

# The Bacterial Toxin RelE Displays Codon-Specific Cleavage of mRNAs in the Ribosomal A Site

Kim Pedersen,<sup>1</sup> Andrey V. Zavialov,<sup>2</sup>  
Michael Yu. Pavlov,<sup>2</sup> Johan Elf,<sup>2</sup>  
Kenn Gerdes,<sup>1</sup> and Måns Ehrenberg<sup>2,\*</sup>

<sup>1</sup>Department of Biochemistry and Molecular  
Biology, OU  
University of Southern Denmark  
Campusvej 55  
DK-5230 Odense  
Denmark

<sup>2</sup>Department of Cell and Molecular Biology, BMC  
Uppsala University  
Box 596  
S-75124 Uppsala  
Sweden

## Summary

The *Escherichia coli relBE* operon encodes a toxin-antitoxin pair, RelE-RelB. RelB can reverse inhibition of protein synthesis by RelE in vivo. We have found that although RelE does not degrade free RNA, it cleaves mRNA in the ribosomal A site with high codon specificity. Among stop codons UAG is cleaved with fast, UAA intermediate and UGA slow rate, while UCG and CAG are cleaved most rapidly among sense codons. We suggest that inhibition of protein synthesis by RelE is reversed with the help of tmRNA, and that RelE plays a regulatory role in bacteria during adaptation to poor growth conditions.

## Introduction

The *relBE* locus (Bech et al., 1985) is common among eubacteria and Archaea (Gerdes, 2000). The *relB* gene was first identified from *E. coli* missense mutants with a “delayed relaxed” phenotype (Lavallé, 1965; Diderichsen et al., 1977; Mosteller, 1978). After amino acid depletion, these mutants initially respond like wild-type cells with a high level of ppGpp that turns off synthesis of stable RNAs (reviewed by Cashel et al., 1996). Then, however, the concentration of ppGpp drops to its basal level and synthesis of stable RNA resumes in a relaxed response, delayed about 10 min in relation to the downshift (Lavallé et al., 1976). The *relBE* gene pair in *E. coli* has the regulatory components and genetic organization that characterize most toxin-antitoxin (TA) operons (Figure 7; Jensen and Gerdes, 1995). The antitoxin gene (*relB*) is located upstream of the toxin gene (*relE*) and both are translated from one mRNA transcript (Bech et al., 1985). RelB and RelE form a non-toxic protein complex (Galvani et al., 2001), which together with free RelB autogenously regulate transcription of *relBE* (Gottfredsen and Gerdes, 1998). During balanced growth RelB is expressed in excess over RelE (Gottfredsen and Gerdes, 1998) but if de novo synthesis of RelB, which is unstable due to degradation by Lon proteases, becomes too

small its concentration will dwindle and free and active RelE toxins will appear in the cytoplasm (Christensen et al., 2001).

The mechanism of action of RelE has remained unclear, but experimental observations suggested that it targets a factor involved in protein synthesis (Galvani et al., 2001; Pedersen et al., 2002). Induction of transcription of the *relE* gene from a plasmid led to instantaneous and complete inhibition of protein synthesis and greatly reduced the fraction of cells that could form colonies on solid medium plates (Christensen et al., 2001). Subsequent blocking of transcription of *relE* and overexpression of *relB* restored protein synthesis and colony formation (Pedersen et al., 2002). When an *E. coli* cell extract was first preincubated with RelE protein synthesis stopped completely, but a reporter mRNA (*hok*) that subsequently was added together with a high concentration of RelB could be translated (Pedersen et al., 2002). From this followed that inhibition of protein synthesis by RelE is bacteriostatic rather than bacteriocidal.

In the present study, we have used an in vitro system with components of high purity for bacterial protein synthesis to clarify the mechanism of action of RelE. It is demonstrated that RelE inhibits protein synthesis by cleaving mRNA codons in the ribosomal A site in a sequence specific way with preference for the stop codon UAG. Our findings suggest that recovery of protein synthesis in vivo after inhibition by RelE is mediated by tmRNA (reviewed by Gillet and Felden, 2001). We discuss the delayed relaxed response of *relB* mutants and suggest how RelE could facilitate rapid adaptation of bacteria to poor growth conditions after a downshift in the medium.

## Results

### Catalytic Inhibition of Recycling Ribosomes by the RelE Toxin

To clarify the inhibitory mechanism of RelE, we used a complete *E. coli* system for protein synthesis with translation factors of high purity (Pavlov et al., 1997) and ribosomes of high activity (Zavialov et al., 2001; 2002; Experimental Procedures). In the first set of experiments, the ribosomes were in recycling mode, each synthesizing many copies of a short peptide in the presence of initiation, elongation, termination, and recycling factors (Pavlov et al., 1997; Karimi et al., 1999; Experimental Procedures). The ribosomes were programmed with an mRNA (XR7) containing a strong Shine and Dalgarno (SD) sequence, AUG, an ORF encoding the peptide sequence MFTI followed by either one of the three stop codons (Experimental Procedures). Incubation was started by mixing preinitiated ribosomes with translation factors for elongation, termination, and recycling back to initiation either in the presence or absence of RelE. Overexpressed his6-RelE (Pedersen et al., 2002) had the same activity as native RelE, was used throughout this study, and is referred to as RelE. The extent of tetrapeptide synthesis was monitored at different time points

\* Correspondence: ehrenberg@xray.bmc.uu.se

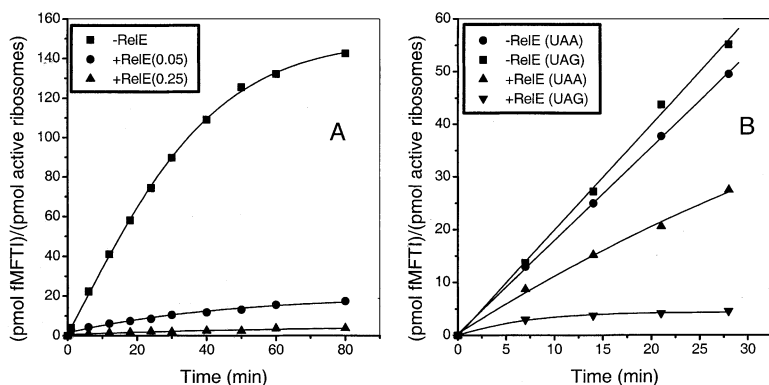


Figure 1. RelE-Dependent Inhibition of Recycling Ribosomes Programmed with XR7 mRNA

(A) RelE inhibition at catalytic concentration. Initiation mixes and elongation mixes were preincubated for 10 min at 37°C and added together at time zero. The translation reactions contained 0.5  $\mu$ M ribosomes (60%–80% active) and 1  $\mu$ M XR7 UAG mRNA. RelE was added to the initiation mixes at time  $-0.5$  min in amounts corresponding to 0.25 or 0.05 pmol per pmol active ribosome. In the control reaction,  $-$ RelE, buffer was added instead of RelE. Accumulation of fMFTI-tetrapeptide was measured by HPLC.

(B) Comparison of inhibition by RelE of trans-

lation of mRNAs with UAG and UAA stop codons. The translation assays were performed as in (A) but with XR7 mRNAs with UAG or UAA as stop codons with or without 0.2 pmol RelE per pmol active ribosome.

by HPLC and online radiometry (Pavlov et al., 1997; Experimental Procedures).

At a RelE concentration four times less than the concentration of ribosomes that were competent in dipeptide formation (Zavialov et al., 2001; Experimental Procedures), peptide synthesis from the mRNA with UAG as stop codon was completely blocked by the toxin. With twenty times less RelE than active ribosomes, the rate of peptide synthesis was reduced to about 10% of the rate of the uninhibited reaction (Figure 1A). Since the concentrations of all translation factors were higher than that of ribosomes, this implies that one RelE molecule can inhibit many target molecules in a catalytic mode of action.

Next, we compared RelE inhibition of ribosomal recycling with mRNAs containing different stop codons. When termination was carried out by the class 1 release factor RF1 and the concentration of RelE was 20% of that of active ribosomes, the inhibition of protein synthesis was very strong with UAG and intermediate with UAA as stop codon (Figure 1B). This result suggested that RelE-inhibition of protein synthesis had codon specificity.

#### RelE Only Inactivates Ribosomal Complexes that Contain mRNA

When, in our *in vitro* system, RelE was added together with RelB in excess to recycling ribosomes there was no inhibition of translation. However, when the recycling ribosomes had already been inactivated by RelE, addition of RelB alone or together with either fresh mRNA or fresh ribosomes did not restore protein synthesis. It was only when RelB was added together with both fresh mRNA and fresh ribosomes that tetrapeptide synthesis was resumed (Figure 2A). This indicated that the target for RelE was either the ribosome, its mRNA, or both.

