

## ORIGINAL RESEARCH

**YoeB toxin is activated during thermal stress**Brian D. Janssen<sup>1,\*</sup>, Fernando Garza-Sánchez<sup>1,a</sup> & Christopher S. Hayes<sup>1,2</sup><sup>1</sup>Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, Santa Barbara, California<sup>2</sup>Biomolecular Science and Engineering Program, University of California, Santa Barbara, Santa Barbara, California**Keywords**

A-site mRNA cleavage, mRNA turnover, ribosome pausing, RNase II, tmRNA.

**Correspondence**

Christopher S. Hayes, Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, Santa Barbara, CA.

Tel: 805-893-2028;

E-mail: chayes@lifesci.ucsb.edu

**Funding Information**

This work was supported by grant GM078634 (to C. S. H.) from the National Institutes of Health.

Received: 8 May 2015; Accepted: 27 May 2015

**MicrobiologyOpen 2015; 4(4): 682–697**

doi: 10.1002/mbo3.272

**\*Current address:**

Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, California 90095-1489

<sup>a</sup>The two authors contributed equally to this work.**Introduction**

In *Escherichia coli*, prolonged translational arrest often leads to mRNA degradation into the ribosome A site (Hayes and Sauer 2003; Sunohara et al. 2004; Li et al. 2006, 2008; Garza-Sánchez et al. 2008). This A-site mRNA cleavage activity results in truncated A-site codons, which prevent further decoding and produce stalled translation complexes. Such non-productive ribosomes are “rescued” by at least three quality control systems in bacteria (Janssen and Hayes 2012). tmRNA-SmpB mediates the primary ribosome rescue pathway, and this system is found in all eubacteria and some plastids (Tu

**Abstract**

Type II toxin-antitoxin (TA) modules are thought to mediate stress-responses by temporarily suppressing protein synthesis while cells redirect transcription to adapt to environmental change. Here, we show that YoeB, a ribosome-dependent mRNAse toxin, is activated in *Escherichia coli* cells grown at elevated temperatures. YoeB activation is dependent on Lon protease, suggesting that thermal stress promotes increased degradation of the YefM antitoxin. Though YefM is efficiently degraded in response to Lon overproduction, we find that Lon antigen levels do not increase during heat shock, indicating that another mechanism accounts for temperature-induced YefM proteolysis. These observations suggest that YefM/YoeB functions in adaptation to temperature stress. However, this response is distinct from previously described models of TA function. First, YoeB mRNAse activity is maintained over several hours of culture at 42°C, indicating that thermal activation is not transient. Moreover, heat-activated YoeB does not induce growth arrest nor does it suppress global protein synthesis. In fact, *E. coli* cells proliferate more rapidly at elevated temperatures and instantaneously accelerate their growth rate in response to acute heat shock. We propose that heat-activated YoeB may serve a quality control function, facilitating the recycling of stalled translation complexes through ribosome rescue pathways.

et al. 1995; Keiler et al. 1996, 2000; Gueneau de Novoa and Williams 2004). tmRNA is a stable RNA with both transfer-RNA and messenger-RNA functions and recycles stalled ribosomes in a process termed *trans*-translation. During *trans*-translation, tmRNA-SmpB enters the ribosome A site, and the nascent peptide is transferred to alanine-charged tmRNA. The truncated message is then released from the ribosome and translation resumes using a short open reading frame within tmRNA. In this manner, tmRNA provides a stop codon in *trans*, thereby allowing normal translation termination and ribosome recycling (Hayes and Keiler 2010). More recently, two alternative ribosome rescue pathways have been identified.

ArfA (alternative rescue factor A) was discovered in a synthetic lethal screen for genes that are essential for the viability of mutants that lack tmRNA (Chadani *et al.* 2010). ArfA is a small peptide that binds the ribosome and allows release factor-2 to catalyze nascent chain release in the absence of an A-site stop codon (Chadani *et al.* 2012; Shimizu 2012). YaeJ (ArfB) is a release factor homolog that lacks the canonical stop codon recognition domain (Baranov *et al.* 2006; Hayes and Keiler 2010). Thus, YaeJ can bind the ribosome A site in the absence of a stop codon and catalyze nascent peptide release (Chadani *et al.* 2011; Handa *et al.* 2011; Gagnon *et al.* 2012; Feaga *et al.* 2014). Because A-site cleavage produces nonstop transcripts in response to translational pausing, this nuclease activity is thought to function in molecular quality control by facilitating ribosome rescue (Hayes and Sauer 2003; Sunohara *et al.* 2004).

In *E. coli* there are at least two enzymes, RelE and YoeB, which have ribosome-dependent A-site nuclease activity (Pedersen *et al.* 2003; Feng *et al.* 2013). RelE and YoeB are encoded by type II toxin–antitoxin (TA) modules together with cognate antitoxins that specifically neutralize nuclease activity. These mRNases have been termed “toxins” because their activities inhibit cell growth and can lead to cell death under some circumstances. In general, toxins are activated under stress or other conditions that prevent continued antitoxin synthesis. Antitoxins are labile to proteolysis and their degradation liberates the cognate toxins to exert growth inhibition activities. The physiological functions of TA systems remain controversial. They have been proposed to play roles in stress-response, persistence, genomic stability and programmed cell death (Engelberg-Kulka *et al.* 2005; Gerdes *et al.* 2005; Magnuson 2007; Tsilibaris *et al.* 2007; Nariya and Inouye 2008; Maisonneuve *et al.* 2011). Though RelE and YoeB have the potential to catalyze A-site mRNA cleavage during translational arrest, mutants lacking these enzymes and other known toxins retain A-site cleavage activity (Hayes and Sauer 2003; Sunohara *et al.* 2004; Garza-Sánchez *et al.* 2009; Janssen *et al.* 2013). Thus, the identity of the A-site nuclease is unknown, but it remains possible that an unidentified toxin catalyzes cleavage in response to the stress of translational pausing.

Though the A-site nuclease has not been identified, the phenomenon clearly requires the 3′-to-5′ exoribonuclease activity of RNase II (Garza-Sánchez *et al.* 2009; Janssen *et al.* 2013). In  $\Delta rnb$  mutants, which lack RNase II, prolonged translational arrest produces transcripts that are truncated 12 nucleotides downstream of the ribosome A site (Garza-Sánchez *et al.* 2009; Janssen *et al.* 2013). This position corresponds to the “toeprint” of the paused ribosome, suggesting that the ribosome physically blocks further mRNA degradation into the A site. However,

purified RNase II only degrades mRNA to the +18 position (with respect to the A-site codon) when incubated with translation complexes *in vitro* (Garza-Sánchez *et al.* 2009). Moreover, the deeply recessed active site of RNase II is incompatible with the ability to directly catalyze A-site mRNA cleavage (Frazao *et al.* 2006; Zuo *et al.* 2006). Because RNase II is unable to degrade the A-site codon itself, the enzyme must play an indirect role in A-site mRNA cleavage. One possibility is that RNase II-mediated mRNA degradation to the 3′-edge of the ribosome is required for subsequent degradation into the A site by an unknown nuclease. This model is supported by experiments showing that secondary structures placed on the 3′-side of paused ribosomes are sufficient to inhibit RNase II activity and also block A-site cleavage (Garza-Sánchez *et al.* 2009).

We previously reported that A-site mRNA cleavage is suppressed in response to heat shock at 42°C (Garza-Sánchez *et al.* 2009). This observation suggests that A-site nuclease activity is heat labile, and therefore we revisited these experiments in an effort to identify the enzyme responsible. Although acute heat shock temporarily inhibits A-site mRNA cleavage, the experiments presented herein demonstrate that prolonged growth at elevated temperature actually restores the activity. This heat-induced mRNase activity is dependent on the Lon protease, suggesting the activation of one or more TA modules during thermal stress. Taking a candidate-gene approach, we discovered that the *yefM-yoeB* TA operon is required for heat-induced A-site mRNA cleavage activity. Remarkably, YoeB mRNase activity remains constant over several hours of culture at 42°C. Cells proliferate rapidly under these conditions, demonstrating that growth arrest is not necessarily concomitant with toxin activation. Moreover, global protein synthesis is not suppressed by the active toxin. Together, these findings show that type II toxins can be activated at low levels in response to environmental stress. We propose that YoeB facilitates the recycling of stalled translation complexes, thereby playing a role in molecular quality control during thermal stress.

## Materials and Methods

### Bacterial strains and growth conditions

All bacterial strains were derived from *E. coli* X90 (DE3) and are listed in Table 1. The  $\Delta clpB::kan$ ,  $\Delta htpG::kan$ ,  $\Delta lon::kan$ , and  $\Delta clpP::kan$  alleles were obtained from the Keio collection (Baba *et al.* 2006) and were transferred between strains using bacteriophage P1-mediated transduction (Moore 2011). The  $\Delta dnaK::kan$  disruption was generated as described (Hayes and Sauer 2003). Briefly, a

