Translation affects YoeB and MazF messenger RNA interferase activities by different mechanisms

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ABSTRACT

Prokaryotic toxin–antitoxin loci encode mRNA cleaving enzymes that inhibit translation. Two types are known: those that cleave mRNA codons at the ribosomal A site and those that cleave any RNA site specifically. RelE of *Escherichia coli* cleaves mRNA at the ribosomal A site *in vivo* and *in vitro* but does not cleave pure RNA *in vitro*. RelE exhibits an incomplete RNase fold that may explain why RelE requires its substrate mRNA to presented by the ribosome. In contrast, RelE homologue YoeB has a complete RNase fold and cleaves RNA independently of ribosomes *in vitro*. Here, we show that YoeB cleavage of mRNA is strictly dependent on translation of the mRNA *in vivo*. Non-translated model mRNAs were not cleaved whereas the corresponding wild-type mRNAs were cleaved efficiently. Model mRNAs carrying frameshift mutations exhibited a YoeB-mediated cleavage pattern consistent with the reading frameshift thus giving strong evidence that YoeB cleavage specificity was determined by the translational reading frame. In contrast, site-specific mRNA cleavage by MazF occurred independently of translation. In one case, translation seriously influenced MazF cleavage efficiency, thus solving a previous apparent paradox. We propose that translation enhances MazF-mediated cleavage of mRNA by destabilization of the mRNA secondary structure.

INTRODUCTION

Toxin–antitoxin (TA) loci encode two components, a ‘toxin’ whose ectopic overproduction inhibits translation or replication and an antitoxin that inhibits the toxin by direct protein–protein contact (1,2). Prokaryotic chromosomes encode a plethora of TA loci that have been grouped into seven independent families based on toxin sequence similarities. Some slowly growing and free-living organisms have particularly many TA loci while obligate intracellular organisms have few or none. For example, *Mycobacterium tuberculosis* has more than 60 TA loci, while *M. leprae* has retained two TA pseudo-loci only (3). These observations are consistent with the proposal that the TA loci function as stress response elements (1,4–11).

The molecular targets of the toxins have been of particular interest. Four independent toxin families that inhibit translation are known (RelE, MazF, HicA and Doc). Doc of prophage P1 inhibits translation by interacting with the ribosomal S30 subunit but probably does not cleave RNA (12). Members of the three other toxin families, RelE, MazF and HicA, are mRNA Interferases (mI) that inhibit translation by mRNA cleavage (13,14) (Jørgensen et al., submitted for publication). RelE was the first mI to be discovered. RelE cleaved mRNA positioned at the ribosomal A site, between the second and the third base of the A site codon, both *in vitro* (13) and *in vivo* (15). RelE did not cleave naked RNA *in vitro* and did not cleave mRNA outside their coding regions *in vivo*. Interestingly, archaean RelE homologues distantly related to RelE of *Escherichia coli* cleaved mRNA positioned at the A site in a pattern very similar to that of RelE (15).

Many RelE homologues have been identified in Bacteria and Archaea (1,6,16). Thus, in a recent bioinformatics survey, we identified 400 relBE loci in ~200 prokaryotic genomes. The RelE family of proteins is highly diverse with very few conserved amino acids. Nevertheless, sequence comparisons and structural analyses together have shown that the members of the mI families HigB, YoeB, YafQ and YhaV all belong to the RelE superfamily (17–21). The crystal structure of YoeB of *E. coli* revealed a microbial RNase fold and sequence alignment suggested that RelE has a similar RNase fold (21). The same study also showed that, in contrast to RelE, YoeB can cleave naked RNA *in vitro*. This difference was suggested to be explained by the fact that RelE lacks several amino acids...
predicted to be essential for catalytic activity. RelE’s lack of catalytic activity \textit{in vitro} may reflect that RelE requires that the substrate is presented at the ribosomal A site.

MazF of \textit{E. coli} is another well-characterized mI. MazF cleaves RNA substrates at ACA sites both \textit{in vivo} and \textit{in vitro} (14,22). MazF and MazF homologues cleave RNA preferentially at single-stranded regions (23,24) although cleavages at sites presumed to be in a double-stranded configuration have also been observed (25,26). However, we observed that MazF cleavage of one model substrate \textit{in vivo} depended on translation (5).

