formation buries ~1,510 A² of solvent-accessible surface area.

**Results**

**Overall architecture of the M. tuberculosis MazEF4 complex**

The MazEF4 complex consists of a dimer of two heterotrimers composed of two MazF4 molecules and one MazE4 molecule (Fig. 1A). The hydrogen bonds that take part in MazE4 and MazF4 heterotrimer formation are clustered on helices α1 and η1 and strands β3 and β6 (β4 for chains B and D) of MazF4. The counterpart residues on MazE4 are clustered on helices α2 and α3 (Fig. 2A, left panel). Residues on loops between strands β1 and β2 (S1–S2 loop) and between strands β3 and β4 (S3–S4 loop) of MazF4 are also involved in MazEF4 heterotrimer formation via hydrophobic interactions (Fig. 2A, right panel). The assembly of MazE4 and MazF4 in the M. tuberculosis MazEF4 complex is highly similar to the assembly of MazE and MazF in the Bacillus subtilis MazEF complex (PDB code 4ME7) (29), which is one of only two MazEF complex structures published to date (Fig. 1B). The other available MazEF complex structure is the E. coli MazEF complex (PDB code 1UB4) (30) in which MazE interacts with MazF via its long C-terminal loop instead of the helix α3 (Fig. 1B) observed in the MazE antitoxins from M. tuberculosis and B. subtilis. Although the overall E. coli MazE structure is completely different from M. tuberculosis and B. subtilis MazEs, His⁶⁶ of E. coli MazE superimposes well with Tyr⁶¹ of B. subtilis MazE and Tyr⁷⁶ of M. tuberculosis MazE, which are essential for MazEF interaction. All MazEF structures form a heterohexamer in a molar ratio of 4 MazFs:2 MazEs.

**Structure of the M. tuberculosis MazF4 toxin in the MazEF4 complex**

A β-barrel-like structure composed of six anti-parallel β strands, which is flanked by three helices defines the global structure of M. tuberculosis MazF4 in MazEF4 complex. Two MazF4 monomers interact with each other through hydrogen bonds and close hydrophobic contacts, mainly formed by residues placed on strands β3, β6 (β4 for chains B and D) and helix α2 (Fig. 2B). The homodimer formation buries ~1,510 A² of solvent-accessible surface area.

**Structure of the M. tuberculosis MazE4 antitoxin in the MazEF4 complex**

The model built for the M. tuberculosis MazE4 antitoxin consists of three α helices (Fig. 1C, right panel), showing an obvious electron density for residues Met¹⁹–Ala³⁹ among a total of 100 residues. Close hydrophobic contacts between two MazE4 N termini allow MazE4 homodimer formation burying ~2,390 A² of solvent-accessible surface area. Overall, the MazE4 structure is highly similar to the MazE structure in the B. subtilis MazEF complex (PDB code 4ME7), which shows a ribbon-helix-helix (RHH) motif at the N terminus. However, strand β1 of the RHH motif is absent in the crystal structure of MazE4 due to a poor electron density for the corresponding residues at the N terminus. The RHH motif is not shown in the crystal structure for MazE in the E. coli MazEF complex (PDB code 1UB4), but it forms an intertwined barrel structure that is also a typical DNA-binding fold. The invisible N-terminal...
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region of MazE4 is believed to form a β strand as part of the RHH motif.

**Structural comparison between homologous MazFs**

The architecture of *M. tuberculosis* MazF4 is defined as a β-barrel-like structure formed by seven anti-parallel β strands flanked by three helices (Fig. 1C, left panel). Homodimer formation is mainly driven by the hydrogen bonds, ionic interactions, and hydrophobic interactions that occur between strand β6, helix α1, and helix α2, burying ~1,835 Å² of solvent-accessible surface area (Fig. 2C). A homolog search using the DALI server (31) found several *M. tuberculosis* MazF4 homologs, which are the PemK-like, MazF-like toxins. The closest structural homologs are (i) MazF from *B. subtilis* (PDB code 4MDX (chains A and B); r.m.s. deviation of 1.2 Å for 101 equivalent Cα positions, Z-score of 16.9, and sequence identity of 29%) (29), (ii) MazF from *M. tuberculosis* (PDB code 5HK3 (chains A and B); r.m.s. deviations of 1.3–1.5 Å for 97 equivalent Cα positions, Z-scores of 16.6–16.7, and sequence identity of 27%) (33). Although *M. tuberculosis* MazF4 shows high Z-scores with its structural homologs, it has a remarkably shorter S1–S2 loop than that of its homologs (Figs. 3, A and B). The less stringent sequence specificity of MazF4 (19) is believed to result from the shorter S1–S2 loop, which is proposed by Hoffer et al. (27) as the loop is not long enough to reach the downstream of the RNA substrate where it is significant for sequence specificity of MazF (34). Interestingly, one of the major MazE-binding pockets (the area named as Site 1 in former studies (33)) of MazF4 is negatively charged, which is opposite to that of structural homologs (Fig. 4, A and B) with the exception of the *M. tuberculosis* MazF3 structure (PDB code 5UCT) (27). The less stringent sequence specificity of MazF4 (19) is believed to result from the shorter S1–S2 loop.

