Characterization of the Interactions within the \textit{mazEF} Addiction Module of \textit{Escherichia coli}

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Running Tile: Interactions within the \textit{mazEF} addiction module
Abbreviation

EMSA, Electrophoretic Mobility Shift Assay

ppGpp, guanosine 3’, 5’-bispyrophosphate

3-AT, 3-amino-1, 2, 4-triazole
ABSTRACT

In bacteria, programmed cell death is mediated through the unique genetic system called “addiction module”, which consists of a pair of genes encoding a stable toxin and an unstable antitoxin. The mazEF system is known as an addiction module located on the Escherichia coli chromosome. MazF is a stable toxin, and MazE is a labile antitoxin interacting with MazF to form a complex. MazE and the MazE-MazF complex can bind to the mazEF promoter region to regulate the mazEF expression. Here we show that the binding of purified (His)$_6$MazE to the mazEF promoter DNA was enhanced by MazF. The site-directed mutations at the conserved amino acid residues in MazE N-terminal region (K7A, R8A, S12A and R16A) disrupted the DNA-binding ability of both (His)$_6$MazE and the MazE-MazF(His)$_6$ complex, suggesting that MazE binds to the mazEF promoter DNA through the N-terminal domain. The ratio of MazE to MazF(His)$_6$ in the MazE-MazF(His)$_6$ complex is about 1:2. Since both MazE and MazF(His)$_6$ exist as a dimer by themselves, the MazE-MazF(His)$_6$ complex (76.9 kDa) is predicted to consist of one MazE dimer and two MazF(His)$_6$ dimers. The interaction between MazE and MazF was also characterized with the yeast two-hybrid system. It was found that the region from residue 38 to 75 of MazE was required for its binding to MazF. Site-directed mutagenesis at this region revealed that Leu55 and Leu58 play an important role in the MazE-MazF complex formation but not in MazE-binding to the mazEF promoter DNA. The present results demonstrate that MazE is composed of two domains, the N-terminal DNA-binding domain and the C-terminal domain interacting with MazF.
INTRODUCTION

In *Escherichia coli*, programmed cell death is proposed to be mediated through the system called “addiction module” (1), which consists of a pair of genes encoding a stable toxin and an unstable antitoxin which are co-expressed. Their expression is auto-regulated either by a complex formed by toxin and antitoxin or by antitoxin alone. When the co-expression is inhibited, the antitoxin is rapidly degraded by protease, enabling the toxin to act on its target. In *E. coli*, extrachromosomal elements are the main genetic system for bacterial programmed cell death. The most studied extrachromosomal addiction modules are the *phd-doc* on bacteriophage P1 (2-6), the *ccdA-ccdB* on factor F (7-13), and the *pemI-pemK* on plasmid R100 (14-17). Interestingly, the *E. coli* chromosome also contains several addition module systems, such as the *relBE* system (18-21) and the *mazEF* system (22).

The *mazEF* system, which consists of two adjacent genes, *mazE* and *mazF*, is located downstream from the *relA* gene on the *E. coli* chromosome. Sequence analysis revealed that they are partly homologous to the *pemI* and *pemK* genes on plasmid pR100 (23). The *mazEF* system has the properties required for an addiction module: MazF is toxic and MazE is antitoxic; MazF is stable, while MazE is a labile protein degraded *in vivo* by the ATP dependent ClpPA serine protease (22); MazE and MazF are coexpressed and interact with each other to form a complex; and the expression of *mazEF* is negatively auto-regulated by MazE and the MazE-MazF complex (24). The expression of *mazEF* is also inhibited by guanosine 3′, 5′-bispyrophosphate (ppGpp)\(^1\), which is synthesized by the RelA protein under extreme amino acid starvation (22). The *mazEF*-mediated cell death can be triggered by extreme amino acid starvation and thymine starvation (25), by
toxic protein Doc (26), and by some antibiotics that are general inhibitors of transcription and/or translation, such as rifampicin, chloramphenicol and spectinomycin (27).

Some crucial aspects of the mazEF system have remained elusive. It is still unknown how MazE or the MazE-MazF complex binds to the mazEF promoter DNA, and how MazE and MazF interact with each other to form a complex. In the present paper, we have investigated the interactions between MazE, MazF, and the mazEF promoter DNA to identify the functional domains in MazE responsible for binding to the mazEF promoter DNA and for interacting with MazF. It is demonstrated that MazE has a DNA-binding domain in its N-terminal region, and that the region from residue 38 to 75 in MazE is required for its binding to MazF, in which Leu55 and Leu58 are essential. The data in the present paper also suggest that the MazE-MazF complex consists of one MazE dimer and two MazF dimers.

EXPERIMENTAL PROCEDURES

Reagents and enzymes — Nucleotides, ampicillin and kanamycin were from Sigma. The restriction enzymes and DNA modifying enzymes used for cloning were from New England Biolabs. Pfu DNA polymerase was from Stratagene. The radioactive nucleotides were from Amersham Pharmacia Biotech.