The specific biological function of mIs are known in a few cases only (4,27) and it is thus important to understand, in depth, their substrate specificities. Here, we describe a method that is generally useful for the analysis of mI activity \textit{in vitro}. We employ the method to analyse the activities of YoeB and MazF and compare them with two other well-characterized mIs, RelE and HigB. We find that the cleavage specificity of all three RelE homologues (RelE and YoeB of \textit{E. coli} K-12 and HigB-1 of \textit{Vibrio cholerae}) depend strictly on translation of the substrate RNA. In contrast, MazF cleavage specificity does not depend on substrate translation. However, the efficiency of MazF cleavage was with one model substrate highly stimulated by translation. Our results show that translation affect different mIs by different molecular mechanisms.

**MATERIALS AND METHODS**

Strains and plasmids used and constructed in this work are listed in Table 1. DNA oligonucleotides are listed in Table 2.

### Table 1. Strains and plasmids

| Strains/plasmids | Genotype/plasmid properties | References |
|------------------|-----------------------------|------------|
| MG1655           | Wild-type \textit{E. coli} K-12 |            |
| MG1655Δlpp       | Δlpp                        | (15)       |
| MG1655ΔΔlpp,Δx44 | Δlpp,Δx44                   | Jørgensen,M.G., unpublished data |
| **Plasmids**     |                             |            |
| pBAD33           | p15; cat araC pBAD          | (29)       |
| pOU254           | R1; bla par mcr-lacZYA      | Jensen,R.B., unpublished data |
| pMCD3           | pBAD33; pBAD::yoeB           | (18)       |
| pMCD3326        | pBAD33; BAD::opt::mазF      | This work |
| pMG3323         | pBAD33; BAD::relE           | (15)       |
| pRK100          | pBAD33; BAD::opt::yoeB      | Ramisetti,B.C.M., unpublished data |
| pKW254T         | R1; terminator from pMG25   | Winther, K., unpublished data |
| pMCD25410       | R1; pKW254T lpp             | This work |
| pMCD25411       | R1; pKW254T lpp             | This work |
| pMCD25412       | R1; pKW254T lpp             | This work |
| pMCD25420       | R1; pKW254T dksA            | This work |
| pMCD25421       | R1; pKW254T dksA             | This work |
| pMCD25422       | R1; pKW254T dksA             | This work |
| pSC710          | R1; bla par lpp             | (15)       |
| pSC711          | R1; bla par lpp ATGA1AAG     | (15)       |
| pSC20           | pBR222, tet, ssx-A and smpB | (15)       |
| pSC21           | pBR222, tet, ssx-A and smpB | (15)       |
| pSC333          | pUC, pGEM3, bla, T3::lpp    | (15)       |
| **Northern blotting analysis**

Cells were grown in LB medium at 37°C. At an OD\textsubscript{450} of 0.5, the cultures were diluted 10 times and grown to an OD of 0.5 and transcription of the mI gene was induced by the addition of 0.2% arabinose. To inhibit translation, chloramphenicol (50 μg/ml) was added. For northern blotting analysis, total RNA was fractionated by PAGE (6% low his acrylamide), blotted to a Zeta probe nylon membrane and hybridized with a single-stranded \textsuperscript{32}P-labelled riboprobe complementary to the RNA of interest. For \textit{lpp} mRNA hybridization, the radioactive probe was generated by T7 RNA polymerase using linearized plasmid DNA of pSC333 as the template. The riboprobe used to detect \textit{dksA} mRNA was transcribed from a PCR fragment containing the partial \textit{dksA} gene and the T7 promoter (constructed using the primers \textit{dksA} probe-1 and \textit{dksA} T7 probe-r).

