Pseudouridylation of messenger RNA emerges as an abundant modification involved in gene expression regulation. Pseudouridylation of stop codons in eukaryotic and bacterial cells results in stop-codon read through. The structural mechanism of this phenomenon is not known. Here we present a 3.1-Å crystal structure of Escherichia coli release factor 1 (RF1) bound to the 70S ribosome in response to the \(\Psi\AA\) codon.

The structure reveals that recognition of a modified stop codon does not differ from that of a canonical stop codon. Our \textit{in vitro} biochemical results support this finding by yielding nearly identical rates for peptide release from \textit{E. coli} ribosomes programmed with pseudouridylated and canonical stop codons. The crystal structure also brings insight into \textit{E. coli} RF1-specific interactions and suggests involvement of L27 in bacterial translation termination. Our results are consistent with a mechanism in which read through of a pseudouridylated stop codon in bacteria results from increased decoding by near-cognate tRNAs (miscoding) rather than from decreased efficiency of termination.

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recent computational analyses suggest that recognition of pseudouridine instead of uridine would require a conformational rearrangement of a stop codon [18]. The rearrangement was proposed to be necessary to align pseudouridine’s dipole moment (which is distinct from that of uridine) with the dipole moment of a release factor’s recognition helix α5 [18].

In this work, we test the hypothesis that modification of the uridine at the first position of a stop codon to pseudouridine affects the efficiency of translation termination. To visualize the mechanism of recognition of a pseudouridylated stop codon, we have determined a 3.1-Å resolution structure of E. coli RF1 bound to the Thermus thermophilus 70S ribosome programmed with the ΨAA codon in the A site (Fig. 1b). The structure of an E. coli RF1 bound to the 70S ribosome brings insights into the termination mechanism in E. coli, which has been the primary bacterial model system. Furthermore, this work allows us to address structural differences between the mesophilic and previously studied thermophilic release factors. The structure reveals that the conformations of the pseudouridylated stop codon

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Termination on a Pseudouridylated Stop Codon

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Results and Discussion

Crystal structure reveals similar mechanisms of recognition of the ΨAA and UAA stop codons

We have determined a crystal structure of the bacterial translation termination complex formed with the ΨAA stop codon, using *T. thermophilus* 70S ribosomes bound with tRNAfMet in the P (peptidyl-tRNA) site and *E. coli* release factor RF1 in the A site (Fig. 1b–e). RF1 and mRNA were resolved in the initial unbiased electron density maps, which allowed unambiguous interpretation of protein and RNA conformations (Fig. 1d and e). The ribosomal 50S and 30S subunits adopt the non-rotated (classical) conformation, similar to that observed in structures of termination complexes formed with the canonical (non-modified) stop codons [9–12]. The conformation of the release factor (Fig. 1c) is similar to that in the previous structures [10,11], but domain 1 of the release factor is disordered. We note that domain 1 was the least resolved part of RF1 in previous termination complexes formed with *T. thermophilus* 70S ribosomes and *T. thermophilus* RF1 [11].

Previous structures demonstrated that release factors employ domain 2 to recognize the stop codon and domain 3 to catalyze the hydrolysis of peptidyl-tRNA (reviewed in Refs. [19,20]). The “recognition head” of domain 2 of RF1 contains the conserved PXT motif critical for codon recognition. The tip of domain 3 contains the universally conserved GGQ motif, required for catalysis. As described below, the structural recognition of the ΨAA stop codon and positioning of the catalytic GGQ motif in the peptidyl-transferase center are nearly identical to those observed in the canonical 70S termination complexes [10,11].

The ΨAA codon is bound in the A site (decoding center) of the 30S subunit and adopts a conformation similar to that of canonical stop codons (Fig. 1f) in the presence of RF1 [10,11] or RF2 [9,13,14]. Specifically, the first two nucleotides, Ψ1 and A2, are stacked, whereas the base plane of A3 is nearly orthogonal to that of A2. A3 is sandwiched between I196 of RF1 and the universally conserved G530 of 16S ribosomal RNA (Fig. 1e). The bases of the codon interact with the recognition head of domain 2.

