YaeJ is a novel ribosome-associated protein in *Escherichia coli* that can hydrolyze peptidyl–tRNA on stalled ribosomes

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**ABSTRACT**

In bacteria, ribosomes often become stalled and are released by a *trans*-translation process mediated by transfer-messenger RNA (tmRNA). In the absence of tmRNA, however, there is evidence that stalled ribosomes are released from non-stop mRNAs. Here, we show a novel ribosome rescue system mediated by a small basic protein, YaeJ, from *Escherichia coli*, which is similar in sequence and structure to the catalytic domain 3 of polypeptide chain release factor (RF). *In vitro* translation experiments using the *E. coli*-based reconstituted cell-free protein synthesis system revealed that YaeJ can hydrolyze peptidyl–tRNA on ribosomes stalled by both non-stop mRNAs and mRNAs containing rare codon clusters that extend downstream from the P-site and prevent Ala-tmRNA•SmpB from entering the empty A-site. In addition, YaeJ had no effect on translation of a normal mRNA with a stop codon. These results suggested a novel tmRNA-independent rescue system for stalled ribosomes in *E. coli*. YaeJ was almost exclusively found in the 70S ribosome and polysome fractions after sucrose density gradient sedimentation, but was virtually undetectable in soluble fractions. The C-terminal basic residue-rich extension was also found to be required for ribosome binding. These findings suggest that YaeJ functions as a ribosome-attached rescue device for stalled ribosomes.

**INTRODUCTION**

Ribosome stalling occurs for a variety of reasons such as aberrant mRNAs lacking a stop codon and particular mRNAs possessing a cluster of rare codons (1). To overcome this problem, bacteria have a unique, elaborate translation mechanism, *trans*-translation, which is mediated by transfer-messenger RNA (tmRNA). tmRNA, encoded by the *ssrA* gene, possesses both tRNA and mRNA properties (2–4). In the tRNA mode, a tmRNA charged with alanine by alanyl–tRNA synthetase enters the A-site of the stalled ribosome via a ternary complex with elongation factor, EF-Tu and GTP, and donates the alanine to the growing polypeptide chain. Then, tmRNA switches from the tRNA mode to the mRNA mode, and translation resumes at the first codon of the internal coding region of tmRNA, followed by normal termination at the stop codon in the coding region. Throughout this process, the SmpB protein remains bound to the tmRNA, playing an essential role at various stages of the translation process (5,6). A tag peptide encoded by tmRNA is added to the C-terminus of the growing polypeptide, and the resulting tagged protein is immediately degraded by several tag-specific proteases (1). This process consequently promotes ribosome recycling and truncated mRNA degradation (7), and prevents accumulation of abortively synthesized polypeptides during normal cell growth (8,9). Additional biological roles of tmRNA include stress management and the regulation of transcriptional circuits (1). This tmRNA system is ubiquitous among bacteria, although it is not essential for cell viability in the majority of cases (8,10).

Recent reports provide direct evidence that in the absence of tmRNA stalled ribosomes are released from non-stop mRNAs. In a *ΔssrA* strain of *Escherichia coli*, a tmRNA-defective strain, non-stop mRNAs are translated efficiently, although the translating ribosomes fail to undergo canonical termination (11,12). In addition, stalled ribosomes containing peptidyl–tRNA and non-stop mRNA have not been detected in the polysome fractions of cells (13). Peptidyl–tRNAs derived from non-stop mRNAs have been detected at significant levels in a cell-free translation reconstituted with purified components, not including tmRNA, in which the stalled ribosomes appear in the polysome fractions (13). Other
in vitro experiments using an optimized poly(Phe) synthesis system containing S100 enzymes show that ribosomes can recycle from the 3′-end of mRNA lacking a stop codon without the help of tmRNA (14). Pulse-chase analysis of peptidyl–tRNA turnover indicates that paused ribosomes recycle efficiently from non-stop mRNA in a ΔssrA strain (12). This recycling process is not mediated by peptidyl–tRNA hydrolase (Pth), which reacts to dropped-off peptidyl–tRNAs to cleave the ester link between the peptide and the tRNA (12,13). These findings suggest the existence of a tmRNA-independent ribosome rescue system mediated by unknown factor(s) that hydrolyze peptidyl–tRNA from non-stop mRNA (1,12,13,15).