### Table 2. DNA oligonucleotides used

| Oligonucleotide name                          | Sequence                                      |
|----------------------------------------------|-----------------------------------------------|
| **mazF-Sall-SD-up**                          | 5’-CCCCCGTCGACTCAAGGAGTTTTA                   |
| **mazF-HindIII-down**                        | 5’-CCCCCAAGCTTTAACAAGCTACCCCA                 |
| **transcR**                                  | 5’-CCCCGGATCGATTTACCAACCCTCTTTCGATC          |
| **term-bamH1-kpm1-sac1-pmlf-xhoI#CCW**       | 5’-CCCCCGGATCGGTACGGGAGGTCCTA                |
| **lpp-BamH1-sac1#CW**                        | 5’-CCCCCGGATCGGCAGTCGAGGAAAGA                 |
| **lpp-pmlf-xhoI#CCW**                        | 5’-CCCCCGGATCGTCGAGGAAAGA                     |
| **lpp 1AAg-1**                               | 5’-GAGGCTATTAAATAGAGAGCTA                    |
| **lpp 1AAg-2**                               | 5’-GTGTTAGACGTTCTATTTTTTATTA                 |
| **lpp 6ACT-cw**                              | 5’-ATGAAAGACTCTAATACCGTACTG                  |
| **lpp 6ACT-cxw**                             | 5’-CGGCCCACAGGCAGTGTATATAG                   |
| **dksA-bamH1-sac1#CW**                       | 5’-CCCCGGAGATGCTGCACGGGCTCGC                 |
| **dksA-pmlf-xhoI#CCW**                       | 5’-ATGAAAGACTCTAATACCGTACTG                  |
| **dksA 1AAg-cw**                             | 5’-ATGAAAGACTCTAATACCGTACTG                  |
| **dksA 1AAg-cxw**                            | 5’-ATGAAAGACTCTAATACCGTACTG                  |
| **dksA 2AA-cw**                              | 5’-ATGAAAGACTCTAATACCGTACTG                  |
| **dksA 2AA-cxw**                             | 5’-ATGAAAGACTCTAATACCGTACTG                  |
| **lpp 26**                                   | 5’-CAGCTGTCGACTATTAGGCGTTC                   |
| **dksA PE1**                                 | 5’-GATATGCGCTACCCCGGCAGG                    |
| **pKW71D-3SpE**                              | 5’-GAATTAGGTAATCTACAGGCTCGCC                  |
| **10SA-2**                                   | 5’-GAATTAGGTAATCTACAGGCTCGCC                  |
| **dksA probe-1**                             | 5’-GAATTAGGTAATCTACAGGCTCGCC                  |
| **dksA T7 probe-1**                          | 5’-GAATTAGGTAATCTACAGGCTCGCC                  |

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mapped with the primer pKW71D-3#PE, which is complementary to the linker RNA of pKW254T. The primer lpp 21 was used to map the 5’-end of lpp RNA. The 5’-end of dksA mRNA was mapped using the primer dksA PE1.

**Plasmids constructed**

pMCD3326. The mazF gene was amplified from chromosomal DNA of MG1655 with primers mazF-SalI-SD-up and mazF-HindIII-down. The PCR product was digested with SalI and HindIII and inserted into pBAD33. The resulting plasmid contains the mazF gene with an efficient SD sequence (from parM of plasmid R1) downstream of the P\textsubscript{BAD} promoter.

pKW254T. The region of pMG25 containing the rnrB\textsubscript{E. coli} yoeB transcriptional terminators and ±100-bp upstream was amplified using primers TRANSFERM#CCW and term-bamHI-kpnI-sacI-pmlI-xhoI#CW. The PCR product was digested with EcoRI and BamHI and inserted into pKW254T. The plasmid therefore expresses an rnrB mRNA with an additional 100-bp linker RNA downstream of the P\textsubscript{BAD} promoter.

pMCD25410. The lpp gene of MG1655 was amplified from chromosomal DNA using the primers lpp-BamHI-sacI#CW and lpp-pmlI-xhoI#CCW. The two overlapping PCR products were used as templates in a second round of PCR using primers lpp-BamHI-sacI#CW and lpp-pmlI-xhoI#CCW. The resulting PCR product was digested with BamHI and XhoI and inserted into pKW254T. The plasmid encodes a frameshift in the first codon.

pMCD25411. The lpp gene of MG1655 was amplified with primers lpp-BamHI-sacI#CW and lpp 1AAG-2 in addition to lpp 1AAG-1 and lpp-pmlI-xhoI#CCW. The two overlapping PCR products were used as templates in a second round of PCR using primers lpp-BamHI-sacI#CW and lpp-pmlI-xhoI#CCW. The resulting PCR product was digested with BamHI and XhoI and inserted into pKW254T. The plasmid encodes a non-translatable lpp gene (AAG replaces the natural start codon).

pMCD25412. The lpp gene of MG1655 was amplified with primers lpp-BamHI-sacI#CW and lpp 6ACT-CCW in addition to lpp 6ACT-cw and lpp-pmlI-xhoI#CCW. The two overlapping PCR products were used as templates in a second round of PCR using primers lpp-BamHI-sacI#CW and lpp-pmlI-xhoI#CCW. The resulting PCR product was digested with BamHI and XhoI and inserted into pKW254T. The plasmid encodes an lpp gene with an extra A inserted between the fifth and sixth codon, thus generating a frameshift in lpp.