**Table 1.** Bacterial strains and plasmids.

Strain or plasmid	Description	Reference
<b>Strains</b>		
X90	F' <i>lac<sup>R</sup> lac' pro/lara Δ[lac-pro] nalA argE[am] rif<sup>R</sup> thi-1</i>	Beckwith and Signer (1966)
CH12	X90 (DE3)	Hayes et al. (2002a)
CH113	X90 (DE3) <i>ssrA::cat</i> , Cm <sup>R</sup>	Hayes et al. (2002a)
CH165	X90 Δ <i>ssrA</i>	This study
CH950	X90 (DE3) Δ <i>lon::kan</i> , Kan <sup>R</sup>	This study
CH951	X90 (DE3) <i>ssrA::cat</i> Δ <i>lon::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH972	X90 (DE3) <i>ssrA::cat</i> Δ <i>relBE::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	Hayes and Sauer (2003)
CH1019	X90 (DE3) <i>ssrA::cat</i> Δ <i>yefM-yoeB::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	Hayes and Sauer (2003)
CH1023	X90 (DE3) <i>ssrA::cat</i> Δ <i>dinJ-yafQ::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	Hayes and Sauer (2003)
CH1129	X90 Δ <i>yefM-yoeB::kan</i> , Kan <sup>R</sup>	Hayes and Sauer (2003)
CH1207	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	Garza-Sánchez et al. (2009)
CH3550	X90 (DE3) <i>ssrA::cat</i> Δ <i>relBE</i> Δ <i>chpB</i> Δ <i>yefM-yoeB</i> Δ <i>mazEF</i> Δ <i>dinJ-yafQ</i> Δ <i>yhaV</i> , Cm <sup>R</sup>	Garza-Sánchez et al. (2009)
CH4646	X90 Δ <i>rnb</i> <i>ssrA::cat</i> , Cm <sup>R</sup>	This study
CH5820	X90 Δ <i>rnb</i>	This study
CH6157	X90 (DE3) Δ <i>ssrA</i> Δ <i>clpPX-lon::cat</i> , Cm <sup>R</sup>	This study
CH6158	X90 (DE3) Δ <i>ssrA</i> Δ <i>clpPX-lon::cat</i> Δ <i>rnb::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH6262	X90 (DE3) <i>ssrA::cat</i> Δ <i>clpP::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH6595	X90 (DE3) <i>ssrA::cat</i> Δ <i>relBE</i> Δ <i>chpB</i> Δ <i>yefM-yoeB</i> Δ <i>mazEF</i> Δ <i>dinJ-yafQ</i> Δ <i>yhaV</i> Δ <i>rnb::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH6608	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>clpP::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH6609	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>lon::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7212	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>relBE::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7213	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>yefM-yoeB::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7214	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>dinJ-yafQ::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7215	X90 (DE3) <i>ssrA::cat</i> Δ <i>mazEF::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	Hayes and Sauer (2003)
CH7216	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>mazEF::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7217	X90 (DE3) <i>ssrA::cat</i> Δ <i>chpB</i> Δ <i>rnb::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	Hayes and Sauer (2003)
CH7218	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>chpB</i> Δ <i>rnb::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7219	X90 (DE3) <i>ssrA::cat</i> Δ <i>yhaV::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7220	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>yhaV::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7360	X90 Δ <i>ssrA</i> Δ <i>yefM-yoeB::kan</i> , Kan <sup>R</sup>	This study
CH7361	X90 Δ <i>ssrA</i> Δ <i>lon::kan</i> , Kan <sup>R</sup>	This study
CH7362	X90 (DE3) Δ <i>yefM-yoeB::kan</i> , Kan <sup>R</sup>	This study
CH7440	X90 Δ <i>rnb</i> <i>ssrA::cat</i> Δ <i>yefM-yoeB::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH7442	X90 Δ <i>rnb</i> <i>ssrA::cat</i> Δ <i>lon::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH12151	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>dnaK::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH12301	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>htpG::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH12314	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>clpB::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH12402	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>yefM-yoeB</i> Δ <i>dnaK::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
CH12403	X90 (DE3) <i>ssrA::cat</i> Δ <i>rnb</i> Δ <i>lon</i> Δ <i>dnaK::kan</i> , Cm <sup>R</sup> Kan <sup>R</sup>	This study
<b>Plasmids</b>		
pSIM6	Temperature-induced expression of phage λ Red recombinase proteins, Amp <sup>R</sup>	Datta et al. (2006)
pCP20	Temperature-induced expression of FLP recombinase, Amp <sup>R</sup> Cm <sup>R</sup>	Cherepanov and Wackernagel (1995)
pCH450	pACY184 derivative carrying arabinose-inducible <i>araBAD</i> promoter and <i>araC</i> , Tet <sup>R</sup>	Hayes and Sauer (2003)
pKAN	pBluescript SK+ with FRT-flanked kanamycin-resistant cassette, Amp <sup>R</sup> , Kan <sup>R</sup>	Hayes et al. (2002a)
pKAN-dnaK	Construct for deletion of <i>Escherichia coli</i> <i>dnaK</i> , Amp <sup>R</sup> Kan <sup>R</sup>	This study
pFLAG-(m)YbeL-PP	Expresses FLAG epitope fused to the C-terminal 49 residues of YbeL(E159P), Amp <sup>R</sup>	Janssen and Hayes (2009)
pFLAG-(m)YbeL(E28Am)-PP	Variant of FLAG-(m)YbeL-PP with amber termination codon at Glu28, Amp <sup>R</sup>	This study
pCH450- <i>lon</i>	Arabinose-inducible expression of <i>lon</i> , Tet <sup>R</sup>	This study

**Table 1.** (Continued).

Strain or plasmid	Description	Reference
pCH450- <i>lon</i> (S679A)	Arabinose-inducible expression of catalytically inactive <i>lon</i> , Tet <sup>R</sup>	This study
pCH450- <i>yoeB</i>	Arabinose-inducible expression of <i>yoeB</i> , Tet <sup>R</sup>	This study
pCH450- <i>yefM</i>	Arabinose-inducible expression of <i>yefM</i> , Tet <sup>R</sup>	This study
pCH450- <i>yefM-yoeB</i>	Arabinose-inducible expression of <i>yefM-yoeB</i> operon, Tet <sup>R</sup>	This study
pCH410- <i>relB</i>	Arabinose-inducible expression of <i>relB</i> , Tet <sup>R</sup>	This study
pCH450- <i>rpoH</i>	Arabinose-inducible expression of $\sigma^{32}$ heat-shock transcription factor, Tet <sup>R</sup>	This study

Amp<sup>R</sup>, ampicillin resistant; Cm<sup>R</sup>, chloramphenicol resistant; Kan<sup>R</sup>, kanamycin resistant; Tet<sup>R</sup>, tetracycline resistant.

region upstream of *dnaK* was amplified with primers (restriction sites are underlined) *dnaK*-Sac (5'-GAT GAG CTC CCA CTA GTT TAC TGC TGA TAA AGA G) and *dnaK*-Bam (5'-AAC GGA TCC ACT ATA TAT TCG GTC ATC ATG TGG); and a downstream region with *dnaK*-Eco (5'-GCT GAA TTC GAA GAA GTC AAA GAC AAA AAA TAA TCG) and *dnaK*-Kpn (5'-AAC GGT ACC AAA AAT ATC GCT GAA GTC TGC GCC). The two polymerase chain reaction (PCR) products were sequentially ligated to plasmid pKAN to generate pKAN-*dnaK*. Plasmid pKAN-*dnaK* was digested with SacI/KpnI and the small fragment was used to delete the *dnaK* gene by Red-mediated recombination (Thomason et al. 2014). Kanamycin-resistant cassettes were removed with flippase (FLP) recombinase to allow the construction of strains carrying multiple gene deletions (Cherepanov and Wackernagel 1995). All mutations were confirmed by locus-specific PCR amplification.

*Escherichia coli* cells were grown in lysogeny broth (LB) media supplemented with the appropriate antibiotics (ampicillin, 150  $\mu\text{g}/\text{mL}$ ; chloramphenicol, 66  $\mu\text{g}/\text{mL}$ ; kanamycin, 50  $\mu\text{g}/\text{mL}$ ; and tetracycline 25  $\mu\text{g}/\text{mL}$ ). Cells from overnight cultures were resuspended at optical density (OD<sub>600</sub>) ~0.05 in fresh LB supplemented with the appropriate antibiotics. Cells were grown to mid-log phase (at the indicated temperatures) with shaking, then *flag-(m)ybeL-PP* expression was induced with isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at a final concentration of 1.5 mmol/L. After 30 min, the induced cultures were poured into an equal volume of ice-cold methanol to arrest growth. The effects of *Lon*, *YefM*, *YoeB*, *RelB*, and  $\sigma^{32}$  on mRNA processing were determined by expressing the corresponding genes from a plasmid-borne P<sub>BAD</sub> promoter (Hayes and Sauer 2003). Cells were grown to mid-log phase at the indicated temperatures and *flag-(m)ybeL-PP* induced with 1.5 mmol/L IPTG. After 30 min, L-arabinose was added to 0.4% and incubation continued for an additional 15 min. Cultures were then poured into an equal volume of ice-cold methanol. Cells were harvested by centrifugation and the cell pellets frozen at  $-80^{\circ}\text{C}$  for subsequent RNA isolation. The growth-rate response to acute

heat shock was determined in LB medium without antibiotics. Cells were grown for 2.5 h at  $30^{\circ}\text{C}$  in an environmental shaker. Cultures were then split in two, with one culture maintained at  $30^{\circ}\text{C}$  while the other was transferred to a shaking-water bath equilibrated at  $42^{\circ}\text{C}$ . The growth of each culture was monitored during the acute heat shock and for an additional 4.5 h. Long-term growth at various temperatures was performed on LB agar without antibiotics. Cells were grown to mid-log phase in shaking LB medium at  $30^{\circ}\text{C}$ . Culture density was adjusted to OD<sub>600</sub> = 1.0, then subjected to 10-fold serial dilutions in LB medium. Samples (2  $\mu\text{L}$ ) from each dilution were spotted onto LB agar and incubated at the indicated temperatures for 15 h.

### Plasmid constructs

Plasmid pFLAG-(m)YbeL-PP has been described previously (Janssen and Hayes 2009; Seidman et al. 2011). An amber stop codon was introduced at codon 28 of pFLAG-(m)YbeL-PP using the megaprimer PCR approach (Aiyar and Leis 1993). Plasmid pFLAG-(m)-YbeL-PP was first amplified with primers Glu28Amb (5'-GGC TGG GAA ATC TGG TCT GCT AGA AAT GTC ACT TCC ATC TCC) and pET-Eco (5'-CGT CTT CAA GAA TTC TCA TGT TTG ACA GC). The resulting product was used as a megaprimer with pET-Sph/Pst (5'-CAA GGA ATG GTG CAT GCC TGC AGA TGG CGC CC) to amplify the *flag-(m)ybeL-PP* coding sequence and T7 promoter. The final product was digested with SphI/EcoRI and ligated to plasmid pET11d. The *relB*, *rpoH*, *yefM*, and *yoeB* genes were all amplified from *E. coli* genomic DNA and ligated to plasmids pCH410 or pCH450 to generate L-arabinose-inducible expression constructs. The *relB* gene was amplified with *relB*-Nde (5' - GAG GTG TAA CAT ATG GGT AGC ATT AAC CTG CG) and *relB*-Sac-rev (5'-AAT GAG CTC TCA GAG TTC ATC CAG CGT CAC ACG), digested with NdeI/SacI and ligated to plasmid pCH410 (Hayes and Sauer 2003). The *rpoH* gene was amplified with *rpoH*-Eco (5'-ATA GAA TTC AAG GAG ATA TCA TAT GAC TGA CAA AAT GCA AAG TTT

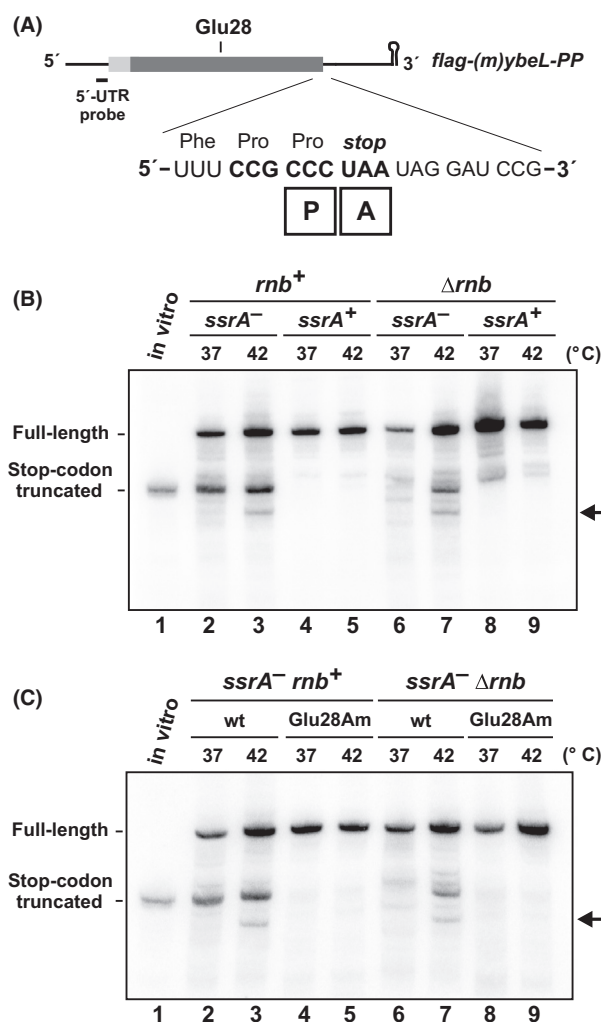
AGC) and rpoH-Xho (5'-TAT CTC GAG AAA TTA CGC TTC AAT GGC AGC), digested with EcoRI/XhoI and ligated to plasmid pCH450. The *yefM* gene was amplified with *yefM*-Eco (5'-TTT GAA TTC CAT ATG AAC TGT ACA AAA GAG G) and *yefM*-Sac (5'-TGA GAG CTC AGA CCA GAT TAG TTT CAC TCA ATG ATG), digested with EcoRI/SacI and ligated to pCH450. The *yoeB* gene was amplified with *yoeB*-Eco (5'-GGA GAA TTC CAT ATG AAA CTA ATC TGG TCT GAG G) and *yoeB*-Sac (5'-ATA GAG CTC CGC TAG CGT ATC AAA ACT GAC AAT TC), digested with EcoRI/SacI and ligated to pCH450. The *yefM-yoeB* operon was amplified with primers *yefM*-Eco and *yoeB*-Sac, digested with EcoRI/SacI and ligated to pCH450. The wild-type and Ser679Ala alleles of *lon* were excised from plasmids pBAD33::*lon* and pBAD33::*lon*(S679A) (Gur and Sauer 2008) by SacI/SbfI digestion, and the fragments ligated to SacI/PstI-digested plasmid pCH450.