Constructions of plasmid — The mazEF gene (including its Shine-Dalgarno sequence region) was amplified by PCR using E. coli genomic DNA as template, and cloned into the XbaI-Nhel sites of pET11a, creating the plasmid pET11a-EF. The mazEF gene (including its Shine-Dalgarno sequence region) was amplified by PCR, and cloned into
the XbaI-XhoI sites of pET21cc to create an in-frame translation with a (His)$_6$ tag at the MazF C-terminus. The plasmid was designated as pET21cc-EF(His)$_6$. The mazE gene was amplified by PCR and cloned into the Ndel-HindIII sites of pET28a. This plasmid was designated as pET28a-(His)$_6$E. MazE was expressed as a fusion with an N-terminal (His)$_6$ tag followed by a Thrombin cleavage site, named (His)$_6$MazE. The full-length mazE gene and various N-terminal and C-terminal deletion constructs of the mazE gene as indicated in Fig. 7, were generated by PCR and cloned into EcoRI-PstI sites of pGAD-C1 vector to create in-frame translation fusions with the Gal4 transcriptional activation domain. These plasmids were designated as pGAD-MazE, pGAD-MazEΔ(1-13), pGAD-MazEΔ(1-24), pGAD-MazEΔ(1-37), pGAD-MazEΔ(1-46), pGAD-MazEΔ(68-82) and pGAD-MazEΔ(76-82). The full-length mazF gene and various N-terminal and C-terminal deletion constructs of the mazF gene were generated by PCR and cloned into EcoRI-BglII sites of pGBD-C1 vector to create in-frame translation fusions with the Gal4 DNA binding domain. These plasmids were designated as pGBD-MazF, pGBD-MazFΔ(1-14), pGBD-MazFΔ(1-25), pGBD-MazFΔ(72-111) and pGBD-MazFΔ(97-111).

Protein purification — pET11a-EF was introduced into E. coli BL21(DE3) strain. The coexpression of MazE and MazF was induced for 4 h in the presence of 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation and lysed using French press. The cell lysis was kept at 37 °C for 30 min to degrade MazE as much as possible, and cell debris and unbroken cells were then removed by centrifugation 8,000×g for 10 min followed by ultracentrifugation at 10,000×g for 1 h to remove membrane and insoluble fractions. MazF was subsequently purified by ammonium
sulfate fractionation, gel filtration on Sephadex G-100 column, DEAE-Sepharose and hydroxyapatite column chromatography. The fractions containing MazF protein were pooled and concentrated. MazF was further purified by gel filtration with a Superdex\textsuperscript{TM} 200 column (Pharmacia Biotech). For purification of (His\textsubscript{6})\textsubscript{6}MazE, pET28a-(His\textsubscript{6})\textsubscript{6}E was introduced into \textit{E. coli} BL21(DE3) strain, and (His\textsubscript{6})\textsubscript{6}MazE expression was induced with 1mM IPTG for 4 h. (His\textsubscript{6})\textsubscript{6}MazE protein was immediately purified by Ni-NTA (QIAGEN) affinity chromatography. pET21cc-EF(His\textsubscript{6}) was also introduced into \textit{E. coli} BL21(DE3) strain. The coexpression of MazE and MazF(His\textsubscript{6}) were induced in the presence of 1 mM IPTG for 4 h. The MazE-MazF(His\textsubscript{6}) complex was immediately purified by Ni-NTA (QIAGEN) affinity chromatography, and further purified by gel filtration. To purify MazF(His\textsubscript{6}) from the purified MazE-MazF(His\textsubscript{6}) complex, MazE in the purified MazE-MazF(His\textsubscript{6}) complex was dissociated from MazF(His\textsubscript{6}) in 5 M guanidine-HCl. MazF(His\textsubscript{6}) was retrapped by Ni-NTA resin (QIAGEN) and refolded by step-wise dialysis. The yield of refolding is approximately 80%. The biochemical activity of MazF(His\textsubscript{6}) was determined with \textit{E. coli} T7 S30 extract system (Promega) for the protein synthesis inhibition.

\textit{Electrophoretic mobility shift assays (EMSA)} — Two single-stranded oligonucleotides (5′-GCTCGTATCTACAATGTAGATTGATATATACTGTATCTACATATGATAGC -3′ and 3′-CGAGCATAGATGTTACATCTAACTATATGACATAGATGTATACTATCG-5′) were synthesized and annealed to get the 50-bp double-stranded DNA containing the \textit{mazEF} promoter sequence. The 50-bp DNA fragment was end-labeled by T4 polynucleotide kinase with \(\gamma^{-32}\)P\textsubscript{ATP} and used to detect the protein-DNA binding by
EMSA. Binding reactions (20 µl) were carried out at 4 °C for 30 min with purified proteins, 2 µl 100 µg/ml poly(dI-dC) and 2 µl labeled DNA fragment in the binding buffer [50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM dithiotheritol and 5% glycerol]. Electrophoresis was performed in TAE buffer at 100 V in 6% native polyacrylamide gel. After electrophoresis, the gel was dried and then exposed to X-ray film.

