mRNA interferases are sequence-specific endoribonucleases encoded by the toxin-antitoxin systems in the bacterial genomes. MazF from *Escherichia coli* has been shown to be an mRNA interferase that specifically cleaves at ACA sequences in single-stranded RNAs. It has been shown that MazF induction in *E. coli* effectively inhibits protein synthesis leading to cell growth arrest in the quasi-dormant state. Here we have demonstrated that *Mycobacterium tuberculosis* contains at least seven genes encoding MazF homologues (MazF-mt1 to -mt7), four of which (MazF-mt1, -mt3, -mt4, and -mt6) caused cell growth arrest when induced in *E. coli*. MazF-mt1 and MazF-mt6 were purified and characterized for their mRNA interferase specificities. We showed that MazF-mt1 preferentially cleaves the *era* mRNA between U and A in UAC triplet sequences, whereas MazF-mt6 preferentially cleaves U-rich regions in the *era* mRNA both in *vivo* and in *vitro*. These results indicate that *M. tuberculosis* contains sequence-specific mRNA interferases, which may play a role in the persistent dormancy of this devastating pathogen in human tissues.

Most bacteria contain "suicidal" or "toxic" genes whose expression leads to growth arrest and eventual death upon exposure to stress. These toxin genes are usually coexpressed with their cognate antitoxin genes present in the same operon (1, 2). The *Escherichia coli* chromosome contains six such operons called toxin-antitoxin (TA) systems. Of these, the MazE (antitoxin)/MazF (toxin) system is one of the most extensively characterized TA systems, and MazF has been shown to be a sequence-specific endoribonuclease that cleaves at ACA sequences in mRNAs (3, 4). Thus, MazF is an mRNA interferase, and its induction causes the effective inhibition of protein synthesis leading to cell growth arrest. However, MazF-induced cells fully retain cellular metabolic activities, including ATP production and amino acid and nucleotide synthesis, as well as RNA and protein synthesis. Therefore, MazF-induced cells are capable of synthesizing a protein, if the gene encoding this protein is devoid of ACA sequences (4). This metabolically active dormant state caused by mRNA interferase induction is called "quasi-dormancy" (4) and has important implications in the physiology of various pathogenic bacteria, including persistent multidrug resistance.

Notably, *Mycobacterium tuberculosis*, one of the most devastating human pathogens, contains nearly forty TA systems on its genome, among which nine have been shown to be homologous to *E. coli* MazF (5). It has been proposed that the TA modules play important roles in bacterial survival and formation of stable persisters in adverse conditions. These phenomena are essential for the persistence of *M. tuberculosis*. Persisters are the few rare pre-existing bacterial cells in a culture at any growth phase that are not growing and are intrinsically resistant to antibiotics by virtue of their subdued metabolism (6, 7).

EXPERIMENTAL PROCEDURES

Strains and Plasmids—*E. coli* BL21(DE3), BW25113 (ΔaraBAD) were used. Plasmids pET-28a-MazF-mt1 and MazF-mt6 were constructed using pET-28a (Novagen) to express His-MazF-mt1 and His-MazF-mt6. The plasmids pBAD-MazF-mt1 to pBAD-MazF-mt7 were constructed from pBAD vector, in which the protein expression can be tightly regulated by the addition of arabinose (0.2%).

**Purification of His₃-tagged MazF-mt1 and MazF-mt6 Proteins Expressed in E. coli**—His₃-MazF-mt1 and His₃-MazF-mt6 proteins tagged at the N-terminal end were purified from strain BL21(DE3) carrying pET-28a-MazF-mt1 and MazF-mt6, respectively, using nickel-nitritoltriacetic acid resin (Qiagen). The proteins were stored in 100 mM NaH₂PO₄, 10 mM Tris-HCl, 0.5% glycerol, 10 mM β-mercaptoethanol buffer, pH 8.0, at −20 °C.