**Figure 1.** Overall structure of *M. tuberculosis* MazF4 and MazF4 in complex with MazE4. A, the structure of the *M. tuberculosis* MazEF4 heterohexamer. The models of the MazF4 toxin are shown as pale orange (chains A and C) and pale cyan (chains B and D), respectively. The models of the MazE4 antitoxin are shown as light pink. The disordered regions (Thr44 to Arg47) of the loop connecting β3 and β4 are depicted as dotted lines. B, structural comparison of the *M. tuberculosis* MazEF4 heterohexamer with other MazEF complexes (*B. subtilis* MazEF (PDB code 4ME7); *E. coli* MazEF (PDB code 1UB4)). The models of the MazF toxin and MazE antitoxin are colored blue and pale pink, respectively. C, schematic presentation of the *M. tuberculosis* MazF4 monomer (pale green) and *M. tuberculosis* MazE4 monomer (light pink) in the MazEF4 complex. The α-helices, β-strands, and a 3_10 helix of the MazF4-fold are labeled α1 to α7, β1 to β7, and η1, respectively. The disordered regions of the loops are depicted as dotted lines. D, the structure of the *M. tuberculosis* MazF4 homodimer. The models of the MazF4 toxin are shown as pale green (chain A) and pale yellow (chain B). The disordered regions (Gly15 to Gly17) of the loop connecting β1 and β2 are depicted as dotted lines.
5), their counterpart MazE4 residues are mainly positively charged.

**Structural changes in *M. tuberculosis* MazF4 upon binding to MazE4 reveal the mode of MazF4 neutralization**

The overall-fold of *M. tuberculosis* MazF4 upon MazE4 binding remains at large. However, the structural comparison between MazE4-bound MazF4 and MazE4-free MazF4 shows noticeable differences on the S1–S2 loop, S3–S4 loop, and dimerization interface. The S1–S2 loop, which shows no clear electron density map for MazE4-free MazF4, is believed to be flexible (Fig. 1C, left panel). In contrast, the electron density for the S1–S2 loop is visible in the MazEF4 complex. The S1–S2 loop of each MazF4 monomer forms hydrophobic contacts with MazE4 helix α3 (Ala86 and Ala99, respectively). These interactions induce S1–S2 loop closure and mask the entrance that leads into a hydrophobic pocket, where the active site is located (Figs. 2A, right panel, 3B, and supplemental Fig. S1). In contrast to the disordered-to-ordered transition of the S1–S2 loop upon binding to MazE4, the S3–S4 loop of two MazF4 monomers (chains B and D) becomes flexible (Figs. 1A and supplemental Fig. S1). Although, the S3–S4 loop of the other two monomers (chains A and C) still shows a clear electron density map, it moves toward helix H9251 of MazE4. These interactions are mainly attributed to hydrophobic interactions among residues Ile48, Pro49, and Trp51 of MazF4 and helix α2 of MazE4 along with the salt bridges formed between Arg46 of MazF4 and Asp72 of MazE4 (Fig. 2A). Apart from residues
placed on the S1–S2 and S3–S4 loops, residues comprising the toxin dimerization interface undergo changes as two toxin molecules rotate about their symmetric axis. The residues on strand H6 (Asn68, Ile69, and Thr71) are no longer involved in dimer formation, but a hydrogen bond is formed between Asn26 (chain A) and Thr98 (chain B) after MazE4 binding. This rearrangement increases the angle between two MazF4 monomers. However, the toxin dimerization interface does not directly interact with RNA. In addition to conformational changes, several hydrogen bonds are formed between MazE4 and residues on helix H2 and S5–S6 loop of MazF4 upon binding to MazE4. Structural equivalents of residues located on these 4 major MazF4-binding sites (S1–S2 loop, S3–S4 loop, helix H2, and S5–S6 loop) in the structures of the B. subtilis MazF–RNA complex (PDB code 4MDX) (29), S. aureus MazF–RNA complex (PDB code 5DLO), and M. tuberculosis MazF–RNA complex (PDB code 5HJZ) are involved in RNA substrate binding. This is consistent with our NMR titration result against uncleavable RNA (Fig. 6, A–C).

