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The Inhibitory Mechanism of Protein Synthesis by YoeB, an *Escherichia coli* Toxin*

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YoeB is a toxin encoded by the *yefM-yoeB* antitoxin-toxin operon in the *Escherichia coli* genome. Here we show that YoeB, a highly potent protein synthesis inhibitor, specifically blocks translation initiation. In *in vivo* primer extension experiments using two different mRNAs, a major band was detected after YoeB induction at three bases downstream of the initiation codon at 2.5 min. An identical band was also detected in *in vitro* toeprinting experiments after the addition of YoeB to the reaction mixtures containing 70 S ribosomes and the same mRNAs, even in the absence of $tRNA_f^{Met}$. Notably, this band was not detected in the presence of YoeB alone, indicating that YoeB by itself does not have endoribonuclease activity under the conditions used. The 70 S ribosomes increased upon YoeB induction, and YoeB was found to be specifically associated with 50 S subunits. Using tetracycline and hygromycin B, we demonstrated that YoeB binds to the 50 S ribosomal subunit in 70 S ribosomes and interacts with the A site leading to mRNA cleavage at this site. As a result, the 3′-end portion of the mRNA was released from ribosomes, and translation initiation was effectively inhibited. These results demonstrate that YoeB primarily inhibits translation initiation.

YoeB is one of the toxins encoded by the genome of *Escherichia coli*, which is co-expressed with YefM, its cognate antitoxin (1). It has been shown that YefM is highly unstable in the cell and purified YefM exists as a denatured form without secondary structure (2). However, when YefM is co-expressed with YoeB, a dimer of YefM forms a stable complex with a single molecule of YoeB. The x-ray structure of this heterotrimer has been determined (3). Under normal growth conditions, YoeB presumably exists as a heterotrimer and is thus unable to exert its cellular toxicity. Lon is an ATP-dependent protease that is induced under various stress conditions, and has been implicated to be responsible for the degradation of YefM and release of YoeB. This consequently leads to inhibition of protein synthesis. These authors demonstrated that the *yefM-yoeB* system is involved in Lon-dependent lethality (1). They also showed that induction of YoeB leads to cleavage of the *lpp* mRNA at a few sites and concluded that YoeB functions similar to other *E. coli* toxins such as RelE, MazF, and ChpBK by cleaving translated mRNAs (1). Interestingly, RelE by itself has no endoribonuclease activity (4) and has been proposed to be a ribosome-associated factor, which stimulates the endogenous ribonuclease activity of ribosomes (4, 5). On the other hand, MazF is a ribosome-independent endoribonuclease that cleaves mRNAs specifically at ACA sequences, and is thus termed an mRNA interferase (6). ChpBK was also found to be another mRNA interferase specifically cleaving mRNAs at UAC sequences (7). Therefore, it remains to be determined if YoeB is a ribosome-associating factor like RelE or if it functions as a ribosome-independent mRNA interferase like MazF and ChpBK or if it inhibits translation by a yet-unknown mechanism. Notably, purified YoeB has been shown to have an intrinsic endoribonuclease activity specific for purine residues in RNA (3).

In the present paper we demonstrate that YoeB is a 50 S ribosome-associating factor that inhibits translation initiation using a mechanism that is different from the mechanism proposed earlier by Christensen *et al.* (1) and Kamada and Hanaoka (3). The former authors proposed that YoeB induces cleavage of the translated mRNA and the latter authors concluded that YoeB is a purine-specific endoribonuclease. In *in vivo* primer extension experiments carried out with two different mRNAs, a major band was detected after YoeB induction at a position three bases downstream of the initiation codon at 2.5 min. An identical band was also detected in *in vitro* toeprinting experiments when YoeB was added to the reaction mixtures containing 70 S ribosomes and the same mRNAs even in the absence of initiator $tRNA_f^{Met}$. When $tRNA_f^{Met}$ was added to the reaction mixtures in the absence of YoeB, toeprinting sites were shifted to a position 13 to 14 bases downstream of the initiation codon. When YoeB forms a complex with 70 S ribosomes and mRNA *in vitro*, partial cleavage of mRNAs is also observed at positions three and four bases downstream of the initiation codon as observed in *in vivo* experiments. Notably, this partial cleavage was not observed when YoeB was incubated with mRNA in the absence of ribosomes. However, when a nuclease-negative YoeB mutant (H83Q) (3) was used in *in vitro* toeprinting experiments, partial cleavage was observed. Using tetracycline and hygromycin B, we demonstrated that YoeB binds to the 50 S ribosomal subunit in 70 S ribosomes and interacts with the A site leading to mRNA cleavage at this site. As a result, the 3′-end portion of the mRNA was released from ribosomes. These results indicate that YoeB associates with 50 S subunits in 70 S ribosomes to effectively inhibit translation initiation by preventing the formation of the translation initiation complex on mRNAs.

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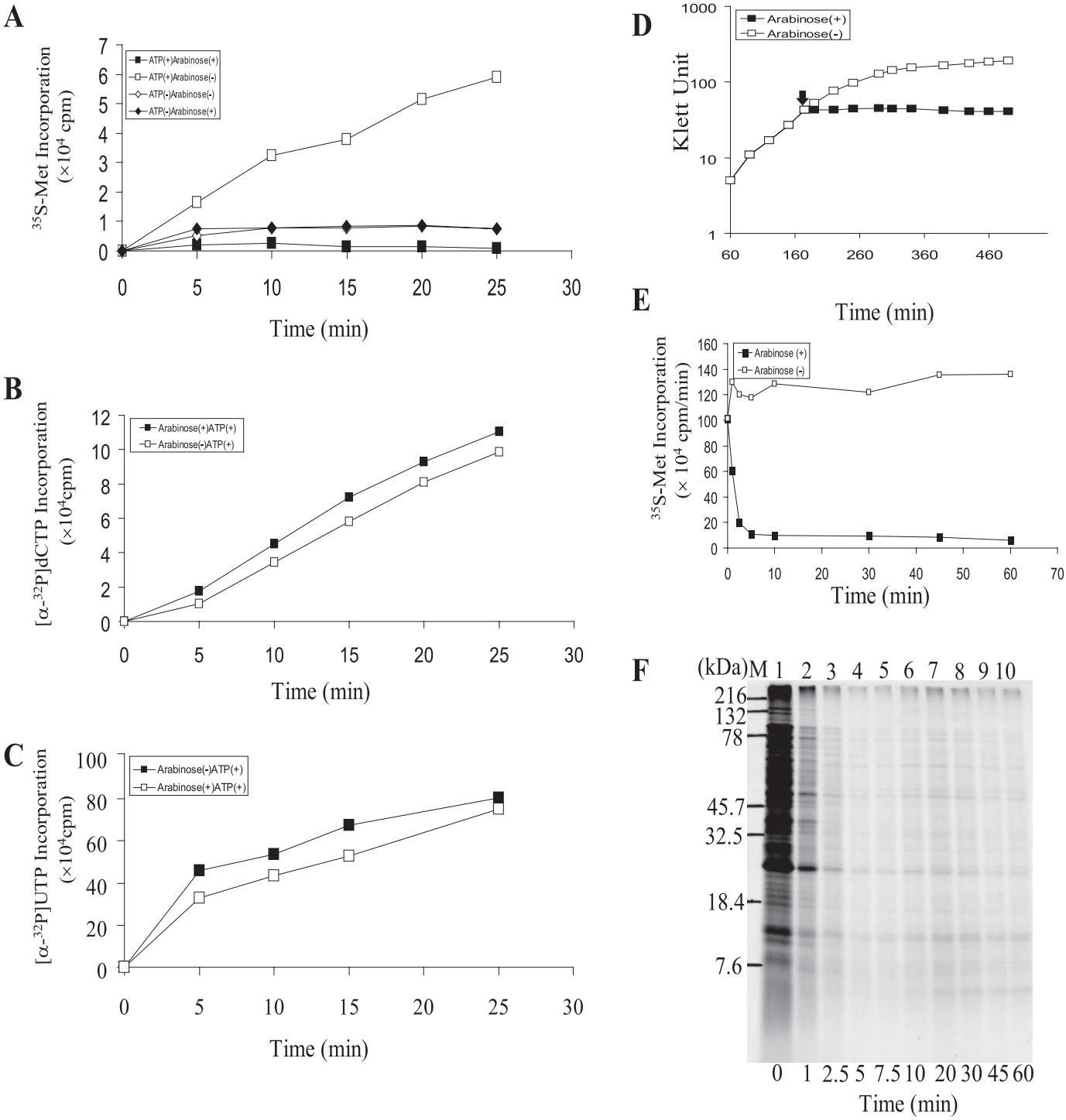


FIGURE 1. **Effect of YoeB on protein, DNA, and RNA synthesis.** *A*, effect of YoeB on [^{35}S]methionine incorporation in toluene-treated cells. *E. coli BW25113* cells containing pBAD-YoeB were grown at 37 °C in glycerol-M9 medium. When the A_{600} of the culture reached 0.4, arabinose was added to a final concentration of 0.2%. After incubation at 37 °C for 10 min, the cells were treated with toluene (10, 11). Using toluene-treated cells, ATP-dependent protein synthesis was carried out with [^{35}S]methionine as described previously (10). *B*, effect of YoeB on [α-^{32}P]dCTP incorporation in toluene-treated cells (12). *C*, effect of YoeB on [α-^{32}P]UTP incorporation in toluene-treated cells (13). *D*, growth curve of BW25113 cells containing pBAD-YoeB plasmid in LB medium. Arabinose was added at 175 min as indicated by the *solid arrow*. *E*, effect of YoeB on the rate of [^{35}S]methionine incorporation *in vivo*. [^{35}S]Methionine incorporation into *E. coli* BW25113 cells containing pBAD-YoeB was measured at various time points after YoeB induction as indicated. *F*, SDS-PAGE analysis of *in vivo* protein synthesis after the induction of YoeB. The same cultures in *E* were used.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—E. coli BL21(DE3), BW25113 (Δ*ara*BAD) (8), and *MRE600* (9) were used. The *yefM-yoeB* operon was amplified by PCR using *E. coli* genomic DNA as template and cloned into the NdeI-XhoI sites of pET21c (Novagen). This construction created an in-frame translation fusion with a $(His)_6$ tag at the *yoeB* C-terminal end. The plasmid was designated as pET21c-YefM-YoeB$(His)_6$. The *yoeB* gene was

cloned into pBAD creating pBAD-YoeB to tightly regulate *yoeB* expression by the addition of arabinose (0.2%). The *yefM* gene was cloned into pGEX-4T-1 (Amersham Biosciences) creating pGEX-4T-1-YefM.