The Watson–Crick face of Ψ1 forms hydrogen bonds with the backbone atoms of G120 and Q123 at the N-terminus of α5. The O2 atom of pseudouridine forms a hydrogen bond with the hydroxyl group from threonine of the PXT motif (residues 189–191), which also hydrogen bonds with the N6 atom on the second stop-codon nucleotide (Fig. 1f). The Hoogsteen face of A3 forms a hydrogen bond with T198 of domain 2 (Fig. 1f). Domain 3 connects domain 2 and the large ribosomal subunit (Fig. 1b). The catalytic GGQ motif of domain 3 is docked into the peptidyl-transferase center, ~80 Å away from the PXT motif of domain 2. The glutamine of the GGQ motif abuts the ribose of the 3′-terminal nucleotide of the P-site tRNA, consistent with its catalytic function, as described previously [10,11].

Our structure provides insight into the differences between the mesophilic and extremophilic release factors and reveals interaction of protein L27 with the release factor, which we discuss in a subsequent section (Fig. 2). Previous structural analyses of bacterial termination were based on 70S complexes obtained with *T. thermophilus* release factors [9–11,13,14]. The functional centers of the mesophilic release factors (e.g., *E. coli* RF1 in this work) are distinct from those of the thermophilic release factors. The PXT codon-recognition motif contains alanine (i.e., PAT) in *E. coli* and valine (i.e., PVT) in *T. thermophilus*. The alanine of the PAT motif of *E. coli* RF1 packs on L126 (Fig. 2a). In *T. thermophilus* RF1, the valine of PVT is positioned similarly and interacts with L122, which in turn packs on L152 (H156 in *E. coli*). The more hydrophobic environment of the codon-recognition head in the thermophilic release factor likely contributes to conformational stability of this key functional center [21].

In domain 3, the GGQ catalytic motif is followed by a histidine (H236) in both *E. coli* RF1 and RF2 and by glycine in *T. thermophilus* RF1 and RF2 (Fig. 2b). The GGQH motif is highly conserved in mesophilic bacteria, including pathogens, and in the human mitochondrial release factor mRF1a. The histidine forms hydrogen bonds with the backbone of nucleotide G2494 and with the N6 atom in the nucleobase of the universally conserved A2602 of 23S rRNA (Fig. 2b). A2602 is buried in a pocket of domain 3 of RF1 and is critical for translation termination [22]. H236 therefore likely contributes to stabilization of the GGQ motif and peptidyl-transferase center conformations for catalysis in mesophilic release factors.

Pseudouridylation does not affect kinetics of peptide release

We next asked whether the structural similarity of the 70S-RF1 structures formed in the presence of ΨAA and UAA codons reflects the similarity of stop codon recognition mechanisms. To this end, we tested the efficiency of peptide release by both *E. coli* release
factors, RF1 and RF2, on canonical and pseudouridylation of stop codons (Table 1). We employed a standard in vitro assay to measure the rates of release of \[^{35}\text{S}\]-N-formyl-methionine from E. coli 70S ribosomes bound with fMet-tRNA\[^{\text{fMet}}\] [23].

The efficiencies of peptide release catalyzed by RF1 and RF2 were similar for E. coli 70S complexes formed on UAA, ΨAA, UAG or ΨAG codons (Table 1). Apparent \(K_m\) values for RF1-catalyzed reactions were in the range of 5 to 10 nM (Fig. 3a and b), consistent with the \(K_m\) of 8.3 nM determined by Freistroffer and colleagues for the canonical stop codons [17].