This study focuses on the yaeJ gene product as a putative candidate for peptidyl–tRNA hydrolysis (PTH). YaeJ homologs have been identified in bacteria and in eukaryotes, but few studies regarding YaeJ function have been reported. In E. coli, YaeJ is known to be dispensable for growth under normal laboratory growth conditions (16). YaeJ is a candidate enzyme for PTH because its sequence contains the Gly–Gly–Gln (GGQ) motif that invariably occurs in the class I polypeptide chain release factor (RF) catalytic site. In translation termination, RF, which comprises four domains, enters the A-site of a ribosome where it recognizes stop codons and hydrolyzes the peptidyl–tRNA at the P-site to release the nascent polypeptide chain with the GGQ motif (17–19). Domain 3 containing the GGQ motif is the catalytic domain that directly interacts with the peptidyltransferase center (PTC) of the large ribosomal subunit to trigger PTH. In addition to sharing the GGQ motif, YaeJ from Pseudomonas syringae and a eukaryotic homolog from the mouse were also reported to be similar in structure to domain 3 of RF (20,21). However, YaeJ (140 residues) is shorter than RFs (for E. coli, 365 residues), and completely lacks domains 2 and 4 that are required for stop codon recognition.

Recently, it has been reported that the human YaeJ homolog, ICT1, is a component of the mitochondrial ribosome and that it has a PTH activity via the GGQ motif in a codon-independent manner (22). These findings suggest that ICT1 is involved in the rescue of stalled ribosomes in mitochondria.

In this study, we show that YaeJ can hydrolyze peptidyl–tRNA on a ribosome stalled by non-stop mRNA and a rare codon cluster in vitro. In addition, we demonstrate that YaeJ is associated with 70S ribosomes and polysomes, and free YaeJ is rare. Our findings demonstrate a novel tmRNA-independent rescue system of stalled ribosomes mediated by the ribosome-attached enzyme, YaeJ.

**MATERIALS AND METHODS**

**Plasmids and E. coli strains**

The wild-type E. coli strain MG1655 and the yaeJ-deficient strain JW0187 derived from BW25113 were provided by the National BioResource Project (NIG, Japan): E. coli (23). The yaeJ-deficient (AyaeJ) strain used in this study was constructed by P1 transduction using MG1655 as a recipient and JW0187 as the donor strain followed by kanamycin selection. The double deletion strain of the yaeJ and ssrA genes was constructed by P1 transduction using the ssrA-deficient strain derived from MG1655 (a gift from Dr Toshimasa Tadaki) as a recipient and the AyaeJ strain as the donor strain followed by kanamycin selection. The absence of the YaeJ protein and tmRNA was confirmed by western blotting or PCR.

**In vitro translation using PUREsystem technology**

PUREExpress (New England Biolabs) based on PUREsystem technology and FluroTect Green_{38} in vitro translation labeling system (Promega) were used for in vitro translation experiments. Template DNA fragments were prepared using a two-step PCR reaction. For example, to prepare wild-type mRNA of the crp gene, stop primers were used for the first PCR, followed by universal primer and stop (R) primer for the second PCR (Table 1). The primers used for each mRNA were:

- **Wild-type mRNA of the crp gene:**
  - First: stop (F) and (R) primers
  - Second: universal and stop (R) primers

- **Non-stop mRNA:**
  - First: stop (F) and non-stop (R) primers
  - Second: universal and non-stop (R) primers

- **Wild-type mRNA and GGQ mutant of the yaeJ gene:**
  - First: 1–140 (F) and (R) primers
  - Second: universal and 1–140 (R) primers

The C-terminal truncation mutants of YaeJ (residues 1–130, 1–119 and 1–100):