**Catalytic core of MazF4**

Superposition with the structures of B. subtilis MazF in complex with RNA (PDB code 4MDX) (29), E. coli MazF in complex with a substrate analog (PDB code 5CR2) (34), and S. aureus MazF in complex with a substrate analog (PDB code 5DLO) strongly suggests that two residues (Lys19 and Thr42) in M. tuberculosis MazF4 are involved in RNA catalysis (Fig. 3B). The recently deposited crystal structures of M. tuberculosis MazF9–RNA complex (PDB code 5HJZ) and M. tuberculosis MazF6–RNA complex (PDB code 5HK0) also show the residues that take part in RNA binding are similarly positioned. Additionally, the sequence alignment result shows that the two residues (Lys19 and Thr42) of M. tuberculosis MazF4 are well conserved in the structural homologs (Fig. 3A). In our NMR titration result against an uncleavable RNA substrate (5'-AUA-(dU)CGCUAUG-3'), Lys19 shows a significant intensity reduction (Fig. 6, A and B) and Thr42 shows moderate chemical shift perturbations (CSP, Fig. 6, A and B). Moreover, two mutants (MazF4 K19A and MazF4 T42A) show a reduction in the ribonuclease activity compared with the wild-type thus, Lys19 and...
Thr\textsuperscript{42} are believed to be the critical residues in catalysis (Fig. 7A). It is quite clearly stated by Zorzini et al. (34) that \textit{E. coli} MazF Thr\textsuperscript{52} (the equivalent of Thr\textsuperscript{42} in \textit{M. tuberculosis} MazF4) acts as general acid/base during charge-relay, whereas \textit{E. coli} MazF Arg\textsuperscript{29} residue (the equivalent of Lys\textsuperscript{19} in \textit{M. tuberculosis} MazF4) serves as a charge stabilizer along with Thr\textsuperscript{53} (the equivalent of Thr\textsuperscript{43} in \textit{M. tuberculosis} MazF4). The contribution of the Thr\textsuperscript{53} as a charge stabilizer in \textit{E. coli} MazF is supported by the fact that Thr\textsuperscript{53} is within hydrogen bonding distance to a non-bridging oxygen of the phosphate of DNA analog in \textit{E. coli} MazF–substrate analog complex structure (PDB code 5CR2). Thus, we assume that Thr\textsuperscript{43} and/or Thr\textsuperscript{44} of \textit{M. tuberculosis} MazF4 may also compensate the role of Thr\textsuperscript{42} in T42A mutant. This explains why T42A mutant shows only a little decrease in ribonuclease activity, whereas K19A shows a significant ribonuclease activity reduction. The NMR titration result also revealed other residues involved in RNA recognition (Fig. 6, A and B). The residues showing CSP and/or peak intensity reductions are located on the deep crevice between two MazF4 molecules and a cleft between the S1–S2 loop and S3–S4 loop (Fig. 6C). Leu\textsuperscript{60} and Glu\textsuperscript{70} are the residues, which display the largest CSP in our study. Leu\textsuperscript{60} is the structural equivalent of Phe\textsuperscript{69} in \textit{B. subtilis} MazF, which interacts with RNA via hydrophobic and van der Waal interactions.
Glu^{70} is located on β6, which corresponds to the β5–β6 loop, the major RNA recognition site in *B. subtilis* MazF in complex with the RNA structure (PDB code 4MDX) (29). This also agrees well with the trajectory of the RNA substrate in the crystal structures of the MazF homolog–RNA complexes (Fig. 3B).

Figure 6. *M. tuberculosis* MazF4 residues involved in RNA recognition. *A*, overlayed [^{1}H,^{15}N]-HSQC titration spectra of 0.1 mM ^{15}N-labeled MazF4 titrated with uncleavable RNA. Examples of peaks that exhibit large chemical shift changes following RNA titration are boxed and enlarged. The peak corresponding to Thr^{42} is circled. The arrows in the diagram indicate the direction of the chemical shift. Lys^{19} is indicated by an arrow. *B*, examples of peaks that exhibit chemical shift changes and intensity losses following RNA titration. Chemical shift changes of residues in MazF4 produced by RNA binding are plotted. Thr^{42} is indicated by an arrow. *C*, the ratio of the cross-peak intensities of residues in MazF4 produced by RNA binding is plotted against the residue number. Lys^{19} is indicated by an arrow. *B*, upper, the ratio of the cross-peak intensities of residues in MazF4 produced by RNA binding is plotted against the residue number. Lys^{19} is indicated by an arrow. *B*, lower, the ratio of the cross-peak intensities of residues in MazF4 produced by RNA binding is plotted against the residue number. Lys^{19} is indicated by an arrow. *C*, the chemical shift changes and reductions in the signal intensities produced by RNA titration were mapped onto the crystal structure of the MazF4 homodimer. The residues that showed the largest chemical shift changes are indicated in red, and the residues that showed moderate chemical shift changes are indicated in sky blue (*C*, upper). The residues that showed reductions in their signal intensities are indicated in purple (*C*, lower). Only one set of affected residues is colored for clarity.
The crystal structure of *M. tuberculosis* MazEF4

**In vitro ribonuclease assays in the presence of the EDF homolog**

The effect of the EDF homolog (ELWDR) on MazF4 ribonuclease activity was evaluated using fluorometric assays. For the assay, an 11-base long mRNA substrate that contains the MazF4-specific sequence (UCGCU) was selected based on a previously published report as UCGCU is the most common cleavage sequence of MazF4 confirmed by their primer extension (19). The fluorometric assay result showed an elevation of MazF4 activity in the presence of the EDF homolog (Fig. 7B). MazF4 mixed with MazE4 in the presence of the EDF homolog showed a higher ribonuclease activity than without the EDF homolog (Fig. 7C). The mixture of MazF4 and MazE4 in the absence of the EDF homolog still showed ribonuclease activity, which is probably due to a small fraction of free MazF4 dissociated from the MazEF4 complex. The increase in a ribonuclease activity upon EDF homolog addition is believed to result from both the elevated intrinsic MazF4 activity and inhibited MazEF4 complex formation.