We also tested whether our heterologous release complex (E. coli RF1 and T. thermophilus ribosome) used for crystallography behaves similarly to E. coli 70S complexes. Although E. coli RF1 on the T. thermophilus ribosome is almost three times slower than on E. coli ribosomes, the rates of release on the ΨAA and UAA codons are nearly identical \((0.020 \pm 0.003 \text{ s}^{-1} \text{ and } 0.023 \pm 0.002 \text{ s}^{-1}, \text{ respectively})\). Similarly, at concentrations between 10 nM and 2 \(\mu\)M, E. coli RF2 (K12 strain) on ΨAA and UAA codons catalyzes peptide release with nearly identical rates (Table 1; Fig. 3c). Since the rates of peptide release are sensitive to pH [24], we have measured reaction rates at pH 6.5 (Table 1) and pH 7.5. Consistent with previous work [24], the release is faster at higher pH; however, the rates remain similar for ΨAA and UAA \((0.21 \pm 0.04 \text{ and } 0.18 \pm 0.03 \text{ for } 100 \text{ nM RF1}; (9.0 \pm 1.0) \cdot 10^{-4} \text{ and } (9.5 \pm 0.9) \cdot 10^{-4} \text{ for } 100 \text{ nM RF2})\). As expected, negative control 70S complexes formed with a sense codon AAA or in the absence of mRNA are inefficient in N-formyl-methionine release (Table 1 and Fig. 3a).
Our data demonstrate that RF1 does not distinguish between the ΨAA and UAA codons, or between the ΨAG and UAG codons on E. coli and T. thermophilus ribosomes. Furthermore, RF2 does not distinguish between the ΨAA and UAA stop codons.

### Interaction with L27

Bacterial ribosomes contain a conserved ribosomal protein L27, whose N-terminal tail reaches the 3′-terminal nucleotide of the P-site tRNA in an elongation complex [25]. The function of this interaction is not fully understood. Deletion of three N-terminal amino acids of L27 inhibits cell growth and impairs peptidyl-transferase activity [26]. Recent work, however, argues against the involvement of L27 in the peptidyl-transferase activity, as ΔL27 ribosomes were found to catalyze peptidyl transfer similarly to wild-type ribosomes in vitro [27]. Because the peptidyl-transferase centers of archaeal and eukaryotic ribosomes do not contain an L27 homolog, and their P-site tRNA recognizes AA stop codon with the ribosome and RF1 are similar to those of the UAA codon, bacterial release factors catalyze peptide release on pseudouridylated and non-modified stop codons with similar rates. Rather than decreasing the efficiency of termination, pseudouridylation may exert its effect through increased miscoding. Previous crystallographic analyses suggest that the structural mechanism of miscoding of a pseudouridylated stop codon by tRNA<sup>Se</sup> is the same as that of a canonical stop codon [8], which echoes our findings for the termination mechanism. Biochemical studies are required to test whether the efficiency of miscoding is affected by pseudouridylation. Furthermore, structural and biochemical analyses of eukaryotic translation termination complexes will reveal whether pseudouridylation modulates translation termination in eukaryotes.

### Table 1. Rates of [35S]-fMet release mediated by E. coli RF1 and RF2 from E. coli 70S ribosomes programmed with canonical or pseudouridylated stop codons