In a different type of recycling experiments, RelE was first preincubated 10 min with free mRNA, with free ribosomes without translation factors or, finally, with ribosomes, mRNA, and all factors necessary for initiation of protein synthesis. Then, all complementary factors required for ribosomal recycling were in each of the three cases added in the absence or presence of an excess amount of RelB. In the absence of RelB, protein synthesis was strongly and equally inhibited in all three

cases as in Figure 1A. In the presence of RelB, protein synthesis was inhibited when RelE had been preincubated with initiating ribosomes but not with only mRNA or only ribosomes (Figure 2B). These experiments showed that RelE could inactivate initiated ribosomes but neither free mRNA nor ribosomes lacking mRNA.

#### RelE Inhibits Peptide Release in a Stop Codon Dependent Way

The observation that RelE inhibited recycling ribosomes more strongly when the stop codon was UAG than UAA (Figure 1B) suggested that RelE could impair peptide release. To study this phenomenon further, ribosomes were initiated with XR7 mRNAs containing either one of the three stop codons. Translation of the mRNAs in the absence of release factors and subsequent gel filtration to remove translation factors and other small components led to three different ribosomal complexes (RCs) with tetrapeptidyl-tRNA in the P site and a stop codon in an otherwise empty A site (Experimental Procedures; Zavialov et al., 2001; 2002).

In a first experiment, the three RCs were incubated in the presence of a catalytic amount of RelE. After different incubation times, the fraction of tetrapeptides that could be released from peptidyl-tRNA by addition of RF1 (UAG, UAA) or RF2 (UGA, UAA) in large excess was monitored. The results (Figure 3A; RF2 on UAA not shown) showed that RelE inhibits termination rapidly for UAG, with intermediate rate for UAA (identical results were obtained when termination was carried out by RF1 and RF2) and very slowly for UGA codons. To quantify the inhibition of termination by RelE, another set of experiments was performed on the RCs. Here, each of the three RCs was exposed to varying concentrations of RelE during 1 min before a large excess of either RF1 (stop codons UAG or UAA) or RF2 (stop codon UGA) was added and the fraction,  $r_p$ , of releasable peptide monitored (Figure 3B). When the logarithm of  $r_p$  was plotted against the concentration of RelE in the incubation mixture, straight lines were obtained for all three stop codons in the RCs (Figure 3C). The slopes of the lines showed that RelE inhibited termination at UAG ten times faster than at UAA and a thousand times faster than at UGA (Figure 3C, insert). Peptide release by transfer to the antibiotic puromycin was not inhibited by RelE

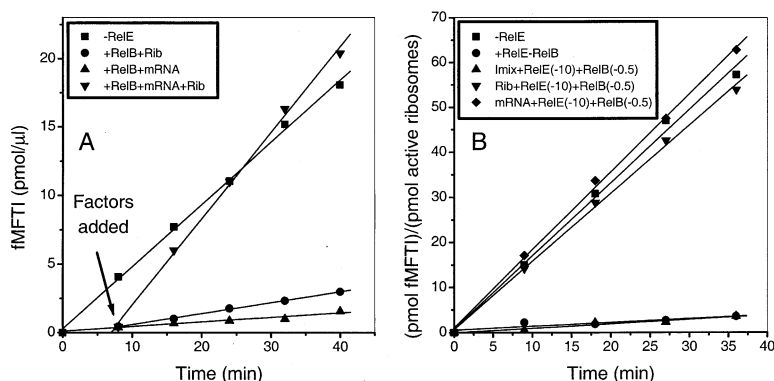


Figure 2. RelB-Dependent Neutralization of RelE-Dependent Inhibition of Recycling Ribosomes

(A) Restarting translation by adding RelB, new mRNA, and/or new ribosomes (Rib). RelE was added to the initiation mixes except in the control, -RelE, at time -0.5 min. Concentrations in the translation reactions were 0.06  $\mu\text{M}$  RelE, 0.5  $\mu\text{M}$  ribosomes, and 1  $\mu\text{M}$  XR7 UAG mRNA. After 8 min incubation with RelE, the reaction was split in three aliquots and to them were added the different combinations of indicated factors to final concentrations of 1.2  $\mu\text{M}$  RelB, 0.5  $\mu\text{M}$  Rib, and/or 1  $\mu\text{M}$  XR7 UAG mRNA. Synthesis of fMFTI-tetrapeptides was measured by HPLC.

(B) Neutralization of RelE-inhibition of recycling ribosomes by addition of RelB before elongation start. RelE was added either to a complete initiation mix (Imix), free mRNA or free ribosomes (Rib) at time -10 min except in the control reaction, -RelE. The samples were split in two and one set received RelB at time -0.5 min. At time zero, the preincubated samples received the rest of the translation factors necessary for complete translation. The amount of fMFTI synthesized was identical in the three controls and in the three reactions without RelB (-RelB) so that only one of each is shown. Concentrations in the reactions were 0.06  $\mu\text{M}$  RelE, 1.2  $\mu\text{M}$  RelB, 0.5  $\mu\text{M}$  ribosomes, and 1  $\mu\text{M}$  XR7 UAG mRNA.

for any choice of stop codon and there was no drop off of peptidyl-tRNA that could account for the inhibition of termination (not shown).

To decide whether the differences in the rates originated in the specificity parameter  $k_{cat}/K_m$  (Fersht, 1999) for the interaction between RelE and the ribosome, the same type of experiment was performed at ten times higher concentration of RC. When the logarithm of  $r_p$  was plotted against the concentration of RelE, straight lines identical to those in Figure 3C were obtained (not shown). This implied that the rates of the inhibition reactions in all cases were proportional to the RelE concentration and thus determined by  $k_{cat}/K_m$  rather than  $k_{cat}$  values. Inhibition of termination at UAG had a  $k_{cat}/K_m$  value of about  $3 \cdot 10^7 \text{ M}^{-1}\text{s}^{-1}$  (Figure 3C, insert) close to  $k_{cat}/K_m$  for termination by RF1 (Freistoffer et al., 2000).

The fact that termination, but not peptidyl-transfer to the aminoacyl-tRNA analog puromycin, was inhibited by RelE indicated that the toxin did not damage the peptidyl-transferase center in the 50S ribosomal sub-

unit. This is essential not only for peptidyl-transfer but also for release factor dependent termination (Zavialov et al., 2002; reviewed by Kisselev and Buckingham, 2000). Instead, the strong codon specificity of the rate of inhibition (Figure 3) suggested that the target for RelE could be the mRNA itself.

#### RelE Cleaves mRNA at UAG in the Ribosomal A Site

To test if RelE degraded mRNA we prepared gel filtered RCs programmed with 5'-end-( $^{33}\text{P}$ )-labeled XR7 mRNA containing the stop codon UAG in the A site and tetrapeptidyl-tRNA in the P site (Experimental Procedures). The RCs were incubated for 3 min in the presence of RelE, in the presence of RelE plus an excess of RelB or in the absence of both toxin and antitoxin. After phenol extraction, the RNA fragments were fractionated on a denaturing sequencing gel, which revealed (Figure 4) that XR7 mRNA had been cleaved between the A and the G of the stop codon in the presence (Lane 3) but