### RNA isolation and analysis

Total RNA was isolated from frozen *E. coli* cell pellets using guanidinium isothiocyanate-phenol extraction as described (Garza-Sánchez et al. 2006). RNA was quantified by absorbance at 260 nm and 10 µg run on Tris-borate-ethylenediamine tetraacetic acid, 10% polyacrylamide gels containing 50% urea. Gels were electroblotted onto Nytran Supercharge nylon membranes and subjected to northern blot hybridization using oligonucleotide probes as described (Hayes and Sauer 2003; Garza-Sánchez et al. 2006). Radiolabeled oligonucleotide T7-SD probe (5'-GTA TAT CTC CTT CTT AAA GTT AAA C) was used as a probe to detect *flag-(m)ybeL-PP* transcript. Endogenous transcripts were detected with radiolabeled oligonucleotides: *lpp* 5'-probe (5'-CAT TAT TAA TAC CCT CTA GAT TGA G); *lpp* 3'-probe (5'-CTT GCG GTA TTT AGT AGC CAT G); *ompA* 5'-probe (5'-CAT TTT TTG CGC CTC GTT ATC ATC); *grpE* 5'-probe (5'-CAT GAA TTT CTC CGC GTT TTT TTC G); and *ibpB* 5'-probe (5'-CAT AGT CAT TTC TCC TTC TAA GAA GC). All northern blots were imaged with an FX phosphorimager using the Quantity One software package (Bio-Rad, Hercules, CA USA).

### Immunoblot analysis

Protein was extracted from frozen cells using freeze-thaw in urea lysis buffer (50% urea, 10 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl) and lysates clarified by centrifugation at 15,000 × g for 15 min (Hayes et al. 2002b). Protein was quantified by the Bradford method and equal amounts of total urea-soluble protein were run on Tris-tricine sodium dodecyl sulfate (SDS) 10% polyacrylamide gels. Immunoblot analysis was performed as described



**Figure 1.** A-site nuclease activity is induced at elevated temperature. (A) The *flag-(m)ybeL-PP* reporter transcript is presented schematically. The sequence expansion depicts the P- and A-site codons during translation termination, and the position of the glutamate-28 codon is indicated. The 5'-UTR northern blot probe hybridizes immediately upstream of the start codon. (B) Northern blot analysis of A-site mRNA cleavage. *flag-(m)ybeL-PP* transcripts were expressed in cells of the indicated genotype at 37°C and 42°C, and total RNA was analyzed by northern hybridization. (C) Northern blot analysis of *flag-(m)ybeL-PP* transcripts carrying the Glu28 amber mutation (Glu28Am). Wild-type (wt) and Glu28Am transcripts were expressed in the indicated genetic backgrounds at 37°C and 42°C and analyzed by northern hybridization. The migration positions of stop codon truncated messages in (B) and (C) are indicated by control transcripts prepared by *in vitro* transcription. The horizontal arrows in (B) and (C) indicate an additional truncated transcript that is produced during growth at 42°C.

(Janssen and Hayes 2009), and blots were imaged using a Odyssey® infrared imager (LI-COR, Lincoln, NE, USA). Lon antigen was detected with rabbit polyclonal antisera and IRDye® 680 (LI-COR, Lincoln, NE, USA) labeled anti-rabbit secondary antibodies.

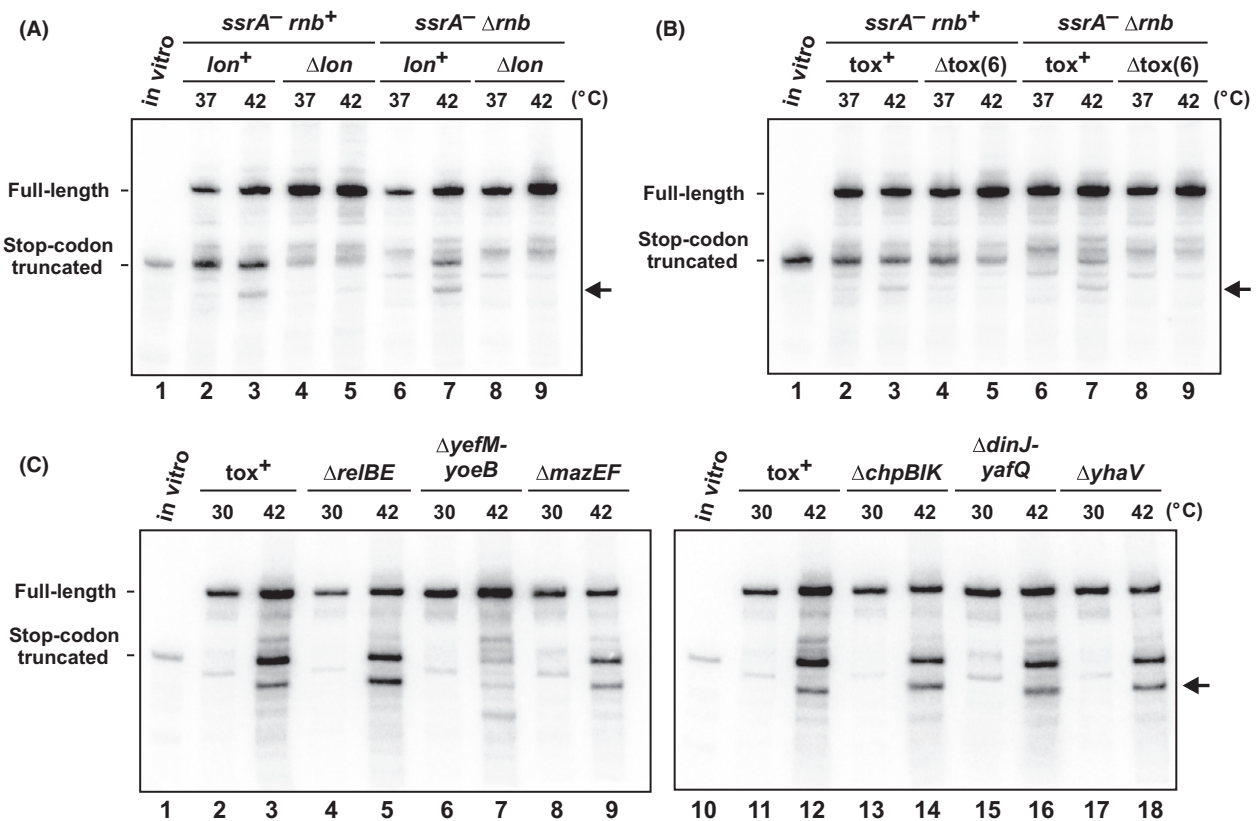


## Results

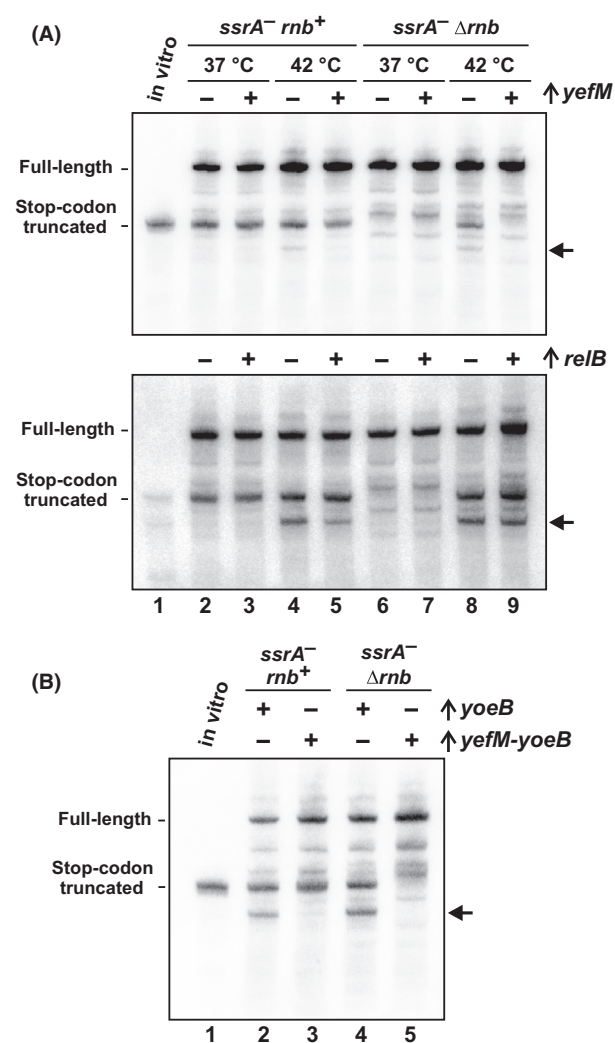
### A-site nuclease activity is induced at elevated temperature

We previously reported that A-site mRNA cleavage is suppressed during heat shock (Garza-Sánchez et al. 2009), suggesting that the A-site nuclease is thermolabile. Alternatively, abrupt increases in temperature could disrupt protein synthesis temporarily and therefore indirectly affect A-site cleavage. To test this possibility, we revisited these experiments using the previously described *flag-(m)ybeL-PP* transcript as a reporter of A-site mRNA cleavage (Fig. 1A) (Garza-Sánchez et al. 2009; Janssen and Hayes 2009). This transcript encodes a C-terminal Pro-Pro peptide motif, which interferes with translation termination and induces cleavage at the A-site stop codon (Mottagui-Tabar et al. 1994; Bjornsson

et al. 1996; Hayes et al. 2002a; Hayes and Sauer 2003; Garza-Sánchez et al. 2008). A substantial proportion of *flag-(m)ybeL-PP* transcripts is truncated in the stop codon when expressed in *ssrA*<sup>-</sup> mutants, which lack tmRNA, but not in *ssrA*<sup>+</sup> cells (Fig. 1B, compare lanes 2 and 4). Presumably, mRNA processing also occurs in wild-type cells, but the truncated messages are rapidly degraded once released from the stalled ribosome through tmRNA activity (Hayes and Sauer 2003; Yamamoto et al. 2003). As reported (Garza-Sánchez et al. 2009), RNase II (encoded by the *rnb* gene) is required for this activity because truncated transcripts were not detected in *ssrA*<sup>-</sup>  $\Delta$ *rnb* cells (Fig. 1B, lane 6). We next examined RNA isolated from cells that had been grown at 42°C for 2 h, and unexpectedly found that A-site mRNA cleavage was not suppressed (Fig. 1B, lane 3). In fact, growth at 42°C actually restored A-site cleavage activity to *ssrA*<sup>-</sup>  $\Delta$ *rnb* cells (Fig. 1B, compare lanes 6



**Figure 2.** Lon and YoeB are required for temperature-induced A-site mRNA cleavage. (A) *flag-(m)ybeL-PP* transcripts were expressed in the indicated genetic backgrounds at 37°C and 42°C and analyzed by northern hybridization. (B) *flag-(m)ybeL-PP* transcripts were expressed in cells that lack six characterized toxin-antitoxin modules ( $\Delta$ *tox*(6)) at 37°C and 42°C, and compared to background that retain these toxin-antitoxin genes (*tox*<sup>+</sup>). (C) *flag-(m)ybeL-PP* transcripts were expressed in *ssrA*<sup>-</sup>  $\Delta$ *rnb* cells that carry deletions in the indicated toxin-antitoxin genes. Growth at 42°C induces truncated mRNA in all cells except those deleted for *yefM-yoeB*. The migration positions of stop codon truncated messages are indicated by control transcripts prepared by in vitro transcription. The horizontal arrows indicate an additional truncated transcript that is produced during growth at 42°C.