Assay of Protein, DNA and RNA Synthesis in Toluene-treated Cells—A 70-ml culture of *E. coli* BW25113 containing pBAD-YoeB plasmid was grown at 37 °C in M9 medium with 0.5% glycerol (no glucose) and all the amino acids except for methionine and cysteine (1 mM each). When the A_{600} of the culture reached 0.4, arabinose was added to a final concentration of 0.2%. After incubation at 37 °C for 10 min, the cells were treated with 1% toluene (10, 11). Using toluene-treated cells, protein synthesis was carried out with [^{35}S]methionine as described previously (10, 11). The toluene-treated cells were washed once with 0.05 M potassium phosphate buffer (pH 7.4) at room temperature, and then re-suspended into the same buffer to examine DNA synthesis using [α-^{32}P]dCTP as described previously (12). For assaying RNA synthesis, the toluene-treated cells were washed once with 0.05 M Tris-HCl buffer (pH 7.5) at room temperature, and then re-suspended into the same buffer to measure [α-^{32}P]UTP incorporation into RNA as described previously (13).

Assay of in Vivo Protein Synthesis—E. coli BW25113 cells containing pBAD-YoeB were grown in M9 medium with 0.5% glycerol (no glucose) and all the amino acids except for methionine and cysteine (1 mM each). When the A_{600} value of the culture reached 0.4, arabinose was added to a final concentration of 0.2% to induce YoeB expression. Cell cultures (0.6 ml) were taken at time intervals as indicated in Fig. 1*F* and mixed with 30 μCi of [^{35}S]methionine. After a 1-min incubation at 37 °C, the rate of protein synthesis was determined as described previously (6). For SDS-PAGE analysis of the total cellular protein synthesis, samples were removed from the [^{35}S]methionine incorporation reaction mixture (500 μl) at time intervals indicated in Fig. 1*F* and added into chilled test tubes containing 100 μg/ml each of non-radioactive methionine and cysteine. Cell pellets collected by centrifugation were dissolved into 50 μl of loading buffer and subjected to SDS-PAGE followed by autoradiography.

Purification of YoeB(His)$_6$ and GST-YefM Proteins—YoeB(His)$_6$ tagged at the C-terminal end was purified from strain BL21(DE3) carrying pET-21cc-YefM-YoeB. The complex of YoeB(His)$_6$ and YefM was first trapped on nickel-nitrilotriacetic acid resin. After dissociating YefM from YoeB-(His)$_6$ in 6 M guanidine HCl at 4 °C, YoeB(His)$_6$ was re-trapped by nickel-nitrilotriacetic resin and refolded by step-by-step dialysis against buffer (50 mM NaH_2PO_4, 500 mM NaCl, pH 8.5) at 4 °C overnight. GST-YefM tagged at the N-terminal end was purified from strain BL21(DE3) carrying pGEX-4T-1-YefM with use of glutathione-Sepharose 4B resin (Amersham Biosciences).

Effect of YoeB and MazF on Protein Synthesis in Prokaryotic and Eukaryotic Cell-free Systems—Prokaryotic cell-free protein synthesis was carried out with an *E. coli* T7 S30 extract system (Promega). The reaction mixture consisted of 10 μl of S30 premix, 7.5 μl of S30 extract, and 2.5 μl of an amino acid mixture (1 mM each of all amino acids except methionine), 1 μl of [^{35}S]methionine, and different amounts of YoeB(His)$_6$ and GST-YefM in a final volume of 29 μl. The different amounts of YoeB(His)$_6$ and GST-YefM were preincubated for 10 min at 25 °C before the assay was started by adding 1 μl of pET-11a-MazG plasmid-DNA (0.16 μg/μl) (15). The reaction was performed for 1.5 h at 37 °C, and proteins were then precipitated with acetone and analyzed by SDS-PAGE followed by autoradiography. Eukaryotic cell-free protein synthesis was carried out with a rabbit reticulocyte lysates system, TnT® T7 Coupled Reticulocyte Lysate System (Promega). A luciferase T7 control DNA was used as DNA template. The reaction was performed for 1.5 h at 30 °C, and proteins were then precipitated with acetone and analyzed by SDS-PAGE.

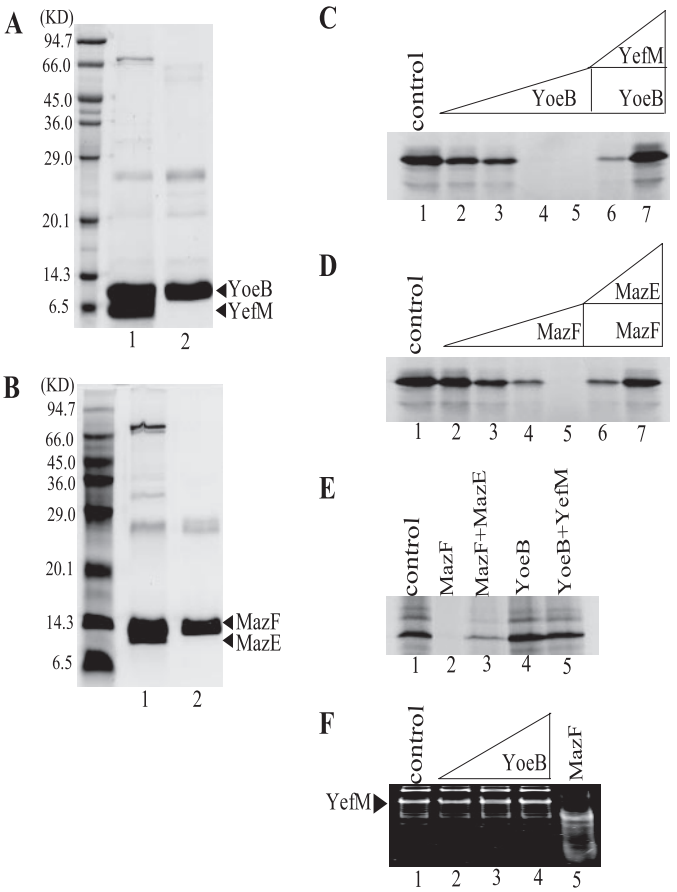


FIGURE 2. **Effect of purified YoeB(His)$_6$ and MazF(His)$_6$, on cell-free protein synthesis.** *A*, purification of YoeB(His)$_6$. *Lane 1*, purified YefM-YoeB(His)$_6$ complex, and *lane 2*, purified YoeB(His)$_6$. *B*, purification of MazF(His)$_6$. *Lane 1*, purified MazE-MazF(His)$_6$ complex, and *lane 2*, purified MazF(His)$_6$. *C*, effect of YoeB(His)$_6$ on MazG synthesis in a prokaryotic cell-free protein synthesis system. *Lane 1*, without YoeB(His)$_6$; *lanes 2–5*, 30, 60, 240, and 960 nM YoeB(His)$_6$ were added, respectively; and *lanes 6* and *7*, 960 nM YoeB(His)$_6$ plus GST-YefM in its ratios to YoeB(His)$_6$ of 2.8 and 4.2, respectively. *D*, effect of MazF(His)$_6$ on MazG synthesis in a prokaryotic cell-free protein synthesis system. *Lane 1*, without MazF(His)$_6$; *lanes 2–5*, 161, 644, 1288, and 2576 nM MazF(His)$_6$ were added, respectively; *lanes 6* and *7*, 2576 nM MazF(His)$_6$ plus MazE(His)$_6$ in its ratios to MazF(His)$_6$ of 1.7 and 11.9, respectively. *E*, effect of MazF(His)$_6$ and YoeB(His)$_6$ on the synthesis of luciferase in a eukaryotic cell-free protein synthesis system. *Lane 1*, without protein; *lane 2*, 2.57 μM MazF(His)$_6$; and *lane 3*, 31.5 μM MazE(His)$_6$ and 2.57 μM MazF(His)$_6$ (the ratio of (His)$_6$MazE to MazF-(His)$_6$, 11.9:1); *lane 4*, 1.92 μM YoeB(His)$_6$; and *lane 5*, 8.1 μM GST-YefM and 1.92 μM YoeB(His)$_6$ (the ratio of GST-YefM to YoeB(His)$_6$, 4.2:1). In both cell-free systems, the reactions were carried out in the presence of [^{35}S]methionine according to the instructions given by the manufacturer. Reaction products were analyzed by SDS-PAGE followed by autoradiography. *F*, effect of YoeB on the *yefM* mRNA *in vitro*. *Lane 1*, no protein was added; *lanes 2–4*, 40, 80, and 160 nM YoeB were added; *lane 5*, 80 nM MazF was added.