**Expression and Purification of MazF-mt1 Using Mycobacterium smegmatis mc²155 as a Host**—The wild-type MazF-mt1 gene was PCR-amplified and cloned into an *E. coli*-M. smegmatis shuttle vector downstream of an acetamidase gene promoter (cloned from *M. smegmatis* genomic DNA) and upstream of a hexahistidine tag. This vector, carrying a hygromycin resistance gene, was constructed in our laboratory. *M. smegmatis*-competent cells carrying the construct were used to inoculate 2 ml of 7H9 medium, and this starter culture was incubated at 37 °C with shaking for 2 days and then used to inoculate larger volumes of 7H9 medium supplemented with Middlebrook oleic acid-albumin-dextrose-catalase enrichment and 2% acetamide (Sigma). The culture was grown for three days and cells were then harvested and resuspended in equilibration buffer (25 mM imidazole, 400 mM NaCl, and 50 mM NaPi, pH 8.0). The cells were then lysed with a French press. Cell debris was removed by one round of centrifugation at 25,000 rpm and at 4 °C. The lysate was added to Ni²⁺-nitritoltriacetic acid-agarose (Qiagen) for 1 h, and the resin was then transferred to a column and washed with 50 ml of 50 mM NaPi, pH 8.0, 400 mM NaCl, and 20 mM imidazole. The His-tagged proteins were eluted with 50 mM NaPi, pH 8.0, 400 mM NaCl, and 20 mM imidazole.

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NaCl, and 250 mM imidazole. Protein fractions were checked at A280 and purity was assessed by SDS-PAGE analysis. The highly purified fractions were collected and dialyzed against 10 mM HEPES buffer containing 5% glycerol, 0.1 mM dithiothreitol, and 500 mM NaCl. The protein aliquots were stored at -80 °C.

**Primer Extension Analysis in Vivo and in Vitro**—For primer extension analysis of mRNA cleavage sites in vivo, pIN-Era plasmid (8) was transformed into E. coli BW25113 cells containing pBAD-MazF-mt1 or MazF-mt6 plasmids. The era mRNA transcription was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside. After a 30-min induction, the toxin protein was induced by the addition of arabinose (a final concentration of 0.2%). Total RNA was isolated at time intervals as indicated in Figs. 3 and 4. Primer extension was carried out using different primers as described previously (3). For in vitro primer extension analysis, the full-length era mRNAs were synthesized in vitro by T7 RNA polymerase from the DNA fragment containing a T7 promoter sequence and the era open reading frame (ORF) using the RiboMAX™ T7 large scale RNA production system (Promega). The

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**FIGURE 1.** Sequence alignments of MazF homologues from M. tuberculosis with E. coli MazF. A, alignment of MazF-mt1 (Rv2801c) with E. coli MazF. β-strand (S) and helical (H) region are assigned according to Kamada et al. (9). Identical and homologous residues are shown in black and shaded backgrounds. B, alignments of MazF-mt2–mt7 to MazF-mt1. The consensus sequence is shown at the bottom. In MazF-mt2, the 19-residue sequence in blue is immediately downstream of the codon for Thr-88 with a -1 frameshift. MazF-mt2–mt7 correspond to Rv0456c, Rv1991c, Rv0659c, Rv1942c, Rv1102c, and Rv1495, respectively. C, upstream ORFs for MazF-mt1–mt7. These overlap with downstream MazF ORFs by 14, 14, 7, 14, 4, 29, and 4 bases for MazF-mt1–mt7, respectively. Total residue numbers and pI values are shown on the right.
era DNA fragments were amplified using the forward primer (5’-CCCGCGAATTAATACGACTCACTATAG-3’ T7 promoter) and the reverse primers starting from the 3’-end of the era ORF. RNA substrates were partially digested with purified toxin protein at 37 °C for 15 min. The digestion reaction mixture (10 μl) consisted of 1 gRNA substrate, 0.2 μg of E. coli His6MazF-mt1 or 1 μg of M. smegmatis His6MazF-mt1 or E. coli His6MazF-mt6 and 0.5 μl of RNase inhibitor (Roche Applied Science) in 10 mM Tris-HCl (pH 7.8). Primer extension was carried out at 42 °C for 1 h in 20 μl of the reaction mixture as described previously (8). The reactions were stopped by adding 12 μl of sequence loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF (C25H27N2NaO7S2)). The samples were incubated at 90 °C for 5 min prior to electrophoresis on a 6% polyacrylamide and 36% urea gel. The primers were 5’-labeled with [γ-32P]ATP using T4 polynucleotide kinase.