**Figure 3.** Overexpression of *yefM* suppresses temperature-induced A-site mRNA cleavage. (A) *flag-(m)ybeL-PP* transcripts were expressed in *ssrA*<sup>-</sup> *rnb*<sup>+</sup> and *ssrA*<sup>-</sup>  $\Delta$ *rnb* backgrounds at 37°C and 42°C. Where indicated (+), the *yefM* or *relB* antitoxin genes were overexpressed from a plasmid-borne arabinose-inducible promoter. (B) *flag-(m)ybeL-PP* transcripts were expressed in *ssrA*<sup>-</sup> *rnb*<sup>+</sup> and *ssrA*<sup>-</sup>  $\Delta$ *rnb* backgrounds at 37°C. Where indicated (+), the *yoeB* or *yefM-yoeB* genes were overexpressed from plasmid-borne arabinose-inducible promoters. The migration positions of stop codon truncated messages are indicated by control transcripts prepared by in vitro transcription. The horizontal arrows indicate an additional truncated transcript that is produced during growth at 42°C (A) or *yoeB* induction without *yefM* (B).

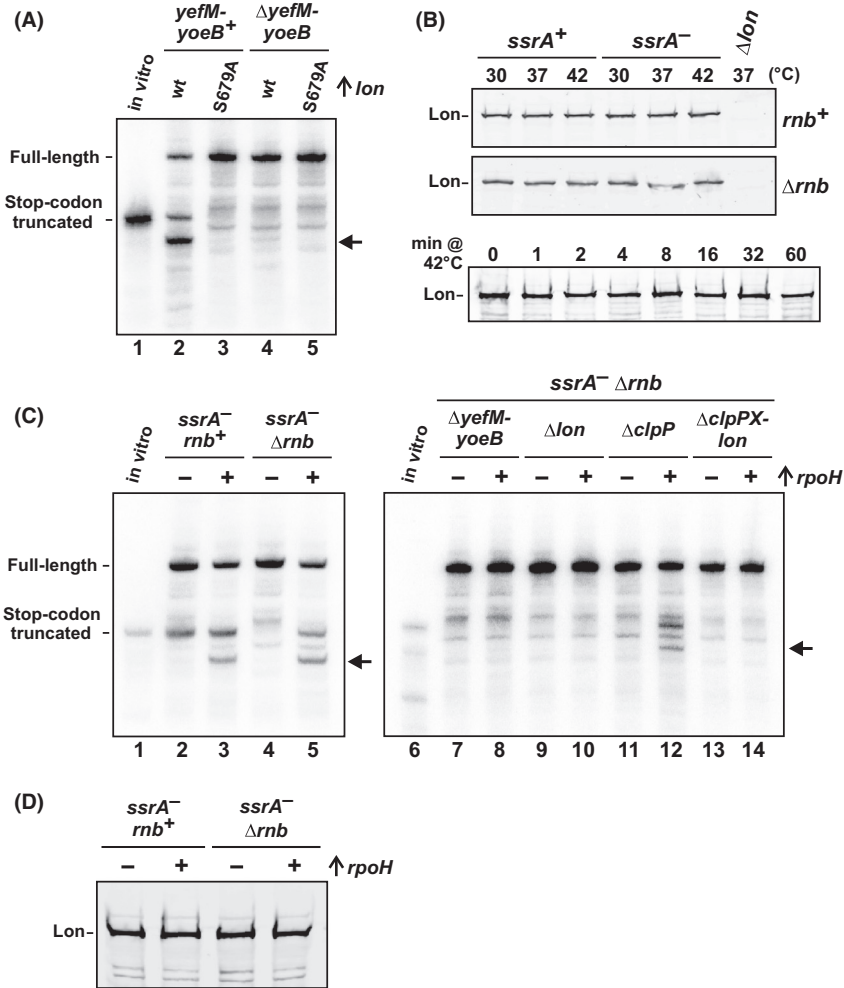
and 7). Another smaller truncated transcript was also detected in *ssrA*<sup>-</sup> cells grown at 42°C (Fig. 1B, marked by an arrow in lanes 3 and 7). These observations suggest that a new RNase activity is induced during growth at 42°C. Because tmRNA promotes nonstop mRNA turnover during ribosome rescue (Yamamoto et al. 2003; Richards et al. 2006; Ge et al. 2010), the apparent

absence of truncated mRNA in *ssrA*<sup>+</sup> cells suggests that cleavage may occur during translation. We tested this hypothesis by introducing an amber stop codon at position Glu28 in the *flag-(m)ybeL-PP* coding sequence (Fig. 1A). Thus, ribosomes terminate translation at codon 28 in the Glu28Am transcript, rather than the original ochre stop at codon 61. Truncated Glu28Am transcripts were not detected in any of the examined backgrounds (Fig. 1C), indicating that translation to the tandem Pro codons is required for the heat-induced mRNA activity.

### YoeB mediates A-site mRNA cleavage at elevated temperature

Some type II TA modules encode ribosome-dependent RNases that cleave A-site codons (Pedersen et al. 2003; Prysak et al. 2009; Feng et al. 2013). Toxins are typically activated through Lon protease-mediated degradation of antitoxins (Gerdes and Maisonneuve 2012; Brzozowska and Zielenkiewicz 2013); therefore we examined *flag-(m)ybeL-PP* transcript processing in  $\Delta$ *lon* cells. We observed less truncated mRNA in  $\Delta$ *lon* compared to *lon*<sup>+</sup> backgrounds even at 37°C (Fig. 2A, lanes 2 and 4), indicating that Lon influences A-site cleavage during translational pauses. But more importantly, we failed to detect heat-induced mRNA activity in  $\Delta$ *lon* mutants (Fig. 2A, lanes 7 and 9). The latter result suggests that a toxin is responsible for heat-induced mRNA activity, which led us to test an *E. coli* strain that lacks multiple TA genes. The *E. coli*  $\Delta$ *tox(6)* strain lacks six validated TA systems encoded by the *relBE*, *mazEF*, *yefM-yoeB*, *dinJ-yafQ*, *chp-BIK* and *yhaV* genes. As reported previously (Garza-Sánchez et al. 2009), *flag-(m)ybeL-PP* transcripts still undergo A-site cleavage in the *ssrA*<sup>-</sup>  $\Delta$ *tox(6)* background at 37°C (Fig. 2B, lanes 2 and 4). However, *ssrA*<sup>-</sup>  $\Delta$ *tox(6)*  $\Delta$ *rnb* cells did not exhibit heat-induced mRNA activity (Fig. 2B, lanes 7 and 9), strongly suggesting that one (or more) of the deleted toxins is responsible for activity. Further analysis of *ssrA*<sup>-</sup>  $\Delta$ *rnb* strains carrying individual TA gene deletions revealed that  $\Delta$ *yefM-yoeB* mutants lack the heat-induced activity (Fig. 2C).

The YefM antitoxin specifically binds to YoeB toxin and neutralizes its RNase activity (Cherny et al. 2005; Kamada and Hanaoka 2005; Feng et al. 2013). Therefore, if heat-induced transcript cleavage is mediated by YoeB, then the activity should be specifically blocked by *yefM* overexpression. We cloned *yefM* under control of the P<sub>BAD</sub> promoter and induced expression in cells that coexpress *flag-(m)ybeL-PP*. In cells grown at 37°C, *yefM* expression had no discernible effect on mRNA cleavage (Fig. 3A, lanes 2, 3, 6, and 7). However, *yefM* expression suppressed mRNA cleavage in *ssrA*<sup>-</sup>  $\Delta$ *rnb* cells at 42°C



**Figure 4.** Overexpression of *lon* and *rpoH* induces A-site mRNA cleavage. (A) Overexpression of *lon* induces A-site mRNA cleavage. *flag-(m)ybeL-PP* transcripts were expressed in *ssrA<sup>-</sup> Δrnb* backgrounds at 37°C. Where indicated, the *lon* or *lon*(S679A) genes were overexpressed from a plasmid-borne arabinose-inducible promoter. (B) Lon immunoblot analysis. Urea-soluble protein was isolated from cells of the indicated genotype that had been cultured at 30°C, 37°C or 42°C for 2.5 h. The bottom panel shows Lon levels in *ssrA<sup>-</sup> Δrnb* cells that had been cultured at 30°C for 1.5 h, then shifted to 42°C for the indicated number of minutes. (C) Overexpression of *rpoH* induces A-site mRNA cleavage. *flag-(m)ybeL-PP* transcripts were expressed in the indicated genetic backgrounds at 37°C. Where indicated (+), *rpoH* was overexpressed from a plasmid-borne arabinose-inducible promoter. In (A and C), the migration position of *flag-(m)ybeL-PP* transcript that is truncated at the stop codon is indicated, and horizontal arrows indicate an additional *yoeB*-dependent transcript. (D) Immunoblot analysis of Lon. Urea-soluble protein was isolated from cells of the indicated genotype that had been grown at 37°C. Where indicated (+), the  $\sigma^{32}$  heat-shock transcription factor (*rpoH*) was overexpressed. Samples were analyzed by immunoblot using polyclonal antisera to Lon protease.