Native PAGE — Different amounts of (His)₆MazE and MazF were mixed in binding buffer [50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM dithiotheritol and 5% glycerol] at 4 °C for 30 min, and then 2 × loading solution [40 mM Tris-HCl (pH 7.5), 80 mM β-mercaptoethanol, 0.08% bromophenol blue and 8% glycerol] were added to the mixtures before loading on a native gel. The composition of the stacking gel was 5% acrylamide-bis (29:1) in 62.5 mM Tris-HCl (pH 7.5), and the composition of the separation gel was 10% acrylamide-bis (29:1) in 187.5 mM Tris-HCl (pH 8.9). The running buffer contains 82.6 mM Tris-HCl (pH 9.4) and 33 mM glycine. Electrophoresis was performed at constant voltage (150 V) at 4 °C. Protein bands were visualized by Coomassie brilliant blue.

Resolution of low molecular weight proteins by tricine SDS-PAGE — Tricine SDS-PAGE was carried out according to the method described previously (28) with some modifications as following: stacking gel, 5% acrylamide-bis (48:1.5) in 0.75 M Tris-HCl (pH 8.45) and 0.075 % SDS; spacer gel, 10% acrylamide-bis (48:1.5) in 1.0 M Tris-HCl (pH 8.45) and 0.1% SDS; resolving gel: 16.5% acrylamide-bis (48:1.5) in 1.0 M Tris-HCl (pH 8.45) and 0.1% SDS. The anode running buffer was 0.2 M Tris-HCl (pH 8.9), and
the cathode running buffer was 0.1 M Tris base, 0.1 M tricine and 0.1% SDS. After running the gel at constant current (20 mAmp) at room temperature, protein bands were visualized by Coomassie brilliant blue.

**Assays of MazE-MazF interaction in the yeast two-hybrid system** — The yeast two-hybrid reporter strain PJ69-4A \([MATa \ trp1-901 \ leu2-3,112 \ ura3-52 \ his3-200 \ gal4\ gal80LYS2::GAL1-HIS3 \ GAL2-ADE2 \ met::GAL7-lacZ]\) and vectors pGAD-C1 and pGBD-C1 were used for two-hybrid assays (29). In order to localize the MazF-binding region in MazE, a series of N- and C-terminal deletions of the *mazE* gene were constructed in pGAD-C1, and cotransformed with the pGBD-MazF plasmid into the PJ69-4A cells, as shown in Fig. 7. In order to localize the MazE-binding region in MazF proteins, a series of N- and C-terminal deletions of the *mazF* gene were constructed in pGBD-C1 and cotransformed with the pGAD-MazE plasmid into the PJ69-4A cells. Assays of the interactions were performed by monitoring growth of cotransformants on synthetic dropout (SD) minimal medium (Clontech) lacking Trp, Leu, His and adenine (Ade). The medium was supplemented with 1mM 3-amino-1, 2, 4-triazole (3-AT) and incubated at 30 °C for 5 days.

**Results**

* MazE and MazF form a complex in a 1:2 ratio — Tricine SDS-PAGE patterns of purified MazE-MazF(His)₆, MazF and (His)₆MazE are shown in Fig. 1, lanes 2, 3 and 4, respectively. The sizes of MazF and (His)₆MazE agree with theoretical molecular weight 12.0 kDa and 11.4 kDa, respectively (Fig. 1, lanes 3 and 4). The MazE-MazF(His)₆
complex was separated into 9.3 kDa MazE and 13.2 kDa MazF(His)$_6$ (Fig. 1, lane 2), and the ratio of MazF(His)$_6$ to MazE is approximately 2 as determined by densitometer.

When (His)$_6$MazE and MazF were mixed together and the mixture was subjected to native PAGE, a new band appeared at position a near the top of the gel (position a in Fig. 2). The gel corresponding to the new band was cut out and incubated in a reducing buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 50 mM β-ME] for 30 min at room temperature, and then the gel was placed on the top of SDS-PAGE gel to run a second dimensional electrophoresis to analyze the protein components. After staining the gel with Coomassie brilliant blue, two bands corresponding to (His)$_6$MazE and MazF were observed, while (His)$_6$MazE moved slower than MazF on the SDS-PAGE. These results demonstrated that the new band was the complex consisting of (His)$_6$MazE and MazF. If the gel cut from the native PAGE was not treated in the reducing buffer, three protein bands were observed after it was subjected to the SDS-PAGE, (His)$_6$MazE, MazF and the MazF dimer (data not shown). Three bands appeared for the purified MazF on the native PAGE, but only one peak was observed when the purified MazF protein was assayed by HPLC (data not shown). The reason for the multi-band formation of MazF is not known at present.