pMCD25420. The dksA gene of MG1655 was amplified with primers dksA-bamHI-sacI#CW and dksA 1AAG-ccw in addition to dksA 1AAG-cw and dksA-pmlI-xhoI#CCW. The two overlapping PCR products were used as templates in a second round of PCR using primers dksA-bamHI-sacI#CW and dksA-pmlI-xhoI#CCW. The resulting PCR product was digested with BamHI and XhoI and inserted into pKW254T. The plasmid encodes a non-translatable dksA gene (AAG replaces the natural start codon).

pMCD25421. The dksA gene of MG1655 was amplified with primers dksA-bamHI-sacI#CW and dksA 1AAG-ccw in addition to dksA 1AAG-cw and dksA-pmlI-xhoI#CCW. The two overlapping PCR products were used as templates in a second round of PCR using primers dksA-bamHI-sacI#CW and dksA-pmlI-xhoI#CCW. The resulting PCR product was digested with BamHI and XhoI and inserted into pKW254T. The plasmid encodes a frameshift in the second codon, thus creating a non-translatable dksA gene.

**RESULTS**

Development of a novel strategy to analyse mI activity

We found it advantageous to generate plasmids that express model RNAs suitable for the analysis of mI activity in vivo. Such plasmid derivatives have two advantages: first, the RNA can be expressed at increased levels as compared to the chromosome-encoded RNA, thereby increasing the resolution and sensitivity of the analysis. Second, specific mutations are easily introduced into plasmid-encoded test genes. When we analysed a model RNA expressed from a plasmid, the corresponding chromosomal gene was deleted thus to avoid interfering signals from the endogenous mRNA. We employed two model mRNAs, lpp and dksA, both of which encode non-essential proteins. DNA fragments encoding the wild-type lpp and dksA genes and their native promoters were inserted into pKW254T, a plasmid that encodes an efficient primer annealing site adjacent to a multiple cloning region (Figure 1A), thereby resulting in pMCD25410 and pMCD25420 (Figure 1B and C). Plasmids pMCD25411 and pMCD25421 carry point mutations in the start codons of lpp and dksA reading frames, respectively, thereby preventing translation of the reading frames. In plasmid pMCD25412, an A base was inserted between the fifth and sixth codons of lpp. This frameshift mutation generates a novel small reading frame that terminates prematurely as indicated in the figure. Similarly, in pMCD25422, an A base was inserted between the second and third codons of dksA that also in this case generates a novel small reading frame that stops prematurely.

YoeB activity depends on translation

The relE and yoeB genes of E. coli, K-12 and the higB-1 gene of V. cholerae were inserted into plasmid pBAD33 that carries an arabinose-inducible promoter. Next, the mI production plasmids were transformed into strains MG1655Δlpp and MG1655ΔdksA carrying the reporter
plasmids shown in Figure 1B and C. Finally, we followed the decay patterns of lpp and dksA mRNAs after mI induction (Figure 2A and B, respectively). As seen, the levels of the translated wild-type versions of lpp and dksA mRNAs decreased rapidly after induction of relE and higB-1. In contrast, the non-translated versions of the lpp and dksA mRNAs (denoted lpp0 and dksA0 in Figure 2) were much more stable. This result was in agreement with previous analyses showing that RelE and HigB-1 cleave translated RNAs only (15,18). Addition of chloramphenicol, that also inhibits translation rapidly and efficiently, did not mediate decay of any of the model mRNAs (Figure 2, left panels).

Next, we analysed the effect of yoeB induction. As seen from Figure 2, yoeB induction also destabilized...
versions of extension analysis on the wild-type and non-translated gate YoeB cleavage specificity, we performed primer requires the substrate RNA to be translated. To investi-

cleavage pattern

Translation reading frame determines the YoeB mRNA cleavage pattern

The above result indicated that YoeB activity in vivo requires the substrate RNA to be translated. To investi-
gate YoeB cleavage specificity, we performed primer extension analysis on the wild-type and non-translated versions of lpp and dksA mRNAs (Figures 3 and 4). We included frameshift variants of lpp and dksA mRNAs in the analysis (denoted lpp” and dksA”). For comparison, we included RelE and HigB-1 in the analysis. RelE induced the strongest cleavages in wild-type lpp and dksA mRNAs, but the cleavage patterns per se were similar for all three mIs: in all three cases, the non-translated 5'- and 3'-regions of the mRNAs were not cleaved at all. In contrast, the second codons and the UAA stop codons exhibited strong cleavages in all three cases. All three mRNAs were also cleaved at internal codons usually between the second and the third bases of the codons.