<table>
<thead>
<tr>
<th>Stop codon</th>
<th>UAA</th>
<th>UAG</th>
<th>ΨAA</th>
<th>ΨAG</th>
<th>AAA</th>
<th>No mRNA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nM RF1</td>
<td>0.031 ± 0.004</td>
<td>0.039 ± 0.005</td>
<td>0.032 ± 0.005</td>
<td>0.028 ± 0.004</td>
<td>(1.7 ± 0.2) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>(9.1 ± 1.0) · 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>30 nM RF1</td>
<td>0.045 ± 0.004</td>
<td>Not measured</td>
<td>0.044 ± 0.005</td>
<td>Not measured</td>
<td>(2.8 ± 0.3) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>(1.7 ± 0.3) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 nM RF1</td>
<td>0.058 ± 0.008</td>
<td>0.046 ± 0.005</td>
<td>0.048 ± 0.005</td>
<td>(7.9 ± 0.6) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>(2.5 ± 0.2) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>(4.2 ± 0.1) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>500 nM RF1</td>
<td>0.055 ± 0.006</td>
<td>0.059 ± 0.006</td>
<td>0.059 ± 0.006</td>
<td>(1.8 ± 0.1) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>10 nM RF2</td>
<td>(5.5 ± 0.8) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>(5.7 ± 1.0) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>25 nM RF2</td>
<td>(4.9 ± 1.0) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>(5.9 ± 0.7) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
<tr>
<td>2 μM RF2</td>
<td>(4.3 ± 0.3) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>(5.4 ± 0.4) · 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>Not measured</td>
<td>(1.2 ± 0.2) · 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>(6.0 ± 1.9) · 10&lt;sup&gt;-6&lt;/sup&gt;</td>
</tr>
<tr>
<td>T. thermophilus 70S + 500 nM E. coli RF1</td>
<td>0.023 ± 0.002</td>
<td>Not measured</td>
<td>0.020 ± 0.003</td>
<td>Not measured</td>
<td>Not measured</td>
<td>Not measured</td>
</tr>
</tbody>
</table>
Fig. 3. Kinetics of peptide release from 70S ribosomes programmed with pseudouridylated stop codons are similar to those with non-modified stop codons. (a) Michaelis–Menten curves for RF1-mediated release of $^{35}$S-fMet from *E. coli* ribosomes programmed with the stop codons UAA (filled circles) or ΨAA (clear circles, red curve), sense codon AAA (clear squares), or not bound with an mRNA (clear triangles). (b) Michaelis–Menten curves for RF1-mediated release of $^{35}$S-fMet from *E. coli* ribosomes programmed with the UAG (filled circles) or ΨAG (clear circles, red curve) codon. (c) Time progress curves for RF2-mediated release of $^{35}$S-fMet from *E. coli* ribosomes programmed with the UAA (filled circles) or ΨAA (clear circles, red curve) codon at 10 nM RF2.
Materials and Methods

Purification of ribosomes and ligands

70S T. thermophilus ribosomes were purified from the HB27 strain as described [11]. E. coli 70S ribosomes were purified from MRE600 cells [35], and E. coli RF1 (C-terminally His-tagged) and E. coli (K12) RF2 (N-terminally His-tagged) were purified essentially as described [9,11]. Messenger RNAs GCCAAAGGAGGUAAAAUG-XYZAAAAAAA, where XYZ stands for a stop codon used in this study, were purchased from IDT (non-modified) and Dharmacon (pseudouridylated).

Crystallization and structure determination

Crystallization of the 70SΨAARF1 complex was performed essentially as described [36]. 4 μM (all concentrations are given for the final crystallization solution) T. thermophilus ribosome was incubated with 12 μM ΨAA-containing mRNA, 10 μM tRNAfMet, and 16 μM E. coli RF1 in buffer containing 25 mM Tris acetate (pH 7.0), 50 mM potassium acetate, 10 mM ammonium acetate, 10 mM magnesium acetate, and 2.8 mM Deoxy Big Chaps (Soltec Ventures). Crystallization drops were obtained by mixing 3 μl of the 70SΨAARF1 complex and 3 μl of crystallization solution containing 0.1 M Tris–HCl (pH 7.5), 4% (v/v) PEG 20000 (Hampton Research), 8% (v/v) 2-methyl-2,4-pentanediol (Hampton Research), and 0.2 M KSCN. Crystallization was carried out via hanging-drop vapor diffusion over 300 μl of 0.4–0.6 M NaCl. Crystals were cryoprotected by gradually adding 4 μl different concentrations of 1.5 M Tris–HCl (pH 7.5), 4.5% (v/v) PEG 20000, 10% (v/v) PEG 200 (Hampton Research), 30% (v/v) 2-methyl-2,4-pentanediol, and 0.2 M KSCN. Crystals were frozen by plunging into liquid nitrogen.