- First: 1–140 primer (F); 1–130 (R), 1–119 (R) and 1–100 (R) primers, respectively
- Second: universal primer; 1–130 (R), 1–119 (R) and 1–100 (R) primers, respectively

The DNA fragments contained T7 RNA polymerase promoter sequences, and the transcription and translation reactions were coupled in this system. The DNA fragments (0.1 pmol) were incubated with 5 μl of reaction mixture at 37°C, and the resultant samples were mixed with RNAsecure (Applied Biosystems), treated with 2× sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% glycerol, 5% 2-mercaptoethanol and 0.1% bromophenol blue] and then analyzed by NuPAGE (Invitrogen). Direct ‘in-gel’ detection of the proteins containing fluorescently labeled lysine residues was accomplished using a laser-based fluorescent gel scanner, Molecular Imager FX Pro (Bio-Rad).

**PTH assay**

YaeJ was expressed in the in vitro translation system described above. Independently, using a stop or non-stop template as shown in Figure 1A, an in vitro translation reaction was performed for 1 h to produce stalled ribosomes with peptidyl–tRNAs, to which the in vitro translation mixture containing YaeJ was directly added. The resulting mixture was incubated for 10 min and then analyzed by NuPAGE. The gel was visualized on a laser-based fluorescent gel scanner. To determine the
yield of YaeJ and its mutants, the protein, along with the positive control protein, dihydrofolate reductase (DHFR), was expressed and analyzed by SDS–PAGE. When the yield of DHFR was 200 μg/ml, the yield of YaeJ was determined according to the relative band intensities, as quantified using the laser-based fluorescent gel scanner. Although some of the YaeJ protein probably bound to ribosomes in the reaction solution, sufficient amounts of free YaeJ were obtained for the next stalled ribosome rescue reaction. The ribosome concentration in the reaction solution of PURExpress was 2.5 μM.

Protein expression and purification of recombinant His-tagged YaeJ protein

The DNA sequence encoding the entire YaeJ protein was PCR-amplified using genomic DNA from strain MG1655 as a template. The amplified DNA fragment was digested with XbaI and NcoI and then cloned into the expression vector pET15b (Novagen) as a fusion protein with a C-terminal 6× His-tag followed by a thrombin cleavage site plus a vector-derived sequence (in total, 43 residues). This recombinant protein was cleaved by thrombin, and the resultant protein had a 17-residue artificial site plus a vector-derived sequence (in total, 43 residues).

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Site-directed mutagenesis

Mutations were introduced into the yaeJ gene using the overlap PCR method (21) and the outside primer and different complementary mutagenic oligonucleotides as the inside primers [e.g. for the GGE mutant, the GGE (F) primer was used as the outside primer and the GGE (R) primer was used as the inside primer] (Table 1). Amplification was performed with high-fidelity Pfu DNA polymerase (Roche) and pET15b-yaeJ plasmid DNA as the template. The amplicons were digested with DpnI. Transformation into E. coli strain DH5α was performed according to the CaCl2 procedure.

Sucrose density gradient centrifugation

Wild-type strain (MG1655) or the ΔyaeJ strain of E. coli were grown in LB medium to an OD600 of 0.6–0.8 for log phase or 1.8–2.0 for stationary phase. Cells harvested at either log or stationary phase were then resuspended in lysis buffer [20 mM Tris–HCl (pH 7.6), 15 mM magnesium acetate, 100 mM sodium chloride, 1 mM AEBSF and 6 mM 2-mercaptoethanol], mixed with an equal volume of