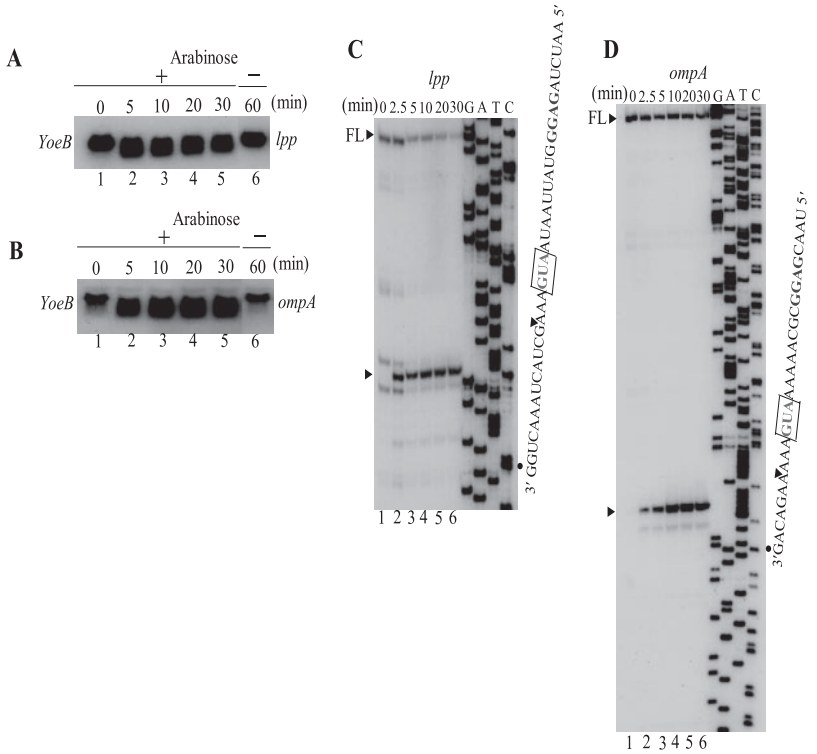


FIGURE 3. **Effect of YoeB on cellular mRNAs and identification of YoeB cleavage sites in the chromosomally encoded *lpp* and *ompA* mRNAs.** Total cellular RNA was extracted from *E. coli* BW25113 cells containing pBAD-YoeB at various time points as indicated after the addition of arabinose and subjected to Northern blot analysis using radiolabeled *lpp* and *ompA* open reading frames as probes shown in *A* and *B*, respectively. For *in vivo* primer extension, the *lpp* and *ompA* mRNAs were prepared from *E. coli* BW25113 cells containing pBAD-YoeB at various time points as indicated in *C* and *D* before and after the induction of YoeB. The sequence ladders for *lpp* and *ompA* were obtained using pCR®2.1-TOPO®-*lpp* and pCR®2.1-TOPO-*ompA* as template, respectively. The sequences around the major cleavage sites are shown at the *right-hand* side and the major primer extension stop sites are indicated by *arrowheads*. On the *top* of the gels, the full-length (*FL*) RNA bands are shown with an *arrowhead* and *FL*. *C*, primer extension analysis of the *lpp* mRNA. *D*, primer extension analysis of the *ompA* mRNA. *FL*, full-length.

Effect of YoeB on the yefM mRNA in Vitro—Different amounts of YoeB were incubated with 1 μg of the *yefM* mRNA at 37 °C for 15 min in a 10-μl reaction mixture containing 10 mM Tris-Cl (pH 7.8), 60 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM DTT[2] as indicated in Fig. 2*F*. The reaction products were analyzed by 8% acrylamide native gel electrophoresis followed by ethidium bromide staining.

Preparation of E. coli 70 S Ribosomes—70 S ribosomes were prepared from *E. coli* MRE 600 as described previously (16–18) with minor modifications. Bacterial cells (2 g) were suspended in buffer A (10 mM Tris-HCl (pH 7.8) containing 10 mM $MgCl_2$, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol). Cells were lysed by French Press. Cell debris was removed by centrifugation two times at 30,000 × *g* for 30 min at 4 °C with a Beckman 50Ti rotor. The supernatant (three-fourths volume from the top) was then layered over an equal volume of 1.1 M sucrose in buffer B (buffer A containing 0.5 M NH_4Cl) and centrifuged at 45,000 × *g* for 15 h at 4 °C with a Beckman 50Ti rotor. After washing with buffer A, the ribosome pellets were resuspended in buffer A and applied to a linear 5 to 40% (w/v) sucrose gradient prepared in buffer A, and centrifuged at 35,000 × *g* for 3 h at 4 °C with a Beckman SW41Ti rotor. Gradients were fractionated and the 70 S ribosome fractions were pooled, pelleted at 45,000 × *g* for 20 h at 4 °C with a Beckman 50Ti rotor. The 70 S ribosome pellets were resuspended in buffer A before they were stored at −80 °C.

Ribosome Profile Analysis—For polysome profile analysis, cells containing pBAD-YoeB plasmid were grown at 37 °C in 150 ml of LB medium and at A_{600} of 0.6, the arabinose was added to a final concentration of 0.2%, after 10 min induction, chloramphenicol was added to a final concentration of 200 μg/ml. Cell pellets were suspended in 1 ml of buffer A (10 mM Tris-Cl, pH 7.8, 60 mM NH_4Cl, 10 mM $MgCl_2$, and 1 mM DTT). Extracts were prepared using liquid nitrogen freezing and thawing four times. After centrifugation at 45,000 × *g* for 10 min at 4 °C with a Beckman 100.3 Ti rotor, about 10 A_{260} units of the supernatant were layered onto a 5–40% sucrose gradient in buffer A and centrifuged at 35,000 × *g* for 3 h at 4 °C in a Beckman SW41 rotor. Gradients were analyzed with continuous monitoring at 254 nm. To analyze a 50 S and 30 S ribosome profile, another 10 A_{260} units of supernatant as prepared above were dialyzed overnight against buffer B (10 mM Tris-HCl, pH 7.8, 60 mM NH_4Cl, 0.5 mM $MgCl_2$, and 1 mM DTT) (dialysis buffer was changed once) and then layered onto 10–30% sucrose gradients in buffer B and centrifuged at 35,000 × *g* for 3 h at 4 °C in a Beckman SW41 rotor. Gradients were analyzed with continuous monitoring at 254 nm.

Primer Extension Analysis in Vivo—For primer extension analysis of mRNA cleavage sites *in vivo*, total RNAs were extracted from the *E. coli* BW25113 cells containing pBAD-YoeB at different time points as indicated in Fig. 3, *C* and *D*. Primer extension reactions were carried out for 1 h using different primers at 42 °C using reverse transcriptase-avian myeloblastosis virus (2 units) as described previously (6).

Toeprinting Assays—Toeprinting was carried out as described previously (19) with a minor modification. The mixture for primer-template annealing containing mRNA and ^{32}P-end labeled DNA primer was incubated at 70 °C for 5 min, and then cooled slowly to room temperature. The ribosome-binding mixture contained 2 μl of 10× buffer (100 mM Tris-HCl, pH 7.8, containing 100 mM $MgCl_2$, 600 mM NH_4Cl, and 60 mM 2-mercaptoethanol), different amounts of YoeB$(His)_6$, 0.375 mM dNTP, 0.05 μM 70 S ribosomal subunits, 1 μM $tRNA_f^{Met}$, and 2 μl of the annealing mixture in a final volume of 20 μl. The final mRNA concentration was 0.035 μM. This ribosome-binding mixture was incubated at 37 °C for 10 min, and then reverse transcriptase (2 units) was

[2] The abbreviations used are: DTT, dithiothreitol; TP, toeprinting.

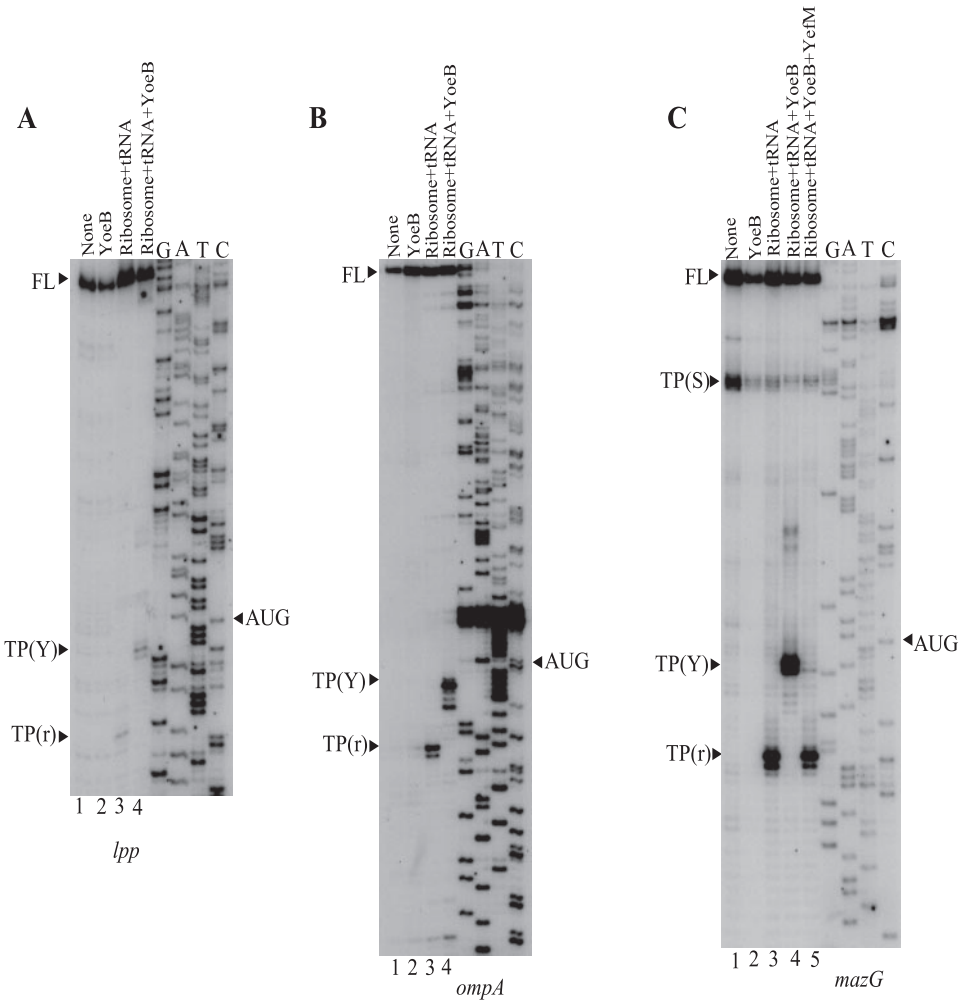


FIGURE 4. **Toeprinting of the *lpp, ompA,* and *mazG* mRNA.** *A*, toeprinting of the *lpp* mRNA in the presence of YoeB(His)$_6$. *B*, toeprinting of the *ompA* mRNA in the presence of YoeB(His)$_6$. *C*, toeprinting of the *mazG* mRNA in the presence of YoeB(His)$_6$. For each panel, *lane 1*, without YoeB(His)$_6$, 70 S ribosomes, and tRNA$_f^{Met}$; *lane 2*, 3.4 μM YoeB(His)$_6$ alone; *lane 3*, 0.05 μM 70 S ribosomes and 1 μM tRNA$_f^{Met}$; *lane 4*, 0.05 μM 70 S ribosomes and 1 μM tRNA$_f^{Met}$ with 3.4 μM YoeB(His)$_6$; *lane 5*, 0.05 μM 70 S ribosomes and 1 μM tRNA$_f^{Met}$ with 3.4 μM YoeB(His)$_6$ and 13.6 μM glutathione *S*-transferase-YefM. The sequence ladders shown at the *right-hand* side in all cases were obtained using the same primers used for toeprinting with their corresponding genes cloned in pCR 2.1-TOPO as template. All mRNA sequences shown are complementary to the sequencing ladders. The initiation codon, AUG, is indicated with an *arrowhead*. *TP*(*Y*) is the band where toeprinting was stopped in the presence of YoeB(His)$_6$. *FL*, the full-length of the mRNA; and *TP*(*r*), the toeprinting site due to normal ribosome binding to mRNA in the absence of YoeB(His)$_6$. The band at TP(S) is likely to be formed due to a secondary structure of the mRNA.

added. The cDNA synthesis was carried out at 37 °C for 15 min. The reaction was stopped by adding 12 μl of the sequencing loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol EF). The sample was incubated at 90 °C for 5 min prior to electrophoresis on a 6% polyacrylamide sequencing gel. The *lpp* and *ompA* mRNAs were synthesized *in vitro* from their individual DNA fragments containing a T7 promoter and part of their opening reading frames using T7 RNA polymerase. These ~200-bp DNA fragments for *lpp* (194 bp) and *ompA* (248 bp), all of which had the initiation codon at the center, were amplified by PCR using appropriate primers using chromosome DNA as templates, respectively. The 5′-end primers for *lpp and ompA* contained the T7 promoter sequence. The *mazG* DNA fragment containing a T7 promoter and part of the *mazG* open reading frame as described earlier (6) was obtained by PCR amplification using the pET-11a-MazG plasmid (15) as DNA template. mRNA produced from this DNA fragment was 151 bases in length.