**EDF homolog-binding site on MazF4**

NMR titration experiments of 0.1 mM 15N-labeled MazF4 with the EDF homolog were carried out. The result displayed chemical shift changes for several residues (Fig. 8A). The most perturbed residues were Ile69, Glu70, Thr71, and Gly73 on strand β6 (Fig. 8, B and C). The split peaks that correspond to Glu70 indicate a slightly different conformation of the two monomers constituting MazF4 homodimer in solution. *B. anthracis* MoxT-EDF models suggest a negatively charged residue of EDF, placed in proximity to Arg81 and Arg87 of MoxT, which are structural equivalents of Glu70 and Glu76 in MazF4, strongly increases the MoxT activity (33). It is interesting that the arginine residues that are likely to interact with EDF in the structural homologs are replaced with glutamates in MazF4. Moreover, the new EDF homolog (ELWDR) presented here bears an arginine residue at the fifth position, which possibly recognizes the glutamate residues. It is believed that the Glu70 residue is perturbed upon addition of the EDF homolog due to its interaction with Arg5 of the EDF homolog. As the third tryptophan...
residue of EDF is conserved across the species, the EDF homolog seems to bind to MazF4 in a similar conformation as other EDFs but with a swapped charge distribution. This was further investigated by generating E70R and E76R mutants. Our NMR titration experiments using the E70R and E76R mutants against Arg to Glu swap peptide (ELWDE) confirmed that there are interactions between the mutants and the Arg to Glu swap peptide (Fig. 9A). Moreover, we measured the ribonuclease activity of E70R and E76R mutants with increasing amounts of EDF homolog. As expected, ribonuclease activities of E70R and E76R mutants were not elevated upon addition of EDF homolog (Fig. 9B). The results support the idea that the electrostatic interaction between MazF4 and the EDF homolog is critical in increasing MazF4 activity. The peak shifts without resonance broadening on the 2D-[1H-15N]-HSQC spectra of MazF4 indicate that the EDF homolog binds to MazF4 in a fast exchange mode on the NMR time scale. This observation implies a weak interaction between MazF4 and EDF homolog, which is consistent with our in vitro ribonuclease assay results in that a large amount of EDF homolog was required to increase the activity of MazF4.

**Backbone assignments of the MazF4**

The backbone 1H, 13C, and 15N resonance assignments for 78 of 97 residues of *M. tuberculosis* MazF4 were carried out. There were no assignments for the S3–S4 loop and S7–H3 loop, where no secondary structural elements are present in the crystal structure of MazF4. Point mutations to alanine were introduced on residues Arg11 and Tyr16 (MazF4 R11A/Y16A) to improve the solubility of MazF4 for the acquisition of better 3D spectra. The 2D-[1H-15N]-HSQC (heteronuclear single quantum coherence spectroscopy) spectra of MazF4 and MazF4 R11A/Y16A are overlaid (supplemental Fig. S2). The 2D-[1H-15N]-HSQC spectrum of MazF4 R11A/Y16A with peak assign-
The crystal structure of \textit{M. tuberculosis} MazEF4

ments is shown in supplemental Fig. S3A. Based on the assignment, the secondary structures with regard to the MazF residues were predicted by the TALOS+ program (35). The result revealed the presence of three helices and seven β-strands in MazF4 (supplemental Fig. S3B).

Oligomeric state of the MazEF4 complex in solution

Oligomeric states of the MazEF4 complex were investigated using equilibrium analytical ultracentrifugation techniques at various speeds and concentrations. Supplemental Fig. S4 shows the data (circles) and fits for the homogeneous heterotrimer (3×, dotted line) and heterohexamer (6×, solid line) models, respectively. The weighted r.m.s. errors for the homogeneous 3× and 6× fits were 2.55 × 10⁻² and 7.05 × 10⁻³, respectively, indicating the superiority of the 6× model. In the the inset of supplemental Fig. S4, residual plots are also shown. Although the 6× model shows a random residual distribution, the 3× model shows systematic deviations at the beginning and end of the radial positions. The velocity sedimentation data (50,000 rpm) that showed a single c(s)² distribution and the equilibrium sedimentation data that were measured at different speeds (20,000 and 25,000 rpm) and concentrations (2.3 and 2.7 mM) also support the homogeneous heterohexameric (6×) model (data not shown). These data confirm that the MazEF4 complex exists as a heterohexamer in solution. This result is consistent with our crystal structure of \textit{M. tuberculosis} MazEF4.

Discussion

The significance of MazF4 and EDF from \textit{M. tuberculosis} for a new drug design

\textit{M. tuberculosis} MazF4 is known to specifically cleave pentad sequences that are relatively less presented in several genes such as PPE55 and PPE56 (19), thus the cleavage of the genes by MazF4 is believed to occur only in a certain condition. We speculate that suppression of the PPE protein expression requires hyperactive MazF4, which is accomplished by quorum sensing molecules such as EDF. PPE55 is reported to trigger immune responses in host and be expressed during subclinical TB infection but not in latent TB infection. PPE56 is highly homologous (67%) to PPE55 (36). As PPE proteins are reported to be the marker of the progression to active TB (36), it is believed that hyperactive MazF4 under high bacterial density prevents the progression to active TB. In other words, MazF4 is believed to serve as a safety precaution before switching to active TB from latent TB because it is not beneficial to move on to active TB in high bacterial density. The ability of MazF4 as a quorum sensor is expected to be exploited by incorporating EDF externally so that MazF4 exhibits hyperactivity even in low bacterial density. In that environment, the pathogen is unable to enter the active TB state even in favorable conditions. That would keep the bacterial density low and then eventually cause the clearance of the pathogen. We expect that modifications based on the sequence of the EDF homolog could conceve new anti-tuberculosis drug candidates.