| Primers used with the template for the PUREsystem: |  |
|---|---|
| stop (F) | 5'-TAAGAAGGAGATATACCAATGGTGCTGCTCGAGGAAACCGC-3' |
| stop (R) | 5'-GTCTGCGCCACATCGGGGGAAGAAACAAATTGG-3' |
| non-stop-1 (R) | 5'-CGCGTGGCCGCGGTAAACACAGGACTG-3' |
| non-stop-2 (R) | 5'-GGGTGCCCGCTGAAACACAGGACTG-3' |
| non-stop-3 (R) | 5'-CGTTGCGCTGAAACACAGGACTG-3' |
| R4L4 (R)-Stop | 5'-TATTTTATTAGTTAAAATTTTGTATTTAAATTTTAGAAGGATATACCA-3' |
| R4L8 (R)-Stop | 5'-AGAAATTTAATACGACTCACTATAGGAGACACCAACCGTGT-3' |
| 1-140 (F) | 5'-GAAATTAATACGACTCACTATAGGAGACACCAACCGTGT-3' |
| 1-140 (R) | 5'-AGAAATTTAATACGACTCACTATAGGAGACACCAACCGTGT-3' |
| 1-119 (R) | 5'-GAAATTAATACGACTCACTATAGGAGACACCAACCGTGT-3' |
| 1-100 (R) | 5'-GAAATTAATACGACTCACTATAGGAGACACCAACCGTGT-3' |
| universal | 5'-GAAATTAATACGACTCACTATAGGAGACACCAACCGTGT-3' |

| Primers used for mutation of the yaeJ gene: |  |
|---|---|
| GGE (F) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| GGE (R) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| GAQ (F) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| GAQ (R) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| GAE (F) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| GAE (R) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| VAQ (F) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |
| VAQ (R) | 5'-GGGCCGCGGGCGGGCGGTACGTAATAGAAGACC-3' |

Table 1. Primers used in this study

Forward (F) and reverse (R) primers
glass beads (250–425 microns, Fuji Rika Kogyo) and vortexed 10 times for 0.5 min. After centrifugation at 12 000 g for 30 min, the absorbance of the supernatant at 260 nm was measured using a DU-640 spectrometer (Beckman). The supernatants were layered on a 5–20% linear sucrose density gradient made with association buffer [10 mM Tris–HCl (pH 7.5), 50 mM ammonium chloride and 10 mM magnesium chloride], such that the optical density at 260 nm times volume (ml) (ODV) values were equal among all of the samples. The samples were then analyzed by centrifugation in a P28S rotor (Hitachi Koki) at 112 700 g for 3 h at 4°C. The absorbance of each fraction was measured at 260 nm using a DU-640 spectrometer.
Polysome profile analysis

The wild-type strain was grown in LB medium to an OD_{600} of between 0.6 and 0.8 for log phase. Chloramphenicol was added to a final concentration of 100 µg/ml and incubated for 5 min before harvesting to avoid polysome run-off. After extraction, the supernatants were layered on a 10–40% linear sucrose density gradient made with association buffer, such that the ODV values were equal. The samples were then analyzed by centrifugation in a P28S rotor at 112 700 × g for 3 h at 4°C. The absorbance of each fraction at 260 nm was measured using a DU-640 spectrometer.

Immunoblotting

The total protein concentrations of the lysates were determined using the Quick start Bradford Dye Reagent (Bio-Rad). The proteins (20 µg/lane) were separated by 15% SDS–PAGE and subsequently transferred to nitrocellulose membrane. The membrane was then blocked with 1% nonfat dried milk in PBS containing 0.2% Tween 20 at room temperature for 1 h before incubation with anti-YaeJ polyclonal antibody (1:500; anti-rabbit, operon) or anti-His_{6} antibody (1:500; anti-mouse, GE Healthcare), and horseradish peroxidase-conjugated immunoglobulin G (1:4000; anti-rabbit or mouse, Cell Signaling Technology). The protein was visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Sequence alignment

Sequence alignment of the YaeJ proteins from various bacteria was performed with the ClustalW program (21). The accession numbers of the sequences used in the alignment are as follows: E. coli yaeJ, P40711; Salmonella typhimurium STM0240, NP_459245.1; Pseudomonas aeruginosa, NP_249559.1; Zymomonas mobilis yaeJ, CAA63804.1; Chlorobaculum tepidum, NP_662839.1; Streptomyces avermitilis SAV3946, NP_825123.1; Prochlorococcus marinus, NP_896036.1; Synechocystis sp., NP_942271.1.