Next, we attempted to determine if the ratio of (His)$_6$MazE to MazF is stable in the complex. As shown in Fig. 2A, different amounts of (His)$_6$MazE were added into the solution containing a constant concentration of MazF (2 μM) to make the (His)$_6$MazE:MazF ratios as 1:1, 2:1, 4:1, 6:1 and 8:1, while in Fig. 2B, different amounts of MazF were added into the solution containing a constant concentration of (His)$_6$MazE (2 μM) to make the (His)$_6$MazE:MazF ratios as 1:1, 1:2, 1:4, 1:6 and 1:8. The mixtures
were incubated for 30 min at 4 °C and then analyzed by native PAGE. The gel corresponding to the new band (at position a) was cut out and incubated in the reducing buffer for 30 min at room temperature, and then subjected to 15% SDS-PAGE. The second dimensional gel was stained with Coomassie brilliant blue to detect protein bands. Relative protein amounts in each lane were determined by densitometer using purified (His)$_6$MazE and MazF as controls. The ratios of MazF to (His)$_6$MazE in the complex were maintained almost constant at 1.8 whenever (His)$_6$MazE or MazF was added in excess in the mixtures (Fig. 2). As mentioned above, the MazE-MazF(His)$_6$ complex was separated to MazE and MazF(His)$_6$ on a tricine SDS-PAGE, and the ratio of MazF(His)$_6$ to MazE is approximately 2 (Fig. 1, lane 2). The molecular mass of the purified MazE-MazF(His)$_6$ complex and MazF were determined as 76.9 kDa and 27.1 kDa by gel filtration with a Superdex$^\text{TM}$ 200 column (Pharmacia Biotech) (Fig. 3). MazF(His)$_6$ was purified from the MazE-MazF(His)$_6$ complex. MazF(His)$_6$ was able to inhibit the protein synthesis in an E. coli cell-free system (E. coli T7 S30 extract system, Promega), and the protein synthesis was rescued by the co-addition of (His)$_6$MazE (data not shown). The molecular mass of MazF(His)$_6$ was determined to be 28.3 kDa with light scattering, suggesting MazF(His)$_6$ exists as dimer. The structure of MazE has been demonstrated as a dimer (30). Therefore, the MazE-MazF(His)$_6$ complex (76.9 kDa) may consist of one MazE dimer (predicted to be around 18.6 kDa as the MazE molecular weight is 9.3 kDa) and two MazF(His)$_6$ dimers (predicted to be 56.6 kDa).

*MazF enhances MazE binding to the mazEF promoter* — The 50-bp mazEF promoter fragment prepared as described under “Experimental Procedures” was end-labeled by T4
polynucleotide kinase with $\gamma^{32}\text{P}]\text{ATP}$. Using electrophoretic mobility shift assays (EMSA), (His)$_6$MazE, MazF and the MazE-MazF(His)$_6$ complex were tested separately for their binding abilities to the $mazEF$ promoter DNA fragment. (His)$_6$MazE was able to shift the $mazEF$ promoter fragment at 2 $\mu$M or higher concentrations (Fig. 4A, lanes 7-12). At 0.4 to 1.0 $\mu$M (His)$_6$MazE, no discrete mobility-shifted bands were observed, although the signals of the DNA fragment started to smear upward (Fig. 4A, lanes 3-6), indicating that some unstable (His)$_6$MazE-DNA complexes were formed at these concentrations. At 2 to 20 $\mu$M (His)$_6$MazE, we observed discrete mobility-shifted complexes, which move slower at the higher concentrations of (His)$_6$MazE (Fig. 4A, lanes 7-12), suggesting that the number of (His)$_6$MazE molecules bound to the DNA fragment increased at higher MazE concentrations. It is possible that there are more than one (His)$_6$MazE binding sites in the 50-bp $mazEF$ promoter fragment. On the other hand, MazF protein could not bind to the 50-bp $mazEF$ promoter DNA even at 20 $\mu$M concentration (Fig. 4B). Increasing amounts of both (His)$_6$MazE and MazF proteins were added with a constant (His)$_6$MazE/MazF (1:2) ratio. Compared with (His)$_6$MazE alone, MazF significantly enhanced (His)$_6$MazE binding to the $mazEF$ promoter. Under these conditions, the 50-bp $mazEF$ promoter fragment was shifted at a (His)$_6$MazE concentration of as low as 0.2 $\mu$M (Fig. 4C), and supershifting was observed at higher concentrations of the (His)$_6$MazE-MazF complex, which indicates that more (His)$_6$MazE-MazF complexes bind to the DNA fragment at higher concentrations, suggesting there are multiple binding sites for the (His)$_6$MazE-MazF complex in the $mazEF$ promoter.
Conserved Amino acid sequence in MazE homologs — The MazE homologs were identified by the BLAST search, and their amino acid sequence alignments are shown in Fig 5. Although generally MazE is not highly conserved in bacteria, there are still some conserved boxes in MazE. First, the N-terminal region of MazE is more conserved than other regions in MazE. MazE is an acidic protein with a pI of 4.7, but there are a few conserved basic residues (K7, R8 and R16) in its N-terminal region, named the N-box (Fig. 5). Since MazE is able to bind the mazEF promoter DNA, the N-box may be responsible for the DNA binding. Secondly, there is a conserved C-terminal region, named the Hp-box (Fig. 5), which contains several conserved hydrophobic residues.