The distinct charge distribution of MazF4 implies the independence of \textit{M. tuberculosis}

MazF4 displays a negatively charged pocket where positively charged MazE4 residues bind. This charge distribution is opposite in \textit{B. subtilis} and \textit{E. coli} MazEF complexes, which show a positively charged pocket on MazF where negatively charged MazE residues bind. Interestingly, the new EDF homolog from \textit{M. tuberculosis} we present here has an arginine residue at the fifth position. The positively charged cluster near the S1–S2 loop formed by Arg⁸¹ and Arg⁸⁷ in \textit{B. anthracis} MoxT is required to be occupied by a negatively charged residue for the activity enhancement (33). As the cluster is substituted with negatively charged residues in \textit{M. tuberculosis} MazF4 (Glu⁷⁶ and Glu⁷⁸), arginine residue at the fifth position is believed to be critical for enhancement of the MazF4 activity. The swapped charged MazF-EDF interaction shown in the MazF4 case suggests that \textit{M. tuberculosis} have developed a novel EDF and its corresponding MazF for the advantage of its own survival without being disturbed by other species.

Biological implication of \textit{M. tuberculosis} MazF4 activity enhancement by the EDF homolog

Our NMR result shows that the MazF4 residues involved in binding to the EDF homolog are located at the bottom of the S1–S2 loop (Figs. 5 and 8C). This region corresponds to an area called Site 1 (Fig. 4), which was named in previous MazF studies (30, 33). This agrees well with the \textit{E. coli} EDF and EDF-like peptide docking studies performed on \textit{E. coli} MazF and \textit{B. anthracis} MoxT (26, 33). In the peptide docking model of MoxT, the opening of the S1–S2 loop is required for peptide binding at the bottom of the S1–S2 loop of MoxT, as the area is buried under the S1–S2 loop (33). Therefore, the S1–S2 loop opening is believed to be a mechanism by which the peptide increases the ribonuclease activity of MoxT. The \textit{M. tuberculosis} EDF homolog probably opens the S1–S2 loop in a similar manner to that shown in the MoxT-peptide model. Considering the size of the EDF homolog, the hydrophobic cavity wherein the catalytic residues are located is unlikely to be affected. Taken together, the EDF homolog placed at the bottom of the S1–S2 loop is believed to keep the loop from masking the catalytic site, but not to interact with the catalytic residues. We assume that reorientation of the S1–S2 loop renders the catalytic cavity more exposed, and this allows the substrate RNA to access the active site of MazF4 more easily and subsequently increase the ribonuclease activity of MazF4. Additionally, Site 1 is where the major interaction between MazE and MazF is observed (Fig. 5). This supports our assumption that the EDF homolog hinders MazEF4 complex formation by occupying the MazE4-binding site on MazF4. Overall, our in vitro data suggest that \textit{M. tuberculosis} MazF4 with an elevated activity by the EDF homolog probably induce the bactericidal stage to lessen the bacterial density. As the effect of EDF is advantageous in high population density, treatment of the pathogen in low population density such as LTBI with EDF would disrupt the bacterial survival.
The tight regulation of *M. tuberculosis* in LTBI

Interestingly, the *E. coli* G6PD gene also shows a high similarity with the *M. tuberculosis* G6PD 2 gene (zwf2) in its nucleotide sequence, but the *M. tuberculosis* G6PD 2 gene does not contain an *M. tuberculosis* MazF4-cleavable nucleotide sequence behind the EDF-like sequence (PIWNA) codon. Therefore, this peptide is believed to not be generated by MazF4. Although it is unclear whether or not the peptide fragment (PIWNA) that exists in the *M. tuberculosis* G6PD 2 sequence is generated by other MazF toxins from *M. tuberculosis*, our NMR titration result shows the absence of an interaction between MazF4 and the peptide (supplemental Fig. S5). Therefore, the interaction between MazF4 and the EDF homolog is specific. However, two G6PD isozymes and the relatively high number of maezEF pairs present in *M. tuberculosis* suggest a low likelihood of complete deprivation of G6PD, which is a key enzyme for nucleotide precursor production (37) upon maezEF pair activation in *M. tuberculosis*. Consequently, *M. tuberculosis* is believed to have a very complex regulation during the latently infected state for a long-term survival.

Experimental procedures

**Strains**

The *E. coli* strains used in this study included *E. coli* DH5α™, *E. coli* Rosetta™ 2 (DE3) pLysS, and *E. coli* C41 (DE3).

**Cloning**

The gene encoding *M. tuberculosis* (strain *H37Rv*) MazE4 (Rv1494) was cloned into expression vectors pET28a(+) (Novagen) and pET29a(+) (Novagen) to enable the production of His₆-tagged and intact MazE4, respectively. The gene encoding *M. tuberculosis* MazF4 (Rv1495) was cloned into modified expression vector pET28a(+) (Novagen) to enable the production of MazF4 fused with His₆-encoded soluble protein GB1 with a TEV cleavage site inserted between two proteins. The oligonucleotide primers used for PCR are listed in supplemental Table S1. Each MazE4 recombinant plasmid was transformed into *E. coli* Rosetta™ 2 (DE3) pLysS, and MazF4 and the MazF4 mutants were transformed into *E. coli* C41 (DE3).