RNA Isolation and Northern Blot Analysis—E. coli BW25113 containing pBAD-YoeB was grown at 37 °C in LB medium. When the A_{600} value reached 0.6, arabinose was added to a final concentration of 0.2%. The samples were taken at different intervals as indicated in Fig. 3, *A* and *B*. Total RNA was isolated using the hot-phenol method as described previously (20). Northern blot analysis was carried out as described previously (21).

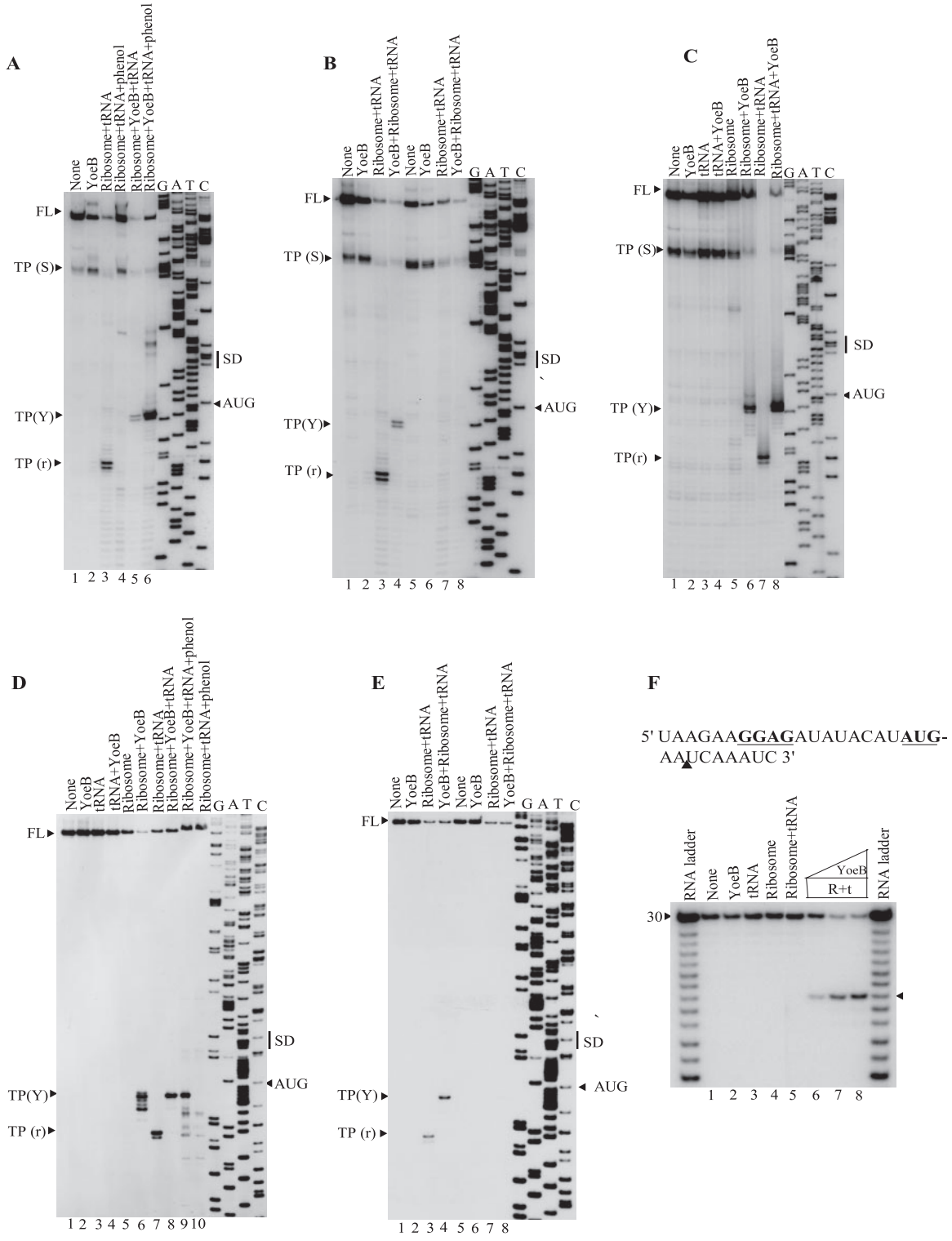
Binding of [^{35}S]Met-tRNA$_f^{Met}$ to the mRNA-70 S Ribosome Complex—Reactions were carried out with *ompA* mRNA that was used for the toeprinting experiments. The reaction buffer used was buffer A (10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 60 mM NH_4Cl, and 6 mM β-mercaptoethanol). 70 S ribosomes (1 pmol) were first incubated with the *ompA* mRNA (0.7 pmol) and [^{35}S]Met-tRNA$_f^{Met}$ (20 pmol) for 10 min at 37 °C, and then different amounts of YoeB were added in a final reaction volume of 20 μl. The mixture was incubated for an additional 10 min at 37 °C. Reactions mixtures were applied to nitrocellulose filters (Millpore 0.45 μM HA) and the filters were washed twice with 2 ml of buffer A before measuring the radioactivity. [^{35}S]Met-tRNA$_f^{Met}$ was synthesized in a buffer containing 30 mM Hepes, pH 7.6, 1 mM DTT, 0.1 mM EDTA, 3 mM ATP, 5 mM $Mg(OAc)_2$, 30 mM KOAc, 350 μCi of [^{35}S]methionine, and 62.3 μM tRNA$_f^{Met}$ (Sigma) using aminoacyl-tRNA synthetases, which was prepared as described previously (25).

Effect of YoeB(His)$_6$ on the 30-Base RNA in the Presence 70 S Ribosomes and tRNA$_f^{Met}$—The 30-base RNA was 5′-end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (2 units). The mixture of 0.05 μM 70 S ribosomes, 1 μM tRNA$_f^{Met}$, and different amounts of YoeB (0.16, 0.64, and 2.56 μM in *lanes 6–8*, respectively, in Fig. 5*F*) were preincubated at 37 °C for 10 min, and then 0.035 μM ^{32}P-labeled 30-base RNA was added. The reaction mixture was incubated at 37 °C for another 10 min and then the reaction product was loaded on a 20% sequencing gel.

RESULTS

YoeB Inhibits Protein Synthesis Immediately After Its Induction—To identify the cellular functions inhibited by YoeB, a cell-free system prepared from *E. coli* BW25113 cells

carrying arabinose-inducible pBAD-YoeB vector was used. The cells were permeabilized by toluene treatment (10, 11). ATP-dependent [^{35}S]methionine incorporation was completely inhibited when cells were preincubated for 10 min in the presence of arabinose before toluene treatment (Fig. 1*A*). However, the incorporation of [α-^{32}P]dCTP (Fig. 1*B*) and [α-^{32}P]UTP (Fig. 1*C*) was not significantly affected under similar conditions (12, 13). These results demonstrate that YoeB inhibits protein synthesis, but not DNA replication or RNA synthesis. Notably, cell growth was inhibited almost immediately after the addition of arabinose (Fig. 1*D*). The *in vivo* incorporation of [^{35}S]methionine was also dramatically inhibited within 5 min after YoeB induction (Fig. 1, *E* and *F*). MazF is another potent *E. coli* toxin that functions as an mRNA interferase that specifically cleaves mRNAs at ACA triplet sequences and consequently leads to inhibition of protein synthesis (6). However, in contrast to



YoeB, complete inhibition of protein synthesis by MazF requires significantly longer time (15–20 min) after its induction (6).

Inhibitory Effect of Purified YoeB on Cell-free Protein Synthesis—Next, we examined the effect of purified YoeB on *E. coli* cell-free protein synthesis. YoeB was purified from cells co-expressing both YefM and YoeB as described under "Experimental Procedures" (Fig. 2*A*). MazF was purified (Fig. 2*B*) as described previously (6). The synthesis of MazG protein (30 kDa) (15) from plasmid pET-11a-MazG was tested at 37 °C for 1 h in the absence and presence of YoeB using an *E. coli* T7 S30 extract system (Promega) (Fig. 2*C*). MazG synthesis was almost completely blocked at YoeB concentrations of 240 nM or above. Similar inhibition was observed when purified MazF was added (Fig. 2*D*) consistent with the previous result (6).