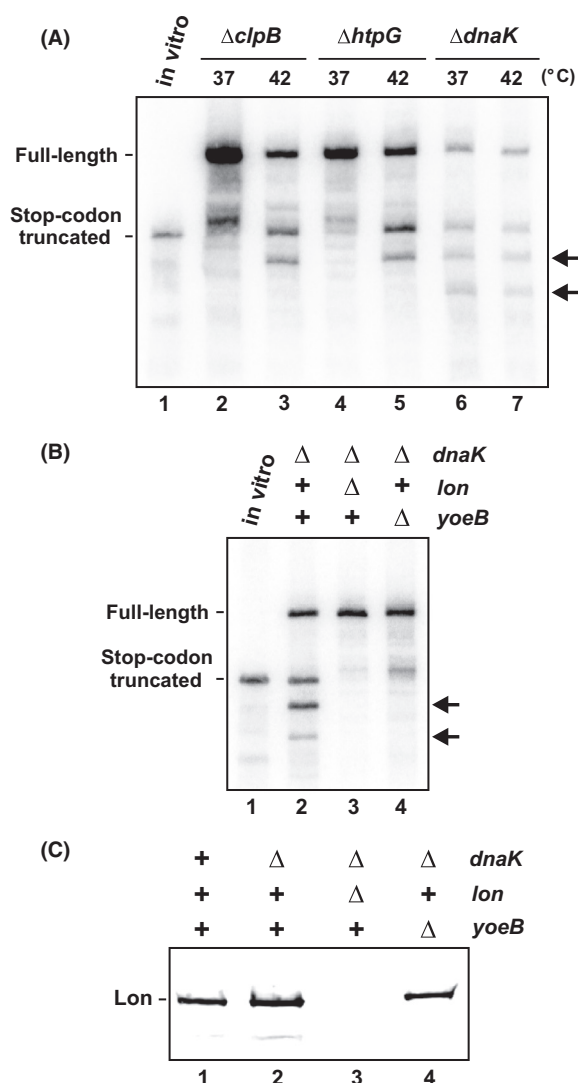
(Fig. 3A, lane 8 and 9). This suppressive effect was specific because induction of *relB*, which encodes the anti-toxin for RelE toxin, had little effect on heat-induced mRNA activity (Fig. 3A, bottom blot). We then expressed *yoeB* from a plasmid-borne P<sub>BAD</sub> promoter to determine whether the toxin cleaves *flag-(m)ybeL-PP* transcripts. The two major truncated species that accumulate during thermal stress were also produced in response to *yoeB* induction at 37°C (Fig. 3B, lanes 2 and 4). Thus, the heat-induced mRNA activity can be recapitulated by *yoeB* expression at lower temperature. In contrast, expression

of the entire *yefM-yoeB* operon from the same plasmid vector did not induce mRNA cleavage (Fig. 3B, lanes 3 and 5). Taken together, these data indicate that YoeB is responsible for the heat-induced mRNA activity.

### YoeB is activated during heat-shock stress

Christensen et al. 2004 have shown that overproduced Lon inhibits cell growth largely by activating YoeB. These findings indicate that YefM is particularly susceptible to proteolysis and suggest that thermal activation may be



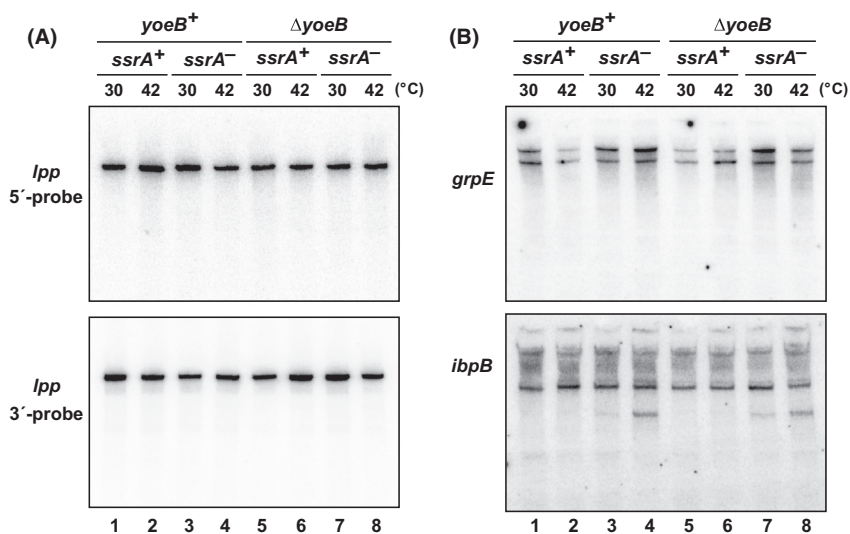


**Figure 5.** YoeB is activated in  $\Delta dnaK$  mutants. (A) Deletion of *dnaK* induces A-site mRNA cleavage activity. *flag-(m)ybeL-PP* transcripts were expressed in *ssrA*<sup>-</sup>  $\Delta rnb$  cells that carry additional deletions in *clpB*, *htpG*, or *dnaK*. The  $\Delta clpB$  and  $\Delta htpG$  cells were grown at 37°C or 42°C as indicated. The  $\Delta dnaK$  cells were grown at 37°C then maintained at 37°C or shifted to 42°C for the final 30 min of culture. (B) YoeB is activated in  $\Delta dnaK$  mutants. *flag-(m)ybeL-PP* transcripts were expressed in *ssrA*<sup>-</sup>  $\Delta rnb$  cells carrying additional gene deletions as indicated. All cells were grown at 37°C and transcripts were detected by northern hybridization. In (A and B), the migration positions full-length and truncated *flag-(m)ybeL-PP* mRNA are indicated. The horizontal arrows indicate additional *yoeB*-dependent truncated transcripts. (C) Immunoblot analysis of Lon in  $\Delta dnaK$  backgrounds. Urea-soluble protein was isolated from *ssrA*<sup>-</sup>  $\Delta rnb$  cells carrying additional gene deletions as indicated. All cells were grown at 37°C and Lon antigen was detected using polyclonal antisera.

due to increased Lon levels. To address this possibility, we first confirmed that truncated *flag-(m)ybeL-PP* transcripts accumulate in cells that overexpress *lon* at 37°C

(Fig. 4A, lane 2). This mRNA processing was not observed when *lon* was induced in  $\Delta yefM$ -*yoeB* cells (Fig. 4A, lane 4). We also determined that protease activity is required for this effect, because truncated messages were not detected when catalytically inactive Lon(Ser679-Ala) was overproduced (Fig. 4A, lane 3) (Botos et al. 2004). Having established that increased Lon is sufficient to activate YoeB in our system, we then examined endogenous Lon levels by immunoblot. Given that Lon is considered to be a heat-shock protein (Phillips et al. 1984), we were surprised to find that Lon antigen levels were remarkably constant in cells cultured for 2.5 h at 30°C, 37°C, and 42°C (Fig. 4B). Lon levels were also unchanged regardless of *ssrA* or *rnb* genetic background (Fig. 4B). We further examined whether Lon levels increase transiently in response to temperature up-shift, but observed no significant increase over the first few minutes of heat stress (Fig. 4B, lower blot). These data show that Lon overproduction can activate YoeB, but this mechanism does not account for activation at elevated temperature.

Heat shock activates the transcription of several genes that facilitate adaptation to thermal stress. We reasoned that an additional heat shock factor may collaborate with Lon to degrade YefM and therefore account for activation at high temperature. To test this hypothesis, we induced the heat-shock regulon through ectopic expression of *rpoH*, which encodes the  $\sigma^{32}$  heat-shock transcription factor (Nonaka et al. 2006; Guisbert et al. 2008). Expression of *rpoH* at 37°C induced an mRNAse activity that is indistinguishable from that observed in cells grown at 42°C (Fig. 4C, lanes 3 and 5). This activity was dependent on *lon* and *yefM*-*yoeB*, but not *clpP* (Fig. 4C, lanes 8, 10, 12, and 14). We tested whether Lon increases in response to *rpoH* expression and found that protease levels remained constant (Fig. 4D). Because YoeB is activated at lower temperature through induction of the heat-shock regulon, we tested whether known ATP-dependent chaperones facilitate activation. ClpB, HtpG, and DnaK are all induced during heat shock and have been implicated in the degradation and/or refolding of proteins at high temperature (Sherman and Goldberg 1992; Mogk et al. 1999; Thomas and Baneyx 2000; Huang et al. 2001). Therefore, we examined *flag-(m)ybeL-PP* transcripts expressed in *ssrA*<sup>-</sup>  $\Delta rnb$  cells that carry additional deletions of the *clpB*, *htpG*, or *dnaK* genes. The  $\Delta clpB$  and  $\Delta htpG$  mutations had no effect on transcript processing at either 37°C or 42°C, but the  $\Delta dnaK$  mutant showed increased mRNAse activity even at 37°C (Fig. 5A). In fact, yet another truncated *flag-(m)ybeL-PP* transcript accumulated in *ssrA*<sup>-</sup>  $\Delta rnb$   $\Delta dnaK$  cells (Fig. 5A, indicated by lowest arrow in lanes 6 and 7). This additional truncation product presumably arises from YoeB-mediated cleavage at an



**Figure 6.** Thermal-induced YoeB activity is not detected on endogenous transcripts. (A) Total RNA from cells grown at 30°C and 42°C was analyzed by northern hybridization using oligonucleotide probes to the 5'- and 3'-untranslated regions of *lpp* mRNA in *yoeB*<sup>+</sup> and  $\Delta$ *yefM-yoeB* ( $\Delta$ *yoeB*) backgrounds. (B) The same RNA samples from (A) were analyzed by northern hybridization using probes to the 5'-UTRs of *grpE* and *ibpB* messages.

upstream codon within the *flag-(m)ybeL* message. *dnaK* mutations are known to induce the heat-shock regulon at low temperature (Straus et al. 1990), suggesting that YoeB is activated in  $\Delta$ *dnaK* cells by the same mechanism that underlies toxin activation during ectopic *rpoH* expression. In accord with this model, we found that *lon* and *yefM-yoeB* are both required for increased mRNAse activity in the  $\Delta$ *dnaK* background (Fig. 5B, lanes 3 and 4). Additionally, immunoblot analysis showed that Lon protease levels do not increase dramatically in  $\Delta$ *dnaK* mutants (Fig. 5C). Collectively, these results demonstrate that YoeB is activated under three conditions – high temperature, *rpoH* expression and *dnaK* mutation – that induce the heat-shock regulon.

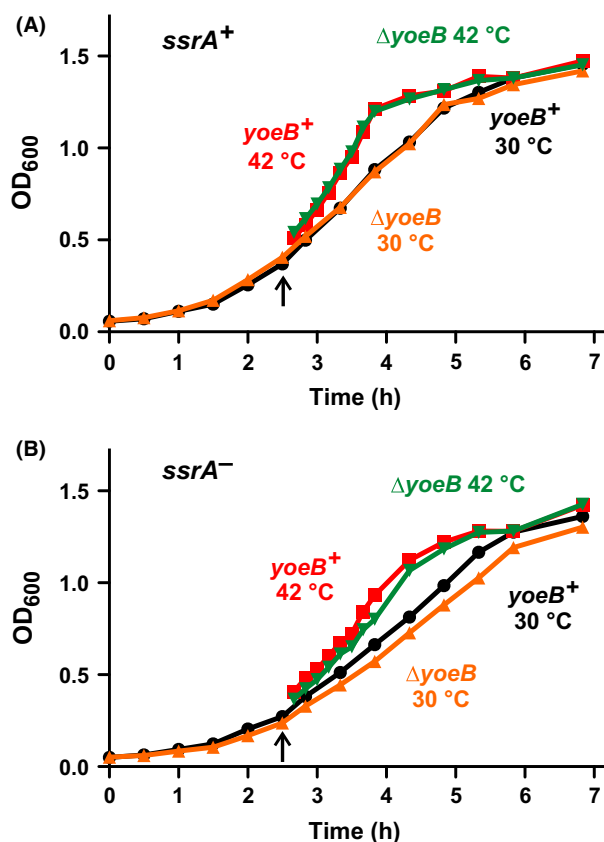
### Thermal activation of YoeB does not inhibit global protein synthesis

Previous work has shown that YoeB cleaves *ompA* and *lpp* transcripts in *E. coli* (Winther and Gerdes 2009; Zhang and Inouye 2009). Therefore, we tested whether YoeB cleaves these endogenous mRNAs in response to elevated temperature. We were unable to detect truncated *lpp* transcripts using probes to the 5'- and 3'-untranslated regions (Fig. 6A). Similarly, the *ompA* transcript was not cleaved during culture at 42°C (data not shown). We also considered the possibility that YoeB may preferentially cleave heat-shock transcripts and provide a mechanism to fine-tune their translation. However, we did not detect *yoeB*-dependent cleavage in *grpE* and *ibpB* transcripts in

response to growth at 42°C (Fig. 6B). Although smaller *ibpB* transcripts were detected at 42°C in *ssrA*<sup>-</sup> cells, these fragments accumulated to similar levels in both *yoeB*<sup>+</sup> and  $\Delta$ *yoeB* backgrounds (Fig. 6B). These data are seemingly at odds with the observation that *flag-(m)ybeL-PP* reporter transcripts are efficiently cleaved under the same growth conditions. We hypothesize that YoeB activity is focused on the reporter transcript due to a combination of overexpression and inefficient translation termination. We have shown previously that the entire pool of release factor-1 (RF-1) is sequestered on paused ribosomes during *ybeL-PP* overexpression (Janssen and Hayes 2009). RF-1 depletion then causes other ribosomes to stall at the *ybeL-PP* termination codon with unoccupied A sites (Janssen and Hayes 2009). Because YoeB must compete with translation factors to gain access to its A-site codon substrate, we propose that these latter ribosomes with unoccupied A sites are preferentially targeted by the nuclease.