**Growth conditions**

Each colony was separately subcultured at 37 °C overnight in 5 ml of LB media supplemented with kanamycin and subsequently transferred to 1 liter of LB media supplemented with kanamycin. Each culture was induced by the addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at an OD of 0.5. The cultures of MazF4 and its mutants were grown at 37 °C for 4 h, and the cultures of MazE4 were grown at 15 °C for 24 h after induction. For the preparation of selenomethionine (SeMet)-substituted MazF4, cells were grown in M9 cell culture medium containing extra amino acids, including SeMet. For the preparation of ¹⁵N-labeled MazF4 and ¹⁵N,¹³C-labeled MazF4 mutants, cells were grown in M9 medium containing ¹⁵NH₄Cl and/or [¹³C]glucose as stable isotope sources. Each culture was harvested by centrifugation at 5,600 × g. The resulting pellets were stored at −80 °C.

**Protein purification**

Each culture of MazF4 and its mutants was lysed by sonication in buffer A (20 mM Tris-HCl, pH 8.0, and 500 mM NaCl) containing 10% (v/v) glycerol and protease inhibitor mixture tablets (Roche Applied Science) and centrifuged to remove cellular debris at 17,900 × g. The supernatant was applied to an affinity chromatography column of nickel-nitrilotriacetic acid-agarose (Novagen) equilibrated in buffer A. The protein was eluted with buffer A containing 500 mM imidazole following a wash with 100 ml of buffer A containing 40 mM imidazole. Then, the eluted protein was concentrated to 3 mg ml⁻¹ prior to the addition of 100 units of TEV protease (Sigma) for His₆-tagged GB1 removal. After the cleavage was completed, the mixture was dialyzed against buffer A and subjected to an affinity chromatography column of nickel-nitrilotriacetic acid-agarose (Novagen) equilibrated in buffer A once again to obtain intact MazF4 in the eluate. The sample was further purified by size-exclusion chromatography on a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer, pH 7.8, containing 150 mM NaCl. The cultures of intact MazE4 and GB1-fused MazF4 were mixed together for MazEF4 complex production, and the aforementioned purification procedure for MazF4 production was applied. To acquire MazE4 alone, the supernatants of His₆-tagged MazE4 was also applied to an affinity chromatography column of nickel-nitrilotriacetic acid-agarose (Novagen) after lysis in buffer A. The His₆-tagged MazE4 was eluted with buffer A containing 500 mM imidazole following a wash with 100 ml of buffer A containing 40 mM imidazole. MazE4 was dialyzed against 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl for further experiments. The recombinant MazEF4 complex substituted with SeMet was obtained through the same procedure applied for native MazEF4 complex production. Isotope-labeled samples were subjected to the same purification protocol used to produce native MazF4.

**Site-directed mutagenesis**

The mutations in MazF4 (K19A, T42A, E70R, E76R, and R11A/Y16A) were introduced by site-directed mutagenesis using mutagenic oligonucleotide primers (supplemental Table S1) and Rv1495 in modified expression vector pET28a(+) as a template. The R11A/Y16A mutant construct was obtained using a stepwise procedure. Reaction components were mixed and subjected to PCR guided by the manufacturer’s protocol (QuickChange Lightning Site-directed Mutagenesis Kit, Agilent). The PCR products were incubated with DpnI to digest the template DNA, and the products were transformed into competent DH5α cells. All point mutations were confirmed by DNA sequencing (Cosmogenetech, Seoul, Korea).

**Equilibrium sedimentation**

Equilibrium sedimentation studies were performed in 6-sec- tor cells using a Beckman ProteomeLab XL-A analytical ultra-centrifuge in 20 mM Tris-HCl buffer, pH 7.8, containing 500 mM NaCl and 1 mM DTT at 20 °C. The MazEF4 complex was measured at three different speeds (20,000, 25,000, and 30,000 rpm) and concentrations (2.3 μM (0.080 mg/ml), 2.7 μM (0.092 mg/ml), and 3.0 μM (0.10 mg/ml)). All measured data fit well to a homogeneous heterohexamer (6 ×, 4 MazF4–2 MazE4 com-
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plex) model, and a representative result measured at 30,000 rpm using a 3.0 µM (0.10 mg/ml) protein concentration is presented. The concentration of the protein complex (heterotrimer (3 × 2, MazF4–1 MazE4)) was calculated using $e_{280\text{nm}} = 56,380 \text{m}^{-1} \text{cm}^{-1}$, and a molecular mass of 34,692 daltons was calculated from its amino acid composition. The time required for the attainment of equilibrium was established through a run at a given rotor speed until the scans were invariant for 4 h: this was achieved by 48 h at most in the 6-sector cells. The partial specific volume of the heterotrimer complex and buffer density was achieved by 48 h at most in the 6-sector cells. The partial specific volume at 20 °C was 0.7295 cm$^3$/g, and the buffer density was 1.01924 g/cm$^3$. For data analysis by mathematical modeling using non-linear least-squares curve fitting, the following fitting function was used,

$$C_r = C_b \exp \left( A_M (r^2 - r_i^2) \right) + e_A (1 - \nu) w^2 / 2RT$$

(Eq. 1)

where, $C_r$ is the total concentration at the radial position $r$, $C_b$ is the concentration of protein at the cell bottom, $M_p$ is the molecular weight of the heterotrimer (3 × 2, MazF4–1 MazE4), $\nu$ and $r$ are the partial specific volume and the solution density, respectively. $w$ is the rotor angular velocity, and $\epsilon$ is a baseline error term. The selection of the model was made by examining the numbers of the weighted sum or square values and weighted root mean square error values. Further data handling and analysis by mathematical modeling were performed using MLAB$^2$ operating on the data analysis computer.