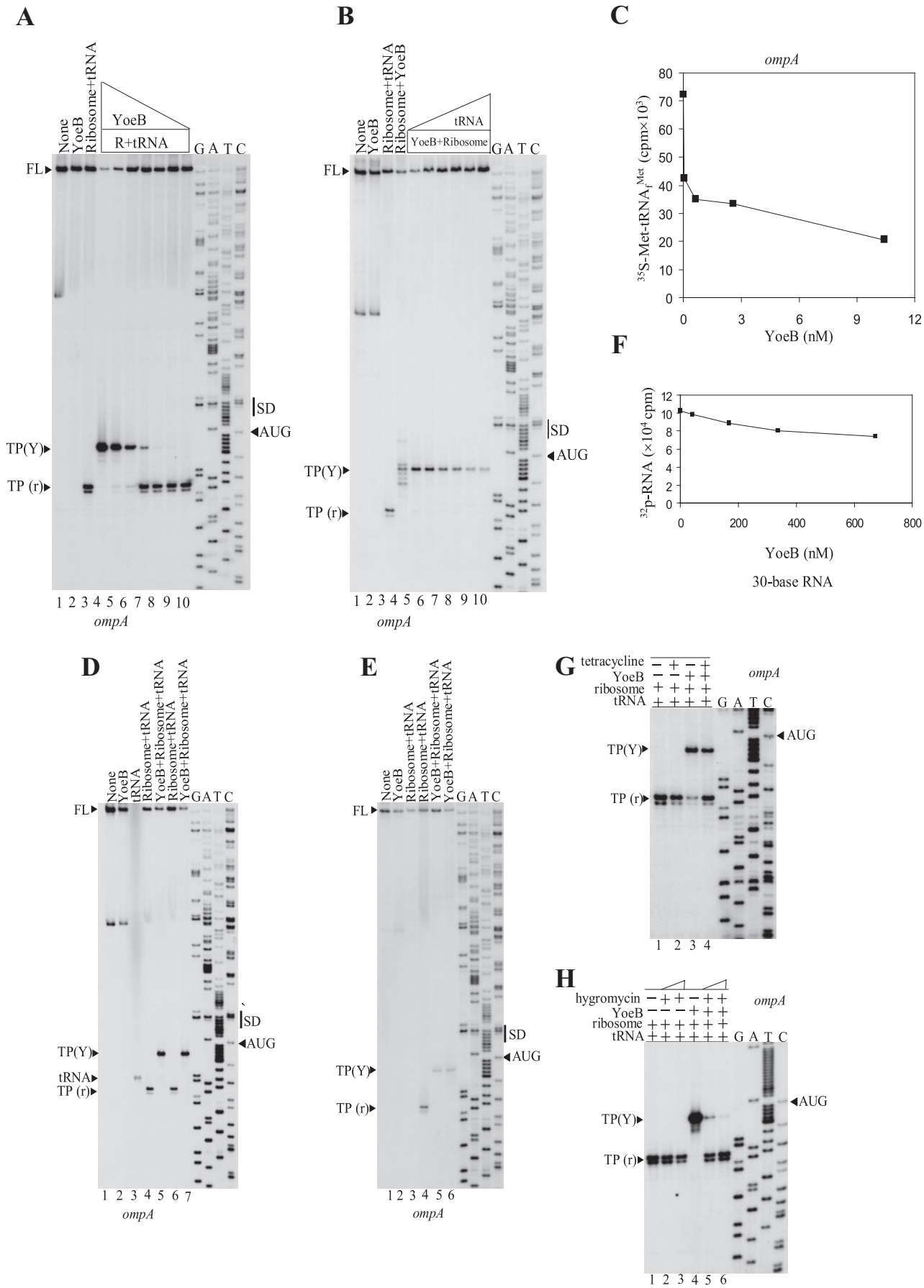
We then tested the effect of YefM antitoxin on the YoeB-mediated inhibition of MazG synthesis. The addition of GST-YefM rescued MazG synthesis in a dose-dependent manner (Fig. 2*C*). Importantly, YoeB did not inhibit eukaryotic cell-free protein synthesis (Fig. 2*E*, *lane 4*) in contrast to MazF (Fig. 2*E*, *lane 2*), which is known to inhibit both prokaryotic, and eukaryotic protein synthesis (6). These results indicate that YoeB is a protein synthesis inhibitor specific to prokaryotes. This is also consistent with the observation that YoeB induction in yeast cells had no effect on cell growth, whereas MazF induction blocked yeast cell growth (data not shown). It should be noted that YoeB alone could not degrade YefM mRNA under our experimental condition (Fig. 2*F*).

YoeB Induction Does Not Affect the Stability of Cellular mRNAs—Despite the abrupt inhibition of protein synthesis by YoeB, its induction did not severely affect the stability of cellular mRNAs. On the other hand, the induction of MazF significantly decreased stability of cellular mRNAs (6). Most significantly, full-length *lpp* and *ompA* mRNAs very quickly disappeared after MazF induction (10 min) (6), whereas all these mRNAs appeared to be more stable after YoeB induction (Fig. 3, *A* and *B*).

In Vivo Cleavage of the lpp and ompA mRNAs—As shown in Fig. 3, *A* and *B*, cellular mRNAs were partially degraded upon induction of YoeB. Therefore we next attempted to identify the cleavage sites of the chromosomally encoded *lpp* and *ompA* mRNAs by primer extension experiments. For this purpose, total RNA was extracted from *E. coli* BW25113 cells harboring pBAD-YoeB at different time intervals following induction of YoeB. The primer extension analyses of *lpp* (Fig. 3*C*) and *ompA* (Fig. 3*D*) mRNAs demonstrated that the distinct major bands exhibiting the specific cleavage sites in each mRNA appeared as early as 2.5 min after YoeB induction (Fig. 3, *C* and *D*, *lane 2*). The band intensities for *ompA* (Fig. 3*D*) mRNA further increased from 2.5 to 10–20 min after YoeB induction. Interestingly, in both cases, the major bands resulted from cleavage of the mRNAs at three bases downstream of AUG, and most notably no other bands were observed in the regions between 5′-ends of the mRNAs and the initiation codon suggesting that YoeB may function only when it associates with the ribosomal translation machine. It is interesting to note that the substantial amounts of the full-length mRNAs for *lpp* (Fig. 3*C*) and *ompA* (Fig. 3*D*) mRNAs remained uncleaved even 30 min after induction of YoeB. This observation is again consistent with the fact that unlike MazF, YoeB does not significantly affect the stability of cellular mRNAs.

YoeB Binds to the Translation Initiation Complex in Vitro—Next, we examined if similar cleavage of *lpp* and *ompA* mRNAs was observed *in vitro* by toeprinting (TP) (19). In this experiment, we also used the *mazG* mRNA (Fig. 4*C*), which we previously used in our experiments with MazF (6) in addition to *lpp* (Fig. 4*A*) and *ompA* (Fig. 4*B*) mRNAs. Primer extension analysis of the mRNAs alone yielded the full-length bands (Fig. 4, *A*–*C*, *lane 1*). Importantly, the addition of YoeB alone did not result in formation of new bands indicating that YoeB by itself has no endoribonuclease activity under the conditions used (Fig. 4, *A*–*C*, *lane 2*). When 70 S ribosomes and initiator $tRNA_f^{Met}$ were added to the reaction mixtures, typical toeprinting band (TP(r)) downstream of the initiation codon appeared (Fig. 4, *A*–*C*, *lane 3*). On the other hand, when YoeB was added together with 70 S ribosomes and initiator $tRNA_f^{Met}$, a new band TP(Y) (Fig. 4, *A*–*C*, *lane 4*) appeared with concomitant disappearance of the TP(r) band in all cases. This new band, TP(Y), is 10 bases upstream of the normal toeprinting band (13–14 bases downstream of the initiation codon) as judged from the sequence

FIGURE 5. **Effect of a ribosome-binding site mutation in *mazG* and *ompA* mRNAs on the inhibitory activity of YoeB(His)$_6$.** *A*, toeprinting of the *mazG* mRNA after phenol extraction. The experiment was carried out in the same way as described in the legend to Fig. 4*C* except that reaction products in *lanes 4* and *6* were phenol-extracted to remove proteins before primer extension. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 3.4 μM YoeB(His)$_6$ alone; *lanes 3* and *4*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$; and *lanes 5* and *6*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$ with 3.4 μM YoeB(His)$_6$. *B*, effect of a mutation (GGAG to AAUG) at the Shine-Dalgarno sequence of the *mazG* mRNA on the YoeB(His)$_6$ activity. *Lanes 1* and *5*, without YoeB(His)$_6$; *lanes 2* and *6*, 3.4 μM YoeB(His)$_6$; *lanes 3* and *7*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$; *lanes 4* and *8*, 3.4 μM YoeB(His)$_6$, 0.05 μM 70 S ribosomes, and 1 μM $tRNA_f^{Met}$. *Lanes 1–4*, the wild-type *mazG* mRNA was used; *lanes 5–8*, the mutated *mazG* mRNA was used. *C*, toeprinting of the *mazG* mRNA in the presence of YoeB(His)$_6$ and 70 S ribosomes with and without $tRNA_f^{Met}$. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 3.4 μM YoeB(His)$_6$; *lane 3*, 1 μM $tRNA_f^{Met}$; *lane 4*, 3.4 μM YoeB(His)$_6$ and 1 μM $tRNA_f^{Met}$; *lane 5*, 0.05 μM 70 S ribosomes; *lane 6*, 0.05 μM 70 S ribosomes with 3.4 μM YoeB(His)$_6$; *lane 7*, 0.05 μM 70 S ribosomes with 1 μM $tRNA_f^{Met}$; and *lane 8*, 0.05 μM 70 S ribosomes with 1 μM $tRNA_f^{Met}$ and 3.4 μM YoeB(His)$_6$. The band at TP(S) in Fig. 5, *A*–*C*, is likely to be formed due to a secondary structure of the mRNA. *D*, toeprinting of the *ompA* mRNA after phenol extraction and toeprinting of the *ompA* mRNA in the presence of YoeB(His)$_6$ and 70 S ribosomes with and without $tRNA_f^{Met}$. The experiment was carried out in the same way as described in the legend to Fig. 5, *A* and *C*. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 3.4 μM YoeB(His)$_6$; *lane 3*, 1 μM $tRNA_f^{Met}$; *lane 4*, 3.4 μM YoeB(His)$_6$ and 1 μM $tRNA_f^{Met}$; *lane 5*, 0.05 μM 70 S ribosomes; *lane 6*, 0.05 μM 70 S ribosomes with 3.4 μM YoeB(His)$_6$; *lane 7*, 0.05 μM 70 S ribosomes with 1 μM $tRNA_f^{Met}$; and *lane 8*, 0.05 μM 70 S ribosomes with 1 μM $tRNA_f^{Met}$ and 3.4 μM YoeB(His)$_6$; *lane 9*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$ with 3.4 μM YoeB(His)$_6$; *lane 10*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$. Reaction products in *lanes 9* and *10* were phenol-extracted before primer extension. *E*, effect of the mutation (GGAG to GAAA) at the Shine-Dalgarno sequence of the *ompA* mRNA on the YoeB(His)$_6$ activity. *Lanes 1* and *5*, without YoeB(His)$_6$; *lanes 2* and *6*, 3.4 μM YoeB(His)$_6$; *lanes 3* and *7*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$; *lanes 4* and *8*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$ together with 3.4 μM YoeB(His)$_6$. *Lanes 1–4*, the wild-type *ompA* mRNA; *lanes 5–8*, the mutated *ompA* mRNA. *F*, the RNA sequence of a 30-base synthetic RNA and the effect of YoeB(His)$_6$ on the 30-base RNA in the presence 70 S ribosomes and $tRNA_f^{Met}$. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 2.56 μM YoeB(His)$_6$; *lane 3*, 1 μM $tRNA_f^{Met}$; *lane 4*, 0.05 μM 70 S ribosomes; *lane 5*, 0.05 μM 70 S ribosomes and 1 μM $tRNA_f^{Met}$; and *lanes 6–8*, 0.05 μM 70 S ribosomes, 1 μM $tRNA_f^{Met}$, and 0.16, 0.64, and 2.56 μM YoeB(His)$_6$, respectively. An *arrowhead* indicates the cleavage product, which migrated at the position seven bases shorter than the 30-base RNA on the basis of the ladder (a partial alkaline hydrolysate of the 30-base RNA). *SD*, Shine-Dalgarno; *FL*, full-length.