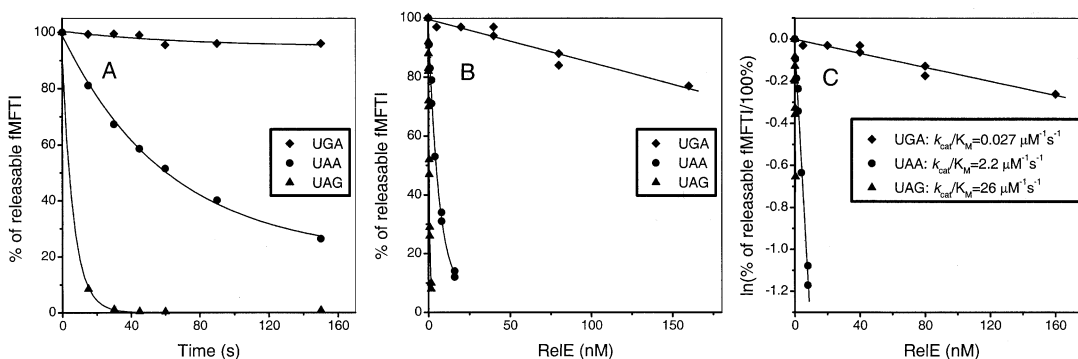
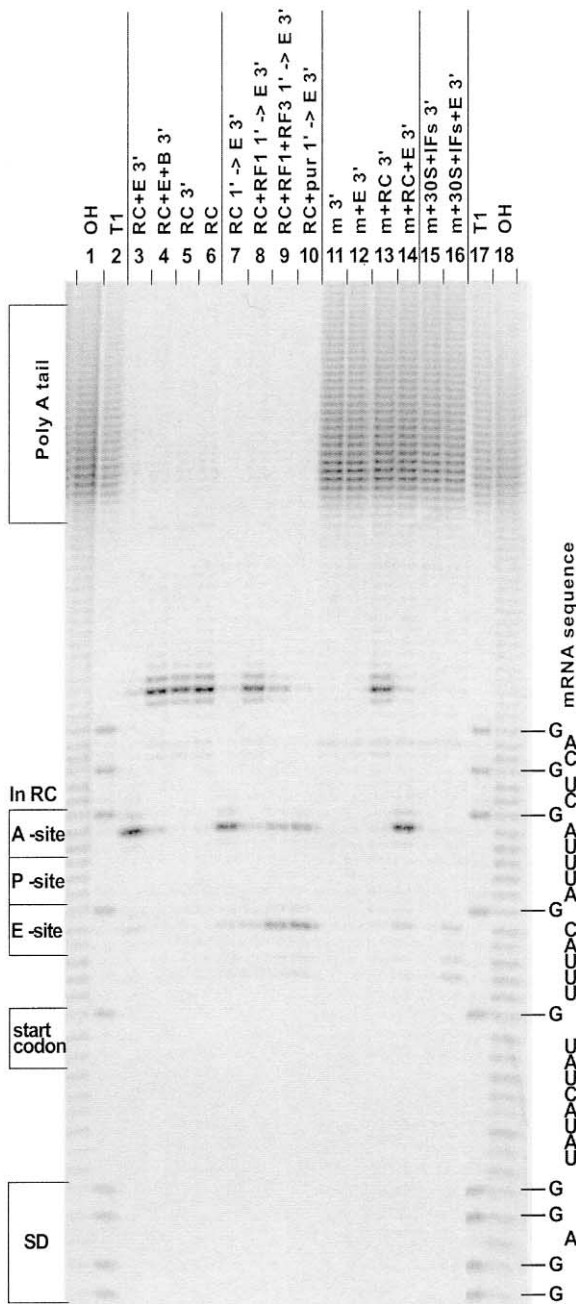


Figure 3. RelE Inhibition of Peptide Release from Ribosomes with a Tetrapeptidyl-tRNA in the P Site and a Stop Codon in the A Site

(A) Time dependent inhibition by RelE of peptide release from 30 nM RCs with different stop codons. Y-axis: fraction of fMet-Phe-Thr-Ile peptides that are released by RF1 (UAA and UAG) or RF2 (UGA). X-axis: time of exposure of RCs to 5 nM RelE before addition of RF1 or RF2. (B) RelE concentration dependent inhibition of peptide release from 40 nM RCs with different stop codons. Y-axis: fraction of fMet-Phe-Thr-Ile peptides that are released by RF1 (UAA and UAG) or RF2 (UGA). X-axis: RelE concentration during the 1 min exposure of RCs to the toxin before addition of RF1 or RF2. (C) Log plot of the data in (B). The  $k_{cat}/K_m$  values in the insert were calculated as the slopes of the straight lines divided by the incubation time.



**Figure 4. RelE Cleavages of mRNA**  
XR7 UAG mRNA was labeled in the 5'-end with  $\gamma$ [ $^{32}$ P]-ATP and T4 PNK. Ribosome complex (RC) consisting of 70S, [ $^{32}$ P]mRNA, [ $^3$ H]fMet-tRNA in the P site and UAG stop codon in the A site was purified by gel filtration. Cleavage of the mRNA in the RC was tested by incubating 1 pmol RC with 10 pmol RelE (lane 3), 10 pmol RelE + 200 pmol RelB (lane 4) or buffer (lane 5) at 37°C for 3 min followed by phenol extraction. As a control, 1 pmol RC was phenol extracted without prior incubation (lane 6). Competition for A site access was tested by preincubating 1 pmol RC for 1 min with buffer (lane 7), 20 pmol RF1 (lane 8), 20 pmol RF1 + 20 pmol RF3 + 1 mM GTP (lane 9) or 10 nmol Puromycin (lane 10) followed by 3 min incubation with 10 pmol RelE and then phenol extraction. Cleavage of free mRNA was tested by incubating 5 pmol XR7 UAG mRNA with buffer (lane 11), 10 pmol RelE (lane 12), 1 pmol RC (lane 13) or 10 pmol RelE + 1 pmol RC (lane 14) for 3 min before extraction with phenol. Cleavage of mRNA bound to the 30S ribosomal subunit was tested by incubating 5 pmol XR7 UAG mRNA with 10 pmol fMet-tRNA<sup>Met</sup>, 5 pmol IF1,

not in the absence (Lane 5) of RelE and that the presence of RelB in excess over RelE completely inhibited the reaction (Lane 4). When the RC had been preincubated with RF1, which binds very stably to the ribosomal A site (Zavialov et al., 2002), there was little cleavage of the mRNA by RelE (Lane 8). However, when the RC had been preincubated with RF1 and the G protein RF3 that removes RF1 or RF2 from the ribosome after peptide dissociation (Zavialov et al., 2001; 2002), or with just the antibiotic drug puromycin, that accepts nascent peptide chains from peptidyl-tRNA (Monro et al., 1969), the mRNA was cut by RelE in both the ribosomal A and E sites (Lanes 9 and 10). These findings suggested that RelE could efficiently attack mRNA in the E site after, but not before, removal of the peptide from peptidyl-tRNA in the P site. Control experiments showed that RelE was unable to cleave free mRNA in the absence as well as in the presence of RCs (Lanes 11–14), in line with the result shown in Figure 2B.

We also checked if RelE could cleave mRNA bound to the small ribosomal subunit by preincubating it with IF1, IF2, IF3, fMet-tRNA<sup>Met</sup>, and 5'-end-labeled XR7 mRNA. A 3 min long incubation was started by addition of RelE and the mRNA fragments were analyzed as above. The results (Lane 16) showed that RelE cleaved the mRNA with intermediate efficiency in the first (UUU) and second (ACG) codon after AUG.

The translation factors that were used to construct the RC contained an exonuclease activity that removed the heterogeneous polyA tail of the XR7 mRNAs before gel filtration (compare lanes 3–10 with lanes 11–16). This activity disappeared completely in the gel filtration step and did not interfere with the study of the endonucleolytic activity of RelE.

### RelE Cleaves mRNAs Bound to Ribosomes in a Codon Specific Manner

The codon specificity of RelE cleavage of mRNAs was studied more systematically with RCs programmed with twenty-one mRNAs of XR7 type, which were identical except for the fifth codon of their ORFs. This was either one of the three stop codons or a sense codon that can be reached from anyone of the stop codons by a single mutation (Freistroffer et al., 2000). These RCs with the fifth codon of the mRNA in the A site were gel purified and aliquots of each complex were incubated during a fixed time with different concentrations of RelE. The mRNA cleavage was analyzed with sequencing gels as in Figure 4, and results obtained with the stop codons are shown in Figure 5A. The mRNAs with UAG and UAA stop codons were cleaved only between the A in the middle position of the codon and its last nucleotide, while the UGA codon was cleaved after both the G and

2.5 pmol IF2, 5 pmol IF3, 20 pmol 30S, and 1 mM GTP in the absence (lane 15) or presence of 10 pmol RelE (lane 16). Markers for mapping the 3'-end of the mRNA fragments is the XR7 UAG mRNA subjected to limited alkaline hydrolysis (lane 1 and 18) or partially cut with RNase T1 under denaturing condition (lane 2 and 17). After the phenol extraction, the samples were analyzed on a 7% polyacrylamide sequence gel and the result was visualized with a phosphorimager.