The non-translated lpp” and dksA” mRNA variants exhibited a strikingly different pattern: the only specific cleavages seen with these model mRNAs occurred at the second codon. Most notably, the very strong cleavages seen at the stop codons in the wild-type RNAs were completely absent in the lpp” and dksA” mRNAs. These results show that the YoeB, RelE and HigB-1 cleavages in the model mRNAs generally required the RNAs to be translated. The cleavages seen at the second codons of lpp” and dksA” may be a consequence of the presence of strong Shine and Dalgarno sequences upstream of the mutated start codons (AUG was changed to AAG in lpp” and dksA”). The cleavages in the non-translated lpp” and dksA” mRNAs were abolished (Figures 3B and 4B) concomitantly with the appearance of strong cleavages in the new stop codons in the “frameshifted” lpp” and dksA” mRNAs (marked as UAA in Figures 3A and 4A). Moreover, the cleavage patterns seen with the ‘frameshifted’ mRNAs were in general strikingly different from those of the wild-type mRNAs. As expected, the primer extension bands reflecting cleavages at the second codons of lpp” and dksA” mRNAs were shifted one base up (as compared to the wild-type RNAs), consistent with the mutational insertion of one base at the 5'-end of the reading frames (Figures 3A and 4A).
Figure 3. mI-induced cleavage patterns of wild-type and mutated lpp mRNAs. The strains MG1655Δlpp/pMCD25410 (wild-type lpp), MG1655Δlpp/pMCD25412 (lpp')—contains a frameshift mutation in the lpp gene) or MG1655Δlpp/pMCD25411 (lpp''—the start codon has been changed from ATG to AAG) were co-transformed with the plasmids, pMG3323 (pBAD::relE), pMCD3 (pBAD::higB-1), pRB100 (pBAD::yoeB) and pMCD3326 (pBAD::SDopt::mazF). These strains were grown exponentially in LB medium at 37°C and 0.2% arabinose was added at time 0 to induce transcription of the mIs. The Figure shows primer extension reactions. (A) The 5'-ends of lpp mRNAs were mapped using the primer lpp26. (B) The mRNA regions close to the stop codon of lpp were mapped using the primer pKW71D-3#PE. Numbers are time points of cell sampling relative to the addition of arabinose. Significant cleavage sites are indicated with black dots. These experiments were accomplished at least three times.
Figure 4. mI-induced cleavage patterns of wild-type and mutated dksA mRNAs. The strains MG1655ΔdksA/pMCD25420 (wild-type dksA), MG1655ΔdksA/pMCD25422 (dksAΔ—contains a frameshift mutation in the dksA gene) or MG1655ΔdksA/pMCD25421 (dksAΔ—the start codon has been changed from ATG to AAG) were co-transformed with the plasmids, pMG3323 (pBAD::relE), pMCD3 (pBAD::higB-1), pRB100 (pBAD::yoeB) and pMCD3326 (pBAD::SDopt::mazF). The strains were grown exponentially in LB medium at 37°C and 0.2% arabinose added at time 0 to induce transcription of the mIs. (A) The 5’-ends of the dksA mRNAs were mapped using the primer dksA PE1. (B) The mRNA regions close to the stop codon of dksA were mapped using the primer pKW71D-3#PE. Numbers are time points of cell sampling relative to the addition of arabinose. Significant cleavage sites are indicated with black dots. These experiments were accomplished at least three times.
We note that the cleavage patterns mediated by relE, higB-1 and yoeB induction were very similar on all six model mRNAs. That all three mIs preferred specific codons rather than specific sequences was further supported by the observation that the cleavage patterns of the wild-type mRNAs were strikingly different from that of the frameshifted mRNAs. Thus, it is the translational reading frame of a given mRNA rather than the sequence itself that determines the cleavage pattern.

In agreement with the northern blotting analyses (Figure 2), our primer extension analyses confirmed that the non-translatable lpp' and dksA' model mRNAs were more stable than the translated versions of the mRNAs. This conclusion comes from the observation that the amount of full-length mRNA clearly decreased in cases of the translated mRNAs (lpp, lpp', dksA and dksA'); whereas, this was not the case for the non-translated versions (lpp' and dksA') (see arrows pointing at mRNA 3'-ends in Figures 3A and 4A). Again, the YoeB-mediated mRNA cleavage patterns were very similar to those mediated by RelE and HigB-1.