A
None
YoeB
Ribosome+tRNA
YoeB
R+tRNA
G A T C
FL
SD
AUG
TP(Y)
TP (r)
1 2 3 4 5 6 7 8 9 10
ompA
B
None
YoeB
Ribosome+tRNA
Ribosome+YoeB
tRNA
YoeB+Ribosome
G A T C
FL
SD
AUG
TP(Y)
TP (r)
1 2 3 4 5 6 7 8 9 10
ompA
C
ompA
35S-Met-tRNAfMet (cpm×10³)
YoeB (nM)
F
32p-RNA (×10⁴ cpm)
YoeB (nM)
30-base RNA
D
None
YoeB
tRNA
Ribosome+tRNA
YoeB+Ribosome+tRNA
Ribosome+tRNA
YoeB+Ribosome+tRNA
G A T C
FL
SD
AUG
TP(Y)
tRNA
TP (r)
1 2 3 4 5 6 7
ompA
E
None
YoeB
Ribosome+tRNA
Ribosome+tRNA
YoeB+Ribosome+tRNA
YoeB+Ribosome+tRNA
G A T C
FL
SD
AUG
TP(Y)
TP (r)
1 2 3 4 5 6
ompA
G
tetracycline
YoeB
ribosome
tRNA
ompA
G A T C
AUG
TP(Y)
TP (r)
1 2 3 4
H
hygromycin
YoeB
ribosome
tRNA
ompA
G A T C
AUG
TP(Y)
TP (r)
1 2 3 4 5 6

ladder at the right-hand side. It should be noted that this band is observed only in the presence of 70 S ribosomes, initiator $tRNA_f^{Met}$, and YoeB (Fig. 4, *A–C*, *lane 4*). Interestingly, this TP(Y) band disappeared with concomitant appearance of the TP(r) band when YefM, the YoeB antitoxin, was added together with YoeB (Fig. 4*C*, *lane 5*). The band below the full-length band shown by TP(S) was probably due to the secondary structure present in the *mazG* mRNA because this band was detected in the absence of any factors (Fig. 4*C*, *lane 1*).

Next, we tested whether the TP(Y) and TP(r) bands resulted from the cleavage of mRNAs using *mazG* and *ompA* mRNAs. These mRNAs were first preincubated without (Fig. 5, *A*, *lane 3*, and *D*, *lane 7*) or with YoeB (Fig. 5, *A*, *lane 5* for the *mazG* mRNA, and *D*, *lane 8* for the *ompA* mRNA) in the presence of 70 S ribosomes and initiator $tRNA_f^{Met}$. RNAs were then phenol extracted and used for primer extension shown in Fig. 5*A* (*lanes 4* and *6*) for the *mazG* mRNA and Fig. 5*D* (*lanes 9* and *10*) for the *ompA* mRNA. The TP(Y) band was observed even after phenol extraction (compare Fig. 5, *A, lanes 5* and *6* for the *mazG* mRNA, and *D*, *lanes 8* and *9* for the *ompA* mRNA), indicating that the TP(Y) band resulted from at least partial cleavage of the *mazG* and *ompA* mRNAs. In contrast, the TP(r) band disappeared after phenol extraction, indicating that this band was not due to mRNA cleavage, but was caused by binding of ribosomes to the mRNAs. It is important to note that the mRNAs were cleaved three and four bases downstream of the translation initiation codon only in the presence of ribosomes, initiator $tRNA_f^{Met}$, and YoeB, but not in the presence of YoeB alone. Therefore, the latent endogenous endoribonuclease activity of 70 S ribosomes (5) may be enhanced only when YoeB binds to the ribosome initiation complex. The ribosome-dependent endoribonuclease activity induced by RelE preferentially cleaves mRNA at stop codons (UAG > UAA > UGA) and at certain sense codons (UCG and CAG) in *in vitro* experiments (4). Notably, the primary sequence of YoeB has only 15% identity with that of RelE. Comparison of the x-ray structures of the YoeB-YefM complex (3) and the RelE-RelB complex (26) demonstrates little homology between these complexes, although they share a microbial RNase fold (3). The possibility that YoeB may function as an endoribonuclease when it binds to the ribosome initiation complex is unlikely because a nuclease-negative YoeB mutant H83Q (3) was still able to block protein synthesis as shown later.

When the Shine-Dalgarno (SD) sequences, GGAG in the *mazG* mRNA (Fig. 5*B*, *lanes 1–4*) and GGAG in the *ompA* mRNA (Fig. 5*E*, *lanes 1–4*) were mutated to AAUG (Fig. 5*B*, *lanes 5*-8) and GAAA (Fig. 5*E*, *lanes 5–8*), respectively, both TP(Y) and TP(r) completely disappeared (compare Fig. 5, *B, lanes 4* and *8*, for the *mazG* mRNA, and *E,* for the *ompA* mRNA). These results indicate that the binding of 70 S ribosomes to the mRNA is absolutely required for the YoeB-mediated cleavage of mRNAs.

$tRNA_f^{Met}$ Is not Required for YoeB-Mediated A Site Cleavage—Next we examined if the initiator $tRNA_f^{Met}$ is required for YoeB-mediated mRNA cleavage in the presence of YoeB. Intriguingly, the *mazG* mRNA was cleaved even in the absence of $tRNA_f^{Met}$ if YoeB and 70 S ribosomes were included in the reaction, yielding identical toeprinting bands (compare Fig. 5*C*, *lane 6* with *8*). It should be noted that in the absence of YoeB, the addition of $tRNA_f^{Met}$ was required for the detection of the toeprinting band at the TP(r) position (compare *lane 7* with *5*, where toeprinting in *lane 7* was carried out in the presence of $tRNA_f^{Met}$, whereas that in *lane 5* was performed in the absence of $tRNA_f^{Met}$). The addition of YoeB alone (*lane 2*), $tRNA_f^{Met}$ alone (*lane 3*), or YoeB plus $tRNA_f^{Met}$ (*lane 4*) did not yield any bands either at TP(Y) or TP(r) positions. The identical cleavage pattern was observed with the *ompA* mRNA (Fig. 5*D*). These results suggest that $tRNA_f^{Met}$ was not required for YoeB-mediated A site cleavage. Note that both TP(Y) bands for *mazG* and *ompA* (Fig. 5, *C* and *D*, *lane 6*, respectively) were still detectable after phenol extraction (data not shown).

Cleavage of a Synthetic 30-Base RNA—To further unambiguously demonstrate that the mRNA cleavage occurring downstream of the initiation codon requires both ribosomes and YoeB, we repeated a similar experiment as above using a 30-base synthetic RNA that contains an SD sequence and AUG (see Fig. 5*F*). When YoeB was incubated with 70 S ribosomes, $tRNA_f^{Met}$, and the 5′-end ^{32}P-labeled 30-base RNA at 37 °C for