RESULTS

YaeJ can hydrolyze peptidyl–tRNA of non-stop mRNA on stalled ribosomes in vitro

To confirm whether YaeJ can hydrolyze peptidyl–tRNA in stalled ribosomes, we performed in vitro translation experiments using the cell-free protein synthesis system reconstituted from purified components, PUREsystem (24). In this system, translation is coupled with transcription using a linear DNA fragment as the template. This in vitro translation system can effectively produce stalled ribosomes using mRNA lacking a stop codon (13,25). In this study, the in vitro translation products were analyzed using the NuPAGE Bis–Tris electrophoresis system, since one of the products, peptidyl–tRNA, is stable in this neutral gel (11). Fluorescent labeling of the products through the use of tRNA_{Lys} charged with a fluorescently-labeled lysine was detected using a laser-based fluorescent scanner.

The use of the crp gene coding for cAMP receptor protein was based on a report by Kuroha et al. (13). The mRNA constructs are shown in Figure 1A. When mRNA of the non-stop template, non-stop-1, was translated, two nucleotides are thought to be located at the decoding region of the A-site. The absence of a stop codon prevents RF-mediated termination and the ribosomes stall. All constructs used in this study were also designed to have no stop codons around the 3' end even if frameshifting occurred. The products of a 1-h in vitro translation reaction from the non-stop mRNA were examined. Peptidyl–tRNA (CRP–tRNA) was mainly detected, while the CRP product was detected at low levels (Figure 1B). These results confirmed the findings of Kuroha et al. (13), who explained the existence of the CRP product by the fact that some ribosomes translating non-stop mRNAs are dissociated from the 3' end of mRNA and that the peptidyl–tRNA is only weakly hydrolyzed in the monosome.

We attempted to overexpress YaeJ in E. coli, but the protein became aggregated and insoluble during the purification process (data not shown). Instead, soluble YaeJ was successfully expressed on a small scale using the PUREsystem. The reaction mixture containing YaeJ was directly added to another solution, in which a preliminary 1-h in vitro translation reaction had been performed using the non-stop template, and the resultant sample was incubated for another 10 min. This PTH assay showed that YaeJ decreased the level of CRP–tRNAs along with a concomitant increase in the level of CRP in a concentration-dependent manner (Figure 1B). Similar results were obtained in the other non-stop templates, non-stop-2 and -3 (Figure 1A), in which one and no nucleotides were assumed to lie at the decoding region, respectively (data not shown).

Recombinant YaeJ protein with a His-tag at the C-terminus was overexpressed in E. coli and successfully purified. When His-tagged YaeJ was added to the sample in which the translation reaction had preliminarily proceeded with the non-stop-1 template, similar results were observed (Figure 1C, left). To examine whether YaeJ had any effect on translation efficiency of the normal template with a stop codon, His-tagged YaeJ was added to the mixture for translation of the crp gene with a stop codon. YaeJ protein had no significant effect on translation efficiency (Figure 1C, right).

These results demonstrated that YaeJ can hydrolyze peptidyl–tRNA of non-stop mRNA in a concentration-dependent manner in vitro. It was also found that YaeJ does not affect translation of normal mRNA with a stop codon, showing that YaeJ can function only when ribosomes are stalled.