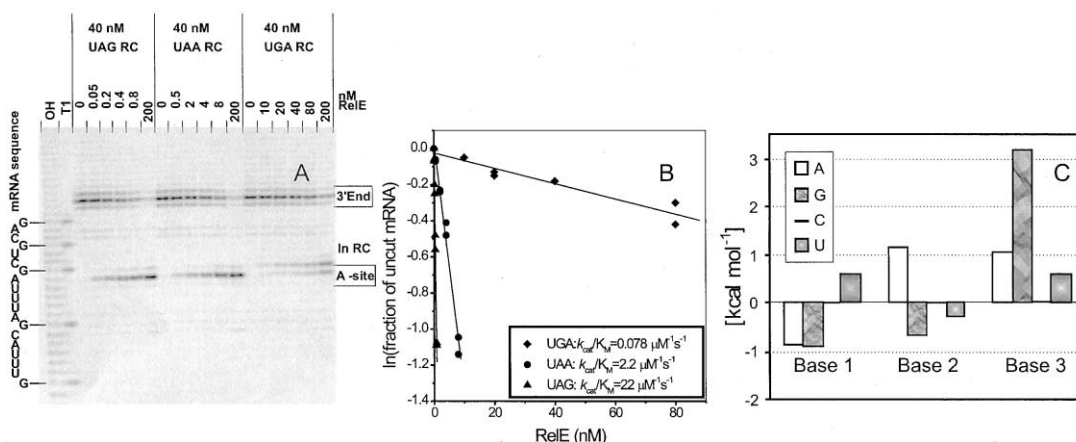


Figure 5. Estimates of the Rates of Cleavage by RelE of mRNAs with Different Stop Codons in the Ribosomal A Site (A) Cleavage in UAG (left side), UAA (middle) or UGA (right side) stop codons. The reactions were started by incubating 40 nM RC with increasing amounts of RelE for 1 min at 37°C followed by phenol extraction. Samples were analyzed on a 7% polyacrylamide sequence gel. (B) The results in (A) were quantified with a phosphor-imager and manufacture supplied software. The plots show the result of two independent experiments and the  $k_{cat}/K_m$  values in the insert were calculated from the slopes of the lines. (C) The parameter  $\Delta G$  (in kcal/mol) is the contribution to the standard free energy function of the different nucleotides in different positions of the codon used for the predictions of  $k_{cat}/K_m$  values for RelE cleavage of mRNAs shown in Table 1A.

the A with similar rates. When the logarithm of the fraction of noncleaved mRNA was plotted against the concentration of RelE, straight lines were obtained in all three cases (Figure 5B). The  $k_{cat}/K_m$  values calculated from the data in Figures 3C and 5B are very similar which allowed us to interpret the termination assay in Figure 3C as follows. When the mRNA is cut, the class 1 RFs cannot bind to the ribosome, which inhibits termination. Puromycin, which interacts with the 50S subunit more than 70 Å away from the codon in the A site of the 30S subunit, is unaffected by the disrupted stop codon.

The  $k_{cat}/K_m$  values for RelE dependent cleavage of sense codons could be estimated for thirteen of the eighteen codons tested, while the cleavage rates were below the detection limit for the other five. The measured  $k_{cat}/K_m$  values for the stop and sense codons are given in Table 1B. There is a marked codon dependence of

the RelE toxicity in this subset of base triplets. The RelE-dependent rates of cleavage of all possible codons were estimated by fitting measured  $k_{cat}/K_m$  values to a simple exponential model with ten unknown standard free energy parameters. Its physical rationale is that  $k_{cat}/K_m$  is determined by the standard free energy difference between RelE in activated complex with a ribosome and a ground state with free RelE and ribosome (Experimental Procedures; Table 1A; Figure 5C).

#### Rescue of Protein Synthesis after Exposure to RelE

We knew from the results in Figure 2A that recycling ribosomes that had been inhibited by RelE could not be rescued by addition of mRNA together with RelB in excess over RelE. One requirement for rescue is that

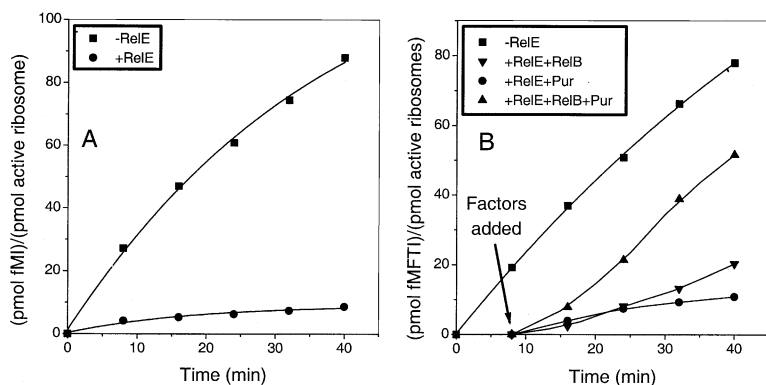
Table 1A. Predicted  $k_{cat}/K_m$  Values ( $s^{-1}\mu M^{-1}$ ) for RelE Cleavage of Codons in the Ribosomal A Site

Predictions	U	C	A	G	
U	0.046	0.072	0.51	0.024	U
U	<b>0.098</b>	<b>0.16</b>	<b>1.1</b>	<b>0.052</b>	<b>A</b>
U	0.016	0.026	0.18	0.0087	C
U	<b>3.6</b>	<b>5.7</b>	<b>40</b>	<b>1.9</b>	<b>G</b>
C	0.016	0.026	0.18	0.0087	U
C	0.035	0.055	<b>0.39</b>	<b>0.019</b>	<b>A</b>
C	0.0058	0.0092	0.065	0.0031	C
C	1.3	2.0	<b>14</b>	0.68	<b>G</b>
A	0.0038	0.0061	0.043	0.0020	U
A	0.0082	0.013	<b>0.092</b>	<b>0.0044</b>	<b>A</b>
A	0.0014	0.0022	0.015	0.0007	C
A	0.30	0.48	<b>3.4</b>	<b>0.16</b>	<b>G</b>
G	0.0037	0.0059	0.041	0.0020	U
G	0.0079	0.013	<b>0.089</b>	<b>0.0042</b>	<b>A</b>
G	0.0013	0.0021	0.015	0.0007	C
G	0.29	0.46	<b>3.2</b>	0.15	<b>G</b>

Bold face means that experimental values exist in Table 1B.

Table 1B. Measured  $k_{cat}/K_m$  Values ( $s^{-1}\mu M^{-1}$ ) for RelE Cleavage of Codons in the Ribosomal A Site

Experimental	
C G A	0.0061
U G C	0.026
U G U	0.026
U A C	0.061
U G A	0.08
A A A	0.1
U A U	0.48
C A A	0.77
U G G	1.2
U A A	2.7
A A G	3.1
G A G	3.6
U U G	4.4
U C G	11
U A G	17
C A G	22
G G A	<0.08
U U A	<0.08
U C A	<0.08
G A A	<0.08
A G A	<0.08



were added either puromycin (Pur), RelB, or RelB + Pur. The final concentrations were 1  $\mu$ M XR7 UAG mRNA, 1.2  $\mu$ M RelB, and/or 1.5  $\mu$ M Pur. The reaction, -RelE, is a control where XR7 mRNA is translated without RelE from time 0. The amount of fMFTI-tetrapeptide synthesized was measured by HPLC.

ribosomal complexes with peptidyl-tRNA in P site and an mRNA truncated in the A site codon can be brought back to initiation by ribosomal recycling factor RRF and elongation factor EF-G. Since removal of the peptide from peptidyl-tRNA in the ribosomal P site by RF1 or RF2 activates ribosome splitting by RRF and EF-G (Karimi et al., 1999), one reason for the results in Figure 2A could be that the remaining peptide prevented dissociation of the ribosome into subunits. Since RelE dependent truncation of a stop codon in the A site did not inhibit peptidyl-transfer to puromycin, we used this drug to remove the peptide from ribosomes stalled on truncated mRNAs in a rescue experiment. Ribosomes were first initiated with an mRNA (barI; Guzman and Guarneros, 1989) with a SD sequence of intermediate strength, an ORF encoding the dipeptide fMet-Ile and with a UAG stop codon (Experimental Procedures). Synthesis of fMet-Ile by recycling ribosomes was rapid in the absence but very slow in the presence of RelE (Figure 6A). Both the rate of recycling and the degree of inhibition by RelE were similar for ribosomes programmed with barI and XR7 mRNA (Figure 1A).

In the rescue experiment, the ribosomes were synthesizing fMet-Ile in the presence of RelE exactly as in Figure 6A during 8 min. Then, XR7 mRNA was added together with RelB in excess over RelE, with only puromycin in slight excess over ribosomes or with both RelB and puromycin. When only RelB or only puromycin was added with mRNA, there was little synthesis of tetrapeptides. However, when both RelB and puromycin were present fMFTI peptides were synthesized at a rate similar to normal translation of XR7 (Figure 6B). These data suggested that RRF and EF-G can promote ribosome splitting into subunits also when the A site codon is degraded and demonstrated that removal of the peptide from P site bound peptidyl-tRNA can lead to ribosome rescue in the presence of RelB and new mRNAs.

## Discussion

### The Mechanism by which RelE Inhibits Bacterial Protein Synthesis

The chromosomally expressed *E. coli* toxin RelE inhibits protein synthesis by truncating ribosome bound, but not

free, mRNAs codon specifically (Figures 2 and 7). RelE normally cuts the mRNAs between the second and third base of the codon in the ribosomal A site (Figures 4 and 5). The fastest cleavage occurs for UAG codons where the RelE activity ( $k_{cat}/K_m$ ) approaches that of release factor RF1 (Freistoffer et al., 2000), and cleavage at UAA and CAG codons takes place with intermediate rates (Tables 1A and 1B). The codon specificities of RelE are reminiscent of those of RF1. The major difference is that RF1 discriminates extremely well (Freistoffer et al., 2000) but RelE poorly (Tables 1A and 1B) between U and C in the first position of a codon. RelE truncates ribosome bound mRNAs in competition either with termination by class 1 release factors or with aminoacyl-tRNA binding and peptide elongation. Our data suggest that protein synthesis in cells exposed to free RelE will be inhibited by disruption of UAG stop codons, but cleavage of starved sense codons sensitive to RelE will also occur (Tables 1A and 1B). Our results show that mRNA cleavage can occur in the ribosomal E site after release of the peptide from peptidyl-tRNA in the P site and that mRNA bound to the free 30S subunit can be degraded by RelE (Figure 4).