FIGURE 6. **Competition of [^{35}S]Met-$tRNA_f^{Met}$, tetracycline, and hygromycin B binding to the mRNA-70 S ribosome complex with YoeB, and release of the 3′-end portion of the *ompA* mRNA cleaved by the addition of YoeB from ribosomes.** *A*, effect of YoeB on the formation of the *ompA* mRNA-70 S ribosomes-$tRNA_f^{Met}$ complex. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 20.7 nM YoeB(His)$_6$; *lane 3*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lanes 4–10*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes and 20.7, 10.4, 2.58, 0.64, 0.16, 0.04, and 0.01 nM YoeB(His)$_6$, respectively. *B*, effect of $tRNA_f^{Met}$ on the activity of YoeB. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 20.7 nM YoeB(His)$_6$; *lane 3*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lane 4*, 0.05 μM 70 S ribosomes and 20.7 nM YoeB(His)$_6$; *lanes 5–10*, 0.05 μM 70 S ribosomes and 20.7 nM YoeB with 1, 4, 8, 16, 32, and 64 μM $tRNA_f^{Met}$, respectively. *C*, binding of [^{35}S]Met-$tRNA_f^{Met}$ to 70 S ribosomes at 37 °C in the absence and presence of different amounts of YoeB(His)$_6$. 70 S ribosomes (0.05 μM) were first incubated with [^{35}S]Met-$tRNA_f^{Met}$ (1 μM) and the *ompA* mRNA (0.035 μM) for 10 min at 37 °C, then 0, 0.04, 0.64, 2.58, and 10.4 nM YoeB was added. The reaction mixtures were incubated for an additional 10 min at 37 °C and then applied to nitrocellulose filters (Millpore 0.45 μM HA), which were washed twice with 2 ml of buffer A before measuring the radioactivity. *D*, toeprinting experiment with the *ompA* mRNA and [^{35}S]Met-$tRNA_f^{Met}$. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 20.7 nM YoeB(His)$_6$; *lane 3*, [^{35}S]Met-$tRNA_f^{Met}$; *lane 4*, 1 μM [^{35}S]Met-$tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lane 5*, 1 μM [^{35}S]Met-$tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, and 20.7 nM YoeB(His)$_6$; *lane 6*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lane 7*, 1 μM Met-$tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, and 20.7 nM YoeB(His)$_6$. *E*, release of the 3′-end portion of the *ompA* mRNA cleaved by the addition of YoeB from ribosomes. *Lane 1*, without YoeB(His)$_6$; *lane 2*, 20.7 nM YoeB(His)$_6$; *lanes 3* and *4*, with 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lanes 5* and *6*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes and 20.7 nM YoeB(His)$_6$. *Lanes 3* and *5*, the samples were centrifuged at 90,000 × *g* for 1 h at 4 °C, and the supernatant was taken for the primer extension. *F*, effect of YoeB on the 30-base RNA binding to the $tRNA_f^{Met}$-70 S ribosome complex. 70 S ribosomes (0.05 μM) were first incubated with 0, 42, 168, 336, and 672 nM YoeB(His)$_6$ and $tRNA_f^{Met}$ (1 μM) for 10 min at 37 °C. The 5′-end labeled 30-base RNA (35 nM) was then added. The reaction mixture was incubated for an additional 10 min at 37 °C, and then applied to nitrocellulose filters (Millpore 0.45 μM HA), which were washed twice with 2 ml of buffer A before measuring the radioactivity. *G*, effect of tetracycline on the activity of YoeB-mediated mRNA cleavage. *Lane 1*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lane 2*, 1 μM $tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, and 160 μM tetracycline; *lane 3*, 1 μM $tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, and 10.4 nM YoeB(His)$_6$; *lane 4*, 1 μM $tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, 10.4 nM YoeB(His)$_6$, and 160 μM tetracycline. *H*, effect of hygromycin B on the activity of YoeB-mediated mRNA cleavage. *Lane 1*, 1 μM $tRNA_f^{Met}$ and 0.05 μM 70 S ribosomes; *lanes 2* and *3*, 1 μM $tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, and 0.64 and 1.6 mM hygromycin B, respectively; *lane 4*, 1 μM $tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, and 20.7 nM YoeB(His)$_6$; *lanes 5* and *6*, 1 μM $tRNA_f^{Met}$, 0.05 μM 70 S ribosomes, 20.7 nM YoeB(His)$_6$, and 0.64 and 1.6 mM hygromycin B, respectively. *SD*, Shine-Dalgarno; *FL*, full-length.

10 min, a new band appeared (Fig. 5*F*, *lanes 6–8*) and its intensity increased with the increasing amounts of YoeB. This band was not formed when only YoeB (*lane 2*), or only $tRNA_f^{Met}$ (*lane 3*), or only 70 S ribosomes (*lane 4*) or only 70 S ribosomes plus $tRNA_f^{Met}$ (*lane 5*) were incubated with the 30-base RNA. As judged by using an RNA ladder, the new band is 7 bases shorter than the 30-base RNA. This result is consistent with the results seen with *mazG* (Fig. 5, *A–C*) and *ompA* (Fig. 5*D*) mRNAs, because the 30-base RNA was cleaved only when YoeB, $tRNA_f^{Met}$, and 70 S ribosomes were added together. Judged from the RNA ladder, the cleavage of the 30-base RNA occurred at two bases downstream of AUG. The result confirmed the notion that the RNA cleavage occurs only in the presence of both 70 S ribosomes and YoeB.

YoeB Competes with [^{35}S]Met-$tRNA_f^{Met}$ for 70 S Ribosome Binding—Next, we tested if YoeB is able to displace fMet-$tRNA_f^{Met}$ from the ribosome *in vitro*. For this purpose, YoeB was added at the minimal concentration that is just enough to produce the TP(Y) band without forming the TP(r) band. As the amounts of YoeB added in the reaction mixture were reduced (Fig. 6*A*, from *lanes 4* to *10*), the intensities of the TP(Y) band were progressively reduced, whereas the intensities of the TP(r) band increased gradually to reach the normal level.

We also tested if $tRNA_f^{Met}$ bound to the ribosomes is displaced with YoeB under the condition as that used in Fig. 6*A*. For this purpose, [^{35}S]Met-$tRNA_f^{Met}$ was first incubated with 70 S ribosomes and mRNA at 37 °C for 10 min, and then different amounts of YoeB as used in Fig. 6*A* were added. The reaction mixtures were incubated at 37 °C for another 10 min. The reaction mixtures were then applied to nitrocellulose filters (Millpore 0.45 μM HA) and the filters were washed twice with 2 ml of buffer A before measuring the radioactivity retained on the filters. As shown in Fig. 6*C*, [^{35}S]Met-$tRNA_f^{Met}$ was displaced in a YoeB concentration-dependent manner. Over 70% of [^{35}S]Met-$tRNA_f^{Met}$ was released at 10 nM YoeB (the ratio of YoeB to ribosome is 1:5). It should be noted that ribosome-bound initiator tRNA drops from 72 to about 42, upon addition of 0.04 nM YoeB and further to about 30 upon addition of 3 nM YoeB. Because the major part of the decrease in ribosome bound initiator tRNA occurred at substoichiometric additions of YoeB, this result is not due to direct competition between initiator tRNA and YoeB. It is more likely due to indirect competition caused by mRNA cleavage. The binding stability of initiator tRNA to 70 S ribosomes is known to be coupled to that of mRNA, and therefore if mRNA is cleaved, the binding of mRNA to 70 S ribosomes should be reduced, which in turn, likely destabilizes initiator tRNA binding (Fig. 6*C*).

In a reciprocal experiment, where increasing amounts of $tRNA_f^{Met}$ were added in the reaction mixture, the YoeB-mediated mRNA cleavage activity was diminished (Fig. 6*B*), However, the TP(r) band did not appear, further suggesting that YoeB and initiator tRNA appear to indirectly compete for binding to 70 S ribosomes. It should be noted that the use of [^{35}S]Met-$tRNA_f^{Met}$ instead of uncharged $tRNA_f^{Met}$ did not affect the toeprinting results (Fig. 6*D*, compare *lane 4* with *6*, and *lane 5* with *7*).

Tetracycline and Hygromycin B Inhibit YoeB-mediated mRNA Cleavage—To identify the exact YoeB-acting site, we next examined the effect of tetracycline and hygromycin B on YoeB-mediated mRNA cleavage. Both tetracycline and hygromycin B are known to bind to the A site in ribosomes (23). $tRNA_f^{Met}$ was first incubated with 70 S ribosomes and mRNA at 37 °C for 10 min to form the translation initial complex, and then tetracycline (Fig. 6*G*, *lanes 2* and *4*) and different amounts of hygromycin B (Fig. 6*H*, *lanes 2*, *3*, *5*, and *6*) were added. The reaction mixtures were incubated at 37 °C for another 10 min. YoeB was then added to the reaction mixtures for another 10 min (Fig. 6, *G*, *lanes 3* and *4*, and *H*, *lanes 4–6*). Reverse transcriptase was finally added to initiate cDNA synthesis. As shown in Fig. 6*G*, 160 μM tetracycline (the ratio of tetracycline to 70 S ribosomes is 3250 to 1) inhibited YoeB-mediated mRNA cleavage at the A site with concomitant appearance of the TP(r) band (Fig. 6*G*, *lane 4*). With hygromycin B, two different concentrations (0.64 mM for *lanes 2* and *5* and 1.6 mM for *lanes 3* and *6*) were used. As shown in Fig. 6*H*, 1.6 mM hygromycin B had little inhibitory effect on the translation initiation complex formation (compare *lane 1* with *lane 3* in Fig. 6*H*). However, 1.6 mM hygromycin B (the ratio of hygromycin B to 70 S ribosomes is 32500) almost completely blocked YoeB-mediated mRNA cleavage activity (Fig. 6*H,* compare *lane 4* with *6*). At 0.64 mM hygromycin B, the mRNA cleavage was partially inhibited with concomitant appearance of the TP(r) band (Fig. 6*H*, *lane 5*). Note that at 1.6 mM hygromycin B, the intensity of the TP(r) band was almost identical to that in the absence of both hygromycin and YoeB (Fig. 6*H*, compare *lane 6* with *1*). These results are consistent with the result obtained with tetracycline in Fig. 6*G*, suggesting that hygromycin B binding to A sites interferes with YoeB-mediated mRNA cleavage at the A site.

Cleavage of mRNA at the A Site Codons Releases the 3′-End Portion of the mRNA from 70 S Ribosomes—To examine if the cleavage of mRNA results in the release of the 3′-end portion of the mRNA from 70 S ribosomes, the reaction mixture after complex formation with the use of the *ompA* mRNA was centrifuged at 90,000 × *g* at 4 °C for 1 h to pellet ribosomes. Subsequently, we determined if the 3′-end portion of the mRNA was released into the supernatant by primer extension reaction. As shown in Fig. 6*E*, the TP(r) band was not detectable in the supernatant fraction (*lane 3*), whereas the TP(Y) band was almost fully recovered in the supernatant fraction (*lane 5*). These results indicate that the TP(Y) band detected in the toeprinting experiments was derived from the 3′-end portion of the *ompA* mRNA, subsequently released into the soluble fraction (or into the cytoplasmic fraction in the cells). Using the same 30-base synthetic RNA as used in Fig. 5*F*, we examined if the 5′-end portion of the RNA dissociates from ribosomes in the presence of YoeB. Although the RNA is cleaved at 7 bases from the 3′ end (see Fig. 5*F*), the remaining 23-base RNA (labeled at its 5′ end with ^{32}P) was bound to the ribosomes even at a very high concentration of YoeB (70 times higher concentration than that used in Fig. 6*C*; see Fig. 6*F*), indicating that the 5′-end portion of mRNA stays bound to 70 S ribosomes even after cleavage of the mRNA.