The N-box of MazE is responsible for the DNA-binding of both MazE and the MazE-MazF complex — Various site-directed mutations were constructed in the mazE gene on pET21cc-EF(His)₆ plasmid, converting the conserved amino acid residues in the N-box to Ala. The complexes formed by MazE mutant proteins and MazF(His)₆ were purified. These complexes were tested for their binding ability to the mazEF promoter DNA by EMSA respectively. As shown in Fig. 6A, the complexes formed by MazE mutants with mutation in the N-box (K7A, R8A, S12A or R16A) and MazF(His)₆ were unable to bind to the mazEF promoter DNA (Fig. 6A, lanes 3, 4, 5 and 6). However, the substitution mutations on the conserved amino acids outside the N-box, such as MazE I43N and E57Q, did not affect the DNA binding of the complex (Fig. 6A, lanes 7 and 8, respectively). Various of substitution mutations were also constructed in the mazE gene on pET28a-(His)₆E plasmid. All of the (His)₆MazE mutants with the substitution mutation in the N-box (K7A, R8A, S12A and R16A) lost their DNA-binding ability (Fig.
6B, lanes 3, 4, 5 and 6, respectively), while the wild-type (His)$_6$MazE was able to bind to the mazEF promoter (Fig. 6B, lane 2). The (His)$_6$MazE mutants with the substitution mutation outside the N-box (R48A, F53A, L55A/L58A and E57Q) were able to bind the mazEF promoter DNA (data not shown). These results indicate that the DNA-binding ability of the MazE-MazF complex is due to MazE protein in the complex, and that the N-box is responsible for the DNA binding of MazE.

Interaction between MazE and MazF — Yeast two-hybrid assays were performed to examine the interaction between MazE and MazF. In order to demonstrate which region of MazE is required for its interaction with MazF, the full-length mazE gene and various N-terminal and C-terminal deletion constructs of the mazE gene, as indicated in Fig. 7, were generated by PCR and cloned into the EcoRI-PstI sites of pGAD-C1 vector to create in-frame translation fusions with the Gal4 transcriptional activation domain, and then each of these plasmids was cotransformed with the pGBD-MazF plasmid into PJ69-4A yeast cells respectively. The cotransformants harboring pGAD-MazE, pGAD-MazEΔ(1-13), pGAD-MazEΔ(1-24), pGAD-MazEΔ(1-37) or pGAD-MazEΔ(76-82) with pGBD-MazF were able to grow on the on a synthetic medium (SD medium, Clotech) lacking Trp, Leu, His and Ade, while the cotransformants harboring pGAD-MazEΔ(1-46) or pGAD-MazEΔ(68-82) with pGBD-MazF were not. These data demonstrate that the full length MazE, MazEΔ(1-13), MazEΔ(1-24), MazEΔ(1-37) and MazEΔ(76-82) are able to interact with MazF, while the further N-terminal deletion mutant MazEΔ(1-46) and the further C-terminal deletion mutant MazEΔ(68-82) are unable to do so. These
results indicate that the region from residue 38 to 75 of MazE is responsible for the interaction with MazF.

A series of truncation mutations from the N- and C-terminal ends of MazF were constructed in pGBD-C1 and cotransformed with pGAD-MazE into PJ69-4A cells. All of these cotransformed yeast cells were unable to grow on a complete synthetic medium in the absence of Trp, Leu, His, and Ade, indicating that all of these MazF mutants were unable to interact with MazE. Therefore both N- and C-terminal regions of MazF may be involved in the interaction with MazE, or the deletion mutations disrupt the MazF structure responsible for interaction with MazE. However, it is possible that the negative results of the yeast two-hybrid assays may be due to degradation of the fusion proteins.

Site-directed mutations were also performed on plasmid pET28a-(His)$_6$E to construct (His)$_6$MazE R48A, F53A, L55A/L58A and E57Q mutants. The complex formations with these (His)$_6$MazE mutants and MazF were examined by native PAGE. As shown in Fig. 8A, (His)$_6$MazE mutants R48A, F53A and E57Q were able to form the complex with MazF (Fig. 8A, lanes 5, 6 and 7, respectively), while (His)$_6$MazE L55A/L58A mutant was not (Fig. 8A, lane 4). By EMSA, it was found that both the wild-type (His)$_6$MazE and (His)$_6$MazE L55A/L58A mutant were able to bind to the mazEF promoter DNA (Fig. 8B, lanes 2 and 3, respectively). When MazF was added, the wild-type (His)$_6$MazE was able to interact with MazF to form the complex resulting in a supershifted band near the top of the gel (Fig. 8B, lane 4) compared to the lane with wild-type (His)$_6$MazE alone (Fig. 8B, lane 2). However, the addition of MazF to (His)$_6$MazE L55A/L58A did not cause the supershifting of the DNA fragment, confirming that the (His)$_6$MazE L55A/L58A mutant cannot interact with MazF to form a complex.
DISCUSSION