Cleavage of Synthetic RNA by MazF-mt1—Oligoribonucleotides (11 bases in length; see Fig. 3) were commercially synthesized and 5’-labeled with [γ-32P]ATP using T4 polynucleotide kinase. Endoribonuclease activity was assayed in 10 μl of the reaction mixture containing 0.5 μl (20 units) of ribonuclease inhibitor (Roche Applied Science), 0.2 μg of E. coli MazF-mt1 or 1 μg of M. smegmatis MazF-mt1 and 32P-labeled oligonucleotides in 10 mM Tris-HCl (pH 7.8). Reactions were carried out at 37 °C for 30 min and stopped by the addition of the loading buffer as described above. The reaction mixtures were then subjected to 20% sequence gel electrophoresis followed by autoradiography.

RESULTS

Blast Search for MazF Homologues in M. tuberculosis—A blast search of all known ORFs in the M. tuberculosis (H37Rv) genome with E. coli MazF identified the gene \( \text{Rv2801c} \) that encodes a 118-residue protein.
that has 32.5% identity and 44% homology to *E. coli* MazF (Fig. 1A). Using this protein MazF-mt1 for further blast search, six more MazF homologues with 20% identity to MazF-mt1 (mt2 to mt7) were found (Fig. 1B). Notably, MazF-mt1, -mt2, and -mt3 have the putative 11-residue S1–S2 loop similar to *E. coli* MazF (9), whereas the others have much shorter S1–S2 loops (Fig. 1, A and B). The putative helix H1 is longer by five residues in MazF-mt1 and -mt2 and by three residues in -mt3, compared with that of *E. coli* MazF, whereas it is shorter by one residue in MazF-mt4, -mt5, -mt6, and -mt7 as compared with that of *E. coli* MazF (Fig. 1, A and B). MazF-mt2 lacks the C-terminal region encompassing β-strand S7 and helix H3. This seems to be due to a 28-bp deletion after the codon for Thr-88, because the 19-residue sequence highly homologous to helix H3 of MazF-mt1 is found immediately downstream of Thr-88 in a different reading frame (68% identity) (Fig. 1B). Interestingly, all MazF homologues appear to be co-translated with a short upstream ORF, most of which overlaps with the MazF ORFs (Fig. 1C). None of the upstream ORFs, however, show homology to *E. coli* MazE, antitoxin of MazF. As observed with MazE and MazF, most of the pairs consist of acidic and basic proteins, except for the pair of MazF-mt2 and its upstream ORF, both of which are basic proteins (Fig. 1C). It remains to be elucidated whether these upstream ORFs function as antitoxins for their cognate downstream ORFs.

Four of the Seven MazF Homologues in *M. tuberculosis* Show Toxicity to *E. coli*—All ORFs were tested for their toxicity in *E. coli* using the pBAD expression plasmid. In the presence of 0.2% arabinose, cells expressing MazF-mt1, -mt3, -mt4, and -mt6 could not grow, whereas cells expressing MazF-mt2, -mt5, and -mt7 proteins were able to grow on an agar plate (Fig. 2A). Inhibitory effects of MazF-mt1, -mt3, -mt4, and -mt6 were also observed in M9-CAA liquid medium in the presence of 0.2% arabinose (Fig. 2B). In the case of cells expressing MazF-mt4 protein, severe growth inhibition was seen only after one generation of cell growth.