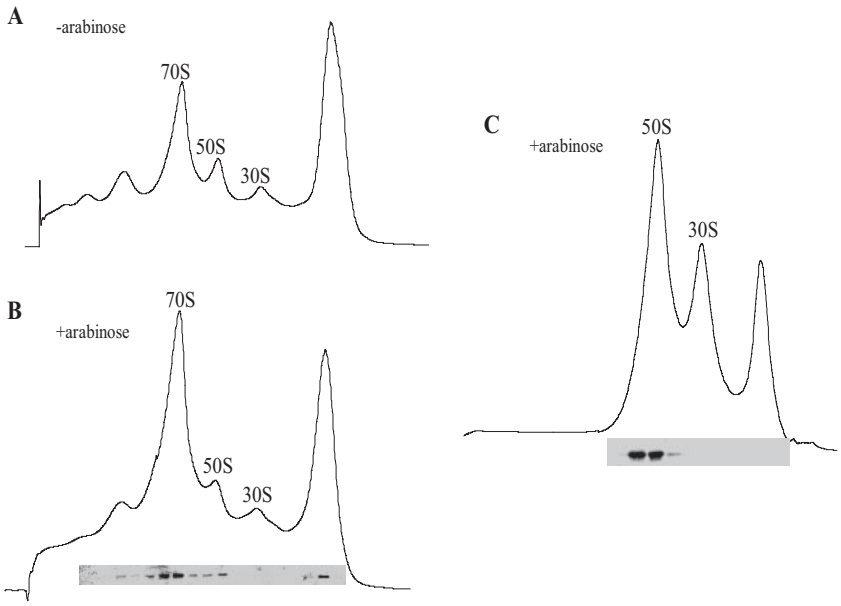


FIGURE 7. **YoeB associates with the large 50 S ribosomal subunits.** Ribosome profiles were analyzed by sucrose density gradient centrifugation as described under "Experimental Procedures." *A*, polysome profiles of *E. coli* BW25113 containing pBAD-YoeB without YoeB induction. *B*, polysome profiles of *E. coli* BW25113 containing pBAD-YoeB at 10 min after YoeB induction. *C*, 70 S ribosomes of the same lysate used in *B* were disassociated into 50 S and 30 S ribosomal subunits in 0.5 mM Mg^{2+}. In *B* and *C*, Western blot analysis was carried out to detect YoeB in each gradient fraction.

Effect of a Nuclease-negative Mutation (H83Q) in YoeB on the Cell Growth and Protein Synthesis—H83Q, a nuclease negative YoeB mutant, previously reported by Kamada and Hannoka (3) was also constructed to examine its effect on cell growth. Induction of the YoeB(H83Q) mutant inhibited cell growth and the addition of YoeB(H83Q) also inhibited MazG protein synthesis *in vitro* (data not shown). When the toeprinting experiment was carried out using the purified YoeB(H83Q), toeprinting bands (TP(Y)) three and four bases downstream of the initiation codon were detected in the manner identical to that of the wild-type YoeB (data not shown). Thus, YoeB(H83Q), a nuclease negative mutant, does not affect YoeB-mediated mRNA cleavage at the A site. Notably a higher concentration of the mutant YoeB than the wild-type YoeB was required to result in the same toeprinting effect as caused with the wild-type YoeB, indicating the mutant YoeB seems to have a weaker affinity to 70 S ribosomes than the wild-type YoeB.

YoeB Associates with 50 S Ribosomal Subunits—Because YoeB binds to the translation initiation complex (Fig. 4), we examined whether YoeB affects the polysome profiles and also specifically associates with one of the ribosomal subunits. When the polysome pattern of *E. coli* BW25113 cells carrying the pBAD-YoeB plasmid was analyzed by sucrose density gradient at 10 min after induction of YoeB by arabinose, a significant increase in the 70 S ribosomal fraction was observed without change in the 30 S and 50 S ribosomal profiles (compare Fig. 7*B* with Fig. 7*A*). Western blotting analysis showed that YoeB was associated with both 70 S ribosome and 50 S ribosome fractions, but not with 30 S ribosome (Fig. 7*B*). Cell lysates were prepared in 0.5 mM Mg^{2+} to dissociate 70 S ribosomes to 50 S and 30 S ribosomes and analyzed by sucrose density gradient centrifugation (Fig. 7*C*), where YoeB was detected only in the 50 S ribosome fraction, demonstrating that YoeB is a 50 S ribosome associating protein.

DISCUSSION

In the present paper, we demonstrated that YoeB is a potent inhibitor of protein synthesis, which functions in a manner that is distinctly different from that of MazF, an mRNA interferase (6). Upon YoeB induction, [^{35}S]methionine incorporation into cellular proteins was abruptly stopped within less than 5 min (Fig. 1, *E* and *F*). This is in sharp contrast to MazF induction, which stops [^{35}S]methionine incorporation only after 15–20 min of its induction (6). Another important difference between YoeB and MazF is that significant amounts of full-length cellular mRNAs remain in the YoeB-induced cells even 30 min after its induction (Fig. 3, *A* and *B*), whereas they rapidly disappear after 10 min upon MazF induction (6). In addition, MazF inhibits protein synthesis in both prokaryotes and eukaryotes as expected from its function as an mRNA interferase (6), whereas YoeB was found to be a prokaryote-specific inhibitor (Fig. 2, *C* and *E*).

On the basis of these results, we speculated that YoeB does not primarily function as a sequence-specific endoribonuclease or an mRNA interferase as originally proposed by Christensen *et al.* (1) and Kamada and Hanaoka (3). To substantiate our hypothesis, we carried out *in vivo* primer extension experiments using chromosomally encoded mRNAs as targets rather than mRNAs encoded by genes cloned in a multicopy plasmid to avoid artifacts (Fig. 3, *C* and *D*). Interestingly, both mRNAs tested were detected either as the corresponding full-length mRNAs or as a product cleaved at three bases downstream of the initiation codon (Fig. 3, *C* and *D*). Notably, in the ~200-nucleotide 5′ leader sequences for both mRNAs, no other cleavage bands were detected. These mRNAs encoded outer membrane proteins and were used in our experiment because of their abundance in the cell. However, the mRNA from the *glnH* gene that encodes a cytoplasmic protein was also cleaved at three bases downstream of AUG (data not shown), indicating that the cleavage of mRNAs by YoeB does not depend on the type of these mRNAs.

Importantly, this *in vivo* observation was consistent with the *in vitro* toeprinting experiments using 70 S ribosomes, $tRNA_f^{Met}$, purified YoeB, and the same mRNAs (Fig. 4). We demonstrated that the addition of YoeB caused the upstream shift of the stop site for primer extension (toeprinting site) from normal 13 and 14 bases downstream of AUG to three and four bases from AUG. This apparent 10-base upward shift resulted from the cleavage of the mRNA at the A site mediated by YoeB bound to 70 S ribosomes (Fig. 5, *A*, *lane 6*, and *D*, *lane 9*, and Fig.

6, *E*, *lane 5*), although the exact mechanism for this cleavage remains to be determined. YoeB specifically binds to 50 S ribosomes (Fig. 7, *B* and *C*), whereas hygromycin B and tetracycline bind to 30 S ribosomes. Therefore it is reasonable to speculate that YoeB locates at the interface between 50 S and 30 S ribosomes to interact with the A site to cleave mRNA at that site. This interface is known to play important roles in translation initiation (22).

Importantly, these antibiotics (tetracycline and hygromycin B), which are known to inhibit protein synthesis by binding to the A site (23), block YoeB-mediated mRNA cleavage at the A site (Fig. 6, *G* and *H*). This supports the notion that YoeB binds to the 50 S ribosomal subunit in 70 S ribosomes and interacts with the A site leading to mRNA cleavage. It is also apparent that mRNA cleavage upon YoeB binding makes the translational initial complex unstable as [^{35}S]Met-tRNA$_f^{Met}$ was released (Fig. 6*C*). On the other hand, it should be noted the mRNA is able to form a complex with 70 S ribosomes even in the absence of tRNA$_f^{Met}$ if YoeB is added (Fig. 5, *C* and *D*, *lane 6*).

It is important to note that YoeB alone did not show any endoribonuclease activity to several mRNAs under the conditions used in the toeprinting experiments (Fig. 4, *lane 2*). Only when it was added along with 70 S ribosomes, mRNAs were cleaved at three and four bases downstream of AUG (Fig. 5, *C* and *D*, *lane 6*), suggesting that YoeB is a ribosome-associating factor (Fig. 7, *B* and *C*), which may stimulate the intrinsic endogenous endoribonuclease activity of ribosomes or YoeB itself. Although purified YoeB has been shown to have a purine-specific endoribonuclease activity, albeit very weak (3), the fact that the nuclease-negative YoeB(H83Q) was able to inhibit cell growth and protein synthesis supports the notion that the endoribonuclease activity of YoeB is not primarily required for its inhibitory function for protein synthesis. It should be noted that even though protein synthesis was inhibited *in vivo* in 5–10 min after YoeB induction (1), purified YoeB only partially cleaved mRNA even 60 min after its incubation with the mRNA substrate (3). Notably, YoeB stimulates only partial ribosome-mediated mRNA cleavage at three bases downstream of AUG as seen in the *in vivo* primer extension experiments for all mRNAs tested (Fig. 3, *C* and *D*), whereas protein synthesis is completely inhibited (Fig. 1, *E* and *F*). This further suggests that the primary inhibitory function of YoeB for protein synthesis is not its endoribonuclease activity, but is to prevent the formation of the initiation complex. However, the binding of YoeB to 70 S ribosomes seems to stimulate their latent endoribonuclease activity, resulting in partial cleavage of mRNAs at three bases downstream of the initiation codon (Fig. 5, *A*, *lane 6*, and *D*, *lane 9*). As a result, the 3′-end portion of the mRNA is dissociated from the ribosomes and released to the cytoplasm (Fig. 6*E*, *lane 5*).

In summary, YoeB binds to 50 S ribosomal subunit in 70 S ribosomes and interacts with the A site (Fig. 6, *G* and *H*) to very effectively prevent the translational initiation complex formation resulting in the inhibition of protein synthesis almost immediately after YoeB induction. This apparently leads to activation of the latent endoribonuclease activity of either ribosomes or YoeB resulting in the cleavage of mRNA at the A site, and release of the 3′-end portion of mRNA from ribosomes (Fig. 6*E*).

Recently, it has been reported that *E. coli* cold-shock protein Y (22) and an antibiotic GE82221 (24) cause specific inhibition of translation initiation. Both of these, however, bind to 30 S ribosomes to directly prevent tRNA$_f^{Met}$ binding to the P site, which is quite different from the mechanism of YoeB-mediated inhibition of translation. It is quite interesting to point out that *E. coli* uses different strategies to regulate translation; at the level of initiation (YoeB and protein Y), or at the level of elongation by mRNA interferases (MazF and ChpBK) and termination (RelE).

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