The mazEF addiction system in *E. coli* consists of two genes, *mazE* and *mazF*, encoding liable antitoxin MazE and stable toxin MazF, respectively (22). The toxic effect of MazF is activated by ppGpp, the signal produced by RelA protein due to amino acid starvation (22), by certain antibiotics (27), and by the toxic protein Doc (26). In each case, the degradation of labile MazE results in free stable MazF that exerts toxic effect to the cell. The regulation of the MazE cellular concentration is a major determinant of cell death. MazE forms a complex with MazF to inhibit its toxic effect and is also involved in the autoregulation of the *mazEF* expression by binding to the *mazEF* promoter (24). Therefore, MazE is considered to consist of at least two functional domains: the DNA-binding domain and the MazF-binding domain.

(His)_6MazE is able to interact with MazF and bind to the *mazEF* promoter. MazF(His)_6, like MazF, forms a dimer and inhibits the *in vitro* protein synthesis, and the protein synthesis is rescued by co-addition of (His)_6MazE (data not shown). Therefore the His-tags appear to have no effects on the function of MazE and MazF *in vitro*. Using highly purified (His)_6MazE and MazF, we demonstrated that (His)_6MazE can bind to the *mazEF* promoter by itself and the addition of MazF enhances (His)_6MazE-binding to the *mazEF* promoter DNA by more than 10 times. At the higher concentrations of (His)_6MazE or the (His)_6MazE-MazF complex, supershiftings are observed in the electrophoretic mobility shift assays with the *mazEF* promoter DNA, indicating that both (His)_6MazE and the (His)_6MazE-MazF complex have more than one binding sites on the *mazEF* promoter DNA. A previous fluorescent study suggests that there may be three MazE-binding sides in the *mazEF* promoter region (30). It is interesting to note that the
bands are not shifted in a step-wise manner. The reason for such shifting patterns is unknown at present. The site-directed mutations in the conserved N-box of MazE (K7A, R8A, S12A and R16A) disrupted the DNA-binding ability of both (His)$_6$MazE and the MazE-MazF(His)$_6$ complex (Fig. 6), suggesting that MazE is responsible for the DNA-binding ability of the MazE-MazF(His)$_6$ complex, and that the highly conserved N-terminal region in MazE is the DNA-binding domain. So far, the precise targets in the promoter sequence for the binding of MazE and the MazE-MazF complex have not been identified.

Yeast two-hybrid assays were performed to identify the region responsible for the MazE-binding to MazF. It was found that the region from residue 38 to 75 in MazE was required for its binding to MazF. There is a conserved C-terminal region in MazE named the Hp-box, which is rich in hydrophobic residues. The Hp-box mutations at the conserved Lue55 and Lue58 (L55A/L58A) disrupted the interaction between (His)$_6$MazE and MazF. The yeast two-hybrid experiments also indicated that the entire structure of MazF protein may be required for its interaction with MazE, since deletions from either the N- or C-terminal end of MazF disrupted the interaction between MazE and MazF.

The molecular mass of the MazE-MazF(His)$_6$ complex was determined to be 76.9 kDa by gel filtration. When the purified MazE-MazF(His)$_6$ complex was subjected to tricine SDS-PAGE, the ratio of MazE to MazF(His)$_6$ was found to be approximately 1:2 (Fig. 1, lane 2). Even in the presence of excess amounts of (His)$_6$MazE or MazF, the ratio of (His)$_6$MazE to MazF in the (His)$_6$MazE-MazF complex was stably maintained at around 1:1.8 (Fig. 2). Since both MazE (30) and MazF(His)$_6$ exist as dimers, the MazE-MazF(His)$_6$ complex (76.9 kDa) may consist of one MazE dimer (predicted to be around
18.6 kDa as the molecular weight of MazE is 9.3 kDa) and two MazF(His)$_6$ dimers (predicated to be around 56.6 kDa as the molecular mass of MazF(His)$_6$ dimer is 28.3 kDa).