### Rescue of Protein Synthesis after Exposure to RelE

The fact that RelE degrades mRNA rather than a more stable component of the translational machinery can explain rescue of protein synthesis after RelE inhibition by overexpression of the antitoxin RelB in living cells or by addition of RelB to cell extracts (Pedersen et al., 2002). The rescue requires that ribosomal complexes with a peptidyl-tRNA in the P site and a disrupted codon in the A site split into subunits and reinitiate. However, peptide release by RFs cannot occur with disrupted stop codons (Figure 3) and ribosome splitting by RRF and EF-G requires a deacylated tRNA in the P site (Karimi et al., 1999). The results in Figure 6B show that addition of RelB and intact mRNA to ribosomes inhibited by RelE can rescue protein synthesis, provided that the peptide is removed from peptidyl-tRNA by transfer to the antibiotic puromycin. Therefore, recycling of ribosomes back to initiation by RRF and EF-G can be carried out after peptide release even when the codon in the A site has been disrupted.

Figure 6. Rescue of RelE-Inhibited Ribosomes by RelB, Uncleaved mRNA, and Puromycin

(A) Inhibition of ribosomes translating barI mRNA in recycling mode. Conditions as described in Figure 1A except that RelE was added to the initiation mixes at time -10 min. The concentrations in the reactions were 0.06  $\mu$ M RelE, 0.5  $\mu$ M ribosomes, and 1  $\mu$ M barI UAG mRNA. The amount of fMII-dipeptide synthesized was measured by HPLC.

(B) The amount of tetrapeptide synthesized from a second mRNA added to the translation reaction +RelE in (A). After 8 min, a sample was removed to which XR7 mRNA was added. Then, it was split in aliquots to which

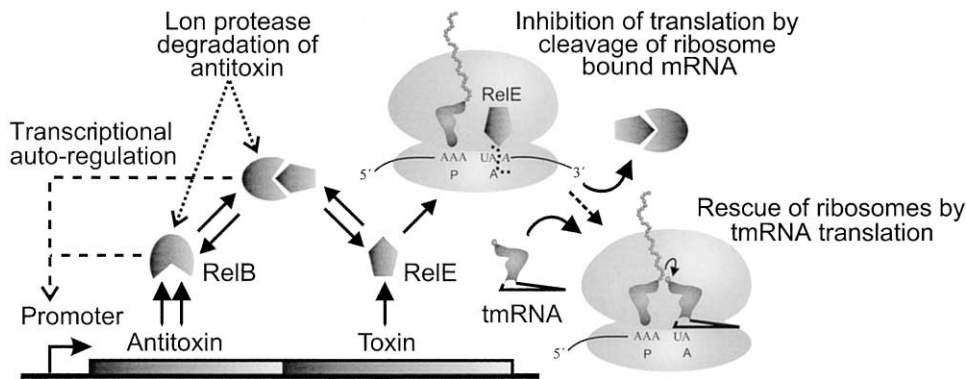


Figure 7. Genetic Organization and Regulatory Components of the *relBE* Toxin-Antitoxin Operon (Left), the Site of mRNA Cleavage by RelE (Middle), and Ribosome Rescue by tmRNA after mRNA Truncation by RelE (Right).

### Ribonucleolytic Inhibitors of Protein Synthesis Other Than RelE

RelE is one of several toxins with ribonuclease activity that inhibit protein synthesis but its codon specific cleavage of mRNA makes it special. However, the prophage P1 *phd-doc* and *E. coli* chromosomal *mazEF* (*chpA*) TA loci could possibly inhibit protein synthesis in the same way (Hazan et al., 2001; Pedersen et al., 2002). Accordingly, these and other similar toxin-antitoxin systems might act by codon specific mRNA degradation on ribosomes.

Another set of ribonucleases that inhibit protein synthesis is a subgroup of the colicins, which are plasmid-encoded toxins, programmed to kill *E. coli* cells that lack the plasmid (reviewed by Riley and Gordon, 1999). Colicins in one group (e.g., E3) cleave 16S rRNA near its 3' end in the ribosomal A site. The cut in 16S rRNA abolishes peptidyl-transfer, possibly by disrupting the contact between tRNA and mRNA. The bacteriocidal effects of another group of colicins depend on their cleavage of anticodon loops of different tRNA isoacceptors (Ogawa et al., 1999; Soelaiman et al., 2001).

A different and extensively characterized group of toxins contains the fungal sarcin and sarcin-like toxins. Sarcin cleaves rRNA of the large ribosomal subunit (between G4325 and A4326 in rat numbering), thereby inhibiting the binding of translation factors to the ribosome (Wool et al., 1992). Ricin-like toxins in plant and bacteria deadenylate base A4324 with a similar effect on ribosome function as the adjacent sarcin cleavage (reviewed by Peumans et al., 2001; Nielsen and Boston, 2001).

In contrast to inhibition of translation by RelE, the inactivation of ribosomal complexes by colicins, sarcin-, and ricin-like toxins is essentially irreversible and rescue requires de novo synthesis of ribosomes or translation factors. This might reflect different biological functions of RelE and irreversibly acting toxins.

### What is the Physiological Role of RelE in Bacteria?

It was previously known that RelE becomes activated when protein synthesis is strongly inhibited (Christensen et al., 2001), for the reason that the neutralizing RelB antitoxin is unstable and rapidly disappears from the cytoplasm as soon as its continuous synthesis is interrupted (Gotfredsen and Gerdes, 1998; Christensen et

al., 2001). Here, it has been established that RelE cleaves mRNAs in the ribosomal A site with high specificity (Tables 1A and 1B), in the E site after peptide release and also on free 30S subunits (Figure 4).

The fact that RelE cleaves mRNAs in the A site of the ribosome suggests a functional link between the toxin and tmRNA (Figure 7), known to rescue ribosomes that are stalled on truncated mRNAs (Keiler et al., 1996; Karzai et al., 2000). This hypothesis has now been verified in two different ways. Biochemical experiments have shown that transfer of a tetra-peptide from peptidyl-tRNA in the ribosomal P site to tmRNA is greatly stimulated by preincubation with RelE when the A site codon is UAA and UAG but not UGA (N. Ivanova, unpublished data). Furthermore, it was shown in vivo that cells with tmRNA deficiency have slower recovery after RelE-poisoning than wild-type (S. Christensen and K.G., unpublished data). From this connection with tmRNA, we now suggest that RelE promotes fast adaptation after medial downshifts that lead to amino acid starvation. In such cases, amino acid synthesizing enzymes suddenly become essential and must be synthesized rapidly, when the only amino acid supply is via slow degradation of existing proteins (Brunschede and Bremer, 1971; Kuroda et al., 2001; Gottesman and Maurizi, 2001). After a downshift, RelE could be activated and cut ribosome bound mRNAs with toxin-sensitive codons in the A site (Tables 1A and 1B). The subsequent action of tmRNA would then tag nascent peptides on stalled ribosomes for degradation and rapidly make the amino acids from these peptides available for de novo synthesis of essential proteins that did not exist in the cell before the shift. It can be noted that UAG codons, that are most sensitive to cleavage by RelE, are greatly underrepresented as stop signals among mRNAs for amino acid synthesizing enzymes (GenBank NC\_000913, Blattner et al., 1997). This feature could have been selected to facilitate synthesis of these essential proteins in the presence of RelE after a downshift.

The most well-known event after medial downshifts is the stringent response (reviewed by Cashel et al., 1996), when a global inhibitor of stable RNA synthesis, ppGpp, rapidly increases to high values (Block and Haseltine, 1975). It is known that ppGpp strongly inhibits protein synthesis in the absence of amino acid starva-

tion and that this “intrinsic” inhibition relieves translational errors that otherwise would occur at a high frequency at starved codons during amino acid deprivation (O’Farrell, 1978). There are two principally different ways by which bulk synthesis of proteins can be inhibited. The first is that a kinetic constant in one of the elongation steps of every ribosome is slowed down and the second that the fraction of ribosomes engaged in elongation is reduced. For the decrease of translational errors during amino acid starvation, it does not matter how protein synthesis is slowed down, but for rapid adaptation to amino acid depletion the second way is superior. The reason is that the delay between initiation of transcription of a gene for an amino acid synthesizing enzyme and the emergence of active proteins will be much shorter when few, rather than many, ribosomes tap in on a rate limiting supply of amino acids. Accordingly, we suggest that a second function of RelE is to reduce the number of elongating ribosomes after a downshift, thereby speeding up elongation on the ones that remain active. The inhibition could result from attacks by RelE on sensitive codons in the A site, but it is conceivable that the toxin will degrade also mRNAs on the small subunit, thereby slowing down initiation of translation. If these two suggestions were true, the term toxin for RelE would be misleading since its effects would then be beneficial, rather than harmful, for the cell.