The MazF-mt1 Has Endoribonuclease Activity—We examined whether these MazF homologues have sequence-specific mRNA interferase activity, both in vivo and in vitro. When MazF-mt1 was induced by the addition of 0.2% arabinose, a specific cleavage of the *era* mRNA...
between U and A was detected in vivo by primer extension in a time-dependent manner (Fig. 3A). The identical cleavage site was detected with use of the era mRNA synthesized with T7 RNA polymerase and purified MazF-mt1 tagged with His6 at the N-terminal end (Fig. 3B, lane 2). In addition to the same site reported for the in vivo cleavage site (CU ↓ ACC), a weak cleavage site (UU ↓ ACA) was also detected. Using five primers covering almost the entire era mRNA, CU ↓ ACC and UU ↓ ACA were the only major cleavage sites detected, even when era mRNA contained six other UAC sequences (UUACU, AUACU, CUACG, AUACA, GUACU, and UUACG). Importantly, identical sites were cleaved by His6MazF-mt1, which was expressed and purified from M. smegmatis, a weak cleavage site (UU ↓ ACA) was also detected. Using five primers covering almost the entire era mRNA, CU ↓ ACC and UU ↓ ACA were the only major cleavage sites detected, even when era mRNA contained six other UAC sequences (UUACU, AUACU, CUACG, AUACA, GUACU, and UUACG). Importantly, identical sites were cleaved by His6MazF-mt1, which was expressed and purified from M. smegmatis (Fig. 3B, lane 3), indicating that the observed cleavage is due to MazF-mt1. We further confirmed that the cleavage activity was not due to contaminating enzymes, because a purified His6MazF-mt1 (E19A) mutant protein showed a substantially reduced cleavage activity (not shown). This mutation in E. coli MazF has recently been shown to dramatically reduce the MazF mRNA interferase activity (10).

For further biochemical characterization, purified His6MazF-mt1 was used to cleave a synthetic 15-base RNA (AGAUAU ↓ ACAUA-UGAA), which was also shown to be cleaved between U and A of the UAC sequence (Fig. 3C, lane 2 and arrows). A 19-base DNA with a sequence identical to that of the RNA substrate in the center could not be cleaved (lane 4). When the DNA and RNA were mixed and treated with the enzyme, only RNA was cleaved (lanes 5), indicating that MazF-mt1 is an endoribonuclease. To further test the cleavage specificity, five 11-base RNA substrates were synthesized, one with the UACG sequence in the center (AUACUACAUUG) and the others with a G residue in each one of the UAC sequences. The first U and the second A residues could not be replaced with G (Fig. 3D, lanes 3–6). The third C residue also appears to be important, as the cleavage between U and A (lanes 7 and 8) was significantly reduced when this C was replaced with G. On the other hand, the fourth A residue was replaceable with G (lanes 9 and 10), confirming that MazF-mt1 is an endoribonuclease that specifically cleaves UAC sequence.

The MazF-mt6 Is an mRNA Interferase—Because MazF-mt6 showed toxicity in E. coli (Fig. 2), we examined whether it, too, is a sequence-specific RNA interferase. We speculated that other MazF homologues are not toxic in E. coli, possibly because they may have highly specific cleavage activities and thus cleave only a very limited number of sequences in E. coli mRNAs or they are not efficiently expressed in E. coli. We carried out in vivo and in vitro primer extension experiments using the era mRNA and the MazF-mt6 protein. As shown in Fig. 4, a number of in vivo cleavage sites were detected in a time-dependent manner after its induction, all of which were also detected in an in vitro experiment with purified His6MazF-mt6 protein. Notably, a new cleavage site was also observed in an in vitro experiment in addition to those found in an in vivo (Fig. 4B, lane 10) experiment. These cleavages preferentially occurred in U-rich regions and after a U residue. Therefore, (U/C)U ↓ (A/U/C/U/C) may be assigned as a consensus cleavage sequence for MazF-mt6 mRNA interferase. The cleavage also occurred after G or A residues in some cases, although all cleavage sites contained UU, UC, or CU residues.

DISCUSSION

In this report, we have demonstrated that M. tuberculosis contains a number of MazF homologues, some of which were toxic to E. coli cells when induced. It has been shown that E. coli MazF causes complete cell growth arrest; however, the cells retain full metabolic activ-
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M. tuberculosis is one of the most devastating human pathogens, as one third of the world’s population is infected with this pathogen and eight million people develop active disease each year. Two phenotypic properties of this pathogen account for these statistics: 1) latent infection sometimes referred to as dormancy and 2) persistence of infectious bacteria (14, 15). Although the mechanisms of latency and persistence are not well understood, recent studies of the TA systems in this pathogen suggest that these TA systems may be responsible for the dormancy of this pathogen in human tissues (16). Our results demonstrating the existence of multiple mRNA interferases in M. tuberculosis support this notion, and further biochemical and genetic characterization of M. tuberculosis toxins will shed light into our understanding of their roles in the pathogenesis of this persistent human pathogen.

Acknowledgment—We thank Dr. Hirofumi Nariya for suggestion of a deletion mutation in the gene for MazF-mt2.

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