While preparing this manuscript, the crystal structure of the MazE-MazF complex was determined by Kamada et al (31). The crystal structure of the MazE-MazF complex confirmed our results in its following aspects: 1) In the crystal structure, MazE and MazF form a 2:4 heterohexamer, consisting of alternating MazF and MazE homodimers (MazF$_2$-MazE$_2$-MazF$_2$). It is important to note that the 2:4 stoichiometric complex formation between MazE and MazF appears to be very stable, since the ratio between (His)$_6$MazE and MazF in the (His)$_6$MazE-MazF complex was found irrespective of which protein was added in large excess (Fig. 2). 2) The C-terminal region of MazE interacts with MazF homodimer in the structure of MazE-MazF complex. The Hp-box region identified in this study is involved in the seemingly most stable interface between MazE and MazF (Fig. 9). The sidechains of hydrophobic amino acid residues (Leu55, Leu58, Val59 and Ile62) in the Hp-box contact with a cluster of hydrophobic residues in the MazF homodimer. Indeed (His)$_6$MazE L55A/L58A mutant was not able to form a complex with MazF (Fig. 8), suggesting that these hydrophobic interactions are essential for the MazE-MazF complex formation. 3) Based on the similarity between MazE and other addiction module antidotes and the distribution of the basic regions on the electrostatic surfaces of MazE and MazF, Kamada et al (31) proposed that Lys7 and Arg8 in MazE serve as the primary DNA anchoring sites in the MazE-MazF complex. In the present paper, we showed that the DNA-binding abilities of (His)$_6$MazE and the MazE-MazF(His)$_6$ complex were disrupted not only by the site-directed mutations at
Lys7 and Arg8 but also by mutations at other conserved amino acid residues (Ser12 and Arg16) in the N-box (Fig. 9). It is possible that, since MazE exists as a dimer, the two N-boxes in the MazE dimer may be involved together in DNA-binding.

The coexpression of MazE and MazF is not only negatively auto-regulated by MazE and MazE-MazF complex, but also inhibited by ppGpp. It has been demonstrated that the global regulatory nucleotides ppGpp affects the activity of RNA polymerase on specific promoters. A conserved GC-rich consensus sequence localized immediately downstream of the –10 promoter element has been recognized as an important cis-element for negative stringent control (32). A GC-rich sequence (GCGG) exists immediately downstream of the –10 box of the major mazEF promoter P2 (24). It is possible that the inhibition of mazEF expression by ppGpp is due to its effect on RNA polymerase, while the auto-regulation of the mazEF operon by MazE and the MazE-MazF complex may be due to their binding to the mazEF promoter region to block RNA polymerase from binding to the promoter. At present, there is no evidence suggesting that ppGpp has any effects on the protein-protein or protein-DNA interaction within the mazEF addiction module.

The cellular effects of the toxins in the addiction modules have been studied quite extensively. CcdB, the toxin in the ccdA-ccdB system, interacts with DNA gyrase to induce DNA cleavage and block transcription (9,13), and RelE, the toxin in the relBE system, has a ribosome-dependent codon-specific mRNA cleavage activity at the ribosome A site (21). MazF has been shown to block translation as well as DNA replication (20). The detail mechanism of MazF function is currently under investigation.
ACKNOWLEDGMENTS

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REFERENCES


Figure legends

Fig. 1. Purification of the MazE-MazF(His)₆ complex, MazF, and (His)₆MazE. The MazE-MazF(His)₆ complex, MazF, and (His)₆MazE protein were purified as described under “Experimental Procedures”. The purified proteins were analyzed by tricine SDS-PAGE and visualized with Commassie brilliant blue. Lane 1, protein molecular weight markers; lane 2, MazE-MazF(His)₆ complex; lane 3, MazF; and lane 4, (His)₆MazE.

Fig. 2. Stoichiometric complex formation between (His)₆MazE and MazF. (His)₆MazE and MazF were mixed at different molar ratios as indicated. The mixtures were incubated for 30 min at 4 °C, and then subjected to native PAGE. The gel corresponding to the band of the complex was cut out and incubated in the reducing buffer [20 mM Tris-HCl (pH 7.5), 100 mM NaCl and 50 mM β-ME] for 30 min at room temperature, and then was subjected to 15% SDS-PAGE for second dimensional electrophoresis. (His)₆MazE and MazF in the complex are separated as shown in the gels in the lower panels. Relative protein amounts in each lane were determined by densitometer with the (His)₆MazE and MazF as controls. A. Different amounts of (His)₆MazE were added into 20-µl 2 µM MazF solution. Lanes 1-5, the (His)₆MazE:MazF ratios are 1:1, 2:1, 4:1, 6:1 and 8:1, respectively. B. Different amounts of MazF were added into 20-µl 2 µM (His)₆MazE solution. Lanes 1-5, the (His)₆MazE:MazF ratios are 1:1, 1:2, 1:4, 1:6 and 1:8, respectively. The upper panels in A and B are the results of native PAGE. The position of the (His)₆MazE-MazF complex is indicated by an arrow a. The lower panels in A and B are the results of SDS-PAGE for the second dimensional electrophoresis. Purified
(His)_6MazE (40 pmol) and MazF (40 pmol) were applied to the first and the second lanes as controls.

Fig. 3. Determination of the molecular masses of MazF and the MazE-MazF(His)_6 complex. The molecular masses of MazF and the MazE-MazF(His)_6 complex were determined by gel filtration with a Superdex 200 column. The protein molecular weight standard curve includes Thyroglobulin (669 kDa), Apoferritin (443 kDa), β-Amylase (200 kDa), BSA (66 kDa), Ovalbumin (45 kDa) and Carbonic Anhydrase (29 kDa), on which the vertical arrows indicate the positions of MazF and the MazE-MazF(His)_6 complex.