YaeJ can release stalled ribosomes caused by a rare codon cluster in vitro

Ribosomes are believed to be stalled at a rare codon cluster due to the deficiency of cognate aminoacyl–tRNAs, and the tmRNA system plays a major role in
the rescue of such ribosomes (26). To examine whether YaeJ can hydrolyze peptidyl–tRNA in ribosomes stalled by a rare codon cluster, we designed templates in which a tandem repeat of four rare Arg codon (AGG) and an additional tandem repeat of four or eight major Leu codons (CTG) were aligned downstream of the crp sequence (Figure 1A). After a 1-h in vitro translation reaction using the template containing the rare Arg codon cluster, modified CRP–tRNAs were detected along with equivalent amounts of modified CRP products, which were terminated at the stop codon (Figure 1D). These results indicated a population of stalled ribosomes caused by a rare codon. The multiple bands observed in the gels were likely due to heterologous translation products released from ribosomes stalled at the four rare Arg codons. The YaeJ-containing reaction mixture obtained by the PUREsystem was added to the 1-h in vitro translation reaction sample using each template. This resulted in a significant decrease in the level of peptidyl–tRNA (CRP–tRNA) and a concomitant increase in the level of CRP from both templates (Figure 1D). These results showed that YaeJ can rescue ribosomes stalled by a rare codon cluster.

**GGQ motif is involved in PTH activity**

To clarify whether the GGQ motif of YaeJ contributes to PTH activity, we changed the GGQ residues to GAQ, GGE, GAE or VAQ by site directed mutagenesis. Each mutant-containing reaction mixture obtained by the PUREsystem was added to the 1-h in vitro translation reaction sample using each template. This resulted in a significant decrease in the level of peptidyl–tRNA (CRP–tRNA) and a concomitant increase in the level of CRP from both templates (Figure 1D). These results showed that YaeJ can rescue ribosomes stalled by a rare codon cluster.

**YaeJ plays no role in ribosome assembly or maturation**

Some ribosomal proteins and ribosome-associated proteins play important roles in ribosome assembly and/or maturation in *E. coli* (29). To confirm whether YaeJ is involved in these processes, we compared the ribosome profiles of wild-type and *ΔyaeJ* strains by sucrose density gradient centrifugation. The ribosome profiles in log phase, polysomes and stationary phase were essentially the same between the wild-type and *ΔyaeJ* strains (Figure 4). These results showed that YaeJ does not significantly contribute to ribosome assembly, maturation or 100S formation. This is consistent with the finding that the growth rates of the wild-type and *ΔyaeJ* strains are essentially the same (data not shown).

**The C-terminal extension of YaeJ is required for ribosome binding and PTH activity**

A striking feature of many ribosomal proteins is a long, basic extension stretching from the structured domain, which becomes structured when penetrating deeply into ribosomes (30). Similarly, YaeJ has an unstructured region at the C-terminus, which is rich in basic residues (29,31) (Figure 5A). To examine whether this C-terminal extension plays a role in PTH activity, we constructed three YaeJ mutants in which the C-terminal region was truncated from three different positions (1–130, 1–119 and 1–100). The PTH assay using the YaeJ mutants expressed by the in vitro translation system showed that none of the three mutants displayed any PTH activity (Figure 5B). These results showed that the C-terminal 10 residues are required for the PTH activity of YaeJ.

To verify whether the YaeJ mutant (1–130) has the capacity to bind to ribosomes, we performed sucrose density gradient centrifugation analysis. We constructed a His-tagged YaeJ mutant (1–130) that retained its native 130 residues plus an artificial 6×His-tag sequence (17 residues), which can be detected by western blot analysis using an anti-His antibody. The circular dichroic spectrum of the YaeJ mutant protein (1–130) overexpressed in *E. coli* was similar to that of the

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**Figure 2. Mutation of the GGQ motif of YaeJ affected PTH activity.** Wild-type YaeJ and four YaeJ mutants in which the GGQ residues were changed to GAQ, GGE, GAE or VAQ were expressed using the in vitro translation system. The in vitro translation reaction mixture containing a YaeJ mutant was directly added to another solution in which a preliminarily 1-h in vitro translation reaction had been performed using the non-stop-1 template. The final concentration of YaeJ and the mutants was 5 μM.