Crystallization and X-ray data collection

Crystals of native *M. tuberculosis* MazF4 and native, SeMet-labeled *M. tuberculosis* MazEF4 were grown using the sitting-drop vapor diffusion method at 20 °C. Each sitting-drop was prepared by mixing equal volumes (0.5 µl each) of the protein solution and the reservoir solution. The best crystals of native *M. tuberculosis* MazF4 were obtained using a reservoir solution of 0.2 M sodium malonate, pH 7.0, and 20% (w/v) polyethylene glycol 3,350. The best crystals of native *M. tuberculosis* MazEF4 were obtained using a reservoir solution of 0.1 M citric acid/sodium hydroxide, pH 4.0, and 0.8 M ammonium sulfate. The best crystals of SeMet-labeled *M. tuberculosis* MazEF4 were obtained using a reservoir solution of 0.2 M calcium acetate, 0.1 M Tris-HCl, pH 7.0, and 20% (w/v) polyethylene glycol 3,000.

The crystals of native MazF4 were mounted without any additional cryoprotectant, but the crystals of native and SeMet-labeled MazEF4 were transferred to a cryoprotectant solution containing 23% (v/v) glycerol in the reservoir solution prior to mounting. X-ray diffraction data for the native and SeMet-labeled crystals were collected at 100 K using an ADSC Quantum 315r CCD detector system (Area Detector Systems Corp., Poway, CA) at the BL-5C experimental station of Pohang Light Source, Korea. For each image, the crystal was rotated by 1°, and the raw data were processed and scaled using the program suite HKL2000 (39).

Structure determination and refinement

Initially, phase calculation with data from the SeMet-labeled MazEF4 crystal was attempted using Autosol of PHENIX (40), but the acquisition of a reasonable electron density map and model was not successful. Alternatively, molecular replace-

ment was carried out by Molrep (41) to find a solution using the crystal structure of the Fic protein from *Neisseria meningitidis* (PDB code 3SE5) as a template. Molecular replacement was conducted by Molrep (41) using chain A of the MazEF4 complex as a template for the structural determination of MazE4-free MazF4. Subsequent manual model building was conducted using Coot (42), and the models were refined using Refmac5 (43) and PHENIX (40), including the bulk solvent correction. Five percent of the data were randomly set aside as the test data for the calculation of $R_{\text{free}}$ (44). Water molecules were added using Coot (42) and were manually inspected. Superimpositions and calculations of structural deviations were performed using the secondary structure matching function in Coot (42). Solvent-accessible surface areas were calculated using PISA (45). The refined model was visualized and drawn using PyMOL (46) and Chimera (47). Sequence alignments were performed using ClustalX 2.0 (48) and visualized using ESPript 3.0 (49). The *M. tuberculosis* MazF4 crystal diffracted to a 2.01-Å resolution and was refined to $R_{\text{work}}/R_{\text{free}}$ values of 0.204/0.228. The *M. tuberculosis* MazEF4 complex crystal diffracted to a 2.30-Å resolution and was refined to $R_{\text{work}}/R_{\text{free}}$ values of 0.219/0.268. Ninety-seven percent of the residues in the MazF4 model lie in favored regions of the Ramachandran plot with no outliers, and 96.38% of the residues in the MazEF4 model lie in favored regions of the Ramachandran plot with no outliers. The crystallographic and refinement statistics are summarized in Table 1. The MazF4 crystal belongs to space group C222$_1$ with unit cell dimensions of $a = 49.475 \AA$, $b = 65.548 \AA$, $c = 57.390 \AA$, $\alpha = 90.00^\circ$, $\beta = 90.00^\circ$, and $\gamma = 90.00^\circ$. One asymmetric unit is present in 1 unit cell, which contains a single copy of the MazF4 molecule. The MazF4 model consists of residues Met$^1$ to Pro$^{105}$ (except Gly$^{15}$–Gly$^{17}$), two additional residues (Glu$^1$ and Phe$^9$) that are introduced onto the N terminus for a TEV cleavage site insertion, and 59 water molecules. The electron density is clear for all residues of the MazF4 toxin except residues Gly$^{15}$ to Gly$^{17}$, which appear to be disordered. The MazEF4 crystal belongs to space group $P2_1_1$ with unit cell dimensions of $a = 60.473 \AA$, $b = 70.018 \AA$, $c = 76.754 \AA$, $\alpha = 90.00^\circ$, $\beta = 113.275^\circ$, and $\gamma = 90.00^\circ$. Two asymmetric units are present in 1 unit cell. Each asymmetric unit consists of a heterotrimer of two MazF4 toxin molecules and one MazE4 antitoxin molecule (chains ABE or chains CDF) that are related by a 2-fold symmetry to form a heterohexamer (Fig. 1A). The MazEF4 model contains 568 amino acid residues of four MazF4 toxin molecules and two MazE4 antitoxin molecules with 70 water molecules. The electron density is clear for all residues of the four MazF4 toxins except residues Thr$^{44}$ to Ile$^{48}$ in chains B and D, which appear to be disordered. The elongated C terminus of each MazE4 molecule projects outward from the crystallographic 2-fold symmetry axis and interacts with the MazF4 homodimers.