Previously, tmRNA was used as a model substrate in the analysis of mI activity (15). Thus, RelE cleaves within the reading frame of wt tmRNA; whereas, RelE did not cleave a non-translated version of tmRNA. To compare our results with these earlier observations, we decided to investigate how expression of HigB-1 and YoeB affects the wild-type and the non-translated version of tmRNA (residue codon GCA was changed to UAA). As seen from Figure 5, RelE, HigB-1 and YoeB cleaved the translated but not the non-translated version of tmRNA, and tmRNA cleavage was confined to its coding region. These results support the conclusion that HigB-1 and YoeB cleavage depends on translation.

MazF exhibits translation-dependent and -independent mRNA cleavage

It has not been resolved whether MazF cleavage of mRNA in vivo depends on translation. To address this question, we first compared the decay patterns of wild-type and non-translated lpp and dksA mRNAs after induction of mazF. As seen from Figure 2A, the amount of wild-type lpp mRNA decreased rapidly after mazF induction. However, as with the RelE family of mIs, the non-translated lpp mRNA was not affected by MazF expression. In contrast, the translated and non-translated versions of the dksA mRNA were both very rapidly cleaved by MazF (Figure 2B).

Translation affects MazF cleavage efficiency but not cleavage specificity

Next, we employed primer extension analyses to investigate how translation affects MazF-induced mRNA cleavage. It has previously been reported that MazF cleaves specifically at ACA sites, independently of translation (14). Consistently, induction of MazF mediated cleavage at two ACA sites located upstream of the stop codon of lpp (Figure 3B). The lpp mRNA contains one additional ACA site at its very 5'-end. As seen, MazF also cleaved this ACA site (Figure 3A). Thus, all ACA sites in lpp mRNA were cleaved by MazF, independently of their location in the RNA. MazF also mediated cleavage at an ACC site just downstream of the stop codon of lpp mRNA (Figure 3B), suggesting that MazF cleavage is not absolutely restricted to ACA sites.

We then performed primer extension analysis on the frameshifted and non-translated versions of lpp mRNA after induction of mazF. Strikingly, abolition of translation did not change the MazF-mediated cleavage pattern per se (Figure 3). However, the efficiency of cleavage was significantly reduced in the 3'-end of the lpp' mRNA variant as compared to the wild-type (Figure 3B). Cleavage of the lpp' mRNA was even more reduced, consistent with the high degree of stability of the full-length lpp' mRNA observed by northern blotting analysis (Figure 2A). In contrast, the strengths of the cleavages at the 5' ACA sites of the three lpp mRNA variants were very similar (Figure 3A, top).

The dksA mRNA has three ACA sites, one located seven codons downstream of the start codon, one four codons upstream of the stop codon and one just downstream of the stop codon. Ectopic induction of mazF mediated cleavage at all three sites in the wild-type dksA mRNA (Figure 4). Moreover, the dksA' and dksA'' variant mRNAs were cleaved in a pattern that was indistinguishable from that of the wild-type mRNA. Thus, translation does not affect MazF cleavage of the dksA mRNA.

For completion, we included tmRNA in our analysis of MazF-mediated RNA cleavage (Figure 5) even though tmRNA is devoid of bona fide MazF cleavage sites (5'-ACA-3'). Unexpectedly, we observed a weak MazF-mediated cleavage in an ACU site in the coding region of tmRNA that occurred in both the translated and non-translated forms of the RNA. This was unexpected since MazF has been shown to be highly specific for ACA sites. One possible explanation for this observation is that MazF in this particular case had a relaxed cleavage site specificity. Another, more likely explanation, is that the tmRNA cleavage was a secondary effect caused by the MazF-mediated global inhibition of translation.