### Cell growth is not inhibited during YoeB activation

The stress-response model of TA function postulates that environmental stress activates mRNAse toxins to temporarily inhibit translation while transcription is redirected to stress-response genes (Gerdes et al. 2005). Because YoeB is activated at high temperature, we asked whether acute heat shock leads to temporary growth arrest as predicted by the stress-response model. We monitored the growth of wild-type *yoeB*<sup>+</sup> cells and observed no inhibi-



**Figure 7.** Cell growth is not arrested during heat shock. (A) & (B) *E. coli* cells with the indicated genotypes were grown in shaking LB broth at 30°C for 2.5 h, then shifted to 42°C (indicated by the upward arrow) for continued culture. Cell growth was monitored by optical density at 600 nm ( $OD_{600}$ ).

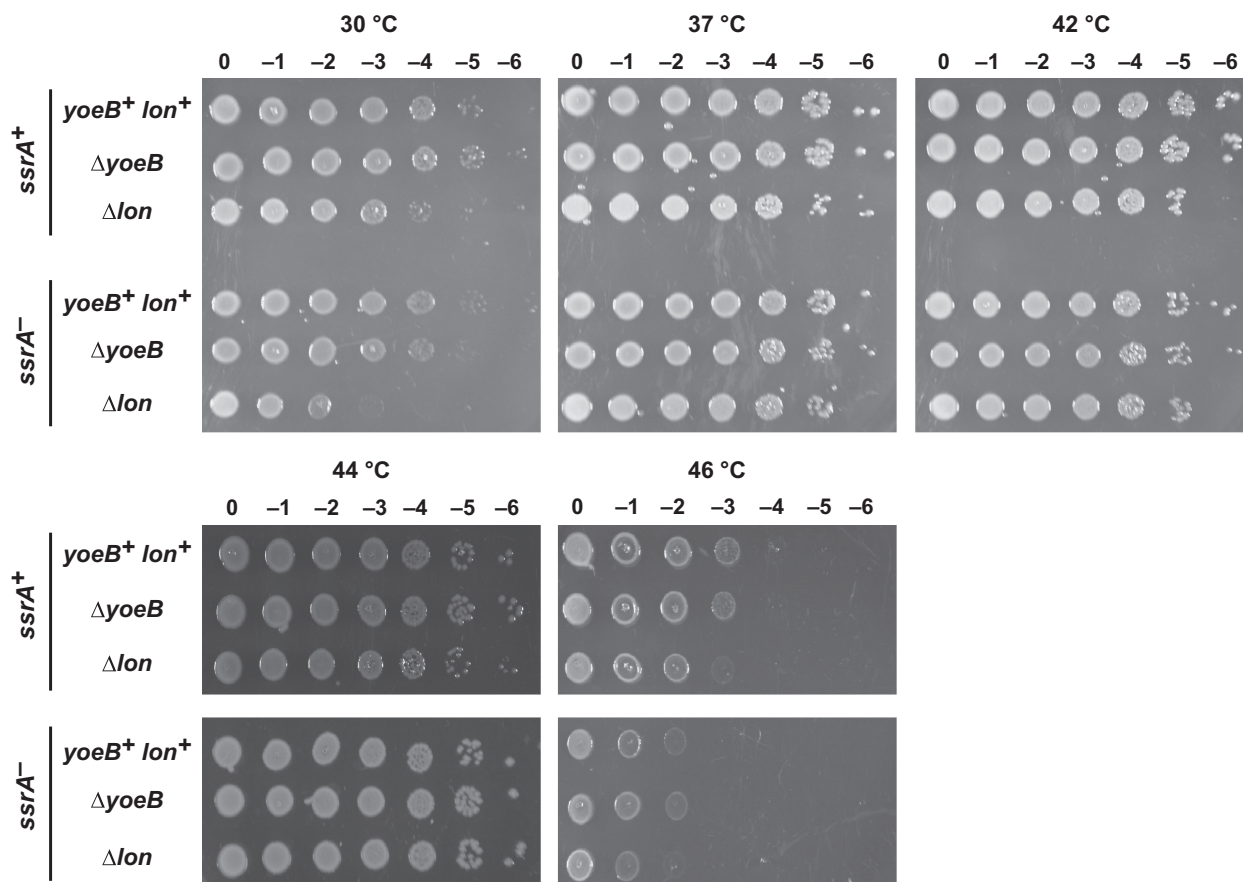
tion of growth during the transition from 30°C to 42°C (Fig. 7A). Moreover,  $\Delta yefM$ -*yoeB* mutants grew along the same trajectory as *yefM*-*yoeB*<sup>+</sup> cells during the heat-shock treatment (Fig. 7A). These results indicate that YoeB activity is not sufficient to arrest growth, consistent with the absence of detectable cleavage within endogenous messages at 42°C. tmRNA facilitates ribosome recycling after YoeB-mediated A-site cleavage, so we also examined the response of *ssrA*<sup>-</sup> cells to heat shock. Although *ssrA*<sup>-</sup> mutants grow more slowly than *ssrA*<sup>+</sup> cells at 30°C and 42°C, the  $\Delta yefM$ -*yoeB* mutation had little effect in this background (Fig. 7B). Because YoeB activity persists over at least 2 h of culture at 42°C, we examined whether the toxin influences cell growth over longer time scales. We spotted *yefM*-*yoeB*<sup>+</sup> and  $\Delta yefM$ -*yoeB* cells onto LB agar for overnight incubation at temperatures ranging from 30°C to 46°C, but observed no differences in cell growth in various *ssrA* and *lon* backgrounds (Fig. 8). In fact, the most prominent growth defects were observed with *ssrA*<sup>-</sup>

cells at 46°C and  $\Delta lon$  cells at 30°C. The *ssrA* mutation reduced cell growth at 46°C about 10-fold in the *yefM*-*yoeB* and *lon* backgrounds (Fig. 8). Additionally,  $\Delta lon$  cells grew more slowly than *lon*<sup>+</sup> cells on solid medium at 30°C, and this effect was even more pronounced in the *ssrA*<sup>-</sup> background (Fig. 8). Together, these results indicate that tmRNA and Lon contribute to fitness at high and low temperatures, but YefM and YoeB do not confer a discernible advantage under these conditions.

## Discussion

The results presented here reveal temperature-induced mRNAse activity in *E. coli* cells. Several observations argue that YoeB is responsible for this nuclease activity. First, transcripts must be actively translated in order to be cleaved. This is in accord with evidence that YoeB binds the ribosome and only cleaves translated messages *in vivo* (Christensen-Dalsgaard and Gerdes 2008; Feng *et al.* 2013). Second, temperature-induced mRNAse activity requires Lon, consistent with the well-established role for this protease in antitoxin degradation (Gerdes *et al.* 2005; Tsilibaris *et al.* 2006; Gerdes and Maisonneuve 2012; Goeders and Van Melderen 2014). Third, expression of *yoeB* at lower temperatures recapitulates heat-induced mRNAse activity; and *yefM* overexpression is sufficient to suppress mRNAse activity at elevated temperature. Finally,  $\Delta yefM$ -*yoeB* mutants do not exhibit heat-induced mRNAse activity. Collectively, these results indicate that growth at elevated temperatures results in Lon-mediated degradation of YefM and concomitant activation of YoeB. These findings reinforce the conclusions of several previous studies showing that environmental stresses activate TA modules. Gerdes and colleagues have shown that RelE, MazF and other *E. coli* toxins become activated in response to amino acid starvation (Christensen *et al.* 2001; Christensen and Gerdes 2003). Additionally, the *E. coli* *dinJ*-*yafQ* operon contains a LexA box in its promoter, and its transcription is de-repressed in response to DNA damage (Pryszak *et al.* 2009). Thus, our data are broadly consistent with a stress-response function and show that temperature stress is yet another environmental stimulus that activates TA modules.

Although YoeB activity becomes manifest at higher temperatures, the mechanism by which YefM is degraded preferentially over other antitoxins is not clear. Van Melderen and colleagues reported that gratuitous overproduction of Lon is sufficient to activate YoeB, but other known *E. coli* toxins were activated to a much lesser degree (Christensen *et al.* 2004). Similarly, the data presented here suggest that YoeB is the only toxin activated in response to elevated temperature. Together, these observations indicate that YefM is particularly sensitive to



**Figure 8.** YoeB confers no growth advantage at elevated temperatures. *Escherichia coli* X90 cells of the indicated genetic backgrounds were adjusted to  $OD_{600} = 1.0$ , then serially diluted in LB medium and spotted onto LB agar for overnight growth at the indicated temperatures.

Lon activity. Although Lon is considered a heat-shock protein and *lon* transcription is upregulated during heat shock (Goff et al. 1984; Phillips et al. 1984), we find that Lon levels are essentially the same in cells grown at different temperatures. This argues that increased Lon concentration cannot account for thermal activation. It is also possible that YefM and YoeB dissociate in response to increased temperature, though it appears that YefM-YoeB is no more thermolabile than other TA complexes (Cherny et al. 2005). Moreover, we find that YoeB is activated at 37 °C by inducing the heat-shock regulon through ectopic *rpoH* expression. This latter observation raises the possibility that an additional heat-shock factor may collaborate with Lon to degrade YefM. Indeed, there are reports of ATP-dependent chaperones working in concert with Lon to specifically degrade misfolded and damaged proteins (Sherman and Goldberg 1992; Huang et al. 2001). However, the ClpB, HtpG, and DnaK chaperones are not required for the thermal activation of YoeB. In fact,  $\Delta$ *dnaK* mutants show constitutive activation of YoeB even at low temperature. This finding is consistent

with recent data from the Chien and Laub laboratories showing that *Caulobacter crescentus*  $\Delta$ *dnaK* mutants have increased Lon protease activity (Jonas et al. 2013). They find that unfolded proteins stimulate Lon activity allosterically. Because proteins tend to aggregate and misfold during thermal stress, the findings of Jonas et al. provide an explanation for increased protease activity in the absence of Lon overexpression. Their model may also account for YoeB activation in response to  $\sigma^{32}$  overproduction. DnaK binds to  $\sigma^{32}$  (Gamer et al. 1992; Liberek et al. 1992), and therefore overproduced transcription factor could potentially sequester the chaperone, preventing it from refolding other client proteins. Thus, super-physiological levels of  $\sigma^{32}$  could paradoxically lead to more unfolded proteins and increased Lon activity. Though this model accounts for Lon activity at high temperature, it does not explain why YefM is preferentially degraded while other antitoxins appear resistant to proteolysis. Presumably, YefM carries a unique recognition determinant that allows facile degradation. Given that Lon and YefM/YoeB are distributed widely throughout Gram-negative



and Gram-positive bacteria (Nieto *et al.* 2007; Kumar *et al.* 2008; Yoshizumi *et al.* 2009; Sevillano *et al.* 2012), it will be of interest to determine whether thermal activation is conserved in other species.