**Figure 3A and B.** These results showed that the C-terminal 10 residues are required for the PTH activity of YaeJ.
His-tagged full-length YaeJ protein (140 plus 17 residues), showing that the truncation mutation had no significant effect on the structured region of YaeJ (data not shown). The full-length or mutant YaeJ proteins were added to the lysates of the ΔyaeJ strain and sucrose density gradient centrifugation was performed. His-tagged full-length YaeJ protein was associated with 70S ribosomes (Figure 5C), indicating that the extra 17 residues did not impair the binding properties or PTH activity of YaeJ. In contrast, the His-tagged YaeJ mutant (1–130) failed to bind to any ribosome subunits or to 70S ribosomes (Figure 5C). Similar results were obtained in another His-tagged YaeJ mutant (1–100) (data not shown). Thus, the lack of PTH activity in the YaeJ mutants can be explained by the failure to bind to ribosomes. These results show that both PTH activity and binding to ribosomes require the full-length C-terminal extension of the YaeJ protein.
In this study, we revealed that YaeJ can release a peptidyl-tRNA from ribosomes stalled by non-stop mRNA or a rare codon cluster, with the GGQ motif acting as the catalytic site. These results suggest that YaeJ is involved in a novel tmRNA-independent rescue system for stalled ribosomes in *E. coli*. Furthermore, we demonstrated that YaeJ is associated with 70S ribosomes or the 30S subunits, while free YaeJ is rare. YaeJ was also observed in the fractions of polysomes during translation and 100S ribosomes that are formed by translational inactivation at the ribosome resting stage (28). Thus, YaeJ could be regarded as a ribosome-attached rescue device for stalled ribosomes.

YaeJ proteins can be divided into two parts: an N-terminal structured region, which closely resembles that of domain 3 of RF, and a C-terminal basic residue-rich region, which is presumably unstructured in solution (21). When ribosomes stall, the domain 3-like structured part of YaeJ is thought to interact with the PTC of the 50S subunit, hydrolyzing peptidyl-tRNA at the P-site via the GGQ motif, as observed in translation termination mediated by RF. As a consequence, a complex similar to that of the post-termination complex consisting of a ribosome, mRNA and a deacylated tRNA is thought to be produced. Subsequently, this post-stalled complex would be disassembled into components for a new round of translation. Whether YaeJ stays attached to the 30S subunit after this process has yet to be determined. The apparent absence of YaeJ in the soluble fractions implies almost no turnover of YaeJ into another 30S subunit for initiation of translation, or into another stalled complex.

How is YaeJ bound to ribosome? During normal translation elongation and termination, it was found that ribosome-bound YaeJ is inactive. Thus, during these processes, YaeJ would be positioned outside the A-site so as not to disturb the entry of aminoacyl–tRNAs or RFs, whereas in a ribosome-stalled state, at least the domain 3-like part of YaeJ would be allowed to enter the A-site to interact with the PTC. We speculate that when a ribosome is stalled, a specific conformational change of the ribosome would allow the introduction of the domain 3-like part of YaeJ into the A-site, with the C-terminal extension being fixed somewhere in the 30S subunit. In light of the RF-bound ribosome structures

**DISCUSSION**

In this study, we revealed that YaeJ can release a peptidyl-tRNA from ribosomes stalled by non-stop mRNA or a rare codon cluster, with the GGQ motif acting as the catalytic site. These results suggest that YaeJ is involved in a novel tmRNA-independent rescue system for stalled ribosomes in *E. coli*. Furthermore, we demonstrated that YaeJ is associated with 70S ribosomes or the 30S subunits, while free YaeJ is rare. YaeJ was also observed in the fractions of polysomes during translation and 100S ribosomes that are formed by translational inactivation at the ribosome resting stage (28). Thus, YaeJ could be regarded as a ribosome-attached rescue device for stalled ribosomes.