DISCUSSION

Here, we developed a rapid and straightforward method to analyse mI activity on two model mRNAs. The coverage of the 3'-region of a given reading frame by primer extension analysis requires that a primer can anneal to the non-translated mRNA 3'-end. Many bacterial 3'-ends are short and fold into stable secondary structures (such as Rho-independent transcriptional terminators) that may prevent efficient annealing of a DNA primer used in the primer extension analysis. To circumvent this problem, we constructed a low copy number plasmid (pKW254T) carrying a primer annealing site flanked by a multiple cloning region at the 5'-side and a transcriptional terminator at the 3'-side (Figure 1A). Using this plasmid, we were able to analyse the entire reading frames of two model mRNAs. This experimental set-up should be generally useful for the in vivo analysis of mIs.
Two conclusions can be drawn from the analyses presented here. First, YoeB-induced mRNA cleavage in vivo required that the mRNA is translated and cleavage occurs only within translated regions of the RNA (Figures 3–5). This requirement was very similar to that exhibited by RelE from *E. coli* and RelE homologues from *V. cholerae* (HigB-1 and -2) and Archaea (14,15,18,30). In contrast, YoeB was able to cleave naked RNA in vitro, independent of the presence of ribosomes (21). We can only speculate about the reason for this observation. A remote possibility that we could not exclude was that the mRNA cleavages seen after ectopic production of YoeB in vivo was caused by fortuitous induction of a chromosome-encoded mI whose activity depends on translation. To rule out this possibility, we repeated the experiments shown in Figures 3–5 in an *E. coli* K-12 strain devoid of the three known relBE homologues and the two known mazEF homologues (data not shown). RNAs prepared from the multiple deletion strain exhibited YoeB-mediated cleavage patterns indistinguishable of those seen with RNA from the wild-type strain (MG1655/C1lpp) (data not shown). A more likely explanation invokes that YoeB has a high affinity for RNA substrates presented at the ribosomal A site and that the presumed few YoeB molecules that are required to inhibit translation are titrated by the ribosomes. In turn, such titration would prevent random cleavage of cellular RNAs.

The second conclusion was that MazF cleavage specificity did not depend on translation. Previously, we observed that a non-translated version of *lpp* mRNA was cleaved much slower than the isogenic wild-type mRNA (5). This observation was seemingly at variance with the in vitro activity of MazF (14,23). However, our new analyses can now explain the discrepancy. We observed that MazF cleaved at ACA sites in both *lpp* and *dksA* mRNAs. The three versions of *dksA* mRNA were cleaved with equal efficiencies, thus ruling out that translation plays any role in the reaction in this case (Figure 4). In contrast, the cleavages in *lpp* mRNA were much weaker in the non-translated *lpp* mRNA than in the wild-type mRNA (Figures 2 and 3). The *lpp* mRNA carrying the frameshift mutation exhibited an intermediary susceptibility to MazF cleavage (Figure 3). These results show that MazF cleavage does not strictly depend on translation, that is, MazF cleavage occurs whether or not the substrate mRNA is translated. However, translation enhanced MazF-mediated *lpp* cleavage. The most likely explanation for this observation is that ribosomes translating the target mRNA disrupts secondary structure that is inhibitory to cleavage by MazF that prefers single-stranded RNA substrates (23). A recent analysis of two MazF homologues supports this interpretation (24). These authors showed that addition of CspA, the major cold-shock protein of *E. coli* that prevent the formation of RNA secondary structures (31), stimulated MazF homologue cleavage at target sites predicted to be folded into RNA secondary structure. These results are consistent with the proposal that the ACA sites in *lpp* mRNA are shielded by secondary structure and the ribosomes disrupt these interactions during translation.

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**Figure 5.** Primer extension analysis of wild-type and mutated tmRNA before and after mI induction. Strains MG1655/ΔssrA::pSC320 (wt ssrA) and MG1655/ΔssrA::pSC321 (ssrA*—the resume codon of tmRNA was changed from GCA to TAA) also carrying one of the plasmids pMG3323 (pBAD::relE), pMCD3 (pBAD::higB-1), pRB100 (pBAD::yoeB) or pMCD3326 (pBAD::SDopt::mazF) were grown exponentially at 37 °C. At time 0, translation was inhibited by the addition of 50 μg ml⁻¹ chloramphenicol or by induction of mI transcription with 0.2% arabinose. Numbers are time points of cell sampling relative to the addition of arabinose. Significant cleavage sites are indicated with black dots. The RNA was mapped using the primer 10SA-2. This experiment was performed at least three times.
The translation-independent MazF cleavage of dskA mRNA indicated that the ACA sites in this case was present in single-stranded configurations. The biological function of most TA loci is still unknown. However, the observations presented here show that mRNAs have very different requirements for mRNA target cleavage, thus raising the possibility that relBE and mazEF loci play different biological roles.

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