Verification of these proposals requires observation of lag times before fast growth is resumed after downshifts in *relE* and wild-type strains, which remains to be done. However, there exist a number of experiments on *relB* mutants with the delayed relaxed phenotype that are relevant. When these mutants are depleted of an amino acid, the stringent response is initially induced as in wild-type cells (Lavallé et al., 1976). However, the level of RelB will drop much more rapidly in *relB* than in wild-type cells, presumably due to a much shorter life time of the mutant protein (Bech et al., 1985; Gotfredsen and Gerdes, 1998). This, we suggest, will induce a burst of active RelE toxin in the cytoplasm and lead to extensive truncation of ribosome bound mRNAs so that protein synthesis and consumption of amino acid stops completely. This will cause the tRNA-charging levels to rise, the RelA-dependent synthesis of ppGpp to be shut down and the concentration of ppGpp to drop to its basal level (Lavallé et al., 1976) by the action of SpoT (Fiil et al., 1977; Somerville and Ahmed, 1979); i.e., to the delayed relaxed response. When the missing amino acid is readded to the medium, slow resynthesis of RelB will create a long delay before ribosomes become active again, as observed (Diderichsen and Desmarez, 1980). The difference, we propose, between *relB* mutants and wild-type cells is that the RelB level is much lower and the RelE activity much higher after amino acid depletion in the former case. Therefore, the RelE activity does not remove the stringent response in wild-type cells and their recovery after readdition of a missing amino acid is comparatively fast.

#### Experimental Procedures

##### Chemicals, Enzymes, and Buffers

Calf intestine alkaline phosphatase (AP) and T4 polynucleotide kinase (PNK) were from Pharmacia. Amino acids and myokinase (MK)

were from Sigma. Radioisotopes were from Amersham. RNase T1 and pyruvate kinase (PK) were from Boehringer Mannheim. RelE and RelB proteins were purified according to Pedersen et al. (2002) and Christensen et al. (2001), respectively, dialyzed in polymix buffer (Jelenc and Kurland, 1979) and concentrations determined by UV measurements.

##### Synthetic mRNAs

DNA templates for T7 RNA polymerase (T7RNAP) synthesis of mRNAs were prepared by filling out two partially aligned DNA oligos with taq polymerase. The full sequence of XR7 UAG mRNA was: GGG AAU UCG GGC CCU UGU UAA CAA UUA AGG AGG UAU ACU AUG UUU ACG AUU UAG CUG CAG (A)<sub>21</sub> (SD sequence and fMFTI reading frame are underlined). The full sequence of the barI UAG mRNA was: GGG AAG CUG AAC GAG AAA CGU AAA AUG AUC UAG AUA UCA AUA UAC UGC AG (A)<sub>21</sub> (SD sequence and fMI reading frame are underlined). The mRNAs were prepared by in vitro transcription with T7RNAP and purified using poly-dT column from Pharmacia as described in Pavlov et al. (1997). The mRNAs with a sense codon in fifth position but otherwise identical to XR7 UAG mRNA were prepared as described in Freistroffer et al. (2000).

##### Labeling of mRNAs

To prepare 5'-end-labeled mRNAs, they were first dephosphorylated by AP treatment, extracted with buffer saturated phenol [pH 8], and then precipitated by adding 2 volumes EtOH and 0.1 volumes 3 M NaAc [pH 5]. This was followed by  $\gamma^{32}\text{P}$ -ATP + PNK labeling, phenol extraction, and purification on a micro bio-spin P30 column (Bio-Rad). AP, PNK, and P30 were used according to the supplier's protocols. Recovery of the mRNA was checked from the OD<sub>260</sub> value. The alkali ladder was prepared just before gel loading by incubation of 1  $\mu\text{l}$  (approximately 50 pmol) end-labeled mRNA with 1  $\mu\text{l}$  1 M NaOH, and 20 mM EDTA at 95°C for 5–10 s. The reaction was then placed on ice and stopped by addition of 2  $\mu\text{l}$  9.5 M urea, 85 mM NaAc, 1% (W/V) glacial acetic acid, and 12  $\mu\text{l}$  formamide loading buffer. The G ladder was obtained by partial digestion of 1  $\mu\text{l}$  end-labeled mRNA in 1  $\mu\text{l}$  tRNA (5  $\mu\text{g}$ ) and 20  $\mu\text{l}$  7.2 M urea, 120 mM NaCl, 24 mM NaAc [pH 4.7] with 2  $\mu\text{l}$  RNase T1 (diluted a 100-fold in 100 mM NaCl, and 20 mM NaAc [pH 4.7]) at 37°C for 4 min. Digestion was stop by phenol extraction followed by EtOH/NaAc precipitation and the RNA was resuspended in 16  $\mu\text{l}$  loading buffer.

##### In Vitro Translation with Recycling Ribosomes

Initiation and elongation mixes for the translation experiments were prepared with the following final concentrations of translation components. Initiation mix: 1.7  $\mu\text{M}$  70S ribosomes (60%–80% active according to dipeptide assays), 1.7  $\mu\text{M}$  IF1, 0.83  $\mu\text{M}$  IF2, 1.7  $\mu\text{M}$  IF3, 3.3  $\mu\text{M}$  mRNA, 3.3  $\mu\text{M}$  [<sup>3</sup>H]Met-tRNA<sup>Met</sup>, 1 mM GTP, and 0.3% RNA guard in 1  $\times$  polymix buffer. Elongation mix: 17  $\mu\text{M}$  EF-Tu, 1.7  $\mu\text{M}$  EF-Ts, 0.71  $\mu\text{M}$  EF-G, 0.71  $\mu\text{M}$  RF1 or RF2, 0.71  $\mu\text{M}$  RF3, 0.71  $\mu\text{M}$  RRF, 0.21 U/ $\mu\text{l}$  each of aa-tRNA synthetases (MetRS, PheRS, ThrRS and IleRS), 140  $\mu\text{M}$  total tRNA (containing 2.9  $\mu\text{M}$  tRNA<sup>Phe</sup>-isoacceptor), 0.29 mM of each of the unlabeled amino acids (Met, Thr, and Ile, or Met, Phe and Thr), 0.14 mM of [<sup>14</sup>C]Phe or [<sup>14</sup>C]Ile, 64 ng/ $\mu\text{l}$  PK, 3.6 ng/ $\mu\text{l}$  MK, 1.4 mM ATP, 14 mM PEP, 0.29 mM THF, 0.071 U/ $\mu\text{l}$  FMT 1 mM GTP, and 0.3% RNA guard in 1  $\times$  polymix buffer. The free Mg<sup>2+</sup> concentration was adjusted to 4 mM with MgAc<sub>2</sub>. Initiation (30% of final volume) and elongation (70% of final volume) mixes were preincubated for 10 min and then added together to start incubation. RelE was included in initiation mixes as indicated in the Figure Legends. Neutralization mixes containing different translation components and/or RelB were prepared in 1  $\times$  polymix buffer and added to the translation reaction in a ratio of 1:14 (typically 18  $\mu\text{l}$  + 250  $\mu\text{l}$ ) after 8 min incubation. Aliquots of 50  $\mu\text{l}$  were withdrawn and quenched with 18  $\mu\text{l}$  50% cold formic acid after different incubation times. Translational components and determination of the amount of free peptide in the samples were as described in Pavlov et al. (1997).