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lethality screening experiments have shown that the
in the absence of the two genes. Very recently, synthetic
another factor that is involved in stalled ribosome rescue
However, the double deletion mutant was found to grow
although each single deletion mutation is viable.

between the roles of these two rescue systems
functions of tmRNA requires the absence of mRNA at
The efficiency of tmRNA action decreases
rapidly with increasing the length of the mRNA down-
stream from the P-site of stalled ribosomes and
and to the 3OS subunit.
The results of this study suggest that E. coli has at least
two rescue systems for stalled ribosomes, one mediated by
tmRNA and the other by YaeJ. What are the distinctions
between the roles of these two rescue systems in vivo?
The function of tmRNA requires the absence of mRNA at
the A-site (33). The efficiency of tmRNA action decreases
with the increase in length of mRNA downstream from
the P-site of stalled ribosomes and
approaches zero when the length exceeds 15 bases
(34,35). In contrast, our findings showed that unlike the
tmRNA system, the YaeJ system does not depend on the
length of mRNA downstream from the P-site. Thus, a
particular role of YaeJ appears to be the rescue of ribo-
somes stalled at the sense codon which tmRNA cannot
enter. It has been reported that such ribosome stalling
on an intact mRNA induces mRNA cleavage at the
A-site in vivo, producing non-stop mRNA that can be
recognized by tmRNA (36).

When non-stop mRNAs are overexpressed in E. coli,
mot if not all of the encoded proteins are tagged by
tmRNA (4), indicating that tmRNA-mediated rescue
occurs at a faster rate than alternative rescue systems
(1). In addition to ribosome rescue, the tmRNA system
includes an excellent degradation system for incompletely
synthesized proteins in which the tmRNA-encoded
peptides are tagged for a protease recognition site (1).
Thus, YaeJ may function as a complementary rescue
system for situations in which tmRNA cannot function.
In fact, YaeJ is not conserved among all bacteria, unlike
tmRNA. YaeJ homologs have been identified in many
Gram-negative bacteria, with some exceptions such as
Thermus thermophilus, but not in Gram-positive
bacteria. Interestingly, no YaeJ homologs have been
found in Mycoplasma genitalium (Gram-positive) or
Neisseria gonorrhoeae (Gram-negative), in which disrup-
tion or deletion of the genes encoding tmRNA and/or
SmpB is lethal (8,10).

In E. coli, it was anticipated that a double deletion mu-
tations of the yaeJ and tmRNA genes would be lethal,
although each single deletion mutation is viable.
However, the double deletion mutant was found to grow
as well as the single deletion mutants in LB medium (data
not shown). These results suggest the presence of yet
another factor that is involved in stalled ribosome rescue
in the absence of the two genes. Very recently, synthetic
lethality screening experiments have shown that the
yhdL gene product is essential for the viability of E. coli in
the absence of mRNA, indicating that it is involved in
a tmRNA-independent ribosome rescue (37). The viability
of the double deletion mutant can probably be explained
by the functioning of YhdL. It is notable that YhdL has
neither a GGQ motif nor PTH activity and that it is
conserved only among enterobacteriaceae. In addition, a
putative candidate is the prfH gene product, which is
another truncated RF containing the GGQ motif,
although little is known about its function (38).

Homologous genes are also found in not only many
Gram-negative bacteria, but also some groups of
Gram-positive bacteria such as Clostridium, unlike YaeJ.
In bacteria, the entire stalled ribosome rescue system
appears to be more complicated than was previously
thought, and to be diverse among species.

In contrast to bacteria, whereas tmRNA has not been
found in eukaryotes with the exception of some organelles,
YaeJ homologs are completely conserved in the genomes
of all eukaryotes from fungi to vertebrates. The human
YaeJ homolog, ICT1, was reported to be a component of
mitochondrial ribosomes, displaying codon-independent
PTH activity via the GGQ motif (22). Unlike E. coli
YaeJ, ICT1 is essential for cell viability (21,22).
Considering that mitochondria are thought to descend
from ancient Gram-negative bacteria (specifically
α-proteobacteria) (39), it is plausible that the bacterial
YaeJ and mitochondrial ICT1 systems have evolved
from a common ancestor. It is also likely that after the
loss of the complicated tmRNA system, the YaeJ/ICT1
system became indispensable for stalled ribosome rescue
in mitochondria. Further studies are required to reveal not
only the molecular mechanism of YaeJ function in stalled
ribosomes but also to elucidate a broader picture of
ribosome rescue systems depending on the organism.

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