Sequence alignment between *E. coli zwf* and *M. tuberculosis* zwf genes

Sequence alignment using BLAST (50) allowed the nucleotide sequence comparison between the *E. coli* glucose-6-phosphate 1-dehydrogenase gene (*zwf*) and the *M. tuberculosis* glucose-6-phosphate 1-dehydrogenase genes (*zwf1* and *zwf2*).
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Table 1
Data collection and refinement statistics

|                      | MazF4 crystal | MazEF4 crystal |
|----------------------|---------------|---------------|
| Space group          | C2221         | P21           |
| Cell dimensions      |               |               |
| a, b, c (Å)          |               |               |
| α, β, γ (°)          |               |               |
| Wavelength (Å)       |               |               |
| Rsym                 |               |               |
| I/σI                 |               |               |
| Completeness (%)     |               |               |
| Redundancy           |               |               |
| Resolution (Å)       | 30.00–2.01 (2.04–2.01)* | 30.00–2.30 (2.34–2.30)* |
| No. reflections      | 6119          | 23785         |
| Rwork/Rfree          | 0.2037/0.2280 | 0.2226/0.2680 |
| No. atoms            | 1606          | 8706          |
| Protein              | 23.80         | 44.70         |
| Water                | 33.10         | 38.40         |
| R.m.s. deviations    |               |               |
| Bond lengths (Å)     | 0.007         | 0.014         |
| Bond angles (°)      | 1.182         | 1.677         |
| Residues in favored region of the Ramachandran plot (%) | 97.00 | 96.38 |
| Residues in allowed region of the Ramachandran plot (%) | 3.00 | 3.62 |

* Highest resolution shell is shown in parenthesis.

The nucleotide sequence around the predicted EDF codon of *M. tuberculosis* zwf1 mRNA is shown in supplemental Fig. S6.

Ribonuclease assay

The ribonuclease activity of MazF4 and its enhancement by the EDF homolog were assessed by a fluorometric method (51) using 11-base-long mRNA (5' -AUAUCGUAG-3') as a substrate. Mixtures of MazF4 with the EDF homolog were prepared in several ratios and incubated at 37 °C for 1 h. To confirm the inhibition of MazEF4 complex formation, MazEF4 was added to the preincubated mixture of MazF4 and the EDF homolog, and further incubated at 37 °C for 30 min. This assay adopted the fluorescence quenching method to measure ribonuclease activity using a fluorescence-labeled synthetic mRNA substrate. Briefly, a fluorophore (6-FAM) was covalently attached to one end of the mRNA molecule and quenched by a quencher group (BHQ) attached on the other end. When ribonuclease was added to the synthetic mRNA that contained a fluorophore-quencher pair, digestion of the RNA caused the separation of the fluorophore and quencher pair, resulting in a subsequent escalation of fluorescence at 520 nm after excitation at 490 nm by a light source. Fluorescence (RFU) was detected using a SPECTRAmax M5 spectrofluorometer. The graphs were drawn after subtracting the value obtained from a reaction containing only buffer and mRNA substrate. Both the peptide (Peptron, Inc., Daejeon, Korea) and mRNA substrates (Bioneer, Daejeon, Korea) were commercially ordered. The ribonuclease activity of MazF4 mutants (K19A, T42A, E70R, and E76R) was also measured by the protocol described above.

NMR spectroscopy and titrations

The 2D-[^1]H-[^15]N]-HSQC spectra of 15N-labeled wild-type MazF4, E70R, and E76R mutants (final concentration of 0.1 mM) were obtained on a Bruker Avance 800 spectrometer equipped with a cryoprobe. The average CSP values (Δδave) for the 15N and 1H nuclei were calculated as follows,

\[
\Delta \delta_{ave} = \left\{ 0.5 \left[ (\Delta \delta_{HN})^2 + (\Delta \delta_{N})^2 \right] \right\}^{0.5}
\]

where, ΔδHN and ΔδN are the CSP values of the amide proton and nitrogen, respectively. For the backbone resonance assignments, the MazF4 R11A/Y16A mutant was chosen due to the poor solubility of the wild-type protein. The comparison between the HSQC spectra of the mutant and wild-type showed the conservation of the overall fold (supplemental Fig. S2). The three-dimensional HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCB, and HN(CO)CACB spectra of the 15N,13C-labeled MazF4 R11A/Y16A mutant were obtained on a Bruker Avance 800 spectrometer equipped with a cryoprobe. All NMR samples were dissolved in the final buffer (20 mM MES, pH 6.5, containing 1 mM DTT and 200 mM NaCl) and the spectra were recorded at 308 K. The NMR samples contained 10% D2O for the lock signal. All spectra were processed and analyzed using NMRPipe/NMRDraw (52) and NMRView (53), respectively.

Author contributions—D.-H. A. and K.-Y. L. designed the research; D.-H. A. and S. J. L. performed the research; H.-J. Y. solved a phasing problem for the crystal structures; S.-J. K. performed the analytical ultracentrifugation; D.-H. A. and S. J. P. analyzed the data; D.-H. A. and B.-J. L. wrote the paper.

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