Early biochemical studies showed that purified YoeB has intrinsic RNase activity *in vitro* (Kamada and Hanaoka 2005; Christensen-Dalsgaard and Gerdes 2008); but *in vivo*, the toxin only cleaves actively translated messages that are bound to the ribosome (Christensen-Dalsgaard and Gerdes 2008; Zhang and Inouye 2009; Feng *et al.* 2013). YoeB binds to the ribosome A site, where it cleaves the A-site codon to produce a truncated nonstop mRNA (Kamada and Hanaoka 2005; Feng *et al.* 2013). Although YoeB is generally accepted to be a ribosome-dependent A-site nuclease, there are conflicting reports about which stage of translation is affected. Inouye and colleagues have shown that YoeB acts during translation initiation (Yoshizumi *et al.* 2009; Zhang and Inouye 2009), whereas Gerdes and colleagues report YoeB-dependent cleavage in termination codons (Winther and Gerdes 2009). Both studies monitored the same endogenous *lpp* and *ompA* transcripts, but the experimental approaches differed. Zhang & Inouye overproduced YoeB from a plasmid vector, whereas the Gerdes group detected endogenous YoeB activity in response to ectopic VapC toxin overexpression (Winther and Gerdes 2009; Zhang and Inouye 2009). Our data show temperature-induced cleavage at two sites, one in the stop codon and another at an unidentified upstream position. Both cleavages are not observed when codon Glu-28 is mutated to an amber stop, indicating that the upstream cleavage site is well downstream of the start codon. Thus, our results are closer to those of Winther *et al.* and we conclude that endogenous YoeB activated at elevated temperature does not affect translation initiation.

The stress-response model of TA function postulates that activated toxins facilitate changes in gene expression in response to environmental stress (Christensen *et al.* 2003; Gerdes *et al.* 2005). The classic example is RelE activation in response to starvation. In this model, amino acid starvation slows protein synthesis, leading to depletion of RelB antitoxin due to its intrinsic instability. RelE then acts as an A-site nuclease to cleave translated messages and inhibit translation. The resulting stalled ribosomes are recycled by tmRNA-SmpB, which provides a burst of amino acids through proteolysis of *ssrA*-tagged nascent chains (Christensen and Gerdes 2003). The starved cells then alter transcription to express genes for amino acid biosynthesis and a new steady-state is eventually achieved. In principle, YoeB could function in the same manner to accelerate mRNA decay so that new heat-shock transcripts are translated more rapidly. However, we were unable to detect YoeB-dependent cleavages

within endogenous messages. YoeB must compete with translation factors for access to the ribosome A site, and therefore it is more likely to cleave mRNA in the context of arrested ribosomes. Because heat shock does not inhibit global translation, YoeB activity is presumably limited to a small population of ribosomes that encounter difficulties during protein synthesis. In contrast, amino acid starvation stress not only activates RelE toxin, but also blocks protein synthesis to produce stalled ribosome complexes for RelE-mediated mRNA cleavage. Another discrepancy between our results and the stress-response model is the absence of growth arrest upon YoeB activation. In fact, *E. coli* cells show no growth lag in response to temperature up-shift, but rather accelerate their growth rate immediately. Moreover, YoeB activity is detectable over several hours of growth at elevated temperature. These data demonstrate that toxins can be activated at low levels, suggesting that they may actually promote more efficient protein synthesis under stress conditions. Heat shock and temperature stress has been shown to damage translation complexes and disrupt protein synthesis (Korber *et al.* 2000; Jiang *et al.* 2009). In our model, YoeB activity would perform a quality control function to help recycle translation complexes that stall stochastically during thermal stress. This is still adaptation to environmental stress, yet the proposed function is distinct from previously established roles for toxins in growth arrest and persistence (Maisonneuve *et al.* 2011; Gerdes and Maisonneuve 2012; Maisonneuve and Gerdes 2014).

## Acknowledgments

We thank Bob Sauer for providing plasmids pBAD33-*lon* and pBAD33-*lon*(S379A), and Tania Baker for *Lon* antisera. This work was supported by grant GM078634 (to C. S. H.) from the National Institutes of Health.

## Conflict of Interest

None declared.

## References

- Aiyar, A., and J. Leis. 1993. Modification of the megaprimer method of PCR mutagenesis: improved amplification of the final product. *Biotechniques* 14:366–369.
- Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, *et al.* 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2:2006.0008.
- Baranov, P. V., B. Vestergaard, T. Hamelryck, R. F. Gesteland, J. Nyborg, and J. F. Atkins. 2006. Diverse bacterial genomes encode an operon of two genes, one of which is an unusual

- class-I release factor that potentially recognizes atypical mRNA signals other than normal stop codons. *Biol. Direct* 1:28.
- Beckwith, J. R., and E. R. Signer. 1966. Transposition of the *lac* region of *Escherichia coli*. I. Inversion of the *lac* operon and transduction of *lac* by phi80. *J. Mol. Biol.* 19:254–265.
- Bjornsson, A., S. Mottagui-Tabar, and L. A. Isaksson. 1996. Structure of the C-terminal end of the nascent peptide influences translation termination. *EMBO J.* 15:1696–1704.
- Botos, I., E. E. Melnikov, S. Cherry, J. E. Tropea, A. G. Khalatova, F. Rasulova, et al. 2004. The catalytic domain of *Escherichia coli* Lon protease has a unique fold and a Ser-Lys dyad in the active site. *J. Biol. Chem.* 279:8140–8148.
- Brzozowska, I., and U. Zielenkiewicz. 2013. Regulation of toxin-antitoxin systems by proteolysis. *Plasmid* 70:33–41.
- Chadani, Y., K. Ono, S. Ozawa, Y. Takahashi, K. Takai, H. Nanamiya, et al. 2010. Ribosome rescue by *Escherichia coli* ArfA (YhdL) in the absence of *trans*-translation system. *Mol. Microbiol.* 78:796–808.
- Chadani, Y., K. Ono, K. Kutsukake, and T. Abo. 2011. *Escherichia coli* YaeJ protein mediates a novel ribosome-rescue pathway distinct from SsrA- and ArfA-mediated pathways. *Mol. Microbiol.* 80:772–785.
- Chadani, Y., K. Ito, K. Kutsukake, and T. Abo. 2012. ArfA recruits release factor 2 to rescue stalled ribosomes by peptidyl-tRNA hydrolysis in *Escherichia coli*. *Mol. Microbiol.* 86:37–50.
- Cherepanov, P. P., and W. Wackernagel. 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.
- Cherny, I., L. Rockah, and E. Gazit. 2005. The YoeB toxin is a folded protein that forms a physical complex with the unfolded YefM antitoxin. Implications for a structural-based differential stability of toxin-antitoxin systems. *J. Biol. Chem.* 280:30063–30072.
- Christensen, S. K., and K. Gerdes. 2003. RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. *Mol. Microbiol.* 48:1389–1400.
- Christensen, S. K., M. Mikkelsen, K. Pedersen, and K. Gerdes. 2001. RelE, a global inhibitor of translation, is activated during nutritional stress. *Proc. Natl. Acad. Sci. USA* 98:14328–14333.
- Christensen, S. K., K. Pedersen, F. G. Hansen, and K. Gerdes. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *J. Mol. Biol.* 332:809–819.
- Christensen, S. K., G. Maenhaut-Michel, N. Mine, S. Gottesman, K. Gerdes, and L. Van Melderen. 2004. Overproduction of the Lon protease triggers inhibition of translation in *Escherichia coli*: involvement of the *yefM-yoeB* toxin-antitoxin system. *Mol. Microbiol.* 51:1705–1717.
- Christensen-Dalsgaard, M., and K. Gerdes. 2008. Translation affects YoeB and MazF messenger RNA interferase activities by different mechanisms. *Nucleic Acids Res.* 36:6472–6481.
- Datta, S., N. Costantino, and D. L. Court. 2006. A set of recombineering plasmids for Gram-negative bacteria. *Gene* 379:109–115.
- Engelberg-Kulka, H., R. Hazan, and S. Amitai. 2005. *mazEF*: a chromosomal toxin-antitoxin module that triggers programmed cell death in bacteria. *J. Cell Sci.* 118:4327–4332.
- Feaga, H. A., P. H. Viollier, and K. C. Keiler. 2014. Release of nonstop ribosomes is essential. *MBio* 5:e01916.
- Feng, S., Y. Chen, K. Kamada, H. Wang, K. Tang, M. Wang, et al. 2013. YoeB-ribosome structure: a canonical RNase that requires the ribosome for its specific activity. *Nucleic Acids Res.* 41:9549–9556.
- Frazao, C., C. E. McVey, M. Amblar, A. Barbas, C. Vonnrhein, C. M. Arraiano, et al. 2006. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature* 443:110–114.
- Gagnon, M. G., S. V. Seetharaman, D. Bulkley, and T. A. Steitz. 2012. Structural basis for the rescue of stalled ribosomes: structure of YaeJ bound to the ribosome. *Science* 335:1370–1372.
- Gamer, J., H. Bujard, and B. Bukau. 1992. Physical interaction between heat shock proteins DnaK, DnaJ, and GrpE and the bacterial heat shock transcription factor sigma 32. *Cell* 69:833–842.
- Garza-Sánchez, F., B. D. Janssen, and C. S. Hayes. 2006. Prolyl-tRNA<sup>Pro</sup> in the A-site of SecM-arrested ribosomes inhibits the recruitment of transfer-messenger RNA. *J. Biol. Chem.* 281:34258–34268.
- Garza-Sánchez, F., J. G. Gin, and C. S. Hayes. 2008. Amino acid starvation and colicin D treatment induce A-site mRNA cleavage in *Escherichia coli*. *J. Mol. Biol.* 378:505–519.
- Garza-Sánchez, F., S. Shoji, K. Fredrick, and C. S. Hayes. 2009. RNase II is important for A-site mRNA cleavage during ribosome pausing. *Mol. Microbiol.* 73:882–897.
- Ge, Z., P. Mehta, J. Richards, and A. W. Karzai. 2010. Non-stop mRNA decay initiates at the ribosome. *Mol. Microbiol.* 78:1159–1170.
- Gerdes, K., and E. Maisonneuve. 2012. Bacterial persistence and toxin-antitoxin loci. *Annu. Rev. Microbiol.* 66:103–123.
- Gerdes, K., S. K. Christensen, and A. Lobner-Olesen. 2005. Prokaryotic toxin-antitoxin stress response loci. *Nat. Rev. Microbiol.* 3:371–382.
- Goeders, N., and L. Van Melderen. 2014. Toxin-antitoxin systems as multilevel interaction systems. *Toxins (Basel)* 6:304–324.
- Goff, S. A., L. P. Casson, and A. L. Goldberg. 1984. Heat shock regulatory gene *htpR* influences rates of protein degradation and expression of the *lon* gene in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 81:6647–6651.