Crystals were screened at the Argonne National Laboratory (beam lines 23 ID-B, 23 ID-D), Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25). The data sets that resulted in the crystal structure were collected at the Argonne National Lab (beam line 23 ID-D, Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25). The data sets that resulted in the crystal structure were collected at the Argonne National Lab (beam line 23 ID-D, Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25). The data sets that resulted in the crystal structure were collected at the Argonne National Lab (beam line 23 ID-D, Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25). The data sets that resulted in the crystal structure were collected at the Argonne National Lab (beam line 23 ID-D, Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25). The data sets that resulted in the crystal structure were collected at the Argonne National Lab (beam line 23 ID-D, Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25). The data sets that resulted in the crystal structure were collected at the Argonne National Lab (beam line 23 ID-D, Stanford Synchrotron Radiation Lightsource (beam line 12-2), and Brookhaven National Laboratory (NSLS-I, beam line X25).

Table 2. Data collection and structure refinement statistics.

| Space group | P2_1;2_1;2_1 |
| Cell dimensions | a, b, c (Å) |
| Completeness (%) | 100 (100) |
| Resolution (Å) | 70–3.1 (3.2–3.1) |
| Redundancy | 37.4 (36.6) |
| Rfree/a,b | 0.237 (1.99) |
| Rmerge | 70% (90, 90) |
| l/αl | 82.8 (1.01) |
| Completeness (%) | 100 (100) |
| Resolution (Å) | 50–3.1 |
| No. reflections | 1,064,941 |
| Bond angles (°) | 0.007 |
| Bond lengths (Å) | 0.222/0.256 |
| Total no. of atoms | 301,021 |
| Values in parentheses are for the high-resolution shell. |
| a Rfree/a,b (precision-indicating merging R factor) [45] was calculated using SCALA, which is part of the CCP4 [46] package. |
| b CC(1/2) is the percentage of correlation between intensities from random half-datasets as defined by Karplus and Diederichs [47]. |

Peptide release assay

[35S]-methionine (Perkin Elmer) was used to aminoacylate E. coli tRNAfMet (Chemical Block), as previously described [44]. Termination assays were carried out essentially as described [23]. Pre-termination complex was formed by incubating [35S]-Met-tRNAfMet with 1.5-fold molar excess of E. coli 70S ribosome for 15 min at 37 °C in buffer containing 20 mM Tris acetate (pH 6.5; Table 1) or 20 mM Tris chloride (pH 7.5; as described in Results and Discussion), 100 mM ammonium acetate, and 20 mM magnesium acetate. The solution was diluted 22-fold with the same buffer containing 0.05% Triton X-100 (Omni Pur), resulting in 10 mM pre-termination complex (10 nM [35S]-Met-tRNAfMet and 15 nM ribosome). 1 mM mRNA (final) was added to the pre-termination complex. The complex was incubated for 5 min at 37 °C and cooled to room temperature; 4.5 μl of the mixture was extracted in 30 μl of 0.1 M HCl to represent the zero-time point. 45 μl of the pre-termination complex was mixed with 5 μl of a 10× solution of release factor in the buffer used to prepare the pre-termination complex, and 5 μl aliquots collected at different time points were quenched in 30 μl of 0.1 M HCl. [35S]-N-formyl-methionine was extracted with 700 μl of ethylacetate, and 600 μl of extract was mixed with 3.5 ml of scintillation cocktail (Econo-Safe). Samples were counted using a scintillation counter (Beckman).

Rate constants (kobs) were obtained by single exponential fitting of the time progress curves. Values for kobs were plotted as a function of release factor concentration and fit to a hyperbola, yielding apparent Kn values (Fig. 3a and b.). All experimental data were obtained from experiments performed at least twice. Fitting and visualization of kinetic data were carried out using GraphPad Prism 6.

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Termination on a Pseudouridylated Stop Codon

Accession numbers
Coordinates and structure factors were deposited in Protein Data Bank (PDB ID: 5J4D).

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Author Contributions:
E.S. and A.A.K. designed the project. R.M. purified proteins, ribosome and optimized the release assay. E.S. performed crystallization and peptide release experiments. E.S. and A.A.K. processed the crystallographic data and refined the structure. E.S. and A.A.K. wrote the manuscript.

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