##### Ribosome Complexes

Ribosomes paused with the fifth codon of the mRNAs in the A site and the fMFTI-tetrapeptidyl-tRNA in the P site (Ribosome Complexes; RCs) were prepared from an initiation mix and an elongation



mix according to Freistroffer et al. (1997) with modifications. Initiation mix (200  $\mu$ l): 3 mM PEP, 1 mM GTP, 45 ng/ $\mu$ l PK, 7.5  $\mu$ M mRNA, 15  $\mu$ M f<sup>3</sup>H]Met-tRNA<sup>Met</sup>, 5  $\mu$ M 70S ribosomes (typically around 75% active in dipeptide formation), 1.25  $\mu$ M IF1, 0.63  $\mu$ M IF2, and 1.25  $\mu$ M IF3 in 1  $\times$  polymix buffer. Elongation mix (300  $\mu$ l): 1 mM ATP, 2.5 ng/ $\mu$ l MK, 3 mM PEP, 1 mM GTP, 45 ng/ $\mu$ l PK, 0.2 mM of each aa (Phe, Thr and Ile), 6.66  $\mu$ M tRNA<sup>Leu</sup> in tRNA bulk, 0.3 U/ $\mu$ l of each aa-tRNA synthetases (PheRS, ThrRS and IleRS), 10  $\mu$ M EF-Tu, 2.5  $\mu$ M EF-Ts, 4  $\mu$ M EF-G in 1  $\times$  polymix buffer. The free Mg<sup>2+</sup> concentration was adjusted to 4 mM with MgAc<sub>2</sub>. The two mixes were preincubated at 37°C for 10 min and then added together to start peptide elongation. After 1 min incubation, the reaction tube was placed on ice for 10 min before gel filtration (Sephacryl S300, Pharmacia). The ribosome concentration was determined from OD<sub>260</sub> (1 OD<sub>260</sub> unit = 23 pmol 70S/ml) and the concentration of f<sup>3</sup>H]Met by scintillation counting. The purified fraction contained between 0.1 and 0.2  $\mu$ M f<sup>3</sup>H]Met (70%–75% of the concentration of 70S). In most preparations, around 5% of the f<sup>3</sup>H]Met was hydrolyzed from tRNA, 5% was not elongated to fMFTI-tetrapeptide, and 10% had dropped off as peptidyl-tRNA during handling. The remaining 80% of the f<sup>3</sup>H]Met was incorporated as fMFTI-tRNA in the P site of active ribosome complexes.

#### Inhibition of Peptide Release

Inhibition of peptide release from the fMFTI-tRNA:mRNA:70S RCs with a stop codon in the A site was determined as follows. For the time dependence of inhibition (Figure 3A), one mixture containing RCs with UAA, UAG, or UGA stop codons (Mixture A) and one with a catalytic amount of RelE (Mixture B) were preincubated separately for 4 min at 37°C. Then the A and B mixtures were combined and 40  $\mu$ l aliquots taken at varying times and added into 10  $\mu$ l of RF1 or RF2. The resulting mixture (C) was incubated for 1 min and the reaction was stopped by addition of 20% formic acid (900  $\mu$ l). The amount of peptide released from the RCs was determined from supernatant counting after centrifugation. Mixture C contained 25 nM RC, 4 nM RelE, and 120 nM RF1.

For the RelE concentration dependence of inhibition (Figure 3B), RCs (2 pmol) were incubated in 46  $\mu$ l 1  $\times$  polymix buffer at 37°C for 1 min and then 4  $\mu$ l RelE (diluted in 1  $\times$  polymix buffer + 20  $\mu$ g/ml BSA) was added. After 1 min incubation, peptide-release was initiated by addition of 10  $\mu$ l 4  $\mu$ M RF1/RF2 and the reaction was stopped after 1 min by addition of 60  $\mu$ l ice-cold 26% formic acid. Remaining peptidyl-tRNA was pelleted by centrifugation (Eppendorf) for 15 min at 14,000 rpm and released peptide was measured by scintillation counting of the supernatant. In the calculations of peptide-release, the background value (set as 0% release) was obtained from a control where 10  $\mu$ l 1  $\times$  polymix buffer was added instead of RF1/RF2 and was the same with or without addition of RelE. The maximum amount of peptide-release (set as 100%) was determined in a control with addition of 4  $\mu$ l 1  $\times$  polymix buffer without RelE.

#### Cleavage of mRNA

Cleavage of mRNA in the A site of fMFTI-tRNA:mRNA:70S RC by RelE was determined by incubating 1 pmol RC in 23  $\mu$ l 1  $\times$  polymix buffer at 37°C for 1 min, adding 2  $\mu$ l RelE (diluted in 1  $\times$  polymix buffer + 20  $\mu$ g/ml BSA), and continuing incubation for 1 min. The reaction was stopped by addition of 25  $\mu$ l 40 mM EDTA and 0.5% SDS + 25  $\mu$ l phenol [pH 8] and vortexed. Before centrifugation, 25  $\mu$ l chloroform and isoamylalcohol (24:1) were added to reduce the loss of mRNA in the phenol phase. After centrifugation in an Eppendorf centrifuge for 5 min at 14,000 rpm RNA in the water phase was collected, EtOH/NaAc precipitated and resuspended in 6  $\mu$ l formamide loading buffer. The samples were incubated at 95°C for 5 min and then placed on ice before loading onto a 7% polyacrylamide 8 M urea sequencing gel. To calculate the fraction of cleaved mRNAs, we first normalized the intensities of bands originating from each mRNA using the "ImageQuant5.0" program. The background intensity was determined from the lane with the RC sample incubated without RelE. The intensity of the product in a lane where the RC had been incubated with 400 nM RelE was set as 99% in the case of the sensitive codons (UAG, UAA, UAU, UGG, CAA, CAG, AAG, GAG, UCG, and UUG) and to 71% for the less sensitive codons

(UGA and AAA). These percentages were based on the information obtained in the peptide release assay described above. For the other, least sensitive, codons (UAC, UGU, UGC, CGA, GGA, UUA, UCA, GAA, and AGA) we estimated that 50% of the mRNA was associated with a RC and had its fifth codon in the A site ("cleavable RC"). The percentage of cleavage was then calculated from an incubation with 400 nM RelE.

#### Chemometric Analysis of the Codon-Specificity of RelE-Cleavage of mRNA

Model: The  $k_{cat}/K_m$  value ( $y$ ) for RelE dependent cleavage of mRNA in the A site with bases XYZ in positions 1, 2, and 3 is assumed to depend on standard free energies according to:

$$y_{mod}^{XYZ} = ke^{(\Delta G_{ix} + \Delta G_{2y} + \Delta G_{3z} + \hat{G}_0)/RT} = e^{(\Delta G_{ix} + \Delta G_{2y} + \Delta G_{3z} + \hat{G}_0)/RT} \quad (1)$$

The exponent is essentially the standard free energy difference between free RelE and ribosomes on one hand and, on the other, RelE molecules in activated complex with ribosomes.  $\hat{G}_0$  is a reference standard free energy corresponding to the CCC codon.  $\Delta G_j$  is the difference between the standard free energy of any base  $j$  ( $= A, U, C$  or  $G$ ) and  $C$  in position  $i$  ( $= 1, 2, 3$ ) of a codon.  $R$  is the gas constant and  $T$  is the temperature. When  $k$  ( $\mu\text{M}^{-1}\text{s}^{-1}$ ) is put equal to one,  $\hat{G}_0 = G_0$ . It is assumed that the standard free energy contribution from one base in a codon does not depend on the identity of the other two. There are ten unknown model parameters ( $G_0$  and nine  $\Delta G_j$  values) in the parameter vector  $\bar{G}$ . Knowledge of  $\bar{G}$  allows prediction of the  $k_{cat}/K_m$  values for RelE cleavage at all sixty-four codons.  $\bar{G}$  was estimated by minimizing the mean squared error of the logarithm of 21 experimentally measured  $k_{cat}/K_m$  values ( $\ln y_{exp}^{XYZ}$ ) and the logarithm of those predicted by the model ( $\ln y_{mod}^{XYZ}$ ):

$$\bar{G}_{est} = \underset{\bar{G}}{\operatorname{argmin}} \sum_{\text{codon}=1}^{21} [\ln(y_{exp}^{\text{codon}}) - \ln(y_{mod}^{\text{codon}}(\bar{G}))]^2 \quad (2)$$

Five of the twenty-one experimental values were upper limits (Tables 1A and 1B) and the corresponding terms only contributed to  $\bar{G}_{est}$  when  $y_{mod}^{XYZ} > y_{exp}^{XYZ}$ . Estimates for  $\Delta G_j$  are illustrated in Figure 5C and predictions of the 64  $k_{cat}/K_m$  values are given in Table 1A.

Validation: The relative training error,

$$\frac{1}{21} \sum_{\text{codon}=1}^{21} [|\ln(y_{mod}^{\text{codon}}) - \ln(y_{exp}^{\text{codon}})|/y_{exp}^{\text{codon}}],$$

was 0.53. This shows that the problem was well structured for prediction since a relative training error of 47.7 was obtained when all codon-measurement pairs had been randomized before determination of  $\bar{G}_{est}$ . Leave one out crossvalidation (Hastie et al., 2001), where one codon-measurement pair at the time was left out in the determination of  $\bar{G}_{est}$ , gave an average relative prediction error of 1.78. This corresponds to an uncertainty of a factor of two in estimated  $k_{cat}/K_m$  values; good compared to the span of three orders of magnitude of the observed values.