- Gueneau de Novoa, P., and K. P. Williams. 2004. The tmRNA website: reductive evolution of tmRNA in plastids and other endosymbionts. *Nucleic Acids Res.* 32:D104–D108.
- Guisbert, E., T. Yura, V. A. Rhodius, and C. A. Gross. 2008. Convergence of molecular, modeling, and systems approaches for an understanding of the *Escherichia coli* heat shock response. *Microbiol. Mol. Biol. Rev.* 72:545–554.
- Gur, E., and R. T. Sauer. 2008. Recognition of misfolded proteins by Lon, a AAA(+) protease. *Genes Dev.* 22:2267–2277.
- Handa, Y., N. Inaho, and N. Nameki. 2011. YaeJ is a novel ribosome-associated protein in *Escherichia coli* that can hydrolyze peptidyl-tRNA on stalled ribosomes. *Nucleic Acids Res.* 39:1739–1748.
- Hayes, C. S., and K. C. Keiler. 2010. Beyond ribosome rescue: tmRNA and co-translational processes. *FEBS Lett.* 584: 413–419.
- Hayes, C. S., and R. T. Sauer. 2003. Cleavage of the A site mRNA codon during ribosome pausing provides a mechanism for translational quality control. *Mol. Cell* 12:903–911.
- Hayes, C. S., B. Bose, and R. T. Sauer. 2002a. Proline residues at the C terminus of nascent chains induce SsrA tagging during translation termination. *J. Biol. Chem.* 277:33825–33832.
- Hayes, C. S., B. Bose, and R. T. Sauer. 2002b. Stop codons preceded by rare arginine codons are efficient determinants of SsrA tagging in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 99:3440–3445.
- Huang, H. C., M. Y. Sherman, O. Kandrór, and A. L. Goldberg. 2001. The molecular chaperone DnaJ is required for the degradation of a soluble abnormal protein in *Escherichia coli*. *J. Biol. Chem.* 276:3920–3928.
- Janssen, B. D., and C. S. Hayes. 2009. Kinetics of paused ribosome recycling in *Escherichia coli*. *J. Mol. Biol.* 394: 251–267.
- Janssen, B. D., and C. S. Hayes. 2012. The tmRNA ribosome-rescue system. *Adv. Protein Chem. Struct. Biol.* 86:151–191.
- Janssen, B. D., F. Garza-Sanchez, and C. S. Hayes. 2013. A-site mRNA cleavage is not required for tmRNA-mediated ssrA-peptide tagging. *PLoS One* 8:e81319.
- Jiang, L., C. Schaffitzel, R. Bingel-Erlenmeyer, N. Ban, P. Korber, R. I. Koning, et al. 2009. Recycling of aborted ribosomal 50S subunit-nascent chain-tRNA complexes by the heat shock protein Hsp15. *J. Mol. Biol.* 386:1357–1367.
- Jonas, K., J. Liu, P. Chien, and M. T. Laub. 2013. Proteotoxic stress induces a cell-cycle arrest by stimulating Lon to degrade the replication initiator DnaA. *Cell* 154:623–636.
- Kamada, K., and F. Hanaoka. 2005. Conformational change in the catalytic site of the ribonuclease YoeB toxin by YefM antitoxin. *Mol. Cell* 19:497–509.
- Keiler, K. C., P. R. Waller, and R. T. Sauer. 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* 271:990–993.
- Keiler, K. C., L. Shapiro, and K. P. Williams. 2000. tmRNAs that encode proteolysis-inducing tags are found in all known bacterial genomes: a two-piece tmRNA functions in *Caulobacter*. *Proc. Natl. Acad. Sci. USA* 97:7778–7783.
- Korber, P., J. M. Stahl, K. H. Nierhaus, and J. C. Bardwell. 2000. Hsp15: a ribosome-associated heat shock protein. *EMBO J.* 19:741–748.
- Kumar, P., B. Issac, E. J. Dodson, J. P. Turkenburg, and S. C. Mande. 2008. Crystal structure of *Mycobacterium tuberculosis* YefM antitoxin reveals that it is not an intrinsically unstructured protein. *J. Mol. Biol.* 383:482–493.
- Li, X., R. Hirano, H. Tagami, and H. Aiba. 2006. Protein tagging at rare codons is caused by tmRNA action at the 3' end of nonstop mRNA generated in response to ribosome stalling. *RNA* 12:248–255.
- Li, X., M. Yagi, T. Morita, and H. Aiba. 2008. Cleavage of mRNAs and role of tmRNA system under amino acid starvation in *Escherichia coli*. *Mol. Microbiol.* 68: 462–473.
- Liberek, K., T. P. Galitski, M. Zylicz, and C. Georgopoulos. 1992. The DnaK chaperone modulates the heat shock response of *Escherichia coli* by binding to the sigma 32 transcription factor. *Proc. Natl. Acad. Sci. USA* 89:3516–3520.
- Magnuson, R. D. 2007. Hypothetical functions of toxin-antitoxin systems. *J. Bacteriol.* 189:6089–6092.
- Maisonneuve, E., and K. Gerdes. 2014. Molecular mechanisms underlying bacterial persistence. *Cell* 157:539–548.
- Maisonneuve, E., L. J. Shakespeare, M. G. Jorgensen, and K. Gerdes. 2011. Bacterial persistence by RNA endonucleases. *Proc. Natl. Acad. Sci. USA* 108:13206–13211.
- Mogk, A., T. Tomoyasu, P. Goloubinoff, S. Rudiger, D. Roder, H. Langen, et al. 1999. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* 18:6934–6949.
- Moore, S. D. 2011. Assembling new *Escherichia coli* strains by transduction using phage P1. *Methods Mol. Biol.* 765:155–169.
- Mottagui-Tabar, S., A. Bjornsson, and L. A. Isaksson. 1994. The second to last amino acid in the nascent peptide as a codon context determinant. *EMBO J.* 13:249–257.
- Nariya, H., and M. Inouye. 2008. MazF, an mRNA interferase, mediates programmed cell death during multicellular *Myxococcus* development. *Cell* 132:55–66.
- Nieto, C., I. Cherny, S. K. Khoo, M. G. de Lacoba, W. T. Chan, C. C. Yeo, et al. 2007. The yefM-yoeB toxin-antitoxin systems of *Escherichia coli* and *Streptococcus pneumoniae*: functional and structural correlation. *J. Bacteriol.* 189:1266–1278.
- Nonaka, G., M. Blankschien, C. Herman, C. A. Gross, and V. A. Rhodius. 2006. Regulon and promoter analysis of the

- E. coli* heat-shock factor, sigma32, reveals a multifaceted cellular response to heat stress. *Genes Dev.* 20:1776–1789.
- Pedersen, K., A. V. Zavialov, M. Y. Pavlov, J. Elf, K. Gerdes, and M. Ehrenberg. 2003. The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal A site. *Cell* 112:131–140.
- Phillips, T. A., R. A. VanBogelen, and F. C. Neidhardt. 1984. *lon* gene product of *Escherichia coli* is a heat-shock protein. *J. Bacteriol.* 159:283–287.
- Pryszak, M. H., C. J. Mozdziej, A. M. Cook, L. Zhu, Y. Zhang, M. Inouye, et al. 2009. Bacterial toxin YafQ is an endoribonuclease that associates with the ribosome and blocks translation elongation through sequence-specific and frame-dependent mRNA cleavage. *Mol. Microbiol.* 71:1071–1087.
- Richards, J., P. Mehta, and A. W. Karzai. 2006. RNase R degrades non-stop mRNAs selectively in an SmpB-tmRNA-dependent manner. *Mol. Microbiol.* 62:1700–1712.
- Seidman, J. S., B. D. Janssen, and C. S. Hayes. 2011. Alternative fates of paused ribosomes during translation termination. *J. Biol. Chem.* 286:31105–31112.
- Sevillano, L., M. Diaz, Y. Yamaguchi, M. Inouye, and R. I. Santamaria. 2012. Identification of the first functional toxin-antitoxin system in *Streptomyces*. *PLoS One* 7:e32977.
- Sherman, M., and A. L. Goldberg. 1992. Involvement of the chaperonin dnaK in the rapid degradation of a mutant protein in *Escherichia coli*. *EMBO J.* 11:71–77.
- Shimizu, Y. 2012. ArfA recruits RF2 into stalled ribosomes. *J. Mol. Biol.* 423:624–631.
- Straus, D., W. Walter, and C. A. Gross. 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of sigma 32. *Genes Dev.* 4:2202–2209.
- Sunohara, T., K. Jojima, Y. Yamamoto, T. Inada, and H. Aiba. 2004. Nascent-peptide-mediated ribosome stalling at a stop codon induces mRNA cleavage resulting in nonstop mRNA that is recognized by tmRNA. *RNA* 10:378–386.
- Thomas, J. G., and F. Baneyx. 2000. ClpB and HtpG facilitate de novo protein folding in stressed *Escherichia coli* cells. *Mol. Microbiol.* 36:1360–1370.
- Thomason, L.C., Sawitzke J. A., Li X., Costantino N. and D. L. Court. 2014. Recombineering: genetic engineering in bacteria using homologous recombination. *Curr. Protoc. Mol. Biol.* 106:1, 16.1–1.16.39.
- Tsilibaris, V., G. Maenhaut-Michel, and L. Van Melderen. 2006. Biological roles of the Lon ATP-dependent protease. *Res. Microbiol.* 157:701–713.
- Tsilibaris, V., G. Maenhaut-Michel, N. Mine, and L. Van Melderen. 2007. What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? *J. Bacteriol.* 189:6101–6108.
- Tu, G. F., G. E. Reid, J. G. Zhang, R. L. Moritz, and R. J. Simpson. 1995. C-terminal extension of truncated recombinant proteins in *Escherichia coli* with a 10Sa RNA decapeptide. *J. Biol. Chem.* 270:9322–9326.
- Winther, K. S., and K. Gerdes. 2009. Ectopic production of VapCs from Enterobacteria inhibits translation and trans-activates YoeB mRNA interferase. *Mol. Microbiol.* 72:918–930.
- Yamamoto, Y., T. Sunohara, K. Jojima, T. Inada, and H. Aiba. 2003. SsrA-mediated *trans*-translation plays a role in mRNA quality control by facilitating degradation of truncated mRNAs. *RNA* 9:408–418.
- Yoshizumi, S., Y. Zhang, Y. Yamaguchi, L. Chen, B. N. Kreiswirth, and M. Inouye. 2009. *Staphylococcus aureus* YoeB homologues inhibit translation initiation. *J. Bacteriol.* 191:5868–5872.
- Zhang, Y., and M. Inouye. 2009. The inhibitory mechanism of protein synthesis by YoeB, an *Escherichia coli* toxin. *J. Biol. Chem.* 284:6627–6638.
- Zuo, Y., H. A. Vincent, J. Zhang, Y. Wang, M. P. Deutscher, and A. Malhotra. 2006. Structural basis for processivity and single-strand specificity of RNase II. *Mol. Cell* 24:149–156.