Fig. 4. Binding of (His)_6MazE and/or MazF to the mazEF promoter DNA determined by EMSA. A 50-bp [³²P]-labeled DNA fragment containing the mazEF promoter region was incubated with increasing concentrations of (His)_6MazE (A), with increasing concentrations of MazF (B), and with increasing concentrations of both (His)_6MazE and MazF at the constant (His)_6MazE/MazF ratio of 1:2 (C).

Fig. 5. Alignments of the amino acid sequences of MazE homologs. Sequence alignments of eight MazE family proteins are shown. The ClustalW program was used for alignment analysis. Identical residues among eight different proteins are shown by black boxes. Similar residues are shown by gray boxes. Gaps (indicated by dashes) are introduced to optimize the alignment. The sequences are: MazE in Deinococcus radiodurans (GenBank accession no. NP_294139); MazE in Bacillus halodurans (NP_244587); PemI on plasmid
R100 (NP_052993); PemI on plasmid R466b (AAC82515); MazE in *Escherichia coli* (NP_289337); ChpB in *Escherichia coli* (NP_290856); MazE in *Pseudomonas putida* KT2440 (NP_742931); MazE in *Photobacterium profundum* (AAG34554). The numbers correspond to amino acid residue numbers.

Fig. 6. MazE N-terminal domain is responsible for DNA binding of both MazE-MazF(His)₆ complex (A) and (His)₆MazE protein (B). DNA binding of the proteins was determined by EMSA with a 50-bp [³²P]-labeled DNA fragment containing the mazEF promoter region. A. The DNA fragment was incubated with 1 µM each complex indicated in a 20-µl mixture at 4 °C for 30 min. *Lane* 1, control without protein; *lane* 2, MazE-MazF(His)₆ complex; *lane* 3, MazE(K7A)-MazF(His)₆ complex; *lane* 4, MazE(R8A)-MazF(His)₆ complex; *lane* 5, MazE(S12A)-MazF(His)₆ complex; *lane* 6, MazE(R16A)-MazF(His)₆ complex; *lane* 7, MazE(I43N)-MazF(His)₆ complex; and *lane* 8, MazE(E57Q)-MazF(His)₆ complex. B. The DNA fragment was incubated with 4 µM (His)₆MazE or (His)₆MazE mutant indicated in a 20-µl mixture at 4 °C for 30 min. *Lane* 1, control without protein; *lane* 2, wild-type (His)₆MazE protein; *lane* 3, (His)₆MazE(K7A) mutant; *lane* 4, (His)₆MazE(R8A) mutant; *lane* 5, (His)₆MazE(S12A) mutant; and *lane* 6, (His)₆MazE(R16A) mutant.

Fig. 7. Yeast two-hybrid assays of the interaction between MazE and MazF. The full-length mazE gene and all of the truncated mazE genes were constructed in pGAD-C1. Numbers refer to the amino acid positions in MazE. The plasmids were cotransformed with pGBD-MazF into yeast PJ69-4A cells. Protein-protein interactions were tested on
SD medium (Clotech) plates containing 1 mM 3-AT in the absence of Trp, Leu, His and Ade. +, visible colonies formed in 5 days; -, no visible colonies formed in 5 days.

Fig. 8. Assays of the interactions between MazE mutants and MazF. A. Interactions between MazF and (His)$_6$MazE or (His)$_6$MazE mutants were determined by native PAGE. Lane 1, wild-type (His)$_6$MazE; lane 2, MazF; lane 3, wild-type (His)$_6$MazE and MazF; lane 4, (His)$_6$MazE L55A/L58A mutant and MazF; lane 5, (His)$_6$MazE R48A mutant and MazF; lane 6, (His)$_6$MazE E57Q mutant and MazF; and lane 7, (His)$_6$MazE F53A mutant and MazF. B. Interactions between MazF and (His)$_6$MazE or (His)$_6$MazE L55A/L58A mutant were determined by EMSA with the 50-bp $[^{32}P]$-labeled DNA fragment containing the mazEF promoter region. Lane 1, control without protein; lane 2, 4 µM wild-type (His)$_6$MazE; lane 3, 4 µM (His)$_6$MazE L55A/L58A mutant; lane 4, 2 µM wild-type (His)$_6$MazE and 4 µM MazF; and lane 5, 2 µM (His)$_6$MazE L55A/L58A mutant and 4 µM MazF.

Fig. 9. Conserved amino acid residues essential for MazE function(s) on the X-ray structure of the MazE-MazF complex determined by Kamada et al (31). Only a part of the MazF$_2$-MazE$_2$-MazF$_2$ complex is shown, in which one MazE molecule (blue) is interacting with two MazF molecules of the MazF homodimer (purple and red). In the MazE molecule, the N-box and the Hp-box are shown in green and yellow, respectively. Positions of Lys7, Arg8, Ser12 and Arg16 in the N-box and Leu55 and Leu58 in the Hp-box are shown, substitution mutations of which resulted in the loss of MazE function(s).
Fig. 1  Zhang J. *et al*
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