#### Acknowledgments

We thank Hans Bremer, Richard Buckingham, Johan Paulsson, and Tanel Tenson for valuable comments on the manuscript. We are grateful to Natalia Ivanova (Uppsala) and Susanne Christensen (Odense) for making unpublished data available and Daniel Nilsson for help with the bioinformatics. This work was supported by The Danish Biotechnology Instrument Center (DABIC), the European Union Contract BIO4-98-0283 and QLK3-CT-2001-00277, the Swedish Foundation for Strategic Research, the National Graduate School for Scientific Computing, and the Swedish Research Council.

Received: October 21, 2002

Revised: November 25, 2002

#### References

- Bech, F.W., Jorgensen, S.T., Diderichsen, B., and Karlstrom, O.H. (1985). Sequence of the *relB* transcription unit from *Escherichia coli* and identification of the *relB* gene. EMBO J. 4, 1059–1066.
- Blattner, F.R., Plunkett, G., III, Bloch, C.A., Perna, N.T., Burland, V.,

- Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., et al. (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* 277, 1453–1474.
- Block, R., and Haseltine, A.W. (1975). Purification and properties of stringent factor. *J. Biol. Chem.* 250, 1212–1217.
- Brunschede, H., and Bremer, H. (1971). Synthesis and breakdown of proteins in *Escherichia coli* during amino-acid starvation. *J. Mol. Biol.* 37, 35–57.
- Cashel, M., Gentry, D., Hernandez, V.J., and Vinella, D. (1996). The stringent response. In *Escherichia coli and Salmonella*, F.C. Neidhardt, ed. (Washington, D.C.: ASM Press), pp. 1458–1496.
- Christensen, S.K., Mikkelsen, M., Pedersen, K., and Gerdes, K. (2001). RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl. Acad. Sci. USA* 98, 14328–14333.
- Diderichsen, B., and Desmarez, L. (1980). Variations in phenotype of *relB* mutants of *Escherichia coli* and the effect of *pus* and *sup* mutations. *Mol. Gen. Genet.* 180, 429–437.
- Diderichsen, B., Fiil, N.P., and Lavalle, R. (1977). Genetics of the *relB* locus in *Escherichia coli*. *J. Bacteriol.* 131, 30–33.
- Fersht, A. (1999). Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding (New York: W.H. Freeman & Co).
- Fiil, N.P., Willumsen, B.M., and von Meyenburg, K. (1977). Interaction of alleles of the *relA*, *relC* and *spoT* genes in *Escherichia coli*: analysis of the interconversion of GTP, ppGpp and pppGpp. *Mol. Gen. Genet.* 150, 87–101.
- Freistroffer, D.V., Pavlov, M.Y., MacDougall, J., Buckingham, R.H., and Ehrenberg, M. (1997). Release factor RF3 in *E. coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner. *EMBO J.* 16, 4126–4133.
- Freistroffer, D.V., Kwiatkowski, M., Buckingham, R.H., and Ehrenberg, M. (2000). The accuracy of codon recognition by polypeptide release factors. *Proc. Natl. Acad. Sci. USA* 97, 2046–2051.
- Galvani, C., Terry, J., and Ishiguro, E.E. (2001). Purification of the RelB and RelE proteins of *Escherichia coli*: RelE binds to RelB and to ribosomes. *J. Bacteriol.* 183, 2700–2703.
- Gerdes, K. (2000). Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. *J. Bacteriol.* 182, 561–572.
- Gillet, R., and Felden, B. (2001). Emerging views on tmRNA-mediated protein tagging and ribosome rescue. *Mol. Microbiol.* 42, 879–885.
- Gotfredsen, M., and Gerdes, K. (1998). The *Escherichia coli relBE* genes belong to a new toxin-antitoxin gene family. *Mol. Microbiol.* 29, 1065–1076.
- Gottesman, S., and Maurizi, M.R. (2001). Surviving starvation. *Science* 293, 614–615.
- Guzman, P., and Guarneros, G. (1989). Phage genetic sites involved in lambda growth inhibition by the *Escherichia coli rap* mutant. *Genetics* 121, 401–409.
- Hastie, T., Tibshirani, R., and Friedman, J. (2001). The elements of statistical learning (New York, USA: Springer).
- Hazan, R., Sat, B., Reches, M., and Engelberg-Kulka, H. (2001). Postsegregational killing mediated by the P1 phage “addiction module” *phd-doc* requires the *Escherichia coli* programmed cell death system *mazEF*. *J. Bacteriol.* 183, 2046–2050.
- Jelenc, P.C., and Kurland, C.G. (1979). Nucleoside triphosphate regeneration decreases the frequency of translation errors. *Proc. Natl. Acad. Sci. USA* 76, 3174–3178.
- Jensen, R.B., and Gerdes, K. (1995). Programmed cell death in bacteria: proteic plasmid stabilization systems. *Mol. Microbiol.* 17, 205–210.
- Karimi, R., Pavlov, M.Y., Buckingham, R.H., and Ehrenberg, M. (1999). Novel roles for classical factors at the interface between translation termination and initiation. *Mol. Cell* 3, 601–609.
- Karzai, A.W., Roche, E.D., and Sauer, R.T. (2000). The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat. Struct. Biol.* 7, 449–455.
- Keiler, K.C., Waller, P.R., and Sauer, R.T. (1996). Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* 271, 990–993.
- Kisselev, L.L., and Buckingham, R.H. (2000). Translational termination comes of age. *Trends Biochem. Sci.* 25, 561–566.
- Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H., and Kornberg, A. (2001). Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* 293, 705–708.
- Lavallé, R. (1965). Nouveaux mutants de regulation de la synthese de l'ARN. *Bull. Soc. Chim. Biol.* 47, 1567–1570.
- Lavallé, R., Desmarez, L., and deHauwer, G. (1976). Natural messenger translation impairment in an *E. coli* mutant. In *Control of Ribosome Synthesis*, Alfred Benzon symposium IX, N.O. Kjeldgaard, and O. Maaloe, eds. (Copenhagen: Munksgaard), pp. 408–416.
- Mosteller, R.D. (1978). Evidence that glucose starvation-sensitive mutants are altered in the *relB* locus. *J. Bacteriol.* 133, 1034–1037.
- Monro, R.E., Staehelin, T., Celma, M.L., and Vazquez, D. (1969). The peptidyl transferase activity of ribosomes. *Cold Spring Harb. Symp. Quant. Biol.* 34, 357–368.
- Nielsen, K., and Boston, R.S. (2001). RIBOSOME-INACTIVATING PROTEINS: a plant perspective. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 785–816.
- O'Farrell, P.H. (1978). The suppression of defective translation by ppGpp and its role in the stringent response. *Cell* 14, 545–557.
- Ogawa, T., Tomita, K., Ueda, T., Watanabe, K., Uozumi, T., and Masaki, H. (1999). A cytotoxic ribonuclease targeting specific transfer RNA anticodons. *Science* 283, 2097–2100.
- Pavlov, M.Y., Freistroffer, D.V., MacDougall, J., Buckingham, R.H., and Ehrenberg, M. (1997). Fast recycling of *Escherichia coli* ribosomes requires both ribosomal recycling factor (RRF) and release factor RF3. *EMBO J.* 16, 4134–4141.
- Pedersen, K., Christensen, S.K., and Gerdes, K. (2002). Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Mol. Microbiol.* 45, 501–510.
- Peumans, W.J., Hao, Q., and Van Damme, E.J. (2001). Ribosome-inactivating proteins from plants: more than RNA N-glycosidases? *FASEB J.* 15, 1493–1506.
- Riley, M.A., and Gordon, D.M. (1999). The ecological role of bacteriocins in bacterial competition. *Trends Microbiol.* 7, 129–133.
- Soelaiman, S., Jakes, K., Wu, N., Li, C., and Shoham, M. (2001). Crystal structure of colicin E3: implications for cell entry and ribosome inactivation. *Mol. Cell* 8, 1053–1062.
- Somerville, C.R., and Ahmed, A. (1979). Mutants of *Escherichia coli* defective in the degradation of guanosine 5'-triphosphate, 3'-diphosphate (ppGpp). *Mol. Gen. Genet.* 169, 315–323.
- Wool, I.G., Gluck, A., and Endo, Y. (1992). Ribotoxin recognition of ribosomal RNA and a proposal for the mechanism of translocation. *Trends Biochem. Sci.* 17, 266–269.
- Zavialov, A.V., Buckingham, R.H., and Ehrenberg, M. (2001). A post-termination ribosomal complex is the guanine nucleotide exchange factor for peptide release factor RF3. *Cell* 107, 115–124.
- Zavialov, A.V., Mora, L., Buckingham, R.H., and Ehrenberg, M. (2002). Release of peptide promoted by the GGQ motif of class 1 release factors regulates the GTPase activity of RF3. *Mol. Cell* 10, 789–798.